Clinical Chemistry 44:1
72–77 (1998)

Exon 5 of the *p53* gene is a target for deletions in ovarian cancer

Katerina Angelopoulou,[1,2] Michael A. Levesque,[1,2] Dionyssios Katsaros,[3]
Rob Shipman,[4] and Eleftherios P. Diamandis[1,2]*

Missense point mutations, leading to inactivation of the p53 tumor suppressor gene product, are currently the most frequent alterations in human cancer. Little, however, is known about small intragenic deletions or insertions occurring in this locus of chromosome 17. We have analyzed 56 primary ovarian tumors for the presence of such abnormalities. The analysis was based on multiplex PCR amplification of exons 1 through 11 of the *p53* gene and fragment analysis of the generated PCR products. Mutations were detected in 14% (8 of 56) of the tumors. Deletions were much more prevalent than insertions (seven vs one). Six of the deletions and the insertion affected exon 5, and the other deletion was in exon 7. Two deletions and the insertion did not disrupt the reading frame; the protein product was expressed in the tumor at high concentrations in all three cases. The other five deletions generated a frameshift, which is predicted to result in the production of a truncated protein product. In the case of the deletions, a 2–5-bp repeat was present close to the detected deletion, whereas the insertion duplicated the sequence immediately upstream of the insertion site. Overall our findings indicate that small intragenic *p53* deletions/insertions are not rare events in ovarian cancer, and that *p53* exon 5 is the target in the vast majority (88%) of the cases.

The nuclear phosphoprotein p53 is currently the most studied molecule in human tumorigenesis. *p53*, mutated in ~25% of tumors of virtually every origin, has been accepted to be the most frequently mutated gene in cancer. Although the exact mechanisms by which *p53* exerts its biological functions have not been clearly elucidated, its ability to bind specific DNA sequences *[1, 2]* and to activate transcription *[2–4]* suggests that this molecule plays an important role in the regulation of cell proliferation. p53 is believed to be central to the cellular response to DNA damage. After such damage, p53 concentrations increase, leading to either cell cycle arrest at the G1 phase *[5–7]* or to apoptosis *[8]*. Both pathways aim to prevent cells that carry DNA abnormalities from proliferating, thereby protecting the genome from accumulating deleterious mutations. Loss of p53 function may abrogate this response to DNA damage and may increase the likelihood of genomic abnormalities *[9]*.

The most common mechanism of *p53* inactivation is somatic mutation. Inactivating mutations are confined to a portion of the gene, spanning codons 132–281 (corresponding to exons 5–9), which includes four highly conserved domains *[10]*. The most frequently observed mutations are missense mutations leading to single amino acid substitutions. These mutations have been extensively studied, and their correlation to environmental carcinogen exposure in particular tumor histotypes has been observed in some cases. The most striking paradigm is the relation between aflatoxin B1 exposure and the G-to-T transversion in codon 249 in 50% of Southeast Asian patients with hepatocellular carcinoma *[11]*.

To date, small *p53* intragenic deletions and insertions, which can disrupt the reading frame and lead to the production of a truncated protein product, have not received much attention. However, a review that compiled 740 *p53* mutations from a wide variety of cancers showed that 10% of the mutations were either deletions or insertions *[12]*. Insertions ranged from 1 to 14 nucleotides in length and in 88% of the cases the inserted nucleotides duplicated the sequence adjacent to the insertion. Deletions were observed more frequently and ranged from 1 to 37 nucleotides in length. In the majority of the cases, a direct repeat of 2–8 bp was present in the unaltered sequence in the vicinity of the deletion. Both *p53* gene

[1] Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, 600 University Ave., Toronto, ON, Canada M5G 1X5.
[2] Department of Laboratory Medicine and Pathobiology, University of Toronto, 100 College St., Toronto, ON, Canada M5G 1L5.
[3] Department of Gynecologic Oncology, Institute of Obstetrics and Gynecology, University of Turin, Turin, Italy.
[4] Visible Genetics Inc., 700 Bay St., Ste. #1000, Box 333, Toronto, ON, Canada M5G 1Z9.
*Send correspondence to this author at the Mount Sinai Hospital address. Fax 416-586-8628; e-mail ediamandis@mtsinai.on.ca.
Received May 7, 1997; revision accepted August 17, 1997.

deletions and insertions can be explained by a slipped mispairing mechanism during DNA replication *[12, 13]*.

Deletions and insertions affecting the *p53* gene are unlikely to be tumor specific because they appear in a variety of human tumors *[12]*. In ovarian cancer, investigations of *p53* gene alterations have mainly focused on missense point mutations in exons 5–9 *[14–16]*. Transitions predominate over transversions *[17, 18]*, suggesting that *p53* mutations in this type of cancer most likely arise from spontaneous errors in DNA synthesis and repair rather than from exposure to carcinogens. A recent study reported a high incidence of *p53* deletions/insertions (15.6%) in this type of cancer *[19]*.

In this study, we systematically searched for *p53* gene deletions and insertions in all 11 exons and intron/exon boundaries in a series of 56 primary epithelial ovarian tumors. The method involved multiplex PCR amplification of all 11 p53 exons and fragment analysis of the PCR products on a high-resolution polyacrylamide sequencing gel *[20]*. Our results revealed that deletions or insertions occur with a frequency of 14% in ovarian cancer, the most frequent being deletions. All aberrations but one were found in exon 5, suggesting that mutational inactivation of *p53* via this mechanism may be a significant event in ovarian cancer.

Materials and Methods

TUMOR SPECIMENS

Fifty-six primary epithelial ovarian tumors were collected at the Department of Obstetrics and Gynecology, University Hospital, Turin, Italy. Immediately after surgery, a representative portion of each tumor was selected during quick section procedures in the operating room, snap-frozen in liquid nitrogen, and stored at −80 °C until analysis. Genomic DNA was extracted with a phenol/chloroform-based procedure *[21]*.

MULTIPLEX PCR AMPLIFICATION AND FRAGMENT ANALYSIS

A multiplex PCR method that amplifies two or more exons of the *p53* gene in the same reaction mixture was used *[20]*. Four different PCR reactions were performed. The exons that were simultaneously amplified in each reaction were as follows: 4, 8, and 11; 1, 5, 9, and 10; 3 and 6; 2 and 7. One of the two primers used for amplifying each *p53* exon was labeled with the fluorescent dye Cy-5 to facilitate detection of the PCR product. The primers and the PCR conditions are described in detail elsewhere *[20]*. The PCR-amplified DNA fragments were separated according to size on an ALF-Express automated DNA sequencer *[20]*.

SEPARATION OF NORMAL AND ABNORMAL FRAGMENTS

The PCR products for which an abnormality was identified with fragment analysis were subjected to individual (nonmultiplexed) PCR amplifications. Target DNA (500 ng) 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 deoxynucleoside triphosphates (dNTPs), and 1.25 U of Taq polymerase (Boehringer Mannheim). The PCR primers used for exons 5 and 7 amplification are shown below:

Exon 5, E5–5′: 5′AAGCTCCTGAGGTGTAGACG-3′, E5–3′: 5′TTTCCTTCCACTCGGATAAG-3′

Exon 7, E7–5′: 5′GGCGACAGAGCGAGATTCCA-3′, E7–3′: 5′GGGTCAGCGGCAAGCAGAGG-3′

All the primers were used at concentrations of 0.4 μmol/L. The temperature cycling consisted of denaturation at 94 °C for 20 s, annealing at 59 °C (exon 5) or 63 °C (exon 7) for 30 s, and extension at 71 °C for 30 s. The cycling was repeated 30 times. Each PCR was initiated with a 3-min denaturation at 94 °C and terminated with a 3-min extension at 71 °C.

The PCR products were electrophoresed on 6% polyacrylamide minigels with 1× Tris-borate/EDTA (TBE) electrophoresis buffer (Novex). Bands corresponding to heteroduplex DNA products (normal DNA annealed to abnormal DNA strand) were excised from the gel and eluted in 100 μL of 10 mmol/L Tris-HCl, pH 7.4, overnight at 37 °C. A 5-μL aliquot of the eluate was subjected to a new round of PCR as described above but with two differences: (*a*) Only one of the two primers was used, and (*b*) the cycling was repeated 40 times to generate sufficient single-stranded DNA for direct sequencing.

AUTOMATED SEQUENCING

The primers used for sequencing of exons 5 and 7 were designed with the computer software Oligo 5.0 (National Biosciences) according to the *p53* sequence deposited in the GenBank by Chumakov et al. (accession # 54156). Sequencing primers were labeled at the 5′-end with the fluorescent dye Cy-5 (National Biosciences).

Exon 5, E5–5′S: Cy-5-TCTTTGCTGCCGTGTTCC, E5–3′S: Cy-5-CCTGGGGACCCTGGGCAA

Exon 7, E7–5′S: Cy-5-CTCCCCTGCTTGCCACA

Single-stranded DNA products were subjected to exonuclease I digestion and shrimp alkaline phosphatase hydrolysis according to the ThermoSequenase sequencing protocol (Amersham Life Sciences). The sequencing reactions were performed with the Thermo Sequenase fluorescent labeled primer cycle sequencing kit (Amersham) according to the manufacturer's directions. Sequence analysis was performed with an automated laser fluorescence sequencing apparatus (A.L.F. Express DNA Sequencer™; Pharmacia Biotech).

Results

FRAGMENT ANALYSIS

With a combination of multiplex PCR amplification and fragment analysis techniques *[20]* we analyzed genomic DNAs from 56 primary ovarian tumors for the presence of deletions and insertions in the *p53* gene (exons 1–11). This method detects gene deletions and insertions on the basis of changes in the size of DNA fragments produced by PCR. Length changes of PCR products are reflected as

changes in the electrophoresis times. With this method, length changes of 1 bp or more can be readily detected. Point mutations, however, which result in single nucleotide substitutions, cannot be detected.

The analysis revealed eight abnormalities detected as novel peaks after electrophoresis of the multiplex PCR reactions. Deletions were identified when the new peak appeared earlier than expected compared with the wild-type fragment elution time, whereas insertions were suggested when the peak appeared later. To localize the mutation to a particular exon, the PCR was repeated with exon-specific PCR primers and the products were subjected to gel electrophoresis on the sequencer. The results (Fig. 1) were suggestive of six deletions (tumors 61, 80, 82, 87, 93, 97) and one insertion (tumor 120) in exon 5, and one deletion in exon 7 (tumor 68; data not shown). In all cases, both the abnormal and the normal peaks were present, suggesting either heterozygosity or contamination of the tumor DNA with DNA from adjacent normal tissue.

P53 DELETIONS AND INSERTIONS IN OVARIAN TUMORS

To characterize the genetic abnormalities identified, we applied direct DNA sequencing. The normal and abnormal DNA fragments, which coexisted in the PCR reaction mix (Fig. 1), were separated with the following strategy: PCR products of exon 5 (for tumors 61, 80, 82, 87, 93, and 120) and exon 7 (for tumor 68) were electrophoresed on 6% polyacrylamide minigels (data not shown). The heteroduplex DNA products (consisting of one normal and one abnormal strand annealed together) were excised from the gel, and DNA was eluted. This DNA was subjected to another round of thermocycling with one primer to achieve linear amplification of one of the two strands (either the normal or the abnormal). These single-stranded DNAs were used for sequencing.

The seven deletions and the one insertion identified are presented in Table 1, and partial sequencing results are shown in Fig. 2. All abnormalities but one (tumor 93, 29-bp deletion; Fig. 2A) affected only three to eight nucleotides. In all the identified small deletions (3–8 nucleotides) a repeat of 2–5 bp was present in the unaltered sequence close to the deletion, as previously observed by Jego et al. *[12]*. The 6-bp insertion, on the other hand, was a duplication of the sequence immediately upstream of the site of the insertion (Fig. 2C). An unusual abnormality found in two cases (tumors 82 and 97; Fig. 2B) involved 7 bps and consisted of a tandem 3-bp deletion and a 4-bp deletion separated by one nucleotide. Tumors 61 and 80 contained an in-frame deletion in exon 5, which would be expected to yield a predicted protein product of 392 amino acids. The insertion in exon 5 (tumor 120) was also in frame and would result in a predicted protein product of 395 amino acids. In all three

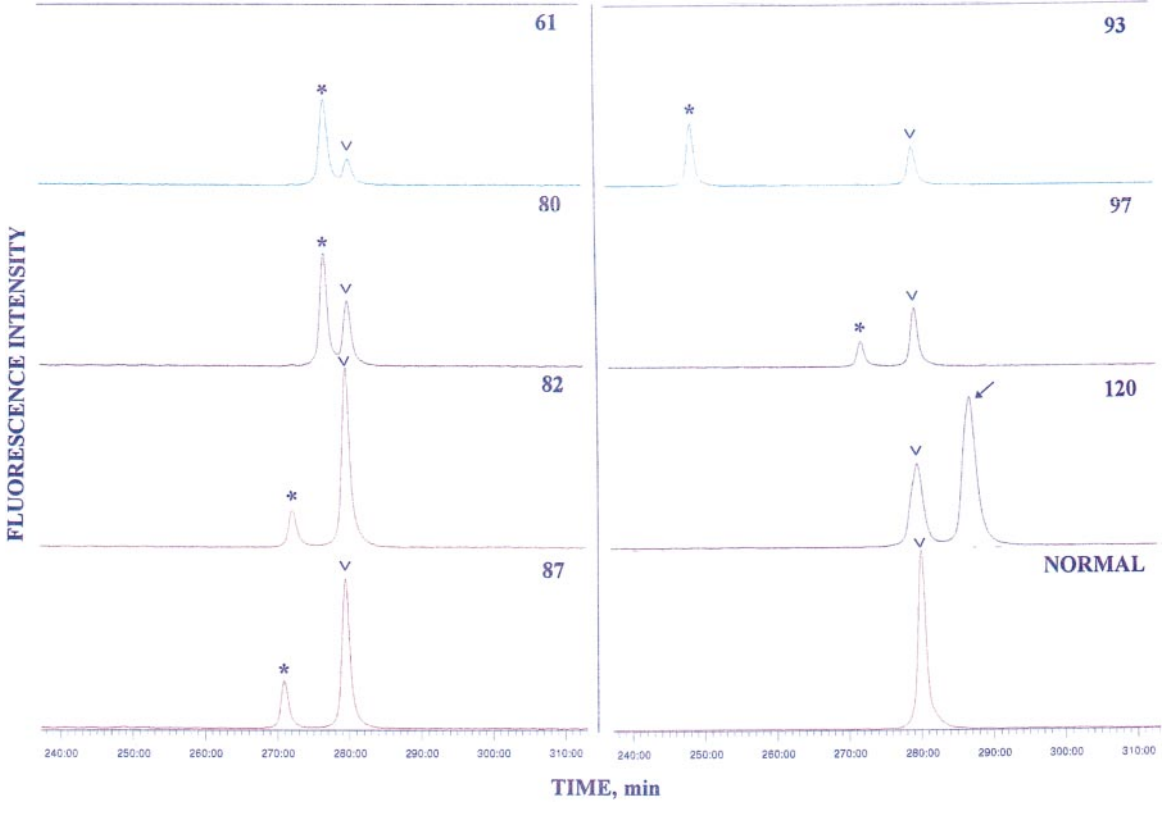

Fig. 1. Abnormalities in exon 5 of the *p53* gene identified with fragment analysis.
Arrowheads denote the normal fragments. *Asterisks* denote fragments with deletions; the *arrow* indicates the fragment with the insertion.

Table 1. *p53* deletions/insertions identified in ovarian tumors.

Tumor code	Exon	Abnormality	Sequence	Codons affected	Predicted protein size (amino acids)	p53 protein concentrations (U/g)
61	5	Deletion	CAG**C**<u>**TG**</u>**TG**G	145	392	28
68	7	Deletion	TC**TG**<u>AC</u>**TG**TACC	22–229	244	0.8
80	5	Deletion	T**CAA**<u>**CAA**</u>GAT	131–132	392	32
82	5	Deletion	**CAA**CTGGC<u>**CAA**</u>GA<u>**CCT**</u>GC**CCT**GT	138–141	166	0.4
87	5	Deletion	CAC<u>**CCGCG**</u>T<u>**CCGCG**</u>CC	155–158	176	0.9
93	5	Deletion	<u>GATTCCACACCCCCGCCCGGCACCCGCGT</u>	148–157	169	0.6
97	5	Deletion	**CAA**CTGGC<u>**CAA**</u>GA<u>**CCT**</u>GC**CCT**GT	138–141	166	0.3
120	5	Insertion	G<u>**ACGGAG**</u>**ACGGAG**GTT	171–172	395	24

Deleted nucleotides are underlined and the direct repeats are in bold.

p53 protein concentrations were quantified in ovarian cancer cytosolic extracts by using an immunofluorometric procedure described previously [*25*]. Concentrations <3 U/g classify tumors as p53 protein-negative.

cases, the abnormalities resulted in p53 protein overexpression in the tumors (Table 1). In the remaining cases, where the deletion created a frameshift, a predicted truncated protein product would be produced. The p53 concentrations in the corresponding tumor, as measured by immunofluorometry *[22]*, were very low (Table 1). In Table 2 some clinical information on the patients whose tumors contained *p53* gene abnormalities are presented.

Discussion

Genetic aberrations affecting the *p53* gene locus in cancer patients have been extensively studied since the characterization of this gene as a tumor suppressor. Most studies have examined missense point mutations within exons 5–9. Little is known about genetic alterations involving losses or gains of genetic material that commonly lead to premature arrest in translation and therefore the production of a truncated protein product. One study, however, which compiled data published between 1989 and 1992, revealed that the frequency of these abnormalities in the *p53* gene in human tumors is ~10% with no obvious clustering within certain tumor types *[12]*.

The presence of these genetic aberrations in ovarian cancer was specifically examined in 11 newly established ovarian carcinoma cell lines *[23]*. Data collected from previously published reports *[14–16]* demonstrate that small *p53* gene deletions have been observed with a frequency of 4.7% in a total of 127 ovarian tumors analyzed. Only one of 66 cases had an insertion *[15]*. A more recent study demonstrated that 25% of the abnormalities found in ovarian tumors were deletions or insertions *[19]*.

Using a combination of multiplex PCR and fragment analysis techniques *[20]*, we analyzed 56 primary ovarian tumors for the presence of *p53* gene deletions and insertions in all 11 exons of p53. The results revealed that these abnormalities are found with a frequency of 14% in our series of ovarian tumor specimens. Of the eight abnormalities identified, seven were deletions and one was an insertion. Six of the deletions and the insertion were detected in exon 5, and the other deletion was in exon 7. Previous studies reported three deletions in exon 4 *[16, 19]*, four in exon 5 *[14, 19]*, one in exon 6 *[15]*, five in exon 7 *[15, 19]*, and two in exon 8 *[19]*. Unlike the study from Skilling et al. *[19]*, who found these abnormalities to be homogeneously spread between exons 4 and 8, in our study 88% (7 of 8) of the abnormalities were found in exon 5. Exon 5 is known to be a hotspot for missense mutations. In a series of 30 ovarian tumors, Mazars et al. *[14]* found that all *p53* mutations detected were clustered in exons 5 and 7, and they suggested that mutations in these two exons may reveal a pattern particular to this cancer. Our results support this speculation.

The deletions found in this study were flanked by short direct repeats, and the insertion duplicated the sequence where it was inserted, in agreement with previous reports *[12, 13]*. The presence of the repeats suggest that a slipped mispairing mechanism, which takes place during DNA replication, might be responsible for the creation of these gene abnormalities. This mechanism involves a misalignment of the template DNA strands during replication, which leads either to deletion if the nucleotides excluded from pairing are on the template strand, or to insertion if they are on the primer strand. When direct repeat sequences mispair with a complementary motif nearby, the intervening oligonucleotide sequence may form a loop between the two repeat motifs and be deleted *[12]*. More lengthy runs and sequence repeats are more likely to generate frameshift mutations. In our series, two of the deletions and the insertion were in frame and gave rise to 392 and 395 predicted amino acid products that were overexpressed in the corresponding tumors. The rest of the abnormalities resulted in a frameshift in the gene sequence; a stop codon was generated, and predicted truncated protein products were produced that were not recognized by immunofluorometry of p53 protein.

The possibility that these genetic lesions in *p53* consist of a marker for a novel form of DNA replication error should not be excluded. Other candidate target genes in the genome of such tumors should be sought to investigate whether the same underlying mechanism is involved.

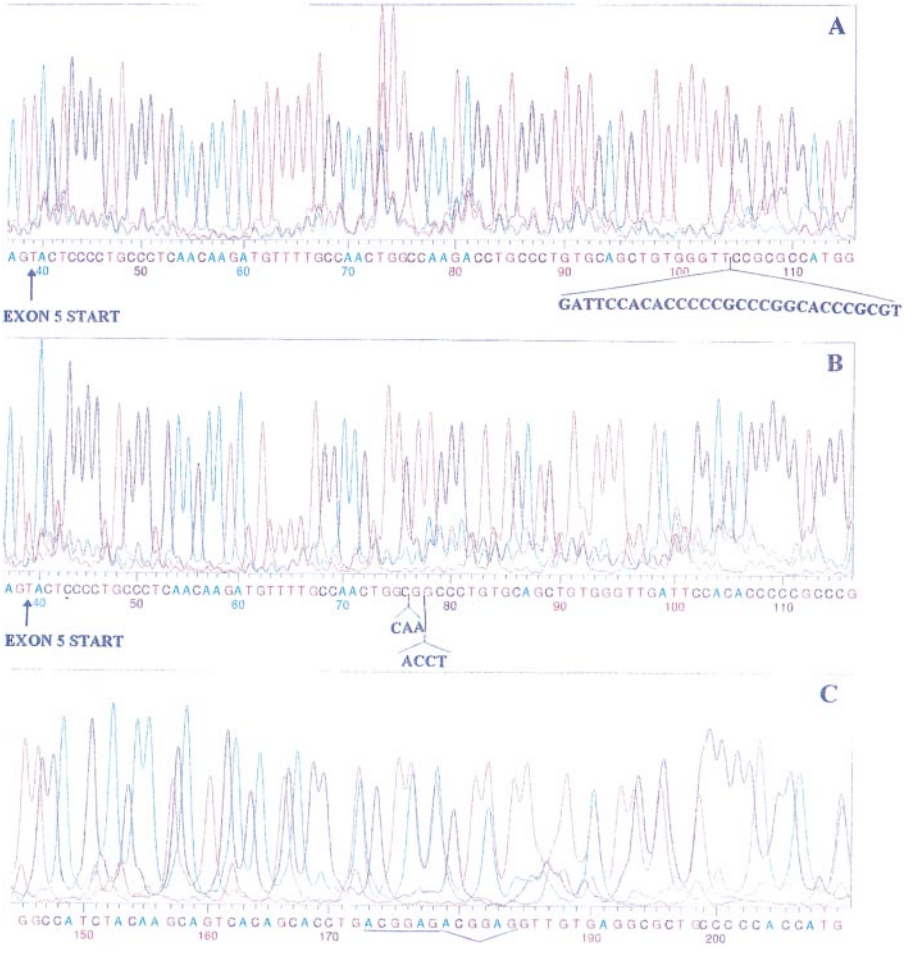

Fig. 2. Sequencing of *p53* exon 5.

(*A*) 29-bp deletion identified in tumor 93; (*B*) a 7-bp deletion, consisting of a 3-bp and a 4-bp deletion separated by one nucleotide detected in tumors 82 and 97; (*C*) A 6-bp insertion identified in tumor 120. The start of exon 5 and the abnormalities detected are indicated.

In all cases where deletions/insertions were identified, both normal and abnormal *p53* alleles were present. This may be due to patient heterozygosity or may simply indicate tumor heterogeneity or contamination with normal tissue. Unfortunately, the status of the second *p53* allele in these tumors is not known. However, even if only one allele is involved, the mutants may act in a dominant, negative fashion. The biochemical effects of such mutants should clearly be further evaluated in this regard.

In Table 2, we present clinicopathological features of the tumors with *p53* gene abnormalities. We could not identify any relations between the genetic abnormalities and tumor features or patient survival because the number of patients was too small for statistical analysis.

In conclusion, our results show that deletion and insertion mutations of the *p53* gene are not rare events in ovarian tumors. Of the 56 cases studied, eight (14%) harbored deletions or insertions, of which seven were localized in exon 5. These data suggest that exon 5 of the *p53* gene may be a hotspot for such intragenic aberrations in ovarian cancer. Mutations in exon 5 may play an important role in the clinical outcome of this cancer as previously shown for colon cancer *[24]*.

Table 2. *p53* deletions/insertions and clinicopathological features of the patients.

Tumor code	Abnormality	Age	Menopausal status	Stage	Grade	Residual tumor (cm)	Histology	Survival (months)	Status
61	Deletion/exon 5	61	Post	III	G2	5–10	Serous	31.9	Expired
68	Deletion/exon 7	54	Post	IV	G3	2–5	Serous	10.4	Expired
80	Deletion/exon 5	71	Post	III	G3	0	Serous	31.7	Remission
82	Deletion/exon 5	41	Pre	III	G3	<2	Undifferentiated	23.7	Remission
87	Deletion/exon 5	76	Post	III	G3	>10	Undifferentiated	11.6	Expired
93	Deletion/exon 5	60	Post	I	G2	0	Unclassified	16.4	Remission
97	Deletion/exon 5	49	Pre	I	G1	0	Mucinous	16.3	Remission
120	Insertion/exon 5	62	Post	III	G3	0–2	Clear	4.7	Remission

We thank D. Tsuyuki and J. Dunn for valuable advice. This work was supported by an Industrial Research Assistance Program from the National Research Council of Canada and Visible Genetics Inc., Toronto, Canada.

References

1. El-Deiry WS, Kern SE, Pietenpol JA, Kinzler KW, Vogelstein B. Definition of a consensus binding site for p53. Nature Genet 1992;1:45–9.
2. Funk WD, Pak DT, Karas RH, Wright WE, Shay JW. Definition of a consensus binding site for p53. Mol Cell Biol 1992;12:2866–71.
3. Kern SE, Pietenpol JA, Thiagalingam S, Seymour A, Kinzler KW, Vogelstein B. Oncogenic forms of p53 inhibit p53-regulated gene expression. Science 1992;256:827–30.
4. Unger T, Nau MM, Segal S, Minna JD. p53, a transdominant regulator of transcription whose function is ablated by mutations occurring in human cancer. EMBO J 1992;11:1383–90.
5. El-Deiry WS, Tokino T, Velculescu VE, et al. WAF1, a potential mediator of p53 tumor suppression. Cell 1993;75:817–25.
6. El-Deiry WS, Harper JW, O'Connor PM, et al. WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. Cancer Res 1994;54: 1169–74.
7. Nelson WG, Kastan MB. The DNA template alterations that trigger p53-dependent DNA damage response pathways. Mol Cell Biol 1994;14:1815–23.
8. Lane DP. A death in the life of p53. Nature 1993;362:786–7.
9. Lane DP. p53, guardian of the genome. Nature 1992;358:15–6.
10. Caron de Fromental Soussi T. Tp53 tumor suppressor gene: a model for investigating human mutagenesis. Genes Chrom Cancer 1992;4:1–15.
11. Hsu IC, Metcalf RA, Sun T, Welsh JA, Wang NJ, Harris CC. Mutational hotspot in the *p53* gene in human hepatocellular carcinomas. Nature 1991;350:427–8.
12. Jego N, Thomas G, Hamelin R. Short direct repeats flanking deletions, and duplicating insertions in the *p53* gene in human cancers. Oncogene 1993;8:209–13.
13. Greenblatt MS, Grollman AP, Harris CC. Deletions and insertions in the *p53* tumor suppressor gene in human cancers: confirmation of the DNA polymerase slippage/misalignment model. Cancer Res 1996;56:2130–6.
14. Mazars R, Pujol P, Maudelonde T, Jeanteur P, Theillet C. p53 mutations in ovarian cancer: a late event? Oncogene 1991;6: 1685–90.
15. Millner BJ, Allan LA, Eccles DM, et al. p53 mutation is a common genetic event in ovarian carcinoma. Cancer Res 1993;53:2128–32.
16. Okamoto A, Sameshima Y, Yokoyama S, et al. Frequent allelic losses and mutations of the *p53* gene in human ovarian cancer. Cancer Res 1991;51:5171–6.
17. Kupryjanczyk J, Thor AD, Beauchamp R, et al. *p53* gene mutations and protein accumulation in human ovarian cancer. Proc Natl Acad Sci U S A 1993;90:4961–5.
18. Liu FS, Kohler MF, Marks JR, Bast RC, Boyd J, Berchuck A. Mutations and overexpression of the *p53* tumor suppressor gene frequently occurs in uterine and ovarian sarcomas. Obstet Gynecol 1994;83:118–24.
19. Skilling JS, Sood A, Niemann T, Lager DJ, Buller RE. An abundance of p53 null mutations in ovarian carcinoma. Oncogene 1996;13: 117–23.
20. Angelopoulou K, Diamandis EP. Identification of deletions and insertions in the *p53* gene using multiplex PCR and high resolution fragment analysis: Application to breast and ovarian tumors. J Clin Lab Anal (in press).
21. Ausubel FM, et al., eds. Current protocols in molecular biology. New York: John Wiley and Sons, 1995:2.2.1–2.2.2.
22. Levesque MA, Katsaros D, Yu H, Zola P, Sismondi P, Giardina G, Diamandis EP. Mutant p53 protein overexpression is associated with poor outcome in patients with well or moderately differentiated ovarian carcinoma. Cancer 1995;75:1327–38.
23. Runnebaum IR, Tong XW, Moebus V, Heilmann V, Kieback DG, Kreienberg R. Multiplex PCR screening detects small *p53* deletions and insertions in human ovarian cancer cell lines. Hum Genet 1994;93:620–4.
24. Goh HS, Yao J, Smith DR. *p53* point mutations and survival in colorectal cancer patients. Cancer Res 1995;55:5217–21.
25. Hassapoglidou S, Diamandis EP, Sutherland DJA. Quantification of p53 protein in tumor cell lines, breast tissue extracts and serum with time-resolved immunofluorometry. Oncogene 1993;8: 1501–9.