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REVIEW

p53 alterations in human cancer: more questions than answers

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The strongest and undisputed fact about p53 is the high frequency of p53 alterations in human cancer and that mutant p53 proteins constitute a complex family of several hundred proteins with heterogeneous properties. Beyond these observations, the p53 pathway and its regulation in a normal cell is like a desert trail, always moving with the wind of novel findings. The field is full of black boxes that are often ignored for sake of simplicity or because they do not fit with the current dominant view. Mutant p53 protein accumulation in tumours is the best example of a preconceived idea, as there is no experimental evidence to explain this observation. In this review, we will discuss several questions concerning the activity or selection of p53 mutations. The central domain of the p53 protein targeted by 80% of p53 mutations is associated with the DNA-binding activity of the p53 protein, but it is also the binding site for several proteins that play a key role in p53 regulation such as ASPP proteins or BclxL. The role of impaired DNA binding and/or protein interactions in tumour development has not been fully elucidated. Similarly, novel animal models carrying either missense p53 mutations or inducible p53 have provided abundant observations, some of which could challenge our view on p53 function as a tumour suppressor gene. Finally, the possible clinical applications of p53 will be discussed.
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Keywords: p53 mutations; apoptosis; cancer; cell cyle; tumour suppressor gene

> ….These results demonstrate that p53 can be activated by mutational changes in the cellular gene and suggest that mutation leading to an increased stability of a short half-life protein is a novel mechanism by which a cellular oncogene can participate in multistage carcinogenesis.
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> Jenkins *et al*. (1985) *Nature* **317**: 816–818.

The history of p53 is a chaotic voyage from the world of oncogenes to the world of tumour suppressor genes, while retaining a certain degree of individuality (Lane and Benchimol, 1990). Apart from artefactual problems related to involuntary cloning of mutant p53, this ambiguity is also due to our propensity to over categorize in order to satisfy our Cartesian and over-simplistic view of science.

In fact, review of a number of old publications combined with novel data clearly show that wild-type p53 cannot be confined to the single category of tumour suppressor gene and that mutant p53 is not a single entity, but a complex collection of proteins, each with a unique set of properties defined by a combination of heterogeneous loss of activity, dominant-negative activity (antimorphic) and gain of function (neomorphic). Several layers of complexity can also be added to mutant p53 function by arguing that tissue specificity or tumour genetic background can also modulate their function.

One of the advantages of an invited review is the freedom to approach various topics that are not always open for discussion and to raise questions that I personally feel are either biased by dominant-preconceived ideas and/or underestimated due to lack of interest. The explanation for p53 accumulation in tumour cells is a perfect example (see below). As insight is obtained from discussions and exchanges, all questions raised in this review will be available as topics for discussion in the p53 forum that will open in Spring 2007.

The spectrum of p53 mutations is not a strong argument to infer neomorphic or antimorphic mutations

Unlike most other tumour suppressor genes that are inactivated by frameshift or nonsense mutations leading to disappearance or aberrant synthesis of the gene product, almost 80% of *p53* gene mutations are missense mutations leading to the synthesis of a stable protein, lacking its specific DNA-binding activity and accumulating in the nucleus of tumour cells (Soussi, 2005). Whether this high frequency of missense mutations is specific for the *p53* gene during tumourigenesis or corresponds to the normal pattern of mutation in human cancer is difficult to assess. However, the recent analysis of the genome of colorectal and breast cancer may shed new light on this question. Sequencing of more than 13 000 genes in eleven breast and colorectal tumours led to the detection of 1672 mutations (Sjoblom *et al*., 2006). As the sequencing strategy did not specifically focus on

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oncogenes or tumour suppressor genes, we can expect that the spectrum of mutations will reflect a general mutagenesis mechanism. First of all, for both colorectal and breast cancer, the frequency of missense mutations was 80%: 7% were nonsense, 4% were splice mutations and 8% were insertions and deletions (Figure 1). This figure is strikingly similar to the spectrum of *p53* mutation in human cancer with 80% of missense mutations, 10% of insertions/deletions and 8.8% of *missense mutations*. This figure is observed for both the entire p53 database (2006 version comprising 24, 151 mutations) and for breast or colorectal cancer (Figure 1). On the other hand, this pattern is totally different from those observed for other oncogenes or tumour

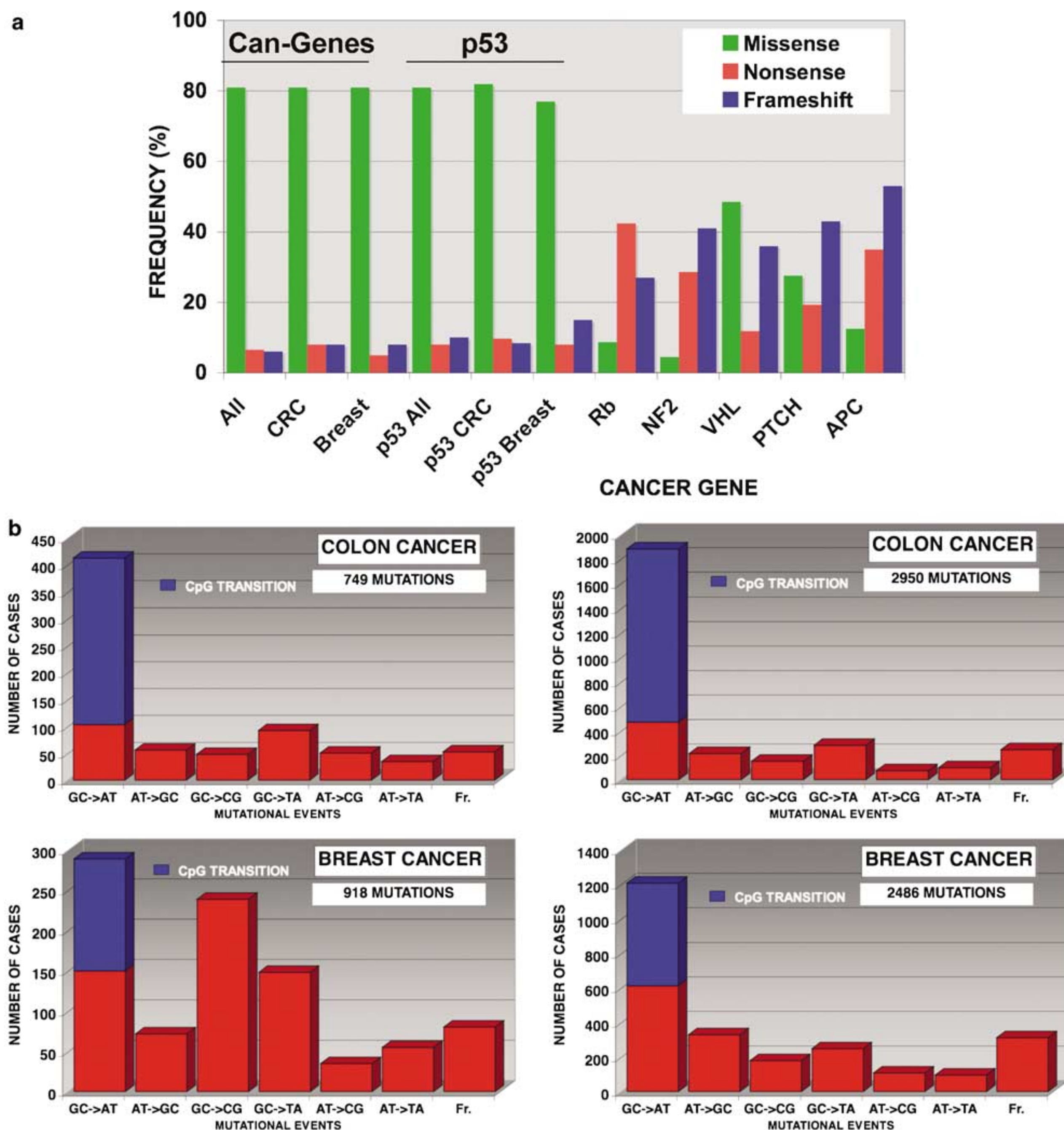

Figure 1 Mutational spectrum of the *TP53* gene in human cancer: (**a**) frequency of missense, nonsense and frameshift mutations: Can-Genes, data obtained by Sjoblom *et al.* (2006), for colorectal cancer (CRC), breast and both (All). p53 data obtained from the UMD p53 database (http://p53/free/fr) for all cancers (All), breast and CRC. Rb: data from the retinoblastoma database; NF2: data from the type 2 Neurofibromatosis database; VHL: data from the Von Hippel Lindau mutation database; PTCH: data from the xx mutation database; APC: data from adenomatous polyposis coli mutation database. (**b**) Pattern of mutational events in CRC and breast cancer from the Can-Genes study (left) and p53 mutation database (right). Data for oncogenes have not been included as most of them lead to the detection of more than 90% of missense mutations.

suppressor genes (Figure 1). Somatic mutations in oncogenes are missense mutations restricted to specific codons in which an alteration leads to a gain of function. For tumour suppressor genes, small deletions, insertions or nonsense mutations are far more frequent in agreement with the idea that their primary consequence is to disrupt protein function. The spectrum of *p53* mutation can be explained by the observation that the central region (CR) of the p53 protein, residues 100–300 encoded by 600 nucleotides, is very fragile and each residue in this region has been found to have been the target of at least one mutation in human cancer. A recent large-scale mutagenesis study of each residue of the p53 protein indicates that each modification in this region leads to a loss of p53 transcriptional activity (Kato *et al*., 2003). It is therefore not surprising that the pattern of *p53* mutation is similar to the general spectrum demonstrated by analysis of the entire genome.

The observation that missense mutations are predominant in the *p53* gene was a strong argument to support an oncogenic activity of mutant p53 in malignant transformation. It is generally advanced that the 'particular selection' for missense mutations in tumour cells could have two consequences: (i) a dominant-negative role by hetero-oligomerization with wild-type p53 expressed by the second allele, or (ii) a specific gain of function of mutant p53. Although, both functions cannot be excluded for mutant p53, it is clear that missense mutations are the most frequent genetic alterations in human cancer (excluding gross genetic rearrangements) and that it cannot infer any specific biological selection.

Heterogeneity of p53 mutants and gain of function: a complex intertwined problem

The structural difference between the various p53 mutants was initially identified using monoclonal antibodies able to discriminate mutations that change p53 folding and mutations in the residues involved in DNA recognition (Milner, 1995). Two classes of mutations have been distinguished on the basis of various *in vitro* assays and the three-dimensional structure of the protein (Cho *et al*., 1994): class I mutations, exemplified by mutants at codon 248 (7.6% in the p53 database, http://p53.free.fr/), affect amino acids directly involved in the protein–DNA interaction. They have a wild-type conformation as probed by conformational monoclonal antibodies and they do not bind to the chaperone hsp70. Class II mutations, exemplified by mutants at codon 175 (4.9% in the database), have an altered conformation with intense binding to hsp70. The amino acids altered in this class of mutants are involved in stabilizing the tertiary structure of the protein. Class II mutations are associated with a more severe *in vitro* phenotype than class I mutations. This biochemical and biological heterogeneity has been confirmed and refined by structural studies. For example, NMR spectroscopy suggests that mutations in the L3 domain can induce either limited or extensive conformational changes, depending on their position or the type of substitution (Wong *et al*., 1999).

Biochemical analyses have shown that p53 mutant proteins can be heterogeneous in terms of loss of DNA-binding activity and transactivation (Soussi and Lozano, 2005). The DNA-binding site recognized by p53 is highly degenerated and the affinity of p53 for the various biological sites is variable (Resnick and Inga, 2003). Using a library of 2500 different p53 mutants, Kato *et al*. (2003), showed a marked heterogeneity in the loss of function of the various mutants. Although hot spot mutants found in human cancer present complete loss of their transactivating properties on all target genes, other mutants retain either a partial activity on all genes or on a subset of genes leading to a wide range of mutant activity (Soussi *et al*., 2005).

The idea that some *p53* mutations can actively participate in cellular transformation was already postulated in 1990 (Eliyahu *et al*., 1984; Lane and Benchimol, 1990). Many studies have tried to distinguish between the dominant negative and the gain of function properties of mutant p53 without reaching any clear-cut conclusions (see the animal model section below for more insight) (Blagosklonny, 2000; Deppert *et al*., 2000; Sigal and Rotter, 2000). This task is further complicated by the marked heterogeneity of mutant p53 as already described. Transfection of various mutant *TP53* genes into cells devoid of endogenous p53 leads to an increase in their carcinogenicity, which varies according to the type of mutation (Halevy *et al*., 1990; Dittmer *et al*., 1993). This research into the oncogenic potential of certain *p53* mutations is not purely theoretical, but can have obvious clinical implications, as it could explain the marked disparity of the results of studies trying to demonstrate a relationship between the presence of a *p53* gene mutation and various clinical parameters, such as survival or response to treatment. In breast cancer patients, the response to adriamycin is very strongly correlated with the presence of a mutation specifically localized in the loop domains L2 or L3 of the p53 protein (Aas *et al*., 1996). *In vitro*, the expression of *p53* mutations in position 175 (R175H) specifically induces resistance of cells to etoposides compared to other *p53* mutations (Blandino *et al*., 1999).

The two homologous genes of *p53*, *p63* and *p73* express many isoforms due to alternating use of transcription promoters and alternative splicing (Yang *et al*., 2002). Long isoforms (TA-p73 or TA-p63) are able to transactivate the same target genes as p53 and induce apoptosis, while short forms (DNp63 or DNp73) have an opposite activity via dominant-negative mechanisms. p63 and p73 are able to cooperate with p53 to induce apoptosis, suggesting the existence of a complex network of interactions between the products of these three genes (Melino *et al*., 2002). Although wild-type p53 does not interact with p73 or p63, some mutant p53 proteins bind strongly to the two p53 homologues via their DNA-binding domains. This interaction leads to inactivation of p73 and p63 function (DiComo *et al*., 1999; Marin *et al*., 2000; Strano *et al*., 2000; Gaiddon

et al., 2001). Studies by Crook and Kaelin showed that resistance to anticancer agents involves inactivation of the apoptotic function of p73 protein by a subset of mutant p53 that have sustained a change of conformation (Bergamaschi *et al.*, 2003; Irwin *et al.*, 2003).

p53 accumulation in normal and tumour cells: many preconceived ideas and few clues

For a long time, regulation of p53 in normal cells was summarized by a bipolar model in which the ubiquitin ligase mdm2 interacts with wild-type p53 leading to its degradation and any interference with this interaction after DNA damage will lead to p53 accumulation and cellular outcomes such as apoptosis or growth arrest (Freedman *et al.*, 1999). The recent discovery that other ubiquitin ligases also regulate the fate of p53 suggests that the circuitry is more complex (Levine *et al.*, 2006). More interesting is the observation that the increase of p53 half-life due to absence of degradation is not sufficient to account for its stabilization. A more sophisticated mechanism that involves an increase of p53 translation via binding of ribosomal protein RPL26 and nucleolin to the 5'UTR of *p53* RNA has been revealed (Takagi *et al.*, 2005). Whether this mechanism accounts for all of the p53 accumulation observed after DNA damage and whether it is associated with all pathways that activate p53 are unknown. As these regulators are potential targets that could modulate p53 activation, their genetic study in normal or tumour cells could reveal novel regulatory mechanisms.

p53 accumulation in tumour cells was one of the observations that led to the belief that wild-type p53 was an oncogene. The subsequent discovery that only mutant p53 is stable in the nucleus of tumour cells led to the development of immunohistochemical analysis of p53 status (Dowell *et al.*, 1994). However, no truly satisfactory explanation for mutant p53 accumulation has been found to date. Several non-exclusive possibilities have been proposed: (i) absence of induction of *mdm2* or other p53 regulated genes which can no longer regulate p53 stability; (ii) conformational change and decreased sensitivity to degradation; (iii) stability or overtranslation of *p53* messenger RNA. All of these hypotheses depend on a change of p53 behaviour. Unfortunately, several observations indicate that the situation is much more complex. Fifteen years ago, Lavigueur *et al.* (1989), described the first model of p53 transgenic mice. This strain overexpresses a mutant p53 and has been shown to be prone to the development of lung and bone tumours. Although the exogenous mutant p53 is expressed in all cells, tumour cells express a much higher level of the mutant protein. A similar situation is observed in Li–Fraumeni (LFS) patients who carry a germline mutation of the *p53* gene. Normal cells from LFS do not accumulate p53, in contrast with tumours from the same patient. In several cases, both the wild-type and mutant allele are expressed in tumour cells ruling out specific modifications due to a change in p53 dosage. The fact that the spectrum of germline mutations in LFS does not differ from that of somatic mutations suggests that the tumour context is an important component for stabilization of the p53 protein. A similar situation is observed in novel mouse models (Lang *et al.*, 2004). Normal tissues from knock-in mice heterozygous for the R172H (equivalent to human R175H) and R270H (equivalent to human R273H) mutations do not have stable mutant p53. Stable p53 was observed in some, but not all, tumours in heterozygous mice. Mutant p53 instability was not due to the presence of wild-type p53, as loss of wild-type p53 was not a common event in the genesis of tumours in these mice. Moreover, mice engineered with one mutant and one *p53* null allele also had unstable mutant p53. The instability of mutant p53 in tumours indicates that secondary events in the genesis of the tumours contribute to mutant p53 stability and potentially to the gain of function phenotype. This remains an unexplored field of investigation, which would certainly provide useful information and kill several old unsatisfactory dogmas.

The central domain of the p53 protein: a crowded area

p53 missense mutations are predominantly found in the CR of the p53 protein. It is noteworthy that mutations outside this region are more frequently nonsense mutations or deletions and insertions that lead to either synthesis of a truncated protein or complete absence of the protein (Figure 2). The CR of the p53 protein is not only devoted to recognition of the p53 response element found in p53 target genes. Older and more recent data have shown that this region is also an important domain

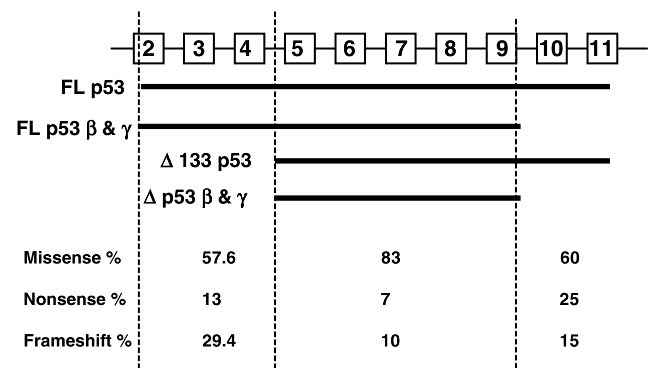

Figure 2 Distribution of *Tp53* mutations in the *p53* gene. Only codons 2–11 are translated. FL p53: Full-length p53 obtained after 'classical' splicing of *p53* RNA that retains all exons. FL p53 β and γ: Carboxy-terminus truncated p53 obtained after two different alternative splicings in intron 9: both forms contains residues encoded by exons 2–9, but are slightly different for the penultimate 10 residues. All Δ133 p53 forms are derived from RNA transcribed from a promoter localized in intron 4. They contain either a classical (Δ133 p53) or truncated carboxy-terminus (Δ133 p53 β, γ). For the sake of simplicity, the Δ40 p53 isoforms are not depicted. The frequency of *p53* mutations in exons 2–4, 4–9 and 9–10 are taken from the UMD p53 mutation database. The higher frequency of frameshift mutations in exons 2–4 compared to 5–8 is statistically significant ($P < 0.0001$).

for specific protein–protein interactions. Everything began with the discovery that the CR is the recognition site of SV40 large T antigen (LT) and that this interaction is conserved in all *p53* from humans to frogs (Jenkins *et al*., 1988; Soussi *et al*., 1989). The recent crystal structure of LT complexed with p53 confirms that the viral protein shields the entire CR region and impairs any interaction with either DNA or other proteins (Lilyestrom *et al*., 2006). Cellular proteins also interact with the CR region, but each protein requires a specific set of p53 residues that partially overlap. p53 interaction with BclXl is specifically associated with transcription-independent p53-induced apoptosis (TIPA as acronymed by Chipuk and Green (2003)). A fraction of p53, localized at the mitochondrial membrane directly induces permeabilization of this outer membrane by disrupting the protective role of BclXL (Mihara *et al*., 2003). The interface of p53 associated with BclXl involves the loop domain L3 (residues 239–248) with the help of two other p53 regions, residues 135–141 (part of loop L1) and residues 173–187 (part of loop L2). The protein 53BP2/ASPP2 also binds to the CR of p53 (Gorina and Pavletich, 1996). This interaction involves the C-terminal part of loop L3 (residues 243–249) and part of loop L2 (residues 165; 167 and 181). 53BP2/ASPP2 interaction with p53 specifically enhances transactivation of apoptotic genes such as *bax* or *PIG3* but has no effect on *p21* (Samuels-Lev *et al*., 2001).

This intricate promiscuity of various functions in the CR of p53 raises a number of questions concerning the interplay between the loss of these functions and mutant p53. No systematic analysis of the loss of protein–protein interaction of various p53 mutants has been performed and only partial data are available. As shown in Table 1, several residues in the CR are hot spot mutations found in human cancers. Some mutants, such as R175H, are totally defective for transactivation and protein binding. On the other hand, some p53 mutants retain the capacity to bind apoptosis stimulating protein of p53 (ASPP) despite a loss of their transactivation activity and a negative TIPA (Tidow *et al*., 2006).

These observations raise the question of whether loss of the transcriptional activity of p53 via impaired DNA-binding activity is the only consequence of *p53* mutations selected during transformation or whether loss of TIPA or other activities related to protein binding are also important in this selection. Furthermore, the heterogeneity of the binding interface of these various p53 partners once again results in marked variability of p53 mutant properties.

Spectrum of p53 mutations: are frameshift and nonsense mutant really null and what about p53 'silent mutations'?

In several genes, such as *APC*, *BRCA1* or *MBD4*, low levels of expression of truncated proteins can have a dominant effect on proliferation or chromosomal stability (Bader *et al*., 1999; Rowan *et al*., 2000; Fan *et al*., 2001). Although the majority of *p53* alterations are missense mutations, there are also 8% of nonsense mutations and 11% of small deletions or insertions. The frequency of frameshift mutations is higher in exon 4 (30%) and exon 9 and 10 (22 and 13%) compared to exons 5–8 (9.5%) that encode the CR of the protein. A similar situation is observed for nonsense mutations (Figure 2). Functional analysis of missense mutants at each amino-acid residue in exons 4, 9 and 10 showed that the transcriptional activity of p53 is not severely impaired. These missense mutants are therefore not selected during transformation, which explains the predominance of frameshift mutations in these exons.

Analysis of truncated p53 mutants has not received a great deal of attention, as it is generally assumed that these mutations are null. The majority of immunohistochemical studies showed an absence of p53 accumulation in tumours expressing these frameshift or missense p53 mutants. Nevertheless, we cannot exclude the possibility that some truncated p53 are expressed at low levels using the main transcription promoters as a transcription start. In a recent analysis of *p53* alterations in a panel of 56 colorectal cancer cell lines, Liu *et al*. (2004) found that among the 22 cell lines that carried either missense or frameshift mutations, 12 expressed a truncated protein. The p53+/m mouse developed by Tyner *et al*. (2002) is also a good example of a possible dominant effect of truncated p53. This mouse expresses one wild-type p53 allele while the second allele has a deletion of the first six exons. Although the expression

Table 1 Mutant p53 and protein binding in the CR of p53

	Transactivation[a]	*Binding to ASPP2*[b]	*Binding to BclXL/cytochrome* C *release*[c]	*Frequency in human tumours*[d]
R181E	Unknown[e]	–	ND/ND	0
G245S	Totally defective	–	ND/–	401
R273H	Totally defective	+	–/–	731
R175H	Totally defective	–	–/–	1072
R249S	Totally defective	–	ND/–	412
F270L	Totally defective	+	ND/ND	29
R282W	Totally defective	+	ND/–	556
R248W	Totally defective	ND[f]	ND/–	682
Wild-type p53	Active	+	+/+	Irrelevant

[a]Data from Kato *et al*. (2003). [b]Data from Tidow *et al*. (2006). [c]Data from Tomita *et al*. (2006). [d]Data from http://p53.free.fr. [e]Inactive for DNA binding to a CONS sequence. [f]Another substitution at codon 248 (R248A) totally impairs p53 and ASPP2 interaction.

of a short RNA has been detected, the putative truncated protein has never been observed. p53+/m mice are associated with a profound early ageing phenotype that could be due to constant activation of the wild-type protein by the short protein. *In vitro*, overexpression of short C-terminal p53 can oligomerize with the wild-type protein and enhance its transcriptional activity.

About 5% of *p53* mutations are silent (excluding germline polymorphisms at codons 36, 72 and 213 which are often erroneously reported as mutations). Among these silent mutations, 12% are found in skin cancers as a tandem mutation associated with a missense mutation on the adjacent nucleotide. They should not be considered to be silent mutations as they originate from a single lesion that targeted the two adjacent residues, usually pyrimidines. The remaining silent mutations are found with an equal frequency in all cancer types, although they can be passenger mutations coselected with a mutant p53.

p53 mutations and p53 isoforms

The observation that the *p53* gene can express multiple protein isoforms adds a novel and unexpected level of complexity to the outcome of *p53* mutations (Prives and Manfredi, 2005). Δp53 is produced by alternative splicing that partially deletes exon 8 (Rohaly *et al.*, 2005). Δp53 transcriptional activity is restricted to a subset of p53 target genes such as *p21* and *14-3-3-σ* and is functionally associated with the intra-S checkpoint when DNA replication is impaired.

Bourdon *et al.* (2005) described nine p53 isoforms including full-length p53 (Figure 2). Three amino-termini, beginning at codons 1, 40 and 133, can shuffle with three different carboxy-termini by a combination of alternative splicing and the use of alternative transcription promoters (Figure 2). The function of these various isoforms is still unclear and characterization of their expression at the protein level remains to be performed and will be an important step to gain knowledge about their interrelations.

The transcription promoter in intron 4 leads to the synthesis of three different isoforms that begin translation at codon 133 in exon 5 (Δ133p53, Δ133P53β and D133p53γ) (Bourdon *et al.*, 2005). They contain part of the DNA-binding domain and the oligomerization domain (Figure 2). They have no transactivating activity *per se* but their overexpression leads to a dominant-negative activity of p53 transcriptional activity. Mutations localized in exons 2–4 do not target these short isoforms and only modify full-length p53. Similarly, other isoforms in the carboxy-terminus of p53 lack exons 10 and 11 and are not targeted by mutations localized in these exons. The situation is more complex for isoform Δp53 that lacks part of the DNA-binding domain including codon 273, a hot spot for *p53* mutations.

Whether or not a change in the ratio between inactive full-length p53 and active p53 isoforms is an important player in cell transformation is still an open question that deserves further study. If the tissue specific expression of these isoforms is also confirmed, the situation will therefore prove to be very complex.

Methylation of the *p53* gene

DNA methylation, the addition of a methyl group to the 5-position of cytosine in the context of a CpG dinucleotide, is a DNA modification that is important for normal development of the organism (Baylin *et al.*, 2000). Areas of genes that are rich in CpG dinucleotides (so-called CpG islands) can be found at the vicinity of many transcription promoters. They are usually not methylated in normal tissues, but frequently become hypermethylated in cancer. This hypermethylation can be associated with gene silencing and is an important mechanism for the inactivation of tumour suppressor genes such as *APC*, *p16* or *MLH1* (Baylin *et al.*, 2000).

The *p53* promoter does not display a CpG island that could regulate p53 expression via an epigenetic mechanism. It can be hypothesized that *p53* gene expression is always active and that no selection for negative regulation has occurred during evolution. On the one hand, no reproducible description of epigenetic modification of *p53* gene expression in human cancer has been reported to date. On the other hand, the coding sequence of the *p53* gene contains 42 CpG dinucleotides that are scattered along the molecule. It has been reported that all of these CpG's are methylated in various cell types (Tornaletti and Pfeifer, 1995). Several arguments strongly suggest that this methylation is an important feature in the spectrum of *p53* mutations, as most hot spots for *p53* mutations are found at CpG dinucleotides (Soussi and Béroud, 2003). It is generally assumed that the higher deamination rate of 5-methylcytosine leading to a T/G mismatch that is not efficiently repaired could lead to this high rate of transition in the *p53* gene. Deamination of cytosine leads to a U/G mismatch that could be removed more efficiently. Although attractive, this hypothesis has not been formally demonstrated and several lines of evidence suggest that other models should also be investigated. Several studies have demonstrated that exogenous carcinogens, such as benzoaphrene diol exoxide or ultraviolet sunlight, have a higher affinity for methylated CpG dinucleotides than their unmethylated counterparts (Denissenko *et al.*, 1997; You *et al.*, 1999). It is conceivable that endogenous mutagens, derived from an altered cell metabolism, could also target methylated CpG dinucleotides leading to a high rate of transition. The recent sequencing of the cancer genome confirms that these CpG sites are preferential targets for mutations (Sjoblom *et al.*, 2006) (Figure 1).

The function of *p53* gene methylation has never been addressed neither *in vitro* nor *in vivo*. It is completely unknown whether or not *p53* gene methylation is associated with the fine-tuning of *p53* gene expression. Although the gene is methylated in normal cells, its status in human tumours is also unknown. Most *p53* hot

spot mutations are localized at CpG sites with a mutation spectrum compatible with 5-methylcytosine deamination. These hot spot codons, CGN, encode arginine residues important for either p53 structure and/or activity. It is interesting to note that arginine can also be encoded by AGG and AGA that have the same frequency of usage in humans, but are not targeted by methylation. The existence of specific selection to keep CGN is a controversial issue. Mutations of the *p53* gene have always been explained at the protein level. It is unlikely that a change of methylation of a single CpG dinucleotide induced by a hot spot mutation has any tumour-specific effect, but biology is always full of surprises and epigenetic regulation is still in its infancy.

How do acute and chronic p53 responses contribute to tumour prevention?

Acute DNA damage triggers a rapid p53 response that starts with p53 accumulation, p53 post-translational modifications and culminates with either apoptosis or growth arrest 24–72 h after triggering transcription-dependent and/or -independent pathways. Determinants that define the fate of the cell are currently unknown and involve multiple parameters including cell type, intensity of DNA damage and presence of cofactors such as p300, ASPP or myc (Helton and Chen, 2006). Furthermore, whether TIPA, which is a very fast response that precedes the transcriptional response, is also part of this choice remain to be determined. It is generally accepted that the apoptotic activity of p53 is the main target of *p53* gene mutations. The absence of this apoptotic activity could therefore account not only for tumour progression according to the established rules of multi-hit carcinogenesis, but could also explain treatment resistance phenomena.

Using an elegant mouse model, in which p53 can be reversibly switched on or off *in vivo*, Christophorou *et al.* (2006) made the surprising observation that the acute apoptotic p53 response after gamma irradiation is not associated with tumour protection, when p53 is switched on at the time of DNA damage. But then, restoration of p53 expression 8 days after the injury led to a protective effect with a significant delay in tumour development. This protective effect was dependent on p19ARF. The mechanisms that contribute to this delayed protection are unknown, but these observations suggest that p53 has a subtle tumour suppressor effect unrelated to the acute apoptotic response.

Among the various ‘underestimated’ activities of p53, DNA repair has never been fully explored. There is a plethora of publications associating p53 with all DNA repair pathways, that is NHEJ, HR, BER, NER and MMR (Sengupta and Harris, 2005; Gatz and Wiesmuller, 2006). It is possible that, following a burst of genotoxic stress and massive apoptosis, there is a sustained persistence of DNA damage that is not eliminated via cell death or growth arrest. This long-lasting DNA repair activity of p53 could be associated with an outcome, which is not phenotypically obvious and difficult to monitor in an *in vitro* assay. These studies could also be a good opportunity to review some of the unusual properties of p53 such as its exonuclease activity (Mummenbrauer *et al.*, 1996).

Similarly, the antioxidant function of p53 that protects the genome from oxidation by reactive oxygen species (ROS) also deserves greater attention. The expression of several p53 targets genes, such as *PIG3* or *FDXR*, is associated with an increased production of ROS and p53-induced apoptosis (Polyak *et al.*, 1997). There is now a concordant volume of data indicating that p53 also has an antioxidant function. Firstly, several p53 target genes, such as *SOD2* or *GPX1*, can act as antioxidants. Sablina *et al.* (2005) showed that, in the absence of any genotoxic stress, removal of p53 leads to a rise in intracellular ROS associated with an increased mutation rate and chromosomal instability. Dietary supplementation with antioxidants such as *N*-acetylcysteine had a substantial effect on tumour incidence on p53−/− mice. By the age of 6 month, 90% of untreated mice died from lymphoma, whereas treated mice were all alive and developed solid tumours at later ages. By using the well-known antioxidant Selenomethionone (SeMet), Seo *et al.* (2002) demonstrated that SeMet activates p53 in a different way from the ‘classical pathway’. Via an interaction with the redox factor Ref-1 and thioredoxin, SeMet alters the redox state of p53 cysteines, leading to a change of conformation of p53 and an increase of DNA excision repair, but no phosphorylation of p53, growth arrest or apoptosis were observed (Seo *et al.*, 2002).

It is therefore possible that p53 acts both as a killer and a healer, as elegantly described by Gudkov (2002). Following acute injury, p53 activation occurs via the canonical pathway that leads to growth arrest or apoptosis. In a more lifelong situation, p53 is associated with continuous stimulation of DNA repair, which is necessary to maintain genome integrity in response to the multiple daily DNA damage derived from endogenous processes. It is not yet clear whether alteration of the acute and/or chronic p53 activity is the target for cancer development.

The pros and cons of mouse models

Animal models for p53 fall broadly into two main categories. The first category includes alterations that reproduce the situation found in human cancers, such as p53 knockout (KO) mice or knock-in models expressing mutations equivalent to hot spot positions in human cancer. The second category concerns mutations targeting residues, which have been shown to be important for the regulation of p53 *in vitro*. A complete description of all these models is beyond the scope of this review (see Lozano and Zambetti (2005); Johnson and Attardi (2006) for more information). We will focus on the two aspects that have been unravelled by these models, the oncogenic behaviour of mutant p53 (the pros) and the marked discrepancy between *in vitro* and *in vivo* studies (the cons).

p53−/− mice have a different spectrum of tumours compared to knock-in mice expressing various p53 hot spot mutants (Lang *et al.*, 2004; Olive *et al.*, 2004). Knock-in mice have a higher frequency of solid tumours with a high potential for metastasis, a feature not seen in KO animals. This particular spectrum of tumours is also observed in mice expressing one mutant allele in a p53 null background, which is one of the strongest arguments for a gain of function of p53. Analysis of these knock-in models in mice defective for p63 and p73 suggests that alteration of the activity of the entire p53 family is an important feature for the gain of function of mutant p53.

In vitro analysis has identified numerous properties of the p53 protein, each associated with specific residues/domains in the protein. Many of these residues are the target of post-translational modifications such as phosphorylation, acetylation or ubiquitination (Appella and Anderson, 2001). Mutational analysis of these key residues usually leads to well defined impaired phenotypes associated with the biological activity analysed (Table 2). However, mouse models generally do not confirm the results of these *in vitro* studies (Table 2). Mouse phenotypes are always more attenuated or even nonexistent and are infrequently associated with tumour formation.

Analysis of p53 response in cell lines after various types of stress has led to the general belief that stabilization and activation of p53 always occur regardless of the cell type. Unfortunately, this universal p53 response does not apply to whole animals. As early as 1995, Midgley *et al.* (1995) demonstrated a marked tissue-specific restriction of p53 response after gamma irradiation. Accumulation of p53 protein following whole body irradiation was associated with a strong apoptotic response in the spleen and thymus, while no response was observed in hepatocytes. Using *in situ* hybridization with probes corresponding to various p53 target genes, Fei *et al.* (2002) extended these observations and also showed a strong tissue specificity with distinct regulation of various p53 target genes in different tissues. In liver cells, only the *p21* gene involved in growth arrest was induced, whereas none of the apoptotic genes were detected. This observation is the inverse of what occurs in the spleen with specific induction of the apoptotic gene *PUMA*, whereas *p21*or other apoptotic genes such as *noxa* or *DR5* were barely detectable. Gottlieb *et al.* (1997) used another approach based on transgenic mice expressing a *lacz* reporter gene fused to the *mdm2* promoter. They showed that the pattern of p53 response was fairly homogenous in the early embryo but become more restricted with increasing embryo age and with differentiation of the tissues. A predominant p53 response was observed in the spleen, thymus and small intestine after DNA damage. We should also keep in mind the elegant work conducted by Norimura *et al.* (1996), who found that the incidence of radiation-induced malformations of mouse embryos in p53 KO mouse was 70% compared to 20% in normal mice, whereas the number of *in utero* deaths associated with apoptosis was 60% in wild-type and 10% in mutant animals.

Although the possibility of redundant pathways and the complexity of animal models is often suggested, it is also possible that our *in vitro* analyses have shaped our view on p53 activity, creating a blinker that impairs our vision. Studies using more physiological *in vitro* or *in vivo* models will be necessary to obtain better insight into this complex p53 pathway and perhaps, as already suggested by Brash (1996) the complexity of p53 activity could be narrowed around a key role in maintaining cellular homeostasis.

p53 and clinical practice: no future ?

This question will only deal with the status of p53 or other genes as clinical markers. It does not address their potential as therapeutic targets which is still an open and promising field of investigation as discussed in Selivanova and Wiman (2007, in this issue).

Answers to the various questions raised in this manuscript would certainly provide a better knowledge of the regulation of the p53 pathway, but whether this improved knowledge will have any impact on clinical practice is still a subject of debate. Our reductionist analysis of p53 status has led to the discovery that *p53* mutations *per se* do not have any real meaning, as each p53 mutant is a different protein with a specific behaviour. Furthermore, the penetrance of each p53 mutant may be influenced by various factors such as normal genetic background, other genetic alterations in the tumour, tissue specificity and other unknown factors. Finally, it is also important to keep in mind that ‘only’ 50% of human tumours carry *p53* mutations, but it is usually assumed that the whole pathway is inactivated via inactivation of upstream regulators or downstream targets (Vogelstein and Kinzler, 2004). Whether the clinical value of ‘p53 pathway inactivation’ will be more important than *p53* mutations remains an open question. It is therefore likely that each situation is different and tumour-specific, an observation that is difficult to apply in clinical practice. Furthermore, the contribution of this heterogeneity to the major clinical issues such as prognosis and tumour response to therapy is quite controversial. It is perhaps time to consider the limits of reductionism in medicine, which can be biased by the focus of studies with popular candidate genes. We are now at the crossroads of a technological revolution with the development of high-throughput methodology. Global analysis of tumours at the level of DNA, RNA or proteins may possibly lead to improved cancer classifications and improved *patient management*. Moreover, high throughput methodologies will lead to a change in clinical diagnosis, as we can now perform multiple analyses (DNA, RNA and/or protein) on a single clinical sample. Although our reductionist studies have led to the discovery of a handful of genes with sufficient power to be used as clinical markers, many of them have failed because of insufficient power.

Table 2 Heterogeneity of phenotype in mice expressing different p53 mutants

Mutation[a]	*Mutant properties/ in vitro studies*	*Mouse status*	*Phenotype*	*Reference*
P53 gene knock-out	Total deletion of the gene	Heterozygote	High susceptibility to spontaneous tumours Mean survival times of 15–16 months High frequency of T-cell lymphoma and sarcoma with frequent aneuploidy Rare metastasis	Donehower *et al.* (1992)
		Homozygote	High susceptibility to early spontaneous tumours Mean survival times of 4–5 months High frequency of T-cell lymphoma and sarcoma with frequent aneuploidy Rare metastasis	
Knock-in R172P (R175P)	Wild-type for growth arrest; impaired for apoptosis; activate *p21* but not *bax* transcription	Heterozygote	No data available	Liu *et al.* (2004)
		Homozygote	High susceptibility to spontaneous tumours Mean survival times lower than for p53−/− mice (12 months) High frequency of T-cell lymphoma and sarcoma The majority of tumours are diploid	
Knock-in R172H (R175H) (C57BL/6) Hot spot mutant found in human tumours	Conformational mutant; impaired for growth arrest and apoptosis	Heterozygote	High susceptibility to spontaneous tumours Mean survival times of 15-16 months High frequency of T-cell lymphoma and sarcoma with frequent aneuploidy High frequency of metastasis	Lang *et al.* (2004)
		Homozygote	High susceptibility to early spontaneous tumours Mean survival times of 4–5 months High frequency of T-cell lymphoma and sarcoma with frequent aneuploidy Rare metastasis (Similar to p53−/− phenotype so far)	
Knock-in R172H (R175H) 129S4/SVJae Hot spot mutant found in human tumours	Conformational mutant; impaired for growth arrest and apoptosis	Heterozygote	High susceptibility to spontaneous tumours Mean survival times of 15–16 months High frequency of metastatic sarcomas	Olive *et al.* (2004)
		R172H/KO[b]	High susceptibility to early spontaneous tumours Mean survival times of 4–5 months High frequency of metastatic carcinomas	
Knock-in R270H (R273H) 129S4/SVJae Hot spot mutant found in human tumours	DNA contact mutant; impaired for growth arrest and apoptosis	Heterozygote	High susceptibility to spontaneous tumours Mean survival times of 15–16 months High frequency of metastatic carcinomas	Olive *et al.* (2004)
		R172H/KO[b]	High susceptibility to early spontaneous tumours Mean survival times of 4–5 months High frequency of metastatic carcinomas	
S18A (S15A)	Phosphorylation site of ATM/ATR; impaired p53 accumulation after DNA damage	Homozygote	No obvious tumour-prone phenotype; impaired apoptosis in thymus and spleen; normal p53 accumulation after DNA damage	Sluss *et al.* (2004)
S23A (S20A)	Phosphorylation site; impaired interaction of p53 and mdm2; impaired p53 accumulation after DNA damage	Homozygote	Very late onset of B-cell tumours; impaired apoptosis in thymus and spleen; normal p53 accumulation after DNA damage	MacPherson *et al.* (2004)
S389A (S392A)	Phosphorylation site after UV irradiation	Homozygote	No obvious tumour-prone phenotype; slight reduction of p53 accumulation and impaired apoptosis after UV irradiation	Bruins *et al.* (2004)
K7R	Ubiquitination and/or acetylation site (7 Lysine in the carboxy-terminus); impaired mdm2-dependent degradation	Homozygote	No obvious tumour-prone phenotype; normal growth arrest and apoptosis; normal degradation by mdm2; increased activation after DNA damage	Krummel *et al.* (2005)

[a]The site of the mutation corresponds to the mouse *p53* gene with the site in human *p53* shown in parentheses. [b]The p53 mutant is expressed on a p53 null background leading to the expression of one mutant p53 allele without wild-type p53. As the genetic background of the mouse strain has a profound influence on the phenotype of mice with similar p53 mutations, each study is presented separately, indicating the mouse strains used. This table summarizes only mouse models expressing missense mutant p53. Other mouse models that target p53-regulated genes or expressing other types of p53-modified construct regulatory domains are not described.

Analysis of p53 status falls into this second category. Since the first description of p53 mutation in 1989, several thousands of papers have described clinical studies in which p53 has been tentatively linked to response to treatment or patient survival. As early as 1994, the usefulness of these studies was questioned in terms of strategy (too few patients enrolled for correct statistical analysis) and methodology (heterogeneity of immunohistochemical analysis) (Hall and Lane, 1994; Soussi and Béroud, 2001). The low power, the lack of independence and the complexity of p53 status have been obvious since 1998–2000 and a lot of money and effort have been wasted on studies that were mostly doomed to fail. Does this mean that there is no future for p53 in clinical practice? As stated throughout this review, p53 is only a component of a giant surveillance network whose efficiency is modulated by many other elements, some of which are genetically different in various individuals. Investigation of this network will require a combination of SNP analysis, expression profiling and mutational studies on a large number of patients in order to answer this question. The usefulness of this type of gene- or pathway-oriented studies compared to phenotype-oriented studies with unbiased large-scale analysis of patient tumours with well-defined clinical outcome is unclear at the present time.

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