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# GENETIC ENGINEERING RECENT RESEARCH ADVANCEMENT

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## ABSTRACT

The TP53 gene is one of the most studied genes in human cancer. In recent years, considerable interest was focused on mutant p53, the abnormal protein product of TP53 somatic or germline alleles with missense mutations that often accumulate in cancer cells. There is now compelling experimental evidence that many mutations can exert mutant-specific, gain-of-function effects by perturbing the regulation of expression of multiple genes. This notion is supported by the observation that targeted mutant p53 expression enhances the formation of specific cancers in the mouse even in the absence of wild-type p53 expression. In addition, clinical studies are producing a wealth of functional pathway data demonstrating correlations between specific TP53 mutations and gene expression patterns identified by transcriptome studies. These correlations imply that alteration of p53 function is critical in shaping gene expression patterns in cancer. Finally, progress is being made in the development of new therapeutic approaches targeting p53 alterations. Key advances regarding the structural, biochemical and functional properties of normal and mutant p53 proteins, their abnormal regulation and distribution in human cancers, and their associations with clinical and pathological cancer characteristics are reviewed. New opportunities for translational research for improving cancer detection, prognosis, prevention and therapy based upon the integration of this knowledge are described. Cancer Gene Therapy (2009) 16, 1–12; doi:10.1038/cgt.2008.69; published online 19 September 2008.

**KEYWORDS:** TP53; mutation; loss of function; Li-Fraumeni syndrome; prognosis; therapy.

## INTRODUCTION

The tumor suppressor TP53 is one of the most frequently altered genes in human cancer.[1] It encodes the p53 protein that exerts multiple antiproliferative functions through the transcriptional control of many different target genes and through protein–protein interactions. The p53 protein is maintained at low levels in cells because of its active degradation by the proteasome mediated by the E3 ubiquitin ligase MDM2. Various forms of stress, in particular genotoxic events, stabilize p53 through post-transcriptional modifications that allow p53 to escape degradation. Once stabilized, p53 regulates the expression of many target genes involved in cell cycle control, apoptosis and DNA repair either by direct or indirect transcriptional activation or by repression. Loss of p53 function in cells leads to uncontrolled proliferation and promotes cancer development. In human cancers, p53 is frequently altered by mutation of the gene, which results in the expression of a mutated protein that differs from the wild type by a single amino-acid change.

TP53 gene mutations have been found in almost all types of cancers at various frequencies and are very diverse in their nature. p53 may also be altered by protein–protein interactions with other cellular proteins or with viral proteins. Understanding the biological consequences of p53 alterations, how they impact the pathological characteristics and clinical outcome of cancer, and how they might be used for therapeutic intervention requires a global approach that integrates knowledge on the structural, biochemical and functional properties of mutant proteins, their distribution in human cancers and their associations with clinical and pathological characteristics of cancer. Recent advances in these different disciplines have allowed the development of new targeted therapies and new strategies for improving the management of cancer patients. We provide in this article a review of key recent advances presented at the Third International Workshop on Mutant p53, held at the International Agency for Research on Cancer in Lyon in November 2007 (http://www-p53.iarc.fr/P53meeting2007/ P53meeting2007.html), and

propose some perspectives for translational research based upon this new knowledge.

**p53 functions and mechanisms**

Although the p53 protein and its mutated forms have been the subject of intense study over the last 25 years, many questions still remain regarding the range of activities that wild-type and mutant p53 proteins may exert and their related molecular mechanisms. Current knowledge concerning the key activities mediated by both wild-type and mutant p53 are summarized below.

**Wild-type p53 activities and mechanisms influencing them**

The mechanisms underlying p53 selectivity toward target genes have been studied by several investigators in recent years. Resnick and co-workers[2] showed that the transactivation (TA) capacity of p53 toward different target genes was greatly influenced by the overall structure of the p53 response element (RE) in addition to its primary sequence and by the level of p53 protein expression. Notably, the number of spacer nucleotides separating the two palindrome sequences that constitute a consensus p53-RE greatly impacted on DNA binding. Increasing the size of this spacer reduced binding efficiency. A novel functional motif containing a p53-RE half-site and an estrogen receptor RE half-site has been identified in the VEGFR1/FLT1 gene promoter at a polymorphic site (C/T).[3] This site confers responsiveness to both p53 and estrogens. In carriers of T allele, VEGF-R/flt-1 is under the control of both p53 and estrogen receptors, and VEGF-R expression is strongly activated in presence of both factors. Although the occurrence of such motifs in the entire genome and their functional impacts remain to be determined, these examples show that the p53 regulatory network may be more complex than expected from the canonical p53-RE. Furthermore, p53 half-sites can also be recognized by some p53 mutants, a feature that may expand their biological effects.[4]

Although p53 activities rely mainly on its capacity to regulate gene expression, p53 may exert effects through impact on the stability of other cellular proteins. Milner[5] showed that SIRT1, a deacetylase that reduces p53 transcriptional activity, is degraded by 5-FU treatment in a p53-dependent manner. As SIRT1 is a survival factor, its degradation would favor cell death and might contribute to p53-dependent apoptosis. The absence of SIRT1 degradation in cancer cells that have lost p53 may thus contribute to resistance to chemotherapeutic drugs such as fluorouracil.

The best-characterized cellular responses induced by p53 are apoptosis and cell cycle arrest. However, the question of what directs the choice between cell cycle arrest and cell death is still not fully answered. Recent studies suggest that this choice may be influenced by the nature of p53-regulated target genes and the timing of their regulation by p53. Vousden[6] showed that p53 target genes that mediate cell cycle arrest (WAF1 and TIGAR) were rapidly induced by genotoxic stress (adriamycin) and that the p53 target genes that mediate apoptosis (PUMA) were induced at later time points when stress is maintained. Induction of PUMA correlated with apoptosis and downregulation of WAF1 and TIGAR. Moreover, p53-dependent apoptosis mediated by PUMA occurred through a mitophagy (mitochondria autophagy) mechanism that depends on BAX and BAK mitochondrial proteins. Indeed, PUMA led to the activation of BAX and BAK proteins, which was followed by perinuclear clustering of mitochondria. The resulting mitophagy induced cytochrome c release and subsequent apoptosis.[7,8]

Among p53 biological effects, the induction of senescence has recently emerged as an important contributor to TP53 tumor suppressor activity. In a TP53þ/m mouse model generated in Donehower's lab, the expression of a mutant allele (m) in which the first six exons of TP53 are deleted giving rise to a truncated p53 protein resulted in a decreased number of spontaneous tumors observed in these mice but induced an accelerated aging compared with wild-type mice. This phenotype was shown to be because of permanent p53 activation ('super p53') resulting from the interaction of the m protein with wild-type p53.[9] TP53þ/m mice irradiated with high doses of g radiations showed a decrease in DNA repair associated with senescence rather than apoptosis.[10] These results suggest that senescence is an important biological consequence of p53 activation by genotoxic stress. In another model, Harris et al.[11] described a new p53 downstream effector involved in senescence. They observed that during cellular response to inflammation induced by nitric oxide donors, p53 is activated and initiates senescence by inducing the expression of the microRNA miR-34a. miR-34a and p53 knockdown induced a similar delay in replicative senescence in these conditions. Moreover, overexpression of miR-34a in human IMR90 lung fibroblasts caused growth arrest and cellular senescence.[12]

Another tumor suppressive activity of p53, which is still poorly understood, is its ability to modulate cell migration. Roux and co-workers[13] have shown that p53 inhibits Cdc42-induced filapodia formation. Absence of p53 in mouse embryonic fibroblast induced rounded blebbing movements through overactivation of RhoA and ROCKdependent translocation of RhoA to membrane blebbing structures.[14] Thus, by altering cellular movements, loss of p53 (as well as the expression of one of the p53 isoforms, D133p53) increased cell motility and may thus contribute to tumor invasiveness.

**Mutant p53 properties and activities**

The most frequent TP53 alterations found in human cancers are single-nucleotide substitutions leading to the production of a mutant protein that differs from the

wildtype protein by one amino acid (missense mutations). These substitutions usually lead to structural changes that destabilize p53 structure and alter its DNA-binding capacity. To study the DNA-binding capacity of specific mutants, Fersht and co-workers[15] produced a stabilized p53 core domain by introducing four mutations (M133L, V203A, N239Y and N268D). This stable core domain retained the ability to bind to DNA similarly to wild-type p53. Introduction of the p.R249S mutation in this stable core domain induced a distortion in the L3 loop that resulted in the loss of DNA binding.[16] The same effect was observed by introducing the p.Y220C mutation. This mutation is located outside the DNA-binding surface and increases the surface of the crevice between S7–S8 b sheets. In contrast, the p.R273H mutation had no drastic effect on the tertiary structure. In this case, the contact with DNA was lost because of the replacement of the arginine residue 273 that directly interacts with DNA.[16] As p53 acts as a homotetramer, Fersht and co-workers used nuclear magnetic resonance to explore the quaternary structure of full-length tetrameric p53 complex. They showed that oligomerization had only limited impact on the core domain structure. The full-length tetrameric p53 complex had a flexible structure but became rigid after binding to DNA.[17] These observations support earlier results on the structure of mutant and wild-type p53 based from the analysis of the core domain only. Thus, this stable mutant is a powerful tool for the analysis of the structural impact of missense mutations.

Missense mutations show various degree of loss of TA capacities[18] and are often accumulated in the nuclei of tumor cells. In experimental systems, missense mutations can cooperate with HRAS in cell transformation. These observations have led to the notion that some mutants carry pro-oncogenic activities, often referred to as 'gain of function' (GOF). By definition, these properties are not shared by wild-type p53 and are independent of their ability to exert a dominant-negative effect toward wildtype p53. Although the mechanism responsible for GOF properties is still a matter of debate, recent data are accumulating that suggest that mutant proteins can modulate gene expression in a specific way, distinct from wild-type p53, which may provide a basis for GOF. Deb and co-workers[19] showed that p.R175H, p.R273H or p.D281G was capable of inducing the survival gene nuclear factor (NF)-kB2. Indeed, in cellular models, p53 mutants or NF-kB2 knockdown rescued cells from resistance to chemotherapeutic drugs such as etoposide, cisplatin and carboplatin, suggesting a direct involvement of these mutants in chemoresistance through the NF-kB2 pathway. Using H1299 cells expressing an inducible p.R175H mutant, Blandino[20] showed that this mutant could upregulate the ID4 gene and the induction of ID4-dependent neoangiogenesis in mouse xenografts. Moreover, ID4 expression was increased after genotoxic treatment in cells carrying an endogenous TP53 mutant but not a wild-type gene, and this effect was suppressed by silencing TP53 using siRNA. These two examples show how abnormal gene expression mediated by mutant p53 can favor tumor development and aggressiveness.

A common hypothesis for the molecular mechanisms by which p53 mutants may regulate gene expression has emerged from several studies. These investigations suggest that p53 mutants may be tethered to chromatin through protein–protein interactions with one or more sequencespecific DNA-binding proteins. Several examples of mutant-dependent regulation of transcription through interaction with such proteins were recently described, including NF-Y factors, p65 or E2F1, the latter helping p.R175H to bind two regions of the ID4 promoter.[20] Along the same lines, the groups of Oren and Rotter provided two examples of cross talk between mutant p53 and antiproliferative pathways.

Oren[21] showed that p.R175H mutant coimmunoprecipitated with vitamin D3 receptor and was bound to vitamin D3 receptor response element, leading to an increase in both vitamin D3 receptor nuclear localization and TA activity. This increase in vitamin D3 receptor activity was accompanied by an antiapoptotic effect (protection from etoposide-induced apoptosis), contrasting with the known pro-apoptotic effect of vitamin D3. In fact, vitamin D3 induced apoptosis in a wild-type p53 background, but had antiapoptotic effects in some, but not all, cell lines expressing a mutant p53. Expression array experiments suggested that the presence of a mutant p53 altered vitamin D3 target gene selectivity and converted vitamin D3 from a pro-apoptotic into an antiapoptotic agent. As vitamin D3 and its derivatives are currently being investigated as cancer chemopreventive and therapeutic agents, further studies are needed to address the influence of TP53 status on the effects of vitamin D3 treatment.

Rotter and co-workers[22] recently described the cross talk between mutant p53 and transforming growth factor TGFB1 signaling pathway. TGFB1 is known to have a critical role in preventing the initiation and progression of cancer. Both p.R175H and p.R248W mutants, but not wild-type p53, inhibited the expression of TGFBR2 in some mammalian cells, leading to a decrease in the global TGFB1-induced biological responses. Even though the mechanism of TGFBR2 downregulation remains to be elucidated, this novel in vitro GOF may help to explain the relationship between mutant p53 expression and resistance to TGFB1.

Using a global and ambitious approach to address the underlying mechanisms involved in GOF, Tolstonog and co-workers[23] presented studies on the 'mutant p53 interactome' by using a combination of genome-wide experiments and bioinformatics. The aim was to map the interactions of p.R273H mutant on DNA, RNA and proteins. Overall, they showed that this mutant may operate as an epigenetic factor affecting the functional organization of chromatin thereby regulating the

transcription of physically linked genes. As p53 mutant was shown to interact with several RNA-binding proteins, it was suggested that it may participate in the spatial organization of transcription/processing factories providing a growth and survival advantage to tumor cells.

In vivo evidence for GOF has been provided by recent mouse models using different approaches by overexpression of an exogenous mutant and by the inhibition of an endogenous mutant. Deppert and co-workers[24] showed the effect of the expression of p.R245W and p.R270H mutants on mammary adenocarcinoma development in a double-transgenic mouse model, in which SV40 Large T and a p53 mutant were under the control of the murine whey acidic protein (WAP) promoter. This system allows mammary tissue-specific expression of the transgenes and to study mutant p53 activities independently of its dominant-negative effect over wild-type p53, which is considered to be inactivated by SV40 LT. In this model, the presence of mutant p53 led to an overall increase in the number and invasiveness of mammary tumors, but did not increase genomic instability. At the molecular level, both monotransgenic and bi-transgenic mice were characterized by similar genetic alterations, suggesting that p.R245W and p.R270H mutants exert their effect on tumor progression at a quantitative rather than qualitative level. Bossi et al.[25] used an inducible shRNA in a lentivirus vector to study the effect of inhibiting endogenous p.R175H or p.R273H mutants on tumor xenograft development. In both cases, mutant silencing led to a delay in tumor formation and a reduction in tumor vascularization. These two studies provided additional proof for GOF exerted by mutant p53. However, it should be kept in mind that p53 loss of function is sufficient to promote cancer development, as shown by earlier studies on p53 knockout mouse models. Indeed, Roux and co-workers[14] showed that loss of wild-type p53 function was enough by itself to confer an increased migratory capacity to cells. Also, in Li-Fraumeni patients, Bougeard et al. showed that large TP53 deletions could confer a tumor phenotype similar to the one of missense hotspot mutations.[26]

Interesting new findings may provide some perspectives regarding the ongoing debate concerning the relative importance of GOF versus loss of function in the contribution of mutant p53 to carcinogenesis. Using a transgenic p.R172H mouse model (equivalent to human p.R175H), Lozano[27] demonstrated that mutant p53 is regulated in a manner similar to wild-type p53 and that it must be stabilized to exert its GOF activity. This may explain why the contribution of GOF has been repeatedly observed in experimental systems where p53 is artificially overexpressed, whereas its contribution in human tumors remains controversial (see also section on p53 regulation below).

Table 1 summarizes GOF activities attributed to p53 mutants and described during the 'p53 Marathon 2007' meeting in Lyon 2007.

**p53 regulation**

p53 activities related to tumor suppression are required to maintain genetic integrity in response to genotoxic and non-genotoxic stresses. On the other hand, p53 must be tightly regulated to make sure that its antiproliferative activities are induced in a timely and controlled manner. Several levels of control are responsible for this regulation, including post-translational modifications, protein relocalization and stabilization, and protein–protein interactions. New findings regarding these mechanisms of p53 regulation are summarized below.

p53 stabilization and relocalization As mentioned above, Lozano[27] introduced an interesting new concept demonstrating that mutant p53 is regulated in similar ways as wild-type p53 in vivo. Using a previously established transgenic mouse model, she showed that the p.R172H mutant (equivalent of human p.R175H) exhibited higher expression in an MDM2/ background.[27] In addition, the survival of p.R172H homozygous mutant mice was reduced in a MDM2/ background, because of an increase in tumor aggressiveness. As MDM2 is one of the main negative regulators of p53 protein, these observations indicated that this p53 mutant is negatively regulated by MDM2 and that it needs to be stabilized to exert a dominantnegative effect or GOF activity. Thus, dominant-negative effect or GOF may arise only after mutant p53 stabilization in response to genotoxic or oncogenic stress, revealing the importance of these cooperative events for cancer development in a p53-altered background.

**Table 1: Some gain of function (GOF) activities attributed to p53 mutants[a].**

| Mutant | Cellular model | GOF | Mechanism |
|---|---|---|---|
| p.R175H | R175H-H1299 | Resistance to chemotherapeutic agents | NFKB2 upregulation |
| p.R273H | R273H-H1299 | | |
| p.D281G | D281G-H1299 | | |
| p.R175H | Inducible R175H-H1299, SKBr3 | Neoangiogenesis in vivo | ID4 upregulation |
| p.R273H | R273H-H1299, HT29 | Not specified | ID4 upregulation |
| p.R273H/R309S | SW480 | | |

| | | | |
|---|---|---|---|
| p.R280K | MDA-MB-231 | | |
| p.R175H | SKBr3, R175H-H1299 | Alteration of Vit D3 transcriptional network | VDR binding |
| p.R175H | R175H-H1299, SKBr3 | Decrease in TGFB1-induced biological responses | TGFBR2 downregulation |
| p.R248W | R248W-H1299 | | |
| p.R273H | Inducible R273H-H1299 | Regulation of some gene clusters | Epigenetic-like factor |

a To study their GOF properties in vitro, these mutant p53 were either overexpressed in p53-null cells (the lung adenocarcinoma cell line H1299) or endogenous mutants were inhibited by RNA interference (in SkBr3, SW480 or HT29). Shading indicates low delimitation.

MDM2 and MDM4 (MDMX) have crucial roles in the regulation of p53 cellular levels and activities. Alterations in these genes can thus impact on p53 tumor suppressor function as shown by Jochemsen.[28] Whereas alterations in both TP53 and RB1 pathways are found in most types of cancers, only RB1 pathway is apparently affected in retinoblastoma. However, double TP53/RB1 knockout mice display a higher risk of developing retinoblastoma than single RB1 knockouts, suggesting that inactivation of the TP53 pathway is important for retinoblastoma development. In fact, an amplification of MDM4 was found in retinoblastoma samples concomitant with a low rate of TP53 alterations.[29] In addition, overexpression of MDM4 in RB1/ retinal cells was sufficient for cell survival and clonal expansion. Taken together, these observations suggest that MDM4 could act as an oncogene by inactivating p53.

Wahl and co-workers[30] demonstrated the importance of the ratio between MDM2/MDM4 and p53 proteins on p53-dependent responses. An excess of both MDM2 and MDM4 limited p53 function in basal conditions, because of proteasome-mediated p53 degradation by MDM2 and inhibition of transcriptional p53 activity by MDM4. In a DNA-damage context, the decrease in MDM4 expression in normal embryonic fibroblasts correlated with an increase of both p53 Ser15 phosphorylation and WAF1 expression. In addition, in the presence of Nutlin, an inhibitor of MDM2/p53 interaction, MDM4 induced the nuclear accumulation of p53. Thus, there is a synergy between HDM2 and HDM4 to maximize p53 inactivation.

Although MDM2 and MDM4 negatively control p53 levels and activities, some proteins have been described that may enhance wild-type p53 response and activate mutant p53 GOF. One protein among them is the propylisomerase, PIN1, that binds and catalyzes cis/trans isomerization of Ser/Pro or Tyr/Pro motif.[31] Del Sal and co-workers[32] showed that PIN1 could interact with p53 after DNA damage and stimulate p53 TA by increasing both p300 recruitment on p53 and p53 acetylation, and the dissociation between p53 and the apoptotic inhibitor iASPP. The first event increased p53 DNA-binding capacity, whereas the second increased p53-dependent apoptosis. PIN1 also affected the activity of several cancer-associated p53 mutants. Indeed, the absence of PIN1 in mouse embryonic fibroblast cells expressing both activated RAS and p.R172H reduced the number of colonies formed in soft agar. These results indicate that PIN1 could act as a global enhancer of wildtype p53 response to DNA damage and also contribute to mutant p53 GOF activity.[33] Another protein, previously shown to inhibit MDM2-mediated p53 degradation, the promyelocytic leukemia protein, was shown to induce the phosphorylation of p53 on Thr18 and Ser20 (two residues located in the Mdm2-binding site) by CK1 and CHEK2, respectively, which induced p53 accumulation and nuclear relocalization.[34] As for PIN1, promyelocytic leukemia protein also interacted with some p53 mutants. Indeed, promyelocytic leukemia protein was shown to regulate the phosphorylation of p.R248W mutant upon stress, suggesting that promyelocytic leukemia protein may enhance the activity of both wild-type and mutant p53.

In normal conditions, p53 is present at low levels in the cytoplasm and nucleus. Under stress conditions such as DNA damage, p53 accumulates in the nucleus. A new mechanism responsible for p53 nuclear relocalization upon DNA damage has been described by Moll.[35] She showed that importin a-3 bound p53 on the main nuclear localization domain and facilitated the import of p53 into the nucleus. This mechanism was strongly dependent upon lysine residues 319–321, which were also the targets of MDM2-mediated ubiquitination. In addition, in non-stressed cells, the cytoplasm contained both non-ubiquitinated and ubiquitinated p53, whereas in stressed cells, cytoplasmic p53 was mainly non-ubiquitinylated. Thus, cytoplasmic non-ubiquitinated p53 might be selectively imported by importin a-3 upon stress, resulting in a rapid accumulation of p53 in the nucleus.

**Modulation of p53 transcriptional activity**

Several cellular factors that modulate p53 transcriptional activity and affect p53-dependent responses have been recently described. Beckerman[36] demonstrated that acetylation of lysine residues 351 and 357 affected the switch between cell cycle arrest and apoptosis, after induction of p53 expression in the H1299 p53-null cell line. The substitution of lysine with arginine inhibited p53 acetylation. The DNA-binding and transcriptional capacities of the resulting mutant were conserved but led to G1 arrest only. However, when the lysine residues were changed to glutamine, mimicking a constitutive acetylation, DNA binding was impaired, but a

transcription-independent p53-dependent apoptosis response was induced.

Le Cam et al.[37] showed that E4F1, a cellular transcription factor, has an important role in the proliferation/ survival balance. E4F1 has an atypical E3 ligase activity on p53 leading to its activation instead of its degradation. Ubiquitination of lysine residues by E4F1 impaired acetylation by PCAF and leads to a higher p53 transcriptional activity on the target genes involved in cell cycle arrest. Another regulator of p53, TWIST1, was described that blocks NMYC-induced apoptosis by inhibiting p53 transcriptional activity. Overexpression of TWIST1 occurs in a wide variety of cancers and correlated with NMYC amplification in neuroblastomas, a type of tumor that presents with a very low rate of TP53 mutations.[38] In addition, cooperation between TWIST1 and NMYC, HRAS or ERBB2 oncogenes was observed in colonyformation assays, suggesting that TWIST1 has oncogenic properties through these mechanisms.[39]

Another mechanism of regulation of p53 transcriptional capacities involves the isoforms of the p53 protein itself. Nine p53 isoforms have been identified to date.[40] Two of them, D40p53 and D133p53, differ from fulllength p53 in their N terminus. D40p53 lacks the TA domain and D133p53 the TA and the proximal part of the DNA-binding domain. Each of these three isoforms can be combined with three different C termini (a, b and g) generated by alternative splicing. The formation of hetero-oligomers between p53 and these isoforms can modulate p53 transcriptional capacity and modify some p53-dependent biological responses. Bourdon[41] showed that coexpression of D133p53 and p53 resulted in an increase of WAF1 promoter activity and in a decrease of BAX promoter activity as compared with p53 alone.

**Table 2: Effectors of p53 regulation and their biological properties.**

| Effectors | Mechanism | p53 regulation | Biological impact |
|---|---|---|---|
| PIN1 | p300 recruitment dissociation | mTA | — |
| | iASPP | — | mApoptosis |
| | interaction with mutant p53 | — | m GOF activity |
| PML | T18-S20 phosphorylation | mAccumulation | — |
| | Interaction with wild-type p53 | Nuclear localization | — |
| | Interaction with mutant p53 | — | mGOF activity |
| MDM2 | Wild-type p53 ubiquitination | mDegradation | — |
| | Mutant p53 ubiquitination | mDegradation | mGOF activity |
| HDM4 | Wild-type p53 interaction | Nuclear localization | — |
| IMPORTIN ALPHA 3 | NLS ubiquitination | Nuclear localization | — |
| | C-ter acetylation on Lysine | kDBA | mG1 arrest |
| | No C-ter acetylation on Lysine | mDBA+TA | mApoptosis |
| E4F | Wild-type p53 ubiquitination | mTA | mCell cycle arrest |
| TWIST1 | Wild-type p53 transcription | kTA | kApoptosis |
| p53 isoforms | Heterooligomers 133p53 | | mCell cycle arrest (p21) |
| | | TA modulation | kApoptosis (Bax) |
| | Heterooligomers p53b | | kApoptosis |

Abbreviations: C-ter, C-terminal domain; DBA, DNA-binding activity; GOF, gain of function; N-ter, N-terminal domain; NLS, nuclear localizing site; T, Threonine; TA, transcriptional activity. Shading indicates low delimitation.

**This suggests that D133p53 expression in a wild-type** p53 background favors cell cycle arrest instead of apoptosis. Other results suggest that p53b has a role in the regulation of p53-dependent apoptosis. Indeed, Kim[42] showed that p53b overexpression in wild-type p53-expressing cells decreased BAX and CASPASE3 expression, and that p53b-expressing cells exhibited resistance to pro-apoptotic drugs. In both examples, p53b seemed to downplay p53 capacity to induce apoptosis.

**p53 and molecular carcinogenesis**

In many cancers, mutations in the TP53 gene have been identified as early events in premalignant tissues, in particular those exposed to environmental carcinogens. Data from liver and esophageal cancers showed that TP53 mutations may occur early in the natural history of these cancers.[43] Rather than initiating events, TP53 mutations may help cells and tissues to cope with stressinduced tissue remodeling constraints, providing a shortterm proliferative advantage, which ultimately backfires with enhanced risk of progression to cancer. Two hypotheses related to this may be proposed: (1) TP53 p.R249S mutation (the most prevalent mutation found in liver cancer from chronic hepatitis B carriers exposed to aflatoxin) may become selected in chronically infected cells, perhaps as an adaptive mechanism to resist hepatitis B-induced cell death and cirrhosis; (2) early TP53 mutations in esophageal cancers may provide a selective advantage in the context of tissue remodeling under stress conditions, a process in which the TP53 paralog TP63 has an essential role. The latter hypothesis was substantiated by two experimental examples. First, inhibition of TP63 in cultured cells has profound effects on cell-adhesion complexes.[44] Second, in esophageal

cells, degradation of TP63 isoforms by the proteasome occurs in cells exposed to acid/bile stress, the main condition inducing the formation of intestinal metaplasia and adenocarcinoma in lower esophagus.[45]

In breast cancer, five gene-expression profiling subtypes have been identified: normal breast-like, ERBB2þ, basallike, luminal-B and luminal-A.[46] These subtypes have been reproduced in many other data sets and with different array platforms, and have been found in breast cancers of different stages.[47] They have different genetical and biological characteristics and were associated with different patient outcomes. Recently, it has been shown that TP53 mutations were strongly associated with the Basal-like and ERBB2þ profiles,[48] suggesting that p53 inactivation by gene mutation is confined to these specific subtypes, which may reflect different routes to cancer development.

The role of DNA double-strand breaks in early stages of cancer development under oncogene-induced DNA replication stress has been recently highlighted.[49] Bartek[50] developed experiments showing the recruitment of DNA repair proteins at sites of DNA damage in two successive waves. They showed that the oncogene-induced DNA-damage response is activated according to a threshold that correlates with the risk of invasive carcinoma, leading to a selection pressure that inactivates the proteins involved in both checkpoint and repair processes during early stages of tumor development, such as p53.

Using the humanized p53 gene knock-in (Hupki) mouse model, Reinbold et al.[51] studied the role of environmental mutagenesis in senescence bypass. In mice, senescence control is regulated by the p19ARF/p53 pathway, and escape from this control can occur through TP53 inactivation by point mutation. Exposure of mouse embryonic fibroblasts to carcinogens produced immortal cell lines carrying missense TP53 mutations. TP53 'signature mutations' obtained using this approach revealed a remarkable degree of concordance between mutations that allow senescence bypass in vitro and mutations that arise in vivo.[51]

Overall, these data show that the origin of TP53 mutations is greatly influenced by cancer-initiating events such as DNA-damage stress and that the effect of these mutations is to promote cancer development through loss of antiproliferative activities including apoptosis and senescence.

Translational research for diagnostic, prognostic and therapeutic applications Assessment of p53 mutations and isoforms for diagnosis and prognosis.

To use p53 as a biomarker for diagnosis and prognosis or as a target for therapy, it is important to accurately assess TP53 mutation status in tumor samples. A microarray-based test for high-throughput detection of TP53 gene mutations, the AmpliChip p53 test, was shown to correctly detect all single-base substitutions and 1bp deletions along the entire coding sequence of TP53, including splice junctions. AmpliChip p53 was shown to work on formalin-fixed samples and with small DNA quantities. Three pilot studies that compared AmpliChip p53 with single-strand conformation polymorphism sequencing and sequencing methods showed good concordance, except for insertions and deletions of more than 1bp that could not be detected by AmpliChip p53.[52] Although this approach allows for a faster analysis of the complete TP53 coding sequence, the non-detection of insertions and deletions may be a problem in cancers that show high frequencies of these mutations such as head and neck cancers.

The prognostic value of TP53 gene mutations has been investigated in several types of cancer. A large number of studies have been performed in breast cancer and have shown consistent association with poor prognosis. As mentioned before, TP53 mutation status in breast cancer was strongly associated with the two subtypes that carry the poorest prognosis, basal-like and ERBB2þ. Langerod et al.[48] showed that, in multivariate analysis, the prognostic value of TP53 mutation was similar to the gene profile molecular subtypes. Moreover, different types of TP53 mutations were associated with different outcomes, non-missense mutations being associated with the worst outcome in several cohorts.[53] Thus, TP53 mutation status and gene expression profiles are powerful prognostic markers in breast cancer.

Recent data on the expression of p53 isoforms in tumor samples showed that in primary breast tumors, the presence of wild-type TP53 was associated with high expression of p53b mRNA, whereas the presence of mutant p53 correlated with the expression of D133p53b, the latter association being correlated with poor overall survival.[41] Poor survival was also correlated with a high expression of both estrogen receptor and D133p53b. The prognostic value of TP53 mutations in breast cancer may thus be modulated by the expression of specific isoforms whose origin and regulation remain to be fully characterized.

**Targeting p53 for therapy**

Small molecule and peptide p53 therapies. Several small molecules that target p53 have been identified, including molecules that reactivate mutant p53 into a form with wild type-like activities, molecules that block mutant p53 GOF, molecules that prevent wild-type p53 degradation by disrupting MDM2–p53 interaction or inhibiting MDM2-E3 ubiquitin ligase activity.

PRIMA-1 (p53 reactivation and induction of massive apoptosis -1) and RITA (reactivation of p53 and induction of tumor cell apoptosis) are two small molecules that have been shown to reactivate mutant p53 suppressive functions in tumor cells.[54,55] In stably transfected Saos2p.R273H and H1299-p.R175H cells,

both mutants were refolded into a wild-type conformation by PRIMA-1.[56] PRIMA-1 completely inhibited the growth of Saos2p.R273H cells, but only caused a minor reduction in the growth rate of Saos2 cells. This correlated with the induction of PUMA and CASPASE3/8 in response to PRIMA-1 treatment in mutant p53 but not in p53-null cells. Other p53 target genes, such as GADD45 and NOXA, were also activated by PRIMA-1, demonstrating the reactivation of wild-type p53 activity in this context. The effect of PRIMA-1 was also demonstrated in vivo by the p53-dependent reduction in the size of tumor xenografts obtained with these cell lines in severe combined immunodeficiency mice. PRIMA-1 is thought to directly bind mutant p53 and refold it, allowing the activation of p53 targets. A phase I clinical study with PRIMA-1 is ongoing to test its efficacy in acute myeloid leukemia. RITA acts through a different mechanism as it was shown to restore wild-type p53 function in tumor cells by preventing the degradation of p53 through the disruption of its interaction with MDM2. p53 TA activity could be reactivated in cultured cells of different origins, leading to a p53-dependent apoptosis through inhibition of the expression of many antiapoptotic proteins such as BCL2, MAP4, MCL1 and blockage of growth signaling (IGF1R, PI3K). In contrast to PRIMA-1, the effect of RITA was not limited to p53 reactivation, this molecule appearing to be capable of other effects through the downregulation of oncoproteins.[57] Other p53-targeting drugs that have been developed for therapeutic use are available, such as Nutlin-3 that has been designed to modulate p53/MDM2 interactions and is extensively used in fundamental research to study p53 regulation.

A drug that targets a specific p53 mutant, p.Y220C, has been designed from p53 structural studies. The p53 mutant p.Y220C is frequently found in various types of human cancers. Residue 220 is located in a crevice at the opposite of the DNA-binding surface, and the Y4C substitution increases the surface of the crevice. A small molecule drug (Phikan059) was designed to bind this crevice.[58] This drug rescued mutant p53 in a noncompetitive manner toward wild-type p53. Although the benefits of designing mutant-specific drugs might be questioned, based on cancer incidence and the overall frequency of this mutant reported in the International Agency for Research on Cancer TP53 database, up to 76000 new annual cancer cases worldwide may carry the p.Y220C mutant. This strategy might thus have a significant impact on therapy for the most frequent mutants.

Another area of research concerning p53-targeted therapy is the understanding of the regulation of p53 functions through the characterization of post-translational modifications and protein-interaction profiles. Using a two-hybrid strategy, Del Sal[33] has recently isolated five peptide aptamers (Pas) able to interact specifically and strongly with several p53 mutants, including p.R175H. The observed interaction was mediated by an unknown process but led to the inhibition of the TA capacities of mutant proteins, inducing to cell death exclusively in cells expressing mutant p53.

p53 immunotherapy or gene therapy. As aberrant p53 overexpression is often observed in tumors, it may provide an opportunity for T cells to discriminate between malignant and normal somatic cells. Indeed, peptides derived from the p53 protein and presented by major histocompatibility complex molecules for T-cell recognition could serve as universal tumor-associated antigens for cancer immunotherapy. However, because p53 normally functions as a ubiquitously expressed self-protein, it represents a paradigm target molecule for tumor-reactive self-antigen-specific T cells. Tailoring p53-based cancer immunotherapy thus requires overcoming both interference with p53-specific self-tolerance and induction of the appropriate repertoire of p53-reactive T cells. Theobald[59] is exploiting this concept and exploring the transfer of selected p53-specific T-cell receptor genes into human T cells as a novel and appealing strategy to meet these requirements. He showed that genetic modification of T cells by a p53 antigen-specific T-cell receptor gene can be used to circumvent self-tolerance of autologous T lymphocytes to p53.

Another gene therapy strategy is based on vectormediated expression of a wild-type p53 protein that is currently being tested in the clinic. Advexin is a replication-incompetent adenovirus type 5 vector containing a normal TP53 tumor suppressor gene. In the treatment of a patient with Li-Fraumeni syndrome (LFS), local intratumoral injection of Advexin resulted in complete and durable remission of the injected lesion by 18-fluorodeoxyglucose-positron emission tomography scan with improvement of tumor-related symptoms.[60] Advexin has also demonstrated clinical activity in various types of sporadic tumors including head and neck squamous cell carcinomas, non-small cell lung carcinomas and prostate cancers. In patients with recurrent head and neck cancer, tumor response and increased survival were associated with specific p53 biomarker profiles. p53 profiles predictive of Advexin efficacy were based upon TP53 gene sequence analyses and p53 protein expression determined by immunohistochemistry. The absence of high-level protein expression of 'dominant negative' p53 DNA-binding domain mutations that could block Advexin activity were predictive of Advexin efficacy.[61]

**Recent findings and developments on Li-Fraumeni syndrome**

Li-Fraumeni syndrome was first described in 1988 as a clustering of early onset tumors with a predominance of sarcomas (a proband with sarcoma and two close relatives with cancer under 45 years of age).[62] In 1990, it was found that this cancer syndrome may be caused by germline mutations in the TP53 gene.[63] Seventeen years

later, it is clear that TP53 gene alterations are the main cause of LFS and that different types of mutations are associated with different penetrance and phenotypes. The spectrum of tumors observed in TP53 mutation carriers is wider than initially described, including sarcomas, breast carcinomas, brain tumors, childhood adrenocortical carcinoma and several other types of cancers that occur at earlier ages or at greater frequencies than expected (gastric cancers, Wilms' tumors, malignant phyllodes tumors). It was proposed by Dr Birch that the diagnosis of LFS should be based on the presence of a germline TP53 mutation in an individual or a family and that the original clinical definition of LFS, although clinically useful, should be revisited to better reflect tumor spectra observed in TP53 mutation carriers and to select families for TP53 testing.[64]

Analyses of several LFS families collected through different networks have revealed interesting findings. Frebourg[65] showed that in a large series of French LFS families (n¼474), four carried large deletions or gene rearrangements. The identification of such TP53 gene deletions in LFS provided strong evidence that LFS may result from a loss of function at the TP53 locus.[66] Nonetheless, missense mutations were the most frequent (78 cases) and were associated with earlier age at onset of tumors, providing in vivo evidence for a potential gain of oncogenic functions with these mutations.

In the Brazilian population, 45 unrelated Brazilian subjects whose family histories matched LFS or LFL clinical definitions were identified. TP53 mutation screening revealed a high frequency of the same mutation, R337H, in probands of unrelated kindreds (six cases).[67] Although this mutation was reported earlier to be exclusively associated with ADC in Brazilian children,[68] evidence was presented that showed a large spectrum of tumors associated with this mutation. Moreover, this mutation resided on the same allele, rare in the general population but more common in the Brazilian population, demonstrating a founder effect.[69] This Brazilian situation is quite unique as there is no other example of such a frequent germline TP53 mutation in the International Agency for Research on Cancer TP53 database of LFS (see below).

The International Agency for Research on Cancer TP53 database compiles data on LFS families reported in the scientific literature and represents a worldwide collection of the clinical and molecular characteristics of the syndrome. This database has revealed an overrepresentation of families from Europe and Northern America, suggesting the possibility of less frequent diagnosis and/or reporting of LFS in other parts of the world.[70]

Recent analysis of genotype–phenotype associations using this large data set showed that the degree of loss of function of missense mutations is related to the age at onset of some tumor types.[71] Overall, these analyses have revealed several genotype–phenotype associations that may help the clinical management of TP53 germline mutation carriers. Potential surveillance strategies for members of LFS kindreds based on tumors observed in US families recruited at DNA Farber/NCI were proposed.[72] In this cohort, gastrointestinal malignancies were higher than expected suggesting that TP53 mutations may confer some susceptibility to these malignancies.[73] On the basis of this observation, Syngal[72] proposed to consider endoscopic surveillance for these kindreds. Garber showed the results of a pilot study aimed at gathering preliminary data on the use of 18-fluorodeoxyglucose-positron emission tomography-computerized tomography scan imaging as a potential surveillance strategy for LFS tumors in TP53 germline mutation carriers. Asymptomatic cancers were detected in 3 of 14 LFS individuals, showing potential efficacy of 18-fluorodeoxyglucose-positron emission tomography-computerized tomography scan imaging in these kindreds. A larger trial is needed to evaluate safety, sensitivity, specificity and observer variability of the screening.

In the United Kingdom, a new program has been launched by Rahman[74] to perform a broad analysis of TP53 gene on families and individuals selected according to all criteria suggestive of LFS and including isolated cases of appropriate cancers, to better assess the phenotypes associated with TP53 mutations. Indeed, as mutation analysis is traditionally undertaken in highly selected cases based on prior assumptions, genotype–phenotype association studies are subjected to strong biases.

Compelling evidence for modifier effects on phenotypes associated with TP53 germline mutations has been recently uncovered. Strong[75] described modifier effects of MDM2 SNP309 polymorphism on cancer risk in US kindreds selected for childhood sarcomas. TP53 germline mutations were found in 5–7% of childhood sarcoma patients. In TP53 mutation carriers, the cumulative cancer risk was found to be higher in women than in men at all ages and was attributable in part to SNP309. This association between SNP309 polymorphism (G allele) in the MDM2 gene and earlier cancer onset in TP53 germline mutation carriers was also described in the French series.[76] Genetic anticipation (when cancers occur at an earlier age of onset in subsequent generations) was observed in the US kindreds and was related to telomere shortening as measured by quantitative fluorescent in situ hybridization. In another cohort, new data obtained with comparative genomic hybridization and single-nucleotide polymorphism arrays on genomic DNA from TP53 mutation carriers showed that copy number variations were more frequent in TP53 mutation carriers compared to non-carriers and were even more frequent in TP53 mutation carriers with cancers compared with those without cancer. Telomere shortening was also observed in these patients.[77] These results suggest that genetic anticipation may be because

of telomere dysfunction and accumulation of genetic alterations caused by TP53 haploinsufficiency.

As LFS patients are prone to develop secondary cancers after conventional anticancer treatments (radioor chemotherapies), new treatment strategies are highly needed. A gene therapy approach that may be used for LFS patients, Advexin, has recently been tested on one LFS patient.[60] Advexin is a replication-incompetent adenovirus type 5 vector containing a normal TP53 tumor suppressor gene. Local intratumoral injection of Advexin in a progressing embryonal carcinoma of an LFS patient resulted in complete and durable remission of the injected lesion by positron emission tomography scan evaluation. Further trials are needed to confirm that Advexin is a suitable and safe treatment for LFS patients.

Overall, these data showed the progress that has been made in our understanding of the molecular basis of the syndrome and provide perspectives for improving LFS patient management and treatment.

**Perspectives for further research and translational applications**

The Third International Workshop on Mutant TP53 has highlighted several developments in our understanding of the impact of TP53 mutations in cancer. First and foremost, there is now a consistent and increasing body of mechanistic evidence for the role of at least some mutant p53 proteins in conferring a 'gain of function' phenotype, supporting earlier findings that mutant p53 may somehow behave as an oncogene. The mechanism of action of mutant p53 appears to be complex. It is most likely to involve interactions with other transcription factors and modulation of their activities, and appears to be critically dependent upon the stability and accumulation of mutant p53 proteins in tumor cells. In this respect, the observation that many mutants are still responsive to MDM2/ MDM4-controlled induction in response to stress is of particular relevance, as this implies that tumor treatment with cytotoxic drugs may activate or enhance these 'oncogenic' properties.

A second major highlight relates to wild-type p53 and its emerging role as a critical factor in stress-induced senescence. In fact, the picture emerging from different lines of work is that replicative senescence (permanent cell cycle arrest) and tissue aging are interconnected through a pathway in which hyperproliferative/oncogenic stress and DNA damage cooperate to activate p53 by a p14/19arfdependent pathway. Thus, excess of p53 activity appears to induce premature aging. Senescence and aging may be seen as a normal suppressive response to DNA damage induced by stress or as a byproduct of normal DNA replication. In contrast, the maintenance of an active replication capacity may maintain cell renewal and prevent senescence, but at the expense of an increased risk of cancer. Thus, cancer or senescence may be seen as two alternative fates in aging organisms, the secret of longevity being to find the best possible trade-off between these two options.

A third major development is the emergence of p53 isoforms at the forefront of the p53 scene. The trend is a well-known one in p53 research. p53 isoforms are as old as the p53 field itself, but are only recently 'rediscovered' and correctly interpreted. This area of research is still young, but it is clear that several types of p53 isoforms may exist, and it will be important to assess in detail how each of these are regulated, their impact on p53 functions and possible contribution to p53 downregulation in cancers. Interestingly, the N-truncated isoforms (D40p53 and D133p53) lack critical regions for both protein degradation and transcriptional activation. It should be expected that these proteins have regulatory and functional properties that are very different from and possibly even opposite to those of wild-type p53. In this respect, this Third International Workshop provided an opportunity for an informal working group on p53 isoforms to meet and to discuss collaborations, exchanges of reagents and joint efforts to examine the multiple biological challenges raised by these findings.

Finally, the workshop has provided a forum to highlight p53 translational research efforts that are advancing to clinical applications. The data on TP53 mutations in breast cancer are now solid and coherent enough to recommend the use of TP53 mutations as prognostic biomarkers in clinical practice. Progress in preclinical work on small molecule drugs such as PRIMA-1 or RITA makes it justifiable to move these drugs into phase I and II clinical trials. p53-restoring gene therapy, an approach that earlier showed its promise in the work of Roth and co-workers[78] almost 15 years ago, is now being evaluated as an adenoviral p53 gene therapy product, Advexin, in phase III clinical trials. It is clear after 25 years of p53 investigations, the importance of basic and clinical research to progress hand in hand. Further integrated efforts are needed to insure that the knowledge discovered in basic p53 research will find its most rapid and effective translation into clinical practice.

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p53.iarc.fr/P53meeting2007/P53meeting2007. html; p53 Gateway: http://www.p53gateway.org/

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