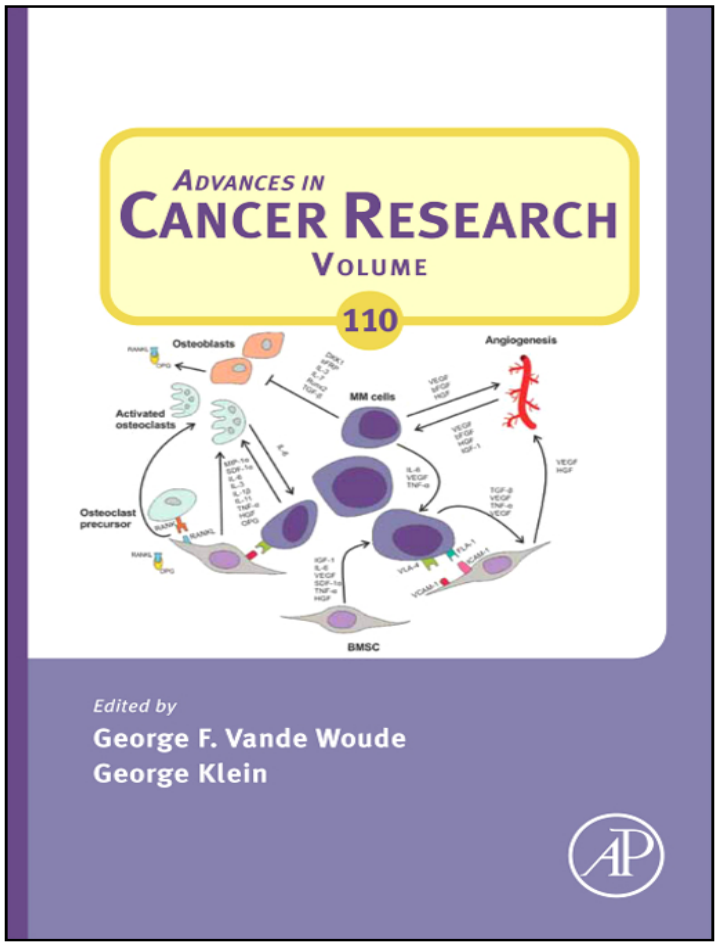

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TP53 Mutations in Human Cancer: Database Reassessment and Prospects for the Next Decade

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TP53 mutations are the most frequent genetic alterations found in human cancer. For more than 20 years, TP53 mutation databases have collected over 30,000 somatic mutations from various types of cancer. Analyses of these mutations have led to many types of studies and have improved our knowledge about the TP53 protein and its function. The recent advances in sequencing methodologies and the various cancer genome sequencing projects will lead to a profound shift in database curation and data management. In this paper, we will review the current status of the TP53 mutation database, its application to various fields of research, and how data quality and curation can be improved. We will also discuss how the genetic data will be stored and handled in the future and the consequences for database management. © 2011 Elsevier Inc.

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I. INTRODUCTION

Several large-scale screens for genetic alterations in human cancers using Next Generation Sequencing (NGS) have been recently published (Meyerson *et al.*, 2010). Identification of novel gene mutations associated with tumor growth provides new insight into the biology of cancer development. These studies should also identify whether some of these mutated genes could be efficient targets for anticancer drug development. The prevalence of missense somatic mutations has been much higher than expected and constitute the most frequent genetic alterations detected in tumor genomes (Greenman *et al.*, 2007). These observations are complicated by the discovery that the genome of cancer cells is polluted by somatic passenger mutations (or hitchhiking mutations) that have no active role in cancer progression and are only coselected by the driver mutations, which are the true driving force for cell transformation (Haber and Settleman, 2007). Passenger mutations can be found in coding or noncoding regions of the genome and distinguishing them from driving mutations will be a difficult but necessary task in order to obtain an accurate picture of the cancer genome. Several statistical approaches have been developed to solve this problem, such as comparing the observed to expected ratios of synonymous:nonsynonymous variants. Alternatively, various bioinformatics methods are used to give an indication about whether an amino acid substitution is likely to damage protein function on the basis of either conservation through species or whether or not the amino acid change is conservative.

Reporting, storing, classifying, and analyzing these mutations also constitute a major challenge (Horaitis and Cotton, 2004). For a long time, locus-specific databases (LSDB) were developed for this purpose. Although each LSDB has been developed for a single gene, they are very accurate as they are curated manually by experts in the field (Claustres *et al.*, 2002). They provide information that can be used for large-scale analysis and often include structural, functional, or evolutionary data that allow easy distinction between passenger and driving mutations. TP53 mutation databases (TP53; MIM# 191170) are a paradigm, as they constitute the largest collection of somatic mutations (31,000 mutations from 29,000 patients) for a single gene.

TP53 mutations are found in approximately 50% of human cancers (Soussi and Wiman, 2007). 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. The TP53 protein is a transcription factor that binds a very loose DNA response element (TP53RE) found in several hundred genes that are

differentially activated depending on the cell type, identity, and extent of damage, and various other parameters that have yet to be identified (Harris and Levine, 2005). The unique feature of TP53 compared to other tumor suppressor genes is its mode of inactivation. While most tumor suppressor genes are inactivated by frameshift or nonsense mutations leading to absence of protein synthesis (or production of a truncated product), more than 80% of TP53 alterations are missense mutations that lead to the synthesis of a full-length protein that accumulates in the nucleus of the tumor cell (Fig. 1). This selection to maintain mutant TP53 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 (Soussi and Wiman, 2007).

This 30th anniversary of the discovery of TP53 in 2010 was associated with the publication of a large number of reviews describing the complexity of this gene (as it codes for multiple isoforms), its family (the TP63 and TP73 genes also code for multiple isoforms with opposite properties), the various signaling pathways associated with these proteins (repair, response to stress, and modulation of the growth of stem cells or embryonic development) and various epistemologic aspects (Junttila and Evan,

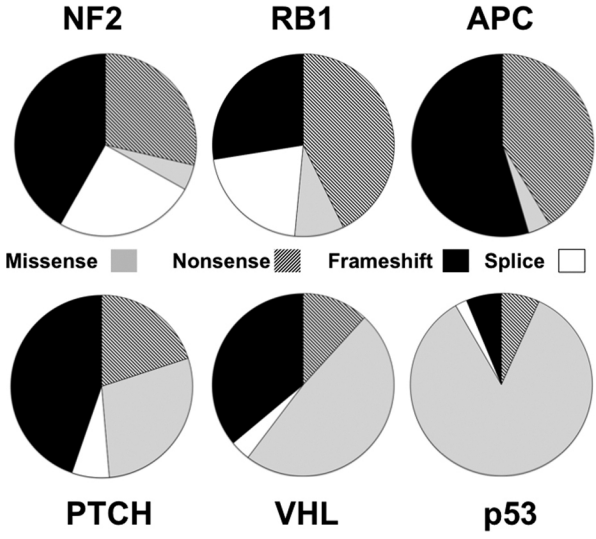

Fig. 1 Mutation spectrum in human cancer: frequency of missense, nonsense, splice, and frameshift mutations. TP53 data obtained from the UMD TP53 database (http://p53/free/fr) for all cancers (2011 vR0.9, unreleased). Data for other tumor suppressor genes were taken from their respective mutation databases available from the web. RB1: retinoblastoma gene; NF2: type 2 neurofibromatosis gene; VHL: Von Hippel Lindau gene; PTCH: patched, *Drosophila* homolog gene; APC: adenomatous polyposis coli gene.

2009; Khoury and Bourdon, 2010; Krizhanovsky and Lowe, 2009; Levine and Oren, 2009; Soussi, 2010; Vousden and Ryan, 2009). In this review, I will focus on the evolution of TP53 mutation databases, how this information has been used to increase our knowledge on the TP53 protein, the questions raised by the heterogeneity of these mutations, how the quality of data can be improved and how this database (and other LSDB) will change with growing volume of data generated by NGS.

II. TREND IN TP53 MUTATION PUBLICATIONS AND THE FUTURE OF LSDB

Since the first description of a TP53 mutation in 1989 (Baker *et al.*, 1989; Takahashi *et al.*, 1989), there has been a constant growth of TP53 mutation analysis, culminating in 1998 with the publication of 190 articles describing TP53 mutations (Fig. 2, blue column) (Takahashi *et al.*, 1989). The trend in the number of TP53 mutations described remained roughly parallel and culminated in 2000 with the description of 2076 mutations (Fig. 2, red column). The slight discrepancy between the publication trend and the number of mutations published is due to the

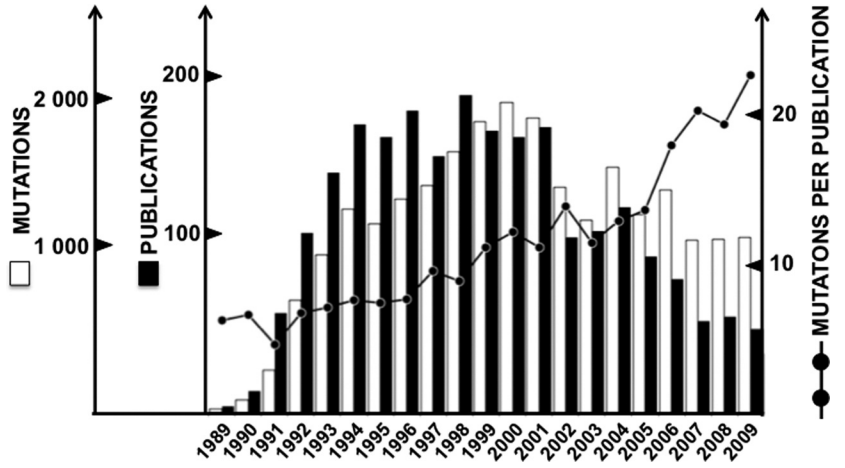

Fig. 2 Trends in TP53 mutation publications. Blue column: number of publications describing TP53 mutations published each year; red column: number of mutations published per year; black line: mean number of TP53 mutations per publication. Only publications describing molecular analysis of TP53 mutations were included in this analysis. Analyses using immunohistochemistry were not taken into account. TP53 data obtained from the UMD TP53 database (2011 vR0.9, unreleased). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this book.)

description of increasing numbers of TP53 mutations in each publication (Fig. 2, black line). The decline in TP53 mutations, first observed in 2000, is continuing and is similar for both publications and the number of TP53 mutations published. These trends are not due to lack of interest in TP53, but to the difficulty of publishing TP53 mutations in peer-reviewed journals due to their lack of novelty. Furthermore, in recent publications, TP53 mutations are not fully described due to journal space considerations. There are either listed as supplementary materials or, increasingly frequently, not described at all. This problem is not specific to TP53 and applies to many other genes, raising an important issue related to the publication of somatic mutations and the future growth of LSDB. It should be noted that the rate of discovery of novel TP53 mutations, that is, TP53 mutations at novel positions, is also now very low as the majority of hot spot and frequent mutations have been described for more than 20 years.

DNA sequencing has been dominated for more than 30 years by the chain-terminator method (or Sanger sequencing), which is easy to perform and rapidly interpreted (Ziebolz and Droege, 2007). The development of automatic sequencing using dye-terminator labeling has changed the throughput of this methodology, but data interpretation and format remain similar. The development of NGS (second and third generation) and whole tumor genome sequencing will result in an enormous increase in the volume data, but extraction of specific gene information will be a very challenging task for LSDB curators (Mardis, 2008; Metzker, 2010). Although some recently obtained whole tumor genome sequencing data can be easily analyzed, as they have been partially interpreted, these data can be expected to be no longer available in the near future. NGS will generate exabytes of raw data (1 gigabyte (GB) = 10^9 bytes; 1 exabyte (EB) = 10^{18} bytes) and sophisticated software will be required for their interpretation (Schadt *et al.*, 2010). As an example, the amount of data generated by the pilot analysis of the 1000 genomes project is currently 50 terabytes of raw data. Handling, accessing, and analyzing this amount of data will also require novel computing methodology such as cloud or heterogeneous computing (Schadt *et al.*, 2010). Manual curation of publications for gene mutations will come to an end and computerized data mining of repositories will become a necessity. Unfortunately, this will not be an easy task for several reasons. First, the formats of raw data differ considerably between the various companies that provide NGS technology and data analysis will require high-level bioinformatic expertise. Second, quality control analyses are currently very difficult to assess, as these methodologies are very new and accurate data filtering will require more extensive experience. Third, data will be scattered around the globe and large-scale data repositories have yet to be developed. The Catalogue of Somatic Mutations in Cancer (COSMIC) is the first attempt to gather

such information in a single environment (Forbes *et al.*, 2010). This database contains more than 150,000 somatic mutations involved in the development of cancer. This database collects information from two major sources. First, mutations in known cancer genes are collected from the literature and second, data from whole genome sequencing studies of cancer samples performed by the Cancer Genome Project. Other cancer genome databases will certainly be developed in the near future.

This will have a profound impact on LSDB and it is currently unclear whether or not they will survive this major change. The flow-through between data and curator will obviously change, as publications will not be the primary material. Mining large databases for data on specific genes will be required provided these data are available and that authorization is granted, which is not the case today.

III. MOLECULAR EPIDEMIOLOGY: TP53 MUTATION DATABASE AND CANCER GENOME PROJECTS

One of the greatest contributions to the study of TP53 mutations has been provided by molecular epidemiology and its applications (Greenblatt *et al.*, 1994; Soussi and Beroud, 2003). We will not discuss these epidemiologic studies in detail, as they have been the subject of many detailed reviews. The most important findings of molecular epidemiology are summarized in Table I. These studies demonstrate a link between exposure to various types of carcinogens, specific mutational events in the TP53 gene and the development of specific cancers. The most striking example is that of tandem mutations, specifically induced by ultraviolet radiation, which are only observed in skin cancers. The relationships between G → T transversion and lung cancer in smokers or mutation of codon 249 observed in aflatoxin B1-induced liver cancers are also very demonstrative. These studies were possible because TP53 was the only gene that combined several specific features used to study the origin of carcinogenesis in a human population, (1) it is mutated in many types of cancers; (2) the mutation frequency is high; (3) the gene is predominantly modified by point mutations; (4) the gene is small enough to be relatively easy to analyze.

Two recent lines of study have expanded these observations and confirmed the association between mutations of TP53 (and other genes) in human cancer and carcinogen exposure. The first line is a novel experimental mouse model developed to assess the mutagenicity of various physical or chemical carcinogens. The Hupki mouse model system was constructed using gene-targeting technology and contains human wild-type TP53 gene from exons 4 to 9 in place of the homologous murine DNA

Table I Relationship Between TP53 Gene Mutations and Exposure to Carcinogens

Type of cancer	Characteristics of TP53 mutations[a]	Genotoxic agent incriminated	Comments	References
Hepatocellular carcinoma	Specific G → T transversions in codon 249	Aflatoxin B1	Aflatoxin B1 binds specifically to codon 249	Puisieux *et al.* (1991), Staib *et al.* (2003)
Hepatic angiosarcoma	High frequency of A:T → T:A transversions	Vinyl chloride		Hollstein *et al.* (1994)
Lung cancer	High frequency of G → T transversions[b]; hot spot on codons 157 and 158	Benzo(*a*)pyrene (cigarette smoke)	Benzo(*a*)pyrene has a particular affinity for codons 157 and 158	Denissenko *et al.* (1996), Toyooka *et al.* (2003)
Skin cancer (BCC and SCC)	Very high frequency of mutations on pyrimidine dimers; high frequency of tandem mutations	Ultraviolet radiation	Photo-induced mutations	Brash *et al.* (1991), Tornaletti *et al.* (1993)
Wilson's disease, hemochromatosis	Specific G > T transversions in codon 249	In these diseases related to iron or copper overload, overproduction of free radicals leads to high oxidative stress	Exposure of cells to a carcinogen derived from lipid peroxidation leads to alterations on codon 249 of the TP53 gene	Hussain *et al.* (2000), Marrogi *et al.* (2001)
Urothelial malignancy	High frequency of A:T → T:A transversions	Aristolochic acids	Exposure of cells to aristolochic acid leads to similar mutational events	Grollman *et al.* (2007)

Only the most striking observations are summarized in this table. For more details, the reader can refer to the review by Hofseth *et al.* (2004).
[a]Compared to TP53 mutations observed in the absence of exposure to the agent incriminated.
[b]This high frequency of transversion is also observed in cancers of the esophagus and head and neck cancers associated with drinking and smoking.

sequences in both copies of the mouse TP53 gene (Luo *et al.*, 2001b). The human region encodes the proline-rich domain and the DNA binding region. This chimeric gene remains under normal transcription regulation at the mouse locus. No dysfunction of TP53 activity including nuclear accumulation of TP53 protein after exposure to DNA-damaging agents, transcriptional activation of known TP53 downstream targets has been observed. This Hupki mouse develops normally, exhibits no apparent physiologic defects, remain fertile, and shows no susceptibility to spontaneous cancer (Luo *et al.*, 2001b).

Exposure of Hupki mouse embryonic fibroblasts to the tobacco-derived carcinogen, benzo[*a*]pyrene leads to a high frequency of TP53 missense mutations and G → T transversions are the most frequent mutational events (Fig. 3A and E) (Liu *et al.*, 2005). Furthermore, these mutations are localized at codons 157 and 158 in exon 5, the exact same hot spot observed in lung cancer in heavy smokers (Liu *et al.*, 2005). Similarly, treatment of these fibroblasts with aristolochic acid (AA) elicits a high frequency of A → T transversions similar to those observed in patients with urothelial cancer from populations exposed to AA (Feldmeyer *et al.*, 2006). Hupki mice can also be used *in vivo*. Exposure of these mice to UVB leads to specific features known to be associated with skin cancer such as cellular patches with TP53 nuclear accumulation (Luo *et al.*, 2001a). The overall pattern of TP53 mutations found in these patches corresponds to the unique features of TP53 mutations in sunlight-associated human skin tumors such as a high frequency of C → T transitions at dipyrimidine sites and the presence of tandem mutations. On the other hand, exposure of Hupki mice to aflatoxin B1 leads to the development of a high frequency of hepatocellular carcinoma, but no TP53 mutations were detected emphasizing certain differences between humans and the mouse model (Tong *et al.*, 2006).

The second line of study is related to the various large-scale sequencing analyses of cancer genomes, as they expand molecular epidemiology to the entire genome. Two types of analysis have been performed: (i) exome sequencing that targets regions of interest such as exons. These studies started with the sequencing of specific gene families such as kinases (kinome) and phosphatases (phosphatome) and were more recently extended to the entire set of coding exons (McLendon *et al.*, 2008; Sjoblom *et al.*, 2006; Stephens *et al.*, 2005; Wang *et al.*, 2004). (ii) The second type of analysis consists of whole cancer genome sequencing (Baudot *et al.*, 2009) for a review. Eight complete sequences of cancer genomes together with matched normal genomes have been reported to date, but this number will grow very rapidly (December 2010). Comparison with matched normal tissue is a key feature in order to distinguish natural germline SNP from somatic mutations. Nucleotide

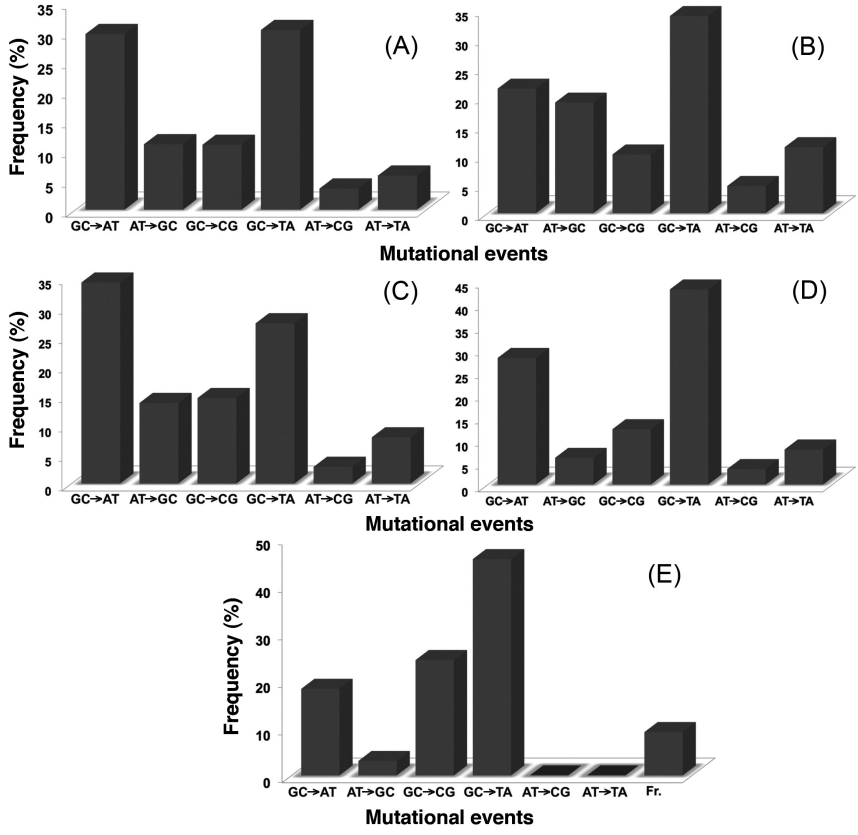

Fig. 3 Frequency of mutational events detected in lung cancer. (A) Data from the UMD TP53 database (2011 vR0.9, unreleased). (B) Data from Pleasance *et al.* (2010). (C) Data from Kan *et al.* (2010). (D) Data from Ding *et al.* (2008). (E) Data from Liu *et al.* (2005).

substitutions are the most common known somatic genomic alteration in cancer, typically occurring at the rate of about one somatic nucleotide substitution per million nucleotides, but this number can vary according to the type of cancer. Nevertheless, this implies that cancer genomes harbor thousands of mutations but only a few of them will be drivers associated with neoplastic transformation. As discussed in the introduction, most of the remaining mutations are passenger mutations coselected during transformation and are considered to be ''neutral'' during transformation. Whether or not these mutations occur during the normal division of these cells or only during tumorigenic progression remains to be evaluated.

Analysis of the mutational events found in these cancer genomes (both passenger and driver mutations) has shown that they are very similar to those observed for the TP53 gene (Greenman *et al.*, 2007). In lung cancer, regardless of the methodology used to sequence the cancer genome (exome or whole genome sequencing), the patterns of mutational events are very similar and show a striking similitude to the mutational events observed in the TP53 gene (Fig. 3A–D). The predominance of GC → TA transversions is a typical signature of carcinogen exposure indicating that both passenger and driver mutations share a similar origin. For other types of cancer, such as colorectal cancer (Fig. 4A–C), breast cancer (Fig. 5A–D), and brain cancer (data not shown), the pattern of TP53 mutations found in the entire genome is similar to that described for TP53. The only exception is the high frequency of GC → CG transition observed in the breast cancer genome, which has not yet been explained. These observations support the various molecular epidemiology studies performed in driving mutations from the TP53 database and indicate that global analysis of mutational events in cancer genomes, including both driving and passenger mutations, will expand molecular epidemiologic studies performed in TP53. Furthermore, the large number of mutations per tumor will allow novel studies that were not possible with analysis restricted to a single gene. The possibility to analyze and compare pre- and posttreatment

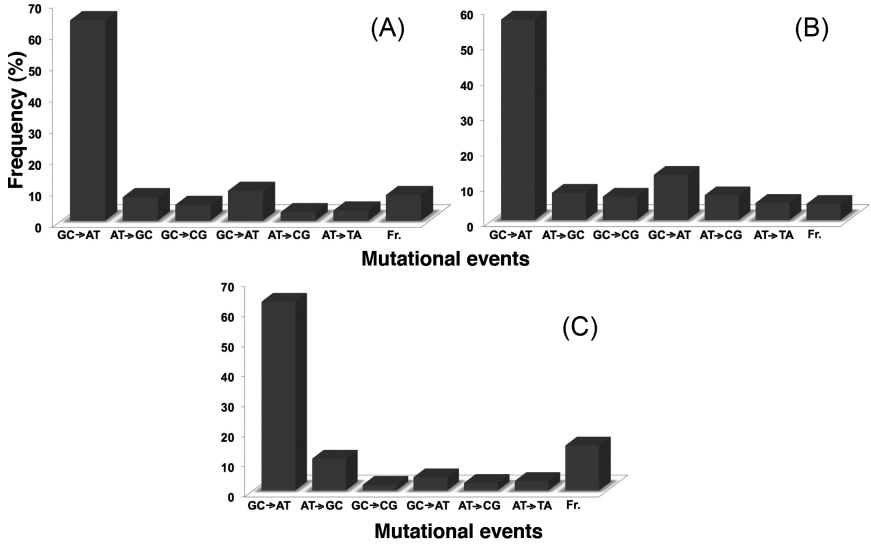

Fig. 4 Frequency of mutational events detected in colorectal cancer. (A) Data from the UMD TP53 database (2011 vR0.9, unreleased). (B) Data from Sjoblom *et al.* (2006). (C) Data from Greenman *et al.* (2007).

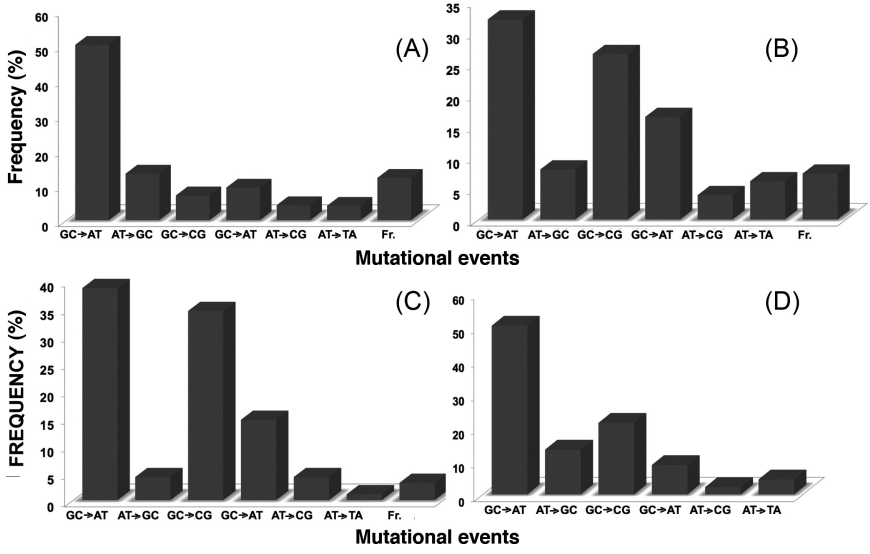

Fig. 5 Frequency of mutational events detected in breast cancer. (A) Data from the UMD TP53 database (2011 vR0.9, unreleased). (B) Data from Sjoblom *et al.* (2006). (C) Data from Greenman *et al.* (2007). (D) Data from Kan *et al.* (2010).

tumors from the same patient may also reveal a novel pattern of mutations induced by chemotherapy. This type of finding has already been reported in glioma patients treated with temozolomide, an alkylating agent that is known to be mutagenic (Greenman *et al.*, 2007).

IV. THE TP53 MUTATION DATABASE AS A FRAMEWORK FOR STRUCTURE–FUNCTION STUDIES OF THE TP53 PROTEIN

TP53 mutations found in human cancer are not random. They have been selected during neoplastic transformation indicating that they target essential function(s) of the TP53 protein as a "tumor suppressor." The first TP53 mutations were identified in the conserved domain of the protein in 1989 before TP53 was identified as a transcription factor (1990) and before the discovery of its specific DNA binding activity (in 1992) (Table II for a detailed history). This natural reservoir of thousands of TP53 mutations has been tremendously useful to further our knowledge on the TP53 protein and demonstrate how clinical studies can generate data that translate to basic research.

Table II Relationship Between TP53 Mutations and Structure–Function Analysis

Year	Observation	References
1989	First description of TP53 mutations in human cancer localized in a highly conserved domain	Nigro *et al.* (1989)
1990	P53 is a transcription factor: mutant TP53 does not transactivate	Raycroft *et al.* (1990)
1990	Transforming activities of various mutant TP53 is heterogeneous	Halevy *et al.* (1990)
1990	Discovery of the first thermosensitive TP53 mutant	Michalovitz *et al.* (1990)
1991	P53 protein is a DNA binding protein: mutant TP53 does not bind to DNA	Kern *et al.* (1991)
1991	Identification of two class of mutant TP53 based on conformation change probed with monoclonal antibodies	Gannon *et al.* (1990)
1993	Specific DNA binding activity of TP53 is localized in the highly conserved domain	Bargonetti *et al.* (1993), Pavletich *et al.* (1993), Wang *et al.* (1993)
1994	X-ray analysis of TP53 provide a framework to understand how mutant TP53 are deficient for DNA binding	Cho *et al.* (1994)
1994	Analysis of 30 TP53 hot-spot mutants distinguishes two classes of mutant TP53 based on structure and function	Ory *et al.* (1994)
1995	Mutant TP53 acts as a dominant negative protein in a mouse model	Harvey *et al.* (1995)
1995–1996	Specific TP53 mutations are associated with poorer survival or response to treatment in cancer patients	Aas *et al.* (1996), Goh *et al.* (1995)
1996	First observation of mutant TP53 with loss of apoptotic activity and normal growth arrest function	Friedlander *et al.* (1996)
1997	Biophysical analysis of mutant TP53 defines several classes of mutant TP53 based on stability and folding	Bullock *et al.* (1997)
1998	First description of intragenic suppressor mutations that reverse the effect of TP53 mutations	Brachmann *et al.* (1998)
1999	A subset of mutant TP53 binds to p73 and inactivates its transcriptional activity	Di Como *et al.* (1999)
2000	Analysis of the TP53 mutation database reveals that domain L1 of the TP53 protein is a mutation cold spot	Soussi *et al.* (2000)
2004	Mice models expressing mutant TP53 display a gain of function	Lang *et al.* (2004), Olive *et al.* (2004)
2005	Hupki mice expressing human TP53 hot-spot mutant provide a new system to analyze mutant TP53 gain of function	Liu *et al.* (2005)
2009	Novel structural studies of the TP53 protein provide an improved model for TP53 mutation studies	Brown *et al.* (2009)

A. Heterogeneity of TP53 Mutations in Human Cancers

The selection to maintain mutant *TP53* in tumor cells is believed to be required for both a dominant negative activity of wt *TP53* expressed by the remaining allele as discussed previously and/or a gain of function for certain mutant TP53 (Soussi and Lozano, 2005). An important feature of the *TP53* protein is the extreme flexibility and fragility of the DNA binding domain (DBD, residues 90–300), as more than 200 of the 393 residues have been found to be modified and several residues have sustained multiple types of substitutions (arginine 273 can be substituted for a Trp, Gln, Gly, Leu, or Pro). This observation has led to a bias in *TP53* mutation analysis, as more than 80% of *TP53* mutation studies focus on exons 5–8 (residues 126–306). On the other hand, TP53 alterations found in the amino terminus (1–80) or carboxy terminus (300–393) are predominantly null mutations (frameshift or nonsense) strongly suggesting that these two regions are more robust than the central domain (Hamroun *et al.*, 2006).

The structural difference between the various missense *TP53* mutations was initially identified using monoclonal antibodies able to discriminate mutations that change *TP53* folding and mutations in the residues involved in DNA recognition (Milner, 1995). Two classes of mutations have been distinguished on the basis of various *in vitro* assays and the three-dimensional structure of the protein (Cho *et al.*, 1994; Gannon *et al.*, 1990; Ory *et al.*, 1994): class I mutations, exemplified by mutants at codon 248 (7.6% in the TP53 database, http://TP53.free.fr), affect amino acids directly involved in the protein–DNA interaction. They have a wild-type conformation as probed by conformational monoclonal antibodies and they do not bind to the chaperone protein hsp70. Class II mutations, exemplified by the mutant at codon 175 (4.9% in the database), have an altered conformation with strong binding to the chaperone protein H. The amino acids altered in this class of mutants are involved in stabilizing the tertiary structure of the protein. Class II mutations are associated with a more severe phenotype *in vitro* than class I mutations. Due to an irreversible change of conformation, class II mutants cannot be restored to the wild-type conformation by activating antibodies or peptides. Such heterogeneity can also be due to the nature of the resulting residue. Mutant R273H has a wild-type conformation, whereas mutant R273P is unfolded. This biochemical and biological heterogeneity has been confirmed and refined by structural studies (Bullock *et al.*, 1997; Joerger and Fersht, 2008). Nuclear magnetic resonance (NMR) spectroscopy suggests that mutations in the L3 domain can induce either limited or extensive conformational changes, depending on their position or the type of substitution. Analyses using more sophisticated biophysical techniques have revealed that the DBD of the *TP53* protein can adopt

at least five thermodynamic states and have defined the unfolding properties of the various hot-spot mutants (Bullock *et al.*, 1997; Joerger and Fersht, 2008).

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 (Menendez *et al.*, 2009; Riley *et al.*, 2008). Using a library of 2500 different TP53 mutants, Kato *et al.* (2003) showed a marked heterogeneity in the loss of function of the various mutants. Although hot-spot mutants found in human cancer present complete loss of their transactivating properties on all target genes, other mutants such as R181L or R283H retain either a partial activity on all genes or on a subset of genes leading to a wide range of mutant activity.

The idea that some TP53 mutations can actively participate in cellular transformation was already postulated in 1990 (Halevy *et al.*, 1990). Many studies have tried to distinguish between the dominant negative and gain of function properties of mutant TP53 without reaching any clear-cut conclusions. This task is further complicated by the marked heterogeneity of mutant TP53, as already described. Transfection of various TP53 mutants into cells devoid of endogenous TP53 leads to an increase in their carcinogenicity, which varies according to the type of mutation (Soussi and Lozano, 2005). TP53 null mice have a different spectrum of tumors compared to knock-in mice expressing various TP53 hot-spot mutants (Broz and Attardi, 2010; Donehower and Lozano, 2009). Knock-in mice expressing various hot-spot mutants have a higher frequency of solid tumors with a high potential for metastasis, a feature not seen in KO animals. This particular spectrum of tumors is also observed in mice expressing one mutant allele in a TP53 null background, which is one of the strongest arguments for a gain of function of TP53 (Lang *et al.*, 2004; Olive *et al.*, 2004). Analysis of these knock-in models in mice defective for p63 and p73 suggests that alteration of the activity of the entire TP53 family is also an important feature for the gain of function of mutant TP53 (Flores *et al.*, 2005).

This research into the oncogenic potential of certain TP53 mutations is not purely theoretical, but can have obvious clinical implications, as it could explain the marked disparity of the results of studies trying to demonstrate a relationship between the presence of a TP53 gene mutation and various clinical parameters, such as survival or response to treatment. In breast cancer patients, the response to adriamycin is very strongly correlated with the presence of a mutation specifically localized in the loop domains L2 or L3 of the TP53 protein (Aas *et al.*, 1996). *In vitro*, the expression of TP53 mutant in position 175 (R175H) specifically induces

resistance of cells to etoposides compared to other TP53 mutants (Blandino *et al.*, 1999).

The two homologous genes of TP53, p63, and p73, express many isoforms due to alternating use of transcription promoters and alternative splicing (Collavin *et al.*, 2010; Yang and McKeon, 2000). Long isoforms (TA-p73 or TA-p63) are able to transactivate the same target genes as TP53 and induce apoptosis, while short forms (DN-P63 or Dnp73) have an opposite activity via dominant negative mechanisms. p63 and p73 are able to cooperate with TP53 to induce apoptosis, suggesting the existence of a complex network of interactions between the products of these three genes. Although wt TP53 does not interact with p73 or p63, some mutant TP53 proteins bind strongly to the two TP53 homologs via their DNA binding domains (Di Como *et al.*, 1999; Marin *et al.*, 2000). This interaction leads to inactivation of p73 and p63 function. Studies by T. Crook and B. Kaelin showed that resistance to anticancer agents involves inactivation of the apoptotic function of p73 protein by a subset of mutant TP53 that have sustained a change of conformation (Bergamaschi *et al.*, 2003; Irwin *et al.*, 2003).

B. Mutant TP53 with Low or Specific Penetrance

As discussed above, mutant TP53 is heterogeneous and displays a high degree of penetrance depending on the position of the mutation. Several mutations have been shown to have either a low degree of penetrance or are linked to a specific type of cancer. We will focus our discussion on two TP53 mutants that have been extensively analyzed: R175P and R337H.

Despite the fact that the R175P mutation is localized on a hot-spot codon (codon 175), it is very rarely detected in human cancers (eight times) in contrast with mutant R175H, which has been reported 1353 times (Soussi and Beroud, 2003). The R175P mutation has a normal cell cycle arrest and gene p21 induction behavior, but is deficient for apoptotic activity and does not transactivate bax or PIG3 genes (Friedlander *et al.*, 1996; Ory *et al.*, 1994). The reasons for this heterogeneity are unknown at the present time, but could be related to a difference of interaction with various coactivating molecules. Mice homozygous for the R172P mutation (equivalent to the human R175P alteration) are defective in TP53-dependent apoptosis, but retain a partial cell cycle checkpoint function (Liu *et al.*, 2004). These mice have a very low predisposition to develop tumors compared to mice not expressing TP53. Furthermore, these tumors do not present the chromosomal instability revealed in TP53−/− or mice expressing the hot-spot mutant R175H (Liu *et al.*, 2004). These results derived from purely basic research, but based on a clinical observation, suggest that the apoptotic activity may not be the

primary activity targeted by TP53 gene alterations. This represents a major challenge in relation to current models, which define apoptosis as being the fundamental activity of TP53.

The R337H mutation was found as a germline mutation specifically associated with pediatric adrenal cortical carcinoma in southern Brazil in several families that were not predisposed to other tumors (Ribeiro *et al.*, 2001). Genetic analyses strongly suggested the existence of a founder effect and that all these patients are related, indicating that a single mutational event led to this mutation (Pinto *et al.*, 2004). Analysis of the last version of the mutation database indicates that this mutant is very infrequent in sporadic cancer (nine tumors). This mutation is localized in the oligomerization domain of the TP53 that is not frequently mutated in human cancer (see below). In every transactivation assay, the R337H mutant showed a wt behavior. Precise biochemical analysis revealed that the mutant protein 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 specific association with adrenal cortical carcinoma has not been explained. As the adrenal gland is known to undergo extensive apoptosis during pre- and postnatal development, it has been postulated that an increased intracellular pH may lead to TP53 inactivation and impair apoptosis specifically in these cells. This type of observation emphasizes an important aspect of the TP53 protein, its *in vitro* and *in vivo* flexibility and the influence of this flexibility on its properties.

C. What is the Target of TP53 Mutations?

Older and more recent data have shown that TP53 DBD is also an important region for specific protein–protein interactions such as the proapoptotic protein Bak and BclXl or the ubiquitin ligase mdm2 (Leu *et al.*, 2004; Mihara *et al.*, 2003; Shimizu *et al.*, 2002). Everything began with the discovery that is region is the recognition site of SV40 large T antigen (LT) and that this interaction is conserved in all TP53 from humans to frogs. The crystal structure of LT complexed with TP53 confirms that the viral protein shields the entire DBD region and impairs any interaction with either DNA or other proteins (Soussi *et al.*, 1989). Cellular proteins also interact with the DBD region, but each protein requires a specific set of TP53 residues that partially overlap (Gorina and Pavletich, 1996; Iwabuchi *et al.*, 1994; Thukral *et al.*, 1994). TP53 interaction with BclXl is specifically associated with transcription-independent TP53-induced apoptosis (TIPA as acronymed by Chipuk and Green (2006). A fraction of TP53, localized at the mitochondrial membrane, directly induces permeabilization of this outer membrane by disrupting the protective role of BclXL (Mihara *et al.*, 2003). The interface

of TP53 associated with BclXl involves the loop domain L3 (residues 239–248) with the help of two other TP53 regions, residues 135–141 (part of loop L1) and residues 173–187 (part of loop L2). The 53BP2/ASPP2 protein also binds to the CR of TP53. This interaction involves the C-ter part of loop L3 (residues 243–249) and part of loop L2 (residues 165, 167, and 181) (Gorina and Pavletich, 1996). 53BP2/ASPP interaction with TP53 specifically enhances transactivation of apoptotic genes such as bax or PIG 3, but has no effect on p21 (Samuels-Lev *et al.*, 2001). Although, the primary binding site of mdm2 to TP53 is situated in HCD I (residues 15–29), other docking regions have been identified, one in the central region of the protein (HCD V, residues 261–270) and more recently in the carboxy terminus of the protein (residues 367–392) (Poyurovsky *et al.*, 2010; Shimizu *et al.*, 2002).

This intricate promiscuity of various functions in the DBD of TP53 raises a number of questions concerning the interplay between the loss of these functions and mutant TP53. No systematic analysis of the loss of protein–protein interactions of various TP53 mutants has been performed and only partial data are available. Some hot-spot mutants, such as R175H, are totally defective for transactivation and protein binding. On the other hand, other hot-spot mutants such as R282W or R173H retain the capacity to bind ASPP despite loss of their transactivation activity and a negative TIPA (Sot *et al.*, 2007).

These observations raise the question of whether loss of the transcriptional activity of TP53 via impaired DNA binding activity is the only consequence of TP53 mutations selected during transformation or whether loss of TIPA or other activities related to protein binding are also important in this selection. Furthermore, the heterogeneity of the binding interface of these various TP53 partners once again results in marked variability of TP53 mutant properties.

D. TP53 Mutation Cold Spots

Three important regions of TP53 are devoid of mutations and have been defined as mutation ''cold spots'': the amino terminus regions that include the mdm2 binding region and the transactivation domain, the L1 loop in the central region of the protein and the oligomerization domain (Soussi and Beroud, 2003). These three regions have a very low frequency of missense mutations (Fig. 6). Large-scale analyses of artificial TP53 mutants in the transactivation domain have shown that this region is very robust and that a single missense mutation does not abolish the activity of the protein (Lin *et al.*, 1994). Total deletion of highly conserved domain I (HCD I, residues 13–23) does not alter the activity of TP53 (Kubbutat *et al.*, 1998). This region also contains one of the binding

domains of the mdm2 protein and three TP53 residues (19, 23, and 26) are essential for this interaction. Not a single missense mutation has been found in any of these three residues, which is only to be expected as such mutations would abolish TP53–mdm2 interactions and lead to either permanent growth arrest or cell death.

The L1 region (residues 113–123) contains residue 120, which is essential for DNA binding as it is directly in contact with a guanine base in the major groove of the DNA (Cho *et al.*, 1994). Artificial mutations in the L1 domain change the specificity of DNA binding and several mutants at positions 120, 121, or 123 display an increase affinity for certain p53RE and an increased apoptotic activity (Saller *et al.*, 1999; Zupnick and Prives, 2006). Although this observation explains why such mutants are not selected during tumorigenesis, the modular role of this L1 loop to distinguish various TP53 target genes has not yet been resolved.

The third mutation cold spot is the oligomerization domain (Fig. 6). This domain forms a dimer of dimers and is composed of a short monomeric region consisting of a β-strand (residues 326–333) followed by an α-helix (residues 335–355). The primary dimers are stabilized by an intermolecular β-sheet and mainly hydrophobic helix packing interactions (Clore *et al.*, 1995). In addition, the primary-dimer interface is stabilized by a salt bridge between residue R337 and D352 in two monomers. It must also be remembered that this region also includes a nuclear export signal essential for TP53 trafficking (residues 340–351) (Stommel *et al.*, 1999).

Only a few mutations have been observed in this region, but a recent thorough analysis of all of these mutants suggested that they have a wild-type behavior and could be passenger mutations. The only exception is mutant R337H found in patients with pediatric ACC, as discussed above. The lack of mutation in this region suggests that they could be counter-selected in human cancer, keeping the TP53 protein as a tetramer either for the dominant negative activity or for gain of function.

V. QUALITY OF THE DATABASE

As discussed previously, inclusion of novel TP53 mutations in the database has slowed down over recent years, a trend that was already observed 10 years ago and which is continuing today (Soussi *et al.*, 2006). It is therefore now possible to focus on the content and quality of the database. In 2001 and then in 2005, we expressed several reservations concerning the biological significance of some of these mutations (Soussi and Beroud, 2001; Soussi *et al.*, 2005). Although an unbiased database should contain all publications of the literature, we were very concerned by the inclusion of dubious reports.

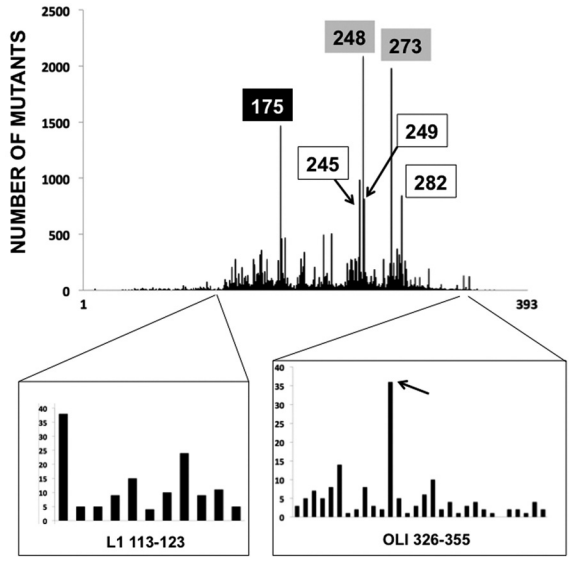

Fig. 6 Distribution of TP53 mutations. The distribution of missense mutations is shown along the 393 amino-acid sequence of TP53 protein. The six most common hot spot positions are highlighted in blue for DNA-contact mutations (248 and 273), orange for locally unfolded mutants (245, 249 and 282) and red for globally denatured mutants (175). Data for the two cold spots, region, L1 loop (codons 113 to 123) and oligomerization domain (codon 326 to 355), are also displayed below. Data from the UMD TP53 database (2011 vR0.9, unreleased). For residue R337 (red arrow), the frequency shown here (34) is much lower than that reported in other analyses. As a founder effect has been shown for the germline mutation identified in Brazil, it was only included as a single genetic event in the database which more accurately reflects the frequency of this mutation. Other mutations are somatic mutations at the same position found in other types of cancer. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this book.)

Analysis of the TP53 mutation database and TP53 publications reveal three unusual features: (i) publications with a large number of TP53 mutations with no loss of activity; (ii) publications with a large number of synonymous mutations (Syn) (i.e., mutations that do not change the amino acid); (iii) publications with a large number of TP53 mutations per tumor.

A. Publications with a Large Number of TP53 Mutations with no Loss of Activity

There is a marked difference in the frequency between the various mutations, with occurrences ranging from 1 (401 mutants) to 1353 times (1 mutant, R175H, Fig. 7A). Analysis of loss of activity of TP53 mutations in relation to their frequency in the database has led to unexpected

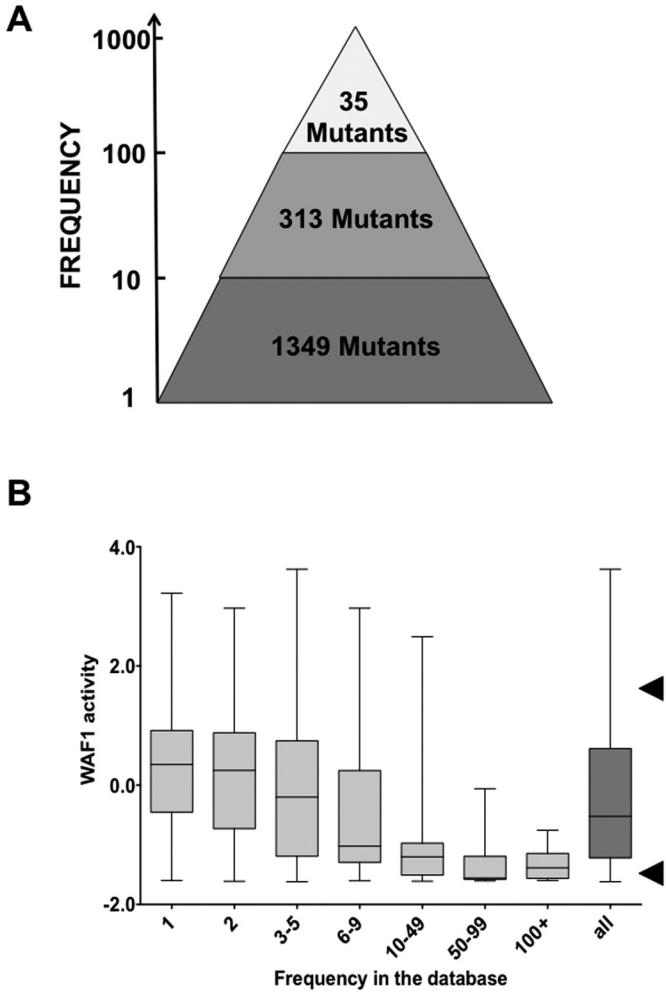

Fig. 7 (A) Frequency of p53 mutants. There are 1697 different p53 missense mutants in the database, but their frequency is heterogeneous. One thousand three hundred and forty-nine mutants are found at frequencies ranging from 1 to 10 times, with 401 mutants described only once. Three hundred and thirteen mutants have been described at intermediate frequencies (between 11 and 99 times). Only 35 mutants are found more than 100 times, with the highest frequency of 1353 times for the R175H mutant (B) Activity of mutant TP53 according to their frequency in various subsets of the database. Mutant TP53 were classified into eight categories according to their frequencies. Box-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); analysis was performed all missense mutants found in human tumors. Black triangles corresponds to 0% (bottom) and 100% (top) TP53 activity. (For the *Y*-axis, the values are given in log scale.)

observations (Soussi *et al.*, 2005). For hot-spot mutants, the remaining activity is always low, ranging from 0% to 20% compared to the normal protein (Fig. 7B). Rare mutants present a very heterogeneous scatter, ranging from 0% to 160%. There is a clear inverse correlation between the frequency of TP53 mutants and their activity. Approximately one half of the mutants that have only been reported once have an activity greater than 50% compared to wild-type TP53, indicating the very limited importance of these mutations. More detailed functional analyses of some of these mutants with wt TP53 activity (Ps^{Wt} for pseudo wild type) clearly show that they have growth arrest and apoptotic activities similar to those of wt TP53.

This observation raises an important question about these infrequent TP53 mutations, that is, what is their significance in neoplastic transformation? Are they only coselected passenger mutations? Sequencing artifacts? Mutants with undiscovered loss of activity? As discussed below, all three options are possible.

B. Publications with a Large Number of Synonymous Mutations (Syn)

Syn mutations are usually associated with passenger mutations, as they do not change the amino acid sequence of the protein. However, they should be considered with caution, as they can lead to alterations in RNA processing such as splicing, translation efficiency, or mRNA stability. The T125T silent mutation in the TP53 gene was previously considered to be a neutral mutation before it was observed that it induces aberrant splicing (Holmila *et al.*, 2003; Varley *et al.*, 2001). Unfortunately, this mutation is still erroneously considered to be a natural SNP in ref SNP database (rs55863639). The frequency of Syn mutations in the entire TP53 database is low, as they are observed only in 4.1% of tumors. Although their distribution should be random throughout all published studies, a small number of publications present a very high frequency of Syn mutations (Table III). This feature is not associated with either a particular type of cancer, a specific type of chemotherapy, or a specific geographical localization.

C. Publications with a Large Number of TP53 Mutations per Tumor

Multiple mutations (MM) of the TP53 gene in the same tumor is also a very unusual feature observed in the database (Table IV). The majority of human tumors harbor only a single mutation but a few of them display two or more mutations in the same tumor. Analysis of the residual

Table III Frequency of Syn Mutations in the TP53 Gene in the Literature[a]

Number of publications	Frequency of Syn mutation (%)[a]
677 (485)[b]	<10 (0)[b]
84	10–20
29	20–50
15	>50

[a]Only publications describing more than 10 mutations were taken into account for this analysis.
[b]Brackets: number of publications without Syn mutations.

transcriptional activity of TP53 mutants identified in cell lines that displayed two different TP53 mutations has demonstrated a high frequency of weak mutations that are paired with more potent mutations suggesting a driver/passenger configuration (Berglind *et al*., 2008). A similar finding has been observed for tumors with two mutations (T. Soussi, unpublished observations). The finding of more than two mutations in the same tumor is more difficult to explain and is rarely discussed in the literature. An increase of genetic instability, a defect in DNA repair or treatment-induced mutagenesis could also lead to an increased number of mutations, but an increased frequency of TP53 mutations has never been observed in any of these *in vivo* settings. The observation that multiple mutations are not random but associated with specific publications could be due to a specific geographical factor associated with a particular type of environmental exposure.

D. Unusual Pattern of Mutations or Sequencing Artifacts?

Integration of these three independent features (Ps^{Wt}, Syn, and MM mutations) into a single analysis indicates they are not random and strongly associated. Ten publications accounting for 700 mutations

Table IV Number of Mutations per Tumor

Tumors	Number of mutation per tumor
25,447	1
1,766	2
269	3
98	4
20	5
39	>5

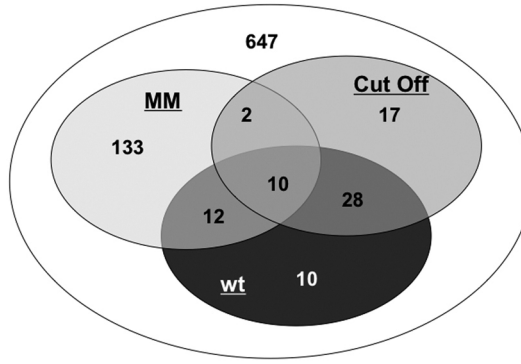

Fig. 8 Venn diagram illustrating the relationship between the three parameters used to analyze the quality of the TP53 mutation database.

report a large number of Ps^{Wt}, Syn, and MM mutations (Fig. 8 and Table V). Further examination of these articles reveals other unusual features, such as the use of formalin fixed paraffin embedded (FFPE) tissue (7/10) or nested PCR (5/10). In two publications, other unusual genetic alterations were observed in other genes (Table V). FFPE samples raise problems for genomic analyses because they contain fragmented DNA and crosslinks between DNA, RNA, and proteins. Several studies have now clearly demonstrated that sequencing DNA from FFPE is prone to sequencing artifacts and leads to a high rate of false mutations. Furthermore, FFPE sections or cells obtained after Laser Capture Microdissection can result in very small amounts of DNA that would require nested PCR, a method that is also known to be fraught with artifacts when it is not performed very carefully. All of these features indicate that these 10 publications are not trustworthy and they have been tagged accordingly in the UMD TP53 database.

Forty-two articles included two of these three unusual features (Fig. 8) and also raise a doubt about their validity, but it has yet to be determined whether these features are due to passenger mutations or sequencing artifacts. Taken together, these analyses shows that the TP53 mutation database contains a nonnegligible number of mutations that are not directly associated with neoplastic transformation. Dealing with these problems is not harmless. Inclusion of these reports in various types of analysis can lead to erroneous interpretation. One study in breast cancer described an unusual number of clustered mutations at positions never previously described and a large number of mutations that did not change the amino acid

Table V Description of the 10 Discordant Studies

Reference value	Cancer	Strategy	Tissue	Freq. wt[a]	Freq. MM[b]	Comments
607	Bladder	Nested PCR followed by asymmetric PCR and DNA sequencing	Sections of PFE	17/44 (39%)	12/15 (80%)	
1207	HNSCC metastasis	PCR followed by cloning and sequencing individual clones	Cell lines	5/16 (31%)	6/9 (67%)	Discrepancy of the TP53 status in various publications
1266	Breast	Nested PCR followed by cloning and sequencing individual clones	Sections of PFE	22/58 (38%)	24/28 (86%)	
1546	HNSCC	Nested PCR followed by direct sequencing	LCM on PFE section	7/24	6/8	Exons 5 and 6 only
1659	NSCLC	PCR followed by cloning and sequencing individual clones	Frozen	31/104 (29%)	16/18 (89%)	Multiple ras mutations scattered outside codon 12 and 13
1745	Prostate	PCR followed by SSCP, elution, PCR amplification and sequencing of bands with altered mobility	LCM on PFE section	10/24 (42%)	7/11 (64%)	

1838	NHL	PCR followed by SSCP, elution, PCR amplification and sequencing of bands with altered mobility	Sections of PFE	5/19	6/10	Multiple syn mutations found in c-kit and β-catenin
2125	Breast	Nested PCR followed by cloning and sequencing individual clones or nested PCR followed by direct sequencing	Sections of PFE	75/206 (36%)	55/61 (90%)	
2305	Ovarian	cDNA amplification followed by cloning and sequencing individual clones	Tissue collected directly in RNA later	14/34 (41%)	10/17 (59%)	
2395	Breast	Nested PCR followed by sequencing	Sections of PFE	37/167 (22%)	37/53 (70%)	
All data[c]				1,313/30,593 (4.3%)	2,196/27,647 (7.9%)	

[a]Frequency of "silent" mutations.
[b]Frequency of tumors with more than two mutations.
[c]Data for the whole database.

sequence (Patocs *et al.*, 2007). This constituted a serious problem, as this highly controversial manuscript was published in the *New England Journal of Medicine* and raised the important question of the existence of genetic alterations in stromal cells from breast cancer patients, a highly debated field (Campbell *et al.*, 2008; Roukos, 2008; Soussi and Soussi, 2008; Zalcman *et al.*, 2008). TP53 mutation databases should therefore be used with caution and mutations should be selected according to the type of analysis. As discussed above, the inclusion of passenger mutations in epidemiologic studies should not raise any problems, but their inclusion in clinical studies could lead to erroneous results.

VI. FUTURE PROSPECTS: WHAT CAN BE EXPECTED FROM A TP53 MUTATION DATABASE IN THE FUTURE?

The TP53 mutation database has been an invaluable tool. It is a unique example of an LSDB due to the high rate of missense mutations and the high frequency of TP53 alterations in human cancer and demonstrates how exchanges between clinical studies and basic research is beneficial to both fields. Changes in the flowthrough of data acquisition in the near future will change all LSDB and particularly LSDB for cancer genes and somatic mutations. In the near future, it will be important to centralize mutation databases associated with cancer genome projects to allow expert curators to perform accurate data mining. This will ensure the survival of LSDB and will provide the scientific community with invaluable tools for the study of old or novel genes. We must bear in mind that one of the oldest LSDB, the lacI mutation database, that collected all mutations in the LacI repressor, was established more than 40 years ago and has been a paradigm for the study of dominant negative mutations and molecular epidemiology and is still in use with BigBlue transgenic mice.

The large number of natural TP53 mutations that have already been published indicates that we have nearly reached mutation saturation and it is unlikely that novel mutations will be identified. Although cancer genome sequencing will allow large-scale analyses of mutational events, it could still be possible to narrow down the effect of a specific carcinogen on TP53. A good example is the specific effect of aflatoxin B1 at position 249 of the TP53 gene. Such a highly specific mutation hot spot identified for a single type of cancer has never been observed for any other genes. It has not been reproduced in animal models, indicating that TP53 codon

249 is a specific target in human liver cells for an as yet unknown reason. An important, but still unresolved issue, is the relationship between TP53 mutation and clinical parameters such as response to therapy or survival. This question has been raised for TP53 and other genes for a long time since the discovery of the first oncogenes and tumor suppressor genes more than 20 years ago. Is this type of question still relevant today? Over the last few years, genetic analysis of tumors has undergone a profound shift from analysis of single events to global studies comprising expression profiling, and analysis of mutational and chromosomal changes. The international cancer genome consortium project to sequence 25,000 cancer genomes will lead to an enormous volume of data that should allow global analysis. Analysis of pathway alterations, based on integration of structural and expression data, instead of a single gene analysis will be more accurate and will allow more comprehensive clinical studies.

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