Frequent Allelic Losses and Mutations of the p53 Gene in Human Ovarian Cancer

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ABSTRACT

The p53 gene on chromosome 17p is considered to be a tumor suppressor gene, and frequent mutations of the p53 gene have been found in a wide variety of human cancers. We examined 31 ovarian cancers for allelic losses and mutations of the p53 gene by polymerase chain reaction-single strand conformation polymorphism analysis as well as restriction fragment length polymorphism analysis. Allelic loss of the p53 gene was detected in 16 of 20 cases (80%). Mutations were detected in 9 of 31 cases (29%): 2 cases in exon 4; 5 cases in exons 5–6; and 2 cases in exons 7–8. In 8 of 9 cases, p53 mutations were accompanied by losses of the normal allele. These alterations of the p53 gene were commonly detected from stage I to stage IV. These results suggest that alterations of the p53 gene play an important role in the development of human ovarian cancers.

INTRODUCTION

It is widely accepted that both activation of protooncogenes and inactivation of tumor suppressor genes are involved in the genesis or progression of various kinds of human tumors. Although ovarian cancer is the most lethal tumor of the female genital tract, molecular mechanisms of tumor development have not yet fully been understood. At present, little information is available on the molecular genetic alterations associated with either genesis or progression of ovarian cancer. For instance, overexpression and amplification of the erbB-2/HER-2/neu gene have been reported to be associated with poor prognosis of the patients with this disease (1,2). In addition, loss of heterozygosity on chromosomes 3, 6, 11, 17p, and 17q has been reported to occur frequently in ovarian cancer (3–7), suggesting the involvement of tumor suppressor gene inactivation in the development of this tumor. The p53 gene is located on chromosome 17p13.1 (8) and the accumulating evidence suggests that it acts as a tumor suppressor (9–11). This gene is considered to be constitutionally homozygous by this method.

In this article, we investigate structural alterations of the p53 gene in 31 primary ovarian tumors obtained at surgery by PCR-SSCP analysis which is a rapid and sensitive method for detection of mutations and polymorphisms in a given sequence (12). RFLP analysis was also performed to examine for allelic loss of the p53 gene in these tumors. RFLP and PCR-SSCP analyses showed allelic loss in 16 of 20 informative cases (80%). Mutations of the p53 gene were detected in 9 of 31 cases (29%) by PCR-SSCP analysis. These results strongly suggest that allelic losses and mutations of the p53 gene are critical genetic alterations for the development of ovarian cancer.

MATERIALS AND METHODS

Human Tissue Samples and DNA Extraction. Thirty-one primary tumors were obtained at surgery and ten of them were with adjacent noncancerous tissue or peripheral blood to provide sources of these patients’ normal tissue DNA. The tumors were histologically classified according to the Histological Typing of Ovarian Tumors by the World Health Organization (13) and staged according to the International Federation of Gynecology and Obstetrics staging system. Twenty-nine of the tumors were of epithelial origin: ten serous adenocarcinomas; ten clear cell adenocarcinomas; four endometrioid carcinomas; one mesodermal mixed tumor; and four mixed adenocarcinomas. The other two tumors were germ cell tumors: one mature teratoma; and one embryonal carcinoma. Nine tumors were classified as being stage I, 4 as stage II, 12 as stage III, and 6 as stage IV (Table 1). High-molecular-weight DNA was prepared by proteinase K digestion and phenol/chloroform extraction as described (14).

RFLP Analysis. Ten μg of DNA were digested with BanII or BglII endonuclease, electrophoresed on 0.8% agarose gel, transferred to nylon filters, and hybridized to 32P-labeled p53 cDNA probe pR4-2 (15), prepared by random primer labeling. In ten cases with corresponding normal tissue, allelic loss was considered to have occurred when the hybridization intensity of one allelic fragment was less than 40% that of the other allelic fragment measured by FUJIX BAS 2000 SYSTEM (17). Since a residual faint signal, probably due to contamination of normal cells in the tumor specimen, was observed in most of the tumors showing allelic loss, we were able to determine the presence of allelic loss in the other 21 cases without corresponding normal tissue by means of loss of intensity of 1 of 2 allelic fragments; i.e., allelic loss was considered to have occurred if tumors showed a heterozygous genotype and the signal intensity of one of the allelic fragments was less than 40% that of the other allelic fragment. Although this approach may underestimate the occurrence of allelic loss in tumor, it never overestimates the incidence of allelic loss, because complete loss is considered to be constitutionally homozygous by this method.

PCR-SSCP Analysis. Oligonucleotide primers used for PCR to amplify coding exons of the p53 gene were designed based on the published sequence (18): 2U, GGAATTCCTTCTCTGAGCAAGCCA; 3D, GGAATTCCTTCTCTGAGCAAGCCA; 4U, GGAATTCCTTCTCTGAGCAAGCCA; 5U, GGAATTCCTTCTCTGAGCAAGCCA; 6D, GGAATTCCTTCTCTGAGCAAGCCA; 7D, GGAATTCCTTCTCTGAGCAAGCCA; 8D, GGAATTCCTTCTCTGAGCAAGCCA; 9D, GGAATTCCTTCTCTGAGCAAGCCA; 10D, GGAATTCCTTCTCTGAGCAAGCCA. For instance, 2U and 3D are upstream and downstream primers, respectively, spanning exons 2–3 of the p53 gene. Sizes of PCR fragments are 266 base pairs (exons 2–3, codons 1–32), 317 base pairs (exon 4, codons 33–125), 416 base pairs (exons 5–6, codons 126–224), 623 base pairs (exons 7–8, codons 225–306), 104 base pairs (exon 9, codons 307–311), 145 base pairs (exon 10, codons 332–367). The abbreviations used are: PCR-SSCP, polymerase chain reaction-single strand conformation polymorphism; RFLP, restriction fragment length polymorphism.
buffer containing 1.0 µl of [32P]dCTP (3000 Ci/mmol; 10 Ci/ml). Thirty cycles consisting of 40 s at 94°C (denaturation), 40 s at 55°C (annealing), and 90 s at 72°C (extension) were performed. Two µl of the PCR product were diluted 100-fold by a buffer consisting of 20 mM EDTA, 96% formamide, 0.05% bromophenol blue, and 0.05% xylene cyanol. Forty-five µl of this diluted sample were heated at 80°C for 2 min and then 5-6, 7-8, 9, and 10. Electrophoresis was performed at 30 W for 1.5-12 h with cooling by fans at room temperature or at 4°C. The gel was gelled and transferred to Kodak XAR film at −80°C for 10-24 h with an intensifying screen.

Cloning and Sequencing. Since the primers used for PCR had EcoRI sites at their 5’ ends, the products were purified, digested with EcoRI, and ligated into the EcoRI site of pGEM1 plasmid. Sequencing was performed by the dideoxy chain termination method with a 7-DEAZA MspI, TaqI, or PvuII endonuclease; electrophoresed on 0.8% agarose gel; transferred to nylon filters; and hybridized to 32P-labeled probes: pHH35 (second exon of the c-myc gene subcloned from pHSR-1); pNB1 (N-myc); pCERNH2 (erbB-2/HER-2/neu); pE7 (EGFR); SS6 (INT2); and probe AA (HST1) (19-24).

RESULTS

Allelic Losses of the p53 Gene Detected by RFLP Analysis. The polymorphic DNA fragments digested with the restriction endonuclease BanII hybridized to the pR4-2 probe were 2.9 kilobases long (allele 1) and 1.5 and 1.4 kilobases long (allele 2) (25); three constant fragments (1.2, 0.9, and 0.7 kilobases) were also detected. We measured the intensity of 2.9- and 1.5-kilobase fragments and obtained a ratio of 2.9 to 1.5 or 1.5 to 2.9 kilobases which was always expressed as the lesser value over the represented allele; thus the ratio was always smaller than 1. Eleven of 31 cases showed heterozygous genotypes by BanII digestion of DNA hybridized to the pR4-2 probe. As shown in Table 2, the ratio of signal intensity of allelic fragments in 4 normal tissue DNAs ranged from 0.877 to 0.947, while that in 11 tumor DNAs ranged from 0.043 to 0.991. Because allelic loss was considered to have occurred when the ratio was less than 0.4, it was concluded that the allelic loss had occurred in 8 tumors (Cases 2, 4, 12, 14, 23, 24, 25, and 29) in which the ratio was below 0.373 and that heterozygosity was retained in 3 tumors (Cases 6, 7, and 30) in which the ratio was between 0.926 and 0.991. The hybridization pattern of tumor DNA showing loss is shown in Fig. 1. The p53 cDNA probe, pR4-2, also detected BglIII polymorphisms, and two allelic fragments, 12 (allele 1) and 9 (allele 2) kilobases long (25), in addition to one constant 3-kilobase band were detected. Nine of 31 tumors showed heterozygous genotypes and the intensity ratio of allelic fragments was less than 0.4 in 6 of 9 tumors: Cases 2, 4, 12, 14, 17, and 24 (Table 2). As a total, 12 of 31 cases were informative and allelic loss the the p53 gene was observed in 9 of the 12 informative cases (75%).

Allelic Losses and Mutations of the p53 Gene Detected by PCR-SSCP Analysis. The results of PCR-SSCP analysis on exons 2-11 of the p53 gene are summarized in Table 1. It was previously shown that several types of DNA polymorphisms could be efficiently detected by PCR-SSCP analysis (13, 26, 27). By this method, it was shown that there are DNA polymorphisms in the regions within exons 2-3, within exon 4, and within exons 7-8, so that we were able to detect allelic loss as a reduced intensity of allelic fragments by PCR-SSCP analysis (Fig. 2). As in the case of RFLP analysis, allelic loss was considered to have occurred if the signal intensity of a paired allelic fragment was less than 40% of the other paired fragments by PCR-SSCP analysis. In exons 2-3, the heterozygosity was represented by 4 bands with different mobility; the inner 2 bands (allele 1, A1) and outer 2 bands (allele 2, A2) corresponded to each allele of the complementary single strand DNA (Fig. 2A). Allelic loss was observed in 8 of 12 tumors with heterozygous genotypes: Cases 2, 4, 12, 14, 24, 25, 27, and 29. In exon 4, 3 bands were observed in patients with heterozygous genotypes (Fig. 2B). The band on the top (A2) and the band in the middle (A1) were allelic single strand DNA molecules, and

### Table 1 Alterations of the p53 gene in ovarian cancer

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*CCA, clear cell adenocarcinoma; MT, mature teratoma; EA, endometrioid adenocarcinoma; SA, serous adenocarcinoma; MA, mixed adenocarcinoma; MMT, mesodermal mixed tumor; EC, embryonal carcinoma; 1, loss of smaller allele by a RFLP analysis or loss of lower or outer band by a PCR-SSCP analysis; 2, loss of larger allele by a RFLP analysis or loss of upper or inner band by a PCR-SSCP analysis; 1.2, retention of heterozygosity; LON, loss of normal allele by a PCR-SSCP analysis; -,-, mutation negative or constitutional homozygosity.

The ratio of signal intensity of two allelic fragments was calculated as described in "Materials and Methods."
Fig. 1. Allelic loss of the p53 gene detected by RFLP analysis. DNA from ovarian carcinoma (T) and corresponding normal tissue (N) was digested with BanI endonuclease and hybridized to the p53 cDNA probe, pR4-2. Top abscissa, patient numbers; right ordinate, the size of each hybridizing band in kilobase pairs (kbp). Alleles are designated A1 or A2 (left ordinate).

Fig. 2. Allelic losses of the p53 gene detected by PCR-SSCP analysis. Top abscissa, patient numbers; polymorphic bands are designated A1 or A2 in exons 2-3 (A), exon 4 (B), and exons 7-8 (C). The signal intensity of either A1 or A2 reduced in tumor samples (T) compared with that in normal tissue samples (N).

the band at the bottom corresponded to each of the complementary strands which were co-migrated under this condition. Signal intensity of 1 of the upper 2 bands was reduced in 9 of 11 heterozygotes: Cases 2, 4, 12, 14, 24, 25, 27, 29, and 31. Typical cases with allelic losses in this region are shown in Fig. 2B. In exons 7-8, 4 fragments were detected in the case of heterozygous patients as in the case of exons 2-3. Cases 2, 4, 12, 14, 23, 24, 25, and 29 showed allelic loss by a PCR-SSCP analysis as well as by a RFLP analysis of BanII-digested DNA probed to pR4-2 (Figs. 1 and 2C), because the polymorphic site in intron 7 was represented not only as a BanII polymorphism by RFLP analysis but also as different mobilities by PCR-SSCP analysis of exons 7-8 (28). Moreover, we were also able to determine allelic loss in five additional cases with mutations by a reduced intensity of the corresponding normal fragments as described below (Cases 3, 10, 11, 15, and 28). Allelic loss of the p53 gene was detected in 15 of 19 cases (79%) by PCR-SSCP analysis. In total, allelic loss of the p53 gene was detected in 16 of 20 cases (80%) by PCR-SSCP and RFLP analyses.

In 9 of 31 tumors, different mobilities were observed in 1 of 7 PCR fragments (Fig. 3). If a normal allele is retained in the tumor, the intensity of bands for the mutated allele should be the same as or less than that of bands for the normal allele. The intensity of bands with different mobilities was higher than that of the corresponding normal bands in 8 of 9 cases. This result indicates the loss of normal allele in these tumors. Bands with the same mobility could originate from normal cell contamination in tumor tissue or may be due to heterogeneity of tumor cells with respect to allelic loss of the p53 gene. In Case 26, the intensity of the band with different mobility was lower.
than that of the band with no different mobility (Fig. 3C1). Moreover, information on allelic loss was not available in this case because of constitutional homozygosity either by RFLP or PCR-SSCP analysis. Therefore, we were not able to determine whether the normal allele of the \( p53 \) gene was lost in this tumor.

Mutations in Exon 4 of the \( p53 \) Gene. Since mutations in exon 4 have not yet been reported to occur in tumors, we further sequenced exon 4 of the \( p53 \) gene in the tumors from Cases 10 and 25, which showed a mobility shift in exon 4. A 10-base pair deletion between nucleotides 215 and 224 (GGCTGGCCCCC) was detected in Case 10 (Fig. 4A), and this deletion resulted in a premature termination codon (TGA) at nucleotides 365–367 in exon 4. These nucleotide numbers are based on numbering 1 at A of the start codon ATG in \( p53 \) complementary DNA (16). In Case 25, a 1-base pair deletion between nucleotides 333 and 335 (GGG) was detected (Fig. 4B) and this deletion also resulted in a novel stop codon (TGA) at nucleotides 365–367 in exon 4.

Oncogene Amplification. The amplification of six oncogenes, \( erbB-2/HER-2/neu \), \( EGFR \), \( c-myc \), \( N-myc \), \( HST1 \), and \( INT2 \), was examined in these 31 tumors by Southern blot analysis. Although \( HST1 \) and \( INT2 \) were coamplified in 1 of 31 tumors (Case 28), amplification of other 4 oncogenes was not detected in these tumors. The Case 28 tumor showed both loss and mutation of the \( p53 \) gene.

Clinical and Pathological Features of Tumors with \( p53 \) Alterations. Staging and pathological typing of tumors were reviewed with respect to the presence or absence of \( p53 \) abnormalities (Table 1). Allelic loss was observed in three of five informative cases in stage I, three of four in stage II, six of the six in stage III, and four of four in stage IV. We compiled statistics on allelic loss in the histological type and detected losses in six of seven serous adenocarcinomas, four of six clear cell adenocarcinomas, two of two endometrioid adenocarcinomas, two of two mixed adenocarcinomas, one of one mature teratoma, one of one mesodermal mixed tumor, but none of one embryonal carcinoma. Mutations were detected in one of nine cases in stage I, two of four in stage II, three of 12 in stage III, and three of six in stage IV. Histologically, 3 of 10 serous adenocarcinomas, 2 of 4 endometrioid adenocarcinomas, 3 of 4 mixed adenocarcinomas, and 1 of 1 malignant mixed mesodermal tumor showed \( p53 \) mutations, but none of 10 clear cell adenocarcinomas and neither the mature teratoma nor the embryonal carcinoma carried mutated \( p53 \) genes.

DISCUSSION

We show here frequent allelic loss and mutations of the \( p53 \) gene in ovarian cancer by using PCR-SSCP analysis, a sensitive method for detection of DNA polymorphisms and mutations in a given sequence (13). Eccles et al. (7) previously reported that allelic loss of the \( p53 \) gene was observed in 54.5% (6 of 11) of ovarian cancer. In our study, allelic loss of the \( p53 \) gene was detected in 16 of 20 cases (80%) examined and it was commonly observed from clinical stage I to IV. Therefore, it is likely that allelic losses of the \( p53 \) gene in ovarian cancer are early events in the clinical course of tumor development. Moreover, we found 9 cases with mutation of the \( p53 \) gene by PCR-SSCP analysis in these 31 ovarian cancers (29%), and in at least 8 of these 9 tumors the normal allele of the \( p53 \) gene was lost. These results strongly suggest that loss of normal function of the \( p53 \) gene is one of the critical events for the development of ovarian cancer.

Although amplification of \( erbB-2/HER-2/neu \) was reported to occur in 20–30% of ovarian cancer and to be associated with poor survival of patients with this disease (1, 2), no amplification was detected in these 31 tumors. Moreover, two other oncogenes, \( INT2 \) and \( HST1 \), were coamplified only in a case with stage IV (Case 28). Therefore, alterations of the \( p53 \) gene probably occur prior to amplification of these oncogenes.

We analyzed various histological types of ovarian cancer. Although alterations of the \( p53 \) gene were not confined to one histological type, no mutation was detected in ten clear cell adenocarcinomas, while allelic losses were detected in four of these six tumors. It is possible that molecular mechanisms for the development of clear cell adenocarcinoma are different from those of other types of ovarian cancer.

The incidence of allelic loss was higher than that of mutation. There are several possible explanations for this result: (a) mutations may exist in the noncoding regions of the \( p53 \) gene in some tumors, because only coding exons of the \( p53 \) gene were analyzed by PCR-SSCP analysis; (b) there are undetectable \( p53 \) mutations by a PCR-SSCP analysis, since DNA fragments with different sequences sometimes comigrate each other under certain conditions as in the case of exon 4; (c) there are other tumor suppressor genes than the \( p53 \) gene on chromosome 17, because it has been shown by RFLP analysis that the distal portion of 17p is lost in breast carcinoma (29) and 17q is lost in ovarian carcinoma (6, 7).

Although mutations in exons 5–10 of the \( p53 \) gene have been reported to occur frequently in various types of tumors (30), mutations in exon 4 have not yet been reported. We performed sequence analysis in two cases which showed a mobility shift in exon 4 and found novel mutations in exon 4 of the \( p53 \) gene. One was a 10-base pair deletion and the other was a 1-base pair deletion in exon 4; both mutations resulted in a premature stop.
Fig. 4. Intragenic deletion in exon 4 of the p53 gene. A 10-base pair deletion and a 1-base pair deletion were detected in Case 10 (A) and Case 25 (B), respectively, by sequencing using templates from several single clones. Although sequences of some clones, which were probably derived from normal cells contaminated in tumor tissue, were identical to those of the wild-type p53 gene published previously (N) (18), most of the clones carry these deletions (T).

codon in exon 4. Since these two cases also showed allelic loss of the p53 gene by PCR-SSCP and RFLP analyses, it is obvious that no normal p53 protein was produced in these tumors. Although the sites of mutations in these tumors were not within the highly conserved regions of the p53 gene, it is noted that these deletions eliminate the synthesis of normal p53 proteins as a result of the appearance of new stop codon before the regions with conserved sequences.

PCR-SSCP analysis revealed the presence of DNA polymorphisms in the regions of exons 2–3, 4, and 7–8 of the p53 gene. By sequence analysis of introns 2 and 7, it was concluded that polymorphic patterns represented by PCR-SSCP analysis were completely associated with the sequence polymorphisms in introns 2 and 7 (28). Polymorphic patterns of exon 4 fragments were due to sequence polymorphism in codon 72 [arginine (CGC) or proline (CCG)] (31, 32). These different patterns for exons 7–8 by PCR-SSCP analysis completely cosegregated with BanII polymorphic patterns by RFLP analysis, and the intensity ratio of two alleles by PCR-SSCP analysis was almost equal to that by RFLP analysis. Therefore, we were able to evaluate the presence of allelic loss of the p53 gene by PCR-SSCP analysis. Moreover, 19 of 31 cases (61%) were informative by PCR-SSCP analysis, although information was available in only 12 of these 31 tumors (39%) by RFLP analysis.

The p53 gene has been shown to be mutated and lost frequently in a wide variety of human cancers, and in this study we also showed that it is mutated or lost in a high population of ovarian cancer. A further study should focus on the determination of biological significance of the p53 mutations for the development of ovarian cancer.

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