

## Meta-analysis of the p53 Mutation Database for Mutant p53 Biological Activity Reveals a Methodologic Bias in Mutation Detection

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**Abstract** **Purpose:** Analyses of the pattern of p53 mutations have been essential for epidemiologic studies linking carcinogen exposure and cancer. We were concerned by the inclusion of dubious reports in the p53 databases that could lead to controversial analysis prejudicial to the scientific community. **Experimental Design:** We used the universal mutation database p53 database (21,717 mutations) combined with a new p53 mutant activity database (2,300 mutants) to perform functional analysis of 1,992 publications reporting p53 alterations. This analysis was done using a statistical approach similar to that of clinical meta-analyses. **Results:** This analysis reveals that some reports of infrequent mutations are associated with almost normal activities of p53 proteins. These particular mutations are frequently found in studies reporting multiple mutations in one tumor, silent mutations, or lacking mutation hotspots. These reports are often associated with particular methodologies, such as nested PCR, for which key controls are not satisfactory. **Conclusions:** We show the importance of accurate functional analysis before inferring any genetic variation. The quality of the p53 databases is essential in order to prevent erroneous analysis and/or conclusions. The availability of functional data from our new p53 web site (<http://p53.free.fr> and <http://www.umd.be:2072/>) will allow functional prescreening to identify potential artifactual data.

*p53* mutations are found in ~50% of human cancers (1). 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 are oncogenic and have been the subject of extensive studies providing a better understanding of their origin (2, 3).

The unique feature of *p53*, compared with other tumor suppressor genes, is its mode of inactivation. Although most tumor suppressor genes are inactivated by mutations leading to absence of protein synthesis (or production of a truncated

product), >80% of *p53* alterations are missense mutations that lead to the synthesis of a stable full-length protein (1). This selection to maintain mutant *p53* in tumor cells is believed to be required for both a dominant-negative activity to inhibit wild-type *TP53* expressed by the remaining allele, and for a gain of function that transforms mutant *TP53* into a dominant oncogene (4–6). An important feature of the *TP53* protein is the extreme flexibility and fragility of the DNA binding domain (residues 90–300; ref. 7), as all these residues have been found to be modified and several residues could sustain multiple alterations. Another puzzling aspect of mutant *p53* proteins is their structural, biochemical, and biological heterogeneity.

The universal mutation database (UMD) p53 database contains 21,717 mutations, i.e., ~30% of all mutations found in human diseases reported thus far (April 2005 release). In 2001, and then in 2003, we expressed several reservations concerning the biological significance of some of these mutations (1, 8). Although an unbiased database should contain all publications of the literature, we were very concerned by the inclusion of dubious reports. The very marked difference of frequency between the various mutations suggested that rare mutations did not have the same biological significance as hotspot mutations. Unfortunately, it is difficult to prove this hypothesis in the absence of functional analysis.

Recently, Kato et al. constructed a library of mutants and analyzed the transactivational activity of >2,300 p53 mutations (9). After combining this new information and all mutations of the UMD p53 locus-specific mutation database (LSDB), we did a functional analysis on all mutations of the database. By using

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**Table 1.** Description of studies outside the reference range

Cancer	Study ID	Mean and 95% CI	Methodology	Comments
Breast carcinoma	all breast carcinoma 1266-40*	-1.187 (95% CI, -1.220 to -1.154) 0.451 (95% CI, -0.774 to -0.128)	nested PCR followed by cloning and sequencing of pooled plasmids	31 tumors with 2 p53 mutations 3 tumors with 3 p53 mutations 1 tumor with 5 p53 mutations 1 tumor with 6 p53 mutations 37 mutations do not change the amino acid
	403-24	-0.794 (95% CI, -1.146 to -0.422)	sequencing of cloned PCR products from purified SSCP gel	10 tumors with 2 p53 mutations
	547-18	-0.722 (95% CI, -1.161 to -0.283)	direct sequencing	3 tumors with 3 p53 mutations 6 tumors with unique mutation with wild-type p53 activity
Colorectal carcinoma	all colorectal carcinomas 1459-49*	-1.235 (95% CI, -1.262 to 1.208) -0.652 (95% CI, -0.927 to -0.377)	direct sequencing	2 tumors with 2 p53 mutations 4 tumors with 3 p53 mutations 2 tumors with 4 p53 mutations 3 tumors with 5 p53 mutations 2 tumors with 6 p53 mutations 1 tumor with 8 p53 mutations 1 tumor with 9 p53 mutations 1 tumor with 10 p53 mutations 13 mutations do not change the amino acid Several tumors have multiple K-ras mutations outside codons 12 and 13
	1386-24*	-0.313 (95% CI, -0.730 to 0.103)	nested PCR and sequencing	4 tumors with 2 p53 mutations 1 tumor with 4 p53 mutations 8 mutations do not change the amino acid
	2010-17	-0.168 (95% CI, -0.811 to 0.476)	sequencing of eluted SSCP products after second amplification	Multiple weak mutations found only in early colorectal carcinomas; advanced colorectal carcinomas displays only single strong mutation
	1924-14	-0.343 (95% CI, -1.097 to 0.410)	direct sequencing of eluted SSCP products	4 tumors with 2 p53 mutations 1 tumor with 3 p53 mutations 5 tumors with an very rare G76A mutation
Non – small cell lung cancer	all non – small cell lung cancers 1659-70†	-1.105 (95% CI, -1.144 to -1.066) -0.690 (95% CI, -0.955 to -0.424)	sequencing of cloned PCR products	The majority of tumors have between 2 to 14 p53 mutations 4 tumors with 1 p53 mutation 1 tumor with 2 p53 mutations 1 tumor with 3 p53 mutations 3 tumors with 4 p53 mutations 3 tumors with 6 p53 mutations

(Continued on the following page)

**Table 1.** Description of studies outside the reference range (Cont'd)

Cancer	Study ID	Mean and 95% CI	Methodology	Comments
Hepatocellular carcinoma	all hepatocellular carcinomas 378-15	−1.080 (95% CI, −1.129 to −1.031) 0.565 (95% CI, 0.07 to 1.052)	direct sequencing	1 tumor with 7 p53 mutations 1 tumor with 8 p53 mutations 1 tumor with 12 p53 mutations 1 tumor with 14 p53 mutations 23 mutations do not change the amino acid the majority of tumors have multiple K-ras mutations outside codons 12 and 13
Gastric carcinoma	1972-6 all gastric carcinomas 1039-19	−0.145 (95% CI, −0.950 to 0.660) −1.135 (95% CI, −1.229 to −1.077) −0.586 (95% CI, −1.11 to −0.069)	direct sequencing sequencing of eluted SSCP products after second amplification	12 of the 15 mutations are similar (S166T) with a wild-type p53 activity. This mutant has never been described elsewhere mutant G293R with wild-type p53 activity is found in 4 tumors
	1706-20 1850-12	−0.612 (95% CI, −1.03 to −0.193) −0.427 (95% CI, −1.091 to 0.236)	nested PCR and sequencing cloning and sequencing of pool of 4 plasmids	1 tumor with 2 p53 mutations 1 tumor with 3 p53 mutations
Esophageal squamous cell carcinoma	all esophageal squamous cell carcinomas 1270-12	−1.209 (95% CI, −1.249 to −1.170) −0.427 (95% CI, −1.041 to 0.186)	direct sequencing	mutant Q331P with a wild-type p53 activity is found in 3 tumors. This mutant has not been described elsewhere. Unusual tandem mutation in 3 tumors
Head and neck squamous cell carcinoma	all head and neck squamous cell carcinomas 1201-35 924-16 1546-15*	−1.091 (95% CI, −1.131 to −1.052) −0.635 (95% CI, −1.055 to −0.216) −0.356 (95% CI, −0.750 to 0.039) −0.339 (95% CI, −0.833 to 0.155)	nested PCR and sequencing nested PCR and sequencing nested PCR and sequencing	several tumors with multiple p53 mutations 20% of mutations do not change the amino acid 3 tumors with 2 p53 mutations. The mutation P191T described in 3 tumors has never been reported elsewhere 2 tumors with 1 p53 mutation 1 tumor with 2 p53 mutation 2 tumors with 3 p53 mutation 4 tumors with 4 p53 mutation 1 tumor with 5 p53 mutations 1 tumor with 6 p53 mutations 7 (32%) mutations do not change the amino acid

(Continued on the following page)

**Table 1.** Description of studies outside the reference range (Cont'd)

Cancer	Study ID	Mean and 95% CI	Methodology	Comments
	461-13	-0.235 (95% CI, -0.911 to 0.442)	direct sequencing	mutant H296D found in 3 tumors and N288T found in 2 tumors have a wild-type activity
	870-12	-0.442 (95% CI, -0.919 to 0.035)	cloning and sequencing of individual clones	the majority of tumors display only one mutation
	1933-39*	-0.323 (95% CI, -0.691 to 0.043)	Sequencing of eluted SSCP products after second amplification	4 tumors with 2 p53 mutation 3 tumors with 3 p53 mutations 3 tumors with 4 p53 mutations 1 tumor with 6 p53 mutation
	1594-17	0.541 (95% CI, -1.083 to -0.047)	direct sequencing of eluted SSCP products	none of the mutation is localized in any hotspot. This can reflects exposure to specific carcinogen in betel nuts
Brain	all brains	-1.269 (95% CI, -1.304 to -1.233)		
	1377-11	0.276 (95% CI, -0.415 to 0.967)	nested PCR and sequencing	1 tumor with 4 p53 mutations 50% of p53 mutations found in axon 4 which usually accounts for less than 5% of all mutations
Bladder	all bladder carcinoma	-1.159 (95% CI, -1.211 to -1.107)		
	607-26*	-0.554 (95% CI, -0.987 to -0.121)	nested PCR and sequencing	8 tumors with 2 p53 mutations 1 tumor with 4 p53 mutations different sections of primary tumors harbor different p53 mutations
	1992-19*	-0.439 (95% CI, -1.204 to -0.145)	direct sequencing of eluted SSCP products	13 (33%) mutations do not change the amino acid 6 tumors with 2 p53 mutations 1 tumor with 3 p53 mutations 3 (10%) mutations do not change the amino acid 6 mutations change 2 nucleotides of the codon 1 mutation change the 3 nucleotides of the codon
Ovarian	all ovarian carcinoma	-1.263 (95% CI, -1.299 to -1.228)		
	1596-43	-0.9267 (95% CI, -1.190 to -0.663)	direct sequencing	10 tumors with 2 p53 mutations 1 tumor with 3 p53 mutations

NOTE: Brain, includes all astrocytomas and glioblastomas.

\* Publications included in the IARC LSDB (<http://www-p53.iarc.fr/index.html>), but excluded from the UMD database because of their inconsistencies.

† This publication is not included in both the UMU and IARC LSDB.

an approach similar to that of clinical meta-analyses, we clearly showed that several published studies have a p53 mutant activity profile that differs significantly from the normal distribution observed in other studies and can have a profound effect on the analysis of the p53 mutation database.

## Materials and Methods

**Analysis of the biological activity of p53 mutants.** p53 mutant activity has been described in detail in a previous report (9). Briefly, 2,314 haploid yeast transformants containing p53 mutations and a green fluorescent protein reporter plasmid were constructed. p53 mutant activity was tested by measuring the fluorescent intensity of green fluorescent protein that is controlled by the WAF1 promoter sequence of the plasmid after 3 days of growth at 37°C. The activity of the yeast without p53 or with wild-type p53 was -1.58 and 2.03, respectively. The activity of the majority of p53 mutants was situated between these two values.

**Data analysis.** The UMD p53 database used for this study contains 21,717 mutations derived from 1,992 publications (2005 version,

which has been available since April 2005). For this analysis, we also added 30 publications that were previously excluded because of inconsistencies (Table 1). Mutations described in cell lines, in normal skin, or in patients suffering from rheumatic arthritis were not included in order to incorporate only somatic mutations detected in primary tumors. All frameshift and nonsense mutations were also excluded, as their biological significance has not been clearly established. Nevertheless, an analysis of colorectal cancers including these mutations (giving them a null biological activity) led to similar results to those described in Fig. 2 (data not shown).

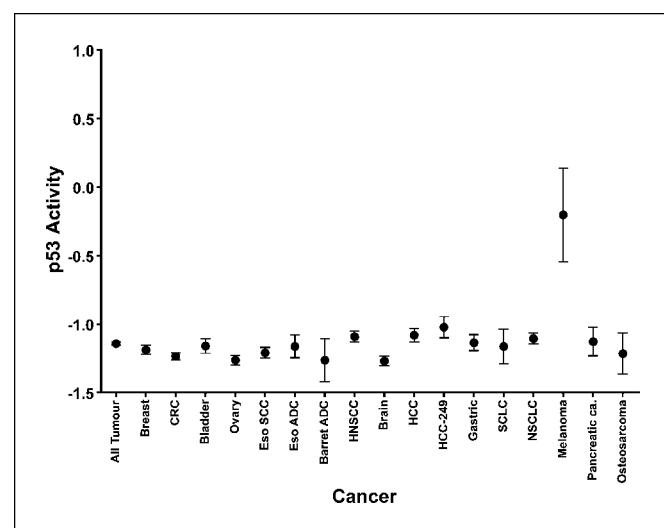
The mean and 95% confidence interval (CI) of the biological activity of all mutants was calculated by using the transactivational activity measured on the WAF1 promoter. Similar results were obtained with the activity measured on seven other promoters of transcription (Supplementary Fig. S1 online). This study was done by integrating all biological activities of mutant p53 into the UMD p53 database, and by developing new statistical routines in order to analyze and export data. All data are now available to the scientific community with the new version of the p53 database.

For data analysis and presentation of the results, we used a similar approach to that used for meta-analyses comparing clinical trials. For

each cancer, the mean and 95% CI of p53 activity in each publication were graphically displayed. The reference value corresponds to the mean and 95% CI of all studies for the specific cancer. Although the mean value of the entire database can be used as the reference value, we believe that the use of an individual reference value for each cancer type more closely reflects the heterogeneous etiology and pattern of p53 mutations in various cancers. To verify the accuracy of this reference value, we checked the p53 database for reliable studies, in which p53 mutation analysis was done objectively by two different methodologies. Studies using yeast assay were excluded from this validation analysis in order to obtain independent information. We found six studies satisfying these criteria, including one study in breast cancer in which the DNA or RNA of two samples of the same tumors were analyzed in two different laboratories. The mean and 95% CI of the biological activity of mutant p53 found in all of these studies were within the same range as the reference value defined for each individual cancer type (Supplementary Fig. S2 online). In this statistical analysis, the width of the 95% CI depends on both the scatter of the individual values (SD) and the sample size: the width of the 95% CI increases as the sample size decreases (Supplementary Fig. S3 online). Only publications reporting 10 or more mutations were analyzed in this study in order to ensure significant results. Exclusion of these data does not alter the results, as no additional "out-of-range" study was found (Supplementary Fig. S3 online). Cancers with >500 published mutations were analyzed, corresponding to the 10 most frequent cancers. For brain tumors, astrocytomas and glioblastomas were pooled, as they present an identical pattern of p53 mutations. Statistical analyses were done with PRISM software (GraphPad Software, Inc., San Diego, CA) on a Mac OS X platform.

## Results

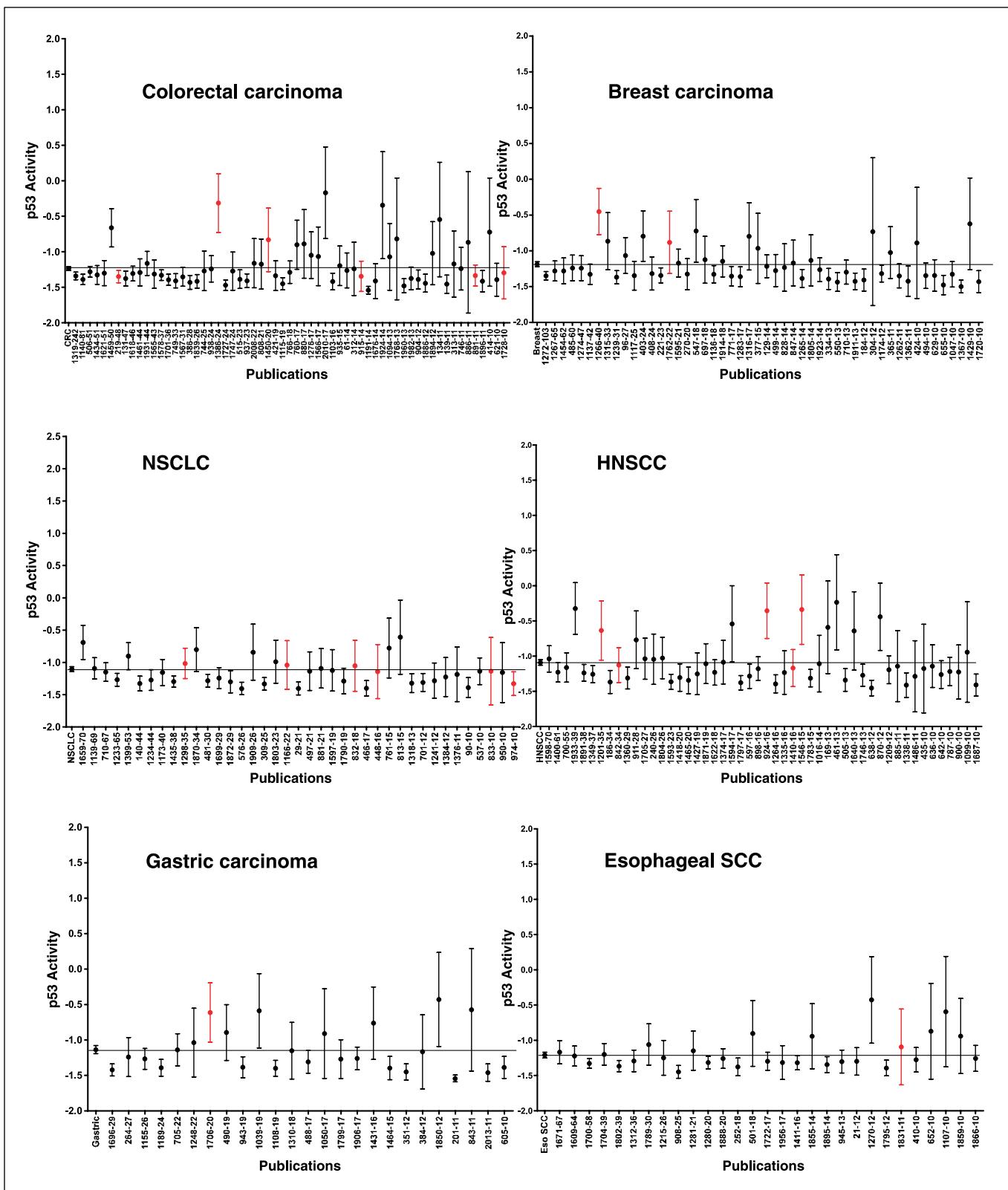
The UMD p53 database contains 21,717 mutations representing 1,300 p53 variants with occurrence ranging from once (401 mutants) to 979 times (mutant R175H). To study the significance of each p53 mutant, we combined information provided by the functional database that we have developed and correlated these data with all mutations of the UMD p53 LSDB (see Materials and Methods). The analysis shows that, for all cancers, except malignant melanomas, the mean activity was situated around -1.2 with a narrow 95% CI, demonstrating an apparent homogeneity of p53 mutant activity for all of the mutations included in the database (Fig. 1). This value corresponds to a residual transactivational activity of ~10% compared with wild-type p53. The underlying reasons for the abnormal profile of the p53 gene mutations associated with malignant melanomas have not been elucidated (see ref. 10 for discussion). To refine these results, we individually analyzed each publication for cancer types with >500 reported p53 mutations (Fig. 2), corresponding to the 10 most frequent cancers found in the human population. The distribution of p53 mean activity in each report was compared with that of all studies for a given cancer (global mean value, see Materials and Methods for a detailed explanation of the choice of the global mean value). Most reports display a homogeneous distribution with a 95% CI, which includes the global mean value. However, for several publications, the distributions significantly differ from the average as their CI does not include the global mean value. Although only publications which describe 10 or more p53 mutations were included in this analysis, the addition of publications including fewer mutations does not reveal additional out-of-range data, indicating that these observations are not nonspecifically related to the number of p53 mutations analyzed (Supplementary Fig. S3 online).



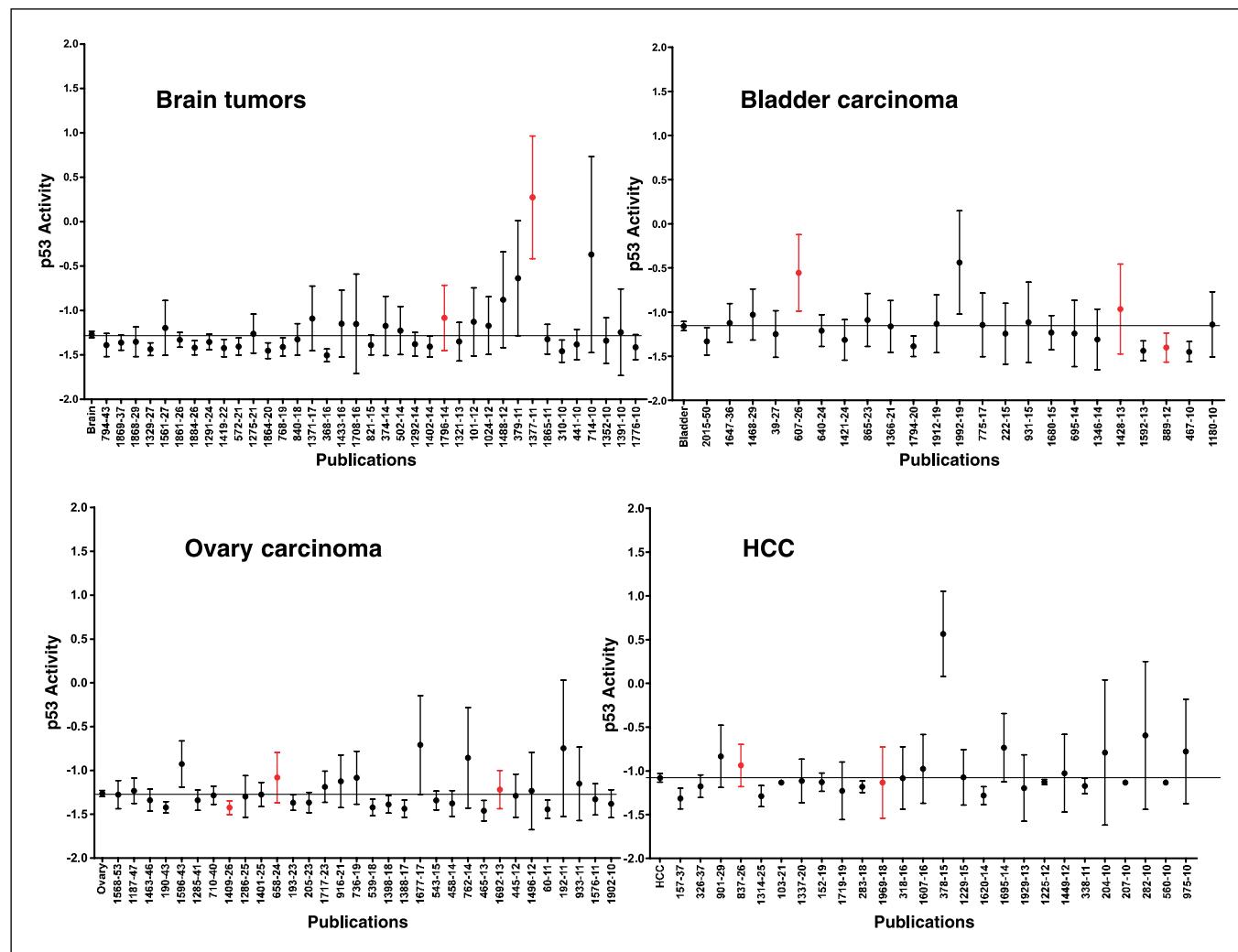
**Fig. 1.** Distribution of p53 loss of function in various types of cancer. Points, mean p53 activity as measured by transactivation with the WAF1 promoter; bars, 95% CI. The mean and 95% CI of p53 activity for all studies in the database is also included (all tumors). The y-axis corresponds to p53 transactivation activity, with a value of -1.5 for the negative control and a value of 2.5 for 100% of wild-type p53 response genes (data not shown). CRC, colorectal carcinoma; HNSCC, head and neck squamous cell carcinoma; NSCLC, non–small cell lung cancer; HCC, hepatocellular carcinoma; HCC-249, hepatocellular carcinoma excluding the hot spot mutation at codon 249.

In colorectal cancers, two studies present a different behavior when a lower limit of their 95% CI is above the global mean value of all studies (Fig. 2; Table 1). Analysis of these two studies reveals various characteristics that are not generally described in colon and other cancers (Table 1): (a) many tumors present several p53 gene mutations (as many as ten), (b) virtually no mutations are described at hotspot codons, (c) there is a high proportion of "neutral" mutations, which do not change the amino acid, (d) one of these studies also reported multiple mutations of the Ki-ras gene in codons other than codons 12 and 13. Analysis of other cancer types reveals similar results to those observed for colorectal cancer (Fig. 2; Table 1). The majority of these out-of-range studies share a large number of tumors with multiple mutations and neutral mutations. These findings are not observed in other studies. Furthermore, they also describe p53 mutants that are very rarely found in other publications, some of them being exclusively described in these reports (Supplementary Fig. S4 online).

The 1659-70 study in lung cancer (Table 1) shows how these out-of-range studies can lead to serious problems of interpretation. Apart from the observation described in Table 1, this study found a higher frequency of G→T transversion in nonsmokers compared with smokers, a unique finding in the literature (Supplementary Fig. S5 online). Inclusion of this study in a previous release of the IARC database was one of the factors that led some authors to question the link between smoking and p53 gene mutations in a previous release of lung cancer (11). It was subsequently shown that the mutation profile of lung cancers was biased by this study, which accounted for almost 20% of the mutations associated or not associated with known exposure to tobacco (12). Another analysis excluding the data from this study clearly confirmed the links between tobacco exposure and p53 gene mutations (12).



**Fig. 2.** Meta-analysis of p53 loss of function. *Points*, mean p53 activity as measured by transactivation with the WAF1 promoter; *bars*, 95% CI. The mean and 95% CI of p53 activity for all studies combined for a specific type of cancer is shown on the far left of each graph. *Horizontal line*, mean of the combined studies. The publication code is indicated on the x-axis: the first number is an anonymous ID for the publication and the second number indicates the number of p53 mutants included in this study. Studies are presented from left to right in decreasing order of number of p53 mutants. The y-axis corresponds to p53 transactivation activity, with a value of -1.5 for the negative control and a value of 2.5 for 100% of wild-type activity (see Materials and Methods). Only studies with 10 or more p53 mutations are shown on the graph. Graphs with a larger scale or including all publications are shown in Supplementary Fig. S2. Studies using nested PCR are in red.

Fig. 2. *Continued.*

In breast cancer analysis, we removed tumors derived from BRCA1 or BRCA2 patients. Previous investigations have suggested that p53 mutations arising in BRCA1- or BRCA2-associated tumors occur at a higher frequency compared with sporadic tumors. Functional characterization of these mutants in mammalian cells revealed that they frequently possess properties not commonly associated with those occurring in sporadic cases: they retain apoptosis-inducing, transactivating, and growth-inhibitory activities similar to the wild-type protein, but are compromised in terms of transformation suppression and also possess an independent transforming phenotype (13). In the yeast assay, the activity profile of these mutations is also different from those observed in sporadic breast cancers, confirming the particular loss of function of these p53 mutants and underscoring the biological and functional relevance of the yeast functional assay (Campomemosi, 2001 13012; Supplementary Fig. S6 online). Therefore, the analysis shown in Fig. 2 only displays tumors of sporadic origin. Two of the three out-of-range studies displayed an abnormal pattern of p53 mutations (Table 1).

During this meta-analysis, we noticed that many of the out of range studies used a nested PCR approach. We therefore

analyzed the methodology used in each publication. Statistical analysis revealed a significant association between studies using nested PCR and out of range studies ( $P = 0.0003$ , two-tailed exact Fisher's test; Table 2). In addition, the majority of these studies used paraffin-embedded tissue as a starting material. It is well known that such material could lead to the detection of false mutations if controls are not done adequately (14). A recent analysis of the *BRCA1* gene in ovarian tumors using nested PCR and paraffin-embedded tissue described a pattern of artificial mutations similar to those described above: multiple mutations, no mutations at the classic hotspot, and >30% of neutral mutations (15).

## Discussion

The quality of the data included in a LSDB must obviously be the primary objective and a race to report the largest number of mutations would only be harmful to the scientific community (16). Inclusion of artificial results has a number of harmful effects, both intellectually when they are quoted indiscriminately by nonspecialists, but also for the integrity of the databases. This is well illustrated by the debate on the origin

**Table 2.** Distribution of p53 studies according to the methodologies used

	Classical PCR	Nested PCR	
Normal distribution	483	41	
"Out of range" distribution	15	8	$P = 0.0003$

NOTE: Hepatocellular carcinomas were not used for this analysis, as many studies only focused on exon 8, the site of codon 249, the hotspot for aflatoxin-linked liver cancer.

of p53 mutation in lung cancer challenged by the tobacco industry as recently discussed by Bitton et al. in a recent issue of *Lancet* (17).

Inclusion of artifactual data in LSDBs can also mask other original studies describing real differences in p53 mutation profiles. Quality control must therefore be applied at all levels (ref. 18; Supplementary Annex online). Therefore, we believe that the statistical analysis described here can be used by anyone as a prescreening for potentially poor quality data during the course of their studies. The functional data are available at our p53 web site. We have also organized an international curator committee to monitor the integrity of data included in the UMD p53 database. This independent committee is composed of p53 specialists in various types of cancers. The role of this committee is to examine all articles presenting an abnormal mutation profile and define how these data will be included in the database. Although this second reviewing solution may seem complicated and redundant to the work of reviewers, it nevertheless constitutes a solution at the present time to provide the scientific community with reliable and good quality data. Application of simple rules can only be beneficial for the entire scientific community (Supplementary Annex online). Apart from ensuring the author's compliance with a rigorous scientific and technological approach, reviewers and editors must also act as gatekeepers to ensure that the

quality of the information published is maintained at a level of excellence.

We also consider that this problem is not limited to p53 and must be extended to all mutations recorded in all LSDBs. A recent analysis revealed 262 LSDB for 29,000 mutations (excluding p53; ref. 19). Not only will the number of these LSDB continue to rapidly increase, but their value for clinical practice and basic research will also continue to develop. It is important to keep in mind that all these LSDBs constitute an enormous reservoir of natural mutants that have been selected in the context of a particular pathologic phenotype. The recent discovery that dominant-negative mutations of the kinase domain of epidermal growth factor receptor are associated with increased sensitivity to treatment with Iressa is a good example of translation between basic science and clinical practice (20, 21). Not only could the presence of these mutations allow a better selection of patients to be treated, but basic analysis of these mutations could also provide a better understanding of the signaling pathways involved. Similarly, the finding that p63 gene mutations localized in two distinct regions of the protein are associated with two different developmental syndromes suggests the need to study the various properties of this protein in more detail (22). The biological significance of these mutations may vary with accumulation of information about the protein, but also as a function of our basic knowledge about the function of these signaling pathways and their interconnections. It is therefore essential to ensure the optimal quality of data stored in these LSDBs and only the use of quality control procedures by all persons involved in the publication of these data (authors, reviewers, editors, and publishers) can prevent their "pollution" by irrelevant data. We have also established a curator committee of p53 specialists that will propose guidelines to improve the quality of the information contained in the LSDB.

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