ABERRATIONS IN THE RETINOBLASTOMA SUSCEPTIBILITY GENE IN TUMOURS FROM SOUTH AFRICAN OESOPHAGEAL CANCER PATIENTS

by

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To my parents

DECLARATION

I, the undersigned, hereby declare that **"Aberrations in the Retinoblastoma susceptibility gene in South African oespohageal cancer patients "**, is my own work and has not previously in its entirety, or in part, been submitted at any university for a degree. All the sources that I have used or quoted have been indicated and acknowledged by means of complete references.

J. Gamieldien

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LIST OF ABBREVIATIONS

AC	Adenocarcinoma
APC	Adenomatous Polyposis Coli gene
bp	base pairs
CDK	Cyclin Dependent Kinase
cDNA	complementary DNA
CI	chloroform-isoamylalcohol
DCC	Deleted in Colorectal Cancer gene
DNA	Deoxyribonucleic Acid
GITC	Guanidinium isothiocyanate
HD	Heteroduplex Analysis
kb	kilobasepairs
LOH	Loss of heterozygosity
M	Mitotic phase
MCC	Mutated in Colorectal Cancer gene
MDE	Mutation Detection Enhancement gel solution
MSI	microsatellite instability
MTS1	Multiple Tumour Suppressor 1 gene
N	normal
NP40	Nonidet P40
PCR	Polymerase Chain Reaction

R	Rands
Rb	Retinoblastoma gene
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
RNAses	ribonucleases
rpm	revolutions per minute
RT	Reverse transcription
RTPCR	Reverse transcription-polymerase chain reaction
S	DNA synthesis phase
SCC	Squamous Cell Carcinoma
SDS	Sodium dodecyl sulphate
SSCP	Single Strand Conformation Polymorphism
T	tumour
Tm	melting temperature
UV	Ultra Violet
VNTR	Variable Number of Tandem Repeats
v/v	volume per unit volume
w/v	weight per unit volume

ABSTRACT

Little is known about the genetic events occuring in oesophageal cancer and very few studies have been undertaken to analyse oesophageal tumours from South African patients in this regard. Inactivation of numerous tumour suppressor genes, including the *Rb* gene, has been implicated in oesophageal tumourigenesis in different populations. This study had two objectives. The first was to develop a procedure for the simultaneous extraction of DNA and RNA from small (ca. 25mg) oesophageal biopsy samples. The procedure developed here has proven to be rapid, cost effective and consistently produced excellent yields of high quality DNA and RNA. It has to be determined, however, whether long-term storage affects the integrity of the isolated RNA. The second and primary objective of this study was to determine whether the *Rb* gene is involved in oesophageal tumourigenesis in South African patients. Loss of Heterozygosity analysis using a VNTR marker in intron 20 and a microsatellite marker in intron 4 of the *Rb* gene revealed that *Rb*-allelic loss had occurred in 50% of the thirty-three patients analysed. Furthermore, microsatellite instability was demonstrated at the intron 4 marker in 15% of the patients analysed. Mutation screening of exons 17 and 21 of the *Rb* gene, frequently mutated in oesophageal tumours from Chinese patients, in twenty samples using the mutation screening techniques of SSCP and heteroduplex analysis, followed by DNA sequencing of putative positives, revealed no positive mutations. However, the high percentage of allelic loss found suggests that the *Rb* gene is inactivated in the progression of South African oesophageal tumours.

Furthermore, the microsatellite instability suggests that defective DNA repair may also play a role in oesophageal tumourigenesis.

CHAPTER 1

GENERAL INTRODUCTION

1.1 Oesophageal cancer statistics

Oesophageal cancer is one of the most prevalent cancers in South Africa with incidences of 24.9 and 11.5 per 100 000 men and women, respectively (Sitas , 1992). Although these figures are much lower than for China, where it has been estimated that nearly half of all oesophageal cancer cases occur (Parkin *et al.*, 1988), certain regions of South Africa have extremely high incidences of the disease. In the Transkei region, for example, the incidence is 60 to 70 oesophageal cancer cases per 100 000 (Sitas, 1992). In addition, there is an upward trend in the incidence of the disease in the Western Cape, with an increase of 50% recorded between 1977 and 1987 for Coloured men (Bradshaw and Harington, 1987) bringing the incidence to approximately 21 cases per 100 000.

1.2 Oesophageal cancer - histological classes

There are two major classes of oesophageal cancer, adenocarcinoma and squamous cell carcinoma.

Adenocarcinoma occurs most frequently in Caucasians, with a male to female ratio of approximately 9:1 (reviewed by Altorki *et al.*, 1992) and is extremely rare in South Africa (reviewed by Walker, 1987). Patients are usually in their fifth decade of life and most have a history of gastrointestinal reflux which leads to Barrett's oesophagus. This premalignant condition, in which the normal squamous epithelium of the oesophagus is replaced by columnar epithelium, is so well documented that diagnostic oesophagectomy is routinely performed in suitable surgical candidates with this condition. (Hamilton *et al.*, 1988)

Squamous cell carcinoma is the most frequently occurring type of oesophageal cancer, accounting for approximately 60% of all cases and is the class seen almost exclusively in South Africa. This form predominantly occurs in people between 50 and 70 years of age. These tumours arise from the squamous epithelium lining the oesophagus and are most often located in the middle third of the oesophagus. Unlike adenocarcinoma, the aetiological factors in oesophageal squamous cell carcinoma are still poorly defined, although a number of epidemiological studies have identified potential risk factors. However, no premalignant histological markers for oesophageal squamous cell cancer have yet been identified. (reviewed by Altorki *et al.*, 1992)

1.3 Aetiological factors implicated in South African oesophageal cancer

Several epidemiological case studies have been carried out worldwide to identify environmental factors which contribute to oesophageal carcinogenesis. A number of these studies have identified alcohol consumption and tobacco usage as the major contributing factors (De Stefani *et al.*, 1990; Blot, 1994; Cheng, 1994). Furthermore, reports by Tuyns *et al.*(1977) and Day (1984) have suggested that the combined use of alcohol and tobacco significantly increases risk. Besides alcohol and tobacco, a number of other potential factors have also been identified. In China dietary habits include large amounts of pickled vegetables known to be contaminated by fungi, as well as foods containing large amounts of nitrosamines, which are themselves powerful carcinogens. Extracts from these pickled vegetables have been demonstrated to induce cancer in rats (Cheng, 1984). In Iran, the chewing of opium residues, which contain a number of potent mutagens, has been suggested as a risk factor (Chadirian *et al.*, 1985). In addition to the aforementioned factors, dietary deficiencies are common in all the high risk areas, further increasing the risk of developing oesophageal cancer, possibly due to lack of essential micronutrients (reviewed by Altorki *et al.*, 1992).

Similarly, a number of epidemiological studies have been carried out in areas of high oesophageal cancer incidence in South Africa. The results of these studies have linked squamous cell carcinoma to a number of possible aetiological factors previously reported in other high incidence areas of the world. Alcohol consumption, tobacco usage (Van Rensburg *et al.*, 1985; Segal *et al.*, 1988; Sumeruk *et al.*, 1992) and dietary deficiencies

(Van Rensburg, 1981) appear to be the major risk factors associated with the disease in South Africa. In the high incidence area of Transkei, alcohol is consumed mainly in the form of traditional beer. There is also evidence that the frequent fungal infection of food plays a major role in oesophageal tumourigenesis in Transkei (Marasas, 1982). Extracts from *Fusarium moniliforme*, the most frequent fungal contaminant of Transkeian maize (Marasas, 1982), were found to enhance tumour development in rats significantly (Jasckiewitz *et al.*, 1987), which further supports the epidemiological findings.

1.4 Major clinical problems associated with oesophageal cancer

1.4.1 Diagnosis

Most patients with carcinoma of the oesophagus present with advanced disease and are very rarely cured (reviewed by Altorki *et al.*, 1992). Delay in presentation is the main reason why prognosis is poor in South Africa, since the high risk population groups are also largely economically disadvantaged and have limited access to medical care. The mean time in which Blacks die of the disease after diagnosis is two months, compared to 18 months for Whites. This can be attributed to late presentation and therefore more advanced tumours in Blacks (reviewed by Walker,1987). Endoscopy-histology, which is almost exclusively used for the diagnosis of oesophageal carcinoma in South Africa, essentially detects only the advanced forms of the cancer which are generally incurable. There is a need for more efficient diagnostic tools, with an emphasis on early detection.

1.4.2 Therapy - present and future

The most important types of therapy for carcinoma of the oesophagus are oesophagectomy, chemotherapy and radiotherapy (reviewed by Altorki *et al.*, 1992). The tolerance of most patients to these types of therapy is poor (Ilson and Kelson, 1994) probably due to the severe wasting caused by the disease in its later stages. Furthermore, advanced oesophageal tumours frequently demonstrate extreme resistance to therapeutic drugs and radiotherapy. Late diagnosis is thus the major factor which thwarts the success of these intervention methods (Smalley *et al.*, 1994).

It is evident that an effective screening procedure is needed for the high risk areas of South Africa to facilitate early diagnosis and thus improve prognosis. This notion is supported by similar strategies adopted in high risk areas of China, where improved post-therapy survival rates have been reported (Cheng, 1994). A molecular diagnostic tool would be a useful addition to existing screening methods to improve cancer detection sensitivity, with limited effects on cost-effectiveness and practicality. Even the limited information on the genetic events involved in oesophageal tumourigenesis suggest possible therapeutic guides as well as prognostic markers. It has been suggested that mutations in specific genes are possible indicators for the response of a tumour to therapy and predictors for a patient's survival (Rosen, 1994). Limited studies have been carried out to elucidate the genetic events involved in the development of the disease in South Africa. More importantly, little is known about the *early* events in worldwide oesophageal cancer, knowledge of which may provide valuable molecular markers for

early detection, facilitate appropriate choices of therapy and improve the worldwide prognosis of the disease. The development of a screening strategy using genetic markers would require an extensive study of the molecular mechanisms of South African oesophageal cancers.

1.5 The colorectal cancer model: a case for studying molecular mechanisms of oesophageal cancer

An important thrust of current molecular cancer research is to associate specific mutational events with stages of tumour formation, a goal that is far from being realised for most cancers. Human colon cancer provides the clearest picture of the association between specific genetic events and tumour progression. The wealth of data accumulated has allowed the development of a molecular model for colon cancer development which can be applied to both the hereditary and spontaneous forms of the disease (Vogelstein *et al.*, 1988; Fearon and Vogelstein, 1990). In this model, the early, or initiating event, is loss of the APC tumour suppressor gene, which results in hyperproliferative epithelium. This is followed by alterations in oncogenes and tumour suppressor genes, which result in full-blown carcinoma. Subsequent events cause the tumour to metastasize and colonize other sites. (Figure1). Most of the molecular events identified during the development of colorectal cancer contribute to uncontrolled growth which is the major characteristic of cancer cells. These changes lead to the disruption of the normally strict control of the cell cycle, a series of events in which cells divide .

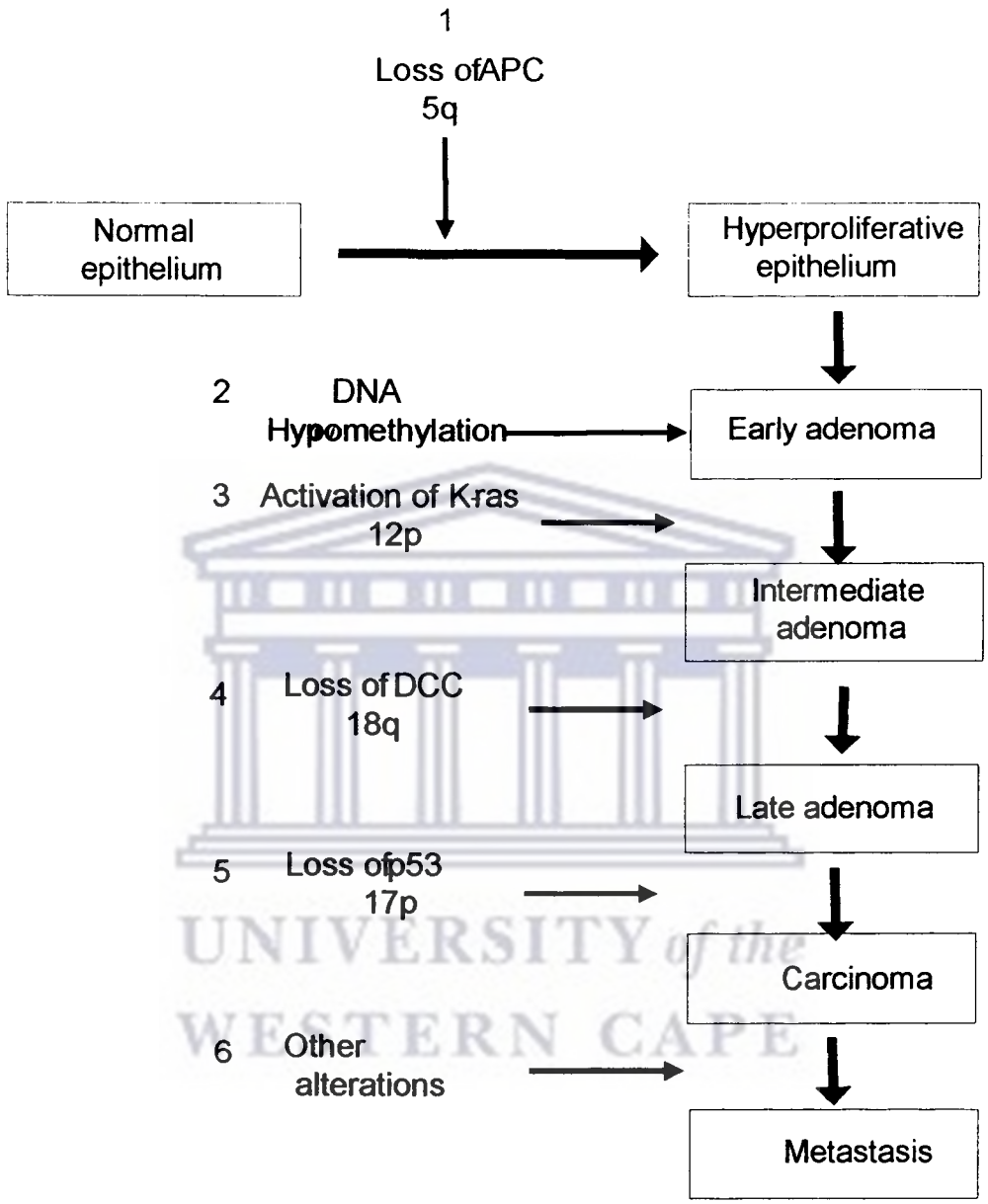

Fig 1.1 The genetic changes statistically associated with various phases in the evolution of a typical colon cancer (adapted from Varmus, 1993)

1.6. Molecular mechanisms of cancer: disruption of the cell cycle

1.6.1 The cell cycle

The homeostasis of a normal tissue relies on the ability of its cells to repopulate (mitosis), differentiate, or reduce cell number by apoptosis. The sequence of events in which a eukaryotic cell replicates into two daughter cells is called the cell cycle (Figure 2). There are two major phases in the cell cycle, the DNA synthesis (S) phase in which the genome is copied and the mitotic (M) phase in which the cell divides. Between these central phases are two phases called G1 and G2. G1 precedes the S phase and during this phase, the cell accumulates all the necessary building blocks for DNA synthesis, as well as other important factors required by the daughter cells. The G2 phase follows the S phase and precedes the M phase and serves as a preparation for mitosis as well as a checkpoint for "quality control" of DNA synthesis. Another vital component of the cell cycle is a rest-stop phase (Go) which cells can enter after mitosis and become quiescent. The inability to enter Go and thus entrapment in the active growth cycle is the basis for the inappropriate growth of a tumour cell (reviewed by Varmus and Weinberg, 1993).

The cell cycle is normally tightly controlled with non-cycling cells remaining quiescent in Go. Starved or DNA damaged cells arrest at a restriction point in late G1 (Rosen, 1994). From a cancer perspective, positive and negative control of the cell cycle are mediated by the products of oncogenes which act as accelerators and tumour suppressor

genes which act as brakes (Figure 2). Disruption of these control elements occurs at a genetic level. (reviewed by Varmus and Weinberg, 1993)

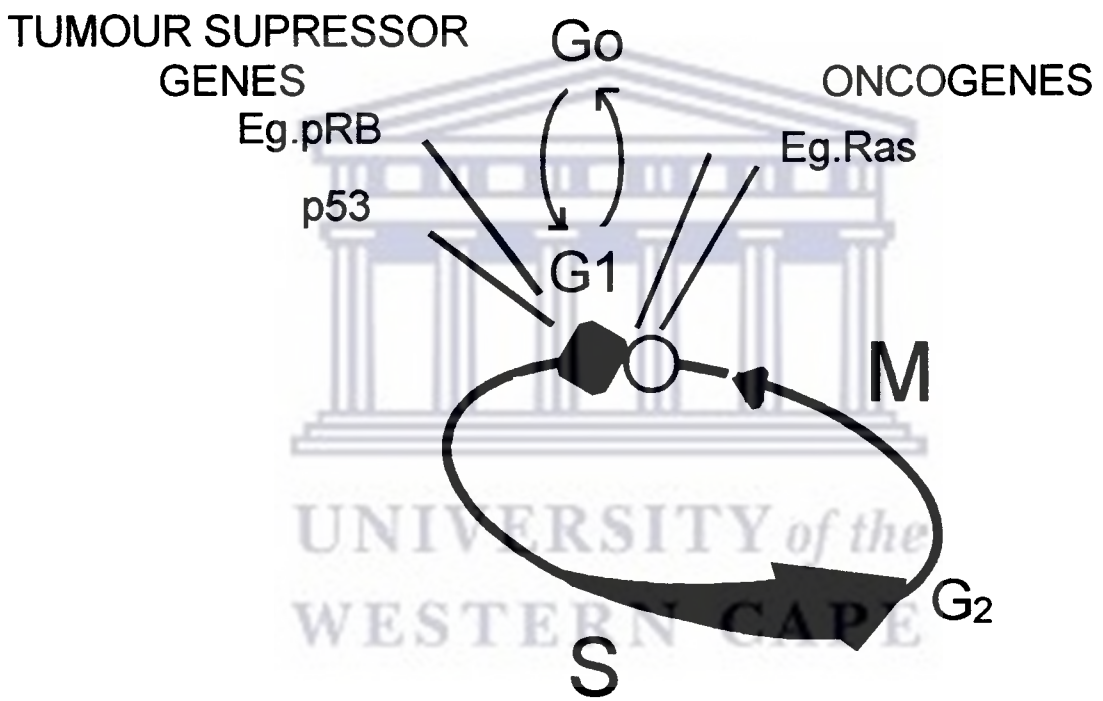

Figure 1.2 The cell cycle (adapted from Varmus and Weinberg, 1993)

a. Oncogenes

Protooncogenes do not lead to cancer in their normal state, but do so only after a mutational event and are then called oncogenes. These "cancer genes" were first discovered when it was found that certain tumour-forming viruses needed only a single gene to transform cells (Doolittle *et al.*, 1983; Downward *et al.*, 1983). It was soon discovered that these oncogenes were homologs of normal mammalian genes which are essential for the normal activity of cells. The viral forms were activated as compared to their normal (unmutated) cellular counterparts. It has been suggested that these viruses acquired the oncogenes from host cells during their evolution and incorporated them into their own genomes (reviewed by Evans, 1993). Similarly, human tumour-derived DNA segments containing single genes also showed the ability to transform cultured cells. Analysis of these genes identified them as human homologs of viral oncogenes and suggested that activation occurs in a similar manner to tumour-forming viruses, by gain of function mutation, constitutive overexpression or deregulation of expression (reviewed Rosen,1994). Mammalian oncogenes are rarely able to transform truly normal cells such as normal embryonic cells, which have undergone no genetic alterations. This phenomenon suggests that tumourigenesis is a multistep process involving more than one gene.

b. Tumour suppressor genes

The existence of tumour suppressor genes was demonstrated in somatic cell hybridization experiments, in which cancerous cells fused with normal cells lost their tumourigenicity (Harris, 1988). This occurred even though the hybrids retained most of the characteristics of cancer cells, which suggested that normal cells harbour genes capable of suppressing the tumorigenic phenotype (Harris, 1988; reviewed by Levine, 1993; reviewed by Rosen, 1993). Specific chromosomes containing tumour suppressor genes were identified by hybridizing normal single chromosome containing microcells to cancer cells (Ponder, 1988). Many tumour suppressor genes have been identified and characterised since these initial findings (reviewed by Levine, 1993). Examples of these are the *Rb*, *p53*, *APC*, *MCC* and *DCC* genes which were first implicated in hereditary cancers and later in spontaneous cancers (reviewed by Weinberg, 1992).

Unlike oncogenes which require only a single mutational event for activation, tumour suppressor genes generally require two independent mutational events, each affecting a different allele. Mutations in tumour suppressor genes are usually loss of function mutations that act in a recessive manner to the wild type, allowing such mutations to be inherited through the germ line (reviewed by Levine, 1993). Such mutations can thus be found in genetic predispositions to cancer and *act* in a dominant manner because of the high frequency of second genetic events such as allelic loss or reduction to homozygosity, which leaves only the mutant allele (Dryja *et al.*, 1989). Somatic tumour suppressor gene inactivation most commonly arises by a mutation in

one allele, followed by loss of the remaining allele (reviewed by Levine, 1993).

A number of tumour suppressor genes show loss of heterozygosity (LOH) in oesophageal cancer, including *Rb* (48%), *p53* (55%), *APC* (66%) and *DCC* (24%) (Huang *et al.*,1992). It has been shown that the existence of allelic loss in tumour suppressor genes is frequently accompanied by mutations in the remaining allele (Weinberg, 1989). Of these suppressors, only *p53* and *Rb* fulfill all the requirements of a true tumour suppressor gene. This has been demonstrated by experiments in which the wild type tumour suppressor gene is returned to cell lines defective for that gene. When these cells were injected into test animals they were unable to form tumours (reviewed by Levine, 1993). Additionally, *p53* and *Rb* have been shown to collaborate in their tumour suppressing activities (Williams *et al.*, 1994; Taya, 1995).

1.7 The *Rb* tumour suppressor gene

The *Rb* gene is located on chromosome 13q14 in the human genome and is expressed in all cells. The gene was named after the first cancer in which it was implicated, namely retinoblastoma, a childhood ocular tumour. The hereditary form of the disease is inherited as an autosomal dominant trait and Knudson hypothesized that functional inactivation of both *Rb* alleles is required for retinoblastoma formation (Knudson, 1971).

The *Rb* protein has a molecular weight of approximately 110 kDa and its main function seems to be binding and functionally inactivating the transcription factor E2F (Figure 3), thereby regulating E2F function (reviewed by Levine, 1993 and Varmus and Weinberg, 1993). The observation that E2F activity is increased in cells in response to mitogens suggests that it enhances the transcription of genes involved in the positive regulation of the cell cycle (Picksley and Lane, 1994). The *Rb* protein also binds and functionally inactivates a host of other transcription factors, including the product of the protooncogene *myc*, a powerful growth stimulant (Taya, 1995). Additionally, the *Rb* protein is also targeted by many tumour virus oncoproteins, supporting its importance in suppressing transformation (reviewed by Levine, 1993). The gene fulfills all the criteria of a true tumour suppressor gene as is demonstrated by its ability to reverse the tumourigenic phenotype of *Rb* deficient cells (Bookstein *et al.*, 1990). Analysis of these cells revealed that they were arrested in G1. The observed increase in cell size indicated that *Rb* did not affect their cytoplasmic growth, but prevented G1 to S transition (Wang *et al.*, 1994).

The first clue to the role of *Rb* in cell cycle regulation came from the observation that its phosphorylation status oscillates in proliferating cells (Figure 1.3). Several studies have shown that *Rb* is underphosphorylated in G1 and has growth suppressing activity. Conversely, it is hyperphosphorylated from late G1 to M and demonstrates no growth suppressing ability (Wang *et al.*, 1994). *Rb* phosphorylation has been shown to be mediated by a group of cyclin dependent kinases (cdks), namely cdk 2, 4 and 6. These kinases are activated by the binding of proteins called cyclins, resulting in the

phosphorylation of *Rb* and the subsequent release of active E2F. (Figure1. 3) (Wang *et al.*, 1994; Taya, 1995). In this phosphorylated state *Rb* also loses its ability to bind and inactivate viral oncoproteins (reviewed by Levine, 1993). A number of cyclin dependent kinase inhibitors have been identified which prevent *Rb* phosphorylation and thereby G1 to S transition. These proteins are activated in response to growth inhibitory signals. Examples of these proteins are *p21*/Waf1, which is activated by *p53* in response to DNA damage and *p15*, which is activated by TGFβ-mediated response to contact inhibition (Taya, 1995). The cdk inhibitor, *p16* interacts specifically with cdk4 and is frequently targeted for reduction to homozygosity in many cancers (Brenner and Aldaz, 1995; Graña and Reddy, 1995; Yang *et al.*, 1995). The gene coding for *p16* has been named the multiple tumour suppressor 1 (*MTS1*) gene because of the wide range of cancers in which it is altered.

It follows from the preceding discussion that both the integrity and proper regulation of the *Rb* gene and other components of the *Rb* pathway are clearly imperative for correct cellular response to growth suppressing signals.

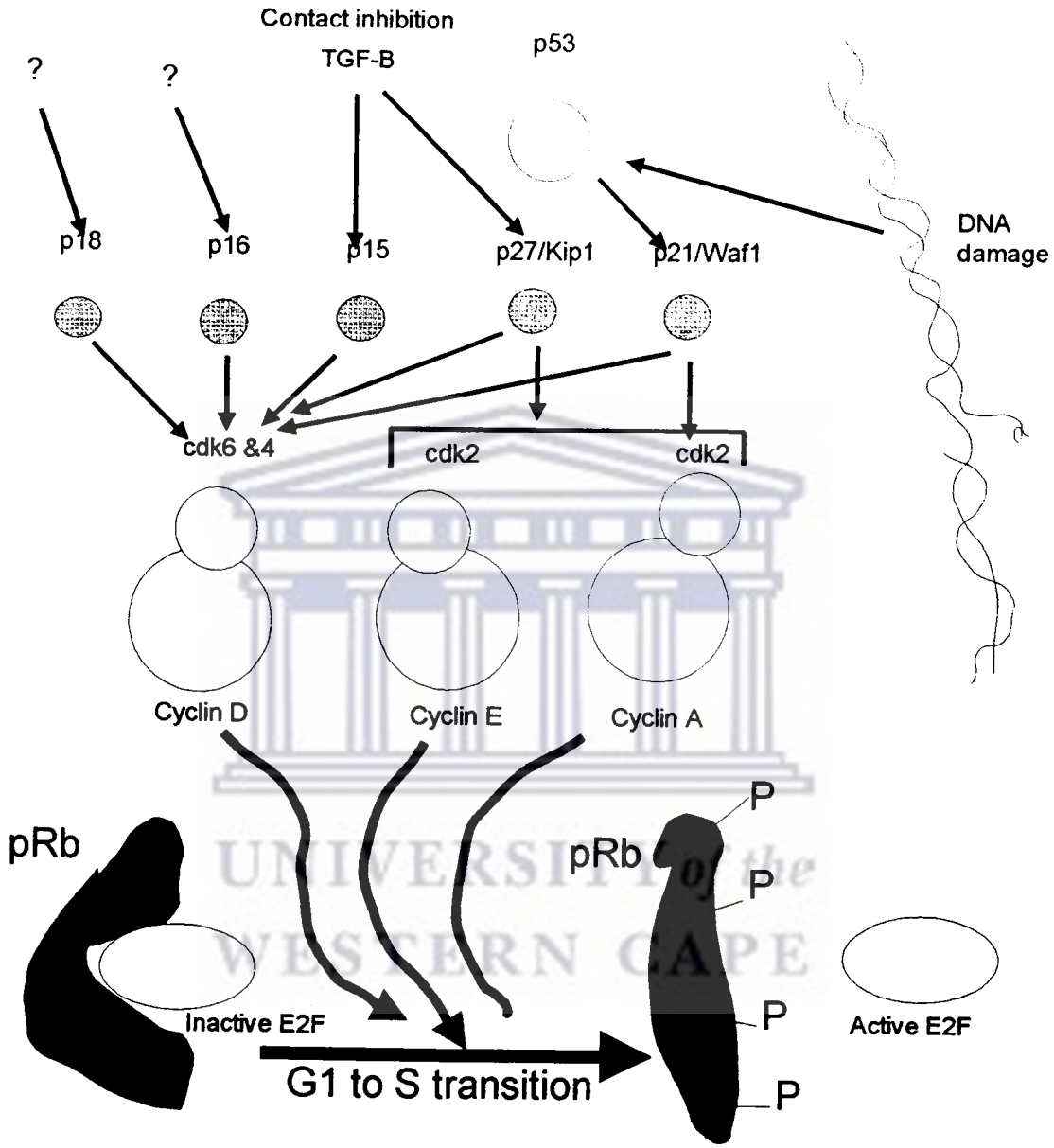

Fig 1.3 Regulation of p*RB* phosphorylation at the G1/S border by cyclin-dependent kinases and cyclin-dependent kinase inhibitors (adapted from Taya, 1995)

1.8 Motivation for evaluating *Rb* integrity in South African oesophageal cancers

Rb inactivation has been observed in several other tumours besides retinoblastoma as high frequencies of allelic loss and point mutations within the gene (Hensel *et al.*, 1990; Xu *et al.*, 1993; Reissman *et al.*, 1993). Furthermore, a high frequency of *Rb* allelic loss has been demonstrated in oesophageal tumours of American patients (Boynton *et al.*, 1991; Huang *et al.*, 1992) and point mutations have been reported in oesophageal tumours from Chinese patients (Li, 1993). These findings suggest involvement of the *Rb* gene in oesophageal tumourigenesis in those populations.

In this study, the status of the *Rb* gene in South African oesophageal tumours was investigated as such a study has not previously been undertaken in South Africa.

CHAPTER 2

DEVELOPMENT OF A PROCEDURE FOR THE CONCURRENT ISOLATION OF DNA AND RNA FROM OESOPHAGEAL BIOPSY SAMPLES

2.1 INTRODUCTION

An understanding of the molecular changes associated with oesophageal cancer requires analysis at both the DNA and RNA levels, since changes frequently occur at both levels. Molecular analysis at the DNA level is important to screen for mutations such as allelic loss, genomic instability, gross deletions and insertions, as well as point mutations. In addition, certain mutations in oncogenes may result in their overexpression and a subsequent increase in the steady state level of the specific mRNA. Similarly, novel oncogenes or tumour suppressor genes may be identified using techniques that can detect genes differentially expressed in tumour and normal tissue. RNA analysis also facilitates the screening of exons by polymerase chain reaction (PCR)-based techniques. Exons of many mammalian genes are often interspersed with very large introns, which makes their analysis laborious and expensive if standard PCR methods are used. If RNA is available, groups of small exons can be analysed by reverse transcription-PCR of a specific mRNA. The

abovementioned techniques all rely on the isolation of DNA and RNA of high quality.

Samples of oesophageal tissue are mostly available as small biopsies (ca.25mg), necessitating the simultaneous extraction of DNA and RNA in order to maximize the use of the material, while striving to obtain satisfactory yields of both these nucleic acids. Several procedures have been developed for the separate isolation of DNA and RNA from mammalian tissue samples (Sambrook et. al, 1989; Ausubel *et al.*, 1995). The procedures available for the simultaneous extraction of DNA and RNA rely on costly reagents and/or equipment. In this study, a simple, inexpensive method for the concurrent extraction of DNA and RNA from small amounts of animal tissue was developed.

The final procedure is based on a modification of two popular RNA isolation methods. The first uses a gentle nonionic detergent, Nonidet P40 (NP40), to lyse the cell membranes and separate the cytoplasmic (RNA) fractions from the intact nuclei by low-speed centrifugation for subsequent isolation of RNA (Ausubel *et al.*, 1995). Although RNA is usually targeted, it is possible to isolate DNA from the nuclear fraction. While this method has been used successfully for isolation of RNA from cultured cells (Berk and Sharp, 1977; Favarolo *et al.*, 1980), it takes no special precautions against ribonucleases (RNAses) which may result in partially degraded RNA. Tissues harbour more endogenous RNAses than cultured cells and we have not been able to apply the latter technique successfully to biopsy material. The second standard procedure on which the technique described in this study is based, relies on

the powerful protein denaturant, guanidinium isothiocyanate (GITC). Cells or tissues are homogenised in a GITC-containing cell lysis buffer, immediately exposing cellular contents to a denaturing environment, and thereby protecting RNA from RNAse degradation (Sambrook *et al.*, 1989; Ausubel *et al.*, 1995). When the recovery of both DNA and RNA is required, the fractions are subsequently separated by cesium chloride ultracentrifugation (Glisin *et al.*, 1974), which is expensive, relies on specialized equipment and is not feasible for small quantities of tissue.

The method described in this study separates the cytoplasmic (RNA) and nuclear (DNA) fractions using NP40. DNA is isolated from the nuclear pellet using a common technique, while RNA is protected during isolation by the addition of GITC to an appropriate concentration.

2.2 MATERIALS AND METHODS

2.2.1 Tissue preparation

a. Rat tissues

Rat tissue was used for the development and optimization of the concurrent DNA/RNA extraction procedure. Rats were anaesthetized with ether and sacrificed by decapitation. Tissue samples of approximately 20 to 50 mg were quickly (<5 minutes) removed from liver, lung, pancreas, skeletal muscle and small intestine and immediately frozen in liquid nitrogen to prevent nucleic acid degradation. Samples were stored at -70°C until used. Pancreatic tissue was chosen because it is rich in nucleases, especially RNAses and would be an effective test of the procedure.

b. Human oesophageal biopsy material

Oesophageal tumour and where possible, adjacent normal biopsies were kindly provided by the Gastrointestinal Unit at Groote Schuur Hospital in Cape Town. All oesophageal biopsy samples were immediately frozen in liquid nitrogen to prevent any DNA or RNA degradation. Samples were stored at -70°C until DNA and RNA were extracted.

2.2.2 DNA and RNA extraction procedure

The composition of all solutions is given in the Appendix

a. Separation of DNA and RNA fractions

Tissues (< 40mg) were ground in liquid nitrogen and 400ul of NP40 lysis buffer was added. The tissue suspension was mixed by vortexing to disperse clumps and then placed on ice for 5 minutes, with intermittent vortexing. This ensured complete lysis of the cell membranes while leaving the nuclei intact. The cell lysate was transferred to a microfuge tube and centrifuged at 3000 x g for 30 seconds at 4° C to pellet the nuclei and tissue debris. The supernatant was used for RNA isolation and the pellet for DNA isolation as summarised in Figure 2.1.

b. RNA isolation

A microfuge tube containing the following components was prepared for each sample before starting the isolation:

400 µl GITC solution

100 µl 2M Sodium Acetate, pH 4

50 µl ß-mercaptoethanol

The supernatant from 2.2.2a (ca. 400 µl) was added to the tube, mixed by gentle

inversion and left at room temperature for 10 minutes to dissociate nucleoprotein complexes. Guanidinium isothiocyanate, together with ß-mercaptoethanol, causes instant denaturation of proteins (including RNAses) and protects RNA from degradation throughout the entire extraction. The acidic environment also causes both proteins and DNA to become soluble in phenol while the RNA remains in the aqueous phase.

After the incubation, 100 µl of pure chloroform was added to each tube and the contents mixed by inversion. The aqueous and organic phases were separated by centrifugation at 10000 x g for 5 minutes at room temperature. The aqueous phase was carefully removed, avoiding the interphase and transferred to a fresh tube. 500 µl of chloroform:isoamyl alcohol (24:1) was added to the tube containing the aqueous phase to remove residual phenol and the contents mixed well before separation of the two phases by centrifugation. The RNA in the aqueous phase was precipitated by the addition of an equal volume of isopropanol and storage at -70°C for at least 30 minutes. The RNA was pelleted by centrifugation at 15000 x g for 20 minutes at 4°C. The supernatant was removed and the RNA pellet washed in 1ml of 70% ethanol at room temperature to remove salts. After repelleting the RNA by centrifugation and removal of the ethanol, the RNA pellet was dried under vacuum and resuspended in sterile diethylpyrocarbonate (DEPC)-treated water. The absorbance of each sample was measured at 260nm and 280nm. The A_{260} was used to determine RNA concentration and the A_{260} : A_{280} ratio calculated to assess RNA quality (Sambrook *et al.*, 1989).

c. DNA isolation

The isolation of DNA was based on a popular technique for the extraction of chromosomal DNA from mammalian cells and tissues (Gross-Bellard *et al.*, 1973).

The nuclear pellet obtained in 2.2.2a was used for the extraction of DNA. 400 µl of Proteinase-K buffer was added to the nuclear pellet and the contents mixed gently to resuspend the pellet. 20 µl of a 10mg/ml Proteinase-K stock solution was then added and the tube placed at 55°C for a minimum of 3 hours. SDS lyses the nuclear membrane while inhibiting nucleases and proteinase-K digests proteins, releasing smaller peptides.

After incubation, an equal volume of TE-saturated Phenol:Chloroform:Isoamyl alcohol, PCI, (25:24:1) was added to the tube and the contents mixed well by inversion. The tube was centrifuged at 10000 x g for 5 minutes at room temperature to separate the organic and aqueous phase. This serves to remove the digested proteins which are dissolved in phenol and trapped in the organic phase. The aqueous phase was carefully removed and the PCI extraction step repeated. An equal volume of chloroform:isoamyl alcohol (24:1) was added to the aqueous phase after the second PCI extraction to remove residual phenol. After centrifugation, the upper (aqueous) phase was transferred to a fresh tube.

DNA was precipitated by adding 2.5 volumes of ice-cold absolute ethanol to the aqueous phase. The DNA was visualised as a white, stringy precipitate. The tubes were placed at -20°C for 30 minutes to ensure complete precipitation. When no precipitate could be seen after addition of ethanol, one tenth volume of 3M sodium acetate, pH 5.5, was added and the tubes left at -20°C overnight. After precipitation, the tubes were centrifuged at 10000 x g at room temperature for 2 minutes to pellet the DNA. The ethanol was removed and the DNA pellet washed with 1ml of 70% ethanol at room temperature. The pellet was partially dried at room temperature (overdrying causes difficulty with resuspension), an appropriate volume (usually 250 µl) of 1xTE added and the DNA pellet allowed to dissolve slowly at 65° C. Absorbances were measured at 260nm and 280nm. The A_{260} was used to determine DNA concentration and the A_{260} : A_{280} ratio calculated to assess DNA quality (Sambrook *et al.*,1989).

2.2.3 DNA and RNA quality assessment

DNA and RNA integrity was assessed by agarose gel electrophoresis. Both the isolated DNA and RNA were used in PCR- based techniques to test their suitability for use in sensitive molecular applications.

a. Electrophoresis

Both nucleic acids were electrophoresed using agarose minigels in 0.5 x TBE. DNA was electrophoresed on a 0.7% gel at 80 volts for 1 hour and RNA on a 1.5% gel at 100 volts for 30 minutes. Gels were stained with ethidium bromide at a final concentration of 0.5 µg/ml in water. Intact chromosomal DNA migrates as one high molecular weight band, while an indicator of RNA integrity is the presence of 28S and 18S ribosomal RNA bands.

b. PCR Amplification of DNA

100 ng of isolated human chromosomal DNA was subjected to PCR amplification using primers that amplify a VNTR region in intron 20 of the *Rb* gene using specific PCR conditions as described in Chapter 3. The PCR products were electrophoresed on an 8% polyacrylamide gel and visualised by staining the gel with ethidium bromide as described in Chapter 3.

c. Reverse transcription-PCR (RT-PCR) amplification of RNA

1 µg of human RNA was reversed transcribed (20 µl reaction) using standard methods with antisense primers designed for the *Rb* gene (procedure described in chapter 4). 1 µl of the resulting cDNA was subjected to PCR amplification using primers that amplify a region of the *Rb* cDNA corresponding to exons 13 to 16 of

the gene. RT-PCR products were electrophoresed on a 1.5% agarose gel and visualized by staining the gel with ethidium bromide as described in Chapter 3.

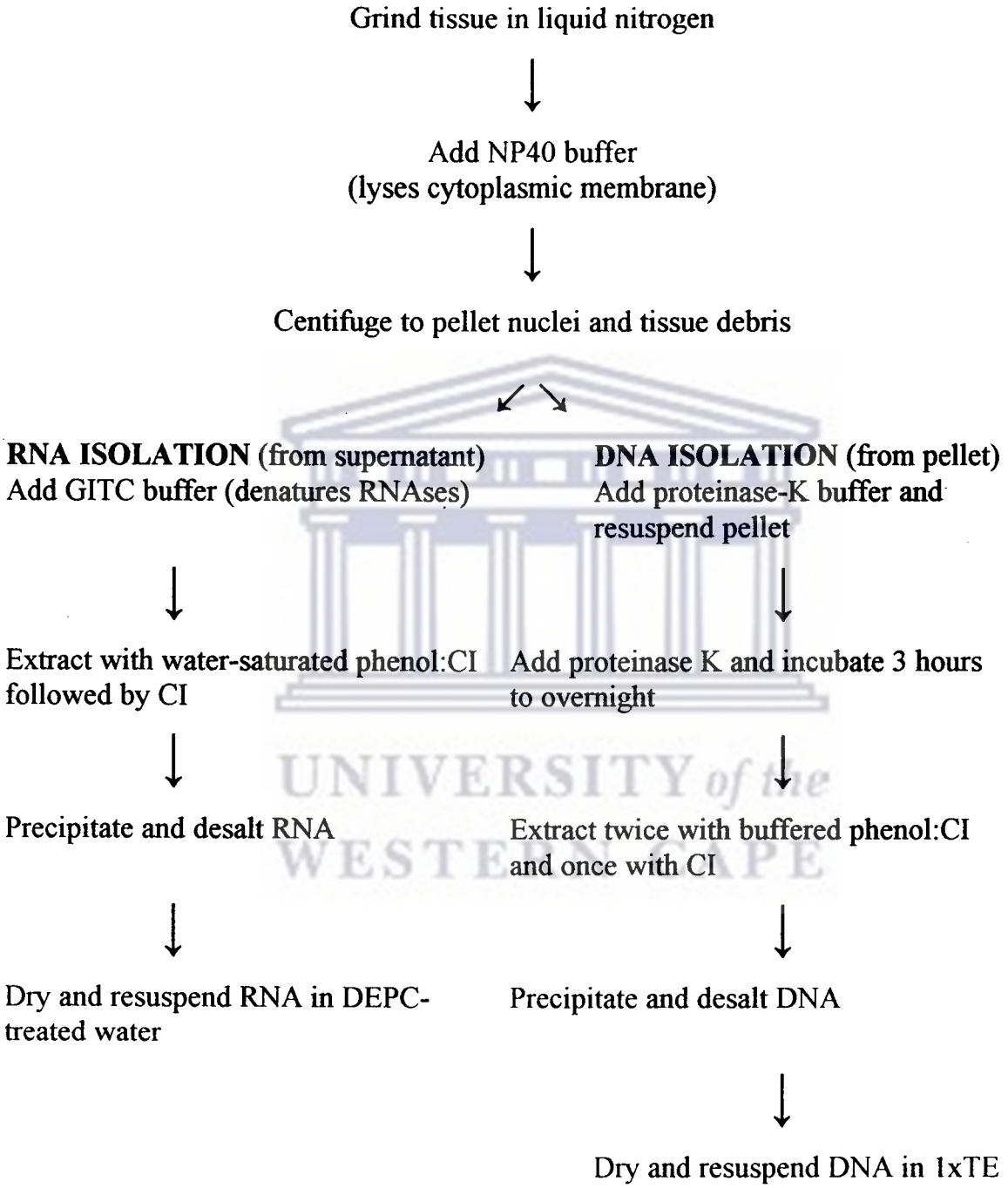

Figure 2.1 Flow diagram of the procedure for concurrent isolation of DNA and RNA from small amounts of animal tissue

2.3 RESULTS

2.3.1 DNA and RNA yields

The yields obtained with this method of concurrent DNA and RNA isolation were similar to those reported for popular techniques where only one of the nucleic acids is targeted (Table 2.1). For example, skeletal muscle gave an average yield of 6 µg DNA and 1 µg RNA per mg of tissue. An exceptionally high average yield of DNA (10 µg/mg tissue) was obtained from oesophageal tumour tissue. The ratio of A_{260} to A_{280} were also consistently greater than 1.8 for both DNA and RNA, which implied minimal contamination with proteins (Sambrook *et al.*, 1989).

2.3.2 DNA and RNA integrity

All tissue types yielded intact DNA and RNA as was assessed using agarose gel electrophoresis. A high molecular weight band was always visualised for DNA isolated from all tissue types (Figure 2.2). Similarly, 28S and 18S ribosomal RNA bands were always present on the RNA gels (Figure 2.3). Even pancreatic tissue which is rich in ribonucleases, consistently yielded intact RNA (Figure 2.3, lane 3).

Table 2.1 Average yields of DNA and RNA obtained from different tissues

TISSUE	YIELDS (µg/mg tissue)	
	DNA	RNA
Rat Liver	5	5
Rat Skeletal Muscle	6	1
Rat Lung	3.5	4
Rat Intestine	3	2
Rat Pancreas	3.5	5
Human oesophagus	3	1
Human Oesophageal tumour	10	1

2.3.3 PCR and RT-PCR analysis

PCR amplification of DNA consistently yielded the expected product as is shown by the single band at approximately 300bp in lane 2 of Figure 2.4, when the primer-specific parameters (Chapter 3) were used. The band at 323bp in lane 3 of Figure 2.4 indicates that the RT-PCR amplification of RNA was also successful. These results demonstrated that the RNA could be reverse transcribed to yield high quality cDNA. This could, however, not be repeated in subsequent experiments using the same RNA samples and RT-PCR system (Chapter 4). This may suggest that even though the RNA isolated using this method appeared to be intact after long-term storage at -

70°C, it may have been rendered unsuitable for use in sensitive molecular applications.

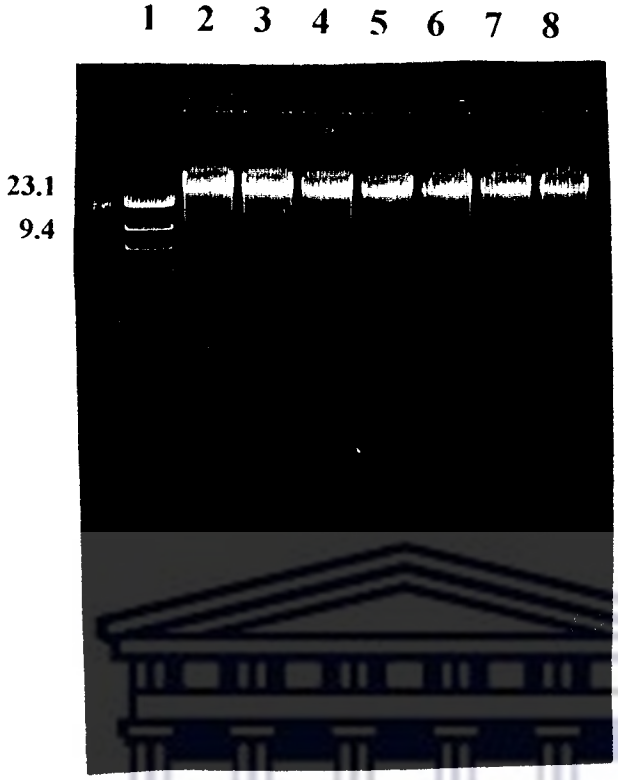

Figure 2.2 Agarose gel electrophoresis of isolated chromosomal DNA. Intact chromosomal DNA was isolated from various rat tissues and from human oesophageal tissue. To assess quality, 1ug of each isolated DNA sample was electrophoresed on a 0.7% agarose gel at 80V for 1 hour and stained with ethidium bromide.

1. Lambda DNA digested with *Hind* III
2. Rat liver DNA
3. Rat lung DNA
4. Rat pancreas DNA
5. Rat small intestine DNA
6. Rat skeletal muscle DNA
7. Human oesophagus DNA
8. Human oesophageal tumour DNA

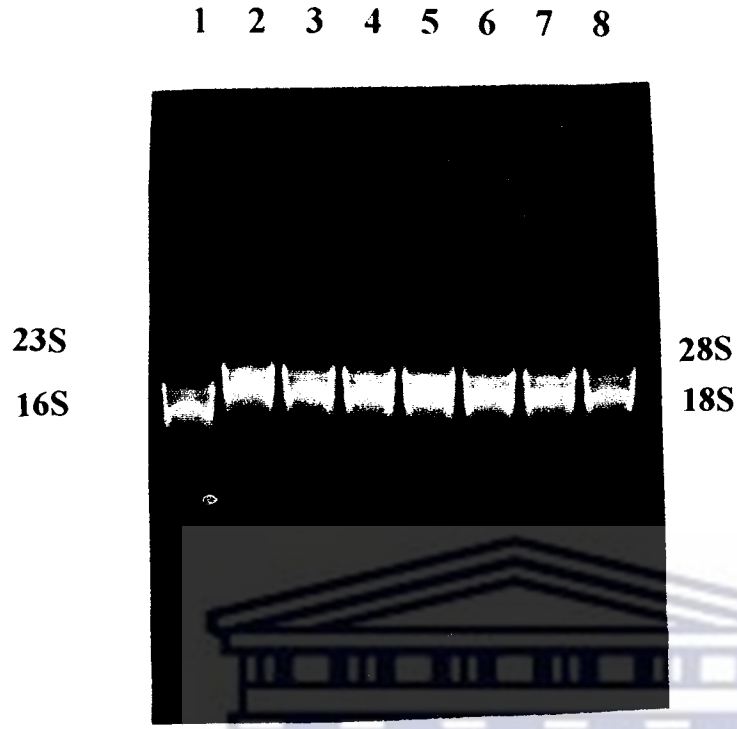

Figure 2.3 Agarose gel electrophoresis of isolated cytoplasmic RNA. Cytoplasmic RNA was isolated from various rat tissues and from human oesophageal tissue. Rat tissue was used for the optimization of the extraction procedure. 2ug of each RNA sample was electrophoresed on a 1.5% agarose gel at 100V for 30 minutes and stained with ethidium bromide.

1. Bacterial 23S and 16S ribosomal RNA marker
2. Rat liver RNA
3. Rat lung RNA
4. Rat pancreas RNA
5. Rat small intestine RNA
6. Rat skeletal muscle RNA
7. Human oesophagus RNA
8. Human oesophageal tumour RNA

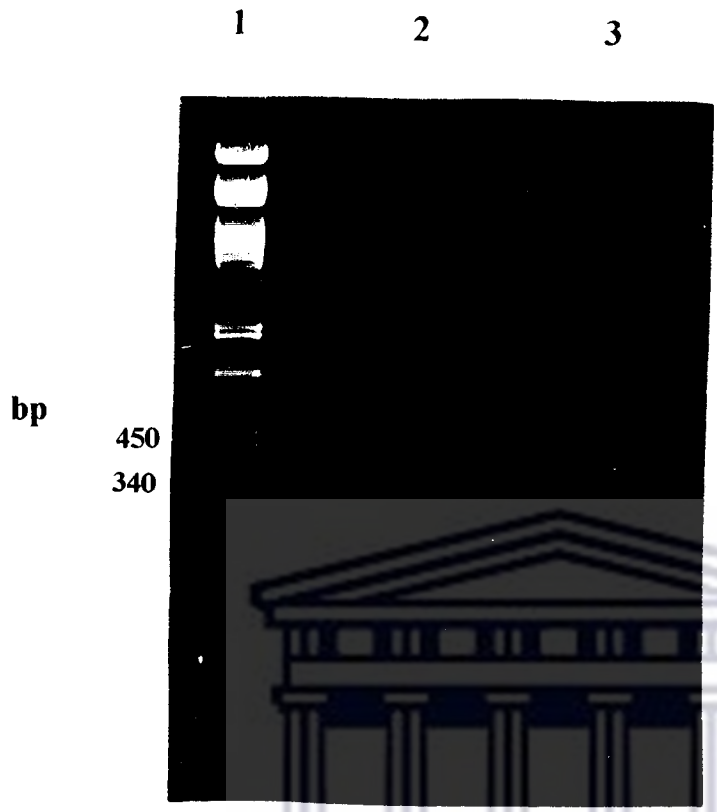

Figure 2.4 Agarose gel electrophoresis of PCR and RT-PCR products. Isolated DNA and RNA were tested for their suitability for use in molecular applications using PCR. Human oesophageal DNA was subjected to PCR using primers flanking a VNTR region in intron 20 of the *Rb* gene. Human RNA was subjected to reverse transcription-PCR using primers that amplify a region of the *Rb*-cDNA corresponding to exons 13 through to 16 of the gene. 5ul of each product was resolved on a 1.5% agarose gel.

1. Lambda DNA digested with *Pst*I
2. PCR product from isolated human DNA
3. RT-PCR product from isolated human RNA

2.4 DISCUSSION

A problem often encountered in medical research projects which investigate the genetic basis of diseases of specific organs, is the availability of tissue. Occasionally, tissue is only available as small biopsies, which is a major problem if both DNA and RNA are required from the same sample. It is necessary to isolate these nucleic acids simultaneously in order to maximize the use of precious tissue samples. Currently, this can only be achieved with the use of expensive reagents. This type of project also often requires screening of a large number of samples, further necessitating a cost-effective isolation procedure.

A procedure has been developed in this study that allows the simultaneous isolation of both DNA and RNA from small quantities of mammalian tissue. One advantage of this method is its cost effectiveness, with a cost of R3,00 per isolation compared to R12,00 for a commercially available reagent.

The integrity of the DNA and RNA isolated using this method has been confirmed by electrophoresis and even tissues rich in ribonucleases such as pancreas, yielded intact RNA. Yields are comparable to those previously described for procedures that target one nucleic acid only (Ausubel *et al.*, 1995; Sambrook *et al.*, 1989), as well as those claimed by expensive commercial products. Unusually high DNA yields of 10ug DNA/mg tissue were often obtained from the tumour tissue, which were probably due

to the increased DNA content often found in cancer cells (Varmus and Weinberg, 1993). Furthermore, both the isolated DNA and RNA have been used successfully in sensitive PCR-based techniques. These results could, however, not be repeated when using the RNA in later experiments using the same RT-PCR system (Chapter 4), suggesting that even though the RNA appeared to be intact after long-term storage at -70°C, it may have been rendered unsuitable for use in sensitive molecular applications. The RNA would have to be tested in another RT-PCR system, using appropriate controls to confirm its integrity.

The procedure described here has definite advantages over existing methods and promises to be useful in those applications which require the simultaneous isolation of DNA and RNA from small tissue samples, with the added benefit of low cost. It is however, important to determine whether the RNA isolated using this technique is affected by long term storage at -70°C.

CHAPTER 3

LOSS OF HETEROZYGOSITY ANALYSIS OF THE *Rb* GENE

3.1. INTRODUCTION

Frequent allelic deletions which affect individual tumour suppressor genes, have been demonstrated in a number of human tumours. For example, allelic loss affecting the *p53* gene has been demonstrated in most cancers (Levine, 1993). Loss of heterozygosity (LOH) has been demonstrated for the tumour suppressor genes, *p53*, *APC*, *DCC* and *Rb*, in American oesophageal cancers (Huang *et al.*,1992). Furthermore *Rb*-LOH has been found in a number of other cancers (Hensel *et al.*, 1990; Xu *et al.*, 1993).

There are several approaches aimed at the investigation of allelic loss. One of these is Southern blotting, which has a number of shortcomings in that the technique requires a large amount of DNA and is laborious and time consuming. The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) approach eliminates the need for large amounts of target DNA initially. This approach relies on the presence or absence of a polymorphic restriction site at a specific locus as an allelic marker. The disadvantage of this approach is that heterozygosity or informativity at

such sites is low, which makes LOH analysis inefficient. This is illustrated by an *Xba*I polymorphism in intron 17 of the *Rb* gene, which has been shown to be informative in only 48% of the American population (Mc Gee *et al.*, 1990). Furthermore, inefficient restriction enzyme action would result in underestimation of the number of heterozygotes, as well as possible false LOH positives.

Microsatellites and VNTRs are polymorphic stretches of DNA that are highly informative and have been used successfully in many allelotyping assays, including LOH analysis (Osborne and Leech, 1994). Microsatellites are short di-, or tri-, or tetranucleotide repeats, usually $(CA)_n$, which are highly polymorphic in terms of the number of repeats and are randomly distributed in the genome (Weber and May, 1989). These markers are easily typed by PCR, using primers flanking the repeats and analysis of the products on high resolution gels. This allows efficient resolution of the maternal and paternal allelic fragments, which may differ in size by as little as two nucleotides (Hauge and Litt,1993). LOH can easily be detected as the loss of a band or of a group of bands. Microsatellites have also been used effectively as markers for genomic instability or DNA repair deficiency (Thibodeau *et al.*, 1993). The nucleotide repeats are "hotspots" for replication errors that are normally rectified by an efficient DNA repair system. Aberrations in this repair system can be detected on high resolution gels as a change in PCR product size when compared with a control sample.

VNTRs (Variable Number of Tandem Repeats) are similar to microsatellites but they have larger repeats of five or more nucleotides eg. $[GATCC]_n$. They are as informative

as microsatellites and are easier to analyse since the resulting PCR products have larger size differences (Osborne and Leech, 1994).

Conveniently, the *Rb* gene has a microsatellite region in intron 4 and a VNTR in intron 20 (McGee *et al.*, 1987; Toguchida *et al.*, 1993), which could be used as markers for the 5' and 3' ends of the gene, respectively. This is particularly important in view of the large size of the Rb gene, which spans approximately 180kb. In this study, both markers were used to determine the frequency of *Rb*-LOH in South African oesophageal cancer. Such a study has not previously been undertaken in South Africa.

3.2 MATERIALS AND METHODS

(The composition of solutions is given in the appendix)

3.2.1 Oesophageal tumour samples

Tumour and paired normal biopsy samples were obtained from oesophageal cancer patients undergoing routine endoscopy at Groote Schuur Hospital in Cape Town. The availability of normal samples was important since LOH analysis is interpreted in relation to normal DNA samples from the same patient. DNA and RNA were extracted from both tumour and normal samples as described in Chapter 2.

3.2.2 VNTR analysis

a. Polymerase Chain Reaction (PCR) conditions

The VNTR in intron 20 of the *Rb* gene was analysed by PCR to examine LOH using primers described previously (Table1) and standard PCR procedures (Boynton *et al.*, 1991; Ausubel *et al.*, 1995). Optimal amounts of all individual components were determined empirically and specificity enhancing agents were added as required. The final concentrations or amounts of each component used per 20µl PCR reaction is indicated below:

Genomic DNA	50 ng
Primers	4 pmol of each
dNTPs	100 µM of each
$MgCl_2$	4 mM
Taq DNA polymerase	0.25 units

Formamide was added to a final concentration of 5% v/v to prevent nonspecific annealling of primers (Sambrook *et al.*, 1989). When required, 3µCi of ^{32}P-dCTP or ^{32}P-dATP was added to the PCR reactions.

PCR cycling parameters were as follows (thirty cycles): 96° C for 10 seconds, 53° C for 30 seconds, 72° C for 30 seconds, followed by one final extension step at 72° C for 5 minutes to ensure full-length PCR products.

b. Electrophoresis

Nonradiolabelled products: 10µl of each nonradiolabelled product was electrophoresed on an 8% acrylamide slab gel in 1xTBE for approximately four hours at 150V, or until the xylene cyanol of the loading buffer ran off the gel. Gels were stained with ethidium bromide (0.5ug/ml in water) and viewed on an ultraviolet transilluminator.

Labelled products: 4% denaturing sequencing gels containing 7M urea were used to resolve PCR products since the non-denaturing slab gels were inefficient in this regard and underestimated the number of informative samples.

Sequencing gels were prepared according to standard methods (Ausubel *et al.*, 1995), using well- forming combs. The gels were pre-electrophoresed in 1xTBE at 40W until the gel temperature was approximately 45°-50°C. The temperature and presence of urea ensured that no renaturing of the PCR products occurred or secondary structures formed. Both would have resulted in ambiguous bands and affected interpretation of results. Equal volumes of the PCR reaction and formamide loading buffer were mixed and the products denatured at 90°C for 1 minute before snapcooling on ice to prevent reannealling. 5ul of each sample was loaded and the gels electrophoresed at 40W for 3-3.5 hours or until the xylene cyanol had reached the bottom of the gel. Gels were dried and exposed to X-ray film overnight at room temperature.

3.2.3 Microsatellite analysis

a. PCR conditions

The PCR primers (Table1) for the microsatellite region ($(CA)_n$ repeat) in intron 4 of the *Rb* gene were designed using the *Rb* gene sequence (Toguchida *et al.*, 1993). The optimal amounts and concentrations of individual PCR components were

determined empirically and were found to be the same as for the VNTR analysis, with the exclusion of formamide. ^{32}P-dCTP or ^{32}P-dATP was included in all PCR reactions.

PCR cycling parameters were as follows (usually thirty cycles): 96°C for 10 seconds, 54°C for 30 seconds, 72°C for 30 seconds. This was followed by a final extension step at 72°C for 5 minutes to ensure full-length PCR products.

b. Electrophoresis

PCR products were electrophoresed on sequencing-type gels (6%) which were prepared according to standard microsatellite analysis methods (Weber and May,1989), using well-forming combs. Electrophoresis and autoradiography was performed as described in 3.2.2(b).

Table 3.1 Intronic polymorphisms in the *Rb* gene used as allelic markers for loss of heterozygosity analysis

Location	Primer pairs (sense/antisense)	Type of polymorphism	Reference
Intron 4	5'-TCCTTTGGGTATTGTTTCAC-3' 5'-TTTAGAAAGACACAATTAGG-3'	Microsatellite $(CA)_n$-repeat	Toguchida *et al.*, 1993**
Intron 20	5'-CTCCTCCCTACTTACTTGT-3' 5'-AATTAACAAGGTGTGGTGG-3'	VNTR*	Boynton *et al.*, 1991

* VNTR-variable number of tandem repeats ** primers designed from *Rb* gene sequence

3.2.4 Special considerations and precautions

The polymerase chain reaction amplifies any suitable DNA substrate (Ausubel *et al.*, 1995) and so special care was taken to prevent contamination with foreign DNA. All tubes and tips used for PCR were autoclaved to destroy any nucleases and proteases that may be present, stored separately and used for PCR only. A set of micropipettes was designated for use with PCR only and their shafts periodically treated with 1N Hydrochloric acid to depurinate any DNA that may have been introduced during use.

When ^{32}P-labelled nucleotides were used, PCR reactions were prepared in a radioactive laboratory and the necessary precautions taken. A radioactivity dosimeter was worn at all times.

3.3 RESULTS

3.3.1 VNTR analysis

The use of 5% formamide in the VNTR-PCR reactions eliminated nonspecific products. Initially, PCR products were resolved on 8% polyacrylamide slab gels. Resolution was inadequate and caused inaccurate scoring since products of alleles having similar sized VNTRs did not resolve well and were scored as uninformative (homozygous). Electrophoresis of ^{32}P-labelled PCR products on 4% denaturing sequencing-type gels gave better resolution than the non-denaturing gels and this system was used exclusively for the rest of the study. Representative results are shown in figure 3.1. For the sequencing gels, a patient was scored informative when two doublets were present (each doublet representing one allele) for the normal DNA sample as seen in figure 3.1(b-f). LOH was scored when tumour DNA from an informative patient yielded only one doublet (Fig1, c-e), or when one set of bands showed marked reduced intensity (Fig1, f). The faint bands present in such tumour samples may be due to contamination with normal cells. The VNTR marker proved to be very informative, with twenty-six heterozygote patients out of thirty-three analysed (79%). Of these, thirteen of the informative cases (50%) demonstrated LOH. Only one of these tumours demonstrated "partial" loss with the bands representing the lost allele much fainter in comparison to those representing the remaining allele (Figure 1, f). This phenomenon was probably caused by the

presence of normal cells in the tumour biopsy sample.

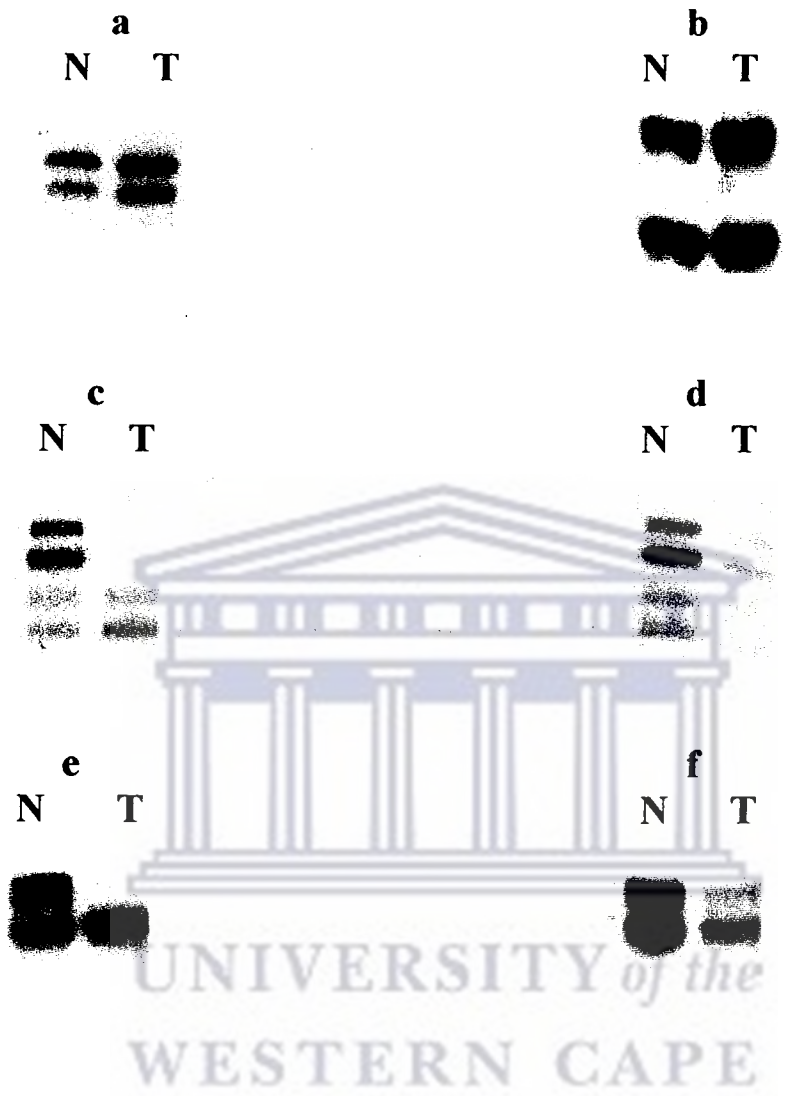

Figure 3.1: VNTR-Loss of heterozygosity (LOH) analysis. The VNTR in intron 20 of the *Rb* gene was used as an allelic marker for LOH analysis and was amplified using the polymerase chain reaction (PCR). ^{32}P-radiolabelled PCR fragments were electrophoresed on 4% sequencing gels at 40W for 3-3.5 hours to resolve the fragments representing both alleles. **a**, **b**, **c**, **d**, **e** and **f** represent paired normal (N) and tumour (T) samples of patients 22 , 13, 24 , 25 , 48 and 49 respectively. Homozygous patient DNA samples produced a single doublet (**a**). Heterozygotes produced four bands, each pair representing an allele (**b**). When loss of heterozygosity occurred in a tumour sample, one pair of bands was either completely absent (**c-e**) or less intense than the remaining pair (**f**).

3.3.2 Microsatellite analysis

The primer set designed for this assay consistently yielded specific products using standard PCR procedures. A number of faint bands were often present in addition to the specific bands obtained when products were resolved on 6% denaturing polyacrylamide gels. This often occurs when microsatellites are analysed using the PCR and has been attributed to "slippage" or mistakes made by *Taq* DNA polymerase when dinucleotide repeats are the template (Hauge and Litt, 1993). In this study, the most intense bands were chosen to represent the alleles and informativity was scored when two bands were present for normal samples. LOH was scored when any one of the two bands was absent or reduced in intensity by more than 50%.

This marker demonstrated 100% informativity using standard microsatellite electrophoresis techniques. No LOH was demonstrated in thirty-three patients analysed, even for patients that demonstrated LOH using the VNTR marker (Table 3.2). Microsatellite instability was demonstrated in five of thirty-three (15%) patients analysed (Table3.2). This could be visualised as a shift of one (figure 3.2b) or both (figure 3.2 c) of the major bands in the tumour DNA sample when compared to its paired normal, indicating either an increase or decrease in the number of CA-repeats. An interesting result was that patients 14 and 25 demonstrated both LOH (only at VNTR) and microsatellite instability (Table 3.2).

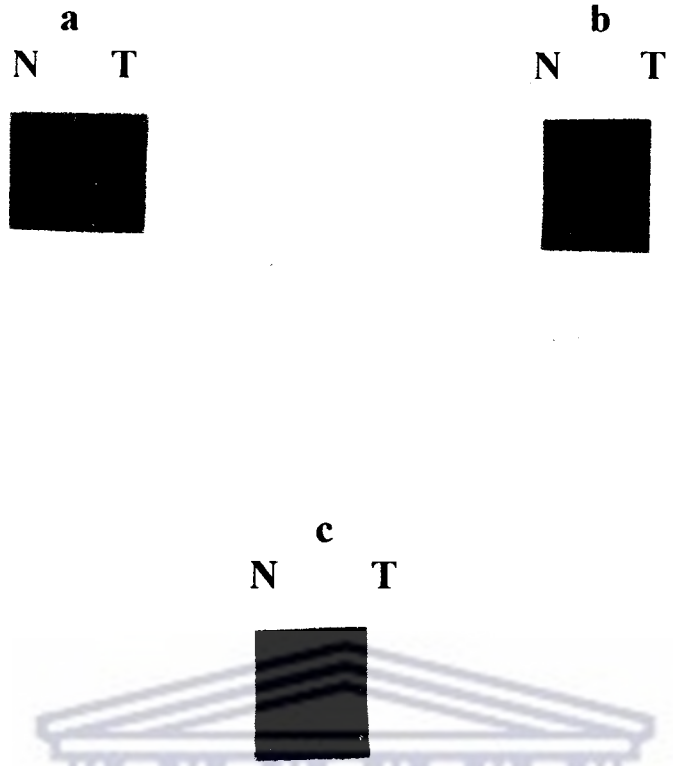

Figure 3.2 Microsatellite-LOH analysis: The microsatellite region in intron 4 of the *Rb* gene was used as an allelic marker for LOH analysis and was amplified using the polymerase chain reaction (PCR). ^{32}P-radiolabelled PCR products were electrophoresed on 6% sequencing gels to resolve the fragments representing both alleles. **a**, **b** and **c** represent paired normal (N) and tumour (T) samples of patients 24, 14 and 25 respectively. Heterozygous patient DNA samples produced two intense bands, each representing an allele (**a-c**). Although no LOH was detected at this marker, microsatellite instability was demonstrated in five of thirty-three patients (15%) screened. This could be seen as either a shift of one (**b**) or of both (**c**) of the major bands in the tumour sample, indicating either an increase or decrease in the number of CA-repeats.

Table 3.2 Summary of loss of heterozygosity (LOH) results

Patient	VNTR (intron 20)		Microsatellite (intron 4)		
	Informativity	LOH	Informativity	LOH	MSI*
1			+		
5	+	+	+		
7			+		
8	+		+		
9	+		+		
12	+		+		
13	+		ND**	ND	ND
14 §	+	+	+		+
15	+	+	+		
16	ND	ND	+		
17	+	+	+		
19	+		+		+
20	+	+	+		
22			+		
23	+	+	+		
25 §	+	+	+		+
27	+		+		
28	+		+		
32	+		+		
34	+	+	+		
35	+		+		
37	+		+		
39			+		
41	+		+		
43	+		+		
44			+		
45	+	+	+		
48	+	+	+		
49	+	+	+		
50			+		
57	+	+	+		
58	+		+		+
59			+		+
62	+	+	+		

* MSI - Microsatellite instability

** ND - Not done

§ patients 14 and 25 show both LOH and MSI

3.4. DISCUSSION

Frequent loss of heterozygosity at the *Rb* gene locus has been demonstrated for oesophageal cancer in many parts of the world (Boynton *et al.*, 1991; Huang *et al.*, 1992). This suggests that the *Rb* gene is involved in the progression of oesophageal tumourigenesis in those populations, since it has been frequently shown that LOH is an indication of mutational inactivation of a tumour suppressor gene (Dryja *et al.*, 1989; Osborne and Leech, 1994).

In this study of South African oesophageal cancer, allelic loss was analysed using a VNTR marker and a microsatellite marker. Loss of heterozygosity analysis using the VNTR marker in intron 20 of the *Rb* gene was highly informative and twenty-six of the thirty-three patients (79%) assayed were heterozygous for this marker. Of these, 50% demonstrated LOH at the *Rb* gene locus. Boynton *et al.* (1991) and Huang *et al.* (1992) reported 54% and 48% *Rb*-LOH, respectively, in oesophageal squamous cell cancer tested in American patients. In both studies, flow cytometry or gross microscopic microdissection was used to select for tumour cells from the tumour biopsy, thus minimising misinterpretation due to the presence of normal cells. In contrast, Wagata *et al.* (1991) detected *Rb*-LOH in only 13% of unsorted samples from Chinese oesophageal cancer patients.

The microsatellite region in intron 4 of the *Rb* gene was also very informative (100%) but strikingly, no patients demonstrated LOH at this marker. An unexpected result was the absence of LOH using the microsatellite marker at the 5' end of the gene in patients who demonstrated LOH at the VNTR marker at the 3' end of the gene. Wadayama *et al.* (1994) demonstrated that when *Rb*-LOH was detected at one informative site, LOH could also be detected at all other informative sites analysed, except in those cases where partial deletions of the gene was found. This suggests that a partial deletion of one allele of the *Rb* gene may have occurred in the patients that displayed LOH in this study. However, additional markers between introns 4 and 20 would have to be screened to determine whether the deletion occurs in the same position in the gene in these samples. While no LOH was detected in intron 4, microsatellite instability was observed in five of thirty three samples (15%). This suggests malfunction of DNA repair in these patients, since microsatellite instability is a marker for defective DNA repair (Thibodeau *et al.*, 1993). These results are especially interesting since defective DNA repair may in itself cause other major genetic events such as activation of oncogenes and the inactivation of tumour suppressor genes. An interesting result was that both *Rb*-LOH and microsatellite instability was detected in patients 14 and 25 (Table 3.2). This suggests that oesophageal tumourigenesis may involve not only the inactivation of multiple tumour suppressor genes (Huang *et al.*, 1992), but disruption of other processes such as normal DNA repair as well.

The low incidence of background LOH at random loci demonstrated in another study in our laboratory (unpublished results - < 1.5%) supports the postulation that the high percentage of *Rb*-LOH found in this study is non-random and due to mutational inactivation of the *Rb* gene. Additionally, the microsatellite instability demonstrated in this study suggests that dysfunctional DNA repair systems, along with tumour suppressor gene inactivation, may also play a role in the progression of oesophageal tumourigenesis.

CHAPTER 4

MUTATIONAL ANALYSIS OF THE *Rb* GENE

4.1. INTRODUCTION

Inactivation of the *Rb* gene frequently occurs by point mutations that produce a mutant protein (reviewed by Levine, 1993) in addition to frequent allelic loss (Boynton *et al.*,1991; Wagata *et al.*, 1991; Huang *et al.*, 1992 this study, Chapter 3). These mutations occur most frequently in exons 13 to 22 which code for amino acids 393 to 772. This region of the protein contains a binding site for a large number of viral and cellular proteins. *Rb* proteins harbouring mutations in this region fail to bind these proteins and thereby lose their tumour suppressing ability. (reviewed by Levine, 1993)

In this study, the initial approach was to examine exons 13 to 23 of the *Rb* gene.The use of the reverse transcription-PCR technique (Kawasaki, 1991) would allow the screening of exon groups instead of individual exons. This results obtained using this technique were not reproducible and subsequent analyses focused on exons 17 and 21 of the *Rb* gene, as these exons have been reported to harbour mutations in 40% of Chinese oesophageal tumours (Li, 1993).

Two mutation screening methods, PCR-Single Strand Conformational Polymorphism analysis (PCR-SSCP) (Orita *et al.*, 1989) and PCR-Heteroduplex Analysis (PCR-HD) (Nagamine *et al.*, 1989), were used for primary screening of samples. Both these methods differentiate between the wild-type PCR product and mutated forms on the basis of altered electrophoretic mobility.

4.2 MATERIALS AND METHODS

(The composition of solutions are given in the Appendix)

4.2.1 DNA and RNA samples

DNA and RNA were simultaneously extracted from both tumour and normal oesophageal samples as described in Chapter 2. RNA was also isolated from blood donated by a healthy individual for the optimization of the RT-PCR procedure.

4.2.2 Reverse transcription-polymerase chain reaction (RT-PCR)

Primers were designed using the *Rb*-cDNA sequence (Friend *et al.*, 1986), dividing the "hotspot" region into four groups of exons: 13 to 16; 17 and 18; 19 and 20; 21 to 23 (Table 4.1). Primers were designed to anneal to sequences in flanking exons eg. primers for exons 13 to 16 annealed in exons 12 and 17.

Reverse transcription (RT): A standard reverse transcription protocol was used for production of *Rb*-cDNA (Kawasaki, 1991) using all the antisense primers to ensure that the entire region of interest was represented in the resulting cDNA. 1 µg of cytoplasmic RNA was reverse transcribed in a 20 µl reaction volume at 42°C for 1 hour.

Exact amounts of each RT component in 20 µl:

RNA	1 µg
Each primer	5 *p*moles
dNTPs	1mM of each
Reaction buffer (5x)	1x concentration
RNAse inhibitor	20 units
M-MuLV Reverse transcriptase	20 units

After incubation, the reverse transcriptase was denatured by heating the samples at 95°C for 10 minutes to prevent the reverse transcriptase from binding to the cDNA and interfering with the PCR step.

PCR amplification of cDNA: 1 µl of the RT reaction was used for amplication of the specific exon groups by PCR. The final amounts and concentrations of each component (50 µl reaction) were determined empirically and is given below:

Primers	5 *p*mol of each
dNTPs	100 µM
$MgCl_2$	3 mM
Taq DNA polymerase	0.5 units

When required, 3 µCi of ^{32}P-dCTP or ^{32}P-dATP was added to the PCR reactions to radiolabel the products.

The following PCR cycling parameters (forty cycles) yielded specific products for all primer sets: 96°C for 10 seconds, 52°C for 10 seconds and 72°C for 30 seconds,

followed by one final extension step at 72°C for 5 minutes to ensure full-length PCR products.

4.2.3 PCR amplification of exons 17 and 21

Primers for the amplification of exons 17 and 21 (Table 4.1) were designed using the *Rb* gene sequence (Toguchida *et al.*, 1993). Primer sets for both exons were designed to have similar average melting temperatures (Tm) so that they could be used in a multiplex PCR system.

The optimal amounts and concentrations of individual components were determined empirically for normal and multiplex PCR and increasing the $MgCl_2$ concentration from 2mM to 3mM improved yields when using the multiplex system. The final concentrations and amounts of each component used per 50 µl PCR reaction are given below:

DNA	100 ng
Primers	10 *p*mol of each
dNTPs	100 µM
$MgCl_2$	3 mM
Taq DNA polymerase	0.5 units

When required, PCR products were radiolabelled by adding 3 µCi of ^{32}P-dCTP or ^{32}P-dATP.

PCR cycling parameters were as follows (thirty cycles): 97° C for 5 seconds, 53° C for 5 seconds and 72°C for 20 seconds.This was followed by one final extension step at 72° C for 5 minutes to ensure full-length PCR products.

Table 4.1 Primers used for *Rb* mutational analysis of exons 13-23 by RT-PCR (A) and exons 17 and 21 by PCR analysis (B)

Targeted region	Product size	Primer pairs (sense/antisense)	Reference
A.		5'-ATGATGATTTTAAATTCACG-3'	Friend *et al.*, 1986*
Exons 13-16	323bp	5'-AAGATTCTGAGATGTACTTC-3'	
Exons 17&18	370bp	5'-ACATTTTTCATATGTCTTTA-3'	"
		5'-ATCTTACAGGAGAAAGATAC-3'	
Exons 19&20	370bp	5'-CTTGAATCTGCTTGTCCTCT-3'	"
		5'-TACATGGAACACATCATAAT-3'	
Exons 21-23	450bp	5'-AATGAGTATGAACTCATGAG-3'	"
		5'-CACCAATTGATACTAAGATT-3'	
B.		5'-AGCTCAAGGGTTAATATTTC-3'	Toguchida *et al.*, 1993**
Exon 17	295bp	5'-GCACATGAATGAATTTCTTC-3'	
Exon 21	200bp	5'-CCATGTAATAAAATTCTGAC-3'	"
		5'-GTTATGGATATGGATTTATC-3'	

* primers designed from *Rb*-cDNA sequence

** primers were designed from *Rb* gene sequence and annealled approximately 40 bases into the intron to facilitate sequence analysis of the intron-exon boundaries

4.2.4 Single Strand Conformation Polymorphism (SSCP) analysis

a. Principle

The SSCP technique relies on each single strand of a DNA molecule having a unique secondary conformation which is determined by its primary nucleotide sequence. Every double-stranded PCR product thus has two single-stranded conformers which can be resolved using defined gel electrophoresis conditions. A PCR product containing even a single base substitution would be identified by a change in mobility compared to the normal DNA sequence (Orita *et al.*, 1989) (Fig 4.1).

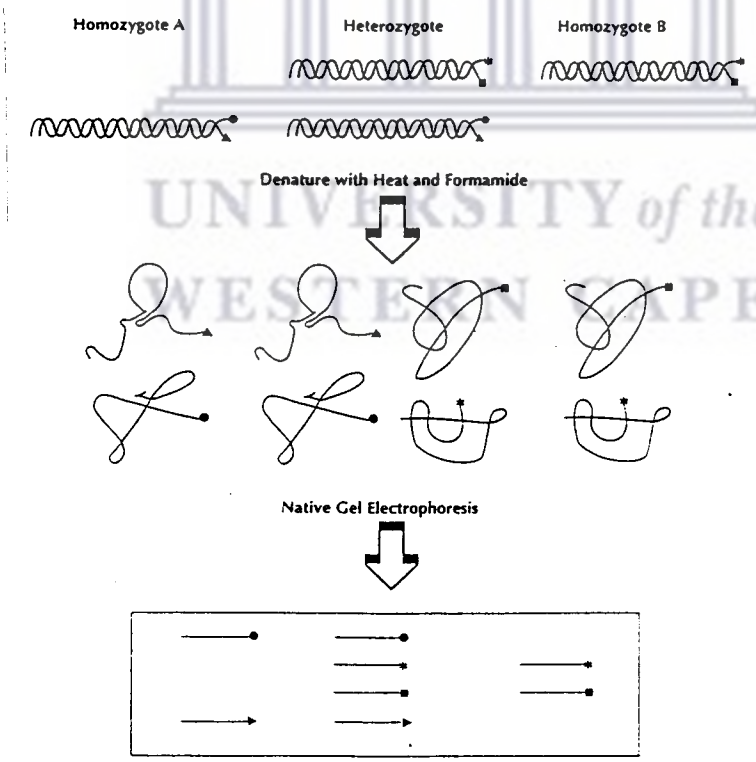

Figure 4.1 Principle of the SSCP technique

b. SSCP procedure

(All samples were assayed twice and any "positives" reassayed twice)

PCR products were radioactively labelled by the addition of 3 μCi of ^{32}P-dCTP or ^{32}P-dATP to the PCR reaction for detection by autoradiography as described in section 4.2.3. Equal volumes of PCR product and formamide loading buffer were mixed together and the samples denatured at 95°C for 5 minutes and cooled immediately on ice for at least 5 minutes before electrophoresis.

0.5xMDE™ (Mutation Detection Enhancement) acrylamide-based gels were used to resolve the single stranded conformers. Gels used routinely were 40cm long, 20cm wide and 0.4mm thick and contained 10% glycerol to improve resolution. 5*u*l of each denatured sample was electrophoresed in 1xTBE at 20W for 6 hours, or at 5W overnight. Samples were electrophoresed both at room temperature or at 4°C, since changes in secondary structure are often dependent on temperature.

After electrophoresis the gels were dried and exposed to X-ray film for at least 18 hours at room temperature.

4.2.5 PCR-Heteroduplex analysis

a. Principle

This mutation screening procedure relies on the decreased electrophoretic mobility of a heteroduplex, which is formed when normal and mutant single strands anneal, as compared to that of the normal homoduplex PCR product. For example, when a mutation is present in one allele of a gene, two products will be generated by PCR. In addition to the two homoduplexes, two heteroduplexes will form when the products re-anneal after heat denaturation. These heteroduplexes can be visualised as an extra, slower migrating band on acrylamide gels (Nagamine *et al.*, 1989) (Fig 4.2).

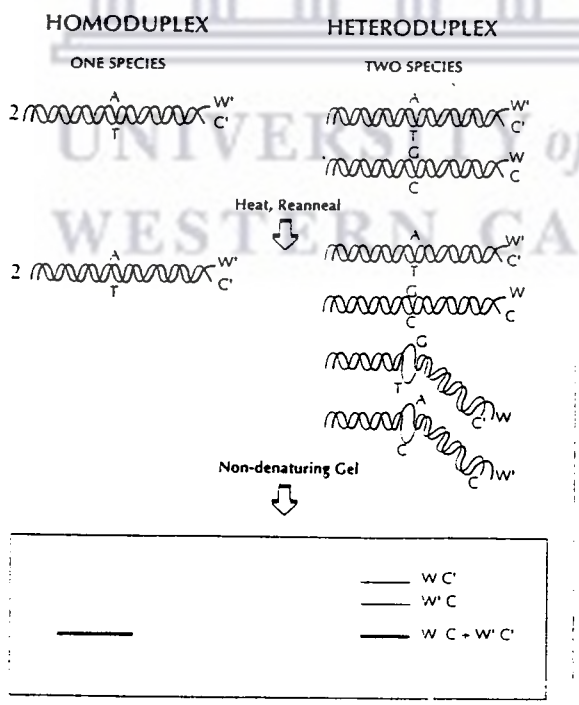

Figure 4.2 Principle of Heteroduplex analysis

b. Procedure

(All samples were assayed twice, and "positives" were re-assayed twice)

For patient tumour samples which demonstrated allelic loss, normal DNA from the same patient was added to the PCR reaction to enable the formation of heteroduplexes in cases where a mutation is present in the remaining allele. PCR products were denatured at 95°C for 5 minutes and left to cool slowly to room temperature to allow reannealing. 10 µl of each sample were mixed with 5 µl of formamide loading buffer (samples were not heated before loading as this may dissociate heteroduplexes).

Samples were electrophoresed in 0.6xTBE on 40cm long, 20cm wide and 1mm thick 10% heteroduplex polyacrylamide gels (1% crosslink) containing a nondenaturing concentration of urea (15% w/v) to prevent non-specific doublets. After loading, the samples were prerun into the gel matrix at 500V for 10 minutes and the gels electrophoresed at 200V for approximately 18 hours. Gels were then stained with ethidium bromide and viewed on a ultraviolet transilluminator.

4.2.6 DNA sequencing

Additional conformers obtained in the SSCP assay were extracted from the gel, reaplified and then sequenced. For the heteroduplex positives, the entire PCR sample was sequenced.

PCR products were sequenced using the Sequenase™ PCR product sequencing kit (USB). 7 µl of the required PCR product was used for sequencing and primer annealling was peformed as suggested. Labelling (with ^{35}S-dATP) was carried out on ice instead of at room temperature to facilitate the reading of sequences close to the primer and the termination reactions were carried out as suggested. Samples were resolved on 6% sequencing gels containing 7M urea in 1xTBE at 50W, using long (4 hours) and short (2 hours) runs to maximise the amount of sequence data obtained per sequencing reaction. Gels were dried and exposed to X-ray film for at least 18 hours at room temperature. DNA sequences were compared to the published *Rb* gene sequence (Toguchida *et al.*, 1993).

4.3 RESULTS

4.3.1 Mutational analysis of the *Rb* gene using RT-PCR

RT-PCR products of the expected size were obtained for each primer set (Fig 4.3). An annealling temperature of 52°C and a $MgCl_2$ concentration of 3mM was found to be optimal for all primer sets.

Of three patients screened (numbers 3, 4 and 6 of total patient sample), one (patient 3) exhibited an altered exon 13 to 16 PCR product of approximately 250bp (Fig 4.4, lane 3), instead of the normal 323 bp (Fig 4.4, lane 2), suggesting that a large deletion had occurred in that region of the *Rb* gene. When this product was sequenced, however, it was found to be homologous to a rat mitochondrial gene (GenBank entry: NCBI gibbsq 631637).

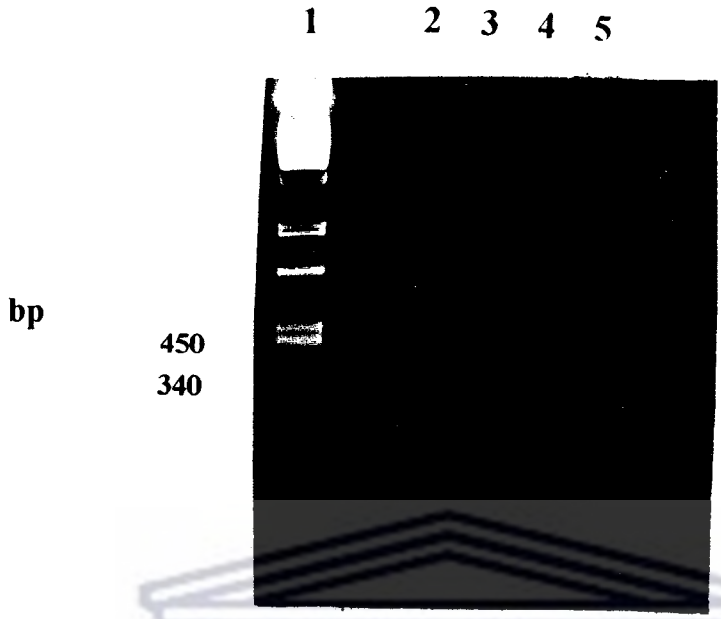

Figure 4.3 Reverse-transcription PCR products. 1 µg of cytoplasmic RNA was reverse transcribed in a 20ul reaction using antisense primers to the Rb gene. 1 µl of cDNA was used for PCR amplification of exon groups 13 to 16, 17 & 18, 19 & 20 and 21 to 23. Products were electrophoresed on a 1.5% agarose gel at 70V for 1.5 hours, stained with ethidium bromide and viewed on an ultraviolet transilluminator:

1. Lambda marker digested with *PstI*
2. exons 13-16 (323 bp)
3. exons 17 and 18 (370 bp)
4. exons 19 and 20 (370 bp)
5. exons 21 to 23 (450 bp)

After the first three patients were screened, no specific RT-PCR products could be obtained for any of the primer sets. All primer sets produced only smears with product bands either very faint or completely absent . Even cDNA that had previously produced specific PCR products, yielded smears. However, when a specific PCR-product for exons 13-16, which had been eluted from a polyacrylamide gel, was included as a positive template control for all subsequent experiments, a specific product of the correct size was always produced. All attempts to re-optimize the assay were unsuccessful. The parameters/steps that were changed were as follows:

RT - repeated RT reactions for all samples

- RNA samples which were used successfully in other RT-PCR based assays were included as controls for RNA quality. Even these RNA samples failed to produce specific PCR products. These RNA samples were isolated by Mr H. Donninger (Medical Biochemistry, UCT) from cultured cells using the single step guanidinium thiocyanate method (Chomczynski and Sacchi, 1987)
- cDNA was purified by phenol-chloroform extractions and ethanol precipitation
- new stocks of all RT components were used

PCR - decreased amounts of cDNA template were used

- formamide or dimethylsulfoxide was added to reactions to increase specificity
- decreased amounts of dNTPs and *Taq* DNA polymerase were used to minimise extension of nonspecifically bound primers
- decreased amounts of primers used to as little as 5 *p*mol each

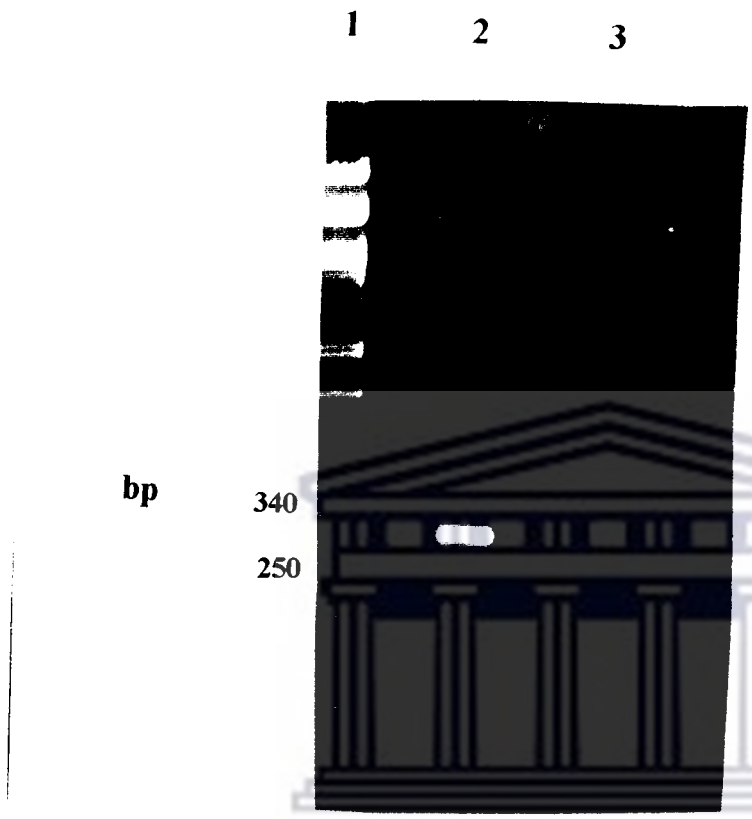

Figure 4.4 Patient 3 RT-PCR product with "deletion".

RT-PCR amplification of exons 13-16 using cytoplasmic RNA isolated from tumour tissue from patient 3 consistently produced a product of approximately 250 bp (lane3) instead of the normal 323 bp (lane 2). The RT-PCR products were electrophoresed on a 1.5% agarose gel at 70V for 1.5 hours, stained with ethidium bromide and viewed on an ultraviolet transilluminator. Lane 1 is lambda DNA digested with *Pst* I.

4.3.2 Mutation analysis of exons 17 and 21 of the *Rb* gene using PCR-SSCP and PCR-Heteroduplex analysis

The primer sets for both exons 17 and 21 were designed to have similar annealling temperatures for use in multiplex PCR (both primer sets in one reaction tube). Both primer sets were found to work best at an annealling temperature of 54°C when used separately and produced the expected products of 295bp and 200 bp, respectively (Fig 4.5, lanes 2 and 3). These primers also produced specific products when used in a multiplex system (Fig 4.5, lane 4).

SSCP analysis of PCR products was performed using 0.5xMDE gels containing 10% glygerol as these conditions resulted in the best resolution. When the multiplex PCR system was used, two sets of bands were observed. Of the twenty patients screened by SSCP analysis, no mutations were detected in exon 21 and only one patient's exon 17 product had an altered electrophoretic pattern, suggesting a possible mutation. This can be seen by the additional slower migrating conformers (Fig 4.6, lane 2), when compared with the normal (Fig 4.6, lane 1). These conformers were only present on gels that were electrophoresed at 4°C. These results were reproduced in two independent experiments.

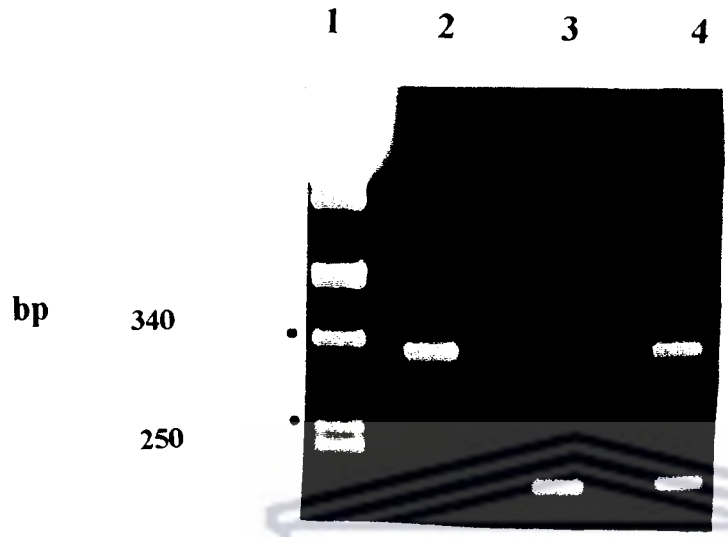

Figure 4.5 Rb exon 17 and 21 PCR products. 50 ng of normal human chromosomal DNA was used for the PCR amplification of exons 17 and 21 of the Rb gene. Specific products were obtained when individual primer sets were used as well as in multiplex PCR. Products were electrophoresed on a 2% agarose gel at 80V for 2.5 hours, stained with ethidium bromide and viewed on an ultraviolet transilluminator:

1. Lambda DNA digested with *Pst*I
2. exon 17 (295 bp)
3. exon 21 (200 bp)
4. multiplex products

Since heteroduplexes migrate slower than homoduplexes, a patient was scored positive when two bands were present, the normal band and a slower migrating band as is shown in Fig 4.7, lanes 2-6. A number of negative controls (products from normal patient DNA) were included on each gel to identify the homoduplex band. Of the twenty patients screened by heteroduplex analysis, twelve patients showed patterns indicative of a mutation in exon 17 whereas normal patterns were observed in all patients tested for exon 21 mutations. These results were reproduced in three independent experiments.

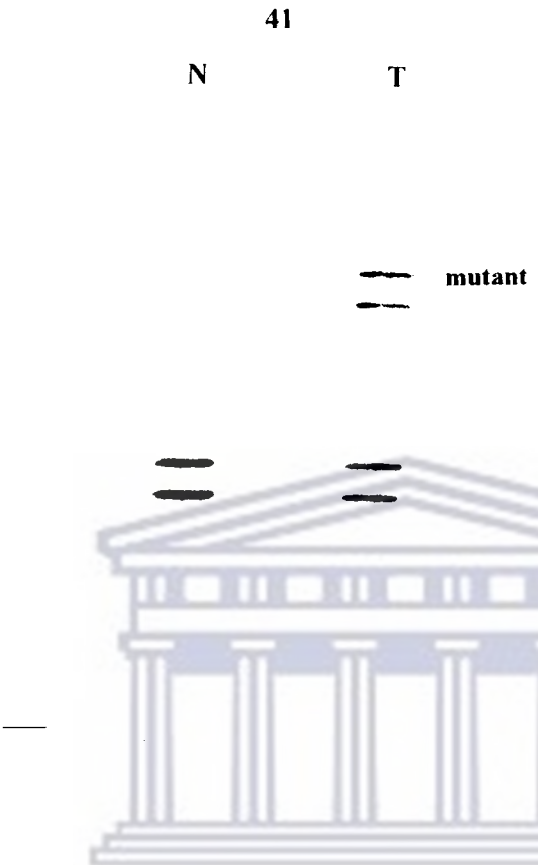

Figure 4.6 Examples of normal and mutant SSCP patterns.

Denatured, radiolabelled exon 17 PCR products were electrophoresed on 0.5 x MDE™ gels containing 10% glycerol to separate the single stranded conformers. Additional conformers were present for the tumour DNA sample of patient 41, suggesting that the PCR product harboured a mutation that had occured in one of the alleles.

(N = normal/control DNA, T = tumour DNA)

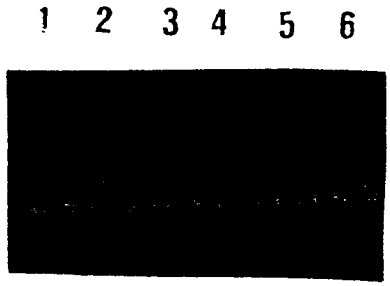

Figure 4.7 Heteroduplex analysis of patients 5, 15, 41, 58 and 62.

PCR products were denatured and allowed to reanneal . Samples were electrophoresed on a 10% polyacrylamide gel containing a non-denaturing concentration of urea, stained with ethidium bromide and viewed on an ultraviolet transilluminator. A wild-type PCR product was included on the gel as a negative control (Lane 1). Heteroduplexes could be visualised as an extra, slower migrating band (lanes 2-6). Lanes 2-6 represent PCR products from tumour DNA of patients 5, 15, 41, 58 and 62, respectively.

4.3.4 DNA Sequencing Analysis

All exon 17 PCR products that were positive for SSCP or Heteroduplex Analysis were sequenced in order to confirm and identify the mutation. The entire exon and parts of the flanking introns were examined and none of the sequences obtained demonstrated mutations when compared to the published sequence.

4.4 DISCUSSION

Besides frequently being a "target" for allelic loss in oesophageal cancer (Boynton *et al.*,1991; Huang *et al.*, 1992; this study chapter 3), the *Rb* gene is also inactivated by point mutations that produce a dysfunctional protein (reviewed by Levine 1993). The aim of this study was to determine whether point mutational inactivation of the *Rb* gene is involved in oesophageal tumourigenesis in South African patients.

It was initially decided to screen the *Rb* mutation "hotspot" exons 13 to 23 using the reverse transcription-polymerase chain reaction (RT-PCR) technique in conjunction with the mutation screening techniques, SSCP and heteroduplex analysis. Only three patients were screened because the results obtained using the RT-PCR component of the assay were not reproducible. One of those patients appeared to have a deletion in the exon 13 to 16 region, as shown by the smaller PCR product in Fig 4.4, lane 3. However, when this product was sequenced, it was found to be a non-specific PCR artifact. All subsequent RT-PCR experiments were unsuccessful and produced only smears. Even those cDNA templates that had previously produced specific PCR products yielded smears.In contrast, a gel purified RT-PCR product included as a control PCR template, always yielded a specific product. Since the assay was successful initially, a number of steps were taken to re-optimise the procedure. However, none of the strategies followed were successful. Even RNA templates, which had been used successfully in other RT-PCR based assays, yielded non-specific smears. Because all attempts to solve the RT-

PCR problem were unsuccessful, it was decided to screen the oesophageal tumour DNA directly using PCR.

Exons 17 and 21 of the *Rb* gene were analysed directly by PCR-SSCP and PCR-heteroduplex analysis, since mutations in these exons were reported in 40% of oesophageal tumours in Chinese patients (Li, 1993). In this study, only one of twenty patients screened by SSCP analysis was found to harbour a possible mutation in exon 17 in three independent experiments. However, when the additionalconformers (Fig. 4.6) were extracted from the SSCP gel, reamplified and sequenced, no mutation was detected. In contrast to SSCP, analysis of exon 17 by heteroduplex analysis, revealed twelve patients with putative mutations (one of these were also positive for SSCP), repeated in two independent experiments. These results proved to be false positives as no mutations were found when the positive PCR products were examined by DNA sequencing of the entire exon as well as the intron-exon boundaries.. No mutations were detected in exon 21 in twenty patients analysed using SSCP and heteroduplex analysis. The absence of mutations in those samples that appeared positive in the screening assays was unexpected, suggesting that the false positives were probably an artifact of the system used in this study. The fact that all the mobility shifts detected in the heteroduplex assay were identical to those demonstrated in figure 4.7, also suggests that they are false positives. It is expected that some mutations would not be detected using SSCP and heteroduplex analysis, since not all mutations produce extra bands. It is thus possible that mutations may have been missed in this study due to false negative SSCP results. However, a high proportion of false positives, as obtained here for heteroduplex analysis, has not been

reported previously. These findings suggest that direct sequencing of PCR products, albeit time consuming and expensive, is probably the best method of mutation analysis. Alternatively, when the techniques of SSCP and heteroduplex analysis are employed, any positives should always be confirmed by DNA sequencing of the PCR products. This is in contrast to studies where mutation frequencies in tumour suppressor genes are calculated from mutation screening results alone (Chang *et al.*, 1995). Also, the Hydroxylamine Osmium Tetroxide chemical cleavage method (HOT) for mutation detection (Cotton *et al.*, 1988) has been described as being 100% efficient (Grompe, 1993). This technique, however, requires the use of extremely hazardous chemicals.

Although the sample size in this study was small (n=20), the results suggest that inactivation of the *Rb* gene does not occur through mutations in exons17 and 21 in South African oesophageal tumours, in contrast to the results reported by Li (1993). This may be due to different aetiological factors contributing to tumourigenesis in the Chinese and South African populations. *Rb* mutation studies in other cancers indicate that point mutations are spread mostly throughout the "hotspot" exons of the gene (Hogg *et al.*, 1992; Wadayama *et al.*, 1994). This further suggests that the mutational profile reported in the abovementioned Chinese study is probably unique for that population and that the samples analysed here may harbour mutations in the other "hotspot" exons. Alternatively, the high percentage of allelic loss affecting the *Rb* gene found in South African oesophageal tumour samples (this study, Chapter 3) may have been sufficient for inactivation of the gene since *Rb* loss of heterozygosity has been correlated with loss of *Rb* protein expression in bladder cancers and osteosarcomas (Xu *et al.*, 1993; Wadayama

et al., 1994).

CONCLUSION

One objective of this study was to devise a method to extract DNA and RNA simultaneously from small (ca. 25mg) oesophageal biopsies. The final procedure (Chapter 2) was based on two popular RNA isolation techniques. The first employs NP40 to separate the nuclear and cytoplasmic fractions and the second protects the RNA from degradation by using the powerful denaturant GITC. DNA is isolated from the nuclei using a standard isolation protocol. This procedure has consistently yielded intact DNA and RNA from a number of tissue types, even pancreatic tissue which is rich in ribonucleases, and promises to be invaluable in those projects where both nucleic acids have to be isolated from small tissue samples. It is however, important to determine whether the isolated RNA is suitable for sensitive molecular applications after long-term storage at -70°C.

The primary objective of this study, however, was to determine whether inactivation of the *Rb* gene played a role in tumourigenesis in South African oesophageal cancer patients. In Chapter 3, tumour samples from oesopageal cancer patients were analysed for *Rb* allelic loss using a microsatellite marker in intron 4 and a VNTR in intron 20 of the *Rb* gene. The VNTR proved to be very informative and 79% of the thirty three patients were informative at this marker.

Of the twenty-six informative cases, thirteen (50%) demonstrated allelic loss. The low frequency of allelic loss detected at random loci in another study in our laboratory suggests that the high frequency of *Rb*-LOH is due to mutational inactivation of the *Rb* gene. The microsatellite marker had an informativity ratio of 100%. Strikingly, no LOH was detected at this marker, even in those patients demonstrating LOH at the VNTR. This may suggest that a partial (3') deletion of one allele of the *Rb* gene had occurred in those tumour samples. While no LOH was detected at the microsatellite, five of the thirty-five (15%) patients showed genomic instability. Of these, two also demonstrated *Rb*-LOH at the VNTR marker.

In Chapter 4, the *Rb* gene was analysed for point mutations in the exons 17 and 21 using the mutation screening assays SSCP and Heteroduplex Analysis (Chapter 4). Putative mutations were detected in exon 17 in twelve of the twenty tumours using these assays. However confirmation of the positives by DNA sequencing indicated that no mutations had occured in those samples. It was therefore, apparent that putative mutants identified using the screening techniques should be confirmed by DNA sequencing to prevent overestimation of mutation frequency due to false positives.

The high percentage of LOH found in the samples analysed does, however, implicate inactivation of the *Rb* gene as one of the steps of oesophageal tumourigenesis in South African patients, since a number of studies have correlated *Rb*-LOH with loss of *Rb* protein expression.

FUTURE PERSPECTIVES

1. In order to determine whether the 3' deletion of the *Rb* gene found in Chapter 3 occurs at a common site in all the LOH+ tumours, a number of other allelic markers located between introns 4 and 20 would have to be screened.

2. A number of other microsatellites should be examined to approximate the prevalence of microsatellite instability, and thus defective DNA repair mechanisms, in South African oesophageal tumours. Positive tumour DNA samples could then be analysed for inactivation of candidate DNA repair genes to propose a mechanism for the genomic instability displayed here.

3. It would also be interesting to determine whether inactivation of the *Rb* gene (and other tumour suppressor genes) correlates with a certain tumour

grade or with patient survival status to determine. This would require screening of these tumour suppressor genes in a larger sample to be able to make statistical correlations between these phenomena. The results obtained in such a study may provide valuable molecular diagnostic and prognostic markers that may assist in oesophageal cancer therapy.

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APPENDIX

A. DNA AND RNA ISOLATION SOLUTIONS

GITC solution

4M Guanidinium isothiocyanate

20mM Sodium Acetate pH 5

Dissolve all components in distilled water - stir at approximately 65°C. Make up to the required volume and autoclave.

STABLE FOR ONLY 3 MONTHS.

NP40 solution

2% Nonidet P40

50 mM Tris pH 8.0

100 mM NaCl

5 mM $MgCl_2$

Add Nonidet P40 after mixing and autoclaving the other components. Store at 4°C.

Proteinase K buffer (prepare from concentrated stock solutions)

100mM NaCl

10 mM Tris pH 8

25 mM EDTA pH 8

0.5% SDS

Mix all components, make up to the required volume and autoclave.

Water-saturated phenol

Dissolve 25g phenol in distilled water in a microwave and shake thoroughly. Place in a refrigerator and allow phases to separate. Aspirate the upper water phase and transfer phenol solution to a dark bottle. Store at 4°C for up to two weeks.

Proteinase K

10mg/ml of lyophilized proteinase K in 1 X TE

10 X TE

0.01 M Tris

0.001 M EDTA

pH to 7.5 and autoclave

B. ELECTROPHORESIS SOLUTIONS

40% acrylamide stock

38% acrylamide

2% bis-acrylamide

Dissolve components in water by stirring at room temperature. Make up to the required volume. Store in a dark bottle at 4°C.

40% heteroduplex acrylamide stock

39.5% acrylamide

0.5% bis-acrylamide

Dissolve components in water by stirring at room temperature. Make up to the required volume. Store in a dark bottle at 4°C.

10 X TBE

108g Tris-(hydroxymethyl) amino methane

55g Boric acid

EDTA

Ethidium bromide - 10mg/ml in distilled water

Gel loading buffer

0.25% bromophenol blue

0.25% xylene cyanol

in 30% glycerol in sterile distilled water

Formamide loading buffer

0.25% bromophenol blue

0.25% xylene cyanol

in deionised formamide

C. PCR REAGENTS

Taq DNA polymerase

Thermoprime plus™ (5U/µl), 10X reaction buffer and 25 mM $MgCl_2$ supplied with the enzyme was used according to manufacturers specifications. Typically 0.5 units was used for a 50µl PCR reaction.

Deoxynucleotide mix (dNTPs)

The four dNTPs (100mM) were mixed to a final concentration of 2.5mM of each.

Primers

Primers were diluted to a 5*p*mol/µl working concentration in sterile distilled water.

D. LIST OF CHEMICAL SUPPLIERS

ITEM	SUPPLIER
Tris	Merck
EDTA	Merck
Phenol	Merck
Orthoboric acid	Merck
Mineral oil	Sigma
Urea	Sratagene
Acrylamide - general use	Merck
Acrylamide - heteroduplex	Stratagene
Bis-acrylamide	Merck
Agarose	Sigma
0.5 ml thin-walled PCR tubes	Whitehead Scientific
1.5 ml microcentrifuge tubes	Whitehead Scientific
Restriction enzymes	Boehringer Mannheim
dNTPs	Boehringer Mannheim
Thermoprime plus™ Taq polymerase	Southern Cross Biotech
Proteinase K	Boehringer Mannheim
Lambda DNA	Boehringer Mannheim
Sequenase™ PCR product sequencing kit	Weil Organization

X-ray film	Agfa
Xray developer	Agfa
Xray fixer	Agfa
Polaroid 667™ film	Fotolens
^{32}P-dCTP and ^{35}S-dATP	Whitehead Scientific