Simultaneous immunohistochemical localization of β-catenin and cyclin D1 in differentiated but not in undifferentiated human endometrial carcinoma

F. Narita, A. Sato, S. Hamana, M. Deguchi, T. Otani, T. Maruo

Department of Obstetrics and Gynecology, Kobe University Graduate School of Medicine, Kobe (Japan)

Summary

Purpose: β-catenin plays dual important roles in epithelial cell-cell adhesion in cytoplasm as well as in the nuclear T-cell factor (TCF)/lymphoid enhancing factor-1 (LEF-1) signaling pathway. Abnormal nuclear accumulation of β-catenin promotes colorectal carcinogenesis by triggering the expression of cyclin D1 gene through the TCF/LEF-1 pathway. The purpose of this study was to investigate the possible involvement of the TCF/LEF-1 pathway in endometrial carcinogenesis.

Methods: Immunohistochemical localization of β-catenin and cyclin D1 in normal endometrium, hyperplastic endometrium and endometrial carcinoma were assessed on serial tissue sections.

Results: Nuclear accumulation of β-catenin was observed in endometrial carcinomas compared with normal endometria. Cyclin D1-positive endometrial cancer cases were β-catenin-positive in the nuclei, especially in 70% (7/10) of G1 and 55.6% (5/9) of G2 differentiated endometrial carcinomas, but never in G3 undifferentiated ones.

Conclusions: These results imply that the simultaneous nuclear accumulation of β-catenin and cyclin D1 – suggesting the activation of the TCF/LEF-1 pathway – may be a potential marker for the progression of Type 1 endometrial carcinogenesis.

Keywords: β-catenin; Cyclin D1; TCF/LEF pathway; Uterine endometrial carcinoma.

Introduction

The protein β-catenin was originally identified as a submembranous component of the cadherin-mediated cell-cell adhesion system [1]. β-catenin directly binds to the intracellular domain of E-cadherin as well as α-catenin and this complex links to the actin cytoskeleton [1]. This cadherin-catenin linkage plays important roles in the formation and maintenance of tissues, and disorder of this linkage thus correlates with increased invasiveness and metastasis of tumors [2]. In addition, β-catenin translocates into the nucleus [3-7], where it can complex with transcription factors of the T cell factor/lymphoid enhancing factor-1 (TCF/LEF-1) and regulate the expression of specific genes [8, 9], such as cyclin D1 [10]. By playing several roles, β-catenin controls the structural organization, cell signaling and gene expression [1, 11-13].

Intracellular distribution of β-catenin is regulated by its association with the tumor suppressor molecule; adenomatous polyposis coli (APC) [14, 15]. APC forms a complex with β-catenin as well as glycogen synthase kinase-3β (GSK-3β) and mediates the phosphorylation of β-catenin [16].

Phosphorylation of β-catenin by the APC-axin-GSK-3β complex leads to the degradation of β-catenin by the ubiquitin-proteasome system [6, 17-19]. It is reported that APC mutations in colon cancer result in truncated APC proteins with the loss of β-catenin regulatory activity and thus cause accumulation of β-catenin [20]. β-catenin mutations, especially at the multiple serine/threonine residues in exon 3 targeted for APC-dependent phosphorylation by GSK-3β, are also reported in colorectal carcinoma, melanoma and ovarian cancer [21-23]. These mutations increase the stability of β-catenin and induce the intracellular accumulation of β-catenin [21, 24]. Recently β-catenin mutations in exon3 and aberrant nuclear localization have been demonstrated in endometrial carcinoma [25-29]. Abnormal aggregation of β-catenin may contribute to neoplastic transformation by causing accumulation of cyclin D1 through TCF/LEF pathway [10, 30].

Cyclin D1 is associated with the onset of cell progression in the G1 phase of the cell cycle [31, 32]. Cyclin D1 interacts with a catalytic subunit $p34^{PSK-J3}$/cdk4, and this complex, possessing kinase activity to the retinoblastoma gene product (pRB), accumulates during the G1 and declines in the S phase in murine macrophages [10]. Amplification and overexpression of the gene encoding cyclin D1 has frequently been observed in several types of human neoplasia, such as head and neck carcinoma [33], esophageal carcinoma [34], hepatocellular carcinoma [35] and breast cancer [36]. Expression of cyclin D1 is elevated in about 30% of human colorectal cancer, and expression of anti-sense cyclin D1 cDNA abolished the growth of SW480 colon cancer cells in nude mice, indicating a critical role for cyclin D1 in tumorigenesis [37]. Abnormal expression of cyclin D1 has also been reported in uterine endometrioid carcinoma [38].

However, to our knowledge, concomitant expression of β-catenin and cyclin D1 has not been reported in endometrial carcinoma. To investigate the possible involve-

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ment of the TCF/LEF-1 pathway in the development of endometrial carcinoma, we analyzed the expression of β-catenin and cyclin D1 by immunohistochemical staining and assessed the possible relationship between their localization and histopathological differentiation or clinical stages of endometrial carcinomas.

Materials and Methods

Tissue specimens

Endometrial tissues were obtained from 51 women who underwent abdominal hysterectomy with uni- or bilateral salpingo-oophorectomy for a variety of gynecological conditions. These specimens included 16 normal endometria (proliferative: 6 cases, secretory: 7 cases and postmenopausal: 3 cases), 6 hyperplastic endometria (complex without atypia: 3 cases and atypical: 3 cases) and 29 endometrial carcinomas. The collection of these tissues has been approved by the Institutional Review Board. These patients ranged in age from 30 to 78 years, with a mean age of 51.8 years. Informed consent was obtained from each patient before surgery for the use of endometrial tissues for immunohistochemical studies. Each specimen was examined by pathologist for histological evaluation. Endometrial tissues were categorized into endometrial carcinoma, hyperplastic endometrium and normal endometrium. Endometrial carcinomas were graded into G1-G3 (G1: 10 cases, G2: 9 cases, G3: 10 cases), and staged according to the criteria of the International Federation of Gynecology and Obstetrics (FIGO). The endometrial specimens obtained were fixed in 10% buffered neutral formalin, dehydrated and embedded in paraffin. Sections, 4 μm in diameter, were deparaffinized and examined using standard histologic techniques.

Immunohistochemical staining

Immunohistochemical staining was performed by the universal immuno-enzyme polymer method with the use of a Histofine Simple Stain MAX-PO (MULTI) kit (Nichirei, Tokyo, Japan). Mouse monoclonal antibody against human β-catenin (Zymed Laboratories, San Francisco, CA) or cyclin D1 (Zymed Laboratories, San Francisco, CA) was used as the primary antibody. The sections were incubated with anti-β-catenin antibody diluted at 1: 50 or anti-cyclin D1 antibody (ready to use), followed by the incubation with secondary antibody conjugated to horseradish peroxidase. Subsequently, the sections were incubated with substrate solution of amino-ethylcarbazole. Sections were counterstained with Mayer hematoxylin and examined microscopically.

The following control procedures were undertaken to assure the specificity of the immunological reactions. Adjacent control sections were subjected to the same immunohistochemical method, except that the primary antibodies to β-catenin and cyclin D1 were substituted for nonimmune murine IgG. In the above-mentioned controls, no positive immunostaining was observed.

The intensity of the immunostaining was evaluated by two independent observers. Although some variation was found in the intensity of the nuclear and cytoplasmic immunostaining in different positive cells, each immunostained nucleus and cytoplasm was scored as positive. Statistical analysis was taken with a StatView system (Abacus Concepts, INC., Berkeley, CA) using Fisher's test. The significant difference was considered if the p value was < 0.05.

Results

Abnormal nuclear accumulation of β-catenin in endometrial carcinomas.

β-catenin staining was detected in endometrial epithelial cells but not in stroma cells (Figure 1- A, B, C). Cytoplasmic localization of β-catenin was observed in 100% (16/16), 83.3% (5/6) and 96.6% (28/29) of normal endometria, hyperplastic endometria and endometrial carcinomas, respectively (Tables 1, 2 and 3). There was no significant difference in cytoplasmic β-catenin positivity among three groups.

In normal endometrium, nuclear localization of β-catenin was detected only in 18.8% (3/16) of the cases, without any correlation between the nuclear staining and the phases of the menstrual cycle (Table 1). On the other hand, positive nuclear staining for β-catenin was observed in 66.6% (4/6) and 58.6% (17/29) of hyperplastic endometria and endometrial carcinomas, respectively, and the latter was significantly higher than that in normal endometria ($p < 0.05$) (Tables 2 and 3). Especially differentiated G1 and G2 carcinoma cases demonstrated significantly higher nuclear β-catenin positive rate than normal endometria ($p < 0.01$). In endometrial carcinomas, no significant correlation was observed between the nuclear expression of β-catenin and

Table 1. — *Immunohistochemical localization of β-catenin and cyclin D1, and phase of menstrual cycle in normal endometrium samples.*

No.	Age(yrs)	Cycle	β-catenin		Cyclin D1
			Cytoplasmic	Nuclear	
1	30	Proliferative	+	–	+
2	40	Proliferative	+	–	–
3	47	Proliferative	+	–	–
4	48	Proliferative	+	–	–
5	49	Proliferative	+	+	–
6	51	Proliferative	+	–	–
7	38	Secretory	+	–	–
8	40	Secretory	+	+	–
9	42	Secretory	+	–	–
10	45	Secretory	+	–	–
11	45	Secretory	+	–	–
12	47	Secretory	+	+	–
13	48	Secretory	+	–	+
14	53	Postmenopausal	+	–	–
15	54	Postmenopausal	+	–	–
16	56	Postmenopausal	+	–	–

+ positive; – negative.

Table 2. — *Immunohistochemical localization of β-catenin and cyclin D1, and histopathological type of hyperplastic endometria.*

No.	Age(yrs)	Pathologic grade	β-catenin		Cyclin D1
			Cytoplasmic	Nuclear	
1	36	Complex	+	+	+
2	43	Complex	+	+	–
3	45	Complex	+	+	–
4	36	Atypical	+	–	+
5	39	Atypical	+	+	+
6	39	Atypical	–	–	–

+ positive; – negative.

Table 3. — *Immunohistochemical localization of β-catenin and cyclin D1 and clinicopathological features of endometrial carcinoma cases examined.*

No.	Age(yrs)	Pathologic grade	FIGO stage	β-catenin		Cyclin D1
				Cytoplasmic	Nuclear	
1	39	G1	I b	+	+	+
2	44	G1	I b	+	–	–
3	54	G1	I b	+	–	–
4	54	G1	I b	+	+	+
5	61	G1	I c	+	+	+
6	35	G1	II b	+	+	+
7	47	G1	II b	+	+	+
8	64	G1	II b	+	+	+
9	57	G1	III a	+	+	+
10	47	G1	IV a	+	+	–
11	78	G2	I a	+	–	–
12	46	G2	I b	+	+	+
13	57	G2	I b	+	+	–
14	66	G2	I b	+	+	+
15	61	G2	II b	+	+	–
16	75	G2	II b	+	–	–
17	52	G2	III a	+	+	+
18	69	G2	III c	+	+	+
19	72	G2	III c	+	+	+
20	51	G3	I b	+	+	–
21	54	G3	I b	+	+	–
22	56	G3	I b	+	–	–
23	61	G3	I b	+	–	–
24	57	G3	I c	+	–	–
25	66	G3	I c	+	–	–
26	58	G3	II a	–	–	–
27	64	G3	II a	+	–	–
28	63	G3	IV b	+	–	–
29	65	G3	IV b	+	–	–

FIGO: International Federation of Gynecology and Obstetrics
+ positive; – negative.

Table 4. — *Relation between nuclear immunoreactivity of β-catenin and cyclin D1 in endometrial carcinoma cases.*

		Nuclear β-catenin		
		Positive	Negative	Total
Cyclin D1	positive	12	0	12
	negative	5	12	17
	Total	17	12	29

$p < 0.001$

advance of clinical stages. Poorly differentiated G3 carcinoma cases demonstrated a significantly lower nuclear β-catenin positive rate than differentiated G1 and G2 cases ($p < 0.05$) (Table 3). Replacement of the primary antibody with non immune murine IgG resulted in the lack of positive staining (Figure 1 - G, H, I).

Abnormal localization of cyclin D1 in hyperplastic endometria and endometrial carcinomas.

Cyclin D1 was immunohistochemically localized in the nuclei of endometrial epithelial cells but not in those of stroma cells (Figure 1-D). Expression of cyclin D1 was observed only in 12.5% (2/16) of normal endometria (Table 1), whereas it was apparent in 50.0% (3/6) and 41.4% (12/29) of hyperplastic endometria and endometrial carcinomas, respectively (Tables 2 and 3). Cyclin D1 positive rate in G1 cases (70%, 7/10) was significantly higher than that in normal endometria ($p < 0.01$). None of the G3 cases (0/10) demonstrated cyclin D1 immunostaining, the rate was significantly lower than that of G1 or G2 (55.6%, 5/9) ($p < 0.01$, and $p < 0.05$, respectively) (Table 3).

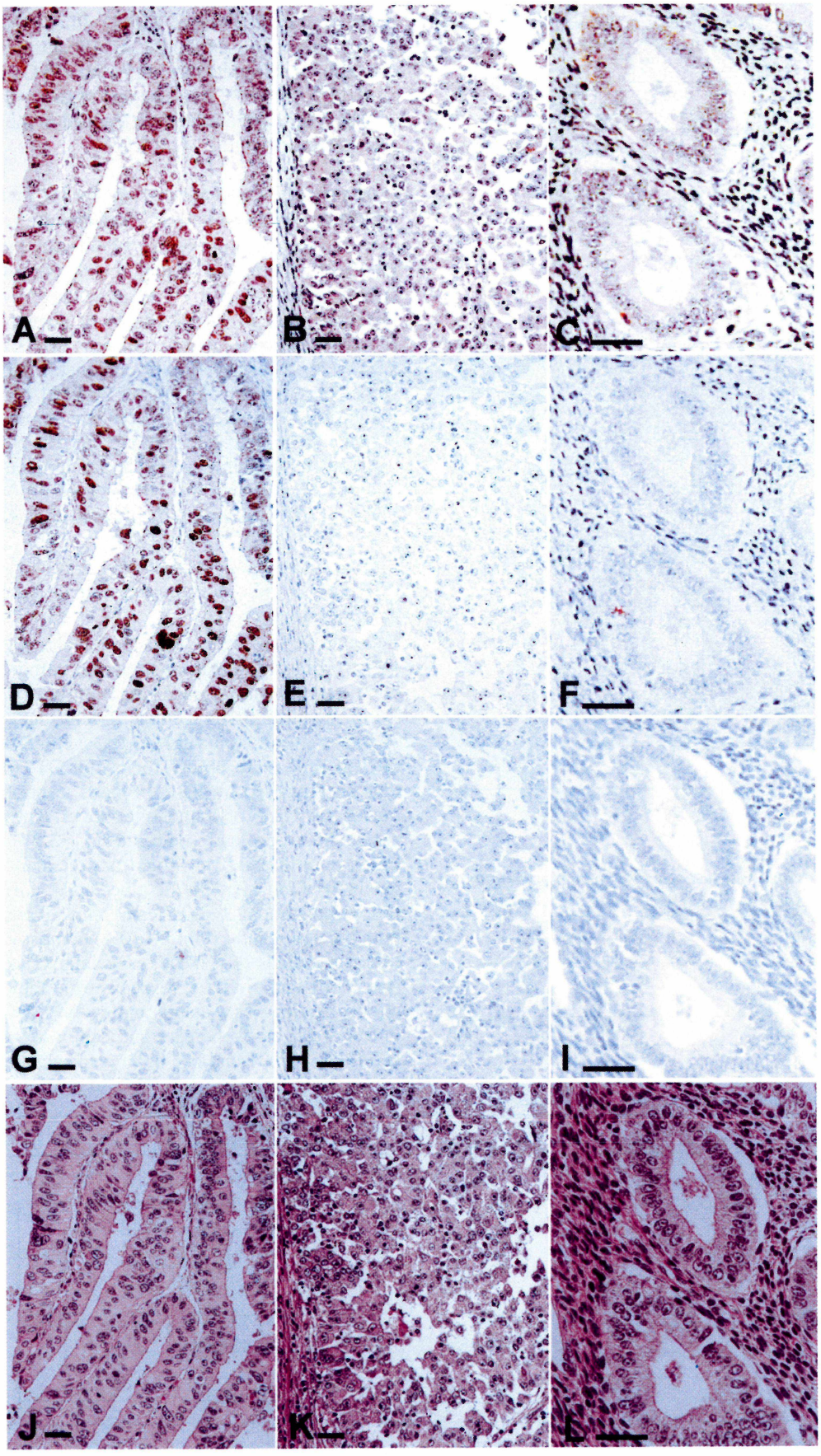
Simultaneous nuclear localization of β-catenin and cyclin D1 in hyperplastic endometria and endometrial carcinomas.

To investigate the possible involvement of the TCF/-LEF-1 pathway during endometrial carcinogenesis, we compared the nuclear localization of β-catenin and cyclin D1 on the serial tissue sections. Twelve out of 29 (41.4%) endometrial carcinoma cases were cyclin D1-positive and nuclear immunoreactivity for cyclin D1 coincided with that for β-catenin in these 12 cases, especially in seven out of ten G1 (70%) and five out of nine G2 (55.6%) cases. In hyperplastic endometria, 33.3% (2/6) of the cases demonstrated co-localization of β-catenin and cyclin D1 (Table 5). On the other hand, normal endometria demonstrated a significantly lower positive rate of both β-catenin and cyclin D1 (0%) compared to endometrial carcinomas ($p < 0.01$), especially G1 and G2 cases ($p < 0.001$ and $p < 0.01$, respectively) (Table 4). Nuclear co-localization of β-catenin and cyclin D1 was observed in 0% of G3 cases, being significantly lower than in G1 and G2 ($p < 0.01$ and $p < 0.05$, respectively) (Table 5).

Table 5. — *Pathologic features of both nuclear β-catenin and cyclin D1 positive staining cases.*

	Grade	Number of both nuclear β-catenin and cyclin D1 positive cases (%)
Endometrial carcinomas	Total	12/29 (41.4%)
	G1	7/10 (70.0%)
	G2	5/9 (55.6%)
	G3	0/10 (0.0%)
Hyperplastic endometria	Total	2/6 (33.3%)
	Atypical	1/3 (33.3%)
Normal endometria	———	0/16 (0.0%)

*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.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Discussion

In the present study, accumulation of β-catenin in the nuclei was frequently detected in endometrial carcinomas. In normal endometria, nuclear localization of β-catenin was detected only in 18.8% (3/16) of the cases, without any correlation with the phases of the menstrual cycle. Saegusa et al. [25] reported that nuclear β-catenin immunoreactivity was not found in any normal endometrial tissues and Nei *et al.* [39] observed weak nuclear β-catenin staining in the mid-proliferative, late-proliferative and early secretory phase of the endometrium. It is not clear why the nuclear β-catenin staining in normal endometria varies between the studies. Further investigation will be needed.

Fujimoto *et al.* [40] reported that as endometrial dedifferentiation, myometrial invasion and clinical staging advanced, the expression of E-cadherin and β-catenin mRNAs decreased. Thus, nuclear localization of β-catenin seems unlikely due to the enhanced transcription of β-catenin genes. Since β-catenin does not contain a nuclear localization signal, intracellular distribution of β-catenin is regulated, in part, by its tyrosine phosphorylation associated with EGF receptor and c-erb B2 [1]. Tyrosine phosphorylation of β-catenin has been implicated in the dissociation of the cadherin-catenin complex from the actin cytoskeleton and a concomitant decrease in intercellular adhesion [2]. β-catenin distribution is also controlled by the APC-GSK-3β phosphorylation complex and subsequent ubiquitin proteasome system [16, 20]. β-catenin mutations in colon cancer, especially in exon 3 which includes the residues targeted for phosphorylation by GSK-3β, induce the intracellular accumulation of β-catenin [30 31]. Recently several studies have demonstrated the mutations in exon 3 of β-catenin in endometrial carcinoma and the association with nuclear localization of β-catenin [25-29]. APC mutations in colorectal cancer have been shown to result in an increase and subsequent nuclear translocation of β-catenin [20]. However, unlike colon cancer, mutations in APC have not been detected in endometrial carcinomas to date [26].

Nuclear translocation of β-catenin is also affected by LEF-1 which is a functional component of wnt-1 signaling pathway [5]. Overexpression of β-catenin and LEF-1 in Neuro2A cells leads to translocation of β-catenin to the nucleus [5]. Nuclear accumulation of β-catenin in endometrial hyperplasia and endometrioid carcinoma may reflect the activation of the TCF/LEF-1 cell signaling pathway.

Cyclin D1 is a target of the TCF/LEF-1 pathway and a major regulator of the progression of cells into the proliferative stage of the cell cycle [31, 32]. Nikaido *et al.* [38] demonstrated that cyclin D1 was detected in 40% of endometrial carcinomas, and its staining pattern was correlated with advances of the clinical stages and histological grades. On the contrary, Ito *et al.* [41] reported that cyclin D1 staining in well differentiated carcinoma was higher than that of moderately and poorly differentiated tumors, and that there were no significant correlations between the cyclin D1 expression and the survival or clinical outcome of the patients. Actually, in the present study, cyclin D1 expression increased in endometrial carcinomas, especially in G1 and G2 differentiated carcinoma but not in G3 poorly differentiated carcinoma. Moreover, in G1 and G2 endometrial carcinomas, nuclear cyclin D1-positive endometrial cancer cases were also β-catenin-positive in the nuclei.

In recent studies it has been proposed that there are at least two different pathways of endometrial carcinogenesis [42-45]. One is an estrogen-driven pathway, which develops G1 and G2 endometrioid carcinomas through hyperplastic endometria (Type 1). The other is best represented by serous carcinoma, which is thought to be developed from endometrial intraepithelial carcinoma (EIC) in atrophic endometrium unrelated to estrogen (Type 2). Type 1 endometrial carcinogenesis resembles the proposed model for colorectal carcinogenesis [42]. These data imply that the accumulation of β-catenin may activate the cyclin D1 promoter through the TCF/LEF-1 pathway in Type 1 endometrial carcinomas.

In breast cancer, it has been shown that cyclin D1 directly binds to the hormone binding domain of the estrogen receptor and potentiates transcription of estrogen receptor-regulated genes [46]. Cyclin D1 overexpression reduces the growth requirement for mitogens in estrogen-responsive breast tumors [47]. Moreover, this novel role of cyclin D1 is independent of complex formation to cdk4 [48]. Thus, nuclear localization of β-catenin and cyclin D1 in differentiated endometrial carcinomas implies not only the cell cycle progression but also the direct stimulation of estrogen receptor-mediated cell proliferation.

On the other hand, none of the G3 cases demonstrated either nuclear β-catenin or cyclin D1-positivity, suggesting the existence of another oncogenic mechanism in Type 2 endometrial carcinogenesis.

Taken together, this is the first study to demonstrate the simultaneous immunohistochemical localization of β-catenin and cyclin D1 in differentiated endometrial carcinoma, suggesting the activation of the TCF/LEF-1 pathway, may be a potential marker for the progression of Type 1 endometrial carcinogenesis.

Figure 1. — Immunohistochemical localization of β-catenin and cyclin D1 in well differentiated (G1) endometrial carcinoma (A, D, G, J), poorly differentiated (G3) endometrial carcinoma (B, E, H ,K) and normal endometrium (C, F, I, L). In G1 endometrial carcinoma, immunostaining for β-catenin was observed in the cytoplasm as well as in the nucleus (A), while immunostaining for cyclin D1 was observed in the nucleus (D). In G3 endometrial carcinoma, immunostaining for β-catenin was observed only in the cytoplasm but not in the nucleus (B), while nuclear staining for cyclin D1 was not observed (E). In normal endometrium in the proliferative phase, immunostaining for β-catenin was observed only in the cytoplasm but not in the nucleus (C), while nuclear staining for cyclin D1 was not observed (F). The control sections (G, H, I) showed negative immunostaining in which primary antibodies against β-catenin and cyclin D1 were replaced with nonimmune murine IgG. HE stained slides were also presented (J, K, L). Bars represent 10 μm. (Original magnification A, B, D, E, G, H, J, K: x 200, C, F, I, L: x 400).

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Address reprint requests to:
T. MARUO, M.D., Ph. D.
Dept. Obstetric/Gynecology
Kobe Univ. Graduate School of Med.
7-5-1 Kusunoki-cho, Chuo-ku
Kobe 650-0017 (Japan)