Pergamon

European Journal of Cancer Vol. 33, No. 9, pp. 1479–1483, 1997
© 1997 Elsevier Science Ltd. All rights reserved
Printed in Great Britain
0959-8049/97 $17.00 + 0.00

PII: S0959-8049(97)00136-6

Original Paper

An Intronic Deletion in *TP53* Gene Causes Exon 6 Skipping in Breast Cancer

G. Voglino,[1] S. Castello,[1] L. Silengo,[2] G. Stefanuto,[2] O. Friard,[2] G. Ferrara[3] and L. Fessia[1]

[1]Department of Clinical Pathology, Service of Histopathology and Diagnostic Cytology, Molecular Biology Laboratory, Ospedale Sant' Anna, via Ventimiglia 1, 10126 Turin; [2]Department of Genetics, Biology and Medical Chemistry, University of Turin; and [3]Advanced Biotechnology Centre-IST, Laboratory of Immunogenetics, Genoa, Italy

Six hundred and thirty primary breast cancer were screened for abnormalities in exons 5, 6, 7 and 8 of the *TP53* tumour suppressor gene. Analysis of the structure of the *TP53* gene exons was performed with the polymerase chain reaction–single strand conformation polymorphism (PCR–SSCP) method and with direct sequencing of amplified DNA. In a breast tumour case from a postmenopausal patient, we found a deletion of 36 bp in intron 5 and no immunohistochemical staining for p53. We amplified and sequenced the cDNA region between exons 4 and 7 and showed that the deletion causes the skipping of exon 6. The resulting mRNA sequence had a frameshift that yields an inactive protein with a truncated C terminus. These results show the first example of intronic deletion causing exon skipping at the *TP53* gene level. © **1997 Elsevier Science Ltd.**

Key words: breast cancer, p53, mutational analysis, RT–PCR, exon skipping

Eur J Cancer, Vol. 33, No. 9, pp. 1479–1483, 1997

INTRODUCTION

MUTATION OF the *TP53* tumour suppressor gene is the genetic marker most frequently detected in human cancer [1, 2]. Although the exact mechanism by which such a gene carries out is biological function is not compeltely clear, there is evidence that it plays two important roles in genomic stability: (i) blocking cell replication after DNA damage until it has been repaired, and (ii) starting apoptosis if the damage is too extensive [3].

The product of the *TP53* gene acts as a transcriptional regulator promoting expression of those genes that contain specific p53 binding sites and inhibiting the expression of other genes by its interaction with different transcriptional factors [4]. The loss of the normal function of p53 leads to a selective advantage in cell growth and increases the resistance to antitumoral therapy; thus the cells containing mutated p53 are clinically more aggressive [5].

Different studies have been carried out to show the link between prognosis and p53 expression in breast cancer [6–8]. Recently molecular biology techniques (PCR–SSCP, PCR product direct sequencing) have allowed a significant correlation between *TP53* mutation and poor prognosis in patients with breast cancer both in disease-free and overall survival [9]. More than 4200 cases of *TP53* gene mutation have been described of which so far 350 are related to breast cancer [10]. In exon 5 to exon 8, the mutations reported are as follows: 62% missense, 22% insertions or deletions, 13% nonsense and 3% splicing site; only one inframe deletion has been described at the junction of intron and exon 4 [11]. The latter normally involves the highly conserved 5′ GT (donor) and 3′ AG (acceptor) splicing sites.

Although the mechanism of splicing has still not been completely clarified, there is clear evidence that all pre-mRNA intronic sequences in vertebrate genomes are spliced similarly via the formation of a branched intermediate, and that they interact with small nuclear RNA (snRNA) conserved sequences. One of these branch sites in the primary transcript paired with U2 snRNA has been identified close to the 3′ intron splice site and presents the following consensus sequence: TNCTNAC where N can be one of the four nucleotides (A, C, G, T) and A is the bulged branch site [12]. Here we describe a deletion of 36 bases in the

Correspondence to G. Voglino.
Received 16 Sep. 1996; revised 10 Feb. 1997; accepted 27 Feb. 1997.

human *TP53* gene intron 5, which alters the branch site sequence and gives rise to a mature mRNA lacking exon 6.

MATERIALS AND METHODS

Biopsies

Six hundred and thirty primary breast cancer tissues were analysed during the last two years. All the biopsies were submitted to tumour/node/metastasis (TNM) staging and grading as reported by the Standard International Committee of Cancer. In addition oestrogen receptor (ER), progesterone receptor (PR), DNA ploidy and S-phase were assayed.

Immunohistochemical analysis

Five μm thick sections from fixed, paraffin-embedded tumours were stained with three different monoclonal antibodies for p53 (D0-1, D0-7 and Pab1801) detected by a streptavidin–biotin complex and immunoperoxidase labelling. Tumours with 5% or more cells expressing p53 to any of the antibodies were considered immunohistochemically (IHC) positive.

Mutational analysis

DNA was extracted from fresh breast tumour samples and blood samples by conventional phenol/chloroform methods.

Oligonucleotide primers used for the PCR amplification of *TP53* exon 6 were as follows: Ex6 Forward 5′-CTGGAGAGACGACAGGGCTGGTTG-3′; Ex6 Reverse 5′-CCAGAGACCCCAGTTGCAAAC-3′.

The PCR mixture contained 10 mM Tris-HCl (pH 8.5), 50 mM KCl, 2 mM $MgCl_2$, 0.1% (v/v) Triton X-100, 0.2 mM dNTPs, dinucleotide triphosphates 0.5 μM primers, 1 U Taq polymerase in 50 μl reaction. DNA template (50 ng) was amplified in a thermal cycler (Omnigene, Hybaid, U.K.) using a touch-down programme: 2 min at 94°C for an initial denaturation; 94°C for 30 s, 62°C for 30 s, 72°C for 30 s for 4 cycles; 94°C for 30 s, 60°C for 30 s, 72°C for 30 s for 4 cycles; 94°C for 30 s, 58°C for 30 s, 72°C for 30 s for 4 cycles; 94°C for 30 s, 55°C for 30 s, 72°C for 30 s for 30 cycles; finally 72°C for 5 min.

10 μl of PCR products were electrophoresed on a 2% TRIS-Bonate-EDTA (TBE)–agarose gel and visualised with ethidium bromide (0.5 μg/ml(. For the SSCP analysis 1 μl of PCR product was added to 5 μl of a denaturing solution containing 95% formamide, 20 mM EDTA (pH 8.0), 0.05% xylene cyanol and 0.05% bromophenol blue. Immediately before electrophoresis, the samples were heated to 95°C for 5 min and placed on ice. Two μl of denaturated samples were loaded on to a 0.75 mm thick 12% polyacrylamide (29:1 ratio of acrylamide to bisacrylamide) gel with 0.5× TBE buffer (pH 8.4) using a Protean II slab cell (BioRad, U.S.A.). Electrophoresis was carried out at room temperature at 5 mA constant current for 3–4 h. Single-strand DNA was visualised by silver staining (BioRad).

mRNA analysis

The mRNA of the mutated *TP53* gene was analysed by reverse transcription–polymerase chain reaction (RT–PCR). Specific primers covering the open-reading frame from the 3′ region of exon 4 to the 5′ region of exon 7 have the following sequence: R1 sense 5′-AAGTCTGTGACTTGCACG-3′; R2 antisense 5′-CTGGAGTCTTCCAGTGT-3′. Total RNA was extracted from frozen tissue with a FastTrack 2.0 mRNA Isolation Kit (Invitrogen, The Netherlands) and 5 μg were processed for reverse transcription in 50 μl of reaction volume, using R2 primer (0.5 μM) and reagents supplied by the GeneAmp RNA PCR Core Kit (Perkin-Elmer, U.S.A.). PCR was carried out in the same conditions described above and using the primers R1 an R2.

DNA sequencing

PCR products were sequenced with PRISM Ready Reaction Dye Deoxy Terminator Cycle Sequencing Kit Protocol and using an ABI PRISM 377 DNA Sequencer Apparatus (Perkin-Elmer) according to manufacturer's instructions. Manual sequence was performed using the AmpliCycle kit according to manufacturer's instructions (Perkin-Elmer). Results were analysed using a recent database [13].

RESULTS

Six hundred and thirty primary breast cancer tissues were analysed over the last two years. Thirty per cent mutations were found mainly due to missense (70%), followed by deletion/insertion (18%), nonsense (10%) and splice site (2%). Here we report a breast tumour case (pT2, pN0, pM0, G3, ER = 50%, PR = negative p53 IHC = negative, DNA index = 1.89 and S phase = 6.6%) found in a postmenopausal patient (Patient 568).

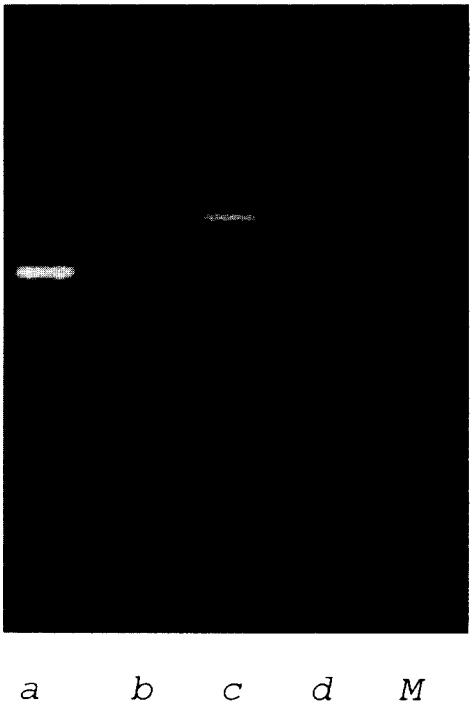


Figure 1. Agarose electrophoresis of ethidium bromide stained p53 amplification products. (a) Normal exon 6 (206 bp); (b) 568 exon 6 (206 and 170 bp); (c) product of RT–PCR on normal RNA (485 bp); (d) product of RT–PCR on 568 RNA (485 bp and 372 bp). M, marker DNA size standard (AmpliSize Bio-Rad) From the top 2000, 1500, 1000, 700, 500, 400, 300, 200, 100 and 50 bp.

PCR products obtained on DNA from biopsies with specific primers for exons 5, 6, 7 and 8 gave an anomalous amplification pattern for exon 6 when analysed on agarose gel. Two distinct bands were present: one very faint corresponding to the expected size and a stronger one, approximately 40 base pairs shorter (Figure 1, lane b).

Control amplifications carried out on DNA from a blood sample of the same patient showed a normal pattern, suggesting that the normal faint band, present after DNA tissue amplification was due to contamination with germline DNA (e.g. from lymphocytes or normal tissue). SSCP assay on biopsies and other tissues indicated a loss of heterozygosity (LOH) in the DNA from neoplastic cells only.

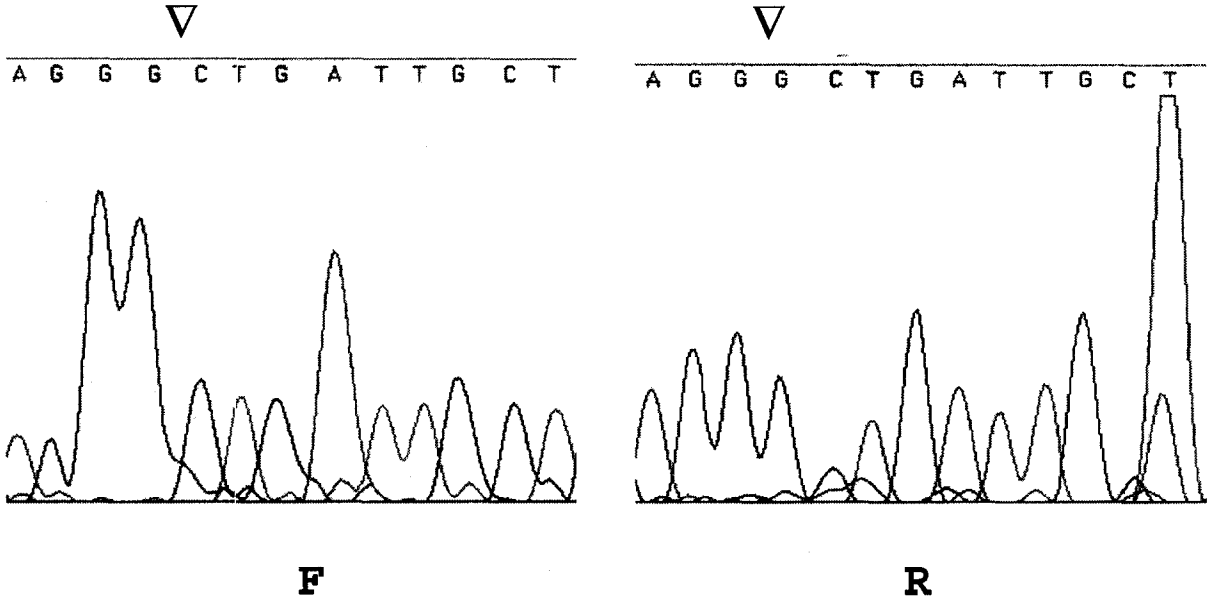
Direct sequences were performed on the purified amplificated DNA corresponding to the shorter band (Figure 1). A deletion of 36 bp was found in intron 5 starting from base 32. The singular feature of this mutation is that despite the fact that the deletion affected 7 bases in the 3′ forward primer, these deleted bases were replaced in the mutated form by an almost identical sequence (except one mismatch) normally present at the other end of the deletion (Figure 2).

The 36 base deletion comprises an adenosine (A) suspected to be the bulged branch site (Figure 2) and shortens the intron 5 below the 80 bp, the limiting length of an eukariotic intron, Wieringa and associates [14].

To verify the effects of this mutation on the maturation of the primary transcript, we amplified and sequenced the corresponding cDNA, designing primers in the 3′ of exon 4 and the 5′ of exon 7.

A two-band pattern was observed: one weak band with a 485 bp size like a normal product and a stronger one of about 100 bp less (Figure 1, lane c). The direct sequence of the purified shorter band showed that the deletion caused the skipping of exon 6 (Figure 3). Therefore, the shorter band we saw in RT–PCR is a product of 372 bp from an mRNA lacking exon 6, equal to a loss of 113 bp (Figure 1, lane d).

These results show the first example of intronic deletion causing exon skipping at the *TP53* gene level, the most stu-



CTGGAGAGACGACAGGGCTGGTTG

Exon5 . GTGAGCAGCTGGGGCTGGAGAGACGACAGGG**CTGGTTGCCC**

↓ *

AGGGTCCCCAGGCCTCTGATTCCTCACTGATTGCTCTTGA Exon6

TRCTR**A**C

(Branch site)

Figure 2. Automated sequencing of PCR product of p53 exon 6. ▽= indicates the point of deletion in forward (F) and reverse (R). In the complete sequence of intron 5, bold letters indicate the 36 bp deletion, while the forward primer sequence used for exon 6 PCR amplification is underlined. *mismatch (G → A) in repeated intronic sequence downstream of the 36 bp deletion). ↓ adenosine (A) of the splice branch site.

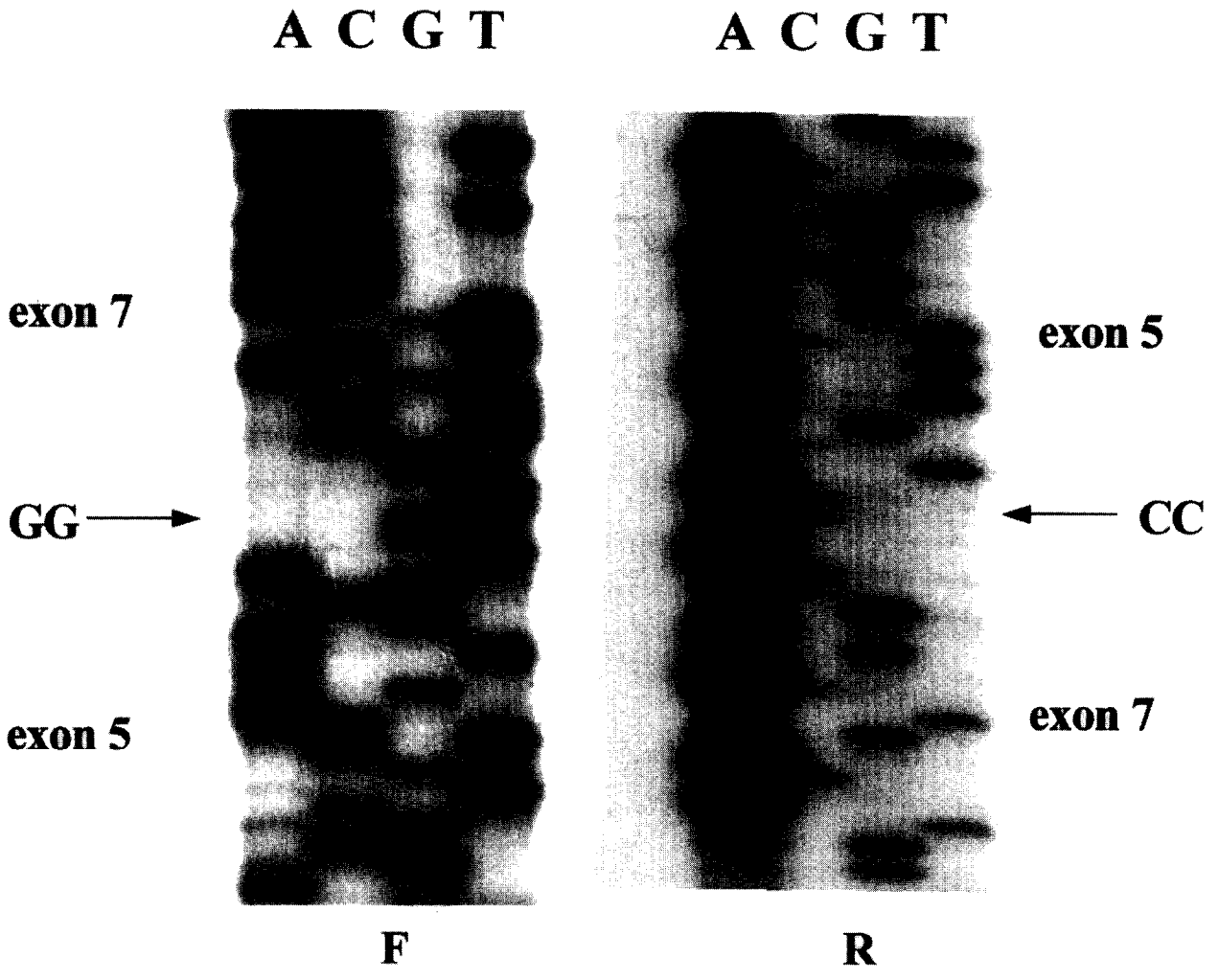


Figure 3. Manual sequence of RT-PCR of the DNA in the deleted sample (568, Figure 2, lane d). Bases GG in forward (F) and reverse (R) sequences are found at the junction between exons 5 and 7.

died oncosuppressor as far as the prognosis of breast cancer is concerned.

DISCUSSION

The mutation we described causes a frameshift starting from codon 187 giving a nonsense codon in position 190 that yields a protein with a truncated C terminus. This implies loss of the p53 sequence-specific DNA binding domain, the tetramerisation domain and three nuclear localisation sequences. This mutated protein should be inactive and probably not able to reach the cell nucleus. Our results of immunohistochemical staining, performed with antibodies that recognise the N terminus region of the p53 codified by exons 2–5 were negative possibly due to the degradation of this mutated protein.

In conclusion, we think that this finding has implications for analytical approaches. When the PCR–SSCP method is used, the choice of primers is very critical. In this case the particular situation of a duplicated sequence after the deletion in the *TP53* gene intron 5 allowed primer annealing and has led to the finding of the mutation. A similar deletion with one more or one less base would have given only the normal genomic DNA amplification which is always present in small quantities in these kind of samples. Such a situation would have resulted in a false-negative, in other words no mutuation would have been recorded for this patient.

1. Hollstein M, Sidransky D, Vogelstein B, Harris CC. p53 mutation in human cancer. *Science* 1991, **253**, 49–53.
2. Harris CC. p53: at the crossroads of molecular carcinogenesis and risk assessment. *Science* 1993, **262**, 1980–1981.
3. Clarke AR, Purdie CA, Harrison DJ, *et al.* Thymocyte apoptosis induced by p53-dependent and independent pathway. *Nature* 1993, **352**, 849–852.
4. Marx J. How p53 suppresses cell growth. *Science* 1993, **262**, 1644–1645.
5. Zambetti GP, Levine AJ. A comparison of biological activities of wild-type and mutant p53. *FASEB J* 1993, 7, 855–865.
6. Allred DC, Clark GM, Elledge R, *et al.* Association of p53 protein expression with tumour cell proliferation rate and clinical outcome in node-negative breast cancer. *J Natl Cancer Inst* 1993, **85**, 200–206.
7. Andersen TI, Holm R, Nesland JM, Heimdal KR, Ottestad L, Børresen AL. Prognostic significance of TP53 alteration in breast carcinoma. *Br J Cancer* 1993, **68**, 540–548.

8. Tsuda H, Iwaya K, Fukutomi T, Hirohashi S. p53 mutations and c-erbB-2 amplification in intraductal and invasive breast carcinoma of high histological grade. *Jpn J Cancer Res* 1993, **84**, 394–401.
9. Kovach JS, Hartmann A, Blaszyk H, Cunningham J, Schaid D, Sommers SS. Mutation detection by highly sensitive method indicates that p53 gene mutations in breast cancer can have important prognostic value. *Proc Natl Acad Sci USA* 1996, **93**, 1093–1096.
10. Beroud C, Verdier F, Soussi T. p53 gene mutation: software and database. *Nucleic Acids Res* 1996, **24**, 147–150.
11. Hartmann A, Blaszyk H, McGovern RM, *et al.* p53 gene mutations inside and outside of exons 5-8: the patterns differ in breast and other cancers. *Oncogene* 1995, **10**, 681–688.
12. Hall SL, Padgett RA. Requirement of U12 snRNA for *in vivo* splicing of minor class of eukariotic nuclear pre-mRNA introns. *Science* 1996, **271**, 1716–1718.
13. Cariello NF, Douglas GR, Soussi T. Databases and software for the analysis of mutation in human p53 gene, the human hprt gene and lacZ gene in transgenic rodents. *Nucleic Acids Res* 1996, **24**, 119–120.
14. Wieringa B, Hofer E, Weissmann C. A minimal intron length but no specific internal sequence is required for splicing the large rabbit β-giobin intron. *Cell* 1984, **37**, 915–925.

Acknowledgements—We thank Steve S. Sommer and Thierry Soussi for critical reading of the manuscript. We are also grateful to V. Maulà, C. Benedetto V. Asnaghi, A. Gillio Tos, C. Marchi and B. Serafino for technical support. Particular thanks to A. Serra (Dept Scienze Biomediche e Oncologia Umana, University of Turin) for her collaboration on RNA analysis and M. Giai and P. Sismondi who kindly provided tissue samples. We are also indebted to the patient whose cooperation made this study possible.