Up-regulation and nuclear localization of β-catenin in endometrial carcinoma in response to progesterone therapy

Makoto Saegusa,[1] Mieko Hamano,[2] Takeshi Kuwata,[1] Tsutomu Yoshida,[1] Miki Hashimura,[1] Fumiyuki Akino,[1] Jun Watanabe,[1] Hiroyuki Kuramoto[3] and Isao Okayasu[1]

[1]Department of Pathology and [2]Division of Cell and Tissue Culture, Kitasato University School of Medicine and [3]Department of Clinical Cytology, Kitasato University School of Allied Health Science, 1-15-1 Kitasato, Sagamihara, Kanagawa 228-8555

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Ovarian hormones are considered to be capable of regulating expression of β-catenins. A possible role of β-catenin in alteration of cell morphology has been proposed, but little is known about β-catenin expression during changes in the tumor morphology of endometrial carcinomas induced by progesterone therapy. To clarify changes in expression of β-catenin and their relation to morphological alteration, expression of hormone receptors and several cell kinetic markers, sequential biopsy and hysterectomy specimens of 23 endometrial carcinoma and 6 complex hyperplasia with atypia (atypical hyperplasia) cases receiving progesterone therapy were investigated. *In vitro* assay was also conducted using two endometrial carcinoma cell lines (HEC265 and Ishikawa) expressing progesterone receptors (PRs). An increase of nuclear β-catenin accumulation was evident during progesterone therapy in endometrial carcinomas and atypical hyperplasias. The nuclear labeling indices were significantly associated with gene mutations and alteration in morphological features in response to progesterone, independently of the status of Ki-67, $p21^{WAF1}$ and $p27^{Kip1}$, and hormone receptors. In HEC265 having a *β-catenin* gene mutation (A32V), cytoplasmic β-catenin levels were elevated by progesterone treatment, linked to down-regulation of PR expression, but such changes were relatively minor in Ishikawa without the gene alterations. These findings demonstrate a possible role of progesterone in regulation of β-catenin expression in endometrial tumors. Moreover, nuclear β-catenin accumulation, like gene abnormalities, is associated with the alteration of tumor morphology due to progesterone, indicating that β-catenin may be a clinically useful marker of hormone therapeutic effects. (Cancer Sci 2003; 94: 103–111)

The most successful approach to the treatment of endometrial carcinoma is hysterectomy with bilateral salpingo-oophorectomy, combined with cytotoxic chemotherapy and radiation therapy employed for advanced or recurrent tumors. Hormone therapy on the basis of the profound maturation-stimulating effects of progesterone is also applied for tumors in young patients to avoid the necessity of hysterectomy.[1)] Such therapy alone, however, is not considered to be sufficient to eradicate carcinomas from the endometrium, since alterations in tumor morphology through cell maturation or differentiation are induced, but not cell death.[2, 3)]

β-Catenin was originally identified as a major component of the cadherin adhesion system, binding to both E-cadherin cytoplasmic and α-catenin amino-terminal domains.[4)] The cytoplasmic level of β-catenin is tightly regulated through degradation via the ubiquitin-proteasome pathway, whereby serine and threonine residues in exon 3 are phosphorylated by glycogen synthetase kinase (GSK)-3β and ubiquitinylated by binding to proteins such as adenomatous polyposis coli (APC), and axin.[5)] Stabilization of cytoplasmic β-catenin due to up-regulation of wingless/wnt signalling or abnormalities of either APC or β-catenin proteins can lead to its interaction with nuclear transcription factors of the T cell factor-lymphoid enhancer factor (TCF/LEF) family, resulting in activation of target genes.[6–8)]

Recently, ovarian hormones have been reported to be capable of regulating several cell adhesion-related molecules, including E-cadherin, α- and β-catenins and cad-11.[9–11)] Moreover, changes in expression level and subcellular distribution of the β-catenin occur during morphological alterations through cell differentiation in several colorectal carcinoma cell lines.[12, 13)] Our previous findings indicated that β-catenin abnormalities may play an important role in relatively early events during the endometrial hyperplasia-carcinoma sequence.[14)] In the present study, to clarify possible associations between β-catenin and alteration of morphology in response to ovarian hormones, we investigated changes in endometrial carcinomas and complex hyperplasias with atypia during progesterone therapy. We also examined the status of hormone receptors and cell kinetic-related markers, including Ki-67, $p21^{WAF1}$ (p21) and $p27^{Kip1}$ (p27). In addition, an *in vitro* assay of the association between alteration in β-catenin and progesterone treatment was examined using endometrial carcinoma cell lines.

Materials and Methods

Cases. A total of 23 patients with endometrial carcinomas (endometrioid type, grade 1) who received progesterone therapy before hysterectomy, as well as 6 cases of complex hyperplasia with atypia (atypical hyperplasia), were selected based on their charts at Kitasato University Hospital from the period 1988 to 2000, according to the histological criteria of the World Health Organization histological classification (1994). These patients were diagnosed as clinical FIGO (International Federation of Gynecology and Obstetrics) stage I based on the findings of both physical and radiographic examinations, their mean age being 32.2±7.1 years (mean±SD). All patients received 400 to 600 mg daily of medroxyprogesterone acetate (MPA) via oral administration. Biopsy samples were taken before and during hormone therapy, and all contained sufficient carcinoma elements to allow examination of sequential morphological changes. A total of 37 samples of carcinomas and 13 of atypical hyperplasias taken before, 99 and 21 taken during and 12 and 5 taken after progesterone therapy were available. Of the 29 patients investigated, 15 finally underwent hysterectomy after hormone therapy due to a positive diagnosis of malignancy on histological and cytological evidence. None of the cases underwent chemotherapy or radiotherapy (Table 1). In addition, areas of squamous differentiation (SqD) within tumors were identified in 19 (82.6%) of 23 carcinomas and 5 (83.3%) of 6 atypical hyperplasias.

E-mail: msaegusa@med.kitasato-u.ac.jp

Table 1. Summary of data for endometrial carcinoma and atypical hyperplasia cases undergoing progesterone therapy

Case No.	Age (yrs)	MPA dosage (mg/day)	Before hormone therapy								During hormone therapy									Post hormone therapy								FIGO stage at operation	Total period of therapy
			Hist	β-Cat. ex. 3 mutation	Average LI values							β-Cat. ex. 3 mutation	Average LI values							Average TE	β-Cat. ex. 3 mutation	Average LI values								
					β-Cat.	ER	PR	Ki-67	p21	p27	*n*		β-Cat.	ER	PR	Ki-67	p21	p27	*n*			β-Cat.	ER	PR	Ki-67	p21	p27	*n*		
C1	30	600	G1	ACC 41 ATC	3.7	98.1	94.9	60.6	7.8	48.2	1		11.4	86.4	1.2	11.8	1.5	84	10	3.9	ACC 41 ATC* ACC 41 ATC*	2.9	97.4	63.9	35.6	2.8	71.3	3	II	4 yrs
C2	33	600	G1	GGA 34 GAA	16.4	82.8	66.1	15.3	1.8	63.6	3	GGA 34 GAA (36 w)	5.9	90.4	0	9.4	0.8	91.5	7	1.6									ND	36 w
C3	28	400	G1	GGA 34 AGA	6.4	95.7	54.1	17.5	1.5	51	5	GGA 34 AGA* Wild*	17.1	87.9	11.3	4.9	0.7	36.1	4	1									I	24 w
C4	41	400	G1	GAC 32 TAC	0.6	76.4	82	33.1	1.1	14.7	1	Wild (4 w)	12.8	88.4	0	5.3	3.4	81.8	5	4									ND	64 w
C6	30	600	G1	GAC 32 TAC	20.3	79.8	60	7.9	1.6	52.4	2	Wild (12 w) Wild (16 w)	11.3	88.7	72.6	5.8	0.7	74.1	2	1									ND	16 w
C7	28	600	G1	NE	16.5	97.8	98.3	27.7	3.3	51.7	2	NE	24.5	67.7	0	11.5	7.1	71.5	6	3.5									I	15 w
C8	37	400	G1	GAC 32 GCC	13.2	87.7	86.7	13.4	2.5	60.4	1	GAC 32 GCC* GAC 32 GCC*	23	88.6	43.2	5.5	1.8	77.1	4	2.5									II	12 w
C9	38	400	G1	Wild	0	96.1	76.8	46.5	5.6	73.7	2	Wild (24 w)	0	90	0	16.6	0.8	NE	1	1									I	24 w
C10	24	600	G1	Wild	8.2	90	89.9	35	32.7	39.3	1	Wild*	23.6	88.7	5.1	10.9	1.1	46.9	8	2.1									I	29 w
C11	35	400	G1	Wild	0	97.8	99	69.3	2.1	24.5	2	Wild*	0	92.5	22.1	41.7	18.4	51	3	1									I	12 w
C12	27	800	G1	NE	NE	NE	NE	NE	NE	NE	0	Wild (4 w) Wild (52 w)	5.1	90	2.9	2.7	0	86.7	2	1									I	60 w
C13	34	600	G1	Wild	0	99.2	99.3	4.2	0.9	48.9	1	Wild (18 w)	5.1	88.8	0	0.8	0.5	70	1	4									ND	18 w
C15	29	400	G1	GAC 32 TAC	9.7	87.5	75.5	11.5	0	72.2	3		26.9	NE	0	NE	NE	NE	1	3	GAC 32 TAC*	9.4	90	98.9	23.9	2.1	29.8	2	I	2 yrs
C16	34	600	G1	TCT 37 TGT	0	90	90.7	49.6	19.1	93.3	2	TCT 37 TGT (20 w)	1.8	96.9	0	41.9	10.6	95.6	3	1									ND	20 w
C17	25	600	G1	NE	NE	NE	NE	NE	NE	NE	0	TCT 37 TGT (4 w)	14.6	74.9	0	5.9	1.6	74.6	3	4									ND	20 w
C18	33	600	G1	NE	NE	NE	NE	NE	NE	NE	0	Wild (12 w)	8.8	89.9	0	3.8	11.3	59.7	1	4									ND	12 w
C20	26	600	G1	GAC 32 TAC	17	93.2	67.9	26.1	3.1	37.3	2	Wild (2 w) Wild (10 w)	22.1	85.8	32.6	12.9	1.4	47.6	3	2		0	96.1	98.7	28.9	NE	56.3	1	ND	62 w
C21	31	600	G1	GAC 32 TAC	10.4	97	99	29.6	1.9	60.7	2	Wild (36 w)	20.2	87.8	46.1	16.3	1.7	55.9	2	2		11.2	91.1	91.3	36.5	3.9	37.1	2	ND	2 yrs
C22	29	600	G1	GAC 32 AAC	4.2	95.4	90.1	23.9	11	77.5	3	GAC 32 AAC (6 w)	32.5	95.5	0	6.5	3.9	95.2	4	4	Wild*	9	87.2	64.7	31.3	2.1	46.9	2	I	40 w
C23	24	600	G1	Wild	0	93.6	86.6	54.7	5.9	32.4	1	Wild (48 w)	0	93.3	0	24.5	8.4	70.5	4	1.3	Wild*	0	72.3	20.1	45.2	19.1	16.8	1	I	60 w
C24	59	400	G1	NE	NE	NE	NE	NE	NE	NE	0	Wild (16 w)	0	70	0	3.1	0.6	85.5	3	3.7									I	16 w
C25	32	600	G1	ACC 41 ATC	0.9	98.7	98.7	31.7	2.9	10.6	1		28.7	97.2	7.5	9.7	2.8	73.2	7	3.4	ACC 41 ATC*	29.7	92.4	43.7	20.1	1.6	55.9	1	I	42 w
C28	30	600	G1	TCT 37 TGT	3	95.6	33.4	26.3	0.8	15.1	1	TCT 37 TGT* TCT 37 TGT*	2.2	98.2	0	4.9	1.6	76.2	6	4									II	37 w
AH5	35	400	AH	Wild	0	92.3	99	3.3	0	40	1	Wild (10 w)	23	77.2	0	NE	0	NE	2	4									ND	14 w
AH14	38	400	AH	ACC 41 ATC	11.1	95.4	88	29.9	0.6	63.4	3	ACC 41 ATC* ACC 41 ATC*	24	93.3	2.6	12.7	0.4	61.7	5	3									I	45 w
AH19	32	400	AH	NE	NE	NE	NE	NE	NE	NE	0		27	86.1	42.8	5.2	0.8	51.4	1	2	TCT 37 TTT	6.5	91.4	74.6	16.9	3.4	50.7	1	ND	48 w
AH26	22	400	AH	Wild	0.5	97.7	98.8	30.3	0.2	54.7	4		25.8	88.7	0	5	0	79.4	3	3	Wild	5.1	92.3	96.7	39.3	1.1	44.6	2	ND	30 w
AH27	40	400	AH	Wild	1.2	98.5	97.1	21.3	0.5	60.3	3	Wild (8 w)	8.4	92.3	0	2.9	0.2	87.4	3	4		0	97.5	97.7	35.7	2.1	10.3	2	ND	16 w
AH31	31	400	AH	Wild	0	98.6	98.2	16.2	2.1	47.7	2	Wild (4 w) Wild (8 w)	0	95.8	0	3.5	2.7	88.9	2	2									ND	16 w

β-Cat., β-catenin; Hist, histology; G, grade; ex. 3, exon 3; w, week; *n*, numbers; TE, therapeutic efficacy; LI, labeling index; NE, not examined; *, mutation analysis using DNAs obtained from two different parts within a hysterectomy sample.
Date the section taken during hormone therapy was also indicated in the results of mutation analysis.

Biopsy and hysterectomy specimens were routinely fixed in 10% formalin, embedded in paraffin wax and stained with hematoxylin and eosin. Histological diagnoses were performed according to the criteria of the FIGO classification.

Histopathological analysis of endometrial tumors during progesterone therapy. Evaluation of morphological changes that occurred during hormone therapy was performed in accordance with methods described previously.[3, 15] Briefly, the sections obtained from tumors were examined in terms of the following four parameters: 1) cellularity, 2) nuclear rearrangement, 3) eosinophilia in the cytoplasm, and 4) the nuclear/cytoplasmic ratio. Therapeutic efficacy (TE) was graded by counting the numbers of altered parameters. For example, when reduced cellularity, a change in nuclear rearrangement from multiple- to mono-integrated layers, and an increase of eosinophilia in the cytoplasm were found in more than 50% of the area in a biopsy specimen after hormone therapy, in comparison with before treatment, the TE value was defined as grade 3 (1+1+1). On the basis of the correlations among the TE grades, cases were subdivided into two categories, as follows: grade 3 or 4, good response; grade 0, 1, or 2, poor response.

Immunohistochemistry. Immunohistochemistry was performed using a combination of microwave-oven heating and standard streptavidin-biotin-peroxidase complex (LSAB kit, Dako, Copenhagen, Denmark) methods. Briefly, slides were heated in 10 m*M* citrate buffer (pH 6.0) for two 10-min cycles using a microwave oven and then incubated overnight at 4°C with an anti-β-catenin monoclonal antibody (×1000 or ×2000 dilution, Transduction Lab., Lexington, KY), anti-estrogen receptor (ER) mouse monoclonal antibody (×100 or ×200 dilution, Novocastra Lab., Ltd., Newcastle, UK), anti-progesterone receptor (PR) mouse monoclonal antibody (×100 or ×200 dilution, Novocastra Lab., Ltd.), anti-human Ki-67 antigen rabbit polyclonal antibody (×300 dilution, Dako), anti-WAF 1 mouse monoclonal antibody for p21 protein (×300 dilution, CALBIOCHEM, Cambridge, MA), or anti-Kip 1 mouse monoclonal antibody for p27 protein (×1000 dilution, Transduction Lab.).

As a positive control, membrane β-catenin staining of capillary endothelial cells within tumors was used. For p21, a uterine cervical adenocarcinoma known to express p21 was employed.[16] For the two hormone receptors and p27, nuclei of stromal cells were also applied as internal controls. To confirm the specificity of binding, normal mouse or rabbit serum (×100 dilution) was supplied instead of primary antibody as a negative control.

Labeling indices (LIs) for nuclear β-catenin immunoreactivity were calculated after examining signals in at least 500 cells (average values, 934 nuclei, range 510–2003) in three to five randomly selected fields, using high-power (×40 objective and ×10 ocular) magnification. A similar approach was also applied for Ki-67, ER, PR, p21 and p27.

Extraction of DNA and RNA and sequencing of β-catenin. DNA was extracted and purified from 2 or 3 tumor biopsy or hysterectomy samples before or after hormone therapy, using proteinase K/phenol-chloroform methods. With five cases (C1, 3, 8, and 28 and AH14 in Table 1), extraction of DNA from two different blocks within hysterectomy samples was also performed. Six serial 4 μm thick paraffin wax sections were cut from each case and reviewed microscopically, and then lesions containing at least 80% tumor cells were microdissected.

Total cellular RNA and genomic DNA from two endometrial carcinoma cell lines, HEC265 and Ishikawa, were also ex-

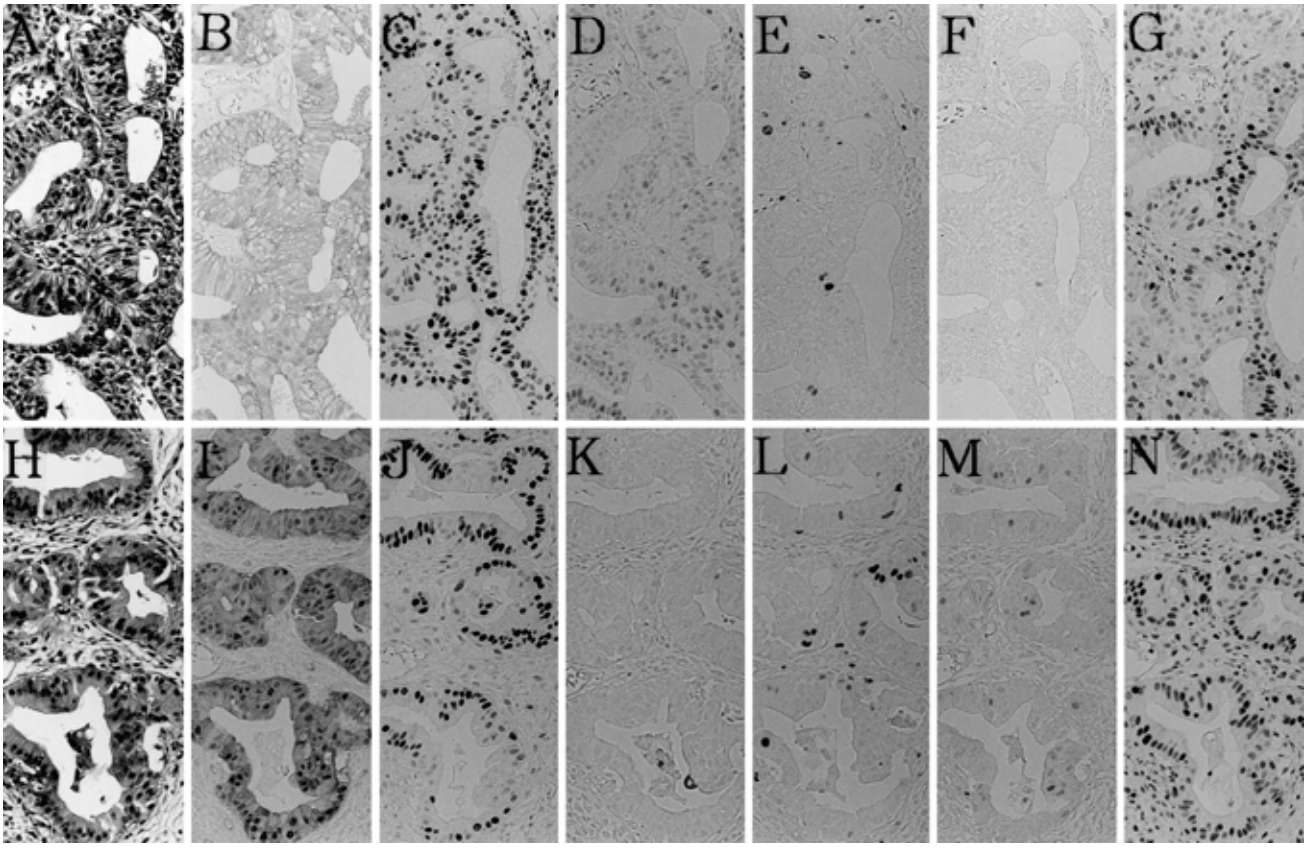

Fig. 1. Semi-serial sections of the endometrial carcinoma in case 22. A–G) Initial biopsy specimen. H–N) Biopsy taken after 10 weeks of progesterone treatment. A,H) H&E staining. Immunohistochemistry for β-catenin (B,I), ER (C,J), PR (D,K), Ki-67 (E,L), p21 (F,M) and p27 (G,N). Note the increased nuclear β-catenin accumulation (B vs. I) and disappearance of PR (D vs. K) immunoreactivity during progesterone therapy, in line with the alteration in tumor morphology (A vs. H, TE score=3). Original magnification, ×150.

tracted using Isogen (Nippon Gene Co., Tokyo). cDNAs were synthesized from 5 μg of total RNA using RAV-2 reverse transcriptase (TaKaRa, Shiga).

Sequence analysis of exon 3 of the *β-catenin* gene was performed using PCR or RT-PCR products, as described previously.[14, 17] Clinical samples were defined as positive for *β-catenin* gene abnormality when a mutation was detected in at least one sample investigated in each case.

Cell lines and culture conditions. Of 12 endometrial carcinoma cell lines, HEC 265 and Ishikawa cells were selected, since these cells are known to express PR.[18, 19] Cells were maintained in Eagle's MEM with 10% fetal bovine serum (FBS), then in phenol red-free Bio-Rich (1:1 mixture of Dulbecco's MEM and Ham's F12) containing 10% dextran-charcoal stripped FBS. Forty-eight hours later, cells were treated with 0.5, 1, and 10 μM MPA (Pharmacia UpJohn, Tokyo) or the vehicle (0.1% ethanol) for 0, 24 or 48 h.

Western blot assay. Cytoplasmic and membrane/nuclear fractions were prepared according to a protocol described previously.[20] Briefly, cells were scraped off the plates and lysed with a hypotonic lysis buffer [1 mM $NaHCO_3$ and 0.2 mM phenylmethylsulfonyl fluoride (PMSF)] for 30 min on ice. The lysate was passed through a 20-gauge needle (10 times), then centrifuged (15 000g). The supernatants and the pellets were collected as the cytoplasmic and membrane/nuclear fractions. Total cellular proteins were also isolated using RIPA buffer [50 mM Tris/HCl (pH 7.2), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) and 0.2 mM PMSF].

Antibodies against β-catenin (×5000 dilution, Transduction Lab.), GSK-3β (×4000 dilution, Transduction Lab.), PR (×500 dilution, Novocastra Lab.), and β-actin (×5000 dilution, Sigma Chemical Co., St. Louis, MO) were used. Proteins (1–20 μg) were separated by SDS-polyacrylamide gel electrophoresis, transferred to Hybond-P (Amersham Pharmacia Biotech, Tokyo) and incubated with the primary antibodies. Antibody-binding was detected using enhanced chemiluminescence reagents according to the manufacturer's instructions.

Immunofluorescence. The HEC265 and Ishikawa cells were cultured with phenol red-free Bio-Rich containing 10% dextran-charcoal stripped FBS on glass slides. After treatment with 1 μM MPA (Pharmacia UpJohn) or the vehicle (0.1% ethanol) for 48 h, the monolayers were fixed with 3.7% paraformaldehyde for 10 min and were permeabilized in 0.2% Triton X-100 for 10 min and incubated with anti-β-catenin monoclonal antibody (×250 dilution, Transduction Lab.) for 1 h at room temperature. The second antibody was FITC-labeled rabbit ant-mouse IgG (Molecular Probes, Leiden, Netherlands).

Transient transfection and luciferase reporter assay. Cells were plated to form a 60% to 80% confluent culture in a 24-well dish. A combination of one of two luciferase reporter constructs, TOP-FLASH and FOP-FLASH (Upstate Biotechnology, Lake Placid, NY), and pRL-TK (Promega, Madison, WI), was transfected into HEC265 and Ishikawa cells using the Lipofectamine PLUS method (Invitrogen, Groningen, the Netherlands), in duplicate or triplicate, in accordance with the manufacturer's instructions. TOP-FLASH contains three copies of the TCF/LEF binding site (AAGATCAAAGGGGGT) upstream of the thymidine kinase minimal promoter, and FOP-FLASH contained a mutated TCF/LEF binding site (AAGGCCAAA-

GGGGGT). After 24 h with or without MPA treatment, luciferase activity was measured with the Dual-luciferase reporter assay system (Promega), with the Renilla luciferase activity as an internal control.

Statistics. Comparative data were analyzed using the Mann-Whitney *U* test, the χ^2 test, and Pearson's correlation coefficient. The cut-off for statistical significance was set as $P<0.05$.

Results

Nuclear β-catenin accumulation during hormone therapy. Data on changes in LIs for the 6 parameters investigated, TE grading and exon 3 mutations of the *β-catenin* gene during hormone therapy of the 23 carcinoma and 6 atypical hyperplasia cases are summarized in Table 1.

In the good-response group (TE grade≥3), tumor cells showed marked changes in their morphological appearance within the first 10 weeks of hormone therapy, often coming to resemble normal endometrial glandular elements in the secretory phase, while such alterations were only sporadic and relatively minor in the poor-response group (TE≤2), as previously demonstrated.[3, 14)]

In initial biopsy tissues, nuclear β-catenin immunoreactivity, with or without cytoplasmic stainings, was sporadically observed in 14 (73.7%) of 19 carcinomas and 3 (60%) of 5 atypical hyperplasias, while nuclear immunostaining was dramatically increased during hormone therapy (Fig. 1). After interruption of the progesterone therapy, however, decreased nuclear immunopositivity was evident in 8 of 10 cases, together with reversion of the tumor morphology. In contrast, no apparent changes in membrane β-catenin immunoreactivity were observed during prolonged progesterone therapy (data not shown).

Average nuclear (N)-LI values for β-catenin were significantly higher in carcinomas during hormone therapy, even within 10 weeks, as compared to those of tumors before or post treatment, being significantly associated with the TE grading. Similar findings were also apparent for atypical hyperplasias (Fig. 2).

Sequence analysis of PCR products of exon 3 of the *β-catenin* gene detected heterozygous substitution mutations in at least one sample before or during hormone therapy in 14 (63.6%) of 22 informative carcinomas and 2 (33.3%) of 6 atypical hyerplasias: six GAC to TAC (Ser to Tyr) and one GAC to GCC (Ser to Ala) change in codon 32; a GGA to GAA (Gly to Glu) and a GGA to AGA (Gly to Arg) in codon 34; three TCT to TGT (Ser to Cys) and a TCT to TTT (Ser to Phe) in codon 37; and three ACC to ATC (Thr to Ile) in codon 41 (Fig. 3). An identical gene status before and after hormone therapy was observed for 19 (82.6%) of 23 informative cases, while 4 (17.4%) tumors showed discrepant results (Table 2). Among 5 cases (C1, 3, 8, and 28 and AH14), for which *β-catenin* gene mutations could be analyzed in two different parts of hysterectomy samples, heterogeneity within the tumor was identified in case 3 (Table 1 and Fig. 3).

Average β-catenin N-LI values were significantly higher in the gene mutation-positive than -negative groups in both tumor categories before and after therapy. In the mutation-positive group, a significant increase in the N-LI value was noted during hormone therapy. A tendency for the same association was also apparent in the mutation-negative category, but this did not reach significance ($P=0.14$). Similar results were also obtained for atypical hyperplasias (Fig. 4).

As shown in Table 3, there was a significant association between TE grading and exon 3 mutations of the *β-catenin* gene in endometrial carcinomas.

Expression of hormone receptors and cell kinetic-related molecules during hormone therapy. In initial biopsy specimens, diffusely strong immunoreactivity for ER and PR and a heterogeneous or

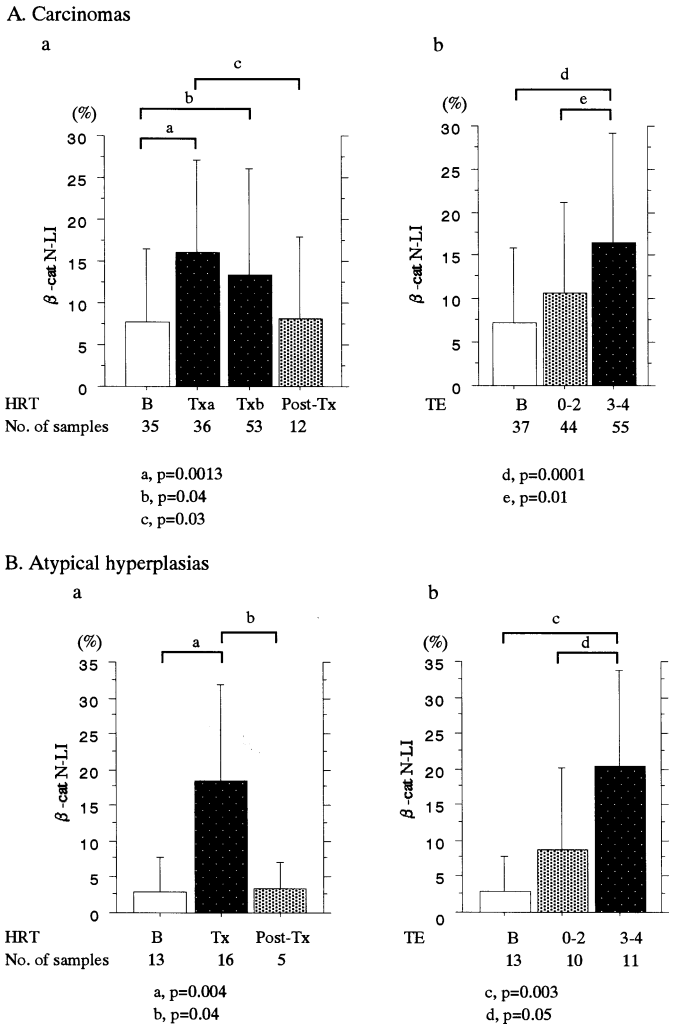

Fig. 2. a) Changes in nuclear β-catenin accumulation during hormone therapy. N-LI, nuclear labeling index; HRT, hormone therapy; B, before therapy; Txa, during hormone therapy within 10 weeks; Txb, during therapy over 10 weeks; Post-Tx, post-hormone therapy. b) Relation between nuclear β-catenin accumulation and therapeutic efficacy (TE). The data are mean values±SD.

sporadic distribution of immunopositive cells for Ki-67, p21 and p27 were found for most tumors. During hormone therapy, reduction or loss of PR and Ki-67 immunoreactivity was evident in endometrial carcinomas, in contrast to increased p27 and no alteration in ER or p21 immunoreactivity (Fig. 1). There were significant associations of TE grading with PR, Ki-67, and p27 LIs (Fig. 5). Similar findings were also noted in atypical hyperplasias (data not shown).

Correlations among β-catenin, hormone receptors and cell kinetic-related molecules during hormone therapy. As shown in Table 4, the N-LI values for β-catenin, as well as the gene mutation status (data not shown), did not correlate with any of the other parameters investigated, with the exception of the hormone receptor status in the atypical hyperplasia category.

PR, but not ER, LI values were positively correlated with Ki-67 and inversely with p27 LIs in both carcinomas and atypical hyperplasias. Significant correlations for LIs between Ki-67 and either p21 or p27 were also noted.

Effects of progesterone on endometrial carcinoma cell lines. PCR-direct sequence assays demonstrated an identical exon 3 mutation of the *β-catenin* gene with mRNA and genomic DNA samples from HEC265, featuring a GAC to GTC (Asp to Val)

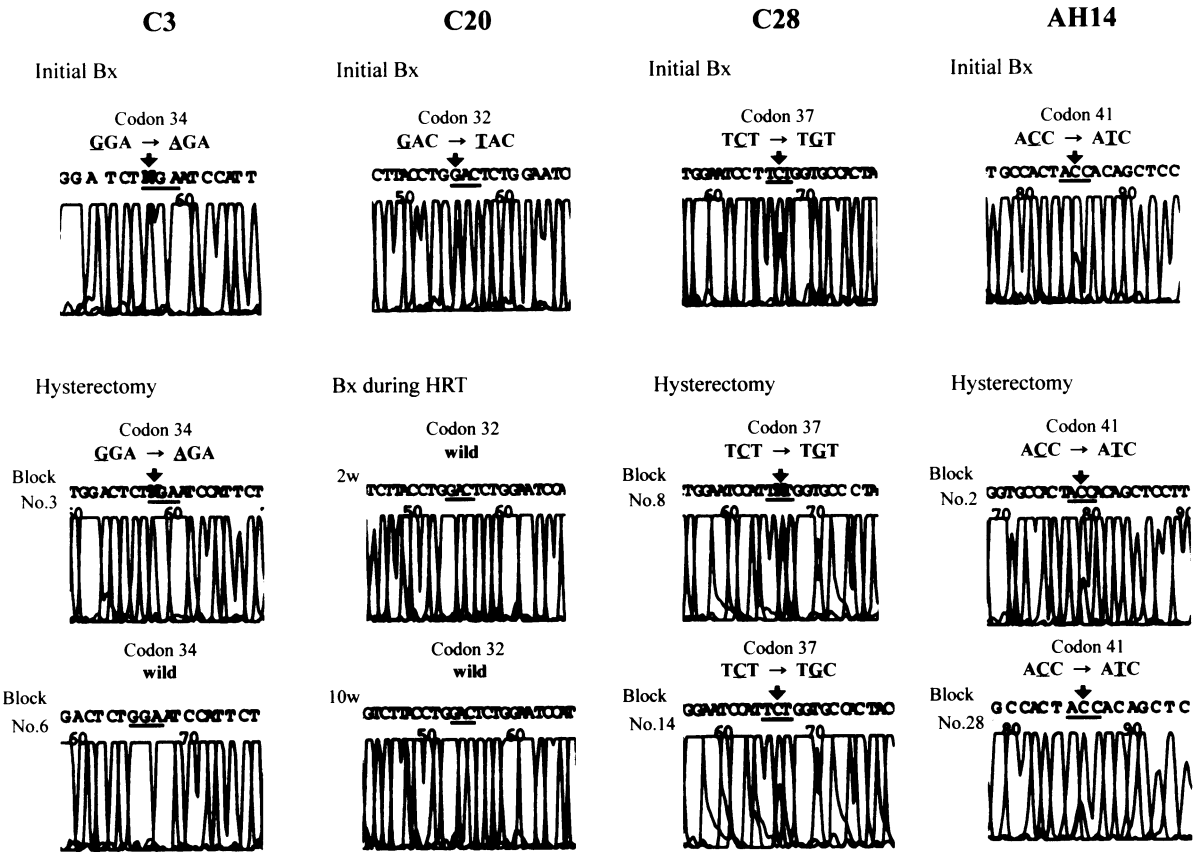

Fig. 3. Examples of sequence analyses of exon 3 of the *β-catenin* gene in endometrial carcinomas (C3, C20 and C28) and atypical hyperplasia (AH14). Note identical *β-catenin* gene mutations among sections at initial biopsy (Bx) and two different parts of the hysterectomy samples in C28 and AH14, in contrast to C3, which shows heterogeneity of the gene status in hysterectomy samples. C20 also demonstrates discrepant results between samples taken before and after 2 and 10 weeks of progesterone therapy.

change at codon 32, in contrast to no mutation in Ishikawa (data not shown). Immunofluorescence findings also showed more frequent nuclear β-catenin stainings in HEC265, but not Ishikawa (Fig. 6A).

Upon MPA treatment, no apparent morphological changes were observed in HEC265 and Ishikawa under phase-contrast microscopy. Western blot assays revealed that lysates obtained from total cell or membrane/nuclear fractions did not exhibit any significant changes in β-catenin levels (data not shown). The cytoplasmic protein level, however, was increased significantly during MPA treatment in HEC265, together with down-regulation of PR, while there was no apparent change in GSK-3β expression. In Ishikawa, PR expression levels were also suppressed by MPA treatment, but the effect was relatively minor (Fig. 6B). No apparent induction of nuclear β-catenin accumulation was evident in HEC265 and Ishikawa cells (data not shown). In addition, β-catenin transcriptional functions as assessed with TCF-responsive luciferase reporter constructs were not observed in either cell line before or after MPA treatment (data not shown).

Discussion

Although a biological role of ovarian hormones in regulation of cadherin and catenin expression has already been postulated, the published results are conflicting. For example, progesterone is capable of increasing the levels of mRNA transcripts encoding α- or β-catenins in human endometrial stromal and T47D breast carcinoma cells.[9, 21] Furthermore, in human endometrium, β-catenin mRNA levels have been shown to increase in the secretory phase of the menstrual cycle, in line with increase of progesterone levels.[11, 22] In contrast, Fujimoto *et al.*[10] reported that progesterone did not exert any effect on mRNA expression of E-cadherin or α- and β-catenins in Ishikawa endometrial carcinoma cells.

Table 2. Presence of β-catenin mutations before and after hormone therapy in endometrial carcinomas and atypical hyperplasias

	Before HRT	After HRT	No. of cases
β-Catenin exon 3 mutation	Mutant	Mutant	10
	Mutant	Wild	4
	Wild	Mutant	0
	Wild	Wild	9

HRT, hormone therapy; No., numbers.

The present study clearly demonstrated increased nuclear β-catenin accumulation in endometrial carcinomas and atypical hyperplasias during progesterone therapy, and disappearance on its interruption. To our knowledge, this is the first report to indicate that changes in expression or subcellular distribution of β-catenin are induced by progesterone therapy in human endometrial tumors. The lack of alteration in the membrane status of β-catenin may be explained by the fact that all of the cases investigated were categorized as grade 1 tumors, since membrane immunoreactivity in well-differentiated lesions is very high, even before treatment.[14] This idea is supported by our finding of no apparent change in β-catenin levels in total cell and membrane/nuclear fractions because of the large endogenous expression.

The *β-catenin* gene mutations reported in several human ma-

lignancies have been found to be concentrated at specific GSK-3β phosphorylation sites, Ser-33, Ser-37, Thr-41, Ser-45 and residues flanking codon 33 (codons 32 and 34).[23, 24] Our previous work demonstrated the presence of such mutations in 16 (22.9%) of 70 endometrial carcinomas and 3 (12.5%) of 24 atypical hyperplasias.[14] In the present study, mutations were detected in 63.6% and 33.5% of informative carcinomas and atypical hyperplasias, respectively, some involving codons 32, 34, 37 and 41. The higher frequency of mutations might simply be a reflection of their higher incidence (82.8%) of SqD areas within tumors and the age (32.2±7.1 years) of the patients investigated, since gene abnormalities are relatively common in association with SqD features and in early onset cases.[17, 25] Of 14 tumors determined to be positive for *β-catenin* gene mutations, identical single nucleotide substitutions before and after hormone therapy were observed in 10. The discordant results in the remaining 4 cases may have been due to genetic heterogeneity, as demonstrated for case 3 (Table 1 and Fig. 3). Similar findings have also been described for hepatocellular, gallbladder and prostatic carcinomas.[26–28]

It has been documented that mutant β-catenin can alter the cellular morphology of murine L cells from a fibroblast-like appearance to a more cuboidal shape,[29] similar to that of v-src-transformed Madin-Darby canine kidney (MDCK) cells.[30] Nuclear β-catenin accumulation has further been noted during endometrial tumor cell differentiation towards squamoid features.[17] Consistent with these observations, the present study demonstrated a significant association between increased nuclear β-catenin accumulation, as well as gene mutations, and altered tumor morphology due to progesterone therapy, suggesting that β-catenin abnormalities may play an important role in promotion of a more terminally differentiated phenotype in endometrial carcinomas, as well as atypical hyperplasias.

Our *in vitro* findings further showed progesterone to significantly suppress PR protein expression in HEC265 and Ishikawa cells. Given that the actions of progesterone are mediated by PR,[31] this may be simply a reflection of the negative feedback system. Moreover, the increase of cytoplasmic β-catenin levels by the treatment in HEC265 detected by western blot assay was in line with the results of our *in vivo* study, although no changes were found in nuclear β-catenin accumulation by immunofluorescence analysis. This may be due to the difference in sensitivity between the two assays. Similar findings have also been demonstrated in epithelial MDCK cells, which were stably transfected with constitutively expressed *β-catenin* transgenes (S37A).[32] In addition, the reason for the difference from the Ishikawa case, where such findings were relatively minor, may include variations in the status of *β-catenin* gene abnormalities, level of PR expression, and sensitivity to progesterone. Since no alteration was evident in levels of GSK-3β expression, the kinase activity rather than the regulation of transcription may be affected by progesterone. Inactivation of GSK-3β kinase activity on oocyte maturation in response to progesterone is known to be necessary and rate-limiting for the cell cycle response to this hormone and the subsequent accumulation of β-catenin.[33]

Activation of β-catenin-TCF signaling is considered to upregulate expression of c-myc and cyclin D1, resulting in promotion of tumor progression by stimulating cell proliferation.[6–8] Several studies, however, have indicated that overexpressed β-catenin can be imported into the nucleus without being complexed with TCF4 proteins.[34, 35] In addition, variable molecules, such as ICAT and Duplin, have been reported to negatively regulate the *β-catenin*-dependent gene expression by interfering with the complex formation of β-catenin, TCF4, and DNA.[36, 37] In agreement with these findings, we observed that an increase in cytoplasmic β-catenin levels induced by MPA treatment was not accompanied with its transcriptional activation in HEC265 cells. Our preliminary results also revealed that stabilization of β-catenin in response to transient transfection of mutant β-cate-

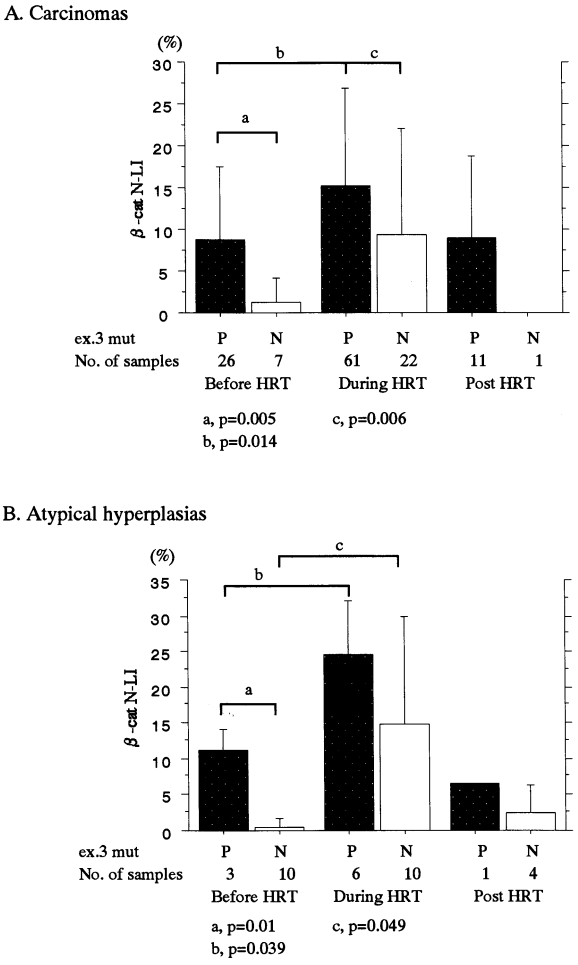

Fig. 4. Relation between nuclear β-catenin accumulation and mutations. ex. 3 mut, exon 3 mutation; No., numbers; P, positive; N, negative; HRT, hormone therapy. The data are mean values±SD.

Table 3. Relation between therapeutic efficacy and β-catenin mutation in endometrial carcinoma and atypical hyperplasia

	Carcinoma			Atypical hyperplasia		
	β-Cat. ex. 3 mutation		*P* value	β-Cat. ex. 3 mutation		*P* value
	Positive	Negative		Positive	Negative	
TE Grade 0–2	27	16	0.008	4	6	0.76
Grade 3–4	44	7		3	6	

β-Cat. ex. 3, β-catenin exon 3; TE, therapeutic efficacy.

nin (a deleted Ser-45) construct did not always result in inducing active β-catenin-TCF complex in several endometrial carcinoma cell lines (data not shown).

Recently, Damalas *et al.*[38] have reported that an excess of β-catenin (S37Y) can activate a p53 pathway and trigger the p53-mediated anti-proliferative response, which in primary fibroblasts takes the form of a growth inhibition associated with senescence-like morphological features. Mao *et al.*[34] have also indicated the possibility that a sustained retention of β-catenin in the nucleus can signal for p53 induction and cell cycle arrest. In this study, the increase of nuclear β-catenin accumulation appeared to be in line with suppression of tumor cell proliferation characterized by low Ki-67 and high p27 LI values during progesterone therapy. Considering our previous findings of fre-

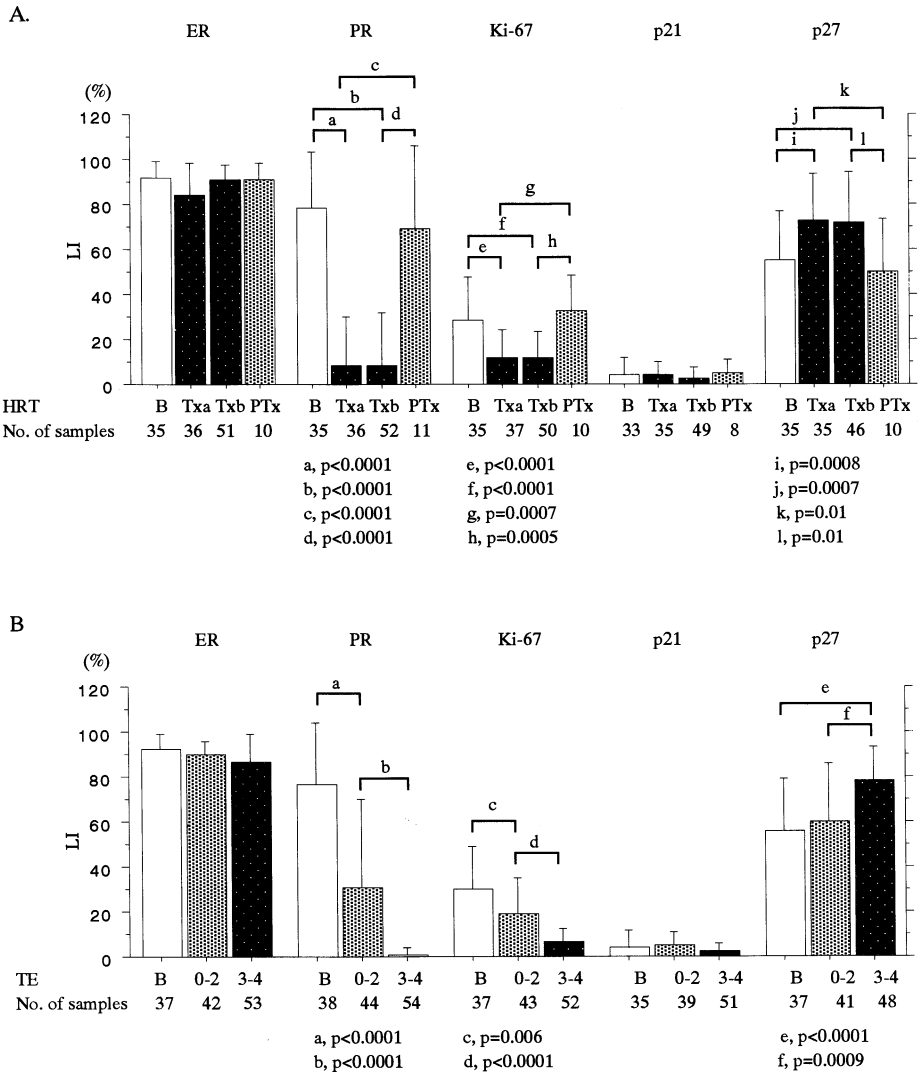

Fig. 5. a) Changes in ER, PR, Ki-67, p21, and p27 labeling indices (LIs) during hormone therapy in endometrial carcinomas. HRT, hormone therapy; B, before therapy; Txa, during hormone therapy within 10 weeks; Txb, during therapy over 10 weeks; PTx, post-hormone therapy. b) Relations between LIs of ER, PR, Ki-67, p21 and p27 and therapeutic efficacy (TE) in endometrial carcinomas. The data are mean values±SD.

Table 4. Correlation of labeling indices for nuclear β-catenin, hormone receptors and cell kinetic markers in endometrial carcinomas and atypical hyperplasias

		β-Catenin vs.					ER vs.				PR vs.			Ki-67 vs.		p21 vs.
	n	ER	PR	Ki-67	p21	p27	PR	Ki-67	p21	p27	Ki-67	p21	p27	p21	p27	p27
		r	*r*	*r*	*r*	*r*	*r*	*r*	*r*	*r*	*r*	*r*	*r*	*r*	*r*	r
Overall	163	−0.25	−0.37	−0.37	−0.17	0.1	0.3	0.23	0.06	0.005	0.56	0.07	−0.46	0.45	−0.4	0.08
		P=0.002	*P*<0.0001	*P*<0.0001	*P*=0.03	*P*=0.2	*P*<0.0001	*P*=0.003	*P*=0.47	*P*=0.95	*P*<0.0001	*P*=0.4	*P*<0.0001	*P*<0.0001	*P*<0.0001	*P*=0.36
Em Ca	130	−0.2	−0.28	−0.39	−0.2	0.07	0.25	0.24	0.09	0.02	0.55	0.13	−0.41	0.49	−0.4	−0.1
		P=0.02	*P*=0.0009	*P*<0.0001	*P*=0.02	*P*=0.4	*P*=0.004	*P*=0.006	*P*=0.27	*P*=0.84	*P*<0.0001	*P*=0.15	*P*<0.0001	*P*<0.0001	*P*<0.0001	*P*=0.3
AH	33	−0.47	−0.61	−0.3	−0.3	0.2	0.49	0.29	0.21	0.1	0.7	0.16	−0.67	0.05	−0.56	0.12
		P=0.006	*P*=0.0001	*P*=0.1	*P*=0.09	*P*=0.28	*P*=0.004	*P*=0.13	*P*=0.26	*P*=0.58	*P*<0.0001	*P*=0.38	*P*<0.0001	*P*=0.78	*P*=0.002	*P*=0.5

Pearson's correlation coefficient. Em Ca, endometrial carcinoma; AH, atypical hyperplasia.

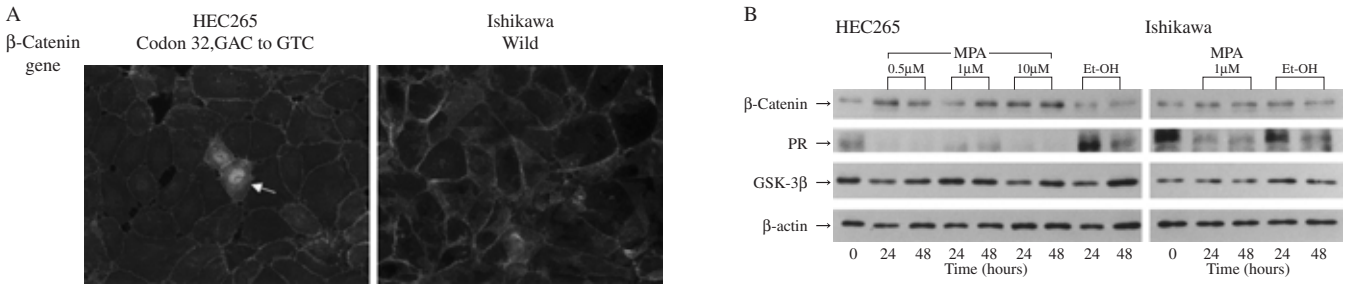

Fig. 6. A) Immunofluorescence for β-catenin in HEC265 and Ishikawa before MPA treatment. Note the strong nuclear stainings (indicated by arrows) surrounded by weak nuclear immunoreactivity in HEC265, in contrast to no nuclear accumulation in Ishikawa. Frequent membrane stainings are also observed in both cells. Original magnification, ×400. B) Western blot assays for expression of cytoplasmic β-catenin, PR and GSK-β during progesterone treatment in HEC265 and Ishikawa cells.

quent β-catenin nuclear accumulation in SqD areas lacking proliferative activity within endometrial carcinomas,[15, 17] an inhibition of cell proliferation may also be linked to dysregulation of β-catenin expression. Further studies in this area are clearly warranted.

Although the exact reason for the lack of a significant correlation between LIs of nuclear β-catenin and p21 or p27 in this study is unclear, it seems likely that β-catenin may not affect the expression levels of these markers. Dai *et al.*[39] have recently demonstrated up-regulation of p21 and p27 by progesterone in Hec50 human endometrial carcinoma cells infected with progesterone receptors. Our previous study also indicated high p27 expression in endometrial carcinomas adjacent to secretory non-neoplastic endometrium as compared to those surrounded by atrophic or proliferative tissues.[40] Considering that GSK-3β activity is altered by progesterone,[33] it is thus possible that stabilization of β-catenin and p27 in response to progesterone may occur in parallel through different mechanisms.

In conclusion, the present study demonstrated a significant role of progesterone in the regulation of β-catenin expression in endometrial tumors. Moreover, β-catenin abnormalities are associated with alterations of tumor morphology by progesterone, indicating that the molecule may be a clinically useful marker of hormone therapeutic effects.

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