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β-Catenin Mutation Is a Frequent Cause of Wnt Pathway Activation in Gastric Cancer[1]

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ABSTRACT

Studies of Wnt activation in gastric cancer have yielded conflicting results. The goals of this study were to determine the frequency of Wnt pathway activation and *β-catenin* mutation in these tumors. Three hundred eleven gastric cancers were examined for β-catenin expression by immunostaining and dissected using laser capture microscopy to obtain DNA from those tumors with nuclear β-catenin. Exon 3 of *β-catenin* was amplified using PCR and sequenced. Ninety gastric cancers (29%) displayed nuclear β-catenin. DNAs from 73 tumors were amplified and sequenced; 19 (26%) contained mutations in exon 3 of *β-catenin*, whereas no mutations were detected in 19 tumors negative for β-catenin nuclear staining ($P < 0.05$). Most mutations were adjacent to or abolished known regulatory phosphorylation sites. Mutations in exon 3 of *β-catenin* are common in gastric cancer that display nuclear β-catenin. These results suggest that Wnt pathway activation contributes to carcinogenesis in a subset of gastric adenocarcinomas.

INTRODUCTION

The Wnt pathway was initially characterized for its role in development; however, recent studies have revealed that dysregulation of Wnt signal transduction plays an important role in human tumor development. β-catenin, a M_r 92,000 protein that also functions in cadherin-based epithelial cell adhesion, is a key regulator of Wnt signaling. Elevation of the cytoplasmic pool of β-catenin occurs by the binding of Wnt ligands or by gene mutation in either *APC*, *axin*, or *β-catenin*. Accumulation of cytoplasmic β-catenin results in its binding to the members of the Tcf/Lef family of architectural transcription factors and its entry into the nucleus (1, 2). On their own, Tcf-Lef proteins are weak transcriptional activators; binding of β-catenin induces a significant increase in transcriptional activity (2, 3). Gene targets of Tcf/β-catenin include growth-promoting genes such as c-*myc* and *cyclin D1*, the matrix-remodeling enzyme *matrilysin*, the nuclear receptor *PPARδ*, as well as *gastrin*, *connexin 43*, *WISP1*, and *WISP2* (4–10).

Several lines of evidence implicate the Wnt signaling pathway as a contributor to gastric carcinogenesis. Persons with germ-line mutation of the *APC* tumor suppressor gene have a 10-fold increased risk of developing gastric cancer as compared with normal persons (11). Mutations in the *APC* gene have also been found in sporadic gastric cancers. Nakatsuru *et al.* (12) reported *APC* mutations in 12 of 46 gastric cancers. Ten of the 12 mutations were noted in intestinal and signet-ring carcinomas. Other investigators noted lower rates of *APC* mutation in diffuse-type gastric cancers. There have been five studies examining the incidence of *β-catenin* mutation in gastric cancer. The initial report by Candidus *et al.* (13) reported no mutations in 16 diffuse- and 5 intestinal-type tumors. Park *et al.* (14) reported mutations in 7 of 26 intestinal-type gastric cancers, but no mutations in 17 diffuse tumors. Woo *et al.* (15) described β-catenin nuclear staining in 81 of 303 tumors (27%). Seventy-seven tumors with nuclear β-catenin were analyzed, and 4 (5%) β-catenin mutations were detected. Another recent report examined diffuse tumors only and noted nuclear staining for β-catenin in 23%; however, no mutations were detected from a total of 35 specimens (16). Thus, although *β-catenin* mutations have been detected in gastric cancer, the incidence has varied greatly among studies. The incidence of Wnt activation in intestinal- *versus* diffuse-type tumors remains unclear.

The goals of this study were to determine: (*a*) the incidence of β-catenin nuclear localization in gastric cancer; (*b*) the incidence of *β-catenin* mutations in tumors with and without nuclear β-catenin; and (*c*) the incidence of *β-catenin* mutation in diffuse- *versus* intestinal-type gastric cancers. We report that β-catenin nuclear localization occurs in approximately one-third of gastric tumors, that the presence of β-catenin nuclear localization is a useful indicator of Wnt pathway activation in gastric cancer, and that *β-catenin* mutations occur in both diffuse- and intestinal-type gastric cancer at a higher rate than has been recognized previously.

MATERIALS AND METHODS

Tumor Samples and Immunohistochemistry. Three hundred eleven gastric adenocarcinomas were collected as part of Southwestern Oncology Group (SWOG) Study 9008 and stored in the Southwestern Oncology Group gastrointestinal tumor repository at the University of Cincinnati. Formalin-fixed, paraffin-embedded tissue blocks from these 311 gastric adenocarcinomas were cut in 4-μm-thick sections and placed onto positively charged slides. Slides were incubated overnight, deparaffinized in xylene, and rehydrated in decreasing concentrations of ethanol:water. Epitope unmasking was accomplished by microwaving in 10 mM citrate buffer pH 6.0 for 15 min, followed by cooling for 30 min at room temperature. The sections were incubated with an anti-β-catenin monoclonal antibody (Transduction Laboratories, Lexington, KY) at a dilution of 1:200 for 2 h at 37°C. Immunohistochemical staining was performed using an indirect biotin streptavidin 3,3′-diaminobenzidine method and the Ventana 320 ES automated immunostainer (Ventana Medical Sytems, Tucson, AZ). Slides were lightly counterstained with Mayer's hematoxylin. Adjacent normal-appearing gastric tissues were used as controls. Staining was scored in a semiquantitative manner by two independent observers (O. J. K. and C. F. P.). Nuclear staining was scored for intensity (0, 1 = weak and 2 = strong) and the percentage of cells stained (0, 1 = 1–25%; 2 = 26–50%; 3 = 51–75%; 4 = >75%). The presence of any degree of nuclear staining was deemed positive.

LCM[3] and DNA Extraction. All tumors that displayed nuclear staining for β-catenin were selected for LCM. Normal-appearing lymphocytes within the same tissue sections were used as internal controls. An additional 19 randomly selected tumors that were negative for nuclear β-catenin were also

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[3] The abbreviation used is: LCM, laser capture microdissection.

subjected to LCM. Using the β-catenin-stained slides as road maps, serial sections were stained with H&E and left without coverslips. LCM was then used to microdissect the cells in the region of the nuclear staining cells (Arcturus, Mountain View, CA). All cases were dissected by a dedicated pathologist (O. J. K.). The microdissected tissues were placed in a 0.5 ml microcentrifuge tube and treated with digestion buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 1.0% Tween 20] containing 400 μg/ml proteinase K at 55°C for 12 h. Samples were then heated to 95°C for 10 min to inactivate the proteinase K and used for PCR.

PCR Amplification and Sequence Analysis. After LCM and DNA extraction, exon 3 of *β-catenin* was amplified by PCR. The primers used to amplify exon 3 of *β-catenin* were forward primer 5′-TAACATTTCCAATCTACTAATGC-3′ and reverse primer 5′-AGCTACTTGTTCTTGAGTGAAG-3′. The PCR reaction was performed in a 12.5-μl reaction volume containing 1 μl of template DNA, 6 mM of each primer, 200 μM deoxynucleotide triphosphates, 2.0 mM $MgCl_2$, 4% DMSO, 0.6 unit of AmpliTaq DNA polymerase, and 1.25 μl of 10× PCR buffer containing 100 mM Tris-HCl (pH 8.3) and 500 mM KCl (Applied Biosystems, Forest City, CA). The reaction mixture was preincubated for 5 min at 95°C and then incubated for 35 cycles of 94°C for 1 min, annealing at 54°C for 2 min, and extension at 72°C for 3 min on a MJ systems thermal cycler (MJ Research). The final extension was continued at 72°C for 7 min. An aliquot of the PCR product was electrophoresed on a 1.5% Seakem GTG agarose gel in TBE buffer (FMC Bioproducts, Rockland, ME). The PCR products were visualized with ethidium bromide and purified using the Qiaquick PCR purification kit (Qiagen, Valencia, CA). Sequence analysis was performed using the USB ThermoSequenase Radiolabeled Terminator Cycle Sequencing kit (USB, Cleveland, OH) in both the sense and antisense directions using the same primers as for the PCR amplification. All mutations were verified by repeat sequence analysis in the sense and antisense directions.

RESULTS

Subcellular Localization of β-catenin. In normal gastric mucosa, β-catenin staining localized to the cell membrane, while cytoplasmic and nuclear staining was generally absent (Fig. 1*A*). In 90 gastric cancers (29%), β-catenin nuclear staining was present (Fig. 1*B*). In the remaining 221 tumors (71%), β-catenin localized to the cell membrane only. The adjacent gastric mucosa was also negative for nuclear β-catenin staining. The 90 tumors that stained positive for nuclear β-catenin were selected for LCM and mutation analysis (Fig. 1, *C–E*).

Mutational Analysis of β-Catenin. In 73 of 90 tumors, DNA was of suitable for quality for PCR and sequence analysis. Mutations in exon 3 of *β-catenin* were detected in 19 of 73 tumors (26%), whereas no mutations in this exon were detected in lymphocyte DNA extracted from these same tumors or in 19 tumors negative for nuclear β-catenin staining ($P < 0.05$; Table 1). One intestinal-type tumor contained three separate mutations at codons 25, 37, and 48 sampled from different areas of the tumor. Thus, a total of 21 mutations were detected. Intestinal-type tumors contained mutations in 10 of 43 (23%) tumors, whereas diffuse-type tumors had mutations in 9 of 24 (38%; P = not significant). No mutations were noted in 6 tumors of other histological subtypes. Five mutations occurred at codon 37, a mutational hotspot described in numerous tumor types. There were

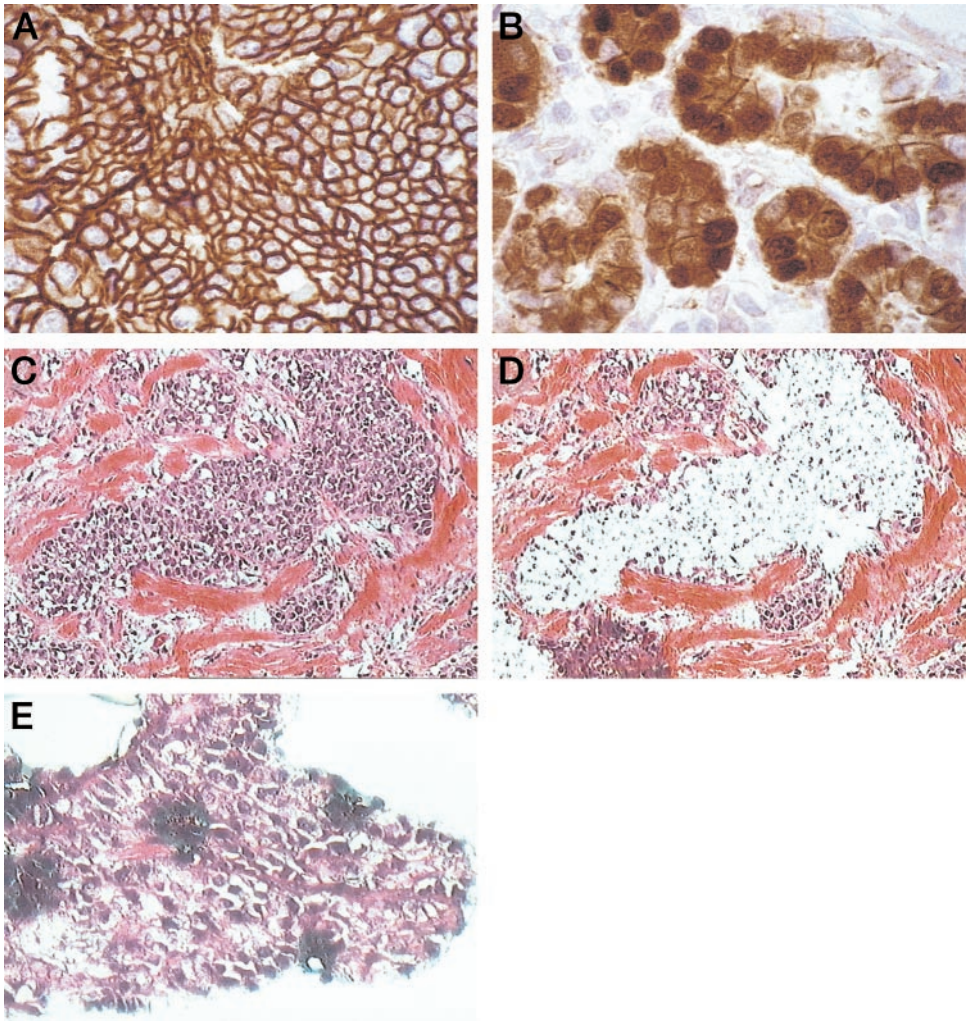

Fig. 1. Examples of β-catenin immunostaining and H&E-stained tissue sections used for LCM in gastric adenocarcinomas. *A*, normal gastric mucosa immunostained for β-catenin. Note the presence of pure membranous staining. *B*, a gastric adenocarcinoma with nuclear localization of β-catenin. *C*, the adjacent tissue stained with H&E. *D*, the same section after LCM microdissection. *E*, a high power view of the sample obtained from laser capture. Note the pure population of gastric cancer cells.

Table 1 *β-catenin exon 3 mutations in gastric cancer*

Sample	Codon	Mutation	Amino acid change	Histology	Location of mutation relative to phosphorylation site
1	8	ATG → ATA	Met → Ile	Diffuse	None
2	11	GAC → AAC	Asp → Asp	Intestinal	None
4	13	GCC → ACC	Ala → Thr	Diffuse	None
5	21	GCT → ACT	Ala → Thr	Intestinal	None
6	24	CAC → TAC	His → Tyr	Intestinal	None
7	25	TGG → TTG	Trp → Leu	Intestinal	None
8	28	CAG → TAG	Gln → Stop	Diffuse	Adjacent
9	29	TCT → GCT	Ser → Ala	Intestinal	Phosphorylation site
10	32	GAC → TAC	Asp → Tyr	Intestinal	Adjacent
11	32	GAC → AAC	Asp → Asp	Diffuse	Adjacent
12	37	TCT → TTT	Ser → Phe	Intestinal	Phosphorylation site
13	37	TCT → GCT	Ser → Ala	Diffuse	Phosphorylation site
13[a]	55	GAG → GGG	Glu → Gly	Diffuse	None
14[b]	37	TCT → GCT	Ser → Ala	Diffuse	Phosphorylation site
15	37	TCT → GCT	Ser → Ala	Intestinal	Phosphorylation site
16	39	GCC → GGC	Ala → Gly	Diffuse	Adjacent
17	47	AGT → ACT	Ser → Thr	Diffuse	Phosphorylation site
18	48	GGT → GTT	Gly → Val	Intestinal	Adjacent
19[c]	25	TGG → TTG	Trp → Leu	Intestinal	None
	37	TCT → GCT	Ser → Ala	Intestinal	Phosphorylation site
	48	GGT → GTT	Gly → Val	Intestinal	Adjacent

[a] Sample was a metastasis from primary tumor 13.
[b] Sample was a metastasis; primary tissue was unavailable.
[c] Multiple foci were analyzed within the same tumor.

two instances of mutations at codons 13, 32, and 48. Mutation at codon 48 has only recently been described in a rectal carcinoid tumor (17). Additionally, previously undescribed mutations at codons 8, 11, 13, and 39 were identified. Codon 39 sits adjacent to a threonine phosphorylation site at codon 40. In all, 14 of the 21 mutations occurred at, or adjacent to, serine-threonine phosphorylation sites. One diffuse tumor revealed a serine to alanine missense mutation in the primary tumor. A metastatic focus from this tumor was also analyzed and found to contain a different mutation, glutamate to glycine mutation at codon 55.

DISCUSSION

Activation of Wnt signaling is an important step in the development of numerous human tumors. In this study, we sought to determine the incidence of Wnt pathway activation and *β-catenin* mutation in gastric adenocarcinoma. The final common end point of Wnt activation, whether related to mutation in *APC*, *β-catenin*, or *axin*, is elevation of cytoplasmic β-catenin levels and its translocation to the nucleus. We used β-catenin nuclear localization to estimate the frequency of Wnt pathway activation in a large number of gastric tumors and report that the Wnt pathway is activated in nearly one-third of gastric tumors. These findings are similar to previously published smaller series that found nuclear β-catenin in 27 and 31% of tumors, respectively (15, 18). Mutational analysis revealed a 26% incidence of *β-catenin* mutation, significantly higher than has been reported in most series. We attribute this primarily to two factors:

(*a*) Our strategy of immunohistochemical screening for nuclear β-catenin was successful in focusing our analysis on those tumors more likely to harbor mutations in Wnt pathway genes. The hypothesis that nuclear β-catenin predicts Wnt pathway activation is especially strengthened by our finding that no mutations were present in 19 tumors negative for nuclear β-catenin.

(*b*) The use of LCM allowed us to sample pure populations of tumor cells for mutations in the *β-catenin* gene. Most other published studies have relied on the less precise method of manual microdissection. It is likely that the use of laser capture greatly decreases the incidence of false-negative results that can occur when normal, contaminating tissue DNA is amplified by PCR.

Exon 3 of the *β-catenin* gene encodes serine-threonine phosphorylation sites for the glycogen synthase kinase-3β kinase that regulate degradation of β-catenin by the ubiquitin-proteasome pathway. Mutations in exon 3 of *β-catenin* and alteration of these phosphorylation sites confer resistance to phosphorylation and lead to the accumulation of cytoplasmic and nuclear β-catenin and subsequent changes in gene expression. Nearly all β-catenin mutations reported in human cancers are localized to exon 3, and most occur at serine-threonine sites or on adjacent residues (19). Although changes at phosphorylation sites directly impair phosphorylation itself, it is theorized that mutations adjacent to serine/threonine residues alter the DSG amino acid motif that serves as a recognition sequence for ubiquitination (2). The resulting decrease in phosphorylation and alteration of the ubiquitin DSG amino acid targeting sequence leads to diminished β-catenin degradation. In this study, 14 of 21 mutations were located at or adjacent to phosphorylation sites. Mutation at codon 37 was most common and was detected in 5 tumors. The serine to alanine mutation at codon 37 (S37A) is common in colon carcinoma and has been described in numerous other tumor types as well (19). The significance of the S37A mutation has been documented by pulse-chase analysis that demonstrated an extended half-life of the β-catenin protein. In addition, it has been reported that S37A mutant β-catenin is not ubiquitinated in SKBR3 cells treated with a proteosomal inhibitor (2, 3).

Five of the mutations detected are predicted to affect regions not involved in phosphorylation or ubiquitination. It is less clear how these mutations might affect the phosphorylation of β-catenin; however, such mutations have also been reported in anaplastic thyroid carcinoma (20). It is possible that such alterations may alter protein secondary structure and thereby the interaction of β-catenin with the APC/axin complex. Alternatively, they may, in some other manner, alter the recognition site of phosphorylation by glycogen synthase kinase-3β or simply prevent phosphorylation in an unknown manner.

In 54 tumors, nuclear β-catenin was noted; yet no β-catenin mutations were found. Our study was technically biased by the limited examination of *β-catenin*. We could have missed mutations in other exons of the gene or failed to detect a deletion of exon 3. It is also possible that these tumors may harbor an alteration in another Wnt pathway regulatory gene. *APC* is a likely candidate because mutations in gastric cancer have been described previously (12). Hypermethy-

lation of the *APC* promoter has also been described in gastric tumors and thus could potentially contribute to down-regulation of APC and subsequent Wnt activation (21). Other recent reports have described over expression of various Wnt ligands and altered frizzled receptors in gastric cancer (22–26). Axin is a known negative regulator of β-catenin and is mutated in hepatocellular carcinomas with β-catenin accumulation and without *β-catenin* mutation (27). A recent report noted multiple different mutations were responsible for activation of the Wnt pathway in ovarian endometrioid adenocarcinomas (28).

Gastric cancer can be divided pathologically into two broad groups, intestinal-type and diffuse-type, as classified by Lauren (29). The intestinal-type is well differentiated and is composed of neoplastic gland-forming cells, whereas the diffuse-type is poorly differentiated and is composed of scattered groups of loosely associated cells without much cell-to-cell adhesion. A previous study found no *β-catenin* mutations in diffuse-type cancer but found that 27% of intestinal-type cancers carried a mutation in exon 3 of *β-catenin* (14). We did not identify any significant difference in the incidence of either β-catenin nuclear staining or *β-catenin* mutation between diffuse- and intestinal-type tumors. We believe this suggests that Wnt activation is likely an early event in gastric carcinogenesis and that other molecular events may play a more critical role in determining diffuse- *versus* intestinal-type histology.

In conclusion, Wnt pathway activation occurs in nearly one-third of gastric adenocarcinomas as defined by β-catenin nuclear localization. *β-catenin* mutation is a frequent cause of pathway activation in those tumors with nuclear β-catenin. Our results demonstrate that *β-catenin* mutation occurs in both diffuse- and intestinal-type cancers. This suggests that in the stomach as in the colon, Wnt pathway activation may be an early inciting event in multistep carcinogenesis.

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