

• GASTRIC CANCER •

Mutation analysis of APC gene in gastric cancer with microsatellite instability

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Abstract

AIM: To evaluate the role of APC mutation in gastric carcinogenesis and to correlate APC mutation with microsatellite instability (MSI) in gastric carcinomas.

METHODS: APC mutation was measured with multiplex PCR, denaturing gradient gel electrophoresis and DNA sequencing; and MSI was analyzed by PCR-based methods.

RESULTS: Sixty-eight cases of sporadic gastric carcinoma were studied for APC mutation at exon 15 and MSI. APC mutations were detected in 15 (22.1 %) gastric cancers. Frequency of APC mutation (33.3 %) in intestinal type of gastric cancer was significantly higher than that in diffuse type (13.1 %, $P < 0.05$). On the contrary, no association was observed between APC mutation and tumor size, differentiation, depth of invasion, metastasis or clinical stages. Using five microsatellite markers, MSI in at least one locus was detected in 17 of 68 (25 %) of the tumors analyzed. APC mutations were all detected in MSI-L (only one locus, $n=9$) or MSS (tumor lacking MSI or stable, $n=51$), but no mutation was found in MSI-H (≥ 2 loci, $n=8$).

CONCLUSION: APC mutation is involved in carcinogenesis of intestinal type of gastric cancer and is independent of MSI phenotype but related to the LOH pathway in gastric cancer.

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INTRODUCTION

The mechanisms of carcinogenesis in the gastric mucosa remain unclear. Adenomatous polyposis coli or APC, has been characterized as a tumor suppressor gene and considered a "gatekeeper" because alterations in this gene occur as an early event in the neoplastic transformation^[1-3]. The APC gene was first identified in the germline of individuals with the condition known as familial adenomatous polyposis or FAP^[4] and has been shown to play a role in the development of sporadic

colorectal cancer^[5,6]. Studies have attempted to characterize the mutation of APC in sporadic gastric cancer^[7-10] and in a limited number of familial gastric cancer^[11,12]. It has been found that inactivation of APC plays a role in the development of some gastric cancers, particularly very well differentiated adenocarcinomas and signet-ring cell carcinomas^[13,14], and that the mutations of the APC gene, similar to those in colorectal tumorigenesis, occur during the early stages of gastric adenoma development^[15,16].

Genetic instability is strongly involved in neoplastic transformation and progression^[17]. In gastrointestinal carcinomas, such genetic instability may be classified into two different forms in which hypermutability occurs either by means of chromosomal instability or microsatellite instability (MSI)^[18-23]. MSI represents an important new form of genetic alteration characterized by widespread instability in repetitive nucleotide sequences. MSI has been found in the majority of tumors associated with hereditary non-polyposis colorectal cancer (HNPCC)^[24,25] in which germ-line mutation occurs within the mismatch repair genes hMSH2, hMLH1, hPMS2 or hMSH6^[26-28]. Mutations of the transforming growth factor type β receptor gene (TGF- β R II), and BAX gene are strongly correlated with MSI^[29-31]. MSI is also a distinctive feature in about 10-15 % of sporadic colorectal tumors and to a varying degree in tumors of other organs, including the stomach^[32-34]. Although mutations of APC have been reported in gastric cancer, less clear, however, is the relevance of APC mutation as a potential factor in MSI-positive gastric cancer. The aim of the present study is to correlate APC mutation with MSI in gastric carcinomas.

MATERIALS AND METHODS

Tissue samples

Sixty-eight cancer and corresponding normal tissues were obtained from surgically resected gastric carcinoma in Southwest Hospital. Each specimen was frozen immediately and stored at -80°C until analyzed. A $5\mu\text{m}$ section was cut from each tissue and stained with hematoxylin/eosin in order to confirm whether the cancer cells in tissues were predominant or not. Genomic DNA was isolated by standard proteinase-K digestion and phenol-chloroform extraction protocols. Of the 68 patients with gastric cancer, 45 were men and 23 were women with an age range of 30-76 years (mean age of 56.2 years at diagnosis). None of the patients included in the present series had a family history suggestive of HNPCC and had received chemotherapy or radiation therapy.

Multiplex PCR

Multiplex PCR was utilized in order to visualize the product of several regions simultaneously. Singleplex PCR was also used to help confirm cases and for regions that were difficult to amplify. Multiplex PCR analysis of APC exon 15 was performed using primers with a GC clamp (Table 1). Genomic DNA (100-400 ng), 0.25 μM primer for multi-primer reaction,

1×buffer, 1.5 mM MgCl₂, 0.25 mM of each dNTPs, and 0.1 U/μl Amplitaq Gold were combined in 10 μl reaction. The PCR reaction consisted of a denaturation step at 95 °C for 12 minutes followed by 32 cycles of 40 seconds at 94 °C, 60 seconds at 49 °C, and 90 seconds at 72 °C. Elongation occurred through 10 minutes at 72 °C, 10 minutes at 98 °C, 30 minutes at 50 °C and 30 minutes at 37 °C.

Denaturing gradient gel electrophoresis (DGGE)

DGGE analysis was performed for scanning mutations at 15 exon of APC gene. 8 μl of each sample was loaded on the gels consisting of 10 % polyacrylamide and 10-60 % urea-formamide. The gels were run at 90V for 17 hours in tanks of buffer at 50 °C. Gels were stained with 0.5× Sybr Green I and Sybr Green II for 20 minutes and visualized by UV transillumination. Heteroduplex samples were further analyzed by manual sequencing.

Manual sequencing

Samples were amplified for sequencing with PCR primers that did not include the GC clamp (Appendix A). The PCR reaction included 200-400 ng of genomic DNA, 0.25 μM primer, 1×buffer, 1.5mM MgCl₂, 0.25mM of each dNTPs, and 2.5U Amplitaq Gold were combined in a 10 μl reaction. The PCR reaction consisted of a denaturation step at 94 °C for 3 minutes followed by 35 cycles of 60 seconds at 94 °C, 60 seconds at 55 °C, and 60 seconds at 72 °C. A final extension step included 10 minutes at 72 °C. 1.5 % agarose gels were run at 150V to confirm the existence of PCR product. The product was purified following the instructions of the Wizard PCR Preps DNA Purification System (Promega, Madison, WI). Following the purification process, the product was run on a 1.5 % agarose gel at 150V to verify the product. Sequencing was performed following the instruction of the system. The film was read independently by two individuals according to the APC sequence.

Table 1 Primer Sequences used in APC mutation analysis

Exon	GC Clamp	Primer Sequences	Size (bp)
15.1	2/0	F-TTCAGGCAAATCCTAAGAGA/R-TTGAGCCAGGAGACATAATA	329
15.2	0/2	F-GGAATCTCATGGCAAATAGG/R-TCATCATGTCGATTGGTGTGTC	284
15.3	2/0	F-AGCAAAGTCTCTATGGTGAT/R-TGACACTTCTTCCATGACTT	387
15.4	0/2	F-CTACCATCCAGCAACAGAAA/R-TGGCATAAGGCATAGAACAT	312
15.5	2/0	F-AAGCTCTGCTGCCCATAACAC/R-CTAGGTCGGCTGGGTATTGA	282
15.6	2/5	F-CTTATGCCAAATTAGAATAC/R-ACTGCTCATCTGAATATTTA	306
15.7	7/5	F-ATGATGGAGAACTAGATACA/R-ATCAGTGCTCTCAGTATAAAA	253
15.8	2/5	F-AAGACCCAAACACATAATAG/R-TAATTGGTAGGCTTATCATC	327
15.9	2/0	F-AAATCGAGTGGGTTCTAATC/R-TTCTCACTGCTTGAAGACAT	355
15.10	2/6	F-CAGCCTATTGATTATAGTTT/R-AACATATTGGAGTATCTTCT	344
15.11	2/0	F-TTAACCAAGAAACAATACAG/R-CTTCGCTCACAGGATCTT	320
15.12	2/0	F-GGAAGCAGATTCTGCTAATA/R-ATAGTGTTTCAGGTGGACTTT	287
15.13	2/5	F-AGAATCAGCCAGGCACAAAG/R-CATGGTTTGTCCAGGGCTAT	310
15.14	2/6	F-TGGTAAGTGGCATTATAAGC/R-AGTATCAGCATCTGGAAGAA	279
15.15	5/2	F-AAACCAAGCCAGAAGTACCT/R-AGGCTGCTCTGATTCTGTTT	335
15.16	2/5	F-CGATGAGCCATTTATACA/R-TTTCGTTTACGTGATGAC	291
15.17	4/2	F-ATTATTTCTGCCATGCCAAC/R-TTCGATTGTTAGATCACTTA	300
15.18	2/6	F-TGCCACGGGTGTATTGTGTT/R-TTTCCTTTGGGCATAGCAG	351
15.19	4/2	F-GGTGATATTCTTGCAGAATG/R-CACGTGCTCTATATTCAGTA	250
15.20	2/0	F-GAAACCAACTTCACCAGTAA/R-AATAGGCGTGTAATGATGAG	378
15.21	2/0	F-AGTCCCAAATAATGAAGAT/R-GATTGTTGGTTGGAGGTTAG	342
15.22	2/6	F-GCTAAAGTTACCAGCCACAC/R-TTGGTCAATGTCACTGAGAG	
15.23	2/0	F-CCAGTCATCCAAAGACATAC/R-CAACAGGTCATCTTCAGAGT	
15.24	2/5	F-TCACAGGGAGAACCAAGTAA/R-TTGCACCTTCCTGAATAGC	
15.25	2/3	F-ATGGGTGGCATATTAGGTGA/R-TCGTGGGCGCTTTATTACTTG	

GC clamp: 1=CGCCCCGCGCGCCCCGCGCCCGTCCCGCGCGCCCCGCGCCG;

2=CGCCCCGCGCGCCCCGCGCCCGGCCCCGCGCCCCGCGCCG;

3=CG; 4=GCGCG; 5=CGTCCCGC; 6=GCGCCCGTCCCGCCCC;

7=CGCCCCGCGCGCCCCGCGCCCGTCCCGCGCGCCCCGCGCCGCCCCG;

MSI detection

MSI analyses included five microsatellite markers: BAT25, BAT26, BAT40, D2S123, and D5S346. PCR was performed as previously described^[35]. MSI was defined as the presence of band shift in the tumor DNA that was not present in the corresponding normal DNA. Based on the number of mutated MSI markers in each tumor, carcinomas were characterized as MSI-H if they manifested instability at two or more markers, MSI-L if unstable at only one marker, and MSS if they showed no instability at any marker.

Statistical analysis

Chi-square test with Yates' correction was used. A *P* value <0.05 was considered significant.

RESULTS

Multiplex PCR and DGGE were used to scan the mutation at the exon 15 of APC in 68 gastric cancers (Figure 1). The samples that showed alterations by DGGE were further analyzed through manual sequencing (Figure 2). APC mutations were detected in 15 samples (22.1%) held APC mutations that lead to premature protein truncation (Table 2). The truncation of the protein occurred as a result of a stop codon created through a base pair change, an insertion, or a deletion.

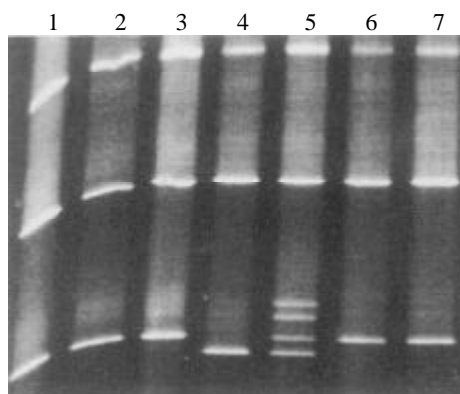


Figure 1 Abnormal four-band patterns in lane 5 after multiplex PCR and denaturing gradient gel

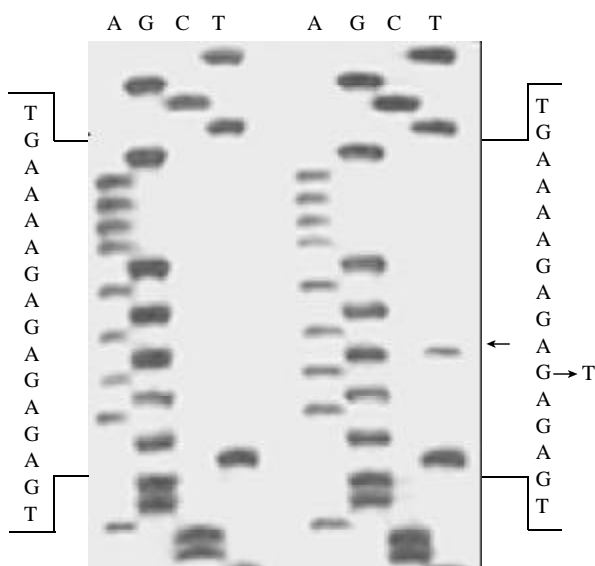


Figure 2 DNA sequencing shows a G→T change at 1464 codon

We compared mutation of exon 15 of APC gene in gastric cancer with various clinicopathological parameters including tumor size, differentiation, TNM status, clinical stage and Lauren's types. Table 3 shows the association of mutations of APC to the clinicopathological parameters of 68 gastric carcinomas. Mutations of APC gene were observed more common in intestinal type than in diffuse type tumors (*P*<0.05).

Alterations of electrophoretic patterns of PCR products of five microsatellite markers were compared between tumor and normal DNA in each patient. MSI affecting at least one locus was observed in 17 (25 %) of 68 tumors, among which eight (11.8 %) were MSI-H, nine (13.2 %) were MSI-L, and fifty-one (75 %) were MSS. Table 4 shows the association of mutations of APC to MSI of 68 gastric carcinomas. Three mutations were found in 9 tumors with MSI-L and 12 were found in 51 tumors with MSS, and no mutation was found in 8 tumors with MSI-H.

Table 2 APC mutation in 12 gastric carcinomas

Exon region	Nucleotide alteration	Nucleotide location	Codon number
15.4	C→A	2883	940
15.5	insA	2866	935
15.10	del A	3835 or 3836	1258
15.11	del AAAA	3981	1307
15.12	del AAAGA	3988	1309
15.12	G→T	4010	1317
15.13	delT	4146	1362
15.13	delGT	4254	1398
15.13	delT	4282	1407
15.14	C→T	4410	1450
15.14	G→T	4452	1464
15.15	delAG	4455	1465
15.19	insCT	5326	1755
15.21	insT	5596	1845
15.22	delG	5940	1960

Table 3 The relationship between the APC mutation and clinicopathological parameters

Parameters	No. of cases	APC mutation
Size		
<5cm	31	7
>5cm	37	8
Differentiation		
Well	14	4
Moderate	20	6
Poor	28	3
Mucinous	6	2
Lauren classification		
Intestinal type	30	10 ^a
Diffuse type	38	5
Serosal invasion		
Absent	30	6
Present	38	9
Metastasis		
Absent	33	8
Present	35	7
Clinical stage		
I and II	36	9
III and IV	32	6

^a*P*<0.05 vs diffuse type

Table 4 The relationship between MSI and APC mutation

MSI status	No. of cases	APC mutation
MSI-H	8	0
MSI-L	9	2
MSS	51	13

DISCUSSION

Mutations of APC gene has been shown to play an important role in colorectal tumorigenesis. A germline mutation in APC contributes significantly to the development of colitis-associated neoplasia^[36]. In the current study, we investigated mutations of the APC gene in 68 gastric carcinomas obtained surgically. Mutations at exon 15 of APC gene were detected in 22.1 % of gastric cancers. This finding is similar to the previous studies^[14,15]. This proportion may be an underestimate because the mutation of exons 1 through 14 were not detected in this study. Previous investigations have found that the 5' half of APC holds the majority of the germline mutations^[3,37]. The mutations identified in the study were spread throughout the exon 15 of APC sequence. Sequencing analysis confirmed that the mutations resulted in truncation of the gene products or in an amino acid change. The APC gene encodes a large protein with multiple cellular functions and interactions. Mutations in this gene would lead to alterations in signal transduction of cell, differentiation, mediation of intercellular adhesion, stabilization of the cytoskeleton and possibly regulation of the cell cycle and apoptosis^[38,39]. Our results imply that APC plays a crucial role in gastric carcinogenesis, as was observed in colorectal carcinogenesis.

In the current study, we did not find an obvious relationship between the APC mutation and tumor size, depth of invasion, node metastasis or clinical stages, indicating a limited role of the APC mutation in predicting prognosis of gastric carcinomas. This finding is in agreement with the recently published data on cholangiocarcinoma and breast cancer^[40,41]. Gastric carcinomas can be divided into "intestinal" type and "diffuse" type. The intestinal type of gastric cancer is the predominant type in elderly population at high risk and preceded by well-defined precancerous lesions, such as intestinal metaplasia and atrophic gastritis. The diffuse type is relatively more frequent in low risk populations and is not as often preceded by intestinal metaplasia. A distinct genetic pathway exists in gastric carcinogenesis of different histological subtypes and their tumor progression^[42-45]. Increased beta-catenin mRNA levels and mutational alterations of the APC and beta-catenin gene were present in intestinal type gastric cancer^[46,47], whereas epigenetic inactivation of E-cadherin via promoter hypermethylation may be an early critical event in the development of undifferentiated tumors^[48-51]. In this study, marked difference in APC mutation was noted in gastric cancer by histological type. APC mutations were significantly more frequent in intestinaltype gastric cancers as compared with diffusetype gastric cancers, suggesting that APC gene is not only a predisposing gene in colorectal cancer but also a predisposing gene in intestinal type of gastric cancer. The mutation of APC gene may be considered makers for intestinal or colonic differentiation. The mutation of APC in the majority of intestinal type cancers also supports the theory that these cancers result from transformation of intestinal metaplasia.

There is evidence that MSI cancer comprises distinctive MSI-H and MSI-L categories^[14]. MSI-H cancers are distinguished clinicopathologically and in their spectrum of genetic alterations from cancers showing MSI-L and MSS cancers^[14]. Our previous studies indicated that MSI-H gastric cancers often show lower frequency of LOH of APC, MCC and DCC genes than do MSI-L and MSS cancers^[1]. In present study, 15 APC mutations were all detected in MSI-L and MSS, but no mutation was found in those showing MSI-H. This result indicates that APC is mutational target in MSI-L and MSS tumor cells and support the notion that APC mutation-positive tumors may identify an alternative pathway which is probably

different from MSI-related phenomenon observed in HNPCC. Our analysis of APC mutation should further provide some clues to the molecular mechanisms underlying the profound genomic instability in the MSI and LOH pathway for gastric carcinoma.

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