Expression of p53 in endometrial polyps with special reference to the p53 signature

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Summary. We herein examined the significance of the p53 expression in endometrial polyps (EMPs). A total of 133 EMPs, including 62 premenopausal and 71 postmenopausal women with EMP, were immuno-histochemically studied for the expression of estrogen receptor (ER)-alpha, Ki-67 and p53. Apoptotic cells were identified using a TUNEL assay. A DNA sequence analysis of *TP53* exons 5 to 9 was performed. Among the premenopausal EMPs, a multivariate analysis showed the labeling index (LI) for Ki-67 to correlate significantly with that for p53 (P<0.001), but not that for apoptosis. On the contrary, among the postmenopausal EMPs, the LI for Ki-67 correlated significantly with that for apoptosis (P<0.001). The p53 signature (p53S) was defined by endometrial epithelial cells, which are morphologically benign in appearance but display 12 or more consecutive epithelial cell nuclei with strong p53 immunostaining. The p53S was found in nine (12.7%) postmenopausal EMPs (mean age: 70.2 years). The median Ki-67 index for the p53S was 7%, with no significant difference from that of the glands of the postmenopausal EMPs without the p53S (P=0.058). The median apoptotic index for the p53S was 0%, which was significantly lower than that of the postmenopausal EMPs without the p53S (P=0.002). Two of four p53Ss showed *TP53* mutations according to the DNA sequence

analysis. The presence of the p53S is not rare in postmenopausal EMPs with an advanced age. Among postmenopausal EMPs, the LI of Ki-67 significantly correlates with that of apoptosis. However, such a positive correlation between the LI of Ki-67 and apoptosis is not observed in p53S.

Key words: Endometrial polyp, p53 signature, p53, Ki-67, Estrogen receptor, Apoptosis

Introduction

A previous systematical review and meta-analysis of the medical literature regarding the oncogenic potential of endometrial polyps (EMPs) showed both symptomatic vaginal bleeding and the postmenopausal status in women with EMPs to be associated with an increased risk of endometrial malignancy (Lee et al., 2010). In another study examining age-related differences in the incidence of EMP at the time of diagnosis, the frequency of malignant EMPs increased with age and reached statistical significance in women 65 years of age or older (Hileeto et al., 2005). Although the most common histologic subtype of malignant EMPs was endometrioid adenocarcinoma (Hileeto et al., 2005), several clinicopathologic studies investigating minimal uterine serous carcinomas (USCs) showed most of them to have developed in the EMPs (Wheeler et al., 2000; Hui et al., 2005).

Recent morphologic and molecular genetic studies of women with a hereditary mutation in *BRCA1* and

BRCA2 have reported new findings regarding the carcinogenesis and histogenesis of pelvic serous carcinoma (Folkins et al., 2008). It appears that a significant subset of pelvic serous carcinomas originates from the distal fallopian tube rather than the ovarian surface epithelium. In addition, the p53 signature (p53S) is associated with serous tubal intraepithelial carcinoma (STIC), and has been reported to be the earliest lesion in the development of tubal serous carcinoma (Crum et al., 2012).

In cases of endometrial tumors, the p53S was found in seven of ten EMPs involved with intraepithelial and/or invasive serous carcinomas and six (4%) of 137 benign EMPs (Jarboe et al., 2009). The *TP53* mutation was found in three p53Ss associated with intraepithelial and/or invasive serous carcinomas. The authors hypothesized that the endometrial p53S may represent a potential precursor to some cases of USC. Another study showed that the p53S was specifically associated with USC and only rarely associated with either the endometrium adjacent to the endometrioid adeno-carcinoma or normal endometrium (Zhang et al., 2009).

In this report, we describe the expression of p53 in EMPs along with the expression of ER-alpha, Ki-67 and apoptotic cells. The immunohistochemical characteristics of the p53S in EMPs were shown with the *TP53* mutation status.

Materials and methods

Case selection

A total of 133 EMPs, including 62 premenopausal and 71 postmenopausal women with EMP, were retrieved from the database of the Pathology Department at the University of Occupational and Environmental Health Hospital for the 17-year-period from 1994 to 2011. The histological diagnosis of an EMP was assessed by two authors using new hematoxylin/eosin (H&E)-stained slides. Surgical specimens were collected from 62 women undergoing hysterectomy, 62 women undergoing hysteroscopy, and 9 women undergoing an endometrial biopsy. No women received hormones for the management of menopausal symptoms. Seven tamoxifen (TAM) and four toremifene (TOR) users after undergoing surgery for breast cancer were included in this study. The study was approved by the Review Board of the University Hospital of Occupational and

Table 1. Clinicopathologic data for premenopausal and postmenopausal endometrial polyps.

Variable	Endometrial polyps		p-value
	premenopausal	postmenopausal	
Number of cases	62	71	
Age (years)			
mean (range)	39.5(23-47)	65.7(54-88)	
vaginal bleeding			0.119
presence	36	31	
absence	26	40	
BMI			0.287
median (range)	21.6(16.6-39.6)	23.7(17.1-36.2)	
Parity			0.002
0	22	6	
1-2	27	44	
>2	13	21	
Hypertension			0.002
presence	6	23	
absence	56	48	
Diabetes mellitus			0.029
presence	2	10	
absence	60	61	
Tamoxifen or Toremifene use			0.010
presence	1	10	
absence	61	61	
Diameter of the polyp (cm)			0.162
median (range)	1.4(0.5-3.1)	1.5(0.5-5.1)	
p53 (%)			0.934
median (range)	4(0-32)	3(0-98)	
Ki-67 (%)			0.037
median (range)	25(0-81)	12(0-76)	
Apoptosis (%)			0.001
median (range)	2(0-11)	0(0-9)	

BMI; body mass index [weight (kg)/height (m) X height (m)].

Table 2. Incidence rate ratios for the expression of Ki-67 in endometrial polyps.

	Univariate				Multivariate			
	IRR	95%CI		p	IRR	95%CI		p
premenopausal								
p53	1.05	1.05	1.05	<0.001	1.05	1.05	1.05	<0.001
apoptosis	1.01	0.99	1.02	0.481	1.01	0.99	1.03	0.189
postmenopausal								
p53	0.99	0.99	1.00	<0.001	1.00	1.00	1.00	0.175
apoptosis	1.24	1.22	1.26	<0.001	1.24	1.21	1.26	<0.001

IRR; incidence rate ratio, CI; confidence interval.

Environmental Health on Ethical Issues (H24-058).

Immunohistochemistry

For the immunohistochemical analyses, 4-µm sections were cut from formalin-fixed paraffin-embedded tissue blocks, deparaffinized in xylene, and rehydrated through sequential changes of alcohol and distilled water. Ki-67 and p53 were detected using ready-to-use monoclonal antibodies against Ki-67 and p53 (clones MIB-1 and DO-7, respectively; DAKO, Kyoto, Japan). ER-alpha was detected using an antibody against ER (clone 6F11, diluted 1:50,; Novocastra, Fukuoka, Japan). The slides were heated in an autoclave at 120°C for 5 min in 0.01M citrate buffer (pH=6.0) before immunostaining. The slides were incubated with the abovementioned antibodies for 2 hours at room temperature. Antibody binding was visualized using the EnVision+ Dual link system and diaminobenzidine as a chromogen (Dako Cytomation, Kyoto, Japan). 3'-endlabeling of apoptotic cell DNA was performed using an Apop Tag in situ apoptosis detection kit (Millipore Corporation, Tokyo, Japan) according to the manufacturer's instructions. The slides were counterstained with methyl green or hematoxylin and mounted. The p53S was defined by the endometrial epithelial cells, with either glandular or surface growth patterns, which are morphologically benign in appearance but display 12 or more consecutive epithelial cell nuclei with strong p53 immunostaining (Folkins et al., 2008). The interpretation of the immunohisto-chemical preparations and presence of apoptotic cells
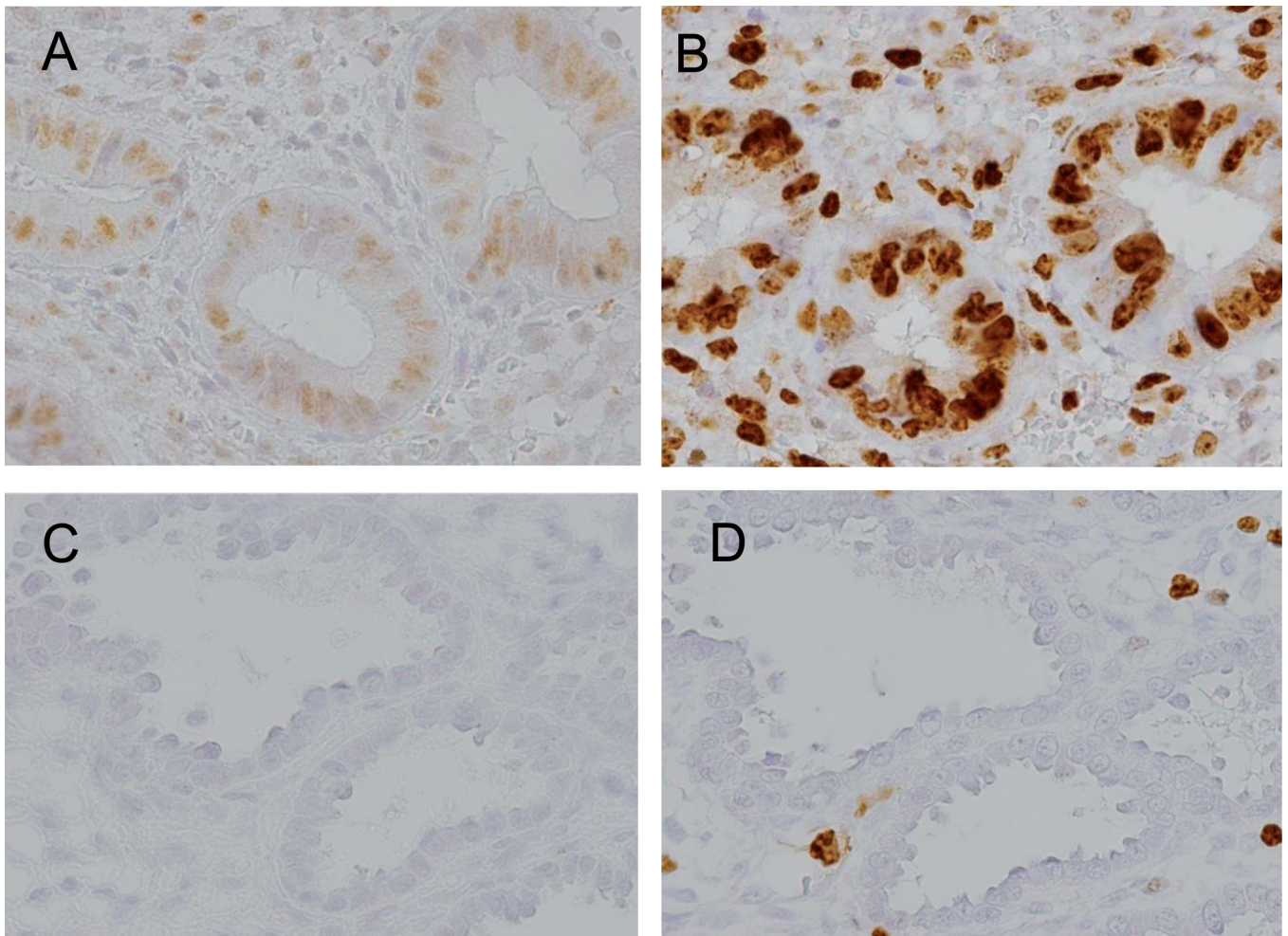

Fig. 1. Glands of an endometrial polyp in the proliferative phase of a 36-year-old woman showed positivity for p53 **(A)** and Ki-67 **(B)**. Glands of an endometrial polyp in the secretory phase of a 32-year-old woman showed negativity for p53 **(C)**, and Ki-67 **(D)**. x 40

was assisted with the WinROOF image processing software program (Mitani Corp., Tokyo, Japan) (Koi et al., 2015).

Microdissection and DNA sequence analysis for TP53 exons

The formalin-fixed paraffin-embedded tissue sections (6 μm) were used for DNA extraction. A section of each specimen was then stained with hematoxylin/eosin (H&E) and examined microscopically to confirm the diagnosis. Targeted epithelial cells were selectively microdissected under a microscope using a micromanipulator with reference to the p53 staining section. These samples were submitted to the facility of the genomic analysis of LSI Medience Corporation (Life Science Institute, Tokyo, Japan) for the DNA sequence analysis of *TP53* exons 5 to 9 using a PRISM3130 sequencer. Potential *TP53* gene mutations were examined by comparison to the GenBank database (accession no. AF136270).

Statistical analysis

Statistical analyses were carried out using the SPSS for Windows, version 20.0.0 software program (SPSS, Chicago, IL, USA). Data for the clinicopathological factors and immunohistochemical markers were evaluated using the Chi-square test or Mann-Whitney *U*-test. The mean age of the patients was assessed using Student's *t*-test. The Poisson regression analysis was used to examine the association between p53 and apoptosis on Ki-67. Statistical significance was considered to exist at a value of $P<0.05$.

Results

Clinicopathological and immunohistochemical findings

The clinicopathological data for premenopausal and postmenopausal EMPs are presented in Table 1. Women with postmenopausal EMPs showed more frequent incidences of parity, hypertension, diabetes mellitus (DM), or the use of TAM or TOR compared with women with premenopausal EMPs. Among premenopausal EMPs, the median LI for Ki-67, p53, and apoptosis was 49%, 7% and 3% in 41 EMPs during the proliferative phase and 0%, 0%, 3% in 21 EMPs during the secretory phase ($P<0.001$, $P<0.001$ and $P=0.58$), respectively (Fig. 1). Univariate and multivariate analyses showed the LI for Ki-67 to correlate significantly with p53 ($P<0.001$), but not with apoptosis (Table 2). The glands of premenopausal EMPs during the proliferative phase were positive for ER-alpha. A marked reduction in the expression of ER-alpha was found in the glands of premenopausal EMPs during the secretory phase.

Among postmenopausal EMPs, a univariate analysis showed the LI for Ki-67 to inversely correlate with p53 ($P<0.001$), and it significantly correlated with apoptosis ($P<0.001$). According to a multivariate analysis, the significance of the correlation between the LI of Ki-67 and apoptosis was preserved, whereas the significance of the inverse correlation between the LI of Ki-67 and p53 disappeared.

The clinicopathological data of postmenopausal EMPs with or without the p53S are presented in Table 3. The areas with the p53S could not be histologically recognized without p53 immunostaining (Fig. 2). Immunohistochemical staining for p53S was positive in nine (12.7%) postmenopausal EMPs (Fig. 3). The mean age was 70.2 years in women with EMPs associated with the p53S. Vaginal bleeding and an overweight status were significantly more frequently observed in postmenopausal women with the p53S than those without the p53S. The median Ki-67 index for the postmenopausal EMPs with the p53 (7%) was not different from that for the postmenopausal EMPs without the p53S (12%), ($P=0.058$). The median apoptotic index for the postmenopausal EMPs with the p53S (0%) was significantly lower than that of the

Table 3. Clinicopathologic data for postmenopausal endometrial polyps.

Variable	p53 signature		p-value
	presence	absence	
Number of cases	9	62	
Age (years)			0.094
mean (range)	70.2(60-83)	65.4(54-88)	
vaginal bleeding			0.027
presence	7	24	
absence	2	38	
BMI			0.033
median (range)	26.8(21.2-33.3)	23.7(17.1-36.2)	
Parity			0.135
0	0	6	
1-2	4	40	
>2	5	16	
Hypertension			0.910
presence	3	20	
absence	6	42	
Diabetes mellitus			0.904
presence	2	8	
absence	7	54	
Tamoxifen or Toremifene use			0.784
presence	1	9	
absence	8	53	
Diameter of the polyp (cm)			0.160
median (range)	2.4(1.1-4.5)	1.4(0.5-5.2)	
Ki-67 (%)			0.058
median (range)	7(3-23)	12(0-76)	
Apoptosis (%)			0.002
median (range)	0	1(0-9)	

BMI; body mass index [weight (kg)/height (m) X height (m)].

postmenopausal EMPs without the p53S (1%), (P=0.002). All postmenopausal endometrial glands were positive for ER-alpha, regardless of the presence or absence of the p53S.

TP53 mutation status

Of 10 cases, including four p53Ss in postmenopausal EMPs, three postmenopausal EMPs without p53S, and three premenopausal EMPs during the proliferative phase, microdissected materials were obtained for the DNA analysis and genomic DNA amplification was successful. One p53S showed both a silent mutation in exon 5 at codon 145 from CTG to CTA (Leu to Leu) and a missense mutation in exon 5 at codon 169 from ATG to ATA (Met to IIe) in an EMP of a 71-year-old TOR user (Fig. 4). One p53S showed a silent mutation in exon 5 at codon 169 from GCC to GCT (Ala to Ala) in an EMP of an 82-year-old woman. The other two p53Ss were *TP53* wild type in an EMP of an 82-year-old and a 72-year-old woman, respectively. Three postmenopausal

EMPs without the p53S and three premenopausal EMPs during the proliferative phase were *TP53* wild type.

Discussion

The endometrium undergoes cyclical changes of proliferation, differentiation and apoptosis in response to the rise and fall in the ovarian estrogen and progesterone levels. The expression of Ki-67 is detected with a higher frequency during the proliferative phase than during the secretory phase in the normal endometrium. Similar findings were observed in premenopausal EMPs (Maia et al., 2004). The previous study using the TUNEL method showed that apoptotic cells in the normal endometrium were most frequently detected in glandular epithelial cells during the late secretory phase and rarely in glandular epithelial cells during the proliferative phase (von Rango et al., 1998; Udou et al., 2004). However, apoptotic cells were often found in the glands of EMP during the proliferative phase, and the incidence of apoptotic cells was not significantly different from
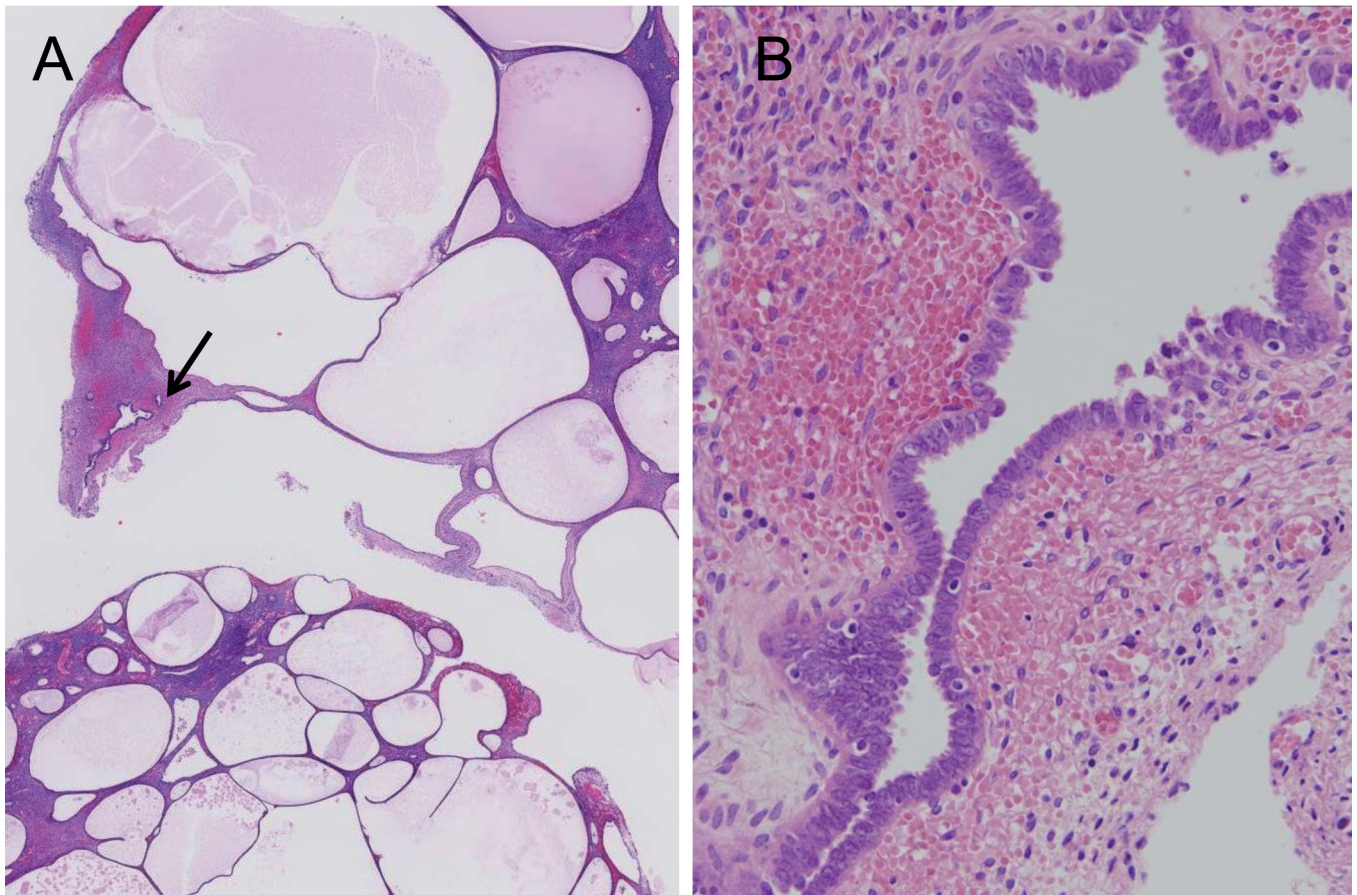

Fig. 2. The endometrial polyp of a 70-year-old woman with the p53 signature (**A**: arrow and **B**). A, x 2; B, x 20

that of EMPs during the secretory phase in the present study.

In both the normal endometrium and EMP, focal p53 positivity was found in the late proliferative phase, which disappeared in the late secretory phase (Maia et al., 2004). Shin et al. reported that p53 may play a dual role, including both the elimination of irreparably genetically damaged cells and the proliferative response necessary for their replacement, in response to physical-chemical damage (Shin et al., 1995). *In vitro* studies using breast cancer cells suggested the presence of a functional link between wild type p53 and the growth-promoting ER signaling pathways (Shirley et al., 2009). Among the premenopausal EMPs, the expression of p53 correlated with that of ER. These findings suggest that the correlation between p53 and ER plays a role in the proliferative activity of the endometrial glands in the cyclic change of EMP. Among the postmenopausal EMPs, the LI for Ki-67 significantly correlated with the LI for apoptosis, while it inversely correlated with that for p53 in the univariate analysis. The gland of the postmenopausal EMPs consistently showed the expression of ER-alpha.

The p53S was found in nine (12.7%) of 71 postmenopausal EMPs, but not in the premenopausal EMPs. The mean age was 70.2 years in women with EMPs associated with the p53S. Jarboe et al. detected the p53S without expression of DNA damage (γ-H2AX) in six (4%) of 137 benign EMPs (Jarboe et al., 2009). *TP53* mutations were found in two of four p53Ss examined by the sequence analyses for p53 exons in the present study.
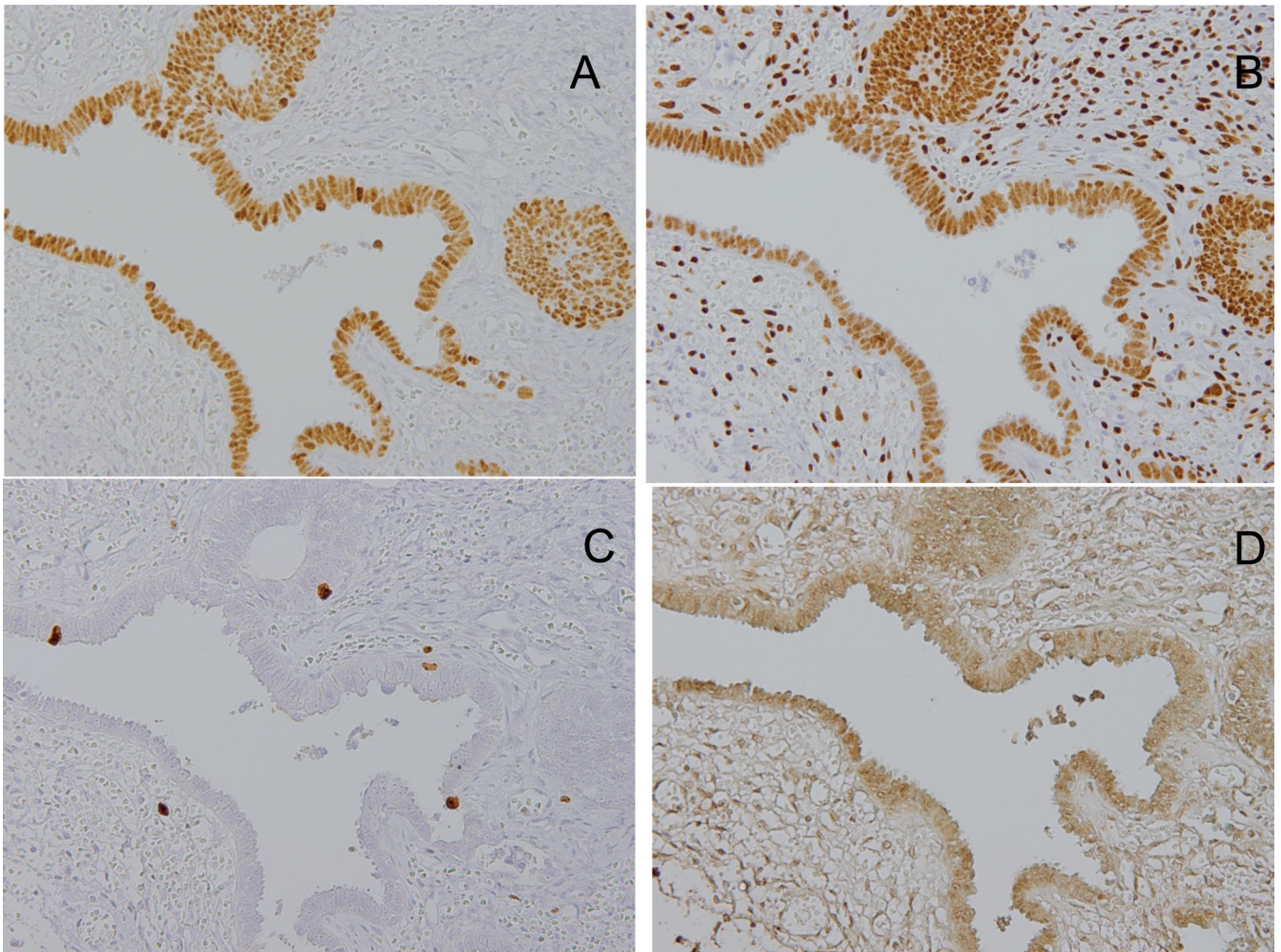

Fig. 3. The same case as in Figure 2 with positivity for p53 **(A)** and estrogen receptor-alpha **(B)**, a low Ki-67 labeling index **(C)**, and no apoptotic cells **(D)** in the p53 signature. x 20

USC typically occurs in elderly women, often arising against a background of an inactive or atrophic endometrium, with frequent *TP53* mutations and the overexpression of p53 protein (Ellenson et al., 2011). In a recently proposed model of USC carcinogenesis, the p53S is thought to be responsible for latent precancerous lesions of USC (Zheng et al., 2011). Zhang et al. speculated that the p53S may be operational, and a strategy to eradicate the latent precancerous lesion, such as the p53S, may offer an effective method for cancer prevention (Zhang et al., 2009). In our previous study, a focal glandular overexpression of p53 was found in eight (11%) of 72 postmenopausal endometrium tissue specimens (Koi et al., 2015). The authors suggested that overexpression of p53 may be responsible for the high proliferative activity of postmenopausal endometrial glandular cells associated with conditions of low apoptotic cell death. The incidence of glands with an overexpression of p53 in postmenopausal endometriums was not different from that of the p53S in the postmenopausal EMPs. The p53S was also associated with conditions of low apoptotic cell death. Furthermore, in a study regarding the presence of the p53S in the background endometrium of endometrial cancer (Nguyen et al., 2015), 12 (8%) of 152 cases of endometrioid adenocarcinomas were p53S positive. Of 21 cases of serous carcinoma, five (24%) were p53S positive. The p53S in the background endometrium of endometrial cancer also expressed ER-alpha.

Among the endometrial alterations reported in TAM users, polyps are the most common (Leao et al., 2013). TAM and TOR both produced a similar increase in the endometrial thickness of postmenopausal breast cancer patients (Hachisuga et al., 2005). Many studies have demonstrated that TAM use increases the risk of endometrial cancer and malignancy in EMPs (Hoogendoorn et al., 2008; Jones et al., 2012). The high endometrial cancer mortality of TAM users was associated with the excess of non-endometrioid histological subtypes and advanced tumor stages. These data suggest that postmenopausal EMPs in TAM or TOR users are more frequently associated with the p53S than that in non-users. However, no difference was observed in the incidence of the p53S between postmenopausal EMPs of TAM or TOR users compared with non-users.

In conclusion, the gland of the postmenopausal EMP showed a differential expression of p53 from that of the premenopausal EMP. The p53S was not rare in postmenopausal EMPs associated with an advanced age. Among postmenopausal EMPs, the LI of Ki-67 significantly correlated with that of apoptosis. However, such a positive correlation between the LI of Ki-67 and apoptosis was not observed in the p53S. Some cases of p53S showed *TP53* mutation, however, it is not proven that these *TP53* gene mutations have significance for carcinogenesis for USC. Further investigation is necessary to confirm that the p53S in EMPs is responsible for the imbalance between proliferation and apoptosis of endometrial epithelial cells with advanced age and/or a latent precancerous lesion of USC.
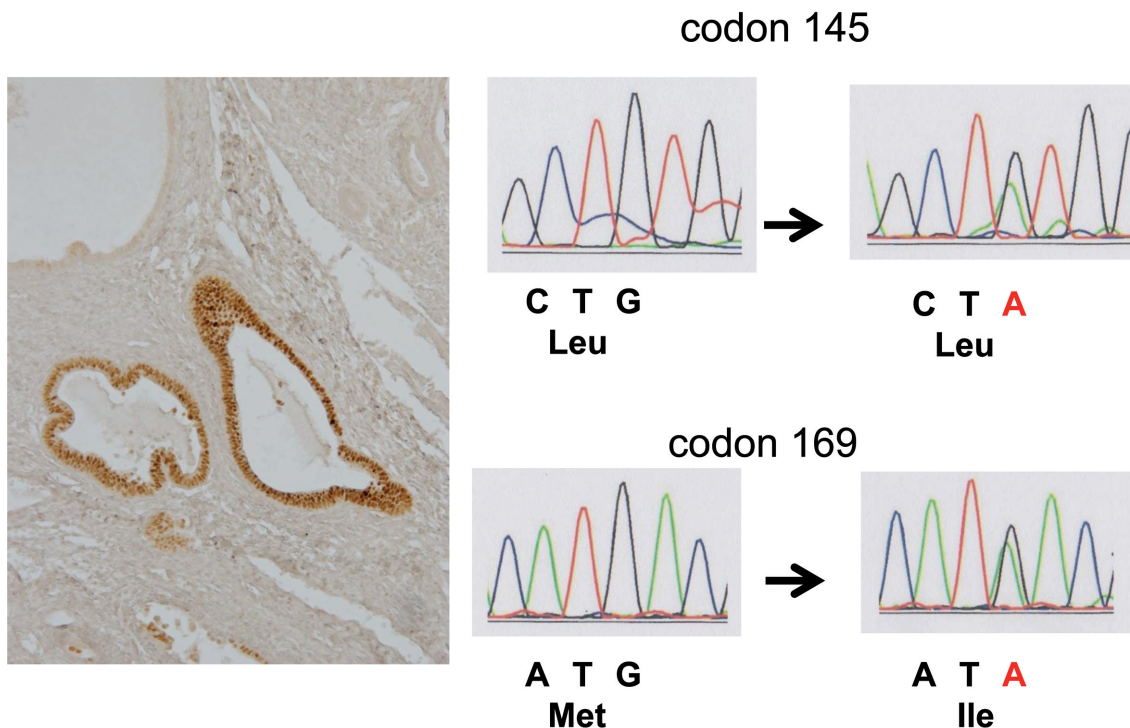

Fig. 4. TP53 gene sequencing result from a microdissection sample. Left, The p53 signature. Right, Corresponding DNA sequencing results showing TP53 mutations in exon 5 at codon 145 from CTG to CTA (Leu to Leu) and at codon 165 from ATG to ATA (Met to Ile).

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