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ΤΜΗΜΑ ΙΑΤΡΙΚΗΣ

ΕΘΝΙΚΟ ΙΔΡΥΜΑ ΕΡΕΥΝΩΝ
ΙΝΣΤΙΤΟΥΤΟ ΧΗΜΙΚΗΣ ΒΙΟΛΟΓΙΑΣ

ΔΙΙΔΡΥΜΑΤΙΚΟ ΠΡΟΓΡΑΜΜΑ ΜΕΤΑΠΤΥΧΙΑΚΩΝ ΣΠΟΥΔΩΝ

«Ογκολογία απο την Ογκογένεση έως τη Θεραπεία»

ΔΙΠΛΩΜΑΤΙΚΗ ΕΡΓΑΣΙΑ

ΙΣΤΟΡΙΚΗ ΑΝΑΔΡΟΜΗ ΤΟΥ ΓΟΝΙΔΙΟΥ P53 ΚΑΙ ΝΕΕΣ ΠΡΟΟΠΤΙΚΕΣ

ΕΠΙΒΛΕΠΩΝ ΚΑΘΗΓΗΤΗΣ: ΔΙΔΑΚΤΩΡ, ΖΟΥΜΠΟΥΡΛΗΣ ΒΑΣΙΛΕΙΟΣ

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MASTER THESIS

Historical retrospective analysis of the *p53* gene and new perspectives

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Η παρούσα διπλωματική εργασία εκπονήθηκε στο πλαίσιο σπουδών για την απόκτηση του Μεταπτυχιακού Διπλώματος Ειδίκευσης που απονέμει το Τμήμα Ιατρικής του Πανεπιστημίου Κρήτης, σε συνεργασία με το Ινστιτούτο Χημικής Βιολογίας του Εθνικού Ιδρύματος Ερευνών.

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Abstract

The tumor suppressor gene, TP53, is the most frequently mutated gene in human cancers. Although this gene was firstly identified in simian virus 40- transformed mouse cells in 1979, it took several years to be established as a tumor suppressor. Finally, p53 protein was proved to play a crucial role in many cellular processes and induce apoptosis whenever is necessary, deservedly dubbed as the 'guardian of genome'. Research revealed that p53 protein comprises of three main domains: N-terminal domain (NTD), DNA-binding domain (DBD) and C-terminal (CTD) which are responsible for its function as a transcription factor. p53 has both transcription-dependent and -independent functions. It is involved in a wide range of cellular processes such as cell cycle arrest, apoptosis, DNA damage repair, cellular senescence, angiogenesis, metabolism, ROS regulation, differentiation and development. TP53 is mutated in approximately 50-60% of human cancer cases and most of these mutations are missense mutations localized in 6 specific sites of DNA-binding domain. This results in loss-of-function (LOH) because mutated p53 has no longer the ability to bind DNA and act as a transcription factor. In some cases, besides LOH, mutated p53 protein acquires new properties, known as Gain-of-function (GOF), which include protein-protein interactions as it is unable to bind DNA. Significant role in p53 research had the discovery of MDM2/MDM4 proteins which block the activity of p53, by binding on the p53's transactivation domain (N' terminal) under normal conditions and lead to proteasome-mediated degradation of p53. This procedure was found to be disrupted in many cancers leading to continuous p53 inhibition which is destructive for the cells. The above knowledge on p53 functions and mutants led to the discovery of p53-targeted anti-cancer therapies such as gene therapies, therapies of p53 restoration, use of MDM2/MDM4 inhibitors, GOF targeting, immunotherapy, etc. Many of these treatments are currently tested in clinical trials, with few compounds having reached phase III (e.g. ARP-246, a p53 reactivator).

Keywords: p53 protein; tumor suppressor; p53 functions; p53 structure; mutant p53 proteins; MDM2-p53 interaction; p53-related anti-cancer treatments

Περίληψη

Το ογκοκατασταλτικό γονίδιο TP53 είναι ένα από τα πιο συχνά μεταλλαγμένα γονίδια στον καρκίνο του ανθρώπου. Παρόλο που το γονίδιο ταυτοποιήθηκε για πρώτη φορά το 1979 σε μεταλλαγμένα κύτταρα ποντικού με ιό Simian 40, χρειάστηκαν αρκετά χρόνια για να καθιερωθεί η ογκοκατασταλτική λειτουργία του γονιδίου. Τελικά, η πρωτεΐνη p53 αποδείχθηκε ως μία από τις πιο σημαντικές πρωτεΐνες του ανθρώπινου γονιδιώματος και δικαιωματικά πήρε τον τίτλο «Φρουρός του Γονιδιώματος». Διαπιστώθηκε ότι η πρωτεΐνη p53 αποτελείται από τρεις κύριες δομές: ένα αμινοτελικό άκρο, μια περιοχή ειδική για την πρόσδεση στο DNA και ένα καρβοξυτελικό άκρο τα οποία είναι υπεύθυνα για τη λειτουργία της πρωτεΐνης ως πυρηνικός μεταγραφικός παράγοντας. Πέρα από τις λειτουργίες της p53 οι οποίες εξαρτώνται από τη δράση της ως μεταγραφικός παράγοντας, υπάρχουν και λειτουργίες οι οποίες είναι ανεξάρτητες από αυτή. Εμπλέκεται σε ένα μεγάλο αριθμό κυτταρικών διαδικασιών όπως στη διακοπή του κυτταρικού κύκλου, την απόπτωση, την επιδιόρθωση βλαβών στο DNA, την κυτταρική γήρανση, την αγγειογένεση, το μεταβολισμό, τη ρύθμιση των δραστικών μορφών οξυγόνου, τη διαφοροποίηση και την ανάπτυξη. Το γονίδιο TP53 είναι μεταλλαγμένο στο 50-60% των περιπτώσεων ανθρώπινου καρκίνου και οι περισσότερες μεταλλάξεις είναι παρανοηματικές οι οποίες βρίσκονται σε έξι συγκεκριμένες θέσεις στην περιοχή πρόσδεσης του DNA. Γεγονός που οδηγεί σε απώλεια λειτουργίας λόγω του ότι η μεταλλαγμένη p53 πρωτεΐνη δε είναι ικανή να προσδεθεί στο DNA και να ασκήσει τη λειτουργία της ως μεταγραφικός παράγοντας. Ωστόσο, σε μερικές περιπτώσεις πέρα από την απώλεια λειτουργείας, η μεταλλαγμένη πρωτεΐνη αποκτά και νέες ιδιότητες όπως αλληλεπιδράσεις πρωτεΐνης-πρωτεΐνης. Σημαντικό ρόλο στην έρευνα του p53 είχε η ανακάλυψη των πρωτεϊνών MDM2/MDM4 οι οποίες μπλοκάρουν τη λειτουργία της πρωτεΐνης p53 μέσω παρεμπόδισης της περιοχής που σχετίζεται με την ενεργοποίηση της μεταγραφής υπό φυσιολογικές συνθήκες και οδηγούν σε αποδόμηση της p53 από το πρωτεάσωμα. Η διαδικασία αυτή βρέθηκε διαταραγμένη σε πολλές περιπτώσεις καρκίνου οδηγώντας σε συνεχόμενη αναστολή της πρωτεΐνης p53, το οποίο είναι καταστροφικό για τα κύτταρα. Η παραπάνω γνώση για τις λειτουργίες και τις μεταλλάξεις της πρωτεΐνης p53 οδήγησαν στην ανακάλυψη αντικαρκινικών θεραπειών που στοχεύουν τη συγκεκριμένη πρωτεΐνη όπως γονιδιακές θεραπείες, θεραπείες επαναφοράς λειτουργίας της p53, εκμετάλλευση του φαινομένου σύνθετης θνησιμότητας, χρήση αναστολέων των πρωτεϊνών MDM2/MDM4, στόχευση των μεταλλάξεων που προσδίδουν νέες ιδιότητες στην μεταλλαγμένη πρωτεΐνη, ανοσοθεραπεία κλπ.

Πολλές από αυτές τις θεραπείες βρίσκονται τώρα σε κλινικές δοκιμές και λίγες από αυτές τις ενώσεις έχουν φτάσει στο επίπεδο III των κλινικών δοκιμών (π.χ. το ARP-246, ένωση που επαναφέρει τις λειτουργίες της p53 πρωτεΐνης).

Λέξεις κλειδιά: Πρωτεΐνη p53; Ογκοκασταλτικό γονίδιο; Λειτουργίες της p53 πρωτεΐνης; Δομή της p53; Μεταλλαγμένες p53 πρωτεΐνες; Αλληλεπίδραση των πρωτεϊνών MDM2-p53; Αντικαρκινικές p53-θεραπείες

Contents

1. Introduction

The history of p53 research begins in 1979 when it was first identified as a transformation-associated protein. Through the years, p53 was shown to play a pivotal role in tumor suppression and in the cellular response to a variety of stress conditions, most notably those that put the cellular genome at risk. Nowadays, TP53 is proved to be the most frequently mutated tumor suppressor gene in human cancer and in combination with MDM2, can control a wide range of cellular responses induced by stress signals. In human cancer, the function of p53 to control cell homeostasis or induce death, when it is required, is disrupted and thus, scientists try to target this gene/protein in order to accomplish a successfully cancer treatment. The aim of this review is to relate how p53 research has evolved until now and to discuss new perspectives on p53 functions and therapies. It would be impossible to cover all aspects of p53, therefore this review will be restricted in some parts of this huge research.

2. First years of p53

In the 1970's, many cancer researchers, focused on cancer-causing viruses and discovered that such viruses carried oncogenes[1]. It was shown that RNA tumor viruses "steal" a cellular gene which is reintroduced into the cell that they infect. Therefore, this triggers the overexpression of the cellular protein and eventually leads to transformation[2]. On the other hand, DNA tumor viruses were found to contain their own oncogenes not related to the cellular oncogenes of the RNA tumor viruses[1]. To study how these DNA viruses, which don't carry cellular oncogenes, transform cells and produce tumors in animals, tumors were induced in animal models by DNA tumor viruses such as Simian Virus 40 (SV40)[3].

Tumors induced by SV40 were expected to express viral encoded proteins in order to induce normal cells to increase transcription and synthesize DNA so that they can use the cell machinery for their replication[4]. Subsequently, these proteins were detected by the immune system of the host causing the production of antibodies against them[5]. By the mid-1970's, such antibodies were used as tool to study and identify these proteins called viral tumor antigens. Genetic analysis revealed that these tumor antigens are responsible for the transformation of normal cells turning their name to viral oncoproteins[1]. Serum from tumor bearing experimental animals was used to

detect tumor antigens induced by SV40 infection or transformation. The result was the identification of two viral proteins, the large T-antigen and small t-antigen[5].

In 1979, while research was directed towards these two viral proteins, researchers stumbled on the p53. Several groups simultaneously, identified p53 in SV40 mouse cells by immunoprecipitation of T-serum[6]. Lane and Crawford were one of these groups that discovered a complex between the large T-antigen and a host protein with a molecular mass of 53 kDa by immunoprecipitating the sera from SV40-transformed animals. Further analysis revealed that this protein was complexed with SV40 T- antigen[7]. Consequently, it was found that SV40 T-antigen was not the only protein responsible for the transformation of the cells but a non-viral protein was the major key for induced tumors. Apart from the research above, other scientists discovered simultaneously the p53 protein. Daniel Linzer and Arnold Levine followed a similar immunological procedure with SV40 transformed cells and described a cellular 53 kDa protein that formed a bond with the large T-antigen[8]. In 1979, many other groups from UK, New York and France came up with the same observations[9,10,11]. Notably, Linzer and Levine noticed that immunoprecipitation with the anti-T-serum to uninfected embryonal carcinoma cells detected the same 53 kDa protein without the presence of SV40 infection[8]. This result indicates that the immune response (anitbodies) of the host does not act only against SV40 large T-antigen but also against this cellular protein. Independent research showed that 53 kDa protein was also highly expressed in chemically induced tumors[12]. Interestingly, it was noticed that tumors induced by the Abelson murine leukemia retrovirus triggered the production of the same protein in high levels[13]. Later, it was discovered that viral oncoproteins from other DNA viruses like adenovirus E1B[14], human papillomavirus HPV[15], Epstein-Barr virus[16] and hepatitis B virus[17], bound p53 protein. Thus, high levels of p53 expression were present not only in SV40-transformed cells but also in other types of tumor cells whereas the p53 level in non-transformed cells was generally very low[1].

In 1979, after p53 had been discovered, many researchers focused their study on this “promising” non-viral protein. In 1983, during the first p53 Workshop in Oxted, it was suggested the name of p53 and since then is the predominant nomenclature. The reason of this decision was the molecular mass of the protein that was 53 kDa, based on its migration in SDS gel. Later the molecular mass was proved to be wrong and it was found that a proline-rich region slowed down the migration of it. So, the correct molecular mass of human p53 was proved to be 43.7 kDa but the name p53 remained[1].

3. Oncogene or Tumor Suppressor

Firstly, it was observed that SV40 leads to overexpression of p53 protein in transformed cells[1]. A temperature sensitive mutant of SV40 large T antigen was the key to believe that p53 was responsible for the transformation. When T-antigen was functional the levels of p53 were high whereas the levels of p53 were quite lower when T-antigen was non-functional[18]. This led to the conclusion that p53 was an oncogene. As mentioned before, scientists found more DNA viruses that bind p53 and lead to cell transformation. In addition, it was shown that in many tumors, the level of p53 expression was very high, while this was not observed in normal cells[12,19].

In order to examine the properties and the functions of p53, there was a need to isolate p53 cDNA and genomic clones[1]. In the early 1980's, this procedure was not so simple though some groups managed to overcome the difficulties and clone p53 from mouse and human. Gene cloning till that time was not quite developed so p53 cDNA cloning was the key to increase the insight into the function of p53 in cell transformation[20]. The first p53 cloning was conducted using RNA from cancer-derived cells because p53 protein was abundant in those cells[1]. One of the most important findings, that enhanced the belief that p53 was an oncogene, was a mutation of this gene that shown to increase the transformation efficiency. This finding was fitted with the thought that p53 was an oncogene possibly activated by a mutation[21]. Also, a series of studies revealed that transfected p53 amplified the function of established oncogenes, such as H-Ras, to transform primary cells in culture[22,23]. In general, by the mid 1980's, p53 was believed to be an oncogene whose significance and function remained unknown[1].

4. P53 as a tumor suppressor

The ascertainment that p53 was a tumor suppressor took a long time to establish. Nevertheless, evidence that refuted this theory started to come out. Firstly, in 1984, it was reported the inactivation of p53 by an insertion of Moloney murine leukemia virus-like DNA sequences in an Abelson murine-transformed mouse cell line[24]. Moreover, the loss of normal p53 expression and function found to strengthen the transformation rate in mouse spleen tumors induced by Friend erythroleukemia virus[25,26,27]. Another clue for this assertion was that p53 gene found to be rearranged in the HL60 cell line derived from human leukemia[28].

The strongest evidence came out when two different labs compared the DNA sequences of various p53 clones employed for many experiments above[1]. The results showed that the clones differed from one sequence to another, leading to the conclusion that most of clones used in those experiments carried mutations within a conserved region of p53, responsible for its biological activity[29]. This fact was proven when the sequence of wild-type p53, derived from normal-murine cells, was established[30]. After this, it was realized that p53 mutations are responsible for promoting cell transformation and are usually present in cancer-derived cells, in contrast with wild-type p53 that acts in an opposite way[31]. Thus, in the late 1980's, researchers started to realize that p53 is a tumor suppressor rather than an oncogene.

In 1989, scientists showed that there was a lack of wild-type p53 alleles in human colorectal tumors replaced by p53 mutations and deletions[32]. The importance of this discovery was enhanced by a parallel detection that the overexpression of wild-type p53 can suppress cell transformation by mutant TP53 and RAS gene[33,34]. Apart from human colorectal tumors, p53 was found to be lost or to contain negative mutations in about half of all human cancer types[35,36,37]. In addition, germline p53 mutations were proved to be responsible for hereditary Li-Fraumeni syndrome leading to early appearance of cancer[38]. P53 is now known to be the most commonly mutated gene in human tumors and the most frequently analyzed one, but its practical clinical diagnostic value is still limited[39].

5. Interaction of viral oncogenes with p53

During this period, scientists found that the oncoproteins from DNA tumor viruses like SV40 T-antigen and adenovirus E1A protein bind retinoblastoma protein (pRb), a tumor suppressor protein[40,41]. These oncoproteins interact with pRb and inactivate its function that is to regulate the mammalian cell cycle with binding E2F transcription factors[42]. As a result, E2F transcription factors are free to lead the cell into S-phase[43,40]. Generally, this happens because DNA tumor viruses use some of the cell's functions in order to support their own DNA replication. However, the increasing rate of replication is detected by p53 protein that attempts to stop this action leading the cell into apoptosis. In order to achieve their target, the DNA tumor viruses bind and inactivate p53 protein with the large T-antigen or the E1B protein. As a result of inactivated and accumulated p53 protein, the apoptosis could not be occurred and the cell remains at S-phase[1].

6. Structure and domains of p53

In 1985, the cloned human p53 cDNA was used as a probe to detect where p53 is located. After *in situ* hybridization, the human p53 gene was found to span about 20 kb of DNA and be located on the p arm of chromosome 17 (17p13). This gene contains 11 exons, the first of which is a non-coding exon and is 8-10 kb away from exon 2[44,45,46]. The transcription of human p53 gene initiates from 2 different sites: one upstream of exon 1 and one from an internal promoter located in intron 4. The second promoter leads to the expression of p53 protein lacking the 1st transactivation domain of N-terminal. Moreover, there are locations for alternative splicing in intron 9, resulting in three isoforms: p53, p53β, p53γ. Isoforms p53β and p53γ lack the oligomerization domain. As a consequence, the human p53 gene can encode at least 9 different p53 protein isoforms due to alternative initiation of translation and splicing. Figure 1 depicts the structure of TP53 gene and its 8 different transcripts. It contains 11 (green) exons the first of which is non-coding. In addition, there are different sites for promoters P1/P1'/P2 and splicing. Exons 9β (red) and 9γ (blue) lead to different transcripts through alternative splicing. Transcripts 1 to 4 is produced by P1 and P1' promoters (upstream to the gene), while transcript 5 to 8 by P2 promoter[47]. P53 variant mRNA is differently expressed in normal human tissues indicating that alternative promoters and splicing can be regulated [48].

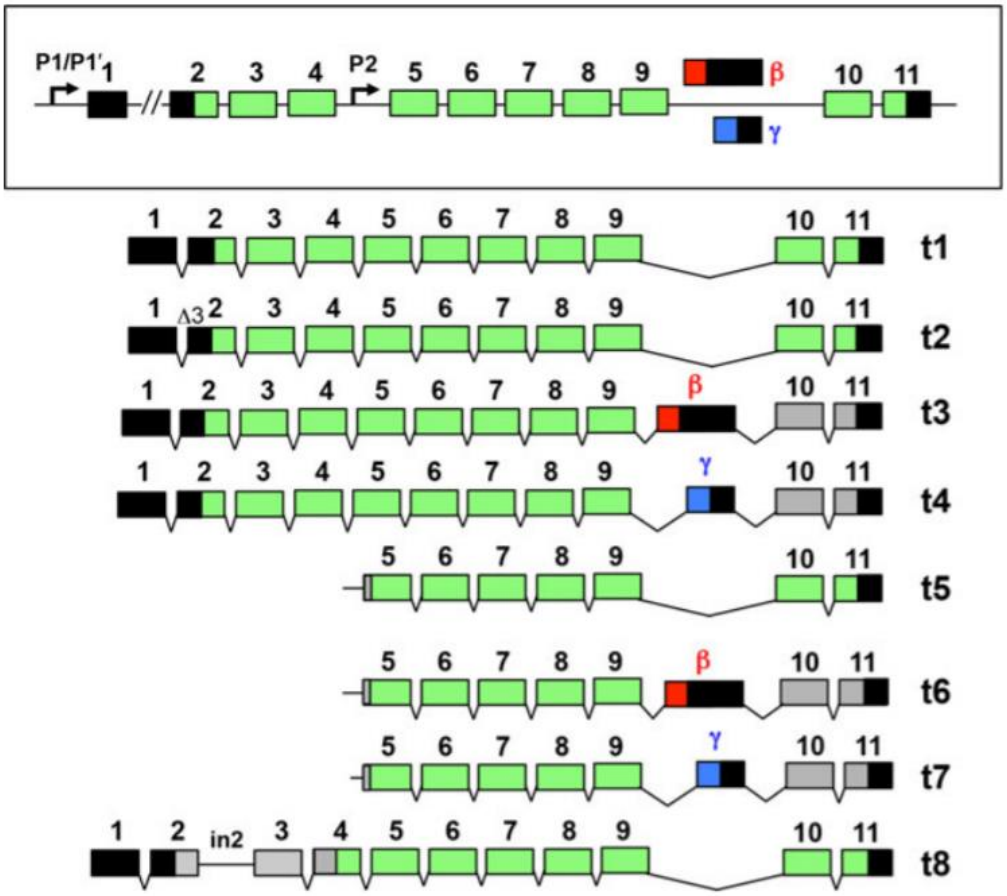

Figure 1: Structure of human p53 gene[47].

Most tumors are related to false expression of the gene p53. It was found that during evolution, p53 has been conserved. Specifically, there are some conserved amino acid sequences (Box motifs) in p53 proteins from a variety of species. These regions are within the residues 13-23, 117-142, 171-181, 234-258, 270-286[49,50]. Human full length p53 protein consists of 393 amino acids and has three domains: N-terminal domain (NTD), DNA-binding domain (DBD) and C-terminal (CTD). NTD domain comprises of two transcriptional activation domains -TAD1 & TAD2- and a proline-rich domain (PRD), CTD domain consists of a tetramerization domain (TD), a hinge domain (HD) and a C-terminal α domain (CTDα) and DBD domain contains a sequence-specific DNA-binding domain (Figure 2)[51]. The conserved amino acid residues (Box I-V) found to be part of these domains, underline the significance of these regions at p53 functions (Figure 3)[52]. Box I conserved region is localized in TAD1, whereas the rest Box regions (II-V) in DBD. Figure 3 shows with more details the structure of p53, the conserved regions, the alternative initiation of p53 mRNA translation and the cell stress-dependent regulation caused by this [52].

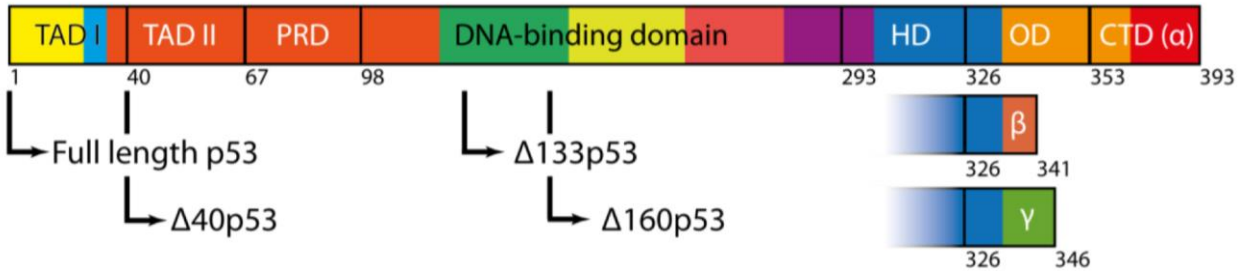

Figure 2: p53 protein structure, its domains and its different isoforms[51].

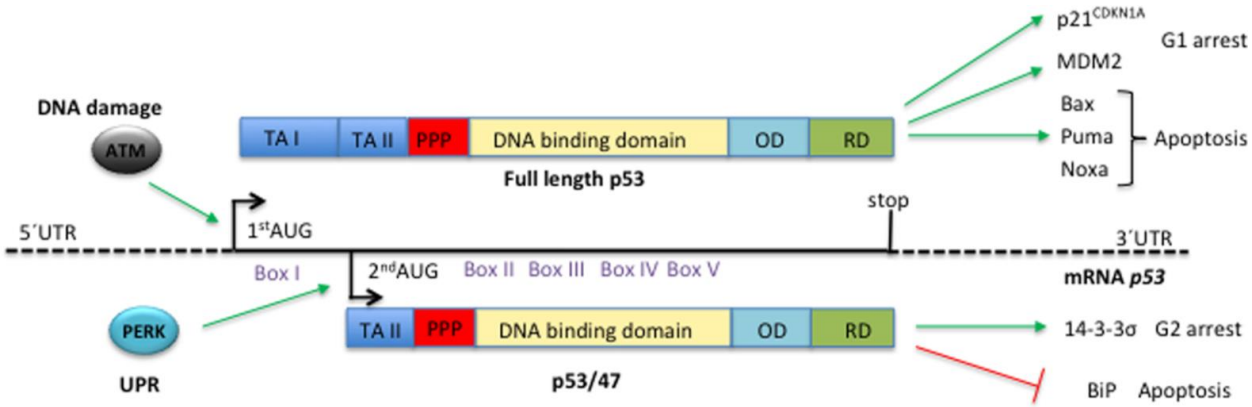

Figure 3: The alternative splicing of p53 mRNA produces two protein isoforms: the full length p53 (p53FL) and the p53 lacking TA 1 domain (p53/47). 1st AUG and 2nd AUG are the two sites of mRNA translation. When DNA damage occurs, ATM kinase (Ataxia Telangiectasia Mutated) is activated resulting in the induction of p53FL synthesis from 1st AUG. On the other hand, when Unfolded Protein Response (UPR) pathway is activated in case of stress to endoplasmic reticulum, PERK kinase is activated and mRNA synthesis initiates at 2nd AUG forming p53/47 isoform. In the first case, the p53 target genes are the G1 cell cycle kinase inhibitor $p21^{CDKN1A}$ or apoptotic factors such as Bax ,Puma, Noxa of the Bcl-2 family whereas in the second case, G2/M arrest is induced via induction of 14-3-3σ. Box I in TAD I and Box II-V in DNA binding domain are the conserved regions of p53 protein[52].

6.1. N-terminal domain (NTD)

The N-terminal domain consists of a transcriptional activation domain (TAD) and a proline-rich domain (PRD/PPP). The entire TAD domain contains 61 residues and is divided into two subdomains which are residues 1-39 and 40-61[53]. The first N-terminal subdomain contains residues 1-39 and the second N-terminal subdomain contains residues 40-61. They are also called transactivation domain 1 (TAD1) and transactivation domain 2 (TAD2), respectively[54]. TAD1 consists of the strongly amphipathic helix and its role is to activate transcription factors and regulate pro-apoptotic genes[6]. At this subdomain there are two hydrophobic residues L22 and W23 that are critical for p53 transactivation and are highly conserved through the species (Figure

4)[54] . Conversely, TAD2 consists of weakly amphipathic helix[53]. The major highly-conserved and hydrophobic residues at this subdomain are W53 and F54 (Figure 4) that contribute significantly to p53 transcriptional activity[6]. Studies indicated that the TAD1 and TAD2 act synergistically rather than additively[54] and when the target protein interacts with the entire p53 TAD, the bonds are stronger due to multiple hydrophobic surfaces[53]. Interestingly, the BOX-I motif of the TAD1 of p53 is one of the most conserved regions of p53 protein and recently was proved to have a crucial -dual role in the interaction of MDM2 with p53 TA1 domain both in mRNA and protein levels. The p53 transactivation activity is mediated via TAD1 and TAD2 of N-terminal region, transactivating different target genes and cellular pathways[52]. For example, stress-induced ATM kinase leads to the translation of full length p53, indicating that TAD 1-induced transactivation is required for DNA-damage response. On the contrary, the activation of Unfolded Protein Response (UPR) pathway leads to the translation of p53/47 protein, lacking TAD1 region. p53-47 protein transactivates genes related to G2 arrest and inhibition BiP apoptosis through TAD 2[52].

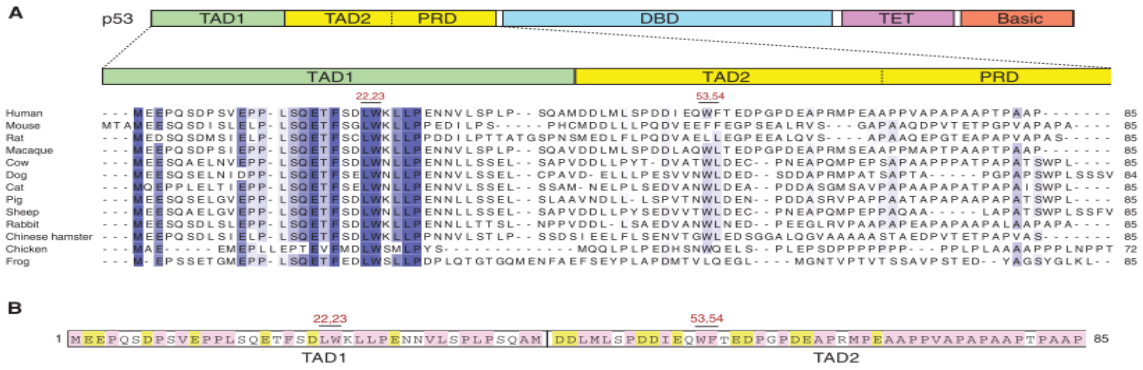

Figure 4: A) Depiction of p53 Transactivation Domains (TADs) sequences in various species. Blue shading indicates the highly-conserved residues. **B)** The entire human TAD domain (1-61 residues) with acidic (yellow) and hydrophobic amino acids (pink) underlined. It also highlights the most critical residues in TADs: 22, 23, 53, 54[54].

TAD contains binding sites for other viral and cellular proteins like transcription factors. It allows p53 to activate the transcription machinery consisting of TATA box binding protein (TBP) and TBP-associated factors (TAF) that are part of multiprotein assembly, TFIID[55,56]. Apart from interaction with transactivation domain (TAD), TBP interacts also with carboxyl terminus[57]. Furthermore, adenovirus E1B-55 kDa protein, human MDM2 protein and hepatitis B virus X protein bind to transactivation domain and inhibit its function[58,59,60].

The N-terminal region contains also a proline-rich domain (residues 62–93) that is crucial for p53's apoptotic function[61]. It contains five PXXP motifs which can bind to SH3 domains[6]. Scientists produced a p53 protein lacking this proline region (Δ pro) and led to the result that Δ pro protein affects the expression of other promoting proteins like MDM2 and WAF1. This means that proline-rich region plays a major role in apoptotic activity, reactive oxygen species and other functions of p53[62].

6.2. DNA-binding domain (DBD)

The central domain of p53 protein is a DNA-binding region which comprises of 94–290 residues. It contains four conserved regions (Figure 3), one zinc atom and several arginine residues[6]. The majority of tumor mutations are found at DBD and most commonly at sites 175, 248, 249, 273 and 282[63]. In 1990, it was found that wild type p53 inhibit the replication activities of SV40 large antigen opposite to mutant p53[64] and in 1991, it was discovered that only wild-type p53 binds specifically to DNA sequences like SV40[65]. Scientists started to realize that p53 achieves its activity by binding to specific DNA sequences[66]. Hence, through the testing of a large number of human genomic cDNA, many clones were found to contain specific sites for p53 binding. These cDNAs proved to have two copies of 10bp motif with palindromic structures each containing half of the sequence[67]. After these findings they realized that the p53-binding sites have obvious symmetry forming head-to-head quarter sites and it became clear that p53's role as activator of transcription depends on these specific DNA-binding sites[67].

6.3. C-terminal domain (CTD)

The C-terminal domain contains 291-393 residues and has three domains. The hinge domain (HD), the homo-oligomerization domain (OD) which is also called tetramerization domain (TD) and the C-terminal-α domain (CTDα), also called negative regulatory domain (NRD). The hinge domain (HD) is a short linker sequence of amino-acids (291–324) between DBD and OD which offers a structural flexibility. Its role is to facilitate the binding of p53 response elements. Mutations in HD lead to loss of p53-mediated transcriptional activity underlining the necessity of HD in the proper function of the protein[68]. The OD consists of residues 325–356 and has two symmetric

dimers each containing two antiparallel α helices and one antiparallel β sheet[6]. It was proved that mutated p53 lacking oligomerization domain was unable to bind to DNA and activate transcription[69]. Interestingly, p53 binds DNA as a homo-tetramer and tetramerization is achieved through the OD. This homo-tetramer is required for site-specific DNA binding, posttranslational modifications, as well as interaction between proteins[70]. The p53 response region comprises of 4 repeats with 5 nucleotides and each p53 DBD is connected to one of these repeats[71]. CTDα/NRD consists of 357–393 amino acids and it is rich in positively charged amino acids which interact with negatively charged nucleic acids (RNA and DNA). The p53 α domain affects the DNA binding and the transcriptional activity of p53 and due to post-translational modifications can regulate the protein degradation, tetramerization as well as the interaction between p53 and transcriptional machinery[68].

7. Functions of p53: both transcription-dependent and -independent

P53 is characterized by its function as a transcription factor. The transactivation domain contains binding sites for transcription co-regulators and the DNA-binding domain binds specific sequences regulating the transcription of many genes. This is also the function that distinguishes wtp53 from mutated forms. Through the years hundreds of p53 target genes have been identified and found to be activated by p53 binding on specific DNA sequences within or upstream to these genes[1]. Apart from its role as a transactivator, p53 is also a transcriptional repressor, mainly using mechanisms without direct binding on the target gene[72]. In addition, research unraveled non-transcriptional activities of p53 in the cytoplasm which brought a new perspective of its functions[73,74]. Generally, p53 has various mechanisms in order to regulate a wide range of cellular processes. It has been found that this protein is involved in cell-cycle arrest, DNA repair, cellular senescence, differentiation, apoptosis and angiogenesis[75]. Moreover, p53 guards the cell from DNA damage and stress signals, like ionizing radiation, UV, tumor virus, hypoxia, overexpression of oncogene, which can lead to tumor formation[76]. These explain the reason that p53 dubbed the “guardian of the genome”[77].

7.1. Discovery of p53val135

In 1990, a temperature-sensitive mutant of p53, that enhanced the research of this protein, was discovered. This particular mutant, called p53val135, elicits transformation at 37.5°C and suppresses the proliferation of transformed cells at 32.5°C. Of note, the proliferation is controlled at the permissive temperature and this action is reversible. Through the use of p53val135, it was found that the role of wild-type p53 is to cause growth arrest at either G1 or G2/M[78–80]. In 1991 scientists detected another function of p53val135 in the murine leukemia cell line. After restoring the expression of wild-type p53, all cells died in a way characteristic of apoptosis[81]. Similar findings of apoptosis were detected in a human colon tumor-derived cell line while wild-type p53 was expressed[82]. These results led to the conclusion that apoptosis could be a mechanism of tumor suppression. Later, p53 was found to activate replicative senescence in both human and rodent cells/cell lines as an additional mechanism for controlling immortalization[83,84]

7.2. Functions

7.2.1. Cellular Senescence

Scientists found an association of p53 with cell senescence. Senescent cells have no longer the ability to proliferate as a response to damaging stimuli like telomere shortening, DNA damage and tumor activity. Opposite to apoptosis, senescence just inhibits the proliferation of cells without destroying them. The cells undergo morphological changes and are irreversibly arrested at the G1 phase of the cell cycle but they are still metabolically active[6]. In 1997, scientists found that expression of oncogenic ras in primary human or rodent cells and rat cell line triggers a permanent G1 arrest[83]. This arrest is accompanied by accumulation of p53 and $p16^{INK4a}$, two major tumor suppressors, and decreased levels of cyclin A and CDK2 kinase activity. Moreover, the cells undergo morphological changes phenotypically indistinguishable from cellular senesence[83]. Whereas if p53 or $p16^{INK4a}$ is inactivated, the ras-induced arrest is prevented, leading to the conclusion that p53 plays a major role in cellular senesence[83]. Scientists suggested that p53 is involved in cellular senescence after noticed that levels of p53 are increased transiently and then dropped to the normal rate during this procedure[6].

7.2.2. Regulation of cell cycle

P53 responds in any cellular stress situations and stimuli by inhibiting further replication of transformed cells. There are some major checkpoints in the cell cycle acting as a surveillance mechanism when DNA is damaged. These checkpoints are at phase G1/S and G2/M and provoke cell division arrest until positive regulator molecules allow the cell cycle to finish the procedure[85]. At phase G1/S the replication of impaired DNA is inhibited until the damage is restored. Mutated forms of p53 found to alter cell cycle arrest and gene amplification potential, underlining the importance of this protein at the regulation of cells division[86]. P53-inducing cell arrest is mostly mediated by the transcriptional activation of p21/WAF1[87,88]. Protein p53 binds to two sites upstream of p21 promoter and activates the synthesis of p21 protein[87]. Afterwards, p21 binds to cyclin E/Cdk2 and cyclin D/Cdk4 complexes and inactivates them leading to G1 arrest[87]. This event blocks pRb phosphorylation and the active pRb binds to E2F1 and stops its activity as a transcription factor[89]. Without active E2F1 form, its target genes that are critical for DNA replication, remain in silence[89]. Furthermore, p21 binds to PCNA, a nuclear cofactor for DNA polymerase δ, and blocks its activity as a replication factor[90]. When the damage is restored MDM2 blocks the p53 activity and allows cell to reenter the S phase[91]. Mouse cell lines lacking p21/WAF1 showed failure to control the cell cycle progression after DNA damage, indicating the importance of p21 and, by extension, p53 in cell-cycle regulation[92]. In addition, p21 controls checkpoint G2/M, an extremely significant phase before mitosis, by binding cyclin A and B[93].

7.2.3. Cell apoptosis

As mentioned above, the first clue that p53 can trigger apoptosis came from the discovery of p53val135 in a myeloid leukemia cell line[78]. This temperature sensitive mutant behaves as a mutant at 37.5^{o}C whereas at 32.5^{o}C acts like wild-type p53 protein. After restoring the expression of wt-p53, scientists observed that all cells died in an apoptotic way. The ability of p53 to induce apoptosis was confirmed in several studies using different cell lines[82,94,95].

In mammalian cells there are two distinct pathways to apoptosis, the BCL-2 regulated pathway and the Death Receptor pathway. BCL-2 pathway, which is also called mitochondrial or intrinsic pathway, is activated by stress conditions such as DNA damage[96]. On the other hand, Death Receptor pathway ,also called extrinsic, is activated via death receptors in cell membrane when conditions in the extracellular environment determine that a cell must die[96]. The p53 protein

induces apoptosis through the BCL-2 regulated pathway (Figure 5). There are pro-apoptotic and anti-apoptotic genes which regulate this apoptotic pathway. The pro-apoptotic genes contain Bax, Bak, Noxa while the anti-apoptotic genes contain Bcl-2, Bcl-XL, Bcl-B, MCL-1 and other genes[96]. Bcl-2 and Bax are homologous proteins with opposite functions and Bcl-2 forms heterodimers with Bax while Bax forms homodimers with itself[6]. P53 is involved in regulation of these genes either directly or indirectly. The promoter of pro-apoptotic Bax has binding sites for p53 protein meaning that p53 transactivate this gene directly[97]. Additionally, the anti-apoptotic function of Bcl-2 can be overcome by p53-induced BAX indicating that BAX determines the apoptotic activity frequency[98]. However, this is not the only way that p53 affects apoptosis. It was found that the proapoptotic 'BH3-only' class of Bcl-2 family members, which comprises of Puma, Noxa and other genes, has an association with p53 protein[98]. The p53 protein transactivate these genes directly[99] and 'BH3-only' proteins act upstream of Bax inducing the activation of multi-domain proapoptotic proteins[100]. Furthermore, IGF-IR, an insulin-like growth factor 1 receptor, was found to be a molecular target of p53. Elevated IGF-IR expression can promote cell proliferation and lead to cancer while wt-p53 suppresses this activity by acting as a transcription repressor[101]. In addition to its ability to activate pro-apoptotic or inactivate anti-apoptotic targets via transactivation, p53 has additional transactivation-independent strategies[98]. Cytoplasmic p53 binds to multi-domain proapoptotic Bcl-2 family proteins and triggers permeabilization of mitochondria and apoptosis[74]. Thus, p53 can lead to apoptosis via several mechanisms and its role is crucial for the cell fate.

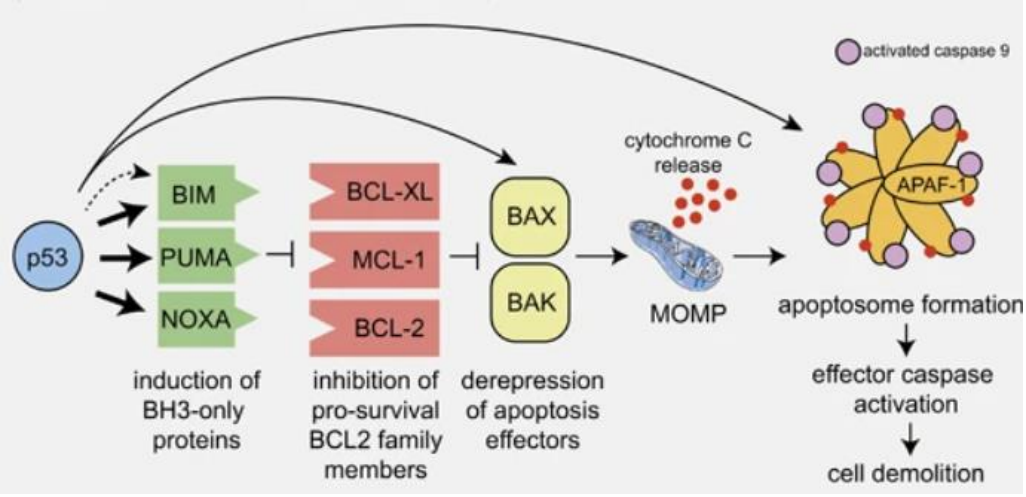

Figure 5: p53 target genes in mitochondrial apoptotic BCL-2 pathway[96].

7.2.4. Angiogenesis

Angiogenesis plays a pivotal role in the growth of cancer and is essential for the transformation of normal to tumor cells. There is an inhibitor of angiogenesis called thrombospondin-1 (TSP-1) whose gene is activated by p53[6]. It was shown that wild-type p53 inhibits angiogenesis by activating TSP-1. In addition, p53 found to down-regulate vascular endothelial growth factor (VEGF), while mutated forms of p53 were proved to increase the expression of VEGF leading to angiogenesis[102]. Similarly, p53 directly represses expression of basic fibroblast growth factor (bFGF), which is also a positive modulator of angiogenesis[102]. Last but not least, hypoxia inducible factor (HIF) as its name suggests, responds to hypoxic conditions in cancer cells. This heterodimeric transcription factor HIF comprises of two subuntis HIF-α and HIF-β and is a positive regulator of angiogenesis. p53's role is to repress its activation under extreme hypoxic environment and DNA damage but not in normal conditions[102].

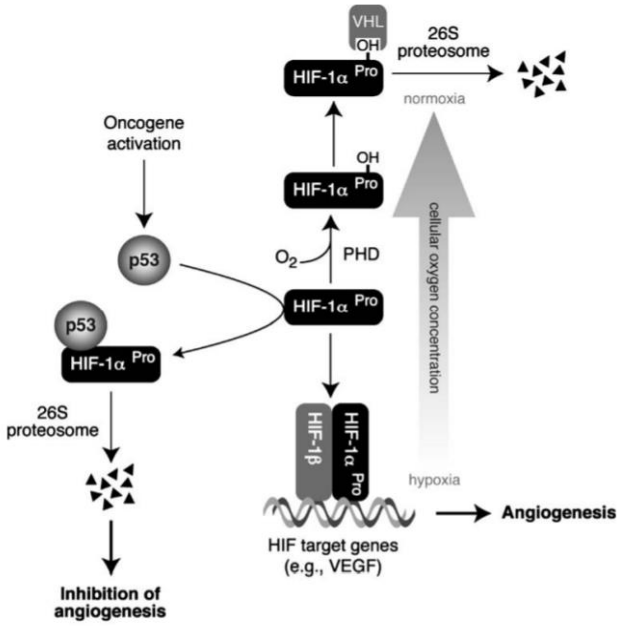

Figure 6: p53 regulation of hypoxia inducible factor, HIF[102]

7.2.5. DNA repair

Remarkably, p53 plays also a major role in DNA repair. In case of DNA damage in cells with wild-type p53, GADD45 (growth arrest and DNA-damage inducible) gene family is activated. In contrast, GADD45 is inactivated in cells with mutation or loss of p53, leading to tumorigenesis. GADD45 enhances Nucleotide Excision Repair (NER) to remove DNA damage (thymine dimers and photoproducts) induced by UV. In case of p53 mutation or loss, this mechanism does not work resulting in devastating consequences for the cell[103,104]. Also, p53 regulates DNA polymerase β and APE1 in Base Excision Repair (BER), another kind of repair that removes chemically modified bases[105].

7.2.6. Differentiation

p53 was found to be involved in normal development and differentiation pathways. It was discovered that during mouse embryonic development there is a differential expression pattern of p53[106]. Furthermore, high levels of p53 are detected both in several points of B cell differentiation pathway and in the process of spermatogenesis underlining the significant role of p53 in genome integrity[107].

7.2.7. Metabolism

Another significant role of p53 appears to be metabolism regulation which plays a crucial role in many diseases such as cancer. Through both transcriptional activation and non-transcriptional means, p53 is involved in glycosis, oxidative phosphorylation, glutaminolysis, insulin sensitivity, nucleotide biosynthesis, mitochondrial integrity, fatty acid oxidation, antioxidant response, autophagy and mTOR signalling[108]. Metabolic stress activates p53 which either positively or negatively regulates these pathways. For example, p53 can control glucose metabolism in many ways. It can suppress the transcription of glucose transporters GLUT1 and GLUT4 to block glucose uptake when it is necessary[108]. Moreover, p53 has the ability to suppress glycolysis via the degradation of phosphoglycerate mutase (PGM), an essential enzyme for the glycolytic procedure[108]. Another example, is the regulation of IGF-AKT-mTORC1 pathway which is hyperactivated in cases such as cancers. Metabolic stress signals activate p53 which in turn,

inhibits mTORC1 signalling in order to stop protein synthesis and by extension, cell proliferation and tumor formation[109].

7.2.8. Inflammatory Microenvironment

Significantly, p53 also affects bioenergetic balancing, inflammation and epithelial- mesenchymal transition (EMT) via the downregulation of transcription nuclear factor-κB (NF-κB). NF-κB family of transcription factors consist of five proteins with DNA-binding domain which induce chronic inflammation and in contrast to p53, promote resistance to apoptosis. The chronic inflammation increases the risk of tumor formation and chemo-resistance[110]. When inflammation is occurred in normal cells with wild-type p53, p53 blocks the NF-κB transcription factor and leads to DNA repair, growth arrest or cell apoptosis. Whereas in p53-deficient or mutant cancer cells, this kind of stress leads to activation of NF-κB and the continuously exposure to inflammatory cytokines resulting in tumor-promoting inflammation and cancer stem cells generation (Figure 7)[111].

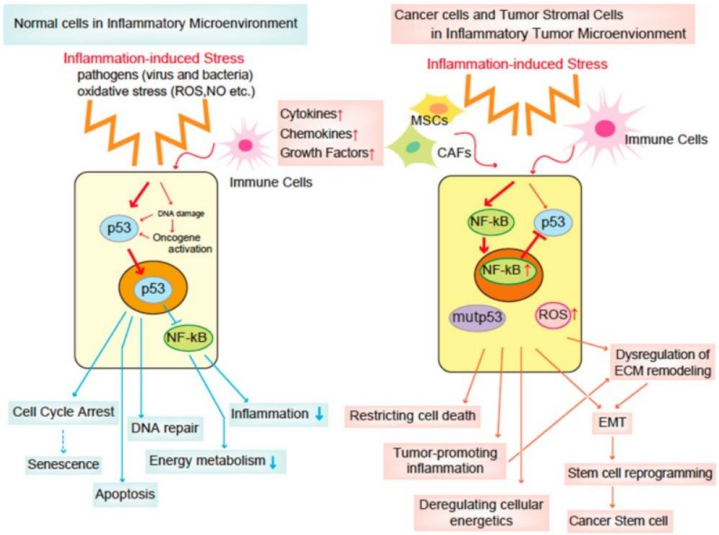

Figure 7: Inflammatory Microenvironment in both normal and tumor cells[111]

7.2.9. ROS regulation

Reactive oxygen species (ROS) are produced by cells and can act as signalling molecules or cellular toxicants. ROS are found to function both as an upstream signal that induces p53 activation and as downstream-mediator of apoptosis[112]. Oxidative stress, occurred due to imbalance between production and accumulation of ROS, leads to p53 activation through phosphorylation by ROS-activated kinases such as ATM (ataxia telangiectasia mutated) and AMPK (AMP-activated protein kinase)[113]. ATM and AMPK phosphorylate p53 which surpasses MDM2 control and is free to transactivate target-genes (Figure 8) [113]. Apart from ROS-mediated regulation of the protein, p53 can act in reverse. This means that p53 has the ability to regulate ROS depending on the stress conditions. If the stress levels are low, p53 upregulates the expression of antioxidant genes which block ROS production. Such antioxidant genes are Sens1/2, GPx1, TIGER, GSL2, ALDH4 etc. Conversely, high stress-levels hyper-activate p53 which in turn activates pro-oxidant genes such as Puma, Bax, PIG3. The activation of these genes triggers ROS production and by extension, cell death (Figure 9) [113].

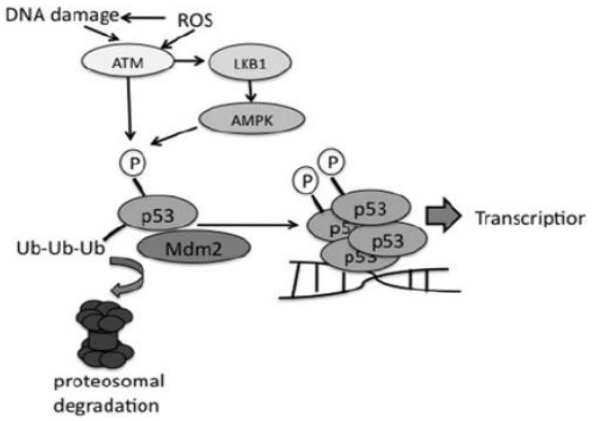

Figure 8: ROS function as an upstream signal for p53 activation[113].

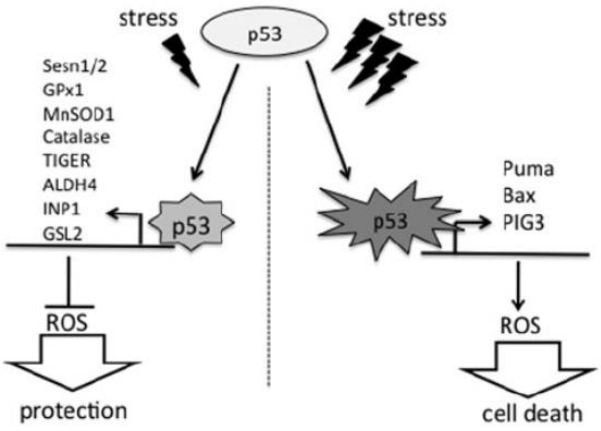

Figure 9: ROS regulation by p53 depends on the stress levels[113].

8. MDM2 and p53 interactions

In 1992, the mdm2 gene was found to transform an immortalized cell line, BALB/c 3T3[60,114,115]. It was proved that MDM2 is a key cellular regulator of p53 that binds tightly to this protein and inhibits its role as a transactivator[60]. Further research has shown that human MDM2 is a 491-amino acid long phosphoprotein that interacts via its hydrophobic NH2 terminal domain with an α-helix present in the NH2 terminal transactivation domain 1 of p53, inhibiting its transcriptional activity[116]. Additionally, MDM2 protein has a RING (really interesting new gene) domain at C-terminus which confers a E3 ubiquitin ligase activity to the protein (Figure 10)[116]. MDM2 acts as p53-specific E3 ubiquitin ligase promoting the ubiquitylation and proteasomal degradation of p53[117]. Importantly, mdm2 gene is also a direct transcriptional target of p53. This means that MDM2 and p53 form a negative loop: p53 elicits the expression of MDM2 and MDM2 blocks p53 activity and leads to degradation[118,119](Figure 11)[120]. The regulation of this loop depends on whether the cells are stressed or not. Under normal conditions, MDM2 translocates the p53 protein out of the nucleus for proteasomal degradation via protein-protein interaction between MDM2 protein and BOX I motif of p53 TAD1. Recently it was shown that in case of DNA damage, ATM-kinase (stress sensor) is activated by double-stranded breaks and phosphorylates p53 at S15 and MDM2 at S395. S15 phosphorylation of p53 prevents MDM2-p53 protein interaction

while S395 phosphorylation of MDM2 protein provokes a conformational change of MDM2 which leads to interaction between MDM2 protein and nucleus p53 mRNA. The translocation of MDM2 to the nucleolus switches MDM2 into a positive regulator of p53 by increasing p53 synthesis and simultaneously inhibits MDM2-induced degradation of p53. During this stress-induced synthesis of p53, ATM kinase phosphorylates the nascent peptide resulting in stabilization and activation of p53 protein towards DNA damage response. This phosphorylation by ATM requires the interaction between phosphorylated MDM2 and the ribosomal proteins RPL5 and RPL11. When DNA damage signalling stops, there is inactivation of ATM kinase and dephosphorylation of MDM2 by wild-type p53-induced phosphatase 1 (Wip1). As a result, MDM2 switches again from a positive into a negative regulator leading to p53 degradation. Thus, MDM2 is now shown to be both a negative and a positive regulator of p53 (Figure 12)[121,122].

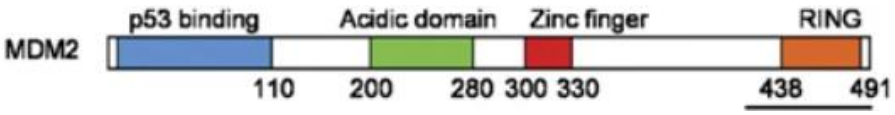

Figure 10: Depiction of MDM2 protein domains[116]

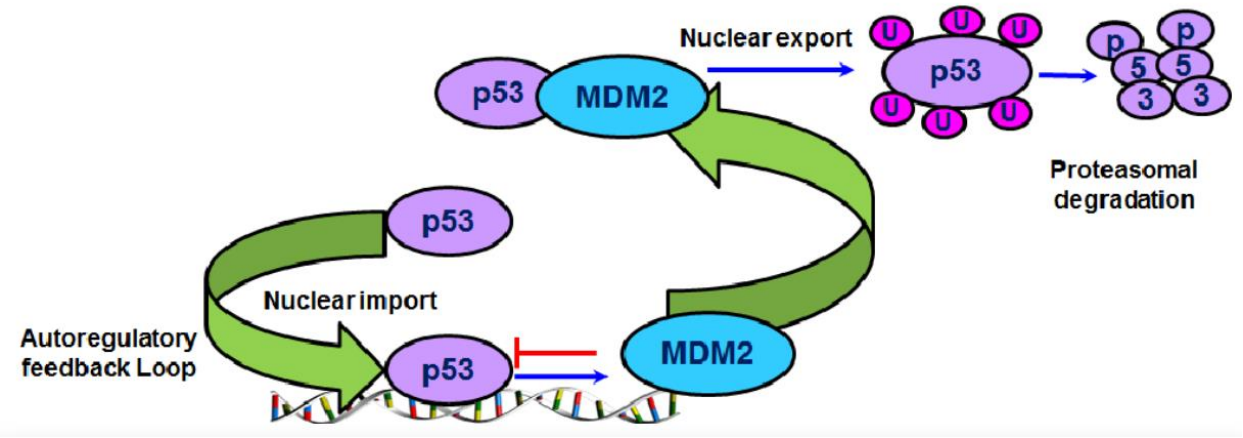

Figure 11: MDM2-p53 pathway: an autoregulatory feedback loop. p53 induces the transcription of MDM2 which in turn, blocks p53 functions by a variety of means: it binds to TAD1 of p53 N-terminal domain and blocks its transcriptional activity directly, or it uses its E3-ligase activity for proteasomal degradation of p53[120].

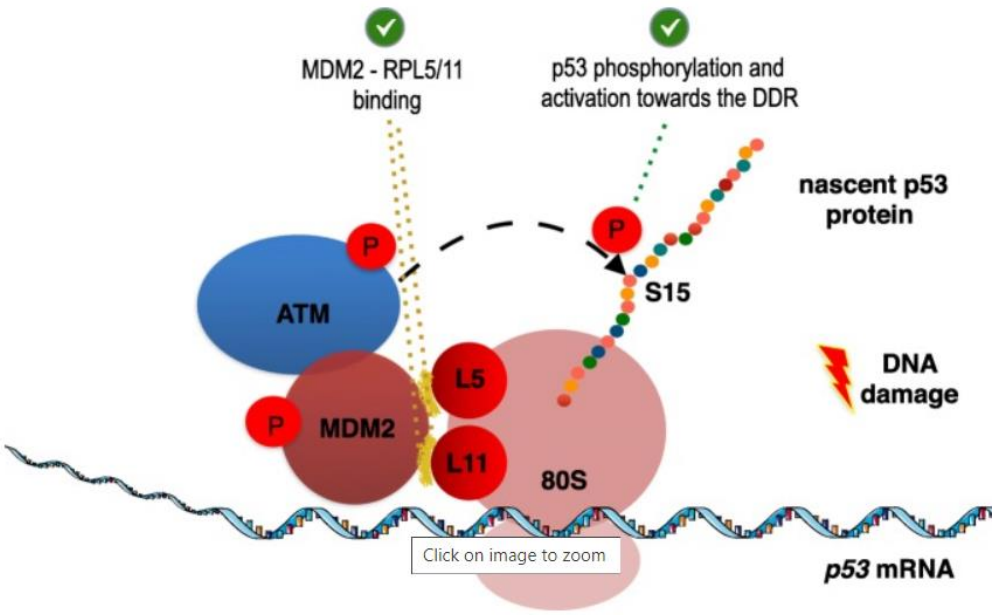

Figure 12: Ribosomal translation of p53, following by DNA damage. ATM phosphorylates the nascent p53 peptide via the interaction between phosphorylated MDM2 and ribosomal proteins (L5 and L11). The phosphorylated p53 protein is activated towards the DNA Damage Response (DDR)[121].

In 1996, a new p53-associating protein with structural similarity to MDM2, came up. Due to the similarity with MDM2, it was called MDMX and was first isolated and found as a complex-partner with p53 in a cDNA library[123]. Subsequently, it was proved that MDMX also binds to the N-terminal region of p53 and acts synergically with MDM2 to amplify its E3 ligase activity and lead to degradation of p53 but in contrast to MDM2, MDMX is not under the transcriptional control of p53 and does not possess its own E3 ligase activity[124–126]. Under normal conditions, MDM2 and MDMX inhibit p53's transcriptional activity via protein-protein interaction or act synergically in order to promote polyubiquitination via MDM2-E3 ligase activity (Figure 13). Under stress conditions, recently it was proved that both proteins act as a positive regulators of p53 via their phosphorylation by ATM-kinase[127].

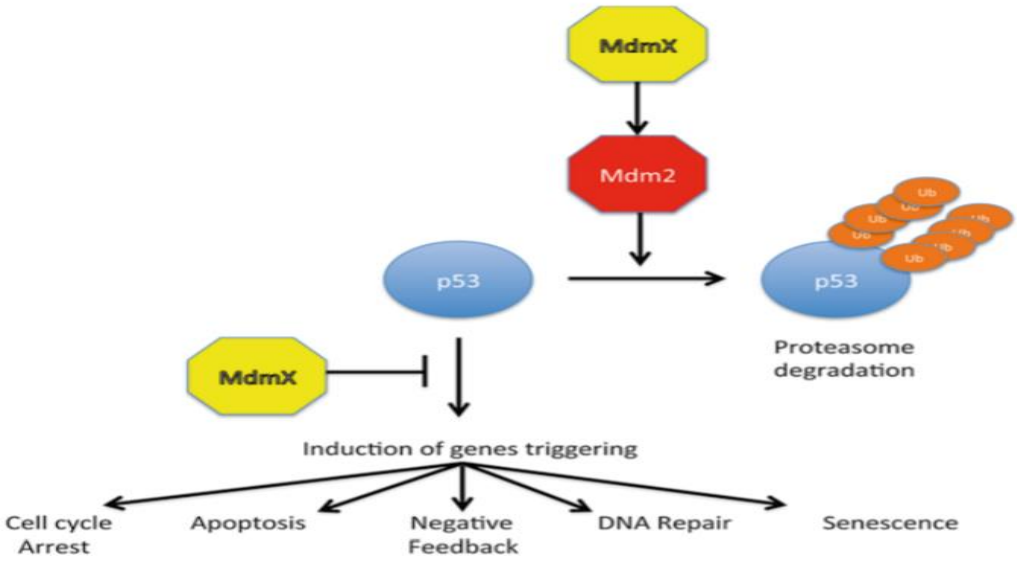

Figure 13: MDM2-MDMX-p53 signaling pathway: MDM2 and MDMX act synergically for blocking p53 in two ways. MDMX enforces the E3-ligase activity of MDM2 and in addition, blocks its transactivation domain[126].

MDM2-p53 interaction is a central node connected to several pathways in cellular network which decides the type of response in about 10 different stress signals (Figure 14), underlining the significance of p53 protein in many cell processes and explaining why TP53 is a tumor suppressor gene[128]. Stress signals activate specific protein mediators whose role is to regulate the E3 ubiquitin-ligase activity of MDM2 (Figure 14). Inactivation or degradation of MDM2 due to these proteins, results in p53 increased activity and response to stress signals. For example, DNA damage-induced ATM kinase leads to MDM2 inactivation, p53 stabilization and response towards damage (Figure 14)[128].

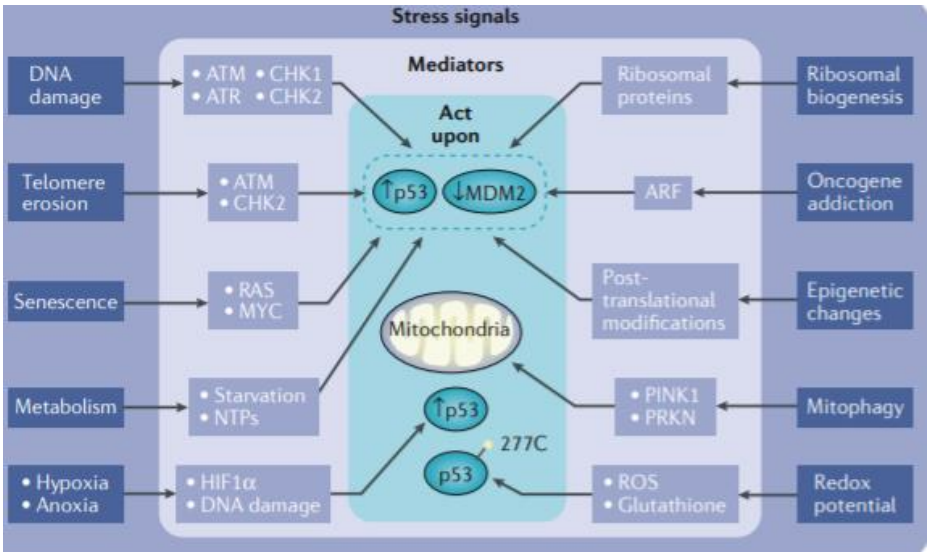

Figure 14: This figure depicts a wide range of stress signals which modulate MDM2-p53 interaction by inhibiting MDM2 protein and, thus, allow p53 to respond to stress[128].

9. Isoforms of p53: p63 and p73

In 1997, two p53-family genes, p63 and p73, were identified and proved to have significant structural similarity with the tumor suppressive transcription factor p53[129]. However, these two isoforms do not act as tumor suppressors and usually, they are not mutated in human cancers[130]. Due to the homology in structure with p53, p63 and p73 bind and activate transcription in some p53-targets. There are three major highly conserved domains among the p53 family members: TAD, DBD and OD. The major similarities with p53 domain were detected in DNA-binding region and this is why p63 and p73 transactivate p53-responive genes leading to cell arrest or apoptosis (Figure 15) [130,131]. It was found that the residues of p53 related to interaction with DNA are the same among p53 family[131]. Apart from the overlapping functions of these isoforms, p63 and p73 knock-out mice were shown to have difficulties in controlling development and differentiation (skin, nervous system, etc.) meaning that these isoforms are essential for the procedures above. Consequently, when cells are stressed p53 isoforms regulate tumorigenesis like p53 protein but in no-stress situation, p63 and p73's role is to control differentiation and development [131]. Interestingly, studies have shown that MDM2, a key regulator of p53 activity, binds also to p73 and more weekly or at all to p63. MDM2 inhibits p73 transcriptional activity without targeting it for degradation. In addition, loss of N-terminal region of MDM2 has showed no consequence on p73-

MDM2 interaction in contrast to p53-MDM2 interaction which is based on both N-terminal domains of p53 and MDM2. This means that p73 interacts with different region of MDM2[132].

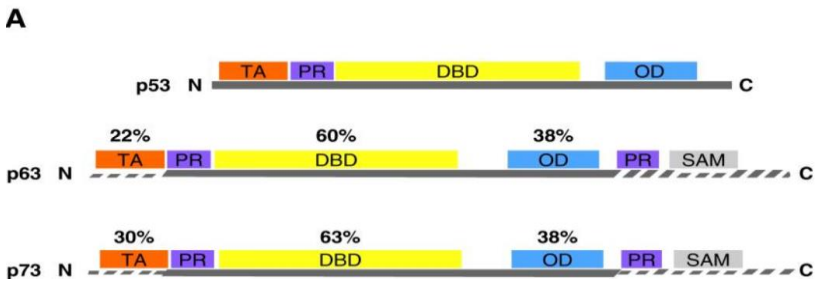

Figure 15: Structural representation of p53 family members. The highly conserved domains among p53 family members are: TAD (orange region), DBD (yellow region) and OD (blue region). The percentages show the similarity rate of p63 and p73 with p53. DBD domain of both p63 and p73 is the most similar with p53's DBD domain (60% and 63%, respectively)[131].

10. Inactivation of p53 in cancer

10.1. Alterations in TP53 gene

10.1.1. Mutations and Loss of Function (LOF)

TP53 is the most frequently altered gene in human cancers. Its inactivation can occur through mutations or p53 allele/-es deletion[133]. TP53 is proved to be mutated in more than 50% of all human cancers[134]. However, there are some specific types of cancer like small-cell lung cancer, squamous cell lung cancer or triple-negative breast cancer where TP53 is mutated in over 80% of the cases[135–137]. One major difference between TP53 and other tumor suppressor genes is the kind of mutations occurred in cancer. Mutations in tumor suppressor genes, such as RB1 or APC, are mostly non sense or deletions and usually result in low or no protein expression[134]. In contrast, the most common mutations in p53 are missense which lead to modified or faulty protein and are most notably detected in 6 specific sites (hotspots) of DNA-binding domain (approximately in 80-90% of cases)[134]. Figure 16 depicts these 6 codons which are usually found to be mutated: 175, 245, 248, 249, 273, 282[138]. Missense mutations can be divided in two categories: structural or

contact alterations. Structural type of mutations lower the melting temperature of the protein resulting in misfolded protein while contact type elicits faulty protein-DNA interactions[139]. Of note, there are missense mutations that affect both the structure and the interaction with DNA[140]. Regardless of the missense mutation type, p53 mutant proteins cannot target the same genes leading to loss of wild-type function (LOF) and, by extension, to tumorigenesis. Moreover, missense mutations can result in oncogenic activity that affect any remaining wild-type p53 protein of the cell with a dominant-negative way. This happens through the formation of mixed tetramers (wt-p53 and mutated p53 proteins) in heterozygous cells. These mixed tetramers are proved to have impaired p53 function, concluding that one mutated allele of p53 is enough to impact the other wild-type p53 allele either with inhibition or attenuation of its fucntions[141]. Generally, p53 is a haploinsufficient tumor suppressor explaining the fact that just a small reduction of wild-type p53 amount is able to promote cancer formation[133].

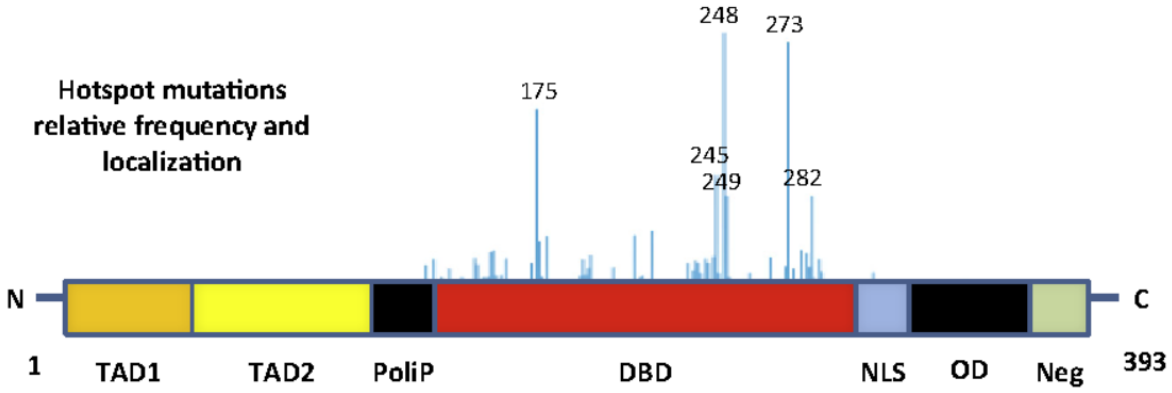

Figure 16: The 6 most-frequently mutated sites, which are located in DBD of p53, are the codons**:** 175, 245, 248, 249, 273, 282[138].

10.1.2. Mutations and Gain of Function (GOF)

It was found that some missense mutations located at DBD not only lead to loss of function but also give new properties in p53 protein, a phenomenon called gain-of-function (GOF)[142]. In GOF mutations, due to the inability of mutant p53 to bind DNA and transactivate target-genes, mutant GOF p53 protein achieves its goal through interactions with other transcription factors and chromatin-modifying proteins[133]. These protein-protein interactions result in new properties such as inhibition of apoptosis, uncontrolled cell transformation, resistance to anti-cancer elements, tumorigenesis, immunosuppression and immune evasion[133,134,143,144]. Studies have shown that stabilization of mutant p53 is major prerequisite for GOF manifestation[145]. Of note, research has

revealed that intact p53 transactivation domain is required in GOF p53 mutated-proteins, implying the need of other transcriptional regulators in order to reach their GOF target[146,147]. However, not all the missense mutations in TP53 provoke GOF activities. For example, in myeloid tumors, gain-of-function mutation is not present[145].

There are some proposed mechanisms by which GOF p53 mutant obtains new functions. One mechanism is by binding and regulating transcriptional factors such as p63, p73, ETS1/2, SMAD 2/3, SREPB, SWI/SNF, SP1, NRF2, nuclear factor Y, vitamin D receptor[145,148–150]. GOF p53 mutants recruit the transcriptional factors above and lead to up- or downregulation of their target-genes that they would be otherwise differently regulated (Figure 17)[133]. For example, GOF p53 mutants can negatively regulate the target genes of p53 family members by downregulating p63 and p73, resulting in inhibition of apoptosis and tumor cell invasion[151]. An additional mechanism is the activation of chromatin regulatory genes by mutant p53 proteins triggering the accessibility of transcription factors and by extension, gene expression. Such regulatory genes are the methyltransferases, MLL1 and MLL2, and the acetyltransferase, MOZ[152].

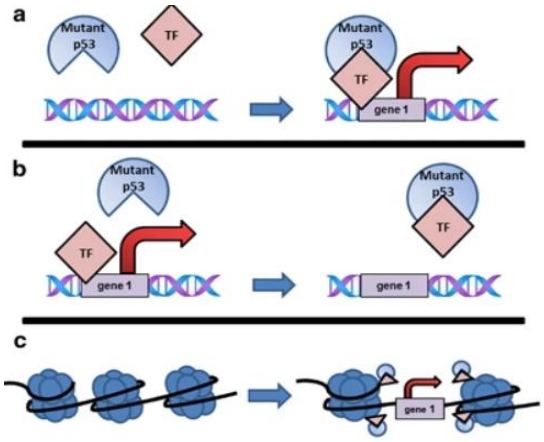

Figure 17: Different mechanisms of GOF p53 mutants. **A)** Mutant p53 protein binds to a transcription factor (TF) and activate the transcription of gene. **B)** Mutant p53 protein binds to a transcription factor (TF), remove it from its target and inactivate the transcription of the target-gene. **C)** Mutant p53 proteins (light blue circles) bind and activate chromatin-modifying proteins (orange triangles) which, in turn, regulate the chromatin structure and activate gene expression at the area between the nucleosomes[133].

10.1.3. Loss of heterozygosity (LOH)

In more than 90% of tumors bearing p53 mutations, there is loss of both functional wild-type alleles of TP53[134]. Apart from missense mutations that are the most common in TP53 gene, another alteration that plays crucial role in exertion of p53 mutant activities is the loss-of-heterozygosity deletion (LOH). Among tumors with missense mutations, approximately 60% present concomitant deletion of the remaining allele, a phenomenon called loss of heterozygosity (LOH)[133]. Research has revealed a two-hit mechanism: mutations in one allele of TP53 gene in early stages and then, loss of the other wild-type allele, during cancer formation[153]. p53 LOH is also a following mechanism in Li-Fraumeni syndrome where inherited or early-arisen germline mutations are present[154]. Notably, LOH is a frequent event in many human cancers with missense mutated p53 allele. The LOH-related human cancers include 61% of sarcoma cases, up to 82% breast cancer with or without HER2 amplification as well as 75% of ovarian cancer cases[155]. In addition, studies with mouse tumors carrying one GOF mutated allele and one wild-type p53 (-/+) have proved that LOH is a prerequisite for mutant p53 protein stabilization and by extension, for *in vivo* GOF exertion[155]. This stabilization mainly refers to mutant p53 protection from E3 ubiquitin ligase activity of MDM2[145].

11. P53 and cancer treatment

The significance of p53 in human cancer necessitates the research of anti-cancer therapies based on this protein. However, targeting mutant p53 has not been an easy task up till now and there are many reasons to explain why. First of all, most of mutant p53 proteins present loss of function which is a difficult drug-target. Secondly, because of different mutations occurred in p53, there is a need for targeting each and every mutant with a single drug. Such a procedure takes a long time to be accomplished and in fact, it is impossible to target all the mutants that have been discovered. Furthermore, p53 is a tumor suppressor with a crucial role in several pathways as it regulates diverse functions and unfortunately, there is a little experience in targeting this kind of genes. Till now, the increased understanding of p53 functions has led to the development of various anti-cancer p53-based therapies[142]. There are several approaches to p53-mediated cancer treatment, including gene therapy, virus therapy, gain-of-function inhibitors, wild-type p53

enhancement in tumors cells, restoration of p53 loss-of-function, synthetic lethal inhibitors in mutant p53 cells and reactivation of immune responses to mutant p53 cancer cells[158].

11.1. Gene therapy via viruses

One of the most promising approach for treating p53-mutant cancers is the virus-mediated gene therapy. As mentioned above, viruses such as SV40, human adenoviruses and papillomaviruses encode oncoproteins (T-antigen, E1B-55Kd, E6, respectively) that block p53's activity and prevent the cell from p53-mediated apoptosis or growth arrest. Scientists exploited this virus mechanism by creating mutant viruses lacking oncogenes and used them as means to introduce a wild-type cDNA into human tumor cells[158]. In China, Shenzhen SiBiono GeneTech created for the first time a recombinant human p53 adenovirus lacking E1A and B oncogenes (rAD5-p53) (Gendicine), which is the first approved gene therapy medicine for cancers with p53 mutated genes[159]. Gendicine was approved in 2003 by China Food and Drug Administration (CFDA) for head and neck cancer treatment, but it has not got approval in the other countries yet[160–162]. Though, there were recent evidence that this recombinant adenovirus (rAD5-p53) has benefits in other type of cancers as well[159]. Gendicine is injected directly into the tumor and afterwards, irradiation is used in order to damage the DNA of the cells and activate the injected wild-type p53 to induce apoptosis or growth arrest[158]. The dominant effect of mutant p53 against wild-type p53 in cells is surpassed by the excessive amount of wild-type p53. The combination of wild-type p53 injection and irradiation leads to cell death which is the desirable result[158]. Nevertheless, a similar gene therapy (Advexin), developed by Introgene, used the same mechanism but it was rejected by US-FDA in 2008[163]. Besides the benefits, there are also drawbacks in the reintroduction of p53 via recombinant adenovirus. One of them is that rAD5-p53 may also repress tumors carrying wild-type p53 gene because of the dysregulation of p53 pathway[163]. Moreover, it is impossible for adenovirus to be transduced in every cancer cell which increases the rate of relapse after treatment[158].

Another way of adenovirus-mediated gene therapy is the exploitation of their oncolytic properties. ONYX-015, a mutant adenovirus lacking E1B gene, selectively kills tumor cells with defective p53 in vivo because these cells have not protection against the adenovirus's damage. While in tumor cells with wild-type p53, adenovirus ONYX-015 cannot be proliferated into the cell due to p53-mediated apoptosis or growth arrest. Of note, research showed that is not certain whether the existence of wild-type p53 affects ONYX-015's amplification in the cell or not[164–167]. However,

ONYX-015 development as a gene therapy was stopped because of financial issues[158]. Apart from ONYX-015, another oncolytic gene therapy was developed, this time with the adenovirus H101. In contrast to ONYX-015, H101 was approved by CFDA for head and neck treatment but like Gendicine, has not received approval in the other countries[168].

11.2. Targeting mutp53 GOF

As mentioned above, there are some missense mutations located at DBD that lead to new p53-related properties, known as gain-of-function mutations. Some p53 mutant proteins form aggregates in order to exert their functions. An attractive approach for mutp53 GOF treatment is the degradation of these aggregates which are formed in hypoxic conditions. C-terminus Hsc70-interacting protein (CHIP), which has both chaperone and E3 ligase activity, promotes this autophagy-mediated degradation (occurred in hypoxic conditions) whereas in the absence of this protein, the amount of aggregates remains at the same levels allowing mutp53 GOFs to exert their abnormal activities[169]. Exploiting CHIP's activity may have a great impact on mutp53 GOFs treatment and open the way for new therapeutic strategies.

Another approach is related with the mutp53 GOF stabilization that is required for GOF exertion. Mutant p53 protein forms a complex with heat shock proteins (HSPs), HSP90/HSP70, and histone deacetylase 6, HDAC6, which are upregulated in tumor cells in comparison to normal cells[158]. This complex makes the mutant p53 protein stable[158]. It was found that inhibitors of HSP90 and HDAC6 decrease the level of mutant p53 in tumor cells whereas the overall survival rate is increased[170]. Scientists have developed some inhibitors of HSP90 (17AAG, 17DMAG, Ganetespib) and HDAC6 (SAHA)[170]. Inhibition of HDAC6 using SAHA weakens the interaction between mutp53 and HSPs resulting in mutp53 degradation by MDM2 and CHIP (Figure 18)[171].However, the combination of 17AAG and SAHA therapy was examined and the results showed that there is a significant level of cytotoxicity in tumor cells due to broadly destabilization through HSP90/HDAC6 inhibition[170]. On the other hand, Ganetespib was shown to be more potent in degrading mutant p53 than 17AAG or 17AAG/SAHA and it has proved to have a more safety profile in cancer patients. In addition, Ganetespib is p53-mutant specific and leads to cell growth arrest or apoptosis[170].

Besides HSP90 chaperone machinery (HSP90, HSP70 & HDAC6) which interacts with mutp53 GOF and stabilize both types of missense p53 mutations (DNA-contact and structural type), there is also HSP40-DNAJA1, another molecular chaperone for mutp53's protection. In contrast to HSP90 chaperone machinery, DNAJA1 protects only the structural type of missense p53 mutations from degradation by CHIP[172]. The mutp53-DNAJA1 interaction is controlled and protected by the mevalonate-5-phosphate (MVP) which is part of mevalonate biosynthetic pathway (MVA) and leads to cholesterol production[173]. Scientists noticed that statins, inhibitors of HMG-CoA reductase -a major enzyme in mevalonate pathway- reduce the level of MVP and by extension, affect the mutp53-DNAJA1 interactions[174]. As a result, DNAJA1 liberates mutp53 and, CHIP mechanism is free to degrade the mutated gain-of-function protein (Figure 18)[172]. These data indicate that apart from HSP90 chaperone machinery inactivation by inhibitors of HSP90 and HDAC6, there is another promising anti-mutp53 therapy using inhibitors of MVA (statins) to block HSP40-DNAJA1 interaction whose normal role is to provide stabilization to structural mutp53 proteins[158]. Conversely, one of mutp53 gain-of-functions is the upregulation of MVA pathway (increase the levels of cholesterol) via activation of MVA-related genes[147]. This procedure is achieved by p53 binding to SREBP (sterol regulatory element-binding protein) which is a major transcription factor of sterol biosynthesis. Hence, mutp53 auto-regulates its stability by activating MVA pathway which in turn, results in DNAJA1 activation and DNAJA1-mutp53 complex formation[172]. Using molecules, such as statins, to disrupt p53 interactions with other proteins such as SREBP, p63, p73 etc., is a major key in blocking p53 gain-of-function pathways and suggests a new ways for therapeutic strategies[158]. Figure 19 depicts gain-of-functions of p53 protein that they would be promising targets for anti-mutp53 GOF therapies.

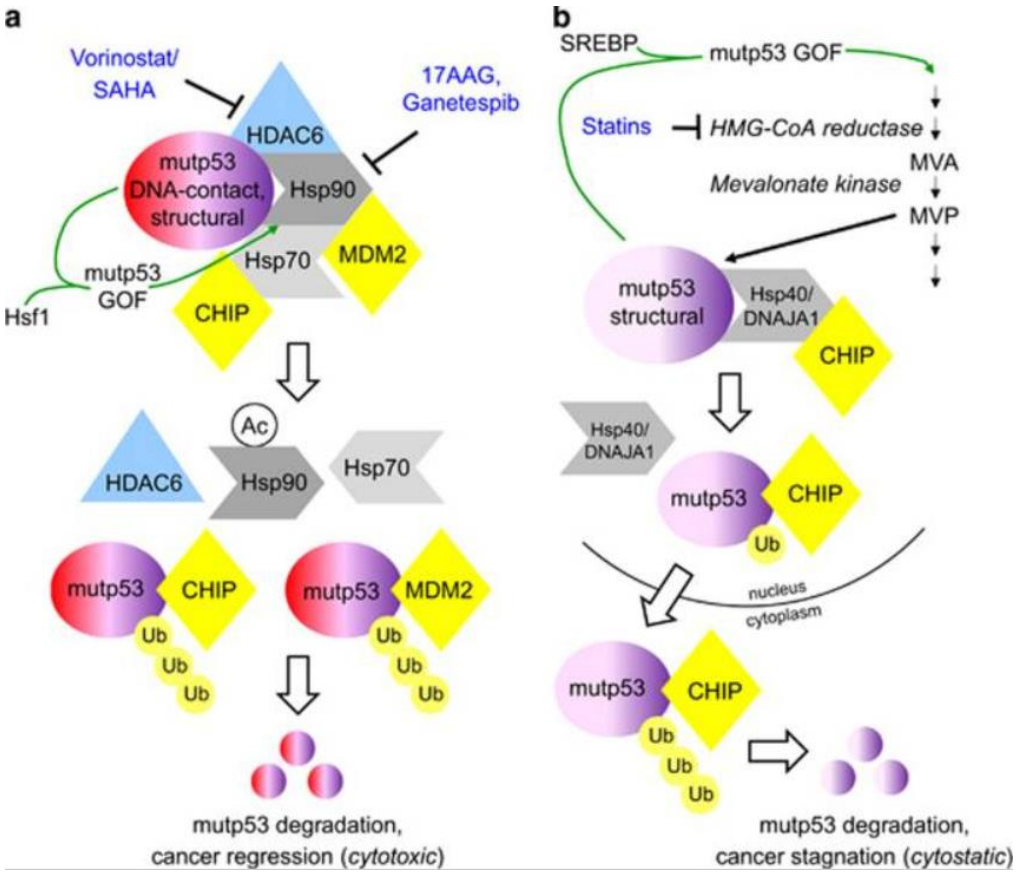

Figure 18: Degradation of mutp53 by targeting heat shock proteins (chaperones). Two parallel pathways: HSP90/HDAC6 chaperone machinery and HSP40/DNAJA1 chaperone. They both promote the stabilization of mutp53 in cancer and prevent CHIP- and MDM2-mediated degradation. Targeting these chaperones directly or indirectly with specific inhibitors (SAHA, 17AAG, Ganetespib, Statins) is a promising anti-mutp53 therapy[172].

Gain-of-function	Molecular mechanism	Mutant version	Type of cancer	References
Proliferation	Increasing receptors translocation through the RCP complex	R175H R273H	Breast cancer	Muller et al., 2009
	Increasing the PI3K/AKT axis through inhibition of DAB2IP	R280K R175H	Breast and prostate cancer	Valentino et al., 2017
	Increasing the active state of K-Ras	R175H	Pancreatic cancer	Escobar-Hoyos et al., 2020
Migration and metastasis	Interacting with p63 and downregulating its anti-metastatic activities	R175H	Breast cancer	Gaiddon et al., 2001; Adorno et al., 2009
	Increasing Rac1 activity	R175H R248W R273H	Colorectal cancer	Yue et al., 2017
	Interacting with HIF1α and favoring secretome activity and metastatic capabilities	R175H R273H R280K	Breast cancer	Capaci et al., 2020
Metabolic reprogramming	Increasing activity of RhoA/ROCK axis and translocation of glucose transporters to membrane	R175H R248Q R273H	Lung and breast cancer cells	Zhang et al., 2013
	Interacting with SREBP and increasing the MVA pathway	R273H	Breast cancer	Freed-Pastor et al., 2012
	Inhibiting AMPK activity and increasing anabolic pathways	R175H G245C R282W	Head and neck squamous cell carcinoma	Zhou et al., 2014
Immune evasion	Increasing inflammation and favoring NF-kB activity	R273H	Colorectal cancer.	Cooks et al., 2013
	Augmenting pro-inflammatory activity in the tumor microenvironment by interaction with MAFF	R273H	Colon and breast cancer cells	Ubertini et al., 2015
	Reprogramming macrophages from M1 to M2	R245 R248 R175 R273 R282	Colorectal cancer	Cooks et al., 2018
Stemness	Increasing CSC surface markers and ALDH enzymatic activity	R175H R273H	Colorectal cancer	Solomon et al., 2018
	Increasing activity of YAP/TAZ pathways and promoting self-renewal of CSC	R175H R273H	Glioblastoma and breast cancer cells	Escoll et al., 2017
	Inducing a repressive state of chromatin through PRC2 activity	R175H R248W R273H	Hematopoietic stem cells	Chen et al., 2019
Chemoresistance	Favoring changes in transcriptional regulation by mutp53/YAP/β-arr1 and promoting cisplatin resistance	R273H	Ovarian cancer	Tocci et al., 2019
	Up-regulating DNA repair pathways	R280K	Breast cancer	Lin et al., 2019
	Downregulating procaspase-3 by increasing miRNA-128-2	R175H	Non-small-cell lung cancer	Donzelli et al., 2012

Figure 19: Mutant p53 Gain-of-functions and the type of cancer that are present[175].

11.3. MDM2/MDM4 inhibitors

As mentioned above, MDM2/MDM4 promote degradation of p53 protein through ubiquitin ligase activity. In many cancers with intact p53 protein, the levels of wt-type p53 protein remain low due to the interaction with negative regulators (MDM2, MDM4) which are upregulated in some specific types of tumors[176,177]. Subsequently, many researchers attempted to block the interaction between p53 and MDM2/MDM4 in order to protect p53 from degradation and in the aftermath, suppress tumorigenesis[177]. Studies of this interaction revealed the binding-sites of p53-MDM2 protein complexes. The N-terminal region was found to interact with 3 hydrophobic amino

residues (Phe19, Trp23, Leu26) of p53's transactivation domain[178]. Thus, small molecule antagonists, that imitate this interaction, were synthesized and utilized resulting in inhibition of this interaction and increased levels of p53 in the tumor cells[179]. In addition, 2 more binding sites between p53 and MDM2 were discovered, one at the DNA binding domain and the other at the C-terminal region of p53[180,181].

The first compounds shown to inhibit MDM2 from binding to p53 sites and specifically to the 3 amino acids of p53's transactivation domain were the nutlins (also known as cis-imidazoline group of molecules) and mainly nutlin-3a[179]. Although nutlin-3a was shown to interact perfectly with MDM2, block its ability to interact with p53 and trigger the normal p53 response in cancer, adverse effects on preclinical studies, such as cytotoxicity, limited efficacy and bioavailability, prevented the use of this molecule in clinical trials[182,183]. Beyond of these compounds, there are other more potent MDM2 or MDM2/4 inhibitors that underwent or finished clinical trials. One of them, nutlin-3a analogue, also known as RG7112, showed great potentials to clinical trials but the significant cytotoxicity prevented its further investigation[184,185]. A more potent and selective nutlin analogue, idasanutlin (RG7388, RO5503781), was designed and is currently recruiting for several clinical trials[186]. Recent findings suggest that the idasanutlin-related response of leukemic blasts depends on the levels of MDM2 protein before the treatment[187]. Notably, idasanutlin has been currently tested in combination with cytarabine for the therapy of relapsed or refractory Acute Myeloid Leukemia (AML) and is one of the few compounds that reached phase III clinical trials[188].

Apart from MDM2, another attractive therapeutic target for cancer treatment is the MDMX/MDM4 protein. The inhibitors of MDM2 mentioned above, have low affinity for MDM4 so they might be inadequate in tumors with highly-expressed MDM4. In addition, MDM4 overexpression may lead to MDM2-inhibitor-resistance. Hence, MDM4 or MDM2-MDM4 inhibitors have been developed in order to inhibit MDM4's function[189]. The major MDM4 inhibitors are the SAH-p53-8 (a cell-penetrating, stabilized, α-helical peptide) and XI-001[190,191]. Furthermore, a significant discovery was the peptide ALRN-6924 whose role is to prevent both MDM2 and MDM4 from interacting with p53[192]. The results in AML (acute myeloid leukemia) cell lines treated with ALRN-6924 have showed that ALRN-6924 peptide is potent to induce cell cycle arrest and apoptosis[192]. Results from ALRN-6924 clinical trials confirmed these findings and notably, showed that this type of treatment has less adverse effects from the inhibitors mentioned above[193]. Another promising novel therapy is Protoporphyrin IX (PpIX), a metabolite of aminolevulinic acid (ALA) that has already been approved for photodynamic diagnosis and therapy[194,195]. Recently, it was shown that

exogenous Protoporphyrin (exo-PpIX) is a dual inhibitor of p53/MDM2 and p53/MDMX interactions and triggers apoptosis in B-cell chronic lymphocytic leukemia cells[196]. In addition, Verteporfin, an analog of PpIX, is also possible for drug repurposing in cancer, as it was previously approved by FDA for treatment of age-related macular degeneration. Latterly, it was found that Verteporfin induces p73 activation in p53-mutated tumor cells by inhibiting p73/MDM2 interactions in a Nutlin-way[197]. This analog of PpIX is proposed for the improvement of pancreatic cancer therapy and has still to be studied[197].

11.4. Synthetic Lethality in mutant p53 cells

A new approach for cancer treatment, and more specifically for p53-mutated tumors is Synthetic Lethality (SL). Synthetically lethal genes are a pair of genes whose both perturbation leads to cell death whereas perturbation of only one gene is related to cell viability[198]. New types of anti-cancer therapies based on this SL phenomenon were discovered resulting in highly-selective therapies that affect only p53-mutated tumor cells as distinct from p53-wild type cells[199]. The mechanism of these therapies is to inhibit specific genes in cells which bear mutated p53 genes and induce cell death in cancers due to Synthetic Lethality phenomenon (Figure 20)[199].

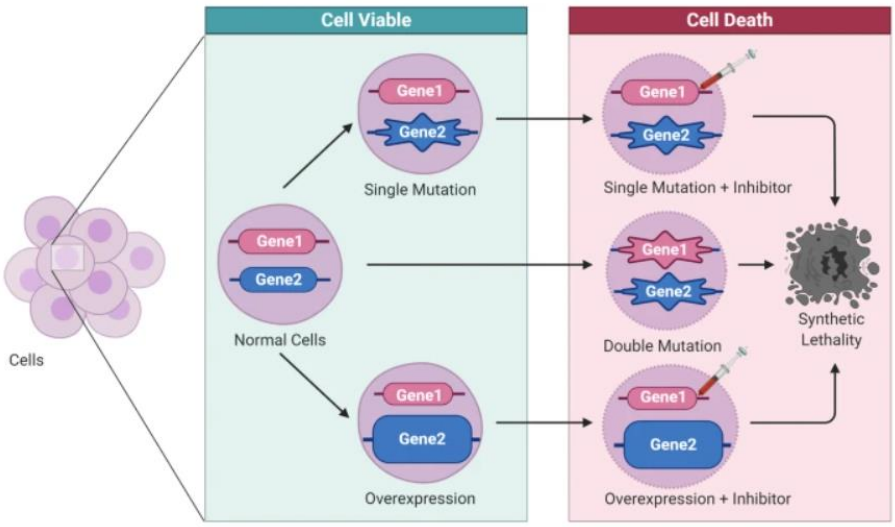

Figure 20: The phenomenon of Synthetic Lethality. The left table depicts normal cells with 2 wild-type genes. These cells are tolerant to a single mutation or overexpression of one gene. On the other hand, the right table shows that an

inhibitor of the second gene can provoke cell death through the Synthetic Lethality phenomenon, indicating that occurs only when a pair of synthetically lethal genes has concurrent deficiencies[199].

Many screens have been made in order to find potential synthetic lethal partners of p53 for targeting p53-deficient tumors[200]. The most important detected genes include WEE1, PLK1, PKC, SGK, ATM, CHK1, GEFH1, PAK3 etc. which are all involved in several p53-mediated pathways (G2/M and G1 checkpoints, DNA damage, etc.)[201–204]. Of note, WEE1 is a protein kinase that activates G2/M checkpoint by inhibiting CDK1 and CDK2, resulting in cell cycle arrest and DNA damage repair. Inhibitors of WEE1 protein kinase lead to accumulation of DNA damage and in combination with p53-deficient cells, which have also decreased ability in G2/M checkpoint regulation, cell death is occurred[205]. Hence, inhibiting WEE1 protein is a significant type of Synthetic Lethality therapy (SL). Adavosertib is the only WEE1 inhibitor that is now recruiting for clinical trials with encouraging results[199].

11.5. Restoring p53 activity

A different approach in anti-cancer therapies is to restore wild-type p53 activity through the development of low molecular weight compounds. The proper function of p53 requires the binding of a single zinc atom and thus, missense p53 mutations which block this binding, provoke the destabilization of zinc interaction and the formation of misfolded p53 proteins[206]. Researchers observed that addition of zinc in media culture of zinc-deficient tumor cells, restore the wild-type p53 structure and function[207]. Evidence has shown that drugging Zinc, for example through Zn-cur (Zinc-curcumin complex) treatment, restores the wild-type structure of the protein and reactivates p53's normal functions[207]. Interestingly, Zinc-curcumin complex treatment has the ability to cross blood-brain barrier and is indicated for glioblastoma cases[208]. Recently, COTI-2, a novel thiosemicarbazone derivative was proposed to modulate p53 protein function by binding to the misfolded mutp53 forms. Preclinical studies have shown that COTI-2 reactivates the target genes of p53 such as p21, PUMA and NOXA and induces DNA damage and stress that lead to apoptosis or senesence[209]. Phase I clinical trial of COTI-2 is currently tested in gynecological tumors and head/neck squamous cell carcinoma (HNSCC) (NCT02433626). Notably, COTI-2 has also p53-independent properties, as it was found to be involved in AMPK pathway activation and mTOR pathway inhibition in HNSCC-related studies (Head and Neck Squamous Cell Carcinoma)[210].

Moreover, PRIMA-1 and its methylated version ARP-246, another low molecular weight compounds, were found to restore p53 activity through methylene quinuclidinone (MQ) conversion product[211]. This MQ product is able to interact and modulate the thiol chemical groups of mutp53 reactivating p53 protein[211]. Despite the benefits of PRIMA-1 use, there is significant cytotoxicity that prevents it from further clinical studies, whereas its analog ARP-246 appears to be more potent compound as a reactivator of p53 with less toxicity[211]. ARP-246 is currently recruited for several clinical trials, even a phase III clinical trial[175].

More recently, it was proved that the acetylation of GOF mutp53 with the specific Arg[158] mutation -common in lung carcinomas- represses the oncogenic nuclear factor kappa-B (NF-κB) and elicits apoptosis[212]. This unique mechanism exploits the properties of a specific GOF mutation and restores wtp53 activity opening the way for new type of anti-cancer therapies which will depend on the specific GOF mutation[212]. Furthermore, phenethyl isothiocyanate (PEITC), derived from cruciferous vegetables was shown to inhibit growth in cells with mutp53 forms by restoring p53 conformation and transactivation functions[213]. A new treatment mechanism focused on glutamine deprivation under stress conditions in tumor cells suggests that IκB-kinase β (IKKβ) reactivates p53 to promote survival and metabolic adaptation but not apoptosis[214].

11.6. Immunotherapy

Studies have revealed that p53 mutant proteins which are common in cancer cells induce the production of antibodies against them both in mice and humans[12,215]. It has been showed that these p53 antibodies recognize epitopes localized in the amino- and carboxy-terminal of p53 protein[215]. Statistical analysis of 9489 patients with various types of cancer proved that p53-Abs is a specific marker in cancer patients. Furthermore, it was found that the presence of p53-Abs is related with the frequency of p53 mutations, indicating that mutations of p53 result in immune response. The ascertainment of this fact came when cancers lacking p53 mutations, such as hepatoma, melanoma, testicular carcinoma, proved to be negative for p53-Abs (Figure 21)[216–218].

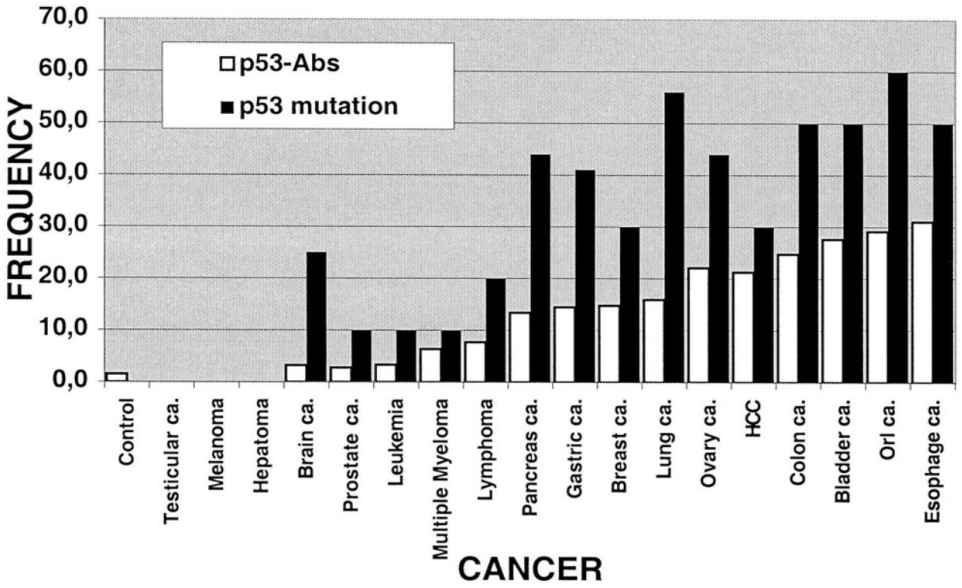

Figure 21: Correlation between p53 mutations and p53-Abs in various cancer types. Testicular carcinoma, Melanoma and Hepatoma which lack p53 mutations, display absence of antibodies production[215].

It has been also proved that only the missense mutations can result in antibodies production[219]. Despite the fact that p53 missense mutations -related to antibodies production- usually occur in DBD of p53 (hot-spot mutations), the epitopes detected by p53-Abs were located in NH2- and COOH- terminal regions. This, in combination with the similarity of human immune response in various types of cancer, suggest that missense mutations indirectly affect the production of Abs via p53 accumulation in the nucleus of tumor cells[215]. The potential role of mutant p53 protein as an antigen led to the idea that mutant p53 could be a druggable target in immunotherapy[220]. In the future, perhaps it will be possible that an antibody against a specific mutant p53 will be an effective treatment for cancers carrying this particular mutant. Recent studies showed that a T-cell receptor-like antibody (TCLR) was able to interact in vitro with mutant p53-expressing tumors and not with wild-type p53 ones, blocking the tumor growth of mice that were previously inoculated with mutan-p53 cells than with non-p53-mutated cancer cells[221].

12. Discussion and Future

This review presents a comprehensive overview of TP53 history and how this gene has achieved to be in the center of biology research reaching the list with the most analyzed genes of all time. Over the past 40 years, there has been tremendous development in p53 field resulted in better understanding of p53 structure and how this affects its functions, p53-mediated responses due to stress signals, p53 inhibition and degradation by MDM2 protein, p53 family genes and the relation between p53 and cancer. Remarkably, MDM2-p53 interaction plays a central role in defining the fate of many stress signals and regulating many cellular processes. Hence, p53 is such an important protein due to its role to repair and restore homeostasis or induce cell death when it is necessary. All this knowledge has led to the development of anti-cancer p53-related therapies which are promising tools for cancer elimination. However, few of them, such as ARP-246 and idasanultin, have reached the clinical trials while many of the discovered p53-targeted therapies presented toxicity or low efficacy in preclinical studies. More research should be done in order to acquire a better picture of p53 pathways and how gain-of-function mutants of p53 act. Additionally, the involvement of p53 family genes in these pathways and how these can be exploited in therapies need to be explored in the future. Time and more research on this evolving and complex field will answer these questions and clarify the crucial role of p53 in both normal and cancer cells.

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