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Immunohistochemical analysis of p53 protein over-expression in endometrial carcinomas: inverse correlation with 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 over-expression of p53 protein. To examine the significance of p53 gene alteration in endometrial carcinomas, we studied the immunohistochemical reactivity with a monoclonal antibody against p53 (PAb 1801) in 30 endometrial carcinomas as well as in 64 normal endometria. The presence or absence of correlation of p53 over-expression with the clinicopathological features and with the immunohistochemical expression of sex steroid receptors (oestrogen receptors; ER, progesterone receptors; PR) was also analysed. Expression of p53 was found in none of 64 normal endometria, but was identified in 5 of the 30 (16.7%) endometrial carcinomas. All 5 of the p53-positive tumours developed in women more than 3 years post-menopause, whereas the carcinomas in 5 pre-menopausal women and 3 women less than 3 years post-menopause were p53-negative. None of the 5 p53-positive carcinomas was associated with adjacent endometrial hyperplasia. Two of the 5 p53-positive tumours showed non-endometrioid histology: serous papillary and clear cell carcinomas. In contrast, 6 carcinomas accompanied by adjacent hyperplasia were p53-negative. In addition, ER and/or PR expression was found in none of the 5 p53-positive tumours, but was present in 21 of the 25 p53-negative tumours ($p < 0.01$). These clinicopathological features of p53-positive carcinomas and the inverse correlation of p53 immunoreactivity with sex steroid receptor status suggest that p53 over-expression is frequent in a specific category of endometrial carcinoma, presumably oestrogen-unrelated tumours.

Key words: Endometrial carcinoma – p53 – Immunohistochemistry – Oestrogen receptor – Progesterone receptor

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Introduction

Endometrial carcinomas are believed to develop in the context of a specific hormonal milieu such as that occurring in oestrogen replacement therapy or in the presence of unopposed oestrogen due to polycystic ovary or perimenopause (Gurpide 1991). However, several lines of epidemiological and clinicopathological evidence suggest that there is an another distinct form of endometrial carcinoma that has non-endometrioid histology and no association with endometrial hyperplasia, that is unrelated to oestrogenic stimulation which occurs in older postmenopausal women (Bokhman 1983; Deligdish and Cohen 1985; Kurman and Norris 1987). Although little is known about the molecular genetic events that contribute to the development of endometrial carcinoma, mutation of the tumour suppressor p53 gene has recently been reported in this neoplasm (Okamoto et al. 1991; Kohler et al. 1992). The p53 protein is believed to play a role in regulating the cell cycle (Reich and Levine 1984), and wild-type p53 is capable of suppressing the growth of a variety of cancer cell lines (Finlay et al. 1989; Chen et al. 1990). Loss of wild-type p53 is associated with cell transformation (Hollstein et al. 1991; Levine et al. 1991). The significance of p53 mutation in the two types of endometrial carcinogenesis, however, remains unclear. The recent development of a monoclonal antibody against p53 protein has made possible the analysis of its immunohistochemical over-expression in human tumours (Banks et al. 1986). The half-life of wild-type p53 is too short for immunohistochemical detection but mutant p53 has a longer half-life, which leads to higher steady-state p53 levels (Finlay et al. 1988). Therefore, the immunohistochemical expression of p53 is usually associated with p53 gene alteration (Banks et al. 1986; Davidoff et al. 1991; Kohler et al. 1992). Accordingly, we examined the expression of p53 in endometrial carcinomas immunohistochemically, and analysed the presence or absence of correlation with their clinical and histological features, especially with reference to the two distinct forms of endometrial carcinoma. Since a differ-

ence of sex steroid receptor status has been reported between these two types of endometrial carcinomas (Deligdish and Holinka 1986), we also analysed the immunohistochemical expression of oestrogen receptors (ER) and progesterone receptors (PR) in our series.

Materials and methods

Fresh surgical specimens of endometrial carcinoma were obtained from 30 women who underwent hysterectomy and bilateral salpingo-oophorectomy with pelvic and para-aortic lymphadenectomy. Five of the 30 patients were pre-menopausal and the remaining 25 patients were post-menopausal; 3 patients were less than 3 years post-menopause, and 22 were 3 years or more post-menopause. Two pre-menopausal patients were given medroxyprogesterone acetate 300 mg/day for 3 weeks before operation. According to the International Federation of Gynecology and Obstetrics (FIGO, 1988) classification, the 30 cases of endometrial carcinoma consisted of 16 stage I, 5 stage II, and 9 stage III. Histologically, 27 of the 30 cases were endometrioid type carcinoma, 2 were papillary serous carcinoma, and the remaining 1 was clear cell carcinoma. Of the 27 endometrioid type carcinomas, 12 were well-differentiated (G1), 11 were moderately differentiated (G2), and 4 were poorly differentiated (G3). In 6 of the 30 endometrial carcinomas, cryostat sections revealed the presence of an area of endometrial hyperplasia in the vicinity of carcinomatous glands.

Specimens of histologically normal endometrium were obtained from 64 women with benign gynaecological diseases who underwent hysterectomy. Of the 64 patients, 39 had regular menstrual cycles. At the time of operation, 23 of these were in the proliferative phase and 16 were secretory phase; the remaining 25 patients were post-menopausal. The materials, obtained immediately after the surgical procedure, were snap-frozen in OCT compound (Ames, Elkhart, Inn., USA) and stored at −70° C. Serial cryostat sections were stained with haematoxylin and eosin for light microscopy. The menstrual cycle of patients with normal endometria was estimated by endometrial dating, according to the method of Noyes et al. (1950).

Immunostaining for p53 protein on the cryostat 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. The sections 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. For positive controls, we used cryostat sections of ovarian carcinomas with p53 gene mutations.

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, 4 μm cryostat sections were fixed in 3.7% formaldehyde in phosphate-buffered saline (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-positive and PR-positive cells. In the endometrial carcinomas, localization of ER and PR was heterogenous, and therefore 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 the chi-square test and the Fisher's 2-tailed exact test, on the correlation of p53 immunohistochemical positivity with the menstrual state of the patient, FIGO stage, grade of differentiation, and sex steroid receptor expression in endometrial carcinoma.

Results

Specific staining with anti-p53 antibody, anti-ER antibody, and anti-PR antibody was exclusively confined to the nuclei, with no cytoplasmic staining being observed. Patient age, menstrual state, FIGO stage, histological type, grade of differentiation, site of extra-uterine spread, and the immunohistochemical expression of p53, ER, and PR in endometrial cancer cases are listed in Table 1.

Immunohistochemical expression of the p53 protein was observed in none of the 64 specimens of normal endometrium, but was found in 5 of the 30 (16.7%) endometrial carcinomas. In the p53-positive cases (Cases 26–30), 30%–80% of the nuclei were strongly stained for p53 protein. The clinical and histological features of the p53-positive patients were as follows; Case 26 was a 69-year-old, post-menopausal woman who underwent simple hysterectomy with pelvic and para-aortic lymphadenectomy. The tumour was a small lesion of the endometrioid type, G1 carcinoma, and was confined within the endometrium. Case 27 was a 43-year-old woman whose menopause had occurred at the age of 39. Hysteroscopy and endometrial curettage revealed poorly differentiated adenocarcinoma of the endometrium, and the hysterectomy specimen showed no myometrial invasion or metastasis. Case 28 was a 60-year-old, post-menopausal patient who had papillary serous carcinoma of the endometrium with myometrial invasion of less than 1/2 of the uterine wall (Fig. 1). Case 29 was a 66-year-old, post-menopausal woman having clear cell carcinoma of the endometrium with superficial endocervical involvement (Fig. 2). Case 30 was a 64-year-old, post-menopausal patient with stage IIIC, endometrioid type, G2 carcinoma, which involved the left parametrium and lymph nodes. None of the 5 patients showed clinical evidence of exposure to unopposed oestrogen, either exogenously or endogenously.

The clinical and histological features of the p53-positive and p53-negative endometrial carcinomas were compared. With respect to menstrual state, all 5 p53-positive carcinomas had developed in patients who were more than 3 years post-menopause, whereas the 25 p53-negative cases included all 5 of the pre-menopausal women and 3 women who were less than 3 years post-menopause. With respect to the clinical stage, p53-positive tumours were observed in 3 of the 16 (18.8%) stage I patients, in 1 of the 4 (25%) stage II patients, and in 1 of the 8 (12.5%) stage III patients. There was no

Table 1. Age, menstrual state, FIGO stage, histological type, grade of differentiation, site of extra-uterine spread, and immunohistochemical expression of p53, ER, and PR in endometrial carcinomas.

Patient no.	Age (yr)	FIGO stage	Histological type	Grade of differentiation	Extra-uterine spread	Presence of hyperplasia	Immunohistochemical expression		
							p53	ER	PR
Premenopausal patients									
1	39[a]	IA	E	G1	−	+	−	+	++
2	41	IA	E	G1	−	+	−	++	++
3	43[a]	IB	E	G1	−	+	−	+	++
4	47	IB	E	G1	−	+	−	+	++
5	43	IIA	E	G1	−	−	−	+	++
Patients <3 years after menopause									
6	51	IC	E	G2	−	−	−	−	−
7	59	IIIA	E	G1	Ovary	−	−	++	++
8	53	IIIC	E	G2	Peritoneum Lymph nodes	−	−	+	++
Patients ≧3 years after menopause									
9	61	IA	E	G1	−	+	−	−	++
10	51	IA	E	G2	−	+	−	+	++
11	75	IA	UPSC	/	−	−	−	−	−
12	65	IB	E	G1	−	−	−	++	++
13	67	IB	E	G1	−	−	−	+	++
14	63	IB	E	G2	−	−	−	+	++
15	56	IC	E	G1	−	−	−	+	++
16	68	IC	E	G2	−	−	−	+	+
17	69	IIB	E	G1	−	−	−	+	+
18	71	IIB	E	G2	−	−	−	+	+
19	59	IIB	E	G3	−	−	−	−	−
20	57	IIIA	E	G2	Peritoneum	−	−	+	+
21	59	IIIC	E	G2	Parametrium Lymph nodes	−	−	−	++
22	63	IIIC	E	G2	Lymph nodes	−	−	++	++
23	70	IIIC	E	G2	Lymph nodes	−	−	+	++
24	57	IIIC	E	G3	Lymph nodes	−	−	−	−
25	63	IIIC	E	G3	Lymph nodes	−	−	−	++
26	69	IA	E	G1	−	−	+	−	−
27	43	IA	E	G3	−	−	+	−	−
28	60	IB	UPSC	/	−	−	+	−	−
29	66	IIA	Clear cell	/	−	−	+	−	−
30	64	IIIC	E	G2	Parametrium Lymph nodes	−	+	−	−

[a] Medroxyprogesterone acetate was administered before operation
[b] Histological type: E, endometrioid type; UPSC; papillary serous carcinoma; Clear cell, clear cell carcinoma

significant relationship between p53 expression and the FIGO disease stage. Histologically, 2 of the 3 non-endometrioid type carcinomas were p53-positive, whereas 24 (88.9%) of the 27 endometrioid type carcinomas were negative for p53 (Fig. 3) ($p<0.05$). In the endometrioid type tumours, there was no significant relationship between p53 expression and grade of differentiation; p53 positivity was found in 1 of 12 (8.3%) G1 cases, 1 of 11 (9.1%) G2 cases, and 1 of 4 (25%) G3 cases. An area of endometrial hyperplasia was identified in the vicinity of carcinomatous lesions in 6 of the 30 cases, and all of the 6 hyperplasias and carcinomas were negative for p53. Endometrial hyperplasia was not associated with any of the 5 p53-positive carcinomas.

In the functional layer of normal endometrium, glandular cells were strongly positive for both ER and PR during the proliferative and early secretory phases. However, the expression of ER and PR in glandular cells was weakly positive or negative in the mid-secretory phase, and negative in the late secretory phase and during pregnancy. That is, there were changes in the expression of ER and PR during the menstrual cycle in normal glandular cells. In the post-menopausal endometrium, glandular cells were positive for ER and PR.

In the endometrial carcinomas, ER positivity was found in 17 of the 30 patients (56.7%), and PR positivity was found in 21 of the 30 (70%). Nine of the 30 patients (30%) were negative for both ER and PR. With regard to the menstrual status, ER and/or PR positivity of tumour cells was found in all 5 pre-menopausal patients (100%), in 2 of the 3 patients who were less than 3 years post-menopause (66.7%), and in 14 of the 22 pa-

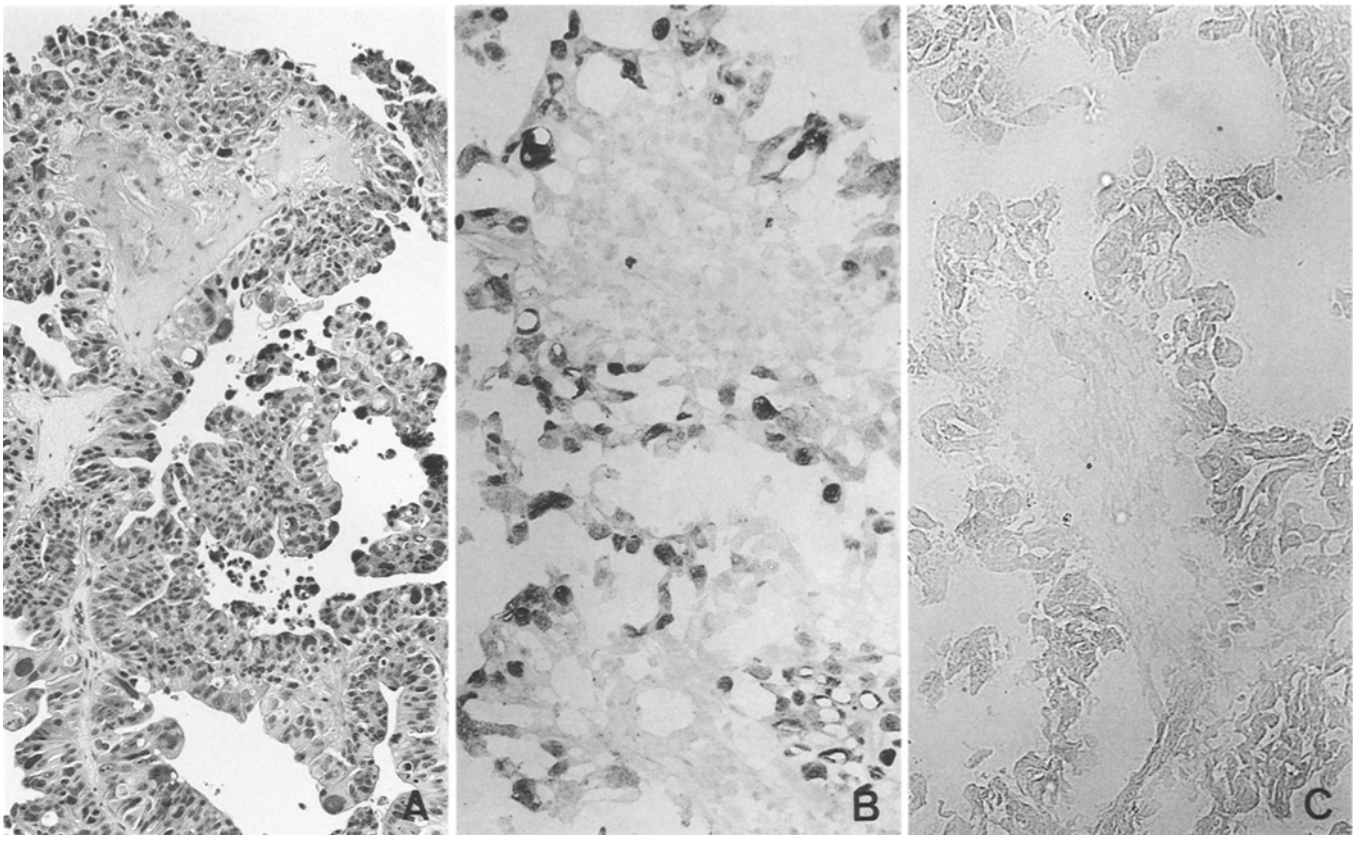

Fig. 1 A–C. Histological features (**A**) and immunohistochemical expression of p53 (**B**), and PR (**C**) in papillary serous carcinoma of the endometrium (Case 28). Tumour cells are positive for p53 and negative for PR. **A**: ×200, **B, C**: ×400

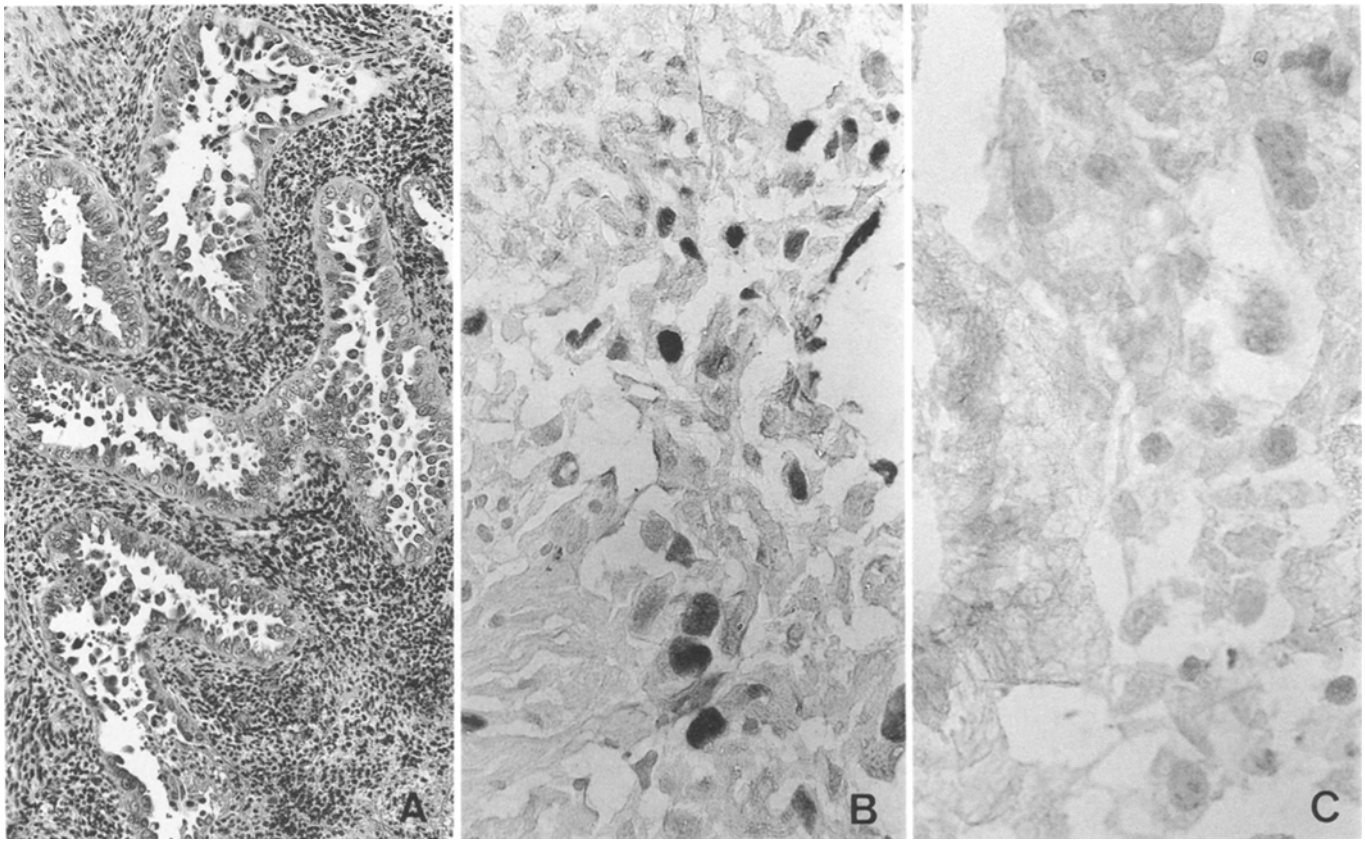

Fig. 2 A, C. Histological features (**A**) and immunohistochemical expression of p53 (**B**), and PR (**C**) in clear cell carcinoma of the endometrium (Case 29). Tumour cells are positive for p53 and negative for PR. **A**: ×200, **B, C**: ×400

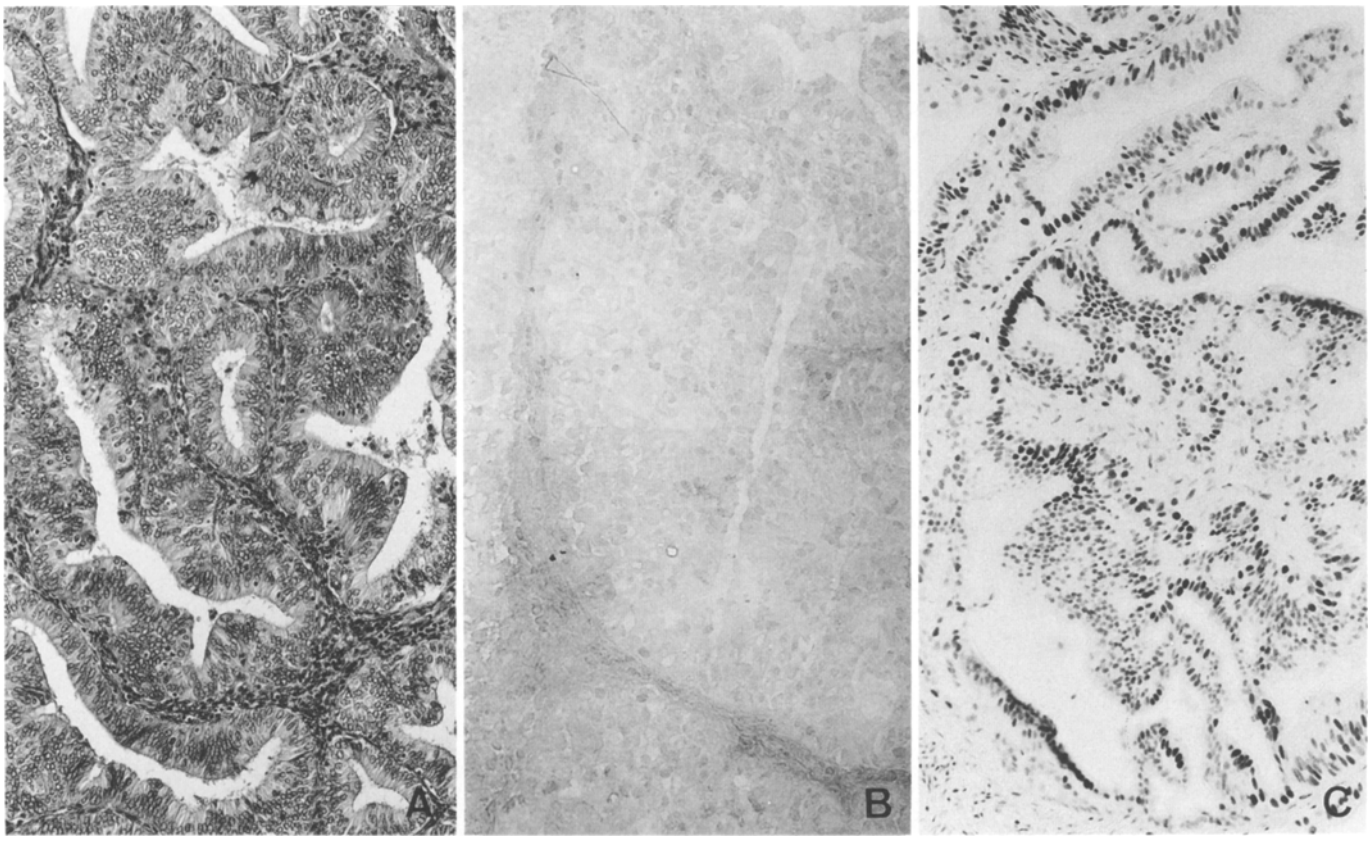

Fig. 3A–C. Histological features (**A**) and immunohistochemical expression of p53 (**B**), and PR (**C**) in endometrioid type, G1 carcinoma of the endometrium (Case 4). Tumour cells are negative for p53 and positive for PR. A, B, C: ×200

tients who were more than 3 years post-menopause (63.6%). Regarding the FIGO stage, positivity for ER and/or PR was observed in 10 of the 16 (68.8%) stage I patients, 3 of the 5 (60%) stage II patients, and 7 of the 9 (77.8%) stage III patients. With respect to the histological type, ER and/or PR positivity was observed in 21 of the 27 (77.8%) endometrioid type tumours, but in none of the 3 non-endometrioid type carcinomas ($p < 0.05$). Of the 27 endometrioid type carcinomas, positivity for ER and/or PR was seen in 11 of the 12 (91.7%) G1 tumours, 9 of the 11 (81.8%) G2 tumours, and 1 of the 4 (25%) G3 tumours ($p < 0.05$).

Of the 30 endometrial carcinomas, none of the 5 p53-positive cases were positive for either ER or PR (Figs. 1, 2), whereas 21 (84%) of the 25 p53-negative tumours showed positivity for ER and/or PR (Fig. 3). There was an inverse relationship between the p53 expression and sex steroid receptor status ($p < 0.01$).

Discussion

Our study revealed that immunohistochemical reactivity for the p53 protein was observed in 5 of 30 cases (16.7%) of endometrial carcinoma. In other malignant tumours, immunohistochemical over-expression of p53 or mutation of the p53 gene has been reported in 42%–50% of colorectal carcinomas (Rodrigues et al. 1990; Purdie et al. 1991; Scott et al. 1991), in 46%–70% of lung cancers (Iggo et al. 1990; Caamamo et al. 1991), and in 50%–57% of ovarian carcinomas (Okamoto et al. 1990; Marks et al. 1991; Eccels et al. 1992; Naito et al. 1992). The relatively lower rate of p53 positivity in our series of endometrial carcinoma is consistent with previous studies of p53 over-expression or mutation in this tumour; the rate being reported as 12.5% by Okamoto et al. (1991), 20.6% by Kohler et al. (1992), 23% by Enomoto et al. (1993), and 9.5% by Honda et al. (1993); this feature was found in 1 of the 7 cases in our previous study using polymerase chain reaction-single strand conformation polymorphism analysis (Naito et al. 1992). Although carcinomas expressing immunohistochemically undetectable levels of p53 protein may have acquired non-sense mutations or may have lost both copies of the p53 gene, these mechanisms of p53 inactivation appear to be relatively uncommon (Hollstein et al. 1991). Therefore, it is likely that p53 over-expression or p53 gene alteration in endometrial carcinomas occurs in a minority of cases.

With respect to the relationship between p53 expression and the stage of disease, Kohler et al. (1992) reported that p53 expression was more frequently observed in stage III/IV than in stage I/II patients, and they suggested that p53 mutation may be a late event during endometrial carcinogenesis. However, another report by Enomoto et al. (1993) did not support this asso-

ciation. In our series, 4 of the 5 p53-positive cases were stage I or II, and 2 of these tumours were confined in the endometrium with no myometrial invasion. Therefore, p53 over-expression does occur even in early stage carcinomas; however, further studies are necessary to analyse the relationship between p53 gene mutation and the progression of endometrial carcinomas.

It is suggested that there are at least two distinct forms of endometrial carcinoma, one being oestrogen-related, frequently showing endometrioid histology with squamous metaplasia, and occurring in younger, perimenopausal women; the other being oestrogen-unrelated, often showing non-endometrioid histology, and developing in older, post-menopausal patients (Bokhman 1983; Deligdish and Cohen 1985; Kurman and Norris 1987). Our p53-positive endometrial carcinomas developed only in post-menopausal patients who were more than 3 years post-menopause, while the carcinomas in women who were pre-menopausal or less than 3 years post-menopause were p53-negative. Histologically, 2 of the 5 p53-positive tumours were non-endometrioid type carcinomas; papillary serous carcinoma and clear cell carcinoma. Significant correlation of p53 expression with non-endometrioid histology has been also reported (Kohler et al. 1992). An unopposed oestrogen environment due to exogenous or endogenous oestrogen sources leads to endometrial carcinoma via its precursor, endometrial hyperplasia (Gambrell et al. 1983; Kurman et al. 1985). In our study, both endometrial hyperplasias and the carcinomas accompanied by adjacent endometrial hyperplasia were p53-negative; this is consistent with the data by Okamoto et al. (1991). Although mutation of Ki-*ras* protooncogene in endometrial hyperplasias has recently been reported (Sasaki et al. 1993), occurrence of p53 mutation seems to be independent from Ki-*ras* mutation in endometrial carcinomas (Enomoto et al. 1993). These data suggest that p53 over-expression is less frequent in oestrogen-related, endometrial hyperplasias and carcinomas.

Sex steroid receptor status in endometrial carcinoma has been reported to correlate with a variety of clinicopathological variables such as stage, histological type, grade of differentiation, prognosis, and patient survival (Kurman and Norris 1987). Association of ER and/or PR expression with histological type and with grade of differentiation was also observed in our series of endometrial carcinomas. In addition, we found a significant inverse correlation of sex steroid receptor status with p53 over-expression in endometrial carcinomas; ER and/or PR expression was absent in all of the 5 p53-positive tumours, but was present in 21 of the 25 (84%) p53-negative tumours ($p < 0.01$). Such an inverse relationship has been also reported in another hormone-related neoplasm, breast carcinoma (Cattoretti et al. 1988). Oestrogen-related hyperplasias of the endometrium have frequently been reported to express sex steroid receptors (Janne et al. 1979; Bergeron et al. 1988; Brustein et al. 1989). Our previous study demonstrated that constitutive expression of ER and PR irrespective of the change of hormonal milieu is one of the characteristic features of well-differentiated carcinoma in pre-menopausal women (Wang et al. 1993). However, expression of sex steroid receptors is negative or very weak in non-endometrioid type carcinomas (Carcangiu et al. 1990) or in oestrogen-unrelated carcinomas (Deligdish and Holinka 1986). Therefore, an inverse relationship between p53 immunoreactivity and sex steroid receptor status suggests that p53 over-expression occurs more frequently in ER- and PR-negative endometrial carcinomas, which may be categorized as oestrogen-unrelated tumours. However, further studies are necessary to verify the significance of p53 gene mutation and over-expression in different pathways of endometrial carcinogenesis.

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