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Frequent Occurrence of p53 Gene Mutations in Uterine Cancers at Advanced Clinical Stage and with Aggressive Histological Phenotypes

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The clinical and pathological significance of mutation of the p53 tumor-suppressor gene was examined in 108 cases of primary uterine cancers using single-strand conformation polymorphism and direct DNA sequencing analyses. Mutation of the p53 gene was detected in 19 (31%) of 62 cases of cancer of the uterine corpus and was more frequent in groups at an advanced clinical stage and/or with aggressive histology. Among four adenocarcinomas arising in the lowest portion of the uterine corpus, three showed integration of human papillomavirus (HPV) types 16 and/or 18 DNA, and two of them also showed p53 mutation. In cancer of the uterine cervix, p53 mutations were rare; 7% (3/46) in total, 3% (1/30) of cases with integration of HPV types 16 and/or 18 DNA and 13% (2/16) of cases without HPV DNA integration. Three mutations were detected among two cases at clinical stage IV and two cases of undifferentiated cervical carcinoma. Immunohistochemically, all five cases of uterine cancer which showed diffuse (>50% of cancer cells) nuclear staining of p53 protein also carried the p53 mutation. Therefore, p53 alterations were suggested to be involved in the development of uterine cancers showing aggressive biological behavior. Although a high incidence of HPV DNA integration and a low incidence of p53 mutation were confirmed in cancer of the uterine cervix, there was no inverse association between integration of HPV types 16 and/or 18 DNA and p53 mutation.

Key words: Cancer of uterine corpus — Cancer of uterine cervix — Tumor-suppressor gene — Immunohistochemistry — Human papillomavirus

Alterations of tumor-suppressor genes are considered to be critically involved in human carcinogenesis. In particular, mutations of the p53 tumor-suppressor gene have been frequently detected in human cancer cell lines and tissues. In the majority of human cancers with p53 mutation, loss of the other allele of the p53 gene occurs concurrently.[1,2] Immunohistochemically detectable p53 protein has been shown to be in its stabilized form, which results from p53 gene mutation,[3,4] and, in surgically resected cases of various cancer types, a positive immunohistochemical reaction for p53 protein is correlated with mutation of the p53 gene[5–7] and is also associated with poor prognosis, aggressive histological type and/or advanced clinical stage in breast and lung cancers.[8,9]

In endometrial cancer, Okamoto *et al.* postulated that p53 gene mutation might be involved in the carcinogenesis,[10] and Kohler *et al.* suggested that immunohistochemical overexpression of p53 protein is associated with aggressive clinical behavior.[11] Therefore, mutation of the p53 gene also seems to be associated with aggressive behavior of uterine cancers.

In the present study, we examined mutation of the p53 gene in uterine cancers by polymerase chain reaction (PCR)-single strand conformation polymorphism(SSCP) and direct DNA sequencing analyses, and its association with clinical and histological parameters. Furthermore, association of p53 mutation with nuclear immunoreaction for p53 protein and, mainly in cervical cancer, with integration of human papillomavirus (HPV) DNA was also examined.

Abbreviations used: HPV, human papillomavirus; MMMT, malignant mixed müllerian tumor; PCR, polymerase chain reaction; SSCP, single-strand conformation polymorphism.

MATERIALS AND METHODS

Tissue samples Tumor and non-tumor tissue samples were obtained from 108 primary uterine cancers, comprising 62 cancers of the uterine corpus, including four adenocarcinomas arising in the lowest portion of the uterine corpus, and 46 cancers of the uterine cervix, which were surgically resected from 108 patients at the National Cancer Center Hospital, Tokyo, between March 1989 and August 1991. Clinical staging was performed according to the staging system of the International Federation of Gynecology and Obstetrics (1988). Histological classification was based on that of the World Health Organization,[12] except for serous papillary carcinoma of the uterine corpus.[13]

PCR-SSCP analysis and direct DNA sequencing DNA was isolated from tissue samples of 108 patients using the method described by Sambrook, Fritsch and Maniatis.[14] Each exon (exons 4 to 9) of the p53 gene in a 0.1-μg sample of genomic DNA was enzymatically amplified by PCR using 1 μM primers and a GeneAmp kit (Perkin-Elmer Cetus, Norwalk, CT) under the conditions recommended by the supplier. Primers for PCR used in this

study were; 4L, TTTTCACCCATCTACAGTCC and 4R, CTCAGGGCAACTGACCGTGC for exon 4; 5L, TTCCTCTTCCTGCAGTACTCC and 5R, GCCCCAGCTGCTCACCATCG; 6L, CGATGGTGAGCAGCTGGGGC and 6R, AGTTGCAAACCAGACCTCA for exon 6; 7L, TCCTAGGTTGGCTCT and 7R, AAGTGGCTCCTGACCTGGA for exon 7; 8L, CCTATCCTGAGTAGTGGTAA and 8R, CCTGCTTGCTTACCTCGCT for exon 8; and 9L, TTGCCTCTTTCCTAGCA and 9R, CCCAAGACTTAGTACCTG for exon 9. The amplified DNA was subjected to SSCP analysis according to the method described by Orita *et al.*[15] The gel containing a band of PCR product DNA showing a mobility shift by SSCP analysis was cut out,[16] and subjected to asymmetrical PCR and direct DNA sequencing[17] using a Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical Corporation, Cleveland, OH).

Southern blot analysis In the 46 cases of cancer of the uterine cervix and four cases arising in the lowest portion of the uterine corpus, DNA was digested with *Bam*HI (Takara, Kyoto), and integration of HPV DNA was examined by Southern blot analysis.[14] The probes used were HPV 16 DNA (7.9 kilobase, *Bam*HI fragment)[18] and HPV 18 DNA (7.9 kilobase, *Eco*RI fragment).[19]

Immunohistochemistry In the 38 cases of cancer of the uterine corpus and 38 cases of cancer of the uterine cervix, a portion of tumor tissue was processed by the AMeX method and embedded in paraffin,[20] cut into 5-μm sections, deparaffinized in xylene, rehydrated in acetone and fixed with 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) for 10 min. Immunohistochemistry was performed as described previously.[8] A mouse monoclonal antibody against human p53, PAb-1801 (Oncogene Science Inc., Manhasset, NY), which recognizes an epitope between amino acids 32 and 79, was used for immunohistochemical study.[21]

According to the proportion of cancer cells showing a positive nuclear immunoreaction for p53 protein, cases were classified into four groups: 1) diffuse (+++), when more than 50% of cancer cells were positive; 2) partial (++), when 10–50% of cancer cells were positive; 3) sparse (+), when less than 10% of cancer cells were positive; and 4) negative (−), when no cancer cell nuclei were stained.

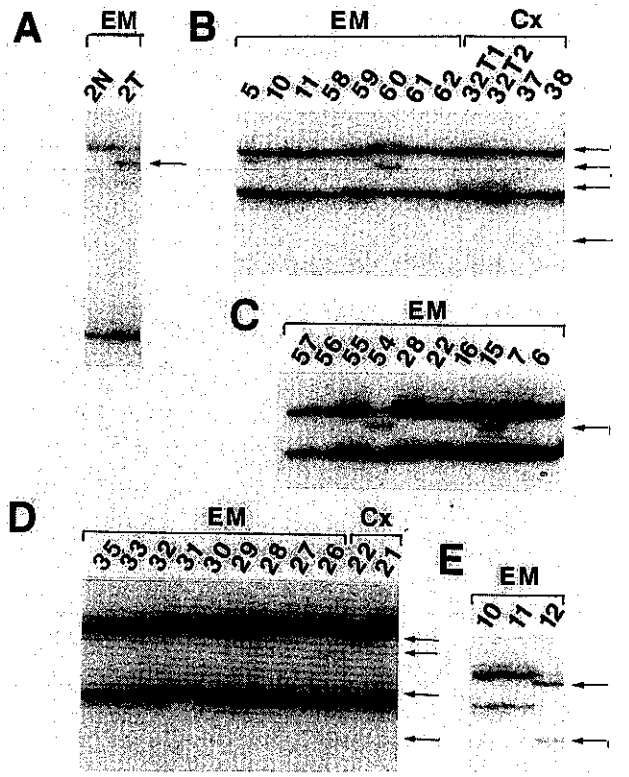

Fig. 1. Detection of mutation in exons 4, 5, 6, 7 and 8 of the p53 gene by SSCP analysis in cases of cancer of the uterine corpus (EM) and cancer of the uterine cervix (Cx). Arrows indicate bands showing a mobility shift. A. Mutation in exon 4. Electrophoresis was performed using non-denaturing polyacrylamide gel without glycerol at room temperature.[15] In case EM2, cancer DNA (T) shows a mobility shift, whereas non-cancer DNA (N) does not. B. Mutation in exon 5. Electrophoresis was performed at room temperature with 5% glycerol. Cancer DNAs from cases EM 60, Cx32 and Cx37 show mobility shifts. In case Cx32, T1 refers to the primary tumor and T2, to a metastatic ovarian lesion. C. Mutation in exon 6. Conditions of electrophoresis were identical to those for exons 5 and 7. Cancer DNAs from cases EM15 and EM54 show mobility shifts. D. Mutation in exon 7. Cancer DNAs of cases Cx22, EM26 and EM31 show mobility shifts. E. Mutation in exon 8. Conditions of electrophoresis were identical to those for exon 4. In case EM12, cancer DNA reveals a mobility shift.

RESULTS

p53 alteration in cancer of the uterine corpus Mutations of the p53 gene were detected in 19 (31%) of the 62 cases of cancer of the uterine corpus (Figs. 1 and 2). The incidence of the mutation was significantly higher in the cases at clinical stage III (7/13, 54%) than in those at stages I (10/41, 25%) and II (2/8, 25%) (Table IA). Mutations of the p53 gene were most frequent in poorly differentiated adenocarcinoma (3/5, 60%) and special histological types (6/9, 67%): adenosquamous carcinoma (2/4, 50%), clear cell adenocarcinoma (1/1), serous papillary carcinoma (1/1), and malignant mixed müllerian tumor (MMMT, 2/3, 67%). On the other hand, the incidence was lower in well differentiated (4/31,11%) and moderately differentiated (6/17, 35%) adenocarcinomas (Table IIA).

Sites of mutations were distributed between codons 62 and 285, and 12 of 19 mutations were detected in the evolutionarily conserved regions (II, III, IV and V, corresponding to codons 117–142, 171–181, 234–258 and 270–286, respectively, in Fig. 3).[22] Base substitutions were mainly transitions; from G:C to A:T in 8 cases, four of which were detected at CpG sites, and from A:T to G:C in five (Table IIIA). Transversions were detected in three cases, from G to T in Case EM2, T to G in Case EM38, and G to C in Case EM68. Three cases revealed small deletions: in Case EM12, six bases were deleted in exon 8; in Cases EM55 and EM57, one base deletion generated a termination codon at the 122th and at the 246th position, respectively.

In 13 cases with mutation in evolutionarily conserved regions, eight were aggressive histologic types and six were at clinical stage III, whereas in six cases which showed mutation in exon 4 or 6, outside the conserved regions, five were at clinical stage I and were well or moderately differentiated adenocarcinoma.

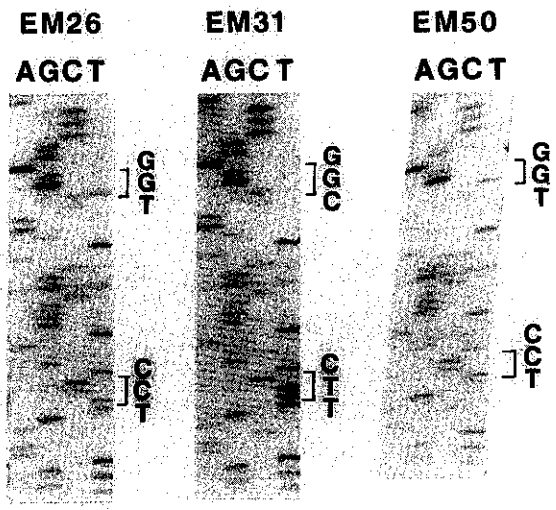

Fig. 2. Detection of mutation in exon 7 of the p53 gene by direct DNA sequencing. Three cases of cancer of the uterine corpus (EM26, EM31 and EM50) are shown. In EM26 and EM50, cancer DNA reveals a point mutation at codon 248 from CGG to TGG, and in case EM31, cancer DNA reveals a point mutation at codon 241 from TCC to TTC.

Table I. Association of Mutation of the p53 Gene with Clinical Stage of Uterine Cancers

	Number of cases (%)			
	Mutation of p53 gene		Total	*P*-value[a]
	Positive	Negative		
A. Cancer of the uterine corpus				
Stage I	10 (25)	31	41	
II	2 (25)	6	8	<0.05
III	7 (54)	6	13	
Total	19 (31)	43	62	
B. Cancer of the uterine cervix				
Stage I	0 (0)	18	18	
II	1 (4)	25	26	
IV	2 (100)	0	2	
Total	3 (7)	43	46	

a) *P* value was calculated by means of the chi-square test.

Table II. Association of Mutation of the p53 Gene with Histologic Type of Uterine Cancer

	Number of cases (%)						
	Mutation of p53 gene				Total		*P*-value
	Positive		Negative				
A. Cancer of the uterine corpus							
Adenocarcinoma							
Well differentiated	4	10 (21)	27	38	31	48	<0.005
Moderately differentiated	6		11		17		
Poorly differentiated	3		2		5		
Adenosquamous carcinoma	2		2		4		
Clear cell adenocarcinoma	1	9 (64)	0	5	1	14	
Serous papillary carcinoma	1		0		1		
MMMT	2		1		3		
B. Cancer of the uterine cervix							
Squamous cell carcinoma	0	(0)	33		33		
Adenocarcinoma							
Endocervical type	1	1 (11)	6	9	7	10	
Endometrial type	0		3		3		
Undifferentiated carcinoma	2	(100)	0		2		
Adenosquamous carcinoma	0		1		1		

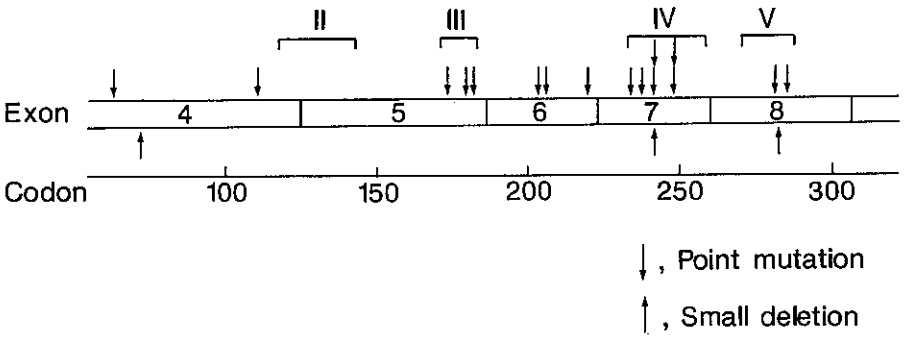

Fig. 3. Site of p53 mutation in cancer of the uterine corpus. At the top, evolutionarily conserved regions (II to V)[22] are indicated. Sites of p53 point mutation are indicated by each downward arrow, which represents a case carrying this type of mutation. Sites of small deletion causing a frame shift of the p53 gene sequence are indicated by each upward arrow, which represents a case carrying this type of mutation.

Table III. Clinical and Histological Profiles of Uterine Cancer Cases Revealing Mutation of the p53 Gene

Patient Case No./Age	Stage	Histologic type[a]	Mutation of p53 gene		Nuclear p53 immunoreaction[b]
			Codon	Base substitution (amino acid)	
A. Cancer of the uterine corpus					
EM 2/56	Ic	W/D adenocarcinoma	62	GAA→TAA (Glu→term)	+
EM 5/70	IIIc	Clear cell adenocarcinoma	179	CAT→CGT (His→Arg)	−
EM12/42	Ib	P/D adenocarcinoma	282–283	(6-base deletion)	++ (20%)
EM14/76	Ic	Adenosquamous carcinoma	173	GTG→ATG (Val→Met)	ND
EM15/43	Ib	M/D adenocarcinoma	203	GTG→GCG (Val→Ala)	ND
EM17/39	IIIa	W/D adenocarcinoma	234	TAC→TGC (Tyr→Cys)	ND
EM26/48	IIIa	MMMT	248	CGG→TGG (Arg→Trp)	+++ (80%)
EM31/68	IIb	P/D adenocarcinoma	241	TCC→TTC (Ser→Phe)	+++ (80%)
EM38/37	IIIb	Adenosquamous carcinoma	237	ATG→AGG (Met→Arg)	ND
EM41/64	IIIa	MMMT	110	CGT→TGT (Arg→Cys)	ND
EM46/61	Ic	M/D adenocarcinoma	219	CCC→TCC (Pro→Ser)	ND
EM50/54	IIIc	M/D adenocarcinoma	248	CGG→TGG (Arg→Trp)	ND
EM54/46	Ib	M/D adenocarcinoma	205	TAT→TGT (Tyr→Cys)	+++ (80%)
EM55/55	Ic	W/D adenocarcinoma	71 or 72	(1-base deletion)	+
EM57/75	IIIa	Serous papillary carcinoma	241	(1-base deletion)	−
EM60/62	IIb	M/D adenocarcinoma	181	CGC→TGC (Arg→Cys)	+
EM61/55	Ib	M/D adenocarcinoma	281	GAC→GGC (Asp→Gly)	++ (30%)
EM64/80	Ia	P/D adenocarcinoma	241	TCC→TTC (Ser→Phe)	+++ (90%)
EM68/45	Ib	W/D adenocarcinoma	285	GAG→CAG (Glu→Gln)	+
B. Cancer of the uterine cervix					
Cx22/72	IVa	Undifferentiated carcinoma	254	ATG→ATC (Met→Ile)	−
Cx32/51	IVb	Adenocarcinoma	161	GCC→GAC (Ala→Asp)	+++ (60%)
Cx37/29	IIa	Undifferentiated carcinoma	128–136	(27-base deletion)	++ (20%)

a) W/D, well differentiated; M/D, moderately differentiated; P/D, poorly differentiated; MMMT, malignant mixed müllerian tumor; term, termination codon. Criteria for judgement of nuclear immunoreaction of p53 protein are described in the text.

b) Parentheses indicate percentage of cancer cells showing nuclear immunoreaction for p53 protein. ND, not done.

In four (11%) of 38 cases of cancer of the uterine corpus, a diffuse nuclear immunoreaction for p53 was evident (Table IVA). Various nuclear immunoreaction patterns in cancer of the uterine corpus are shown in Fig. 4A–4C. Mutation of the p53 gene had a tendency to be more frequent in cases with a higher proportion of cancer cells showing nuclear immunoreaction for p53 protein, and all four cases showing a diffuse immunoreaction and two of four showing a partial immunoreaction carried the mutation (Table IVA). However, among 30 cases

Table IV. Association between Pattern of p53 Nuclear Immunoreaction and Mutation of the p53 Gene in Uterine Cancer

Nuclear immunoreaction of p53 protein[a]	Number of cases (%)		
	Mutation of p53 gene		Total
	Positive	Negative	
A. Cancer of the uterine corpus			
−	2 (14)	12	14
+	4 (25)	12	16
++	2 (50)	2	4
+++	4 (100)	0	4
Total	12 (32)	26	38
B. Cancer of the uterine cervix			
−	1 (7)	13	14
+	0 (0)	16	16
++	1 (14)	6	7
+++	1 (100)	0	1
Total	3 (8)	35	38

a) Pattern of immunoreaction was divided into four categories, +++ (diffuse), ++ (partial), + (sparse) and − (negative). The criteria are given in the text.

with a sparse or negative pattern, only six (20%) revealed the mutation.

p53 alteration in cancer of the uterine cervix Mutations of the p53 gene were detected in only three (7%) of 46 cases of cancer of the uterine cervix (Tables IB, IIB and IIIB). Mutation was detected in only one of 44 stage I or II cases, one of 10 adenocarcinomas and none of 33 squamous cell carcinomas. The three cases of cervical cancer showing mutation of the p53 gene were clinically far advanced and/or unusually aggressive histologically. Case Cx22 was an undifferentiated carcinoma invasive to the urinary bladder mucosa (stage IVa). Case Cx32 was an endocervical adenocarcinoma disseminating to the ovaries and peritoneum (stage IVb), and both the primary and metastatic lesions showed the mutation (Fig. 1B). The third case, Cx37, was also an undifferentiated carcinoma at clinical stage IIa. In the former two cases, the mutation was a transversion in exon 5 or 7, and in the third case, deletion of 27 bases in exon 5.

Cervical cancer cases with various nuclear immunoreaction patterns are shown in Fig. 4D–4F. Among 25 squamous cell carcinomas, none showed a diffuse pattern (Table IVB). Three (12%) cases revealed a partial immunoreaction pattern. and the other 22 revealed a sparse or negative pattern. Among 10 adenocarcinomas, a diffuse immunoreaction pattern was detected in one case (Cx32), which carried the mutation, and a partial immunoreaction pattern was detected in three, in which the mutation was not detectable. In two cases of undifferentiated carcinoma with p53 mutation, cases Cx22 and Cx37 showed a negative and partially positive immunoreaction, respectively, and one adenosquamous carcinoma revealed a sparsely positive pattern.

Integration of HPV DNA and p53 mutation Integration of HPV 16 DNA and HPV 18 DNA was detected in 23 (50%) and 8 (17%) of the 46 cases of cervical cancer, and in two and two cases of cancers arising in the lowest portion of the uterine corpus, respectively. In total, HPV 16 and/or 18 DNA was detected in 30 (65%) of the cervical cancers and three (75%) of the cancers arising in the lowest portion of the uterine corpus. The incidence of p53 mutation was low in both the cervical cancer groups with and without integration of HPV types 16 and/or 18 DNA: only one (Cx37) (3%) of 30 cases with integration of HPV DNA, and only two (Cx22 and Cx-32) (13%) of 16 cases without integration. Among the three (EM17, EM31 and EM38) mutation-positive cases occurring in the lowest portion of the uterine corpus, two showed the HPV-DNA integration (Table III).

DISCUSSION

In this series of cases of cancer of the uterine corpus, the incidence of p53 mutation was 31% and mutation was more frequent in cases at an advanced clinical atage (stage III) and of an aggressive histological type, i.e., poorly differentiated adenocarcinoma and other special histological types including adenosquamous carcinoma, MMMT, clear cell adenocarcinoma and serous papillary carcinoma.[13, 23–26] On the other hand, p53 mutation was not considered to be involved in the development of the majority (>70%) of stage I cases and/or cases of well differentiated adenocarcinoma.

The site of the mutation was also suggested to be of importance because mutation within the evolutionarily conserved regions in p53 seemed to be associated with an advanced atage and/or aggressive cancer types arising in the uterine corpus, whereas mutation outside these regions did not. The conserved regions III, IV and V are known to be mostly included in regions which bind to simian virus 40 large T antigen and to be important in cell transformation.[22, 27] The pattern of the base change in cancer of the uterine corpus was similar to that of colorectal cancer, in that G:C-to-T:A transitions were most common whereas G-to-T transversions were very rare.[28] Although mutations at CpG sites were infrequent in the former, all four mutations at CpG sites in cancer of the uterine corpus were C to T transitions.

In cancer of the uterine cervix, the incidence of p53 mutation was only 7%, and all three mutations were detected in cases showing unusually aggressive clinical behavior and/or histologic phenotype. Squamous cell carcinoma and adenocarcinoma at clinical stage I or II did not show mutation of the p53 gene, except for one case. Therefore, pathways not associated with the p53

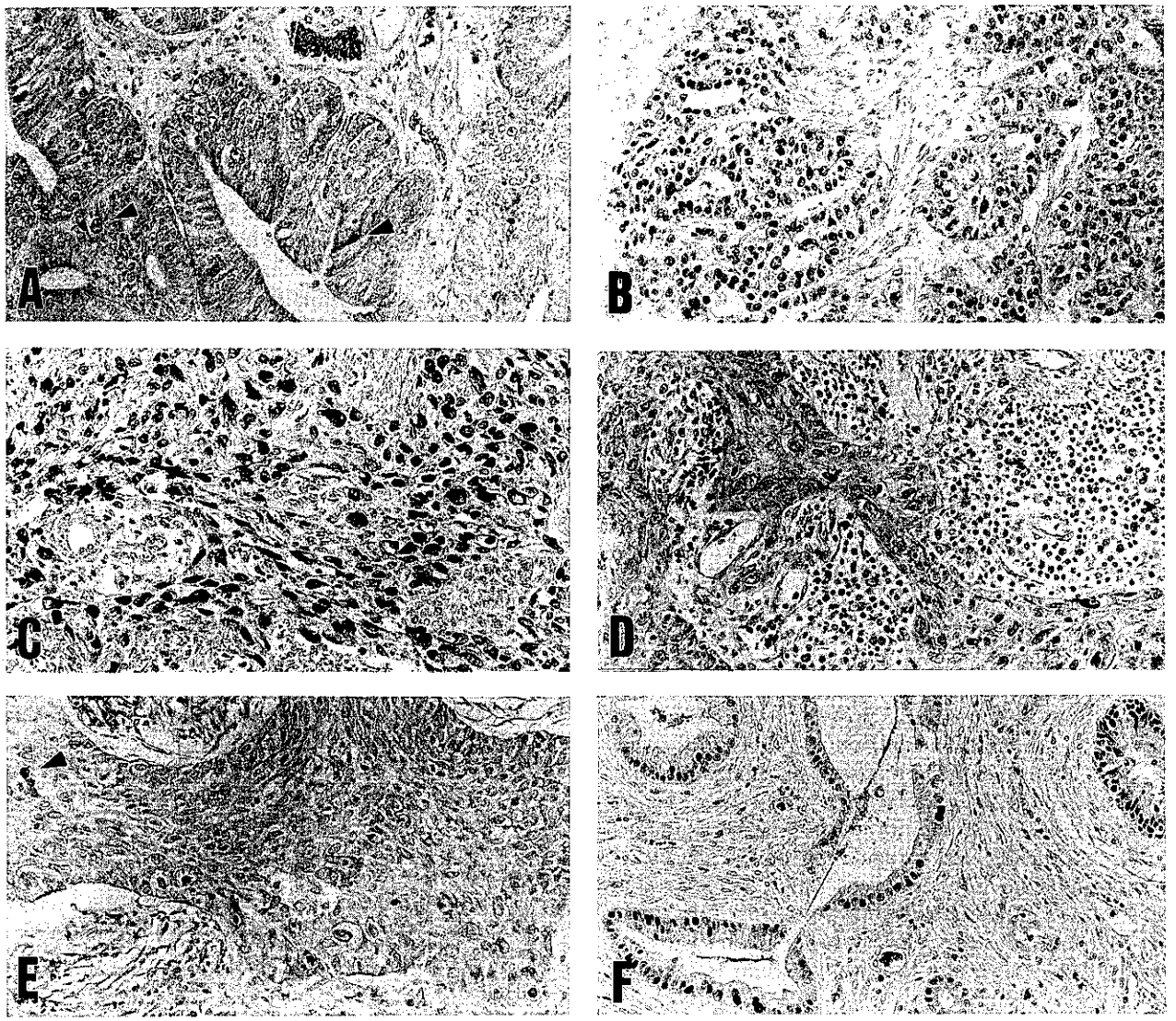

Fig. 4. Nuclear immunoreaction of p53 protein in uterine cancers of various histologic types. A–C, cancer of the utrine corpus; D–F, cancer of the uterine cervix (immunoperoxidase staining, ×200). A. A case (EM32) of well differentiated adenocarcinoma showing a sparse pattern. p53 protein is detected in a small portion of cancer cell nuclei (arrowheads). B. A case (EM31) of poorly differentiated adenocarcinoma arising in the lowest portion of the uterine corpus, showing a diffuse immunoreaction pattern. p53 protein is positive in >50% of cancer cell nuclei. C. A case (EM26) of MMMT showing a diffuse immunoreaction pattern. D. A case (Cx30) of squamous cell carcinoma showing a partial immunoreaction pattern, where the intensity of the immunoreaction is somewhat weaker than that in 4B and 4C, although nearly 40% of cancer cells are positive. E. A case (Cx6) of squamous cell carcinoma showing a sparse nuclear immunoreaction pattern (arrowhead). F. A case of adenocarcinoma (Cx16) showing a partial immunoreaction pattern. Nuclear immunoreaction for p53 is evident in nearly 20% of the cancer cells.

gene alterations seemed to be involved in common cervical carcinogenesis and progression. In the present study, we did not examine an adequate number of clinically advanced stage III and IV cases. Therefore, it is still undetermined whether p53 mutation in cervical cancer is an exceptional event or more frequent in aggressive-type cancers and/or those at advanced clinical stages.

HPV DNA was shown to be commonly integrated not only in cancer of the uterine cervix but also in adenocarcinoma arising in the lowest portion of the uterine corpus. Since cervical carcinogenesis is considered to be mostly associated with HPV infection and/or integration of the HPV genome,[18, 19] it is probable that cervical cancer cells carrying the p53 mutation do not have a

special growth advantage because degradation of p53 protein is promoted by the E6 protein of HPV.[29] Indeed, 65% of cervical cancer cases in the present study revealed integration of HPV type 16 and/or 18 DNA, and the majority of them did not carry the p53 mutation. However, in the cases with no integration of HPV type 16 or 18 DNA, the incidence of p53 mutation was also low. Including two cases of cancer arising in the lowest portion of the uterine body, three cases showed both p53 mutation and HPV DNA integration. Therefore, an inverse association between integration of HPV type 16 and/or 18 DNA and presence of p53 mutation was not evident, in contrast to the data obtained by Crook *et al.*,[30] and degradation of p53 protein by E6 protein might not be a sole cause of the low incidence of p53 mutation in cervical cancers.

We classified the pattern of nuclear p53 immunoreaction into four categories according to the population of cancer cells showing the immunoreaction. The nuclear p53 immunoreaction pattern revealed a tendency to be associated with the presence of the mutation. Expression of wild-type p53 protein has been shown to increase at the late G1 phase after mitogenic stimulation of 3T3 cells, and is suggested to play a role in the initiation of DNA synthesis.[31] Normal p53 protein is, therefore, considered to function at a restriction point in the cell cycle.[32, 33] Cancers showing a sparse or negative nuclear immunoreaction usually carried the wild-type p53 gene and seemed to express the normal p53 protein of short half-life periodically under regulation of the cell cycle. Immunohistochemically, only cells which had expressed the p53 protein immediately before resection of the tissue seemed to show positive nuclear localization. The percentage of cells expressing p53 protein seems to increase in accordance with the speed of proliferation, since a larger number of cancer cells are about to start DNA synthesis.

The cases showing a strong or partial immunoreaction pattern frequently carried p53 mutation. Once mutation occurs in the p53 gene, it seems to accelerate proliferation of cancer cells. The mutant p53 protein with a longer half-life will be expressed unrestrictedly and seems to be recognizable as diffuse nuclear deposition.[4] However, a proportion of mutation-positive cases revealed a sparse or negative immunoreaction. Thus a difference in the site or pattern of mutation might influence the immunoreaction pattern of p53 protein.

Mutation of the p53 gene and diffuse nuclear immunoreaction of p53 protein are suggested to be associated with aggressive biological behavior of cancer cells, and the presence of these alterations in p53 is potentially a significant prognostic indicator in uterine cancers.

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