MOLECULAR BIOLOGY INTELLIGENCE UNIT

p53

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LANDES BIOSCIENCE
AUSTIN, TEXAS
USA

SPRINGER SCIENCE+BUSINESS MEDIA
NEW YORK, NEW YORK
USA

p53

Molecular Biology Intelligence Unit

Landes Bioscience
Springer Science+Business Media, LLC

ISBN: 978-1-4419-8230-8 Printed on acid-free paper.

Springer Science+Business Media, LLC, 233 Spring Street, New York, New York 10013, USA.
http://www.springer.com

Please address all inquiries to the Publishers:
Landes Bioscience, 1806 Rio Grande, Austin, Texas 78701, USA.
Phone: 512/ 637 6050; FAX: 512/ 637 6079
http://www.landesbioscience.com

Printed in the United States of America.

9 8 7 6 5 4 3 2 1

Library of Congress Cataloging-in-Publication Data

p53 / [edited by] Ayeda Ayed, PhD, Ontario Cancer Institute, University of Toronto, Toronto, Ontario, Canada, Theodore Hupp, PhD, Institute of Genetics and Molecular Medicine, CRUK p53 Signal Transduction Laboratories, University of Edinburgh, Edinburgh, Scotland, UK.
p. ; cm. -- (Molecular biology intelligence unit)
Includes bibliographical references and index.
ISBN 978-1-4419-8230-8 (alk. paper)
1. p53 antioncogene. 2. p53 protein. I. Ayed, Ayeda, 1962- editor. II. Hupp, Theodore, 1969- editor. III. Series: Molecular biology intelligence unit (Unnumbered : 2003)
[DNLM: 1. Cell Transformation, Neoplastic--genetics. 2. Genes, p53. QZ 202]
RC268.44.P16P15 2011
572.8'6--dc22

2010046107

About the Editors...

AYEDA AYED is on leave from the Ontario Cancer Institute at the University of Toronto where she worked as a Postdoctoral Fellow and Associate Scientist on structural aspects of p53 using nuclear Magnetic Resonance Spectroscopy. She was the recipient of the Governor General's Award in Leukemia Research and a National Cancer Institute Fellowship. She obtained her PhD in Chemistry at the University of Manitoba in Winnipeg and currently resides in Toronto, Canada.

About the Editors...

THEODORE HUPP was trained in Chemistry as an undergraduate at Bowling Green State University in Ohio working with Bill Scovell and applied developing interests in life sciences towards a PhD degree at Michigan State University under the mentorship of Jon Kaguni. Interests in enzymology was applied to the cancer field working with Sir David Lane during the time when the p53 field was discovering key p53-inducible genes like *p21*, transgenic technologies showed the key role of p53 as a tumor suppressor, and the p53 protein was found to amenable to activation by post-translational modifications such as phosphorylation, ubiquitination, peptide ligands, or small molecules. The Hupp lab is now based at the University of Edinburgh (UK) funded by the Cancer Research UK charity, where enthusiastic students and colleagues continue to study fundamental enzymological aspects of p53 control by ubiquitination, acetylation, and phosphorylation with the hope of developing novel therapeutics for activating the p53 pathway in human cancers.

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PREFACE

Our understanding of human cancer in the past 40 years has been driven by linking innovative concepts and cutting edge technologies to key problems identified by clinical research. Some of the successes in cancer genetics identified from clinical work have been the identification of specific gene deletions in human chromosomes, the use of PCR-based cloning methodologies to identify and clone human cancer genes, the validation of the human cancer genes using transgenetic technologies in the mouse, and the ability to sequence whole genomes that has recently allowed a collation of all somatic and germline mutations in a human genome. In the same generation, entirely different disciplines involved in basic life science research have used model organisms like yeast, flies, worms, and cancer causing animal viruses as tools to develop windows to see into the machinery of the cell life cycle. The discoveries of pro-apoptotic genes, oncogenes, and covalent control mechanisms like phosphorylation and ubiquitination using the tools of science and technology have all been awarded Nobel prizes for their contribution to our understanding of how cells work. The discovery of p53 using the tumor causing animal virus SV40 falls into this pioneering period of biological and medical research. Now, at the 30th year anniversary following the discovery of p53, the international community has demonstrated the fundamental role of p53 in cancer suppression, reproduction, ageing, and anti-viral immunity, further cementing the fundamental role of p53 as a key gene maintained by natural selection to contribute to fitness and health. Although knowledge on p53 continues to advance in leaps and bounds, and is all revealed in international journals, it is relatively difficult for students entering the cancer field or p53 field to get a historical or practical grasp on fundamentals of p53.

This volume, *p53*, was developed primarily as a resource for students to have access to key ideas in the field that have developed over the years including how transgenics have been used to study p53, how clinical genetics have identified and studied mutations in p53 found in human cancers, how p53 can be regulated by post-translational modification, and how key drug targets have been defined, namely MDM2, which has provided fundamental approaches for defining how p53 can be activated with potentially therapeutic effect. This book is by no means comprehensive and the large number of reviews published in peer-review journals always provide the cutting edge ideas developing in the field. However, the key concepts in the chapters included provide a perspective on key paradigms in the p53 field.

Theodore Hupp, PhD

CHAPTER 1

TP53 Mutations in Human Cancers:
Selection versus Mutagenesis

Magali Olivier,* Audrey Petitjean, Claude Caron de Fromentel and Pierre Hainaut

Abstract

The tumor suppressor gene *TP53* differs from most other cancer-related genes by the very high prevalence of missense mutations which result in the expression of a mutant protein. Considerable variations are observed between mutation patterns from different types of cancer and from different population groups, reflecting both mutagenesis and selection processes. These mutations are compiled in a database which includes information on tumor histology and patient characteristics, allowing the analysis of *TP53* mutation patterns according to various parameters (http://www-p53.iarc.fr/). *TP53* mutations are also observed in the germline and are associated with a syndrome of early onset cancers, the Li-Fraumeni syndrome. Germline and somatic mutations are very similar and affect codons located in the DNA-binding domain of the protein. Six major hotspot codons account for 30% of all mutations. Most mutations lead to proteins with impaired transactivation activities. However, all mutations are not equivalent. In addition to the loss of wild-type activity, some mutants exert dominant-negative effects and/or acquire new pro-oncogenic activities. Our understanding of the behavior of mutant p53 functions is expanding and holds promises for applications to cancer risk assessment, early diagnosis, prediction of disease outcome, as well as for development of new therapeutic strategies.

Introduction

Cancer growth involves the sequential accumulation of genetic alterations in genes controlling cell proliferation, lifespan, responses to stress, relationships with neighbours and gene homeostasis.[1] Amongst these alterations, the *TP53* tumor suppressor gene (OMIM #191170) represents a focal point, irrespective of the tissue and cellular origin of the tumor.[2] *TP53* encodes the p53 protein, a transcription factor that controls the expression of several proteins involved in cell-cycle control, DNA-repair, apoptosis and differentiation. The p53 protein acts by inhibiting the growth of cells exposed to chemical or physical stress, including cancer cells. Thus, loss of p53 functions promotes cell growth under conditions which suppress the proliferation of normal cells. The special role of *TP53* in cancer protection is also illustrated by the fact that Li-Fraumeni Syndrome (LFS), a familial syndrome of predisposition to multiple cancers, is caused by germline *TP53* mutation.[3]

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p53, edited by Ayeda Ayed and Theodore Hupp.

TP53 alterations typically include loss of alleles, gene mutations and inactivation of the protein by sequestration by viral or cellular proteins.[4] A database of mutations reported in human cancers is maintained at the International Agency for Research on Cancer (http://www-p53.iarc.fr/).[5] The nature and distribution of mutations vary among cancer types and population groups. Two main factors contribute to the shaping of a tumor-specific "mutation pattern". The first is mutagenesis: the type of damage caused by a mutagen can be specific in its nature and DNA sequence context, and the rate of mutation formation is limited by the cell's capacity to repair DNA lesions. The second is biological selection: only those mutants that have significant changes in their functional properties will induce a proliferative advantage and contribute to cancer. Weighting the contribution of these two factors provides interesting clues on the molecular mechanisms involved in the etiology and pathogenesis of human cancers. Mutations are also useful biomarkers in epidemiological and clinical studies and for patient management.

TP53 Alterations in Human Cancers

TP53 *Mutation Databases*

Soon after the identification of *TP53* as a frequent target gene for mutation in cancer,[6] it became evident that mutation patterns could significantly differ from one cancer to an other.[7,8] This observation led to the compilation of computerized lists of mutations that have now evolved into complex databases. The *TP53* database, maintained and developed at the International Agency for Research on Cancer (IARC *TP53* Database, http://www-p53.iarc.fr/), includes somatic and inherited mutations or variations that have been reported in the literature since 1989. Independent datasets or mirror datasets are maintained by other groups, providing a variety of analysis tools for data mining (see list at http://www-p53.iarc.fr/p53databases.html).

The information compiled in the IARC *TP53* database includes precise identification of the mutation, detailed description of tumor specimen, patient demographics and, when available, individual risk factors, genetic background and clinical parameters. It is thus possible to search for associations between mutation patterns and individual risk factors. Curated data also include information on biological activities and structural properties of p53 mutant proteins and provide a list of mouse models with engineered *TP53* (Fig. 1).

It should be noted that the database is affected by several intrinsic biases.[9] First, only a minority of publications describes molecular epidemiological studies with adequate controls and exposure groups. Second, as the database is exclusively based on peer-reviewed literature, it reflects changing trends in reporting and publishing of mutations. Other biases may result from the use of different methods for mutation detection. Despite these limitations, the database is a powerful tool to retrieve and analyze large sets of mutation data and generate hypotheses about their causes and consequences.

Sequence Variations

Several polymorphisms in the coding and noncoding regions of the *TP53* gene have been identified in human populations (see list at http://www.p53.iarc.fr/PolymorphismView.asp). Most polymorphisms are located in introns, outside consensus splicing sites, and the functional consequences of these variations remain largely unknown. With the recent discovery of p53 isoforms that are generated by use of an alternative promoter or alternative splicing,[10] some of these variations may affect the production or stability of some isoforms. Indeed, an intronic polymorphism which consists of a 16 bp duplication in intron 3, p53PIN3 (rs17878362; A1: nonduplicated; A2: duplicated), has been shown to affect p53 mRNA levels, with the presence of the A2 allele being correlated with lower p53 mRNA levels and lower p53 activity.[11] Adding to the complexity of p53 regulation, a recent study showed that the p53 regulatory protein MDM2, which mainly regulates p53 through protein-protein interactions, is also able to bind p53 mRNA and facilitate its translation, and that silent mutations within the N-terminus of p53 can abrogate this effect.[12] Thus, synonymous polymorphisms may affect p53 function through this new mechanism.

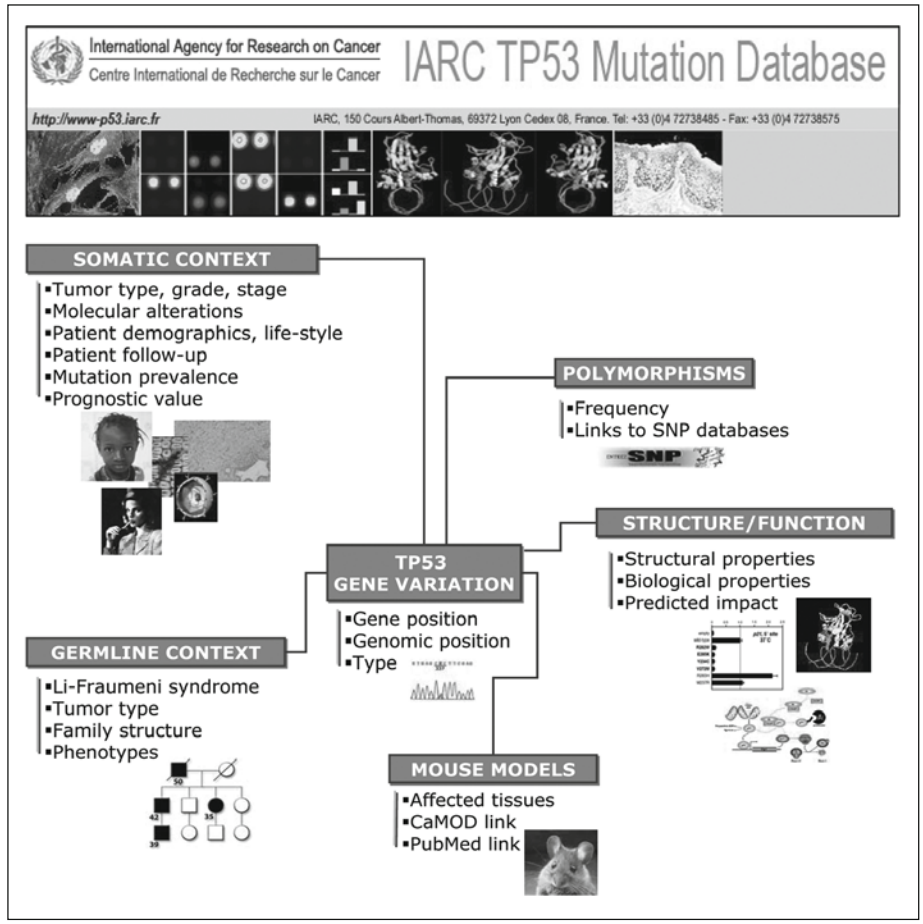

Figure 1. IARC TP53 Database online search system. The IARC *TP53* database can be searched and analyzed through a web interface (http://www-p53.iarc.fr/). Entire datasets, or sets of data selected according to user's queries, can be displayed and downloaded in tabular as well as graphical formats. A user guide is available that describes database and web site contents.

In the coding sequence, four polymorphisms alter the amino acid sequence of p53. There is sufficient molecular evidence that p53 function is affected by these polymorphisms for two of them only. One is a nonsynonymous variation in exon 4 (rs1042522; G/C) that leads to an arginine (R) to proline (P) amino-acid substitution at codon 72 (p53R72P). This residue is located in the proline-rich domain that is thought to be essential for a full p53 apoptotic response. p53 proteins containing a R or P allele display subtle changes in biochemical and functional properties that result in a more potent capacity of the R allele to induce apoptosis, while the P allele is more efficient in inducing cell cycle arrest.[13-15] However, the tissue specificity of these functional differences and their in vivo significance remains to be demonstrated. The other functional polymorphism is a rare C/T variation reported in African populations (rs1800371) that leads to a proline (P) to serine (S) amino-acid substitution at codon 47 (p53P47S). This codon is located in the transactivation domain and is close to a serine residue important for p53-dependent apoptosis induced by DNA damage as well as cellular senescence induced by oncogenic stress.[16] P47S was shown to be a poorer substrate for phosphorylation of serine 46 by p38 MAPK. However, the

consequence on protein transactivation capacity has shown conflicting results. While yeast assays showed a more potent transactivation capacity of 47S compared to 47P, other assays showed a decreased ability to transactivate two p53 target-genes, p53AIP1 and PUMA, but not other p53 response genes, which is correlated with a lower capacity to induce apoptosis.[17,18]

Residue 72, although not conserved, is located within the proline-rich region and may affect the structure of the putative SH3-binding domain. Sharp ethnic differences in codon 72 allele frequencies have been observed.[19] In the Northern hemisphere, the Pro72 allele shows a North-South gradient, from 0.17 in Swedish Saamis to 0.63 in African Blacks (Nigerians). In Western Europe (France, Sweden, Norway), North America (USA), Central and South America (Mexico, Costa-Rica, Peru) and Japan, the most common allele is Arg72, with frequencies ranging from 0.60 to 0.83. However, frequencies of Pro72 superior to 0.40 have been observed in African-American and Chinese populations.

Many studies have investigated the association of *TP53* polymorphisms with increased risk of cancer. p53R72P and p53PIN3 have been the most extensively studied, however no consistent results have been found. For example, a meta-analysis of 13 studies on the association of 3 *TP53* polymorphisms, including p53R72P and p53PIN3, and lung cancer risk failed to find any significant association.[20] In breast cancer, a large recent study has found that none of the frequent *TP53* SNPs (Single Nucleotide Polymorphisms) were associated with breast cancer risk.[21]

Overall, the functional significance and clinical impact of *TP53* polymorphisms is far from being understood.[22]

Somatic Alterations

Gene Mutations

Inactivation of p53 tumor suppressor functions by gene mutations is one of the most frequent alterations found in human cancers. Mutations are found in almost every type of cancer by the time the capacity for invasive growth has been acquired. The overall mutation frequencies range from 5% to 50% depending on the tumor type (Fig. 2). Malignancies with high mutation frequencies (40-55%) include ovarian, esophageal, colorectal, head and neck and lung cancers. Tumors of the brain, breast, stomach and liver show an intermediate mutation frequency (20-35%). Malignancies with low mutation frequency include cervical cancer, neuroblastoma, leukemia, sarcoma, testicular cancer and malignant melanoma.

Protein Interactions

In several cancers that do not carry *TP53* mutations, inactivation of p53 occurs by protein-protein interactions that either promote p53 degradation or inhibit its activity. In Human Papilloma Virus (HPV) related cervical cancers, the *TP53* gene is often wild-type, but the protein is inactivated by the HPV protein E6. E6 binds p53 in association with the cellular protein E6AP and targets it for proteasome-mediated degradation.[23,24] In soft tissue sarcomas, the *HDM2* gene is amplified and over expressed without evidence of *TP53* gene mutation in about 30% of the cases, leading to destabilization and inactivation of p53.[25] Amplification of *HDM2* is also observed in other tumors, but not always correlated with the presence of wild-type *TP53*.[26] In retinoblastoma, caused by Rb1 deficiency, a recent study showed that amplification of the *HDMX* gene and increased expression of HDMX protein was responsible for the suppression of the p53 apoptotic response triggered by Rb1 deficiency.[27] In neuroblastoma, Twist1, a protein involved in development, has been found to interact with and inhibit p53 activities.[28-30]

Other Alterations

Other modes of inactivation of structurally normal p53 have been proposed in testicular cancers, but the mechanism is unknown.[31] Loss of function through cytoplasmic retention has been observed in neuroblastoma and in inflammatory breast cancer.[32,33] A similar phenomenon has been proposed in some hepatocellular carcinomas associated with Hepatitis B Virus (HBV) infection, since transgenic expression of the HBx protein in mouse liver blocks p53

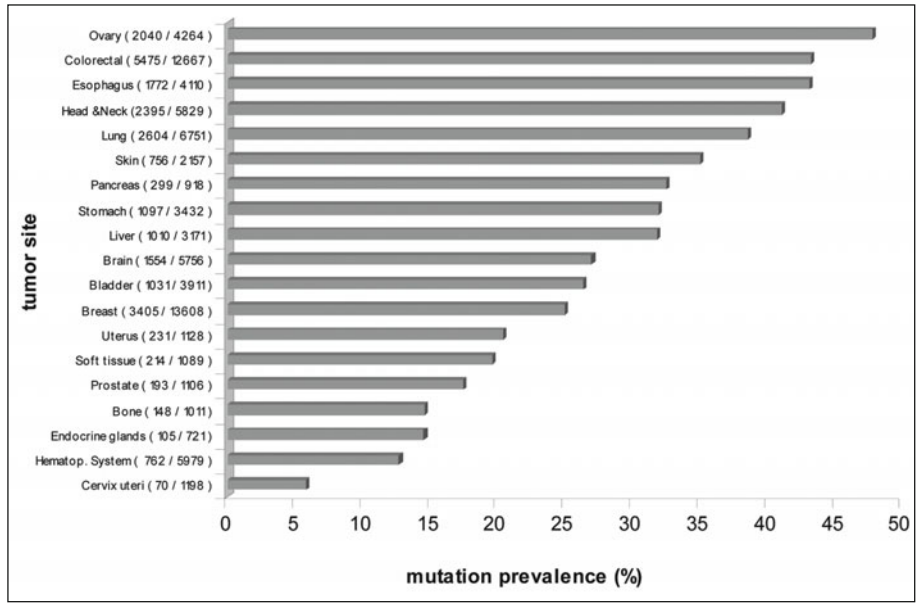

Figure 2. TP53 mutation prevalence in sporadic cancers. The proportion of tumors with somatic *TP53* mutations is indicated. Data from IARC *TP53* Database (R13, November 2008).[9]

entry into the nucleus.[34] Mutations in p53 downstream effectors have been searched for, but have produced largely negative results.

Germline Mutations

Inherited *TP53* mutations are associated with a rare autosomal dominant disorder, the Li-Fraumeni syndrome (LFS). LFS is characterized by familial clustering of tumors diagnosed before 45 years of age, mostly sarcomas, breast, brain and adrenocortical cancers.[35] Families with incomplete features of LFS are referred to as Li-Fraumeni-like Syndrome (LFL), for which several clinical definitions have been proposed.[36] In LFS/LFL patients, normal cells are heterozygous (*TP53* wild-type/mutant), but in cancer cells the wild-type allele is usually lost or inactivated by somatic mutation.

Although breast cancers, sarcomas (soft tissue sarcomas and osteosarcomas), brain tumors and adrenocortical carcinomas account for about 80% or all tumors arising in *TP53* germline mutation carriers, the spectrum of tumors observed in mutation carriers is wide (Fig. 3). This heterogeneous tumor patterns in LFS/LFL families may be explained in part by differences in *TP53* mutation types and their functional consequences.[36] In addition, polymorphisms in the *TP53* pathway have been shown to have modifier effects on *TP53* germline mutations.[37,38]

Types of Mutations

The type and distribution of inherited and somatic *TP53* mutations are very similar (Fig. 4). In contrast to many tumor suppressors such as *RB1*, *APC* or *BRCA1*, which are often inactivated by deletion, frameshift or nonsense mutations, most *TP53* alterations are missense mutations (73%) (Fig. 4A). About 35% of them fall within five "hotspot" codons (Fig. 4B) detectable in almost every types of cancer (codons 175, 245, 248, 273, 282). The corresponding residues are located in the DNA-binding domain of the protein. This domain has a complex structure made of two beta-sheets (forming a sandwich) bridged by flexible loops and helixes.[39] These loops are kept in place by the binding of an atom of zinc. The hotspot residues play important roles either

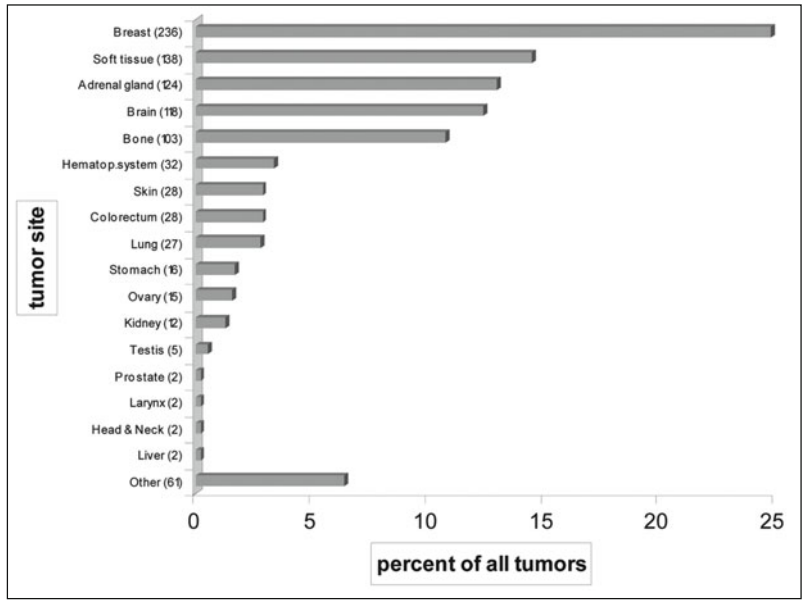

Figure 3. Tumor spectrum in individuals with a germline TP53 mutation. The proportion of specific tumor types among all tumors reported in confirmed *TP53* germline mutation carriers is indicated. Data from IARC *TP53* Germline Database (R13, November 2008, http://www-p53.iarc.fr/Germline.html).[9]

in protein-DNA contacts (codons 248 and 273) or in maintaining the conformation of the protein (175, 245, 282) (Fig. 5), explaining their high mutation frequency in cancer. However, all codons within the DNA-binding domain have been reported to be mutated in cancer and 80% of all mutations fall within this domain, reflecting its importance in the tumor suppressor function of p53. The main function of this domain is to interact with specific DNA sequences that regulate the transcription of p53 target genes. The main consequence of these mutations is thus a loss of p53 capacity to regulate its target genes. However, different types of mutations show different degrees of loss of function. Kato et al[17] have performed, in yeast assays, a systematic analysis of the transactivation capacity of all possible point mutants on several p53 responsive-elements. They showed that mutants that are found in cancer display severe loss of function, while mutants that retain some activity are rarely found in human tumors. These results show the importance of p53 transactivation capacity in its role as a tumor suppressor.

Sequence Variation and Phenotype

Several lines of evidence suggest that germline mutations may illicit tissue specific effects. The most striking example is the R to H mutation at codon 337 (R337H) that has been found in the Brazilian population and shown to predispose preferentially (although not exclusively) to childhood adrenocortical carcinoma.[40,41] Functional analysis revealed that this mutant is pH-sensitive, i.e., inactive (mutant-like) at pH>7.7 and active (wild-type-like) at pH<7.7.[42] The protein may thus adopt a mutant or unfolded conformation only under particular physiological conditions. Although this effect does not explain the tissue-specificity of the R337H mutant, this example illustrates the fact that mutant p53 protein function may depend on the cellular context.

Other evidence come from genotype-phenotype analysis of a large dataset of *TP53* germline carriers.[36] These analyses have shown that missense mutations affecting residues located in the L2 and L3 loops of the p53 structure, that bind to the minor groove of DNA, were preferentially associated with brain tumors, whereas those outside the DNA-binding surface (in the non-DNA-binding loops, beta-sheets and oligomerization domain) were associated with

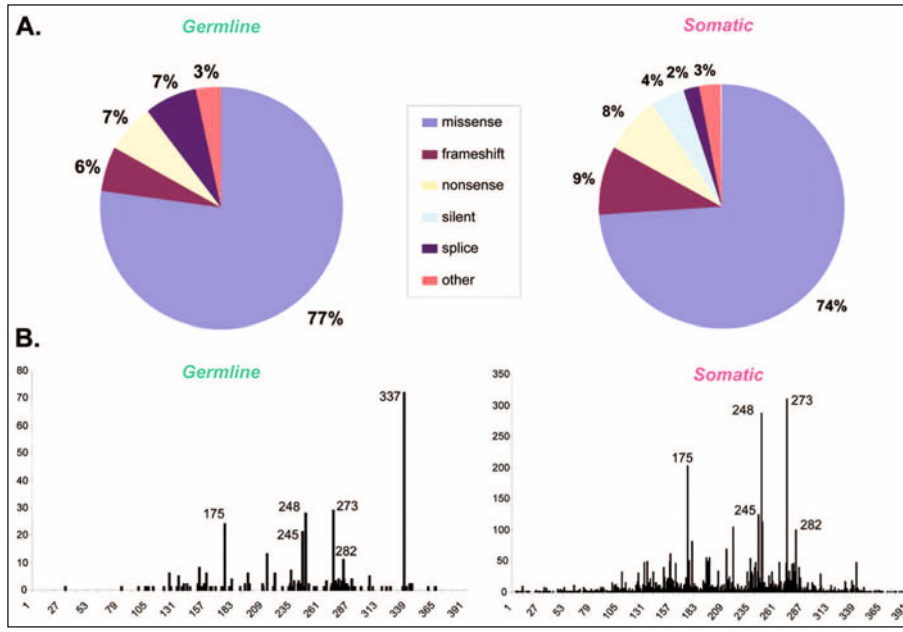

Figure 4. Comparison of germline and somatic TP53 mutations. A) Pie charts showing the proportion of the different types of *TP53* germline and somatic mutations. B) Histograms displaying the position of the germline and somatic point mutations in the coding sequence of the *TP53* gene. Data from the IARC *TP53* Database (R13, November 2008).[9]

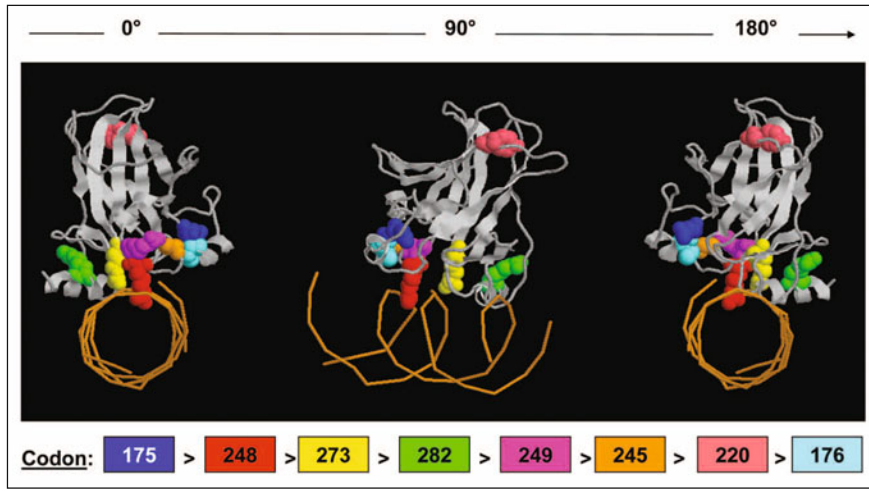

Figure 5. Structural localization of the most frequent TP53 mutations. 3D view of the core domain of the p53 protein in complex with DNA. This domain has a complex structure made of two beta-sheets (forming a sandwich) bridged by sets of loops and helixes. These loops are kept in place by the binding of an atom of zinc (zinc coordination by codons 176, 179, 238 and 242), which is essential for the stability of the whole structure. Codon 248 makes contact with DNA in the minor groove of the helix, whereas codon 273 makes contact in the major groove. Codons 175, 245, 249 and 282 play important roles in the conformation of the protein. Structure from Cho et al.[39]

adrenocortical carcinoma. Mutations resulting in a p53-null phenotype (frameshift deletions or insertions and nonsense mutations) were associated with early onset of brain tumors. Another analysis that used annotations derived from functional assessment of p53 mutants transactivation capacities, showed that the degree of loss of function was associated with age of onset of breast and colorectal cancers.[43] Thus, although the functional basis of these observations remains to be fully elucidated, the degree of loss of function may affect mutation penetrance in a tissue specific manner.

The Case for Mutagenesis

TP53 somatic mutation patterns look extremely similar from one cancer to the next. This similarity results from the fact that many mutations, in particular transitions at CpG sites, are common in all cancers. Nevertheless, several cancers show distinct patterns that indicate the presence of mutations induced by exogenous carcinogens. An "induced" mutation profile is suspected when the following features are present: (1) tumor-specific or exposure-specific "hotspot" mutations; (2) unusual predominance of a particular type of base substitution; (3) preferential accumulation of the mutation on the nontranscribed strand of DNA (strand bias).[44] Strand bias is the consequence of the preferential repair of DNA adducts on the transcribed strand by transcription-coupled repair systems.[45] This phenomenon results in the preferential accumulation of certain types of mutations on the nontranscribed strand.

TP53 *Mutations as Carcinogen Fingerprints*

The most distinctive mutation patterns have been observed in studies on populations exposed to high levels of mutagens. In general, the spectrum of *TP53* mutations is in keeping with mutation patterns generated experimentally by the suspected agents. A well-documented example is that of lung cancer, where a high prevalence of G>T mutations at specific residues have been correlated with exposure to tobacco and with a site of DNA adduct by benzo(a)pyrene, a major carcinogen contained in tobacco smoke.[46] Other examples include hepatocellular carcinoma (dietary aflatoxins) and nonmelanoma skin cancers (solar UVs). In several other cancers, such as bladder and esophageal carcinomas, specific mutation profiles have been observed, but the mutagens have not been clearly identified. These questions have been extensively addressed in recent reviews.[46-48]

Recently, cells derived from human p53 knock-in mouse models have been used to examine induced human p53 gene mutations in cell cultures exposed to mutagenic factors.[49] Mutations observed in these models were very similar to the ones observed in human tumors. Thus, these models provide a basis for generating experimental mutation patterns in human p53 and, together with the analysis of mutation patterns in human tumors, may help to identify carcinogens and mutagenic processes involved in the development of cancer with specific mutation patterns.

Mechanisms of Mutation

Table 1 presents a simple key for the the interpretation of different types of mutations found in sporadic tumors. The most frequent mutations (25%) are transitions (purine to purine or pyrimidine to pyrimidine) at cytosines within CpG sites. These transitions can, in the first instance, be considered as resulting from an endogenous mutagenic process. Spontaneous deamination of methylated cytosine occurs frequently at CpG sites, leading to a substitution to thymine. This process is greatly enhanced by oxyradicals, in particular nitric oxide (NO), that are generated endogenously during processes such as inflammation or bacterial infection.[50,51] In colon cancer, NO production has been correlated with the presence of transition mutations at CpG sites in *TP53*.[52] In contrast, transversions (purine to pyrimidine or vice-versa) at G bases (G:C to T:A or G:C to C:G) are often caused by bulky carcinogens in various experimental systems. G:C to A:T transitions at non-CpG sites can be induced by many different agents, in particular N-nitroso compounds, oxidizing agents and alkylating agents (for review see ref. 53). Altogether, non-CpG transitions and transversions at G bases represent around 40% of all mutations. About 10% of mutations are deletions with the majority being small deletions. Micro deletions, in particular in CG base repeats, are thought to primarily result from polymerase slippage during replication.[54]

Table 1.* TP53 *mutations in sporadic cancers and suspected mechanisms of mutagenesis

Mutation Type	Cancer with High Prevalence	Suspected Agents or Mechanisms
Insertions	Head and neck, Esophagus	
Deletions	Head and neck	Polymerase slippage; Irradiation?
CC tandem	Skin (other than melanoma)	UV
A:T bases	Esophagus (SCC), Head and neck	Acetaldehyde?
A:T>T:A	Liver (Hemangiosarcoma)	Vinyl chloride
G:C>A:T	Bladder, Many other cancers	Alkylating agents? Aromatic amines? Radiations?
G:C>A:T at CpG	Colon, Brain, Stomach, other cancers	Spontaneous deamination of methylated cytosines
G:C>C:G		
G:C>T:A	Lung	PAH (Benzo(a)pyrene)
	Hepatocellular carcinoma (hotspot at codon 249)	Aflatoxin B1

Data from IARC *TP53* database and references 44,53,91. SCC= squamous cell carcinoma.

Nucleotide substitution rates[55] derived from human-mouse aligned sequence of chromosomes 21 and 10 have been applied to *TP53* wild-type and mutated sequences to estimate the propensity of each mutation to occur as a neutral process from replication error or endogenous mutagenesis.[5] The comparison of these mutation rates with frequency of occurrence in cancers shows that rare mutants have the lowest median nucleotide substitution rates while frequent mutants have the highest rates (Fig. 6). Thus, underlying substitution rates are highly associated with mutation frequency, showing that mutagenesis (spontaneous or carcinogen-induced) plays a major role in shaping mutation patterns.

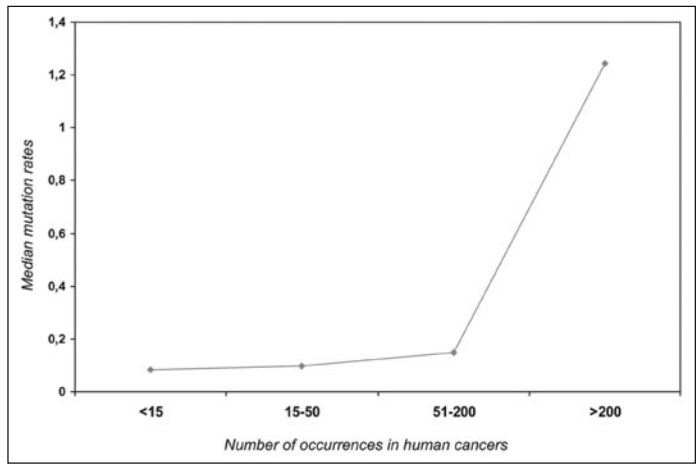

Figure 6. *TP53* mutation rates and frequency in cancer. Single amino-acid substitutions were grouped into four categories according to their frequency in the somatic dataset of the IARC TP53 database (R13, November 2008). The median mutation rates were calculated for each group of mutants. These rates were derived from dinucleotides substitution rates calculated for all point mutations according to Lunter et al.[55] Only mutations detected by DNA sequencing and located within the DNA-binding domain were included. Data from the IARC *TP53* Database (R13, November 2008).[9]