

Bringing p53 into the Clinic

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Abstract

The use of p53 as a biological marker to predict chemotherapeutic outcome has been challenging, with clinical research showing positive as well as negative correlations with p53 mutations. Recent research reveals the complexity that underlies the use of p53 as a single predictive marker as well as the challenges associated with classifying p53 mutations. It is becoming clear that various p53 mutations are associated with differential treatment response and outcomes. In addition, different drug regimens could also play a role in modifying the effects of p53 mutations on therapeutic outcome. Finally, we discuss improvements in the diagnostic detection of p53 mutations and gene signatures that may better reflect p53 functionality in tumors, which may serve as a more reliable tool in correlating p53 mutations to clinical response.

Keywords: p53; Chemotherapy; Diagnostic; Prognostic; Gain-of-function

Introduction

Detecting cancer specific mutations in tumors provides valuable clinical information for both the diagnosis and treatment of the disease. Biomarkers that emerged from studying cancer-specific mutations in Kras [1] and BRCA [2] have been used successfully to guide therapy in the clinic and have spurred an immense interest in the field of oncology to develop novel biomarkers with the potential to tailor treatments to each individual patient.

Mutations in the p53 tumor suppressor gene occur frequently in all human cancers, underscoring the importance of p53 in maintaining genome stability. Approximately 70% of breast cancer alterations in the p53 gene are missense mutations, including hotspot mutations in the DNA binding domain of p53 [3]. The significance of p53 mutations as a potent driver of the pathological development of breast cancer is emphasized by the high frequency of mammary carcinomas in Li-Fraumeni patients, a hereditary cancer predisposition syndrome due to causative p53 germline mutations [4]. The biochemical functions of p53 explain its importance in maintaining cellular integrity in response to various stresses. The p53 protein is a 393 amino acid protein that comprises a N-terminal transactivation domain (residues 1-42), a proline rich domain (residues 40-92) which also contains a second transactivation domain, a sequence specific DNA binding domain (residues 103-306), a tetramerization domain (residues 307-355) and a C-terminal regulatory domain (residues 356-393) [5]. Most hotspot mutations rendering p53 inactive occur within its DNA binding domain, underscoring the important role of p53 as a transcription factor. Its interaction with MDM2, an E3 ubiquitin ligase and a transcriptional target of p53, forms a negative feedback loop to keep p53 activity in check. Upon stress stimuli (DNA damage, oncogenic signals), p53 stabilization triggers the transcription of a wide plethora of genes involved in DNA repair, cell cycle arrest and senescence [6]. Mutation of p53 therefore leads to abrogation of checkpoints, impaired DNA repair, attenuated apoptosis and tolerance of genomic alterations and instability. Recent data also demonstrate important roles of p53 in metabolism, autophagy, epithelial to mesenchymal transition and stem cell renewal [7].

Effects of Different p53 Mutations

Given the wide mutational spectrum of p53 in cancers, a substantial amount of research remains focused on elucidating the distinct oncogenic properties of specific p53 mutations. Frequent mutations in p53 gene identified in human cancers are also known as “hotspot” mutations, and these tend to cluster within the central most conserved DNA binding domain of p53, most commonly targeting residues 175, 245, 248, 249, 273 and 282. Mutations R175H, G245S/D, R249S are classified as structural mutants as they result in loss of wildtype p53 conformation while R248W/Q, R273H/C and R282W are regarded as contact mutants as they disrupt sequence specific DNA binding. Unlike mutation of other tumor suppressor genes (e.g. VHL, APC, RB1, etc.) which results in deletions and low or no expression of the tumor suppressor proteins, hotspot mutations of p53 result in a non-functional full length mutant protein which is often stabilized in tumor tissues. High levels of mutant p53 protein can lead to either a dominant-negative effect by forming oligomers with the wildtype p53 protein, or, gain-of-function properties, as in the case of LOH (loss of heterozygosity) where the second wildtype allele is lost. Hotspot mutations in the p53 gene are of particular interest as in addition to abrogating its transactivation function through disruption of DNA binding functions, these mutations also confer oncogenic gain-of-function properties resulting in loss of cell polarity, increased invasiveness, angiogenesis and chemoresistance [8-20].

It is clear that mutant p53 can exert oncogenic or gain-of-function activity independent of its effects on wildtype p53. Over-expression of tumor-derived mutants of p53 on a p53 null background increases

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their ability to form tumors in mice compared to parental cells, distinguishing gain-of-function from dominant negative effects, which abrogate wildtype p53 functions. Therefore to fully demonstrate gain-of-function in mutant p53, the main experimental approaches adopted in studies should include the expression of mutant forms in a p53 knockout background or knockdown of mutant p53 by RNAi in cancer cells that have lost the wildtype allele (LOH), to avoid misinterpreting dominant-negative effects for gain-of-function. Mouse models demonstrate convincingly that the gain-of-function properties of mutant p53 are not equivalent to a mere loss of wildtype p53 functions. Mice expressing two frequent hotspot mutations, R172H and R270H (equivalent to R175H and R273H in human), displayed an altered tumor spectrum with increased metastatic cancers compared to p53 knockout mice, demonstrating that the missense mutations exhibit bona-fide gain-of-function [21-23]. Furthermore, LFS patients experience an earlier onset of cancers compared to patients with germline p53 deletions [24].

The effects of p53 mutations have been widely studied in tissue culture systems and mouse models. Numerous reports supported the role of mutant p53 in conferring increased invasiveness and metastatic potential in tumors. The underlying mechanisms are thought to involve enhanced receptor signaling through transforming growth factor β (TGF- β) receptor, epidermal growth factor receptor and MET receptor, mediated in part by increased integrin/RCP driven recycling and expression of growth factor receptors [12,25]. Although both R273H and R280K promote invasiveness in response to EGF, the specific interaction between mutant R273H and nardilylin (NRD1) is required for an invasive response to HB-EGF growth factor and this is independent of p63 and Rab coupling protein [11]. The ability of mutant p53 (R270H) in mice in augmenting and prolonging the response of epithelial cells to low levels of inflammatory cytokine and thereby enforcing a state of persistent NF- κ B activation may be related to the physical interactions between NF- κ B and mutant p53 [26,27]. Chronic inflammation combined with increased tissue damage, genome instability and ability of mutant of mutant p53 cells to evade apoptosis, lead to rapid accelerated inflammation-drive colon cancer in mutant p53 but not wildtype p53 mice. Increased secretion of cytokines, hormones and growth factors (including HGF and TGF- β) could also contribute to this p53-dependent invasion and metastasis.

While most mutant p53 have lost the wildtype transcriptional activities, it is reported that they may have gained new transcriptional functions through selective binding to DNA, direct modulation of gene expression or through interactions with p53-related proteins, p63 and p73, and other transcription factors [28]. TAp63 and TAp73 are reported to interact with mutant but not wildtype p53 [28,29]. Interactions with p63 or p73 negate their transcription activities thus deregulating apoptotic pathways or spindle assembly checkpoints, leading to chemoresistance, increased migration, invasion and metastasis [30]. However, not all mutants exhibit the same gain-of-function mechanism or property, and only a subset of tumor-derived forms of p53 down-regulate p63/p73-dependent activities [29]. An altered tumor spectrum, along with a more metastatic phenotype seen in p53^{+/+}p63^{-/-} and p53^{+/+}p73^{-/-} double mutant is reminiscent of that observed in the mutant p53 knock-in mice, suggesting that the gain-of-function p53 mutants may act in part by inhibiting p63/p73 functions [31]. Furthermore, polymorphism at p53 residue 72 (R72) favors binding to p73, adding yet another layer of complexity [32]. Extrinsic cell signals also drive gain-of-function events, as illustrated by the formation of a ternary complex between mutp53, p63 and Smad proteins, which is driven by TGF β signaling and oncogenic Ras [12].

In addition, p53 mutants (R248W and R273H) compromise genomic stability through reported interactions with MRE11 exonuclease, thereby impairing MRN (Mre11-Rad50-Nbs1) complex recruitment to sites of DNA damage and inhibiting ATM activation (ataxia-telangiectasia-mutated) [33]. This results in the perpetuation of unrepaired DNA breaks and unwanted recombinogenic events. In addition, mutant p53 interaction with TDP2 was reported to increase resistance to topoisomerase poisons and may contribute to chemoresistance [34]. Other proteins that interact with mutant p53 include PML (promyelocytic leukemia protein), which was reported to interact with and induce mutant p53 and its target, prolyl isomerase Pin1 [35,36]. Pin1 cooperates with mutant p53 (R280K) to promote a mutant p53-dependent inhibition of p63 and the induction of transcriptional events that lead to aggressive tumor phenotypes including increased migration and invasiveness [36]. Pin1 also enhanced tumor growth in a mutant p53 knock-in Li Fraumeni mouse model, and may work in a synergistic manner with mutant p53 as suggested by observations that outcome was worse in patients with combined Pin1 over-expression and p53 mutation, compared to those with either Pin1 over-expression or p53 mutation alone [36].

Other molecular mechanisms that may contribute to the gain-of-function phenotype include interactions of mutp53 with NF-Y, SRBEP, the vitamin D3 receptor and can evoke activities that are different from wild-type p53 [37-40]. This is further exemplified in many different scenarios, including the reported up-regulation of myosin-X which promotes breast cancer invasion and metastasis [41], and the up-regulation of transcription factors such as Twist, ZEB-1/2 which lead to epithelial to mesenchymal transition [42-44].

However, not all mutants are equal in their oncogenic properties and it is becoming clear that different mutations affect p53 functions to different extents regardless of their subclassification as contact or structural mutants [45,46]. The p53R172H and p53R270H mutations produce gain-of-function properties and increase tumorigenesis in mice [22,23]. Another mutation, R246S, which abrogates the transactivation potential of p53, did not confer any gain-of-function properties, but instead exerted a dominant negative effect on wild-type p53 to inhibit radiation-induced cell death [47]. The extent of tumorigenesis and survival in the R246S mutant mice are equivalent to the p53 knockout mice, suggesting that the R246S mutation results only in a loss of wild-type p53 function. Li Fraumeni patients harboring R248Q mutation appear to have an earlier onset of disease and a shorter lifespan compared to inherited R245S or null mutations [47]. Such an effect was recapitulated in humanized mutp53 knock-in models harboring R248Q and G245S alleles: R248Q p53 knock-in in mice showed a faster tumor onset and decreased overall survival [47], compared to G245S or null mutations. However, unlike p53(R248Q) mice, the age of tumor onset is similar in p53(R248W) and p53^{-/-} mice [33]. Given that both R248Q and R248W are structural mutations at the same residue, it is indeed surprising that a functional difference could be observed. Therefore, the broad classification of p53 mutations into contact and structural mutants provides information on the biochemical effects of mutations on wild-type p53 protein structure and DNA binding capability, but gives little insight into any gain-of-function properties. To thoroughly examine the effects of the gain-of-function mutations, one needs to take into account the mechanism(s) that promote the specific gain-of-function property and systematically examine whether different p53 mutations engage the same mechanisms (Table 1).

To further complicate matters, it is becoming known that p53 mutations have tissue-specificity, an important factor to consider in the

p53 mutation	Genomic instability	Chemoresistance/ Antiapoptotic	Increased invasion/ Altered cell migration	Altered cell cycle function/ Promote proliferation	Anchorage dependency	Cell polarity	Stem cell differentiation	EMT	Angiogenesis	Clinical Relevance
V143A	✓[30]			✓[81]	✓[82]		✓[83]	✓[84]		Promotes proliferation and disrupts neuronal differentiation in pheochromocytoma [85]
R175H	✓[86,87]	✓[39,88-92]	✓[9,10,93]	✓[81,90]	✓[82,94]	✓[44]	✓[15,83]	✓[12,44,95]	✓[13]	Potentiates invasiveness in squamous cell carcinoma of the oesophagus [25]; Induces EMT in breast carcinoma [38]
G245S					✓[94]	✓[44]		✓[44]		Alters cell polarity and induces EMT in breast carcinoma [44]
R248Q		✓[88]	✓[65]					✓[88]		Associated with reduced overall survival in breast carcinoma [64]
R248W	✓[33,87]	✓[16,37,91]	✓[11]	✓[96]	✓[82]	✓[44]		✓[44]		Associated with reduced overall survival in breast carcinoma [64]
R249S	✓[9]		✓[92]							Induced by aflatoxin in hepatocellular carcinoma on background of chronic hepatitis B infection [97]
R273H	✓[98]	✓[16,90-92]	✓[9 - 11]	✓[81,90,99]	✓[82,94]	✓[38,44]	✓[83]	✓[44,84,95]		Potentiates invasion and metastasis in endometrial carcinoma [100]
R273C		✓[37,92]								Associated with reduced overall survival in breast carcinoma [64]
R280K			✓[12,36]			✓[38]				Inhibits apoptosis in breast carcinoma [101]
D281G		✓[102]		✓[81]	✓[82]					Induces tumor growth and migration in lung carcinoma [103,104]
R282Q	✓[94]									Possible role in squamous cell carcinoma by interfering with keratinocyte differentiation [105]
R282W	✓[81]									Associated with chemoresistance in locally advanced breast carcinoma [106]

*We apologies to authors whose work we have inadvertently missed in this review because of the broad scope of the topics covered.

Table 1: List of p53 mutations and their reported gain of function properties.

clinic. In several cancers, tumor-specific p53 signature gene mutations have been found. The R249S mutation is common in liver cancers, leading to speculation that liver tumors harbouring the mutation result from pathways that are unique to the R249S mutation [48]. Interestingly, a rare germline p53 mutation, encoding an arginine instead of a histidine at codon 337 (R337H), is found in much higher frequency in pediatric adrenal cortical carcinomas (ACC) [49]. Inheritance of this mutation is not associated with increased incidence of other cancers suggesting an exclusive predisposition only to ACC. In mice, hotspot mutations such as p53R270H and p53R172H gave rise to a separate tumor spectrum which includes hemangiosarcomas, compared to p53^{-/-} mice, supporting a gain-of-function effect towards the development of epithelial and endothelial tumors [22]. Furthermore, analysis of the most frequent missense mutants observed in specific cancer types

show that R273C is most frequently observed in brain and prostate cancers, while R175H, R248Q/W and R273H occur preferentially in other cancers [50]. While the reasons underlying these mutation-specific occurrences are unclear, understanding the molecular basis of these tissue specific mutations could have far reaching implication for chemotherapy recommendations as these mutations could potentially modify chemotherapeutic outcome in a tissue-specific manner.

Given the importance of p53 in influencing clinical outcome and survival, any therapeutic strategy should consider the impact on 1) wild-type p53 tumors, 2) loss of function p53 mutations and 3) specific gain-of-function mutations.

Clinical Significance of p53 Mutations

Mutations in the p53 gene occur in about a third of all human

cancers and is the second most frequent gene mutation found in breast cancers where it can be found in about a quarter of tumors. The majority of p53 mutations are missense mutations that occur within the DNA binding domain [3]. A higher frequency of p53 mutations occur in breast tumors with a basal or HER2-over-expressing subtype, both of which are associated with less favourable clinical outcomes [51]. Several studies have reported correlations with chemoresistance and poor survival, not only in breast cancers but in several other malignancies as well [50]. However, there are a handful of studies that have reported findings to the contrary. For instance, p53 mutations conferred a more favourable prognosis in both primary and secondary glioblastomas [52] and also reduced disease progression and improved short-term survival in advanced ovarian carcinomas [53,54]. The correlation with chemotherapy response is also conflicting and the effect of p53 mutations appears to be specific to the particular chemotherapy regimen. Several reports have found p53 mutations to confer resistance to doxorubicin, [55-57] while on the other hand, pathological complete response was observed more frequently in tumors harbouring p53 mutations treated with epirubicin-cyclophosphamide, another anthracycline-based regimen [58]. Although this may appear contradictory, p53 mutations frequently result in cell cycle deregulation and may therefore increase susceptibility to chemotherapeutics which target rapidly dividing cells. Other reports have also linked p53 deficiencies to chemosensitivity. Mutations in p53 correlated with increased cisplatin sensitivity in head and neck squamous cell carcinomas and in BRCA-related cancers [59,60]. However, p53 mutations did not predict response to gemcitabine or paclitaxel, suggesting that different DNA damage repair pathways may be involved [60]. Preclinical and early clinical studies also found p53 mutations to increase tumor sensitivity to paclitaxel, [61,62] but this was not confirmed in a large phase-3 clinical trial [63]. In a separate extensive clinical study that explore the prognostic value of p53 mutations, specific mutations at codon 220 and 245 appear to correlate with a better prognosis than other missense mutations, although most loss-of-function p53 mutants correlated with poorer prognosis compared to the wild type p53 cases [64].

These inconsistent data are the main reason why p53 analysis has not been incorporated into routine clinical practice. There are two likely explanations for these conflicting reports. Firstly, it is increasingly being recognized that the functional impact of p53 mutations is highly dependent on the specific mutation involved. Mutations in p53 can result in loss of function, a dominant negative effect or gain of function properties and consequently, not every p53 mutation produces a similar biological outcome [47,65]. As a corollary of this, p53 mutations have been associated with differential survival outcomes. Missense mutations occurring within the DNA binding domain (DBD) were associated with a lower 10-year mortality compared to missense mutations occurring elsewhere [64]. Even among hotspot DBD missense mutations, the prognostic impact difference according to the specific mutation involved; mutations at codon 179 and R248W had the worse prognosis, while G245S and Y220C mutations fared better [64]. Secondly, this complexity is further complicated by the different methods used to detect p53 mutations in clinical samples.

Diagnostic Detection of p53 Mutations

Earlier studies, and even many of the more recent ones, relied solely on immunohistochemistry (IHC) to detect mutant p53 proteins. Positive staining with anti-p53 antibody served as indirect evidence of p53 mutation, since wild-type p53 protein is expected to be rapidly destabilized and degraded. However, null mutations, and even some missense mutations, do not result in stabilization and accumulation of

mutant proteins and result in a falsely negative IHC analysis [66,67]. Indeed, studies comparing p53 gene sequencing and IHC found an almost three-fold under-estimation of p53 null mutations when IHC was used alone [67]. Furthermore, most anti-p53 antibodies do not differentiate between mutant and wild-type p53 proteins and positive staining will still be observed should wild-type p53 protein accumulate due to impaired degradation mechanisms or persistent stress signals. Finally, IHC results depend, to a significant extent, on the specific protocol and cut-off thresholds used and the lack of standardization may account for why some studies have reported insignificant or sometimes even contradictory associations with prognosis.

More consistent results were obtained with p53 gene sequencing, which provide direct evidence of p53 mutation. However, whole gene sequencing remains too costly to perform on a routine basis and focusing on the DNA binding domain alone, which was done in most published studies, will miss up to 20% of p53 mutations [66]. Gene sequencing also fails to take into account post-transcriptional modifications that may affect the structure and function of mutant p53 proteins, and thus some studies have found that including IHC analysis to p53 gene mutational analysis improves prognostication [68-70]. This has led to more studies that have focused not solely on the specific p53 mutation sequence itself but also on the functional effects of the mutation.

Another method to assess p53 functional status is the FASAY test (Functional Analysis of Separated Alleles in Yeast) [71]. This assay screens for p53 mutations that alter the transcriptional activity in comparison to wild-type p53 and p53 functionality is assessed with a phenotypic assay based on colony pigmentation. This was used to screen tumor and blood samples in a large scale study where mutated p53 was found to be required for complete pathological response to dose-dense epirubicin-cyclophosphamide [61,72,73]. Potential limitations of this method include an inability to detect mutations which occur in the promoter sequence of p53 gene or within the 3' or 5' untranslated regions. Nevertheless, the combination of a functional assay with p53 IHC analysis and deep sequencing can provide a sensitive and robust approach to determine p53 functionality in the tumors.

Some have used functional analysis to further stratify p53 mutations by their predicted structure and function [74-76], while others have combined p53 gene analysis with evaluation of the expression levels of downstream transcriptional targets, such as phosphorylation-dependent prolyl isomerase Pin1 [36]. In fact, the prognostic power of p53 mutational analysis was found to increase when more parameters, such as standard clinicopathological parameters, and related factors such as EGFR, Rb, MDM2 SNP309, were included into the predictive model [77-79]. Unique gene expression profiles associated with p53 mutations have also been identified. Miller and colleagues identified a 32-gene signature that could differentiate mutant p53-bearing tumors from those expressing wild-type p53 [80]. This signature not only stratified patients into low- and high-risk groups for disease recurrence and survival, but also predicted response to chemotherapy, hormonal therapy and radiotherapy. Interestingly, none of the 32 genes were known p53 targets or factors involved in p53-dependent pathways, though a significant number of the genes were known to be involved in cell growth and proliferation. This provides evidence that assessing a marker that reflects the functional outcome of the p53 mutation may be of greater clinical value than detecting the specific mutation itself. This in fact may be more practical since it removes the need to test for each individual known p53 mutation and will make detection less tedious and more affordable.

Conclusion

Despite a large body of evidence that p53 mutations have a clinically significant impact on survival and treatment response, there remains no clear conclusion to date. This is largely due to the variability of functional outcomes resulting from specific p53 mutations, as well as the different methods of detection, which has made comparisons across studies difficult. This lack of consensus has prevented p53 from being developed into a useful biomarker to guide clinical decisions. There thus remains a need for a more accurate and cost effective means of assessing p53 mutations in clinical samples. While IHC remains the simplest and least expensive means of detecting p53 mutations, it has several limitations. More reliable and accurate results have been obtained through gene sequencing and by incorporating other molecular parameters into the predictive model, but this increases the complexity of the detection techniques required and can render the analysis too costly and impractical to be offered as part of routine care. But perhaps because of the multitude of p53 mutations and the wide variation in the properties of mutant p53 proteins, it may be more important to assess the functional outcome of the mutation rather than simply detecting the mutation per se. Such a factor or gene signature can be used as a surrogate of p53 mutation and may more reliably reflect the biological impact of the mutation. The evidence so far shows that further work on p53 is certainly of value since p53 mutations can provide better prognostication, and more importantly, it can potentially predict therapeutic response and guide clinical decisions to improve treatment outcomes.

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