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Identification of Deletions and Insertions in the p53 Gene Using Multiplex PCR and High-Resolution Fragment Analysis: Application to Breast and Ovarian Tumors

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We have developed a simple and highly efficient method to detect deletions and insertions in the p53 gene. All 11 exons of the p53 gene were amplified along with a control sequence in four multiplex PCR reactions in the presence of fluorescein-labeled primers. The PCR products were resolved on an automated sequencing gel and the DNA fragments were detected by fluorescence. Using this method, we screened 7 DNA specimens from ovarian tumors, 19 from breast tumors, and 26 from normal breast tissues. No abnormality was found in any of the DNA samples extracted from the normal tissues. A 19 base pair deletion in exon 5 of the p53 gene was detected in one ovarian tumor. Insertions were identified in two breast and two ovarian tumors. The insertions were identical in 3 of these tumors and consisted of a 16 bp repeat within intron 3 of the p53 gene. It appears that the insertion within intron 3 may represent a hot spot for duplication of the normal sequence at that site. J. Clin. Lab. Anal. 12:250–256, 1998. © 1998 Wiley-Liss, Inc.

Key words: p53 gene; gene deletions; gene insertions; fragment analysis; DNA sequencing; ovarian cancer; breast cancer

INTRODUCTION

Many studies have examined the role of the p53 tumor suppressor gene in human carcinogenesis. Currently, research is focusing on the following areas: (a) identification of mutations in the p53 gene and examination of their role in cancer initiation and progression; (b) elucidation of the p53 protein structure and function and its interactions with other cellular proteins and DNA; (c) designing of gene therapy which aims to restore the loss of wild-type p53 function in cancer cells and; (d) identification of ways for cancer diagnosis, prognosis, monitoring, and cytotoxic therapy based on current knowledge of the p53 gene and protein.

Among the best-established functions of p53 are the regulation of the cell cycle at the G1/S boundary and the induction of apoptosis following DNA damage (1–3). These functions are believed to be at least partially mediated by the ability of p53 to act as a transcription factor. Target genes regulated by p53 include the WAF1/CIP1 (4), GADD45 (5), MDM2 (6), HIC-1 (7) and IGFBP-3 (8). Other described functions of p53 involve transcriptional repression of genes (9,10), and direct reannealing of single-stranded DNA (11).

The most common genetic feature among human malignancies is the deletion of one p53 allele on the short arm of chromosome 17, accompanied by a mutation in the other allele (12,13). This strongly suggests that p53 gene inactivation plays a role in tumorigenesis. The frequency of p53 gene mutations is high in cancers of the colon (14), breast (15), lung (16), ovary (17), and brain (18), as well as in leukemias (19) and osteosarcomas (20). The most common abnormalities observed are missense point mutations that are clustered between exons 5–8. Genetic alterations of the p53 gene which either lie outside exons 5–8 or constitute gain or loss of genetic material have been studied less frequently. In a recent review of the p53 mutational spectrum, it was reported that about 10% of the mutations are deletions or insertions (21). These abnormalities did not associate with any specific tumor type. Insertions ranged from 1 to 14 nucleotides in length and in 88% of the cases, the inserted nucleotides duplicated the sequences of the neighbouring region. Deletions were observed more frequently and ranged from 1 to 37 nucleotides. More recently, another study demonstrated the presence of deletions/insertions in 6 out of 11 newly established ovarian carcinoma cell lines (22).

In this article, we describe a method that identifies deletions/insertions within p53 exons and in intron/exon boundaries. The method involves multiplex PCR amplification and size analysis of the amplified fragments on a high-resolution gel. Application of this method for the analysis of tumors of

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ovarian and breast origin revealed that deletions/insertions in the p53 gene could be detected in about 20% of the tumors but in none of the control DNA samples from healthy individuals.

EXPERIMENTAL SECTION

Tissue Specimens

Breast tumor tissues were obtained from the University of Texas Health Science Center at San Antonio, Texas. The ovarian tumors and normal breast tissues (from breast reduction surgery) were obtained from The Toronto Hospital, Toronto, Canada. Immediately following surgery, a representative portion of each primary tumor was selected during quick section procedures in the operating room, snap frozen, and stored at -80°C until analysis. DNA was extracted with a phenol/chloroform-based procedure described in detail elsewhere (23). The ethanol-precipitated DNA was redissolved in TE buffer (10 mmol/L Tris, pH 7.4, 1 mmol/L EDTA). The extracted DNA was quantified by absorbance measurements at 260/280 nm and kept at 4°C until analysis.

PCR Primers

A detailed description of the PCR primers used is given in Table 1. The primers were designed using the computer software Oligo version 5.0 (National Biosciences, Inc., Plymouth, MN). Primers anneal within intronic sequences of the p53 gene with two exceptions: primer-encoded E1-5 anneals in a region upstream of exon 1; primer-encoded E11-3F anneals within exon 11. All primers were synthesized by phosphoramidite chemistry on the Gene Assember Plus (Pharmacia Biotech, Uppsala, Sweden). 5'-fluorescein-labeled primers were synthesized with fluorescein isothiocyanate amidite using the Fluoro-Prime Kit (Pharmacia). For primer design, the p53 sequence deposited by Chumakov et al. (GenBank accession #54156) was used.

PCR Amplification

A multiplex PCR amplification method was developed for the simultaneous amplification of two or more exons of the p53 gene in the same reaction mixture. Four different PCR

TABLE 1. Primer Sequences Used for PCR Amplification of p53 Exons

Exon	Primer code[1]	Sequence	Primer location[2]	Fragment size	Exon location[2]	Primer concentration mmol/L
1	E1-5 E1-3 F	5′-CGGATTACTTGCCCTTACTTGTCA-3′ 5′-F-CCCCAGCCCCAGCGATTTT-3′	711 1041	331	843-949	0.4
2	E2-5 F E2-3	5′-F-CCAGGGTTGGAAGCGTCTC-3′ 5′-GACAAGAGCAGAAAGTCAGTCC-3′	11641 11899	259	11689-11790	0.9
3	E3-5 E3-3 F	5′-CATGGGACTGACTTTCTGCT-3′ 5′-F-ATGGGTGAAAAGAGCAGT-3′	11874 12014	141	11906-11927	0.8
4	E4-5 E4-3 F	5′-CTGGTCCTCTGACTGCTCTTTTCA-3′ 5′-F-AAAGAAATGCAGGGGGATACGG-3′	11986 12367	382	12021-12299	0.48
5	E5-5 F E5-3	5′-F-TGTTCACTTGTGCCCTGACT-3′ 5′-CAGCCCTGTCGTCTCTCCAG-3′	13005 13272	268	13055-13238	0.2
6	E6-5 E6-3 F	5′-CTGGGGCTGGAGAGACGACA-3′ 5′-F-GGAGGGCCACTGACAACCA-3′	13247 13493	274	13320-13432	0.14
7	E7-5 F E7-3	5′-F-CTCCCCTGCTTGCCACA-3′ 5′-AGGGGTCAGCGGCAAGCAGA-3′	13933 14177	245	14000-14109	0.4
8	E8-5 E8-3 F	5′-GACAAGGGTGGTTGGGAGTAGATG-3′ 5′-F-GCAAGGAAAGGTGATAAAAGTGAA-3′	14350 14669	320	14452-14588	0.2
9	E9-5 F E9-3	5′-F-GCGGTGGAGGAGACCAAGG-3′ 5′-AACGGCATTTTGAGTGTTAGAC-3′	14609 14817	209	14681-14754	0.1
10	E10-5 E10-3 F	5′-TGATCCGTCATAAAGTCAAACAA-3′ 5′-F-GTGGAGGCAAGAATGTGGTTA-3′	17477 17866	390	17572-17678	0.3
11	E11-5 E11-3 F	5′-GGCACAGACCCTCTCACTCAT-3′ 5′-F-TGCTTCTGACGCACACCTATT-3′	18540 18795	256	18599-18876	0.4
C4	C4-5 C4-3 F	5′-GCCCCTCACCCGCACCTAAGT-3′ 5′-F-GCCGTCCCTCCAAGCACTG-3′		282	—	0.06

[1]F = Fluorescein labeled primer.

[2]Nucleotide numbers are as described in the sequence of p53 (GeneBank Accession #54156) submitted by Chumakov et al.

reactions were performed. The exons that were simultaneously amplified in each reaction are as follows: reaction P1, exons 4, 8, and 11; reaction P2, exons 1, 5, 9, and 10; reaction P3, exons 3 and 6; and reaction P4, exons 2 and 7. In each of the reactions we also incorporated primers for amplification of a region of the C4 gene which was used as a positive internal control. One of the two primers used for amplifying each p53 exon or C4 was labeled with fluorescein in order to facilitate detection of the PCR product. Each PCR reaction was performed in a final volume of 50 μL containing ~300 ng of genomic DNA, 10 mmol/L Tris buffer (pH 8.3), 50 mmol/L KCl, 3 mmol/L $MgCl_2$, 300 μmol/L deoxynucleoside triphosphates (dNTPs), 0.5 mg/mL bovine serum albumin, and 1.25 units of Taq DNA polymerase (Boehringer Mannheim). The primers were used at concentrations between 0.1–0.9 μmol/L as shown in Table 1. The temperature cycling protocol for multiplex PCR on the Perkin-Elmer 2400 PCR instrument consisted of denaturation at 94°C for 15 sec, annealing at 62°C for 10 s followed by 59°C for 10 sec, and extension at 71°C for 40 sec. This cycling was repeated 18 times. Each multiplex reaction was initiated by a 2 min denaturation step at 94°C and terminated by a 3 min extension at 71°C.

For individual PCR amplification of exons 3 and 5,500 ng of target DNA was added in a PCR mix containing 10 mmol/L Tris (pH 8.3), 50 mmol/L KCl, 2 mmol/L $MgCl_2$, 200 μmol/L dNTPs, and 1.25 units of Taq polymerase. In the case of exon 3, the concentrations of the primers were 0.4 μmol/L (for primer E3-5) and 0.8 μmol/L (for primer E3-3F). For exon 5, we used 0.4 μmol/L of primer E5-5F and 0.4 μmol/L of primer E5-3. The temperature cycling consisted of denaturation at 94°C for 20 s, annealing at 60°C for 30 s, and extension at 71°C for 30 s. The number of cycles was 30. Each PCR was initiated with a 3 min denaturation at 94°C and terminated with a 3 min extension at 71°C.

Gel Electrophoresis

Electrophoresis of PCR amplified fragments was performed by using 2% agarose gels. The electrophoresis buffer was a 0.5x Tris-acetate/EDTA (TAE) buffer (20 mmol/L Tris, pH 7.2; 10 mmol/L sodium acetate; 0.5 mol/L EDTA). The PCR products for which an abnormality was identified by fragment analysis were electrophoresed on 6% polyacrylamide minigels using Tris-borate/EDTA (TBE) elactrophoresis buffer (Novex, San Diego, CA, 92121). After staining with ethidium bromide, the PCR products (usually two closely positioned bands with 16–19 base pair difference, see results) were cut from the gel, extracted in 10 mM Tris.HCl, pH 7.4 and reamplified by PCR. The reamplified PCR products were cloned as discussed below.

DNA Cloning and Plasmid Purification

The TA Original Cloning Kit (Invitrogen Corporation, San Diego, CA) was used for cloning of the PCR products into the pCR™II Vector. The manufacturer's protocol was followed throughout. At least two white colonies were obtained from each agar plate and cultured overnight at 37°C in Luria-Bertani medium (LB broth) with ampicillin. Plasmids were extracted and purified using the Plasmid Midi Kit from Qiagen (QIAGEN Inc., Chatsworth, CA).

Plasmid Digestion

1 μg of each plasmid was digested with 10 units EcoR1 enzyme (Boehringer Mannheim) at 37°C for 1 h and it was subsequently electrophoresed on agarose gels for verification of the presence of the insert.

Automated Sequencing

The primers used for sequencing of the cloned PCR products were specific for regions of the vector flanking the insert. All DNA inserts were sequenced using the M13 (-20) Forward Primer (5´-GTAAAACGACGGCCAG-3´), labeled with fluorescein. If sequencing with this primer did not provide information on the complete sequence of the insert, sequencing was also performed with fluorescein-labeled SP6 Primer (5´-TAAATCCACTGTGATAT-3´) which anneals upstream of the inserted PCR product, at the region of the plasmid SP6 promoter. In one case, sequencing was also performed with fluorescein-labeled M13 Reverse Primer (5´-GTCCTTTGTCGATACTG-3´) which is specific for a region of the plasmid further upstream of the SP6 promoter. For sequencing reactions, the Sequitherm Kit (Cedarlane Laboratories, Hornby, Ontario, Canada) was used. Each reaction mixture consisted of 250–300 ng of DNA plasmid, 5 pmoles of primer, and 5 units of the Sequitherm polymerase. All reactions were performed by thermocycling using a PCR thermal cycler. The temperature cycle consisted of denaturation at 94°C for 20 s, annealing at 55°C for 20 s, and extension at 72°C for 40 s. This cycle was repeated 36 times. Each thermocycling was initiated with a 2 min denaturation at 94°C and terminated with a 10 min extension at 72°C.

Sequencing and fragment analysis were performed with an automated laser fluorescence sequencing apparatus (A.L.F. DNA Sequencer™; Pharmacia, Uppsala, Sweden).

RESULTS

Multiplex Amplification and Fragment Analysis

A multiplex PCR was developed for amplification of two or more of the eleven p53 exons at the same time. Four groups of primers were used which simultaneously amplified exons 4, 8, and 11 (group P1), 1, 5, 9, and 10 (group P2), 3 and 6 (group P3), and 2 and 7 (group P4). Each one of the four groups also contained a set of primers responsible for the amplification of the C4 gene which served as an internal control to indicate optimal amplification reaction. With this protocol, p53 exons and exon/intron boundary sequences were

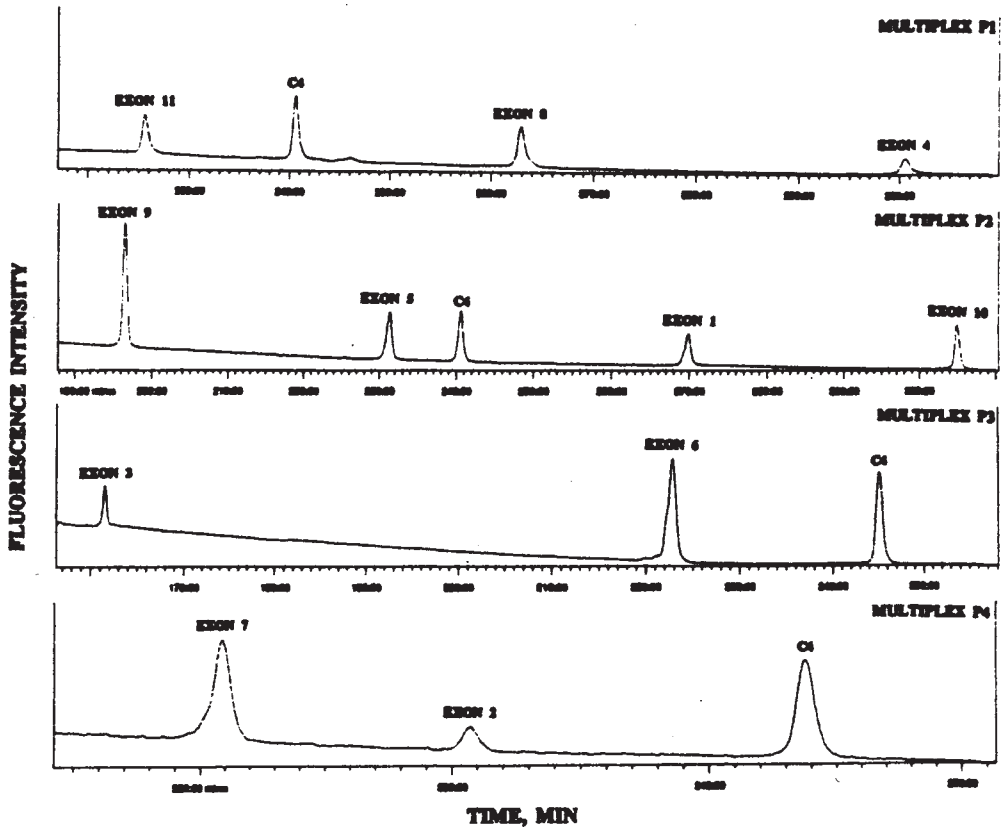

Fig. 1. Fragment analysis of normal DNA. The electrophoresis times and the intensity of the peaks for each of the 11 p53 exons and the control (C4) are shown.

amplified, since the primers used anneal at intronic sequences. One of the two primers for each exon was labeled with fluorescein in order to facilitate detection of the PCR product. The p53 amplified DNA fragments, as obtained from the multiplex PCR, were directly subjected to fragment analysis. This method detects gene deletions and insertions based on changes in the size of DNA fragments since during gel electrophoresis, DNA fragments move through the gel according to their size. Length changes of PCR products are reflected as changes in the electrophoresis times. With this method length changes of one base or larger can be readily detected (unpublished data). Missense mutations, however, cannot be identified.

In Figure 1, we present fragment analysis of multiplex PCR amplified products generated by using DNA extracted from lymphocytes of a healthy blood donor. The peaks and electrophoresis times corresponding to exons 1–11 of p53, as well as the peak generated by the control reaction (C4) are indicated. This "normal" pattern was used to compare all other patterns obtained with DNA extracted from the tumor specimens.

Fragment Analysis of Normal and Pathological Tissues

Twenty-six DNA samples extracted from malignant tissues were subjected to multiplex PCR amplification and subsequent fragment analysis. Seven of these DNA samples were from ovarian tumors, 19 from breast tumors. Five abnormalities were identified; 3 were found in ovarian tumors and 2 in breast tumors. The abnormalities manifested as newly appearing peaks in the multiplex reactions. When an abnormality was identified, the DNA was reamplified in separate PCR reactions in order to localize the abnormality in one exon. Some results are illustrated in Figure 2. Case 8 (ovarian carcinoma) was suggestive of a deletion in the DNA fragment corresponding to exon 5, since a new peak appeared earlier than the normal peak for exon 5. Case 10 (ovarian carcinoma), 65 (breast carcinoma), 59 (breast carcinoma), and 92 (ovarian carcinoma; results not shown) presented with an extra peak of higher molecular weight than the normal PCR product for exon 3. This was suggestive of an insertion. The putative insertion seemed to be of the same size in all four tumors. In tumor 10 only the abnormal fragment was detected. In all other cases, the abnormal as well as the normal peaks were seen, suggesting either heterozygosity or contamination of the tumor DNA with DNA from adjacent normal tissue.

In order to investigate if the detected insertions were due to polymorphisms of p53 intronic sequences, we performed complete fragment analysis with DNA extracted from 26 normal breast tissues obtained from healthy women during breast

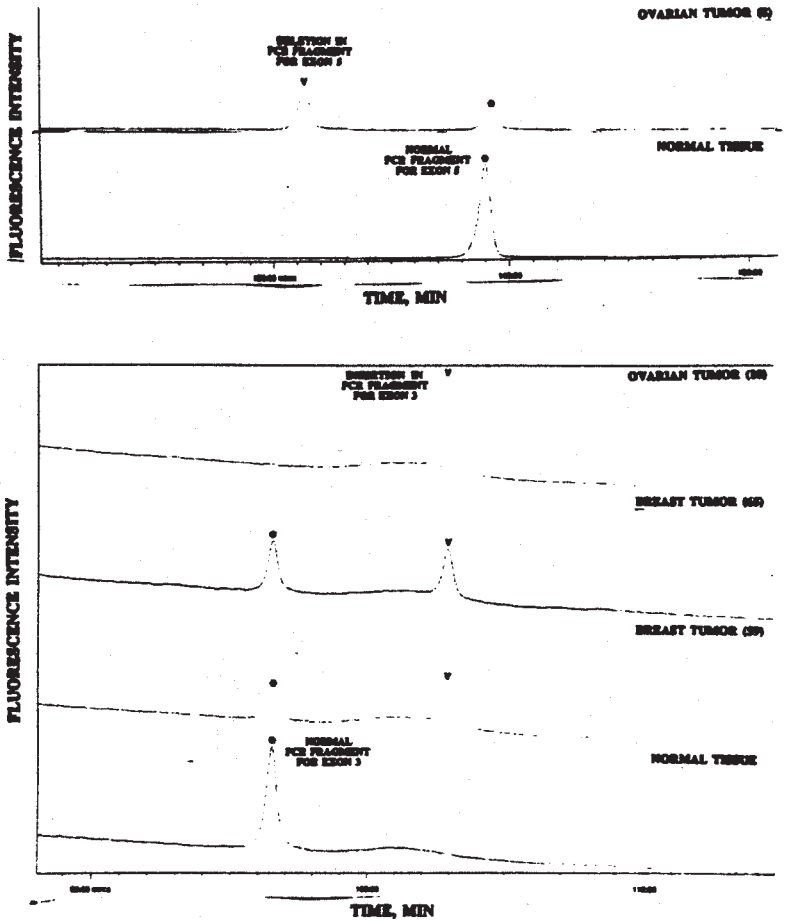

Fig. 2. Abnormalities identified with fragment analysis. Asterisks denote the normal fragments and arrowheads denote the abnormal fragments.

reduction surgery. No abnormalities were detected in any of the tested tissues.

Cloning of PCR Products and Sequencing

PCR products for exon 5 (DNA sample 8) and 3 (DNA samples 10, 59, 65, and 92) were electrophoresed on 6% polyacrylamide gels in order to separate the normal and abnormal amplified fragments which appeared as two distinctly moving bands on the gel. Each DNA fragment was then extracted from the polyacrylamide gel and further reamplified. The PCR products were subsequently subcloned into the pCRII vector. Recombinant pCRII plasmids were purified, tested for inserts and sequenced using dideoxy protocols and automated sequencing with fluorescently labeled primers annealing to vector sequences.

Sequencing of the p53 gene in DNA sample 8 revealed a 19 bp deletion within exon 5 (Fig. 3, Table 2). This deletion leads to premature termination of translation with production of a predicted p53 protein with 162 instead of 393 aminoacids, which may account for the p53 low levels (p53 negative) detected in this tumor. When DNA fragments corresponding to exon 3 from tumor samples 10, 65, and 92 were sequenced, the same 16 bp insertion was found in all of them (Fig. 3, Table 2). The insertion was located outside exon 3, within the third intron of the p53 gene. Comparison with the normal sequence showed that the insertion was a 16 bp repeat of the normal sequence right upstream of the site of the abnormality. Tumor 10 was strongly positive, while tumors 65 and 92 were negative for p53 protein.

DISCUSSION

p53 gene alterations occur in about 40–50% of all cancers. The most common abnormalities are missense mutations localized in exons 5–8. Deletions and insertions are thought to represent ~10% of all alterations but the literature reports may be biased for three reasons. First, most investigators sequence only exons of genomic DNA or cDNA. Mutations at intron/exon boundaries may thus be missed. Second, others sequence only exons 5–8 based on the false premise that mutations outside these exons are very rare. New reports find frequent

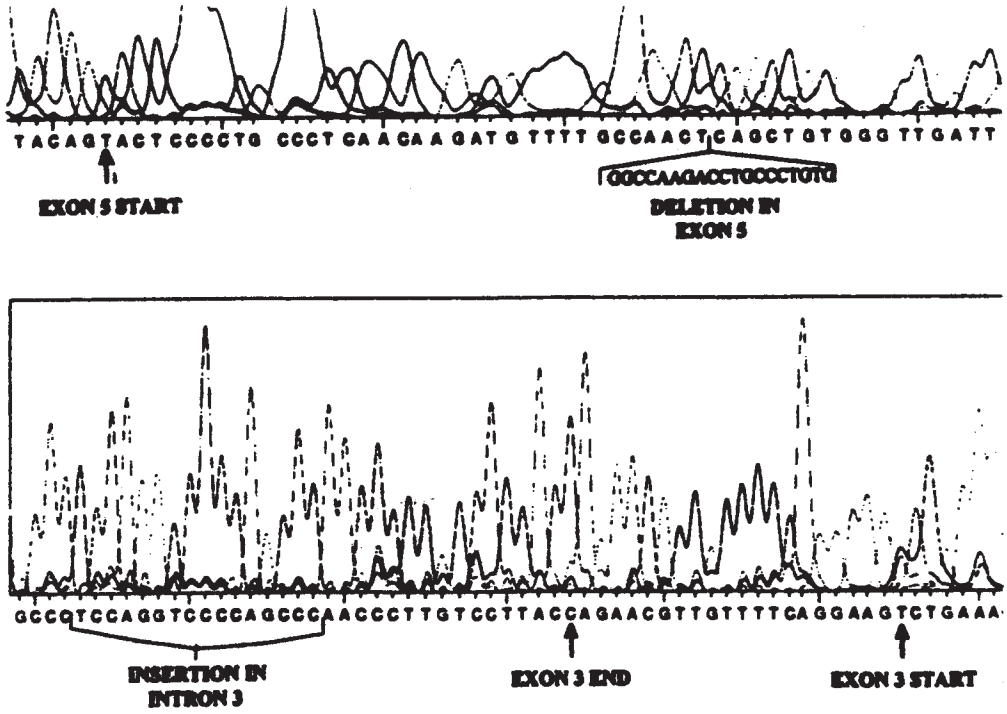

Fig. 3. Sequencing of abnormal DNA fragments. **Upper panel**: A 19 bp deletion identified in p53 exon 5 of an ovarian cancer patient. The start of the exon and the detected deletion are indicated. **Lower panel**: A 16 bp insertion within intron 3 of the p53 gene detected in one ovarian and two breast cancer patients. Exon 3 boundaries are indicated.

mutations in exons other than 5–8 (24). And finally, mutations in intronic sequences are not usually sought and when found, they are considered polymorphisms.

This article presents a simple optimized method for detecting insertions/deletions in the p53 gene by utilizing multiplex PCR and fluorometric detection of product size on sequencing gels. DNA samples from 26 tumors (7 ovarian and 19 breast) were examined with this method and 5 abnormalities were detected; one deletion and four insertions. The deletion consisted of a 19 bp loss in exon 5 and belonged to an ovarian tumor. The insertions were identified in two ovarian and two breast tumors. One insertion was homozygous; the others appeared to be heterozygous but we do not exclude the possibility that the normal allele was co-amplified due to contamination of tumor DNA by DNA from adjacent normal tissue. Sequencing revealed that 3 of the insertions consisted of the same 16 bp gain of genetic material within intron 3 of the p53 gene. This 16 bp insertion was a duplication of the normal sequence right upstream of the site of the abnormality (Fig. 3). No abnormalities were found in any of the 26 DNA extracts from normal breast tissue of healthy individuals. Normal tissue DNA of the patients with this genetic abnormality was not analyzed. This 16 bp intronic insertion, however, has previously been reported to represent a polymorphism (25). It still remains to be investigated whether this polymorphic sequence within the p53 gene has any value as a marker for predisposition to ovarian cancer.

The deleted sequence was almost identical to the 18 bp sequence TGGCCAAGACCTGCCCTG which was previously reported to be deleted in an ovarian tumor (Mazars et

TABLE 2. Detection of p53 Gene Alterations with Multiplex PCR and Fragment Analysis

Code	Tumor site	p53 Protein expression[1]	Abnormality	Location	Sequence of abnormality
8	ovary	negative	19bp deletion	Exon 5	GGCCAAGACCTGCCCTGTG
10	ovary	positive	16bp insertion	Intron 3	TCCAGGTCCCCAGCCC
59	breast	positive	insertion	Fragment 3[2]	Not Sequenced
65	breast	negative	16bp insertion	Intron 3	TCCAGGTCCCCAGCCC
92	ovary	negative	16bp insertion	Intron 3	TCCAGGTCCCCAGCCC

[1]p53 levels were measured in tumor extracts as described elsewhere (26).
[2]DNA fragment which consists of p53 exon 3 and part of intron 3 as obtained after PCR amplification.

al., 1991). The 16 bp inserted sequence was found in 1 breast and 2 ovarian carcinomas. Recently, the same 16 bp insertion was reported in an ovarian carcinoma cell line (22).

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