THE RELATIVE CONTRIBUTION OF THE p53 TUMOUR SUPPRESSOR GENE AND THE HUMAN PAPILLOMA VIRUS TO CANCER OF THE CERVIX IN BLACK WOMEN

by

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DEDICATED WITH LOVE TO MY FAMILY WITH A SPECIAL DEDICATION TO MY SISTER AMAYA

Cancer stands at what might be the meeting place, but what is in fact the no-man's land between the three disciplines of hereditary, development and infection.

Darlington, 1948

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ABSTRACT

The p53 tumour suppressor gene which is involved in the regulation of DNA replication and transcription, is reportedly mutated in approximately 50% of all human malignancies. Comparatively little information is available however, on the status of this gene in tumours of the uterine cervix, particularly in high risk populations.

In this study cervical tumours from 101 Black South African women were examined for evidence of p53 mutations using loss of heterozygosity (LOH) as a screening procedure. The technique used was based on differences in PCR amplified variable numbers of tandem repeat sequences. Tumours were also examined for the presence of the human papilloma virus (HPV) using PCR and Southern analysis. Those tumours identified as having an increased probability of harbouring a mutation, were then subjected to further analysis by PCR-single strand conformational polymorphism (SSCP), a technique which relies on conformational differences between normal and mutant single strand DNA to detect mutations.

The results revealed that only 2 out of 53 informative tumours demonstrated LOH of the p53 gene. Both these tumours were HPV negative. Using PCR-SSCP no evidence of mutations in exons 5 to 8 was detected in either of these tumours, or in any of 16 other HPV negative tumours examined. These results are in agreement with the low incidence of p53 mutations in cervical tumours of other populations reported in the literature. They also support the hypothesis that HPV E6 inactivation of the p53 protein is the predominant contributory factor to carcinogenesis of the cervix and that p53 mutations are more frequently involved in the absence of HPV infection.

Since no mutations were detected by PCR-SSCP in exons 5 to 8, it is possible that mutations could be present in other exons. These exons were subsequently analysed by Dr Tim Crook of the Rayne Institute, Kings College School of Medicine, London who reported SSCP band mobility in exons 2/3 in both cervical tumours which showed LOH.

These findings suggest firstly, that LOH as determined by the technique described in this study is a useful primary screen for mutations in the p53 gene and secondly, that Black South African women may have a novel pattern of p53 mutations in carcinoma of the cervix.

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ABBREVIATIONS

A	adenine
A_{260}	absorbance at 260 nm
AML	acute myeloid leukemia
ALL	acute lymphoblastic leukemia
APC	adenomatous polyposis coli
α	alpha
ATCC	American Type Culture Collection
β	beta
bis	bisacrylamide
bp	base pair
BPB	bromophenol blue
BSA	bovine serum albumin
C	cytosine
Ci	Curie
cm	centimetre
CML	chronic myelogenous leukemia
c-onc	cellular oncogene
CpG	cytosine-guanine doublet
°C	degrees Celsius
dATP	deoxyadenine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanidine triphosphate
DIG-ddUTP	digoxigenin-dideoxyuridine triphosphate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNA-MTase	deoxyribonucleic acid methyltrasferase
dNTP	deoxynucleotide triphosphate
dpm	disintegrations per minute
dTTP	deoxythymine triphosphate

EDTA	ethylenediaminetetraacetate
G1	gap 1
g	gram
g	gravity, accelaration due to
G	guanine
GTP	guanosine 5′-triphosphate
GTPase	guanosine 5′-triphosphatase
h	hour
HA	heteroduplex analysis
HPV	human papilloma virus
ISH	*in situ* hybridization
kb	kilobase
l	litre
LB	Luria Berti
LOH	loss of heterozygosity
mRNA	messenger ribonucleic acid
μg	microgram
μl	microlitre
μCi	microCurie
MDE	mutation detection enhancement
MEN	multiple endocrine neoplasia
μmol	micromole
mg	milligram
min	minute
ml	millilitre
mm	millimetre
mmol	millimole
mol	mole
NBT	nitroblue tetrazolium salt
NF1	neurofibromatosis type 1
nm	nanometre

OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
pH	negative log of hydrogen ion concentration
^{32}P	radiolabelled phosphorus
Rb	retinoblastoma
RE	restriction enzyme
RFLP	restriction fragment length polymorphism
RNAse	ribonuclease
s	second
S	synthesis
SDS	sodium dodecyl sulphate
SSCP	single strand conformational polymorphism
SV40	simian virus 40
T	thymine
t	translocation
Taq	*Thermus aquaticus*
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
TEMED	N, N, N′, N′-tetramethylenediamine
Tm	melting temperature
TMAC	tetramethylammonium chloride
UV	ultraviolet
v	volume
V	Volt
VNTR	variable number of tandem repeat
v-onc	viral oncogene

PREFACE

This study represents original work by the author and has not been submitted in any form to another University. Where use was made of work of others it has been duly acknowledged in this text.

The research described in this thesis was carried out in the Department of Chemical Pathology, University of Natal, under the supervision of Dr RJ Pegoraro.

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Date:	Date:

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CHAPTER 1

A REVIEW OF THE LITERATURE

1.1 GENETIC ASPECTS OF MALIGNANCY

Cancer is essentially a disorder of cellular growth. It often manifests as a mass of cells, the visible tumour being the end result of a sequence of events known as carcinogenesis. This is a complex multistep process which is believed to involve initial changes at the genetic level; these changes can be either congenital or acquired (Cotran *et al*, 1989).

In normal cells cellular proliferation is under the control of both growth-promoting and growth-suppressing genes (Goldberg *et al*, 1991). This genetic control can be lost, for example, by damage to the DNA which the cell's repair mechanisms fail to correct. Thus alterations in growth-promoting genes may result in the stimulation of tumour cell growth. On the other hand, genetic defects that inactivate growth-suppressing genes liberate the cell from the constraints imposed by these genes, and thereby may also contribute to the carcinogenic process.

In recent years the application of molecular biological techniques to the study of various human cancers has demonstrated that defects in a relatively small number of genes occur repeatedly in tumours. A deeper understanding of these genes, as well as the various types of genetic alterations that occur and their frequency and distribution, may lead to an elucidation of the aetiology and prevention of malignant tumours. Furthermore this knowledge may aid in the diagnosis and monitoring of cancer and in the future may lead to advances in therapy (Goldberg *et al*, 1991; Lee, 1991; Harris, 1993; Roth, 1993).

i CARCINOGENESIS AS A MULTISTEP PROCESS

The development of a carcinoma is generally believed to occur following a complex succession of changes in a particular cell population. This process is far from clearly understood, and there is no single model that explains the development and aberrant growth of all tumours.

The changes that occur in the transformation of normal cells to malignant cells involve several factors such as loss of regulated growth, dedifferentiation and synthesis of abnormal proteins. The process of carcinogenesis also involves several secondary events such as angiogenesis and metastasis. All these changes are now thought to be initiated at the genetic level (Craft and Harris, 1994).

Early studies on mouse skin showed that tumour development can be induced following exposure to various agents termed initiators and promoters (Berenblum and Shubik, 1947). In these experiments a single, low dose of initiating agent produced no tumours during the life span of the animal. However when the low dose of initiating agent was followed by repetitive doses of a promoting agent, high yields of papillomas and ultimately carcinomas were obtained. No tumours developed when only the promoting agent was applied or when the promoting agent was applied before the initiating agent. Among the initiators are various chemical and physical agents such as, hydrocarbons, viruses, ionizing radiation and UV light, and among the promoters are hormones and normal growth promoters (Franks, 1991). Chemicals that initiate carcinogenesis are extremely diverse in structure, but they all fall into one of two categories; direct-acting compounds, which do not require chemical transformation for their carcinogenic effect, and indirect-acting compounds which require metabolic conversion to produce carcinogens capable of transforming cells. Both types of chemical carcinogens form covalent adducts (addition products), are highly reactive and may attack several electron rich sites in the target cells, including DNA, RNA and proteins, sometimes resulting in lethal damage (Cotran *et al*, 1989).

The steps in the development of neoplasia are now attributed to defects or mutations commonly occurring in a class of genes broadly described as oncogenes (these are discussed fully in Section iii). All known oncogenes are involved to some degree in growth regulation and several oncogenes are now known to act in collaboration with one another in tumorigenic transformation. For example, oncogene products acting in the nucleus co-operate with those that act in the cytoplasm to transform normal cells

into tumour cells. This strongly suggests that each oncogene makes a unique contribution to the process of carcinogenesis. This is clearly illustrated in a recent model proposed by Fearon and Vogelstein (1990) for human colon cancer (Fig 1.1), which shows how discrete genetic changes correlate with specific steps in tumour development. The changes have been statistically associated with each step in tumour progression, and although individual tumours may take a slightly different path to the endpoint, the model demonstrates that tumour formation is the consequence of a series of genetic changes. The precise factors influencing these changes are still to be clearly elucidated. Although this model has been proposed for a specific tumour type, it is likely that most tumours arise following a similar sequence of genetic alterations, which may be the result of inherited, socioeconomic or enviromental factors.

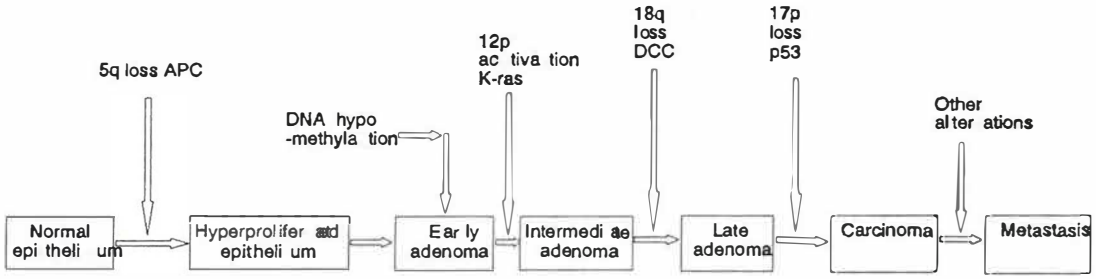

Fig 1.1. Genetic changes occurring during evolution of a typical colon carcinoma (reproduced from Fearon and Vogelstein, 1990).

ii INHERITED AND SPORADIC CANCERS

As alluded to previously, almost all types of tumours occur in both a familial and a sporadic form. In the rarer familial form which accounts for less than 5% of all cancers, the tumour itself is not inherited, but rather the genetic susceptibility to develop cancer.

Studies carried out by Knudson (1971) in both the inherited and sporadic forms of the

childhood malignancy retinoblastoma (Rb), gave rise to the 'two hit' hypothesis in which he proposed that at least two mutations are necessary for tumour formation. Knudson suggested that in the inherited form a mutation is carried in the germ line. For the disease to manifest itself, however, a second somatic mutation must subsequently occur in the other allele. On the other hand, in the sporadic form of the disease, mutations in both alleles appear to occur in somatic tissue.

Following these observations Knudson proposed a class of genes in which the mutated gene is recessive because both copies of the gene must be defective before the tumour manifests itself clinically. However upon investigation, in some instances no specific second mutation was detected, but rather a complete loss of the corresponding allele. These findings suggested that the complete absence of any normal gene product is a prerequisite for tumour formation and hence the name tumour suppressor gene was adopted. It is now believed that most inherited cancers involve genes which fall into this category.

iii ONCOGENES AND TUMOUR SUPPRESSOR GENES

At the beginning of this review it was mentioned that the development of neoplasia is attributed to alterations in a relatively small number of genes, all of which are involved in some way in the function of normal cellular growth (Lane, 1991). These genes are divided into two major categories: oncogenes (proto-oncogenes) which act in a dominant gain-of-function fashion to promote tumour growth, and tumour suppressor genes which act in a recessive loss-of-function manner.

a Oncogenes/proto-oncogenes

Oncogenes are normal regulatory genes which have undergone a mutation and consequently may contribute to tumorigenesis. Prior to a mutation they are known as proto-oncogenes. It would appear that a single mutation is sufficient to cause activation, the mutated gene acting as dominant to the wild-type allele

(non-mutated or normal allele). The dominant nature of oncogene mutations does not permit these mutations to be inherited through the germ line, since their expression in the fetus is likely to disrupt development and be lethal (Levine, 1993). A recent report however, indicates that this may not always be true for all oncogenes; a unique clearly inherited mutation in the *ret* proto-oncogene has been identified in 48 of 50 patients from 34 families with MEN (multiple endocrine neoplasia) type 2 (Lips *et al*, 1994).

Oncogenes/proto-oncogenes comprises a group of genes whose products can be divided into 5 different classes of molecules according to their function and cellular location. These are:

* growth factors eg. fibroblast growth factor related (*int-2*), platelet-derived growth factor related (*sis*).

* growth factor receptors eg. truncated epidermal growth factor receptor (*erb-B*), mutant colony-stimulating factor receptor (*fms*).

* protein kinases eg. nonreceptor protein-tyrosine kinase (*src*), truncated receptor-like protein-tyrosine kinase (*ret*).

* nuclear DNA-binding proteins or transcription factors eg. sequence-specific DNA-binding protein (*myc*), thyroid hormone receptor (*erb A*).

* G proteins eg. membrane-associated GTP-binding/GTPase (*K-ras, N-ras*), mutant-activated form of G_s (*gsp*).

All these genes form part of signal transduction pathways or serve as part of the intracellular regulatory machinery, or both.

b Tumour suppressor genes

These are normal growth regulatory genes which can undergo mutation to result in a loss of regulatory properties. Mutations in tumour suppressor genes generally cause a loss of function but act as recessive to the wild-type. This means that these genes require two independent mutations to manifest a loss of growth regulatory properties. It would appear that this most commonly arises by a mutation in one allele associated with a loss of part, or the whole of the corresponding allele. This phenomenon is known as loss of heterozygosity (LOH), loss of allele or reduction to homozygosity (Levine, 1992).

There are however several ways in which the normal allele can be inactivated. These include chromosome loss, chromosome duplication, translocation, deletions, point mutations, as illustrated in Figure 1.2.

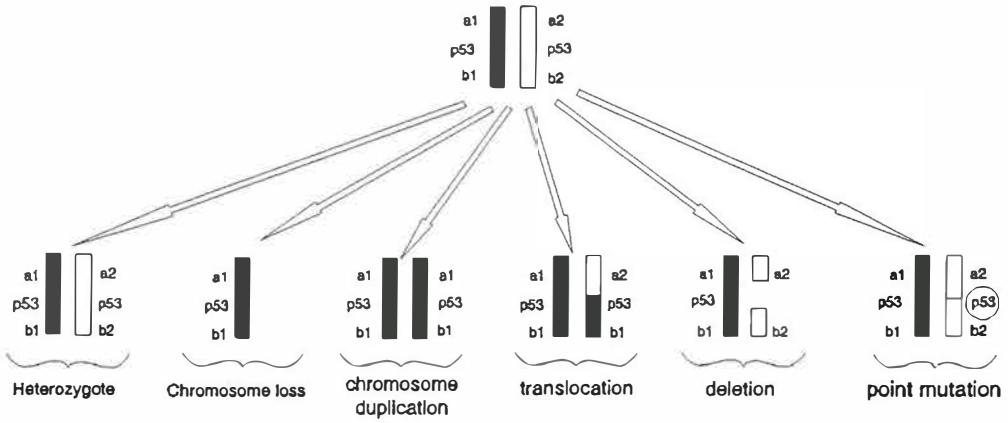

Fig 1.2. Examples of different ways in which a normal allele can be inactivated.

iv TYPES OF GENETIC ALTERATIONS

Several different types of genetic alterations can occur in malignancy. These include:

a Point mutations

This is a single base change in a specific codon which results in an amino acid substitution.

These mutations can be classified as:

* Silent mutations within a gene - these occur if a point mutation takes place at the third nucleotide position of a codon, and changes the codon but not the amino acid.

* Missense - this is a change in a codon which results in a new amino acid.

* Nonsense - this is a change of an amino acid codon into a termination codon, resulting in a truncated protein.

b Insertion/deletion

This is the addition or deletion of genetic material ranging from one base to large pieces of DNA. An insertion or deletion of a number of bases not divisible by three is known as a frameshift. Such mutations usually result in the generation of termination codons.

c Translocation

This is a rearrangement of a chromosome in which a segment is moved from one location to another, either within the same chromosome or to another

chromosome. It can also be reciprocal when one fragment is exchanged for another.

d Gene amplification

This is selective replication of a DNA sequence resulting in multiple copies of that sequence or gene.

e Viral activation

Several direct mechanisms of viral activation exist and include:

* Retroviral transduction - retroviruses contain transforming genes that are capable of inducing tumours. These viral oncogenes (v-onc) are mutated versions of their cellular counterparts (c-onc) (Sleeman, 1992).

* Retroviral insertion - many retroviruses do not contain oncogene sequences but initiate tumorigenesis by inserting viral DNA, which has been copied from the viral RNA by the enzyme reverse transcriptase into the host genome. This may alter the expression of the adjacent cellular genes, leading to point mutations, amplifications, truncations or hybrid transcripts with aberrant coding sequences (Sleeman, 1992).

Other mechanisms of viral involvement are not direct but are based on the interactions of viral oncoproteins with the proteins produced by tumour suppressor genes, for example, the interaction of the E7 oncoprotein of the human papilloma virus (HPV) with the product of the Rb gene (Lee, 1991). This is discussed in more detail in Section 1.3.

f Methylation

DNA methylation is the transfer of methyl groups to cytosine residues on the cytosine-guanine doublet (CpG) by an enzyme called cytosine DNA methyl

transferase (DNA-MTase) to form 5-methylcytosine. This process is known to play a role in the regulation of gene activity (Goodman and Counts, 1993) by providing a chemical signal that is recognised by transcriptional regulation factors.

In normal cells, the function of DNA methylation may be to ensure that areas of transcriptionally active chromatin replicate earlier than the transcriptionally inactive chromatin. Thus any alteration in DNA-MTase may create changes in the methylation status and alter the expression of the gene. Global DNA hypomethylation has been observed in some human neoplasms and has been implicated as an important factor in carcinogenesis (Kim *et al*, 1994).

v GENETIC ALTERATIONS IN SPECIFIC CANCERS

A number of cancers both familial and sporadic, have been shown to have specific aberrations. Amongst the familial forms are: alterations on a gene located on chromosome 11p13 producing *Wilm's tumour* of the kidney which occurs in infants and young children (Lee, 1991). Mutations on a gene located on the long arm of chromosome 17 are responsible for *neurofibromatosis type 1 (NF1),* which is associated with an increase risk of malignancies, principally neurofibrosarcoma and optic glioma (Vile, 1992). Alterations on a gene known as APC located on chromosome 5 commonly occur in *familial adenomatous polyposis,* a disorder in which affected individuals develop multiple colonic and rectal polyps. Alterations in loci mapped to the long arm of chromosome 11 and to the pericentromeric region of chromosome 10 produce respectively, *multiple endocrine neoplasia types 1 and 2A*: this disease includes disorders with hyperfunction of two or more endocrine tissues. Alterations on the p53 gene, which is located on the short arm of chromosome 17 are responsible for a rare familial cancer called *Li-Fraumeni syndrome* characterized by the occurrence of diverse mesenchymal and epithelial neoplasms at multiple sites. The spectrum of cancers in this syndrome include breast carcinomas, soft tissue sarcomas, brain tumours,

osteosarcomas, leukemia, adrenocortical carcinoma, and possibly other tumours types such as melanoma (Lee, 1991). Alterations on two genes namely, BRCA1 and BRCA2 located on chromosome 17q21 and 13q12, respectively, are reported to be involved in familial *breast cancers* (Futreal *et al*, 1994; Miki *et al*, 1994; Wooster *et al*, 1994).

Sporadic cancers are also linked to specific mutations. Although defects in the p53 tumour suppressor gene are reported to be the most commonly occurring genetic abnormality in all cancers in general, clearly differing frequencies of p53 involvement are observed in different tumour types. For example, the frequency of mutations of the p53 gene is reported to be 33% in breast carcinomas (Sommer *et al*, 1992), 61% in hepatocellular carcinomas (Hsia *et al*, 1992) and 72% in ovarian carcinomas (Kohler *et al*, 1993).

Amplification of the *neu* (also called *c-erbB-2* or *HER-2*) proto-oncogene is reported in approximately 20% of all breast tumours and some ovarian tumours but is not widely observed in other malignancies. Similarly, amplification of the *mdm-2* (murine double minute) oncogene on chromosome 12q is reported in approximately 30% of all soft tissue sarcomas (Harris and Hollstein, 1993).

Translocations are common occurrences in certain types of leukaemias and lymphomas such as the t(9:22) crossover which results in the generation of the Philadelphia fusion gene (Russell, 1992). Several other examples exist, such as, the t(8:21) crossover in acute myeloid leukaemia (AML), the t(4:11) in acute lymphoblastic leukaemia (ALL) (Hoffbrand and Pettit, 1994) and the t(8:14) in Burkitt's lymphoma (Wolfe, 1993). Translocations are also frequent in soft tissue sarcomas (Sreekantaiah *et al*, 1994).

vi POSSIBLE CLINICAL USEFULNESS

The knowledge of the precise defects that occur in different tumours holds potential for future intervention in several ways:

a Aetiology

Studies on cancer-related genes have revealed some of the possible environmental and endogenous molecular processes that may lead to carcinogenesis. For example, specific p53 mutations at codon 249 in hepatocellular carcinoma, commonly occur in patients residing in high-risk regions of the world (China, Mozambique) where there is high intake of dietary aflatoxin B1 and increased risk of infection with the hepatitis B virus (Hsu *et al*, 1991; Hsia *et al*, 1992). Information of this type might be useful in prevention.

b Diagnosis

Studies of some genes, particularly those involved in haematological tumours are being used to aid diagnosis, monitor response to therapy and detect minimal residual disease. Examples include the t(9:22) translocation in CML (chronic myelogenous leukaemia) and the t(4:11) translocation in ALL (Russell, 1992). In cancer of the breast, *c-erbB2* amplification has been associated with more aggressive tumour growth and is now being used to identify subgroups of patients with a particularly poor prognosis (Russell, 1992; Knyazev *et al*, 1993; Muss *et al*, 1994).

In familial cancers detection of germline mutations has obvious uses in prenatal diagnosis and counselling and in the identification of individuals at risk (Malkin, 1994; Russell, 1992).

c Future developments

The frequent failure of currently available cancer therapies is largely attributable to two characteristics of human malignancies; tumour cell heterogeneity and genetic instability of the malignant clone. Combination therapies targeted to the various steps in the transformation process of carcinogenesis may be invaluable.

There are several possible approaches to therapy, for example antisense oligonucleotides targeted to mRNA, proteins as targets for specifically designed antibodies, and corrective gene therapy (Roth, 1993; Tari and Lopez-Berestein, 1993).

1.2 THE p53 TUMOUR SUPPRESSOR GENE

The p53 tumour suppressor gene is one of the main foci of this thesis and will therefore be reviewed in detail here.

i HISTORY

Mutations in the p53 gene are reported to be the most common genetic alteration in human cancers. Yet until recently, little was known about the functions of the p53 gene product.

The nuclear p53 protein was originally reported to form a complex with the simian virus 40 (SV40) oncoprotein, the large T antigen, in cells infected by this virus. Because large T antigen is needed to maintain the transformed cells, it was suggested that this interaction was important for transformation. The p53 protein was therefore described as a tumour antigen (Eliyahu *et al*, 1984).

The gene encoding p53 was later shown to transform rodent cells *in vitro*, either alone or in co-operation with an activated *ras* gene (Eliyahu *et al*, 1984; Lamb and Crawford, 1986). These transfection experiments, together with the demonstration that p53 gene expression was increased in a variety of chemically, virally and genetically transformed human and rodent tumour cells (Nigro *et al*, 1989) suggested that the p53 gene was an oncogene that could exert its growth-promoting effects if expressed at high level. At this point the p53 gene was classified as an oncogene.

However subsequent attempts to repeat these experiments were unsuccessful and it was realized that the early transformation studies were performed with a mutated form of the p53 gene. Further studies showed that the normal p53 gene behaves as a tumour suppressor gene, regulating the cell cycle and requiring to become mutated in order to lose these regulatory properties. At the same time mutations and deletions of various p53 alleles in several human tumours were reported (Levine *et al*, 1991). Thus convergent lines of research indicated that the wild-type (normal) p53 gene functions as a tumour suppressor gene and today it is recognised as such.

ii STRUCTURE

The p53 gene comprises 20 kb of DNA and is located on the short arm of human chromosome 17, at position 17p13.1. The gene contains 11 exons, the first of which (213 bases) is noncoding. A notable feature is that intron 1 is 10 kb long, which is approximately 50% of the entire gene (Lamb and Crawford, 1986). Little is known about the nucleotide sequences regulating the transcription of this gene. The mRNA for p53 is expressed in all cells of the body with the highest levels being found in the spleen and thymus (Levine, 1993).

iii p53 PROTEIN

The protein encoded by the p53 gene comprises 393 amino acids in humans and is a nuclear phosphoprotein. The protein is composed of three functional domains: an alpha-helical stretch of 75 amino acids at the amino-terminal end, followed by a hydrophobic region rich in proline residues (codons 75-150) and finally, at the carboxy-terminal end of the protein (codons 319-393), a basic region of alpha helix conformation. The regions of the protein that frequently carry activating mutations are spread through the protein in certain 'hot-spots'. These are at codons 111-136, 165-175, 230-252 and 264-280. The functional significance of these areas of the protein is not fully described but they show a high degree of conservation between p53 proteins of different species (Levine, 1993).

iv FUNCTION

The p53 protein exerts its growth regulatory properties in several ways:

a Cell cycle arrest

Studies have shown that the wild type p53 protein introduced directly into tumour cells arrests growth of the tumour cells in the late G1 phase of the cell cycle. This allows the cell sufficient time to repair damage. The role of p53 is therefore, postulated as a monitoring protein that determines whether the cell enters the S phase to continue through the cell cycle. It is reported to be synthesised in response to DNA damage, and has important implications in the maintenance of the cell's genetic stability (Donehower, 1994).

b Transcriptional regulation

The p53 protein binds to specific DNA sequences to act as a transcription factor in regulating the expression of particular genes which include the p21 and GADD45 (growth-arrest-and-DNA-damage inducible) genes. The p53 protein may halt DNA synthesis through p21 allowing time for the damage to be repaired. It would appear that p53 can also stimulate the repair mechanism through the GADD45 gene which is found in cells exposed to stress (Marx, 1994).

c Apoptosis

Apoptosis, or programmed cell death, was first described in the 1970s (Lane, 1993). Cells that do not have the ability to undergo apoptosis continue to grow uncontrolledly. Furthermore, defects in genes that regulate this event hold a double threat for the cell: as well as the absence of normal cell death, they can also confer tolerance to chemotherapy and radiotherapy.

A number of experiments support a role for p53 in apoptosis. It has been demonstrated that wild-type p53 mediates apoptosis of both normal and tumour

cells. This is possibly a defence mechanism to protect the organism against cells that have undergone transformation (Cohen, 1993).

Other evidence for the role of p53 in apoptosis was obtained following ionizing radiation experiments, in which cells from mice containing wild-type p53 entered apoptosis after treatment with ionizing radiation (Lane, 1993). By contrast, cells with two defective p53 alleles did not undergo apoptosis following similar treatment (Donehower, 1994). It would also appear that DNA damage that is incapable of being properly repaired may induce a p53-mediated apoptosis program and has led to the concept that p53 might be part of a damage-control pathway in addition to a normal-control pathway (Lane, 1993).

Further studies suggest that p53 may also induce apoptosis in response to an imbalance in growth signals. For example, if positive growth signals are activated through *c-myc* oncoprotein deregulation in the absence of appropriate growth factors, negative growth regulators such as p53 may detect the resulting imbalance, and induce an apoptotic pathway (Lane, 1993).

v DETECTION OF p53 MUTATIONS

A large variety of human tumours are reported to contain a mutated p53 tumour suppressor gene. The majority of these mutations are found in 4 highly conserved regions of exons 5 to 8 (Hollstein *et al*, 1991).

Several different methods of detection have been developed. The definitive method is direct nucleic acid sequencing, but this can be laborious and expensive when large number of samples are involved, even with the use of an automatic sequencer. There are however several indirect methods which can be used as screening techniques. Amongst these are:

a *The detection of loss of heterozygosity (LOH)*

As mentioned previously (Section 1.1 iii), mutations in tumour suppressor genes are commonly associated with the loss of the corresponding allele. This loss is apparently necessary for carcinogenesis, reflecting the recessive nature of these genes. About 80% of mutations in the p53 gene are thought to be associated with LOH. Thus this technique is a useful means of screening for mutations in the p53 gene.

To detect loss of heterozygosity paired samples of DNA from blood and tumour are compared to identify loss of allele in the tumour DNA. The methodology is described fully in Chapter 3.

b *Single strand conformational polymorphism (SSCP)*

This technique relies upon the fact that, under non-denaturing conditions, single-strand DNA molecules carrying mutations fold into unique conformations (Hongyo *et al*, 1993), which under appropriate electrophoretic conditions migrate differently to wild-type DNA. This technique is described in Chapter 5.

c *Heteroduplex analysis (HA)*

This method is based upon the observation that a hybrid between two single-stranded DNA molecules with sequences which differ from each other by a single nucleotide (heteroduplex), has an altered conformation. This may be detected as a reduction in electrophoretic mobility on a denaturing gel (Ganguly *et al*, 1993).

Several other methods exist for the detection of mutations, such as restriction site analysis, band shift assay, the use of allele-specific oligonucleotide primers and probes. To make use of these techniques however, the precise location and nature of the mutation must be known.

vi RELATIONSHIP OF p53 TO VIRAL ONCOPROTEINS

The p53 protein has been shown to interact with several viral oncoproteins including the SV40 large T antigen, the E1A adenovirus and the E6 protein product of the HPV types 16 and 18 (Levine, 1993). As discussed previously (Section 1.2 i), the inactivation of a normal suppressor gene protein by complexing with viral oncoproteins is believed to play an indirect role in carcinogenesis of certain tumours, particularly those of the uterine cervix. This relationship between the p53 gene and its product, the HPV and cancer of the cervix is discussed in greater detail in the following section.

1.3 CANCER OF THE CERVIX AND HUMAN PAPILLOMA VIRUSES

There is overwhelming evidence that human papillomaviruses are involved in the aetiology of cervical cancers, with HPV DNA being reported in approximately 70 to 90% of all cervical tumours (Levine, 1992; Jha, 1993).

Furthermore it is recognised that HPV infection of the cervix, specifically the HPV 16 and 18 types, progresses to malignancy in a significant number of patients. It was estimated following a study of 530 women from Finland, that 80% of women with HPV 16 infection finally progressed to malignancy (Jenkins, 1994).

The nature of this relationship between HPV and cancer of the cervix has been better understood recently with the demonstration that the E6 oncoprotein product of the HPV 16 and 18 types binds to wild type p53 protein (Scheffner *et al*, 1990). The complex formed results in an altered half life of the p53 protein (Band *et al*, 1993) and in addition, targets the p53 protein for attack by proteolytic enzymes (Scheffner *et al*, 1990). The result is the functional inactivation of the p53 tumour suppressor protein. As a consequence, it has been hypothesized that cervical tumours which contain HPV 16 and 18 DNA are less likely to have mutations in the p53 tumour suppressor gene (Scheffner *et al*, 1990).

This concept has been supported by cell line studies, in which mutant forms of the p53 gene were found only in those cervical cells without HPV DNA. Investigations carried out by Crook *et al* (1992) and Scheffner *et al* (1991) revealed that two cervical carcinoma cell lines, C33a and HT3, in which HPV DNA was not detected, contained mutations in codon 273 and codon 245 respectively. These studies were confirmed by Iwasaka *et al* (1993).

It may be that in tumours of the cervix, allele loss and/or mutations in the p53 gene are a rare event, and have little role in the carcinogenic process, in contrast to other malignancies. This hypothesis has been addressed by a handful of researchers (Crook *et al*, 1992; Kaelbling *et al*, 1992; Busby-Earle *et al*, 1993; Paquette *et al*, 1993). For example, Crook and his associates, analysed tumour tissue samples from 28 women with primary cervical tumours for the presence of HPV sequences and for somatic mutations of the p53 gene. Twenty five of the tumours contained HPV sequences without any evidence of p53 mutation. Point mutations were detected in the remaining three HPV negative tumours. Similarly Paquette *et al* (1994) undertook a study of 42 cervical carcinomas, 42 of which were HPV positive with normal p53. A missense mutation was detected in one of the three HPV negative tumours. Kaelbling *et al* (1992) analysed 27 cervical carcinomas for the presence of HPV and showed that 21 had HPV infection. LOH of the p53 gene was found in 4 of the 27 cases, two of which had no evidence of HPV.

To date there would appear to be a degree of consensus concerning the likelihood that

p53 mutations are not common in carcinoma of the cervix. However, these findings are based on very small numbers of patients and therefore require confirmation. Furthermore, the complete absence of information on the p53 gene in populations groups known to be at high risk for cancer of the cervix, such as Black women of Southern Africa (Cooper *et al*, 1991), suggests that further studies are essential.

1.4 INTRODUCTION TO THE PRESENT STUDY

This review of the literature on the genetic aspects of malignancy has revealed several salient features. Firstly, the p53 tumour suppressor gene appears to be of major significance in the maintenance of normal cellular growth and control. There have been numerous reports of genetic alterations of the p53 gene in a large variety of human tumours including those of the colon, rectum, lung, breast, brain, bone, and bladder.

The second point to emerge from the review, was the fact that while p53 involvement has been extensively studied in some tumours, others, more specifically tumours of the uterine cervix have been largely overlooked. The high prevalence of this cancer in the Black South African population makes this malignancy a particular focus of interest.

Thirdly, it would appear that with the exception of one study reporting variations in p53 mutations in a cohort of Black Americans with breast cancer (Blaszyk *et al*, 1994), no studies on the p53 gene have been carried out in racial groups other than whites and Japanese.

Given these three factors, a study was undertaken on the prevalence of p53 abnormalities in carcinoma of the cervix amongst Black South African women. At the same time an analysis was made of the HPV status and the specific type identification

of HPV present in the tumours in the study group.

The study was approached in the following way:

a Examination of tumour and blood DNA for loss of heterozygosity of the p53 gene as a primary screen for mutations.

b Examination of the tumours for HPV 16 and 18 types.

c Analysis by PCR-SSCP of DNA from tumours showing LOH as well as HPV negative tumours as a secondary screen for mutations.

d Evaluation of all results to assess the relative contribution of p53 mutations and HPV infection to cervical carcinogenesis.

CHAPTER 2

MATERIALS AND METHODS

2.1 INTRODUCTION

The purpose of this chapter is to describe the principles and practical details of the molecular biological techniques used in this study. Specific details of the more complex methodologies such as the polymerase chain reaction (PCR) and single strand conformational polymorphism (SSCP) are described in the relevant chapters.

2.2 CLINICAL SAMPLES

i COLLECTION AND STORAGE

Tumour tissue was collected at surgery. Samples were placed on ice for transport to the laboratory, and then stored in liquid nitrogen (-196°C) until isolation of DNA. All tumour samples were histologically confirmed on routine analysis.

Blood was collected into tubes containing EDTA in the post-operative period and stored as whole blood at -20°C.

ii ETHICAL PERMISSION

All specimens were obtained in compliance with the Ethics Committee of the Faculty of Medicine of the University of Natal. Verbal consent for the use of samples for analysis was obtained from patients.

2.3 LABORATORY METHODS

i ISOLATION OF DNA

There are several methods available for the extraction of DNA from a variety of sources. The choice of method is dependent largely on the nature of the source, and on the use to which the DNA is to be put. When DNA of high purity is required, as in the case of cloning procedures, the classical phenol/chloroform extraction method is recommended (Sambrook *et al,* 1989b). However other procedures, for example PCR, do not require very pure DNA, reducing the need for extensive purification. In some cases a rapid alkaline boiling method can be used (Williamson B, personal communication).

Principle

The DNA isolation procedure chosen for this study is a modification of a salting out procedure (Miller *et al*, 1988) which involves the use of a strong salt solution such as 6 mol/l NaCl. The technique is based on the fact that DNA, unlike proteins, is soluble in high concentrations of salt. The cell homogenate is suspended in this solution, the proteins removed by centrifugation and DNA is recovered from the supernatant. This method was selected because with appropriate modifications it can be used to isolate nucleic acids from different sources. In addition it yields DNA which can be used for both PCR amplification and hybridisation techniques.

Procedure

Extraction of DNA from tumour tissue

Proteinase K was used to deproteinise tumour tissue homogenates. The steps involved in this extraction procedure were:

a Frozen tumour tissue (approximately 100 mg) was finely cut with a razor blade and lysed with RNAse (15 μg/ml), proteinase K (15 μg/ml) and 10% SDS for 24 h at 37°C, or 5 h at 65°C. The tubes were shaken from time to time, until all the tissue was disrupted.

b 6 mol/l NaCl was added to the tissue digest to a final concentration of 1 mol/l and shaken gently for 10 min at 24°C.

c The mixture was centrifuged at 2500 *g* for 15 min at 24°C to remove the proteins.

d The supernatant was extracted with a 1:1 volume of chloroform-isoamylalcohol, and centrifuged at 2000 *g* for 15 min at 4°C.

e The supernatant was transferred to a glass tube and the DNA precipitated using isopropanol in a proportion of 1:1.

f The DNA strands were removed using a bent Pasteur pipette, washed in 70% ethanol and redissolved in TE buffer. The buffer comprises 10 mmol/l Tris-HCl; 1 mmol/l EDTA (pH 8).

g The concentration of DNA was determined (see Section 2.3 ii), and aliquots were stored at either -20°C or -70°C. Storage at -70°C, especially for longer periods was preferred because it avoided the possibility of pockets of unfrozen liquid of high salt concentration which can damage the DNA.

Extraction of DNA from whole blood

The extraction of DNA from blood was carried out as follows:

a Equal volumes of **fresh** blood and solution A (6 g dextran T70 in 0.9% NaCl and 200μl of 0.5 mol/l EDTA per 10 ml of blood) were added to narrow plastic or glass tubes. These were left to stand at an angle of 45° for 1 h at ambient temperature or 20 min at 37°C.

b **Frozen** blood was thawed at 37°C and diluted with an equal volume of 0.9% NaCl.

c The blood (**fresh or frozen**) mixture was washed twice with 0.9% NaCl by centrifugation at 3500 *g* at 4°C for 15 min.

d The white cell pellet was then resuspended in 2 ml 0.9% NaCl, 2 ml 0.5 mol/l EDTA (pH 8) and 880 μl mol/l $NaClO_4$ and mixed well.

e To this was added 0.4 ml of 10% SDS and mixed gently. The solution of lysed cells was left to stand for 10 min.

f Five ml of a 24:1 chloroform-isoamylalcohol mix was added. The two phases were mixed by shaking slowly for 15 min; if the phases did not form an emulsion, the tubes were shaken mechanically for 1 h.

g The two phases were separated by centrifugation at 5000 *g* for 10 min at 4° C.

h The viscous aqueous phase containing the DNA was transferred to a polypropylene tube, and the extraction repeated with 5 ml of chloroform.

i The DNA solution was centrifuged at 5000 *g* for 15 min at 4° C to precipitate the proteins. The supernatant was transferred to a glass tube, and DNA precipitated by adding 1:1 isopropanol.

j The DNA was washed using 70% ethanol and redissolved in TE buffer.

k The DNA concentration was determined (Section 2.3 ii) and aliquots stored at -20°C or -70°C.

ii MEASUREMENT OF DNA

Principle

The UV absorbance peaks of nucleic acids and proteins are at 260 nm and 280 nm, respectively; the absorbance at 260 nm reflects the concentration of nucleic acid in the sample, while the absorbance at 280 nm the amount of protein present in the DNA solution. A ratio of these two readings gives an estimate of the amount of impurities, a ratio greater than 1.8 generally being considered acceptable (Wenham, 1992).

It is estimated that double stranded DNA at a concentration of 50 μg/ml and single stranded DNA at a concentration of 33 μg/ml each have an OD of 1. These values are used to calculate the concentration of DNA in any solution (Sambrook *et al*, 1989).

Procedure

The absorbance of a diluted solution of DNA (usually 1:100) was measured in a Beckman DU®-5 spectrophotometer (Beckman Instruments Inc, Fullerton, California) at the wavelengths 260 nm and 280 nm. The ratio of these two readings was calculated and the concentration of DNA estimated as follows:

A_{260} x 50 μg/ml DNA x dilution factor.

The final concentration was expressed as μg/μl.

iii SEPARATION AND DETECTION OF DNA

It is frequently necessary to separate and detect different DNA fragments, either for identification or isolation. This is generally performed using electrophoretic techniques. Two common supports are used, namely agarose and polyacrylamide, and are selected on the basis of the size of the DNA fragment to be detected and the resolution required.

a Agarose gel electrophoresis

Principle

At neutral pH, DNA is negatively charged and in the presence of an electrical current, DNA loaded into a well at the cathode end of a gel moves through the gel towards the anode. The electrophoretic mobility of DNA fragments in agarose is dependent on fragment size and is fairly independent of base composition or sequence. The resolving power of an agarose gel is a function of its concentration (Sambrook *et al*, 1989e), with the concentration being inversely proportional to the size of the DNA fragment to be detected.

Procedure

Electrophoresis was carried out using the Hoefer Mighty Small (Hoefer Scientific Instruments, San Francisco, California) horizontal apparatus. The running buffer was a Tris-borate (TBE) buffer made up as a 5 x concentrated stock solution and diluted to a 0.5 x working solution. The 5 x TBE buffer was prepared as follows: 0.4 mol/l Tris; 0.4 mol/l boric acid; 1 mmol/l EDTA (pH 8). The loading buffer was 2 g Ficoll; 3.2 ml 0.5 mol/l EDTA (pH 8); 20 mg bromophenol blue; made up to 8 ml in 0.5 x TBE.

The gel was cast by melting the appropriate amount of agarose in TBE buffer (in a microwave oven) until a transparent solution was obtained. The melted

solution was first cooled to approximately 50°C, to prevent damaging the gel tray, then poured into a mould and allowed to set for approximately 30-45 min.

After removal of the comb, the gel submerged in running buffer was loaded with the required amount of DNA preparation or PCR product mixed with 2 μl loading buffer. The Ficoll in this buffer makes the DNA solution more dense so that it sinks to the bottom of the well, and the bromophenol blue is used as a tracking dye. In addition, an appropriate molecular weight marker was included in every run. The gel was run at a constant voltage of 8 V/cm at room temperature until the dye front reached the bottom of the gel. After electrophoresis the gel was viewed and documented as described below.

Staining and imaging of the gel

The most convenient method to visualise DNA in an agarose gel is staining with the fluorescent dye ethidium bromide. This substance intercalates between the bases, binding to DNA and this is visualized as a fluorescent band under ultraviolet radiation (Sambrook *et al*, 1989e).

Ethidium bromide was prepared as a stock solution of 10 mg/ml in water, and kept in bottles wrapped in aluminium foil in a fume cupboard at room temperature. After electrophoresis was completed, the gel was immersed in water containing ethidium bromide (0.5 μg/ml) for 30-45 min at room temperature. The gel was destained by soaking in water for 10 min at room temperature; this reduced the excess of background fluorescence caused by unbound ethidium bromide.

Alternatively the dye was incorporated into the gel and/or the electrophoresis buffer at a concentration of 0.5 μg/ml. This allowed examination of the gel directly under ultraviolet light during or at the end of the run, but resulted in a loss of resolution.

Initially, gels were photographed on a UV transilluminator after a 5 s exposure using a Polaroid CU-5 Land Camera and polaroid film type 667 (camera settings: speed t=1/8; aperture f:11).

Subsequently a GDS 5000 Gel documentation system (Ultraviolet Products, Ltd), attached to a Sony thermal printer (Ultraviolet Products Ltd) and a computer was used. The optimal camera settings were an 8.5 mm lens with a f 1.5 aperture and a 16 mm UV filter. Once the image was captured on the screen it was enhanced if necessary and a text annotation added using the Imagestore 5000. The image was then printed and/or stored on to a computer disk. The stored images could then be analyzed, if required, using a gelbase software package (Gelbase/Gelblot™Windows Software, UVP Ltd).

b Polyacrylamide gel electrophoresis (PAGE)

Principle

Polyacrylamide gels are formed by the polymerization of acrylamide monomers in a chain reaction, and the linking of these chains with N,N′-methylenebisacrylamide (bis). The polymerization is catalysed by ammonium persulphate and stabilised by TEMED (N,N,N′,N′-tetramethylenediamine) (Smith and Nicolas, 1983). When the bifunctional agent N,N′-methylenebisacrylamide is included in the polymerization reaction, the chains become cross-linked to form a gel, the porosity of which is determined by the length of the chains and the degree of cross-linking. The pore size of the resultant three dimensional network is determined by the concentration of acrylamide and the ratio of acrylamide to bis.

Polyacrylamide gels are more tedious to prepare and run than the agarose gels, but PAGE is particularly useful for the separation of small fragments of DNA.

Procedure

PAGE was carried out using the Hoefer Mighty Small® (Hoefer Scientific Instruments, San Francisco, California) vertical electrophoresis apparatus. A spacer thickness of 0.5-0.75 mm was found to be most suitable in terms of resolution. The bottom of the assembled gel mould was sealed with 2% agarose before pouring the gels, to prevent leakage of the acrylamide solution.

A series of experiments using different polyacrylamide concentrations was carried out to find the concentration that showed an optimal resolution for each application. A 30% polyacrylamide stock solution was made up with 29 g of acrylamide and 1 g of N′-N′-methylenebisacrylamide dissolved in 100 ml of water and the mixture was filtered and stored at 4°C in a dark bottle. The TBE buffer was made up as described previously in section 2.3 iii and 10% ammonium persulphate was freshly prepared before use. Just prior to pouring the gel, 5.5 µl of TEMED was added to the solution and mixed. Using a pasteur pipette the solution was transferred into the gel mould. The volume of reagents used to cast a 18% polyacrylamide gel are given below as an example.

Reagents	Volume	
30% polyacrylamide (stock)	3	ml
TBE 5 x	1	ml
distilled water	1	ml
10% ammonium persulphate	35	µl
TEMED	5.5	µl

The gel polymerised in approximately 30 min at room temperature. The comb

was removed and the wells were washed to remove any remaining acrylamide solution which could produce distorted DNA bands. The gel was attached to the electrophoresis tank and the reservoir of the tank was filled with 1 x TBE buffer.

The reaction mixture containing the sample and the appropriate loading buffer (0.25% xylene, 0.25% bromophenol blue, 40% sucrose in 0.5 x TBE buffer) was loaded into the wells using a Hamilton syringe. The gel was electrophoresed at the required voltage and temperature for the appropriate time.

The gels were then stained with ethidium bromide and documented as described in the previous section.

iv RESTRICTION DIGESTION

Principle

Genomic DNA can be cumbersome and it is often necessary to work with more manageable fragments of DNA. These can be generated by cutting the DNA with bacterial enzymes known as restriction endonucleases (REs). These enzymes bind to and cleave double stranded DNA at specific sites within or adjacent to a particular sequence known as a recognition site, which may be from 4 to 7 bases long. Over five hundred different restriction endonucleases have been characterized and purified for use (Brown, 1992b).

Restriction enzymes are very useful in the analysis of differences occurring in the human genome; modifications of bases in the genome may produce new restriction sites or remove pre-existing ones. This variability is inherited and the restriction fragment length polymorphisms (RFLPs) generated by these sites, provide a large number of markers for tracking mutant genes that may be involved in disease.

Restriction enzymes have a number of other uses, for example, isolation of cloned DNA from vectors, cleavage of genomic DNA for hybridisation procedures, restriction mapping of DNA, preparation of DNA for cloning (Brown, 1992a).

Procedure

Samples of DNA were restricted with the appropriate enzyme for the purpose required. In most instances 5 μg of DNA were digested using the concentration of enzyme and reaction buffer recommended by the manufacturer. Care was taken in diluting the enzyme so that the final glycerol concentration was no greater than 5% (v/v), to prevent the inhibition of the enzyme activity (Sambrook *et al*, 1989c). The digestion reaction was usually carried out at 37°C overnight.

The reaction was terminated by the addition of EDTA (0.5 mol/l) and the restricted DNA fragments were then separated on agarose gel as described in Section 2.3 iii.

v POLYMERASE CHAIN REACTION

Principle

The PCR was developed in 1987 by Kary Mullis in an effort to imitate the replication of genetic material which occurs in nature when cells divide (Wolfe, 1993). The technique is based on the *in vitro* amplification of a specific nucleic acid fragment of defined length and sequence. It depends upon the principle that when DNA is heated above a certain temperature, its two component strands separate and that when cooled, the strands come together and anneal. With this procedure amplification of $>10^6$ fold can be obtained from very small amounts of template. In practical terms this is achieved by means of two flanking oligonucleotide sequences called primers (20-30 bases in length), and repeated cycles of amplification in the presence of the enzyme DNA polymerase and deoxynucleotide triphosphates (dNTPs) (Saiki, 1990). It has been possible to automate this procedure with the use of a thermostable enzyme isolated from the bacterium *Thermus aquaticus*, namely Taq DNA polymerase.

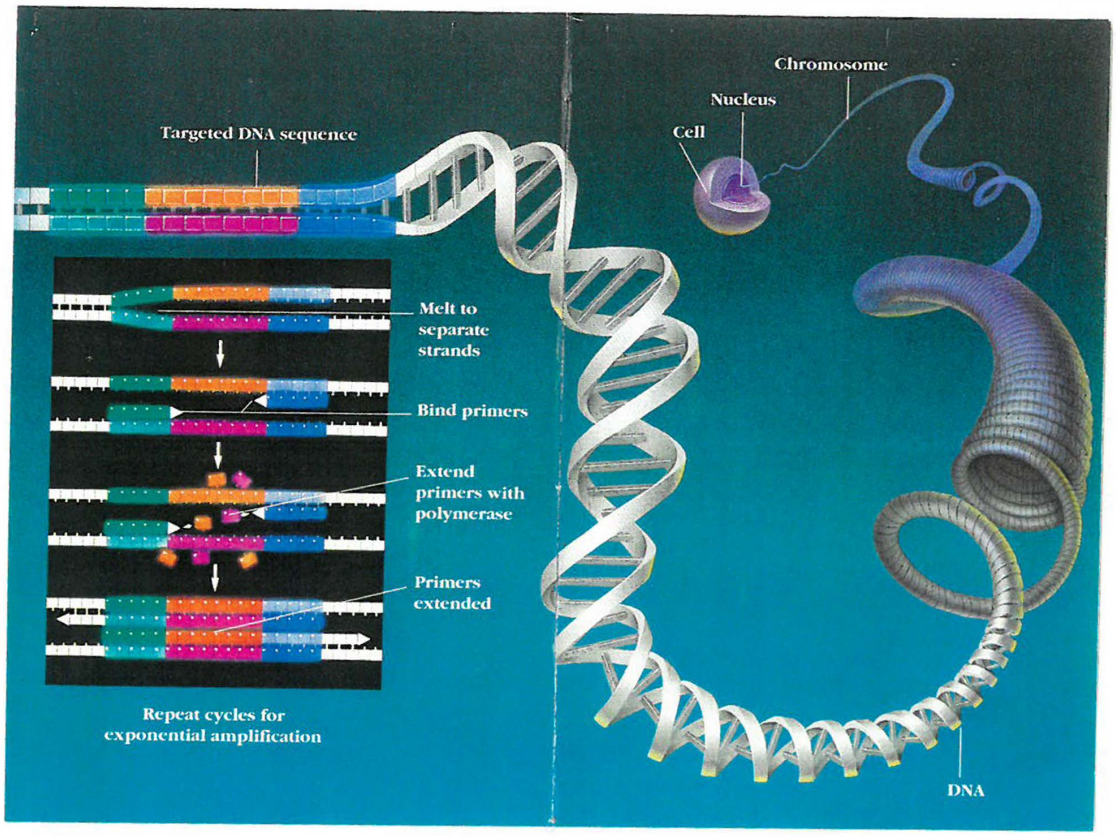

Fig 2.1. Schematic representation of PCR in which the targeted DNA sequence is subjected to three temperature dependent steps (reproduced with permission from Roche Diagnostic Systems).

This technique is now used in many laboratories worldwide. It has a number of advantages: it is fast, sensitive with a high specificity, and has wide applications in a number of areas such as prenatal diagnostics, forensic medicine, detection of foreign organisms or many others. The PCR however also has some disadvantages. It relies upon knowledge of the sequence in order to design the primers, extreme care needs to be taken to avoid contamination, and because of limitations on the size of the DNA fragment that can be conveniently amplified, usually only a small part of a gene can be examined at any one time.

Factors affecting the PCR

Many complex interactions occur during the PCR and it is therefore unlikely that there will be one set of conditions optimal for all reactions. Minor adjustments to the PCR

parameters are often required to improve the specificity and yield. Hence it is usually necessary to optimize the PCR for a given application, by varying certain parameters:

a Magnesium chloride

The concentration of $MgCl_2$ influences the specificity and yield of an amplification reaction; it may affect primer annealing, strand dissociation of template, product specificity, enzyme activity and fidelity and may cause formation of primer-dimer artefacts.

Magnesium chloride may particularly affect Taq DNA polymerase activity because this enzyme requires free magnesium apart from that bound by template DNA, primers, and dNTPs (Innis and Gelfand, 1990). The amount of magnesium chloride free in the PCR mixture depends on the amount of dNTPs present, because dNTPs apparently bind Mg^{2+} quantitatively. Consequently, if the dNTP concentration is changed significantly, a compensatory change in $MgCl_2$ may be necessary. The $MgCl_2$ concentration in the final reaction mixture should be between 0.5 and 5 mmol/l higher than the total dNTP concentration.

In addition, high concentrations of EDTA in the PCR solution decrease the amount of $MgCl_2$ available for the Taq polymerase. Care should be taken when using DNA stored in TE buffer to optimise the concentration of $MgCl_2$ in the reaction. In general $MgCl_2$ in the range of 1.5 to 4 mmol/l is used (Saiki, 1990).

b Deoxynucleotide triphosphates (dNTPs)

The deoxynucleotide triphosphates (dATP; dCTP; dGTP and dTTP) are usually added at concentrations of 200 μmol/l for each dNTP in the reaction mix, to give an optimal balance between yield, specificity, and fidelity. The four dNTPs are used at equivalent concentrations to minimize misincorporation errors.

c Taq DNA polymerase

The recommended concentration range for Taq DNA polymerase is between 1 and 2.5 units per 100 μl of reaction mixture. For amplification reactions involving DNA samples with high sequence complexity, such as genomic DNA, the optimum increases from 1 to 4 units per 100 μl of reaction mixture.

Addition of excess enzyme may lead to amplification of non-target sequences (Sambrook *et al*, 1989d).

d Primers

The design of oligonucleotide primers is very important to the success of the PCR reaction and is usually carried out according to certain guidelines. In general, the primers should be between 20 and 30 nucleotides in length, allowing a reasonably high annealing temperature to be used (Newton and Graham, 1994b); this in turns decreases the risk of non-specific binding. Primer pairs should avoid complementarity at the 3′ end as this promotes the formation of primer-dimer artefacts reducing the yield of the desired product.

The optimal concentration of primers is 0.1 to 0.5 μmol/l; higher concentrations promote mispriming and non-specific bands, and may create primer-dimers (Innis and Gelfand, 1990).

The melting temperatures (Tms) for a given primer pair should be similar. The Tm is calculated using the formula 2 (A+T) + 4 (G+C); annealing temperatures are generally chosen to be approximately 5°C below the Tm. The percentage of GC content should be no higher than 50-60%.

e Other components

Other substances reported to enhance the efficiency and/or specificity of a PCR can be added. These include: formamide, dimethyl sulfoxide (DMSO), tetramethylammonium chloride (TMAC), polyethylene glycol 6000 (PEG), glycerol and Tween 20 (Newton and Graham, 1994b). The optimum concentration of these substances has to be determined empirically.

The major drawback of these components is that no single one can be said to have a beneficial effect in all PCRs.

f Cycling conditions

As mentioned previously the PCR is performed by incubating the reaction mixture at three different temperatures corresponding to the three steps of denaturation, annealing and extension.

Double-stranded DNA is denatured at 94°C. Incomplete denaturation allows the DNA strands, to snap back reducing product yield or can even result in PCR failure. Thus it is sometimes helpful to carry out an initial denaturation step at 93-95°C for a few minutes, prior to the start of the PCR.

The primers are allowed to anneal to their complementary sequences by briefly cooling to 40-60°C. This is followed by heating to 70-75°C to extend the annealed primers with the Taq polymerase. The primer extension time depends upon the length and concentration of the target sequence and upon temperature. Primer extensions are usually performed at 72°C (Fig 2.2).

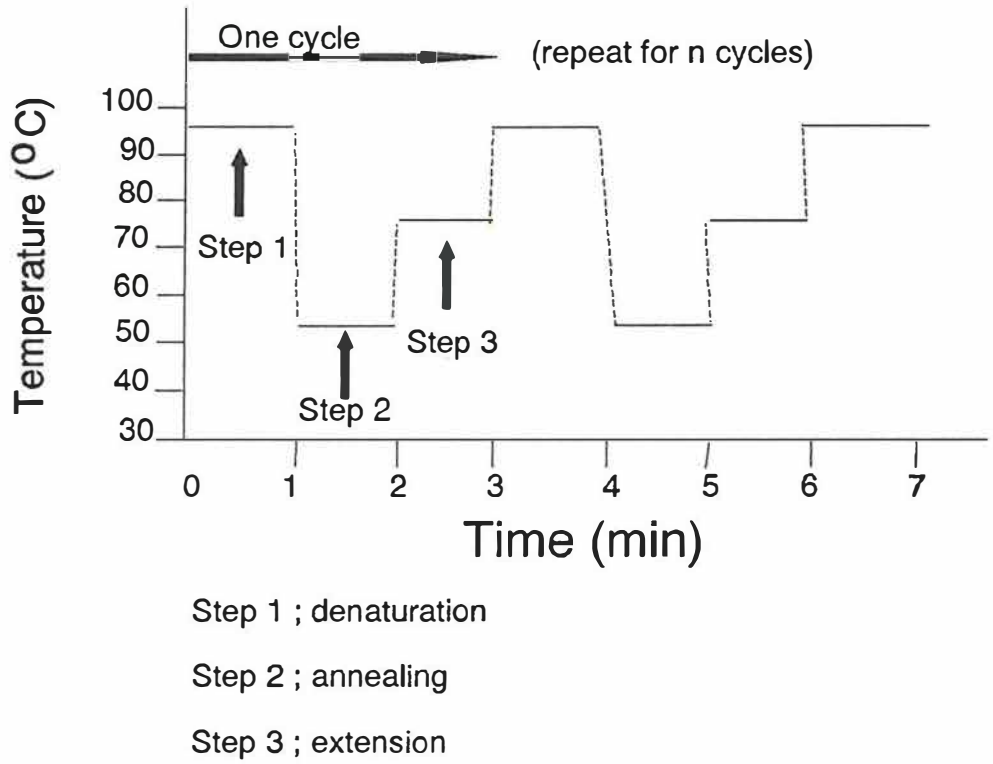

Fig 2.2. PCR temperature cycling profile. ***Step 1****: Denaturation of DNA template at around 93°C-95°C.* ***Step 2****: Oligonucleotide primers anneal to the denatured template at temperature 40-60°C.* ***Step 3****: Primer is extended at 72°C.* ***Steps 1 to 3*** *constitute one cycle of the PCR. This process is then repeated for the appropriate number of cycles for a given PCR.*

The optimum number of cycles depends mainly upon the starting concentration of target DNA when all other parameters are optimized. Too many cycles may increase the amount and complexity of non-specific background products and may also result in product inhibition.

The amplification reaction is not infinite. After a certain number of cycles the desired amplification fragment gradually stops accumulating exponentially; this is called the plateau effect. The point at which a PCR reaction reaches its plateau depends primarily on the number of copies of target originally present in the sample and on the total amount of DNA synthesized. Some of the possible causes of the plateau effect are substrate excess, competition by non-specific products and product reassociation.

Denaturation of DNA template

The first few rounds of a PCR are very important since they determine the final yield. Thus it is vital that the DNA is completely denatured particularly when using high molecular weight DNA as a template. Boiling of the DNA for up to 10 min and immediate chilling on ice prior to being added to the PCR mixture has been shown to greatly enhance the amplification process. Boiling the DNA before PCR also reduces the amount of primer oligonucleotide required (Abbott *et al*, 1988).

All PCRs in this study were carried out using a Hybaid®DNA Thermal Cycler

(Hybaid Ltd. UK). Exact details of primer sequences, reagent concentrations and cycling conditions are described in the relevant sections.

vi SOUTHERN HYBRIDISATION

Principle

Fragments of DNA which have been separated by electrophoresis are rendered single stranded and transferred from the gel on to a nylon or nitrocellulose membrane. After fixing the DNA on the membrane by crosslinking under UV radiation it is hybridised with a suitably labelled probe in order to detect a specific sequence (or gene) in the DNA. The transfer method was originally developed by Ed Southern (Mathew, 1983) for DNA molecules (Southern blot) but has been adapted for RNA (northern blot) and proteins (western blot).

Procedure

The procedure used for Southern hybridisation has several steps such as transfer of the DNA to a membrane, preparation and labelling of the DNA probes, and finally the hybridisation and visualization. These are described separately in the following sections.

Both oligoprobes (short 20-30 base sequences) and large DNA probes can be used for Southern hybridisation. The choice is dependent on the application. Oligoprobes are

usually synthesized on a DNA synthesizer while DNA probes are obtained from the culture of *E.coli* which harbour plasmids in which the DNA of interest has been cloned. Details of the probes used are provided in the relevant sections.

a Preparation of DNA probes

Culture of E.coli

Bacteria (2 to 3 colonies picked off an agar plate) containing the plasmid/probe of interest were grown in 3 ml of Luria-Berti (LB) broth (10g/l Tryptone, 5 g/l yeast extract, 10 g/l NaCl) containing the appropriate antibiotic in a 5 ml Bijou bottle by incubating overnight at 37°C with vigorous shaking.

Greater amplification of the plasmid was obtained by treating the cells in late log phase with an inhibitor of protein synthesis, such as chloramphenicol (10-20 μg/ml). This inhibits bacterial chromosomal DNA replication but allows continued replication of the plasmid DNA (Sambrook *et al*, 1989a).

Isolation of plasmid DNA

The culture was spun at 1200 *g* for 5 min to harvest the bacteria. The medium was drained off to leave the bacterial pellet as dry as possible.

A Wizard™DNA purification system (Promega Corporation, USA) for the

isolation of plasmid DNA was used according to the manufacturer's instructions. Briefly, the bacteria cells were lysed in an alkali solution and the free plasmid DNA bound to a silica-based resin at a high salt concentration. The resin was then washed to remove salts or contaminants and the DNA eluted from the resin at low salt concentration by centrifugation, and stored at 4°C or -20°C.

Extraction of the DNA probe from the plasmid

The DNA insert was removed from the plasmid by restriction with the appropriate RE as described in Section 2.3 iv. A one hour incubation at 37°C was found to be sufficient to cut the plasmid DNA. The restricted DNA was then electrophoresed on a 1% agarose gel with the appropriate molecular weight marker.

The band corresponding to the DNA insert was identified, and extracted from the gel using a Qiaex Kit (Qiagen Ltd, Promega Corporation, Madison, USA). The principle of this kit is based on solubilisation of agarose and selective adsorption of nucleic acids to silicagel particles in the presence of high concentrations of salt. This was followed by the elution of the nucleic acids at low salt concentrations using centrifugal force.

b Labelling of probes

The choice of label and labelling technique depends on the size of the probe to be labelled. In this study the DNA probe was labelled with α-^{32}P-dCTP using the Megaprime™ DNA Labelling Systems Kit (Amersham, International plc, England) employing the random priming reaction. For oligoprobes a non-radioactive DIG Oligonucleotide 3′-End Labeling Kit was used (Boehringer Mannheim GmbH, Germany).

b.1 Random priming method

Principle

This method is based on the use of a random sequence of nonanucleotides, used as primers, to start DNA synthesis on a denatured template DNA. This occurs at numerous sites along its length. The Klenow polymerase in the reaction mix adds randomly a ^{32}P radiolabeled nucleotide (in this case ^{32}P-dCTP) and unlabelled dNTPs to the single stranded DNA template and thus the newly synthesized DNA is radioactively labelled.

The Klenow fragment of DNA polymerase is used specifically because it lacks the 5′-3′ exonuclease activity associated with DNA polymerase I and thus ensures that labelled nucleotides incorporated by the polymerase are not

subsequently removed. The presence of exonuclease activity can be troublesome because it degrades the 5′terminus of primers that are bound to DNA templates (Sambrook *et al*, 1989c).

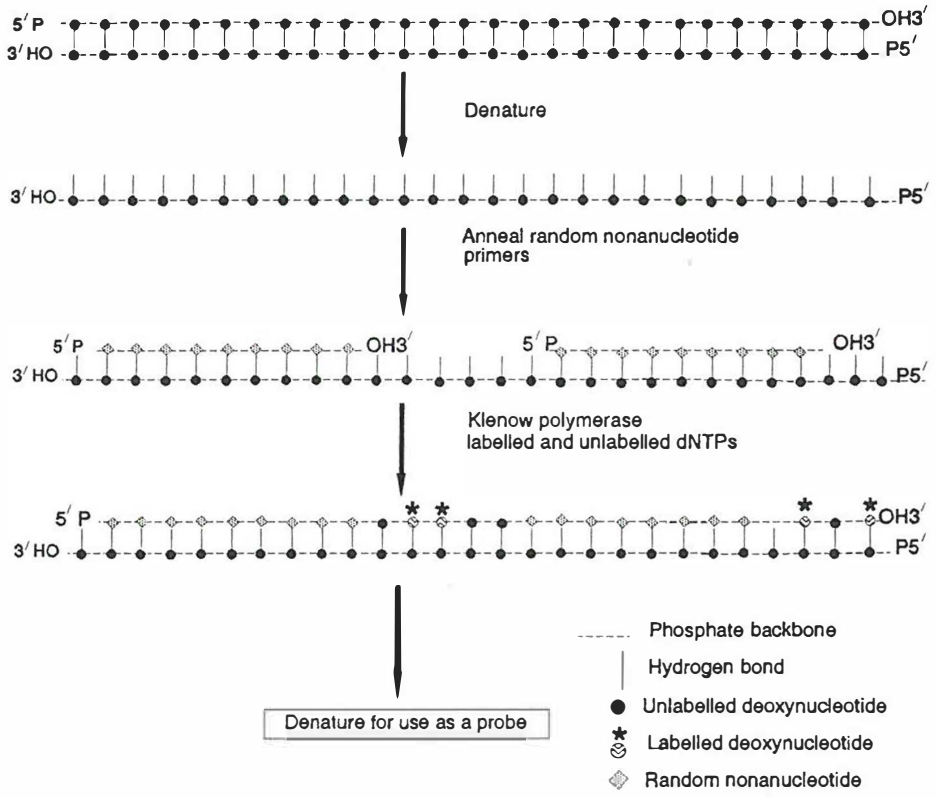

Fig 2.3. Schematic diagram showing the random primer reaction which allows incorporation of radiolabelled bases using Klenow polymerase.

Procedure

The labelling procedure was carried out according to the manufacturer's instructions.

The percentage of incorporation of ^{32}P-dCTP varied between 30-60% and the specific activity was between 1.3-2.5 x 10^9dpm/μg.

b.2 3′-End labelling method

Principle

The principle is based on the addition of digoxygenin dideoxyuridine triphosphate (DIG-ddUTP) to the 3′ end of the oligonucleotide DNA template (probe) with the aid of the terminal transferase enzyme.

The procedure was carried out according to the manufacturer's instructions.

c DNA transfer

Procedure

There are several methods for the transfer of DNA on to a solid support such as nylon or nitrocellulose, namely capillary, electrophoretic or vacuum transfer. In this study transfer by vacuum was chosen because of its efficiency and the short period of time required for complete transfer.

The vacuum transfer was carried out as follows:

The gel was placed in contact with a nylon membrane (Hybond, Amersham) supported on a porous screen over a vacuum chamber, taking care to ensure that the vacuum was applied evenly over the entire surface.

A depurination solution (0.2 mol/l HCl) was washed through the gel for 20 min under vacuum. This allowed the DNA to be broken into smaller pieces to facilitate the transfer process.

A denaturation solution (1.5 mol/l NaCl and 0.5 mol/l NaOH) was washed through the gel for 30 min to denature the double stranded DNA.

Finally the DNA was transferred to the membrane by washing the gel under vacuum with 10 mol/l CH_3COONH_4 and 10 mol/l NaOH for 30 min.

d Hybridisation

Principle

When single-stranded DNA is placed in contact with a complementary single-stranded DNA sequence, the two DNA molecules bind to each other by hydrogen bonding between the bases. This binding, or hybridisation, forms the basis of the techniques used for the detection and semi-quantitation of specific nucleic acid sequences.

Procedure

DNA probe

The initial step was a prehybridisation (without labelled probe) to block the non-specific binding sites on the membrane. This was carried out by rotating the membrane for 2 h at an appropriate temperature in 30 ml of hybridisation solution containing 0.5 mol/l $NaHPO_4$ (pH 7.2), 0.001 mol/l EDTA, 7% SDS and 1% BSA.

This was followed by overnight hybridisation in 20 ml of the same solution containing labelled probe. Usually 12.5 ng/blot (25 μCi) was used. The probe was previously denatured by boiling for 5 min and chilling on ice to ensure the separation of the double-stranded DNA.

After hybridisation, the membrane was washed twice for 15 min under conditions of higher stringency in a solution of 0.04 mol/l $NaHPO_4$ (pH 7.2) containing 1% SDS (Meling *et al*, 1993).

Oligoprobes

Conditions used for hybridisation of short sequences of DNA were based on an adaptation of the 3′-End Labelling and Hybridisation Kit (Boehringer) instructions. The nylon membranes were carefully cut to include only the region where the bands were expected in order to reduce the amount of hybridisation solution required. No prehybridisation step was necessary and the duration of the hybridisation (approximately 1 h) was much shorter than for the DNA probes.

e Visualisation

e.1 DNA probes

The hybridisation of the ^{32}P labelled DNA probe to the DNA of interest was visualized by means of autoradiography, in which the radioactive isotope reacts with an X-ray film resulting in a visible signal.

Procedure

The wet nylon membrane (containing the hybridised DNA) was wrapped in Glad Wrap plastic, and placed in an X-ray cassette. In a darkroom the membrane was covered with a sheet of X-ray film (Hyperfilm™MP, Amersham) and an intensifying screen to increase the signal. The cassette was then placed at -70°C to stabilize the ions that form the latent image of the radioactive source. The length of exposure depended on the intensity of the signal and was usually from 1 to 2 weeks.

The X-ray film was developed manually as follows: the film was placed in a developer solution diluted 1:3 for 1 min, washed in water for 1 min and finally in a fixer solution for 1 min (FX-Universal Agfa chemicals).

After development of the X-ray film, the position of the visible signal or band was examined relative to an image of the original gel to determine the size of DNA fragment to which the probe was hybridised.

e.2 Oligoprobes

The visualization of the DIG-labelled oligoprobes was carried out as instructed by the manufacturer. Briefly the hybridized molecules were visualized by the production of an enzymatically derived insoluble blue precipitate following the reaction in which the oligonucleotide labelled at the 3′ end with DIG-ddUTP reacts with an antibody conjugate.

vii SINGLE STRAND CONFORMATIONAL POLYMORPHISM (SSCP)

Principle

Single strand conformational polymorphism analysis depends on the principle that the electrophoretic mobility of a molecule within a gel matrix is sensitive to the size, charge and shape of the molecule. Under non-denaturing gel conditions single stranded DNA has a folded structure that is imposed by intramolecular interactions dictated by its sequence. In SSCP analysis a single nucleotide difference between similar sequences is sufficient to alter the folded structure of one sequence relative to the other. This conformational change is detected as a mobility difference upon gel electrophoresis. The sensitivity of the method allows detection of most single nucleotide differences in a fragment composed of several hundred nucleotides (Newton and Graham, 1994).

Practical considerations

Several factors contribute to the success of SSCP analysis. These are dealt with individually in this section.

a Polymerase chain reaction

The first step in the preparation of DNA for SSCP analysis is the amplification of the region(s) of interest. Since optimal sensitivity for SSCP is obtained with DNA sequences between 300 to 400 bases in length (Hongyo *et al*, 1993; Newton and Graham, 1994a), it is sometimes necessary to examine the region of interest one section at a time. In the case of the p53 gene the exons 5 to 8 in which more than 90% of mutations are found, all comprise less than 183 bp and therefore each exon can be amplified intact. In order to ensure that the entire exon is covered, primers were chosen complementary to regions in the introns flanking each exon.

The primer sequences and details of the PCRs are described in detail in Chapter 5.

b Analysis of single stranded DNA

Several approaches to the analysis of single stranded DNA are available. The original SSCP protocol uses radiolabelled nucleotides to generate a radiolabelled PCR product, which is then diluted, denatured by heat, and electrophoresed at high voltage on a large formatted (40 x 20 cm) non-denaturing polyacrylamide gel (Orita *et al*, 1989). The method depends heavily on experimental conditions that optimize migration differences of the single stranded DNA conformation. Thus, factors such as the addition of glycerol to the polyacrylamide, modification of the temperature and electrophoretic conditions may result in greater levels of sensitivity. Others supports such as Hydrolink MDE and Hydrolink D-5000 have also been used in an attempt to improve the sensitivity of SSCP (Soto and Sukumar, 1992).

Recently the use of non-radioactively labelled primers has been introduced.

Following PAGE, the single stranded fragments can be visualised with chemiluminescence, silver nitrate or ethidium bromide (Orita *et al*, 1989; Hongyo *et al*, 1993; Dockhorn-Dworniczak *et al*, 1994).

The approach adopted in this study is that of Hongyo *et al*, (1993) in which non radio-labelled primers are used. Single stranded fragments are detected using ethidium bromide, on a small polyacrylamide gel run at high voltage.

c Factors affecting SSCP analysis

The factors discussed here are specific for the SSCP analysis used in this study. However, the principles are applicable to SSCP carried out using a range of different electrophoresis and detection systems.

Temperature

The control of the temperature within the gel unit is important for consistent detection of single strand bands (Hongyo *et al*, 1993). Thus recirculation of water through a thermostatically controlled system is recommended to maintain the running buffer at constant temperature. The ideal temperature is dependent on the conformation of the DNA fragment and is determined empirically for each product.

PCR product concentration

The concentration of PCR product loaded on to the gel affects the resolution of the bands. It would appear that at least a 1:3 dilution is required to avoid significant amounts of double stranded DNA and blurred single stranded bands in the PAGE gel. This phenomen is probably due to the partial reannealing that occurs at high DNA concentrations (Hongyo *et al*, 1993).

Denaturants

The use of a denaturant to keep the two DNA strands separated is important to the success of the method. One denaturant used is methylmercury hydroxide, which prevents excessive reannealing of the DNA strands prior to entry into the gel. The use of less hazardous compounds such as sodium hydroxide is also recommended (Hongyo *et al*, 1993).

Loading buffers

The composition of the loading buffer would appear to affect resolution. Buffers containing 15% Ficoll with or without formamide generally result in the sharpest bands (Hongyo *et al*, 1993).

Buffer type and concentration

The type and concentration of the running buffer in the gel chamber can also affect the clarity of the SSCP bands. In most cases 1-1.5 x TBE yields the sharpest SSCP bands when using polyacrylamide gels (Hongyo *et al*, 1993).

Voltage

A constant voltage between 300 or 400 V produces the best sensitivity and sharpness of the bands in SSCP analysis (Hongyo *et al*, 1993). Under these conditions however temperature control is essential. Gels can also be satisfactorily run at lower voltages for longer periods, usually overnight at a low temperature.

Details of the gel concentrations, electrophoresis conditions and other factors used in this study for SSCP analysis of exons 5, 6, 7 and 8 of the p53 gene are documented in Chapter 5.

CHAPTER 3

LOSS OF HETEROZYGOSITY AS A SCREEN FOR p53 MUTATIONS

3.1 INTRODUCTION

Reports in the literature indicate that 80-90% of mutations in the p53 gene are associated with loss of the corresponding allele, also known as loss of heterozygosity (LOH) or reduction to homozygosity (Sidransky *et al*, 1991). This relationship between LOH and mutations can therefore be used to screen tumours for mutations (Delattre *et al*, 1989; Sidransky *et al*, 1991) and has been shown to be useful in several tumour types. For example, in human hepatocellular carcinoma an association with LOH was found in 80% tumours manifesting p53 mutations (Fujimoto *et al*, 1994), in breast tumours this relationship was found to be 83% (Tsuda and Hirohashi, 1994), and in oesophageal squamous cell carcinoma 89% (Maesawa *et al*, 1994).

In tumours of the cervix very little information is available on LOH in the p53 gene. Three studies have been reported on a small number of patients which indicate that both LOH and p53 mutations in this tumour type occur with a much lower frequency (Crook *et al*, 1992; Fujita *et al*, 1992; Busby-Earle *et al*, 1993). The purpose of this study therefore, was to undertake a survey of tumours of the cervix from black South African women for LOH of the p53 gene as an indication of the prevalence of p53 aberrations in a local population.

The most commonly used technique for the identification of LOH is Southern analysis in which appropriately restricted DNA is hybridized with a ^{32}P-labelled DNA probe specific to the gene of interest or an adjoining region (Eccles *et al*, 1992). In this way restriction fragment length polymorphism (RFLPs) or large variable number of tandem repeats (VNTRs) can be detected. This method, however, is cumbersome, expensive, often uninformative and requires the use of radio-isotopes. Consequently other approaches have been sought, for example, the amplification of genetic regions in which dinucleotide, triplet or other VNTRs are common.

This chapter describes one such technique in which a VNTR region in intron 1 of the p53 gene is amplified by PCR and LOH determined in heterozygotic individuals. For the purpose of comparison, a subset of tumours was also analysed by conventional Southern hybridisation. The results obtained using these two approaches are presented.

3.2 LOSS OF HETEROZYGOSITY BY AMPLIFICATION OF A VNTR POLYMORPHISM

i INTRODUCTION

The method used is based on a published technique in which a VNTR region in intron 1 of the p53 gene was amplified by PCR and subjected to polyacrylamide gel electrophoresis (PAGE) (Hahn *et al*, 1993). Normal and tumour DNA from individuals showing allele heterozygosity was examined for the loss of one allele.

ii PRINCIPLE

Different numbers of repeats in a VNTR region of the genome result in different length fragments which can be detected on PAGE following PCR (Fig 3.1 a). When two alleles are homozygous with respect to the number of repeats (AA), only one band is detected on PAGE. Heterozygous alleles with differing numbers of repeats (AB,

AC) result in two bands. By making comparisons between DNA from tumour tissue and normal cells, such as lymphocytes, loss of one allele can be clearly demonstrated in heterozygous but not in homozygous individuals (Fig 3.1 b).

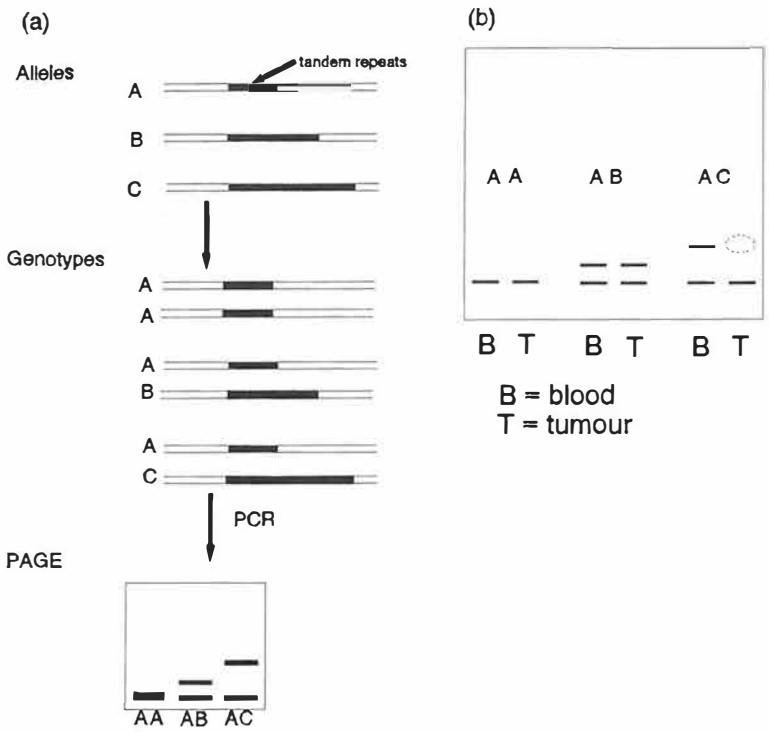

Fig 3.1. Schematic representation of ***(a)*** *a VNTR region of a gene with 'A', 'B', 'C' representing examples of different alleles and* ***(b)*** *loss of allele in the tumour DNA from a heterozygous individual.*

iii METHODS

Clinical material

Cervical tumours and blood from 123 patients were collected, stored and the DNA isolated as described in Section 2.3. Special care was taken to avoid contamination of the tumour with surrounding normal tissue or blood.

All patients were black women aged between 20 and 91 years with primary squamous carcinoma of the cervix. Tumours were histologically confirmed before acceptance into

the study. A total of 22 patients was subsequently excluded because insufficient tumour tissue was available, or tumour or blood DNA did not amplify satisfactorily. Thus results are reported on 101 patients.

Polymerase chain reaction

The general principles and factors influencing the PCR are outlined in Chapter 2. In the present chapter the protocol used to amplify a VNTR region in intron 1 of the p53 gene is described in detail.

The primers used were designed by Hahn *et al*, (1993) and have the following sequences:

Primer 1

5′ ACTCCAGCCTGGGCAATAAGAGCT 3′

Primer 2

5′ ACAAAACATCCCCTACCAAACAGC 3′

These primers flank the following region of tandem repeats in intron 1 of the p53 gene: (Arrows and numbers show the orientation and position of the PCR primers which are indicated by capital letters)

8946 →

5′ **ACTCCAGCCTGGGCAATAAGAGC**tgagactccgtctcaaaataaaataaaa.....taaaataaaa

←9076

taaaataaaaaaagaaaagagcctgccattaaagga**GCTGTTTGGTAGGGGATGTTTTGT** 3′

Primer 1 is a sense strand sequence which binds to the complementary antisense DNA strand running from 3′ to 5′. Primer 2 is an antisense sequence and binds to the complementary sense DNA strand running from 5′ to 3′. The expected PCR product

is in the region of 131 bp, depending on the number of repeat sequences present. The melting temperatures (Tms) are 72°C and 70°C for the first and second primers, respectively, and were calculated as described in Section 2.3 v.

The primers were synthesized on a Milligen Oligonucleotide Synthesizer in the Department of Biochemistry at the University of Cape Town.

Optimization of the PCR

a DNA integrity

The integrity of the DNA was checked by running 1 μg of DNA from blood and tumour samples on a 1% agarose gel with Marker II (Boehringer) as described in Section 2.3 iii. The DNA was of high molecular weight and showed no degradation as can be seen in Fig 3.2.

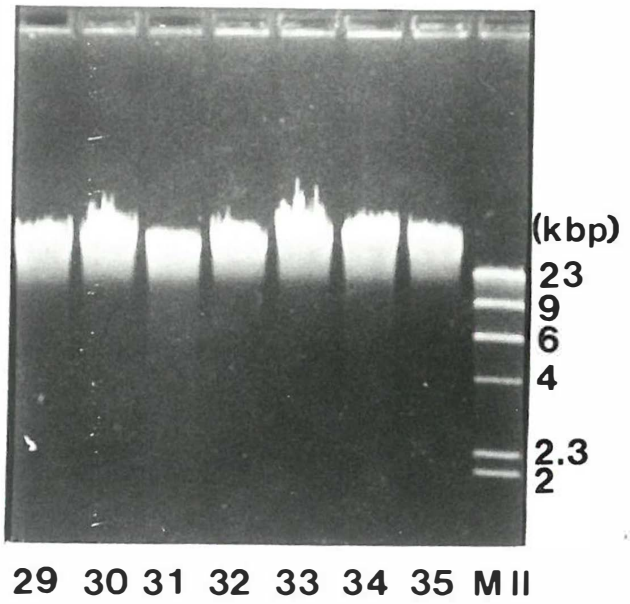

Fig 3.2. Agarose gel (1%) showing 1 μg blood DNA from seven individuals.

b Concentration of $MgCl_2$

A magnesium chloride concentration of 1.5 mmol/l is generally adequate for the PCR. However, the DNA used in this study was stored in a buffer containing EDTA and it was therefore necessary to optimise the concentration of $MgCl_2$. This was done by carrying out a series of reactions with concentrations of $MgCl_2$ ranging from 2 to 4 mmol/l. Results indicated that 2 mmol/l $MgCl_2$ was optimal for the concentration of reagents used. Higher concentrations resulted in an increase of non specific bands (Fig 3.3). Some amplification reactions were also carried out with 1.5 mmol/l of $MgCl_2$ (results not shown) resulting in a very faint product or none at all.

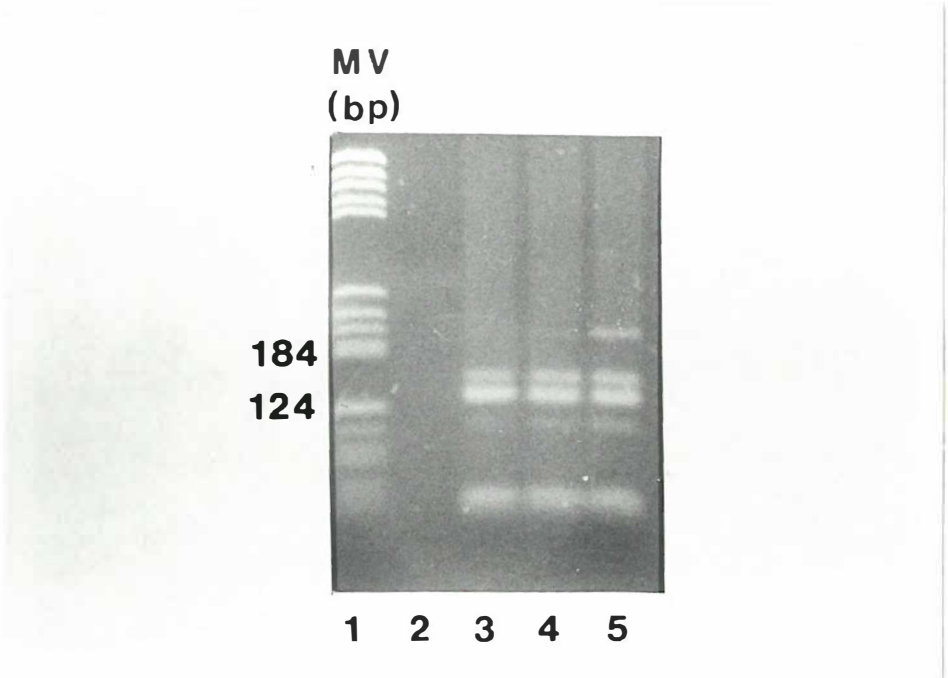

*Fig 3.3. Agarose gel (2%) showing the PCR products obtained with different concentrations of $MgCl_2$. **Lane 1**, molecular weight marker V (Boehringer); **lane 3**, 2 mmol/l $MgCl_2$; **lane 4**, 3 mmol $MgCl_2$; **lane 5**, 4 mmol/l $MgCl_2$.*

c Concentration of primers and Taq DNA polymerase

The optimum concentration of primers was determined by carrying out different experiments using 100 nmol/l, 200 nmol/l, 400 nmol/l of each primer and 1 or 2 units of Taq DNA polymerase. Results indicated that the optimal concentration of primers was 200 nmol/l using 1 unit of Taq (lane 6, Fig 3.4).

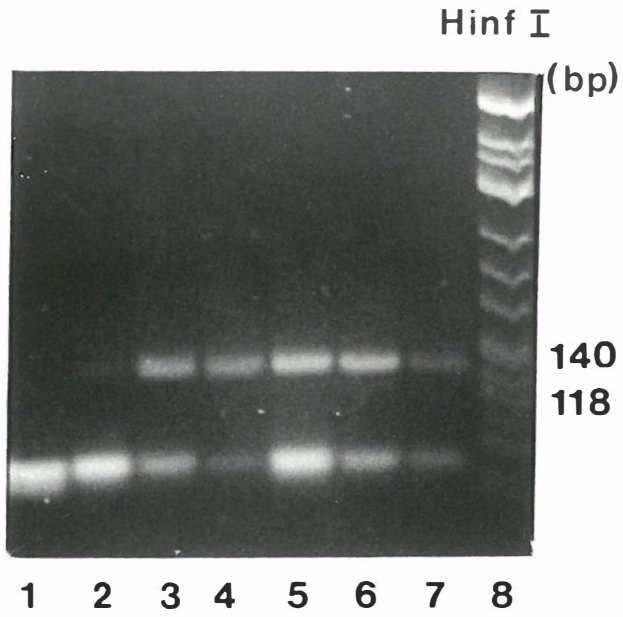

Fig 3.4. Agarose gel (2%) showing the PCR products obtained with different concentrations of primers and amounts of Taq polymerase. The concentrations of primers were ***lane 2****, 400 nmol/l;* ***lane 3****, 200 nmol/l;* ***lane 4****, 100 nmol/l with 2 units of Taq polymerase;* ***lane 5****, 400 nmol/l;* ***lane 6****, 200 nmol/l; lane 7, 100 nmol/l with 1 unit of Taq polymerase;* ***lane 8****, molecular weight* marker *HinfI (Promega);* ***lane 1****, no DNA.*

d Concentration of DNA

To determine the optimum concentration of DNA different reactions were carried out using between 10 and 29 ng of boiled DNA in a total PCR mixture of 25 μl. The concentration of $MgCl_2$ was 2 mmol/l with 200 nmol/l of primers and 1 unit of Taq. The total volume for the PCR mixture was 25 μl except for one reaction (lane 7, Fig 3.5) in which the amount of all reagents used was doubled and the amount of boiled DNA was 14 ng. The optimal amount of DNA for successful amplification was found to be approximately 14 ng of boiled DNA with 200 nmol/l of primers, 2 mmol/l $MgCl_2$ and 1 unit of Taq in a total PCR mixture of 50 μl (lane 7, Fig 3.5). Boiling of the DNA prior to use clearly enhanced the yield of PCR product (compare lanes 5 and 8).

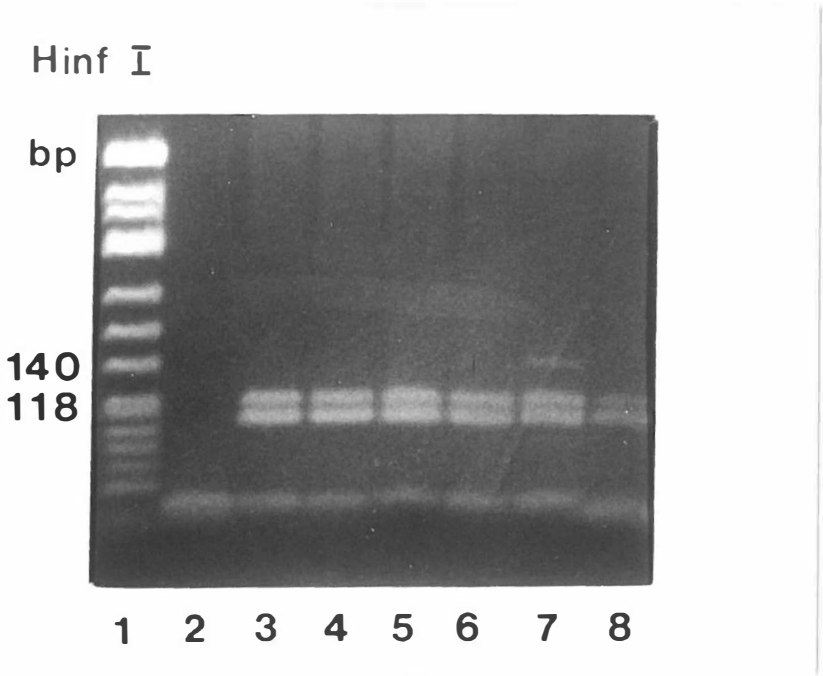

Fig 3.5. Agarose gel (2%) showing the PCR products obtained with different concentrations of DNA. ***Lane 1****, molecular weight marker HinfI (Promega);* ***lane 2****, no DNA control, the concentrations of DNA were as follows,* ***lane 3****, 29 ng/25 μl reaction mixture;* ***lane 4****, 19 ng/25 μl;* ***lane 5****, 14 ng/25 μl;* ***lane 6****, 10 ng/25 μl;* ***lane 7****, 14 ng/50 μl;* ***lane 8****, 14 ng/25 μl. All the DNA samples were boiled prior to being added to the PCR mix except the one in* ***lane 8****. The total volume in the PCR mixture was of 25 μl except for* ***lane*** *7 which was 50 μl.*

PCR procedure

Having optimized the PCR as described above, tumour and blood DNA from the patients was amplified as follows: each PCR reaction contained 14 ng of DNA, 5 μl of Taq polymerase buffer, 2 mmol/l of $MgCl_2$, 200 nmol/l of each primer, 200 μmol/l of each dNTP and 2 units of Taq DNA polymerase (Promega) made up to 50 μl with sterile deionized water.

A master mix of the reagents common to the PCR was made up in a 1.5 ml eppendorf before each run. The mixture (excluding the DNA) was then aliquoted to each reaction tube using a positive displacement pipette. This procedure reduces tube to tube variation and minimises the risk of contamination.

The DNA to be amplified was boiled for 10 min and quickly chilled on ice prior to being added to each PCR mixture. After spinning briefly a few drops of mineral oil were added to each tube to seal the reaction mix and prevent condensation. Silica gel was used in the heating block holes to ensure good thermal contact. Each run included a reaction mix without DNA to monitor for contamination of the reagents.

The PCR was carried out in a Hybaid®DNA Thermal Cycle (Hybaid Ltd. UK). After an initial denaturation step at 93°C for 5 min each reaction mix was subjected to 35 cycles at the following temperatures:

93°C for 1 min (denaturation)
64°C for 1 min (annealing)
72°C for 1 min (extension)

A final 10 min extension at 72°C completed the reaction. The annealing temperature chosen was approximately 5°C below the lowest primer Tm.

Analysis of the PCR amplified product by polyacrylamide gel electrophoresis (PAGE)

A 25 μl volume of the PCR product mixed with 2 μl of loading buffer was loaded on to an 18% polyacrylamide gel (as described in Section 2.3 iii). The gel was

electrophoresed at 4°C for 16 h at 60 V. Two molecular weight markers (Marker V-Boehringer and Hinf1-Promega) were included on each gel.

Precautions taken during the PCR procedure

There are many potential sources of contamination in the PCR. In this study several precautions were taken including:

* Reactions were set up in a designated area in a laminar flow cabinet.
* Equipment and reagents used for the PCR were kept separately from general laboratory equipment.
* DNA was added to the reaction mixture in a separate room.
* Gloves were changed frequently.
* Reagents such as buffers and dNTPs were prepared in batches and aliquoted for storage in convenient lots to prevent contamination of stock solutions.
* Positive displacement pipettes and pipette tips containing barriers to prevent aerosols were used.
* Each run included a control with no DNA and regular inclusion of a positive control or a well characterized sample.

iv RESULTS

Allele frequency

Five different alleles were detected, each differing by the number of tandem repeats. These were designated A, B, C, D and E. The allelic frequency was: A = 0.20, B = 0.56, C = 0.12, D = 0.07 and E = 0.05.

Using the molecular weight markers, the sizes of the 5 bands seen on the polyacrylamide gel were estimated to range from 118 to 144 bp.

A number of different genotypes were detected, examples of which are shown in Fig 3.6. The most common genotypes were BB and AB which accounted for 38% and 11%, respectively, of the samples studied.

Fifty three (52%) of individuals were found to be heterozygous for the VNTR polymorphism while the remaining 48 (48%) were homozygous.

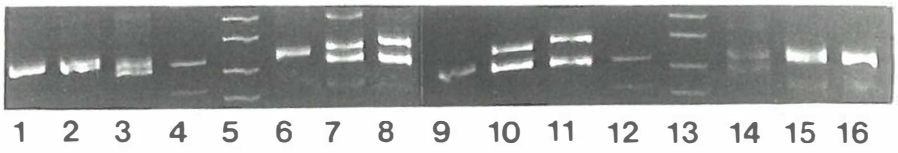

Fig 3.6. Polyacrylamide gel (18%) showing different genotypes for the VNTR in intron 1 of the p53 gene. ***Lanes 4 and 12*** *are Marker V and* ***lanes 5 and 13*** *are Marker HinfI.*

Non specific bands were occasionally detected in some individuals. However these were always larger than the VNTR bands and therefore did not constitute a problem.

Loss of allele

Of the 53 pairs of tumour and blood DNA which were heterozygous and potentially informative, two (4%) showed clear loss of one allele as indicated on the polyacrylamide gel by the absence of one band in the tumour samples (Fig 3.7). Sample 107 showed a very faint band in the position of the second allele. This most likely reflects contamination with normal tissue or blood.

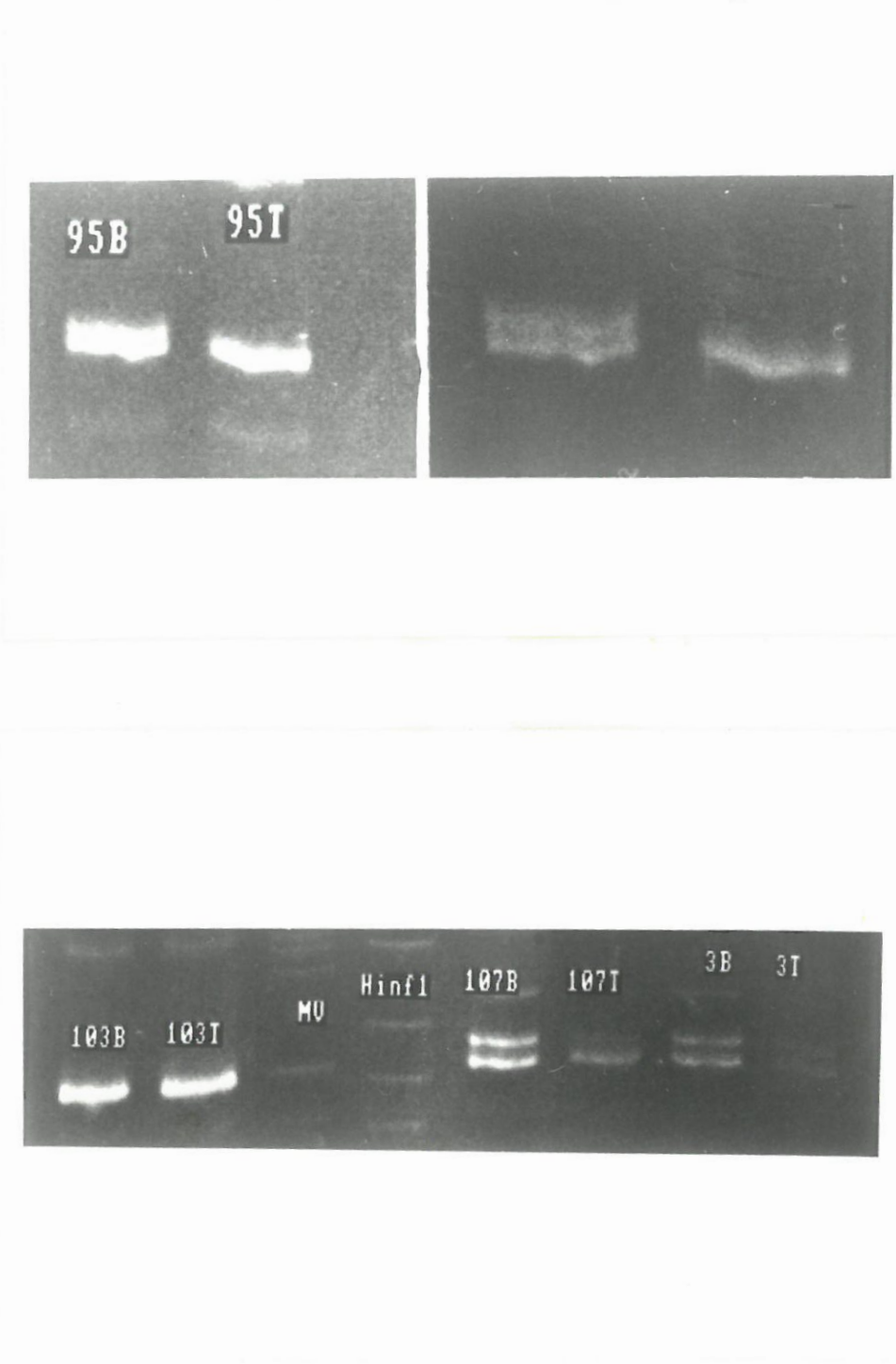

Fig 3.7. Polyacrylamide gels (18%) showing paired samples of blood and tumour. ***Samples 95 and 107*** *show LOH as indicated by the loss of one band in the tumour DNA (T) compared with the blood DNA (B). An enlarged image of* ***sample 95*** *is also shown.*

3.3. LOSS OF HETEROZYGOSITY USING SOUTHERN HYBRIDISATION

i INTRODUCTION

Southern hybridisation is the most commonly used technique for the determination of LOH. Restricted fragments of DNA are transferred to a nylon membrane and hybridised with a ^{32}P-DNA probe. Comparison of bands seen in normal and tumour DNA allows detection of the loss of heterozygosity in tumour tissue.

ii PRINCIPLE

The principle of Southern hybridisation is described in full in Section 2.3 vi.

Loss of heterozygosity is generally defined as a greater than 40% decrease in signal intensity of at least one allele in the tumour sample when compared with the respective blood (Dalbagni *et al*, 1993).

iii METHODS

Clinical material

Thirty seven (37%) of the 101 tumours in which allele loss was determined by PCR detected VNTR polymorphism, were chosen randomly for Southern hybridization analysis.

DNA probe

The genomic DNA probe used to detect LOH of the p53 gene is designated pYNZ22. It is a 1.6 kb fragment which recognises a VNTR sequence within the 17p13 locus telomeric to the p53 gene (Fig 3.8). The probe purchased from the American Type

Culture Collection (Rockville, Md) was available as a BamHI insert in the plasmid vector pBR322 and was received as a freeze-dried *E. coli* preparation.

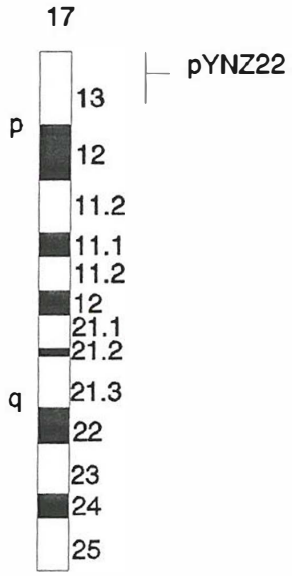

Fig 3.8. Schematic diagram of ***chromosome 17*** *showing the localization of the pYNZ22 probe.*

a Preparation of the DNA probe

The culture of *E.coli* and isolation of the plasmid DNA was described in Chapter 2. Briefly, the *E.coli* were grown for 24 h in LB medium and treated with chloramphenicol to obtain a high copy number of plasmids containing the DNA probe. The bacterial cells were then ruptured at an alkali pH to liberate the plasmids from which DNA was extracted. Finally, the DNA insert was separated from plasmid DNA by restriction with the BamHI enzyme as follows: approximately 6 μg of plasmid DNA were incubated with 9 units of restriction enzyme/μg DNA at 37°C for 1 h. The reaction was stopped by adding 0.5 mol/l EDTA (pH 8).

The DNA mix was separated by electrophoresis on a 1% agarose gel (Fig 3.9). The 1.6 kb fragment representing the pYNZ22 DNA probe was extracted from the gel using a Qiaex DNA Extraction Kit as described in Section 2.3 vi.

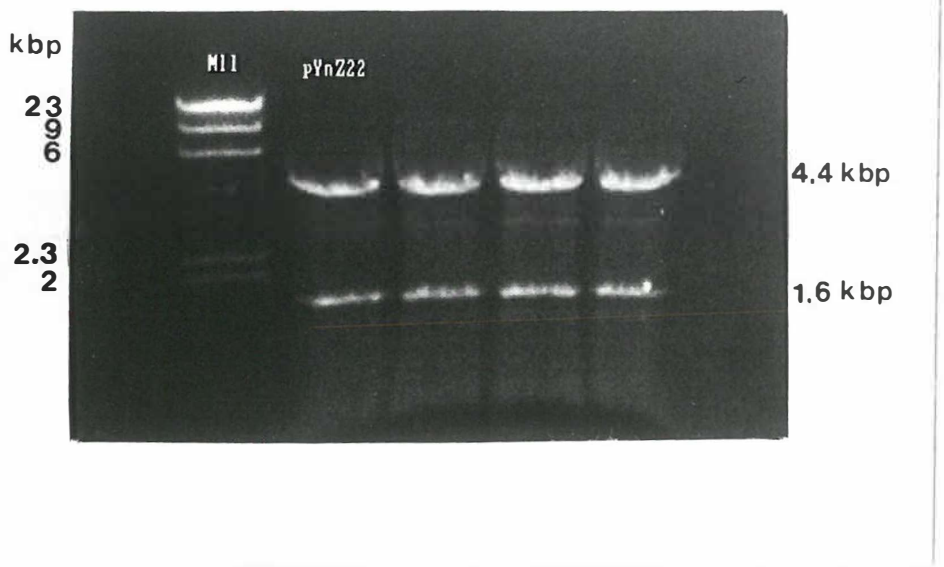

Fig 3.9. Agarose gel (1%) showing BamHI ***restricted pBR322 plasmid DNA****. The 1.6 kb fragment corresponds to the pYNZ22 insert and the 4.4 kb fragment to the pBR322 vector.*

b Labelling of the DNA probe

The pYNZ22 probe was labelled with α^{32}P-dCTP using a Megaprime-Random Priming Kit (Amersham) following the instructions of the manufacturer. Incorporation of radioactivity ranged from 30 to 40% and the specific activity was of the order of 2.5 x 10^9dpm/μg.

Southern blot

Genomic DNA (5μg) from blood and tumour was digested with 20 units of BamHI by incubating at 37°C overnight (Section 2.3 vi). The reaction was stopped with 0.5 mol/l EDTA (pH 8). The restricted DNA was transferred on to a nylon membrane by vacuum transfer as described in Section 2.3 vi.

Hybridisation

A 2 hour prehybridisation at 64°C in 30 ml of hybridisation buffer (0.5 mol/l $NaHPO_4$ pH 7.2, 0.001 mol/l EDTA, 7% SDS and 1% BSA) was followed by hybridisation (overnight) at the same temperature, in 10 ml of the same buffer which also contained 25 μCi of ^{32}P-DNA probe per blot. The hybridisation procedure was carried out in a Hybaid Mini Hybridization Oven (Hybaid Ltd, UK).

After hybridisation the membranes were washed for 15 min (2x) at 64°C in 0.04 mol/l $NaHPO_4$ (pH 7.2) containing 1% SDS.

The membranes were kept moist by wrapping in Glad Wrap plastic and exposed to an X-ray film for 7-14 days at -70°C using an intensifying screen before being developed as described in Section 2.3 vi.

Optimization of DNA concentration

The minimum amount of restricted DNA that could be efficiently hybridised and visualised was determined in a series of experiments in which amounts of DNA ranging from 4 μg to 10 μg were analysed. It was found that the minimum amount of restricted DNA that yielded satisfactory results was 5 μg (Fig 3.10).

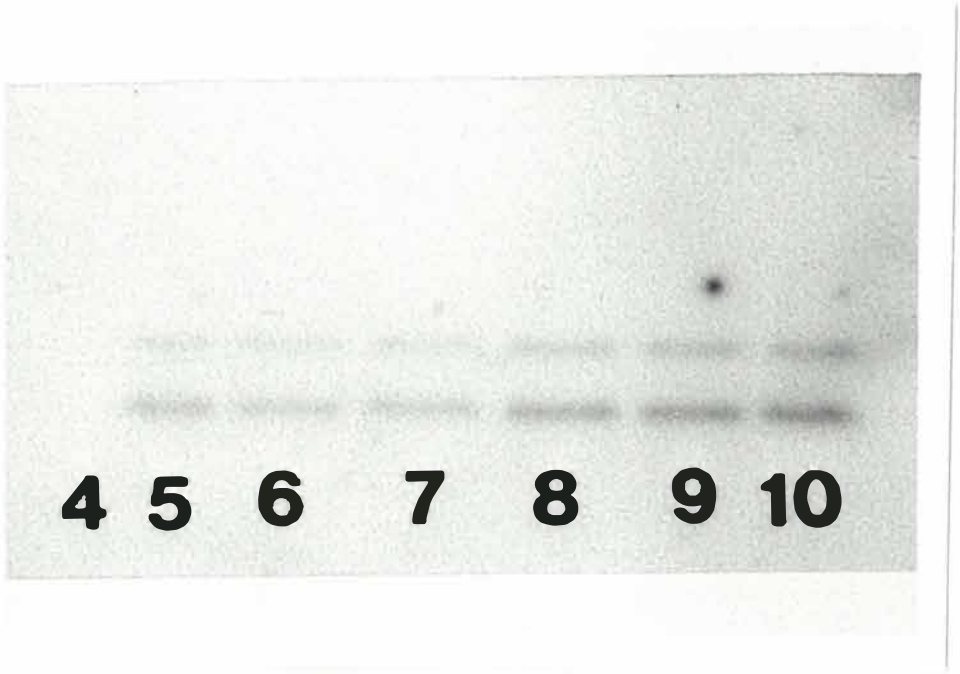

Fig 3.10. Autoradiograph showing hybridisation of ***DNA, ranging from 4 μg to 10 μg,*** *to* ^{32}*P-pYNZ22 DNA probe.*

iv RESULTS

Of the 37 paired samples of blood and tumour that were analyzed, 23 were heterozygous as indicated by two bands in the autoradiograph. The fragment sizes ranged from 0.5 to 1.3 kb. The remaining 6 tumours and bloods showed single alleles. There were 8 samples which were impossible to assess due to insufficient DNA transfer and/or inadequate hybridisation. In none of the 23 heterozygous tumours was there any evidence of loss of allele and neither was there any indication of loss of signal intensity in any of the 6 homozygous tumours (Fig 3.11).

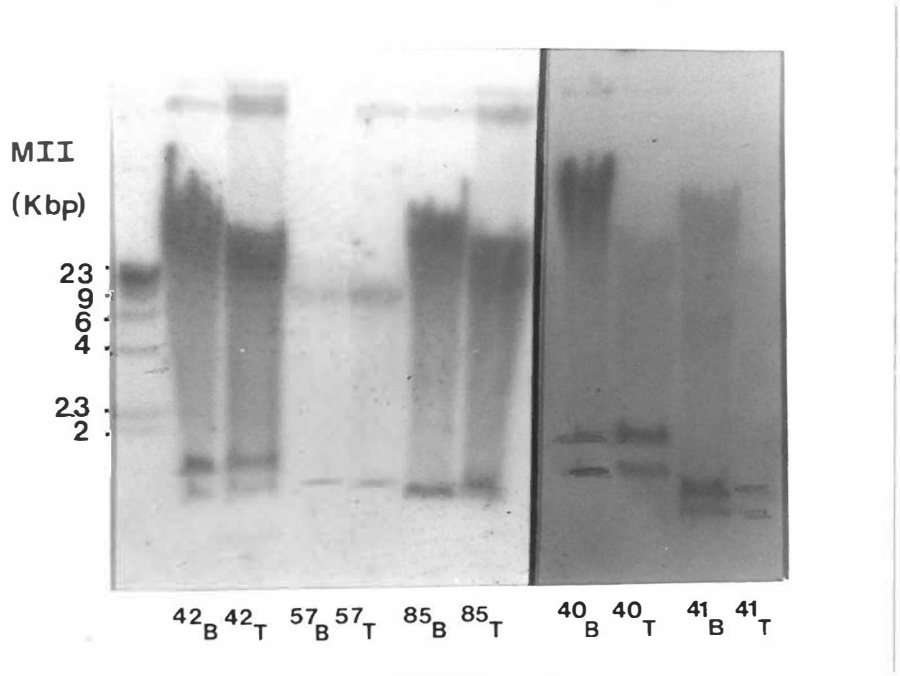

Fig 3.11. Autoradiograph showing VNTRs detected by ^{32}***P-pYNZ22*** *DNA probe in paired samples of blood and tumour. Samples* 40_B-40_T, 41_B-41_T *and* 42_B-42_T *were heterozygous and samples* 57_B-57_T *and* 85_B-85_T *were homozygous.*

3.4 DISCUSSION

i LOSS OF ALLELE

The results of this study suggest that the incidence of LOH of the p53 gene in cervical carcinomas from Black South African women is low. Only two (4%) out of 53 informative tumours demonstrated LOH as detected in a polymorphic region of intron 1 of the p53 gene while none of a subset of 29 tumours showed evidence of LOH using Southern hybridisation to a VNTR region telomeric to the p53 gene.

These findings contrast strongly with the frequency of LOH reported in other tumour types. For example, allelic deletions on chromosome 17p have been reported in 48%

cases of ovarian tumours (Frank *et al*, 1994), 58% of hepatocellular carcinomas (Fujimoto *et al*, 1994) and 76% of osteosarcomas (Toguchida *et al*, 1989). However the low frequency of LOH in carcinoma of the cervix reported here is in excellent agreement with the incidence reported in the three studies that have been published on this particular tumour type. Some authors (Fujita *et al*, 1992; Crook *et al*, 1992) did not find any evidence of LOH of the p53 gene in studies on 10 and 17 squamous cervical tumours, respectively, while another study (Busby-Earle *et al*, 1993) reported LOH in 3 out of 20 informative cases evaluated. Recently a Japanese study has reported a 35% frequency of allelic loss on chromosome 17p in a variety of uterine tumours; however these results were obtained using a selection of genomic probes (Jones *et al*, 1994).

The low incidence of LOH in carcinoma of the cervix, implies that if tumour suppressor genes are involved in the aetiology of this tumour, they may be found on chromosomes other than chromosome 17, as has been suggested in the literature (Riou *et al*, 1988; Yokota *et al*, 1989; Jones *et al*, 1994). On the other hand, the low frequency of LOH in carcinoma of the cervix, and by implication the low rate of p53 mutations suggests that tumours of the cervix may arise through an alternate mechanism. One possibility is a viral involvement. It is known that HPV which commonly infects cervical epithelium produces an oncoprotein that complexes to the normal p53 protein. This inactivation of the normal protein results in unregulated cell growth. To determine the possibility of a relationship between the p53 mutation status and HPV infection, all tumours examined for LOH of the p53 gene in this study were also examined for the presence of HPV DNA. The results are presented in the next chapter.

With respect to the two tumours in this study which showed LOH, reports in the literature (Baker *et al*, 1989; Crook *et al*, 1992; von Deimling *et al*, 1992; Nishida *et al*, 1993) suggest that they have a high probability of containing a p53 mutation. These tumours were therefore examined for the presence of mutations by single strand

conformational polymorphism (SSCP). This technique which allows rapid detection of point mutations is described together with the results in Chapter 5.

ii Methodology

In this study a novel approach has been used to detect LOH in which a short VNTR polymorphic region was amplified and examined by PAGE. The results, reflecting a low incidence of LOH, would appear to compare well with other studies on carcinoma of the cervix using Southern hybridisation. In addition there was a 100% agreement in the results obtained in the subset of tumours in this study which were examined by both methods. Due to the small tumour size and limited DNA available, a direct comparison of the tumours exhibiting LOH by the PCR based VNTR method with the Southern hybridisation technique was not possible.

From the point of view of convenience, the PCR based method has distinct advantages over the conventional DNA hybridisation technique. These include improved resolution, reliability, time taken to obtain results (24 h as opposed to at least 10 days), cost factors and a move away from the use of radioactivity. However the PCR based method also has disadvantages, in particular the fact that only approximately 50% of individuals are heterozygous and thus potentially informative for this particular polymorphism. One approach to increasing the number of informative cases would be to examine other polymorphic regions along the p53 gene and to establish additional PCRs and possibly even a multiplex PCR so that amplification of several loci could be carried out simultaneously. This approach should result in an increase in the number of informative cases and holds potential as a simple routine screen for the detection of LOH in the p53 gene (Runnebaum *et al*, 1994).

CHAPTER 4
HUMAN PAPILLOMA VIRUS (HPV) IN CERVICAL CARCINOMAS

4.1 INTRODUCTION

The results presented in Chapter 3 as well as evidence obtained by other workers (Lo *et al*, 1992; Busby-Earle *et al*, 1994) suggest that p53 mutations may not play an important role in tumorigenesis of the cervix. It is therefore relevant to investigate other factors implicated in this type of tumour.

There is a great deal of evidence that viruses, in particular the HPVs, are involved in the aetiology of cervical cancer (zur Hausen, 1987; Jenkins, 1994). It is reported that different types of HPVs can infect the female genital tract but not all of these are associated with progression to malignancy. Thus, depending on the capacity to induce a benign lesion or one with malignant potential, HPVs have been classified as low risk or high risk (Levine, 1991). Both classes of viruses have a similar genome organization. However, the high risk HPV types 16 and 18 encode major transforming proteins E6 and E7, expression of which can lead to *in vitro* immortalization of cell lines (Vousden *et al*, 1991). The E6 and E7 proteins produced by low risk HPV types on the other hand, fail to show this property in cells. The E6 oncoprotein encoded by HPV 16 and 18 is now known to bind to the p53 tumour suppressor protein to render its inactive, resulting in the loss of normal p53 functions within the cell (Werness *et al*, 1990). The net effect of this inactivation may be the same as a somatic mutation in the p53 gene.

Numerous studies have demonstrated the high incidence of HPV DNA in cervical

tumours (zur Hausen, 1987; Riou *et al*, 1990; Cooper *et al*, 1991; Das *et al*, 1992). Thus the purpose of this part of the study was to establish the incidence of HPV infection in the tumours already examined for LOH of the p53 gene and to compare these data. Several different methods have been used for the detection of HPVs such as Southern hybridisation with DNA probes and direct *in situ* hybridisation (ISH) (Blomfield, 1991; Cooper *et al*, 1991). These methods, however, sometimes lack sensitivity and specificity. The technique used for the detection of HPV DNA in this study addresses these shortcomings by first amplifying the HPV DNA using consensus primers, followed by Southern hybridisation with probes specific for the various HPV types.

4.2 AMPLIFICATION OF HPV DNA USING PCR

i PRINCIPLE

Since HPV DNA may not be present in tumours in abundant amounts, a region in an open reading frame which is common to all HPV types is amplified to provide sufficient DNA on which analyses can be made. The common region generally used is the conserved HPV protein coat gene L1, which is targeted for amplification by a set of consensus primers that are able to amplify a wide range of HPV types. This is possible by the substitution of different bases in the primers to render them degenerate. The level of degeneracy is such that the primers are complementary to the DNA of all so far sequenced HPV genomes (Chetsanga *et al*, 1991). In rare cases in which viral integration disrupts the L1 gene preventing its amplification, the early E6 open reading frame could be used.

ii METHOD

Clinical material

The DNA from 98 of the 101 patients on whom LOH studies were performed was included in this study. The remaining 3 were not analysed because of insufficient tumour DNA.

Polymerase chain reaction

The general principle and factors influencing the PCR are outlined in Chapter 2. In the present chapter details of the protocol to amplify HPV DNA are provided.

The primers and reaction conditions were those described by Manos *et al* (1989) and Resnick *et al* (1990). The primers have the following sequences:

Primer 1 (MY11)

5′ GC**M**CAGGG**W**CATAA**Y**AATGG 3′

Primer 2 (MY09)

5′ CGTCC**MARR**GGA**W**ACTGATC 3′

M = A + C

R = A + G

W = A + T

Y = C + T

Primer 1 is a sense strand sequence which binds to the complementary antisense DNA strand running from 3′ to 5′. Primer 2 is an antisense sequence and binds to the complementary sense DNA strand running from 5′ to 3′. These primers are degenerate at the positions indicated, at which one or other nucleotide is substituted to render them complementary to the DNA of different HPV types.

The amplified DNA fragment expected is approximately 450 bp in length and is common to all known HPV types, as well as to several undetermined types.

All primers were synthesized on a Milligen Oligonucleotide Synthesizer in the Department of Biochemistry at the University of Cape Town.

PCR procedure

Tumour DNA from the patients in this study was amplified as follows:
Each PCR reaction contained 16 μl DNA (ranging from 9 ng to 75 ng), 5 μl of Taq polymerase buffer, 2 mmol/l $MgCl_2$, 1 μmol/l of each primer, 200 μmol/l of each dNTP, and 1.25 units of Taq polymerase made up to a final volume of 50 μl with sterile

deionized water. The mixture (excluding DNA) was then aliquoted to each reaction tube using a positive displacement pipette.

The DNA was boiled for 10 min and quickly chilled on ice prior to being added to the PCR mixture. After spinning briefly a few drops of mineral oil were added to each tube. Silica gel was used in the heating block holes to ensure good thermal contact. Each run included a reaction mix without DNA to monitor for contamination of reagents.

The PCR was carried out in a Hybaid®DNA Thermal Cycle (Hybaid Ltd, UK) for 35 cycles of:

93°C for 1 min (denaturation)
55°C for 1 min (annealing)
72°C for 1 min (extension)

A final 5 min extension at 72°C completed the reaction.

PCR control

Human papillomavirus DNA may not be present in every tumour sample analyzed, and the use of a control PCR is therefore essential to monitor the integrity of the DNA analysed and to check for the presence of inhibitors of the PCR. The control is usually a pair of primers which amplify a single-copy human gene that is certain to be present in the sample. In this case a pair of primers which amplify the β-globin gene was used. The PCR product of the control primer needs to be sufficiently different in size from that of the target product to enable a clear distinction between the two bands.

The concentration of the reagents and the cycling conditions for the amplification of the β-globin gene were the same as for the HPV amplification, with the exception that a lower primer concentration (100 nmol/l of each primer) was used.

The β-globin primer sequences are as follows:

Primer 1 (GH20)

5′ GAAGAGCCAAGGACAGGTAC 3′

Primer 2 (PCO4)

5′ CAACTTCATCCACGTTCACC 3′

The expected PCR product is 300 bp.

Analysis of PCR product

A 5 μl aliquot of the PCR product was electrophoresed on a 2% agarose gel at room temperature for 1 h at 100 V. The molecular weight marker VI (Boehringer) was also included on the gel. Following staining with ethidium bromide, a band of 300 bp which corresponds to the β-globin product and a band of 450 bp representing HPV amplification were seen (Fig 4.1).

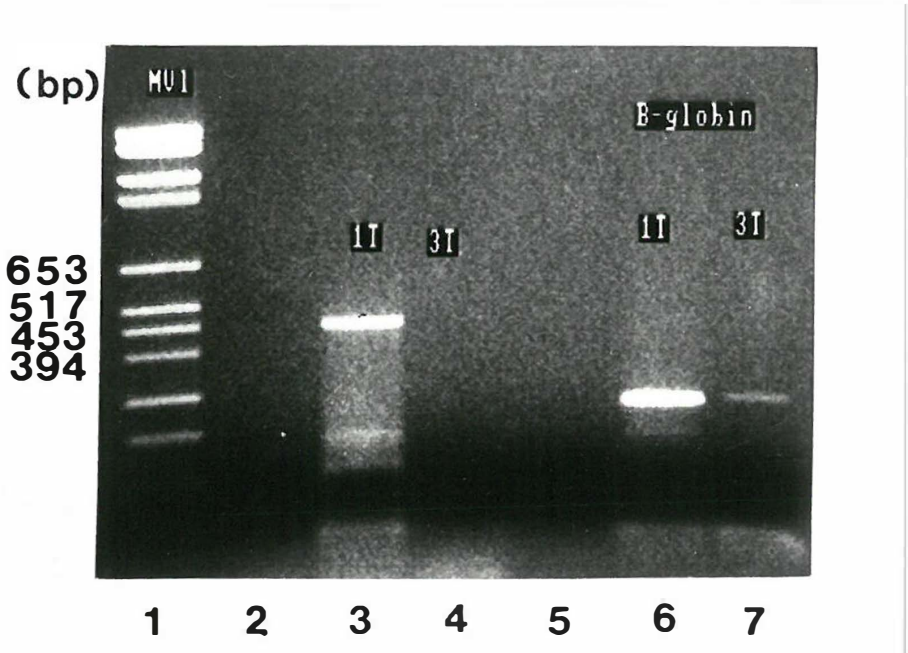

Fig 4.1. Agarose gel (2%) showing the PCR product of HPV amplification and β-globin amplification. ***Lane 1****, molecular weight marker VI (Boehringer);* ***lane 3****, HPV amplification in sample 1T;* ***lane 4, absence of*** *HPV amplification in sample 3T;* ***lane 6****, β-globin amplification in sample 1T;* ***lane 7****, β-globin amplification in sample 3T.*

Initially primers for both HPV and β-globin amplification were included in the same PCR mixture. In some reactions however, inhibition of amplification apparently occurred in the presence of both sets of primers (lane 7, Fig 4.2). Thereafter separate PCR reactions were carried out for each set of primers, β-globin amplification being performed on those samples which did not amplify HPV DNA.

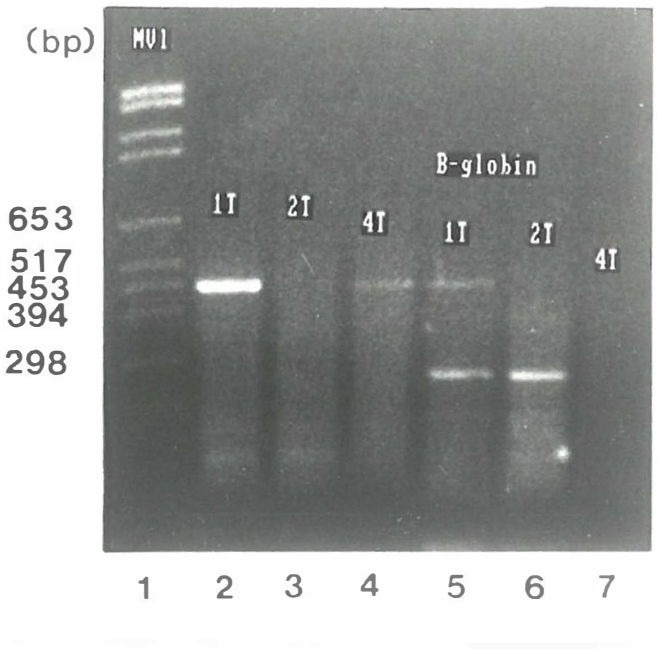

Fig 4.2. Agarose gel (2%) showing the amplification of HPV and β-globin DNA. ***Lane 1****, molecular weight marker VI (Boehringer);* ***lane 2****, HPV amplification in sample 1T;* ***lane 3****,* ***absence*** *of HPV amplification in sample 2T;* ***lane 4****, HPV amplification in sample 4T;* ***lane 5****, HPV and β-globin amplification in sample 1T;* ***lane 6****, β-globin amplification in the absence of HPV amplification in sample 2T;* ***lane 7****,* ***absence*** *of both HPV and β-globin amplification in sample 4T.*

Precautions taken during PCR procedure

Precautions taken during the PCR procedure were the same as those described in Chapters 2 and 3. Despite these precautions the use of cloned DNA control material gave rise to some difficulties. Because of the extreme sensitivity of the PCR technique

to contamination by minute amounts of template, previously amplified products or cloned DNA which are easily spread by aerosol can present a serious problem when amplifying samples (McCance and Hollingworth, 1990), as is seen in Fig 4.3.

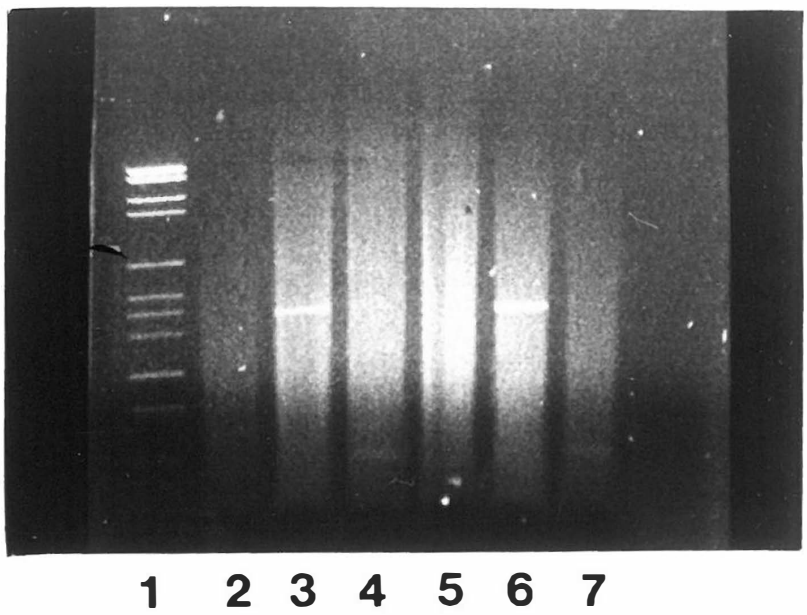

Fig 4.3. Agarose gel (2%) showing intense smearing believed to be due to contamination with cloned DNA. ***Lane 1****, molecular weight marker VI (Boehringer);* ***lane 2****, no DNA control;* ***lanes 3 to 7****, HPV amplifications of different samples.*

This contamination was successfully eliminated only by vigorous decontamination procedures including UV irradiation of all pipettes, racks and similar equipment, and the disposal of all reagents in use at that time.

The use of cloned HPV DNA as a control for HPV types is therefore not advisable on a regular basis (A. Williamson, personal communication) and it is recommended that to avoid recurrent problems of contamination these controls are used only once in order to verify PCR product.

4.3 HPV TYPE IDENTIFICATION USING SOUTHERN ANALYSIS

Agarose gel electrophoresis of the amplified product, followed by ethidium bromide staining merely allows visualization of the PCR product. It is therefore always necessary to confirm the presence of the target-DNA and in this case to identify the specific HPV type. This was performed by Southern analysis using specific non-radioactively labelled oligoprobes.

i PRINCIPLE

The principle and procedures of Southern blot analysis have been discussed previously (Chapters 2.3 vi and 3.3 iii). Slight modifications to the procedure were made because of the use in this instance of small synthetic nucleotide probes as opposed to large DNA probes.

ii METHOD

The probes used to confirm the presence of HPV and to identify the HPV type were oligoprobes which were a gift from Dr Anna-Lise Williamson (Dept of Medical Microbiology, University of Cape Town).

* *Generic probe.*

This probe detects DNA from the common L1 region of the HPV, the presence of which indicates infection by one or more types of HPV. The sequence is as follows:

5′ CTGTTGTTGATACTACACGCAGTAC 3′

* *Type specific probes for HPV 16 and HPV 18.*

Each of these type specific probes recognises a region that is unique to the correspondent HPV type. The sequences of these probes are:

HPV 16 (MY14)
5′ CATACACCTCCAGCACCTAA 3′

HPV 18 (WD74)
5′ GGATGCTGCACCGGCTGA 3′

All probes were labelled with a 3′ End Labelling Kit (Boehringer) according to the manufacturer's instructions. The transfer of DNA to nylon membranes, hybridisation and visualization are described in detail in Chapter 2.3 vi. Briefly, the blotted membranes were hybridized in Bijou bottles for 1 h at 55°C in 2.5 ml of hybridisation buffer containing 5 μl of labelled probe. After hybridisation the filters were washed and the membranes exposed to 10 ml of a colour reactant to allow development of a visible blue precipitate, the presence of which indicates successful hybridisation to a particular HPV probe.

iii RESULTS

Of the 98 cervical tumour DNA samples amplified using consensus primers, 78 (80%) exhibited a PCR product while 20 (20%) did not show any evidence of amplification. These 20 samples were also analysed for the presence of the β-globin gene to ensure that there were no inhibitors present and that there was amplifiable DNA. All 20 DNA samples showed amplification of the β-globin gene.

The presence of HPV DNA was confirmed in all 78 samples demonstrating amplification by the successful hybridisation with a generic HPV probe. In 76 samples (97%) the HPV DNA was typed as HPV 16 and in 6 (8%) samples as HPV 18. Four tumours were found to contain DNA for both HPV 16 and HPV 18.

No evidence of HPV infection was found in tumours **95** and **107**, both of which were previously shown to have LOH of the p53 gene (Fig 4.4).

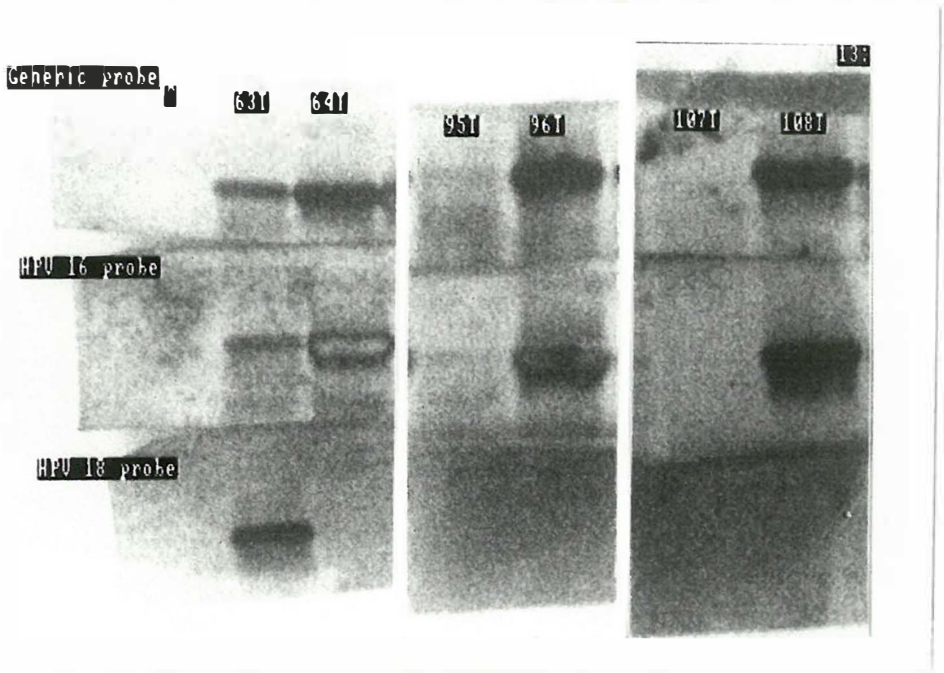

Fig 4.4. Nylon blots showing hybridisation of generic, HPV 16 and 18 DNA probes. ***Tumour 63****, positive for the generic HPV and both HPV 16 and HPV 18;* ***tumours 64, 96 and 108****, positive for only the generic and the HPV 16 probe; tumours* ***95 and 107*** *no evidence of HPV infection.*

4.4 DISCUSSION

i INCIDENCE OF HPV INFECTION

The 80% incidence of HPV 16 and/or HPV 18 infection in the cervical tumours in this study is in concordance with other reports of a 79% to 98% incidence (Riou *et al*, 1990; Higgins *et al*, 1991; Das *et al*, 1992; Arends *et al*, 1993). There are however some studies which indicate a slightly lower frequency in the range of 65 to 70% (Cooper *et al*, 1991; Hagmar *et al*, 1992). In this study the HPV 16 was found to be the more prevalent HPV type in squamous cervical tumours which is also in agreement with findings reported in the literature.

The reasons for the variations in reported frequencies of HPV in cervical tumours are unclear. One reason could be the lack of sensitivity and specificity of some of the earlier methods which were based on cytological and histological identification of HPV in cervical smears (Chetsanga *et al*, 1991) and later methods which detected HPV by Southern hybridisation. Nowdays sensitivity has been improved by the use of PCR to detect even very small amounts of HPV DNA and specificity is increased by the use of oligonucleotide probes under stringent hybridisation conditions. Another possible explanation could be variations in patterns of social behaviour between study groups. Since HPV is a sexually transmitted virus, its frequency in sexually promiscuous groups may be expected to be higher (Blomfield, 1991). A further reason could relate to the disease stage at which tumours were biopsed, since is known that HPV plays a role in the early intraepithelial stages of tumour progression but that viral DNA sequences can be lost at later stages (Riou *et al*, 1990).

In this study the method employed is the same as that being used in several centres worldwide and is reported to be both sensitive and specific. Further precautions were taken by the inclusion of a control PCR to eliminate the possibility of inadequate DNA template and /or PCR inhibitors.

ii RELATIONSHIP OF HPV TO LOH IN THE p53 GENE

The most striking feature to emerge from this study was the fact that neither tumours which exhibited LOH of the p53 gene had any evidence of HPV infection. This finding clearly supports the hypothesis that in the absence of HPV infection somatic mutations of the p53 gene may contribute to cervical carcinogenesis, and is in complete accord with the observations of Crook *et al* (1991), Busby-Earle *et al* (1992), Crook *et al* (1992), Kaelbling *et al* (1992) and Paquette *et al* (1993). All these workers consistently reported higher rates of p53 abnormalities in HPV negative tumours when compared with HPV positive tumours. However the study groups were small, ranging between about 20 and

42 patients each. The current study examined 101 cervical tumours of which the HPV status was known in 98% of cases.

As mentioned previously (Chapter 3.4 i), the two tumours manifesting LOH which have now been shown to be HPV negative have a high probability of harbouring a p53 mutation. They were therefore screened for mutations by single strand conformational polymorphism (SSCP) analysis together with the other tumours which did not contain HPV. The results are presented in the next chapter.

The converse to these findings is that none of the 78 tumours which were found to contain DNA for HPV 16 and/or 18 showed any evidence of p53 mutation as determined by LOH studies. It is therefore more likely that any p53 involvement in these tumours is through viral oncoprotein inactivation of the normal p53 protein. In the future it is intended to confirm these findings by screening all the HPV positive tumours for the absence of p53 mutations using SSCP analysis.

CHAPTER 5

DETECTION OF MUTATIONS BY PCR-SINGLE STRAND CONFORMATIONAL POLYMORPHISM (SSCP)

5.1 INTRODUCTION

Single strand conformational polymorphism is reported to be a convenient and rapid method for the detection of mutations (Newton and Graham, 1994a). It was therefore decided to use this technique to examine the two tumours which demonstrated LOH of the p53 gene for evidence of mutations. In addition, since reports in the literature indicate that tumours which do not contain HPV are more likely to carry p53 mutations (Crook *et al*, 1991; Scheffner *et al*, 1991), most of the HPV negative tumours identified in this study were also examined for mutations using this technique. Exons 5, 6, 7 and 8 were selected for screening as more than 90% of the p53 mutations in human cancers are believed to occur in well conserved regions of these exons (Hollstein *et al*, 1991).

The original SSCP protocol makes use of radiolabelled PCR primers to generate a product sensitive enough to allow visualisation of the single stranded fragments (Orita *et al*, 1989). This approach, however, is both expensive and cumbersome. In a move away from the use of radioisotopes, the technique chosen for this study was a rapid 'cold' SSCP analysis in which unlabelled PCR primers were used and the single stranded DNA fragments were visualized on polyacrylamide gel using ethidium bromide (Hongyo *et al*, 1993).

5.2 PCR-SSCP

i PRINCIPLE

The principle of SSCP has already been discussed in Chapter 2. Briefly, this technique depends upon the fact that under non-denaturing gel conditions, single-stranded DNA molecules fold into unique conformations which are determined by their nucleotide sequence. Thus by comparison of differences in mobility of wild-type (normal) and mutation-bearing single strand DNA fragments through a solid support, such as polyacrylamide gel, different patterns can be visualised with the aid of radioisotopes, ethidium bromide, silver nitrate or chemiluminescence (Orita *et al*, 1989; Hongyo *et al*, 1993; Faille *et al*, 1994; Niwa *et al*, 1994). Evidence of a mutation is usually defined as a distinct shift of one or both of the wild-type bands or the presence of at least one additional band.

ii METHOD

Clinical material

The two cervical tumours (**95** and **107**) which showed evidence LOH of the p53 gene were subjected to PCR-SSCP analysis. A further 16 tumours were included in the analysis, none of which had any evidence of HPV infection. In addition, DNA from a **breast tumour** (**3T**) in which LOH had been demonstrated previously, was included.

DNA control

DNA samples from two HPV negative cervical cancer cell lines, HT3 and C33A, known to carry point mutations in exons 7 (codon 245) and 8 (codon 273), respectively, were also analyzed. These were a gift from Dr Tim Crook of the Rayne Institute, Kings College School of Medicine, London.

Polymerase chain reaction

As mentioned previously the first step in the preparation of DNA for analysis by SSCP is amplification of the region of interest. The general principle and the factors influencing the PCR are outlined in Chapter 2. In the present chapter the protocols used to amplify exons 5 to 8 of the p53 gene are described in detail.

a Design of the primers

Although numerous publications are available describing procedures for PCR-SSCP analysis of the p53 gene, there is little uniformity on the sequences of the primers used or explanation as to the choice of these sequences. This made the selection of primers difficult. It was therefore decided to choose primers for this study based on the following specific criteria (Bangham, 1991):

* Similar primer pair length (18-21 bases) and composition, so that Tms are within 5°C of each other.
* Similar GC content for both primers (ideally 50-60%).

* No self-complementarity or complementarity with the other primer.
* No runs of three or more Gs or Cs at the 3′ end of the primer.
* Amplified product not greater than 300 bp in size.
* Primer sequences selected flanking introns so that the complete exon sequence is included in the PCR product.

The primers were synthesized on a Gene Assembler (Pharmacia, LKB Biotechnology, Sweden) in the Faculty of Medicine Molecular Biology Unit of the University of Natal.

The sequences of each primer pair are shown below:

Exon 5	bp	A+T	G+C	Tm	GC%
5′ TCTGTTCACTTGTGCCCTGAC 3′	21	10	11	64	52
5′ ATCAGTGAGGAATCAGAGGC 3′	21	10	11	64	52

These primers flank the following region in exon 5:

13003→

TCTGTTCACTTGTGCCCTGACtttcaactctgtctccttcctcttcctacagtactcccctgccctca

←13310

.....acgacagggctggttgcccagggtccccaGGCCTCTGATTCCTCACTGAT

The expected size PCR product is **308** bp.

Exon 6	bp	A+T	G+C	Tm	GC%
5′ AGAGACGACAGGGCTGGTT 3′	19	8	11	60	58
5′ AAAGCCCCCCTACTGCTCA 3′	19	8	11	60	57

These primers flank the following region in exon 6:

13257→

AGAGACGACAGGGCTGGTTgcccagggtccccaggcctctgattcctcactgattgctcttagg.....

←13516

.....ggggttaagggtggttgtcagtggccctccgggTGAGCAGTAGGGGGGCTTT

The expected size is **259** bp.

Exon 7	bp	A+T	G+C	Tm	GC%
5′ AAAAAAGGCCTCCCCTGCTTG 3′	21	10	11	64	52
5′ GGTGGATGGGTAGTAGTATGG 3′	21	10	11	64	52

These primers flank the following region in exon 7:

13924→

AAAAAAGGCCTCCCCTGCTTGccacaggtctccccaaggcgcactggcctcatcttg.....

←14222

.....tgacccctgggcccacctcttaccgatttctt**CCATACTACTACCCATCCACC**

The expected size is **298** bp.

Exon 8	bp	A+T	G+C	Tm	GC%
5′ GGACCTGATTTCCTTACTGCC 3′	21	10	11	64	52
5′ TGCACCCTTGGTCTCCTCCA 3′	20	8	12	64	60

These primers flank the following region in exon 8:

14402→

GGACCTGATTTCCTTACTGCCtcttgcttctcttttcctatcctgagtagtggtaatct.....

←14632

.....aagcaagcaggacaagaagcgg**TGGAGGAGACCAAGGGTGCA**

The expected size is **231** bp.

b Annealing temperature and cycling conditions

All primers have similar Tms which allowed the use of the same annealing temperature to amplify exons 5, 6, 7 and 8. After an initial denaturation step at 94°C for 5 min, each reaction mix was subjected to 30 cycles at the following temperatures:

94°C for 1 min (denaturation)

59°C for 1 min (annealing)

72°C for 1 min (extension)

A final 10 min extension at 72°C completed the reaction.

c PCR procedure

Each reaction contained 16 μl DNA (approximately 9 to 75 ng), 5 μl of Taq polymerase buffer, 2 mmol/l $MgCl_2$, 200 nmol/l each primer, 200 μmol/l

dNTPs and 1.5 units of Taq DNA polymerase made up to a final volume of 50 μl with sterile deionized water. The exact procedure followed to set up the reaction was the same as that described in Section 3.2 iii.

The DNA was boiled for 10 minutes and quickly chilled on ice prior to being added to the PCR mixture. After spinning briefly a few drops of mineral oil were added to each tube. The reaction mixes were then subjected to the cycling conditions described above. Each run included a reaction mix without DNA to monitor for contamination of reagents.

d Precautions taken during the PCR procedure

The precautions taken to avoid contamination in the PCR were the same as those described in Section 3.2 iii.

e Identification of the PCR products

A 6 μl aliquot of the PCR product mixed with 1.5 μl of loading buffer (0.25% bromophenol blue, 40% sucrose, 1 mg/ml of ethidium bromide made up to 2 ml with 0.5 x TBE) was loaded on to a 2% agarose gel (as described in Section 2.3 iii). The gel was electrophoresed at room temperature for 1 h at 90 V. A molecular weight marker (HinfI-Promega) was included in each gel.

The successful amplification of each exon was confirmed by the identification of a band of the appropriate base pair size (Fig 5.1). The presence of unambiguous bands made purification of the PCR product unnecessary.

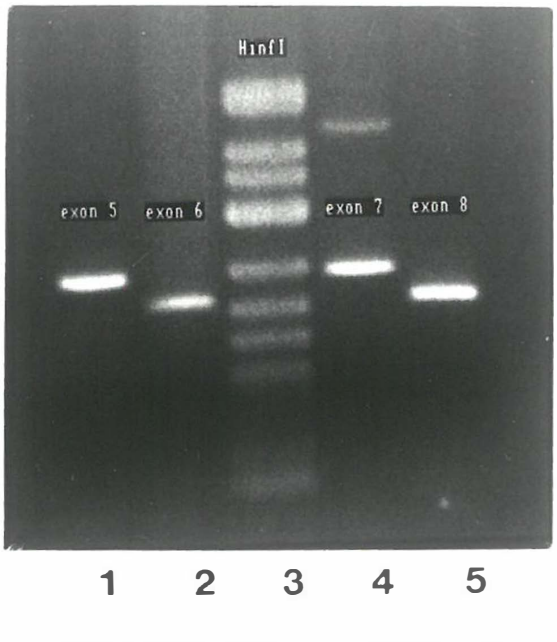

Fig 5.1. Agarose gel (2%) showing the amplification products of the various exons. ***Lane 1****, amplification product (308 bp) of exon 5;* ***lane 2****, amplification product (259 bp) of exon 6;* ***lane 3****, molecular weight HinfI (Promega);* ***lane 4****, amplification product (298 bp) of exon 7;* ***lane 5****, amplification product (231 bp) of exon 8.*

f Optimization of the PCR

All 4 exons amplified satisfactorily in the first instance under the conditions described above. It was therefore unnecessary to carry out extensive

optimization procedures. Slight adjustments were however made to the annealing temperature to eliminate minor non specific bands.

SSCP analysis

As suggested in Chapter 2 (Section 2.3 vii) several factors are critical for successful SSCP analysis. Each of these was addressed carefully in an attempt to obtain optimal separation of the single strands. The final validated procedures are presented below.

a Denaturation of the PCR product

A mixture consisting of 5 μl of PCR product, 1 μl of loading buffer (0.25% bromophenol blue, 0.25% Xylene-Cyanol, 30% Ficoll in 1 x TBE buffer), 13.6 μl of 1 x TBE and 0.4 μl of 1 mol/l NaOH was prepared to yield a total volume of 20 μl. This was heated at 98°C for 5 min and then immediately placed on ice prior to loading the entire 20 μl on to the gel.

Adequate denaturation of the PCR product was vital to the detection of single strand bands. It was found that in the presence of an insufficient amount of NaOH, only the double stranded product was visualized (results not shown).

b Electrophoresis conditions

A 10% polyacrylamide mini gel (8 cm x 8 cm x 0.75 mm) containing 4% glycerol was used. This was made as described in Section 2.3 iii. The buffer chamber of the electrophoresis apparatus was filled with 1 x TBE buffer and the apparatus was connected to a thermostatically controlled circulating water bath (Ephortec™, Haake Buchler Instruments, Inc. Saddke Brook, NJ), to maintain a constant temperature during electrophoresis.

After loading the gel with the PCR mixture prepared as described above, electrophoresis was carried out at 300 V at the temperature appropriate for the exon being examined, until the bromophenol blue marker reached the bottom of the gel. The running time was approximately 45 min.

A polyacrylamide gel of 10% was found to give the sharpest bands under the electrophoretic conditions described above (results not shown).

c Temperature

Each exon required a different temperature which was determined empirically, for optimal separation of the single stranded fragments. The optimal temperatures were determined as 27°C for exon 5, 14°C for exon 6, 15°C for exon 7 and 4°C for exon 8.

d Visualisation and documentation

The polyacrylamide gel was stained with a 0.5 μg/ml solution of ethidium bromide in 1 x TBE for 20 min, and then destained in distilled water for 5 min and documented as described in Section 2.3 iii.

iii RESULTS

SSCP analysis

Each of the four exons examined showed two single strand bands on PAGE under the conditions described above (Fig 5.2a,b). These bands represent the normal or wild-type single strand conformations. Samples of DNA carrying known mutations in exons 7 and 8 (HT3 and C33A, respectively) clearly demonstrated band shifts on SSCP analysis of the appropriate exon (Fig 5.2b). No DNA with mutations in exons 5 or 6 was available for validation.

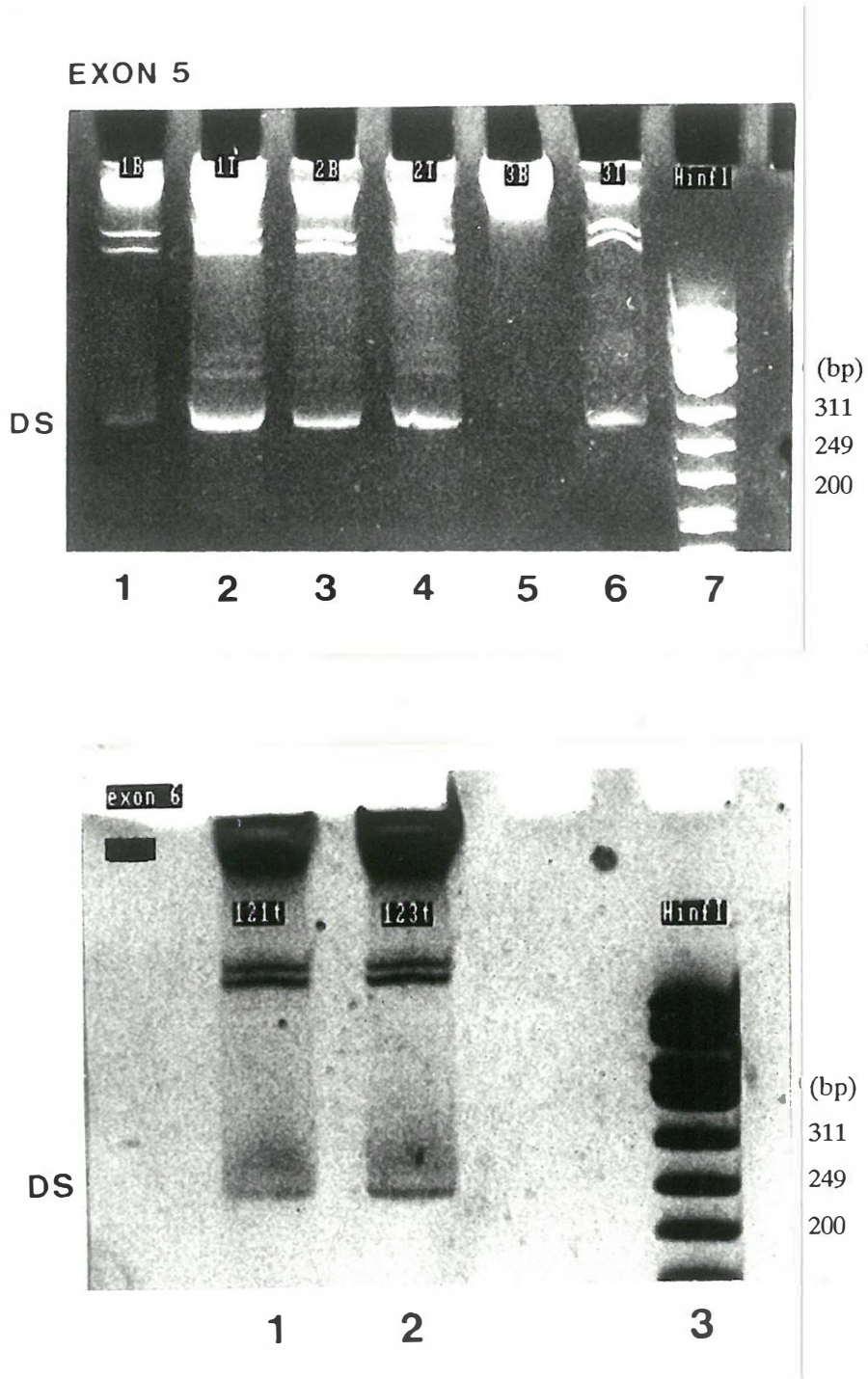

Fig 5.2a. Polyacrylamide gel (10%) showing single strand fragments of exons 5 and 6 following amplification by PCR. Doubled stranded DNA is indicated by DS. The numbers on each lane indicate blood (B) or tumour (T) DNA from patients with cervical tumours.

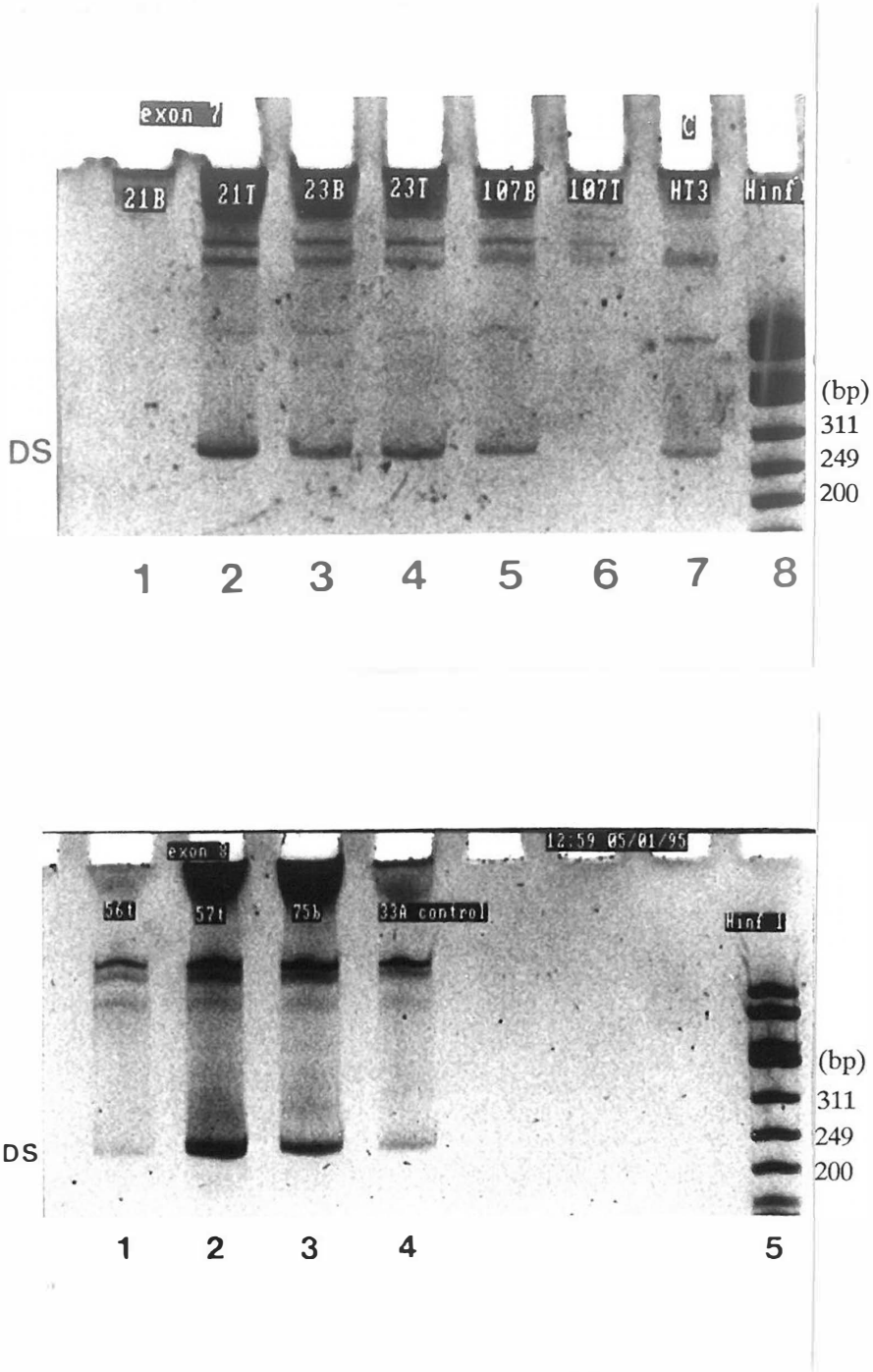

Fig 5.2b. Polyacrylamide gel (10%) showing single strand fragments of exons 7 and 8 following amplification by PCR. Note the distinct band shifts in samples ***HT3 (lane 7)*** *and* ***C33A (lane 4)*** *which contain known mutations in* ***exons 7 and 8****, respectively. Double stranded DNA is indicated by DS. The numbers on each lane indicate blood (B) or tumour (T) DNA from patients with cervical tumours.*

The temperature at which each exon was subjected to PAGE was found to be critical to within one or two degrees. This can be seen in Fig 5.3, in which the upper single

strand of exon 6 appears to have an alternative conformation at a suboptimal temperature (12°C). At 14°C only two single strand bands are visible. This phenomenon was also observed in exon 5.

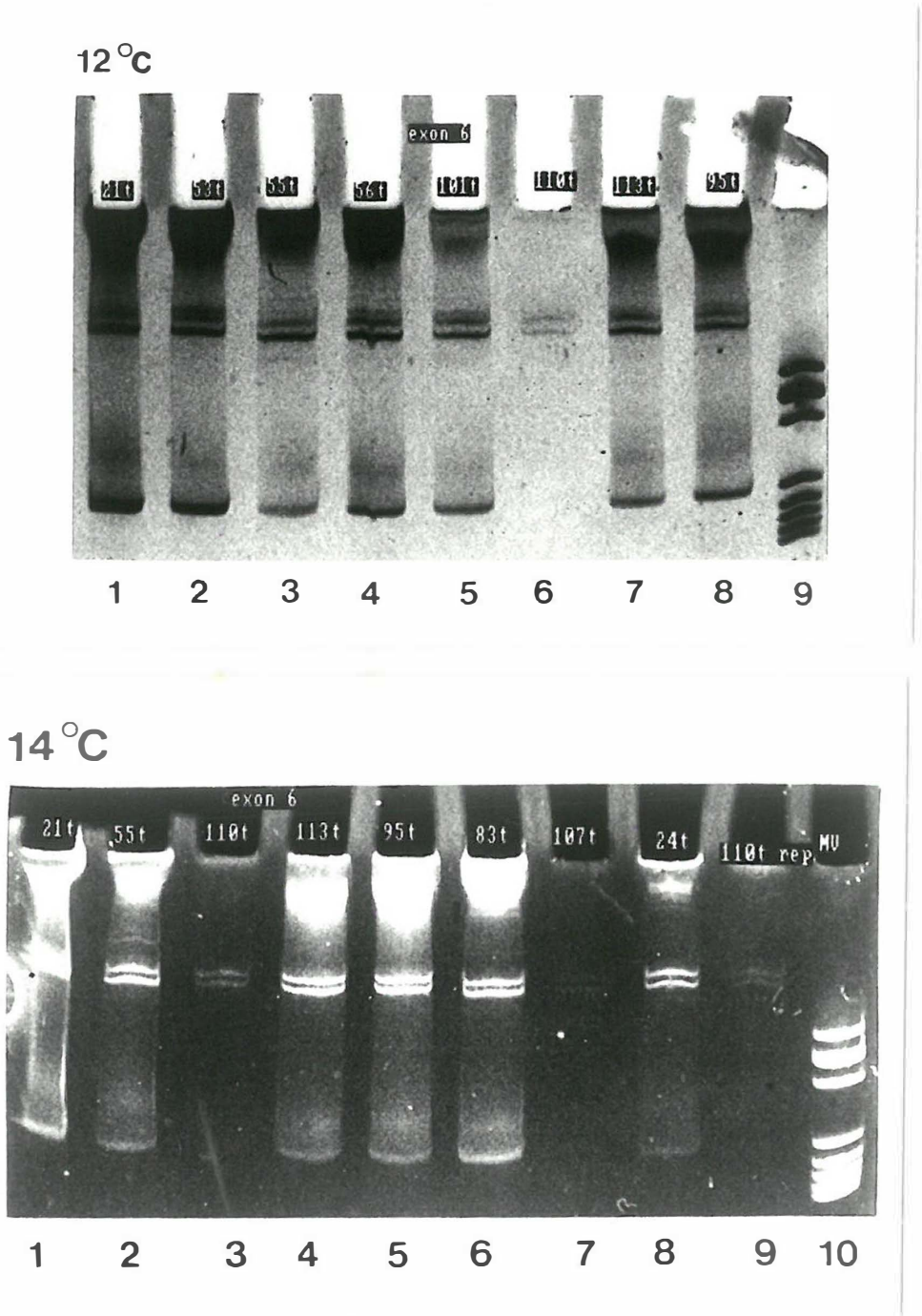

Fig 5.3. Polyacrylamide gel (10%) showing different conformations of exon 6 following PAGE at 12°C and 14°C. Note that the same PCR product from tumours ***21, 55, 110, 113*** *and* ***95*** *was run on each gel.*

Detection of mutations

No evidence of mutations as determined by band shifts or additional bands in exons 5, 6, 7 or 8 of the p53 gene was detected in either of the two tumours (**95** and **107**) which demonstrated LOH of the p53 gene. Neither was there evidence of mutations in any of the 16 HPV negative cervical tumours.

The DNA from a breast tumour (**3T**) which was included in the SSCP analysis because it demonstrated LOH, was found to exhibit a slight shift downwards in both bands of exon 5 (Fig 5.4).

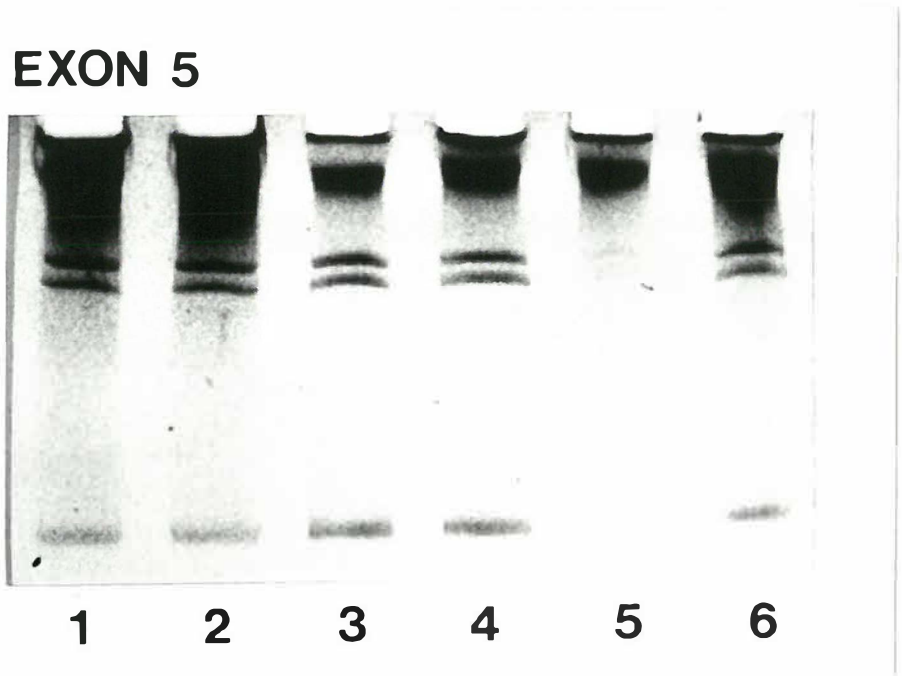

Fig 5.4. SSCP analysis of exon 5 which shows downward band shifts of both single strand fragments from the breast tumour ***(3T)*** *in* ***lanes 1 and*** *2 when compared with wild-type in* ***lanes*** *3* ***and*** *4.*

5.3 DISCUSSION

i METHODOLOGY

The conventional approach to PCR-SSCP analysis as first described by Orita *et al* (1989) made use of radio-labelled primers and large formatted polyacrylamide gels, which allow good resolution and sensitive detection of single strand fragments. Recently however, there have been attempts to move away from the use of isotopes with the introduction of chemiluminescence, silver nitrate and ethidium bromide for the detection of single strand fragments (Hongyo *et al*, 1993; Arai et al, 1994; Niwa *et al*, 1994). At the same time simplification of the technique in terms of the use of readily available and easily handled small polyacrylamide gels has been a convenient innovation. The method adopted for use in this study is based on one such approach, that of Hongyo *et al* (1993).

The primers used for the amplification of exons 5 to 8 were selected specifically for this study because on analysis of the literature, ambiguities were found in a number of the published primer sequences and it was deemed advisable to apply the same set of criteria to the choice of all primers. The primers selected appear to have been satisfactory with respect to the PCR product obtained since all 4 exons amplified, resulted in clear unambiguous bands.

Under appropriate conditions the analysis by SSCP of the 4 exons yielded two single strand bands in each instance. A direct comparison of these bands with those reported in the literature was impossible due to the variety of conditions under which SSCP is applied. Comparison with the method of Hongyo *et al* (1993) however, revealed apparently similar single strand bands, at least for the exons illustrated. In addition the behaviour of the single strand fragments observed in this study, particularly with respect to the temperature at which electrophoresis was carried out, was consistent with that reported by Hongyo *et al* (1993).

With respect to the two DNA controls for exons 7 and 8, both demonstrated clear band shifts, as did the DNA from the breast tumour (3T) in exon 5. The breast tumour DNA was sent for analysis to Dr Tim Crook of the Rayne Institute, Kings College School of Medicine, London who confirmed band shifts in exon 5 (personal communication). This sample was of particular interest because, having been shown to have loss of one allele, it would be expected to have only two bands in the apparently mutated form, as was found to be the case. Similarly, in view of the fact that neither of the two controls showed more than two bands, it seems likely that both these cell lines exhibit a 17p LOH.

Thus the PCR-SSCP technique described here would appear to fulfil the criteria of a convenient, economical, rapid (less than two hours), and sensitive method for the detection of mutations in the p53 gene, bearing in mind that it is a screening technique and not a definitive identification. Potential problems due to factors such as poor optimization and inadequate denaturation of the DNA have been addressed. Failure of SSCP analysis in some instances could possibly be due to non-controllable factors, such as the position and nature of the mutation since this, together with the sequence of the fragment being analysed, affects the ultimate conformation assumed and consequently the mobility upon gel electrophoresis (Weghorst and Buzard, 1993).

ii p53 MUTATIONS

Of a total of 18 HPV negative tumours screened for mutations in exons 5 to 8 of the p53 gene, none showed any evidence of a mutation. These results reflect once more the apparently low frequency of p53 mutations in cancer of the cervix in Black South African women. Only two other studies are reported in which SSCP was used as a screen for mutations in HPV negative tumours: Fujita *et al* (1992) who observed no abnormalities in 7 tumours and Park *et al* (1994) who identified SSCP abnormalities in 2 out of 21 tumours. Both these tumours were subsequently confirmed by direct sequencing to have missense mutations in exons 5 and 8. In addition Crook

et al (1992) showed point mutations by direct sequencing in 3 out of 3 tumours which lacked HPV. In a recent publication Park *et al* (1994) reviewed the literature and reported a 15% incidence (6/41) of p53 mutations in HPV negative tumours. In contrast, in HPV positive tumours an incidence of only 3% (7/206) was recorded. It is interesting to note that all 13 mutations so far reported in tumours of the cervix, as well as the two cell lines, were found in exons 5 to 8.

It was disappointing that in this study it was not possible to demonstrate evidence of p53 mutations on the basis of SSCP mobility shifts in any of the 18 HPV negative tumours. This was particularly so in the case of the two tumours which exhibited LOH of the p53 gene, a feature associated with mutations in the corresponding allele in over 80% of tumours (Hollstein *et al*, 1991). However, it should be borne in mind that this study undertook SSCP analysis in exons 5, 6, 7 and 8 only, and the possibility exists that there may be mutations present elsewhere in the gene.

To address this possibility DNA from tumours **95** and **107** (those showing LOH) was sent to Dr Tim Crook of the Rayne Institute for further SSCP analysis. He confirmed the absence of any aberrant SSCP bands in exons 5 to 8, but reported mobility shifts in exons 2/3 in both these tumours. These findings will in due course be validated by direct sequencing (personal communication).

In view of this communication, it would appear therefore that LOH as detected by the PCR based VNTR technique described here does in fact predict the presence of a

mutation as assessed by SSCP analysis. This is further substantiated by the identification of band shifts in exon 5 of the breast tumour which exhibited LOH.

If these abnormalities are indeed confirmed by sequencing, this will constitute a very interesting finding since all p53 mutations so far reported in cervical tumours are in exons 5 to 8 (Park *et al*, 1994). It is possible that in the Black South African population, a novel pattern of p53 mutations exists in tumours of the cervix, and perhaps in other tumour types. A similar observation has been reported recently in a cohort of Black American breast cancer patients who show a different pattern of p53 mutations and have a high mortality (Blaszyk *et al*, 1994).

CHAPTER 6

CONCLUSIONS

The primary objective of this project was to undertake a study into the possible involvement of the p53 tumour suppressor gene in tumours of the cervix amongst Black South African women. A study of this nature is timely, both because the p53 gene has not been widely researched in this malignancy, and because cancer of the cervix is the most prevalent tumour in this population group.

The results obtained using LOH of the p53 gene as a screen for mutations suggest that p53 mutations are not a common event in squamous carcinoma of the cervix since only a 4% incidence of LOH was detected in informative tumours. This observation was substantiated by the absence of p53 mutations as determined by PCR-SSCP analysis in exons 5 to 8 of tumours without any evidence of HPV infection. Abnormalities on PCR-SSCP analysis were subsequently shown in exons 2/3 of the two tumours exhibiting LOH, which led to the conclusion that LOH of the p53 gene does indeed predict the presence of a mutation as assessed by SSCP. These findings reflect reports in the literature which indicate a composite 5% incidence of p53 mutations in a total of 247 cervical tumours studied (Park *et al*, 1994); all these mutations occurred in exons 5 to 8.

Thus the results of this study suggest firstly, that the apparent low incidence of p53 mutations in tumours of the cervix in Black South African women is similar to that seen in other populations. Secondly, the two cases of LOH of the p53 in this study occurred exclusively in HPV negative tumours, a group which comprised only 20% of the total study. This implies that mutations in the p53 gene may not play a major role in carcinogenesis of the cervix and that the more significant contributory factor is likely to be a viral involvement, probably through the HPV E6 inactivation of the normal p53 protein.

Thirdly, the presence of SSCP abnormalities in exons 2/3 of the two tumours with LOH, suggests that the aetiology of cancer of the cervix involving genetic alterations in the p53 gene in Black women, may differ from that in other ethnic groups.

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