Nucleotide sequence variation and expression levels of *TP*53 in cancers of the upper gastro-intestinal tract

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

Signature. Date.

SUMMARY

The work presented in this thesis deals with the association between cancers of the upper gastro-intestinal tract and the tumor suppressor gene, *TP*53, and can be divided into three parts: (i) the analysis of the mutational spectrum of *TP*53 with respect to laryngeal cancer, (ii) the analysis of the mutational spectrum of *TP*53 with respect to esophageal cancer and (iii) the analysis of *TP*53 transcriptional levels in esophageal cancer.

Laryngeal cancer (LC) is the 6th most common cancer in the world and the 2nd most common respiratory cancer, with approximately 500 000 new cases per annum detected worldwide. Over the last few years, LC has become increasingly prevalent within the Coloured Community of the Western Cape. The mechanisms of tumorigenesis in LC remain unknown, although smoking and alcohol consumption are considered to be major risk factors. Mutations within the gene *TP*53 have been strongly implicated as playing a role in cancer development, as they are frequently found in several cancer types. We therefore screened exons 5 – 8 of *TP*53 for mutations in DNA from tumor biopsies (n=44) and blood samples (n=42) from Coloured LC patients, using polymerase chain reaction – single strand conformation polymorphism (PCR-SSCP) analysis and direct sequencing. Blood samples from a healthy, matched control group (n=40) were included in the study as controls. Significant correlations were found between the occurrence of LC and age and smoking, whereas daily meat consumption was a possible protective factor. In tumor-derived samples, mutations were found in 3 of the exons under investigation, representing 25% of the samples. The mutations were unique to the tumor biopsies, indicating a somatic origin for mutations. The data confirms that the region between codons 175 and 273 of *TP*53 is a mutational hotspot for cancers in general. This study reports 6 novel mutations within this same region.

Esophageal cancer (EC) has a very high incidence in South Africa, relative to the rest of the world, and is particularly common amongst the Black Transkei population. The goal of this study was to determine whether there are differences in the *TP*53 mutational pattern observed in the Coloured Western Cape community as compared to that observed in the Black Transkei community. This required the analysis of the molecular structure of *TP*53, specifically exons 5 – 8, in a group of Coloured EC patients (n=44) treated at Tygerberg Hospital, Cape Town, South Africa. DNA obtained from tumor biopsies and blood (from patients) as well as from apparently healthy surrounding tissue was screened via PCR-SSCP and direct sequencing analysis. Only 4 nucleotide changes were observed from a total of 124 sequences obtained, of which two were novel to esophageal squamous cell carcinoma. These 4 nucleotide alterations were found only within the tumor biopsy sample set, representing 9% of the tumors investigated. This study revealed that the mutational spectrum of *TP*53 within the Coloured population of the Western Cape greatly differs from that of the Black community of the Transkei. This suggests that a different set of etiological factors are involved in the tumorigenic process for each of these distinct geographical communities, which is the subject of an epidemiological study undertaken by the MRC.

The final part of this thesis deals with the quantification and comparison of *TP*53 transcription levels in esophageal cancer tumor tissue to the *TP*53 levels in healthy esophageal tissue obtained from patients from a unique geographical and ethnic background. The cohort used in this study consisted of Coloured patients (n=2) treated at Tygerberg Hospital. The LightCycler system was implemented in order to try to accurately quantify *TP*53 mRNA levels. Unfortunately, the desired results were unattainable due to unforeseen difficulties encountered during the study. These difficulties included the insufficient preservation of samples for RNA based studies. Several recommendations were made concerning future similar studies, including an improved planning strategy as well as the employment of an RNA stabilizing agent. Additionally, a few

important contributions were made through this study, including the design and optimization of *TP*53 primers specifically intended for future RNA studies. These primers would enable the identification of the presence of *TP*53 RNA species as well as the absence of DNA contamination in a single PCR amplification step. Other contributions include the development of a well-optimized RNA extraction method for the extraction of RNA from tough tissues (such as the human esophageal tissue used in this study). This method makes the extraction of large quantities of RNA from small amounts of tough tissue types possible.

In conclusion, this study has made a significant contribution to the field of cancer research, by shedding light on the *TP*53 mutational spectrum with regards to laryngeal as well as esophageal cancer in a population unique to the Western Cape.

The first part of this thesis has been published in Cancer Genetics and Cytogenetics (Barnard, D., K. Lehmann, E.G. Hoal, P.D. van Helden, and T.C. Victor. 2003. The spectrum of mutations in *TP*53 in laryngeal cancer patients from a high-incidence population shows similarities to many of the known mutational hotspots. Cancer Genetics and Cytogenetics 145:126-132), of which a copy can be found in Appendix I. This work has also been presented (by D. Barnard) at an international conference entitled "Cancer of the Esophagus and Gastric Cardia: From Gene to Cure", held in Amsterdam, the Netherlands during the period 13 – 15 December 2002.

OPSOMMING

Die werk wat in hierdie tesis voorgelê word handel oor die assosiasie tussen kankers van die boonste gastrointestinale weg en die tumor suppressor geen, *TP*53, en kan in 3 dele gedeel word, (i) die analise van die mutasiespektrum van *TP*53 in laringiale kanker (LK), (ii) die analise van die mutasiespektrum van *TP*53 in slukderm kanker (SK) en (iii) die analise van die transkripsievlakke van *TP*53 in SK.

Laringeal kanker (LK) is die 6de algemeenste kanker in die wêreld en die 2de algemeenste respiratoriese kanker, met 'n benaderde 500 000 nuwe gevalle jaarliks wêreldwyd. Oor die afgelope paar jare het LK 'n toenemende probleem geraak, veral in die Kleurling gemeenskap van die Wes Kaap. Die meganismes van die tumorvorming in LK is onbekend, alhoewel rook-en alkoholgebruik vername risiko faktore is. Die voorkoms van mutasies in *TP*53 is verskeie kere aangetoon in verskillende kanker tipes en daar word vermoed dat dit 'n rol speel in tumorvorming. In hierdie studie is dus na mutasies in eksons 5 – 8 van *TP*53 gesoek in tumor biopsie weefsel (n=44) en bloed isolate (n=42) van Kleurling LK pasiënte d.m.v. polimerase ketting reaksie - enkelstring konformasie polimorfisme (PKR-ESKP) analisering en direkte volgorde bepaling. Bloed monsters van 'n vergelykbare groep (n=40) is ook in die studie ingesluit as 'n kontrole. Betekenisvolle positiewe korrelasies is gevind tussen die voorkoms van LK en ouderdom sowel as rook. Daarmee saam is daaglikse vleisinname as potensiële beskermende faktor gevind. In tumor biopsies is mutasies in 3 van die ondersoekte eksons gevind, wat 25% van die biopsie monsters verteenwoordig. Hierdie mutasies is uniek aan die tumor biopsie weefsels en dui op 'n somatiese oorsprong van mutasies. Hierdie bevindinge bevestig dat die gedeelte tussen kodons 173 – 273 van *TP*53 'n hipermuteerbare gebied geassosieer met kankers is. Hierdie studie bevestig 6 nuwe mutasies.

Daar is 'n hoë insidensie van slukderm kanker (SK) in Suid Afrika relatief tot die res van die wêreld. Hierdie soort kanker word veral gevind by die Swart populasie van die Transkei. Die doel van hierdie studie was om verskille tussen die *TP*53 mutasie patroon van die Kleurling gemeenskap van die Wes Kaap en die Swart gemeenskap van die Transkei te vergelyk. Hiervoor is die molekulêre struktuur van *TP*53, veral eksons 5 – 8, in 'n groep Kleurling SK pasiënte (n=42) wat behandel is by Tygerberg Hospitaal, Kaapstad, Suid Afrika, geanaliseer. Analisering is gedoen deur DNS van tumor, bloed en ook oënskynlike gesonde aangrensende weefsel van dieselfde pasiënte te onderwerp aan PKR-ESKP analise en direkte volgorde bepaling. Slegs 4 nukleotied veranderings is gevind in 124 volgorde bepalings, waarvan 2 nuwe veranderings is in SK. Hierdie 4 nukleotied veranderinge verteenwoordig 9% van al die tumors wat ondersoek is in die studie. Hierdie studie bewys dat die mutasiespektrum van *TP*53 in die Kleurling gemeenskap van die Wes Kaap grootliks verskil van die Swart gemeenskap van die Transkei. Dit impliseer dat verskillende etiologiese faktore moontlik 'n rol mag speel op die tumorvormingsproses in die 2 afsonderlike geografiese gemeenskappe. Hierdie is die onderwerp van 'n epidemiologiese studie wat deur die MNR onderneem word.

Die laaste deel van hierdie tesis handel oor die kwantifisering en vergelyking van *TP*53 transkripsievlakke in SK tumor weefsel teenoor *TP*53 vlakke in gesonde slukderm weefsel van pasiënte in 'n unieke geografiese en etniese agtergrond. Die studie populasie in hierdie projek het bestaan uit Kleurling pasiënte (n=2) wat by Tygerberg hospitaal behandel is. Die "LightCycler" sisteem is gebruik vir die akkurate kwantifisering van *TP*53 boodskapper RNS vlakke. Ongelukkig is die verlangde resultate nie gekry nie as gevolg van onvoorsiene probleme wat ondervind is tydens die studie. Hierdie probleme sluit in die onvoldoende preserv

RNS studies. Hierdie inleiers maak dit nou moontlik om die teenwoordigheid van *TP*53 RNS spesies sowel as die afwesigheid van DNS kontaminasie in een PKR amplifikasie stap te kan identifiseer. 'n Ander belangrike bydrae is die ontwikkeling van 'n goed geoptimaliseerde RNS ekstraksie metode vir moeilike starre weelfsel tipes (soos menslike slukderm weefsel in hierdie studie) en maak die ekstraksie van groot hoeveelhede RNS uit klein hoeveelhede van moeilik hanteerbare weefsel tipes moontlik.

Om saam te vat, hierdie studie het betekenisvolle bydraes gemaak tot die veld van kankernavorsing deur die ontrafeling van die *TP*53 mutasiespektrum in beide laringeale sowel as slukderm kanker, in 'n populasie uniek aan die Wes Kaap.

Die eerste deel van hierdie tesis is gepubliseer in Cancer Genetics and Cytogenetics (Barnard, D., K. Lehmann, E. G. Hoal, P. D. van Helden, and T. C. Victor. 2003. The spectrum of mutations in TP53 in laryngeal cancer patients from a high-incidence population shows similarites to many of the known mutational hotspots. Cancer Genetics and Cytogenetics 145:126-132) en 'n afskrif van die artikel is ingesluit in Appendix I. Hierdie werk is ook voorgedra (deur D. Barnard) by 'n internasionale kongres getiteld "Cancer of the Esophagus and Gastric Cardia: From Gene to Cure", wat in Amsterdam, Nederland gehou is gedurende 13 – 15 Desember 2002

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LIST OF ABBREVIATIONS

A	Adenine
*ABL*1	v-abl Abelson murine leukemia viral oncogene homolog 1
*ABL*2	v-abl Abelson murine leukemia viral oncogene homolog 2
*AKT*1	v-akt Murine thymoma viral oncogene homolog 1 gene
APC	Adenomatous polyposis coli gene
*ARAF*1	v-raf Murine sarcoma 3611 viral oncogene homolog 1
*ARAF*2	v-raf Murine sarcoma 3611 viral oncogene homolog 2
Arg	Arginine
Asn	Asparagine
BAX	BCL2-associated X protein
BC	Before Christ
*BCL*2	B-cell CLL/lymphoma 2 gene
Bp	Base pairs
*BRCA*1	Breast cancer 1 gene
*BRCA*2	Breast cancer 2 gene
BSA	Bovine serum albumin
C	Cytosine
CANSA	Cancer Association of South Africa
cc	Correlation coefficient
cDNA	Complementary DNA
cm	Centimeters
CM	Coloured male
C-terminal	Carboxyl-terminal
*CSF*1*R*	Colony stimulating factor 1 receptor, formerly McDonough feline sarcoma viral (v-fms) oncogene homolog
CYP	Cytochrome P450
Cys	Cysteine
DCC	Deleted in colon cancer gene

DEPC	Diethyl Pyrocarbonate
dH_2O	Deionized pure water
DNA	Deoxyribonucleic acid
dsDNA	Double stranded deoxyribonucleic acid
EC	Esophageal cancer
ESCC	Esophageal squamous cell carcinoma
EDTA	Disodium ethylenediaminetetraacetate dehydrate
e.g.	*Exempli gratia* for example
EH	Epoxide hyrdrolases
EGFR	Epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian
ELK2P1	ELK2, member of ETS oncogene family, pseudogene 1
ELK2P2	ELK2, member of ETS oncogene family, pseudogene 2
ERBB	v-erb-b2 Erythroblastic leukemia viral oncogene homolog
ERBB2	v-erb-b2 Erythroblastic leukemia viral oncogene homolog 2, neuro/glieoblastoma derived oncogene homolog (avian)
ERG	v-ets Erythroblastosis virus E26 oncogene like (avian)
et al.	*Et allii / et alia* and others
ESCC	Esophageal squamous cell carcinoma
ETS1	v-ets Erythroblastosis virus E26 oncogene homolog 1 (avian)
ETS2	v-ets Erythroblastosis virus E26 oncogene homolog 2 (avian)
F	Forward
FES	Feline sarcoma oncogenes
FGF4	Fibroblast growth factor 4 (heparin secretory transforming protein 1, Kaposi sarcoma oncogene)
FGR	Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene homolog
FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog
G	Guanine
G1	Gap 1
G2	Gap 2
GADD45	Growth arrest and DNA-damage-inducible

GLI	Glioma-associated oncogene homolog (zinc finger protein)
Gly	Glycine
GST	Glutathion-S-transferase
hGAPDH	Human glyceraldehyde-3-phosphate dehydrogenase
His	Histidine
HIV	Human immunodeficiency virus
*hMLH*1	PMS2 postmeiotic segregation increased 2 gene
*hMSH*2	human MutS homolog 2
*hPMS*1	human Postmeiotic segregation increased 1 gene
*hPMS*2	human Postmeiotic segregation increased 2 gene
HPV I	Human papilloma virus type I
HPV II	Human papilloma virus type II
HRAS	v-Ha-ras Harvey rat sarcoma viral oncogene homolog
HSP70	Heat shock protein 70kDa
HSV-1	Herpes simplex virus type 1
IARC	Internation Agency for Research on Cancer
i.e.	I*d est* that is
Ile	Isoleucine
IRB	Institutional Review Board
JUN	v-jun Sarcoma virus 17 oncogene homolog (avian)
kDa	kilo Dalton
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
*KRAS*2	v-Ki-ras2 Kirsten rat sarcoma 2 viral oncogene homolog
*KRAS*1*P*	v-Ki-ras1 Kirsten rat sarcoma 1 viral oncogene homolog, processed Pseudogene
L	Liter
LC	Laryngeal cancer
LCO	Liver cancer oncogenes
LED	Light emitting diode
LOH	Loss of heterozygosity
LTR	Long terminal repeats

LYN	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog
M	Molar
mA	Milliamp
*MAS*1	MAS1 oncogene
*MDM*2	Murine double minute 2
MEL	Mel transforming oncogene (derived from cell line NK14)- RAB8 homolog
MET	Met proto-oncogene (hepatocyte growth factor receptor)
mg	Milligrams
$MgCl_2$	Magnesium cloride
ml	Milliliters
mm	Millimeters
mmol	Millimolar
MOS	v-mos Moloney murine sarcoma viral oncogene homolog
MRC	Medical research council
mRNA	Messenger ribonucleic acid
MYB	v-myb Myeloblastosis viral oncogene homolog (avian)
MYC	v-myc Myelocytomatosis viral oncogene homolog (avian)
*MYCL*1	v-myc Myelocytomatosis viral oncogene homolog 1, lung carcinoma derived (avian)
MYCN	v-myc Myelocytomatosis viral related oncogene, neuroblastoma derived (avian)
n	Total number
NaCl	Sodium Chloride
NCBI	National Centre for Biotechnology Information
NF-1	Neurofibromatosis1 gene
NF-2	Neurofibromatosis 2 gene
ng	Nanogram
nm	Nanometer
NO	Nitric oxide
*NRAS*1	Neuroblastoma RAS viral (v-ras) oncogene homolog

NR2F6	Nuclear receptor subfamily 2, group F, member 6
NR2F1	Nuclear receptor subfamily 2, group F, member 1
NRASL1	Neuroblastoma RAS viral (v-ras) oncogene homolog-like 1
NRASL2	Neuroblastoma RAS viral (v-ras) oncogene homolog-like 2
OD	Optical density
OD_{260}	OD at 260 nanometers
OD_{280}	OD at 280 nanometers
p	Significance level
P1	Promoter 1
P2	Promoter 2
p53	Tumor protein p53
$p16^{INK4a}$	Cyclin-dependent kinase inhibitor 2A
$p21^{WAF1}$	Cyclin-dependent kinase inhibitor 1A
PAH	Polycyclic aromatic hydrocarbon
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDGFB	Platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene homolog
Phe	Phenylalanine
PIM1	pim-1 Oncogene
Pro	Proline
Pu	Purine
PVT1	Pvt1 oncogene homolog, MYC activator (mouse)
Py	Pyrimidine
R	Reverse
RAF1	v-raf-1 Murine leukemia viral oncogene homolog 1
RAF1P1	v-raf-1 Murine leukemia viral oncogene homolog 1 pseudogene 1
RALA	v-ral Simian leukemia viral oncogene homolog A (ras related)
Rb1	Retinoblastoma 1 gene
REL	v-rel Reticuloendotheliosis viral oncogene homolog (avian)
RNA	Ribonucleic acid

ROS	Reactive oxygen species
*ROS*1	v-ros UR2 Sarcoma virus oncogene homolog 1 (avian)
rpm	Revolutions per minute
RRAS	Related RAS viral (r-ras) oncogene homolog
S	Synthesis
SCC	Squamous cell carcinoma
SDS	Sodium dodecyl sulphate
SEA	S13 erythroblastosis oncogene homolog (avian)
Ser	Seronine
SKI	v-ski Sarcoma viral oncogene homolog (avian)
SPI	Spleen focus forming virus (SFFV) proviral integration oncogene spi1
*SPI*1	Spleen focus forming virus (SFFV) proviral integration oncogene spi1
SPSS	Statistical Package for the Social Sciences
SRC	v-src Sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)
SSCP	Single strand conformation polymorphism
SV40	Simian virus 40
T	Thymidine
Taq	*Thermus aquaticus*
TBE	Tris, Boric acid, EDTA
TBP	TATA-binding protein
TE	Tris-EDTA buffer
TGFR	Transforming growth factor receptor
*THRA*1	Thyroid hormone receptor, alpha (erythroblastic leukemia viral (v-erb-a) oncogene homolog, avian)
THRB	Thyroid hormone receptor, beta (erythroblastic leukemia viral (v-erb-a) oncogene homolog 2, avian)
Tm	Melting temperature
TNF	Tumor necrosis factor

*TP*53	Tumor protein p53 gene
Trp	Tryptophan
tRNA	Transfer ribonucleic acid
UV	von Hippel-Lindau gene
WT-1	Wilms' Tumor gene
*YES*1	Yamaguchi sarcoma viral (v-yes) oncogene homolog 1
YESP	v-yes-1 Yamaguchi sarcoma viral oncogene homolog pseudogene
UCT	University of Cape Town
V	Volt
W	Watt
WM	White male
μg	Micrograms
μl	Microlitres
μmol	Micromolar
°C/s	Degrees Celsius per second
18S rRNA	Ribosomal ribonucleic acid with a sedimentation coefficient of 18 Svedberg units
28S rRNA	Ribosomal ribonucleic acid with a sedimentation coefficient of 28 Svedberg units

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Chapter 1

Introduction

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"Nobody knows what the cause is, though some pretend they do; it is like a hidden assassin waiting to strike at you. Childless women get it, and men when they retire; it is as if there had to be some outlet for their foiled creative fire"

W.H. Auden

1.1 The History of Cancer

1.1.1 Centuries of Cancer

The word 'cancer', accredited to the "Father of Medicine", Hippocrates (460 – 370 BC), is derived from the Greek words *carcinos* and *carcinoma*, referring to a crab, apparently due to the resemblance between the veins surrounding tumors and the appearance of a crab crawling out from its shell (American Cancer Society ::The History of Cancer 2002; Cancer Association of Southern Africa 2002). Some of the earliest evidence of the presence of cancer comes from human mummies in ancient Egypt and fossilized bone tumors displaying growths suggestive of osteosarcoma and head and neck cancer (American Cancer Society ::The History of Cancer 2002). Cancer has also been recorded in ancient Chinese and Arabic medical manuscripts (Higginson et al. 1992), dating as far back as 1600 BC. The earliest recorded cure for cancer can also be found in the Ramayana, the Hindu epic, where arsenic pastes are recommended for prevention (Through the Years...Brief History of Cancer 1998).

Through the years many theories have existed on the causes of cancer. The ancient Egyptians believed the Gods to be responsible (American Cancer Society ::The History of Cancer 2002). Hippocrates proposed the *Humoral Theory*, whereby it was believed that the body contains 4 *humors* (body fluids): blood, phlegm, yellow bile and black bile. As long as these 4 fluids were in balance, the body was in a natural, healthy state, however, when an imbalance occurred, the consequences included diseases such as cancer (American Cancer Society ::The History of Cancer 2002).

Due to prohibition of autopsies during the Middle Ages very little was known about the function of the human body and consequently, the Humoral theory was believed for more than 1300 years (American Cancer Society ::The History of Cancer 2002). As the years passed, scientists gained more and more knowledge and one theory replaced another, with the list including theories such as the *Lymph Theory*, the *Blastema Theory*, and the *Parasite theory*, which was wrongly awarded a Nobel Prize in 1926 (American Cancer Society ::The History of Cancer 2002).

It was scientists such as Galileo and Newton who were responsible for laying the foundation for scientific research as early as the beginning of the 15th century, during the Renaissance period (American Cancer Society ::The History of Cancer 2002). In 1628, Harvey was the first to perform a novel procedure, known as an autopsy, in order to unravel mysteries such as the circulation of blood throughout the human body (American Cancer Society ::The History of Cancer 2002). Giovanni Morgagni in turn, was the first to perform an autopsy in an attempt to relate cause of death to post-mortem findings (American Cancer Society ::The History of Cancer 2002). Soon after, Rudolf Virchow was the first to implement microscopy in the pathological study of cancer (American Cancer Society ::The History of Cancer 2002). This laid the basis for what is today known as the science of oncology and both these procedures are routinely performed in current medical practices (American Cancer Society ::The History of Cancer 2002).

The first cancer hospital was then founded during the 18th century in Reims, France, based on the belief that cancer was a contagious disease (Through the Years...Brief History of Cancer 1998). However, it was only during the next century that great surgeons such as Bilroth (Germany), Handley (London) and Halsted (Johns Hopkins) (Through the Years...Brief History of Cancer 1998), now renown for their contribution to the science of cancer surgery, laid the basis for

tumor removal as well as surrounding lymph node excision (American Cancer Society ::The History of Cancer 2002). Accredited to the French gynecologist, Josheph Recaimer, metastasis was the term given to the process whereby cancer cells spread throughout the body via the bloodstream, a further complication originally concluded by Paget (Through the Years...Brief History of Cancer 1998).

During the 19th century, Wilhelm Roentgen discovered the x-ray (1895) and only three years later, Pierre and Marie Curie, two French scientists, discovered an element called radium. It was found that this element is capable of discharging intense radiation, which sadly, led to their eventual death (Through the Years...Brief History of Cancer 1998).

But is was Jean Bergonie, a fellow French researcher, who made it possible for 21st century doctors to destroy cancer cells, without damaging the surrounding healthy tissues, by describing the law of radio sensitivity. He too, sacrificed his life for the progression of cancer research and died in 1925, due to cancer caused by his research (Through the Years...Brief History of Cancer 1998). Today cancer treatments included procedures such as hormone therapy, radiation, chemotherapy and immunotherapy (American Cancer Society ::The History of Cancer 2002).

1.1.2 Historical Cancer Epidemiology

The science of epidemiology can be defined as the study of the distribution and variables involved in human diseases and the application of such results to developing control or prevention programs (Higginson et al. 1992).

Three major observations allowed the launch of the field of cancer epidemiology during the 18th century; in 1761, John Hill, was the first to identify tobacco as a carcinogen (any agent that can induce cancer), and in 1775, Percival Pott

demonstrated the relationship between cancer of the scrotum in chimney sweeps and soot collection under their scrotum (Higginson et al. 1992; Bishop et al. 1996; King 1996). Similarly, in 1775, Bernardino Ramazzini observed the total absence of cervical cancer as well as a relatively high incidence of breast cancer in nuns and postulated that it be attributed to their celibate lifestyle (American Cancer Society ::The History of Cancer 2002). In 1915, Yamagiwa and fellow scientists at the Tokyo University managed to induce cancer in laboratory rabbits by applying coal tar to their skin (American Cancer Society ::The History of Cancer 2002; Bishop et al. 1996). In 1968, Peyton Rous from the Rockefeller Institute in New York was awarded a Nobel Prize for describing how the Rous sarcoma virus was responsible for inducing sarcoma in chickens (Higginson et al. 1992; Bishop et al. 1996).

Today, countless numbers of these carcinogens have been identified, with the list including substances such as benzene, certain hydrocarbons, aniline, asbestos, arsenic and many more. A number of viruses have also been identified as participants in the tumorigenic process of certain cancer types (Higginson et al. 1992).

1.2 Defining Cancer Genetically

1.2.1 The Cell Cycle, Oncogenes, Tumor Suppressor Genes and DNA Repair Genes

During the growing years of a child, cells divide at a more rapid pace, to the point where the child becomes an adult (American Cancer Society ::The History of Cancer 2002). Once an adult, cells are no longer required for growth, but rather for maintenance, where new cells replace old or dead cells (American Cancer Society ::The History of Cancer 2002). However, if one cell escapes this control, an entire mass of tumor cells can originate through the cloning of the original progenitor (Bishop et al. 1996; Fialkow 1979; Fearon et al. 1987). Otherwise

stated, cancer cells act in an abnormal fashion by continuously dividing, irrespective of whether there is a requirement for new cells. Cancer can therefore simply be defined as cells that have escaped cell cycle (Figure 1.1) control, or as once stated 'a disease of the cell cycle (American Cancer Society ::The History of Cancer 2002). (It should also be kept in mind that tumors originating from multiple cells do also exist, such as those induced by transforming RNA tumor viruses (Bishop et al. 1996)).

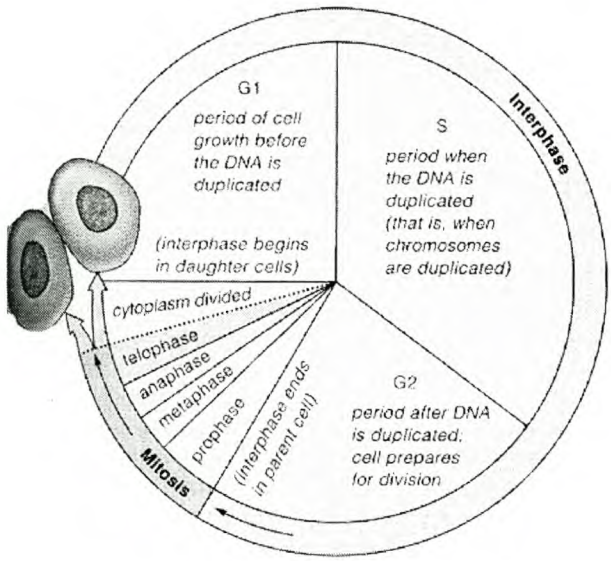

Figure 1.1 The cell cycle starts in the G1 phase and is responsible for the division of a single parent cells into two identical daughter cells. Failure to monitor for DNA damage at checkpoints within the cell cycle will result in the accumulation of mutations, which eventually lead to the development of tumors (Thomas 2003).

Generally, mitosis can be regulated in one of two ways. Firstly, by genes that normally acts to suppress cell division. And secondly, by genes that function to promote cell division. The first class is referred to as tumor suppressor genes and represses cell division/proliferation (Table 1.1) (Steele et al. 1998; Bishop et al. 1996; Pergament and Fiddler 2001c). The protein products of these tumor suppressor genes must be absent or inactive for normal cell division to occur. Once these tumor suppressor genes or their protein products become permanently inactive, cell cycle control is lost and the cells proliferate in an uncontrolled fashion, resulting in a malignancy (Klug and Cummings 1997; Macdonald and Ford 1991; Steele et al. 1998; Pergament and Fiddler 2001c). In the case of mutations occurring in such tumor suppressor genes, one mutant allele is not sufficient for manifestation and the normal allele generally acts in a

dominant fashion (Steele et al. 1998; Pergament and Fiddler 2001c). It can therefore be said that only when loss-of-function mutations occur in such tumor suppressor genes, are they involved in tumorigenesis (Table 1.2) (Bishop et al. 1996; Pergament and Fiddler 2001c). An example of such a tumor suppressor gene is *TP*53 (Table 1.1) (Steele et al. 1998; Imazeki et al. 1992; Hinds et al. 1989; Eliayahu et al. 1984; Pergament and Fiddler 2001c). The second class of cell division promoting genes associated with the regulation of cell growth and differentiation is called proto-oncogenes (Steele et al. 1998; Pergament and Fiddler 2001b). Proto-oncogene products can usually be categorized into four types: growth factors, growth factor receptors, intracellular signal transducers and transcription factors (Bishop et al. 1996; Macdonald and Ford 1991; Klug and Cummings 1997). These genes occur in an "on/off" state and need to be in an "on" state in order to promote cell division. If proto-oncogenes become permanently switched "on", permanent cell division/differentiation occurs and these genes are then referred to as oncogenes (Klug and Cummings 1997; Bishop et al. 1996; Ross 1998; Steele et al. 1998; Pergament and Fiddler 2001b). There are several mechanisms of proto-oncogene conversion to oncogene, for example, the gene sequence could be altered by mutation, which may code for a protein with abnormal activity. Another example of proto-oncogene conversion to oncogene is with the incorporation of viral genomes, often placing these genes under the control of foreign promoters or enhancers, which my lead to excess or inappropriate production of the product (Alberts et al. 1994; Lewin 1994; Pergament and Fiddler 2001b). An oncogene typically acts in a dominant fashion so that a mutation in only one allele is sufficient for manifestation (Table 1.2) (Steele et al. 1998; Bishop et al. 1996; Klug and Cummings 1997; Pergament and Fiddler 2001b). To date, over 50 oncogenes have been identified to be associated with several cancer types (Table 1.3) (Klug and Cummings 1997; Macdonald and Ford 1991).

Table 1.1 Tumor Suppressor Genes and their functions

Cancer Syndrome	Gene	Primary Tumors	Cellular Location	Mode of Action
Li-Fraumeni	*TP53*	Sarcomas, breast and brain tumors	Nucleus	Transcription factor/regulator
Rentinoblastoma	*Rb*	Retinoblastoma, osteosarcoma	Nucleus	Transcriptional Regulator
Familial adenomatous polyposis	*APC*	Colon cancer	Cytoplasm	Unknown
Neurofibromatosis type 1	*NF-1*	Neurofibromas	Cytoplasm	$p21^{ras}$.GTPase activator
Neurofibromatosis type 2	*NF-2*	Schwannomas, meingniomas	Inner membrane	Cytoskeleton-membrane link
Wilms' Tumor	*WT-1*	Nephroblastoma	Nuclues	Transcription factor?
von Hippel-Lindau	*VHL*	Renal cell Carcinoma	Cytoplasm?	Inhibits transcription elongation
Familial breast cancer	*BRCA1*	Breast, ovary	Nucleus?	Transcription factor?
	BRCA2	Breast cancer	Unknown	Unknown
Colorectal cancer	*DCC*	Colon cancer	Membrane	Cell adhesion Molecule
Familial melanoma	*p16*	Melanoma, many Others	Nucleus	Cyclin-dependent kinase inhibitor
Hereditary nonpolyposis colon cancer	*hMSH2*, *hMLH1*, *hPMS1*, *hPMS2*	Colon cancer	Nucleus	Nucleotide mismatch repair

Table 1.2 Properties of Tumor Suppressor Genes and Proto-oncogenes

Property	Tumor Suppressor Genes	Proto-oncogenes
Alleles mutated in cancer	Both alleles	One allele
Germ line transmission of mutant allele	Frequently seen	Rare
Somatic mutation involved in tumor formation	Yes	Yes
Function of mutant allele(s)	Loss of function (recessive)	Gain of function (dominant)
Effects on cell growth	Inhibits cell growth	Promotes cell growth

Table 1.3(a) Human oncogenes and their chromosomal location

Oncogene	Chromosomal location	Oncogene	Chromosomal location
ABL	9q34	*KRAS2*	12P12.1
ABLL	1q24-q25	*LCO*	2Q14
AKT1	14q32.3	*LYN*	8Q13-qter
ARAF1	Xp11.4-p11.2	*MAS1*	6q24-q27
ARAF2	7p14-q21	*MEL*	19p13.2-cen
BCL2	18	*MET*	7q31-q32
CSF1R	5q33-q34	*MOS*	8q11 or 8q21-q23
EGFR	7p13-p12	*MYB*	6q22-q23
ELK1	Xp22.1-p11	*MYC1*	8q24
ELK2	14q32.3	*MYCL1*	1p32
ERBA2L	17q21-q22	*MYCN*	2p24
ERBAL2	19	*NRAS1*	1p22 and/or 1p13
ERBAL3	5	*NRASL1*	9p
ERBB2	17q11-q12	*NRASL2*	22
ERG	21q22.3	*PDGFB*	22q12.3-q13.1
ETS1	11q23.3	*PIM1*	6p21
ETS2	21q22.3	*PVT1*	8q24
FES	15q25-qter	*RAF1*	3p25
FGR	1p36.2-p36.1	*RAF1P1*	4p16.1
FOS	14q24.3	*RALA*	7p22-p15
GLI	12q13	*REL*	2p13-cen
HRAS	11p15.5	*ROS1*	6q21-q22
HRASP	Xpter-q26	*RRAS*	19
HRAS2P	Xp11.4-p11.2	*SEA*	11q13
HSTF1	11q13.3	*SKI*	1q21.1-q24
INT1	12q13	*SPI1*	11p12-p11.2
INT2	11q13	*SRC*	20q12-q13
INT4	17q21-q22	*THRA1*	17q11-q12
JUN	]1p32-p31	*THRB*	3p24.1-p22
KIT	4p11-q22	*YES1*	18q21.3
KRAS1P	6p12-p11	*YESP*	22q11-q12

Table 1.3(b) Human tumors associated with certain oncogenes

Tumor	Associated oncogenes(s)
Bladder	*HRAS, KRAS*
Brain	*ERBB*
Breast	*ERBB, HRAS, MYC*
Cervical	*MYC*
Colorectal	*HRAS, KRAS, MYB, MYC*
Gastric	*ERBB, MYB, MYC, NRAS, YES*
Lung	*ERBB, HRAS, KRAS, MYC, MYCL, MYCN*
Melanoma	*HRAS*
Neuroblastoma	*MYCN*
Ovarian	*ERBB, KRAS*
Pancreas	*KRAS, MYC*
Prostate	*MYC*
Testicular	*MYC*

In addition to proto-oncogenes and tumor suppressor genes, genes involved in the DNA repair mechanism are vital in the process of inhibiting tumorigenesis. When DNA damage is detected at critical checkpoints, the cell cycle comes to a halt. In order to prevent the proliferation of the detected damage, DNA repair mechanisms come into play. The normal functioning of the genes involved in these repair mechanisms are essential and it is clear that when these genes themselves are damaged, the cells are incapable of any DNA repair. Defects in genes coding for proteins that regulate DNA repair have recently been identified as the causative factor in syndromes such as xeroderma pigmentosum, Bloom's syndrome, ataxia telangiectasia and Fanconi's anemia, which have all been linked with an increased risk for the development of leukemia and lymphoma (Macdonald and Ford 1991; King 1996; Cleaver and Karentz 1987; Pergament and Fiddler 2001a).

1.2.2 Types of Cancer

Generally speaking, all cancers can be divided into 5 major groups: carcinomas, sarcomas, leukaemias, lymphomas and myelomas. Carcinomas are the most common form of cancer, and originate in tissues covering a surface or lining the internal organs. Sarcomas are known to develop in connective tissue and therefore affect bones and soft tissues. Leukaemias occur in the blood and blood-forming tissues. Lymphomas affect the lymphatic system and myelomas originate in the plasma cells of bone marrow (Cancer Association of Southern Africa 2002). These can then be further subdivided into 3 categories, defined by the highly variable nature of an individual's genetic makeup, lifestyle and external environment. The first category is heritable cancers, where the individual's genetic background is the major contributor and the environment is largely uninvolved. Such cancers are usually found to have an earlier onset than non-inheritable cancers. The second category can be described as cancers occurring in individuals genetically predisposed i.e. those with an increased risk for developing a certain type cancer under the appropriate conditions. The third category is that where the cancer is mainly induced by environmental agents, e.g. lung cancer as a result of smoking, or bladder cancer as a result of aromatic amine exposure (Higginson et al. 1992). However, recent studies suggest that a genetic component is also involved in this last category, resulting in a modification of the cancer.

It is therefore widely accepted that cancer is mostly likely the consequence of a multi-factorial, multi-step process, with the outcome being the reflection of a individuals lifetime-lifestyle (Sur and Cooper 1998; Chang et al. 1992; Bishop et al. 1996; Ross 1998; Imazeki et al. 1992;).

Nonetheless, one thing remains common to all 3 of these categories: DNA damage remains the basis for tumorigenesis (American Cancer Society ::The History of Cancer 2002). This damage includes phenomena such as point

mutations, gene insertions, gene activation and repression. When these damaged cancer cells make their way into the bloodstream or lymph vessels, they are capable of spreading to other parts of the body, away from the site of primary tumor development (American Cancer Society ::The History of Cancer 2002). This process is known as metastasis and allows for these cancer cells to replace normal tissue elsewhere, leading to the formation of secondary tumors (American Cancer Society ::The History of Cancer 2002).

1.3 Causes of Cancer

1.3.1 DNA Damage

Epidemiological data collected over the past two centuries has proven that external factors are responsible for the development of most cancers today (Bishop et al. 1996; Wynder and Gori 1977; Doll and Peto 1981; Henderson et al. 1991; Ames et al. 1995). The common end result of any form of DNA damage is a disturbance in the genetic material carried within the genome. DNA damage to any one of a host of genes could potentially result in the initiation of tumorigenesis; genes involved in cell cycle control, DNA repair, tumor suppressing, chaperoning of other proteins, cell wall structure and integrity, detoxification of carcinogens, the list is endless.

There are six main types of damage experienced by DNA; oxidation, radiation, UV light, misinterpreted codes, cytosine methylation and carcinogens (King 1996). Firstly, oxidation of guanine by reactive oxygen species (ROS) produces 8-hydroxyguanine, which is eventually replaced by thymine during the next round of DNA synthesis, resulting in a base pair substitution. ROS are formed as by-products of mitochondrial electron transport or by radiation exposure. ROS can also be produced from other sources such as phagocytic cells and lipid metabolism. Other oxidizing agents such as nitric oxide found in tobacco smoke are also capable of forming ROS (King 1996). Secondly, radiation is responsible

for the formation of strand breaks which eventually result in deletions (King 1996). Thirdly, UV light has the ability to form cross-links between pyrimidines leading to thymidine replacements (King 1996). Fourthly, during normal DNA synthesis, the genetic code is very often misinterpreted. This can happen if the wrong concentration of a specific nucleotide is present and incorporates the incorrect base or when the DNA polymerase macromolecule slips on the template and incorporates additional bases (King 1996). Fifthly, during normal cellular differentiation, genes are switched off by the methylation of cytosines, a process that is catalyzed by C-DNA methyltransferases (Mtases). Such methylated cytosines are capable of being deaminated to uracil, which is an abnormal constituent of DNA. DNA polymerase therefore treats this as a thymidine and the end result is a thymidine substitution for a guanine (King 1996). Although it is not yet fully understood exactly how cytosine methylation results in DNA damage, oxygen radicals have thusfar been suggested as being involved . However, the largest contributor to DNA damage is the sixth group, namely carcinogens.

1.3.2 Carcinogens

We live in world where the universe of chemicals is believed to consist of 9 to 10 million compounds, with more than 60,000 being in general commercial use (Higginson et al. 1992). Many of these chemicals fall in the category of carcinogens, and can occur in association with the work place, drug therapy, air and water pollution as well as contaminants in food and beverages, such as herbicides and pesticides (Higginson et al. 1992). All carcinogens can broadly be classified into one of two groups; genotoxic and non-genotoxic. Agents that directly damage DNA are classified as genotoxic carcinogens, whereas those that exert their carcinogenic effect in any other way are non-genotoxic agents (King 1996). Often, the genotoxic chemical must first be converted to the harmful state by the incorporation of epoxide or hydroxyl groups, which is catalyzed by cytochrome P450-dependant enzymes. The general reaction involves the

conversion of the initial carcinogen to a proximate carcinogen and finally to the ultimate carcinogen, which then reacts with the DNA (Figure 1.2) (King 1996).

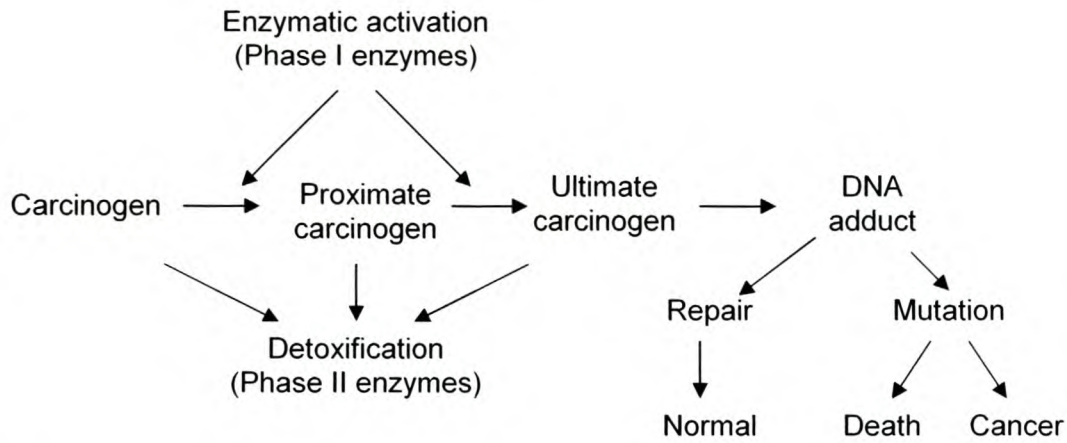

Figure 1.2 The conversion of a carcinogen into a DNA adduct (King 1996).

Under the category of genotoxic carcinogens, four major classes of compounds exert their effects by forming covalent adducts with bases of DNA; the polycyclic aromatic hydrocarbons (PAHs), the aromatic amines, nitrosamines and alkylating agents. These carcinogens can be found in a number of places, in everyday life; PAHs are generated in tobacco, whisky, grilled meat and by incomplete combustion of fossil fuels; aromatic amines were used in dyes and in the rubber industry as well as in margarine production (however, such uses are banned today); nitrosamines can be found in smoked meats and fish, and alkylating agents, such as mustard gas, were used during the first world war. The common feature to all these compounds is that they all contain electrophilic groups or groups capable of being metabolically converted to electrophilic. Such groups then form covalent bonds with nucleophilic groups, such as amino, sulphydryl and hydroxyl groups, which in turn, can be found as part of macromolecules such as DNA and RNA. In normal cells, DNA occurs in the double stranded form, however, in proliferating cells, the two DNA strands separate in preparation for DNA synthesis, and it is during this particularly vulnerable phase that these

compounds attack. The resulting complex between DNA and carcinogen, known as a DNA adduct, distorts the DNA to such an extent that if DNA repair is not possible, an incorrect base (mutation) is incorporated.

The non-genotoxic carcinogens include agents such as hormonal steroids, which do not directly damage DNA, but act as tumor promoters. These hormones are mitogens, which increase cell proliferation by binding to transcription factors. DNA occurs naturally in the double stranded form, but when cell proliferation is stimulation (by mitogens), the DNA enters the single-stranded form more often, thus exposing the bases to the actions of genotoxic carcinogens more often (King 1996). A list of some of the known carcinogens can be found in Tables 1.4(a) to 1.4(c) (Higginson et al. 1992).

Table 1.4(a) Chemicals or occupations recognized as representing a carcinogenic hazard to humans

Ambient exposures	Magenta, manufacturing of
Arsenic an arsenic compounds	Mineral oils, untreated and mildly treated
Erionite	Mustard gas (Sulfur gas)
Cultural habits	2-Naphthylamine
Betel quid with tobacco	Nickel and nickel compounds
Tobacco products, smokeless	Rubber industry
Tobacco smoke	Shale oils
Ethanol	Soots
Maté	Vinyl chloride
Dietary contaminants	**Therapeutic use**
Aflotoxins	Analgesic mixtures containing phenacetin
Arsenic and arsenic compounds	Azathioprine
Occupational	1,4-Butanediol dimethane sulphonate (Myleran 1-(2-Chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea
Aluminium production	
4-Aminobiphenyl	Cyclophosphamide
Auramine, manufacture of	Diethylstilbestrol
Benzene	Melphalan
N,N-Bis(2-Chloroethyl)-2-naphthylamine	8-Methoxypsoralen plus ultra violet radiation
Bis ether and chloromethyl methyl ether	MOPP (combined therapy with nitrogen mustard, vincristine procarbazine and prednisone) and other combined chemotherapy including alkylating agents
Boot and shoe manufacture and repair	
Chromium compounds, hexavalent	
Coal gasification	
Coal-tar pitches	
Coal-tars	
Coke production	Estrogen replacement therapy
Furniture and cabinet-making	Estrogen, steroidal
Hematite mining, underground with exposure to radon	Oral contraceptives, combined oral contraceptives, sequential
Iron and steel founding	Treosulphan
Isopropyl alcohol manufacture, strong acid process	

Table 1.4(b) Chemicals recognized as representing a probable carcinogenic hazard to humans

Acrylonitrile	Formaldehyde
Adrianmycin	5-Methosypsoralen
Androgenic steriods	4,4'-Methylene *bis* (2-chloroaniline)
Benz[*a*]anthracene	*N*-Methyl-*N*'-nitrosoguanidine
Benzidine-based dyes	Nitrogen mustard
Beryllium and beryllium compounds	*N*-Nitrosodiethylamine
*Bis*chloroethyl nitrosourea	*N*-Nitrosodiemethylamine
Cadmium and cadmium compounds	Phenacetin
1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea	Polychlorinated biphenyls
Cisplatin	Probacarbazine hydrochloride
Creosotes	Propylene oxide
Dibenz[*a*,*h*]anthracene	Silica, crystalline
Diethyl sulphate	Styrene oxide
Dimethyl sulphate	Tris(1-aziridinyl)phosphine sulphine
Epichlorohydrin	Tris(2,3-dibromopropyl) phosphate
Ethylene bromide	Vinyl bromide
Ethylene oxide	
N-Ethyl-*N*-nitrosourea	

Table 1.4(c) Chemicals or occupations recognized as representing a possible carcinogenic hazard to humans

A-a-C (2-Amino-9*H*-pyrideo[2,3-b]indole)	1,2-Dichloroethane
Acetaldehyde	Dichloromethane
Acetamide	1,3-Dichloropropene
AF-2[2-(2-Furyl)-3-(5-nitro-2-furyl)]	Diepoxybutane
acrylamide	Di(2-ethylhexy)phthalate
para-Aminoazobenzene	1,2-Diethylhydrazine
ortho-Aminoazotoluene	Diglycidyl resorcinol ether
2-Amino-5-(5-nitro-2-furyl)1,3,4thiadiazole	Dihydrosafrole
Amitrole	3,3'-Dimethoxybenzidine
ortho-Anisidine	*para*-Dimethylaminoazobenzene
aramite	*trans*-2-[(Dimethylamino)-methylimino]-5-[2-(5-nitro-2-furyl)vinyl]1,3,4-oxadiozole
Auramine	3,3'Dimethylbenzidine
Azaserine	1,1-Dimethylhydrazine
Benzo[*b*]fluoranthene	1,2-Dimethylhydrazine
Benzo[*j*]fluoranthene	1,4-Dioxane
Benzo[*k*]fluoranthene	Ethyl acrylate
Benzyl violet 4*B*	Ethylene thiorurea
Butamens, extracts of steam and air-refined	Ethyl methanesulphonate
Bleanmycins	2-(2-Formylhydrazino)-4-(5-nitro-2-furyl)thiazole
Bracken fern	Glu-P-1 (2-Amino-6-methyldipyrdio[*1,2a:3',2'-d*]imidazole
1,3-Butadiene	Glu-P-2 (2-Aminodipyrdio[*1,2-a:3',2'-d*]imidazole
Butylated hydroxyanisole	Glycidaldehyde
B-Butyrolactone	Hexachlorobenzene
Carbon-black extracts	Hexachlorocyclohexanes
Carbon tetrachloride	
Carpentry and joinery	
Carrageenan, degraded	
Chloramphenicol	
Chlorodecone	
Chlorophenols	

Continued...

Chlorophenoxy herbicides
4-Chloro-*ortho*-phenylenediamine
para-Chloro-*ortho*-toluidine
Citrus Red No.2
para-Cresidine
Dicarbazine
Daunomycin
DDT
N,N'-Diacetylbenzidine
2,4-Diaminoanisole
4,4'-Diaminodiphenyl ether
2,4-Diaminotoluene
Dibenz*[a,h]*acridine
7H-Dibenzo[*c,g*]carbazole
Merphalan
2-Methylaziridine
Methylazoxymethanol and its acetate
5-Methylchrysene
4,4'-Methylene *bis*(2-methylaniline)
4,4'-Methylenedianiline
Methyl methanesulphonate
2-Methyl-1-nitroanthraquinone
N-Methyl-*N*-nitrosourethane
Methylthiouracil
Metronidazole
Mirex
Mitomycin C
Monocrotaline
5-(Morpholinomethyl)-3-[(5-nitro-furfurylidene)]amino-2-imidazolidinone
N-[4-(5- Nitro-2-furyl)-2-thiaxolyl]acetamide
Nitrogen mustard *N*-oxide
Hexamethylphosphoramide
Hydrazine
Indeno[*1,2,3-cd*]pyrene
IQ (2-Amino-3-metylimidazo [*4,5-f*]quinoline
Iron-dextran complex
Lasiocarpine
Lead and lead compounds, inorganic
MeA-a-C (2-Amino-3-methyl-9H-pyrido[*2,3,b*]indole)
Medroxyprogesterone acetate
N-Nitrosopiperdine
N-Nitrosopyrrolidine
N-Nitrososarcosine
Oil Orange SS
Panfuran S (containing dihydroxymethylfuratrizine)
Phenazopyridine hydrochloride
Phenobarbitol
Phenoxybenzamine hydrochloride
Phenytoin
Polybrominated biphenyls
Ponceau MX
Ponceau 3R
Potassium bromate
Progestins
1,2-Propane sultone
β-Propiolactone
Propylthiouracil
Saccharin
Safrole
Sodium *ortho*-phenylphenate

Continued...

2-Nitropropane
N-Nitrosodi-n-butylamine
N-Nitrosodiethanolamine
N-Nitrosodi-n-propylamine
3-(*N*-Nitrosomethylamino)propionitrile
4-(N-Nitrosomethylamino)-1-(3-pyridyl)-1-butanone
N-Nitrosomethylethylamine
N-Nitrosomethylvinylamine
N-Nitrosomorpholine
N'-Nitrosonornicotine
Dibenzo[*a,e*]pyrene
Dibenzo[*a,h*]pyrene
Dibenzo[*a,i*]pryene
Dibenzo[*a,l*]pryene
1,2-Dibromo-3-chloropropane
para-Dichlorobenzene
3,3'-Dichlorobenzene
3,3-Dichloro-4,4'-diaminodipheyl ether
Sterigmatocystin
Streptozotocin
Styrene
Sulfallate
2,3,7,8-Tetrachlorodibenzo-para (TCDD)
Tetrachloroethylene
Thioacetamide
4,4'-Thiodianiline
Thiourea
Toluene diisocyanates
ortho-Toluidine
Toxa (Polychlorinated camphenes)
Trp-P-1 (30Amino-1,4-dimethyl-5H-pyrido[*4,3-b*]indole)
Trypan blue
Uracil mustard
Urethane

Rofo first demonstrated the carcinogenic qualities of tobacco tar in the 1920's, and the first epidemiological data confirming smoke as a lung cancer carcinogen came from Müller shortly after, in 1939 (Higginson et al. 1992). Today it is known that cancers caused by smoking include lung, oral cavity, pharynx, larynx, esophagus, urinary bladder, renal pelvis and pancreas, with lung cancer being the most prevalent (Higginson et al. 1992; IARC 1995). It is known that cigarette smoking accounts for more cancer deaths that any other known factor (Higginson et al. 1992), and even side-stream tobacco smoke, known as passive smoking or second-hand smoking, may pose a cancer risk (O'Neill et al. 1987; Higginson et al. 1992).

Cigarette smoke has been defined as a highly complex mixture of a large number of chemicals, with more than 3 000 already having been identified (Table 1.5) (Higginson et al. 1992).

Table 1.5 Several of the biologically active agents found in mainstream smoke[a]

Smoke constituent	Biological Effect
Total particulate matter	T, HC
Carbon monoxide	T
Nicotine	T
Acetaldehyde	CT
Acetone	CT
NO_x	T
Formic Acid	CT
Hydrogen cyanide	CT,T
Catechol	CoC
Ammonia	T
Benzene	HC
Acrolein	CT
Acrylonitrile	C
Phenol	TP
Formaldehyde	C
Carboxole	C?
2-Nitroporpane	C
N'-Nirosonornicotine	C
4-(Methylnitrosamino)1-(3-pyridyl)-1-butanone	C
N'-Nirosonabasine	C?
N-Nirosodiethanolamine	C
N-Nirosophyrrolidine	C
N-Nirosodimethylanime	C
N-Niroso diethylamine	C
N-Nirosodi-n-propylamine	C
N-Nirosodi-n-butylamine	C
N-Nirosopiperidine	C
N-Nirosopyrrolidine	C
Hydrazine	C
Urethane	C
Vinyl chloride	C

Continued...	
Benz[*a*]anthracene	HC
Benzo[*a*]pyrene	C
5-Methylachrysene	C
Dibenz[*a,j*]acridine	C
2-Naphthylamine	C
4-Aminobiphenyl	HC
2-Toluidine	HC

[a] Quantitative data refer to non-filter cigarettes

T = toxic-agents

HC = human carcinogen

CT = ciliatoxic agent

CoC = co-carcinogen

TP = tumor promoter

C = animal carcinogen

It is due to this chemical complexity that it has thus far been impossible to identify a single agent as being responsible for inducing tumorigenesis (Higginson et al. 1992). However, considerable progress has been made in identifying the compounds most likely to have such carcinogenic properties and the effects of tobacco smoke and tobacco tar have been intensely studied in animal models (O'Neill et al. 1987; Hecht and Hoffman 1989; Higginson et al. 1992). It has also been shown that approximately 80% of the tar inhaled from mainstream smoking is deposited within the respiratory tract (Higginson et al. 1992).

The damaging effect of smoking is further complicated by the fact that alcohol, which has also been found to have carcinogenic properties, acts as an accomplice to smoke. This is further aggravated by the observation that many smokers tend to consume excess amounts of alcohol (Higginson et al. 1992). It has been noted that many cancers of the oral cavity, pharynx, esophagus and larynx, occur among individuals working in the alcoholic beverage industry (Higginson et al. 1992; Clemmesen 1941). This was confirmed by the observation that residents of Normandy in France, who are heavy drinkers of

distilled apple cider show and unusually high incidence of esophageal cancer. In contrast, religious and other groups against the consumption of alcohol have shown lower frequencies of these cancers (Phillips et al. 1980; Enstrom 1980). It comes as no surprise then that the two predominant carcinogens considered to be the major contributors to the development of both esophageal cancer as well as laryngeal cancer are tobacco smoking and alcohol consumption (Osborne et al. 1997; Schottenfeld et al. 1974; Saunders et al. 1999; Jussawalla and Deshpande 1971; Gorgoulis et al. 1994; Hirvikoski et al. 1997; Rowley et al. 1998; The Voice Centre at Eastern Virginia Medical School - The Larynx 2001; Altieri et al. 2002; Bagnardi 2001; Tuyns and Audigier 1976; Chilvers et al. 1979; Segal et al. 1988; Hollstein et al. 1991b; Negri et al. 1992; Tavani et al. 1993; Muñoz and Castellsaguè 1994; Franceschi et al. 1994; Klimstra 1994; Montesano et al. 1996; Castellsaguè et al. 1999; Pacella-Norman et al. 2002; Levi et al. 2000; Zambon et al. 2000; Gallus et al. 2001; Higginson et al. 1992; Peto 2001).

1.3.3 Diet

In addition to those cancers inherited or those induced through previously mentioned mutagens, it is known that one third of cancers in the United States can be accounted for by an unbalanced diet. This section will discuss only 2 examples. It has been found that the intake of fruit is indirectly correlated to the occurrence of cancer. This protective effect could possibly be due to the high levels of antioxidants present in such foods, which, in correct doses are believed to have beneficial qualities. A decreased calorie intake has also been shown to significantly decrease tumor incidence in animal studies. This could possibly be due to the fact that calorie intake rate is linked to the mitotic rate of some tissue types. In other words, diet can affect cancer susceptibility and protection by either stimulating cell proliferation, or by providing advantageous antioxidants respectively (Bishop et al. 1996).

1.3.4 Viruses

For more than 30 years, it has been known that DNA viruses such as SV40 and RNA viruses such as Rous sarcoma, are capable of transforming those cells infected to a cancerous state (American Cancer Society ::The History of Cancer 20020; Plachy et al. 2001; Macdonald and Ford 1991; Vilchez et al. 2003; Garcea and Imperiale 2003; Diamandopoulos 1972; Eddy 1962; Eddy et al. 1961; Girardi et al. 1963; Girardi et al. 1962; Rubin 2003). Other examples include the hepatitis virus, which is associated with liver cancer (Hiraoka et al. 2003; Boldogh et al. 1983; Hashiro et al. 1979; Sanford et al. 1977; Huang and Roche 1978). A number of the viruses belonging to the human herpes family have been incriminated as etiological factors for several human cancer types, including cervical carcinoma, prostate and colon adenocarcinoma as well as Kaposi's sarcoma (McDougall et al. 1980; McDougall et al. 1984; Rawls et al. 1977; Zafiropoulos et al. 2003; Pacsa et al. 1975; Stoian et al. 1982; Boldogh et al. 1983; Sanford et al. 1977; Hashiro et al. 1979; Huang and Roche 1978; Boldogh et al. 1981; Giraldo et al. 1980; Giraldo et al. 1978). The Epstein-Barr virus has also been implicated in nasopharyngeal cancer and African Burkitt's lymphoma (Zafiropoulos et al. 2003; Higginson et al. 1992; zur Hausen et al. 1970). The latest epidemic, human immunodeficiency virus (HIV) is also known to be associated with an increased risk for the development of cancers such as Kaposi's sarcoma (Newton et al. 2003). Cancers of the penis, vulva and cervix have also been associated with the human papilloma viruses (HPVs) (Buffet et al. 2002; Waggoner 2003; Higginson et al. 1992; Rodriguez-Pinilla et al. 2003; Canavan and Cohen 2002).

Furthermore, retroviruses are also known to activate proto-oncogenes by a process known as insertional mutagenesis, whereby the insertion of a DNA copy of the retrovirus into the host genome close enough to a proto-oncogene, is sufficient to induce abnormal activation of that gene, resulting in malignancy.

1.4 Laryngeal Cancer

The larynx, also known as the voicebox, is situated in the neck and plays a central role in speech as well as breathing. It is at this point of the throat that the common aerodigestive tract splits into the trachea (for breathing) and the esophagus (for swallowing) (The Voice Centre at Eastern Virginia Medical School - The Larynx 2001). Furthermore, the vocal chords (vocal folds) are positioned in the center of the larynx (The Voice Centre at Eastern Virginia Medical School - The Larynx 2001). Cancer of the larynx (Figure 1.3), which is sometimes simply referred to as throat cancer, falls in the category of head and neck cancers.

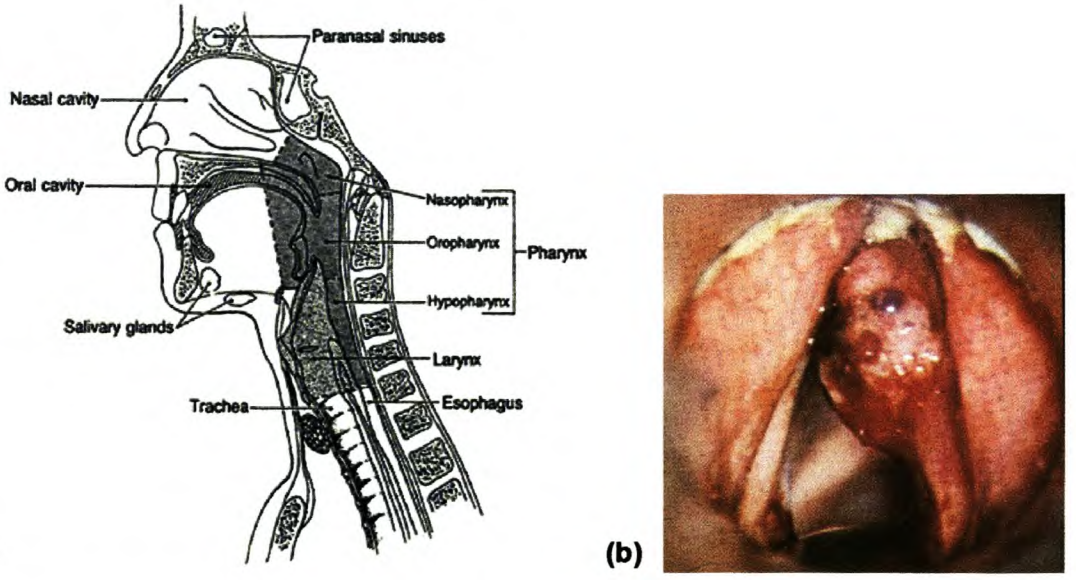

Figure 1.3 The larynx (a) The larynx is situated where the common aero-digestive tract divides into the trachea (for breathing) and the esophagus (for swallowing) (INTERNATIONAL ASSOCIATION OF LARYNGECTOMEES 2003). **(b)** A photo of a typical laryngeal cancer tumor seen on the right vocal chord (Lutte contre le Tabac

This is the sixth most common cancer type in the world (Vokes et al. 1993), and after lung cancer, it is the second most common type of respiratory cancer (Cattaruzza et al. 1996), affecting approximately 500 000 new individuals annually (Dahse et al. 1999). Nearly all are squamous cell carcinoma (SCC) (Higginson et al. 1992; Gorgoulis et al. 1994; Batsakis 1979), a type of cell that lines a portion of the larynx and when a malignant tumor arises within this lining, it is referred to as laryngeal squamous cell carcinoma (The Voice Centre at Eastern Virginia Medical School - The Larynx 2001). Typical symptoms of laryngeal cancer (LC) include throat pain, hoarseness, ear pain, breathing difficulty, coughing blood and lumps in the neck (The Voice Centre at Eastern Virginia Medical School - The Larynx 2001). Commonly recognized prognostic factors include the site of the primary tumor, the stage and histological grade of the tumor, and the sex of the patient. A combination of these factors determines the appropriate therapy for each individual. To date, the leading cause of treatment failure, specifically after surgical removal, remains recurrences due to the presence of tumorigenic cells within the surgical margins and the cervical lymph nodes (Gasparotto et al. 1995). Treatments of LC include radiation therapy, chemotherapy and surgical procedures such as a laryngectomy, where the larynx is partially or totally removed (The Voice Centre at Eastern Virginia Medical School - The Larynx 2001). Total removal of the larynx renders the patient incapable of what is known as laryngeal speech. In such cases, the patient has a choice of three modes of alaryngeal speech; electrolaryngeal speech, esophageal speech and tracheo-esophageal speech (The Voice Centre at Eastern Virginia Medical School - The Larynx 2001).

Even in high incidence populations, LC is very unusual before the age of 40 and has been found to be rarer in females than in males (The Voice Centre at Eastern Virginia Medical School - The Larynx 2001; Cattaruzza et al. 1996). Generally, the overall 5-year survival rate for LC patients is approximately 60% (Higginson et al. 1992; Rowley et al. 1998; Snow 1990). It has been shown that within the South African population, LC occurs predominantly in the Coloured

community (Hille et al. 1996) (The Coloured population is of mixed ancestry, consisting mainly of Khoi origins, but also including Caucasian, African Malay and San origins (Marais 1957)). Although there is little genetic information concerning the linkage between xenobiotic agents and LC, there is strong evidence that smoking and alcohol consumption (Osborne et al. 1997; McCoy and Wynder 1979; Schmidt and Popham 1981; Tuyns 1991; Wynder and Stellman 1977; Schottenfeld et al. 1974; Saunders et al. 1999; Jussawalla and Deshpande 1971; The Voice Centre at Eastern Virginia Medical School - The Larynx 2001; Altieri et al. 2002; Bagnardi 2001; Gorgoulis et al. 1994; Rowley et al. 1998; Hirvikoski et al. 1997) are involved in the development of LC, and together account for 80-90% of all LC (Higginson et al. 1992). This has been further confirmed by studies done in the UK where it was shown that LC is common among occupations such as barmen where constant exposure to both alcohol and tobacco smoking is inevitable (Higginson et al. 1992).

Furthermore, there is evidence that exposure to asbestos and nickel as well as certain aspects of ethanol production and mustard gas manufacturing are risk factors in the development of LC (Higginson et al. 1992). Although it has never been confirmed, the possible role of a virus (e.g. HPV I and II) in the development of LC cannot be excluded (Higginson et al. 1992; Barnard et al. 2003; Dyson et al. 1989; Scheffner et al. 1990; Nadal and Cardesa 2002; Brandsma and Abramson 1989; Brandwein et al. 1993; Pérez-Ayala et al. 1990; Gorgoulis et al. 1999; Lindeberg and Krogdahl 1999).

As mentioned earlier, tobacco smoke is one of the main culprits in the development of LC and most tobacco-related carcinogens are activated by phase I enzymes to the ultimate reactive intermediate. This ultimate reactive intermediate is then capable of binding and damaging DNA directly, as explained in section 1.3.2. It is clear then that defects in phase I activation enzymes could confer increased susceptibility to the development of LC. The same argument could be applied to phase II detoxification enzymes. Examples of such metabolic

enzymes that either have been or are currently under investigation with respect to LC susceptibility are glutathione-S-transferases (GST's), microsomal epoxide hydrolase (EH) and cytochrome P450 (CYP) (To-Figueras et al. 2002; Strange et al. 1998; Jahnke et al. 1995; Hong et al. 2000; Jourenkova et al. 1998; Gronau et al. 2003; Jahnke et al. 1997).

Countless other studies have been conducted to determine the involvement of genes that possibly confer increased susceptibility to the development of LC. A summary of some of the most frequently investigated genes to date, in relation to laryngeal cancer development or susceptibility, can be found in Table 1.6, accompanied by their references.

Table 1.6 A summary of the most frequently investigated genes in relation to LC development or susceptibility (Nadal and Cardesa 2002)

Gene	Reference(s)
c-myc	(Marcu et al. 1992; Dolcetti et al. 1991; de la Guardia et al. 2001; Fracchiolla et al. 1995; Haughey et al. 1992)
EGFR	(Irish and Bernstein 1993; Scambia et al. 1991; Almadori et al. 1999)
HRAS, KRAS, NRAS	(Anwar et al. 1993; Shidara et al. 1994; Fracchiolla et al. 1995; Cazorla et al. 1998; Saranath et al. 1991; Yarbrough et al. 1994; Scambia et al. 1994)
*Cyclin D*1	(Somers et al. 1990; Berenson et al. 1989; Jares et al. 1994; Dong et al. 2001)
Cyclin E	(Dong et al. 2000)
Rb	(Scholnick et al. 1994; Scholnick et al. 1994; Jares et al. 1997)
*TP*53	(Maestro et al. 1992; Dolcetti et al. 1992; Nadal et al. 1995; Xu et al. 1994; Casey et al. 1996; Nadal et al. 1997; Ogawa et al. 2003; Liu et al. 2003; Vielba et al. 2003)

continued...	
$p21^{WAF1}$	(Harper et al. 1993; El-Deiry et al. 1993; Nadal et al. 1997)
$p16^{INK4a}$	(Serrano et al. 1993; Kamb et al. 1994; Merlo et al. 1995; Wing Yuen et al. 2002; Jares et al. 1997; Jares et al. 1999; Kamijo et al. 1997)
UDP-glucuronosyltransferase 1A7 (UGT1A7)	(Zheng et al. 2001)
TNF	(Matthias et al. 2001; Matthias et al. 1998)
TGFR	(Massagué 1998; Franchi et al. 2001; Sardi et al. 2001)
Telomerase	(Luzar et al. 2001; Hohaus et al. 1996)
Adhesion molecules and extracellular matrix components	(Goussia et al. 2000)
Matrix metalloproteinases	(Abramson et al. 1975; Osmak et al. 1999; Osmak et al. 2001)

1.5 Esophageal Cancer

The esophagus (gullet) is a long, muscular tube connecting the throat to the stomach, stretching at least 10 cm in an adult (cancerBACUP 2000) (Figure 1.4). Typical symptoms of esophageal cancer (EC) include swallowing difficulty, unexplained weight loss, pain during swallowing, hoarseness, chronic coughing, vomiting and hiccups with treatments including surgery, radiotherapy and chemotherapy (cancerBACUP 2000). There are two histological types of EC, the first one known as squamous cell carcinoma or SCC (Ribeiro et al. 1998; Higginson et al. 1992), accounting for approximately 80-90% of EC (Higginson et al. 1992). The other form of EC is known as adenocarcinoma, and accounts for the remaining 10-20% (Higginson et al. 1992). Approximately half of the SCC's occur in the middle of the esophagus, while the majority of adenocarcinoma occur in the lower third and are usually associated with Barrett's esophagus caused by acid reflux (Higginson et al. 1992; cancerBACUP 2000; Montesano et al. 1996; Spechler et al. 1994). Individuals with chronic gastro-esophageal reflux are most at risk for the development of this type of cancer (Montesano et al. 1996; cancerBACUP 2000; Thomas and Sobin 1995).

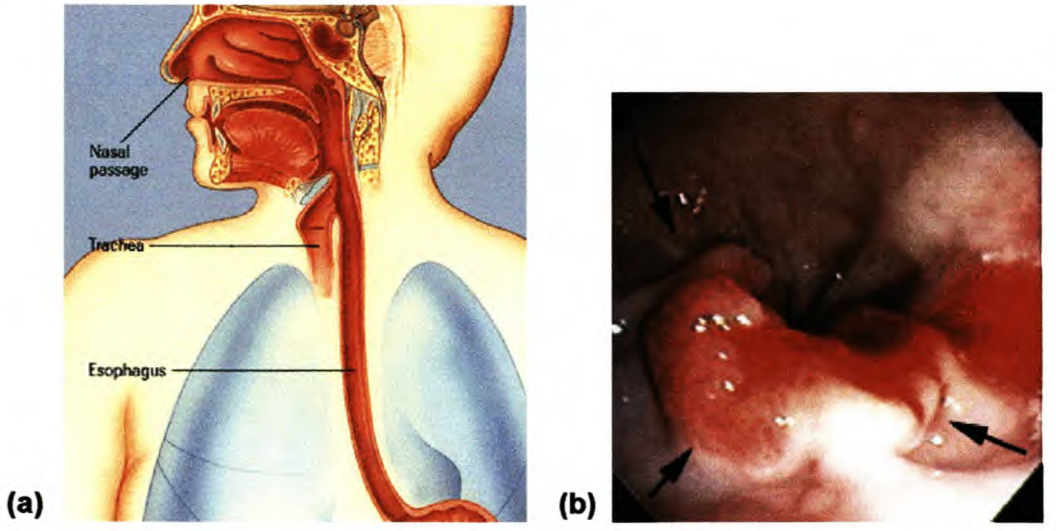

Figure 1.4 The esophagus (a) The esophagus is situated behind the trachea and leads to the stomach (Healthcommunities.com 2003). **(b)** A photo of a typical esophageal tumor (gihealth.com 1997).

By 1980 EC was the 6th most common cancer in the world, taking 4th place in developing countries and 15th place in developed countries (Higginson et al. 1992). Today it is ranked amongst the 10 most frequent cancer types in the world with more than 300 000 new cases annually (Montesano et al. 1996; Parkin et al. 1993). Known as one of the most lethal cancer types, it boasts with a 5-year survival rate of less than 10%, a statistic that has remained unchanged over the last three decades (Ribeiro et al. 1998; Hendricks and Parker 2002; Berrino et al. 1995; Montesano et al. 1996). It has also been shown that males are at a higher risk for the development of EC, with the average age of presentation being at 56 years (Mannell and Murray 1989). Notorious for occurring mostly in developing countries (Montesano et al. 1996; Parkin et al. 1993), the highest incidence and mortality rates can still be found in the 'Asian esophageal cancer belt', stretching from Eastern China to Russia and Turkey (Higginson et al. 1992; Yang 1980) as well as northern Iran, and parts of Iraq (Canon 1997; Day 1994; Joint Iran International Agency for Research on Cancer group Study 1977). Certain black communities of Southern Africa (Transkei, Uganda and Zimbabwe) also show very high rates, with South Africa showing the highest incidence (Canon 1997) (Figure 1.5).

Figure 1.5 The distribution of the highest incidence rates of esophageal cancer (Poon 2003).

It was first noticed in 1957 that there was a high incidence of EC amongst the Xhosa speaking people of the Transkei and by 1992, EC was reported to be a serious health problem in South Africa, affecting both males and females (Sitas and Mqoqi 1992). Today, this disease is the most common cause of cancer-related deaths in South African black males (Gamieldien et al. 1998; Vizcaino et al. 1995; Sitas and Mqoqi 1992; Sitas et al. 1998). In general it can be said that this disease preferentially attacks groups with a lower socio-economic status or those afflicted by poverty (Mannell and Murray 1989), possible due to the contribution of malnutrition.

As for most other tumors, EC tumorigenesis is a complex, multi-factorial process, involving numerous suspected risk factors. (Ribeiro et al. 1998; Sur and Cooper 1998; Montesano et al. 1996; Gamieldien et al. 1998). The following section will briefly discuss merely a few of these suggested risk factors.

Tobacco smoking, alcohol consumption, betel chewing and some dietary habits have been identified as the major risk factors in ESSC, where tobacco and alcohol are relevant in Europe and North America. However, in the high-risk areas of China and northeast Iran, dietary habits are more closely involved than tobacco and alcohol consumption (Bagnardi 2001; Bagnardi et al. 2001; Gallus et al. 2001; Pacella-Norman et al. 2002).

These risk factors can be used as criteria for further sub classifications of ESCC; those arising as a result of alcohol and tobacco consumption, which is the predominant group, and those due to other factors (Higginson et al. 1992). Some studies have tried to further categorize this by showing a possible higher risk for smokers of pipes and hand-rolled cigarettes (Tuyns and Estève 1983; Segal et al. 1989) or an increased risk for smokers of high-tar cigarettes (La Vecchia et al. 1986; Higginson et al. 1992). Other observations suggest that the swallowing of smoke condensates is worsened by alcohol, which acts as a solvent for such condensates (Higginson et al. 1992).

The involvement of fumonisin (a family of mycotoxins produced by *Fusarium moniliforme* and *F. proliferatum*) contaminated maize has also been suggested to play a major role in the development of ESCC (Marasas 1996; Chu and Li 1994; Yoshizawa et al. 1994).

Even viral agents, such as the human papilloma virus (HPV) have been implicated in the development of ESCC (Peto 2001; Lavergne and de Villiers 1999; Matsha et al. 2002).

Other factors have also been researched with regards to their potential involvement in cancer development, and include iron status (Isaacson et al. 1985; MacPhail et al. 1979) allelic imbalance and microsatellite instability (Naidoo et al. 1999), vitamins and lipotrope deficiencies (Jaskiewicz et al. 1988) and several geochemical factors (Kibblewhite et al. 1984) .

Furthermore, numerous studies have been conducted on genes, or the expression of their respective protein products, suspected of conferring genetic susceptibility to the development of EC, such as the ras protooncogenes (Victor et al. 1990), cyclin A (Chetty and Simelane 1999), cyclin D1 and retinoblastoma (Chetty and Chetty 1997) as well as the androgen receptor gene (Dietzsch et al. 2003). Several more such examples can be found listed in Table 1.7 (Mandard et al. 2000).

Table 1.7 A summary of the most frequently investigated genes in relation to EC development or susceptibility

Gene	Reference(s)
*TP*53	(Hollstein et al. 1991a; Gao et al. 1994; Montesano et al. 1996) (Vos et al. 2003)
p16/CDKN2,p15 (MTS1)	(Xing et al. 1999; Montesano et al. 1996; Busatto et al. 1998)
Cyclin D1	(Jiang et al. 1993; Montesano et al. 1996)
EGFR	(Hollstein et al. 1988; Lu et al. 1988; Montesano et al. 1996)
c-myc	(Montesano et al. 1996)
Rb	(Montesano et al. 1996; Jiang et al. 1993; Busatto et al. 1998)
TOC	(von Brevern et al. 1998; Iwaya et al. 1998)
FEZI	(Ishii et al. 1999)
DLC1	(Daigo et al. 1999)
RARβ	(Xu et al. 1999)
GST's	(van Lieshout et al. 1999; Hori et al. 1997; Lin et al. 1998a; Lin et al. 1998b; Tan et al. 2000; Lee et al. 2000; Tan et al. 2001; Wang et al. 2002; Adams et al. 2003; Lin et al. 1998a)
CYP	(Lin et al. 1998b; Tan et al. 2000; Wang et al. 2002; Itoga et al. 2002)
Cytoplasmic-β-catenin	(Kimura et al. 1999)
iNOS	(Tanaka et al. 1999)
SULT1A1	(Wu et al. 2002)
ADH's	(Hori et al. 1997; Boonyaphiphat et al. 2002; Ballo et al. 2003)
ALDH's	(Matsuo et al. 2001; Watanabe et al. 2002; Boonyaphiphat et al. 2002)
XRCC1,ERCC1, XPC,XPD,XPF, SRCC3	(Lee et al. 2001; Xing et al. 2002; Goode et al. 2002)
*TP*73	(Nimura et al. 1998)

These are all merely a few examples of the most frequently investigated genes that have been investigated based on the grounds of suspicion of their involvement in conferring susceptibility to the development of EC. This goes to prove that the genetic component of EC research is a large one, encompassing numerous facets and remains to be solved.

One of the genes found in both Tables 1.6 and 1.7 is *TP*53. In 1989, the first *TP*53 mutations associated with cancer were identified and within the following ten years, 10 000 mutations were described. Today, over 14 000 *TP*53 mutations are known (Hainaut 2001) in more than 10 000 different tumors (Greenblatt et al. 1994), with EC and LC (head and neck cancer) ranking amongst the top four cancer types with the highest *TP*53 mutation prevalence (Figure 1.6).

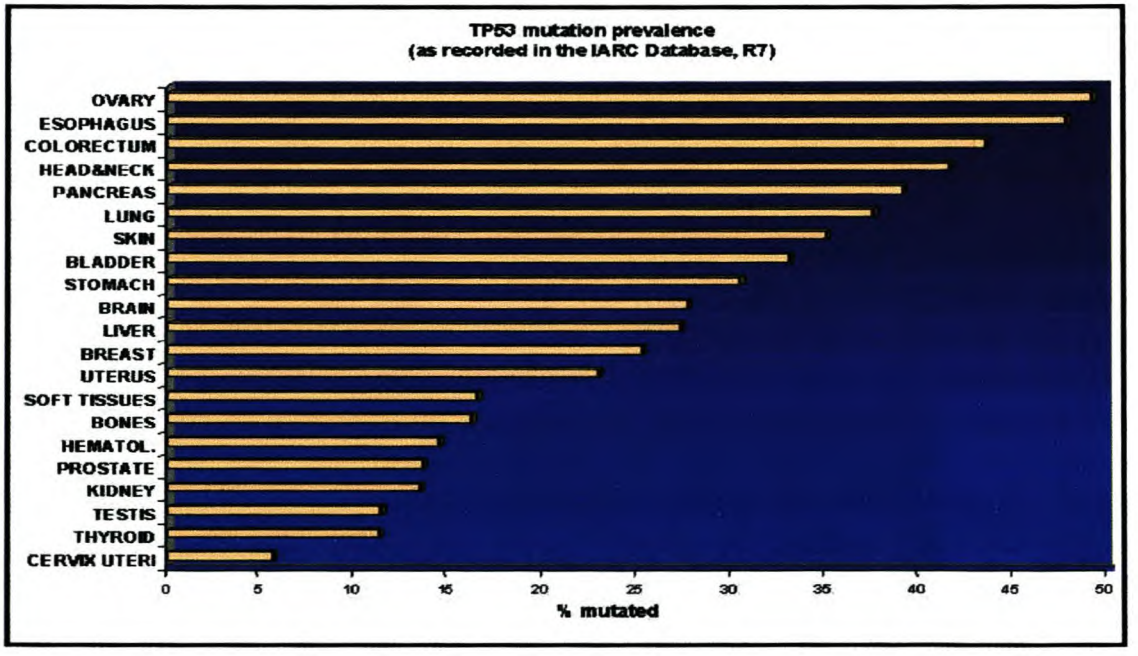

Figure 1.6 The prevalence of *TP*53 mutations in several of the major cancer types (IARC Database 2003).

To date, more than 13 000 publications have come from this topic (Midgley and Lane 1997; Soussi 2000).

1.6 *TP*53 and Friends

Tumor progression is associated with cumulative genetic changes as a result of a loss of cell cycle control. The progression of the cell cycle is constantly monitored for DNA damage at the G1-S and G2-M boundaries and genetic analysis has revealed in the involvement of tumor suppressor proteins in this process. An example of such a tumor suppressor protein is p53, which has earned it the renown title of 'guardian of the genome' (Sherbet and Lakshmi 1997; Sun.Y. et al. 1999; Adams and Kaelin 1998b). This protein has been found to respond to a number stressful stimuli, including DNA damage (for example, due to X-rays, gamma-rays, and UV oxidative stress) hypoxia, heat shock, metabolic changes, certain cytokines and certain oncogenes, such as c-myc, E1F1 and E1A (Steele et al. 1998; Hainaut 2001; Klug and Cummings 1997). The consequence of p53 activation is the activation of a series of events resulting in the cell being destined for one of two routes; either the DNA damage is repaired, where possible, or the cell undergoes programmed cell death, a process known as apoptosis (Adams and Kaelin 1998b; Pai et al. 1998; Hainaut 2001; Steele et al. 1998) (Figure 1.7).

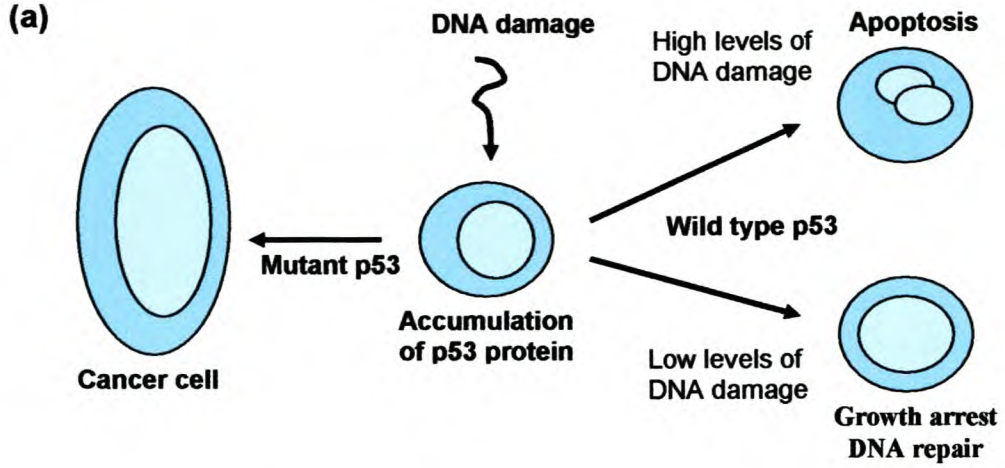

Figure 1.7 The course of DNA damage. When DNA damage is detected, the cells are destined for one of two routes; either the DNA damage is repaired, where possible, or the cell undergoes programmed cell death, a process known as apoptosis. p53 has been shown to be involved in this process (Rotter et al. 2003).

First identified more than 20 years ago, it is widely accepted today that *TP*53 mutations are frequent events in the tumorigenesis of several cancers with the mutational spectrum varying greatly amongst different types of cancers (Yamada et al. 2003; Gottschlich et al. 2000; Soussi et al. 2000; Gamieldien et al. 1998).

Li-Fraumeni cancer family syndrome, interestingly, is caused by a germline mutation in the core domain of *TP*53 (exons 5 to 8) (Steele et al. 1998; Bishop et al. 1996; Midgley and Lane 1997; Adams and Kaelin 1998b) and leaves affected individuals predisposed to the development of a range of cancer types, including breast carcinoma, soft tissue sarcoma, brain tumors, osteosarcoma, leukemia, and adrenocortical carcinoma (Srivastava et al. 1990; Malkin et al. 1990; Bishop et al. 1996). *TP*53 is located on chromosome 17 at locus p13.1 (Imazeki et al. 1992; Toguchida et al. 1992; Soussi et al. 2000; Gorgoulis et al. 1994) and codes for a 2,8kb mRNA transcript (Sherbet and Lakshmi 1997). Named the "molecule of the year" in 1996 by Newsweek magazine, the product of *TP*53, known as p53, is a 53-kDa phosphoprotein consisting of 393 amino acids (Bishop et al. 1996; Imazeki et al. 1992; Soussi et al. 2000; Steele et al. 1998) which functions

as a transcription factor (Toguchida et al. 1992; Bishop et al. 1996; Gorgoulis et al. 1994), capable of inducing cell cycle arrest in the G1 phase should any DNA damage be detected (Ribeiro et al. 1998; Soussi et al. 2000; Kastan et al. 1991; Smith et al. 1994). Furthermore, p53 has also been found to play a role in DNA synthesis and repair (Harris 1996; Ribeiro et al. 1998; Hainaut 2001; Kastan et al. 1991), maintenance of DNA stability (Harris 1996; Ribeiro et al. 1998; Lane 1992), as well as cell proliferation and apoptosis (Harris 1996; Ribeiro et al. 1998; Hainaut 2001; Soussi et al. 2000). Highly conserved in all species studied to date, this gene consists of 11 exons, of which the first one is non-coding. This is followed by a large 10kb intron (Sherbet and Lakshmi 1997; Imazeki et al. 1992; Lamb and Crawford 1986b). Two regulatory promoter sites control the transcription of this gene; the first (P1) occurs 400bp upstream of exon 1 and the second (P2) occurs within intron 1, approximately 1kb downstream of P1 (Harlow et al. 1985; Hainaut 2001; Reisman et al. 1988; Tuck and Crawford 1989; Sherbet and Lakshmi 1997). Furthermore, 5 highly conserved regions have been identified; region I (codons 13-19), region II (codons 120-143), region III (codons 172-182), region IV (codons 238-259) and region V (codons 271-290), i.e. in exons 4, 5, 7 and 8 (Sherbet and Lakshmi 1997; Macdonald and Ford 1991; Levine et al. 1994; Vogelstein and Kinzler 1992). It has been shown that mutations are prone to occur in clusters, or hotspot regions, specifically in conserved regions II-V (Sherbet and Lakshmi 1997; Hainaut 2001; Ribeiro et al. 1998; Steele et al. 1998).

As suspected, it was later shown that these regions do in fact code for an important functional domain of the final protein product (Figure 1.8), representing the sequence-specific DNA-binding site which stretches from amino acid residue 90 to 290 (Sherbet and Lakshmi 1997; Bishop et al. 1996; Cho et al. 1994; Hainaut 2000).

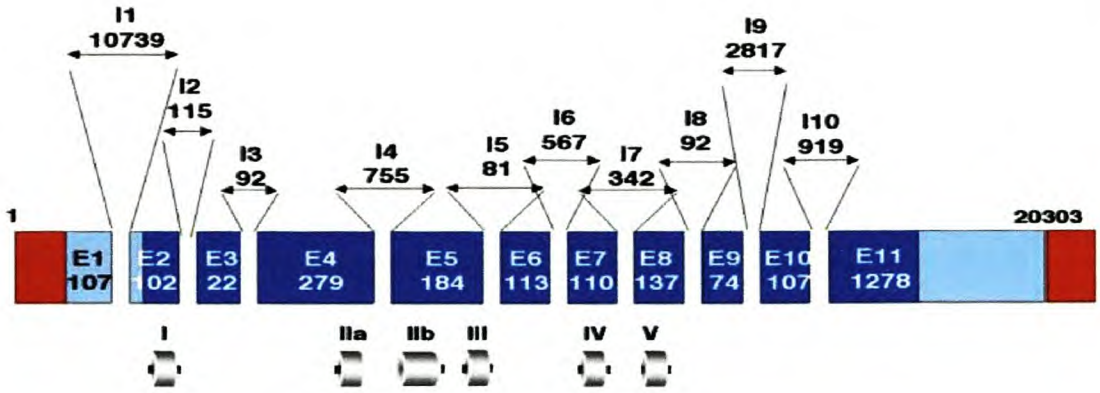

Figure 1.8 The structural organization of *TP53*; where E and I respectively indicate the exon and intron number followed by their length in number of nucleotide bases (Soussi 2001).

This central DNA binding domain contains 10 cysteine residues, which suggest the involvement of metal ions (Sherbet and Lakshmi 1997). This has in fact been shown to be true by proving that chelating agents abolishes DNA binding. It has therefore been suggested that p53 is a metalloprotein (Sherbet and Lakshmi 1997; Pavletich et al. 1993) which occurs and functions predominantly as a tetramer. It therefore follows that oligomerization is an essential step prior to DNA binding (Sherbet and Lakshmi 1997; Stenger et al. 1992; Friedman et al. 1993). A 53-amino acid C-terminal domain, referred to as the oligomerization domain, mediates the formation of these stable tetramers (Sherbet and Lakshmi 1997; Macdonald and Ford 1991; Hainaut 2001; Pavletich et al. 1993). DNA-protein binding is subsequently allosterically regulated by changes in the conformation of these tetramers between states of high and low affinity for DNA (Hupp et al. 1992; Sherbet and Lakshmi 1997; Halazonetis et al. 1993; Halazonetis and Kandil 1993). Hetero-oligomers have also been found to form between wild type and mutant p53, which are unable to bind to DNA (Sherbet and Lakshmi 1997; Bishop et al. 1996; Milner and Medcalf 1991; Bargonetti et al. 1992) and thus one mutant allele is enough to completely eliminate p53 activity, acting in a "dominant negative" fashion.

The biochemical basis for p53 function lies in its ability to regulate transcription by direct binding to DNA via the DNA binding domain (Zubay 1993; Sherbet and Lakshmi 1997), which lies between residues 90 and 290 (Sherbet and Lakshmi 1997). The consensus sequence required for p53 binding to DNA is 5'–Pu–Pu-Pu-C-A/T-A/T-G-Py-Py-Py-3' (Sherbet and Lakshmi 1997; El-Deiry et al. 1992; Funk et al. 1992; Kern et al. 1992) and p53 will enhance the transcription of any gene that contains a p53 responsive element (Sherbet and Lakshmi 1997; Farmer et al. 1992; Zambetti et al. 1992). It has been shown that the TATA-binding protein (TBP) interacts with p53 and that this TBP-p53 complex binds to the TATA-box, which is known to be one of the genetic elements controlling the transcription of most genes. It has been further suggested that the nature of this complex could possibly determine the success of p53 induced transcription (Sherbet and Lakshmi 1997; Martin et al. 1993). This has been confirmed by showing that *in vitro* p53 binds only when nuclear extracts are present, indicating that either p53 undergoes post-translational modification, or, that it may need to form complexes with other nuclear proteins (Sherbet and Lakshmi 1997; Funk et al. 1992), such as TBP (Sherbet and Lakshmi 1997).

A number of genes have been shown to contain the appropriate regulatory sequences capable of binding p53 and whose activity is altered by that interaction, and depending on the gene involved, the effect could be either stimulatory or inhibitory (King 1996). In general, four cellular responses are influenced by p53 interaction with such genes, namely; cell proliferation, apoptosis, DNA repair and angiogenesis (the growth of new blood vessels) (Figure 1.9) (King 1996).

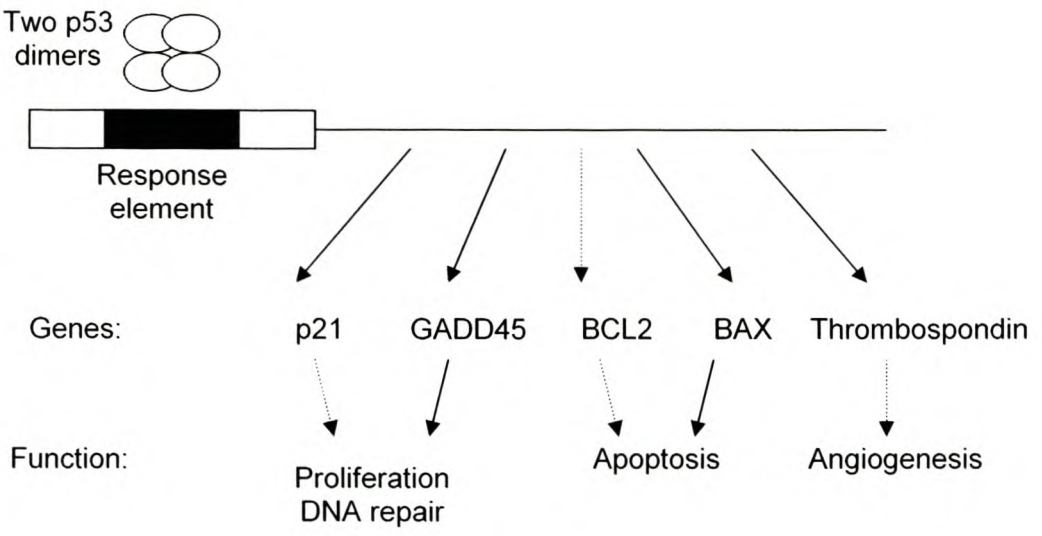

Figure 1.9 p53 Regulates transcription of several genes; solid arrows indicate activation and broken arrows indicate inhibition (King 1996).

Further examples of genes that are regulated by p53 interaction include: *β-actin*, *HSP70*, *JUN*, *FOS*, and *PCNA* (Sherbet and Lakshmi 1997; Chin et al. 1992; Subler et al. 1992; Zastawny et al. 1993). Some genes are completely repressed by p53, and include SV40 early promoter enhancer, herpes simplex virus type 1 (HSV-1), thymidine kinase, T-lymphoblastic virus type 1 and certain LTR promoters of Rous sarcoma virus and human immunodeficiency virus (HIV) (Sherbet and Lakshmi 1997; Subler et al. 1992).

Several viral as well as cellular proteins have been found to interact with and inactivate p53, such as E1B encoded by the adenovirus, E6 encoded by the human papilloma virus, the X protein encoded by the hepatitis B virus and *MDM2* (murine double minute 2), found in human tissues (King 1996; Bishop et al. 1996; Sherbet and Lakshmi 1997).

The *MDM2* gene has been mapped to chromosome 12q13 and the expression of its phosphoprotein product has been found to be associated with highly tumorigenic potential (Bishop et al. 1996; Sherbet and Lakshmi 1997; Cahilly-Snyder et al. 1987). Multiple transcripts of *MDM2* have been identified in breast epithelial cells, with the protein products ranging from 54 – 68 kDa to 90 – 10 kDa (Sherbet and Lakshmi 1997; Gudas et al. 1995). Furthermore, *MDM2* has been found to be transcriptionally activated by p53, resulting in *MDM2* targeting p53 for destruction, forming an auto regulatory mechanism. (Sherbet and Lakshmi 1997; Steele et al. 1998; Momand et al. 1992). If p53 is mutated, *MDM2* transcription cannot be induced and thus p53 accumulates (Steele et al. 1998; Midgley and Lane 1997). This indicates that p53 stability depends more on binding to *MDM2* than by any mutation contained (Steele et al. 1998; Lane and Hall 1997).

The C-terminal domain also contains a signal for nuclear localization (Sherbet and Lakshmi 1997; Dang and Lee 1989; Addison et al. 1990; Shaulsky et al. 1990), and thus p53 is seen to be a nuclear protein. This has been confirmed by showing that in cases where mutations occur within this C-terminal region, the protein was found to be localized within the cytoplasm (Sherbet and Lakshmi 1997; Sturzbecher et al. 1988). Some groups have even found it to be present in both locations (Sherbet and Lakshmi 1997; Moll et al. 1992; Stenmark-Askmalm et al. 1994). Similarly, it has been found that mutations occurring even within the proximity of the nuclear localization signal domain could affect the eventual localization of p53 (Sherbet and Lakshmi 1997; Diller et al. 1990).

In general, it can be said that p53 is expressed at very low levels in normal cells (Steele et al. 1998; Soussi et al. 2000; Gottschlich et al. 2000; Sherbet and Lakshmi 1997) and it has a short half-life, approximately 2-15 min (Montesano et al. 1996; Sherbet and Lakshmi 1997). However, high levels of this protein have been found in many tumors, resulting from an increase in p53 stability leading to

a longer half-life, which is usually associated with *TP*53 mutations (Steele et al. 1998; Midgley and Lane 1997; Pai et al. 1998; Hainaut 2001;).

Several other functional domains have also been identified within *TP*53, showing similarity to heat shock proteins, *MDM*2 and SV40 large T-antigen-binding domains (Figure 1.10).

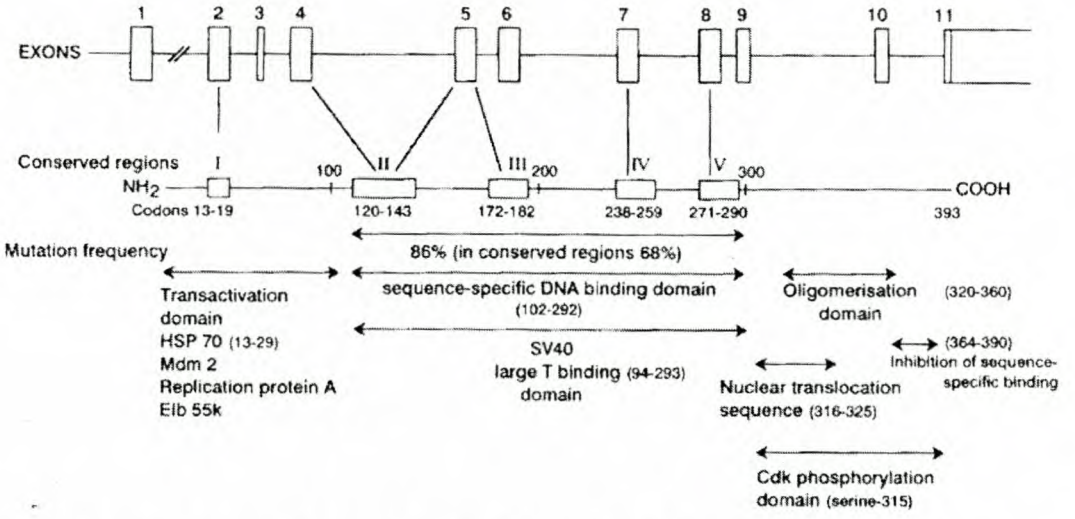

Figure 1.10 The structural and functional domains of p53 (Sherbet and Lakshmi 1997).

It has been known for several years that one the mechanisms of gene regulation is the generation of splice variants. Although no alternative splice variants of p53 have been identified in rat tissues, p53 psuedogenes have been found, which is suggested to have been the result of the incorporation of spliced mRNA's into the germline (Sherbet and Lakshmi 1997; Lin and Chan 1995). Furthermore, the expression of splice variants have been reported in mouse epidermal cells, where the predominant form is said to be expressed during the G1 phase and an alternative splice variant, with an additional 96 nucleotides, is expressed mainly in the G2 phase (Sherbet and Lakshmi 1997; Kulesz-Martin et al. 1994; Bayle et al. 1995). Also, spliced p53 transcripts have been found to occur in normal human lymphocytes. It has therefor been suggested that p53 splice variants may be expressed in a tissue- or even a species-specific fashion and may possibly

have a role in the regulation of the function of this gene (Sherbet and Lakshmi 1997; Will et al. 1995).

As mentioned earlier, p53 function may be impaired by mutations or even by alternative splicing, but there are several other factors that also need to be considered.

By now it is clear that genetic alterations of *TP*53 and its dysfunction as a consequence leads to the deregulation of cell proliferation and eventually to tumorigenesis (Sherbet and Lakshmi 1997; Zambetti and Levine 1993; Gorgoulis et al. 1994; Steele et al. 1998). In general, approximately 60% of all human cancers show *TP*53 mutations together with a loss of heterozygosity (LOH) which results in the total loss of wild type alleles (Sherbet and Lakshmi 1997; Levine et al. 1991). It has also been found that the conserved regions II-V carry 86% of all the known mutations of p53 and that 68% are missence mutations (Sherbet and Lakshmi 1997). Such missence mutations lead to the production of a defective protein product that cannot bind to its target genes via their p53 responsive elements (Sherbet and Lakshmi 1997). It has further been found that the consequence of mutations such as allelic deletions, rearrangements or base pair substitutions within *TP*53 results in a protein which often has not only lost its tumor suppressor function, but rather has gained several new functions which manifest themselves phenotypically within tumors (Sherbet and Lakshmi 1997). In addition to loss of function mutations, there is evidence to suggest that some *TP*53 mutations may enhance tumorgenicity (Montesano et al. 1996). Over 2000 inactivating mutations have been identified in *TP*53 (King 1996) and the malfunctioning of this popular protein has been regarded as a significant event in the pathogenesis of a number of cancers, including breast, colon, stomach, bladder, ovary, endometrium, testicular tumors, soft tissue sarcomas, melanomas and hematological malignancies (Sherbet and Lakshmi 1997; Klug and Cummings 1997; Berchuck et al. 1994; Liu et al. 1994). The most frequent mutation is found in codon 248, which codes for an arginine, which binds directly

to DNA. This codon has been found to not only be affected in somatic cells, but also in inherited germline mutations, such as in the case of Li-Fraumeni syndrome in which patients experience multiple cancers (King 1996). Mutations in the very next codon, 249, have been found in aflotoxin-induced hepatic carcinomas (King 1996). In breast cancer, *TP*53 mutations occur at an incidence of 25-40%, with the frequency of G-T transversions being higher than expected, and occurring predominantly within the conserved regions of exons 5-8 (Sherbet and Lakshmi 1997). Another mutational hotspot occurs at codon 273 in ovarian and pancreatic tumors (King 1996). In lung cancer, hotspots are found at codons 157, 248, 249 and 273 (King 1996). In esophageal and lung cancers, *TP*53 mutations arise earlier in disease progression (Sherbet and Lakshmi 1997; Bennet et al. 1992; Sozzi et al. 1992) and it has been shown that a low frequency of point mutations occur in approximately 1 in every 10 SCC and in one in every 14 adenocarcinomas as well as in 4 out of every 7 Barrett's epithelium adjacent to adenocarcinomas in the esophagus (Sherbet and Lakshmi 1997).

In summary, the codon and type of p53 mutation varies according to the cancer type as well as the geographical distribution (King 1996) and with this study, we intend to obtain clues as to the type of environmental factor(s) that may play a role within our unique South African communities.

1.7 The Present Study

The aim of the present study was to analyze the molecular structure of *TP*53 as well as the transcription levels thereof, using DNA and RNA obtained from patients suffering from esophageal and laryngeal cancer, two cancers of the upper gastro-intestinal tract known to occur at high frequencies in South Africa. These studies were approved by the IRB of Stellenbosch University (95-069 and 97/030).

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Chapter 2

*TP*53 in Laryngeal Cancer

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2.1 Overview

The motivation for this study lies in the finding that laryngeal cancer in South Africa occurs at a high incidence, relative to the rest of the world, and is particularly common among the Coloured males. The aim of this study was thus to analyse the molecular structure of *TP*53, specifically exons 5 – 8, in a group of laryngeal cancer (LC) patients treated at Tygerberg Hospital, Cape Town, South Africa. This included screening the DNA of patients as well as a control group via PCR-SSCP and direct sequencing analysis. The participants of this study were also asked to complete questionnaires in order to determine possible etiological factors, and this, together with the data obtained from the molecular screening, was used to establish correlations between the occurrence of LC, the type of mutations observed, and the various etiological factors under investigation. The more long term goals of this study would be to determine whether *TP*53 mutations in primary laryngeal cancer tumors have any implication in the recurrence rate of these tumors even after complete surgical removal. The value of such knowledge has far reaching effects in practice, placing physicians in an advantageous position in determining the appropriate therapies for each individual patient.

The institutional ethics committee approved this study and informed consent was obtained from all subjects.

The work discussed in this chapter has been submitted and accepted for publication in Cancer Genetics and Cytogenetics (Barnard et al. 2003) and a copy of the pre-print can be found in Appendix I. Furthermore, this work has been presented (by D.Barnard) at an international conference entitled: Cancer of the Esophagus and Gastric Cardia: From Gene to Cure, held in Amsterdam, the Netherlands during the period 13 – 15 December 2002.

2.2 Materials and Methods

2.2.1 Collection of tumor samples

Fresh tumor biopsies were obtained from 44 LC patients. They consisted of 22 Coloured males, 8 Coloured females, 6 African Black males, 2 White males and 6 were of an unidentified ethnicity. All 44 patients received treatment and underwent surgery at Tygerberg Hospital, Cape Town, South Africa, during the period from January to December 2000. The age of the patients ranged between 41 and 78 years of age, with a mean of 58 years.

Upon removal, each biopsy was divided into two equal parts. One half was used to histologically confirm poorly differentiated to well differentiated squamous cell carcinoma. The second half of the tissue was snap-frozen in liquid nitrogen immediately after surgical removal and stored at -70ºC for further molecular analysis in disposable polypropylene cryogenic vials (Appendix II).

2.2.2 Collection of blood samples

Blood samples were collected in 5 ml disodium ethylenediaminetetraacetate dehydrate (EDTA) tubes (Appendix II) from 42 of the same patients and frozen for further molecular analysis. Data obtained from the blood samples would serve as a control to test for a loss of heterozygosity (LOH). This would serve as an indication as to whether any nucleotide alterations observed within the tumor samples was somatic in origin, or whether they were germline mutations.

In addition, blood was collected in 5ml EDTA tubes (Appendix II) from a healthy control group consisting of 40 volunteers who were matched to the patients according to age, gender and ethnic group. This would enable us to distinguish true nucleotide alterations from polymorphisms known to occur within the population.

2.2.3 Collection of demographic data

Questionnaires were compiled in order to evaluate various aspects of the participants' lifestyle, including physical as well as dietary exposures. Both the patients as well as control group completed these questionnaires. An example of this questionnaire can be seen in Appendix IV.

The data obtained from these questionnaires were used to construct a database, which would be used for future correlation studies.

2.2.4 DNA extractions

2.2.4.1 Tumor Samples

DNA was extracted from the tumor biopsy samples using the QIAmp DNA minikit. A glass slide (Appendix II) was weighed and, using a new, sterile scalpel blade (Appendix II) for each individual biopsy, the frozen biopsies were cut into smaller fragments on the glass slides (on ice) and weighed again. The weight of the biopsy was taken as the difference between these two weight measurements. The protocol for the DNA extraction was then followed according to the specifications of the manufacturer, except in the case of a biopsy sample weighing more than 25mg, where all the reagent quantities were doubled.

2.2.4.2 Blood Samples

DNA was extracted from the blood samples according to an adaptation of the salting-out procedure for human DNA extraction (Miller et al. 1988). Essentially, each blood sample was transferred from the 5 ml EDTA tubes (Appendix II) into 50 ml Falcon tubes (Appendix II) to which 5 – 6 volumes of ice-cold cell lysis buffer (Appendix III) was added. The tubes were inverted 10× and this was followed by a 15-minute centrifugation (Appendix II) step at 4°C and 3000 rpm. A pellet was formed in the base of the Falcon tube which

was then resuspended in 9 ml nuclei lysis buffer (Appendix III), 1 ml 10% sodium dodecyl sulphate (SDS; Appendix II), and proteinase K (Appendix II) (to a final concentration of 100 μg/ml). After an overnight incubation period at 37°C, 3 ml saturated NaCl solution (> 6 M) (Appendix III) was added. This was then followed by another 20-minute centrifugation (Appendix II) step at 4°C and 3000 rpm. The DNA was then precipitated by the addition of 100% ethanol (Appendix II), and washed by adding 500 μl 70% ethanol (Appendix III) and pipetting (Appendix II) up and down several times. A glass Pasteur pipette was heated over a Bunsen burner in order to seal the tip. This sealed tip was then used to remove the precipitated DNA strands and transfer it to a clean 1,5 ml Eppendorf microfuge tube. The DNA was finally resuspended in 200 μl TE buffer (Appendix III).

The optical density (OD) of the DNA was then determined using a Milton Roy series 120l spectrophotometer at 260 nm (OD_{260}). The concentrated DNA was then diluted by adding 10 μl concentrated DNA to 490 μl dH_20 and the DNA concentration determined by multiplying the OD_{260} by a factor of 2.5. This gave an answer of DNA concentration in μg/μl. The purity of the DNA was also determined by calculating the OD_{260}/ OD_{280} ratio, which is optimally at approximately 1.8 for pure DNA.

2.2.5 Polymerase Chain Reaction/PCR

2.2.5.1 Principals of PCR

Polymerase chain reaction (PCR) is a technique by which a specific target region of known or unknown sequence is exponentially amplified. This is achieved by designing oligonucleotide (primers) capable of complementary binding to the regions flanking the region of interest (Figure 2.1).

2.2.5.2 Oligonucleotide primers

PCR has the ability to exponentially amplify very specific regions of DNA. This is achieved due to the specificity of the primers used. These primers are usually designed in such a way that they are complementary to the sequences flanking the region of interest, allowing for them to bind these sites. When the DNA is denatured into its single stranded form, an enzyme known as DNA polymerase seeks these primers, and initiates a copying process in the 5' – 3' direction, whereby the primers serve as a starting point, and the original DNA serves as a template.

For the purpose of this study, primers were synthesised as previously described (Toguchida et al. 1992) (Table 2.1) using a Beckman Instruments Oligo 1000M DNA synthesiser (Appendix II) at the Department of Biochemistry, University of Cape Town (UCT), Cape Town, South Africa.

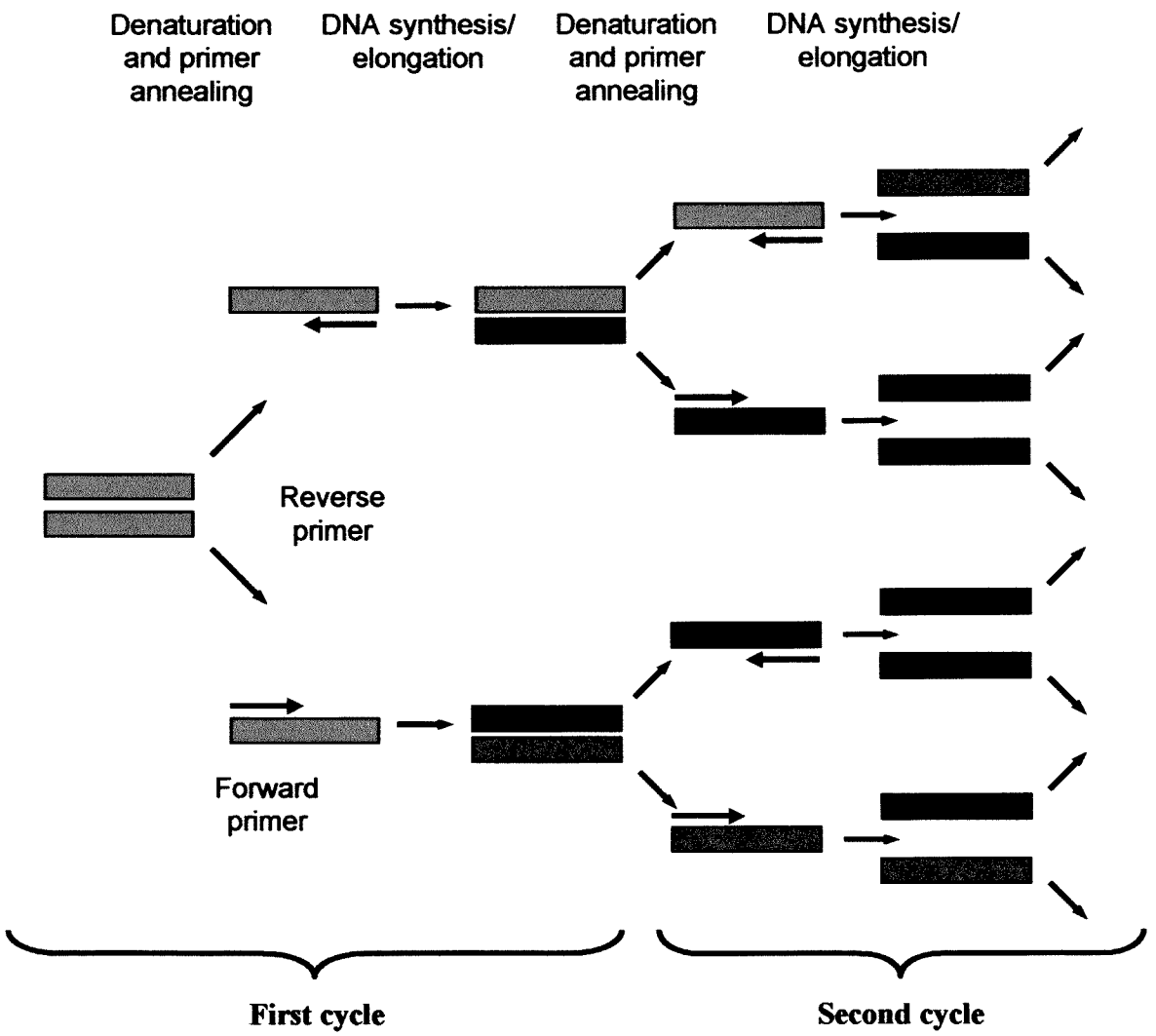

Figure 2.1 Polymerase chain reaction (PCR) exponentially amplifies a specific target DNA sequence with each passing cycle. Adapted from Microbiology 2nd edition.

Table 2.1 Forward (F) and reverse (R) primers for polymerase chain reaction (PCR) amplification of exons 5 to 8 of *TP*53

Primer[a]	Sequence[b]	*Tm* (°C)[c]
Exon 5-5'F	TTA TCT GTT CAC TTG TGC CC	58
Exon 5-5'R	TCA TGT GCT GTG ACT GCT TG	58
Exon 5-3'F	TTC CAC ACC CCC GCC CGG CA	68
Exon 5-3'R	ACC CTG GGC AAC CAG CCC TG	68
Exon 6F	ACG ACA GGG CTG GTT GCC CA	64
Exon 6R	CTC CCA GAC ACC CCA GTT GC	64
Exon 7F	GGC CTC ATC TTG GGC CTG TG	64
Exon 7R	CAG TGT GCA GGG TGG CAA GT	64
Exon 8F	CTG CCT CTT GCT TCT CTT TT	58
Exon 8R	TCT CCT CCA CCG CTT CTT GT	58

[a]Exon 5 was amplified as two PCR products due to its large size, where primers set 5-5'F and 5-5'R amplified the 5' end and primer set 5-3'F and 5-3'R the 3' end.

[b]Primer sequences obtained from (Toguchida et al. 1992)

[c]*Tm* is the optimal melting temperature for each primer set used in PCR amplification during this study.

2.2.5.3 Methodology

The primers that were used to amplify each of exons 5 – 8 of *TP*53 using the DNA obtained from all three sample sets (tumor biopsy, corresponding patient blood as well as blood from the healthy control group) are listed in Table 2.1. Each PCR mix contained the following: 10% Thermophilic DNA polymerase 10× buffer (Appendix IIII), Bovine Serum Albumin (BSA) (Appendix II) to a final concentration of 1mg/ml, dNTP's to a final concentration of 2.5 mmol each (Appendix II), $MgCl_2$ to a final concentration of 1,5 mmol (Appendix II), forward and reverse primers to a final concentration of 0.2 μmol each (Appendix II), 1 – 2 units of *Taq* DNA polymerase (Appendix II), 250 ng DNA template and deionised pure water (dH_20) up to a final volume of 100 μl. All the PCR's were performed in 500 μl tubes (Appendix II) in a Biometra Thermocycler (Appendix II) at a standard heating lid of 105°C, an initial

denaturation period of 3 minutes at 93°C, a subsequent denaturation period of 1 minute at 93°C, an annealing period of 2 minutes at each of the respective melting temperatures (Table 2.1), an elongation period of 2 minutes at 72°C, and a final elongation period of 5 minutes at 72°C. For each PCR reaction, 35 cycles were performed. Extreme care was taken to prevent PCR contamination, and all experiments included a control reaction tube in which template DNA was replaced by dH_20.

2.2.6 Gel Electrophoresis

2.2.6.1 12% Non-denaturing polyacrylamide gel electrophoresis

Successful PCR amplification as well as the absence of contamination was verified by the electrophoresis of 10 µl of the amplified product on 12% polyacrylamide minigels (Appendix III).

2.2.6.1.1 Casting the gel

The gels (Appendix III) were cast between two 100mm x 80mm glass plates. The plates were first washed with Cal-liquid hand soap (Appendix II) and rinsed with tap water. A wash with 70% ethanol (Appendix III) and drying with paper towel followed this. One millimetre spacers where then placed on either side of one of the back plate and the front plate placed on the spacers. The plates were then inserted into the casting apparatus (Appendix II) and the gel solution (Appendix III) poured between the two plates. The well-forming comb was inserted and the gel was left to set for 30 minutes prior to loading and electrophoresis.

2.2.6.1.2 Electrophoresis

After the gels had set, they were placed into the electrophoresis apparatus (Appendix II) and the buffer chambers were filled with 1× Tris Boric acid EDTA (TBE) (Appendix III) electrophoresis buffer.

Loading dye (5 μl) (Appendix III) was added to 10 μl of each sample and loaded into the wells. A DNA marker (Appendix II) was always loaded in one of the wells as a method of verifying the correct amplified product size.

The apparatus was then connected to an electrophoresis power supply and electrophoresed at 150V for 1 to $1^1/_2$ hours.

2.2.6.1.3 Silver staining

After completion of electrophoresis, the electrophoresis apparatus was disassembled. The gel was then removed and placed in a container containing 100 ml of solution B (Appendix III) and gently shaken for 10 – 15 minutes. Solution B was then discarded and the gel rinsed in dH_20. The gel was then covered with 100 ml solution C (Appendix III) and once again gently shaken, until the bands became visible (approximately 20 – 30 minutes). The gel was then visually inspected on a white light illuminator and photographed using a video printer (Appendix II).

2.2.7 Single-strand conformational polymorphism (SSCP) analysis

2.2.7.1 Principals of SSCP

Single-strand conformational polymorphism analysis is a rapid method of detecting sequence variations. Under normal conditions, DNA has a double-stranded helix conformation, however, when denatured, the DNA separates into two single strands. These single strands, however, have the ability for form secondary structures by folding and binding to itself via intrastrand interactions. Each secondary structure is unique in that its conformation determines its electrophoretic mobility. This leads to the conclusion that the conformation and mobility of each single strand is directly dependant on its nucleotide sequence, meaning that a single base change is enough to alter this unique characteristic, resulting in a difference in electrophoretic mobility.

Thus SSCP analysis provides a means of rapid detection of variations in nucleotide sequence based on electrophoretic mobility.

For each of the three panels of samples analysed, a 6% mildly denaturing polyacrylamide gel with 5% glycerol (Appendix III) was used for SSCP analysis.

2.2.7.2 Methodology

2.2.7.2.1 6% polyacrylamide SSCP gels

2.2.7.2.1.1 Casting the gel

The gel was cast between two 445mm × 330mm glass plates. The plates were first washed with Cal-liquid hand soap (Appendix II) and rinsed with tap water. A wash with 70% ethanol (Appendix III) and drying with paper towel followed this. Gelbond™ PAG polyester film (Appendix II) was attached to the back plate by its hydrophobic side using 70% ethanol (Appendix III). The front plate was also silinised with Gelslick (Appendix II), which would allow for easier removal of the gel after electrophoresis. One-millimetre spacers where then placed on either side of the back plate (with the hydrophilic side of the Gelbond now facing up) and the front plate placed on the spacers, with the silinised side facing towards the hydrophilic Gelbond surface. The plates were then sealed together on all four corners using gel-sealing tape (Appendix II) and the plates were inserted into the casting rubber boot (Appendix II). The gel solution (Appendix III) was then poured between the two plates onto the hydrophilic side of the Gelbond, allowing the gel to stick to it, thus facilitating the handling of it. The well-forming comb was inserted in the top and held in place by clamps. The gel was left on a horizontal surface to set for approximately 30 minutes. Sufficient paper towel was wet and placed over the well-forming combs and this was then covered by cling wrap (Appendix II) and left in the horizontal position overnight.

2.2.7.2.1.2 Electrophoresis

After allowing the gel to set overnight, the cling wrap (Appendix II), wet paper towel and the casting boot (Appendix II) were removed. The gel was placed in the electrophoresis apparatus (Appendix II) and sufficient 0.6× TBE buffer (Appendix III) was poured in both the upper and lower buffer reservoir chambers. The well-forming comb was removed the wells were washed with the 0.6 × TBE (Appendix III) and a syringe in order to rid of any residual gel solution.

Loading dye (7 μl) (Appendix III) was added to 7 μl of each sample and heat denatured at 96 °C for 5 minutes. This was followed by immediately snap-freezing the samples on ice for 5 minutes. The samples were loaded, accompanied by a DNA marker (Appendix II) in one of the wells as a method of verifying the correct amplified product size. The gels were electrophoresed at a 1500V, 300mA and 50W for a period of 4 hours.

2.2.7.2.1.3 Silver staining

After completion of electrophoresis, the electrophoresis apparatus (Appendix II) was disassembled. The gel-Gelbond structure was removed and stained as discussed in section 2.2.6.1.3, except that 500 ml of solution B (Appendix III) was used and 1 L of solution C (Appendix III).

2.2.7.2.2 Scoring of the 6% polyacrylamide SSCP gels

Each of the 6% SSCP gels (Appendix III) for exons 5 – 8 were scored individually according to the various banding patterns. Scoring condition were stringent, and to eliminate the possibility of overlooking a mobility shift caused by a nucleotide change, bands of a very low intensity were often included in the scoring procedure, as a precautionary measure. For each exon, PCR products obtained from tumor biopsy samples and from blood samples of the

same patients were run adjacent to each other on addition 6% SSCP gels (Appendix III). Representative samples from each unique banding pattern were selected for DNA sequencing analysis.

2.2.7.2.3 Reproducibility of 6% polyacrylamide SSCP gels

To prove that these results were reproducibly, these representative samples were again amplified and the amplicons were again analysed under identical SSCP conditions, as described in section 2.2.7.2.1.

2.2.7.2.4 Second set of SSCP conditions: 8% polyacrylamide SSCP gels

The same used in section 2.2.5.3 were evaluated under a second set of SSCP conditions. This was done to improve the chances of detecting all possibly nucleotide changes and to further prove reproducibility. The same approach was used as described in section 2.2.7.2.1 with the exception that an 8% polyacrylamide gel solution (Appendix III) was used instead of a 6% polyacrylamide gel solution (Appendix III).

2.2.8 Sequencing analysis

2.2.8.1 Amplicon purification

Samples were subsequently selected for sequencing from all three of the sample sets (tumor biopsies, blood from same patients and blood from healthy control individuals) to represent at least one of each of the unique banding patterns observed across exons 5 to 8. Amplicons were purified using the Nucleospin kit (Appendix II), following the instructions recommended by the manufacturer.

2.2.8.2 Automated sequencing

The PCR products were sequenced using the primers described in section 1.2.5.2 for the relevant exons. The Department of Genetics performed automated sequencing at the University of Stellenbosch, Stellenbosch, South Africa, using an ABIprism analyser (model 3100) (Appendix II).

2.2.9 Correlation studies

Dr. E.G. Hoal performed all mathematical calculations concerning the correlation studies. The non-parametric Spearman rank correlation was performed to determine any significant correlations between parameters measured under demographic data and laryngeal carcinoma. SPSS version 10 software (Appendix II) was used and $P \leq 0.05$ was considered statistically significant.

2.3 Results

2.3.1 12% Non-denaturing polyacrylamide gel electrophoresis

Before performing the SSCP analyses, successful amplification of all samples were verified on 12% non-denaturing polyacrylamide gels, as can be seen in Figure 2.2.

2.3.2 6% polyacrylamide SSCP gels

Mobility shifts were detected in exons 5, 7 and 8, but none in exon 6. The results obtained from the various SSCP gels indicate that mobility shifts were detected only within the patient tumor biopsy sample set and not within the blood from the same patients sample set, neither in the blood from the healthy control subjects. Representative examples of these SSCP gels can be seen in Figures 2.2 – 2.12. This suggests that the nucleotide changes observed have somatic origins and are not germline mutations neither population based polymorphisms.

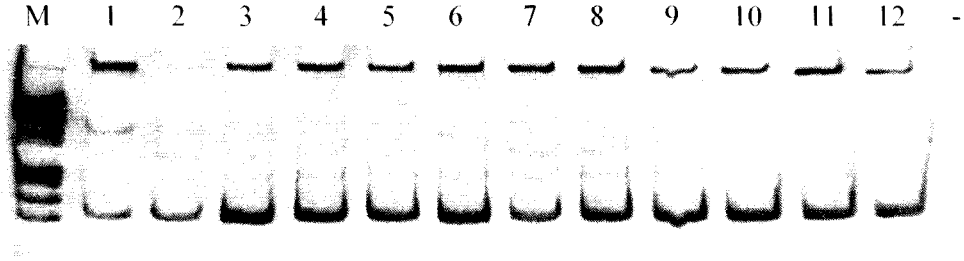

Figure 2.2 A representative example of one of the 12% non-denaturing polyacrylamide gels that were used to verify the presence of amplicons as well as the absence of DNA contamination during PCR. This gel demonstrates the successful amplification of exon 8 for samples as well as the absence of contamination of the negative lane (-). A DNA marker (Appendix III) was loaded in lane M.

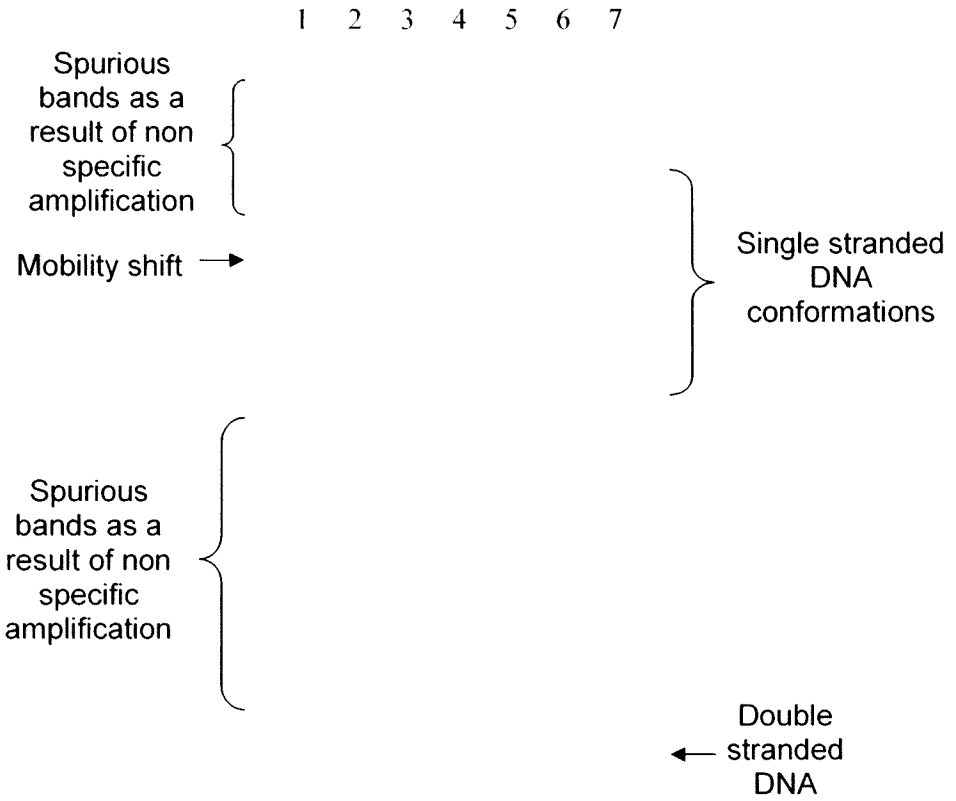

Figure 2.3 (a) A representative example of the SSCP banding patterns detected in exon 5 of *TP53* in DNA obtained from the tumor biopsy samples of 7 representative patients; on a 6% polyacrylamide SSCP gel.

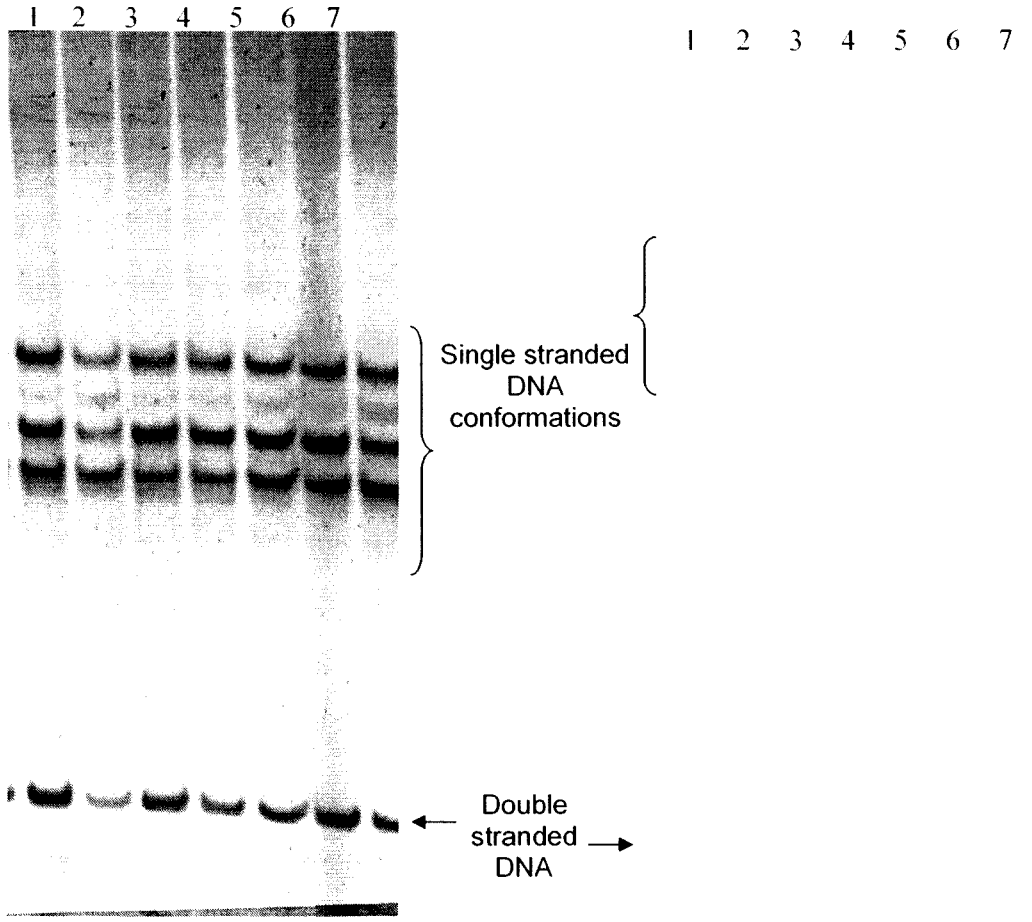

Figure 2.3 (b) A representative example of the SSCP banding patterns detected in exon 5 of *TP*53 in DNA obtained from the corresponding blood of 7 of the patients (left) and from the blood of 7 members of the healthy matched control group (right); on a 6% polyacrylamide SSCP gel.

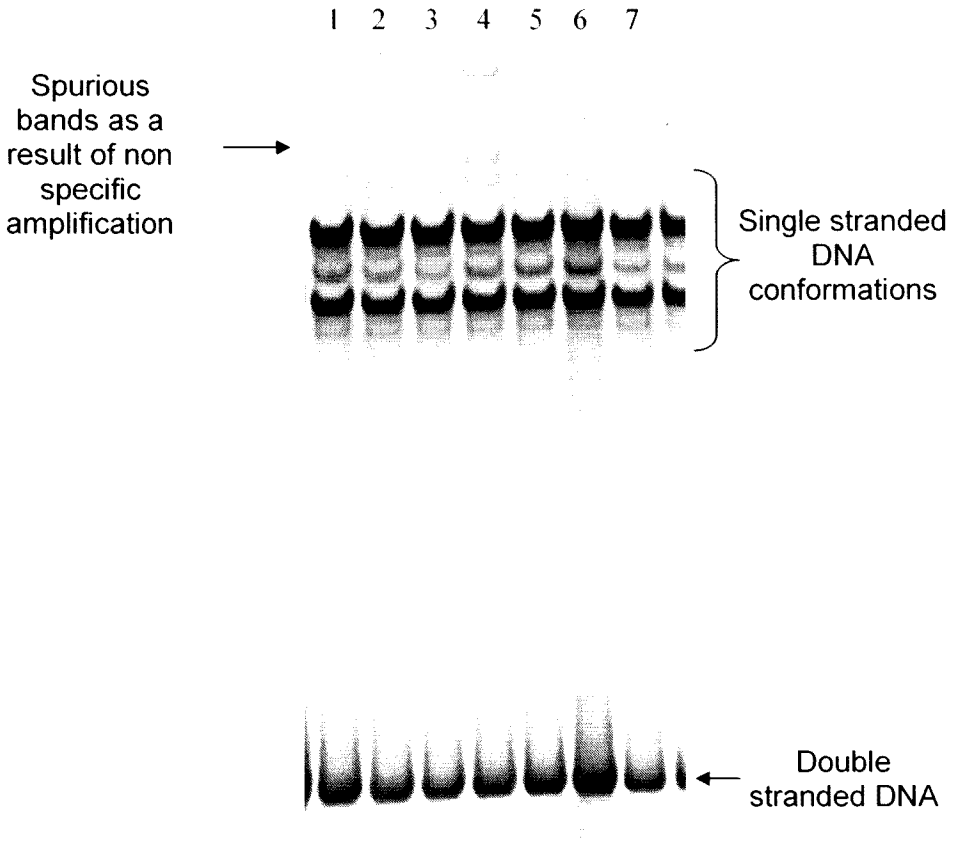

Figure 2.4 (a) A representative example of the SSCP banding patterns detected in exon 6 of *TP*53 in DNA obtained from the tumor biopsy samples of 7 representative patients; on a 6% polyacrylamide SSCP gel.

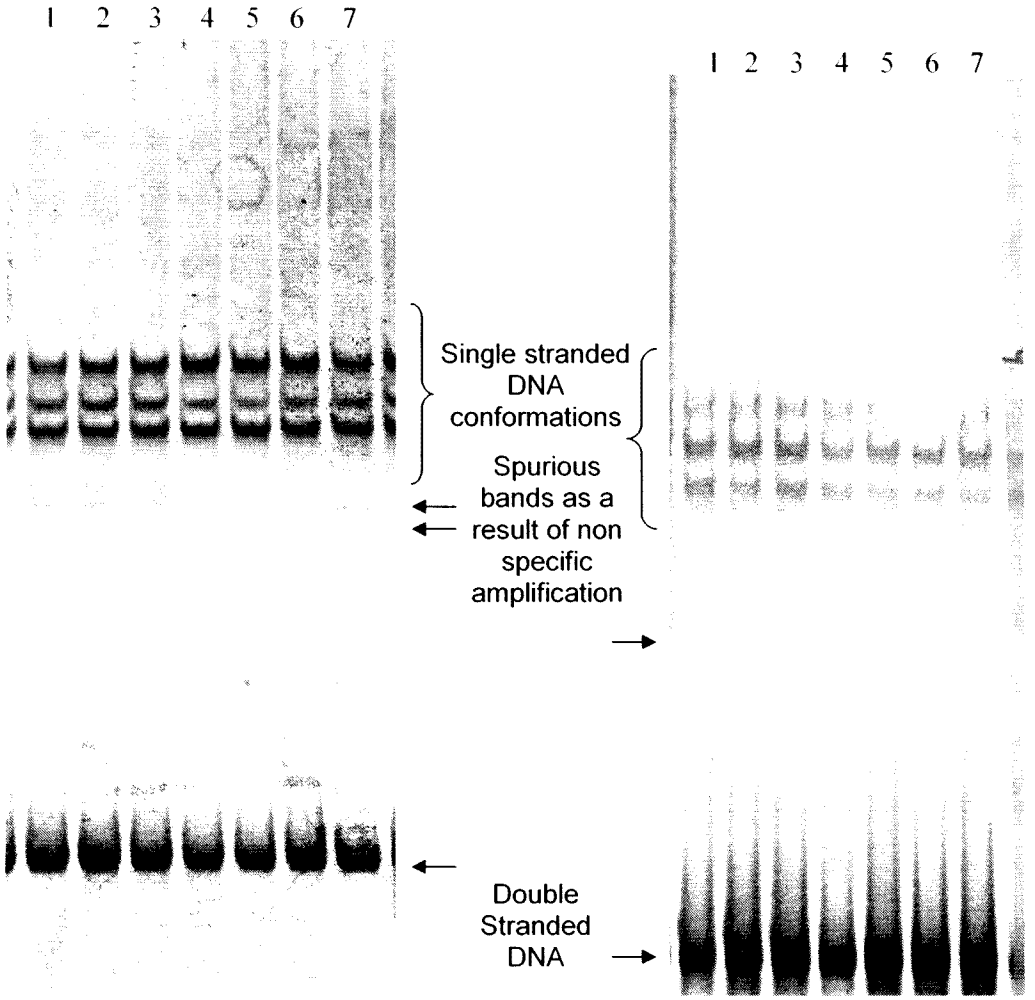

Figure 2.4 (b) A representative example of the SSCP banding patterns detected in exon 6 of *TP*53 in DNA obtained from the corresponding blood of 7 of the patients (left) and from the blood of 7 members of the healthy matched control group (right); on a 6% polyacrylamide SSCP gel.

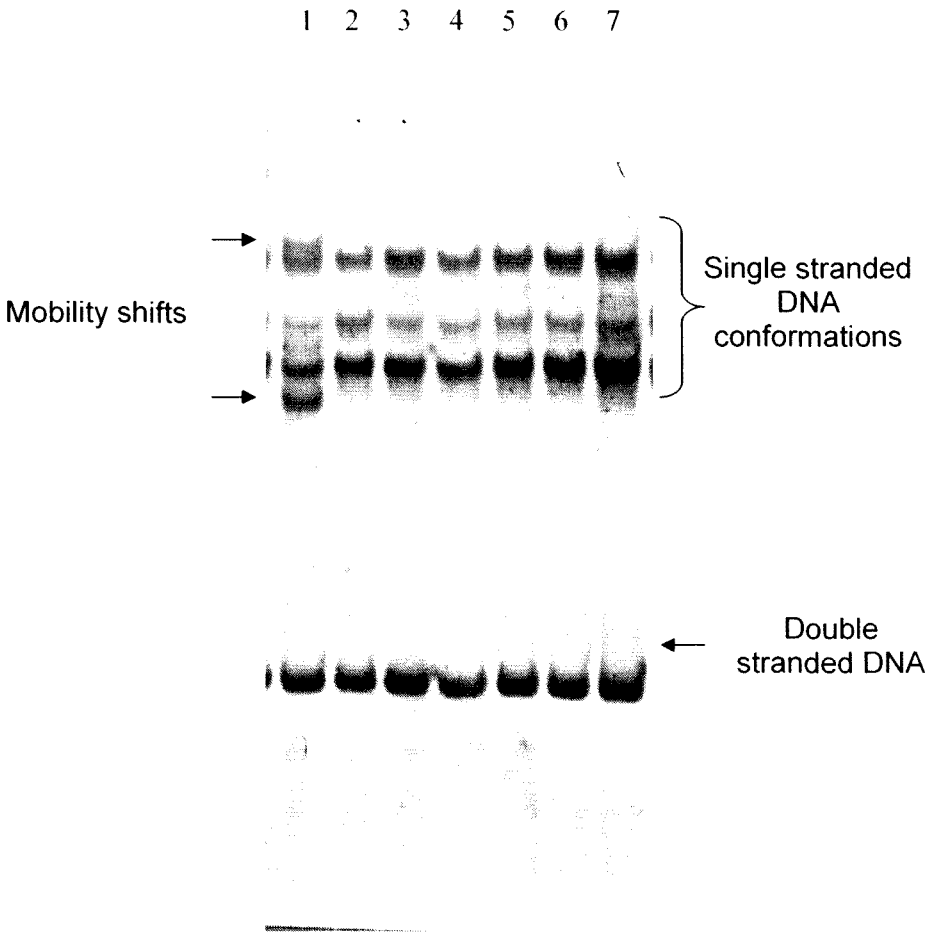

Figure 2.5 (a) A representative example of the SSCP banding patterns detected in exon 7 of *TP*53 in DNA obtained from the tumor biopsy samples of 7 representative patients; on a 6% polyacrylamide SSCP gel.

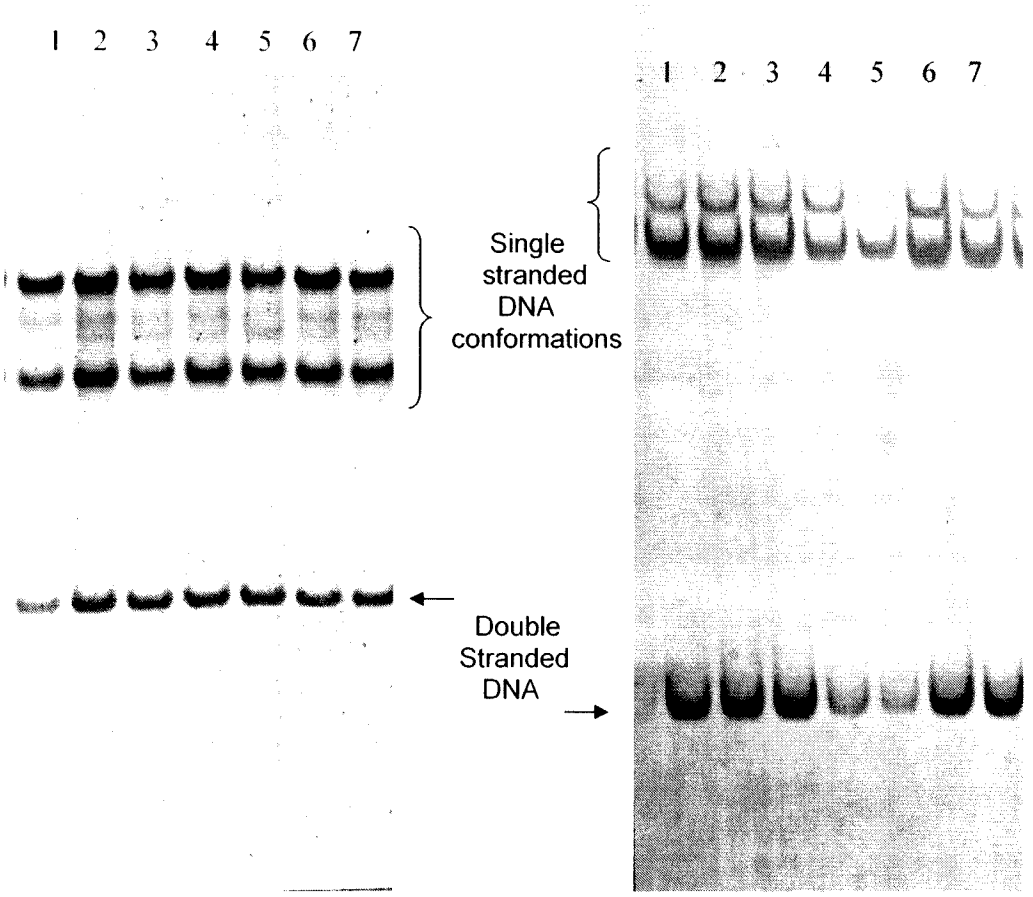

Figure 2.5 (b) A representative example of the SSCP banding patterns detected in exon 7 of *TP*53 in DNA obtained from the corresponding blood of 7 of the patients (left) and from the blood of 7 members of the healthy matched control group (right); on a 6% polyacrylamide SSCP gel.

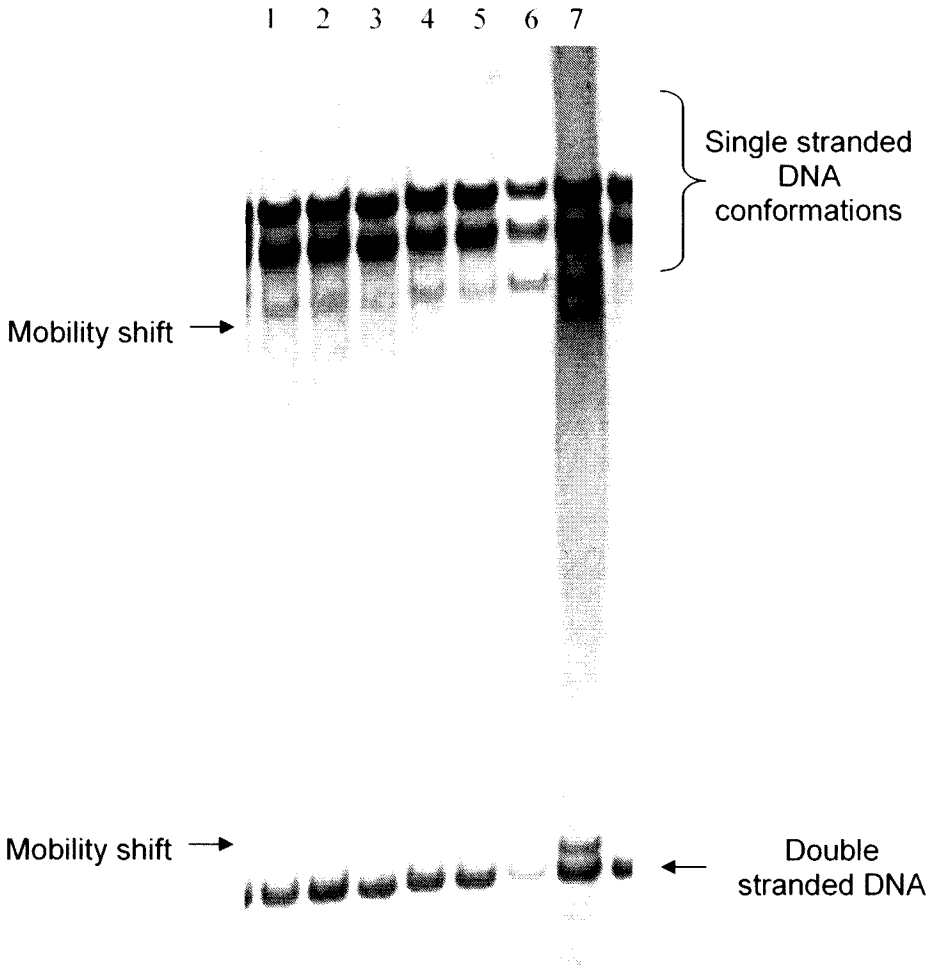

Figure 2.6 (a) A representative example of the SSCP banding patterns detected in exon 8 of *TP*53 in DNA obtained from the tumor biopsy samples of 7 representative patients; on a 6% polyacrylamide SSCP gel.

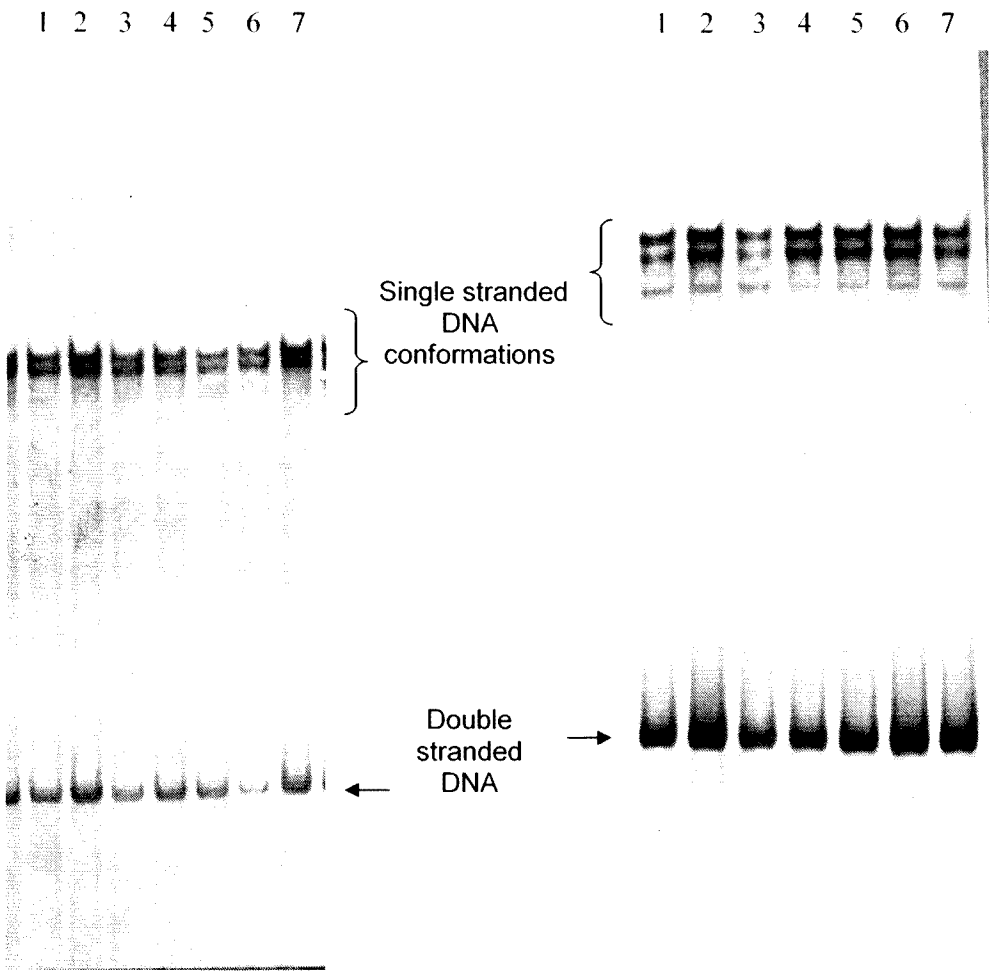

Figure 2.6 (b) A representative example of the SSCP banding patterns detected in exon 8 of *TP*53 in DNA obtained from the corresponding blood of 7 of the patients (left) and from the blood of 7 members of the healthy matched control group (right); on a 6%

Upon closer analysis, slight differences can be found in the banding patterns of each gel. These differences occur between the different sample sets within the same exon (e.g. when comparing the overall banding pattern of exon 8 in Figure 2.6(a) to that of Figure 2.6(b)). This can be accounted for by accepting that no two gels are identical. Often, when running a gel, a difference of 10 minutes can alter the appearance of the banding patterns. One also needs to take into account factors such as slight differences in the temperatures at which the gels run. Furthermore, fluctuations in the current can also have a significant effect on the appearance of the gels. Further complications are brought about by the natural tendency of human error i.e. certain fluctuations in concentrations are prone to occur due to inaccurate measuring (i.e. acrylamide, TBE and urea).

2.3.3 Automated sequencing

In total, 75 sequences were obtained from the selected samples, which were then compared with the wild-type *TP*53 sequence (National Centre for Biotechnology Information [NCBI] nucleotide database accession number X54156) and it was found that the nucleotide changes observed were unique to only the tumor biopsy samples, as was expected from the SSCP analysis interpretation. All sequences obtained from the representative controls correlated with the same wild-type *TP*53 sequence (i.e. NCBI accession number X54156). In total, 11 nucleotide changes were found in three of the four exons under investigation, representing 25% of the tumors investigated in this study (Figure 2.7) (Table 2.2). Each of the 11 nucleotide changes observed occurred in 11 different patients. In total there were G to T (n=4), C to T (n=2), G to A (n =2), G to C (n =1) and T to A (n =1) nucleotide substitutions, which translates to 4 transitions and 6 transversions. Only in one case was a T-insertion found. Thus, in summary, exon 7 was mutated in 6 of the 11 cases (55%), with the G to T (n =3; codon 249), C to T (n =1;

codon 248) and T to A (*n* =1; codon 232) substitutions, all resulting in amino acid alterations. The one T-insertion mentioned earlier was also found in exon 7 (codon 255) and resulted in a frameshift. Exon 8 mutations were detected in 3 of the 11 cases (27%), with the G to A (*n* =1; codon 266), G to C (*n* =1; codon 273) and C to T (*n* =1; codon 273) substitutions also resulting in amino acid alterations. Exon 5 contained the remaining two substitutions, namely, G to T (*n* =1; codon 176) and G to A (*n* =1; codon 175) which also resulting in amino acid alterations. No nucleotide changes were observed within exon 6.

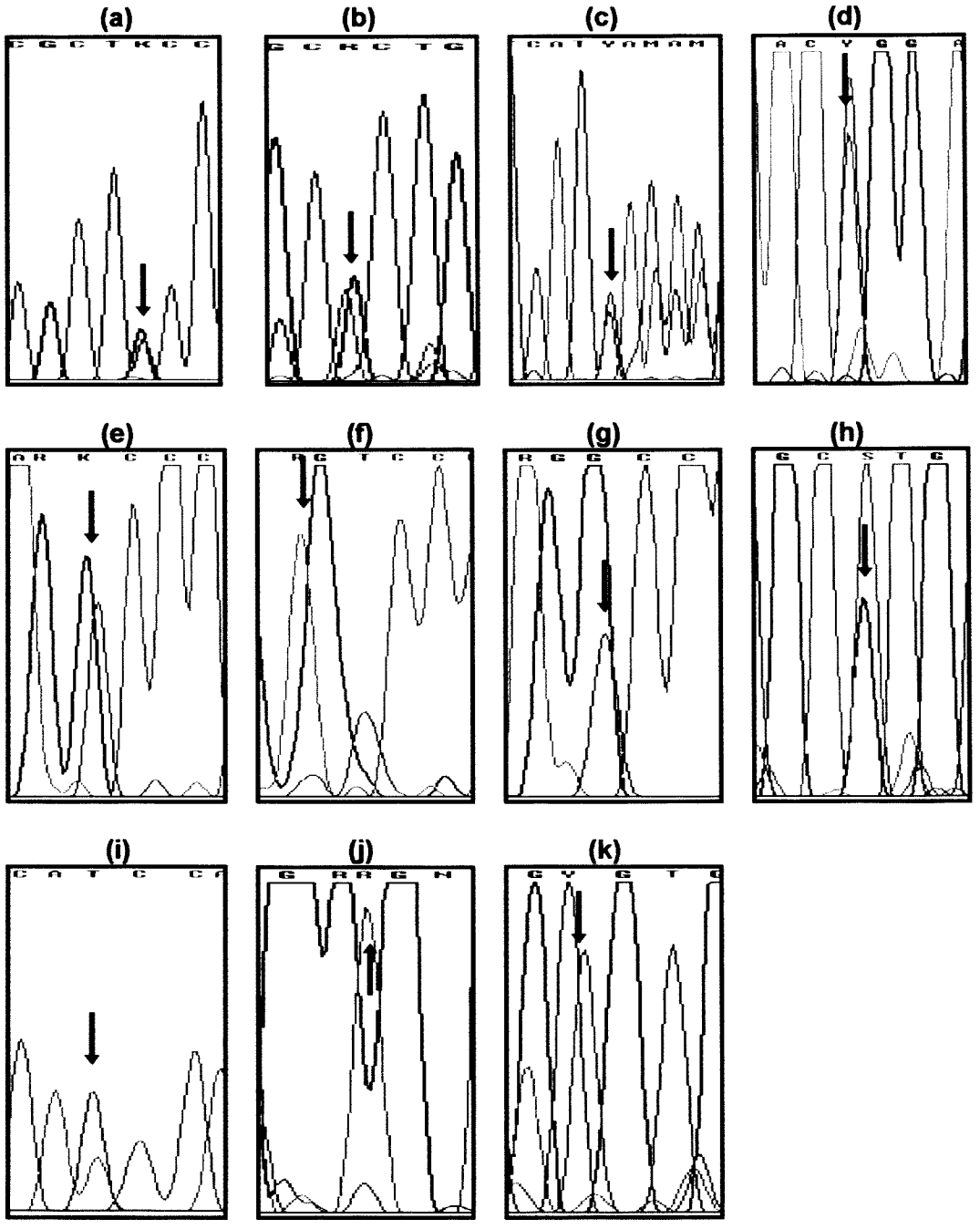

Figure 2.8 Automated sequencing results of patients **(a)** 4 **(b)** 33 **(c)** 1 **(d)** 8 **(e)** 9 **(f)** 19 **(g)** 32 **(h)** 39 **(i)** 12 **(j)** 23 and **(k)** 24. The arrows indicated the nucleotide changes observed in each of these 11 cases.

Table 2.2 A summary of the nucleotide changes, codon positions as well as the final amino acid alterations found in exons 5 to 8 of *TP*53 in various laryngeal cancer (LC) tumor biopsies investigated in this study

Exon	Patient no	Ethnic group and sex/age	Codon	Mutation	Amino Acid Alteration
5	4	CM/48	176	TGC→TTC	Cys→Phe
	33	CM/45	175	CGC→CAC	Arg→His
6	-	-	-	-	-
7	1	N/A	255	ATC→ATTC	Frameshift
	8	CM/66	248	CGG→TGG	Arg→Trp
	9	CM/69	249	AGG→AGT	Arg→Ser
	19	N/A	249	AGG→AGT	Arg→Ser
	32	CM/62	249	AGG→AGT	Arg→Ser
	39	CM/46	232	ATC→AAC	Ile→Asn
8	12	N/A	273	CGT→CCT	Arg→Pro
	23	CM/51	266	GGA→AGA	Gly→Arg
	24	WM/65	273	CGT→TGT	Arg→Cys

Abbreviations: CM, Coloured male; N/A, not available at time of study; WM, white male.

2.3.4 Association of observed nucleotide changes to etiologic factors

All patients and members of the control group completed questionnaires. Aspects covered by the questionnaires included ethnic group, gender, date of birth, place of birth, alcohol and tobacco consumption, family history of cancer, source of cigarettes and average number of cigarettes per day, source of tobacco for pipe smoking, chewing tobacco, drinking and source of beer, drinking and source of spirits, use and source of tribal medicine, induced vomiting and method of induction, and numerous aspects of their dietary habits.

The data compiled for both the patients and the control group were used to test for correlations between possible etiologic factors and the occurrence of

mutations within *TP*53 and to test for a correlation between LC and the type of mutation observed. Spearman rank correlations were determined to detect any significant associations. Significant correlations were found between the occurrence of LC and the following: age, smoking, eating meat on a daily basis and drinking milk on a daily basis (Table 2.3). The results were then further stratified by dividing the groups (patients and controls) into two categories (smokers and non-smokers), where after the Spearman rank correlation was repeated (Table 2.4). It was found that 3 of 23 non-smokers (13%) and 30 of 55 smokers (55.4%) were diagnosed with LC. Both milk and meat consumed on a daily basis resulted in negative correlations with the occurrence of LC (Table 2.3), thus indicating that they may be protective factors against the development of LC. In contrast, smoking and age displayed positive correlations with the occurrence of LC, thus indicating these as risk factors for the development of LC. However, a negative correlation between daily milk consumption and cigarette smoking was noted (Table 2.3). From Table 2.4, where results were stratified according to smoking, it can be seen that the association between daily milk consumption and the occurrence of LC was not significant in either group (smoking or non-smoking). The association in Table 2.3 is therefore spurious, and due to the correlation with cigarette smoking, which exerts a major effect. In non-smokers, therefore, age is the only risk factor identified. In smokers, age is a risk factor, but with a lower correlation coefficient than in non-smokers, and high daily meat consumption remains correlated with protection against the development of LC (Table 2.4).

Table 2.3 Interrelationships between possible etiological factors and laryngeal cancer

Characteris-tics	Laryngeal Cancer	Pathology Report	Age	Smoking Cigarettes	Ever smoked cigarettes	Meat Daily
Pathology Report	n=78					
	cc=-0.970					
	p=0.000					
Age	n=80	n=74				
	cc=0.479	cc=-0.445				
	p=0.000	p=0.000				
Smoking Cigarettes	n=78	n=73	n=77			
	cc=0.304	cc=-0.229	cc=0.136			
	p=0.007	p=0.051	p=0.238			
Smoking Cigarettes ever	n=78	n=73	n=77	n=78		
	cc=0.383	cc=-0.317	cc=0.229	cc=0.851		
	p=0.001	p=0.006	p=0.045	p=0.000		
Meat Daily	n=81	n=75	n=80	n=78	n=78	
	cc=-0.436	cc=0.453	cc=-0.257	cc=-0.132	cc=-0.180	
	p=0.000	p=0.000	p=0.021	p=0.250	p=0.114	
Milk Daily	n=81	n=75	n=80	n=78	n=78	n=81
	cc=-0.264	cc=0.275	cc=-0.053	cc=-0.331	cc=-0.293	cc=-0.35
	p=0.017	p=0.017	p=0.6390	p=0.003	p=0.009	p=0.757

n = total number of subjects with information on the particular parameter; out of a total of 84 subjects in study
cc = correlation coefficient
p = significance level

Table 2.4 Interrelationships between possible etiological factors and LC

Group	Laryngeal Cancer	Age	Milk Daily
Smokers			
Age	n=54		
	cc=0.317		
	p=0.019		
Milk Daily	n=55	n=54	
	cc=-0.214	cc=0.000	
	p=0.116	p=1.000	
Meat Daily	n=55	n=54	n=55
	cc=-0.413	cc=-0.078	cc=-0.080
	p=0.002	p=0.576	p=0.560
Non-smokers:			
Age	n=23		
	cc=0.584		
	p=0.003		
Milk Daily	n=23	n=23	
	cc=-0.233	cc=0.049	
	p=0.284	p=0.8255	
Meat Daily	n=23	n=23	n=23
	cc=-0.259	cc=-0.303	cc=-0.012
	p=0.232	p=0.160	p=0.957

n = total number of subjects out of a total of 84 subjects with information on the particular parameter;
cc = correlation coefficient; p, significance level.

2.4 Discussion

In this study, *TP53* was examined in a set of laryngeal cancer patients (n=44) all having received treatment at Tygerberg Hospital, Cape Town, South Africa. To date, the majority of *TP53* mutations have been found within exons 5-8, the most evolutionary conserved region coding for the DNA binding domain (Hainaut 2001), and it is for this reason that we decided to focus on this region. However, it is possible that mutations may occur within the intronic sequences, as well as in exons 1-4 and 9-11, which were not investigated here. A few studies (Bradford et al. 1997; Zhang et al. 1994; Sarkar et al. 1996; Gottschlich et al. 2000) have been conducted to investigate *TP53* mutations in LC using PCR-SSCP analysis combined with direct sequencing. The results indicate that the tumors contain mutations within exons 5-8 at varying frequencies, ranging from $^{4}/_{32}$ (12,5%) (Gottschlich et al. 2000), $^{19}/_{61}$ (31,3%) (Sarkar et al. 1996), $^{7}/_{44}$ (39%) (Bradford et al. 1997) to $^{13}/_{40}$ (32,5%) (Zhang et al. 1994). This represents an average of 25%. Early reports (Jego et al. 1993) indicated that 90% of the mutations in *TP53* from a variety of human cancers were single-point mutations (n=740) and the remainder consisted of small insertion/deletions. This study identified mutations in *TP53* sequence in 25% of the cases of which 91% were single-base changes, suggesting a unifying molecular mechanism. Factors possibly responsible for this include environmental agents as well as dietary components. It has also been reported that small insertion/deletions within *TP53* are common in ovarian cancer (Skilling et al. 1996) and oesophageal cancer (Vos et al. 2003) and a slippage mechanism due to mismatch DNA-repair deficiency can be suggested as being responsible for the T-insertion observed in this study. Data obtained from questionnaires completed by the patients, as well as the control group, allowed correlation studies to identify smoking and age as risk factors for the development of LC. Furthermore, daily consumption of meat (excluding fish) has been identified as being a protective factor against the development of LC. This protective effect of high meat consumption could be either a direct nutritional effect, or meat may be a marker for higher socio-economic status, and therefore an indication of other factor(s) involved in

protection against the development of LC. The larynx is where the common aero-digestive tract splits into the trachea and the oesophagus and smoking has been identified as a risk factor for oesophageal cancer (Montesano et al. 1996), as well as for lung cancer (Steele et al. 1998; Soussi et al. 2001; Soussi et al. 2001). The typical lung cancer *TP53* mutations associated with smoking (G → T transversions and G → A and C → T transitions) (Zhang et al. 1994) accounted for 73% of the mutations found within this study. Although the mutations observed in this study fall within a known *TP53* hotspot for cancers in general, it is interesting to note that the particular mutations at codons 175, 176, 248, 249, 266 and 273, accounting for 82% of the mutations observed in this study, are known lung cancer hotspots (Soussi et al. 2001). Similarly, the particular mutations at codons 175, 176, 248, 249, 255 and 273 are known oesophageal cancer hotspots (Soussi et al. 2001), and also account for 82% of the mutations observed in this study. Thus, the *TP53* mutations common to laryngeal cancer (this study), oesophageal cancer and lung cancer are codons 175 (G → A), 176 (G → T), 248 (C → T), 249 (G → T), 273 (G → A) and 273 (C → T) (Soussi et al. 2001). Furthermore, the overlap between LC and lung cancer (82%) and between LC and oesophageal cancer (82%) implies that, in terms of mutations observed, there is a 73% overlap between all three of these cancers. Comparing the results of this study to those reported elsewhere (Zhang et al. 1994; Maestro et al. 1992; Boyle et al. 1993; Somers et al. 1992; Maestro et al. 1992; Boyle et al. 1993; Somers et al. 1992) we find that the mutations at codons 175, 176 and 248 have been previously identified as *TP53* mutations in LC in other geographical areas. However, the mutations at codons 232, 249, 255, 266 and 273 have not been previously reported, making them novel mutations. It was also found that 100% of mutations in this study occurred between codons 175 and 273, confirming the presence of a known hotspot region within *TP53*. It should also be noted here that the nucleotide changes observed within this study are not necessary the primary cause of malignancy and could merely be a secondary event, implying that such nucleotide changes are not necessarily disease causing. The predominant mutation at codon 249 (G → T) is associated with aflotoxin B exposure in conjunction with hepatitis B viral

infection in hepatocellular carcinoma (Dominquez-Malagon and Gayton-Graham 2001).Although there is no data to suggest the involvement of either of these two etiological factors in the carcinogenesis of LC, this study has shown that the involvement of a virus or a toxin, within this unique geographical area, is a possibility, and requires further investigation.

2.5 Acknowledgements

We would to acknowledge the Cancer Association of South Africa (CANSA) for funding the project via the Oesophageal Cancer Consortium as well as aiding in personal bursaries and travel fees for the attendance of Cancer of the Esophagus and Gastric Cardia: From Gene to Cure", held in Amsterdam, the Netherlands during the period 13 – 15 December 2002.

We would also like to acknowledge PROMEC for providing a basis for the questionnaires used in this study.

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Chapter 3

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3.1 Overview

The aim of this study was thus to analyse the molecular structure of *TP*53, specifically exons 5 – 8, in a group of Coloured esophageal cancer (EC) patients treated at Tygerberg Hospital, Cape Town, South Africa. This involved the screening of DNA obtained from the patient tumor biopsies as from blood obtained from the same patients, via PCR-SSCP and direct sequencing analysis. The participants of this study were also asked to complete questionnaires in order to determine possible etiological factors (a study undertaken by the MRC). The goal of this study would be to determine whether there are differences in the *TP*53 mutational pattern observed in the Coloured Western Cape community as compared to that observed in the Black Transkei community. The value of the knowledge obtained from this genetic study in alliance with that obtained from the epidemiological study undertaken by the MRC has far reaching effects in practice, placing epidemiologists in an advantageous position in their pursuit to unravel the influence of various environmental factors on carcinogenesis. This could allow educational programs to be initiated within communities at higher risk for the development of esophageal cancer, promoting an understanding of the causes and risks for cancer development within each unique population.

The institutional ethics committee approved this study and informed consent was obtained from all subjects.

3.2 Materials and Methods

3.2.1 Collection of tumor samples

Fresh tumor biopsies were obtained from patients over the period from 1997 to 2003. A variety of population groups are represented in this sample collection, including the Indian, Caucasian, Black and Coloured (discussed in section1.4) communities native to South Africa. However, for the purposes of this study, 44 Coloured patients were selected to form part of an initial screening. All patients received treatment and underwent surgery at Tygerberg Hospital, Cape Town, South Africa.

During the surgical procedure of obtaining a biopsy for diagnostic purposes, a second biopsy was taken for research purposes. This second biopsy (for research) was snap-frozen in liquid nitrogen immediately after surgical removal and stored at -70°C for further molecular analysis in disposable polypropylene cryogenic vials (Appendix II). Only the biopsy obtained for diagnostic purposes were originally diagnosed histologically and the resulting diagnosis was assumed for the second biopsy (obtained for research purposes).

3.2.2 Collection of surrounding tissue samples

During the surgical procedure of obtaining a biopsy for diagnostic purposes, a second piece of apparently healthy surrounding tissue was taken for research purposes. This apparently healthy surrounding tissue sample was snap-frozen in liquid nitrogen immediately after surgical removal and stored at -70°C for further molecular analysis in disposable polypropylene cryogenic vials (Appendix II). Only the biopsy obtained for diagnostic purposes were originally diagnosed histologically and the surrounding tissue was assumed to be healthy and free of any tumorigenic cells.

3.2.3 Collection of blood samples

Blood samples were collected in 5 ml disodium ethylenediaminetetraacetate dehydrate (EDTA) tubes (Appendix II) from the same patients (discussed in section 3.2.1) and frozen for further molecular analysis. Data obtained from the blood samples would serve as a control to test for a loss of heterozygosity (LOH). This would serve as an indication as to whether any nucleotide alterations observed within the tumor samples were somatic in origin, or whether they were germline mutations.

3.2.4 Collection of control samples

Due to difficulties encountered with ethical approval at the time of the study, it was not possible to collect any external control samples for this study.

3.2.6 DNA extractions

3.2.6.1 Tumor biopsy and surrounding tissues

DNA extractions were performed on the tumor biopsy samples as well as the apparently healthy surrounding tissue samples as discussed in section 2.2.4.1.

3.2.6.2 Blood Samples

DNA extractions were performed on the blood samples as discussed in section 2.2.4.2.

3.2.7 Polymerase Chain Reaction/PCR

3.2.7.1 Principals of PCR

See section 2.2.5.1.

3.2.7.2 Oligonucleotide primers

See section 2.2.5.2.

3.2.7.3 Methodology

PCR was performed on all three sample sets discussed in sections 3.2.1 to 3.2.3. For more detail see section 2.2.5.3.

3.2.8 Gel Electrophoresis

3.2.8.1 1% DNA Agarose gel electrophoresis

Successful PCR amplification as well as the absence of contamination was verified by electrophoresing 10 µl of the amplified product on 1% DNA agarose gels (Appendix III).

3.2.8.1.1 Preparation of the gel

The gels (Appendix III) were cast in 85mm × 150mm to a thickness of approximately 5mm. The Perspex moulds were first washed with Cal-liquid hand soap (Appendix II) and rinsed with tap water. A wash with 70% ethanol (Appendix III) and drying with paper towel followed this. After pouring the gel

solution (Appendix III), the well-forming combs were inserted and the gels were left to set for 30 minutes prior to loading and electrophoresis.

3.2.8.1.2 Electrophoresis

See section 2.2.6.1.2.

3.2.9 Single-strand conformation polymorphism (SSCP) analysis

3.2.9.1 Principals of SSCP

See section 2.2.7.1.

3.2.9.2 Methodology

3.2.9.2.1 8% Polyacrylamide SSCP gels supplemented with 10% urea

3.2.9.2.1.1 Casting the gel

See section 2.2.7.2.1.1.

3.2.9.2.1.2 Electrophoresis

See section 2.2.7.2.1.2.

3.2.9.2.1.3 Silver staining

See section 2.2.7.2.1.3.

3.2.9.2.2 Scoring of the 8% polyacrylamide SSCP gels supplemented with 10% urea

See section 2.2.7.2.2

3.2.9.2.3 Reproducibility of the 6% polyacrylamide SSCP gels supplemented with 10% urea

See section 2.2.7.2.3

3.2.9.2.4. Second set of SSCP conditions: 10% polyacrylamide SSCP gels supplemented with 5% glycerol and 17% urea

See section 2.2.7.2.4. The same approach was used as described in section 2.2.7.2.1 with the exception that a 10% polyacrylamide gel solution (Appendix III) was used instead of a 6% polyacrylamide gel solution (Appendix III).

3.2.10 Sequencing analysis

3.2.10.1 Amplicon purification

Samples were subsequently selected for sequencing from all three of the sample sets (tumor biopsies as well as surrounding tissue and blood from the same patients) to represent at least one of each of the unique SSCP banding patterns observed across exons 5 to 8. Amplicons were purified using the Nucleospin kit (Appendix II), following the instructions recommended by the manufacturer.

3.2.10.2 Automated sequencing

See section 2.2.8.2.

3.2.11 Solving potentially discrepant results

In cases where SSCP results were non-reproducible or contradictory to sequencing results obtained, the following 3 approaches were taken:

i. The discrepant samples were re-amplified using *Taq* DNA polymerase (Appendix II) as described in section 2.2.5.3. The PCR products were then purified using the Nucleospin kit (Appendix II) and this was followed by a second round of automated sequencing as described in section 2.2.8.2

ii. The same discrepant samples were re-amplified using HotStarTaq™ DNA Polymerase (AppendixII) as described as section 2.2.5.3. These PCR products were once again purified using the Nucleospin kit (Appendix II) and succeeded by a second round of automated sequencing as described in section 2.2.8.2.

iii. Simultaneously, the discrepant SSCP bands of interest were excised from the SSCP gel, using a sterile scalpel blade (Appendix II). The DNA was eluted by mashing the gel pieces with a pipette-tip in a 1,5ml Eppendorf tube (Appendix II) and adding 10-20 μl dH_2O. This was then left overnight at 4°C and incubated at 37°C for 10 minutes prior to its use as a template for a subsequent PCR reaction (again using Taq Polymerase as described in section 2.2.5.3). Once amplified, the PCR products were purified using the Nucleospin kit (Appendix II), followed by another round of automated sequencing as described in section 2.2.8.2.

3.2.12 Histological re-evaluation of tumorigenic cells

Based on uncertainties observed in the appearance of the SSCP banding patterns, 20 tumor samples were randomly selected to be histologically re-analyzed for the presence of tumorigenic cells as previously described (Alan Stevens James Lowe 1997).

3.3 Results

3.3.1 1% Agarose gel electrophoresis

Before starting the SSCP analyses, all amplicons were verified on 1% agarose gels in order to confirm the presence of the expected product as well as the absence of any contamination, as can be seen in Figure 3.1.

3.3.2 8% Polyacrylamide SSCP gels supplemented with 10% urea

All SSCP gels were scored individually and samples were selected for sequencing to represent each uniquely scored banding pattern. Mobility shifts were detected in exons 6 and 8 of the tumor biopsy sample set (Figures 3.3 (a) and 3.5 (a)), but none in exons 5 and 7. The results obtained from the various SSCP gels (Figures 3.2 – 3.5) indicate that no mobility shifts were detected within the blood from the same patients sample set, neither within the apparently healthy surrounding tissues from the same patients. This suggests that the nucleotide changes observed have somatic origins and are not germline mutations. All SSCP analyses were repeated once under identical conditions as well as under a different set of conditions, as described in sections 3.2.9.2.3 and 2.3.9.2.4, however, only representative examples of the initial SSCP screen are given in Figures 3.2 – 3.5.

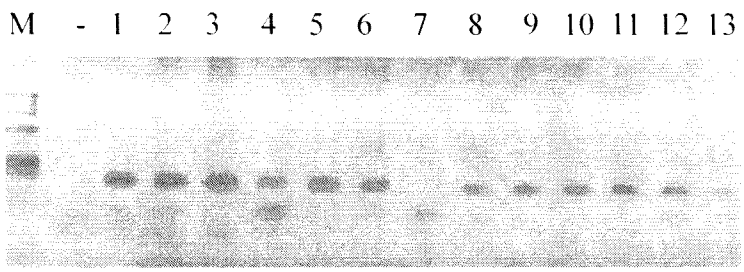

Figure 3.1 A representative example of one of the 1% agarose gels that were used to verify the presence of amplicons as well as the absence of DNA contamination during PCR. This gel demonstrates the successful amplification of exon 8 for samples as well as the absence of contamination of the negative lane (-). A DNA marker (Appendix III) was loaded in lane M.

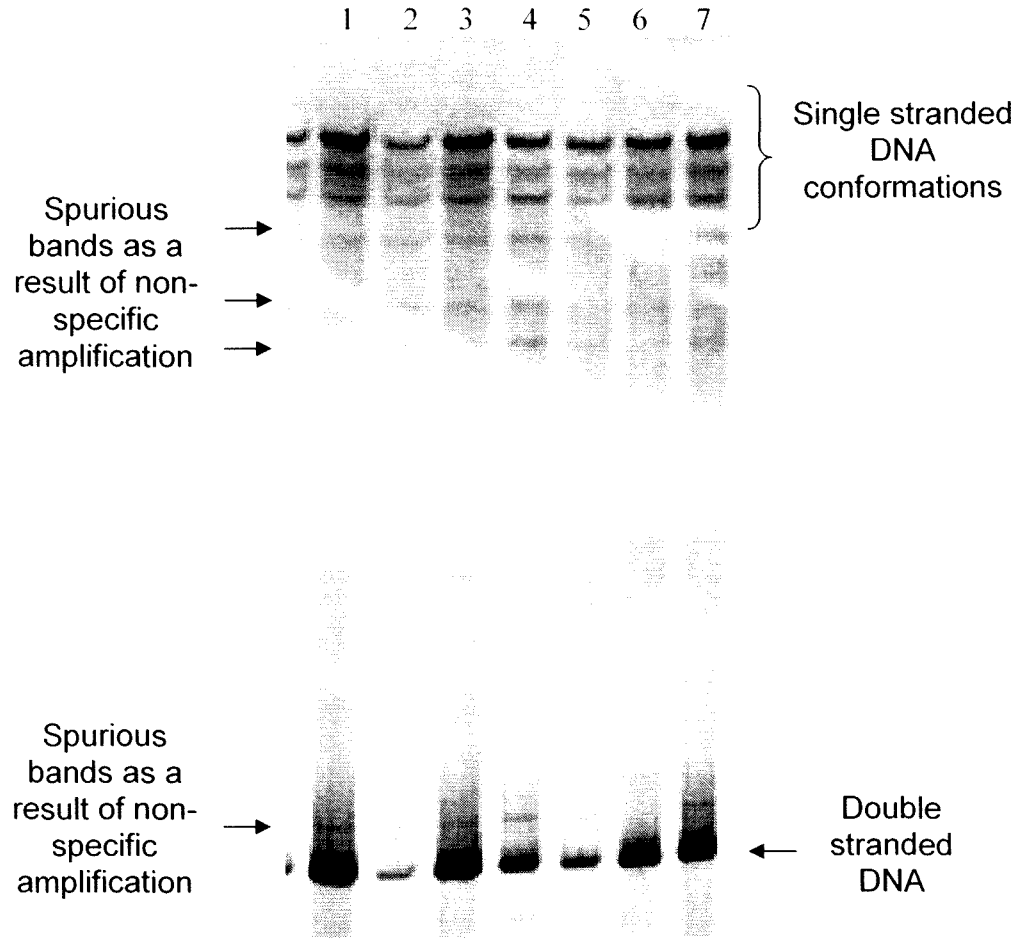

Figure 3.2 (a) A representative example of the SSCP banding patterns detected in exon 5 of *TP53* in DNA obtained from the tumor biopsy samples of 7 patients; on a 6% polyacrylamide SSCP gel supplemented with 10% urea.

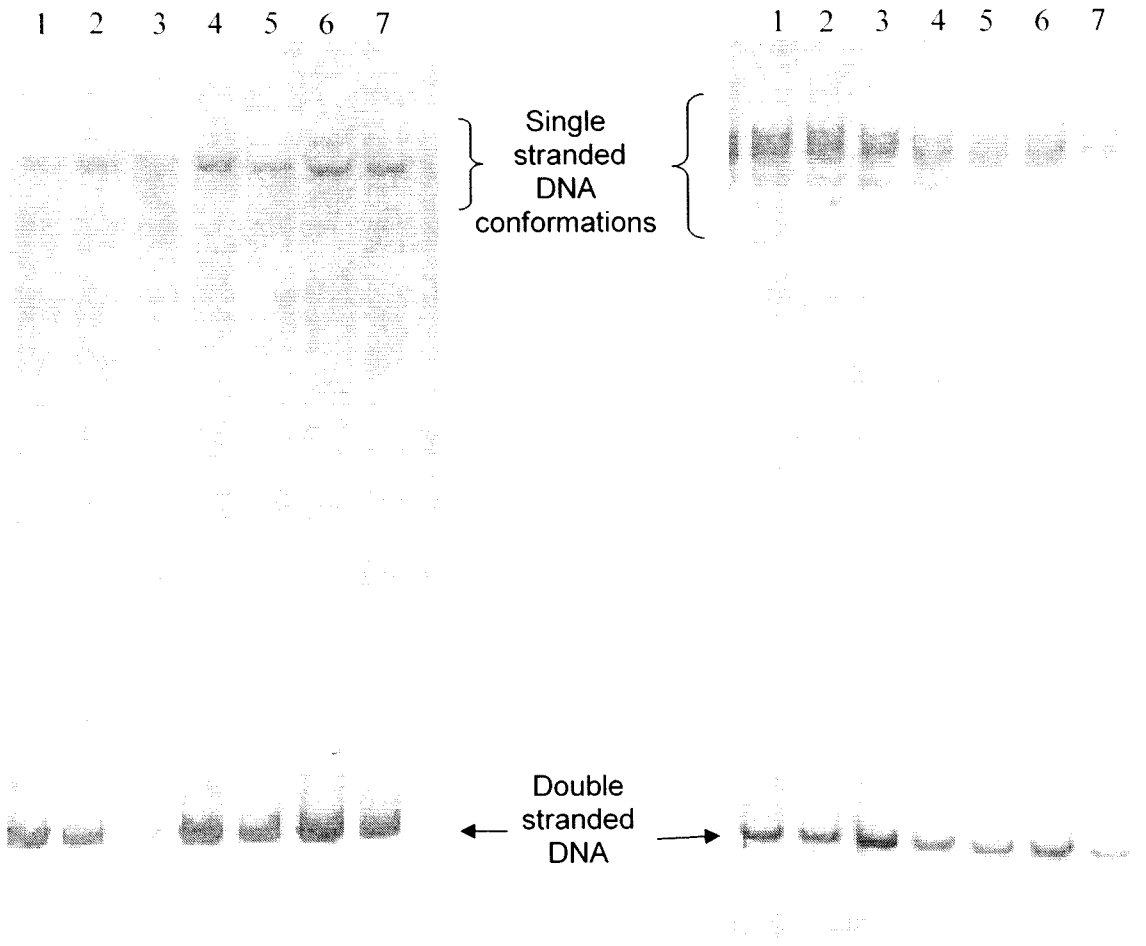

Figure 3.2 (b) A representative example of the SSCP banding patterns detected in exon 5 of *TP*53 in DNA obtained from the apparently healthy surrounding tissue of 7 patients (left) as well as from the corresponding blood of 7 of the patients (right); on a 6% polyacrylamide SSCP gel supplemented with 10% urea.

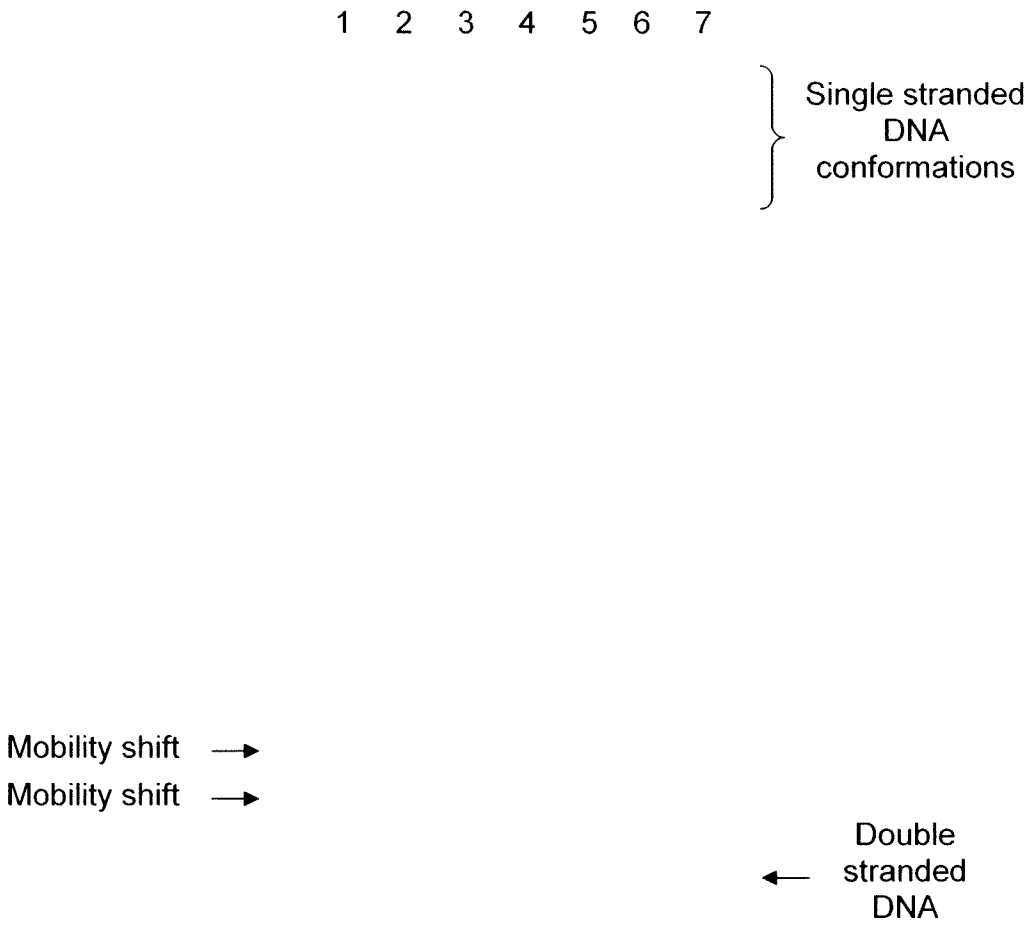

Figure 3.3 (a) A representative example of the SSCP banding patterns detected in exon 6 of *TP*53 in DNA obtained from the tumor biopsy samples of 7 patients; on a 6% polyacrylamide SSCP gel supplemented with 10% urea.

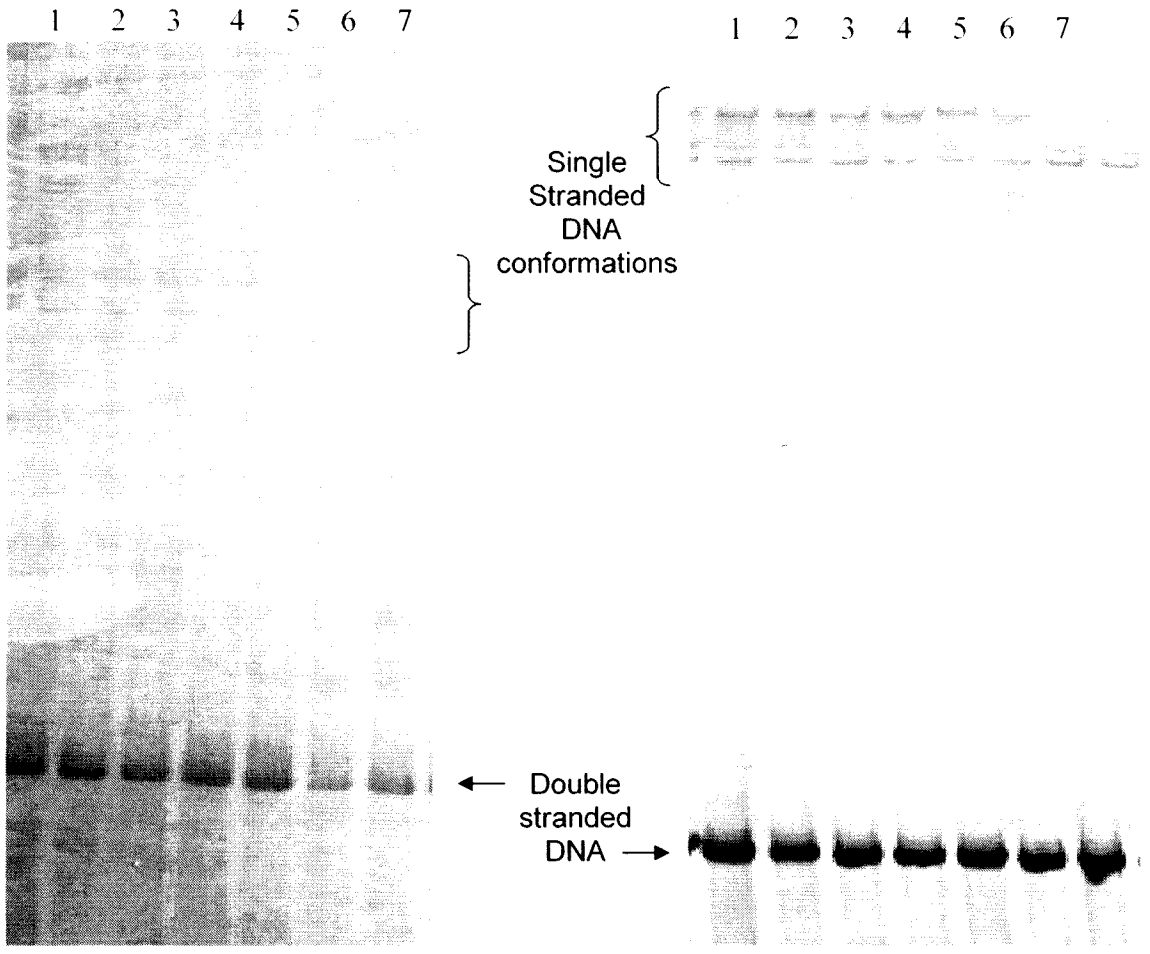

Figure 3.3 (b) A representative example of the SSCP banding patterns detected in exon 6 of *TP*53 in DNA obtained from the apparently healthy surrounding tissue of 7 patients (left) as well as from the corresponding blood of 7 of the patients (right); on a 6% polyacrylamide SSCP gel supplemented with 10% urea.

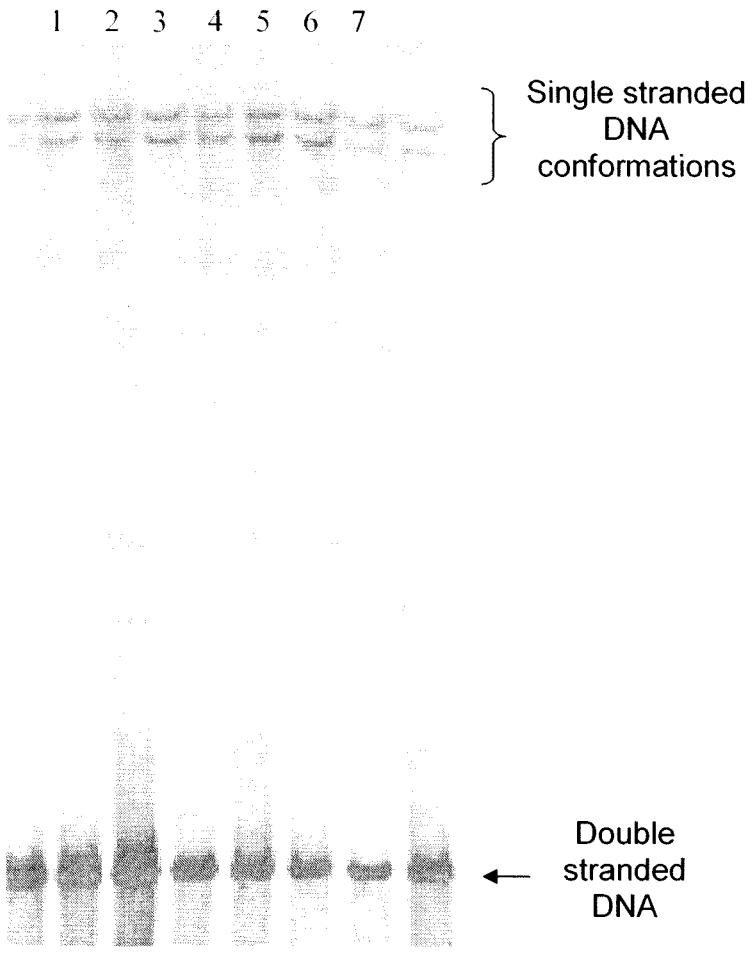

Figure 3.4 (a) A representative example of the SSCP banding patterns detected in exon 7 of *TP*53 in DNA obtained from the tumor biopsy samples of 7 patients; on a 6% polyacrylamide SSCP gel supplemented with 10% urea.

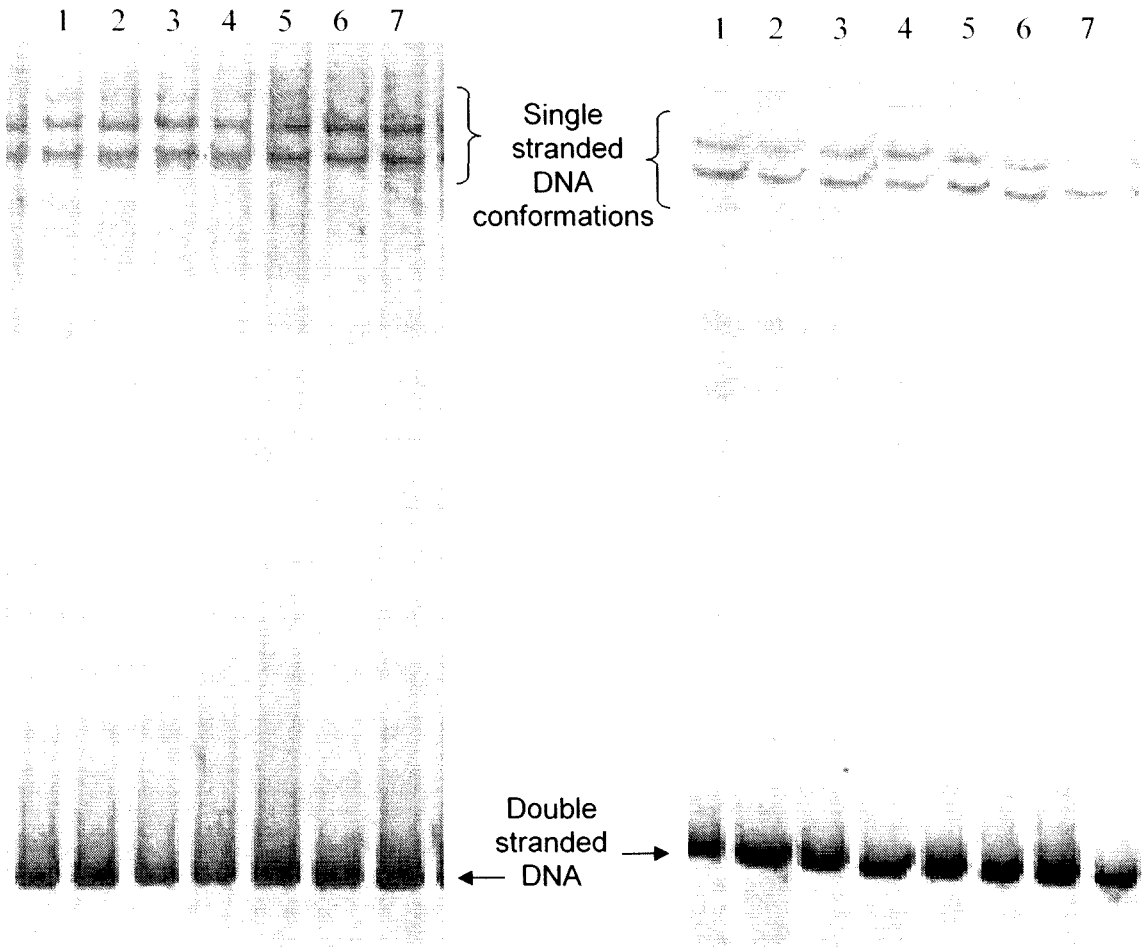

Figure 3.4 (b) A representative example of the SSCP banding patterns detected in exon 7 of *TP*53 in DNA obtained from apparently healthy surrounding tissue of 7 patients (left) as well as from the corresponding blood of 7 of the patients (right); on a 6% polyacrylamide SSCP gel supplemented with 10% urea.

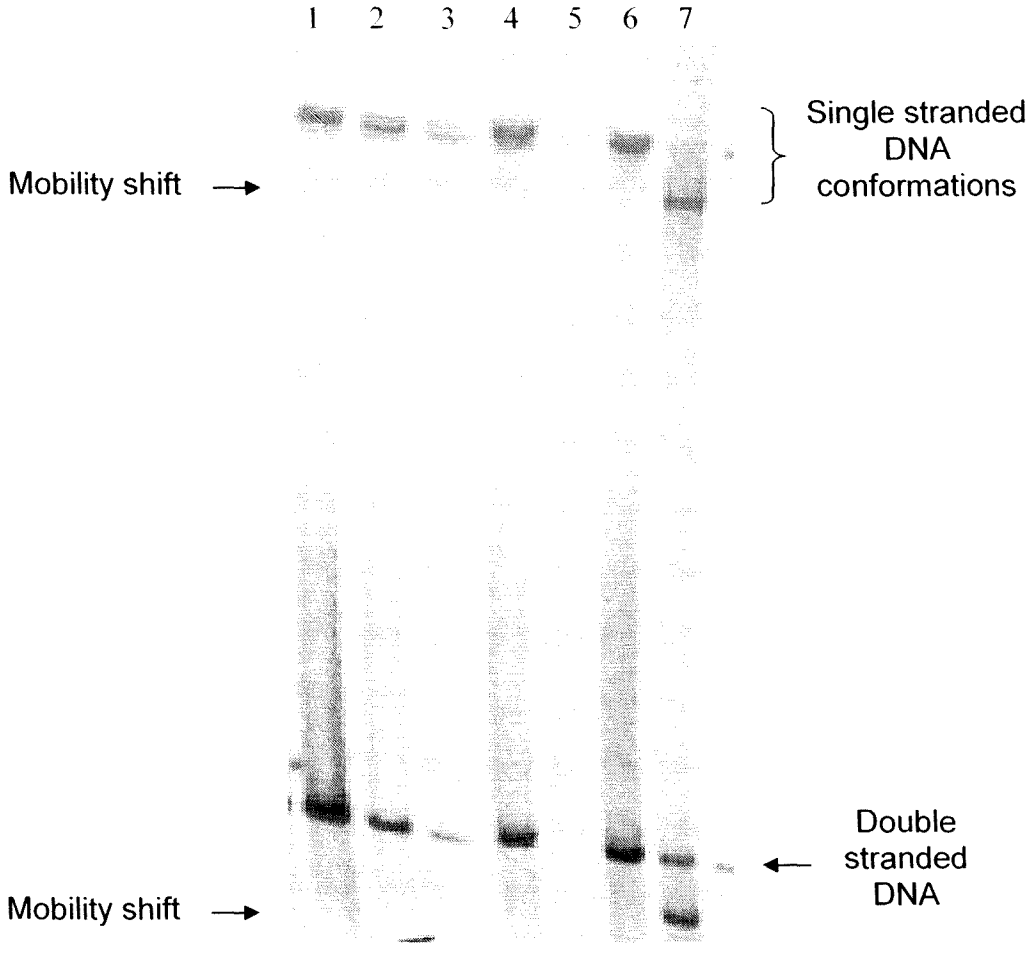

Figure 3.5 (a) A representative example of the SSCP banding patterns detected in exon 8 of TP53 in DNA obtained from the tumor biopsy samples of 7 patients; on a 6% polyacrylamide SSCP gel supplemented with 10% urea.

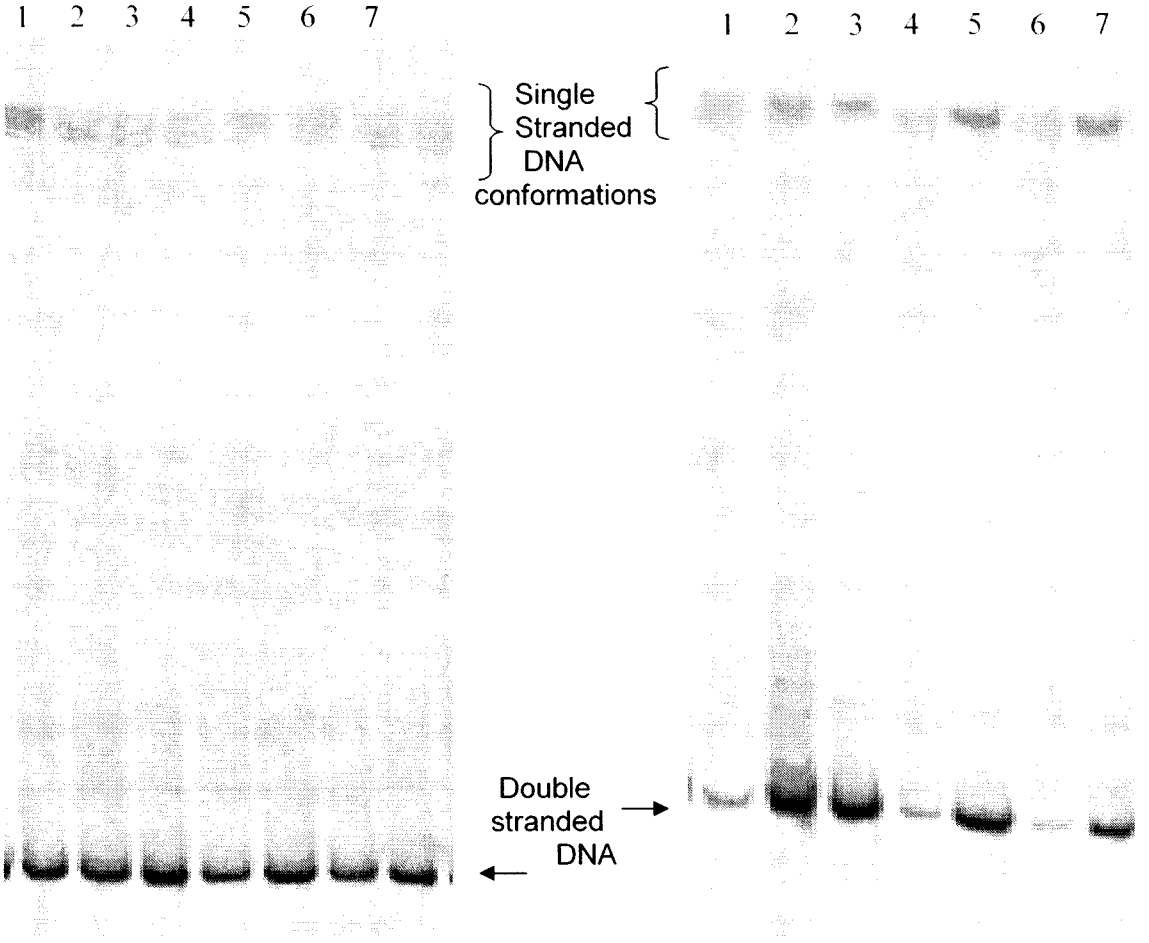

Figure 3.5 (b) A representative example of the SSCP banding patterns detected in exon 8 of TP53 in DNA obtained from apparently healthy surrounding tissue of 7 patients; on a 6% polyacrylamide SSCP gel supplemented with 10% urea.

3.3.3 Automated sequencing

From 124 sequences obtained, only 4 nucleotide changes were observed in 3 different tumor samples (Figures 3.6 (a) to (c)). One single nucleotide change (C – T) was found in codon 192 of exon 6 resulting in a stop signal. One GA deletion was found in codon 209 of exon 6 which resulted in an early stop signal at codon 214. Furthermore, a 15bp deletion was found in codon 276 of exon 8, resulting in a frameshift. Interestingly, another homozygous nucleotide change was found within the same sample, further downstream, at codon 283 (C – T). In each case, both forward and reverse sequences were obtained in order to confirm such nucleotide changes. These can be found summarized in Table 3.1.

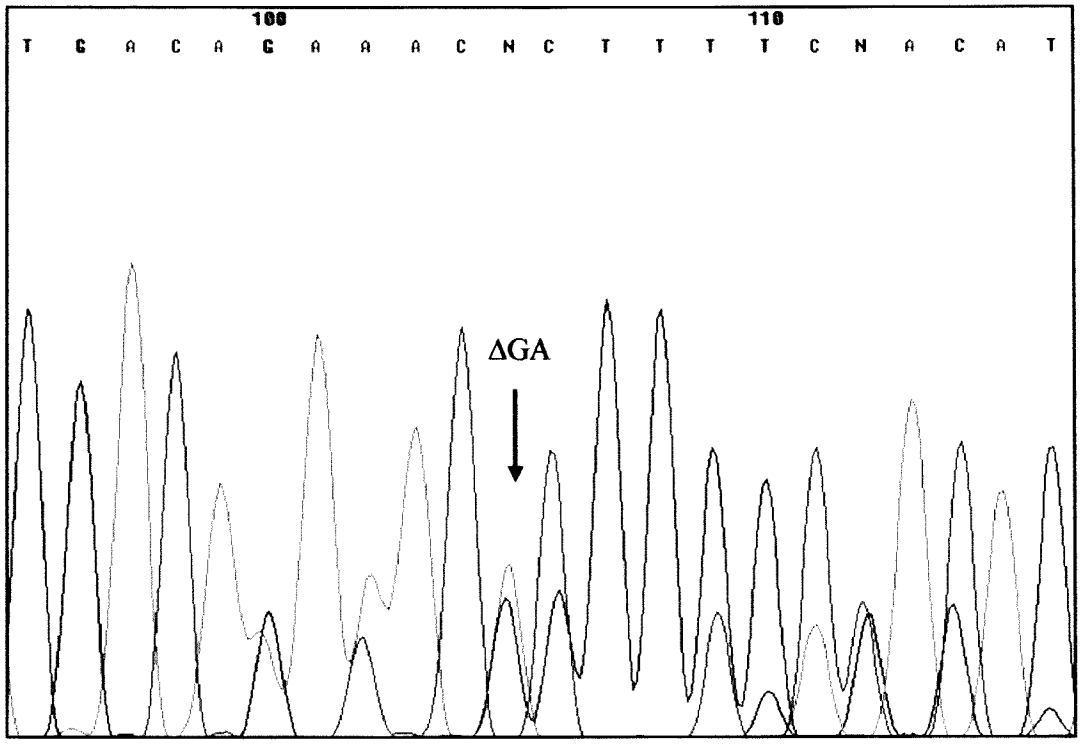

Figure 3.6 (a) Automated sequencing results obtained from the DNA extracted from the tumor biopsy sample of patient no 49. This chromatograph demonstrates a 2bp (GA) deletion – indicated as ΔGA - in exon 6 of TP53 (as compared to the wild type sequence deposited in the National Centre for Biotechnology Information [NCBI] nucleotide database (accession number X54156)).

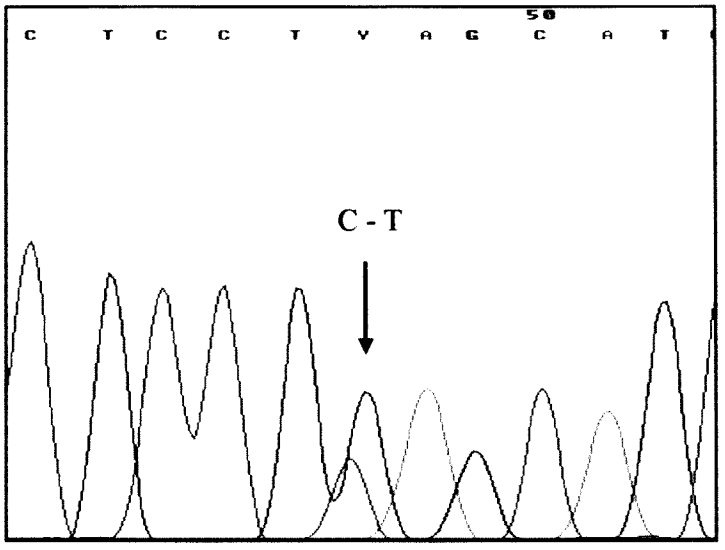

Figure 3.6 (b) Automated sequencing results obtained from the DNA extracted from the tumor biopsy sample of patient no 59. This chromatograph demonstrates a single nucleotide alteration (indicated by an arrow) within exon 6 of TP53 (as compared to the wild type sequence deposited in the National Centre for Biotechnology Information [NCBI] nucleotide database (accession number X54156)).

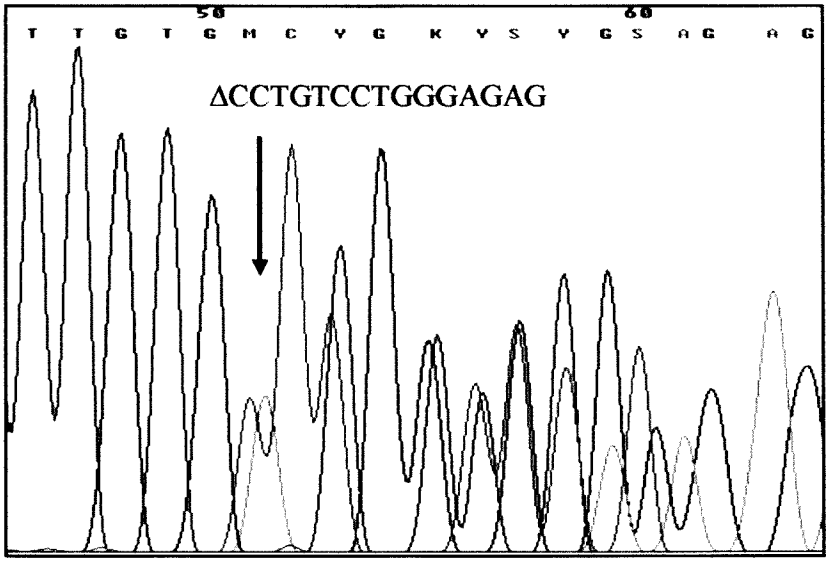

Figure 3.6 (c) Automated sequencing results obtained from the DNA extracted from the tumor biopsy sample of patient no 73. This chromatograph demonstrates a 15bp deletion - indicated as ΔCCTGTCCTGGGAGAG - in exon 8 of TP53 (as compared to the wild type sequence deposited in the National Centre for Biotechnology Information [NCBI] nucleotide database (accession number X54156)).

Table 3.1 A summary of the nucleotide changes confirmed with sequence analysis

Exon	Patient no.	Sex/ Age	Codon	Nucleotide alteration	Amino Acid Alteration
6	49	F/58	209	GA deletion	Frameshift – stop at codon 214
6	59	M/n/a	192	CAG - TAG	Stop codon
8	73	M/41	276	15bp deletion	Frameshift
8	73	M/41	283	CGC – TGC	Arg - Cys

Abbreviations: F, Female; M, Male; n/a, not available at time of study;

3.3.4 Solving potentially discrepant results

In an attempt to clarify the discrepant results obtained from the initial SSCP analysis of exon 6 of *TP*53 (Figure 3.7(a)), several approaches were taken, as described in section 3.2.11. Initially, the original amplicons were re-analyzed on a second SSCP gel under identical conditions (Figure 3.7(b)), however, these results proved to be non-reproducible. Another approach was to re-amplify the samples in question, using *Taq* DNA polymerase (Appendix II) as well as HotStarTaq™ DNA Polymerase (AppendixII) as described as section 2.2.5.3, following by purification using the Nucleospin kit (Appendix II). These purified samples were then used for a second round of automated sequencing, which revealed the samples to be identical to the wild type sequence deposited in the National Centre for Biotechnology Information [NCBI] nucleotide database (accession number X54156). Lastly, the discrepant bands of interest were excised from the original SSCP (Figure 3.7(a)) as described in section3.2.11, and used as template DNA in a further PCR reaction. These amplicons were then purified using the Nucleospin kit (Appendix II) and used in an automated sequencing reaction. The sequence obtained was identical to the wild type sequence deposited in the National Centre for Biotechnology Information [NCBI] nucleotide database (accession number X54156).

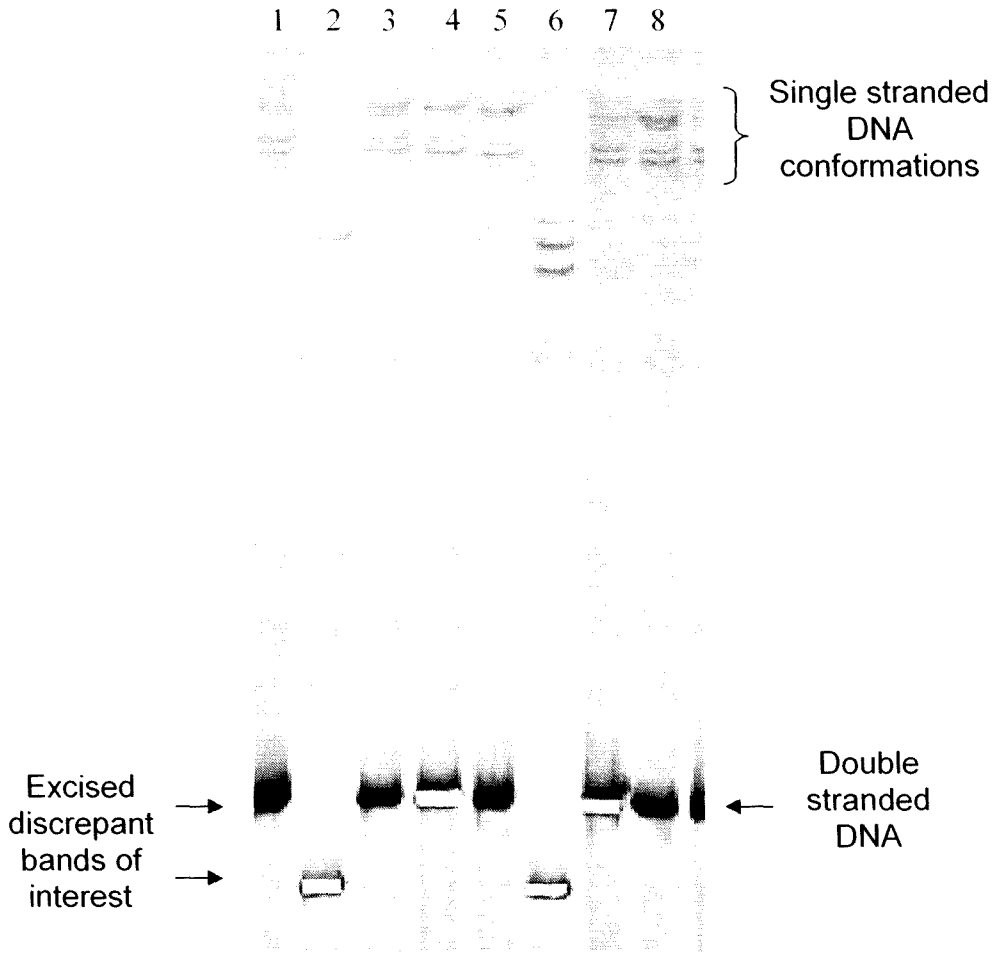

Figure 3.7 (a) A representative example of the discrepant SSCP banding patterns detected in exon 6 of TP53 in DNA obtained from tumor biopsy tissue of 7 of the patients; on a 6% polyacrylamide SSCP gel supplemented with 10% urea. This photo demonstrates how the discrepant bands of interest were excised from the SSCP gel for use as DNA template in a further PCR reaction as described in section 3.2.10.

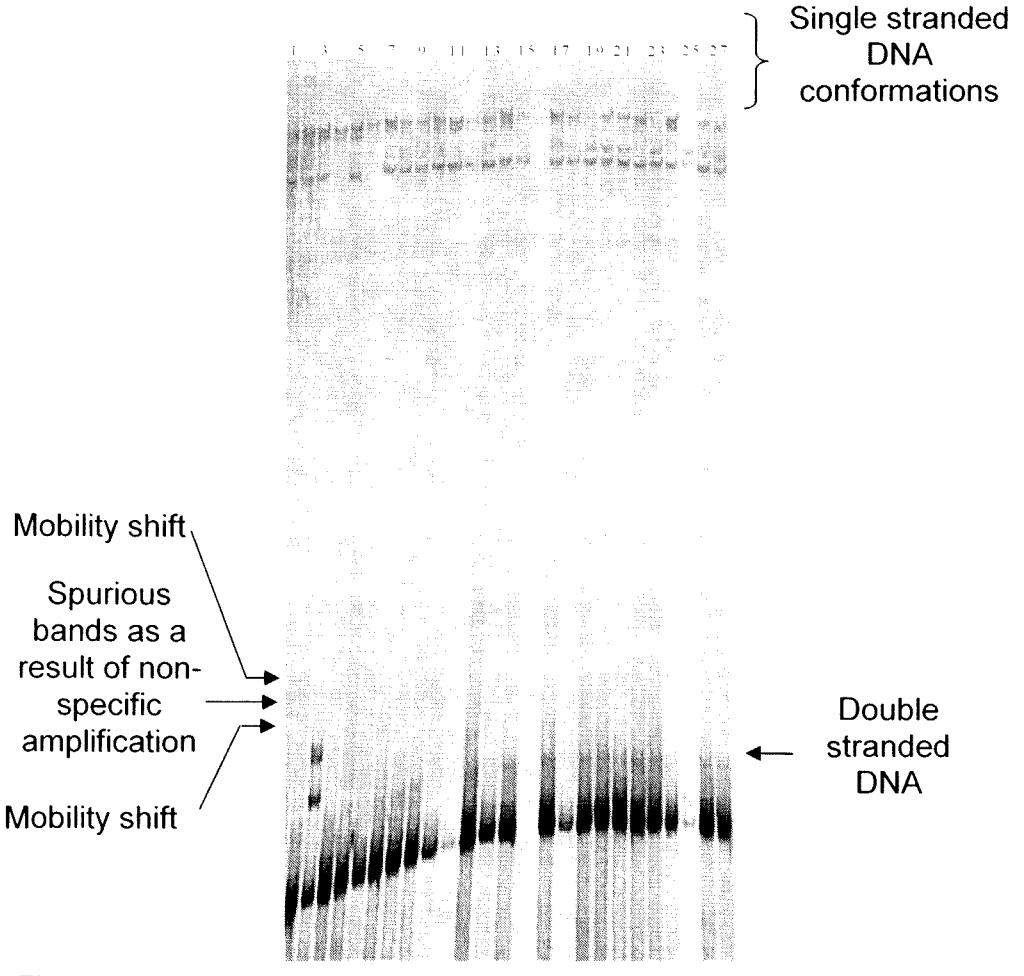

Figure 3.7 (b) A representative example of the SSCP banding patterns detected in the repeat SSCP gel of the discrepant samples, under identical conditions to those of Figure 3.3(a) of exon 6 of TP53 in DNA obtained from the tumor biopsy samples; on a 6% polyacrylamide SSCP gel supplemented with 10% urea.

3.3.5 Comparison of the *TP*53 mutation spectrum of the Black community vs. the Coloured community

In doing a comparison between the nucleotide changes observed in the Black community of the Transkei and those nucleotide changes observed in the Coloured community of the Western Cape (Table 3.2), it was found that the only similarities are the deletions resulting in frameshifts (codons 209 and 276) and

that, in terms of codon position, there are no nucleotide changes common to both these groups under investigation.

Table 3.2 A summary of the nucleotide changes observed in the Black Community of the Transkei vs. those nucleotide changes observed in the Coloured Community of the Western Cape

	Exon	Codon	Mutation	Amino acid change
Black Community of the Transkei (Gamieldien et al. 1998)	5	137	1bp deletion	Frameshift
	5	148-149	2bp insertion	Frameshift
	5	157	1bp insertion	Frameshift
	5	158	4bp insertion	Frameshift
	5	159	1bp deletion	Frameshift
	6	191	2bp insertion	Frameshift
	7	239-241	6bp deletion	Frameshift
	7	239-241	6bp deletion	Frameshift
	7	243-246	8bp deletion	Frameshift
	8	296	TCA - TCT	Silent
	8	303	GAG - GTG	Glu - Val
Coloured Community of the Western Cape (present study)	6	209	GA deletion	Frameshift – stop at codon 214
	6	192	CAG - TAG	Stop codon
	8	276	15bp deletion	Frameshift
	8	283	CGC – TGC	Arg - Cys

3.3.6 Histological re-evaluation of tumorigenic cells

Histological evaluation revealed that 4 of the 20 (20%) randomly selected samples, appeared to never have been positive for squamous cell carcinoma. These were rather attributed to adenocarcinoma (ID 75), chronic inflammation (ID 84), neuro-endocrine carcinoma (ID 183) and a complete lack of any neoplasm (ID 88). Of the remaining 16, histological re-evaluation indicated 5

(31%) to be absent of squamous cell carcinoma. Otherwise stated, 9 of the 20 (45%) samples re-evaluated were negative for the presence of squamous cell carcinoma. The results from the histological re-evaluation of tumorigenic cells can be found in Table 3.2. Furthermore, of the samples listed in Table 3.2, numbers 49, 75, 76, 80, 84, 88 and 89 were included in the molecular analysis of TP53 performed in this study. Based on their histological stats, numbers 49, 76 and 80 were expected to be genetically altered, however, genetic analysis revealed that only sample number 49 was altered within the *TP*53 region under investigation.

Table 3.3 Tumor biopsy samples' originally histologically diagnosed as compared to the re-evaluated diagnosis performed during this study

Study sample number	Original Histological diagnosis	Histologically re-evaluated diagnosis	Study sample number	Original Histological diagnosis	Histologically re-evaluated diagnosis
18	+	-	85	+	+
49	+	+	86	+	+
69	+	-	88	-	-
70	+	-	89	+	-
75	-	-	183	-	-
76	+	+	187	+	+
80	+	+	188	+	+
81	+	+	202	+	-
82	+	+	204	+	+
84	-	-	205	+	+

\+ Indicates a positive diagnosis for the presence of squamous cell carcinoma within the tumor biopsy sample

\- Indicates a negative diagnosis i.e. the absence of any squamous cell carcinoma within the tumor biopsy sample

3.4 Discussion

The aim of this study was to identify similarities between the nucleotide changes observed in the high incidence Black community of the Transkei (Gamieldien et al. 1998) and the Coloured community of the Western Cape, in order to possibly identify mutagens or carcinogens common to both these areas. This was done using PCR-SSCP and direct sequencing analysis whereby the DNA binding domain of *TP*53 (exons 5 – 8) was screened in DNA obtained from the tumor biopsy samples of 44 Coloured EC patients originating from the Western Cape. As controls, the corresponding blood as well as apparently healthy surrounding tissue from the same patients were also included in this screening process.

Based on the information obtained from the SSCP gels performed on DNA obtained from tumor biopsy tissue, corresponding blood as well as apparently healthy surrounding tissue from the group of Coloured EC patients, it appeared that very few nucleotide changes were present within exons 5 – 8 of *TP*53. It also appeared that the nucleotide changes observed were present only within the DNA obtained from the tumor biopsy tissues, with no indication of any such changes within the corresponding blood or the apparently healthy surrounding tissues, as seen in the Transkei study as well (Gamieldien et al. 1998).

Upon further investigation, it was confirmed that from 124 sequences obtained, only 3 samples contained nucleotide changes (Table 3.1). The nucleotide alteration at codon 209 is a 2bp deletion (ΔGA) which results in an early stop signal at codon 214 and has been found in numerous cancer types, including esophageal squamous cell carcinoma (SCC), head and neck cancers, ovarian carcinoma, breast carcinoma, colorectal carcinoma, melanomas, ampullary tumors, thyroid carcinoma, B-chronic lymphocytic leukaemia, pancreatic cancer, astrocytoma, myelodysplastic syndrome, gliomas, rectal cancer, oligodendrioglioma and glioblastomas (Soussi et al. 2001).

Similarly, the nucleotide alteration at codon 192 results in an early stop codon and has been found in esophageal squamous cell carcinoma, head and neck squamous cell carcinoma, ovarian carcinoma, breast carcinoma, colorectal carcinoma and melanoma. However, in addition to these, also mentioned in the previous paragraph for codon 214, the alteration at codon 192 has also been found in esophageal adenocarcinoma, vulvar SSC, cervical cancer, basal cell carcinoma, plasma cell leukaemia, gallbladder cancer, bladder carcinoma, acitinic keratoses, hepatocellular carcinoma, skin SSC, non-small cell lung cancer, skin dysplasia, non Hodgkin's lymphoma, gastric carcinoma and cutaneous lymphoma (Soussi et al. 2001).

The nucleotide alteration at codon 283 results in an amino acid alteration as has also been found in colorectal carcinoma, breast carcinoma, B-chronic lymphocytic leukaemia, chronic myelocytic leukaemia, bladder carcinoma, ovarian carcinoma, vulvar SCC and non-small cell lung cancer. However, it has never been reported in any form of esophageal carcinoma, thus making it novel to esophageal SCC.

Likewise, the 15 bp deletion at codon 276, has never been reported and is thus a novel mutation. Two of these 4 nucleotide alterations were C – T transitions and it has been suggested that oxidized, deaminated cytosines are the main cause of such events. Furthermore, the deletions noted in this study could be accounted for by the a slippage mechanism as a result of a mismatch DNA-repair deficiency, as discussed in section 2.4

There were, however, some discrepant results that needed clarification. A certain mobility shift pattern that occurred repetitively throughout exon 6, could not be reproduced under identical conditions, neither under the second set of SSCP conditions. Sequencing analysis of these samples revealed the sequence to be identical to the wild type *TP*53 sequence as deposited in the

National Centre for Biotechnology Information [NCBI] nucleotide database (accession number X54156).

In order to eliminate any potential enzymatic errors, the very same samples were re-amplified using both *Taq* DNA polymerase (Appendix II) as well as HotStarTaq™ DNA Polymerase (Appendix II). These amplicons were also purified using the Nucleospin Kit (Appendix II) and used for automated sequencing. These results were also identical to the wild type *TP*53 sequence as deposited in the National Centre for Biotechnology Information [NCBI] nucleotide database (accession number X54156).

In a further attempt to solve this obscurity, the bands of interest were excised from the SSCP gel (as described in section 3.2.10) and used as template in a further PCR reaction. Automated sequencing of these amplicons once again resulted in a wild type *TP*53 sequence as compare to the sequence deposited in the National Centre for Biotechnology Information [NCBI] nucleotide database (accession number X54156).

It is generally accepted that a result has to be reproducible at least under identical conditions and preferably under a second set of conditions in order for it to be considered a true result. If these criteria are not met, as is commonly seen with the technique of SSCP analysis, the result is simply considered to be an artefact. In all the above attempts, the discrepant results discussed in section 3.3.4 were not reproducible. This goes to prove that they were merely artefacts, either of the SSCP gel or of the PCR.

In achieving the aim of this study, a comparison was made between the *TP*53 mutational spectrums within the previously studied Black community of the Transkei (Gamieldien et al. 1998) vs. the Coloured community of the Western Cape. It was subsequently found that there are no similarities in the observed mutational spectrum of the DNA binding domain of *TP*53 within these two

population groups (Table 3.2). Nucleotide changes were observed in 9% (4/44) of the Coloured EC tumor biopsies investigated, opposed to 14,5% (11/76) in the Black Transkei population (Gamieldien et al. 1998), which, for the most part, identified insertions and deletions of varying size.

None of the nucleotide changes identified are common to both studies, indicating the possibility different carcinogens being involved. It has been previously identified that EC in South Africa is largely dietary related, where maize and wheat are the central food sources. Fungal contamination of these foods by *Fusarium moniliforme* are believed to play a major role in the tumorigenesis of EC once ingested. In addition, it is hypothesized that a nutritional deficiency is a further complicating factor (Marasas et al. 1988). However, the culture of harvesting one's own maize and wheat crops is traditionally accepted and practiced by the Black population of the Transkei and is very rare within the Coloured Western Cape community. If contamination by *Fusarium moniliforme* is the leading cause of EC development within the Black community of the Transkei, it can, almost with certainty, be ruled out as the leading carcinogen in the Coloured Western Cape community.

Furthermore, the involvement of various human papillomavirus types have also been suggested and cannot be ruled out, although the tumorigenic mechanism remain unclear (Montesano et al. 1996).

Of the 20 samples that were histologically re-evaluated, 6 were included in the molecular analysis of *TP*53. According to the results obtained from the histological re-analysis of these selected samples, 3 were identified as squamous cell carcinoma. However, the molecular analysis of the DNA binding domain of *TP*53 revealed that, of these three sampes, only 1 was genetically altered. This could possibly be explained by the fact that only the DNA binding domain of *TP*53 was investigated. No other upstream or downstream exons or any other regions (known to include regulatory elements such as promoters, enhancers,

silencers, etc) were investigated. Furthermore, no intronic sequences were investigated for the purposes of this study, although it has been found that similar regulatory elements are also know to occur within these regions. This could also be the reason for the overall low frequency of nucleotide alterations within *TP*53 observed within this study (9%).

Furthermore, the results found in Table 3.2 indicate that out of 20 randomly selected samples, only 55% were histologically confirmed to contain squamous cell carcinoma. This could also be a major contributing factor to the overall low frequency (9%) of nucleotide changes observed in this study. For similar future studies, it would be recommended to perform such a histological evaluation prior to the initiation of the molecular aspect of the study. Any samples which prove to be absent of the desired species (such as squamous cell carcinoma in this study), should be immediately excluded from the study. This would minimize the number of complicating factors and allow for a more accurate interpretation of results.

This study has indicated that only 4 nucleotide alterations were found within the DNA binding domain of *TP*53 in a group of 44 (9%) Coloured Western Cape EC patients, a very low frequency. Furthermore, based on the type of nucleotide alterations observed, we have demonstrated that different carcinogenic mechanisms are involved as compared to the high EC incidence Black Transkei community.

Further investigation is required in order to identify the factors responsible for the development of EC within the Western Cape Coloured community. Suggestions would include analyzing the structure of the intronic as well as promoter regions of *TP*53 and subsequently finding correlations between the nucleotide alterations observed and possible etiological factors responsible. Furthermore, analyzing the activity of the *TP*53 promoters could help to further unravel the ambiguity of the

high prevalence of EC within the Western Cape Coloured community vs. the low mutation frequency observed within the DNA binding domain of *TP*53.

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Chapter 4

*TP*53 transcription levels in Esophageal Cancer

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4.1 Overview

The aim of this study was to quantitate and compare the levels of TP53 transcription in esophageal cancer tumor tissue to the TP53 levels in healthy esophageal tissue obtained from patients from a unique geographical and ethnic background. The cohort of patients in this study consisted of Coloured patients, all having been treated at Tygerberg Hospital, Cape Town, South Africa. There is no evidence for any such analyses having been performed within the available literature to date, not within this unique population, neither using the technology used within this study.

Following the principal of the central dogma whereby DNA is transcribed into mRNA which is in turn translated to protein, the measuring of TP53 transcription levels within tumor biopsy samples as compared to the TP53 transcription levels within the corresponding surrounding tissue of the same patients, could shed some light on the status of TP53/p53. This could possibly lead to the identification of errors occurring between these two final stages of transcription (to mRNA) and translation (to the final protein product), complementing the existing bank of knowledge on p53 protein levels.

To date, countless studies have been performed in order to determine the levels of p53 expression within tumor cells. Immunohistochemistry has thus far proven to be the most popular technique employed for these purposes, providing greatly contrasting data; with p53 detected in normal esophageal mucosa at frequencies ranging from 0% (Monges et al. 1996) to 11,7% (Fagundes et al. 2001) and p53 over expression detected in 45% (Chyczewski et al. 1999), 48,6% (Mizobuchi et al. 2000), 51,3% (Monges et al. 1996), 53% (Rosa et al. 2003), 53,9% (Saeki et al. 2002), 67,4% (Shi et al. 1999) and 100% (Fagundes et al. 2001), within esophageal squamous cell tumor samples. By determining the mRNA levels of *TP*53 within this study, it could help to generate a more complete picture and aid in the unraveling of these contrasting results.

Bearing in mind that immunohistochemistry is a technique used for the detection of final protein levels, several techniques are currently available for the quantification of mRNA levels and include methodologies such as Northern analysis, microarrays, the RNase protection assay system (Promega) as well as Taqman technology (Appendix II). However, it was decided that in order to accurately quantify *TP*53 mRNA levels, the LightCycler system (Appendix II) would be implemented. This decision was based on several advantages presented by the LightCycler system, including aspects such as the renowned speed at which the analysis is performed (each run taking approximately 40 minutes); the extremely accurate as well as rapid temperature control based on air heating (maximum of 20°C/s); the optimal size of the product being analyzed varying greatly, from 100 up to 500 bp can be accurately analyzed and generally the software provided is more user friendly and flexible. Furthermore, the University of Stellenbosch had recently acquired such a LightCycler System, implying that the application thereof has already been established at this facility thus enabling easy access.

All the information within this chapter referring to the LightCycler system was obtained from the following website: http://www.lightcycler-online.com.

4.2 Materials and Methods

4.2.1 Collection of samples

Tumor samples were collected and preserved as described as section 2.2.1.

4.2.2 Sample selection

This study was performed on biopsy samples obtained from 2 patients (patient numbers 204 and 205) and corresponding surrounding tissue biopsies obtained from the same sample selection described in chapter 3. Samples were selected

based on the following criteria: firstly, tumor biopsy samples were histologically confirmed to be malignant, and secondly, the tumor biopsy samples, as well as the corresponding surrounding tissue from the same patient had to weigh 25 mg or more, in order to extract sufficient amounts of RNA. Due to ethical difficulties encountered, it was not possible to include tissues from healthy individuals as controls.

4.2.3 RNA extractions

4.2.3.1 Preparation for RNA extractions

Based on the sensitivity of the molecular analysis of all RNA species, certain criteria had to be met prior to the start of RNA extractions. These criteria include factors such as ensuring an RNase free workspace. Due to the fact that DNA, just as RNA, is a nucleic acid and therefor possesses many of the same chemical properties, it is crucial to prevent DNA contamination. Thus, wiping all surfaces and instrumentation with RNase Away (Appendix II) expelled of both RNases and DNA contamination. In a further attempt to eliminate RNases and DNA, disposable apparatus were used as far as possible. Any Perspex apparatus used was washed using DEPC (Appendix II) water. Furthermore, RNase and DNA free water (Appendix II) replaced regular distilled water.

4.2.3.2 Methodology

RNA was extracted from tumor biopsy samples and the corresponding surrounding tissue of the same patient as well as from cells obtained from a cultured NG 63 human osteoblastic cell line (used as a control for the success of the study), using the High Pure RNA Isolation Kit (Appendix II).

For the tissue samples, a mortar and pestle were initially cooled with liquid nitrogen (Appendix II), while, simultaneously, the tumor and corresponding

surrounding tissues were frozen in liquid nitrogen. Once the mortar and pestle were cold enough, the recommended volumes of lysis buffer from the High Pure RNA Isolation Kit were added to each of the tissue samples individually. This mixture was then homogenized to a fine powder. After allowing the powder to return to a liquid phase at room temperature, the protocol for 'Isolation of total RNA from cultured cells' was further followed as advised by the manufacturer, with the following exceptions. The DNase incubation period was extended to 1 hour. The elution buffer (eluted in a final volume of 60 μl) incubation period was also extended to 2 hours and followed by centrifugation as per prescribed protocol. This step was repeated by again pipetting the same eluent onto the glass filter fleece and allowing a second incubation period of 2 hours. This was once again followed by a centrifugation step as per prescribed protocol.

The cells from the cultured NG63 osteoblastic cell line were extracted as recommended by the manufacturer for 'Isolation of total RNA from cultured cells'.

4.2.3.3 Gel electrophoresis

4.2.3.3.1 1% RNA agarose gels

The successful extraction of RNA from the tumor tissue as well as corresponding surrounding tissues as well as the control NG 63 human osteoblastic cell line was confirmed on 1% RNA agarose gels.

4.2.3.3.1.1 Casting the gels

The Perspex gel casting apparatus (15cm × 10cm) was first rinsed with DEPC water (Appendix II), followed by 70% ethanol (Appendix III) and 10% bleach (Appendix III). Finally, the apparatus was wiped with paper towel spotted with RNase Away (Appendix II). The gel solution (Appendix III) was then poured and

the 50 µl Perspex well combs inserted. The gel solution was then allowed to set for approximately 30 minutes.

4.2.3.3.1.2 Electrophoresis

For each of the tumor and surrounding samples, as well as the positive control, 2 volumes of RNA sample buffer (Appendix III) was added to 1 volume of extracted RNA. This was then incubated at 65°C for a period of 5 minutes. This was followed by placing the samples on ice for 2 minutes during which 2 µl RNA loading dye (Appendix III) and 2 µl ethidium bromide (Appendix III) was added. The samples were then loaded and electrophoresed at 100V and 10W until the dye front had migrated across 70 – 75% of the length of the gel.

4.2.3.3.1.3 Visual inspection

The presence of successfully extracted RNA was then visualized under a ultra violet light using a UV™ Transilluminator apparatus (Apparatus II).

4.2.3.4 Determining the RNA concentrations and purity

The optical density (OD) of the RNA was determined using a Milton Roy series 120l spectrophotometer at 260 nm (OD_{260}). The concentrated RNA was diluted by adding 3 µl concentrated RNA to 297 µl dH_20 and the RNA concentration determined by multiplying the OD_{260} by a factor of 4. This gave an answer of RNA concentration in µg/µl. The purity of the RNA was also determined by calculating the OD_{260}/ OD_{280} ratio, which is optimal between 1.6 and 1.9 for pure RNA.

4.2.4 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

4.2.4.1 Principals of RT-PCR

Reverse transcription polymerase chain reaction (RT-PCR) works on the same principal as conventional PCR, whereby a template is amplified with the aid of oligonucleotide primers and a polymerase enzyme. The main differences between RT-PCR and conventional PCR lie in the fact that PCR amplifies DNA from a DNA template. RT-PCR, however, amplifies DNA from an RNA template, resulting in complementary DNA (cDNA). Furthermore, RT-PCR is not an exponential amplification of the original RNA template, but rather creates a scenario whereby the amount of original RNA template is directly proportional to the amount of final cDNA product, i.e. each template RNA molecule is copied into one cDNA molecule. This is achieved with the aid of an enzyme known as reverse transcriptase (Figure 4.1). RT-PCR therefor allows one to apply conventional PCR to a form of template that is a direct reflection of the original RNA present.

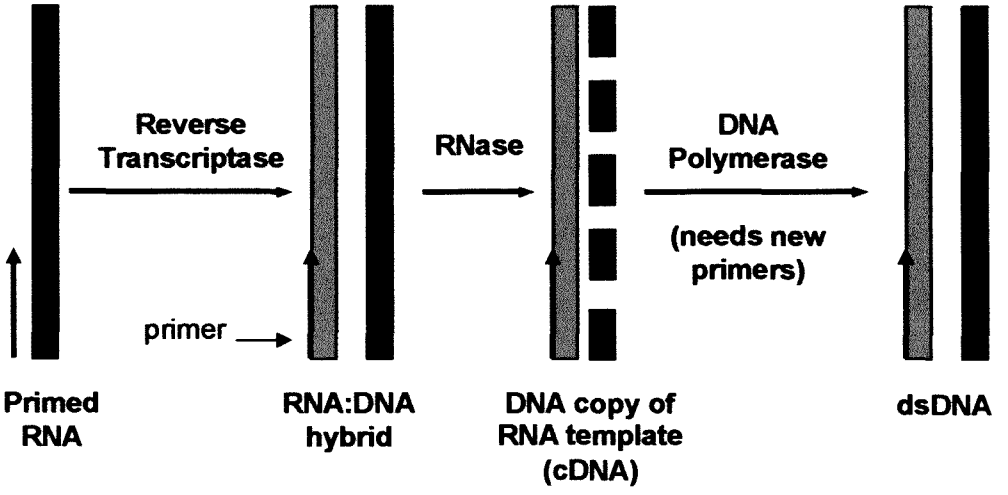


Figure 4.1 Reverse transcription polymerase chain reaction (RT-PCR) copies each original RNA template into a complementary DNA (cDNA) template, which can then be used in a conventional PCR reaction.

Thus, an example of such an application would be to use mRNA as a template for cDNA synthesis. This would enable one to investigate a number of facets concerning mRNA, which could answer several questions concerning the events between initiation of gene expression and the final protein product of such an initiation.

4.2.4.2 Preparation for RT-PCR

Preparations were done as discussed in section 4.2.3.1.

4.2.4.3 Methodology

RT-PCR was performed with the First Strand cDNA Synthesis Kit (Appendix II) as prescribed by the manufacturer using the random primers provided. In each case, approximately 1 μg RNA was used template (as determined in section 4.2.4).

4.2.4.4 Gel electrophoresis

4.2.4.4.1 1% DNA agarose gels

The successful reverse transcription of mRNA into cDNA was confirmed on 1% DNA agarose gels.

4.2.4.4.2 Casting the gels

After washing the Perspex gel casting apparatus (20cm × 10cm) with Cal-liquid hand soap (Appendix II) and rinsing with tap water, the apparatus was dried with paper towel and the gel solution (Appendix III) poured. The 20 Perspex well

combs were placed in the appropriate positions and the solution allowed to set for approximately 30 minutes.

4.2.4.4.3 Electrophoresis

For each sample, 2.5 μl loading dye (Appendix III) was added to 5 μl cDNA and loaded. The gels were then electrophoresed at 150V and 150W until the dye front had migrated 70 – 75% across the length of the gel.

4.2.4.4.4 Visual inspection

The cDNA was visually inspected under ultra violet light using a UV™ Transilluminator apparatus (Appendix II).

4.2.5 Quantitative analysis of *TP*53 mRNA levels using the LightCycler system

4.2.5.1 Principals of the LightCycler system

One of the many applications of the LightCycler system (Appendix II) is the accurate and reproducible quantification of gene expression. In addition to the many applications, the LightCycler system has another major advantage over conventional PCR, namely ultra-rapid cycling. This is achieved by the use of air, which allows for a virtually massless process. The air is warmed by a heating coil, which is controlled by thermocouples in a thermal chamber, which allows for a programmed temperature accuracy of ±0.3 °C. Furthermore, a fan distributes the temperature evenly to ensure identical PCR conditions for each capillary. Rapid temperature transition rates of up 20 °C /second can be achieved in this way.

PCR occurs in specially designed borosilicate glass capillaries which can hold a maximum of 20 µl (Figure 4.2). These capillaries have a high surface-to-volume ration, which ensures that equilibrium is rapidly reached between the outer air and the inner PCR components. This system is accompanied by software that allows one to follow amplification in real-time.

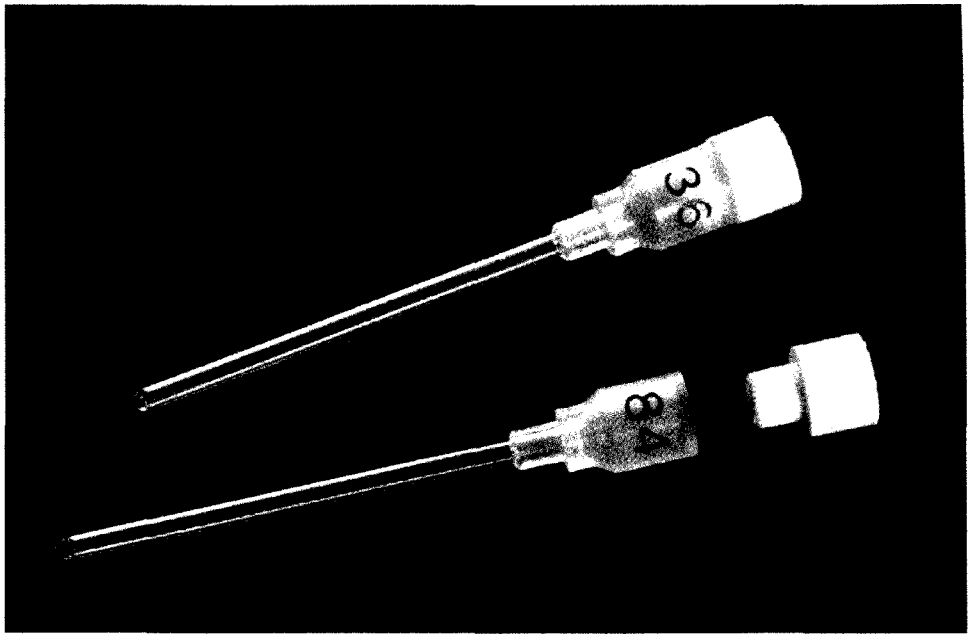


Figure 4.2 Borosilicate glass capillaries used in the LightCycler carousel.

The combined advantage of the high surface-to-volume ratio of the capillaries and the air used in thermocycling allows for a complete PCR run of 30 – 40 cycles to be completed within 20 – 30 minutes.

Furthermore, the borosilicate glass capillaries are ideally suited at cuvettes for fluorescence measurement, due to its optical properties. This allows for the direct application of fluorescence chemistry, whereby the intensity of the signal produced by a fluorophore is relative to the amount of PCR product. This fluorescence is detected from the tip of the capillaries at preprogrammed interval during PCR cycling (on average once every cycle) and can be monitored visually with the aid of the provided software.

A light emitting diode (LED) serves as the source of light with the optical unit having three detection channels which can measure at 530 nm, 640 nm and 710 nm respectively. The LED focuses light (at the appropriate wavelength) on the capillary tips and excites the fluorophore. The resulting fluorescence light is conducted back into the optical unit, where a set of filters and mirrors separates the light into different wavelengths that can be detected by one of the three channels. It is result of this fluorescence that is plotted on a graph to give real-time visualization.

The LightCycler carousel is capable of holding 32 capillaries (Figure 4.3), which are continuously rotated in the thermal chamber during PCR. The mechanics are designed in such a way that the optimal position for the carousel as well as the optical unit is determined for each capillary, prior to initiation of the PCR run.

Figure 4.3 A LightCycler carousel accommodating 32 borosilicate capillaries.

By monitoring the PCR on cycle-to-cycle basis, it is possible to identify exactly when the PCR is in the log-linear phase, i.e. where the PCR product doubles with each cycle (Figure 4.4). As can be seen in Figure 4.4, the start of the increase in fluorescence is determined by the initial concentration of each sample, with higher concentrations increasing first.

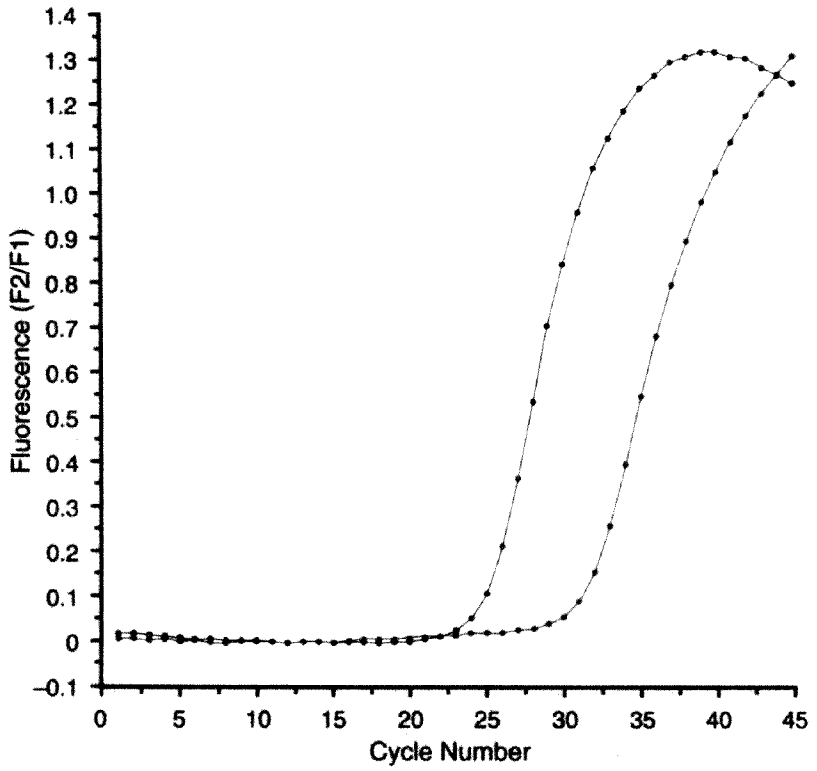


Figure 4.4 Amplification results in an increase in flouresence. A typical graph demonstrating the increase in fluorescence during amplification of a 300 ng (blue line) and 3 ng (red line) of human genomic DNA.

This monitoring of the PCR is achieved with the aid of a dye known as SYBR Green I, which binds to the minor groove of double-stranded DNA (dsDNA) (Figure 4.5). SYBR Green I is thus more specific and sensitive than ethidium bromide, due to the fact that fluorescence is greatly enhanced when bound to dsDNA. This would mean that as the PCR progresses, different intensities of fluorescence signals can be detected, increasing as the amount of dsDNA increases. It is for this reason that the fluorescence is measured at the end of every elongation step, at a wavelength of 530 nm, which is optimal for SYBR Green I. It is this fluorescence from cycle to cycle, that is monitored visually with the accompanying software (Figure 4.4).

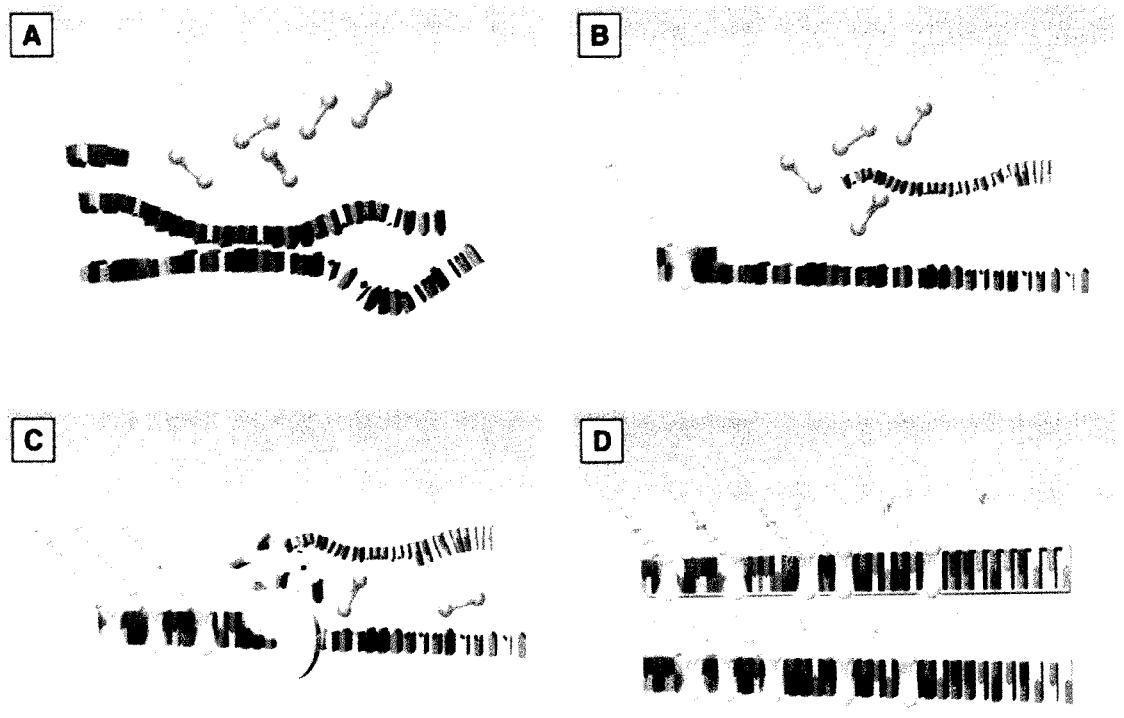


Figure 4.5 PCR and SYBR Green I (A) During denaturation, all template DNA becomes single-stranded which does not allow for the binding of SYBR Green I, resulting in a very low level of fluorescence. **(B)** When primers anneal, a certain degree of dsDNA is present, to which SYBR Green I will bind, increasing the level of fluorescence slightly. **(C)** During the elongation period, the primers are extended, allowing more SYBR Green I to bind, further increasing the level of fluorescence. **(D)** At the end of the elongation, all the DNA is in the double-stranded form and therefor the maximum amount of SYBR Green I is bound as fluorescing.

Another application of the LightCycler system is the determination of each amplicons melting curve via a process known as melting curve analysis (Figure 4.6). Each dsDNA has its own specific melting temperature (Tm; which is defined as the temperate at which half the DNA is in the double-stranded form and the other half in the single-stranded form) which in mostly determined by the GC content and the length of that fragment. Determining the Tm of PCR products is achieved with SYBR Green I and allows the confirmation of PCR product identity as well as differentiating between different fragments, such as non-specific PCR products and primer-dimers.

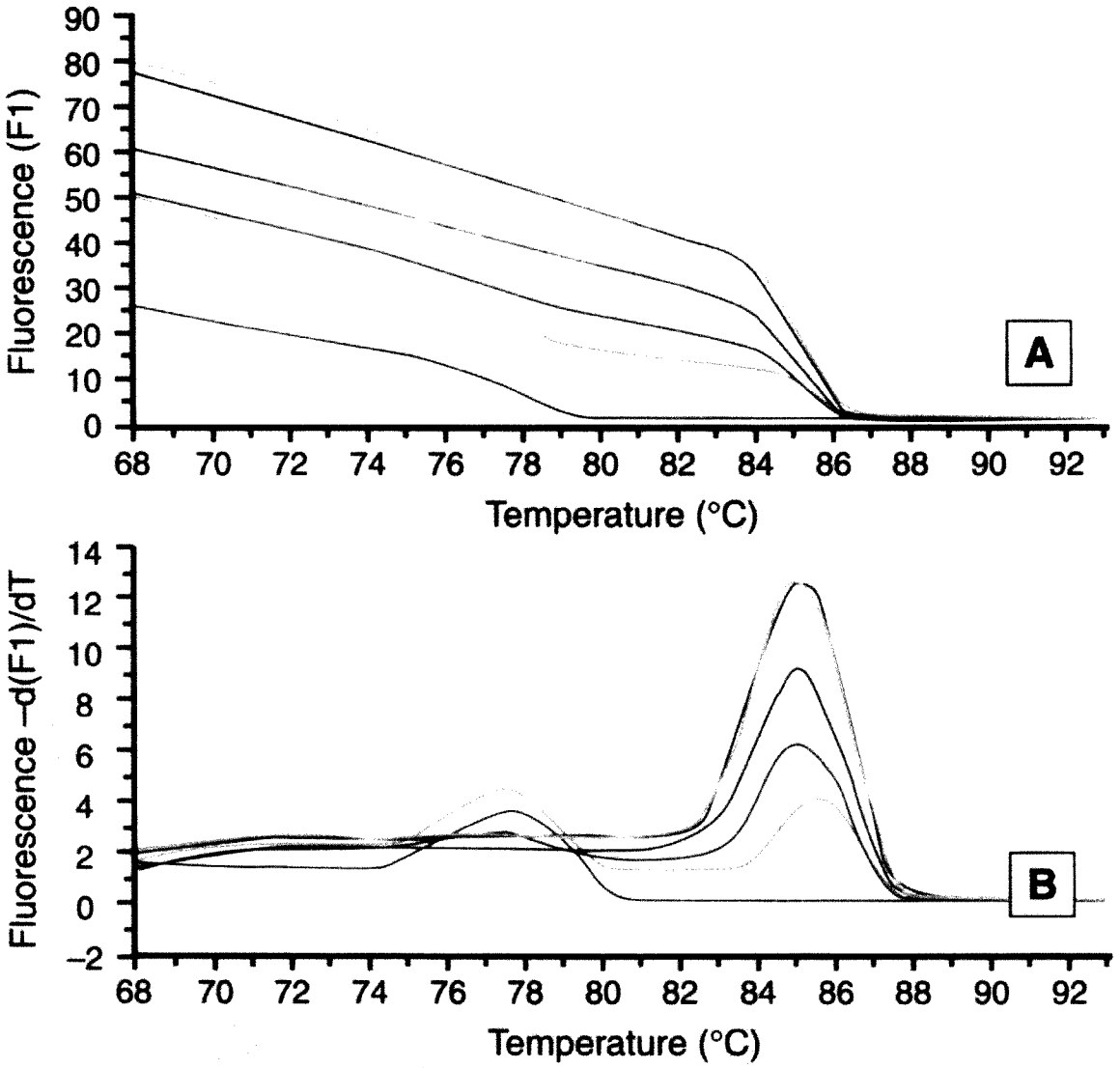


Figure 4.6 Melting curve analysis of a β-actin example; (a) demonstrating a typical graph obtained when plotting the fluorescence vs. temperature and (b) demonstrating a typical graph obtained when plotting the log of the fluorescence obtained vs. temperature. This figure then also demonstrates how different sets of peaks can be obtained such as the first one seen at approximately 77°C which could merely be the result of non-specific amplification of primer-dimer formation. However, the peak of interest i.e. the melting point, can be seen at approximately 85°C and can be identified by the high levels of fluorescence of each sample, at exactly the same temperature.

As can be seen from Figure 4.6 (A), the fluorescence decreases as the temperature increases. Melting peaks are derived from this data (Figure 2.6 (B)), which indicate that at lower temperatures, non-specific products show a peak, but

no peaks are seen at higher temperatures. The inverse can be seen for the β-actin fragments, showing how one can distinguish target products from non-specific ones.

One also needs to consider that not samples contain the same amount of nucleic acids and therefor not all PCR reactions will contain the same amount of nucleic acid as a starting template. This is compensated for by inclusion of an endogenous reference as control. This endogenous control must be chosen in such a way that the conditions of the study (e.g. induction) do not influence the expression of this endogenous control.

Finally, the data obtained from the LightCycler system can be interpreted in one of two ways: absolute quantification or relative quantification.

When performing absolute quantification, it is necessary that the absolute quantities of the standard be known and accurate pipetting is absolutely essential. This type of quantification enables one to determine the exact copy number of any given molecule (e.g. mRNA transcript).

When performing relative quantification, however, the approach is slightly different. It is not necessary to have a standard of known concentrations. Rather, target quantity is expressed relative to an endogenous reference. This is achieved by firstly generating a standard curve for both the target as well as for the endogenous reference. The amounts of the target and endogenous reference are then determined from the respective standard curves. Finally, the target amount is divided by the endogenous reference amount, resulting in a normalized target value.

4.2.5.2 Oligonucleotide primers

4.2.5.2.1 Endogenous Reference

Human glyceraldehyde-3-phosphate dehydrogenase (hGAPDH) is a glycolytic enzyme that catalyzes the conversion of glyceraldehydes-3-phosphate to 1,3-diphosphoglycerate. GAPDH mRNA as well as protein are known to be abundant in most cells types and was chosen as an endogenous control, based on previous quantitative studies done on various cancer types, including esophageal cancer (Ikeguchi et al. 2003; Li et al. 2003; Ikeguchi et al. 2002; Liu et al. 2002; Li et al. 2002; Park et al. 2002; Hu et al. 2001; Shen et al. 2001; Oka et al. 2001; Yajima et al. 1998). The primers (Table 4.1) were designed and optimized by Dr. Heidi De Wet and synthesized by Whitehead Scientific.

4.2.5.2.2 *TP*53

*TP*53 primers were designed and optimized for *TP*53 analysis (by D.Barnard), according to the following criteria: the PCR product should range between 100 and 250 bp. The primers were also designed to lie within neighboring exons. This would allow for easy verification of DNA contamination of RNA; a PCR product arising from mRNA template (cDNA) would be contain the intron between the two primers as thus be larger than the PCR product arising from the mRNA (cDNA) product which contains no intron. Contamination could therefor be verified on conventional agarose gels as well as on the LightCycler system, where two melting curves could be observed in the case of two PCR products being present.

Table 4.1 hGAPDH and *TP*53 primers used in this study

Primer[1]	Sequence	Tm (°C)[2]	DNA product [3]	cDNA product[4]
hGAPDH_F	5' AAG GTC GGA GTC AAC GGA TT 3'	53	1954 bp	226 bp
HGAPDH_R	5' CTC CTG GAA GAT GGT GAT GG 3'	53	1954 bp	226 bp
*TP*53_F	5' AAT CTC CGC AAG AAA GGG GA 3'	58	218 bp	126 bp
*TP*53_R	5' GGT GAA ATA TTC TCC ATC CA 3'	58	218 bp	126 bp

[1]F refers to forward primer and R refers to reverse primer

[2]The melting temperature used for each primer pair during PCR amplification

[3]Size of PCR product obtained from a DNA template

[4]Size of PCR product obtained from a cDNA product

4.2.5.3 Methodology

4.2.5.3.1 hGAPDH and *TP*53 melting curve analysis

Melting curve analyses of both hGAPDH and *TP*53 were performed with each PCR amplification in order to confirm the absence of unwanted products such as those arising from DNA contamination or primer dimers.

4.2.5.3.2 hGAPDH and *TP*53 standard curves

Based on the relative quantification method described earlier (section 4.2.5.1), tumor biopsy number 204 was selected at random for the generation of the hGAPDH and *TP*53 standard curves. This was done by creating a series of cDNA dilutions as follows: undiluted, 1:10 dilution, 1:2 dilution and 1:100 dilution. Lightcycler-PCR was then performed for each of the two primer pairs, using each of the above mentioned dilutions as template. LightCylcer-PCRs were performed in the appropriate capillaries (section 4.2.5.1) using the LightCycler – FastStart DNA master SYBR Green I kit (Appendix II) under the following conditions: each mix contained $MgCl_2$ to a final concentration of 3 mmol, 1.0 μl of the LightCycler

– FastStart DNA Master SYBR Green I mix, forward and reverse primers to a final concentration of 0.5 mmol, approximately 1 μg cDNA template and the provided sterile water up to a final volume of 10 μl. Using the provided software, the LightCycler run was programmed as follows. An activation step at 95°C for 9 minutes initiated the run. This was followed by the PCR step which consisted of a denaturation step at 95°C for 5 minutes, an annealing step at the appropriate melting temperatures for each primer pair for a period of 5 minutes, followed by an elongation step at 72°C for 8 minutes. A total of 50 such cycles were performed and where the acquisition mode was set to measure fluorescence only once after every elongation step. The PCR step was followed by a melting curve step whereby the reaction was kept at the appropriate melting temperatures for a period of 5 minutes, followed by an increase in temperature to 95°C at a constant transition rate of 0.20°C/s. The acquisition mode was set to measure fluorescence continuously during this increase to 95°C. The final cooling step was performed at 38°C for a period of 10 minutes. A constant transition rate of 20.0°C/s was maintained throughout the run, except in cases where specified otherwise. Extreme care was taken to prevent PCR contamination, and all experiments included a control reaction tube in which template DNA was replaced by dH_20.

4.2.5.3.3 hGAPDH and *TP*53 measurements

The levels of both hGAPDH and *TP*53 were then measured in the undiluted samples of each of the 4 samples discussed in section 4.2.2 by performing LightCycler-PCR as discussed in section 4.2.5.3. The relative quantities were then determined from the corresponding standard curves.

4.3 Results

4.3.1 Sample selection

Samples were histologically confirmed to be positive for malignant cells and can be seen in Figure 4.7. The results deomonstrated in Figure 4.8 are those obtained after performing an RNA extraction on fresh cultured cells from a NG63 human osteoblastic cell (lane 2) versus those results obtained after performing an RNA extraction from the poorly preserved tumor and surrounding tissues selected for this study (lanes 1 and 3). The RNA in lane 2 demonstrates the typical profile seen in genomic RNA, with the upper band representing the 28S rRNA and the middle band representing the 18S rRNA. In some cases, an even lower band can be seen, representing the tRNA species. The faint smear seen across the length of the gel, represents the mRNA species, varying in molecular weight. As can been seen here, the intensity of the 28S rRNA is twice that of the 18S rRNA species, which is typically seen in an ideal RNA extraction. This would indicate that the technique employed for the RNA extraction was without fault. Although there appears to be a general smearing of the gel, this can be accounted for by the fact that the agarose used was fairly old, and does not imply any negativity in terms of the techniques employed.

In contrast to the expected bands at their expected intensities seen in lane 2, lanes 1 and 3 rather appear as gross smears, with no defined bands. This already served as the first clear indication of severe RNA degradation resulting from the insufficient sample preservation discussed earlier.

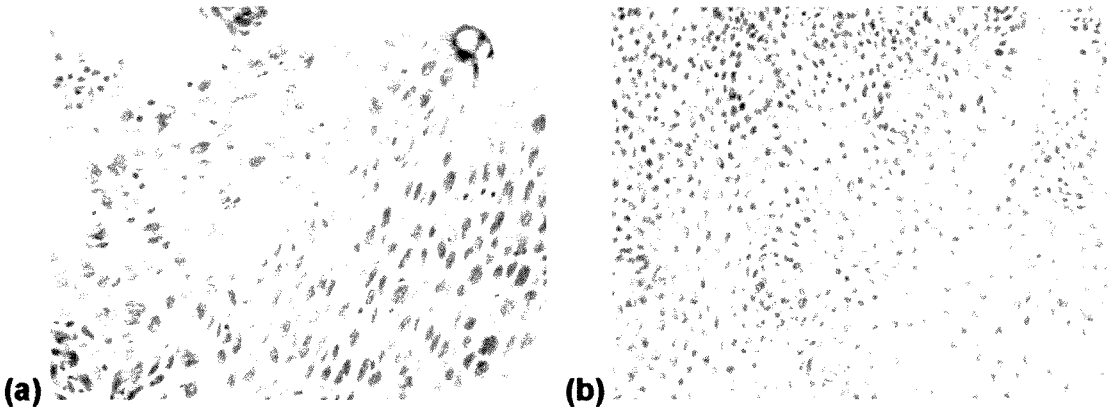


Figure 4.7 Cross section of a typical tumor biopsy sample (not one of the two used in this study) used to histologically confirm the presence of malignant cells where **(a)** illustrates the presence of malignant tissue and **(b)** illustrates normal healthy esophageal tissue.

4.3.2 Confirmation of successful RNA extraction on 1% RNA agarose gels

1 2 3

Figure 4.8 A representative example of a 1% RNA agarose gel demonstrating the RNA extracted from the two tumor biopsy samples (lane 1 – patient 204 and lane 3 – patient 205) as well as from cells from a cultured NG63 human osteoblastic cell line as a positive control (lane 2).

4.3.3 Concentration and purity of extracted RNA

The RNA concentrations for the 5 samples (2 tumor biopsy tissues, 2 corresponding surrounding tissues and HG63 human osteoblastic cell line as control) varied from approximately 0.6 μg/μl to 1.2μg/μl with the OD_{260}/ OD_{280} ratio averaging at 1.7

4.3.4 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

1 2 3 4 - - +

Figure 4.9 A 1% DNA agarose gel illustrating the cDNA obtained after performing RT-PCR on RNA obtained from both of the two tumor biopsy samples (lane 1 – patient 205 and lane 3 – patient 204) as well as from the corresponding surrounding tissues from the same patients (lane 2 – patient 205 and lane 4 – patient 204). Two negative controls (-; containing water as template) and one positive control (+; provided in the kit) were also included.

In Figure 4.9, the results of the RT-PCR performed on the RNA extracted from the tumor biopsy as well as the corresponding surrounding tissue from the same patients can be seen. Here again, the effects of the severe RNA degradation resulting from the poor preservation techniques can be seen in the form of smears rather than defined bands at particular intensities

Even though the RNA obtained (from the two tumor biopsy as well as the corresponding surrounding tissue samples selected for this study) proved to be of an insufficient quality to obtain any recognizable results, the study was taken one step beyond to further prove this. By attempting to apply the generated RNA to procedures performed on the LightCycler system. This was approached by initially performing a melting curve analysis on cDNA obtained from the RNA extracted from the freshly cultured cells of a NG63 human osteoblastic cell line. The results can be found in Figure 4.10 and represent an excellent example of an hGAPDH optimized primer pair, showing only one peak at the expected temperature, with no non-specific products or any primer-dimers.

4.3.5 hGAPDH melting curve analysis

Figure 4.10 demonstrates a typical melting curve under optimal conditions, were are the samples denature at the same temperature, resulting in all the peaks lying on top of one another, at one particular temperature.

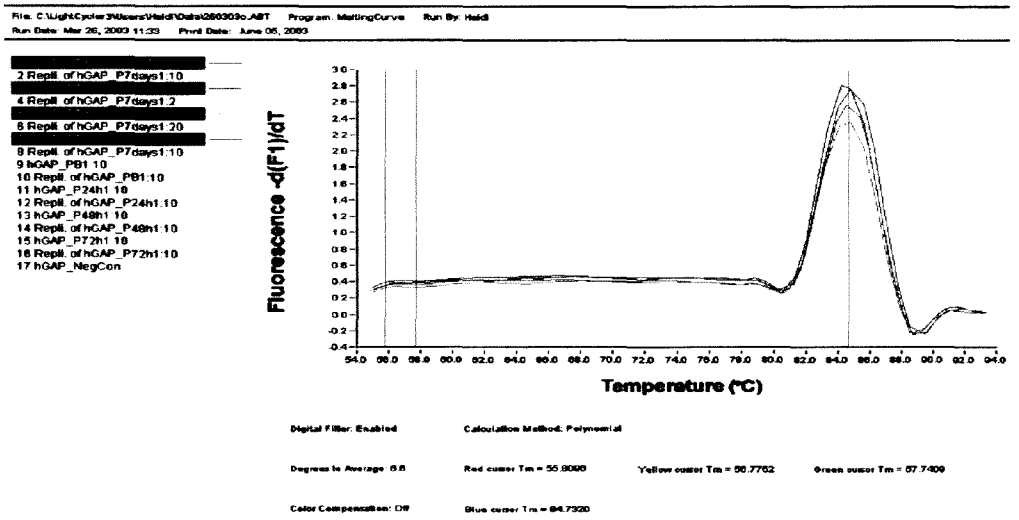
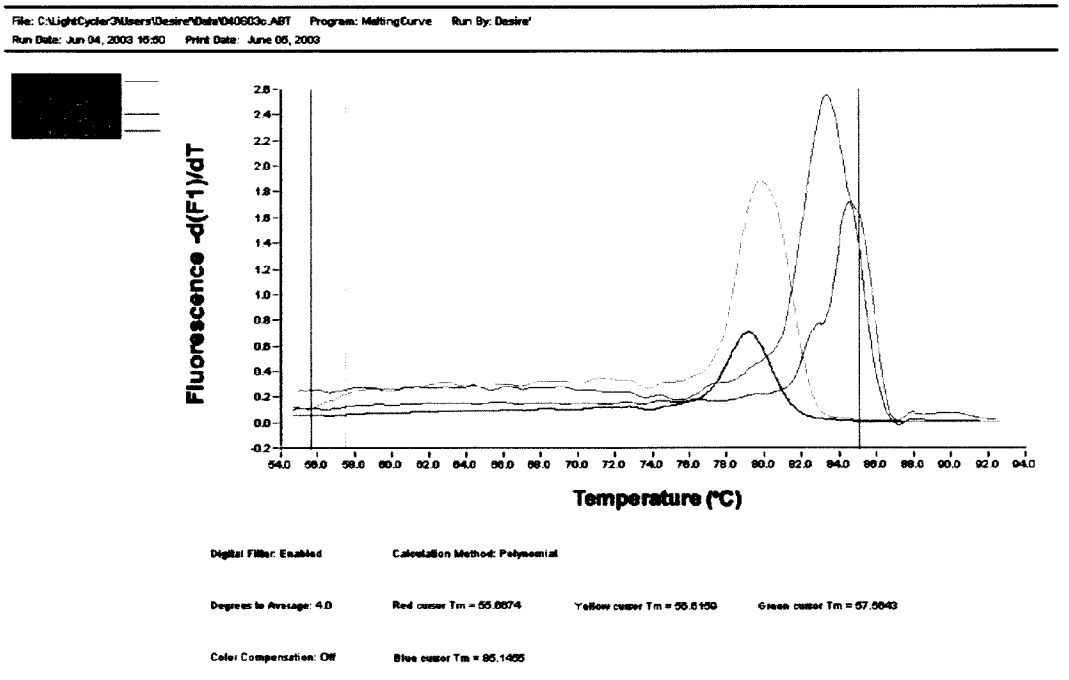
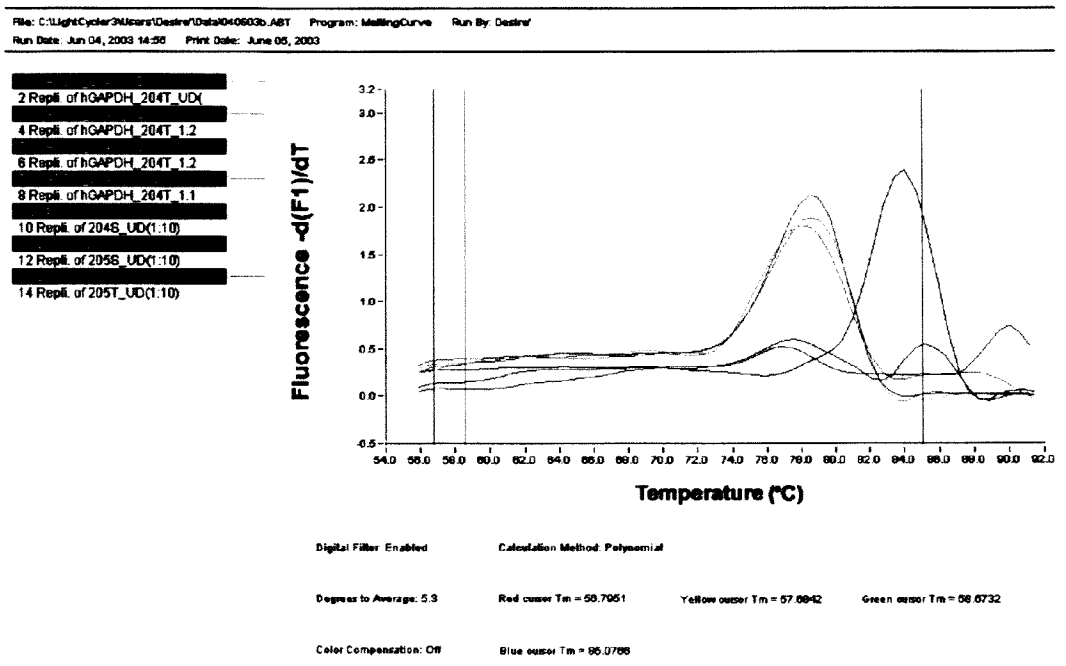


Figure 4.10 A representative example of a hGAPDH melting curve under optimized conditions, using cDNA generated from RNA obtained from cultured cells of a NG63 human osteoblastic cell line as template.

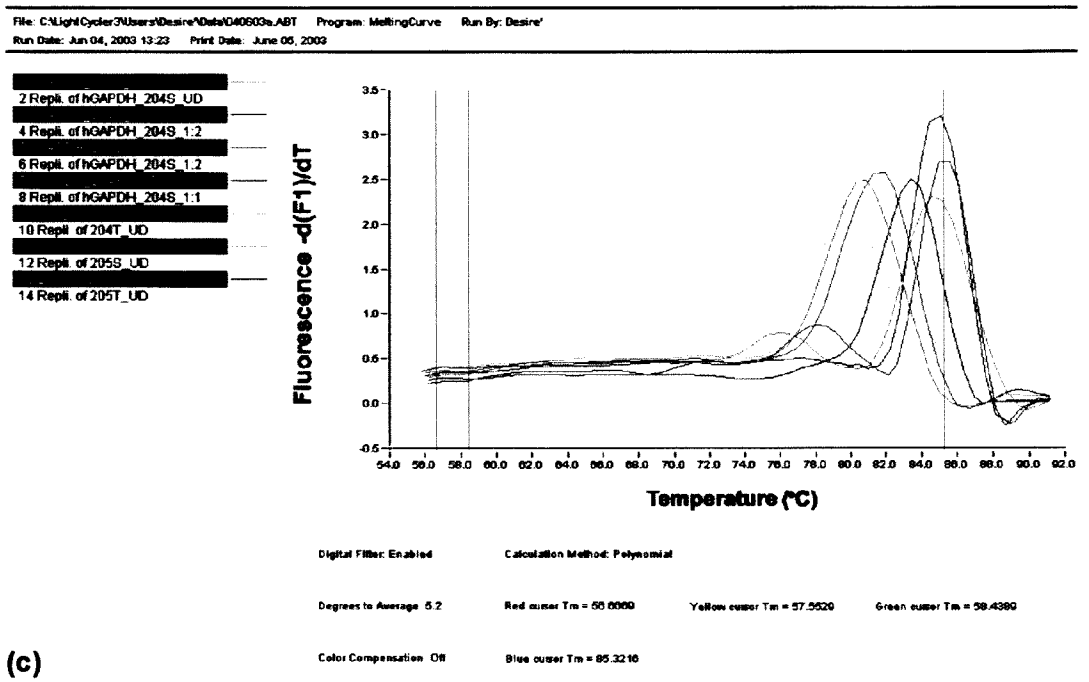
In Figure 4.11 (a - c), the very same primers (hGAPDH) and LightCycler program were used to perform a melting curve analysis on the cDNA generated from the RNA obtained from one of the two tumor biopsy samples. Initially, the sample dilution series was prepared in single, however, later duplicate series were prepared. The reason for this melting curve analysis being performed three times at three separate occasions and by two different individuals was to eliminate the possibility of inaccurate pipetting being the cause of the discrepant results. The only conclusion that can be drawn from these final results are that the RNA extracted from the tumor biopsy and surrounding tissues were of such a poor condition that no results were obtainable, irrespective of the technique or technology applied.



(a)



(b)



(c)

Figure 4.11 (a – c) hGAPDH melting curve analysis performed on cDNA generated from RNA extracted from sample 204 tumor biopsy. This analysis was performed three times, two of which were done in duplicate.

4.3.6 hGAPDH standard curve

Finally, a standard curve was generated using hGAPDH primers and cDNA generated from RNA obtained from freshly cultured cells from a NG63 human osteoblastic cell line (Figure 2.12). This was simply to once again demonstrate that the hGAPDH primers were efficiently optimized and to prove that the RNA extraction method employed in this study was sufficient and not the reason for the poor quality of the RNA extracted from the tumor biopsy and surrounding tissue samples used in this study.

It was then for the above mentioned reasons that the study was halted here, and no further experiments were performed on these sample.

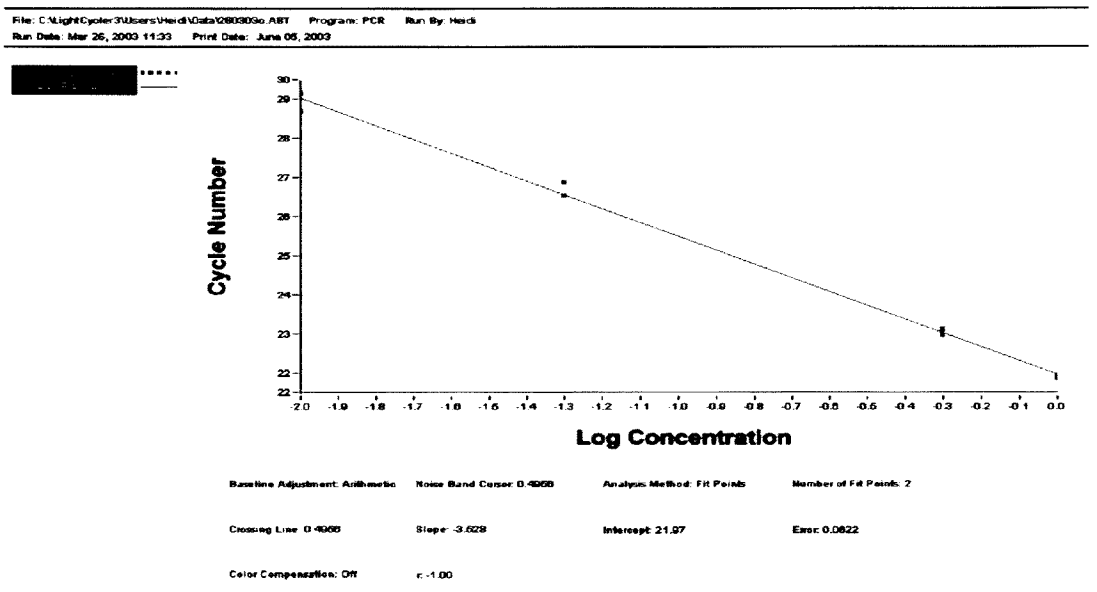


Figure 4.12 A representative example of a hGAPDH standard curve under optimized conditions, using cDNA generated from RNA obtained from cultured cells of a NG63 human osteoblastic cell line, as template.

4.4 Discussion

The conclusion of this study is that RNA obtained for the purposes of this study were of a poor condition and not suitable for the applications attempted in this study. This section will therefore explain why this was the case, and furthermore discuss recommendations for future attempts at such a study.

The first point to note is that the samples used in this study were selected based on their immediate availability and were collected and stored at -80°C in disposable polypropylene cryogenic vials. The original intended uses of these samples were for the application of molecular analyses on a DNA level. This method of preservation may be insufficient for any molecular analyses involving RNA. This could be due to the renowned short half-life of RNA as well as its tendency to degrade rather easily.

In future, a recommended solution for this problem would be to start with an improved planning strategy. If samples are collected for molecular analysis, and one of the intended uses could include RNA related studies, the samples should be preserved more appropriately. This would mean that immediately after obtaining the sample, be it blood or tissue, it should be immersed in an RNA stabilizing agent. Examples of such products include the RNA/DNA Stabilization Reagent for Blood/Bone Marrow (Roche) and RNA*later* Stabilization Reagent (QIAGEN).

Furthermore, although no recognizable results were obtained from this study, three important contributions were made to possibly similar such studies in future. Firstly, if one intends to perform molecular analyses on any RNA species, the correct method of preservation from the very beginning is essential. By employing RNA stabilizing agents in this preservation process, one dramatically increases the half-life of the RNA species, enable its safeguarding for extended periods.

Secondly, a very well optimized RNA extraction method from tough tissues (such as the human esophageal tissue used in this study) has been developed. By making the minor adjustments discussed in section 4.2.3 to the High Pure RNA Isolation Kit (Appendix II), and also keeping in mind the precautionary measures discussed in the same section, it is now possibly to extract large quantities of RNA from small amounts of tough tissue types.

And finally, for future *TP*53 studies involving RNA analysis, primers have now been designed and optimized, enabling one to both identify the presence of *TP*53 RNA species as well as the absence of DNA contamination in a single PCR amplification step.

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The spectrum of mutations in *TP53* in laryngeal cancer patients from a high-incidence population shows similarities to many of the known mutational hotspots

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Abstract Laryngeal cancer (LC) is the sixth most common cancer in the world and the second most common respiratory cancer, with approximately 500,000 new cases worldwide, annually. The mechanisms of tumorigenesis in LC remain unknown, although smoking and alcohol consumption are considered to be major risk factors. Mutations within *TP53* have been strongly implicated as frequent events in several cancers. We screened exons 5–8 of *TP53* for mutations in DNA from tumor biopsies ($n = 44$) and blood samples ($n = 42$) from the same LC patients, and blood samples from a healthy, matched control group ($n = 40$), using polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) analysis and direct sequencing. Significant positive correlations were found between the occurrence of LC and age and smoking, whereas daily meat consumption was a possible protective factor. In tumor-derived samples, mutations were found in three of the exons under investigation, representing 25% of the samples. The mutations were unique to the tumor biopsies, indicating a somatic origin for mutations. The data confirm that the region between codons 175 and 273 of *TP53* is a mutational hotspot region for cancers in general; six novel mutations were found within this same region.

1. Introduction

Laryngeal cancer (LC), often referred to simply as throat cancer, is the sixth most common cancer in the world [1] and, next to lung cancer, it is the second most common respiratory cancer [2]. Being prevalent in males over the age of 55, it occurs at a global incidence of approximately half a million new cases each year [3] and presents with a 60% overall 5-year survival rate [4]. Despite advances in current therapies, one of the leading causes of treatment failure, specifically after surgical removal of the tumor, remains recurrences due to the presence of tumorigenic cells within the surgical margins or cervical lymph nodes [3]. According to the upper aerodigestive tract (UADT) cancer figures for 1990–91, South African men and women fall in the highest incidence category for LC [5], with Colored males displaying the highest number of new cases per annum (10.36/100,000/year) [5]. For a definition of Colored, see section 2.

The exact causes of LC remain unknown; reported risk factors include smoking and heavy alcohol consumption [6].

Mutations within *TP53* have been observed as frequent events in many cancers [7]. This gene is located on the short arm of chromosome 17 and encodes a 393 amino acid, 53 kDa protein, which is expressed in the nucleus of the cell [8]. This phosphoprotein [8,9] is a tumor suppressor protein and is responsible for negative cell cycle regulation [10]. It has also been found to play an important role in genomic stability as well as in DNA repair [11]. More than 95% of the observed nucleotide alterations within *TP53* are point mutations, leading to the synthesis of a mutant protein [12] that loses its transactivational activity [13].

Exons 5 through 8 have been found to encode the DNA binding domain of p53 [14], and most mutations reported occur in this region. The aim of this study was to search for nucleotide changes within exons 5 to 8 of *TP53* and to test

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for correlations with possible causative etiologic factors in a group of LC patients treated at Tygerberg Hospital, Cape Town, South Africa.

2. Materials and methods

2.1. Samples

Fresh tumor sample biopsies (6–54 mg) were obtained from 44 LC patients: 22 Colored males, 8 Colored females, 6 African Black males, 2 White males, and 6 of unidentified ethnicity. The Colored group is of mixed ancestry, consisting mainly of Khoi origins, but also including Caucasian, African, Malay, and San origins [15]. All patients underwent investigation at Tygerberg Hospital, Cape Town, South Africa, during the period from January to December 2000. The age range of the patients was 41–78 years (with a mean of 58 years). Each biopsy was divided into two sections, and histologic evaluation of the one section confirmed poorly differentiated to well differentiated squamous cell carcinoma. The second section of tissue was snap-frozen in liquid nitrogen immediately after surgical removal and was stored at −70°C for further molecular analysis. Blood samples were available from 42 of the same patients as controls. A comparison of the data obtained from these sample sets was done to determine the occurrence of loss of heterozygosity (LOH), which would indicate whether the observed mutations are genetically inherited or somatic in origin. In addition, blood was obtained from a healthy control group ($n = 40$), matched according to age, gender, and ethnic group, to distinguish mutations from possible polymorphisms. The study was approved by the institutional ethics committee and informed consent was obtained from all subjects.

2.2. Demographic data

Questionnaires were completed by all patients and by members of the control group, covering aspects such as age, gender, ethnic group, place of birth, tobacco and alcohol consumption, and various dietary habits. Data obtained from these questionnaires were used to construct a database for further correlation studies.

2.3. Tissue samples and DNA extractions

DNA was extracted from the tumor biopsy samples using the QIAmp DNA minikit (Qiagen, Valencia, CA, USA). Using a new, sterile scalpel blade for each sample, the frozen biopsies were cut into smaller fragments (on ice) and weighed. The protocol was followed according to the specifications of the manufacturer, except that all the solution volumes were doubled for biopsies weighing 25–50 mg. DNA was obtained from the blood samples according to an adaptation of the salting-out procedure for human DNA extraction [16]. Essentially, ice-cold cell lysis buffer (320 mmol/L sucrose, 1% Triton X-100, 5 mmol/L $MgCl_2$, 10 mmol/L pH 7.6 Tris) was added to each blood sample (5–6 volumes), followed by a 15-minute centrifugation step at 4°C and 3000 rpm. The pellet was then resuspended in 9 mL nuclei lysis buffer (400 mmol/L NaCl, 10 mmol/L pH 8.2 Tris, 2 mmol/L pH 8.2 EDTA), 1 mL 10% sodium dodecyl sulfate (SDS), and proteinase K (to a final concentration of 100 µg/mL). After an overnight incubation period at 37°C, 3 mL of saturated NaCl (>6 mol/L) solution was added, followed by another 20-minute centrifugation step at 4°C and 3000 rpm. The DNA was then precipitated by addition of 100% ethanol, washed in 70% ethanol, and resuspended in 200 µL TE buffer (10 mmol/L pH 7.5 Tris, 2 mmol/L pH 7.5 EDTA).

2.4. Polymerase chain reaction (PCR) amplification

Primers used to separately amplify exons 5 to 8 of *TP53* were as previously described [17] (Table 1) and synthesized using a Beckman Instruments (Fullerton, CA, USA) Oligo 1000M DNA synthesizer. All PCR amplifications were performed at a standard heating lid temperature of 105°C, an initial denaturation of 3 minutes at 93°C, a subsequent denaturation period of 1 minute at 93°C, an annealing period of 2 minutes at the respective melting temperatures (Table 1), an elongation period of 2 minutes at 72°C, and a final elongation period of 5 minutes at 72°C. In total, 35 cycles were performed for each PCR reaction, with each 100-µL mix containing the following: 10× buffer (Promega, Madison, WI, USA; 500 mmol/L KCl, 100 mmol/L Tris, and Triton X-100 to a final concentration of 0.1%), $MgCl_2$ (Promega; to a final concentration of 1.5 mmol/L), primers forward and reverse (to a final concentration of 0.20 µmol/L each), Taq polymerase (Promega; 1–2 units per reaction), and deionized

Table 1
Forward (F) and reverse (R) primers for polymerase chain reaction (PCR) amplification of exons 5 to 8 of *TP53*

Primer[a]	Sequence[b]	T_m (°C)[c]
Exon 5–5′F	TTA TCT GTT CAC TTG TGC CC	58
Exon 5–5′R	TCA TGT GCT GTG ACT GCT TG	58
Exon 5–3′F	TTC CAC ACC CCC GCC CGG CA	68
Exon 5–3′R	ACC CTG GGC AAC CAG CCC TG	68
Exon 6 F	ACG ACA GGG CTG GTT GCC CA	64
Exon 6 R	CTC CCA GAC ACC CCA GTT GC	64
Exon 7 F	GGC CTC ATC TTG GGC CTG TG	64
Exon 7 R	CAG TGT GCA GGG TGG CAA GT	64
Exon 8 F	CTG CCT CTT GCT TCT CTT TT	58
Exon 8 R	TCT CCT CCA CCG CTT CTT GT	58

[a] Exon 5 was amplified as two PCR products due to its size, where primer set 5–5′F and 5–5′R amplified the 5′ end and primer set 5–3′F and 5–3′R the 3′ end.
[b] Primer sequences obtained from Toguchida et al. [17].
[c] T_m is the melting temperature for each primer set used in PCR amplification during this study.

pure water (dH_2O; up to a total volume of 100 µL). DNA template was added to a final concentration of 250 ng per sample. Extreme care was taken to prevent PCR contamination, and all experiments included a control reaction tube in which no template DNA was added. The presence of DNA products and the absence of contamination were verified by ethidium bromide staining after electrophoresis of 10 µL of amplified DNA on 12% polyacrylamide minigels (Bio-Rad Laboratories, Hercules, CA, USA) [18].

2.5. *Single-strand conformational polymorphism (SSCP) analysis*

Amplicons obtained from exons 5–8 were analyzed with SSCP gels consisting of 6% polyacrylamide (Bio-Rad; 30% acrylamide-bis solution, 29:1), 5% glycerol, 6% 10× TBE buffer (108 g Tris, 55 g boric acid, 7.4 g EDTA in a total volume of 1000 mL dH_2O; pH 8.30), 0.06% ammonium persulfate, and 100 µL Temed, in a total volume of 200 mL dH_2O. GelBond film was used to facilitate the handling of the gels, which were then electrophoresed at 1500 V, 300 mA, and 50 W and room temperature for 3 hours 30 minutes, using a 0.6× TBE running buffer. This was done both for amplicons obtained from tumor biopsy samples ($n = 44$) and for those obtained from the two blood sample sets (control blood from patients for LOH, $n = 44$, and control blood for polymorphisms, $n = 40$) to determine whether LOH or polymorphisms occurred.

Each SSCP gel (e.g., Fig. 1A) was scored individually according to the various banding patterns (*a* to *e*). Scoring conditions were stringent, and to eliminate the possibility of overlooking a mutation, bands of a very low intensity or with the slightest mobility shifts were often included in the scoring procedure, as a precautionary measure. For each exon, PCR products obtained from tumor biopsy samples and from blood samples of the same patients were run adjacent to each other on SSCP gels. Representative samples from each unique pattern were then selected for DNA sequencing analysis. To prove that these results were reproducible, these representative samples were then reamplified and the amplicons were again analyzed under identical SSCP Gel conditions, as described above. The same amplicons were also evaluated under a second set of SSCP conditions: 8% polyacrylamide, 5% glycerol (Bio-Rad; 30% acrylamide-bis solution, 29:1), 6% 10× TBE Buffer (108 g Tris, 55 g boric acid, 7.4 g EDTA in a total volume of 1000 mL dH_2O; pH 8.30), 0.06% ammonium persulfate, and 100 µL Temed in a total volume of 200 ml dH_2O. This was done to prove that the results were reproducible and also to improve the chances of detecting all possible mutations.

2.6. *DNA sequencing*

Samples were subsequently selected for sequencing (Fig. 1B) from each of the three DNA sample sets to represent at least one of each of the various banding patterns for the four exons under investigation. Amplicons were purified using the NucleoSpin kit (Macherey-Nagel, Düren, Germany), following the instructions recommended by the manufacturer. The PCR products were then sequenced using an ABIPrism analyzer (model 3100; Applied Biosystems, Foster City, CA, USA).

2.7. *Correlation studies*

The nonparametric Spearman rank correlation was performed to determine any significant correlations between

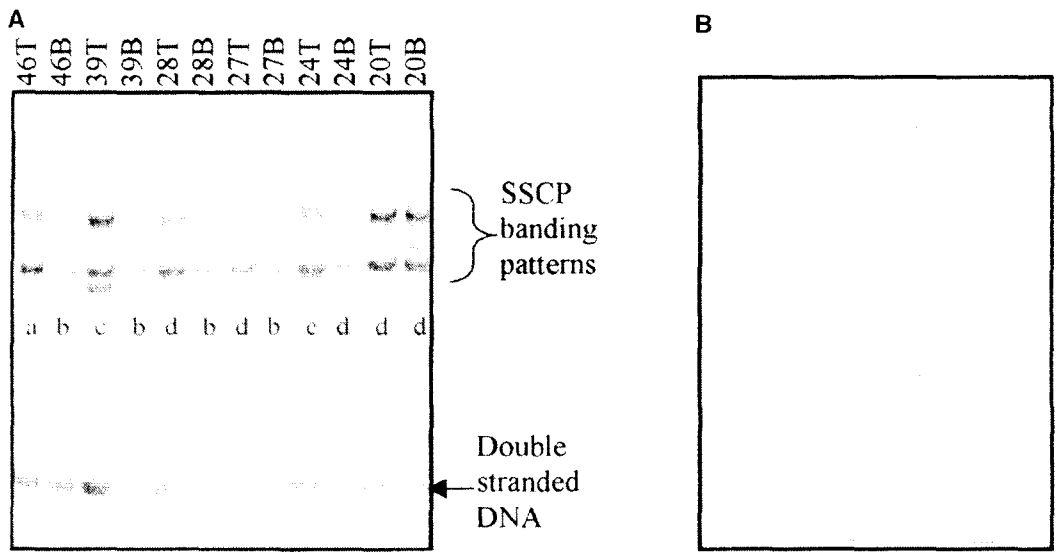

Fig. 1. (Panel A) An example of a 6% polyacrylamide (PAGE) gel, 5% glycerol SSCP gel run for 3 hours 30 minutes at 1500 V and room temperature. This gel contains representative examples of PCR products of exon 7, obtained from tumor biopsy DNA (T) and DNA derived from the patient's blood (i.e., the control for LOH sample set B). The scoring system is demonstrated here, from *a* to *e*, indicating the various mobility shifts detected. Variations in banding patterns between a T lane and a B lane would indicate that any nucleotide changes observed within the tumor DNA is probably somatic in origin. This was verified with sequencing: (panel B) representation of the sequence obtained from the tumor DNA for exon 7 of patient 39, indicating a mutation at position 30, where both a T and A can be seen. The sequence deposited in NCBI (accession no. X54156) shows a T→A mutation (wild-type is a T).

parameters measured under demographic data and laryngeal carcinoma. SPSS version 10 software was used (SPSS, Chicago, IL, USA), and $P \leq 0.05$ was considered statistically significant.

3. Results

3.1. Mobility shifts detected by SSCP analysis

Results obtained from SSCP gels indicate that mobility shifts were detected only within the patient sample set and not within the control blood for LOH set, suggesting a somatic origin for the mutations observed.

3.2. DNA sequencing

In total, 75 sequences were obtained from the selected samples, which were then compared with the wild-type *TP53* sequence (National Center for Biotechnology Information [NCBI] nucleotide database accession number X54156) and it was found that the mutations observed were unique to only the tumor biopsies. All sequences obtained from the representative controls correlated with the same wild-type *TP53* sequence (i.e., NCBI accession number X54156). In total, 11 nucleotide changes were found in three of four exons under investigation, representing 25% of the tumors investigated in this study (Table 2). The 11 mutations observed occurred in 11 different patients. In total, there were G→T ($n = 4$), C→T ($n = 2$), G→A ($n = 2$), G→C ($n = 1$), and T→A ($n = 1$) mutations, which represents four transitions, six transversions, and a T-insertion which was found in only one case. Exon 7 was mutated in 6 of 11 patients (55%), with G→T ($n = 3$; codon 249), C→T ($n = 1$; codon 248), and T→A ($n = 1$; codon 232) changes, all resulting in amino acid alterations, as well as one T-insertion (codon 255) resulting in a frame shift. Exon 8 mutations were detected in 3 of 11 patients (27%), with G→A ($n = 1$; codon 266), G→C ($n = 1$; codon 273), and C→T ($n = 1$; codon 273) changes; exon 5 contained the remaining two mutations, with G→T ($n = 1$; codon 176) and G→A ($n = 1$; codon 175) changes, all resulting in amino acid alterations. No mutations were found in exon 6.

Table 2
Summary of the mutations obtained in exons 5–8 of *TP53* in LC tumor biopsies

Exon	Patient no.	Ethnic group and sex/age	Codon	Mutation	Amino acid alteration
5	KLC4	CM/48	176	TGC→TTC	Cys→Phe
	KLC33	CM/45	175	CGC→CAC	Arg→His
6					
7	KLC1	n/a	255	ATC→ATTC	Frameshift
	KLC8	CM/66	248	CGG→TGG	Arg→Trp
	KLC9	CM/69	249	AGG→AGT	Arg→Ser
	KLC19	NA	249	AGG→AGT	Arg→Ser
	KLC32	CM/62	249	AGG→AGT	Arg→Ser
	KLC39	CM/46	232	ATC→AAC	Ile→Asn
8	KLC12	NA	273	CGT→CCT	Arg→Pro
	KLC23	CM/51	266	GGA→AGA	Gly→Arg
	KLC24	WM/65	273	CGT→TGT	Arg→Cys

Abbreviations: CM, colored male; NA, not available; WM, white male; —, absence of exon 6 mutations.

3.3. Association of observed nucleotide changes to etiologic factors

Questionnaires were completed by all patients and members of the control group. Aspects covered by the questionnaires included ethnic group, gender, date of birth, place of birth, alcohol and tobacco consumption, family history of cancer, source of cigarettes and average number of cigarettes per day, source of tobacco for pipe smoking, chewing tobacco, drinking and source of beer, drinking and source of spirits, use and source of tribal medicine, induced vomiting and method of induction, and numerous aspects of dietary habits.

The data compiled for both the patients and the control group were used to test for correlations between possible etiologic factors and the occurrence of mutations within *TP53* and to test for a correlation between LC and the type of mutations observed. Spearman rank correlations were determined to detect any significant associations. Significant correlations were found between the occurrence of LC and the following: age, smoking, eating meat on a daily basis, and drinking milk on a daily basis (Table 3). The results were then further stratified by dividing the groups (patients and controls) into two categories (smokers and nonsmokers), whereafter the Spearman rank correlation was repeated (Table 4). It was found that 3 of 23 nonsmokers (13%) and 30 of 55 smokers (55.4%) were diagnosed with LC. Both milk and meat consumed on a daily basis resulted in negative correlations with the occurrence of LC (Table 3), thus indicating that they may be protective factors against the development of LC. In contrast, smoking and age displayed positive correlations with the occurrence of LC, thus indicating these as risk factors for the development of LC; however, a negative correlation between daily milk consumption and cigarette smoking was noted (Table 3). From Table 4, where results are stratified according to smoking, it can be seen that the association between daily milk consumption and the occurrence of LC was not significant in either group (smoking or nonsmoking). The association in Table 3 is therefore spurious, and due to the correlation with cigarette smoking, which exerts a major effect. In nonsmokers, therefore, age is the only risk factor identified; in smokers, age is a risk factor, but with a lower correlation coefficient than in nonsmokers, and high daily meat consumption remains correlated with protection against the development of LC (Table 4).

4. Discussion

In this study, *TP53* was examined in a set of laryngeal cancer patients ($n = 44$) all having received treatment at

Table 3
Interrelationships between possible etiologic factors and laryngeal cancer

Characteristics	Laryngeal cancer	Pathology report	Age	Smoking cigarettes	Ever smoked cigarettes	Meat daily
Pathology report	n = 78 cc = −0.970 P = 0.000					
Age	n = 80 cc = 0.479 P = 0.000	n = 74 cc = 0.445 P = 0.000				
Smoking cigarettes	n = 78 cc = 0.304 P = 0.007	n = 73 cc = −0.229 P = 0.051	n = 77 cc = 0.136 P = 0.238			
Ever smoked cigarettes	n = 78 cc = 0.383 P = 0.001	n = 73 cc = −0.317 P = 0.006	n = 77 cc = −0.229 P = 0.045	n = 78 cc = 0.851 P = 0.000		
Meat daily	n = 81 cc = −0.436 P = 0.000	n = 75 cc = 0.453 P = 0.000	n = 80 cc = −0.257 P = 0.021	n = 78 cc = −0.132 P = 0.250	n = 78 cc = −0.180 P = 0.114	
Milk daily	n = 81 cc = −0.264 P = 0.017	n = 75 cc = 0.275 P = 0.017	n = 80 cc = −0.053 P = 0.6390	n = 78 cc = −0.331 P = 0.003	n = 78 cc = −0.293 P = 0.009	n = 81 cc = −0.35 P = 0.757

Abbreviations (variables): n, total number of subjects with information on the particular parameter; out of a total of 88 subjects in study; cc, correlation coefficient; P, significance level.

Tygerberg Hospital, Cape Town, South Africa. To date, the majority of *TP53* mutations have been found within exons 5–8, the most evolutionarily conserved region coding for the DNA binding domain [19], and it is for this reason that we decided to focus on this region. It is possible, however, that mutations may occur within the intronic sequences, as well as in exons 1–4 and 9–11, which we did not investigate. A few studies [7,20–22] have investigated *TP53* mutations in LC using PCR-SSCP analysis combined with direct sequencing. The results indicate that the tumors contain mutations within exons 5–8 at varying frequencies, ranging from 4 of 32 (12.5%) [7], 19 of 61 (31.3%) [20], 7 of 44 (39%) [21], to 13 of 40 (32.5%) [22]. This represents an average of 25%. Early reports [23] indicated that 90% of the mutations in *TP53* from a variety of human cancers were single-point mutations (n = 740) and the remainder consisted of small insertion/deletions. This study identified mutations in *TP53* sequence in 25% of the cases, of which 91% were single-base changes, suggesting a unifying molecular mechanism. Factors possibly responsible for this include environmental agents and dietary components. It has also been reported that small insertion/deletions within *TP53* are common in ovarian cancer [24] and esophageal cancer [25], and a slippage mechanism due to mismatch DNA-repair deficiency can be suggested as being responsible for the T-insertion observed in this study.

Table 4
Interrelationships between possible etiologic factors and LC

Group	Laryngeal cancer	Age	Milk daily
Smokers			
Age	n = 54 cc = 0.317 P = 0.019		
Milk daily	n = 55 cc = −0.244 P = 0.116	n = 54 cc = 0.000 P = 1.000	
Meat daily	n = 55 cc = −0.413 P = 0.002	n = 54 cc = −0.078 P = 0.576	n = 55 cc = −0.080 P = 0.560
Nonsmokers			
Age	n = 23 cc = 0.584 P = 0.003		
Milk daily	n = 23 cc = −0.233 P = 0.284	n = 23 cc = 0.049 P = 0.8255	
Meat daily	n = 23 cc = −0.259 P = 0.232	n = 23 cc = −0.303 P = 0.160	n = 23 cc = −0.012 P = 0.957

Abbreviations (variables): n, total number of subjects with information on the particular parameter; out of a total of 88 subjects in study; cc, correlation coefficient; P, significance level.

Data from questionnaires completed by the patients and by the control group allowed correlation studies to identify smoking and age as risk factors for the development of LC. Furthermore, daily consumption of meat (excluding fish) has been identified as a protective factor against the development of LC. This protective effect of high meat consumption could be either a direct nutritional effect, or meat may be a marker for higher socioeconomic status, and therefore an indication of some other factor or factors involved in protection against the development of LC. The larynx is where the common aerodigestive tract splits into the trachea and the esophagus, and smoking has been identified as a risk factor for esophageal cancer [26] and for lung cancer [27,28].

The typical lung cancer *TP53* mutations associated with smoking, G→T transversions and G→A and C→T transitions [22], accounted for 73% of the mutations found in the present study. Although the mutations observed in the present study fall within a known *TP53* hotspot for cancers in general, the particular mutations at codons 175, 176, 248, 249, 266, and 273, accounting for 82% of the mutations we

Appendix II

Product	Supplier/Manufacturer
Absolute ethanol	Merck (Pty.) Ltd.
Agarose Seakem LE	BMA
$AgNO_3$	Merck (Pty.) Ltd.
Ammonium per sulfate	Merck (Pty.) Ltd.
Bleach	Snobrite
Boric Acid	Merck (Pty.) Ltd.
Bromophenol Blue	Merck (Pty.) Ltd.
BSA	Promega
Cal-liquid hand soap	Cal-Chem
Cling wrap	Pick 'n Pay
Deionized formamide	Sigma
Disposable 0.5ml, 1.5ml and 2ml tubes	Eppendorf
DNA marker IX	Roche
EDTA	Promega
EtBr	Merck (Pty.) Ltd
Formaldehyde	Merck (Pty.) Ltd
Gelbond	BMA
Gel sealing tape	3M
Gelslick	FMC Bioproducts
Glycerol	FMC
Heraeus Biofuge Pico	MSE Multex
HotStart Taq DNA Polymerase	Qiagen
$MgCl_2$	Promega

Mini-gel casting and electrophoresis apparatus	Bio-Rad Laboratories
Mini-gel power supply – EPS 3500	Pharmacia
MOPS	Promega
NaCl	BDH Chemicals
$NaBH_4$	Sigma
NaOH	Sigma
Nuclease-free dH_2O	SABAX
Nucleospin Kit	Macherey-Nagel
Oligo 1000M DNA synthesizer	Beckman Instruments
Pipettes	Eppendorf, Pipetman, Nichipet, Labsystems
Primers	UCT, Whitehead Scientific
Proteinase K	Roche Diagnostics
Rubber bootie	Life Technologies
SDS	BDH
Sequence analyzer 3100	ABIprism
Sodium Acetate	Merck (Pty.) Ltd.
Sucrose	Merck (Pty.) Ltd
Taq DNA polymerase	Promega
TEMED	Sigma
Tris	Merck (Pty.) Ltd.
Triton X-100	Sigma
UNOII Thermocycler	Biometra
Urea	BDH Chemicals
Video Graphic Printer UP- 860 CE	Sony
5ml EDTA tubes	BD Vacutainer Systems
50ml Falcon tubes	Greiner bio-one

10× PCR buffer	Promega
30% Polyacrylamide	BioRad
40% Polyacrylamide (37:5:1)	BioRad

Appendix III

Buffers and Solutions

Cell lysis buffer

Sucrose	320mM
Triton X-100	1%
$MgCl_2$	5mM
Tris, pH 7.6	10mM

Nuclei lysis buffer

NaCl	400mM
Tris, pH8.2	10mM
EDTA, pH 8.2	2mM

Saturated NaCl Solution (>6M)

NaCl	23.4g
dH_2O	1L

TE Buffer

Tris	0.1M
EDTA (pH 8.0)	0.01M
dH_2O	150ml

10×TBE buffer

Tris	108g
Boric Acid	55g
EDTA	7.4g
dH_2O	1L

1×TBE buffer

10×TBE buffer	100ml
dH_2O	900ml

0.6×TBE buffer

10×TBE buffer	60ml
dH_2O	940ml

12% Polyacrylamide minigels (2 gels)

dH_2O	4.8ml
10×TBE buffer	1ml
30% Polyacrylamide (29:1)	4ml
3% Ammonium per sulfate	210µl
TEMED	20µl

Loading dye

Bromophenol blue	0.2%(w/v)
Glycerol	50%
Tris, pH 8.0	10mM

Solution B

$AgNO_3$	1g
dH_2O	1L

Solution C

NaOH	15g
$NaBH_4$	0.1g
Formaldehyde	4ml
dH_2O	1L

6% Polyacrylamide SSCP gels

dH_2O	134ml
10×TBE buffer	12ml
Glycerol	10ml
30% Polyacrylamide	40ml
3% Ammonium per sulfate	4.2ml
TEMED	100µl

8% Polyacrylamide SSCP gels supplemented with 10% urea

dH_2O	120ml
10×TBE buffer	20ml
40% Polyacrylamide (37:5:1)	40ml
Urea	20g
3% Ammonium per sulfate	4.2ml
TEMED	100µl

10% Polyacrylamide SSCP gels supplemented with 17% urea

dH_2O	80.5ml
10×TBE buffer	9ml
Glycerol	7.5ml
30% Polyacrylamide (29:1)	50ml
Urea	25.5g
3% Ammonium per sulfate	3.15ml
TEMED	75µl

70% Ethanol

Absolute ethanol	70ml
dH_2O	30ml

10% Bleach

Bleach	10ml
dH_2O	90ml

5× MOPS Buffer

MOPS, pH 7.0	0.2M
Sodium Acetate	0.05M
EDTA, pH 8.0	0.005M

1% RNA agarose gels

5× MOPS Buffer	20ml
DEPC-treated or Nuclease-free dH_2O	72ml
Agarose Seakem LE	1g
37% Formaldehyde	17.6ml
EtBr (10mg/ml)	5µl

RNA sample buffer

Deionized formamide	10ml
37% Formaldehyde	3.5ml
5×MOPS, pH 7.0	2.0ml

RNA loading buffer

Glycerol	50%
EDTA	1mM
Bromophenol blue	0.4%

1% DNA agarose gels

Agarose Seakem LE	1g
dH_2O	100ml

QUESTIONNAIRE: OESOPHAGEAL CARCINOMA

PROMEC
PO Box 19070
Tygerberg
7505

1. **Patient general information** Official Use Only

Name and address: _____

_____

_____

_____

_____

Telephone no: _____ (h)

_____ (w)

2. **Hospital/Institution information**

2.1 **Name Hospital/Institution**

2.2 **File Number**

2.3 **Diagnosis**

2.4 **Classification on diagnosis (stage)**

Day Month Year

2.5 **Final classification of tumor**

	Description

2.6 **Questionnaire completed by:**

Name
Tel. (w)

Date completed

3. **Patient Demographic Information** Official Use Only

	3.1 **Sex**	Male = 1; Female = 2
	3.2 **Date of Birth**	Day Month Year Age
	3.3 **Occupation**	

	Previous year
	Location
	Previous 5 years
	Location

3.4 Anthropometry

	Weight in kg
	Length in m

3.5 Place of birth

	Region:
	District:
	Location:

3.6 Where did you spend most of your time for the period:

1. Age up to 18 years:

	Region:
	District:
	Location:

2. Age 19 to 35 years:
Region:
District:
Location:

Official Use Only

3. Age 36 to 64 years:
Region:
District:
Location:

4. Age > 64 years:
Region:
District:
Location:

4a. Family history (Cancer)

Father/mother
Brother/sister
Other

4b. Suffers loss in Body Weight

Yes= 1; No = 2
Period

4. Habits

4.1 Smoking cigarettes
Never = 1; Yes = 2; Yes, but stopped = 3
Average number per day
Shop-bought = 1; Home-grown = 2; Both = 3

4.2 Smoking pipe (tobacco)
Never = 1; Daily = 2; Weekly = 3; Seldom = 4
Shop-bought = 1; Home-grown = 2; Both = 3

4.3 Chewing tobacco
Never = 1; Daily = 2; Weekly = 3; Seldom = 4
Shop-bought = 1; Home-grown = 2; Both = 3

4.4 Drinking beer
Never = 1; Daily = 2; Weekly = 3; Seldom = 4
Shop-bought = 1; Home-made = 2; Both = 3

4.5 Drinking spirits
Never = 1; Daily = 2; Weekly = 3; Seldom = 4

Official Use Only

Shop-bought = 1; Home-made = 2; Both = 3

4.6 Tribal medicines

Never = 1; Daily = 2; Weekly = 3; Seldom = 4
Shop-bought = 1; Home-made = 2; Both = 3

4.7 Inducing vomiting

Self-inflicted
By emetics
Specify
Never = 1; Daily = 2; Weekly = 3; Seldom = 4
Description

5. Dietary Information

5.1 Main cereal in diet

Maize = 1; Sorghum = 2; Wheat = 3; Other = 4

5.2 Maize meal intake

Seldom = 1; Daily = 2; Weekly = 3; Monthly = 4
Home-grown = 1; Bought = 2; Both = 3
Amount in tea-cups per day: one = 1; two = 2; etc.

5.3 Maize meal used for home-made beer

Yes = 1; No = 2

5.4 Home-made beer intake

Seldom = 1; Daily = 2; Weekly = 3; Monthly = 4
Amount in tea-cups per day: one = 1; two = 2; etc.

5.5 Maize meal for home-made beer

Shop-bought = 1; Home-grown = 2; Both = 3

5.6 Other food components added to daily diet

Mark clearly the type of food only:
Never/Seldom = 1; Almost Daily (≥5x/week) = 2;
1-2x/Week = 3

Green, leafy vegetables
Green pod vegetables
Imifino (wild/veld greens)
Beans in Umngqusho
Fruit
Milk
Margarine or butter

☐ **Meat (beef/mutton/pork/chicken/mince/sausage)**
☐ **Fish**

☐ **5.6 Suffers from heartburn**

6.0 General:

6.1 Bronchoscope

6.2 Barium meal

6.3 Description of lesion (position etc.)

6.4 Fistula

6.5 CXR

6.6 Hb

6.7 K^+

VOLUNTEER CONSENT FORM

1. I, the undersigned, voluntarily agree to take part in the following study:

   THE VALUE OF p53 MUTATION IN PATIENTS WITH SQUAMOUS CELL CARCINOMA OF THE HEAD AND NECK

2. I have been fully informed of the purpose and nature of the study as well as the advantages and possible adverse effects resulting from the undermentioned procedures and/or treatment, as explained (see attached sheet - "summary of information given to patients") and I understand what it says.

3. Nature of the procedure and/or treatment to be taken is:

   i) Biopsies from tumor to be taken during surgery. ii) blood samples (10 ml for molecular analyses) are to be collected in separate tubes.

4. The procedure will be carried out by/or under the supervision of a qualified nurse (blood collection) and a surgeon (tissue collection).

5. I understand that I can recall my consent at any time.

Name of volunteer: ----------

Postal address: ----------

Telephone Number: ----------(h) ----------(w)

Signed at ----------this ----------day of ----------

Volunteer

Person who informed volunteer

As witness

SUMMARY OF INFORMATION GIVEN TO PATIENT

a) **Tissue sampling:** Tissue samples (tumour) to be taken only when surgery is performed. Therefore, there is no additional risk beyond that encountered during the normal surgical procedures.

b) **Blood samples:** 10 ml blood for molecular analyses.

c) **Questionnaire:** To be completed to provide additional information about possible contributing factors that could have been involved in the development of the disease. From these data, information will be obtained whether environmental factors and/or the traditional lifestyle are associated with the occurrence of the disease.