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The role of p16-cyclin D/CDK-pRb pathway in the tumorigenesis of endometrioid-type endometrial carcinoma

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Summary We analysed p16 gene alteration and p16, cyclin-dependent kinase 4 (CDK4), CDK6, cyclin D1, cyclin D2, cyclin D3 and retinoblastoma protein (pRb) expression in ten normal endometriums (PE), 18 endometrial hyperplasias (EH) and 35 endometrial cancers (EC). Two of ten PE (20%), nine of 18 EH (50.0%) and 29 of 35 EC (82.9%) exhibited p16 nuclear staining. p16 expression was significantly higher in EC than EH (P = 0.0119). In the six p16 (–) EC, one was considered to have reduced gene dosage consistent with possible homozygous deletion of the *CDKN2* gene and three had methylation in 5′CpG island in the promoter region of the *p16* gene, whereas none showed such reduced gene dosage and four had methylation in the nine p16 (–) EH. Strong CDK4 staining was observed in 12 of 35 EC (34.3%) and one of 18 EH (5.6%). The strong expression of CDK4 was higher in EC than in EH (P = 0.0399). The expression of CDK4 was higher in EH than PE (P = 0.0054). The abnormalities of p16-cyclin D/CDK-pRb pathway were detected in 18 of 35 EC (51.4%). In conclusion, the expression of p16 and CDK4 may be an early event in the neoplastic transformation of endometrial cancer. © 2000 Cancer Research Campaign

Keywords: p16[INK4]; cyclin D; CDK; pRb; endometrial cancer

Recent genetic and biochemical investigations of the molecular mechanisms governing the G1 to S progression in mammalian cells have demonstrated a central role for D-type cyclins and their partner kinases cyclin dependent kinase (CDK4) and CDK6 (Kamb et al, 1995; Sherr et al, 1995; Strauss et al, 1995; Weinberg et al, 1995; Hall et al, 1996). When activated by cyclin D, CDK is able to phosphorylate retinoblastoma protein (pRb) leading to the release of associated proteins like E2F that have the capability to activate genes necessary for cell progression through the G1 phase (Weinberg et al, 1995). p16 controls cell cycle proliferation during G1 by inhibiting the ability of cyclin D/CDK4 and cyclin D/CDK6 complexes to phosphorylate pRb (Serrano et al, 1993). The components of the p16-cyclin D/CDK-pRb pathway are frequently found to be altered in various types of cancer (Cirns et al, 1995; Nakagawa et al, 1995; Kinoshita et al, 1996; Barbieri et al, 1997; Zhang et al, 1997).

In solid tumours, there is a reciprocal correlation between genetic alterations of single members of the p16-cyclin D/CDK-pRb pathway (He et al, 1994; Schauer et al, 1994; Bartkova et al, 1996). It has been reported that hypophosphorylated active pRb can repress p16 expression, whereas inactivation of pRb by phosphorylation leads to p16 expression (Li et al, 1994). Consistent with these findings, *CDKN2* gene deletion and Rb deficiency are reported to be inversely correlated in many types of tumours (Bartkova et al, 1996; Kinoshita et al, 1996). In addition, a strong association between altered cyclin D1 and pRb expression has been reported in oesophageal tumours (Jiang et al, 1993). Muller et al have demonstrated that the cell cycle-dependent expression of cyclin D1 in tumour cell lines requires the presence of a functional pRb (Muller et al, 1994). The expression levels and activities of these proteins can modulate each other. It is thought to be important to evaluate these four elements simultaneously.

Endometrial carcinoma is the most common malignant neoplasm of the female genital tract; however, its molecular pathogenetic events are not fully understood. There are few reports about the abnormalities of p16-cyclin D/CDK-pRb pathway in endometrial cancer (Peiffer et al, 1995; Niemann et al, 1997; Shiozawa et al, 1997). Additionally, to our knowledge, there are no reports in which p16, cyclin D, CDK and pRb were examined simultaneously in endometrial cancer. In this study, we have examined all these key components of this G1 checkpoint mechanism by a combination of genetic and immunohistochemical approaches in normal endometrium (PE), endometrial hyperplasia (EH) and endometrial cancer (EC).

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MATERIALS AND METHODS

Clinical samples

Paraffin-embedded tissue of 35 endometrioid-type endometrial cancers: (EC) (stage 1: 23; stage 2: five; stage 3: six; stage 4: none; recurrence: one) and 18 endometrial hyperplasia: (EH) (simple hyperplasia: 12, atypical hyperplasia: six) were collected at Osaka City General Hospital, Osaka, Japan. For control specimens, normal endometrial tissue (proliferative phase endometrium, PE) was selected from ten patients who had benign uterine leiomyoma. Histological diagnosis was confirmed by microscopic examination of the haematoxylin and eosin (H&E)-stained sections according

to World Health Organization criteria. Clinical stages were determined according to the International Federation of Gynecology and Obstetrics (FIGO) system. Regions of more than 80% tumour density were marked on H&E-stained slides to be used as guidelines for microdissection. Two 5-μm sections were cut from the paraffin block and the neoplastic tissue was microdissected away from contaminating normal tissue. The DNA was extracted using a DEXPAT™ kit (TaKaRa, Japan) and was finally precipitated in cold ethanol with sodium acetate and resuspended in Tris–EDTA buffer, pH 7.5.

PCR-SSCP analysis

Polymerase chain reaction (PCR) was performed using 100 ng of genomic DNA as template in a 50 μl reaction volume containing 10 pmol of each rhodamine-labelled oligonucleotide primer, 5 μl of 10 × *Taq* DNA polymerase buffer (Perkin-Elmer, Cetus, CT, USA), 200 μM each dNTP (Perkin-Elmer, Cetus), 5% (v/v) dimethyl sulphoxide (DMSO) and 1.25 units *Taq* Gold DNA polymerase (Perkin-Elmer, Cetus). The primers used to amplify the regions of the *CDKN2* genes are the following (Hussussian et al, 1994): for exon 1, 5′-GGGAGCAGCATGGAGCCG-3′ (X1.31F)/5′-CTGGATCGG-CCTCCGACCGT-3′ (MK50) and 5′-AGCAGCATGGATCC GG-CGGCGG-3′ (MK49)/5′-AGTCGCCCGCCATCCCCT-3′ (X1.26R); for exon 2, 5′-AGCTTCCTTCCGTCATGC-3′ (X2.62F)/5′-GCAGCACCACAGCGTG3′ (286R), 5′-AGCCCAACT-GCGC-CGAC-3′ (200F)/5′-CCAGGTCCACGGGCAGA-3′ (346R) and 5′-TGGACGTGCGCGATGC-3′ (305F)/5′-GGAAGCTCTCAGG-GGTACAAATTC-3′ (X2.42R). After a 10 min initial denaturation step at 94°C, 40 cycles of 30 s at 94°C, 30 s at an annealing temperature (55–60°C), and 1 min at 72°C were performed in a thermal cycler (Perkin-Elmer, Cetus).

For single-strand conformational polymorphism (SSCP) analysis, 1 μl of each PCR product was mixed with 9 μl of SSCP loading buffer [98% (v/v) formamide, 10 mM EDTA] and incubated at 80°C for 5 min, followed by rapid cooling on ice. Two microlitres of the sample solution were loaded on a 6% acrylamide gel containing 1 × TBE (86 mM Tris–borate, 2 mM EDTA) and 5% glycerol. Following electrophoresis at 30 W at room temperature for 2–3 h, the gel was analysed using an FMBIO 100 fluorescent image analyser (TaKaRa, Japan). The presence of bands with variant migration pattern was confirmed by repeating PCR-SSCP at least once prior to extraction of the band for DNA sequence analysis.

DNA sequencing

PCR products that revealed mobility shifts on SSCP analysis were cut from the gels and recovered. Re-amplification by PCR was followed by subcloning into pAMP1 vector (Gibco-BRL, MD, USA) and sequencing using M13/pUC primer. The sequence analysis for each product was performed with an ABI 310 genetic analyser (Perkin-Elmer, Cetus) and the Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin-Elmer, Cetus).

Multiplex PCR of *CDKN2* gene

The *CDKN2* gene homozygous deletion was investigated by PCR for the ability to amplify a region of the gene compared with the ability to amplify, as an internal control, the human β-globin gene. The sequence of the primers used for multiplex PCR were *CDKN2* exon 2 (Marchini et al, 1997), sense 5′-TCTGACCATTCT-GTTCTCTC and antisense 5′-AGCACCACCAGCGTGTC; β-globin, sense 5′-CAACTTCATCCACGTTCACC and antisense 5′-GGTTGGCCAATCTACTCCCAGG. This p16 primer pair amplifies on the 5′ half of exon 2, where the majority of p16 mutations in human cancer cells have been described (Maestro et al, 1995). These primers amplify fragments of 166 bp and 205 bp respectively. One hundred nanograms of genomic DNA was amplified in a final volume of 50 μl containing 10 pmol of each oligonucleotide primer, 5 μl of 10 × *Taq* DNA polymerase buffer (Perkin-Elmer, Cetus), 200 μM each dNTP and 3.75 units *Taq* Gold DNA polymerase (Perkin-Elmer, Cetus). The cycles of amplification were as described previously (Kamb et al, 1994) and reduced to 30 cycles in the β-globin co-amplification to maintain the amplification in the exponential phase (Campbell et al, 1995). Samples were loaded on 4% MetaPhor agarose gel and visualized by ethidium bromide staining. The signal intensity was analysed using an FMBIO 100 fluorescent image analyser (TaKaRa, Japan). The *CDKN2* signal intensity was normalized against the β-globin signal intensity. We considered a tumour to have reduced gene dosage consistent with possible homozygous deletion of *CDKN2* gene in the tumour cells when the normalized *CDKN2* signal intensity in the tumour sample was reduced to less than 80% of that in the normal uterine control. In order to ascertain *CDKN2/p16* homozygous deletion, each experiment was repeated two times.

Analysis of methylation in 5′CpG island in the promoter region of the *p16* gene

The methylation status of the 5′CpG island in the promoter region of the *p16* gene was determined with the CpGWiz™ p16 Methylation Kit (Oncor, Inc.). Briefly, 0.5–1.0 μg of DNA was denatured with 3 M sodium hydroxide at 50°C for 10 min and treated with sodium bisulphite following the manufacturer's protocol. After completion of the DNA modification, the DNA was purified by precipitation. The dissolved DNA was amplified by PCR, utilizing primers specific for the methylated (M) or unmethylated (U) sequences. A 5-μl aliquot of template (corresponding to treated DNA, positive control for methylated DNA, positive control for unmethylated sequences, and distilled water as negative control) was amplified in the presence of 10 × Universal PCR buffer, 2.5 mM dNTP mix, U or M primers, and AmpliTaq Gold™ (Perkin-Elmer), under the following conditions: preheating (95°C, 12 min), followed by 35 cycles (95°C, 45 s; 65°C, 45 s; 72°C, 1 min). The PCR product was analysed on a 2% agarose gel. The presence of DNA methylation was determined by the identification of one 145 bp fragment in those samples amplified with the M primers. All the cases were evaluated for the presence of an unmethylated specific fragment (154 bp), which served as an internal control for the quality of the treated DNA.

Immunohistochemistry of p16, CDK4, CDK6, cyclin D1, cyclin D2, cyclin D3 and pRb proteins

Histological sections (4 μm) were affixed to glass slides, dewaxed and rehydrated. Autoclave unmasking process (10 min at 121°C in 10 mM citrate buffer, pH 6.0) was used. The sections were then incubated in 3% hydrogen peroxide for 10 min at room tempera-

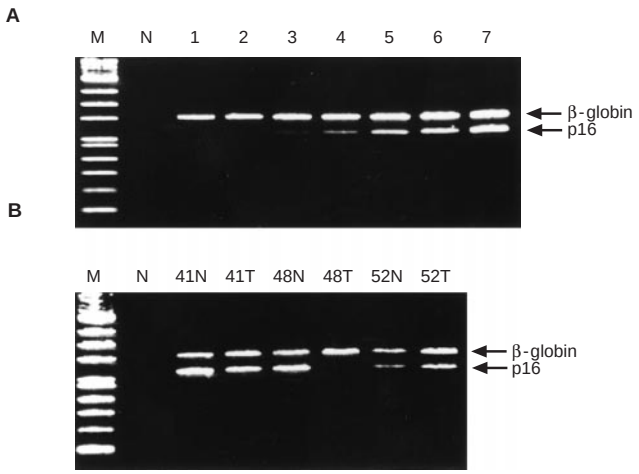

Figure 1 (**A**) The comparative multiplex PCR assay using serial mixtures of normal human DNA and DNA from a central nervous system cell line (SF295) with homozygous deletion of *CDKN2/p16* locus. M: size marker; N: negative control; lane 1: SF295 DNA:normal DNA = 10:0; lane 2: 9.5:0.5; lane 3: 9:1; lane 4: 8:2; lane 5: 7:3; lane 6: 6:4; lane 7: 0:10. Reduction of *CDKN2/p16* amplification product was detected when normal DNA constituted less than 20% of the total DNA. (**B**) Multiplex PCR of *CDKN2/p16* locus in cases 41, 48 and 52. *CDKN2/p16* homozygous deletion was observed in case 48

ture to quench endogeneous peroxidase activity. The sections were reacted with one of the following primary antibodies at 4°C overnight: (a) rabbit anti-p16 polyclonal antibody (Pharmingen, San Diego, CA, USA), (b) mouse anti-pRb monoclonal antibody (Pharmingen), (c) rabbit anti-CDK4 polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), (d) rabbit anti-CDK6 polyclonal antibody (Santa Cruz Biotechnology, Inc.), (e) mouse anti-cyclin D1 monoclonal antibody (Medical and Biological Laboratories Co., Nagoya, Japan), (f) rabbit anti-cyclin D2 polyclonal antibody (Santa Cruz Biotechnology, Inc.), (g) rabbit anti-cyclin D3 polyclonal antibody (Santa Cruz Biotechnology, Inc.), or nonimmunized rabbit serum. After rinsing, the sections were incubated for 30 min with mouse or rabbit EnVision + Peroxidase (Dako, CA, USA). The peroxidase activity for p16, pRb, CDK4, CDK6, cyclin D1, cyclin D2 and cyclin D3 was visualized by applying diaminobenzidine chromogen containing 0.05% hydrogen peroxide for 2–10 min at room temperature. The sections were then counterstained with haematoxylin. Positive and negative control experiments were performed for each tumour staining.

Interpretation of immunohistochemical staining

Evaluation of p16, pRb, CDK4, CDK6, cyclin D1, cyclin D2 and cyclin D3 positives was performed with semiquantitative analyses. The following scale was used: 0 = no immunoreactive tumour cells detectable or less than 5% of the tumour cells were positive with a weak intensity; 1 = 5–50% of the tumour cells were positive with a strong intensity; 2 = more than 50% of the tumour cells were positive with a strong intensity. Tumours were scored as p16-negative if less than 5% malignant cells had positive nuclear staining, and surrounding normal stromal cells showed adequate nuclear staining as a positive internal control. Tumours were regarded as p16-positive if more than 5% malignant cells had nuclear staining.

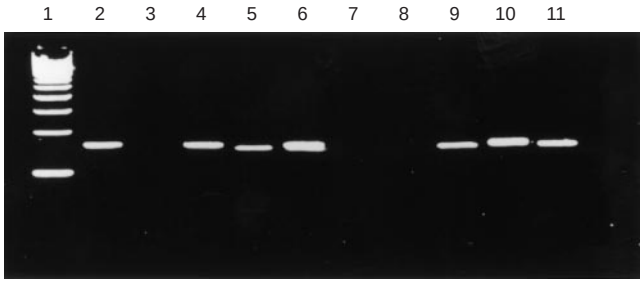

Figure 2 Analysis of methylation in 5′CpG island in the promoter region of the *p16* gene. Primer sets used for amplification are designed as unmethylated (U) or methylated (M). Lane 1: size marker; lanes 2, 4, 6 and 8: PCR products with U primer in cases 2, 14, 24 and 37, respectively; lanes 3, 5, 7 and 9: PCR products with M primer in cases 2, 14, 24 and 37, respectively; lane 10: unmethylated DNA with U primer; lane 11: methylated DNA with M primer

Small lymphocytes, which showed no nuclear staining of p16, were used as a negative internal control (Kratzke et al, 1996). These criteria were used for evaluation of pRb staining. Negative nuclei staining (0) was considered as abnormal expression in p16 and pRb. Strong nuclear staining (2) was considered as abnormal expression of CDK4, CDK6, cyclin D1, cyclin D2 and cyclin D3 (Michalides et al, 1995).

Statistical analysis

The abnormalities of p16, pRb, CDK4, CDK6, cyclin D1, cyclin D2 and cyclin D3 were analysed using Fisher's exact probability test to compare the different clinicopathologic groups with each other.

RESULTS

PCR-SSCP analysis of the *CDKN2* genes

SSCP analyses were performed for two *CDKN2* coding exons. One SSCP variant pattern was detected in assay for exon 1B in case 27. DNA sequence analysis of clones of this abnormally migrating SSCP fragment revealed a point mutation in intron 2. No mutations of either exons 1 or 2 were found in any samples (data not shown).

Detection of homozygous deletion of the *CDKN2* genes

The comparative multiplex PCR assay was titrated using serial mixtures of normal human DNA and DNA from a CNS cell line (SF295) with homozygous deletion of *CDKN2*/p16 locus. Reduction of *CDKN2/p16* amplification product was indicated when normal DNA constituted less than 20% of the total DNA (Figure 1A). In case 48, *CDKN2/p16* homozygous deletion was observed (Figure 1B).

Analysis of methylation in 5′CpG island in the promoter region of the *p16* gene

Forty-two samples DNA were available for p16 methylation analysis. Fifteen of 42 samples (35.7%) had methylation in 5′CpG island in the promoter region of the *p16* gene. Five of 15 EH (33.3%) and ten of 27 EC (37.0%) had methylation (Figure 2). Of

Table 1 Endometrial hyperplasia

Case	Age	Histology	SSCP	Methylation	HD	p16	CDK4	CDK6	CCND1	CCND2	CCND3	pRb
1	78	SH	N	M	N	1	0	0	0	0	0	1
2	46	SH	N	U	N	1	1	0	0	0	0	1
3	66	SH	N	U	N	1	1	0	0	0	0	1
4	45	SH	N	U	N	1	1	0	0	0	0	1
5	49	SH	N	ND	N	1	1	1	0	0	0	1
6	46	SH	N	U	N	1	1	0	0	0	0	1
7	48	SH	N	ND	N	1	2	0	0	0	0	1
8	47	SH	N	M	N	0	0	0	0	0	0	1
9	52	SH	N	M	N	0	0	0	0	0	0	1
10	54	SH	N	U	N	0	1	0	0	0	0	1
11	70	SH	N	ND	N	0	1	0	0	0	0	2
12	51	SH	N	U	N	0	1	0	0	0	0	1
13	82	AH	N	U	N	1	1	0	0	0	0	1
14	83	AH	N	M	N	0	1	1	0	0	0	1
15	22	AH	N	U	N	1	1	0	0	0	0	1
16	52	AH	N	U	N	0	1	0	0	0	0	1
17	50	AH	N	M	N	0	1	0	0	0	0	2
18	74	AH	N	U	N	0	0	0	0	0	0	1

AH: atypical hyperplasia; SH: simple hyperplasia; N: normal; ND: not done.

Table 2 Endometrial cancer

Case	Age	Stage	Grade	SSCP	Methylation	HD	p16	CDK4	CDK6	CCND1	CCND2	CCND3	pRb
19	59	1a	1	N	U	N	1	0	0	0	0	0	2
20	39	1a	1	N	U	N	1	2	0	0	0	0	1
21	53	1a	2	N	M	N	1	1	0	0	0	0	1
22	55	1a	2	N	ND	N	1	2	1	1	0	0	2
23	67	1a	2	N	M	N	2	1	0	0	0	0	1
24	67	1b	1	N	U	N	1	1	0	0	0	0	1
25	51	1b	1	N	M	N	1	0	0	0	0	0	2
26	73	1b	1	N	U	N	1	1	0	0	0	0	1
27	60	1b	1	A	U	N	1	0	0	0	0	0	0
28	56	1b	1	N	U	N	1	2	0	0	0	0	2
29	54	1b	1	N	U	N	1	1	0	0	0	0	1
30	44	1b	1	N	ND	N	1	2	0	0	0	0	2
31	59	1b	2	N	ND	N	2	0	0	0	0	0	1
32	58	1b	2	N	M	N	0	1	0	1	0	0	1
33	52	1b	2	N	U	N	1	2	1	0	0	0	1
34	77	1b	2	N	U	N	1	1	0	1	0	0	1
35	65	1b	2	N	U	N	1	1	0	0	0	0	1
36	55	1b	2	N	U	N	1	0	0	0	0	0	2
37	54	1b	3	N	M	N	0	1	0	0	0	0	1
38	62	1b	3	N	ND	N	2	2	0	0	0	0	0
39	42	1c	1	N	M	N	1	1	0	1	0	0	1
40	54	1c	2	N	U	N	1	0	0	1	0	0	1
41	52	1c	2	N	M	N	0	0	0	1	0	0	0
42	45	2a	1	N	ND	N	1	2	0	1	0	0	1
43	48	2a	1	N	M	N	1	1	0	1	0	0	1
44	52	2a	2	N	ND	N	1	2	0	1	0	0	1
45	68	2b	1	N	M	N	1	1	0	0	0	0	2
46	80	2b	3	N	ND	N	1	2	0	1	0	0	1
47	37	3a	1	N	U	N	1	2	0	0	0	0	0
48	72	3a	1	–	ND	HD	0	0	2	0	1	0	1
49	60	3a	3	N	U	N	1	1	0	1	0	0	1
50	53	3c	1	N	U	N	1	1	0	0	0	0	1
51	60	3c	1	N	U	N	0	1	0	0	0	0	1
52	56	3c	3	N	U	N	0	2	1	0	0	0	1
53	57	RE	2	N	M	N	1	2	0	0	0	0	1

N: normal; A: abnormal; U: unmethylated; M: methylated; ND: not done; HD: homozygous deletion

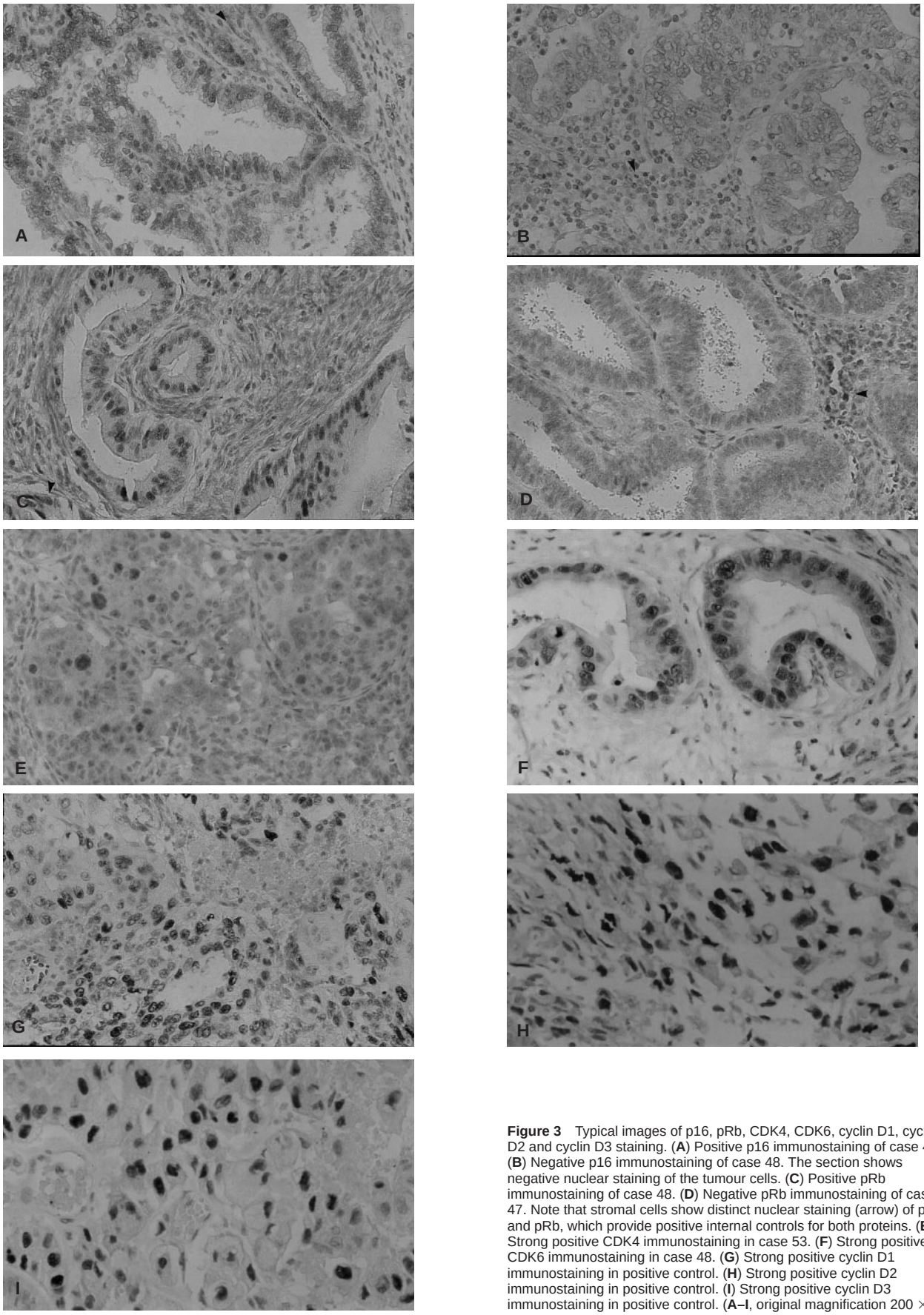

Figure 3 Typical images of p16, pRb, CDK4, CDK6, cyclin D1, cyclin D2 and cyclin D3 staining. (**A**) Positive p16 immunostaining of case 47. (**B**) Negative p16 immunostaining of case 48. The section shows negative nuclear staining of the tumour cells. (**C**) Positive pRb immunostaining of case 48. (**D**) Negative pRb immunostaining of case 47. Note that stromal cells show distinct nuclear staining (arrow) of p16 and pRb, which provide positive internal controls for both proteins. (**E**) Strong positive CDK4 immunostaining in case 53. (**F**) Strong positive CDK6 immunostaining in case 48. (**G**) Strong positive cyclin D1 immunostaining in positive control. (**H**) Strong positive cyclin D2 immunostaining in positive control. (**I**) Strong positive cyclin D3 immunostaining in positive control. (**A–I**, original magnification 200 ×)

Table 3 Correlation of expression of p16, CDK4, CDK6, CCND1, CCND2, CCND3 and pRb and clinicopathologic features

Clinicopathologic features	NO	p16	pRb	CDK4		CDK6	CCND1	CCND2	CCND3	Pathway
				1+ or 2+	2+	1+ or 2+	1+	1+	1+	
Proliferative	10	2 (20.0%)	10 (100%)	2 (20.0%)**	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	–
Hyperplasia	18	9 (50.0%)*	18 (100%)	14 (77.8%)**	1 (5.6%)***	2 (11.1%)	0 (0%)****	0 (0%)	0 (0%)	–
Cancer	35	29 (82.9%)*	31 (88.6%)	27 (77.1%)	12 (34.3%)***	4 (11.4%)	10 (28.6%)****	1 (2.9%)	0 (0%)	18 (51.4%)
Clinical stage										
I	23	20 (87.0%)	20 (87.0%)	16 (69.6%)	6 (26.1%)	2 (8.7%)	5 (21.7%)	0 (0%)	0 (0%)	10 (43.5%)
II III RE	12	9 (75.0%)	11 (91.7%)	11 (91.7%)	6 (50.0%)	2 (16.7%)	5 (41.7%)	1 (8.3%)	0 (0%)	8 (66.7%)
Histologic grade										
G1	17	15 (88.2%)	15 (88.2%)	13 (76.4%)	5 (29.4%)	1 (5.9%)	3 (17.6%)	1 (5.9%)	0 (0%)	8 (47.1%)
G2	13	11 (84.6%)	12 (92.3%)	9 (69.2%)	4 (30.8%)	2 (15.4%)	5 (38.5%)	0 (0%)	0 (0%)	6 (46.2%)
G3	5	3 (60.0%)	4 (80.0%)	5 (100%)	3 (60.0%)	1 (20.0%)	2 (40.0%)	0 (0%)	0 (0%)	4 (80.0%)

Pathway: p16-cyclin D/CDK-pRb pathway. *P = 0.0119, **P = 0.0054, ***P = 0.039, ****P = 0.0118.

nine p16 (–) EH and six p16 (–) EC, four (44.4%) and three (50.0%) had methylation respectively. Of five methylated EH, four (80%) cases had no expression of p16 and of ten methylated EC, three (30%) cases had no expression of p16.

Immunohistochemistry of p16, pRb, CDK4, CDK6, cyclin D1, cyclin D2 and cyclin D3 proteins

The results of immunohistochemical analysis are shown in Tables 1 and 2. Two of ten PE (20%), nine of 18 EH (50.0%) and 29 of 35 EC (82.9%) had p16 nuclear staining. In the six p16 (–) EC, one was considered to have reduced gene dosage consistent with possible homozygous deletion of the *CDKN2* gene and three had methylation in 5′CpG island in the promoter region of the *p16* gene, wheres none showed such reduced gene dosage and four had methylation in the nine p16 (–) EH. p16 expression was significantly higher in EC than EH (P = 0.0119). pRb was detectable in all ten of ten PE, all 18 of 18 EH and 31 of 35 EC; pRb was not detectable in four of 35 EC (11.4%). The EC cases with loss of either p16 or pRb expression are 1bG2, 1bG3, 1cG2, 3aG1, 3cG1, 3cG3, 1bG1, 1bG3, and 3aG1. Typical images of p16 and pRb staining are shown in Figure 3 A–D.

In total, two of ten PE (20%), 14 of 18 EH (77.8%) and 27 of 35 EC (77.1%) exhibited positive nuclear staining with CDK4 antibody. Strong CDK4 staining was observed in 12 of 35 EC (34.3%) and one of 18 EH (5.6%). The strong expression of CDK4 was higher in EC than in EH (P = 0.0399). The weak and strong expression of CDK4 was higher in EH than PE (P = 0.0054). The strong expression of CDK4 was higher in cases with grade 3 tumours (60.0%) than with grade 1 tumours (29.4%), but this difference was not significant. Ten cases of EC exhibited weak positive nuclear staining of cyclin D1 and its expression was higher in EC than EH (P = 0.0118). In contrast, the expression of CDK6, cyclin D2 and cyclin D3 was rare in EC. Representative pictures of CDK4, CDK6, cyclin D1, cyclin D2 and cyclin D3 staining are shown in Figure 3 E–I. The abnormalities of p16-cyclin D/CDK-pRb pathway were detected in 18 of 35 EC (51.4%). Correlation of expression of p16, pRb, CDK4, CDK6, cyclin D1, cyclin D2 and cyclin D3 and clinicopathologic features are shown in Table 3.

DISCUSSION

It was reported that the alterations of *CDKN2* were uncommon and late events in endometrial cancers (Peiffer et al, 1995; Milde-Langosch et al, 1999). In contrast, Shiozawa et al (1997) reported that in endometrial cancer, 65.8% of examined cases were negative for p16 protein. In our study, the rate of absence of p16 expression in EC was 17.1%. Based on our study, p16 alterations are thought to be rare in endometrial cancer. In our studies cases, the rate of absence of pRb expression in EC was low (11.4%). It was reported that alterations of pRb were rare events (Niemann et al, 1997; Milde-Langosch et al, 1999). In particular, Yaginuma et al (1996) demonstrated that Rb gene abnormality was a rare event in endometrial cancer. Geradts et al (1996) reported that studies on the expression of Rb in neoplasms will include an unknown, but probably small, number of positive stains despite the lack of functional pRb. The pRb alterations are thought to be rare in endometrial cancer. Further examination will be required to solve this problem.

There were no cases exhibiting overexpression of cyclin D1 in this study. Cyclin D1 was reported to be overexpressed in 30–50% of invasive primary breast cancers and to play a key role in mediating mitogenic response to steroids and growth factor in breast cancer cells in vitro (Buckley et al, 1993; Dutta et al, 1995; McIntosh et al, 1995). In addition, it was reported that the abnormal expression of tumour suppressor genes such as p16 or pRb was present in 40% of breast cancer (Cairns et al, 1995; Herman et al, 1995; Pietilainen et al, 1995). Both breast cancer and endometrial cancer are sex steroid-dependent tumours. Studies in mice have demonstrated that cyclin D1 plays a pivotal role in normal mammary gland development. Cyclin D1 knockout mice fail to undergo the lobular proliferative changes normally seen in the breast epithelial compartment under the influence of steroid hormones in pregnancy (Sicinski et al, 1995), whereas overexpression of cyclin D1 in the mammary gland of transgenic animals results in premature lobulo-alveolar development, abnormal epithelial proliferation in pregnancy, and the late development of adenocarcinoma (Wang et al, 1994). It was demonstrated that cyclin D1 and oestrogen receptor (ER) gene expression are positively correlated in primary breast cancer (Zuberberg et al, 1995; Hui et al, 1996) and that oestrogen stimulation increases cyclin D1 gene expression in an ER-positive cultured breast cancer cell line (Prall et al, 1997). The mechanism of development and carcinogenesis of endometrium may differ from that of mammary gland tissue.

In this study, expression of CDK4 significantly increased from PE to EC. However, it did not significantly increase with the

progression from stage 1 to 3. These data suggest that the overexpression of CDK4 protein is important in the early stages of endometrial oncogenesis.

The meaning of loss of p16 expression in EH remains unclear. The loss of p16 protein in EH may be precursors to tumorigenesis. Four of nine p16 (–) EH (44.4%) had methylation in 5′CpG island in the promoter region of the *p16* gene. The methylation of the 5′CpG island of *p16/CDKN2* is thought to be associated with inactivation of this tumour suppressor gene. However, the fact that the loss of p16 protein was significantly higher in EH than EC is contradictory. It is possible that in EH, the loss of p16 protein with methylation may differ from that without methylation. Another speculation is that the loss of p16 protein may be involved in normal cell function. The loss of p16 protein was significantly higher in EH than in EC and one of the six p16 (–) EC was considered to have reduced gene dosage consistent with possible homozygous deletion of the *CDKN2* gene, whereas none of the nine p16 (–) EH showed such reduced gene dosage. The fact that five of 15 EH (33.3%) had methylation in 5′CpG island in the promoter region of the *p16* gene may be contradictory; however, Gonzalez reported that *p16/CDKN2* was methylated and not expressed in the 50% of normal colon tissue (Gonzalez et al, 1995). It has been reported that overexpression of p16 might be an important early event in neoplastic transformation (Bartkova et al, 1996; Shiozawa et al, 1997; Shigemasa et al, 1997). Yao et al (1998) reported that primary soft tissue sarcoma overexpressing CDK4 also had high levels of pRb and/or p16, which may reflect the onset of feedback to counteract the overexpression of CDK4. It may be that the loss of p16 in EH is a reversible change of the feedback system between CDK4 and p16. Further examination will be required to determine if the transcriptional silencing of *p16/CDKN2* observed in EH is involved in normal cell function or is a precursor to tumorigenesis.

In conclusion, the expression of p16 and CDK4 may be an early event in the neoplastic transformation of endometrial cancer.

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