Functional implications of a rare patient-derived mutation in tumor suppressor gene *TP53*

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By

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Declaration

I hereby declare that this thesis entitled "**Functional implications of a rare patient- derived mutation in tumor suppressor gene *TP53*"** is an authentic record of research work carried out by me under supervision of Prof. Tapas K. Kundu at Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India and this work has not been submitted elsewhere for the award of any other degree.

In keeping the general practice of reporting scientific observations, due acknowledgements have been made wherever the work is based on the finding of other investigators. Any omission, which might have occurred by over sight or misjudgment is deeply regretted.

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Certificate

This is to certify that the work described in this thesis entitled, **"Functional implications of a rare patient-derived mutation in tumor suppressor gene *TP53* ",** is the result of investigation carried out by Mr. Siddharth Singh, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research Bangalore, India, under my supervision, and that the results presented in this thesis have not previously formed the basis for the award of any other diploma, degree or fellowship.

Prof. Tapas K. Kundu

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Dedicated to PAPA

Contents

Abbreviations

Ab	Antibody
APS	Ammonium persulphate
ATP	Adenosine triphosphate
Bp	Base pair
BSA	Bovine serum albumin
DBD	DNA binding domain
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphatase
DTT	Dithiothreitol
E.coli	*Escherichia coli*
EDTA	Ethyldiaminetetraacetate
FBS	Fetal Bovine serum
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kbp	kilobasepair
kDa	kilodalton
LB	Luria Bertani
mg	Milligrams
Mut	Mutant
M.W.	Molecular weight
ng	Nanogram
nm	Nanometer
nt	Nucleotide
O.D.	Optical density
P152L	Proline to leucine mutation at 152^{nd} position

PBS	Phosphate buffered saline
PMSF	Phenyl methyl sulphonyl fluoride
RPMI	Roswell Park Memorial Institute medium
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
TBE	Tris Borate EDTA buffer
TEMED	N,N,N'N' Tetramethyl ethylenediamine
Tris	Tris hydroxymethyl amino ethane
UV	Ultraviolet
Wt	Wildtype
β- Me	Beta- mercaptoethanol
μg	Micrograms
μM	Micromolar
μL	Microlitre

Chapter 1 Introduction

This chapter introduces you to the field of cancer biology with special emphasis on tumor suppressor genes, their classes and how their mutation leads to cancer. It presents a detailed account of a tumor suppressor gene p53 - its domain architecture, structure, function. Being the most frequently mutated tumor suppressor gene in cancer, mutations in p53, their behaviour and consequences are also elaborated in this chapter.

CHAPTER OUTLINE:

1.1. Cancer - an overview :

Cancer is defined as the group of related disease which involves abnormal cell growth with the potential to invade surrounding tissues and other parts of the body. Many cancers form solid tumors which are malignant. There are many differences between a cancerous cell and a normal cell. Cancer cells are less specialised than the normal cells. While normal cells mature into very distinct cell types, cancer cells do not and hence continue to divide without stopping. Cancer cells are able to ignore signals such as apoptosis or programmed cell death, which the body uses to get rid of unwanted cells. Cancer cells are able to influence the normal cells, molecules or blood vessels that surround and feed the tumor (tumor microenvironment). They also acquire the ability to evade immune system response.

Cancer is a genetic disease and the genetic changes that cause cancer can be either inherited from parents or are acquired during a person's lifetime as a result of errors that occur during cell division or due to DNA damage caused by certain environmental exposures like radiation such as UV rays or substances such as chemicals in tobacco smoke or infections such as viral infections.

There are more than 100 types of cancer which are either named for the organs or tissues where the cancers form, for e.g., lung cancer, liver cancer, blood cancer, brain cancer; or they are named by the type of cell that formed them. These type include carcinoma (derived from epithelial cells), sarcoma (derived from connective tissues), lymphoma (derived from lymphocytes - B cells or T cells), germ cell tumor (derived from pluripotent cells in the testicle or the ovary), blastoma (derived from immature "precursor" cells or embryonic tissue.

There are two groups of genes, namely proto-oncogenes and tumor suppressor genes, whose genetic alterations leads to cancer. Proto-oncogene is a normal gene that code for protein involved in cellular growth and differentiation. But due to mutations or increased expression these proto-oncogene becomes oncogene or a tumor inducing agent allowing cells to survive when they should not. Few examples are - *RAS, MYC, WNT.* Tumor suppressor genes are also involved in controlling cell growth and division. Mutations in tumor suppressor genes render cells to divide in an uncontrolled manner.

1.2. Tumor suppressors :

Tumor suppressors are the genes which code for the proteins that inhibit the formation of tumors. They normally function by inhibiting the cell proliferation. Mutations in tumor suppressors genes causes reduction or loss in their functions, thus contributing to development of cancer, usually in combination with other genetic changes (1). Tumor suppressor genes can be grouped into three categories : Caretaker genes, Gatekeeper genes and Landscaper genes (2).

Caretaker genes:

These are the genes which encode products involved in maintaining genetic stability and preventing accumulation of undue mutations. These are the genes involved in DNA repair pathways and telomere metabolism. Few examples include DNA mismatch repair gene *(MLH1, MSH2, PMS1, PMS2)*, nucleotide-excision repair genes *(ERCC2), ATM* gene. Mutations in these genes leads to genetic (changes in nucleotide sequence of DNA) and chromosomal (improper arrangement of chromosomes) instability. Caretaker genes do not directly regulate cell proliferation, rather mutations in these genes predisposes cells to cancer by increasing the DNA mutation rate.(2)

Gatekeeper genes :

These are the genes which code for products that directly regulate the growth of potential cancer cells by inhibiting cell proliferation or promoting cell death. Each cell type has only one (or a few) gatekeepers, inactivation of a given gatekeeper leads to a very specific tissue distribution of cancer. Few examples include retinoblastoma *(RB),* adenomatus polyposis coli *(APC),* von Hippel-Lindau *(VHL),* multiple endocrine neoplasia *(MEN)* type I.

Landscaper genes :

These genes act by modulating the microenvironment in which the tumor cells grow. They do so by direct/indirect regulation of extracellular matrix proteins, cell surface markers, adhesion proteins or secreted growth/survival factors. Landscaper genes, when mutated, would cause the microenvironment to become conducive to unregulated cell proliferation.

Gatekeeper genes differ from that of 'caretakers' and 'landscapers' in three aspects : first, their loss of function is rate limiting for a particular step in multistage tumorigenesis; second, they act directly to prevent tumor growth; and third, restoring gatekeeper function to tumor cells suppress neoplasia (2,3).

Tumor suppressor p53 can be classified as either a 'caretaker' or a 'gatekeeper' tumor suppressor gene as its functions justifies its categorization in both the groups. As an apoptotic inducer, p53 act as a gatekeeper but due to its involvement in DNA damage repair and cell cycle arrest, it has a role of a "Guardian of the genome" whose major function is to prevent genomic instability. Hence p53 can be defined as a 'caretaker gene' also. Other examples in this context is *BRCA1, BRCA2*

Pathways to cancer :

In case of 'gatekeeper' genes, predisposed individual carrying one mutant copy of the gatekeeper gene (somatic or germ line mutation) need only one additional mutation (inactivation of second gatekeeper allele) to initiate neoplasia. But inactivation of 'caretaker' gene does not promote tumor initiation directly. The

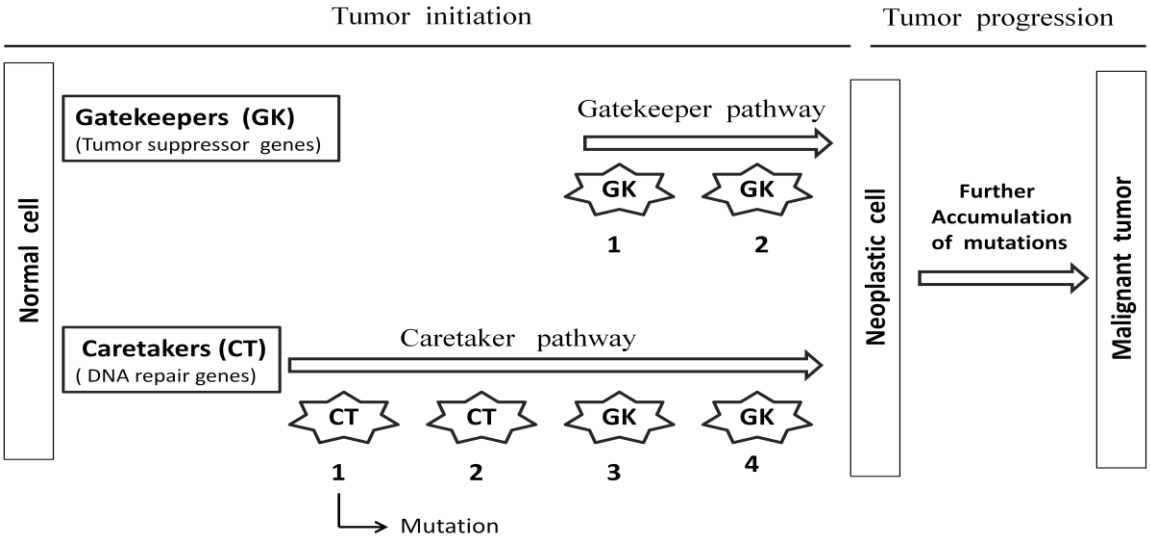


Figure 1.1 : Schematic representation of two different pathways of tumor initiation : Mutations of both the gatekeeper alleles are required to initiate oncogenesis whereas in case of caretaker pathway, a total of four mutations - two alleles of a caretaker gene (leading to genomic instability) and two alleles of a gatekeeper gene (leading to loss of growth control), is necessary to initiate oncogenesis.

predisposed individual requires three additional mutations to initiate neoplasia. Mutation in second 'caretaker' allele leads to its inactivation and genetic instabilities which result in increased mutation of all genes, including gatekeepers. When two somatic mutations in gatekeeper gene causes its inactivation, which leads to loss of growth control, neoplastic transformation occurs (3,4). p53 is one of the exception where single dominant negative mutation can substitute for two inactivation mutations of a gatekeeper gene. (Figure 4.1)

1.3. Tumor suppressor p53 :

p53, voted molecule of the year in 1993 by Science magazine, has several names accredited to it like "Guardian of the genome", "Death star", "Good and bad cop", "Cellular gatekeeper for growth and division" , "An acrobat in tumorigenesis". It was discovered in 1979 as a 53-kDa host protein found to be associated with the oncogenic Simian Virus 40 (SV40) large T antigen in transformed cells when sera from animals with SV40 induced tumors was used to immunoprecipitate SV40 large T antigen (5-7). Although it was initially discovered as an oncogene due to the use of mutated cDNA obtained after purification of tumor cell mRNA. Its character as a tumor suppressor gene was revealed in 1989 (8). Human p53 gene is located on the short arm of chromosome number 17 and it encodes 53 kDa protein (actual molecular weight - 43.7 kDa) contains 393 amino acids. *p53* gene is 20 kbp long, with 11 exons coding for 2.2 kb mRNA. p53 has twelve isoforms which arises due to alternative splicing or alternative transcription initiation with their molecular weights ranging from 28 kDa - 53 kDa. These isoforms are expressed in a tissue dependent manner. It has two other family members - p63 and p73. The anticancer functions of p53 include DNA repair, cell cycle arrest and apoptosis.

1.4. Domain organization in p53 :

Human p53 protein is 393 amino acids long and consists of five protein domains, each corresponding to specific functions : the transactivation domain, proline rich domain, central DNA binding domain (DBD), tetramerization domain and C-terminal regulatory domain (CTD). (Figure 4.2)

Transactivation Domain (1-42 amino acids) :

It is acidic N-terminal domain which spans the first activation domain (AD1). The second activation domain (AD2) spans from 43-63 amino acids and is included in proline rich domain. The NMR structure of p53 activation region (AD1 and AD2) reveals this region to be unstructured and natively unfolded which confers this domain the ability to undergo folding upon binding to a target protein and hence providing functional diversity to this domain. Transactivation domain interacts with basal transcription machinery proteins such as TFIID and TFIIH and activates transcription. It also interacts with other transcription factors like p300/CBP which promotes transcriptional activation by p53 and with MDM2, an E3 ubiquitin ligase and negative regulator of p53. The transactivation domain is also indispensible for cell cycle arrest function of p53.(9)

Proline Rich Domain (43-92 amino acids) :

PRD contains five PXXP motifs which serve as the consensus sequence for binding to Src-homology 3 (SH3) domains. Additionally it has 15 other proline residues, rendering this domain proline rich. It contains the AD2 from 43-63 amino acid residues. This domain is required for p53 dependent apoptosis as deletion or mutation of this domain is unable to induce apoptosis.

DNA Binding Domain (102-292 amino acids) :

The DNA binding domain region is involved in sequence specific DNA binding of p53 transcriptional targets. This domain is protease resistant and consist of two structures - a β sandwich scaffold and a DNA binding surface. The DNA binding surface consists of loop-sheet-helix motif and two loops L2 and L3, bound by a Zn^{2+} atom. Loss of Zn^{2+} results in abrogation of site specific DNA binding activity of p53 (9). Amino acid residues Lys120, Ser241, Arg248, Arg273, Ala276 and Arg283 are important for sequence specific DNA binding ability. DBD region is the most mutated region in *TP53* gene with more than 90% of the tumor derived mutations in p53 localize in DBD (10). The fact that majority of these mutations are missense mutations highlights the importance of sequence specific DNA binding for p53 to function as tumor suppressor (11).

Oligomerization domain (326-355 amino acids) :

p53 exists as tetramer in solution and oligomerization domain / tetramerization domain is required for it (12). The structure of tetramer is a dimer of a dimer with two β sheets and two α helices. The two dimers are held together by a large hydrophobic surface of each helix pair. p53 tetramerization is essential for DNA binding and for its tumor suppressive functions. Tetramerization domain is linked to DBD through a flexible linker of 37 residues (13).

C- Terminal Domain (356-393 amino acids) :

CTD is a regulatory domain rich in basic amino acid residues. It is involved in both positive and negative regulation of p53 functions which is likely due to the fact that nearly every residue in domain is subjected to post translational modification. CTD as a negative regulator binds non specifically to DNA, thus contributing to p53 latency. Of the two proposed p53 latency models, allosteric model proposes that CTD inhibits the core domain via conformational changes whereas competitor DNA model suggests that when long ssDNA binds non-specifically to CTD, binding of p53 core domain to its consensus sites on target DNA is inhibited (14). In contrast CTD also enables DNA binding in a sequence dependent manner which is drastically altered by its modification or deletion. CTD also ensures p53 binding to a diverse set of response elements and the stable binding to these response elements is through DNA induced conformational changes within the DBD itself (15) .

Figure 1.2 : Schematic representation of p53 domain organization : p53 has 393 amino acids with a N-terminal transactivation domain (TAD), proline rich domain (PRD), central DNA binding domain (DBD), followed by oligomerization domain (O.D.) and C-terminal regulatory domain (CTD). Two nuclear export signals - NES 1 (a.a. 11-27) and NES 2 (a.a. 340-351) ; and three nuclear localizing signal NLS 1 (a.a. 305-322), NLS2 (a.a. 370-376) and NLS3 (a.a. 380-386) are marked in the respective domains

Nucleocytoplasmic translocation in p53 :

Shuttling of p53 between nucleus and cytoplasm is facilitated by nuclear import and export signals. p53 has three nuclear localizing signals (NLS) located in the C-terminus of *TP53* gene - Bipartite NLS1 (a.a. 305-322) located in the linker region between DBD and OD, NLS2 (a.a. 370-376) and NLS3 (a.a. 380-386) located in the CTD. p53 has two nuclear export signals - NES1 (a.a. 11-27) in the N-terminal TAD and NES2 (a.a. 340-351) in the C-terminal tetramerization domain. p53 is cytoplasmic in most of the normal cells. However in rapidly growing normal cells and in transformed cells p53 is primarily found to be localized in nucleus. p53 activity can be spatially regulated during cell cycle which is seen in normal fibroblast cells where p53 accumulates in the cytoplasm during the G1 phase and is shuttled into the nucleus during G1/S transition and is retained there until S phase begins when p53 cycles back to cytoplasm. p53 is also translocated in the nucleus during cellular stress responses. p53 is normally maintained at low basal levels in the cytoplasm due to ubiquitination and subsequent nuclear export by its negative regulator Mdm2 which first binds to N and C terminal NES of p53 and targets it for ubiquitination and translocation from nucleus to cytoplasm. NES and NLS of p53 are also subjected to post translational modification which alters their localization (16).

1.5. p53 dependent transcription - anti cancer functions :

p53 mediated cell cycle arrest : Cell cycle is a highly regulated process by which cellular growth and proliferation are controlled. The cell cycle is catalyzed by cyclins and cyclin-dependent kinases (CDKs). Cyclin-CDK complexes phosphorylate serine or threonine residues on several transcription factors required to activate downstream target necessary for cellular growth and proliferation. p53 negatively regulates the cell cycle at both the G1/S and G2/M phases in response to DNA damage recognized by ATM and ATR and to oncogenic stress signalled by the ARF tumor suppressor. Progression from G1- S phase is specifically inhibited by the p53 downstream target p21. p21 is a cyclin dependent kinase inhibitor (CKI), which binds to CDK-cyclin complexes and prevents the cell from entering S- phase. p21 null mice are viable and embryonic fibroblasts from these mice show partial cell cycle arrest, when they are subjected to DNA damage,

suggesting the presence of alternate downstream targets activated in response to p53. The cyclin B and CDK2 complex promote cellular progression from G2 to M phase. They are in an inactive complex due to phosphorylation by the CKIs, WEE1 and MYT1. p53 inhibits this complex by inducing downstream targets GADD-45 and 14-3-3σ, in response to DNA damage. GADD-45 promotes dissociation of Cyclin B and CDK2 whereas 14-3-3σ prevents nuclear import of the CDK2/Cyclin B complex, blocking entry into mitosis and hence cell division.(17)

p53-mediated Apoptosis :

Apoptosis, or programmed cell death, requires the activation of either the extrinsic or intrinsic pathways. p53 promotes the intrinsic pathway by transactivation of BCL-2 family proapoptotic genes, BAX and BAK. BCL-2 antagonist/killer (BAK) and BCL-2 associated X protein (BAX) contain the BCL-2 homology domain-3 (BH-3 domain) (18). These proteins regulate the mitochondrial outer membrane permeability, leading to the release of cytochrome c from the mitochondria into the cytosol. Cytochrome c, caspase 9 and APAF-1 form the apoptosome within the cytosol. Once procaspase-9 is cleaved, active caspase-9 activates effector caspases (procaspases -3,-6 and 7) leading to morphological changes that occur during apoptosis. p53 also directly transactivates other pro-apoptotic factors such as Puma and Noxa. Both the proteins translocate to the mitochondria to induce apoptosis. In response to DNA damage, Puma and Noxa are both activated to block pro survival signals from the anti-apoptotic Bcl-2 protein family members, Bcl-2 and Bcl-XL. Another model of p53 induced apoptosis suggests that p53 binds antiapoptotic factors such as Bcl-2 and Bcl-XL in the cytoplasm at the mitochondria. Under cellular stress, p53 cytoplasmic sequestration by Bcl-2 and Bcl-XL is relieved by nuclear p53 transactivation of pro-apoptotic proteins such as Puma. Puma then competes with p53 for binding of Bcl-XL in the cytoplasm which results in release of p53 to promote oligomerisation of pro-apoptotic genes such as BAX and BAK (19). (Figure 1.3)

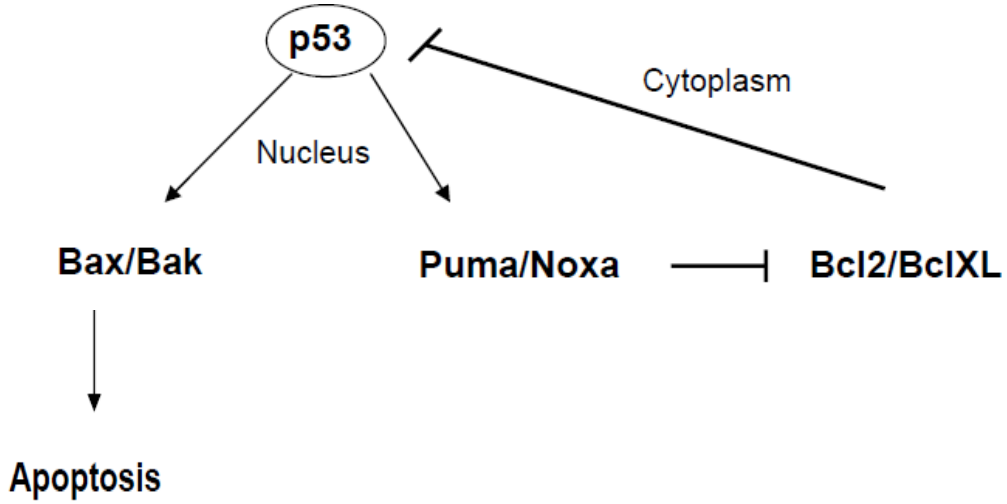


Figure 1.3 : p53 mediated apoptosis : p53 directly transactivates pro-apoptotic proteins such as Bax, Bak, Puma, Noxa. p53 binds Bcl-2/Bcl-XL in the cytoplasm. Under cellular stress p53 competes with proapoptotic proteins such as Puma for binding of anti-apoptotic proteins such as Bcl-XL. Once p53 is released from Bcl-2 / Bcl-XL, it is free to possibly promote oligomerisation of pro-apoptotic genes including Bax and Bak, which induce apoptosis.

1.6. p53 - Structure :

p53 consist of folded DNA binding and tetramerization domains, flanked by intrinsically disordered regions at both the amino- and carboxy- termini which has posed a great challenge to obtain a full length crystal structure of p53 till date. Several studies have revealed the structural details of the individual domains of p53 such as the DNA binding domain and the tetramerization domain.

Structure of the DNA binding domain :

Human p53 core domain has low intrinsic thermodynamic stability and it rapidly unfolds at body temperature. This has important implication as far as protein turnover and its binding to partner protein is concerned. Low kinetic and thermodynamic allows rapid cycling between folded and unfolded states, which is yet another way of regulation of functionally active cellular protein levels (20). Low intrinsic stability can also be implicated in the structural plasticity required to facilitate binding to different partner proteins. The susceptibility of p53 to deleterious mutations and cancer development can also be linked to this instability

(21,22). p53 functions primarily as a transcription factor and is biologically active as a homotetramer.

p53 tetramer binds cooperatively to its target DNA in a sequence specific manner. The target binding site consists of two decameric half sites of the form RRRCWWGYYY (R – A/G, W- A/T, Y- C/T), separated by 0-13 bp (23). Genome wide mapping of p53 binding sites show that most p53 response elements have consecutive half sites. It has been shown in the case of TIGAR, Noxa and p21 response element that an increase in the spacer length between two decamer half sites causes decrease in the p53 affinity and transactivation. Further structural and biochemical studies show that p53 binds each decameric half site as a dimer and the two dimers from the two decameric half sites constitutes a tetramer (dimer of dimer) (24).

A recent study where crystal structure of a p53 core domain tetramer assembled on a full consensus site was solved, revealed that DNA binding by the p53 core domain is a cooperative self-assembling process accompanied by the structural changes in the p53 dimer and the DNA. A novel dimer-dimer interface was identified which is the cause of assembly of p53 core tetramer on a contiguous decameric half site. Moreover it is not the DNA bending, but the structure of the p53 dimer that undergoes most significant structural change. (Figure 1.4) . In the structure each subunit of the tetramer is trapped by DNA binding and protein-protein interactions with two neighbouring monomers. This results in an enclosed structure which is kinetically and thermodynamically more stable (Figure 1.4). The extensive and interconnected protein-protein and protein-DNA interactions enhances the stability of the core domain which might function together with tetramerization domain in the full protein to enhance p53 binding. Major determinant of the protein-protein interaction at the dimer-dimer interface is the shape complementarity. Therefore most residues at the dimer-dimer interface are conserved among species (25,26).

Structure of full length p53 protein :

There are some major hindrance in obtaining the full-length structure of p53 : p53 is too flexible for crystallographic studies, too big for conventional NMR and small enough to be at the limits of cryo-electron microscopy. Small angle X-ray

scattering experiments (SAXS) and computation has been used to reconstruct the structure of p53 and its complexes in the solution (11). An important aspect of these experiments is the design of an engineered stabilized variant of p53 containing four mutations in the core domain (M133L / V203A / N239Y / N268D) which significantly increases the overall stability of the protein (27). Using cryoelectron microscopy, three dimensional structure of full length p53 tetramer has also been solved. The new revelation of this structure is the contact between N- and C- terminus. According to this model two specific DNA binding sites are formed from the tetramer, one from the core domain of each dimer. The distance between core domain of each dimer can be altered using linking domains as levers which provides structural plasticity to the structure to bind to various target sequences (28).

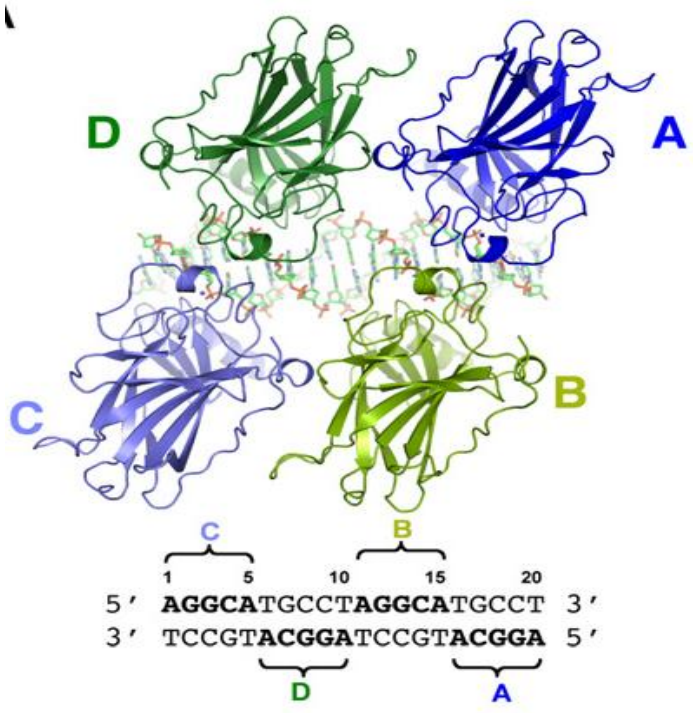


Figure 1.4 : The structure of the p53 core domain bound to DNA as a tetramer : The four monomers are shown coloured in blue (A), light green (B), light blue (C), and green (D). The DNA is in stick model with its sequence shown below. The four pentameric motifs and their corresponding monomers are indicated in the sequence. [Adapted from Chen *et al*, Crystal structure of p53 core domain bound to a full consensus site as a self assembled tetramer. *Stucture*. 18, 246-256 (2010)]

Mutations in p53 :

Although there is huge diversity in the genes implicated in tumorigenesis, p53 protein clearly stands out as a key tumor suppressor and a master regulator of various signalling pathway involved in this process. *TP53* mutations has been reported in almost every type of cancer with varying rates. At least 50% of all tumor exhibit mutation of p53, and in those that retained wild type p53, its activity can be attenuated by several other mechanisms. Unlike majority of the tumor suppressor genes, which are usually inactivated during cancer progression by deletions or truncating mutations, vast majority (75%) of cancer associated mutations in TP53 are missense mutations, in which single nucleotide is substituted by another (29,30). Hence full length protein containing only a single amino acid substitution is produced. While wt p53 protein under unstressed condition, is a very short lived protein, these missense mutations lead to the production of full-length altered p53 protein with a prolonged half life. Although cancer associated p53 mutations are very diverse in their locations within the p53 coding sequence, a great majority (more than 90%) of these missense mutations are clustered within the central most conserved region of p53 that spans the DNA-binding domain and among these are small number (approximately six) of residues that occur with unusually high frequency. These residues are termed as "hotspot" residues (10,31). Out of all the missense mutations in DNA-binding domain there are about eight mutants which alone account for about 30% of the total p53 core domain mutants.

1.7. Types of p53 mutations :

p53 mutants can broadly be classified into two structural subgroups - "DNA contact" mutants (e.g. R273, R248) or "Conformational" mutants (e.g. R175, G245, R249, R282). DNA contact (or Class I) mutants arise due to the missense mutations in the amino acid residues that normally make direct contact with target DNA sequences whereas Conformational mutants arise due to those missense mutations that disrupt the structure of p53 protein on either a local or global level (32-34).

The DNA binding domain structure of several common mutants has been elucidated by NMR spectroscopy or X-ray crystallography. DNA contact mutants does not alter the overall conformation of p53 molecule whereas conformational mutants cause partial or complete abrogation of the wild type conformation of the DBD exposing residues or interfaces that are normally buried within the DBD. DNA contact mutants actively prevent DNA binding if a large hydrophobic side chain is introduced (e.g. R248W) or when zinc binding mutants affect the zinc coordination sphere (e.g. R175H). Since conformational mutants affect the residues that contribute to the maintenance of the tertiary structure or conformation of protein, these mutants cause distortions that create internal cavities or surface crevices in the protein scaffold, inducing conformational changes in the DNA binding surface (14,22,35).

1.8. Behaviour of p53 mutants :

In addition to their classification as a DNA contact or a conformational mutant, p53 mutants show some intrinsic differences in their biological activities (22,36). These differences may or may not depend upon them being a DNA contact or a conformational mutant. For e.g. R273H (DNA contact) and R175H (conformational) mutant differ in many aspects listed below :

- R273H prevents DNA binding by the isolated core domain, although other contact may enable full length p53 to bind weakly whereas both the core domain as well as full length p53 with R175H mutation fails to bind DNA due to highly destabilized structure.
- Increased migratory and invasive activity was observed in cells expressing R273H mutant as compared to cells expressing R175H whose migratory and invasive capability was insignificant as compared to parental cells (37).
- Core domain of R273H is thermodynamically more stable than that of R175H mutant (20).
- Conformational mutant such as R175H but not DNA contact mutant such as R273H increased cellular resistance to etoposide, although both the mutants elicited similar response to cisplatin treatment - both mutants conferred resistance at low concentration of cisplatin but not at high concentration (38).

- When treated with mitotic spindle inhibitor, cells expressing conformational mutants like R175H caused genomic instability by abrogating the mitotic spindle checkpoint thereby facilitating the generation of polyploid cells by promoting cells to re-enter S phase with no apparent arrest whereas contact mutant like R273H caused tetraploid G1 arrest thereby prevented the cells to enter into S phase (39).

The differential effects is also seen in the interaction of mutant p53 with p63 or p73 where the structural mutants bind p63 or p73 with much higher affinity than the contact mutants, although both groups of mutation seem equally capable of inhibiting p63 and/or p73 to promote invasion and metastasis or to prevent apoptosis. Besides these, the differences also exists in terms of affinity with which mut p53 binding partners bind to a subset of tumor-derived p53 mutants. In fact even different amino acid substitutions at the same position in the p53 protein can have dramatically different phenotypic effects. For example p53-R248Q but not p53-R248W, is able to confer invasive ability when overexpressed in p53 null cells. There are other mutants which show partial defects. For example mice expressing the murine equivalent of R175P (R172P) is able to activate the cell cycle arrest but not apoptotic programs of p53 and demonstrate a delay in spontaneous tumor formation (40,41). In addition, in humanized mutant p53 knock in models, p53R248Q/- and p53R248Q/Q, but not p53G245S/- and p53G245S/S, mice show an acceleration of tumor development and shorter survival as compared to p53-/- mice. Furthermore, different human tumor types show different spectra of p53 mutations. For example in human pancreatic tumors, mutations at codon 248 of p53 are mostly observed whereas in breast tumors codons 175 and 275 are most frequently mutated (42). Also when prognostic impact of *TP53* mutations in breast cancer was examined, it was observed that specific missense mutations (R248W and mutations at codon 179) actually correlated with a significantly poorer prognosis compared with other missense mutations (43,44).

1.9. Consequences of mutation in p53:

Mutation in p53 within a cell can have three consequences which are not mutually exclusive. First, the mutation abrogates the tumor suppressor function of the affected TP53 allele, thereby reducing the overall capacity of the cell to

mount a proper p53 response. But if in a cell, both the alleles are mutated or the remaining allele is lost, the cell becomes totally deprived of anticancer protection by p53. Secondly, many of the stable mutant isoforms can exert a dominant negative effects over coexpressed wt p53, largely by forming a mixed tetramers that are incapable of DNA binding and transactivation or by the incorporation of wtp53 into the mutant p53 supratetrameric aggregates (45). Hence even if one wt allele is retained, the cell can be rendered devoid of wtp53 function through this mechanism, particularly if the mutant protein is expressed in excess over its wild-type counterpart (33). Although it has been observed that missense mutation in p53 in human tumors are usually followed by loss of heterozygosity at the corresponding locus, suggesting that there is a selective advantage conferred by losing the remaining wtp53, even after one allele has been mutated. The third outcome of the mutation in p53 is the oncogenic gain of function activities exerted by mutant p53 which is independent of its effects on wild type p53 and these activities can contribute to various aspects of tumor progression. According to the gain-of-function hypothesis - "the mutation of p53 is not equivalent to simply losing wild-type p53 function; rather, the strong selection for maintained expression of a select group of mutant p53 protein suggests a positive role for certain p53 mutants in tumorigenesis." In short GOF encompasses any activity of mutant p53 exerted in the absence of coexpressed wild-type p53 (32,40,46).

1.10. Gain-of-function effects of mutant p53 :

Gain of function of mutant p53 leads to increase in tumorigenesis through increase in cell proliferation, cell survival, increased cell migration and invasion, metastasis, angiogenesis, increased tumorigenicity, gene amplification, abnormal chromosomal and spindle check points, antiapoptotic activity and increased therapy resistance. Some of these effects are discussed in detail below.

Cell migration and invasion : It has been established that in cultured cells, mutant p53 can augment cell migration and cell invasion whereby cells acquire ability to invade adjacent tissue, migrate towards distant site and thus seed metastases. Induction of cell migration by mutant p53 is cell context dependent and additional signals such as oncogenic Ras in combination with TGF-β might be required to unleash this effect. It has been shown further that mutant p53 isoforms

cooperate synergistically with oncogenic Ras to induce a precancerous gene cluster consisting of chemokines, interleukins and ECM related molecules which are major contributors to tumor progression and invasion (37,47-49).

Antiapoptosis : Many p53 gain of function mutants confers on cells an increased resistance to a variety of proapoptotic signals, thereby providing a selective advantage to aspiring tumor cells within a competitive microenvironment. Interestingly, over expression of several tumor associated mutant p53 isoforms render cells more resistant to killing by a variety of anticancer agents like γ irradiation, doxorubicin, cisplatin, etoposide. Knock down of endogenous mutp53 sensitizes cancer cells to killing by such agents. Hence antiapoptotic / chemoresistance activity not only accelerates tumor progression but also hinder the response of cancer patients to anticancer therapy (50,51).

Genomic Instability : Mutp53 can disrupt normal spindle checkpoint control, leading to accumulation of cells with polyploid genome. Higher mutation rates, increased frequency of centrosome amplification, aberrant mitosis, aneuploidy are few other genomic instability effects manifested by mutp53 (52). Disruption of cellular genome integrity by mutp53 has drastic consequences on tumor progression, particularly in advanced stages of the disease where gross genomic alterations are quite frequent (39,51).

1.11. Mechanism of gain of function :

There are broadly four different types of molecular mechanism by which mut p53 function to elicit its transcriptional effects. There can be alterations in the DNA binding ability of mutant p53 where amino acid substitutions in within the DNA-binding domain of the majority of tumor derived p53 mutations may change, rather than abolish, the sequence specific DNA binding. However consensus mut p53 specific DNA response element has so far not been characterized. Mutant p53 can also interact with other sequence specific transcription factors and thereby get recruited to their respective cognate binding sites and either strengthen or dampen the target response (41). One of the example of inhibitory interaction with the transcription factor is the interaction of mutant p53 with its family member proteins p63 and p73 and thereby preventing them from binding to DNA. Mut

p53 can also interact with the proteins which are not directly involved in transcription and modulates their function (53) (Figure 1.5)

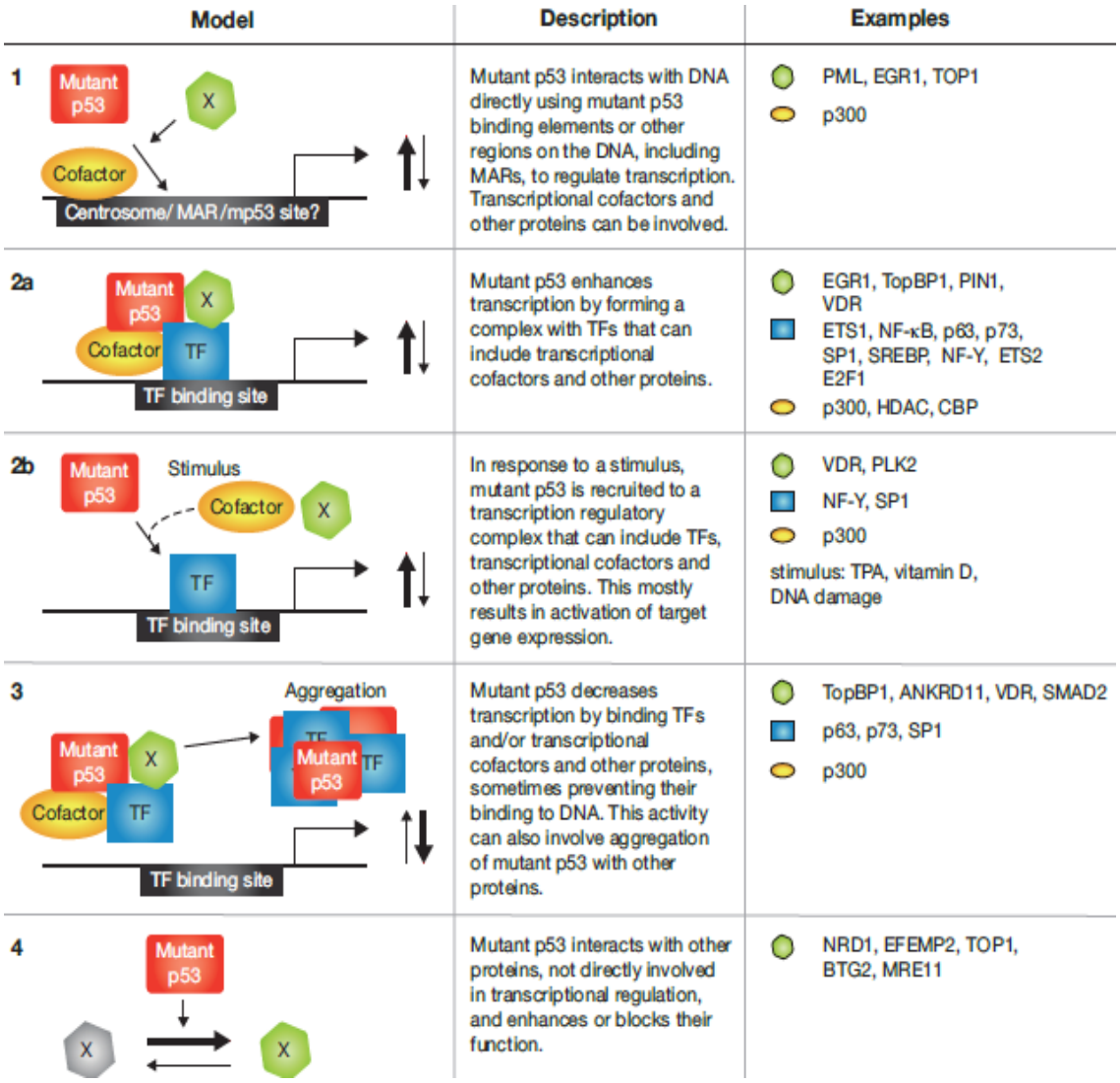


Figure 1.5 : Models of mechanisms through which mutant p53 functions : As a part of its gain of function, mutant p53 interacts with different proteins to enhance or inhibit their activities. TF, transcription factor ; X, any protein other than transcription factor or transcriptional cofactor ; MAR, matrix attachment region DNA element ; mp 53, mutant p53. [Adapted from Patricia A.J. Muller and Karen H. Vousden. p53 mutations in cancer. *Nature cell biology*. 15, 2-8 (2013)]

1.12. Mouse models for mutant p53 GOF study :

Nude mice, transgenic mouse model, gene knockin mouse model are the animal models being used to elucidate the biological manifestations of mutp53 GOF. Several hotspot p53 mutants enhances tumor formation in nude mice when overexpressed in various p53 null cell lines, thus establishing the generality of mutp53 GOF. Transgenic mouse models driving tissue specific mutp53 overexpression showed cooperation of mutp53 with additional oncogenic events in accelerating tumor development and in rendering the tumors more invasive and aggressive. Also in both the mouse models, overexpressed mutp53 was found to augment experimental metastasis in mice (54). These results were further validated by using either stable or conditional shRNA mediated knockdown of mutp53 in a number of human cancer cell lines implanted in nude mice where down-regulation of the endogenous mutp53 rendered those cells significantly less tumorigenic. Such knockdown also sensitized the tumors to chemotherapy in vivo. The most significant advancement in animal model study of mutp53 GOF effect is the generation of mutp53 knockin mice, in which the endogenous wt p53 allele was replaced by mutant versions mimicking common human hotspot mutations. When compared with p53 knockout animals, mice carrying mutp53 alleles developed more aggressive, metastatic tumors, as well as higher frequency of tumor types that are associated with p53 mutations in human cancers. The advantage of this model is that the mutp53 proteins are expressed at the physiological levels and are under physiological regulation which provides more credibility to the distinct cancer-associated features exhibited by mutp53 proteins (42,43).

1.13. Aim and Scope of study :

Tumor suppressor *p53*, described as the "guardian of the genome" is the most frequently mutated gene in human cancer and probably the most well studied tumor suppressor gene in context of cancer. It maintains the genomic integrity by regulating several cellular processes mainly by acting as a transcription factor. Mutations in p53 gene are predominantly of missense type which leads to structural alterations in full length protein at the local or global level and results in loss of its DNA binding ability. Earlier observations from our lab identified a rare proline to leucine mutation at 152^{nd} position in DNA binding domain region of p53 in an oral cancer patient sample. Although this mutation is not a hotspot mutation but it still features among the top 50 missense mutation in cancer and is functionally uncharacterised yet.

The present study was aimed to determine the functional implication of this mutation, where we looked upon the effect of this mutation in DNA binding ability of p53 protein and also identified that at what level, local or global, the distortion is created in the protein structure. The mutant p53 forms often acquire oncogenic gain of function properties which enable them to promote metastasis, proliferation, invasion and cell survival in cancer cells. Hence we went ahead to delineate the gain of function effects conferred by this mutation. In addition to these gain of function phenotypes, mutant p53 proteins exhibit differences in their biological activities. These intrinsic differences are manifested at the level of their structure, tumorigenic potential, prevalence in specific tumor types, genes they activate or repress, their effect on downstream signalling pathways etc. Hence it becomes important to understand the uniqueness of p53 mutations in driving different tumor types in different ways.

Chapter 2 Materials and methods

This chapter presents the information on experimental protocols followed and the techniques utilized along with the reagents used for the work done in this thesis.

CHAPTER OUTLINE:

2.1. General Methods

2.2 Subcloning of DBD of wt p53 and mut p53 (P152L) from pRSET(A) vector into pET28a+ vector containing cleavable His Tag

2.3 Protein Purification

2.4 DNA-Protein interaction study

2.5 Gain of function experiments

2.6 Gluteraldehyde crosslinking experiment

2.1 General Methods:

2.1.1 Preparation of competent cells:

E.coli strain XL-10 Gold cells were streaked on LB agar plate containing Tetracycline (10 ng/µl) and Chloramphenicol (34 ng/µl) and was incubated at 37°C overnight. A single colony was inoculated in 10ml of LB medium containing tetracycline and chloramphenicol and grown overnight at 37°C under shaking conditions. $O.D._{600}$ was measured till it reached 1 and accordingly inoculation was done in 50 ml of secondary culture so that the final O.D. becomes 0.1. Culture was then grown at 37°C under shaking conditions for 2 hours. $O.D._{600}$ was measured again till it reached 1. Culture was then transferred to 18°C for 1 hr

under shaking conditions for acclimatization. $O.D._{600}$ was measured and accordingly inoculation was done in 200 ml of LB media so that the final O.D. became 0.05. Culture was then grown overnight at 18°C under shaking conditions till its O.D. reached 0.45. Culture was then kept in ice for 30 minutes and was pelleted down at 4°C, 4000rpm for 10 minutes. Pellet was resuspended in 80 ml of freshly prepared pre-chilled transfer buffer (10mM PIPES buffer pH 6.7, 15mM $CaCl_2$, 250 mM KCl, 55 mM $MnCl_2$ filter sterilized) inside hood and kept on ice for 1 hr. Previous step was repeated again and the pellet was resuspended in 9.1 ml of transfer buffer and 0.9 ml of filter sterilized DMSO was mixed homogenously to get 10 ml of suspension. Culture was immediately aliquoted on ice, freezed in liquid nitrogen and stored at -80°C.

2.1.2 DNA purification:

DNA was isolated from transformed E.coli DH5α cells with either individual plasmid or cloned constructs using GenElute Plasmid miniprep kit (Thermo Scientific) as per manufacture's protocol. Briefly, transformed bacterial colonies were inoculated in 5ml of LB media containing appropriate selectable antibiotic and cells were harvested and resuspended using resuspension buffer. Cells were lysed using lysis buffer and gently mixed for maximum 5 minutes. Appropriate volume of neutralization buffer was added and was mixed thoroughly. The cell lysis mixture was centrifuged at 13000 rpm for 10 minutes and supernatant was collected. Meanwhile the plasmid column was prepared by passing column binding solution followed by centrifugation to remove the supernatant. The cell lysate obtained was passed through the column (twice for effective binding of plasmid to the column) followed by centrifugation at 13000 rpm for 1 minute. Washes were given using wash buffer containing ethanol and finally eluted with desired volume of elution buffer by centrifugation.

2.1.3 Transformation:

Competent cells were thawed on ice for 5 min just before the addition of 100 ng of DNA to be transformed followed by incubation on ice for 30 min. The cells were given heat shock for 90 seconds at 42°C and immediately incubated on ice for 5 min. The cells were kept for recovery after adding 1 ml of LB medium,

and grown at 37°C under shaking conditions for 1 hour. The cells were then centrifuged at 11000 rpm for 2 min. Pellet was resuspended in 100µl of residual medium and the cells were plated on to LB agar plate containing appropriate antibiotic and incubated at 37°C overnight.

2.1.4 Estimation of nucleic acid and proteins:

- Nucleic acid concentration estimation was done spectrophotometrically by measuring the absorbance of DNA solution dissolved in autoclaved MilliQ water at 200mm wavelength (A_{260}). The concentration was calculated according to Beer-Lambert's Law.

$$C = A_{260}\,\theta$$

Where, C is the concentration of the nucleic acid in ng/µl. For DNA θ is 50 ng/µl, for oligonucleotide it is 33 ng/µl, whereas for RNA it is 40 ng/µl.

- Densitometric estimation was done for the recombinant proteins by running different concentrations of BSA along with the protein of interest in a SDS- PAGE gel.

2.1.5 Agarose gel electrophoresis:

Agarose gel electrophoresis was performed to analyze DNA/RNA samples. Indicated percentage of agarose was added to 1X TBE (0.09M Tris Borate and 0.002M EDTA) and dissolved by melting in microwave. Samples were prepared in 1X loading buffer (0.25% Bromophenol blue, 0.25% Xylene cyanol in 40% sucrose) and loaded on to the gel. Samples were electrophoresed at different voltages for varying time periods as required. Gels were stained with Ethidium Bromide (10µg/100ml H_2O) with gentle rocking. Nucleic acids were visualized on U.V. transilluminator in gel documentation system (Biorad).

2.1.6 Poly acrylamide gel electrophoresis:

a. Native PAGE: Native PAGE was employed to separate small oligonucleotides according to molecular weight of the samples. 6% gel was prepared. Samples were prepared in 1X EMSA buffer (20mM HEPES pH 7.9, 25mM KCl, 0.1mM

EDTA, 10% Glycerol, 2mM $MgCl_2$) along with 0.5% NP40, 1µg/µl BSA, 3µg/ml Poly d[I-C] and loaded on preequilibrated (0.5X TBE) gels. The gels were then dried and were kept for exposure against X-ray film.

b. SDS-PAGE: SDS – PAGE was performed to separate the proteins according to their molecular weight to check the induction profile and analyze the purity of the protein samples. Resolving gels were made in various percentages of acrylamide (Stock : 30%, Acrylamide : Bis acrylamide – 29:1) along with 0.375 M Tris pH 8.8, 0.1% SDS, 0.1% APS and 8 % TEMED. Stacking gels were made with 5% acrylamide along with 0.375 M Tris pH 6.8, 0.1% SDS, 0.1% APS and 8 % TEMED above the separating gel. Protein samples were made in 1X sample buffer (50 mM Tris-HCl pH 6.8, 100 mM DTT, 0.1 % bromophenol blue, 10% glycerol) boiled at 90°C for 5 min before loading on to the gel. Gels were electrophoresed using Tris-Glycine buffere (25 mM Tris, 250 mM Glycine pH 8.3, 0.1% SDS). Gels were visualized by staining with coomassie (45% methanol, 10% acetic acid, 0.25% bromophenol blue) followed by destaining with destaining solution (30 % methanol, 10 % acetic acid in H_2O)

2.1.7 Western Blot Analysis:

The separated proteins on the SDS-PAGE gel were blotted to PVDF membrane using semidry western apparatus. Initially the gel was washed with transfer buffer (25mM Tris, 192mM glycine, 0.038% SDS, 20% methanol v/v) for 30 minutes on a rocker. The PVDF membrane was activated by soaking in methanol for 1 minute followed by a wash with transfer buffer. The proteins were transferred to the membrane at 25V for appropriate time period according to the size and nature of the protein. The nonspecific sites were blocked by using 5% skimmed milk at 4°C at room temperature for 2 hours. The blot was then incubated with primary antibody in 2.5% skimmed milk in 1X PBS at 4°C overnight. The blot was washed with wash buffer according to standardized conditions for each primary antibody. Further the blot was incubated with appropriate secondary antibody conjugated with HRP in 2.5% skimmed milk in 1X PBS for 2 hours at room temperature. The membrane was washed and the blot was developed using Pierce Super Signal West Pico Chemiluminiscent kit as described by the manufacturer.

The blot was exposed to X-ray films for different time points and developed using Developer-Fixer.

2.1.8 Mammalian cell culture:

H1299 ($p53^{-/-}$) and UMSCC ($p53^{-/-}$) cells were maintained at 37°C in Roswell Park Memorial Institute medium (RPMI) and Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and appropriate antibiotics in 5% CO_2 incubator. For cell storage, approximately 2 million cells were suspended in 1ml of 50% FBS, 10% DMSO and 40% incomplete media. The temperature of the cells was brought down gradually to -80°C with the help of thermo cooler and finally cells were stored in liquid nitrogen for long term storage. Cells were revived by keeping the vials at 37°C for 3 minutes followed by washing with 10 ml of RPMI/DMEM to remove DMSO. Cells were further seeded in 25mm flask for maintenance. the protein.

2.1.8 Immunofluorescence:

Cells were grown on cover slips coated with poly-Lysine at 37°C in a 5% CO_2 incubator. After the transfection cells were washed with PBS and fixed with 4% paraformaldehyde (in PBS) for 20 min at room temperature. Cells were then permeablized using 1% Triton X-100 (in PBS) for 10 min and subsequently washed with PBS for 10 min, 3 times. Nonspecific sites were blocked using 5% FBS (in PBS) for 45 min at 37°C. Immunostaining was done with primary antibody at appropriate dilutions for 1 hour at room temperature. The cells were washed with wash buffer (1% FBS in PBS) 4 times, 3 min each. Primary antibody stained cells were incubated with secondary antibody tagged with fluorescent dye at appropriate dilutions for 1 hour at room temperature. After washes with wash buffer the nuclei were stained with Hoechst (1:1000 dilution) for 20 min. Two times PBS washed cover slips were inverted on to a microscope slide over a 2µl of 70% glycerol (in PBS) and visualized using confocal microscope.

2.1.9. Silver staining:

SDS PAGE Gel was fixed in fixing solution (40% methanol, 10% acetic acid in double distilled water) overnight. Gel was rinsed in 50% ethanol (in ddH_2O) for 10

min thrice followed by sensitising it for 2 min in Sensitising solution (0.02% sodium thiosulphate in ddH_2O). Gel was rinsed in ddH_2O for 1 min followed by incubation for 20 min in Staining solution (0.1% silver nitrate in ddH_2O) and rinsing in water for 10 sec thrice. Gel was developed in Developing Solution (0.04% Formalin, 2% sodium carbonate in ddH_2O). Development was arrested by 1% acetic acid (in ddH_2O)

2.2 Subcloning of DBD of wt p53 and mutant p53 (P152L) from pRSET(A) vector into pET28a+ vector containing cleavable His-Tag:

Untagged DBD of wild type and mutant p53 (P152L) was cloned into the pET28a+ bacterial expression vector for the expression of cleavable His_6 tag wild type and mutant p53 (P152L) DBD protein. Wt and mt p53 DBD insert was released from pRSET(A) vector using NdeI and EcoRI as restriction enzymes. pET28a+ vector was linearized using the same restriction enzymes. The released insert was ligated using T4 DNA ligase and the ligated product was transformed into XL-10 Gold competent cells and clones were obtained. Confirmation of the clones were done by insert release after restriction digestion of cloned pET28a+ vector and also by sequencing.

2.3 Protein purification:

2.3.1 Purification of FLAG-p53 and FLAG-p53 P152L :

FLAG tagged wt and mutant FLAG-p53 (P152L) were purified using M2- Agarose affinity chromatography. BL 21 DE3 cells transformed with clones containing full-length wild type p53 and mutant p53 P152L nucleotide sequences were grown overnight at 37°C in 100 ml LB containing 100 µg/ml ampicillin. Secondary inoculation was done with the overnight 100 ml culture in to 900 ml LB containing 100 µg/ml ampicillin and grown at 30°C till the O.D. reached 0.6. The culture was then induced with IPTG (1 mM) and grown at 30°C for further 5 hours. Cells were harvested by centrifuging at 6000 rpm for 10 min at 4°C. The cell pellet was resuspended in BC 300 and the sonication was done at an amplitude of 35 for 1 min (3 sec ON / 5 sec OFF) for 3 times with a gap of 5

min between each sonication cycle. Lysates were cleared of debris by centrifuging at 16,000 rpm for 30 min and the supernatant was centrifuged again for 30 min at 16,000 rpm. The cleared cell lysates were incubated with pre- equilibrated M2-Agarose (with BC 300) for 4 hours at 4°C on a rotary shaker. Beads were pelleted by centrifugation at 2000 rpm for 5 min and washed with 10 ml of BC 300 for 5 times followed by 10 ml of BC 100 for 5 times. The beads were then collected in vials and spun to remove residual buffer. Elution of the FLAG- tagged protein was done by mild vortexing of the beads with elution buffer (BC 100 buffer containing 0.4 mg / ml FLAG peptide) at 4°C for 30 min followed by quick spin. Same process was repeated again to ensure complete elution of the protein.

2.3.2 Purification of Native p53 DBD:

Untagged recombinant p53 DBD protein was purified from *E.Coli*. BL 21 (DE3) cells that were transformed with pRSET(A) vector containing wt p53 DBD construct, using heparin sepharose ion exchange chromatography. BL 21 (DE3) cells transformed with clone containing wt p53 DBD were grown overnight at 37°C in 100 ml of LB containing 100µg/ml ampicillin. Secondary inoculation was done with overnight 100 ml culture in to 900 ml LB containing 100µg/ml ampicillin and grown at 37°C till the OD_{600} reached 0.8. The culture was then induced with IPTG (1 mM) and grown at 30°C for further 5 hours. Cells were harvested by centrifuging at 6000 rpm for 10 min at 4°C. The cell pellet was resuspended in BC 300 and the sonication was done at an amplitude of 35 for 1 min (3 sec ON / 5 sec OFF) for 3 times with a gap of 5 min between each sonication cycle. Lysates were cleared of debris by centrifuging at 16,000 rpm for 30 min and the supernatant was centrifuged again for 30 min at 16,000 rpm. The lysate was poured into 50 ml falcon containing activated heparin sepharose swollen beads (0.4 gm of heparin sepharose was incubated in 5 ml of H_2O overnight) and kept for binding on end to end rotor at 4°C for 5 hours. Content of the falcon was poured into the column containing filter and beads were allowed to settle down. Entire content was allowed to pass through the column filter and flow through was collected. The column was the washed twice with 10 ml each of BC 300 buffer and eluted with 2.5 ml of BC 500 in five fractions of 500 µl each. 1 ml of BC

500 was again poured into the column and eluate was collected after 4 hours (two fractions of 500µl each).

2.3.3 Purification of mutant p53 (P152L) DBD:

Untagged recombinant mutant p53 DBD protein was purified from *E.Coli*. BL 21 (DE3) cells that were transformed with pRSET(A) vector containing mt p53 DBD construct, using SP sepharose ion exchange chromatography. BL 21 (DE3) cells transformed with clone containing mt p53 DBD were grown overnight at 37°C in 100 ml of LB containing 100µg/ml ampicillin. Secondary inoculation was done with overnight 100 ml culture in to 900 ml LB containing 100µg/ml ampicillin and grown at 37°C till the OD_{600} reached 0.8. The culture was then induced with IPTG (1 mM) and grown at 22°C for 9 hours. Cells were harvested by centrifuging at 6000 rpm for 10 min at 4°C. The cell pellet was resuspended in BC 0 and the sonication was done at an amplitude of 35 for 1 min (3 sec ON / 5 sec OFF) for 3 times with a gap of 5 min between each sonication cycle. Lysates were cleared of debris by centrifuging at 16,000 rpm for 30 min and the supernatant was centrifuged again for 30 min at 16,000 rpm. 0.5 ml of SP sepharose slurry was poured into the column and washed twice with 5 ml of BC 0 buffer for equilibration. Cell lysate was mixed with SP sepharose and kept for binding in end to end rotor for 5 hours at 4°C. Column was fixed vertical and beads were allowed to settle down. Cell lysate was made to flow through and collected. Beads were then washed with 10 ml of BC 0 and BC 100 buffer. Elution was done with 600 µl (3 fractions each of 200 µl each) of BC 200, 300, 500, 850 with intermittent washing with 1 ml of the respective buffer after elution was performed with same buffer.

2.3.4 Purification of His-p53 DBD and His-p53 DBD (P152L) :

N-terminal His_6-tagged recombinant p53 DBD protein (wild type and mutant) was purified from *E.Coli*. BL 21 (DE3) cells that were transformed with pET28a+ vector containing wt p53 or mutant p53 (P152L) DBD construct, using Ni-NTA column chromatography. BL 21 (DE3) cells transformed with clone containing wt/mt p53 DBD were grown overnight at 37°C in 100 ml of LB containing 50µg/ml kanamycin. Secondary inoculation was done with overnight 100 ml culture

in to 900 ml LB containing 50µg/ml kanamycin and grown at 37°C till the OD_{600} reached 0.8. The culture was then induced with IPTG (1 mM) and grown at 22°C for 9 hours. Cells were harvested by centrifuging at 6000 rpm for 10 min at 4°C. The cell pellet was resuspended in homogenization buffer (20mM Tris HCl pH 7.4, 20% Glycerol, 0.2mM EDTA, 300 mM KCl, 0.1% NP-40, 20mM Imidazole, 2 mM PMSF, 2 mM β-mercaptoethanol) and the sonication was done at an amplitude of 35 for 1 min (3 sec ON / 5 sec OFF) for 3 times with a gap of 5 min between each sonication cycle. Lysates were cleared of debris by centrifuging at 16,000 rpm for 30 min and the supernatant was centrifuged again for 30 min at 16,000 rpm. The cleared cell lysate was incubated with pre-equilibrated (homogenization buffer) Ni-NTA beads for 4 hours at 4°C. Beads were pelleted by centrifugation at 2000 rpm for 5 min and washed with 10 ml of wash buffer (20mM Tris HCl pH 7.4, 20% Glycerol, 0.2mM EDTA, 600 mM KCl, 0.1% NP-40, 40mM Imidazole, 2 mM PMSF, 2 mM β-mercaptoethanol) for 10 times. After the last wash some amount of wash buffer was kept in the falcon in which beads were mixed and collected in microfuge tube. The tube was given a brief spin and buffer was discarded. 200µl of elution buffer (20mM Tris HCl pH 7.4, 20% Glycerol, 0.2mM EDTA, 100 mM KCl, 0.1% NP-40, 250mM Imidazole, 2 mM PMSF, 2 mM β-mercaptoethanol) was added to the beads and they are incubated at 4°C for 15 min in an end to end rotor. After incubation beads were centrifuged at 2000rpm for 1 min and supernatant was collected as eluate. Four more elutions were taken likewise.

2.4 DNA- Protein interaction study :

2.4.1. Electrophoretic Mobility Shift Assay (EMSA):

a. DNA probe labelling:

T4 Poly Nucleotide Kinase and PNK buffer was added in water followed by the addition of DNA probe (40 p moles) and ^{32}P- γ- ATP and incubated at 37°C for 30 min. Enzyme was heat inactivated at 65°C for 20 min. To 50µl of reaction mixture, 150µl of Tris-EDTA (pH 8.0) was added. To this equal volume of phenol:chloroform was added. Reaction mixture was then centrifuged at 13,000 rpm for 10 min. To the supernatant 1µl of tRNA and 1/10 th volume (18.5 µl) of 3M sodium acetate and 600µl of absolute ethanol was

added. Reaction mix was incubated at -20°C for 3-4 hours or in -80°C for 1 hour. Pellet was obtained by centrifugation at 13,000 rpm for 30 min. Pellet was dried and resuspended in 30 µl of 1X annealing buffer to which 1µl of complementary oligo was added and allowed to anneal from 85°C decreasing to room temperature overnight.

b. Purification of labelled probe:

Sephadex G-50 was dissolved in H_2O (0.5 gm in 50 ml of H_2O) and was left for soaking overnight. Glass wool was packed upto 1mm of the 1ml syringe and 200 µl of 50% slurry was added and the syringe was centrifuged at 1000 rpm for 2 min. This was repeated till 1ml of column was packed. Syringe column was washed with water thrice and centrifuged for 10 min so that no residual water is left behind in the column. The labelled probe was then added and centrifuged at 2000 rpm for 2 min.

c. Sample preparation and Native PAGE :

6% native polyacrylamide gel was casted and allowed to polymerize for 2 hours. Gel was mounted in the vertical electrophoresis apparatus and tank reservoirs were filled with 0.5 X TBE buffer. Pre electrophoresis was conducted at 10 V / cm of the gel length for 1 hr at 4°C during which samples were prepared. 30 base pair radiolabeled oligonucleotide (concentration – 10000 cpm / µl) containing consensus p53 binding site (GADD 45 promoter sequence) was incubated in a reaction volume of 40 µl containing 8 µl of EMSA buffer (100 mM HEPES pH 7.9, 125 mM KCl, 0.5 mM EDTA, 50% glycerol, 10 mM $MgCl_2$), 2 µl of 60 µg / ml double stranded poly dI- dC, 4 µl of BSA (1 mg / ml), 2 µl of NP 40, 2 µl of DTT with the proteins as indicated for 30 min at 30°C. Samples were analyzed on 6% native PAGE containing 0.5 X Tris-Borate- EDTA buffer and electrophoresed at 4°C for 2 hours (200V). The gel was dried at 85°C for 1 hour and exposed to X-ray film at - 80°C overnight and film was then developed.

2.5. Gain of function experiments:

2.5.1 Generation of H1299 stable cell line

Antibiotic Kill Curve - Cells were seeded in 6 well plate and allowed to grow till 60-80% confluency. Increasing amount of antibiotic G418 (200µg/ml- 1000 µg/ml). RPMI media was replaced every 3 days for about a week. Concentration of antibiotic at which all the cells in a plate are dead is taken for positive antibiotic selection for transfection.

Stable Transfection - Cells were seeded in 30 mm dish to grow till 70% confluency. Media change was given just before transfection. Both plasmid (1µg) and lipofectamine were diluted in equal volume (75µl) of incomplete RPMI media. Diluted DNA was added to diluted lipofectamine 2000 reagent after 5 min and the reaction mix was incubated for 20 min after which DNA-lipid complex was added to the cells.

Selection and Expansion - 48 hrs post transfection G418 antibiotic was added and media containing G418 was changed in every 3 days and cells were examined for visual toxicity daily for 14 days. Remaining live cells were expanded in 60 or 100mm dish with continuous antibiotic treatment. Protein levels were checked by western blot and immunofluorescence.

2.5.2. Scratch assay:

Cells were seeded in 30mm dishes to create a confluent monolayer. After the confluency was achieved, cell monolayer was scrapped in a straight line to create a "scratch" with a p20 pipette tip. Cell debris was removed and the edge of the scratch was smoothened by washing the cells once with 0.5 ml of growth medium (RPMI) and then was replaced with 2 ml of complete medium. Scratches of similar size were created in both assessed cells and control cells. To obtain same field during image acquisition, markings were created to be used as reference points. Dishes was placed in the 5% CO_2 incubator at 37°C and images were acquired at time point of 0hr, 8hr, 16hr and 24 hr post scratch.

2.5.3. Colony formation Assay:

Cells harvested were counted using a haemocytometer and diluted such that 500 cells were seeded into 60mm dishes which were incubated for 2 weeks in a 5%

CO_2 environment at 37°C for colony formation. After 2 weeks of incubation, media was gently removed and the plates were washed with 1X PBS. Colonies were then fixed with 5ml of ice cold 70% methanol for 30 min and stained with 5ml of 0.01% (w/v) crystal violet in distilled water for 1 hr. Excess staining was removed by washing the dishes with distilled water. The dishes were allowed to dry and colonies were counted.

2.5.4. Apoptotic assay:

Cells were seeded in a 6 well plate and allowed to grow till they become 60-70 % confluent. Cells were treated with 100µM of 5-fluorouracil (5-FU) for 12 hours. After treatment, cells were harvested, spun and supernatant was discarded. Cells were resuspended in 1ml of 1X PBS and centrifuged at 1000rpm for 5 min. Cells were resuspended in 1 ml of Annexin V binding buffer and centrifuged for 5 min at 1000 rpm and resuspended again in 100µl of Annexin V binding buffer. 1 µl of Annexin V- FITC and 1 µl of Propidium Iodide (2µg/ml) was added to each of the sample. Reaction tubes were incubated for 15 min at 37°C. 500 µl of 1X Annexin V binding buffer was added and the cells were washed. Cells were resuspended in 500 µl of 1X Annexin V binding buffer and the samples were analyzed.

2.5.5. Non-Orthotopic xenograft mouse model study:

H1299 cells were subcutaneously injected into 8 week old female nude mice on left and right flank region with 1 million and 2 million cells respectively. Growth in tumor was observed for about a month followed by tumor extraction.

2.6. Gluteraldehyde cross linking:

200ng of recombinant protein was incubated for 30 min at room temperature in Buffer H containing 20 mM HEPES pH 7.9, 100 mM KCl, 0.5 mM EDTA, 10% glycerol, 0.5 mM PMSF with 0.005% Gluteraldehyde in a final reaction volume of 10 µl. Reaction was terminated by adding SDS gel loading dye and the reaction products were analyzed by 12% SDS PAGE followed by silver staining.

Chapter 3 Expression and Purification of Full length and DNA binding domain of wt and mutant p53 (P152L)

This chapter is about the protein expression and purification strategies used for the purification of untagged wt and mutant p53 DBD protein and flag tag full length wt and mut p53 protein. Also successful cloning of DBD construct into the vector with cleavable His-tag followed by its protein expression and purification is discussed.

CHAPTER OUTLINE:

3.1. Purification of untagged wt and mut p53 (P152L) protein

3.2 Purification of full length wt and mut p53 (P152L) protein

3.3 Cloning of wt and mut p53 DNA binding domain gene in pET28a+ vector

3.4 Purification of 6X His-tag wt and mut p53 DBD protein.

3.1. Purification of untagged wt and mut (P152L) p53 protein :

Modified pRSET(A) vector (without His-tag) containing wild type and mutant p53 DBD construct was transformed into bacterial strain BL21 DE3 designed for protein expression. First, the induction conditions for protein expression were standardized. Briefly, single colony was inoculated in a 5ml LB medium containing ampicillin as a resistant marker and grown overnight at 37°C. With 10% of the primary culture as the inoculum, the secondary culture of 100 ml was grown till the $O.D_{600}$ reaches 0.8 . 5 ml of the secondary culture was distributed in each glass tube and induced at two different concentrations of IPTG (0.5 mM and 1 mM) in four different temperatures of 30°C, 25°C, 22°C and 18°C for 3, 6, 9 and 12 hrs respectively. The expression was checked by comparing the laemelli

lysates with the uninduced culture upon SDS- PAGE. For wild type p53 DBD protein expression was seen at the induction condition of 30° / 1mM / 3hr and 22° / 1mM / 12hr whereas for mutant p53 (P152L) DBD expression was seen at the induction condition of 22° / 1mM / 12hr.

Since the p53 DBD protein is untagged, ion exchange chromatography technique was used for its purification. The pI of the DBD protein was found to be 8.4. So the protein is positively charged in the resuspension buffer whose pH is 7.4. Therefore, cation exchanger beads were used for DBD purification. For the wt p53 DBD protein heparin sepharose beads were used for purification whereas for mut p53 DBD SP sepharose beads were used. Following the induction conditions standardized, the protein was purified and checked for its concentration and integrity on 12 % SDS-PAGE. Figure 3.1 shows the purification profile of untagged wt and mut p53 DBD protein. From the figure, it can be evident that though the amount of protein is adequate, some amount of contamination can also be seen suggesting that the protein is not purely homogenous.

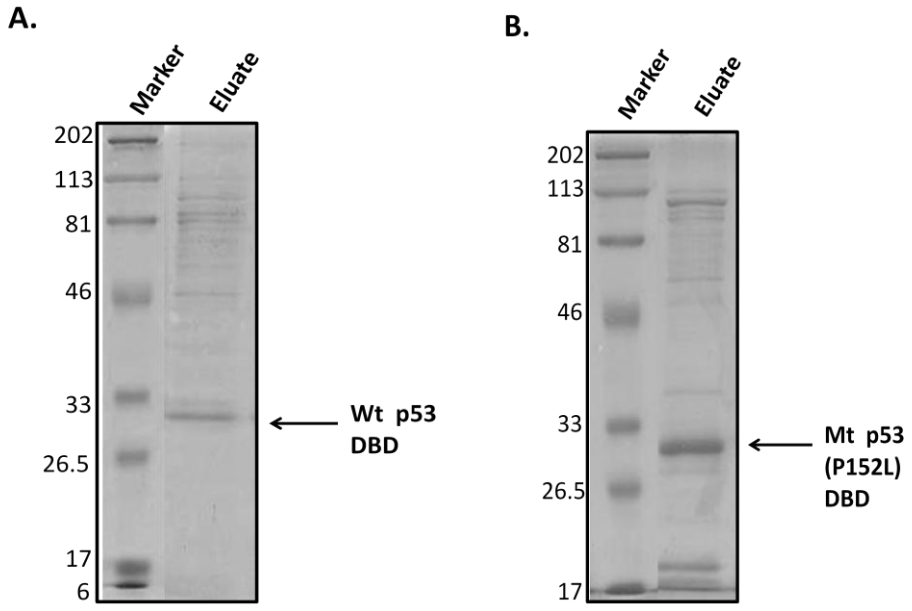


Figure 3.1 : Protein purification profile of (A) Untagged wt p53 DBD protein purified by heparin sepharose ion exchange chromatography and **(B)** Untagged mut p53 (P152L) DBD protein purified by SP sepharose ion exchange chromatography**.**

3.2 Purification of full length wt and mut (P152L) p53 protein :

Next purification of full length wt and mut (P152L) p53 protein was carried out for which induction conditions were standardized beforehand. The culture was induced with 1mM IPTG at 30°C for 4 hrs. Since the gene for full length wild type and mutant p53 protein was present in the pET 22b(+) vector containing flag tag, M2 agarose affinity chromatography was used to purify the protein.

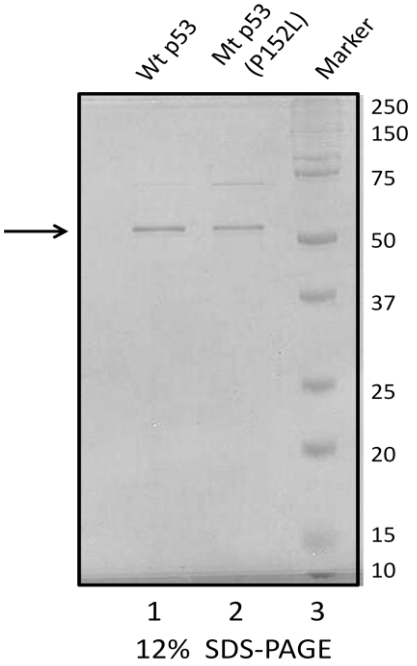


Figure 3.2 : Protein purification profile of flag-tagged wt and mut (P152L) p53 protein purified by M2 agarose affinity purification.

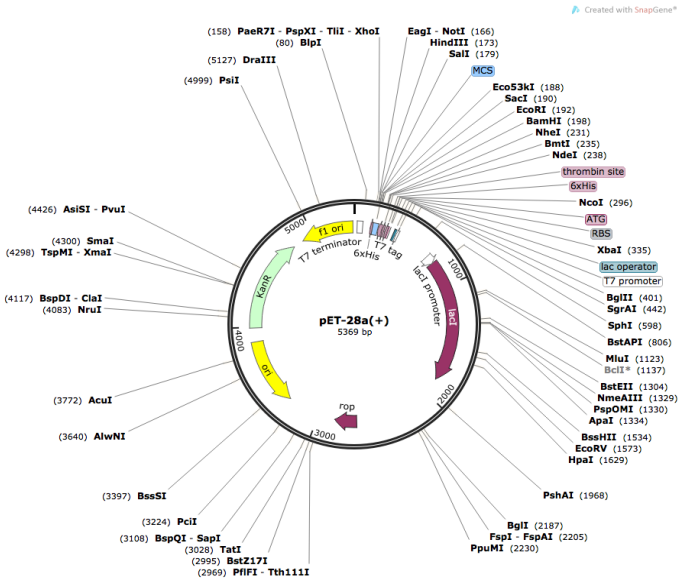
As shown in figure 3.2, the amount of both wt and mut p53 full length protein (flag - tagged) is adequate and the quality of the protein is in near homogeneity indicating its integrity and minimal contamination.

3.3 Cloning of wt and mut p53 DNA binding domain gene in pET28a+ vector :

Since the purification of untagged wt and mut p53 DBD protein yielded protein which was not purely homogenous (Figure 3.1), these two constructs were

subcloned from pRSET(A) vector into the pET 28a+ (Figure 3.3) bacterial expression vector. Briefly, in the pRSET(A) vector NdeI and EcoRI restriction site was present just upstream and downstream of start and stop codon respectively.

A.



B.

pRSET(A) vector

NdeI Ser Thr EcoRI
94 312
.....CATATGTCA.....ACCTGAATTC.....

pET28a+ vector (cloning/expression region)

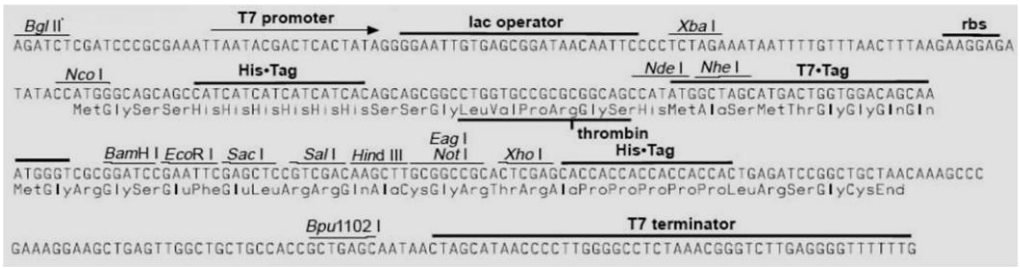


Figure 3.3 : Schematic representation of (A) pET 28a+ bacterial cloning and expression vector **(B)** Cloning / expression region of pET 28a+ vector and location of NdeI and EcoRI restriction sites in pRSET(A) vector

The same two restriction sites were also present in pET28a+ vector downstream of six histidine residues and thrombin cleavage site (Figure 3.3). Hence without any amplification of the construct, both the pRSET(A) vector and pET 28a+ vector were digested with NdeI and EcoRI restriction enzymes for the release of DBD insert and vector linearization respectively. The released insert from the pRSET(A) vector

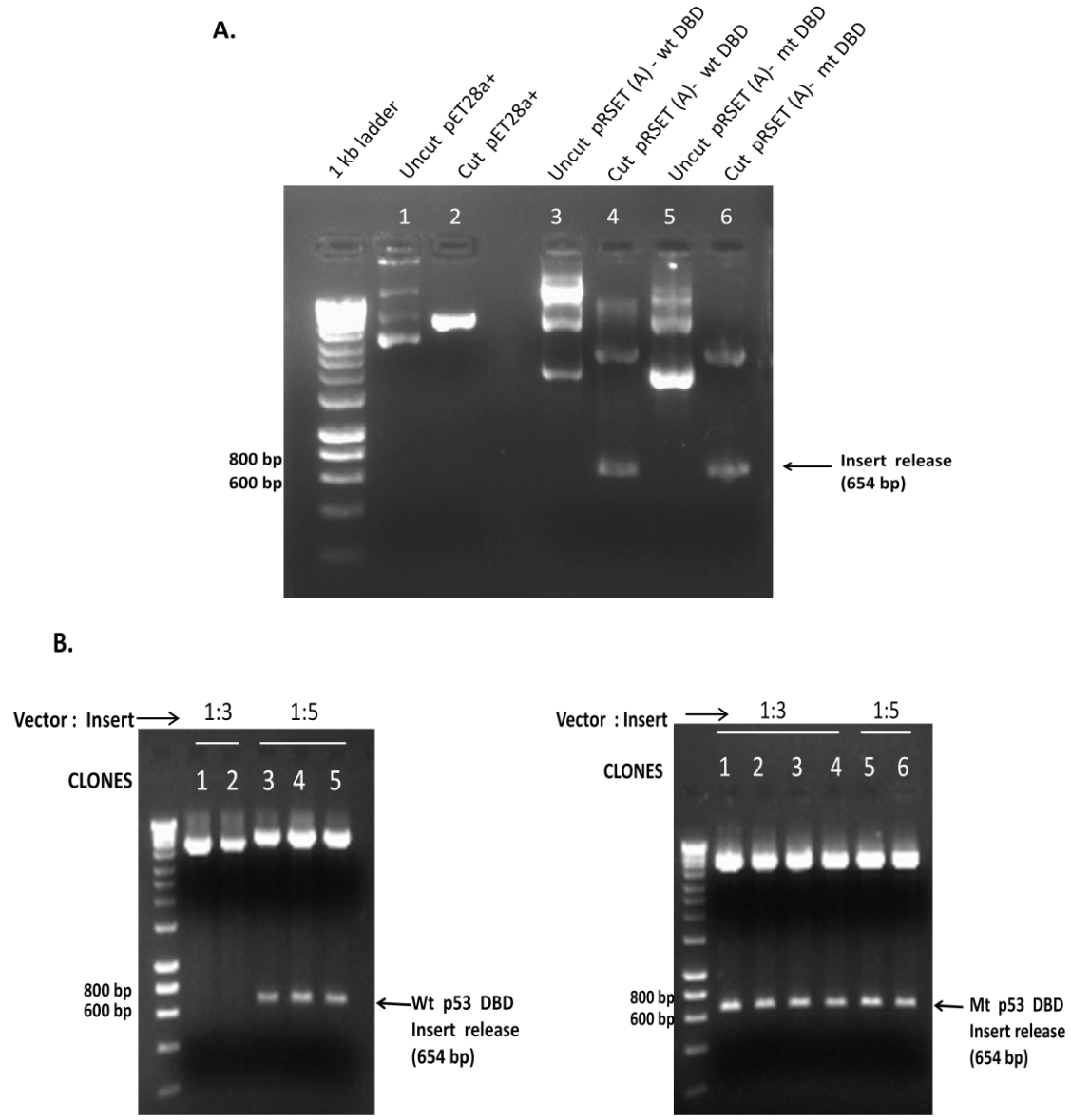


Figure 3.4 : Cloning of wt and mut p53 DBD : (A) Restriction digestion of pRSET(A) and the pET 28a+ vector by NdeI and EcoRI **(B)** Conformation of clones by restriction digestion and insert release

was ligated. The released insert from pRSET(A) vector was ligated (vector : insert - 1:3 and 1:5) into linearized pET 28a+ vector using T4 DNA ligase and the ligated product was transformed into XL10 Gold competent cells and clones were obtained. Conformation of the clones were done by both restriction digestion and checking for the insert release (Figure 3.4) and also by sequencing.

3.4 Purification of 6X His-tag wt and mut p53 DBD protein :

After successful cloning, purification of His-tag wild type and mut p53 protein was carried out using Ni-NTA column chromatography. Induction conditions for protein expression was kept the same as used in the purification of untagged wt and mut p53 DBD protein. After purification protein samples were dialyzed for imidazole and were analyzed on 12% SDS-PAGE. Figure 3.5 shows the purification profile of his_6- wt and his_6-mut p53 DBD protein. It is clear from the figure that the amount of protein obtained is adequate and the protein is homogenous with no visible contamination.

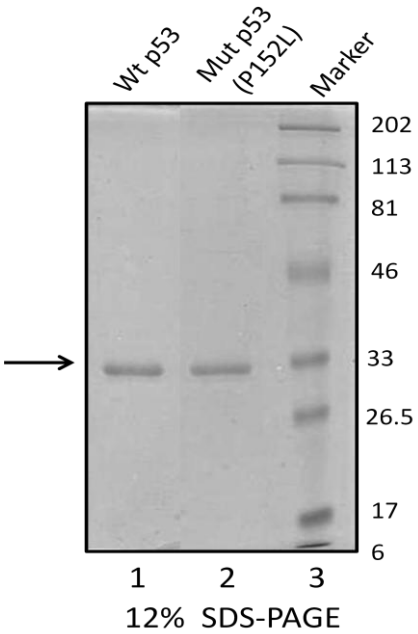


Figure 3.5 : Protein purification profile of 6X Histidine-tagged wt and mut p53 (P152L) DNA binding domain protein purified by Ni-NTA column chromatography.

Chapter 4 DNA BINDING PROPERTIES OF MUTANT p53 (P152L)

This chapter unravels the impact of P152L mutation in p53 in terms of its effect on the DNA binding ability of p53 protein, the kind of distortion in protein structure it undergoes due to this mutation. Additionally, tetramer formation ability of this protein has also been looked upon.

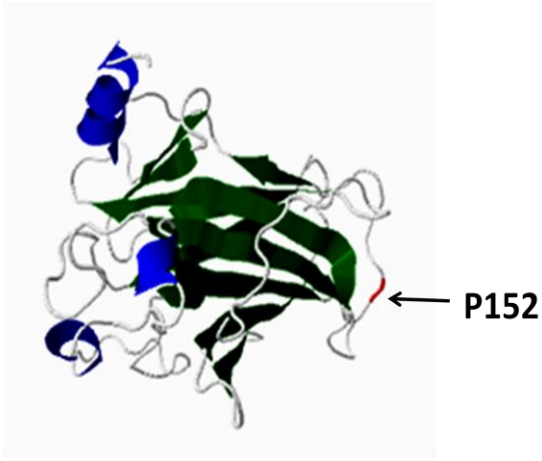
CHAPTER OUTLINE:

4.1. Introduction :

While screening the cancer patient samples for the mutation in p53, a rare proline to leucine mutation at 152nd position in the DNA binding domain region of p53 was identified in an oral cancer patient sample. The overexpression of mutant p53 harbouring this mutation was picked up by PAb 240 antibody which recognizes only the conformational mutant of p53 under non denaturation condition. This is because this antibody is raised against 156-214 amino acids of p53 of human origin which get exposed only if there is a distortion in the structure of the p53 protein, thereby exposing these buried residues to be recognized by PAb 240. From the location of P152L mutation in the three dimensional structure of p53 core domain-DNA complex, it can be clearly deduced that although this mutation resides in the DBD region of p53, still the location of this mutant residue is in

the loop region (figure 4.1) which is far from the protein surface which is in contact with the DNA. (Roberta et al, 2010) (55,56)

A.



B.

Info about mutation R273H:

Accessibility [0-1]	0.78
Conservation [0-1]	1.00
Average energy [0-1]	0.47
Specific energy [0-1]	0.28
Similar amino acids [-1/-0.5/0]	-1.00
Size change [0-1]	0.03
Hydrophobicity change [0-1]	0.14
Surrounding amino acids [0-1]	0.44
2D-structure [0/1]	1
Polarity change [0/1]	0
Pocket [0/1]	1
DNA or zinc-binding [0/1]	1
Severity score (cut-off 60.18)	87.26
Predicted class	Severe

Info about mutation R175H:

Accessibility [0-1]	1.00
Conservation [0-1]	1.00
Average energy [0-1]	0.76
Specific energy [0-1]	0.54
Similar amino acids [-1/-0.5/0]	-1.00
Size change [0-1]	0.03
Hydrophobicity change [0-1]	0.14
Surrounding amino acids [0-1]	0.33
2D-structure [0/1]	0
Polarity change [0/1]	0
Pocket [0/1]	1
DNA or zinc-binding [0/1]	0
Severity score (cut-off 60.18)	92.55
Predicted class	Severe

Info about mutation P152L:

Accessibility [0-1]	0.67
Conservation [0-1]	1.00
Average energy [0-1]	0.13
Specific energy [0-1]	0.13
Similar amino acids [-1/-0.5/0]	0.00
Size change [0-1]	0.22
Hydrophobicity change [0-1]	0.60
Surrounding amino acids [0-1]	0.45
2D-structure [0/1]	0
Polarity change [0/1]	0
Pocket [0/1]	0
DNA or zinc-binding [0/1]	0
Severity score (cut-off 60.18)	78.37
Predicted class	Severe

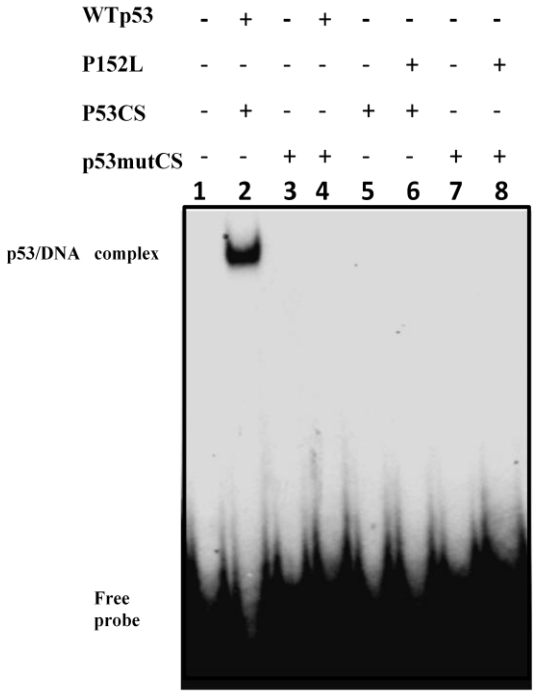
Figure 4.1 : (A) Cartoon representation of p53 protein showing position of the mutated residue marked in red in the 3-D structure model generated by JSmol based on PDB id 1TSR. **(B)** Table showing the structural effects of mutation on p53 (Table generated from p53 mutational browser - http://persson-group.scilifelab.se/p53/)

From the table (Figure 4.1) showing the structural effects of three mutations - R273H (DNA contact mutant), R175H (Conformational mutant) and P152L, it is clearly evident that the hydrophobicity change on scale of 0-1, which is 0.6 for P152L, is significantly more than that of other two hotspot mutations (0.14). This

is an indicative of a significant distortion in the protein structure due to P152L mutation. Taking all above observations into consideration we hypothesized that P152L mutation is a conformational mutant rather than DNA contact mutant and performed DNA binding experiments to confirm this hypothesis as well as to look upon the level at which (local or global) the distortion in protein structure occurs.

4.2. P152L mutation abrogates the DNA binding ability of the full length mutant p53 protein

Upon DNA damage, p53 induces the expression of its downstream target genes such as GADD-45 and 14-3-3σ to elicit cell cycle arrest. In order to assess the impact of P152L mutation on the DNA binding ability of the full-length p53 protein, EMSA was performed with a radiolabelled oligonucleotides of 30bp of GADD45 promoter containing p53-responsive elements.



GADD45 - p53 CSFP : 5' TACAGAACATGTCTAAGCATGCTGGGGACT 3'

Figure 4.2 : Abrogation of the DNA binding ability of mut p53 (P152L) : [γ- ^{32}P] - labelled oligonucleotide containing a p53 binding site was incubated with 100ng of flag-tag wt p53 (lane 2) and flag-tag mut p53 (lane 6). Similarly [γ- ^{32}P] - labelled oligonucleotide containing mutant consensus sequence was incubated with 100ng of flag-tag wt p53 (lane 4) and flag-tag mut p53 (lane 8). Lane 1 and 5 contains [γ- ^{32}P] - labelled p53 consensus sequence alone whereas lane 3 and 7 contains [γ- ^{32}P] - labelled mutant consensus sequence alone. Below the figure GADD45 consensus sequence is shown in 5' – 3' direction.

Bacterially expressed flag tagged wild type and mutant p53 (P152L) protein were used. GADD45 mutant consensus sequence was also tested as a negative control to rule out non specific DNA binding of the protein. As seen clearly from the figure 4.2, flag-tag wt p53 formed the sequence specific DNA-protein complex, whereas flag-tag mut p53 (P152) did not (lane 2 versus lane 6). Both the protein did not form any complex with the mutant oligonucleotide indicating that the binding of wt p53 protein is sequence specific. Therefore, P152L mutation leads to abrogation of DNA binding ability of p53.

4.3. DNA binding ability of the DBD of p53 is unaffected by P152L mutation :

In continuation with the above result we decided to examine the effect of P152L mutation on the DNA binding ability of DNA binding domain of p53. For this,

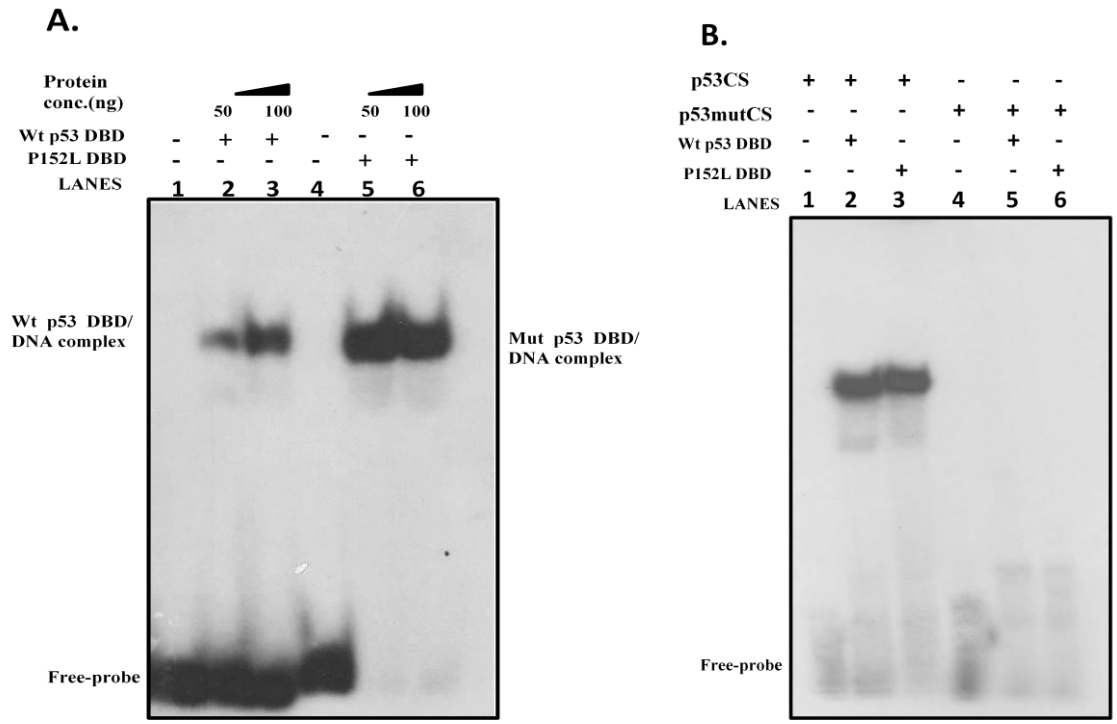


Figure 4.3 : DNA binding ability of the p53 DBD unaffected by P152L mutation : (A) [γ- ^{32}P] - labelled oligonucleotide containing a p53 binding site was incubated with increasing concentrations (50 ng and 100 ng) of untagged wt p53 DBD (lane 2 and 3) and untagged mut p53 DBD (lane 5 and 6). Lane 1 and 4 contains [γ- ^{32}P] - labelled oligonucleotide alone. **(B)** [γ- ^{32}P] - labelled oligonucleotide containing a p53 binding site was incubated with 50ng of untagged wt p53 DBD (lane 2) and untagged mut p53 DBD (lane 3). Similarly [γ- ^{32}P] - labelled oligonucleotide containing mutant consensus sequence was incubated with 50ng of untagged wt p53 DBD (lane 5) and untagged mut p53 DBD (lane 6). Lane 1 contains [γ- ^{32}P] - labelled p53 consensus sequence alone whereas lane 4 contains [γ- ^{32}P] - labelled mutant consensus sequence alone.

EMSA was performed with a 30-bp GADD45 promoter containing p53-responsive elements. Bacterially expressed untagged wild type and mutant p53 (P152L) DBD protein were used. GADD45 mutant consensus sequence was also tested as a negative control to rule out non specific DNA binding of the protein. As shown in figure 4.3 (A) both wild type and mutant p53 DBD were able to form DNA-protein complex (lane 2,3 and lane 5,6). As clearly evident from figure 4.3 (B) both the wt and mut p53 DBD protein did not form any complex with the mutant oligonucleotide sequence indicating that binding of both the proteins is sequence specific.

From these observations, it becomes evident that although the full-length mutant p53 (P152L) loses its DNA binding ability, the structural changes due to the proline to leucine mutation in the DBD region is not sufficient to abrogate the DNA binding ability of DNA binding domain. But as we can see from figure 4.2 that the DNA binding ability of full-length p53 is lost upon P152L mutation. Hence it can be deduced that P152L mutation results in global distortion instead of creating any significant local structural alterations.

4.4. Tetramer formation ability of full length p53 protein is unaltered by P152L mutation :

Since p53 binds to DNA as a homotetramer, so we wanted to examine the impact of P152L mutation on the tetramer formation ability of the mutant p53 protein. For this we performed gluteraldehyde cross linking experiment followed by silver staining. The principle behind this assay is that gluteraldehyde would cross link two monomeric protein subunits only if they interact and bind with each other. First we determined the lowest concentration of gluteraldehyde required to induce tetramer formation in full-length wild type p53. Various concentrations of gluteraldehyede were checked - 0.025% , 0.010% , 0.005% , 0.0025% , 0.0010% . As shown in figure 4.4 (A), tetramer formation was seen when the gluteraldehyde concentration was 0.005% and above (lanes 4, 3 and 2) whereas no band corresponding to tetramer was visible in untreated control. Therefore, 0.005% was chosen as a threshold concentration of gluteraldehyde for tetramer formation. The incubation time after treatment was 30 minutes.

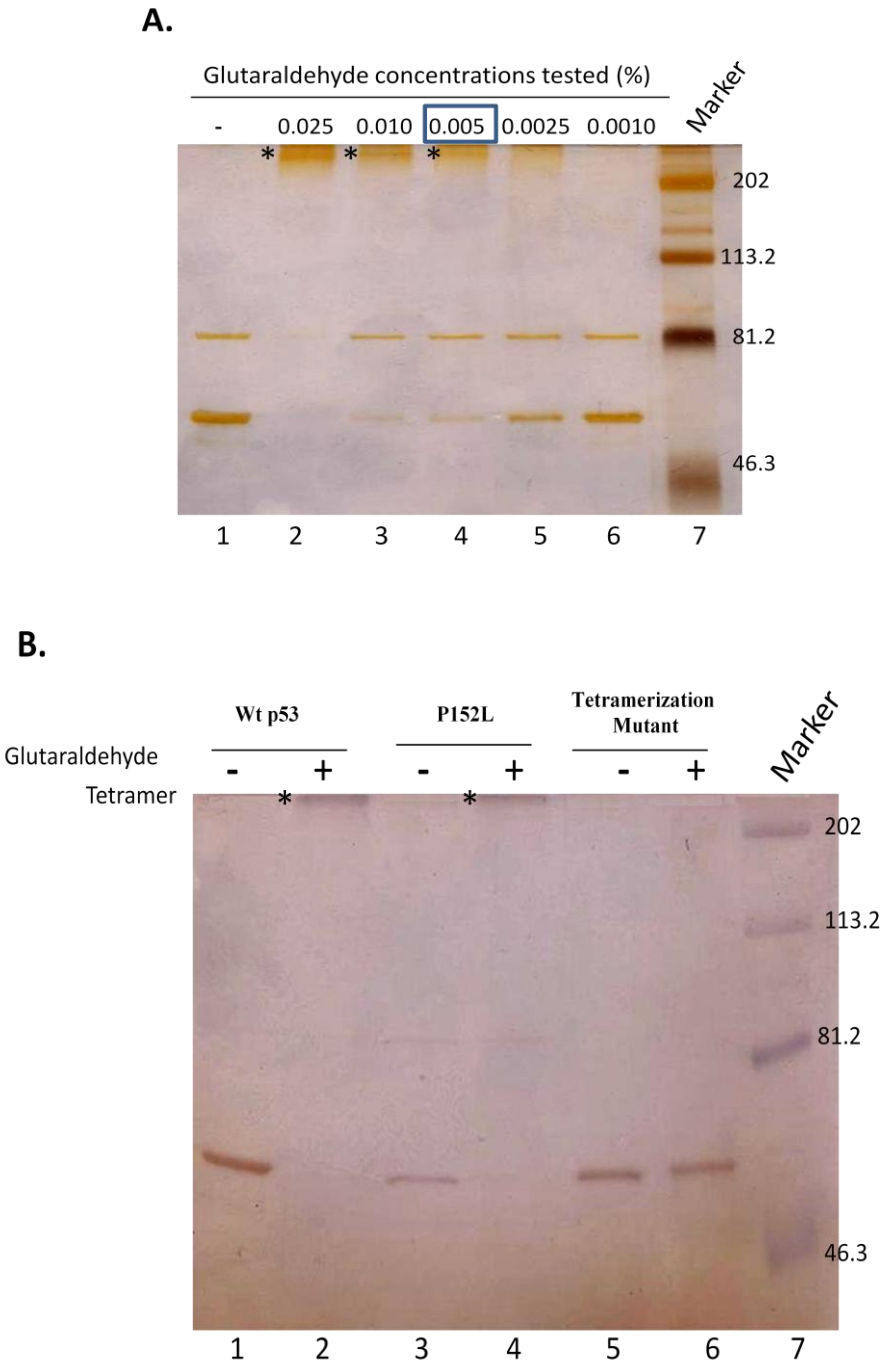


Figure 4.4 : Full- length mutant p53 (P152L) retains the ability to form tetramer : (A) Gluteraldehyde cross linking profile of full-length wt p53 protein with decreasing concentrations of gluteraldehyde (lane 2 - lane 6). Lane 1 contains wt p53 without gluteraldehyde. Position of tetramer is marked with asterisk (*). **(B)** Gluteraldehyde cross linking profile of full-length wt (lane 1 and lane 2) and mut p53 (lane 3 and lane 4) protein. A tetramerization mutant of p53 (L344A) was taken as a negative control (lane 5 and lane 6). Gluteraldehyde concentration used in the assay was 0.005% (+, treated ; -, untreated). Position of tetramer is marked with asterisk (*)

In the next experiment using the gluteraldehyde concentration of 0.005% , cross linking experiment was performed for both full-length wild type and mutant p53 (P152L) protein. A tetramerization mutant of p53 which has a single point mutation from leucine to alanine at 344^{th} position (tetramerization domain) was taken as a negative control, since this mutant is unable to form tetramer.

Interestingly, from the figure 4.4 (B), for both full-length wild type and mutant p53 (P152L), the disappearance of bands in the lanes treated with gluteraldehyde (lane 2 and 4) was observed and a band corresponding to the p53 tetramer was visible above 202kDa marker. Thus it can be inferred that in spite of the mutation, mut p53 (P152L) retains the ability to form a tetramer. This is a peculiar observation in the view of its DNA binding ability which is completely lost upon P152L mutation (figure 4.2). The negative control mut p53 (L344A) did not show any tetramer formation as expected.

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Chapter 5 Gain-of-function effects conferred by P152L mutation

This chapter is about the delineation of the gain-of-function effects imparted by P152L mutation to the p53 protein. Gain of function properties like cell proliferation, cell migration, antiapoptosis and tumorigenic potential have been characterized for mutant p53 (P152L). Generation of p53 null stable cell line is also discussed.

CHAPTER OUTLINE:

5.1. Generation of p53 null H1299 stable cell line expressing wild type p53, mutant p53 (P152L) and vector control pCMV10

5.2. Wound healing assay to assess cell migration of cells expressing mut p53 (P152L)

5.3. Colony formation assay to assess cell proliferation of cells expressing mut p53 (P152L)

5.4. Apoptotic assay to assess chemoresistance of the mut p53 (P152L) expressing cells

5.5. Non-orthotopic xenograft mouse model study to assess the tumorigenic potential cells expressing mut p53 (P152L)

5.1. Generation of p53 null H1299 stable cell line :

In order to investigate whether mutant p53 (P152L) has any gain of function properties, we decided to generate a stable cell line of H1299 cells expressing wild type p53, mutant p53 (P152L). H1299 is a p53 null human lung carcinoma cell line. Cells stably transfected with pCMV10 null vector (vector without wild type p53 or mutant p53 construct) were taken as control. Figure 5.1 (A) shows the schematic representation of the procedure followed for generation of stable cell.

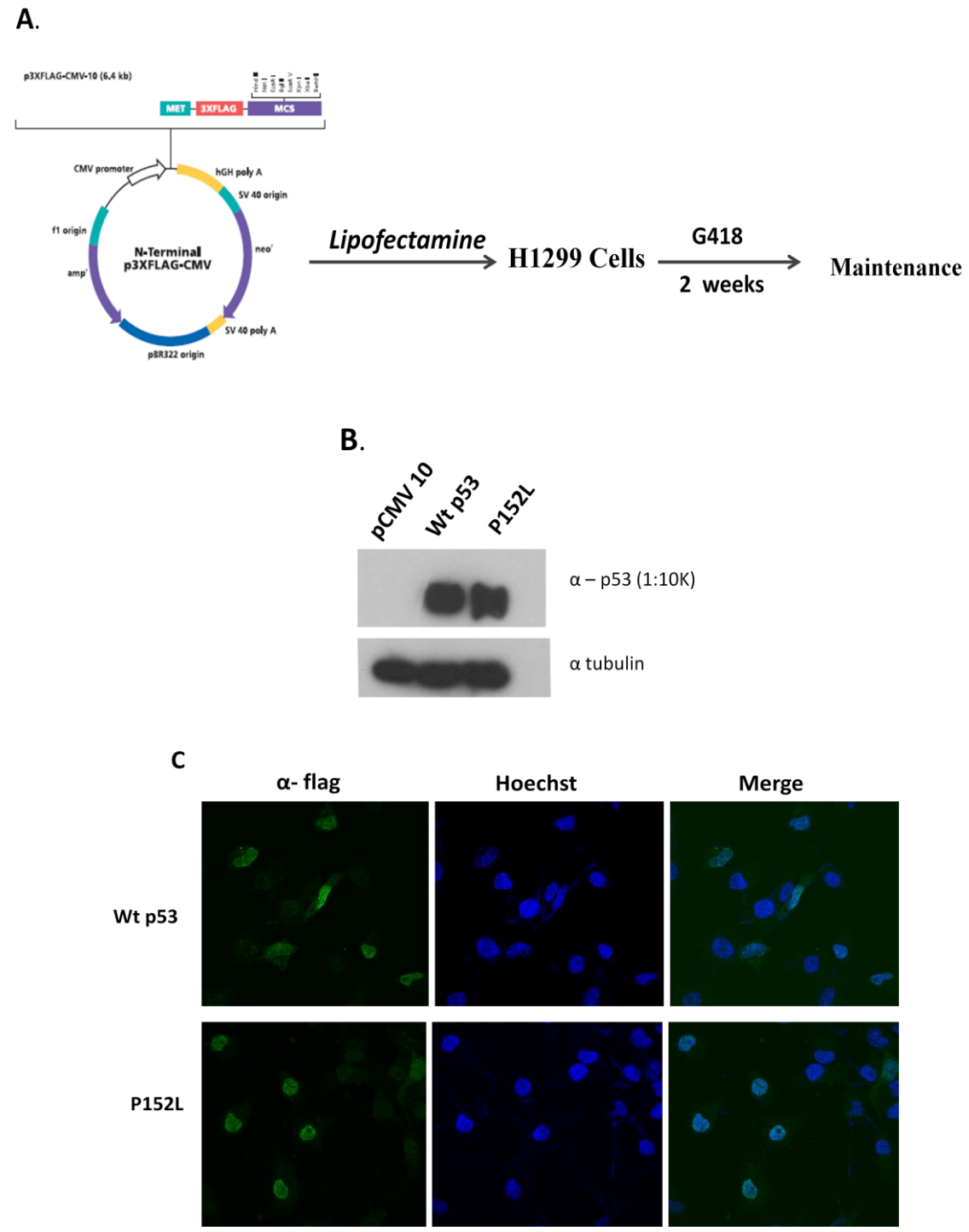


Figure 5.1 : Generation of H1299 stable cell line and its conformation : (A) Schematic representation of H1299 stable cell line generation. **(B)** Conformation of wt and mut p53 protein expression in H1299 stable cell line through western blotting and probing with α-p53 (DO-1) **(C)** H1299 cells were fixed and probed with α-flag antibody (1:1000) followed by Alexa fluor 488 secondary anti-mouse fluorescently tagged antibody and imaged under confocal microscope.

line. After about a month of maintenance of stable cell line in antibiotic, the protein levels were checked by western blot and immunofluorescence . Figure 5.1 (B) shows western blot conformation of cells expressing wt p53 and mut p53 (P152L) protein whereas no band was seen in pCMV10 vector control. Similarly fluorescence intensity was observed in cells stably transfected with pCMV10 vector containing wild type or mut p53 (P152L) construct as seen in figure 5.1 (C).

5.2. Wound healing assay to assess cell migration of cells expressing mut p53 (P152L) :

To start with delineating the gain-of-function effects imparted by P152L mutation, we first performed the wound healing assay in which the scratch healing is

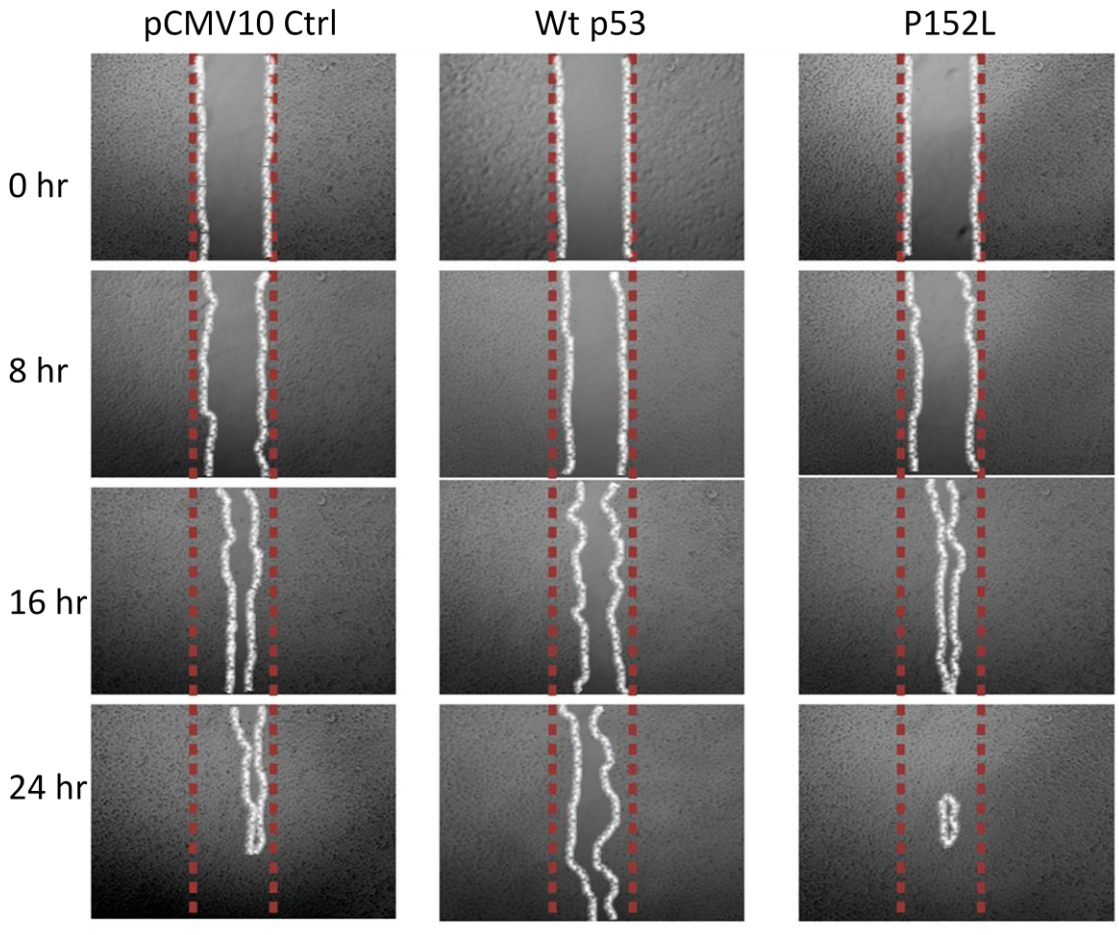


Figure 5.2 : Increased cell migration observed for mut p53 (P152L) expressing cells : Extent of wound enclosure observed for cells stably expressing wt p53, mut p53 (P152L) and vector control till 24 hrs. Images are shown for four different time points - 0 hr, 8 hr, 16 hr, 24 hr.

considered as a parameter to assess the cell migration / proliferative capability of cells. As seen clearly from the figure 5.2 wound healing was observed to be the fastest in case of P152L mutant expressing cells followed by the pCMV10 vector control and slowest for cells expressing wt p53. Hence wound healing ability when extrapolated to cell migratory / proliferative potential was found to be in decreasing potential as : P152L > pCMV10 Ctrl > wt p53.

5.3. Colony formation assay to assess cell proliferation of cells expressing mut p53 (P152L) :

Next, we assessed the cell proliferative capability through colony formation assay in which number of colonies obtained determines the proliferative potential of the cell. It is clearly evident from figure 5.3 that the number of colonies are more in P152L mutant expressing cells when compared to wt p53 expressing cells. The colonies obtained were quantitated and the difference between colony number was found to be significant. Colonies obtained with pCMV10 Ctrl cells were more or less equal in number as were obtained with wt p53 expressing cells.

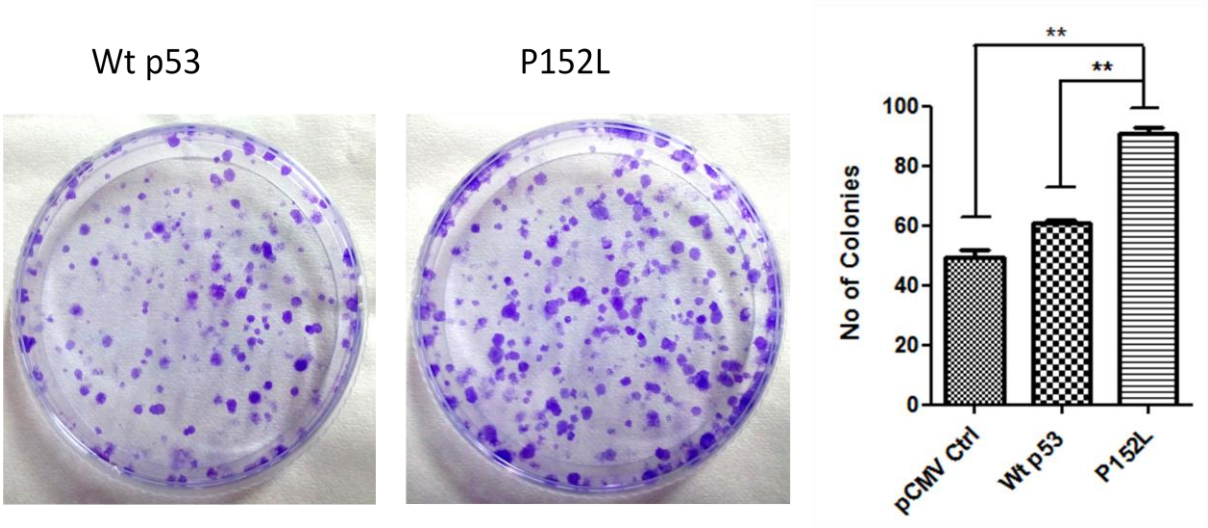


Figure 5.3 : Increased cell proliferation observed in cells expressing mut p53 (P152L) : Images of colonies obtained after staining with crystal violet for wt p53 and P152L mutant expressing cells. The colonies were counted and plotted in a bar graph and the difference in colonies were found to be statistically significant as determined by two tailed test.

5.4. Apoptotic assay to assess chemoresistance of the mut p53 (P152L) expressing cells :

Some of the mutant p53 acquires oncogenic gain-of-function property to evade apoptosis even after administration of anti-cancer drugs which can induce apoptosis. This gain-of-function effect is called chemoresistance. In order to determine whether P152L mutant has chemoresistance property, H1299 cells stably expressing wt and mut p53 (P152L) protein were treated with 5- fluorouracil (5-FU) for 12 hrs and FACS analysis was carried out.

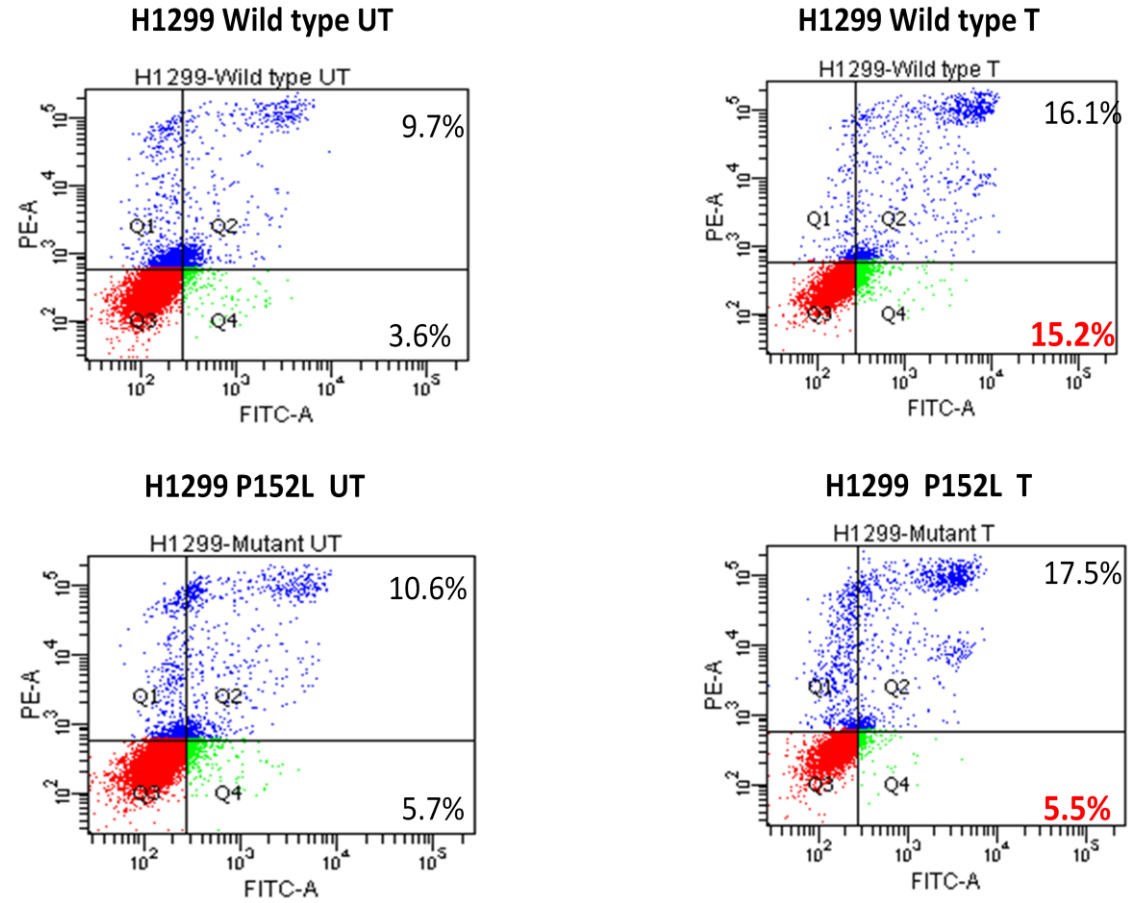


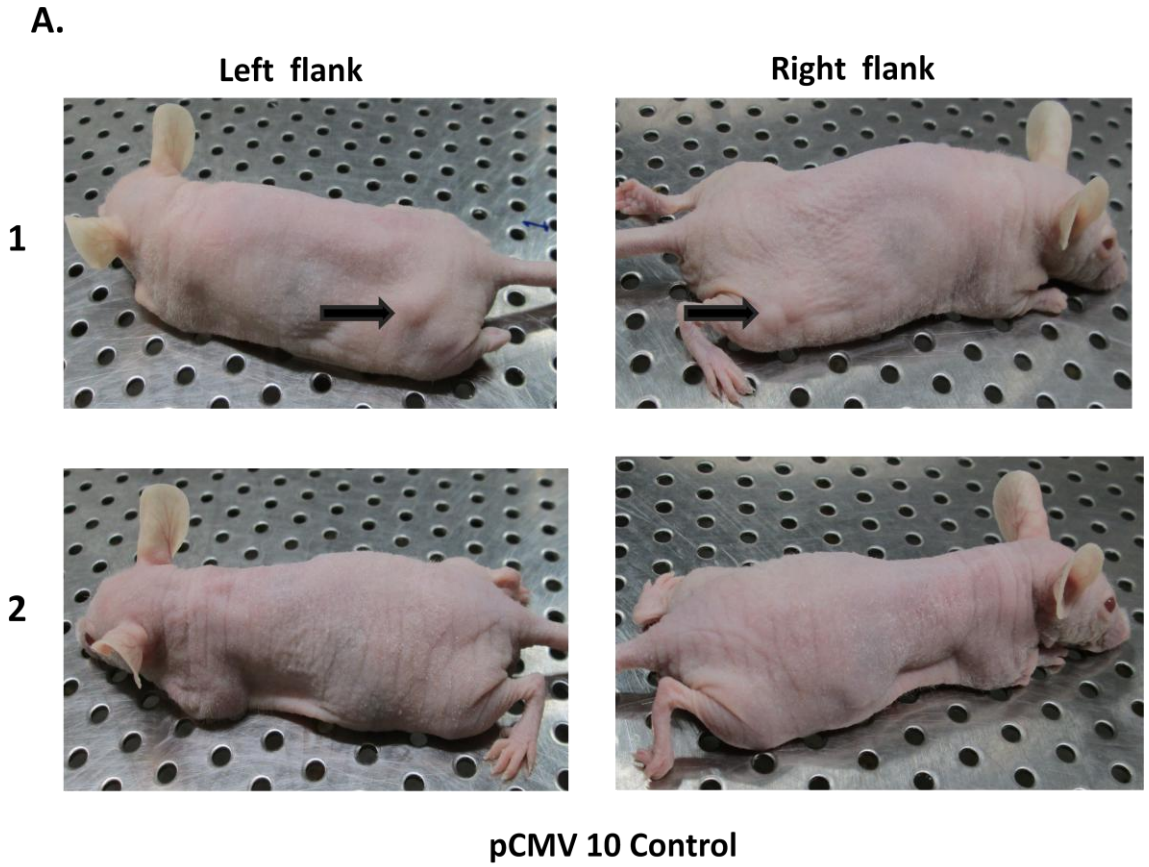
Figure 5.4 : Apoptotic assay and FACS analysis : Dot plot representation of FACS analysis for apoptotic assay showing the percentage of H1299 (stably expressing wild type or mutant p53-P152L protein) cells stained with propidium iodide (Q1), Annexin V (Q4) and both (Q2) [UT - untreated , T - treated]

In the figure 5.4, Q1 represents cells stained with propidium iodide and are indicative of dead cells whereas Q4 represents cells stained with annexin-V and are indicative of cells in early apoptotic stage. Q2 represents cells stained with

both propidium iodide and annexin-V and are indicative of necrotic/ late apoptotic cells. Since there is three fold increase in the number of cells (3.6% -15.2%) undergoing early apoptosis in case of wt p53 expressing cells whereas no difference could be seen (5.7% - 5.5%) for cells expressing mut p53 (P152L). Hence it can be deduced that mut p53 (P152L) provides resistance against early apoptosis to the cells although both the wt p53 and mut p53 (P152L) showed similar increase in late apoptotic cells indicating late apoptosis being unaffected due to P152L mutant.

5.5. Non orthotopic xenograft mouse model study to assess the tumorigenic potential of cells expressing mut p53 (P152L) :

In order to examine the tumorigenic potential as gain-of-function effect, non orthotopic xenograft mouse model study was carried out in which H1299 cells (human lung carcinoma cell line) expressing mut p53 (P152L) and pCMV10 Ctrl cells were injected subcutaneously into the left (1 million cells) and right (2 million cells) flanking region of mice.



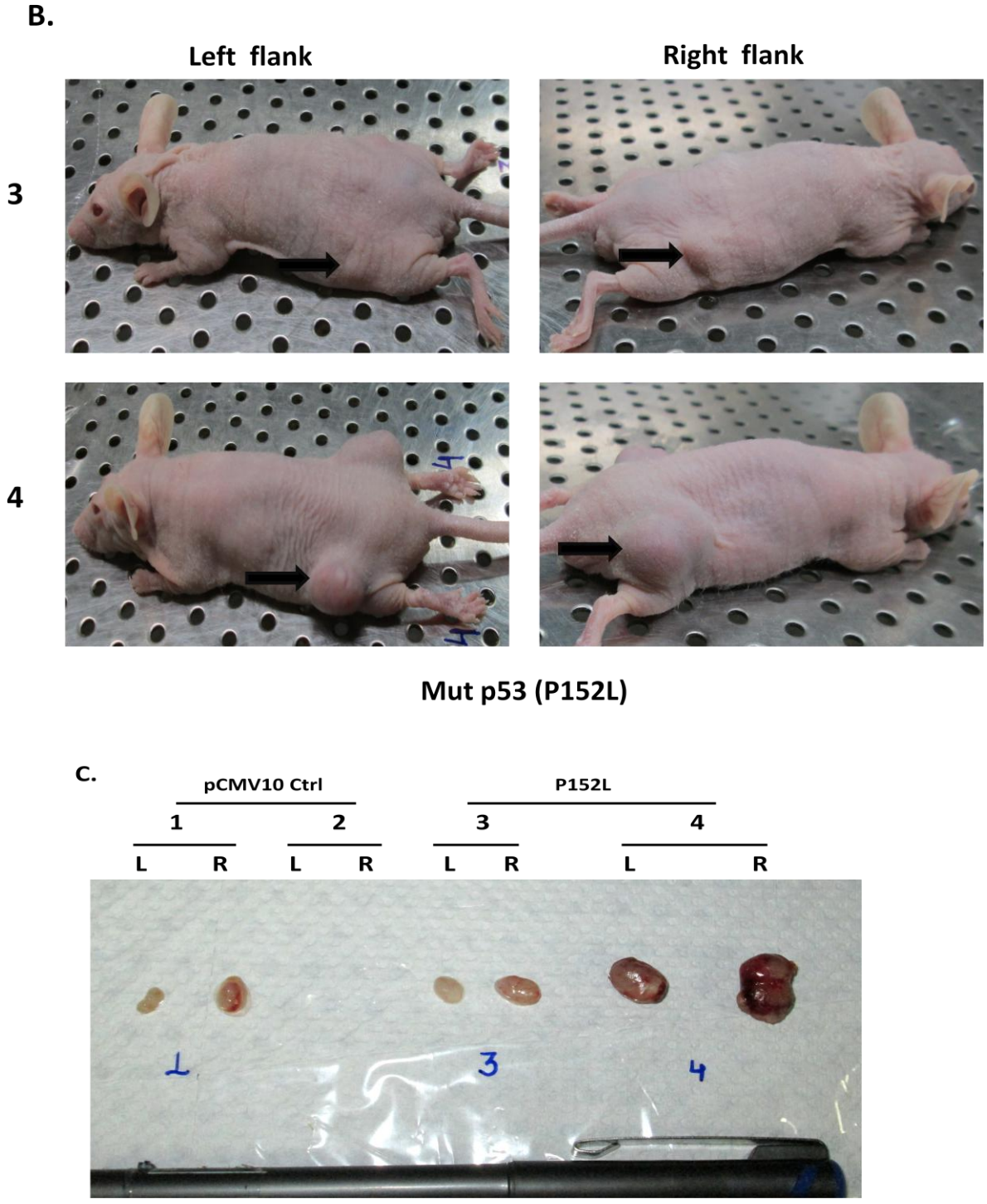


Figure 5.5 : Cells expressing mut p53 (P152L) exhibit tumorigenic potential : (A) Photographs of mice injected subcutaneously in duplicates (1 and 2) in the left and right flank region with 1 and 2 million of H1299 pCMV10 vector control cells respectively. **(B)** Photographs of mice injected subcutaneously in duplicates (3 and 4) in the left and right flank region with 1 and 2 million of H1299 mut p53 (P152L) expressing cells respectively. **(C)** Image shown is of the extracted tumors from both the flank region of those mice in which the tumor was visible. Visible tumors are marked with arrow.

The tumor was allowed to grow for about 40 days and were then extracted after mice sacrifice by cervical dislocation. As can be seen from figure 5.5 tumor was visible in both the flanking region in one of the two mouse injected with pCMV10 Ctrl cells whereas significant tumor growth was observed in both the mice injected with P152L mutant p53 expressing cells. Size of the tumor extracted out were compared. It is evident from figure 5.5 (C) that size of the tumor extracted from P152L mice were quite big as compared to tumor formed in pCMV10 Ctrl mice. Hence, it can be inferred that P152L mutation has tumorigenic potential in it.

Chapter 6 Discussion, Summary and Future directions

This chapter summarizes the research work presented in the thesis along with the discussion and future perspectives

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OUTLINE OF THE WORK DONE SO FAR :

- Untagged wild type and mutant p53 (P152L) DNA binding domain and Flag-tag full-length wild type and mutant p53 (P152L) protein was expressed and purified.
- Cloning , expression and purification of His-tag wild type and mutant p53 DNA binding domain.
- Impact of P152L mutation on the DNA binding properties of both full-length and DNA binding domain of wild type and mutant p53 protein was assessed
- Tetramer formation ability of p53 found unaffected upon P152L mutation
- Increased cell migration and proliferation was observed upon P152L mutation
- P152L mutation was found to confer chemoresistance to the cells expressing mutant p53 (P152L)
- P152L mutation was found to have tumorigenic potential.

Our present work is an attempt to understand the functional implications of a rare proline to leucine mutation in DNA binding domain region of p53 protein. Since p53 mainly acts as a transcription factor, its mutation causes loss of its DNA binding ability to the consensus sequences on the gene it activates to elicit its functions. Hence we attempted to understand the effect of P152L mutation on the DNA binding ability of mutant p53 protein. For this full-length flag-tagged wild

type and mutant p53 (P152L) protein were purified. Through DNA binding experiments we observed that P152L mutation in p53 leads to abrogation of its DNA binding ability. Earlier work done in the lab indicated that mutant p53 (P152L) is a conformational mutant rather than DNA contact mutant since it was found to be over expressed in an oral cancer tissue sample by PAb240 antibody which recognizes only conformational mutants. In order to test the hypothesis that P152L mutant is a conformational mutant and to determine whether the loss of the DNA binding ability of full- length mutant p53 (P152L) protein is due to a local structural change or global distortion in the protein structure, we looked into the DNA binding ability of mutant p53 DNA binding domain for which untagged wild type and mutant p53 (P152L) DBD was expressed and purified. It was observed through DNA binding experiments that P152L mutation has no effect on the DNA binding ability of the DNA binding domain of p53. Binding of mutant p53 (P152L) DBD to DNA supported our hypothesis and also indicated that the abrogation of full-length p53 DNA binding ability due to P152L mutation is due to the global distortion in protein structure rather than the local structural change. Since p53 binds to DNA as a homotetramer, we determined the impact of P152L mutation on the tetramer formation ability of p53. Interestingly, it was observed that tetramer formation ability of p53 is unaffected due to P152L mutation.

Since, mutant p53, in addition to the loss of its canonical functions of wild type p53, acquires new oncogenic gain-of-functions which helps in tumor growth and progression, we went ahead to investigate whether this rare p53 (P152L) mutation has any gain-of-function properties. Increased cell migration and proliferation in cells stably expressing mutant p53 (P152L) was observed when assessed by wound healing assay and colony formation assay respectively. When the chemoresistance property was checked through apoptotic assay followed by FACS analysis, it was observed that mutant p53 (P152L) provided resistance to cells against early apoptosis, although no effect was seen on late apoptosis; when the cells were administered with chemotherapeutic drug. When tumorigenicity of P152L mutation was checked by non orthotopic, xenograft mouse model study, tumorigenic potential was observed in cells expressing mutant p53 (P152L). Hence it can be inferred that P152L mutation imparts several gain-of-function properties to the cells.

With regard to understanding the implications of proline to leucine mutation in the DNA binding domain region of p53, study of structural alterations in protein structure through biophysical experiments is one of the major things to be looked upon. Since P152L mutation abrogated the DNA binding ability of p53 but has no effect on the tetramer formation ability, it would be interesting to understand the structural difference between the tetramer of full-length wild type and mutant p53 (P152L) protein. Some more gain-of-function effects can be examined for mutant p53 (P152L). Finally at the cellular context, using high throughput data analysis by experiments such as RNA sequencing or microarray, the differential gene expression pattern due to mutant p53 (P152L) will be useful to understand gene regulatory pathways.

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