p53 Mutations and Microsatellite Instabilities in the Subtype of Intestinal Metaplasia of the Stomach

To investigate the potential implication of the subtype of intestinal metaplasia in the progression to the gastric carcinoma, we analyzed the mutations of the *p53* gene and microsatellite instability (MSI) both in the complete type (type I) and in the sulphomucin-secreting incomplete type (type III) intestinal metaplasia located adjacent to the gastric carcinoma. *p53* mutations were observed in 13.3% of type I, in 6.6% of type III intestinal metaplasia, and in 40% of gastric carcinoma. The difference between *p53* mutations observed in type I and type III intestinal metaplasia was not statistically significant. No identical mutation of the *p53* gene was found in the intestinal metaplasia and carcinoma specimens from the patients. There was no case of intestinal metaplasia showing MSI. In gastric carcinomas, MSI was observed in six cases (40%). The cases harboring *BAT-26* instability did not have the mutation of the *p53* gene. These data suggest that intestinal metaplasia adjacent to gastric carcinoma, irrespective of its subtype, do not have the genetic alterations as showing in their carcinoma tissues.

Key Words : *Intestines; Metaplasia; Subtype, Genes p53; Mutation; Microsatellite Repeats*

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INTRODUCTION

Gastric carcinoma is the most common malignant cancer in the Korean population (1) and one of the most frequent cancers in the world (2). Cancer is caused by the accumulation of genetic alterations such as the activation of oncogenes and inactivation of tumor suppressor genes (3). Gastric adenocarcinoma, especially of the intestinal type, is believed to arise via a multistage process that includes chronic gastritis, gastric atrophy, intestinal metaplasia, and finally dysplasia (4).

Intestinal metaplasia is classified into complete (I) and incomplete (II & III) types depending on the secreted mucin and mucosal characteristics, and these subtypes may exist concomitantly in a patient (5, 6). Intestinal metaplasia has long been considered to play an important role in the development of gastric carcinoma. Type III incomplete intestinal metaplasia was found to be more common in the mucosa of gastric carcinoma than in the mucosa of chronic gastritis and greater number of genetic mutations developed in incomplete metaplasia (6-9). The rate of evolution into gastric carcinoma was 4.58 times higher in type III intestinal metaplasia than in type I intestinal metaplasia (10). These findings suggest that incomplete intestinal metaplasia carry a higher risk for development of gastric carcinoma than complete intestinal metaplasia. However, several studies did not show the relation between type III intestinal metaplasia and gastric carcinoma

(11, 12). Therefore, further studies are necessary to determine the role of the subtypes of intestinal metaplasia as a marker for gastric carcinoma.

The mutation of the *p53* gene is one of the most representative genetic abnormalities in gastric carcinoma. *p53* mutation could also be observed in intestinal metaplasia with 2.5-50% incidence reported (13-15). The mutation was more commonly observed in incomplete intestinal metaplasia than in complete intestinal metaplasia.

Microsatellite instability (MSI) is a mutator phenotype that occurs through a loss of the mismatch repair system in cells, and contribute to the generation of cancer by inducing mutations of oncogenes and tumor suppressor genes (16, 17). In gastric carcinoma, MSI is noted in the early stages of cancer development and is shown in 13-44% of cases (18-20). The frequency of MSI in intestinal metaplasia was variable, ranging from 0% to 48% (18, 21-24).

However, these reports made their comparison on different specimens from different patients. There have been few reports that studied these genetic abnormalities according to the subtypes of intestinal metaplasia in a single patient.

The present study was aimed to investigate the potential implication of the subtype of intestinal metaplasia in the relationship between intestinal metaplasia and gastric carcinoma by comparing the mutations of the *p53* gene and MSI in the complete type (type I) intestinal metaplasia and those in the

sulphomucin-secreting incomplete type (type III) intestinal metaplasia located adjacent to the gastric carcinoma.

MATERIALS AND METHODS

Tissue specimens

The study subjects were 15 patients with intestinal type gastric carcinoma operated at St. Mary's Hospital of Catholic University of Korea. The tissue specimens of both complete (type I) and incomplete (type III) intestinal metaplasia were acquired from resected normal tissues adjacent to the gastric carcinoma. The neutral mucin and acidic sialomucin in intestinal metaplasia was confirmed by alcian blue (pH 2.5)/ peri-odic acid Schiff stain and the sulphomucin was confirmed by high iron diamine/alcian blue (pH 2.5) stain. Type I intestinal metaplasia was determined by the presence of goblet cells that secrete acidic sialomucin (stained blue) between nonsecreting absorptive cells as well as the presence of brush border and in some cases, Paneth cells at the crypt base. Type III intestinal metaplasia was determined by presence of abundant immature columnar cells and also secretion of sulphomucin (stained brown) by cells (5). The type III intestinal metaplasia, although it was scarse in non-cancerous stomach, was detected frequently in the mucosa adjacent to gastric carcinoma. We selected the specimens contained much of type III intestinal metaplasia enough to extract DNA. The formalin-fixed, paraffin-embedded specimens of type I, type III intestinal metaplasia, and carcinoma were sectioned twice
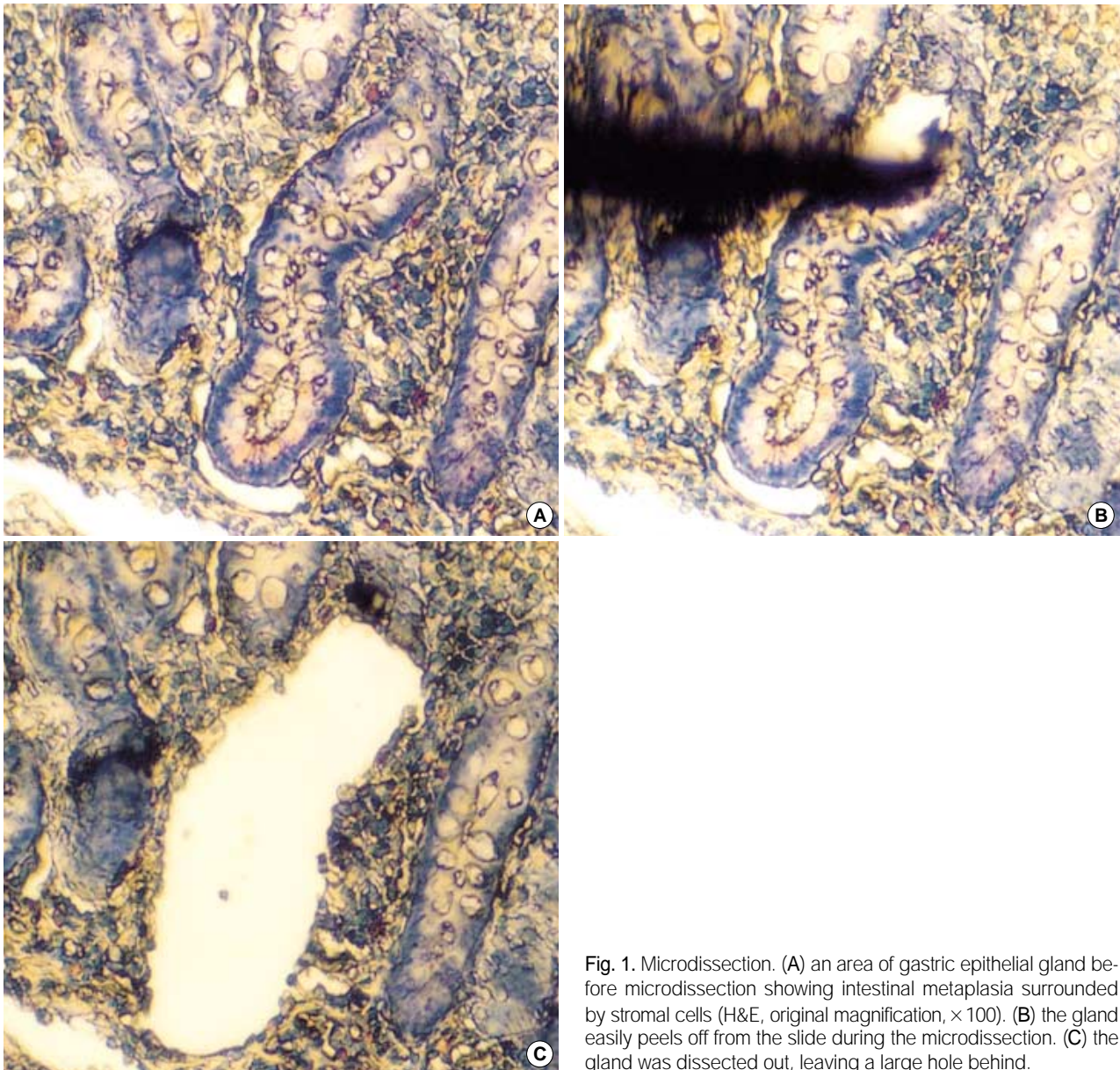
Fig. 1. Microdissection. (A) an area of gastric epithelial gland before microdissection showing intestinal metaplasia surrounded by stromal cells (H&E, original magnification, ×100). (B) the gland easily peels off from the slide during the microdissection. (C) the gland was dissected out, leaving a large hole behind.

with 7-μm thickness for DNA extraction. The lymph node was used as negative control.

DNA extraction

After deparaffinization, the tissue specimens were H&E stained and placed in glycerol buffer [2% glycerol in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA)] for 2 min. After wiping the back of the slide, the specimens were observed under a light microscope with 40-100-fold magnification. Tissues were extracted with 31-gauge (Becton Dickinson, Franklin Lake, NJ, U.S.A.) needles (Fig. 1), and were placed in a 30-μL lytic solution (0.5% Tween 20, 1 mM EDTA, pH 8.0, 50 mM Tris-HCl pH 8.5). The process was repeated until 1,500-2,000 cells were collected. After proteinase K (2 mg/mL of lytic solution; Promega, WI, U.S.A.) was added, the mixture was incubated for 48 hr at 37°C.

Sequencing analyses of the *p53* gene

Exon 5 to 8 of the *p53* gene were amplified by the polymerase chain reaction (PCR). The PCR premix included 2.5 μL of the template DNA, 5 μL of 5 pmol/μL primer (25), 3 μL of 1.25 mM MgCl$_2$, 5 μL of 1.25 mM dNTP, 0.5 μL of 5 units/μL of *Taq* DNA polymerase (TaKaRa Biomedicals, Shiga, Japan), 5 μL of 10×buffer, and distilled water to a final volume of 50 μL. PCR reaction was performed immediately using the MJR thermal cycler (MJ Research Inc., Watertown, MA, U.S.A.); 1 cycle at 99°C for 5 min for denaturation of the template DNA and 32 cycles at 94°C for 50 sec, 62°C for exons 5, 7, 8 and 66°C for exon 6 for 50 sec, and 72°C for 1 min. The amplified DNA were confirmed by electrophoresis on 2% agarose gel (Sigma Chemical Co., St. Louis, MO, U.S.A.) (Fig. 2).

The DNA sequencing was performed on both DNA strands using the enzymatic dideoxy chain termination sequencing chemistry with the four different nucleotides labeled with different fluorescent dyes (BigDye terminator cycle sequencing kit; Perkin Elmer, U.S.A.). Automated gel reader (Auto DNA Sequenser 377XL, Applied Biosystem, U.S.A.) using argon laser beam read the sequence trace. The sequence traces were transferred to a computer file where they can be manually edited and further analyzed with dedicated software. The terminator premix included the thermally stable enzyme,
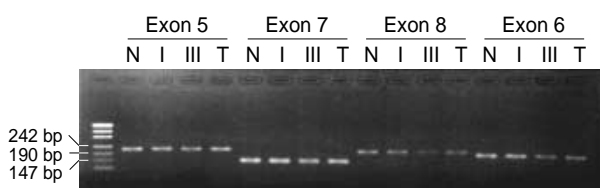
Ampli *Taq* DNA polymerase FS, dITP, 8 μL of BigDye premix, 300 ng of template DNA, 5 pmoles of primer, and distilled water to a final volume of 20 μL. It was mixed well by vortexing. PCR reaction was performed immediately using the Perkin Elmer GenAmp 9700 thermal cycler (U.S.A.); 1 cycle at 95°C for 1 min for denaturation of the template DNA and 30 cycles at 96°C for 15 sec, 55°C for 15 sec, and 60°C for 4 min. After PCR amplification, excess BigDye terminators were removed from the sequencing reaction by using spin columns or ethanol washing. The purified samples were placed onto a sequencing gel on one lane.

Analyses of microsatellite instability

Mononucleotide markers *BAT-25* and *BAT-26*, and dinucleotide markers *D2S123*, *D5S346*, *D13S170*, *D17S250*, and *TP53* were used in the analyses of MSI (26-30). PCR premix included 1 μL of the template DNA, 0.4 μM of primer, 125 μM of dNTP, 1.5 mM of MgCl$_2$, 0.4 unit of *Taq* DNA polymerase, 0.5 mCi of [^{32}P]dCTP (Amersham, Buckinghamshire, United Kingdom) and 1 μL of 10×buffer mixed to a total reaction volume of 10 μL. The mixture was amplified using the MJR thermal cycler (MJ Research Inc.); 1 cycle at 95°C for 5 min for denaturation of the template DNA, 35 cycles at 95°C for 1 min, annealing at defined temperatures on the references for 1 min, and 72°C for 1 min, and 1 cycle at 75°C for 5 min. Two microliters of the PCR product was mixed with 10 μL of loading dye (95% formamide, 20 mM EDTA, 0.05% xylene cyanol FF, and 0.05% bromophenol blue) and denatured at 95°C for 5 min. The sample was loaded in a denaturing gel (8.3 M urea and 8% acrylamide) and electrophoresed for 3 hr. After electrophoresis, the gel was fixed and dried onto 3 mm Whatmann paper and DNA abnormality was analyzed through autoradiography using Kodak-OMAT film (Eastman Kodak, Rochester, NY). DNA bands that were different in size from those obtained from normal lymph node were considered to harbor MSI. *BAT-26* instability was regarded as the representative marker of high frequency MSI.

RESULTS

Mutation of the *p53* gene

Of the 15 cases, mutation in the *p53* gene was found in 2 cases of type I intestinal metaplasia (13.3%), one case of type III intestinal metaplasia (6.6%), and in 6 cases of gastric carcinoma (40%). There was no identical mutation between intestinal metaplasia and carcinoma. All the detected mutation of the *p53* gene was missense mutation in which an amino acid is replaced by another amino acid. Case No. 11 had a silent mutation in which the altered codon coded for the same protein (CTG→TTG, Leucine). The case No. 5 had a muta-

	Exon 5	Exon 7	Exon 8	Exon 6
	N I III T	N I III T	N I III T	N I III T

242 bp
190 bp
147 bp

Fig. 2. Agarose gel electrophoresis of amplified products of the *p53* gene, exon 5 to 8, in the DNA from normal (N), type I (I) and III (III) intestinal metaplasia, and gastric carcinoma (T).

tion in the splice junction between intron 7 and exon 8 (AG →AT) in the type III intestinal metaplasia. Of the nine *p53* gene mutations, four were observed in exon 5, two in exon 6, two in exon 7, and one in exon 8 (Table 1 and Fig. 3).

Microsatellite instability

In the intestinal metaplasia, no case showed microsatellite instability. Microsatellite instabilities were observed in 6 cases

(40%) of gastric carcinoma. Among these, case No. 5, 9, and 10 showed high frequency MSI, including *BAT-26* instability. In case No. 1 and 7, the instability was detected in single dinucleotide marker as in *D17S250* and *D13S170*, respectively. Case No. 4 showed instabilities in two dinucleotide markers, *D17S250* and *TP53*. Loss of heterozygosity was observed in 4 cases of gastric carcinoma (26%) (Table 2 and Fig. 4).

Table 1. *p53* mutations in the intestinal metaplasia type I and III, and gastric carcinoma

Case No.	IM type I	IM type III	Carcinoma
1	-	-	-
2	-	-	E8, N[†]263*D[†] (AAT→GAT)
3	E6, L194F (CTT→TTT)	-	-
4	-	-	E7, G245D (GGC→GAC)
5	-	In7/E8 (AG→AT) : splicing mutation	-
6	-	-	E6, H193R (CAT→CGT)
7	-	-	-
8	-	-	-
9	-	-	-
10	-	-	-
11	E5, L145L (CTG→TTG) : silent mutation	-	-
12	-	-	E5, Q136E (CAA→GAA)
13	-	-	-
14	-	-	E5, R175H (CGC→CAC)
15	-	-	E5, C176F (TGC→TTC)

IM, intestinal metaplasia; E, exon; In, intron.
*, codon number. [†], amino acid codes (C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; L, leucine; N, asparagine; Q, glutamine; R, arginine; V, valine).
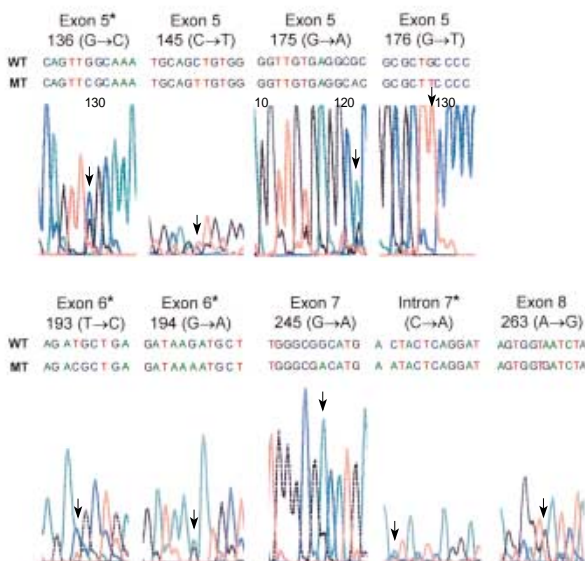
Table 2. Microsatellite instabilities and loss of heterozygosity in the intestinal metaplasia and gastric carcinoma

Case No.	IM type I	IM type III	Carcinoma
1	-	-	MSI-L (D17S250)
2	-	-	-
3	-	-	-
4	-	-	MSI-L (D17S250, TP53)
5	-	-	MSI-H (BAT-25, BAT-26, D13S170, D17S250, TP53)
6	-	-	-
7	-	-	MSI-L (D13S170) LOH (TP53)
8	-	-	-
9	-	-	MSI-H (BAT-25, BAT-26, TP53)
10	-	-	MSI-H (BAT-25, BAT-26, D5S346, D13S170, TP53)
11	-	-	-
12	-	-	-
13	-	-	LOH (TP53)
14	-	-	LOH (D13S170)
15	-	-	LOH (D13S170, D17S250, TP53)

IM, intestinal metaplasia; LOH, loss of heterozygosity; MSI-L, low frequency microsatellite instability; MSI-H, high frequency microsatellite instability.
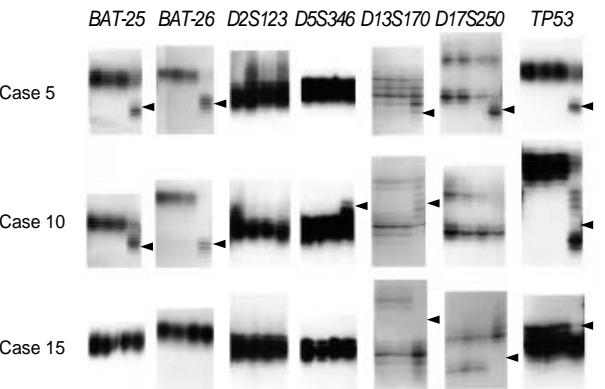

Fig. 3. Sequencing histograms of *p53* mutation. Arrows, site of mutation; WT, wild type sequence; MT, mutant sequence; *, reverse-strand histogram.

Fig. 4. Microsatellite instability analysis. For each of the 7 mono- and dinucleotide markers, autoradiograms of microsatellite instability and loss of heterozygosity for three selected cases are shown. Each autoradiogram has four lanes indicating normal, type I and type III intestinal metaplasia, and carcinoma, respectively. Case No. 5 exhibits microsatellite instability in 5 loci (*BAT-25, BAT-26, D13S170, D17S250,* and *TP53*), case No. 10 reveals microsatellite instability in 5 loci (*BAT-25, BAT-26, D5S346, D13S170,* and *TP53*), and case No. 15 shows loss of heterozygosity in three loci (*D13S170, D17S250,* and *TP53*).

Table 3. The relation between microsatellite instabilities and *p53* mutations in gastric carcinoma

Case No.	*p53* mutation	Microsatellite instability
1	-	MSI-L
2	+	-
3	-	-
4	+	MSI-L
5	-	MSI-H
6	+	-
7	-	MSI-L
8	-	-
9	-	MSI-H
10	-	MSI-H
11	-	-
12	+	-
13	-	-
14	+	-
15	+	-

MSI-L, low frequency microsatellite instability; MSI-H, high frequency microsatellite instability.

The relationship between microsatellite instabilities and *p53* mutations

The case harboring MSI tended not to have mutation of the *p53* gene. Of the total six cases with MSI, only one case (case No. 4) with low frequency MSI had a mutation of the *p53* gene in the gastric carcinoma. Cases with the *BAT-26* instability had no concomitant mutation of the *p53* gene (Table 3).

DISCUSSION

Mutation of the *p53* gene and microsatellite instability have been previously studied in subtypes of intestinal metaplasia by immunohistochemical and molecular genetic techniques, and much of genetic abnormalities were observed in the incomplete type intestinal metaplasia (9, 13-15). However, these reports compared the intestinal metaplasia from different specimens of different patients. In this study, DNA of type I and type III intestinal metaplasia was extracted from the same patient, and *p53* mutation and MSI were analyzed. The results revealed no genetic differences between type I and type III intestinal metaplasia adjacent to their carcinoma tissues.

The incidence of *p53* mutation in intestinal metaplasia was reported as 2.5-50% (13-15). Shiao et al. reported that *p53* mutations occurred in four out of eight (50%) intestinal metaplasia by the polymerase chain reaction-single stranded conformational polymorphism (PCR-SSCP) and nucleotide sequence analysis (13). Gomyo et al. reported two cases with *p53* mutations from 21 cases with intestinal metaplasia (9.5%), and they were all incomplete type intestinal metaplasia (14). Ochiai et al. have demonstrated that 2.5% of positive immunohistochemical stain for p53 was incomplete type

intestinal metaplasia (15).

Cancers often harbor the same genetic abnormalities with the premalignant lesions, which provide evidence of monoclonal expansion of mutated cells. This finding can be observed in various cancers, such as actinic keratosis and skin melanoma as well as dysplasia and gastric carcinoma (13, 31). In limited studies on intestinal metaplasia, the same *p53* mutation was found both in intestinal metaplasia and gastric carcinoma. However, this was not always the case. In some cases, *p53* mutation in intestinal metaplasia was different from that in carcinoma. In this study, there was no identical mutation of the *p53* gene between intestinal metaplasia and carcinoma. Shiao et al. showed that out of 4 cases of intestinal metaplasia with *p53* mutation, one case did not have the same mutation in the gastric carcinoma (13). Gomyo et al. reported that one of two cases did not have same mutation of the *p53* gene in intestinal metaplasia and in carcinoma (15). As suggested by Shiao et al., these findings can be explained by genetic alteration of a small population of cells due to instability of the *p53* gene, which may not be detected by DNA sequencing (13). The other possibility may be that not all the *p53* mutations exhibited on intestinal metaplasia portend a progression to carcinoma. Some cases of intestinal metaplasia may be fatal before they proceed to cancer, or may be at an ongoing step for development of carcinoma.

In this study, MSI was observed in 40% (6/15) of cases with gastric carcinoma, and these result was comparable to those reported in literature (18-20). However, intestinal metaplasia showed no MSI. In the literature, MSI in the intestinal metaplasia has been frequently observed, ranging from 27% to 48% (18, 21-23). MSI reported in intestinal metaplasia was present at a few dinucleotide microsatellite markers. There was no *BAT-26* instability, which was known as the marker of mismatch repair defects, in intestinal metaplasia. MSI at a few dinucleotide microsatellite markers may not necessarily be the result of defective mismatch repair, if it is not associated with *BAT-26* instability (24). In a recent study using *BAT-25*, *BAT-26*, *D2S123*, *D5S346* and *D17S250*, there was no instability of these markers in intestinal metaplasia adjacent to gastric carcinoma (24). This study was similar with our study in the tissue specimens used, MSI markers, and the results. The discrepancies in MSI frequencies in the reports may result from differences in sample selection, data interpretation and/or microsatellite markers used.

In this study, among the six cases of gastric carcinoma with MSI, only one case had an accompanying mutation of the *p53* gene. Cases having *BAT-26* instability, which is regarded as a representative marker for MSI, had no concomitant mutation of the *p53* gene. This finding is in line with those from other studies. MSI often accompanies other genetic abnormalities, mainly of the *β-catenin* and *transforming growth factor β receptor type II* genes. On the other hand, mutations of the *adenomatous polyposis coli (APC)* gene and the *p53* genes appeared to be rarely accompanied by MSI (32-35). A recent study

showed that there was no correlation between MSI and mutation of the *p53* gene, suggesting that *p53* mutation might not be generated by MSI (35).

The present study suggests that intestinal metaplasia adjacent to gastric carcinoma, irrespective of its subtype, do not have the genetic alterations as showing in their carcinoma tissues. Further studies may be needed with the other genetic markers, and with the larger amount of samples for the purpose of discrimination of potential role of intestinal metaplasia as a precancerous lesion.

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