DATABASES

Reassessment of the *TP53* Mutation Database in Human Disease by Data Mining With a Library of *TP53* Missense Mutations

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TP53 alteration is the most frequent genetic alteration found in human cancers. To date, more than 15,000 tumors with TP53 mutations have been published, leading to the description of more than 1,500 different TP53 mutants (http://p53.curie.fr). The frequency of these mutants is highly heterogeneous, with 11 hotspot mutants found more than 100 times, whereas 306 mutants have been reported only once. So far, little is known concerning the biological significance of these rare mutants, as the majority of biological studies have focused on classic hotspot mutants. In order to gain a deeper knowledge about the significance of all of these mutants, we have cross-checked each mutant of the TP53 mutation database for its activity, derived from a library of 2,314 TP53 mutants representing all possible amino acid substitutions caused by a point mutation. The transactivation activity of all of these mutant was analyzed with respect to eight transcription promoters [Kato S, et al., *Proc Natl Acad Sci USA* (2003)100:8424–8429]. Although the most frequent TP53 mutants sustain a clear loss of transactivation activity, more than 50% of the rare TP53 mutants display significant activity. Analysis in specific types of cancer or in normal skin patches demonstrates a similar distribution of TP53 loss of activity, with the exception of melanoma, in which the majority of TP53 mutants display significant activity. Our data indicate that TP53 mutants represent a highly heterogeneous population with a large diversity in terms of loss of transactivation activity that could account for the heterogeneous tumor phenotypes and the difficulty of clinical studies. Hum Mutat 25:6–17, 2005.

KEY WORDS: TP53; cancer; functional analysis; Li-Fraumeni; transactivation; database

DATABASES:
TP53 – OMIM: 191170, 151623 (LFS); GDB: 120445; GenBank: X54156, NM_000546.2
http://p53.curie.fr (The p53 database, Institut Curie, France)
www.lf2.cuni.cz/projects/germline_mut_TP53.htm (p53 database with germline mutations)

INTRODUCTION

TP53 mutations (*TP53*; MIM# 191170) are found in 50% of human cancers [Soussi and Béroud, 2001]. Apart from the fact that tumor cells must select for inactivation of the *TP53* network that safeguards the cell from various types of insults, these mutations have been the subject of extensive studies, providing a better understanding of their origin [Greenblatt et al., 1994; Soussi, 1996]. *TP53* protein is a transcription factor with a very loose recognition sequence, which can be found in several hundred genes that are differentially activated depending on the cell type, identity, and extent of damage, as well as on various other parameters that have yet to be identified [Oren, 2003; Vogelstein et al., 2000; Vousden and Lu, 2002]. The unique feature of *TP53* compared to other tumor suppressor genes is its mode of inactivation. While most tumor suppressor genes are inactivated by mutations leading to absence of protein synthesis (or production of a truncated product), more than 80% of *TP53* inactivations are missense mutations that lead to the synthesis of a stable full-length protein [Soussi and Béroud, 2001]. This selection to maintain mutant *TP53* in tumor cells is believed to be required for both a dominant negative activity of wild-type (wt) *TP53* expressed by the remaining allele, but also for a gain of function that transforms mutant *TP53* into a dominant oncogene [Dittmer et al., 1993; Lane and Benchimol, 1990; Soussi and Béroud, 2003]. An important feature of the *TP53* protein is the extreme flexibility and fragility of the DNA binding domain (residues 90 to 300) [Milner, 1995], as more than 200 of the 393 residues have been found to be modified and several residues have sustained multiple alterations. Most *TP53* mutations are localized in the DNA binding domain of the protein (residues 100 to 300),

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leading to a bias of *TP53* mutation analysis, as more than 80% of *TP53* mutation studies focus on exons 5 to 8 (residues 126 to 306) [Soussi and Béroud, 2001]. One of the most puzzling aspects of mutant *TP53* proteins is their structural, biochemical, and biological heterogeneity. The structural difference between the various mutant *TP53* proteins was initially identified using monoclonal antibodies able to discriminate mutations that change *TP53* folding and mutations in the residue involved in DNA recognition [Gannon et al., 1990; Legros et al., 1994]. Recent analyses using more sophisticated biophysical techniques have revealed that the central region of the *TP53* protein can adopt at least five thermodynamic states [Bullock and Fersht, 2001]. Biochemical analyses have shown that *TP53* mutant proteins can be heterogeneous in terms of loss of DNA binding activity and transactivation. The DNA binding site recognized by *TP53* is highly degenerated and the affinity of *TP53* for the various biological sites is variable [El-Deiry et al., 1992]. Some mutant *TP53* proteins display only partial loss of their DNA binding activity, allowing the mutant to bind only to a subset of *TP53* response elements [Friedlander et al., 1996; Rowan et al., 1996]. This feature is linked to a differential transactivation activity. Biologically, these mutants have lost their apoptotic properties, but their growth arrest activity remains close to that of the wt protein. The biological significance of these mutants has not been fully elucidated.

In a recent report, we raised a number of questions concerning mutant *TP53* found in the *TP53* databases [Soussi and Béroud, 2003]. The 15,000 mutations are distributed between 1,400 different mutants. The frequency of these mutants is very variable. The most common mutants are: c.542G>A (pR175H), which is found 688 times; c.743G>A (pR248Q), found 548 times; and c.818G>A (pR273H), found 468 times. At the other end of the spectrum, 585 variants are found only once, 265 variants are found twice, and 156 variants are found three times. It should be noted that only 12 variants are found more than 100 times, 194 variants are found between 11 and 99 times, and 1,240 variants are found less than 10 times. This series of rare variants also corresponds to 29% of all mutations in the database. Common variants are clearly real *TP53* mutants. Biochemical and biological studies have demonstrated that they have impaired DNA binding activity, leading to a protein that is inactive for transactivation [Forrester et al., 1995; Ory et al., 1994]. They have also lost their growth arrest or proapoptotic properties. However, the significance of the infrequent mutants remains unclear.

In a recent study, Kato et al. [2003] described the construction and characterization of more than 2,000 *TP53* mutations. The biological activity of each mutant was evaluated in vitro in a yeast system using eight different transcription promoters. This raises the possibility of performing a cross-analysis between the activity of the various mutants and their occurrence in the database. Our results clearly show that although mutants frequently described in the literature are true mutants that display loss of function, more than 50% of the rare *TP53* mutants have an activity indistinguishable from that of wt *TP53*. These analyses raise a number of concerns about analysis of *TP53* mutations and indicate the need for caution in global analysis of *TP53* mutations from the database. Our analysis leads to the proposal of several recommendations for rigorous analysis of *TP53* status in human tumors.

ANALYSIS OF THE DATABASE

For this analysis, we used the latest version of the *TP53* database maintained at the Institut Curie (http://p53.curie.fr). The database contains 16,055 entries corresponding to *TP53* mutations found in tumors, normal skin, and noncancerous diseases such as rheumatoid arthritis (RA). It also includes germline mutations found in Li-Fraumeni syndrome (LFS) and LFS-like syndrome. (This version of the database, 2004-R1, will be available for download in July 2004.) For each analysis, only missense mutations were selected. Frameshift mutations, nonsense mutations, and mutations that do not change the amino acid residues were discarded (except for cell lines, see below). Tandem mutations, predominantly found in skin cancer, were maintained, provided they led to amino acid substitution. For the global analysis, we also excluded germline mutations, mutations found in cell lines, and in nonneoplastic tissue such as skin or RA, in order to focus to human tumors only. This led to the selection of 10,449 entries, corresponding to 1,004 mutants that were found with a frequency ranging from once (306 mutants) to 643 times (one mutant, c.542G>A [p.R175H]). About 6% of tumors displayed more than one mutation. These mutations were included in the analysis.

TP53 mutant activity has been described in detail in a previous report [Kato et al., 2003]. Briefly, 2,314 haploid yeast transformants containing *TP53* mutations were constructed. *TP53* mutant activity was tested by mating each transformant with a compatible yeast strain harboring a reporter plasmid controlled by a specific *TP53* response element. Promoter activity was assessed after 3 days of growth at 37°C.

CLUSTERING ANALYSIS OF MUTANT *TP53* FOUND IN HUMAN TUMORS

First, we evaluated whether the transactivation assay used in our analysis is truly representative of *TP53* loss of function. The functional analysis of separated alleles in yeast (FASAY) developed by Ishioka et al. is widely used for the detection of *TP53* mutations in human tumors, and a very strong correlation has been demonstrated between *TP53* mutations detected by sequencing and by FASAY [Flaman et al., 1995; Ishioka et al., 1993; Meinhold-Heerlein et al., 2001]. As the assay used by Kato et al. [2003] is slightly different from FASAY, we analyzed seven large published or unpublished series of *TP53* mutations detected by FASAY. The majority of the 92 different mutants analyzed (corresponding to 130 tumors, as the same mutation was published several times), displayed a significant loss of activity (Supplementary Fig. S1, available online at www.interscience.wiley.com/jpages/1059-7794/suppmat). For the WAF1 promoter, all but one mutant had an activity less than 50% of that of wt *TP53*.

For the database analysis, we selected missense mutations as described above, and removed germline mutations and mutations found in cell lines and in nonneoplastic tissues, such as skin or RA. This led to the selection of 10,449 entries corresponding to 1,004 mutants that were found as somatic events in various types of human cancers. Each mutant was cross-checked with the activity in relation to the eight transcription promoters. Clustering analysis defined three gross classes of mutant *TP53* (Fig. 1). The two larger classes (A and C) correspond to *TP53* mutants with either low (class A, 359 mutants) or high (class C, 464 mutants) activity on the majority of promoters. Class B (182 mutants) corresponds to *TP53* mutants that have low activity toward WAF1, mdm2, or bax, but which retain partial or total activity toward the other promoters. Although the number of *TP53* mutants in class C is high, it must be remembered that this category contains predominantly infrequent mutants (76% of the total database) compared to class B (12.7%) and class A (11.3%).

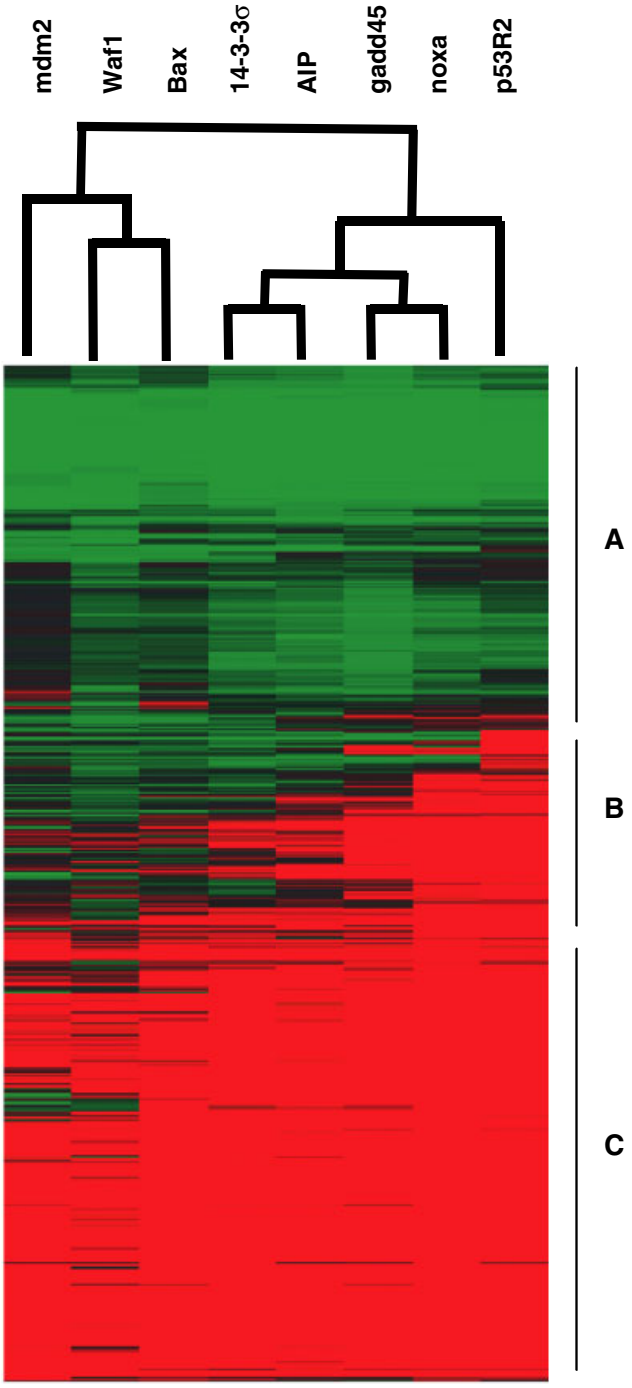

FIGURE 1. **Clustering analysis of 1,004 mutant *TP53*s. Each column represents a transcription promoter and each row represents a mutant *TP53*. Activities are displayed from green (lowest) to red (highest). Visualization was performed with CLUSTER and TREVIEW software.**

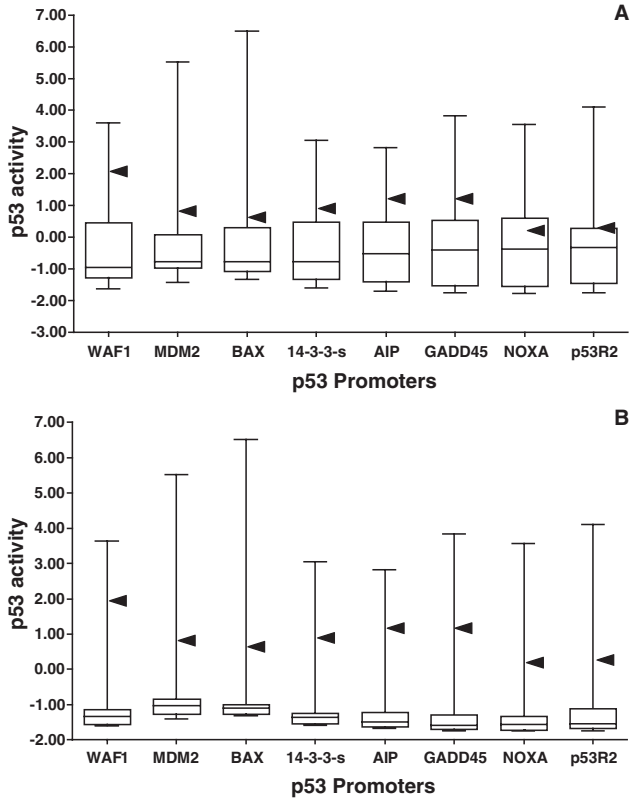

FIGURE 2. **Activity of mutant *TP53* toward each transcription promoter. Boxes and whisker plots show the upper and lower quartiles and range (box), median value (horizontal line inside the box), and full range distribution (whisker line). Differences were analyzed by the Mann-Whitney U test. The black arrow indicates the value of wt p53 activity for each promoter. A: Analysis was performed with the 1,004 mutants. B: Analysis was performed with the 10,449 entries of the database. Due to the high redundancy of inactive hotspot mutants, the range is more homogeneous in B than in A.**

The activity of each *TP53* mutant toward the eight transcription promoters is shown in Figure 2A and B and in Supplementary Figure S2. It is clear that a large number of mutants do not display any loss of transactivation function and several of them display a superior activity to that of wt *TP53* (Fig. 2A and B). The 1,004 mutants have been divided into seven categories according to their frequency (Fig. 3A). The activity of mutants found very frequently in the *TP53* database (categories >99, 50–99, 10–49, and 6–9) is usually low, ranging from 0 to 20%, with only a few mutants with higher activity in the 10–49 and 6–9 categories (Fig. 3A; Supplementary Fig. S3; Supplementary Table S1). For categories 3–5, 2, and 1 (mutants found only once), the scatter is highly heterogeneous, ranging from 0 to 160% compared to wt. A similar pattern is observed for each transcription promoter (Supplementary Fig. S3). There is a clear inverse correlation between the frequency of *TP53* mutants and their activity. Depending on the promoters used, 44 to 60% of mutants found only once have an activity greater than 50% compared to wt *TP53*. Nonparametric statistical analysis using a Mann-Whitney test did not reveal any statistical difference between the three categories >99, 50–99, and 10–49. However, comparison of each of these three categories with each of the low frequency categories (6–9, 3–5, 2, and 1) showed a highly significant difference for each analysis ($P < 0.0001$). Analysis of the various types of cancer revealed similar results (Fig. 3B; data not shown).

The ">99" category includes 11 well-defined hotspot mutants that have been extensively analyzed biologically and biochemically and demonstrated to be inactive for transactivation, growth arrest, and apoptosis [Forrester et al., 1995; Ory et al., 1994]. In the present study, their activity ranged from 0 to 20% compared to wt *TP53*, suggesting that an arbitrary cutoff of 20% could be used to determine the significance of the negative effect of the change. In

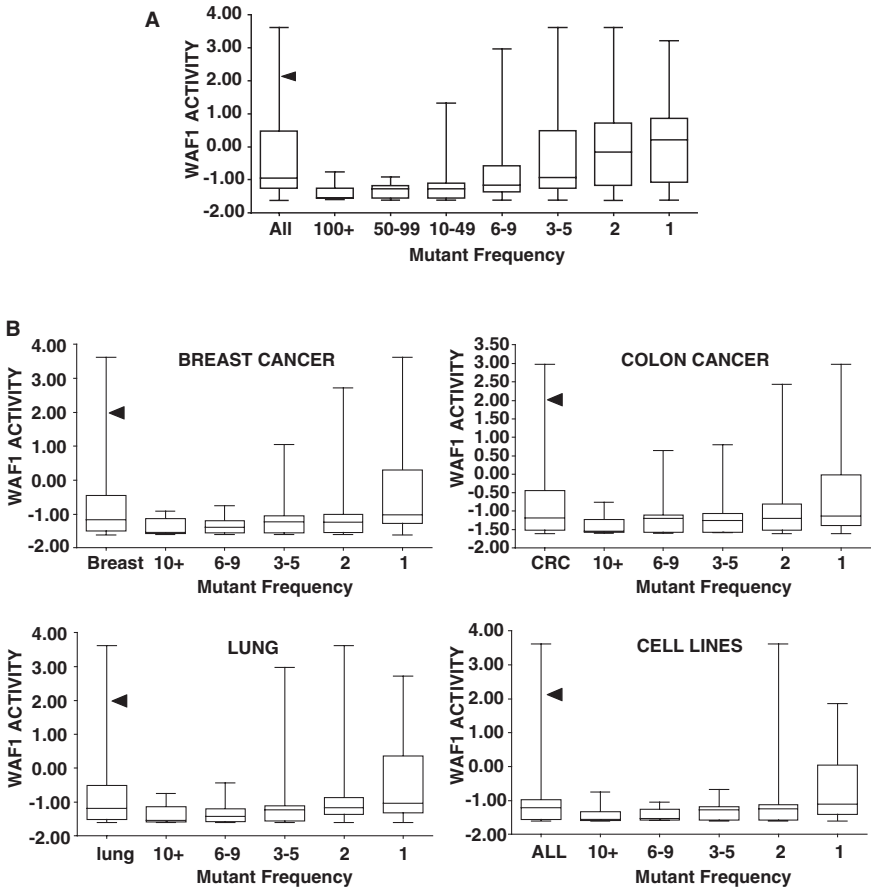

FIGURE 3. **Activity of mutant *TP53*s according to their frequency in various subsets of the database. Mutant *TP53* have been classified into seven categories according to their frequencies. Box and whisker plots are similar to those of Figure 2. A: Analysis was performed for the 1,004 mutants found in tumors. B: Same analysis as in A, except that it was performed for specific cancers: breast (381 mutants); colon (305 mutants); lung (348 mutants), and cell lines (234 mutants). Analysis was performed using the activity of the WAF1 promoter, but similar results were obtained with the other promoters (see Supplementary Fig. S3).**

terms of their activity, 100% of mutants from the >99 and 50–99 categories can be considered to be inactive, with less than 20% of wt *TP53* activity (Fig. 2; Supplementary Fig. S3).

In the 10–49 category, nine mutants (6%) had an activity in the 21 to 50% range, and six mutants (4%) had an activity greater than 50%. These mutants are described in detail in Table 1. Four of these mutants (p.E286K (c.856G>A), p.R175L (c.524G>T), p.R181H (c.542G>T), and p.A138V (c.413C>T)) are known to be temperature-sensitive, with wt activity at 30°C and mutant behavior at 37°C in mammalian cells [Shiraishi et al., 2004 and references therein]. Temperature sensitivity of *TP53* mutants has been extensively described for the murine or human protein, and is due to the highly sensitive folding of the DNA binding region of the protein [Michalovitz et al., 1990; Zhang et al., 1994]. To date, 140 human *TP53* mutants have been described as temperature sensitive, but it is possible that others remain to be discovered [Shiraishi et al., 2004]. These four mutants have an activity ranging from 20 to 40% compared to wt *TP53* in yeast. Extensive studies of p.A138V (c.413C>T) in mammalian cells have shown that this mutant is totally deficient at 37°C. Mutant p.R290H (c.869G>A) has been independently identified 11 times in human tumors, but it has also been described as a germline mutation in a LFS family with an unusual compound heterozygosity [Quesnel et al., 1999]. The p.R290H (c.869G>A) mutation was found on the paternal allele, whereas a double mutation, p.R156H (c.467G>A);p.R267Q (c.800G>A) was found on the maternal allele. Although the maternal family displays an LFS-like syndrome, no information was available for the paternal family. Individual analysis of each mutant indicates that they have only a weak mutant phenotype. On the other hand, the p.R156H (c.467G>A);p.R267Q (c.800G>A) double mutant has a strong mutant behavior, with total loss of transactivation activity and growth suppression [Quesnel et al., 1999]. This observation suggested that some *TP53* mutants may have only a partial defect and that total inactivation would require a second mutation in the same allele in order to fully inactivate the protein. Due to the tetrameric structure of the protein, it is also possible that two weak mutations in two different alleles could lead to a fully inactive protein. A similar situation has been described and studied by Fulci et al. [2002] for a single germline mutation (p.R283H [c.848G>A]) found to be weakly inactive. Analysis of the patients' tumors showed that the mutant allele acquired a second mutation leading to a p.R267W (c.799C>T);p.R283H (c848G>A) double mutant that was fully inactive. Furthermore, analysis of tumor recurrence showed that a single inactivating mutation (p.E258D [c.774A>T]) occurred on the remaining wt allele. Altogether, these observations suggest that there is a marked heterogeneity of mutant *TP53* behavior. Analysis of the latest version of the *TP53* database indicates that about 6% of tumors display more than one mutation. Unfortunately, in the

TABLE 1. Properties of Mutant *TP53**

Mutation	Codon change (p.)	Frequency	WAF	MDM2	BAX	Properties of the mutant	Reference
c.706T>A	Y236N	10	80	84	320	No information available	
c.869G>A	R290H	11	67,37	132	149	Active for growth arrest in saos cells active for transactivation with RGC sequence; weak activation of p21 in saos Associated with other mutations (5x)	Quesnel et al. [1999]
c.704A>G	N235S	14	73	58	128	No information available associated with other mutations (6x)	
c.496T>A	S166T	13	71	89	99	No information available[a]	
c.532C>T	H178Y	13	66	72	40	No information available	
c.469G>A	V157I	11	34	40	90	No information available	
c.523C>T	R175C	17	62	34	37	Active for growth suppression in saos; active for p21/mdm2 transactivation in saos cells; associated with other mutations (5x) differential activity to various promoters in yeast	Flaman et al. [1998]; Ory et al. [1994]
c.748C>T	P250S	11	37	8	78	No information available	Campomenosi et al. [2001]
c.413C>T	A138V	29	32	56	25	First thermosensitive mutant found for human *TP53*	Zhang et al. [1994]
c.524G>T	R175L	12	13	44	41	Active for growth suppression in saos; active for p21 transactivation in saos cells differential activity to various promoters in yeast; temperature sensitive	Flaman et al. [1998]; Ory et al. [1994]; Shiraishi et al. [2004]
c.772G>C	D258Q	11	16	24	37	No information available	
c.542G>A	R181H	21	34	23	19	Differential activity to various promoters in yeast; temperature sensitive	Campomenosi et al. [2001]; Flaman et al. [1998]; Shiraishi et al. [2004]
c.847C>T	R283C	12	25	0	151	No information available	
c.405C>G	C135W	17	12	28	31	Thermosensitive mutant	Shiraishi et al. [2004]
c.541C>T	R181C	14	26	21	18	No information available 181 H and 181G are thermosensitive	
c.715A>G	N239D	25	20	20	21	No information available	
c.856G>A	E286K	44	11	26	24	Thermosensitive mutant	Campomenosi et al. [2001]
c.713G>A	C238Y	44	15	20	26	No information available	
c.840A>T	R280S	21	22	21	18	No information available	

[a]Twelve out of 13 of these mutants originate from a single publication and should be considered with caution.
*The nomenclature for TP53 mutation uses the cDNA as a reference (RefSeqNM_000546.2). Protein reference: RefSeq NP 000537. For numbering, +1 is A of the ATG initiation codon in the correct RefSeq (NM_000546.2).

majority of the cases, it is impossible to determine whether mutations are located on the same allele or on different alleles. It is also likely that this number is underestimated, as the majority of studies have focused on exons 5 to 8, where the majority of potent mutants are localized. In 5 out of 11 tumors, the p.R290H (c.869G>A) mutant is associated with a second mutation, indicating that a second hit may be necessary for full *TP53* inactivation. A second degree of heterogeneity has been described, with the description of mutant *TP53* with differential loss of activity on different transcription promoters. These mutants, such as p.R175P (c.524G>C) and p.R181L (c.542G>T), are defective for transactivation of bax genes and display a defective apoptosis, whereas transactivation of p21 and growth arrest activity appear to be normal [Friedlander et al., 1996; Rowan et al., 1996]. The p.R181H (c.542G>A), p.R175C (c.523C>T), and p.R175P (c.524G>C) mutants shown in Table 1 have already been described to present a different behavior with different transcription promoters [Campomenosi et al., 2001]. Altogether, these observations suggest that the majority of mutant *TP53* proteins found more than 10 times in the *TP53* database sustain a significant loss of activity.

Analysis of mutants that are found only once or twice reveals another pattern. More than 50% of these mutants display an activity greater than 50% of wt *TP53* activity and less than 10% of them have an activity corresponding to 10%. Several explanations, which are not mutually exclusive, can be proposed for the occurrence of these mutations with wt activity. The most prosaic explanation is a laboratory artifact ranging from PCR or sequencing errors to typing errors or errors of codon assignment. In a recent analysis of the 1,500 articles describing *TP53* mutations, we observed typing errors in the identity of the wt codon in about 5% of publications, with a notable increase in recent years, with several publications presenting multiple errors. Although these errors are easily detected, as the sequence of wt *TP53* is well known, it is impossible to detect such errors in the description of mutations, but we can assume that they occur with a similar frequency. We estimate that at least 2 to 5% of *TP53* mutations in the database are incorrect, but there is no way of identifying them.

The pattern of mutational events that inactivates the *TP53* gene is specific from one cancer to another [Soussi, 1996]. In colon cancer, there is a high rate of transition at CpG dinucleotides, whereas in lung cancer, GC>TA transversion is the leading mutational event. These differences are due to the heterogeneity of the mutagenic process that inactivates *TP53* in these two cancers. This feature has been largely described and discussed over the last 10 years [Greenblatt et al., 1994; Soussi, 1996]. Analysis of the mutational events of the unique mutant

reveals two important findings: 1) they are identical from one cancer to another, and 2) there is no specific mutational event compared to the analysis performed for frequent variants in the same cancer (data not shown, see Soussi and Béroud [2003]). Once again, these data suggest that the majority of rare mutants are probably laboratory artifacts, although it is impossible to exclude the possibility that some of them are true *TP53* inactivating mutations and their rarity could be due either to the difficulty of the mutational event to occur in vivo or the low penetrance of the *TP53* mutation that could need a specific genetic background to be effective. Both hypotheses could also be simultaneously true. The genetic background of the cell must also be taken into account, because the severity of *TP53* mutations may be different according to the presence of other alterations in various pathways. The presence of the wt allele could also be an important feature in the penetrance of these mutations.

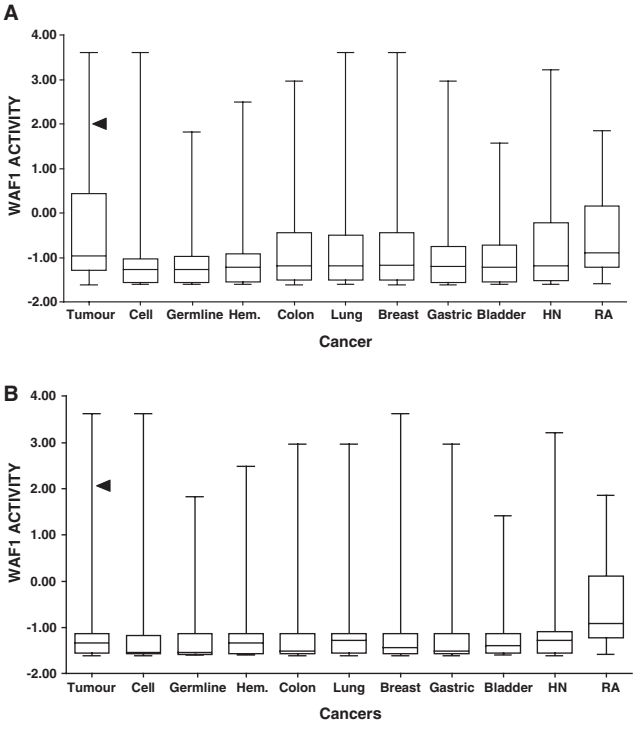

FIGURE 4. **Activity of mutant *TP53* in various types of cancer or rheumatoid arthritis. Box and whisker plot are similar to those of Figure 2. A: Analysis was performed with p53 mutants. B: Analysis was performed with the total number of patients for each cancer. Due to the high redundancy of inactive hotspot mutants, the range is more homogenous in B than in A. Analysis was performed using the activity of the WAF1 promoter, but similar results were obtained with the other promoters. Cell, cell lines; Hem, hematologic malignancies; HN, head and neck squamous cell carcinoma; RA, rheumatoid arthritis.**

TP53 MUTANTS IN LI-FRAUMENI SYNDROME (LFS)

Germline mutations of *TP53* are associated with LFS, a familial disorder with a high predisposition to various types of cancer [Varley, 2003]. The description of germline mutations is less prone to laboratory errors. The mutation is always present in 50% of the cells, a feature that leads to unambiguous results with the various sequencing technologies. The same mutation is often found in various members of the family. Furthermore, due to the ethical implications of finding germline *TP53* mutations, these investigations are usually performed according to a well-defined protocol in a genetic laboratory. The distribution of p53 mutant activity in LFS is one of the most homogeneous, with a low variability and the lowest median value (Fig. 4A and B; Table 2). A total of 78% of *TP53* mutants found in LFS have an activity less than 20% and only 10% of these mutants have an activity greater than 50% (Table 2), and most of them are also infrequently found in sporadic tumors, except for the p.R290H (c.869G>A) mutation discussed above. Altogether, these observations indicate that *TP53* mutation analysis, when it is performed rigorously, allows the detection of a higher rate of true inactive mutant *TP53*.

TP53 MUTANTS IN CELL LINES

The *TP53* mutation status in cell lines is important, as cell lines are used in all laboratories as biological "test tubes" for many experiments. As in LFS, the detection of mutant *TP53* in cell lines should be easier than in tumors. The distribution of p53 mutant activity is also very homogenous, with a low activity for the majority of p53 mutant (Fig. 4A and B; Table 2). The higher full range of activity is due to the coselected mutants included in the analysis (see below). A total of 71% of *TP53* mutants found in cell lines have an activity less than 20%, one of the highest frequencies (Table 2). In order to obtain a more accurate picture of the *TP53* mutation status of cell lines, the 727 different cell lines included in our database were analyzed individually. A total of 668 of these cell lines had a single mutation with an activity less than 20% and can be considered to be mutants. Several of these cell lines have been fully characterized biologically for *TP53* pathways that were shown to be inactive. Among the remaining 59 cell lines, 19 presented more than one mutation. It is very interesting to observe that in all but 1 of these 19 cell lines (95%), one of the mutants always presented an activity less than 20%, whereas the other mutant(s) had an activity greater than 20%, and, in many cases, presented an activity similar to that of wt *TP53* (Table 3). This observation supports the idea suggested above that weak mutants can often be associated with strong mutants. We would also like to mention

TABLE 2. **Frequency of *TP53* Mutants Among the Four Categories of Activity**

Frequency/activity	<10%	11–20%	21–50%	>50%
Colon cancer	32.1	33.8	13.1	21.0
Breast cancer	30.1	34.6	12.6	22.8
Lung cancer	30.5	34.8	14.9	19.8
Skin cancer	31.4	23.3	15.1	30.2
Melanoma	23	17	10	50
Cell lines	35.2	36.5	11.6	16.7
Germline	44.9	33.3	11.5	10.3
Normal skin	45.5	18.2	15.2	21.2
Rheumatoid arthritis	16.1	26.8	19.6	37.5

TABLE 3. *TP53* Mutations and Activity in Cell Lines With Multiple Mutations*

Name	Mutation	Codon change (p.)	Frequency in DB	Cancer	CLIa	WAF1
A2	c.718A>T	S240C	3X	T-cell leukemia	52.3	13.7
A2	c.715_717del3	Frameshift[b]		T-cell leukemia	0	0
JURKAT	c.778T>G	S260A	4X	T-cell acute lymphoblastic leukemia	97.7	66.3
JURKAT	c.766A>G	T256A	6X	T-cell acute lymphoblastic leukemia	34.3	10.8
JURKAT	c.776A>G	D259G	3X	T-cell acute lymphoblastic leukemia	18.6	17.3
JURKAT	c.586C>T	R196X[b]	123X	T-cell acute lymphoblastic leukemia	0	0
MOLT16	c.710T>G	M237R	4X	T-cell acute lymphoblastic leukemia	25.0	5.7
MOLT16	c.730G>T	G244C	12X	T-cell acute lymphoblastic leukemia	0.0	0.0
SCMC-L9	c.1072G>A	E358K	1X	B-acute lymphoblastic leukemia	74.7	54.4
SCMC-L9	c.523C>T	R175C	18X	B-acute lymphoblastic leukemia	44.2	61.6
SCMC-L9	c.743G>A	R248Q	552X	B-acute lymphoblastic leuemia	0.4	0.0
SNU-C5[c]	c.652G>C	V218L	1X	Colorectal carcinoma	62.7	54.8
SNU-C5	c.742C>T	R248W	467	Colorectal carcinoma	0.0	0.0
SW480	c.925C>T	P309S	4X	Colorectal carcinoma	109.2	43.6
SW480	c.818G>A	R273H	468X	Colorectal carcinoma	0.0	1.0
MDA 1686	c.653T>G	V218G	7X	Head and neck SCC	98.9	61.5
MDA 1686	c.768delA	Frameshift		Head and neck SCC	0	0
OSC-6	c.283T>C	S95P	1X	Head and neck SCC	44.8	63.5
OSC-6	c.378C>G	Y126X		Head and neck SCC	0	0
OSC-9	c.925C>A	309T	1X	Head and neck SCC	65.0	60.3
OSC-9	c.818G>A	R273H	468X	Head and neck SCC	0.0	1.0
DU145	c.668C>T	P223L	4X	Prostate carcinoma	24.5	8.5
DU145	c.820G>T	V274F		Prostate carcinoma	0.0	0.8
GT9	c.748C>G	P250A		Glioblastoma	65.2	46.6
GT9	c.747G>T	R249S		Glioblastoma	18.5	12.4
H676	c.649G>T	V217L		Lung (NSCLC)	57.8	50.2
H676	c.743G>T	R248L	70X	Lung (NSCLC)	0	0
J82	c.960G>C	K320N	3X	Bladder carcinoma	25.8	30.3
J82	c.811G>A	E271K		Bladder carcinoma	18.6	8.5
J82	c.820G>T	V274F		Bladder carcinoma	0.0	0.8
KYSE 1170	c.763A>G	I255V		Esophageal SCC	132.3	80.3
KYSE 1170	c.578A>T	H193L		Esophageal SCC	13.3	11.0
MKN74	c.751A>C	I251L		Gastric carcinoma	0.5	0.0
MKN74	c.812A>C	E271A		Gastric carcinoma	40.2	17.0
NUC-1	c.72A>T	K24N	1X	Choriocarcinoma	87.4	75.0
NUC-1	c.51A>T	E17D	1X	Choriocarcinoma	57.7	49.6
SNU-449	c.416A>G	K139R		Hepatocellular carcinoma	74.1	48.9
SNU-449	c.481G>A	A161T		Hepatocellular carcinoma	17.5	13.3
SNU475	c.823T>C	C275R		Hepatocellular carcinoma	2.54	0.37
SNU-475	c.863A>G	N288S	4X	Hepatocellular carcinoma	113.5	63.8
SNU-475	c.715A>G	N239D		Hepatocellular carcinoma	20.2	20.4
UPT-29	c.486C>G	I162M		Unknown primary tumor	36.0	31.5
UPT-29	c.548C>G	S183X		Unknown primary tumor	0	0

[a]Mean of waf1, mdm2, and bax promoters.
[b]We assume that frameshift and nonsense mutations are inactive.
[c]Previous analysis have shown that the two mutations are located in different alleles. The pV218L mutant displays a wt transactivation activity. The *TP53* pathway of this cell line is fully inactive indicating that the pR248W mutant displays a strong dominant negative behavior [Rand et al., 1996].
*For numbering, +1 is A of the ATG initiation codon in the correct RefSeq (NM_00546.2). WAF1 and CII: activity (%) toward the waf1 and cluster CII.

that, in several cases, as in the HCT-15 colorectal carcinoma cell line, there is a discrepancy concerning the localization of the mutation in the literature. The two mutations, p.P153A (c.457C>G) (active *TP53*) or p.C241F (c.722C>T) (inactive *TP53*), have each been published once, so it is unclear which is the true mutation. In other cases of discrepancy, one of the genotypes has been described more often than the other, suggesting that the most frequent phenotype could be the true phenotype. Altogether, these observations indicate that *TP53* is not functional in the majority of these cell lines.

TP53 MUTANTS IN DISTINCT CANCERS (INTERNAL TUMORS)

Lung, breast, and colorectal cancers are among the most frequent cancers in the world and have been the subject of many studies concerning *TP53* alterations. A total of 348, 381, and 305 mutants have been analyzed for 1,169, 1,398 and 1,452 lung, breast, and colorectal cancers, respectively (Table 2; Fig. 4A and B; Supplementary Fig. S4). The lower number of *TP53* mutants for colorectal cancer is due to the fact that a greater proportion of mutants are located in the hotspot codon at the CpG dinucleotide in this cancer compared to other cancers. Although the mutants frequently display a clear loss of activity, rare mutants display a more heterogeneous phenotype in the entire database (Fig. 4A and B). There is a striking similarity of loss of activity among the various categories of mutant *TP53* in these three types of cancer. A total of 65% of mutant *TP53* proteins have less than 20% of activity, whereas about 20% of them display an activity greater than 50%. A similar picture is also observed in other types of cancer (Fig. 4A and B; Supplementary Fig. S4). The highest loss of activity in p53 mutants found in hematologic malignancies can be explained by the quality of the clinical samples, which are less prone to contamination and therefore easier to analyze (Fig. 4A and B). The marked similarity of mutant phenotype in various

types of cancer for which the etiology of *TP53* mutations is clearly different excludes any influence of the origin of mutation in the heterogeneity of the mutant phenotype. The distribution of these p53 mutants with a slight loss of activity is not restricted to a specific region of the p53 protein, as they are scattered all over the molecule (Supplementary Fig. S5, Supplementary Table S1).

TP53 MUTATIONS IN SKIN CANCER

The etiology of *TP53* mutations in skin cancer has been clearly linked to ultraviolet (UV) exposure [Giglia-Mari and Sarasin, 2003]. UV radiation induces tandem mutations that affect two contiguous nucleotides in the same DNA strand. When these two residues belong to the same codon, a single mutant is produced. On the other hand, when the adduct targets two nucleotides contained in two contiguous codons, it leads to a tandem mutation. Depending on the codon sequence, the first residue may either be not modified by the mutation (i.e., the tandem mutation, [c.741C>T; c.742C>T], [p.N247N; p.R248W]) or it may change the two residues (i.e., [c.603G>T; c.604C>T], [p.L201F; p.R202C]). In the latter case, only one of the two changes in amino acid residues would need to be deleterious and the second would be coselected. Therefore, in a primary analysis, all tumors with tandem mutations found in skin cancer were analyzed for their pattern of activity. A total of 88% of tandem mutations that target two codons contain a wt residue associated with a mutant with either less than 20% of activity or a stop codon. Only two tandem mutations (3%) led to the synthesis of a double mutant, with one mutant displaying an activity close to wt activity. This analysis indicates that there is no specific bias due to tandem mutations; the analysis of *TP53* mutants was performed for internal tumors for every skin cancer (Fig. 5). The distribution of p53 mutants in BCC and XP-associated skin cancer was not different from that observed in internal tumors. The higher variability observed for SCC and KA is certainly due to the greater number of tumors with nontandem double mutations, in which a weak mutant is coselected with a stronger mutant. An independent analysis was performed for melanoma and provided unexpected results (Table 3; Fig. 5). A total of 50% of *TP53* mutants found in melanoma have an activity greater than 50% of wt *TP53* activity, and 25% of mutants have an activity equivalent to or greater than that of the wt protein. This observation is not due to the small number of *TP53* mutants, as other cancer types with similar numbers of mutants behave in the same way as the internal tumors described in Table 3. This feature is also specific for melanoma tumors, as it is not found in melanoma cell lines, in which 12 out of 14 mutants display an activity less than 20% compared to wt *TP53* (data not shown). A careful review of the literature concerning *TP53* mutations in melanoma did not reveal any particular bias able to explain this finding. Analysis of the latest publication describing a large series of *TP53* mutations in melanoma suggests that a large number of "neutral mutations" can be found in this type of cancer [Ragnarsson-Olding et al., 2002]. Among the 22 mutations described in this article, seven (35%) were silent and seven (35%) corresponded to mutant *TP53* with an activity close to that of wt *TP53*. The origin of these mutations is unclear, as they are not related to UV exposure.

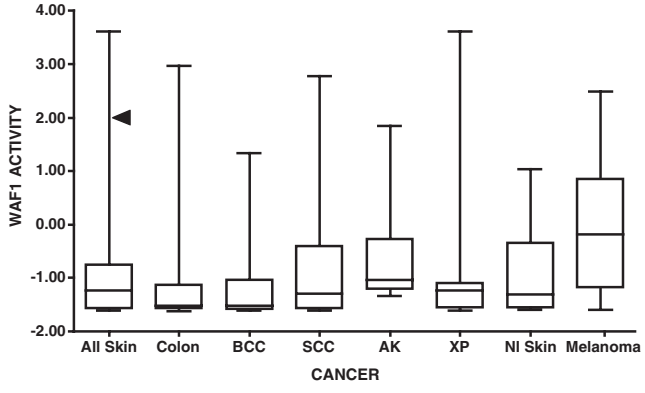

FIGURE 5. **Activity of mutant *TP53* in skin cancer. Box and whisker plot are similar to those of Figure 2. Analysis was performed with p53 mutants. The black arrow indicates the value of wt p53 activity. Analysis was performed using the activity of the WAF1 promoter, but similar results were obtained with the other promoters. BCC, basal cell carcinoma; SCC, squamous cell carcinoma; AK, actinic keratosis; XP, xeroderma pigmentosum–associated skin cancer; Nl Skin, normal skin (p53 patches).**

TP53 MUTANTS IN NORMAL SKIN

TP53 mutation is a very early event in skin tumorigenesis and several studies have shown that *TP53* mutations can be found in area of normal skin in individuals not presenting any cancer [Brash and Ponten, 1998]. Most of these mutations bear the hallmark of UV exposure, i.e., predominantly localized at dipyrimidine sites and with a high frequency of tandem mutations. It has been proposed that when a mutation confers apoptosis resistance, as induced by *TP53* mutations, subsequent UV exposure is more likely to kill normal cells than mutant cells, which can consequently expand to form a clone, and only one cell of this clone needs to undergo another mutation. As these mutations in normal skin are usually found only in a small number of cells using sensitive methodologies, it is possible that these mutations correspond to background *TP53* mutations induced by sun exposure. As shown in Figure 5 and Table 2, *TP53* mutants found in normal skin of individuals free of tumors display a loss of activity similar to that observed for other tumors, with 63% of mutants presenting an activity less than 20%. This observation reinforces the notion that *TP53* inactivation is indeed an important feature in early skin cancer tumorigenesis.

TP53 MUTANTS IN RHEUMATOID ARTHRITIS

Several reports have described *TP53* mutations in RA [Sun and Cheung, 2002; Yamanishi et al., 2002]. This chronic inflammation and destruction of cartilage and bone of systemic joints is far from being a neoplastic disease, but several studies suggest that *TP53* could be an important factor in the inflammatory process. Analysis of *TP53* mutants found in RA indicates that 57% of them display an activity greater than 20%, compared to 35% in human tumors, and only 16% have an activity less than 10% (Table 3; Fig. 2). Although this observation could suggest that *TP53* inactivation is not important in RA, we cannot exclude alternative explanations. As RA is a different disease, it is possible that loss of transactivation is specific for gene promoters that have not been tested in our studies. Furthermore, it is also conceivable that transactivation activity is not the target of *TP53* mutations and that the real target of these mutations concerns other properties of *TP53* such as protein–protein interactions.

PREDICTION OF THE BEHAVIOR OF *TP53* MUTATIONS

Prediction of the significance and behavior of missense mutations has always been a difficult task [Steward et al., 2003]. For germline mutations, true polymorphisms are difficult to distinguish from rare deleterious mutations and a similar situation is observed for somatic mutations, in which "random mutants" without an associated phenotype are coselected in a genetically unstable background. In the absence of a functional assay, the evolutionary approach, based on the study of the phylogenetically conserved and therefore important functional region of a protein, has been widely used. A recent study on the BRCA1 gene detected 38 new potential deleterious mutations [Fleming et al., 2003]. Unfortunately, such studies cannot provide definitive results. They also cannot be applied to proteins with marked diversity of their functional region, such as immunoglobulin-like proteins or proteases. For *TP53*, the situation is more delicate. Many studies have used an evolutionary sequence analysis to predict the functional consequences of mutations, but they have not provided any robust conclusions. The most advanced published study is the work by Martin et al. [2002]. Using a series of structural and phylogenetic criteria, they identified a set of mutations that could be explained in structural terms using nine different criteria (Fig. 6). Three criteria (zinc, clash, and cavity) apparently lead to a good guess for inactivation of the *TP53* protein, whereas the others are less discriminating (Fig. 6). The phylogenetic conservation criterion leads to a relatively poor discrimination between wt and mutant phenotype. We believe that this criterion should be interpreted cautiously in the case of *TP53*. Conserved residues are important for the DNA binding activity of the protein, but also for its folding, which is an essential feature of the *TP53* protein because many mutations affect this folding. Recent studies in our laboratory with Xenopus *TP53* emphasize this point. The frog protein is only active at 25°C, its physiological temperature, but is inactive at 37°C due to denaturation of the core region (amino acids 100 to 300) [Bensaad et al., 2003; Ridgway et al., 1994]. This situation mimics that observed for thermosensitive human *TP53* mutant. This thermosensitivity of *TP53* has also been observed for Drosophila *TP53* and it is reasonable to suppose that all *TP53* proteins derived from animals with an optimal temperature below 37°C, such as fish or insects, would present a similar behavior [Waddell et al., 2001]. Therefore, residues involved in maintaining a wt folding at 20°C have evolved differently in mammalian *TP53*, whereas residues involved in DNA binding and other functional properties are more likely to be conserved. Inactivating mutations at these nonconserved residues are selected in human tumors, but cannot be predicted if phylogenetic studies include all *TP53* species. We have also used other predictive software, such as SIFT (http://blocks.fhcrc.org/sift/SIFT.html) or specific alignment software that scores residue conservation with multiple parameters such as Scorecons (www.ebi.ac.uk/thornton-srv/databases/cgi-bin/valdar/scorecons_server.pl) without success in efficient *TP53* mutation behavior. Although the crystal structure of the core region of *TP53* has been identified, the structure of the entire protein has not been established and no structural data are available to explain the heterogeneity of wt *TP53* in discriminating various types of promoters. The situation is certainly highly complex, as it is very possible that other cellular proteins may modulate *TP53* DNA binding activity and the effect of these interactions on various mutations is totally unknown. On the other hand, it is now clear that several monogenic mutant *TP53* proteins can gain new activity via a specific interaction with either p73 or p63. The observation that this gain of function is modulated by the polymorphism of the *TP53* at codon 72 adds another level of complexity.

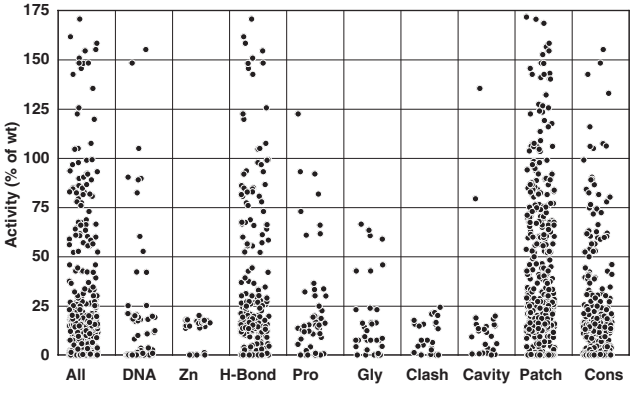

FIGURE 6. ***TP53* mutant activity in mutations that were structurally explained by Martin et al. [2002] (see that article's Materials and Methods section). DNA, mutations involving DNA binding; Zn, mutations involving zinc binding; H-Bond, mutations affecting hydrogen bonding; Pro, mutations to proline residues (may result in distortion of the protein); Gly, mutations from glycine (can affect protein folding); Clash, residue clashes (may result in incorrect folding); Cavity, cavity-creating mutations; Patch, mutations most likely to affect protein–protein or domain–domain interactions; Cons, mutations in conserved residues. In each column, the activity of the mutants is given after standardization (0% for yeast without p53 and 100% for wt p 53). For this analysis, the activity corresponds to the activity of a single mutant, and its X position in the column is random.**

CONCLUSIONS

This analysis raises a large number of unresolved questions. A total of 127 *TP53* mutants (13% of mutants corresponding to 128 reports) and 245 mutants (24% of mutants corresponding to 577 reports) display an activity greater than that of wt *TP53* or greater than 50% of wt *TP53* activity, respectively (Fig. 2; Supplementary Fig. S2). It is difficult to believe that these mutants are simply due to a random background of neutral mutations arising in an unstable genetic background. In fact, several studies have shown that the rate of *TP53* mutation is lower in tumors with high levels of microsatellite instability [Cottu et al., 1996; Kim et al., 1994]. Other explanations, therefore, need to be found and could concern the method of analysis itself.

The fact that activity analysis was performed in yeast could lead to a bias, as it does not correspond to the mammalian cell environment of wt *TP53* and, to date, no *TP53* has been found in yeast. Also, the optimal temperature for yeast is 30°C and not 37°C. Several arguments contradict this hypothesis. First, in the present analysis, we used data obtained from yeasts grown at 37°C in order to minimize the potential bias due to thermosensitive *TP53* mutants. Second, we and other authors have previously demonstrated a very good correlation between the activity of wt and mutant *TP53* in yeasts and mammalian cells in a transient expression system.

Another criticism that can be made in relation to the present analysis concerns the cutoff value used to define normal vs. abnormal *TP53* activity. In fact, without applying any cutoff value, 127 mutants presented an activity identical to or greater than that of wt *TP53*. Only a minority (less than 10%) were localized at the

CpG dinucleotide, ruling out "hitch-hiking" mutations selected by increased spontaneous deamination at cytosine residue.

Therefore, beyond the problems associated with spurious reports, we would like to discuss the present observations with the notion of penetrance of *TP53* mutations. The major problem associated with all studies on *TP53* activity is that they are performed with the use of a transient transfection assay, raising a number of questions concerning the limits of this type of study. Does overexpression of mutant *TP53* in response to a strong transcription promoter in a fully transformed recipient cells accurately reproduce the situation observed in precancerous cells, in which a low level of *TP53* is expressed in response to its own transcription promoters? Furthermore, is the penetrance of these mutations similar in different cell types and at different stages during cell transformation as other genetic events are able to modulate *TP53* defects. The other question concerns the defect induced by *TP53* mutations. Although it is clear that the DNA binding activity (and therefore the transcriptional activity) is the major defect selected during cell transformation, we cannot exclude the possibility that other rare alterations could also be selected. Such defects could be more specific to particular cancer subtypes or other diseases. The c.1010G>G (p.R337H) mutation is the best example of this type of mutation. This alteration was found as a germline mutation specifically associated with pediatric adrenal cortical carcinoma in southern Brazil in several independent families that were not predisposed to other tumors, a feature which is totally different from LFS [Ribeiro et al., 2001]. In every transactivation assay, this mutant showed a wt behavior. Precise chemical analysis revealed that this c1010G>G (p.R337H) mutant is highly sensitive to pH in the physiological range, leading to folding changes depending on the protonated state of the protein [DiGiammarino et al., 2002]. The observation that the syndrome associated with this mutation has been predominantly found in Brazil suggests that it could be linked to other modifier genes that could influence folding of *TP53* or cell pH. In the same line of evidence, mutants with subtle changes such as folding, nuclear localization, efficiency of ubiquitination, or other post-translational modifications could be missed by a transient transfection assay. One important aspect concerning the function of wt and mutant *TP53* is the amount of protein expressed in the cells. Mice with only one *TP53* allele are prone to cancer in a similar way to that observed in LFS, suggesting that a 50% loss of function of *TP53* is sufficient to inactivate the *TP53* pathway. Although in LFS it is difficult to distinguish between the effect of simple loss of function of one allele and a dominant negative activity that simultaneously inactivates most of the *TP53* function, the recent description of a case of LFS with a complete heterozygous deletion of the *TP53* gene confirms that a threshold of wt *TP53* is essential to maintain normal *TP53* function in humans [Bougeard et al., 2003].

In a recent study, Resnick and Inga [2003] nicely addressed the question of mutant *TP53* threshold. Using an inducible and regulatable promoter for *TP53* expression, they demonstrated that there is a very marked heterogeneity in the behavior of mutant *TP53* towards various transcription promoters according to the level of *TP53* protein expressed. Therefore, it is possible that mutant *TP53* that expresses nearly normal wt activity in a transient assay may nevertheless carry normal function when expressed from its own promoters.

Finally, the last important issue is the correlation between *TP53* mutations and clinical outcome, such as response to therapy and survival. Focusing only on molecular analysis, the literature is quite controversial on this matter and there is no unanimity on any single tumor type. Several studies have shown that only mutations localized in specific structural components of the *TP53* protein were associated with poor outcome (for review see Soussi and Béroud [2001]). It should be highly interesting to retrospectively determine whether other types of correlation can be demonstrated between clinical data and *TP53* mutations stratified by loss of function. The importance of the transcription promoter used for analysis would be critical in these types of studies. For response to therapy, it seems obvious that emphasis must be placed on the genes that are important for apoptosis. It would also be easy to check the library for other transcription promoters that could be clinically relevant, such as PUMA, which appears to be an important factor in apoptosis [Jeffers et al., 2003; Yu and Zhang, 2003]. On the other hand, it is also possible that clinical data may lead to the discovery of a link with the activity of a particular promoter. We are, therefore, now in a position to be able to achieve a better assessment of *TP53* alterations in human tumors and to decide whether *TP53* status has a clinical relevance.

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