Assessment of the beneficial loci and prognostic implications of microsatellite instability in gastric carcinoma

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Abstract. Microsatellite instability (MSI) is a hyper-mutable phenotype caused by the loss of DNA mismatch repair activity, and plays a crucial role in gastric carcinogenesis. To clarify the role of genetic instability in relation to clinicopathological variables, and to identify predictive MSI markers that facilitate the early detection and improve the classification of gastric carcinomas (GCs), 13 microsatellite (MS) loci, including the National Cancer Institute (NCI) panel of MS markers (*D2S123*, *D5S346*, *D17S250*, *BAT25* and *BAT26*) and 8 dinucleotide repeats (*D3S1260*, *D5S107*, *D5S409*, *D17S261*, *D17S520*, *D17S855*, *D18S34* and *D18S61*) were studied in GC patients. MSI was found in 88.2% (30/34) of GC cases and the number of high-frequency MSI (MSI-H, 23.5%, 8/34), low-frequency MSI (64.7%, 22/34), and stable MSI (11.8%, 4/34), was calculated. Among the MS loci analyzed, *D18S34* and *D17S261* (15/34, 44.1%) exhibited the highest frequency of MSI, followed by *D2S123* (14/34, 41.2%), *D5S107*, *D5S346*, *D5S409* and *D18S61* (12/34, 35.3%) (MSI>35%). MSI-H was particularly prevalent in older patients and was mainly found in the antrum and poorly differentiated tumors. Furthermore, MSI-H was significantly associated with lymph node involvement cases in females. One notable finding in this analysis was that the markers, *D17S250* and *D17S520,* exhibited a significantly higher percentage of MSI in advanced gastric carcinomas than in early gastric carcinomas (P=0.046 and 0.046, respectively), and the *D17S520* and *BAT26* loci represented significant different correlations between the tumor stages (P=0.038 and 0.042, respectively). This study indicates that the novel markers, *D18S34* and *D17S261,* perform more favorably than the NCI panel for the detection of MSI, and the *D17S520* locus presents a potential target for predicting the clinical impact of GC. These novel MS loci may prove to be beneficial and independent tools for the construction of a comprehensive genetic classification for GC cases.

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Introduction

Gastric carcinoma (GC) is responsible for the highest number of cancer-related deaths worldwide (1). Genetic instability in GC represents a key molecular step that occurs early in the carcinogenesis process (2,3). Although numerous studies have been performed to evaluate the genetic events associated with the development and progression of GC (4,5), its molecular mechanism still remains to be understood, and identification of the predictive markers is crucial.

Microsatellite instability (MSI) is a phenomenon caused by a mismatch repair gene deficiency, and plays an important role in the development and progression of tumor cells. The detection of the MSI frequencies or the degrees of the MSI at the specific microsatellite (MS) loci in the tumors might reveal the presence of DNA mismatch repair genes, which may be used as a prognostic indicator of GC (6).

Previous studies (2-5) have revealed that colorectal cancers exhibit distinctive clinicopathological characteristics and prognoses according to the MSI status. However, there is a wide variation in results between MSI status and clinicopathological parameters in GCs due to a lack of standardized and comparable parameters (7). Although the potential of MSI as a molecular prognostic marker in GC has gained prominence over the years, most reports in the literature are prone to arbitrariness, and the potential limitations of the National Cancer Institute (NCI)-recommended panel of MS markers for MSI testing have been recognized (8-11).

In this study, we therefore examined the 13 MS loci, including the NCI panel of MS markers and 8 additional dinucleotide repeats scattered over 7 chromosome arms (2p, 3p, 4q, 5q, 17p, 17q and 18q) to assess the frequency of MSI at various genomic loci according to clinicopathological parameters, and to identify predictive MSI markers that facilitate the early detection or improved classification of GC.

Materials and methods

Patient specimens. The paraffin-embedded tissues from 7 early gastric carcinomas (EGCs) and 27 advanced gastric

Table I. MS loci analyzed in GCs.

Locus symbol	Chromosome	Gene	Sequence (5'→3')
D2S123	2p21-2p16	*hMSH2*	5'-6FAMAAACAGGATGCCTGCCTTTA-3' 5'-GGACTTTCCACCTATGGGAC-3'
D3S1260	3p23-3p21 3p24.2-3p22		5'-AGCTACCAGGGAAGCACTGT-3' 5'-CTATGCAATCACCTGCCATT-3'
D5S107	5q11.2-5q13.3	*APC/MCC*	5'-6FAMGATCCACTTTAACCCAAATAC-3' 5'-GGCATCAACTTGAACAGCAT-3'
D5S346	5q21-5q22	*APC*	5'-ACTCACTCTAGTGATAAATCGGG-3' 5'-AGCAGATAAGACAGTATTACTAGTT-3'
D5S409	5q14-5q15 5pter-5qter		5'-6FAMGGGATGAAGTGTGGATAAAC-3' 5'-TAGGATGGCAGTGCTCTTAG-3'
D17S250	17q11.2-17q12		5'-GGAAGAATCAAATAGACAAT-3' 5'-GCTGGCCATATATATATTTAAACC-3'
D17S261	17p12-17p11.2		5'-CAGGTTCTGTCATAGGACTA-3' 5'-TTCTGGAAACCTACTCCTGA-3'
D17S520	17p12-17p12	*Tp53*	5'-6FAMGTACTGGGCCTCTAAACTCTA-3' 5'-TTAGGAGAAAGTGATACAAGG-3'
D17S855	17q-17q		5'-GGATGGCCTTTTAGAAAGTGG-3' 5'-ACACAGACTTGTCCTACTGCC-3'
D18S34	18q12.2-18q12.3	*DCC*	5'-6FAMCAGAAAATTCTCTCTGGCTA-3' 5'-CTCATGTTCCTGGCAAGAAT-3'
D18S61	18q22.3-18q22.3		5'-ATTTCTAAGAGGACTCCCAAACT-3' 5'-ATATTTTGAAACTCAGGAGCAT-3'
BAT25	4q12-4q12	*c-kit*	5'-TCGCCTCCAAGAATGTAAGT-3' 5'-TCTGGATTTTAACTATGGCTC-3'
BAT26	2p16-2p16	*hMSH2*	5'-TGACTACTTTTGACTTCAGCC-3' 5'-AACCATTCAACATTTTTAACC-3'

carcinomas (AGCs) were obtained from the Chungnam National University Hospital (Taejeon, Republic of Korea). The patients were classified according to the WHO histological typing of gastric tumors and the UICC tumor-node-metastasis (TNM) staging system. All cases were reviewed by pathologists to verify the original histopathological diagnosis, tumor stage, tumor differentiation, and lymph node metastasis.

The neoplastic and adjacent non-neoplastic areas were microdissected individually. The genomic DNA was extracted using a genomic DNA purification kit (Promega, Madison, WI, USA), with some modifications as previously described (12). This study was reviewed and approved by the Institutional Review Board of the Chungnam National University Hospital. Written informed consent was obtained from each patient according to the institutional regulations.

Polymerase chain reaction (PCR). A total of 20 μl of the PCR mixture (AmpliTaq Gold PCR master mix, Applied Biosystems, Foster City, CA, USA) containing 50 ng/μl of the DNA template was prepared for a 30-cycle amplification. The optimal conditions for each primer to react were selected in order to avoid the formation of multiple artifacts as previously reported (13).

The reaction was performed in an automated GeneAmp PCR thermal cycler (Perkin Elmer, Waltham, MA, USA). The thermocycling was programmed as follows: denaturation at 95˚C for 10 min in the early stage and at 96˚C for 10 sec in the late stage followed by annealing at an optimally conditioned temperature for 30 sec, extension at 70˚C for 3 min, and 30 min only in the last cycle.

MSI analysis. Matched tumor and normal tissue samples from 34 GC patients were analyzed using 13 MS loci with an ABI Genetic Analyzer (Applied Biosystems). The reaction mixture for electrophoresis was composed of 0.3 μl of the PCR product, 0.5 μl of the Genescan size standard, and 14.2 μl formamide (Applied Biosystems). The samples were then injected for capillary electrophoresis. The electrophoresis was confirmed with a log file, set using Genescan software (Applied Biosystems) and calculated by the Genotype software. MSI was evident when the tumor DNAs exhibited new bands compared to their normal counterparts. Genomic DNA from K562 cells and nuclease-free water were used as positive and negative controls, respectively.

MSI was classified as an MSI genotype based on the ratio of the number with a positive MSI to the total number of examined MS loci. Cases that were MSI-positive (MSI^+) in ≥40% of the tested markers were designated as high-frequency MSI (MSI-H), and cases that were MSI^+ in <40% of the tested markers were designated as low-frequency MSI

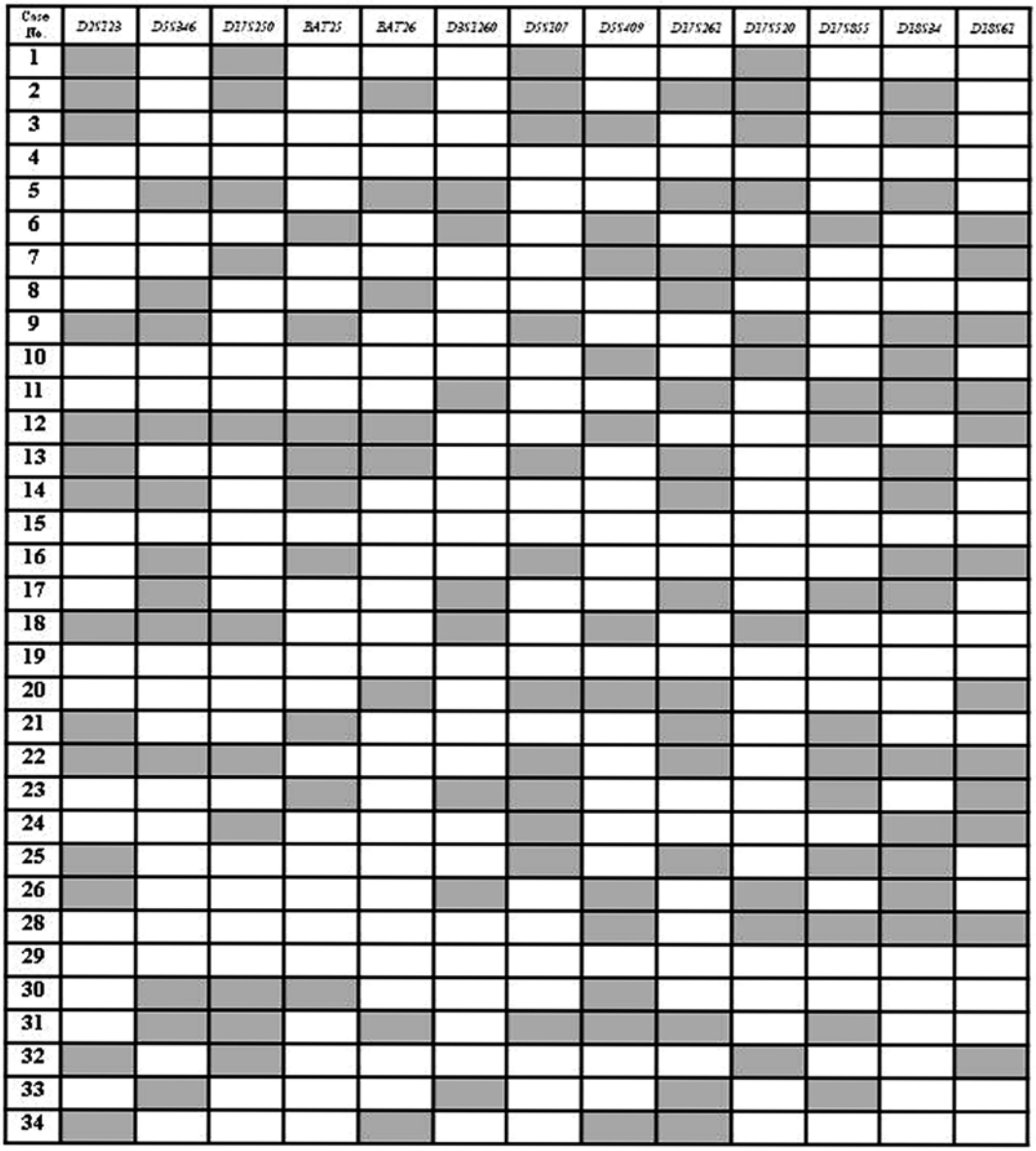

Figure 1. A graphic representation of the MSI frequencies from 13 MS markers. The status of each MS locus is indicated as follows: gray, MSI positive; white, MSI negative.

(MSI-L) (14-16). A non-detectable MSI was assessed as stable MSI (MSS).

Statistical analysis. Associations between clinicopathological features and the degree of MSI were analyzed using either the Chi-square test or the one-way ANOVA test. P-values correspond to two-sided tests. Differences were considered statistically significant if $P<0.05$. Calculations were performed using the 13.0 SPSS software package.

Results

MSI analysis. MSI was assessed by comparison of paired tumor and normal samples using 13 MS loci including the 5 NCI panel of markers (*D2S123*, *D5S346*, *D17S250*, *BAT25* and *BAT26*) and 8 dinucleotide repeats (*D3S1260*, *D5S107*, *D5S409*, *D17S261*, *D17S520*, *D17S855*, *D18S34* and *D18S61*) using an ABI PRISM Genetic Analyzer. The primers for the 13 MS markers are presented in Table I.

The 13 MS markers were successfully amplified in samples from the GC cases. The presence of unequivocal changes when compared to the matching normal samples (shifts in fragments and appearance of new peaks) was classified as MSI. The overall frequency of MSI^+ cases (allelic shifts in at least one marker) was 88.2% (30/34) with a range from 23.5% to 44.1% on each locus. Fig. 1 shows a graphic representation of the MSI frequencies from 13 different MS markers.

Among the NCI panel of MS loci analyzed, *D2S123* (41.2%, 14/34) presented the most frequently altered target of MSI, followed by *D5S346* (35.3%, 12/34), *D17S250* (32.4%, 11/34), *BAT25* (26.5%, 9/34) and *BAT26* (23.5%, 8/34). Among the 8 dinucleotide panels, *D18S34* and *D17S261* exhibited the highest polymorphic rate at 44.1% (15/34), followed by *D5S107*, *D5S409* and *D18S61* (35.3%, 12/34),

Table II. The correlation between MSI status and clinicopathological variables in GCs.

Variable	MSS (n=4)	MSI-L (n=22)	MSI-H (n=8)	Total (%) (n=34)	P-value	P-value (MSI-H vs. MSS/MSI-L)
Age (mean)						
<55	1 (12.5)	5 (62.5)	2 (25.0)	8/34 (23.5)	0.496	0.259
55-64	1 (8.3)	10 (83.4)	1 (8.3)	12/34 (35.3)		
>65	2 (14.3)	7 (50.0)	5 (35.7)	14/34 (41.2)		
Gender (M/F)						
M	3 (13.0)	17 (74.0)	3 (13.0)	23/34 (67.6)	0.113	**0.050**
F	1 (9.0)	5 (45.5)	5 (45.5)	11/34 (32.4)		
Borrmann's type						
EGC	0 (0.0)	7 (100.0)	0 (0.0)	7/34 (20.6)	0.029	0.160
AGC	4 (14.8)	15 (55.6)	8 (29.6)	27/34 (79.4)		
Location						
Antrum	2 (10.0)	12 (60.0)	6 (30.0)	20/34 (58.8)	0.617	0.401
Body	0 (0.0)	3 (100.0)	0 (0.0)	3/34 (8.8)		
Cardia	0 (0.0)	1 (50.0)	1 (50.0)	2/34 (5.9)		
Mixed	2 (22.2)	6 (66.7)	1 (11.1)	9/34 (26.5)		
Differentiation						
WD/MD	0 (0.0)	10 (83.3)	2 (16.7)	12/34 (35.3)	0.089	0.402
PD	4 (18.2)	12 (54.5)	6 (27.3)	22/34 (64.7)		
LN spread						
Absent	1(9.1)	10 (90.9)	0 (0.0)	11/34 (32.4)	0.018	**0.027**
Present	3 (13.0)	12 (52.2)	8 (34.8)	23/34 (67.6)		
Metastasis						
Absent	3 (11.5)	19 (73.1)	4 (15.4)	26/34 (76.5)	0.116	0.066
Present	1 (12.5)	3 (37.5)	4 (50.0)	8/34 (23.5)		
TNM stage						
I/II	1 (6.3)	13 (81.1)	2 (12.6)	16/34 (47.1)	0.163	0.153
III/IV	3 (16.7)	9 (50.0)	6 (33.3)	18/34 (52.9)		

WD, well differentiated; MD, moderately differentiated. Bold, statistically significant.

D17S520 and *D17S855* (32.4%, 11/34) and *D3S1260* (23.5%, 8/34) in the tumor samples. Fig. 2 shows the MSI frequencies from the 13 MS markers. The representative example of (A) a normal case and (B) the allelic shifts at the *D17S855* (top) and *D18S34* (bottom) loci are shown in Fig. 3.

Genotype-phenotype and clinicopathological associations. Clinicopathological variables of the GC cases were comparatively analyzed according to the MSI genotypes: MSI-H (23.5%, 8/34), MSI-L (64.7%, 22/34) and MSS (11.8%, 4/34). Given the preponderance of evidence indicating that tumors with MSI-L are not biologically distinct from those that are MSS (17,18), we combined two subtypes of MSI (MSI-L/MSS) into a MSI-negative (MSI^-) classification to compare the clinical significance and prognostic value with a MSI-H group (MSI^+).

The prevalence of MSI revealed a significant difference in terms of gender in which MSS/MSI-L were primarily observed in males (males, 87.0% vs. females, 54.5%), whereas MSI-H was found at a significantly higher frequency in women (females, 45.5% vs. males, 13.3%; P=0.050). The difference in MSI status also showed a large discrimination with the status of lymph node (LN) metastasis. The prevalence of MSI-H was significantly higher in patients with LN metastasis than in those without (34.8% vs. 0%, respectively; P=0.018).

Most clinicopathological variables, including age, tumor location, Borrmann's type and histological differentiations were also closely related to the MSI genotype, although this association was not always statistically significant. Older patients (62.5%, 5/8), antral location (85%, 6/8), AGC cases (100%, 8/8), and poorly differentiated (PD) tumors (85%, 6/8) strongly emerged as MSI-H cases. A comparison of clinicopathological features according to the MSI status is shown in Table II.

Clinicopathological significance of MSI status on each MS locus. In order to further clarify critical MS markers

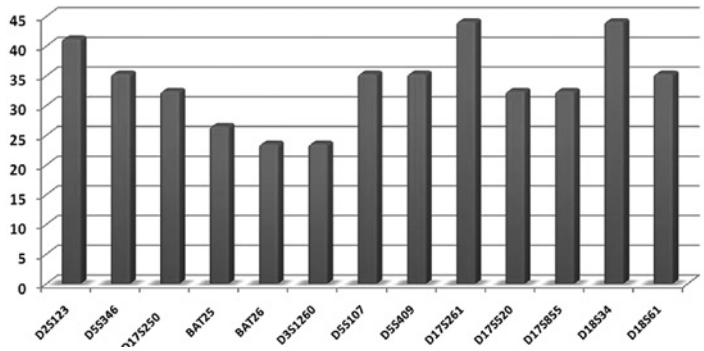

Figure 2. The frequency of MSI detected at 13 different MS markers from GC patients. The percentage of MSI was calculated by dividing the number of MSI at the specific MS marker by the number of informative cases.

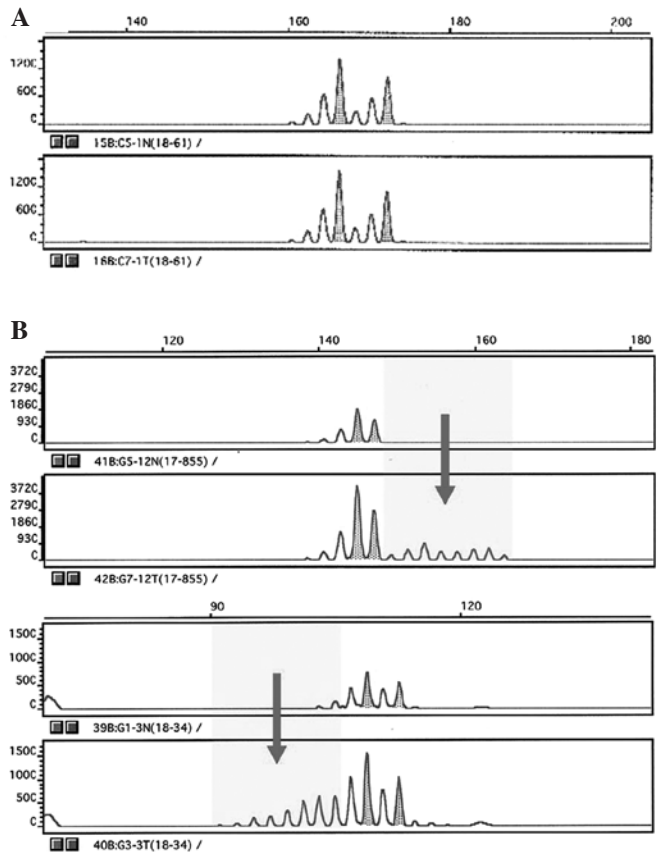

Figure 3. Examples of MSI analysis in GC cases. (A) MSS. (B) MSI analysis using the *D17S855* (top) and *D18S34* (bottom) markers revealed different allelic shift peaks (arrowhead) compared to those of the normal control DNA.

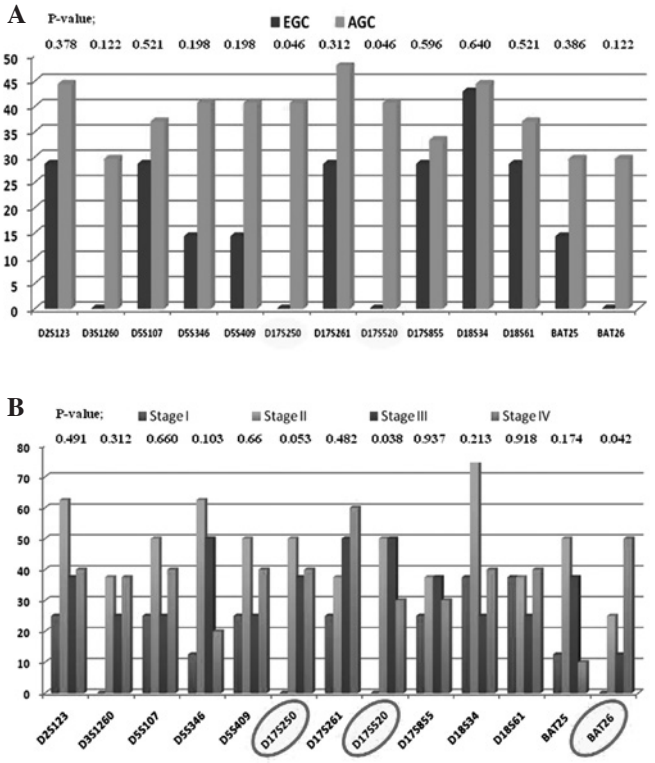

Figure 4. Comparison of MSI frequency on each locus by Borrmann's type and tumor stages. (A) Frequency of MSI between Borrmann's type on each locus in 34 GC cases. (B) Frequency of MSI among different tumor stages on 13 MS markers. The frequencies indicated refer to the proportion of MSI^+ neoplasms on each locus.

associated with GC progression, each MSI frequency from 13 different MS loci was compared to histological classifications and various tumor stages of GC.

The significant associations between MSI incidence and histological classifications of GC were identified. The incidence of MSI for the markers, *D17S250* and *D17S520*, showed significantly higher frequencies in AGC than in EGC (100% vs. 53.8%, respectively; P=0.046). Fig. 4A represents the comparison of the MSI frequency between histological classifications and the 13 MS loci.

A significantly different association was also noted between tumor stages and MSI prevalence for the two markers, *D17S520* and *BAT26* (P=0.038 and 0.042, respectively), indicating progressive accumulation of these loci during GC development. The frequency of various tumor stages detected with the different MS markers in GC cases is shown in Fig. 4B.

Discussion

Gastric cancer remains a leading cause of cancer mortality worldwide (1). During the last decade, numerous studies have described the potential of MSI as a molecular prognostic marker in GC (2-5). However, in absence of established criteria and methodology, the data on the prevalence and significance

of MSI in GC are still not conclusive and literature on this field remains ambiguous.

In this study, we examined an additional 13 MS loci to assess the clinicopathological implication of MSI to identify the critical MS loci, which will facilitate the comparison of results from different studies. Our analysis demonstrated that a significant proportion of GC samples exhibited MS alteration in 88% of the patients, and 23.5% had the MSI-H phenotype.

In previous reports (2,4,8,19), GC with MSI-H had a significantly higher frequency in older patients and women, and in antrally located and PD tumors, but a low incidence in patients with lymph node (LN) metastasis in comparison with MSI-L or MSS cases. In the current analysis, although not significant, MSI-H was somewhat more frequent in older patients, and was mainly found in the antrum and AGC patients, and exhibited a trend toward a more favorable prognosis in PD tumors.

Interestingly, MSI-H GCs were significantly associated with LN involvement in females. Previous reports considered the low frequency of LN metastasis to be an indication of good prognosis in MSI-H patients (20). However, in the analysis of Korean patients, the incidence of LN metastasis was more frequent in MSI-H tumors. Oki *et al* (7) noted that LN metastasis occurred frequently in MSI-H tumors, and a large degree of lymph vessel invasion was also observed in GC cases. Therefore, MSI cannot be regarded as a prognostic factor in GC patients. The high incidence of LN metastasis in MSI-H tumors was also reported in GC cases (21). Accordingly, it is unclear whether MSI is a prognostic or a predictive factor in GC. GCs with and without MSI-H appear to represent distinctive pathways of carcinogenesis (22). Future integrative applications with a larger series and expanded cases are warranted to verify the clinical impact of MSI on this outcome for GC patients.

Even though the data consistently support the possible correlation between the degree of the MSI and clinical outcome (2,23,24), there is no consistent consensus regarding the MSI genotypes. Thus, widely variable results on the frequency and definition of MSI in GC have been reported depending on the type and number of MS markers used (2). In this study, we therefore assessed an additional 8 dinucleotide repeat MS loci to further clarify the critical MS markers in GC. The highest frequency of MSI was simultaneously noted at the *D17S261* and *D18S34* (44.1%, 15/34) loci; this is superior to the NCI panels of MS markers. The DCC gene at the *D18S34* locus is thought to be one of the prime target genes on 18q, and its hyper-methylation or reduced expression in GC has been described in previous studies (23-25), indicating that the *D18S34* locus is critically involved in initiating gastric carcinogenesis and may be a valuable MS marker for GC screening.

The TNM stage has been considered to be the most important prognostic determinant in GC (9). To clarify the critical MSI loci in relation to GC progression, each MSI frequency from 13 different loci was compared to various tumor stages. We identified significantly different associations between tumor stages and MSI prevalence for the two markers, *D17S520* and *BAT26* (P=0.038 and 0.042, respectively), and a borderline significance for the *D17S250* locus (P=0.053), indicating that these markers may be another independent genetic determinant with GC prognostic value. Moreover, the *D17S250* and *D17S520* markers exhibited a significantly higher percentage of MSI in AGC than in EGC patients in terms of histological classifications of GC (P=0.046 and 0.046, respectively). The *D17S520* marker has thus far not been documented in GC. However, the high frequency of MSI or the loss of heterozygosity at this locus has been established in different cancer types. Uchino *et al* (25) reported the high frequency of MSI at *D17S520* (41%) in oral carcinoma, and others have demonstrated sequence deletions at *D17S520* (52%) in squamous cell carcinomas of the lung (26). These findings support our hypothesis that *D17S520* may be a potential MSI marker in GC and may be useful for predicting disease progression and prognosis.

In this study, we confirm previous findings that GC with MSI-H exhibits specific clinicopathological characteristics. Moreover, we identify the novel MS markers *D17S261* and *D18S34* as screening MSI loci, and *D17S520* as having a role in predicting a poor prognosis in GC. Future investigations are expected to validate these MS loci in a translational application in the GC field aiming to predict responses to treatments or outcomes, and to eventually use them as therapeutic targets.

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