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Molecular mechanisms of apoptosis in lung cancer: A role for Retinoblastoma Binding protein 6 (RBBP6) and its protein products.



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A Thesis submitted to the Faculty of Science under the school of Molecular and Cell Biology, University of the Witwatersrand, in partial fulfilment of the requirements for PhD.

Gauteng, Johannesburg, 2009

Declaration

I declare that "**Molecular mechanisms of apoptosis in lung cancer: A role for Retinoblastoma Binding protein 6 (RBBP6) and its protein products**." is my own work that has not been submitted for any degree or examination in any other university and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Lesetja Raymond Motadi April 2009

Signed:…….

Abstract

RBBP6 (retinoblastoma binding protein 6) is a 250-kDa multifunctional protein that interacts with both p53 and pRb and has been implicated in mRNA processing. It has also been identified as an E3 ubiquitin ligase due to the presence of a RING finger domain and also assumed to have a regulatory role of p53 due to the presence p53BD through MdM2, although no substrate has been identified up to now. RBBP6 gene mutants are reported to be resistant to apoptosis inducers, which led to a belief that mutation of this gene might result in the development of lung cancer. Earlier localization and expression studies have shown that RBBP6 expression and apoptosis levels are indirectly proportional. The purpose of this study is to establish the expressional pattern of the RBBP6 gene in lung cancer at both mRNA and protein levels. The objective is also to characterize the role of this gene and apoptosis in diverse lung diseases. An understanding of the role of RBBP6 in the development of lung diseases may lead to insights into developing new therapeutic measures for those lung diseases in which apoptosis plays a prominent part.

This thesis elucidate the possible role of RBBP6 in lung cancer and apoptosis, to establish tissue distribution and expression levels of RBBP6 at protein and mRNA levels in lung cancer by immunocytochemistry (ICC), *in situ* hybridization (ISH) and confirm findings by quantitative RT-PCR. RBBP6 mRNA transcripts were expressed in the cytoplasm of normal and tumour lung epithelium. In general, expression was highest in the cytoplasm of well-differentiated carcinoma and invasive carcinoma that showed signs of cells undergoing mitosis. Immunolabelling results further showed high level of expression in all lung cancer types except in Small and large cell carcinomas. The immunolabeling were confirmed by ISH experiments and RT-PCR.

In relation to p53, RBBP6 mRNA expression was higher in lung cancer cell lines that had p53 silenced and apoptosis induced by TRAIL and camptothecin. There was no notable change in the levels of p53 expression following RBBP6 silencing and apoptosis induction. However, there was a little correlation between RBBP6 expression and apoptosis levels in both lung cancer tissues by TUNEL and lung cancer cell line following apoptosis induction by TRAIL. The ratio of Bax/Bcl-2 was seen to be upregulated following p53 and RBBP6 silencing after apoptosis induction. The most common mutation notable after RBBP6 DNA sequencing was point mutations where only single nucleotide was mutated and mostly they were observed in lung cancer tissues.

This was the first demonstration that RBBP6 is expressed in lung cancers. Because of the ubiquitin-like nature of the protein and its localized up-regulation and corresponding pro-apoptotic activity in lung cancer cells, it is possible that further characterization of this gene could lead to its manipulation as a diagnostic marker and a potential therapeutic target for cancer treatment.

Key Words: RBBP6, Apoptosis, Bax, lung cancer and p53 protein

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ABBREVIATIONS

Amp	Ampicillin
Apaf-1	Apoptotic protease-activating factor-1
APS	Ammonium persulphate
ATP	Adenine triphosphate
Bad	Bcl-x_L/Bcl-2 associated death protein
Bax	Bcl-2-asssociated x protein
Bid	B cell leukaemia-2
BIRs	behavioural IAP repeat domain
BLAST	Basic Local Alignment Search Tool
bp	base pair
BCL-2	B cell leukaemia lymphoma-2
BSA	Bovine serum albumin
CAD	Caspase-3-activated depxyribonuclease
CARP	Caspase-8/10 associated RING domain protein
Caspase	Cysteine aspartic-specific proteases
cDNA	complementary DNA
cdk	Cyclin-D dependent kinase
cFLIP	FADD like interleukin
cIAP	cellular inhibitor of apoptosis
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DR	Death receptor
DTT	Dithioreitol

DWNN	Domain With No Name
DEPC	Diethyl pyrocarbonate
DED	death effector domain
DMEM	Dulbecco's modified medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotides
EDTA	Ethylene diamine tetra acetic acid
FACS	Fluorescence activated cell sorter
FADD	Fas-associated death domain
Fas	Fibroblast-associated
FasL	Fas Ligand
FBS	Foetal bovine serum
FCS	Foetal calf serum
FISH	Flouresence *in situ* hybridization
FAP	familial adenomatosis polyposis
ICC	Immunocytochemistry
ISH	in situ hybridization
IAP	Inhibitors of apoptosis protein
IkB	Inhibitors of Kappa B
Kb	Kilobase
kDa	kilo Dalton
l	litre
LB	Luria Broth
MCS	Multiple cloning site

MW	Molecular weight
MDM2	Murine double minute clone 2
min	minute
MOPS	4-Morpholine-propanesulfonic acid
mRNA	messenger RNA
NF1	neurofibromatosis type 1
NF-Kb	Nuclear Factor Kappa B
PACT	p53 associated cellular-protein testis-derived
PAGE	Polyacrylamide gel electrophoresis
P2P-R	Proliferation potential protein-related
P53/Tp53	protein 53/tumour protein 53
P53BD	p53 binding domain
PBS	Phosphate buffered saline
pRb	Retinoblastoma protein
PVDF	polyvinylidene difluoride
PCR	Polymerase chain reaction
Rb	Retinoblastoma
RbBD	Retinoblastoma binding domain
RBBP6	Retinoblastoma binding protein 6
RNA	Ribonucleic acid
RING	Really Interesting New Gene
Smac/DIABLO	Second mitochondira derived activator of caspase
SDS	Sodium dodecyl sulphate
Sec	seconds

TEMED	*N, N, N', N'*-Tetramethylethylenediamine
TNF	Tumour necrosis factor
TRADD	TNFR-associated death domain
tBID	truncated BID
TRAIL	TNF-related apoptosis-inducing ligand
UDPs	Ubiquitin domain protein
UV	Ultra violet
V	Volts
VNR	von Hippel-linday syndrome
WTI	wil's tumours
XIAP	X-linked IAP

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Chapter 1

Introduction and Literature review

1 Introduction

1.1 Retinoblastoma binding protein 6 (RBBP6)

Retinoblastoma Binding Protein 6 (RBBP6) is a novel gene, believed to control apoptosis cascade or cell proliferation in the cell during cancer development (Mbita, 2004 thesis). Through its encoding protein products, RBBP6 has been suggested to bind p53 and other genes such as Retinoblastoma binding protein (pRb) leading to their ubiquitination (Pugh *et al* 2006). RBBP6 is located at chromosome 16p12.2 and it encodes three protein isoforms (Scott *et al* 2003; Gao *et al* 2002). RBBP6 encode from two transcripts, 1.1 kb and 6.1kb. Isoform 1 codes for 18 exons, isoform 2 for 17 exons because it lacks exon 16 due to alternative splicing and isoform 3 codes for only three exons (Pugh *et al* 2006).

RBBP6 consists of seven domains with all the three isoforms having a highly conserved N-terminal novel domain called Domain With No Name (DWNN). As shown in figure 1.1, isoform 3 comprises of DWNN domain only with the two other isoforms consisting of the RING finger, zinc finger, retinoblastoma binding (RbBD) and p53-binding domains (P53BD) and nuclear localisation signal (NLS) linked to the DWNN domain (Pugh *et al*., 2006). In humans, a fly homologue of RBBP6, SNAMA has in it the DWNN domain also, which is considered to possess an ubiquitin-like function (Mather *et al*., 2005). The functions of ubiquitin ligases are well documented in cell homeostasis and protein degradation in a human, this lead to suggestions that RBBP6 through its domains, DWNN, p53BD and RbBD domains may be involved in degradation of both p53 and pRb proteins (Pugh *et al*., 2006; Sakamoto, 2002; **Fig 1.1**).

1.1.1 Role of RBBP6 during cell homeostasis

During certain conditions such as apoptosis and cell cycle arrest or decease, the cell responds to such events by activating a cascade of several genes involved in the homeostasis pathway. RBBP6, through its two domains RbBD and p53 BD can interact with both p53 and pRB. Simons *et al*. (1995) have shown that P2P-R which is a truncated form of RBBP6 lacks the DWNN domain that binds both p53 and pRB (Sakai *et al*., 1995; Simons *et al*., 1997; Witte and Scott, 1997). The role of p53 during cell homeostasis is well documented and any regulation of this gene either by an increase or decrease in its levels can have a positive or a negative effect on the survival of that particular cell (Benedict *et al*., 1990a; 1990b; Sherr and McCormick, 2002; Motoyama *et al*., 2004; Oren, 2003). The role of p53 and that of pRb in cancer development has been reported, however little is known about the mechanism that is involved their binding to RBBP6. Understanding the role played by RBBP6 during p53 degradation will be crucial towards understanding how cells respond to cancer development.

1.2 Ubiquitination

Critical cellular processes are regulated by maintaining appropriate intracellular levels of proteins. Proteins are rapidly degraded at a rate compatible with the control of cell cycle transitions and cell death induction. The main pathway for protein degradation is initiated by the addition of multiple 76-amino acid ubiquitin monomers via a three step process of ubiquitin activation and substrate recognition. Ubiquitin is an abundant and essential cellular 9kd protein that is conserved across evolution from prokaryote to mammals. Ubiquitin is used by cells as a covalent modifier of other proteins both to activate their function and to target them for degradation, depending on the degree of ubiquitin ligation (Goldstein *et al*., 1975). Ubiquitin protein ligases are defined as proteins or protein complexes required to facilitate the ubiquitination of proteins, which are marked for degradation, by tagging them with

polyubiquitin of specific substrates (Hochstrasser, 1995; Kevin *et al*., 1999; Martinez-Noel, 2001). Ubiquitin protein ligases catalyze the formation of an isopeptide bond between the carboxyl terminus of ubiquitin (8.5kd polypeptide) and the ϵ-amino acid group of lysine residues on the target protein (Scheffner, *et al*., 1995).

1.2.1 Ubiquitin ligases pathway

The ubiquitin-proteasome protein degradation pathway comprises: ubiquitin, a three-enzyme ubiquitination complex, intracellular protein ubiquitination targets, and the proteasome that is the organelle for protein degradation. Ubiquitination is activated by ubiquitin-activating enzyme, E1 which requires a single ATP to initiate ubiquitin ligation by adenylating ubiquitin that results in the formation of E1-ubiquitin linkage (Adam, 2003). The ubiquitin molecule is then transferred to the ubiquitin-conjugating enzyme E2, which transiently carries ubiquitin. Next ubiquitin ligase E3, which is responsible for conferring substrate, works in conjunction with E2 to ensure that the reaction eventuates. E3 mediates the transfer of ubiquitin to an internal lysine of the target protein (Mani, and Gelmann, 2005). The polyubiquitinated target protein undergoes proteolytic cleavage by the proteasome, which is a large, cylindrical multisubunit complex. After degrading the peptides, the ubiquitin molecules are recyclable (Adam, 2003).

Ubiquitin activating enzyme together with ubiquitin ligase transfers ubiquitin to target proteins. There are two major types of ubiquitin ligases, those that possess RING finger motif indicating that RING finger-containing proteins are mostly involved in ubiquitination and Hect domain, which is found in ubiquitin ligases and is homologous with the E6-COOH terminus. The RING E3 ligases are crucial since they mediate the direct transfer of E2-bound ubiquitin to substrates without thioester bond formation, and it has been reported that

mutation in RING finger apparently abolishes this interaction (Martinez-Noel *et al*., 2001 and Lorick *et al*., 1999). RING finger domains found in these ubiquitin ligases are classified into C3H2C3, C3HC4 and the least abundant C2H2C4 (Farukawa *et al*., 2002). Because of the RING E3 ligases, ubiquitin-proteasome pathway is crucial in controlling levels of different proteins for normal cell homeostasis. This pathway is involved in the regulation of protein levels, which may have a negative effect as reported to play a role in tumourigenesis (Maki *et al*., 1999; Phillip-Staheli *et al*., 2001; Sakamoto, 2002). Degradation of key regulators of apoptotic machinery has recently become a target for drug discovery and development against cancer.

1.2.2 Apoptosis, cancer and Ubiquitin ligase

The central importance of ubiquitination and proteasomal degradation raises the possibility that targeting these processes may represent an effective therapeutic strategy for certain diseases. It is well documented that inhibition of proteasome activity causes apoptosis in proliferating cells (Burger and Seth, 2004). Considering that cancer cells are characterized by defects in apoptosis, proteasome inhibitors form interesting therapeutic modalities in cancer. Under normal conditions, normal cells have intact checkpoint mechanisms that cause cell cycle arrest in response to cell cycle regulators but this protective mechanism is lost in most cancers. It has been reported that by inhibiting the proteasome, cancer cells are able to undergo apoptosis by stabilizing cell cycle regulators such as p21, cyclins and cyclin-dependent kinase inhibitors and p53 (Fang *et al*., 2003; Burger and Seth, 2004).

One of the most recently studied targets of proteasome inhibition is NFκB which upon activation stimulates cell proliferation and reduces the efficacy of chemotherapy and radiation. Proteasome inhibition stabilizes IκB, an inhibitor of NFκB activation (Burger and

Seth, 2004). Hu *et al.* (2006) have shown that E3 ligases are important in preventing hyperproliferation that is due to NFκB driven lymphocyte activation. These authors have shown that cIAP2 that is lost in lymphomas whereupon this loss leads to up-regulation of its target due to deregulation of its ubiquitination and degradation. A group of proteins called ubiquitin domain proteins (UDPs) is related to ubiquitin but they have not been shown to modify any other proteins. These proteins include the *Xenopus* protein Scythe involved in the induction of apoptosis and another called Parkin, that is defective in Parkinson's disease and has an N-terminal ubiquitin like domain and a RING finger flanked by two IBR (In-between-RING finger) domains (Zhang *et al*., 2000). In their report, Huang *et al*. 2006) have shown that the p53-inducible RING-finger protein (p53RFP), a p53-inducible E3 ubiquitin ligase, induces p53-dependent but caspase-independent apoptosis. P53RFP is said to interact with E2 ubiquitin-conjugating enzymes UbcH7 and UbcH8 and the interaction is mediated through the RING-IBR-RING domain of p53RFP (Zhang *et al*., 2000; Huang *et al*., 2006). The highly conserved C-terminal domain of p53RFP is sufficient for p53RFP to mediate apoptosis, suggesting p53RFP-mediated apoptosis does not require its E3 ubiquitin ligase activity. In addition p53RFP is said to be involved in ubiquitination and degradation of p21, a p53 downstream protein promoting growth arrest and antagonizing apoptosis (Fang *et al*., 2003; Huang *et al*., 2006).

Another well studied E3 ubiquitin which is implicated in cancer is Mdm2 is over-expressed in 5–10% of all tumours (Toth *et al*., 2006; Gershon and Oren., 1999). Mdm2 targets wild type p53 for proteasomal degradation by binding to p53 through its N-terminus and mediating its ubiquitination and itself through an atypical C-terminal RING finger (Fang *et al*., 2003). Prior to the identification of Mdm2 as an ubiquitin. The complexing of p53 with Mdm2 has been considered to be a mechanism for stabilizing p53. And it is now believed to substantially contribute to tumourigenesis and this view is supported by evidence that shows

that lowering Mdm2 levels using antisense approaches accumulates and activates p53 leading to apoptosis of Mdm2 over-expressing tumour cells (Okamoto *et al* ., 2006; Ringshausen *et al*., 2006). Recently with the knowledge that Mdm2 is a RING finger E3, specific inhibitors of their E3 activity represent attractive ways of activating p53-mediated apoptosis in cancer. RBBP6 gene is associated with both the RING Finger and p53-binding domains, which opens the possibility that RBBP6 is involved in p53 dependent apoptotic pathway as an ubiquitin-ligase enzyme. Ubiquitin protein ligases, of which RBBP6 may be one, are a very important group of enzymes that play a significant role in the pathogenesis of many human diseases through deregulation of targeted proteolysis.

1.3 Tumour suppressors genes

Tumour suppressor genes play a critical role in regulating cell division. When DNA damage is detected in a cell, some tumour suppressor genes can stop the cell from multiplying until the damage is repaired. In some cases, specific tumour suppressor genes stimulate cells with damaged DNA to commit "cell suicide". When tumour suppressor genes don't function correctly, the cells with DNA damage continue to divide and can cause further DNA damage that can eventually lead to the formation of a cancer cell. Tumour suppressor genes protect a cell from one step on the path to cancer, and were first identified by making cell hybrids between tumour and normal cells (Knudson, 1971; Sherr, 2004). Several familial cancers have been shown to be associated with the loss of function of a tumour suppressor gene (**Table 1.1**). There are several types of these tumour suppressor genes which include the retinoblastoma susceptibility gene (RB), Wilms' tumours (WT1), neurofibromatosis type-1 (NF1), familial adenomatosis polyposis coli (FAP), von Hippel-Lindau syndrome (VHL), and those identified through loss of heterozygosity such as in colorectal carcinomas (called DCC for deleted in colon carcinoma) and P53 which was originally thought to be a proto-oncogene (Yoshida, 2000; Sherr, 2004).

1.3.1 Tp53

The TP53 gene is considered to be 'the guardian of the cell genome", since it determines whether a cell dies or survives following DNA damage, hypoxia, Nucleotide depletion or radiation (Toyooka *et al*., 2003; Rodin and Rodan., 2005; Pfeifer *et al*., 2002; Yu and Zhang., 2002) The protein product of the TP53 gene is an oligomeric transcription factor of 53 kilodaltons (hence its name) (Wikman and Kettunen., 2006). The p53 protein assembles as a homo-tetramer, and by binding to specific DNA target sequences either activates or represses expression of a relatively large number of genes with different biological functions, which include regulation of cell cycle, cell differentiation, DNA repair and apoptosis (Figure 1.3).

1.3.1.1 The Role of TP53 in cell cycle

Cell cycle control is a fundamental cellular process that governs cellular proliferation. It is also well documented so that progression of the cell cycle is controlled by the cyclin-dependent kinase (CDK)/cyclin complex and counter balanced by CDK inhibitors (CKI). The protein product, of CDKN1A, p21, is an important inhibitor of p53, and when cells over-express p21, the cell cycle is arrested in the G_1 and G_2 phases. The cell cycle gene, stratifin (SFN, 14-3-3 sigma), which is induced by p53 plays, an important role in the cell cycle at the G_2 check point by sequestering the key mitotic kinase cdc2/cyclinB1 as well as silencing aberrant genes. The expression of the sigma gene is frequently lost in small cell lung carcinoma (SCLC) due to DNA hypermethylation (Wan *et al*., 2006).

1.3.1.2 The role of TP53 in apoptosis

TP53 is an essential protein that controls the apoptotic machinery. The ability of p53 to initiate apoptosis was first demonstrated in the early 90's by Yonish-rouach *et al.* (1995) and Johnson *et al.* (1994) in cultured cell lines transfected with p53 expression constructs.

Experiments continued in knock-out mice showed that p53-/- thymocytes pre-B-cells and mature B and T cells were resistant to death induced by Y-radiation and DNA damage-inducing drugs. Although most tumour genes have p53 response elements, and are activated in a p53- dependent manner in response to genotoxic stress, their involvement in p53-dependent apoptosis pathway it is still unclear.

p53 is involved in both the intrinsic and extrinsic apoptotic pathways (Irene *et al*., 2005). There is substantial evidence in support of the view that p53 regulates apoptosis by activating its down-stream targets which are involved directly in apoptosis control. In the extrinsic pathway, p53 promotes apoptosis through activation of the death receptors located at the plasma membrane, which include Fas, DR4 and DR5, known to be regulated by p53, The death receptors, TRAIL and chemotherapeutic agents direct p53 to initiate apoptosis (**Figures 1.2 and 1.3**). In the intrinsic pathway, several bcl-2 family proteins and mitochondrial proteins are implicated in the p53-dependent cascade. Wild-type -p53 together with STAT-1 (which acts as a co-activator of p53) induces apoptosis in injured or DNA damaged cells in order to prevent the generation and proliferation of transformed cells. This control is affected through direct action on the Bax promoter (Shabnam *et al.,* 2004; Fujioka *et al.,* 2004). p53 accumulates also in the mitochondria of some tissues following DNA damage. The mitochondrial p53 activates BAK to induce apoptosis by interacting with MCL-1 which is an anti-apoptotic protein of the Bcl-2 family (Chipuk *et al.,* 2004). Dysfunctional p53 together with a loss Bax activity has been associated with accelerated tumour growth in a brain model of cancer. Several reports have suggested that bax was responsible for almost half of the p53-dependent apoptosis induced by 5-fluorouracil (5-FU) in colorectal cancer cells (Zhang *et al.,* 2005).

Figures 1.1 and 1.2: showing the RBBP6 domains and its involvement in cell proliferation and apoptosis

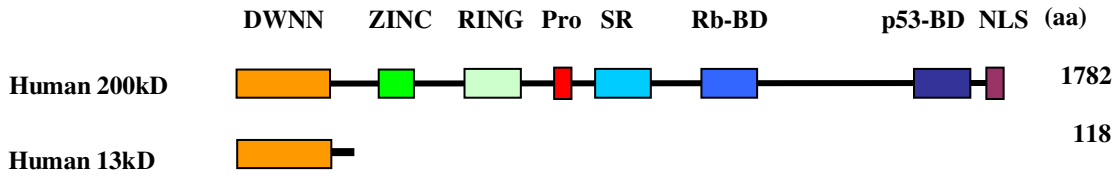

Figure 1.1: The structure shows domain arrangement in the RBBP6 from *Homo sapiens* (Pugh *et al* 2006).

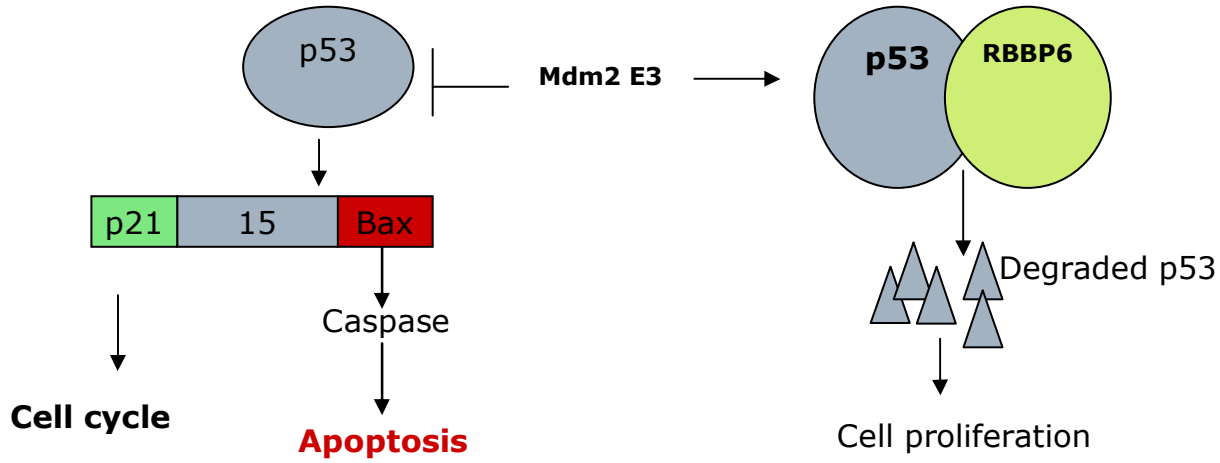

Figure 1.2: Cellular interactions of the tumour suppressor gene protein, p53. Cellular p53 interact with RBBP6 that have a RING finger domain to facilitate its degradation/ ubiquitination thereby promoting cell proliferation.

1.3.1.3 TP53 in lung cancer

As a key coordinator of cellular defence, p53 is the most frequently altered gene in cancer (Rodin and Rodin, 2005). In lung cancer, approximately 50% of all human lung cancer harbour dysfunctional p53 protein (Viktorsson *et al.,* 2005). P53 is found to be frequently mutated in human cancers, namely lung, colon and breast. The most frequent p53 mutations which have been detected in almost 90% of SCLC and 50% of NSCLC (Rodin and Rodin, 2005). Most common p53 mutations are seen in squamous carcinoma and small-cell carcinoma of the lung and the type of mutations commonly seen are represented at G:C → T:A transversions (Rodin and Rodin, 2000). In these tumours p53 activity is inhibited mostly by its negative regulator mdm2 that binds to p53 to facilitate its transfer to the nucleus and simulates its degradation. p53 binds to DNA in a sequence-specific manner to activate its downstream targets. The great majority of tumour derived p53 mutants show functional defects (Yu and Zhang, 2005).

There are several reports suggesting that the different p53 mutations have a different effect on survival of patients with lung cancer. Marchetti *et al.* (1996) first tried to establish the relationship between p21 expression and p53 gene status in lung carcinoma but failed to observe any relationship. Then Caputi and colleagues (1998) found that p21 play a productive role in the clinical behaviour of NSCLC in which they found that patients with p53+/p21 died within a year of surgical NSCLC resection. In contrast, Caffo *et al.* (1996) found that patients with breast carcinoma expressing p21 survived longer than those who lacked p21 protein. However, further studies by Tomizawa *et al.* (1999) suggested that p21 protein expression had no correlation with survival of stage I NSCLC, but the presence of p53 mutation in these tumours was significantly associated with shortened survival. Cancer cells undergo apoptosis, and it is widely thought that activation or restoration of the components of the apoptosis that

are altered during tumour progression may provide a mechanism for specific killing of cancer cells. Attempts to understand the defective p53-mediated apoptosis signalling pathway of tumour cells has been the main focus of recent studies.

1.3.2 Retinoblastoma protein (Rb)

The retinoblastoma tumour suppressor gene, *Rb*, was the first tumour suppressor gene to be identified. It plays a fundamental role in regulating the cyclical changes that occur during cell division. The retinoblastoma tumour suppressor gene (*Rb*) was initially identified as the causative agent whose loss resulted in the development of retinoblastoma, a paediatric tumour of the eye (Morris and Dyson, 2001; Shimizu *et al*., 1994). RB protein is a 928 amino acid, a nuclear phospho-protein that harbours no catalytic activity, and possesses weak, nonspecific DNA binding activity. In 1988, it was found that RB is sequestered by viral oncoproteins of DNA tumour viruses, including SV40 large T antigen, adenovirus E1A, and human papilloma virus E7 (Johnson *et al*., 1993; Yamazaki *et al*., 2005, Batsche *et al*., 2005). It was correctly hypothesized that sequestration by viral oncoproteins disrupts the ability of RB to exert its tumour suppressor function, thus exposing one mechanism by which viral oncoproteins initiate tumour formation. Subsequent efforts to identify cellular proteins that associate with RB showed that the cohort of RB interacting proteins belong to a wide array of disparate cellular processes (Zitting *et al*., 2002; Franklin. 2000; Terasaki *et al*., 2003), thus providing for RB in multiple biological pathways.

1.3.2.1 The RB Pathway and Cancer

Cell cycle control is a fundamental cellular process that governs cellular proliferation, and the retinoblastoma tumour suppressor protein (RB) is a critical component of the cell cycle control machinery. RB loss or inactivation is a major mechanism by which cancer cells attain

a growth advantage during tumourigenesis (Batsche *et al*., 2005). Because cells must progress through the cell cycle to proliferate, there must molecular steps that regulate RB and antagonize its transcriptional repressor function. Such an effect is achieved through a series of phosphorylation events that regulate RB function in relation to cell cycle position and in response to mitogenic stimulation (Yamazaki *et al*., 2005, Batsche *et al*., 2005). Based on the model of RB function, mutations or alterations that disrupt the ability of RB to form transcriptional repressor complexes can be predicted to impair its tumour suppressor activity (Smith, 1996). In cervical cancer, excessive expression of CDK4 is known to occur with relatively high frequency in selected tumour types which results in enhanced RB phosphorylation (Xing *et al*., 1999; Morris and Dyson, 2001). In the same cancer, the human papilloma virus E7 protein can also sequester RB and preclude association with E2F, thus compromising its ability to modulate gene transcription (Xing *et al*., 1999; Bartek *et al*., 1997). In lung cancer, Rb mutation is observed at high frequency in small cell lung cancer, while it is a relatively rare event in non–small cell lung cancer. Rb when mutated or lost contributes to unchecked E2F activity (Bartek J *et al*., 1997) It has long been observed that while almost all human tumours inactivate RB function, the mechanisms by which this is achieved is tissue specific.

1.4 Rational of Retinoblastoma Binding Protein 6 (RBBP6)

Tight regulation of p53 is essential for maintaining normal cell growth. RBBP6 is a 250-kDa nuclear protein with a conserved N-terminal Ring-finger domain that is considered to be involved in protein degradation (Pugh *et al*., 2006; Yoshitake *et al*., 2004). RBBP6 interacts with both p53 and Rb *in vitro* and *in vivo* (Saijo *et al*., 1995; Zhou *et al*., 1999). Yoshitake *et al*. (2004) reported the presence of highly up-regulated RBBP6 in esophageal cancer suggesting that RBBP6 was acting to promote cell proliferation (**Fig 1.2**). p53 is controlled

primarily by Hdm2-mediated ubiquitination and degradation by the proteasome. Recent studies suggest that additional proteins may regulate Hdm2-mediated p53 ubiquitination (Zitting *et al*., 2002; Franklin. 2000; Terasaki *et al*., 2003). Several reports have suggested that RBBP6 homologues (P-2-PR, RQ1 and PACT) are the third protein that interacts with p53-Hdm2 thereby promoting its degradation; however, the mechanism is still unclear (Saijo *et al*., 1995; Zhou, *et al*., 1999; Yoshitake *et al*., 2004).

1.5 Apoptosis

Apoptosis is a tightly regulated process that is characterized by specific morphological and biochemical properties. Apoptosis is also said to play major role in many diseases, cell development and tissue homeostasis. This section will cover apoptosis and its role in lung cancer development and progression. Apoptotic pathways and their involvement in lung cancer prevention and drug targeting.

1.5.1 Biology and molecular mechanisms

Apoptosis or Programmed Cell Death (PCD) is a regulated pathway that is essential in multicellular organisms to eliminate unwanted cells during development, tissue homeostasis, and immune system function. Apoptosis is characterized by specific morphological and biochemical properties. Morphologically, apoptosis involves a series of structural changes in dying cells such as condensation of the cytoplasm and nucleus, degradation of cytoskeletal filaments and cellular fragmentation into membrane apoptotic bodies (Colucci *et al*., 2007). Biochemically, apoptosis is characterized by the degradation of chromatin, initially into large and subsequently into small fragments. It is known that the induction of apoptosis by extracellular signals involves ligands, related to the TNF family proteins, and death receptors, belonging to the TNF receptor superfamily. Some death receptors have been described in

detail, including CD95 (Fas) that binds the CD95 ligand (FasL) and TRAIL receptors that bind the TNF-related apoptosis-inducing ligand (TRAIL) (Du *et al*., 2000; Verhagen *et al*., 2000).

However, the Initiation and regulation of apoptosis is highly controlled through specific protein-protein interactions and by a family of proteolytic enzymes called caspases that collaborate in a proteolytic cascade to activate each other (Reed, 2004). Within the proteolytic cascade, caspases can be positioned as either upstream initiators or downstream effectors of apoptosis. In mammals, signalling cascades culminating in apoptotic cell death can be divided into two broad categories: **i)** Extrinsic pathway which is said to be initiated by the tumour necrosis factor (TNF) –family receptors including p53 and **ii)** Intrinsic pathway which is mostly controlled by the mitochondria (see **Fig 1.3**). Cancer-associated defect in apoptosis play a crucial role in chemoresistance and radioresistance. Mechanisms to overcome apoptosis resistance that has been reported in most cancers include direct targeting of antiapoptotic molecules expressed in tumours as well as re-sensitization of cells to previously resistant tumour cells by re-expressing of all pro-apoptotic molecules and counteracting apoptosis inhibitory molecules. A characteristic feature and a major cause of lung cancer is an in built resistance to programmed cell death (Nachmias *et al.*, 2004). Apoptosis is therefore considered to play a significant role in the prevention and progression of lung cancer. The most promising approach to eradicating cancerous cells should be to involve apoptosis as the therapeutic target (Kiechle and Zang, 2002; Fulda and Debatin, 2004a).

1.5.1.1 Extrinsic pathway-death receptor orchestrated apoptosis

The extrinsic pathway mediates apoptosis in response to Fas ligand (FasL) and Apo2L/TRAIL (Apo2 ligand or tumour necrosis factor (TNF)-related apoptosis-inducing ligand), which signal respectively through the death domain (DD)-containing receptors Fas or death receptor DR4 and DR5 (Li *et al*., 1998; Luo *et al*., 1998; Wang *et al*., 2007; see Figure 2) . Death receptors are classified as TNF/nerve growth factor superfamily of cell surface receptors. The death receptors are characterized by their cytoplasmic protein motif called death domain that are able to activate extrinsic apoptotic pathway. Death receptors are said to be activated by the binding of corresponding ligand or agonist antibodies that results in receptor trimerization and clustering of the receptors' death domains (see Figure1.3). The cytosolic domains of the death receptor form a death inducing signalling complex (DISC) that attracts and then links up with the adaptor molecule termed FADD (Fas-associated death domain containing protein). The DISC-FADD complex through their death effectors domains (DED) bind to initiator caspases (caspase-8 and -10), thereby causing the autocatalytic cleavage of the procaspase-8. The activated caspase-8, in turn activates downstream executioner caspases (see Figure 1.3). These proteases cleave cellular death substrates, resulting in morphological and biochemical changes observed in apoptotic cells (Hengartner, 1997, 2000; Krammer, 2000).

1.5.1.1.1 TNF-alpha mediated apoptosis

TNF-alpha is a well known cytokine, which is involved in the various cellular activities including cell activation, differentiation and apoptosis. Depending on the type of stimuli, TRADD can activate either FADD which is a caspase-8 activator leading to the induction of extrinsic and intrinsic apoptotic pathways or it can stimulate NF- κB activity leading to the recruitment of TRAF1, TRAF2 and RIP genes which interact with anti-apoptotic proteins

such as Clap-1 and Clap-2 to prevent apoptosis (Basile *et al.*, 2001; Kulik *et al.,* 2001; Chinnaiyan *et al.,* 1996; Roy *et al.,* 1997). TNFR1-induced apoptosis involves two sequential signalling complexes with the initial stage being the assembling of TNFR1, kinase RIP1 and TRAF2 that will activate anti-apoptotic genes by activating NF-kB signalling complex caspase -8 inhibitor c-flips, which will counter react apoptosis resulting in cell survival. Complex II is a cytoplasmic complex, consisting of TRADD, RIP1, FADD and Caspase-8 which induces apoptosis.TNRF1-mediated signal transduction cascade possesses a checkpoint in which if complex I fails to activate NF- κB pathway then activation of complex II induces cell death through caspase cascade activation. Previously, it has been reported that following the treatment of cells with TNF- α induced apoptosis, thereby, increasing the expression of TRAF1, TRAF2, c-IAP1, and c-IAP2 and that overexpression of these proteins protected RelA-deficient cells which are sensitive to TNF-α– induced apoptosis from cell death (Wang *et al.,* 1998). Recently, it has been reported that up-regulation of TNF receptors sensitized ATRA treated human lung cancer cell line to TNF-alpha induced apoptosis (Aggarwal, 2003).

1.5.1.1.2 Fas-mediated apoptosis

Fas (also known as CD95/APO-1) is a cell surface protein that belongs to the tumour necrosis factor receptor superfamily, which is expressed in a variety of normal and neoplastic cells including lung cancer (Ungefroren *et al.*, 1998; von Bernstorff *et al.*, 1999; Huang *et al.*, 2008). Triggering of Fas signalling by FasL or agonistic antibodies results in rapid induction of apoptosis in susceptible cells *(*Huang *et al.*, 2008). Fas/FasL-induced apoptosis was initially recognized as a critical mechanism in the regulation of immunohomeostasis (Sun *et al.*, 2000).

The CD95 has been implicated in chemotherapy-induced tumour death in a controversial way with several studies suggesting that expression levels of CD95 were found to be upregulated following chemotherapeutic treatment which resulted in the activation of the CD95 pathway (Muller *et al.,* 1998; Fulda *et al.,* 2000, Fulda *et al.,* 2001), whereas others have stated that CD95 overexpression has no effect on protecting tumour cells following chemotherapy (Petak and Houghton, 2000; Villunger *et al.*, 1997). Because FasL expressed on T lymphocytes induces apoptosis in Fas-expressing lung cancer cells, the Fas/FasL system plays an important role in the cell-mediated cytotoxicity against lung carcinomas. It has been reported that malignant cells escape the immune system by the downregulation of the FasL, and by killing lymphocytes.

In recent studies Chosa and colleagues (2004) examined the effect of various caspase inhibitors on the apoptosis induced by CH-11 and they found that Fas-mediated apoptosis of human salivary gland (HSG) cells was slightly inhibited by the caspase-9 inhibitor although it was mainly inhibited by caspase-8; based on this results they concluded that CH-11 -induced apoptosis in HSG cells appeared to be mainly mediated by type I death signalling pathway expression of FasL (Bramhall, 1997). Recent studies have shown that commonly used chemotherapeutic drugs induce apoptosis and Fas expression on lung cancer cells (Volm and Rittgen, 2000). Recently Shimizu *et al.* (2004) reported high levels of soluble Fas and FasL in the peripheral blood of lung cancer patients. Furthermore, the over-expression of Fas-associated death domain and the up-regulation of Fas/Apo-1 have been reported to induce apoptosis in lung cancer cells (Kim *et al.,* 2003; Kuo *et al.*, 2005; Fujita *et al.*, 2002). However, the role of soluble Fas and FasL in the proliferation and cell death of lung cancer cells has yet to be clarified and may open new opportunities for potential therapy.

1.5.1.1.3 TRAIL-induced apoptosis

Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) is a potential anticancer agent that selectively induces apoptosis in a variety of cancer cells by interacting with death receptors DR4 and DR5. TRAIL can also bind to decoy receptors (DcR1, DcR2, and osteoprotegerin receptor) that cannot induce apoptosis (Tur *et al*, 2008). TRAIL has been shown to induce apoptosis in a variety of transformed or tumour cells by binding to its two proapoptotic receptors DR4 and DR5 leading to the recruitment of the adaptor and induction of apoptosis through activation of caspases (Pitti *et al.,* 1996). Unlike DR4 and DR5, TRIDD lacks an intracellular domain and TRUNDD has a truncated death domain. These two receptors act as decoy receptors to antagonize TRAIL-induced apoptosis by competing for ligand binding (Ashkenazi and Dixit, 1998). In a study by Yeh *et al.* (1998) following knockout of FADD in some cells, it was evident that FADD is not required for TRAIL-mediated apoptosis. However, several reports have suggested recently that the mechanism of TRAIL-mediated apoptosis involves activation of FADD and caspase-8 in human NSCLC. TRAIL induced apoptosis is effected through cleavage of caspases 8,9,7,3 and BID, which results in the release of cytochrome c from the mitochondria and cleavage of poly(ADP-ribose) polymerase (PARP) (Bodmer *et al.,* 2000).

Tumours that overexpressed Bcl-2 protein may not be sensitive to TRAIL induced cell death (Sun *et al.,* 2001). Importantly, the release of cytochrome c and the cleavage of caspase-7 triggered by TRAIL were both blocked in Bcl-2 overexpressing cells, when compared to vector control cells. Moreover, inhibition of TRAIL- mediated cytochrome c release and caspase-7 activation by Bcl-2 correlated with the inability of PARP to be cleaved and the inability of the Bcl-2 transactivation to undergo apoptosis. Activation of caspase-7 but not caspase-3 was completely blocked by overexpression of Bcl-2 during TRAIL-mediated

apoptosis. TRAIL is known to activate NF-kB in a number of lung cancer cells. It has been reported also that TRAIL exerts a relatively selective cytotoxic effect on human tumour cell lines without much effect on normal cells.

1.5.1.2 Intrinsic pathway- mitochondrial-dependent apoptosis

The mitochondrial apoptotic pathway is a highly regulated biological mechanism which determines cell fate. It is defined as a cascade of events, going from an apoptotic stimulus to the mitochondrial membrane permeabilization, resulting in the activation of the so-called executive phase (Verhagen *et al*., 2000; Lalier *et al*, 2007). The intrinsic pathway is engaged when cells that are challenged by stress and the apoptotic signal mediated by mitochondria. The Bcl-2-family proteins are key regulators of this pathway. Interactions between the family members control the release of apoptogenic proteins, including cytochrome c, SMAC/Diablo, Omi/HtrA2, AIF and EndoG, which lead eventually to cell death through caspase-dependent and caspase-independent systems (Du *et al*., 2000; Verhagen *et al*., 2000). Mitochondria are regarded as life-supporting components of all eukaryocytes; contrastingly play a crucial role in inducing apoptosis of the very cells they are meant to sustain.

The cascade is commenced by Intracellular death signals such as those initiated by chemotherapeutic or other cytotoxic agents that modulate mitochondrial function by expressing either one or both of pro-apoptotic and anti-apoptotic proteins. Activated pro-apoptotic proteins attach to the mitochondrial outer membrane to form conducting channels through homo-oligomerization or hetero-oligomerization, allowing cytochrome c that resides in the intermembrane matrix to translocate into the cytoplasm. Cytosolic cytochrome c combines with ATP, Apaf-1 and procaspase 9 to form apoptosomes which activate caspase 9, and subsequently caspase 3 resulting in cell death (Liu *et al*., 1996; Li *et al*., 1997; Zou *et al*.,

1997). Bcl-2 and Bcl-x_L can inhibit the initiation of apoptosis by interfering with the interaction of activated Bax, Bak and BID with the mitochondrial membrane at many levels and thus preventing the release of cytochrome c. The mechanism by which cytochrome c is released from mitochondria to the cytosol is still unclear. More recent reports indicated that cytochrome c is released from mitochondria by special mechanisms under conditions of preserved intactness of the outer mitochondrial membrane (Halestrap *et al*., 2000; Martinou *et al*., 2000). A decisive role in this process is played by the mitochondrial permeability transition pore (PTP) and the pro-apototic protein Bax (Crompton, 1999).

A central checkpoint of apoptosis is the activation of Caspase-9 by mitochondrial cytochrome c. The BH4 domain of Bcl-2 and Bcl-$_{XL}$ binds to the C terminal part of Apaf-1, thus inhibiting the association of Caspase-9 with Apaf-1. The pro-survival proteins appear to protect mitochondria, since Bcl-2 prevents the release of cytochrome c. However, the pro-apoptotic BH3 subfamily member, Bid has been reported to mediate the release of cytochrome c without evoking mitochondrial swelling and permeability transition, and further it has been shown to bind to pro-apoptotic members of the Bcl-2 family, such as Bax , Bcl-2 and Bcl-$_{XL}$ (Lindsten *et al*., 2000; Wei *et al*., 2001). Bcl-2 cell survival activity was reduced by substitutions in two of ten conserved BH4 residues. Deletion of BH4 rendered Bcl-2 (and Bcl-xL) inactive but did not impair either Bcl-2 homodimerization or ability to bind to Bax or five other pro-apoptotic relatives (Bak, Bad, Bik, Bid or Bim). Hence, association with these death agonists is not sufficient to promote cell survival. The BH1 and BH2 domains of Bcl-2 family members (Bcl-2, Bcl-$_{XL}$ and Bax) form pores in organelles such as mitochondria; Bax and Bak induce cell death, whereas Bcl-2 protect by preventing mitochondrial disruption. Bax and Bax-like proteins may mediate caspase-independent death via channel-forming activity (Halestrap *et al*., 2000; Martinou *et al*., 2000).

1.5.1.2.1 Involvement Bcl_2 family in the induction of apoptosis

The primary regulatory step for mitochondrial-mediated caspase activation may be at the level of cytochrome c release. The known regulators of cytochrome c release are the Bcl-2 family proteins. According to their function in apoptosis, the mammalian Bcl-2 family can be divided into pro-apoptotic and anti-apoptotic members (Cory and Adams, 2002). The pro-apoptotic members include Bax, Bcl-Xs, Bak, Bok/Mtd, which contain 2 or 3 Bcl-2 homology (BH) regions, and molecules such as Bad, Bik/Nbk, Bid, Hrk/DP5, Bim/Bod, and Blk, which contains only the BH3 region. The anti-apoptotic Bcl-2 family members include Bcl-2, Bcl-XL, Bcl-w, A1/Bfl-1, Mcl-1, and Boo/Diva, which contain three or four regions with extensive amino acid sequence similarity to Bcl-2 (**Table 1.2**) BH1-BH4Bcl-2 cleavage and activation is an important signal for mitochondrial cytochrome c release upon specific cellular insults (Suzuki *et al*., 2000; Cheng *et al*., 2001).

The Bcl-2 pro-apoptotic family members encourage cells to undergo apoptosis by permeabilizing mitochondrial membrane to cytochrome c, whereas anti-apoptotic Bcl-2 family members prevent its translocation and activation of Bax on mitochondria. Bax in viable cells resides as a monomer in the cytosol or loosely attached to intracellular membranes and about 10% in the endoplasmic reticulum (ER) (Scorrano and Koromeyer, 2003). In healthy cells or in its inactive form, it has been shown that Bax's c-terminal α-helix that is essential for mitochondrial targeting is bound in the hydrophobic pockets formed by the BH1, BH2, and BH3 domains of the molecules. Following its activation by several stimuli, a structural change occurs and α-9 is displaced from the BH3 pockets (Suzuki *et al*., 2000; Cheng *et al*., 2001; Scorrano and Korsmeyer, 2003). Once activated, Bax translocates to the mitochondria and oligomerizes causing cytochrome c release through the permeabilized mitochondria while some evidence suggest that changes in mitochondrial

membrane potential induce the activation of Bax (Kiechle and Zhang, 1998, Jurgensmeier *et al*., 1998; Chandra, 2002; Willis and Adam, 2005).

A variety of intrinsic death signals direct BH3-only proteins to undergo post-translocation modification that results in its activation. Following its activation, BH3 only proteins cause the release of cytochrome c, interact and inhibit anti-apoptotic Bcl-2 family members, activate Bax and Bak. Additionally, they interact with intrinsic multidomain proteins such as the adenine nucleotide exchanger to initiate apoptosis. BH3-only proteins are restrained from initiating inappropriate cell death through post-transcriptional constraints that i) induces sequestration of phosphorylated Bad by 14-3-3 proteins and ii) increases synthesis of Bid which cleaved by caspase 8 to generate the active truncated, tBid. In healthy cells, Mcl-1 has been reported to sequester Bak and initiate its degradation, whereas in apoptosis noxa displaces Bak from Mcl-1 and Bad displaces Bcl-xL to induce apoptosis by Bak (Figure 3). Following cytotoxic stimuli, binding of Bad or Bik to Bcl-2 displaces tBid (Bim), allowing it to engage Bax and trigger its oligomerization (Letai *et al*., 2002). The intact Bid protein is located in the cytoplasm but upon cleavage by caspase 8 truncated Bid translocates to mitochondria where it initiates the release of cytochrome c. (Schendel *et al.,* 1999). PUMA and Bim targ*et al*l pro-survival proteins and are said to be more potent killers than other BH3-only proteins that target only a subset (Chen *et al.,* 2005). In most healthy cells, apoptosis requires to be neutralized by Bcl-2, Bcl-x_L Bcl-w, Mcl-1 and A-1, and the sequestration of cytoplasmic p53 by Bcl-x_L, Following apoptotic stimulation, PUMA engages Bcl-x_L and releases p53 allowing it to activate Bax, which then activates caspases. Bim is crucial in eliminating lymphocytes that recognize self-antigens during the development of both T Cells and B cells, and it has been shown to mediate death induced by physiological stimuli, signal induced by activated oncogenes, DNA damage, chemotherapy drugs and γ-

irradiation (Willis and Adams, 2005). In further experiments, Thomas *et al.* (2001) reported that granzyme B (cytotoxic T-lymphocyte-associated serine protease, GzmB) was able to initiate cytochrome c release independent of caspases by cleaving Bid at asp75 during GzmB-mediated apoptosis. Additionally Gzm-B was able to cause a rapid mitochondrial depolarization with features that distinguished it from other depolarization events described previously during apoptosis. This finding provided greater understanding of molecules that can induce cytochrome c release in the absence of Bid, Bad and Bax.

BH3 proteins initiate cell death by inhibiting the anti-apoptotic Bcl-2 family members or activating the pro-apoptotic proteins (Schendel *et al.,* 1999; Thomenius *et al.*, 2003). In contrast, Bcl-2 and Bcl-x_L prevent all the mitochondrial changes that lead to the release of cytochrome *c* (Tsujimoto, 2000; Reed, 1997, see **Fig 1.3**). Upon activation, anti-apoptotic 28 kD Bcl-2 is said to be cleaved by caspase 3 to a 23 kD pro-apoptotic Bax-like death effectors that prevents caspase activation by sequestering Apaf-1, and thereby preventing disruption of the apoptosome (Cheng *et al*., 1997). This effect prevents downstream activation of executioner caspases, and thus apoptosis is prevented (Narita *et. al*., 1998). Antiapoptotic Bcl-2 family members have the potential of inhibiting apoptosis induced by BH3-only proteins at a location distinct from the mitochondrion. This was shown in mutant Bcl-2 and Bcl-xL that were deficient in Bax but can still function (Cheng *et al*., 2001). In support of the above statement, Thomenius *et al.* (2003) reported that Bcl-2 protects the mitochondrial membrane Following dephosphorylation, Bcl-2 has been reported to sequester Bad, by preventing unbound Bad from accumulating on the mitochondrial membrane and inducing apoptosis. Evidence therefore suggests that Bcl-2 and Bcl-x_L may serve as check points for BH3 mediated apoptosis. Because BH3 by binding to Bcl-2 or Bcl-x_L initiates apoptosis, it has been proposed that small inhibitor molecules could bind to this hydrophobic pocket in Bcl-2 resistant cells (Pisoni *et al*., 2005). In view of this finding it has been suggested that

small inhibitor molecules which could block the interaction between Bcl-2/ Bcl-x_L and proapoptotic proteins (peptide) such as Bak, Bad and Bax, and thereby inhibit the biological function of Bcl-2/ Bcl-x_L could provide a new approach to developing anti-cancer drugs.

1.5.1.2.1.1 Bcl-2 family and carcinogenesis

The Bcl-2 family proteins play a central role in maintaining the balance between death and life of cancerous cells. In lung cancer 50% -90% overexpression of Bcl-2 has been reported suggesting that Bcl-2 play a crucial role in genesis of the lung. Unlike in normal cells, Bcl-2 promote tumourigenesis by attenuating cell death as opposed to promoting cell proliferation, thus Bcl-2 allows tumour cells to ignore the environmental cues which signal normal cells to undergo apoptosis. Overexpression of the anti-apoptotic 28 KD Bcl-2 in cancer cells may results in drug and chemotherapeutic resistant despite the presence of an intact p53 (Wan *et al*., 2005, Teixeira *et al*., 1995). Bcl-2 in overexpressing cells (Hl-60) has been shown to be resistant to triptolide-induced cell death which indicates that Bcl-2 might play a protective role on triptolide-induced apoptosis (Wan *et al*., 2005). During triptolide treatment cleavage of Bcl-2 acts as a positive amplification loop for the release of cytochrome c and full activation of caspase which might suggest that Bcl-2 cleavage serves as a alternative mechanism for p53 independent apoptosis in p53 deficient cells (Wan *et al*., 2005).

Recently, the effects of the Bcl-2, Bcl-x_L and Bax proteins have been investigated on the regulation of cancer cell apoptosis induced by chemotherapy, and it has been reported that in human cancer cell lines, the expression levels of Bcl-2 and Bcl-x_L demonstrated a positive correlation with the prevention of apoptosis induced by various cytotoxic drugs. Cytosolic cytochrome c release, caspase 3 activation and the expression of Bcl-2 family protein play an essential role in the apoptotic process in cancer cells. Several studies have shown that

overexpression of Bcl-2 and Bcl-x_L prevents the mitochondrial release of cytochrome c and cells from death induced by a variety of agents such as UV irradiation cytotoxic drugs, growth factor withdrawal, p53 and c-myc overexpression, thereby inhibiting the activation of caspases cascade and thus inhibiting apoptosis and protecting some cell types from Fas and TNFR1-mediated apoptosis (Liu *et al.,* 1996; Palozza *et al.,* 2003, 2004).

Overexpression of Bax protein sensitizes cancer cell to several chemotherapeutic agents (Chou *et al.*, 2003). In recent study, Kaliberov and colleagues (2002) using a single adenoviral vector to target expression of the Bax gene in cancer cells reported that overexpression of Bax induces apoptosis in lung cancer cell lines but not in normal cell lines. Furthermore, the results have demonstrated that under hypoxic conditions the regulatory activity of the human VEGF promoter in lung cancer cells increases apoptosis and growth suppression. Interestingly, recent studies have shown that the combined addition of β-carotene and tobacco smoke condensate prevented the increase in Bax expression and minimized apoptosis induction caused by tobacco smoke alone due to decreased intracellular expression of p53 which is known to regulate Bax levels (Palozza *et al.,* 2004). This recent report provides a promising molecular mechanism on how tobacco smoke transforms cells into cancerous cells, and how one may target the Bax gene to induce apoptosis in lung cancer cells. Loss of Bax has been associated with accelerated tumour growth which resulted from the loss of p53 in a brain model. Zhang *et al.* (2000) reported that Bax was responsible for almost half of p53-dependent apoptosis induced by 5-FU in colorectal cancer cells. However the induction of Bax is not strictly dependent on p53 in many tissues.

1.5.1.2.1.2 Role of caspase in apoptosis and carcinogenesis

The caspases are a family of cysteine proteases that are the main effectors of apoptosis or programmed cell death (PCD) and their activation leads to characteristic morphological changes of the cell such as shrinkage, chromatin condensation, DNA fragmentation and plasma membrane blebbing. They belong to a group of enzymes known as cysteine proteases and exist within the cell as inactive zymogens (Boatright and Salvesen, 2003). These zymogens can be cleaved to form active enzymes following the induction of apoptosis (Boatright and Salvesen, 2003). Deletion of genes that encode murine caspases suggests that caspases are involved not only in apoptosis but also in cytokine maturation and cell growth and differentiation. Among them, caspase-1 and caspase-11 are primarily involved in the processing of pro-inflammatory cytokines. Caspase-3 and caspase-9 are essential for apoptosis during brain development. Caspase-8 is required for the development of heart muscle, cell proliferation in the hematopoietic lineage and death-receptor-mediated apoptosis.

Induction of apoptosis via death receptors typically results in the activation of an initiator caspase such as caspase 8 or caspase 10 (Thornberry *et al.*, 1997). These caspases can then activate other caspases in a cascade. This cascade eventually leads to the activation of the effector caspases, such as caspase 3 and caspase 6. There are other mechanisms in which caspases can be activated e.g Granzyme B can be delivered into cells by cytotoxic T lymphocytes and can directly activate caspases 3, 7, 8 and 10 (Fuentes-Prior and Salvasen, 2004). The mitochondria are also key regulators of the caspase cascade and apoptosis. Release of cytochrome C from mitochondria leads to the activation of caspase 9 and caspase 3. This effect is mediated through the formation of an apoptosome, a multi-protein complex consisting of cytochrome C, Apaf-1, pro-caspase 9 and ATP (Fuentes-Prior and Salvasen, 2004). These apoptosomes are responsible for the cleavage of the key cellular proteins, such

as cytoskeletal proteins, that leads to the typical morphological changes observed in cells undergoing apoptosis. Caspase activation can also be mediated by intrinsic factors such as Bcl-2 on the mitochondrial membrane. Bcl-2 is normally found associated with Apaf-1. Damage causes Bcl-2 to disassociate from Apaf-1 leading to the release of cytochrome c into the cytosol and formation of apoptosome.

Cancer cells are characterized by their resistance to undergo apoptosis which maybe due to failure of some initiator caspases to activate the caspase cascade. From the 14 caspases identified to-date, only caspase 10 shares the homologous death effector domains with caspase 8, which suggests that caspase 10 may also function by interacting with death receptors (Wang *et al*., 2001). If this view proves to be true, caspase 8 could be inactivated by virally encoded inhibitors or through a process of malignant transformation to promote cell longevity, and thus caspase 10 could provide an alternative pathway for cell elimination by apoptosis. Several studies have also proposed that caspase 10 may induce apoptosis following death receptor signaling, because of recruitment of caspase10 into the death receptors signaling complex; however, the molecular mechanism involved is still unclear (Kischkel *et al.,* 2001, Wang *et al.*, 2001). In support of this concept, Wang *et al*. (2001) reported impaired apoptosis following signaling by death receptor associated with increased caspase 10 mutant proteins. They further reported that FADD interacts much more strongly with caspase 10 than with caspase 8. In support of the latter view, expression of caspase-10 sensitizes MCF-7 breast carcinoma cells to TRAIL- but not tumour necrosis factor (TNF)-induced apoptosis (Engels *et al*., 2005). Caspase 10 mediated sensitization for TRAIL-induced apoptosis appears to be dependent on caspase 3, as expression of caspase 10 in MCF-7/caspase 3 cells but not in caspase 3-deficient MCF-7 cells overcomes TRAIL resistance.

Neutralization of TRAIL-R2, but not TRAIL-R1 impaired apoptosis in a caspase 10-dependent manner, indicating that caspase 10 enhances TRAIL-R2-induced cell death. Several other studies showed also that caspase 10 was able to induce apoptosis in a caspase 3-dependent manner in a number of caspase 8-deficient tumour cells (Deng *et al.,* 2002; Teitz *et al*., 2001).

1.5.2 Apoptosis inhibitors

Members of the inhibitor of apoptosis (IAP) gene family (Zitting *et al*., 2002) are found in the genomes of all metazoans, and are structurally characterized by the presence of 1-3 copies of a ~70 amino acid zinc finger fold, designated Baculovirus IAP Repeat (BIR) (Zitting *et al*., 2002). Other structural features found in certain, but not all IAPs include a caspase-recruitment domain (CARD), a RING finger, an ubiquitin-conjugating domain, and a nucleotide-binding P loop motif (Deveraux and Reed, 1999). The inhibitors of apoptosis (**IAPs**) comprise a family of anti-apoptosis proteins which directly bind and inhibit the functional activity of caspases, the cell death regulators (Roy *et al*., 1997; Wang *et al*., 2004; Schimmer, A.D., 2004). Growing evidence also indicates that IAPs also modulate cell division, cell cycle progression, and signal transduction pathways. The inhibitor of apoptosis proteins (IAP) are found in both vertebrates and invertebrates originally found in baculoviruses. There are currently eight human IAP proteins which have been identified to-date (Shin *et al.,* 2003). Many of the IAPs also contain a RING finger domain, and like other RING finger proteins, these IAPs can function as ubiquitin ligases. Ubiquitin ligases work in conjunction with ubiquitin-activating and ubiquitin-conjugating enzymes to covalently link ubiquitin to the amino acid lysine, present in the target protein. Sequential linkage of multiple ubiquitin moieties then results in targeting of the ubiquitinated protein for destruction by the

proteasome. Among all of the IAP members, Survivin and xIAP (X-linker IAP) are highly expressed in cancer and in transformed cells, but to a lesser extent in normal cells, which suggests that these members may contribute to the development and progression of cancers. IAP overexpression is a poor prognostic marker in a variety of solid tumours and hematologic malignancies. Finally, IAPs are attractive therapeutic targets, and efforts are under way to develop antisense and chemical IAP inhibitors that may be useful for the treatment of a variety of malignancies. For all of these potential clinical applications, however, the challenge remains to incorporate these findings into actual clinical practice.

1.5.2.1 Role of xIAP and Smac in apoptosis inhibition

xIAP is the most potent inhibitor of caspases (Lin *et al*., 2000; Shin *et al.,* 2003, 2005; Tamm *et al.,* 2003). Recent studies on the structure of xIAP-BIR2 complexed with caspase 3 or 7 (Chai *et al.*, 2001), and xIAP-BIR3 complexed with caspase 9 have elucidated the mechanism of caspase inhibition by these two behavioural IAP repeat domains (BIRs). In the xIAP-BIR2 mechanism, it has been shown that the linker molecule between BIR1 and BIR2 of xIAP inhibits activated caspase 3 or 7 by binding tightly to their active sites, thereby preventing substrate entry and catalysis (Huang *et al.,* 2001; Shin *et al.,* 2003, 2005). In addition interactions have been observed also between the N-terminus of caspase 3 and the peptide-binding groove of xIAP-BIR2 (Riedl *et al.,* 2001; Chai *et al*., 2001), whereas in xIAP-BIR3 the peptide-binding groove makes contact with the N-terminus of the small subunit of caspase 9 (Shiozaki *et al.,* 2003). ML-IAP (melanoma inhibitor of apoptosis) is also a potent anti-apoptotic molecule which was considered to inhibit caspase 3 and 7, although its mechanism of action was unclear. Recently, Vucic *et al*. (2005) have suggested that ML-IAP regulates apoptosis by sequestering Smac, resulting in xIAP mediated inhibition of caspases being antagonized, rather than directly inhibiting caspases. Yang *et al.* (2003) reported that

expression levels of xIAP were associated with chemoresistance in NSCLC NCL-H460 cells lines as a result of dysfunctional cytochrome c/dATP-dependent caspase activation. Since caspases play a crucial role in eliminating unwanted cells, small molecules that targets caspase inhibitors could be of therapeutic value against cells resistant to apoptosis.

Smac/DIABLO was the first natural inhibitor of IAPs and most studies has shown that it directly binds to IAPs. After activation, Smac translocates to the intermembrane space of mitochondria where the N-terminal 55 amino acids constitute the mitochondrial localization signal are removed to reveal the RHG (Reaper/HID/Grim- pro-apoptotic) motif, and Smac is then released from the mitochondria into the cytoplasm in response to many apoptotic stimuli, both those that activate death receptors and the intrinsic pathway. Following its translocation, the N-terminal peptide of Smac/DIABLO binds to a substrate groove on the xIAP-BIR domain thereby preventing it from binding with caspase 3, 6 or 7 (see **Figure 1.3**). Even though several reports have suggested that Smac alone could not induce apoptosis but it is shown to have the potential to serve as a therapeutic target in cancer cells as it was capable of potentiating the proapoptotic activity of epothilon B, TRAIL and wide range of chemotherapeutic drugs (McNeish *et al.,* 2004). Smac was able to induce apoptosis in cancerous cells following transfection with recombinant adenovirus encoding the mature processed form of Smac and a Smac mutant (GVP1-Smac) in which the first alanine was replaced by glycine to prevent IAP binding (McNeish *et al.,* 2004). It was shown that Smac not GVPI-Smac was a potent inducer of apoptosis in ovarian carcinoma cells. However, this was not observed in normal cells and that Smac interacted with survivin ubiquitination and proteasomal degradation (McNeish *et al.,* 2004). This verified the earlier results that Smac peptides in which the initial alanine of the RHG motif is mutated to glycine are incapable of binding to either the BIR2 or BIR3 of xIAP and that survivin does not play a role in Smac-

mediated apoptosis. In other studies targeting Smac/DIABLO-derived peptides Yang *et al*. (2003) and Franklin *et al*. (2003) demonstrated that peptides were able to sensitize a number of different tumour cell lines to apoptosis induced by a variety of pro-apoptotic drugs. Recently, small molecule inhibitors of Bcl-2 or mirror molecules of Smac/DIABLO have been shown to promote anti-neoplastic activity of conventional cytotoxic drugs (Harada and Grant, 2003). Chosa and colleagues (2004) have examined the effect of various caspase inhibitors on the apoptosis induced by CH-11, and they found that Fas-mediated apoptosis of human salivary gland cells (HSG) cells was slightly inhibited by the caspase-9 inhibitor although it was mainly inhibited by caspase-8 based on this results they concluded that CH-11 -induced apoptosis in HSG cells to be mainly mediated by type I death signalling pathway. In summary, the identification of small molecules that directly inhibit IAPs could provide a potential mechanism for the induction of apoptosis in these cells.

1.5.2.2 Role of Survivin in Cell Death

Survivin is a 16 kDa protein member of the inhibitor of apoptosis (IAP)/BIRP gene family, which includes xIAP, c-IAP-1, c-IAP-2, ILP-2, NAIP, Livin and Apollon. Survivin is structurally characterized by the presence of a single baculovirus IAP repeat (BIR) domain, consisting of an approximately 70 amino acid zinc finger fold, and a RING finger domain (Altieri, 2003). Survivin is a bifunctional IAP that has been implicated in protection from apoptosis and cell division. Survivin's function in cell division has been linked to centrosomal function, metaphase and anaphase microtubule assembly and spindle checkpoint regulation, however, the mechanism by which survivin inhibits apoptosis has remained elusive. Functionally, survivin is considered to inhibit apoptosis by binding to microtubules of the mitotic spindles (Li, 2003; Li *et al*, 1998; Tamm *et al.,* 2003). Some reports have suggested that it is an IAP with a purely anti-apoptotic role due to the presence of a BIR

domain that makes it capable of directly inactivating caspase 3 and 7 (Tamm *et al*., 1998; Tamm *et al.,* 2003) . Survivin blocks the proteolytic processing and enzymatic activity of the initiator and effector caspases by direct interaction (Tamm *et al*., 1998). There is still no concurrence between the apoptotic function of surviving and its modulatory role in pathogenesis (Li *et al*, 2003). Understanding the mode of action of survivin has become a priority due its bifunctional activity and proposed role in human tumour biology.

Survivin is a unique inhibitor of apoptotic proteins (IAP), in that it is practically undetectable in most normal tissue but overexpressed in most human cancers including adenocarcinoma- and squamous carcinomas of lung (Ambrosini *et al.*, 1997). The overexpression of survivin in cancer and transformed cells but low expression in normal differentiated tissues was confirmed by Gazzaniga *et al.* (2003). In their report Ichiki *et al*. (2005) suggested that survivin is anti-apoptotic molecule, and therefore may contribute to the development and progression of cancer. In other studies, high levels of survivin expression have been detected in non-small-cell lung cancer using RT-PCR (Tanabe *et al*., 2004). Furthermore, Ling *et al.* (2005) found that high levels of survivin expression correlated with poor survival in NSCLC patients. Similarly Ikehara *et al.* (2002) reported that the expression of survivin protein in tumour cells was a poor prognostic factor in patients with Survivin may be an independent of the tumour stage. adenocarcinoma of the lung Moreover, suppression of survivin by antisense or dominant negative mutant or small interference RNA (siRNA) results in spontaneous apoptosis. Therapeutic molecule for some cancers, since it inhibits proliferating breast cancer MCF-7 cells (Li *et al*., 2005).

Figure 1.3: Showing the mechanism of apoptosis in mammalian cell

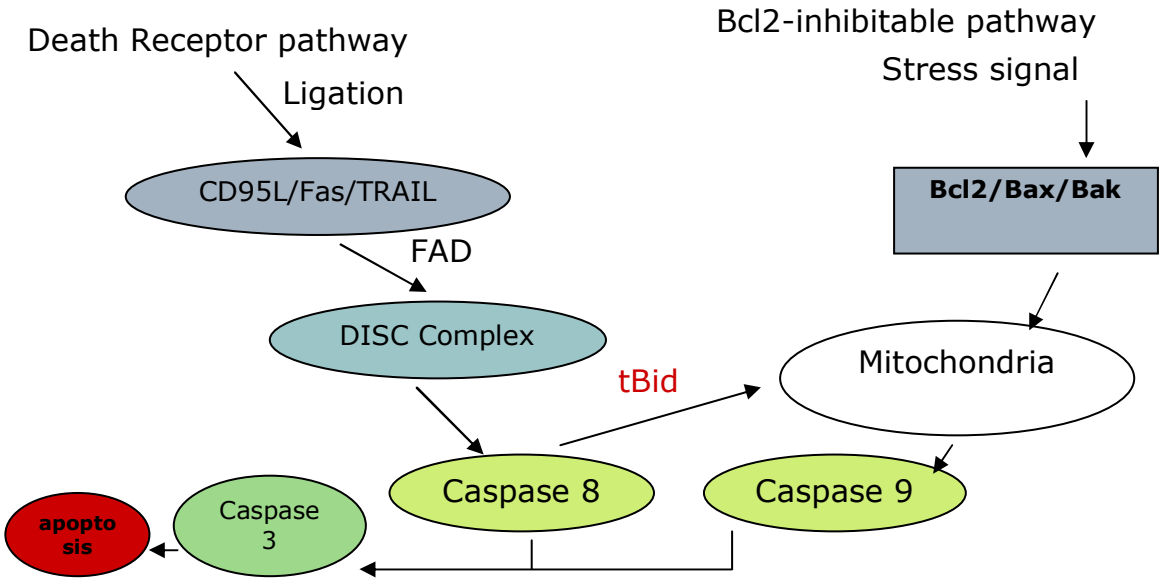

Figure 1.3: Schematic representation of the mitochondrial-mediated apoptotic pathway. Upon apoptotic stimulus, the mitochondrial permeability transition pore is formed, resulting in the release of the cytochrome c that initiate a cascade of caspapes that lead to cell death by apoptosis. The release of these pro-apoptotic molecules is controlled by the Bcl-2 family of pro-apoptotic members. When the ratio is in favour of apoptosis, these pro-apoptotic molecules are released. Receptor mediated apoptosis can enhance this pathway through Bid cleavage by caspae-8, enhancing the cytochrome c release.

1.5.2.3 Role of NF- κB in apoptosis

Nuclear factor kappa B (NF-κB) is a pleiotropic transcriptional factor involved in the inducible expression of a wide variety of genes, particularly those that promote cell growth and survival and that protect cells from apoptotic death stimuli (Chang, 2002). NF-κB is a heterodimer most frequently comprising a 50-kDa protein called p50 or NF-κB1 and a 65-kDa protein referred to as p65. In most mammalian cells the NF-κB heterodimer resides in the cytoplasm in the inactive form, bound to an inhibitor protein, I-κB. Upon exposure of the cell to a variety of stimuli such as growth factors, bacterial lipopolysaccharide (LPS, endotoxin), or cytokines such as TNF-α, inhibitory kappa B is phosphorylated and ubiquinated, allowing it to be degraded by the 26S proteosome (Chandra *et al*., 1998; Jeremias *et al*., 1998; Orlowski *et al*., 1998). These results in release of the active NF-κB heterodimer, which then translocates to the nucleus and mediates the upregulation of specific pro-survival and anti-apoptotic genes by binding to the κB consensus sequence in their regulatory regions (Barkett and Gilmore, 1999).

NF-κB pathway has been implicated in the pathogenesis of some human cancers, the abnormalities resulting in quantitatively high levels of NF-κB in the nucleus of a variety of tumours due to mutations that inactivate the IκB protein (Baldwin, 1996; Ghosh *et al.,* 1998). When NF-kB activation is induced by chemotherapeutic agents, it has been reported to provide an anti-apoptotic function by promoting cell survival of colorectal carcinoma and NSCLC cell lines (Jones *et al*., 2000). Ectoside, a chemotherapeutic agent, increases NF-κB levels and by inducing A1/Bf1-1 prevents the release of cytochrome c from mitochondria and the activation of caspase 3 (Wang *et al.,* 1998). By increasing the expression of anti-apoptotic cellular proteins NF- κB activation can therefore reduce apoptosis in response to treatment

with different chemotherapeutic agents. Chang *et al.* (2004) reported that following treatment with gemcitabine caused the cells to survive, whereas treatment with NF-kB inhibitors enhanced gemcitabine-induced apoptosis. A recent hypothesis states that blocking the NF- κB pathway by the IκB-super-repressor enhances the sensitivity of cells to apoptosis inducing stimuli. In testing this hypothesis Sanglioglu and colleagues (2004) infected human lung carcinoma cells with Ad.EGFP prior to treatement with TNF-apha, and found that AdIKB-alphaSR-mediated gene transfer completely blocked the TNF-alpha mediated nuclear translocation of NF- κB. Kuroda *et al.* (2000) in their study on the anti-tumour effects of human TNF-alpha mutant RGD-V29 reported that RGD V29 showed potent anti-tumour activity against human lung cancer (Mqnu-1 cells) xenografted nude mice without severe organ toxicity even at the maximal tolerated dose (MTD). On the contrary, severe toxicity at the MTD level was observed when recombinant hTNF was used. Kim *et al*. (2000) have demonstrated that TNF-alpha induced NF-kB activation was related to resistance of lung cancer cells to TNF-alpha mediated apoptosis based on the fact that they observed adenovirus-mediated overexpression of IkB-alpha-SR blocked NF-kB activation and sensitized lung cancer cells to TNF-alpha. By blocking the degradation of endogenous IkB-alpha pre-treated with a proteasome inhibitor suppressed NF-kB activation and increased TNF-alpha mediated apoptosis. Under specified circumstances, the up-regulation of NF-κB activity is responsible for the anti-apoptotic effects of TNF-alpha. So, one of the potentially useful strategies of gene therapy approach involves induction of apoptosis in lung cancer cells by inhibiting TNF-alpha–mediated NF-κB activity, thereby shifting the balance of TNF-alpha stimulation towards cell death.

1.5.3 Molecular targets in Apoptosis associated with human lung cancer

Many advanced cancers have cells with alterations in cell cycle kinetics, survival pathways, and apoptosis effector mechanisms that cause resistance to cytotoxic agents. Optimal anticancer drug combinations need to kill malignant cells in all cell cycle phases, while sparing normal cells. A new cancer therapy should be bi-directional, one including chemotherapy or radiation, and a key drug that targets a specific apoptotic molecules that are presently ineffective in cancer cells with low or no toxicity effect on the normal surrounding cells (Dlamini *et al*., 2005; Kim *et al*., 2002; Sarantopoulos *et al.,* 2005)

1.5.3.1 Gemcitabine-induced apoptosis

Gemcitabine is a novel antimtabolite, and is structurally related to deoxycytidine with two fluorine substitution of the two hydrogen atoms in position 2 of the deoxyribose sugar (Alberts *et al*., 2003; Apostolidou *et al*., 2003; Berlin and Rothenberg, 2003). In several different studies, Gemcitabine as a single agent or in combination with other drugs, which have known antitumour activity in the clinical setting, has been shown to be a very useful and promising drug in the treatment of human cancers of various types (Chen *et al*., 2003; Karasek *et al*., 2003; *Kralidis et al*., 2003; Lehman *et al*., 2003; Lim *et al*., 2003; Tomek and Manegold, 2003; Natale, 2005). Gemcitabine enters the cell via a nucleoside transport system and is activated to its difluoro deoxycytidin triphosphate before is incorporated into DNA, and in consequence causes chain termination (Graham *et al.,* 2000). Till to date there is no clearly defined relationship between gemcitabine activity in vitro and NSCLC, and the relationship between gemcitabine sensitivity and p53 status in NSCLC.

It is well documented that disruption of p53 function is coupled with resistance to chemotherapy and other drugs in some tumour cell lines (Achanta *et al.,* 2001; Galmarini *et*

al., 2002; Chang *et al*., 2004). Furthermore, some studies have shown that reintroducing wild-type p53 lead to a variety of effects in tumour cells including growth arrest and apoptosis (Chang *et al.,* 2000; Pirollo *et al.,* 2000), but the result obtained for most of tumour cell types was controversial. Galmarini *et al.* (2002), they found that cancer cells carrying a wild-type p53 were more sensitive to gemcitabine than cells with a mutated p53 while other reports found that over-expression of p53 severely compromised treatment with gemcitabine (Chang *et al.*, 2004). In comparison with other apoptotic molecules, gemcitabine induced NF-kB activation in NSCLC in a p53-independent manner (Arlt *et al.,* 2003). Furthermore, treatment with the NF-kB inhibitor, PDTC, significantly enhanced gemcitabine-induced cell. These suggest that activation of NF-kB provides a cell survival signal following gemcitabine treatment. Other studies have indicated that inhibition of NF-kB sensitized tumour cells to gemcitabine-induced apoptosis (Jones *et al.,* 2000). Overexpression of Bcl-2 protein has also been shown to block gemcitibine-mediated apoptotic cell death. If gemcitibine has to be used as potentially successful cancer therapy, further studies need to be conducted to target reintroduction of wild-type p53 into tumour cells.

1.5.3.2 Targeting cancer stem cells as a means to eradicate cancer cell

There is increasing evidence that both hematologic malignancies and solid tumours may originate in and be driven by a cellular subcomponent that maintains or reacquires stem cell properties. These tumour initiating or cancer stem cells may undergo clonal evolution and contribute to therapeutic resistance and relapse following therapy. A number of strategies are being developed to target this cell population for cancer prevention and therapy. Identification of the molecular factors that regulate survival function of stem cells may represent a novel therapeutic approach. Stem cell research has escalated significantly in recent years with the focus on increased understanding of natural role of stem cells in cell and

developmental biology, as well as their potential use for biomedical application. Clinical development of a gene therapy approach will depend on challenges in the development of faster and higher efficiency vectors with validation in animal studies relevant to human diseases (Agrawal and Schaffer, 2005).

1.5.3.3 Combination therapy between chemotherapy and radiation

Combination chemotherapy with radiation remains the standard of care for a majority of solid malignancies. Numerous randomized clinical trials across a wide variety of solid tumour sites have demonstrated that the combination of chemotherapy and radiation improves local-regional control, distant metastasis, and overall survival. The optimal chemotherapy regimens and radiation fractionation schemes; how best to integrate chemotherapy, radiation and surgery; and how to maximize the therapeutic gain while maintaining acceptable toxicity profiles and quality of life remains a constant challenge as we strive to further improve patient outcomes. The recent introduction of effective biological targeted agents provides exciting avenues and new challenges for integrating local and systemic treatment to further enhance the therapeutic ratio. Resistance to chemotherapy is still one of the most difficult problems in lung cancer treatment. In order to overcome this problem, the identification of the molecular prognostic markers predictive of the response to specific therapies may improve treatment profiles.

1.6 Lung Cancer

Lung cancer, one of the most common forms of cancer that affects adults in the world, is the leading cause of cancer deaths worldwide, accounting for approximately 20% of all cancer deaths. In developed countries, lung cancer accounts for 24% of all cancers with almost 1.3 million cases reported annually. Lung cancer is a disease of uncontrolled cell growth in

tissues of the lung. This growth may lead to metastasis, which is invasion of adjacent tissue and infiltration beyond the lungs. The vast majority of primary lung cancers are carcinomas of the lung, derived from epithelial cells. The main types of lung cancer are small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). This distinction is important, because the treatment varies; non-small cell lung carcinoma (NSCLC) is sometimes treated with surgery, while small cell lung carcinoma (SCLC) usually responds better to chemotherapy and radiation. The most common cause of lung cancer is long-term exposure to tobacco smoke. The occurrence of lung cancer in nonsmokers, who account for as many as 20% of cases, is often attributed to a combination of genetic factors, asbestos, and air pollution, including exposure to cigarette smoke.

1.6.1 Subtypes of lung cancer

Classification of lung cancers has presented many challenges, and a basis for the classification of human lung cancers has been critically reviewed (Zitting *et al*., 2002; Hodgson *et al*., 2000).

1.6.1.1 Squamous cell (epidermoid) carcinoma

Squamous cell carcinoma is a common form of lung cancer, accounting for approximately one-third of all cases of bronchogenic carcinomas. Unlike adenocarcinoma, it is strongly linked with a history of cigarette smoking. Its histogenesis may be related to chronic inflammation and injury of the bronchial epithelium, which leads to replacement of the normal ciliated columnar epithelium by a squamous epithelium (Boffetta. 2004; Brambilla *et al*., 2001). This transformation from a glandular epithelium to squamous epithelium is known as squamous metaplasia. Most squamous cell carcinomas arise centrally from either the main, lobar or segmental bronchi and ulcerate through the mucosa into the surrounding lung

parenchyma. Their central location also tends to produces symptoms at an earlier stage than tumours located peripherally (Boffetta. 2004).

Squamous cell carcinomas are graded according to their degree of differentiation and designated as well, moderately, or poorly differentiated. Well differentiated tumours are recognized as exhibiting orderly stratification, obvious cellular bridges, and keratin pearl formation. In contrast, poorly differentiated squamous cell carcinomas are noted for their lack of keratinization and lack of intercellular bridges. Moderately differentiated tumours fall somewhere in between. Tumours are graded with respect to their least differentiated areas (Brambilla *et al*., 2001).

1.6.1.2 Adenocarcinoma

Adenocarcinoma is a type of non-small cell lung cancer that 35-40% of all lung cancers, usually occurring in a peripheral location within the lung and arising from bronchial mucosal glands. The progression of adenocarcinoma is quite unpredictable. In most cases, adenocarcinoma spreads slowly and causes very few lung cancer symptoms. But it can also be extremely invasive, aggressively spreading through the body and causing death before it can be treated. The incidence of adenocarcinoma is higher in women than in men and it is the most frequent type in people who have never smoked. Adenocarcinoma has been linked to interstitial pulmonary fibrosis and to asbestos exposure because scarring is considered to cause malignant transformation of hyperplastic epithelial cells. Most adenocarcinoma cases originate in the outer lungs, but roughly 33 percent first surface in the lungs' central regions. Once adenocarcinoma cancer cells develop, they form thick tumours that inhibit breathing and lung function. Sometimes, these tumours spread to the liver, adrenal glands, and bones, making adenocarcinoma much more difficult to treat.

Adenocarcinomas arise peripherally, frequently occurring as single or multiple nodules of variable size and often in subpleural mucous glands. Invasion of the pleura and the chest wall is seen in about 15% of patients. The cells retain some of the tubular, acinar or papillary differentiation and mucus secretion. Histologically, they show a pattern of spread in which well differentiated, mucin- or non-mucin-producing cells grow alongside the pre-existing septal architecture of the lung (Brambilla *et al*., 2001). The tumour cells are usually arranged singly or in combination as clusters, papillae, acini or as sheets of cells. In some cells the feature visualized is either abundant homogenous or granular cytoplasm, but in others foamy or mucin filled vacuoles are seen. The nuclei are usually single and round to oval in appearance with granular chromatin. A significant number of adenocarcinomas comprise bronchioalveolar and invasive carcinomas (Travis *et al*., 2004).

1.6.1.3 Large cell undifferentiated, anaplastic carcinoma

Large cell carcinoma is the uncontrolled growth of abnormal cells in the lungs. This cancer is a type of non-small cell lung cancer that represents 10% to 20% of all tumours that start in the bronchi, which are the main branches of the trachea that lead to the lungs. With 40% of cases associated strongly with smoking. Large cell carcinoma is usually large at the time of diagnosis. They tend to be accompanied by extensive bleeding and tissue damage. They often are called undifferentiated tumours because the cells lack the specific architecture found in other types of cancer cells. Large cell carcinoma tends to grow quickly and metastasize at an earlier stage than other forms of non-small cell lung cancer.

The cytology shows a lack of discriminatory features and the tumours show aggregation of the transformed cancer cells. The cytoplasm is basophilic with large nuclei. Many large cell carcinomas have cytological and architectural features similar to those of poorly

differentiated squamous carcinomas or adenocarcinomas. The major subtypes are the neuroendocrine, basaloid, clear cell, rhabdoid and lymphoepithelioma-like phenotypes. These tumours arise from a common progenitor reserve cell or basal bronchial epithelial stem cell with the capacity to differentiate and transform into cancer cells with cytology similar to any of the subtypes (Franklin. 2000).

1.6.1.4 Small cell undifferentiated carcinoma

Small cell carcinoma is a type of cancer that always affects the lung. Small cell carcinoma may also be referred to as oat cell carcinoma and in some cases, is a mixed cell carcinoma. Small cell carcinoma is frequently caused by smoking, but exposure to large amounts of asbestos is also a risk factor. Small cell carcinoma usually affects men more than women and whiles not a common type of lung cancer, is considered very deadly. Unlike other types of cancer, small cell carcinoma is not staged on a numerical scale but rather as simply limited or extensive. Limited stage refers to cancer that is contained within the lungs or bronchial tubes only.

This tumour has an incidence of about 20%, and shows very high correlation with smoking and uranium mining. Histologically, the tumour comprises sheets of darkly staining, densely packed cells with scanty cytoplasm, ill defined cell membrane, fine granular cytoplasm and absence of nucleoli (Brambilla *et al*., 1993). Fusiform or spindle shaped cells are also common. The cancer cells contain cytoplasmic granules that immunolabel for neuron-specific enolase, CD56, chromogranin and synaptophysin (Nicholson and Ryan. 2000). Small cell neoplasia arises from the basal (stem) cells of the bronchial epithelium and has the capacity to differentiate into each of the major histological types of lung cancer comprised of either endocrine or non-endocrine epithelial cells. The morphology and genomics of small cell and

large cell neuroendocrine phenotypes is very similar but they show less commonality with carcinoid tumours.

1.6.1.5 Carcinoid tumours

Carcinoid tumours of the lung are a fascinating but uncommon group of pulmonary neoplasms. In the past, these tumours were grouped with benign or less aggressive malignant pulmonary tumours (DeLellis. 2001). Typical carcinoid tumours represent 2% of lung tumours and occur equally in both sexes. The mean age of incidence is younger than that for lung cancer with many tumours presenting in young adulthood. Carcinoid tumours are unrelated to cigarette smoking. Bronchial carcinoid can be a component of a multiple endocrine neoplasia syndrome. As no predisposing factors are known prevention and screening do not have a role in this disease.

Together they were grouped as a category of neoplasms called bronchial adenomas. Recent study has revealed that carcinoid lung tumours represent the most indolent form of a spectrum of bronchopulmonary neuroendocrine tumours that includes small cell carcinoma of the lung as its most malignant member and several other forms of intermediately aggressive tumours, such as atypical carcinoid (Pu *et al*., 2001). Carcinoid tumours represent the less malignant end of a spectrum of lung tumours showing neuro-endocrine characteristics which includes typical carcinoid (TC), atypical carcinoid (AC), large cell neuro-endocrine carcinoma (LCNEC) and small cell carcinoma (SCLC) (Nicholson and, Ryan. 2000; Brambilla *et al*., 2000; Soga and Yakuwa. 1999; Cagle *et al*., 1989).They range from benign to frankly malignant but their main effect on the lungs is generally of an obstructive nature. Most can be cured by surgical resection with a low recurrence rate. Chemotherapy and radiotherapy are rarely required. Carcinoid tumours are made up of nests, trabeculae and

mosaic patterns of polygonal cells with round uniform nuclei, granular "salt and pepper" chromatin, and inconspicuous nucleoli. The stroma shows numerous thin-walled small blood vessels. On electron microscopy numerous membrane-bound dense core neuro-secretory granules are apparent. Immuno-histochemical staining can help differentiate carcinoid tumours from NSCLC but not SCLC.

1.6.1.6 Bronchial carcinoid tumour

Bronchial carcinoid tumours make up the more benign, well differentiated, low grade neuro-endocrine tumours. The terms typical for those with benign pathological features and atypical for those with more sinister features are commonly used but may be misleading. A "typical" carcinoid may metastasise and thus be clinically atypical, while an atypical carcinoid may be cured by simple excision. More recently the term well differentiated neuro-endocrine carcinoma has been introduced to emphasise that all these tumours have malignant potential, and therefore should be excised, and that there is a spectrum from benign behaviour to malignancy.

This is a neoplasm of low malignancy that arises from the Kulchitsky cell of the bronchial mucosa. When it involves the major airways, the carcinoid tumour shows two growth patterns, endobronchial or peribronchial (Fong *et al.*, 2003). Lymph node and distal metastases occur in less than 10% of cases. The tumours occur as submucosal lesions that protrude intraluminally. They contain abundant neurosecretory granules, and the neuroendocrine markers, enolase and chromogranin. The tumours may secrete serotonin that results in the clinical carcinoid syndrome. Atypical carcinoid tumours arise centrally; they show striking nuclear pleomorphism and mitotic activity and greater metastatic potential.

1.6.1.7 Bronchoalveolar carcinoma

Bronchoalveolar carcinoma (BAC) is considered a subtype of adenocarcinoma. It has been called by a variety of different names (alveolar cell carcinoma, bronchiolar carcinoma, and BAC). BAC represents between 1% - 9% of lung cancers and nearly 2:1 ratio of women to men (DeLellis. 2001). There have been claims of correlation between certain clinical, radiographic, and pathologic findings and survival. Some researchers have reported BAC in women and no correlation to cigarette smoking (Rolen, *et al* 2003). The characteristic pathologic features of BAC are the presence of aerogenous spread and evidence of advancement along the alveolar wall. BAC can coexist with adenocarcinoma, and overlap within the same tumour often occurs.

Innovative research for new therapeutic targets in apoptosis is essential to increasing our understanding of cancer etiology. Defects in the regulation of apoptosis and cell death mechanism make important contributions to cancer development, progression, and resistance to therapy, most of the promising strategies for exploiting knowledge about apoptosis targets for cancer drug discovery have resulted in fruitful benefits; however that has not been entirely successful in all cases.

Table 1.1: Different tumour suppressors/oncogene and their related cancers

Oncogene/Tumor Suppressor Gene	Related Cancers
BRCA1, BRCA2	Breast and ovarian cancer
bcr-abl	Chronic myelogenous leukemia
bcl-2	B-cell lymphoma
HER2/neu (erbB-2)	Breast cancer, ovarian cancer, others
N-myc	Neuroblastoma
EWS	Ewing tumor
C-myc	Burkitt lymphoma, others
p53	Brain tumors, skin cancers, lung cancer, head and neck cancers, others
MLH1, MSH2	Colorectal cancers
APC	Colorectal cancers

Table1.2: Bcl-2 family of pro- and anti-apoptotic proteins

Pro-apoptotic members	Anti-apoptotic members
Bax, Bak, Bok, Bcl-Xs and BH3 only (Bad, Noxa, Bik/Nbk, Bid, Bim/Bod, Hrk/DP5)	Bcl-2, Bcl-X_L, Mcl-1,Bcl-w, A1/Bfl-1, Boo/Diva

1.7 OBJECTIVES AND RATIONALE

The purpose of this study is to establish the expressional pattern of the RBBP6 gene in lung cancer at both mRNA and protein levels. The objective is also to characterize the role of this gene and apoptosis in diverse lung diseases. An understanding of the role of RBBP6 in the development of lung diseases may lead to insights into developing new therapeutic measures for those lung diseases in which apoptosis plays a prominent part.

- To investigate mutations of the following RBBP6 domains, DWNN domain, RING finger domain and p53 Binding Domain (p53BD) in lung cancers. Ring finger is know for its ubiquitin function, any mutations in these domains might influence RBBP6 at protein level leading to disruption protein-protein interactions during lung cancer development.
- Determine the effect of RBBP6 on apoptosis and lung cancer following RBBP6 knock-down. This would light some light on its function in apoptosis because this study targeted the use of known apoptotic inducers i.e. Camptothecin and staurosporine
- Determination of expression pattern of the gene products i.e. mRNA and protein. This was done to determine whether the RBBP6 is expressed or not in human cancers. Localisation of the gene was conducted in both cancer tissues and cancer cell lines.

Chapter Two

Materials and Methods

2.1 Introduction

In this chapter, we will describe all the materials and methods used in this study to obtain the results that will be discussed in subsequent chapters. All procedures that were conducted several times will only be described once but any adjustment made will be duly mentioned in order to avoid repetition. Principles of each technique will be discussed before writing a full protocol of the technique. The following techniques were used included: Polymerase Chain Reaction (PCR), reverse transcription, Immunocytochemistry (ICC), *in situ* hybridisation, quantitative Real Time Polymerase Chain Reaction (qRT-PCR), Cloning, RNA interference, Western Blotting, apoptosis detection and sequence alignment. Chemicals, their suppliers and recipes for the solutions are given in the Appendix A. A list of equipment used is given in appendix B.

2.2 Materials

2.2.1 Ethics Approval

Ethic clearance was applied for from the University of the Witwatersrand Human Ethics Committee. The approved ethics certificate is enclosed as Appendix C.

2.2.2 Sample Materials

Tumour and healthy samples from South Africa patients were diagnosed and classified clinicopathologically by Dr J Murry, Consultant Pathologist from the National Health Laboratories Service (NHLS) and were processed onto slides by their Technicians at NHLS. Some other lung cancer and normal lung samples were purchased from American company

Cybrdi which provided us with total RNA for some of the cases. For interest, some lung carcinoma samples were provided by Prof Bhoola (Lung Institute of Western Australia).

2.2.3 Human Lung Cell lines

Different cell lines were used for this study, primarily for the evaluation of the role of Retinoblastoma Binding Protein 6 (RBBP6) during lung cancer development. The following human cell lines were used in this study A549 Adenocarcinoma cell line, MRC5 fibroblast and Lsqr1 Squamous cell carcinoma.

2.2.4 Primers

The following sets of primers were designed to amplify a fragment of the translated regions of each gene mentioned below.

RBBP6 primers:

Forward primer: 5' GGC TCC ACA TCT CCC TCT G 3'

Reverse primers: 5' GCA TTA TCA TCA GTA TAT TCT TCT TTC G 3'

DWNN Primers

Forward primer: 5' TTG GAC CGT CTG AAT GAA C 3'

Reverse primer: 5' TGG AAC TTG AAT ACT CTC TGG 3'

p53 Binding Domain (p53BD)

Forward primer: 5' GGT CCT TCG GTG TCT TTG 3'

Reverse primers: 5' AGG TGA CGG TAT CAT AGT TG 3'

2.2.4.1 quantitative Real Time –PCR primers

The following primers were used to measure the expression of the following genes.

DWNN Primers

Forward primer: 5' TTG GAC CGT CTG AAT GAA C 3'

Reverse primer: 5' TGG AAC TTG AAT ACT CTC TGG 3'

p53 Binding Domain (p53BD)

Forward primer: 5' GGT CCT TCG GTG TCT TTG 3'

Reverse primers: 5' AGG TGA CGG TAT CAT AGT TG 3'

BCl_2

Forward primer: 5' CCC TCC AGA TAG CTC 3'

Reverse primer: 5' CTA GAC AGA CAA GGA AAG 3'

Bax

Forward primer: 5' ATG GAC GGG TCC GGG GAG 3'

Reverse primer: 5' TCA GAA AAC ATG TCA GCT GCC 3'

2.2.4.2 RNAi Oligos

RNAi targets were generated by using the sequence of all the 3 RBBP6 variants and p53 in RNAi design software from Ambion. The RNAi oligos were synthesized in a manner that after annealed they were ready to use. The table below shows the targeted regions:

Table 2.1: A table showing different RBBP6 RNAi target oligos

Oligos	**Oligo sequence**
Isoform 1	Sense target sequence loop region Antisense target region 5'TCAAGACTTGGTTCAACACGTTCAAGAGACGTGTTGAACCAGATCTTGA TTTTTGGAAA3'
Isoform2	Sense target sequence loop region Antisense target region 5'TCTCCCTATAGTGGTTCTTCGTATTCAAGAGATACGAAGAACCACTATA GGGAGA TTTTTTGGAAA3'
Isoform3	Sense target sequence loop region Antisense target region 5'TCTCAGACTTTTTCTACACATTGCTTCAAGAGAGCAATGTGTAGAAAAA GTGTGAGATTTTTTGGAAA3'

This table shows RNAi oligos for targeting RBBP6 mRNA variants. The Isoform 3 was used to knock down variant 3, the DWNN which targets all the other two variants. Different regions are coloured with different colours, sense target sequence (red), loop region (blue) and antisense target sequence (green).

2.2.5 Cloning Vector

pGEM-T-Easy

The pGEM-T easy vector supplied in a PCR cloning system is engineered to have thymidine extentions on this blunt-end-digested vector. These TT extensions make it possible to clone PCR products which conventionally have poly-adenine nucleotide added at ends of PCR products. This vector was used to clone PCR products for probe synthesis as well as for sequencing. Attached appendix 4 is the vector map of pGEM-T Easy.

Figure 2.1: pGEM-T Easy vector

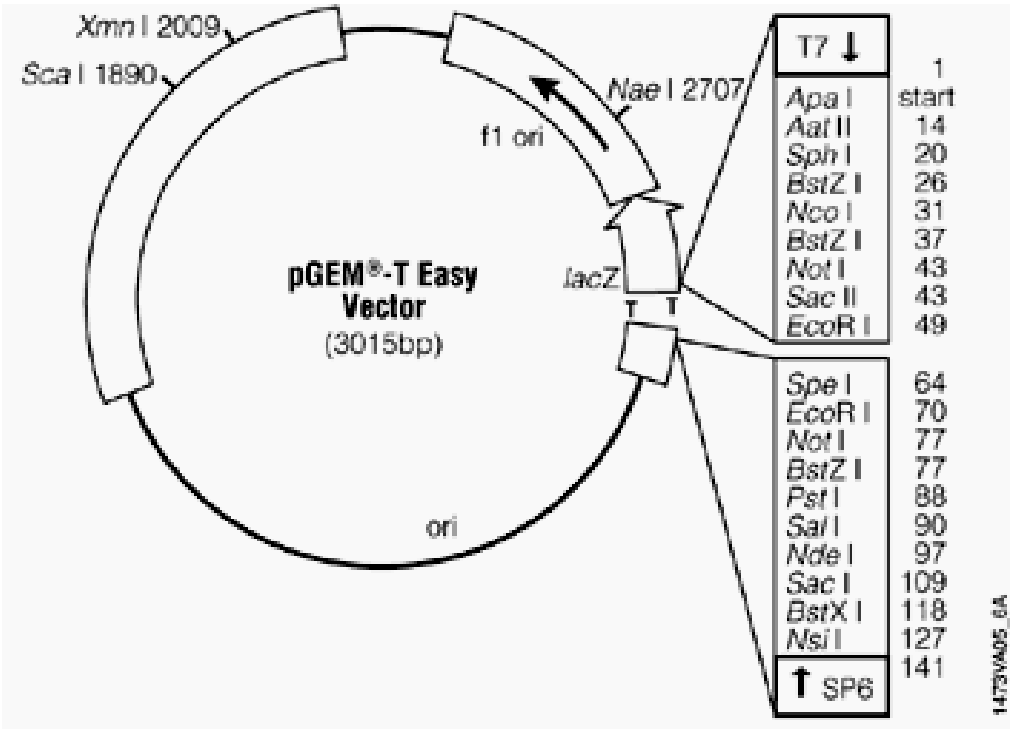

Figure 2.1: pGEM-T Easy vector: the map shows the different restriction endonuclease cut/digest sites in the pGEM-T Easy multiple cloning sites (MCS).

2.3 Methods

2.3.1 Tissue culture room

The culturing of mammalian cell has become a very important research technique in medical sciences. It has since its early development become more popular in molecular and cell biology. The tissue culture room was in closed room with no window fitted, with UV light that was turned on automatically at 19H00 to 05H00 am. The room was also washed with sulfuric acid every 10 week to remove all possible contaminants. Equipment required for use during cell culture was stored in the tissue culture room and all autoclaved glassware was rapped with the foil and stored safely in the tissue culture room.

2.3.1.1 Preparation of complete culture media and cell culturing

The complete (Dulbecco's Modified Eagle's Medium) DMEM, Hams F-12 and Eagle's Minimum Essential Medium (EMEM) consisted 10% fetal calf serum (FCS), 1% Pen-Strep. The media were then stored at 4 °C and pre-warm before culturing of cells. All the media, Phosphate buffer saline and Trypsin were purchased from Sigma and Highveld Biological. Cells were grown up to 70-80% confluence as a monolayer before any experiment can be conducted on them.

2.3.1.2 Trypsinization

Confluent cells were washed with Phosphate Buffered Saline (PBS) and trypsinised. Media containing 10% FCS was added to deactivate trypsin followed by centrifugation at 3000 rpm in a bench top centrifuge for 4 minutes. The supernatant was discarded and the cells were resuspended in desired media. The cells were either split into 3 flasks or frozen in complete medium containing 10% DMSO.

2.3.2 Total Ribonucleic Acid (RNA) Extraction

When cells were confluent or following any form of manipulation or induction of apoptosis using chemical agents, total RNA was isolated using RNA extraction kit from Roche Biochemicals (German-RSA). Briefly as per manufacturer's instruction, cultured cell were trypsinized, pelleted and resuspended in 200 µl PBS. Resuspended cells were then lysed in 400 µl of lysis/-Binding buffer by vortexing for 15seconds. High Pure Filter tube plus collection tube was assembled as described in the manufacturer's manual. The entire sample was transferred into the High Pure Filter tube-assemble and incubated for one minute and then centrifuged for 15 seconds at 8000 x g at room temperature. The flow-through liquid was discarded and the High Pure Tube + Collection tube were reassembled. The High Pure Tube + collection tube were incubated with a mixture of Incubation buffer and DNAase I for 15 minutes at room temperature. The High Pure Tube + collection tube were washed with 500 µl of wash buffer I and centrifuged for 15 seconds at 8000 x g. The flow-through was discarded and the tubes reassembled and the washing was repeated with 500 µl wash buffer II. A third wash was done with 200 µl Wash Buffer II at 12000 x g for 2 minutes at room temperature. The High Pure Tube was transferred into a sterile 1.5 ml micro centrifuge tube. Then 50 µl of RNA Elution Buffer was added to the assembled micro centrifuge tube + High Pure Tube to elute the total RNA and incubated for 1 minute. The elution was carried out by centrifuging at 12 000 x g for 2 minutes. The total RNA was then measured with the nano drop and quality accessed with RNA electrophoresis. Remaining RNA was aliquoted into 10 µl of aliquots and stored at – 80 ºC for future use.

2.3.2.1 RNA Gel Electrophoresis and preparation

Gel electrophoresis is a procedure that is used for the separation of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), or protein molecules using an electric current applied to a gel medium like agarose for RNA and DNA and acrylamide for protein. A 1% agarose solution was prepared in 1X MOPs (prepared in DEPC water), 0.6% (37% v/v formaldehyde), 0.3 µl/ml ethidium bromide (EtBr). RNA sample (1µg) was mixed with 10 µl of freshly prepared formaldehyde gel loading buffer. The mixture was heated at 65 ºC for five minutes, cooled on ice and was loaded on a 1X MOPS equilibrated gel. The RNA was electrophoresed at 100 volts for one hr in 1X MOPS.

2.3.2.2 RNA quantification

Quantification of both RNA and cDNA was done using a Nanodrop (NanoDrop technologies, USA). Reading was taken at 260 and 280 nm as described by the manufacturer. A 260/280 ratio of more than 2.0 were regarded as pure quality.

2.3.3 Reverse Transcription (RT)

In order to have deoxyribonucleic acid (DNA) for further experiments i.e. expression studies; cloning; probe synthesis and DNA sequencing, cDNA was synthesized using Promega's cDNA synthesis kit for RT-PCR (Promega, USA). The kit uses the ability of reverse transcriptase, which synthesizes a new cDNA strand at the site determined by the type of primer used: at the 3'-end of the poly (A) mRNA when oligo-p $(dT)_{15}$ is used, at non-specific points along the RNA template when using an oligo $(dT)_{15}$ primers. The resulting cDNA is then used as a DNA template for normal PCR.

Reserve transcript was performed as per manufacturer's instructions as follows. 2 µg of total RNA sample was mixed with 0.5 µg/reaction oligo $(dT)_{15}$ primer and incubated at 70 ºC for five minutes to denature any secondary structures and to allow the primers to anneal. The oligo $(dT)_{15}$ primer was used was used for this reaction. This was followed by chilling the template preparation on ice for 5 minutes. This preparation (table 2.2a) was then mixed with the rest of the components of the cDNA synthesis reaction tabulated in table 2.2b. The sample were mixed and briefly centrifuged to collect everything at the bottom of the tube. Following this samples were incubated at 25 ºC for 10 minutes to allow the primers to anneal at their sites. This was followed by incubation of samples at 42 ºC for 60 minutes to allow reverse transcription. After this incubation the reverse transcriptase was inactivated by incubation at 70 ºC for 10 minutes and the sample were cooled on ice. The sample were either used immediately for qRT-PCR or PCR or stored at -20 ºC for later use.

Table 2.2a: Template preparation for Reverse transcription

Total RNA	1 µg
Oligo $(dT)_{15}$	0.5 µg
Nuclease free water	5 µl
Final reaction volume	5 µl

This table shows a tabulation of RNA preparation for reverse transcription.

Table 2.2b: Cocktail of the reverse transcription (RT) mix

	Volume	Final concentration
5 X Improm II reaction buffer	4 µl	1X
Magnesium Chloride 25mM)	5 µl	5mM
dNTP mix	1 µl	0.5 mM each dNTP
RNA inhibitase	0.5 µl	20u
Nuclease free water	-	Up to 15 µl
Improm II Reverse transcriptase (1u/ µl)	1.0 µl	1u
Final volume	**15 µl**	

This table shows all the components for the RT reaction with volumes needed to make up the final concentrations given on the last column.

2.3.4 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is a technique that allows logarithmic amplification of short DNA sequences of between 100 to 600 bases pair within a longer double stranded DNA molecule. Primers for genes of interest were designed using Nucleotide sequences from NCBI. Designed primes were entered into the NCBI blaster programme to establish if any homologues existed with other genes. Once primer sequences were identified, they were sent to Inqaba biotech, SA for synthesis. The cDNA obtained through reverse transcription was used as a DNA template using sequence specific primers for different fragments of the RBBP6 gene. The PCR was performed using a master mix from Promega (USA). The master mix already contains deoxyribonucleotides, 2XPCR buffer, $MgCl_2$ and Taq Polymerase. For 25 µl reaction, 12.5 µl 2X master mix; 1 µl 10pmoles of each of the sequence specific

primers; 2 µl 25mM $MgCl_2$; 1 µl template DNA (1pg-1 µg) and 7.5 µl nuclease-free water were added. The reactions were then subjected to approximately 30 cycles of the three PCR steps (denaturation, annealing and extension) after the initial denaturation step and followed by a final extension step. The steps were as follow with some variation in annealing temperature and extension time depending on the nucleotide sequence of the gene of interest:

95 ºC for 2 Min

94 ºC for 30 sec

Tm-5 for 30 sec — 30-35 cycles

72 ºC for 30 sec

72 ºC for 10 Min

The products were either stored at 4 ºC for further use or elctrophoresed on 0.8 -1.5 % agarose gel.

2.3.4.1 Agarose gel electrophoresis of DNA

The principle of agarose gel electrophoresis involves separation of nucleic acids according to their size and conformation. During this technique the DNA fragments can be visualized by staining the gel with ethidium bromide which intercalates between bases of DNA and illuminating the gel under UV light on a transluminator.

Agarose gel electrophoresis is a technique used for separation of nucleic acids according to size and charge. The DNA fragments were visualized by staining the gel with ethidium bromide (1 µg/ml), which intercalates between bases of DNA and illuminating the gel under UV light using a transluminator. Agarose gel (1%) was prepared in 1 X TBE and contained 3

µl ethidium bromide. The DNA samples were electrophoresed on 1% agarose gel. The DNA molecular weight marker used was 100 base pair ladder from Inqaba Biotech-fermentous., USA. Gels were then subjected to 70 volts for 90 minutes in 1X TBE buffer.

2.3.5 CLONING OF PCR PRODUCTS IN pGEM-T-Easy VECTOR

The principle of a DNA cloning experiment involves inserting a DNA to be cloned by in vitro techniques into a cloning vector. The recombinant plasmid is then introduced by transformation into competent cells of *E.coli*.

2.3.5.1 DNA Ligation

pGEM-T-Easy vector allows the cloning of PCR products. Ligafast cloning system from Promega was used to clone PCR products into pGEM-T-Easy vector for both sequencing and probe synthesis. For the experimental ligation, 2µg PCR product was added to 5 µl of 2X ligation buffer; 10 ng of pGEM-T-Easy vector; 3 u of T4 DNA ligase and sterile distilled water to get a final volume of 10 µl. Background control was also set up as well as a positive control supplied with the kit. The components were mixed and quickly centrifuged all components to the bottom. The ligation mixture was incubated at room temperature for a minimum of 60 minutes. After 60 minutes ligation, the ligation mixture was then transformed into MC1061 *Escherichia coli (E.coli)*. Cloning strain competent cells prepared as follows:

Table 2.3: Cocktail of DNA ligation mixture

PCR Product	2µg
2X Ligation Buffer	5µl
pGEM-T- Easy Vector	10 ng
Distilled water (H_2O)	Up to 10µl
Final Volume	**10 µl**

This table shows all the components for DNA ligation reaction mixture.

2.3.5.2 Preparation of competent cells

The desired strain of *E.Coli* cells was streaked on a LB plate containing 10 mM Mg Cl_2 a night before. All glassware used in the preparation of competent cells was washed with concentrated HCL, rinsed with distilled water and autoclaved. A single fresh colony of the desired strain was grown for 2 hours in 20 ml TYM broth in 200-500 ml flask with shaking at 300 rmp, at 37 °C up to an OD_{550}= 0.2. The cells were transferred to a 100ml of fresh TYM broth in a 2 litre flask. This was shaken for a further 2-3 hours until the OD_{550}= 0.2 and 400ml of fresh TYM broth was added to the 2 litre flask. This was then shaken for a further 2-3 hours until the OD_{550}= 0.4-0.6. The cells were rapidly cooled in ice water with swirling and collected by centrifugation at 1400 x g for 10 minutes into 125 ml polypropylene tubes. The media drained and discarded and the cells were resuspended in 125 ml of Tfb 1. The cells were then incubated for 30 minutes and centrifuged for 10 minutes at 4 °C. The cell pellets were gently resuspended in 30 ml of Tfb 2. Resuspended cells were split into 0.3 ml aliquots in 1.5 ml centrifuge tubes and frozen in dry ice. The cells were stored at -70 °C in aliquots until in use.

2.3.5.3 Transformation

Competent MC1061 E.coli cells thawed on ice, after which 100 µl of these cells were mixed with 1 ng of plasmid and incubated at 4 °C for 30 minute. This mixture was immediately incubated at 37 °C for five min to heat shock the cells and then followed by incubation on ice for 2 min. Luria broth (LB) lacking ampicillin was added to the mixture. This was followed by incubation at 37 °C for 1 hour to allow all the cells to grow. After 1 hour, 100 µl of each transformation mix was plated on pre-warmed LB plates containing ampicillin. The plates were incubated at 37 °C overnight.

2.3.5.4 Screening for positive clones

Successful cloning of an insert can usually be identified by colour screening on indicator plates or by using colony PCR. The characteristics of PCR products cloned into a vector can significantly affect the ratio of blue: white colonies obtained following transformation of competent cells. Clones that are obtained from PCR products produce a white colour in most cases. However, we have used colony PCR to obtain colonies that contain a plasmid and our insert. Positive colonies were picked and dissolved in 10 µl of sterile distilled water and was used as our template DNA for our Colony PCR.

Colony PCR is a technique that uses PCR technology to find colonies that contain a plasmid and an insert of interest. Instead of a template DNA, individual colonies were picked from LB agar plates and resuspended in 10 µl sterile water. One microlitres of the mixture was then used as a template for a PCR reaction as tabulated on table 2.4. The PCR conditions were dependent on the fragment of interest and the universal primers (M13) were used to amplify the product. Those colonies that showed the presence of the DNA fragment of the

expected size were selected for plasmid extraction procedure. The remainder of the 10 µl of the colony mix used as an inoculum for making an overnight culture for plasmid extraction.

Table 2.4: Coctail for Colony PCR

Reagent	**Volume**	**Final Conc.**
Master Mix (2X)	10 µl	1X
Forward Primer (10 pmole)	1.0 µl	0.5pmoles
Reverse Primer (10pmoles)	1.0 µl	0.5 pmoles
Magnasium Chloride ($MgCl_2$ 25 mM)	2.0 µl	
Nucleas free water	5 µl	
Colony Mix	1 µl	
Total final volume	**20.0 µl**	

The table shows the components of a colony PCR reaction in a single PCR tube.

2.3.5.5 SEQUENCING

Following transformation and colony PCR, plates were then sent to Inqaba Biotech in Pretoria, SA for sequencing. The sequences were analyzed using DNAMAN and BLAST basic alignment tool (www.ncbi.nlm.nih.gov).

2.3.5.6 Plasmid DNA extraction-Miniprep.

In order to get high quality plasmid DNA for further experiments such as restriction digest and probe synthesis, plasmid DNA was extracted using Wizard Plus SV Miniprep DNA Purification System from Promega, USA. And as per manufacturer's instructions, colony containing a plasmid of interest was grown in 6 ml of Luria Broth (LB) containing ampicillin

(100 µg/ µl). The overnight culture was centrifuged at 12000 x g for 5 min at room temperature. The supernatant was discarded and the pellet was resuspended in 250 µl Resuspension buffer. Cells were then lysed by adding 250 µl of Lysis buffer and by inverting the tube 4-5 times to mix. Ten microlitres alkaline protease solution was added, followed by 5 min incubation at room temperature. Chromosomal DNA and degraded proteins were precipitated by addition of 350 µl Neutralization buffer accompanied by inverting the tube 4-5 times to mix. The mixture was then centrifuged for 10 min at 8000 x g at room temperature. The follow through was discarded and column assembly was reassembled. The column was washed with 750 µl wash solution and centrifuged at 8000 x g for 1 min at RT. The wash was repeated with 250 µl Wash Solution. The spin column was transferred to a 1.5ml centrifuge tube. The plasmid DNA was eluted by the addition of 50 µl of nuclease free water followed by centrifugation at 12000 x g. The spin column was discarded and the DNA was immediately used or stored at -20 °C.

2.3.5.7 Restriction digestion of plasmid DNA

Following plasmid DNA purification, two restriction endonucleases (REN) were used for restriction analysis of the clones. PstI or Apa I were used to linearize the clones while EcoR1 was used to release the insert. 2 µg of plasmid DNA of each clone was restriction digested with these three RENs. The restriction digests are shown in table 2.5.

Table 2.5: Linearization of pGEM-T Easy-DWNN plasmid DNA *with EcoRI, ApaI and PstI*

	EcoRI digest	**Pst I digest**	**Apa I digest**
pGEM-T-Easy Plasmid (2 µg/ µl)	10.0 µl	10.0 µl	10.0 µl
Buffer B 10X	5.0 µl	5.0 µl	5.0 µl
REN (3u)	1.0 µl	1.0 µl	1.0 µl
Sterile distilled water	34.0 µl	34.0 µl	34.0 µl
Total Volume	**50.0 µl**	**50.0 µl**	**50.0 µl**

The table shows the components of the restriction digests of pGEM-T Easy vector clones with *EcoRI, ApaI and PstI*

2.3.6 Quantitative Real Time –PCR using SYBR Green I

There are three techniques for RNA measurement: Reverse transcription PCR, Northern blot analysis and RNase protection assay. Reverse transcription PCR is the most sensitive technique for mRNA detection and can be used to quantify mRNA levels from much smaller samples. RT-PCR principle is based on the properties of the PCR reaction kinetics. A quantification of the PCR products synthesized during the PCR is obtained at each cycle. From the PCR cycle number curves obtained for each sample, a threshold is defined. The cycle threshold (C_T) corresponds to the intersection of the threshold and the PCR amplification curve. The threshold is chosen to intersect with all the PCR amplification curves during their exponential phases.

RT-PCR was developed from the PCR technique that measures the amplification of small DNA amount. For RT-PCR, mRNA or total RNA is isolated from a particular sample before producing a DNA copy of complementary DNA (cDNA) of each mRNA molecule. The gene

expression levels are then further amplified from the cDNA mixture together with a housekeeping gene (internal control). Housekeeping genes are those whose expression levels remain roughly constant in all samples and include such genes as actin and glyceraldehyde phospho-dehydrogenase (GAPDH). For the RT-PCR principle, more mRNA is in a sample, the earlier it will be detected during repeated cycles of amplification.

In this experiment I used the SYBR Green that provides the simplest and most economical format for detecting and quantitating PCR products in real-time reactions. SYBR Green binds double-stranded DNA, and upon excitation emits light. Thus, as a PCR product accumulates, fluorescence increases. The disadvantage is that SYBR Green will bind to any double-stranded DNA in the reaction, including primer-dimers and other non-specific reaction products, which results in an overestimation of the target concentration; however the products can be verified through gel electrophoresis. Quantitative RT-PCR primers are specifically designed with special software that gives optimal results. The following primers were designed using Biorad (SA) and Quagen (UK) primer design software and send to Inqaba for sequencing.

2.3.6.1 Real-time efficiency by standard amplification

The cDNA with known concentrations were diluted by a dilution factor of 10. These dilutions were then used in the real-time PCR set up. Real-time efficiencies were obtained from the Roche Lightcycler 1.5 (Roche Diagnostic, Germany).

2.3.6.2 Real-time quantification setup

The cDNAs from different responses were quantified using a Nanodrop spectrophotometer (NanoDrop Technologies, USA). Equal concentrations of 2 µg were subjected to real-time

reactions. The SYBR Green technology (Roche Applied Science, Germany) was used and the reaction was prepared as in table 2.6.

Table 2.6: Cocktail for qRT-PCR

Components	**Volume**
Lightcycler DNA Master SYBR Green I	10 µl
Forward Primer (10 pmoles)	1.0 µl
Reverse Primer (10 pmoles)	1.0 µl
$MgCl_2$ (25 mM)	1.0 µl
Nuclease free water	2.0 µl
Template DNA	5.0 µl
Final Total Volume	**20.0 µl**

The table shows quantities of different components in areal-time reaction.

2.3.7 Immunohistochemistry (ICC) of lung tissue sections using primary antibodies

2.3.7.1 Principle of the technique

The technique involves highly specific binding of an antigen to avidin-biotin. The antigen is adhered to a microscope glass slide and fixed and endogenous peroxidase blocked. A primary antibody is reacted with the immobilized antigen to form an antigen-antibody complex. A species specific second biotinylated antibody is next reacted with the complex. Next streptavidin conjugated to Horseradish Peroxidase is reacted with this complex localizing the peroxidase at the site of the antigen. Substrate is then added producing a coloured precipitate to form on the location of the antigen.

2.3.7.2 Antibodies

The following antibodies were kindly donated by Professor Jasper Rees, Department of Biotechnology, University of the Western Cape**,** a polyclonal rabbit antibody against human Retinoblastoma Binding protein 6 (RBBP6) and Domain With No Name (DWNN. A monoclonal mouse antibody against human p53, Bax and Bcl2 were all purchased from Santa Cruz, USA.

2.3.7.3 Immunoperoxidase labelling

An Immunocytochemistry kit, DAKO LSAB+KIT Peroxidase (DAKO, DenMark) was used. The tissue sections were dewaxed in xylene for 20 min with two changes of 10 min each at RT. The slides were then rehydrated in 100% absolute ethanol (Merck) for 10 min, two changes of 5 min each. The slides were subsequently incubated with 100% absolute methanol (Merck) for 20 min at RT to quench endogenous peroxidase. Sections were again rehydrated in 95% ethanol for 8 min, 2 changes of 4 minutes each at RT. Dehydration was repeated with 70% ethanol at RT for 3 min. Antigen retrieval was done at 80 °C for 5 min at low microwave setting in 0.1M Sodium Citrate (pH 6.0) until bubbles started to form. The slides were allowed to cool to RT for approximately 20 min. The sections were washed in PBST/TBST containing 1% BSA for 2 min at RT. Endogenous peroxidises were blocked in PBS containing 3% H_2O_2 for 20 min at RT. The sections were washed in PBST/TBST containing 1% BSA for 2 min at RT. The antibodies were diluted 1:4000 in PBST/TBST containing 1% BSA. The antibody aliquot was centrifuged at 3000 x g in a bench top microcentrifuge for 1 min. Hundred microlitres of the diluted primary antibody (Anti-human DWNN) preparation was applied to each processed slide and this was then incubated overnight at °C in a Ominislide humid chamber (Hybaid Ltd, UK). The sections were washed with PBST/TBST containing 1% BSA for 2 min. An antibody linker (DAKO, Denmark) was applied on each slide and incubated for 5 minutes at RT. This was followed by a wash in

PBST/TBST containing 1% BSA for 2 min. A secondary anti rabbit IgG-conjugated with perixidase (DAKO, Denmark) was applied as per manufacturer's manual and incubated for 30 min at RT. This was then washed with PBST/TBST containing 1% BSA for 5 min. Chromogen was prepared for detection by adding one drop of the chromogen [Diaminobenzidine-DAB] (DAKO, Denmark) in substrate buffer and was covered with foil and put on ice after mixing. The antibody preparation (100 µl) was applied to each section, allowed to develop colour change for 30 seconds and immediately put in water. These were counterstained with Mayer's hematoxylin for 10 min and then washed in tap water for 5 min. The slides were then dehydrated in the solution starting from 70% ethanol to xylene skipping methanol for 1 min each. The slides were mounted with xylene-based entellan and allowed to completely dry. The slides were examined under the Axtioplan 2 imaging system (Zeiss, Germany) and images were captured with Axio Version 3.0 software. They were then stored in a dark place at RT for future referencing.

2.3.8 *In Situ* HYBRIDAZATION (ISH)

Tissue arrays were used for in situ hybridization (ISH). Digoxigenin (DIG) labeling system from (Roche diagnostic, Germany) was used for labeling RNA probes for both ISH and FISH.

2.3.8.1 DWNN Probe synthesis

Different DWNN fragments were amplified and cloned into pGEM-T Easy vector. The clones were then sequenced and linearized with appropriate enzymes to generate antisense and sense RNA probes as shown in **table 2.5**. The enzymes used to linearize the DWNN clones were PstI and ApaI generating antisense and a sense probe respectively. The direction of cloning (5' to 3' and 3' to 5') was assessed from the sequence results. Based on this either

T7 or Sp6 promoter was used to generate sense or antisense probes. The linearized clones were electrophoresed on a 1% agarose gel. The DNA bands of interest were visualized using a UV lamp and cut out from the gel using a sterile blade. The linearized vectors were purified from the gel using a Promega Wizard SV gel and PCR Clean-up system using the manufacturer's instructions. The gel pieces were put into weighed 1.5ml centrifuge tubes and were weighed to obtain the weight of the gel slices. The gel slices were dissolved in membrane binding solution [10 µl of membrane binding solution for every 10mg of the gel slice]. These were incubated at 65 °C for 10 min with occasional mixing until the gel completely dissolved. Once dissolved, SV mini-column was inserted into a collection tube. The dissolved gel mixes were transferred onto the SV mini column assembles and these were incubated at RT for 1 min. This was followed by centrifugation for 1 min 6000 x g. The SV mini columns were removed and the flow through was discarded and the columns were reassembled. The mini columns were washed with 700 µl of membrane Wash solution and centrifugation for 5 min at the same speed. The SV mini column assembles were carefully removed from the centrifuge making sure that the column did not touch the flow through. The columns were then transferred into 1.5 ml centrifuge tube. Fifty microlitres (50 µl) of nuclease-free DEPC treated water was applied into the centre of each column. This was incubated at RT for 1 min, followed by centrifugation at RT at 6000 x g for 1 min. The SV mini columns were discarded and the DNA was quantified spectrophotometrically stored at -20 °C for labeling at a later stage.

2.3.8.2 DIG Labeling of the DWNN probes

The linearized plasmids containing DWNN fragments were used as templates for the labeling reaction to generate antisense and sense DWNN RNA transcripts as tabulated in **table 2.7**. About 2 µg of linearized and purified plasmid containing DWNN gene was incubated with a

mixture containing DIG dUTP (deoxyuridine triphosphate) for 2 hours at 37 °C with T7 RNA polymerase or Sp6 RNA polymerase to generate both antisense and sense probes respectively. The specificity of the labeling reaction was determined by generating a DIG labeled control cRNA probe from DNA PSPT 18-Neo/Pvu II supplied in the labeling kit. The reaction mixture of DIG labeling of antisense, sense and control RNA probes was generated by adding the reagents as tabulated in **table 2.7** into 1.5 ml centrifuge tube. These mixtures were incubated in a thermocycler for 2 hours at 37 °C. The reactions were then stopped with the addition of 4 µl of 0.2 M EDTA (pH 8.0). The reactions were then mixed and pulse-centrifuged to collect everything in the bottom of the tubes. Precipitation each of the labeled RNA probes was achieved by the addition of 4.4 µl 4 M lithium chloride and 150 µl cold absolute ethanol. These were mixed, pulse-centrifuged and incubated at -20 °C for 2 hour. The precipitate was pelleted by centrifugation at 8000 x g for 5 min at 4 °C and decanting the supernatant. The pellet was washed with 200 µl cold 70% ethanol and centrifuged at 12 000 x g for 5 min at 4°C after which the supernatant was removed with a pipette to avoid dislodging the pellet. The pellet was dried under the laminar flow. It was then dissolved in 50 µl sterile DEPC treated water. This was left for 1 hour at 4 °C to allow the pellet to completely dissolve. The DIG labeled probes were stored at -70 °C in aliquots of 10 µl in 0.2 ml tubes. The concentrations of the probes were then estimated using guidelines described in DIG user's guide (Roche, Germany).

Table 2.7: Cocktail mix of DIG labeling

Reagent	**Antisense RNA**	**Sense RNA**	**Control RNA**
DWNN cDNA-PstI/ApaI digested	25.0 µl	25.0 µl	-
Control DNA PSTT 18-Neo/PvuII	-	-	8.0 µl
10X NTP labeling mix	4.0 µl	4.0 µl	4.0 µl
10X Transcription buffer	4.0 µl	4.0 µl	4.0 µl
T7 RNA polymerase 20u/ µl	2.0 µl	-	-
Sp6 RNA polymerase 20u/ µl	-	2.0 µl	4.0 µl
DEPC-treated water	5.0 µl	5.0 µl	20 µl
Final total volume	**40. µl**	**40.0 µl**	**40.0 µl**

The table shows the amounts that were added in each reaction for the generation of different probes as advised by the manufacturer.

2.3.8.3 Estimation of probe concentration

To estimate the probe concentration of the synthesized probes, dilutions were made as tabulated in table 2.7. Dilutions for control labeled RNA (supplied in the labeling Kit); antisense and sense DWNN RNA and DIG labeled cRNA to control DNA pSPT-18-Neo. DIG user's guide was used. Spot points were marked lightly with a pencil on a nylon membrane (Hybond, Amersham, USA).One microlitres of each dilution was spotted onto the membrane and this was air-dried for 10 min. The probes were fixed to the placing it under a UV light for 5 min. The membrane was washed with 1X washing buffer while shaking for 5 min. The membrane was then incubated in 1X blocking buffer with shaking for 30 min. this was incubated in Anti-DIG Alkaline phosphotase diluted 1:10000 in 1X blocking buffer for 30 min. NB: throughout the experiment the membrane was washed or incubated with

sufficient volume of reagent to cover the membrane. The membrane was washed twice with 1X washing; it was immersed in 1X detection buffer with shaking for 2 min. It was then incubated in NBT/BCIP (diluted 1:50). This was allowed to develop in the dark overnight. The reaction was terminated by placing the membrane in 50 ml TE buffer (pH 8.0) for 5 min with shaking. The membrane was air-dried and the probe concentrations were estimated from the known DIG-labeled control RNA probe by comparing the intensity of the colour spot that develop on the membrane.

Table 2.8: Dilutions for the estimation of the probe

Dilution	DIG-labeled cRNA	Control DNA-Pspt-18 Neo	DWNN probes
Initial Conc.	0.1 µg/ µl	0.005 µg/ µl	0.2 µg/ µl
Dilution 1	1:10	1:10	1:10
Dilution 2	1:10	1:10	1:10
Dilution 3	1:10	1:10	1:10
Dilution 4	1:10	1:10	1:10
Dilution 5	1:10	1:10	1:10
Dilution 6	1:10	1:10	1:10
Dilution 7	1:10	1:10	1:10

The table shows how the concentration dilutions were conducted.

2.3.8.4 ISH procedure on tissue sections

Before starting the procedure, 4% paraformaldehyde (PFA) was prepared. PFA was dissolved at 60 ºC with stirring, not allowing the temperature of the solution to exceed 60 ºC. Once the solution became clear, it was allowed to cool to RT.

2.3.8.4.1 Pre hybridization treatment of sections

All equipment and glasswares used during this experiment were washed with 70% alcohol in DEPC-treated water and rinsed with RNAase inhibitors. The tissue array sections were dewaxed in clean xylene for 30 min at RT. The sections were rehydrated in fresh in fresh ethanol for 6 min with 2 changes of 3 min each at RT. The sections were then further rehydrated in 90%, 70%, 50% and DEPC-treated water for 3 min each at RT. The array sections were fixed in freshly prepared 4% PFA for 20 min at RT. They were rinsed 3 times in fresh TBS for 1 min at RT. The proteins were then denatured in 0.1M HCL for 10 min at RT. They were rinsed three times in fresh TBS for 1 min at RT. Non-specific labeling was limited with freshly prepared 0.5% Acetic Anhydride in 100mM Tris (pH 8.0) for 10 min at RT. Sections were rinsed in TBS for 6 min with 2 changes for 3 min. Cell membranes were permeabilised with 20 µg/µl Proteinase K in 1X TBS for 20 min at RT. Sections were then was rinsed in TBS for 3 min. Proteinase K activity was terminated in chilled TBS for 5 min at 4 ºC. The sections were then dehydrated in 50%, 70% and 90% for 1 min in each followed by dehydration in absolute ethanol for 2 min with 2 changes of 1 min each. They were then dried in chloroform for 10 min in the fume hood.

2.3.8.4.2 Probe preparation and hybridization

The estimated probe concentration from section 2.2.7.3 was 0.1ng/µl. Herring sperm DNA/HSD (0.01 µg/ µl) was added to 990 µl of hybridization buffer and vortexed to thoroughly mix them. Each probe 1µl was added to 1000 µl (hybridization buffer + HSD). The hybridization buffer+ HSD (100 µl) was used as a negative control. Each probe preparation (100 µl) was spread on each slide limited by an adhesion square/gene frame. To limit evaporation of the hybridization mix a solution of 5X SSC buffer and 50% formamide

was prepared and poured into the hybridization chamber. Hybridization was done in a Hybaid Omnislide flat Block humidity chamber at 55 ºC overnight.

2.3.8.4.3 Post-hybridization

Following overnight incubation, slides were removed from the humid chamber and excess probe were discarded. The slides were subjected to various concentration of SSC. Firstly the probe was washed off in 2 XSSC for 30 min at 37 ºC in a hybridization oven. Washing in 2X SSC, 1X SSC and 0.1 SSC at 55 ºC for 20 min each followed. The slides were rinsed in TBS for 3 min with 3 changes of 1 min each at RT. Non-Specific binding was blocked by 100 µl of 1X blocking buffer to each slide in a humid chamber for 2 hours. The slides were incubated with anti-DIG IgG conjugated with alkaline phosphate diluted 1:500 in 1X blocking buffer and 100 µl per slide. The slides were incubated in a humid chamber for 1 hour at RT. Afterwards, the slide were rinsed in TBS for 3 min with a 3 changes of 1 min each. The slides were the incubated with chromogen NBT/BCIP (Roche Diagnostic, Germany) diluted 1:50 in 1X detection buffer. 100 µl of this preparation was added to each slide. The slides were incubated with the NBT/BCIP to develop in a dark humid chamber overnight at RT and the following morning the reaction was stopped in 1 X TE for 5 min at RT. The slides were washed in running tap water for 5 min at RT. The slides were viewed under Axioplan 2 imaging system (Zeiss, Germany) and image captured using an Axio Version 3.0 software.

2.3.9 Western Blot Analysis Using Sodium dodecyl sulphate (SDS PAGE)

2.3.9.1 Total protein extraction

Total protein was extracted using Cytobuster protein extraction (). Briefly, cultured cells were washed with PBS and scrapped off in 200 µl cytobuster protein extraction. Detached cells were placed on ice for 5 min and then vortexed for 30 seconds. The cells were placed on ice for 30 min with occasional vortexing. The samples were centrifuged at 12 000 x g at RT for 5 min. The soluble proteins were in the supernantant and were transferred to a new 1.5 ml centrifuge tubes. The protein concentrations were quantified using the NanoDrop technology. The samples were mixed with 2X sample buffer containing 10mM DTT and stored at -70 ºC for further use.

2.3.9.2 Sodium dedocyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were separated on a 12% separating PAGE. For SDS PAGE, these samples were thawed and boiled, 20 µg of protein were loaded onto a 12% SDS PAGE. The protein electrophoresis system was then set at a constant voltage of 120V and allowed to run for 1 hour in 1 X SDS-PAGE running buffer.

2.3.9.3 Preparation of 12% SDS PAGE

To prepare two plates of SDS PAGE, 10ml of the gel was prepared as follows, 3.75 mls of bis/acrylamide (40%-37:5:1), 2.5 ml of 1.5M Tris-Cl (pH 8.8); 100 µl of 10% SDS; 500 µl of 10% freshly prepared Ammonium Persulphate (APS) and 3.15 ml of water were added to a 15 ml tube. 5 µl of TEMED was then added to solidify the gel. The mixture was then poured into protein casting apparatus (BioRad, USA).It was then overlaid with water and after solidifying, stacking gel (prepared as follows 3.7 ml of water; 0.625 ml of 0.5M Tris, pH 6.8; 0.5 ml of Acrylamide/Bis-30%; 50 µl of 10% SDS; 100 µl of 10% APS; 5 µl TEMED) was

prepared and added on top of this separating gel. Denatured Samples together with low range protein marker were loaded in duplicate onto SDS gel. The samples were run at 150 mV for about 1-2 hour.

2.3.9.4 Electrophoretic Transfer

Upon completion of antigen separation, one SDS gel was stained and the other three, antigens were transferred onto the polyvinylidene fluoride membrane (PVDF) [Sigma] in transfer buffer for 2 hours. The electro-blotting cassette (BioRAD, USA) was assembled according to the manufacturer's instructions. Briefly, the polyvinylidene fluoride membranes and the gels were placed between the two filter papers and ran at 100 V at 4 ˚C for 2 hours.

2.3.9.5 Immunobloting

Upon completion of the protein transfer, one the membrane was stained with ponceou stain. For immunobloting, the membranes were washed with TBS for 5 minutes at RT, blocked with blocking buffer (TBSMT) for 2 hours at RT. Then the membrane was probed with diluted primary anti-human DWNN or p53 or GADPH (1:100- 1:10 000 in TBSMT) with gentle agitation at 4ºC overnight. After overnight incubation, the membranes were washed three times, (10 min for each), with 1X PBS containing 0.1% Tween 20. After the washes, the membranes were incubated with secondary antibody, anti-mouse IgG peroxidase conjugated (1:1000 dilution in TBSMT) [DAKO] for 1 hour. This was followed by washing the membrane six times with 1X PBS containing 0.1% Tween 20 for 10 min each.

2.3.9.6 Detection and exposure

Detection was done using autoradiography. To visualize the bands that the primary antibodies bound to, the membranes were immersed in a solution containing Super Signal Wes Pico

Chemiluminescent Substrate (PIERCE, USA) for 5 min. The blot was then exposed to CL X-Posure film (Thermo Scientific, USA) for an appropriate time (e.g 30 Sec, 1 min to 10 min). The x-ray film was then developed in the dark.

2.3.10 Apoptotic detection

Apoptosis was detected using different technique depending on the sample that was used. One example of such kits is a Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labelling (TUNEL) kit (Promega Corp, USA). The other way of detecting apoptosis in this study was through the ANNEXIN V APOPercentage (Merck, USA). The two protocols uses different principles with the TUNEL used to detect DNA fragmentation while APOPercentage detect the externalization of phosphatidylserine found in apoptotic cells. In this study both the TUNEL and APOPercentage assay using a FACScan were used to detect apoptosis in both paraffin fixed Tissues and cultured cells under both normal growth and apoptosis stimuli respectively.

2.3.10.1 Detection of Apoptosis using the TUNEL method

Before the experiment 4% PFA was prepared and allowed to cool at RT. Tissue sections were dewaxed in clean xylene for 20 minutes with 2 changes of 10 min each. The sections were dehydrated in 100% ethanol for 10 minutes with 2 changes of 5 minutes each. This was followed by incubated in 3% H_2O_2 in absolute methanol to quench endogenous peroxidase. Sections were washed in deionized water for 5 minutes. The sections were rehydrated in a series of ethanol grades (95%, 85%, 70 % and 50 %) for 3 minutes in each. The sections were then washed in 0.85% NaCl for 5 minutes at RT. The sections were washed with 1X PBS for 5 minutes at RT followed by fixing in 4% PFA for 15 minutes at RT. The sections were then washed three times in 1X PBS for 5 minutes at RT. The sections were permeabilized with

Proteinase K (20 µg/µl) prepared as follows (1:500 dilution in PBS) for 20 min at RT. After permeabilization, sections were washed in 1X PBS for 5 minutes at RT. The sections were refixed in 4% PFA for 5 minutes and washed twice in PBS for 5 minutes each at RT. The sections were equilibrated for 10 minutes with equilibration buffer (Enough equilibration buffer was applied appox. 100 µl). The bionylated nucleotide mix was prepared as per manufacturer's instructions (Promega, USA). Briefly, 100 µl of bionylated mix was applied onto the section and sections were incubated at 37ºC for 60 min in a humid chamber to allow labelling. The reaction was terminated by immersing the slides in 2XSSC for 15 minutes at RT then sections were washed in PBS three times for 5 minutes for each wash. The endogenous peroxidises was quenched in 0.3% hydrogen peroxide for 5 minutes at RT then washed three times in PBS for 5 minutes each. The sections were incubated for 30 minutes in diluted streptavidin (diluted 1: 500 with PBS). The sections were incubated with 100 µl DAB substrate (1.6 ml + 1 drop of DAB) until brownish colour developed. The sections were washed 3 times for 2 minutes each with distilled water and dehydrated with graded alcohol, 50%, 70%, 95% and 100% ethanol for 1 min each. The sections were cleared with xylene 4 times for 2 minutes each and were mounted with polymount mounting media. The slides were observed under the microscope.

2.3.10.2 APOPercentage Assay using ANNEXIN V

The experiment was carried out as per manufacturer's instruction. Briefly, from culture plates, the supernatant was removed and stored on ice. The cells were washed with PBS and trypsinized. The cells were washed off in trypsin and added to stored supernatant media. These were centrifuged for 3 min at 6000 x g. The cell suspension concentration was adjusted to approximately 1x 10^6 cells/ ml and 0.5 ml of cell suspension were transferred to 1.5 ml centrifuge tube. Ten microlitres (10 µl) of Media Binding Reagent was added to the cell

suspension followed by addition of 1.25 µl of Annexin V-FITC into the sample and incubated for 15 minutes at room temperature in the dark. The samples were centrifuged at 1000 x g for 5 minutes at room temperature and media removed. The cells were resuspended in 0.5 ml cold 1 x Binding Buffer and 10 µl of Propidium Iodide was added to cell suspension. The samples were placed on ice away from light. The FASCan instrument equipped with 488 nm argon laser as a light source was used to analyze the samples.

2.3.11 Ribonucliec Acid Interference (RNAi)

Introduction of an engineered DNA into a mammalian cells has become a challenging exercise and this has attracted a lot of attention from companies to develop effective and yet safe reagents to facilitate this process. In research communities transfection has been depending on the use of lipid technology to transfer a foreign DNA. In this research siPort NeoFX which is less toxic reagent (Ambion, England) was used to transfect the siRNA construct into MRC5, Lsqr 1 and A549 cell lines.

2.3.11.1 Seed cells in plates and culture flasks

Cells were resuspended to 1x 10^5 cells/ml in respective media containing 10% FBS, 1% L-glutamine and 1% pen/strep and cells were mixed by gently inverting the tube several times. 25 ml of cell mixture was transferred to a sterile disposable reagent reservoir for plating.4 x 96 well OptiLux tissue culture plates were labeled for p53 siRNA and the other for RbBP6 siRNA. Using a multi-channel pipette, 100 µl of the mixture was transferred into each well. The cells were distributed throughout the wells by rocking the plate for 20-30 seconds. Then flasks were left at room temperature for 15 minutes to allow the cells to adhere to the surface. The cells were placed in a container box with little bit of water and slightly opened corners of

the container to allow CO_2 entry. The container boxes were placed in 37 ºC incubator with 5% CO_2.

2.3.11.2 Transfection of cells with siRNA

In brief, the OPTI-MEM I Media (Invitrogen, USA) and transfection reagent were brought to RT and the transfection agent was centrifuged to collect to the bottom. The following mixtures as indicated in table 2.9 and 2.10 were prepared and were incubated at room temperature for 10 minutes each. Following this incubation, the reagents were mixed together and incubated for a further 10 min. For, plates that were to be transfected, cells were gently mixed to resuspend those that have already attached to the surface. The cells and transfection agent complex were gently mixed by rocking the plate gently back and forth. The transfection mixtures were incubated at 37ºC for 24 hours before the media could be removed and new 100 µl or 5 ml of warm media to each well or culture flask respectively was added. The experiments were assayed for transfection efficiency and cytotoxicity after 48 and 24 hours respectively.

Table 2.9: Concentration dilutions of the transfection reagents

Reagent	96 well	25mm^2 plate
siPort NeoFX(µl)	**0.5 µl**	**20 µl**
OpTi-MEM I to (µl)	**10 µl**	**400 µl**

The table shows the mixture of the siPort NeoFX transfection reagent and the OpTi-MEM I medium.

Table 2.10: Concentration dilutions of the transfection reagents

Reagent	96 well	25mm^2 plate
siPort Amine(µl)	0.3 µl	12 µl
OpTi-MEM to (µl)	10 µl	400 µl

The table shows the mixture of the respective siRNA and the OpTi-MEM I medium.

2.3.12 Apoptosis induction

In this study, apoptosis was induced with staurosporine, Camptothecin and TRAIL. These had been used and proven to induce high levels of apoptosis (Charlot, *et al*., 2006; Mei, *et al*., 2007). Negative control was untreated cells grown for 24 hours. To make sure that equal numbers of cells were obtained at the start of induction or any treatment, the number of cells plated on the wells or plates were counted using Fuchs haemocytomeyers. Approximately equal numbers of cells were then plated in the wells. The media containing apoptosis inducers were prepared as follows (5 µl/ml TRAIL, 0.8 µM Camptothecin and 25nM Steresporine).The cells were removed from the incubator and 100 µl of media containing apoptosis inducers pipetted to the wells. After 24 hours the cells were washed and 100 µl media containing alamarBlue was pipetted and plates placed in the incubator for 3 hours. The plates were read at a wavelength of 590nm.

2.3.13 Cell cytotoxicity assay

Fifty millilitres of alamarBlue media was prepared by adding 5 ml of warm alamarBlue to 45mls of warm media in a 50 ml falcon tube. Twenty five millilitres of the alamarBlue media was transferred into a sterile disposable reagent reservoir. The plates were removed from the incubator and checked for any bacterial contamination. The wells containing positive control siRNA were examined to verify the efficiency of siRNA. The cells were washed twice with PBS and 100 µl of alamarBlue media was pipetted into each well. The cells were placed back into the incubator for 3 hours. The plates were read at a wavelength of 590nm at 37 ˚C.

2.3.14 Statistical analysis

Data were expressed as means F standard errors. Statistical comparisons of the results were made using analysis of variance (ANOVA). Significant differences (pb 0.05) between the means of control and treated cells were analyzed by Dunnett's test. For ICC, association among biological factor values was assessed with the Spearman rank correlation coefficient. Protein expression and categorical data were compared using χ2-test.

Chapter Three

Results

3.1 Introduction

The primary hypotheses of the study are: i) Retinoblastoma Binding Protein 6 (RBBP6) through its Ring finger domain is involved in the ubiquitination of p53 that results in the degradation of p53 and development of carcinogenesis ii) RBBP6 causes apoptosis of cancer cells. To evaluate the question that RBBP6 enhances the development of lung cancer a variety of experimental procedures were used; these involved fluorescence *in situ* hybridization (FISH), immunocytochemistry (ICC), gene silencing (siRNA) and induction of the apoptosis.

3.1.1 Total RNA and cDNA Synthesis

Total RNA isolated from MRC5 fibroblast and A549 Adenocarcinoma cell lines was subjected to electrophoresis before it was used to synthesize cDNA by reverse transcription. The RNA was of good quality as demonstrated in **figure 3.1** with distinct 18S and 28S rRNA bands as indicated the arrows.

Figure 3.1: Agarose gel electrophoresis showing total RNA

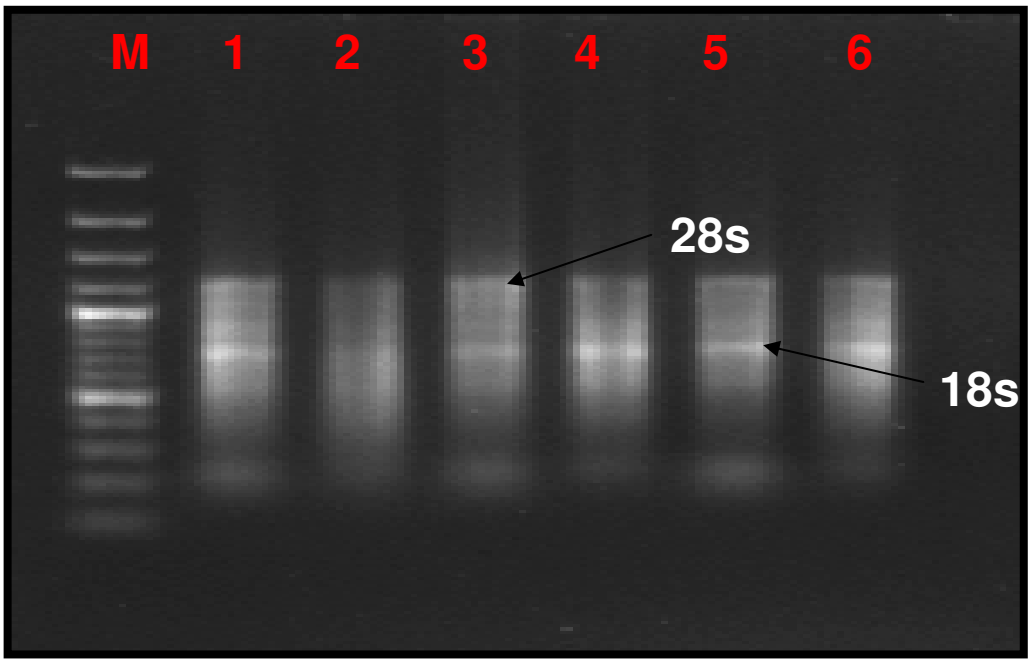

Figure 3.24: Gel electrophoresis indicating the integrity of the RNA extracted from the cell lines, Lane-M represent the RNA molecular marker while lanes 1-6 represent MRC5 cell line RNAs. Note the two bands indicated by the arrows as 28s and 18s.

3.1.2 Amplification of DWNN and p53BD by PCR

The following domains were amplified using appropriate sets of primers. The amplification of the two domains was successful. Based on the sequence analysis the designed primers should amplify about 200 base pair (bp) fragments. **Figure 3.2** shows the amplification of a 200 bp for all the domains.

Figure 3.2: DNA agarose gel showing PCR products.

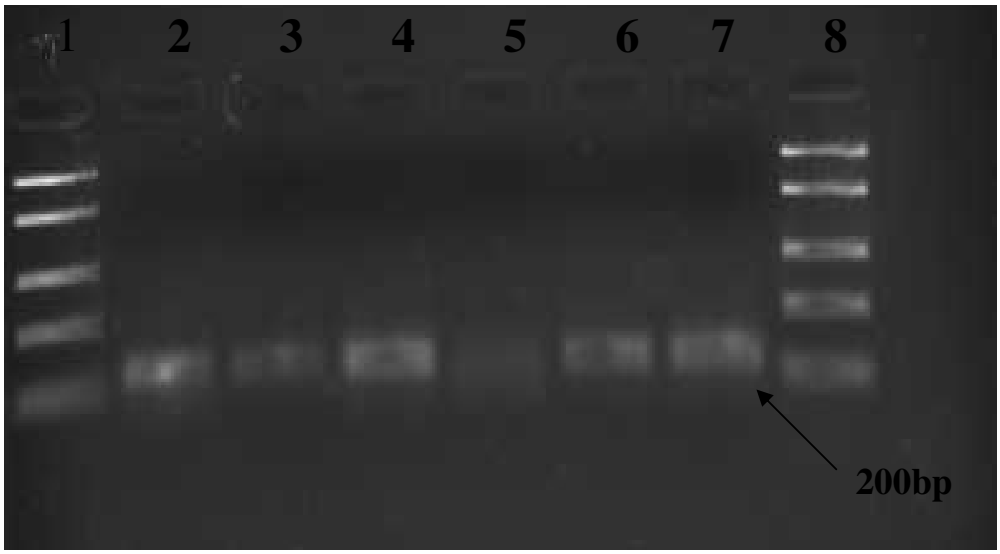

Figure 3.2: DNA agarose gel shows a successful amplification of both the DWNN and p53BD. DWNN- normal lung (lane 2), DWNN lung Tumor (lane 3), DWNN A549 cell line (lane 4); p53BD-Tumour lung (lane 6), p53BD-normal lung (lane 7) and Molecular DNA ladder (lane 1 and 8).

3.1.3 Screening of colonies containing DWNN and p53BD using colony PCR

The PCR products from the amplification of RBBP6 domains (DWNN and p53BD) were cloned into pGEM-T Easy vector for sequencing. Cloning of the DWNN and p53BD domains from different cell lines was successful as illustrated in **figure 3. 3 and 3.4.**

Figure 3.3: Agarose gel showing colony PCR products of DWNN.

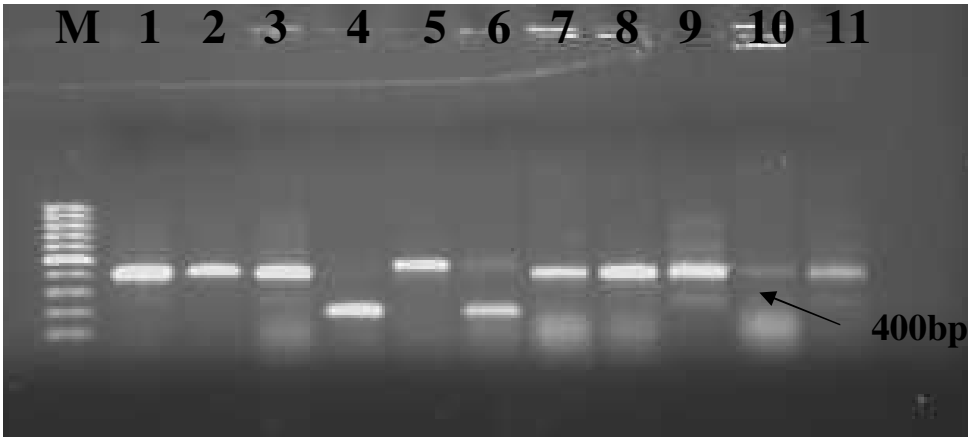

Figure 3.3: Agarose gel electrophoresis showing PCR products screening for DWNN domain: lanes 1-11 show colony PCR screen of 11 different colonies obtained after the cloning of DWNN domain. Lane 1-4 shows colony PCR products from normal lung, lane 5-8 shows colony PCR product from A549 cell lines and lane 9-11 that shows colony PCR product from lung tumour. Lane M shows a DNA molecular weight marker. **Note** lane 4 and 6 colonies did not have the DWNN fragment.

Figure 3.4: Agarose gel showing colony PCR products of p53BD.

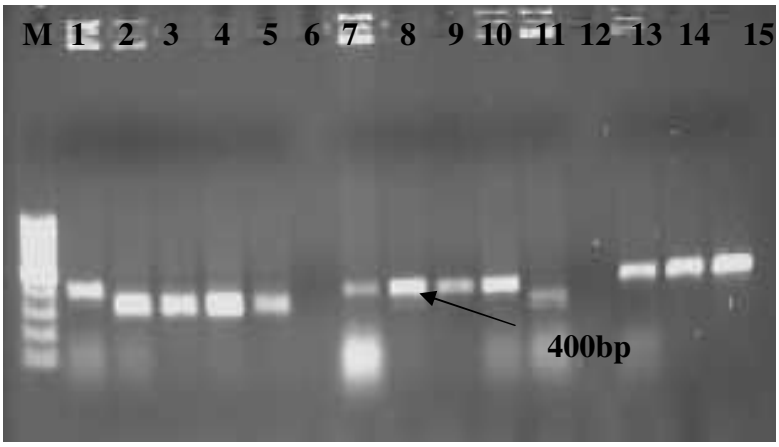

Figure 3.4: Agarose gel electrophoresis showing PCR products screening for p53BD domain: lanes 1-15 show colony PCR screen of 13 different colonies obtained after the cloning of p53BD domain. Lane 1-5 shows colony PCR products from normal lung, lane 7-11 shows colony PCR product from A549 cell lines and lane 13-15 that shows colony PCR product from lung tumour. Lane M shows a DNA molecular weight marker.

3.2 Mutational Analysis of Retinoblastoma Binding protein 6 (RBBP6)

It is well documented that mutations of oncogenes and tumor suppressor genes play a crucial role in cancer development and progression (Antoniou, *et al*., 2006). Mutations of these genes have been implicated in the disruption in cell cycle checkpoint regulation that is required for keeping cell division in check and apoptosis induction in response to DNA damage (Pal, *et al*., 2004). In this study, mutation of RBBBP6 i.e Domain With No Name (DWNN) and p53-Binding Domain (p53BD) were investigated in lung A549 Adenocarcinoma cell line, Lung MRC5 fibroblast, Normal Lung and Lung Tumor. In previous studies it has been suggested that RBBP6 may contribute to cancer development and that it may be mutated or a target of mutagenesis (Gao and Scott, 2002).

Different experiments were conducted to achieve the objective of this study. Briefly, cells were cultured and total RNA extracted from cultured cells. Some total RNA from normal lung and lung tumor were purchased from Ambion (England). After reserve transcription and PCR of the RNAs, PCR product was cloned into pGEM T-Easy vector and sequenced. DNA sequences generated were analysed using both chromas and DNAMAN and NCI BLAST tools.

3.2.1 Sequence analysis of the RBBP6 p53BD

Figures 3.5-8 shows hits from a BLAST search obtained using the p53BD domain sequence from A549 adenocarcinoma cell line (**figure 3.5**), lung tumour (**figure 3.6**), normal lung (**figure 3.7**) and MRC5 fibroblast cell line (**figure 3.8**). The sequence matched the RBBP6 variant 1 and 2. This demonstrated that the primers used were specific to RBBP6. This also

indicated that p53BD and RBBP6 is expressed in all lung cancer cell lines and normal lung cells. This study was aimed at investigating whether there were any mutations in the p53BD of RBBP6 and from the BLAST results, the sequence alignments showed no alterations in the p53BD in all lung cancer cases and normal cases.

3.2.2 Sequence analysis of RBBP6's DWNN domain

Figures 3.9-13 shows the BLAST search results of DWNN domain of RBBP6 in A549 cell line (**figure 3.9 and 3.10**), normal lung (figure 3.11), MRC5 fibroblast (**figure 3.12**) and lung tumour (figure 3.13) which contained no mutations. On average there was a 97-99% identity to RBBP6 transcripts observed when sequenced with both Sp6 and T7. In some cases there was either 98% identity when sequenced with T7 but when the same was done with Sp6 there was 100% identity which if they were the same could have been called mutation. These results suggest that the sequenced clones had no mutations and DWNN may not be harbouring mutations. More clones might need to be sequenced in order to precisely confirm that there are no mutations of DWNN in lung cancer.

3.2.3 Summary of mutational analysis

Sequencing of RBBP6 exons 1–3 and exon 14 was carried out on lung cancer tumour, normal lung tissue, and MRC5 and A549 cell lines. Four single base changes, two single base deletions and one double base change were identified as indicated by red and pink colours in figures 3.5-3.13. The mutations found were mostly transitions, with a marked strand bias: C→T at position 116 of exon 2 in normal lung tissue, C→A at position 45 of exon 14 in lung tumour tissues and two G→A in both position 40 and 43 of exon 2 in lung tumour tissues.

Only two transversions were identified: A→T at position 123 in A549 cell line and C→G at position 42 on exon 2 in lung tumour tissues. There was further two single base deletion of an A base at exon 14 (p53BD) position 8 as indicated by pink colour. While in normal lung tissue, A549 and MRC5 cell lines there was no any mismatch or point mutations recorded. If the results are an indication of protein justification these changes will result in the following amino acid change in lung tumour tissues on exon 2 there will be a change from Asp (GAC) → Glu (GAG) and Val (GUU) → Ile (AUU). In exon 14 of lung tumor there will be alteration from Phe (UUC) → Leu (UUA) and Try (UAU) → Phe (UUU). While in exon 2 of A549 cell line there was no alteration of protein sequence but just a change in base from Arg (CGU) → Arg (CGA).

```
Features in this part of subject sequence:
   retinoblastoma-binding protein 6 isoform 2
   retinoblastoma-binding protein 6 isoform 1


 Score =  200 bits (108),  Expect = 3e-49
 Identities = 112/114 (98%), Gaps = 0/114 (0%)
 Strand=Plus/Minus
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A

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Sbjct  15864959
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TTGGCACCATGTCCTGTGTGCATTATAAATTTTCCTCTAAACTCAACTATGATACCGTCA  15865078
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                  |||
Sbjct  15865079   CCT  15865081
```

B

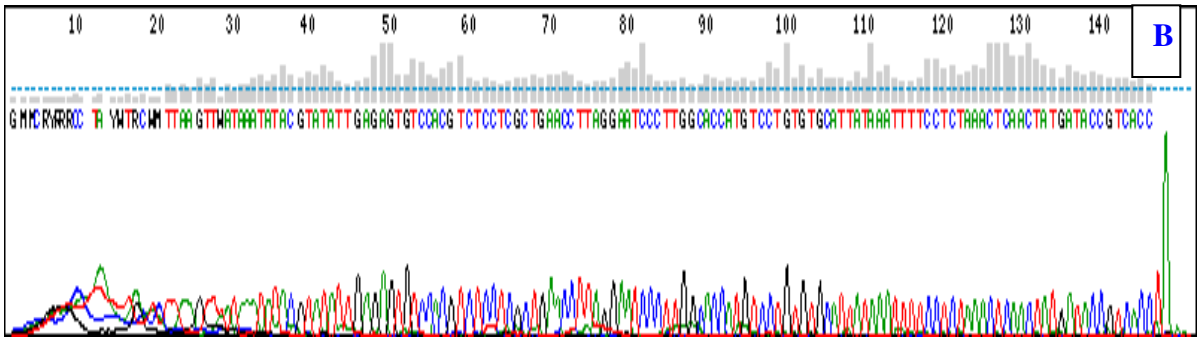

Figure 3.5: Representation of P53BD (exon 14) sequence analysis for mutational analysis in A549 cell lines, which show no mutations and 98 % alignment with against NM_006910.4 and NM_018703.3 RBBP6 mRNA. (A) Blast sequence comparing RBBP6 mRNA on NCBI and our DNA query. (B) chromas analysis of our sequence. Note alteration at position 116 of a base C→ T.

Features in this part of subject sequence:
retinoblastoma-binding protein 6 isoform 2
retinoblastoma-binding protein 6 isoform 1

Score = 196 bits (106), Expect = 4e-48
Identities = 111/114 (97%), Gaps = 1/114 (0%)
Strand=Plus/Minus

A

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Sbjct  15895865
Query  60
AAGATTGAGACAGACTTTTAAAATTTCCTTGTTCATTCAGACGGTCCAAA  109
||||||||||||||||||||||||||||||||||||||||||||||||||
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Sbjct  15895805
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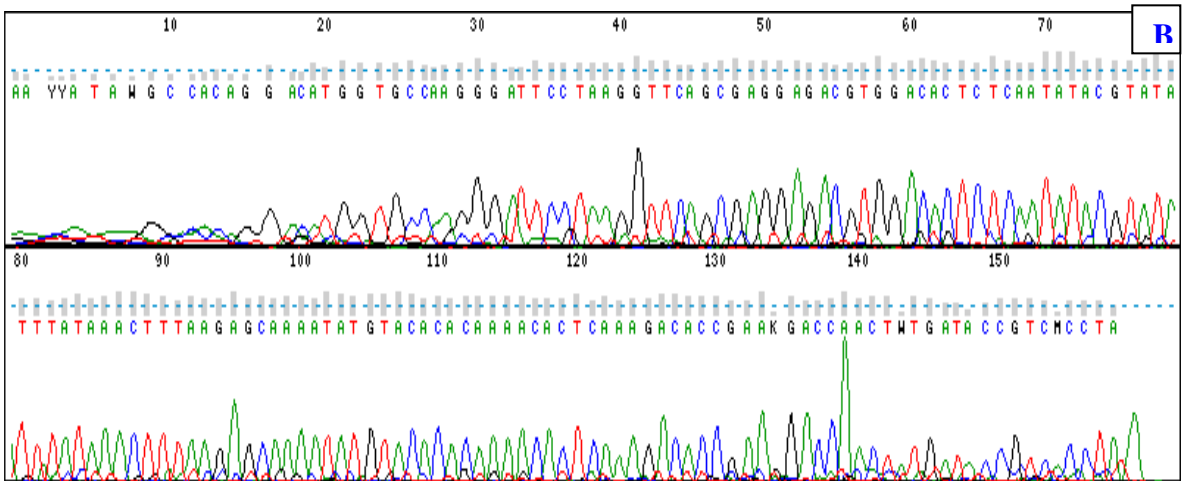

Figure 3.6: Representation of P53BD (exon 14) sequence analysis for mutational analysis in lung tumour, which show no mutations and 98 % alignment with against NM_006910.4 and NM_018703.3 RBBP6 mRNA. (A) Blast sequence comparing RBBP6 mRNA on NCBI and our DNA query. (B) chromas analysis of our sequence. Note the alteration at position 45 from a C→A

```
Features in this part of subject sequence:
   retinoblastoma-binding protein 6 isoform 2
   retinoblastoma-binding protein 6 isoform 1


 Score =  200 bits (108),  Expect = 3e-49
 Identities = 112/114 (98%), Gaps = 0/114 (0%)
 Strand=Plus/Minus
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A

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AAATATACGTATATTGAGAGTGTCCACGTCTCCTCGCTGAACCTTAGGAATCCCTTGGCA  15865024
Sbjct  15864965
Query  61
CCATGTCCTGTGTGCATTATAAATTTTCCTCTAAACTCAACTATGATACCGTCACCT  117
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CCATGTCCTGTGTGCATTATAAATTTTCCTCTAAACTCAACTATGATACCGTCACCT  15865081
Sbjct  15865025
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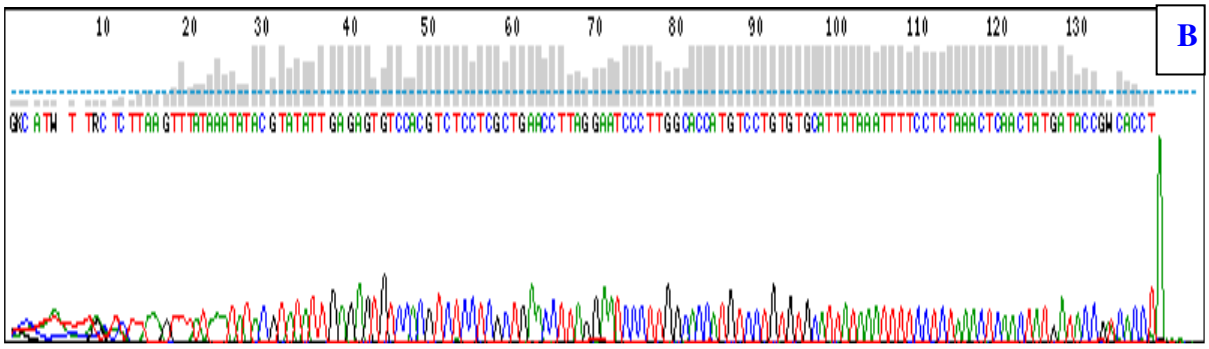

Figure 3.7: Representation of P53BD (exon 14) sequence analysis for mutational analysis in normal lung tissue, which shows no mutations and 98 % alignment with against NM_006910.4 and NM_018703.3 RBBP6 mRNA. (A) Blast sequence comparing RBBP6 mRNA on NCBI and our DNA query. (B) chromas analysis of our sequence.

Features in this part of subject sequence:
retinoblastoma-binding protein 6 isoform 2
retinoblastoma-binding protein 6 isoform 1

Score = 124 bits (117), Expect = 9e-27
Identities = 117/117 (100%), Gaps = 0/117 (0%)
Strand=Plus/Minus

A

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GCCAAGGGATTCCTAAGGTTCAGCGAGGAGACGTGGACACTCTCAATATACGTATATTTA  15864964
Sbjct  15865023
Query  61
TAAACTTTAAGAGCAAAATATGTACACACAAAACACTCAAAGACACCGAAGGACC  115
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TAAACTTTAAGAGCAAAATATGTACACACAAAACACTCAAAGACACCGAAGGACC  15864909
Sbjct  15864963
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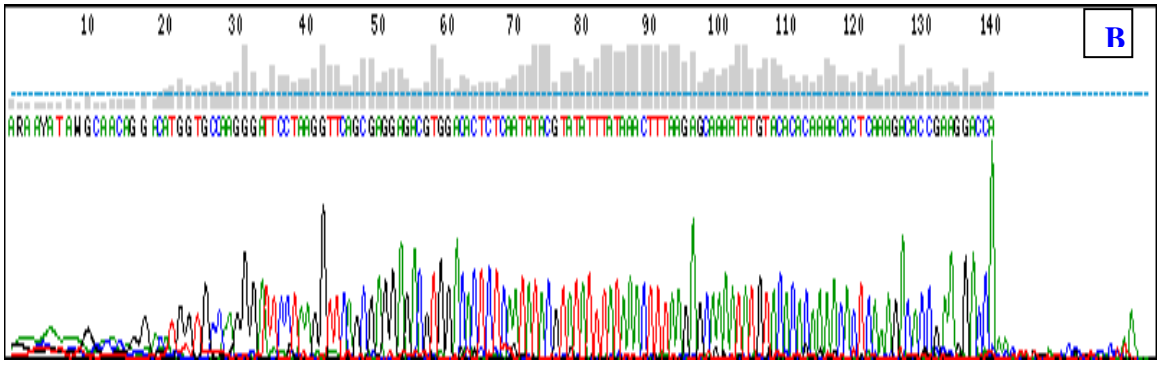

Figure 3.8: Representation of P53BD (exon 14) sequence analysis for mutational analysis in MRC5 cell lines, which show no mutations and 100 % alignment with against NM_006910.4 and NM_018703.3 RBBP6 mRNA. (A) Blast sequence comparing RBBP6 mRNA on NCBI and our DNA query. (B) chromas analysis of our sequence.

.

Features in this part of subject sequence:
retinoblastoma-binding protein 6 isoform 2
retinoblastoma-binding protein 6 isoform 1

Score = 187 bits (101), Expect = 2e-45
Identities = 104/107 (97%), Gaps = 0/107 (0%)
Strand=Plus/Minus

A

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TCTTTATTTGAGGAAGCACGAGTGGAATCATGTTTATCTGACGTTCTAGCCTCTTTGGAA  15895804
Sbjct  15895863
Query  61
GATTGAGACAGACTTTTAAAATTTCCTTGTTCATTCAGACGGTCCAA  107
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Sbjct  15895803
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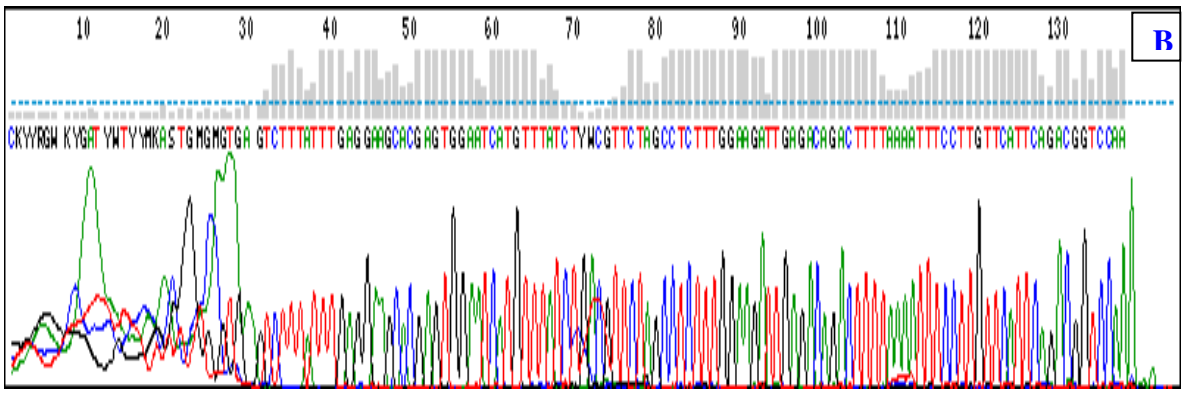

Figure 3.9: Representation of RBBP6 (exon 2 (DWNN)) sequence analysis for mutational analysis in lung tumour tissues, which show no mutations and 97% alignment with against NM_006910.4 and NM_018703.3 RBBP6 mRNA. (A) Blast sequence comparing RBBP6 mRNA on NCBI and our DNA query. (B) chromas analysis of our sequence. The results shows a single base point mutations of A→G at position 40 and CG→GA at position 42 and 43

```
Features in this part of subject sequence:
   retinoblastoma-binding protein 6 isoform 2
   retinoblastoma-binding protein 6 isoform 1


 Score =  196 bits (106),  Expect = 4e-48
 Identities = 111/114 (97%), Gaps = 1/114 (0%)
 Strand=Plus/Minus
```

A

```
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 CTTAAAGTTTATAAATATACGTATATTGAGAGTGTCCACGTCTCCTCGCTGAACCTTAGG  15865012
Sbjct  15864953

Query  60
AATCCCTTGGCACCATGTCCTGTGTGCATTATAAATTTTCCTCTAAACTCAACTATGATA  119
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AATCCCTTGGCACCATGTCCTGTGTGCATTATAAATTTTCCTCTAAACTCAACTATGATA  15865072
Sbjct  15865013
Query  120        CCGWCACCT  128
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Sbjct  15865073   CCGTCACCT  15865081
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B

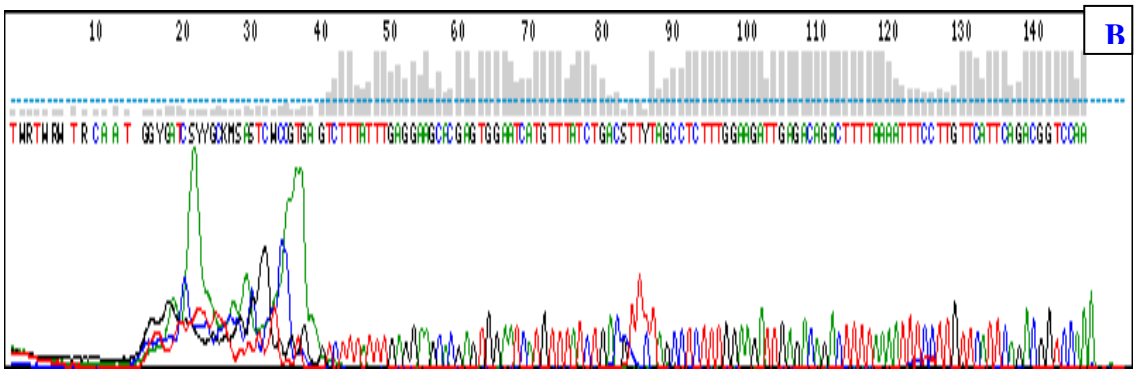

Figure 3.10: Representation of RBBP6 (exon 2 (DWNN)) sequence analysis for mutational analysis in A549 cell lines, which show no mutations and 97 % alignment with against NM_006910.4 and NM_018703.3 RBBP6 mRNA. (A) Blast sequence comparing RBBP6 mRNA on NCBI and our DNA query. (B) chromas analysis of our sequence. The results show a transversion of T→A at position 123.

```
Features in this part of subject sequence:
   retinoblastoma-binding protein 6 isoform 2
   retinoblastoma-binding protein 6 isoform 1


 Score =  200 bits (108),  Expect = 3e-49
 Identities = 112/114 (98%), Gaps = 0/114 (0%)
 Strand=Plus/Minus
```

A

```
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Sbjct  15864958
Query  61
CTTGGCACCATGTCCTGTGTGCATTATAAATTTTCCTCTAAACTCAACTATGATACMGTC  120
|||||||||||||||||||||||||||||||||||||||||||||||||||||| |||
CTTGGCACCATGTCCTGTGTGCATTATAAATTTTCCTCTAAACTCAACTATGATACCGTC  15865077
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                  ||||
Sbjct  15865078   ACCT  15865081
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B

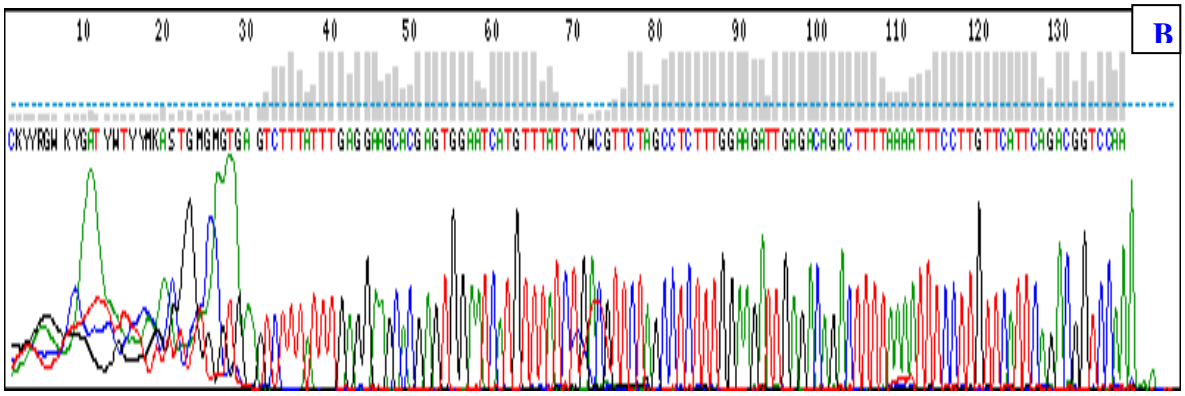

Figure 3.11: Representation of RBBP6 (exon 2 (DWNN)) sequence analysis for mutational analysis in normal lung tissues, which show no mutations and 98 % alignment with against NM_006910.4 and NM_018703.3 RBBP6 mRNA. (A) Blast sequence comparing RBBP6 mRNA on NCBI and our DNA query. (B) chromas analysis of our sequence. The results show a transition of C→ T at position 117.

Features in this part of subject sequence:
retinoblastoma-binding protein 6 isoform 3
retinoblastoma-binding protein 6 isoform 2

Score = 226 bits (122), Expect = 7e-57
Identities = 123/124 (99%), Gaps = 0/124 (0%)
Strand=Plus/Minus

A

```
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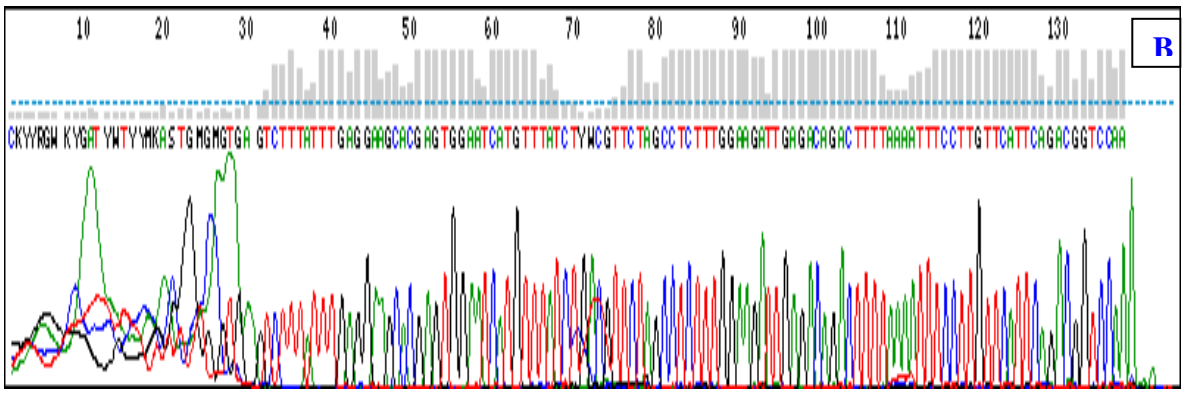

Figure 3.12: Representation of RBBP6 (exon 2 (DWNN)) sequence analysis for mutational analysis in MRC5 cell lines, which show no mutations and 99 % alignment with against NM_006910.4 and NM_018703.3 RBBP6 mRNA. (A) Blast sequence comparing RBBP6 mRNA on NCBI and our DNA query. (B) chromas analysis of our sequence.

```
Features in this part of subject sequence:
   retinoblastoma-binding protein 6 isoform 2
   retinoblastoma-binding protein 6 isoform 1


 Score =  200 bits (108),  Expect = 3e-49
 Identities = 112/114 (98%), Gaps = 1/114 (0%)
 Strand=Plus/Minus
```

```
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Sbjct  15895869
Query  60
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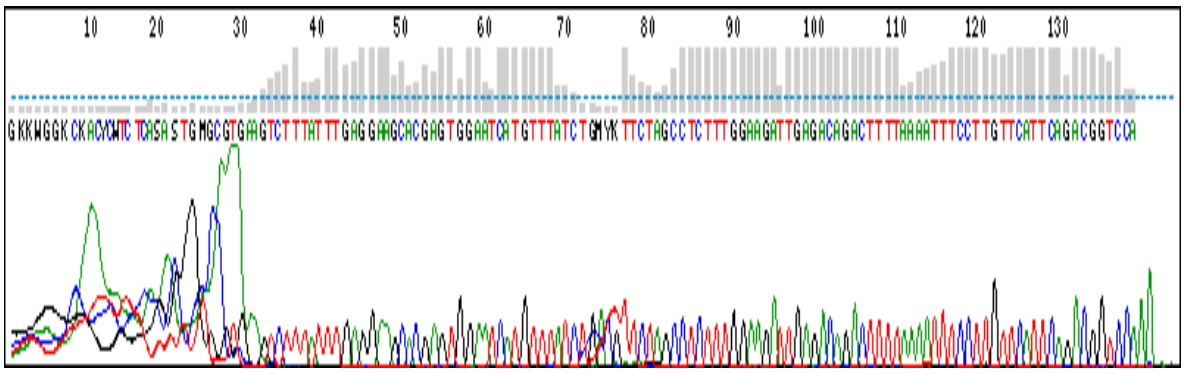

Figure 3.13: Representation of RBBP6 (exon 2 (DWNN)) sequence analysis for mutational analysis in lung tumour, which show no mutations and 98 % alignment with against NM_006910.4 and NM_018703.3 RBBP6 mRNA. (A) Blast sequence comparing RBBP6 mRNA on NCBI and our DNA query. (B) chromas analysis of our sequence.

3.3 Fluorescence in situ Hybridazation (FISH) study of lung cancer sections

Tissue arrays were purchased from cybrdi, Inc (USA) and other slides were supplied by Dr G Murray from National Health Laboratories Service (NHLS, SA). The pathological re-examination and confirmation on each tissue core of array slides including digital image capture were conducted before the IHC and FISH tasks were performed. Tissue sections used in this task were formalin fixed, paraffin embedded and viewed on a fluorescence microscope.

In this study, expression pattern analysis of the DWNN and that of RBBP6 was investigated using *in situ* hybridization (ISH). DWNN RNA probes were used to detect RBBP6 mRNA. Variant 3 which are also known as DWNN (**figure 3.14**) was cloned into pGEM-T Easy vector and sequenced at Inqaba Biotech (**figure 3.15-17**). A DWNN domain coding region was shown to have been successfully labelled with digoxigenin (DIG).

Figure 3.14: Agarose gel showing the PCR product of DWNN

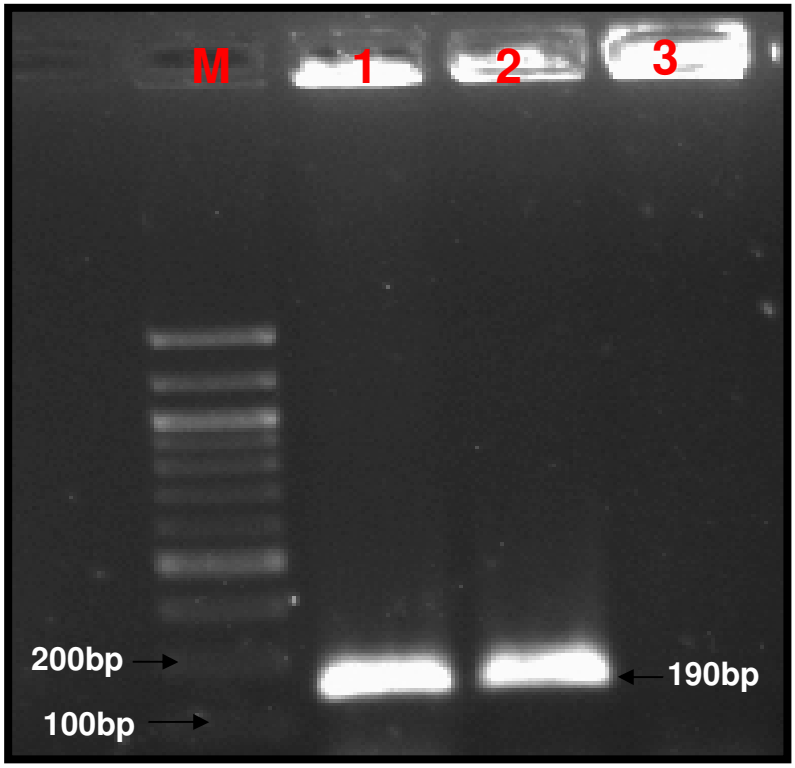

Figure 3.14: Gel electrophoresis representing amplification of RBBP6 from MRC5 cell line with a fragment size of ~ 190bp as shown by lanes 1 and 2. Lane M represents the 100 bp marker. Lane 3 represents negative control (no DNA added).

Figure 3.15: Agarose gel showing PCR products screening for DWNN domain for probe synthesis.

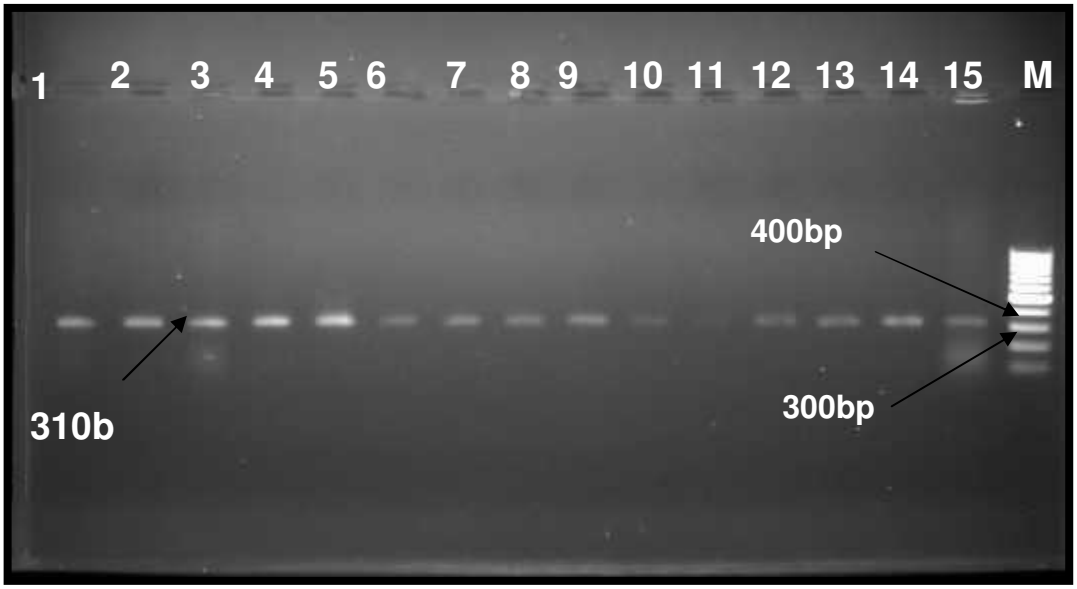

Figure 3.15: Results of recombinant screening using colony PCR gel electrophoresis technique, representing MRC5 cell line PCR product. Lane M represents the 100 bp marker while lanes 1-15 represent 15 different colonies which confirmed successful cloning of the DWNN fragment.

3.3.1 Conformation of RBBP6 clones by sequencing

Following successful cloning in pGEM, products were sent to Inqaba Biotech (South Africa) for sequencing to check whether the cloned sequence was the correct clone using M13 primers. The sequence results from Inqaba were aligned 99% when aligned against 6.1kb RBBP6 into cloned cDNA on NCBI (Figure 3.16). The sequences have also shown to be cloned in a 5' ⟶3' direction.

```
Query  1
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Sbjct  15864972
Query  121       GACC  124
                  ||||
Sbjct 15864912        GACC 15864909
```

Figure 3.16: The diagram show a sequence analysis of RBBP6. The sequence shows a 99 % compatibility with the NCBI mRNA sequence.

3.3.2 Linearization of plasmid DNA

The plasmid DNA was linearized with Pst 1 for antisense strand and Sp6 for sense strand restriction enzyme which cut the DNA in a way such that RNA polymerase fell off at the end of the gene of interest and resulted in the fragments as indicated in figure 3.17.

Figure 3.17: agarose gel showing linearized plasmid DNA for probes

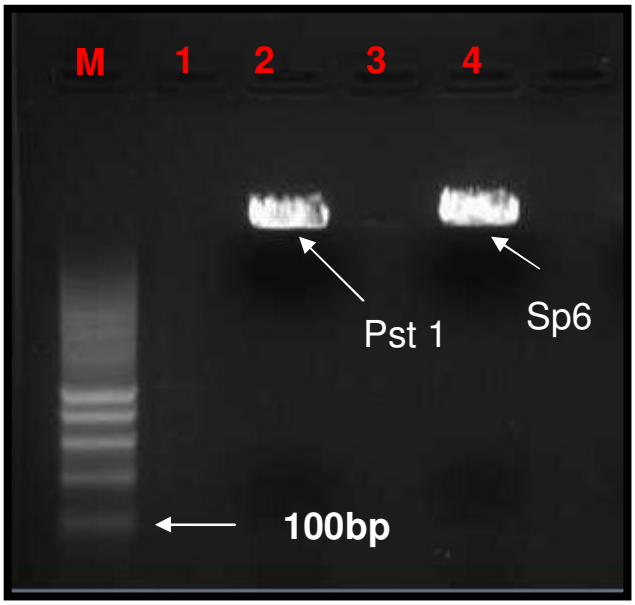


Figure 3.17: The results of restriction enzyme digest on gel electrophoresis showing linearized plasmid DNA cut with Pst 1 for antisense strand and Sp6 for sense strand. Lane-M represents the marker.

3.3.3 Determining probe concentration

It is important to optimise the concentration of the probe in localizing and detecting the mRNA. From the original concentration of 2.8µg/µl for antisense strand and of 0.38µg/µl for the sense strand, concentration of the probe was estimated using nylon membrane. As indicated in lane 2 of figure 3.18 the chosen dilution was 1:5 (10 ng/µl) for antisense and for the sense strand the undiluted concentration (10.0 ng/µl; lane 3).

Figure 3.18: The figure show the probe concentration estimation.

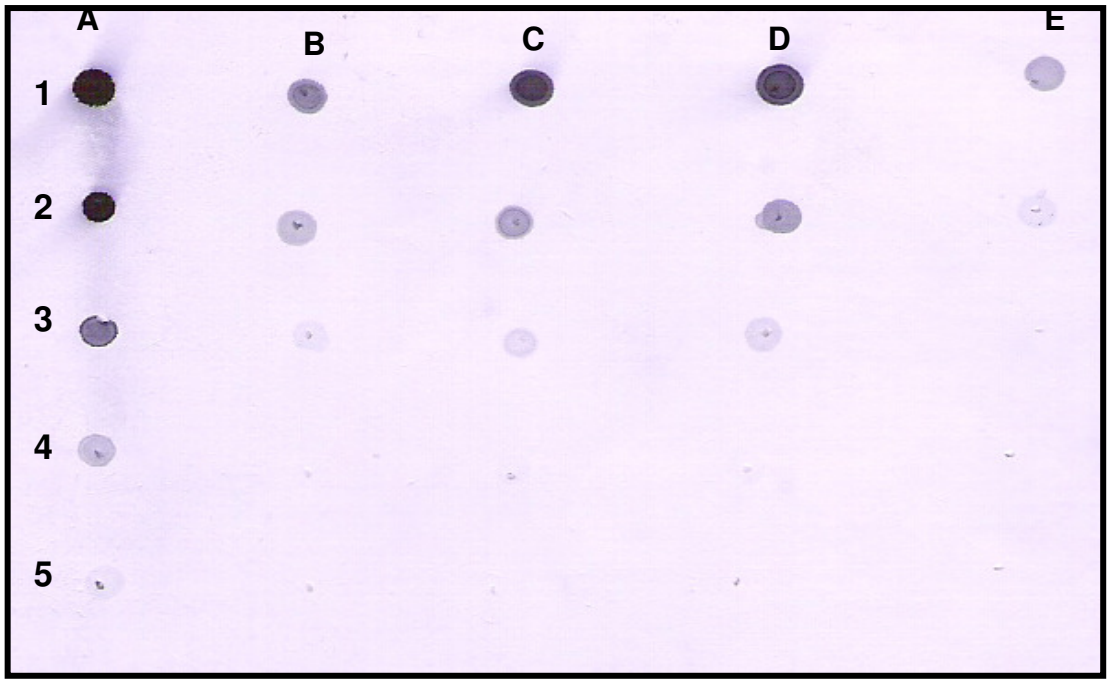


Figure 3.18: A representation of probe estimation results which indicates selected dilution concentration of 10.0ng/ml on lane 2 D. Lanes 1 (A-E) represent the control experiment while lanes 3A represented the sense strand probes concentration of 10.0 ng/ml which was used undiluted. Lane 5 represents the negative control.

3.3.4 Localization of RBBP6 mRNA by in situ hybridization (FISH)

No hybridization was observed in the negative control sections where the sense strand probe was used, whereas in normal lung tissue there was selective localization of RBBP6 probes (**figure 3.19B**). The expression of RBBP in normal tissue was lower in comparison to cancerous tissues (**figure 3.19A**). Localization studies in adenocarcinoma revealed some staining in the cytoplasm with more intensity in the papillae of well differentiated adenocarcinomas. The expression of RBBP6 mRNA further varied from poorly to well-differentiated lung adenocarcinoma with low to nil expression in poorly differentiated and fairly to upregulated in well-differentiated lung adenocarcinoma (**figure 3.20**). Furthermore most inflammatory foreign cells attracted to the tumour showed upregulation of RBBP6 mRNA (**figure 3.20B**).

In squamous cell carcinoma, there was low to nil expression of RBBP6 mRNA in poorly to moderately differentiated tumours. The micrographs showed upregulation of RBBP6 mRNA in well-differentiated squamous cell carcinoma (**figure 3.21**). The localization indicates increased abundance of the RBBP6 mRNA in the cytoplasm of squamous cell carcinoma with more staining in keratinized regions. In bronchoalveolar carcinoma (BAC), there was upregulation of RBBP6 mRNA in the cubodial cells. The uprequlation of RBBP6 mRNA in cells that show signs of undergoing mitosis (Mi) supports the view expressed in chapter one that RBBP6 may be a proliferation gene (**figure 3.23**). In Small cell and large cell carcinomas, there was nil to low levels of RBBP6 expression (**figure 3.24B**).

RBBP6 mRNA was detectable by *in situ* hybridization in adenocarcinoma, squamous cell carcinoma and BAC. The lung tissue from case BAC gave a hybridization signal that was

overall more intense than that of both adenocarcinoma and squamous cell carcinoma cases. The hybridization signal was ambiguous in small cell carcinoma sections, whereas in large cell carcinoma sections no specific hybridization signal was detected this finding was similar to the immunolabeling observed in large cell carcinoma. Hybridization was mostly observed in cancerous cell that seemed to be undergoing mitosis in BAC and more around the cytoplasm of glandular and papillae cells of adenocarcinomas. Hybridization was further observed to be more intense with progression of the tumour, in well-differentiated tumours there was greater intensity of RBBP6 mRNA labeling than in poorly differentiated. In most cases, the highest grain densities were detected in bronchiolar epithelial cells. In cuboidal epithelium, the hybridization signal was more intense than in columnar epithelium.

Figure 3.19: Represents hybridization of normal lung tissue with RBBP6 probe and negative control using the sense probe.

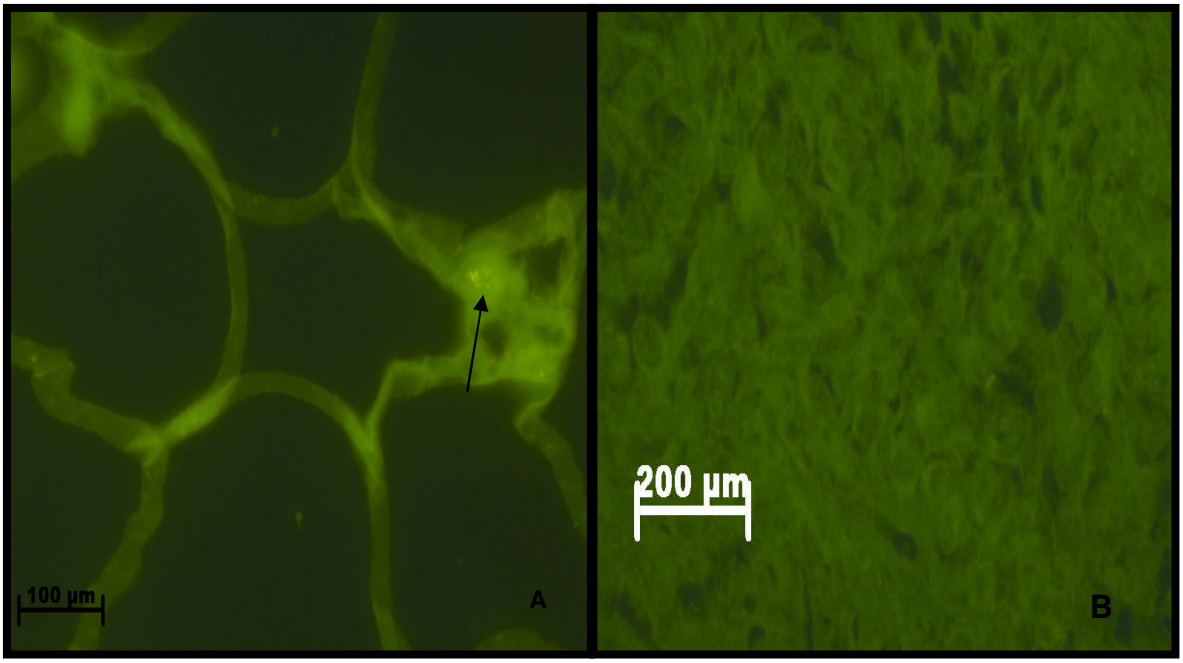


Figure 3.19: Expression of RBBP6 mRNA in normal lung has shown cytoplasmic localization in normal lung (**arrow in figure 3.19 A**). Note type I alveolar cells and squamous alveolar cells, these are squamous epithelial cells. Negative control results of sense probe showing no localization (**figure 19 B**). The micrographs were taken at a 100X (A) and 40 X (B) magnifications.

Figure 3.20: Represents hybridization of RBBP6 probes in lung Adenocarcinoma

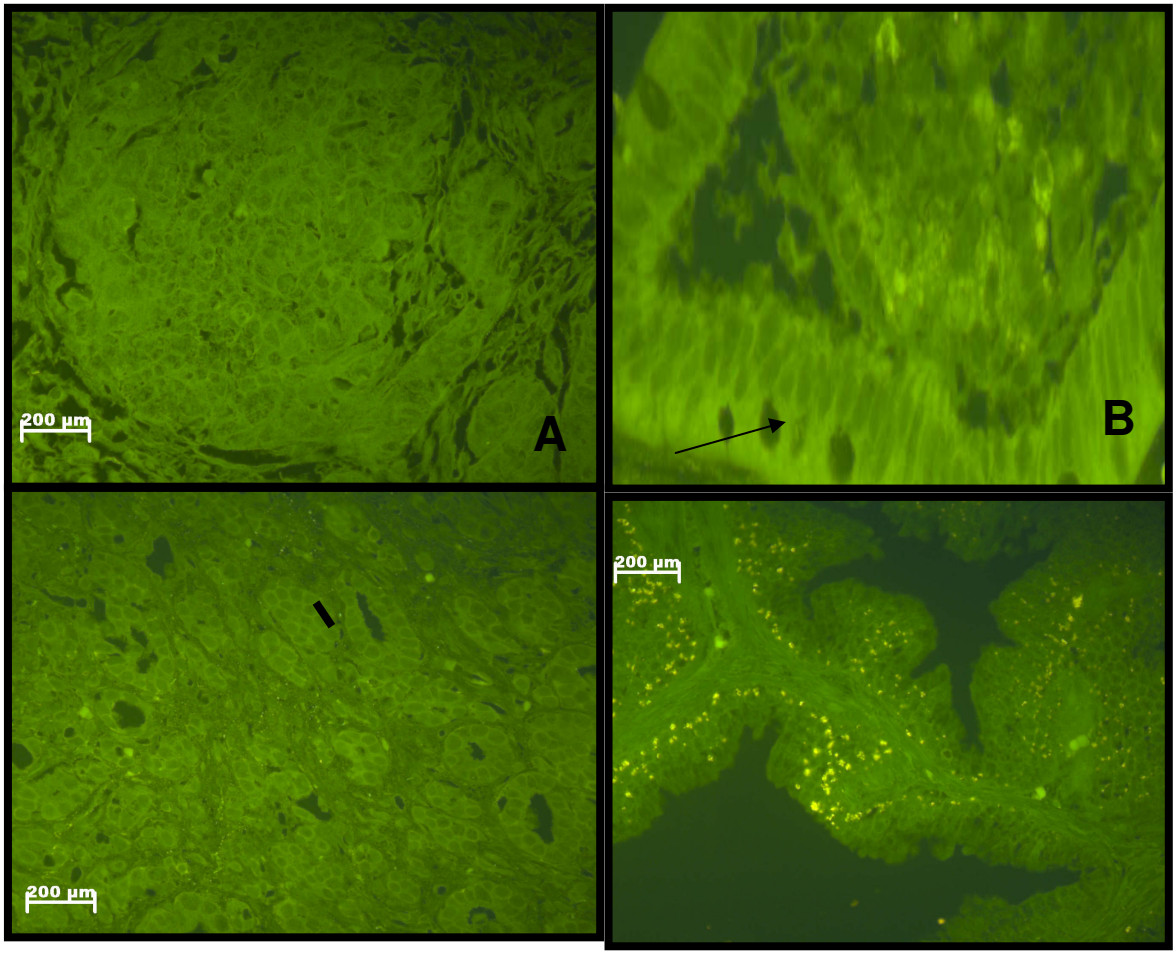


Figure 3.20: The figure shows the localization of the RBBP6 in lung adenocarcinoma. (**A**) Expression of RBBP6 in poorly differentiated lung adenocarcinoma which showed low to nil expression of RBBP6. **(B**) Upregulation of RBBP6 mRNA in papillary cells of adenocarcinoma of the lung. (**C & D**). **Note** high intensity of stain around the cytoplasm of the papillary cells (**arrow**). Expression of RBBP6 mRNA in moderate and well-differentiated lung adenocarcinoma showing upregulation of RBBP6 (**C & B**). **Note** some staining in the cytoplasm of glands (**G shown by a bar**). The sections were taken at 40X magnification.

Figure 3.21: Represents hybridization of RBBP6 probes in Lung Squamous cell carcinoma

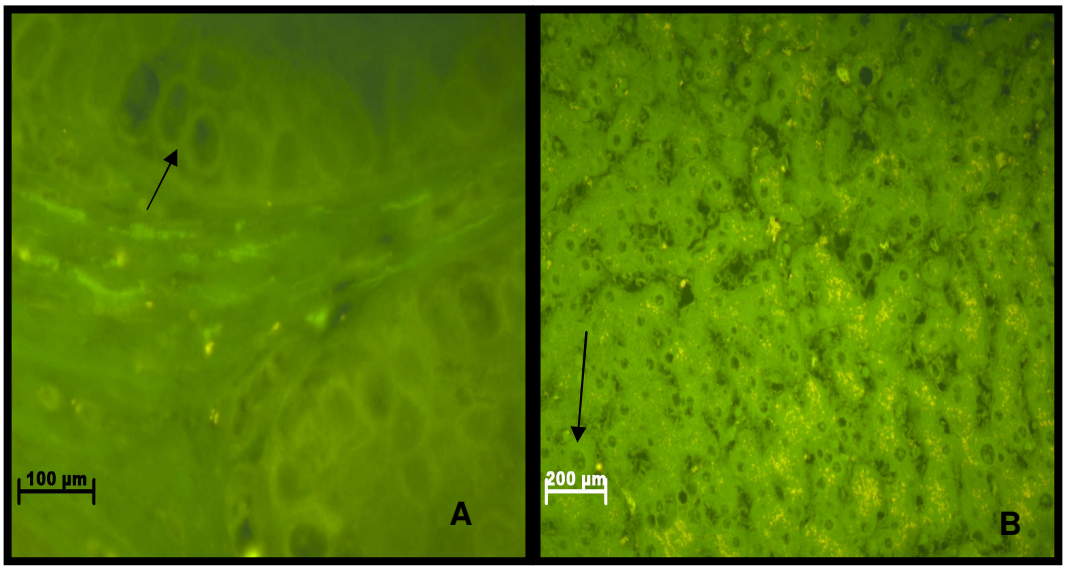


Figure 3.21: A micrograph showing localization of the RBBP6 mRNA in squamous cell carcinoma of the lung. The figure shows the mild staining in the cytoplasm of both poorly (A) and well-differentiated cells (B). The sections were taken at 100 X (A) magnifications (B) and 40X.

Figure 3.22: Represents hybridization of RBBP6 probes in Bronchioloalveolar carcinoma (BAC)

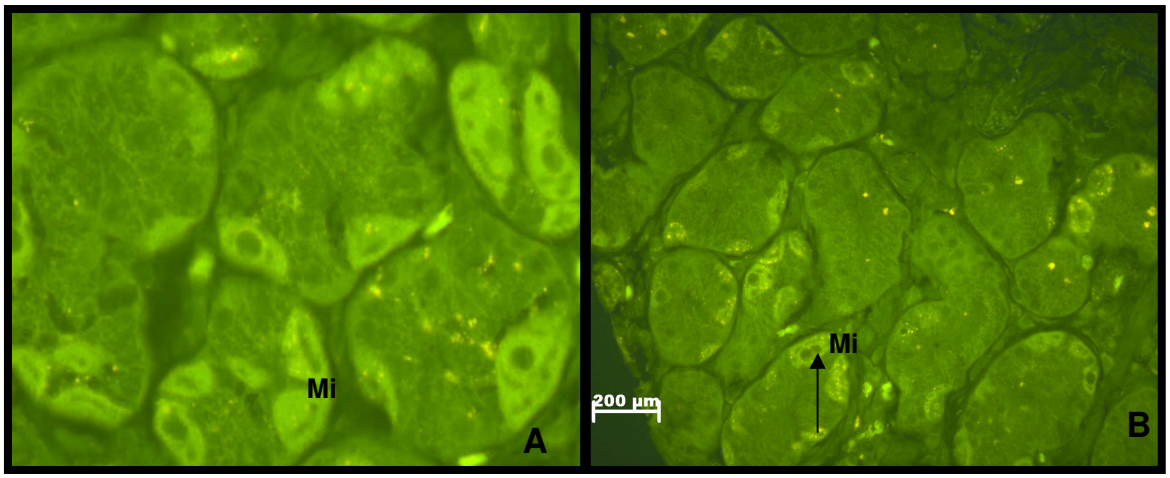


Figure 3.22: The micrograph shows expression of RBBP6 mRNA in the BAC with high intensity of staining visible in the cytoplasm of cells that seem to under go mitosis (**Mi and arrow**). **Note** high intensity of RBBP6 antisense probe in and around mitotic cells. The sections were taken at 40X magnification.

Figure 3.23: Represents hybridization of RBBP6 probes in Small and large Cell Carcinoma

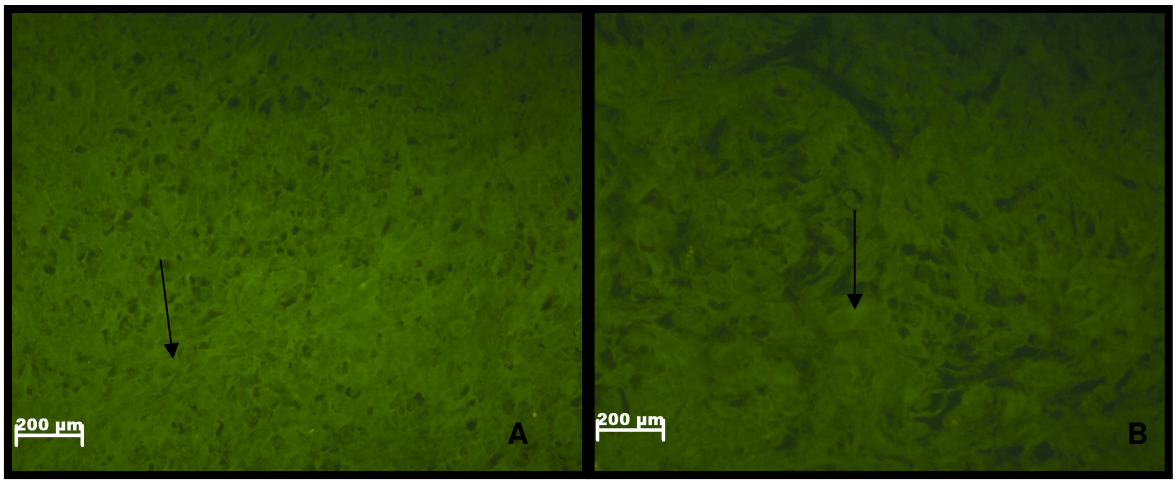


Figure 3.23: The micrograph shows expression of RBBP6 mRNA in small cell carcinoma of the lung, **Figure 3.23A** shows that RBBP6 mRNAs are not localized in the tumour. (B) Shows expression of RBBP6 mRNA in large cell carcinoma of the lung, the figure 3.22B show no localization of RBBP6 mRNA. The sections (A&B) were taken at 40X magnification.

3.4 Retinoblastoma binding protein 6 (RBBP6) protein expressions in lung cancer by immunocytochemistry

Proper and correct localization of cellular molecules is crucial for biochemical and physiological reaction that these molecules partake in. It is very important to know the localization of the DWNN-containing RBBP6 proteins to be able to understand its cellular function. Currently, there is no clear function attributed to RBBP6 in malignancy; however it has been suggested that it might be involved in cell proliferation or apoptosis during carcinogenesis. In order to examine this question one approach was to determine the subcellular localization of the endogenous RBBP6 protein using specific antibodies raised against RBBP6 in the rabbit and mouse at the University of Stellenbosch and supplied to use by Prof J Rees's Laboratory from University of the Western Cape.

3.4.1 Experimental Controls

Cellular expression of RBBP6 was determined in formalin embedded, paraffin, and fixed, lung cancer and normal lung tissues. Negative controls were carried out by incubating sections with normal serum and PBS or without 1º antibody. As illustrated in figure 3.23, no labelling was observed in the absence of the primary antibody (**figure 3.24**). Since RBBP6 is proposed to be a proliferative protein it may be found in abundance in tissues that are highly proliferative such as testis, placenta and the brain. An abundant amount of RBBP6 localizes in the cytoplasm semineferous tubules of the testis (**T**)(**figure 3.25**).

Figure 3.24: Represents the negative control of ICC

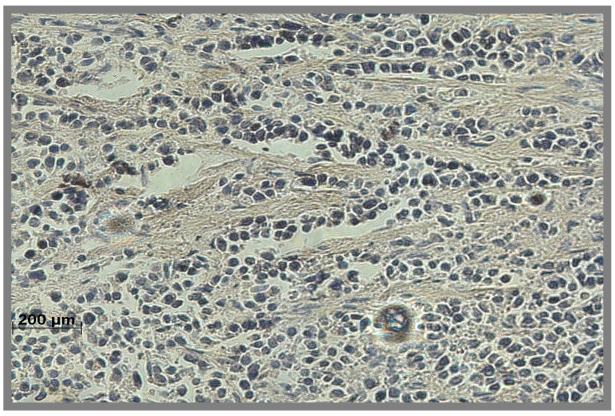


Figure 3.24: Negative control for immunocytochemistry, squamous cell carcinoma. Magnification: X40.

Figure 3.25: The figure showing the positive control of ICC

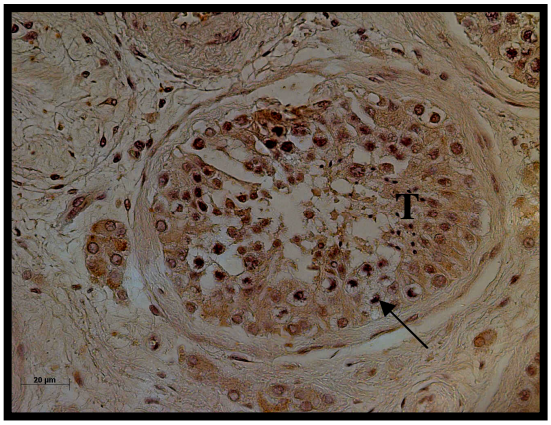


Figure 3.25: The micrograph shows expression of RBBP6 protein in the seminiferous tubules (T and arrow) of the testis. Note abundant localization and expression as indicated by the arrow in the cytoplasm. The sections were taken at 40X magnification.

3.4.2 Normal lung Tissue control

Expression patterns of RBBP6 protein was examined in normal lung tissue. Cytoplasmic localization of RBBP6 is shown in the alveoli cells of normal lung tissue (**figure 3.26**, see arrow). In the bronchioles there was localization in the cytoplasm (Br) indicating moderate expression of RBBP6. The nuclei are indicated by the blue staining produced by the counterstain.

Figure 3.26: The figure showing Immunolocalization of RBBP6 in normal lung tissue control.

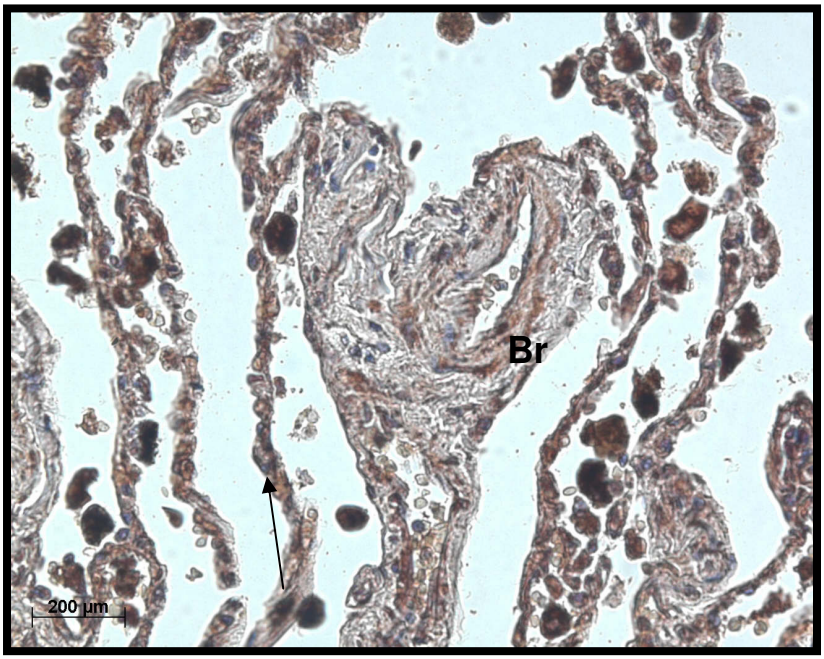


Figure 3.25: The micrograph shows expression of RBBP6 protein in the alveoli cells of (**arrow**) and bronchiole (**Br**) of normal lung. Note localization and expression as indicated by the arrow in the cytoplasm of type I alveolar cells. The sections were taken at 40X magnification.

3.4.3 Localization of RBBP6 protein in lung cancer

The pattern of localization of the RBBP6 protein does not differ in different lung tumours. Exception are Small cell carcinoma and large cell carcinoma which showed no expression and in adenocarcinoma and squamous cell carcinoma which showed more cytoplasmic expression. These were shown in figure 3.26-30 and table 3.1.

Table 3.1: Lung cancer type and immunohistochemical staining for p53 and RBBP6

Histo-Pathology	p53				RBBP6		
	-ve	+ve	+ve Rate (%)		-ve	+ve	+ve Rate (%)
Adenocarcinoma	30	20	40		30	20	40
Squamous cell carcinoma	16	24	60		6	34	85
Normal lung tissue	12	0	0		10	2	20
Small cell carcinoma	12	4	50		14	4	22
Papillary alveolar cell carcinoma	14	0	0		10	4	29
Large cell carcinoma	6	2	33		6	2	25

Figure 3.27: Immunolocalization RBBP6 by ICC in Adenocarcinoma of the lung

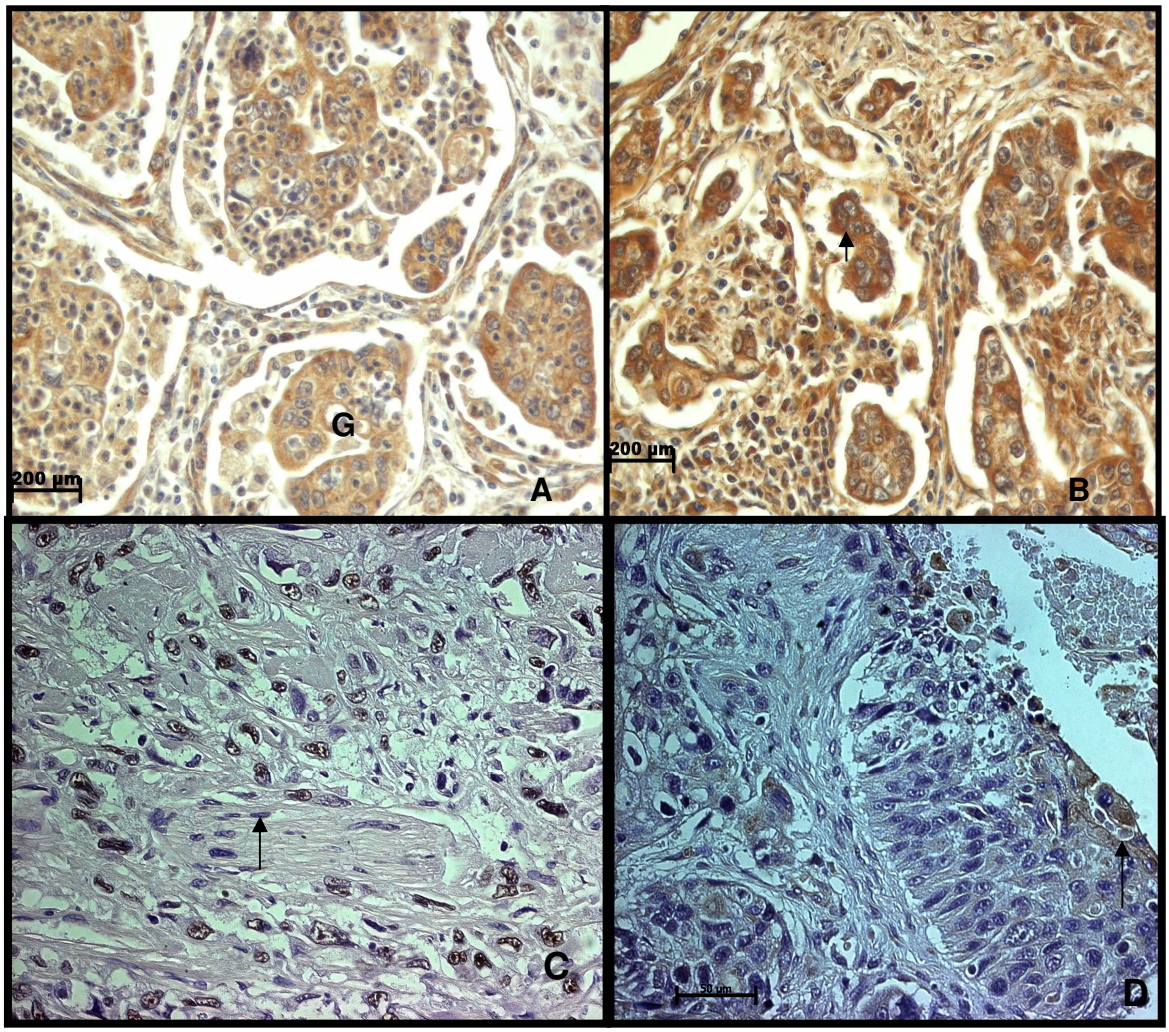


Figure 3.27: Well-differentiation adenocarcinoma of the lung (A & B) composed of glandular regions showing higher expression of RBBP6 around the glandular regions (**G**) particularly in the cytoplasm. (C) A poorly-differentiated and (D) a moderately-differentiated adenocarcinoma showing less or lower levels of RBBP6 staining or expression in the cytoplasm. The sections were taken at 40X magnification.

Figure 3.28: Immunolocalization RBBP6 by ICC in Adenosquamous and pulmonary Adenocarcinoma.

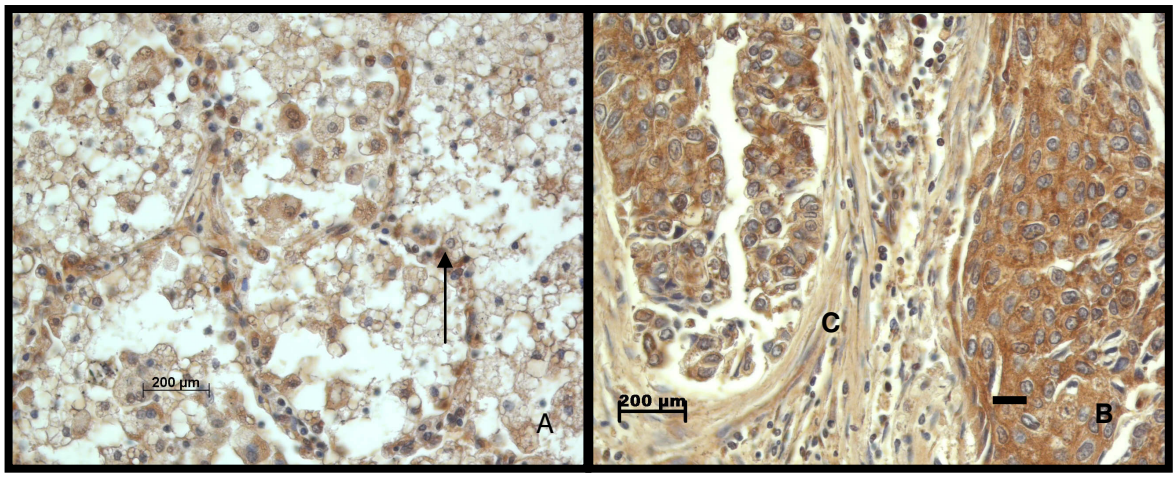


Figure 3.28: (A) Immunohistochemistry of pulmonary adenocarcinoma indicating cytoplasmic staining for RBBP6. Note positive staining of RBBP6 in alveoli cell type1 as indicated by arrow. (B) Immunocytochemistry of adenosquamous cell carcinoma showing cytoplasmic expression of RBBP6 in mucin producing cell (C) and the squamous region indicated by a bar. The sections were taken at 40X magnification.

Figure 3.29: Immunolocalization RBBP6 by ICC in Squamous Cell Carcinoma

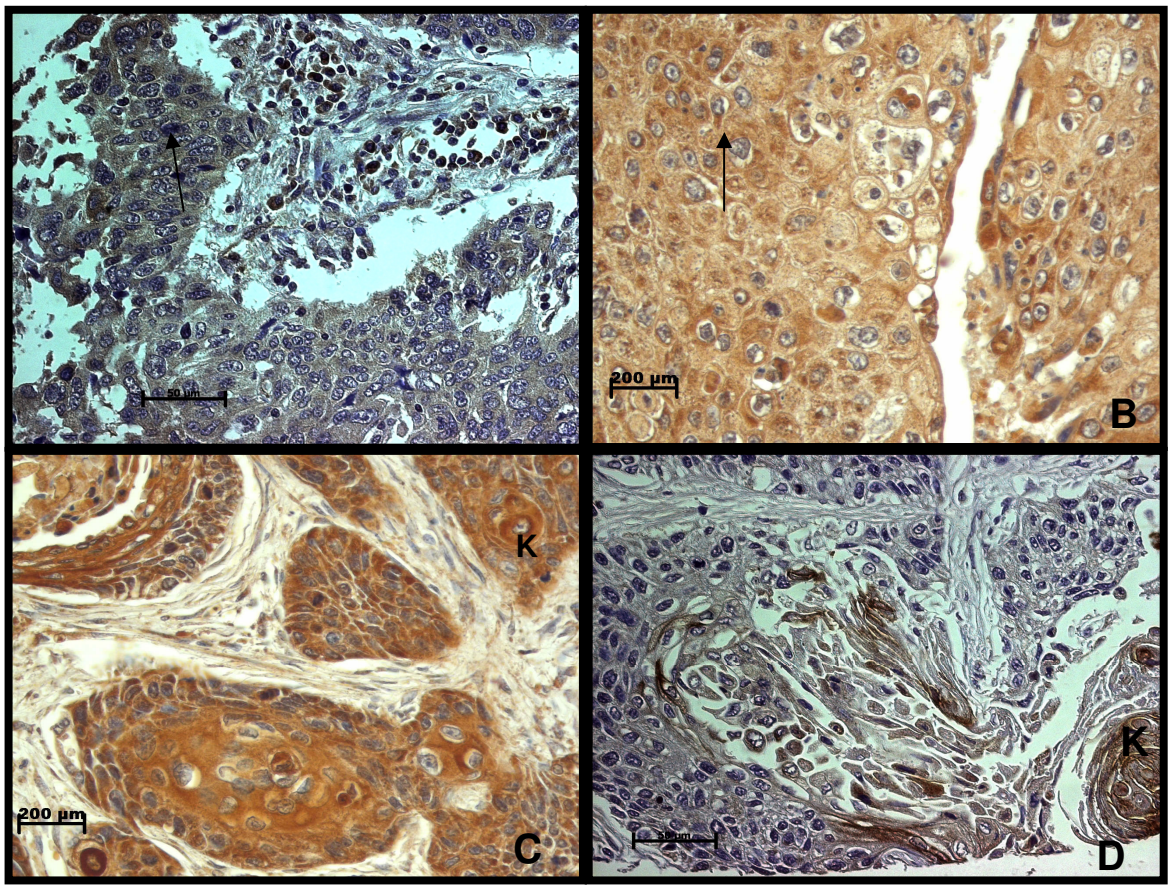


Figure 3.29: RBBP6 protein expression in squamous cell carcinoma. (A) Partial RBBP6 expression over part of the cytoplasm in squamous cell carcinoma. (B) RBBP6 expression was seen over the entire cancerous cell's cytoplasm. RBBP6 expression in moderately squamous cell higher expression was observed around keratinized regions (K). (D) RBBP6 expression in well differentiated squamous cell carcinoma with poor expression in most cell but higher expression around the keratin regions (**k**). The sections were taken at 40X magnification.

Figure 3.30: Immunolocalization RBBP6 by ICC in cytology and lung muscles

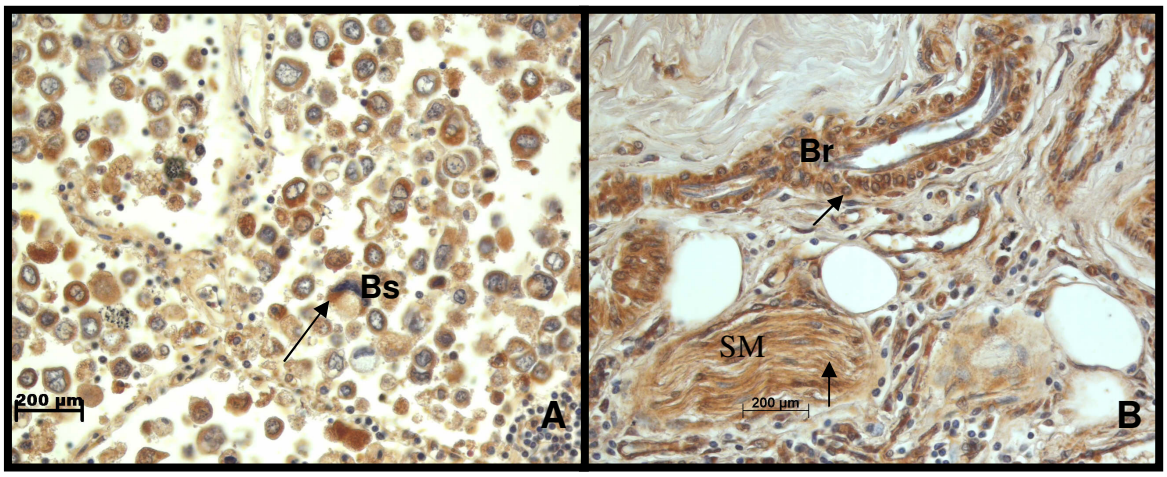


Figure 3.30: (A) Cytologic appearance of squamous cell carcinoma showing cytoplasmic staining of RBBP6 in basophils (Bs). Note high expression of the RBBP6 protein in immune cells recruited to the site of the tumour. (B) Squamous cell carcinoma showing RBBP6 protein expression in the smooth muscles (SM) and bronchioles (Br). Note high intensity of the stain around both the muscle cells and bronchioles which indicate high expression of RBBP6 in these tissues. The sections were taken at 40X magnification.

Figure 3.31: Immunolocalization RBBP6 by ICC in Small cell and large cell carcinomas

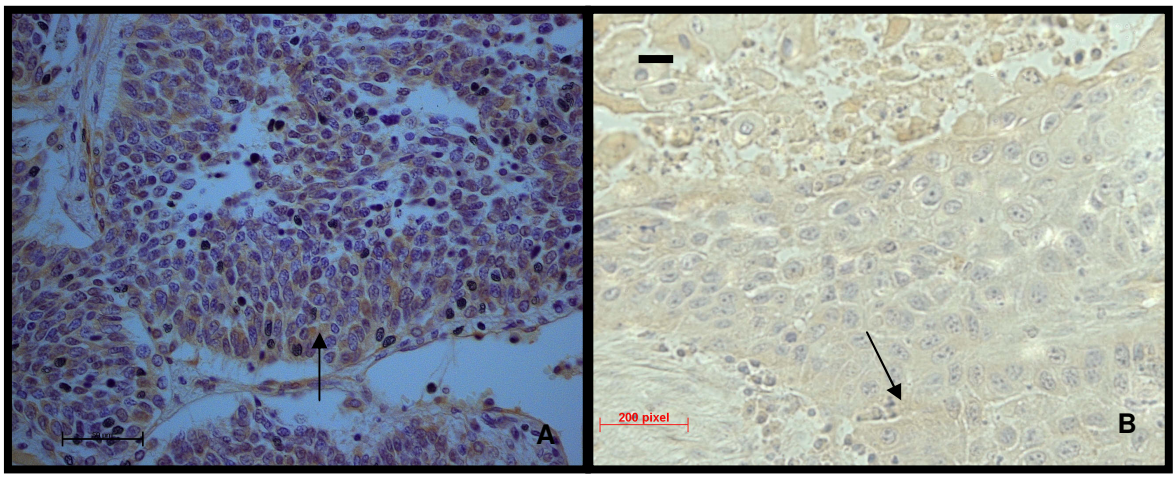


Figure 3.31: Immunohistochemistry in (A) Small cell carcinoma showing blue stained oval to spindle nuclei and RBBP6 protein expression in extremely cytoplasm (arrow) and (B) Expression of RBBP6 protein in the cytoplasm of large cell carcinoma tissue. The sections were taken at 40X (A&B) magnifications.

Summary of RBBP6 protein expression by immunocytochemistry (ICC)

The immunolabeling data (**table 3.1**) showed that in 20/50 (40%) of the carcinomas RBBP6 was over-expressed ($p<0.001$). The sections of adenocarcinoma with tubular and papillary type showed strong membranous expression for RBBP6 protein on the luminal surface, but weaker expression in the cytoplasm of tumour cells (**figure 3.27 D**). The luminal contents also stained strongly for RBBP6. It is clear from micrographs 3.26 A and B that the cytoplasmic localization and expression of RBBP6 protein was higher in the malignant cells of adenocarcinoma when compared to grade II adenocarcinoma which showed moderate to

less expression of RBBP6. RBBP6 protein was localized also in the cytoplasm of the pulmonary cells/alveoli cells type 1. Whereas expression of RBBP6 protein was similar between adenosquamous carcinoma and squamous cell carcinoma, the latter showed greater immunoreactivity in the cytoplasm of glandular or mucin producing cells (Mc).

Of the 40 cases analyzed, 34 stained positively for RBBP6 protein which represented a high frequency of 85%. In comparison to the normal tissue, there was significantly higher expression of RBBP6 antigen ($p>0.01$) in squamous cell carcinoma when compared to normal lung tissue (**table 3.1**). Based on degree of tumour differentiation, RBBP6 protein was highly expressed predominantly in well-differentiated than in poorly-differentiated squamous cell carcinoma (**figure 3.28**). Localization in these tumours was more evident in the cytoplasms and membranes of the tumour cells. More positive staining was observed around and within the keratinized regions (**figures 3.28D**). Further the RBBP6 protein was found to be abundantly localized within the cytoplasm of basophils (**Bs**). The smooth muscles (SM) associated with the tumour showed high expression of RBBP6 protein within sarcomeres, and there was also clear RBBP6 protein in the bronchioles (**Br**) (**figure 3.30**).

Of the 18 cases of small cell carcinoma, only 4 (22%) stained positively for the RBBP6 protein. In comparison with squamous cell carcinoma, there was lower level of expression in the cytoplasm of small cell carcinoma as for large cell carcinomas, immunolabeling data (**table 3.1**) revealed that 2/8 (25%) of the carcinomas expressed the RBBP6 protein (**figure 3.31**). As in the other carcinomas, RBBP6 protein was expressed in the cytoplasm of the tumour cells however expression was much lower.

Overall, RBBP6 protein values varied among 142 tissues of lung cancer examined (**appendix 1**). Loss of RBBP6 protein expression was more frequently observed in small cell and large cell carcinomas when compared to adenocarcinoma and squamous cell carcinomas. There was a strong correlation between loss of RBBP6 expression and the tumour stage or grade, with the well-differentiated showing greater expression than the poorly-differentiated carcinomas. Strikingly, staining intensity was stronger in neoplastic cells relative to adjacent normal cells.

3.5 RBBP6 expression in A549 cell and MRC5 cell line using western blotting

The protein concentrations of A549 cell line and MRC5 cell lines were determined using the Nano drop meter and Bradford assay and the protein samples were electrophoresed on SDS PAGE as described in Chapter 2. The proteins were transferred unto a PVDF membrane (Amersham Pharmacia) using a Mini Protean II™ system (Biorad) and screened by Western blot analysis using the polyclonal anti-rabbit RBBP6 protein antibody and p53 monoclonal antibodies purchased from Biochom Biotech (SA). As shown in **figure 3.32A**, testis cell lysated expressed higher levels of RBBP6 protein. Thus, the two cell lines showed RBBP6 varying expression profiles (**figure 3.32 B & D**). RBBP6 expression in testis cell lysate was also associated with high expression levels of cytoplasmic RBBP6 in the testis tissue section, which is a key organ in proliferation. In contrast, lower RBBP6 expression levels were observed in A549 and MRC5 cells in comparison to the testis cell lysates. Further lower expression were observed in cells treated with RBBP6 siRNA (**figure 3.32 C (A549) & E (MRC5)**) which showed successful silencing of RBBP6 in the two cell lines. As for p53 there was low to total silencing of p53 as indicated by **figure 3.33 D** in A549 cell lines

treated with sip53 but to the less extend in MRC5 cell lines. This result was in line with most results observed in other studies.

Figure 3.32: Western Blot analyses of RBBP6 in A549 and MRC5 cell lines.



Figure 3.32: Shows the results of Western blot, which detected a ~200 kDa protein. This corresponds to the expected size. The Western blot also demonstrates that the expression level of RBBP6 is higher in testis (A) and fairly expressed in A549 (B) and MRC5 (D). In cells transfected with RBBP6 siRNA, there was low expression of RBBP6 protein in A549 (C) and MRC5 (E).

Figure 3.33: Western Blot analyses of p53 in A549 and MRC5 cell lines.



Figure 3.33: Western blot result showing a p53 protein. The Western blot also demonstrates the expression of p53 protein in (A) MRC5 cell fribroblast, (B) A549 cell line; (C) MRC5 cell lines silenced with sip53 and (D) A549 cell line silenced with sip53. Note the expression of p53 was almost totally silenced in A549 cell line.

3.6 Apoptosis detection in lung cancer

DNA fragmentation is a powerful tool for studying the apoptotic process *in vitro* and *in vivo*. DNA fragmentation associated with apoptotic cells has been detected using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) assay. The TUNEL assay has been the most widely used for identifying apoptotic cells *in situ*. In this assay, biotinylated dUTP is incorporated at the 3' OH ends of fragmented DNA using TdT, and detected by a variety of methods involving brightfield or fluorescence microscopy. The labeling of fragmented DNA also facilitates detection of condensed chromatin, another characteristic morphological change associated with apoptotic cells.

The quantity of apoptotic cells and bodies, apoptotic index (AI %), has been defined as a percentage of apoptotic cells in the entire tumour cell population. Table 3.2 summarises the apoptotic indices as assessed by TUNEL immunoreactivity in different lung cancer tissues. The mean apoptotic index in normal epithelial mucosa was 0.2 ± 0.05 %, which was lower than in normal mucosa adjacent to squamous cell carcinomas: 1.8 ± 0.23 %. The mean apoptotic index in adenocarcinoma was 1.5 ± 0.18 %, lower than the apoptotic index of 1.8 ± 0.23 % in squamous cell carcinomas. There was positive correlation between the frequency of apoptosis as assessed by TUNEL immunoreactivity and different grades of dysplasia or growth type.

Table 3.2: Overview of studies on the detection of apoptosis in lung cancer tissues.

Lung Cancer type	Number of Cells	Apoptotic Index (AI) %
Adenocarcinoma	59	1.5 ± 0.18 %
Squamous carcinoma	62	1.8 ± 0.23 %.
Small cell carcinoma	23	0.9 ± 0.03 %
Large cell carcinoma	16	0.3 ± 0.02
Normal lung	9	0.2 ± 0.05 %

In adenocarcinoma, the degree of apoptosis was higher in well differentiated adenocarcinomas than in poorly differentiated tumours. In addition the immunoreactivity was seen mainly in cancerous cells (**figure 3.34 A**). In squamous cell carcinomas, the degree of apoptosis was greater in late stages of the tumour than in the early stages with the immunoreactivity observed mainly in the cancerous cells (**figure 3.34C**). In the large cell and small cell carcinomas, there was lesser apoptotic index as compared to normal cells (**figure 3.34B**). The immunostaining of RBBP6 revealed diffuse, cytoplasmic staining and was present in most of the malignant cells examined. There was no statistically significant association between the extent of apoptosis and the expression of RBBP6.

Figure 3.34: The figure showing apoptosis detection by TUNEL in lung cancer.

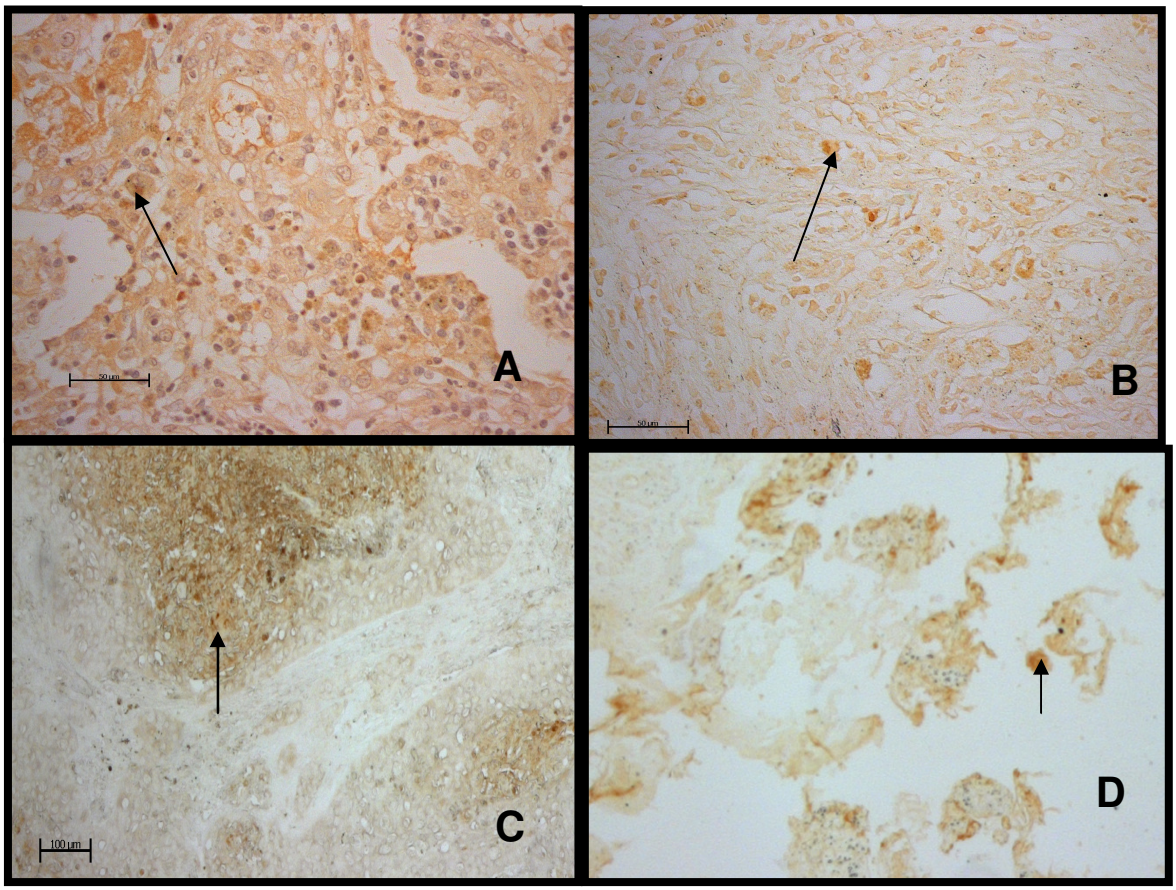


Figure 3.34: TUNEL representation in (A) adenocarcinoma, (B) large cell carcinoma, (C) squamous cell carcinoma and (D) Mesothilia. The sections were taken at 40X (A, B & D) and 10X (C) magnification.

3.6.1 Apoptosis in Cell lines (MRC 5 and A549 cell lines)

The activation of a particular apoptotic pathway is dependant on the reagent used to activate apoptosis, its concentration and to some extend also the cell type. The objective was to first identify the concentration of staurosporine and camptothecin that would be suitable to induce apoptosis in A549 cell lines but to a lesser extend in MRC5 cell lines. Following activation of

apoptosis with these inducers, RNA was extracted to measure and correlate the expression of RBBP6 and p53.

To measure the effects of apoptosis inducers; cells were harvested by trypsinisation, stained with propidium iodide and annexin FITC for flow cytometer analysed at 10 000 events (number of cells). Cells that were necrotic did pick propidium iodide and were located at the Upper left of the flow cytometer chart while those that were at their early, late and did show apoptosis will be located at lower right, upper right and lower left respectively. From flow cytometer observations, there was about less than 1 % apoptosis in cells that were not treated with any apoptosis inducers (staurosporine and camptothecin). In cell treated with 25nM Staurosporine (**figure 3.35 A**), 0.8 -1 µM camptothecin (**figure 3.35 D**) there was a shift towards apoptosis with about 9.2% and 3% respectively in both A549 and MRC5 cell lines. However, at 35nM and 50nM there was significant shift toward necrosis (**figure 3.35 C&B**) respectively. From both flow cytometer and cell cytotoxicity assays, the following concentrations were chosen as appropriate for further studies, 25nM Staurosporine, 0.8 µM camptothecin, and 0.5 µM TRAIL.

Figure 3.35: The figure showing apoptosis in A549 and MRC5 cell lines by FACScan

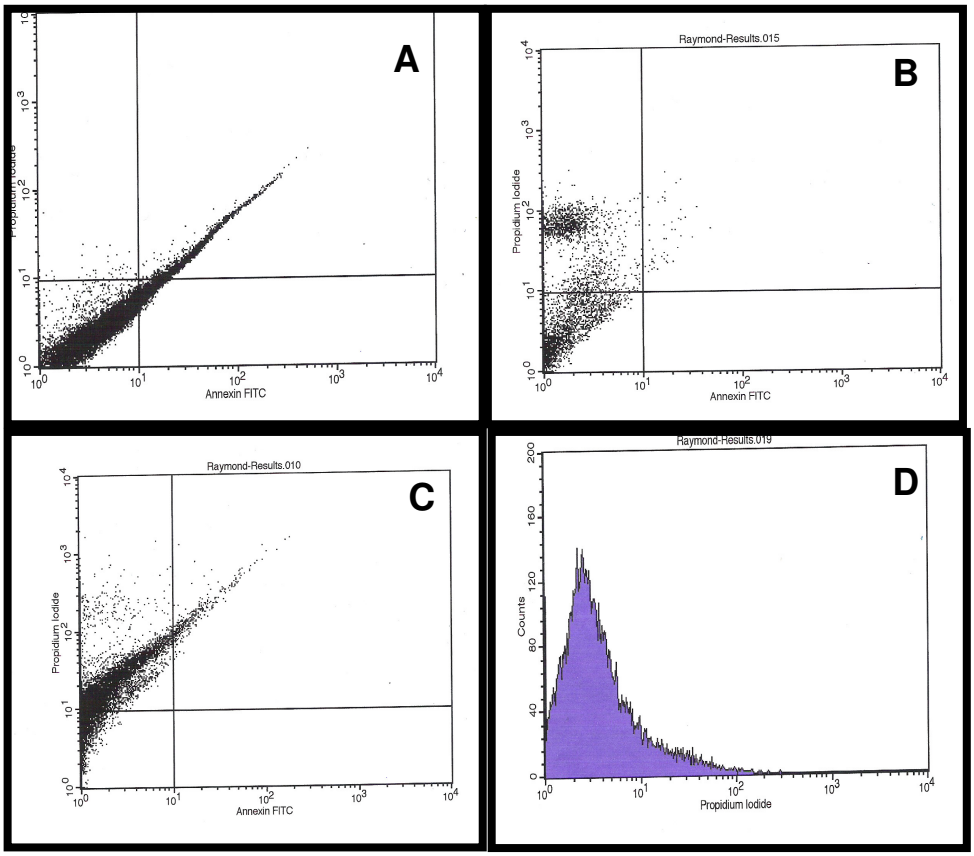


Figure 3.35: Illustrates the results of flow cytometer determination of appropriate concentration of staurosporine. (A) 25nM staurosporine showing 9.2% apoptosis; (B) 35nM showing 3% apoptosis and (C) 50nM showing 4% apoptosis in both A549 and (D) 0.8µM camptothecin showing 15% apoptosis in A549.

3.6.1.1 Measuring apoptosis in Cell lines (MRC 5 and A549 cell lines) following RBBP6 and p53 gene silencing and treatment with apoptosis inducers.

The objective of this section was to evaluate the effect of RBBP6 silencing on apoptosis, its correlation with p53 and its effect on cancer cell lines. Following both p53 and RBBP6 gene silencing and the induction of apoptosis with 25nM staurosporine, there was about 2.7% shift towards apoptosis and 17% towards necrosis in RBBP6- MRC5 and A549 cell lines (**figure 3.36 C & D**) while in p53-MRC5 treated cells there was about 2.55 % apoptosis which was not significant in comparison to cells treated with staurosporine only (**figure 3.36 A**) and there was also a 57% shift towards apoptosis in p53-A549 treated cells which was significant as compare to the same treatment without gene silencing (**figure 3.36 B**). For cell treated with camptothecin, there was about 3% apoptosis in p53-siRNA MRC5 treated cells and 54% apoptotic cells in p53-siRNA treated A549 cells which were significant in the latter as compared to A549 cells treated with camptothecin only (**figure 3.37 A & B**) and there was 11.92% necrotic cells in p53-siRNA treated A549 cells. As for RBBP6-siRNA camptothecin treated cells there was 8.51 % and 2.74% apoptotic cells in A549 and MRC5 cells respectively which showed a little shift as compared to the respective treatment with camptothecin only (**figure 3.37 D&C**). In p53-siRNA TRAIL induced apoptosis, there was 8.92% and 54.34% apoptosis in MRC5 and A549 cells respectively (**figure 3.38 A & B**) while in RBBP6-siRNA TRAIL induced apoptosis; there was 3.98% apoptosis in MRC5 and 2.74% in A549 cell lines (**figure 3.38 C &D**).

Figure 3.36: The figure showing the results of apoptosis induction by Stuarosporine

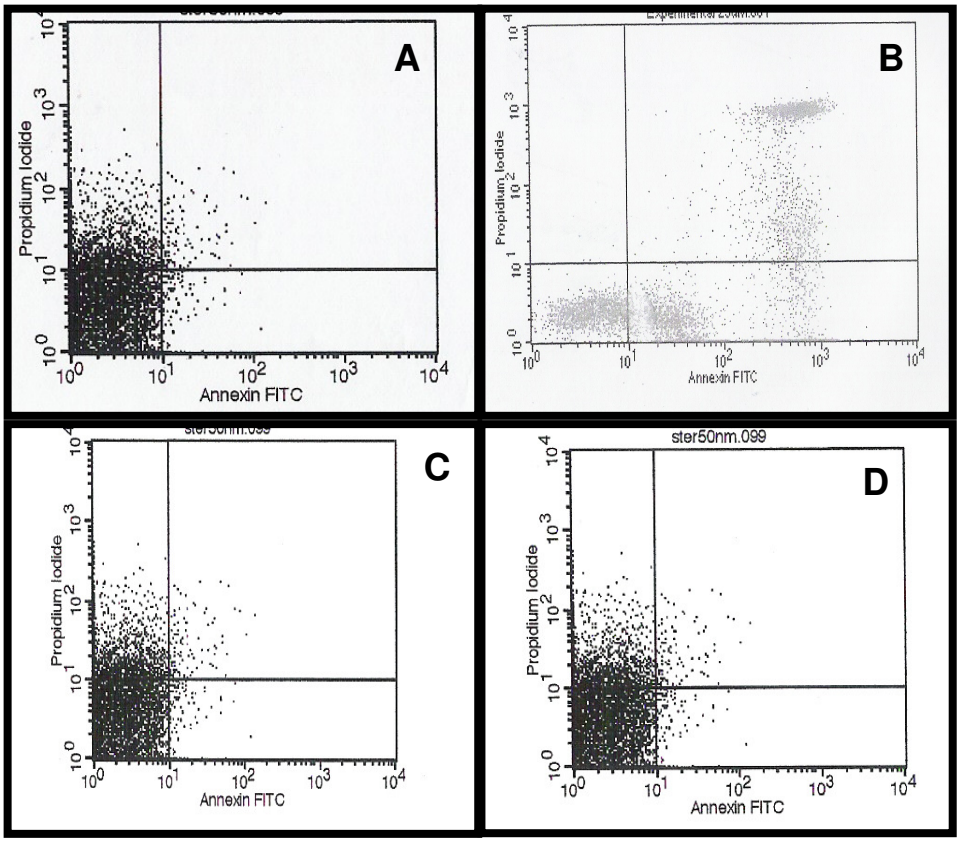


Figure 3.36: Illustrates Staurosporine treated cells with (A) p53-siRNA MRC5 showing more necrosis than apoptosis, (B) p53-siRNA A549 showing 54% apoptosis, (C) RBBP6-siRNA showing more necrosis in MRC5; (D) A549 treated with RBBP6-siRNA showing more necrosis.

Figure 3.37: The figure showing apoptosis induction by camptothecin

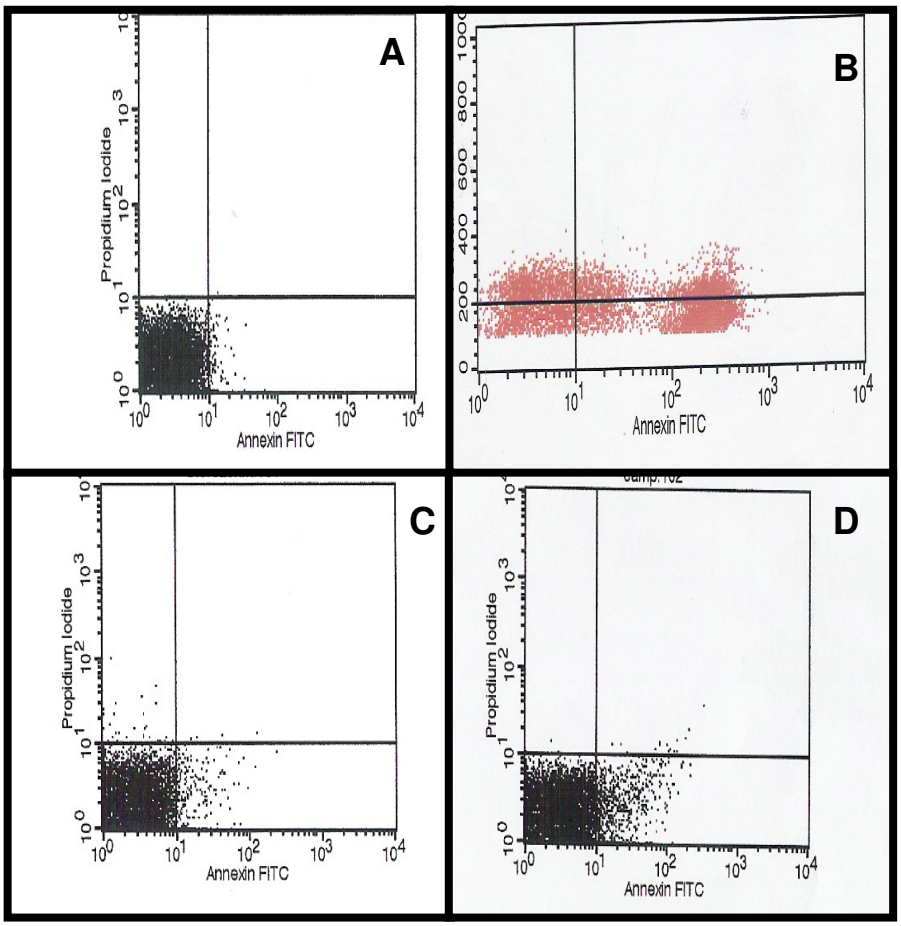


Figure 3.37: Illustrates camptothecin treated cells with (A) p53-siRNA MRC5 showing little to nil apoptosis, (B) p53-siRNA A549 showing 54% apoptosis, (C) RBBP6-siRNA showing 3% apoptosis in MRC5; (D) RBBP6-siRNA showing 8.5% apoptosis.

Figure 3.38: The figure show results of apoptosis induction by TRAIL.

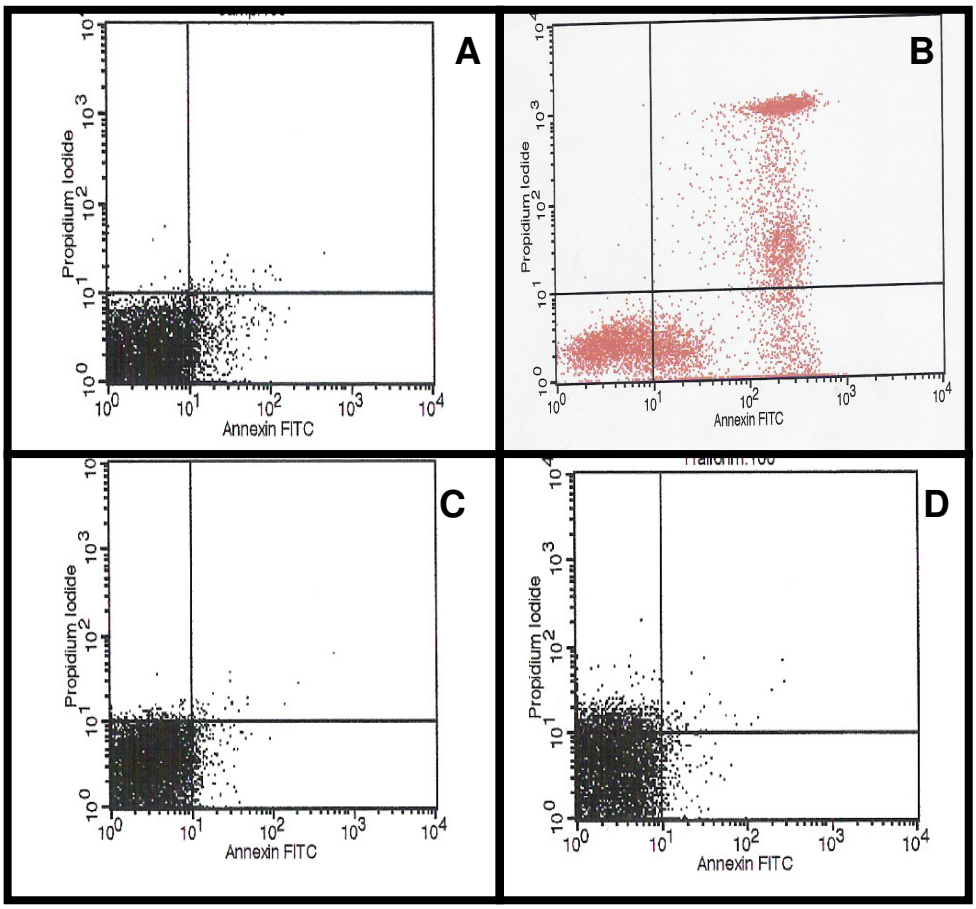


Figure 3.38: Illustrates TRAIL induced apoptosis (A) p53-siRNA MRC5 showing 8.9% apoptosis, (B) p53-siRNA A549 showing 54.3%, (C) RBBP6-siRNA showing 4% in MRC5; (D) RBBP6-siRNA showing 2.7% apoptosis.

3.7 Cell viability and cytotoxicity

Cytotoxicity is a prime problem with chemicals used to induce apoptosis and needs to be studied carefully for its successful clinical application. We evaluated cell cytotoxicity of camptothecin and staurosporine on different concentrations by both MTT and DHL cell cytotoxicity assays. In camptothecin, 0.8 µM displayed 72.6 % cell viability compared to 81.18% in 0.5 µM. In 1.6 µM and 2 µM there was excessive cell cytotoxicity with cell viability at 19.6% and 3 % respectively (**figure 3.39**). In staurosporine there was 72.3 % cell viability at 25nM and excessive cell cytotoxicity at 26.7% and 4 % at 35nM and 50 nM concentration respectively (**figure 3.40**). It is to note that cell viability was above 70% in 0.8 µM and 25nM concentrations of camptothecin and staurosporine respectively which corresponded with about less than 30% of cell cytotoxicity. In contrast, concentrations of above 1.6 µM showed 70% cell cytotoxicity.

Figures 3.39 & 3.40: Shows the Cell viability and cytotoxicity

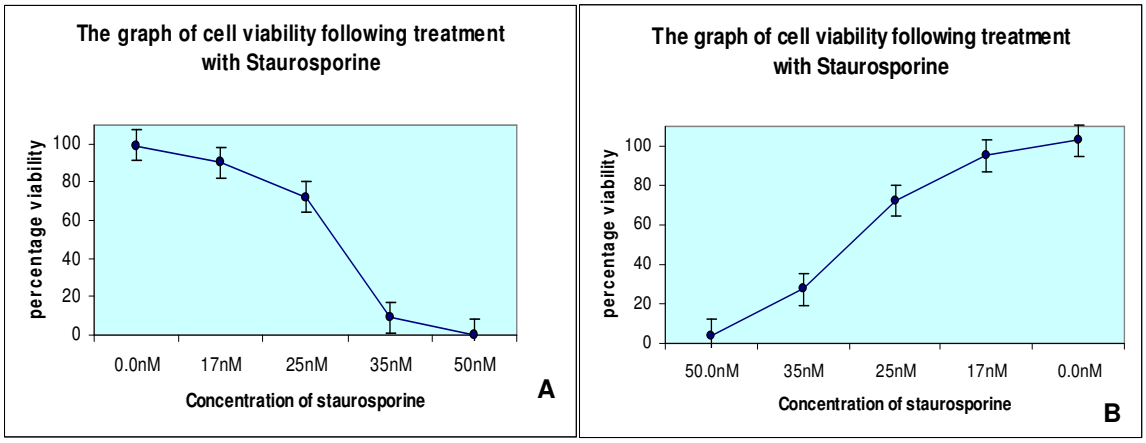


Figure 3.39: Illustration of percentage cell viability following treatment of A549 cell (A) and MRC5 (B) cell lines with different concentration of staurosporine. Note 72.3 % cell viability at 25nM.

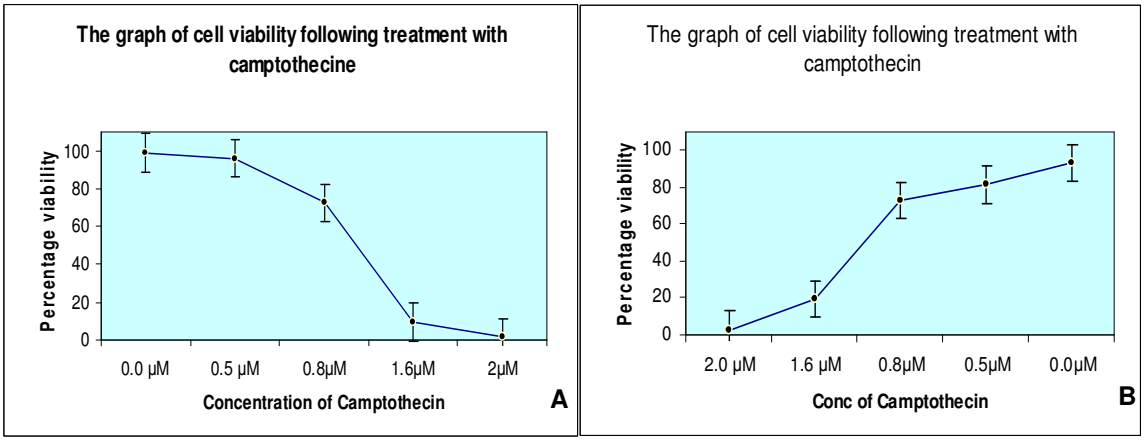


Figure 3.40: Illustration of percentage cell viability following treatment of A549 cell (A) and MRC5 (B) cell lines with different concentration of camptothecin. Note 72.3 % cell viability at 0.8µM camptothecin.

3.7.1 Concentration optimization for p53 and RBBP6 siRNA

As siRNA concentration is a defining parameter in establishing silencing efficiency, different concentrations of p53 (0.01–1.5 µg), RBBP6 (0.01–1.5 µg) were screened for the maximal p53 and RBBP6 knock-down. In order to choose an effective knock-down concentration that is not toxic to the cells, we performed cell viability test to determine optimal concentration of sip53 and siRBBP6 using both A549 cells and MRC5 cell lines.

In case of sip53 and siRBBP6 treatments, post-normalization, 15% reduction in A549 and MRC 5 cell survival was observed at 0.5 µg sip53 and 1.0 µg siRBBP6 concentrations, respectively. Further decrease in sip53 or siRBBP6 concentration to below 0.5µM did not have any effect on the two genes eventhough cell survival was high and concentration above 1.5 µg displayed significant increase in toxicities as indicated by the bar graph (**figure 3.41 & 3.42**). The efficiency differences between sip53 and siRBBP6 were noticeable at 0.5 and 1.0 µg respectively. From these results we infer that 0.5 µg of sip53 and 1.0 µg siRBBP6 are optimal concentrations for silencing the respective genes in both A549 and MRC5 cell lines.

Figures 3.41 & 3.42: Showing cell viability following transfections.

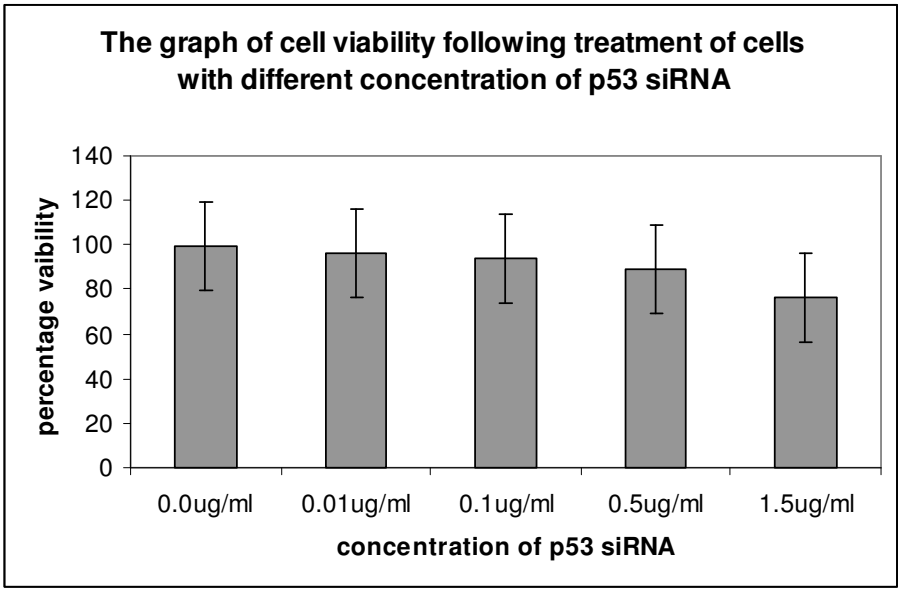


Figure 3.41: Cell viability assay for optimization of sip53 concentration by cell toxicity of different concentrations on A549 and MRC5 cell lines

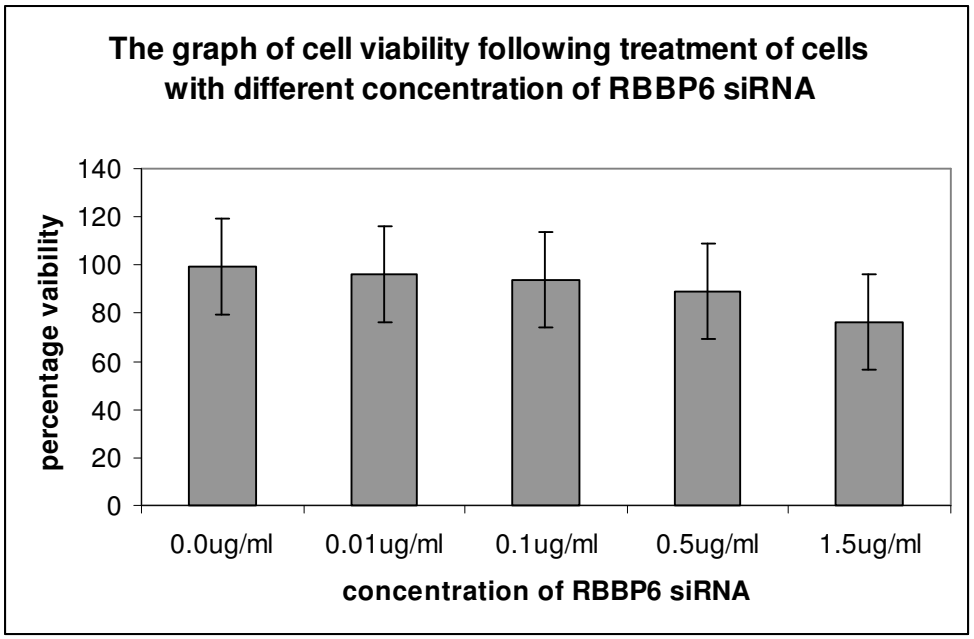


Figure 3.42: Cell viability assay for optimization of siRBBP6 concentration by cell toxicity of different concentrations on A549 and MRC5 cell lines.

3.8 Quantitative Real-Time PCR analysis of the RBBP6, p53-Binding Domain, p53, Bax and BCl_2.

In order to analyse the expressional levels and relationship between RBBP6, p53-Binding Domain, p53, Bax and BCl_2 in Normal Lung Tissues, Lung cancer Tissue sections, MRC5 fibroblast and A549 Cell lines following gene silencing and apoptosis induction, quantitative Real Time PCR which is the current method of choice was employed to answer the following questions: (i) Is my gene of interest present? (ii) Does the level of my gene of interest change due to an experimental condition? (iii) To what degree does the treatment change the level of expression of my gene of interest? Standard curves, melting curves and quantitation curves were used to analyse the results using the Roche 1.5 light cycler.

3.8.1 Assessment of assay efficiency and optimisation using standard curves

In an attempt to answer these questions, qRT-PCR specific primers were designed as in chapter 2 using the quantitative Real Time PCR probe design software from BioRad and Quagen biotech and both the RNA and cDNA were quantified using the nano drop (appendix 4 table 3.2). In order to assess the efficiency of the assay, relative standard curves for the genes under investigation was prepared with 10 fold serial dilutions (undiluted, 1:10, 1:100, 1:1000, and 1:10000) of the cDNA in **figures 3.43-3.48**. Amplification efficiency was calculated using the slope obtained from the standard curve using the following formula:

$$E \text{ (Efficiency)} = 10^{-1/\text{slope}}$$

The mRNA expressional levels were normalized to the levels of GADPH transcript, which served as our housekeeping genes.

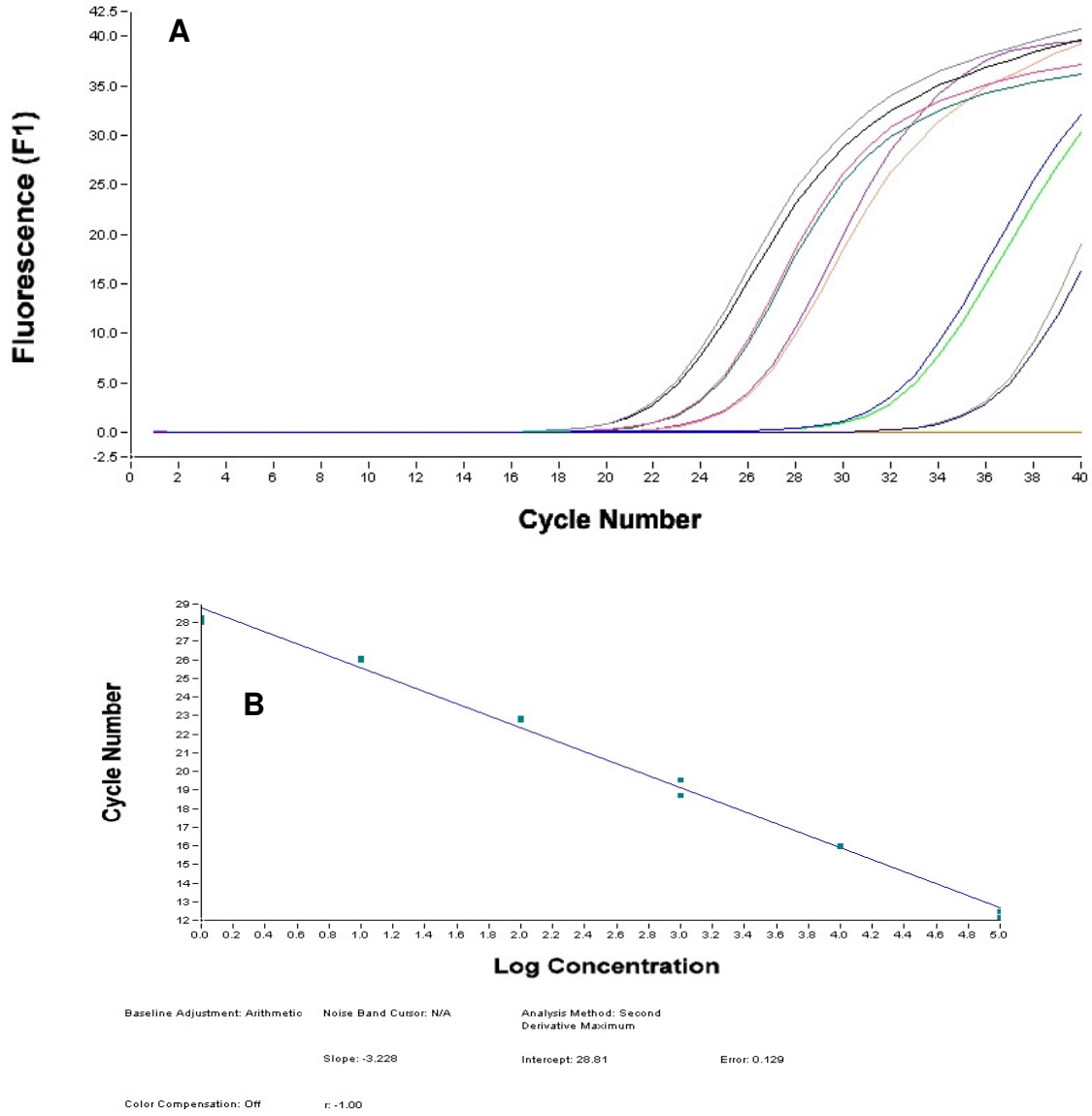


Figure 3.43: GADPH standard curve to access the efficiency of the primers.

Amplification curves (**A**) for GADPH gene using SYBR Green I dye illustrate the fold dilutions series. Standard curve C_T values obtained from the amplification curve plotted against the Log concentration of the template starting quantity for each dilution. (**B**)The slope of the curve was found to be -3.229 and the efficiency was 100%.

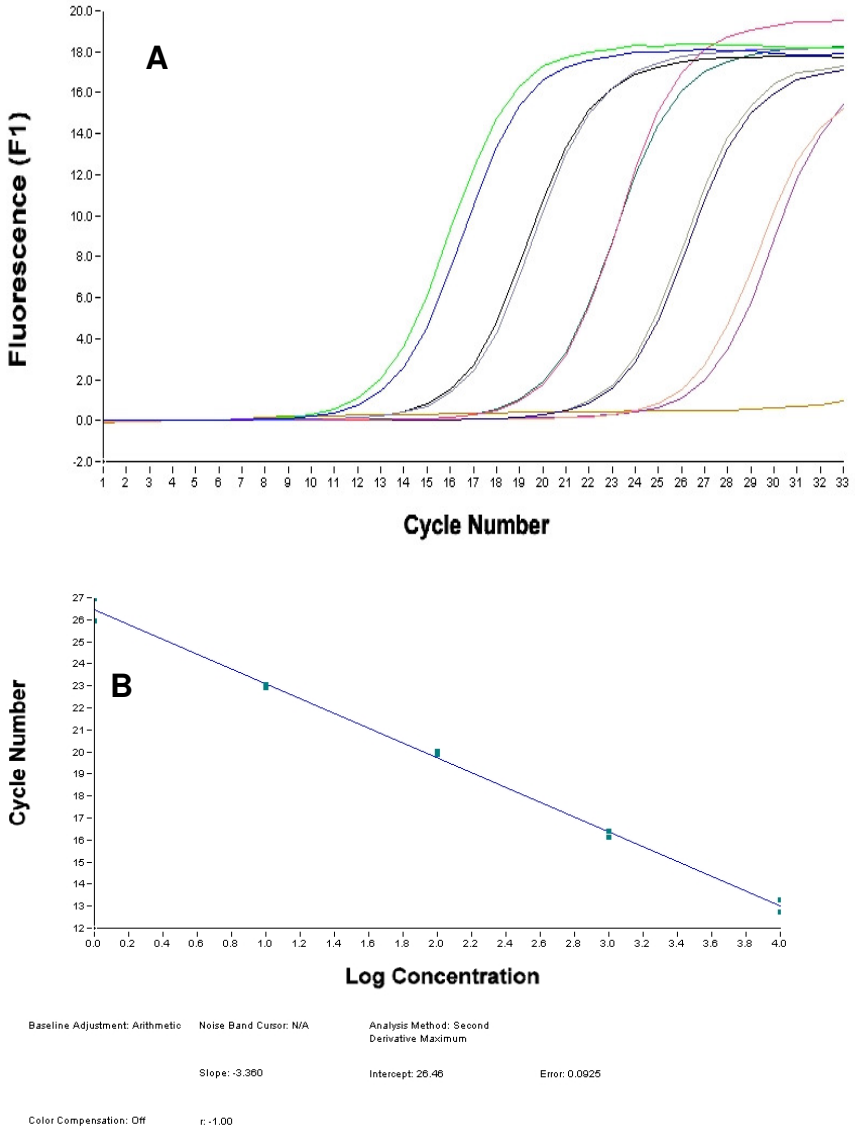


Figure 3.44: RBBP6 standard curve to access the efficiency of the primers.

Amplification curves (**A**) for RBBP6 gene using SYBR Green I dye illustrate equidistance between the fold dilutions. Standard curve (C_T) points are based on serially diluted cDNAs of a mixture of MRC5 cell lines. All samples were performed in duplicates. (**B**) The slope of the curve was found to be -3.3 and the efficiency was 100%.

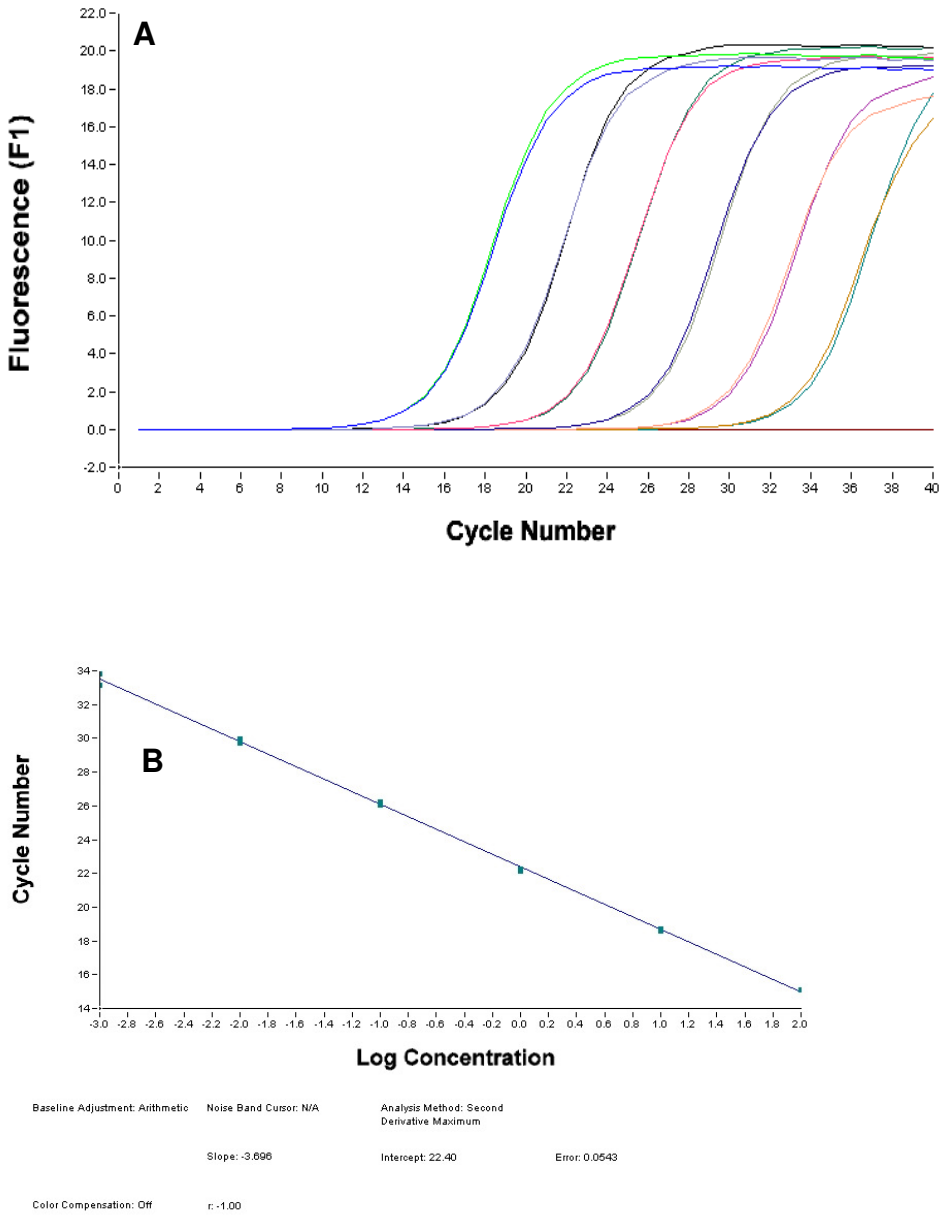


Figure 3.45: Bax standard curve to access the efficiency of the primers.

Amplification curves (**A**) for Bax gene using SYBR Green I dye illustrate equidistance between the fold dilutions series. (**B**) The slope of the curve was found to be -3.69 and the efficiency was 100%.

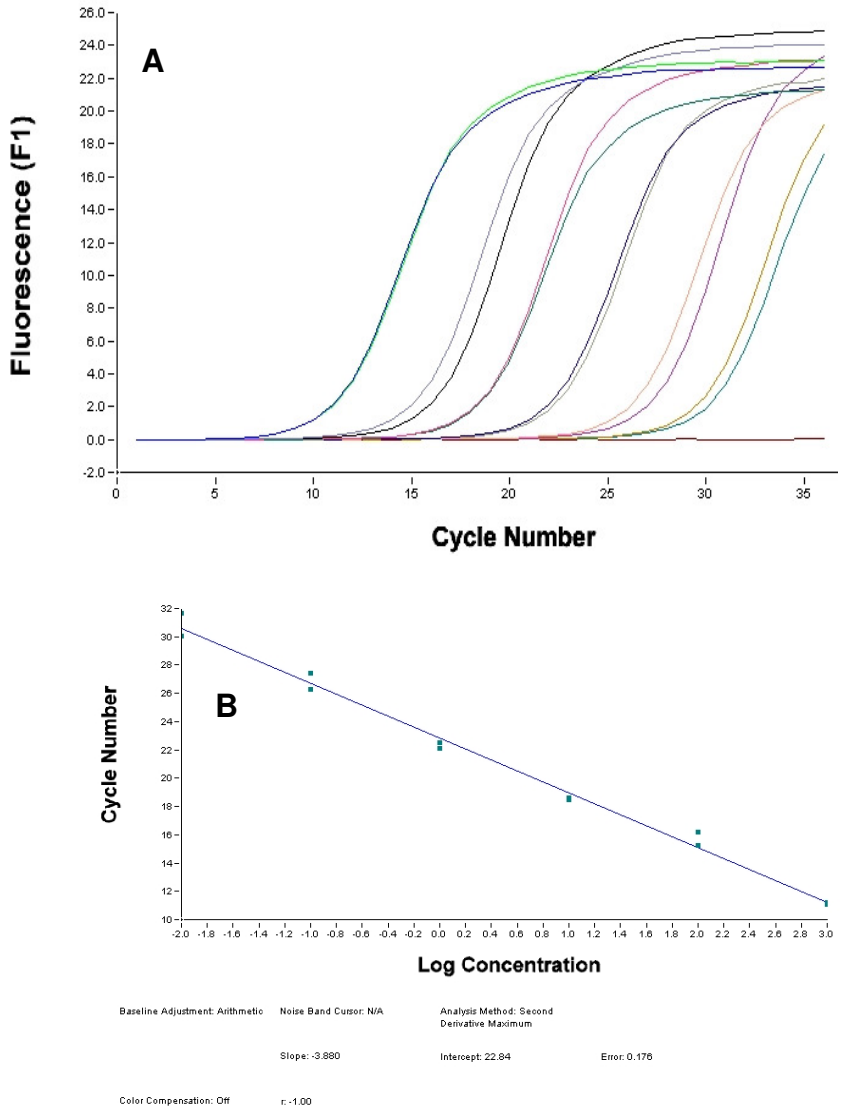


Figure 3.46: Bcl_2 standard curve to access the efficiency of the primers.

Amplification curves (**A**) for BCl_2 gene using SYBR Green I dye illustrate equidistance between the fold dilutions series. (**B**) The slope of the curve was found to be -3.690 and the efficiency was 100%.

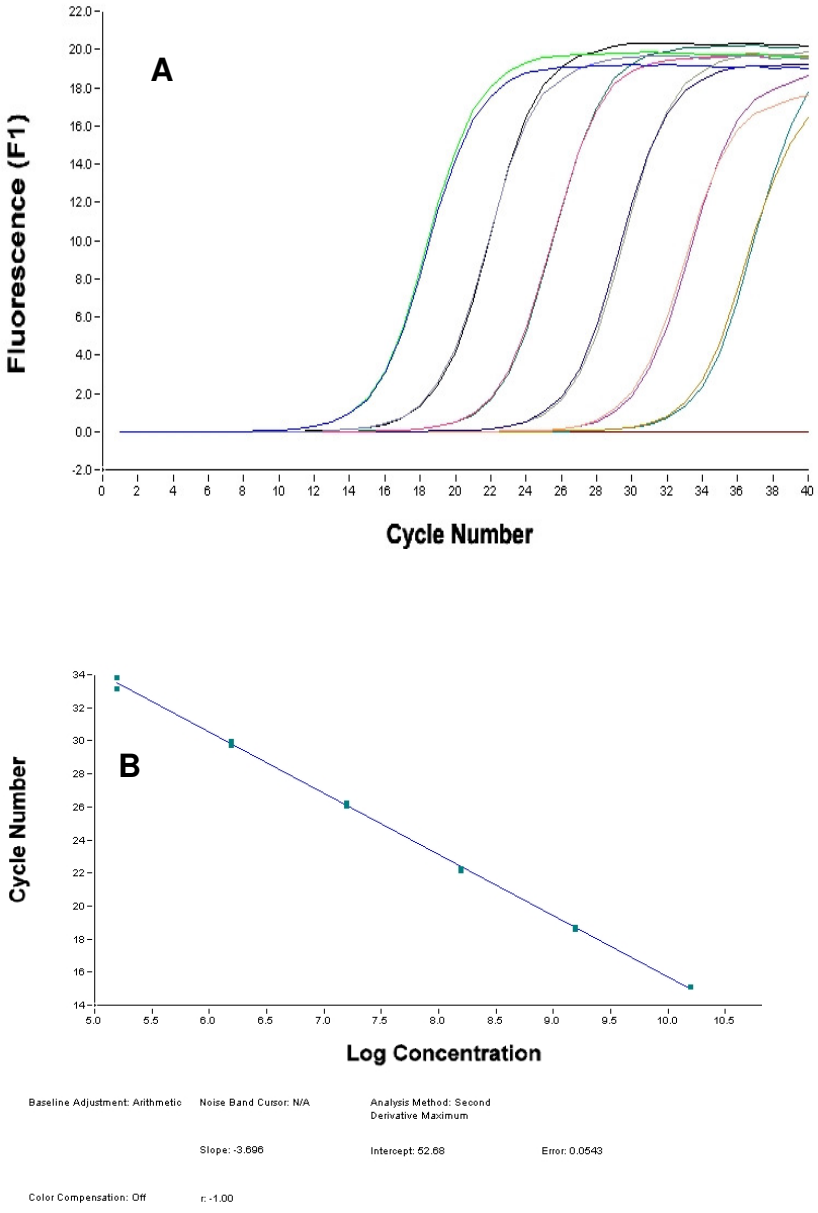


Figure 3.47: p53BD standard curve to access the efficiency of the primers.

Amplification curves (**A**) for p53BD gene using SYBR Green I dye illustrate equidistance between the fold dilutions series. (**B**) The slope of the curve was found to be -3.690 and the efficiency was 100%.

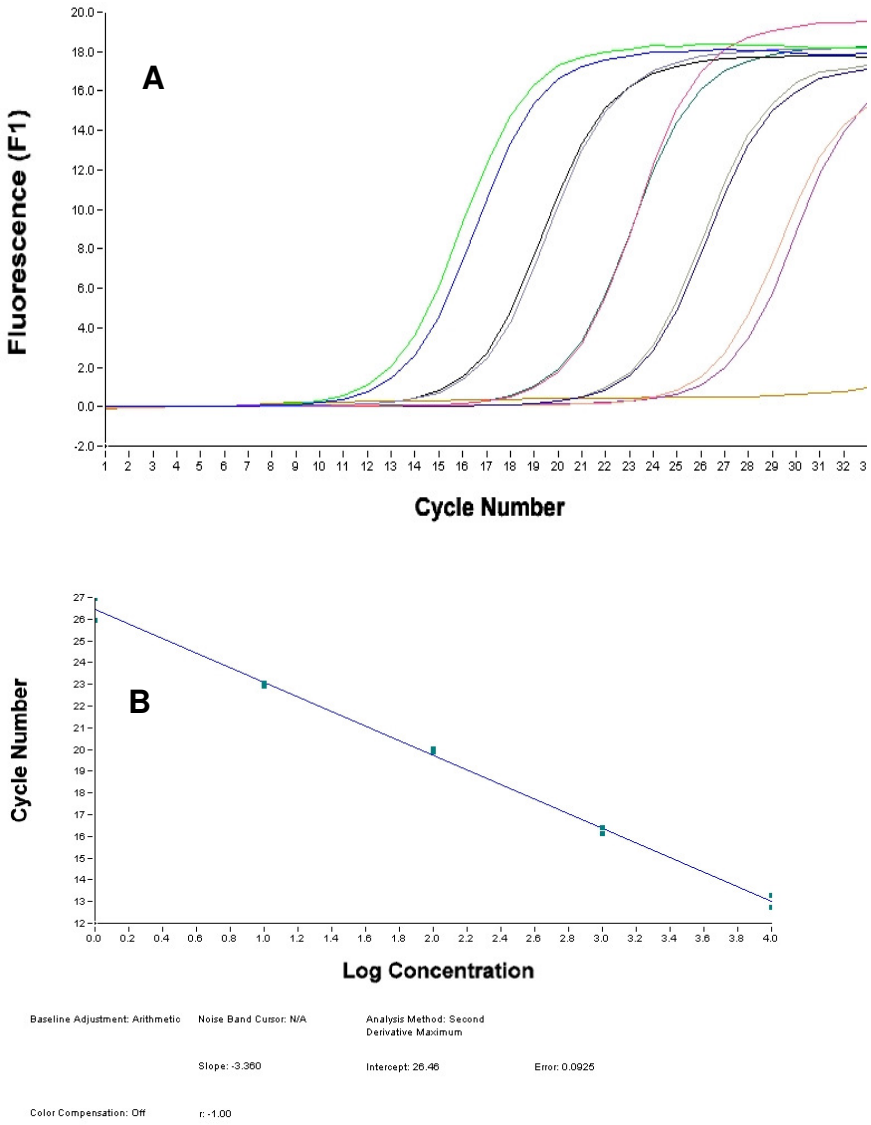


Figure 3.48: p53 standard curve to access the efficiency of the primers.

p53 standard curve by qRT-PCR. Standard curve plotting showing CO (concentration) in log scale *versus* Ct (Cycle threshold). Amplification curves (**A**) for GADPH gene using SYBR Green I dye illustrate equidistance between the fold dilutions series. (**B**) The slope of the curve was found to be -3.360 and the efficiency was 100%.

3.8.2 Amplification and Melting curves

Melting curves was also assessed for non-specificity of the primers or presence of primer dimmers. The quantified product was allowed to melt at a constant increase in temperature of 1°C and allowed to hold for 30 seconds from 55 °C to 99°C. As shown in the diagrams below the presence of a single pick on each graph signified the presence of a single identical product (**figures 3.49- 3.53**). Since elimination of primer dimers is very crucial for the accuracy of real time PCR using SYBR Green dye, melting curves presents a very useful tool for gene expression analysis.

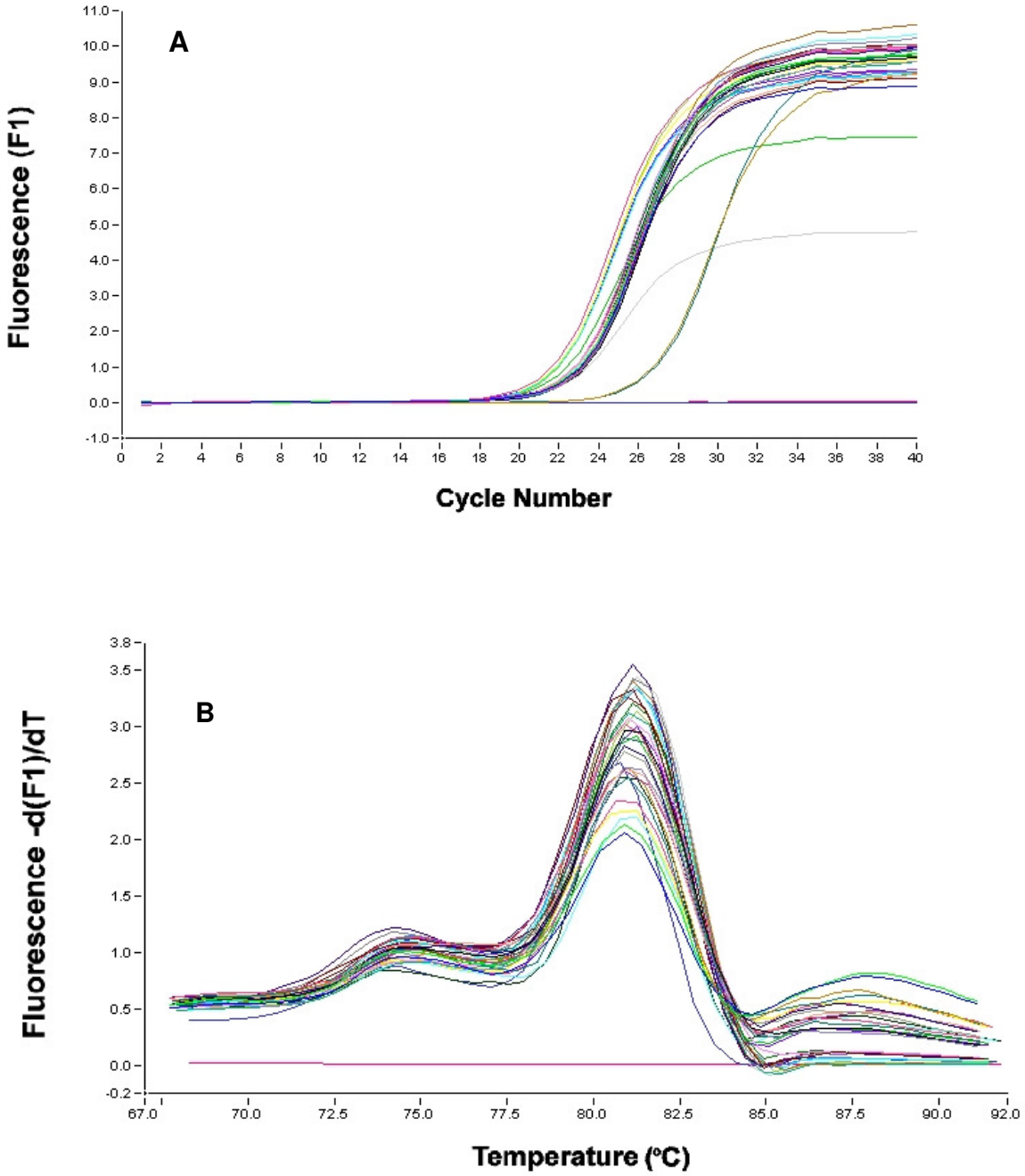


Figure 3.49: RBBP6 Quantification curves; The above figures illustrate the expression level of RBBP6 in (**A**) lung Tumour, normal lung and several treatment of A549, MRC5 and Lsq cell lines with different apoptosis inducers and gene silencing which gene is silenced?. (**B**) Melting RBBP6 product at ~ 81 °C.

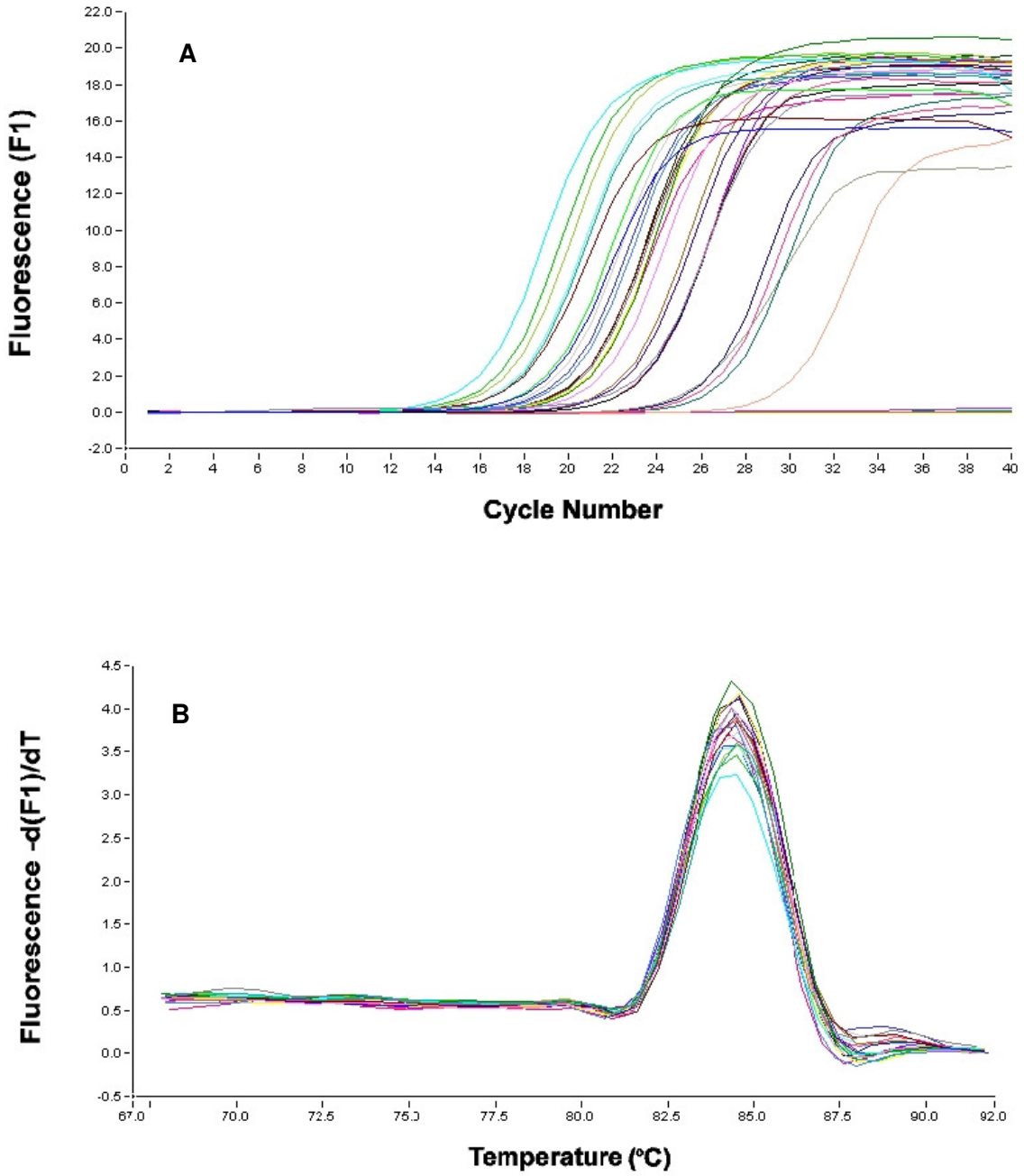


Figure 3.50: GADPH Quantification curves; The above figures illustrate the expression level of GADPH in (A) lung Tumour, normal lung and several treatment of A549, MRC5 and Lsq cell lines with different apoptosis inducers and gene silencing which gene is silenced?. (**B**) Melting RBBP6 product at ~ 84 °C.

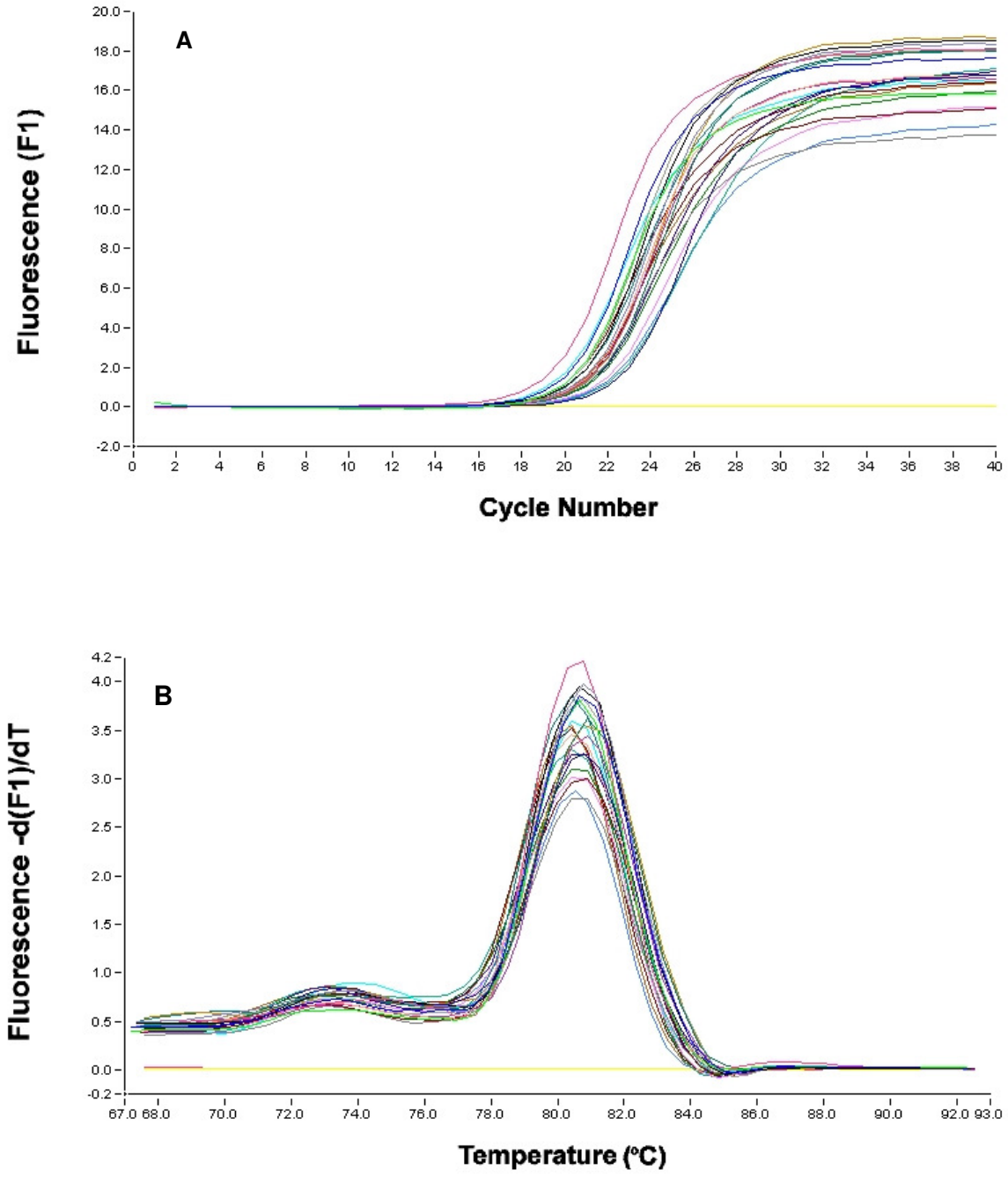


Figure 3.51: p53 Quantification curves; the above figures illustrate the expression level of p53 in (A) lung Tumour, normal lung and several treatment of A549, MRC5 and Lsq cell lines with different apoptosis inducers and gene silencing which gene is silenced?. (B) Melting RBBP6 product at ~ 80.5 °C.

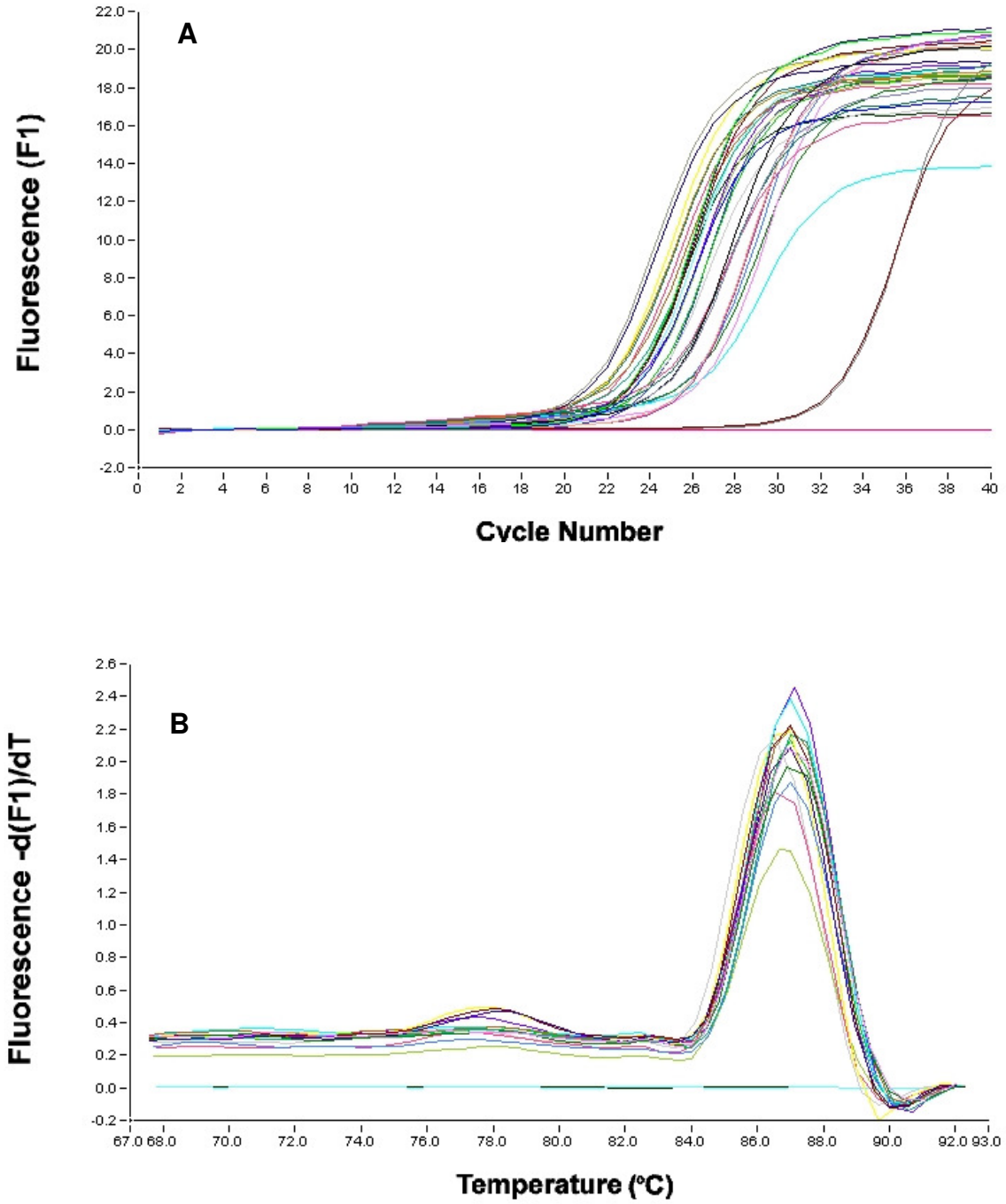


Figure 3.52: Bax Quantification curves; The above figures illustrate the expression level of Bax in (**A**) lung Tumour, normal lung and several treatment of A549, MRC5 and Lsq cell lines with different apoptosis inducers and gene silencing which gene is silenced?. (**B**) Melting RBBP6 product at ~ 87 °C.

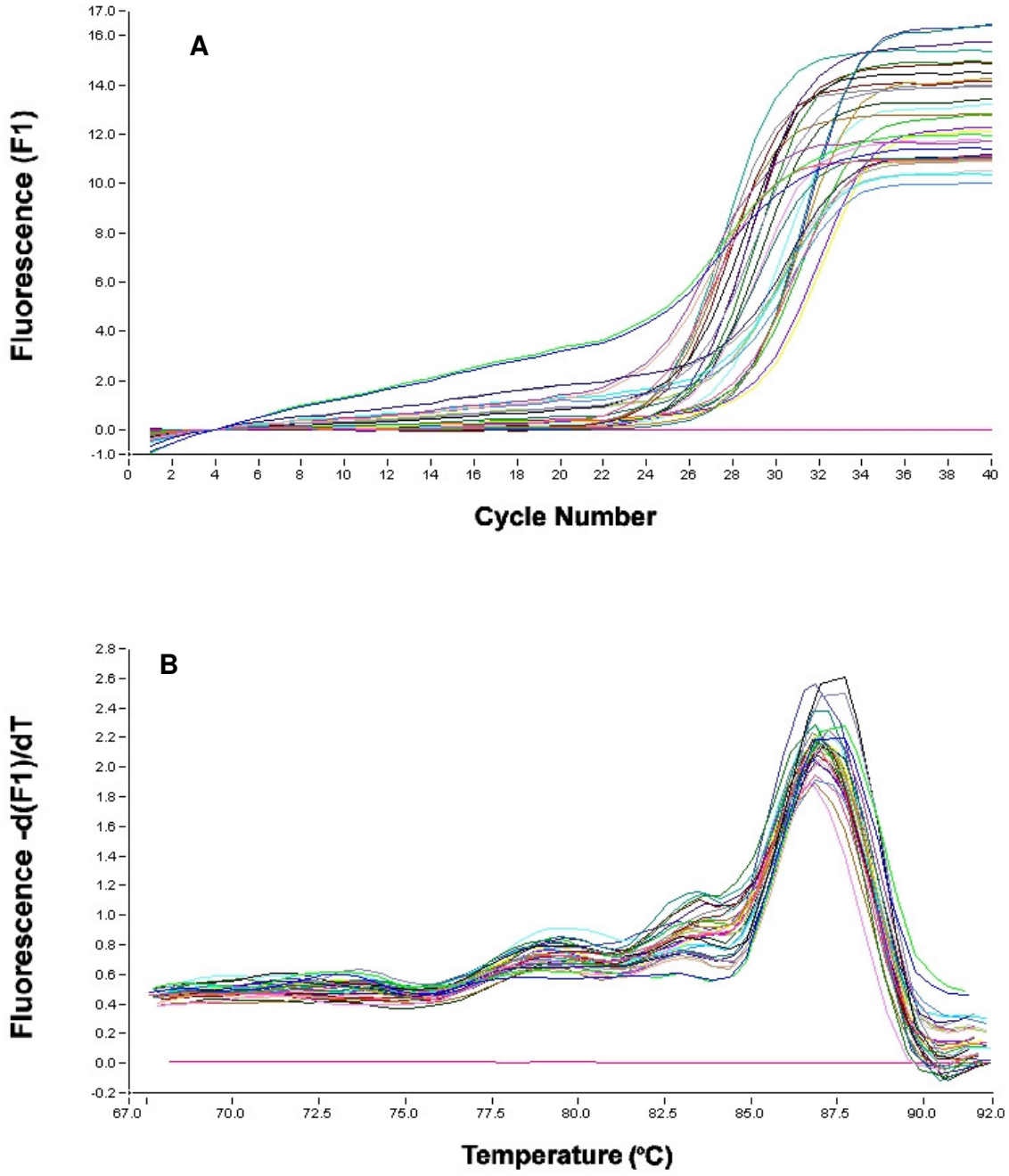


Figure 3.53: BCl_2 Quantification curves; The above figures illustrate the expression level of BCl_2 in (**A**) lung Tumour, normal lung and several treatment of A549, MRC5 and Lsq cell lines with different apoptosis inducers and gene silencing which gene is silenced?. (**B**) Melting RBBP6 product at ~ 87.5 °C.

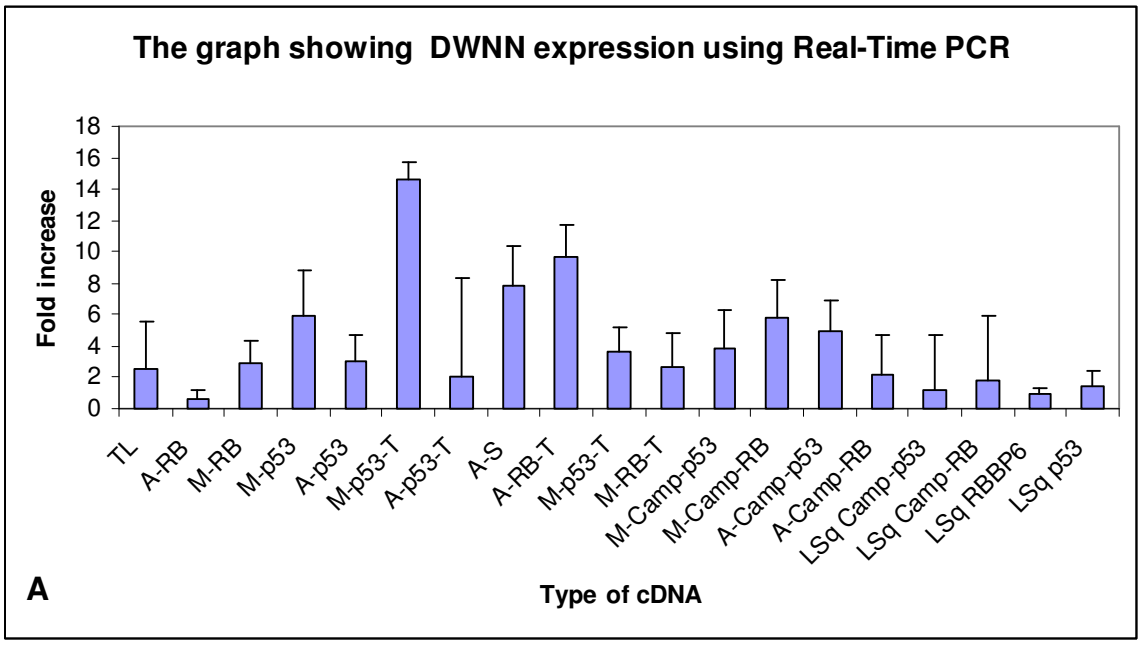
3.8.3. Relative Quantitative Real Time Polymerase reaction

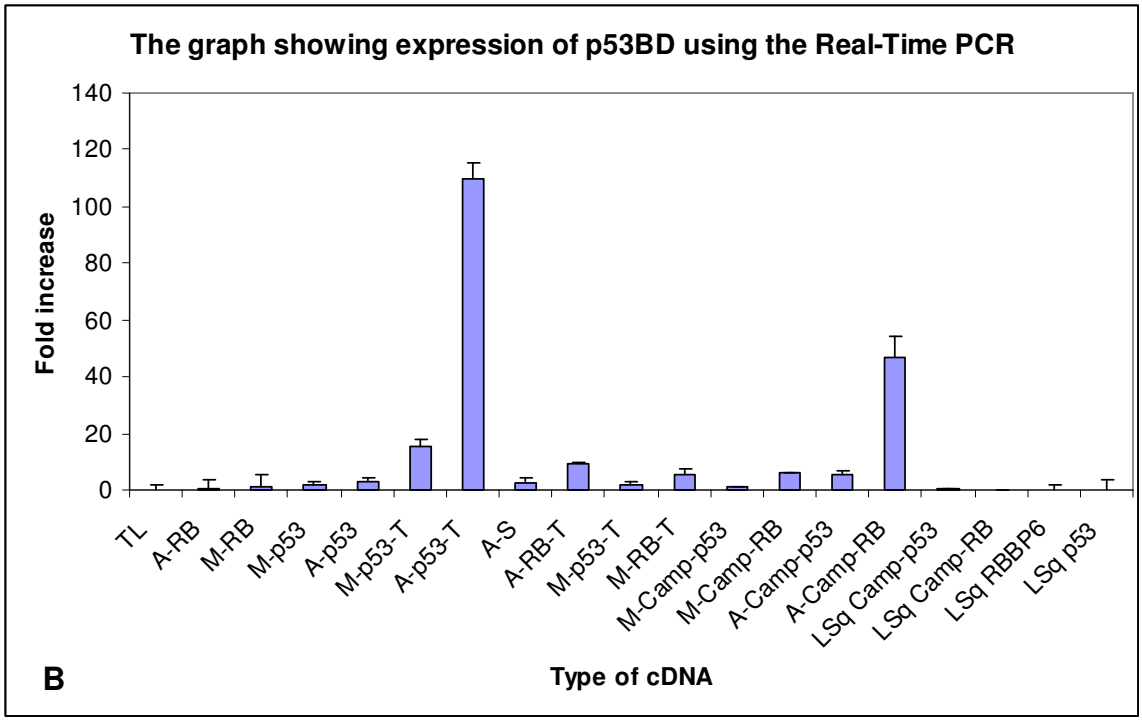
quantitative RT-PCR revealed that the levels of the transcript of RBBP6 and p53BD following normalization with GADPH in both lung tumour and normal lung tissues have expressed various levels of the genes.RBBP6 in tumour tissue have shown a 2.5 fold increase while that of p53BD was 0.23 fold decrease (**appendix 4 and figure 3.54**). However, following treatment of cells with apoptosis inducers and gene silencing, the levels of RBBP6 was significantly reduced in A549 and Lsq cell lines and was significantly increased up to 14.6 fold in cells treated with apoptosis inducers such as Staurosporine (**appendix 4 and table 3.7**). The ratio of p53/RBBP6 indicated decreased levels of expression from 2.86 in normal lung to 0.63 in lung tumour that was associated with apoptosis increase from 0.23 to 1.5 apoptosis in lung tumours. Our studies have further shown that (*i*) the level of p53 mRNA is slightly identical in A549, MRC5 and Lsq following transfection with RBBP6 siRNA and p53 siRNA, (*ii*) the level of RBBP6 and p53BD is slightly decreased following treatment of A549, MRC5 and Lsq cell lines with p53 siRNA (**appendix 4 tables 3.7-3.9**). The results also have shown that silencing of RBBP6 did not have much effect on p53 in all lung cancers. It also further showed that induction of apoptosis with any of the inducers did not have any influence on the levels of p53 (**Appendix 4 table 3.6**).

In terms of the relationship between RBBP6 and apoptosis, there results as shown in figure 3.53A and B, when apoptosis was induced with camptothecin the value of RBBP6 was increased from 1 to about 7 fold in almost all cell line including the MRC5. When treated with Trail the level of RBBP6 increased to about 14 times as compared to the cells that were not treated with anything. As for p53BD level seemed to be at lower levels in all cases except in A549 cell line that were silenced with p53 and then treated with both Trail and camptothecin that showed over 100 x and upto 42 x increase respectively. The fragments of

qRT-PCR are represented in figure 3.54 with different intensity that correlated with the concentration of the product.

Using qRT-PCR, the ratio of Bax/BCl_2 was determined and ranged from 0.033 in lung tumour to 4.28 in cell treated with Trail (**table 3.10**). Bax/Bcl2 ratio regulate whether the cell survive or undergo apoptosis, in cells treated with Trail the ratio of Bax was at 4.28 which correlated with apoptosis that was at 54%. The ratio of Bax/Bcl_2 was increased in all cell types when RBBP6 was silenced but reduced when p53 was silenced (table 3.10).





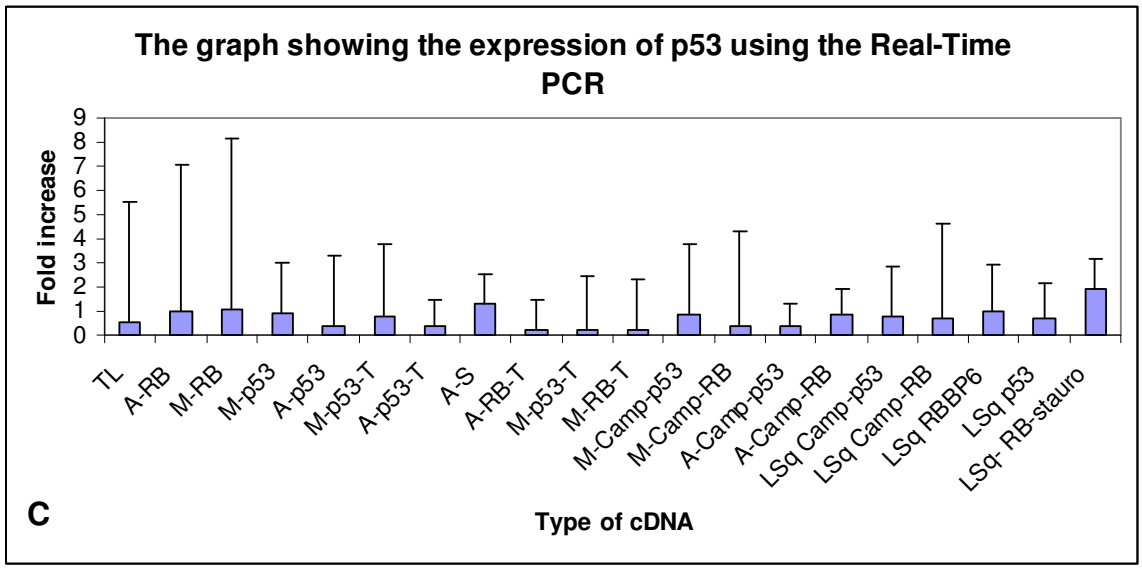


Figure 3.54: Correlation between mRNA expression level of RBBP6, p53BD and p53 in relation to different lung cancer cell lines, Tumour tissues and normal lung tissue. Both RBBP6 (A) and p53 (C) show a wide range of mRNA expression in tumour tissues and cell lines with significant increase of RBBP6 in tumour tissue and cell lines treated with TRAIL and p53 siRNA. There was no significant correlation between RBBP6 silencing and p53

levels in all cell line. (B) Indicate the mRNA expression level in the same cell lines that show significant increase in mRNA in cells treated with TRAIL and p53 siRNA.

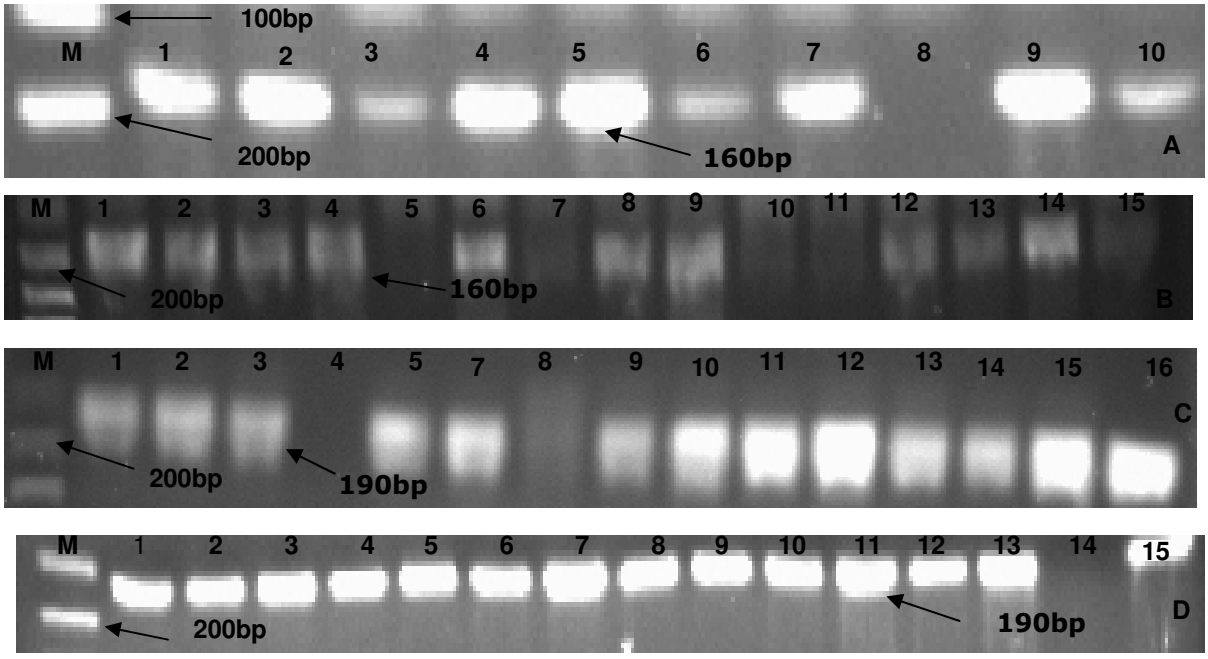


Figure 3.55 Results of the qRT-PCR for RBBP6, p53BD, p53 and GAPDH with RNA from different types of cell lines treated with the following TRAIL, camptothecin, Staurosporine and (p53 and RBBP6 siRNA). (A) Gel electrophoresis of p53BD showing a fragment of about 160 bp. **Note lanes 3, 6 and 10** representing silenced p53BD lanes 6 treated with both RBBP6 siRNA and Staurosporine and lane 10 RBBP6 siRNA and camptothecin. (B) Electrophoresis of p53 showing a fragment of 160bp. **Note lanes 5, 7 10 and 11**, representing silenced p53 treated with sip53, staurosporine, camptothecin and TRAIL respectively. (C) Gel electrophoresis of RBBP6 showing 190bp. Note lanes 4, 8 and 14, representing silenced RBBP6 treated with staurosporine (4), TRAIL (8) and camptothecin (14). (D) Showing electrophoresis of GADPH showing a fragment of 190bp note less intensity shown by lane 14 which show s reduced GADPH mRNA following GADPH silencing.

Chapter Four

Discussion

4.1 Introduction

Retinoblastoma binding protein 6 (RBBP6) also known as p53-associated cellular protein testes derived (PACT), proliferation potential-related protein (P2P-R or PP-RP) and retinoblastoma binding Q protein 1 (RBQ-1) is a 250-kDa multidomain protein that has been reported to interact with both p53 and pRb, and has been implicated in tumouregenesis (Chibi *et al* 2008; Scott *et al* 2003, Gao *et al* 2002). While a number of studies suggest that it plays a role in regulation of the cell cycle, enhanced expression levels correlated with poor clinical progression in oesophageal cancer (Scott *et al* 2003, Gao *et al* 2002). RBBP6 binds to wild-type p53 but not to DNA binding-deficient mutants and interferes with the binding of p53 to DNA. RBBP6 also plays a role in facilitating the interaction between p53 and its primary regulator mdm2/Hdm2, leading to ubiquitination of p53 and its degradation. Although RBBP6 contains its own RING finger domain, it has been reported that RBBP6 does not itself ubiquitinate p53 but plays the role of a scaffold on which the interaction between p53 and Hdm2 takes place (Li *et al* 2007)

4.2 RBBP6 mRNA localization and expression in lung cancer

RBBP6 is considered to act as an E3 ubiquitin ligase due to the presence of the RING finger domain. However it is tempting to suggest that RBBP6 is involved in mRNA processing which is required for protein synthesis in the cytoplasm (Vo *et al* 2001). The fact that RBBP6 is known to be localized in the cytoplasm of the kidney cells opens up the possibility that it

may be involved in cell homeostasis (Ledwaba M.Sc Thesis, 2005; Mbita M.Sc thesis, 2004). In this study expression of RBBP6 *mRNA* was examined in human lung cancer tissues and normal lung tissues, the results have shown markedly up-regulated RBBP6 in well-differentiated lung adenocarcinoma tissues and squamous cell carcinoma, but down to nil expression in moderately and poorly differentiated subtypes of these lung cancers respectively. More positive results were also recorded in well differentiated regions of BAC but less in their poorly-differentiated regions. However, in contrast there was down-regulation of RBBP6 mRNA in both small cell and large cell carcinomas (**Fig 3.23**). These findings suggest a potential role of the overexpression of RBBP6 in lung adenocarcinoma, squamous cell carcinoma and bronchial adenocarcinoma carcinoma progression, although a larger number of cases need to be studied to confirm the association between expression of RBBP6 mRNA and tumour differentiation and progression. RBBP6 is also thought to play a crucial role during cell proliferation and these results are not surprising as indicated in **figure 3.22** whereby cell that looked to be undergoing mitosis seemed to overexpress RBBP6 especially around the dividing cells. These results further support the results of Chibi *et al* who reported increased RBBP6 mRNA in H1299 cell line.

Human oncogenesis involves multistep genetic alterations, including activation of dominant oncogenes and inactivation of tumor-suppressor genes Homer *et al* 2005; Lasham *et al* 2003; Roth *et al*., 1998). However, the question as to which genetic changes are correlated with the morphologic changes associated with tumour progression remains poorly understood. In lung tumorigenesis, inactivation or mutation of the p53 gene is considered an initiation step, whereas activation of Bcl_2 genes is considered to be involved in the process of tumour progression (Gewirtz *et al.,*1998; Baker *et al.,* 1997; Stein *et al.,* 1988). It has been previously reported that up-regulation of RBBP6 mRNA is closely correlated with tumour

progression in cervical cancer and HIV associated nephropathy (HIVAN), suggesting that RBBP6 gene plays a crucial role in the expression of malignant phenotypes in human cancer (Mbita MSc 2004; ledwaba MCS 2006). In this study we further show that there was increased expression of RBBP6 mRNA in lung cancer tissue section which is an indicator of RBBP6 protein synthesis. Taking into consideration little clarity into the mechanism of p53 inactivation, RBBP6 which is suggested to play a key regulatory role through Mdm2 might be responsible for the inactivation of p53 (Bianco *et al*., 2005; Zhang *et al*., 2004; Vassilev *et al*., 2004; Chene *et al*., 2000).

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The gene whose deletion is associated with a poor degree of differentiation of lung adenocarcinoma has not been determined. Few genes have been associated with differentiation of human cancers (Momand *et al*., 1992; Thut *et al*., 1997). The up-regulation of RBBP6 mRNA in well- differentiated lung adenocarcinoma tissue in the present study is similar to the up-regulation of RBBP6 mRNA in other cancers (Yang *et al*., 2005; Deb *et al* 2003; GAO e*t al* 2003). p53 is mostly singled out as a major factor during lung cancer development and the simplest explanation for this increased expression of RBBP6 mRNA in well-differentiated lung adenocarcinoma is that RBBP6 might be recruited to the tumour site to regulate the levels of p53 thereby end up leading to inactivation of p53.

4.3 RBBP6 protein expression in lung cancer

It is generally accepted that the main physiological mechanism by which cancer cells die is by apoptosis, yet the precise mechanisms behind the induction of apoptosis has still have to be identified (Bianco *et al*., 2005; Zhang *et al*., 2004; Vassilev *et al*., 2004; Chene *et al*., 2000). Changes in the degree of apoptosis as lung cancer progresses have been reviewed recently (Motadi *et al* 2008; Zhang *et al*., 2004). Variations in extent of apoptosis-may are

explained by changes in apoptosis regulating p53 pathways. In this regard, the expression of RBBP6 which is said to regulate p53-dependent pathway through mdm2 might be of relevance to study its role during lung cancer development.

In order to gain insight into the potential use of RBBP6 as a therapeutic tool in lung cancer, the expression of RBBP6 was examined in subtypes of human lung (adenocarcinomas, squamous cell carcinoma, small cell and large cell carcinomas as well as normal lung tissue). The expression and localisation of RBBP6 were investigated by immunohistochemistry in normal lung (n = 12), adenocarcinoma (n = 50), squamous cell carcinoma (n= 40), small cell carcinoma (n=18) and large cell carcinoma (n = 8). In addition, correlations between expression of RBBP6, p53 and the degree of apoptosis (assessed by TUNEL) and histopathological characteristics were explored. The results are described in Chapter 3 has revealed expression of RBBP6 in all cases studied but varied normal lung to lung adenocarcinoma. Expression of RBBP6 was demonstrated in squamous and adeno-carcinomas but by comparison less expression was found in the poorly-differentiated tumours (**figure 3.27 & fig 3.29**). RBBP6 expression was less in both small cell carcinoma and large cell carcinomas when compared to normal lung cancer (**figure 3.31**). Higher expression occurred more frequently in squamous cell carcinoma when compared to adenocarcinoma. Intensity of RBBP6 and localization of the staining was stronger in the cytoplasm of tumour cells compared to adjacent normal cells, and was accompanied by a higher degree of apoptosis in those cells.

There was no statistical significance for the correlation of the expression between p53 and RBBP6 in squamous cell carcinoma and adenocarcinoma. The stronger expression of RBBP6 in the cytoplasm of neoplastic cells as opposed to normal cells holds promise for the

future use of RBBP6 as cancer therapeutic agent. (**figure 3.28**). The interesting observation that immune cells which were attracted towards a tumour also stained positively for RBBP6 (**figure 3.30**). In view of these results, we investigated further the level of expression of RBBP6 protein in A549 (human lung adenocarcinoma epithelial cells) and MRC5 (foetal lung fibroblasts) cell lines using the western blot. No significant difference was found in RBBP6 expression between A549 and MRC5 cells.

4.4 Correlation between apoptosis and RBBP6 expression.

Several studies have investigated the mechanisms underlying tumour development, progression and drug resistance in cancer, and current information suggests that in the majority of cells, defects in apoptosis signaling are the most critical determinants (Fisher, 1994; Thompson, 1995). Some of the molecular and genetic abnormalities that antagonize apoptosis in lung-cancer cells have been unveiled, providing information for more specifically targeting these lesions.

The p53 can induce apoptosis after interacting with other pro-apoptosis genes like bax, and loss of function of p53 may result in increased progression of tumour cells. As it is reported RBBP6 together with its two domains i.e. p53 binding domain and RING finger domain may play a role in preventing p53 from binding other pro-apoptosis genes thereby resulting in defective apoptosis of tumour cells. Therefore, RBBP6 could be considered as a biomarker for cells that are predisposed to apoptosis. The purpose of these experiments was to clarify whether the apoptotic changes that had occurred could be related to the progressive tumourigenesis of lung cancer. We found a higher degree of apoptosis in the malignant cells

areas of adenocarcinoma and squamous cell carcinoma when compared to the adjacent normal tissue (**figure 3.34**). Our results with TUNEL in both adenocarcinoma and squamous cell carcinomas concur with other studies using this method to assess apoptotic cell death (Ikenaga *et al* 1998; Koike, M., 1996). In line with TUNEL results, we found increased RBBP6 expression in 40 % of adenocarcinomas and 85% of squamous cell carcinomas, while staining was intact in adjacent normal cells. The degree of apoptosis did not differ between RBBP6 positive and RBBP6 negative. Expression patterns of RBBP6 have recently been reported in other tumour types and diseases. In cervical tumours, RBBP6 expression did correlate with the degree of apoptosis (Ledwaba, 2005 Thesis). In colon cancer tissues RBBP6 expression was also correlated with apoptosis (Rupnarain, 2005).

Concerning the degree of apoptosis in normal lung, squamous cell carcinoma and adenocarcinoma, we have found apoptotic indices varied between 0.2, 1.8 and 1.5 % respectively. When studying the transition from normal lung epithelial to well- differentiated carcinomas, most studies have indicated a shift towards higher apoptotic indices. However, there is contradiction about the transition from poorly to well-differentiated carcinoma. Most studies have reported that the frequency of apoptotic cell death in well-differentiated was higher than in poorly-differentiated (Hsieh *et al* 1999; Vaux, D.L. and A. Strasser, 1996). However in other studies no differences or even opposite results have been reported. The distribution of apoptotic cells during transition of the tumour from a poorly to well differentiated one has been described previously (Hsieh *et al* 1999; Vaux, D.L. and A. Strasser, 1996). This report concurs with our observation with regard to expression of RBBP6 at specific stages of cancer development (**figure 3.27 & 3.29**). Regarding the prognostic value of apoptotic indices it was found that most studies suggest that lung tumours

with high apoptotic indices were associated with better prognosis and survival (Compton *et al* 2000; Hsieh *et al* 1999; Liu *et al* 1999).

4.5 Targeting p53-dependent pathway by inducing apoptosis using camptothecin and staurosporine.

p53 is an attractive target for the development of therapeutic drugs in lung cancers. In tumours expressing mutant p53, small molecules that can rescue p53 function have been developed (Bassett, *et al.* 2008). In tumours with wild-type p53, inhibition of MDM2 has been proposed because it will lead to the induction of p53 activity. Small compounds that bind either to p53 or to MDM2/Mdm2 have been isolated and have been shown to induce a p53-dependent apoptosis in tumour cell lines (Selivanova, G. and Wiman, K.G. (2007), Bassett, *et al*. 2008). The downside of this is that targeted treatment of tumours initially expressing wild-type p53 might lead to resistance mechanisms owing to the selection of cells that express high levels of mutant p53 and are unregulated by MDM2 and secondly that exposure to this kind of treatment could be toxic to adjacent normal epithelial cells. Our aim here was first to identify the appropriate concentration that could induce apoptosis in cancerous as well as in normal epithelial cells, and then to silence RBBP6 and p53 prior to induction of apoptosis.

Following this approach, we have demonstrated that camptothecin-treatment in A549 cell lines induced apoptosis, in a manner that implicated mitochondria as the central integrating organelle in apoptotic cell death, which has the capacity to directly activate the programmer cell death execution pathways. In particular, the translocation of cytochrome *c* from the mitochondrial membrane into the cytoplasm is thought to be a critical step in the activation of caspases (Kluck *et al*, 1997). The cells that were treated with both RBBP6 siRNA and

camptothecin and those that were treated with camptothecin alone showed no increase in the number of cells undergoing either early or late apoptosis suggesting that activation of apoptosis pathway by camptothecin was not dependent on RBBP6 expression (**figure 3.36-38**). In contrast an increased number of apoptotic cells were seen in those cells that were treated with p53 siRNA and camptothecin, whereas the effect in MRC-5 fibroblast was minimal.

In the present study, we used both approaches of knockdown and apoptosis induction by staurosporine on A549 and MRC-5 cells to demonstrate that down-regulation of RBBP6 increases the susceptibility of the cells to staurosporine-induced apoptosis. Our findings demonstrated that RBBP6 plays a role in the negative regulation of cell apoptosis in A549 cells (**figure 3.36**). Our experiments demonstrated that up-regulation of RBBP6 promoted cell proliferation and survival. Similarly, it has been indicated that loss or down-regulation of MDM2 expression in cancer cells results in significantly reduced cancer tumourigenic and metastatic potential with increased apoptosis (Hsieh *et al* 1999).Importantly, evidence demonstrating the pro-apoptosis action of p53 was observed in p53 down-regulation experiment which showed more necrotic cells. It has been shown that loss of p53 in lung cancer is associated with cancer progression and development (Ryan *et al* 2001). TRAIL induced significant apoptosis in A549 cells treated with p53 which suggested that even if there was down-regulation of p53; apoptosis could still be induced through TRAIL-induced pathway (**figure 3.38**). This finding is consistent with the observation that a minority (27%) of tumours of hematological origin are sensitive to TRAIL-induced apoptosis (Snell *et al* 1997). This was reported for cancers such as breast cancer in MCF10A and MDA-MB-231 cells (Keane *et al* 1996). In down-regulated RBBP6 the trend was the same as that seen in down-regulated p53 cells sensitive to TRAIL-induced apoptosis. Does not seem to make sense.

Although RBBP6 has been suggested to be an E3 ubiquitin ligase due to the presence of the RING finger domain, it is tempting to assume that RBBP6 is involved in mRNA processing which is required for protein synthesis in the cytoplasm (Vo *et al* 2001). The fact that RBBP6 was reported to be localized in the cytoplasm in the kidney and cervical cancer opens the possibility that it may be involved in cell homeostasis (Ledwaba M.Sc Thesis, 2005; Mbita M.Sc thesis, 2004). In this study we examined the expression of RBBP6 *mRNA* in human lung cancer tissues and found that the RBBP6 gene was markedly up-regulated at the mRNA level (using *in situ* hybridization) in well-differentiated lung adenocarcinoma tissues and squamous cell carcinoma, but it was not what in most of poorly and moderately differentiated lung cancer tissues. The results in **figure 3.22** concurred with these results, because up-regulation of RBBP6 mRNA was observed in well differentiated regions of BAC but not in poorly-differentiated regions of a lung squamous carcinoma (**figure 3.21**). However, in contrast there was down-regulation of RBBP6 mRNA in both small cell and large cell carcinomas (**figure 3.23**). Although further studies with other cancers need to be investigated to prove an association between expression of RBBP6 mRNA and tumour progression and differentiation, these findings suggest the potential role of up-regulated RBBP6 gene in the progression of lung adenocarcinoma, squamous and BAC, although a larger number of cases need to be studied. Human oncogenesis involves multistep genetic alterations, including activation of dominant oncogenes and inactivation of tumour-suppressor genes (Kernek *et al*, 2004). However, which genetic changes are correlated with the morphologic changes associated with progression remains poorly understood. In lung tumourigenesis, inactivation or mutation of the p53 gene is considered a major initiator of tumour development, whereas activation of anti-apoptosis genes like Bcl_2 is involved in the process of tumour progression (Baker *et al.,* 1997; Gewirtz *et al.,*1998; Stein *et al.,* 1988).

It has been previously shown that up-regulation of RBBP6 mRNA is closely correlated with tumour progression in cervical and HIVAN, suggesting that RBBP6 gene plays a crucial role in the expression of malignant phenotypes in human cancer (ledwaba, Thesis, 2005; Mbita, Thesis, 2004). In human lung carcinogenesis, loss of heterozygosity, which suggests the presence of tumour-suppressor genes, has been reported in many chromosomes (Kernek *et al*, 2004). However, the gene whose deletion is associated with a poor degree of differentiation of lung adenocarcinoma has not been determined. Few genes have been associated with differentiation of human cancers. The up-regulation of RBBP6 mRNA in well- differentiated lung adenocarcinoma tissue in the present study resembles the up-regulation of RBBP6 mRNA in other cancers (Gao *et al* 2003). The simplest explanation for the increased expression of RBBP6 mRNA in lung carcinomas is that RBBP6 mRNA expression is up-regulated by activation of other endogenous oncogenes that are required to suppress the function of p53. p53 activity is regulated in great part by Mdm2. Direct interaction of p53 with the N-terminal region of Mdm2 squelches p53 transcriptional activity (Momand *et al*., 1992; Thut *et al*., 1997), while the Mdm2 Ring finger E3-ubiquitin ligase maintains p53 at a low level in normal cells by targeting it for proteasomal degradation (Honda *et al*., 1997; Kubbutat *et al*., 1997). Mdm2 may also target p53 for nuclear exclusion (Roth *et al*., 1998). Since the RBBP6 consist of both RING finger and p53 Binding domains, the two short-lived proteins constitute an autoregulatory loop whereby p53 maintains expression of its own functional inhibitor (Deb, 2003). For tumour cells that retain wild-type p53, one therapeutic strategy to reactivate p53 is to target Mdm2. Aside from direct inhibition of mdm2 expression by antisense, several other approaches have sought to interfere directly with the p53-Mdm2 interaction, including peptidomimetics and more recently, the cis-imidazoline derivatives dubbed Nutlins (Bianco *et al*., 2005; Zhang *et al*., 2004; Vassilev *et al*., 2004; Chene *et al*.,

2000). An alternative approach is to inhibit the Mdm2 E3 ubiquitin ligase activity thought necessary to restrain accumulation of activated p53 (Yang *et al*., 2005).

4.6 RBBP6 protein expression in lung cancer

It is generally accepted that the main physiological mechanism by which cancer cells die is by apoptosis, yet the precise mechanisms behind the induction of apoptosis still have to be identified (Brabletz *et al*, 2000). Changes in the degree of apoptosis in the course of lung cancer sequence have been studied extensively and were recently reviewed (Motadi *et al* 2008). From these studies, it appears that the proportion of cells undergoing apoptosis increases with tumour progression, possibly as a mechanism to delete cells with sustained DNA damage. Alterations in the degree of apoptosis may be explained by changes in apoptosis-regulating p53 pathways. From the currently known mechanism of apoptosis in lung cancer, RBBP6 has not been previously linked to lung cancer development. In this regard, the expression of RBBP6 and its domains in the course of the lung cancer development may be relevant.

To investigate the potential use of RBBP6 as therapeutic targets in lung cancer neoplasms, the expression of RBBP6 was studied in lung cancer tissue sections of different types of lung cancer development, i.e. normal lung, adenocarcinomas, squamous cell carcinoma, small cell and large cell carcinomas. The expression and localisation of RBBP6 was investigated by immunohistochemistry in normal lung (n = 12), adenocarcinoma (n = 50), squamous cell carcinoma (n= 40), small cell carcinoma (n=18) and large cell carcinoma (n = 8). In addition, correlations between expression of RBBP6 and p53 and the degree of apoptosis (assessed by TUNEL immunoreactivity) and histopathological characteristics were explored. In normal lung cells, both RBBP6 and p53 expression was observed. In lung cancers, expression was

seen in larger population of squamous cell carcinoma and adenocarcinoma. However, RBBP6 expression was lower in poorly-differentiated tumours in comparison to well differentiated (**figure 3.29 & 3.27**). Furthermore there was loss of RBBP6 expression in both small cell carcinoma and large cell carcinoma in comparison to normal lung cancer (**figure 3.31**). The higher expression occurred more frequently in squamous cell carcinoma than in adenocarcinoma ($p < 0.001$). Intensity of RBBP6 and localization of the staining was stronger in the cytoplasm of tumour cells compared to adjacent normal cells, and was accompanied by a higher degree of apoptosis in adjacent normal mucosa cells.

There was no statistical significance for the correlation of the expression ($p>0.05$) between p53 and RBBP6 in squamous cell carcinoma and adenocarcinoma (**figure 3.29**). The stronger expression of RBBP6 in cytoplasm of underlying cancer cells as opposed to adjacent normal cells holds promise for the future use of RBBP6 as therapeutic target in lung cancer development (**figure 3.27**). There was also interesting observation around macrophages and basophil cells attracted to the sight of tumour which also stained more positive for RBBP6 (**figure 3.28**). Given these encouraging results, we further investigated the level of expression of RBBP6 protein in A549 and MRC5 cell lines using the western blot and found that there was actually no significant change in the RBBP6 expression between A549 cell lines and MRC5 cell lines. We further need to take note that this correlates well with our observation in immunocytochemistry but further data need to be collected from other types of cancers to be able to present a conclusive involvement of RBBP6 in cancer development.

4.7 Correlation between apoptosis and RBBP6 expression.

Several studies have investigated the mechanisms underlying tumour development, progression and drug resistance in cancer, and current information suggests that in the

majority of cells, defects in apoptosis signalling are the most critical determinants (Fisher, 1994; Thompson, 1995). Some of the molecular and genetic abnormalities that antagonize apoptosis in lung-cancer cells have been unveiled, providing information for more specifically targeting these lesions.

The p53 can induce apoptosis after interacting with other pro-apoptosis genes and loss of function of p53 results in increased progression of tumour cells. As it is reported RBBP6 together with its two domains i.e. p53BD and RING finger domain might play a role in preventing p53-dependent apoptosis thereby binding it and leading to its degradation resulting in defect in apoptosis in these tumour cells. Therefore, RBBP6 might as well be a marker for cells that are predisposed to apoptosis. The aims of this experiment were to clarify the changes occurring in the degree of apoptosis during the gradual development of lung cancer. We found a higher degree of apoptosis in adenocarcinoma and squamous cell carcinoma areas compared to adjacent normal tissue (**figure 3.34**). Our results with TUNEL in both adenocarcinoma and squamous cell carcinomas are in accordance with other studies using this method to assess apoptotic cell death (Elder *et al* 2000; Fang *et al* 2009). In line with TUNEL results, we found increased RBBP6 expression in 40 % of adenocarcinomas and 85% of squamous cell carcinomas, while staining was intact in adjacent normal cells. The degree of apoptosis did not differ between RBBP6 positive and RBBP6 negative stained cells. Expression patterns of RBBP6 have recently been reported in other tumour types and diseases. In cervical tumours, RBBP6 expression did correlate with the degree of apoptosis (Ledwaba, 2005 Thesis). In colon cancer tissues RBBP6 expression was also found to be correlating with apoptosis (Rupnarain, 2005 Thesis). The loss of p53 expression in a subset of lung cancer suggests that these tumours could have evaded defects of apoptosis due to up-regulation of RBBP6.

Concerning the degree of apoptosis in normal lung, squamous cell carcinoma and adenocarcinoma, we have found apoptotic indices varying between 0.2, 1.8 and 1.5 % respectively. When studying the transition from normal lung epithelial to well- differentiated carcinomas, most studies indicated a shift towards higher apoptotic indices. However, there is contradiction about the transition from poorly to well-differentiated carcinoma. Most studies reported that the frequency of apoptotic cell death in well-differentiated was higher than in poorly-differentiated (Sugikawa *et al* 1999; Chiu *et al* 2005). However, in other studies, no differences or even opposite results were found because they argued that in early stages of tumours, cells are trying hard to maintain homeostasis as much as it happens in well-differentiated tumours. The distribution of apoptotic cells during transition from poorly to well differentiated is that the poorly –differentiated apoptosis predominates at the base of the epithelial cells, with proliferative activity prevailing at the luminal surface (Carr. N.J, 2000; Nitsch *et al* 2000). This is more in line with our observation which showed increased RBBP6 expression along those regions and at that particular stage of cancer development (**figure 3.27C & 3.31C**). Regarding the prognostic value of apoptotic indices it was found that most studies suggest that lung tumours with high apoptotic indices were associated with better prognosis and survival (Carr. N.J, 2000; Nitsch *et al* 2000).

4.8 Induction of apoptosis using camptothecin and staurosporine.

p53 is an attractive target for the development of therapeutic drugs in lung cancers. In tumours expressing mutant p53, small molecules that can rescue p53 function have been developed. In tumours with wild-type p53, inhibition of MDM2 has been proposed because it will lead to the induction of p53 activity. Small compounds binding either to p53 or to MDM2/Mdm2 have been isolated and have been shown to induce a p53-dependent apoptosis

in tumour cell lines (Selivanova, G. and Wiman, K.G. (2007), Bassett, E.A. *et al*. 2008). The downside of this is that targeted treatment of tumours initially expressing wild-type p53 might lead to resistance mechanisms owing to the selection of cells that express high levels of mutant p53 and are unregulated by MDM2 and secondly that exposure to this kind of treatment could be toxic to adjacent normal epithelial cells. In this study we aimed to first identify the appropriate concentration that could induce apoptosis in cancerous cell but to a lesser extend in normal epithelial cells and secondly to silencing RBBP6 and p53 then induce apoptosis in this these cells.

Following this approach, we have showed that camptothecin-treatment in A549 cell lines induced apoptosis. In many other studies a mechanism that implicated the mitochondria as a central integrating organelle in apoptotic cell death, which has the capacity to directly activate the cascade execution pathways, was described (Kluck *et al*, 1997). In particular, the release of cytochrome *c* into the cytoplasm is thought to be a critical step in the activation of caspases (Kluck *et al*, 1997). The cells that were treated with both RBBP6 siRNA and camptothecin and those that were treated with camptothecin only did not show any increase in the number of cells undergoing either early or late apoptosis which might suggest that the activation of apoptosis by camptothecin was not dependent on RBBP6 expression pattern of the cell lines studied (**figure 3.37D**). In contrast there was increased number of cells that underwent apoptosis in cells that were treated with p53 siRNA and camptothecin but the effect in MRC5 fibroblast was minimal.

In the present study, we used both approaches of knockdown and apoptosis induction by staurosporine in A549 cell lines and MRC5 cell line to demonstrate that down-regulation of RBBP6 increases the susceptibility of the cells to staurosporine-induced apoptosis. Our

findings demonstrated that RBBP6 plays a role in the negative regulation of cell apoptosis in A549 cell lines. Similarly, it has been indicated that loss or down-regulation of MDM2 expression in cancer cell results in significantly reduced cancer tumourigenic and metastatic potential with increased apoptosis (Ringshausen *et al* 2006; Chene *et al* 2002. TRAIL induced significant apoptosis in A549 cell lines treated with p53 which suggested that even if there is down-regulation of p53; apoptosis can still be induced through TRAIL-induced mechanism (**figure 3.38**). This is consistent with the finding that a minority (27%) of tumours of hematological origin are sensitive to TRAIL-induced apoptosis (Snell *et al* 1997). This was reported in other cancers such as breast cancer in the following cell lines MCF10A and MDA-MB-231 (Keane *et al* 1996). In down-regulated RBBP6 the trend was the same with that in down-regulated p53 with cells sensitive to TRAIL-induced apoptosis.

4.9 RBBP6 mutational analysis in lung cancer

Mutations of the p53 gene are the most common genetic alterations in human lung cancers (Ballal *et al* 2002) and might have a negative or positive effect on the expression of RBBP6 and similarly mutation of RBBP6 might have clinical implications during cancer development and treatment. In this study we analysed RBBP6 mutations in adenocarcinoma lung tumour tissues, normal lung tissue and the following cell lines (A549 and MRC5). The pattern of point mutations found in this study is remarkably homogeneous, with 3/5 (40%) being C→A, C→T and G→A transitions and the rest 2/5 being C→G and A→T transversion. A predominance of G→A transitions has been reported in other studies though usually at a lower frequency (Singh *et al* 2008). On the other hand, we could not demonstrate an association between the presence of RBBP6 point mutations and the p53 expression, no significant RBBP6 mutations were detected in lung tumours (**figure 3.5-3.13**). However,

when we examined the reverse primers we observed about 10% of a single point. Thus, the point mutation may be the critical element in RBBP6 in lung cancer in relation to p53 expression.

4.10 quantitative analysis of RBBP6 and p53 expression by qRT-PCR

Using qRT-PCR, the expression level of both p53, RBBP6, p53BD and the ratio of Bax/Bcl2 mRNA were determined in lung tumours, Normal lung and both squamous, adenocarcinoma and MRC5 fibroblast cell lines. All isoforms were present in the Universal Human Reference of 3 human cell lines mixture and in the human lung tumour samples. The p53, RBBP6, p53BD and the ratio of Bax/Bcl2 mRNA levels were determined by standard curve measurements in 1.0 orders of linear dynamic dilution range. All the genes showed almost similar slopes and accordingly have equivalent target efficiencies (**figure 3.43-48**).

p53 gene therapy is currently being studied in clinical trials for various cancers (Nemunaitis J, 2000; Swisher SG 2003). In preclinical models p53 has been shown to have therapeutic efficacy against a wide range of human tumour types containing nonfunctional *p53* both *in vitro* and *in vivo* (Watanabe *et al* 1998; Gurnani *et al* 1999). Little is known, however, about the biological mechanism of exogenous *p53* once it becomes active in tumours. Thus far, the antitumour effect of *p53 in vivo* has usually been assessed by simply measuring tumour volume or survival duration. In this study, we tried to analyze the mechanism of p53 in relation to RBBP6 in performing the antitumour effect by qRT-PCR, which may provide a key to identifying the mechanism of RBBP6 gene in cancer development. Our experiments

included quantitative assessment of p53- RBBP6 targeted gene expression following apoptosis induction and gene silencing.

Several groups of genes are transcriptionally regulated by p53, including p21 for cell cycle arrest; MDM2 for G_0/G_1 switch (Zhao *et al* 2000); p21 is one of the most important proteins in the p53 pathway, acting immediately downstream and mediating many of the actions of *p53* (Yu *et al* 1999). Our quantitative analysis demonstrated a reduction in the expression of p53 mRNA in lung tumour tissues which has been report in many other cases of lung cancer due to p53 mutations (Lasham *et al* 2003 and Motadi *et al* 2007) and slight increase in squamous cell lines following a combination of both RBBP6 silencing and apoptosis induction with camptothecin (**figure 3.54C and table 3.9**). These results correlated with increase in apoptotic cells that was due to camptothecine that assist in permeabilizing the mitochondria leading to cytochrome release rather than increase level of p53. The increased apoptosis associated with camptothecin further showed an increase in the ratio of bax/bcl2 ratio which suggested that camptothecin might have a role in bax activation. RBBP6 is a novel gene that is thought to play an important role in mediating *p53*-dependent apoptosis through interaction with MDM2 (Pugh *et al* 2006). In quantitative analysis to establish any relationship between apoptosis, p53 and RBBP6; maximal induced RBBP6 mRNA expression was higher among the p53-silenced genes tested and lowest in RBBP6 silenced gene but higher also in lung tumour tissues and MRC5-RBBP6 Staurosporine treated cells as compared to normal lung tissues. RBBP6 is thought to be a proliferative gene and most proliferative genes are recruited to the site of tumour during cancer development resulting in the increased levels of these genes in proliferating cells. However, it is also possible that RBBP6 might me recruited to the site of tumour cells to bind to p53 thereby leading to its degradation through ubiquitination. RBBP6 is reported to regulate p53 through MDM2 which is also recruited to cancer cells for cell cycle arrest (Zhao *et al* 2000; Pugh *et al* 2006) and

since p53 is silenced this resulted in the accumulation of RBBP6 in these cells. In relation to apoptosis and bax/bcl2, the increased level of RBBP6 was evident in Trail induced apoptosis that reported 54% in apoptotic work and the bax/bcl2 ratio was not significantly increased in these cells which was inline with other reports that showed increased apoptosis following Trail-induced apoptosis (Snell *et al* 1997).

We demonstrated that *in vitro* maximal expression of RBBP6 mRNA was achieved after 48 hours of p53 silencing in both squamous and adenocarcinoma cell lines (**figure 3.54A**). Notably, RBBP6 silencing, like that of p53, did not have much effect on the expression of p53 mRNA, while if apoptosis was induced with camptothecin in squamous cell lines, p53 was seen to be up-regulated. Knockdown of RBBP6 increased the ratio of bax/bcl2 in most cases. In contrast to Lsq tumour cell lines, A549 human cell line, which are relatively resistant to p53-mediated apoptosis, showed no increase in p53 expression (**figure 3.54C**) as well as apoptosis despite silencing of RBBP6 which is seen as binding to p53 leading to its degradation. These findings suggest that antitumour efficacy is associated with the cell type rather than p53 expression levels, although little is known about the molecular machinery underlying these interactions.

Chibi *et al* reported interaction between YB-1 and RBBP6 both *in vitro*, demonstrating that no intermediary protein is involved during the interaction, and *in vivo* they demonstrated that it is physically realized within the context of the cell. They further indicated that the interaction was not affected by the presence of mRNA, indicating that the interaction is direct and not mediated by mRNA. As a result of its interaction with RBBP6, they reported that YB-1 was ubiquitinated and degraded by the proteosome, leading to knockdown of YB-1 levels in a dose dependent fashion. Knockdown of exogenously introduced YB-1 was produced by the RING finger alone, as well as by full-length exogenous RBBP6. Blocking of

the proteosome with MG132 abolished the effect, indicating that the knockdown is the result of degradation by the proteosome. We have also indicated in this study that knockdown of RBBP6 did not have much effect on the p53 level which is inline with other observation that degradation might not be due to mRNA but other mechanism like binding to RING finger domain (Lasham *et al* 2003; Homer *et al* 2005).

BCL-2 and BAX proteins play a critical role in programmed cell death pathways at a checkpoint that seems to be upstream of caspases (Falleni *et al* 2004). The active form of BCL-2, which promotes cell survival, forms a heterodimer with BAX protein, which promotes cell death by allowing the activation of caspases (Oltvai *et al* 1993). The ratio of BCL-2 to BAX protein seems to determine the susceptibility of cells to apoptotic stimuli (Hinz *et al* 2002; Gillardon *et al* 1996). Overexpression of BAX is known to promote programmed cell death; conversely, overexpression of BCL-2 protects cells from apoptotic death after a variety of adverse stimuli, including hypoxia in cell cultures (Falleni *et al* 2004). Therefore, BAX and BCL-2 proteins play opposite roles in the physiological regulation of cell survival and cell death, an active process during normal wound repair and healing. In our study, we could demonstrate low Bax/Bcl-2 ratio values in normal/fibrotic pleurae and significantly increased ratio values in lung tumour tissues and cell lines treated with RBBP6 siRNA and camptothecin. A strong Bax expression in cells treated with camptothecin is in fact a common finding as camptothecin act by permeabilizing the mitochondria to cytochrome. The high Bax/Bcl-2mRNA ratios detected in lung cancer are in line with the observation of high Bax/Bcl-2 ratio in mesothelioma cell lines by immunoblotting (Narasimhan *et al* 1998). In most studies, low Bcl-2 to Bax ratios are associated with tumours with low grade histology and better outcome (Brambilla *et al* 1996; Gazzaniga *et al* 1996), slower progression of disease (Aguilar-Santelises *et al* 1996) and sensitivity to chemotherapy

(Chresta *et al* 1996). This observation and our results suggest that cancer biological characteristics have to be explained with other mechanisms that could counteract the pro-apoptotic effects of Bax (Narasimhan *et al* 1998), or in alterations of other genes involved in the regulation of apoptosis, rather than with Bcl-2 gene alterations.

4.11 Summary

Retinoblastoma binding protein 6 (RBBP6) is perceived to control apoptosis cascade or cell proliferation in the cell during cancer development (chibi *et al* 2008). Through its encoding protein products, RBBP6 has been suggested to bind p53 and other genes such as Retinoblastoma binding protein (pRb) leading to their ubiquitination (Pugh *et al* 2006). RBBP6 is located at chromosome 16p12.2 and it encodes three protein isoforms, 1; 2; 3. RBBP6 encode from two transcripts, 1.1 kb and 6.1kb. Isoform 1 code for 18 exons, isoform 2 coding 17 exons because it lacks exon 16 due to alternative splicing and isoform 3 codes for only three exons (Pugh *et al* 2006).

The aims of this thesis were to clarify the changes occurring in the degree of RBBP6 level during the gradual development of lung cancer and apoptosis. Furthermore, the mechanism of RBBP6 during p53-dependent pathway was targeted as a therapeutic agent in lung cancer. Following a brief introduction and outline of this thesis in Chapter 1, current knowledge of mechanisms of apoptosis and changes in the degree of apoptosis in the development of lung cancer was reviewed in Chapter 2. Although there is a multitude of studies available on apoptosis in lung tissue at different stages of lung cancer development, results from these studies concentrated on their relationship with RBBP6 expression in these tissues. Considerable controversy exists as to whether the expression of RBBP6 increases or

decreases during lung cancer development. Therefore, a systematic review of available studies, which addressed changes in frequency, and distribution of RBBP6 in lung cancer was performed.

Mean apoptotic indices assessed by TUNEL in normal epithelial mucosa was 0.2 ± 0.05 %, which was lower than in normal mucosa adjacent to squamous cell carcinomas: 1.8 ± 0.23 %. The mean apoptotic index in adenocarcinoma was 1.5 ± 0.18 %, lower than the apoptotic index of 1.8 ± 0.23 % in squamous cell carcinomas. There was positive correlation between the frequency of apoptosis as assessed by TUNEL immunoreactivity and different grades of dysplasia or growth type.

To investigate the potential use of RBBP6 as therapeutic agents in lung cancer, the expression of the RBBP6 was studied in lung tissue sections and the following cell lines (A549, Lsq1 and MRC5). The expression and localization of RBBP6 were investigated by immunohistochemistry in normal mucosa (n = 12), adenocarcinomas (n = 50), small cell carcinoma (n=18); large cell carcinoma (n=8) and squamous cell carcinomas (n = 40). Tissues were obtained from patients diagnosed with different stages of lung cancer. In addition, correlations between expression of RBBP6 and p53 and the degree of apoptosis were explored. The results are described in Chapter 3.

In normal mucosal epithelial cells, RBBP6 expression was observed. In lung cancer tissues, expression of RBBP6 was seen. However, RBBP6 expression was lost in a subset of both small cell carcinoma and large cell carcinoma in comparison to adjacent normal mucosa. Intensity of RBBP6 staining was stronger in neoplastic cells compared to adjacent normal epithelial cells, and was accompanied by a higher degree of apoptosis. No differences were

found between neoplastic and normal cells regarding RBBP6 expression. There were a certain notable correlations between RBBP6 expression and lung cancer stages with increased expression in well-defferentiated as compared to poorly to moderately differentiated. The results from qRT-PCR further indicated that the expression of RBBP6 was increased at mRNA level in lung cancer tissues which is an indication of protein synthesis. Induction of apoptosis with several apoptosis inducers, have also shown varying expression of RBBP6 and p53 with RBBP6 increased in TRAIL and Camptothecin induced apoptosis and no significant change of p53 levels. When RBBP6 was silenced followed by apoptosis induction, these had insignificant effect on apoptosis and p53 expression. However, when p53 was silenced followed by induction of apoptosis with TRAIL and camptothecin, apoptosis increased to about 54% and level of RBBP6 expression increased to about 46 folds.

4.12 Conclusions and future perspectives

Several genes that regulate apoptosis are inappropriately expressed or mutated in lung cancer. Although the idea of decreased apoptosis during lung cancer development is conceptually attractive, the available evidence from *in vivo* material suggests that the proportion of apoptotic cells increases gradually during the adenoma-carcinoma sequence. *In vitro*, tumour progression is usually associated with resistance to apoptosis induction. Although these data seem contradictory at first sight, and potentially confusing, they are not conflicting. The contradiction resolves when one realizes that *in vivo* studies describe *spontaneous* apoptosis whereas *in vitro* studies describe *induced* apoptosis, whether by chemotherapy or other agents. Considering the limitations of the current methods of *in situ* end labeling of DNA fragments, used in the vast majority of *in vivo* studies, improved diagnostic criteria for the

identification of apoptotic cells are needed and can be expected in the near future. The results from this thesis using the method of TUNEL support its use in the study of apoptosis in lung tissues.

Successful nonsurgical elimination of cancer cells ultimately involves apoptosis. Essentially all cytotoxic drugs work by induction of apoptosis, usually not only of malignant cells but also of normal cells, manifested by toxicity. Over the last years, knowledge has increased of mediators of apoptosis in lung cells, which are dysregulated in tumour cells, such as Bcl-2 family members and IAPs (Reed 2003). These suppressors of apoptosis have become targets for drug development aimed at restoring apoptosis sensitivity of tumour cells. Alternatively, one can imagine strategies aimed at direct activation of agonists of apoptosis, via the extrinsic pathway. RBBP6 is associated with regulatory mechanism of p53-dependent during lung cancer development through MdM2. Based on preclinical toxicity and activity data and the results described in this thesis showing broad expression of the RBBP6 in lung cancer cell lines following p53 silencing and apoptosis induction by TRAIL, recombinant human (rh) TRAIL is considered of great interest for clinical use. Agonistic TRAIL receptor antibodies, directed towards mitochondria have shown to exert the same effects as rhTRAIL (Pukac *et al* 2005; Motoki *et al* 2005).

Apart from the possible future application of RBBP6 in lung cancer, there may also be a potential role in treatment or prevention of cancer development through the p53-dependent pathway. Recent data show that RBBP6 plays a pivotal role in regulating p53 expression during cancer development (chibi *et al* 2008). The finding in Chapters 3 of this thesis of expression of RBBP6 in lung cancer cell lines supports further investigation of RBBP6 in regulating p53.

In conclusion, new treatment options for lung cancer have been developed based on insights into mechanisms involved in the regulation of apoptosis. The potential clinical application of Ad-p53 agents in the treatment of lung cancers, not only in p53 mutated tumours but even more tumours that singled out apoptosis as the main defects should be further explored. Results from this thesis indicate that RBBP6 in involved in cancer development and that its level of expression vary when apoptosis inducers are applied in vitro cells suggesting that this RBBP6 is required by these apoptosis inducers in inducing apoptosis and should guide future therapeutic strategies.

Chapter Five

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