Frequent β-Catenin Mutation and Cytoplasmic/Nuclear Accumulation in Pancreatic Solid-Pseudopapillary Neoplasm

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Abstract

Significance of Wnt signaling with β-catenin mutations on solid-pseudopapillary neoplasm (SPN) of the pancreas was studied by immunohistochemistry and molecular analysis. On immunohistochemistry, all 18 SPNs tested showed diffuse cytoplasmic/nuclear positivity for β-catenin. Upon direct DNA sequencing of exon 3 of the β-catenin gene, 15 (83%) of the 18 SPNs showed 1-bp missense mutation in codons 32 (5 cases), 33 (3 cases), 34 (3 cases), 37 (3 cases), and 41 (1 case). Immunoreactivity for cyclin D1, one of the intranuclear targets of β-catenin complexes, was found in tumor cells of more than half the tumor cells of all the 18 SPNs. The present study strongly suggested a significant role of Wnt signaling, mostly associated with β-catenin mutations in the tumorigenesis of SPN.

Introduction

SPN2 of the pancreas is a rare and distinct tumor classified in tumors of the exocrine pancreas in the current WHO classification (1). SPN is known for its indolent biological behavior and predilection in young women (1–3). The cell of origin in SPN remains uncertain (1–3). Although several studies have referred to molecular/cytogenetic characteristics of pancreatic tumors (4–6), few reports have been concerned with molecular changes of SPNs. Molecular changes often detected in pancreatic DCs, such as alterations of p53 and K-ras, have not been detected in SPNs (3, 6–8). The protein β-catenin was originally identified as a submembranous element of the cadherin-mediated cell-cell adhesion complex. β-Catenin acts as a downstream transcriptional activator of Wnt signaling, by accumulating in the nucleus and forming complexes with the DNA-binding proteins such as Tcf and Lef-1 (9). Recent studies have identified the target genes of these transcriptional factors, including one of the cell cycle regulators, cyclin D1 (10). During the systemic immunohistochemical study of β-catenin in pediatric tumors, we found a consistent cytoplasmic/nuclear reactivity for β-catenin in SPNs. This novel finding prompted us to perform a large-scale study of β-catenin immunoreactivity and mutation analysis of the β-catenin gene in SPNs.

Materials and Methods

Case and Tissue Selections

Age and sex of the 18 patients with SPN is shown in Table 1. Cases 12–14 had been included in the previous immunohistochemical study (11). The ages ranged from 11 to 61 years (mean, 33 years; 13 females and 5 males). All tumors were completely resected and have not recurred or metastasized without additional therapy. No case had a history of FAP. The tumor sample was fixed in 10% buffered formalin, processed routinely, and embedded in paraffin. Four-μm sections were stained with H&E. Histopathological diagnosis was confirmed by the authors, including at least four pathologists.

Immunohistochemistry

Immunohistochemical studies were performed on paraffin sections, which were deparaffinized, hydrated, and immersed in 0.3% hydrogen peroxide in methanol for 30 min to block the endogenous peroxidase activity. For the detection of β-catenin and cyclin D1, monoclonal antibodies against each protein (clone 14, Transduction Laboratories, Lexington, KY; and clone P2D11F11, Novocastra, Newcastle, United Kingdom, respectively) were applied at a dilution of 1:100 and 1:200, respectively; followed by peroxidase-conjugated antimouse immunoglobulin antibody (MBL, Nagoya, Japan; 1:50). Antibody retrieval was performed by microwave treatment for two 10-min cycles with 180 W of power in 10 mm citrate buffer (pH 6.0) and in 1 mM EDTA (pH 8.0) for β-catenin and cyclin D1, respectively. Final visualization was carried out by development in PBS (pH 7.6) containing 20 mg/100 ml of diaminobenzidine tetrachloride and 0.1% H2O2 for 10 min. Sections incubated with PBS instead of the primary antibodies served as negative controls. The sections were counterstained by Mayer’s hematoxylin.

Genomic DNA Preparation

Sufficient numbers of formalin-fixed, paraffin-embedded sections were available in 17 cases (all cases other than case 9). Tumor areas were dissected from 5 to 10 4-μm sections, which were deparaffinized in xylene, rehydrated in ethanol, and air dried. Adjacent nontumorous tissue was found in 8 cases and was separately dissected. Fresh frozen tumor samples were available in 2 cases (cases 6 and 9). DNA extraction was performed according to the methods described previously (12, 13). DNA was dissolved in 20 μl of 10 mM Tris-HCl (pH 8.0).

Mutational Analysis

Genomic DNAs from the sections were amplified by seminested PCR, using 5′-BCTN (5′-TGATTTGATGGAGTTGGACATG-3′) and 3′-BCTN (5′-CCTCTTCTCAGAGTGCCTTCTT-3′) primers for the first PCR and using the same 5′-BCTN and 3′/2nd (5′-TCAGAGTGGC-GCTTTACCACCTA-3′) primers for the second PCR. These primers were designed to amplify a 144-bp DNA fragment of β-catenin gene exon 3, containing the sequence for phosphorylation by glycogen synthase kinase-3β. The thermal cycler (Perkin-Elmer Cetus 480; Applied Biosystems, Foster City, CA) was programmed as follows: initial denaturation at 94°C for 10 min and 35 amplification cycles for the first PCR; and initial denaturation at 94°C for 10 min and 30 amplification cycles for the second PCR. One amplification cycle comprised denaturation at 94°C for 30 s, annealing at 66°C for 30 s, and elongation at 72°C for 1 min. Because DNAs from 4 tumors (cases 13, 14, 16, and 17) were not amplified by this procedure, additional experiments were done to amplify shorter fragments (95 bp) using 5′-BCTN (short) (5′-TGTTAGTCACTGCGGACAAAGC-3′) and 3′-BCTN (short) (5′-CCTCTTCTCAGAGTGCTTTACCACCTA-3′) primers for the first PCR and, for the second PCR, the same 5′-BCTN (short) and 3′-BCTN (2nd short) (5′-CAGAGTGGCCCTTTACCACCTA-3′) primers under the same PCR schedule. Genomic DNAs from frozen samples were amplified using 5′-BCTN exon 3 (5′-CTGATTGATGGAGTTGGACATG-3′) and 3′-BCTN exon 3 (5′-CCTCTTCTCAGAGTGCTTTACCACCTA-3′) primers to amplify a 222-bp fragment. The PCR was carried out according to the same procedure of the first PCR for the paraffin sections. The PCR products were confirmed by agarose gel electrophoresis, purified using GFX Gel Band Purification kit (Amersham Pharmacia), and

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2 The abbreviations used are: SPN, solid-pseudopapillary neoplasm; DC, ductal carcinoma; FAP, familial adenomatous polyposis; NET, neuroendocrine tumor.

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performed a large-scale activity (Fig. 1, nonneoplastic pancreatic parenchyma showed only membranous reactivity. Acinic cells, duct cells, and some islet cells in the tumors examined (Fig. 1, found in the cytoplasm and the nuclei of almost all tumor cells of 18 cases). Membranous reactivity was in- 

duced with ABI Genetic Analyzer Model 310 (Applied Biosystems). Each primer was amplified using the ABI Big Dye Terminator Cycle Sequencing kit (Applied Biosystems) and the primers used for PCR for amplifying shorter fragments.

**Results**

**Conventional Histopathology.** H&E-stained sections of each tumor sample showed typical histopathology of SPN, characterized by the growth of uniform polygonal cells arranged around minute fibrovascular stalks, exhibiting pseudo-papillary pattern (Fig. 1a). The tumors also shows a solid monomorphous pattern, sclerosis, and cystic degeneration in variable degree.

**Immunohistochemistry.** Intense β-catenin immunoreactivity was found in the cytoplasm and the nuclei of almost all tumor cells of 18 tumors examined (Fig. 1, b and c). Membranous reactivity was inconspicuous. Acinic cells, duct cells, and some islet cells in the nonneoplastic pancreatic parenchyma showed only membranous reactivity (Fig. 1, b and d). More than half the tumor cells in each tumor showed nuclear reactivity for cyclin D1 (Fig. 1e).

**Mutational Analysis.** Fifteen of 18 tumors (83%) showed 1-bp missense mutations in the region between codons 34 and 41 of exon 3 of the β-catenin gene. The mutations were detected in paraffin sections of 14 of 17 tumors and in fresh-frozen samples of 2 of 2 tumors. The involved codons are as follows: codon 32 (5 cases), codon 33 (3 cases), codon 34 (3 cases), codon 37 (3 cases), and codon 41 (1 case; Fig. 2). No mutation was found in any corresponding nontumorous tissue.

**Discussion**

Although there have been several molecular studies on pancreatic tumors, they were mostly focused on DC and NET, each being a major component of pancreatic tumors (4–6, 14). Gerdes et al. (14) performed a large-scale β-catenin mutation analysis for pancreatic DCs and NETs. In their study, single-strand conformation polymorphism analysis revealed no variant bands in exon 3 of the β-catenin gene in any of the 78 DCs and the 33 NETs nor in the 14 pancreatic cancer cell lines analyzed, and intracellular β-catenin accumulation was not identified in any of the 40 DCs examined. They concluded that Wnt signaling linked with β-catenin mutation does not play a significant role in tumorigenesis of those tumors (14). No attention, however, has been paid to the relationship between β-catenin and SPNs.

We identified cytoplasmic/nuclear immunoreactivity for β-catenin and mutations of exon 3 of the β-catenin gene in 18 (100%) and 15 (83%) of 18 SPNs, respectively. These mutations were all 1-bp missense mutations that lead to loss of serine/threonine sites for glycogen synthase kinase-3β phosphorylation or may interfere with degradation of the β-catenin gene product. These results suggested that Wnt signaling activated mostly by mutations of the β-catenin gene exon 3 has an important role for the development of SPN.

Among patients with FAP, neoplasms typically arise through biallelic (germ-line then somatic) inactivation of adenomatous polyposis coli gene. The present series, however, included no case associated with FAP, and a literature review showed only 1 SPN case with a history of FAP (15). Nuclear β-catenin accumulation can result from somatic mutations in adenomatous polyposis coli or axis inhibitor 1 genes (16, 17). Because of the demonstration of cytoplasmic/nuclear β-catenin accumulation in all 18 SPNs despite the lack of β-catenin gene mutations in 17% (3 of 18 tumors) of the SPNs examined, some of SPNs are presumed to have other molecular changes activating Wnt signaling. Although few SPNs with malignant course have been reported, SPNs usually behave in a benign fashion (1–3). In this study, more than half the tumor cells of all SPNs conducted showed nuclear overexpression of cyclin D1. Cyclin D1, which participates in the cell cycle control at the G1-S transition, has been shown to be one of the target molecules of β-catenin/Lef-1 complexes (10). Although overexpression of cyclin D1 generally indicates aggressive behavior of the tumor, recent studies have shown the contrary observation (18). Cyclin D1 contributes not only to oncogenic transformation but also to growth arrest, and some investigators speculated that the decision for cell growth or arrest is associated with the concentration of cyclin D1 (19). A recent report has shown overexpression of cyclin D1 in two SPNs (20). Nuclear accumulation of β-catenin and possibly subsequent overexpression of cyclin D1 may have some relationship with mostly favorable clinical behavior of SPNs.

**Table 1 Clinical abstract and mutations in the β-catenin gene in the solid-pseudopapillary neoplasms**

<table>
<thead>
<tr>
<th>Case</th>
<th>Age</th>
<th>Sex</th>
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<td>Ser → Pro</td>
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</table>

*In case 6, the mutation was found both in paraffin sections and frozen sample. In case 9, the mutation was found in a frozen sample (paraffin sections were not available). In cases 13, 14, 16, and 17, the mutations were found by direct DNA sequencing after PCR for amplifying shorter fragments.
In summary, the present study revealed the Wnt signaling mostly associated with β-catenin mutations may play an important role in tumorigenesis of SPN for the first time.

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