MUTATION IN BRIEF

Splice Mutations in the p53 Gene: Case Report and Review of the Literature

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Splice mutations in the p53 gene (TP53) are described as rare events that occur at a frequency of less than 1%. Using a functional assay based on the transcriptional activity of p53 and using RNA as starting material, we describe here a p53 splice mutation that could not be detected by genomic sequencing. This lack of detection is due to a deletion of the region complementary to primers commonly used for amplification. Reviewing the literature, we show that p53 splice mutations have been certainly underestimated and that careful strategy should be used for a complete mutational analysis of the p53 gene. Furthermore, some p53 gene mutations described as "neutral" due to the absence of any amino-acid change are truly deleterious, as they affect gene splicing. © 2002 Wiley-Liss, Inc.

KEY WORDS: p53; TP53; tumor suppressor gene; splice mutation; database

INTRODUCTION

The p53 gene mutation is one of the commonest genetic alterations found in human cancers [Soussi and Béroud, 2001]. The majority of these mutations are missense point mutations that are scattered along the entire p53 gene (TP53; MIM# 191170). The high degree of heterogeneity of p53 mutations and their heterogeneous distribution have led to the development of various screening procedures in order to increase the speed, sensitivity and specificity of p53 mutation detection. One of these methodologies, the Yeast Functional assay (FASAY), is based on the loss of p53 transcriptional activity after being introduced into a yeast indicator strain [Flaman *et al*., 1995; Ishioka *et al*., 1995]. As the starting material is RNA, it is potentially possible to detect aberrant transcripts resulting from splicing alterations provided the altered transcript is sufficiently stable and not degraded by a nonsense mediated decay pathway.

During the course of our study on p53 mutations in lung cancer, we have identified a particular p53 mutation detected by FASAY, but not detected by conventional screening procedures using DNA as starting material. Detailed analysis of this mutation revealed a small deletion that removes the junction – intron sequence, which is

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the site of one of the primers commonly used for genomic DNA amplification.

MATERIAL AND METHODS

Patients

108 patients with lung cancer were analyzed for somatic p53 mutations using both FASAY and direct genomic sequencing (C. Fouquet et al. manuscript in preparation). Patient 9098, described in the present study, had a small cell lung cancer. Biopsy was performed at the time of diagnosis as a part of the routine procedure performed in the Tenon Hospital Chest Surgery department. Frozen material was available at the time of definitive pathological diagnosis.

p53 mutation analysis

DNA and RNA extraction were performed simultaneously using the DNA/RNA minikit (QIAGEN) and resuspended either in TE or water in a final volume of 20 and 25 microliters, respectively. The cDNA was obtained from this RNA by reverse transcription and amplification by polymerase chain reaction (PCR) and p53 mutations were analyzed by the yeast functional assay, as previously described [de Cremoux *et al*., 1999]. Transcriptional activation is the critical biochemical function of p53, which underlies its tumor suppressor activity. Mutant p53 proteins fail to activate transcription. This transcriptional activity is also functional in yeasts, and p53 mutants that are inactive in humans are also inactive in yeasts. A yeast strain (yIG397), defective in adenine synthesis because of a mutation in its endogenous ADE2 gene, but containing a second copy of the ADE2 open reading frame controlled by a p53 response promoter, have been developed. Because ADE2-mutant strains grown on low-adenine plates turn red, yIG397 colonies containing mutant p53 are red, whereas colonies containing wild-type p53 are white. For the assay, the yeast strain yIG397 is cotransformed with RT-PCR-amplified p53 and a linearized expression vector, and the p53 cDNA is cloned *in vivo* by homologous recombination. To minimize mutations introduced during PCR, the high-fidelity polymerase Pfu DNA polymerase (Stratagene) is used. In the original assay described by Flaman et al., only one RT-PCR product was amplified and transformed in the recipient yeast [Flaman *et al*., 1995]. More recently, Waridel et al., have introduced an improvement in this assay in order to add an internal control in each transformation experiment [Waridel *et al*., 1997]. In this split FASAY, the p53 cDNA is amplified in 2 PCR overlapping fragments that are independently transformed in the recipient yeast with the adequate vectors, pFW35 and pFW34. The first fragment (P3-P17) corresponds to screening of residues 52 to 236, whereas the second fragment (P4-P16) corresponds to residues 195 to 364. As there is only one mutation per p53 cDNA, the main advantage of this improvement is that, for each sample, one PCR fragment will lead to background colonies, whereas the other fragment will lead to red colonies if a mutation is present.

For yeast transformation, 1 microliter of the RT-PCR product is co-transformed in the competent yeast strain yIG397 with the recipient plasmid, pFW35 or pFW34. After 3 days at 30°C, red colonies are scored. For patients with a suspected p53 mutation, the RT-PCR product was directly processed for sequencing using the Big Dye Read reaction terminator kit (PE Biosystem) and an ABI 3100 genetic analyzer.

RESULTS AND DISCUSSION

We have established a routine procedure for the detection of p53 mutations in lung cancer biopsies using the FASAY procedure. This material is often heavily contaminated with stromal and normal cells, but this is overcome by the sensitivity of the FASAY, which is about 10%. During the validation period of this assay, we observed a sample that gave positive results on the yeast assay (40% of red colonies), but no mutation could be detected by genomic DNA sequencing. Individual clones obtained by FASAY were sequenced leading to the detection of two aberrant cDNA species (Figure 1). The first species corresponded to a transcript in which the entire exon 8 was skipped. The second species resulted from an abnormal transcript including a partially deleted exon 8, part of intron 8 fused to exon 9 (Figure 1).

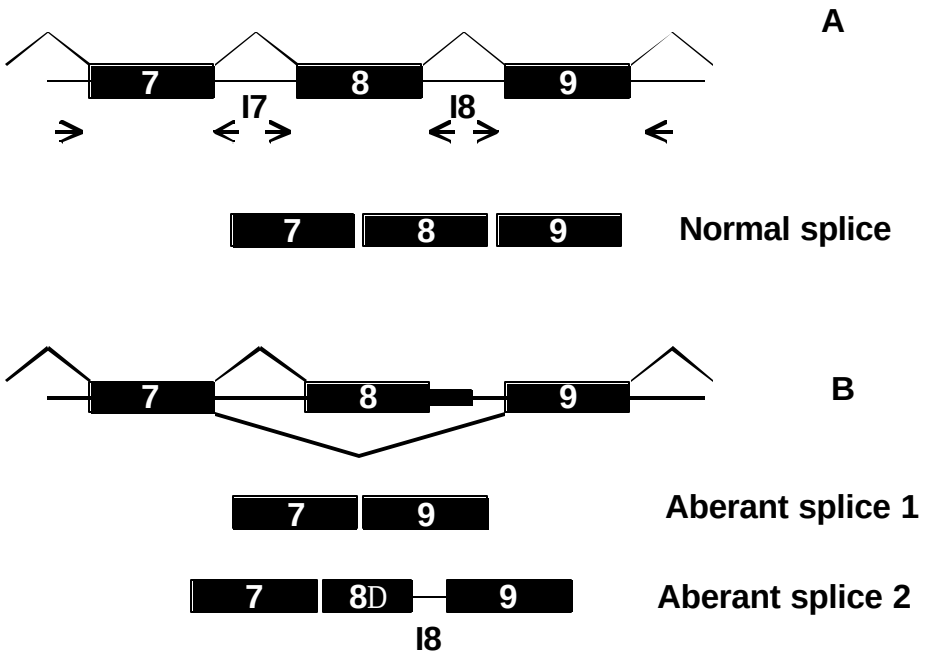

Figure 1: Diagram showing the 2 different splicing products detected in the tumor. **A:** Structure of the wild-type p53 and position of primers used for genomic DNA sequencing (arrows) **B:** Site of the deletion (hatched box) and structure of the two aberrant mRNA.

The sequence of these two cDNA suggests that a deletion removed the 3' end of exon 8 and the beginning of intron 8. PCR amplification of exon 8 from genomic DNA with a primer localized in intron 7 and 8 led to a PCR product with normal size and sequence (data not shown). Examination of the potential deletion deduced from cDNA sequencing suggests that the complementary region of the primer, localized at the 3' end of exon 8 could be deleted. The wild-type PCR product obtained after amplification could be derived from the normal DNA contaminating this biopsy sample. PCR amplification with a couple of primers localized in introns 7 and 9 demonstrated two bands, one with the expected size and a second with a lower molecular weight band (data not shown). Direct sequencing of this PCR product identified the exact boundary of the deletion (Figure 2). This alteration can lead to the synthesis of the two abnormal transcripts detected by the Yeast assay. Non-detection of this mutation by conventional direct sequencing was confirmed to be due to the deletion of one of the complementary sequences of the primer. Furthermore, as conventional direct sequencing uses DNA extracted from biopsies heavily contaminated by normal cells, a PCR product with a normal size was obtained. Consequently, this alteration would not have been detected without the use of the yeast assay. As direct sequencing after PCR amplification is the general methodology used for the detection of p53 mutations, our data suggest that some mutations, such as those described in the present study, could be missed. This mutation would also have been missed if prescreening procedures such as SSCP or DGGE were used, as amplification primers are also located in the deleted intronic region.

Figure 2: Characterization of the 2854_2925delinsC mutation. The region of exon 8 and intron 8 that has been deleted is shown in inverse video characters. Intronic sequences are shown in lower case, while exon sequences are shown in upper case. The underlined intronic sequence corresponds to the site of primers used for genomic amplification and sequencing.

Splice mutations are thought to be rare in the p53 gene, accounting for less than 2% of the database. Nevertheless, it is noteworthy that Varley et al. have reported germline p53 splicing mutations in 7 out of 40 families (18%) with Li-Fraumeni syndrome (LFS) [Varley *et al*., 1999]. Furthermore, in each case, functional analysis of RNA demonstrated that all these mutations induced the synthesis of aberrant transcripts. This high splice mutation rate could be due to a higher rate of splicing mutations in LFS. Nevertheless, it is also possible that detection of p53 germline mutations in LFS is performed under more drastic and rigorous conditions than screening for somatic mutations in a large number of tumors. Table 1 shows a survey from the literature describing splice mutations in the p53 gene. Only studies describing the consequences of aberrant splicing for RNA have been included.

It is fairly difficult to predict a splicing defect from genomic sequence analysis, even for mutations affecting acceptor and donor splices. As RNA is not always available, it is also difficult to establish the consequence of these mutations, especially for tumor tissue. It may be easier for germline mutations, as lymphocytes or lymphoblastoid cell lines constitute a good source for good quality RNA. We have recently added a new routine to the UMD Software for the analysis of the p53 mutation database, which automatically checks for the creation of a putative acceptor or donor splice site according to the consensus sequences described by Senapathy et al. [Harris & Senapathy, 1990]. Among the 652 silent mutations collected in the current release of the UMD-p53 database, we have detected 7 variations resulting in the creation of potential acceptor or donor splice sites. The strength of these sites has been evaluated using the calculation method described by Senapathy et al. [Harris & Senapathy, 1990]. The average score increased from 62.3 to 76.8 (+23%) supporting the hypothesis of the possible pathogenic involvement of these new splice sites. Among the other 14,118 mutations, 753 also result in a putative creation of potential acceptor or donor splice sites (data not shown). None of these mutations have yet been tested experimentally for aberrant splicing. It has already been documented in other genes that such exonic mutations (either silent or coding) could be deleterious for the production of intact RNA in genes such as ATM [Teraoka *et al*., 1999] NF1 [Fahsold *et al*., 2000] or RET [Auricchio *et al*., 1999]. Analysis of the data in Table 1 confirms this hypothesis. In six different cases, including 5 different Li-Fraumeni families, the same 582G>A mutation was shown to induce a splicing defect. This mutation is located in the last codon of exon 4 (125) and does not change the amino-acid residue. Analysis of the latest version of the Institut Curie p53 database (http://p53.curie.fr/) reveals 10 entries with mutations at codon 125, three of which do not change the amino-acid residue, which were described as neutral mutations by the authors. No RNA analysis was performed in these studies.

Table 1: Splice Mutations in the p53 Gene

Cancer	Starting material	Alteration	Mutation nomenclature	Consequence	References
Lung cancer	RNA and DNA	71 bp deletion of 3' exon 8 and 5'intron 8	2854_2925delinsC	2 aberrant transcripts	Present study
B-CLL	RNA only	Base substitution, SA intron 7	IVS7-2A>C	4 aberrant transcripts	[Bromidge *et al.*, 2000]
Myelodysplastic syndrome	DNA and RNA	46 bp deletion in exon 5 + Base substitution, SD intron 5	[1470_1522del + IVS5+2T>C]	1 aberrant transcript	[Kikukawa *et al.*, 1998]
Esophageal carcinoma	Southern and DNA	Deletion of 3' intron 8 and 5' exon 8	IVS7-37del48	1 aberrant transcript	[Huang *et al.*, 1994]
Schwannoma	DNA and RNA	Deletion of 3' exon 5 and 5' intron 6	No sequencing data	1 aberrant transcript	[SchneiderStock *et al.*, 1997]
KE-37R T- cell leukemia	RNA and DNA	Base substitution, SD intron 4	582G>A[1]	1 aberrant transcript	[Soudon *et al.*, 1991]
Lung	RNA and DNA	Base substitution, SA intron 3	IVS3-1G>C	1 aberrant transcript	[Takahashi *et al.*, 1990]
Lung	RNA and DNA	Base substitution, SD intron 7	IVS7+1G>T	1 aberrant transcript	[Takahashi *et al.*, 1990]
HCC	RNA and DNA	Base substitutionSD intron 7	IVS7+1G>T	1 aberrant transcript	[Lai *et al.*, 1993]
HCC	RNA and DNA	Base substitutionSA intron 6	IVS7-2A>T	1 aberrant transcript	[Lai *et al.*, 1993]
HCC	RNA and DNA	Base substitutionSA intron 6	IVS7-2A>G	1 aberrant transcript	[Lai *et al.*, 1993]
HCC	RNA and DNA	Base substitutionSA intron 3	IVS3-2A>T	1 aberrant transcript	[Lai *et al.*, 1993]
Germline mutation in a pediatric patient	DNA and RNA	11 bp deletion in SA intron 5	IVS5-14del11	1 aberrant transcript	[Felix *et al.*, 1993]
HCC	DNA and RNA	Base substitution, SD intron 5	IVS5+1G>A	1 aberrant transcript	[Hsu *et al.*, 1994]
HCC	DNA and RNA	Base substitution, SA intron 7	IVS7-2A>T	1 aberrant transcript	[Hsu *et al.*, 1994]
HCC	DNA and RNA	Base substitution, SA intron 7	IVS7-1G>T	1 aberrant transcript	[Hsu *et al.*, 1994]
HCC	DNA and RNA	Deletion of 2bp of 3' intron 5 and 8bp of 5' exon 6	IVS7-2del10	1 aberrant transcript	[Hsu *et al.*, 1994]
LU-143, SCLC cell line	RNA and DNA	Base substitution, SD intron 7	IVS7+1G>T	1 aberrant transcript	[Sameshima *et al.*, 1990]
Hereditary breast-ovarian cancer	RNA and DNA	Base substitution, intron 5	IVS5-2A>C	1 aberrant transcript	[Jolly *et al.*, 1994]
Bloom's syndrome GM1492 fibroblasts	RNA and DNA	Base substitution, SA intron 5	IVS5-3T>G	1 aberrant transcript	[Magnusson *et al.*, 2000]
LFS	RNA and DNA	Base substitution, SD intron 1	IVS1+1G>T	Absence of expression of the mutated allele in normal cells	[Verselis *et al.*, 2000]

Table 1. ***(continued)***

Cancer	Starting material	Alteration	Mutation nomenclature	Consequence	References
LFS	RNA and DNA	1 bp deletion, SD intron 9	IVS9+1delG	Absence of expression of the mutated allele in normal cells, 3 aberrant transcripts in transformed cells	[Verselis *et al.*, 2000]
LFS	RNA and DNA	Base substitution, SA intron 9	IVS9-1G>C	3 aberrant transcripts	[Verselis *et al.*, 2000]
Heand and Neck SCC	RNA and DNA	SD intron 7	IVS7+1G>A	1 aberrant transcripts	[Yeudall *et al.*, 1997]
LFS	DNA whole gene and promoter sequencing	Base substitution, SD exon 4 *	582G>A[1]	At least 4 aberrant transcripts	[Varley *et al.*, 1999]
LFS	DNA whole gene and promoter sequencing	Base substitution, SA intron 3	IVS3-1G>A	2 aberrant transcripts	[Varley *et al.*, 1999]
LFS	DNA whole gene and promoter sequencing	Short deletion in intron 3	IVS3-11C>G	3 aberrant transcripts	[Varley *et al.*, 1999]
LFS	DNA whole gene and promoter sequencing	Base substitution, SA intron 5	IVS5-1G>A	1 aberrant transcript	[Varley *et al.*, 1999]
Cancer-prone family	RNA and DNA	Base substitution, SD exon 4	582G>A[1]	1 aberrant transcripts	[Warneford *et al.*, 1992]

* 4 four different families

[1]This mutation changes the last nucleotide of exon 4 but does not change the amino acid residue.

Frebourg et al. recently reported an LFS family with an entire deletion of the p53 gene (Bougeard and Frebourg, personnal communication). This molecular event could be detected only by non-conventional methods, such as QMPSF (Quantitative Multiplex PCR of Short fluorescent Fragments) and could not be detected by RNA-based methodology. Taken together, these data emphasize the difficulty of molecular diagnosis of genes involved in tumorigenesis. Although false-negative results will not lead to any change in the global interpretation of mutation rates or molecular epidemiology, it can be critical in two situations: i) analysis of germline mutations and ii) analysis of mutations in relation to clinical parameters, such as response to treatment or survival. In this clinical setting, based on the analysis of a small number of patients, even a low percentage of missed mutations can have a profound impact on the significance of the results.

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