Jpn. J. Cancer Res. 83, 978–984, September 1992

High Incidence of p53 Gene Mutation in Human Ovarian Cancer and Its Association with Nuclear Accumulation of p53 Protein and Tumor DNA Aneuploidy

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Using the poylmerase chain reaction and single-strand conformation polymorphism analysis, p53 gene mutations were examined in 24 cases of ovarian tumor including 14 ovarian carcinomas and 2 borderline cases of common epithelial type, 7 germ cell tumors, and one stromal tumor. Abnormal bands indicating mutations were detected in 12 (50%) of the cases examined, being present most frequently in common "epithelial" ovarian carcinoma (71%, 10/14). One case each of squamous cell carcinoma originating in a dermoid cyst and anaplastic dysgerminoma were positive for mutation. Direct sequencing confirmed 12 mutations and revealed G→A and G→C nucleotide changes in 5 and 3 cases (42% and 25%), respectively. The mutation was localized at the CpG site of the gene in 3 cases. Immunohistochemical examination of p53 protein in 21 cases and DNA flow-cytometrical analysis in 17 cases were also performed. Nuclear accumulation of the p53 protein and DNA aneuploidy pattern were detected in 11 (52%) and 9 (53%) cases, respectively. These were significantly correlated with p53 gene mutation ($P<0.01$ and $P<0.05$, respectively; Fisher's exact test). Neither mutation of the p53 gene, nuclear accumulation of p53 protein nor DNA aneuploidy was detected in borderline cases of common "epithelial" type, typical dysgerminoma and immature teratoma. These results suggest that p53 gene mutation, nuclear accumulation of the protein and the DNA aneuploidy pattern are events occurring almost simultaneously in the progression of ovarian tumors, and that p53 abnormalities seem to be correlated with a high grade of malignancy.

Key words: Ovarian cancer — p53 — Tumor-suppressor gene — DNA ploidy pattern — PCR-SSCP

Although ovarian cancer is the leading cause of death among all cancers of the female reproductive tract,[1] the genetic alterations involved in ovarian cancer remain largely unknown. However, as is the case with other common human cancers, accumulation of multiple genetic alterations must be present in ovarian cancer, playing important roles in carcinogenesis and tumor progression. Amplification of the c-*erb*B-2, c-*myc* and c-Ki-*ras* genes has been detected in ovarian cancer,[2–4] but its incidence is not high, and more work is necessary to elucidate the role of this type of alteration. Recently, mutations of the p53 gene have been detected in many types of human cancer including ovarian cancer.[5–7] The p53 gene lies on the chromosomal locus 17p13, where one allele has been shown to be deleted by restriction fragment length polymorphism (RFLP[5]) analysis.[7–12] Thus, p53 is now considered to be a tumor-suppressor gene, which is usually inactivated by both of two events, mutation of one allele and loss of the other allele. In the present study, p53 gene mutations were examined in DNA samples extracted from surgical specimens of ovarian cancer in order to clarify the extent of involvement of the p53 gene abnormality. Furthermore, clinicopathological parameters and tumor DNA ploidy pattern were examined in relation to p53 gene mutation in order to understand the role of p53 mutation in the development of ovarian cancer. An immunohistochemical study of p53 protein localization was also performed to elucidate the link between p53 mutation and nuclear accumulation of its protein.

MATERIALS AND METHODS

Sample Twenty-four specimens from patients with ovarian tumors (listed in Table I), which were surgically resected at the National Cancer Center Hospital between 1981 and 1990, were examined. Histological classification of the tumors was made according to the criteria of the International Classification of the World Health Organization.[13] Two cases of dysgerminoma were subclassified into typical and anaplastic subtypes according to the criteria of Gillespie and Arnold.[14] The samples were immediately frozen and stored at −70°C. A portion where tumor cells were abundant was chosen by examin-

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[5] Abbreviations: RFLP, restriction fragment length polymorphism; PCR-SSCP, polymerase chain reaction-single strand conformation polymorphism.

ing serial frozen sections stained with hematoxylin and eosin, and from that portion DNAs were isolated by digestion with proteinase K and extraction with phenol/chloroform.[15]

Polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP) analysis Oligonucleotide primers for PCR amplification of all p53 exons were designed as described previously,[16] synthesized using a DNA synthesizer model 380A (Applied Biosystems Japan Co., Tokyo) and purified through an oligomer purification column (Applied Biosystems Japan Co.). The PCR sample volume was 20 μl in total and the reaction mixture contained 0.2 m*M* dATP/0.2 m*M* dGTP/0.2 m*M* dCTP/0.2 m*M* dTTP/0.75–1.0 m*M* $MgCl_2$/10 m*M* Tris-HCl pH 8.3/0.1% gelatine/0.5 μ*M* one set of primers/0.1 μg of DNA/1 μl of [α-^{32}P]dCTP (3000 μCi/mmol, 10 mCi/μl, Amersham) and 0.5 unit of Taq polymerase (Perkin-Elmer Cetus, Norwalk, CA). The samples were subjected to 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, on a DNA thermal cycler (Perkin-Elmer Cetus). For SSCP analysis,[17,18] 2 μl of the PCR product was diluted 1:100 with a solution containing 20 m*M* EDTA, 0.1% SDS, 0.05% xylene cyanol, and 0.05% bromophenol blue, and 1 μl of the diluted sample was electrophoresed on 3% acrylamide neutral gel with or without glycerol at 20–30 W, cooling both sides of the gel with electric fans. The electrophoresis was carried out under three run conditions: 1) on gel without glycerol at room temperature, 2) on gel without glycerol at 4°C, 3) on gel containing 10% glycerol at room temperature, for all PCR products. Then, the gels were dried and exposed to X-ray films at −80°C using an intensifying screen.

DNA direct sequencing Single-stranded DNAs were amplified by the asymmetric PCR method with 55 thermal cycles using primers identical to those used for PCR-SSCP analysis.[19] To recover the amplified single-stranded DNAs, a Centricon 30 microconcentrator (Amicon, Danvers, MA) was used. After the amplified DNAs had been annealed with the internal primers labeled with [α-^{32}P]ATP, the sequencing reaction was carried out using a Sequenase kit (United States Biochemical Co., Cleveland, OH), and the reaction samples were subjected to electrophoresis on 6% acrylamide sequencing gel.[20]

Flow cytometry of tumor DNA ploidy Nuclei were isolated from frozen neoplastic tissue specimens with 0.1% Triton X-100 (Sigma, St. Louis, MO). They were treated with 0.1% RNase (Sigma), stained with 50 μg/ml propidium iodide (Sigma), filtered through nylon mesh, and analyzed immediately using a FACScan (Becton Dickinson, San Jose, CA).[21]

Immunohistochemistry AMeX-processed tissues were prepared according to the method reported previously.[22] In brief, tumor tissues were immersed in acetone at −20°C overnight, cleared with methanol and xylene, and embedded in paraffin. These AMeX-fixed, paraffin-embedded tissues were sliced into sections 5 μm thick and immunostained with a monoclonal antibody against p53 protein, PAb 1801 (Oncogene Science Inc., Manhasset, NY), established by Banks *et al.*[23] using the avidin-biotin-complex immunoperoxidase method, as described previously.[24] Staining of AMeX-fixed SW837, a colon cancer cell line carrying a mutation at codon 248, was used as a positive control.[25] For the negative control staining, the primary antibody was replaced with phosphate-buffered saline containing 2% bovine serum albumin.

RESULTS

Pattern of p53 gene mutations in ovarian tumors We analyzed all exons of the p53 gene for the presence of mutations in DNAs from 24 cases of ovarian tumor by SSCP analysis and direct sequencing (Figs. 1 and 2). Upon SSCP analysis, positive control DNAs from cell lines carrying p53 gene mutation clearly showed a shift of mobility, whereas DNA extracted from normal tissues used as a negative control showed no mobility shift. Thus, DNAs with mobility shifts on SSCP gels were considered to contain a mutant p53 gene. Results of SSCP and subsequent sequence analyses are summarized in Table I. Mutations were detected in 12 tumors, and in two of them, two types of mutation were detected. Direct sequencing confirmed the presence of mutations in 12 of 14 abnormal SSCP bands showing a mobility shift. Nine (75%) of these 12 mutations were located in a highly conserved region of the p53 gene, and six (50%) were on the four hot spots reported by Nigro *et al.*[25] Nucleotide changes are shown in Table I; G→A and G→C changes were found in five and three cases, respectively. Mutations on the CpG site were detected in 25% (3/12).

p53 gene mutations and clinicopathological findings Clinico-pathological findings are also summarized in Table I. Twelve (50%) out of 24 cases of ovarian tumor had p53 gene mutation. The frequency of mutation was very high in ovarian cancers of the common "epithelial" type (71%, 10/14), and the mutation was present in all of its histological types, being most frequent in moderately and poorly differentiated carcinomas (80%, 8/10), and less frequent in well differentiated carcinoma (50%, 2/4). Mutation was not detected in 2 borderline tumors of common "epithelial" type. Since the majority of the cases examined in this study were at an advanced stage, the correlation between p53 mutation and clinical stage could not be examined. Two tumors, one squamous cell carcinoma that had developed in a dermoid cyst and one anaplastic dysgerminoma, were positive for p53 muta-

tions among cases other than those of common "epithelial" type.

Tumor DNA ploidy pattern and p53 gene mutation Nine cases (53%), comprising 8 cases of common "epithelial" type and one case of anaplastic dysgerminoma, were shown to have an aneuploid pattern of tumor DNA. On the other hand, the 2 borderline cases of common "epithelial" type, 2 cases of immature teratoma and one carcinoid tumor, showed a diploid tumor DNA pattern in flow-cytometric analysis (Table I). Tumor DNA

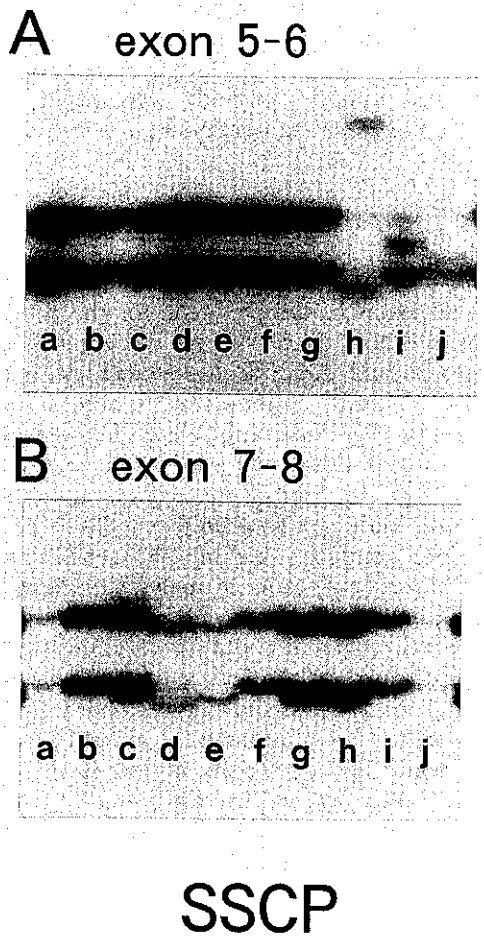

Fig. 1. SSCP analysis of PCR-amplified DNA fragments of p53 gene. A: Analysis of exons 5–6 of the p53 gene using a gel with glycerol at room temperature. Lanes a and b show fragments of normal and tumor DNA from case 2, and lanes c to j show tumor tissue DNA from cases 4, 5, 15, 11, 9, 6, 10 and 22. Mobility shift of single-stranded fragments, differing from normal control DNA in lane a, was detected in lanes h: case 6, i: case 10, and j: case 22. B: Analysis of exons 7–8 of the p53 gene on gel without glycerol at room temperature. Lanes a to j show normal tissue of case 10, and tumor tissue from cases 10, 18, 5, 1, 14, 24, 16, 2 and 12. By SSCP analysis of the PCR product encompassing exons 7 and 8, two or four constitutional bands were observed for each case because of DNA polymorphism within the intron between exons 7 and 8 of the p53 gene. Mobility shift was detected in lanes c: case 18, d: case 5, and j: case 12.

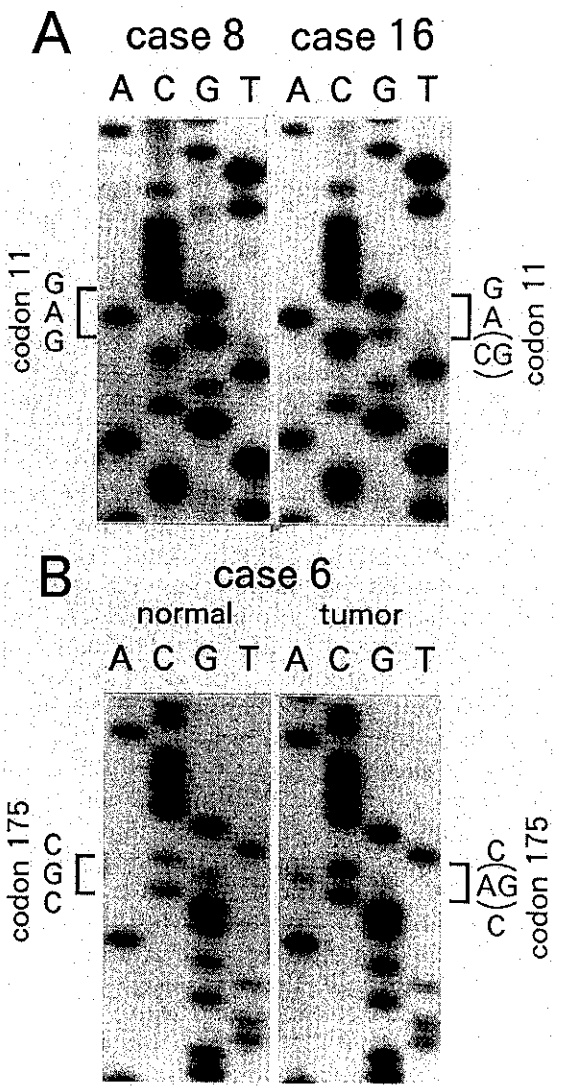

Fig. 2. Examples of direct sequencing analysis of p53 gene mutations. Sequencing of the DNAs of tumor tissues from cases 8 and 16 around codon 11 (A), and sequencing of the DNAs from tumor and normal tissue DNAs from case 6 around codon 175 (B). Point-mutational alterations at codon 11 (sense, CAG from GAG) in case 16 and at codon 175 (sense, CAC from CGC) in case 6 tumor tissue were detected, respectively. The sequencing ladder corresponding to the normal sequence was present, probably due to co-existing normal cells. Case 8 and normal tissue from case 6 showed the wild-type sequence.

Table I. Mutation of p53 Gene, Nuclear Accumulation of p53 Protein, DNA Ploidy Pattern and Clinicopathological Findings of Ovarian Tumors

Case	Histological type	Differentiation	Stage	p53 mutation (exon)	Mutated codon (nucleotide change) : amino acid change	p53 nuclear staining	DNA ploidy pattern
	Common "epithelial" tumors						
1	mucinous tumor of borderline malignancy		I	−		−	D
2	mucinous tumor of borderline malignancy		I	−		−	D
3	serous papillary adenocarcinoma	W	III	−		−	ND
4	serous papillary adenocarcinoma	W	III	−		−	D
5	serous papillary adenocarcinoma	W	IV	+ (8)	281 (GAC-AAC) : Asp-Asn	+	A
6	clear cell carcinoma	W	I	+ (5)	175*(CGC-CAC) : Arg-His	+	A
7	serous papillary adenocarcinoma	M	IV	−		−	A
8	serous papillary adenocarcinoma	M	III	−		+	A
9	serous papillary adenocarcinoma	M	III	+ (2–3)		+	ND
10	serous papillary adenocarcinoma	M	III	+ (6)	207 (GAT-GAG) : Asp-Glu	+	ND
11	serous papillary adenocarcinoma	M	III	+ (7)	234 (TAC-TGC) : Tyr-Cys	+	D
12	serous papillary adenocarcinoma	M	III	+ (7)	245 (GGC-CGC) : Gly-Arg	+	A
13	serous papillary adenocarcinoma	M	III	+ (5)	181*(CGC-CAC) : Arg-His	−	A
14	mucinous adenocarcinoma	M	Rec.	+ (7)	244 (GGC-GAC) : Gly-Asp	+	A
15	endometrioid adenocarcinoma	M	III	+ (7)	242 (TGC-CGC) : Cys-Arg	+	ND
16	serous papillary adenocarcinoma	P	II	+ (2)	11 (GAG-CAG) : Glu-Gln	+	A
	Germ cell tumors						
17	dysgerminoma, typical		I	−		−	D
18	dysgerminoma, anaplastic		I	+ (5, 8)	181*(CGC-CAC) : Arg-His 297 (CAC-CAT) : His-His	−	A
19	immature teratoma		IV	−		−	D
20	immature teratoma		I	−		−	D
21	carcinoid tumor		III	−		ND	D
22	dermoid cyst with malignant transformation (SCC)		II	+ (5, 8)	280 (AGA-ACA) : Arg-Thr	+	ND
23	dermoid cyst with malignant transformation (SCC)		Rec.	−		ND	ND
	Sex cord-stromal tumors						
24	granulosa cell tumor		I	−		ND	ND

Abbreviations used: A, aneuploid; D, diploid; M, moderately differentiated; ND, not done; P, poorly differentiated; Rec, recurrence; SCC, squamous cell carcinoma; W, well differentiated. *: mutation at CpG methylation site.

Table II. Association of p53 Gene Mutation with Nuclear Staining of p53 Protein and Tumor DNA Ploidy Pattern

	Number of cases (%)		
	with p53 gene mutation	without p53 gene mutation	
A. DNA ploidy pattern			
aneuploid	7 (78)	2 (22)	$P<0.05$
diploid	1 (17)	5 (83)	
B. Nuclear staining of p53 protein			
positive	10 (91)	1 (9)	$P<0.01$
negative	2 (25)	6 (75)	

aneuploidy was significantly correlated with the presence of p53 gene mutation ($P<0.05$, Fisher's exact test) (Table IIA).

Nuclear accumulation of p53 protein p53 protein was clearly detected in the nuclei of tumor cells in 11 ovarian tumors out of 21 cases examined (Table I, Fig. 3), but it was not detected in normal cells including stromal cells and lymphocytes. In a case of dermoid cyst with malignant transformation, only the squamous cell carcinoma component showed nuclear staining for p53 protein (Fig. 3). The benign component of the tumor did not show accumulation of the protein. p53 protein was not de-

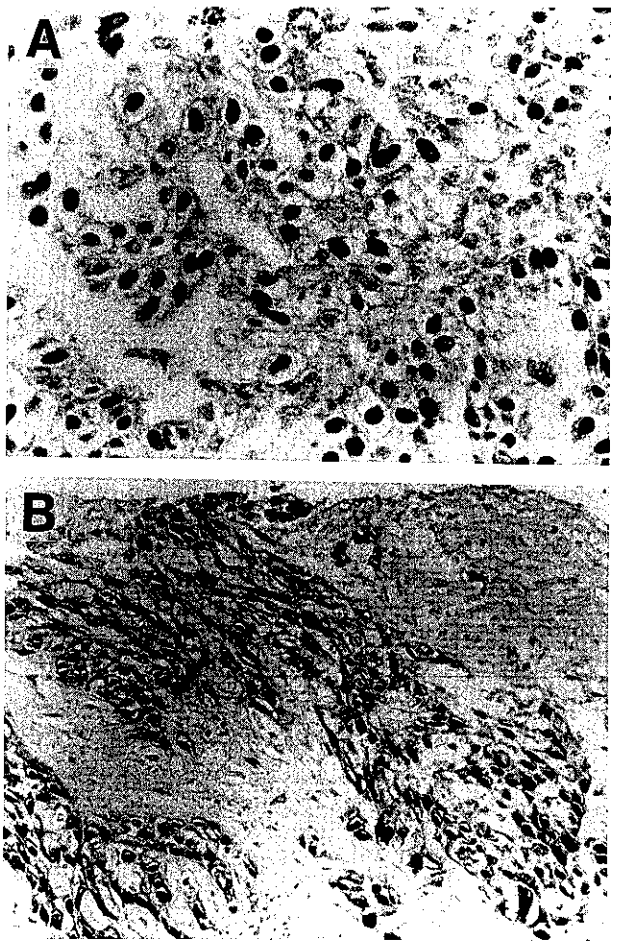

Fig. 3. Immunohistochemical analysis of p53 protein in ovarian cancer. A: Case 8, serous papillary adenocarcinoma (×350). Positive staining in the nuclei of the tumor cells is evident. B: Case 22, squamous cell carcinoma arising in dermoid cyst (×175). Nuclear staining of the p53 protein is present in the squamous cell carcinoma component, but not in the benign component of squamous epithelium.

tected in the two borderline cases of common "epithelial" type. The positivity of nuclear staining for p53 protein was significantly correlated with the presence of mutation of the p53 gene ($P<0.01$, Fisher's exact test) (Table IIB). Two exceptional cases, in which nuclear accumulation of the p53 protein was absent, showed identical mutations of the p53 gene at codon 181.

DISCUSSION

This investigation showed clearly that the incidence of p53 gene mutation is high in ovarian carcinoma. PCR-SSCP analysis is considered to be a very sensitive method for detection of mutations when polyacrylamide gel electrophoresis is performed using three different run conditions in order to improve the detectability of small conformational changes in single-stranded DNAs, although the possibility of some false-negative results for mutation should be kept in mind. Taking our results together with the previous report on frequent loss of heterozygosity of chromosome 17p,[7, 11, 12] it is highly probable that the p53 gene is frequently inactivated by point-mutation on one allele and by loss of the gene on the other allele, and that its inactivation plays an important role in the development of ovarian carcinoma. In the present series, G→A and G→C changes were frequent at the sites of mutation, and mutations were detected at the CpG sites of the p53 gene in one-fourth of all cases. The incidence of mutation at the CpGs site varies among carcinomas of different organs and has been reported to be high in colon carcinomas.[26] It is suggested that mutation at the CpG sites is caused by an endogenous process of deamination of 5-methylcytosine.[27] The pattern of p53 gene mutation in ovarian carcinoma obtained in the present investigation was not the same as those of other carcinomas reported so far,[5] and there is a possibility that this pattern of mutation is unique to ovarian carcinoma.

The correlation between the incidence of p53 gene mutation in ovarian cancer and clinicopathological findings suggests that p53 gene mutation is involved in progression to a high grade of malignancy. Mutation of the p53 gene was more frequent in moderately or poorly differentiated ovarian carcinomas and was absent in borderline cases. It was also detected in an anaplastic dysgerminoma but not in a typical dysgerminoma. A clear correlation was also demonstrated between the presence of p53 gene mutation and an aneuploid pattern of tumor DNA, which has been shown to be a marker of aggressive biological behavior of a tumor, thus also supporting the likelihood that p53 gene mutation is involved in tumor progression. Although the number of cases examined in this study was not sufficient to allow a conclusive statement to be made, mutation of the p53 gene itself could be a marker of aggressive biological behavior of a tumor and poor patient prognosis.

Aneuploidy of tumor DNA is considered to occur through marked enhancement of chromosomal instability. It is suggested that p53 gene mutation is directly or indirectly related to chromosomal instability in tumor cells. If the p53 mutation causes chromosomal instability, or if the mutation occurs in cells with enhanced chromosomal instability, then the remaining allele would be easily lost by chromosome deletion. This is consistent with the hypothesis of Vogelstein *et al.* that the rate-limiting step in p53 inactivation is point-mutation, based on their study of p53 gene mutation and loss of heterozygosity of chromosome 17p in colorectal carcinoma.[26]

Accumulation of p53 protein in nuclei of tumor cells has been reported to be correlated with the presence of mutation in its gene by analysis of a limited number of clinical tumor samples and cultured cell lines. It is speculated that mutated p53 proteins become metabolically stable by binding to heat-shock protein hsp 70.[28] In the present investigation we clearly confirmed this correlation in a large number of clinical samples using immunohistochemistry, and only a few exceptions were detected. Two exceptional cases, in which p53 protein was not accumulated in the nuclei in spite of the presence of mutation in its gene, carried identical mutations at codon 181. Further studies should be done to determine if proteins coded by this mutated gene are not accumulated in the nuclei or are not recognized by the monoclonal antibody used in this investigation. Futhermore, the present immunohistochemical method can detect alteration of the p53 gene in a focal area within one tumor. In a dermoid cyst with malignant transformation, only the squamous cell carcinoma component and not the benign component of squamous epithelium showed positive nuclear staining of p53 protein, indicating that the p53 gene mutation had occurred during the process of malignant transformation. Immunohistochemical analysis of nuclear accumulation of p53 protein is a cheap and easy technique for screening the presence of p53 gene mutation, especially when very large numbers of samples are to be examined.

The present data strongly suggest that mutation of the p53 gene plays an important role in the progression of ovarian cancer. Inactivation of other tumor-suppressor genes may also be involved in the development of ovarian cancer, since ovarian cancer shows multiple loss of chromosomes when studied by RFLP analysis.[11] Further investigation will be required to elucidate the multiple genes, oncogenes and tumor-suppressor genes involved in the early and late stages of ovarian carcinogenesis and to understand their biological and clinical significance.

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid from the Ministry of Health and Welfare of Japan for the Comprehensive 10-Year Strategy for Cancer Control. Toshimasa Kihana was an awardee of a Research Resident Fellowship from the Foundation for Promotion of Cancer Research.

(Received April 24, 1992/Accepted June 18, 1992)

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