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Immunohistochemical analysis of p53 protein and 72 kDa heat shock protein (HSP72) expression in ovarian carcinomas

Correlation with clinicopathology and sex steroid receptor status

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Abstract Mutations of the tumour suppressor p53 gene have been reported in a variety of human malignant tumours, and are frequently associated with overexpression of p53 protein. To examine the significance of p53 gene alteration in malignant epithelial tumours of the ovary, we studied the immunohistochemical reactivity with a monoclonal antibody against p53 (PAb 1801) in 6 ovarian tumours of low malignant potential (LMP) and 32 ovarian carcinomas. The existence of any correlation of p53 overexpression with the clinicopathological features and with the immunohistochemical expression of 72 kDa heat shock protein (HSP72) and sex steroid receptors (oestrogen receptors; ER, progesterone receptors; PR) was also analysed. Expression of p53 was found in 2 of the 6 (33.3%) LMP tumours and in 15 of the 32 (46.9%) carcinomas. Strong expression of HSP72 was observed in 11 of the 17 (64.7%) p53-positive tumours, but only in 2 of the 21 (9.5%) p53-negative ones. Histologically, p53-positivity was observed in 7 of the 10 (70%) serous carcinomas, 4 of the 6 (66.7%) mucinous, 4 of the 10 (40%) endometrioid, and none of the 4 clear cell and 2 transitional cell carcinomas. Distribution of p53-positive cells in the tumour sections was homogenous in serous tumours, but heterogenous in mucinous lesions. All of the 4 carcinomas arising in endometriotic cysts were p53-negative. These differences support the thesis of heterogeneity in ovarian carcinogenesis. There was an inverse relationship between p53-positivity and sex steroid receptor status for ovarian carcinomas; 14 of the 15 p53-positive carcinomas were negative for both ER and PR, whereas 11 of the 17 p53-negative carcinomas were positive for ER and/or PR ($P<0.01$).

Key words Ovarian carcinoma · p53
Sex steroid receptor · Immunohistochemistry
Heat shock protein 72

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Introduction

Epithelial ovarian cancer is believed to arise from the ovarian surface epithelium, but little is known about the early pathogenetic process or about the occurrence of premalignant lesions. This is because more than half of the ovarian cancer patients are diagnosed at advanced stage of the disease [3]. However, histologically benign or borderline malignant lesions are frequently observed adjacent to frankly invasive lesions [22]. Heterogenous pathways of ovarian tumorigenesis have been suggested, based on the histological examination of the limited cases of early ovarian cancer, which demonstrated both carcinomas that arise *de novo* in the ovarian surface epithelium or its inclusion cysts, and those that develop from pre-existing benign lesions [26]. Among the genetic alterations and/or overexpression of oncogene products reported in ovarian carcinomas [8, 30], mutation of the tumour suppressor p53 gene is one of the most common genetic events that have occurred in about 50% of the cases [17, 19, 20]. Nevertheless, the significance of p53 mutation in the possible heterogeneity of ovarian carcinogenesis remains unclear. The development of monoclonal antibodies against p53 protein has made possible the detection of its overexpression in human tumours by immunohistochemical analysis [1]. The half-life of wild-type p53 is too short to be detected by immunohistochemical examination, whereas mutant p53 induces and binds 70 kDa heat shock protein family (HSP70), and has a longer half-life, which leads to higher steady-state p53 levels [7, 28]. Therefore, the immunohistochemical expression of p53 is usually associated with p53 gene alteration [1, 4, 17]. Accordingly, we examined immunohistochemically the expression and distribution of p53 protein as well as a 72 kDa inducible form of HSP70 (HSP72) in both borderline and frankly malignant tumours of the ovary, and analysed for any correlation of these expressions with the clinical and histological features of the respective cases. In addition, our previous study demonstrated an inverse correlation between p53 overexpression and sex steroid receptor status

with reference to the heterogeneous pathways of endometrial carcinogenesis [14]. Sex steroid receptor status in ovarian carcinomas has been reported to correlate with a variety of clinicopathological variables such as histological type, grade of differentiation, and patient survival [10, 25, 27]. Therefore, we examined the relationship between p53 overexpression and immunohistochemical expression of both oestrogen receptors (ER) and progesterone receptors (PR) in our cases.

Materials and methods

Fresh surgical specimens of malignant epithelial tumours of the ovary were obtained from 38 women who underwent bilateral salpingo-ophorectomy and total hysterectomy with or without pelvic and para-aortic lymphadenectomy at Kyoto University Hospital. Informed consent was obtained from each patient according to the guidelines of the Ethical Committee of Kyoto University Faculty of Medicine. Of the 38 patients, 32 had frankly invasive carcinomas and the remaining 6 had tumours of low malignant potential (LMP). According to the International Federation of Gynecology and Obstetrics (FIGO) classification, the 32 carcinomas consisted of 17 stage I, 2 stage II, 10 stage III, and 3 stage IV. Histologically, 10 of the 32 carcinomas were serous type, 6 were mucinous type, 10 were endometrioid type, 4 were clear cell type, and 2 were transitional cell carcinomas. Among these carcinomas, 1 endometrioid and 3 clear cell carcinomas have been suggested to arise in ovarian endometriotic cysts, based on the data from the outpatient clinic before the diagnosis and on the histopathological findings of benign endometriotic lesions adjacent to the carcinomas. The 6 LMP tumours consisted of 2 serous and 4 mucinous types. The materials, two to five specimens from each primary ovarian tumour, obtained immediately after the surgical procedure, were snap-frozen in OCT compound (Ames, Elkhart, Inn., USA) and stored at −80° C. Serial cryostat sections were stained with haematoxylin and eosin for light microscopy.

Immunostaining for p53 protein on cytostat sections was performed by the avidin-biotin-peroxidase complex method, using a Histscan monoclonal detector kit (Biomeda, Foster, Calif., USA). In brief, the sections were fixed in cold acetone for 10 min, treated with 0.3% hydrogen peroxide, and incubated with normal goat serum. The sections were then incubated with mouse monoclonal antibody for a denaturation-resistant epitope of p53 protein, PAb 1801, (p53 Ab-2, diluted 1:100, Oncogene Science, Uniondale, N.Y., USA), or control normal mouse serum, at 4° C overnight. They were then treated with biotinylated goat anti-mouse IgG, followed by treatment with avidin-biotin-peroxidase complex, and stained with diaminobenzidine with 0.15% hydrogen peroxide. Counterstaining was performed with methyl green.

Immunostaining for HSP72 on cryostat sections was also performed using a Histscan monoclonal detector kit (Biomeda). The sections were fixed in 1% paraformaldehyde containing 8% sucrose for 20 min, washed with 0.01 M phosphate-buffered saline (PBS) containing 8% sucrose for 30 min, treated with 0.3% hydrogen peroxide, and incubated with normal goat serum. Then the sections were incubated with mouse anti-72 kDa HSP monoclonal antibody (diluted 1:500, StressGen Biotechnologies Corp., Victoria, B.C., Canada), or control normal mouse serum, at 4° C overnight. The following procedure was the same as that described above. Monoclonal anti-72 kDa HSP antibody (SPA-810), specific for the inducible form of HSP70, which has also been referred as clone C92F3A-5, was produced by StressGen following immunization of BALB/c mice with purified HSP72/73 isolated from HeLa cells. SPA-810 has been used previously for immunohistochemical investigation of HSP72 in normal and abnormal human tissues [11]. The intensity of staining was graded as (−) for no immunostaining, (+) for weak staining, and (++) for strong staining by the evaluation of two independent observers.

Immunostaining for ER and PR was performed on cryostat sections by the peroxidase-antiperoxidase method, using ER-ICA and PgR ICA monoclonal kits (Abbott, North Chicago, Ill., USA). In brief, cryostat sections were fixed in 3.7% formaldehyde in PBS for 10 min. The slides were treated with 0.3% hydrogen peroxide for blocking endogenous peroxidase activity, and incubated with normal goat serum to reduce the non-specific binding of the primary antibody. Then the slides were incubated with anti-ER monoclonal antibody (H222), anti-PR monoclonal antibody (KD68), or control rat IgG for 30 min at room temperature, followed by treatment with goat anti-rat IgG anti-serum, and with peroxidase-antiperoxidase complex. Finally, diaminobenzidine and 0.06% hydrogen peroxide diluted in PBS were applied. Counterstaining was performed with methyl green. For positive controls, we used cryostat sections of breast carcinoma and commercially prepared slides with ER- and PR-positive cells. The percentage of positive cells was graded as (−) when 0% of the nuclei were stained, (+) when less than 50% of the nuclei were stained, and (++) when 50% or more of the nuclei were stained.

Statistical analyses were performed using Student's t test and Fisher's 2-tailed exact test, on the correlation of p53 immunohistochemical positivity with the age and menstrual state of the patient, FIGO stage, HSP72 expression, and sex steroid receptor expression in ovarian carcinoma.

Results

Specific staining with each of the anti-p53, anti-ER, and anti-PR antibodies was exclusively confined to the nuclei, while no cytoplasmic staining was observed. Staining for HSP72 was observed mainly in the nuclei and partly in the cytoplasm of the tumour cells. Patient age, menstrual status, FIGO stage, histological type, and the immunohistochemical expression of p53, ER, PR, and HSP72 in ovarian tumours are listed in Table 1. Table 2 summarizes the relationship between p53 positivity and the other studied variables in ovarian carcinomas.

Expression of p53 and HSP72 in ovarian LMP tumours and carcinomas

Immunohistochemical expression of the p53 protein was observed in 2 of the 6 (33.3%) ovarian LMP tumours. Histologically, p53-positivity was observed in 1 of the 2 serous LMP tumours and 1 of the 4 mucinous LMP tumours. However, the distribution pattern of p53-positive cells was different in the two cases; while p53-positive cells were found homogeneously in the sections from serous LMP tumours, they were quite heterogenous in those from the mucinous LMP tumour. In the mucinous tumour, histologically benign glands were p53-negative, whereas borderline malignant glands showed p53-positivity. With regard to HSP72 expression a case of serous LMP tumour with p53-positivity showed strong expression, and the remaining 5 tumours were weakly positive for HSP72.

Expression of p53 protein was observed in 15 of the 32 (46.9%) ovarian carcinomas. The clinical and histological features of p53-positive and p53-negative ovarian carcinomas were compared. The average age of the patients with p53-positive tumours (61.9±3.7 years;

Table 1 Age, FIGO stage, histological type, grade of differentiation, and immunohistochemical expression of p53, ER, PR, and HSP72 in ovarian low malignant potential tumours and carcinomas (*FIGO*, International Federation of Gynecology and Obstetrics; *ER* oestrogen receptor; *PR* progesterone receptor; *HSP72*, heat shock protein 72 kDa)

Patient no	Age	FIGO stage	Histological type	Grade of differentiation	Immunohistochemistry			
					p53	ER	PR	HSP72
Ovarian LMP Tumour								
1	64	IA	Serous	/	−	++	++	+
2	50	IA	Mucinous	/	−	−	−	+
3	54	IA	Mucinous	/	−	−	−	+
4	79	IA	Mucinous	/	−	−	−	+
5	64	IA	Mucinous	/	+	−	−	+
6	31[a]	IC	Serous	/	+	−	−	++
Ovarian Carcinomas								
1	17[a]	IA	Mucinous	Well	−	−	−	+
2	35[a]	IA	Mucinous	Moderately	−	−	−	+
3	52	IA	Endometrioid	Moderately	−	++	++	+
4	43[a]	IA	Endometrioid[b]	Poorly	−	−	+	+
5	31[a]	IA	Clear cell[b]	Well	−	+	−	+
6	72	IA	Clear cell	Moderately	−	+	+	+
7	59	IC	Endometrioid	Well	−	++	++	+
8	65	IC	Endometrioid	Poorly	−	−	−	++
9	35[a]	IC	Clear cell[b]	Moderately	−	+	+	−
10	39[a]	IC	Clear cell[b]	Moderately	−	+	+	+
11	47[a]	IC	Transitional	Moderately	−	+	++	+
12	58	III	Serous	Poorly	−	+	−	−
13	67	III	Serous	Moderately	−	−	−	+
14	68	III	Endometrioid	Moderately	−	+	−	+
15	44[a]	III	Transitional	Moderately	−	−	−	++
16	46[a]	IV	Serous	Poorly	−	−	−	−
17	49[a]	IV	Endometrioid	Moderately	−	+	−	+
18	62	IA	Serous	Well	+	−	−	++
19	68	IA	Mucinous	Well	+	−	−	+
20	88	IA	Mucinous	Well	+	−	−	++
21	44[a]	IA	Endometrioid	Moderately	+	−	−	+
22	48[a]	IC	Mucinous	Well	+	−	−	+
23	48[a]	IC	Endometrioid	Well	+	−	−	+
24	87	IIB	Mucinous	Well	+	−	−	++
25	68	IIC	Endometrioid	Moderately	+	+	+	++
26	39[a]	III	Serous	Moderately	+	−	−	++
27	52	III	Serous	Poorly	+	−	−	++
28	57	III	Serous	Poorly	+	−	−	++
29	62	III	Serous	Well	+	−	−	++
30	63	III	Endometrioid	Moderately	+	−	−	+
31	67	III	Serous	Moderately	+	−	−	++
32	75	IV	Serous	Poorly	+	−	−	++

[a] Premenopausal patient, [b] Carcinomas arising in ovarian endometriotic cysts

mean±SE) was significantly higher than that of the patients with p53-negative tumours (48.6±3.6 years) ($p<0.01$). Postmenopausal patients were found in 11 of the 15 (73.3%) p53-positive tumours and 7 of the 17 (41.2%) p53-negative tumours, and there was no significant relationship between p53-positivity and the menstrual status of the patient. With respect to the FIGO stage, p53-positive cases were observed in 6 of the 17 (35.3%) stage I patients, all of the 2 (100%) stage II patients, 6 of the 10 (60%) stage III patients, and in 1 of the 3 (33.3%) stage IV patients. There was no significant relationship between p53 expression and the FIGO stage of the disease.

Among the histological types, p53-positivity was found in 7 of the 10 serous (70%), 4 of the 6 mucinous (66.7%), 4 of the 10 endometrioid (40%), and in none of the 4 clear cell and 2 transitional cell carcinomas. There was a significant difference of p53-positivity between the serous/mucinous group and the endometrioid/clear cell group ($P<0.05$). In addition, all of the 1 endometrioid and 3 clear cell carcinomas arising in endometriotic cysts were p53-negative. Histologically, p53-positive cells were usually homogeneously distributed in most tumour sections irrespective of the histological type (Fig. 1A). In 2 of the 4 mucinous tumours with p53-positivity, however, p53-positive tumour cells were unevenly distributed in the same sections; the monolayered, benign mucinous glands were usually p53-negative, whereas the multi-layered, atypical epithelia exhibited p53-positivity (Fig. 2). Expression of HSP72 was found in 29 of the 32 carcinomas, and the remaining 3 were HSP72-negative. Strong expression of HSP72 was observed in 10 of the

Table 2 Summary of the relationship between p53 expression and age, FIGO stage, histological type, HSP72 expression, and sex steroid receptor status in ovarian carcinomas (*FIGO*, International Federation of Gynecology and Obstetrics; *ER* oestrogen receptor; *PR*, progesterone receptor; *HSP72*, 72 kDa heat shock protein)

	Total no. of cases	Immunohistochemical expression of p53	
		p53-positive (%)	p53-negative (%)
Age (mean±SE)		61.9±3.7	48.6±3.6
FIGO stage			
I	17	6 (35.3)	11 (64.7)
II	2	2 (100)	0 (0)
III	10	6 (60)	4 (40)
IV	3	1 (33.3)	2 (66.7)
Histological type			
serous	10	7 (70)	3 (30)
mucinous	6	4 (66.7)	2 (33.3)
endometrioid	10	4 (40)	6 (60)
clear cell	4	0 (0)	4 (100)
transitional	2	0 (0)	2 (100)
HSP72 expression			
strongly positive	12	10 (83.3)	2 (16.7)
weak or negative	20	5 (25)	15 (75)
Sex steroid receptor status			
ER- or PR positive	12	1 (8.3)	11 (91.7)
ER- and PR-negative	20	14 (70)	6 (30)
Total	32	15 (46.9)	17 (53.1)

15 (66.7%) p53-positive tumours (Fig. 1B) but in only 2 of the 17 (11.8%) p53-negative tumours ($P<0.05$). All the 3 HSP72-negative tumours were also p53-negative.

Expression of ER and PR in ovarian LMP tumours and carcinomas

Of the ovarian LMP tumours, 1 of the 2 serous tumours showed immunoreactivity for both ER and PR, but all of the 4 mucinous tumours were negative for both ER and PR.

Of the 32 ovarian carcinomas, ER positivity was found in 10 (31.3%) and PR positivity was detected in 9 (28.1%) tumours. Twelve of the 32 (37.5%) ovarian carcinomas were positive for ER and/or PR, and the remaining 20 (62.5%) were negative for both ER and PR. The average age of the patients with ER and/or PR positive tumours was 51.8±3.9 years, and that of the patients with ER and PR negative tumours was 56.7±3.9 years. Premenopausal status was found in 6 of the 12 (50%) patients with ER and/or PR positive tumour, and in 7 of the 20 (35%) of the patients with ER and PR negative tumour. There was no significant relationship between ER or PR positivity of the tumour and the age or menstrual status of the patients. Regarding the FIGO stage, positivity for ER and/or PR was observed in 8 of the 17 (47.1%) stage I, 1 of the 2 (50%) stage II, 2 of the 10 (20%) stage III, and 1 of the 3 (33.3%) stage IV tumours. No significant relationship was observed between the FIGO stage and sex steroid receptor status. With respect to the histological types, however, ER and/or PR positivity was observed in 1 of the 10 serous (10%), none of the 6 mucinous (0%), 6 of the 10 endometrioid (60%), all of the 4 clear cell (100%), and 1 of the 2 transitional cell (50%) carcinomas ($P<0.01$) (Fig. 3). In addition, in all of

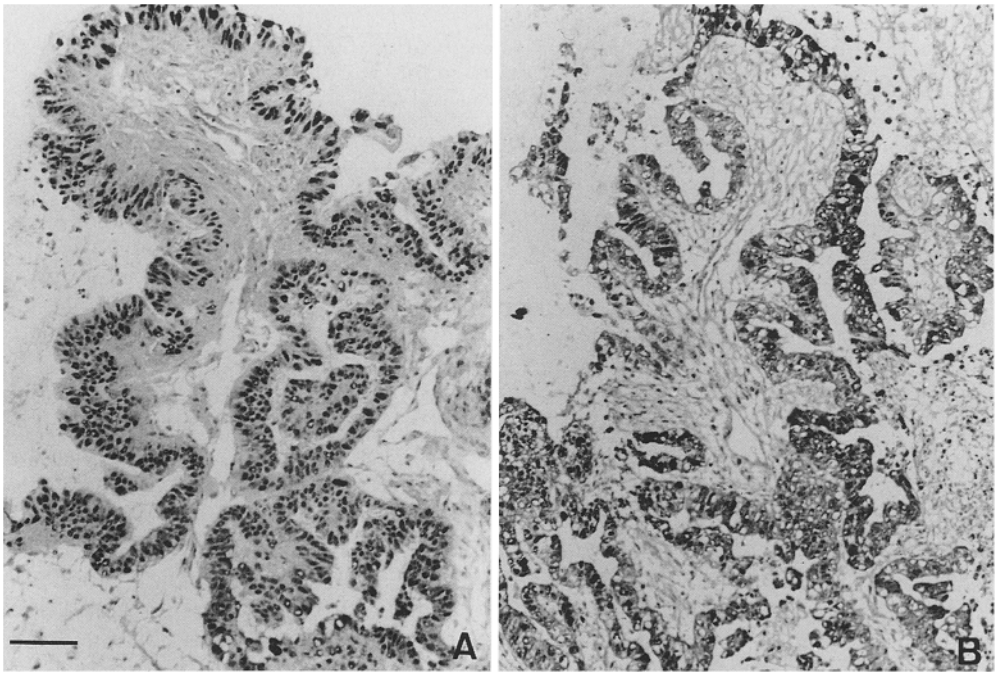

Fig. 1 Immunohistochemical expression of p53 (**A**) and HSP72 (**B**) in ovarian serous cystadenocarcinoma. p53-positive cells are homogeneously distributed and strongly positive for HSP72. ×200 (Scale bar=50 μm)

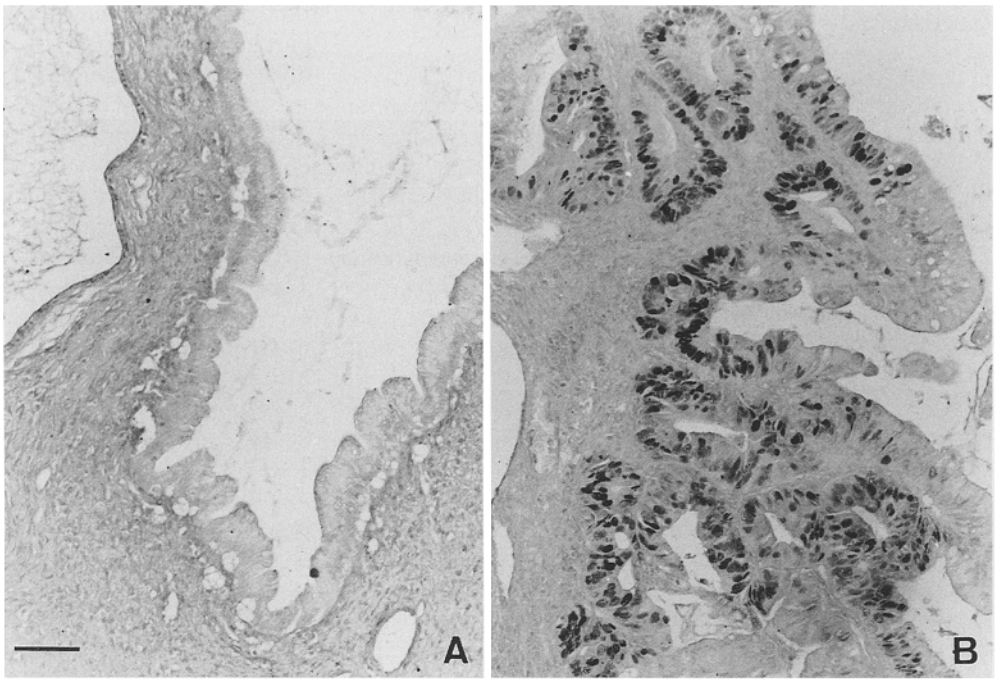

Fig. 2 Immunohistochemical expression of p53 in ovarian mucinous cystadenocarcinoma. Immunoreactivity for p53 is negative in histologically benign epithelia (**A**), but is positive in malignant epithelia (**B**) in the same tumour. ×200 (Scale bar=50 μm)

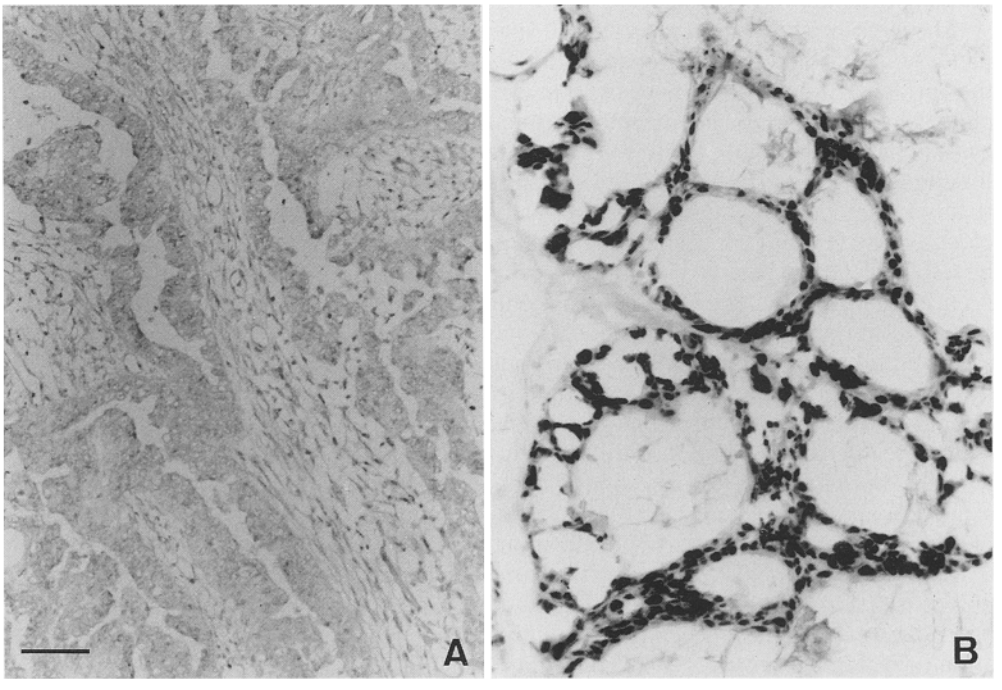

Fig. 3 Immunohistochemical expression of progesterone receptors (PR) in ovarian carcinomas. Representative cases are PR-negative serous carcinoma (**A**) and PR-positive endometrioid carcinoma (**B**). ×200 (Scale bar=50 μm)

the 4 cases, tumours arising in endometriotic cysts were positive for ER and/or PR.

Relationship between the expression of p53/HSP72 and sex steroid receptor status in ovarian LMP tumours and carcinomas

Of the 6 LMP tumours, 1 of the 4 p53-negative tumours was positive for ER and PR, and both of the 2 p53-positive cases were negative for both ER and PR.

Of the 32 ovarian carcinomas, positivity for ER and/or PR was observed in only 1 of the 15 (6.7%) p53-positive cases versus 11 of the 17 (64.7%) p53-negative cases, and in 1 of the 12 (8.3%) cases with strong expression of HSP72 versus 11 of the 20 (55%) cases with weak or negative HSP72 expression. There was an inverse relationship between the sex steroid receptor status and p53-positivity ($P<0.01$) or strong expression of HSP72 ($P<0.05$). All of the 4 carcinomas in endometriotic cysts were p53-negative and ER- or PR-positive.

Discussion

Our study revealed the immunohistochemical reactivity for p53 protein in 15 of the 32 cases (46.9%) of ovarian carcinoma. This is consistent with previous studies on p53 gene mutation and overexpression in ovarian cancer; the rate being reported as approximately 50% of the cases [17, 19, 20, 29] ranging for 29% to 79% [5, 16, 18, 21]. In the present study, the average age of the patients with p53-positive carcinomas was significantly higher than that of the patients with p53-negative tumours. This may be due to the contribution of 4 young women with carcinomas arising in endometriosis, all of which were p53-negative. There was no significant relationship between the p53-positivity of the tumour and the surgical stage of the disease. However, Kohler et al. [13] have recently reported that p53 overexpression was less frequent (15%) in stage IA/IB ovarian cancers than in stage IC/II cases (44%). In ovarian LMP tumours, expression of p53 was observed in 2 of the 6 cases. Recently, p53 overexpression [2] and loss of heterozygosity on chromosome 17p encoding p53 gene [8] have also been reported in LMP tumours. Therefore, it is likely that some LMP tumours possess several genetic alterations including those to the p53 tumour suppressor gene.

The present study demonstrated the difference in the pattern of distribution of p53-positive tumour cells among the various histological types of ovarian carcinoma, especially between serous and mucinous tumours. In serous carcinomas, p53-positive cells were frequent and evenly distributed in the sections, which was consistent with the relative homogeneity of histological features of serous carcinomas. In contrast, mucinous tumours often exhibit histological heterogeneity showing the admixture of benign, borderline, and frankly malignant epithelia even in the same tumour; benign cystadenomas may have small, localized areas of carcinoma [9]. In this study, we found the heterogenous expression of p53 protein in one LMP tumour and in 2 of the 4 carcinomas of mucinous type; histologically benign glands were usually p53-negative, whereas borderline or frankly malignant glands in the same tumour exhibited p53-positivity. This feature resembles the immunohistochemical localization of p53-positive cells of colorectal cancer arising in adenomatous polyposis coli [24]. Such difference in the distribution of p53-positive cells may be consistent with the consideration of different pathways of ovarian tumorigenesis; the great majority of serous carcinomas may arise *de novo* in the ovarian surface epithelium and its inclusion cysts and a sizable proportion of mucinous carcinomas arise from mucinous cystadenomas [26]. Heterogenous distribution of p53-positive cells in some mucinous tumours suggests the possibility that p53 overexpression occurs along with transformation of pre-existing benign cystadenoma into borderline or frankly malignant conditions.

Ovarian carcinoma arising in endometriotic cysts is another representative of malignant transformation of pre-existing benign lesions. We encountered 4 patients with this entity, in which one case was histologically endometrioid type and the remaining 3 were clear cell type carcinomas. Interestingly, all of the 4 tumours were negative for p53 protein and positive for ER and/or PR. In the endometrium, oestrogen-related hyperplasia and carcinoma occurring in young women are characterized by constitutive expression of ER and PR as well as very low rate of p53-positivity [14]. Specific hormonal milieu of unopposed oestrogen has been suggested also in the patients with malignancy arising in endometriosis [23]. Therefore, a common pathway of carcinogenesis may be present in both endometrioid-type carcinomas arising in the endometrium and in ovarian endometriotic cysts. Milner et al. [19] also reported a low p53 mutation rate (15%) in ovarian endometrioid carcinomas, as compared to high p53 mutation rate (56%) in serous carcinomas. These data suggest the difference in the incidence of p53 mutation and overexpression among the different histological types of ovarian carcinomas.

Our study also showed that the sex steroid receptor status in ovarian carcinoma was significantly different among the various histological types; ER- or PR-positivity was low in serous (10%) and absent in mucinous (0%) tumours, whereas high in endometrioid (60%) and clear cell (100%) types. In addition, we found a significant inverse correlation of sex steroid receptor status with p53 overexpression in ovarian carcinomas; 14 of the 15 (93.3%) p53-positive cases were negative for both ER and PR, whereas 11 of the 17 (64.7%) p53-negative cases showed positivity for ER and/or PR. Similar inverse relationship between p53-positivity and sex steroid receptor status has been observed in endometrioid-type versus non-endometrioid type of carcinomas of the endometrium [14]. Mutant p53 protein has been known to induce and bind HSP70 [7, 28], and strong expression of a inducible form of HSP70 (HSP72) has been observed in most cases of the p53-positive carcinoma in our study. HSP70 family is now believed to act as a molecular chaperon which is involved in the folding and unfolding, assembly and disassembly, sorting and translocation of many proteins [12], and is located in both cytosol and nucleus. Immunoreactivity of HSP72 in ovarian cancer cells was observed mainly in the nucleus partly in the cytoplasm, and was consistent with previously reported immunolocalization of HSP72 in thyroid cells of autoimmune diseases [11]. HSP72 has been reported to bind the sex steroid receptors as well [15], or reported to be related to decreased levels of intracytoplasmic steroid receptors [6]. Further studies are needed to examine the possible mechanisms presenting an inverse expression of p53 protein and sex steroid receptors in the tumour cells.

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