

Mutational Analysis of the *CTNNB1* and *APC* Genes in Uterine Endometrioid Carcinoma

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Despite recent studies, the molecular genetic events responsible for the development of uterine endometrioid carcinoma (UEC) remain incompletely characterized. Mutations in the β -catenin (*CTNNB1*) gene have been recently reported in a small percentage of UECs and in the endometrioid variant of ovarian carcinoma suggesting that the Wnt signal transduction pathway is involved in the development of female genital tract tumors with endometrioid morphology. The Wnt pathway is a critical pathway in the development of colorectal cancer (CRC) with mutations occurring in the β -catenin (*CTNNB1*) or adenomatous polyposis coli (*APC*) genes in 10 to 15% and 85% of cases, respectively. Because UEC and CRC share other molecular genetic alterations and histologic features and previous studies of UEC have not reported an analysis of the *APC* gene, we chose to further elucidate the role of the Wnt pathway in UEC. To this end, we analyzed 32 cases of UEC for mutations of the *CTNNB1* and *APC* genes. Mutations of *CTNNB1* were present in six of 32 (18%) cases: four grade 1 carcinomas, one grade 2, and one grade 3 carcinoma. Five missense mutations were identified, three involving Ser/Thr phosphorylation sites and two adjacent to a Ser phosphorylation site. One case contained a deletion encompassing codons 34 to 37, which includes a Ser phosphorylation site. No mutations resulting in truncation of the APC protein were found. Our results support a role for the Wnt signaling pathway via mutation of *CTNNB1*, but not *APC*, in the development of a subset of UECs.

KEY WORDS: APC, β -Catenin, Mutations, Uterine endometrioid carcinoma, Wnt.

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Endometrial carcinoma is the most common malignancy of the female genital tract in the United States with approximately 37,400 newly diagnosed cases and 6400 deaths expected to occur in 1999 (1). Uterine endometrioid carcinoma (UEC) accounts for 85% of all cases of endometrial carcinoma. This subtype is associated with classic risk factors (such as obesity, diabetes mellitus, hypertension, nulliparity, unopposed estrogen stimulation), tends to occur in peri- or post-menopausal women (mean age, 59 years) and has a relatively favorable prognosis as compared to other types of endometrial carcinoma (e.g., serous or clear cell carcinoma). Morphologically, UECs usually present as exophytic tumors composed of glands resembling proliferative endometrium. The histologic features overlap with those of colorectal carcinoma (CRC), often making the distinction between the two entities difficult when they present as metastatic disease. Also, UEC and CRC share several molecular genetic characteristics (e.g., mutations in the *K-ras* and *p53* genes and microsatellite instability) in a significant proportion of cases (reviewed in 2, 3). Recent studies have shown that 85% of CRC harbor a mutation in the adenomatous polyposis coli (*APC*) gene, and of the remaining tumors with wild-type *APC*, approximately 50% have a mutated β -catenin gene (*CTNNB1*) (4). Both the putative oncoprotein β -catenin and the adenomatous polyposis coli (*APC*) tumor suppressor protein are elements of the Wnt signaling pathway that have been implicated in embryonic development and carcinogenesis.

Activation of the Wnt pathway leads to a sequence of protein interactions involving a transmembranous receptor of the frizzled family and the cytoplasmic proteins dishevelled, glycogen synthase kinase 3β (GSK- 3β), APC, axin and β -catenin, resulting in stabilization of β -catenin (5, 6). The stabilization leads to a relative increase in β -catenin levels with subsequent translocation of β -catenin to the nucleus, where it binds to T-cell factor (TCF)-

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class transcriptional factors and induces expression of TCF-responsive genes (7). Recently, the *c-myc* and *cyclin D1* (*PRAD-1*) genes have been identified as target genes for β -catenin/Tcf induced transcription (8, 9). In non-proliferating cells, the cytosolic level of free β -catenin is downregulated through interaction with a multiprotein complex consisting of APC, GSK-3 β , and axin (reviewed in 10, 11, 12) (Fig. 1). APC promotes the phosphorylation of β -catenin at several Ser/Thr residues encoded in exon 3, which in turn targets β -catenin for ubiquitin-dependent degradation. Similarly, alterations of elements in the Wnt signal transduction cascade (e.g., mutations in *APC* or *CTNNB1*) may result in β -catenin stabilization (4, 11). *APC* mutations most commonly occur within the mutation cluster region (MCR) between codons 1286 and 1513 (13) and result in a truncated form of the protein that lacks the ability to promote β -catenin degradation. Stabilizing mutations of β -catenin most frequently affect Ser/Thr phosphorylation sites encoded in exon 3.

Unlike in CRC, *APC* mutations have not been reported in UEC. However, the findings of mutations in *CTNNB1* and immunohistochemical overexpression of β -catenin in a proportion of cases of uterine endometrioid carcinoma (14, 15) and in the endometrioid variant of ovarian carcinoma (16) suggest a role for the Wnt signaling pathway in tumors with endometrioid features. To further investigate this hypothesis we analyzed 32 UECs for mutations in the *CTNNB1* and *APC* genes.

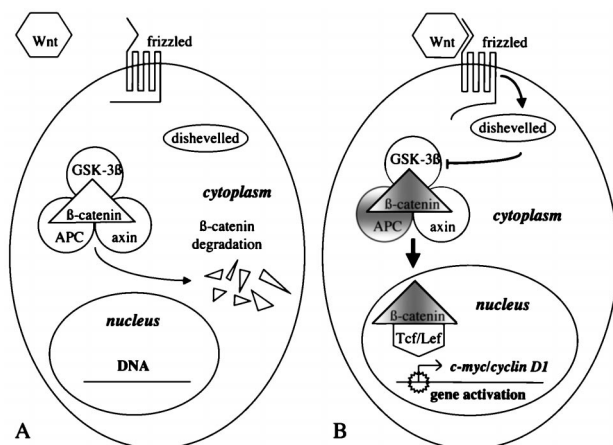


FIGURE 1. A, In the absence of Wnt signaling, β -catenin is degraded through interaction with a multiprotein complex containing APC, GSK-3 β , and axin. B, Wnt signaling via the receptor frizzled and the cytosolic protein dishevelled leads to inhibition of GSK-3 β and thereby suppression of β -catenin degradation. Similarly, stabilizing mutations of β -catenin or truncating mutations of APC result in cytosolic accumulation of β -catenin, its translocation to the nucleus and activation of Tcf responsive genes, such as *c-myc* or *cyclin D1*.

MATERIALS AND METHODS

Samples

Thirty-two cases of uterine endometrioid adenocarcinoma, including 12 grade I tumors, 11 grade II, and nine grade III tumors, were collected from the Johns Hopkins University Tissue Bank. Frozen tumor tissue was microdissected to obtain more than 70% tumor cells. Corresponding normal tissue was microdissected from the same specimens. DNA was isolated and purified by previously described techniques (17, 18).

Mutational Analysis of the CTNNB1 Gene

Sequence analysis of the *CTNNB1* gene was performed by polymerase chain reaction (PCR) amplification of a 159-bp fragment of exon 3 (codons 21 to 73), followed by direct cycle sequencing. PCR amplification of genomic DNA was performed as described previously (16) with slight modifications. In brief, the PCR reaction contained 10 mM Tris-HCl (pH 9.1), 1.5 mM MgCl₂, 75 mM KCl, 0.02% NaOH, 200 μ M dNTPs, 6.25 pmol of each primer (240F: 5' - ATGGAACCAGACAGAAAAGC-3'; 439F: 5'-GCTACTTGTCTTGAGTGAAG-3'), 1.0 U of *Taq* polymerase and 40 ng of genomic DNA in a final volume of 25 μ L. Reaction products were treated with exonuclease I and shrimp alkaline phosphatase using a PCR product presequencing kit (Amersham Life Science, Cleveland, OH) and sequenced with the Thermo Sequenase sequencing kit (Amersham Life Science) with either one of the primers used for the initial amplification. The sequencing reaction was carried out according to the manufacturer's recommendations: 30 cycles at 95° C for 30 seconds, followed by 55° C for 30 seconds and 72° C for 90 seconds. Aliquots of the PCR products were submitted to electrophoresis on a CastAway sequencing device (Stratagene, La Jolla, CA) using precast 6% polyacrylamide gels containing 7 M urea. Gels were exposed to autoradiography film (BioMax, Kodak, Rochester, NY). Mutations were verified by repeating the sequence analysis in both the sense and antisense directions and, when necessary, sequencing cloned products using the Original TA Cloning Kit (Invitrogen Corporation, Carlsbad, CA).

The somatic nature of the mutations was confirmed by analyzing DNA derived from patient matched normal tissue, which showed the wild-type β -catenin sequence in all cases examined.

Mutational Analysis of the Adenomatous Polyposis Coli Gene

To screen for truncation mutations of the *APC* gene, a protein truncation assay (TNT Coupled Reticulocyte Lysate System; Promega, Madison, WI)

was used, following the manufacturer's instructions. Genomic DNA was obtained as described above and used to PCR-amplify two overlapping segments of the *APC* gene, spanning the region from codon 686 to 1693, which includes the mutation cluster region (MCR, codons 1286 to 1513) (13). The following T7-modified sense primers (T7-trans = GGATCCTAATACGACTCACTATAGG-GAGACCACCATGG) were used under the conditions described previously (19):

Segment A (Codons 686 to 1217)

F 5'-[T7-trans]-ATGCATGTGGAACCTTGTGG-3'
R 5'-GAGGATCCATTAGATGAAGGTGTGGACG-3'

Segment B (Codons 1099 to 1693)

F 5'-[T7-trans]-TTTCTCCATACAGGTCACGG-3'
R 5'-GGAGGATCCTGTAGGAATGGTATCTCG-3'

The PCR reaction contained 10 mM Tris-HCl, 1.5 mM Mg Cl₂, 75 mM KCl, 0.0325% NaOH, 40 ng of genomic template DNA, 50 pmol of each primer, 2.5 U *Taq* polymerase and 200 μM dNTPs in a final volume of 50 μL. Amplification was carried out in 35 cycles of 95° C for 60 seconds, 55° C for 90 seconds and 70° C for 120 seconds; mononucleotides were added to the reaction mix during the first cycle at a temperature of at least 80° C.

PCR products were purified by phenol-chloroform extraction and sodium-perchlorate precipitation. The combined *in vitro* transcription/translation based TNT assay was performed using 2 μg of these PCR products and 2 μL [³⁵S]methionine (1000Ci/mmol) in a 50-μL reaction as described by the manufacturer. The reaction product was admixed with protein sample buffer (final concentrations: 60 mM Tris, pH 6.8; 2% SDS; 10% glycerol; 0.7 M 2-mercaptoethanol; 0.025% Bromphenol Blue), and boiled for 5 min. Sample aliquots containing 5 μL of the TNT product were separated by SDS-polyacrylamide gel electrophoresis using 4 to 20% Tris-HCl gels (Ready Gel, BioRad, Hercules, CA). The protein bands representing the generated APC-segments were visualized by autoradiography (BioMax, Kodak, Rochester, NY). Negative controls without DNA were run in parallel for each reaction.

Protein Extraction and Western Blotting

Fresh frozen tumor tissue was pulverized on dry ice and suspended in lysis buffer (final concentrations: 20 mM Tris, pH 7.5; 1 mM EDTA; 100 mM NaCl; 1% Triton-X-100; 0.5% Deoxycholic acid; 0.1% Sodium Dodecyl Sulfate; 1 mM EGTA; 1 mM PMSF; 0.5 μg/mL Leupeptin; 5.0 μg/mL Antipain; 0.7 μg/mL Pepstatin A; 50 mM NaF; 10 mM β-Glycerophosphate; 10 mM Na-pyrophosphate; 1 mM Na-Vana-

date; 10 μM Na-Molybdate). After incubation and centrifugation the supernatant was admixed with Laemmli buffer and submitted to electrophoresis on 10% polyacrylamide Tris-HCl gels in a Ready Gel Cell device (BioRad), following the manufacturer's instructions. Proteins were transferred to Immobilon P membrane (Millipore, Bedford, MA) and probed with antibodies directed against c-myc or cyclin D1 proteins (both antibodies from Santa Cruz, CA; both antibodies were used in a 1:200 dilution). Protein bands were visualized with the ECL kit (Amersham, Arlington Heights, IL) and exposure to autoradiography film (BioMax).

RESULTS

Mutational analysis of the *CTNNB1* was performed by sequencing a 159-bp fragment of exon 3 (codons 21 to 73), which encompasses the sequence for multiple GSK-3β phosphorylation sites. *CTNNB1* mutations were found in six of 32 (18%) cases. Five missense mutations were identified, three involving Ser/Thr phosphorylation sites and two adjacent to a Ser phosphorylation site at codon 33 (Table 1 and Fig. 2). In one case, a deletion of 12 bp, corresponding to an in-frame loss of codons 34 to 37, was detected. This includes the Ser 37 phosphorylation site. In all tumors with *CTNNB1* mutations, the wild-type sequence was also identified, indicating that only one allele was mutated. Of the six cases with mutations, four were grade 1 carcinomas and, one each, a grade 2 and a grade 3 carcinoma.

A protein truncation assay was used to screen for mutations of the *APC* gene between codons 686 and 1693. No mutations resulting in truncation of the APC protein were found (Fig. 3).

Limited amounts of fresh frozen tissue were available from fifteen of the 32 primary tumors in our series, among them four of the six tumors with β-catenin mutations. Protein extracts of those samples were analyzed for c-myc- and cyclin D1 expression by Western blot, using control samples from tumors matched for histologic features, tumor grade and stage, and patient age. However, no cor-

TABLE 1. Mutations of CTNNB1 in Uterine Endometrioid Carcinoma

Case	Tumor Grade	Mutated Codon	Base Change	Amino Acid Substitution
1	1	32	GAC → TAC	Asp → Tyr
2	1	45	TCT → CCT	Ser → Pro
3	1	37	TCT → TTT	Ser → Phe
4	2	32	GAC → TAC	Asp → Tyr
5	3	41	ACC → GCC	Thr → Ala
6	1	34to37	12bpDeletion	4aa Deletion

bp, base pair; aa, amino acid.

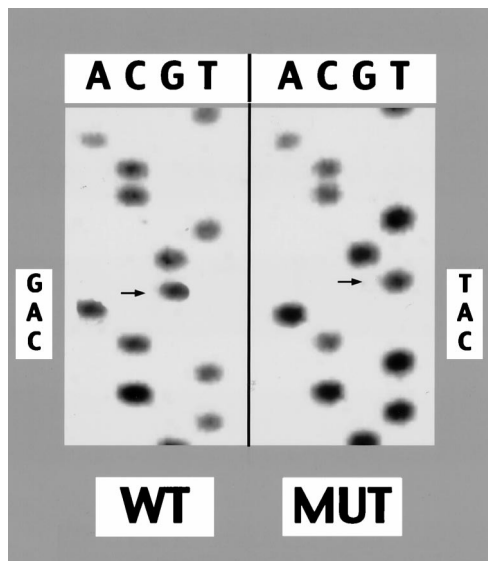


FIGURE 2. DNA sequence from the regulatory domain of the *CTNNB1* gene: G→T substitution at codon 32 (WT, wild type; MUT, case 1 from Table 1).

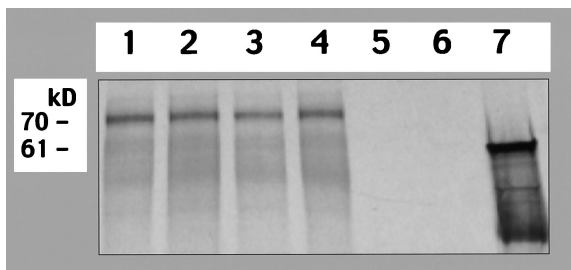


FIGURE 3. Protein truncation assay of APC-segment B. The reaction results in synthesis of a 70 kDa APC protein fragment. Four representative tumor samples (*lanes 1–4*) are shown. No aberrant proteins are identified. Negative controls (*lanes 5–6*), run without DNA, do not express protein. The positive control (*lane 7*), run with DNA encoding luciferase, shows the respective protein product of 61 kDa.

relation with the β -catenin mutational status was evident (data not shown).

The mutational status of the *Ras* oncogene, the *p53* and *PTEN* tumor suppressor genes, the microsatellite instability status, and the *MLH1* promoter methylation status of most of the analyzed cases were known as published previously (18, 20, 21, 22). No statistically significant correlation could be established between any of these parameters and the β -catenin mutational status.

DISCUSSION

β -catenin is a multifunctional protein initially identified as part of a complex connecting the cell adhesion molecule E-cadherin to α -catenin and the cytoskeleton. Subsequently, it was established as an essential protein in the Wnt signaling pathway. Activation of Wnt signaling, resulting from mutations in either the *APC* or β -catenin (*CTNNB1*) gene, has been implicated in the pathogenesis of colorectal

carcinoma (CRC) (4, 23), melanoma (24), medulloblastoma (25), prostate (26), gastric (27), and hepatocellular carcinoma (28, 29). Recently, mutations in *CTNNB1* were also identified in uterine endometrioid carcinoma (UEC) (14, 15) and in the endometrioid variant of ovarian carcinoma (16); however, neither study included analyses of the *APC* gene.

In this study, we analyzed 32 cases of UEC and identified somatic mutations in the regulatory domain of the β -catenin gene in six out of 32 tumors (18%). Mutations similar to those detected in this study have previously been described (4, 14, 16, 23). Our study confirms previous observations that β -catenin mutations occur in about 15% of UECs and tend to be more frequent in low-grade, low-stage tumors (14, 15). β -catenin mutations have been reported so far only in UECs, but not in other subtypes of endometrial carcinoma. Preliminary data from our own laboratory confirm that β -catenin overexpression is present in a subset of UECs, but not uterine serous carcinomas. Thus, molecular analysis suggests that the different morphologic characteristics reflect different underlying genetic alterations. Immunohistochemical studies (14, 15, 16) showed cytoplasmic and/or nuclear overexpression of β -catenin in cases with, but also without, β -catenin mutations. Some authors hypothesize that β -catenin accumulation in the latter cases may be due to *APC*-mutations (16). To our knowledge, we report the first study of the mutational status of *APC* in UEC; however, no truncating mutations of the *APC* gene between codons 686 and 1693 were found. In our analysis, we focused on those regions of the *CTNNB1* and *APC* genes that are known to frequently harbor mutations in CRC. It is possible that UEC is predisposed to a different spectrum of mutations in these genes which was not covered by our methods.

β -catenin has been shown to be a downstream activator of the Wnt signaling pathway by binding Tcf transcription factors. Recently, *c-myc* and *cyclin D1* were identified as targets of β -catenin/Tcf-mediated transcriptional activation (8, 9). Overexpression of β -catenin, attributable to either stabilizing mutations of *CTNNB1* or truncating mutations of the *APC* gene, was shown to upregulate *c-myc* expression in colon cancer cell lines. This finding provides a plausible explanation for the observed overexpression of *c-myc* protein in CRC. *C-myc* overexpression is also found in more than 50% of endometrial tumors (30, 31, 32), which may be the result of upregulation of the Wnt signaling pathway. Western blot analysis of selected cases from our series did not reveal a correlation between *c-myc* expression and β -catenin mutational status. A possible explanation is that *c-myc* expression is determined by multiple factors, of

which β -catenin is only one. In addition, mutations in *CTNNB1* may have a different effect on c-myc in UEC than CRC.

Similarly, we were unable to demonstrate cyclin D1 expression in our tumor samples by Western blot analysis. Immunohistochemical studies had shown increased expression of cyclin D1 in 40 to 56% of UEC (33, 34). Interestingly, cell proliferation after estrogenic stimulation is associated with upregulation of both cyclin D1 and c-myc (35). Conversely, both cyclin D1 and c-myc can mimic estrogen effects on S-phase entry during the cell cycle (36, 37). Thus, unopposed estrogen stimulation of the endometrium may be due to upregulation of cyclin D1 and/or c-myc. Since there is no evidence of interaction between the estrogen and Wnt signaling cascades, it is possible that both pathways converge towards a common step that includes upregulation of cyclin D1 and c-myc to promote proliferation.

In summary, we found mutations in the β -catenin gene in 18% of UEC. These results support a role for the Wnt signaling pathway, via mutation of *CTNNB1* but not *APC*, in the development of a subset of UEC. Future studies of endometrial precursor lesions (e.g., complex atypical hyperplasia) and cell lines are needed to determine the timing and consequence of such mutations in UEC.

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Book Review

Iozzo RV, editor: *Proteoglycans: Structure, Biology, and Molecular Interactions*, 422 pp, New York, Marcel Dekker, Inc., 2000 (\$185).

Even current pathology literature abounds with unfortunate references to mucosubstances or to ground substance. Decades ago that was, perhaps, a defensible way to avoid immersion into a complex topic fraught with unknowns and confusion. The combination of advances in cell and molecular biology has removed the veil of confusion that covered those molecules containing complex carbohydrates (mucosubstances), making proteoglycans and glycosaminoglycans as amenable to study as proteins or any other class of biologic molecules. This first-rate volume does a commendable job in summarizing, in a clear, lucid, and understandable manner, the current knowledge in the field of proteoglycans.

The editor and the 31 authors of this excellent volume deserve congratulations. The 15 book chapters cover all major proteoglycans with clear and up-to-date information on their chemical structure, biosynthesis, catabolism, interactions with the extracellular matrix, cell membranes, cytokines, and the myriad of biologic functions possessed by these fascinating molecules. There are abundant diagrams that help clarify points and solidify information. The only

criticism, and it is a minor one, is the lack of a short introduction clarifying the nomenclature. Initially proteoglycans were named by their constituent glycosaminoglycan chains: thus, chondroitin sulfate, heparan sulfate, etc. Later, it was realized that similar glycosaminoglycan chains could be attached to different proteins, and a qualification was introduced: basement membrane heparan sulfate, cell membrane heparan sulfate. After the isolation of the genes coding for the different core proteins, the excellent decision was made to name the proteoglycans according to the gene product: thus, perlecan, syndecan, etc. To those not following the literature on a regular basis, the nomenclature may introduce some difficulty. Nevertheless, this is a minor point and after reading a few chapters the problems disappear. This book can be enthusiastically recommended, not only to those interested in proteoglycans, but also to all concerned with the extracellular matrix and cell biology. In particular it would be helpful to pathologists and may, perhaps, help to stamp out the old term mucosubstances.

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