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High Frequency of Microsatellite Instability in Intestinal-type Gastric Cancer in Korean Patients

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Background : Although there have been some reports on microsatellite alterations in gastric cancer, findings are inconsistent regarding the associations between histological classification and microsatellite instability (MSI). In the present study, we attempted to determine whether Lauren's histological subtypes are related with MSI status.

Methods : Paraffin-embedded tissue samples from 14 diffuse-type and 14 intestinal-type gastric adenocarcinomas were matched up according to patient gender and age. Mononucleotide markers (*BAT25* and *BAT26*) and dinucleotide markers (*D2S123*, *D5S346*, and *D17S250*) were used for MSI analyses. Microsatellite genotypes were categorized in terms of high MSI incidence (MSI-H, >30% positive marker) or low MSI incidence (MSI-L, <30% positive marker). Losses of *hMLH1* and *hMSH2* protein expression were immunohistochemically studied.

Results : MSI-H was observed in 11 cases (78%) of the 14 intestinal-type cases as compared to 3 (21%) of the 14 diffuse-type cases (p=0.007). In MSI-H tumors, 10 cases (71%) showed losses of *hMLH1* protein expression, while 2 cases (14%) in MSI-L tumors showed losses of *hMLH1* protein expression (p=0.006).

Conclusion : MSI-H tumors are more frequently found in intestinal-type gastric cancer, which suggests the possibility that there are different pathogenic pathways in gastric carcinogenesis according to histologic type.

Key Words : Gastric cancer, Histology, Microsatellite instability

INTRODUCTION

Gastric adenocarcinoma is one of the most frequently observed malignant tumors in the world, contributing significantly to cancer mortality, especially on the Asian continent[1]. Recently, it became clear that gastric carcinogenesis is a multifactorial, multi-step process requiring sequential alterations in genes which codify for tumor suppressors, proto-oncogenes, gate-keeper genes, enzymes, growth factors, and membrane or nuclear receptors (the multi-hit hypothesis)[2]. Among these, mutation carriers of DNA mismatch repair genes exhibit a characteristic phenotype termed microsatellite instability (MSI), which is characterized by an accelerated accumulation of single nucleotide mutations and of alterations in the lengths of simple repetitive microsatellite sequences found throughout the genome[3-7]. In the past, MSI was considered to be restricted to the field of colorectal cancer[3, 4, 8]. However, it has recently been reported that sporadic cancers, including gastric cancer, are also related with MSI[5-8]. In Korea, several studies have suggested that susceptibility to gastric cancer is caused by mutations in one of the genes in the DNA mismatch repair system[9-17].

Gastric adenocarcinoma is classified as intestinal- or diffuse-type according to histologic characteristics[18]. Intestinal-type adenocarcinomas are usually located in the distal stomach and

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Table 1. Primer sequences of the microsatellite instability (MSI) markers

Marker	Size (bp)	Dye	Primer Sequences
BAT25	90	Dye A[1]	5'-TCG CCT CCA AGA ATG TAA GT-3'
			5'-TCT GCA TTT TAA CTA TGG CTC-3'
BAT26	80-100	Dye B[2]	5'-TGA CTA CTT TTG ACT TCA GCC-3'
			5'-AAC CAT TCA ACA TTT TTA ACC-3'
D2S123	197-227	Dye A[1]	5'-AAA CAG GAT GCC TGC CTT TA-3'
			5'-GGA CTT TCC ACC TAT GGG AC-3'
D5S346	96-122	Dye C[3]	5'-ACT CAC TCT AGT GAT AAA TCG GG-3'
			5'-AGC AGA TAA GAC AGT ATT ACT AGT T-3'
D17S250	150-169	Dye A[1]	5'-GGA AGA ATC AAA TAG ACA AT-3'
			5'-GCT GGC CAT ATA TAT ATT TAA ACC-3'

[1]Dye A, 6-carboxyfluorescein; [2]Dye B, 6-carboxy-4, 7, 2′, 4′, 5′, 7′-hexachlorofluorescein; [3]Dye C, 2, 7′, 8′-benzo-5′-fluoro-2′, 4, 7-trichloro-5-carboxyfluorescein

possess a characteristic glandular structure that is believed to arise from the intestinal metaplastic epithelium[18]. In contrast, diffuse-type adenocarcinomas especially invade the cardia and have poor glandular formations that are believed to arise from gastric mucous cells[18]. These two different types are considered to develop through different molecular pathways[19, 20], raising the possibility that they may have different genetic background characteristics, such as MSI rates.

Although previous studies have reported variable MSI rates in the two major types of histological gastric cancer, findings have been inconsistent regarding the association of MSI with these two pathological features. Most reports have suggested that MSI is more frequently observed in the intestinal type[10, 12, 17, 21-25] but, some have also reported the opposite finding[8, 26, 27]. These different results regarding MSI and intestinal/diffuse histotype may reflect ethnic, racial, or geographic differences, in addition to discrepancies due to different definitions of MSI cancer. Moreover, due to the lack of well-defined criteria for microsatellite analysis, different numbers or types of markers were used in each study. Recently, it has been reported that MSI was more frequently seen in gastric cancer from Korea[16]. [MAR1]Therefore, we investigated MSI in gastric cancer in Korea according to Lauren's classification.

MATERIALS AND METHODS

Patients

A total of 28 gastric adenocarcinoma patients were retrieved among the gastric cancer patients who underwent gastrectomy in 1996 at Samsung Medical Center, Seoul, Korea. Fourteen cases of cardiac adenocarcinoma were selected first, and another 14 cases of antral adenocarcinoma were selected, matching for age and gender. None of the patients had any previous history of malignancy. The postoperative stages varied from stage IB to stage IV.

DNA preparation

Serial gastric sections from cancer tissue and adjacent noncancerous tissue were obtained at 5 um thickness and stained with hematoxylin and eosin. Only tissues containing more than 80% of cancer tissue were deemed acceptable for microsatellite analysis. Genomic DNA from tumors and from corresponding normal tissue were obtained from paraffin blocks by microdissection. Deparaffinization was done by xylene for 20 minutes and 40 minutes at room temperature. Rehydration was performed by washing in 100%, 95%, 80%, and 70% ethanol for 10 minutes separately, at room temperature. Tumor tissue was separated from normal tissue with a needle, and was then inserted into an Eppendorf tube. DNA was extracted from the microdissected tissues using 200 uL of proteinase K solution (190 uL of protein kinase digestion buffer with 10 uL of 10 mg/mL proteinase K) in lysis buffer containing 0.5% Tween-20, 1M Tris (pH 8.5), and 500 mM EDTA (pH 8.0). Tissues were incubated overnight in lysis buffer solution at 55℃. After the overnight incubation, centrifugation was performed at 14,000 rpm for 15 minutes at 4℃.

Analysis of MSI

MSI was analyzed by PCR amplification with fluorescent dye-labeled primers of mononucleotide markers (*BAT25* and *BAT26*) and dinucleotide markers (*D2S123*, *D5S346*, and *D17S250*) specific for the microsatellite loci. Primer sequences of the MSI markers are shown in table 1. As previously described, PCR was performed over 35 cycles of: 1 minute at 94℃, 1 minute at 55℃, and 1 minute at 72℃ for the *BAT25* and *BAT26* primers[16]. For *D2S123*, PCR was performed over 35 cycles of: 30 seconds at 94℃, 1 minute at 54℃, and 1 minute at 72℃. For *D5S346*, PCR was performed over 36 cycles of: 30 seconds at 94℃, 30 seconds at 55℃, and 30

Table 2. Clinicopathological findings in high microsatellite instability (MSI-H) and low microsatellite instability (MSI-L) cases

	MSI-H†	MSI-L‡	*p*-value
Male : Female	8 : 6	10 : 4	NS
Age (mean ± SD)	66.7±13.0	67.9±12.5	NS
Intestinal : Diffuse*	11 : 3	3 : 11	0.007
Antrum : Cardia	9 : 5	5 : 9	NS
Stage IB	1	1	NS
II	7	2	
IIIA	2	2	
IIIB	4	6	
IV	0	3	

*Lauren's classification; † MSI-H, >30% positive microsatellite instability marker; ‡ MSI-L, <30% positive microsatellite instability marker

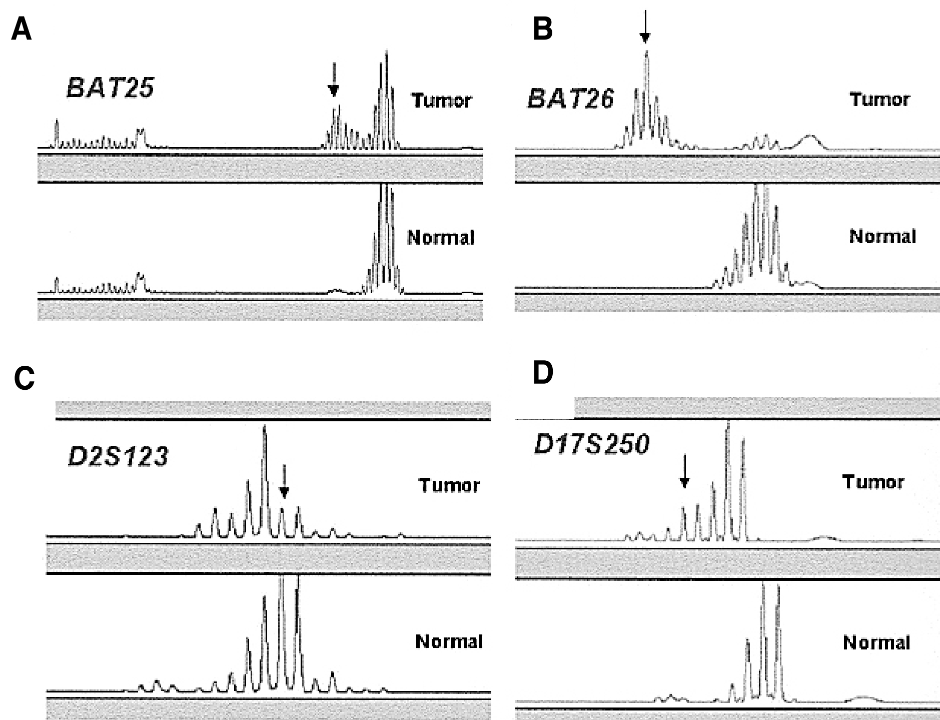

Figure 1. Detection of MSI by analysis of microsatellite markers in tumors and in corresponding normal tissue. Mutant alleles are indicated with arrows in each tumor trace: (A) *BAT25*, (B) *BAT26*, (C) *D2S123*, and (D) *D17S250*.

seconds at 72℃. For *D17S250*, PCR was performed over 38 cycles of: 1 minute at 94℃, 1 minute at 50℃, and 1 minute at 72℃. Fluorescently-labeled PCR products were detected using the ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and Genescan software (Applied Biosystems, Foster City, CA, USA).

Electropherograms were analyzed independently by two different investigators. MSI was defined as a band shift in either of the two alleles or in the appearance of a differently-sized band in the tumor sample (Figure 1). Microsatellite genotypes were categorized as a high incidence of MSI (MSI-H) when instability was detected in more than 30% of markers and as a low incidence of MSI (MSI-L) when instability was detected in less than 30% of markers[28].

Immunohistochemical staining and analysis

Immunohistochemical staining for *hMLH1* and *hMSH2* protein was performed as previously described[29]. Losses of *hMLH1* and *hMSH2* protein expressions were determined by immuno-histochemical staining. Antibody to *hMLH1* (SC-581; Santa Cruz

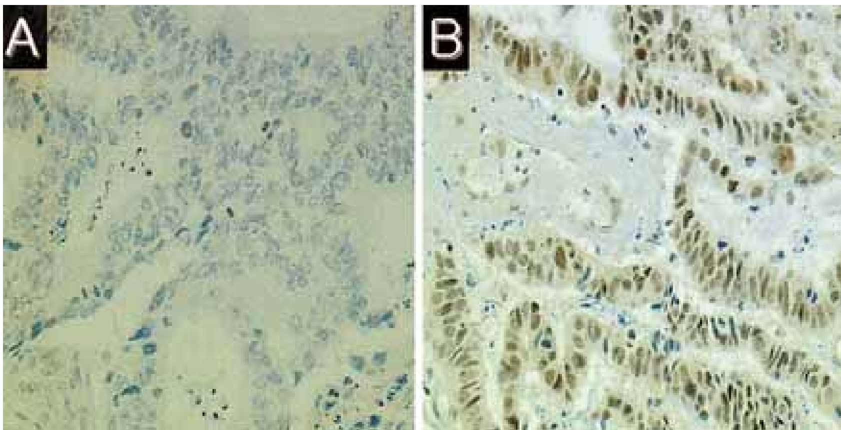

Figure 2. Immunohistochemical analysis of *hMLH1* in gastric cancer mucosa sections. (A) A MSI-H gastric cancer mucosa deficient in *hMLH1* protein (×200). (B) A MSI-L gastric cancer mucosa showing strong *hMLH1* protein expression in the nuclei of cancer cells (×200).

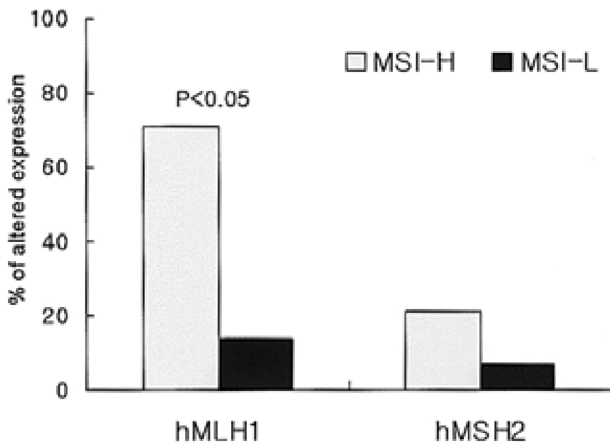

Figure 3. Summary of altered *hMLH1* and *hMSH2* protein expression. A loss of *hMLH1* protein was found to be significantly correlated with MSI status (p=0.006).

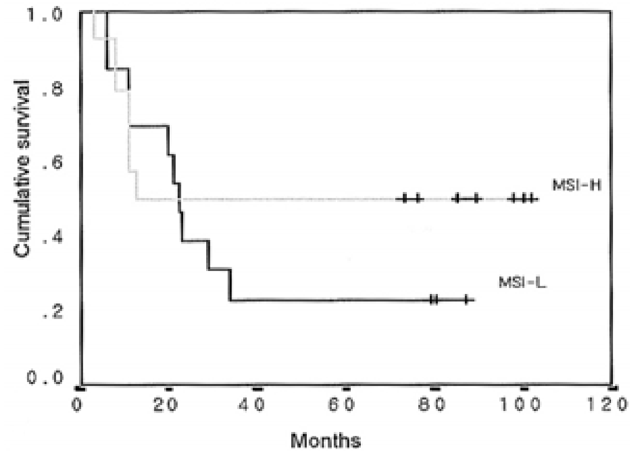

Figure 4. Survival analysis of cause-specific death from gastric cancer. The MSI-H group tended to have better survival than the MSI-L group, but it was not statistically significant (p=0.17).

Biotechnology INC, CA, USA), a rabbit polyclonal antibody, was prepared with a full-length *hMLH1* protein. Another rabbit polyclonal antibody, antibody to *hMSH2* (SC-494; Santa Cruz Biotechnology INC, CA, USA), was generated via a COOH terminal fragment of *hMSH2* protein. As previously described[29], LOVO cells, which express *hMLH1*, were stained simultaneously as a positive control.

Statistics

The possible association between MSI status and histology was assessed using Fisher's exact test. The chi-square test and Fisher's exact test were used for statistical assessment of the association between MSI status and clinicopathological profiles. The Kaplan-Meier method was used to estimate the survival probability as a function of time. The log-rank test (generalized Wilcoxon's test) was performed in order to analyze the differences in patient survival. A p-value less than 0.05 was accepted as statistically significant.

RESULTS

Of the 28 cases, 14 cases (50%) manifested as MSI-H, while the other 14 cases were classified as MSI-L. Among the MSI-L cases, 8 showed no MSI (Table 2). MSI-H was more frequently observed in adenocarcinomas of the intestinal-type (11/14) than in those of the diffuse-type (3/14) (78% and 21%, respectively, p=0.007). There was no statistically significant correlation between MSI status and sex, age, location of the tumor, or tumor stage (Table 2). According to each of the markers, there was no significant difference between the intestinal-type and the diffuse-type in *BAT25* (p=0.42), *BAT26* (p=1.00), *D2S123* (p=0.26), *D5S346* (p=0.21), and *D17S250* (p=0.24).

Immunohistochemical staining results on two mismatch repair proteins revealed strong correlations with MSI status (Figure 2). In MSI-H tumors, 10 cases (71%) showed losses of *hMLH1*

protein expression and 3 cases (21%) showed losses of *hMSH2* protein expression. In MSI-L tumors, 2 cases (14%) showed losses of *hMLH1* protein expression and 1 case (7%) showed a loss of *hMSH2* protein expression (Figure 3). A loss of *hMLH1* protein expression was significantly correlated with MSI status (p=0.006).

After gastrectomy, 8 patients among 14 MSI-H cases and 7 patients among 14 MSI-L cases underwent adjunctive chemotherapy with or without radiotherapy. Follow-up intervals after surgical resection ranged from 73 to 102 months with a median of 87 months in 10 patients who were alive throughout follow-up. Furthermore, follow-up times ranged from 3 to 34 months with a median of 15 months in 18 patients who died of gastric cancer. Using the Kaplan-Meier method, the survival curves of patients at all stages were plotted using MSI status (Figure 4). The MSI-L group exhibited lower survival, whereas the MSI-H group revealed better survival. However, there was no statistically significant difference between the MSI-H group and the MSI-L group in cumulative survival (p=0.17).

DISCUSSION

In the present study, MSI was observed more frequently than in previous reports[10, 12, 17, 21-25], and MSI-H was more frequently observed in the intestinal-type than in the diffuse-type adenocarcinomas. This discrepancy is probably related to the definition and the methods of describing MSI, the patient population that underwent evaluation, and our small sample size. In spite of this unexpected data, our report is supported by previous studies which reported that MSI is more frequently observed in the intestinal-type[10, 12, 17, 21-25]. It is reported that the intestinal-type is prevalent in high-risk populations of gastric adenocarcinoma[19]. Moreover, a previous study concluded that MSI is associated with the intestinal histological type and chromosomal deletion, which in turn lead to an increase in alterations of cancer-related genes[30]. This close relationship between MSI and intestinal-type gastric cancer may also suggest a genetic model common to colon and gastric cancers[17]. Intestinal metaplasia has been considered a precancerous lesion of intestinal-type gastric carcinoma[19], and MSI-associated mutations were detected exclusively in both intestinal-type gastric carcinomas and colon cancers. Thus, these two cancers appear to be closely related to each other histopathologically, as well as genetically[22].

However, these findings are inconsistent in regards to the association between MSI and these two pathological features. Replication error-positive phenotypes were more frequently observed in scirrhous-type gastric cancer (75%) than in other histologic types, which suggests that scirrhous-type gastric cancers may have germline gene mutations in their DNA mismatch repair systems, such as *hMSH2* or *hMLH1*[10]. Moreover, MSI was detected in 57% of the foveolar-type and 8% of intestinal-type[26]. These findings suggest that foveolar-type tumors contain several histopathological problems and are prone to losing their glandular structure and progressing to undifferentiated-type tumors. Thus, they should be regarded as precursors of undifferentiated-type tumors[26]. They concluded that the 'mutator pathway', characterized by MSI, plays an important role in the tumorigenesis of foveolar-type tumors, but not in the complete-type intestinal metastatic phenotype[27]. In addition to discrepancies due to different definitions of MSI cancer, the different results in terms of the MSI and intestinal/diffuse histotype may reflect ethnic, racial, or geographic differences.

The aforementioned study[24] demonstrated that there appear to be three different profiles of carcinogenesis: 1) p53 mutations which accompany the onset of dysplasia and intestinal-type carcinoma; 2) alterations of E-cadherin, both with regards to mutations and abnormal expression; and 3) DNA repair mechanism alterations which condition microsatellite instability seem mutually exclusive with regards to p53 mutations. These alterations are correlated with antrally located intestinal-type carcinoma, with little metastatic tendency and a better prognosis. Some studies have reported that RER-positive cases mostly consisted of intestinal tumors and have been shown to carry relatively good prognoses[21, 31-35]. In the present study, the MSI-H group tended to have better survival and a more favorable prognosis, but this finding was not statistically significant. Although there exists a report which suggests the irrelevance between survival and MSI status[36], our result, like that of previous studies, indicates that MSI seems to improve survival. Unfortunately, we were unable to statistically demonstrate it because the number of patients was too small.

Immunohistochemical staining is a consistent element in the study of MSI. A previous study demonstrated that immunohistochemistry could accurately discriminate between MSI-H and microsatellite stable tumors[37]. Moreover, the majority of germline mutations have been found in key MMR proteins, i.e., *hMLH1* and *hMSH2* proteins[6]. In the present study, immunostaining for the loss of *hMLH1* protein expression revealed a significant correlation between its loss and MSI status (p=0.006), suggesting that promoter hypermethylation of *hMLH1* might be correlated with a loss of *hMLH1* protein expression, which results in MSI, especially in intestinal-type gastric adenocarcinoma.

In summation, MSI was more observed more frequently in adenocarcinomas of the intestinal-type. This suggests that the intestinal- and diffuse-types of gastric adenocarcinoma, by Lauren's classification, are associated with different molecular carcinogenic pathways. Furthermore, our results suggest the

importance of the *hMLH1* promoter in causing MSI-H gastric cancer, and imply that the loss of *hMLH1* protein expression is related with intestinal-type gastric adenocarcinoma. Moreover, our results indicate that *hMLH1* protein expression analysis should be considered for the assessment of MSI-H status.

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