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World J Gastroenterol 2008 March 14; 14(10): 1570-1574
World Journal of Gastroenterology ISSN 1007-9327
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RAPID COMMUNICATION

Mutations in components of the Wnt signaling pathway in gastric cancer

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Supported by State Key Basic Research Program Grant of the Ministry of Science and Technology Foundation of China, G1998051203 and the National Science Foundation of China, G39625016
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Received: November 9, 2007 Revised: November 16, 2007

Abstract

AIM: To explore the contribution of AXIN1, AXIN2 and beta-catenin, components of Wnt signaling pathway, to the carcinogenesis of gastric cancer (GC), we examined AXIN1, AXIN2 exon7 and CTNNB1 (encoding beta-catenin) exon3 mutations in 70 GCs.

METHODS: The presence of mutations was identified by polymerase chain reaction (PCR)-based denaturing high-performance liquid chromatography and direct DNA sequencing. Beta-catenin expression was detected by immunohistochemical analysis.

RESULTS: Among the 70 GCs, 5 (7.1%) had mutations in one or two of these three components. A frameshift mutation (1 bp deletion) in exon7 of AXIN2 was found in one case. Four cases, including the case with a mutation in AXIN2, had frameshift mutations and missense mutations in AXIN1. Five single nucleotide polymorphisms (SNPs), 334 C>T, 874 C>T, 1396 G>A, 1690 C>T and 1942 T>G, were identified in AXIN1. A frameshift mutation (27 bp deletion) spanning exon3 of CTNNB1 was observed in one case. All four cases with mutations in AXIN1 and AXIN2 showed nuclear beta-catenin expression.

CONCLUSION: These data indicate that the mutations in AXIN1 and AXIN2 may contribute to gastric carcinogenesis.

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Key words: AXIN1; AXIN2; β-catenin; Wnt signaling pathway; Gastric cancer

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Pan KF, Liu WG, Zhang L, You WC, Lu YY. Mutations in components of the Wnt signaling pathway in gastric cancer. *World J Gastroenterol* 2008; 14(10): 1570-1574 Available from: URL: http://www.wjgnet.com/1007-9327/14/1570.asp DOI: http://dx.doi.org/10.3748/wjg.14.1570

INTRODUCTION

The Wnt signaling pathway plays an essential role in human cancer development[1-3]. Wnt stabilizes cytoplasmic β-catenin, which stimulates the expression of genes including c-myc, c-jun, fra-1 and cyclin D1[4,5]. AXIN1 and its homologue AXIN2, newly recognized as components of the Wnt signaling pathway, negatively regulate this pathway[6,7]. Other components of the Wnt signaling pathway, including β-catenin and adenomatous polyposis coli (APC), interact with AXIN, and the phosphorylation and stability of β-catenin are regulated in the AXIN complex[5].

AXIN1 is thought to be critical for degrading cytoplasmic β-catenin. Different domains of AXIN1 were shown to interact with APC, GSK-3β, β-catenin, PP2Ac and AXIN itself[8,9]. The function of AXIN1 in promoting β-catenin degradation suggests that it serves as a tumor suppressor. AXIN1 mutations have been reported in a variety of human carcinomas including sporadic medulloblastomas, hepatocellular carcinomas and colorectal cancers[10-13], suggesting that AXIN1 may be involved in the development of these tumors.

AXIN2, which functions as a scaffold protein in the Wnt signaling pathway[14], has been shown to develop frameshift mutations in mononucleotide repeat sequences located in exon7, in colorectal cancer, with defective DNA mismatch repair (MMR)[7]. A recent study has shown that AXIN2 is a transcriptional target of the TCF/LEE transcription factor complex downstream of activated β-catenin[15].

Mutations in CTNNB1 (encoding β-catenin) or APC have been reported in human neoplasms, including colon cancer and gastric cancer (GC)[16-19]. However, AXIN1 and AXIN2, the key players in this pathway, have not been investigated in GC. In this study, we identified mutations of the entire coding region of AXIN1, exon7 of AXIN2 (a hot spot of mutation), and exon3 of CTNNB1 in GC cases, and evaluated if such mutations activate the Wnt signaling pathway during GC development.

MATERIALS AND METHODS

Tissue specimens and DNA isolation

GC samples and their matched normal gastric tissues were obtained from 41 males and 29 females during surgical resections. The study was approved by Institutional Review Board of Peking University School of Oncology, and all subjects gave written informed consent. Fresh samples were collected at the time of surgery, frozen in liquid nitrogen and stored at -80℃. Sections from each specimen were determined by a pathologist, and classified according to Lauren's classification[20]. Among the GC cases, 32 (46%) were intestinal-type, 27 (39%) were diffuse-type and 11 (16%) were mixed-type. High molecular weight genomic DNA was extracted by standard proteinase K digestion and phenol/chloroform extraction.

Primers and PCR

We screened the entire coding sequence of the AXIN1 gene, exon7 of AXIN2 and exon3 of CTNNB1 with PCR-based denaturing high-performance liquid chromatography (DHPLC). The primer sequences are listed in Table 1. PCR was accomplished with a 25 μL reaction mixture containing 50 ng of genomic DNA, 0.4 μmol/L sense and antisense primers for each exon, 200 μmol/L dNTPs (Perkin-Elmer, CA, USA), 0.2 μL of Ampli Taq Gold polymerase (Perkin-Elmer, USA), and 2.0 mmol/L of $MgCl_2$. After initial activation of the enzyme by denaturation at 95℃ for 9 min, PCR amplification was performed for 35 cycles: 94℃ for 30 s, the optimized annealing temperature for 45 s, and 72℃ for 45 s. Final extension was performed at 72℃ for 10 min. The annealing temperatures for various primer sets were: 58℃ for AXIN1 exons 1A, 1B, 1C, 2, 3, 4 and 9; 63℃ for exons 5A, 6, 7, 8 and 10; 62℃ for exon 5B; 60℃ for P3-4; and 59℃ for AXIN2 exon 7.

DHPLC analysis

PCR products were heated to 95℃ for 3 min, then cooled to 25℃ over 45 min. Homozygous mutant DNA was combined with wild-type in an approximately 1:1 ratio prior to hybridization; then, this mixture was examined for heteroduplex content by subjecting 50-100 ng of PCR products to DHPLC (WAVE™ system, Transgenomic, USA) under partial denaturation conditions[21,22]. The mobile phase consisted of a mixture of 0.1 mol/L triethylamine acetate (TEAA, pH 7.0) with or without 25% acetonitrile. The flow rate used in this study was 0.9 mL/min. The column temperatures for various PCR products were: 59℃ for P3-4; 62℃ for AXIN1 exons 1A, 1B, 1C and 3; 63℃ for exons 2 and 10; 64℃ for exons 7 and 9; 65℃ for exons 5A and 6; 66℃ for exons 5B and 8; 68℃ for exon 4; and 66℃ for AXIN2 exon 7.

Sequencing analysis

PCR products were treated with exonuclease and shrimp alkaline phosphatase based on the protocol provided by United States Biochemical Corporation and sequenced by the Mayo Clinic DNA sequencing facility. Sequencing reactions were performed in a GeneAmp PCR System 9600 with fluorescent terminations, and products were analyzed on an ABI 377 sequencer (Perkin-Elmer). All sequence alterations were confirmed by bidirectional sequencing of PCR products generated by at least two independent reactions.

Immunohistochemical staining

Tissue specimens were fixed in 10% neutral-buffered formalin and were paraffin embedded according to standard procedures. Four-micrometer-think sections of representative blocks from each case were deparaffinized in xylene, rehydrated, and treated with 3% H_2O_2 for 10 min to block endogenous peroxidase activity. All sections were subjected to heat-induced epitope retrieval in a microwave oven. Sections were incubated with anti-β-catenin mouse monoclonal antibody (1:50, Santa Cruz, USA) at 4℃ overnight. The sections were treated with polyperoxidase anti-mouse/rabbit immunoglobulin (GBI) for 30 min at 37℃ and antibody-binding sites were visualized by DAB kit (Zhongshan Golden Bridge Co., Beijing, China). Negative controls were performed by omitting the primary antibody.

RESULTS

We evaluated the entire coding region of the AXIN1 gene for mutations by DHPLC followed by sequencing. Two types of mutation were identified in 4 (5.7%) of 70 GC cases, but not in the matched normal tissues. Three cases, including one intestinal-type and two diffuse-type, contained a 1-bp deletion at nucleotide 1076 (Figure 1A). This mutation interrupts the GSK3β and β-catenin binding domains. A point mutation of G to T transition at nucleotide 1578 was observed in one intestinal-type, resulting in a substitution of the amino acid at 489 from glycine to valine (Figure 2). This missense mutation occurs in the β-catenin binding domain. Five single-nucleotide polymorphisms, 334 C>T, 874 C>T, 1396 G>A, 1690 C>T and 1942 T>G, were identified within the coding region. The frequencies of these variant alleles were 0.04, 0.43, 0.10, 0.03 and 0.13, respectively (Table 2). All of them were silent polymorphisms, and the functions of these polymorphisms are unknown. Representative examples of heteroduplexes detected by DHPLC are shown in Figure 1B.

We screened exon7 of the AXIN2 gene for mutations and one intestinal-type revealed a frameshift mutation (1 bp deletion), occurring in the mononucleotide repeat sequences (2083 del G) located in exon7 (Figure 1C). This mutation leads to the elimination of the DIX domain. This case had AXIN2 and AXIN1 (1 bp deletion at nucleotide 1076) mutations.

Table 1 Primers used for AXIN1, AXIN2 and CTNNB1 gene amplification

Exon	Sense primer	Antisense primer
AXIN1		
exon1A	5′-TGGTCGTGTTTCATGGACCC-3′	5′-AATGCAGTGACTCAGCCCACT-3′
exon1B	5′-GGATCTGGACCTGGGGTATG-3′	5′-ATAGTGGCCTGGATTTCGGT-3′
exon1C	5′-ATCATGAAGCAGCTGATCG-3′	5′-GAGGTGAGTACAGAAAGTGGAC-3′
exon2	5′-CTGTTGGCAGGCTGTCACT-3′	5′-GTCCGTGAGGGACTGGGTA-3′
exon3	5′-CTGGCCCTCCTGCTCCTCT-3′	5′-AGGACGATGGGCTGAGGAC-3′
exon4	5′-TTTAGCCTGTGACCTTTCAAC-3′	5′-ATCCCGGCGGCAAGAA-3′
exon5A	5′-AGCTGGTGCTGAGAGGTGATG-3′	5′-CCCTGACTTGGGTACGTGCTT-3′
exon5B	5′-TGGGCACGTGGCCAAGAT-3′	5′-AAGCCCCCTCCTCACTGACAG-3′
exon6	5′-CACCGGAGGCCAGGGCGACT-3′	5′-TGGCAAAGCAGGCCCCACGA-3′
exon7	5′-CCAGGGTGTCGGCCACAGTC-3′	5′-CCCCAGGAGTGGTGCTGTGGT-3′
exon8	5′-GCGCAGCAGCATTGGTCGAG-3′	5′-GGGCAGGACCGGGAGGACC-3′
exon9	5′-AGTCCAAAACCAGGTACCAC-3′	5′-AAACCCTCTTTTCATACCG-3′
exon10	5′-CACGCCGTCCCCTGCCAC-3′	5′-GACACCCGTGCCCGCCAA-3′
AXIN2		
exon7	5′-TCCTTGTTTTGCCCAAAGC-3′	5′-GGTCAGGGGAGGCATCGCAG-3′
CTNNB1		
exon3		
P3-4	5′-GATTTGATGGAGTTGGACATGG-3′	5′-TGTTCTTGAGTGAAGGACTGAG-3′

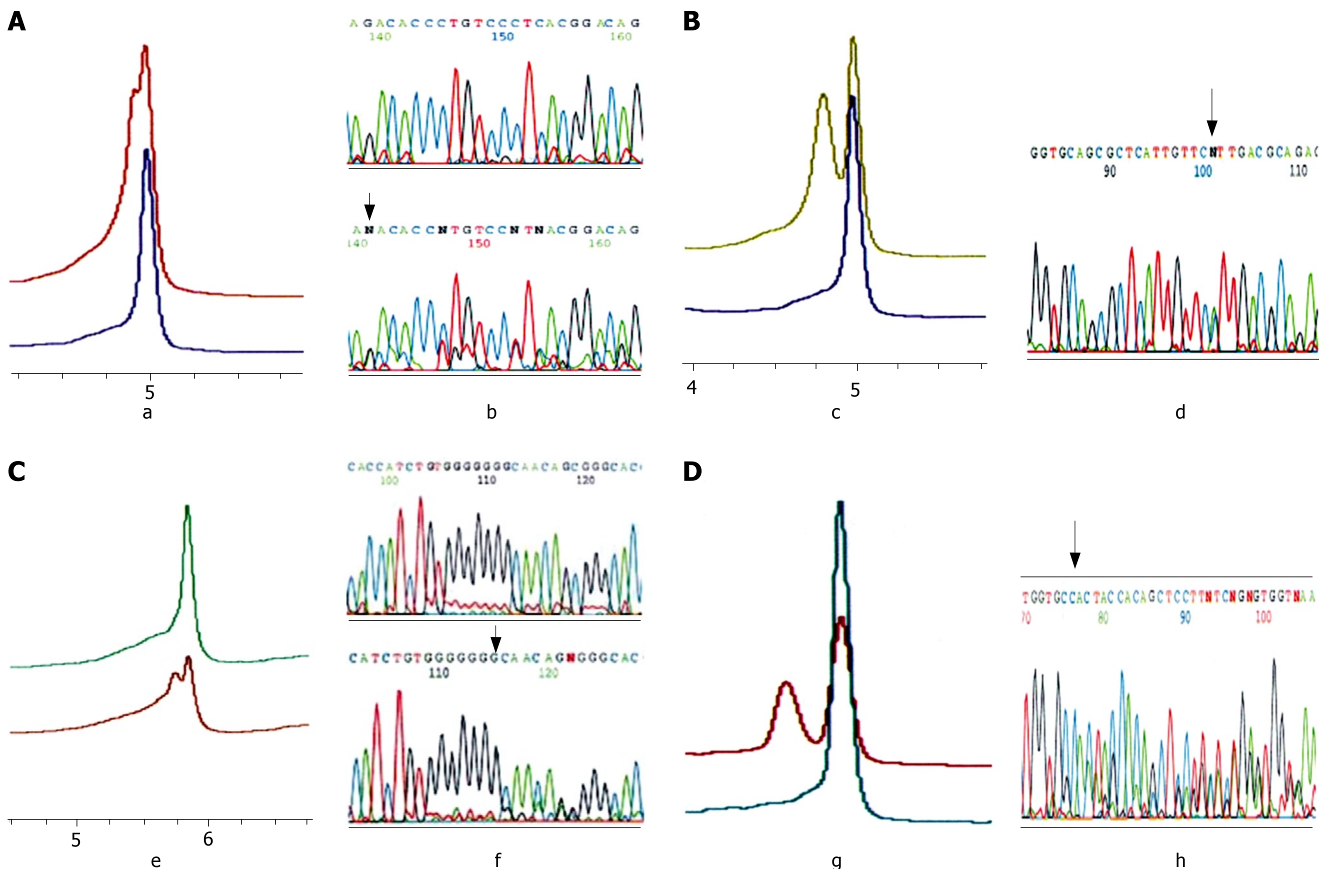

Figure 1 Typical elution profiles of DHPLC analysis and sequencing. **A**: AXIN1 (exon2) mutation. a, DHPLC elution profiles. b, Sequence traces. The upper panel is a normal control; the lower panel is a frameshift mutation (del 1 bp). The arrow points to the mutant nucleotide; **B**: AXIN1 polymorphism. c, Representative DHPLC profiles of heteroduplex for AXIN1. The lower panel is a normal control; the upper panel is a heteroduplex. d, Sequence traces. The arrow points to the mutant nucleotide; **C**: AXIN2 (exon7) mutation. e, DHPLC elution profiles. f, Sequence traces. The upper panel is a normal control; the lower panel is a frameshift mutation (del 1 bp). The arrow points to the mutant nucleotide; **D**: CTNNB1 (exon3) mutation. g, DHPLC elution profiles. The lower panel is a normal control; the upper panel is a frameshift mutation (del 27 bp spanning exon3). h, Sequence traces for the frameshift mutation. The arrow points to the mutant nucleotide.

Somatic mutations in exon3 of the CTNNB1 gene were detected in the same samples. A 27-bp deletion spanning exon3 of CTNNB1 was observed in one of the diffuse-type cases (Figure 1D), which did not contain AXIN1 or AXIN2 mutations. No mutations were detected in any of the normal tissues. This result indicated that AXIN1 and AXIN2 mutations may be solely responsible for the disruption of the Wnt signaling pathway in GC cases.

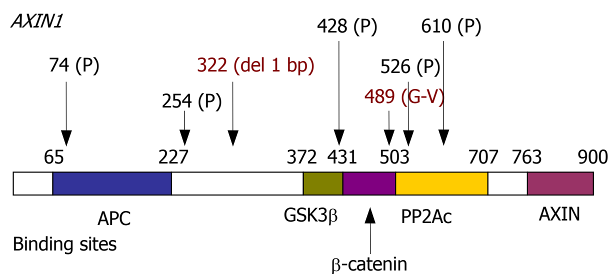

Figure 2 Mutations and polymorphisms found in AXIN1. Bold lines indicate the coding regions corresponding to each protein-binding site. GSK3β: Glycogen synthase kinase 3β; PP2Ac: Protein phosphatase 2Ac; P: Polymorphism.

The functional importance of AXIN1, AXIN2 and CTNNB1 mutations in GC development was further analyzed by immunohistochemical analysis of β-catenin in 50 available GC tissue samples. Ten (20%) of the GCs showed increased nuclear immunoreactivity for β-catenin compared with non-cancerous tissues. All four GCs with AXIN1 and AXIN2 mutations showed nuclear β-catenin expression.

DISCUSSION

Activation of Wnt signaling is an important step in the development of human tumors[23,24]. APC is part of a multiprotein complex in the Wnt pathway which induces β-catenin degradation[25]. AXIN1, AXIN2, GSK-3β, β-catenin, PP2A and PP2C are additional components of this complex[26]. In this complex, AXIN1 and AXIN2 play major roles as scaffold proteins. Mutations in CTNNB1 or APC have been reported in GC[17,18]. To explore if AXIN1 and AXIN2 are also involved in the pathogenesis of GC, we examined mutations in the entire coding region of AXIN1, exon7 of AXIN2 and exon3 of CTNNB1 in 70 GCs. Four (5.7%) of the 70 GCs had mutations in AXIN1, one had frameshift mutations in AXIN2 and one had a mutation in CTNNB1. Our results showed that AXIN1 and AXIN2 are likely to be tumor suppressor genes involved in the carcinogenesis of GC. This is the first study, to our knowledge, to demonstrate AXIN1 and AXIN2 mutations in GC.

AXIN1 mutations have been reported in hepatocellular carcinomas, hepatoblastomas and sporadic medullo-blastomas[10,11]. We have now identified genetic alterations of AXIN1 in GCs. We found a 1-bp deletion at nucleotide 1076, which has previously been reported in hepatocellular carcinomas, in three samples[11]. This mutation interrupts the GSK3β and β-catenin binding domain. Additionally, we discovered a somatic point mutation in GCs, resulting in a substitution of the amino acid at 489 from glycine to valine, a site located within the β-catenin binding domain. It is possible that such alterations may alter protein secondary structure and thereby the interaction of β-catenin with APC/GSK-3β complex. Since AXIN1 is a negative regulator of β-catenin, failure to form a complex with any one of these key players of Wnt signaling is likely to stabilize β-catenin and lead to its accumulation.

AXIN2 mutations were found in one of the 70 GCs. Such mutations resulted in the elimination of the DIX domain, which is necessary for AXIN oligomerization[27-29]. This case had both AXIN1 and AXIN2 mutations, and showed increased nuclear immunoreactivity for β-catenin. AXIN2 mutations have been previously reported in colorectal cancers (in 10 of 11 cases) and were also accompanied by increased nuclear β-catenin staining[7], similar to our results in GC. This study also showed that frameshift mutations in AXIN2 appear to be specifically associated with defective DNA mismatch repair (MMR) in colorectal cancer[7]. To determine the MSI status, we identified two mononucleotide markers, BAT26 and BAT25, by DHPLC[30]. This case was detected as MSI-H. Our results indicate that GCs with MMR deficiency often harbor somatic frameshift mutations in cancer-related genes.

Table 2 The profile of AXIN1 polymorphisms

Exon	Nucleotide changes	Amino acid[1]	Frequency (%)
1	334 C→T	Gly74Gly	4.3
	874 C→T	Asp254Asp	42.8
5	1396 G→A	Ser428Ser	10.0
	1690 C→T	Asp526Asp	2.9
6	1942 T→G	Ala610Ala	12.9

[1]Amino acid number begins from Met at position 113 of GenBank accession, No. AF009674.

CTNNB1 mutations were identified in 70 GCs. A 27-bp deletion spanning exon3 of CTNNB1 was observed in one of the cases, which showed increased membrane and cytoplasmic immunoreactivity for β-catenin. We did not detect any missense mutations of this gene in our GC cases. CTNNB1 mutations occur even more frequently in colon cancer and hepatoblastoma[11,12]. A number of studies have identified CTNNB1 mutations in GCs, but the frequencies of these mutations were significantly different in those studies[17,31]. The different frequencies of CTNNB1 mutations identified could be due to differences in the populations examined or the method of microdissection of tumor cells used. Analysis of β-catenin in a large sample set using a precise method of microdissection is needed to delineate the functional importance of this gene in GC development.

In conclusion, our results indicate that AXIN1 and AXIN2 mutations may contribute to the pathogenesis of GC *via* activation of the Wnt signaling pathway. Further studies will be required to investigate the function of mutated AXIN1 and AXIN2 variants reported in this study.

COMMENTS

Background

The Wnt signaling pathway is known to be involved in tumorigenesis. Recently, AXIN1 and AXIN2, components of Wnt signaling pathway, were characterized as new candidate tumor suppressor genes that may be targeted for deletion or mutation during tumorigenesis.

Research frontiers

Mutations in AXIN1, AXIN2 and CTNNB1 were identified by PCR-based denaturing high-performance liquid chromatography (DHPLC) and direct DNA sequencing. Beta-catenin expression was detected by immunohistochemical staining.

Innovations and breakthroughs

A total of 5 (7.1%) GCs had mutations in one or two of these three components. A frameshift mutation in exon7 of AXIN2 was found in one case. Four cases had frameshift mutations and missense mutations in AXIN1, and 5 single nucleotide polymorphisms (SNPs) were identified in AXIN1. All four cases with mutations in AXIN1 and AXIN2 showed nuclear beta-catenin expression.

Applications

AXIN1 and AXIN2, key players in the Wnt signaling pathway, are involved in gastric carcinogenesis, but the functions of mutated AXIN1 and AXIN2 variants need to be further investigated.

Peer review

This is an interesting report of mutations in components of the Wnt signaling pathway in 70 patients with gastric cancer. They examined AXIN1, AXIN2 and CTNNB1 and found mutations in only 5 (7.1%) of the patients. This is an important and well-written paper.

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