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Detection of p53 Gene Mutations in Human Ovarian and Endometrial Cancers by Polymerase Chain Reaction-Single Strand Conformation Polymorphism Analysis

Mariko Naito,[1] Masanobu Satake,[2] Eiki Sakai,[3] Yasumasa Hirano,[3] Nobuo Tsuchida,[3] Hideharu Kanzaki,[1] Yoshiaki Ito[2,4] and Takahide Mori[1]

[1]*Department of Gynecology and Obstetrics, School of Medicine,* [2]*Department of Viral Oncology, Institute for Virus Research, Kyoto University, Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606 and* [3]*Department of Molecular Cellular Oncology and Microbiology, Faculty of Dentistry, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113*

The presence of mutations in the p53 gene was examined in ovarian cancers by a polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis. The primers were designed to amplify exons 5 through 9 that contain phylogenetically conserved domains of the p53 gene. Mutations were detected in 5 out of 10 cases, one of which contained a deletion in the second allele. A single base substitution was detected in 4 cases at codons 162, 175, 205 and 273 and a single base insertion in one case within codon 315. A high frequency of p53 mutations in ovarian cancers and lack of mutation in 6 benign ovarian tumors and 2 normal ovaries suggested that the mutation of the p53 gene was associated with the genesis and/or progression of ovarian cancer. In 1 of 7 endometrial cancers, two mutations at codons 239 and 254 were detected.

Key words: p53 — Ovarian cancer — Endometrial cancer — PCR-SSCP — HPV

Ovarian cancer is ranked as the most fatal cancer among gynecological tumors, and endometrial cancer of the uterus is the second most common gynecological malignancy in Japan. In contrast to cervical cancer in which human papillomavirus (HPV) is considered to be a main causative agent,[1,2] little is known about genetic changes that contribute to the genesis and progression of ovarian and endometrial cancers. The p53 gene has been implicated as a tumor suppressor gene [3–5] and alterations of the gene are frequently observed in many human cancers such as those of colon, lung, breast and liver.[6–9] A loss of heterozygosity in chromosomes 3, 6, 11, 17p and 17q has been reported in ovarian cancers.[10–14] The p53 gene is located on chromosome 17p13.1.[15] In this study we examined mutations in the p53 gene in ovarian and endometrial cancers by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis, which is a rapid and sensitive method to detect sequence alterations.[16,17]

Twenty-six primary tumors were obtained at surgery (Table I): ten ovarian cancers, seven endometrial cancers, three cervical cancers and six benign ovarian tumors. Histological typing of ovarian tumors was done according to the World Health Organization[18] and their stages were evaluated according to the typing system of the International Federation of Gynecology and Obstetrics.[19] Ovarian cancers that we analyzed were classified into three serous cystadenocarcinomas, two endometrioid adenocarcinomas, two clear cell carcinomas, one mullerian mixed tumor, one mucinous cystadenocarcinoma and one undifferentiated carcinoma. The six benign ovarian tumors included two mucinous cystadenomas, one serous cystadenoma and three dermoid cysts. As for the histology of the endometrial cancers,[20,21] one was serous adenocarcinoma and six were adenocarcinomas of endometrial type. The three cases of cervical cancer were squamous cell carcinomas of non-keratinizing type. Placenta was obtained from normal delivery.

Tissues were cut into small pieces with a razor blade and suspended in 4 ml of buffer containing 100 m*M* Tris-HCl, pH 8.0, 10 m*M* NaCl, and 5 m*M* EDTA per gram of tissue. $NaClO_4$ and SDS were added to final concentrations of 0.5 *M* and 0.3%, respectively. The lysate was rotated overnight at room temperature, then extracted with phenol/chloroform/isoamyl alcohol (25:24:1), and the aqueous layer was dialyzed extensively against buffer containing 20 m*M* Tris-HCl, pH 8.0, 5 m*M* NaCl, and 1 m*M* EDTA at 4°C. DNA was precipitated by adding ethanol and resuspended in buffer containing 10 m*M* Tris-HCl, pH 8.0, and 1 m*M* EDTA. Concentrations of DNAs were quantitated from the absorbancy at 260 nm measured with a spectrophotometer.

The following deoxyoligonucleotides were synthesized and used as primers in PCR: E5S, 5′-TGTTCACTTGTGCCCTGACT-3′; E5A, 5′-CAGCCCTGTCGTCTCTCCAG-3′; E6S, 5′-GCCTCTGATTCCTCACTGAT-3′; E6A, 5′-TTAACCCCTCCTCCCAGAGA-3′; E7S, 5′-ACTGGCCTCATCTTGGGCCT-3′; E7A, 5′-TGT-

[4] To whom correspondence should be addressed.

Table I. Summary of p53 Mutations Identified in 26 Cases of Gynecological Tumors

Case	Stage	Histology[a]	Exon[b]	Codon	Nucleotide change	Amino acid change
Ovarian cancer						
1	Ia	MCA	5	175	CGC→CAC	Arg→His
2	IIIc	EA	5	162	ATC→GTC	Ile→Val
3	IIIc	EA	6	205	TAT→TGT	Tyr→Cys
4	IIIc	MMT	9	315	insertion of T	premature
4′[c]		MMT	9		(TCT→TCTT)	termination
5	IIIc	SCA	8	273	CGT→CAT	Arg→His
5′[c]		SCA	8			
6	Ic	CCA	—			
7	Ia	SCA	—			
8	IIIc	SCA	—			
9	IIIc	CCA	—			
10	IV	UC	—			
Endometrial cancer						
11	Ib	SAC	7	239	AAC→GAC	Asn→Asp
12	II	AC	—	+254	ATC→AGC	Ile→Ser
13	Ia	AC	—			
14	Ib	AC	—			
15	Ia	AC	—			
16	Ia	AC	—			
17	II	AC	—			
Cervical cancer						
18	IIb	SCC	—			
19	IIa	SCC	—			
20	Ib	SCC	—			
Benign ovarian tumor						
21		MC	—			
22		SC	—			
23		MC	—			
24		DC	—			
25		DC	—			
26		DC	—			

a) MCA, mucinous cystadenocarcinoma; EA, endometrioid adenocarcinoma; MMT, mullerian mixed tumors; SCA, serous cystadenocarcinoma; CCA, clear cell carcinoma; UC, undifferentiated carcinoma; SAC, serous adenocarcinoma; AC, adenocarcinoma, endometrial type; SCC, squamous cell carcinoma; MC, mucinous cystadenoma; SC, serous cystadenoma; DC, dermoid cyst.
b) − in the exon column means that p53 gene mutation was not detected.
c) Metastatic lesions obtained from the corresponding case.

GCAGGGTGGCAAGTGGC-3′; E8S, 5′-TAAATGGGACAGGTAGGACC-3′; E8A, 5′-TCCACCGCTTCTTGTCCTGC-3′; E9S, 5′-ACTAAGCGAGGTAAGCAAGC-3′; E9A, 5′-CTGGAAACTTTCCACTTGAT-3′. They were designed to amplify coding exons 5 through 9 of the p53 gene.[22] For example, the pair of E5S and E5A primers hybridize to the sequences in introns 4 and 5, respectively, and amplify exon 5 by PCR. Expected sizes of the PCR fragments are 269 base pairs (exon 5), 181 base pairs (exon 6), 171 base pairs (exon 7), 229 base pairs (exon 8) and 210 base pairs (exon 9), respectively.

The PCR mixture in a total volume of 10 μl contained 100 ng of genomic DNA, 20 pmol of each primer, 500 μM each of deoxynucleotide triphosphates, 20 mM Tris-HCl, pH 8.3, 2 units of *Taq* DNA polymerase and 0.185 MBq of [α-^{32}P]dCTP (110 TBq/mmol).[23] Thirty-five cycles of denaturation (60 s at 95°C), and annealing and extension (80 s at 65°C for exons 5 and 9, and 60 s at 60°C for exons 7 and 8) were performed. For exon 6, annealing (60 s at 55°C) and extension (30 s at 72°C) were done separately.

The PCR products were diluted 50- to 100-fold with buffer containing 0.1% SDS and 10 mM EDTA, and subjected to an SSCP analysis.[16,17] Two μl was mixed with an equal volume of buffer containing 95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol, heated at 85°C for 2 min, quickly chilled on ice and applied to a 6% polyacryl-

amide gel (20×40×0.03 cm) containing 90 m*M* Tris-borate (pH 8.3), 4 m*M* EDTA and 5% glycerol. Electrophoresis was performed at 50 W for 2 h under cooling with a fan at 15°C. The gel was dried on a filter paper and exposed to X-ray film at −80°C for 12 h.

DNA of normal placenta contains the normal p53 gene. The SSCP analysis of the PCR fragments of this DNA exhibited two bands for exons 5, 7, 8 and 9 (see the lanes indicated as hp in Fig. 1). In the case of exon 6, however, three bands were observed for placental DNA.

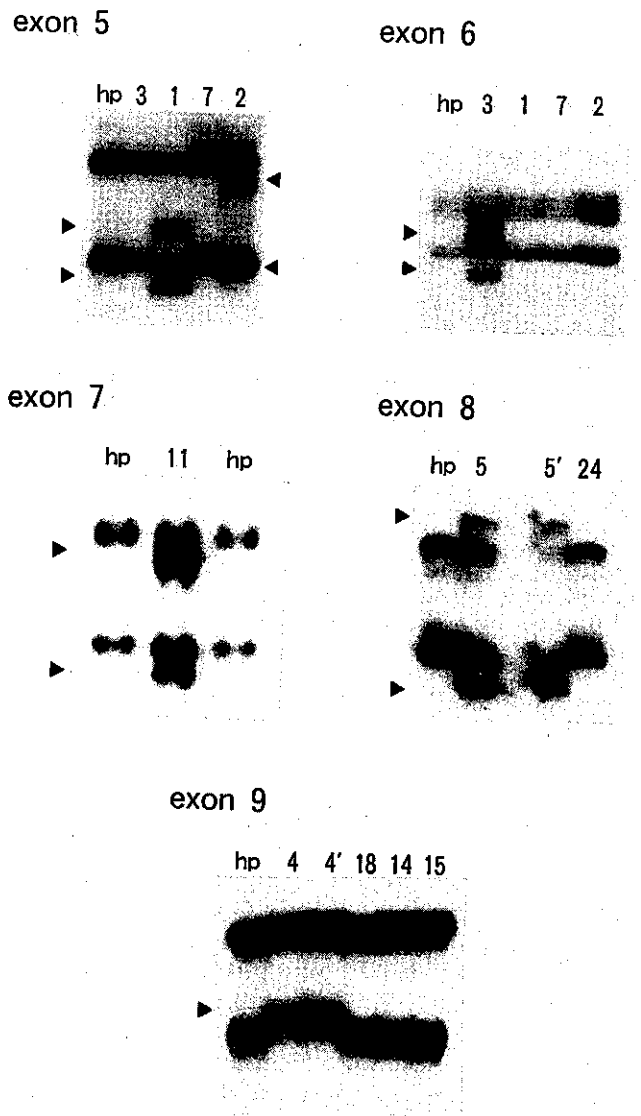

Fig. 1. Detection of p53 mutations by PCR-SSCP analysis. Numbers shown on top of the gels indicate case numbers in Table I. hp stands for normal human placenta DNA. Arrowheads indicate bands which migrated at different positions from normal ones.

The sum of the intensity of the two upper bands appeared to be roughly equal to that of the lowest band. It is likely that one strand of this segment of DNA can take two different conformations in the electrophoresis. Migration of the molecules at locations different from the normal ones indicates the presence of a base change(s) in the primary sequence. In cases 1 (see exon 5), 2 (exon 5), 3 (exon 6), 5 (exon 8) and 11 (exon 7), two more bands in addition to two normal bands were observed, and represented two complementary strands of the mutated allele. In case 11 (see exon 7), however, the upper abnormal band was broader than normal bands. The significance of this observation will be discussed below in connection with the sequence alterations found in these bands. Loss of normal alleles was evident in exon 9 of case 4, where the normal band was not detected at all. In summary, five of ten ovarian cancers and one of seven endometrial cancers contained abnormalities in the p53 gene (Table I). These abnormalities were detected within exon 5 in two cases and in exons 6, 7, 8 and 9, in one case each.

Metastatic lesions were obtained for cases 4 and 5. The SSCP analysis of the DNA samples from metastatic tissues exhibited the same pattern as that of the corresponding primary lesions (compare lanes 4′ and 5′ with 4 and 5 in Fig. 1), indicating the clonality of primary and secondary lesions. In contrast, normal ovaries which were excised simultaneously at surgery in cases 1 and 3 gave no abnormal band in SSCP analysis (data not shown). Six cases of benign ovarian tumors and three cases of cervical cancer did not show any abnormal band, either (Table I).

The sequence alteration of the mutated p53 gene was examined. The PCR products were purified from an agarose gel and cloned into the *Sma* I site of pUC 18. The nucleotide sequences of the cloned PCR products were determined on both strands by a chain elongation termination method using dideoxynucleotides.[24] Single nucleotide substitutions were detected in four cases at codon 162, 175, 205, or 273, resulting in amino acid changes (Table I). In case 4, one nucleotide (T) was inserted within codon 315, causing premature termination of the open reading frame 20 aa downstream of the insertion. The authenticity of the sequence alterations shown in Table I was confirmed by performing the SSCP analysis. The PCR fragment generated from the cloned DNA using the same set of primers migrated to the same positions as the abnormal fragment generated from the respective cellular DNAs (data not shown).

The sequence alterations in case 11 were more complex. Therefore, we also performed direct sequencing. The abnormal bands shown by arrowheads in Fig. 1, exon 7, were isolated separately and sequenced using single-stranded DNAs synthesized by asymmetic PCR as

templates.[22] The results showed that each band seemed to be a mixture of more than one species, consistent with the broadness of the band, especially of the more slowly migrating one. In both cases, 80–90% of the amplified fragments contained the mutation at codon 239 (AAC→GAC). To lesser extents, the mutated sequence at 254 (ATC→AGC) and normal-type sequence were detected. The results suggested that these abnormal bands contained molecules harboring the mutation at codon 239 alone and at codon 254 alone and possibly one harboring the mutations at both codons 239 and 254. When 26 independent clones of the amplified exon 7 fragments were sequenced, we found clones mutated at codon 239 alone, at codon 254 alone, and also at both codons 239 and 254. At the moment, it is not clear whether this last type of molecule containing the double mutations actually existed in the original tumor tissue or whether it was an artefact generated during the PCR and/or cloning. In any case, it is remarkable to find two independent mutations in the same gene in such close proximity in a DNA sample obtained from a single tumor tissue. It is possible that this tumor contained two clonal cell populations, one containing the mutation at codon 239 and the other at codon 254.

Next, we surveyed the prevalence of HPV genome in genital tumors we had collected. To detect HPV genome by PCR, the following primers were used: a sense primer, 5′-GAATATGATTTACAGTTTATTTTTCA-3′ and an antisense primer, 5′-GAAAACTTTTCCTTTAAAT-3′. The primers hybridize to the sequences in a conserved region in L1 open reading frame so that a broad spectrum of genital HPV such as types 6b, 11, 16, 18, 33, 52b and 58 can be detected.[25] Denaturation (60 s at 94°C), annealing (120 s at 32°C) and extension (120 s at 72°C) were performed for thirty-five cycles. The PCR product was electrophoresed through a 2.5% agarose gel and stained with ethidium bromide. As can be seen in Fig. 2, no amplified band could be detected in ten cases of ovarian cancers (lanes 1 through 10) and seven cases of endometrial cancers (lanes 11 through 17). On the other hand, 3 cases of cervical cancer (lanes 18 through 20) and CaSki cells harboring HPV type 16[1] generated the expected fragment of 250 base pairs.

By using a PCR-SSCP method, we demonstrated mutations in the p53 gene in 5 out of 10 ovarian cancers (50%). This frequency is relatively high compared to 37% (11 of 30 cases) reported by Mazars *et al.*[26] and 29% (9 of 31 cases) reported by Okamoto *et al.*[27] both of whom examined the same type of cancer by the same method. It has been reported that the majority of p53 missense mutations found in various malignancies are clustered within the four conserved regions,[28] which are located between amimo acid residues 120 and 286.[29] Moreover, a notable feature of the p53 mutational spectra in human cancers is that transitions at CpG dinucleotides contribute heavily to the mutation frequency in many cancers. Nearly one-third of the 280 human p53 mutations are transitions at hot spot codons with CpG sites (codons 175, 196, 213, 248, 273 and 282).[30] In particular, the three mutational hot spots at codons 175, 248 and 273 are noteworthy.[29, 30] We also detected the mutations at codons 175 and 273. However, the mutations at codons 162 and 315 that we detected have not been reported in the literature.[26, 33–43] We have not studied the significance of these mutations yet. In the case of the mutation at codon 315, however, we speculate as

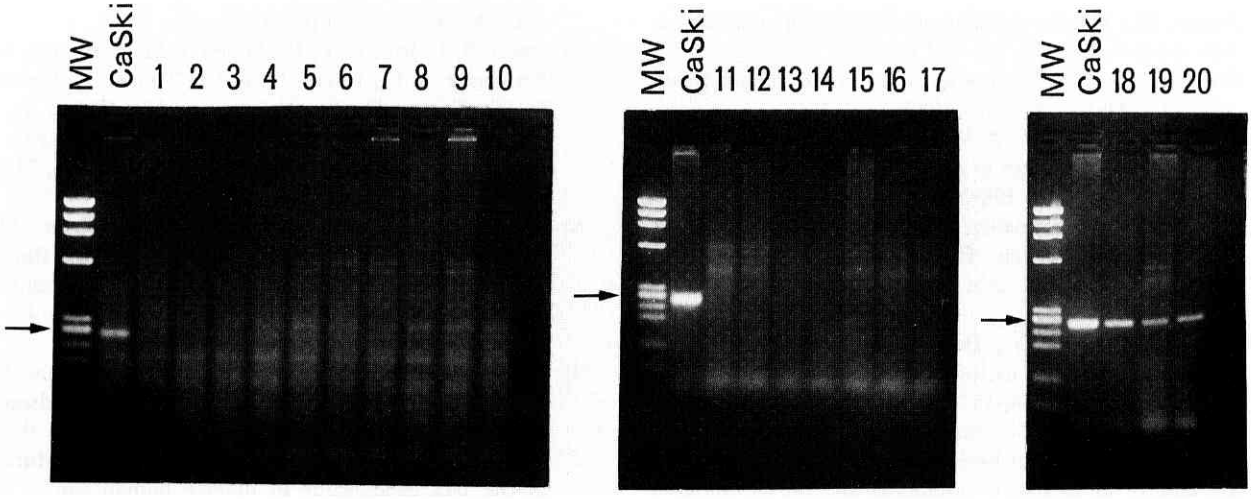

Fig. 2. Examination of HPV genome by PCR. Numbers shown on top of the gels correspond to case numbers in Table I. MW is a molecular weight marker lane containing *Hae*III-digested fragments of ϕX DNA.

follows. p53 contains several nuclear localization signals one of which, NLS I, is located in the region of codons 316–325.[31, 32] A single base insertion at codon 315 eliminates a cluster of lysine residues and terminates the protein 20 aa residues downstream. This would have a significant effect on the properties of the protein. In addition, no oligomerization would take place. The exons examined in this study include most of the phylogenetically conserved domains of p53[28] and the mutations detected did not cluster in a particular exon. The nucleotide changes are not confined to a specific pattern, since two cases each of A to G and of G to A transition and one case of insertion of T have been observed. The expected amino acid changes are both conservative (two cases of Arg to His and one case of Ile to Val) and non-conservative (Tyr to Cys). At the moment, we cannot rule out the possibility that some of these amino acid substitutions may not alter the biological functions of p53.

Mutations have been observed in various histological types of ovarian cancers. The p53 mutations were detected not only with a high frequency but also in the early stages of cancers (see case 1 in Table I). They were not detected in 6 benign ovarian tumors tested, or in unaffected ovaries. These observations strongly suggest that the p53 mutation is associated with the genesis and/or progression of ovarian cancer.

Loss of the normal alleles was detected in exon 9 of case 4. Allelic loss has been reported in 3 of 11[26] or 8 of 9 ovarian cancers.[27] The amplified regions in this study are not informative as to loss of heterozygosity. Polymorphism has been detected in exons 2, 4 and 6 and in introns 2 and 7 of the gene.[27, 44–47] Indeed, Okamoto *et al.* reported loss of heterozygosity in 15 of 19 ovarian cancers as detected by SSCP analysis.[27] Allelic loss was also detected by restriction fragment length polymorphism (RFLP) analysis.[14, 27] The use of appropriate primers in the PCR-SSCP analysis may reveal loss of heterozygosity of the p53 gene in the four cases of ovarian cancers in which we did not detect mutation.

Adenocarcinoma of uterine endometrium is characteristic because of the existence of a precancerous state and progressive development that is similar to that case of colorectal cancer.[20, 21, 48–50] Therefore, it was of interest to study whether the p53 gene mutation could be detected as an early event in this cancer. We detected mutations in 1 out of 7 cases of endometrial adenocarcinoma. This frequencey of mutation is similar to that reported by Okamoto *et al.*[38] (3 out of 24 cases). Clearly, evaluation of the significance of the p53 gene mutation requires a more extensive survey. One serous adenocarcinoma in which we detected the mutation is rather a rare type of endometrial cancer. As for the involvement of HPV in gynecological tumors, we found no evidence of its presence in ovarian and endometrial cancers, in contrast to the 3 cases of cervical cancers.

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