**Dominant-Negative Features of Mutant TP53 in Germline Carriers Have Limited Impact on Cancer Outcomes**

Paola Monti, Chiara Perfumo, Alessandra Bisio, Yari Ciribilli, Paola Menichini, Debora Russo, David M. Umbach, Michael A. Resnick, Alberto Inga, and Gilberto Fronza

**Abstract**

Germline TP53 mutations result in cancer proneness syndromes known as Li-Fraumeni, Li-Fraumeni-like, and nonsyndromic predisposition with or without family history. To explore genotype/phenotype associations, we previously adopted a functional classification of all germline TP53 mutant alleles based on transactivation. Severe deficiency (SD) alleles were associated with more severe cancer proneness syndromes, and a larger number of tumors, compared with partial deficiency (PD) alleles. Because mutant p53 can exert dominant-negative (DN) effects, we addressed the relationship between DN and clinical manifestations. We reasoned that DN effects might be stronger in familial cancer cases associated with germline TP53 mutations, where mutant alleles coexist with the wild-type allele since conception. We examined 104 p53 mutant alleles with single amino acid substitutions described in the IARC germline database for (i) transactivation capability and (ii) capacity to reduce the activity of the wild-type allele (i.e., DN effect) using a quantitative yeast-based assay. The functional classifications of p53 alleles were then related to clinical variables. We confirmed that a classification based on transactivation alone can identify familial cancer cases with more severe clinical features. Classification based on DN effects allowed us to highlight similar associations but did not reveal distinct clinical subclasses of SD alleles, except for a correlation with tumor tissue prevalence. We conclude that in carriers of germline TP53 mutations transactivation-based classification of TP53 alleles appears more important for genotype/phenotype correlations than DN effects and that haplo-insufficiency of the TP53 gene is an important factor in cancer proneness in humans. *Mol Cancer Res; 9*(3); 271–9. ©2011 AACR.

**Introduction**

The spectra of germline TP53 mutations as well as sporadic cancers comprise many different alleles. A vast majority yield single amino acid changes in the DNA binding domain of the protein (1). Functional assays in model systems such as yeast revealed that the mutations could variably impact the p53 sequence-specific transcription factor activity (2, 3). Germ line mutations of TP53 result in cancer proneness syndromes from the more severe known as Li-Fraumeni (LFS), Li-Fraumeni-like (LFL), to the less severe nonsyndromic predisposition with (FH) or without (noFH) family history. We previously explored genotype/phenotype associations by comparing a functional classification of all germline TP53 mutant alleles reported in the public domain (http://www.umd.be:2072/index.shtml) to clinical data from the IARC database R10 release (http://www-p53.iarc.fr/Germline.html). Our analyses revealed that severe deficiency (SD) alleles were associated with more severe cancer proneness syndromes (LFS), whereas partial deficiency (PD) alleles were associated with less severe cancer proneness conditions (FH). These results indicate that the loss of transactivation ability influences clinical manifestations in patients who inherited TP53 mutations and developed cancer (4).

Mutations in TP53 can affect the tumorigenic process through at least 3 different mechanisms: (i) loss of function (LoF; lack of some wild-type function), (ii) gain of function (GoF; acquisition of functions that are absent in the wild-type protein; refs. 5, 6), or (iii) dominant-negative (DN) effects (in heterozygous cells, a DN protein reduces the transactivation capacity of the wild-type protein). Using yeast-based assays we and others (7–9) have found that TP53 alleles have different abilities to abrogate wild-type activity and that this behaviour depends on the...
sequence of the p53 target response element (RE; refs. 9, 10). Even though DN effects were also observed in human cell lines, results were more conflicting (9, 10).

The DN effect of TP53 alleles appears to impact the development and clinical manifestations of at least some sporadic tumors. Among 40 patients with sporadic glioblastomas, the average age at diagnosis was significantly lower in patients with tumors harbouring DN alleles than in those with recessive alleles (i.e., non-DN; see ref. 11 for definition) or in those without mutations, suggesting that DN mutations can accelerate development of glioblastomas. In another study on squamous cell carcinoma (SCC), patients with DN TP53 alleles presented a significantly shorter disease-free survival than those with recessive or with wild-type alleles, suggesting that the presence of a DN TP53 mutation may provide a predictor of early recurrence in oral SCC patients (12).

If the DN effect is biologically important, it may be stronger in familial cancer cases associated with germline TP53 mutations, a situation in which the mutant allele coexists with the wild-type allele since conception. To evaluate this issue, we combined clinical data from the R11 release of the IARC germline database (http://www-p53.iarc.fr/Germline.html) with our own functional data for 104 of the 106 (98%) germline mutant TP53 alleles with a single amino acid substitution. For each allele, we determined transactivation capability and DN effect using a quantitative (luciferase) yeast assay. We also considered a separate group of 52 alleles that can be classified as obligate severe deficiency (O-SD) alleles in terms of transactivation by virtue of nonsense, frameshift, or other mutations that give rise to a truncated protein (4).

We created classifications of TP53 alleles based on transactivation and DN effects (both individually and jointly) and investigated associations between these functional classifications and clinical characteristics of familial tumors.

Materials and Methods

Yeast strains, vectors, and media

The S. cerevisiae haploid yeast strains yLFM-REs (P21-5', MDMDP2, BAX-A + B, PUMA) have a quantitative (luciferase) reporter gene under the control of p53 through a p53 RE in its promoter region (3). The haploid strain ylG397 was used for the gap repair assay (13). The pLS76 (CEN/ARS, LEU2) and pTS76 (CEN/ARS, TRP1; ref. 8) vectors were used for the expression in yeast of the human wild-type p53 cDNA under control of the ADH1 constitutive promoter. The pRS315 (CEN/ARS, LEU2) and pRS314 (CEN/ARS, TRP1) plasmid vectors were used as empty controls. Cells were grown in 1% yeast extract, 2% peptone, and 2% dextrose with the addition of 200 mg/L adenine (YPDA medium) or in selective medium with the addition of adenine (5 mg/L or 200 mg/L). Selective minimal plates were used with the addition of 5 mg/L adenine (gap repair assay) or 200 mg/L adenine (luciferase assay).

Construction of mutant TP53 alleles by 2-step PCR mutagenic approach

Of the 106 alleles present in the IARC R11 germline p53 database, 104 were studied (Gln317His and Lys321Gln were excluded—see the following text). Forty-two alleles were available from previous work (14–20) while 62 alleles were constructed using site-specific mutagenesis. For each mutation, a pair of complementary 30-mer oligonucleotides (which served as forward and reverse primers) was synthesized, with the mutated base adjacent to the central position of the oligonucleotide. These primers were used in 2 separate PCR reactions and paired with P4 and P3 (13), respectively, with pLS76 as template. PCR conditions were: denaturation 40 seconds, annealing 60 seconds, and 80 seconds elongation (Master Taq Kit, 5Prime; Eppendorf). Unpurified aliquots of both PCR products were cotransformed in the ylG397 strain together with HindIII/Stul double digested pRDI-22 plasmid (13; gap repair assay). Plasmid DNA was recovered, expanded, purified, and the presence of the mutation verified by DNA sequencing (BMR Genomics).

Quantitative evaluation of mutant p53 transactivation ability and DN effects

The analysis of transactivation ability was carried out by cotransforming (LiAc method) in 4 strains of the p53 mutant expressing vector and empty vector pRS314. For each reporter strain, the background luciferase activity was measured. Double transformants were selected on minimal plates lacking leucine and tryptophan but containing 200 mg/L adenine. After 3 days of growth at 30°C, transformants were streaked onto the same type of plate and allowed to grow for 2 days. Cells were resuspended in H2O, collected by centrifugation and lysed in 100 μL of cell culture lysis buffer (Glo Lysis Buffer; Promega) using acid washed glass beads (0.4- to 0.6-mm diameter; Sigma). Soluble proteins were purified and quantified using the BCA assay (Pierce, Celbio). Luciferase activity was measured, using a multilabel plate reader (Mithras LB940; Berthold Technologies), following the manufacturer’s protocol (BrightGlo Luciferase Assay; Promega) and normalized to unit of soluble proteins (light unit/mg protein). The transactivation ability of p53 mutants was evaluated as percent luciferase activity with respect to that of the wild-type p53 (cotransformation of pLS76 and pRS314) after subtracting the background of each reporter strain. For each mutant allele, the mean transactivation activity observed in the 4 reporter strains was calculated. Alleles were then classified as SD if their mean transactivation activity was less than 25% of the wild-type allele, and as PD otherwise. Analyses of DN effects were carried out by cotransforming the 4 strains with the p53 mutant expressing vector pLS76 and the p53 wild-type expressing vector pTS76. Double transformants (Leu⁺ Trp⁺) were selected, purified, expanded, and used to extract soluble proteins for luciferase assays as described previously. For each reporter strain the net transactivation was determined. The mean net activity for each mutant in the 4 reporter strains was then
calculated and normalized to the activity of 1 wild-type allele. The net transactivation of a mutant allele that is completely unable to interfere with the wild-type allele must equal (allele does not contribute to net transactivation) or exceed (allele has its own residual activity) the activity of 1 wild-type allele, that is set to 100%. The net transactivation of a mutant allele able to interfere with the transactivation of the wild-type would be less than 100% of 1 wild-type allele [the lowest limit would never reach complete abrogation (i.e., 0% transactivation), as, depending on the mechanism of tetramer formation, a fraction of wild-type homo-tetramers would be formed ranging from 1/16th to 1/4th of the total number of tetramers]. We assessed the DN effect by classifying mutant alleles based on their net transactivation activity in 4 strains. We explored 2 operational definitions for DN alleles. For one definition, DNm90, we classified mutant alleles as DN if, when coexpressed, the mean transactivation activity in 4 strains was less than 90% of the wild-type activity. Thus, the corresponding recessive alleles are those whose mean transactivation activity exceeds 90% of that of 1 wild-type allele alone. We also considered a second, less stringent definition (DN90) where we classified alleles as DN if their transactivation activity, when coexpressed, was lower than 90% in at least one reporter strain. Correspondingly, those mutant alleles whose mean transactivation activity exceeds 90% of that of the wild-type allele in each reporter strain were classified as recessive. Results are presented in Supplementary Table S3. Because the DN effect is RE specific (9, 10), we tend to prefer the second definition. Furthermore, the DNm90 definition results in a highly skewed distribution towards recessive alleles. Consequently, we report results obtained with DN90 in the main text and those obtained with DNm90 in the Supplementary materials (see the following texts, specifically in Supplementary Tables S4 and S5).

Clinical definitions
For clinical definition see Monti et al. (4) and references cited within.

IARC database: criteria for the exclusion/inclusion of mutant TP53 alleles
Germline TP53 mutations result in cancer proneness syndromes such as LFS, LFL, and FH and noFH. This relational database (http://www-p53.iarc.fr/Germline.html) contains information on families with LFS/LFL syndromes (21–23) and those that do not fulfill the clinical definitions of LFS/LFL although they carry a germline mutation in the TP53 gene. Clinical data were downloaded from the database without additional curation with the exceptions cited previously (4). Gln317His (mean transactivation activity 100% of wild-type) and Lys321Gln (mean activity >30% of wild-type) were not examined, but these 2 private alleles should have little or no influence on our analyses [each allele was found in a single subject, classified as NA (data not available), who developed a relatively rare tumor (kidney) with age at diagnosis unavailable]. For the entire database, only affected subjects (at least one diagnosis of cancer reported) were considered. The Arg337His allele was also excluded. Indeed, this allele is observed in a large number of families (R11: 24%; 69/284) but none of them were classified as LFS though there are conflicting views about whether some of them meet the criteria for LFL as opposed to FH (24, 25). The allele is largely associated with a specific tumor type (adenocortical carcinoma) with pediatric onset (median age at diagnosis: 2 years, n = 80). Only 4% of families showed individuals with multiple tumors (3/69). Interestingly, Arg337His has high residual transactivation activity both in yeast (26) and mammalian cells (27). Because Arg337His introduces a strong bias in favour of the correlation between PD status and milder family history, whereas its almost exclusive association with pediatric cancer risk confounds the correlations with age of cancer onset, individuals who inherited an Arg337His allele were excluded from the analyses.

Statistical tests
Statistical comparisons were performed as previously described (4). We regarded families as statistically independent and tested hypotheses about characteristics of families (e.g., clinical class) with Fisher’s exact test. Because individuals within a family are not statistically-independent, we tested hypotheses about characteristics of individuals (e.g., age at diagnosis or tumor site) using the within-cluster resampling approach (28), which accounts for within-family correlations and protects against biases from informative family size. To avoid dependence among multiple tumors from one subject, we analyzed each subject’s first tumor only. The P values are given in the text.

Results
Analysis of transactivation of mutant TP53 germline alleles
We have analyzed TP53 mutations described in the R11 release of the IARC germline database which greatly extends the R10 dataset that we previously investigated (4) in terms of alleles, families, subjects, and tumors with an inherited TP53 mutation. A total of 104 mutant TP53 germline alleles were functionally analyzed. For each mutant allele the transactivation activities were measured in 4 yeast reporter strains and the mean activity was calculated (Supplementary Table S1). Our data confirmed that Arg337His has a high residual transactivation activity, and it was excluded from further analyses for reasons described in the Materials and Methods section. Of the remaining 103 alleles, 69 were classified as SD (mean activity <25% of wild-type) and 34 as PD (mean activity ≥25% of wild-type; Table 1). We obtained similar results using the functional transactivation data obtained by Kato and colleagues (26) and applying the same definition for SD and PD alleles (Supplementary Table S2).

Functional classification based on our transactivation data was then used to query clinical data in the IARC database (R11; http://www-p53.iarc.fr/Germline.html) including the site of the tumor and occurrence of multiple tumors in the same individual (1). In addition, 52 of the p53 mutants could be
classified as O-SD alleles by virtue of nonsense mutations, frameshifts, or other mutations that give rise to a truncated protein. The proportion of families classified as FH was larger among families carrying PD alleles than among those carrying SD alleles (34% vs. 14%; \( P = 0.004 \)). In contrast, the proportion of families classified as LFS was larger among families carrying SD alleles than among those carrying PD alleles (41% vs. 11%; \( P = 0.0002 \)). Similarly, families whose members presented multiple tumors were more common among families carrying SD alleles than among those carrying PD alleles (63% vs. 39%; \( P < 0.006 \); Table 1). Although Kaplan–Meir survival curves (Fig. 1) suggest that age at diagnosis of the first tumor in confirmed carriers tended to be earlier in SD than PD families, the difference was not statistically significant when the within-family correlations were taken into account (\( P = 0.23 \); see Materials and Methods; ref. 28). The discrepancy between the visual impression and the statistical test is attributable in part to correlation in age at first tumor among members of the same family. We also confirmed that the clinical features of carriers of O-SD alleles are similar to those of subjects carrying SD alleles (Table 1 and Fig. 1), as previously reported (4). Overall, these results strengthen the observation that the transactivation properties of p53 mutants can be correlated with distinct clinical history of the patients who inherited those mutations and developed cancer (4).

### Table 1. Relationship of clinical variables to functional classification of TP53 germline alleles, based on the mean transactivation activity

<table>
<thead>
<tr>
<th>Number of alleles</th>
<th>SD</th>
<th>PD</th>
<th>O-SD</th>
<th>SD vs. PD, P</th>
<th>SD vs. O-SD, P</th>
<th>PD vs. O-SD, P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class distribution of families</td>
<td>NA</td>
<td>noFH</td>
<td>FH</td>
<td>LFL</td>
<td>LFS</td>
<td>Total</td>
</tr>
<tr>
<td>Number of alleles</td>
<td>69</td>
<td>34</td>
<td>52</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td>7 (4%)</td>
<td>5 (11%)</td>
<td>2 (3%)</td>
<td>ns</td>
<td></td>
<td></td>
</tr>
<tr>
<td>noFH</td>
<td>25 (15%)</td>
<td>6 (14%)</td>
<td>5 (7%)</td>
<td>ns</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FH</td>
<td>24 (14%)</td>
<td>15 (34%)</td>
<td>6 (9%)</td>
<td>0.004</td>
<td>ns</td>
<td>0.001</td>
</tr>
<tr>
<td>LFL</td>
<td>45 (26%)</td>
<td>13 (30%)</td>
<td>24 (34%)</td>
<td>ns</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>LFS</td>
<td>70 (41%)</td>
<td>5 (11%)</td>
<td>33 (47%)</td>
<td>0.0002</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>171 (100%)</td>
<td>44 (100%)</td>
<td>70 (100%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Families with individuals with multiple tumors</td>
<td>107 (63%)</td>
<td>17 (39%)</td>
<td>40 (57%)</td>
<td>0.006</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>

NOTE: For each allele, transactivation was determined quantitatively (luciferase-based) in 4 different reporter strains. Alleles were classified as SD or PD alleles if their mean residual activity was < 25% or \( \leq 25\% \) of that of the wild-type allele (100% activity), respectively. A separate group of 52 alleles were classified as O-SD alleles in terms of transactivation, by virtue of nonsense mutations, frameshifts, or other mutations that give rise to a truncated protein. The \( P \) values are for testing whether the proportion of families exhibiting a given clinical characteristic differed according to the type of allele carried by the family. Abbreviation: ns, not significant.

**Analysis of DN effects of 103 TP53 germline alleles**

Next, we explored whether DN effects of mutant TP53 alleles can be associated with clinical features of germline carriers who developed cancers. The net transactivation activities for each of the 103 mutant alleles when coexpressed with wild-type p53 were measured in 4 yeast strains. Alleles were classified as DN90 if the transactivation activity of a single wild-type allele; otherwise alleles were classified as recessive (rec) (see Materials and Methods).

Nearly two thirds of the alleles (60/103, 58%; Supplementary Table 3) were classified as DN90, and some significant correlations with clinical features emerged. FH families were overrepresented among families carrying recessive alleles versus those carrying DN90 alleles (30% vs. 13%; \( P = 0.006 \); Table 2). Overall, the proportion of

![Figure 1. p53 functionality versus age at diagnosis of earliest diagnosed tumor. The percentage of tumor-free individuals is plotted as a function of age up to 70 years (Kaplan–Meier method). The analyses were restricted to confirmed germline carriers whose age at diagnosis was reported in the database. For patients with multiple tumors, only the first tumor was considered, as secondary malignancies might be influenced by the nature of the tissue, history, and therapeutic interventions associated with the first tumor. Based on the within-cluster resampling method (Materials and Methods), age at diagnosis did not differ significantly among p53 functional classes.](mcr.aacrjournals.org)
families whose members presented multiple tumors was significantly higher for families carrying DN90 alleles than for those carrying recessive alleles (63% vs. 44%; \( P = 0.015 \)), whereas no significant difference between families carrying DN90 alleles and those carrying O-SD alleles was observed. The distribution of clinical classes also showed some differences between families who carried O-SD alleles and those who carried recessive alleles. The significantly higher proportion of LFS families (\( P = 0.012 \)) and the lower proportion of families with a nonsyndromic condition (FH; \( P = 0.002 \)) among those carrying O-SD compared with recessive alleles were somewhat unexpected, given that O-SD proteins generally cannot form tetramers because they lack the carboxy terminal domain and are thus expected to behave as recessive. However, it is important to note that alleles classified as recessive may be either SD or PD alleles whereas the O-SD alleles are by definition considered SD.

The results described previously argue against a fundamental role for DN interactions per se in the clinical manifestations of subjects who inherited a mutated TP53 allele and developed cancers. However, we note that information on p53 loss of heterozygosity (LOH) should be considered to interpret these results. Indeed, LOH can be a confounding factor since DN could influence the clinical features only when both alleles are present, whereas when the wild-type allele is lost DN effect could not occur. Because there is little information on LOH in familial cancers, the influence of this confounding factor could not be evaluated.

**Subclassification of SD families by DN effects of mutant TP53 alleles**

To assess the value of classifying alleles based on DN independently from their transactivation potential, we next considered the possibility that DN might delineate clinically relevant subclasses within SD alleles carriers. Subclassification of the SD alleles into DN90 and recessive did not reveal any significant associations with clinical features (Table 3). Unexpectedly, however, families whose mutant allele was both SD and rec were overrepresented among LFS families and underrepresented among LFL families compared with families whose mutant allele was both SD and DN90. Thus, among the SD alleles, the DN effect was not associated with a more severe familial clinical history.

**DN effects of TP53 mutant alleles and tissue prevalence of familial tumors**

Using the first tumor identified for each subject and adjusting for family membership (28), we compared the tumor site distribution with allele transactivation properties (SD vs. PD; Table 4A) or DN effect (DN90 vs. rec; Table 4B). Similarly, restricting the analysis to SD allele carriers, we compared the tumor site distribution with DN90 alleles versus recessive alleles (i.e., SD and DN90 vs. SD and rec; Table 4C). Carriers of PD alleles had a higher proportion of lung tumors (\( P = 0.045 \)) but a lower proportion of connective or hematopoietic tumors (\( P = 0.0001 \) and 0.026, respectively) than carriers of SD alleles (Table 4A). Carriers of DN90 alleles had a higher proportion of connective and brain tumors (e.g., the estimated proportion for brain tumors was: DN90 = 0.180 vs. recessive = 0.086, \( P = 0.004 \)) but a lower proportion of breast tumors (e.g., the estimated proportion of breast tumors was: DN90 = 0.197 vs. recessive = 0.356, \( P = 0.003 \)) compared with carriers of recessive alleles (Table 4B). Considering only carriers of SD alleles, those whose alleles were also DN90 had a higher proportion of connective and brain tumors (\( P = 0.002 \) and \( P < 0.0001 \), respectively) but a lower proportion of breast tumors (\( P = 0.004 \)) than those whose allele was also recessive (Table 4C). These results suggest that there is a DN effect of TP53 alleles which can influence the appearance of cancers in a tissue-dependent manner.

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**Table 2. Relationship of clinical variables to functional classification of TP53 germline alleles based on DN90**

<table>
<thead>
<tr>
<th>Number of alleles</th>
<th>DN90</th>
<th>Rec</th>
<th>O-SD</th>
<th>DN90 vs. rec, ( P )</th>
<th>DN90 vs. O-SD, ( P )</th>
<th>Rec vs. O-SD, ( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class distribution of families</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td>7 (5%)</td>
<td>5 (8%)</td>
<td>2 (3%)</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>noFH</td>
<td>22 (14%)</td>
<td>9 (14%)</td>
<td>5 (7%)</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>FH</td>
<td>20 (13%)</td>
<td>19 (30%)</td>
<td>6 (9%)</td>
<td>0.006</td>
<td>ns</td>
<td>0.002</td>
</tr>
<tr>
<td>LFL</td>
<td>44 (29%)</td>
<td>14 (22%)</td>
<td>24 (34%)</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>LFS</td>
<td>59 (39%)</td>
<td>16 (25%)</td>
<td>33 (47%)</td>
<td>ns</td>
<td>ns</td>
<td>0.012</td>
</tr>
<tr>
<td>Total</td>
<td>152 (100%)</td>
<td>63 (100%)</td>
<td>70 (100%)</td>
<td>0.015</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>

Families with individuals exhibiting multiple tumors

NOTE: For each allele, the DN effect was determined quantitatively (luciferase-based) in 4 different reporter strains. Alleles were classified as DN or rec if they reduced (or not) the transactivation activity of the wild-type allele to less than 90% (DN90), respectively, in at least 1 of the 4 reporter strains. The \( P \) values are for testing whether the proportion of families exhibiting a given clinical characteristic differed according to the type of allele carried by the family. Abbreviation: ns, not significant.
DN effects of mutant TP53 alleles and age at diagnosis of first tumor

We also addressed the age of onset of the first tumor in the confirmed carriers of DN90 alleles, using age at diagnosis as a maximum estimate of age at onset. We considered either all first tumors or only first tumors within a specific tissue type. A tendency was observed for a lower age at diagnosis for confirmed carriers of DN90 versus recessive alleles; however, when the within-family correlations were taken into account (28), the difference was not statistically significant (Fig. 2). The curves for confirmed carriers of O-SD and DN90 alleles appeared superimposable. When we considered different tumor types separately, we saw no differences in age at diagnosis for confirmed carriers of DN90 versus recessive alleles (data not shown).

Discussion

Dimerization of p53 seems to occur during the synthesis of the polypeptide chains, whereas tetramerization appears to occur posttranslationally (29). Thus, coexpression of wild-type and mutant p53 is expected to result in only a single category of heterotetrameric species of p53 (i.e., wild-type dimer/mutant dimer), representing approximately 50% of the total pool of p53 proteins. Recently, Natan and colleagues (30) demonstrated that purified wild-type and 273His mutant p53 molecules slowly form wild-type2:wild-type2 (i.e., a tetramer formed by 2 dimers, each formed by 2 wild-type monomers), wild-type2:mutant2 (i.e., a tetramer formed by 2 dimers, 1 dimer with 2 wild-type monomers and the other with 2 mutant monomers), and mutant2:mutant2 (i.e., a tetramer formed by 2 mutant dimers) complexes in the ratio 1:2:1. They further observed that the addition of DNA sequences corresponding to p53 REs dramatically accelerated the formation of the complexes wild-type2:wild-type2:DNA, wild-type2:mutant2:DNA, and mutant2:mutant2:DNA, with relative dissociation constants 1:4:71 and 1:13:85, respectively, for the 3 complex types and 2 REs (P21 and BAX).

Interestingly, according to the relative dissociation constants, these results suggest that the formation of heterotetramers causes a drastic decrease in binding affinity. It is interesting to note that it was previously observed that DN effect was dependent on the sequence of the p53 REs (9, 10).

The mere reduction in functional p53 levels may be sufficient to promote tumorigenesis based on TP53 gene dosage effect seen in mice. Heterozygous p53+/− mice containing a single wild-type TP53 allele develop tumors much earlier than those mice with 2 functional TP53 alleles (31, 32). However, the coexistence of wild-type and mutant p53 can have a stronger impact compared with the reduction in gene dosage and lead to specific phenotypes at the cellular level such as enhanced invasion and migration of the tumor cells (33). Recently, using a model for human mammary epithelial tumorigenesis, Junk and colleagues (34) demonstrated that different mutant/wild-type p53 heterozygous combinations exhibited loss of function, DN effects, and a spectrum of gain of function activities that induced varying degrees of invasive potential.

The development and clinical manifestations of some somatic tumors appear to be influenced by TP53 alleles that have DN effects. DN alleles have been correlated with increased risk of accelerated cancer development (e.g., glioblastomas; ref. 11) and earlier recurrence in oral SCC patients (12). These findings suggest that the clinical consequences of DN alleles may be exacerbated in carriers of TP53 germline mutations, resulting in more severe syndromes and possibly earlier cancer onset when compared with recessive alleles.

Previously, we used the p53 mutant classification proposed in the http://www.umd.be:2072/index.html database that was developed from an early version of transactivation results by Kato and colleagues (26) to interrogate the R10 IARC germline TP53 database (4). For the present study, we determined the transactivation potential of 104 TP53 mutant alleles to drive expression of a luciferase reporter under control of 4 REs in yeast-based assays. Our transactivation data were used to interrogate the R11 germline TP53 database without any restriction on tumor type. Using transactivation capacity and a similar cut-off of residual function to classify alleles as either PD or SD, we confirmed the previously observed correlation between PD alleles and families with a milder family history and lower frequency of multiple tumors (4). The clinical correlation results were comparable when the
Table 4. Tumor site distribution compared between alleles

A) Between SD and PD alleles

<table>
<thead>
<tr>
<th>Tumor site</th>
<th>Estimated proportion (95% CI limits)</th>
<th>Estimated proportion (95% CI limits)</th>
<th>Estimated proportion difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SD</td>
<td>PD</td>
<td>SD–PD difference</td>
<td></td>
</tr>
<tr>
<td>Adrenal</td>
<td>0.033 (0.010–0.056)</td>
<td>0.073 (0.009–0.137)</td>
<td>−0.040 0.029 0.18</td>
</tr>
<tr>
<td>Bone</td>
<td>0.136 (0.088–0.185)</td>
<td>0.061 (0.000–0.130)</td>
<td>0.075 0.041 0.066</td>
</tr>
<tr>
<td>Brain</td>
<td>0.158 (0.107–0.209)</td>
<td>0.141 (0.046–0.235)</td>
<td>0.017 0.05 0.75</td>
</tr>
<tr>
<td>Breast</td>
<td>0.234 (0.175–0.293)</td>
<td>0.278 (0.153–0.403)</td>
<td>−0.044 0.065 0.50</td>
</tr>
<tr>
<td>Connective</td>
<td>0.142 (0.092–0.192)</td>
<td>0.020 (0.000–0.044)</td>
<td>0.122 0.025 &lt;0.0001</td>
</tr>
<tr>
<td>Hematopoietic</td>
<td>0.045 (0.016–0.074)</td>
<td>0.010 (0.000–0.029)</td>
<td>0.035 0.016 0.026</td>
</tr>
<tr>
<td>Lung</td>
<td>0.024 (0.005–0.043)</td>
<td>0.119 (0.023–0.215)</td>
<td>−0.095 0.047 0.045</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.047 (0.017–0.076)</td>
<td>0.061 (0.000–0.131)</td>
<td>−0.014 0.036 0.70</td>
</tr>
</tbody>
</table>

B) Between DN90 and rec alleles

<table>
<thead>
<tr>
<th>Tumor site</th>
<th>Estimated proportion (95% CI limits)</th>
<th>Estimated proportion (95% CI limits)</th>
<th>Estimated proportion difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DN90</td>
<td>rec</td>
<td>DN90–rec difference</td>
<td></td>
</tr>
<tr>
<td>Adrenal</td>
<td>0.030 (0.008–0.053)</td>
<td>0.065 (0.014–0.115)</td>
<td>−0.034 0.023 0.14</td>
</tr>
<tr>
<td>Bone</td>
<td>0.128 (0.079–0.177)</td>
<td>0.118 (0.047–0.188)</td>
<td>0.010 0.039 0.80</td>
</tr>
<tr>
<td>Brain</td>
<td>0.180 (0.123–0.237)</td>
<td>0.086 (0.034–0.138)</td>
<td>0.094 0.033 0.004</td>
</tr>
<tr>
<td>Breast</td>
<td>0.197 (0.139–0.254)</td>
<td>0.365 (0.258–0.473)</td>
<td>−0.169 0.057 0.003</td>
</tr>
<tr>
<td>Connective</td>
<td>0.151 (0.098–0.205)</td>
<td>0.044 (0.009–0.078)</td>
<td>0.108 0.028 0.0001</td>
</tr>
<tr>
<td>Hematopoietic</td>
<td>0.044 (0.014–0.075)</td>
<td>0.027 (0.000–0.060)</td>
<td>0.017 0.021 0.41</td>
</tr>
<tr>
<td>Lung</td>
<td>0.034 (0.010–0.058)</td>
<td>0.051 (0.010–0.092)</td>
<td>−0.017 0.017 0.31</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.051 (0.019–0.084)</td>
<td>0.041 (0.000–0.084)</td>
<td>0.010 0.024 0.68</td>
</tr>
</tbody>
</table>

C) Between SD and DN90 and SD and rec alleles

<table>
<thead>
<tr>
<th>Tumor site</th>
<th>Estimated proportion (95% CI limits)</th>
<th>Estimated proportion (95% CI limits)</th>
<th>Estimated proportion difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SD &amp; DN90</td>
<td>SD &amp; rec</td>
<td>SD &amp; DN90–SD &amp; rec difference</td>
<td></td>
</tr>
<tr>
<td>Adrenal</td>
<td>0.029 (0.005–0.053)</td>
<td>0.061 (0.006–0.116)</td>
<td>−0.033 0.023 0.15</td>
</tr>
<tr>
<td>Bone</td>
<td>0.131 (0.077–0.185)</td>
<td>0.112 (0.031–0.192)</td>
<td>0.020 0.043 0.65</td>
</tr>
<tr>
<td>Brain</td>
<td>0.188 (0.124–0.251)</td>
<td>0.060 (0.018–0.103)</td>
<td>0.128 0.032 &lt;0.0001</td>
</tr>
<tr>
<td>Breast</td>
<td>0.195 (0.131–0.258)</td>
<td>0.400 (0.263–0.538)</td>
<td>−0.205 0.072 0.004</td>
</tr>
<tr>
<td>Connective</td>
<td>0.148 (0.090–0.206)</td>
<td>0.046 (0.001–0.091)</td>
<td>0.102 0.032 0.002</td>
</tr>
<tr>
<td>Hematopoietic</td>
<td>0.026 (0.000–0.052)</td>
<td>0.036 (0.000–0.094)</td>
<td>−0.010 0.031 0.75</td>
</tr>
<tr>
<td>Lung</td>
<td>0.015 (0.000–0.03)</td>
<td>0.024 (0.000–0.051)</td>
<td>−0.010 0.007 0.15</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.034 (0.004–0.063)</td>
<td>0.008 (0.000–0.019)</td>
<td>0.025 0.013 0.049</td>
</tr>
</tbody>
</table>
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Figure 2. p53 functionality including DN versus age at diagnosis for the overall earliest diagnosed tumor. The percentage of tumor-free individuals is plotted as a function of age up to 70 years (Kaplan-Meier method). Analyses were restricted to confirmed germline carriers whose age at diagnosis was reported in the database. For patients with multiple tumors, only the first tumor was considered, as secondary malignancies might be influenced by the nature of the tissue, history, and therapeutic interventions associated with the first tumor. Based on the within-cluster resampling method (Materials and Methods), age at diagnosis did not differ significantly among p53 functional classes.

Functional classification of TP53 alleles based on the transactivation results deposited at p53 databases (http://www-p53.iarc.fr/Germline.html; http://p53.free.fr; ref. 26) was used (Supplementary Table S2) confirming the good agreement between our transactivation results and those previously obtained (26).

Here we explore for the first time whether DN classification of TP53 carrier mutations might uniquely categorize genotype/phenotype associations. The majority of mutant alleles analyzed (~58%) were classified as DN90 and families carrying these alleles were less frequently classified as FH (P < 0.006) and more frequently contained individuals having multiple tumors (P < 0.015; Table 2). However, among SD alleles, the DN classification did not further discriminate the clinical history of families (Table 3). This result has to be taken with caution, as among the 171 SD families only 26 had an allele that was also recessive. Thus, there were few families with SD & recessive alleles in any clinical class, and this sparseness of data limited our ability to detect any hypothesized clinical differences between SD & rec and SD & DN90 families.

It is interesting that carriers of functionally different alleles (PD vs. SD, Table 4A; DN90 vs. rec, Table 4B; SD and DN90 vs. SD and rec, Table 4C) had significantly different proportions of cancers originating at different tissues which is in agreement with previous transactivation assessments (4). The tissue specificity of DN alleles is consistent with previous reports. In a knock-in mouse model, the Arg270His allele expressed in the skin showed no DN effect on spontaneous tumorigenesis (35) whereas it showed a DN effect when expressed in the mammary gland (36), supporting our observation that DN effects of mutant p53 may be highly tissue specific. The reasons for such tissue specificity are presently unknown.

The 2 functional properties of TP53 mutant alleles, transactivation and DN effects, are indeed associated with similar clinical features in that alleles with less residual functionality, are more commonly found in association with more severe syndromes. This observation has led us to consider whether reduced transactivation or DN is the more important factor. Unfortunately, these functional properties are intertwined (i.e., many alleles share both of these characteristics). An indirect hint to answer this question may derive from the behavior of germline carriers of the group of O-SD alleles. The clinical features of the O-SD allele carriers are neither distinguishable from those of the SD (Table 1, Fig. 1) nor from the DN90 allele carriers (Table 2, Fig. 2). Because in terms of transactivation O-SD alleles are per definition SD, the lack of difference in the first comparison (O-SD vs. SD) is understandable. On the contrary, as O-SD are classified as obligatory recessive in term of DN effects (unable to tetramerize), the lack of difference in the second comparison (O-SD vs. DN90) is unclear.

Other approaches to categorizing TP53 mutation carriers into "risk groups" have been described. For example, Shlien and colleagues (37) reported that while mutation carriers have higher frequencies of copy number variations (CNV) than people with wild-type TP53, the carriers with the highest CNV frequencies were more likely to have family histories of cancer. Thus, CNV frequency could also prove useful in assigning TP53 mutation carriers into risk groups, thereby providing additional criteria to be used in screening and genetic counselling.

In conclusion, we have confirmed that the classification of TP53 alleles based on transactivation function can separate familial cancer cases with more severe clinical features. The DN effects were also predictive in general but had limited value in subclassifying clinical features of SD alleles in germline carriers. Based on these results, we conclude that the transactivation features of mutant proteins in germline carriers are a more important overall predictor of genotype/phenotype correlations than DN. However, DN effects may reveal specific tissues where even more subtle variations in functional p53 levels can impact cancer proneness in germline carriers.

Disclosure of Potential Conflict of interests

No potential conflicts of interest were disclosed.

Acknowledgments

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References


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