Frequent Somatic Mutations of the β-Catenin Gene in Intestinal-Type Gastric Cancer

Won Sang Park, Ro Ra Oh, Jik Young Park, Sug Hyung Lee, Min Sun Shin, Young Sil Kim, Su Young Kim, Hyun Kyung Lee, Pun Joon Kim, Seong Taek Oh, Nam Jin Yoo, and Jung Young Lee


Abstract

The increased level of cytoplasmic β-catenin through the mutations to either β-catenin or adenomatous polyposis coli (APC) has been proposed as an important oncogenic step in various tumors. Gastric cancer showed frequent genetic alterations of the APC gene, and the risk for gastric cancer in familial adenomatous polyposis patients is 10 times higher than that in the general population. These findings raise the possibility that mutations of β-catenin may also be associated with the development of gastric cancer. We detected seven somatic mutations in a portion of exon 3 encoding for the glycosyn thase kinase 3β phosphorylation consensus region of the β-catenin gene in 43 gastric cancers. All of these mutations were missense mutations, of which five are in the highly conserved aspartic acid 32 and two are in serine 29; all of these seven mutations were detected exclusively in intestinal-type gastric cancers (7 of 26; 26.9%), but not in the diffuse-type (0 of 17). We concluded that disruption of the APC/β-catenin/T cell factor-lymphoid enhancer binding factor pathway might play an important role especially in the development of intestinal-type gastric cancer.

Introduction

β-Catenin is an ubiquitous intracellular protein that has an important role in the APC/β-catenin/Tcf-Lef pathway and is essential for the intercellular adhesion by linking the cytoplasmic domain of cadherin to α-catenin, which anchors the adhesion complex to the cytoskeleton (1). In the cell, β-catenin is localized to both the cytoplasm where it exists as pools of free monomeric protein and to the lateral cell membranes as part of an adherens junction. β-Catenin protein has also been detected in the nucleus as a complex with the Tcf-Lef family of transcription factors, which transfers cell proliferation signals to the nucleus (2).

Intracellular levels of β-catenin are mainly regulated by degradation, which is probably initiated by interaction with the APC3 protein and GSK3β. The APC protein binds to β-catenin directly and promotes targeted phosphorylation of highly conserved serine and threonine residues in the NH2 terminus by way of GSK-3β, thereby targeting β-catenin for degradation by the proteosome system (3, 4). Therefore, the NH2 terminus of β-catenin (exon 3) is an important area in the regulatory mechanism of β-catenin turnover. Deletion of this sequence, or mutation of four serines or one threonine residues therein, results in the accumulation of β-catenin and, thus, translocates into the nucleus, where it could serve as a transcriptional factor through binding with the Tcf-Lef family (5, 6). Conceivably, then, mutations that stabilize β-catenin, such as APC or β-catenin mutations, may contribute to loss of cell growth control in tumorigenesis of various tumors including colorectal cancer (5–10).

Gastric cancer is one of the leading causes of cancer death in the world. Although its incidence has been decreasing, the risk for this disease in familial adenomatous polyposis patients is at least 10 times higher than that in the general population (11). Furthermore, frequent somatic mutation of the APC gene and loss of heterozygosity on chromosome 5q, where the APC gene is located, have been detected frequently in gastric carcinomas, particularly in intestinal-type gastric cancer (12, 13). All of these findings have also implied a possible association between mutations of β-catenin, resulting in an increased cytoplasmic β-catenin level and carcinogenesis of intestinal-type gastric cancer.

Here, we performed a PCR-based SSCP and sequencing analysis of β-catenin in a series of 43 gastric cancers of intestinal- and diffuse-type to determine whether β-catenin genetic alteration could be involved in gastric cancer development and, if so, to determine to which type it is linked.

Materials and Methods

Samples. A total of 43 formalin-fixed, paraffin-embedded sporadic gastric carcinoma specimens were obtained from Medical College, The Catholic University of Korea. No patient had a family history. H&E-stained histological sections were reviewed in each case. Tumors were classified according to the criteria of Lauren (14): twenty-six carcinomas were of the intestinal-type and 17 tumors were of the diffuse-type. Eight tumors were early gastric carcinomas, which were limited to the gastric mucosa and submucosa. Thirty-five tumors were advanced gastric cancers invading through the muscularis propria into the serosa.

Microdissection and DNA Extraction. Tumor cells were selectively procured from H&E-stained slides using a 30 G1/2 hypodermic needle (Becton Dickinson, Franklin Lake, NJ) affixed to a microdissection device [Simple, Precise, and Economical Microdissection device (SPEM II); BM Korea Co., Seoul, Korea], as described previously (15). We also obtained inflammatory or surrounding normal mucosa cells for corresponding normal DNAs from the same slides in all cases. DNA extraction was performed by a modified single-step DNA extraction method, as described previously (15).

SSCP and DNA Sequencing. Genomic DNA from each tumor sample was amplified for SSCP analysis of exon 3 of β-catenin using the following primer pair: 5'-GATTGTGATGGAGTTGGACATGG-3' and 5'-TGTTCTTGTAGTGAAGGACTGAG-3'. Tumor and corresponding normal DNA from each slide were amplified in a thermal cycler (M. J. Research Institute, Watertown, MA) with primers. Each PCR reaction was generally performed under standard conditions in a 10-μl reaction mixture containing 1 μl of template DNA. 0.4 μM each primer, 125 μM each dNTP, 1.5 mM MgCl2, 0.4 units of Taq polymerase, 0.5 μCi [32P]dCTP (Amersham, Buckinghamshire, United Kingdom), and 1 μl of 10× buffer. The reaction mixture was denatured for 5 min at 95°C and incubated for 35 cycles (denaturing at 95°C for 50 s, annealing at 57°C for 30 s, and extending at 72°C for 30 s). Final extension was continued...
for 10 min. The amplified DNA was mixed with an equal volume of formamide loading dye (95% formamide, 20 mM/liter EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol). The samples were denatured for 5 min at 95°C and loaded onto an MDE gel (AT Biochem, Malvern, PA) with 10% glycerol. After electrophoresis, the gels were transferred to 3 MM Whatmann paper, and autoradiography was performed using X-Omat film. After detection of abnormal bands by SSCP analysis, PCR was performed using DNA eluted from dried gels and sequencing was done using Amplicycle Sequencing Kit (Perkin-Elmer Corp., Branchburg, NJ). All mutations were verified by repeated PCR and gel analyses using different SSCP gel conditions.

Results

A total of 43 primary gastric cancers were screened for the mutations in a portion of exon 3 encoding for the GSK3β phosphorylation consensus region of the β-catenin gene by means of PCR-based SSCP technique.

We detected seven somatic mutations. Two representative cases with aberrant bands and mutations are shown in Fig. 1. All seven mutations were missense mutations, as summarized in Fig. 2. Five mutations are at codon 32 and two mutations are at codon 29. Of these five mutations at codon 32, four patients revealed A to G transitional mutation (GAC to GGC). This nucleotide transition would change a highly conserved amino acid 32 from aspartic acid to a glycine (D32G; samples 34, 35, 37, and 42). The remaining one case revealed G to A transition (GAC to AAC) at codon 32 (Fig. 1a), which would change an aspartic acid to an asparagine (D32N; sample 5). The two mutations at codon 29 were identical mutations (Fig. 1b) containing C

Fig. 1. The autoradiograms show SSCP and sequencing data in two cases of gastric cancer. All of the mutations were missense mutations: an A to G transition at codon D32G in case 34 (a) and C to T transition at codon S29F in case 18 (b).

Fig. 2. Schematic of β-catenin binding domain. GSK3β consensus sequence, its substates, and β-catenin mutations in gastric cancers. β-Catenin has multiple binding sites for GSK3β, α-catenin, APC, and cadherin (a). The second diagram illustrates the GSK3β consensus phosphorylation sites, using the one-letter amino acid code. x, an unspecified amino acid (b). Serine and threonine residues phosphorylated by GSK3β are in boldface print. Three potential matches to this consensus in β-catenin are indicated (c). The seven mutations found in gastric cancers are indicated at the bottom (d): two S29Fs (cases 18 and 43; TCT to TTT) and four D32Gs (cases 34, 35, 37, and 42; GAC to GGC) and one D32N (case 5; GAC to AAC). S, serine; F, phenylalanine; D, aspartic acid; N, asparagine; T, threonine.
to T transitional mutation (TCT to TTT), which changed a serine to a phenylalanine (S29F; samples 18 and 43). All of these seven mutations were detected exclusively in intestinal-type gastric cancer (7 of 26; 26.9%), whereas there were no mutations in 17 cases of the diffuse-type. All of the seven mutations were reproducible through triplicate experiments including tissue microdissection, PCR, SSCP, and sequencing analysis, which ensured the specificity of the results.

Discussion

The increased level of cytoplasmic β-catenin through the mutations to either β-catenin or APC may translocate into the nucleus, where it could serve as a transcriptional factor through binding with the Tcf-
Lef family, which has been proposed as an important oncogenic step in various tumors including colorectal cancer (5–10). In most colorectal tumors (85%), this occurs through mutation in the APC gene and decreased APC-associated degradation of β-catenin. But, in approximately one-half of the remainder of tumors with wild-type APC, it is due to mutations in β-catenin itself (6). Gastric cancer also showed frequent genetic alterations of the APC gene, and the risk for gastric cancer in familial adenomatous polyposis patients is 10 times higher than that in the general population (11–13). These findings raise the possibility that mutations of β-catenin may also be associated with the development of gastric cancer. Most human cancers that involve β-catenin mutations possess changes in amino acid residues in the NH2-terminal region, which provides GSK3β phosphorylation sites (5–10). We, therefore, screened human gastric cancer for the presence of these known mutations.

We identified mutations in exon 3 of the β-catenin gene in 7 of 43 sporadic gastric cancers. All of these mutations are missense mutations, and five mutations are in aspartic acid 32, whereas two mutations are in serine 29 (Figs.1 and 2). Five serines within the NH2 terminus, which are located between β-catenin amino acids 29 and 47, are well conserved among β-catenin, Armadillo, and plakoglobin (γ-catenin; Fig. 2). Mutations of three of these serines (at codons 33, 37, and 45), as well as a conserved threonine 41, result in β-catenin accumulation and have been detected frequently in various tumors in previous studies (5–10). In the present study, we found no missense mutations at these codons. Instead, we detected five missense mutations at aspartic acid 32 (four D32G and one D32N). This acidic amino acid, as well as proline, are highly conserved near GSK3β phosphorylation sites, which are highlighted in Fig. 2. The DSG sequence (Fig. 2., underlined residues) has been characterized as an ubiquitination-targeting motif on the basis of its conservation with IkBα (16), which can also target another protein for degradation by ubiquitin ligases that recognize phosphorylated sequence (16, 17). Therefore, mutations in these adjoining residues of serine or threonine may interfere with ubiquitination, possibly through alteration of the GSK3β kinase recognition sites. In addition, missense mutations at codon 32 have also been detected in various tumors, such as hepatocellular carcinoma, hepatoblastoma, and pilomatoma (7–10).

We detected two missense mutations in serine residue 29, which have not been reported previously in human tumors. There are four SxxxS repeats within β-catenin between amino acids 29 and 47 that are well conserved among β-catenin, Armadillo, glycogen synthase, and plakoglobin (γ-catenin), which are all GSK3β substrates (Fig. 2; Ref. 17). These substrates are usually required before phosphorylation of the SxxxS repeats for recognition by GSK3β (3). For example, the first serine (at codon 641; Fig. 2) in the NH2 terminus of the glycogen synthase is phosphorylated by protein kinase C-like kinase and results in the formation of a phosphorylated SxxxS motif, which is the minimal recognition structural unit recognized by GSK3β as its substrate (18). This phosphorylation changes the substrate conforma-
tion into one recognized by GSK3β kinase and follows phosphorylation of a target serine (at codon 33 in β-catenin) at a fixed spacing to the COOH-terminal side by GSK3β. In this fashion, all of the regularly spaced serine or threonine residues in the SxxxS motifs must be phosphorylated sequentially to target the substrate for degradation (3, 17, 18). Therefore, missense mutation at serine 29 might also be involved in an increase of the β-catenin level despite the lack of mutations at this codon in previous studies.

One interesting aspect of this study is that all of the mutations are exclusively detected in intestinal-type gastric cancer (7 of 26; 26.9%), and not in the diffuse-type (0 of 17). Gastric carcinomas are divided into two general clinicopathological patterns according to the criteria of Lauren (14): (a) intestinal-type or well-differentiated carcinomas; and (b) diffuse-type or poorly differentiated carcinoma. Intestinal-type tumors grow mostly in glandular formations, whereas diffuse-type tumors mostly do not form glands and show scattered cell growth with loose cell-to-cell adhesion. The molecular alterations leading to these two histological patterns may be significantly different enough from each other to consider them two separate entities. For example, loss or mutation of the APC gene is detected in up to 60% of well-differentiated gastric cancers, making it one of the most frequent genetic alterations. But, it is a very rare event in diffuse-type gastric cancer (12, 13). In addition, E-cadherin is mutated in at least 50% of diffuse-type gastric cancers, whereas there is no mutation in intestinal-type (19). Cell-to-cell adhesion is critical to the establishment and maintenance of normal tissue architecture. β-catenin is essential for this cell-to-cell adhesion by linking the cytoplasmic domain of E-cadherin to α-catenin, which anchors the adhesion complex to the cytoskeleton (1, 2). Cells lacking an α-catenin binding site within β-catenin are unable to form stable adherens junctions despite normal E-cadherin and β-catenin expression. For example, the HSC-39 cell line established from a patient with a signet ring cell carcinoma of the stomach has a 321-bp in-frame deletion in the NH2 terminus (at codon 28 to 134), including GSK3β phosphorylation and α-catenin binding sites. This cell line showed a nonfunctional E-cadherin complex because the truncated β-catenin molecule failed to link E-cadherin and α-catenin (20). On the other hand, the mutant having truncation from codon 1 to 89 in the NH2 terminus, ΔN89β-catenin, does not seem to affect binding to E-cadherin, does not affect the domains involved in the binding of α-catenin or Tcf-Lef, and preserves all of the structurally and functionally important armadillo repeats of the protein. Thus, forced expression of ΔN89β-catenin resulted in a marked increase in E-cadherin levels at the adherens junctions and basolateral surfaces of intestinal epithelial cells (21). According to these findings, we can suspect that the α-catenin binding site might be located between codon 90 to 132 in the NH2 terminus of β-catenin. Therefore, mutations in the GSK3β phosphorylation site (at codon 29 to 47) of β-catenin do not perturb the cadherin-catenin complex, but rather increase the stability of adherens junctions (21). Thus, it is reasonable that the activating mutations of β-catenin might be involved only in intestinal-type gastric cancer. These data perfectly matched the findings that genetic alterations of the APC gene were detected solely in well-differentiated adenocarcinoma of the stomach (12, 13) and that no β-catenin mutations were detected in the diffuse-type of gastric cancer in a previous study (22).

Despite the small number of cases, because we frequently observed the activating mutations of the β-catenin gene exclusively in intestinal-type gastric cancer, we concluded that disruption of the APC/β-catenin/Tcf-Lef pathway might play an important role especially in the development of intestinal-type gastric cancer. However, additional studies on a large patient populations will be needed to verify these initial observations.
References

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