THE

ADENOMATOUS POLYPOSIS COLI (*APC*)

AND *p53* GENE STATUS IN SOUTH AFRICAN

OESOPHAGEAL CANCER PATIENTS.

by

Michelle Skelton

DECLARATION

I, _____, declare that " **The Adenomatous Polyposis Coli (*APC*) and *p53* gene status in South African oesophageal cancer patients"** is my own work and that all the sources I have used or quoted, have been indicated and acknowledged by means of complete references.

Date 9 Sept 1996

DEDICATION

This thesis is dedicated to my parents.

ACKNOWLEDGEMENTS

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ABSTRACT

Oesophageal cancer is the most common cause of cancer-related deaths in black males in South Africa. In an effort to understand the molecular nature of oesophageal carcinogenesis in South Africa, two tumour suppressor genes, Adenomatous Polyposis Coli (*APC)* and *p53,* were examined in normal and tumour tissue obtained from 33 oesophageal cancer patients. Several studies have shown that alterations of the *APC* and *p53* genes are associated with the development of cancer. Allelic loss at the *APC* gene locus was examined using two polymorphic markers within the coding region of the *APC* gene. Single stranded conformation polymorphism, heteroduplex and DNA sequencing analyses were used to detect mutations in the mutation susceptible regions of the *APC* and *p53* genes. The "Mutation Cluster Region" (MCR) in exon 15 of the *APC* gene was examined. Only exons 5 and 6 in the "hot spot" region of the *p53* gene were examined. An allelic loss of 21% (4 of 19 informative cases) and an informativity of 59% (19 of 32) of the *APC* gene was demonstrated in patients analysed. No somatic mutations were detected in the MCR in exon 15 of the *APC* gene. Three putative mutations were detected in the *p53* gene using SSCP and HD analysis, two of which were confirmed by DNA sequencing. Analysis of one patient revealed a TCA to TGA base substitution at codon 183 in exon 5 of the *p53* gene, resulting in a stop codon at that position. An eleven base pair deletion in exon 6 of the *p53* gene was detected in another patient. This deletion caused a frame shift mutation and culminated in a premature stop codon 13 codons downstream. Overall, a mutation frequency of 8% (2 of 25 patients analysed) was detected for the *p53* gene with exons 7 and 8 still pending further study. These results suggest that the *APC* gene may not be involved in oesophageal cancer in South Africa and further studies are necessary in order to examine the role of the *p53* gene in this disease in South Africa.

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CHAPTER ONE

Oesophageal Cancer

1.1 Incidence

Oesophageal cancer is one of the ten most common malignancies worldwide (Franceschi *et al.*, 1990) with two major forms recognized based on histological criteria. Squamous cell carcinoma (SCC), which is derived from squamous epithelial cells and is often located in the proximal third of the oesophagus (Wolfman *et al.*, 1994) and adenocarcinoma (AC), which is derived from columnar epithelial cells and is often located in the distal third of the oesophagus (Kruse *et al.*, 1993).

Oesophageal cancer is characterised by a dramatic variation in incidence geographically with high risk areas identified in South Africa, China, Iran, South America and France (Blot, 1994). These high risk areas have incidences in the range of 50-300/100 000, compared to low risk areas such as the United States of America with incidences of less than 5/100 000 (Van Rensburg, 1981; Blot, 1994). China has been reported to harbour 54% of the world's oesophageal cancer cases with SCC as the most common histological type (Parkin *et al.*, 1988; Dawsey *et al.*, 1994). An incidence as high as 470/100 000 has been reported for the Linxian county of China (Li, 1982).

Oesophageal SCC is the most common malignancy in black males in certain parts of South Africa. Males between the ages of 40 and 60 years have a higher risk compared to aged-

matched females, with a ratio of 4:1 (Segal *et al.*, 1988; Sumeruk *et al.*, 1992). Recent demographic data indicated that the national incidence of oesophageal cancer is ~20/100 000, while the high risk area of rural Transkei has an alarmingly high incidence of 50/100 000 (South African National Cancer Registry, 1989).

1.2 Prognosis and Therapy

A disturbing feature of oesophageal cancer in South Africa is the very poor prognosis with less than 10% of the patients surviving beyond 3 months after diagnosis (personal communication, Dr. De Groot, Groote Schuur Hospital, Cape Town, South Africa). This poor survival rate is primarily due to the advanced stage at which individuals present themselves, when displaying swallowing difficulties and weight loss. Oesophageal cancer therapy involves several forms of treatment and a combination of oesophagectomy, chemotherapy and radiation treatment are applied to patients with a favourable prognosis (Mannell *et al.*, 1989; Girvin *et al.*, 1995). Although patients respond to therapy, the mortality rate due to remission is high and the approach frequently adopted is of a palliative nature because of the poor clinical condition of the patients (Girvin *et al.*, 1995).

Ineffective therapeutic strategies and poor prognosis of oesophageal cancer appear to be global problems, highlighting our rudimentary understanding of this disease. Recently, molecular diagnostic and prognostic indicators have been very useful in the treatment of certain cancers, with the identification of genetic lesions associated with specific cancers such as retinoblastoma, colorectal cancer and breast cancer (Wiggs *et al.*, 1988; Sidransky *et al.*,

1992; Harris *et al.*, 1993). These stepping stones encourage the further development of molecular diagnostics at three levels of oesophageal cancer treatment: (i) early detection markers that are economically viable and pragmatic, (ii) markers that assist in defining therapeutic approaches and (iii) prognosis. The development of future therapies depends on the understanding of the risk factors involved and the impact that these factors have on the oesophageal mucosa and the molecular events that trigger tumourigenesis.

1.3 Environmental Factors

In the high risk areas of South Africa, epidemiological studies suggest that oesophageal cancer appears to be associated with mineral and vitamin deficiencies (Van Rensburg, 1981) and is further aggravated by smoking and the consumption of home brewed beer (Segal *et al.*, 1988; Sumeruk *et al.*, 1992). Traditional home brewed beer is frequently prepared using corn infected with the fungus *Fusarium moniliforme* (Rheeder *et al.*, 1992) which produces secondary metabolites (fumonosins) capable of inducing hepatic tumours in rats (Thiel, 1991). The combination of poor diet (linked to socio-economic factors) and exposure to carcinogens apparently contributes to the high incidence of oesophageal SCC in South African black males.

Likewise, no consensus has been reached regarding the causative factors for oesophageal cancer in China. A number of case studies reviewed by Cheng (1994) implicated malnutrition, drinking of traditional beverages at high temperatures and the consumption of pickled vegetables with possible fungal contamination in the development of oesophageal cancer.

However, Yu *et al.* (1993), found no correlation between the consumption of pickled vegetables and oesophageal cancer. Evidence has also been provided which suggests that human papillomavirus infection plays a role in oesophageal cancer (Chang *et al.*, 1994). In Iran, a significant association was demonstrated between the frequent chewing of opium pipe scrapings and the incidence of oesophageal cancer (Ghadirian *et al.*, 1985). In Uruguay, a high risk of oesophageal cancer has been associated with alcohol consumption, the use of black tobacco (which is cheaper than blonde tobacco) and a lack of fresh fruit and vegetables in the diet (De Stefani *et al.*, 1990). Alcohol and tobacco use have also been associated with high risk in France (reviewed by Blot, 1994).

Although current evidence suggests that environmental factors play a major role in the aetiology of oesophageal cancer, the influence of genetic traits should not be excluded. The genetic disorder known as Tylolysis is strongly associated with the development of oesophageal cancer (Marger and Marger, 1993). Irrespective of the causative factors being environmental or hereditary, it is the integrity of the genome that is ultimately compromised by these factors and which eventually results in the neoplastic phenotype. The types of genetic lesions are diverse and include genetic alterations that activate or inactivate genes inappropriately.

1.4 Molecular Biology of Cancer

The molecular basis of cancer is steadily being elucidated and numerous advances have been made in the understanding of the genetic alterations associated with tumourigenesis.

Extensive studies have implicated many genes in the development of cancer, e.g. *p53* (Levine *et al.*, 1991), *APC* (Joslyn *et al.*, 1991), Deleted in Colorectal Cancer (*DCC*) (Thompson *et al.*, 1993), Multiple Tumour Suppressor (*MTS*)1 (Mori *et al.*, 1994); K-*ras* (Keohavong *et al.*, 1996), *myc* (Cole, 1986), Breast Cancer (*BRCA* I) (Miki *et al.*, 1994), Retinoblastoma (*Rb*) (Bookstein *et al.*, 1990), cyclin D1 (Jiang *et al.*, 1993), epidermal growth factor receptor (*EGFR*) and its homologue the *erb* B2 gene (reviewed by Stemmermann *et al.*,1994) and others. These genes are frequently mutated, deleted or overexpressed in tumour tissue compared to normal tissue.

A wealth of information now exists which implicates these genes in the development of cancer. The *p53* gene is mutated in approximately 50% of all human cancers and is thought to play a role in tumourigenesis (Hollstein *et al.*, 1991b). This gene has also been implicated in the development of oesophageal cancer (Wagata *et al.*,1993), prostate cancer (Ittmann *et al.*,1994), colon cancer (Nigro *et al.*,1989) and breast cancer (Hartmann *et al.*,1995). Overexpression of the p53 protein has been demonstrated in a variety of malignancies (Bartek *et al.*,1991) and tumour progression has been correlated with increasing intensity of p53 immunostaining (Younes *et al.*,1993). The germline transmission or inheritance of one inactive *Rb* allele predisposes individuals to the retinoblastoma syndrome (Wiggs *et al.*, 1988; reviewed by Levine, 1993). Since the discovery of the *Rb* gene, mutations in this gene have been associated with other sporadic cancers such as prostate (Bookstein *et al.*, 1990) and oesophageal cancer (Boynton *et al.*, 1991; Huang *et al.*,1993). Mutations in the *ras* gene have been reported in colon cancer (Vogelstein *et al.*, 1988) and lung cancer (Keohavong *et al.*,

1996), but occur infrequently in other cancers. Cyclin D1 gene rearrangement, amplification and overexpression have been reported in several neoplasms including parathyroid (Motokura *et al.*, 1991), breast (Sherr, 1993) and liver cancers (Zhang *et al.*,1993). In addition the coamplification of *hst*, *int 2* and cyclin D1 genes at chromosomal position 11q13 has been reported in oesophageal SCCs (Tsuda *et al.*, 1989; Jiang *et al.*, 1993).

1.4.1 Protooncogenes, Tumour Suppressor Genes and the Cell Cycle

The genes frequently shown to be mutated in cancer can be placed into two broad categories: protooncogenes (*ras, fos, myc* and *jun*) and tumour suppressor genes (*p53, Rb, APC* and *DCC*). These protooncogenes and tumour suppressor genes are involved in regulating the rate at which cells divide. Cell division is a complex process with multiple levels of control and with many signalling pathways and feedback loops. The various phases of the cell cycle are simplistically illustrated in Figure 1.1. The S and M phases represent DNA synthesis and mitosis respectively, separated by G1 (gap 1) and G2 (gap 2), respectively (Howard and Pelc, 1951).

Under normal conditions, a balance exists between those genes which enhance normal cell division and growth ("accelerators"), and those genes which inhibit cell division and growth ("brakes"). Protooncogenes fall into the first category of cell cycle regulators and tumour suppressor genes into the second. This balance is disrupted when mutations in protooncogenes yield oncogenes that encode protein products capable of stimulating target

cells to move through the cell cycle and divide in an uncontrolled manner (Lemoine *et al.*, 1994). Similarly, the loss or mutational inactivation of tumour suppressor genes results in gene products that are no longer capable of regulating cell division and growth and consequently, cell division occurs in an uncontrolled manner. Mutations in protooncogenes and tumour suppressor genes both result in a similar phenotype characterised by uncontrolled proliferation of cells, a prerequisite for the development of cancer.

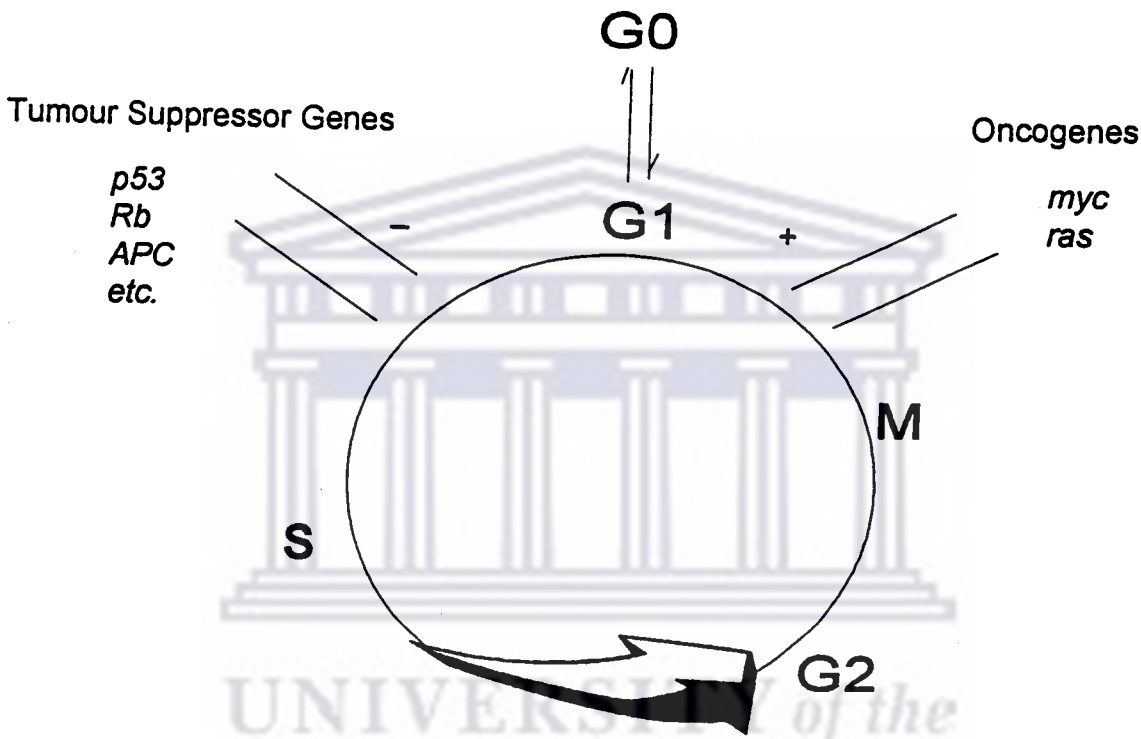


Fig. 1.1 The Cell Cycle
Cell cycle regulation by oncogenes and tumour suppressor genes. The tumour suppressor genes illustrated inhibit cell cycle progression at the G1/S interphase. Oncogenes enhance cell cycle progression resulting in an increase of cell proliferation. S = synthesis, M = mitosis, G0 = quiescence, G1 = gap 1 and G2 = gap 2.

1.5 Characterization of the *APC* and *p53* Tumour Suppressor Genes in Cancer.

Based on current information, it is generally accepted that cancer is caused by the accumulation of mutations in a range of genes which are normally involved in regulating the proliferation of cells, either positively (protooncogenes) or negatively (tumour suppressor genes). The specific genes involved and the sequence of activation or inactivation appear to be tissue specific. The best understood system in this context has been described in colorectal cancer by Fearon and Vogelstein (1990). Several genetic alterations occur during colorectal tumourigenesis: loss and/or mutation of the *APC*, *DCC* and *p53* tumour suppressor genes, activation of the Ki-*ras* oncogene and decreased chromosomal DNA methylation. These events coincide with specific stages of tumour development as illustrated in Fig.1.2. This model has already proven useful in the predictive diagnosis of FAP (familial adenomatous polyposis) individuals who are at risk of developing colorectal cancer (MacDonald *et al.*, 1992). Once early genetic lesions (e.g. loss or mutation of the *APC* gene) have been detected in patients at risk, therapy involving colonic resection may be implemented, thereby decreasing the risk of developing colorectal cancer.

Unlike FAP and colorectal cancer, very little is understood about the molecular biology of oesophageal cancer in South Africa. An understanding of the genes involved in the development of cancer in South Africa may allow us to address issues such as early diagnosis, prognosis and response to therapeutic treatments.

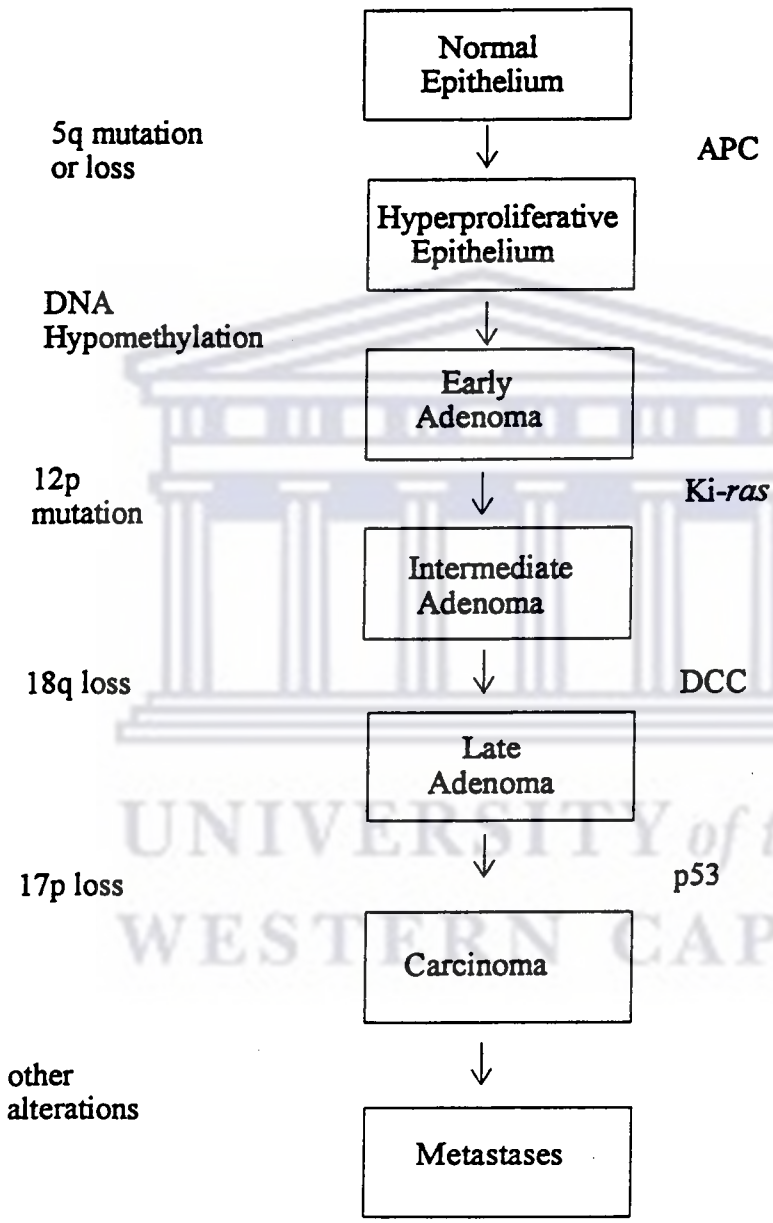


Fig. 1.2 Multistep Colorectal Carcinogenesis
Several genetic lesions occur during colorectal carcinogenesis. Certain genetic lesions are associated with specific tumour stages. Mutations of the Ki-*ras* gene characteristically occur at early adenoma stage whereas loss or mutation of the *APC* gene is the first genetic lesion associated with colorectal cancer (adapted from Fearon and Vogelstein, 1990).

1.5.1 The Adenomatous Polyposis Coli Gene

Consistent loss of a region of chromosome 5q was demonstrated in FAP patients as well as in individuals with sporadic colorectal cancer, which led researchers to investigate the existence of a potential tumour suppressor gene in this region (Okamoto *et al.*, 1988; Vogelstein *et al.*, 1989). After mapping and positional cloning, several potential tumour suppressor genes, including *APC*, were identified at the 5q21-22 locus (Bodmer *et al.*, 1987; Groden *et al.*, 1991; Joslyn *et al.*, 1991). Using the single stranded conformation polymorphism (SSCP) mutational screening technique and DNA sequencing, Groden *et al.* (1991) demonstrated that the *APC* gene was uniquely mutated in several unrelated FAP patients and that specific mutations were inherited by the offspring. Loss or inactivation of this gene in colorectal tissue would therefore predispose individuals to the polyposis phenotype.

Subsequent studies revealed that the *APC* gene is located at chromosomal locus 5q21 and consists of 16 exons with the first exon being non-coding (reviewed by Nakamura, 1993). The *APC* gene encodes a 2844 amino acid protein with a predicted molecular weight of 311.8 kDa (Groden *et al.*, 1991). Exon 15, the largest exon, codes for more than 2000 amino acids (Groden *et al.*, 1991). Very little is known about the structure of the *APC* protein which appears to have no transmembrane domains and is located in the cytosol (Miyashiro *et al.*, 1995). The protein has potential coiled-coil regions within the N-terminal domain, which could facilitate homo-oligomerization (Joslyn *et al.*,1993).

The cellular function of *APC* is unclear. Rubinfeld *et al.* (1993) and Su *et al.* (1993) have shown that *APC* possibly plays a role in cell adhesion through its interaction with β-catenin, an adherence junction protein. *APC* has also been shown to interact with microtubules (Munemitsu *et al.*, 1994). More recently, wild-type *APC* was reported to down-regulate Beta-catenin in colorectal cell lines (Munemitsu *et al.*, 1995). It has also been suggested that *APC* plays a role in blocking the progression of the cell cycle at the G0/G1 to S-phase checkpoint (Baeg *et al.*, 1995).

Increasing interest in the role of the *APC* gene in the development of cancer led to further screening of the gene in several cancers. Allelic loss of the *APC* gene has been demonstrated in 25-50% of colorectal carcinomas (Vogelstein *et al.*,1989), 50-80% of oesophageal SCCs (Boynton *et al.*, 1992; Shibagaki *et al.*,1994), 45% of oral SCCs (Uzawa *et al.*,1994), 63% of prostate (Gao *et al.*, 1995) and 3% of breast tumours (Kashiwaba *et al.*,1994). Intensive mutation screening using a variety of techniques has identified additional novel germline mutations in FAP individuals (Stella *et al.*, 1994), as well as somatic mutations in sporadic colorectal cancer (Powell *et al.*, 1992; reviewed by Nakamura, 1993). Genetic alteration of the *APC* gene appears to be an early event in colorectal tumourigenesis (Powell *et al.*, 1992), preceeding mutations of *ras*, *p53* and *DCC* genes (Fearon and Vogelstein,1990). Miyoshi *et al.* (1992) detected at least one mutation in 80% of colorectal carcinomas, while two mutations were detected in 60% of samples analysed. Somatic mutations have also been reported in oral SCC (12.5%) and breast cancer (6%) (Uzawa *et al.*,1994; Kashiwaba *et al.*, 1994).

The germline and somatic mutational spectrum of the *APC* gene is very similar, with frame shift and nonsense mutations being the most frequent, resulting in the generation of truncated proteins. A "hot spot" for mutations in the *APC* gene has been identified between codons 1281 and 1554 in exon 15 and has been coined the "mutation cluster region" (MCR) (Powell *et al.*, 1992; reviewed by Nakamura, 1993). Fifty percent of all mutations found in colorectal tumours occur within the MCR.

Several studies have implicated the *APC* gene in the pathogenesis of oesophageal cancer. In the United States of America two groups reported varying frequencies of allelic loss or loss of heterozygosity (LOH) of the *APC* gene. Boynton *et al.* (1992) demonstrated LOH in 80% (8 of 10 individuals) of oesophageal SCCs examined, while Huang *et al.* (1992) demonstrated LOH in 66% (23 of 35 individuals) of oesophageal tumour samples examined. Results of a Japanese study showed that 53% of oesophageal squamous cell carcinomas demonstrated allelic loss at the *APC* locus (Shibagaki *et al.*, 1994). No mutations were found in the MCR of the *APC* gene in 60 patients analysed in the latter study. The evidence presented suggests a possible role for the *APC* gene in the pathogenesis of oesophageal cancer.

1.5.2 The *p53* Gene

The *p53* gene is located on the short arm of chromosome 17 at position 17p13.1 and consists of 11 exons that are spread across 16-20kb (Miller *et al.*, 1986). The first exon is non-coding, while the remaining exons encode a 393 amino acid nuclear phosphoprotein with a molecular weight of 53 kDa. The protein has five evolutionary conserved domains (I-V), located

between codons 13-19, 117-142, 171-181, 234-258 and 270-286 (Fig. 1.3) (Soussi *et al.*,1990).

The p53 protein has three functional domains. The amino-terminal domain has transcriptional activation sites, the central domain has sequence-specific DNA binding activity and the carboxy terminal domain is capable of binding DNA non-specifically and is thought to be involved in the detection of damaged DNA (Oberosler *et al.*, 1993). This domain also allows the *p53* gene product to oligomerize with other p53 monomers (Clore *et al.*, 1994).

The p53 tumour suppressor protein is involved in the regulation of normal cellular growth, but also induces cell cycle arrest in response to DNA damage in the cell (Kastan *et al.*, 1991; Kastan and Kuerbitz, 1993; Hartwell and Kastan, 1994). As illustrated in Fig.1.4, p53 induced G1 arrest is most likely mediated by the transcriptional activation of p21 tumour suppressor protein (Harper *et al.*,1993). The p21 protein inhibits cyclin D, cyclin E and cyclin A complexes which normally induce progression through the cell cycle by their inhibitory (phosphorylation) effects on Rb via cyclin dependent kinases (Fig. 1.4). In this way p53 impacts on the G1-S cell cycle checkpoint. Inactivation of the *p53* gene would result in loss of cell cycle arrest in response to DNA damage and would contribute to aberrant proliferation and increased genomic instability (Vogelstein and Kinzler, 1992).

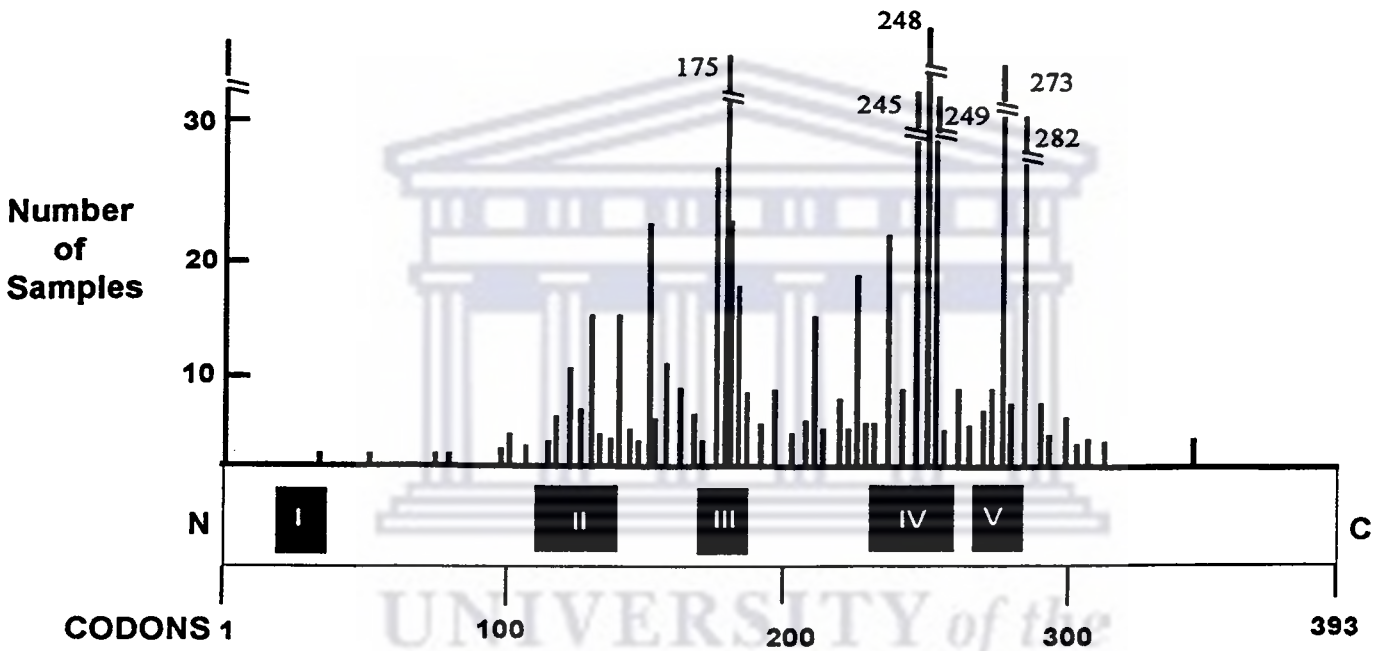


Fig. 1.3 Mutations in the p53 gene

The mutational spectrum of the p53 gene derived from the analyses of 1361 samples as summarized by Harris (1993). The black boxes (I-V) represents the five evolutionary conserved domains. The bar graph shows that substitution mutations are confined between codons 110 to 307 including 4 conserved domains. Hot spot codons occur at 175 (n = 77), 245 (n = 55), 248 (n = 112), 249 (n = 55), 273 (n = 89), and 282 (n = 42). N = Amino-terminus and C = Carboxy-terminus.

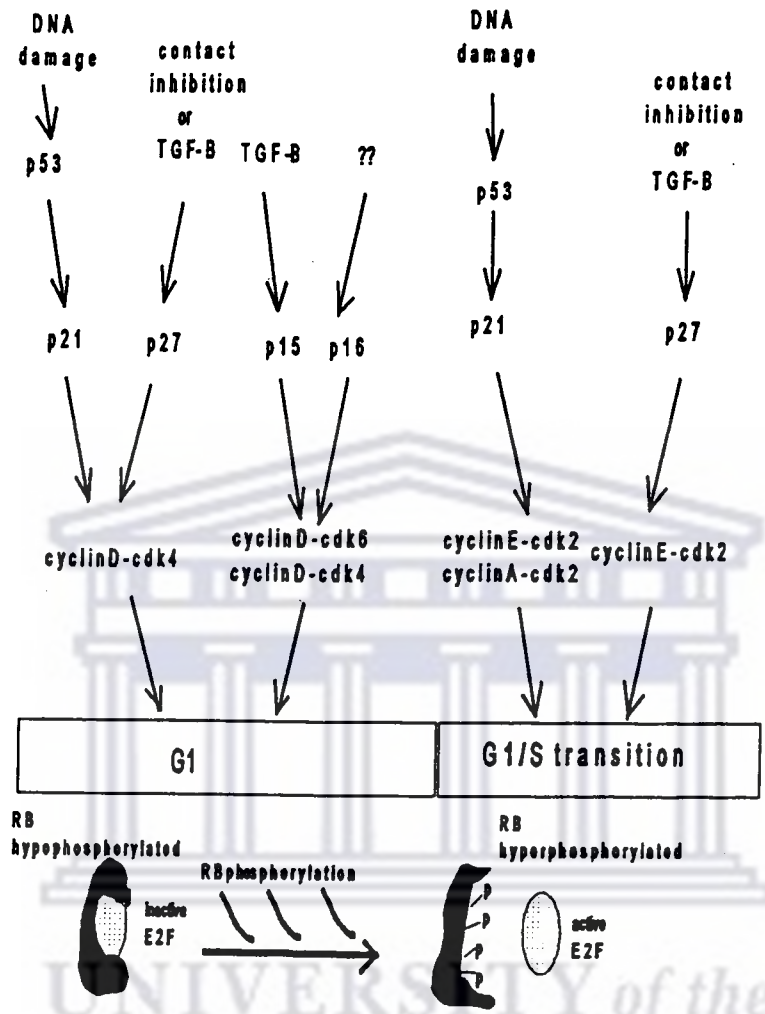


Fig. 1.4 Inhibition of cyclin dependent kinases (CDK) and cell cycle progression
The p53 tumour suppressor effectively inhibits cell cycle progression in response to stress signals or DNA damage within the cell, by activating CDK inhibitor p21 (also known as WAF 1) at the G1/S interphase of the cell cycle (Hartwell and Kastan, 1994). CDK complexes are also inhibited through the activation of p27 (KIP 1) in response transforming growth factor-ß (TGF-ß) and cell-cell contact inhibition signals (Hartwell and Kastan, 1994). Additional CDK inhibitors have been identified recently, p16 (MTS 1 or INK4) and p15 (INK4B) (Sherr, 1994; Hunter and Pines, 1994). Activated CDK complexes in turn enhance cell cycle progression by inhibiting the activity of certain tumour suppressor genes. Hypophosphorylated Rb restricts transit through the cell cycle by inactivation of E2F a transcription factor. Activated CDK complexes hyperphosphorylate Rb causing E2F release and progression through the cell cycle (Wang *et al.*, 1994) (adapted from Coleman and Tsongalis, 1995).

Complete inactivation of the *p53* gene is often achieved through allelic loss and mutational inactivation of the remaining allele, leading to the functional loss of both alleles in affected cells. Loss of heterozygosity at the 17p13.1 locus has been demonstrated in 75% of colorectal carcinomas (Vogelstein *et al.*, 1989; Cunningham *et al.*,1992), 40-80% of oesophageal cancer samples (Meltzer *et al.*, 1991, Huang *et al.*, 1992; Vogelstein *et al.*, 1993; Shibagaki *et al.*, 1994) and many other cancer types examined thus far. Mutations in *p53* have been demonstrated in oesophageal (Wagata *et al.*, 1993), lymphoid (Gaidano *et al.*, 1991), bladder (Sidransky *et al.*, 1991), prostate (Ittman *et al.*, 1994), breast (Hartmann *et al.*, 1995), lung and colon cancer (Nigro *et al.*,1989). Alterations in the *p53* gene have also been associated with a certain percentage of Li-Fraumeni syndrome cases, a rare familial cancer syndrome. This disease is characterised by the development of mesenchymal and epithelial tumours, including carcinomas of the breast, adrenal cortex, brain and soft tissue sarcomas (Garber *et al.*, 1991).

Many different types of mutations have been documented for *p53* including point mutations, deletions and insertions. The majority of mutations are missense mutations occurring mainly between exons 5 to 8 (codons 110-307) which includes 4 of the 5 conserved domains (Fig.1.3) (Hollstein *et al.*, 1991b). Mutational hot spot codons have been identified at codons 175, 245, 248, 249, 273 and 282. CpG dinucleotides are specifically mutated in colon cancer and account for 50% of all mutations reported in other cancers (Harris, 1991). In contrast, oesophageal cancer has a unique *p53* mutational profile with very few mutations at CpG sequences (Wagata *et al.*, 1993). Transversions occur more frequently and with similar

frequencies at both G:C and A:T pairs (Hollstein *et al.*, 1991b and Wagata *et al.*, 1993). According to Harris (1991), 36% of mutations occur at A:T pairs and may be caused by DNA depurination as a result of exposure to chemical carcinogens in alcohol (eg. trace amounts of urethran). Although several studies have implicated p53 in oesophageal cancer in other parts of the world, the involvement of the *p53* gene in the pathogenesis of oesophageal cancer has not been investigated previously in South Africa.

The involvement of the *APC* and *p53* genes in the development of oesophageal cancer in South African patients was investigated in this study. Defining the molecular genetics of oesophageal tumourigenesis will provide us with a better understanding of this disease in South Africa and a challenging opportunity to apply the information to the benefit of the community at risk.

CHAPTER TWO

Analysis of The Adenomatous Polyposis Coli gene

2.1 Introduction

Previous studies have demonstrated the involvement of the *APC* gene in the development of sporadic and inherited colorectal cancer (Okamoto *et al.*, 1988; Vogelstein *et al.*, 1989). This tumour suppressor gene is the first gene to be affected in the progression of colorectal cancer and also appears to be involved in the development of other cancers such as prostate (Gao *et al.*, 1995), oral (Uzawa *et al.*, 1994), pancreatic (reviewed by Nakamura, 1993) and breast (Thompson *et al.*, 1993; Kashiwaba *et al.*, 1994). Other studies have demonstrated allelic loss or loss of heterozygosity (LOH) of the *APC* gene in oesophageal cancer but the role of *APC* in the development of oesophageal cancer is not as clearly defined as in colorectal cancer. Loss of heterozygosity studies carried out by several groups have produced varying results with regard to involvement of the *APC* gene in oesophageal cancer. Boynton *et al.* (1992) demonstrated 80% LOH for oesophageal SCCs, while Huang *et al.* (1992) showed 66% LOH in oesophageal tumours, with no significant differences between SCCs and adenocarcinomas. In another study, Shibagaki *et al.* (1994) demonstrated 52% allelic loss using markers for the 5q chromosomal arm and 39% LOH of the *APC* gene in oesophageal SCCs. Approaches used for this type of analysis often involve the use of polymorphic markers such as Restriction Fragment Length Polymorphisms (RFLP's), Variable Number of Tandem Repeats (VNTR's) and microsatellite repeat regions. These polymorphic markers occur very frequently throughout the human genome, RFLP's for instance, occur at

approximately every 200 base pairs (Emery and Mueller, 1988). Differences in DNA sequences caused by substitution, deletion or insertion mutations are detected by the creation or loss of these restriction sites. Microsatellites and VNTR's are composed of nucleotide motifs which are tandemly repeated numerous times, where the motifs are more than 6 base pairs in VNTR's and 1 - 6 base pairs in microsatellite regions (Jeffreys *et al.*, 1985). Polymorphic markers are useful because they distinguish between the two parental alleles in the samples tested. Variations in DNA at these polymorphic sites are detected as size differences or complete loss of an allele due to genetic alterations. We employed two RFLP's positioned within the *APC* gene to examine allelic loss in this study. The use of markers within a gene is particularly useful since deletions of a specific gene implicate its involvement in the progression of the disease examined. PCR-RFLP analysis was the preferred technique used in this study since lower amounts of DNA (50 ng) are required, as opposed to Southern blotting (5-10μg per reaction).

Mutational screening studies of the *APC* gene in different types of cancer have produced varying results. For example, mutations have been detected in 60% of colorectal carcinomas (Powell *et al.*, 1992), 12.5% of oral SCCs (Uzawa *et al.*, 1994) and 6% of breast carcinomas (Kashiwaba *et al.*, 1994). An interesting finding was the absence of mutations in the mutation cluster region in exon 15 of the *APC* gene reported by Shibagaki *et al.* (1994) for oesophageal cancer, although the group demonstrated 39% LOH in the same sample of patients. These reports suggest a varying degree of involvement of the *APC* gene in different types of cancer and as yet, an undefined role in oesophageal carcinogenesis.

In this study, the status of the *APC* gene in oesophageal cancer in South Africa was examined by LOH analysis and mutation screening. The size of the coding region of the *APC* gene (8.3kb) precluded direct sequencing of the entire gene because of time and cost considerations and so other techniques were used that allow more economical screening for mutations. These techniques included SSCP analysis and heteroduplex analysis of the MCR in exon 15 of the *APC* gene, followed by DNA sequencing of selected samples.

2.2 Results

2.2.1 DNA Isolation and PCR Amplification

The DNA isolation procedure (chapter 4.2.2) consistently produced high molecular weight DNA of good quality from oesophageal tumour and paired normal biopsy samples (Fig. 2.1). Average yields of DNA from tumour tissue were 10μg/mg and normal tissue were 3μg/mg. The DNA was used to PCR-amplify nucleotides 3880 to 4281 (fragment 15A) and nucleotides 4228 to 4779 (fragment 15C) of the *APC* gene (Fig.4.1), yielding products of the expected sizes of 400bp (15A) and 550bp (15C) respectively, as shown in Fig.2.2.

2.2.2 Loss of Heterozygosity Analysis of the *APC* gene

LOH in the *APC* gene was examined using two RFLP's, the *Rsa* I polymorphic site in exon 11 and the *Msp* I polymorphic site in exon 15. Tumour and paired normal DNA isolated from 26 patients was evaluated for LOH at the *Rsa* I site. A representative example of a patient homozygous for the absence of the *Rsa* I polymorphic site is shown in Fig. 2.3 (patient 41), by the presence of only one band corresponding to 133bp in both the uncut and *Rsa* I digested PCR products. Representative examples of patients heterozygous for the *Rsa* I site are shown

in Fig. 2.3 (patients 43 and 14). In these patients, the two bands corresponding to 85bp and 48bp represent the allele containing the *Rsa* I site. Loss of heterozygosity was demonstrated if the tumour DNA showed absence of either uncut band or the cut bands relative to the paired normal (shown in patient 14, Fig.2.3b). Heterozygosity (both alleles present in the same patient) was demonstrated in 7 of the 26 patients (27%). Loss of heterozygosity was demonstrated in 2 (i.e. patients 14 and 37) of the 7 informative cases (29%) using the *Rsa* I marker (Table 2.1 and Table 2.2). The internal control was included in three independent *Rsa* I restriction digestions of exon 11 of the *APC* gene for patient 14. This confirmed that the restriction digestion conditions were appropriate.

In addition, tumour and paired normal DNA from 27 patients was evaluated for LOH at the *Msp* I polymorphic site in exon 15 of the *APC* gene. Representative examples of patients heterozygous for the presence of the *Msp* I site are shown in Fig. 2.4 (patients 1, 17 and 5), by the presence of one band corresponding to 550bp (*Msp* I site absent) and another band corresponding to ~275bp, which represents the allele containing the *Msp* I site. The extra band visible in sample 1 T may represent a nonspecific product generated during PCR (Fig. 2.4). This PCR-RFLP assay required a good yield of PCR product, therefore the thermal cycling conditions were stringent but still managed to produce sufficient PCR product. LOH was demonstrated if either the cut or uncut bands were absent in the tumour sample compared to the paired normal sample (Fig. 2.4, patient 5). The internal control was included initially to confirm that the *Msp* I restriction digestion conditions were appropriate (data not shown). Heterozygosity was observed in 16 of the 27 patients (59%) and LOH was demonstrated in

2 of the 16 informative cases (13%), using the *Msp* I marker (Table 2.1 and Table 2.2). A total of 19 of 32 individuals (59%) were informative for either one or both of the *Msp* I or *Rsa* I markers and LOH was detected in 4 of the 19 informative patients (21%) (Table 2.2).

Table 2.1 Oesophageal cancer patients analysed for LOH of the *APC* gene.

Patient	*Rsa* I	*Msp* I
1	homo uncut	het
5	homo uncut	LOH
7	homo uncut	homo uncut
8	homo uncut	homo uncut
9	het	het
13	homo uncut	het
14	LOH	ND
15	homo uncut	ND
16	homo uncut	het
17	het	het
20	homo uncut	het
22	homo uncut	ND
23	het	ND
25	ND	homo cut
27	homo uncut	het
28	homo uncut	ND
32	homo uncut	het
34	homo uncut	het
35	homo uncut	homo cut
37	LOH	homo uncut
39	homo cut	homo uncut
41	homo uncut	het
43	het	het
44	homo uncut	het

Table 2.1 continued ...

Patient	*Rsa* I	*Msp* I
45	homo uncut	homo uncut
48	ND	homo cut
49	ND	het
50	het	het
57	homo uncut	homo cut
58	ND	homo uncut
59	ND	LOH
62	ND	homo uncut

Two restriction fragment length polymorphic markers, *Rsa* I (exon 11) and *Msp* I (exon 15), situated within the *APC* gene were used to examine LOH of the *APC* gene in oesophageal cancer. Homo uncut, homozygous for the absence of the polymorphic restriction site; homo cut, homozygous for the presence of the polymorphic restriction site; het, heterozygous but no LOH; LOH , loss of an allele and ND, not done or unsuccessful.

Table 2.2 LOH analysis of the *APC* gene using the *Rsa* I and *Msp* I polymorphic sites.

Marker	% Informativity (informative/total)	% LOH (LOH/informative)
Rsa I	27 (7/26)	29 (2/7)
Msp I	59 (16/27)	13 (2/16)
Total	59 (19/32)	21 (4/19)

Summary of LOH results given in Table 2.1. Informativity refers to heterozygosity. The total percentage informativity is the percentage of patients heterozygous at one or both polymorphic sites. LOH refers to the loss of one allele.

2.2.3 SSCP and Heteroduplex analysis

The MCR within exon 15 of the *APC* gene was screened for mutations using SSCP and heteroduplex (HD) analysis in 29 oesophageal cancer patients. PCR primers were designed which amplified the MCR as two overlapping fragments 15A and 15C to facilitate analysis (400bp and 550bp respectively, spanning codons 3880 to 4779) (Fig. 4.1). Small amounts of normal DNA were available from several patients due to the difficulty of obtaining normal biopsy tissue from those patients in poor clinical condition. The WI-38 cell line (normal human embryonic lung fibroblast) was used as a source of normal DNA and was interpreted as the wild-type in several assay. The number of patients analysed varied for SSCP and HD analysis since ambiguous results could not be interpreted, regardless of repetitions of the experiment.

SSCP and HD analyses of exon 15A were performed on 26 and 25 patients respectively, using the same sample of patients. No abnormal SSCP conformers (Fig. 2.5) or heteroduplexes (data not shown) were detected in this region of the *APC* gene. The banding pattern of the tumour samples in Fig. 2.5 was similar to that of a control DNA sample (WI-38). Similarly, 29 individuals were screened for mutations in exon 15C using SSCP analysis and 26 individuals using HD analysis. Representative examples are illustrated in Figures 2.6 and 2.7. No abnormal SSCP conformers or heteroduplexes were detected in this region of the *APC* gene either, suggesting that mutations in the MCR in exon 15 of the *APC* gene are not involved in the development of oesophageal cancer in the patients examined.

2.2.4 DNA Sequence Analysis

The MCR of exon 15 in the *APC* gene was partially sequenced in a small sample of patients. Six samples (i.e. 1, 13, 14, 15, 16 and 17) were chosen randomly for this study. Approximately 200-300 bases of exons 15A and 15C could be read using the foward primers (Table 4.1), respectively. This was achieved by three consecutive loadings per sequencing gel under optimal sequencing conditions. Subsequently SSCP analysis was used to screen the samples for mutations. No somatic mutations were detected in tumour samples of the six patients tested, when compared with paired normal tissue. A polymorphism occurred at codon 1493 in both normal and tumour samples from patients 1, 13, 14, 15 and 17 (Fig. 2.8) compared to the published sequence (Nagase *et al.*, 1992). Both the published sequence of codon 1493 (ACG) and the novel form (ACA) were present in patients 1, 13, 14 and 17, who were heterozygous for this polymorphism. This base substitution did not result in an amino acid change . In addition, patient 17 demonstrated a GCA to GTA substitution at codon 1475 in both tumour and normal tissue, resulting in an alanine to valine substitution. This amino acid replacement may be regarded as a fairly conservative change. Alanine and valine both belong to the same category of amino acids with hydrophobic side chains. The only major difference between alanine and valine are two additional $-CH_3$ groups in valine, making valine slightly more hydrophobic compared to alanine.

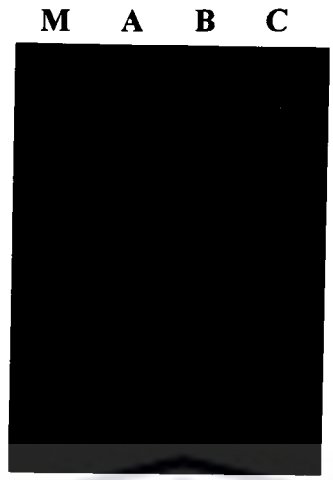


Figure 2.1 Genomic DNA
Genomic DNA was isolated from human oesophageal biopsy tissue using the extraction procedure described in chapter 4.2.2. 1μg of each DNA sample was electrophoresed on a 1% agarose gel containing ethidium bromide at a final concentration of 3μg/ml. M = commercial DNA size marker; (A), (B) and (C) are representative DNA samples.

Figure 2.2 PCR amplification of exon 15 MCR of the *APC* gene.
The MCR in exon 15 of the *APC* gene was amplified from patient genomic DNA using the polymerase chain reaction with two sets of primers (15A and 15C), as described in section 4.4. PCR products (10μl) were analysed on a 6% non-denaturing polyacrylamide gel and stained with ethidium bromide. M1 = Lambda DNA digested with *Pvu* II; M2 = commercial DNA size marker. Numbers at the top of the figure indicate patient identification numbers.

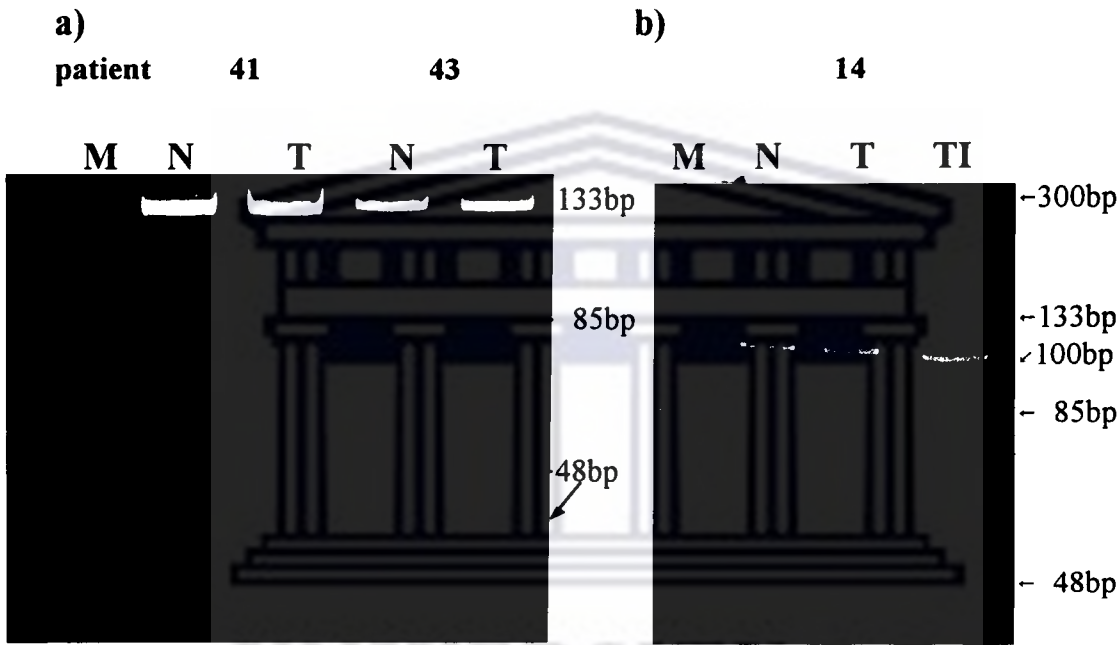


Figure 2.3 PCR-LOH analysis of the *APC* gene using the *Rsa* I polymorphic marker in exon 11.

Tumour (T) and paired normal (N) from oesophageal cancer patients was PCR amplified using primers flanking the *Rsa* I polymorphic site. PCR products (15μl) were digested with *Rsa* I and then electrophoresed on 8% polyacrylamide gels. Gels were stained with ethidium bromide. M = commercial DNA size marker and TI = a PCR product (400bp) with a *Rsa* I restriction site was added to tumour DNA before digestion with *Rsa* I, as an internal control. The internal control yielded products of 300bp and 100bp when digested with *Rsa* I.

Figure 2.4 PCR-LOH analysis of the *APC* gene using an *Msp* I polymorphic marker in exon 15.

Tumour (T) and paired normal (N) DNA from oesophageal cancer patients was PCR amplified using primers flanking the *Msp* I polymorphic site. PCR products (15μl) were digested with *Msp* I and electrophoresed on 6% polyacrylamide gels. Gels were stained with ethidium bromide. M = Lambda DNA digested with *Pvu* II

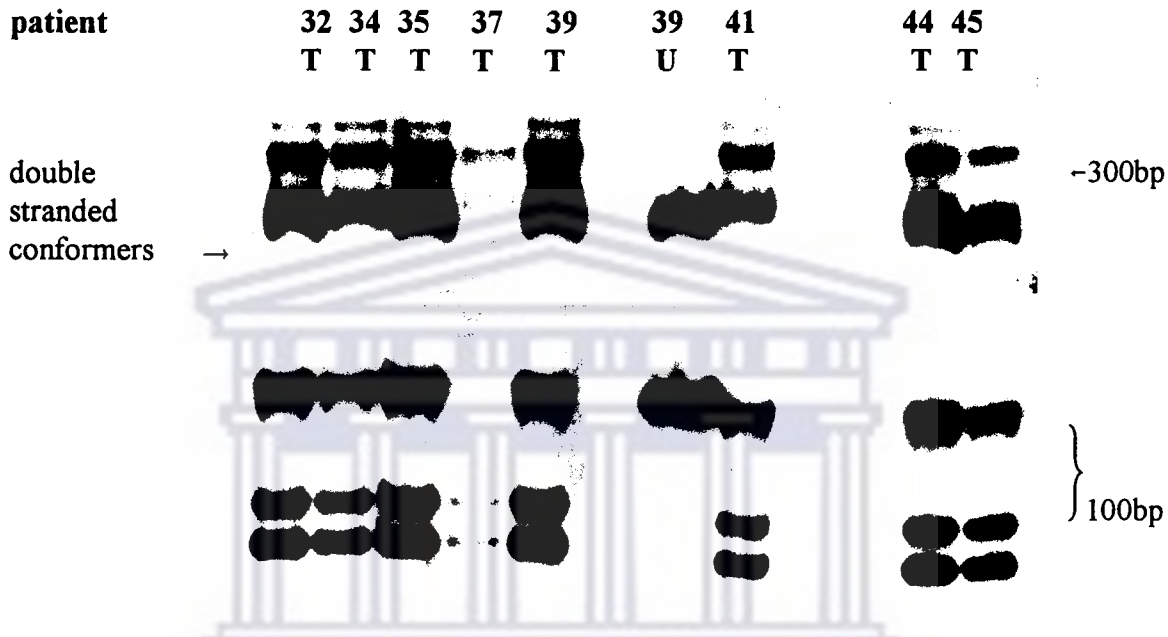


Figure 2.5 PCR-SSCP analysis of exon 15A of the *APC* gene.
Tumour (T) DNA from oesophageal cancer patients was PCR amplified in the presence of ^{32}P-dCTP. The labelled PCR products (10-15μl) were digested with *Rsa* I and the fragments (300bp and 100bp) were electrophoresed on a 6% SSCP gel (room temperature) at 30 Watts for ±4 hours, then autoradiographed. U = undenatured PCR product.

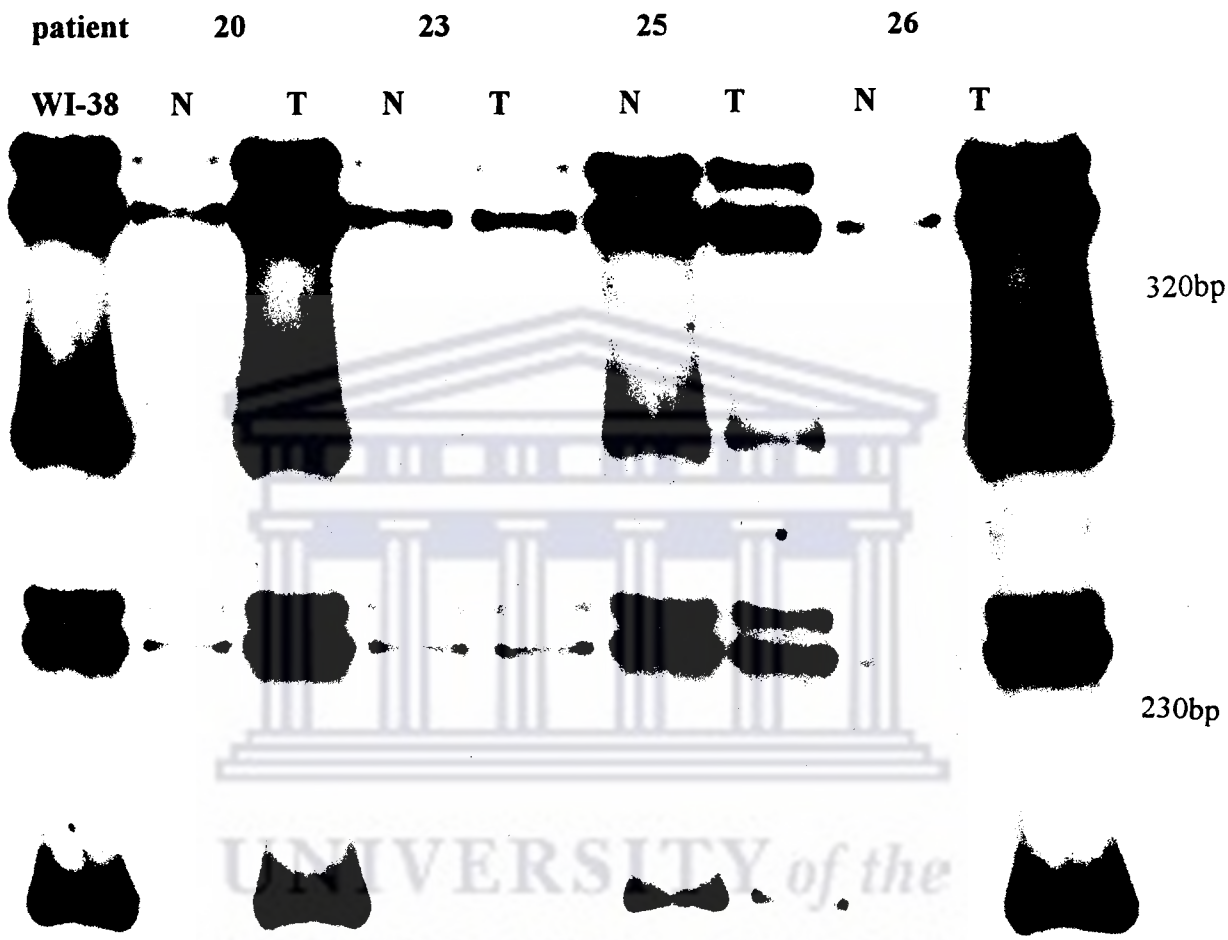


Figure 2.6 PCR-SSCP analysis of exon 15C of the *APC* gene.
Normal (N) and tumour (T) DNA from oesophageal cancer patients and WI-38 (a normal embryonic lung fibroblast cell line) was PCR amplified in the presence of ^{32}P-dCTP. The labelled PCR products were digested with *Taq* I and the fragments (320bp and 230bp) were electrophoresed on a 6% SSCP gel (room temperature) at 30 Watts for ± 4 hours, followed by autoradiography.

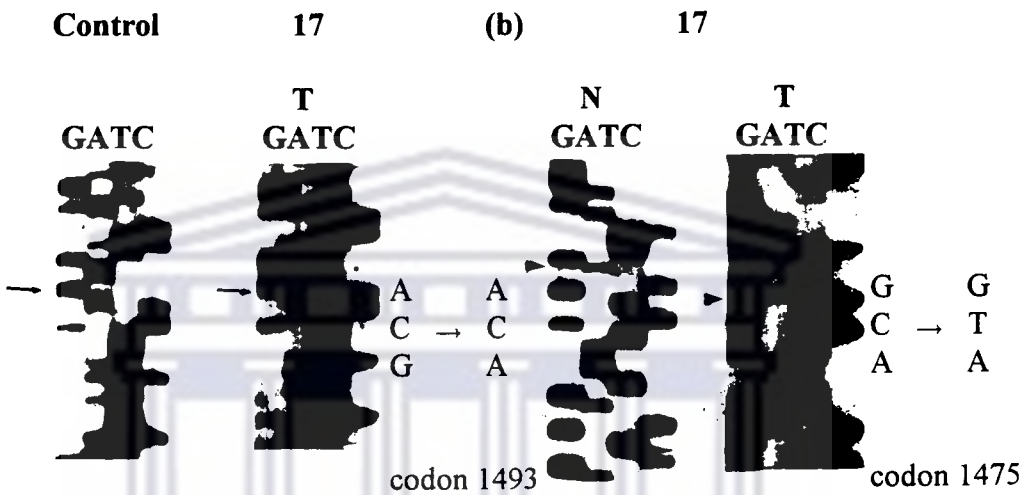


Figure 2.8 Identification of *APC* gene sequence differences in exon 15 of patient material compared to published sequence.
(a) Patient 17 tumour (T) sequence demonstrates an ACA to ACG base substitution at codon 1493 compared to the adjacent control sequence; (b) Patient 17 normal and tumour sequence demonstrates a GCA to GTA base substitution at codon 1475 resulting in an alanine to valine substitution.

2.3 Discussion

In this study we chose to employ two RFLP's positioned within the *APC* gene to examine allelic loss in oesophageal cancer, since these markers may give a better indication of the involvement of the gene than markers located outside the gene itself. The two polymorphic markers (*Rsa* I and *Msp* I) located within the *APC* gene revealed that 21% of the patients analysed displayed LOH of the *APC* gene. Nineteen of the 32 patients (59%) tested were informative at one or both of the polymorphic sites tested. A similar percentage of patients showed informativity in this study when compared to other studies, but a lower percentage of LOH was observed in this study. Other groups who also used the *Rsa* I polymorphic marker include Boynton *et al.* (1992), who demonstrated 80% LOH and 80% informativity (10/12) in SCCs and Huang *et al.* (1992), who demonstrated 66% LOH and 48% (35/72) informativity in oesophageal tumours, with no significant differences between SCCs and adenocarcinomas. Shibagaki *et al.* (1994) reported 52% allelic loss at the 5q21 locus and 39% LOH of the *APC* gene. Boynton *et al.* (1992) and Huang *et al.* (1992) both utilized flow cytometry to separate neoplastic cells from non-neoplastic cells and the patients analysed were from the USA. Shibagaki *et al.* (1994) analysed Japanese patients but did not utilize cell sorting techniques, similar to the present study which examined South African patients. These differences need to be considered when comparing results from different studies. Different populations may be exposed to different aetiological factors that, in turn, may affect the involvement of different genes. An alternative possibility in this study is that the LOH results may be artificially low due to contamination of tumour tissue with normal cells. However, this is unlikely since a high percentage of allelic loss was demonstrated for the *Rb* gene in the

same cohort of patients, in a study performed in our laboratory. Since our sample size (33 individuals) was relatively small, the screening of additional patients and the use of additional polymorphic markers for the *APC* gene may possibly reveal a higher polymorphic frequency and allow the identification of more individuals with allelic loss of the *APC* gene. The results of this study currently suggest that a low level (21%) of allelic loss occurs at the *APC* locus in oesophageal cancer patients in South Africa. The low percentage of LOH demonstrated in this study suggests that *APC* may not play a role in the development of oesophageal cancer in South Africa. This suggestion is supported by the results obtained in the mutation screening studies. No somatic mutations were detected in any of the oesophageal cancer patients when comparing tumour with normal DNA using SSCP, HD and sequencing analyses in this study. Mutations present within the MCR of exon 15 could have been missed. This is supported by the discrepancies demonstrated between sequencing analysis and SSCP analysis of exon 15C in patients 1,13, 14, 15 and 17. While sequencing revealed a polymorphism at codon 1493 in these patients, SSCP did not reveal any mobility shifts for exon 15C. The large size of exon 15A and 15C may have interfered with the sensitivity of the SSCP assay, although fragments of upto 400bp have been screened successfully by SSCP analysis (Orita *et al.*, 1989). Since only exon 15 (MCR) was examined for mutations it is possible that mutations may be present elsewhere in the gene. However, Shibagaki *et al.* (1994) did not detect mutations in the MCR in exon 15 of the *APC* gene in 60 oesophageal SCCs by sequencing analysis. Another finding in an analysis of oral SCCs showed the absence of mutations in the MCR but the presence of mutations beyond the 3' end of the MCR in 12.5% of the patient sample (Uzawa *et al.*, 1994), whereas, 50% of all the somatic mutations in colorectal cancer were confined to the MCR

(Powell *et al.*, 1992). It is possible that the *APC* gene or gene product may be inactivated by mechanisms unrelated to genetic alteration in oesophageal cancer in South Africa, but a more likely explanation is that the *APC* gene is not involved in the development of this disease.

Sequence analysis of the MCR revealed the presence of a known polymorphism at codon 1493 in six patients analysed (Nagase *et al.*, 1992). This alteration was not significant in that no amino acid changes occurred due to this substitution. Patient 17 demonstrated a GCA to GTA at codon 1475 and this resulted in an alanine to valine substitution. This substitution may result in minimal changes to protein structure and function since alanine and valine have similar chemical characteristics. The only difference that may be of any significance is that valine is slightly more hydrophobic compared to alanine.

The results of this study allow us to conclude that the *APC* gene locus may be a target for a small degree of genetic alteration in oesophageal cancer, but the *APC* gene does not appear to play a significant role in the development of this disease in South Africa.

CHAPTER THREE

Analysis of the *p53* Tumour Suppressor Gene

3.1 Introduction

Compelling evidence implicates the *p53* gene in the development of a wide range of human cancers (see chapter 1), reflecting the critical role of *p53* in cell cycle control and the induction of growth arrest or apoptosis in response to DNA damage (Kastan *et al.*, 1991; Yonish-Rouach *et al.*, 1993; Hartwell and Kastan, 1994).

The literature also shows the widespread involvement of the *p53* gene in the development of oesophageal cancer, with a mutation frequency rate (percentage of oesophageal tumour tissue showing mutations compared to paired normal tissue) of 47%, 50%, 56% and 60% in studies in Japan, China, America and Normandy, respectively (Wagata *et al.*, 1993; Huang *et al.*,1993; Liang *et al.*, 1995; Hollstein *et al.*, 1991a). In these studies, most of the mutations in the *p53* gene were located in exons 5 through 8, indicating that the distribution of *p53* mutations in oesophageal cancer conforms to the pattern observed in other cancers (Greenblatt *et al.*, 1994).

In this study, the *p53* gene (in oesophageal tumour and paired normal tissue) was examined for mutations in exons 5 and 6, using SSCP and HD analysis to identify mutated alleles, followed by DNA sequencing to characterise the mutations identified. Initially it was planned

to screen exons 5 through 8 of the *p53* gene, however time constraints curtailed the extent of the study. The hot spot region of the *p53* gene was targeted in this study to identify mutations in South African oesophageal cancer patients, because other reports demonstrated that most of the mutations in the *p53* gene in oesophageal cancer were confined to this region of the gene (Wagata *et al.*, 1993). Extending the mutational analysis to the *p53* gene allowed confirmation that the SSCP and HD analytical approaches were sensitive enough to assay the small biopsy samples used in this study, considering the absence of mutations detected in the *APC* gene described in chapter 2.

3.2 Results

3.2.1 SSCP and Heteroduplex Analysis

In this study exons 5 and 6 of the *p53* gene were screened for mutations using SSCP and HD analysis of DNA isolated from tumour and paired normal biopsy material as described in chapter 4. Only small quantities of normal DNA were available from several patients due to the difficulty of obtaining sufficient normal tissue biopsy material from those patients in poor health. As a consequence, a normal human lung embryonic fibroblast cell line (WI-38) was used as a source of control DNA for several patient analyses. Exons 5 and 6 of the *p53* gene from WI-38 cells were sequenced and the sequence obtained corresponded perfectly with the published sequence (Lamb and Crawford, 1986). The number of patients analysed varied for SSCP and HD analysis because ambiguous results obtained for certain patients could not be interpreted, even after repeating the analysis.

SSCP and HD analyses of exon 5 were performed on 25 and 23 patients respectively, using the same cohort of patients (Table 3.1). SSCP analysis of exon 5 revealed abnormal banding patterns (compared to normal tissue) for patients 11 and 12 (Fig. 3.1), while HD analysis of exon 5 of the same cohort of patients revealed an abnormal banding pattern in patient 12 (Fig. 3.2). The SSCP gel results of patients 11 and 12 could not be repeated regardless of the several experimental attempts made. Patient 12 demonstrated an extra, slower migrating band on a heteroduplex gel, which was confirmed by repeating the experiment three times, using freshly amplified PCR products of exon 5 starting from genomic DNA in each instance. DNA from this patient was then sequenced directly to detect presence of any mutations, as described in the following section. SSCP and HD analyses of exon 6 of the *p53* gene were performed in 33 and 32 patients, respectively, using the same sample of patients (Table 3.1). SSCP analysis of this exon revealed an abnormal banding pattern for patient 16 which are indicated by the arrows in Fig. 3.3. The arrows marked (A) represent the mobility shifts created by the single stranded DNA fragments. The putative mutation was also confirmed by HD analysis (Fig. 3.4). This banding pattern was detected under all SSCP conditions (± glycerol, at room temperature and 4°C). Both the SSCP and HD analyses of patient 16 were repeated three times (using freshly amplified material) and generated the same results.

3.2.2 DNA Sequence Analysis

DNA from samples which produced positive results in preliminary mutation screening assays was sequenced to confirm the presence of mutations. These included patients 11 and 12 (exon 5) and patient 16 (exon 6). Patient 12 was found to have a TCA to TGA substitution at codon 183 (Fig. 3.5) which resulted in a stop codon at this position. Sequencing reactions for

patients 11 and 12 were repeated at least three times for exon 5. Unlike patient 12, the DNA sequencing results obtained for patient 11 were inconsistent and although a suspected mutation or base substitution was detected in two of the three experiments, this result was not scored as a positive mutation. Two separate sequencing reactions were performed to confirm the exon 6 mutation in patient 16. The strategy used to identify the *p53* mutation in patient 16 is illustrated by the flow diagram in Figure 3.6 and differs from the approach used for other samples. Reamplification of the mutant SSCP conformer (illustrated by the arrow in Figure 3.3) resulted in a heterogenous PCR product. The PCR product was separated on a 10% non-denaturing polyacrylamide gel and the smaller mutant band (Fig. 3.7A, lane a, marked Y) was eluted, four of these bands were combined for elution and reamplified (Fig. 3.7A, lane e). The two larger slower migrating bands present in lane (a) marked X, are consistently reproduced in the PCR amplification reaction of exon 6 in patient 16 tumour compared to normal DNA and other samples. It is unlikely that the PCR conditions were nonspecific because this phenomenon was unique to patient 16 tumour. The two smaller bands present in lane (d) appear to be unique to the PCR of sample 16 normal. These nonspecific bands could arise through miss priming during the PCR and because they fail to interfere with the sequencing reaction it indicates that these bands are artifacts of PCR. The PCR product in Fig. 3.7A, lane (e) was used for DNA sequencing using the reverse primer for exon 6. An eleven base pair deletion in exon 6 of the *p53* gene in patient 16 was detected in this manner (Fig. 3.7B). This deletion included the third base of codon 190, codons 191 to 193, and the first base of codon 194, which results in a frame shift mutation generating a premature stop codon 13 codons downstream (Fig 3.7C).

Table 3.1 Summary of mutation screening of exon 5 and exon 6 of the *p53* gene using PCR-SSCP and HD analysis.

Exon	% of patients with mutations (altered patterns/total cases)	
	SSCP	HD
5	8(2/25)	4(1/23)
6	3(1/33)	3(1/32)

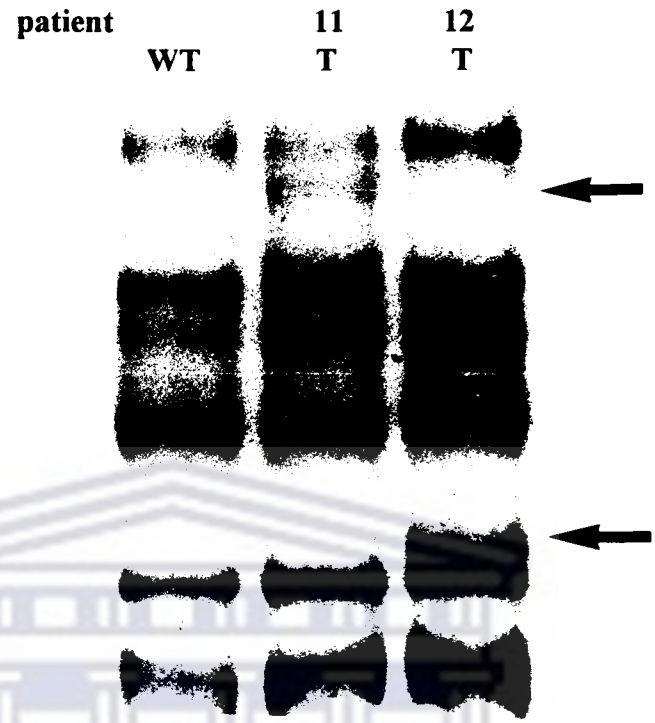


Figure 3.1 PCR-SSCP analysis of exon 5 of the *p53* gene.
DNA from patients 11 and 12 (T = tumour tissue) was amplified by the polymerase chain reaction in the presence of ^{32}P-dCTP, using primers flanking exon 5 of the *p53* gene. Labelled PCR products were electrophoresed on a 6% SSCP gel (room temperature). WT = representative of the wild type exon (patient 10 tumour DNA).

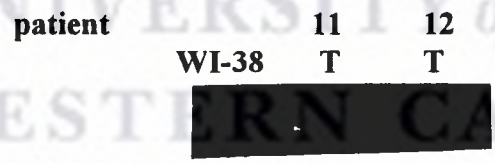


Figure 3.2 Heteroduplex analysis of exon 5 of the *p53* gene.
DNA from patients 11 and 12 (T = tumour) was amplified by the polymerase chain reaction, using primers flanking exon 5 of the *p53* gene. The PCR products were electrophoresed on a 10% heteroduplex gel at 180V overnight and then stained with ethidium bromide.WI-38 = normal human embryonic lung fibroblast cell line used as a control.

Figure 3.3 PCR-SSCP analysis of exon 6 of the *p53* gene.
DNA from patient 16 (N = normal and T = tumour) was amplified by the polymerase chain reaction in the presence of ^{32}P-dCTP, using primers flanking exon 6 of the *p53* gene. Labelled PCR products were electrophoresed on 6% SSCP gels (room temperature). The arrow indicates the putative mutant band. The arrows identify the aberrantly migrating DNA fragments.

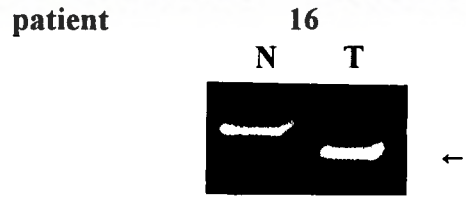


Figure 3.4 Heteroduplex analysis of exon 6 of the *p53* gene.
DNA from patient 16 (N = normal and T = tumour) was amplified by the polymerase chain reaction, using primers flanking exon 6 of the *p53* gene. The PCR products were electrophoresed on a 10% heteroduplex gel (2.3M urea) at 180V overnight and then stained with ethidium bromide.The arrow indicates the putative mutant bands. The arrow identifies the aberrantly migrating DNA fragments.

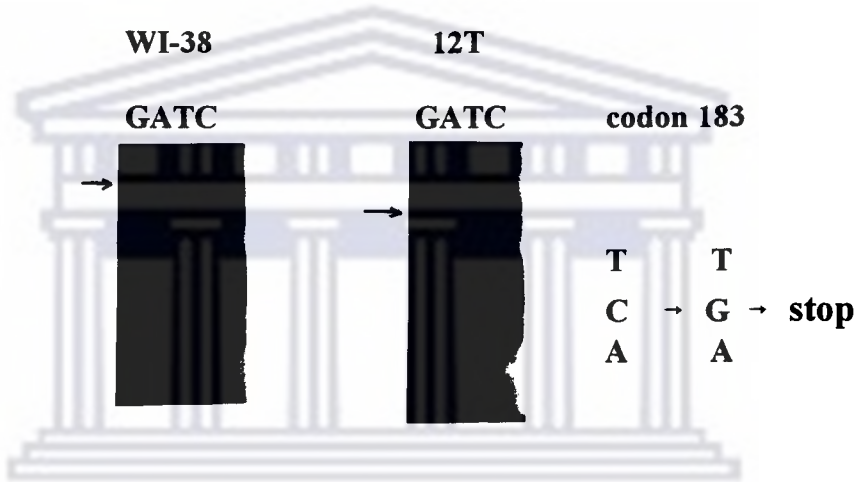


Figure 3.5 Mutation of exon 5 of the *p53* gene in patient 12.
Tumour DNA form patient 12 and DNA from WI-38 cells (control) was sequenced using the forward primer to exon 5 of the *p53* gene. Arrow indicates position of the C to G tranversion at codon 183 in exon 5 of the *p53* gene.

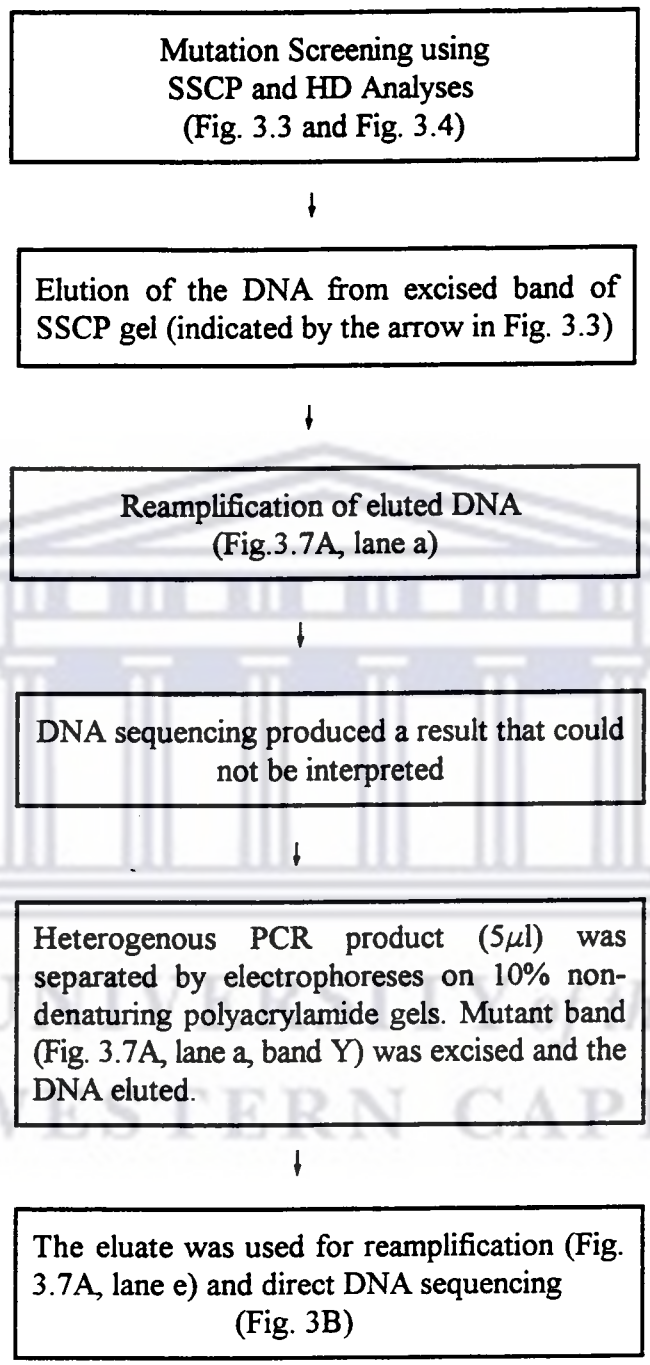
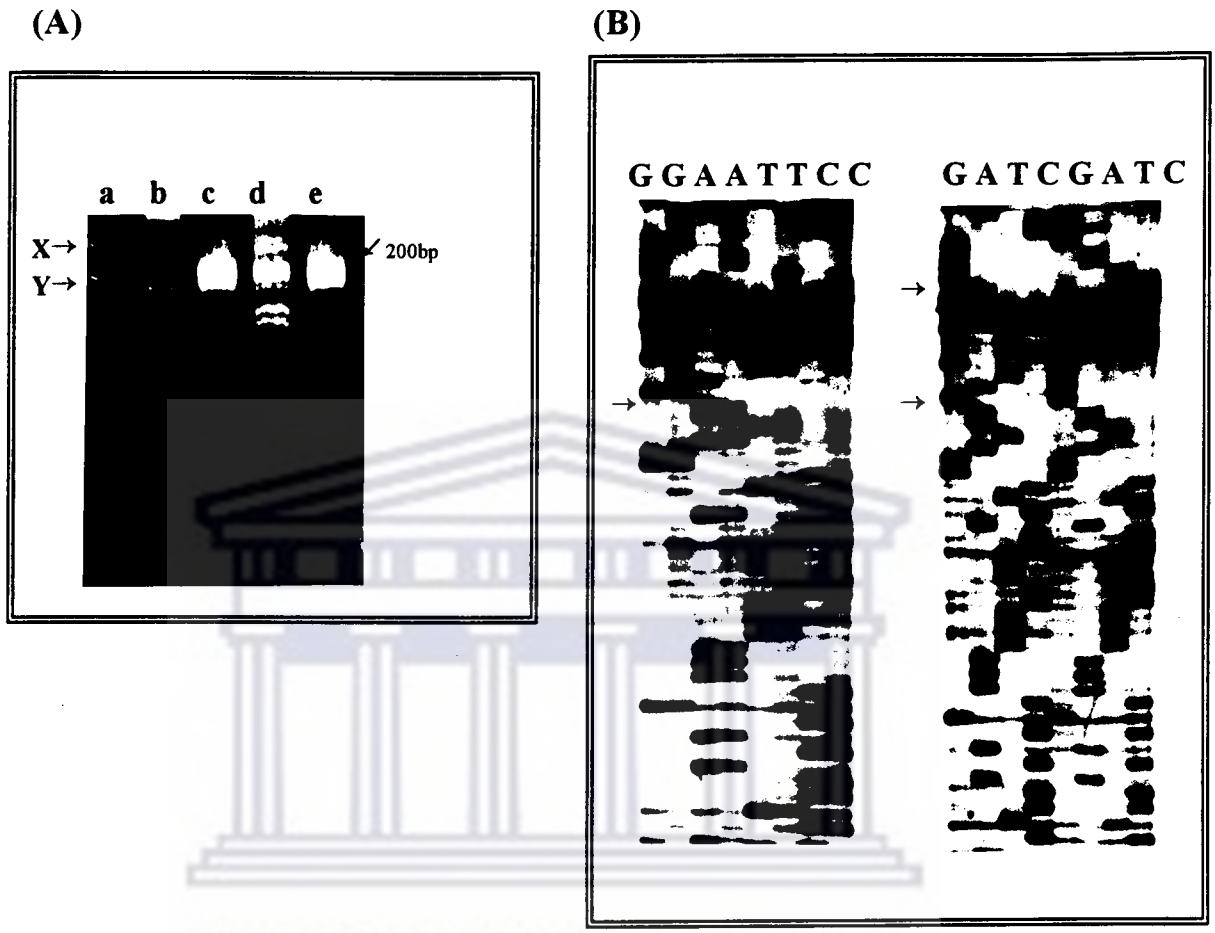


Figure 3.6 A flow diagram illustrating the strategy used to identify the *p53* mutation in exon 6 of patient 16.



(C)

Codon :	189	190	191	192	193	194	195	196	
Normal :	GCC	CCT	CCT	CAG	CAT	CTT	ATC	CGA	
Tumour:	CCG	CCT	TAT	CCG	AGT	GGA	AGG	AAA	→ Stop

Figure 3.7 Characterization of the *p53* mutation in patient 16
(A) Amplification of the *p53* exon 6 using the polymerase chain reaction.(a) Reamplification using the DNA eluted from the SSCP band indicated by the arrow in Figure 3.3. (b) Lambda DNA cut with *Pst* I. (c) PCR amplification of control DNA (WI-38) (d) PCR amplification of patient 16 normal DNA (e) Reamplification using the eluted DNA of the mutant band marked Y in lane a. (B) Identification of *p53* sequence differences in exon 6. Sequences from patient 16 normal (N) and tumour (T) DNA (PCR product in lane e above) using the reverse primer of exon 6.(C) DNA sequencing analysis of exon 6 in patient 16 identified an eleven base deletion from codon 190 to 196 (shown in bold) in tumour compared to normal as illustrated by the arrows.

3.3 Discussion

This study examined the involvement of the *p53* gene in the development of oesophageal cancer in South African patients, using SSCP and HD analyses to screen for putative mutant alleles, followed by DNA sequencing to confirm the mutations. Only exons 5 and 6 of the *p53* hot spot region were screened for mutations.

SSCP or HD analysis of exons 5 and 6 of the *p53* gene in 25 patients identified 3 patients with aberrantly migrating bands, suggesting the presence of mutations. Patient 12 displayed an extra band in exon 5 using both SSCP and HD analyses, whereas the SSCP pattern for exon 5 of patient 11 could not be confirmed with HD analysis. Patient 16 displayed aberrantly migrating bands for exon 6 using both mutation detection procedures. These results were reproducibly repeated three times.

DNA sequencing of exon 5 of patient 11 revealed inconsistent results after repeating the sequencing reaction on three separate occasions. The suspected mutation was detected in two of the three experiments, but was ascribed to an experimental artifact because of poor reproducibility. A total of 2 mutations (exon 5 in patient 12 and exon 6 in patient 16) were confirmed by DNA sequencing. Since normal DNA of patient 12 was not available, it was impossible to determine whether the observed mutation was of germline or somatic origin, unlike the situation with patient 16, where the somatic status of the mutation was confirmed. Overall, a mutation frequency rate of 8% (2 mutations in 25 patients tested) was detected for the *p53* gene in oesophageal cancer in South Africa. This figure is substantially lower than the

47%, 50%, 56% and 60% reported by Wagata *et al.*, (1993), Huang *et al.*, (1993), Liang *et al.*, (1995) and Hollstein *et al.*, (1991a), respectively. In this study only exons 5 and 6 of the mutation hot spot of the *p53* gene were examined, whereas the other studies had examined exons 5 - 8. Although, Wagata *et al.* (1993) examined all the coding regions including intron-exon boundaries of the *p53* gene in oesophageal cancer patients, it was found that mutations were clustered in exons 5 through 9. Since mutations have been detected in exons other than 5 and 6, additional mutations will most likely be discovered if exons 7, 8 and 9 were also examined in the same cohort of patients used in this study. Although our sample size (33 patients) is similar to that of other *p53* studies, screening additional patients could reveal a higher incidence of mutations in the *p53* gene in South African oesophageal cancer patients. Although the results of the present study suggest a less significant involvement of the *p53* gene than reported by other authors, the involvement of the *p53* gene in oesophageal cancer in South Africa will only become clear if exons 7, 8 and 9 are also examined for mutations.

The sequencing analysis defined a TCA to TGA mutation at codon 183 in exon 5 of patient 12 (which results in a stop codon at that position) and an 11 base pair deletion in exon 6 of patient 16. The 11bp deletion causes a frame shift mutation, culminating in a stop codon 39 bases downstream. These nonsense mutations result in the synthesis of truncated p53 proteins, with associated loss of the SV40 large T antigen binding domain and oligomerization domain. These mutants are unable to form homotetramers and therefore cannot bind and activate p53 binding sites in target genes (Farmer *et al.*, 1992; Kern *et al.*, 1992). It appears that nonsense mutations mainly have a quantitative biological effect on *p53*

and not an additional inhibitory effect on the normal *p53* allele, which is often the case with missense mutations (Milner and Medcalf, 1991). Missense mutations often yield full length p53 proteins that are dysfunctional but still able to complex with normal p53. The normal p53 protein is consequently inactivated. This effect is commonly referred to as the dominant negative effect, in that wild type *p53* function is completely destroyed even though only one mutant allele is present. However, all missense mutations do not result in the dominant negative effect.

A recent review claims that *p53* transition mutations are the most common lesions in oesophageal cancer (Greenblatt *et al.*, 1994), whereas previous studies show that transversion and nonsense mutations occur more frequently in oesophageal cancer compared to other cancers (Hollstein *et al.*, 1991b; Huang *et al.*, 1993). Deletion mutations have also been described in oesophageal cancer previously. Liang *et al.*, (1995) identified a 15 bp deletion in exon 7 and Huang *et al.*, (1994) identified a 45bp deletion at the intron 7 boundary. In this study we identify a transversion mutation at codon 183 in patient 12 and a 11bp deletion mutation in exon 6 of patient 16, both of which would result in truncated protein products.

The results of this study show that 8% of the oesophageal cancer tumours examined have a mutation in the *p53* gene. We predict that a higher incidence of *p53* mutations would be detected if exons 7, 8 and 9 were screened as well. These studies are currently being undertaken in our laboratory.

CHAPTER FOUR

Materials and Methods

The composition of all solutions and buffers is provided in the Appendix. Distilled deionized water was used for the preparation of all solutions and all the chemicals used were of analytical grade.

4.1 Patient Samples

The biopsy tissue used in this study was obtained from patients who gave informed consent. Tumour and adjacent normal biopsy samples (where possible) of thirty-three patients were collected during routine oesophagoscopy at the Gastrointestinal Clinic at Groote Schuur Hospital. The tissue samples were immediately placed in liquid nitrogen to prevent degradation of the nucleic acids.

4.2 Nucleic Acid Extraction from Biopsy Specimens

DNA and RNA were extracted from the biopsy samples collected, the former being used in this study, while the latter was used in other studies in the laboratory. The method used for the concurrent extraction of RNA and DNA from the small biopsy specimens was based on procedures described previously (Berk *et al.*, 1977; Favarolo *et al.*, 1980). The final method relied on the separation of cytosolic RNA from intact nuclei, after solubilizing plasma membranes with the nonionic detergent, Nonidet P40 (which leaves nuclear membranes intact). Guanidium isothiocyanate (protein denaturing agent) was

immediately added to the cytosolic fraction to prevent RNA degradation.

4.2.1 RNA Extraction

The biopsy samples were stored at -70°C until required. The sample (ca. 25mg) was pulverized in liquid nitrogen with a glass rod, and the powder suspended in 400μl of NP40 solution (Appendix). The suspension was vortexed for 1 minute, left on ice for 5 minutes and the cell debris and nuclei pelleted by centrifugation in an eppendorf desk top centrifuge at 3000 rpm for 30 seconds at 4°C.

4.2.2 DNA Extraction

The nuclear pellet (see section 4.2.1 above) was mixed with 450μl of digestion buffer (Appendix), vortexed and 50μl of proteinase K (20mg/ml) in TE was added. The sample was incubated at 55°C for 2 hours, with intermittent mixing in the first hour. An equal volume (500μl) of buffered phenol:chloroform:isoamylalcohol, pH 8 (P:C:IAA 25:24:1) was added and the solution mixed gently for two minutes. The aqueous and organic phases were separated by centrifugation at 10 000 rpm for 10 minutes at room temperature. The aqueous phase was collected (avoiding the interphase) and re-extracted with P:C:IAA as described above. After the centrifugation step, the aqueous (top) phase was collected, added to an equal volume of C:IAA (24:1), mixed by inversion and centrifuged at 10 000 rpm for 2 minutes at room temperature. The aqueous phase was collected and the DNA precipitated by the addition of 2.5 volumes of ice-cold absolute ethanol and 0.1 volumes of 3M NaAc, pH 5.5. After mixing by inversion, the tube was placed at -20°C for 5 minutes and then centrifuged at 10 000 rpm for 5 minutes at 4°C. The DNA pellet was washed with 70% ethanol to remove excess salt and dried under vacuum. The dry DNA pellet was dissolved in 100μl of TE (Appendix), dispensed in 20μl

aliquots and stored at -20°C.

4.3 DNA Extraction from Cultured Cells

4.3.1 Cell Culture

DNA was extracted from SNO, an oesophageal squamous cell carcinoma derived cell line (Bey *et al.*, 1976), and WI-38, a normal human embryonic lung fibroblast cell line, obtained from the American Type Culture Collection (ATCC CL-75). The initial optimization of PCR, SSCP and HD analysis, as well as control experiments, were performed using cell line DNA. The cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% heat inactivated fetal calf serum, streptomycin (100μg/ml) and penicillin (10U/ml). The cells were grown to confluence in 100mm petri dishes at 37°C, in a humidified incubator with 5% CO_2.

4.3.2 Harvesting the Cells

All solutions used for harvesting the cells were preincubated at 37°C. The growth medium was removed from the cells, which were then washed with 10ml of phosphate buffered saline. Trypsin (0.05%) containing 10mM EDTA (10ml) was added and the dish placed at 37°C for two minutes. Once the cells had lifted from the dish, they were collected into a 10ml sterile tube and centrifuged at 5000 rpm for two minutes. The supernatant was decanted and the tube containing the cell pellet was placed on ice.

4.3.3 DNA Extraction

The cell pellet was resuspended in 2ml of proteinase K digestion buffer (Appendix),

containing proteinase K at final concentration of 100μg/ml. The suspension was incubated overnight at 55°C with gentle shaking. This was followed by the addition of RNase at a final concentration of 10μg/ml and incubated at 55°C for 30 minutes. An equal volume of P:C:IAA (25:24:1) was added, the contents of the tubes were mixed by inversion and centrifuged at 5000 rpm in a Beckman centrifuge using a JA-20 rotor for 10 minutes at 4°C. The aqueous phase was collected and an equal volume of C:IAA (24:1) was added. The sample was mixed gently and centrifuged as described above. The extraction was repeated if a substantial precipitate of protein was present at the interphase. The aqueous phase was collected and the DNA precipitated by the addition of 2 volumes of absolute ethanol and left for two hours at -20°C. In the absence of a visible white precipitate (DNA) one-tenth volume of 3M NaAc, pH 5.5 was added and the solution left overnight at -20°C. The DNA was recovered by centrifugation at 10 000 rpm in a Beckman centrifuge using a JA-20 rotor for 10 minutes at 4°C. The DNA pellet was washed with 70% ethanol to remove excess salt and thereafter dried under vacuum. The DNA pellet was dissolved in 1ml of TE and left at 65°C for one hour or until it was dissolved. The DNA concentration was determined by measuring the absorbance at 260nm and the A_{260}:A_{280} ratio was used to determine DNA purity, with a ratio equal to or above 1.8 indicative of DNA of acceptable purity. A sample of the DNA (1μg) was electrophoresed on a 1% agarose gel at 40 mA, in 1X TBE buffer for 40-60 minutes to assess the quality of the DNA sample.

4.4 DNA Amplification by the Polymerase Chain Reaction

A variety of polymerase chain reaction (PCR) based techniques, first described by Mullis *et al.* (1986), were used to assess lesions associated with the *APC* and *p53* genes. These

techniques include LOH analysis by PCR-RFLP, Single Stranded Conformation Polymorphism (SSCP) analysis, Heteroduplex (HD) analysis and direct sequencing. The primer sets that were used in these studies are described in Table 4.1.

Table 4.1 Primer Sequences and expected fragment sizes

Primer set	Priming region	Size (bp)	Assay	Sequence 5' - 3'	Reference
1	*APC* exon 11	133	*Rsa* I RFLP	GGACTACAGGCCATTGCAGAA GGCTACATCTCCAAAAGTCAA	Boynton *et al* (1992)
2	*APC* exon 15	550	*Msp* I RFLP	ATGATGTTGACCTTTCCAGGG CTTTTTTGGCATTGCGGAGCT	Cottrel and Bodmer (1992)
3	*APC* * exon 15A	400	MS	GGATGTAATCAGACGACGCAG ACTGGGGCTTATAATGCCACT	Joslyn *et al* (1991)
4	*APC* * exon 15C	550	MS	TCCGTTCAGAGTGAACCATGC TGACTTTGTTGGCATGGCAGA	Joslyn *et al* (1991)
5	*p53* exon 5	310	MS	TGTTCACTTGTGCCCTGACT AGCAATCAGTGAGGAATCAG	**
6	*p53* exon 6	200	MS	TGGTTGCCCAGGGTCCCC TTAACCCCTCCTCCCAGAGA	**

*The "mutation cluster region" of the *APC* gene was divided into two overlapping fragments (15A and 15C) to facilitate screening in this study. Mutation screening (MS); RFLP for LOH analysis. **Primers were designed from the genomic DNA sequence of the *p53* gene (from Genbank, accession number 54156).

4.4.1 PCR Conditions

The PCR components used in this study are given in Table 4.2. Both positive and negative controls were included with each experiment to reduce the risk of scoring false positives. All the samples with a positive score (LOH, SSCP, HD and sequencing) were retested. Positive results were repeated as least three times, while negative and ambiguous results repeated at least once.

Table 4.2 Components of the Polymerase Chain Reaction (50μl).

Reagent	Amount / Concentration in 50μl
dNTP mix (10mM)	200μM
10X PCR Reaction buffer	1X
$MgCl_2$	1.5mM
Forward Primer	0.5μM
Reverse Primer	0.5μM
Taq DNA polymerase	1U
Sterile water	make up to 50μl
Genomic DNA	50ng

The reactions were typically performed in 50μl. Sufficient amplification of the DNA template was achieved in thirty-five consecutive cycles, each cycle including DNA denaturation, primer annealing and polymerase extension. These steps were carried out on an automated thermal cycler (Hybaid Omnigene) using standard cycling parameters as described below (unless otherwise specified).

Standard Cycling Parameters

Initial denaturation	: 95°C for 10 minutes 85°C for 5 minutes	1 cycle
Amplification	: 95°C for 1 minute 58°C for 1 minute 72°C for 1 minute	35 cycles
Final elongation	: 72°C for 10 minutes	1 cycle

4.4.2 Polyacrylamide Gel Electrophoresis

The PCR products were analysed by polyacrylamide gel electrophoresis (PAGE) to determine the product size. The percentage of acrylamide used (6-8%) was determined by the expected fragment size, in order to separate the fragments ranging in size from 133-

550 base pairs (bp). A commercially available DNA size marker (Biomark, BioVentures) or Lambda DNA digested with *Pvu* II, *Hind* III or *Pst* I, was used as a DNA marker. Positive and negative controls were always included on gels. Gels were electrophoresed in 1XTBE at 150V for 90 minutes, followed by ethidium bromide staining (final concentration 1μg/ml) and viewing under UV illumination. The PCR products were stored at -20°C until further analysis.

4.5 Loss of Heterozygosity Analysis using the Polymerase Chain Reaction and Restriction Fragment Length Polymorphism

The PCR-RFLP technique allows one to distinguish between two alleles at a specific chromosomal locus and hence whether allelic loss has occurred at the locus in question. The assay is based on the PCR amplification of a specific fragment of DNA containing a polymorphic site for a restriction enzyme. The two alleles can only be distinguished if they are heterozygous for the restriction site (i.e. one allele is positive for the restriction site and one allele is negative for the restriction site). Digestion of the PCR amplified product, followed by electrophoretic separation of the digested product, confirms the presence or absence of a restriction enzyme site. A comparison of heterozygous normal tissue with the corresponding tumour tissue allows for the identification of patients in which LOH has occurred. In this study two RFLP's located within the *APC* gene were used to assess LOH in tumour tissue, relative to paired normal.

4.5.1 *Rsa* I Restriction Fragment Length Polymorphism

Exon 11 of the *APC* gene contains a *Rsa* I polymorphic site (Fig 4.1) which has previously been used by Boynton *et al.* (1992) for LOH analysis of the *APC* gene. A 133bp fragment was amplified using the polymerase chain reaction with primers spanning the polymorphic site (Table 4.1). Patients were included in this study only if both normal and tumour DNA from that individual was available. The PCR components were prepared as described in Table 4.2 and the standard cycling parameters were used (section 4.4.1). Five microlitres of the PCR product were electrophoresed on an 8% non-denaturing polyacrylamide gel to check the specificity of the amplification reaction. The RFLP analysis was only performed if a single band was visualized. A restriction digest mixture was prepared containing 15μl of PCR product, 3μl of 10X buffer L (Boehringer Mannheim), 0.5μl (5U) of *Rsa* I (Boehringer Mannheim) and 11.5μl of sterile water in a final volume of 30μl and incubated overnight at 37°C. Five microlitres of DNA loading buffer were added to the digestion mixture and the DNA was electrophoresed on an 8% non-denaturing polyacrylamide gel as described in section 4.4.2. Alleles negative for the *Rsa* I polymorphic site were identified by the presence of the intact 133bp fragment, whereas the presence of the *Rsa* I site was inferred from the cleavage of the 133bp product into two fragments of 85bp and 48bp, respectively. A PCR product (400bp) with a *Rsa* I restriction site was often included in the digest as an internal control. *Rsa* I cleavage of the latter PCR product creates fragments of 300bp and 100bp.

4.5.2 *Msp* I Restriction Fragment Length Polymorphism

The *APC* gene also contains a polymorphic *Msp* I site in exon 15 of the gene (Fig. 4.1) which has been previously used to assay for LOH in the *APC* gene (Cottrell and Bodmer, 1992). In this study, a 550bp fragment was amplified using primers spanning the polymorphic site (Table 4.1). Patients were included in this study only if both normal and tumour DNA from that individual was available. The PCR mixture was prepared as described in section 4.4.1, using cycling parameters optimized for this primer set (92°C for 5 minutes, 35 cycles of 1 minute at 92°C, 1 minute at 60°C and 1 minute at 72°C). Five microlitres of the PCR product were electrophoresed on a 6% non-denaturing polyacrylamide gel to assess the specificity of the amplification reaction. A restriction digest incubation mixture was prepared containing 15μl of the PCR product, 3μl of buffer L (Boehringer Mannheim), 0.5μl (5 units) of *Msp* I (Boehringer Mannheim) and 11.5μl of sterile water in a final volume of 30μl and incubated overnight at 37°C. Five microlitres of DNA loading buffer were added to the incubation mixture and the DNA was electrophoresed on an 8% non-denaturing polyacrylamide gel as described in section 4.4.2. Alleles negative for the *Msp* I restriction site were identified by the presence of an intact 550bp fragment, whereas the presence of the *Msp* I site was inferred from the cleavage of the 550bp fragment into two fragments of equal size (275bp). A PCR product (310bp) with an *Msp* I restriction site was often included in the digest as an internal control. *Msp* I cleavage of the latter PCR product creates fragments of 177bp and 133bp.

4.6 MUTATIONAL SCREENING

Mutation Cluster Regions

More than 50% of all mutations associated with the *APC* and *p53* tumour suppressor genes in cancer are located in MCR (Powell *et al.*, 1992) and "hot spot" regions (Nigro *et al.*, 1989; Hollstein *et al.*, 1991b), respectively. The *APC* MCR occurs between codons 1281 and 1554 in exon 15 (Powell *et al.*, 1992; reviewed by Nakamura 1993). The "hot spot" region in the *p53* gene occurs within a phylogenetically conserved region between codons 117 and 286, from exon 5 to exon 8 (Soussi *et al.*, 1990). In this study, these mutation susceptible regions were examined by SSCP and HD as well as by direct DNA sequencing.

4.6.1 Single Stranded Conformational Polymorphism Analysis

This technique was initially described by Orita *et al.* (1989) and allows for the detection of point mutations or single base sequence differences in DNA fragments up to 400bp in length. The method relies on the electrophoretic separation of single stranded DNA fragments on non-denaturing polyacrylamide gels under mild conditions, which permits the single stranded DNA fragments to adopt a secondary conformation determined by their sequence. A single nucleotide change would result in a conformational change which in turn is detected as a change in the banding pattern when compared to control DNA.

The Adenomatous Polyposis Coli gene

Two overlapping primer sets were used to amplify the MCR of exon 15 in the *APC* gene into two fragments, 15A (400bp) and 15C (550bp), respectively (Fig. 4.1 and Table 4.1).

These fragments were amplified using the standard PCR protocol as described in section 4.4.1, with the exception that ^{32}P-dCTP was included in the reaction mixture (3μCi or 0.3μl per reaction). The reaction mixture was then subjected to the standard thermal cycling conditions as described in section 4.4.1. Since the optimum fragment size for SSCP analysis is less than 400bp, both amplified products (15A and 15C) were digested with a restriction endonuclease to generate smaller fragments. Fragment 15A was digested with *Rsa* I and 15C with *Taq* I (in a final volume of 30μl according to the manufacturer's instructions) yielding products of 300bp and 100bp for 15A and 320bp and 230bp for 15C, respectively.

The *p53* gene

In this study, only exons 5 and 6 of the *p53* gene were examined for mutations. Exon 5 was amplified using primer set 5 (Table 4.1), producing a product of 300 bp. The PCR reaction mixture was prepared according to the standard protocol as described in section 4.4, using optimised cycling parameters (35 cycles of: 94°C for 1 minute, 50°C for 1 minute and 72°C for 2 minutes). Exon 6 was amplified using primer set 6 (Table 4.1), generating a product of 200 bp. The PCR reaction mixture was prepared according to the standard protocol as described in section 4.4.1, except that 0.1μM of each primer was used to reduce the occurrence of non-specific products and the cycling parameters were 35 cycles of 94°C for 30 seconds, 58°C for 1 minute and 72°C for 30 seconds.

Sample Preparation and Gel Electrophoresis

The PCR products (*p53* and *APC* genes) were mixed with an equal volume of formamide

loading buffer (Appendix), denatured at 95°C for 5 minutes and placed on ice. A volume of 3μl was normally loaded, or 5μl if the sample had been digested after the PCR step. The samples were electrophoresed on 0.4mm thick, 6% non-denaturing polyacrylamide gels with and without 10% glycerol, at room temperature and at 4°C. The gels were electrophoresed in 1XTBE running buffer at 30W for 4-6 hours or at 4W overnight. Gels were dried at 70°C for 30 minutes and then exposed to X-ray film overnight at -70°C.

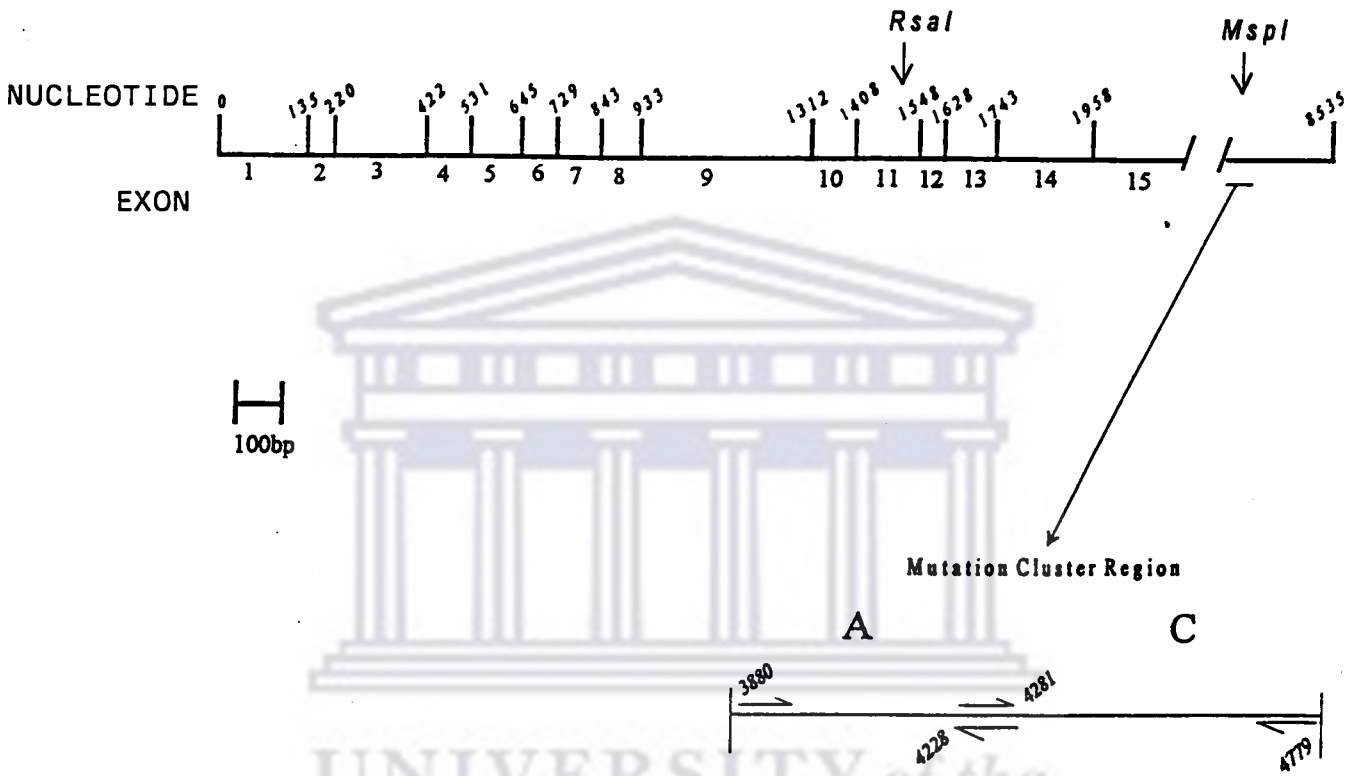


Figure 4.1 Line diagram of the *APC* gene (exons and RFLPs)
Coding exons are indicated by numbers 1 to 15. The polymorphic markers are illustrated by the restriction enzyme sites within exons 11 (*Rsa* I) and exon 15 (*Msp* I). Below, the Mutation Cluster Region with encompassing primer sets (15A and 15C).

4.6.2 Heteroduplex Analysis

The PCR amplification results in the formation of homoduplexes when identical, complementary DNA fragments anneal, while heteroduplexes form when homologous but not identical, complementary fragments anneal. Heteroduplexes have "kinks" where sequence differences occur between two strands (e.g. mutant and wild type strands), resulting in a slower migration and lower intensity relative to the homoduplexes. Mutant homoduplexes may also migrate faster than wild type homoduplexes as a result of deletion mutations (Nagamine *et al.*, 1989). The PCR-amplified fragments generated in SSCP analysis were also screened for mutations by heteroduplex analysis.

Sample Preparation and Gel Electrophoresis

Five microlitres of formamide loading buffer (Appendix) were added to 10μl of the PCR product. The patients who had demonstrated LOH, were analysed by mixing PCR products amplified from normal and tumour DNA to facilitate heteroduplex detection. Equal volumes of normal and tumour PCR product were mixed, denatured at 95°C and allowed to cool at room temperature before loading. The samples were resolved on 1.2mm thick, 10% polyacrylamide gels (1% crosslinked). The gels were electrophoresed in 0.6X TBE running buffer at 600V for 9 hours or 180V overnight. The gels were stained with ethidium bromide (final concentration of 1μg/ml) for 15-30 minutes, destained for 15 minutes and viewed under UV light.

4.6.3 DNA Sequence Analysis

PCR products were sequenced using the Sequenase PCR Product Sequencing Kit (United States Biochemical Corporation). One microlitre of primer (~20pmole) was added to 7μl

of PCR product in a final volume of 10μl. The mixture was denatured by incubation at 100°C for 3 minutes, followed by quick cooling for 5 minutes on ice. The sequencing reactions were carried out according to the "kit specifications" and the samples were electrophoresed on 6% or 8% polyacrylamide 7M urea sequencing gels. The gels were dried under vacuum at 70°C for one hour and exposed to X-ray film overnight or longer at -70°C .

CONCLUSION

South Africa has been described as one of the world's high risk areas for oesophageal cancer and this disease is the most common cause of cancer-related deaths in black males. In an effort to characterise the molecular nature of this disease in South Africa, the genetic integrity of the *APC* and *p53* tumour suppressor genes was investigated in thirty-three South African oesophageal cancer patients. The *APC* gene was initially characterised for its involvement in the early stages of colorectal tumourigenesis, while alterations in the *p53* gene have been found in numerous human cancers. In this study, the *APC* gene was analysed for allelic loss and small mutations in targeted areas previously implicated in the development of cancer. Analysis of allelic loss involved the use of two RFLP markers, *Rsa* I and *Msp* I, within exon 11 and exon 15 of the *APC* gene, respectively. Mutation screening included SSCP, HD and DNA sequencing analyses of the MCR within exon 15 of the *APC* gene. Analyses of the *p53* gene involved SSCP, HD and DNA sequencing of exons 5 and 6.

Analysis of allelic loss of the *APC* gene in the patient sample revealed an informativity of 59% for one or both polymorphic markers and a 21% allelic loss of the *APC* gene. No somatic mutations were detected in the MCR of exon 15 of the *APC* gene. The low frequency of allelic loss of the *APC* gene detected in the sample of patients analysed in this study suggests that the *APC* gene may not play a significant role in oesophageal carcinogenesis. This is further supported by the absence of mutations in the MCR of exon 15 of the *APC* gene. There is a possibility that mutations have been missed due to the SSCP and HD screening techniques used in this study. However, mutations have successfully been identified in this study and elsewhere using the same strategy. Several additional efforts may be undertaken to clearly define the involvement of this

gene in oesophageal cancer, including increasing the number and the type of polymorphic markers used to detect allelic loss, increasing the sample size and examining the entire coding region of the gene for mutations.

The SSCP and HD analyses of exons 5 and 6 of the *p53* gene revealed 3 putative mutations in tumour tissue of three patients, two of which were confirmed by DNA sequencing. One patient displayed a TCA to TGA substitution at codon 183 in exon 5, resulting in a stop codon at that position. Another patient demonstrated an 11bp deletion in exon 6, causing a frame shift mutation and resulting in a premature stop codon 13 codons downstream. Analysis of the *p53* gene revealed an overall mutation frequency of 8% (2 of 25 patients examined), suggesting that the *p53* gene may be involved in the development of oesophageal cancer. To further elucidate the role of *p53* in oesophageal cancer in South Africa several additional studies may be undertaken, including increasing the sample size but, more importantly the examination of exons 7, 8 and 9, which have been implicated in oesophageal tumourigenesis in other studies.

The involvement of two tumour suppressor genes in the development of oesophageal cancer has been examined in this study. Once larger samples of patients are analysed, it would be useful to attempt to correlate any findings with the medical history of the patient. In this manner the relationship between lesions in specific genes and aetiology, prognosis and response to treatment, may be investigated.

In conclusion, the results of this study suggest that the *APC* gene may not be involved in oesophageal cancer, whereas the *p53* gene may play a role in the development of oesophageal cancer in South Africa.

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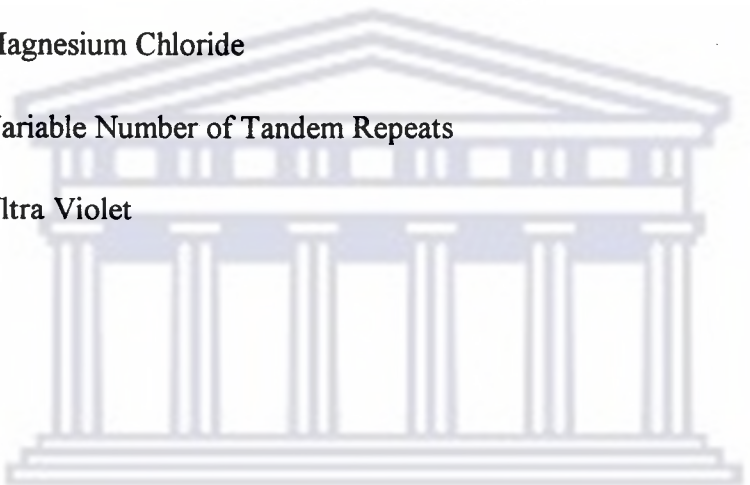
ABBREVIATIONS

AC	Adenocarcinoma
APC	Adenomatous Polyposis Coli
bp	base pairs
BRCA	Breast Cancer
CDK	Cyclin Dependent Kinase
DCC	Deleted in Colorectal Cancer
DNA	Deoxyribonucleic Acid
EGFR	Epidermal Growth Factor Receptor
FAP	Familial Adenomatous Polyposis
G1	Gap 1
G2	Gap 2
HD	Heteroduplex Analysis
kDa	kilodaltons
LOH	Loss of heterozygosity
M	Mitosis
MCR	Mutation Cluster Region
MTS1	Multiple Tumour Suppressor 1
MCR	Mutation Cluster Region
Rb	Retinoblastoma
S	Synthesis
SCC	Squamous Cell Carcinoma
SSCP	Single Stranded Conformation Polymorphism

RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
PCR	Polymerase Chain Reaction
rpm	revolutions per minute
NaAc	Sodium Acetate
$MgCl_2$	Magnesium Chloride
VNTR	Variable Number of Tandem Repeats
UV	Ultra Violet



APPENDIX

Solutions and Buffers

GITC solution
4M Guanidinium Isothiocyanate
25mM Sodium Citrate pH7
0.5% N-Larylsarcosine

NP40 solution
2% Nonidet-P40
50mM Tris pH8
100mM NaCl
5mM $MgCl_2$

PBS
Phosphate Buffered Saline
7.36 mM KCl
1.5 mM KH_2PO_4
137 mM NaCl
8.1 mM Na_2HPO_4

Proteinase K Digestion Buffer
100 mM NaCl
10 mM Tris-Cl pH8
2.5 mM EDTA pH8
0.5% SDS

10X TE
100mM Tris-Cl pH 7.5
10mM EDTA

dNTP mixture
2.5mM of each dNTP (dATP, dCTP, dGTP and dTTP)
final concentration - 10mM

$MgCl_2$
25mM $MgCl_2$

Taq DNA Polymerase
5U/μl - Thermoprime plus supplied Advanced Biotechnology (AB)

10X PCR Reaction Buffer
500mM KCL
100mM Tris-Cl, pH 8.4
1mg/ml gelatin

10X TBE pH 8.3
0.89 M Tris
0.89 M Boric acid
25 mM EDTA

40% Acrylamide (Sequencing and SSCP)
380g Acrylamide
20g Bisacrylamide
Dissolve in 1L of ddH_2O
Deionize with 50g of ion exchanger overnight at 4°C.

40% Acrylamide (Heteroduplex)
80g Acrylamide (Stratagene)
0.8g Bisacrylamide
Dissolve in 200 ml ddH_2O

Formamide Loading Buffer
98% Deionized formamide
0.25% Xylene Cyanol
0.25% Bromophenol Blue
10 mM EDTA

DNA Loading Buffer
0.25% Xylene Cyanol
0.25% Bromophenol Blue
30% glycerol in water

C:IAA (24:1)
24 parts chloroform
1 part isoamylalcohol

P:C:IAA
refer to Aubusel *et al* (1995)
volume 2 section 2.1.5

DEPC
add 0.2ml of diethylpyrocarbonate to 100ml of water
shake until dissolved and autoclave.