Increased β-catenin mRNA levels and mutational alterations of the APC and β-catenin gene are present in intestinal-type gastric cancer

Matthias P.A. Ebert1, Guo Fei, Sabine Kahmann1, Oliver Müller1, Jun Yu2, Joseph J.Y. Sung2 and Peter Malfertheiner

Department of Gastroenterology, Hepatology and Infectious Diseases, Otto-von-Guericke University, D-39120 Magdeburg, 1Max-Planck-Institute for Molecular Physiology, D-44227 Dortmund, Germany and 2Department of Medicine & Therapeutics, Prince of Wales Hospital, Chinese University of Hong Kong, Shatin, Hong Kong

To whom correspondence should be addressed
Email: matthias.ebert@medizin.uni-magdeburg.de

β-Catenin is critical for intercellular adhesion and also plays a role as a transcription activating protein in the Wnt signalling pathway. Increased protein levels and mutation of the β-catenin gene have been demonstrated in various cancers; however, the role of β-catenin in gastric cancer remains largely unknown. Using gastric cancer tissues and normal adjacent gastric mucosa obtained from 20 patients with gastric cancer (eight diffuse-type, 12 intestinal-type) undergoing gastric resection or endoscopy, we assessed the expression of β-catenin by immunohistochemistry and quantitative PCR analysis. Furthermore, the tumour suppressor gene APC, which down-regulates the β-catenin levels was analysed for mutations. Overall mRNA levels of β-catenin were significantly increased in the tumour samples compared with the matched normal gastric mucosa (P < 0.05). Increased β-catenin mRNA levels were significantly more frequent in intestinal-type gastric cancers as compared to diffuse-type gastric cancers (P < 0.01). Six out of 20 tumours exhibited >6-fold increased β-catenin mRNA levels as compared with normal mucosa. APC gene mutations were found in four cases. A β-catenin gene mutation was identified only in one intestinal-type gastric cancer exhibiting a massive overexpression of β-catenin mRNA in the tumour. In intestinal-type gastric cancers β-catenin mRNA levels are greatly enhanced. APC and β-catenin gene mutations are also present primarily in intestinal-type gastric cancers. These findings support the hypothesis that in intestinal-type gastric cancers the accumulation of β-catenin protein may result from impaired degradation of the β-catenin protein due to alterations of the β-catenin and APC genes, as well as from enhanced β-catenin transcription which is present in the great majority of intestinal-type gastric cancers.

Introduction

Gastric cancer is one of the leading causes of cancer-related deaths worldwide. It is believed to develop in a multistep process including the activation and overexpression of onco-
genres, such as K-sam and c-met (1,2), as well as the inactivation of tumour suppressor genes, such as APC and TP53 (3,4). In addition, microsatellite instability occurs in ~30% of gastric cancers as a result of inactivating mutations in DNA repair enzymes (5). Alterations of the expression of adhesion molecules are also common in gastric cancer (6,7). Thus, diffuse-type gastric cancers frequently harbour E-cadherin gene mutations (8). In addition, we and others have reported the frequent down-regulation of E-cadherin and α-catenin expression in gastric cancer (6). β-Catenin is a multifunctional protein that is critical for intercellular adhesion through binding of E-cadherin to α-catenin and, thus, stabilizing the actin cytoskeleton (9). However, β-catenin is also an integral part of the Wnt signalling pathway, involving APC, β-catenin and the Tcf/Lef transcription factor (10,11). Levels of β-catenin are regulated by the interaction of APC, GSK-3β and other proteins, leading to the phosphorylation of its serine and threonine residues at the N-terminal region of the β-catenin protein (11,12). Alteration of β-catenin expression has been reported in several malignancies (13–15). Thus, increased intracellular levels of β-catenin have been reported in colon and hepatocellular carcinomas (13,16). Further analysis has also revealed that inactivation of APC in colon cancer leads to the accumulation of cytoplasmic β-catenin (17). Furthermore, recent reports have also identified β-catenin mutations as a molecular mechanism leading to the accumulation of the β-catenin protein in colon and thyroid cancer (15,18). The presence of β-catenin mutations in gastric cancers has been analysed recently; however, these studies have revealed great differences in the frequency of mutational alterations of the β-catenin gene (19–23). We searched for mutations of the β-catenin and APC genes in diffuse- and intestinal-type gastric cancers and determined the mRNA levels of β-catenin in gastric cancer and the adjacent normal gastric mucosa.

Materials and methods

Subjects

Tissue specimens were obtained by surgical resection or upper gastrointestinal (GI) endoscopy from 20 patients (16 male, four female) with gastric cancer, with a mean age of 63.5 years (range 44–79 years), from the tumour and a tumour-free location which was at least 6 cm distant from the tumour and which was confirmed to be without any tumour cell infiltration by histological assessment.

Immediately after their removal, all tissues for molecular analysis were put in liquid nitrogen and stored at –80°C until use. This study was approved by the Ethics Committee of the University of Magdeburg.

Histology

Formalin-fixed tissues were processed as previously described and sections were stained with haematoxylin and eosin for histological evaluation and for detection of H.pylori (24). Gastric cancer types were classified histologically as intestinal type (n = 12) or diffuse type (n = 8) according to the Lauren system (25). The severity of gastritis and H.pylori colonization of the non-tumorous gastric mucosa was histologically classified according to the updated Sydney system (24).

RNA isolation

Tissue specimens were stored at –80°C and homogenized with an ultrasonic homogenizer (Ultra-Turrax T25; Janke and Kunkel, Köln, Germany) in the
presence of RNAzolB (CINNA/MIRC, Cincinnati, OH) containing RNase inhibitors. Total RNA extracted from frozen tissues by the acid/guanidinium and phenol/chloroform extraction method was quantified by measuring the optical density at 260 nm and separated by gel electrophoresis as previously described (26).

Quantitative analysis of β-catenin mRNA levels
Total RNA (1 μg) was reverse transcribed at 37°C for 1 h in a final volume of 20 μl reverse transcription buffer (50 mM Tris–HCl pH 8.3, 50 mM KCl, 10 mM MgCl₂, 0.5 mM spermidine and 10 mM DTT) containing 4.8 U AMV reverse transcriptase (Promega, London, UK), 16 U RNAse inhibitors, 200 pmol random primer, 1.0 mM dNTPs (Biomeri Feinchemikalien, Hamburg, Germany). The reaction was terminated by incubating the mixture at 95°C for 10 min. PCR amplification of the cDNA was performed as previously described (26). Briefly, PCR primers were designed to amplify a 298 bp cDNA fragment encompassing exon 3 of the β-catenin gene (sense, 5'-ACAAACTGTGTTTGGAAATCCA-3', antisense, 5'-CGAGTTTCAGGATCCTGC-3'). The PCR mixture contained 50 ng cDNA as template in 10 μl reaction containing 4 mM MgCl₂, 0.2 mM each dNTP, 5 pmol each primer and 0.08 μl Taq polymerase (Eppendorf Netheler-Hinz, Hamburg, Germany). Each PCR analysis was performed in duplicate and the mean value was determined. Quantitative PCR was carried out on the Light Cycler (LC24; Idaho Technology, Falls, ID) under the following conditions: 40 cycles of 1 s at 95°C, 3 s at 54°C and 12 s at 72°C. The results were analysed by Idaho LC 24 software (27).

Detection of β-catenin gene mutation
Detection of mutations in exon 3 of the β-catenin gene was carried out by SSCP analysis of cDNA fragments. PCR amplification was carried out as follows: 1 μl reaction containing 200 ng cDNA as template, 10 μl Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 0.2 mM dNTP, 8 pmol each primer and 1.25 U Taq polymerase (Bioster, Köln, Germany). The PCR reaction was performed with 35 cycles at 95°C for 1 min, 58°C for 30 s and 72°C for 30 s, followed by 72°C for 10 min for the final elongation. The PCR product was separated on a 1.5% agarose gel and visualized by ethidium bromide staining (26). Thereafter, the PCR fragment encoding exon 3 of the β-catenin gene was analysed by SSCP. Briefly, 5 μl PCR product was diluted in 3 μl denaturation buffer (0.5 mg bromophenol blue, 0.5 μg xylene cyanol blue in 1000 μl formamide). After denaturation at 97°C for 7 min, it was subsequently chilled on ice; 4 μl of the mixture was loaded on a 0.4 mm thick non-denaturating 0.5× MDE gel (FMC Bioproducts, Rochland, ME). The fragments were separated at 4°C by horizontal electrophoresis and the DNA was visualized using modified silver staining (27).

Detection of APC gene mutations
The mutation cluster region from nucleotides 3570 through 4800 of the APC gene was screened for sequence alterations by PCR of two overlapping fragments and direct sequencing. The analysed region covers >85% of all published somatic APC mutations (3). The first fragment was amplified using the primers 5'-TCCTCATACAGAAGAACGTG-3' and 5'-GCTCGGATTTGTTCTACAGG-3', the second fragment using the primers 5'-GGTCTCAGGTAAGATGTGCTG-3' and 5'-GATGACTTGGTTGCTTGAGCA-3'. Primers were custom-synthesized by MWG Biotech (Munich, Germany). For PCR, 200 μM concentration of primers was used in a final volume of 50 μl containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 30 μM each dNTP, 200 μM each primer, 100 μg/ml BSA and 0.5–1.0 U Taq polymerase (AGS, Heidelberg, Germany). After denaturation at 94°C for 120 s, 30 cycles of PCR were carried out of 60 s at 92°C, 60 s at 62°C and 60 s at 72°C. PCR products were purified from residual primers and non-incorporated nucleotides using standard reagents and protocols (Quanta, Hilden, Germany). Standard cycle sequencing was performed in the presence of fluorescence labelled deoxyxynucleotides using the upstream PCR primer as sequencing primer. Reaction products were analysed on an ABI automated sequencer. PCR and sequence analysis of mutated samples were repeated twice to exclude PCR errors (26).

Immunohistochemistry
Paraffin-embedded sections from eight patients with gastric cancer were deparaffinized and rehydrated by xylene and ethanol. Endogenous peroxidase activity was blocked by incubation for 30 min in 0.3% H₂O₂. After washing twice with PBS, the sections were blocked with normal rabbit serum for 20 min and were incubated with the protein purified anti-β-catenin antibody (1:1000) overnight at 4°C. Anti-β-catenin antibody (c-18) is an affinity-purified goat polyclonal antibody raised against a peptide mapping at the C-terminus of human β-catenin. It reacts with β-catenin of mouse and human origin (Santa Cruz Biotechnology, Santa Cruz, CA). The specificity of the antibody was confirmed by Western blotting (28). Immunohistochemical staining was performed according to the manufacturer’s instructions using the Streptavidin–HRP Systems kit, followed by counterstaining with haematoxylin.

Statistical analysis
The t-test, Fisher’s test and χ²-test were used to determine statistical difference. Values of P < 0.05 were considered statistically significant (29).

Results
Expression of β-catenin in the normal gastric mucosa and gastric cancer
Twenty patients with gastric cancer were studied for β-catenin expression in the tumour and the non-cancerous stomach. All 20 gastric tissue samples from the tumour-free stomach exhibited β-catenin mRNA as determined by the RT–PCR. In addition, all 20 tumour tissue samples also expressed β-catenin mRNA. Quantitative PCR analysis revealed a significant increase of β-catenin mRNA levels in the cancer tissue as compared with the tumour-free tissue (P = 0.0046) (Figure 1). Of the 20 gastric cancer specimens, 12 were gastric cancers of the intestinal type and eight were of the diffuse type (Table I). Only one out of eight diffuse-type gastric cancers exhibited high β-catenin mRNA levels, whereas 11 out of 12 gastric cancers of the intestinal type expressed abundant β-catenin. The difference in β-catenin expression in the different histological types was statistically highly significant (P = 0.0008) (Table II). Using immunohistochemical analysis, the β-catenin protein was detected both at the cell membrane as well as in the cytoplasm of the gastric cancer cells (Figure 2).

Detection of β-catenin gene mutations in gastric cancer
All of the 20 gastric cancers were screened for mutations in exon 3 of the β-catenin gene by SSCP technique. Only one tumour sample exhibited a band shift on the gel (Figure 3A). Sequencing revealed a G→A point mutation at codon 32 of the β-catenin gene which results in an exchange of aspartic acid to asparagine (Figure 3B). This tumour was a gastric cancer of the intestinal type with a 7.67-fold increase in β-catenin mRNA levels in the tumour as compared with normal gastric mucosa (Table I). Immunohistochemical analysis revealed cytoplasmic β-catenin immunoreactivity in this case, too (data not shown).

Detection of APC gene mutations in gastric cancer
Genomic DNA from 13 out of 20 gastric cancer samples was analysed for APC gene mutations in exon 15 (Table I). Four
Table I. Expression of β-catenin in gastric cancer: patient characteristics and molecular changes

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Hp</th>
<th>Relative β-catenin mRNA levels</th>
<th>Histology</th>
<th>APC mutation</th>
<th>β-Catenin mutation</th>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td>2</td>
<td>79</td>
<td>F</td>
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<tr>
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<td>21.76</td>
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<tr>
<td>4</td>
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<tr>
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<tr>
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<td>69</td>
<td>M</td>
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<td>−</td>
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<td>−</td>
</tr>
<tr>
<td>13</td>
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<tr>
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<td>ND</td>
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</tr>
<tr>
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<td>13.29</td>
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<td>−</td>
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<tr>
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<td>F</td>
<td>−</td>
<td>25.17</td>
<td>14.54</td>
<td>Diffuse</td>
<td>ND</td>
</tr>
</tbody>
</table>

Hp, H. pylori; M, male; F, female; ND, not determined; −, negative; +, positive; ins., insertion.

Table II. Correlation of β-catenin expression and histological type

<table>
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<th>No.</th>
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<th>P-value</th>
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<td>1</td>
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<tr>
<td>Intestinal type</td>
<td>12</td>
<td>11</td>
</tr>
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</table>

Fig. 2. Immunohistochemistry. β-Catenin expression in gastric cancer cells was found at the cell membrane (A) and in some cancer cells, β-catenin was also detected in the cytoplasm (B). Magnification, ×400.

of the tumours were found to harbour APC gene alterations, in two cases a 1 bp insertion of adenine at position 4684 was detected leading to a stop codon in both cases. In a third case a missense mutation was detected at position 4407 (AGA→AGT), which leads to an exchange of arginine to serine (sample no. 11). The fourth sample exhibited a mutation at position 3623 (TCT→TGT), which changes serine to cysteine (sample no. 18) (Table I).

Fig. 3. SSCP gel analysis and sequencing. Using SSCP analysis, as outlined in the Materials and methods, only one of the 20 samples obtained from gastric cancer patients exhibited a mobility shift, indicating a somatic mutation in exon 3 of the β-catenin gene (A, arrowhead). M, DNA marker; last lane, normal gastric mucosa. (B) Direct sequencing of the PCR product revealed a point mutation in codon 32 of the β-catenin gene in this sample.

Correlation of β-catenin expression with H. pylori infection
The association of H. pylori infection and increased mRNA levels of β-catenin was studied in the non-tumorous part of the stomach in 15 patients with gastric cancer (Table I). Four of five tissue specimens with H. pylori infection exhibited increased β-catenin expression, whereas in the 10 tissue specimens without H. pylori infection five cases exhibited high β-catenin mRNA levels. Statistical analysis by Fisher’s exact
test could not establish an association of *H. pylori* infection with increased β-catenin mRNA levels (*P > 0.05*).

**Discussion**

The cadherin–catenin complex is the primary cell adhesion complex of epithelial cells. E-cadherin associates with α-catenin, β-catenin and γ-catenin to form a complex that is essential for cell–cell communication and cell adhesion (8,9). Disruption of this complex leads to impaired adhesion contributing to the transformation of epithelial cells. Thus, truncated β-catenin fails to link E-cadherin to α-catenin resulting in a non-functional E-cadherin complex, as previously demonstrated in a gastric cancer cell line (30). Gastric cancers frequently harbour mutations of the *E-cadherin* gene (8). Furthermore, diffuse-type familial gastric cancers have been linked to germline mutations of the *E-cadherin* gene. However, recent studies also demonstrate alterations of catenin expression in sporadic gastric cancer (6). Thus, we and others have demonstrated reduced expression of α-catenin in gastric cancer and in the gastric mucosa of gastric cancer relatives (6,31). β-Catenin is a further player in this adhesion complex and studies by Jawhari *et al.* (6,30) demonstrated a reduction of β-catenin expression in gastric cancer as well. However, β-catenin, apart from its role as a adhesion molecule, also participates in the Wnt signalling pathway (9,11). Inactivation of APC leads to the stabilization of β-catenin protein and thus increased signalling through the Tcf/Lef transcription factors. Besides from APC inactivation, stabilization and thus increase of β-catenin levels also results from mutation of the β-catenin gene itself. Frequent mutations of the β-catenin gene have been found in colorectal, hepatocellular, thyroid, endometrial and ovarian carcinomas (13–16). The N-terminus of β-catenin, encoded in part by exon 3 of the β-catenin gene, contains highly conserved serine and threonine residues. The APC protein in conjunction with GSK-3β leads to the phosphorylation of the residues which target β-catenin for degradation by the proteasome system (10,32). Therefore, apart from APC gene mutations, mutations or deletions in exon 3 of the β-catenin gene lead to the accumulation of the β-catenin protein and increased signalling through the Tcf/Lef family of transcription factors.

Whereas APC gene mutations occur in ~30% of gastric cancers (3), several groups have recently reported large variations in the frequency of β-catenin gene mutations in gastric cancers (19–23). Therefore, we have analysed mutational alterations in the APC and β-catenin genes in our group of patients, with a further focus on possible differences in diffuse- and intestinal-type gastric cancers. Furthermore, in order to determine whether β-catenin mRNA levels may also be altered in cancers without mutational changes in the APC and β-catenin genes, we assessed the levels of β-catenin mRNA in gastric cancers and the matched non-malignant gastric mucosa. Interestingly, we found markedly increased β-catenin mRNA levels in the tumour tissue as compared with the matched non-tumour tissue. These findings are in line with a report by El-Rifai *et al.* (33), who used cDNA arrays to investigate the expression profile of gastric adenocarcinomas. Among other up-regulated genes, they found a marked over-expression of β-catenin in their gastric cancer samples. The enhanced levels of β-catenin mRNA in gastric cancer are, however, in sharp contrast to the reduced β-catenin expression in gastric cancers as determined by immunohistochemistry (6).

Jawhari *et al.* (6) reported loss of membranous staining in 58% of diffuse-type and 38% of intestinal-type cancers. In a study by Sasaki *et al.* (21) nuclear and/or cytoplasmic immunoreactivity was observed in 16 out of 70 gastric cancers and in a further study by Woo *et al.* (23) nuclear accumulation and/or loss of membranous staining was identified in 27% of the gastric tumours. Our immunohistochemical analysis confirmed the various patterns of β-catenin immunoreactivity in gastric cancer cells. However, the number of cases analysed by both PCR and immunohistochemical analysis is too small for a direct comparison of mRNA and protein levels in these cancers. Nonetheless, we found markedly increased mRNA levels in these tumours and, interestingly, the increased β-catenin mRNA levels primarily occurred in intestinal-type gastric cancers. In order to further elucidate the molecular mechanisms underlying the up-regulation of β-catenin mRNA in these cancers, we also analysed mutational alterations of the APC and β-catenin genes in these cancers.

Somatic mutations of the APC gene and loss of heterozygosity of chromosome 5q, the APC gene locus, have also been reported, especially in intestinal-type gastric cancer (3,34). We analysed the mutation cluster region, exon 15, of the APC gene and found four genetic alterations in this region. All of these tumours were again intestinal-type gastric cancers. Interestingly, all four cancers exhibited a >6-fold increase in β-catenin mRNA levels. Since β-catenin gene mutations in exon 3 may also lead to enhanced β-catenin expression, we analysed exon 3 of the β-catenin gene. Using SSCP analysis and direct sequencing of PCR fragments, we detected only one homozygous mutation in exon 3 of the β-catenin gene in 20 gastric cancer tissue samples. This mutation led to an exchange of aspartic acid to asparagine at codon 32, which is located right next to a threonine residue and may interfere with the phosphorylation of its residues and, thus, may lead to the stabilization of the protein. Previously, Candidus *et al.* (19) have reported the absence of β-catenin gene mutations in gastric cancer. In contrast, Park *et al.* (20) identified seven mutations of the β-catenin gene in a series of 26 gastric cancer samples of the intestinal type. However, more recent reports have confirmed that β-catenin gene mutations are rather infrequent in gastric cancers (21–23). Nonetheless, despite the low frequency of mutational changes of the APC and β-catenin genes in our series of gastric cancers, all of these genetic changes were present only in intestinal-type gastric cancers. In addition, in these cancers and in the other intestinal-type gastric cancers without genetic alterations in the APC or β-catenin genes, the β-catenin mRNA levels were markedly enhanced.

Interestingly, gastric cancers with either an APC or β-catenin gene mutation exhibited a >6-fold increase in β-catenin mRNA levels. While the overall analysis revealed that increased β-catenin mRNA levels are present in the majority of intestinal-type gastric cancers independent of genetic alterations of the APC and β-catenin genes, the dramatic increase of β-catenin mRNA levels in cancers harbouring an APC or β-catenin gene mutation points to a possible association between mutational changes of these genes and enhanced β-catenin transcription. The molecular mechanisms underlying this possible association remain largely unknown; however, increased β-catenin mRNA levels could result from the activation of the Wnt signalling pathway due to mutations in either the APC or β-catenin gene, which leads to the accumulation of β-catenin protein. While the activation of β-catenin
transcription through β-catenin itself seems unlikely, since the β-catenin promoter does not contain a TCF-responsive element. β-catenin transcription might be activated through other transcription factors which are induced by the β-catenin–TCF-Lef complex. (35). Apart from this hypothesis, it is also reasonable to assume that the increase of β-catenin mRNA may occur totally independent of the Wnt pathway. Nevertheless, our data indicate that β-catenin mRNA levels are increased in the vast majority of intestinal-type gastric cancers and that this overexpression is independent of H. pylori infection.

Thus, despite the fact that APC and β-catenin gene mutations are not frequent in gastric cancer, the presence of these changes in intestinal-type gastric cancers, together with enhanced β-catenin mRNA levels in intestinal-type gastric cancer, indicate that alterations in the APC/β-catenin pathway may play an important role in the development of gastric cancer and that alterations of this pathway specifically contribute to intestinal-type gastric carcinogenesis.

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References