Genetic Instability in Gastric Epithelial Neoplasias Categorized by the Revised Vienna Classification

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Background/Aims: The aim of this study was to determine the structural chromosomal aberrations, such as loss of heterozygosity (LOH) and microsatellite instability (MSI), at multiple tumor suppressor gene loci in gastric epithelial neoplasia categorized by the revised Vienna classification. **Methods:** All tissue samples were excised by endoscopic mucosal resection. Sixty category 3 (low-grade adenoma) tissue samples and 51 category 4 samples (high-grade adenoma and intramucosal carcinoma with adenoma) were examined at the 7 sets of microsatellite loci linked to the tumor suppressor gene locus. **Results:** For category 3 and 4 tissue samples, there were no differences in the frequencies of LOH-positive chromosomes or the extent of chromosomal loss. The *Helicobacter-pylori* (*H. pylori*)-positive rate was significantly higher in MSI-positive category 4 samples than in category 3 samples ($p=0.04$). The frequency of MSI positivity was significantly higher in category 4 samples than in category 3 samples ($p=0.003$). **Conclusions:** *H. pylori* infection is associated with genetic instability of the premalignant lesion. MSI occurs in the early stages of gastric carcinogenesis and its occurrence increases during malignant transformation. Detection of MSI in premalignant gastric lesions may be a surveillant of risk of malignant transformation. (*Gut Liver* 2010;4: 179-185)

Key Words: Genetic instability; Gastric epithelial neoplasia; Vienna classification

INTRODUCTION

Gastric cancer is one of the most common malignancies and leading causes of death in Asia.¹ Gastric carcinogenesis is characterized by the successive accumulation of mutations in genes that control epithelial growth and differentiation.^{2,3} Theoretically and practically, genetic instability initiates and drives the growth of most cancers. Genetic instability is divided into two categories, chromosomal (CIN) and microsatellite instability (MSI) and whether the instability is at the chromosomal or nucleotide level in a lesion.^{4,5} CIN is defined as the loss of chromosomal material during dysfunctional chromosome replication, repair or segregation⁶ and MSI, which results from an erroneous DNA mismatch repair system, has been well known to be involved in the carcinogenesis of hereditary non-polyposis colon cancers and some of sporadic colorectal cancers.^{7,8}

Gastric epithelial dysplasia or gastric adenomas have been considered to be precancerous lesions with a variable clinical course. Some progress to adenocarcinomas, whereas others persist unchanged for a long time.^{9,10} They are considered benign neoplastic epithelial lesions, histologically distinguishable from invasive carcinoma and regenerative changes. Their features are characterized by cellular atypia, abnormal differentiation and disorganized architecture.¹¹ However, the absence of a uniform classification has resulted in disagreement regarding the differentiating features of preinvasive and early gastric cancer among Western and Japanese histopathologists.¹² Since

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2000, the introduction of the revised Vienna classification^{13,14} has helped to resolve this problem. But this classification is not perfect and it is difficult to discriminate the lesions that progress to the true malignancy.

To date, no study has determined genetic instability of gastric precancerous lesion that divided into two subgroups specific to dysplastic grade. In this study, we aimed to compare the genetic instability of category 3 (low grade dysplasia) with category 4 (high grade dysplasia and intramucosal cancer) lesions, according to the revised Vienna classification.

MATERIALS AND METHODS

All tissues were excised by therapeutic endoscopic mucosal resection. According to the revised Vienna classification, 60 category 3 (low grade adenoma) (32 men, 28 women, mean age 62.0 ± 8.0 years) and 51 category 4 (36 men, 15 women, mean age 63.5 ± 8.3 years) tissue samples were obtained from the department of pathology at the St. Vincent Hospital of the Catholic University of Korea for investigation. Among the category 4 gastric epithelial neoplasia (GEN), 30 tissues were high grade adenomas (category 4.1) and 21 were intramucosal carcinomas with adenomas (category 4.4).

1. DNA extraction

The diagnosis of tissue sample was confirmed by two different histopathologists; when they disagreed, the tissue sample was excluded from the study. All normal tissues had grossly intact mucosa and were at least 1 cm from the mucosal lesion; they were obtained by gastric biopsy just after an endoscopic mucosal resection. The microscopic examination showed no evidence of malignant cells.

Two forty-micrometer-thick tissue sections from cancer and normal tissues were placed on glass slides. The tissue sections were then dehydrated in graded ethanol solutions and dried without a cover glass. The DNA was extracted from the tissues with 20 μ L of extraction buffer (100 mmol/L Tris-HCl; 2 mmol/L ethylene diamine tetraacetic acid (EDTA), pH 8.0; 400 μ g/mL of proteinase K) at 55°C overnight. The tubes were boiled for 7 minutes to inactivate the proteinase K and then 1 μ L of these extracts was used for each polymerase chain reaction (PCR) amplification.

2. PCR-SSCP analysis

Seven loci that are tightly linked to tumor suppressor genes were used to identify significant structural chromosomal aberrations. The DNA was amplified by PCR at mi-

crosatellite loci linked to the adenomatous polyposis coli (APC) locus at 5q21 (D5S505), possible tumor suppressor/senescence gene locus at 10p15 (D10S501 and D10S602), the *p53* locus at 17p13 (TP53), the *BRCA1* locus at 17q21 (D17S855), and the *DCC* locus at 18q21 (D18S58 and D18S61).

A 10% neutral polyacrylamide gel electrophoresis was performed as previously described. In brief, 4 mL 30% acrylamide (29:1) solution, 2.4 mL 5 $\times$ TBE solution, 5.6 mL ddH₂O, 200 μ L 10% ammonium persulfate, and 10 μ L TEMED, were blended adequately and poured into the gel, then concreted for 1 hour at room temperature. Ten μ L of PCR product and 2 μ L of loading buffer (95% formamide, 10 mM NaOH, 0.1% bromophenol blue, 0.1% xylene cyanol) were mixed. The mixture was centrifuged for 15 seconds, denatured at 93°C for 3 minutes, bathed in ice for 10 minutes, placed onto a 10% non-denaturation polyacrylamide gel, and separated with 0.5 $\times$ TBE buffer for 2 hours at RT and 100 V.

3. Detection of DNA fragments - Silver stain

After electrophoresis, the gel was stained using the Bio-Rad Silver Stain kit (Bio-Rad, Philadelphia, PA, USA). Briefly, the gel was fixed with 40% methanol for 30 minutes, oxidized for 5 minutes, rinsed for 5 minutes three times, silver stained for 20 minutes, rinsed for 30 seconds, developed for 1 minutes three times and stopped with 5% acetic acid for 15 minutes. The staining results were analyzed with Gel DocXR (Bio-Rad).

Assessment of loss of heterozygosity (LOH) was assigned when a tumor allele showed at least 50% reduction in the relative intensity of one allele in the cancer

Fig. 1. Representative example of microsatellite instability and loss of heterozygosity. LOH, loss of heterozygosity; MSI, microsatellite instability; N, normal; T, tumor.

tissue compared to the normal matched DNA. MSI in DNA samples was also assessed as positive in cases with additional bands in the tumor sample, which were not observed in the corresponding normal sample or in cases with a band shift in the tumor sample that contrasted with those of the corresponding normal bands (Fig. 1).

4. Statistical analysis

The mean age of the cases was compared using Student's *t*-test. The frequencies of the LOH and MSI in the different histological types were compared using a Chi-square test with SPSS version 12.0 software (SPSS Korea, Seoul, Korea). A *p*-value less than 0.05 was regarded as significant.

RESULTS

The *Helicobacter-pylori* (*H. pylori*) status was evaluated according to the histological results (silver stain or CLO test); there were 50.0% (30/60) of category 3 and 54.9% (28/51) of category 4 samples that were associated with *H. pylori* infection (*p*=0.47). For category 4.1 and 4.4, the rate of *H. pylori* positive was 40.0% (12/30) and 76.2% (16/21) respectively. In category 4.4, *H. pylori* positive rate was significantly high (*p*=0.03).

There were no differences identified in the frequency of LOH-positive chromosomes between category 3 and 4 samples determined by the revised Vienna classification (Table 1). The frequency of LOH for the APC gene was 45.0% (27/60) in category 3. In category 4.1 and 4.4 (intramucosal carcinoma with adenoma), the frequencies of LOH for the APC gene were 36.7% (11/30) and 23.8% (5/21), respectively. The extent of chromosomal loss was scored as the number of LOH-positive chromosomes. According to the classification of chromosomal loss de-

scribed previously, GENs were divided into low-level (LOH-L; 3 or fewer losses) and high level (LOH-H; 4 or more losses).¹⁵ For category 3 and 4, the difference in the frequency of LOH-H was not significant (24/60 [40.0%] vs 21/51 [41.2%]; *p*=0.90). For category 4.1 and 4.4, there was no significant difference in the frequency of LOH-H (15/30 [50.0%] vs 6/21 [28.6%]; *p*=0.13).

The basal characteristics between MSI positive and negative samples were not significantly different. The frequencies of MSI positive were 25% (15/60) and 43.1% (22/51) in category 3 and 4. The basal characteristics between MSI positive category 3 and 4 was not different. In frequencies of MSI positive, there was a statistical difference (*p*=0.003) (Table 2). For category 4.1 and 4.4, the frequencies of MSI positive were 40.0% (12/30) and 47.6% (10/21), respectively. It showed increasing tendency but there was no significant difference in the frequencies of MSI positive. When we evaluated the relationship with MSI state and *H. pylori* infection, *H. pylori*-positive rate in MSI-positive category 4 samples was significantly higher than that in category 3 (*p*=0.04) (Table 2). For category 4.1 and 4.4, *H. pylori* positive rate were 66.7% (8/12) and 50.0% (8/16), respectively. In comparison with the *H. pylori* positive rate in MSI positive category 3 (20.0%, 6/30), the rate in category 4.1 and 4.4 was significantly high (*p*=0.009).

We next investigated the pattern of LOH and MSI state in the gastric epithelial neoplasias. With obtained results, we classified into four subtypes: MSI (-)/LOH (-), MSI (+)/LOH (-), MSI (-)/LOH (+), and MSI (+)/LOH (+). Of the 51 category 4 tissues, 27.5% (14/51) were both LOH (+) and MSI (+). We found that 15.7% (8/51) were LOH (-) and MSI (+), whereas 54.9% (28/51)

Table 1. LOH at Specific Chromosome Loci in GEN Categorized by the Revised Vienna Classification

Location (marker)	Frequency of LOH in category 3 (%; n=60)	Frequency of LOH in category 4 (%; n=51)	<i>p</i> -value
APC (D5S505)	45.0 (27)	31.4 (16)	NS
10p (D10S501)	45.0 (27)	41.2 (21)	NS
10p (D10S602)	48.3 (29)	52.9 (27)	NS
p53 (TP 53)	15.0 (9)	15.7 (8)	NS
BRCA1 (D17S855)	46.7 (28)	43.1 (22)	NS
DCC (D18S58)	53.3 (32)	51.0 (26)	NS
DCC (D18S61)	50.0 (30)	49.0 (25)	NS

LOH, loss of heterozygosity; GEN, gastric epithelial neoplasia; APC, adenomatous polyposis coli; BRCA1, breast cancer gene 1; DCC, deleted in colon cancer; NS, non specific.

Table 2. Positive Frequencies of LOH and MSI, and Basal Characteristics of the Patients

		LOH-positive		MSI-positive	
		Category 3	Category 4	Category 3	Category 4
Frequency		86.7% (52/60)	82.4% (42/51)	25.0% (15/60)	43.1% (22/51)*
Age	≤60	9	14	3	9
	>60	43	28	12	13
Sex	Male	28	28	7	17
	Female	24	14	8	5
Site	Antrum	37	34	11	16
	Body	15	8	4	6
<i>H. pylori</i>	Positive	25	19	6	16 [†]
	Negative	27	23	9	6

LOH, loss of heterozygosity; MSI, microsatellite instability.

**p*=0.003; [†]*p*=0.04.

Table 3. Patterns of LOH and MSI in Category 3 and 4 GEN

	Category 3		Category 4	
	MSI (–)	MSI (+)	MSI (–)	MSI (+)
LOH (–)	3 (5.0%)	5 (8.2%)	1 (2.0%)	8 (15.7%)
LOH (+)	42 (70.0%)	10 (16.7%)	28 (54.9%)	14 (27.5%)

LOH, loss of heterozygosity; MSI, microsatellite instability; GEN, gastric epithelial neoplasia.

were LOH (+) and MSI (–) (Table 3). Dividing the two groups of category 3 according to the diameter of the lesion, frequencies of LOH-positive or MSI-positive, in the group with a diameter less than 15 mm, were the same as the other group. Even if the category 3 were small (<15 mm), the frequencies of LOH positive was 87.5% (28/32) and MSI positive was 18.8% (6/32).

DISCUSSION

Use of the terms ‘adenoma’, ‘dysplasia’, ‘carcinoma *in situ*’ and ‘intramucosal carcinoma’ of gastric epithelium without classification have resulted in confusion with regard to diagnosis. Japanese pathologists diagnose the lesion primarily based on nuclear features and glandular structure, whereas Western pathologists consider the presence of invasion a prerequisite for the diagnosis of gastric carcinoma.^{12,16} In a previous report, pathologists with a Japanese viewpoint diagnosed carcinoma in 91% of sample slides; however, the pathologists with a Western viewpoint diagnosed carcinoma in 43% of the same slide.¹⁷ These inconsistencies lead to confusion with regard to treatment and often have resulted in over-treatment of gastric mucosal neoplasia by surgical resection.^{18,19} The revised Vienna classification has helped to provide guidance for current clinical management. However, there are frequent cases that do not easily fit into the diagnostic categories; 15-30% of low grade adenomas progress to high grade adenomas or adenocarcinomas.^{11,20,21} Therefore, it is difficult for the clinicians to determine which adenoma will progress and which ones will not progress, and the molecular pathogenesis that takes part in the progression will be greatly concerned.

A multi-step adenoma to carcinoma sequence has been established for colon cancer. Many investigators have attempted to identify an analogous multi-step sequence in the development of all types of cancer.²²⁻²⁴ In colon cancer model, the APC gene with CIN could drive most of the somatic evolution to cancer²⁵⁻²⁸ and CIN arises early and is likely to emerge before the inactivation of the first tu-

mor suppressor gene. The consequence of CIN is an imbalance in the number of chromosomes (aneuploidy) and an enhanced rate of loss of heterozygosity. Most cancer cells have abnormal chromosomal content characterized by changes in the chromosomal structure and number.²⁹⁻³¹ Therefore, CIN plays a key role in tumorigenesis. In this study, we determined the LOH at seven chromosomal regions as evidence for CIN linked to common tumor suppressor genes including APC, DCC, p53, 10p and BRCA1. The frequencies of LOH positive chromosomes at these loci showed no significant difference between the low grade adenomas (category 3), high grade adenomas (category 4.1) and the intramucosal carcinomas with adenomas (category 4.4). When the extent of chromosomal loss of category 3 samples was compared with category 4 samples, according to the genetic classification previously reported, there was no significant difference. A recent study showed that the DNA ploidy pattern, of category 3 GEN, was diploid by static cytofluorometry.³² This finding suggests that large-scale changes in the chromosomal number and structure do not occur in category 3 GEN (low grade adenoma), and they are not likely to progress to gastric adenocarcinomas even though they have LOH positive chromosomal regions linked to common tumor suppressor genes. However, the sample included only 17 patients, and these findings must be confirmed in a larger study to determine whether revised Vienna category 3 GEN evolve to malignancies.

In approximately 95% of colon cancers, the early and predominant genetic event is an APC mutation and CIN is predominant.³³ Loss of APC function results in microtubule plus-end attachment defects during mitosis and consequent chromosome misalignment and CIN.^{34,35} Therefore, the APC gene plays a pivotal role in the colon cancer model of carcinogenesis. Studies have attempted to characterize the mutation of APC in gastric cancer. An inverse relationship between an APC mutation in gastric adenoma and the development of adenocarcinoma has been previously reported; a somatic mutation of the APC gene was found to have a limited role in gastric carcinogenesis.³⁶ However, in the development of well differentiated adenocarcinomas and signet ring cell carcinomas, inactivation of APC played a central role, and mutation of the APC gene occurred during the early stages of gastric carcinogenesis, similar to colon cancer.³⁷⁻³⁹ It is not clear whether the APC gene plays a role in the pathogenesis of adenocarcinomas arising from pre-existing adenomas or dysplasia. Therefore, the goal of this study was to determine the role of the APC gene in gastric carcinogenesis. Our results showed that the frequency of LOH for the APC gene was 45.0% in category 3 and 31.4% in

category 4. In category 4.1 and category 4.4, the frequencies of LOH for the *APC* gene were 36.7% and 23.8%, respectively. LOH for the *APC* gene might have a limited role in malignant transformation. The sequential process of colon cancer model has to date only partially explained gastric carcinogenesis.

Microsatellites are repeated sequences of DNA and the length of these microsatellites is highly variable from person to person.⁴⁰ These repeated sequences are normal and the most common microsatellite in the humans is a dinucleotide repeat of cytosine-adenine, which occurs tens of thousands of times across the genome. In cells with mutations in DNA repair genes, however, some of these sequences accumulate errors and become longer or shorter. The appearance of abnormally long or short microsatellites is referred to as microsatellite instability.⁴¹⁻⁴⁴ MSI results in a high rate of point mutation, and has been found to play a role in tumor progression rather than in the initiation phase.^{42,45} MSI is only rarely found in adenomas and there is a significant increase of MSI in benign adenoma with areas of malignancy and in colon cancer.⁴⁶ Previous study on MSI of gastric cancer has been performed in advanced stage, and MSI is reported in 5% to 46% of gastric cancer.^{15,41} On the other hand, MSI is a rare event in intestinal metaplasia in patients who do not have gastric cancer, and sequential accumulation of MSI in the histological progression from intestinal metaplasia to cancer.^{47,48} However, it is unclear whether MSI occurs in dysplastic stage of gastric epithelium. We demonstrated that the frequency of MSI positive was 25% in category 3. The frequency of MSI positive in category 4 is higher than category 3. Considering that gastric malignant lesions have the progressive increase in MSI frequency, our results suggest the detection of MSI is a surveillant marker of risk of gastric cancer development. Moreover in category 3, the frequency of MSI positive is 25%, similar to that in the previous literature for malignant transformation.^{20,21} However, the determination of MSI state is relatively labor-intensive and a simpler test is required for its translation in clinical setting. Recently it is reported that *H. pylori* impairs DNA repair mechanisms, rendering gastric epithelial cells vulnerable to the accumulation of genetic instability and thus contributing to gastric carcinogenesis.^{49,50} In this study, *H. pylori* infection is more frequent in MSI positive category 4 samples than category 3. If *H. pylori* induces genetic instability in early stage of gastric carcinogenesis, further study to determine whether *H. pylori* eradication is beneficial at the dysplastic stage of gastric epithelium, would be planned.

The molecular mechanisms of genomic instability are not independent. With obtained results, we classified the

cancers into four subtypes; MSI (-)/LOH (-), MSI (+)/LOH (-), MSI (-)/LOH (+), and MSI (+)/LOH (+). The MSI (+)/LOH (-) of category 4 GEN were found in 15.7%, whereas MSI (-)/LOH (+) were 54.9%. MSI and LOH events coincided in 27.5% in the present study. In gastric carcinogenesis, it shows overlap between these two mechanisms and it is different from colon carcinogenesis.

Identification of a gastric adenoma that has the potential to advance to cancer is important. In general, the risk of malignant transformation of an adenoma developing depends on its size, histological type and the presence of atrophy.⁵¹⁻⁵³ The tumor size is considered an important prognostic marker, but several studies have not provided evidence to support this. We evaluated revised Vienna category 3 GEN and divided them into two groups according to their size. One group less than 15 mm in diameter and the other more than 16 mm had no significant differences in the frequencies of LOH (+) or MSI (+). Further confirmation of these events in futures studies is needed.

In conclusion, LOH for the *APC* gene might have a limited role in the gastric carcinogenesis. *H. pylori* infection is associated with genetic instability of premalignant lesion, and MSI occurs in early stage of gastric carcinogenesis. MSI pathway may play an early and important role in the gastric carcinogenesis, and the evaluation of MSI state is important to estimate the risk of malignant transformation.

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