MICROSATELLITE INSTABILITY AND CELL CYCLE PROTEIN ANALYSIS IN ENDOMETRIAL CARCINOMA

By

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DECLARATION

The research described in this dissertation was carried out in the Department of Anatomical Pathology and Pfizer Molecular Biology Research Facility, Nelson R. Mandela School of Medicine, Faculty of Health Sciences, University of KwaZulu-Natal, under the supervision of Professor Runjan Chetty.

This study represents the original work by the author and has not been submitted in any form to another university. Use of the work of others has been duly acknowledged in the text.

PADMINI PADAYACHY

This thesis is dedicated to:

MUM AND DAD

WITH LOVE ALWAYS

FOR ALL THE WAYS YOU'VE CARED FOR ME
FOR ALL THE LOVE YOU'VE SHARED WITH ME
FOR ALWAYS BEING THERE FOR ME
THANK YOU
(Build-A-Button Quote)

ACKNOWLEDGEMENTS

Everytime we remember to say "thank you", we experience nothing less than heaven on earth. – Sarah Ban Breathnach
I can no answer make but thanks and thanks and ever thanks. – William Shakespeare

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ABBREVIATIONS

aa	-	Amino acid
AEH	-	Atypical endometrial hyperplasia
AI	-	Allelic imbalance
APC	-	Adenomatous polyposis coli
bp	-	Base pairs
CDK	-	Cyclin dependent kinase
CDKI	-	Cyclin dependent kinase inhibitor
CAK	-	CDK activating kinase
CIP	-	CDK interacting proteins
CRC	-	Colorectal carcinoma
CSA	-	Catalysed signal amplification
CSF1	-	Colony stimulating factor 1
DAB	-	Diaminobenzidine tetrahydrochloride
D&C	-	Dilatation and curettage
DCC	-	Deleted in Colorectal Cancer
DNA	-	Deoxyribonucleic acid
EGF	-	Epidermal growth factor
EIC	-	Endometrial intraepithelial carcinoma
EEC	-	Endometrioid endometrial carcinoma
ER	-	Oestrogen receptor
EWS	-	Ewings sarcoma

FAP	-	Familial adenomatous polyposis
FGF	-	Fibroblast growth factor
FIGO	-	International Federation of Gynaecology and Obstetrics
GADD	-	Growth arrest and DNA damage
$GADD_{45}$-		Growth arrest and DNA damage inducible
GAP	-	GTPase – activating protein
GDP	-	Guanosine diphosphate
GOG	-	Gynaecologic Oncology Group
GTP	-	Guanosine triphosphate
H_2O_2	-	Hydrogen peroxide
H&E	-	Haematoxylin and eosin
HNPCC	-	Hereditary Non-polyposis colorectal carcinoma
HPV	-	Human papilloma virus
HSP	-	Heat shock protein
ICC	-	Immunocytochemistry
IGFI	-	Insulin-like growth factor I
IGFII	-	Insulin-like growth factor II
IGFIIR	-	Insulin-like growth factor II receptor
LOH	-	Loss of heterozygosity
LSAB	-	Labelled Streptavidin-Biotin
LVSI	-	Lymph vascular space invasion
MAPK	-	Mitogen – activated kinase
M-CSF	-	Macrophage-colony stimulating factor

MDM2	-	Murine double minute-2
MLL	-	Myeloid lymphoid leukaemia
MMR	-	Mismatch repair
MSI	-	Microsatellite instability
NAI	-	No allelic imbalance
NE	-	Not expressed
NEEC	-	Non-endometrioid endometrial carcinoma
NER	-	Nucleotide excision repair
NF1	-	Neurofibromatosis type 1
NHL	-	Non – Hodgkins lymphoma
PBS	-	Phosphate buffered saline
PCNA	-	Proliferating cell nuclear antigen
PCR	-	Polymerase chain reaction
PDGF	-	Platelet-derived growth factor
PR	-	Progesterone receptor
pRb	-	Retinoblastoma protein
PTEN	-	Phosphatase & tensin homolog
Rb	-	Retinoblastoma gene
RER	-	Replication error
SSLP	-	Simple sequence length polymorphisms
STR	-	Short tandem repeat
TGF	-	Transforming growth factor
TGFα	-	Transforming growth factor alpha

TGFβ	-	Transforming growth factor beta
TGFβRII	-	Transforming growth factor β receptor II
WHO	-	World Health Organisation
WT - 1	-	Wilms tumour 1 gene

ABSTRACT

As researchers continuously and progressively grow with knowledge of human cancers, there has been an increased awareness regarding genetic alterations and its impact on tumour development. The role of microsatellites and cell cycle proteins has been shown to be particularly important in carcinogenesis. Many human tumours have been observed to have multiple mutations, which the body has no control over. Microsatellite instability and derailments of the cell cycle have been implicated in various human malignancies. Although there seems to be a tremendous advancement in our understanding of endometrial cancer, in South Africa there appears to be a lack of research in this area. In this study, we therefore aimed to investigate the role of microsatellite instability and cyclins A, D1, E, p27, p53 and pRb in endometrial carcinoma.

Fifty-four hysterectomy specimens of endometrial cancer were retrieved from the files of the Department of Pathology at the Nelson R Mandela School of Medicine, University of KwaZulu-Natal. The histological subtype, tumour stage and grade were reviewed. Endometrioid type endometrial cancer formed the bulk (85.2%) of the tumours studied, followed by clear cell carcinoma with 9.3%, serous papillary with 3.7%, and only one case of an adenoacanthoma. Of the 54 cases studied, 27 were Black, 18 were Asian, 3 were Coloured and 6 were White patients. Their ages ranged from 40 to 81 years, while the mean patient age was 57.5 and the median patient age was 63 years.

Immunohistochemical analysis was performed on formalin fixed, paraffin embedded tissue using the CSA and LSAB kits with DAB as the chromogen. The cases were either microwave antigen retrieved or pressure cooked using a 0.01mol/l trisodium citrate buffer at pH 6.0. Monoclonal anti-cyclin A, anti-cyclin D1, anti-cyclin E, anti-p27, anti-p53, and anti-pRb antibodies were used. The immunohistochemical results were correlated with race, age, grade, stage, histological subtype, myometrial

invasion, lymph node metastasis, and the statistical significance was determined by the Chi-square test.

Statistically significant results were obtained with p53 and stage - p=0.030, pRb and grade - p=0.036, cyclin A and myometrial invasion - p=0.009. Close to statistically significant results were obtained with p53 and grade - p=0.052, cyclin E and myometrial invasion - p=0.052 and cyclin D and age -p=0.051.

p53 was expressed in 50% (27/54) of cases. Of the 46-endometrioid tumours, 21 (45.7%) were positive for p53. Both serous papillary cancers expressed p53 as well as 4 of the 5 clear cell tumours. pRb was expressed in 90.7% of cases. 89.1% (41/46) of the endometrioid tumours were positive, as well as all 5 clear cell cancers and both serous papillary tumours. Cyclin A showed expression in 85.2% (46/54) of the cases. The majority, 38 (82.6%) of the endometrioid cancers were positive for cyclin A. All 5 clear cell carcinomas and both serous papillary carcinomas expressed cyclin A. Only 35.2% (19/54) of the cases expressed cyclin D1. Of these, 18 (39.1%) endometrioid cancers stained positive, while none of the serous papillary cancers stained, and only one clear cell carcinoma expressed cyclin D1. Cyclin E was also expressed in majority of cases, 92.6% (50/54). A total of 42 (91.3%) endometrioid cases were positive for cyclin E. All 5 clear cell tumours and both of the serous papillary cancers expressed cyclin E as well. These immunohistochemical stains were also compared against each other. Statistically significant results were obtained with cyclin A and cyclin E with a 100% correlation, cyclin A and p53 with a p value of 0.002, cyclin A and p27 also achieved 100% correlation, cyclin E and p27 with a p value of 0.022, p53 and pRb where p = 0.019, p53 and p27 with a p value of 0.043.

In conclusion we have shown that these protein components of the cell cycle pathway do play a role in the carcinogenesis of endometrial carcinoma. Furthermore, our results add significance to the theory of the two pathogenetic pathways of endometrial carcinoma. This study has observed that cyclin D1, p27

and cyclin A to a certain extent seem to be associated with type I endometrioid endometrial cancer, while cyclin E, p53 and pRb appear to be involved in type II non-endometrioid endometrial cancer.

A molecular study was also performed on the 54 cases studied to investigate the occurrence of the genetic aberrations involved in endometrial cancer. We conducted a microsatellite study using fluorescent-based DNA technology to detect mutations in the microsatellite sequences of the Deleted in Colorectal Cancer (*DCC*) gene, a tumour suppressor gene at 18q21.1, and the mismatch repair gene (MMR) to determine their implications in endometrial carcinoma. The standard proteinase K digestion and phenol-chloroform extraction method was used to isolate normal and tumour DNA from the fifty-four paraffin-wax embedded tissue sections of the endometrial cancer cases. Polymerase chain reactions using 3 microsatellite markers for the *DCC* gene: D18S21, D18S34, D18S58; and three microsatellite markers for the MMR genes: D2S123, D3S659 and BAT 25 were performed. The polymerase chain reaction products were analysed on the ALF Express Automated DNA sequencer. The Chi-square test was used to correlate results with race, age, stage, grade, myometrial invasion, lymph node metastasis, and histological subtype.

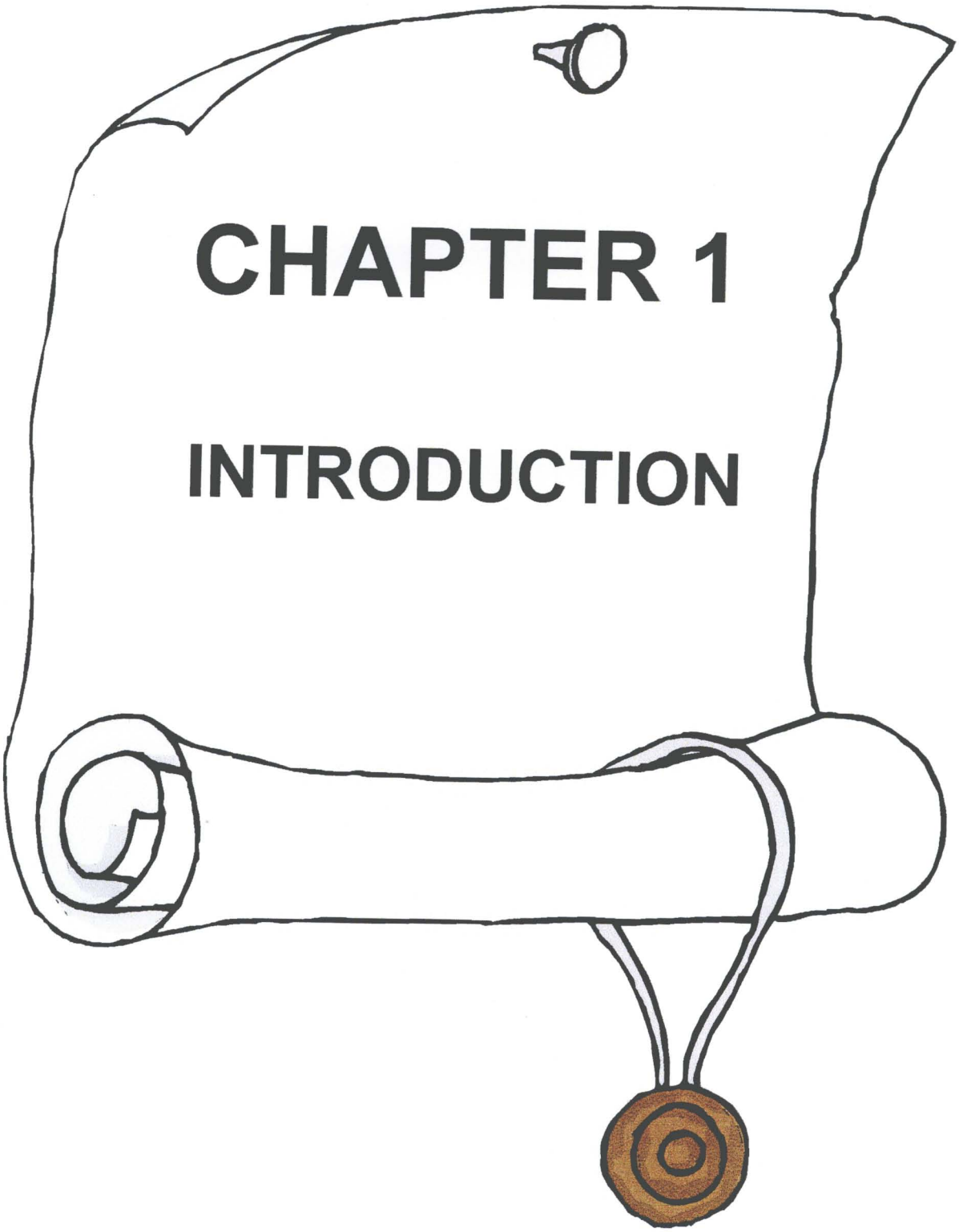
Statistically significant differences were obtained when *DCC* aberrations were compared with stage, $p = 0.024$. The statistics were also significant when *DCC* mutations were compared with stage, $p=0.012$. The total number of cases mutated was 28 (33.3%). Two cases had mutations at two loci. A significant correlation was also obtained with D18S58 and age, $p=0.040$; D18S21 and grade, $p=0.013$; BAT25 and histological subtype, $p=0.049$.

At the MMR gene loci AI/LOH appeared to be a more frequent genetic aberration than MSI. Of the MMR markers employed, D3S659 and BAT 25 both showed the highest percentage of AI/LOH with 7.4% each. The total percentage of AI/LOH was 20.4%. The majority (81.8%) of cases displaying this aberration were endometrioid type endometrial cancer. The MSI+ phenotype was observed for only one of the cancer cases at the MMR gene loci. At the *DCC* gene loci MSI was the more

frequent aberration. The frequency of MSI was the highest with the D18S58 marker. The total rate of MSI at the *DCC* gene was 22.2%. The MSI+ phenotype was observed for one of the tumour cases. At these loci the majority (83.3%) of the MSI+ cases were also endometrioid type tumours.

When these markers were correlated with the immunohistochemical results, statistically significant results were obtained with D3S659 versus Cyclin D, p=0.013; D18S34 versus p53, p=0.021; D18S58 versus pRb, p=0.036; and D18S58 versus p27, p=0.018.

In conclusion, this study has found that multiple aberrations involving the *DCC* and MMR gene loci may play a role in the progression of endometrial carcinoma. We have shown that at the MMR loci AI/LOH is a more frequent aberration whilst at the *DCC* gene loci MSI is the more frequent aberration. Also, the fact that majority of our MSI+ cases were endometrioid type cancers further highlights and supports the two pathogenetic pathways of endometrial carcinoma.



CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

"Nature is nowhere accustomed more openly to display her secret mysteries than in cases where she shows traces of her workings apart from the beaten path; nor is there any better way to advance the proper practice of medicine than to give our minds to the discovery of usual law of Nature by careful investigation of cases of rare forms of diseases. For it has been found in almost all things, that what they contain of useful or applicable nature is hardly perceived unless we are deprived of them, or they become deranged in some way." William Harvey, 1657

Multiple genetic changes occur during the evolution of normal cells into cancer cells. This evolution is facilitated in cancer cells by loss of fidelity in the processes that replicate, repair and segregate the genome. Recent advances in our understanding of the cell cycle, oncogenes, and tumour suppressor genes reveal how fidelity is normally achieved by coordinated activity of cyclin-dependent kinases, checkpoint controls, and repair pathways, and how this fidelity is abrogated by specific genetic changes. These insights suggest molecular mechanisms for cellular transformation and may help to identify potential targets for improved cancer therapies (Hartwell and Kastan, 1994). Another one of these remarkable discoveries is microsatellites. Microsatellites revolutionised the 20th century, increasing our understanding of the processes involved in carcinogenesis. Besides making such a great impact on many diseases, its uses include: personal identification, construction of evolutionary trees and population genetic analysis (Naidoo and Chetty, 1998). One such disease, which has benefited from the study of the above factors is endometrial carcinoma.

It is amazing how we have progressed through the years with all the knowledge and information we now have, about the world of carcinogenesis. 97% of all cancers of the uterus arise from the glands of the endometrium and are known as endometrial adenocarcinomas, the remaining 3% are sarcomas. Carcinomas of the endometrium are malignant epithelial tumours that arise from the endometrium, most of which are adenocarcinomas, but variants with squamous

differentiation can also be seen (Silverberg *et al*, 1992). The most common type is the endometrioid adenocarcinoma. The other types are clear cell, serous papillary and mucinous adenocarcinomas. Endometrial carcinoma occurs primarily in the postmenopausal patient between the ages 50 and 60 years. Only 2-5% of cases occur prior to the age of 40 years, but there is an increasing incidence of this tumour in younger patients. According to reports up to 20% occur in this age group (Ramzy, 1983). Compared to invasive squamous cell carcinoma of the cervix, endometrial carcinoma is known to have a better prognosis. The average survival period is 5 years varying according to the clinical stage, histological grade and depth of myometrial invasion. Endometrial carcinoma may be associated with the Hereditary Non Polyposis Colorectal Carcinoma (HNPCC). HNPCC is a well-characterised autosomal dominant cancer syndrome associated with tumour microsatellite instability and defective mismatch repair (Basil *et al*, 2000). Patients who develop this carcinoma are born with germline mutations in the mismatch repair genes. This syndrome of right -sided colon cancers is also known as the Lynch syndrome II (Mecklin and Jarvinen, 1991; Watson and Lynch, 1993). Endometrial cancer is the second most common neoplasm encountered in patients with HNPCC (Matias-Guiu *et al*, 2001; Watson and Lynch, 1993).

There are many factors involved in the causation of endometrial carcinoma. Endogenous and exogenous oestrogen, obesity, tamoxifen, which is an anti-oestrogen used widely as adjuvant therapy for breast cancer, have all been implicated. It also occurs in patients with low fertility and is common amongst nuns and other nulliparous patients.

Endometrial carcinoma has fast become the most common gynaecological malignancy in many parts of the world (Gusberg 1980, Weiss *et al*, 1976). In the United States of America it is the commonest invasive malignancy of the female genital tract (Boring *et al*, 1991). It is known to be the fourth commonest cancer in women and the most curable of the 10 most common cancers in women (Rose, 1996). In 1996 the United States had an estimated 34000 new cases and approximately 6000 deaths resulting from this neoplasm (Parker *et al*, 1996). World-wide approximately 150 000 cases are diagnosed each year (Parazzini *et*

al, 1991). In the western world adenocarcinoma of the endometrium has increased to such an extent that it has now replaced squamous carcinoma of the cervix as the commonest uterine cancer (Robertson, 1981). However, the high incidence in the industrialised countries of Europe and North America is in contrast to that in the developing countries of Africa, Asia and South America, where its incidence is 4 to 5 times lower (Doll *et al*, 1970).

Through the years, however, it has been heartening to note that the advent of oestrogen-progestagen combinations for postmenopausal hormone replacement has brought about a relative decrease in this high incidence (Austin and Roe, 1982).

In the pathology laboratory the endometrial biopsy constitutes a large percentage of the surgical pathology workload. In certain western countries there is no part of the human body that is examined by histopathological technique, during life, more often than the endometrium. Virtually every general hospital houses a gynaecology unit and even the smallest of these will refer approximately 5 or more biopsies per week to the histopathology laboratory (Robertson, 1981). In one week there are approximately 25 endometrial biopsy samples received in the Histopathology laboratory at the Nelson R Mandela School of Medicine and for a month approximately 100 endometrial specimens are submitted. Approximately 5% of these biopsies are reported as malignant (Unpublished data).

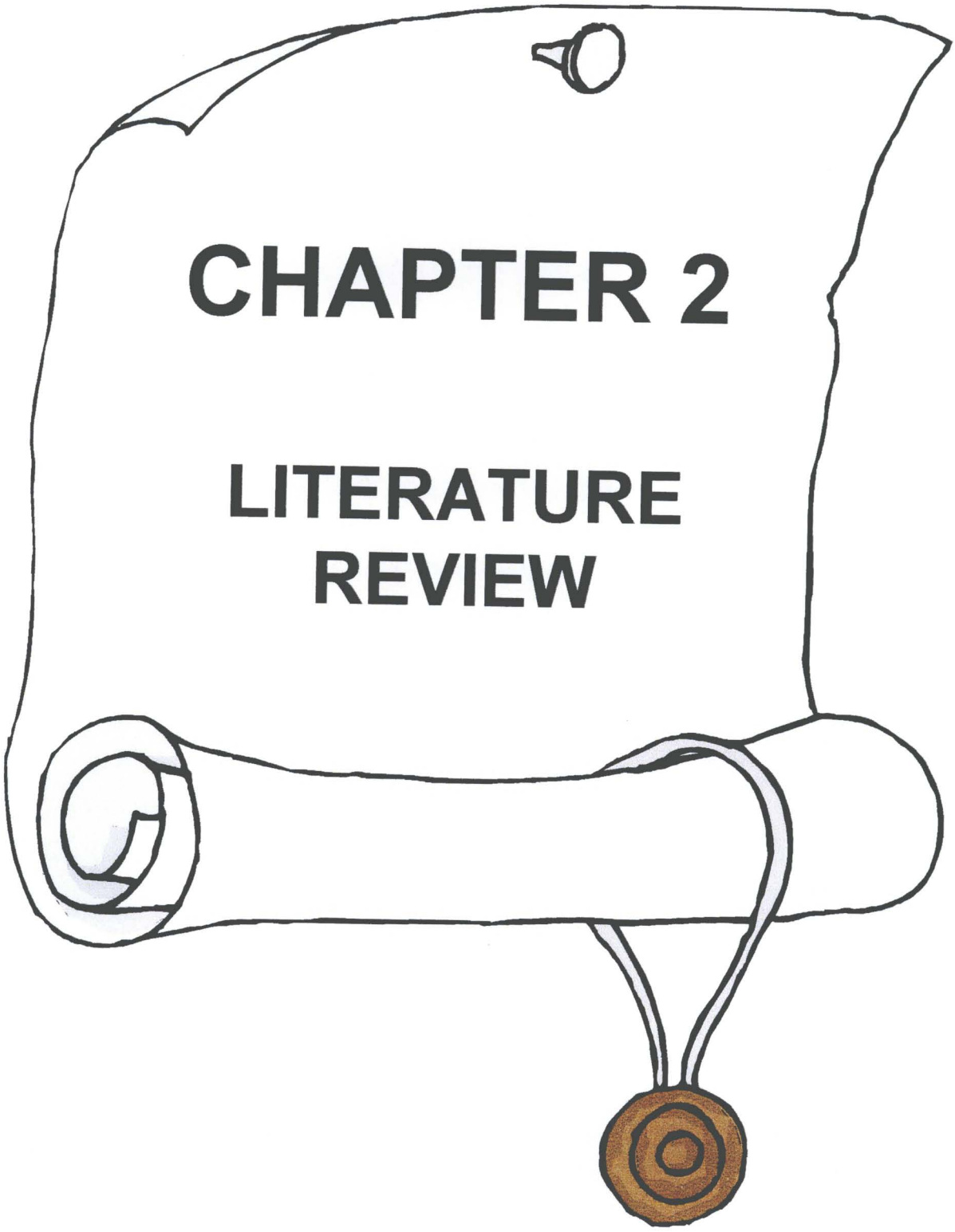
Much focus has recently been placed on the molecular aspects of endometrial carcinoma. However, there are still many questions that are puzzles for the 21st century (Sherman and Kurman, 1998). Although endometrial carcinoma is common in many parts of the world, it has not been that intensely researched in South Africa. Therefore, this research study was undertaken to discover more about the molecular genetic aspects involved in endometrial carcinoma as seen in the KwaZulu-Natal region, and the role played by cell cycle regulators in the pathogenesis of this disease. As yet there is no molecular marker that is as valuable in determining prognosis as the conventional parameters such as tumour type, grade and vascular space involvement (Robertson, 1981). Whether these new tools would be used in therapeutic medicine still remains to be seen.

Recently there has been an increased awareness regarding genetic alterations and its impact on tumour development. The role of microsatellites, cell cycle regulators and the DNA (deoxyribonucleic acid) repair genes, have been shown to be particularly important in endometrial carcinogenesis.
Although Molecular Biology has made a major breakthrough into the world of pathology, there still remains much to be discovered.

1.2. OBJECTIVES

It is clear that endometrial carcinoma has become the most common malignancy of the female genital tract in the western world and the secretive molecular pathway leading to this carcinogenesis has just been opened. However, this carcinoma has not been that extensively researched in Southern Africa. Therefore the objectives of this study were:

1. To investigate the incidence of microsatellite instability in endometrial carcinoma.
2. To examine cell cycle protein expression in endometrial carcinoma.



CHAPTER 2

LITERATURE REVIEW

2.1 CLASSIFICATION OF ENDOMETRIAL CARCINOMA

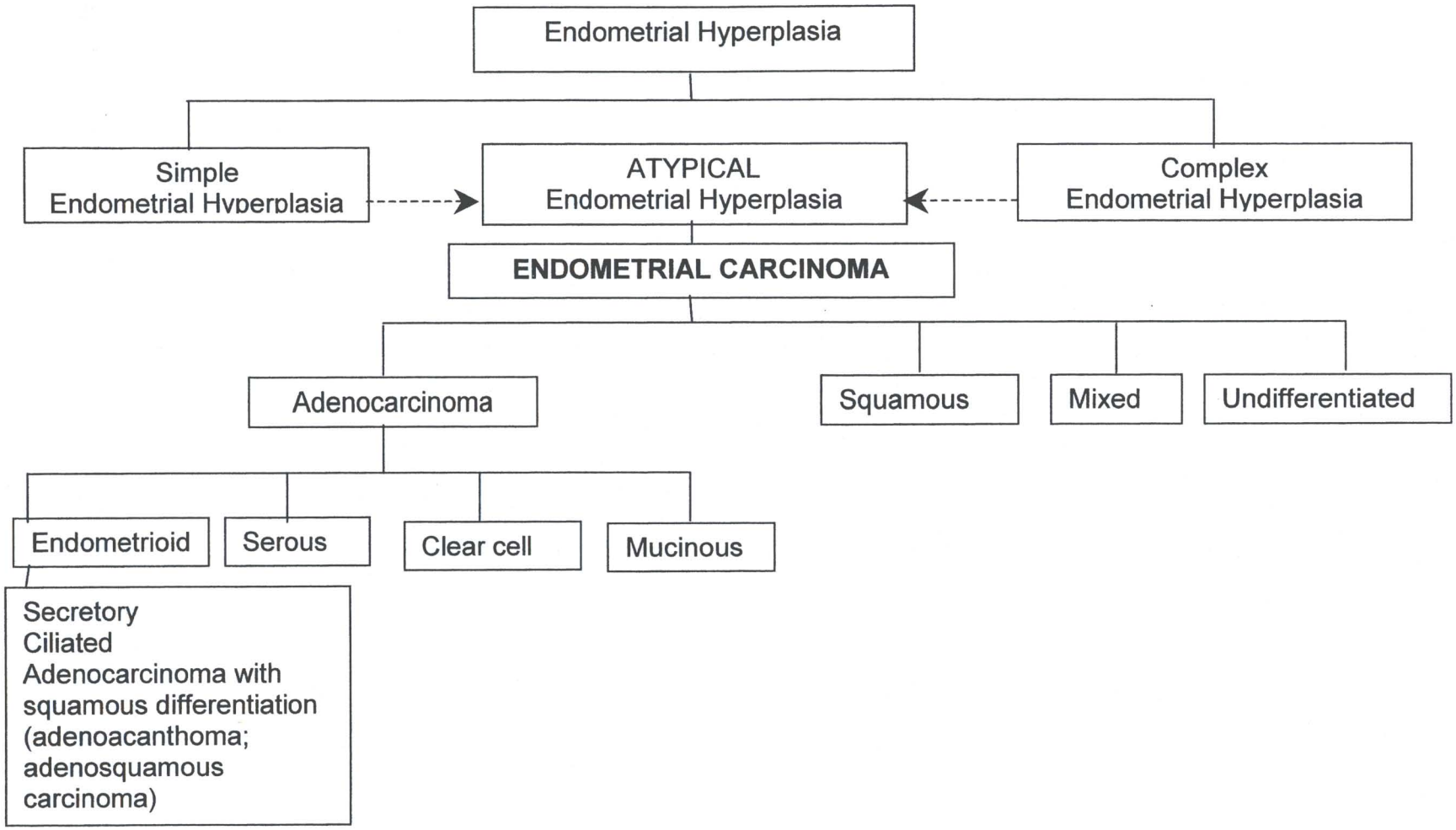


Figure 2.1: The World Health Organisation (WHO) classification scheme of endometrial carcinoma (Scully et al., 1994)

There are a number of different histologic classification schemes that exist for endometrial carcinoma. The comprehensive classification as set out by the World Health Organisation (WHO) is illustrated on the previous page (Figure 2.1), (Adapted from Scully *et al*, 1994). The presentation of the various histological types in this dissertation adheres closely to this classification.

Although there are various other classification shemes that exist, many have certain limitations. It is therefore of vital importance that an appropriate classification system be used as just a simple diagnosis of endometrial adenocarcinoma would represent an inadequate response to the finding of a carcinoma in an endometrial biopsy. Also, some of the histological sub-types of endometrial carcinoma have a particularly poor prognosis and their identification in a diagnostic endometrial sample would be of great importance in the planning of adjuvant therapy.

2.1.1 THE WORLD HEALTH ORGANIZATION (WHO) INTERNATIONAL HISTOLOGICAL CLASSIFICATION OF ENDOMETRIAL CARCINOMA

In the WHO classification (Scully *et al*, 1994), hyperplasias of the endometrium are separated into endometrial hyperplasia and atypical endometrial hyperplasia. Each of these entities is further subdivided into simple and complex, with adenomatous accepted as a synonym for complex.

2.1.1.1 ENDOMETRIAL HYPERPLASIA

This is a proliferation of endometrial glands without cytologic atypia.

- **SIMPLE**

- **COMPLEX (Adenomatous)**

2.1.1.2 ATYPICAL ENDOMETRIAL HYPERPLASIA

This is a proliferation of endometrial glands with cytologic atypia.

- **SIMPLE**

- **COMPLEX (Adenomatous with Atypia)**

Endometrial hyperplasia can be either focal or diffuse. In hyperplastic glands the cytologic atypia is typically focal. The glands of the hyperplastic endometrium are usually of proliferative type, but a focal or diffuse secretory change, metaplastic or related changes, or both may also be present. In simple hyperplasia the endometrial glands are usually increased in number per unit volume, but the endometrial stroma is typically hyperplastic as well. These glands may be cystically dilated and slightly to moderately crowded. In complex hyperplasia the glands are markedly crowded and typically have irregular outlines, thus resulting in a complex pattern. Cytologic atypia is characterised by significant nuclear abnormalities, including loss of polarity and may be superimposed on either simple or complex hyperplasia. There is much confusion regarding the widely used designation "adenomatous". Some authors have used this term to denote architecturally abnormal but cytologically typical endometrial glands and others have used it to signify architecturally abnormal and cytologically atypical glands. The term "atypical adenomatous hyperplasia" has also been employed. In the alternative terminology the word "adenomatous" designates an architectural (complex-glandular) abnormality only, cytologic atypia, if present must be noted additionally. Therefore, the above terminology was selected to avoid confusion regarding this widely used designation "adenomatous". It is important that endometrial hyperplasia be distinguished from an endometrial polyp, glandular alterations accompanying endometritis, epithelial metaplasias, normal or slightly altered cycling endometrium, glandular alterations caused by pregnancy and progestin therapy, and adenocarcinoma.
If not adequately treated, atypical endometrial hyperplasia is capable of progression to carcinoma, but the malignant potential of endometrial hyperplasia without cytologic atypia is much less.

2.1.1.3 ENDOMETRIAL CARCINOMA

2.1.1.3.1 ENDOMETRIOID ADENOCARCINOMA

This carcinoma contains glands that resemble those of the normal endometrium. Endometrioid adenocarcinoma is known to be the most common form of endometrial carcinoma. The glands exhibit varying degrees of cytologic atypia. They are often uniform in size and shape, and also invade the stroma. It is important that when the tumour has a papillary, villoglandular pattern, it must be distinguished from serous papillary and clear cell papillary adenocarcinomas as these are more aggressive tumours.

The grading of the glandular component of all endometrioid carcinomas is as follows (Scully *et al*, 1994):

Grade 1: 5% or less nonsquamous solid growth pattern.

Grade 2: 6% -50% nonsquamous solid growth pattern.

Grade 3: More than 50% nonsquamous solid growth pattern.

Remarkable nuclear atypia, inappropriately severe for the architectural grade of the tumour, raises the grade of otherwise grade 1 or grade 2 tumours by one.

The following are variants of adenocarcinoma:

- **Secretory**

This is an endometrioid adenocarcinoma that contains glands resembling those of an early secretory endometrium.

- **Ciliated Cell**

An endometrioid adenocarcinoma in which the majority of the cells bear cilia.

2.1.1.3.2 ADENOCARCINOMA WITH SQUAMOUS DIFFERENTIATION (ADENOACANTHOMA; ADENOSQUAMOUS CARCINOMA)

This is an endometrioid adenocarcinoma in which there is focal differentiation into other benign (adenoacanthoma) or malignant (adenosquamous carcinoma) appearing squamous epithelium.

The grading of adenocarcinomas with squamous differentiation is according to the grade of the glandular component. Strict criteria have to be applied for the identification of the squamous element in adenocarcinomas with squamous differentiation, because endometrioid carcinomas may contain significant areas of solid growth of either glandular or squamous epithelial cells. A solid focus of tumour in an endometrioid carcinoma should be considered glandular unless at least one of the following features of squamous differentiation is present:

- Keratinization demonstrated by standard staining techniques
- Intercellular bridges
- Three or more of the following four features: sheet-like growth without gland formation or nuclear palisading; sharp cell margins; eosinophilic, dense or glassy cytoplasm; a decreased nuclear-cytoplasmic ratio in comparison with other foci in the tumour

Malignant squamous epithelium is identified by one or more of the following features:

- Standard cytologic features of malignancy
- Mitotic figures
- Destructive stromal infiltration

The malignant squamous elements in these tumours do not require grading. The designation "adenocarcinoma with squamous differentiation", with grading of the glandular component only, is currently preferred over the subdivision of these tumours into "adenoacanthoma" and "adenosquamous carcinoma".

2.1.1.3.3 SEROUS PAPILLARY ADENOCARCINOMA

This variant of adenocarcinoma is characterised by a complex pattern of papillae consisting of cellular budding and the frequent presence of psammoma bodies.
The criteria used to recognise serous papillary adenocarcinoma of the endometrium are similar to those used to diagnose serous papillary adenocarcinoma of the ovary. Irregularly stratified and generally poorly

differentiated cells that form secondary papillae, and cellular buds line the broad fibrovascular connective tissue cores of the papillae. The nuclei are highly atypical, foci of necrosis are often seen, and psammoma bodies are detectable in approximately one third of the cases. Also, solid sheets of undifferentiated cells are commonly present. There is characteristic infiltration of the tumour into the myometrium within lymphatic or blood vascular channels, and at the time of diagnosis is often widely disseminated.

2.1.1.3.4 CLEAR CELL ADENOCARCINOMA

This adenocarcinoma is composed chiefly of clear cells or hobnail cells that are arranged in solid, tubulocystic, or papillary patterns or a combination of these patterns.

Clear cell adenocarcinoma is also noted to have a tendency for early dissemination.

2.1.1.3.5 MUCINOUS ADENOCARCINOMA

Mucinous adenocarcinoma is an adenocarcinoma in which most of the tumour cells, contain abundant intra-cytoplasmic mucin. These are usually well-differentiated adenocarcinomas and may resemble mucinous adenocarcinomas of the cervix. It is therefore important to rule out the possibility of an endocervical adenocarcinoma extending to the endometrium before the diagnosis of a primary mucinous adenocarcinoma of the endometrium is made. It is also important to distinguish the latter from mucinous metaplasia of the endometrium. This is usually accomplished by the finding of stromal invasion, cellular stratification, and nuclear atypia in mucinous adenocarcinoma.

2.1.1.3.6 SQUAMOUS CELL CARCINOMA

This is a carcinoma composed of squamous cells that have varying degrees of differentiation. Squamous cell carcinoma of the endometrium is rare. With this carcinoma as well, it is also important that before making the diagnosis, a squamous cell carcinoma of the cervix extending to the endometrium and

adenocarcinoma of the endometrium with squamous differentiation in which the squamous component is predominant, be excluded.

2.1.1.3.7 MIXED CARCINOMA

Mixed carcinoma is a carcinoma (other than adenocarcinoma with squamous differentiation) in which one or more additional types account for at least 10% of the entire tumour. On examination of a hysterectomy specimen the diagnosis of a mixed carcinoma is optimally made, but if only a smaller specimen is available, any amount of a second tumour category suffices for the diagnosis. The major and minor types and their relative proportions must be specified, when a carcinoma is classified as mixed.

2.1.1.3.8 UNDIFFERENTIATED CARCINOMA

A carcinoma with no or minimal differentiation into any of the above cell types is referred to as undifferentiated carcinoma. This type of carcinoma includes large cell, giant cell, spindle cell and small cell forms. The small cell undifferentiated carcinoma may contain argyrophil cells and should be distinguished from other types of endometrial carcinomas, as the latter may also contain variable numbers of argyrophil cells. The latter tumours should be designated as one of the tumour types already described. The term "argyrophil cell carcinoma" is not warranted, but the content of the argyrophil cells may be commented on separately.

Other types of carcinoma that occur more commonly in other sites have been reported rarely in the endometrium. The following has been recommended:

- "Glassy cell carcinoma" be classified as a subtype of adenocarcinoma with squamous differentiation.
- "Verrucous carcinoma" a subtype of squamous cell carcinoma, and
- "Mucoepidermoid carcinoma" as a variant of mucinous adenocarcinoma (Scully *et al*, 1994).

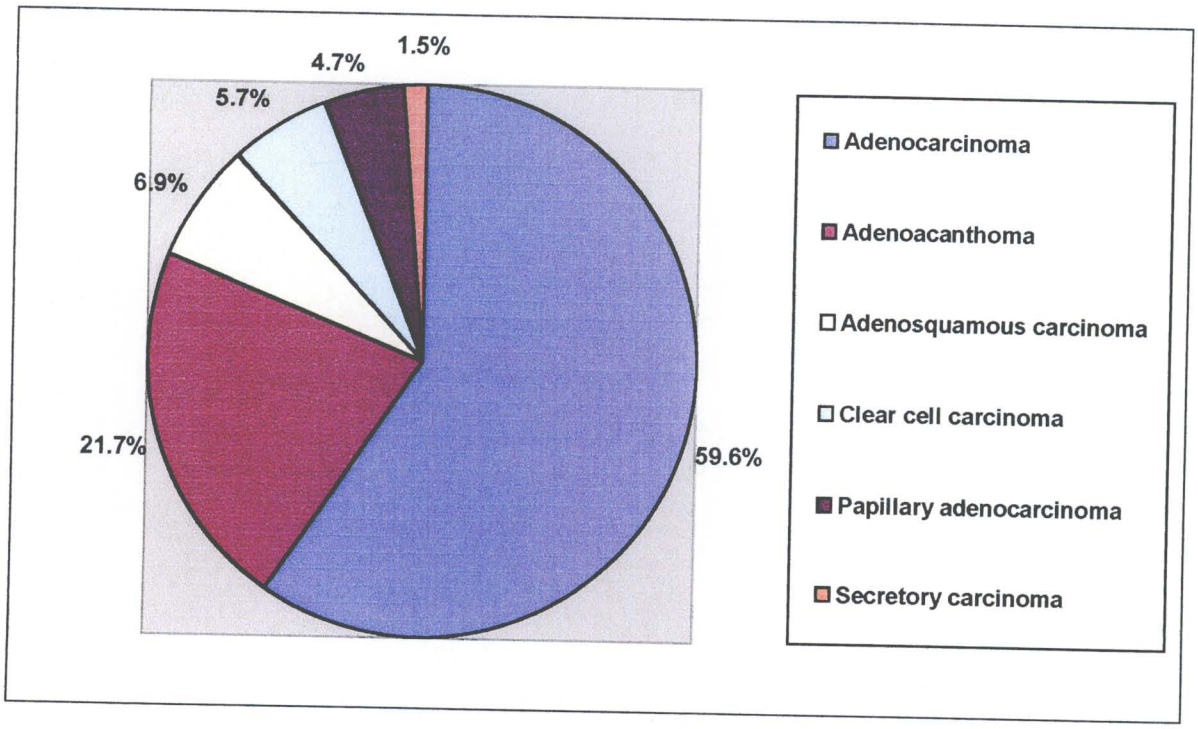


Figure 2.2: The frequency of the common Subtypes of Endometrial Carcinoma from an American population based study (adapted from Christopherson *et al*, 1982a)

The above pie graph (figure 2.2) is a reflection of the results obtained from a population based study of 989 uterine tumours occurring in residents of Louisville, Jefferson County, Kentucky, from 1953 to 1976 (Christopherson *et al*, 1982a).

2.2 HISTORY OF CARCINOMA OF THE ENDOMETRIUM

"A morsel of genuine history is a thing so rare as to be always valuable." - ***Thomas Jefferson***

The Smith Papyrus (plate 2.1) has the earliest description of cancer, which dates prior to 2000 B.C. and is regarded as the oldest nucleus of scientific medical knowledge. This is believed to have been written by the famous Egyptian physician, Imhotep, and lies in the possession of the New York Academy of Medicine.

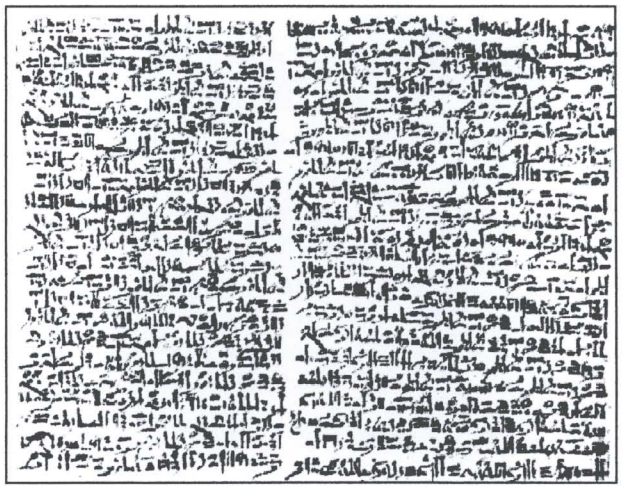


Plate 2.1: The Smith Papyrus, 2000 B.C. (Courtesy of New York Academy of Medicine Library). (Hajdu, 1979).

The Papyrus Ebers, which is housed in the Library of Leipzig, was written in 1552 B.C, a century before the exodus of the Israelites. It is indeed quite an ancient manuscript consisting of a collection of therapeutic suggestions, which was believed true at that time. One such belief was, "Cancer is a tumour which is not to be touched" and "if the tumour goes and comes under your finger, trembling even when your hand is still, it is a fatty tumour, and treat it with the knife," (plate 2.2).

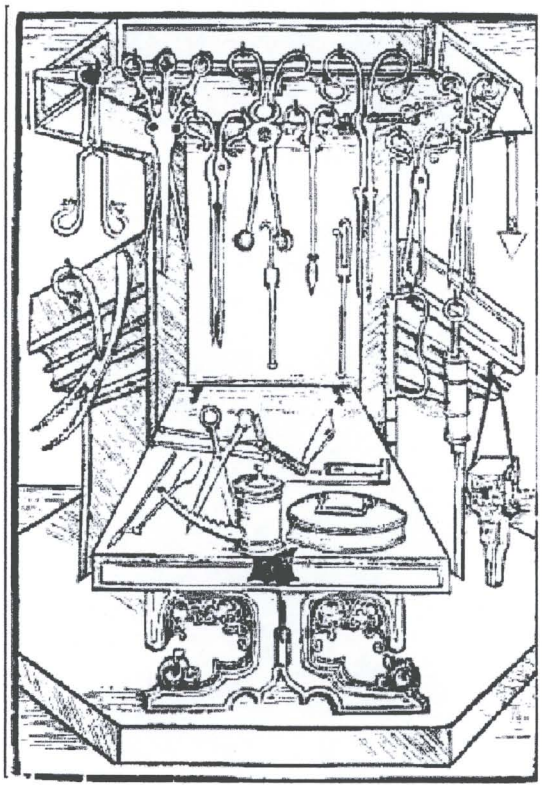


Plate 2.2: Surgical instruments used in operations during the 15th century. (Hajdu, 1979).

Although the Egyptians are regarded as the founders of medicine, they also included many non-neoplastic swellings in their description of tumours. The most fatal view of ancient physicians including that of Hippocrates, was that, "Cancer is incurable because it cannot be cured; if curable, it is not cancer". Hippocrates (460-375 B.C.) also advised never to operate on an ulcerating cancer (Hajdu, 1979).

Through the years carcinoma of the endometrium has fast become the most common invasive neoplasm of the female genital tract (Kurman *et al*, 1994), yet there was a time when reference to this malignancy was rare. This neoplasm has increased in incidence to such an extent that in the Western World it has replaced squamous carcinoma of the cervix as the commonest uterine cancer, (Robertson, 1981). Although reference to carcinoma of the cervix dates back to 1534 when

Aetius of Amida accurately described this tumour in his Tetrabiblion, early references to malignancies of the uterine corpus are rare (Sutton *et al*, 1990).

In 1792, John Leake, had already noted that in post and perimenopausal women, prolonged periods and passage of blood clots indicated a diseased uterus and often ended in cancer. Surgical removal of the diseased uterus was attempted, but as Ricci has written, "the 18th century closed without any serious effort on the part of the profession to deal with this problem" (Ricci, 1943).

However, it was only in 1900 that adenomatous hyperplasia of the endometrium was first described by TS Cullen (Cullen, 1900). Dr Thomas S Cullen, known as the doyen of gynaecologic pathology at Johns Hopkins Hospital, published his seminal work on uterine cancer over a century ago. He considered in detail only one of the variants of endometrial cancer, squamous cell carcinoma, which is one of the rarest subtypes. A short chapter was also devoted to what is most likely a mucinous adenocarcinoma of the uterus. However, Cullen thought it to be primary in the cervix because mucinous adenocarcinoma of the corpus was not a known entity at that time (Cullen, 1900; Young, 1996; Clement and Young, 2004).

There were relatively few advances of great significance for many years subsequent to Cullen's work. It was only in 1957 that clear cell carcinoma was recognised as an entity in the English speaking world by a series of publications beginning with that of Dr Saul Kay (Kay, 1957). The establishment of serous carcinoma of the corpus as a distinct clinicopathologic subtype appeared seventeen years later, when Factor described three cases of papillary adenocarcinoma of the endometrium with psammoma bodies. He, however, did not apply the designation "serous". There had already been previous descriptions of papillary endometrial cancers with psammoma bodies, but his paper was the first that made analogy with serous carcinoma (Factor, 1974). A few years later, in 1980, the influential book "Pathology of the Uterine Corpus, by Dr. Micheal R Hendrickson and Dr. Richard L Kempson of Stanford University was published (Hendrickson and Kempson, 1980b). 1994 was definitely a year of note in the world of endometrial cancer, when serous and clear cell carcinomas, as well as other rare subtypes were included in the classification of endometrial carcinomas by the World Health Organisation and the International Society of Gynaecologic Pathologists (Scully *et al*, 1994; Clement and Young, 2004).

Over the past decades knowledge of this tumour increased enormously, in line with an increased view of hormonal effects on uterine histology and of the effects of hormone therapy in its many forms (Robertson, 1981). The interrelationship between oestrogens and endometrial hyperplasia was described by Gusberg in the late 1940's (Gusberg, 1989). In 1956 Kistner *et al.* reported on the successful use of progestational agents in the treatment of endometrial cancer, and it was in 1970 that the International Federation of Gynaecologists and Obstetricians set out the original FIGO staging system for endometrial cancer, which was revised in 1988 (Kistner *et al*, 1956; FIGO, 1970). In 1975 oral contraceptives were withdrawn from the commercial market owing to reports being made of endometrial carcinoma occuring in young women using these (Lyon, 1975; Silverberg and Makowski, 1975).

There are other milestones in the study of endometrial carcinoma, that need mentioning, and these include, the use of progesterone receptors in endometrial carcinoma by Young and Ehrlich in 1976, the first studies of peritoneal cytology by Creasman and Rutledge and the establishment of papillary serous carcinoma of the uterus as a distinct clinicopathologic entity by Hendrikson *et al* (Ehrlich *et al*, 1981; Creasman and Rutledge, 1981; Hendrickson *et al*, 1982).

Well, as can be seen we have certainly come a long way with our knowledge of endometrial carcinoma. The molecular aspects of endometrial carcinoma, which once seemed to be just an unexplained drop in the ocean, has now opened into a forest of information, that seems to be growing everyday. There are many factors involved in this increased incidence of endometrial carcinoma and this incidence has increased the pressure put on the histopathologist by the gynaecologist, "is it malignant or not" (Robertson, 1981). One thing for sure that has been noted is that early diagnosis is the key to successful treatment of cancer of the endometrium (Barber, 1989).

2.3 EPIDEMIOLOGY AND PATHOGENESIS

2.3.1 EPIDEMIOLOGY

Endometrial carcinoma is the most prevalent primary malignant neoplasm in gynaecologic oncology (Sutton *et al*, 1990). The incidence of endometrial cancer varies widely throughout the world, being lower in Third World, developing countries in Africa, Asia, and South America, compared to the more industrialized countries in North America and Europe (Doll *et al,* 1970).

It is a disease primarily of the postmenopausal woman, usually occurring between the ages of 50 and 65 years, with the median age at diagnosis being 63 years (Ramzy, 1983; Platz and Benda, 1995). 75% of women with endometrial carcinoma are postmenopausal (Gallup and Stock, 1984). Only 2-5% of cases occur prior to the age of 40 years. However, there has been an increasing incidence in relatively young patients with one report indicating that up to 20% of these tumours occur in this age group (Ramzy, 1983). Also interesting to note is that the American Cancer Society reported a rise in the number of new cases during the 1960s and 1970s, reaching a peak of approximately 40,000 new cases (Table 2.1), but during the 1980s, this number slowly declined (Sutton *et al*, 1990).

Table 2.1: The number of new cases and deaths from endometrial cancer amongst the American population for the years 1970 and 1979 to 1984. (Adapted from Silverberg, 1989).

	1970	**1979**	**1980**	**1981**	**1982**	**1983**	**1984**
New cases	42,000	37,000	38,000	38,000	39,000	39,000	39,000
Deaths	3,500	3,300	3,200	3,100	3,000	3,000	2,900

This increase and decline may have been attributed to the use of exogenous oestrogen for hormonal replacement therapy. Other factors such as the prevalence of obesity and the aging of the American population may have also contributed to this trend (Sutton *et al,* 1990). As with many other malignant

neoplasms, there has also been a noteworthy racial difference in the mortality rates for white and black American women (Table 2.2), with the mean survival rate being higher in the white than in the black population (Sutton *et al*, 1990).

Table 2.2: The Endometrial Cancer Survival Rate by Race according to the American Cancer Society statistics. (Adapted from Silverberg, 1989).

	1960-63 (%)	1970-73 (%)	1973-80 (%)
Whites	73	81	88
Blacks	31	44	58

In a study conducted by Platz and Benda in 1995, they also found that the percentage of endometrial carcinoma in blacks (77,9%) is lower than in whites (92,3%), (Platz and Benda, 1995). In another study in which 279 patients with endometrial carcinoma treated at the University of New York Health Science Center and Kings County Hospital, Brooklyn, New York, during 1975 to 1990 were evaluated, the authors found endometrial carcinoma to be more frequent in older black patients (Aziz *et al*, 1996)

2.3.2 PATHOGENESIS

Many factors play a role in the pathogenesis of this disease and they are as follows:

2.3.2.1 Hormonal Stimulation

Reports have shown that excessive oestrogen is associated with most of the risk factors linked to endometrial carcinoma (Dubeau, 1993). Continued stimulation of the endometrium produced by excessive oestrogen can result in endometrial hyperplasia. Women with hyperplasia without atypical cytological findings, have a low risk of uterine cancer. On the other hand, women who have hyperplasia with atypical cytological features have a 23% risk of endometrial carcinoma over the next decade (Kurman *et al*, 1985).

2.3.2.2 Exogenous Oestrogen

Unopposed exogenous oestrogen administered for the treatment of menopausal symptoms, became popular in the late 1960s and 1970s, and was recognized as a pathogenetic factor in endometrial carcinoma (Rose, 1996). When this exogenous oestrogen became available for hormone replacement therapy it was noticed that the incidence of endometrial carcinoma appeared to be rising in women taking this medication (Longacre *et al*, 1995), and was associated with an eightfold increase in incidence (Mack *et al*, 1976; Food and drug administration, 1976). It is generally agreed that oestrogenic stimulation is a growth factor for endometrial glandular epithelium (ie. without the normal counteracting influences of progesterone). Therefore, oestrogenic stimulation resulting in development of endometrial cancer was inevitable (Longacre *et al*, 1995). Since the practice of prescribing unopposed oestrogen has been discontinued and combined oestrogen-progesterone preparations have been adopted, the incidence of endometrial carcinoma has decreased. However, endometrial cancers continue to occur in women treated with combined hormone-replacement regimens that include less than the recommended 12 days of progesterone monthly (McGonigle *et al*, 1994). There has also been a worldwide increase in incidence of endometrial cancer, even in countries where unopposed oestrogen is not prescribed. This increase has been attributed in part to increasing longevity in women (Rose, 1996).

2.3.2.3 Endogenous Oestrogen

Endogenous oestrogen was suspected to play an aetiological role in the development of endometrial carcinoma (Figure 2.3). This suspicion was due to the well-documented increase in incidence of endometrial adenocarcinoma in women who are subjected to prolonged or increased oestrogenic stimulation as a result of anovulatory cycles, oestrogen-producing neoplasms, and polycystic ovaries. The Stein-Leventhal syndrome is an example of such a problem where the patient has secondary amenorrhoea, sterility, bilateral polycystic ovaries and hirsutism occurring in the second or third decades of life (Gusberg, 1989; Wood and Boronow, 1976; Gusberg, 1980). Most investigators agree that there is sufficient evidence to support an association between oestrogen stimulation, hyperplasia and carcinoma, even though many of these studies have been

criticized on a variety of methodological grounds (Gordon *et al*, 1977; Smith *et al*, 1981; Henderson, 1989; Gurpide, 1991).

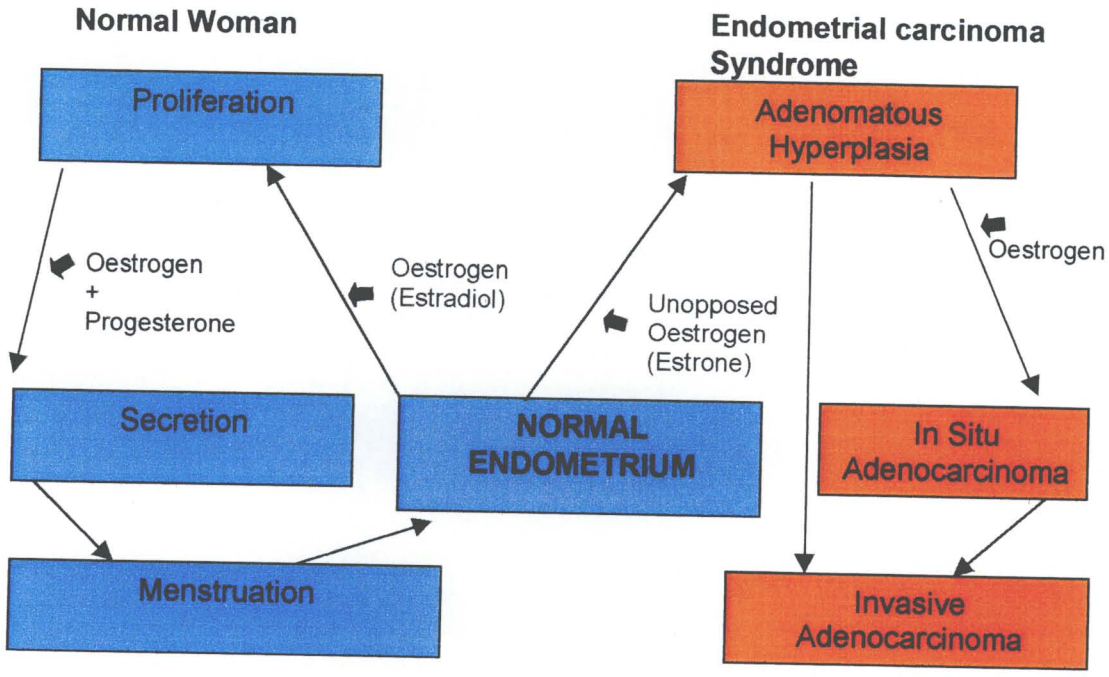


Figure 2.3: Endometrial proliferation in normal and in carcinoma patients- The role of oestrogen . (Adapted from Ramzy, 1983).

2.3.2.4 Obesity

Obesity is another well-known risk factor for the development of endometrial carcinoma. The development of cancer in obese women is believed to be mediated by endogenous oestrogen, through the peripheral conversion of androstenedione to estrone and oestradiol by aromatase in adipose tissue (Longacre *et al*, 1995; Judd *et al*, 1982). This, together with the observation that obese women have decreased levels of serum sex hormone binding globulin, is believed to be the cause for the increased incidence of endometrial carcinoma in overweight women (Siiteri, 1978; Mahboubi *et al*, 1982). This risk is particularly heightened in young women (Henderson *et al*, 1983; LaVecchia *et al*, 1984). The majority of young women with endometrial carcinoma are obese or have high levels of unopposed endogenous oestrogen because they have chronic anovulation, as seen with polycystic ovarian disease (Rose, 1996).

2.3.2.5 Diet / Environmental factors

Many have scrutinized the role of diet in causing this disease, since the worldwide incidence of endometrial carcinoma correlates with estimates of per capita fat consumption (Armstrong and Doll, 1975). It has been noted that Japanese immigrants to the United States have an incidence of endometrial carcinoma twice as high as that of Japanese woman who did not emigrate (Hoenszel and Kurihara, 1968). This suggests that factors such as the high content of animal fat in the Western diet may be an aetiological factor in the development of endometrial carcinoma. Total energy intake and obesity are closely related. There is some evidence that the intake of animal proteins and fat increases the risk of endometrial carcinoma whereas fresh fruit, vegetables and fibres decrease this risk, but the exact role of diet is not clear (Gusberg, 1980; Levi *et al*, 1993).

Also interesting is that cigarette smoking decreases the risk of endometrial cancer by inactivating oestrogen through hydroxylation at the 2-alpha position (Lesko *et al*, 1985; Sutton *et al*, 1990).

2.3.2.6 Nulliparity

Although serum oestrogen and progesterone concentrations increase during pregnancy, progesterone is the predominant hormone of pregnancy. Pregnancy therefore, confers protection from endometrial carcinoma by interrupting the continued stimulation of the endometrium by oestrogen. Nulliparity is thus a risk factor for endometrial carcinoma.

2.3.2.7 Tamoxifen

Tamoxifen is a synthetic anti-oestrogen (oestrogen antagonist) that is widely employed as an adjuvant therapy for breast cancer. In addition, tamoxifen has also been shown to have oestrogenic (agonist) effects on the endometrium and to increase the risk of endometrial carcinoma. This association between tamoxifen and endometrial cancer was first identified in 1985, when endometrial carcinoma developed in three women with breast cancer after only 7 to 14 months of therapy (Killackey *et al,* 1985). This effect of tamoxifen is related to the duration of treatment (De Muylder *et al*, 1991; Seoud *et al*, 1993; Silva *et al*, 1994; Longacre *et al*, 1995). Endometrial monitoring is therefore recommended for patients being

treated with this agent (Gusberg, 1980). A history of long-term tamoxifen treatment is sometimes present and several recent studies have noted an increase in frequency of endometrial carcinoma (Geisler *et al*, 2001; Elit *et al*, 2002).

2.3.2.8 Genetic Aspects

In support of a genetic predisposition several studies have shown a high incidence of both endometrial carcinoma and breast cancer among sisters, mothers and aunts of individuals with endometrial carcinoma (Lynch *et al*, 1967; Musubuchi and Nemoto, 1972; Way, 1954). It has also been recently proposed that there are two forms of heritable endometrial carcinoma:

- A predisposition for endometrial carcinoma only
- A cancer family syndrome (Lynch syndrome II) (Boltenberg *et al*, 1990; Sandles *et al,* 1992).

Endometrial carcinoma is known to be the most common extracolonic cancer in women with the HNPCC syndrome. This syndrome has also been associated with breast and ovarian cancer as well. Endometrial cancer occurs in 4 to 11 % of women in families affected by this syndrome. These women are at a median age of 46 years, which is 15 to 20 years younger than the median age at diagnosis in the general population (Mecklin and Jarvinen, 1991; Watson and Lynch, 1993).

2.3.3 PRECURSORS OF ENDOMETRIAL CARCINOMA

The precursor of endometrial carcinoma has long been noted to be endometrial hyperplasia (Kurman *et al*, 1985). However, hyperplasia without atypia is a manifestation of unopposed oestrogenic stimulation and by itself is not a precursor of endometrial carcinoma. Long term follow up studies by Kurman *et al.* (1985) concluded that only 2% of hyperplasias without atypia progress to carcinoma. This contrasts to the nearly 25% of atypical hyperplasias, which progress to endometrial carcinoma, indicating that this lesion is a precursor of endometrioid carcinoma. Also, no serous carcinomas were found in this study, suggesting that atypical hyperplasia is a precursor of type 1 oestrogen-related carcinoma only (Kurman *et al*, 1985). However, a large cytological screening

study has also indicated that not all endometrioid carcinomas are preceded by atypical hyperplasia (Koss *et al*, 1984). An additional interest was the finding of a lesion called endometrial intraepithelial carcinoma (EIC), also, variously referred to as "uterine surface carcinoma", "noninvasive serous carcinoma", or "endometrial carcinoma in situ". However, none of these terms seem to be entirely satisfactory. Endometrial intraepithelial carcinoma is characterized by replacement of the endometrial surface and glandular epithelium by one or several layers of high-grade malignant cells similar to those of invasive serous carcinoma (Wheeler *et al*, 2000; Ambros *et al*, 1995; Bacgren *et al*, 2001; Clement and Young, 2004). This lesion was found in the adjacent endometrium of nearly 90% of serous carcinomas, but in only 6% of the endometrioid carcinomas, suggesting that intraepithelial carcinoma may be the precursor of serous carcinoma (Kurman *et al*, 1994). It can be associated with high-stage disease and a fatal outcome. This is probably a result of transtubal spread of tumour cells from the endometrial cavity (Wheeler *et al*, 2000; Bacgren *et al*, 2001).

2.3.4 PATHOGENETIC TYPES OF ENDOMETRIAL CARCINOMA

The differences (Table 2.3) noted in the epidemiology, presentation, behaviour, and the molecular pathologic events of endometrial carcinoma suggest that there may be two different forms of the disease (Kurman *et al*, 1994). In 1983 Bockman first described the 2 main clinicopathologic types of endometrial carcinoma (Bockman 1983):

- Type I: oestrogen-related, low-grade endometrioid endometrial carcinomas (EEC) that usually develop in pre- and perimenopausal women. They coexist with, or are preceded by complex and atypical endometrial hyperplasia.
- Type II: nonendometrioid carcinomas (NEEC), mainly papillary, serous and clear cell carcinoma, largely occurring in older women. They are aggressive tumours, unrelated to oestrogen stimulation and occasionally arise in endometrial polyps or from precancerous lesions that develop in

atrophic endometrium (so-called endometrial "intraepithelial" carcinoma) (Bockman, 1983).

Bockman found that type I endometrial carcinoma arose in women with obesity, hyperlipidemia, and signs of hyperestrogenism: anovulatory uterine bleeding, infertility, late onset of the menopause, and hyperplasia of the stroma of the ovaries and endometrium. The frequency of this type was found to be 65%. Type II arose in women who had none of the above signs, or the signs was not clearly defined. The frequency of this type was only 35% (Bockman, 1983). Several other researchers have confirmed the existence of this proposed dualistic model of endometrial carcinogenesis (Sherman *et al*, 1995; Ambros *et al*, 1995, Tashiro *et al*, 1997a, Santin, 2003).

Table 2.3: The major differences between the two pathogenetic types of endometrial carcinoma (Adapted from Kurman *et al*, 1994):

	TYPE I (EEC)	**TYPE II (NEEC)**
Unopposed oestrogen	Present	Absent
Menopausal status	Pre & Perimenopausal	Postmenopausal
Precursor	Hyperplasia	Intraepithelial neoplasia
Race	White	Black
Grade	Low	High
Myometrial invasion	Minimal	Deep
Specific subtypes	Endometrioid	Serous, Clear cell
Behaviour	Stable	Progressive

Recently it has also been shown that the molecular alterations involved in the development of EEC (type I) carcinomas differ from those of NEEC (type II) carcinomas. Matias-Guiu and colleagues found that four different genetic abnormalities may occur in endometrioid adenocarcinomas (microsatellite instability and mutations in the *Pten, k-Ras* and *β-catenin* genes), whereas non-endometrioid carcinomas of the endometrium often had *p53* mutations and loss of heterozygosity on several chromosomes (Matias-Guiu *et al*, 2001). However, occasionally the authors found that a nonendometrioid carcinoma may develop

as a result of dedifferentiation of a preexisting endometrioid carcinoma. In such a case the tumour exhibited overlapping clinical, morphologic, immunohistochemical and molecular features of the 2 types (Matias-Guiu *et al*, 2001). The molecular pathway of endometrial carcinoma, has been researched by many others, who further confirm this dualistic model to endometrial carcinogenesis (Caduff *et al*, 1996; Tritz *et al*, 1997; Tashiro *et al*, 1997a, Swisher *et al*, 1999, Lax *et al*, 2000).

This dual model of endometrial carcinogenesis is now well established (McCluggage, 2004). We have type 1 carcinoma, of which endometrioid adenocarcinoma is the prototype. To contrast with this we have type 2 carcinoma, of which uterine serous carcinoma, also known as papillary serous carcinoma is the prototype (McCluggage, 2004).

Non-endometrioid carcinomas now account for only 10% of endometrial carcinomas (Clement and Young, 2004). To date subtypes such as mucinous, squamous, transitional, undifferentiated, mixed, and poorly differentiated carcinomas with unusual forms of differentiation also fall under this category. These tumours pose a great problem in differential diagnosis, not only their distinction from benign lesions, but also endometrioid carcinoma and various tumours that may secondarily involve the uterine corpus. It is of great importance that a clear distinction is made between type 1 and type 2 carcinomas since management as well as the prognosis of these tumours differ (McCluggage, 2004).

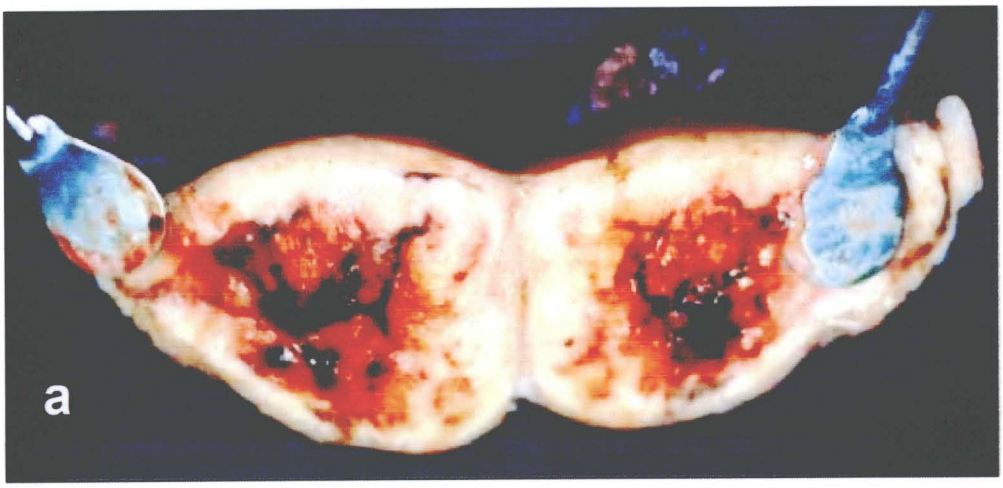
2.3.5 SIGNS AND SYMPTOMS

The most common presenting symptom of endometrial carcinoma is vaginal bleeding. This is most commonly seen as postmenopausal bleeding, however excessive menstrual bleeding and intermenstrual bleeding may also be reported. Most endometrial cancer patients present within 3 months of the first episode of abnormal bleeding, but a small subset has bleeding for greater than 1 year. The Pap smear alone is not considered a reliable screening test for carcinoma of the endometrium due to the high false negative rate. A Pap smear that reveals cells compatible with adenocarcinoma, must be further investigated. Fractional

dilatation and curettage (D&C) should follow failure to identify any source on the vagina or cervix. Some patients present with complaints of pressure, or heaviness in the pelvis, change in bowel or bladder habits, pelvic pain, or vaginal discharge. Rarely, the finding of a mass or cervical lesion on a routine exam may be the first evidence of the disease (Sutton, 1990).

2.3.6 MACROSCOPIC APPEARANCE OF ENDOMETRIAL CARCINOMA

The gross appearance of the different subtypes of endometrial carcinoma is similar. However, there is a possible exception with serous papillary carcinomas (Kurman *et al*, 1994). The surface of endometrial carcinomas is usually shaggy, glistening, and tan (Plate 2.3a). They may also be focally haemorrhagic (Plate 2.3b). Endometrioid carcinomas are almost uniformly exophytic. The neoplasm may be focal or diffuse. Sometimes the tumour may appear to be composed of separate polypoid masses. Necrosis is usually evident macroscopically in poorly differentiated tumours. Invasion of the myometrium by the carcinoma may result in enlargement of the uterus, but a small atrophic uterus may have carcinoma diffusely invading the myometrium. The appearance of myometrial invasion is seen as well-demarcated, firm gray-white tissue with linear extensions beneath an exophytic mass or as multiple, white nodules with yellow areas of necrosis within the uterine wall. On the other hand serous papillary carcinomas are often small and atrophic. The tumour is generally exophytic but has a papillary appearance. The depth of invasion is difficult to assess macroscopically. These tumours frequently develop within a polyp (Kurman *et al*, 1994). Plate 2.4 illustrates a serous papillary tumour.



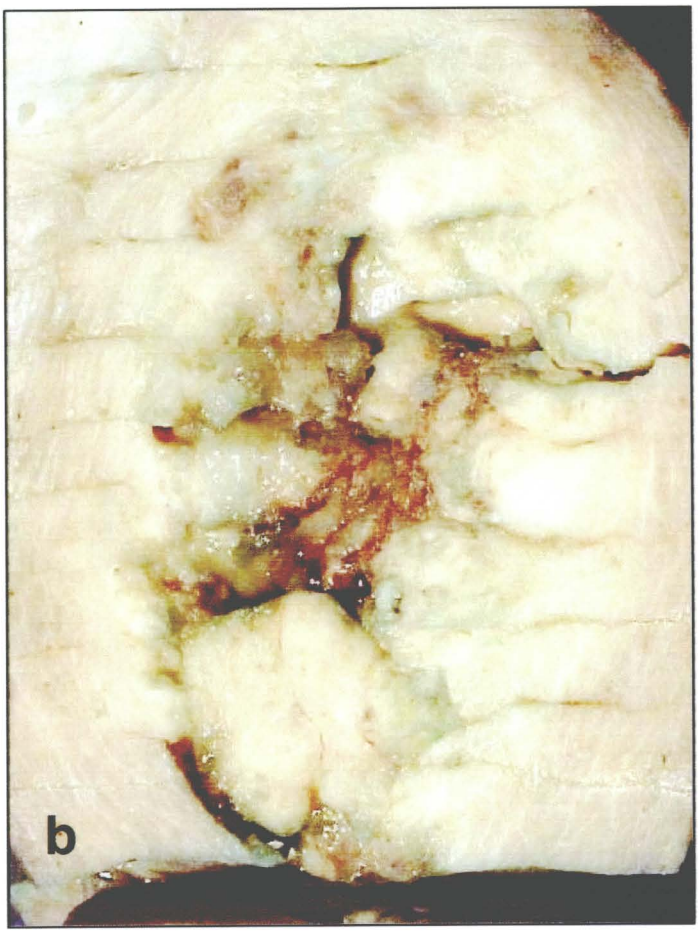


Plate 2.3: Gross appearance of Endometrioid Adenocarcinomas; **a** showing the tumour diffusely invading the endometrial cavity and is also sometimes haemorrhagic; **b** the endometrial surface can be shaggy, glistening and tan.

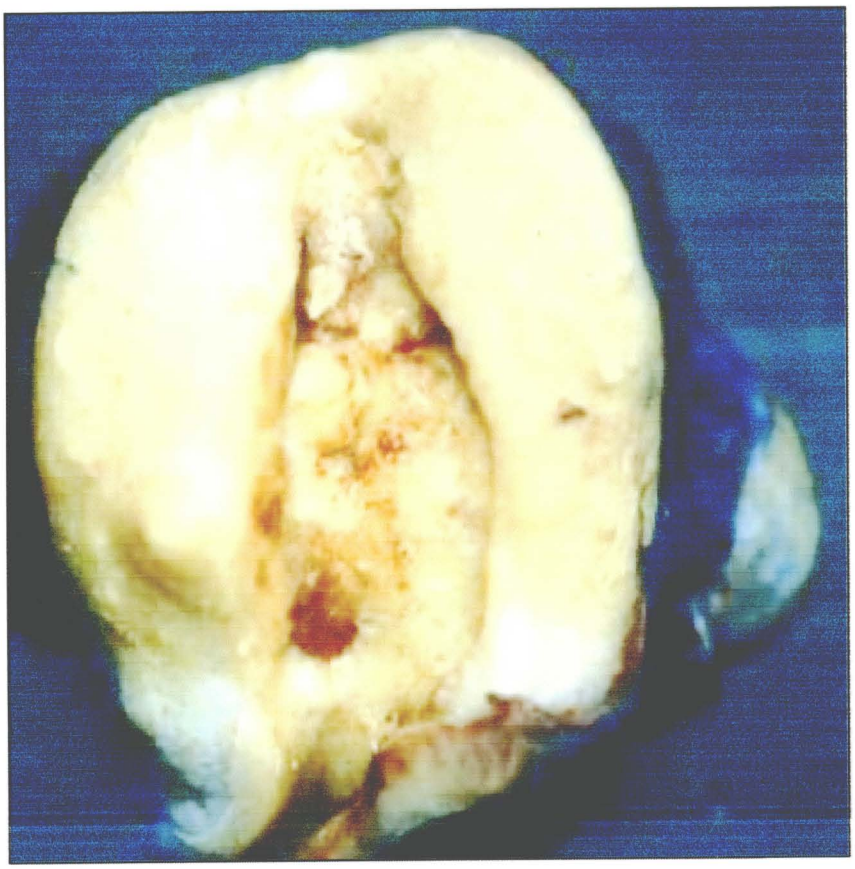


Plate 2.4: Macroscopic appearance of a typical Serous Papillary Carcinoma. The tumour is exophytic and has a papillary appearance.

2.4 PROGNOSTIC FACTORS

It is the pretreatment evaluation of patients with malignant neoplasms together with clinicopathological experience that allows the physician to establish therapy for the best results. Endometrial carcinoma is a common gynaecological malignancy usually associated with a favourable prognosis (Frigerio *et al*, 1999), having a relative 5-year survival rate of 84% (Jemal *et al*, 2005). There are several factors that have been identified for endometrial carcinoma which have significant predictive value and these have been extensively studied so that treatment and follow-up of patients could be improved (Salvesen and Akslen, 2002). The other factors that are also important for determining patient outcome are factors relative to host resistance and modality of therapy. Nolan and Huen (1976) on the basis of clinical experience developed a mathematical model for evaluating prognosis. With this model, prognosis is directly proportional to host resistance factors, inversely proportional to tumour virulence factors, and enhanced by treatment factors.

$$\text{Prognosis} \propto \frac{\text{Resistance}}{\text{Virulence}} \times \text{Treatment}$$

Resistance factors are age and general constitutional condition, and the virulence factors are stage of disease, grade of tumour, and tumour volume (Creasman *et al*, 1992 ; Nolan and Huen, 1976).

To identify women with stage 1 disease who have an increased risk of developing recurrence, is one of the most difficult problems in clinical management (Kurman *et al*, 1994). It is the identification of these high-risk groups that would make it possible to avoid over-treatment of patients with a good prognosis, whilst reserving the most extensive therapy for those with aggressive tumours (Salvesen *et al*, 1998). To date, there are multiple factors that have been identified to have prognostic significance (Frigerio *et al*, 1999). The four principal prognostic variables are depth of myometrial invasion, lymphovascular space invasion, the macroscopic diameter of the tumour or tumour volume, and histological grade. The others are significant but less dominant variables (Southcott *et al*, 2001).

The prognostic factors for endometrial carcinoma can be divided into uterine and extrauterine factors (Table 2.4):

Table 2.4: Prognostic factors of endometrial cancer

Uterine Factors	Extrauterine Factors
• Histological type	• Adnexal involvement
• Grade	• Intraperitoneal metastasis
• Depth of myometrial invasion	• Positive peritoneal cytology
• Cervical involvement	• Pelvic & para-aortic LN metastasis
• Vascular invasion	
• Presence of atypical hyperplasia	
• Progesterone receptor status	
• DNA ploidy & S-phase fraction	
• FIGO Stage	

The patients that have no evidence of extrauterine disease, no cervical involvement, and no evidence of vascular invasion are at a low overall risk of recurrence. It is the grade and depth of invasion that are important prognostic factors for these patients. Women with evidence of extrauterine disease, cervical involvement and vascular invasion constitute a high-risk group. According to a Gynaecologic Oncology Group (GOG) study (Morrow *et al*, 1991), the frequency of recurrence was 20% if one of these 3 factors was positive, 43% for two positive factors, and 63% for three factors. In addition to the above-mentioned pathological risk factors, there are also clinical risk factors that exist, such as, age, weight and race (Kurman *et al*, 1994). There have been many attempts by researchers to identify the most powerful prognostic factors for endometrial carcinoma patients (Salvesen *et al*, 1998). A discussion of these follow:

2.4.1 HISTOLOGICAL TYPE

It is the serous papillary and clear cell adenocarcinomas that are known to be the most aggressive subtypes compared to the other morphologic variants of endometrial carcinoma. Hendrickson *et al.* (1982) in their study of 256 cases

showed that both these histologic subtypes had a poor prognosis. The serous papillary type has a definite tendency for upper abdominal spread, relating to retroperitoneal lymph node metastasis, which differs from the path of spread pattern of the other adenocarcinomas, and therefore requires a different therapeutic approach. Adenosquamous carcinoma also has a bad prognosis. However, this is probably due to the fact that these tumours are usually poorly differentiated. This is in contrast to the excellent prognosis associated with grade 1 endometrioid adenocarcinoma, adenoacanthoma, and secretory carcinoma, and this is attributed to the fact that these are well-differentiated tumours (Rosai *et al*, 1996; Sutton *et al*, 1990). Berman *et al.* (1982) found that the 5 year survival for patients with adenoacanthoma was 88.4% and for those with endometrioid adenocarcinoma 60.3%. Serous papillary adenocarcinoma and clear cell adenocarcinoma are associated with a high relapse rate (Hendrickson *et al*, 1982) and poor survival. However, more recent studies have shown survival rates of up to 70 to 100% for stage 1 serous papillary carcinomas, and 90% survival for stage 1 clear cell carcinomas. This is possibly due to more meticulous staging and or aggressive adjuvant treatment (Lee and Belinson, 1992; Lim *et* al, 2001; Abeler and Kjorstad, 1991). It was unclear whether the prognostic significance of these tumour cell types is independent of retroperitoneal lymph node metastasis and other histopathological prognostic factors. Sakuragi *et al.* (2000) therefore set out to investigate 240 patients with clinical stage 1-3 endometrial carcinoma, to determine the prognostic significance of various histopathological factors, using Cox regression analysis. By this time Greven *et al.* (1993) had already found that patients with clear cell or serous papillary histologic types, a high tumour grade, deep myometrial penetration, or involvement of two or more extrauterine sites were at a high risk for distant recurrence. This result suggested that the extent of the tumour was an important factor for survival of patients with serous papillary adenocarcinoma and clear cell adenocarcinoma. However, the study of Sakuragi and co-workers concluded that the prognosis of patients with endometrial carcinoma was dependent on cell type, and that grade, lymph-vascular space invasion and para-aortic node metastasis were equally notable prognostic factors. More importantly, they also showed that the poor prognosis for patients with serous papillary adenocarcinoma and clear cell adenocarcinoma is independent of lymph node metastasis and other

histopathologic prognostic factors. They suggested that serous papillary adenocarcinoma and clear cell adenocarcinoma should be strictly discriminated from endometrioid adenocarcinoma. The report of Sakuragi et *al.* (2000) was unique as it was based on prospective clinical practices with complete surgical staging of endometrial carcinoma, including systematic pelvic and para-aortic lymph node dissection. Hendrickson and co-workers concluded that special variants of endometrial carcinoma were important to identify, both for prognostic and differential diagnostic purposes (Hendrickson *et al,* 1982).

2.4.2 HISTOLOGIC GRADE

Histologic grading has long been accepted as one of the most sensitive prognostic indicators for endometrial carcinoma, and correlates with outcome (Sorbe *et al*, 1997). The poorer grade tumours are more advanced and are more likely to recur (Creasman *et al*, 1992; Sutton *et al*, 1990). Tumour grade alone is an important predictor of survival. According to a population based study of approximately 2000 patients, from the Norwegian Radium Hospital, the 5 year survival rate for women with grade 1 tumours was 87%, 75% for those with grade 2 tumours and 58% for those with grade 3 tumours (Rose, 1996). Berman and co-workers found in their study that the 5-year survival in grade 1 was 79.3% as compared with 58% in grades 2 and 3 combined. Thus proving that the survival rate for patients with grade 1 tumours was significantly better (Berman *et al*, 1982). This prognostic indicator is also correlated with other factors of prognosis like depth of myometrial invasion and the frequency of lymph node metastasis (Kurman *et al*, 1994).

Except for serous papillary and clear cell adenocarcinomas, the microscopic grade of differentiation for endometrial carcinoma, as defined by the FIGO system (Table 3.1 see page 141) is based primarily on architectural rather than nuclear features (Rosai *et al*, 1996). Nuclear grading takes precedence over histologic differentiation. Some workers also have suggested that nuclear grade is a superior predictor compared to architectural grade (Mittal *et al*, 1988; Sutton *et al,* 1990).

The following Table 2.5 represents a simple grading system adopted by FIGO for the grading of endometrial adenocarcinomas.

Table 2.5 Histologic Grades of Endometrial Adenocarcinomas (adopted by FIGO) (Ramzy, 1983)

GRADE 1	Glandular or papillary pattern. Invasion of stroma but myometrium usually clear.
GRADE 2	Glandular or papillary pattern mixed with solid cores.
GRADE 3	Tumour formed entirely of solid sheets. No attempt at glandular or papillary formation. Atypical mitosis and bizarre cells are common. Myometrial invasion is common.

Grade 1 has a low incidence of myometrial invasion and lymph node metastasis. Bilateral salpingo-oophorectomy and transabdominal hysterectomy is usually adequate treatment. Grades 2 and 3 require irradiation, which is done either preoperatively or postoperatively.

The WHO Histopathological Classification and the FIGO Staging System of uterine carcinoma recommend that both architectural and nuclear criteria be used to grade tumours. This system is more popular than the simple one above.

Although they are used most frequently, reproducibility problems of histologic grading pose an obstacle. Therefore, more objective criteria for both architectural and nuclear grading systems have been introduced through quantitative nuclear

morphometric methods, which will be discussed later on (Selvasen *et al*, 1998). However some still suggest that histological grade be regarded as the "best" prognostic factor, in part by virtue of many years experience and consistency of results. Also the added advantage is that grade can be determined before hysterectomy (Sutton *et al*, 1990).

2.4.3 MYOMETRIAL INVASION

This prognostic factor is known to be a consistent indicator of tumour aggressiveness and many have found it to be a significant predictor of outcome. Watanabe *et al.* (2001) found this to be very useful when they used it to compare uterine isthmic endometrial carcinoma to endometrial carcinoma of the uterine corpus. However, myometrial invasion can only be assessed by examination once the uterus has been removed. It is associated with other poor prognostic factors like poor grade and lymph node involvement. When the tumour invades deeply into the myometrium, there is an increased risk of infiltration of lymphatic vessels, which further adds risk of relapse of the disease. The well-differentiated tumours tend not to invade the myometrium deeply, while the poorly differentiated tumours are known to invade deeply (Creasman *et al*, 1992; Sutton *et al*, 1990). Depth of myometrial invasion is expressed as extension into inner or outer halves. The tumours that are restricted to the endometrium are stage IA, those that involve the inner half of the myometrium are stage IB, and those involving more than half the uterine wall thickness are stage IC (FIGO staging system, Longacre *et al*, 1995). The maximum depth of invasion is measured in millimeters and expressed as a percentage. In a recent study, Cunha *et al.* (2001) compared 2 methods used to measure depth of myometrial invasion. The 2 methods were, magnetic resonance imaging, which is a preoperative, pelvic imaging technique, and gross visual inspection, which is an intraoperative procedure and entails frozen section examination. Magnetic resonance imaging proved to be a reliable, useful method that could replace gross visual inspection, but due to the high accuracy of both, these researchers suggested that both could be used interchangeably (Cunha *et al*, 2001). In endometrial carcinoma the myometrium can be invaded in different forms. It can invade along a broad pushing front, or the myometrium could be diffusely infiltrated with varying size

masses, cords, or in clusters of cells and individual glands. Some authors suggest myometrial invasion to be the single most important predictor of behaviour in stage I and II disease, but there is much controversy regarding this. However, this indicator is still an important predictor of prognosis, independent of tumour grade (Kurman *et al*, 1994). Ambros and colleagues found that in their analysis of 102 cases, myometrial invasion provided one of the most reliable models for predicting outcome (Ambros *et al*, 1992).

2.4.4 UTERINE CERVIX INVOLVEMENT

Cervical involvement has been incorporated into the FIGO staging system and is associated with a worse prognosis. The tumours that are restricted to the uterus but involve the cervix are stage II. If the tumour is confined to the surface epithelium or glands, they are staged as IIA, and if the tumour invades the adjacent stroma, they are staged as IIB. A study conducted by Berman and co-workers. consisted of 97 patients with stage II endometrial carcinoma (Berman *et al,* 1982). Cervical extension reported on cervical punch biopsies was present in 18 patients, and the remaining 79 had occult extension found on endocervical curettage. The 5-year survival was 31.1% in the former group compared with 74.3% in the latter group (Berman *et al*, 1982). This prognostic factor is associated with other indicators of prognosis such as increasing grade, depth of invasion, and tumour volume. It is therefore also associated with a high risk of recurrence (Rosai *et al*, 1996; Kurman *et al*, 1994). Morrow and colleagues confirmed this with their Gynaecologic Oncology Group study consisting of 895 endometrial carcinoma patients (Morrow *et al*, 1991). They found that tumours involving the cervix were clearly unfavourable, and reflected the grade, invasiveness, and volume of the tumour. They also found that these cases had a higher rate of recurrence (Morrow *et al*, 1991). Magnetic resonance imaging is a reliable method to also detect cervical involvement in endometrial carcinoma (Cunha *et al*, 2001).

2.4.5 VASCULAR INVASION

Lymph vessel invasion of the tumour is a poor prognostic sign (Rosai *et al*, 1996). Vascular invasion is an important predictor of recurrence and its presence should therefore be recorded in the surgical pathology report. It is usually associated with high-grade tumours and deep myometrial invasion. The presence of perivascular lymphocytic infiltrates in the myometrium is associated with and biologically related to vascular invasion. Therefore, the presence of unequivocal vascular invasion or perivascular lymphocytic infiltrates alone is termed vascular invasion-associated changes. It is believed that deep myometrial invasion and high tumour grade are likely to increase the probability of vascular invasion and eventual metastasis (Kurman *et al*, 1994). Vascular invasion is thought to be the mechanism by which distal spread and occult metastasis occurs. Ambros *et al.* (1992) who investigated 102 cases of stage 1 endometrioid carcinoma found that survival was closely correlated with age, grade and myometrial invasion, but that vascular invasion and the presence of perivascular lymphocytic infiltrates were the best indicators of prognosis. The perivascular infiltrates may be a reflection of an immune response to tumour cells invading vascular walls (Ambros *et al*, 1992). In another study of 111 patients with stage 1 endometrial carcinoma, Hanson *et al.* (1985) noted lymph vascular space invasion in 16 cases, which were poorly differentiated tumours with deep myometrial invasion. 44% of these patients with lymph vascular space invasion experienced recurrence of tumour, and 6.3% of patients developed extra-pelvic metastasis. They concluded that lymph vascular space invasion by tumour cells is an important prognostic indicator in stage 1 endometrial cancer, which could be considered in treatment planning (Hanson *et al*, 1985).

2.4.6 ENDOMETRIAL HYPERPLASIA AND METAPLASIA

The presence of endometrial hyperplasia and metaplasia, especially ciliated cell and eosinophilic changes are important in recognising patients with a favourable prognosis, and correlates with low tumour grade and absence of myometrial invasion. This is in contrast to high-grade tumours, which are associated more with an atrophic endometrium (Kurman *et al*, 1994).

2.4.7 OESTROGEN AND PROGESTERONE RECEPTORS

Endogenous or exogenous oestrogen is believed to be involved in the aetiology of endometrial carcinoma, and the effects of oestrogen on normal cells, is modulated by other hormones like progesterone. However, if these modulating effects are absent during longer periods of oestrogenic stimulation, disruption of normal cell function may occur (Nyholm, 1996). The tumours that are related to and probably resulting from chronic oestrogenic stimulation are known to have a better prognosis than those that do not. This group includes most tumours in young patients (less than 40 years), those tumours associated with functioning ovarian cancers, the Stein-Leventhal syndrome and exogenous oestrogen stimulation (Rosai *et al*, 1996). A study conducted by Kennedy *et al.* (1982) revealed that women who developed cancer following long-term use of exogenous oestrogen had more localised and better differentiated, less advanced, more localised tumours, without myometrial invasion than patients who had not used oestrogen. This suggested that perimenopausal long-term oestrogen users might develop less aggressive neoplasms. Also, oestrogen use was found to be more common among younger women. They concluded that these patients had an excellent prognosis after conventional therapy, and several other authors noted survival rates of more than 90% (Kennedy *et al*, 1982). Billiet *et al.* (1982) also found high oestrogen receptor (ER) levels in differentiated tumours and that these levels were dependent on the state of tumour differentiation and on the stage of oestrogenic impregnation of the subject. They also noted that the measurement of progesterone receptors (PR) was a sensitive parameter of hormonal dependency (Billiet *et al*, 1982). Researchers have found that progesterone receptor status not only adds useful information in predicting those at risk for recurrence but also in designing the most appropriate therapeutic measures in the event of advanced or recurrent disease (Sutton *et al*, 1990). The amount of ER and PR levels detected in endometrial carcinoma varies, but is lower than the amount found in normal cycling endometrium. Konski and co-workers found that grade 1 tumours were more ER and PR rich than grade 3 tumours (Konski *et al*, 1996), but the PR status seems to be a more important risk factor compared to ER status. Many studies have found that positive PR correlates with endometrioid carcinoma. Also PR is associated with low tumour grade, young patient age, low recurrence rate and greater survival. This is in

contrast to positive ER status, which is generally associated with low recurrence rate, but not survival, grade or histologic type. Neither of these receptors correlates with stage, myometrial invasion, peritoneal cytology or retroperitoneal lymph node metastasis (Kurman *et el*, 1994). In a study of 50 patients Konski and colleagues found that ER and PR status were the more objective prognostic factors than nuclear grade, depth of myometrial invasion and histological subtype (Konski et al, 1996).

Also, some investigations have shown ER and PR positivity to be correlated with a good prognosis (Creasman, 1993; Pertschuk *et al*, 1996; Dahmoun *et al*, 2002).

2.4.8 DNA PLOIDY AND S-PHASE FRACTION

It is known that approximately two thirds of endometrial carcinomas are diploid, and that aneuploidy occurs in only 16% of stage 1 tumours. However, half of the tumours that relapse are aneuploid (Kurman *et al*, 1994). These tumours are associated with high clinical stage, high tumour grade and poor prognosis (Rosai *et al*, 1996). S-phase fraction determines the degree of tumour cell proliferation and was found to be a strong predictor of outcome (Rosai *et al*, 1996). Flow cytometry and image cytometry are methods used for DNA ploidy studies (Lundgren *et al*, 2002). DNA ploidy correlates with hormonal receptor status, myometrial invasion, tumour grade and stage but is still an independent prognostic factor (Kurman *et al*, 1994). Konksi *et al.* (1996) found DNA ploidy and hormonal receptor status to be more objective factors than clinicopathological parameters which were more subjective. Other studies, however, have found S-phase flow cytometry analysis and DNA ploidy not to be entirely consistent (Askensten *et al*, 1990). Also, these are more costly and sophisticated techniques compared to the conventional microscopic analysis. There seems to be much controversy regarding this prognostic indicator. In a recent study, Lundgren and co-workers found that after tumour grade and histological subtype, DNA ploidy was the strongest predictor of outcome and was of value in predicting risk of relapse (Lundgren *et al*, 2002). They found that DNA ploidy had a strong prognostic impact and was an independent factor. In another study, Frigerio *et al.* (1999) found that DNA ploidy was the most important predictor of outcome, compared to myometrial invasion, stage, histologic type,

peritoneal cytology, and lymph node involvement. Lai and colleagues also confirmed the prognostic importance of DNA ploidy (Lai *et al*, 1999). Aneuploidy was found to be common in high-grade endometrioid and serous papillary carcinomas and in tumours with a poor prognosis (Konski *et al*, 1996). The 5 year survival for low grade diploid tumours was 94% and for aneuploid tumours it was 64% (Kurman *et al*, 1994).

2.4.9 FIGO CLINICAL STAGE

Table 2.6: Modified FIGO surgical staging system for Endometrial Carcinoma. (Adapted from Longacre *et al*, 1995).

Stage I	Confined to the uterine corpus **IA:** Tumour limited to endometrium **IB:** Invasion of less than half of the myometrium **IC:** Invasion of more than half of the myometrium
Stage II	Uterine cervix involved **IIA:** Endocervical glandular involvement only **IIB:** Cervical stromal invasion
Stage III	Pelvic extension **IIIA:** Tumour invades serosa &/or adnexa &/or positive peritoneal cytology **IIIB:** Vaginal metastasis **IIIC:** Metastasis to pelvic &/or para-aortic lymph nodes.
Stage IV	Extrapelvic extension **IVA:** Tumour invasion of bladder &/or bowel mucosa **IVB:** Distant metastasis including intra-abdominal &/or inguinal lymph nodes

The FIGO staging system remains the standard by which results are reported worldwide (Sutton *et al*, 1990), and has been used for years by clinicians to estimate the aggressiveness of tumours. In 1988 this FIGO staging system was revised (Table 2.6). Staging of a tumour was designed to have prognostic value

by determining the size and extent of the tumour. Endometrial carcinoma has a 5 year survival rate ranging from 36% to 95% in stage I disease (Rose, 1996). Stage has long been found to be one of the most important prognostic factors in endometrial carcinoma (Barrett, 1995). Stage II disease has a worse prognosis than stage I (Scurry *et al*, 2000). Tumour extension beyond the corpus (Stages II-IV) was present in 25% of patients 60 years and over, and only in 8.8% of patients that were younger (Kennedy *et al*, 1982).

2.4.10 ADNEXAL INVOLVEMENT

Adnexal spread is rarely a sole poor prognostic finding. It is usually associated with pelvic or para-aortic lymph node metastasis. Patients with adnexal spread who are treated by post-operative radiation have a reported survival rate of 85%. On some occasions carcinoma is detected simultaneously in the endometrium and in one or both of the ovaries. It is very rare that different histological types may develop simultaneously in the ovary and in the endometrium. Prognosis usually depends on the type of tumour present, but the spread of tumour beyond the confines of the uterus does confirm a poor prognosis (Longacre *et al*, 1995). This type of spread may represent vascular, transtubal, or lymphatic extension (Sutton *et al*, 1990).

2.4.11 INTRAPERITONEAL METASTASIS

Peritoneal involvement is also very rarely a sole poor prognostic finding. Just like the previous prognostic indicator it is usually associated with pelvic or para-aortic lymph node metastasis (Longacre *et al*, 1995). Serosal penetration is an indicator of the most extreme form of deep myometrial invasion. As mentioned earlier tumour spread beyond the confines of the uterus represents a poor prognosis (Sutton *et al*, 1990).

2.4.12 POSITIVE PERITONEAL CYTOLOGY

The use of cytologic sampling of peritoneal fluids or washings has become common practice. It has been found that in endometrial carcinoma, the presence

of malignant cells in the peritoneum, taken at the time of hysterectomy has an adverse effect on patient survival. Approximately 12-20% of patients with endometrial carcinoma have malignant peritoneal cytology. Malignant peritoneal cytology is incorporated into the current staging system as stage IIIA (Rose, 1996). Although the cytologic analysis of peritoneal washings has been incorporated into the current FIGO staging system, its prognostic value has been controversial for some time. Therefore Benevolo and co-workers set out to investigate the peritoneal washings of 182 patients with endometrial cancer, to verify the prognostic value (Benevolo *et al*, 2000). They analysed samples immunocytochemically (ICC) in parallel with the conventional cytological method and found that there was a significant improvement in diagnostic accuracy. They found that patients with stage 1 disease were under-staged and this could only be shown by ICC analyses. Their studies concluded that immunocytology of peritoneal washings identifies a higher percentage of metastatic free cells than conventional morphology and represents an independent prognostic factor (Benevolo *et al*, 2000).

Preoperative Cervical Cytology: Fukuda *et al.* (1999) set out with their study to assess the significance of malignant or suspicious cervical cytology in preoperative identification of poor prognostic factors in endometrial carcinoma. They discovered that positive preoperative cervical cytology itself is not an independent prognostic factor, but is associated with an increased risk of having known poor prognostic factors (Fukuda *et al*, 1999).

Postoperative Cervical Cytology: The study of Ng and co-workers concluded that the traditional surveillance protocols, particularly vault cytology, were not effective in the detection of recurrences. They suggest that alternative follow-up protocols should be investigated (Ng *et al*, 1997). Another study by Gadducci *et al.* (2000) also discovered that an intensive surveillance protocol, which included a vaginal smear, had no significant impact on the outcome of patients with clinical stage I endometrial cancer. It therefore seems that postoperative cervical cytology cannot be relied upon as a prognostic factor for endometrial carcinoma.

2.4.13 PELVIC AND PARA-AORTIC LYMPH NODE METASTASIS

The presence of positive para-aortic lymph nodes is the most important among the extra-uterine risk factors, in predicting prognosis. Also, pelvic lymph node

metastasis has been reported to be one of the most potent prognostic factors in endometrial carcinoma. Endometrial carcinoma spreads to the pelvic nodes (Plate 2.5) before involving paraaortic nodes (Kurman *et al*, 1994). This prognostic factor has been found to correlate with many other histopathologic factors like myometrial invasion and histologic grade. Therefore, FIGO included pelvic lymph node and paraaortic lymph node status into its staging system. However, it is known that pelvic lymphadenectomy causes deep vein thrombosis, lymphoedema and life-threatening pulmonary embolism. With this in mind pelvic lymph node dissection is therefore recommended only for patients with a high possibility of metastasis. The study of Kamura and colleagues revealed that the combination of degree of myometrial invasion and tumour diameter to be a good predictor of the presence of pelvic lymph node metastasis (Kamura *et al*, 1999). In another study it was found that myometrial invasion correlated strongly with lymph node involvement (Cosa *et al*, 1996). Also, the finding of positive pelvic nodes was a strong predictor of recurrence. These authors concluded that complete pelvic lymphadenectomy was efficient in the management of patients with high-risk endometrial carcinoma. Cunha *et al.* (2001) recommended that those patients who have high risk factors such as high tumour grade, deep myometrial invasion and extension to the cervix are those who should undergo pelvic and paraaortic lymph node dissection.

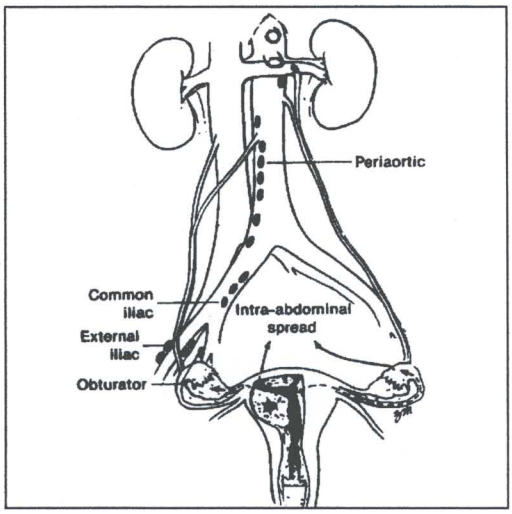


Plate 2.5: Lymph node drainage for endometrial carcinoma (Longacre *et al*, 1995).

2.4.14 AGE

The significance of age, at the time of diagnosis, has been appreciated for years (Creasman *et al,* 1992), but age remains controversial as a prognostic indicator. Some studies have found age to be an important independent risk factor (Kurman *et al*, 1994; Aziz *et al*, 1996), whilst others have not (Mundt *et al*, 2000). The results of Mundt and co-workers revealed that age is not an independent prognostic factor for recurrence (Mundt *et al*, 2000). However, it has been found that younger women have lower grade and less invasive tumours (Kurman *et al*, 1994). Aziz *et al.* (1996) have found that older patients have a higher clinical stage, higher grade, and greater depth of myometrial invasion than younger patients. The study of Berman *et al.* (1982) showed that at the time of diagnosis, no patient less than 51 years old died of endometrial carcinoma. They also found that with advancing age the 5-year survival fell to 62.9% in patients between 51 and 70 years, and 54% in those more than 70 years of age. They had diagnosed 18 patients over the age of 70 years, and of these 8 died of metastatic disease (Berman *et al*, 1982). A recent study by Yamazawa *et al.* (2000) and Sardi *et al.* (1999) confirmed statistically that younger women with endometrial carcinoma had a better prognosis.

Body mass: It has been found that patients who were over 65 years old, and not on exogenous oestrogen were more often slim, but had poorly differentiated tumours. Also noted was that patients of normal body mass or who were moderately obese had better outcomes than those who were morbidly obese or underweight (Sutton *et al*, 1990). Kennedy *et al.* (1982) found that the 3 patients under 40 years of age that were diagnosed with endometrial carcinoma were markedly obese, weighing at 220, 270, and 325 pounds, respectively. Anderson and co-workers carried out a retrospective study of 492 patients with endometrial cancer, whose median weight was 87 kg (Anderson *et al*, 1996). They tested the hypothesis that morbid obesity had no influence on survival in endometrial cancer. Their results showed that morbid obesity did positively affect survival in endometrial carcinoma and was associated with less aggressive disease, and that thinness was associated with a more aggressive disease. Also noted was that lymph node sampling added more important information in the thin patient than in the morbidly obese. The improved prognosis in the obese women was due to the tumours being well differentiated. This was also associated with the

cancers being oestrogen induced, especially in patients taking exogenous oestrogen (Anderson *et al*, 1996).

2.4.15 RACE

In addition to the well-known prognostic factors, studies have identified the black population group as a poor indicator for survival in endometrial cancer (Matthews *et al*, 1997; Christopherson *et al*, 1982b). It was noted that the relative risk of death for black patients with endometrial carcinoma was 4 times that for white patients (Yamada and McGonigle, 1998). Aziz *et al.* (1996) found in their study, that black patients had a higher clinical grade, stage, and greater depth of myometrial invasion than white patients. The median survival for black patients was noted to be 108 months compared to the median survival of 232 months for white patients (Aziz *et al*, 1996). There are many studies that report that black patients fare more poorly than white patients with endometrial carcinoma. Barrett and co-workers believe that cultural, financial, educational and geographic factors may be the main cause of poor outcome in this group of patients as their study confirmed that blacks are diagnosed at later stages than whites (Barrett *el al*, 1995). However, they also suggest that possibly the biologic mechanisms of the tumour in blacks is different to that in whites resulting in a higher stage at the time of diagnosis. Their study revealed that blacks had a more ominous histological type and grade compared to that of white patients, concluding that the biologic mechanisms of this disease is more aggressive in black patients. This aggressiveness plays a role in the overall worse outcome of this group of patients (Barrett *et al*, 1995). In another study it was shown that the incidence of endometrial carcinoma, on the other hand, is lower in black patients than in whites (Sung *et al,* 2000).

2.4.16 NEW PROGNOSTIC FACTORS

Recently, new and possibly better prognostic indicators have been examined, to help determine whether patients will benefit either from adjuvant treatment or a more aggressive primary treatment. Below are a few of these new prognostic factors.

2.4.16.1 Mismatch Repair Genes (MMR):

MMR genes are discussed later in detail under molecular aspects of endometrial cancer.

2.4.16.2 Cyclins, CDKs, CDKIs:

The prognostic significance of these molecules in endometrial cancer has been studied by various researchers and are discussed later in detail under molecular aspects of endometrial cancer.

2.4.16.3 *p53 :*

Discussed later in detail under Molecular aspects of Endometrial carcinoma.

2.4.16.4 *Bcl-2 :*

Discussed later under Molecular aspects of Endometrial carcinoma.

2.4.16.5 *C-erbB-2 (HER-2/neu):*

C-erbB-2 is an oncogene that is usually associated with a poor prognosis. Gassel *et al.* (1998) found that of the 222 endometrial carcinoma cases they studied, 109 cases (49%) demonstrated no immunohistochemical reactivity and 113 (51%) were reactive for this oncoprotein. The low expressing tumours showed a slight tendency towards longer survival. However, they found no significant relation between c-erbB-2 overexpression and tumour stage or survival (Gassel *et al,* 1998). Cases with endometrial hyperplasia also showed c-erbB-2 protein expression. This prognostic indicator still remains controversial (Gassel *et al*, 1998). C-erbB-2 is known to be associated with a higher stage, higher grade and a worse prognosis, but Ioffe and colleagues also found it to be related to a faster cell death rate (Ioffe *et al*, 1998). Berchuck and Boyd found overexpression of the *HER-2/neu* oncogene in 10% of endometrial cancer cases, which also correlated with poor survival (Berchuck and Boyd, 1995). In contrast, Van Nostrand *et al.* (1998) found there to be a statistically better survival in endometrial cancer patients with *HER-2/neu* amplification.

2.4.16.6 Cathepsin D:

Cathepsin D is a lysosomal proteinase. It has been found that low levels of this cytosolic cathepsin-D predicts poor prognosis (Southcott *et al*, 2001). Gassel *et al.* (1998) investigated 225 endometrial carcinoma cases for cathepsin D expression. Of these 225 cases, 120 tumours (53%) showed low levels of cathepsin D expression and 105 cases (47%) were strongly positive. A strong correlation between overall survival and expression of this protein was noted. However, there was no association with tumour stage, depth of infiltration, adjusted survival, or recurrence free survival (Gassel *et al*, 1998).

2.4.16.7 Heat Shock Proteins (HSP):

These are proteins of varying sizes that are able to assist the cell in resisting or surviving stressful conditions, such as heat exposure and physiological conditions. These mechanisms, however, are not well understood (Geisler *et al*, 1998). The HSP70 and HSP90 are also known to be sex steroid receptor associated proteins. HSP70 has been found to be associated with several oncogenes such as the *p53* tumour suppressor gene and the HSP90 has been reported to correlate with sex steroid receptor status in endometrial cancer. The HSP70 was also reported to be a prognostic indicator in several other malignant neoplasms, therefore Nanbu and co-workers investigated 44 endometrial carcinoma cases using immunohistochemical staining for HSP70 and HSP90 (Nanbu *et al*, 1998). They observed the expression of HSP70 in 22 cases (50%) and strong HSP90 expression in 13 cases (30%). It was shown that the patients with HSP70-positive tumours had a significantly poorer survival than the patients with HSP70-negative tumours. In contrast to this, a strong expression of HSP90 was associated with a lower histologic grade and a favourable prognosis. The study of Nanbu *et al.* (1998) was the first to report that the expression of HSP70 had a poor prognostic significance. HSP70 expression correlated with histologic grade but not the FIGO stage and is not an independent prognostic factor (Nanbu *et al*, 1998). Geisler and co-workers, in their study examining HSP27 in endometrial cancer, have associated decreased HSP27 expression with aggressive histologic subtypes and decreased survival. They revealed HSP27 to be an independent prognostic indicator of survival (Geisler *et al*, 2001; Geisler *et al*, 1999). Konishi (1996) also attempted to examine HSP70 and HSP90

expression as a prognostic indicator in endometrial cancer. He was able to conclude that HSP70 expression was associated with loss of sex steroid receptors in non-endometrioid or poorly differentiated endometrial cancer, which often exhibited p53 protein expression. Strong expression of HSP90 was associated with well-differentiated endometrial carcinoma and the presence of sex steroid receptors (Konishi, 1996).

2.4.16.8 Proliferating Cell Nuclear Antigen (PCNA):

Garzetti *et al.* (1996) investigated the prognostic significance of PCNA immunoreactivity in tumour cells of patients with endometrial cancer. A population of 74 patients with FIGO stage 1 endometrioid adenocarcinoma were analysed. They observed a significant increase in PCNA index with respect to nuclear grade and depth of myometrial invasion, and maintained that this was a significant prognostic parameter in stage 1 endometrial cancer for disease-free survival. Nuclear grade 3 tumours had the highest PCNA index. There was also an increase in the PCNA index observed, in progression from complex to atypical hyperplasia to invasive adenocarcinoma. This study found that PCNA immunostaining had an added advantage, as the PCNA index is an objective parameter with simplicity of evaluation that had special reference to nuclear or histologic grade. The most important aspect of this study was that it found PCNA index to have an independent prognostic role in advanced endometrial adenocarcinoma. Finally, they concluded that high-risk endometrial cancer patients present with high nuclear grade, deeply invasive and high PCNA index tumours. They also found the PCNA index useful in differentiating the subgroup of patients that require aggressive adjuvant treatment from those at a low risk of recurrence (Gazetti *et al*, 1996).

2.4.16.9 Thrombocytosis:

In a study of 135 endometrial cancer patients Gucer *et al.* (1998) reviewed the occurrence of thrombocytosis and compared it with other prognostic indicators. Of the 135 patients studied, 19 (14%) had thrombocytosis. Increased platelet count was notably more frequent in advanced disease (stage II-IV), unfavourable grade (grade 2 and 3), lymphovascular space invasion and deep myometrial invasion. The 19 patients with thrombocytosis had a significantly worse 5-year

survival rate than those without thrombocytosis and the recurrence rate was also notably higher. They found thrombocytosis to be a predictor of worse outcome and a prognostic factor for survival in endometrial cancer (Gucer *et al*, 1998). Menczer *et al.* (1996) assessed 66 endometrial carcinoma cases and found that thrombocytosis was associated with an unfavourable grade of differentiation. Those patients with an elevated platelet count were also of an older age, had a poorer rate of survival, higher stage and deep myometrial invasion. However, their results were not statistically significant (Menczer *et al*, 1996). In a recent study the results of Tamussino *et al.* (2001) correlated with that of the other studies (Gucer *et al*, 1998; Menczer *et al*, 1996). They also found thrombocytosis, age, high tumour grade, non-endometrioid histology, advanced FIGO stage to be significantly associated with poor prognosis. These authors were the first to analyse pretreatment haemoglobin as a prognostic factor in endometrial cancer. Their data proved that low pretreatment haemoglobin was a prognostic factor in endometrial carcinoma patients, and that it was associated with thrombocytosis (Tamussino *et al*, 2001).

Extreme care has to be taken when evaluating the prognostic risk factors for patients with endometrial carcinoma, as the ultimate risk in oncology is "death". There are a number of conventional factors to be assessed and many are interrelated (Sutton *et al*, 1990), as demonstrated above. However with the advent of new technology in research, new prognostic factors are being identified. Many of the conventional parameters are somewhat subjective, so new objective factors are now necessary. The conventional parameters such as tumour grade and myometrial invasion usually identify a high-risk group. This group may include many who have a good survival rate, but are receiving unnecessary adjuvant treatment, which has adverse effects. It is therefore important to investigate a better definition of patients with a real high-risk of recurrence, and who definitely require aggressive adjuvant treatment (Garzetti *et al*, 1996). New, objective parameters are now required to fulfill this task. It is also hoped that the information derived from these tumour biomarkers will reduce the need for extensive surgical staging and adjuvant treatment in patients with endometrial carcinoma (Salvesen and Akslen, 2002).

2.5 MOLECULAR ASPECTS OF ENDOMETRIAL CARCINOMA

"Just as our present knowledge and practice of medicine relies on a sophisticated knowledge of human anatomy, physiology and biochemistry, so will dealing with disease in the future demand a detailed understanding of the molecular anatomy, physiology and biochemistry of the human genome. We shall have to have physicians who are as conversant with the molecular anatomy of chromosomes and genes as the cardiac surgeon is with the structure and workings of the heart and circulatory tree." - Paul Berg, MD. Nobel Laureate, 1981.

Cancer has been defined as a disease of the genome that is a consequence of genetic instability (Baker, 1996). It has been previously estimated that there were approximately 10 000 to 100 000 genes found throughout the 46 chromosomes of humans (Gelehrter *et al*, 1990). However, the exact number of genes encoded by the genome is still unknown, even though the Human Genome Project has been completed in 2003. It has now been estimated that there are between 20 000-25 000 human protein-coding genes (Stein, 2004), but it still could be years before a truly reliable gene count can be assessed. The smallest of these genes may be composed of no more than a few hundred base pairs of DNA. The largest however, may be more than a million base pairs in length, so, it is not surprising that this intricate genetic system can go array at times (Gelehrter *et al*, 1990). When the genome is altered in some way, this affects the expression and function of genes that control cell growth and differentiation, and has been found to be the main cause of cancer. These cancer-causing genes are of two distinct types: oncogenes and onco-suppressor genes. Conversion of the normal proto-oncogene into an active oncogene occurs by deletion or point mutation in its coding sequence, gene amplification, and by specific chromosome rearrangements. Mutations and abnormal expression in *ras, myc, c-erb*B-2, and other oncogenes have been discovered in several types of gynaecological cancer. Onco-suppressor genes are also implicated in gynaecological cancer, the functions of which are localized in different phases of the cell cycle. It is the structural changes and deletions of these genes that can cause cancer. In endometrial cancer, mutations in *p53, BRCA1, DCC*, and *PTEN* genes have been

reported. Recently, another type of genetic alteration has been described, "microsatellite instability", which reflects damage in the DNA replication and repair systems of the cell (Spandidos *et al*, 2000). Endometrial carcinoma results from the accumulation of several of these genetic and epigenetic alterations in oncogenes, tumour suppressor genes, or DNA repair genes (Figure 2.4). The transition from normal endometrial cells to hyperplasia and to carcinoma is the result of the accumulative effect of these abnormalities. Several additional molecular abnormalities eg. MSI, occur in different neoplastic subclones, once the tumour has developed. These new alterations are responsible for tumour heterogeneity, tumour invasion, and finally, metastasis (Matias-Guiu *et al*, 2001).

2.5.1 MICROSATELLITES

A milestone that surely needs to be acknowledged is the discovery of microsatellites, which has been the subject of intense study recently. It has created such a great impact in the world of pathology that many diseases have been more widely investigated and understood. Microsatellites are short, simple repetitive DNA sequences that are located in non-coding regions (introns) throughout the human genome, and range from 1-4 bases in length (Sherman *et al*, 1998). They often exist as di-, tri- and tetra-nucleotides but the sequence that is most commonly found in eukaryotes is the dinucleotide (CA)n repeat of which there are approximately 50000 to 100000 such repeats in the entire human genome (Catasus *et al*, 1998). These microsatellites are also referred to as SSLP (simple sequence-length polymorphisms) or STRs (short tandem repeat polymorphisms) (Spandidos *et al*, 2000). Since they are found within the introns, which do not code for any protein, they are therefore referred to as "junk" DNA. It is assumed that this "junk" DNA is an accumulation of uncorrected errors in DNA replication or the abortive evolution of genes (Catasus *et al*, 1998).

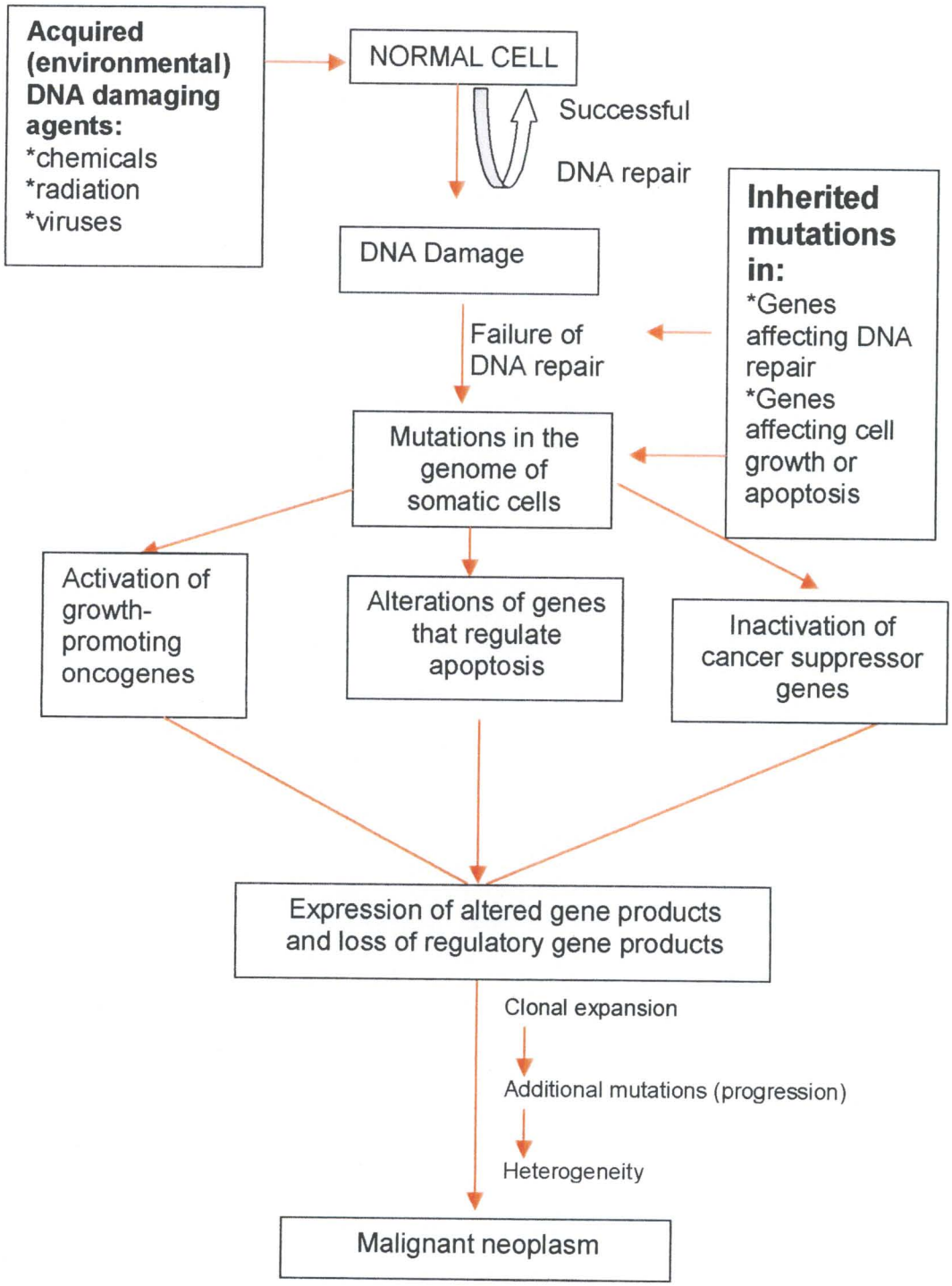


Figure 2.4: Flow-chart illustrating the Molecular Basis of Cancer. (Adapted from Cotran *et al.* 1999).

Microsatellites appear to be a major characteristic of many tumours, the assaying of which seems to be important in identifying many human cancers. The human genome consists of large numbers of these non-essential, repetitive units of DNA segments (over 90%). Microsatellites consist of variations or polymorphisms that are characteristic for an individual, with the result of a unique DNA fingerprint, but they vary between individuals. However, they are the same in different cells from the same individual. It is also very interesting to note, that they are stably inherited and therefore highly conserved from one generation to the next. Microsatellites are found in the heterochromatin, near chromosomal telomeres and centromeres, but are not regularly spaced in the chromosome (Naidoo and Chetty, 1998).

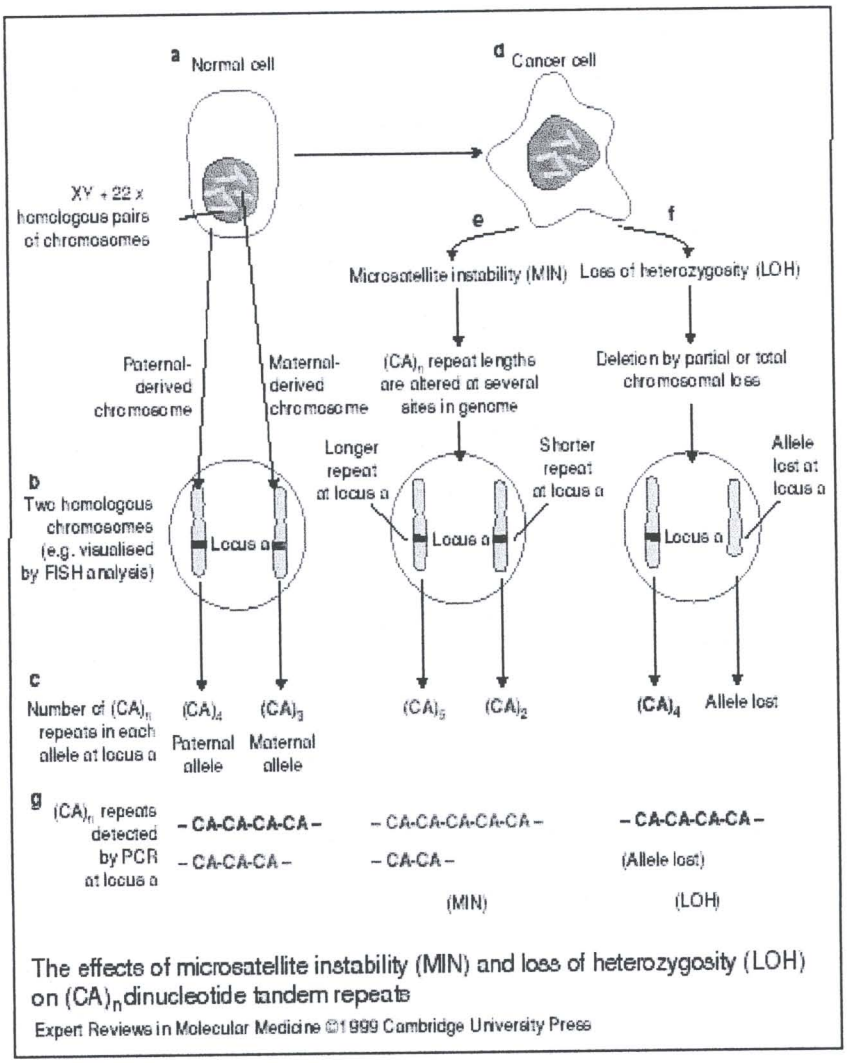
2.5.1.1 MICROSATELLITE INSTABILITY

As research progresses there is an increased awareness that there are multiple mutations present in many human tumours. Based on these advances, the concept that the genomes of cancer cells are unstable emerges, resulting in mutations that the human body has no control over (Loeb, 1994). With regard to the increased chromosomal abnormalities and mutations in cancer, Loeb (1994), hypothesised that cancer is characterised by a mutator phenotype, implying that at some time the mutation rate in human cancers must be greater than in normal cells. He backed this hypothesis up with the demonstration of microsatellite instability in different human tumours, thus providing strong evidence for a mutator phenotype. Due to the highly repetitive nature of microsatellites, these sequences are often prone to replication errors. The accumulation of these replication errors results in microsatellite instability and is thought to represent a type of genomic instability (Burton *et al*, 1998; Sherman *et al*, 1998). Microsatellites contain repetitive nucleotide sequences that are somewhat constant in normal cells but in certain tumours, they vary in length. These variations may not affect the phenotype of the cell but they are defined as mutations (Loeb, 1994). High frequency microsatellite instability is indicative of an underlying "Mutator Phenotype".

Those tumours that are found to possess somatic alterations in the length of their microsatellite loci are termed MSI+ (microsatellite instability positive) or RER+ (Replication error positive), (Tashiro *et al*, 1997a). Several researchers have recently described this new mechanism in the evolution of some tumours (Risinger *et al*, 1993; Fujino *et al*, 1994; Jovanovic *et al*, 1996; Caduff *et al*, 1996; Sirchia *et al*, 1997; Tashiro *et al*, 1997a; Helland *et al*, 1997; Basil *et al*, 2000), which is characterised by a tendency for replication mistakes and by genomic instability of microsatellite repeats (Sirchia *et al*, 1997). Microsatellite instability occurs together with mutated mismatch repair genes in some of these cancers, and is thought to contribute to the mechanisms of cancer (Burton *et al*, 1998; Loeb, 1994). Under normal circumstances, these replication errors are usually repaired by highly robust mechanisms, but defective mismatch repair genes, and mutations in cancer causing genes results in defective proteins that are unable to correct these replication errors.

This mutator phenotype is therefore of major importance, as it signifies that DNA repair mechanisms and other important processes have gone wrong (Sherman, *et al* 1998). Microsatellite instability (Plate 2.6) was initially detected in patients with the HNPCC (hereditary non-polyposis colorectal cancer) syndrome, which is one of at least three major forms of hereditary colorectal cancer (Risinger *et al*, 1993; Katabuchi *et al*, 1995). These patients have right-sided colon cancer and extra-intestinal tumours, including endometrial carcinoma which is the second most common neoplasm encountered in patients with HNPCC (Watson and Lynch, 1993).

Defective mismatch repair was also demonstrated in cell lines derived from these cancers. Thus, linking mismatch repair and microsatellite instability and, providing the explanation for the generation of this phenotype in HNPCC was established. In 1994, Loeb suggested that more experiments were necessary to demonstrate that the expansion of microsatellite sequences, which occurs during tumourigenesis is caused by mutations in mismatch repair genes (Loeb, 1994).



The effects of microsatellite instability (MIN) and loss of heterozygosity (LOH) on $(CA)_n$ dinucleotide tandem repeats

Plate 2.6: Diagram showing microsatellite instability and loss of heterozygosity (Pharoah and Caldas, 1999).

At that time it was already established that microsatellite instability was common in HNPCC (Figure 2.5), but Loeb (1994) questioned whether microsatellite instability was a common event in other types of cancer too. A question we now know the answer to, as microsatellite instability has been detected in many other cancers including endometrial carcinoma. There are many important questions that he had raised, and now through years of research we have some answers.

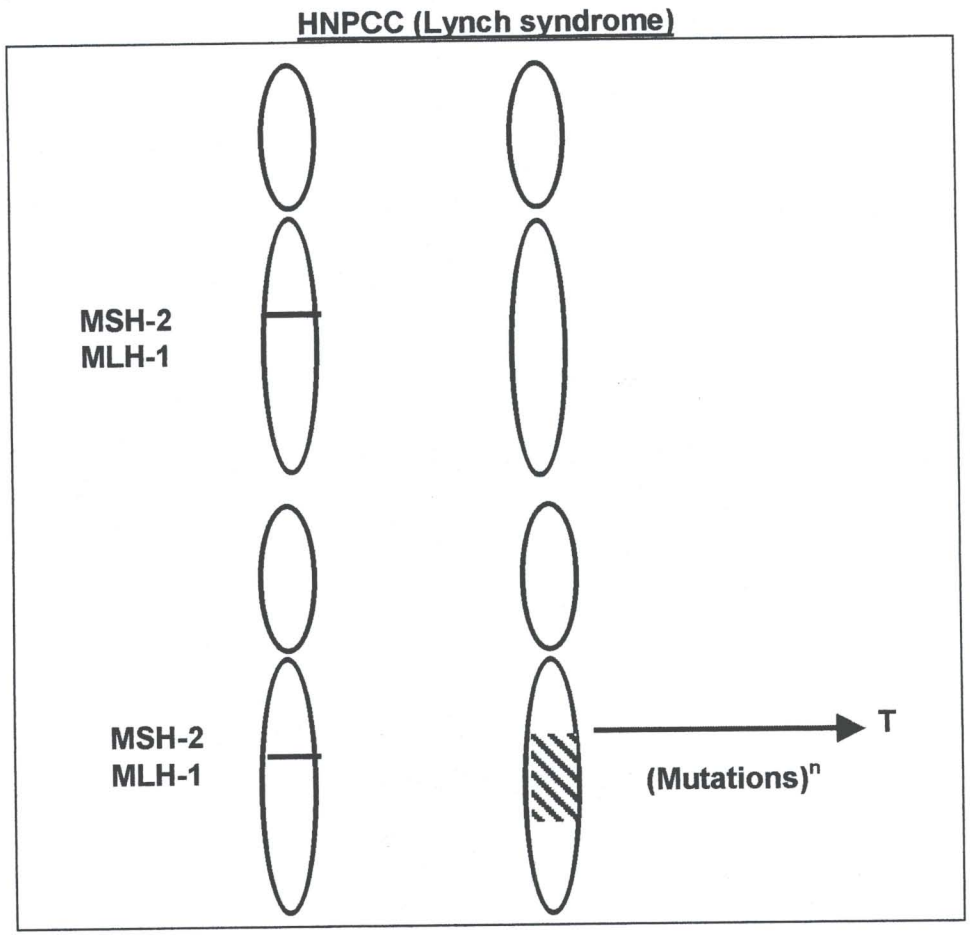


Figure 2.5: HNPCC syndrome. Patients with this syndrome are born with germline mutations in one allele of the mismatch repair genes. The tumour T, however, only develops after deletion or mutation has ocurred in the contralateral allele. This is referred to as the second hit. (Adapted from Matias-Guiu *et al,* 2001)

2.5.1.2 ANALYSIS OF MICROSATELLITES

Microsatellites appear to be a major feature of many tumours, the assaying of which seems to be important in characterising these human cancers. The Polymerase Chain Reaction (PCR) is the method used to assay microsatellite instability. With this method, which makes use of radioactively-labelled primers in the PCR reaction, specific microsatellite sequences in paired DNA samples of normal and tumour tissue from a patient is amplified (Sherman *et al*, 1998). There are approximately 2000 polymorphic microsatellite markers that have been

identified in the human genome (Naidoo and Chetty, 1998). Once amplified, the PCR products obtained are compared using gel electrophoresis. The differences in the electrophoretic pattern of DNA bands of the normal and tumour tissue are identified by their changed lengths. These are seen as altered electrophoretic band patterns, which are actually electrophoretic mobility shifts in microsatellite markers (Burton *et al*, 1998; Sherman *et al*, 1998). Microsatellite instability can be defined as an alteration in the size of at least 2 of the microsatellite loci in tumour DNA when compared with normal DNA (Ohwada *et al*, 1999).

There are many methods used to analyse these microsatellites with several advantages and disadvantages. To date, the most popular and updated, hi-tech method is the use of fluorescently-labelled primers, automated DNA sequencing coupled with a computer software package (Naidoo and Chetty, 1998). This fluorescence based technology, besides being a sensitive and useful tool, has an added advantage in that many microsatellites in large numbers of cases can be analysed simultaneously. With this method fluorescently labelled primers are separated on polyacrylamide gels and the fluorescent products are detected by laser and analysed by a computer software package. After analysing the fluorescently-labelled PCR products, this software package produces electrophoretograms. The areas under the peaks are calculated by the computer, and the ratios comparing normal and tumour DNA are computed.

Advantages of this technique are :

- Fast turnaround of samples
- Accurate and impartial scoring of alleles.
- Easy interpretation of data, which can be captured and stored with the use of the computer package linked to the DNA sequencer.
- Multiple loci can be assayed, as in multiplex PCR (more than one set of primers are used in the same PCR tube, affording co-amplification of multiple products).
- The use of standards eliminates lane to lane variations.
- The fluorescent tag is very sensitive, therefore only 1-3μl of PCR product is required for the run on the gel, which ultimately results in cost saving.

This automated, computer based technology has made microsatellite analysis a valuable tool in the identification of many diseases (Naidoo and Chetty, 1998).

2.5.1.3 THE USES / ADVANTAGES OF MICROSATELLITE ANALYSIS

Microsatellites may not just be referred to as "junk DNA" as they were once thought to be. These short, repetitive DNA seqences display prominent polymorphism (McCarthy, 1995), which is due to DNA slipped strand mispairing, and is also referred to as DNA slippage (Naidoo and Chetty, 1998).

This trait has made them out to be useful in:

- Linkage analysis and genetic mapping because with microsatellite PCR, multiple loci can be examined (Naidoo and Chetty, 1998).
- The diagnosis of tumours in histologically unremarkable specimens; they are a sensitive indicator for genomic hypermutation/genomic instability (Catasus *et al*, 1998).
- As markers of loss of heterozygosity.
- The analysis for replication errors in individuals who clinically fulfil the criteria for HNPCC. Jass *et al.* (1995) found out that determining the RER status makes the diagnosis of HNPCC more accurate, thus affording early family screening and effective targeting of resources.
- Personal identification.
- The construction of the human evolutionary tree.
- Population genetic analysis.
- Also, since they are located in important gene loci, they can be used not only as markers of human diseases, but also in providing information of the mutation, replication and repair of DNA in tumourigenesis (Naidoo *et al*, 1998).
- A good indicator of the state of genes (McCarthy, 1995).
- May signify a genetic defect in DNA repair mechanisms and other processes (Sherman *et al*, 1998).

Previously, microsatellites were regarded as being the "bearers of bad news" as they were related to high tumour grade and poor prognosis, but recently, microsatellite instability positive endometrial carcinomas are diagnosed at earlier stages and are often found to be less aggressive histologic subtypes (Basil *et al*, 2000). The study of microsatellites in endometrial carcinoma has provided the pathologist with an extra tool to help in understanding the pathogenesis of this disease (Burton *et al*, 1998). These studies have more clearly defined the molecular events in endometrial carcinoma, but there still remains much more to be discovered.

2.5.1.4 MICROSATELLITE INSTABILITY IN ENDOMETRIAL CARCINOMA

A microsatellite mutator phenotype, which is also more commonly known as microsatellite instability has often been identified in sporadic endometrial carcinoma (Catasus *et al*, 2000). However, detection of microsatellite instability (MSI) in a tumour does not prove that MSI is causally related to the tumour in question, because MSI may occur after tumour development (Sherman *et al*, 1998). Although MSI does not play a direct role in tumourigenesis its presence indicates a type of genetic instability (Tashiro *et al*, 1997a). In fact, Shibata *et al.* (1996) aptly described microsatellite mutations when they referred to them as being "molecular tumour clocks", as they permit assessment of the sequence and timing of molecular changes.

Carcinoma of the endometrium is the most common noncolorectal carcinoma ocurring in women with HNPCC (Katabuchi *et al*, 1995). The HNPCC syndrome which was once known as Cancer family syndrome and Lynch syndrome II has a familial predisposition to colorectal carcinoma and extracolonic cancers of the urological, gastrointestinal and female reproductive tracts, commonly the endometrium. Instability of dinucleotide repeat sequences is found throughout the genome in HNPCC cases of colorectal carcinomas (Katabuchi *et al*, 1995). Therefore, in 1993 Risinger *et al.* hypothesised that this replication error phenotype may be evident in some sporadic endometrial carcinomas as well as those associated with HNPCC (Risinger *et al*, 1993). In order to address this

hypothesis, Risinger and co-workers studied 36 cases: MSI was found in 17% of sporadic endometrial carcinomas and in 75% of those linked to HNPCC, indicating that the HNPCC gene is involved in heritable and somatic forms of endometrial carcinoma and that this replication error could be the result of chromosome 2p gene defects (Risinger *et al*, 1993). However, the exact mechanism through which the replication error phenotype contributed to carcinogenesis was unknown. At this stage Risinger *et al.* (1993) also hinted that this could be an early event in endometrial cancer. Jovanovic *et al.* (1996) observed MSI in matched atypical endometrial hyperplasias and cancers. In a previous study, it was noted that MSI was not observed in normal endometrium and 'simple hyperplasias' (Duggan *et al*, 1994). The absence of this instability from histologically normal or non-atypical hyperplasias leads to the conclusion that MSI occurs early in the evolution of endometrial carcinogenesis. The data obtained from this research provided additional evidence that a precursor lesion exists prior to well-differentiated endometrial carcinoma (Jovanovic *et al*, 1996). To contradict this, a discovery made by Caduff *et al.* (1996) implied that the RER phenotype did not occur at an early stage. In 1999 Ohwada and colleagues found that RER was not associated with atypical hyperplasia (precancerous lesion of endometrial cancer), but suggested that further investigation was required to confirm this (Ohwada *et al*, 1999). However, several other researchers have confirmed that atypical endometrial hyperplasia displayed the MSI phenotype, supporting this theory that MSI is an early event in carcinogenesis (Mutter *et al*, 1996; Catasus *et al*, 1998; Esteller *et al*, 1999; Ohwada *et al*, 1999; Kiechle *et al*, 2000).

During the past few years MSI has been extensively investigated in endometrial carcinoma. In 1995 Katabuchi *et al.* investigated a total of 65 primary sporadic endometrial carcinomas for MSI (Katabuchi *et al*, 1995). Of these cases, 6 were of the uncommon serous papillary histological subtype and the remaining 59 were of the endometrioid subtype. 8 microsatellite loci were amplified and 12 of the 65 cases were MSI+. The loci amplified were D2S119, D2S123, D2S147, D10S197, D13S175, D18S58, D18S69, and an AT dinucleotide repeat found in an intron of the Deleted in Colorectal Cancer (*DCC)* tumour suppressor gene. Three of these cases showed mutations in all 8 loci and another 3 showed shifts in 7 loci. Shifts

at a minimum of 3 loci were detected in all the MSI + cases and 9 cases had 5 or more affected loci. The MSI + tumours consisted only of the more common endometrioid subtype (Katabuchi *et al,* 1995). Moreover, the incidence of MSI was observed to be higher in FIGO stage III and in more undifferentiated tumours (Muresu *et al*, 2002).

In another study, successful amplification was obtained for 8 markers, these being D2S123, HUMCA 1126, D5S107, D10S197, D11S904, D13S176, and D18S34. This assay consisted of 109 cases, and of these 27 (25%) had instability in at least 1 of the 8 tested loci, and 10 tumours (9%) showed MSI in 2 or more loci. These were considered to be RER + (Caduff *et al*, 1996).
Helland *et al.* (1997) tested 20 endometrial cancer cases for MSI at the following loci, D3S1271, D3S1297, D3S1286, D5S404, D8S255, D10S197, D11S904 and D17S787. They observed MSI in 6 of the 20 (30%) cases. Of the 6 RER + cases, 3 showed a new allele at a single locus, but the other 3 were altered at approximately 5-7 of the 8 loci. Of the 72 endometrial cancer cases that Sakamoto *et al.* examined in 1998 for MSI, 11 tumours (15%) had RERs at two or more microsatellite loci (Sakamoto *et al*, 1998). A study undertaken by Ohwada *et al.* (1999) consisted of 93 endometrial cancer cases. The DNA of these cases were amplified by using 5 sets of microsatellite primers, Tp53, D2S123, D3S1029, D3S1611 and D18S34. Of the 93 cases, 32 (34%) were RER+ at 1 and more than 1 loci, and 22 (24%) were positive at 2 and more than 2 loci. Prognostic factors such as age and histologic grade were not correlated with the RER + rates, but stage and lymph node metastasis showed significant association when compared. Stage 3 and 4 patients and those with lymph node metastasis were found more likely to be RER+ than the stage 1 and 2 patients and those without lymph node metastasis. This difference in RER positivity when comparing the 34% in this study, to previous reports of 17-25%, could be attributed to the different markers and the differing definitions of RER + used. Patients with 1 and more positive loci are considered to be RER+ by some investigators, whilst others consider patients with 2 and more positive loci to be RER positive (Ohwada *et al*, 1999).

Using 13 microsatellite markers Toda *et al.* (2001) examined 60 cases of endometrial cancer for MSI and LOH (loss of heterozygosity). MSI and LOH were noted in 13 of 60 (21.7%) and 20 of 60 (33.3%) cases, respectively. The frequency of both MSI and LOH were highest in stages III and IV (Toda *et al*, 2001). Basil and co-workers were the first to include race as a parameter in the analysis of MSI (Basil *et al*, 2000). They found that 65 of the 70 (93%) patients with MSI positive tumours were of the white race, indicating that this particular race group was more likely to have MSI positive tumours, thereby suggesting an alternative tumourigenic pathway. Also noted was that the overall adverse survival of black patients with endometrial cancer. However, the reason for this was not explained (Basil *et al*, 2000).

2.5.1.5 MICROSATELLITE INSTABILITY AND THE TWO PATHOGENETIC THEORIES OF ENDOMETRIAL CARCINOMA

It has long been accepted that endometrial carcinoma exists in 2 different forms (Tritz *et al,* 1997). Bockman first described the two main clinicopathologic types of endometrial cancer (Bockman, 1983):

- Type 1: An oestrogen dependent 'usual type' and,
- Type 2: An oestrogen independent 'special variant type' (Matias-Guiu *et al*, 2001), as discussed earlier.

These two types correlated with distinctive light microscopic features ie. type 1 has endometrioid features and type 2 consists predominantly of serous papillary type cancers (Tashiro *et al*, 1997a). In 1997 Tritz *et al.* suggested that the molecular characteristics in endometrial carcinoma may differ in the 'usual' and 'variant' types (Tritz *et al*, 1997).

Two different genetic pathways have also been suggested by Tashiro *et al.* (1997a). The endometrioid subtype being the more common type, accounts for approximately 90% of endometrial cancers and therefore has been more frequently analysed for MSI. A few studies on a small number of uterine serous papillary carcinomas have shown them to be MSI negative but these numbers were too small to be of much significance (Tashiro *et al*, 1997a). Tashiro and colleagues, analysed 34 uterine serous papillary carcinomas for MSI. All 34 cases failed to exhibit MSI. In 1996 Caduff *et al.* observed that the RER

phenotype was not identified in serous papillary carcinoma. This finding suggested that serous papillary carcinoma does not only have distinct clinical features such as, aggressive behaviour and occurring in older age, but is also genotypically distinct (Caduff *et al*, 1996). Tritz and co-workers also showed that MSI was more frequently detected in usual endometrial carcinoma (endometrioid) than in special variant (serous papillary) which implied that the clinicopathological phenotypes observed in these two tumour types may be evidence of two different molecular pathways in carcinogenesis (Tritz *et a*l, 1997). Catasus *et al.* in 1998 also noted this. Endometrial intraepithelial carcinoma (EIC) known to be the precursor lesion of serous papillary carcinoma, has not shown MSI, thereby adding further support that MSI does not occur in serous papillary carcinoma (Sherman *et al*, 1998). Lax *et al.* (2000) analysed 57 uterine endometrioid carcinoma cases and found MSI in 16 cases, but none of the 34 uterine serous papillary carcinoma cases showed MSI (Tashiro *et al*, 1997a). These results support the concept that endometrioid and serous papillary cancers have different molecular pathways involved in their pathogenesis (Lax *et al*, 2000), thus leading to the proposal of a dualistic model of endometrial carcinogenesis (Matias-Guiu *et al*, 2001). This hypothetical dualistic model of endometrial tumourigenesis was formed as a result of the association between MSI and endometrioid carcinoma. Matias-Guiu *et al.* (2001) investigated 42 sporadic endometrial carcinomas for the presence of MSI to give further support to this model. The results they obtained from this investigation confirmed what other researchers had already suggested.

With reference to this dualistic model, normal endometrial cells undergo a transformation into endometrioid carcinoma through 4 different molecular pathways, one of them being MSI. The others include mutations of the *pten, k-Ras* and *β catenin* genes (Matias-Guiu *et al*, 2001). The replication errors that occur in MSI result in the accumulation of mutated oncogenes and tumour suppressor genes whilst abnormalities in the *pten, k-ras* or *β-catenin* genes alter several different signal transduction pathways. In contrast, with non-endometrioid carcinoma it is alteration of *p53* tumour suppressor gene and loss of heterozygosity (Figure 2.6) in several chromosomes that drives the neoplastic process (Matias-Guiu *et al*, 2001).

As can be seen, this dualistic model is well explained and therefore very tempting to accept. However, this model is not perfect and exceptions do occur. There are many endometrial carcinomas that are in the overlapping zone with regards to clinical, morphological, immunohistochemical and molecular features. Matias-Guiu and co-workers have postulated a new theory (Matias-Guiu *et al*, 2001).

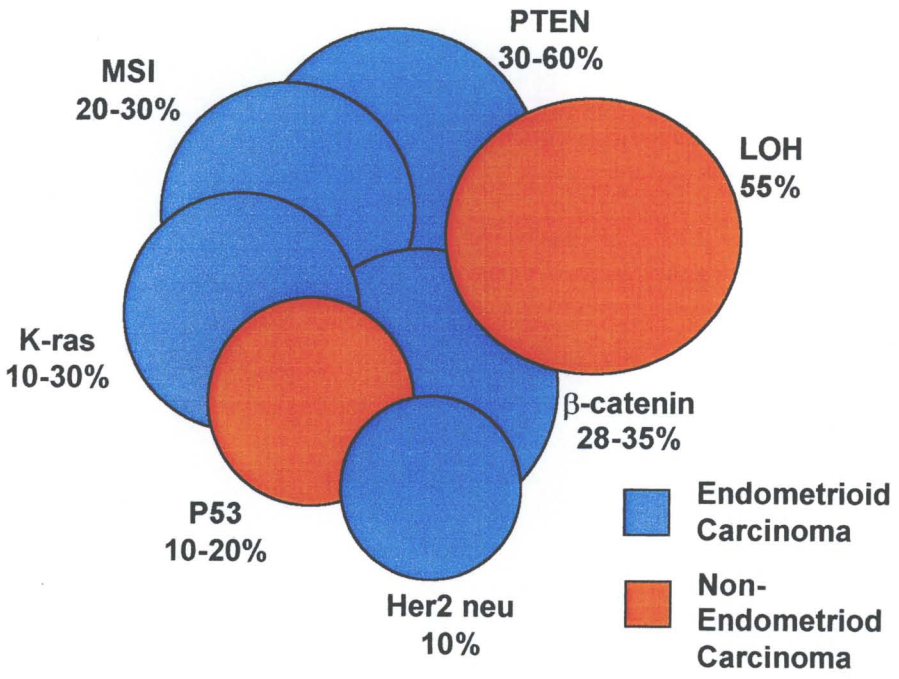


Figure 2.6: The frequency ranges of some of the main molecular genetic alterations found in endometrial carcinoma. (Modified from Matias-Guiu *et al*, 2001; Burton and Wells, 1998).

They suggested that non-endometrioid endometrial carcinoma (NEEC) might develop by following 2 different pathways:

- 1. De novo, through *p53* mutations, LOH at several loci, and other unknown gene mutations.
- 2. Through dedifferentiation of pre-existing endometrioid endometrial carcinoma (EEC) (Matias-Guiu *et al*, 2001).

This hypothesis explains the existence of mixed EEC-NEEC, the presence of MSI, and alterations in *PTEN*, *k-Ras*, or *β-catenin* genes in NEEC and in

carcinosarcomas (malignant mixed Mullerian tumours). Here, *de novo* NEEC would fit the clinicopathologic and molecular features of NEEC, pure papillary serous or clear cell morphology, old age, absence of oestrogen stimulation, lack of endometrial hyperplasia, *p53* mutations, absence of MSI, or PTEN mutations. On the other hand, dedifferentiated NEEC would exhibit overlapping features with EEC, mixed EEC-NEEC morphology, early age of presentation, oestrogen stimulation or pre-existing hyperplasia, coexistence of *p53* mutations and MSI, or *PTEN* mutations (Matias-Guiu *et al*, 2001).

Another recent molecular study has suggested three possible pathways for endometrial carcinogenesis. These being, hyperplasia, metaplasia and de novo pathways (Inoue, 2001). Oestrogen-dependent endometrial carcinoma consists of histopathologically well (G1) or moderately differentiated (G2) endometrioid adenocarcinomas that arise from a series of hyperplastic precursor lesions. This type of tumour occurs in pre- and peri-menopausal women, at an early stage, and generally has a good prognosis. The oestrogen-independent tumours are composed mostly of poorly differentiated endometrioid (G3) or variant types of endometrial carcinomas that often develop in an environment of endometrial atrophy from a nonhyperplastic epithelium. Such tumours often occur in postmenopausal women, present with deep muscular invasion, and have a poor prognosis. The differences recognised have led to the notion that these types of endometrial carcinomas represent different neoplastic processes of the endometrium. The accumulating data on genetic alterations in endometrial cancers also support the notion that endometrial cancers develop through different molecular genetic pathways. Although there is little doubt that atypical hyperplasia is a precursor lesion of oestrogen-dependent endometrioid carcinomas, the precursor lesions of the oestrogen-independent tumours remain unidentified.

Therefore, Inoue (2001) recently proposed that metaplasia is a precursor of the variant types of endometrial carcinomas, including serous papillary adenocarcinoma, mucinous adenocarcinoma, and clear-cell carcinoma.

He based this on the following evidence:

Atypical metaplastic cells can be considered as already initiated and therefore already on the road to carcinogenesis. This is based on the immunohistochemical

findings of p53 accumulation in several metaplastic cells and many cancer cells, and the fact that PCNA can be stained in both cells (Inoue, 2001). Endometrial carcinomas often accompany metaplastic epithelium adjacent to the foci of the carcinoma in the endometrium. Hendrickson and Kempson (1980a) originally described metaplasia as benign proliferative changes of the endometrium. Many different types of lesions are often misdiagnosed as carcinomas because they are cytologically similar and can be found in association with carcinomas. Metaplastic cells with cytologic atypia can be diagnosed as atypical metaplasia. Papillary-type atypical metaplasia may correspond to EIC, the precursor of serous papillary adenocarcinoma. *p53* mutations may play a role early in the metaplastic pathway of carcinogenesis, whereas they represent a late event in the hyperplasia pathway. Mixed types of carcinoma accompanied by heterologous elements such as endometrioid, serous papillary, and clear cells may develop after initiation. *De novo* carcinogenesis, is the other pathway that Inoue (2001) added to his proposal. G3 types of endometrioid carcinomas sometimes appear to have developed directly from the normal lining or atrophic epithelium of elderly women. Surrounding non-neoplastic epithelial cells are usually atrophic and appear to be abruptly transformed into carcinomas. A few transitional cells from cancerous to normal may correspond to EIC because of negative staining of PTEN and sparse positive staining of p53, found in these transitional epithelia. From these findings, Inoue (2001) gathered that endometrial cancer may arise through at least three pathways of carcinogenesis. However, the WHO classification focuses on hyperplasia as the precursor lesions of endometrial carcinoma and does not allude to other potential precursors (Welch and Scully, 1977).

2.5.1.6 THE CAUSE AND CONSEQUENCE OF MICROSATELLITE INSTABILITY

As can be seen MSI certainly plays a major role in endometrial carcinogenesis, but, the burning question is: What is the cause of this mutator phenotype?
Katabuchi and colleagues were the first to carry out and report on mutational analysis of tissues obtained from MSI positive sporadic human tumours. They set out to determine whether MSI in sporadic endometrial carcinomas was

caused by germline or sporadic mutations in DNA MMR genes (Katabuchi *et al*, 1995). Once these genes are mutated, it results in the cell being unable to repair errors produced during DNA replication. Therefore, these cells with the mutated MMR genes, replicate DNA mistakes more often than normal cells (Matias-Guiu *et al*, 2001). Katabuchi and co-workers carried out this assay on four of the known MMR genes, namely: *hMSH2, hMLH1, hPMS1, and hPMS2* (Katabuchi *et al*, 1995). The results obtained suggested that MSI in sporadic endometrial carcinoma was not caused by mutations in these 4 MMR genes. The results of Katabuchi *et al.* (1995) were the first to imply that MSI in most sporadic endometrial cancer was not the result of mutations in these four MMR genes. This is in contrast to several other researchers who believed they were (Fujino *et al*, 1994; Loeb, 1994; Staebler *et al*, 1998). Katabuchi *et al.* (1995) concluded that MSI may merely serve as an indicator of the 'mutator phenotype' and that tumourigenesis may be due to an increase in the rate of mutations of oncogenes and tumour suppressor genes. This increased mutation rate is thought to be due to the same defect that gives rise to MSI. With regards to the belief that MSI may be due to mutated MMR genes, they presumed that perhaps there are additional genes that when mutated, gives rise to MSI in endometrial carcinoma, as with colorectal cancer. They therefore suggested that further research would be beneficial (Katabuchi *et al*, 1995). Mutated MMR genes have been implicated in the pathogenesis of HNPCC, and since RER+ endometrial carcinoma is common in patients with HNPCC, many researchers suggested that mutated mismatch repair genes may be involved in sporadic endometrial cancer as well, and that this may be an early event (Caduff *et al*, 1996). However, there are also others, as mentioned above, who believe they are not, since less than 10% of these mutations, ie. *hMSH2* and *hMLH1* have been reported in these tumours (Tritz *et al*, 1997; Esteller *et al*, 1999). Many studies have observed a different pathway for the inactivation of the hMLH1 gene in colorectal carcinomas with MSI, linked to silencing of this gene by promoter hypermethylation (Esteller *et al*, 1999). Other studies by Esteller *et al.* (1998) have also associated hMLH1 promoter hypermethylation with the presence of the MSI phenotype in sporadic cases of endometrial cancer and, have led to further independent studies. These researchers analysed 27 uterine endometrioid carcinomas that were previously characterised as MSI +, for promoter hypermethylation of hMLH1. 11 of 12

(91%) MSI + cases were hypermethylated. In contrast, none of the 15 MSI negative tumours demonstrated hMLH1 promoter hypermethylation. Also, 21 atypical endometrial hyperplasia (AEH) cases demonstrated hMLH1 abnormal methylation, demonstrating that this is an early event in the development of endometrial cancer, as AEH is the immediate precursor of endometrioid endometrial carcinoma (Esteller *et al*, 1998; Esteller *et al*, 1999). A more recent study by Hirasawa and colleagues found 28% of their endometrioid endometrial adenocarcinomas exhibited high frequency MSI (Hirasawa *et al*, 2003). 92% of these MSI positive cases were observed to have methylation of the *hMLH1* promoter (Hirasawa *et al*, 2003). By 2000, many reseachers were satisfied that mutated *hMLH1*, *hMSH2* or *hMSH6* MMR genes seem to be implicated as being the cause of MSI in endometrial cancers associated with HNPCC, but *hMLH1* methylation and gene silencing seem to account for MSI in sporadic endometrial carcinoma. At this stage another MMR gene, *MBD4*, had also been implicated in endometrial cancer with MSI (Palmieri *et al*, 2000).

In 2001 Matias-Guiu *et al.* explained how deficient MMR function of hMLH1 and hMSH2 causes MSI and eventual development of tumour, with the "2 hit theory" (Matias-Guiu *et al*, 2001): Patients with endometrial carcinoma who have the HNPCC syndrome have an inherited germline mutation in either the *hMLH1 or hMSH2* gene - the first hit. The development of endometrial cancer only occurs after a deletion or mutation in the contralateral hMLH1 or hMSH2 allele – the second hit, in endometrial cells. It is only after the 2 hits have occurred that the deficient MMR function of *hMLH1* or *hMSH2* results in MSI and eventual development of tumour (Matias-Guiu *et al*, 2001).

This implies that DNA hypermethylation plays a causative role in the development of human tumours. DNA methylation occurs as a post-replicative addition of methyl groups to cytosine residues (Baylin *et al*, 1998), and can be reversed by demethylation. However, defects in DNA methylation, which include hypomethylation and hypermethylation have been identified in human tumourigenesis (Goelz *et al*, 1985). There are many studies that support this role of methylation in cancer (Esteller *et al*, 1999; Ellenson, 1999). Although recent studies have identified a link between MSI and hypermethylation of the hMHL1

promoter, there is much doubt about this theory; some feel it may be associated with silencing of the wild type allele of hMLH1 or that it may just reflect a global methylation abnormality (Ellenson, 1999). Some support the theory that hMLH1 hypermethylation is the cause and not the consequence of MSI, and this data seems to imply that this is an epigenetic alteration related to the inactivation of a subset of genes that drives the neoplastic process (Ellenson, 1999).

By 1996, it had already been established that RER in sporadic colorectal carcinomas is associated with high tumour grade and improved prognosis, but its significance in endometrial cancer was not reported. Caduff *et al.* (1996) in their study determined that the RER phenotype in endometrial carcinoma is associated with high grade and poor prognosis (Caduff *et al*, 1996).

What is the molecular consequence of this mutator phenotype, MSI, in endometrial cancer? The development of MSI in one cell has important molecular implications. The mismatch repair deficiency that is associated with MSI results in the accumulation of myriads of mutations in coding and non-coding DNA sequences. Short-tandem repeats eg. microsatellites are prone to mismatch repair alterations, but are predominantly located in non-coding DNA sequences. Therefore, the presence of insertions or deletions, do not have consequences in the production of abnormal proteins. However, some small short-tandem repeats like mononucleotide repeats are occasionally located within the coding sequence of important genes, and as a result may become potential targets in the progression of MSI and endometrial cancer. *hMSH3*, *hMSH6*, *transforming growth factor β receptor II* (*TGF-βRII*), *bax*, and *insulin-like growth factor II receptor* (*IGFIIR*) are possible targets of this phenomenon. These important genes control signal transduction, apoptosis, and mismatch repair, and contain short mononucleotide tracts in their coding sequences. Mutations in these tracts are seen as secondary events in the mutator phenotype pathway in cancers with MSI, and usually alter the open reading frame of these genes. The ultimate consequence being, the production of abnormal or truncated proteins with altered functions (Matias-Guiu *et al*, 2001). In another study, these researchers detected in 17 of 24 endometrial tumours (70.8%), mutations in one or more of these mononucleotide tracts (Catasus *et al*, 2000). Many other

investigators have also shown this (Risinger *et al*, 1993; Katabuchi *et al*, 1995; Caduf *et al*, 1996; Basil *et al*, 2000; Catasus *et al*, 2000).

The role played by microsatellites in endometrial carcinoma has been widely investigated and reported on by many, and as can be seen, MSI is important in endometrial carcinogenesis. Detection of MSI can aid the pathologist in identifying tumours where the clinician can direct appropriate adjuvant therapy, especially in those cases with an unfavourable prognosis (Burton *et al,* 1998).

2.5.2 ONCOGENES

"It is an old experience that through her errors, Nature often grants us unexpected insights into her secrets which are otherwise a closed domain." - A. Loewy and C. Neuberg (From Uber Cystiurie. Hoppe-Seyler's Physiol Chem. 1904; 43:338-354).

Oncogenes are cancer-causing genes that are derived from proto-oncogenes which are cellular genes that promote normal growth and differentiation (Cotran *et al*, 1999). The discovery of these protooncogenes was not a simple process as they were first discovered as "passengers" within the genome of acute transforming retroviruses. This discovery was made by the Nobel Laureates, Varmus and Bishop. These retroviruses are able to rapidly induce tumours in animals and also have the ability to transform animal cells *in vitro*. On molecular dissection of their genomes, unique transforming sequences (viral oncogenes, *v-oncs*) were discovered, that were not found in the genomes of nontransforming retroviruses. Surprisingly, these *v-onc* sequences, revealed by molecular hybridization, were found to be almost identical to sequences in the normal cellular DNA. This resulted in the concept that retroviral oncogenes were captured by the virus during evolution, through a chance recombination with the DNA of a normal host cell, that had been infected by the virus. Proto-oncogenes are named after their viral homologs because they were initially discovered as viral genes. Therefore each *v-onc* is designated by a three letter word that associates the oncogene to the virus from which it was isolated, eg, the *v-onc* contained in feline sarcoma virus is referred to as *v-fes*, and the oncogene in

simian sarcoma virus is *v-sis*. However, the corresponding proto-oncogenes are referred to as *fes* and *sis* (Cotran *et al*, 1999). The word oncogene was derived from the Greek word, onkos, which refers to a mass or tumour (Jameson, 1998). The process by which proto-oncogenes are activated is called insertional mutagenesis. This occurs when there is proviral insertion near a proto-oncogene, which induces a structural change in the cellular gene, thus converting it into a cellular oncogene (*c-onc*), or strong retroviral promoters may be inserted in the vicinity of the proto-oncogenes, which lead to dysregulated expression of the cellular gene. In other words, proto-oncogenes may become oncogenic by retroviral transduction (*v-oncs*) or by influences that alter their behaviour *in situ*, thereby converting them into cellular oncogenes (*c-oncs*) (Cotran *et al*, 1999). When these genes experience amplification, translocation, or mutation, malignant transformation is facilitated by an increased ability of cells to proliferate in an unrestrained fashion. These oncogene products exist as several classes and are involved in transmitting stimulatory growth signals from the periphery of the cell toward the nucleus (Berchuck and Boyd, 1995).

2.5.2.1 CLASSES AND FUNCTIONS OF ONCOGENES AND THEIR ROLE IN ENDOMETRIAL CARCINOMA

Oncogenes can be classed according to their cellular functions (Jameson, 1998), (Figure 2.7).

1. The overproduction of various growth factors in the extracellular environment can influence cell function. Also important are the extracellular effects of angiogenic factors and proteolytic enzymes, which are not shown.
2. At the cell membrane adhesion molecules such as DCC and APC influence tumour invasiveness. Membrane receptors can be activated or amplified.
3. Ras is a proteolytic example of signal transduction molecule that stimulates proliferation when activated.
4. Nuclear proteins include a number of targets for oncogenesis.

Oncogenes encode proteins that are called oncoproteins. These resemble the normal products of proto-oncogenes, but are devoid of important regulatory elements, and their production in the transformed cell does not depend on growth factors or other external signals. Below follows the sequence of events that characterize normal cell proliferation, which will aid in understanding the nature and functions of oncoproteins:

- Binding of a growth factor to its specific receptor on the cell membrane.
- Limited activation of the growth factor receptor, which then activates several signal-transducing proteins on the inner leaflet of the plasma membrane.
- Transduced signal transmission across the cytosol to the nucleus via second messengers.
- Nuclear regulatory factors induction and activation that initiates DNA transcription.
- Entry and progression of the cell, which results ultimately in cell division.

With this information, oncogenes and oncoproteins can be readily identified as altered versions of their normal counterparts, and grouped on the basis of their role in the signal transduction cascade and cell cycle regulation (Cotran *et al*, 1999).

2.5.2.1.1 GROWTH FACTORS

A number of polypeptide growth factors have been described. These stimulate proliferation of normal cells and are suspected of playing a role in carcinogenesis. However, they do not play major roles in human cancer but illustrate their importance in the signalling pathways for the growth of tumours (Jameson, 1998; Cotran *et al*, 1999). Mutations of genes that encode growth factors can render them oncogenic. An example is the proto-oncogene c-*sis* , which encodes the β chain of platelet-derived growth factor (PDGF), first discovered in the guise of the viral oncogene contained in v-*sis*. Several human tumours, like astrocytomas and osteosarcomas, have now been found to produce PDGF. These tumours also express receptors for PDGF and are therefore subject to autocrine stimulation. In most instances the growth factor gene itself may not be altered or

mutated (Cotran *et al*, 1999). Overproduction of growth factors as a consequence of the actions of other oncogenes, displays their more prominent, functional role (Jameson, 1998). The products of other oncogenes, such as *ras* (found along the signal transduction pathway), cause overexpression of growth factor genes. This forces the cells to secrete large amounts of growth factors, like transforming growth factor-alpha (TGF-α), which is related to epidermal growth factor (EGF). This growth factor induces proliferation by binding to the EGF receptors (Cotran *et al*, 1999). The mechanisms that lead to growth factor overexpression are obscure, but many tumours produce growth factors such as insulin-like growth factor-I (IGF-I), IGF-II, EGF and TGF-α (Jameson, 1998). Besides c-*sis,* another group of related oncogenes that encode homologues of fibroblast growth factors (FGFs) (eg. *hst*-1 and *int*-2), is activated in many gastrointestinal and breast tumours. A member of the FGF family (bFGF) is expressed in human melanomas. Bombesin-like peptides are produced by small cell lung carcinomas that stimulate their proliferation (Cotran *et al*, 1999). Growth factors act in the cell cycle to enhance the entry from the quiescent state (G_0) and to stimulate progression to the restriction point in G_1.

Other growth factors like TGF-β, can oppose entry into the cell cycle, by increasing levels of cyclin dependent kinase inhibitors. Therefore, the balance of growth factors and their receptors activity can have profound effects on cell proliferation. The initiation of apoptosis, which is programmed cell death, is also influenced by growth factors. It is the withdrawal of these growth factors, or other nutrients that favours apoptosis, but their presence prevents it (Jameson, 1998). Besides playing a role in enhancing proliferation associated with malignant transformation, growth factors are also involved in development, stromal-epithelial communication, tissue regeneration, and wound healing (Berchuck and Boyd, 1995). Increased growth factor production is not sufficient for neoplastic transformation, but it is the extensive cell proliferation that contributes to the malignant phenotype by increasing the risk of spontaneous or induced mutations in the cell population (Cotran *et al*, 1999).

- **Growth Factors In Endometrial Carcinoma**

With regards to endometrial cancer, these growth factors are not known to be over-expressed.

2.5.2.1.2 GROWTH FACTOR RECEPTOR TYROSINE KINASES

Several oncogenes that encode growth factor receptors have been found to belong to this next group in the sequence of signal transduction (Cotran *et al*, 1999). Besides serving to transduce the signals of extracellular growth factors, these receptors are also important targets of mutations or altered expression that contribute to tumourigenesis. This tyrosine kinase class of receptors includes oncogenes such as *trk* (nerve growth factor class), *kit* (steel receptor), *ret* (a tyrosine kinase receptor that binds glial derived nerve growth factor), *fms* (colony stimulating 1 receptor), *erbB* (epidermal growth factor, EGF, receptor), and *erbB-2/neu* (c-erb B2) (EGF-related receptor). These receptors act primarily by signalling through the *ras*-MAPK (mitogen-activated kinase) cascade (Jameson, 1998). Several growth factor receptors are transmembrane proteins. With the normal forms of receptor kinases, the kinase activity is activated by binding of their specific growth factors. This is followed by receptor dimerization and tyrosine phosphorylation of several substrates that are part of the mitotic cascade. Alternatively, the oncogenic versions of these receptors are associated with persistent dimerization and activation, but without binding to the growth factor. Therefore, the mutant receptors continuously deliver mitogenic signals to the cell. In human tumours growth factor receptors are activated by several mechanisms, which include mutations, gene rearrangements, and overexpression. Point mutations that activate *c-fms*, the gene encoding the colony-stimulating factor 1 (CSF-1) receptor, have been detected in myeloid leukaemias. However, over-expression of the normal forms of growth factor receptors are far more common than mutations of these proto-oncogenes. The ones most commonly involved are three members of the EGF receptor family. The *EGF receptor* gene, which is the normal form of c-erb B1, is overexpressed in up to 80% of squamous cell carcinomas of the lung, but less commonly in carcinomas of the urinary bladder, gastrointestinal tract, and astrocytomas. This contrasts with the second member of the EGF receptor family, the *c-erb B2* gene

(also called *c-neu*), which is amplified in a high percentage of human adenocarcinomas arising within the breast, ovary, lung, stomach, and salivary glands.

- **Growth Factor Receptor Tyrosine Kinases in Endometrial Carcinoma**

***EGF*:** Among the first growth factor receptor tyrosine kinases to be characterized at a molecular level was EGF and its receptor. The endometrium of menstruating women in the proliferative and seceretory phases has EGF receptor present in the glandular and stromal cells. After menopause, expression is also maintained in atrophic endometrium. It is known that some squamous cancers overexpress EGF receptor due to amplification of the *EGF* receptor gene, but amplification has not been noted in endometrial adenocarcinomas. However, during endometrial carcinogenesis, it appears that loss of EGF receptor may occur. EGF receptor expression was also not associated with clinical features or survival (Berchuck and Boyd, 1995).

Her-2/neu (c-erbB2): It has been found that this oncogene product is overexpressed in 10-15% endometrial cancers and this overexpression was associated with poor outcome (Berchuck and Boyd, 1995). Berchuck and Boyd (1995) found high expression in 12 of the 100 cases studied. They found overexpression to be more common in stage III/IV cases (8 of 34, 24%) relative to stage I/II cases (4 of 66, 6%), and was associated with poor progression-free survival in univariate analysis. The multivariate analysis revealed her-2/neu to be an independent variable only if DNA ploidy was excluded (Berchuck and Boyd, 1995). Overexpression of her2/neu is known to be a significant predictor of poor overall and relapse-free survival in endometrial cancers and correlates with advanced stage disease, poorly differentiated tumours and myometrial invasion (Ioffe *et al*, 1998). Nielsen and Nyholm (1994) found that in their study, 15 of 112 (13%) tumours expressed high p185 (Nielsen and Nyholm, 1994). They also found a significant correlation between high c-erbB2 protein and poor outcome.

***Fms*:** This oncogene which was first identified as the transforming gene of a feline retrovirus has also been shown to encode a receptor tyrosine kinase that serves as a receptor for macrophage-colony stimulating factor (M-CSF). Fms expression was found to correlate with advanced stage, poor grade, and deep myometrial invasion (Berchuck and Boyd, 1995).

2.5.2.1.3 SIGNAL-TRANSDUCING PROTEINS

There are several examples of oncoproteins that mimic the function of normal cytoplasmic signal-transducing proteins. Most of these proteins are located on the inner leaflet of the plasma membrane. Here they receive signals from outside the cell (by activation of growth factor receptors) and transmit them to the cell nucleus. The signal transducing proteins are heterogeneous. The most well studied and best example of a signal transducing oncoprotein is the ras family of guanine triphosphate (GTP) binding proteins (Cotran *et al*, 1999). There are three *ras* genes that were initially discovered in the form of viral oncogenes (*N-ras, H-ras, K-ras*). Later, mutant forms of *ras* were identified as the transforming principle in DNA derived from many types of human tumours. *Ras* is now identified as one of the most common genetic alterations in human tumours. In approximately 10 to 20% of all human tumours, mutated versions of ras proteins are found (eg. leukaemias, melanomas, thyroid cancer, lung cancer, colon cancer) (Malumbres and Barbacid, 2003; Minna *et al*, 2002). It also occurs in 90% of certain cancers like pancreatic cancer (Jameson, 1998; Cotran *et al*, 1999). The *ras* gene is amplified in some tumours, but is more commonly activated by point mutations (Jameson, 1998). *Ras* plays an important role in mitogenesis induced by growth factors. Normal ras proteins are tethered to the cytoplasmic aspect of plasma membrane. They flip back and forth between an activated, signal-transmitting form and an inactive, quiescent state. Ras proteins bind guanosine diphosphate (GDP), in an active state. It becomes activated by exchanging GDP for GTP when cells are stimulated by growth factors, or other receptor-ligand interactions. This activated ras then excites the MAP kinase pathway by recruiting the cytosolic protein raf-1. The activated MAP kinases target nuclear transcription factors and thereby promote mitogenesis. The activated signal transmitting stage of ras protein in normal cells is transient because its intrinsic GTPase activity hydrolyzes GTP to GDP, thus returning ras to its quiescent ground state. Two reactions, which are enzymatically regulated, promote the orderly cycling of the ras protein ie. :

a) Nucleotide exchange (GDP by GTP), which activates ras protein, and
b) GTP hydrolysis, which converts the GTP-bound, active ras to the GDP-bound, inactive form.

The GTPase activity intrinsic to normal ras proteins is accelerated by GTPase-activating proteins (GAPs), which bind to the active ras and augment its GTPase activity by more than 1000-fold. This leads to rapid hydrolysis of GTP to GDP and termination of signal transduction. The GAPs therefore function as "brakes" and prevent uncontrolled ras activity. However, when mutations affect the *ras* gene, the response to this braking action of GAPs seems to falter. These mutated ras proteins bind GAP, but their GTPase activity fails to be augmented. The mutant proteins are therefore trapped in their excited GTP-bound form. This in turn causes a pathologic activation of the mitogenic signalling pathway (Cotran *et al*, 1999).

Besides playing a role in transducing growth factor-initiated activating signals, *ras* is also involved in the regulation of the cell cycle. The passage of cells from G_0 to the S phase is modulated by a series of proteins called cyclins and cyclin-dependent kinases (CDKs), and ras seems to be responsible for controlling the levels of these CDKs (to be discussed later). *Ras* having connections with the cell cycle, is involved in the G_0/G_1 transition and is able to cooperate with cyclin D1 in transforming cells (Hunter and Pines, 1994). There has been much effort made to devise a means by which renegade ras can be controlled, since ras is mutated so often in human cancers. To receive activating signals from growth factor receptors, ras must be anchored under the cell membrane close to the cytoplasmic domain of the growth factor receptors. This anchoring is made possible by the attachment of an isoprenyl lipid group to the ras molecule by the enzyme farnesyl transferase, which forms the bridge between ras and the lipid membrane. *Ras* can be disabled by inhibitors of farnesyl transferase, which prevent its normal localization. Using this technique to inhibit *ras*, farnesylation represents a promising therapeutic strategy for blocking the actions of activated forms of ras (Cotran *et al*, 1999; Jameson, 1998).

Several non-receptor associated tyrosine kinases also function in the signal transduction pathways and mutated forms of these are commonly found in the form of v-*oncs* in animal retroviruses (eg. v-*abl*, v-*src*, v-*fyn*, v-*fes*, etc). Except c-*abl*, the others are rarely activated in human tumours. C-*abl* is known to be activated after DNA damage and may play a role in regulating apoptosis (Cotran *et al*, 1999).

- **Signal Transducing Proteins in Endometrial Carcinoma**

The ras proteins are known to be expressed in normal endometrium, and levels may be up-regulated in some endometrial cancers (Berchuck and Boyd, 1995). In a study conducted by Lax *et al.* (2000) 58 endometrioid and 45 serous papillary endometrial tumours were analysed for mutations at codon 12 of the k-*ras* proto-oncogene. They found mutant k-*ras* in 15 of the 58 endometrioid tumours (26%), but in only 1 of the 45 serous papillary tumours (2%), and the frequency of these mutations were especially high in grade 2 endometrioid tumours compared to grade 1 or grade 3 tumours. Also, two endometrioid and one serous papillary endometrial tumour with k-ras mutations harbored mutant *p53* as well. However, p53 mutations seemed to be more dominant in serous papillary carcinomas. K-*ras* mutations occurred more frequently (32%) in the MSI negative endometrioid tumours, compared to the MSI positive tumours (19%). These findings provided substantial further evidence that distinct molecular genetic pathways were responsible for the development of endometrioid and serous papillary endometrial carcinomas and added support for the separate classification of these two histologically distinct tumours (Lax *et al*, 2002). The research study of Caduff *et al.* (1995) also found *k-ras* codon 12 mutations in 13 of the endometrioid tumours (11.6%), but in none of the 17 serous papillary carcinomas they studied. It has been found that in colorectal carcinomas k-*ras* mutations and aneuploidy do not frequently coexist, suggesting that these mutations may not provide an additional growth advantage to aneuploid tumours. This may explain the lack of *k-ras* mutations in serous papillary carcinomas. *K-ras* mutations, just like MSI were detected in grade 1 endometrioid tumours, suggesting that these alterations are an early event during its pathogenesis. However, MSI and *k-ras* mutations were rarely associated, hinting that there are alternative genetic alterations that play a role early in the development of endometrioid endometrial carcinoma (Caduff *et al*, 1995). Mutter *et al.* (1999) also found *k-ras* mutations in the early premalignant phase of endometrial carcinoma. Lax and colleagues found that *k-ras* mutations may contribute to tumour progression, since they were more frequent in tumours of higher histopathologic grade, and it is known that the constitutive activation of the *ras* pathway leads to increased cellular proliferation (Lax *et al*, 2002). From their research findings they were able to conclude that *k-ras* mutations along with MSI were more common in endometrioid carcinoma

while *p53* mutations were confined to a subset of high-grade tumours, ie. serous papillary carcinoma, thus suggesting that different types of genetic instability may underlie the two types of tumours and predispose them to different genetic alterations. With these findings, a hypothetical, pathogenetic model can be built, suggesting that endometrioid carcinoma develops from complex atypical hyperplasia and progresses in a step-like manner by acquiring MSI and *k-ras* mutations early and *p53* mutations late. Endometrioid tumours contrast with the vast majority of serous papillary carcinomas that have *p53* mutations, demonstrate rare *k-ras* mutations, and lack MSI. Only three different molecular genetic alterations were considered in this hypothetical model, with k-ras playing a key role. This clearly provides further evidence and support to the two different molecular genetic pathways in endometrial carcinoma that were suggested by other researchers (Bockman, 1983; Caduff *et al*, 1996; Sherman, 2000). Lagarda *et al.* (2001) reported mutations in 18.9% of their tumours, all of them being endometrioid carcinoma. A higher frequency of *k-ras* mutations were found in MSI positive carcinomas (42.8%) than in MSI negative tumours (11.4%), indicating that *k-ras* mutations are common in endometrial cancer with the microsatellite mutator phenotype, and adding support to the findings of Lax *et al.* (2000). They detected *k-ras* mutations in only 1 of 22 endometrial hyperplasias. Endometrial carcinoma and atypical hyperplasia both exhibited MLH-1 promoter hypermethylation, but had different *k-ras* mutations. Their findings supported the hypothesis that both *k-ras* and MSI are closely related phenomena that may occur simultaneously before and during clonal expansion. There has been recent evidence that altered methylation in several genes occurs in MSI positive carcinomas, suggesting that MSI is a secondary epigenetic alteration that is triggered by an abnormal hypermethylation of hMLH-1 (Ellenson, 1999). Matias-Guiu and colleagues believe that if this hypothesis is accepted, then the finding of methylation-related GC-AT transitions in *k-ras* in MSI positive endometrial carcinomas, and their low occurrence in MSI negative tumours, should provide some basis for the explanation of the occurrence of *k-ras* mutations in MSI positive endometrioid carcinomas (Matias-Guiu *et al*, 2001). Also, the study of Ito and colleagues in 1996 pointed out that *k-ras* mutations in the postmenopausal age group was statistically significantly associated with patients who died or experienced recurrence (Ito *et al*, 1996). These findings suggest a possible role

for *k-ras* activation in the mechanism responsible for a more aggressive behaviour of endometrioid endometrial cancer that is observed in postmenopausal patients (Ito *et al*, 1996).

2.5.2.1.4 NUCLEAR TRANSCRIPTION PROTEINS

The nucleus is where all signal transduction pathways finally enter and impact on many responder genes that orchestrate the cell's orderly advance through the mitotic cycle (Cotran *et al*, 1999). A family of genes, regulate this process of DNA replication and cell division. Their products are localized to the nucleus, where they control the transcription of growth related genes. Specific amino acid sequences or motifs are contained by the transcription factors that allow them to bind DNA or to dimerize for DNA binding. Helix-loop-helix, leucine zipper, zinc-finger and homeodomains are examples of these motifs. These proteins bind DNA at certain sites from which they are able to activate or inhibit transcription of adjacent genes. Therefore, mutations affecting genes that encode nuclear transcription factors are associated with malignant transformation. Many oncoproteins like the products of *myc, myb, jun* and *fos* oncogenes have been localized to the nucleus, but the *myc* gene is most commonly involved in human tumours. Virtually all eukaryotic cells express the *c-myc* proto-oncogene which belongs to the immediate early growth response genes, that are rapidly induced when quiescent cells receive a signal to divide (Cotran *et al,* 1999). They are involved in the G_0/G_1 transition in the cell cycle, and are implicated in the expression of several cyclin genes (Hunter and Pines, 1994). Specific inhibition of *c-myc* expression by antisense oligonucleotides prevents the entry of cells into the S phase. This highlights the importance of *c-myc* in cell proliferation. With regards to cell replication, after translation, this protein is rapidly translocated to the nucleus. Here it forms a heterodimer with another protein called max. This myc-max heterodimer, which is a potent transcriptional activator, binds to specific DNA sequences called E-boxes. Mad, which is another member of the myc superfamily of transcriptional regulators, can also bind max to form the mad-max heterodimer that functions as a transcription repressor. Therefore, the degree of transcriptional activation by *c-myc* is not only regulated by levels of myc protein, but also by the availability and abundance of max and mad proteins. The myc-

max heterodimer favours proliferation, whereas mad-max inhibits cell growth. Therefore, *mad* may be considered as an anti-oncogene or tumour suppressor gene. *Myc* not only functions to control cell growth, but can also drive cell death by apoptosis. When *myc* activation occurs in the absence of survival signals (growth factors), cells undergo apoptosis. This has therefore been named the "conflict" model where apoptosis occurs when there is a conflict between "stop" (no growth factors) and "go" (*c-myc* is activated). Cell growth and cell death are closely interlinked, and the boundary between these two is quite unstable. During normal cell proliferation there is regulated expression of *c-myc*. This is in contrast to oncogenic versions, which are associated with persistent expression, and in some cases, overexpression of the myc protein. The result being sustained transcription of critical target genes and possibly neoplastic transformation. In Burkitt lymphoma, which is a B-cell neoplasm there is dysregulation of *c-myc* expression resulting from translocation of the gene. *C-myc* is amplified in breast, lung, colon and many other carcinomas. The related *L-myc* and *N-myc* genes are amplified in neuroblastomas and small cell cancers of the lung (Cotran *et al*, 1999).

- **Nuclear Transcription Proteins in Endometrial Carcinoma**

There has been no record of abnormal expression of these proteins in endometrial carcinoma.

2.5.3 CYCLINS AND CYCLIN-DEPENDENT KINASES

Cyclins and cyclin dependent kinases are also oncogenes but are discussed independently as they cover a huge section.

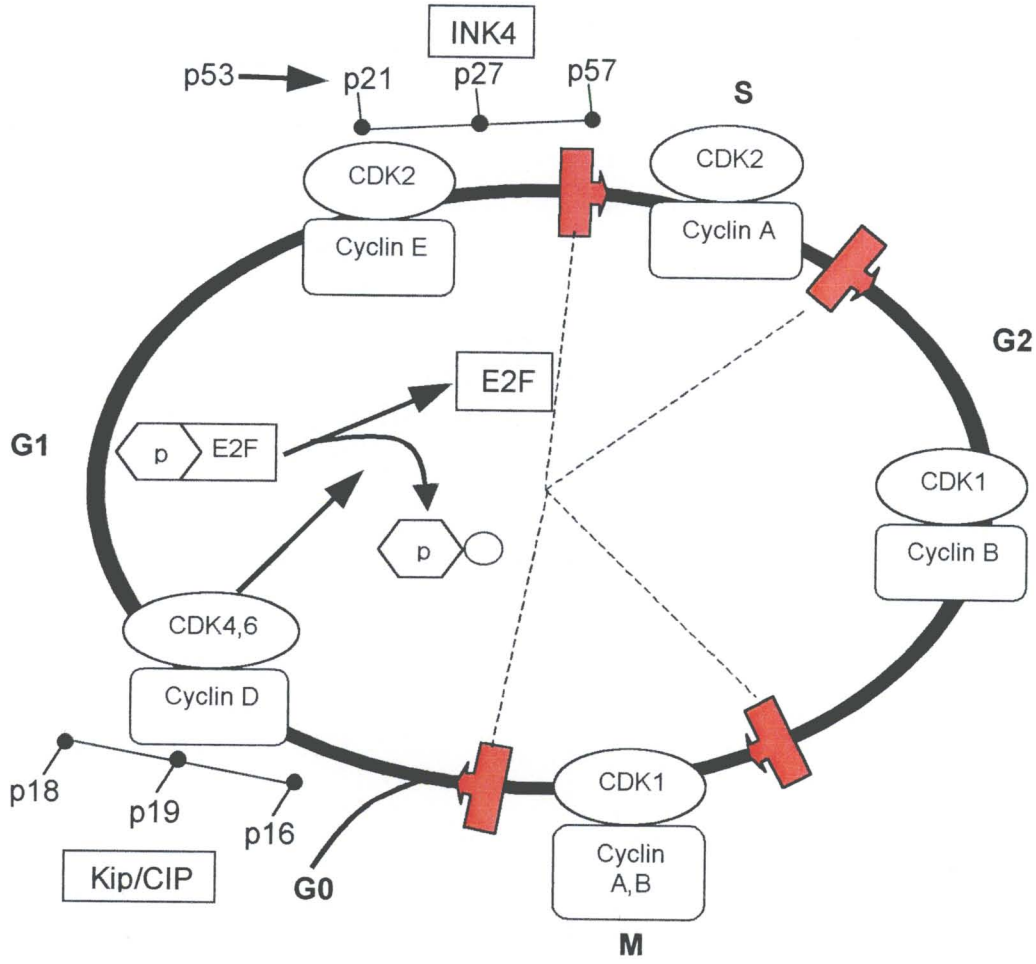


Figure 2.8: The Cell Cycle. (Modified from Clarke and Chetty, 2001).

2.5.3.1 THE CELL CYCLE

The cell cycle (Figure 2.8) is a well-organised sequence of events where repetitive growth and division of cells occur. Molecules involved in the basic machinery of the cell cycle clock include proteins such as cyclins, cyclin dependent kinases (CDKs), regulatory kinases (ie. CDK inhibitors) and phosphatases which are like little workmen that make up this cell cycle (Sherr, 1993; Hirama and Koeffler, 1995; Motokura and Arnold, 1993). These proteins

also include p53 and retinoblastoma (pRb), which will be discussed under tumour suppressor genes. The cell cycle is indeed an impressive machine that describes the series of steps by which a cell coordinates the processes of DNA replication and cell division and is divided into 5 phases:

- **G_1** ("gap_1") phase - (presynthetic), which is a period of growth before DNA replication;
- **S** ("synthesis") phase - (DNA synthesis), as DNA is replicated;
- **G_2** ("gap_2") phase - (premitotic), a period of growth following DNA replication;
- **M** ("mitosis") phase - (mitotic), the period of cell division.

Daughter cells are able to re-enter the G_1 phase once mitosis is complete or they could alternatively proceed to a 5th phase called **"G_0"**. This is a phase where all growth and replication stops. Cells in this G_0 phase are said to be "quiescent", ie. stable. G_0 cells may re-enter the G_1 phase, or eventually die (Benson, 1995; Pines, 1995). Cultured cells have a typical cycle, which lasts approximately 24 hours, but can vary widely. The two leading phases, the S phase (DNA synthesis) lasts 6 hours and the M phase (mitosis) lasts 30 minutes. These two phases are segregated by two gap phases, the G_1 and G_2 phases, which are 12 hours and 6 hours, respectively (Cordon-Cardo, 1995).

The entry of quiescent cells into the cell cycle is the ultimate outcome of all growth-promoting stimuli. The cell cycle is controlled by multiple mechanisms on which exogenous and endogenous stimuli converge. The different cell cycle phases are governed by pathways which are central for the cell's decisions when to commit to DNA synthesis and proliferation versus growth arrest, DNA repair, apoptosis or "suicide" (Funk, 1999). If the cell decides to divide, a set of enzymes and regulatory proteins have to be recruited. These essential tools make up the cell cycle machinery (Hirama and Koeffler, 1995). This orderly progression of cells through the various phases of the cell cycle, is orchestrated by cyclins and cyclin dependent kinases (CDKs), and their inhibitors. Mutations in the genes that encode these cell cycle regulators have been found in several human cancers (Cotran *et al*, 1999). Also, these pathways incorporate various oncogenes and tumour suppressor genes (to be discussed later) and are therefore a central target for genetic alterations in human cancers. Ultimately,

these events may lead to aberrant cell proliferation and increased genetic instability. It is the unraveling of these regulatory networks that provides an important insight into the balance of normal and cancerous cell proliferation and is important for the design of anticancer strategies (Funk, 1999).

It is important to review the normal functions and regulation of cyclins, cyclin dependent kinases and their inhibitors in order to understand the cancer-associated derangements that occur in the cell cycle. It is by phosphorylation of critical target proteins required for progression of the cells to the next phase that cyclin-dependent kinases are able to drive the cell cycle. These CDKs are expressed constitutively in an inactive form during the cell cycle, and are activated by phosphorylation after binding to another family of proteins, called cyclins. Cyclins are only synthesized during specific phases of the cell cycle, in contrast to the CDKs. They function to activate the CDKs, after which their levels decline rapidly. Cyclins arouse the CDKs, while their inhibitors silence them, thus exerting another level of control over the cell cycle. The transition from G_1 to S is a very important checkpoint in the cell cycle clock, as once cells cross this barrier they are committed to progress into S phase. D type cyclins that bind CDK4 and CDK6 are synthesized in the early part of G_1, once a cell receives growth-promoting signals. The synthesis of E cyclin is stimulated later in the G_1 phase, which in turn binds to CDK2. The cyclin D/CDK4, cyclin D/CDK6, and cyclin E/CDK2 complexes phosphorylate the retinoblastoma protein (pRb). The E2F proteins are unshackled by the phophorylation of pRb. They then activate the transcription of several genes whose products are required for the progression through the S phase, ie. DNA polymerases, thymidine kinase and dihydrofolate reductase. The up-regulation of cyclin A facilitates the progress of cells from the S phase into the G_2 phase. Cyclin A binds to CDK2 and CDK1. Cyclin B takes over early in the G_2 phase. It helps the cell move from G_2 to M by forming complexes with CDK1. A variety of proteins required for mitosis are phosphorylated by the cyclin B/CDK1 complex. Two families of cyclin dependent kinase inhibitors (CDKIs) regulate the activity of the CDKs. The one family of the CDKIs is composed of the proteins called, p21, p27 and p57, and inhibits the CDKs broadly. The other family of the CDKI has selective effects on cyclin D/CDK4 and cyclin D/CDK6. P15, p16, p18, p19 are the 4 members of this

family, which are sometimes called INK4 proteins. They are so-called because they are inhibitors of CDK4 and CDK6. From the above information it can be seen that mutations that dysregulate the activity of cyclins and CDKs would favour cell proliferation. Mishaps affecting cyclin D or CDK4 expression are a common event in neoplastic transformation. It is in many cancers, like those of the breast, oesophagus, liver and certain lymphomas, that the *cyclin D* genes are overexpressed. The *CDK4* gene is amplified in melanomas, sarcomas and glioblastomas. However, the mutations of cyclin B, E and other CDKs in malignant neoplasms are much less frequent (Cotran *et al,* 1999).

2.5.3.2 THE CYCLINS

The cyclins are known to be the activating partners of a family of protein kinases, which are the CDKs and are intimately involved with coordinating and regulating DNA replication and cell division. They were originally identified by Tim Hunt in 1983, as proteins that were strongly synthesized after fertilization of marine invertebrate eggs, which were then rapidly and specifically destroyed at each mitosis (Pines, 1995). To date, there are approximately 17 known cyclins, comprising 11 major classes and a few subclasses. These cyclins are labelled A, B1-2, C, D1-3, E1-2, F, G1-2, H, I, K, T1-2 to H (Clarke and Chetty, 2001; Donnellan and Chetty, 1998; Hunter and Pines, 1994; Sherr, 1994). It has been noted that all cyclins contain a characteristic 100 amino acid region of homology, which is called the 'cyclin box', and is located in the amino-terminal region. This part of the protein is involved in binding to the CDK family of protein kinases. Human cyclins also contain a 'destruction box' (D box) or a PEST sequence, which allows protein levels to be regulated through timing-triggered degradation or by constantly rapid turnover. This 'D box', where the proteolysis of cyclins occurs is also located at the amino-terminal region of the proteins (Motokura and Arnold, 1993; Pines, 1995; Clarke and Chetty, 2001; Nguyen, 1998). Two broad classes of cyclins have been defined, according to where they act in the cell cycle, ie. the G_1 or START cyclins and the G_2 or MITOTIC cyclins (Pines, 1995). These different cyclins also attain peak activity during different phases of the cell cycle. Cyclins C, D1-3 and E reach their peak of synthesis and activity during the G_1 phase, and are responsible for regulating the transition from G_1 to S phase,

while cyclins A and B1-2 achieve their maximal levels later in the cycle, during S and G_2 phases, and are regarded as regulators of the transition to mitosis. Cyclin A is actually active in the S and late G_2 phase, and cyclin B1-2 are active in the G_2 phase (Clarke and Chetty, 2001; Donnellan and Chetty, 1998; Cordon-Cardo, 1995; Sherr, 1993).

The role of cyclins and CDKs therefore, seems to be that of sensors and checkpoints for different cells that need to be tightly regulated in cell proliferation. The cyclins and CDKs only allow this orderly progression of a cell through the cell cycle after certain prerequisites have been accomplished, such as mitosis proceeding only after DNA replication has taken place (Sherr, 1994; Nurse, 1994).

2.5.3.2.1 CYCLIN A

Cyclin A is an important positive regulator of the cell cycle (Clarke and Chetty, 2001). This human gene was cloned by its juxtaposition to the chromosomal insertion site of hepatitis B virus DNA in a hepatocellular carcinoma. Cyclin A protein appears just before the beginning of DNA synthesis. It gradually increases until prophase, and is degraded around metaphase, which occurs by way of the ubiquitin-associated pathway (Motokura and Arnold, 1993). During the S phase, cyclin A complexes with CDK2 and with cdc2 in G_2-M transition. It phosphorylates pRb and the nuclear membrane protein, laminin, facilitating nuclear membrane disruption. Cyclin A therefore aids G_1-S and G_2-M phase transition (Clarke and Chetty, 2001). Initially, this cyclin was thought to be one of the mitotic cyclins required for initiation of mitosis. However, the differences between cyclin A and B have now become apparent. The expression of cyclin A precedes that of cyclin B. Cyclin A protein is localized in the nucleus at all times. Even when injected into the cytoplasm of G_1 phase cells, it moves swiftly to the nucleus, while cyclin B on the other hand stays in the cytoplasm early in the cell cycle. Cyclin A binds to $p34^{cdc2}$ and to cdc2-related kinase p33, which is the CDK2 gene product, while cyclin B binds only to $p34^{cdc2}$ kinase (Motokura and Arnold, 1993). In mammalian cells, cyclin A in a complex with CDK2 appears to be required for efficient DNA replication. The following indicates how cyclin A-

cdk2 influences transcription: Cyclin A binds directly to E2F. Through this binding CDK2 is able to phosphorylate the associated DP1 subunit. This phosphorylation event inhibits E2F DNA-binding activity. This suggests that as the cells enter S phase, cyclin A synthesis begins, and this downregulates the transcription of the genes transcribed by E2F that are initiated in late G_1 phase. The synthesis of cyclin A may be influenced by the extracellular matrix. In some respects cyclin A replaces cyclin E, which is rapidly degraded in early S phase. Therefore, cyclin A is also found to be associated with p107 and p130 (which are pRb related proteins) and E2F (transcription factor). These interactions are important for the correct regulation of the cell cycle (Pines, 1995). Cyclin A levels peak later in the cell cycle during the S and M_2 phases. They regulate the S and M phases but not G_1. It does not appear to be essential for the onset of S phase, but disruption of cyclin A function can nonetheless inhibit chromosomal DNA replication and can perturb the normal relationship between S phase and mitosis (Sherr, 1993).

In Endometrial Carcinoma:

Cyclins are known to be overexpressed in human cancers (Kallakury *et al,* 1998). In 1998 Kalakury and colleagues studied 91 endometrial carcinomas to evaluate the immunohistochemical profile of proliferation-associated proteins and to correlate these results with clinicopathologic parameters. Of the 91 endometrial cancers, 74 were endometrioid and 17 were serous papillary carcinomas. The positivity rate of cyclin A was 71% in the serous papillary tumours and 64% in the endometrioid tumours. They also found that cyclin A correlated with patient survival in endometrioid carcinomas on a univariate analysis, but on a multivariate analysis only tumour grade and depth of invasion were independent predictors of outcome (Kallakury *et al,* 1998). Shih and co-workers found that the elevated expression of cyclin A was a characteristic feature of high-grade endometrial carcinomas, which were also associated with p53 expression (Shih *et al*, 2003). This is considered to occur, as the mutated p53 is unable to bind the promoter of cyclin A and may allow uncontrolled expression of cyclin A mRNA. These researchers concluded in their study that positive cyclin A staining was a useful poor prognostic marker for patients with endometrial carcinoma (Shih *et al*, 2003).

2.3.5.2.2 CYCLIN B

The two human B type cyclins are B1 and B2. Just like cyclin A, cyclin B1 and B2 achieve their maximal levels later in the cell cycle, during S and G_2 phases, and are regarded as regulators of the transition to mitosis (Cordon-Cardo, 1995). Cyclin B1 is a 'classical' mitotic cyclin that binds solely to $p34^{cdc2}$ kinase (CDKI), to form the mitosis-promoting factor. It is the primary regulator of mitosis and regulates G_2-M transition. Human cyclin B1 mRNA expression is regulated, in part, transcriptionally through the cell cycle. This protein appears in the cytoplasm late in the S phase and is imported into the nucleus shortly before nuclear envelope breakdown (Clarke and Chetty, 2001; Motokura and Arnold, 1993). This timely import of cyclin B1 might be related to its phosphorylation, which occurs at the onset of M phase. At this crucial time, cyclin B1 appears to navigate $p34^{cdc2}$ kinase to appropriate substrates. Cyclin B1 disappears abruptly at metaphase, through degradation by the ubiquitin pathway. Activation of $p34^{cdc2}$ kinase at the G_2/M boundary not only requires association with cyclin B but also complex modifications by the above mentioned phosphorylation and dephosphorylation steps (Motokura and Arnold, 1993). This cyclin, together with $p34^{cdc2}$, controls both mitotic entry and exit. Removal of the inhibitory phosphates from cyclin B-associated $p34^{cdc2}$ at the G_2/M transition activates the $p34^{cdc2}$ kinase and triggers entry into mitosis. In contrast, exit from mitosis depends upon the abrupt ubiquitin-mediated degradation of cyclin B during anaphase. This results in the release of $p34^{cdc2}$ as an inactive monomer. Just like cyclin A, cyclin B is not available to carry out functions during the G_1 interval. They regulate the S and M phases but not G_1 (Sherr, 1993).

In Endometrial Carcinoma:

Kallakury and colleagues included cyclin B1 as one of the proliferation-associated proteins they evaluated with respect to the immunohistochemical profile and the correlation of these results with clinicopathologic parameters of endometrial carcinomas (Kallakury *et al*, 1998). They found the positivity rate of cyclin B1 to be 24% amongst the 17 serous papillary carcinomas and 26% amongst the 74 endometrioid carcinomas studied (Kallakury *et al*, 1998). To determine which cell cycle regulators are involved in endometrial carcinogenesis, Milde-Langosch and co-workers performed Western blot analysis of five cell-cycle stimulating cyclins.

One of them being cyclin B1 (Milde-Langosch *et al*, 2001). They found upregulation of all analyzed cell-cycle regulators in most tumours compared to normal endometrial tissue. Overexpression of cyclin B1 was associated with a less differentiated phenotype and correlated with cyclin E, cdk2, cdk4, p21, Rb, and Ki67. They concluded that cyclin B1 might be one of the major cell cycle regulators involved in the proliferation and reduced differentiation of endometrial carcinoma (Milde-Langosch *et al*, 2001).

2.5.3.2.3 CYCLIN C

Human cyclin C cDNA was cloned as a result of its ability to rescue yeast mutants defective in G1-cyclins. The encoded protein exhibits a striking conservation between distant species such as *Drosophila* and human (72% identity). Based on this apparently strong constraint from evolutionary divergence, cyclin C may well have a critical role in cell cycle control. Cyclin C mRNA is detected in synchronised HeLa cells, peaking in mid-G_1 well before cyclin A. Thus suggesting a role in the G_1 phase (Motokura and Arnold, 1993). Cyclin C is known to reach its peak synthesis and activity during the G_1 phase and, apparently regulates the transition from G_1 to S phase. Cyclin C levels oscillate only minimally throughout the cell cycle, with very modest increases observed in early G_1 (Cordon-Cardo, 1995; Sherr, 1993). Human G_1 cyclins, ie. cyclins C, D and E, have a PEST sequence at their carboxyl termini. This PEST sequence seems to be important in protein degradation, and is present in the G_1 cyclin, CLN3 of budding yeast, and in a candidate G_1 cyclin cig 1, of fission yeast. It has proved to be necessary for appropriate function in the G_1 phase, probably conferring instability upon these proteins (Motokura and Arnold, 1993). It was already known that cyclin C belongs to the cyclin family of proteins that control cell cycle transitions through activation of specific catalytic subunits, the cyclin dependent kinase (CDKs). Since the role of cyclin C and its partner CDK8 in cell cycle regulation was not known, in 2001 Barette *et al.* set out to investigate this. The cyclin C-CDK8 complex was found to be associated with RNA polymerase II transcription machinery, and their results indicated that cyclin C has apparently diverged from other cyclins in the regulation of its stability by its CDK partner (Barette *et al*, 2001).

In Endometrial Carcinoma:

There are no reports of any significant role played by cyclin C in endometrial carcinoma.

2.5.3.2.4 CYCLIN D

Human *cyclin D1* was originally identified as a putative proto-oncogene (*PRAD 1*) that is clonally rearranged with the parathyroid hormone gene in parathyroid adenomas, therefore the name *PRAD1*, and by its ability to rescue G_1 cyclin-defective yeast. There are three members that make up the D-type cyclin gene family, *cyclin D1, D2, D3*, which are expressed redundantly and differentially in several cell lines (Motokura and Arnold, 1993). D-type cyclins have a very short half-life (30 min). Their synthesis is highly growth-factor dependent and when growth factors are withdrawn, cyclin D synthesis ceases immediately (Pines, 1995). This finding has led to the idea that D-type cyclins act as growth-factor sensors (Sherr, 1993). Not surprisingly, therefore, D-type cyclins are the cell cycle components that have been most closely linked to oncogenesis (Motokura and Arnold, 1993). Unlike the periodically expressed E, A and B type cyclins, D-cyclins do not represent integral components of the cell cycle clock (Sherr, 1993). They exhibit only moderate oscillations during the cell cycle, reaching their peak synthesis and activity during the G_1 phase, and apparently regulating the transition from G_1 to S phase. D-type cyclins generally appear earlier in G_1 than cyclin E (Sherr, 1993; Sherr, 1994; Cordon-Cardo, 1995). Cyclins D1, D2 and D3 are regarded as key regulators of G1 progression in mammalian cells (Sherr, 1994). They link mitogenic stimuli and cell cycle machinery, and act as rate-limiting agents for cell cycle progression. These mitogenic stimuli induce expression of genes encoding the D cyclins, which assemble into holoenzymes with either CDK4 or CDK6, and traverse the cell membrane where phosphorylation by CDK activating kinases (CAK) empowers them to phosphorylate protein substrates and tether some CDKIs (Motokura and Arnold, 1993). The phosphorylation of one such substrate, the retinoblastoma protein, induces its inactive state with subsequent release of E2F transcription factors. Specific proteins are induced by these factors, including those involved in DNA synthesis as well as cyclins E and A (Weinberg, 1995). Cyclin E, which combines

later in G_1 with CDK2, ensures phosphorylation of Rb protein at additional sites, thus augmenting the process. The G_1-phase cyclins also induce cyclin B which, together with cyclin A, mediates G_2-M phase transition and maintains Rb in a hyperphosphorylated form, until mitosis is complete (Krek *et al*, 1995; Guadagno *et al*, 1996). Without being subject to inhibition, cyclin D/CDK4 also tethers CDKIs of the KIP/CIP family, which relieves cyclin E/CDK2 from KIP constraint later in the cycle. This allows it to augment inactivation of pRb and to phosphorylate and degrade p27 (Sherr and Roberts, 1999; Vlach, 1996). Overexpression of D-type cyclins can contract G_1, decrease cell size, and reduce the dependency of the cell on mitogens, with their functions likely being quite different (Ohtsubo and Roberts, 1993). Many differentiated mammalian cells can withdraw from the cell cycle, after removal of mitogens, with an unduplicated DNA content, and persist in a quiescent state (G_0) in which macromolecular synthesis is reduced. Other cells undergo apoptosis. The three D-type cyclins are induced in a cell lineage-specific manner as part of the delayed early response to mitogens. It is unclear whether they carry redundant functions in response to different signals or whether their roles are distinct. They are rapidly degraded when mitogens are withdrawn, regardless of the position of the cell in the cycle. During the G_1 phase their destruction in response to growth factor deprivation, results in the failure of cells to enter S phase. However, their degradation later in the cycle is without effect. Although D-type cyclin synthesis begins during the G_0 to G_1 transition, the associated kinase activity is not manifest until mid-G_1, and increases as cells approach the G_1-S boundary (Sherr, 1994; Matsushime *et al*, 1994). The three types of D cyclin, D1, D2, and D3, are cell type specific. Most cells express D3, and either D1 or D2 (but not all three) (Pines, 1995). *Cyclin D1* is the *CCND1* gene and maps to chromosome 11q13. It also is the most likely candidate for the *Bcl1* proto-oncogene. Wild-type cyclin D1 is overexpressed as *PRAD1* because of a chromosomal inversion, inv 11(p15; q13), such that it comes under the control of the parathyroid promoter. These are benign, noninvasive tumours which suggests that overexpression of cyclin D1 is a purely proliferative phenomenon. Translocation at the *Bcl1* breakpoint, t(11;14)(q13;q32), in mantle cell lymphomas, brings cyclin D1 under the influence of the immunoglobulin heavy-chain enhancer (Pines, 1995). This is characteristic of mantle cell lymphomas, and was originally thought to be identical to the cyclin

D1/PRAD1 gene. However, it has now been shown to reside 110-130kb upstream or centromeric to the *PRAD1* gene (Rosenberg *et al*, 1991). *Cyclin D2*, the *CCND2* gene, maps to chromosome 12p13. It has been identified as the vin-1 site of integration of a murine provirus in retroviral-induced mouse T cell leukaemias, which leads to cyclin D2 overexpression. Human *cyclin D3*, the *CCND3* gene, maps to chromosome 6p21, a region that is rearranged in several lymphoproliferative disorders. Overexpressing D-type cyclins alone is not sufficient to transform a cell. Cooperation from oncogenes is also required for tumourigenesis (Pines, 1995). D-type cyclins are important in the cell cycle regulation of the retinoblastoma (pRb) tumour suppressor protein, and they are able to bind to this protein through an L-X-C-X-E motif in their N-terminus (Dowdy *et al*, 1993). The phosphorylation of pRb during late G_1 phase reverses the growth suppressive effects of pRb by untethering E2F from its inhibitory constraint, thereby allowing the activation of genes required for DNA replication (Nevins, 1992). Cells that lack a functional pRb have significantly lower amounts of cyclin D1 and cyclin D1-CDK4 complexes. This means that the hypophosphorylated pRb is involved in the stimulation of cyclin D1 transcription (Muller, 1994). A negative feedback loop therefore seems to exist in which cyclin D1 synthesis and activation lead to pRb phosphorylation, and this in turn, causes decreased cyclin D1 expression (Lukas *et al*, 1994).

In Endometrial Carcinoma:

In 1996 Nikaido and co-workers investigated the role of cyclin D1 in the development of human uterine endometrial carcinoma. Cyclin D1 expression was restricted only to a few cells of normal and hyperplastic endometrium, whereas it was preferentially expressed in 40% of endometrial carcinomas. The cells that overexpressed cyclin D1 also overexpressed p53. The data they obtained suggested that coabnormal expression of cyclin D1 and p53 protein may contribute to the development of endometrial carcinoma and may also be involved in the progression to malignancy (Nikaido *et al*, 1996). In another study, conducted by Ito *et al.* (1998), immunoreactivity of cyclin D1 was observed exclusively in the nuclei of tumour cells in 56% of endometrioid endometrial carcinoma. They found that in contrast to breast carcinoma, cyclin D1 overexpression was not considered to play an important role in the biological

features of human endometrioid endometrial carcinoma (Ito *et al*, 1998). Since the pattern of cyclin D1 expression is not well defined in normal, hyperplastic, neoplastic, and metaplastic endometrium, Quddus *et al.* (2002) in a recent study, set out to investigate this. They found cyclin D1 to be significantly overexpressed in glands with complex hyperplasia and endometrioid adenocarcinoma compared with proliferative or secretory endometrium and simple hyperplasia (Quddus *et al*, 2002). Significant overexpression was also noted in papillary, syncytial, and squamous epithelia compared with normal surface epithelium or epithelium with tubal metaplasia. With this evidence they concluded that overexpression of cyclin D1 increases from normal endometrium to hyperplasia and carcinoma, suggesting that it may play a role in endometrial carcinogenesis. Also, the overexpression of cyclin D1 in endometrial glands was independent of overexpression of cyclin D1 in surface metaplastic epithelium (Quddus *et al*, 2002). Semczuk and co-workers also noted overexpression of cyclin D1 (Semczuk *et al*, 2004). They found overexpression in 50% of the endometrial tumours tested, suggesting that this cyclin participated in endometrial cancer development in humans (Semczuk *et al*, 2004). In a more recent study, Nishimura *et al.* (2004) found that cyclin D1 expression in endometrioid endometrial adenocarcinoma significantly correlated with histological grade and proliferative activity, but not with prognosis or clinicopathological parameters. This is in keeping with the study of Shih and co-workers who noted positive staining of cyclin D1 to be significantly frequent in advanced-stage and higher grade tumours (Shih *et al*, 2003).

2.5.3.2.5 CYCLIN E

Cyclin E was identified by its ability to rescue G_1 cyclin-defective yeast (Motokura and Arnold, 1993). The E-type cyclins consist of cyclins E1 and E2. They are thought to act after the D-type cyclins at the G_1-S transition and are important in the initiation of DNA replication (Ohtsubo and Roberts, 1993). Cyclin E reaches peak synthesis and activity during the G_1 phase and apparently regulates the transition from G_1 to S phase (Cordon-Cardo, 1995). E-type cyclin expression is periodic, and like the D-type cyclins, overexpressing cyclin E only moderately advances entry into the S phase (Ohtsubo and Roberts, 1993). However, it has

been suggested that cyclin E regulates a different aspect of G_1 phase compared with the D-type cyclins (Pines, 1995). The E-type cyclins bind and activate CDK2, and this complex is essential for the cell to begin DNA replication (Dulic *et al*, 1992). The effect of cyclin E-CDK2 may be on the transcription of genes required for S phase because it is associated with the E2F transcription factor in a complex with the Rb-related proteins p107 and p130 (Lees *et al*, 1992; Hannon *et al*, 1993). Cyclin E is another rate-limiting regulator of the G_1 phase of the cell cycle. Appropriate regulation of this cyclin is esssential for S-phase transition and numerous processes that determine accuracy of chromosome replication (Sherr, 1994). It may induce S phase in a number of ways. By driving the cell cycle through phosphorylation of Rb, p107, and p130 and subsequent release of E2F and transcription of key proteins, it can play a role similar to that of D1. Alternatively, cyclin E may use pathways that do not involve pRb. Cyclin E kinase activity and S-phase transition can be induced by c-myc and ras expression, by antagonizing p27, which is known to inhibit cyclin E/CDK2. Other transcriptional regulators, such as B-Myb and Rpat may then be phosphorylated by the uninhibited complex (Duronio *et al*, 1995, Duronio *et al*, 1996; Leng *et al*, 1997; Lukas *et al*, 1997; Leone *et al*, 1997). The cyclin E-CDK2 complex is the target of a number of negative growth factors, which arrest cells in G1 phase. Prominent among these is TGFβ, which unmasks the CDK inhibitor p27 (Pines, 1995).

In Endometrial Carcinoma:

Aberrant expression of cyclin E has been demonstrated in endometrial carcinoma. Li and colleagues found definite positive staining of cyclin E in cancer cells, and concluded that abnormalities of this cyclin in the develpoment of endometrial carcinoma exists, which may correlate with the progression of the malignant process (Li *et al*, 1993). In another study by Ito *et al.* (1998) immunoreactivity was detected in 37 (95%) of the 39 endometrioid endometrial carcinoma cases studied. In a recent study conducted by Milde-Langosch and co-workers, upregulation of cyclin E was found in most of the tumour cases studied (Milde-Langosch *et al*, 2001). The overexpression of cyclin E was associated with a less differentiated phenotype. From these results they suggested that cyclin E might be a major cell cycle regulator involved in

proliferation and reduced differentiation of endometrial carcinomas (Milde-Langosch *et al*, 2001). A striking association noted by Oshita and colleagues was the increased expression of cyclin E in the tissues from postmenopausal women with endometrial cancer as compared to normal postmenopausal endometrium (Oshita *et al*, 2002). Their study supported the view that increased cyclin E expression plays a role in Type II endometrial cancer formation (Oshita *et al*, 2002). Cassia and co-workers also found that cyclin E overexpression occurs in a subset of endometrial carcinomas (Cassia *et al*, 2003). Their study analysed amplification of the *cyclin E* gene (*CCNE*) and mutation in *hCDC4*, the gene coding for the F-box protein, which tags phosphorylated cyclin E for proteosomal degradation. This was done to ascertain whether these alterations might be responsible for cyclin E overexpression in endometrial cancer. Cyclin E overexpression was found in 32 % of cases and was associated with the histological type of the lesion. 54.5% of the non-endometrioid carcinomas showed overexpression compared to only 27% of endometrioid carcinomas, while none of the atypical endometrial hyperplasias overexpressed cyclin E. Amplification of the *cyclin E* gene (*CCNE*) was found in 16% of endometrial carcinoma cases studied and was also seen more frequently (83%) in the non-endometrioid carcinomas displaying an association with histological type of the lesion. They found *hCDC4* gene mutation in one endometrioid endometrial carcinoma. This study was the first to demonstrate that cyclin E overexpression is associated with gene amplification in endometrial carcinomas. These alterations occurred more frequently in non-endometrioid endometrial carcinomas (Cassia *et al*, 2003). Hubalek and co-workers also have suggested that impairment of cell cycle regulated proteolysis of cyclin E may be linked to carcinogenesis by promoting genomic instability (Hubalek *et al*, 2004).

2.5.3.3 THE CYCLIN DEPENDENT KINASES

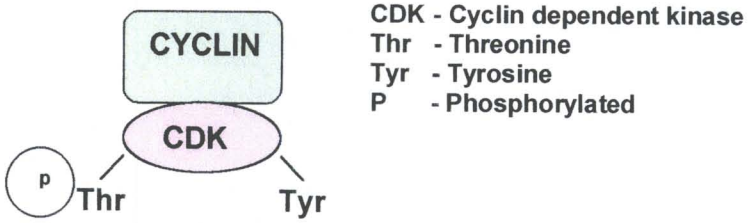


Figure 2.9: Structure of an active CDK. Activation of a CDK requires binding of a cyclin to a catalytic CDK subunit that is phosphorylated on a conserved Thr residue. This subunit also needs to be dephosphorylated on a Tyr residue. (Adapted from Peter and Herskowitz, 1994).

At the centre of the cell cycle engine are a group of protein kinases. These are the cyclin dependent kinases, which move cell proliferation forward by phosphorylating specific substrates in a cell cycle dependent fashion. However, in order to become active protein kinases, a CDK subunit must associate with a cyclin subunit to form a heterodimeric molecule (Figure 2.9). Complexes of CDKs and cyclins ensure progression through various cell cycle transitions (Donnellan and Chetty, 1998; Ohtsubo and Roberts, 1993). The current model for the enzymatically active CDK complexes, consist of a catalytic CDK subunit, typically 34 kDa, and a cyclin as a regulatory molecule. Their regulatory function is achieved by phosphorylation of fundamental elements involved in cell cycle transitions, such as the retinoblastoma protein (to be discussed later) (Peter and Herskowitz, 1994, Cordon-Cardo, 1995). The CDKs are a family of serine/threonine kinases of which nine are currently known (CDK1 to CDK9), and act at different transitions in the cell cycle (Clarke and Chetty, 2001; Nguyen, 1998). They co-ordinate specific transitions that occur at defined times during the cycle. Hartwell originally described CDKs in his studies of the budding yeast *Saccharomyces cerevisiae* (Hartwell, 1978). The genes encoding these proteins were named cell-division cycle or *cdc* genes. *Cdc2* was one of the first *cdc* genes to be isolated, and was found by Nurse during studies of the fission yeast, *Schizosaccharomyces pombe* (Nurse, 1981). This gene encodes a 287 amino acid protein with high homology to protein kinases. The yeast *S. cerevisiae* contains a single cell cycle CDK (cdc28), whereas *S. pombe* (cdc2) and

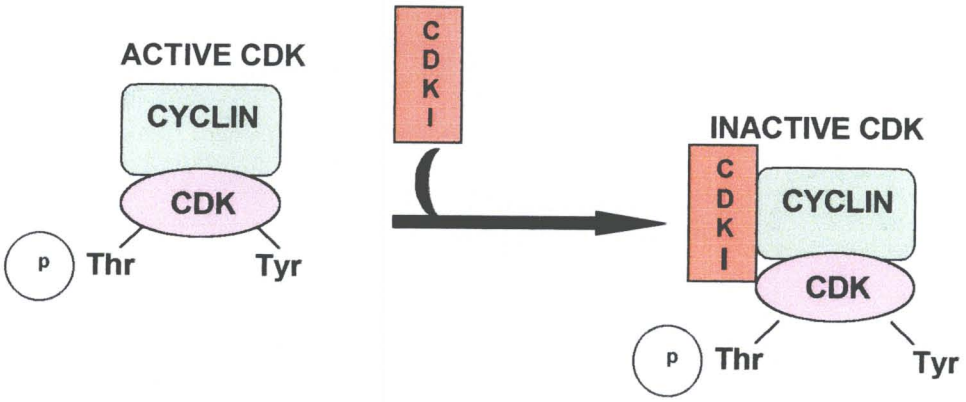
mammalian cells have several (Nguyen, 1998). Ensuing cell rest or division is dependent on the activity of cyclins and their catalytic cyclin-dependent kinases. Events such as DNA replication, segregation, and cell division are initiated by the kinase-mediated phosphorylation reactions, and thus determine cell cycle phase (Clarke and Chetty, 2001). The activities of the nine different CDKs (CDK1-9) acting in concert with their cyclin partners vary with successive phases of the cell cycle. For instance, CDK1, which is also known as cell division control molecule 2 (cdc2), bound to cyclin B has maximal activity at the G2/M transition. CDK2 is important before and during the S phase, binding to cyclin E and A respectively, while CDK4 and CDK6 preferably associate with the D type cyclins during the G1 phase (Donnellan and Chetty, 1998). However during the cell cycle, the CDK levels show minimal variation, suggesting it is the cyclins with their phase dependent expression that drive the cycle (Clarke and Chetty, 2001). For orderly cell division, these CDKs have to be activated and inactivated in an intricate manner at specific time points during the cell cycle. This mechanism of CDK regulation was first analyzed in detail on the cyclin B/Cdc2 complex, which is also known as maturation-promoting factor, and whose activity is necessary for cells to enter the M phase (Hirama and Koeffler, 1995).

2.5.3.4 CYCLIN DEPENDENT KINASE INHIBITORS (CDKI'S)

One of the most significant developments has been considered to be the isolation of a family of small cyclin-dependent kinase (CDK) inhibitor proteins (CDKIs), (Figure 2.10), that bind and inactivate the CDKs (Hunter and Pines, 1994). Studies over the years have converged to identify this additional regulatory subunit for the CDKs, a protein that binds to the CDK-cyclin complex and inhibits its activity. These proteins, termed CDK inhibitory proteins are involved in putting a brake on the cell cycle. Some play a role in response to extracellular signals, whereas others appear to function in intrinsic steps of the cell cycle (Peter and Herskowitz, 1994). They are of utmost interest because by negatively regulating the CDK activities at appropriate time points in the cell cycle, they can play a key role in controlling progression of the cell cycle (Hirama and Koeffler, 1995). The mechanism by which they perform their function appears to be the formation of stable complexes that inactivate the catalytically operative units (Cordon-Cardo,

1995). First, a CDKI associates in vivo with the catalytic subunit, which is the cyclin, or the CDK complex. Second, a CDKI binds to a CDK complex in vitro, inhibits its activity toward exogenous substrates, and interferes with activation of the CDK, or both. Third, a CDKI does not covalently modify either the cyclin or the catalytic subunit (Peter and Herskowitz, 1994). CDKIs form two functional groups based on their structures and CDK targets:

- The **KIP/CIP family,** which comprises **p21, p27** and **p57** and,
- The **INK4 group,** which comprises **p15, p16, p18** and **p19.**



CDK - Cyclin dependent kinase
CDKI – Cyclin dependent kinase inhibitor
Thr - Threonine
Tyr - Tyrosine
P - Phosphorylated

Figure 2.10: The inhibition of an active CDK by a CDKI. CDKIs are able to bind to and inhibit CDK activity when added to fully active CDKs. (Adapted from Peter and Herskowitz, 1994).

The INK4 proteins (inhibitors of CDK4), are so named for their ability to specifically inhibit the catalytic subunits of CDK4 and CDK6. They are composed of multiple ankyrin repeats and bind only to CDK4 and CDK6, but not to other CDKs or to D-type cyclins. These proteins can be contrasted with the more broadly acting inhibitors of the KIP/CIP family whose actions affect the activities of cyclin D, E and A-dependent kinases. All members of the KIP/CIP family contain characteristic motifs within their amino-terminal moieties that enable them

to bind both to cyclin and CDK subunits. P21 also binds PCNA (Proliferating cell nuclear antigen), which is important for DNA replication. As can be seen the two classes of CDKIs appear to exert their inhibiting effects in different ways. The KIP/CIP family are capable of binding to and inhibiting most cyclin-CDK complexes, while the INK4 molecules seem to be specific inhibitors of complexes containing cyclin D. In fact, the INK4 molecules probably compete with the D type cyclins for binding to their CDK partners. Initially, the CDKIs of the KIP/CIP family were thought to interfere with the activities of cyclin D, E and A dependent kinases. However, more recent work has altered this view. It has been revealed that although the KIP/CIP proteins are potent inhibitors of cyclin E and A-dependent CDK2, they act as positive regulators of cyclin D-dependent kinases, by increasing their stability and directing the cyclin D/CDK complex to the nucleus. This helps to explain some enigmatic features of cell cycle control that also involve the functions of the retinoblastoma protein (pRb) and the INK4 proteins. In general, the amount of CDKI present provides a threshold, which the catalytically operative cyclin-CDKs must overcome to drive the cell cycle forward (Donnellan and Chetty, 1998; Sherr and Roberts, 1999; Clarke and Chetty, 2001).

2.5.3.4.1 P21

P21 has been described as the first and best characterised member of the CDKI family, which inactivates cyclin E-CDK2, cyclin A-CDK2, and cyclins D1-, D2- and D3-CDK4 complexes. These are components of the regulatory kinases that target pRb for phosphorylation (Cordon-Cardo, 1995). The p21 protein is also known as WAF1, CAP20, and CIP1 and was identified by its ability to bind to these CDK2-cyclin complexes and functions as a CDKI to inhibit their activity. It was also identified because expression of its gene (*SDI1*) is increased in senescent (old, mature) cells (Peter and Herskowitz, 1994; Harper *et al*, 1993). The *p21/WAF1* gene maps to 6p21.1 and encodes a 164-amino acid protein. (Cordon-Cardo, 1995). The Cip1 mRNA is ubiquitously expressed in all adult human tissues (Harper *et al*, 1993). This gene is a target for p53, the p21 promoter has a p53 binding site, and p21 transcription is activated by wild-type but not mutant p53 (Hunter and Pines, 1994; El-Deiry *et al*, 1993). *P53* (to be

discussed later) is necessary for a G_1 checkpoint that monitors damaged DNA. DNA damage causes the activation of p53, which then induces transcription of the *p21* gene. This leads to transient cell cycle arrest by the inhibition of CDKs (Peter and Herskowitz, 1995). Since *p21* acts downstream to *p53* it has been suggested that this gene might act as a tumour suppressor as well (Hirama and Koeffler, 1995). However, a p53-independent pathway could also accomplish the induction of p21. The results of Cordon-Cardo (1995) have suggested the existence of two separate pathways for the induction of p21, linked to either DNA damage or cellular mitogens. The link between p53 and p21 suggests that CDKIs function in checkpoint control of the cell cycle, at least for the G1/S transition. P21 not only inhibits CDKs, but also proliferating cell nuclear antigen (PCNA). It can directly inhibit DNA replication in vitro (in the absence of CDK) by binding to PCNA. Therefore, p21 may play a dual role in response to DNA damage. By inhibiting CDKs, it prevents entry into S phase and may directly block ongoing DNA replication by inhibiting PCNA (which activates DNA polymerase) (Peter and Herskowitz, 1995). This is an interesting hypothesis concerning the function of p21, as a response to DNA damage during the G1 period, p21 can inhibit cells from entering the S phase by interfering with CDK activities. Also, for DNA damage during the S phase, p21 can stop DNA synthesis by inactivating PCNA, thus allowing cells an opportunity for repairing DNA (Hirama and Koeffler, 1995). The study of Waga *et al.* in 1994 proved that p21 directly inhibits PCNA- dependent DNA replication in the absence of a cyclin/CDK and that it blocks the ability of PCNA to activate the principal replicative DNA polymerase. In addition to its already established role as a CDK inhibitor, these results demonstrated a previously unrecognised role for p21 in the control of DNA replication. This implied that p21 could be a key governor of DNA replication, DNA repair and the cell cycle machinery (Waga *et al*, 1994).

- **In Endometrial Carcinoma:**

In 1997 Ito and colleagues studied 75 patients with endometrial carcinoma to investigate the relationship between p21 expression and the functional status of p53, and the usefulness of p21 as a prognostic marker (Ito *et al*, 1997). They used the polymerase chain reaction and immunohistochemical staining in their study. They detected immunoreactivity for p21, and p53 proteins in 47 (62.7%)

and 37 (49%) patients, respectively. p53 mutations was observed in 23 (31%) patients. However, there were no significant correlations between the presence or absence of p21 immunoreactivity, p53 overexpression and DNA mutations. The survival curves, on the other hand, revealed that patients with p53 overexpression had a poorer prognosis than those without, and those with p53 mutations were worse off than those without. In contrast to the above, patients with p21 expression tended to have a better prognosis than those without p21 expression. The authors therefore concluded that the immunohistochemical analysis of p21 was not useful for evaluating the functional status of p53 in patients with endometrial carcinoma, but both p21 expression and p53 abnormalities were considered as prognostic indicators in patients with endometrioid endometrial carcinoma (Ito *et al*, 1997). In another study of 16 endometrioid endometrial carcinoma cases, Burton *et al*, set out to investigate the relationship between p53 overexpression and p21 expression (Burton *et al*, 1999). Cases selected were devoid of *p53* mutations. They found that factors other than *p53* were involved in the regulation of p21 expression in endometrioid endometrial adenocarcinoma without *p53* mutations. Also, there was no significant correlation demonstrated between p53 and p21 immunoreactivity. However, they suggested follow-up studies with a larger data set for the future (Burton *et al*, 1999).

2.5.3.4.2 P27

P27/Kip1 is another member of the Kip/Cip family that is regarded as a cyclin-dependent kinase inhibitor of the G1 to S cell cycle progression by suppressing the kinase activity of cyclin/cyclin-dependent kinase complex. It is also predicted to act as a tumour suppressor (Watanabe *et al*, 2002). It is a negative regulator that is implicated in G1 phase arrest by TGFβ, cell-cell contact, agents that elevate cyclic AMP, and the growth inhibitory drug, rapamycin. Its expression is increased in numerous circumstances, including loss of cellular adhesion (Cordon-Cardo, 1995; Clarke and Chetty, 2001). P27, which is highly conserved among human, mink and mouse, shares sequence homology with p21/WAF and its encoded product consisting of 198 amino acids. It is the N-terminus that is related to p21, and it also has a putative bipartite nuclear localization signal near

its carboxyl terminus. This CDKI associates with cyclin E-CDK2, cyclin A-CDK2, and cyclin D-CDK4 complexes abrogating their activity. P27 also acts as a stoichiometric inhibitor of G_1 cyclin-CDKs. Even the slightest changes in the relative levels of p27 can have a major effect on G1 progression. This gene has been mapped to 12p12-12p13.1. Overexpression of p27 leads to cell arrest in the G_1 phase. During the cell cycle, its level of mRNA expression does not fluctuate. However, p27 changes its abundance in response to external signals. Research studies on p27 mutations, by Cordon-Cardo (1995) and Hirama and Koeffler (1995) have found that the structural alteration of this gene is either very rare or not found at all.

- **In Endometrial Carcinoma:**

In 1999, Bamberger and co-workers set out to investigate the expression of the cell cycle inhibitor, p27, in endometrial carcinoma. The expression of p27 in endometrial carcinoma was compared to that of the normal endometrium throughout the cell cycle. The normal endometrial cells showed strong nuclear expression of p27 throughout the cycle, being stronger during the secretory phase. However, in the 41 tumour cases analysed, 85.3% were found to have a strongly reduced or abolished expression of p27. All the p27 positive tumours were well-differentiated (grade 1) endometrioid carcinomas (Bamberger *et al*, 1999). Schmitz *et al.* (2000) examined the expression status of p27 amongst other genes in 21 uterine serous papillary carcinoma specimens to determine its role in the development of this disease. They observed a high incidence of p27 alterations, with reduced p27 expression measured in 16 of 21 (76%) tumours. The p27 abnormalities occurred at an early stage of the disease, with 63% of Stage 1 cases displaying reduced p27 expression. They found that the uterine serous papillary carcinomas displayed a high incidence of p27 abnormalities, thus suggesting that p27 abnormalities play an important role in the development of this neoplasm (Schmitz *et al*, 2000). In another study, Nycum *et al.* (2001) examined the prognostic value of p27 protein expression in endometrial cancer in 95 patients. Their study showed decreased p27 protein staining in endometrial cancers compared to normal endometrial tissue. Furthermore, they also found that p27 protein staining showed no correlation with stage, age, or histology and was not prognostic for survival in advanced endometrial cancers. This is a slight

contradiction to the findings of Schmitz *et al.* (2000), but they did suggest that there might be a trend of increased p27 protein staining with advanced grade of tumour (Nycum *et al*, 2001). In a recent study Watanabe and colleagues set out to investigate p27 expression in the normal endometrium and endometrioid adenocarcinoma of the uterine corpus and correlate its expression with cell proliferation and clinicopathological parameters (Watanabe *et al*, 2002). They used tissue samples of 127 endometrioid adenocarcinomas and 15 normal endometria in their study. They observed p27 staining in the nuclei of the glandular cells in the functional layer of the secretory phase endometrium, whereas it was negative in those of the proliferative phase. Paradoxically, in endometrioid adenocarcinomas the p27 expression increased more significantly in the higher histological grades and was correlated with that of Ki-67. They found the high level of p27 expression to be associated with clinicopathological parameters such as FIGO stage, lymph node metastasis, lymphovascular space involvement and myometrial invasion (Watanabe *et al*, 2002). High p27 expression was linked to higher grades of endometrioid adenocarcinoma, cell proliferation and some clinical prognostic factors. These authors concluded that p27 might be an indicator of poor prognosis (Watanabe *et al*, 2002). Another recent study conducted by Masciullo and studied 217 endometrial adenocarcinoma cases (Masciullo *et al,* 2003). Their data indicated that p27 expression could progressively decrease from normal endometrium through hyperplastic endometrium to invasive endometrial carcinomas. Thereby suggesting that loss of this tumour suppressor may represent a distinct molecular alteration involved in oestrogen-related endometrial adenocarcinomas (Type 1). These results supported the study conducted by Oshita and co-workers in 2002, who also found that decreased p27 expression plays a role in type 1 endometrial cancer development and suggested that there was potential for new therapeutic interventions in endometrial cancer treatment and prevention (Oshita *et al*, 2002). However, Masciullo *et al.* (2003) found that p27 was not a predictor of clinical outcome despite there being suggestions of its role in determining the prognosis of several other human tumours. As can be seen, p27 expression in endometrial carcinoma still remains somewhat contradictory.

2.5.3.4.3 P16

P16 ^{INK4a} is another member of the INK4 group, which encodes a 148 amino acid protein. This protein has a large array of names, which includes INK4A (initially INK4), CDK 41, MTS1, and CDKN2 (which is now the formal name), as it is the product of the *CDKN2 (MTS1)* gene (Hirama and Koeffler, 1995; Clarke and Chetty, 2001; Cordon-Cardo, 1995). It forms binary complexes specifically with CDK4 and CDK6, inhibiting their activity and, by doing so, also inhibits pRb phosphorylation. In humans the *p16* gene is adjacent to the *p15* gene, which as mentioned above encodes a very similar protein, and is also mapped on chromosome 9p21 (Cordon-Cardo, 1995; Hunter and Pines, 1994). The amino acid sequence of this gene also comprises four repeats of the ankyrin motif. These ankyrin motifs have been found in proteins that interact with their target macromolecule. With regards to p16 the ankyrin repeats may also be involved in its interaction with CDKs. It plays a role at the G1/S transition phase of the cell cycle and might be responsible for inactivating cyclin D/CDK4/6 at the G1/S boundary, when Rb is phosphorylated enough for the cells to enter the S phase. It has been suggested that p16 can act as a growth inhibitor in vivo (Hirama and Koeffler, 1995).

- **In Endometrial Carcinoma:**

In an immunohistochemical study, using antibodies against CDK4 and $p16^{INK4}$ in 20 normal endometrial specimens and 41 specimens of endometrioid endometrial carcinoma Shiozana *et al.* (1997) showed the following: normal endometrial cytoplasmic staining of CDK4 and $p16^{INK4}$ was observed only in the proliferative phase, while the nuclear staining was negligible. In endometrial carcinomas, 8 (19,5%) and 19 (34,2%) were positive for CDK4 and p16 in the nucleus, respectively. The nuclear CDK4 positive tumour cells were negative for p16 and the nuclear p16 positive tumour cells were found in areas without nuclear CDK4 expression, suggesting an inverse correlation between the two proteins. These researchers therefore concluded that increased expression of nuclear CDK4 associated with loss of p16 expression could be involved in the carcinogenesis of a subset of endometrial carcinomas (Shiozana *et al,* 1997).

2.5.4 TUMOUR-SUPPRESSOR GENES

2.5.4.1 MOLECULES THAT REGULATE NUCLEAR TRANSCRIPTION AND CELL CYCLE

The nucleus is ultimately where all positive and negative signals converge, to make decisions on whether to divide or not. In keeping with this, products of several tumour-suppressor genes (*Rb, WT-1*, and *p53*) are localized to the nucleus (Cotran *et al*, 1999).

2.5.4.1.1 RETINOBLASTOMA *(Rb)* GENE

This was the first tumour-suppressor gene discovered (Kaelin, 1997; Lin, 1996). The retinoblastoma gene encodes an approximately 105 kDa nuclear phosphoprotein and maps to chromosome 13q14 (Friend *et al*, 1987; Lee *et al*, 1987; Fung *et al*, 1987; Cordon-Cardo, 1995). There are three members that make up the retinoblastoma gene family, the *Rb/p105*, one of the most studied tumour-suppressor genes, and two related genes *Rb2/p130* and *p107* (Susini *et al*, 2001; Sanseverino *et al*, 2003). The proteins encoded by these genes, pRb2/p130 and p107 are structurally and functionally similar to pRb (Mayol *et al*, 1993). This product of the *Rb* gene, pRb, is a nuclear phosphoprotein that just like the other members plays a key role in regulating the cell cycle. It is expressed in every cell type examined, where it exists in an active underphosphorylated and an inactive hyperphosphorylated state. pRb, in its active state serves as a brake on the advancement of cells from G1 to the S phase of the cell cycle (Sherr, 1996). The brake is released when the cells are stimulated by growth factors. The cells then traverse the G1→S checkpoint. The cells are committed to divide, without additional growth factor stimulation, once they enter the S phase. The phosphate groups are removed from pRb by cellular phosphatases, during the ensuing M phase, thus regenerating the dephosphorylated form of pRb. The active, hypophosphorylated form of pRb is contained by quiescent cells (in G_0 or early G_1). In this state, pRb prevents cell replication by binding, and possibly sequestering, the E2F family of transcription factors. This elegantly explains the molecular basis of the braking action of pRb (Sherr, 1996). The concentrations of cyclins D and E goes up when quiescent cells are stimulated by growth factors. The resultant activation of cyclin D/CDK4,

cyclin D/CDK6, and cyclin E/CDK2 leads to phosphorylation of pRb. The hyperphosphorylated form of pRb releases E2F transcription factors. The released E2F proteins then form heterodimers with the DP family of proteins, and activate the transcription of several target genes (Cotran *et al*, 1999). Recently, it has been suggested that the pRb-E2F complex binds to DNA and actively inhibits the transcription of the S phase genes (DePinho, 1998). It is clear, however, that the state of pRb phosphorylation is a critical determinant of cell cycle progression. With the above information at hand, it is quite obvious what the outcome would be if the Rb protein is absent, or its ability to regulate E2F transcription factors is derailed by mutations. Ultimately, the molecular brakes on the cell cycle are released, and the cells move blithely into the S phase. The mutations of *Rb* genes found in tumours are localized to a region, called the "Rb pocket" that is involved in binding to E2F. The loss of normal cell cycle control is central to malignant transformation and at least one of the four key regulators of cell cycle (p16, cyclin D, CDK4, Rb) is mutated in the vast majority of human cancers (Hunter, 1997). In cells that harbour mutations in *p16, cyclin D*, or *CDK4*, the function of the *Rb* gene is disrupted even if the *Rb* gene itself is not mutated.

There are several other pathways of cell growth regulation that also converge on pRb:

- The inhibition of cellular proliferation is induced by TGF-β, the effect of which is in part induced by the upregulation of the CDK inhibitors p27 and p15.
- It is by neutralizing the growth inhibitory activities of pRb that the transforming proteins of several oncogenic animal and human DNA viruses seem to act. SV40 and polyoma virus large T antigens, adenoviruses EIA protein, and human papillomavirus (HPV) E7 protein all bind to the hypophosphorylated form of pRb. The same pRb pocket that normally sequesters E2F transcription factors is where the binding occurs. The pRb protein is therefore functionally deleted, as it is unable to bind to the E2F transcription factors. These transcription factors are free to cause cell cycle progression.
- Another well-known tumour-suppresor gene, *p53* (discussed next), exerts its growth-inhibiting effects in part by up-regulating the synthesis of the CDK inhibitor p21 (Cotran *et al*, 1999).

- **In Endometrial Carcinoma:**

The retinoblastoma gene, which was originally described in retinoblastoma, has now been defined in a number of other human neoplasms, and it had also been suggested that the alteration of this gene might play a significant role in the development of endometrial carcinoma as well (Niemann *et al*, 1997). Since then various immunohistochemical studies have been conducted. In 1996 Li and colleagues found that in uterine endometrioid carcinoma, the cancer cells with definite positivity for p53 tended to stain either weakly or negative for Rb, but definitely positive for cyclin E (Li *et al*, 1996). The cells with weak or negative staining for p53 had a tendency to stain positively for Rb, and weakly positive for cyclin E. These researchers therefore suggested that in the development of endometrial carcinoma, stepwise abnormalities of tumour suppressor gene products does exist, and may correlate with the progression of the malignant process (Li *et al*, 1996). In contrast, Niemann and co-workers found that after examining Rb protein expression in a series of cases including normal endometrium, endometrial hyperplasia, and endometrial carcinoma, the alteration of Rb protein expression was uncommon in endometrial adenocarcinoma (Niemann *et al,* 1997). Also, when it does occur, it may represent a late event in carcinogenesis (Niemann *et al,* 1997). Another research group set out to determine the relation between the expression of the retinoblastoma-related gene *Rb2/p130* and outcome in patients with endometrial carcinoma. They were able to conclude that in patients with endometrial carcinoma, who did not receive radiotherapy or chemotherapy before surgery, the presence of decreased levels of pRb/p130 in tumour cells was associated with a significantly increased risk of recurrence and death of disease, independent of tumour stage and ploidy status (Susini *et al,* 1998). In 2000 Tsuda *et al,* detected abnormalities of the p16-cyclin D/CDK-pRb pathway in 18 of the 35 endometrial carcinomas (51.4%) they studied (Tsuda *et al*, 2000). In total contradistinction to the findings of Niemann *et al.* (1997), Semczuk *et al* in 2000, supported the view that pRb is expressed in most human endometrial neoplasms, and the lack of immunoreactivity may correspond with retinoblastoma gene rearrangements in a subset of advanced endometrial carcinomas (Semczuk *et al,* 2000). In 2001 Susini and colleagues conducted another study and found that the outcome was in keeping with their previous results (Susini *et al*, 1998). They also found that *Rb2/p130* might be

diagnostically useful in distinguishing between hyperplastic and atypical hyperplastic endometrium. Moreover, they found a progressive decrease in expression of pRb/p130 from hyperplastic endometrium through atypical hyperplasia to poorly differentiated carcinomas. Thereby, strongly suggesting a role for this negative cell-cycle regulator in endometrial carcinogenesis (Susini *et al*, 2001).

2.5.4.1.2 *p53* GENE

The *p53* gene is another well-studied tumour-suppressor gene, which has earned quite a reputation that dates back many years. By the year 1992 it had already gained the title "Guardian of the Genome" (Lane, 1992) and in 1993 it was declared the 'Molecule of the year', (Culotta and Koshland, 1994). This gene is located on chromosome 17p13.1 and is the most common target for genetic alteration in human tumours (el-Deiry *et al*, 1994). Mutations of this gene are found in over 50% of human tumours. It is in virtually every type of cancer that the homozygous loss of the p53 gene is found. This includes the carcinomas of the lung, colon and breast, which are the three leading causes of cancer deaths. The inactivating mutations affecting both p53 alleles are frequently acquired in somatic cells. However, less commonly, some individuals inherit a mutant p53 allele. The inheritance of one mutant allele predisposes individuals to develop malignant tumours. These individuals are said to have the Li-Fraumeni syndrome, and have a greater chance of developing a malignant tumour by the age of 50. The spectrum of tumours that develop in patients with this syndrome are quite varied, the most common types being sarcomas, breast cancer, leukaemia, brain tumours, and carcinomas of the adrenal cortex. As can be seen, *p53* mutations are common in a variety of human tumours, suggesting that the p53 protein serves as a critical "gatekeeper" against the formation of cancer (Cotran *et al*, 1999). Therefore, p53 can also be called a 'molecular policeman', as it prevents the propagation of genetically damaged cells (Levine, 1997). The p53 protein is localized to the nucleus, and functions by controlling the transcription of other genes, when called into action. This protein has a short half-life (20mins) under physiologic conditions, and in contrast to pRb does not police the normal cell cycle. However, p53 is called in to apply "emergency

brakes" when DNA is damaged by UV light, irradiation, or mutagenic chemicals. This results in the rapid increase in p53 levels and activation of p53 as a transcription factor. The accumulated wild-type p53 binds to DNA. The transcription of several genes is then stimulated, which mediate the two major effects of p53 ie: **cell-cycle arrest and apoptosis** (Figure 2.11).

A recent discovery has shown that *p53* can also prevent tumour growth by yet another mechanism. In addition to DNA damage, it seems that hypoxia can also stimulate the activation of normal p53 (Graeber *et al,* 1996). Hypoxic tumour cells undergo apoptosis if they have normal copies of the *p53* gene. However, if the *p53* gene is mutated, the hypoxic tumour cells are resistant to apoptosis. Hypoxia thus selects for cells in which the *p53* gene is inactive, and the propagation of p53 deficient cells is then favoured. The *p53* gene functions can also be inactivated by other mechanisms. The transforming proteins of several DNA viruses including E6 protein of human papillomaviruses, are able to bind to and degrade p53. *Mdm2,* which normally down-regulates *p53* activity, is overexpressed in a subset of human soft tissue sarcomas, as a result of gene amplification (Cotran *et al*, 1999). By promoting rapid degradation of p53, *mdm2* acts as an oncogene (Graeber *et al*, 1996). *p53* seems to be quite an important gene, as some practical therapeutic implications also exist, with the ability of *p53* to control apoptosis in response to DNA damage.

The two common modalities of cancer treatment, which are radiation and chemotherapy, mediate their effects by inducing DNA damage and subsequent apoptosis. It has been shown that tumours, which carry normal *p53* genes, are more likely to respond to such therapy, than those with mutant *p53* (Cotran *et al,* 1999). Such is the case with testicular germ cell tumours and childhood acute lymphoblastic leukaemias (Chresta and Hickman, 1996).

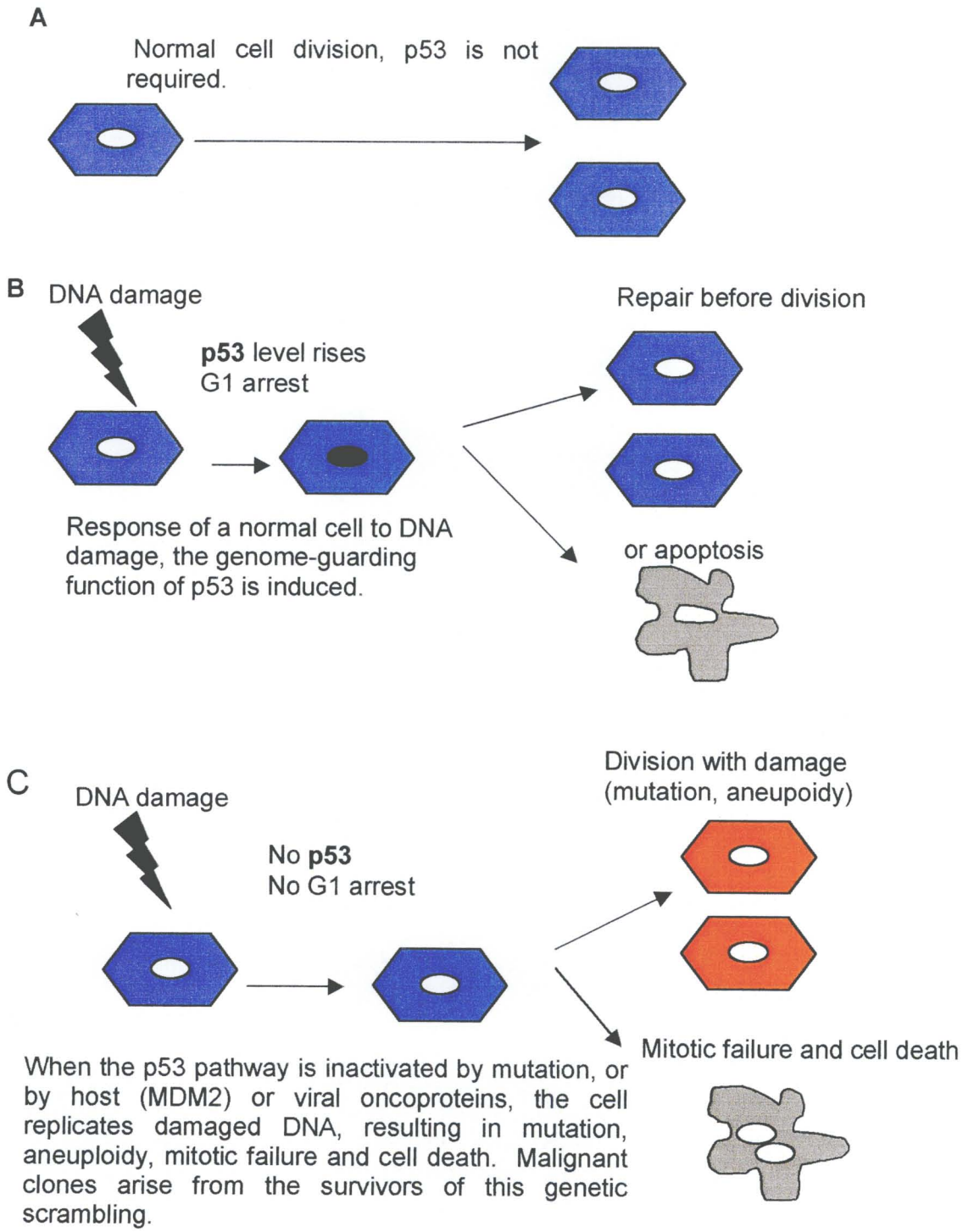


Figure 2.11: The function of p53, guardian of the genome. (Adapted from Lane, 1992).

In total contrast, tumours such as lung cancers and colorectal cancers, which often carry *p53* mutations, are relatively resistant to chemotherapy and radiotherapy.

In 1997 the *p73* gene was discovered (Cotran *et al*, 1999). Until then, *p53* was the only known gene of its kind. *P73* was dubbed the "big brother" of *p53* and is located on 1p36, where it bears many similarities to *p53*. It can cause cell arrest as well as apoptosis. Deletions of 1p36 are common in a variety of tumours, like neuroblastoma, breast and colon cancers. There is now much interest focussed on *p73* (Cotran *et al*, 1999).

- **In Endometrial Carcinoma**

The *p53* tumour suppressor gene seems to play a dominant role in endometrial tumourigenesis. In 1993 Yu *et al* detected p53 immunoreactivity in 47% of invasive endometrial adenocarcinomas and in a much smaller proportion of simple and atypical hyperplasias (Yu *et al*, 1993). Later another study found that p53 was an independent prognostic indicator that correlated with outcome (Ito *et al*, 1994). Recently, the study of Ohkouchi and co-workers also found that p53 overexpression is an independent prognostic factor for endometrial carcinoma (Ohkouchi *et al*, 2002). An interesting finding made by Inoue *et al.* (2001) was that all 5 serous papillary adenocarcinoma cases studied were p53 positive. This added more evidence to the two different pathways that exists between serous papillary adenocarcinoma and endometrioid adenocarcinoma (Inoue *et al*, 2001). The results of Sherman *et al.* (1995) suggested a dualistic model of endometrial carcinogenesis as well. In one pathway, endometrioid carcinomas develop slowly from oestrogen stimulated hyperplasia, *p53* mutations represents a late event related to dedifferentiation in these tumours. In the second pathway, carcinomas develop in an oestrogen-independent fashion from atrophic endometrium. Abnormal *p53* expression occurs early and results in the transformation of endometrial surface epithelium into endometrial intraepithelial carcinoma (EIC), the putative precursor of serous papillary tumours (Sherman *et al*, 1995). Kohler *et al.* (1996) found overexpression of p53 in advanced-stage endometrial cancers. They also found it to be an independent variable, associated with poor survival. This appeared to occur more frequently in black women, which they thought might contribute to the racial disparity in survival (Kohler *et al*, 1996;

Kohlberger *et al,* 1996). A more recent study by Cherchi and colleagues, once again proved that the overexpression of p53 indicated a more malignant tumour phenotype (Cherchi *et al*, 2001). Zheng *et al.* (1996) suggested that p53 alteration may be an early event in the development of uterine serous papillary carcinoma, and may be related to its clinical aggressiveness, while in uterine endometrioid carcinoma it is a late event (Zheng *et al,* 1996). The findings of Tashiro *et al.* (1997b) were in keeping with that of Zheng *et al.* (1996). Their results revealed that *p53* mutations are very common in uterine papillary serous papillary carcinoma and EIC. The presence of *p53* gene mutations in EIC further suggests that *p53* alteration plays an important role early in the pathogenesis of serous carcinoma, possibly accounting for its aggressive biological behaviour (Tashiro *et al*, 1997b). In 1998 the investigation by Kovalev *et al.* added more proof to the above mentioned findings, as they too found *p53* alterations common in uterine papillary serous carcinoma and probably responsible for its aggressive biologic behaviour (Kovalev *et al*, 1998). Also in keeping with these results, a recent study by Lax and co-workers provided further evidence for distinct molecular genetic pathways, as *p53* mutations seemed to be more dominant in uterine papillary serous carcinoma than in uterine endometrioid endometrial carcinoma (Lax *et al*, 2000). So too, were the findings of Mount *et al.* (2000) and Lax *et al.* (2002).

With regards to p53 being a prognostic factor in endometrial cancer the following has been researched:

Sorbe and co-workers, with their investigation of 227 endometrial cancer cases found that 20% of these cases were p53 + (Sorbe *et al,* 1997). This represented a high-risk group of primary advanced tumours with poorly differentiated, nonendometrioid histology. They found that in the patients dying of the disease, 54% were p53+ and that positive staining was more common in older women. P53 was found to be the second most important prognostic factor after nuclear grade, so Sorbe and his team concluded that the immunohistochemical staining for this protein should be included among the previous important, available prognostic factors for endometrial cancer (Sorbe *et al*, 1997). In keeping with the above results Gassel and colleagues found a strong correlation between low expression and better outcome (Gassel *et al*, 1998). Oreskovic *et al.* (2004) also

found that low p53 expression was significantly correlated with better survival in the group of patients with G1 tumours (Oreskovic *et al*, 2004). In 1998, Nanbu and co-workers detected p53 protein expression (mutant protein) in 8 of the 44 cases they investigated. They also found that patients with p53+ tumours had a lower survival rate than p53-ve tumours (Nanbu *et al*, 1998). Kohler *et al.* (1996) observed in their study that race was also associated with p53 staining. They found that p53 overexpression was twice as common in black women than in white women (Kohler *et al*, 1996). In a recent study, Sung *et al.* (2000) confirmed previous reports of an association of p53 overexpression and poor survival/prognosis. Their study was also one of the few studies to find p53 to be an independent risk factor for poor survival (Sung *et al*, 2000). Although there seems to be much controversy regarding p53 and prognosis, Mariani *et al.* (2000), like others, (Lundgren *et al*, 2002; Kohler *et al*, 1996; Ioffe *et al*, 1998; Burton *et al*, 1998) have found that the evaluation of the p53 protein levels does assist in identifying tumours that have a more aggressive course (eg. uterine serous papillary carcinoma).

2.5.4.1.3 *BRCA-1 AND BRCA-2* GENES

The *BRCA-1* and *BRCA-2* genes are two recently discovered tumour-suppressor genes that are located on chromosome 17q12-21 and 13q12-13, respectively. They are associated with breast as well as several other cancers. Individuals who inherit mutations of these genes are highly susceptible to the development of breast cancer. In addition an increased risk of epithelial ovarian cancers, prostate and colon cancers is associated with germline mutations of *BRCA-1* (Cotran *et al*, 1999). Like-wise the *BRCA-2* gene mutations also increase the risk of developing cancers of the male breast, ovary, and possibly prostate, pancreas, and larynx (Gayther and Ponder, 1997). *BRCA-1* and *BRCA-2* aberrations occur in approximately 80% of familial cases of breast cancers, while 5-10% of breast cancers are familial. Mutations of these genes are rarely seen in sporadic breast cancer. The functions of these genes have not been fully defined. However, the protein products of both these genes, which are localized to the nucleus, are believed to be involved in transcriptional regulation. Some data suggest that they are involved in DNA repair (Cotran *et al,* 1999), a conclusion that seems to be

based on the observation of BRCA-1 and BRCA-2 proteins interact with Rad 51. This is a protein implicated in the regulation of recombination and double-stranded DNA repair (Brugarolos and Jacks, 1997). With this view, mutations in *BRCA* genes, do not directly regulate cell growth, rather they predispose to errors in DNA replication. This leads to mutations in other genes that directly affect cell cycle and cell growth. These theories are not entirely consistent, and are currently under active investigation (Cotran *et al,* 1999).

- **In Endometrial Carcinoma:**

In 1999 Hornreich and colleagues presented a breast-ovarian cancer family, including two sisters with advanced serous papillary carcinomas of endometrial and ovarian origin, carrying the same *BRCA-1* mutation. Loss of the wild-type allele was shown on LOH analysis on uterine serous papillary carcinoma tumour DNA, therefore suggesting a causal relationship between the germline *BRCA1* mutation and uterine serous papillary carcinoma. However, they suggested that further studies examining *BRCA-1* mutations in a large number of women with this high-risk endometrial carcinoma is warranted. These authors also proposed that a positive finding may have implications for surveillance and prophylactic surgery in carriers of *BRCA-1* mutations (Hornreich *et al*, 1999).

2.5.4.2 MOLECULES THAT REGULATE SIGNAL TRANSDUCTION

The products of tumour-suppressor genes may also be operative in the down-regulation of growth-promoting signals. The *NF1* (Neurofibromatosis type 1) and *APC* genes fall into this category. Germline mutations at the *NF1* (17q11.2) and the *APC* (5q21) loci are associated with precursors of carcinomas that develop later. Individuals born with one *APC* mutant allele invariably develop hundreds or even thousands of adenomatous polyps in the colon during their teens or twenties (familial adenomatous polyposis, FAP). Unfortunately, one or more of these polyps eventually undergo malignant transformation, giving rise to colon cancer. The APC protein is located in the cytoplasm where it interacts with several other intracellular proteins, like β-catenin, a protein that can enter the nucleus and activate transcription of growth-promoting genes. The APC protein functions to cause degradation of β-catenin, thus maintaining low levels of the

latter in the cytoplasm. Therefore, inactivation of *APC* gene, and the consequent loss of APC protein, increases the cellular levels of β-catenin. This in turn passes to the nucleus and up-regulates cellular proliferation (Cotran *et al*, 1999). *APC* is thus a negative regulator of β-catenin signalling (Fearon, 1997). Dysregulation of the *APC-β catenin* pathway is not restricted to colon cancers. Mutations in either *APC* or *β-catenin* have also been found in about 30% of melanoma cell lines. Both these proteins have other cellular partners, indicating that their functions are multiple. β-catenin also binds to the cytoplasmic aspect of E-cadherin, a cell surface protein that maintains intercellular adhesiveness. However, cancer cells have reduced adhesiveness, possibly resulting from defects in the cadherin-catenin axis.

The *NF-1* gene behaves similar to the *APC* gene. Individuals who inherit one mutant allele develop numerous neurofibromas with inactivation of the second copy of the *NF-1* gene, a condition that is called neurofibromatosis type 1. Later, some neurofibromas develop into neurofibrosarcomas (Cotran *et al,* 1999). Children who have neurofibromatosis-1 also are at an increased risk of developing acute myeloid leukaemia (Side *et al*, 1997). The protein product of the *NF-1* gene is neurofibromin, which functions to regulate signal transduction via the ras protein. Neurofibromin is a GTPase activating protein that facilitates conversion of active ras to inactive ras. Therefore, a loss of *NF-1* results in ras being trapped in an active, signal-emitting state (Cotran *et al,* 1999).

- **In Endometrial Carcinoma:**

Recently mutations in the *β-catenin (CTNNB1)* gene have been reported in a small percentage of uterine endometrioid carcinomas and also in the endometrioid variant of ovarian carcinoma. Thus suggesting that the Wnt signal transduction pathway is involved in the development of female genital tract tumours with endometrioid morphology. The Wnt pathway is known to be a critical pathway in the development of colorectal cancer (CRC), with mutations occurring in the *β-catenin (CTNNB1)* or *APC* genes in 10 to 15% and 85% of cases, respectively. Since uterine endometrioid carcinoma and CRC share other molecular genetic alterations and histologic features and previous studies of uterine endometrioid carcinoma have not reported an analysis of the *APC* gene,

Schlosshauer and colleagues chose to further elucidate the role of the Wnt pathway in uterine endometrioid carcinoma (Schlosshauer *et al,* 2000). 32 cases of uterine endometrioid carcinomas were analyzed for mutations of the *CTNNB1* and *APC* genes (Schlosshauer *et al,* 2000). Mutations of the *CTNNB1* were present in six of 32 (18%) cases. No mutations resulting in truncation of the APC protein were found. Their results therefore support a role for the Wnt signalling pathway via mutation of *CTNNB1,* but not *APC*, in the development of a subset of uterine endometrioid carcinomas (Schlosshauer *et al*, 2000).

2.5.4.3 CELL SURFACE RECEPTORS

There are several types of molecules expressed on the cell surface that can regulate cell growth and behaviour. These molecules include receptors for growth-inhibitory factors, such as TGF-β, and proteins that regulate cellular adhesions, like cadherins. Transcription of growth-inhibitory genes is up-regulated by the binding of TGF-β to its receptors. This effect is mediated by stimulating the synthesis of cyclin-dependent kinase inhibitors, which block the cell cycle by inhibiting the actions of cyclin/CDK complexes. Mutations of *TGF-β* and its signalling pathway have been identified in many cancers (Cotran *et al*, 1999). In approximately 15% of colon cancers, the gene encoding the TGF-β receptor is inactivated. Also, *SMAD2* and *SMAD4* genes, which encode proteins in the TGF-β growth-inhibitory pathway are deleted or inactivated in certain colon and pancreatic cancers (Heldin *et al*, 1997). Cadherins are a family of glycoproteins that act as glue between epithelial cells. Loss of these cadherins can favour the malignant phenotype by allowing easy disaggregation of cells, which can then invade locally or metastasize. Reduced cell surface expression of E-cadherin has been noted in many types of cancers, eg. tumours that arise in the oesophagus, colon, breast, ovary, and prostate (Perl, 1998). Germline mutations of the *E-cadherin* gene can predispose to familial gastric carcinomas (Guilford, 1998). The molecular basis of reduced E-cadherin expression is varied. There are mutations in the *E-cadherin* gene (located on 16q) in a small proportion of cases, while in other cancers E-cadherin expression is reduced,

secondary to mutations in the catenin genes. Catenins bind to the intracellular portion of cadherins and stabilize their expression.

Deleted in colon carcinoma (*DCC*) is a gene located on chromosome 18q21. This chromosome region is frequently deleted in human colon and rectum carcinomas, the *DCC* gene has therefore been considered a candidate tumour suppressor gene (Cotran *et al*, 1999). The *DCC* gene was initially identified because it is affected by somatic mutations in colorectal tumours, including allelic losses in greater than 70% of cancers and localized mutations in a subset of cases (Reale *et al*, 1994; Hsu *et al*, 2001; Candusso *et al,* 2002). It has been proposed that the *DCC* gene may regulate cell growth and differentiation by integrating signals from the cells environment, since its structure resembles other cell-to-cell and cell-to-matrix interactions. There have been serious doubts regarding the likelihood of *DCC* being a tumour-suppressor gene. Instead, it appears that DCC is a cell surface receptor, important in axonal growth (Fazeli, 1997). It therefore seems that some other gene in close linkage with *DCC* on chromosome 18q21 is the real culprit for carcinogenesis (Cotran *et al*, 1999).

- **In Endometrial Carcinoma:**

Many investigators have analyzed the *DCC* locus in endometrial carcinoma (Risinger *et al*, 1993; Katabuchi *et al,* 1995). A study conducted by Gima *et al.* (1994) analyzed LOH at 3 loci on chromosome 18q and *DCC* gene expression. 16 (26%) cases showed allelic losses at one or more chromosome 18q loci. Deletions involved the region within or near 18q21.3 where *DCC* is localized. Moreover, the incidence of altered *DCC* mRNA expression was high in these tumours. However, the histopathological differentiation and clinical stage of disease were not related to LOH frequency or to *DCC* mRNA expression. Their results suggested that the target for allelic loss on chromosome 18q seen in endometrial carcinomas is the *DCC* gene, and that inactivation of this gene may be critical for the development of most endometrial carcinomas (Gima *et al,* 1994). On the other hand, Fujino *et al.* (1994) were unable to detect any mutations of the *DCC* gene in the 60 endometrial carcinomas they studied. They suggested that the observed LOH on chromsome 18q might also occur

subsequent to the mutation of an as yet unidentified tumour suppressor gene other than *DCC* (Fujino *et al*, 1994).

2.5.4.4 OTHER TUMOUR SUPPRESSOR GENES

Despite the fast pace of research today, there is not much doubt that many more tumour-suppressor genes still remain to be discovered. Their location is suspected by the detection of consistent sites of chromosomal deletions or by loss of heterozygosity studies. There are some tumour suppressor genes that are associated with well-defined clinical syndromes, however their functions remain unknown. These genes are briefly discussed below:

2.5.4.4.1 *PTEN*

The phosphatase and tensin homolog *(PTEN)* has been mapped to chromosome 10q23. This gene is frequently deleted in many human cancers, including glioblastomas, prostate cancer, endometrial cancer and breast cancer (Cotran *et al,* 1999). Owing to the structure of this gene it has been suggested that it may negatively regulate cell interactions with extracellular matrix by dephosphorylating undefined substrates (Li *et al*, 1997).

- **In Endometrial Carcinoma:**

PTEN mutations have been reported to be a frequent event in endometrioid carcinomas of the endometrium (Ellenson, 2000; Sherman, 2000). Some correlation has been found between these mutations and the presence of microsatellite instability in endometrioid endometrial carcinoma. Since the cause-effect relationship for such an association has not been convincing, Bussaglia *et al.* (2000) planned to investigate this. Their results confirmed that *PTEN* is an important target gene in endometrial carcinogenesis. The occurrence of *PTEN* mutations in short coding mononucleotide repeats in MSI+ve tumours also suggested that these mutations may be secondary to deficiencies in mismatch repair and gives some explanation for the frequent presence of *PTEN* mutations in these tumours (Bussaglia *et al,* 2000). The study of Matias-Guiu and colleagues in 2001 arrived at a similar conclusion (Matias-Guiu *et al*, 2001). Hirai

et al. (2001) also showed that most MSI+ve endometrial cancers with *PTEN/MMAC1* mutations as well as MSI-ve tumours showed inactivation of both alleles of this gene, which strongly suggested the involvement of this gene in carcinogenesis (Hirai *et al,* 2001). A recent review article by Latta and Chapman (2002) on *PTEN* mutations and evolving concepts in endometrial neoplasia, states that mutations in the *PTEN* gene and microsatellite instability are common genetic abnormalities in endometrioid endometrial carcinomas, which distinguish these from other histological subtypes of endometrial carcinoma. However, these researchers observed that the significance of *PTEN* mutations and MSI in endometrial carcinoma is controversial, and that further study is required to delineate the different pathogenetic pathways of endometrioid endometrial carcinoma and their natural history (Latta and Chapman, 2002).

2.5.4.4.2 *WT1* (*Wilms' Tumour* 1)

WT1 gene is located on chromosome 11p13 and is associated with the development of Wilms' tumour. Mutational inactivation of the *WT-1* locus has been seen in both sporadic and inherited forms of Wilms' tumour. The WT-1 protein is a transcriptional regulator that is presumed to inhibit transcription of growth-promoting genes.

- **In Endometrial Carcinoma:**

A recent study by Makrigiannakis *et al.* (2001) found that in humans the *WT1* gene is expressed in endometrial stromal cells and its mRNA and protein levels remain constant in the proliferative and the secretory phase of the menstrual cycle. Also the *WT1* mRNA and protein expression was found to increase significantly in endometrial stromal cells when cells differentiate into decidual cells (Makrigiannakis *et al*, 2001). However there have been no reports on the role of *WT1* in endometrial carcinoma.

2.5.5 GENES THAT REGULATE APOPTOSIS

It has been accepted for many years that oncogenes and tumour suppressor genes play prominent roles in the molecular basis of tumourigenesis. Genes

from both these classes are known to regulate cell proliferation. However, it is now appreciated that genes that prevent or induce apoptosis are also important factors in the cancer equation (Wyllie, 1997). Human beings may want to live forever, but many cells know better. At certain times they execute a suicide strategy and die with characteristic death rattles. This act, which is called apoptosis or programmed cell death, allows nearby cells to thrive and is crucial for development and immune function. "Apoptosis", which is actually death by another name is a term that was introduced in 1972 by Kerr and his co-workers. This was done to provide a distinction between this cell death that occurs in both animal development and tissue homeostasis and necrosis that occurs in acute lesions from trauma and ischaemia (Schwartz and Bennett, 1995; Kerr *et al,* 1972). Necrosis is a condition resulting from energy independent cell death in response to pathologic changes initiated outside of the cell. It usually occurs at the centre of lesions and cells tend to swell and rupture, resulting in an inflammatory reaction. Being non-programmed, this form of cell death does not involve activation of any intrinsic cellular process. This is in contrast to apoptosis, during which cells usually shrink and condense and macromolecules are programmably degraded through various specific proteases called caspases and nucleases. This results in the dead cells and their fragments being rapidly phagocytosed by neighbouring cells or macrophages, which prevents a possible inflammatory response caused by any leakage of the contents of the cells. The following distinct morphological sequence of events was distinguished during apoptosis (Wang, 1999):

- A cell rounds up with rapid condensation of its DNA resulting in chromatin condensation and DNA fragmentation.
- The nucleus breaks down into discrete masses of a condensed chromatin.
- The cell is fragmented into several membrane bound vesicles (apoptotic bodies)
- These apoptotic bodies are rapidly recognized and phagocytosed by macrophages or neighbouring cells (Wang, 1999).

Cancer progression is the result of an imbalance between the rate of cell proliferation and the rate of cell death. Tumourigenicity is often associated with the activation of several anti-apoptotic events, although virtually every type of tumour cell undergoes a high rate of cell death via apoptosis. Oncogenic tumour

viruses are the best examples, which often carry inhibitors of cell death pathways, eg, several of these viruses encode proteins that functionally inactivate p53 (Wang, 1999). It was found that most malignancies demonstrate abnormalities in programmed cell death. Besides contributing to the immortality of the replicating cell, alterations in the apoptotic pathway also favour the accumulation of additional genetic alterations that would normally induce cell death (Jameson, 1998). A large family of genes that regulate aopoptosis has been identified (McDonnell, 1996). *Bcl2* was the first anti-apoptotic gene identified, and is a member of a large family of homodimerizing and heterodimerizing proteins, some of which inhibit apoptosis (such as b*cl2 itself* and b*cl-xL*), whereas others favour programmed cell death (such as *bax, bad*, and *bcl-xS*) (Cotran *et al*, 1999). *Bcl2* was originally discovered based on its translocation in follicular lymphomas. The recombination of the immunoglobulin locus on chromosome 14 with the *bcl-2* gene on chromosome 18 (18q21) causes persistent overexpression of *bcl-2* in lymphoid cells (Jameson, 1998). This overexpressioin of *bcl-2* protects lymphocytes from apoptosis and allows them to survive for long periods, leading to a steady accumulation of B-lymphocytes, resulting in lymphadenopathy and marrow infiltration. Besides having an unusual function, bcl-2 also has a different location compared to most cancer-associated genes. It is localized on the outer leaflet of the mitochondrial membrane, the endoplasmic reticulum, and the nuclear membrane. Apoptosis is the end point of a cascade of molecular events, which are initiated by several stimuli. This leads ultimately to the activation of proteolytic enzymes responsible for cell death. The bcl-2 family of proteins regulates the activation of these proteolytic enzymes (caspases). The pro-apoptotic and anti-apoptotic members of the bcl-2 family act as a rheostat in regulating programmed cell death. The ratio of death antagonists (*bcl-2, bcl-xl*) to agonists (*bax, bcl-xs, bad, bid*) determines how a cell will respond to an apoptotic stimulus (Yin, 1997). Besides the *bcl-2* family of genes, at least two other cancer-associated genes are also intimately connected with apoptosis: the *p53* gene and the proto-oncogene *c-myc.* The cell death molecular mechanisms of these two intersect with the bcl-2 pathways. The activation of *p53* up-regulates *b*ax synthesis and hence counteracts the anti-apoptotic action of *bcl-2* (Figure 2.12), (Cotran *et al,* 1999).

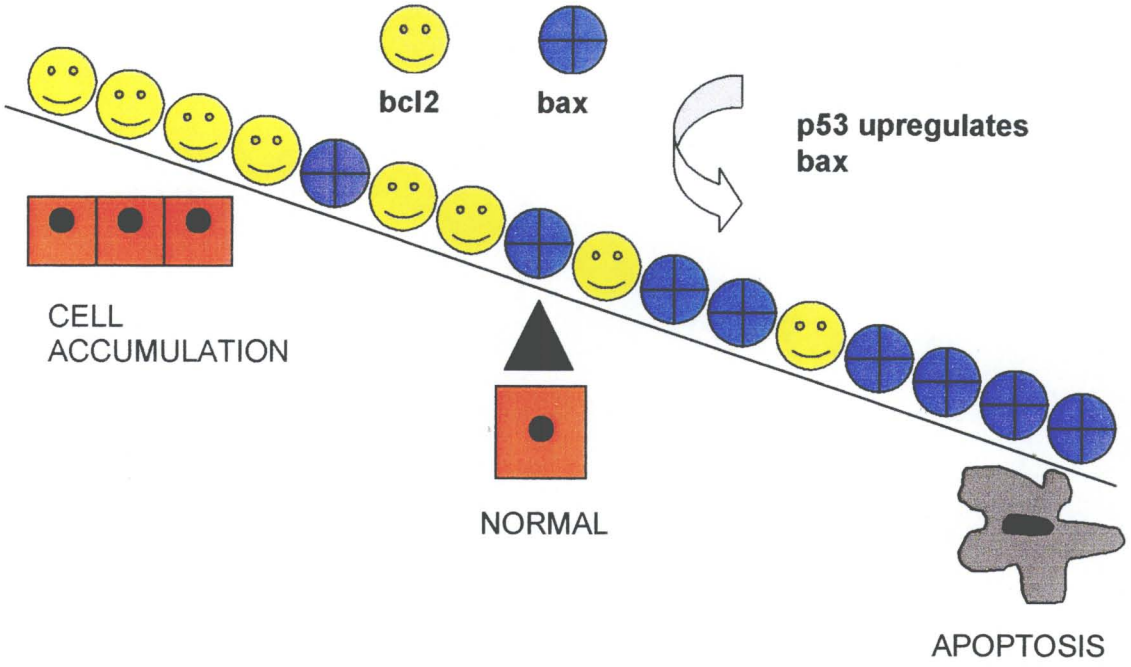


Figure 2.12: Diagram showing regulation of cell death by b*cl2, bax,* and *p53*. The *bcl2* gene favours cell growth by inhibiting apoptosis, whilst the b*ax* gene favours apoptosis. The apoptosis-inducing effect of the normal *p53* gene is mediated in part by increasing the synthesis of bax protein. (Adapted from Cotran *et al*, 1999).

There has also been evidence that radiation and certain anticancer drugs work because they induce apoptosis via the protein product of this tumour suppressor gene *p53* (Culotta and Koshland, 1994). C-myc induces apoptosis when cells are driven by c-myc activation. Confronted by such conflicting signals the cells are therefore programmed to die by up-regulation of p53 and other undefined signals. The overexpression of bcl-2 can rescue cells from c-myc initiated apoptosis. Bcl-2 and c-myc may therefore collaborate in tumourigenesis, as c-myc triggers proliferation, and bcl-2 prevents cell death. This is a perfect example in which two cancer genes cooperate in giving rise to cancer (Cotran *et al*, 1999).

In Endometrial Carcinoma

In 1998 Ioffe *et al* noted that apoptosis is also observed at increasing levels in the hyperplasia-adenocarcinoma sequence in the endometrium (Ioffe *et al*, 1998).

The study of Arends confirmed this, as he concluded that deregulation of apoptosis is seen in the hyperplasia-atypia-adenocarcinoma sequence of the endometrium and is likely to be associated with genetic alterations driving progression along this neoplastic pathway (Arends, 1999). MSI is a frequent occurrence in endometrioid carcinoma of the endometrium. Several genes known to contain mononucleotide short tracts in their coding sequences eg *bax*, are likely targets for mutations in these tumours. A study by Catasus and co-workers confirmed that *bax* is an important target gene in endometrial carcinomas with MSI (Catasus *et al*, 2000). A year later the same researchers hypothesized that altered methylation might be an initial alteration in the genesis of endometrioid endometrial carcinoma (Matias-Guiu *et al*, 2001). hMLH-1 promoter hypermethylation would cause mismatch repair deficiencies, which in turn would produce the phenomenon of MSI and a stepwise progressive accumulation of mutations at coding mononucleotide repeat microsatellites in some important oncogenes and tumour suppressor genes, amongst these being *bax* (Matias-Guiu *et al,* 2001).

The results of Chao *et al.* (Chao *et al*, 2001) suggested that bax expression affects the balance between the endometrial cell proliferation and apoptosis. They also found that *Bax* might play an important role in carcinogenesis and tumour progression of endometrial carcinoma. Also, it could be used as a high-risk factor to evaluate the biological behaviour of endometrial carcinoma (Chao *et al,* 2001). Peiro and colleagues concluded that *bax* plays a role in a small subset of endometrial carcinoma (Peiro *et al*, 2001). A recent study found that a low rate of apoptosis is present in endometrial proliferation and hyperplasia (Vaskivou *et al*, 2002). In Grade 1 carcinoma the rate of apoptosis is decreased, but the rate is subsequently increased in advanced carcinoma. The decrease in the rate of apoptosis in Grade 111 adenocarcinoma may reflect loss of control of cell homeostasis, decreased differentiation, and increased malignancy (Vaskivou *et al*, 2002). In another recent study Sakuragi and colleagues found the *bax* gene frameshift mutation appears to cause a loss of bax expression in endometrial carcinoma. Codon 58 may be a preferred target in *bax* gene mutations in endometrial carcinomas. These researchers believe that the *Bax* gene mutation seems to occur in the early stage of the genesis of a subset of endometrial

carcinomas (Sakuragi *et al*, 2002). In contrast to Catasus *et al.* (2000), who believe it to be a late event. The *bcl2* gene has been entensively researched in relation to being a prognostic factor in endometrial cancer. Geisler *et al.* (1998), set out to investigate whether this protein is related to the traditional prognostic factors. They found that bcl-2 staining was more common in endometrioid (44%) than in nonendometrioid (23.1%) adenocarcinomas of the endometrium. Also, it appeared to be inversely correlated with the well-known prognostic factors such as histologic grade, depth of invasion, and FIGO stage. Their results demonstrated that bcl-2 was an independent predictor of recurrence of disease. They were therefore able to conclude that increased bcl-2 staining correlates with good prognosis (Giesler *et al*, 1998). In another study conducted by Sakuragi and co-workers 49 endometrioid adenocarcinomas were examined (Sakuragi *et al*, 1997). The cytoplasmic expression of bcl-2 was inversely related to depth of myometrial invasion and lymph node metastasis. On the other hand, nuclear expression of Bcl-2 correlated with depth of invasion, lymph node metastasis, stage and higher grade of the tumour. Their results suggested that the decreased cytoplasmic expression of Bcl-2 and the presence of nuclear bcl-2 expression may be a reflection of the malignant progression of endometrioid endometrial carcinoma (Sakuragi *et al*, 1997). Hort *et al.* (1997) found that bcl-2 was a potential prognostic indicator for low grade, stage 1 endometrial carcinoma. The study of Sardi *et al.* (1999) was in keeping to that of Geisler *et al* (1998). However, besides finding increased immunoreactivity of bcl-2 correlating with a good prognosis, they also discovered that bcl-2 expression was more frequent in young patients than in the old patients. 100% of young patients with stage 1 tumours were bcl-2+ compared to the 37% in the older group of patients. In both these groups bcl-2 expression was more frequent in well-differentiated tumours (Sardi *et al*, 1999).

2.5.6 GENES THAT REGULATE DNA REPAIR

It is a known fact that exposure to the naturally occurring DNA damaging agents, such as ionizing radiation, sunlight and dietary carcinogens is relatively common, yet cancer is a rare outcome of such exposure. This happy state of affairs is the result of the ability of normal cells to repair DNA damage and thus prevent

mutations in genes that regulate cell growth and apoptosis. Besides environmental agents being the cause of DNA damage, the DNA of normal dividing cells is also prone to alterations resulting from errors that occur spontaneously during DNA replication. If not repaired promptly, such mistakes can also push the cells along the slippery slope of neoplastic transformation (Cotran *et al,* 1999). In 1994, the DNA repair enzyme earned the title 'Molecule of the Year', as it was seen to serve in a system of like molecules to preserve our health, maintain our species, make evolution possible, and contribute to a sound scientific policy on environmental hazards. The human genome comprises DNA, which provides the blueprint for approximately 60,000 proteins. If this DNA were copied badly, diseases such as cancer would prevail at a much higher frequency, and we would not get a faithful copy of our parental inheritance. On the other hand, if the DNA were copied perfectly, there would be no room for evolution, and the basis for creation of new species with better environmental adaptation would have vanished long ago. Therefore, the DNA repair system allows a happy, balanced medium. The human copying system makes, on the average, only three base pair mistakes when copying the 3 billion base pairs in the human genome (Koshland, 1994). Every second the DNA in each cell of our body is being damaged, chemical bonds break, DNA strands snap, and nucleotide bases misalign. The cell loses more than 10,000 bases per day just from spontaneous breakdown of DNA. Exposure to carcinogens, as mentioned above, adds to the injury. If this persisted, cells would cease to work properly, mutations would accumulate, and the chances of tumours developing would soar. Fortunately, the DNA repair enzymes continually scan DNA for mistakes, slice out damaged pieces, and patch up gaps. Many experiments have revealed the molecular nuts and bolts of the repair machinery, unveiling a system of surprising versatility and power. There are muliple DNA repair pathways; each specializes in a certain kind of damage (Cullota and Koshland, 1994):

- Mismatch repair specializes in errors made when DNA is copied. It scans newly made DNA for mispaired bases, cuts out mistakes, and fills in the gaps with the correct sequences.
- Normal cellular activities injure DNA through oxidation and other common reactions. To repair such damage, cells rely on another pathway, called base

excision repair, which targets single damaged bases eg. DNA polymerase beta.

- Meanwhile, outside agents such as ultra-violet light and chemicals also inflict damage on DNA. Many of these lesions are handled by a third repair pathway, called nucleotide excision repair (NER), which recognizes and repairs large, bulky lesions. This system is crucial to humans, as individuals who lack it, have the rare genetic disease called xeroderma pigmentosum and are so sensitive to sunlight that they often develop skin cancer before age 10 years (Culotta and Koshland, 1994).

Besides evolving this elaborate machinery for repairing DNA after it is damaged, it is already known that *p53* also has a protective function. Research has suggested that this gene may even have more ways of protecting the genome than was originally thought. In addition to blocking cell cycle progression and helping trigger programmed cell death, p53 may directly and indirectly stimulate the DNA repair machinery. This is pursued with the help of another gene, the *GADD45* (growth-arrest-and -DNA-damage-inducible) (Marx, 1994). However, there are several inherited disorders in which genes that encode proteins involved in DNA repair do become defective. This highlights the importance of DNA repair in maintaining the integrity of the cell, as those born with such inherited mutations of DNA repair proteins are at a greatly increased risk of developing cancer (Cotran *et al*, 1999):

2.5.6.1 MISMATCH REPAIR GENES (MMR)

Long before the discovery of its involvement in human cancer, mismatch repair was extensively studied in bacteria and yeast (Wildenberg and Meselson, 1975; White and Fox, 1975). Their human homologues have been implicated in the pathogenesis of a variety of different cancers that exhibit MSI, like hereditary non-polyposis colorectal cancer (HNPCC) (Muc and Naidoo, 2002), as well as endometrial cancer (Loeb, 1994). The DNA mismatch-repair system repairs mismatched base pairs generated by errors that occur during replication, genetic recombination and double strand DNA breaks, which lead to chromosome disruption (Kolodner, 1995; Chu, 1997). These errors arise as a result of

environmental insults that damage specific nucleotides. When a strand of DNA is replicating, mismatch repair genes play the role of "spell checkers" (Cotran *et al,* 1999). These "proof readers" ensure that copies of new DNA produced in the cell, have the same genetic make-up or sequence as the parent strands. The repair of biosynthetic errors such as single base mismatches and alterations in microsatellite length is a highly conserved cellular function, which is carried out by these proteins that recognise defective sequences, excise them and then replace them with the correct ones (Muc and Naidoo, 2002). Sometimes however, these DNA mismatch repair genes somehow misfunction, resulting in a defective DNA repair system. In humans this has been linked to HNPCC as well as to sporadic cancers like endometrial carcinoma that exhibit MSI (Liu *et al,* 1995a; Fujino *et al,* 1994; Loeb, 1994). To date six human DNA mismatch repair genes have been identified (3 homologues of bacterial *MutS*: *hMSH2*, *hMSH6* (*GTBP*), and *hMSH3*; and 3 homolgues of bacterial *MutL*: *hMLH1*, *hPMS1*, and *hPMS2*) (Chung and Rustgi, 1995; Eshleman and Markowitz, 1996).

Genetic defects in mismatch-repair genes play an important role in common cancer-susceptibility syndromes and sporadic cancers (Kolodner, 1995; Papadopoulos *et al,* 1995). MMR defects are integrally involved in carcinogenesis (Eshleman and Markowitz, 1996). The DNA repair genes themselves are not oncogenic, but they allow mutations in other genes during the process of normal cell division (Cotran *et al,* 1999). The HNPCC syndrome dramatically illustrates the role of MMR repair genes in predisposition to cancer (Cotran *et al,* 1999). These genes have been implicated in the pathogenesis of HNPCC, as well as a host of different sporadic cancers that exhibit MSI (Muc and Naidoo, 2002). Mutations in these genes lead to defective DNA repair, resulting in replication errors (mutator phenotype). The first clue that defective MMR is important in carcinogenesis was the discovery of the RER (replication errors) phenotype of microsatellite instability in sporadic and inherited colon cancer. Research has now shown that defective MMR is the underlying defect responsible for both the MSI and the increased mutation rate in at least some RER cancers (Eshleman and Markowitz, 1996). MSI seems to be the hallmark of defective MMR (Cotran *et al,* 1999). Mutations in the genes coding for these proteins results in defective proteins which fail to correct replication errors,

leading to a cascading effect that produces secondary mutations further downstream from the initial mutation site. Oncogenes and tumour suppressor genes are where these secondary mutations may occur (Muc and Naidoo, 2002). *hMSH2*, which is located on chromosome 2p, and *hMLH1* located on chromosome 3p are the two most extensively studied repair genes (Muc and Naidoo, 2002). Both these genes act essentially as tumour suppressor genes with loss of both copies of the gene resulting in abnormalities in the mismatch repair system. These genes are usually mutated in the HNPCC syndrome. However, mutations in *hPMS1* and *hPMS2* have also been implicated in the pathogenesis of endometrial carcinoma (Papadopoulos *et al*, 1997; Muc and Naidoo 2002). Less is known about the *hPMS1*, *hPMS2* and *hMSH6* genes (Fishel and Kolodner, 1995). They are thought to act as a complex, and mutations in any of them may also give rise to MSI. It is believed that these genetic alterations act as a trigger for the initiation and pathogenensis of many tumours (Muc and Naidoo, 2002). MMR defective cell lines from colon, prostate, ovarian, uterine and endometrial tumours have already been identified (Kolodner, 1995).

- **MMR GENES IN ENDOMETRIAL CARCINOMA**

Many studies have come to the conclusion that MSI in tumours from patients with HNPCC is due to the presence of mutations in one of the four known MMR genes (*hMSH2, hMLH1, hPMS1, hPMS2*) (Watson and Lynch, 1993; Jiricny, 1994; Kolodner 1995; de Leeuw *et al*, 2000). Approximately 35-40% of HNPCC family members develop other types of tumours of which endometrial is the most common (Kolodner, 1995; Berchuck and Boyd, 1995). Therefore, the association of endometrial carcinoma with HNPCC hints that such mutations may also account for the MSI observed in the subset of sporadic endometrial carcinomas demonstrating this phenotype. However the results of Katabuchi *et al.* (1995) were the first to suggest that instability of simple repetitive DNA sequences in most sporadic endometrial carcinomas displaying MSI is not caused by mutations in the four MMR genes. Berchuck and Boyd noted that some endometrial cancers have mutations in microsatellite loci, but at this stage several groups were still attempting to identify acquired mutations in DNA repair genes (Berchuck and Boyd, 1995). Staebler and colleagues, in 1998, arrived at the

conclusion that microsatellite instability in sporadic endometrial carcinoma appears to be associated with the lack of protein expression of either *hMLH1* or *hMSH2*. Therefore suggesting that inactivation of these genes may be responsible for MSI in the majority of MSI positive endometrial cancers (Staebler *et al*, 1998). The following year Smith and co-workers assessed hMLH1 and hMSH2 expression in 146 randomly selected carcinomas, including 29 endometrial and 30 colorectal cancers (Smith *et al*, 1999). hMLH1 expression was lost in 24% of endometrial carcinomas. They also found that the loss of hMLH1 expression in endometrial carcinoma was higher than the fraction of tumours attributable to HNPCC. Increased hypermethylation of hMLH1 in hyperplasia would trigger MSI, which in turn would inactivate several essential genes such as bax, IGF-IIR, hMSH3, or hMSH6, involved in apoptosis, cell proliferation, and mismatch repair. However, studies were still on the way to determine whether this high incidence is due to hypermethylation of the *hMLH1* gene, as reported in sporadic colorectal tumours with MSI (Ahuja *et al*, 1997; Smith *et al*, 1999).

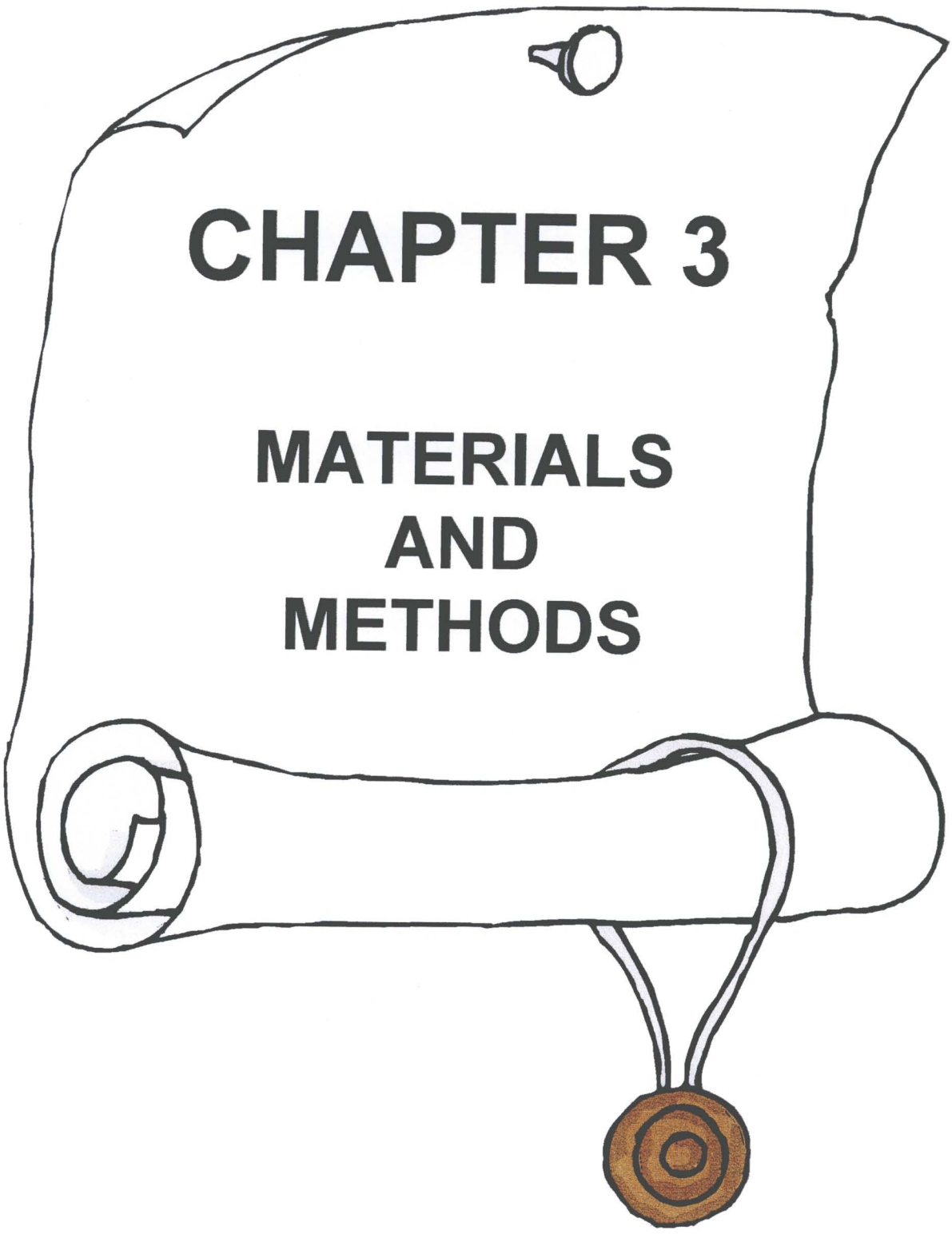
The results of Peiro and co-workers suggested that loss of function of *hMLH1* occurs in a subgroup of endometrial carcinoma and the absence of this expression was associated with better outcome of patients (Peiro *et al*, 2001). Muresu and colleagues found in their study, that inactivation of the *hMLH1* mismatch repair gene may be involved in the majority of the MSI+ sporadic endometrial carcinomas (Muresu *et al*, 2002). However, they suggested a larger study to confirm that this gene plays a major pathogenetic role in sporadic endometrial carcinoma, as their investigation consisted of only 32% of MSI+ cases (Muresu *et al*, 2002). Another study, by Chadwick and colleagues was undertaken to revisit the issue of microsatellite instability and mismatch repair gene mutations in sporadic endometrial cancer. By initially studying tumour tissue, both germline and somatic mutations were evaluated in the *hMSH2*, *hMLH1*, and *hMSH6* genes in a retrospective series of microsatellite stable and microsatellite unstable endometrial cases. They also found that heritable mismatch repair deficiency accounts for a small but definite proportion of sporadic endometrial cancer (Chadwick *et al*, 2001). Early in 2002, the study of Stefaanson and co-workers suggested that the pathological expression of hMLH1

did not seem to account for all tumours with a MSI-positive phenotype, in their population-based series of endometrial carcinomas. Their data indicated that the other mismatch repair genes *hMSH2* and *hMSH6* are also involved, especially in cases with intermediate MSI (Stefaanson *et al*, 2002). At this stage it is already a researched fact that MSI due to replication errors occurs frequently in hereditary tumours. The association with functional inactivation of MMR genes and lack of protein expression has been extensively researched and described. However, in endometrial carcinoma, the prevalence and clinical significance of these phenomena still remains unclear. Therefore, in a recent investigation, Peiro *et al.* (2002) set out to explore this. They concluded that MSI occurs in a small proportion of endometrial carcinoma and is predominantly associated with defects of the *hMLH1* gene. These tumours frequently showed mucinous differentiation, areas of solid-cribiform pattern with necrosis, and higher FIGO stage. Less aggressive clinical behaviour and longer survival was observed in cases with loss of hMLH1 protein expression (Peiro *et al*, 2002). Malfunction in the MMR system is responsible for the MSI phenotype in most of the cases. Only a small proportion show mutations (Katabuchi *et al*, 1995), whereas hMLH1-promoter hypermethylation occurs in the vast majority (Esteller *et al*, 1999). However, in some tumours the cause of this MSI still remains unknown. The combined analysis of MSI status and of MMR protein expression seems to be able to detect most of the tumours with defective MMR genes (Peiro *et al*, 2002). With regards to prognostic implications, Peiro *et al.* (2001) set out to investigate these genes and their relationship with prognosis in endometrial cancer. The findings from this study suggested that loss of hMLH1 expression by immunohistochemical analysis was associated with mucinous differentiation of endometrial cancer. Tumours showing a high expression of *hMLH1* also showed a high *hMSH2* expression. Loss of expression was seen more often for *hMLH1* than for *hMSH2*, indicating that this gene is more susceptible to somatic alteration. Furthermore, these researchers concluded that the absence of *hMLH1* expression was associated with less aggressive clinical behaviour and longer survival. Tumour grade, FIGO stage, histologic type and *hMLH1* expression were identified as independent prognostic factors whereas age, bax and hMSH2 did not reveal significant prognostic information. However, a trend for better outcome was also seen in those patients with low levels of hMSH2 protein (Peiro *et al*, 2001).

2.5.7 LOSS OF HETEROZYGOSITY OR ALLELIC IMBALANCE IN ENDOMETRIAL CARCINOMA

Loss of heterozygosity (LOH) refers to the disappearance of polymorphic marker alleles (Plate 2.6, p54) when normal DNA and tumour DNA from a cancer patient are genotyped. These LOH events are genomic deletions that discard the normal copies of tumour suppressor genes, but uncover or highlight existing tumour suppressor gene mutations (Jameson, 1998). This accumulation of genetic alterations identified through the study of loss of heterozygosity (LOH), gene mutation, and gene activation in tumour DNA has been associated with the establishment and progression of a variety of human malignancies. By the year 1997, a relatively low incidence of LOH had been reported in usual type endometrial cancers, which are the oestrogen-dependent endometrial cancers, (endometrioid cancer). However special variant tumours, which are the oestrogen-independent type (non-endometrioid) had rarely been included in the reported studies. Tritz *et al.* (1997) revealed that both frequency and patterns of LOH differed greatly between the two tumour types. Although LOH was a frequent event in the special variant tumours, it was rare in the usual type tumours. LOH was detected in only 8 of the 18 usual tumours (affecting chromosomes 17, 13 and 2 mostly). In contrast, LOH was detected in all 13 cases of special variant tumours (affecting chromosomes 17p, 14 and 12). Also, they found that LOH was not present in atrophic or hyperplastic endometrium. This study indicated that the frequencies of allelic losses in endometrial carcinoma differ with histological type, occurring more often in special variant compared with usual endometrial types. On the other hand oncogene activation and MSI were more frequently detected in usual endometrial carcinoma than in special variant types. These findings brought them to the conclusion that the clinicopathologic phenotypes observed in these tumour types are likely to be caused by different tumourigenic pathways that reflects alterations of different cancer-controlling genes (Tritz *et al*, 1997). During that same year Sirchia *et al.* (1997) observed LOH in only two of the 23 adenocarcinoma cases they studied for two polymorphic loci of chromosome 16. Those with LOH presented a tumour staging and grading that did not differ significantly from the remaining tumours (Sirchia *et al*, 1997). In contrast Peiffer and colleagues in a previous study consisting of 37 endometrial cancer cases, observed LOH for loci on all

chromosomes examined with the exception of chromosomes 4 and 20 (Peiffer *et al,* 1995). Of the 39 loci examined, 31 showed LOH in an at least 1 tumour specimen. The two most frequent sites of loss were at the marker loci examined on 10q (40%) and 17p (29%) (Peiffer *et al,* 1995). A study by Nagase *et al* (1996) also found that LOH in endometrial cancer occurred most frequently on chromosome 10q25-10q26, and was associated with nuclear grade 1 tumours. Toda *et al.* (2001) observed LOH in 20 (33,3%) of 60 cases studied. The frequency of LOH was highest in stage IV, but did not differ according to grade. These were in non Smad-related regions. However in the Smad-related microsatellite regions, LOH was noted in only 12 (20%) of the 60 cases, with the frequency also being high in stage IV cancers. LOH was seen only in the *Smad 2* gene but not in the *Smad 4* gene. Their results suggested that LOH was associated with middle and late stages of carcinogenesis of endometrial carcinoma. Also, they found that genetic alteration of *Smad 2* gene was more often responsible for endometrial carcinogenesis than that of the *Smad 4* gene (Toda *et al*, 2001). In a recent study LOH was seen at 17 of the 156 loci examined, with the highest rate at D2S123 (chromosome 2p - *hMSH2* locus), followed by BAT26 and BAT25 (Peiro *et al*, 2002). The rate of cases with LOH in association with MSI compared with LOH without MSI, was not different. Furthermore, there was no correlation with clinicopathologic factors. In previous studies LOH of the *p53* gene has been observed most frequently in papillary serous carcinoma with high grade and stage, and those patients had a poor prognosis, suggesting that this specific alteration plays an important role in the development of this subtype (Tritz *et al*, 1997; Lax *et al,* 2000). However, the study of Peiro *et al.* (2002), observed that the prognosis was no different in patients with LOH than those without LOH (Peiro *et al,* 2002). The differences in observed frequencies of LOH in these various studies are probably attributed to the different markers the researchers chose.



CHAPTER 3

MATERIALS AND METHODS

MATERIALS AND METHODS

This study was approved by the Research Ethics Committee at the Nelson R Mandela School of Medicine (Ethics approval number H024/01).

The study consisted of a retrospective analysis of 54 cases of endometrial carcinomas using histological, immunohistochemical and molecular methods that was carried out in the Department of Pathology and Molecular Biology Research Facility at the Nelson R Mandela School of Medicine, University of KwaZulu-Natal.

3.1 CASE SELECTION

Paraffin-wax embedded tissue blocks of the 54 cases (hysterectomy specimens only) were retrieved from the archives of the Department of Pathology. The study period was 1991 to 1999. The patients' demographic details were accessed from the computer files of the Department of Pathology.

3.2 TISSUE PREPARATION

Only paraffin-wax embedded tissue was used for this study. The tissue had already previously been fixed in 10% formal saline, and thereafter processed overnight through a series of formalins, alcohols, xylols and waxes, in the Shandon Hypercentre (see appendix A). The end result being paraffin-wax embedded tissue blocks.

3.3 HISTOLOGICAL ANALYSIS

The haematoxylin and eosin (H&E) (see Appendix B) stained slides were retrieved from the files of the Department of Pathology and reviewed.

3.3.1 GRADING

Table 3.1: The International Federation of Gynaecology and Obstetrics (FIGO) surgical grading system for Endometrial Carcinoma. (Adapted from Kurman *et al*, 1994).

Degree of Architectural Differentiation	
Grade 1	5% or less of a non-squamous or non-morular solid growth pattern
Grade 2	6-50% of a non-squamous or non-morular solid growth pattern
Grade 3	More than 50% of a non-squamous or non-morular solid growth pattern
Cytological features to be used in formulating final grade: • Notable nuclear atypia, inappropriate for the architectural grade, raises the grade of a grade I or grade II tumour by 1 level. • In high-grade papillary adenocarcinomas, clear cell adenocarcinomas and areas of squamous differentiation, nuclear grade takes precedence over architecture. • Adenocarcinomas with squamous differentiation are graded according to the nuclear grade of the glandular component.	
Nuclear grading of endometrial carcinoma	
Grade 1	Cells with oval nuclei and evenly dispersed chromatin
Grade 2	Cells with nuclei that have features between grades 1 & 3
Grade 3	Cells with markedly enlarged, pleomorphic nuclei displaying irregular coarse chromatin and prominent, eosinophilic nucleoli

The WHO Histopathological Classification and the FIGO Staging System of uterine carcinoma recommend that both architectural and nuclear criteria be used to grade tumours (Table 3.1).

3.3.2 STAGING

The tumours were staged according to the International Federation of Obstetrics and Gynaecology (FIGO), staging procedure that was reviewed in 1989 (Table 3.2).

Table 3.2: FIGO Staging for Carcinoma of the Endometrium

Stage IA	**Grade 1,2,3:** Tumour limited to the endometrium.
Stage IB	**Grade 1,2,3:** Invasion to less than one-half the myometrium.
Stage IC	**Grade 1,2,3:** Invasion to more than one-half the myometrium.
Stage IIA	**Grade 1,2,3:** Endocervical glandular involvement only.
Stage IIB	**Grade 1,2,3:** Cervical stromal invasion.
Stage IIIA	**Grade 1,2,3:** Tumour invades serosa and/or adnexa, and/or +ve peritoneal cytology.
Stage IIIB	**Grade 1,2,3:** Vaginal metastases.
Stage IIIC	**Grade 1,2,3:** Metastases to pelvic and/or paraaortic lymph nodes.
Stage IVA	**Grade 1,2,3:** Tumour invasion of bladder and/or bowel mucosa.
Stage IVB	Distant metastases including intraabdominal and/or inguinal lymph nodes

3.4 IMMUNOHISTOCHEMICAL ANALYSIS

Immunohistochemical stains were performed for the immunoexpression of cyclins A, D1, E, pRb, p27 and p53. The Universal Labelled Streptavidin-Biotin (LSAB) and Catalysed Signal Amplification (CSA) kits were used. Antigen retrieval was carried out in a H2500 Microwave Processor (Energy Beam Sciences, Inc, Agawam, Massachusetts) at 85°C for 10 minutes (p27, p53) (see Appendix C). A pressure cooker was also used for antigen retrieval (Cyclins A, D1, E, pRb) (see Appendix C). The Sharp Carousel Domestic Microwave (650 watts), Model Number R7280, was used for the LSAB2 technique, at the low setting.

3.4.1 TISSUE PREPARATION FOR IMMUNOHISTOCHEMICAL STUDIES

Paraffin-wax embedded tissue sections were cut at 2 micrometers, using disposable blades on a rotary microtome. These sections were floated in a water-bath and picked up on poly-L-lysine coated slides (poly-L-lysine, Dako, see Appendix C). Slides were baked on a hotplate at 60°C for 10 minutes.

Sections were then de-waxed through two changes of xylene for 5 minutes each, followed by a passage through two changes of 100% alcohol for 3 minutes each, two changes of 95% alcohol for 3 minutes each and finally hydrated in running tap water for 5 minutes.

3.4.2 KITS USED FOR IMMUNOHISTOCHEMICAL STAINS

3.4.2.1 THE UNIVERSAL KIT (LSAB2) (Code No: 675)

From: Dako Corporation, Carpinteria, CA, USA.

Comprises:

- Link antibody (110ml), which is composed of biotinylated anti-rabbit, and anti-mouse immunoglobulins in phosphate buffered saline (PBS) containing a carrier protein and 0.015M sodium azide.
- Label antibody, which is streptavidin conjugated to horseradish peroxidase in PBS, containing a carrier protein and antimicrobial agent.

This kit was designed for use with primary antibodies from rabbit and mouse.

Used for: The staining procedure of p27 and p53.

3.4.2.2 THE CSA KIT (Code No: 1500)

From: Dako Corporation, Carpinteria, CA, USA..

Comprises:

- 15 ml of 3% hydrogen peroxide.

- 15 ml of protein block, which is serum-free protein in PBS and 0.015 M sodium azide.
- Link antibody, which contains biotinylated rabbit anti-mouse immunoglobulins in Tris-HCl buffer, containing carrier protein and 0.015M sodium azide.
- Reagent A Streptavidin-Biotin complex (1ml), which contains streptavidin in PBS, with an anti-microbial agent.
- Reagent B Streptavidin-Biotin complex, which is made up of biotin conjugated to horseradish peroxidase in PBS, with an anti-microbial agent.
- Streptavidin-Biotin complex (diluent 25ml) in PBS buffer containing carrier protein and an anti-microbial agent.
- Amplification reagent (15ml) composed of biotinyl tyramide and hydrogen peroxide in PBS containing carrier protein and an anti-microbial agent.
- Streptavidin Peroxidase (15ml), containing streptavidin conjugated to horseradish peroxidase in PBS containing carrier protein and an anti-microbial agent.
- Substrate Tablets, DAB chromogen (10 tablets), each tablet has 10mg 3,3'-diaminobenzidine tetrahydrochloride (DAB) packaged with a dessicant.
- Substrate, Tris-HCl buffer concentrate (6ml)-substrate.
- Substrate, (2ml) 0.8% hydrogen peroxide in water.
- Accessories, 5 ml test tube, 10 ml test tube and a pair of forceps.

This kit was designed for use with mouse primary antibodies. The CSA staining system is known to be an extremely sensitive immunohistochemical procedure and has been shown to be 50-fold more sensitive than the standard labelled streptavidin biotin (LSAB) methods.

Used for: The staining procedure for Cyclins A, D1, E and Retinoblastoma.

3.4.2.3 DAB DETECTION SYSTEM (DIAMINOBENZIDINE) (Code No: K3466)

From: Dako A/S Copenhagen, Denmark

Comprises:

- DAB chromogen (5ml), 3.3'-diaminobenzindine in chromogen solution.
- Buffered Substrate (110ml), imidazole-HCl buffer pH 7.5 containing hydrogen peroxide and an anti-microbial agent.

Preparation: 1 drop of DAB chromogen and 1ml of DAB substrate buffer - mixed well.

Used for: This detection system can be used in conjunction with both the CSA and LSAB kits.

DAB is a carcinogenic substance, and therefore extreme care has to be taken when handling this reagent. The staining result using this detection system is a strong brownish colour indicating positivity. These results last a lifetime as the colour does not fade with time, since DPX mounting is permitted with this substrate. Therefore slides can be stored indefinitely

3.4.3 ANTIBODIES USED FOR IMMUNOHISTOCHEMICAL STAINS

From: Cyclins A, D1, E and p27 were obtained from Novocastra laboratories (NCL) Ltd, United Kingdom (Table 3.3).

P53 and Retinoblastoma were from Dako A/S, Copenhagen, Denmark.

These were all monoclonal antibodies (Table 3.3).

Table 3.3: List of Antibodies used:

Antibodies (concentrated)	Clone	Optimal Dilution	Kits used	Staining Pattern
Cyclin A IgG1, kappa	6E6 Mab	1:50 PBS	CSA	Nuclear
Cyclin D1 IgG2a	DCS-6 Mab	1:20 PBS	CSA	Nuclear
Cyclin E IgG2a, kappa	13A3 Mab	1:55 BSA	CSA	Nuclear
P27	1B4 Mab	1:20 PBS	LSAB	Nuclear
P53	DO-7 Mab	1:100 PBS	LSAB	Nuclear
Retinoblastoma	Rb1 Mab	1:50 BSA	CSA	Nuclear

PBS: Phosphate buffered saline (Diluent for antibody) (See appendix D).

BSA: Bovine serum albumin (Diluent for antibody) (See appendix D)

Mab: Monoclonal antibody

3.4.4 LSAB STAINING METHOD FOR p27 and p53

- Slides were prepared as above, under tissue preparation for immunohistochemical studies.
- **Microwave Antigen Retrieval**
  Slides were placed in a thermoresistant plastic coplin jar, filled with 0,01 M buffered sodium citrate solution, pH6, (antigen retrieval solution, see Appendix C) and microwaved at 85°C for 10 minutes in the H2500 microwave processor.
- Thereafter slides were allowed to cool down in the coplin jar for 5 minutes.
- Slides were washed well, but gently in water for 2 minutes.
- Tissue sections were circled with a Dako pen (Dako A/S, Copenhagen, Denmark) to create a well for the staining reagents on the slides.

- The slides were then transferred to a coplin jar of phosphate buffered saline (PBS) at pH 7,4 (Dako A/S, Copenhagen, Denmark). Sections were not allowed to dry.
- Slides were immersed in a coplin jar of 3% hydrogen peroxide (H_2O_2) for 5 minutes at room temperature (See Appendix D).
- They were washed well in three changes of PBS and excess PBS was removed.
- Primary antibody (p27, p53) was placed on the slides (200 microlitres), and incubated in the microwave at a *low setting* for 4 minutes 30 seconds. They were left to stand for 1 hour. (Antibody not in kit).
- Slides were rinsed well in three changes of PBS.
- Excess PBS was removed. Sections were incubated in 1 to 3 drops of Biotinylated Link reagent at *low setting* for 3 minutes and 30 seconds. Slides were allowed 1 minute standing time.
- Slides were rinsed in 3 changes of PBS. Excess PBS was removed. Sections were incubated in 1 to 3 drops of labelling Streptavidin-HRP reagent at *low setting* for 3 minutes and 30 seconds. Allowed 1 minute standing time.
- Slides were rinsed 3 times in PBS. Excess was removed.
- Sections incubated in 1 to 3 drops of prepared DAB Substrate-Chromogen solution at room temperature for approximately 5 minutes. Slides were viewed microscopically. Positive reactivity was indicated by brown coloured end product at the site of the target antigen.
- Slides were rinsed well in running tap water for 3 minutes.
- Sections were counterstained with Mayer's haematoxylin (see Appendix B) for 1-3 minutes.
- Slides were then rinsed in tap water.
- Ammoniated water, (see Appendix D) was used to develop the counterstain.
- Slides were rinsed gently in running tap water.
- Slides were dehydrated in one change of 95% ethanol and then two changes of 100% ethanol through to two changes of xylene.
- Finally, slides were permanently mounted in DPX (Saarchem Unilab, Merck, Gauteng, SA).

3.4.5 CSA STAINING METHOD FOR Cyclin A, D1, E and pRb

- Slides were prepared as above, under tissue preparation.

- **Pressure Cooking Procedure** (see Appendix C)

Slides were slotted in a stainless steel rack and placed into a pressure cooker filled with 2L of 0,01 M buffered sodium citrate solution at pH 6, (antigen retrieval solution). Pressure cooker was placed on a hotplate and slides were pressure cooked for approximately 1 minute.

- Slides were allowed to cool for approximately 15 minutes and washed well in water.
- Tissue sections were circled with a DAKO pen, to create a well.
- Slides were transferred to a coplin jar of PBS, and sections were not allowed to dry.
- Excess PBS was was tapped off slides and 3 % hydrogen peroxide (Bottle 1) was added to slides for 5 minutes at room temperature (RT°).
- Slides were washed in three changes of PBS, excess was removed and 1 - 3 drops of Protein Block (Bottle 2) was added to slides for 5 minutes.
- Protein block was tapped off slides and primary antibody (cyclin A, D1, E, pRb), (step3) was added to sections (200µl) and left to incubate for 1 hour at RT°.
- Tissue sections were washed well in PBS.
- Excess was wiped off and 1-3 drops of Link antibody (Bottle 4) was added to slides for 15 minutes at RT°.
- Slides were washed in PBS. Excess was wiped off.
- Sections were incubated in 1-3 drops of Streptavidin-Biotin Complex (Bottles 5+ 6+ 7), (Prepared 30 minutes prior to use, 1 drop of bottle 5 + 1 drop of bottle 6 was added to 1ml of bottle 7), for 15 minutes at RT°.
- Sections were washed in PBS and the excess wiped off.

  1-3 drops of Amplification Reagent (Bottle 8) was added to sections for 15 minutes at RT°.
- Washed in 3 changes of PBS and the excess wiped off.
- Sections were incubated in 1-3 drops of Streptavidin-Peroxidase (Bottle 9) for 15 minutes at RT°.
- Slides were washed well in PBS and the excess tapped off.

- 1-3 drops of Substrate-Chromogen Solution (Bottles10+11+12), was added to slides for approximately 5 minutes at RT° (Added 10 drops of Tris Buffer concentrate, Bottle 11, in test tube provided, then poured distilled water to reach the 10ml mark, added 1 Substrate tablet, Bottle 10 and mixed well, then added 5 drops of Substrate hydrogen Peroxide, Bottle 12). Slides were examined microscopically. Positive staining viewed as brown coloured end product at the site of the target antigen.
- Slides were washed well in tap water.
- Sections were counterstained in Mayer's Haematoxylin (see Appendix B) for 1-3 minutes.
- Washed in tap water.
- Ammoniated water, (see Appendix D) was used to develop the stain.
- Washed gently in tap water again.
- Slides were then dehydrated through one change of 95% ethanol, two changes of 100% ethanol and two changes of xylene.
- Finally slides were mounted in DPX.

3.4.6 CONTROLS

Both negative reagent and positive tissue controls (pRb–retina; p53-breast tissue, breast duct carcinoma; p27-oesophagus, oesophageal squamous carcinoma; cyclin A-tonsil; cyclin D1-Mantle cell lymphoma, lymph node; cyclin E-breast tissue, breast carcinoma) were used for each immunohistochemical-staining run (Plates 4.6; 4.7; 4.8; 4.9; 4.10; 4.11).

3.4.7 SCORING OF IMMUNOHISTOCHEMICAL STAINS

Immunohistochemical stains were scored as follows:

- <5% positive nuclei: -ve
- 6-25% positive nuclei +1
- 26-50% positive nuclei: +2
- 51-75% positive nuclei: +3
- >75% positive nuclei: +4

3.4.8 ANALYSIS OF IMMUNOHISTOCHEMICAL STAINS

Interpretation of the staining results for each antibody was done independently without prior knowledge of the clinicopathologic parameters. The Olympus BX40 microscope was used for the scoring and grading, which was done manually.

3.5 MOLECULAR ANALYSIS

3.5.1 TISSUE PREPARATION FOR DNA STUDIES

Six-micron sections of paraffin-wax embedded normal and tumour tissues were cut on a rotary microtome using a sterile disposable blade. The tissue sections were floated in a water-bath, picked up on glass slides, and baked on a hot plate at 60°C. The slides were agitated through two changes of xylene, two changes of 100% ethanol, one 95% ethanol, and finally washed thoroughly in running tap water. The slides were then left to air dry.

3.5.2 DNA EXTRACTION

The DNA extraction procedure was carried out as described in Appendix E.

3.5.3 POLYMERASE CHAIN REACTION (PCR) USING THE INSULIN PRIMERS

In order to establish whether the DNA extracted from the paraffin-wax embedded tissue was of good quality for microsatellite analysis, PCR was carried out on all of the cases, using primers for the ubiquitous insulin gene. The primers used for this reaction were for exon 2 region of the insulin gene.

3.5.3.1 INSULIN PRIMERS

The insulin primers were synthesised by the Department of Biochemistry, University of Cape Town, South Africa. The oligonucleotide sequences for these primers were as follows:

2F 5' ACC CAG ATC ACT GTC CTT CTG CC 3'

2R 5'AGG GGC AGC AAT GGG CGG TTG 3'

The expected PCR product size was 236 base pairs.

3.5.3.2 THE ROCHE PCR CORE KIT

The PCR was carried out using the ROCHE PCR core kit (Boehringer Mannheim).

The kit comprised of:

- 50 mM10x PCR reaction buffer (containing 1.5 mM MgCl2)
- 200mM dNTP mix (10 mM dATP, 10 mM dCTP, 10 mM dGTP)
- Taq DNA polymerase (0.2μl).

3.5.3.3 INSULIN PCR REACTION

The insulin PCR was carried out as follows:

The primers were diluted to a concentration of 20 pmoles and were used in a total reaction volume of 25 μl. This contained 5μl template DNA, 200 mM dNTPs, 50 mM PCR buffer containing 1.5 mM MgCl2, and 0.2 units of Taq DNA polymerase. The samples were kept on ice. The PCR was carried out in 200 μl thin-walled reaction tube. The Techne Progene Thermocycler was used for the PCR reaction. The initial denaturing step was carried out at 94°C for 5 min. This was followed by 30 cycles of 1 min at 94°C, 1 min at 64°C and 2 min at 72°C. The final extension step was at 72°C for 10 min. The PCR products (10μl) of this reaction were then analysed on a 2% agarose gel. (see Appendix F and G).

3.5.4 AGAROSE GEL ELECTROPHORESIS

3.5.4.1 PREPARATION OF SAMPLES

2 μl of Bromophenol blue loading dye (see Appendix F) was added to 10 μl of PCR product. These samples were placed on ice.

DNA molecular weight marker preparation: to 2μl of the marker 2 μl of loading dye and 8 μl of sterile water was added.

3.5.4.2 PROCEDURE

A 2% agarose gel (see Appendix F) was made and once this gel completely polymerised, the comb was removed. The gel was then placed into the electrophoretic tank, filled with 500 ml of 1X TBE (see Appendix F). The DNA samples and the molecular weight marker were carefully loaded into the wells using a p20 pipetman. Electrophoresis was carried out at 80V for 45 minutes. at room temperature.

3.5.4.3 PHOTOGRAPHY OF THE GEL

After the electrophoretic run the gel was placed on a Camag UV transilluminator and viewed at long wavelength (300 nm). The gel was then scanned into a computer, using a video camera. The appropriate light and contrast adjustment was made and the picture was printed.

3.5.5 MICROSATELLITE PCR

For this reaction the following was reqiured:

i.) PCR Core Kit

From: Boehringer Mannheim

ii.) CY5 labelled primers for the ***DCC*** gene and **mismatch repair genes**

The primer sequences were obtained from Boland *et al.* (1995) and Liu *et al.* (1995b).

From: Boehringer Mannheim, Penzberg, Germany.

The microsatellite primers for the ***DCC*** gene and **mismatch repair genes** were chosen for this study as they are the most commonly used primers. They are as follows (Table 3.4 and Table 3.5):

3.5.5.1 MICROSATELLITE PRIMERS

Table 3.4: Microsatellite PCR primers used for the DCC gene locus

Locus	Sequence	Microsatellite -type	Product size
D18S21 18q21.1-q21.2 (Fig 3.1)	(F) 5' GTGGTTATTGCCTTGAAAAG 3' (R) 5' GATGACATTTTCCCTCTAG 3'	Dinucleotide repeat	150-210 bp
D18S34 18q12.2-q12.3 (Fig 3.1)	(F) 5' CAGAAAATTCTCTCTGGCTA 3' (R) 5' TCATGTTCCTGGCAAGAAT 3'	Dinucleotide repeat	103-120 bp
D18S58 18q22.3-q23 (Fig 3.1)	(F) 5' GCTCCCGGCTGGTTTT 3' (R) 5' CAGGAAATCGCAGGAACTT 3'	Dinucleotide repeat	144-160 bp

Table 3.5: Microsatellite PCR primers used for the Mismatch Repair loci

Locus	Sequence	Microsatellite type	Product size
D2S123 (2p16) (Fig 3.2)	(F) 5' AAACAGGATGCCTGCCTTTA 3' (R) 5' GAACTTTCCACCTXTGGGAC 3'	Dinucleotide repeat	197-227 bp
D3S659 (3p13)	(F)5'ATTCCAGGGACAAGTTCCCC 3' (R) 5'CTGCAAGGTCTGTTTAACAG 3'	Dinucleotide repeat	110-120 bp
BAT 25 (4q12)	(F) 5'TCGCCTCCAAGAATGTAAGT 3' (R) 5'TCTGCATTTTAACTATGGCTC 3'	Mononucleotide repeat	+/- 90bp

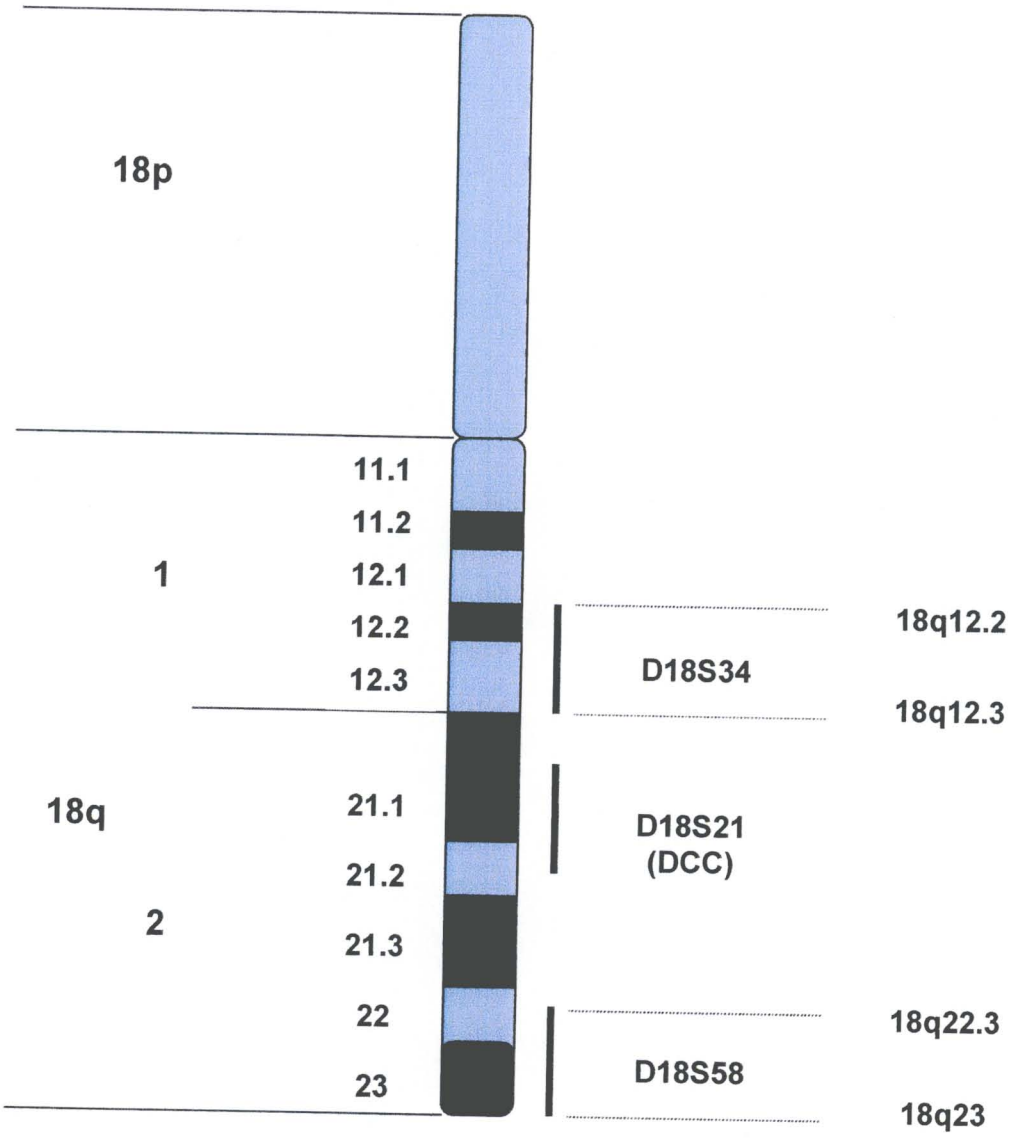


Figure 3.1: Ideogram of chromosome 18, with locations of 3 loci, DCC (D18S21), D18S34 and D18S58

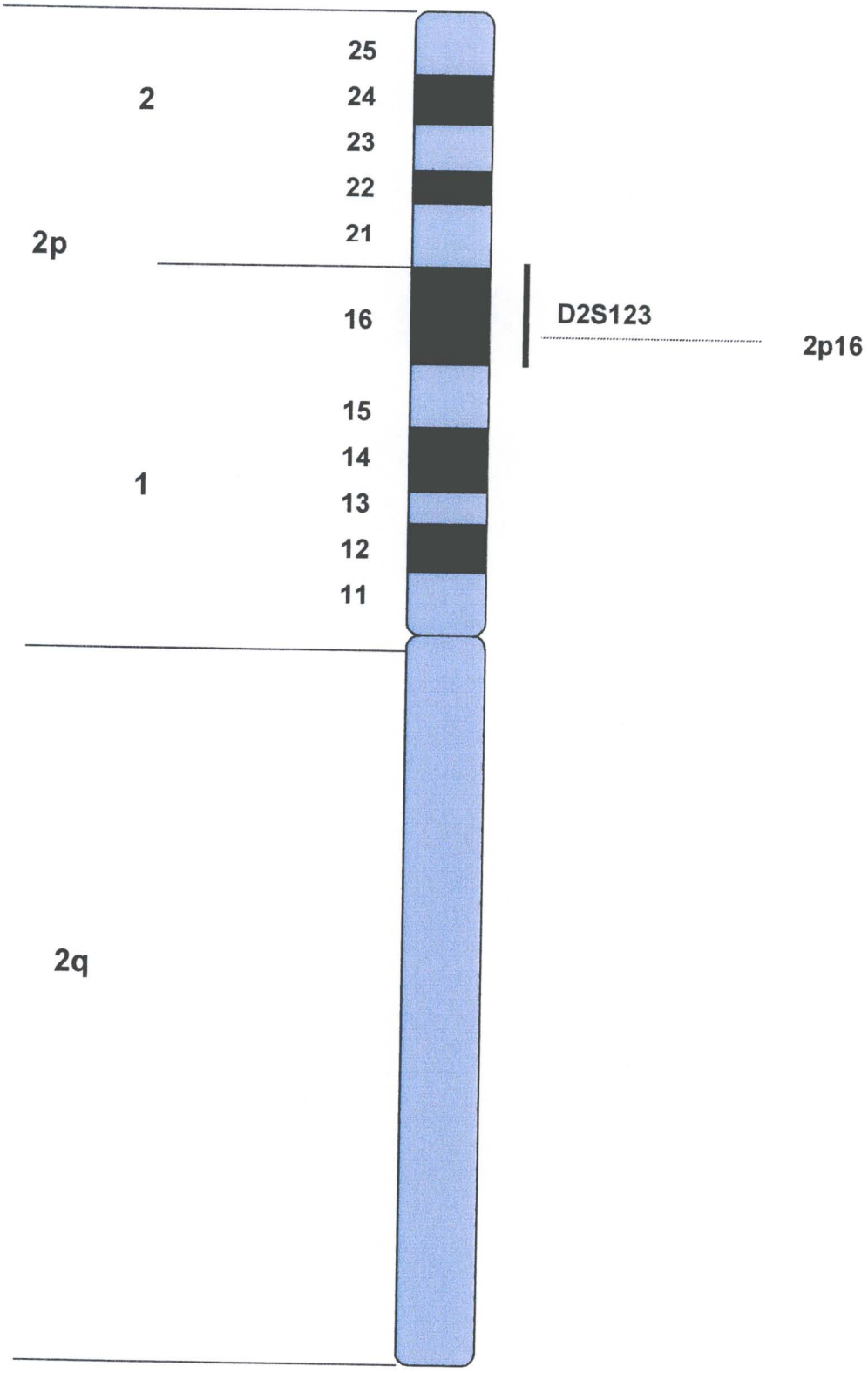


Figure 3.2: Ideogram of chromosome 2, with the location of the D2S123 locus

3.5.5.2 MICROSATELLITE PCR REACTION

Once it was established that good quality DNA was obtained, the microsatellite PCR reaction was carried out. The polymerase chain reactions were carried out in 200 μl PCR tubes, which were kept on ice at all times. The PCR Core Kit (Boehringer Mannheim) was used for this procedure. CY5 labelled primers (20 pmoles) were used in the PCR in a total reaction volume of 25 μl, containing 5 μl template DNA, 200μM dNTPs, 50 mM PCR buffer containing 1.5 mM $MgCl_2$ and 0.2 units of Taq DNA polymerase. The PCR amplification was performed in a Techne Progene Thermocycler, and the programme was adjusted according to the annealing temperature of the markers used (D3S659 - 52°C; D18S58, D2S123, BAT25 - 55°C; D18S21, D18S34 - 58°C). The initial denaturation was at 95°C for 5 min. This was followed by 30 cycles of 1 min at 94°C, 30 seconds at 52°C, 55°C, or 58°C, and 30 seconds at 72°C. The final extension cycle was at 72°C for 10 minutes, (See Appendix H).

The products of this reaction were stored at 4°C, before analysis on a sequencing gel.

3.5.6 AUTOMATED DNA FRAGMENT ANALYSIS

The microsatellite PCR products were analysed using a 6% Longranger sequencing gel (see Appendix F).

3.5.6.1 PREPARATION OF GEL PLATE (SHORT THERMOPLATE)

The short thermoplate (Pharmacia Biotech) was used to cast the gel. The plates, glass spacers (0.5 mm) and the comb (0.5 mm thick) were thoroughly cleaned with milliQ water and 70% alcohol. All the parts of the gel plate were carefully examined under reflected light before assembly, to ensure there were no dust particles present, which would interfere with band migration and broadening during the electrophoretic run. This cleaning procedure was also carried out because the ALFexpress DNA Sequencer is highly sensitive and capable of detecting trace contaminants. The spacers were carefully positioned along the indented edges on the right and left sides of the thermoplates, and gentle pressure was applied to secure them into the silicone - rubber seals.

The glass coverplate was positioned over the thermoplate and bound together with clamps. The comb was positioned between the plates and the gel was cast from the lower edge. The gel solution was allowed to polymerise for approximately 90 minutes.

3.5.6.2 PREPARATION OF SAMPLES AND SIZE MARKER

3 μl of each microsatellite PCR product was mixed with 3 μl of STOP solution (Blue dextran 2000, deionized formamide) (Pharmacia Biotech) and held on ice. The mixture was then denatured at 96°C for 3 mins in a Techne Progene thermocycler and held on ice, just before the samples were loaded on the gel.
A 50-500 CY5 labelled base pair standard was used as the external size marker. This external size marker was purchased from Pharmacia Biotech, Uppsala, Sweden). 1μl of the marker was mixed with 3μl of STOP (see Appendix F) and was loaded in lane one of the gel.

3.5.6.3 PROGRAMMING AND RUNNING CONDITIONS ON THE ALFexpress DNA SEQUENCER

The DNA sequencer was switched on and the running conditions and necessary data were entered using the ALF Manager Software (Pharmacia).

The following running parameters were used for the microsatellite analysis (Table 3.6):

Table 3.6: Running conditions on the ALFexpress DNA sequencer

Sampling interval	1 sec
Voltage	1600 V
Power	25 W
Current	60 mA
Temperature	55°C
Running time	180 mins

3.5.6.4 ATTACHMENT OF THE GEL CASSETTE ON TO THE ALFexpress DNA SEQUENCER

The outer surface of the glass plates were cleaned, especially around the area of the detectors, polymerised gel and urea were removed. The lower buffer chamber was placed in front of the instrument before attaching the gel cassette to the sequencer. The push-fit connectors of the instrument's thermocirculator were attached to the slots on the thermoplate to allow buffer to be automatically pumped through the plates. One litre of TBE buffer (1X concentration) was added to both the upper and lower buffer chambers.

Once the instrument reached 45°C, the comb was removed and the wells cleaned thoroughly of any unpolymerised acrylamide and urea. Squirting buffer into the wells with a syringe helped with the cleaning of the wells. This was done approximately 10X. When the temperature reached 55°C the samples which were held on ice were loaded. Special elongated gel loading tips were used to load 6 μl of sample in each well.

3.5.6.5 VISUALISATION OF RESULTS

The peaks were visualised directly on the screen using the Fragment Manager software, and the results were noted.

The height and area of the peaks were also recorded.

3.6 STATISTICAL ANALYSIS AND CALCULATION OF RATIOS

3.6.1 CALCULATION OF RATIOS FOR ALLELIC IMBALANCE

The method suggested by Canzian *et al.* (1996) was used to calculate the allelic ratios. Peak areas were expressed as ratios ie.: (T2xN1)/(T1xN2), where T/N1 and 2 are the first and second peaks of tumour and normal DNA respectively. A ratio of < 0.6 was regarded as allelic imbalance with loss of the larger allele, and if the ratio was > 1.6, then the smaller allele was lost.

3.6.2 MICROSATELLITE INSTABILITY

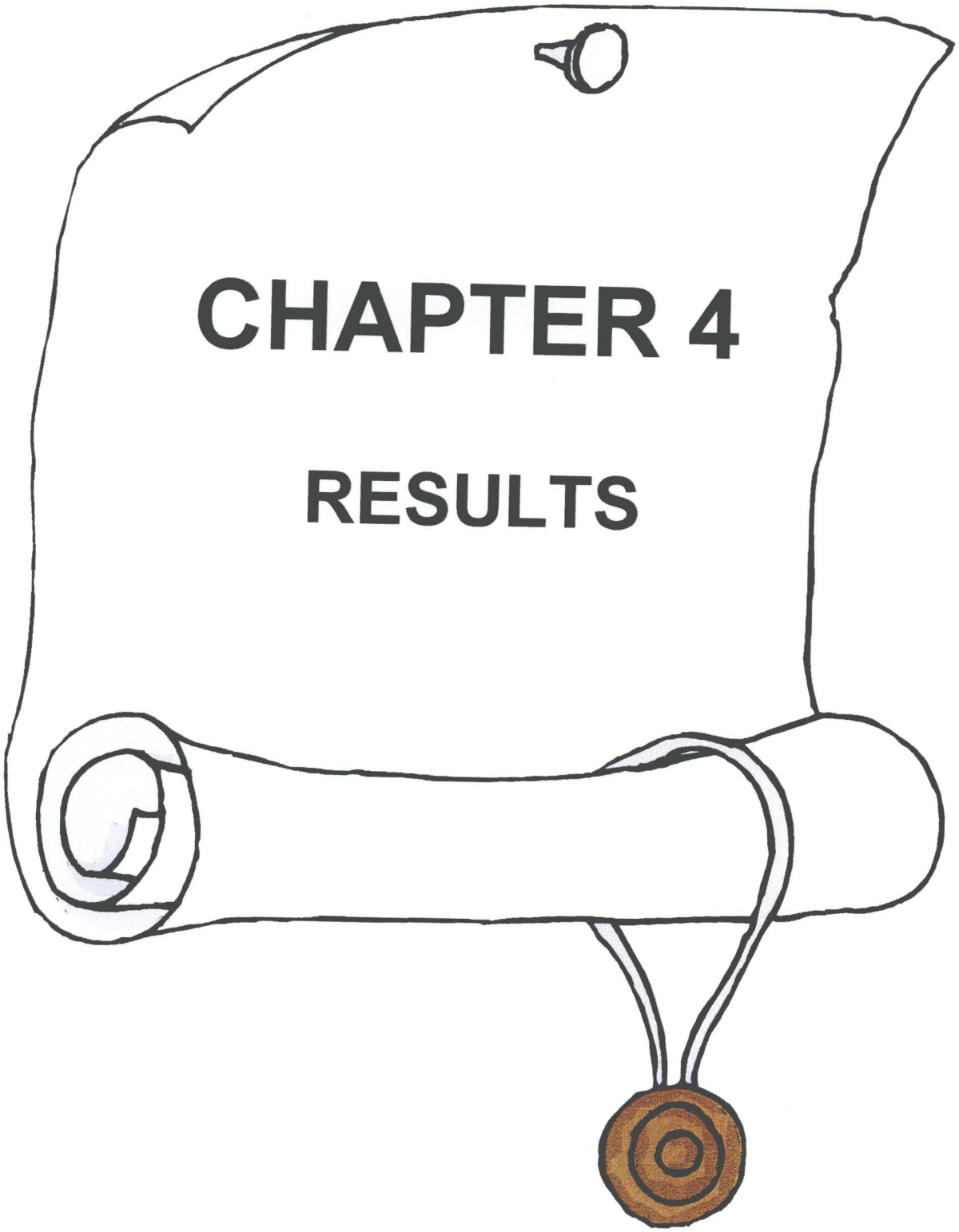
Microsatellite instability was identified by the appearance of a novel allele in the tumour sample (Cawkwell *et al*, 1995).

3.6.3 ANALYSIS OF ELECTROPHORETOGRAMS

Microsatellite analysis was performed with the aid of the Pharmacia Fragment Manager software version 1.0. A filename was given to each experiment before the run on the automated sequencer. Each case had normal and tumour DNA run in sequential lanes on the gel. The data was automatically processed and stored under the relevant filename, once the run was completed. The peaks produced in each lane together with the peak areas and the size of the fragments (in base pairs), was detected by the software, when the external size marker was used.

3.6.4 STATISTICAL ANALYSIS

The Statistical Package for Social Sciences software program (SPSS, Chicago, III, USA) was employed to perform the statistical analysis. Results were correlated with age, race, histological subtypes, grade, stage, myometrial invasion and lymph node involvement. They were assessed by cross tabulation and the Chi-square test was used to determine the significance. Differences were considered significant at $p \leq 0.05$. The Pearson's chi-square test is one of a variety of chi-square tests whose results are evaluated by reference to the chi-square distribution. Pearson's is the original and most widely used chi-sqaure test. It tests a null hypothesis that the relative frequencies of occurrence of observed events follow a specified frequency distribution (Wikepedia, 1995).



CHAPTER 4

RESULTS

RESULTS

"Making a discovery is such a wonderful thing. It's like falling in love and getting to the top of the mountain all in one." - Nobel Laureate Max Perutz, who met Cupid on the top of the mountain called the Structure of Haemoglobin.

4.1 CLINICAL DATA

A total of 54 endometrial cancer patients were studied. All of which had hysterectomies. Light microscopic, immunohistochemistry and molecular analysis were performed on all of these cases (Tables 4.27 and 4.46). However, an in depth clinical patient follow-up was not possible as many of the patients' files were not available.

4.1.1 AGE

The age distribution for the cases studied were as follows: 4 belonged to the 40-49 age group, 12 were in the 50-59 range, 21 were in the 60-69 range, 9 were in the 70-79 range and 3 were in the 80-89 range (Figure 4.1). In 5 patients, ages were unknown. Most patients belonged to the 60-69 age group. The youngest patient was 40 while the oldest was 81 years old. The median patient age was 63 years and the mean patient age was 57.5 years.

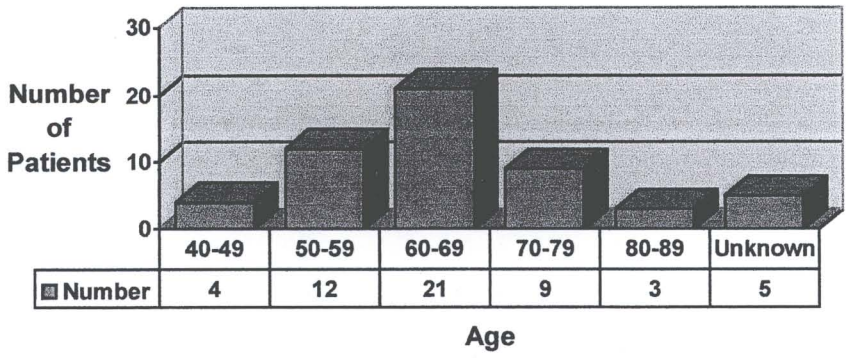


Figure 4.1: Bar graph showing age distribution

4.1.2 RACE

Of the 54 endometrial carcinoma cases studied 17 (31%) were Asian patients, 28 (51.8%) were Black, 2 (3.7%) were Coloured and 7 were White patients (12.9%), (Figure 4.2).

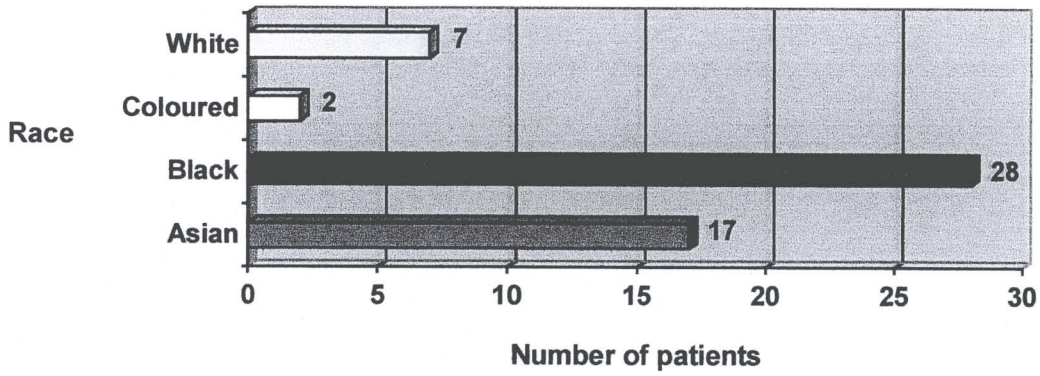


Figure 4.2: Bar graph showing the number of patients with endometrial carcinoma in the different race groups

4.1.3 LYMPH NODE STATUS

The only case that had metastatic nodal disease was a stage IIIC, grade 2-(G2) endometrioid adenocarcinoma. Of the remaining 53 cases, one was negative, while the rest had no lymph node sampling.

4.2 LIGHT MICROSCOPIC EVALUATION OF ENDOMETRIAL CARCINOMA

4.2.1 SUBTYPES OF ENDOMETRIAL CARCINOMA

The 54 endometrial cancer cases studied were classified according to the WHO classification as endometrioid endometrial carcinoma, serous papillary carcinoma, and clear cell carcinoma. Endometrioid carcinoma cases contained glands that resembled those of normal endometrium. They were uniform in size and shape (Plate 4.1). The serous papillary variant of adenocarcinoma was characterised by a complex papillary architectural

growth pattern. Papillae were lined by cuboidal epithelial cells with eosinophillic cytoplasm and vesicular nuclei (Plate 4.2). Psammoma bodies were present (Plate 4.3a,). The clear cell adenocarcinomas were mainly composed of clear cells. These cells were arranged in a solid, tubulocystic/papillary pattern (Plate 4.4). The single adenoacanthoma was seen as an endometrioid adenocarcinoma with focal differentiation into the benign squamous epithelium (Plate 4.5).

Of the 54 cases, 46 were of the endometrioid type, 2 were serous papillary carcinomas, 5 were clear cell carcinomas, and 1 an adenoacanthoma (Table 4.1, Figure 4.3). We did not encounter any other histologic types of endometrial carcinoma.

Table 4.1: The percentage of patients belonging to the different subtypes of endometrial cancer

SUBTYPE	NO OF CASES	%
Endometrioid carcinoma	46	85.2
Serous papillary carcinoma	2	3.7
Clear cell carcinoma	5	9.3
Adenoacanthoma	1	1.9

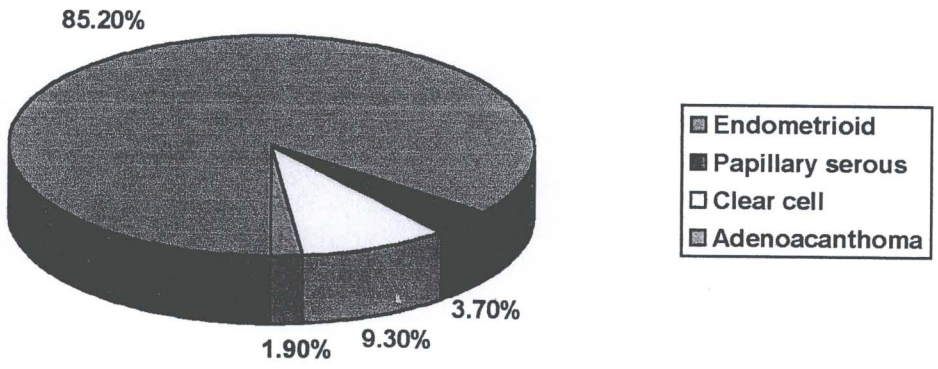


Figure 4.3: Pie Graph showing the different histologic subtypes studied

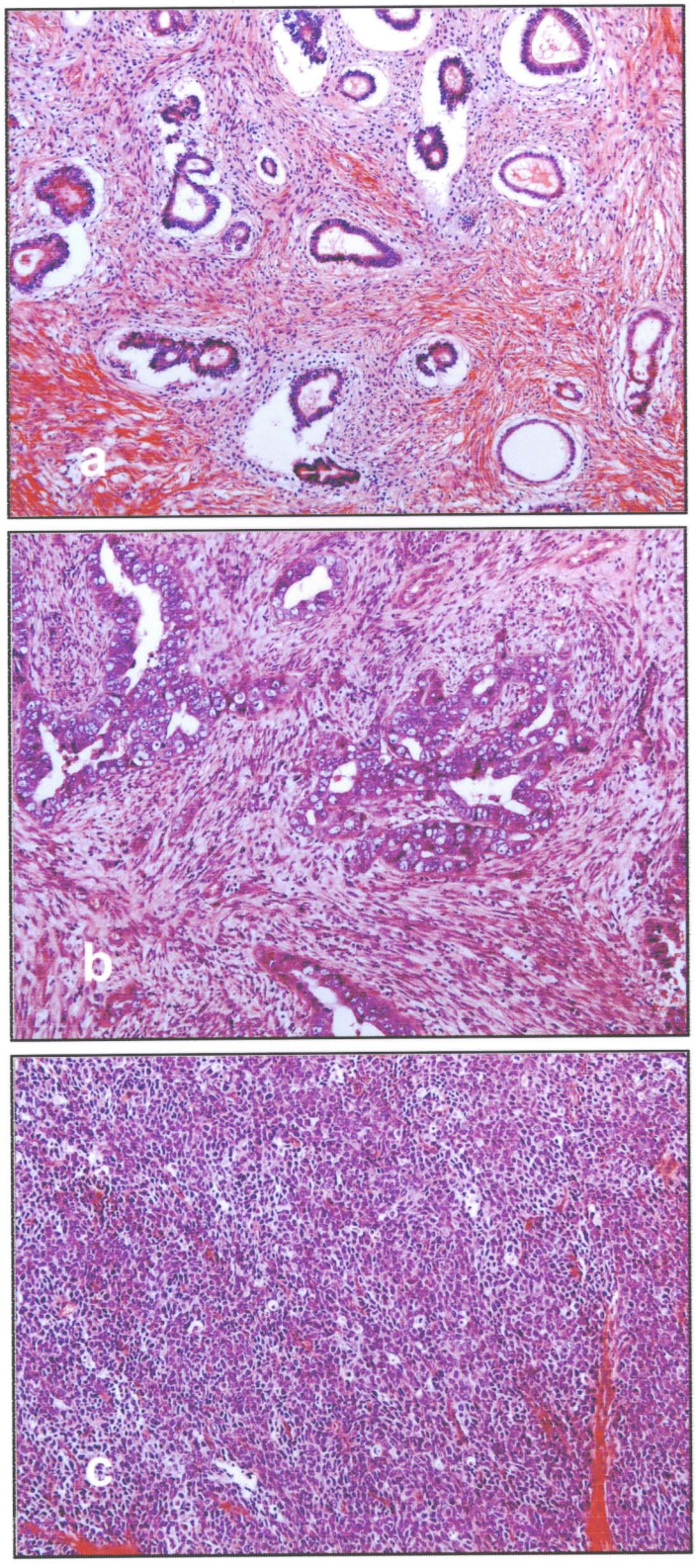


Plate 4.1: Endometrioid Adenocarcinoma; **a** low power showing a grade 1 well differentiated endometrioid adenocarcinoma consisting of well developed glandular structures (H&E, magnification X100); **b** a grade 2 moderately differentiated endometrioid adenocarcinoma (H&E, magnification X100); **c** a grade 3 poorly differentiated endometrial tumour showing little gland formation (H&E, magnification X100)

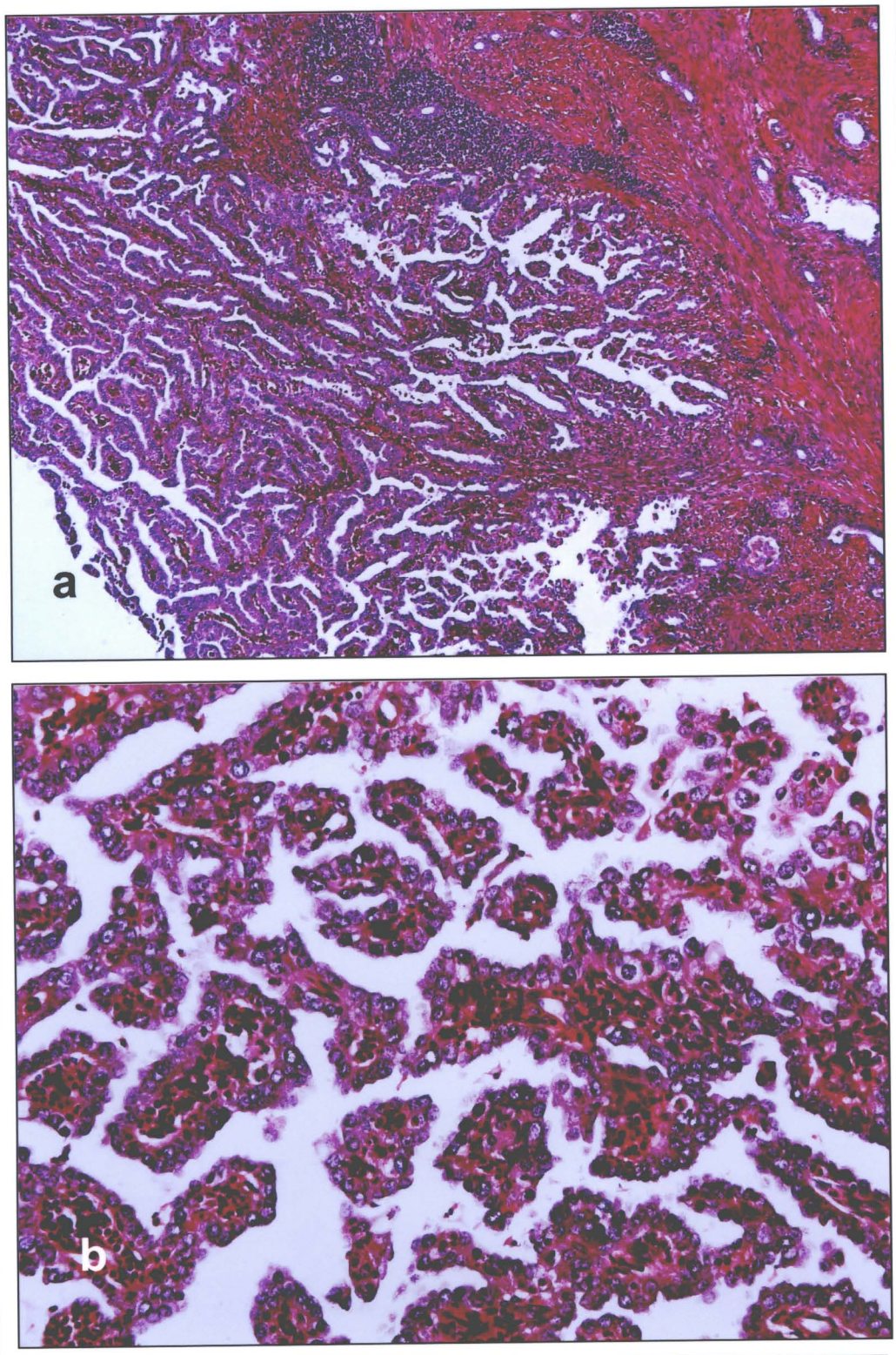


Plate 4.2: Serous papillary endometrial adenocarcinoma, **a** low power showing a papillary architectural growth pattern (H&E, magnification X 50), **b** medium power showing papillae with fibrovascular cores lined by cuboidal epithelial cells with eosinophillic cytoplasm and vesicular nuclei (H&E, magnification X 200)

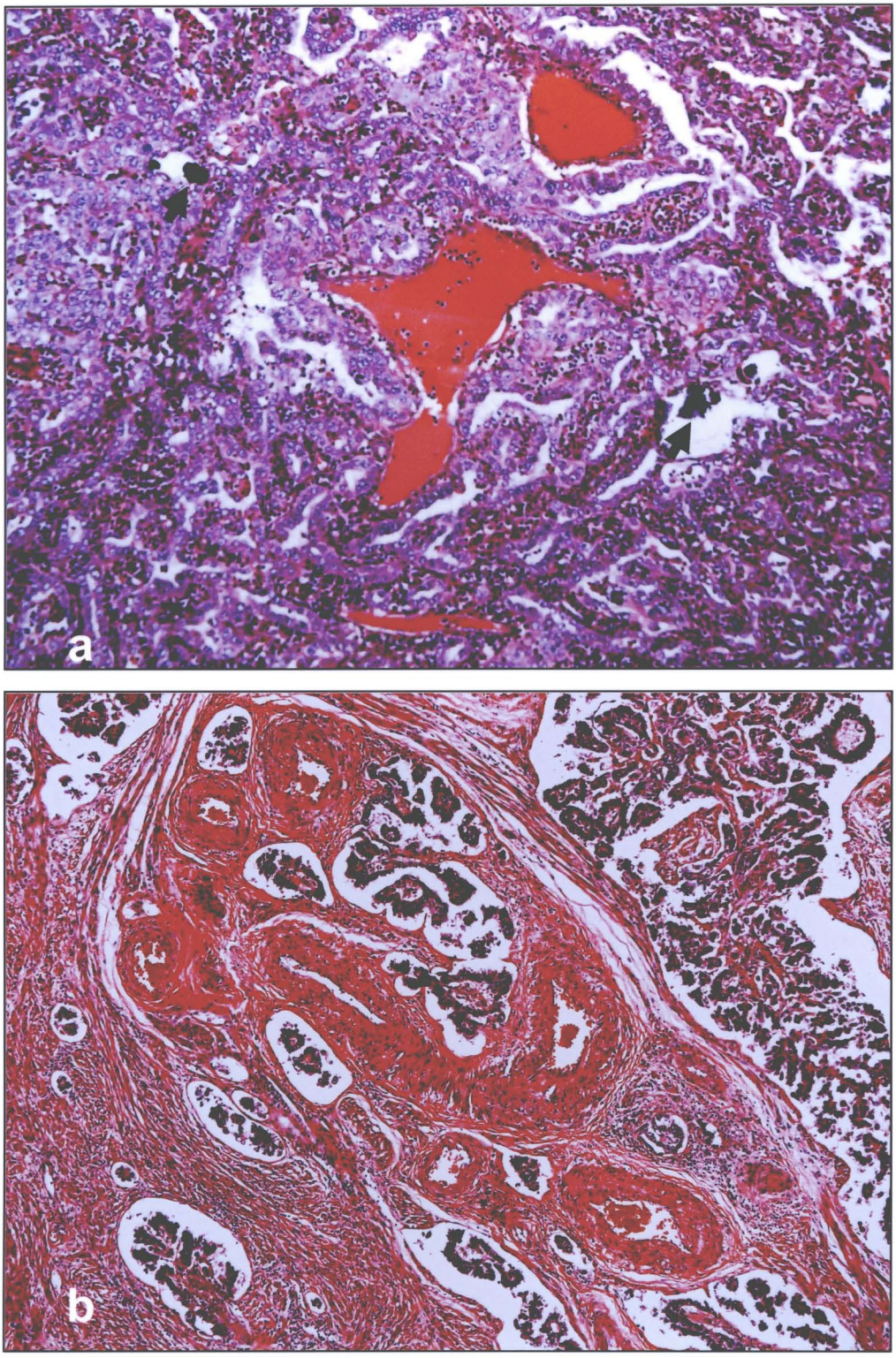
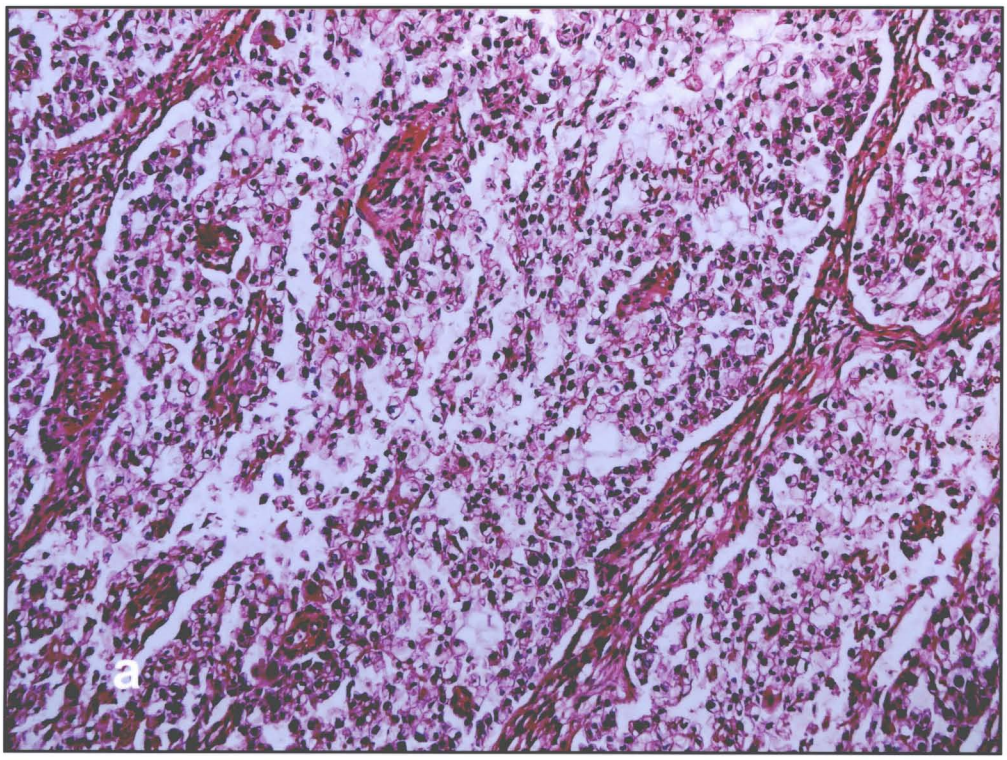


Plate 4.3: Serous papillary endometrial adenocarcinoma with psammoma bodies and muscle invasion, **a** low power showing papillae lined by poorly differentiated cells, consisting of cellular budding and the presence of psammoma bodies (H&E, magnification X100); **b** low power showing the characteristic infiltration of the serous papillary tumour into the myometrium (H&E, magnification X50)



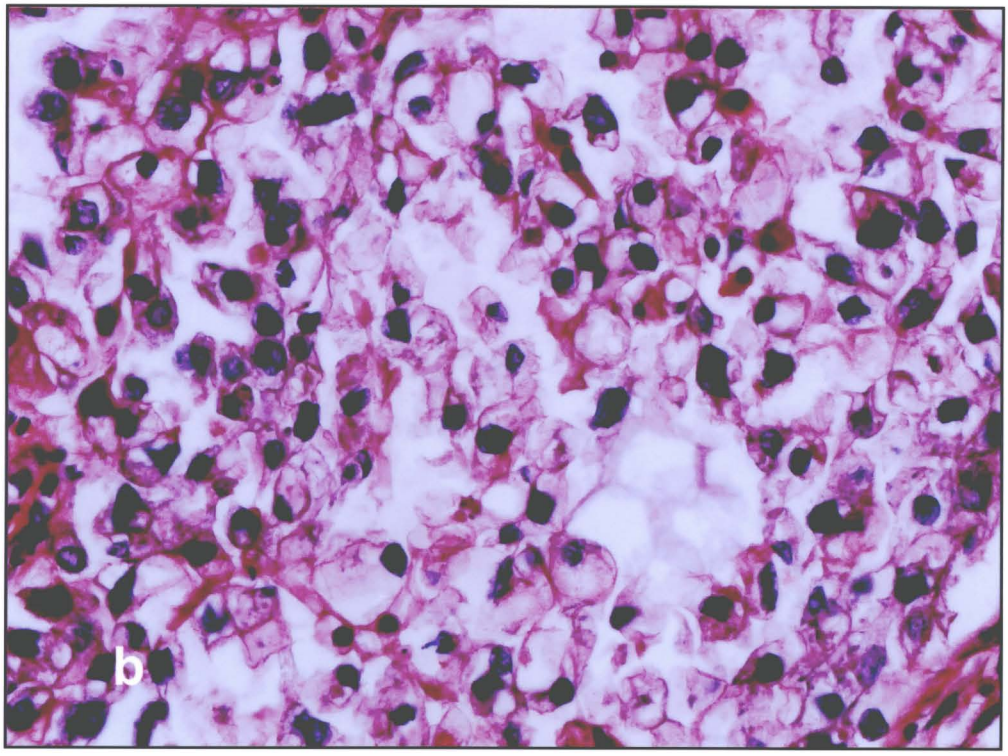


Plate 4.4: Clear cell adenocarcinoma; **a** low power showing clear cells arranged in a solid tubulocystic pattern (H&E, magnification X100); **b** high power showing clear cell adenocarcinoma composed chiefly of clear cells (H&E, magnifcation X400)

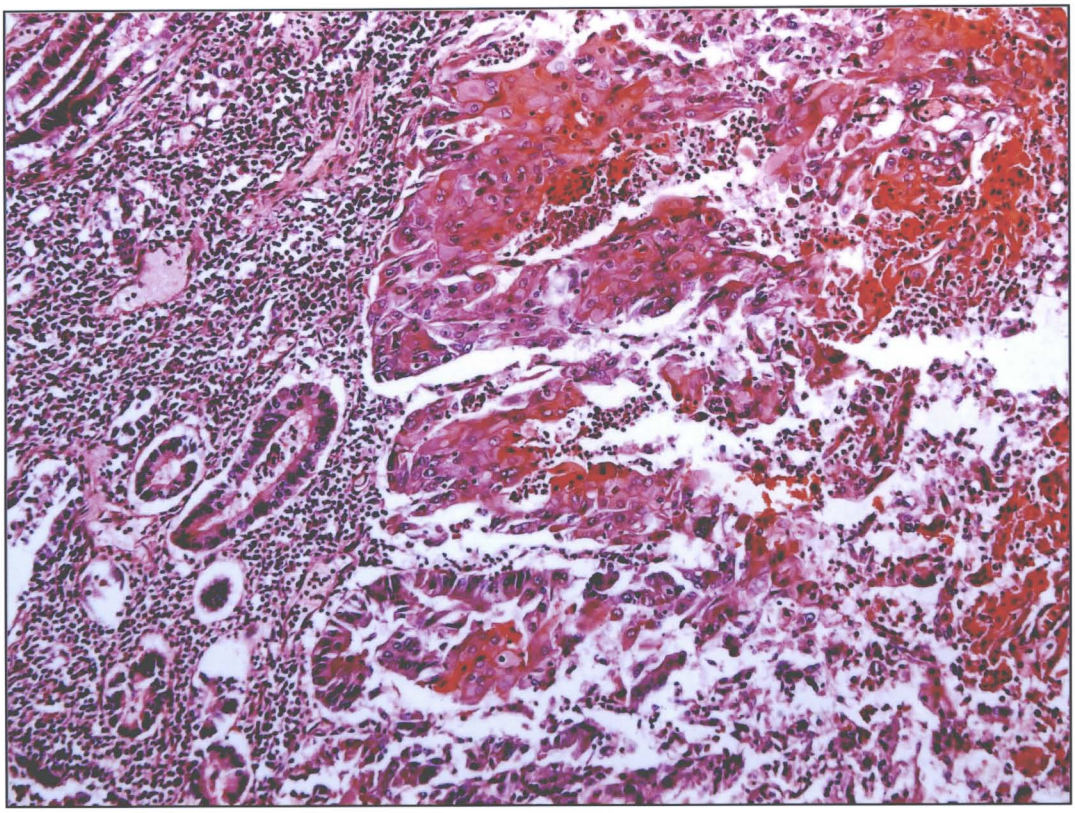


Plate 4.5 : An adenoacanthoma, low power demonstrating an endometrioid adenocarcinoma in which there is focal differentiation into the benign appearing squamous epithelium (H&E, magnification X100)

4.2.2 GRADE

The H&E sections were also graded accordingly and are reflected below. 11 cases were well differentiated, 33 were moderately differentiated while only 10 cases were poorly differentiated (Table 4.2).

Table 4.2: Grading of cases

GRADE	NO OF CASES
1	11
2	33
3	10

Key:

G1-well differentiated

G2-moderately differentiated

G3-poorly differentiated

These cases were graded according to the modified FIGO (International Federation of Gynaecology and Obstetrics) grading system (Plates 4.1a, 4.1b and 4.1c).

4.2.3 MYOMETRIAL INVASION

The depth of myometrial invasion was expressed as a percentage being either less than 50%, 50%, or greater than 50% depending on how deep the tumour invaded the myometrium. In 2 cases the tumour infiltrated 50% of the myometrial wall, 25 cases had less than 50% of tumour invasion, 21 cases showed that the tumour extended more than 50% of the myometrium and 6 cases had no myometrial invasion (Table 4.3). Plate 4.3b demonstrates myometrial invasion in a serous papillary adenocarcinoma.

Table 4.3: Myometrial invasion

MYOMETRIAL INVASION	NUMBER OF CASES
50%	2
< 50%	25
> 50%	21
No myometrial invasion	6

4.2.4 STAGE

Table 4.4: Staging of cases

STAGE	NUMBER OF CASES
IA	6
IB	24
IC	8
IIA	1
IIB	7
IIIA	7
IIIB	0
IIIC	1
IVA	0
IVB	0

The 54 cases studied were staged according to the modified FIGO (International Federation of Gynaecology and Obstetrics) staging system. Of the 54 cases, 6 were stage IA, 24 were IB, 8 were IC, 1 was IIA, 7 were IIB and IIIA, and, 1 was IIIC (Table 4.4).

Table 4.5: The grade, stage, depth of myometrial invasion and lymph node metastasis for the different tumour subtypes.

Case No	Tumour Type	Tumour Grade	Tumour Stage	Depth Of Myometrial Invasion	Lymph Node Status
T_1	Endometrioid	G3	IIB	>50	No nodes
T_2	Endometrioid	G1	1A	No invasion	Neg
T_3	Endometrioid	G3	IB	< 50	No nodes
T_4	Endometrioid	G3	IIB	< 50	No nodes
T_5	Endometrioid	G3	IC	> 50	No nodes
T_6	Endometrioid	G2	IB	< 50	No nodes
T_7	Endometrioid	G2	IIIC	>50	Positive
T_8	Endometrioid	G1	IC	>50	No nodes
T_9	Clear cell	G2	IIB	>50	No nodes
T_{10}	Endometrioid	G1	IA	No invasion	No nodes
T_{11}	Clear cell	G2	IIB	<50	No nodes
T_{12}	Endometrioid	G2	IIIA	>50	No nodes
T_{13}	Endometrioid	G2	IB	<50	No nodes
T_{14}	Endometrioid	G2	IB	<50	No nodes
T_{15}	Endometrioid	G2	IB	50%	No nodes
T_{16}	Endometrioid	G1	IC	>50	No nodes
T_{17}	Endometrioid	G2	IIIA	>50	No nodes
T_{18}	Clear cell	G2	I A	No invasion	No nodes
T_{19}	Endometrioid	G2	IB	<50	No nodes
T_{20}	Endometrioid	G2	IB	<50	No nodes
T_{21}	Endometrioid	G2	IB	<50	No nodes
T_{22}	Endometrioid	G2	IA	No invasion	No nodes
T_{23}	Endometrioid	G2	IIIA	>50	No nodes
T_{24}	Endometrioid	G1	IB	<50	No nodes
T_{25}	Endometrioid	G3	IC	>50	No nodes
T_{26}	Endometrioid	G2	IB	<50	No nodes

T_{27}	Endometrioid	G2	IA	No invasion	No nodes
T_{28}	Endometrioid	G2	IC	>50	No nodes
T_{29}	Endometrioid	G3	IA	No invasion	No nodes
T_{30}	Endometrioid	G2	IB	<50	No nodes
T_{31}	Endometrioid	G3	IB	<50	No nodes
T_{32}	Endometrioid	G2	IB	50%	No nodes
T_{33}	Endometrioid	G1	IB	<50	No nodes
T_{34}	Endometrioid	G3	IIIA	>50	No nodes
T_{35}	Endometrioid	G1	IIB	<50	No nodes
T_{36}	Serous papillary	G2	IIIA	>50	No nodes
T_{37}	Endometrioid	G2	IB	<50	No nodes
T_{38}	Endometrioid	G3	IB	<50	No nodes
T_{39}	Endometrioid	G2	IIB	>50	No nodes
T_{40}	Clear cell	G2	IIIA	>50	No nodes
T_{41}	Endometrioid	G2	IB	<50	No nodes
T_{42}	Serous papillary	G2	IIIA	>50	No nodes
T_{43}	Endometrioid	G1	IB	<50	No nodes
T_{44}	Endometrioid	G1	IC	>50	No nodes
T_{45}	Endometrioid	G2	IB	<50	No nodes
T_{46}	Endometrioid	G2	IB	<50	No nodes
T_{47}	Endometrioid	G2	IC	>50	No nodes
T_{48}	Endometrioid	G2	IC	>50	No nodes
T_{49}	Endometrioid	G3	IB	<50	No nodes
T_{50}	Clear cell	G1	IB	<50	No nodes
T_{51}	Endometrioid	G2	IIA	>50	No nodes
T_{52}	Endometrioid	G1	IB	<50	No nodes
T_{53}	Adenoacanthoma	G2	IB	<50	No nodes
T_{54}	Endometrioid	G2	IIB	>50	No nodes

G1-well differentiated

G2-moderately differentiated

G3-poorly differentiated

4.3 IMMUNOHISTOCHEMICAL ANALYSIS OF ENDOMETRIAL CARCINOMA

In order to facilitate statistical evaluation, a score of 0 was referred to as NE-not expressed, whereas scores of 1-4 were grouped together and placed in the category, E-expressed. The summarized table shows the expression status of each stain (Table 4.6). The Pearson Chi-Square test was used to determine statistical significance and a p value of less than or equal to 0.05 ($p \leq 0.05$) was taken as significant.

4.3.1 p53 IMMUNOEXPRESSION

P53 was expressed in 50% (27/54) of cases. Thirteen percent (7/54) showed 1+ staining, 1.85% (1/54) 2+, 5.6% (3/54) 3+ and 29.6% (16/54) 4+ (Fig 4.5). The positive cases showed a strong nuclear staining pattern (Plate 4.6).

4.3.1.1 p53 IN HISTOLOGICAL SUBTYPES OF ENDOMETRIAL CARCINOMA

Both serous papillary carcinoma cases showed 4+ p53 staining. Of the 5 clear cell carcinomas, 4 showed strong expression of p53, while 1 was negative. The single adenoacanthoma case showed 1+ expression. Twenty one (45.7%) endometrioid adenocarcinoma cases were positive for p53.

4.3.2 RETINOBLASTOMA IMMUNOEXPRESSION

Of the 54 cases studied, 90.7% (49/54) expressed nuclear pRb (Plate 4.7). Five cases were negative for the pRb. 16.7% (9/54) stained 1+; 5.6% (3/54) 2+; 11.1% (6/54) 3+ and 57.4% (31/54) 4+ (Fig 4.5).

4.3.2.1 RETINOBLASTOMA AND HISTOLOGICAL SUBTYPES

All 5 clear cell carcinomas showed 4+ pRb expression. Both serous papillary cases expressed 3+ pRb. The adenoacanthoma case stained weakly 1+. The 5 cases that lacked pRb expression were all of the endometrioid type while the remaining 41 endometrioid cases ie. 89.1% expressed pRb.

Table 4.6: Immunohistochemical staining results

Case no	P53	pRb	Cyclin A	Cyclin D	Cyclin E	P27	P53	pRb	Cyclin A	Cyclin D	Cyclin E	P27
1	4	1	3	0	4	3	E	E	E	NE	E	E
2	0	4	1	4	3	4	NE	E	E	E	E	E
3	4	0	4	0	4	4	E	NE	E	NE	E	E
4	4	4	3	0	4	3	E	E	E	NE	E	E
5	1	0	3	0	3	2	E	NE	E	NE	E	E
6	0	1	0	0	0	0	NE	E	NE	NE	NE	NE
7	1	4	3	0	4	4	E	E	E	NE	E	E
8	3	4	2	0	4	0	E	E	E	NE	E	NE
9	3	4	2	2	4	3	E	E	E	E	E	E
10	0	1	0	1	0	1	NE	E	NE	E	NE	E
11	4	4	3	0	4	2	E	E	E	NE	E	E
12	0	4	2	0	4	2	NE	E	E	NE	E	E
13	4	4	4	0	3	1	E	E	E	NE	E	E
14	4	0	2	0	4	1	E	NE	E	NE	E	E
15	0	4	2	0	2	2	NE	E	E	NE	E	E
16	0	4	4	0	3	4	NE	E	E	NE	E	E
17	4	0	4	0	4	3	E	NE	E	NE	E	E
18	0	4	2	0	4	4	NE	E	E	NE	E	E
19	0	4	1	0	3	0	NE	E	E	NE	E	NE
20	0	1	3	0	3	3	NE	E	E	NE	E	E
21	0	2	1	0	2	4	NE	E	E	NE	E	E
22	0	4	0	0	4	0	NE	E	NE	NE	E	NE
23	2	4	3	1	3	2	E	E	E	E	E	E
24	4	3	2	1	4	2	E	E	E	E	E	E
25	0	4	3	2	4	1	NE	E	E	E	E	E
26	0	4	0	0	0	3	NE	E	NE	NE	NE	E
27	0	4	0	1	2	0	NE	E	NE	E	E	NE
28	0	4	1	0	3	2	NE	E	E	NE	E	E

29	4	4	1	1	4	4	E	E	E	E	E	E
30	1	2	3	4	2	4	E	E	E	E	E	E
31	4	0	4	0	3	2	E	NE	E	NE	E	E
32	0	4	0	3	0	0	NE	E	NE	E	NE	NE
33	3	4	2	1	4	4	E	E	E	E	E	E
34	1	1	4	3	3	4	E	E	E	E	E	E
35	0	2	1	0	3	4	NE	E	E	NE	E	E
36	4	1	3	0	4	3	E	E	E	NE	E	E
37	1	4	1	0	4	4	E	E	E	NE	E	E
38	0	4	4	3	4	3	NE	E	E	E	E	E
39	4	3	3	3	4	2	E	E	E	E	E	E
40	4	4	2	0	4	1	E	E	E	NE	E	E
41	0	1	0	4	4	2	NE	E	NE	E	E	E
42	4	3	3	0	4	3	E	E	E	NE	E	E
43	0	4	1	1	4	4	NE	E	E	E	E	E
44	0	3	2	1	4	4	NE	E	E	E	E	E
45	4	4	2	0	4	4	E	E	E	NE	E	E
46	0	4	0	0	4	0	NE	E	NE	NE	E	NE
47	0	3	1	4	4	4	NE	E	E	E	E	E
48	0	4	2	0	4	4	NE	E	E	NE	E	E
49	1	4	3	1	3	1	E	E	E	E	E	E
50	0	4	3	0	4	4	NE	E	E	NE	E	E
51	0	3	2	0	3	1	NE	E	E	NE	E	E
52	0	1	2	0	2	4	NE	E	E	NE	E	E
53	1	1	1	0	3	1	E	E	E	NE	E	E
54	4	4	2	0	4	2	E	E	E	NE	E	E

Key:

E- Expressed

NE- Not Expressed

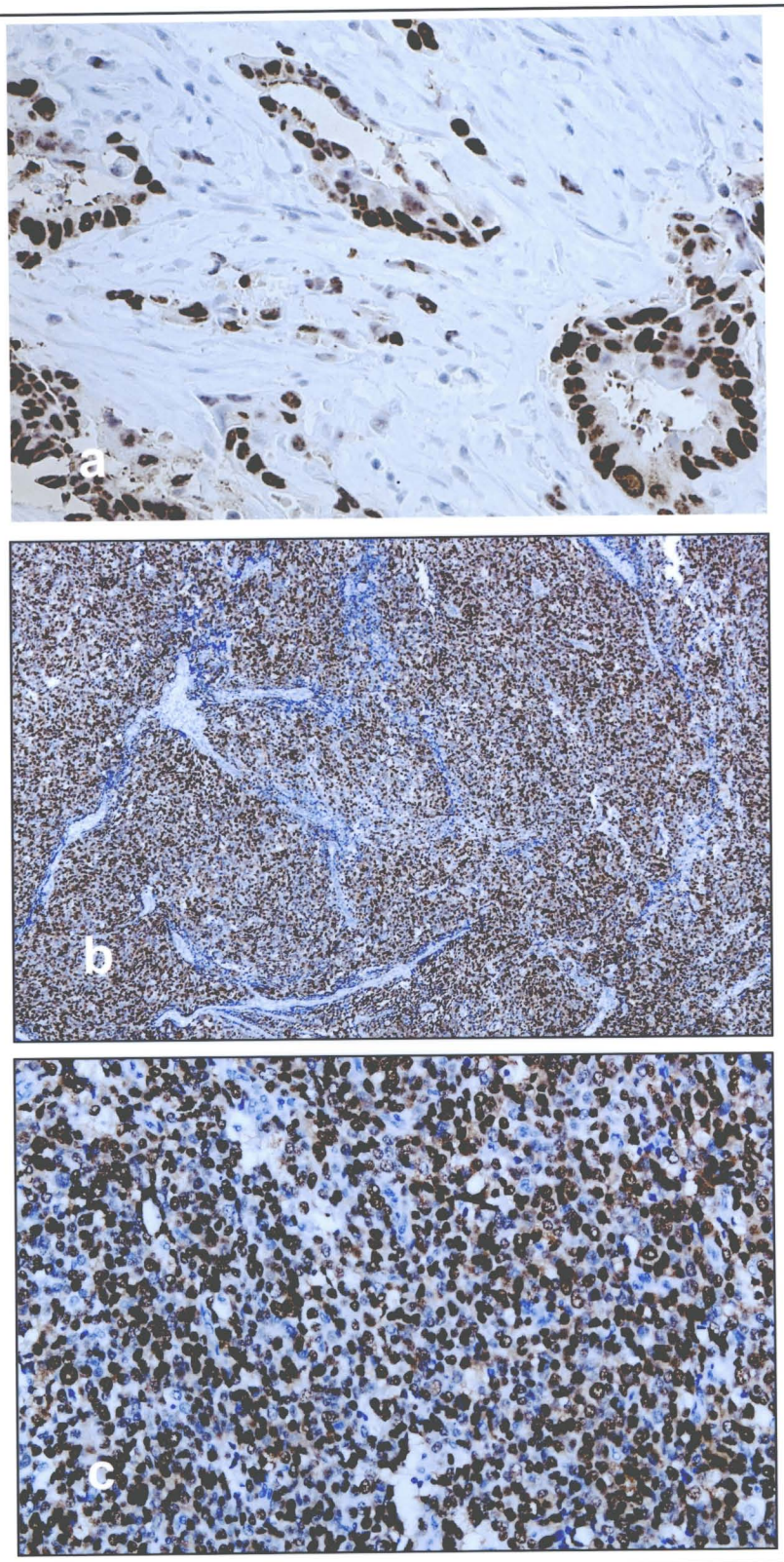


Plate 4.6: Immunohistochemical staining profile for p53; **a** Positive staining control of breast duct carcinoma showing nuclear staining; **b** low power showing diffuse nuclear staining (score 4+, magnification X50); **c** medium power showing dense nuclear staining (score 4+, magnification X200).

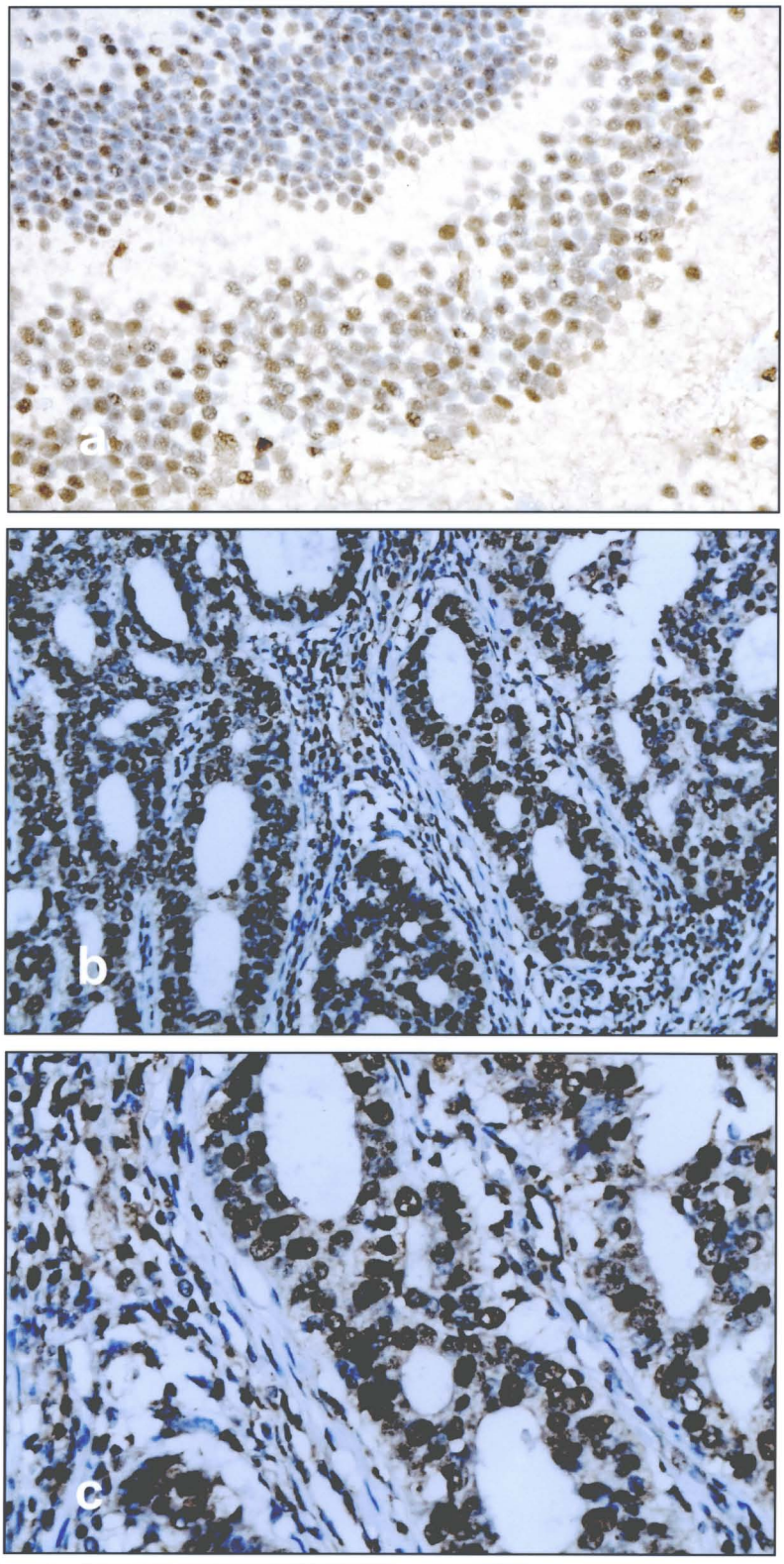
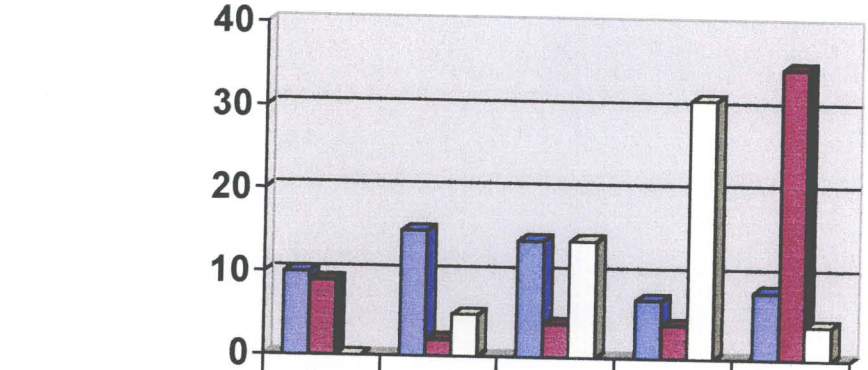


Plate 4.7: Immunohistochemical staining profile for Retinoblastoma protein (pRb); **a** positive staining control of the retina showing nuclear staining; **b** medium power showing diffuse staining in the glands of endometrioid adenocarcinoma (score 4+, magnification X200); **c** high power showing nuclear staining in the glandular epithelium of endometrioid adenocarcinoma (score X40, magnification X400)

4.3.3 CYCLINS



	1+	2+	3+	4+	Neg
CYCLIN A	10	15	14	7	8
CYCLIN D1	9	2	4	4	35
CYCLIN E	0	5	14	31	4

Fig 4.4: Bar graph showing the immunoexpression of Cyclins A, D1, E

4.3.3.1 CYCLIN A IMMUNOEXPRESSION

Cyclin A showed expression in 85.2% (46/54) of the cases. 18.5% (10/54) of the cases stained 1+; 27.8% (15/54) stained 2+; 25.9% (14/54) stained 3+ and 13% (7/54) stained 4+. A diffuse, intense nuclear staining was observed in the positive cases (Plate 4.8). Only 8 cases were negative for cyclin A (Fig 4.4).

4.3.3.1.1 CYCLIN A IN HISTOLOGICAL SUBTYPES

Thirty-eight of the 46 endometrioid cases stained for cyclin A. All 5 clear cell carcinomas and the 2 serous papillary cases, including the single adenoacanthoma also stained.

4.3.3.2 CYCLIN D1 IMMUNOEXPRESSION

Only 35.2% (19/54) of the cases immunoexpressed cyclin D1, with 64.2% (35/54) being negative. 16.7% (9/54) of cases stained 1+; 3.7% (2/54)

stained 2+; 7.4% (4/54) each stained 3+ and 4+ (Fig 4.4). A nuclear staining pattern was observed (Plate 4.9).

4.3.3.2.1 CYCLIN D1 IN HISTOLOGICAL SUBTYPES

Of the 46 endometrioid cases, 39.1% (18/46) were positive for cyclin D1. 4 of the 5 clear cell carcinomas did not stain and both serous papillary and the single adenoacanthoma did not stain as well.

4.3.3.3 CYCLIN E IMMUNOEXPRESSION

Overall cyclin E was immunoexpressed in 92.6% (50/54) of the cases and only 4 cases were negative (7.4%). Cyclin E expression was strong and all cases were 2+ and more; 9.3% (5/54) stained 2+; 25.9% (14/54) stained 3+; and 57.4% (31/54) stained 4+ (Fig 4.4). As can be seen the majority of cases stained strongly positive for cyclin E (Plate 4.10).

4.3.3.3.1 CYCLIN E AND HISTOLOGICAL SUBTYPES

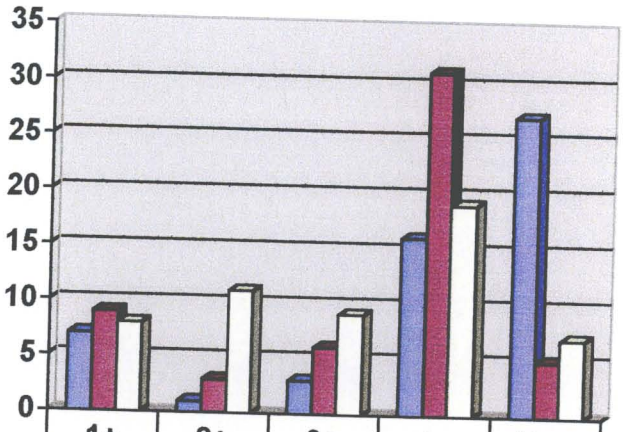
The 4 negative cases were all endometrioid adenocarcinomas. All 5 clear cell carcinomas and both the serous papillary adenocarcinomas stained strongly positive ie. with a 4+. The adenoacanthoma showed 3+ expression.

4.3.4 p27 IMMUNOEXPRESSION

There was an overall 87% (47/54) positive staining for p27. Only 7 (13%) cases were negative. 8 cases (14.8%) stained 1+; 11 (20.4%) cases stained 2+; 9 (16.7%) cases stained 3+ and 19 (35.2%) stained 4+ (Fig 4.5). Intense nuclear staining was observed in the glandular epithelial cells of endometrioid adenocarcinoma (Plate 4.11).

4.3.4.1 p27 AND HISTOLOGICAL SUBTYPES

Thirty-nine of the 46 (84.8%) endometrioid adenocarcinoma cases showed p27 expression. All of the negative p27 cases were of the endometrioid type. The 5 clear cell and both serous papillary adenocarcinomas including the single adenoacanthoma case expressed p27.



	1+	2+	3+	4+	Neg
p53	7	1	3	16	27
pRb	9	3	6	31	5
p27	8	11	9	19	7

Figure 4.5: Bar graph showing comparison of immunoexpression for p53, pRb and p27

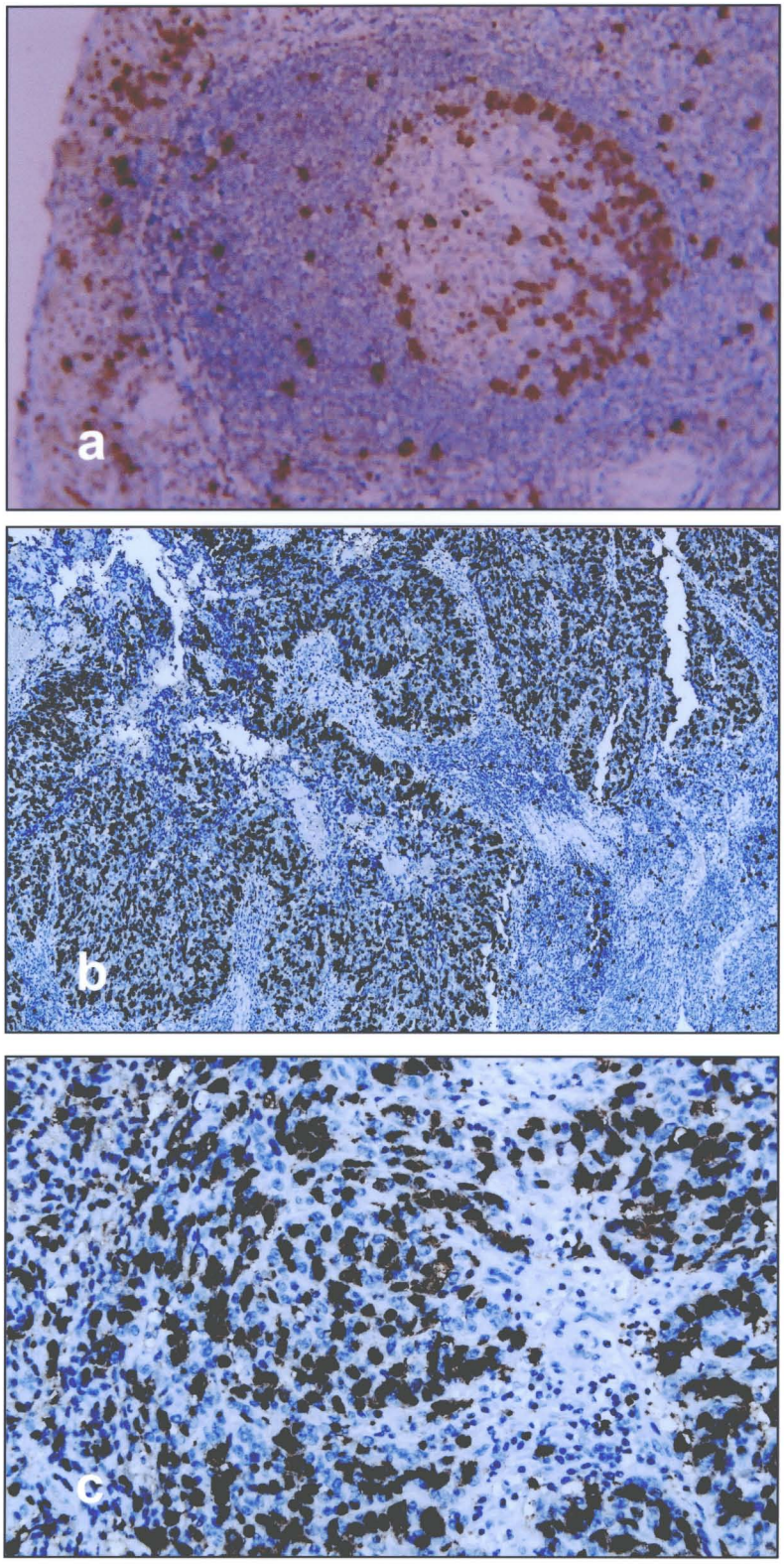


Plate 4.8: Immunohistochemical staining profile for Cyclin A; **a** positive staining control of the tonsil showing a nuclear staining pattern; **b** diffuse staining in endometrial carcinoma (score 3+, magnification X50); **c** intense nuclear staining in endometrial carcinoma (score 3+, magnification X200)

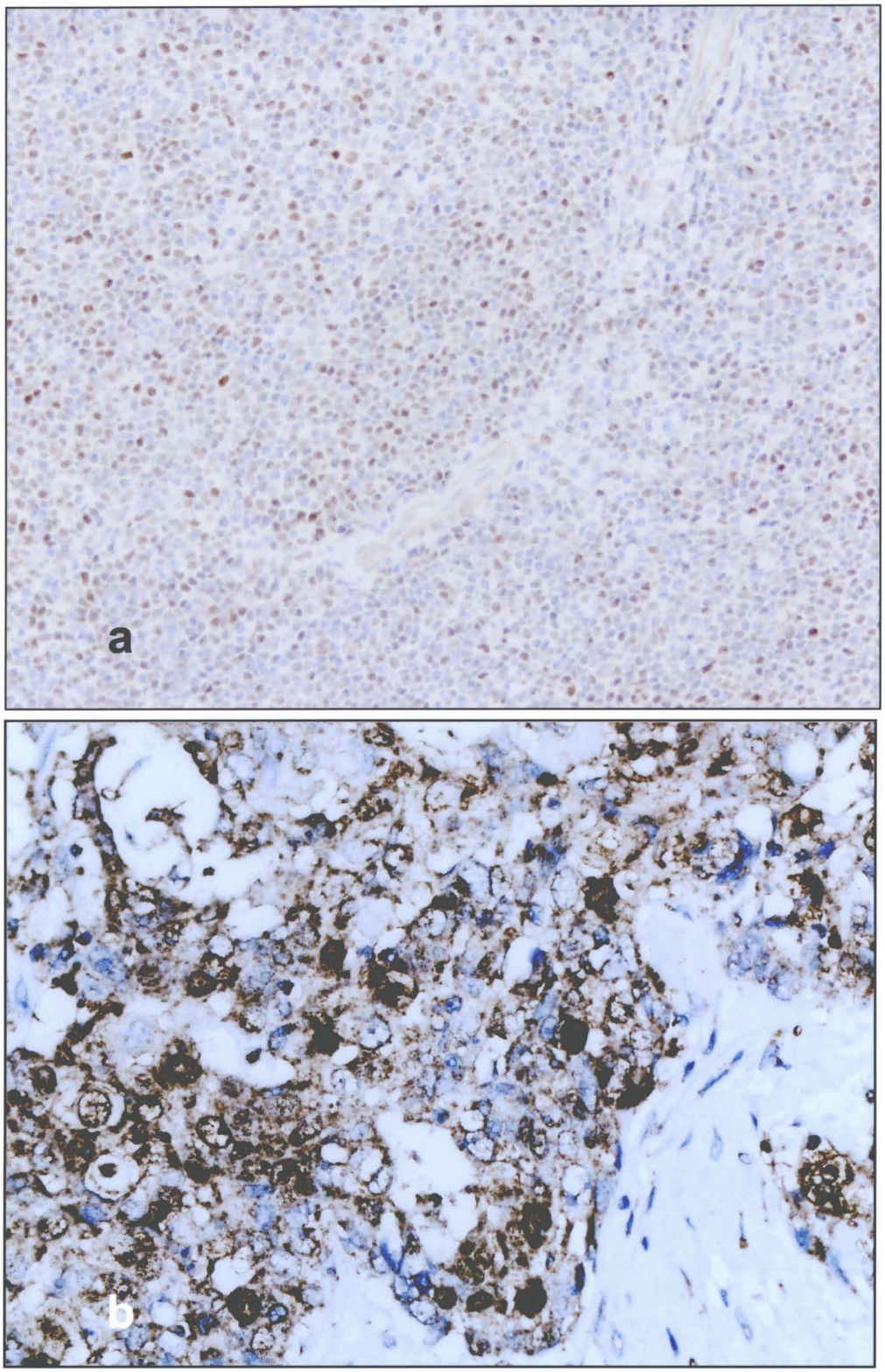


Plate 4.9: Immunohistochemical staining profile for Cyclin D; **a** positive control – mantle cell lymphoma; **b** nuclear staining in endometrial carcinoma (score 2+, magnification X400)

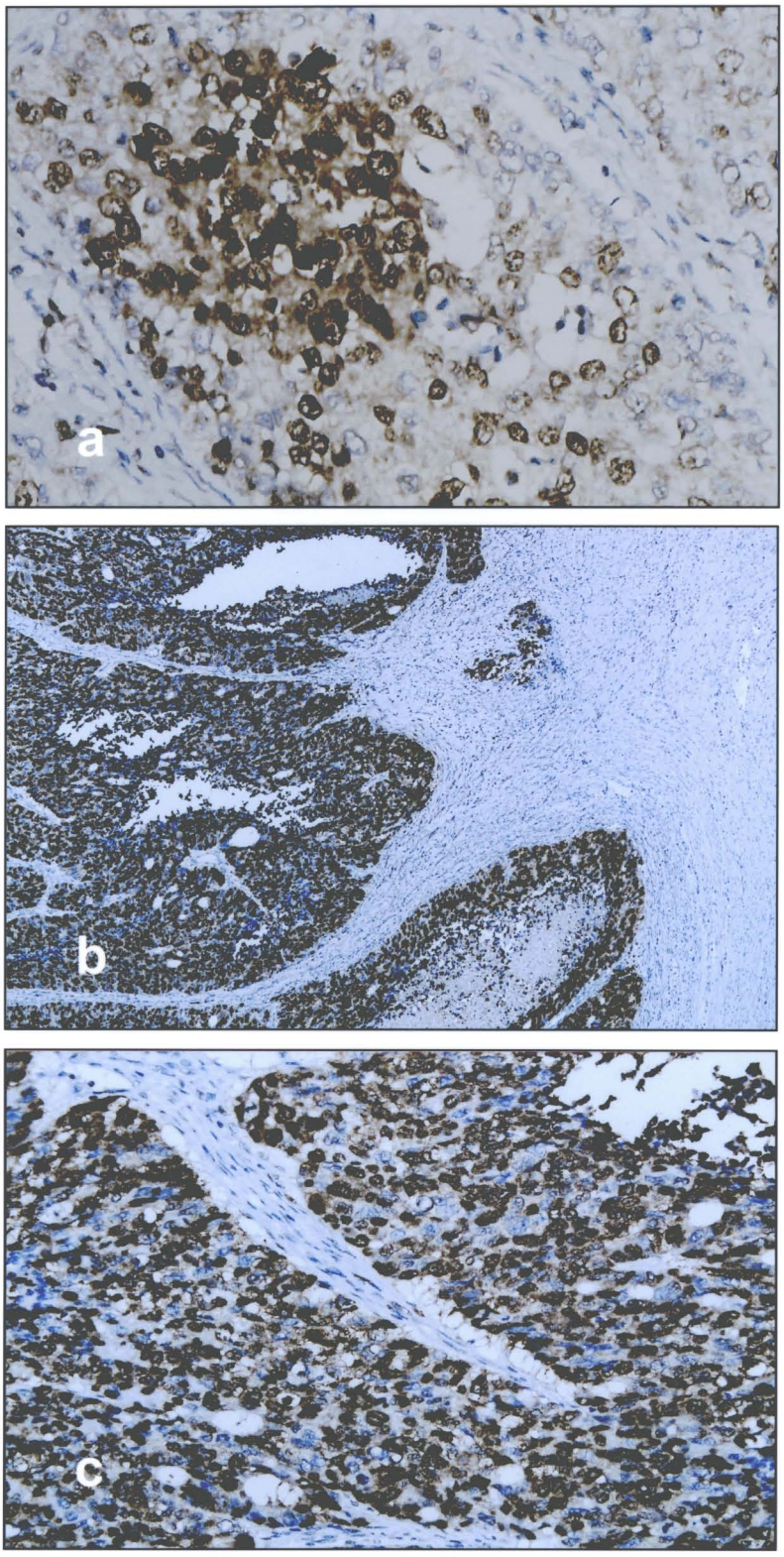


Plate 4.10: Immunohistochemical staining profile for Cyclin E; **a** positive staining control in breast carcinoma ; **b** low power showing diffuse staining in endometrial carcinoma (score 4+, magnification X10); **c** medium power demonstrating positive staining of cyclin E in endometrial carcinoma (score 4+, magnification X200)

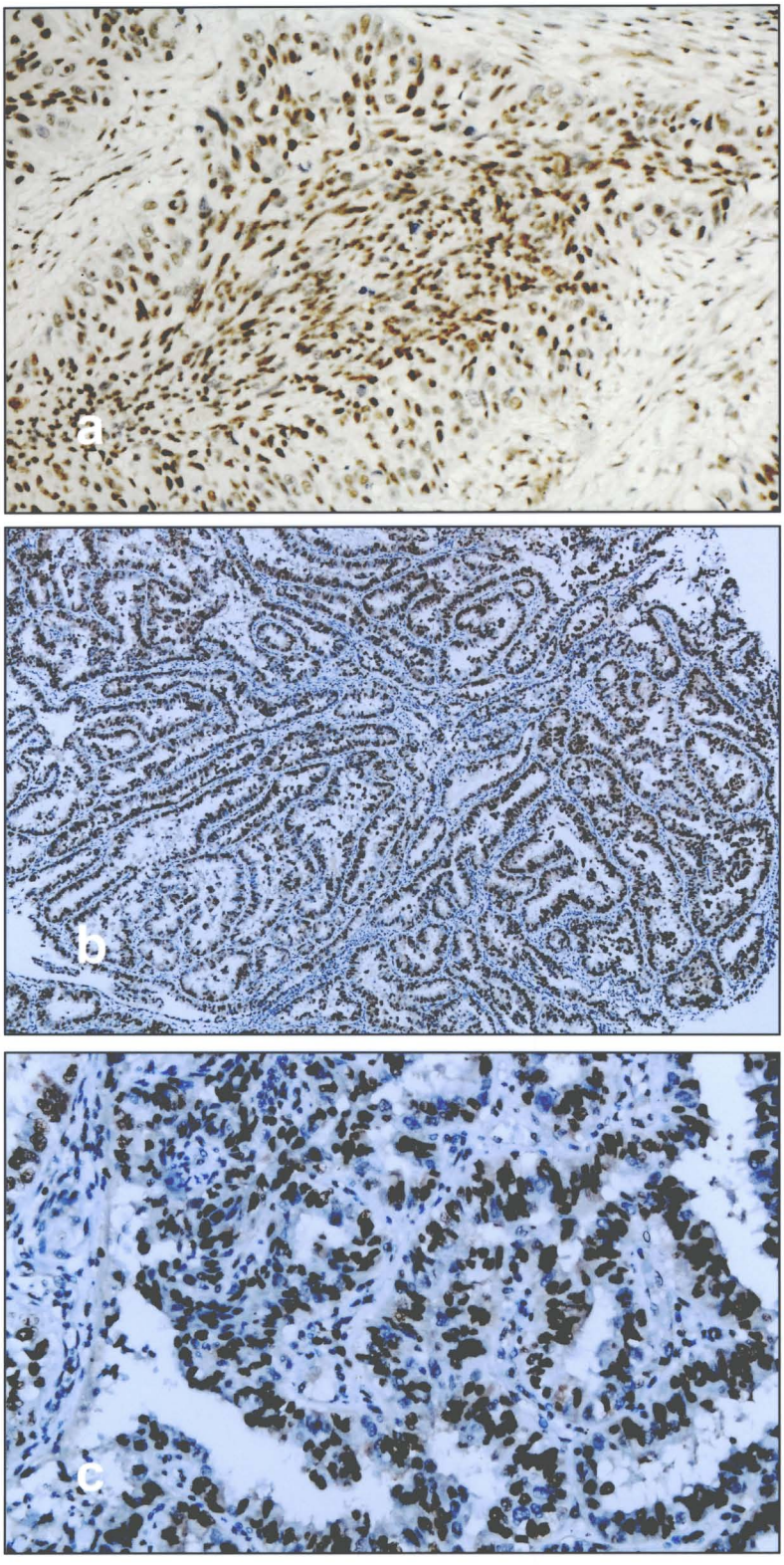


Plate 4.11: Immunohistochemical staining profile for p27; **a** positive staining control of oesophageal squamous carcinoma; **b** low power showing staining in the glandular epithelial cells of endometrioid adenocarcinoma (score 4+, magnification X50); **c** medium power showing the intense nuclear staining in endometrioid adenocarcinoma (score 4+, magnification X200)

4.3.5 COMPARISON OF IMMUNOHISTOCHEMICAL RESULTS

4.3.5.1 CYCLIN A AND CYCLIN D1

Table 4.7: Cyclin A versus Cyclin D1

		Cyclin D1		Total
		E	NE	
Cyclin A	E	15	31	46
	NE	4	4	8
Total		19	35	54

p=0.342

Fifteen cases expressed both cyclin A and cyclin D1, while 4 showed no expression for both. Thirty-one of the 46 cases that expressed cyclin A did not express Cyclin D1 (Table 4.7).

4.3.5.2 CYCLIN A AND CYCLIN E

Table 4.8: Cyclin A versus Cyclin E

		Cyclin E		Total
		E	NE	
Cyclin A	E	46	0	46
	NE	4	4	8
Total		50	4	54

p<0.000

This comparative study was very significant as the p value was less than 0.000. A total of 46 cases expressed both cyclin A and E. Four cases had no expression for both (Table 4.8).

4.3.5.3 CYCLIN A AND p53

Table 4.9: Cyclin A versus p53

		Cyclin A		Total
		E	NE	
P53	E	27	0	27
	NE	19	8	27
Total		46	8	54

p=0.002

Exactly half of the cases studied had expression for both cyclin A and p53, but 19 of the 46 cases that expressed cyclin A had no expression for p53. Eight cases showed no expression for both stains. These results showed statistical significance, the p value being 0.002 (Table 4.9).

4.3.5.4 CYCLIN A AND RETINOBLASTOMA

Table 4.10: Cyclin A versus pRb

		Cyclin A		Total
		E	NE	
pRb	E	41	8	49
	NE	5	0	5
Total		46	8	54

p=0.328

Forty-one cases expressed both cyclin A and pRb while none showed no expression for both (Table 4.10).

4.3.5.5 CYCLIN A AND P27

Table 4.11: Cyclin A versus p27

		p27		Total
		E	NE	
Cyclin A	E	44	2	46
	NE	3	5	8
Total		47	7	54

p<0.0001

Forty-four of the 46 cases that expressed cyclin A also expressed p27. Five showed no expression for both. The p value was significant at less than 0.0001 (Table 4.11).

4.3.5.6 CYCLIN D1 AND CYCLIN E

Table 4.12: Cyclin D1 versus Cyclin E

		Cyclin E		Total
		E	NE	
Cyclin D1	E	17	2	19
	NE	33	2	35
Total		50	4	54

p=0.519

Seventeen cases expressed both cyclin D1 and E. The 33 of the 50 cases that expressed cyclin E had no expression for cyclin D1. Only 2 cases showed no expression for both (Table 4.12).

4.3.5.7 CYCLIN D1 AND p53

Table 4.13: Cyclin D1 versus p53

		Cyclin D1		Total
		E	NE	
p53	E	9	18	27
	NE	10	17	27
Total		19	35	54

P=0.776

This comparison demonstrated no statistical signficance. Nine cases had expression for both while 17 had no expression for both markers (Table 4.13).

4.3.5.8 CYCLIN D1 AND RETINOBLASTOMA

Table 4.14: Cyclin D1 versus pRb

		Cyclin D1		Total
		E	NE	
pRb	E	19	30	49
	NE	0	5	5
Total		19	35	54

p=0.084

Nineteen cases expressed both pRb and cyclin D1 while only 5 had no expression for both (Table 4.14).

4.3.5.9 CYCLIN D1 AND P27

Table 4.15: Cyclin D1 versus p27

		P27		Total
		E	NE	
Cyclin D1	E	17	2	19
	NE	30	5	35
Total		47	7	54

p=0.694

Thirty of the 47 cases that expressed p27 did not express cyclin D1. Seventeen expressed both p27 and cyclin D1 (Table 4.15).

4.3.5.10 CYCLIN E AND p53

Table 4.16: Cyclin E versus p53

		Cyclin E		Total
		E	NE	
P53	E	27	0	27
	NE	23	4	27
Total		50	4	54

p=0.038

All 27 cases that expressed p53 also expressed cyclin E, but cyclin E had a total of 50 cases staining positive. Only 4 cases had no expression for both markers. Half the total number of cases expressed p53 while half did not. Statistical value was significant at p=0.038 (Table 4.16).

4.3.5.11 CYCLIN E AND RETINOBLASTOMA

Table 4.17: Cyclin E versus pRb

		Cyclin E		Total
		E	NE	
pRb	E	45	4	49
	NE	5	0	5
Total		50	4	54

p=0.507

A total of 45 cases stained for both cyclin E and p53. None had no expression for both (Table 4.17).

4.3.5.12 CYCLIN E AND p27

Table 4.18: Cyclin E versus p27

		p27		Total
		E	NE	
Cyclin E	E	45	5	50
	NE	2	2	4
Total		47	7	54

p=0.022

Forty-five cases expressed both p27 and cyclin E while only 2 had no expression for both. Only 5 of the 50 cases that expressed cyclin E had no expression for p27. This finding was significant, the p value being 0.022 (Table 4.18).

4.3.5.13 p53 AND RETINOBLASTOMA

Table 4.19: p53 versus pRb

		pRb		Total
		E	NE	
p53	E	22	5	27
	NE	27	0	27
Total		49	5	54

p=0.019

None of the cases had any expression for both pRb and p53, while 22 were positive for both. Twenty-seven of the 49 cases that expressed pRb did not express p53. Only 5 cases did not express pRb but all of these 5 showed expression for p53. This was of significance, the p value being 0.019 (Table 4.19).

4.3.5.14 p27 AND RETINOBLASTOMA

Table 4.20: p27 versus pRb

		p27		Total
		E	NE	
pRb	E	42	7	49
	NE	5	0	5
Total		47	7	54

p=0.365

Forty-two cases expressed both p27 and pRb while none showed any expression for both (Table 4.20).

4.3.5.15 p27 AND p53

Table 4.21: p27 versus p53

		P27		Total
		E	NE	
P53	E	26	1	27
	NE	21	6	27
Total		47	7	54

p=0.043

Twenty-six of the cases expressed both p27 and p53. Only 1 of the cases that expressed p53 did not express p27. 6 had no expression for both. Twenty-one of the 47 cases that expressed p27 did not express p53. The statistical value was significant, p=0.043 (Table 4.21).

4.3.6 COMPARISON OF IMMUNOHISTOCHEMICAL RESULTS WITH CLINICAL AND LIGHT MICROSCOPIC DATA

4.3.6.1 AGE

When the immunohistochemical expressions were compared to age, there was no significant results noted. The closest value of significance was age and cyclin D with a p value of 0.051.

4.3.6.2 RACE

This comparison showed no statistical significance.

4.3.6.3 LYMPH NODE STATUS

None of the immunohistochemical stains compared statistically with the lymph node status of the patients.

4.3.6.4 STAGE

Table 4.22: P53 versus Stage

		Stage							Total
		IA	IB	IC	IIA	IIB	IIIA	IIIC	
P53	E	1	11	2	0	6	6	1	27
	NE	5	13	6	1	1	1	0	27
Total		6	24	8	1	7	7	1	54

p=0.030

When p53 immunoexpression was compared to stage, a significant statistical value of p=0.030 was achieved. Most of the cases were stage IB. Of these 11 expressed p53 while 13 showed no p53 expression. The single case that was a stage IIIC expressed p53 (Table 4.22).

4.3.6.5 GRADE

Table 4.23: P53 versus Grade

		Grade			Total
		G1	G2	G3	
P53	E	3	16	8	27
	NE	8	17	2	27
Total		11	33	10	54

p=0.052

A value very close to statistical significance was obtained when p53 was compared to grade, p=0.052. Most of the cases were grade 2 ie. 33 cases. Of these, 16 expressed p53 while 17 did not (Table 4.23).

Table 4.24: PRb versus Grade

		Grade			Total
		G1	G2	G3	
pRb	E	11	31	7	49
	NE	0	2	3	5
Total		11	33	10	54

p=0.036

pRb also compared statistically with grade, achieving a p value of 0.036. Of the 33 cases that were grade 2, 31 expressed pRb. All of the 11 grade 1 cases stained for pRb (Table 4.24).

4.3.6.6 MYOMETRIAL INVASION

Table 4.25: Cyclin A versus Myometrial invasion.

		Myometrial invasion				Total
		N	L	E	G	
Cyclin A	E	3	21	1	21	46
	NE	3	4	1	0	8
Total		6	25	2	21	54

p=0.009

Key: N - No invasion
L - <50%
E - 50%
G - >50%

A statistiscal value of p= 0.009 was obtained when cyclin A was compared with myometrial invasion. All of the 21 cases that had greater than 50% of myometrial wall invasion, expressed cyclin A. Also, only 4 of the 25 cases that had less than 50% myometrial wall invasion did not express cyclin A (Table 4.25).

Table 4.26: Cyclin E versus Myometrial Invasion.

		Myo				Total
		N	L	E	G	
Cyclin E	E	5	23	1	21	50
	NE	1	2	1	0	4
Total		6	25	2	21	54

P=0.052

The comparison of cyclin E with myometrial invasion was also very close to statistical significance with a p value of 0.052. Here again, all of the 21 cases that had greater than 50% myometrial invasion expressed cyclin E. Twenty-three of the 25 cases that demonstrated less than 50% of myometrial invasion expressed cyclin E. Five out of the 6 cases that had no myometrial invasion were also positive for cyclin E (Table 4.26).

4.3.6.7 SUBTYPES OF ENDOMETRIAL CANCER

There were no values of statistical significance noted with this comparison of the immunoexpression and histological subtypes.

Table 4.27: Immunohistochemical results together with the clinical data for the 54 cases studied

CASE NO	AGE	RACE	LYMPH NODES	STAGE	GRADE	STAINS						TUMOUR TYPE
						p53	pRb	CYCLIN A	CYCLIN D1	CYCLIN E	p27	
T_1	71	B	No nodes	IIB	G3	+4	+1	+3	-ve	+4	+3	Endometrioid
T_2	56	A	No nodes	IA	G1	-ve	+4	+1	+4	+3	+4	Endometrioid
T_3	60	A	No nodes	IB	G3	+4	-ve	+4	-ve	+4	+4	Endometrioid
T_4	61	B	No nodes	IIB	G3	+4	+4	+3	-ve	+4	+3	Endometrioid
T_5	70	B	No nodes	IC	G3	+1	-ve	+3	-ve	+3	+2	Endometrioid
T_6	71	B	No nodes	IB	G2	-ve	+1	-ve	-ve	-ve	-ve	Endometrioid
T_7	U	A	+	IIIC	G2	+1	+4	+3	-ve	+4	+4	Endometrioid
T_8	65	B	No nodes	IC	G1	+3	+4	+2	-ve	+4	-ve	Endometrioid
T_9	65	B	No nodes	IIB	G2	+3	+4	+2	+2	+4	+3	Clear Cell
T_{10}	67	B	No nodes	IA	G1	-ve	+1	-ve	+1	-ve	+1	Endometrioid
T_{11}	66	B	No nodes	IIB	G2	+4	+4	+3	-ve	+4	+2	Clear Cell
T_{12}	55	B	No nodes	IIIA	G2	-ve	+4	+2	-ve	+4	+2	Endometrioid
T_{13}	61	B	No nodes	IB	G2	+4	+4	+4	-ve	+3	+1	Endometrioid
T_{14}	65	B	No nodes	IB	G2	+4	-ve	+2	-ve	+4	+1	Endometrioid
T_{15}	60	A	No nodes	IB	G2	-ve	+4	+2	-ve	+2	+2	Endometrioid
T_{16}	59	A	No nodes	IC	G1	-ve	+4	+4	-ve	+3	+4	Endometrioid
T_{17}	70	B	No nodes	IIIA	G2	+4	-ve	+4	-ve	+4	+3	Endometrioid
T_{18}	55	A	No nodes	IA	G2	-ve	+4	+2	-ve	+4	+4	Clear Cell
T_{19}	68	W	No nodes	IB	G2	-ve	+4	+1	-ve	+3	-ve	Endometrioid
T_{20}	68	A	No nodes	IB	G2	-ve	+1	+3	-ve	+3	+3	Endometrioid
T_{21}	59	B	No nodes	IB	G2	-ve	+2	+1	-ve	+2	+4	Endometrioid

CASE NO	AGE	RACE	LYMPH NODES	STAGE	GRADE	STAINS						TUMOUR TYPE
T_{22}	U	B	No nodes	IA	G2	-ve	+4	-ve	-ve	+4	-ve	Endometrioid
T_{23}	72	A	No nodes	IIIA	G2	+2	+4	+3	+1	+3	+2	Endometrioid
T_{24}	66	B	No nodes	IB	G1	+4	+3	+2	+1	+4	+2	Endometrioid
T_{25}	81	W	No nodes	IC	G3	-ve	+4	+3	+2	+4	+1	Endometrioid
T_{26}	58	A	No nodes	IB	G2	-ve	+4	-ve	-ve	-ve	+3	Endometrioid
T_{27}	66	A	No nodes	IA	G2	-ve	+4	-ve	+1	+2	-ve	Endometrioid
T_{28}	77	A	No nodes	IC	G2	-ve	+4	+1	-ve	+3	+2	Endometrioid
T_{29}	55	B	No nodes	IA	G3	+4	+4	+1	+1	+4	+4	Endometrioid
T_{30}	49	B	No nodes	IB	G2	+1	+2	+3	+4	+2	+4	Endometrioid
T_{31}	68	B	No nodes	IB	G3	+4	-ve	+4	-ve	+3	+2	Endometrioid
T_{32}	45	B	No nodes	IB	G2	-ve	+4	-ve	+3	-ve	-ve	Endometrioid
T_{33}	61	A	No nodes	IB	G1	+3	+4	+2	+1	+4	+4	Endometrioid
T_{34}	54	B	No nodes	IIIA	G3	+1	+1	+4	+3	+3	+4	Endometrioid
T_{35}	53	B	No nodes	IIB	G1	-ve	+2	+1	-ve	+3	+4	Endometrioid
T_{36}	56	C	No nodes	IIIA	G2	+4	+1	+3	-ve	+4	+3	Serous Papillary
T_{37}	58	C	No nodes	IB	G2	+1	+4	+1	-ve	+4	+4	Endometrioid
T_{38}	48	B	No nodes	IB	G3	-ve	+4	+4	+3	+4	+3	Endometrioid
T_{39}	69	B	No nodes	IIB	G2	+4	+3	+3	+3	+4	+2	Endometrioid
T_{40}	75	B	No nodes	IIIA	G2	+4	+4	+2	-ve	+4	+1	Clear Cell
T_{41}	61	B	No nodes	IB	G2	-ve	+1	-ve	+4	+4	+2	Endometrioid
T_{42}	80	W	No nodes	IIIA	G2	+4	+3	+3	-ve	+4	+3	Serous Papillary
T_{43}	63	A	No nodes	IB	G1	-ve	+4	+1	+1	+4	+4	Endometrioid
T_{44}	40	B	No nodes	IC	G1	-ve	+3	+2	+1	+4	+4	Endometrioid

CASE NO	AGE	RACE	LYMPH NODES	STAGE	GRADE	STAINS						TUMOUR TYPE
T_{45}	79	W	No nodes	IB	G2	+4	+4	+2	-ve	+4	+4	Endometrioid
T_{46}	62	A	No nodes	IB	G2	-ve	+4	-ve	-ve	+4	-ve	Endometrioid
T_{47}	U	A	No nodes	IC	G2	-ve	+3	+1	+4	+4	+4	Endometrioid
T_{48}	80	W	No nodes	IC	G2	-ve	+4	+2	-ve	+4	+4	Endometrioid
T_{49}	60	W	No nodes	IB	G3	+1	+4	+3	+1	+3	+1	Endometrioid
T_{50}	65	B	No nodes	IB	G1	-ve	+4	+3	-ve	+4	+4	Clear Cell
T_{51}	75	B	No nodes	IIA	G2	-ve	+3	+2	-ve	+3	+1	Endometrioid
T_{52}	57	W	No nodes	IB	G1	-ve	+1	+2	-ve	+2	+4	Endometrioid
T_{53}	U	A	No nodes	IB	G2	+1	+1	+1	-ve	+3	+1	Adenocanthoma
T_{54}	U	A	No nodes	IIB	G2	+4	+4	+2	-ve	+4	+2	Endometrioid

4.4 MOLECULAR ANALYSIS OF ENDOMETRIAL CARCINOMA

All of the 54-endometrial carcinoma cases studied had amplifiable normal and tumour DNA (Plate 4.12), and were analysed for the molecular study.

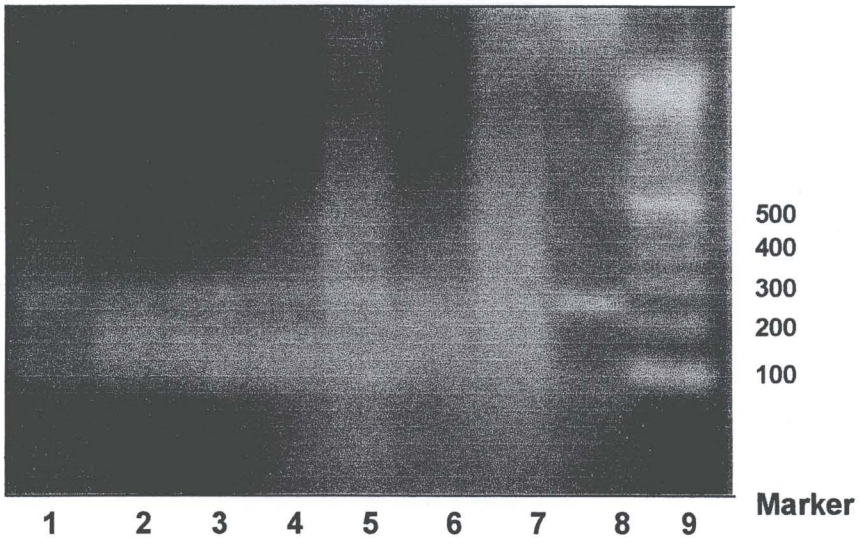


Plate 4.12 shows 2% agarose gel electrophoresis of DNA amplified with insulin primers. Lanes 1-8 contains PCR products of the amplified insulin fragment (size 236 base pairs). Lane 9 shows the molecular weight marker (100 base pair ladder).

Three mismatch repair gene loci and three loci of the DCC (Deleted in Colorectal Cancer) gene were investigated for genetic aberrations. The markers used for the mismatch repair analysis (MMR) were D2S123 (2p16) - (197-227bp); D3S659 (3p13) - (110-120bp); and BAT25 (4q12) - (+/-90 bp). For the DCC analysis, D18S34 (103-120 bp), (18q12.2-q12.3); D18S58 (144-160 bp), (18q22.3-q23) and D18S21/DCC (150-210 bp), (18q21.1-q21.2), were the markers employed. The molecular data for this analysis is shown in the table below (Table 4.28).

Table 4.28: Molecular Data for the MMR and DCC Analysis

Case No	D2S123 MMR	D3S659 MMR	Bat 25 MMR	D18S21 DCC	D18S34 DCC	D18S58 DCC
1	H	NAI	H	H	H	H
2	NAI	H	H	NAI	H	H
3	H	NAI	H	H	H	MSI
4	NAI	NAI	H	NAI	AI/LOH	H
5	NAI	H	H	H	H	AI/LOH
6	H	H	H	H	NAI	NAI
7	H	H	H	H	H	MSI
8	AI/LOH	NAI	NAI	NAI	AI/LOH	NAI
9	H	H	H	H	H	NAI
10	NAI	H	H	MSI	H	H
11	NAI	NAI	AI	H	H	H
12	H	H	MSI	NAI	H	NAI
13	NAI	H	NAI	H	H	H
14	H	NAI	H	AI	AI	NAI
15	AI/LOH	H	H	H	H	H
16	H	NAI	H	MSI	NAI	NAI
17	H	H	H	H	H	MSI
18	NAI	NAI	H	H	H	H
19	NAI	NAI	H	NAI	H	NAI
20	H	H	H	H	H	H
21	NAI	H	H	H	H	H
22	H	H	MSI	NAI	H	H
23	H	AI/LOH	H	H	H	H
24	NAI	H	AI/LOH	H	H	H
25	H	AI/LOH	H	NAI	H	AI/LOH
26	H	NAI	H	NAI	H	H
27	H	NAI	H	H	H	NAI
28	H	H	NAI	H	H	MSI
29	H	H	AI/LOH	H	H	NAI
30	H	AI/LOH	H	H	H	H
31	H	NAI	H	NAI	H	H
32	H	MSI	H	H	H	H
33	H	H	H	MSI	MSI	H
34	H	NAI	NAI	H	H	H
35	H	NAI	H	H	H	H
36	H	NAI	NAI	H	H	H
37	A/LOHI	MSI	H	NAI	H	H

Case No	D2S123 MMR	D3S659 MMR	Bat 25 MMR	D18S21 DCC	D18S34 DCC	D18S58 DCC
38	H	MSI	MSI	NAI	MSI	H
39	H	AI/LOH	H	H	H	H
40	H	NAI	H	H	H	H
41	H	NAI	H	AI/LOH	NAI	H
42	NAI	NAI	H	H	H	H
43	MSI	NAI	H	H	H	H
44	NAI	H	NAI	H	H	NAI
45	H	NAI	H	H	H	NAI
46	NAI	H	H	H	NAI	NAI
47	H	NAI	H	H	H	MSI
48	NAI	NAI	H	NAI	MSI	H
49	H	H	H	H	H	NAI
50	H	H	NAI	NAI	NAI	MSI
51	NAI	H	NAI	H	H	H
52	NAI	NAI	H	NAI	NAI	H
53	NAI	NAI	AI/LOH	H	H	MSI
54	H	H	H	NAI	H	H

Key :

AI/LOH - Allelic imbalance/Loss of heterozygosity

NAI - No allelic imbalance, informative case

H - Homozygous, non-informative case

MSI - Microsatellite instability

4.4.1 INTERPRETATION OF RESULTS

The results were interpreted as follows:

Homozygous: The appearance of a single peak in both normal and tumour tissue DNA on the electrophoretogram was called homozygous or non-informative case.

Heterozygous/NAI: The appearance of two peaks in the normal and tumour tissue, without change in peak area was reflective of a heterozygous or informative case. Also referred to as NAI – no allelic imbalance.

AI/LOH: Loss of heterozygosity or allelic imbalance was shown when there was a loss of one peak/allele in the tumour DNA. This was taken as an informative case.

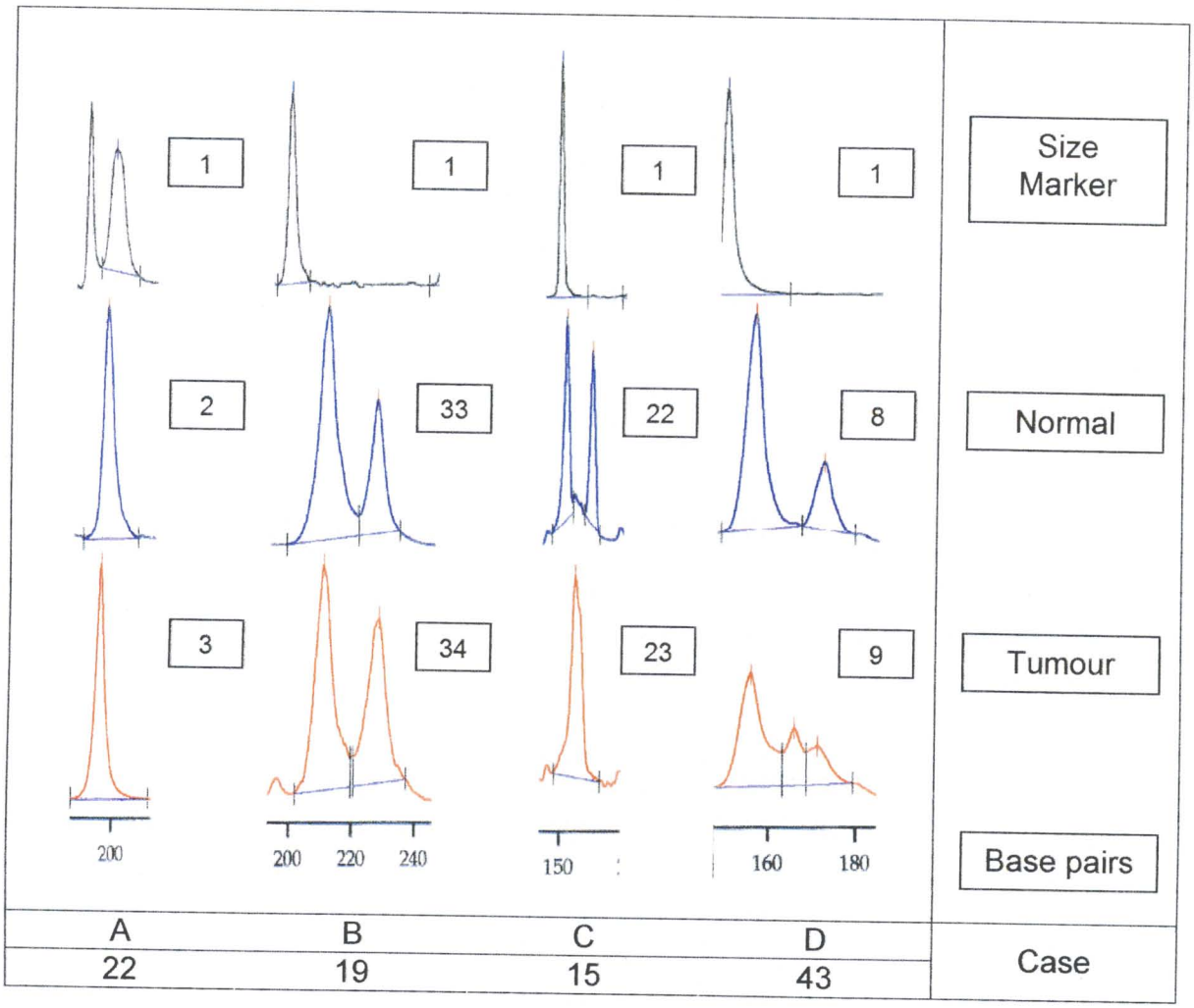
MSI: The appearance of an extra peak in the tumour DNA was regarded as microsatellite instablilty and an informative case.

4.4.2 MICROSATELLITE ANALYSIS OF THE MISMATCH REPAIR GENES

Table 4.29: Microsatellite Data for the MMR Genes

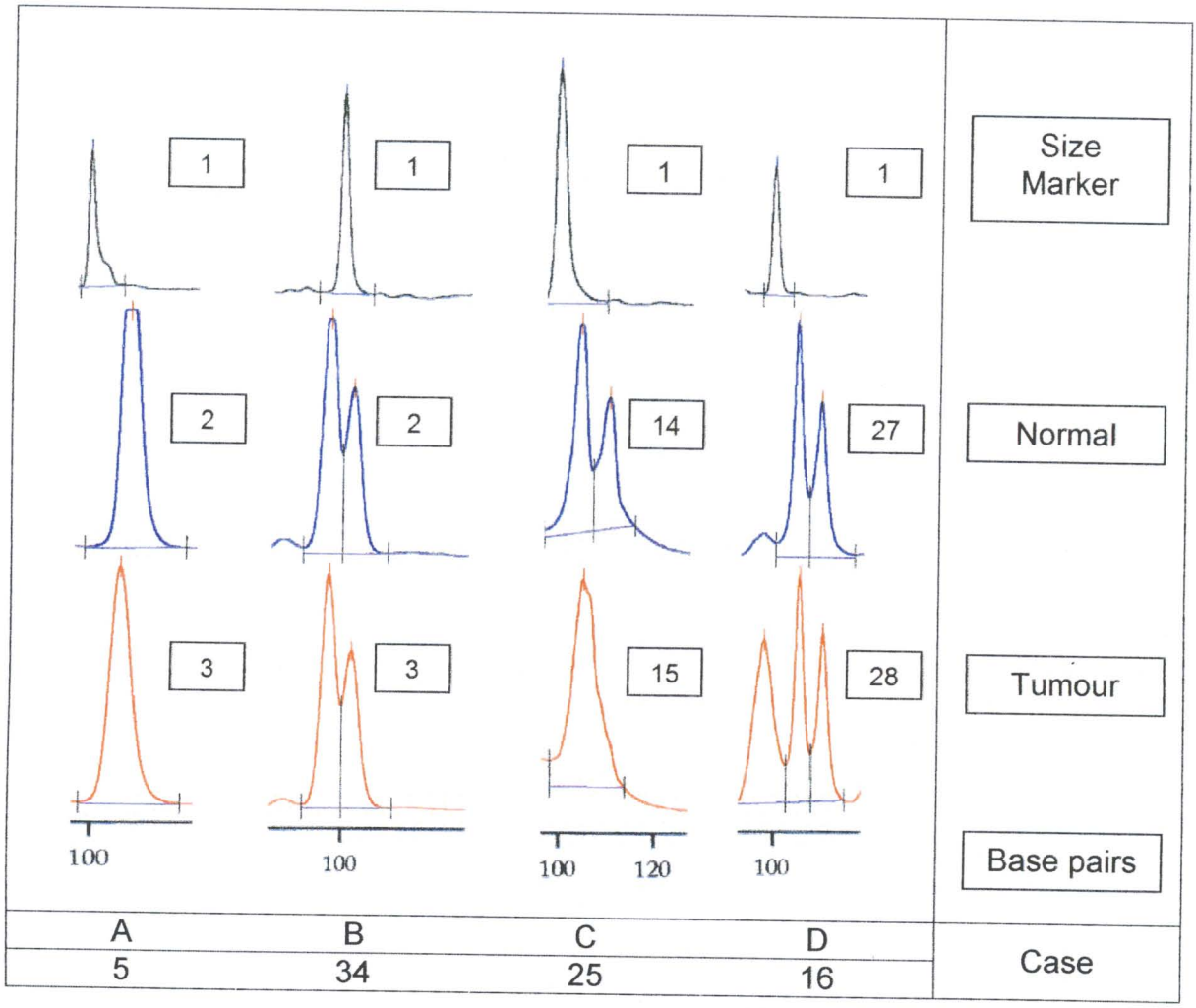
	D2S123	D3S659	BAT25
H	33	23	39
AI/LOH	3	4	4
NAI	17	24	8
MSI	1	3	3
Total cases	54	54	54
Informativity	39%	57%	28%

At the D2S123 locus, AI/LOH was observed in 3 of the 21 informative cases (14%), at the D3S659 locus 4 of 31 cases (13%), and at the BAT25 locus in 4 of the 15 cases (27%). NAI at the D2S123 locus was seen in 17 of the 21 informative cases (81%), at the D3S659 locus 24 of the 31 cases (77%), and at the BAT25 site 8 of the 15 cases (53%). Microsatellite instability was seen in only one of the 21 informative cases (5%) at the D2S123 locus, in the D3S659 region 3 of the 31 cases (10%), and in 3 of the 15 cases in the BAT25 region (20%). The three markers also showed a high rate of non-informativity. At the D2S123 locus 33 cases were non-informative (61%), in the D3S659 region 23 cases were non-informative (43%), and at the BAT25 locus 39 cases were non-informative (72%) (Table 4.29). (Figures 4.6, 4.7, 4.8 show electrophoretograms for markers D2S123, D3S659 and BAT25 respectively).



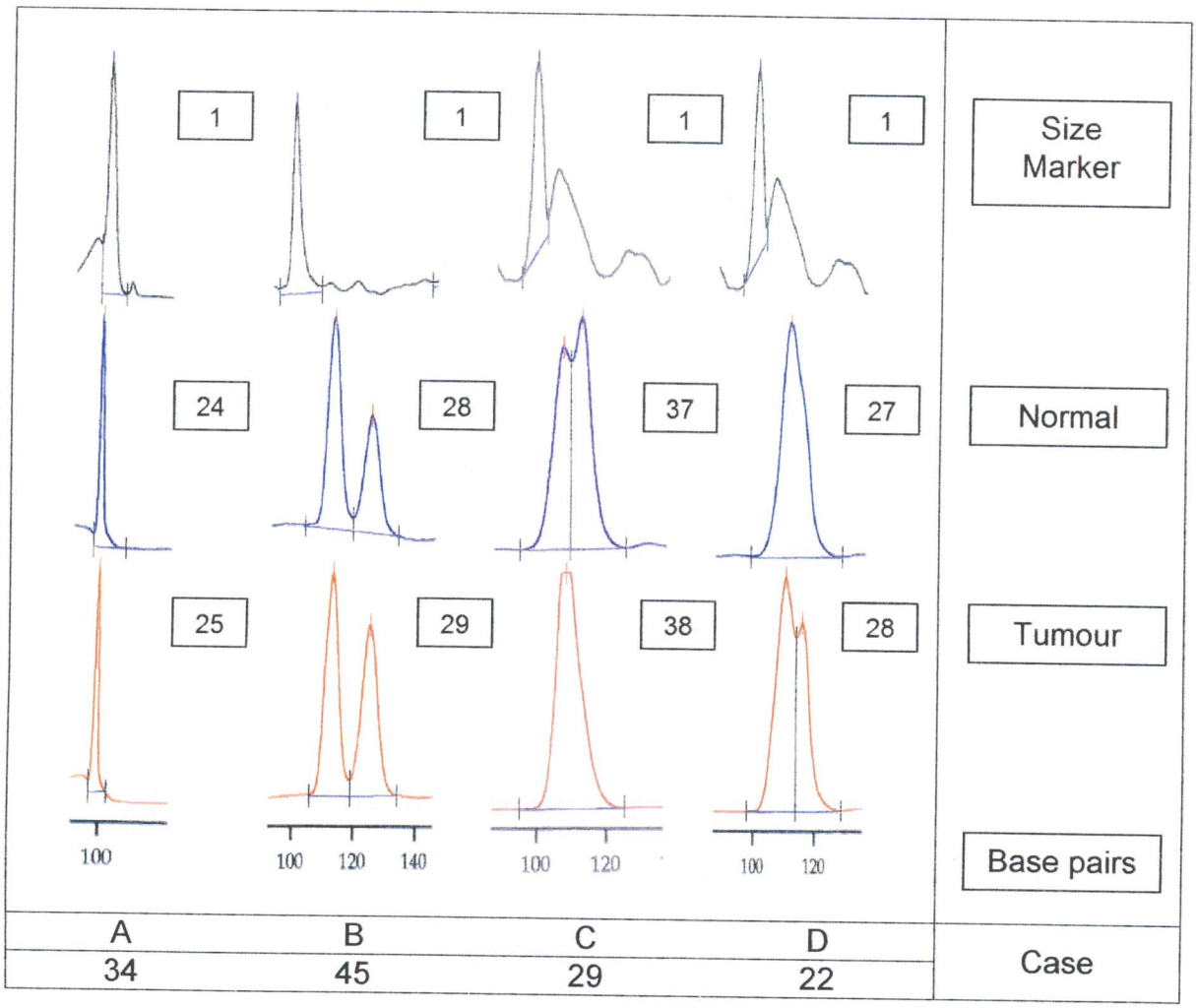
	Lane	Peak area	Size (base pairs)
A	2 (22N)	155.5	197.3
	3 (22T)	2291.4	194.7
B	33 (19N)	326.2	212.3
		144.9	227.9
	34 (19T)	93.5	211.0
		72.6	228.6
C	22 (15N)	31.7	154.3
		25.6	171.7
	23 (15T)	29.8	160.6
D	8 (43N)	219.1	156.9
		63.7	172.6
	9 (43T)	156.9	156
		50.8	165.9
		45.5	171.3

Figure 4.6: Representative electrophoretograms for Marker **D2S123** illustrating **(A)** Homozygous – no change; **(B)** No Allelic imbalance/ Heterozygous – no change; **(C)** Allelic imbalance/Loss of heterozygosity; **(D)** Microsatellite instability



	Lane	Peak area	Size (base pairs)
A	2 (5N)	6671.2	115.9
	3 (5T)	4967.9	111.7
B	2(34N)	4608.3	96.1
		2918.5	103.4
	3 (34T)	3672.3	95.7
		2492.1	103.0
C	14 (25N)	3428.8	104.6
		2330.7	110.6
	15 (25T)	5357.1	105.1
D	27 (16N)	1167.3	110.1
		925.4	119.8
	28 (16T)	290.69	96.6
		210.99	110.8
		169.95	120.6

Figure 4.7: Representative electrophoretograms for Marker **D3S659** illustrating **(A)** Homozygous – no change; **(B)** No Allelic imbalance/ Heterozygous – no change; **(C)** Allelic imbalance/Loss of heterozygosity; **(D)** Microsatellite instability



	Lane	Peak area	Size (base pairs)
A	24 (34N)	437.4	98.2
	25 (34T)	568.4	98.3
B	28 (45N)	113.6	113.1
		75.9	125.3
	29 (45T)	93.6	113.3
		86.6	125.3
C	37 (29N)	974.1	107.5
		1216.4	112.9
	38 (29T)	8698.3	108.3
D	27 (22N)	6218	111.2
	28 (22T)	3912.9	110.2
		2306.5	115.7

Figure 4.8: Representative electrophoretograms for Marker **BAT25** illustrating **(A)** Homozygous – no change; **(B)** No Allelic imbalance/ Heterozygous – no change; **(C)** Allelic imbalance/Loss of heterozygosity; **(D)** Microsatellite instability

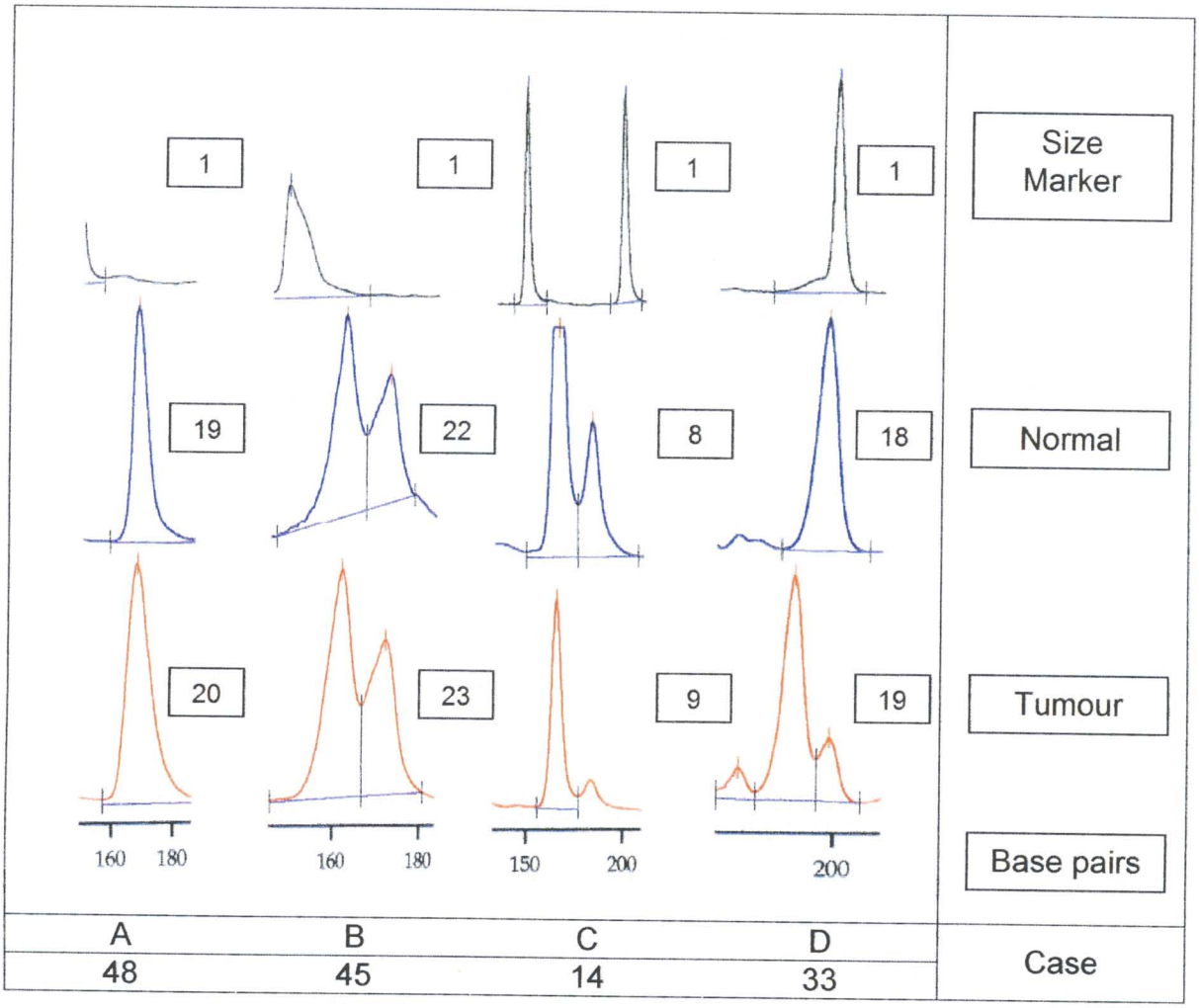
4.4.3 MICROSATELLITE ANALYSIS OF THE *DCC* GENE

Table 4.30: Microsatellite Data for the *DCC* Gene

	D18S21	D18S34	D18S58	Overall cases
H	34	42	32	
AI/LOH	2	3	2	6
NAI	15	6	13	
MSI	3	3	7	12
Total cases	54	54	54	
Informativity	37%	22%	41%	

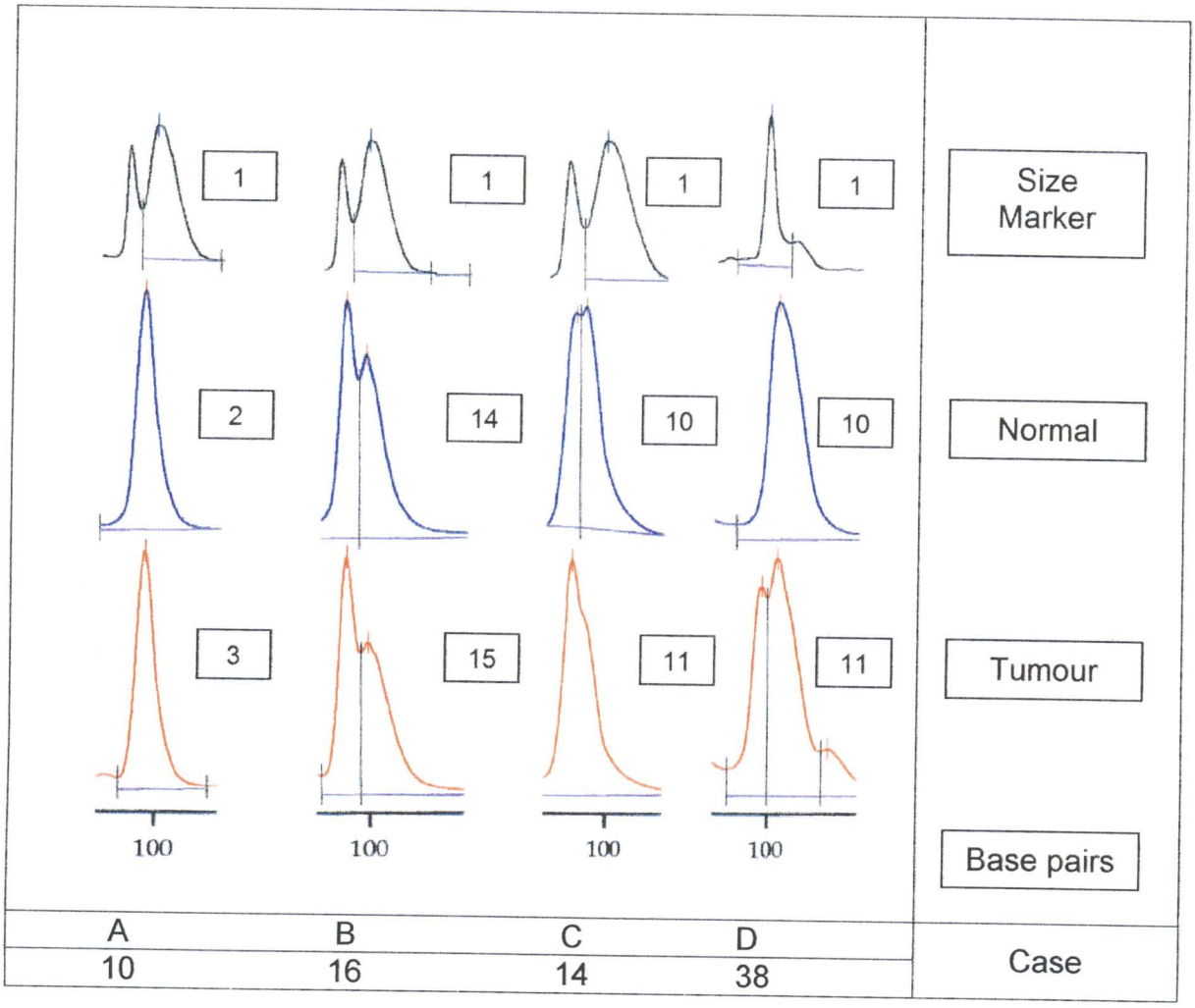
At the D18S21 locus on the *DCC* gene, 2 out of the 20 informative cases showed AI/LOH (10%), at the D18S34 locus 3 of 12 cases (25%), and at the D18S58 locus 2 of 22 cases (9%). NAI was observed in 15 of the 20 informative cases at the D18S21 locus (75%), 6 of 12 cases at the D18S34 locus (50%), and in 13 of 22 cases at the D18S58 locus (59%). Microsatellite instability in the 18q region was observed in 12 cases (22%) in at least one of the three loci investigated. At the D18S21 locus 3 of the 20 informative cases showed MSI (15%), at the D18S34 locus 3 of 12 cases (25%), and at the D18S58 locus 7 of 22 cases (32%). There was also a high rate of non-informativity for all three markers. For the D18S21 locus 34 cases were non-informative (63%), 42 cases for the D18S34 (78%) locus, and 32 cases for the D18S58 locus (59%), (Table 4.30). (Figures 4.9; 4.10 and 4.11 demonstrate electrophoretograms for markers D18S21, D18S34 and D18S58 respectively).

For the *DCC* gene markers AI/LOH and MSI were grouped together as "Genetic Aberrations". This was done in order to facilitate statistical analysis and was possible as all three *DCC* loci are found on one gene. The data was also grouped across all three *DCC* markers, and cases that showed an aberration for any one or more of the 3 markers, was designated mutated (MT). This was not possible for the MMR loci, since these loci are found on different genes (Table 4.31).



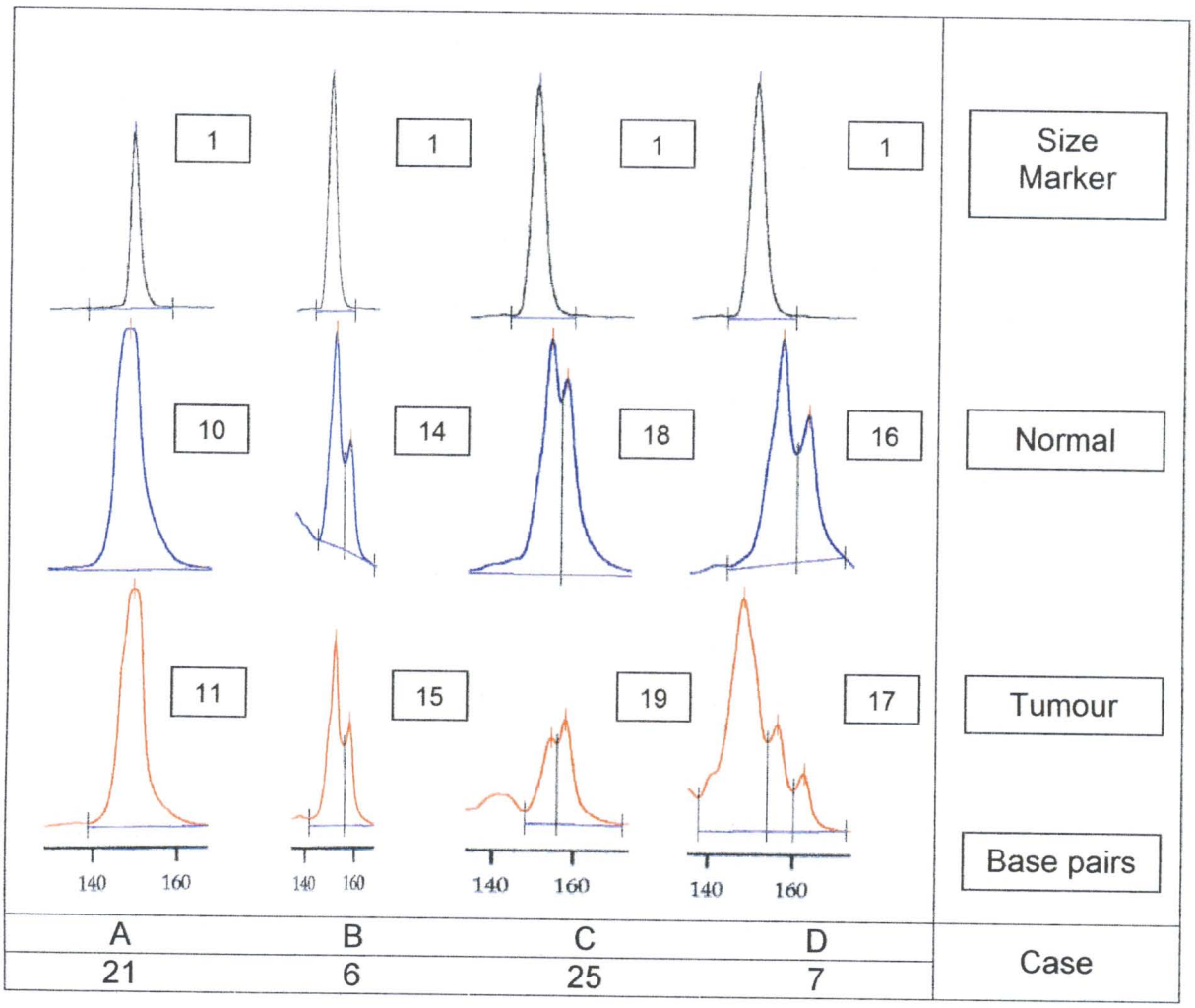
	Lane	Peak area	Size (base pairs)
A	19 (48N)	2100.6	169.9
	20 (48T)	4838	168.8
B	22 (45N)	117.3	163.4
		75.8	173.4
	23 (45T)	126.4	162.4
		905.8	172.3
C	8 (15N)	5915.2	166.4
		3270.9	183.9
	9 (15T)	3254.2	165.7
D	18 (33N)	1887.7	198.0
	19 (33T)	32.5	172.7
		271	188.8
		65.6	198.9

Figure 4.9: Representative electrophoretograms for Marker **D18S21** illustrating **(A)** Homozygous – no change; **(B)** No Allelic imbalance/ Heterozygous – no change; **(C)** Allelic imbalance/Loss of heterozygosity; **(D)** Microsatellite instability



	Lane	Peak area	Size (base pairs)
A	2 (10N)	3673.8	96.5
	3 (10T)	1348	96.4
B	14 (16N)	1249.2	92.0
		1063.3	98.7
	15 (16T)	3196.9	91.7
		2538.4	99.2
C	10 (14N)	1373.2	92.0
		1919.4	94.7
	11 (14T)	2081.2	91.1
D	10 (38N)	4050.7	103.5
	11 (38T)	1123.7	98.2
		2235.6	103.3
		606.3	119.0

Figure 4.10: Representative electrophoretograms for Marker D18S34 illustrating **(A)** Homozygous – no change; **(B)** No Allelic imbalance/ Heterozygous – no change; **(C)** Allelic imbalance/Loss of heterozygosity; **(D)** Microsatellite instability



	Lane	Peak area	Size (base pairs)
A	10 (21N)	6745.9	148.8
	11 (21T)	5847.3	149.8
B	14 (6N)	228.9	152.1
		97.5	157.8
	15 (6T)	985.4	152.3
		419.4	158.0
C	18 (25N)	5626.9	153.4
		2078.5	159.1
	19 (25T)	706.7	150
D	16 (7N)	989.2	156.6
		553.9	162.8
	17 (7T)	465.7	147.9
		116.2	156.2
		60.9	162.6

Figure 4.11: Representative electrophoretograms for Marker **D18S58** illustrating **(A)** Homozygous – no change; **(B)** No Allelic imbalance/ Heterozygous – no change; **(C)** Allelic imbalance/Loss of heterozygosity; **(D)** Microsatellite instability

Table 4.31: Genetic aberration and Mutation status for the 54 cases studied

Case No	D18S21	D18S34	D18S58	D18S21	D18S34	D18S58	Mutation Status	Number of Mutations
1	H	H	H	NGA	NGA	NGA	NM	
2	NAI	H	H	NGA	NGA	NGA	NM	
3	H	H	MSI	NGA	NGA	GA	MT	1
4	NAI	AI/LOH	H	NGA	GA	NGA	MT	1
5	H	H	AI	NGA	NGA	GA	MT	1
6	H	NAI	NAI	NGA	NGA	NGA	NM	
7	H	H	MSI	NGA	NGA	GA	MT	1
8	NAI	AI/LOH	NAI	NGA	GA	NGA	MT	1
9	H	H	NAI	NGA	NGA	NGA	NM	
10	MSI	H	H	GA	NGA	NGA	MT	1
11	H	H	H	NGA	NGA	NGA	NM	
12	NAI	H	NAI	NGA	NGA	NGA	NM	
13	H	H	H	NGA	NGA	NGA	NM	
14	AI/LOH	AI/LOH	NAI	GA	GA	NGA	MT	2
15	H	H	H	NGA	NGA	NGA	NM	
16	MSI	NAI	NAI	GA	NGA	NGA	MT	1
17	H	H	MSI	NGA	NGA	GA	MT	1
18	H	H	H	NGA	NGA	NGA	NM	
19	NAI	H	NAI	NGA	NGA	NGA	NM	
20	H	H	H	NGA	NGA	NGA	NM	
21	H	H	H	NGA	NGA	NGA	NM	
22	NAI	H	H	NGA	NGA	NGA	NM	
23	H	H	H	NGA	NGA	NGA	NM	
24	H	H	H	NGA	NGA	NGA	NM	
25	NAI	H	AI/LOH	NGA	NGA	GA	MT	1
26	NAI	H	H	NGA	NGA	NGA	NM	

Case No	D18S21	D18S34	D18S58	D18S21	D18S34	D18S58	Mutation Status	Number of Mutations
27	H	H	NAI	NGA	NGA	NGA	NM	
28	H	H	MSI	NGA	NGA	GA	MT	1
29	H	H	NAI	NGA	NGA	NGA	NM	
30	H	H	H	NGA	NGA	NGA	NM	
31	NAI	H	H	NGA	NGA	NGA	NM	
32	H	H	H	NGA	NGA	NGA	NM	
33	MSI	MSI	H	GA	GA	NGA	MT	2
34	H	H	H	NGA	NGA	NGA	NM	
35	H	H	H	NGA	NGA	NGA	NM	
36	H	H	H	NGA	NGA	NGA	NM	
37	NAI	H	H	NGA	NGA	NGA	NM	
38	NAI	MSI	H	NGA	GA	NGA	MT	1
39	H	H	H	NGA	NGA	NGA	NM	
40	H	H	H	NGA	NGA	NGA	NM	
41	AI/LOH	NAI	H	GA	NGA	NGA	MT	1
42	H	H	H	NGA	NGA	NGA	NM	
43	H	H	H	NGA	NGA	NGA	NM	
44	H	H	NAI	NGA	NGA	NGA	NM	
45	H	H	NAI	NGA	NGA	NGA	NM	
46	H	NAI	NAI	NGA	NGA	NGA	NM	
47	H	H	MSI	NGA	NGA	GA	MT	1
48	NAI	MSI	H	NGA	GA	NGA	MT	1
49	H	H	NAI	NGA	NGA	NGA	NM	
50	NAI	NAI	MSI	NGA	NGA	GA	MT	1
51	H	H	H	NGA	NGA	NGA	NM	
52	NAI	NAI	H	NGA	NGA	NGA	NM	
53	H	H	MSI	NGA	NGA	GA	MT	1
54	NAI	H	H	NGA	NGA	NGA	NM	

Key:

GA - Genetic aberrations.

NGA - No genetic aberrations. Those cases that showed neither AI/LOH nor MSI were designated as "No Genetic Aberrations".

MT - Mutated.

NM - Not mutated. The case that showed no aberrations for any marker, fell in the NM group

4.4.3.1 GENETIC ABERRATIONS VERSUS NO GENETIC ABERRATIONS

Table 4.32: Genetic Aberrations versus No Genetic Aberrations

	D18S21	D18S34	D18S58
Genetic Aberrations	5	6	9
No Genetic Aberrations	49	48	45

The most genetic aberrations were observed at the D18S58 locus, 17%, followed by locus D18S34, 11%; and locus D18S21 at 9% (Table 4.32).

A statistical analysis was done to also compare genetic aberrations with clinical factors. The results were as follows:

Table 4.33: D18S58 versus Stage

		Stage							Total
		IA	IB	IC	IIA	IIB	IIIA	IIIC	
D18S58	GA	0	3	4	0	0	1	1	9
	NGA	6	21	4	1	7	6	0	45
Total		6	24	8	1	7	7	1	54

P=0.024

This analysis identified a significant correlation between the marker D18S58 and stage with a "p" value of 0.024 (Table 4.33).

Table 4.34: D18S21 versus Grade

		Grade			Total
		G1	G2	G3	
D18S21	GA	3	2	0	5
	NGA	8	31	10	49
Total		11	33	10	54

P=0.059

D18S21 versus grade showed a statistical trend with a p value of 0.059. At the D18S21 locus, 10 of the 49 cases that had no genetic aberrations were grade 3 tumours, while none of the grade 3 cases had genetic aberrations (Table 4.34).

Table 4.35: BAT 25 versus Histological Type

		Type				Total
		ADA	CC	ENDO	SP	
Bat 25	GA	1	1	5	0	7
	NGA	0	4	41	2	47
Total		1	5	46	2	54

P=0.060

Key:
ADA - Adenoacanthoma
CC - Clear cell carcinoma
ENDO - Endometrioid carcinoma
SP - Serous papillary carcinoma

The comparison between BAT25 and histological subtype demonstrated a close statistical significance with a p value of 0.060. At this locus the single adenoacanthoma case showed genetic aberrations. The most number of cases with aberrations were endometrioid type carcinomas (Table 4.35).

4.4.3.2 MUTATED CASES VERSUS NOT MUTATED CASES

Table 4.36: Mutated cases versus Not mutated cases

	DCC LOCI
Mutated	18
Not Mutated	36

Of the 54 cases in this study 18 were mutated while 36 were not mutated (Table 4.36). A comparison was also drawn between mutation status and clinical factors. The only significant correlation was observed between stage and mutation with a p value of 0.012. The other factors did not display any significance.

Table 4.37: Mutation versus Stage

		Stage							Total
		IA	IB	IC	IIA	IIB	IIIA	IIIC	
Mutation	M	1	7	7	0	1	1	1	18
	NM	5	17	1	1	6	6	0	36
Total		6	24	8	1	7	7	1	54

P=0.012

The most mutations were seen with stage IB and IC cases, 7 cases each.
A significant statistical value was reached, p=0.012 (Table 4.37).

4.4.4 COMPARISON OF MOLECULAR RESULTS WITH CLINICAL AND LIGHT MICROSCOPIC DATA

Clinical and light microscopic factors such as race, lymph node status, myometrial invasion and stage did not show any significant correlation when compared to the molecular results. However, with factors such as age, grade

and histological type, there was statistical significance noted with certain of the molecular markers, as follows:

4.4.4.1 AGE

Table 4.38: D3S659 versus Age

		D3S659				Total
		AI/LOH	H	MSI	NAI	
AgeRange	U	0	3	0	2	5
	a	1	1	2	0	4
	b	0	4	1	7	12
	c	1	10	0	10	21
	d	1	5	0	3	9
	e	1	0	0	2	3
Total		4	23	3	24	54

P=0.021

Key:

U - Unknown

a - 40-49 age range

b - 50-59 age range

c - 60-69 age range

d - 70-79 age range

e – 80-89 age range

Two of the three cases that were MSI+ve at the D3S659 locus (MMR) belonged to the 40-49 age group and the other single case was from the 50-59 age range. Each of the age ranges had one case of AI/LOH for D3S659 except the 50-59 age range which had none. A total of 4 AI/LOH +ve cases were observed at the D3S659 locus. Most of the cases that were H or NAI belonged to the 60-69 age group with 10 cases each. There was a significant

correlation between the 3p13 loci and age with a p value of 0.021 (Table 4.38).

Table 4.39: **D18S58 versus age**

		D18S58				Total
		AI/LOH	H	MSI	NAI	
Age Range	U	0	2	3	0	5
	a	0	3	0	1	4
	b	0	9	0	3	12
	c	0	12	2	7	21
	d	1	4	2	2	9
	e	1	2	0	0	3
Total		2	32	7	13	54

P=0.040

Key: refer to page 214

At this *DCC* locus (D18S58), two cases that were MSI +ve belonged to the 60-69 age group and the other two from the 70 -79 age range. With three of the cases, the patients' ages were unknown. This locus had the most number of MSI +ve cases. The two cases with AI/LOH came from the 70-79 and the 80-89 age ranges. This was in contrast to the findings with the MMR marker, D3S659. The most number of cases that were H or NAI were from the 60-69 age group (Table 4.39). This trend was also apparent for the MMR marker D3S659.

4.4.4.2 GRADE

Table 4.40: D18S21 versus Grade

		Grade			Total
		G1	G2	G3	
D18S21	AI/LOH	0	2	0	2
	H	4	24	6	34
	MSI	3	0	0	3
	NAI	4	7	4	15
Total		11	33	10	54

P=0.013

All three cases that were MSI +ve at the D18S21 loci (*DCC*) were grade 1 tumours. Both cases that had AI/LOH were grade 2 tumours. Six of the ten grade 3 cancers were non-informative and the remaining 4 were heterozygous. Most cases at this locus were homozygous and were grade 2 tumours. There was a significant correlation between D18S21 and grade with a p value of 0.013 (Table 4.40).

4.4.4.3 HISTOLOGICAL TYPE

Table 4.41: BAT 25 versus histological type

		Type				Total
		ADA	CC	ENDO	SP	
BAT 25	AI/LOH	1	1	2	0	4
	H	0	3	35	1	39
	MSI	0	0	3	0	3
	NAI	0	1	6	1	8
Total		1	5	46	2	54

P=0.049

At the BAT25 locus all 3 cases that were MSI +ve were endometrioid adenocarcinomas. 4 cases had AI./LOH Of these 2 were endometrioid cancers, 1 was an adenoacanthoma and the other was a clear cell carcinoma. Most of the cases at this locus were homozygous and endometrioid cancers. None of the serous papillary tumours displayed AI/LOH or MSI (Table 4.41).

4.4.5 COMPARISON OF MOLECULAR DATA WITH IMMUNOHISTOCHEMICAL RESULTS

A statistical evaluation was also executed to compare the results obtained with the molecular study to that of the immunohistochemical investigation. A significant correlation was achieved with markers D3S659 and cyclin D1, D18S34 and p53, D18S58 and pRb, D18S58 and p27. Most of the significant values were obtained with the *DCC* marker.

4.4.5.1 Comparison of D3S659 and Cyclin D1

Table 4.42: D3S659 versus Cyclin D1

		D3S659				Total
		AI/LOH	H	MSI	NAI	
Cyclin D1	E	4	8	2	5	19
	NE	0	15	1	19	35
Total		4	23	3	24	54

P=0.013

All 4 cases that had AI/LOH at the D3S659 (MMR) locus expressed cyclin D1. Two of the three cases that were MSI +ve showed expression for cyclin D1. At the D3S659 locus, most of the cases had NAI and did not express cyclin

D1. D3S659 versus Cyclin D1 produced a significant statistical value of p= 0.013 (Table 4.42).

4.4.5.2 Comparison of D18S34 and p53

Table 4.43: D18S34 versus p53

		D18S34				Total
		AI/LOH	H	MSI	NAI	
P53	E	3	23	1	0	27
	NE	0	19	2	6	27
Total		3	42	3	6	54

P=0.021

At the D18S34 (*DCC*) locus most of the cases that expressed p53 were homozygous. All of the cases that had AI/LOH expressed p53 (Table 4.43). These results were similar to the above comparison.

4.4.5.3 Comparison of D18S58 and pRb

Table 4.44: D18S58 versus pRb

		D18S58				Total
		AI/LOH	H	MSI	NAI	
pRb	E	1	31	5	12	49
	NE	1	1	2	1	5
Total		2	32	7	13	54

P=0.036

At this *DCC* locus a total of 12 cases that had NAI also expressed pRb. Of the 7 cases that were MSI +ve, 5-expressed pRb while 2 did not (Table 4.44).

4.4.5.4 Comparison of D18S58 and p27

Table 4.45: D18S58 versus p27

		D18S58				Total
		AI/LOH	H	MSI	NAI	
P27	E	2	30	7	8	47
	NE	0	2	0	5	7
Total		2	32	7	13	54

P=0.018

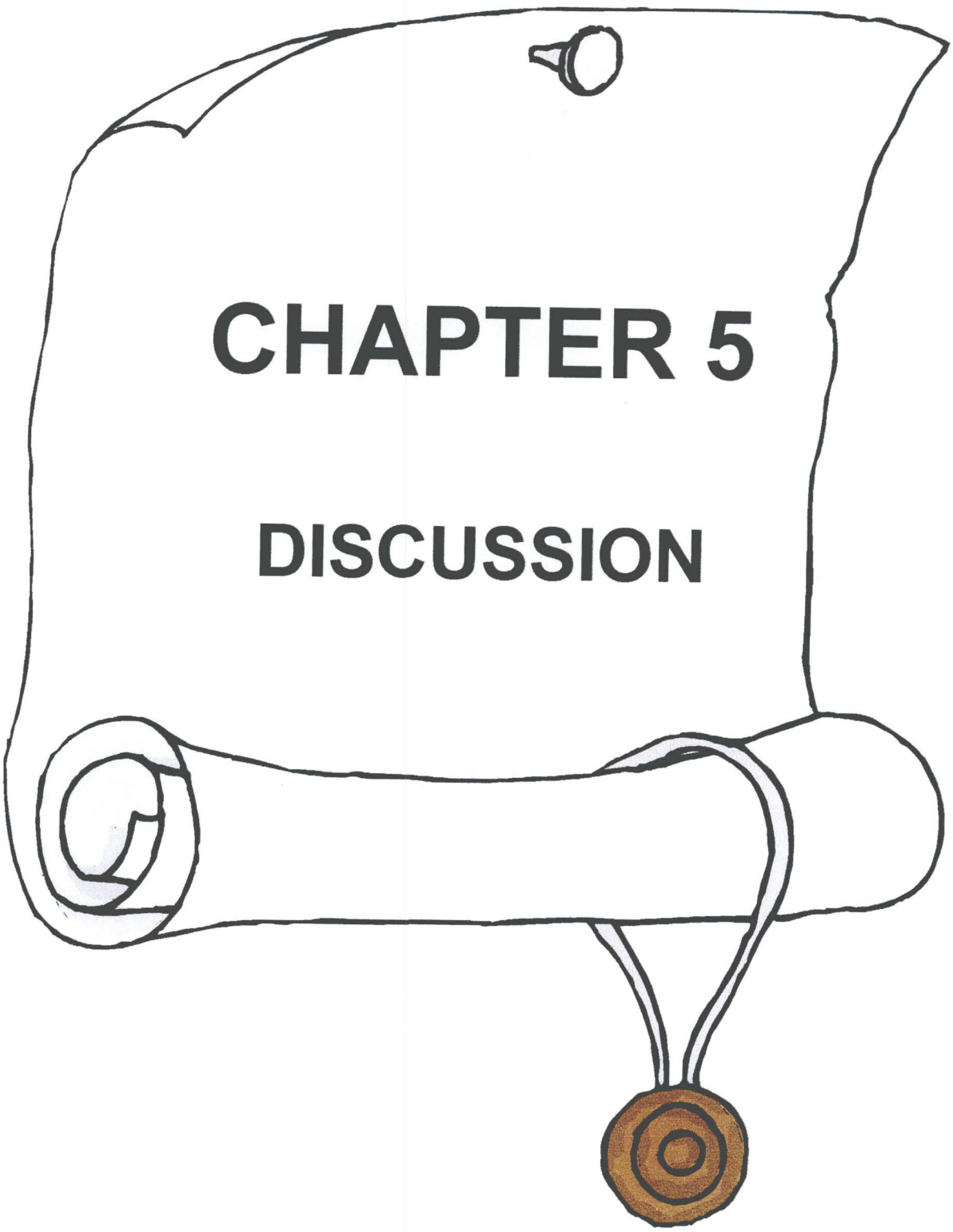
All 7 cases that were MSI +ve at this locus also expressed p27. The 2 cases that had AI/LOH showed expression for p27. This comparison was significant statistical with a p value of 0.018 (Table 4.45).

Table 4.46: Molecular results together with the clinical data for the 54 cases studied

Case no	Age	Race	Lymph	Stage	Grade	Myo	Type	D2S123	3P13	Bat 25	D18S21	D18S34	D18S58
1	71	B	NR	2B	G3	G	ENDO	H	NAI	H	H	H	H
2	56	A	NEG	1A	G1	N	ENDO	NAI	H	H	NAI	H	H
3	60	A	NR	1B	G3	L	ENDO	H	NAI	H	H	H	MSI
4	61	B	NR	2B	G3	L	ENDO	NAI	NAI	H	NAI	AI/LOH	H
5	70	B	NR	1C	G3	G	ENDO	NAI	H	H	H	H	AI/LOH
6	71	B	NR	1B	G2	L	ENDO	H	H	H	H	NAI	NAI
7	U	A	POS	3C	G2	G	ENDO	H	H	H	H	H	MSI
8	65	B	NR	1C	G1	G	ENDO	AI/LOH	NAI	NAI	NAI	AI/LOH	NAI
9	65	B	NR	2B	G2	G	CC	H	H	H	H	H	NAI
10	67	B	NR	1A	G1	N	ENDO	NAI	H	H	MSI	H	H
11	66	B	NR	2B	G2	L	CC	NAI	NAI	AI/LOH	H	H	H
12	55	B	NR	3A	G2	G	ENDO	H	H	MSI	NAI	H	NAI
13	61	B	NR	1B	G2	L	ENDO	NAI	H	NAI	H	H	H
14	65	B	NR	1B	G2	L	ENDO	H	NAI	H	AI/LOH	AI/LOH	NAI
15	60	A	NR	1B	G2	E	ENDO	AI/LOH	H	H	H	H	H
16	59	A	NR	1C	G1	G	ENDO	H	NAI	H	MSI	NAI	NAI
17	70	B	NR	3A	G2	G	ENDO	H	H	H	H	H	MSI
18	55	A	NR	1A	G2	N	CC	NAI	NAI	H	H	H	H
19	68	W	NR	1B	G2	L	ENDO	NAI	NAI	H	NAI	H	NAI
20	68	A	NR	1B	G2	L	ENDO	H	H	H	H	H	H
21	59	B	NR	1B	G2	L	ENDO	NAI	H	H	H	H	H
22	U	B	NR	1A	G2	N	ENDO	H	H	MSI	NAI	H	H
23	72	A	NR	3A	G2	G	ENDO	H	AI/LOH	H	H	H	H
24	66	B	NR	1B	G1	L	ENDO	NAI	H	AI	H	H	H
25	81	W	NR	1C	G3	G	ENDO	H	AI/LOH	H	NAI	H	AI/LOH
26	58	A	NR	1B	G2	L	ENDO	H	NAI	H	NAI	H	H
27	66	A	NR	1A	G2	N	ENDO	H	NAI	H	H	H	NAI
28	77	A	NR	1C	G2	G	ENDO	H	H	NAI	H	H	MSI
29	55	B	NR	1A	G3	N	ENDO	H	H	AI/LOH	H	H	NAI
30	49	B	NR	1B	G2	L	ENDO	H	AI/LOH	H	H	H	H
31	68	B	NR	1B	G3	L	ENDO	H	NAI	H	NAI	H	H
32	45	B	NR	1B	G2	E	ENDO	H	MSI	H	H	H	H
33	61	A	NR	1B	G1	L	ENDO	H	H	H	MSI	MSI	H

Case no	Age	Race	Lymph	Stage	Grade	Myo	Type	D2S123	3P13	Bat 25	D18S21	D18S34	D18S58
34	54	B	NR	3A	G3	G	ENDO	H	NAI	NAI	H	H	H
35	53	B	NR	2B	G1	L	ENDO	H	NAI	H	H	H	H
36	56	C	NR	3A	G2	G	SP	H	NAI	NAI	H	H	H
37	58	C	NR	1B	G2	L	ENDO	AI/LOH	MSI	H	NAI	H	H
38	48	B	NR	1B	G3	L	ENDO	H	MSI	MSI	NAI	MSI	H
39	69	B	NR	2B	G2	G	ENDO	H	AI/LOH	H	H	H	H
40	75	B	NR	3A	G2	G	CC	H	NAI	H	H	H	H
41	61	B	NR	1B	G2	L	ENDO	H	NAI	H	AI/LOH	NAI	H
42	80	W	NR	3A	G2	G	SP	NAI	NAI	H	H	H	H
43	63	A	NR	1B	G1	L	ENDO	MSI	NAI	H	H	H	H
44	40	B	NR	1C	G1	G	ENDO	NAI	H	NAI	H	H	NAI
45	79	W	NR	1B	G2	L	ENDO	H	NAI	H	H	H	NAI
46	62	A	NR	1B	G2	L	ENDO	NAI	H	H	H	NAI	NAI
47	U	A	NR	1C	G2	G	ENDO	H	NAI	H	H	H	MSI
48	80	W	NR	1C	G2	G	ENDO	NAI	NAI	H	NAI	MSI	H
49	60	W	NR	1B	G3	L	ENDO	H	H	H	H	H	NAI
50	65	B	NR	1B	G1	L	CC	H	H	NAI	NAI	NAI	MSI
51	75	B	NR	2A	G2	G	ENDO	NAI	H	NAI	H	H	H
52	57	W	NR	1B	G1	L	ENDO	NAI	NAI	H	NAI	NAI	H
53	U	A	NR	1B	G2	L	ADA	NAI	NAI	AI/LOH	H	H	MSI
54	U	A	NR	2B	G2	G	ENDO	H	H	H	NAI	H	H

KEY:
ADA - Adenoacanthoma
CC - Clear cell carcinoma
ENDO - Endometrial carcinoma
SP - Serous papillary carcinoma
NR - No record



CHAPTER 5

DISCUSSION

DISCUSSION

"Wisdom is the principal thing; therefore get wisdom; and with all thy getting get understanding." – Anonymous.

5.1 DEMOGRAPHICS

5.1.1 INCIDENCE OF ENDOMETRIAL CARCINOMA

Many studies that have investigated cancer incidence in different continents have discovered that endometrial cancer has fast become one of the most prominent gynaecological malignancies (Weiss *et al*, 1976; Gusberg, 1980). This phenomenon has been observed mainly in western countries such as the United States of America (Parker *et al*, 1996). In South Africa it now ranks as the most common pelvic malignancy after cancer of the cervix (health.iafrica.com, 2005), but in the Western world it has been known to have bypassed squamous carcinoma of the cervix as the commonest uterine cancer (Robertson, 1981). Reversal of incidence of these two common cancers in the western world is attributed to aging populations, better genital hygiene, reduction in average parity, and earlier detection and treatment of preinvasive carcinoma of the cervix (Robertson, 1981). The incidence of endometrial cancer seems to vary throughout the world, as it appears to be 4 to 5 times lower in developing countries of Africa, Asia, South America when compared to the more industrialized countries of Europe and North America (Doll *et al*, 1970). The higher rates of the well-differentiated adenocarcinomas observed in the Western world, which is associated with hyperoestrogenism may be related to the common use of oestrogen therapy, a therapy that is seldom prescribed in countries like Japan or Russia (Dallenbach-Hellweg, 1987). The histopathology laboratory at the Nelson R Mandela School of Medicine, where this research study was conducted receives approximately 100 endometrial biopsies per month. Five percent of these biopsies are reported as malignant, which implies that there are probably about 60 malignancies reported per year. It is fortunate that many patients are diagnosed at an early stage and with a favourable grade, which results in most patients being cured. However, stage for stage endometrial

cancer has just as poor a prognosis as any other cancer (Rich, www.personal.u-net.com, 1996).

5.1.2 RACIAL DIFFERENCES IN ENDOMETRIAL CANCER

The incidence of endometrial carcinoma has been noted to differ among races. A study by Sung and colleagues has shown that the occurrence of endometrial cancer is lower in Black patients than in Whites (Sung *et al*, 2000; Steinhorn *et al*, 1986). In fact, it has been found that amongst the American population the incidence in Whites is almost double that in Blacks (Ramzy, 1983). Ramzy has suggested that this incidence appears to be related to high socioeconomic standards (Ramzy, 1983). We have found that of the 54 endometrial carcinoma cases studied, 28 (51.8%) patients were Black while only 7 (12.9%) were White. These findings are at variance with the findings of Sung, Steinhorn and Ramzy (Sung *et al*, 2000; Steinhorn *et al*, 1986; Ramzy, 1983). However, this ratio is not representative of the population as a whole. The attendance at King Edward VIII Hospital is predominantly Black, and this population group formed the vast majority of the cases in this study. Therefore, creating a bias in the population group studied. We also need to take into consideration that South Africa has a majority Black population. In our study 17 (31%) of the patients were Asians, and this could be due to the fact that KwaZulu-Natal has a large Asian population. Only 3.7% of the cases belonged to the Coloured population group.

Although there seems to be a lower prevalence of endometrial cancer in Black as compared with White women recorded, there has been a significant survival advantage noted in white as compared to black patients (Steinhorn, 1986). Kohler and co-workers also noted that black women had a lower survival rate than white women (Kohler *et al*, 1996). This study was unable to obtain sufficient patient follow-up and it was therefore not possible to examine the survival rate of the patients researched. Black patients seem to have a higher proportion of high grade, high stage tumours. They also tend to seek medical attention at a more advanced age compared with white patients (Christopherson *et al*, 1983). The main cause of poor outcome in this race group is believed to be financial, educational, cultural, and geographic factors (Barrett *et al*, 1995). The study of

Barrett and colleagues also confirmed that Black patients are diagnosed at later stages than White patients (Barrett *et al*, 1995). According to the Louisville Uterine Cancer Registry, 92% of White patients had stage I disease compared with 76% of Black patients, and 58% of White patients had grade 1 tumours compared with 39% of Black patients (Christopherson *et al*, 1983). Our study showed 86% of white patients with stage I tumour compared to 61 % of Black patients, and 14% of whites had grade 1 tumours compared to 21% of Black patients, differing slightly from the above findings. Amongst the Asian population in this study, 82% had stage I disease while 24% were grade 1 tumours. Both of the cases that were Coloured patients were grade 2 tumours while one was stage I, and the other a stage III tumour. It has also been suggested that the differences in survival may be related to the types of cancer found in White and Black women (Connelly *et al*, 1982). In the United States, Connelly and colleagues found that a higher frequency of the favourable types of carcinoma like the endometrioid variant occur in White patients than in Black patients, compared with the less favourable types such as serous papillary, clear cell and adenosquamous (Connelly *et al,* 1982). Matthews *et al.* found that Black women had a poorer 5-year survival (56%) than non-Black women (71%). They also found that patients with serous papillary and clear cell endometrial cancer were more likely to be Black and present at an advanced stage of disease, compared to patients with endometrioid adenocarcinoma. This may probably help to explain the poorer survival rate reported in Blacks with endometrial carcinoma (Matthews *et al*, 1997). Our study demonstrated that 4 of the Black patients and 1 Asian patient had clear cell carcinoma while none of the White or Coloured patients did. However, 1 of the 7 White patients had serous papillary carcinoma while the remainder had endometrioid adenocarcinoma. Serous papillary type was also found in one of the Coloured women and an Asian woman had the adenoacanthoma. It seems that the biologic mechanisms of this tumour differ in Black patients leading to a more ominous type of endometrial cancer.

5.1.3 TREND OF ENDOMETRIAL CANCER IN AGE GROUPS

Endometrial carcinoma has been observed to be a disease that predominantly occurs in postmenopausal women (Gallup and Stock, 1984; Kurman, 1984). The

median patient age at diagnosis is usually around 63 years (Platz and Benda, 1995), and the mean patient age is 59 years (Kurman, 1984). The cases in this study had a median age of 63 years and a mean patient age of 57.5 years, which is in keeping with the literature. Endometrial cancer seems to follow a pattern of incidence, which is highly dependent on age. Kosary *et al.* (1995) found that there were 12 cases per 100,000 women at 40 years of age, and 84 per 100,000 at 60 years. Gallup and Stock recorded that 75% of the women were postmenopausal (Gallup and Stock, 1984). This disease therefore seems to be relatively uncommon in young women and is seldom found in patients below 40 years of age (Kurman, 1984). None of the patients we studied were below the age of 40 years, and only 4 belonged to the 40-49 age group. It has been found that the prevalence in women under 40 years is only 3%, and 21 cases were reported in women under the age of 30, the youngest being 15 years (Farhi *et al*, 1986). We found that the majority of our patients belonged to the 60-69 age group with 21 patients from the study falling into this age category. Our findings seem to be in keeping with other studies.

We also noted that in the younger women the tumour was generally low grade and minimally invasive, as did Steinhorn *et al.* (1986). The study of Aziz and co-workers found that older patients have a higher clinical stage, higher grade, and greater depth of myometrial invasion than younger patients (Aziz *et al*, 1996). Our study found that none of the 4 patients in the 40-49 age group had stage III disease, all had stage I tumours. We also observed that the older patients had higher grade and stage of tumours compared to the younger patients, especially those belonging to the 60-69 and 70-79 age ranges. Of the three women who were in the 80-89 age range, 2 had grade 2 tumours and 1 a grade 3 cancer. Also, many of the patients from these age ranges had tumours that invaded more than 50% of the myometrium, which is in keeping to the findings of Aziz and colleagues (Aziz *et al*, 1996). The studies of Yamazawa *et al.* (2000) and Sardi *et al.* (1999) both confirmed statistically that younger women with endometrial carcinoma had a better prognosis. This is understandable because older patients have an increased incidence of tumours with a higher grade, higher stage, and greater depth of myometrial invasion, factors which are associated with a worse

prognosis (Aziz *et al*, 1996). Unfortunately, our studies did not include long term patient follow-up to further investigate this finding.

5.2 HISTOPATHOLOGICAL DATA

5.2.1 HISTOLOGICAL SUBTYPES

In this study endometrioid type endometrial adenocarcinoma formed the bulk of the cases studied: 85.2% of cases. This is in keeping with the general trend, where the majority of endometrial adenocarcinomas lack squamous, mucinous, or papillary features, and is referred to as "typical" endometrioid tumours (Sutton *et al*, 1990). Both clear cell and serous papillary cancers are rare, aggressive tumours associated with a poor clinical outcome. The prognosis of clear cell carcinoma is similar to or slightly better than that of uterine serous papillary carcinoma (Tornos and Silva, 1993; Abeler and Kjorstad, 1991; Carcangiu and Chambers, 1992). Clear cell carcinoma represents 1 to 5.5% of all endometrial carcinomas (Tornos and Silva, 1993). However, we encountered an unusual proportion of clear cell carcinomas, which made up 9.3% of the cases in this study. On the other hand, there were only 2 (3.7%) serous papillary carcinomas in this series.

The histological type of endometrial adenocarcinoma has been proven to be age and race dependent (Hoffman *et al*, 1995; Christopherson *et al*, 1983). In fact, the study of Hoffman *et al.* (1995) has shown that endometrial carcinoma in the age group of 75-92 years is more aggressive, histologically less differentiated, and often non-endometrioid. All the clear cell carcinomas in this study belonged to patients whose ages ranged from 55 to 75 years. Of the 2 serous papillary patients, one was 56 years while the other was 80 years old, and one of these patients was White. With regards to race, as mentioned above, it has been shown that Black patients have more of the less favourable types of endometrial cancer, such as serous, clear cell and adenosquamous (Connelly *et al,* 1982). As stated above, we found that 4 of the black patients studied had clear cell carcinoma compared to none of the white patients. Matthews and colleagues found that 77% of the patients they studied with clear cell cancers were Black

(Matthews *et al*, 1997). Most of the patients researched in this study were Black and this could explain the high percentage of clear cell carcinomas encountered in our study. It would be very interesting to find out whether the Asians and Coloureds also follow the usual trends of endometrial cancer as depicted in the literature. Our study was unable to determine this, as we did not have adequate representation of all the race groups.

5.2.2 GRADE

Histologic grade in endometrial adenocarcinoma correlates with outcome, just like many other malignancies. Since the higher grade tumours are more advanced, they are more likely to recur (Sutton et al, 1990). We were unable to check for any recurrence of the disease, as it was not possible for us to get patient follow-up. Our cases were mostly grade 2 tumours. 61.1% of the cases made up grade 2-moderately differentiated cancers, and 18.5% were grade 3 poorly differentiated tumours, while 20.4% were well-differentiated grade 1 cancers. It has been known that in young women the tumours are usually low grade and minimally invasive (Kurman, 1994). Of the cases that belonged to the 40-49 age range, only one was a grade 1 tumour, and of the remaining cases two were grade 2 and one a grade 3 cancer. However, it should be borne in mind that all the patients from this age group were Black women. Also, the reason for us having a majority of grade 2 tumours could be due to the fact that most of the patients were from the 60-69 age range, and older women are known to have higher grade tumours, as mentioned above (Aziz *et al,* 1996).

5.2.3 STAGE

It is important to surgically stage an endometrial cancer and to obtain information regarding depth, grade and extrauterine spread (Sutton *et al*, 1990). The cases in this study were mostly (44%) stage IB. Only one case was a stage IIIC endometrioid type and 7 were IIIA. Both serous papillary tumours were stage IIIA. None of the cancers belonged to stage IV. This was surprising in that most of our patients were in the age range of 60-69 and were Black patients, and it is a known fact that age and race both have adverse effects on endometrial cancer

(Kohler *et al*, 1996; Gallup and Stock, 1984). Because of the lack of long term patient follow-up, it is uncertain whether some patients subsequently developed distant metastatic (stage IV) disease. A study conducted by Hoffman and colleagues revealed that women in the age group of 75-92 years had only 23% of tumours that were stage I, grade I (Hoffman *et al*, 1995). The majority (77%) of the tumours were deeply invasive or, of advanced stage (IC-IV). These cancers were G2, G3, or "virulent" types of non-endometrioid endometrial carcinoma (undifferentiated, clear cell, squamous cell and serous papillary carcinoma). They concluded that women in this age group had more aggressive, histologically less differentiated, and often non-endometrioid tumours compared to the general population (Hoffman *et al*, 1995). This would explain why our study had more low stage tumours, even though most of our patients were in the 60-69 year age group. Also, most (85.2%) of these tumours were of the endometrioid type, and it is known that non-endometrioid tumours are more often high-stage tumours (Hoffman *et al*, 1995).

5.2.4 MYOMETRIAL INVASION

Myometrial invasion can only be assessed by examination of the excised uterus, when no preoperative radiotherapy has been delivered. It may also be interpreted as a measure of tumour aggressiveness and is associated with grade (Sutton *et al*, 1990). The well-differentiated (grade 1) tumours tend not to invade while the poorly differentiated (grade 3) tumours tend to invade deeply (Sutton *et al*, 1990). The majority of our cases (46.3%) had less than 50% of myometrial invasion and this could be related to the fact that only 18.5% of our cases were grade 3 poorly differentiated tumours. Greater than 50% of myometrial invasion was found in only 38.9% of cases. Serous papillary tumours frequently show deep myometrial invasion and/or extrauterine spread at the time of presentation (Carcangiu *et al,* 1997). This is in keeping with our findings, as both our serous papillary tumours demonstrated more than 50% of myometrial invasion. In endometrioid type endometrial carcinoma depth of myometrial invasion is a powerful predictor of extrauterine disease and survival, but some researchers have found that it is not such a great tool in serous papillary and clear cell carcinomas (Carcangiu *et al*, 1997; Malpica *et al*, 1995).

5.2.5 LYMPH NODE INVOLVEMENT

As with virtually all carcinomas, metastatic lymph node involvement clearly adds risk of relapse of disease in endometrial adenocarcinoma (Sutton *et al*, 1990). We only had a single case that showed metastatic nodal disease. This case belonged to an Asian patient and was a stage IIIC, grade II endometrioid endometrial adenocarcinoma. With regards to relapse or recurrence, our study did not include clinical follow-up, so we were therefore unable to further investigate this.

5.3 IMMUNOHISTOCHEMICAL STUDY

5.3.1 p53 IMMUNOEXPRESSION AND CLINICAL DATA

This "guardian of the genome" mediates many cellular functions that serve to protect against the development of malignancy (Lane, 1992). p53 is a key element in maintaining genomic stability, and during carcinogenesis loss of *p53* function is a common event (Wang, 1999). Most *p53* mutations are missense mutations in conserved regions of the gene. These give rise to structural alterations in the protein that interferes with its function (Soong *et al*, 1996). The conformational changes that occur lead to accumulation/stabilization of p53 protein in the cell such that it becomes detectable by immunohistochemical detection. Whereas, the low concentration of wild-type p53 in normal cells generally cannot be detected by immunohistochemical detection methods (Soong *et al*, 1996). However, there seems to be some controversy about this. Battifora (1994) believes that although the wild-type protein is short lived, it may still be detectable by immunohistochemical methods if overexpressed, when highly sensitive techniques are used. Overexpression of wild-type p53 protein is a normal physiologic condition, as it is a response to slow down the cell cycle at the G1 phase to allow the repair of damaged DNA (Battifora, 1994). Therefore, care has to be taken when interpreting results.

We have found that p53 was expressed in 50% (27/54) of our cases. This is in accordance with other studies. Ioffe and co-workers also found that 50% of endometrial carcinoma cases were positive for p53 and so did Kohler and

colleagues (Ioffe *et al*, 1998; Kohler *et al*, 1996). It is known that p53 is associated with higher stage and histopathologic grade, higher mitotic rate and worse prognosis in a range of tumours (Nielsen and Nyholm, 1994). Inoue *et al.* (1994) and Kohler *et al.* (1996) found p53 protein overexpression in endometrial cancers to also be associated with high malignant potential, including advanced surgical stage, high histological grade, extensive myometrial invasion, lymph node metastasis, and serous papillary tumours. Lax and co-workers with their study of endometrioid type tumours, found that mutations of *p53* were absent in grade 1 tumours, rare in grade 2 tumours, and relatively common (43%) in grade 3 tumours (Lax *et al*, 2000). We found that most of our grade 1 tumours were negative for p53; only two cases stained 3+ and one case 4+. It is interesting to note that two of these patients were Black and one an Asian. Also, expression was rare in our grade 2 tumours, but the majority (80%) of our grade 3 tumours stained up positively for p53. With regards to stage, many of our cases that were positive for p53 were stage IIIA and IIB. Our findings seem to be in keeping with that of the other researchers (Lax *et al,* 2000; Inoue *et al,* 1994; Kohler *et al*, 1996). Most interesting to note is that when p53 was analysed with our clinical data via Pearson's chi square test, we achieved significant results with respect to grade and stage. We had a p value of 0.03 for stage and 0.052 for grade. Burton and colleagues postulated that the high levels of p53 immunoreactivity may be due to the abnormal stabilization of the p53 protein (Burton *et al*, 1999). They also found a correlation between low levels of p53 and apoptosis (Burton *et al*, 1999). Unfortunately, we did not use any apoptotic markers to confirm this.

Kohler and colleagues also found an added variable in their study. They observed a striking racial disparity in survival and p53 overexpression (Kohler *et al*, 1996). It has already been shown that Black women with endometrial cancer more frequently have unfavourable histologic features (Connelly *et al*, 1982), but Kohler *et al.* (1996) found a higher frequency of p53 overexpression in Blacks, which also contributed to the racial disparity in survival. However, they also believe that p53 status is not the sole determinant of the racial disparity in survival (Kohler *et al*, 1996). Most of our cases that expressed p53 belonged to Black patients. About 16 (59.2%) of the cases were Black patients, majority of which stained strongly with a 3+ and 4+. This is somewhat in keeping to the

findings of Kohler *et al.* (1996). However, we have to take into consideration again that the majority of our cases were Black women, and that we did not have a wide representation of the population as a whole. It was interesting to note that of the cases that were negative for p53, 64.7% were Asians, 57.1% were White, and 42.8% were black, while both coloured patients showed expression for p53. Whether this means Asian women follow a less aggressive pathway of endometrial cancer than Blacks, with a course that is similar to White women, is worth pursuing in larger numbers.

None of the researchers seem to have included age in their study of p53 in endometrial cancer. Interestingly, our study found most of the cases that overexpressed p53 belonged to the age range of 60-69 years (44.4%) and 70-79 years (22.2%). It has been discovered that age does play a prominent role with endometrial cancer, as the less favourable types of endometrial cancer with the higher stage and grade seem to occur more often in older women. Perhaps our finding may also lend credence to this, as p53 is associated with the less favourable types and with higher stage and grade of endometrial cancers.

5.3.1.1 p53 IN HISTOLOGICAL SUBTYPES

In 1996 Zheng and colleagues investigated the association of p53 with two histologic subtypes of endometrial cancer (Zheng *et al*, 1996). They found that 71.4% of serous papillary and 38.1% of endometrioid carcinomas overexpressed p53, and that the difference between these subtypes was statistically significant. Unfortunately, we only had two cases of serous papillary tumours and therefore could not make such a comparison. However, both these cases stained strongly for p53. Zheng and co-workers also found that compared to endometrioid carcinoma, serous papillary cancers had a significantly higher frequency of p53 overexpression in stage 1 and 2 tumours, but not in stage 3 and 4 tumours, and the opposite was the case with endometrioid cancers. No p53 positive cases were noted in low stage cancer (Zheng *et al*, 1996). This finding was consistent with our study, only with respect to the endometrioid tumours, but with serous papillary cancers we found the opposite to be true, as both our serous papillary tumours stained strongly positive with a 4+, and both were stage IIIA tumours. Inoue *et al.* (1994); Sherman *et al.* (1995); and Tashiro *et al.* (1997b), all

confirmed the above findings. Furthermore, they also found that the putative precursor of serous papillary tumour, which is endometrial intraepithelial carcinoma (EIC), also demonstrates strong p53 overexpression, whereas endometrial hyperplasia (precursor of endometrioid cancer) does not. They concluded that p53 alteration plays an important role early in the pathogenesis of serous carcinoma, possibly accounting for its aggressive biologic behaviour (Tashiro *et al*, 1997b). We were unable to investigate this, as our study did not include any precursor lesions. The results of their studies lent more evidence to the concept of the dualistic model of endometrial carcinogenesis. To determine whether the pathogenesis of clear cell carcinoma can be accommodated by this dualistic model of endometrial carcinogenesis, Lax and colleagues set out to investigate the immunoexpression of certain antibodies, one of them being p53 in relation to clear cell carcinoma (Lax *et al*, 1998). They found that p53 expression tended to be higher in clear cell carcinoma compared with endometrioid carcinoma, but when compared to serous carcinoma the expression was significantly lower (Lax *et al*, 1998). The results of Lax and colleagues suggested that mutation of *p53* is usually not involved in the initiation of clear cell carcinoma. However the expression of p53 in a group of clear cell cancers with serous features suggests that mutation of *p53* may be a late event that may play a role in tumour progression. It therefore appears that there is another pathway in endometrial carcinogenesis that leads to the development of clear cell cancer (Lax *et al,* 1998). We had only 5 clear cell carcinomas in this study. Of these, 2 were 4+, one was a 3+, and the remaining two were negative. We could say that these results were slightly higher than that of endometrioid tumours, but, with such a small number, it was difficult to make the above comparison, especially with just two serous papillary cancers. An equal number of different subtypes is required to investigate this.

5.3.1.2 p53 OVEREXPRESSION AND PROGNOSIS

The overexpression of p53 has been reported to correlate with poor prognosis in several types of tumours (Ito *et al*, 1994). Ito and co-workers studied 221 cases of endometrioid endometrial cancer with follow-up data to investigate the possible relationship between p53 overexpression and prognosis. They observed a significant correlation between p53 overexpression and decreased survival, and

concluded that p53 immunohistochemical evaluation may be useful as a new prognostic indicator (Ito *et al*, 1994). The study of Kohler and colleagues also found that overexpression of p53 in advanced endometrial cancers was associated with poor survival in both White and Black races, but occurred more than twice as frequently in Blacks (Kohler *et al*, 1996). Our study was hampered by the lack of availability of adequate follow-up data. Therefore, no meaningful comment can be made on p53 expression and prognosis, however, our results did show that overexpression of p53 does correlate with the less favourable types of endometrial tumours, and with a higher grade and stage. We also found that p53 overexpression occurs more often in the Black population.

5.3.2 RETINOBLASTOMA (pRb) IMMUNOEXPRESSION AND CLINICAL DATA

pRb in its active state serves as a brake on the advancement of cells from G1 to the S phase of the cell cycle (Sherr, 1996). This brake is released when the cells are stimulated by growth factors, and the Rb protein is inactivated by phosphorylation (pRb-P), leading to the cells then being traversed through the G1→S checkpoint (Sherr, 1996). This pathway governed by the retinoblastoma protein (pRb) therefore plays a central part in determining whether a cell will proceed through the G1 phase or not (Kaelin, 1999). Derailments of the control mechanisms in the G1/S phase of the cell cycle do occur (Semczuk *et a*l, 2004). These play a fundamental role in the initiation and progression of cancer. However, not many reports have researched the issue of simultaneously occurring abnormalities of Rb-pathway components in malignant endometrial tumours (Semczuk *et al*, 2004).

Our study showed a 90.7 % (49/54) staining for the Rb protein. Most (57.4%) of our cases stained strongly positive with a 4+. This implies that majority of the cases we studied did not have *Rb* gene mutations. Susini *et al.* (2001) found that Rb2/p130, which is a member of the retinoblastoma gene family, was highly expressed in proliferative endometrium and in hyperplasia without atypia, with a mean percentage of staining being 66% and 60% respectively. On the other hand, this expression was downregulated in secretory endometrium, atypical

hyperplasia, and carcinoma, the mean scores being, 38%, 25% and 22%, respectively (Susini *et al,* 2001). However this study did not investigate Rb2/p130. Semczuk and co-workers also investigated the expression of cell-cycle regulatory proteins, namely the Rb-pathway components, (pRb, cyclin D1, $p16^{INK4A}$, and CDK4) in 48 sporadic endometrial cancers (Semczuk *et al*, 2004). They found that 41 of 48 (85%) tumours showed heterogeneous nuclear retinoblastoma protein expression, somewhat similar to our results (Semczuk *et al*, 2004). Other studies have also found the up-regulation of Rb protein to be a common phenomenon in uterine carcinoma, while a subgroup of endometrial neoplasms lacked pRb staining (Milde-Langosch *et al*, 1999; Skomedal *et al*, 1999). Semczuk and co-workers concluded in their study that derailments of the Rb-pathway components, especially cyclin D1 and CDK4, seem to participate in endometrial cancer development (Semczuk *et al*, 2004).

This study also found significant statistical results when grade was compared with pRb. The Pearson's chi square test revealed a p value of 0.036. This is in accordance to the findings of Susini *et al.* (2004). They found that poorly differentiated carcinomas showed a significantly lower immunoreactivity for Rb2/p130 than did well differentiated carcinomas and moderately differentiated carcinomas. We noted that of the 5 cases that were negative for pRb staining, 3 were grade 3 tumours. Also 54.5 % of our grade 1 tumours stained strongly with a 4+, and 57.5% of our grade 2 tumours stained with a 4+. Age and race has not been assessed in these studies, but we found that of the 5 cases that were negative for pRb staining, one was an Asian patient and the rest were Black women. With regards to age, 3 were in the 60-69 year age group and 2 were 70 years old. Interesting to note is that mutations in the *Rb* gene seem to occur in older women and high grade cancers.

5.3.2.1 pRb IN HISTOLOGIC SUBTYPES

We did not come across any significant findings with respect to the staining patterns of the different subtypes. However, it was observed that all 5 of the clear cell tumours stained strongly positive with a 4 + and both the serous papillary cancers also stained positive with a 3+. It was expected that these tumours being unfavourable types of endometrial cancer, would not have stained with

pRb. It has already been found that the oestrogen-independent (less favourable subtypes) pathway is associated with *p53* mutation (Ambros *et al*, 1995; Sherman *et al*, 1995), but the corresponding molecular and genetic alterations underlying oestrogen-related carcinomas have not yet been fully clarified (Susini *et al*, 2001). However, the findings of Susini and colleagues of decreased pRb expression from hyperplastic endometrium through atypical hyperplasia to poorly differentiated carcinomas suggests the involvement of this negative cell-cycle regulator in type 1 endometrial carcinogenesis (favourable type), (Susini *et al*, 2001). This would probably explain the positive pRb staining of our type 2 endometrial cancers (clear cell, serous papillary endometrial cancers). The shift that these researchers observed to lower levels of Rb2/p130 staining in atypical hyperplasia and carcinoma may reflect an alteration of the Rb2/p130 function (Susini *et al*, 2001). As mentioned previously our study did not include any precursors of endometrial cancer.

5.3.2.2 pRb IMMUNOEXPRESSION AND PROGNOSIS

We were unable to establish prognosis since our study did not have adequate patient follow-up, but a previous study of Susini *et al.* (1998) did. They investigated Rb2/p130 expression in a series of 100 endometrial cancers. This study found that decreased levels of Rb2/p130 protein were associated with a significantly increased risk of recurrence and subsequent death from disease, independent of other clinicopathologic features, thereby making Rb2/p130 a strong independent predictor of poor outcome in endometrial carcinoma (Susini *et al*, 1998).

5.3.3 P27 IMMUNOEXPRESSION AND CLINICAL DATA

P27 is a cyclin-dependent kinase inhibitor (CDKI) of the G1 to S cell cycle progression. It inhibits cell-cycle progression by suppressing the kinase activity of cyclin/cyclin-dependent kinase complex (Watanabe *et al*, 2002). Many reports have shown that p27 staining is observed in the nuclei of the glandular cells in the secretory phase but negligible in the proliferative phase of normal endometrium (Bamberger *et al,* 1999; Watanabe *et al*, 2002). The decreased expression of this protein (p27) has been associated with a broad range of human

malignancies, including colorectal (Loda *et al*, 1997); gastric (Mori *et al*, 1997); ovarian (Sui *et al*, 1999) and breast (Porter *et al*, 1997) cancer.

Our study has found an overall p27 expression in 87.3% (47/54) of cases. Most cases stained strongly with a 3+ (16.7%) and 4+ (35.2%). These results are contrary to that of Schmitz *et al.* (2000), Oshita *et al.* (2002), and Nycum *et al.* (2001), who all found a decreased expression of p27 associated with endometrial carcinoma. Oshita and co-workers observed a significantly lower amount of p27 staining in endometrial cancer tissues from premenopausal women than in normal endometrium (Oshita *et al*, 2002). Their results supported the notion that the underexpression of p27 in many endometrial cancers is due to increased p27 protein degradation, which might play an important role in endometrial cancer development in premenopausal women. Our data did not include the patients' menstruation state, however the 4 patients that were in the age group of between 40-49 showed a variable staining pattern. Two stained strongly (4+), one was 3+, and one was negative. Bamberger and colleagues looked at p27 protein in early endometrial adenocarcinomas and noted a significant reduction in expression as the stage and grade progressed (Bamberger *et al*, 1999), whereas Nycum *et al.* (2001) found no significant association of p27 in regard to stage, grade, age, histology, or survival (Nycum *et al*, 2001). This is in keeping with our findings, as when p27 was compared to the clinical data of our patients we found no statistical significant relationship. The results of Watanabe *et al.* (2002) seem to contradict the above researchers to a certain extent, but are somewhat in keeping with the trend of increased p27 protein staining found in this study. They found that p27 expression paradoxically increased more significantly in the higher histological grades (Watanabe *et al*, 2002). This study observed that half (50%) of the grade 3 cases expressed p27 strongly with a 3+ and 4+, which is a similar trend observed with the study of Watanabe *et al,* (2002). Also, they observed that high p27 expression was associated with clinicopathological parameters such as stage, lymph node metastasis, lymphovascular space involvement and myometrial invasion (Watanabe *et al,* 2002). It seems like p27 expression in endometrial cancer still remains quite contradictory and controversial.

5.3.3.1 p27 IN HISTOLOGICAL SUBTYPES

We found no statistically significant association with p27 staining and histologic subtypes, as both serous papillary and all 5 clear cell carcinomas stained with varying extent. However, it is interesting to note that all 7 negative cases were endometrioid endometrial cancers. In relation to this, Oshita and colleagues found their study supported the notion that decreased p27 expression plays a role in type 1 endometrial cancer development (Oshita *et al,* 2002). Decreased expression of p27 was observed in premenopausal women with endometrial cancer, age being an important factor in type 1 and 2 cancers (Oshita *et al,* 2002).

In contrast Schmitz and co-workers observed a high incidence of p27 alterations with reduced p27 expression in 16 of the 21 (76%) serous papillary cancer cases they studied (Schmitz *et al*, 2000). This incidence occurred at an early stage of the disease, with 63% of stage I cases displaying reduced p27 expression, suggesting that this alteration is an early event in the aetiopathogenesis of serous papillary tumours. They found that the serous papillary tumours displayed a high incidence of p27 abnormalities, suggesting that these abnormalities play an important role in the development of this disease (Schmitz *et al*, 2000).

5.3.3.2 p27 IMMUNOEXPRESSION AND PROGNOSIS

The results of Watanabe and colleagues indicated that p27 might be an indicator of poor prognosis (Watanabe *et al*, 2002), whereas Nycum *et al.* (2001) believe that p27 protein staining is not prognostic for survival in advanced endometrial cancers. We were unable to investigate this.

5.3.4 CYCLIN A IMMUNOEXPRESSION AND CLINICAL DATA

Cyclin A is an important positive regulator of the cell cycle, which aids G1-S and G2-M phase transition (Clarke and Chetty, 2001). Although cyclins are known to be overexpressed in human cancers (Kallakury *et al*, 1998), not much literature on the involvement of cyclin A in endometrial carcinoma has been documented. This study found cyclin A to be expressed in 85.2% (46/54) of our cases. There also seemed to be a trend where greater intensity of staining was observed in

higher grades of the tumour. The majority (90%) of our grade 3 tumours stained strongly with a 3+ or 4+, while the grade 1 cancers (81.8%) stained weakly with a 1+ or 2+ and some were negative. This is in keeping to the study of Shih *et al.* (2003). Their study demonstrated that the expression of cyclin A is increased in endometrial carcinomas as compared to normal proliferative endometrium (Shih *et al*, 2003). Although positive staining for cyclin A was present in only 31% of cases, as compared to our 85.2%, they also found a significant correlation with higher histologic grade. Positive rates for cyclin A were significantly higher in grade 2 and grade 3 tumours than in grade 1 tumours (Shih *et al*, 2003). Furthermore, they also found a high correlation between cyclin A and p53 expression (to be discussed later) (*Shih et al*, 2003). Our study observed a statistically significant association with myometrial invasion and cyclin A expression, with a p value of 0.009. Another study by Kallakury and colleagues, also demonstrated a high positivity rate of cyclin A in 64 % of their endometrioid endometrial cancers (Kallakury *et al*, 1998). They found that myometrial invasion and grade were predictors of outcome as well (Kallakury *et al*, 1998).

5.3.4.1 CYCLIN A IN HISTOLOGIC SUBTYPES

All 5 of our clear cell carcinoma cases stained positive for cyclin A, as did the 2 serous papillary cancers. The single adenoacanthoma tumour stained weakly positive (1+). The 8 cases that were negative were all endometrioid endometrial cancers. The study of Shih *et al.* (2003) included 6 cases of carcinoma of non-endometrioid type histology. There were 2 cases each of serous papillary carcinoma, clear cell carcinoma, and squamous cell carcinoma. Both cases of serous papillary carcinoma showed an elevated expression for cyclin A, but in clear cell and squamous cell carcinomas there was no pattern of cyclin A expression characteristic for each histological type (Shih *et al,* 2003). These results are different to the current study as all 5 of our clear cell cancers stained positive (three were 2+ and two were 3+). Kallakury and co-workers also found a high cyclin A positivity rate with their serous papillary tumours. Their study included 17 serous papillary cancers and the positivity rate for cyclin A expression was 71% (Kallakury *et al*, 1998). Both our serous papillary tumours stained strongly with 3+ positivity.

5.3.4.2 CYCLIN A IMMUNOEXPRESSION AND PROGNOSIS

We were unable to determine the prognostic implications of this cell cycle regulator, but Shih and colleagues found that the endometrial cancer cases which stained positive for cyclin A, did not respond to chemotherapy or irradiation. Also, the tumours in 7 of 8 patients who died of disease were positive for cyclin A (Shih *et al*, 2003). They believed that the unfavourable prognosis of these patients may be ascribed to the vigorous growth potential of the tumour due to the overexpression of cyclin A. Their data suggested that accumulation of cyclin A in tumour cells is advantageous for cell cyclin progression. Shih *et al.* (2003) therefore concluded that positive staining for cyclin A could be a useful marker for poor prognosis in patients with endometrial cancer.

5.3.5 CYCLIN D1 IMMUNOEXPRESSION AND CLINICAL DATA

Cyclin D1, which is one of three members that make up the D-type cyclin gene family, is required for completion of the G1/S transition in normal mammalian cells (Motokura and Arnold, 1993; Hirama and Koeffler, 1995). This cyclin is overexpressed in various human cancers and cell lines, and plays an important role in tumourigenesis and cancer progression (Michalides, 1999). Until 1996, abnormal expression of cyclin D1 in endometrial cancer had not been reported on (Nikaido *et al*, 1996). It is only recently that the role of this cyclin in endometrial cancer has been more intensely investigated.

In our study, 19 of the 54 cases examined (35.2%) expressed cyclin D1. Of these, 7.4% stained strongly with a 3+ or 4+ positivity. The remainder showed a weak staining pattern. Nikaido and colleagues found expression of cyclin D1 to be restricted to only a few cells of normal and hyperplastic endometrium, whereas it was expressed in 40% (30/74) of the endometrial cancer cases they investigated (Nikaido *et al*, 1996). These results are similar to our findings of endometrial cancer, when taking into account the percentage of positive cases. However, we did not find cyclin D1 positive rate significantly greater in stage III patients as did Nikaido and colleagues (Nikaido *et al*, 1996). They had a 66.7% positive rate in stage III tumours, and a 33.3% positive rate in stage I cases. In fact, we found quite the opposite, as only 25% of our stage III cases were

positive, compared to 39% of our stage I cases. A similar trend was found with respect to grade. Nikaido *et al.* suggested that cyclin D1 might be affected by mutations in the protein itself, or in a regulatory kinase or phosphatase, and the fact that cyclin D1 can be overexpressed implies that it plays a key role in tumourigenesis (Nikaido *et al*, 1996).

Ito and colleagues observed cyclin D1 immunoreactivity in 56% of endometrial carcinoma cases, but the cyclin D1 labelling index was higher in well differentiated carcinoma than that of moderately and poorly differentiated tumours (Ito *et al,* 1998). We observed a similar staining pattern with grade in our tumours. Semczuk and colleagues reported overexpression of cyclin D1 in 24 of the 48 (50%) endometrial tumours they tested for abnormalities of Rb-pathway components (Semczuk *et al*, 2004). They concluded that derailments of the Rb-pathway components, cyclin D1 and CDK4 in particular, seems to participate in endometrial cancer development (Semczuk *et al*, 2004). Quddus and colleagues have demonstrated that overexpression of cyclin D1 increases from normal endometrium to hyperplasia and carcinoma, also suggesting that it plays a role in endometrial carcinogenesis. They have reported 68% cyclin D1 overexpression in their series of endometrial cancers (Quddus *et al*, 2002). Our study did not incorporate precursor lesions of endometrial cancer and therefore we could not report on this. The latter researchers found cyclin D1 to be overexpressed in hyperplastic lesions, which are considered to be the precursors of endometrioid adenocarcinoma. Their findings suggested that cyclin D1 overexpression may be an early event in endometrial carcinogenesis and also supported the significance of complex hyperplasia as a precancerous lesion (Quddus *et al*, 2002). Although the mechanisms of cyclin D1 dysregulation in endometrial neoplasia are not well defined, it is thought that the dysregulation contributes to an increase in the proportion of cells from the G1 to S phase (Quddus *et al*, 2002).

None of the researchers has incorporated age as a clinical parameter in their research study. When age was statistically compared to the immunohistochemical results in this study, it was found that cyclin D1 and age achieved a value very close to statistical significance. The p value was 0.051. It was also observed that although our staining percentage was only 35.2%, all 4 of

the patients in the 40-49 age group, stained positively for cyclin D1. Three of which, stained strongly with 3+ and 4+. This could add to the suggestion of cyclin D1 playing a role in endometrioid (Type 1) endometrial cancer.

5.3.5.1 CYCLIN D1 IN HISTOLOGIC SUBTYPES

This study found 39.1% of endometrioid endometrial cancers stained positive for cyclin D1, whereas 4 of the 5 clear cell cancers, and both serous papillary tumours, as well as the single adenoacanthoma case were all negative. The single clear cell tumour in this study, stained weakly with a 2+ positivity. Schmitz and colleagues investigated cyclin D1 abnormalities in serous papillary carcinoma (Schmitz *et al,* 2000). Their results indicated that cyclin D1 overexpression is involved in the development of a small number of serous papillary cases. They observed overexpression in only 4 (19%) of the 21 specimens studied, whereas, Nikaido and co-workers found cyclin D1 overexpression in 40% of endometrioid type endometrial cancers (Nikaido *et al*, 1996). This adds more evidence to the suggestion that dysregulation of cyclin D1 probably plays a prominent role in type 1 endometrioid type endometrial cancer.

5.3.5.2 CYCLIN D1 IMMUNOEXPRESSION AND PROGNOSIS

Overexpression of cyclin D1 and its correlation with adverse clinical outcome have been reported in breast carcinoma (Sasano *et al*, 1997), which is also an oestrogen dependent malignancy like endometrioid endometrial carcinoma. However, in contrast to breast carcinoma, cyclin overexpression is not considered to play an important role in the biologic features of endometrioid endometrial cancer. Ito *et al.* (1998) found no significant correlations between the cyclin D1 immunostaining and the actual survival or clinical outcome of patients.

5.3.6 CYCLIN E IMMUNOEXPRESSION AND CLINICAL DATA

Cyclin E is known to contribute to normal cell proliferation and development (Donnellan and Chetty, 1999). In actual fact, it drives the cell cycle forward towards mitosis (Chetty, 2002). Cyclin E has been analysed in many studies in several tumour types (Chetty, 2002), including endometrial cancer, where upregulation has been seen in majority of cases (Ito *et al*, 1998). This study observed up-regulation of cyclin E in 92.6% (50/54) of cases Most of the cases

stained strongly (4+). Ito and colleagues detected cyclin E immunoreactivity in 94.8% (37/39) of endometrial cancer cases examined (Ito *et al*, 1998). They found that the labelling index was more than 80% in all cases, regardless of their grade and stage. Our results were similar in that the histologic grade and stage did not seem to influence the positive staining for cyclin E. However, we found that of the 4 cases that were negative, all were stage I, and 3 were grade 2, while 1 was a grade 1 tumour. There was also no correlation with age of the patients but a value close to statistical significance was noted when cyclin E and myometrial invasion was evaluated. A p value of 0.052 was obtained. Oshita *et al.* (2002) found that cyclin E expression in endometrial cancer tissues from premenopausal women (67%) was higher than that of postmenopausal women (53%), unlike this study, but they also observed a significantly higher amount of cyclin E expression in endometrial cancer tissues from postmenopausal women than in normal postmenopausal endometrium. Their study therefore suggested that upregulation of cyclin E may play an important role in endometrial cancer development in postmenopausal women (Oshita *et al*, 2002). It was also noted in the current study that of the 4 negative cases, 3 were Black patients, while one was an Asian.

5.3.6.1 CYCLIN E IN HISTOLOGIC SUBTYPES

This study noted that all 4 cases that were negative for cyclin E were endometrioid type cancers (type I). Also, all clear cell and serous papillary tumours (type II cancers), stained strongly positive (4+). However, no major correlation could be made from these results, as we did not have an adequate number of type II endometrial cancers to make a comparison, but there does seem to be a trend that cyclin E overexpression is associated more with type II endometrial cancers. The study of Oshita and co-workers supports the notion that increased cyclin E expression plays a role in type II endometrial cancer development (Oshita *et al*, 2002). They found increased expression of cyclin E in majority of postmenopausal women, and these women usually develop type II endometrial tumours (Oshita *et al*, 2002).

5.3.6.2 CYCLIN E IMMUNOEXPRESSION AND PROGNOSIS

It is unclear whether cyclin E analysed on its own is of prognostic significance, but Chetty (2002) observed that looking at the role of one protein at a time is not advisable. He suggested that it is better to look at them in concert, and since cyclin E has an intimate association with p27, these two proteins should be looked at in tandem (Chetty, 2002). Oshita *et al.* (2002) found decreased p27 expression plays a role in type I endometrial cancer development, and that increased cyclin E expression plays a role in type II endometrial cancer development. Their results suggested the potential for new interventions in treatment and prevention of endometrial carcinoma (Oshita *et al*, 2002),

5.3.7 CORRELATION OF IMMUNOHISTOCHEMICAL STAINING

As Chetty suggested it is perhaps more fruitful to look at the role of proteins in association with each other, and obtain the maximum knowledge of their place in cancer, than to examine them one at a time in isolation. Especially when examining something as complex as the cell cycle, which incorporates various different proteins in its intricate operational network (Chetty, 2002). This study looked at the correlation of the different markers and their significance in endometrial cancer.

5.3.7.1 CYCLIN A AND P53

This study has observed a statistical significant correlation between cyclin A and p53. A p value of 0.002 was obtained. Shih and co-workers also found a high correlation between cyclin A and p53 expression (Shih *et al*, 2003). They observed that p53 overexpression occurs more frequently in non-endometrioid type or poorly differentiated (ie. higher-grade) endometrial cancers with p53 mutation (Shih *et al*, 2003). Elevated expression of cyclin A is a characteristic feature of high-grade endometrial cancers associated with p53 expression. It is considered that mutated p53 is unable to bind the promoter of cyclin A and might allow uncontrolled expression of cyclin A mRNA. Therefore, the correlation between the expression of cyclin A and p53 in endometrial carcinomas probably represents the possible interactions of these protein molecules (Shih *et al*, 2003).

5.3.7.2 CYCLIN A AND p27

In many malignancies p27 usually has a decreased expression, but this study found that the 44 cases that expressed cyclin A, also expressed p27, and a 100% correlation in the staining between the 2 markers was obtained. However, it has been observed that higher grades of endometrial cancer, do express p27 (Watanabe *et al*, 2002). Increased expression of cyclin A is also associated with higher grades of endometrial cancer (Shih *et al*, 2003). This study observed both these trends as well. Most of the cases that were positive for both proteins were G2 and G3 tumours, and would therefore explain the statistical significant correlation of both these tumour markers.

5.3.7.3 CYCLIN A AND CYCLIN E

Cyclin A and cyclin E were both expressed in most of the cases in this study and achieved a very significant correlation of $p<0.0001$. During the S phase cyclin A complexes with CDK2 and with cdc2 in G2-M phase transition (Clarke and Chetty, 2001). To influence transcription, cyclin A binds directly to E2F1 (the transcription factor that interacts with pRb) and is able to phosphorylate the DP1 subunit, which inhibits E2F-DNA binding activity, and eventually leads to cyclin A synthesis. Cyclin A is also associated with pRb related proteins (Pines, 1995). Cyclin E regulates transition from G1-S phase and is important in initiation of DNA replication (Ohtsubo and Roberts, 1993). E- type cyclins bind and activate CDK2, which is essential for the cell to begin DNA replication (Dulic *et al*, 1992). Cyclin E, just like cyclin A is also associated with E2F transcription factor in a complex with the Rb related proteins p107 and p130 (Lees *et al*, 1992). MDM2 (murine double minute-2) is a proto-oncogene, which has an oncogenic capacity due to its ability to bind the p53 tumour-suppressor protein and mask its transcriptional activation potential (Momand *et al*, 1992). Martin *et al.* (1995) have shown that MDM2 makes a functional contact with two cooperating transcription factors, E2F1 and DP1, which are involved in S-phase progression. MDM2 uses residues conserved in the activation domain of p53 to contact the activation domain of E2F1. However, MDM2 stimulates the activation capacity of E2F1/DP1 in contrast to its repression of p53 activity. The results of Martin and colleagues indicated that MDM2 not only releases a proliferative block by silencing the tumour suppressor p53, it also positively increases proliferation by

stimulating the S-phase inducing transcription factors E2F1/DP1 (Martin *et al*, 1995). This would probably explain the expression of both cyclin A and E in this study, where 46 of the cases expressed both cyclins.

5.3.7.4 CYCLIN E AND p27

It is well known that cyclin E drives the cell cycle forward towards mitosis, while p27 acts as a brake and prevents cell cycle progression. Many studies have analysed the levels of these two proteins in several tumour types and a trend has emerged (Chetty, 2002). High cyclin E with low p27 seems to be an adverse prognostic indicator in many of the commonly encountered cancers. Furthermore, there has been a stepwise increase of cyclin E and decrease of p27 observed, that correlates with the progression from dysplastic, pre-invasive lesions to full-blown invasive malignant tumours. However, as Chetty (2002) warns there are exceptions to this trend, where the relationship between cyclin E and p27 is not that straightforward, and endometrial cancer appears to be one such exception to the rule. This study has observed most of the cases staining positive for both cyclin E and p27. A statistical significant value of 0.022 was obtained where 45 of the cases studied expressed both cyclin E and p27. This correlation between p27 and cyclin E can therefore not be explained, as the expression of p27 in endometrial cancers still remains quite elusive, since some studies have observed an increased expression of this protein (Watanabe *et al*, 2002), whilst others have noticed a decreased expression in endometrial cancer (Oshita *et al*, 2002).

5.3.7.5 CYCLIN E AND p53

The correlation between cyclin E and p53 achieved a statistical significant value of p=0.038. Cyclin E, just like p53 has an important function in the cell cycle. It is involved in the initiation of DNA replication. The abnormalities of p53 and cyclin E seems to correlate with the progression of the malignant process, as all 27 cases that expressed p53 were also positive for cyclin E. The mutated p53 being unable to cause cell cycle arrest or apoptosis allows the cells to pass through the G1 phase. It is normally at this late G1 phase that an active complex is formed between cyclin E and CDK2, and activated E2F then increases the transcription of cyclin E, needed for DNA replication. However, the overexpression of cyclin E

and p53 is probably due to gene amplification of cyclin E and the enhanced indirect effect of mutated p53 at this late G1 phase.

5.3.7.6 p53 AND pRb

We also obtained a value of statistical significance with p53 and pRb (p=0.019). pRb was overexpressed in this study, while in other cancers it has been known to be suppressed. Li and colleagues found that in their study, the cells with weak or negative staining for p53 had a tendency to stain positively for pRb, whereas the cells with definite positivity for p53 tended to stain either weakly or negative for pRb (Li *et al,* 1996). This study observed similar findings, but not with all the cases. Both these protein molecules are prominent tumour suppressor genes that have a central role in the cell cycle. They regulate the cellular response to external signals. Each of these proteins are regulated by a series of other proteins, but together they constitute a molecular pathway. However, in cancer, disruption of these pathways can occur at multiple points. This leads to mutation or alteration in expression of these components, and both *p53* and *Rb* are known to be frequently mutated in other human cancers (Levine *et al*, 1991). An event that has not been frequently observed in endometrial cancer, but MDM2, an oncoprotein, has been implicated as the culprit in other cancers (Oliner *et al*, 1992). Overproduction of MDM2 is tumourigenic, and has been found to interact physically and functionally with pRb and p53, inhibiting their growth regulatory functions (Xiao *et al*, 1995). Both these proteins can therefore be subjected to negative regulation by the product of a single cellular proto-oncogene, which is MDM2 (Xiao *et al,* 1995).

5.3.7.7 p27 AND p53

This correlation achieved a statistical significant value of p=0.043. It was found that the majority (96.3%) of cases that expressed p53 also expressed p27. Also, the majority of cases that did not express p27 (85.7%) also did not express p53. It was therefore deduced that, if an endometrial cancer expresses p53, it is likely to also express p27 and If an endometrial cancer does not express p27, it is likely that it would not express p53.

5.4 MOLECULAR STUDY

5.4.1 MICROSATELLITE ANALYSIS OF THE MMR GENES

Microsatellite analysis was performed using three MMR markers. The markers used were D2S123, D3S659, and BAT 25. Of these D3S659 proved to be the most informative with an informativity rate of 57%. This was followed by D2S123 with 39%, and BAT 25 with 28%. The informative cases were those normal samples containing 2 alleles. D2S123 showed 61.1% (33/54) homozygosity (non-informative). D3S659 showed 42.6% (23/54) homozygosity (non-informative). BAT25 showed a high rate of homozygosity, 72.2% (39/54), (non-informative). This study achieved a high rate of informativity (57% for the D3S659 marker), compared to other studies, like that of Peiro *et al.* (2002) who only found 11% of informativity. However, another study by Peiffer and co-workers, which incorporated an extensive investigation using 39 microsatellite markers, representing most of the chromosomal arms, found that the number of informative pairs ranged from 45-86% (Peiffer *et al,* 1995).

5.4.1.1 AI/LOH OF MMR GENES

Of the MMR markers used in this study, D3S659 and BAT 25 both showed the highest percentage of AI/LOH with 7.4% each, next was D2S123 with 5.6%. At the MMR loci AI/LOH appears to be a more frequent aberration for endometrial cancer than MSI (discussed below), in this study. The total percentage of AI/LOH observed in this study at the MMR gene loci was 20.4% (11/54).

Peiro and colleagues found that with their study the highest rate of LOH was seen at D2S123 with 16%, and the lowest was at BAT 25 with 0% (Peiro *et al*, 2002). These observations are the total opposite to the present study. The findings of Peiro *et al.* (2002), also implies that the highest LOH rate was observed for chromosome 2p (D2S123 maps an area on chromosome 2p), which is the *hMSH2* gene locus. However, this study found the highest rate of AI/LOH on chromosome 3p (D3S659 maps an area on chromosome 3p), which is the *hMLH1* gene locus. Also, AI/LOH was seen at 2 loci in one tumour (Peiro *et al*, 2002). However, AI/LOH > 2 loci was not observed with this study.

Peiffer *et al.* (1995) found that rate of LOH at D2S123 was also only 5%, which is quite similar to the results of this study of 5.6% (Peiffer *et al*, 1995). They observed the most frequent sites of loss were at the marker loci examined on 10q (40%) and 17p (29%) (Peiffer *et al*, 1995). There seems to be a great variability among studies. This makes comparison difficult, but also reflects the genetic complexity of this carcinoma. The observation of this genetic aberration, AI/LOH in endometrial cancer suggests the presence of tumour suppressor genes.

5.4.1.2 AI/LOH AND CLINICAL DATA OF MMR GENES

Most of the cases that exhibited AI/LOH in this study were grade 2 (63.6%) and stage I (72.7%) tumours. The study of Nagase and colleagues found that LOH, which occurred most frequently on chromosome 10q25-10q26, was associated with grade 1 tumours (Nagase *et al*, 1996). However, the cases of this study were predominantly grade 2 tumours. The patients' ages varied widely. With regards to race there was also no significant correlation. Peiro *et al.* (2002) found that LOH did not correlate with any clinicopathologic factors.

5.4.1.3 AI/LOH AND HISTOLOGICAL TYPE

Of the 11 cases that showed AI/LOH, 9 (81.8%) were endometrioid endometrial carcinomas, while only 1 case was a clear cell carcinoma and the other an adenoacanthoma. Tritz *et al.* (1997) observed the opposite. Their study showed that the special variant tumours (serous papillary and clear cell cancers) had a higher frequency of LOH compared with the usual type tumours (endometrioid cancers). Endometrioid tumours showed an average LOH of 11% among 23 chromosomes analysed, whereas serous papillary and clear cell tumours had an average LOH of 24%. These researchers suggest that these findings may reflect an aneuploidy rate of high-grade tumours, which are more common among the special variant than the usual type tumours. LOH was frequently detected on chromosomes 2, 12, 13, 14, 17. On chromosome 17, serous papillary and clear cell tumours had a much higher frequency of LOH compared with the endometrioid type tumours (87% vs 22%) (Tritz *et al*, 1997). In this study both the serous papillary tumours and the remaining 4 clear cell cancers showed no LOH at the MMR gene loci. However, this current study did not contain an equal

proportion of the different types of endometrial cancer and it was therefore not possible to achieve a good comparison.

5.4.1.4 MSI OF MMR GENES

As mentioned above this study found that at the MMR gene loci, MSI was the less frequent aberration than AI/LOH. The highest frequency of MSI was 5.6% (3) and was observed with both the D3S659 and BAT25 markers. Then followed D2S123 with only 1.9% (1). The total MSI rate for the MMR markers was 11.1% (6/54). MSI was initially observed in patients with the hereditary nonpolyposis colon cancer (HNPCC) (Aaltonen *et al*, 1993). These patients have shown germline and somatic mutations in the *hMSH2* and *hMLH1* genes on chromosomes 2p and 3p respectively (Liu *et al,* 1994). Endometrial carcinoma is known to be the second most common cancer found in HNPCC patients (Watson *et al,* 1994). MSI has been observed in 75% of endometrial carcinomas associated with HNPCC (Risinger *et al,* 1993).

MSI+ PHENOTYPE: Owing to the high frequencies of chromosomal abnormalities and mutations in human cancers, Loeb (1991) has hypothesized that cancer is manifested by a mutator phenotype. He based this hypothesis on the argument that the spontaneous mutation rate in normal cells is insufficient to account for the high frequency of mutations in human cancer cells. He believed that at some time during the life of a tumour, the mutation rate must be greater than in normal cells, ie. cancer cells must exhibit or have exhibited a mutator phenotype (Loeb, 1991). MSI is the accumulation of replication errors due to reduced replication fidelity, and these are observed as electrophoretic mobility shifts in microsatellite markers. However, when these shifts occur in ≥ 2 loci, this aberration is referred to as a MSI+ phenotype. Some have also called this MSI-H (Peiro *et al,* 2002) and RER + (replication error) (Caduff *et al*, 1996). There seems to be some controversy regarding this type of aberration as some investigators consider patients with > 1 positive loci to be RER+, whereas others consider patients with ≥ 2 positive loci to be positive and some even consider ≥ 3 positive loci as positive. We referred to the MSI+ phenotype when MSI was

detected in ≥ 2 loci. In this study, MSI was observed at two loci (D3S659 and BAT25) for only one of the cases.

Caduff and colleagues observed MSI in 27 of the 109 (25%) endometrial carcinomas they studied (Caduff *et al*, 1996). Ten of these 109 tumours (9%) demonstrated MSI in two or more loci, and they were considered to be RER+ (Caduff *et al*, 1996). D2S123 was one of the markers they employed in their study. Peiro *et al.* (2002) found that MSI was present in 32 of the 466 (6.9%) cases analysed. The highest rates were seen with D5S346 and Mfd15 (9 of 32, 28.1%). This was followed by BAT26 (6 of 32, 18.8%); D2S123 (5 of 32, 15.6); and BAT25 (3 of 32, 9.4%). They found that MSI occurred at 2 loci in 7 (7.5%), and at 3 loci in 3 tumours (3.2%). These two groups were classified as MSI-H. MSI-H is highly associated with loss of hMLH1 and hMSH2 expression (Peiro *et al,* 2002). The study of Muresu and colleagues observed that 24 (32%) out of the 74 tumours analysed were MSI+ (Muresu *et al*, 2002). BAT25 and D2S123 were amongst the 5 chromosomal markers they used. Their results revealed that MSI was correlated with the inactivation of *hMLH1* gene expression, whereas the MSH2 protein was found to be normally expressed in all of the MSI+ tumours analysed. This suggests that inactivating mechanisms of hMLH1 plays a major pathogenetic role in sporadic endometrial cancer (Muresu *et al*, 2002). However, a larger study is essential to confirm this. The current study shows some similarity to these findings, as MSI was the highest with the D3S659 marker. This marker maps to chromosome 3p, which is the location of the *hMLH1* gene. Palmieri and colleagues also observed the MSI+ phenotype in 38% (16/42) of sporadic endometrial carcinomas, at the 10q25-q26 chromosomal location (Palmieri *et al*, 2000). Their study revealed a high concordance between down-regulation of the *hMLH1* gene expression and presence of MSI (Palmieri *et al*, 2000).

CAUSE OF MSI PHENOTYPE: It has been suggested that silencing of the *hMLH1* gene by promoter hypermethylation is the mechanism underlying the presence of the microsatellite instability phenotype in endometrial carcinomas, but additional studies are warranted to determine the consequence of such methylation (Ellenson, 1999). To provide support for an important role of

promoter hypermethylation in endometrial tumourigenesis, Esteller *et al.* (1999) evaluated endometrial hyperplasia for hMLH1 methylation. They also included other MMR genes like *hMSH2, hMSH3, and hMSH6* in their study. Their findings revealed that 91% (11/12) of endometrial carcinoma cases displaying MSI, had hMLH1 promoter hypermethylation, whereas aberrant methylation was not observed for any of the other MMR genes (Esteller *et al*, 1999). The current research did not include this aspect in its study. However, to add scepticism to these findings, Katabuchi and colleagues found that their data revealed mutations in the four known MMR genes (*hMSH2, hMLH1, hPMS1, hPMS2*) were not responsible for MSI in the majority of sporadic endometrial cancers displaying this phenotype (Katabuchi *et al*, 1995). They believe that there are additional genes that cause this phenotype in endometrial cancer, but studies that have suggested differently (as mentioned above) are more recent (Esteller *et al,* 1999; Palmieri *et al*, 2000; Muresu *et al*, 2002).

Another study by Sirchia and co-workers indicated that MSI appears to be a relatively rare event in endometrial cancer (Sirchia *et al*, 1997). This study found MSI to be a less common genetic aberration with just 11.1% of cases being MSI+ at the MMR gene loci.

5.4.1.5 MSI AND CLINICAL DATA OF MMR GENES

The majority (66.7%) of the cases that showed MSI at the MMR gene loci were grade 2 tumours. Of the remaining 2, one was a grade 1 and the other a grade 3. All of these cases were stage I. The tumour that showed MSI at two loci was a grade 3 cancer. Hirasawa *et al.* found that 55% of their grade 3 tumours were MSI-H (MSI $\geq$ 2 loci) (Hirasawa *et al*, 2003). Peiro and colleagues observed that most of their MSI+ cases were grade 1 (39%), followed by grade 2 which was 33%, and the stage of these tumours was predominantly I and II (Peiro *et al*, 2002). Also, they found that the depth of myometrial invasion was greater than 50% for 41.6% of tumours (Peiro *et al*, 2002). This study showed only 1 case (16.7%) with more than 50% of myometrial invasion. Most of the MSI+ tumours showed less than 50% of invasion of the myometrium. Muresu and co-workers found a significantly higher incidence of MSI in stage III and in more undifferentiated (G3) tumours (Muresu *et al*, 2002). These findings suggested

that genetic instability might become particularly evident in advanced stage tumours due to the progressive accumulation of errors (Muresu *et al*, 2002). To add substance to these findings, Ohwada and colleagues had previously observed little association between replication errors in endometrial cancer and atypical hyperplasia (Ohwada *et al*, 1999). They, therefore also concluded that MSI might be involved in the advanced rather than the early stages of endometrioid adenocarcinoma (Ohwada *et al,* 1999).

This study found no major correlation with MSI and patient age, except for MMR marker D3S659, where a statistically significant value was achieved (p=0.021). Overall, two patients were from the 40-49 age range, two were from the 50-59 age group, one was in the 60-69 age range and another was of unknown age. Ohwada *et al.* (1999) also found that MSI was not related to patient age. However, Kobayashi and colleagues reported that MSI decreases after a patient age of 50 years (Kobayashi *et al*, 1996).

With regards to race, the current study observed that 4 of the 6 (66.7%) cases that were MSI+ were Black patients, one was Coloured and the other an Asian. Basil and colleagues found that in their study, patients with MSI+ tumours were more likely to be of white race and to present with early stage disease (Basil *et al*, 2000). Of note, they observed that 65 of the 70 (93%) patients with MSI+ tumours were white and only 5 of the 70 (7%) were Black (Basil *et al*, 2000). These findings contradict the current study as none of the patients with MSI+ tumours were White. However, it should be considered that the majority of the patients in this study were Black.

5.4.1.6 MSI AND HISTOLOGICAL SUBTYPE

All of the six MSI+ cases at the MMR gene loci in this study were of the endometrioid type endometrial carcinoma. These results seem to be in keeping with the findings of other researchers. Catasus *et al.* found MSI to be more frequent in endometrioid (33%) than in non-endometrioid (11%) carcinomas (Catasus *et al*, 1998). Tashiro and colleagues observed only three (8.8%) serous papillary carcinomas showing MSI, while 20% of their endometrioid carcinomas were MSI+ (Tashiro *et al,* 1997). Their study found the difference in the MSI

frequency between endometrioid and serous papillary carcinoma statistically significant. This data demonstrates that MSI is uncommon in serous papillary carcinoma and supports the notion that different pathogenetic mechanisms are involved in the development of the two different types of endometrial cancer (Tashiro *et al*, 1997). Furthermore, Caduff and co-workers observed that of the 10 RER+ carcinomas (MSI $\geq$ 2 loci) in their study, 9 were of the typical endometrioid subtype (type I). The tenth RER+ tumour had mixed papillary serous and clear cell components, and was therefore considered a type II endometrial carcinoma (Caduff *et al,* 1996). Finally to further support these findings and to highlight another possible molecular difference in the pathogenesis of these two tumour types, Sherman and Kurman noted that the detection of MSI in EIC (endometrial intraepithelial carcinoma), would provide strong support for a causal relationship between MSI and serous papillary carcinoma (Sherman and Kurman, 1998). However, this has never been shown, but the detection of MSI in both atypical endometrial hyperplasia and in associated endometrioid carcinoma as reported by Catasus *et al.* (1998) and Jovanovic *et al.* (1996) also provides added evidence for the role of MMR defects in the development of endometrioid carcinomas.

5.4.1.7 STATISTICAL CORRELATION BETWEEN MMR GENES AND CLINICAL DATA

5.4.1.7.1 D3S659 VERSUS AGE

This correlation was statistically significant with a p value of 0.021. It was observed that with D3S659, MSI was found in patients from the age ranges of 40-49 and 50-59 years only. None were form the ranges of 60-69, 70-79 and 80-89 years, which is in keeping to the findings of Kobayashi and co-workers who reported a decrease in MSI after the age of 50 years (Kobayashi *et al*, 1996). The opposite seems to be the case with AI/LOH and age. None of the patients with AI/LOH were from the 50-59 age group, yet the other age ranges of 60-69, 70-79, 80-89, all had patients with AI/LOH. An unusual finding was one patient with AI/LOH who fell into the 40-49 age group.

5.4.1.7.2 BAT 25 VERSUS HISTOLOGICAL SUBTYPE

The comparison of BAT 25 with histological subtype showed a statistical value of p=0.049. All 3 MSI + cases at this loci were endometrioid endometrial cancers, which adds support to the two pathogenetic pathways of endometrial cancer.

5.4.2 MICROSATELLITE ANALYSIS OF THE *DCC* GENE

Microsatellite analysis was done on three loci of the *DCC* gene. The markers used for this analysis were D18S21, D18S34 and D18S58. The most informative locus for the *DCC* gene proved to be D18S58 with 41%, followed by D18S21 with 37% and finally D18S34 with 22%. D18S21 showed 62.9% (34/54) homozygosity (non-informative). For D18S34 the rate of homozygosity (non-informative) was 77.8% (42/54), which was quite high. D18S58 had 59.3% (32/54) homozygosity (non-informative). Tritz and co-workers found with their study that the rate of informativity for the D18S34 marker was 13.3% (Tritz *et al*, 1997). These results are close to the 11% observed in this study.

5.4.2.1 AI/LOH OF THE *DCC* GENE

This study observed that of the three DCC markers employed, D18S34 showed the highest frequency of AI/LOH (5.6%). This was followed by D18S21 and D18S58 both with 3.7% each. The overall percentage of AI/LOH observed at the *DCC* gene was 11.1% (6/54). AI/LOH was noted at two loci for only one case, with markers D18S21 and D18S34. Gima *et al.* (1994) noted in their study that among the 61 tumours that were informative, 26% showed allelic losses at one or more chromosome 18q loci. Fearon and colleagues have observed the greatest frequency of LOH at the *DCC* (D18S21) locus on chromosome 18q21, with 33% of the tumours analysed exhibiting allelic deletions in this region (Fearon *et al*, 1990). On the other hand Fujino and colleagues failed to detect any mutations of the *DCC* gene in the 60-endometrial tumours they studied (Fujino *et al*, 1994). They suggested that the observed LOH on chromosome 18q might also occur subsequent to the mutation of an, as yet unidentified tumour suppressor gene, other than *DCC* (Fujino *et al*, 1994). The likelihood of *DCC* being a tumour suppressor gene has created serious doubts amongst researchers. Instead, it appears that DCC is a cell surface receptor, important in axonal growth (Fazeli,

1997). It has therefore been suggested that some other gene in close linkage with *DCC* on chromosome 18q21 is implicated in carcinogenesis (Cotran *et al*, 2000).

5.4.2.2 AI/LOH AND CLINICAL DATA OF *DCC* GENE

Half of the tumours in this study that showed AI/LOH at the *DCC* gene loci were grade 3 tumours. Of the remaining half two were grade 2 and one was a grade 1 tumour. Toda *et al.* (2001) also observed the highest frequency of LOH in grade 3 tumours (46.2%). Most (83.3%) of the cases were stage I cancers, while only one case was a stage II. The study of Gima and colleagues found that clinical stage of the disease was not related to LOH frequency or to DCC mRNA expression (Gima *et al*, 1994), while Toda and colleagues suggested that allelic loss is associated with middle and late stages of carcinogenesis in endometrial carcinoma (Toda *et al*, 2001). This speculation was not consistent with our findings as most of the cases that showed AI/LOH were stage I tumours. The majority (66.7%) of the patients with AI/LOH at the *DCC* gene loci were from the 60-69 age range. Of the remaining two, 1 was from the 70-79 age range and the other from the 80-89 age range. All of these patients were Black except one, who was White.

5.4.2.3 AI/LOH AND HISTOLOGICAL SUBTYPE

This study found that all the cases that showed AI/LOH were of the endometrioid type. Tritz *et al.* (1997) also incorporated D18S34 as one of the markers in their study. As mentioned previously, their research showed that special variant tumours (non-endometrioid cancers) have a higher frequency of LOH compared with usual type tumours (endometrioid tumours) (Tritz *et al,* 1997), which is different from the findings of the current study. This could be attributed to the fact that the majority of the cases in this study were of the endometrioid type. A better representation of the different histological subtypes is required to test their findings.

5.4.2.4 MSI OF THE *DCC* GENE

In contrast to the MMR genes, this study found that at the *DCC* gene loci MSI was the more frequent aberration. The frequency of MSI was the highest at the D18S58 marker with 13%, followed by D18S21 and D18S34 with 5.6% each. Overall the total rate of MSI at the *DCC* gene loci was 22.2%. MSI was observed at two loci (D18S21 and D18S34) for one of the endometrial cancer cases. Toda *et al.* (2001) employed D18S34 as one of the markers in their study but found that the highest frequency of MSI was at the D17S799 locus with 18.3%.

CAUSE OF *DCC* INACTIVATION: It has been noted that the *DCC* (deleted in colorectal cancer) tumour suppressor gene has an AT repeat that was demonstrated to be altered in colorectal cancer (Fearon and Vogelstein, 1992). Furthermore, this tumour suppressor gene seems to be involved in the genesis of endometrial cancer (Imamura *et al*, 1992). It could therefore be hypothesised that the AT repeat instability in *DCC* would be one of the mechanisms that causes the gene inactivation in both tumours (Sirchia *et al*, 1997).

5.4.2.5 MSI AND CLINICAL DATA OF THE *DCC* GENE

Fifty percent of the cases with MSI in this study were grade 2 tumours. Toda and colleagues also found that the frequency of MSI was the highest in cases with grade 2 carcinoma (35.3%) (Toda *et al,* 2001). They found that the frequency of MSI was highest in stage III cancers (50%) (Toda *et al*, 2001). The current study however, found that most of the cases showing MSI were stage I (83.3%). Only two endometrial cancers were stage III, and none were stage II. The single tumour case that had lymph node metastasis also demonstrated MSI at the D18S58 locus. This cancer was a stage III. Ohwada and colleagues observed MSI in 63% of patients with lymph node metastasis (Ohwada *et al*, 1999). These researchers also found that the incidence of MSI was not related to histological tumour grade or patient age (Ohwada *et al*, 1999). We observed that most (33.3%) patients with MSI at the *DCC* gene were from the 60-69 age group. As mentioned above Kobayashi and co-workers have reported that MSI decreases after a patient age of 50 years (Kobayashi *et al*, 1996). With respect to race in this study it was noted that the highest frequency (58.3%) of MSI was found to be in Asian patients. Unlike the MMR gene loci, where it was observed that Black

patients had the highest frequency of MSI, these findings contradict Basil *et al* (2000) who found that patients with MSI positivity were more likely to be White patients.

5.4.2.6 MSI AND HISTOLOGICAL SUBTYPE

This study found that majority (83.3%) of the cases with MSI at the *DCC* loci, were endometrioid tumours. This observation is in keeping with other researchers (Caduff *et al*, 1996; Tritz *et al*, 1997), who also found MSI to be more dominant in the type I endometrioid tumours as compared to the type II special variant cancers (serous papillary, clear cell cancers). In this study only one of the MSI+ cases at the *DCC* loci was a clear cell carcinoma and the other an adenoacanthoma. Catasus *et al* (1998) also observed MSI more frequently in endometrioid (33.3%) than in non-endometrioid (serous papillary) carcinomas.

5.4.2.7 GENETIC ABERRATIONS AT THE *DCC* LOCUS

In order to ensure a good facilitation of statistical analysis this study grouped together AI/LOH and MSI as genetic aberrations (GA). This was convenient and possible as all three *DCC* loci studied are found on one gene. Most genetic aberrations were observed with marker D18S58 (16.7%), followed by marker D18S34 with 11.1% (6/54), and D18S21 with 9.3%. In total the genetic aberration observed was 37% (20/54).

Furthermore, a statistical analysis was done to compare genetic aberrations with clinical factors. A significant correlation was achieved between marker D18S58 and stage ($p=0.024$). Most genetic aberrations were noted with stage IC tumours (44%), while none of the stage II tumours showed any genetic aberrations.

5.4.2.8 MUTATION OF THE *DCC* GENE

This study also looked at the mutation status for each case. The data achieved for genetic aberrations was grouped across all three *DCC* markers, and the cases showing an aberration for any one or more of the three markers were designated as mutated (MT). The total number of cases mutated was 18 (33.3%). Two cases had mutations at two loci. A significant statistical correlation was obtained

again with stage and mutation (p=0.012). The highest rate of mutation was noted with stage IB and IC tumours, with 38.9% (7/18) each.

5.4.2.9 STATISTICAL CORRELATION BETWEEN THE *DCC* GENE AND CLINICAL DATA

5.4.2.9.1 D18S21 VERSUS GRADE

All three cases that were MSI+ at the D18S21 loci were G1 tumours, whereas both tumours that showed AI/LOH at this site were G2 cancers. This correlation achieved a statistical significant value of p=0.013.

5.4.2.9.2 D18S58 VERSUS AGE

Two cases demonstrated AI/LOH at the D18S58 loci. Of the two, 1 was from the 70-79 age range and the other from the 80-89 age group. A total of 7 (13%) cases were MSI+ at this site. Three patients ages were unknown, while two were from the 60-69 age range and two belonged to the 70-79 age range.

5.4.3 AI/LOH VERSUS MSI

This study has observed that endometrial cancer seems to have two subsets of patients, one carrying a specific allelic loss, and the other with preponderance of MSI. Some researchers have proposed that normal endometrial cells would undergo malignant transformation through replication errors, and subsequent accumulation of mutations in oncogenes and tumour suppressor genes (Sakamoto *et al*, 1998), while others suggest that the replication errors could represent a consequence of functional alterations of mismatch repair genes (Modrich, 1994). Tritz *et al.* (1997) concluded in their study that the frequencies of allelic losses in endometrial carcinoma differ with histological subtype. AI/LOH seemed to occur more often in special variant compared with usual endometrial types. In contrast to this, they found oncogene activation and microsatellite instability were more frequently detected in usual endometrial carcinoma than in special variant types. The findings of Tritz and colleagues suggest that the clinicopathological phenotypes observed in these tumour types may reflect

molecular differences of tumourigenesis (Tritz et al, 1997), which lead to the two pathogenetic pathway theory of endometrial cancer.

Although the current study also found that most of the MSI+ tumours were of the endometrioid type cancers, it must be noted that the majority of the cases in this study were endometrioid cancers. Therefore, a fair representation of all the histological subtypes of endometrial carcinoma is required in order to further investigate or confirm the findings of Tritz *et al.* (1997). We observed both AI/LOH and MSI for just one case at the D2S123 and the D3S659 loci respectively.

5.4.4 COMPARISON BETWEEN THE *DCC* AND MMR GENES

This study found that at the MMR loci the frequency of AI/LOH was higher, 20.4% (11/54) than that of MSI, 11.1% (6/54). However, this is in contrast to what was observed at the *DCC* gene loci. It was found that the frequency of AI/LOH was lower, 11.1% (6/54) than that of MSI, 22.2% (12/54). Also at the MMR gene loci only MSI was observed in more than one loci for one case, whereas at the *DCC* loci both AI/LOH and MSI were observed at more than one loci for one case. Stage I, grade 2 tumours were dominant for AI/LOH tumours at the MMR gene loci, but at the *DCC* loci stage I, grade 3 tumours were observed more frequently with AI/LOH cases. With regards to MSI, at both the MMR and *DCC* loci stage I, grade 2 tumours were dominant. The 60-69 age range of patients was common at both loci for both aberrations, except for MSI at the MMR loci, where the 40-49 age range was dominant. Black patients had the highest frequency of both aberrations at the MMR gene loci, but although they were also the dominant race with AI/LOH at the *DCC* gene, MSI was observed more frequently in Asian patients.

5.4.5 CORRELATION BETWEEN IMMUNOHISTOCHEMICAL AND MOLECULAR MARKERS

This study observed that the most significant statistical values were found with the *DCC* markers.

5.4.5.1 D3S659 VERSUS CYCLIN D1

All 4 tumours that showed AI/LOH with the D3S659 marker also expressed cyclin D1 at the MMR gene. Of the three MSI+ cases, only 2 expressed cyclin D1. A statistical significant value of p= 0.013 was achieved with this correlation.

5.4.5.2 D18S34 VERSUS p53

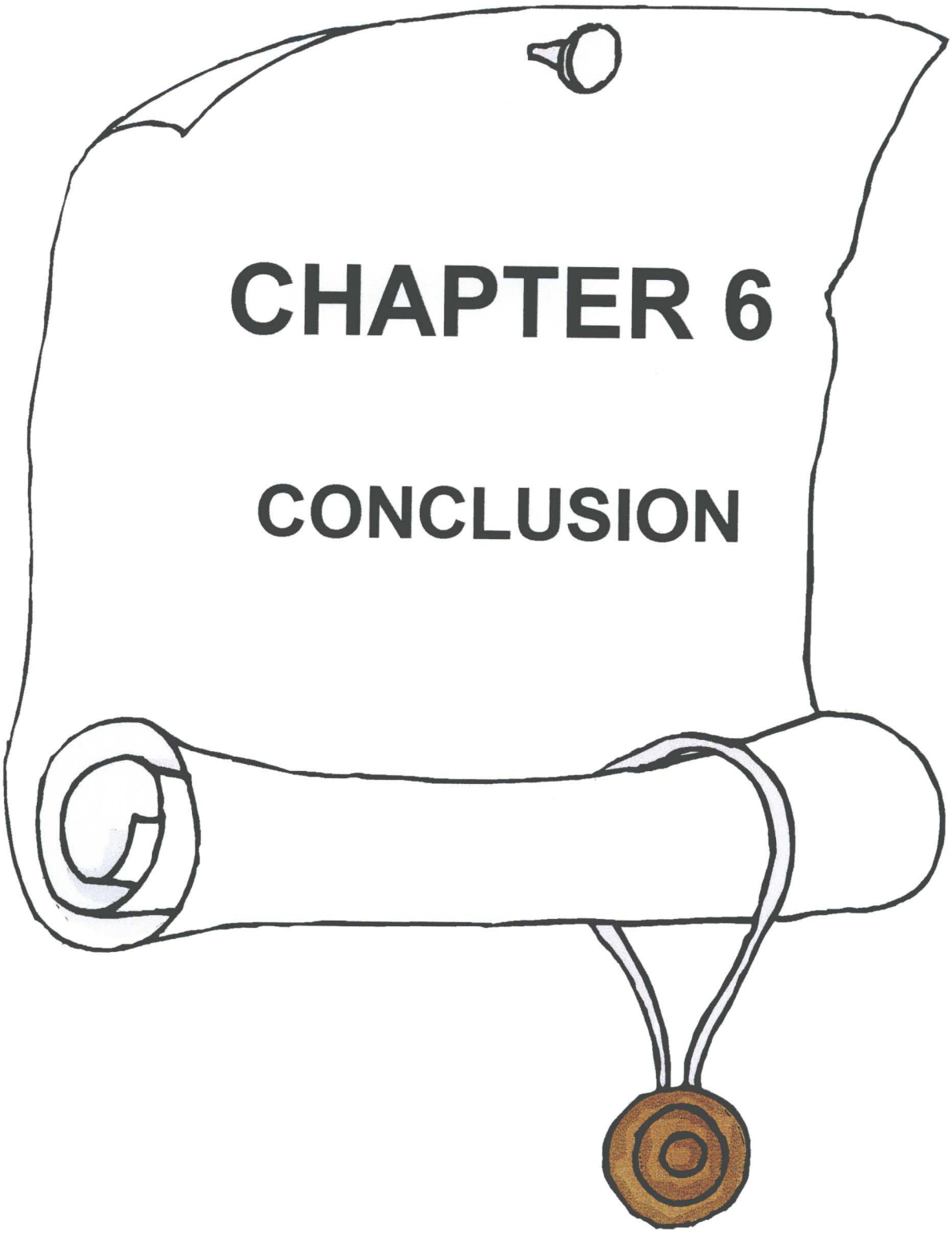
The three cases that showed AI/LOH also expressed cyclin A, and the three cases that were MSI+, expressed cyclin A as well with the D18S34 marker. The statistics were significant (p=0.021).

5.4.5.3 D18S34 VERSUS pRb

One of the cases that showed AI/LOH with the D18S34 marker expressed pRb while the other did not. Of the 7 MSI+ cases, 5 expressed pRb while 2 did not. A significant statistical value was recorded (p=0.036) with this correlation.

5.4.5.4 D18S58 VERSUS p27

The two cases at which AI/LOH was observed with the D18S58 marker, also expressed p27, and all 7 MSI+ cases also expressed p27, which was very interesting to note. This correlation was statistically significant (p=0.018).



CHAPTER 6

CONCLUSION

CONCLUSION

There is gold, and a multitude of rubies: but the lips of knowledge are a precious jewel. ***Proverbs 20:15***

6.1 IMMUNOHISTOCHEMICAL STUDY: Cell cycle protein expression

In conclusion this study has shown that cell cycle protein aberrations do occur in endometrial carcinoma. Derailments of the cell cycle pathway have been considered as one of the most important factors in tumourigenesis of various human malignancies. We have found that in endometrial cancer cyclins A, D1, and E definitely play a prominent role in the progression of carcinogenesis. Furthermore, our results lend credence to the theory of two pathogenetic pathways of endometrial carcinoma, where cyclin D1, p27 and to a certain extent cyclin A seem to be associated with type I endometrioid endometrial cancer, while cyclin E, p53 and pRb have been implicated in the progression of type II endometrial cancer. Although there is not much literature on the prevalence of endometrial carcinoma in Southern Africa, this study has found that the profile among the 54 endometrial cancer cases was in conformity with the established world wide trend. The mean patient age was seen as 57.5 years and the median age as 63 years, which is in keeping with the literature. Endometrioid endometrial cancer formed the majority of the endometrial cancer type in this study. Also, Black patients were seen to have the more aggressive, histologically less differentiated and often non-endometrioid cancers, as noted in previous studies. One discriminating factor that contradicts previous studies is that this study has found that the Black population has a higher incidence of endometrial cancer. However, it has to be taken in to account that this study was hindered by the lack of representation of the population.

6.2 MOLECULAR STUDY: Incidence of microsatellite instability

In conclusion we have shown that at the MMR gene loci, AI/LOH is a more frequent genetic aberration in endometrial cancer than MSI. The observation of this genetic aberration in endometrial cancer suggests the presence of tumour suppressor genes. Although other researchers have observed that the non-endometrioid cancers have a higher frequency of AI/LOH compared with the endometrioid tumours, this study did not. This is probably due to the fact that most of the cases investigated were of the endometrioid type. In contrast, we found that MSI is the more frequent aberration at the *DCC* gene loci. It has been hypothesised that the AT repeat that was demonstrated to be altered in colorectal cancer could be one of the mechanisms that causes the gene inactivation in endometrial cancer as well. This study observed the frequency of MSI to be the highest in endometrioid type cancers, which is in keeping with other researchers. MSI being uncommon in the non-endometrioid tumours (serous papillary, clear cell cancers) further supports the idea that different pathogenetic mechanisms are involved in the development of the two different types of endometrial cancer. Also, at this loci we found that Asian patients have the highest frequency of MSI, whereas previous studies record White patients as being the dominant race group with this aberration. This leaves us with the notion that Asian patients could follow the less ominous pathway of endometrial cancer just as with the White patients.

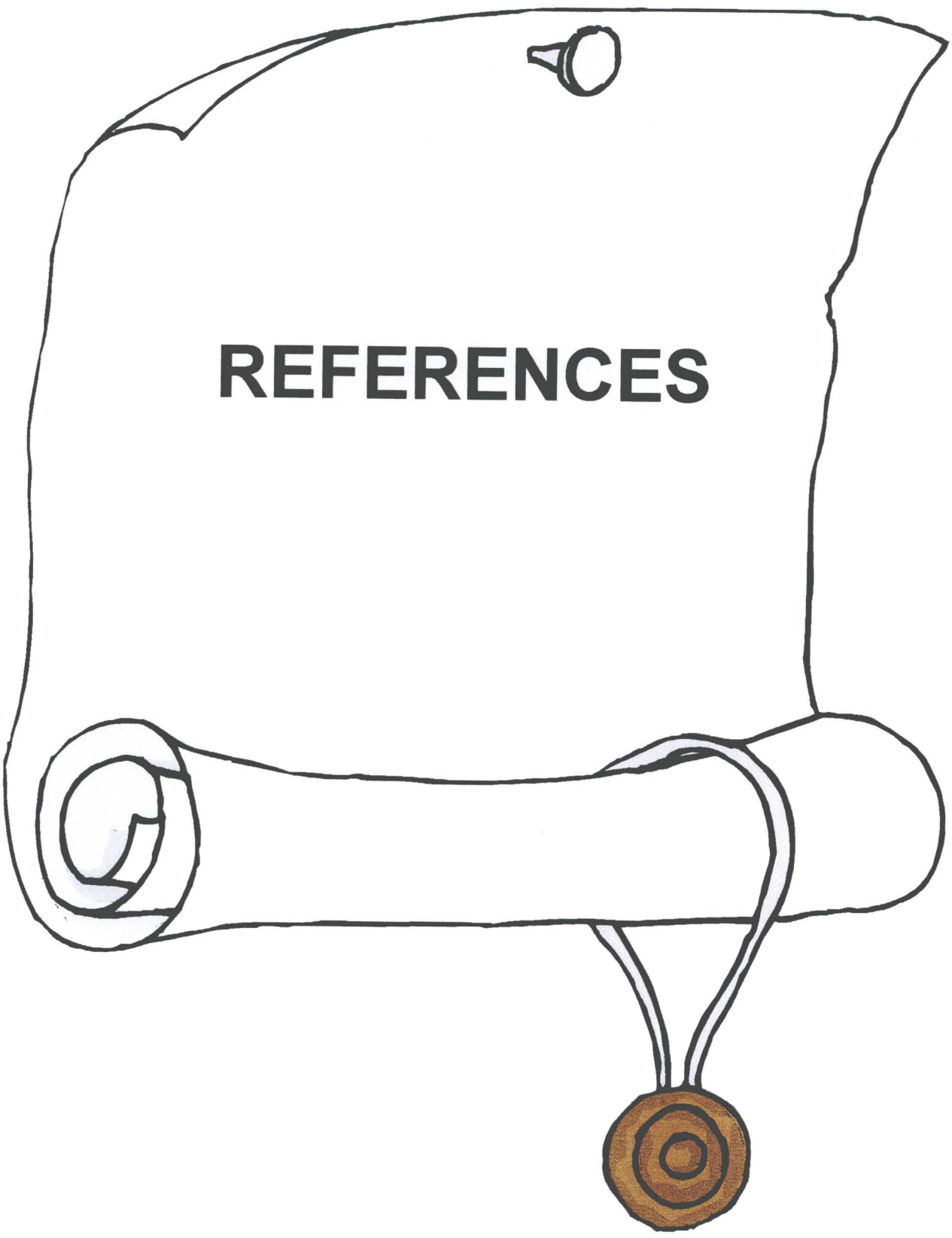
6.3 FUTURE STUDIES

- Larger study groups of endometrial carcinoma should be used, as there do not seem to be many investigations of this cancer in Southern Africa.

- An equal number of patients from all race groups in South Africa should be compared to rule out the race bias. This will provide a more representative population and enable a better understanding of the profile in all race groups.

- Further molecular studies in endometrial cancer are also necessary. Investigations into the cause of MSI in endometrial cancer should also be included.

- Other race groups like Coloureds (mixed race) and Asians should also be further observed to determine whether they follow the usual trends of the Black or White patients in endometrial cancer.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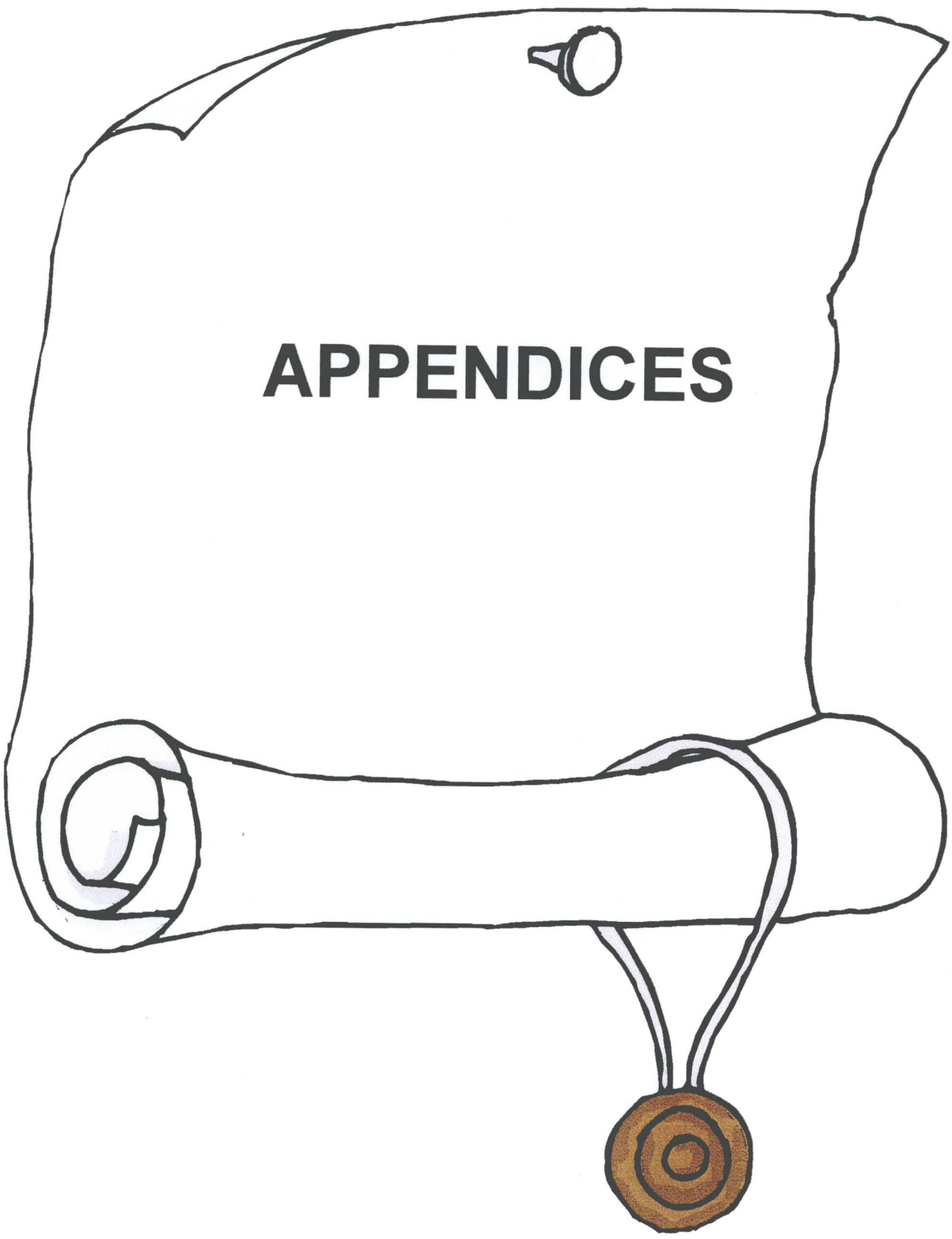
APPENDICES

APPENDIX A: PROCESSING OF TISSUES

Table 3.7: Programme on Shandon Hypercenter for the processing of tissues

Reagent	Temperature in degree Celsius °C	Time in minutes
10% Buffered formal saline	37°C	60 min
10% Buffered formal saline	37°C	60 min
95% Ethanol	37°C	60 min
95% Ethanol	37°C	60 min
95% Ethanol	37°C	60 min
100% Ethanol	37°C	60 min
100% Ethanol	37°C	60 min
100%Ethanol	37°C	60 min
Xylene Pure	37°C	60 min
Xylene Pure	37°C	60 min
Paraffin Wax	60°C	60 min
Paraffin Wax	60°C	60 min
Paraffin Wax	60°C	60 min
Paraffin Wax	60°C	60 min

APPENDIX B: MAYERS H&E STAINING METHOD AND PREPARATION OF REAGENTS

1. Haematoxylin and Eosin Staining Method

- Cut tissue sections at 3 μm on a rotary microtome, pick up on glass slides and bake on a hot plate for 5-10 mins.
- Once baked, bring down through to 2 changes of xylene, 2 changes of absolute alcohol, one change of 95% alcohol, and finally hydrate in water.
- Stain slides in Haematoxylin solution for 5-10 mins.
- Wash sections in water.
- Allow to blue in a lithium carbonate solution, for a few seconds.
- Wash well in water.
- Dip slides in 95% alcohol, and then place directly into a 0.5% Alcoholic Eosin solution for 5 mins.
- Thereafter, bring slides down through one change of 95% alcohol, 2 changes of absolute alcohol, and then two changes of xylene.
- Finally, mount slides in DPX.

Results:

i. Nuclei - Blue

ii. Connective tissue - Varying shades of pink

2. Mayer's Haematoxylin

- In a 2L conical flask dissolve 1g of haematoxylin (Merck) in approximately 950 ml of distilled water.
- To this add 50g of aluminium ammonium sulphate (Merck Lab. Supplies, Gauteng) + 0.2 g of sodium iodate (BDH Chemicals, England) + 1 g citric acid (Saarchem Halpro Analytic, Krugersdorp, RSA) + 50 g chloral hydrate (Associated Chemical Enterprises, Southdale).
- Mix well with the rest of the water to add up to 2L, and leave overnight to dissolve.
- The following morning, boil the solution for 10 mins and then leave to cool.
- Filter Haematoxylin and store at room temperature, until required.

3. 0.5 % Alcoholic Eosin

- Dissolve 20 g of eosin yellow powder (Merck) in 4L of absolute alcohol.
- Mix well.
- Add 0.5ml of glacial acetic acid to 100 ml of the eosin solution just before use.

4. Lithium Carbonate

A saturated lithium carbonate solution is made with distilled water to blue slides after haematoxylin.

APPENDIX C: PREPARATION OF COATED SLIDES AND ANTIGEN RETRIEVAL TECHNIQUES

1. Poly-L-Lysine slide preparation

- Dilute 50 ml of poly-L-lysine (Sigma Diagnostics, St Louis, USA) in 450 ml of distilled water.
- Submerge a clean rack of new glass slides into this solution for 5 minutes.
- Shake slides after removal and place on thick absorbent paper to remove excess solution.
- Allow coated slides to air-dry overnight at room temperature prior to packaging.

Note:

- The solution prepared in step 1 makes up a maximum of approximately 12 packets (50 slides in each packet) of poly-L-lysine slides.
- Employ a 30 second increment for additional racks in step 2.

2. Antigen Retrieval Solution

- Dissolve 2.46 g of tri-sodium citrate in approximately 950 ml of distilled water in a 1000 ml measuring cylinder.
- To this solution, add 400 μl of concentrated HCl.
- Add distilled water into the cylinder to make the 1000 ml mark.
- Mix solution well in the cylinder.
- This would give a 0.01 M buffered sodium citrate solution at a pH of 6.
- Check the pH of this solution prior to commencing staining.

3. Pressure Cooking Technique

- Prepare two litres of 0.01 M buffered tri-sodium citrate (antigen retrieval) solution. (step 2).
- Bring tissue sections down to water (hydrate).
- Heat antigen retrieval solution in the pressure cooker, on a hot plate.

- Stack slides in a clean stainless steel rack, and immerse into the pressure cooker once the solution is boiling, and close the lid.
- Wait until the second red ring on the knob of the lid shows.
- Time 1 minute immediately.
- Remove pressure cooker from the hot plate and place into the sink.
- Run cold tap water over the cooker until the knob descends completely.
- Remove the lid gently.
- Allow slides to cool in the pressure cooker with running tap water.
- After approximately 15-30 mins, slides are ready for staining.

APPENDIX D: PREPARATION OF SOLUTIONS FOR IMMUNOHISTOCHEMICAL ANALYSIS

1. **Phosphate Buffered Saline (PBS)** (Oxoid LTD Basingstoke, Hampshire England, IVD BR0014G, 100 tablets)

- Dissolve 10 PBS tablets in 1 litre of distilled water.
- Shake solution well before use and store at room temperature.

2. **Bovine serum albumin (BSA)** (Delta Bioproducts, JHB SA, 14-501A, 100ml)

- Dispense small quantities of commercially available BSA into tubes and store in the freezer.
- Remove when required and allow to thaw at room temperature,

3. **3% Hydrogen Peroxide** (Saarchem Unilab, Merck chemical supply, Gauteng, SA, PTX, 3063800KP 100ml)

- Add 3 ml of a liquid hydrogen peroxide (Associated Chemical Enterprises Ltd Southdale), to 97 ml of distilled water.
- Store at 4°C.

4. **37 mM Ammonium Hydroxide (Ammoniated Water)**

- Dilute 2.5 ml of 15M ammonium hydroxide solution with distilled water to a total volume of 1000 ml.
- Store at room temperature.

APPENDIX E: DNA EXTRACTION

- Scrape away normal and tumour tissue sections from slides with a sterile scalpel blade.
- Transfer tissue into 1.5 ml Eppendorf tubes.
- Add 40μl of Proteinase K and 360μl of lysis buffer to tubes to digest the tissue. (see Appendix G).
- Incubate for 48 hours at 55°C.
- Heat each tube at 95°C for 20 minutes in a heat block, to inactivate the Proteinase K
- Add 400μl of phenol-chloroform-isoamyl alcohol (see Appendix G), and vortex tubes for 30 seconds.
- Centrifuge the tubes at 12000 rpm for 5 minutes to separate the two phases.
- Add 1μl glycogen + 40μl 3M sodium acetate + 800μl cold (-20°C) absolute ethanol to a clean set of tubes, that are labelled correspondingly. (see Appendix G).
- Add the supernatant (upper aqueous phase) from the spun tubes to this clean set of tubes.
- Vortex and place in a deep freeze (-20°C) overnight.
- The following day, centrifuge the tubes at 14000 rpm for 20 minutes.
- Carefully remove the supernatant without disturbing the pellet (DNA) at the bottom.
- Wash the pellet with 75 μl of 80% ethanol (-20°C), by centrifuging for 3 minutes at 14000 rpm.
- Remove the supernatant and air-dry the pellet for 1 hour.
- Resuspend the pellet in 60 μl of sterile distilled water, and store at 20°C until required.

APPENDIX F: PREPARATION OF REAGENTS FOR DNA EXTRACTION AND MICROSATELLITE ANALYSIS

1. Lysis Buffer

- To approximately 80 ml of distilled water, add the following:
  1 ml 1M Tris-HCL pH 7.5 + 1 ml 0.5 M EDTA pH8 + 8 ml 5M NaCl.
- Top this up to 100 ml with distilled.

2. Proteinase K (20mg/ml)

- Add 100 mg of proteinase K to 5 ml of sterile water.
- Aliquot into little tubes and freeze.

3. TE Buffer (10mM Tris, 1mM EDTA) pH 7.4

- Add 0.16 g Tris-HCL + 0.04 g EDTA to 95 ml of distilled water.
- Mix well.
- Top up to 100 ml, and pH to 7.4
- Store at room temperature.

4. 50mM Tris-HCL

- Add 1.576 g Tris-HCL to 150 ml of distilled water.
- Then add 50 ml of distilled water.
- Mix and store at room temperature.

5. Phenol-chloroform-isoamyl alcohol

- Add 25 ml of phenol + 25 ml of chloroform into a dark bottle.
- To this add 330 µl isoamyl alcohol.
- Mix well.
- Allow to clear and store at 4°C.

6. 3M Sodium Acetate (pH 5.2)

- Add 12.3 g sodium acetate to approximately 40 ml of distilled water and dissolve.
- To this add 7.5 ml of acetic acid.
- Top to 50 ml with distilled water.
- Autoclave and store at room temperature.

7. Absolute ethanol

- Transfer absolute ethanol (Merck chemicals) into an autoclaved bottle.
- Store at -20°C.

8. 80% Ethanol

- Add 60 ml of sterile distilled water to 240 ml absolute ethanol (Merck) in a 500 ml measuring cylinder.
- Mix and store in an autoclaved bottle at -20°C.

9. 10x TBE

- For 1000 ml: Dissolve 108 g Tris base + 55 g Boric acid + 9.3 g EDTA in 900 ml of distilled water using a magnetic stirrer.
- Raise the volume to 1 L.
- Filter and store solution at room temperature.

10. 1x TBE

- Add 100 ml of 10x TBE to 900 ml of distilled water.
- Store at room temperature.

11. Ethidium Bromide

- Dissolve 10 mg of ethidium bromide in 1 ml of distilled water.
- Vortex, cover with aluminium foil and store at room temperature.

- Ethidium bromide has carcinogenic properties, therefore handle with extreme caution (use gloves).

12. 2% Agarose Gel

- Add 1.4 g of agarose to 70 ml of 1x TBE in a glass beaker.
- Mix together gently.
- Place tissue stopper on the mouth of the beaker.
- Microwave for 1 minute on high.
- Mix in between, microwave for another minute, until dissolved, careful not to boil solution.
- Set up casting tray for the gel.
- Position combs into the grooves of the tray.
- Gel must be warm on touch.
- Then add 2 μl ethidium bromide.
- Mix gently, avoiding bubbles.
- Pour gel carefully into casting tray.
- Remove bubbles, by pushing them away from the path of the wells with a pipette tip.
- Leave to set for 45 mins at room temperature.
- Remove combs gently.
- Store in fridge when not used immediately.

13. Bromophenol Blue Loading Dye

- Mix 0.02% bromophenol blue + 0.02% xylene cyanol + 50% glycerol.
- Store at room temperature.

14. 6% Longranger Sequencing Gel (FMC Bioproducts)

- Add 25.2 g urea (ultra-pure) to approximately 40 ml of distilled water, and dissolve on a magnetic stirrer at low speed.
- Add 7.2 ml of 10x TBE + 6 ml of longranger gel.
- Bring volume up to 60 ml with distilled water and mix well.

- Add 300 µl of 10% APS + 30 µl of Temed to 30 ml of the gel in a special pour bottle.

15. Glycogen (20mg/ml)

- Dissolve 200mg of glycogen type II (Sigma, St Louis, Missouri, USA) in 10ml of sterile water.
- Make 200µl aliquots and store at -20°C.

APPENDIX G: PROGRAMME ON PROGENE FOR THE INSULIN PCR REACTION

Table 3.8: PCR Programme on Progene for the Insulin primers:

Temperature °C	Time in minutes	Cycles	Step
95°C	5 mins	1	Denaturation
94°C	1 min	30	Denaturation
64°C	1 min	30	Annealing
72°C	2 mins	30	Elongation
72°C	10 mins	1	Final Extension
4°C			Hold/Store

APPENDIX H: PROGRAMME ON PROGENE FOR THE DCC AND MMR PCR REACTIONS

Table 3.9: Programme on Progene showing the annealing temperatures for the DCC and Mismatch Repair markers used.

Temperture °C	Time in minutes	Cycles	Step
95 °C	5 mins	1 Cycle	Denaturation
94 °C	1 min	30 Cycles	Denaturation
D18S21, D18S34 - 58 °C D18S58, D2S123, BAT 25 - 55 °C D3S659 - 52 °C	1 min	30 Cycles	Annealing
72 °C	2 mins	30 Cycles	Elongation
72 °C	10 mins	1 Cycle	Final Extension
4 °C			Hold/ Store