Chapter 12

ANALISYS OF P53 GENE AALTERATIONS IN CANCER: A CRITICAL VIEW

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> *He threw himself into the water and swam out in search of the swans, who caught sight of him and hurried towards him, their feathers ruffled. "Kill me" cried the poor animal, hanging his head towards the surface of the water, awaiting death. But what did he see in the transparent water? He saw his own image beneath him, no longer that of an ugly, dirty grey duckling, but that of a majestic swan. There's no harm in being born in a farmyard when you hatch from a swan's egg.*
>
> Hans Christian Andersen, *The Ugly Duckling*

INTRODUCTION

"Guardian of the genome" (Lane, 1992), "Death star" (Vousden, 2000), "Good and bad cop" (Sharpless and DePinho, 2002), "An acrobat in tumorigenesis" (Moll and Schramm, 1998), are just a few of the names that have been attributed to the p53 gene over recent years. However, the cameras (and funding) were certainly not present at the time of the discovery of p53 in 1979 (Crawford, 1983). It was only when the first alterations of the p53 gene in human cancers were discovered 10 years later, in 1989, that p53 started to become really popular, with the title of "molecule of the year" attributed by *Science*, in 1993 (Harris, 1993). This title was certainly justified, as the observation that more than one half of human cancers expressed a mutant p53 raised extensive clinical possibilities both for

diagnosis and treatment. As always, during the rapid growth phase of a new field of investigation, great hopes were raised and the pharmaceutical industry became actively involved. Although, from a scientific point of view, research has clearly shown the importance of p53 signalling pathways in the surveillance of the cell after a genotoxic stress (Vogelstein et al., 2000), clinical applications are nevertheless limited at the present time. This situation is not specific to p53, as technology transfer to clinical applications is always a difficult process. The contingencies required to validate a new marker are identical to those used to validate a new therapeutic molecule (see below). The field of diagnosis is also currently undergoing a major revolution with the development of high throughput technologies, such as DNA biochips, proteomic analyses or TMA (tissue microarray). Most of these technologies cannot be applied in routine clinical practice and therefore remain confined to the field of "gene discovery". On the other hand, their discoveries can have applications in routine clinical practice by means of technologies such as high throughput quantitative PCR, detection of mutations on DNA biochips or any other support allowing exhaustive analysis of a large number of samples. In the case of p53, a biochip could be developed allowing the simultaneous definition of the mutational profile of the gene, while also verifying the expression status of the genes involved in upstream or downstream signalling pathways.

In this article, I will review the analysis of p53 gene alterations in human cancers. The first part will discuss the specific properties of the p53 gene which make it an atypical tumour suppressor gene. The second part will evaluate the situations in which the diagnosis of p53 mutations can be useful and the third part will present a critical analysis of the various technological aspects of p53 analysis in human cancers.

THE P53 GENE: A TUMOUR SUPPRESSOR GENE OR AN ONCOGENE? A CARETAKER OR A GATEKEEPER?

The history of p53 is a chaotic voyage from the world of oncogenes to the world of tumour suppressor genes, while retaining a certain degree of individuality (Lane and Benchimol, 1990). Apart from artefactual problems related to involuntary cloning of mutant p53, this ambiguity is also due to our propensity to over-categorize in order to satisfy our Cartesian and oversimplistic view of science .

The idea that some p53 mutations can actively participate in cellular transformation was already postulated in 1990 and several arguments are in favour of such a model (Eliyahu et al., 1990; Lane and Benchimol, 1990). First of all, the mode of "inactivation" of wild-type p53. Unlike most other

tumour suppressor genes that are inactivated by frameshift or nonsense mutations leading to disappearance or aberrant synthesis of the gene product, almost 90% of p53 gene mutations are missense mutations leading to the synthesis of a stable protein, lacking its specific DNA binding function and accumulating in the nucleus of tumour cells (Soussi and Béroud, 2001). This particular selection for accumulation of p53 mutations in tumour cells can have two consequences: i) a dominant negative role by hetero-oligomerization with wild-type p53 expressed by the second allele, or ii) a specific gain of function of mutant p53. Many studies have tried to distinguish between these two hypotheses, with no clear-cut conclusions (Michalovitz et al., 1991; Milner, 1995). This task is further complicated by the fact that not all p53 mutations appear to be equivalent and present a marked heterogeneity of structure or loss of function. Transfection of various p53 mutations into cells devoid of endogenous p53 leads to an increase in their carcinogenicity, which varies according to the type of mutation (Dittmer et al., 1993; Halevy et al., 1990). This research into the oncogenic potential of certain p53 mutations is not purely theoretical, but has 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 p53 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 p53 protein (Aas et al., 1996). *In vitro*, the expression of p53 mutations in position 175 (R175H) specifically induces resistance of cells to etoposides compared to other p53 mutations (Blandino et al., 1999).

The two homologous genes of p53, p63 and p73, discovered 6 years ago, express many isoforms due to alternating use of transcription promoters and alternative splicing (Yang et al., 2002). Long isoforms (TA-p73 or TA-p63) are able to transactivate the same target genes as p53 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 p53 to induce apoptosis, suggesting the existence of a complex network of interactions between the products of these three genes (Melino et al., 2002). Although wt p53 does not interact with p73 or p63, some mutant p53 bind strongly to the two p53 homologs via their DNA binding domains. This interaction leads to the inactivation of p73 and p63 function (DiComo et al., 1999; Gaiddon et al., 2001; Marin et al., 2000) (Strano et al., 2000). Recent studies by T. Crook and B. Kaelin show that the activity of resistance to anticancer agents involves inactivation of the apoptotic function of p73 protein by a subset of mutant p53 that have sustained a change of conformation (Bergamaschi et al., 2003) (Irwin et al., 2003).

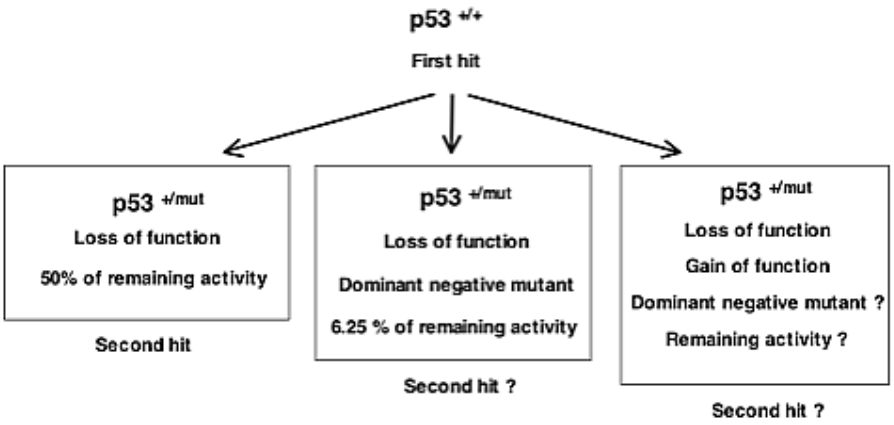

Figure 1. p53 and cancer: in the classical situation (left), the first hit leads to inactivation of p53 without affecting the activity of the second allele. However, this loss of function may have certain consequences for the cell. The second hit leads to complete loss of function of p53. In the case of a dominant negative mutant (middle), an estimated 1/16 of the tetramers theoretically have 4 wild-type monomers if the two proteins are expressed in identical quantities and tetramerization is not affected by the mutation. If the dominant negative effect of a single monomer is sufficient to inactivate p53, then the remaining activity will be 6.25%. In the case of partial penetrance of the dominant negative effect, the activity gradient will be between 6% and 50%. Loss of the second allele may not be mandatory, depending on the remaining activity. In the case of a mutant with a gain of function (right), the situation is more complex with an important combinatorial effect. Loss of the second allele may also be unnecessary in this case. This particular situation of the p53 gene results from: i) its particular mode of inactivation by missense mutations and ii) its tetrameric structure.

It is therefore likely that although the wild-type p53 gene is effectively a tumour suppressor gene, some p53 mutants can be considered to be oncogenes. The distinction between oncogene and tumour suppressor gene, although simple and able to account for the mechanisms of activation or inactivation of these various genes, is probably imperfect and does not allow characterization of all genes involved in the processes of carcinogenesis.

The genes involved can also be classified as a function of the various roles that they play in malignant transformation of a cell when they are altered. Three types of genes are distinguished at the present time(Kinzler and Vogelstein, 1997; Kinzler and Vogelstein, 1998). Gatekeeper genes regulate cellular homeostasis and the cell cycle by controlling the entry of the cell in the various phases of the cell cycle (Rb, VHL or APC). Caretaker genes participate in maintenance of the integrity of the genome and allow the cell to transmit an identical genome during successive cell divisions; they act as caretakers of the genome (MLH1, MSH2, or gene XP). Finally, landscaper genes maintain the integrity and equilibrium of the various cellular components of a tissue (PTEN, Smad4). Once again, the situation is

more ambiguous for the p53 gene, which can be classified as both a gatekeeper and a caretaker gene. Its apoptotic and antiproliferative activity make it an important gatekeeper and re-introduction of a wild-type p53 gene into a tumour cell effectively restores the cell cycle control properties. On the other hand, its possible activity in the control of chromosomal stability would classify p53 as a caretaker gene, whose phenotype cannot be corrected by functional supplementation. p53 probably has a very heterogeneous role in tumour processes as a function of the tissue considered, the chronology of the p53 alteration (early or late), the p53 function targeted (cell cycle or apoptosis) and finally the nature of the other alterations already present in the cell.

WHY ANALYSE P53 GENE ALTERATIONS IN CANCERS?

Before discussing the various approaches to the analysis of p53 gene status in human tumours, it is useful to ask the question of the practical value of this analysis. At the dawn of an age devoted to “high throughput” and “global analyses”, one wonders whether a diagnosis based on a single gene analysis, either p53 or another gene, has any future in clinical practice. Furthermore, as I will discuss below, the p53 protein belongs to a complex signalling network with a high tissue specificity. This has several important consequences in terms of diagnosis: i) the p53 pathway can be inactivated in many ways; ii) the behaviour of p53 mutants can be very heterogeneous according to the organ and the p53 gene status therefore probably does not always reflect the activity of the p53 signalling pathway

Multiple pathways of inactivation of the p53 gene

Inactivation of the p53 gene is essentially due to small mutations (missense and nonsense mutations or insertions/deletions of several nucleotides), which lead to either expression of a mutant protein (90% of cases) or absence of protein (10% of cases)(see the special issue of Human Mutation devoted to p53 published in January 2002 for more information). No inactivation of p53 gene expression by hypermethylation of transcription promoters has been demonstrated at the present time, which supports the hypothesis of a function for p53 mutants. In many cases, these mutations are associated with loss of the wild-type allele of the p53 gene located on the short arm of chromosome 17, which is why the p53 gene is said to behave like a classical tumour suppressor gene with a mutation on one allele and loss of heterozygosity (LOH) of the second allele. This is in line with the

good concordance observed between cancers with a high frequency of p53 gene mutations and the frequency of LOH of the short arm of chromosome 17. However, another tumour suppressor gene, located adjacent to p53, could also be the target of these deletions (Makos Walles et al., 1995). In fact, many studies on murine models show that the haplo-insufficiency of p53 is sufficient to lead to an abnormal cell phenotype (Venkatachalam et al., 2001). Almost 50% of tumours in these mice still express the wild-type allele. It is therefore perfectly possible that inactivation of the remaining wild-type allele is not systematically necessary. As indicated above, the dominant negative activity of some mutants may not always require LOH of p53.

Although the presence of a p53 gene mutation is generally unambiguous, other situations are much more equivocal (Table 1). The mdm2 protein regulates the stability of the p53 protein by ubiquitination and transport towards the proteasome (Iwakuma and Lozano, 2003; Moll and Petrenko, 2003). Abnormal accumulation of the mdm2 protein is observed in many tumours, especially sarcomas (Onel and Cordon-Cardo, 2004). This accumulation can be due to amplification of the mdm2 gene, enhanced transcription of the gene or enhanced translation of its messenger RNA (Michael and Oren, 2002). Although these tumours would be expected to no longer express p53, the opposite situation is generally observed, with a large number of tumours overexpressing both p53 and mdm2. The reasons for this apparent paradox have not been elucidated. No formal exclusion between p53 gene mutation and mdm2 accumulation has been clearly demonstrated, suggesting that this situation could be due to an oncogenic activity of mdm2 independent of p53.

The situation is somewhat clearer for cervical cancer. The E6 viral protein expressed by HPV specifically binds to the p53 protein and induces its degradation (Scheffner et al., 1990). This observation explains the rarity of p53 mutations in cervical cancers (Crook et al., 1992). p53 inactivation by a viral protein has not been formally demonstrated in other human cancers associated with viral infection, such as HCC (associated with HBV) or Burkitt lymphoma (associated with EBV). In inflammatory breast cancers or neuroblastomas, molecular and immunohistochemical analyses demonstrate accumulation of wild-type p53 in the cytoplasm of tumour cells, leading to functional inactivation of p53 (Moll et al., 1995; Moll et al., 1996; Moll et al., 1992).

Insert table 1

Table 1. Multiple pathways of inactivation of p53.

Inactivation	p53 gene status	p53 protein status	Type of cancer	Possible method of analysis*
p53 gene mutation	Mutant	Stable, nuclear	50% of all cancers	Molecular Immunohistochemical Functional
mdm2 amplification	Wild-type	Stable, nuclear	Sarcoma	Molecular Immunohistochemical
Nuclear exclusion (overexpression of the Parc protein)	Wild-type	Cytoplasmic	Neuroblastoma Inflammatory breast cancer	Immunohistochemical**
chk2 gene mutation	Wild-type	Not induced?	LFS (rare)	
Alteration of p14ARF	Wild-type***	Nuclear	20% of cancers, melanomas	
Alteration of Apaf1	Wild-type	Nuclear	Melanomas	
HPV 16 or 18 infection	Wild-type	Degraded	Cervical cancer	

* for p53 status only. For the other genes, the methodology depends on the modes of inactivation (see text for more details);
** the mechanisms leading to accumulation of parc are unknown at the present time;
*** the relationship between p14ARF mutation and absence of p53 mutation is not 100%

Nikolaev et al. recently isolated a new protein, parc, which sequesters p53 in the cytoplasm of cells in the absence of any lesions (Nikolaev et al., 2003). Abnormal accumulation of this protein is observed in neuroblastoma cells and could therefore account for functional inactivation of p53 in this cancer.

p53 mutations are very rare in malignant melanoma, a highly chemoresistant tumour, but are much more frequent in other skin cancers, such as BCC and SCC (Brash and Ponten, 1998). This situation could be due to an alteration of apoptotic pathways upstream and downstream to p53 signals. Alteration of upstream signals corresponds to inactivation of the CDKN2 locus, which expresses the $p14^{ARF}$ protein, an activator of p53 in response to an oncogenic stress and the cyclin kinase inhibitor p16. The interaction of $p14^{ARF}$ with mdm2 blocks MDM2 shuttling between the nucleus and cytoplasm via the nucleolus (Iwakuma and Lozano, 2003; Vousden and Woude, 2000). Sequestration of MDM2 in the nucleolus thus results in activation of p53. Germline alterations (point mutations) and somatic alterations (point mutations and hypermethylation of the promoter) of this gene are frequent in malignant melanoma and impair the induction of p53 after an oncogenic stress (Chin et al., 1998). Alteration of downstream signals corresponds to a marked reduction of expression of the pro-apoptotic apaf1 gene, the absence of which is correlated with resistance to chemotherapeutic agents (Soengas et al., 1999). Decreased apaf1 expression is due to hypermethylation of its promoter.

The role of the hchk2 gene, a kinase activated by ATM following irradiation, is more ambiguous. Many studies have shown that hchk2 is necessary for phosphorylation and stabilization of p53 after genotoxic lesions (Chehab et al., 2000; Shieh et al., 2000). These studies also found a strong supprt with the description of hchk2 germline mutations in families with Li-Fraumeni syndrome (LFS) not presenting any p53 mutations (Bell et al., 1999). However, these results probably need to be interpreted differently, as more recent studies have shown that, in human cells devoid of hchk2, p53 stabilization and induction of its target genes and arrest of the cell cycle after irradiation are perfectly normal (Ahn et al., 2003; Jallepalli et al., 2003).

Many mechanisms are therefore involved in the inactivation of signalling pathways regulated by p53, but the clinical consequences of each of these mechanisms needs to be more clearly elucidated.

Molecular epidemiology

The greatest contribution to the study of p53 mutations has been provided by molecular epidemiology and its applications (Harris, 1991; Soussi, 1996). We will not discuss these epidemiological studies in more detail, as they

have been the subject of many detailed reviews and are discussed in another chapter of this book. The most important findings of molecular epidemiology are summarized in table 2.

These studies demonstrate a link between exposure to various types of carcinogens 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. From a cognitive point of view, these findings are important in that they confirm that a large number of mutations are exogenous and therefore avoidable. On the other hand, these studies will not have any impact in terms of public health as they will not be followed by any political or administrative decisions to modify exposure situations. The only finding that could possibly have a major application concerns the mutation of codon 249 in liver cancer in individuals exposed to aflatoxin B1, a hepatocarcinogenic molecule expressed by a fungus, which frequently contaminates certain agricultural products harvested in tropical or subtropical regions. Contamination of food by this fungus and exposure to aflatoxin B1 are documented facts in several developing countries (Wild et al., 1993). The singularity of the unique mutation is due to the binding specificity of substances derived from aflatoxin B1 on codon 249 of the p53 gene (Puisieux et al., 1991). This specificity has allowed the development of extremely sensitive screening methods for this mutation (1 mutant copy per 10^5 wild-type copies) (Aguilar et al., 1993). By using these approaches, Aguilar et al. demonstrated that the mutation occurs very early and can be demonstrated in the liver of asymptomatic healthy subjects derived from regions exposed to aflatoxin B1 (Aguilar et al., 1994). No mutation was detected in subjects derived from non-exposed regions. This test has subsequently been successfully applied to the detection of p53 mutations in serum DNA from individuals living in high-risk regions (Kirk et al., 2000). This type of analysis should allow very early detection of individuals at high risk of developing hepatocellular carcinoma. However, it can only be effective when it is associated with an infrastructure able to follow these individuals and propose early intervention.

Insert table 2

Table 2. Relationship between p53 gene mutations and exposure to carcinogens. Only the most striking observations are summarized in this table. For more details, the reader can refer to the recent review by Vähäkangas et al. (Vahakangas, 2003)

Type of cancer	Particularity of mutations p53*	Genotoxic agent incriminated	Comments	References
Lung cancer	High frequency of G->T transversions** 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)
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)
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)
Hepatic angiosarcoma	High frequency of A:T -> T:A transversions	Vinyl chloride		(Hollstein et al., 1994)
Wilson's disease, haemochromatosis	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 p53 gene.	(Hussain et al., 2000; Marrogi et al., 2001)

* compared to p53 mutations observed in the absence of exposure to the agent incriminated.

** this high frequency of transversion is also observed in cancers of the oesophagus and head and neck cancers associated with drinking and smoking.

p53 mutations: a new clinical marker?

This is certainly one of the most chaotic subjects in the field of p53. This confusion is due to a number of explanations: diversity of methodologies and strategies used to analyse p53 status, marked heterogeneity in study populations, a very heterogeneous behaviour of mutant p53 and especially our current ignorance concerning all branches of p53 signalling pathways. Research has focussed on the prognostic value of p53 and also on the response to therapy, as it has now been clearly demonstrated that a large number of molecules used in cancer chemotherapy induce p53-dependent apoptosis.

It is essential to avoid confusion about the terms prognostic and predictive. A prognostic marker can be defined as any factor that, at the time of diagnosis, can provide information on the clinical outcome of the patient, such as survival or disease-free survival. The most powerful prognostic factors are tumour size, clinical spread (stage) and histological grade. Among the molecular markers that have been tested during the past decade, *N-MYC* amplification in neuroblastoma remains the best prognostic marker. A predictive factor is defined as any marker that gives information regarding the response to a specific treatment. Prototype predictive markers are the oestrogen and progesterone receptors that mediate the response to the hormone therapy tamoxifen. With a few exceptions, none of the potentially useful prognostic or predictive markers have led to any consistent results in independent clinical studies. Factors that influence these studies include inadequate patient recruitment (sample size, diagnostic entry criteria, heterogeneous treatment) and methodological problems (quality of starting tissue, assay variability). This unsatisfactory situation has led several authors to propose a hierarchy of prognostic and predictive studies, analogous to the hierarchical study design in drug trials (Sullivan Pepe et al., 2001). Such an approach allows logical exploration and step-by-step validation of potential markers. Phase I studies are early exploratory studies of the association between a prognostic marker and important disease characteristics. They should also lead to the definition of a standardized assay. Phase II studies should define the clinical utility of the marker by identifying the optimal cut-off value between high-risk and low-risk patients. Both of these retrospective phases should be performed in carefully controlled (preferably case-controlled) cohorts of well-defined patients. Phase III studies are large, prospective, confirmatory studies in which the marker is evaluated and compared with other well-defined factors. The *TP53* status in human cancer could be considered at the end of Phase I (Bray et al., 1998). Several meta-analyses have indicated that, despite disagreement in the literature, *p53* status could have prognostic significance in non-small-cell lung cancer

(Mitsudomi et al., 2000; Steels et al., 2001) or in breast cancer (Pharoah et al., 1999), so the time is ripe to begin Phase II studies to unravel the true potential of using *p53* status for clinical decision-making. This topic will be extensively developed by Borensen et al. in another chapter of this book.

p53 germline mutations

The discovery of germline mutations in families with LFS was a major argument leading to the classification of p53 as a tumour suppressor gene (Malkin et al., 1990; Srivastava et al., 1990). LFS, with autosomal dominant transmission, is a rare disease of young subjects who present a predisposition to various tumours (Li et al., 1988). The classical and historical definition is based on familial criteria, essentially the observation of a sarcoma in an affected subject before the age of 45 years with a first-degree relative who developed any type of cancer before the age of 45 years or with a second-degree relative with a cancer or sarcoma before the age of 45 years. It is difficult to estimate the incidence of this rare syndrome due to its poorly defined diagnostic criteria. The most characteristic tumours are osteosarcomas, soft tissue sarcomas, breast cancers in young subjects, leukaemias/lymphomas, brain tumours and adrenal cortical carcinomas. However, any type of tumours can be observed. There are an estimated 400 families with LFS in the world at the present time. A germline mutation of the p53 gene is detected in about 70% of LFS families and in certain families or cases suggestive of the syndrome, without strictly satisfying all of the criteria (LFL) (Varley, 2003). A germline mutation of the *hCHK2* gene has also been described in rare families (Bell et al., 1999). A subject with a deleterious mutation of the *TP53* gene has a 15% risk of developing a cancer at the age of 15 years, with a risk of 80% for 50-year-old women and 40% for 50-year-old men; the significant difference between the sexes can be almost completely explained by breast cancer. These patients also present a high risk of second cancers, especially radio-induced cancers.

p53 germline mutations have also been demonstrated in almost 50% of children with adrenal cortical carcinoma with no family history of cancer (Varley et al., 1999b; Wagner et al., 1994). This cancer of the adrenal cortex accounts for 0.2% of all childhood tumours with an international incidence of 0.5/million, and occurs more frequently in girls than in boys (ratio of 1.5:1). The incidence is higher in patients with isolated hemihypertrophy, Wiedemann-Beckwith syndrome, congenital adrenal hyperplasia and LFS. The biological causes of this specific association are unknown.

No international consensus has yet been reached concerning the management of LFS families, which remains subject to local guidelines. These patients have a high risk of developing various types of other primary

malignant tumours, which, because of their very broad spectrum, raise difficult screening problems. There are no data, at the present time, to suggest that a particular constitutional mutation is associated with a specific tumour type, but our knowledge of the biology of p53 suggests that DNA lesions induced by treatment of a first cancer in these patients with a constitutional p53 mutation can induce the emergence of cells with an abnormal genotype and therefore the appearance of other tumours.

No consensus has been reached to define the methodological approach to screening of the p53 gene status in affected families. The most advanced studies have been conducted by J. Varley's team, which clearly showed that only an approach involving exhaustive DNA (exons and introns) and RNA analysis can provide rigorous results (Varley et al., 1999a). The use of the FASAY test can also be considered (see below).

Contribution of p53 gene mutation analysis to basic research into the p53 protein.

This contribution is rarely mentioned, but, in the case of p53, basic research provided an unexpected wealth of data. Historically, the first description of p53 mutations was published in 1989 (Nigro et al., 1989; Takahashi et al., 1989), while the transactivation and the specific DNA binding activity of p53 was only discovered several years later (Bargonetti et al., 1993; Fields and Jang, 1990; Pavletich et al., 1993; Raycroft et al., 1990). Analysis of the mutations detected in human cancers not only allowed definition of the functionally important regions of the protein, but their heterogeneity also allowed us to more precisely dissect the various functions of p53. The establishment of databases combining all of this information provided valuable tools for this research. I would like to cite 3 examples, which appear to be particularly representative of this type of study.

Proline 175 (H175P) mutation

Despite the fact that the H175P mutation is situated on a mutation hot spot (codon 175), it is very rarely detected in human cancers (4 times) in contrast with its little sister (H175R), reported 650 times (Soussi and Béroud, 2003). The H175P mutation has a normal cell cycle arrest and gene p21 induction behaviour (Ory et al., 1994), but is deficient for apoptotic activity and does not transactivate bax or PIG3 genes (Friedlander et al., 1996; Rowan et al., 1996). The reasons for this heterogeneity are unknown at the present time, but could be related to a difference of interaction with various co-activating molecules. A strain of transgenic mice specifically expressing this mutation was recently produced (Liu et al., 2004). These

mice have a very low predisposition to develop tumours compared to mice not expressing p53. However, these tumours do not present the chromosomal instability revealed in p53 -/- mice. These results derived from purely basic research, but based on a clinical observation, suggest that the apoptotic activity cannot be the primary activity targeted by p53 gene alterations (Attardi and DePinho, 2004). This represents a major challenge in relation to current models, which define apoptosis as being the fundamental activity of p53. Only the future will clarify the real role of p53.

3-5-2 Histidine 337 (R337H) mutation

The R337H mutation was found as a germline mutation specifically associated with paediatric adrenal cortical carcinoma in southern Brazil in several independent families that were not predisposed to other tumours (Latronico et al., 2001). In every transactivation assay, this mutant showed a wt behaviour. Precise chemical analysis revealed that this R337H mutant is highly sensitive to pH in the physiological range leading to folding changes depending on the protonated state of the protein (DiGiammarino et al., 2002). The observation that the syndrome associated with this mutation has been predominantly found in Brazil suggests that it could be linked to other modifier genes that could influence folding of p53 or cell pH. This type of observation emphasizes an important aspect of the p53 protein, its *in vitro* and *in vivo* flexibility and the influence of this flexibility on its properties. This type of observation must be considered in the light of the fact that almost 100 p53 mutants have a temperature-sensitive behaviour, i.e. active at 30°C and inactive at 37°C (Shiraishi et al., 2004).

Construction of a p53 mutation library

Many studies have emphasized the quantitative and qualitative heterogeneity of p53 mutations (Forrester et al., 1995; Ory et al., 1994; Resnick and Inga, 2003). In order to obtain a global view of this heterogeneity, Kato et al. constructed a library of 2,500 p53 mutations (Kato et al., 2003). Their transcriptional activity was tested on 8 transcription promoters characteristic of the various activities of p53 (cell cycle arrest, repair or apoptosis). Apart from the technical feat achieved by these authors, this study provides us with a wealth of valuable data to understand the individual activity of each p53 mutation, which can be classified according to the decreasing order of their remaining activity. It remains to be seen whether this classification has any correlation with clinical and laboratory parameters, such as response to therapy.

These three examples clearly illustrate this ping pong game, which can and must exist between basic research and clinical observations.

p53 gene mutations and therapy

Several chapters of this book are devoted to new therapeutic approaches applied to p53 and will not be discussed here. When these approaches are used in clinical trials, the patients' p53 status must be precisely defined in order to measure the efficacy of the new treatment.

3-7 Detection of p53 polymorphism in codon 72

Discovered in 1986, this intragenic polymorphism leads to the expression of two different p53 proteins, with Arginine or Proline in codon 72 in a region rich in proline residues (Harris et al., 1986). This region could be involved in the apoptotic activity of p53 (Walker and Levine, 1996). The distribution of this polymorphism in the general population is heterogeneous with a frequency of the Pro/Pro haplotype of 16% in Scandinavian populations and 63% in Nigerian populations (Beckman et al., 1994). The reason for this North/South gradient is unknown at the present time. Many studies have investigated whether one of the haplotypes could be associated with a higher susceptibility to develop cancers. The results of these studies are very contradictory and have not demonstrated any highly significant findings. In 1998, Storey et al. showed that $p53^{Arg}$ was very sensitive to the degradation activity induced by papillomavirus protein E6, while $p53^{Pro}$ was more resistant. This observation, that has not been contested, was associated with an epidemiological study on cervical cancer, which showed an over-representation of women presenting the $p53^{Arg}$ allele (Makni et al., 2000; Storey et al., 1998; Thomas et al., 1999b). Unfortunately, this second part of the study was strongly contested by many other studies (Helland et al., 1998; Hildesheim et al., 1998). Nevertheless, these two wild-type p53, $p53^{Arg}$ and $p53^{Pro}$, do not have exactly the same properties (table 3).

Some p53 mutants interact with p73 protein and inactivate its apoptotic function. Recently, T. Crook's team showed that this activity is specific to the $p53^{Arg}$ mutant (Bergamaschi et al., 2003). Analysis of a homogeneous population of patients with head and neck cancer demonstrates that the majority of patients expressing a mutant p53 associated with this polymorphism have a poor response to chemotherapy and a shorter survival. If this result is confirmed by other studies, it is therefore possible that analysis of this polymorphism could have a clinical value, but, as discussed below, this analysis is difficult to perform.

Table 3. Comparison of the biological activities of the two polymorphic p53.

	p53Arg72	p53Pro72	Reference
Sensitivity to HPV protein E6	Sensitive	Resistant	(Thomas et al., 1999b)
Induction of apoptosis	High*	Moderate	(Dumont et al., 2003)
Interaction with p73 (in the case of mutant p53)	High	Low	(Marin et al., 2000)
Association with response to treatment	Poor*	Better	(Bergamaschi et al., 2003)
Interaction with transcriptional machinery	Low	High	(Thomas et al., 1999b)
Transactivation	Moderate	Higher	(Thomas et al., 1999b)
DNA binding	identical	identical	(Thomas et al., 1999b)

* In each case, the concept of high/low or poor/better is relative and only concerns the comparison between the two forms of p53.

HOW TO ANALYSE P53 GENE ALTERATIONS IN CANCERS

Molecular analysis

Direct sequencing of the p53 gene after PCR amplification remains the "Gold Standard" of molecular analysis. For the p53 gene, this approach is facilitated by the fact that the 10 coding exons are smaller than 350 bp and can therefore be easily amplified individually. Mutations involving partial or total gene deletions are relatively rare.

Unfortunately, although considerable progress has been made in the field of DNA sequencing in terms of throughput, its sensitivity still remains limited. The major problem of molecular analysis of tumour specimens is the presence of normal cells (lymphocytes, stromal cells) that contaminate the tumour samples. According to the type of tumour or the type of sample, the rate of contamination can range from several percent (surgical tumour sample) to 50% (biopsies) or even more than 95% (urine, stools or bronchial lavage). It is generally accepted that direct sequencing requires at least 20% of mutant alleles, but this can vary considerably according to the quality of the sample. This qualitative aspect is generally underestimated. The quantity and quality of DNA obtained varies considerably according to the origin of the sample (frozen tumour, formalin- or paraffin-embedded tissues). This variability can lead to the generation of PCR artefacts, which can be falsely

interpreted as mutations. In the case of heavily contaminated samples, microdissection can be performed in order to enrich the tumour cell content, but this complicates the manipulations and cannot be performed routinely at the present time. The application of molecular technologies to routine analysis in hospital is a very important aspect. Many extremely sensitive molecular analysis methodologies have been developed, but their clinical application is generally limited because of the complex installation, their low throughput, the use of radioactivity or the need for highly qualified personnel.

Up until now, molecular analyses have been performed on exons 5-8 of the p53 gene, as the majority of mutations are located in these regions. It is generally established that 90% of mutational events are missense mutations leading to the synthesis of an abnormal protein that is not degraded and which accumulates in the nucleus of tumour cells. The remaining 10% of mutational events are nonsense mutations or small deletions that do not lead to accumulation of p53. This type of mutation excludes the possibility of using molecular methodologies such as PTT (Protein truncature test) based on expression of truncated proteins. More recently, molecular studies have been extended to the other exons, as exons 4, 9 and 10 have been found to contain a considerable number of mutations (about 15%) (Soussi and Béroud, 2001). Analysis of molecular events also shows a high proportion of nonsense mutations in these exons. Analysis of the latest version of the p53 gene mutation database shows that about 20% to 25% of mutations do not lead to the synthesis of a p53 protein. These mutations also present a marked variability as a function of the type of cancer: they are more frequent in lung cancers and breast cancers than in colon cancers (Soussi and Béroud, 2001). About 280 of the 393 codons of the p53 gene can be affected by a mutation. Furthermore, as each codon comprises 3 bases, which can each be altered generating a different amino acid, there is a very large number of theoretical combinations. 1,300 different variants have been identified in the p53 mutation database, which comprises more than 15,000 mutations derived from as many tumours (Béroud et al., 2000).

Many prescreening methodologies have been used to increase the sensitivity of detection of mutations and to concentrate the sequencing exclusively on the mutant exon. Unfortunately, many of these methods, possibly with the exception of DHPLC (denaturing high-performance liquid chromatography), remain confined to specialized laboratories and the sensitivity of detection of some of them is incompatible with the needs of clinical diagnosis. However, they present the advantage of being able to detect mutations in samples heavily contaminated by normal DNA.

An important point, rarely discussed, concerns the initial genetic material that can be used for molecular analysis. DNA is obviously a material of

choice, as it is robust and easy to handle, but many studies use RNA and its derived product cDNA for the detection of mutations. This is particularly the case when studying haematopoietic tumours, which are easier to manipulate than solid tumours. In the case of p53, there are several advantages to using RNA as starting material: i) the coding region is small (1,200 bp), allowing analysis of the entire gene; ii) several comparative studies of DNA versus RNA have demonstrated a higher sensitivity of detection of mutations with RNA (Forslund et al., 2002; Williams et al., 1998); iii) the use of RNA can detect the presence of splicing variants not identified on DNA. This last point is important, as the frequency of these splicing mutants of p53 has probably been underestimated. The most striking case is that of the mutation at codon 125 (ACG->ACA) described by many authors as a polymorphic variant. RNA analysis of this mutation, located at the 3' extremity of exon 4, shows that the splicing of the p53 gene is totally aberrant (Warneford et al., 1992).

No major progress in Sanger's sequencing technique has been made over recent years. The only new robust approach that has been developed is pyrosequencing, which does not require electrophoretic separation (Ronaghi, 2001). This approach has been successfully evaluated for the analysis of the p53 gene. However, it has a number of disadvantages at the present time, particularly the unreliable detection of frameshift mutations and the fact that it can only be applied to DNA fragments smaller than 100 bp.

On the other hand, considerable progress has been made in electrophoretic separation methods with the development of multiplexing and automation. Capillary electrophoresis (CE) now allows simultaneous separation of 96 reactions and this number will probably be increased over the next few years. Miniaturization of this approach and the development of new separation matrices and new solid phases have also led to the development of microchannel electrophoresis. The analysis of p53 gene mutations has often been used as a paradigm to evaluate the efficacy of these new approaches (table). We will not describe all of these methods in this review, but it should be noted that they can improve the sensitivity of conventional methods. The combined use of SSCP and HA techniques associated with solid-phase electrophoresis ensures a very high sensitivity (close to 100%) (Kourkine et al., 2002).

Insert table 4

Table 4. Comparison of techniques to identify p53 mutation. Adapted from Kirk et al. with the kind persmission of the author (Kirk et al., 2002).

	Advantages	**Current Limitations**	**References**
Direct sequencing	1) Detects any mutation up to 600 bp /reaction. 2) As rapid as SSCP and DGGE but more accurate. 3) High throughput with capillary electrophoresis	1) Electrophoresis of some specific DNA sequences can be difficult, leading to compression 2) Cannot be applied to tissues with less than 20% of tumour tissue (low level mutations)	many
Pyrosequencing	1) No problem of template compression during electrophoresis 2) High throughput 3) Identify the mutation	1) Does not detect low level mutations. 2) Can only be applied to short DNA fragments (< 100 bp) 3) Poor detection of deletions and insertions	(Garcia et al., 2000)
SSCP	1) Detects low level mutations. 2) Rapid, does not require extra enzymatic steps	1) Misses 30% of possible mutations. 2) Sequencing is necessary to identify the mutation 3) Does not locate the position of polymorphisms. 4) Can miss mutations adjacent to common polymorphisms.	many
DGGE, CDGE, DHPLC	1) Detects low level mutations. 2) Rapid, does not require extra enzymatic steps.	1) Large-scale screen missed 13% of mutations. 2) Sequencing is necessary to identify the mutation 3) Requires GC clamp; limited to small fragments. 4) Some regions can be refractory to analysis	(Breton et al., 2003; Cottu et al., 1996; Smith-Sørensen et al., 1993)
SSCP-HA	1) High throughput 2) Detects low level mutations 3) High sensitivity (close to 100%)	1) Sequencing is necessary to identify the mutation 2) New technique	(Kourkine et al., 2002)
Primer extension	1) Identify the mutation 2) High throughput when combined with a hybridization array	1) Some sequences can be refractory to primer extension 2) Cannot detect mononucleotide repeat insertions and deletions	(Shumaker et al., 2001; Tonisson et al., 2002)
ddF, REF	1) Detects virtually all possible mutations.	1) Does not detect low level mutations. 2) Sequencing is necessary to identify the mutation	(Feng et al., 1999; Kovach et al., 1996)
Cleavase	1) Heteroduplex not required.	1) High background. 2) Sequencing is necessary to identify the mutation 3) Requires optimization for each mutation.	(O. Connell et al., 1999)

	Advantages	Current Limitations	References
T4 endoVII, MutY, CEL I	1) Identifies approximate position of most mutations. 2) Identifies missense, frameshift and nonsense mutations.	1) Difficult to detect low level mutations. 2) High background for T4 endoVII and MutY. 3) Sequencing is necessary to identify the mutation	(Chakrabarti et al., 2000; Zhang et al., 2002)
Thermostable Endonuclease V -	1) Identifies the approximate position of the mutation. 2) Identifies missense, frameshift and nonsense mutations, up to 1,750 bp/reaction. 3) Detects low level mutations; 1 in 20. 4) In combination with sequencing, most rapid screen to directly identify mutation.	1) Does not detect transition mutations in GGCG or RCGC sequences. 2) New technique	(Huang et al., 2002)
LDR	1) Identifies the mutation 2) Detects point mutations, small insertions and deletions 3) High multiplexing capabilities 4) Detects low level mutations; 1 in 20 5) High throughput when combined with a capture array	1) Each mutation requires a specific pair of primers 2) New technique	(Favis et al., 2003; Fouquet et al., 2004)
Padlock probes, rolling circle amplification	1) No PCR amplification required 2) Compatible with in situ applications	1) Requires very long probes 2) Limited multiplexing capabilities	(Thomas et al., 1999a)
Hybridization array	1) Scans for mutations in thousands of positions. 2) Detects some small insertions/deletions 3) High throughput	1) Does not detect low level mutations. 2) Some arrays miss frameshift mutations	(Ahrendt et al., 1999; Okamoto et al., 2000; Wen et al., 2000)
(MALDI-TOF MS)	1) Identifies the mutation 2) High throughput	1) Each mutation requires a specific primer 2) New technique	(Kim et al., 2003)

This table provides information for the detection of small mutations (missense, nonsense and frameshift mutations). These technologies obviously do not detect large deletions or gene rearrangements. Reference are only given for the diagnosis of p53 mutations. The respective advantages and limitations can be different for other genes. Technologies such as PTT (protein truncation test), devoted exclusively to the detection of frameshift mutations, are not included in this table.

CDGE: Constant Denaturing Gel Electrophoresis; ddf: dideoxy Fingerprinting; DGGE: Denaturing Gradient Gel Electrophoresis; DHPLC: Denaturing High Pressure Liquid Chropatography; LDR: Ligase Detection Reaction; MALDI-TOF MS: Matrix-assisted laser resorption/ ionization time-of-flight mass spectrometry; REF: Restriction Endonuclease Fingerprinting; SSCP: Single Stranded Conformation Polymophism. SSCA-HA: SSCP Heteroduplex Analysis.

Multiplexing approaches have also been improved with the development of DNA biochips. They can use various approaches such as mismatch detection, primer extension or hybridization. Many studies have compared the sensitivities of these biochip approaches to those of more conventional methods and have generally shown a good concordance between the various methods (Ahrendt et al., 1999; Schaefer et al., 2002; Wen et al., 2000; Wikman et al., 2000). The only problem concerns deletions greater than 1 bp that are not analysed by these approaches, which can raise a problem in certain cancers such as ovarian or head and neck cancers, presenting a high rate of frameshift mutations.

The LDR technique was recently linked to a biochip binding approach. This combination allows high sensitivity and high throughput. Analysis of bronchial biopsies identified p53 gene mutations not detected by conventional approaches (Favis et al., 2003; Fouquet et al., 2004).

FASAY functional assay

Originally described for the detection of germline mutations in patients with LFS, this methodology has been improved and its current sensitivity allows it to be used for the detection of mutations in tumour samples (Flaman et al., 1995; Ishioka et al., 1993). The initial material used for FASAY (Functional Assay in Yeast) is cDNA obtained from tumour RNA. PCR amplification of this cDNA, using primers corresponding to codons 52 to 364 (68% of exons 4 to 10), followed by introduction of the PCR product into an indicator yeast, where it recombines with an expression vector, can be used to define the transactivating activity of the protein expressed. Red yeast colonies express mutant p53, while white colonies express wild-type p53 (Fronza et al., 2000). The amplified region corresponds to 95 % of the mutations identified to date, which makes FASAY a very good approach for exhaustive analysis of p53 gene mutations. All alterations leading to absence of RNA expression will obviously not be detected, but this is a relatively rare situation for p53. The only criticism that can be formulated in relation to this methodology is that it provides no information about the type of mutation, so that sequencing must always be performed subsequently. As sequencing is performed on DNA extracted from red colonies (mutant p53), problems of sensitivity are eliminated. This methodology can also be used to demonstrate splicing alterations. Waridel et al. have modified the FASAY technique to increase its sensitivity and robustness (Waridel et al., 1997). Recently, we used this approach to detect p53 gene mutations in biopsies containing only 5% of tumour cells (Fouquet et al., 2004). These mutations could not be detected by direct sequencing. In addition to this high level of sensitivity, the FASAY technique also presents the advantage of being

simple and robust. FASAY avoids selecting active variants (see below for problems related to this biological activity). It also has the advantage of being the only method able to rigorously demonstrate codon 72 polymorphism linked to the mutant allele (see beginning of the chapter for the significance of this polymorphism) (Tada et al., 2001). The cloning and sequencing of cDNA in the indicator yeast provide a non-fragmented molecule corresponding to the initial RNA expressed by the p53 gene. Direct sequencing of individual exons would not allow this type of analysis. Contamination of the sample by normal cells and the possibility of loss of heterozygosity prevents any interpretation. At the present time, FASAY remains the only approach suitable for the demonstration of associations between p53 gene mutations and codon 72 polymorphism (Tada et al., 2001).

The first generation FASAY test was based on the transcriptional activity of p53 on the RGC response element exclusively recognized by wild-type p53. This test is generally performed at 30°C, the growth temperature of the yeast. The observation that some p53 mutations are temperature-sensitive (active at 30°C, but inactive at 37°C), has led to the addition of a phase of yeast culture at 37°C to verify the presence of such mutants (Shiraishi et al., 2004). Various authors have also shown that, depending on the response element used (RGC, bax, WAF or PIG3), p53 mutants may have a heterogeneous behaviour (Campomenosi et al., 2001; Flaman et al., 1998). Some mutants that have only lost their apoptotic activity are inactive in relation to the PIG3 or bax elements, but continue to transactivate the WAF or mdm2 genes. On the other hand, the demonstration of pink colonies on the FASAY test shows that not all mutations have the same penetrance. New indicator yeasts allowing more accurate evaluation of p53 activity have been developed. It remains to be seen whether the use of these yeasts can help to increase the sensitivity of this test.

Immunohistochemical analysis

As the p53 protein is the end-product of gene expression, it therefore appears logical to try to directly visualize protein expression by immunohistochemical analysis linked with morphological analysis allowing qualitative evaluation of the cells presenting these defects (Dowell et al., 1994). p53 is a paradigm for this type of study, as most point mutations lead to the synthesis of a stable but “inactive” protein in the nucleus of tumour cells. Although this phenomenon was identified more than 10 years ago, no truly satisfactory explanation for this phenomenon has yet been proposed. Several non-exclusive possibilities have been proposed: i) absence of induction of mdm2 which can no longer regulate p53; ii) conformational

change and decreased sensitivity to degradation; iii) stability or over-translation of messenger RNA. It is interesting to note that normal cells from LFS patients do not overexpress p53, in contrast with the tumours observed in these same patients and in the absence of LOH. It is therefore possible that the tumour context is also important for stabilization of the p53 protein. This remains an unexplored field of investigation, which would certainly provide useful information.

Immunohistochemical studies concerning p53, as for other markers, suffer from a lack of standardization, leading to very heterogeneous results. The sources of heterogeneity are multiple: i) the various antibodies used; ii) methodological aspects (amplification, epitope unmasking); iii) the initial material (paraffin block, frozen tumour) and storage conditions; iv) the positive cut-off value, which can vary from 1% to 20% according to the authors; and v) individual variability of interpretation of the results (McShane et al., 2000; Schmitz-Drager et al., 2000).

Nonsense or frameshift mutations do not lead to accumulation of p53 protein. This is certainly due to instability of truncated proteins, which are generally not detectable despite the use of monoclonal antibodies which recognize an epitope situated in the amino-terminal domain of p53.

The correlation between missense mutations and nuclear accumulation appears to be 80% with variations from one type of cancer to another (Casey et al., 1996). The accumulation of wild-type p53 is more complicated to understand and its clinical and biological significance is unknown. One of the important parameters not revealed by immunohistochemical analysis is the variability of behaviour of p53 mutants (Hashimoto et al., 1999; van Oijen and Slootweg, 2000). Some monoclonal antibodies are described as being specific for p53 mutants (Pab240, HO15.4). However, they must be used cautiously, as: i) not all p53 mutants are recognized by these antibodies; and ii) denaturation of p53 during binding or epitope unmasking procedures leads to recognition of all forms of p53 (Legros et al., 1994b).

It is beyond the scope of this chapter to present an exhaustive review of the literature concerning immunohistochemical analysis of p53 and its clinical applications. The reader can refer to reviews already published on the subject or other chapters of this book (Hall and Lane, 1994; Save et al., 1998).

Like molecular methodologies, immunohistochemistry has also taken a great leap forward with the development of tissue microarrays (TMA), which allow simultaneous analysis of several hundred tumours with a single antibody (Kallioniemi et al., 2001). These TMA can also be used for in situ hybridization or FISH analyses. However, this approach cannot be used for routine diagnostic analyses, but it is very useful for immunohistochemical studies which can analyse several dozen different antibodies on sections

derived from the same TMA block. It would therefore be very easy to analyse combinations of antibodies and to evaluate their clinical significance in terms of prognosis or prediction of response to treatment. It is also possible to analyse the relationships between various antibodies and to study tumours in terms of "pathways" with multiple antibodies in order to determine the clinical significance and independence of each antibody (Hoos et al., 2001; Simon and Sauter, 2003). Analysis of 288 Hodgkin's lymphomas for the expression of 28 proteins involved in regulation of the cell cycle demonstrated that expression of genes involved in apoptosis is strongly correlated with poor prognosis (Garcia et al., 2003). Analysis of 852 small breast cancers (T1N0M0) showed that p53 expression is not correlated with survival, in contrast with c-erb2 overexpression (Joensuu et al., 2003).

Serological analysis of p53 gene alterations

In 1979, DeLeo et al. showed that the humoral response of mice to some methylcholanthrene-induced tumour cells such as MethA was directed against the p53 protein (De Leo et al., 1979). It was subsequently found that animals bearing several types of tumours elicited an immune response specific for p53 (Kress et al., 1979; Melero et al., 1979; Rotter et al., 1980). In 1982, Crawford et al. first described antibodies directed against human p53 protein in the serum of 9% of breast cancer patients (Crawford et al., 1982). No significant clinical correlation was reported, and at that time, no information was available concerning p53 gene mutations. Caron de Fromentel et al. later found that these antibodies were present in serum of children with a wide variety of cancers. The average frequency was 12%, but a frequency of 20% is observed in Burkitt lymphoma (Caron de Fromentel et al., 1987).

Since 1992, a new series of studies has shown that p53-Abs can be found in the serum of patients with various types of cancer, whereas the prevalence of these antibodies in the normal population remains very low. To date, the majority of published studies suggest that most patients with p53 antibodies have a p53 mutation leading to p53 accumulation. It is also clear that not all patients with a p53 alteration develop p53 antibodies. Comparison of the frequency of p53 alterations in the literature indicates that 30% to 40% of patients with an alteration of the p53 gene develop p53 antibodies (Lubin et al., 1995a).

It was initially believed that these antibodies were directed against the central region of the p53 protein, which is the target for the various mutations. Surprisingly, a careful study of the epitope recognized by these p53-Abs indicates that they bind both wild-type and mutant p53 (Labrecque and Matlashewski, 1995; Lubin et al., 1993; Schlichtholz et al., 1992;

Schlichtholz et al., 1994). Using either truncated p53 or synthetic peptides, it has been demonstrated that the epitopes recognized by the p53-Abs are mainly located in the amino and carboxy terminal regions of the protein, regions not corresponding to the p53 mutation hot spots (Lubin et al., 1993; Schlichtholz et al., 1992). These immunodominant epitopes have also been detected in the serum of mice and rabbits hyperimmunized with wild-type p53 (Legros et al., 1994a). Taken together, i) the presence of immunodominant epitopes outside the hot spot region of p53 mutations, ii) the correlation between p53 accumulation (and p53 gene mutation) in tumour cells and p53 antibody responses, iii) the similarity of humoral responses in patients independent of the cancer type and iv) the similarity of antigenic site profiles in patients and hyperimmunized animals, all suggest that p53 accumulation is a major component of the humoral response in patients with cancer. This accumulation could lead to a self-immunization process culminating in the appearance of p53 antibodies. As stated above, the level of p53 proteins in a normal organism is very low, suggesting very weak (if any) tolerance to endogenous p53 (Soussi, 2000).

Numerous studies have tried to determine the clinical value of p53-Abs (Bourhis et al., 1996; Cabelguenne et al., 2000; Lenner et al., 1999; Peyrat et al., 1995; Werner et al., 1997). As for p53 mutations and p53 immunohistochemical analysis, these studies have reported contradictory results and have been recently reviewed (Soussi, 2000). There is a trend towards an association between p53-Abs and poorly differentiated tumours, a feature already observed with p53 mutations.

As p53 accumulation is the main trigger of this humoral response, it was interesting to examine the behaviour of these p53-Abs during therapy to see whether there is a relationship between tumour disappearance and a decrease in p53-Abs. Several studies have addressed this question in various types of cancer (Angelopoulou and Diamandis, 1997; Angelopoulou et al., 1994; Hammel et al., 1999; Saffroy et al., 1999; Zalcman et al., 1998). Zalcman et al. showed that there is a good correlation between the specific time-course of p53-Abs titres and the response to therapy in patients with lung cancer (Zalcman et al., 1998). A similar situation was described in colorectal and ovarian cancer. In several patients, the disappearance of p53-Abs was very rapid, nearly as rapid as the half-life of human IgG (Lubin et al., 1995a). In breast cancer, it is possible to detect the reappearance of p53-Abs two years after initial therapy. This increase in p53-Abs was detected 3 months before the detection of a relapse. In these tumour types, p53-Abs could therefore be a useful tool to determine response to therapy and to monitor certain early relapses before they are clinically detectable.

p53 accumulation is the major component in the appearance of these p53-Abs. It is therefore reasonable to assume that p53-Abs could be used as an

early indicator of p53 mutations in tumours in which such alterations occur early during tumour progression. A good model to test this hypothesis is that of lung cancer and heavy smokers. It is well established that p53 accumulation is an early event in lung cancer and that this cancer is strongly associated with tobacco smoking. In 1994, p53-Abs were found in a heavy smoker in whom no lung cancer could be detected. Two years later, lung cancer was detected in this patient prior to any clinical manifestations of the disease (Lubin et al., 1995b; Schlichtholz et al., 1994). The patient showed good response to therapy in parallel with complete disappearance of p53-Abs (Lubin et al., 1995b). To our knowledge, this is the only prospective study addressing the importance of p53-Abs in individuals at high risk for cancer and using p53-Ab assays for clinical management of the patient. Since this publication, several studies have demonstrated the presence of p53-Abs in the serum of high-risk individuals (Trivers et al., 1995; Trivers et al., 1996).

A similar situation is observed in subjects with premalignant oral lesions (leukoplakia) due to tobacco or betel nut chewing. Such individuals are at high risk of developing oral cancer (5-10%). A high frequency of p53-Abs has been found in patients with premalignant and malignant lesions, suggesting that these antibodies could be used for early detection of cancer (Ralhan et al., 1998). Unfortunately, no follow-up has been performed on these patients. Due to the high frequency of this type of cancer in countries such as India or Pakistan, this type of diagnosis could be particularly useful. The recent discovery that p53-Abs can be found in saliva indicates that a simple screening method could be organized to verify the value of these antibodies (Tavassoli et al., 1998).

The majority of the literature clearly demonstrates the specificity of this serological analysis, as such antibodies are very rare in the normal population. The specificity of this assay can be estimated to be 95%. This high specificity is supported by the fact that p53 specifically accumulates in the nucleus of tumour cells after gene mutation. One of the disadvantages of this assay is its lack of sensitivity, as only 20% to 40% of patients with p53 mutations develop p53-Abs. This lack of sensitivity totally precludes the use of the assay to evaluate p53 alterations in human tumour. Nevertheless, if we estimate that there are 8 million patients with various types of cancer throughout the world, and 50% of them have a mutation in their p53 gene, then we can deduce that about 1 million of these patients would have p53-Abs.

There are several situations in which p53-Abs could be clinically useful. The first situation is that of serum monitoring during therapy. Only prospective studies on various types of cancer in which relapses occur several months or years after treatment would be able to validate this assay.

The use of standardized assays which have been validated for quantitative analysis would be useful for such studies.

The second situation concerns p53-Abs in high-risk individuals. One of the challenges of the next decade is the early detection of tumours using highly sensitive assays with gene probes specific for tumour genetic alterations. These approaches are still under development and remain costly. I believe that there is still room for serological assays of p53 antibody tumour markers. In developing countries, there is an increased burden of tumours due to carcinogen exposure as a result of increasing cigarette consumption, higher pollution caused by political laxity, uncontrolled industrial development and the absence of regulations concerning waste elimination. There may be a high incidence of p53 mutations in cancers related to this type of exposure and the use of an inexpensive assay for the detection of p53-Abs could be of public health benefit in these countries.

CONCLUSIONS

Fifteen years after the first description of mutations of the p53 gene, the clinical situation of p53 is still uncertain. The multiplicity of mutations and their properties makes the situation complex in terms of the clinical, biological and diagnostic significance. Unless the p53 status can provide new data, independent of the information provided by the other markers used at the present time, implementation of routine p53 diagnostic screening would be useless. On the other hand, as indicated throughout this chapter, the main problems related to the study of p53 are: i) heterogeneity of functions of mutant p53 and ii) a far from perfect correlation between p53 mutations and inactivation of the p53 signalling pathway. It is likely that, in the near future, global analysis of this signalling pathway could be evaluated more exhaustively by a mutational approach associated with an expression profile.

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