Mutations of the $\beta$-Catenin Gene in Endometrial Carcinomas

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To investigate the contribution of $\beta$-catenin to the development of endometrial carcinoma, we searched for genetic alterations of the $\beta$-catenin gene in primary endometrial carcinomas. Mutational analysis of exon 3 of the $\beta$-catenin gene, encoding the serine/threonine residues for GSK-3\(\beta\) phosphorylation, was performed for 35 tumors. Nucleotide sequencing analysis revealed that 5 tumors (5/35, 14%) contained mutations (S33C, S37C, S37F, T41A) that altered potential GSK-3\(\beta\) phosphorylation sites. Each of the mutations resulted in the substitution of serine/threonine residues that have been implicated in the down-regulation of $\beta$-catenin through phosphorylation by GSK-3\(\beta\) kinase. Furthermore, the incidence of $\beta$-catenin mutations was significantly higher in early-onset (3 of 5) than that in late-onset tumors (2 of 30) ($P$=0.014, Fisher’s exact test). Replication error (RER)-positive phenotype was not detected in tumors with the $\beta$-catenin gene mutation, although 10 of 35 tumors revealed RER. We performed immunohistochemistry of $\beta$-catenin in 17 cases for which tissue samples were available. We confirmed accumulation of $\beta$-catenin protein in both the nucleus and cytoplasm in 3 tumors, including two in which amino acid alterations had occurred at codon 33 and 37. The other case had no mutation in exon 3. Our results suggested that mutations at serine/threonine residues involved in phosphorylation by GSK-3\(\beta\) affected the stability of $\beta$-catenin. Accumulation of mutant $\beta$-catenin could contribute to the development of a subset of endometrial carcinomas, particularly those of the early-onset type.

Key words: $\beta$-Catenin — Human endometrial carcinoma — $\beta$-Catenin-Tcf pathway — Somatic mutations

Recent reports have demonstrated that $\beta$-catenin is a multifunctional protein involved in two apparently independent processes, acting as a cell-cell adhesion regulator when coupled with cadherin¹ and in the wingless/Wnt signal transduction pathway.²³ The binding of $\beta$-catenin to adenomatous polyposis coli (APC) requires phosphorylation of $\beta$-catenin by GSK (glycogen synthase kinase)-3\(\beta\) on serine/threonine residues, all of which are encoded in exon 3 of the gene.³¹ In colorectal cancers, mutations of APC or $\beta$-catenin result in stabilization of $\beta$-catenin and a significant increase of this protein within the cell. Furthermore, accumulated $\beta$-catenin may translocate to the nuclei, and could serve as a transcriptional factor through binding to the Tcf-Lef family.⁴⁻⁷ These results suggested that the association of $\beta$-catenin and members of the Tcf-Lef family might have a transactivation function, transferring cell proliferation signals to the nucleus. In the APC-$\beta$-catenin-Tcf pathway, accumulated free cytoplasmic $\beta$-catenin would behave as an oncoprotein. In fact, mutations of $\beta$-catenin have been identified in colon cancer with normal APC gene,⁸ melanoma,⁹ ovarian cancer,¹⁰ medulloblastoma¹¹ and hepatocellular carcinoma (HCC).¹²⁻¹³ To investigate more fully whether $\beta$-catenin mutation plays an important role in the development of endometrial carcinomas, 35 endometrial carcinomas were examined for mutations of the $\beta$-catenin gene. Furthermore, immunoeexpression of $\beta$-catenin protein was characterized in 17 of these endometrial carcinomas.

MATERIALS AND METHODS

Preparation of DNAs Materials used in this study were obtained during the course of surgical treatments at Sapporo Medical University. All samples were diagnosed histopathologically as endometrial adenocarcinomas. DNAs were extracted from these tumors according to methods described elsewhere.¹⁴

Polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) and mutational analysis of $\beta$-catenin To detect variant sequences in exon 3 of the $\beta$-catenin gene, genomic DNA from each tumor was analyzed for SSCP as described previously¹⁵ with some modifications. A genomic PCR fragment including exon 3 was amplified using primers G-F, 5′-CCAATCTACTAAAGTCATCGTAATCTG-3′ and G-R, 5′-CTGCATTCTGACTTTCAGTAAGG-3′.¹⁵ In this study, 20 µl of reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5
mM MgCl₂, 0.2 mM of each dNTP, 0.4 mM of each primer, 50 ng of genomic DNA and 0.5 units of TaKaRa Taq (recombinant Taq DNA polymerase, Takara, Ohtsu). Each reaction was carried out for 2 min at 94°C for initial denaturing followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. The PCR product was diluted with formamide-dye solution and electrophoresed in 12% polyacrylamide gels containing 10% glycerol at 17°C. After electrophoresis, gels were stained and visualized with SYBR green II (FMC BioProducts, Rockland, ME). The nucleotide sequence of the aberrant PCR product was directly determined using an Applied Biosystem model 373 DNA sequencer (Perkin-Elmer Cetus, Norwalk, CT) with the primer used for PCR and a Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems, Forster, CA). Each mutation was verified in both the sense and antisense directions.

Analysis of rearrangement To detect genomic rearrangements involving exon 3 of the β-catenin gene, we performed PCR amplifications using genomic DNA with primer sets C-F (5′-CCACGCTGGACATGCTTTAG-3′) and C-R (5′-TGAGCTCGAGTCATTGCATTGCATAC-3′), corresponding to the nucleotide sequences of exons 2 and 4, respectively.

**Immunohistochemical staining for β-catenin** Immunohistochemical studies were performed on formalin-fixed, paraffin-embedded tissue samples with a β-catenin monoclonal antibody (Transduction Laboratories, Lexington, KY), as described previously.

**RESULTS**

Exon 3 of the β-catenin gene was subjected to PCR-SSCP and rearrangement analyses, because it encodes the regulatory domain of β-catenin previously found to contain activating mutations. Five of 35 primary endometrial carcinomas tested showed aberrant bands by PCR-SSCP analysis and were further analyzed for β-catenin alterations. Nucleotide sequencing analysis revealed that these five tumors (T32, T53, T60, T67 and T79) contained mutations that altered potential GSK-3β phosphorylation sites (T41A, S37C, T41A, S33C and S37F, respectively).
respectively; examples shown in Fig. 1). The somatic nature of each mutation was established by sequencing DNA derived from normal tissue of the same patient (data not shown), indicating that each mutation appeared to affect one of the two \( \beta \)-catenin alleles. The mutation in T79 detected by direct sequencing was confirmed using RFLP analysis, since the intensity of the mutational band in the tumor was lower than that of the normal band in both the sense and antisense directions. Exon 3 PCR products from tumor T79 and the normal DNA of the same patient were digested with \( XmnI \), since mutations at codon 37 eliminated the \( XmnI \) restriction. Digestion of the normal PCR product resulted in 129-bp and 181-bp fragments. In tumor T79, a larger 310-bp fragment resulting from loss of the restriction site, along with the normal-length product from the unaltered allele, was seen after digestion (Fig. 1E).

To detect interstitial deletions involving exon 3, we performed a PCR experiment to amplify both exon 3 and the entire intronic sequences derived from genomic DNAs of all 35 tumors. We detected no aberrant band smaller than the normal 931-bp product. In a previous analysis of these same samples for replication error (RER) phenotype, none of these tumors with \( \beta \)-catenin mutations (T32, T53, T60, T67 and T79) showed RER. In contrast, no mutations of the \( \beta \)-catenin regulatory domain (exon 3) were found among the 10 samples with the RER-positive phenotype, although this difference was not statistically significant.

Because paraffin-blocked samples were available for 17 of the 35 cases, immunohistochemical staining of these tumors was performed using an anti-\( \beta \)-catenin antibody to analyze the localization and accumulation of \( \beta \)-catenin in the cell. We detected accumulation of \( \beta \)-catenin protein in cytoplasm and nuclei in three cases (T39, T67 and T79; Fig. 2). The other 14 cases showed intense membranous \( \beta \)-catenin expression. Two of these 3 cases (T67 and T79) had missense mutations in exon 3 of the \( \beta \)-catenin gene.

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**Table I. Genetic and Immunohistochemical Study of \( \beta \)-Catenin in Endometrial Carcinomas**

| Tumor | Aberrant SSCP | Affected codon | Mutation Status | Status | IHC |
|-------|---------------|----------------|-----------------|--------|-----|
| T32   | positive      | 41             | ACC(Thr) → GCC(Ala) | heterozygous | n.a. |
| T39   | negative      | none           | none            | n.a.   |     |
| T53   | positive      | 37             | TCT(Ser) → TGT(Cys) | heterozygous | n.a. |
| T60   | positive      | 41             | ACC(Thr) → GCC(Ala) | heterozygous | n.a. |
| T67   | positive      | 33             | TCT(Ser) → TGT(Cys) | heterozygous | (+)  |
| T79   | positive      | 37             | TCT(Ser) → TTT(Phe) | heterozygous | (+)  |

*a) Single strand conformation polymorphism.
*b) Immunohistochemistry.
*c) Not applicable.*
(Table I). We did not find β-catenin mutation in another case (T39) with nuclear and cytoplasmic β-catenin expression. Three cases (T32, T53 and T60) with β-catenin mutation were not available for this immunohistochemical staining.

To characterize the spectrum of β-catenin mutations in endometrial carcinomas, the clinicopathological status of the five patients with mutations was analyzed. It was interesting that the incidence of β-catenin mutation was 3 of 5 cases in the early-onset group (age <40), but only 2 of 30 in the late-onset group (age ≥40) (P=0.014 by Fisher’s exact test). The mean age of patients with β-catenin mutation, 44.6 years, was younger than that of patients negative for β-catenin mutation, 56.0 years. The histological diagnosis of one case (T53) was an adenocarcinoma, and the 4 other cases (T32, T60, T67, T79) were well differentiated endometrioid adenocarcinomas. As for surgical stage, 3 cases (T32, T60, T67), one case (T53) and one case (T79) were stage Ib, IIIc and IIIb, respectively. However, no significant association of these two clinicopathological factors with β-catenin mutation was seen.

**DISCUSSION**

In this study, we examined the status of β-catenin mutation and this protein in endometrial carcinomas. Five of 35 (14%) tumors had genetic alterations in exon 3 of the β-catenin gene. These five mutations were somatic and heterozygous, and occurred at serine/threonine residues in exon 3, which are essential phosphorylation sites for GSK-3β. The three types of mutations we identified (S33C, S37C and T41A) have been recently reported in endometrial carcinomas, and one mutation (S37F) has been reported in an endometrioid ovarian carcinoma. The incidence of β-catenin mutations in endometrial carcinomas was similar to the reported value. These results imply that the development of about 15% of endometrial carcinomas is associated with genetic alterations of the β-catenin gene. Interstitial deletions, which were detected in about 3% of sporadic colorectal cancers and sporadic HCC, were not found in our endometrial samples. To clarify the incidence of deletions in the β-catenin gene in the development of endometrial carcinomas, we should investigate a larger number of tumors in a further study.

Five cases with β-catenin mutations did not show RER. This is not consistent with the suggestion that β-catenin mutations might occur more frequently in RER-positive colorectal cancer. However, we performed genetic analysis of only exon 3 of β-catenin, and to investigate the possible association with a DNA mismatch repair system, we must screen for mutation of all regions of the β-catenin gene. Furthermore, we found a high frequency of β-catenin mutations in early onset endometrial carcinomas. Again, though, our genetic analysis of β-catenin was limited, and the number of cases in the early-onset group in our study was small. To study whether β-catenin mutations play an important role in the development of early-onset endometrial carcinomas, we should screen for mutations in other regions of β-catenin, especially in a large number of younger patients.

Three of 17 cases studied immunohistochemically (18%) showed accumulation of β-catenin protein in the cell nuclei and cytoplasm; the three included two tumors with amino acid alterations, but the other one case (T39) had no mutation in exon 3. The frequency of accumulation of this protein was slightly lower than that (38%) reported previously, although the difference is not statistically significant. Our results of immunohistochemical analysis of β-catenin indicate that these mutations are responsible for the up-regulation of cytoplasmic β-catenin and its distribution in the nuclei of cells. We did not find β-catenin gene mutations in another case (T39) with nuclear and cytoplasmic β-catenin expression; however, our mutation analysis was limited to exon 3, and genetic alterations outside the regulatory domain have been described by others. Taken together, our results suggested that β-catenin with mutation at a serine/threonine residue potentially available for GSK-3β phosphorylation is accumulated in nuclei, and has a dominant oncogenic effect in tumorigenesis of a subset of endometrial tumors, particularly those of the early-onset type.

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