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Original Article

Comprehensive Study of Microsatellite Instability Testing and Its Comparison With Immunohistochemistry in Gastric Cancers

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ABSTRACT

Purpose: In this study, polymerase chain reaction (PCR)-based microsatellite instability (MSI) testing was comprehensively analyzed and compared with immunohistochemistry (IHC) for mismatch repair (MMR) protein expression in patients with gastric cancer (GC).
Materials and Methods: In 5,676 GC cases, PCR-based MSI testing using five microsatellites (BAT-26, BAT-25, D5S346, D2S123, and D17S250) and IHC for MLH1 were performed. Re-evaluation of MSI testing/MLH1 IHC and additional IHC for MSH2, MSH6, and PMS2 were performed in discordant/indeterminate cases.
Results: Of the 5,676 cases, microsatellite stable (MSS)/MSI-low and intact MLH1 were observed in 5,082 cases (89.5%), whereas MSI-high (MSI-H) and loss of MLH1 expression were observed in 502 cases (8.8%). We re-evaluated the remaining 92 cases (1.6%) with a discordant/indeterminate status. Re-evaluation showed 1) 37 concordant cases (0.7%) (18 and 19 cases of MSI-H/MMR-deficient (dMMR) and MSS/MMR-proficient (pMMR), respectively), 2) 6 discordant cases (0.1%) (3 cases each of MSI-H/pMMR and MSS/dMMR), 3) 14 MSI indeterminate cases (0.2%) (1 case of dMMR and 13 cases of pMMR), and 4) 35 IHC indeterminate cases (0.6%) (22 and 13 cases of MSI-H and MSS, respectively). Finally, MSI-H or dMMR was observed in 549 cases (9.7%), of which 47 (0.8%) were additionally confirmed as MSI-H or dMMR by re-evaluation. Sensitivity was 99.3% for MSI testing and 95.4% for MMR IHC.
Conclusions: Considering the low incidence of MSI-H or dMMR, discordant/indeterminate results were occasionally identified in GCs, in which case complementary testing is required. These findings could help improve the accuracy of MSI/MMR testing in daily practice.

Keywords: Microsatellite instability; DNA mismatch repair; Immunohistochemistry; Gastric cancer

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Conflict of Interest
No potential conflict of interest relevant to this article was reported.

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INTRODUCTION

Defective DNA mismatch repair (MMR) leads to alterations in the length of repetitive sequences, a molecular phenomenon known as microsatellite instability (MSI) [1]. The key to DNA MMR defects is the inactivation of MMR genes by hypermethylation and epigenetic silencing of *MLH1* in most sporadic tumors [2] or by germline mutations in one of the MMR genes in most Lynch syndrome cases [3]. MSI has been identified as a molecular subgroup in gastric cancer (GC) [4] and is mostly caused by hypermethylation of the MLH1 promoter [5].

MSI-high (MSI-H) status can predict tumor prognosis and response to adjuvant therapy in patients with GC. For example, MSI-H tumors were associated with a favorable prognosis in patients with Stage II and III gastric cancer treated only with surgery. However, MSI-H GC appears to be unchanged or even negatively affected by adjuvant chemotherapy [6]. Similar results have been observed in two recent phase III randomized controlled trials [7]. In addition to their prognostic capacities, MSI-H or MMR-deficient (dMMR) protein expression has been proven to be a biomarker for predicting the response to immune checkpoint inhibitors in advanced solid cancers [8]. Pembrolizumab has been approved by the U.S. Food and Drug Administration as a cancer type- or site-agnostic treatment for patients with advanced MSI-H or dMMR solid tumors [9].

dMMR protein expression is generally assessed by immunohistochemistry (IHC) for 4 MMR proteins (MLH1, MSH2, MSH6, and PMS2), and MSI status is examined by polymerase chain reaction (PCR)-based analysis. IHC is easily available, cost-effective, and can be used to identify affected MMR genes directly. However, various staining patterns, such as weak, heterogeneous, or equivocal staining, contribute to uncertain interpretations of IHC results [10,11]. Most institutions accept IHC results alone for MMR proteins, and the discordance rate between IHC and PCR ranges from 1%–10% in many cases [12]. Some studies have reported low discordance rates between IHC and PCR in GC [13,14]. We compared the IHC data for MMR proteins and PCR-based analyses of MSI in our large-scale GC cohorts. We evaluated the discordance rate and the frequency of uncertain IHC interpretation.

MATERIALS AND METHODS

Patients and samples

In total, 5,676 patients with GC who underwent surgical resection at Seoul National University Bundang Hospital (Seongnam, Korea) between 2008 and 2018 were evaluated in this study. Specimens resected surgically were formalin-fixed and paraffin-embedded. In all cases, tumor tissues and their corresponding non-neoplastic mucosal tissues were selected from representative paraffin-embedded blocks. This study was approved by the institutional review board (IRB) of Seoul National University Bundang Hospital (IRB number: B-1905/538-307) with anonymization and performed in accordance with the Declaration of Helsinki for biomedical research involving human subjects.

PCR-based MSI testing

To evaluate the MSI status, macro-dissection and DNA extraction (InstaGene Matrix; Bio-Rad, Hercules, CA, USA) were performed on the same specimens' tumor and non-neoplastic normal tissues. DNA samples were subjected to PCR using the National Cancer Institute (NCI) panel of five markers, including 2 mononucleotide (MT) markers (BAT-26 and BAT-25)

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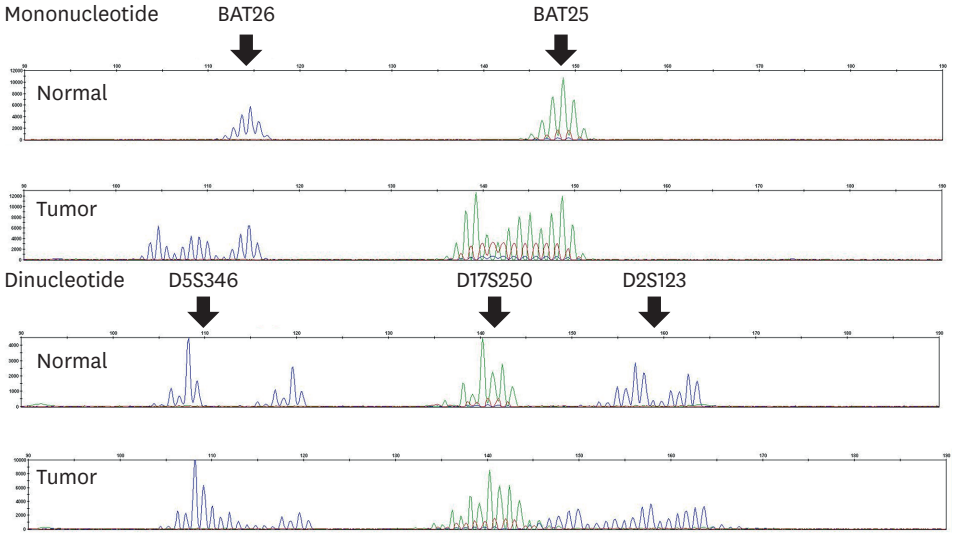

Fig. 1. Representative images of MSI-H result from PCR-based MSI analysis.
MSI-H = microsatellite instability-high; PCR = polymerase chain reaction.

and 3 dinucleotide (DT) markers (D5S346, D17S250, and D2S123). The PCR products were analyzed using an automated sequencer (ABI 3731 Genetic Analyzer; Applied Biosystems, Foster City, CA, USA). Microsatellite status was assessed to determine changes in the allele profiles of tumor cells compared with those of the corresponding non-neoplastic normal tissues. MSI-H was defined as two or more NCI markers with unstable peaks (**Fig. 1**), and MSI-low (MSI-L)/microsatellite stable (MSS) was defined as one or no NCI markers with unstable peaks [15]. MSI indeterminate status was defined as 1) 2 or 3 unstable DTs and 2) unstable BAT25 and 1 or more unstable DTs (**Supplementary Fig. 1**). Repeated examinations were performed if PCR failed, serious background noise was observed, or the results were MSI indeterminate status.

Analysis of MMR protein expression by IHC

Immunohistochemical staining for MLH1, MSH2, MSH6, and PMS2 was performed according to the manufacturer's instructions, with primary antibodies against MLH1 (mouse monoclonal primary antibody, prediluted, clone M1; Ventana, Tucson, AZ, USA), MSH2 (mouse monoclonal primary antibody, 1:200, clone G219-1129; Cell Marque, Rocklin, CA, USA), MSH6 (mouse monoclonal primary antibody, 1:100, clone 44; Cell Marque), and PMS2 (mouse monoclonal primary antibody, prediluted, clone ERP3947; Ventana) used for staining using a Benchmark XT staining and ULTRA system (Ventana).

Interpretation of IHC for MMR protein expression

MMR protein expression was assessed by nuclear staining of tumor cells and compared to that of internal positive control tissues, such as non-neoplastic epithelial cells, stromal cells, and lymphocytes, in the vicinity of the tumor. Each case was classified into one of four categories according to the intensity and proportion of nuclear staining of tumor samples in the whole slide as follows: 1) "intact expression" was defined as unequivocal nuclear staining in all tumor cells with clear staining of internal control tissue adjacent to tumor cells (**Fig. 2A**); 2) "total loss of expression" was defined as an unequivocal loss of nuclear staining in all tumor cells with clear staining of internal control tissue in the vicinity of tumor (**Fig. 2B**); 3) "sub-clonal loss of expression" was defined as clearly demarcated regional loss of nuclear staining of tumor samples with internal control tissue adjacent to tumor cells showing clear staining (**Fig. 2C**),

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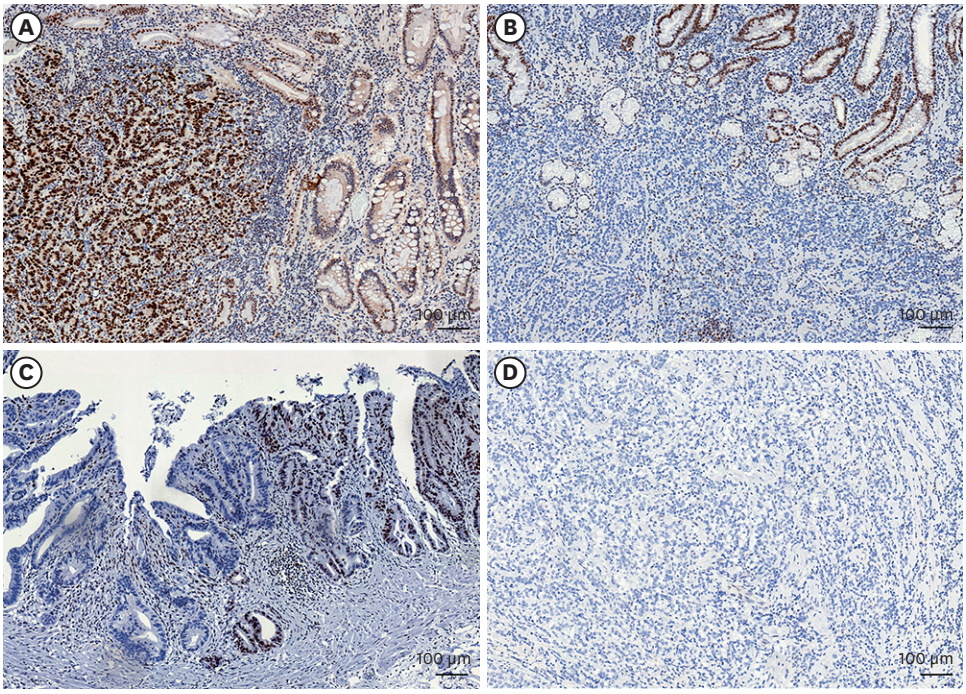

Fig. 2. Representative IHC images of MMR protein expression. Intact MLH1 expression (A). Complete loss of MLH1 expression (B). Sub-clonal loss of MLH1 expression (C). Equivocal MLH1 expression (D). All images are captured at 100× magnifications.
IHC = immunohistochemistry; MMR = mismatch repair.

and 4) "equivocal staining" was defined as a case where tumor and internal control tissue stains were very faint or unstained (**Fig. 2D**) [16,17]. MMR-proficient (pMMR) expression was defined as the intact expression of all four MMR proteins, and dMMR expression was defined as the loss of both MLH1 and PMS2, MSH2 and MSH6, or isolated loss of MSH6 or PMS2 [11]. Sub-clonal loss of expression or equivocal staining cases were classified as indeterminate IHC cases.

Comparison of data between IHC for MMR protein expression and MSI analysis by PCR

All the cases were tested for PCR-based MSI. In all cases, IHC for MLH1 expression was performed because most MSI-H GCs are associated with MLH1 hypermethylation [5]. MSI testing and IHC of MLH1 were re-evaluated in patients exhibiting discrepancies between IHC and MSI status or indeterminate IHC or MSI testing results. The original immunohistochemically stained slides were reviewed, and IHC and PCR were performed on the tested blocks. If necessary, the same tests were performed for additional blocks of the same specimen. Discordant or indeterminate IHC results were further analyzed using IHC for MSH2, MSH6, and PMS2.

Statistical analysis

All analyses were conducted, and graphs were prepared using R.4.0.1, R-studio 1.2.5033, and GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA, USA).

RESULTS

Comparative analysis between PCR- based MSI testing and IHC for MMR proteins.

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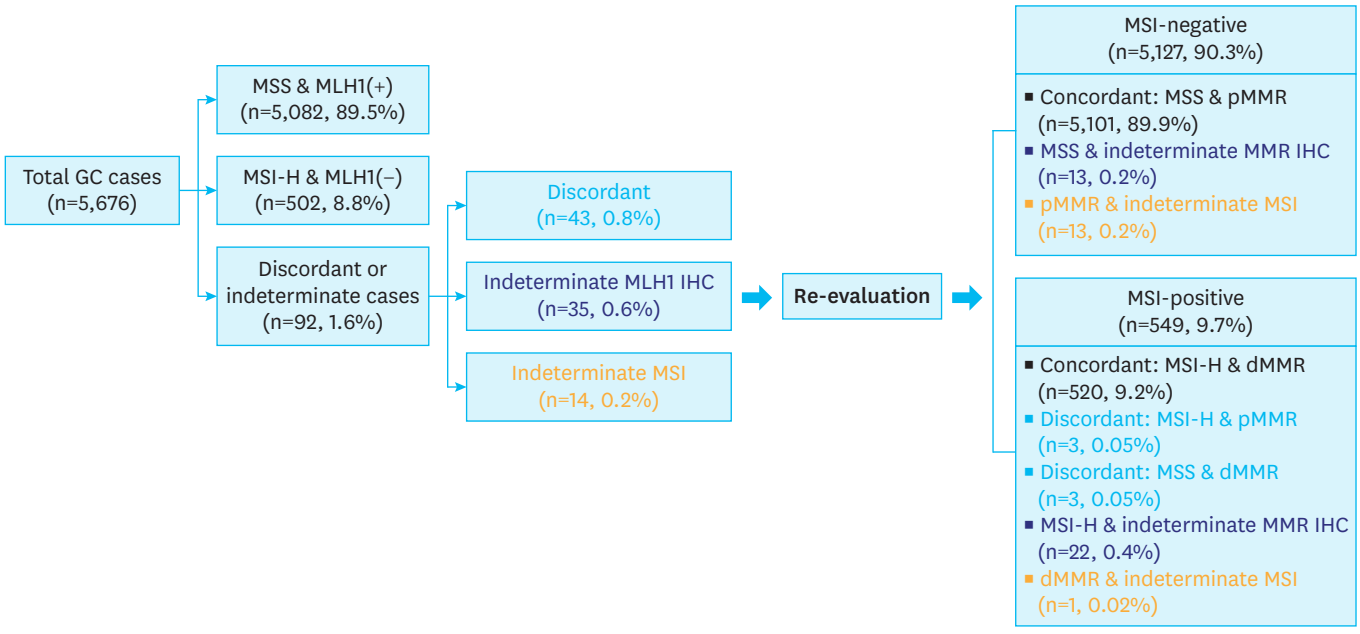

Fig. 3. Flow chart. The results of PCR-based MSI and IHC for MMR protein were compared for 5,676 cases of GCs, and cases with discordant or indeterminate results were re-evaluated.
PCR = polymerase chain reaction; MSI = microsatellite instability; IHC = immunohistochemistry; MMR = mismatch repair; GC = gastric cancer; MSS = microsatellite stable; MSI-H = microsatellite instability-high; pMMR = mismatch repair-proficient; dMMR = mismatch repair-deficient.

A comparative analysis between IHC for MLH1 expression and PCR-based MSI testing was performed for 5,676 GCs. A total of 5,082 cases (89.5%) showed MSS/MSI-L and intact MLH1 and were excluded from further consideration. Among the remaining 594 cases (10.5%), 502 (8.8%) had MSI-H and loss of MLH1 expression, and 92 cases (1.6%) had discordant or IHC/MSI indeterminate results, including 43 discordant cases (0.8%), 14 MSI indeterminate cases (0.2%), and 35 IHC indeterminate cases (0.6%) (**Fig. 3**).

Re-evaluation of MSI testing and IHC for MMR proteins

We re-evaluated 92 patients with a discordant/indeterminate status. In the case of the PCR-based MSI test, if PCR fails/background noise was severe, repeated examinations were performed, and additional tests were performed repeatedly for cases with discordant/indeterminate status. IHC results for discordant/indeterminate status were also reviewed, and repeated IHC or additional MMR IHC tests were performed, if necessary. As a result, it was confirmed that 37 cases (0.7%) (18 and 19 cases of MSI-H/dMMR and MSS/pMMR, respectively) were concordant. The remaining 55 cases were identified as: 1) 6 discordant cases (0.1%) (3 cases each of MSI-H/pMMR and MSS/dMMR), 2) 14 MSI indeterminate cases (0.2%) (1 case of dMMR and 13 cases of pMMR), and 3) 35 IHC indeterminate cases (0.6%) (22 and 13 cases of MSI-H and MSS, respectively) (**Fig. 3**).

After re-evaluation of 37 concordant cases, 17 resulted from misinterpretation (**Fig. 4A and B**) and 12 were affected by problems in the immunohistochemical process (**Fig. 4C and D**). The remaining eight cases displayed total loss of expression of other MMR proteins (6 cases of MSH2/MSH6 loss, 1 case each of MSH6 and PMS2 loss).

Discordant results were detected in three cases, each of MSI-H/pMMR (**Supplementary Fig. 2**) and MSS/dMMR (**Supplementary Table 1**). Discordant results were observed for the four MMRs despite repeated MSI and IHC examinations.

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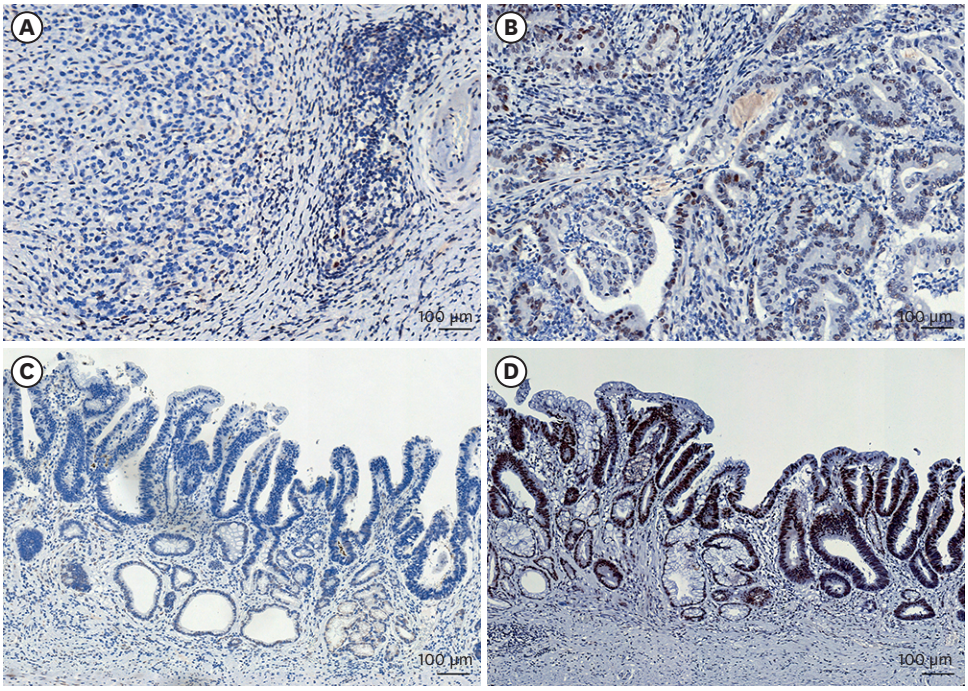

Fig. 4. Representative IHC images from re-evaluated cases. At first, MLH1 expression of this MSI-H case was classified as equivocal because the tumor cells and adjacent stromal cells were not stained. However, the adjacent stromal cells were weakly stained, and the final determination showed complete loss (A). This MSS case with abnormal MLH1 expression patterns, including mixed variable loss and strong staining in tumor cells, was initially classified as equivocal. However, it was corrected to intact MLH1 expression because no area of regional loss was evident (B). At first, this MSS case with equivocal MLH1 expression displayed that both the tumor cells and adjacent stromal cells were not stained (C). However, IHC for MLH1 was performed on other paraffin-embedded blocks of the same tumor, and intact MLH1 expression was confirmed (D). All images are captured at 100× magnification.
IHC = immunohistochemistry; MSI = microsatellite instability; MSS = microsatellite stable.

Indeterminate IHC and MSI cases

When MMR IHC tests were used to validate 14 MSI indeterminate cases, 13 cases (92.9%) were pMMR, and only one case (7.1%) was dMMR. In 1 case of dMMR, the result of the PCR-based MSI test was 2 stable MTs and 3 unstable DTs (**Fig. 5A**).

Of the 35 GC cases with indeterminate IHC results, 22 (62.9%) were MSI-H, and 13 (37.1%) were MSS/MSI-L by PCR-based MSI testing. There were 24 cases (68.6%) with subclonal loss of MMR protein, 14 (40.0%) of which were MSI-H. Sub-clonal losses were observed only for MLH1 and PMS2 (**Fig. 5B**). Despite repeated and additional IHC testing, equivocal MMR IHC results were observed in 11 cases (31.4%), of which 8 (8.5%) were MSI-H. The most frequently observed histological type, grade, and Lauren classification in cases of subclonal loss of expression were tubular adenocarcinoma (17 cases, 73.9%), moderately differentiated (60.9%), and intestinal type (87.0%), respectively (**Supplementary Table 2**).

Finally, MSI-positive GCs that were MSI-H or dMMR were observed in 549 of 5,676 cases (9.7%); 47 cases (0.8%) were additionally confirmed as MSI-H or dMMR by re-evaluation, and MSI-negative GCs were 5,127 cases (90.3%). The sensitivities of MSI testing and MMR IHC were 99.3% and 95.4% for MMR IHC (**Fig. 3**).

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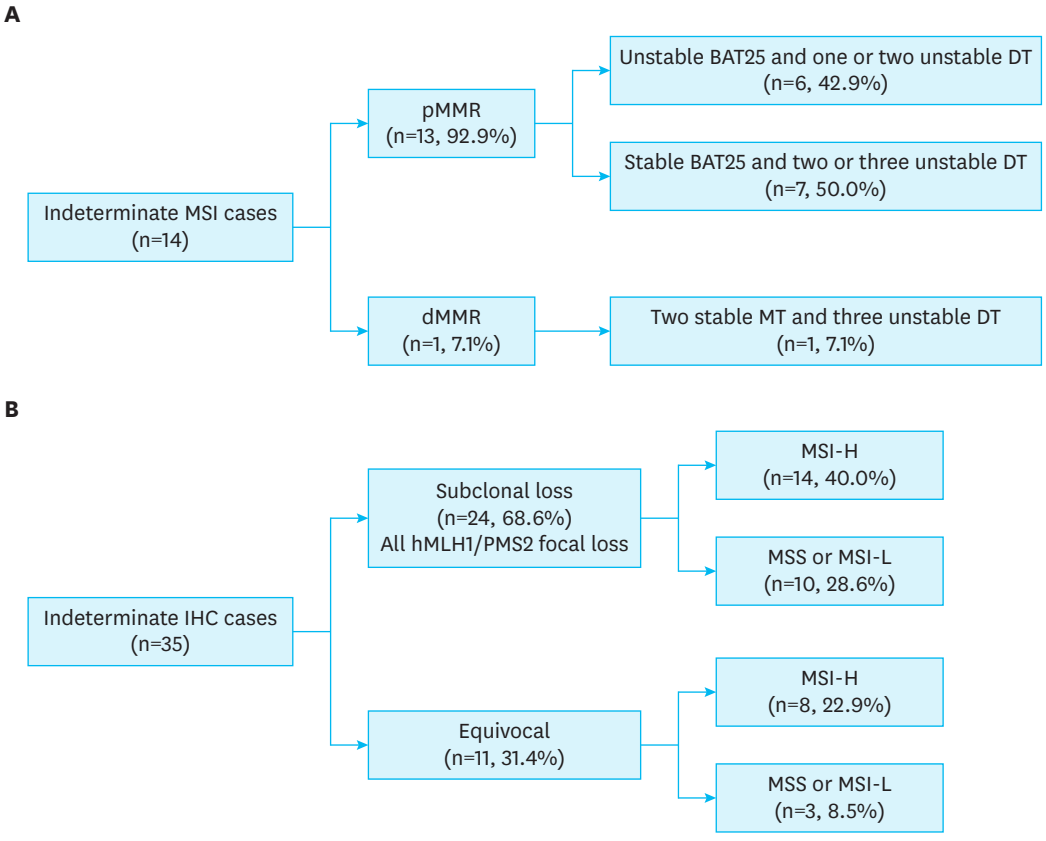

Fig. 5. MSI (A) and IHC (B) indeterminate results after re-evaluation.
MSI = microsatellite instability; IHC = immunohistochemistry; pMMR = mismatch repair-proficient; dMMR = mismatch repair-deficient; DT = dinucleotide; MT = mononucleotide; MSI-H = microsatellite instability-high; MSI-L = microsatellite instability-low; MSS = microsatellite stable.

DISCUSSION

MSI is a molecular phenotype observed in various sporadic cancers [18]. In GCs, MSI status has been classified into molecular subgroups included in The Cancer Genome Atlas [4] and the Asian Cancer Research Group [19] studies with increasing importance for prognosis and response to chemotherapy. Furthermore, in all advanced solid cancers, both tests have become even more important, as tumors with either MSI-H or dMMR are candidates for immunotherapy [11]. The purpose of this study was to evaluate the reliability and accuracy of IHC staining for MMR deficiency compared with PCR-based MSI testing with clinical significance in large-scale GC cohorts.

MSI-H or dMMR GCs have been reported at various rates [11]. The overall proportion of MSI-H or dMMR GCs varies depending on the patient cohort, markers used for PCR-based MSI testing, types of tissues used in IHC analysis, and types of antibodies. In this study, PCR-based MSI tests were performed on both normal and tumor tissues, and IHC for MMR protein expression was performed on whole tissue blocks, not on tissue microarrays. MSI-H or dMMR protein expression was observed in 9.7% of the GCs.

The discordance rate was 0.8% (43/5,676) at initial testing and 0.1% after review and re-testing between IHC for MMR protein expression and PCR-based MSI testing in GC. These were

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remarkably lower than published rates of 4%–9% [13,14]. As in our study, this discrepancy between MSI testing and IHC for MMR proteins may result from various causes, such as misinterpretation and IHC staining issues [17]. However, even after reevaluation, several discordant cases were observed. Rare missense mutations affecting protein functions other than protein translation and antigenicity are mainly observed in *MLH1* and *MSH6,* with pMMR expression observed by IHC [20,21]. Additional tests, such as next-generation sequencing, are required to confirm these missense mutations. Further examination of the clinical outcomes of discordant cases, such as MSS/MSI-L, dMMR, and MSI-H/pMMR, is required.

The present study showed that 0.6% (35 of 5,676 cases) displayed indeterminate staining patterns for MMR protein expression, such as sub-clonal loss of expression and equivocal staining. The apparent incidence of uncertain staining patterns remains unclear, but <10% of such cases have been reported in colorectal and endometrial cancers [16,22]. Twenty-four cases of indeterminate pattern staining in our GC cases displayed a sub-clonal loss of MLH1 expression. Regions with sub-clonal loss of MLH1 and PMS2 expression have been commonly associated with MLH1 hypermethylation, suggesting intra-tumoral heterogeneity in sporadic tumors [16]. Previous studies have reported different MSI results in cases where MMR protein expression was heterogeneous, in which PCR-based MSI testing was performed separately for the region where MMR protein expression was lost and the region where expression was preserved. There have been studies in which heterogeneous MSI test findings were consistent with heterogeneous MMR expression [23,24], but there were also homogeneous MSI test results [24,25]. In this instance, homogenous MSI findings were predominantly MSI-H; however, MSS was also observed in a few cases [24,25]. The association between the sub-clonal loss of MMR protein expression and patient prognosis and treatment response to conventional chemotherapy and immunotherapeutic drugs has not been studied, and further investigations are needed.

Importantly, the proportion of disagreement between IHC and PCR and indeterminate staining patterns for IHC comprised approximately 15% of cases, excluding unequivocal MSS/MSI-L and pMMR cases. In cases of indeterminate IHC results, repeat testing is recommended [10]. However, suboptimal fixation is also a major cause of indeterminate staining patterns, such as equivocal staining; therefore, it may be difficult to correctly interpret the results even after repeated tests [26]. Accurate determination is critical because, as previously stated, MSI-H or dMMR GC cases are candidates for immunotherapy. Therefore, as the importance of identifying MSI-H or dMMR increases, recent studies recommend performing PCR-based MSI tests in cases with indeterminate IHC staining [11,12,17]. Misdiagnosed MMR expression and MSI status are the primary negative factors for the response to immune checkpoint inhibitors; therefore, both IHC and PCR tests should be performed for treatment planning with immune checkpoint inhibitors and evaluation of discordant results between IHC and PCR [27]. In our study, 40.2% of the discordant or indeterminate IHC/MSI cases were concordant through re-evaluation; however, 58.8% of the discordant or indeterminate cases were not completely resolved. PCR-based MSI and MMR IHC tests should be performed in a complementary manner for accurate diagnosis.

In a previous study comparing MSI detection using an NCI panel of two MT and three DT markers versus a panel of only five MT markers (BAT-26, BAT-25, NR-21, NR-22, and NR-24), the latter panel was comparatively more sensitive and had a higher value for positive prediction. Additionally, IHC for MMR protein expression in all MSI-H CRC cases consisted of unstable DT markers and one or no unstable MT markers among the NCI

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panel, confirming normal MMR protein expression [28]. The NCI follow-up workshop also recommends that MSI-H cases consist of only mutated DT markers, which can be examined using a secondary panel of MT markers [15]. In our study, indeterminate MSI was defined as unstable DTs or unstable BAT25 and DTs. pMMR was also observed in most indeterminate MSI cases, but one out of two cases with three unstable DTs showed dMMR. Therefore, complementary MMR IHC should be performed for cases in which only the BAT-25 MT marker is unstable among the MT markers or only the DT markers are unstable.

This study had some limitations. First, only the surgical specimens were compared using IHC for MMR protein expression and PCR-based MSI testing. In clinical practice, both tests are performed on biopsies, and their interpretation is more difficult; therefore, a comparative test between the 2 assays using biopsy specimens of GCs is necessary. Second, a uniform process for interpreting the prognostic significance and molecular basis of discordant cases and indeterminate staining results should be developed. Third, MSH2/MSH6 protein IHC staining was performed only for discordant and indeterminate results, contributing to the low discordant rate. Nonetheless, this is a comparative study of large-scale GC cohorts. PCR-based MSI tests were performed to compare tumor and normal tissues in all cases to ensure the reliability of the results as much as possible.

In conclusion, the overall results showed a very low discordance rate between PCR-based MSI testing and MMR IHC in GC cohorts. However, the discordant or indeterminate rate was higher in GC cases, excluding unequivocal MSS/MSI-L and pMMR expression cases, because MSS and pMMR are usually observed in more than 90% of GC patients. Complementary tests are required for indeterminate MSI or IHC cases for accurate and reliable diagnoses, as discrepancies or uncertain interpretations of MSI testing, and MMR IHC have occasionally been identified in GCs. These findings could help improve the accuracy of MSI/MMR testing in daily practice.

SUPPLEMENTARY MATERIALS

Supplementary Table 1

MMR-IHC and PCR-based MSI results in discordant cases

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Supplementary Table 2

Histological type, histological grade, and Lauren classification in patients with focal expression loss of mismatch repair protein

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Supplementary Fig. 1

Representative images of MSI indeterminate results by PCR. PCR result: stability of 2 mononucleotide (BAT26 and BAT25) and 1 dinucleotide (D17S250); instability of 2 dinucleotide (D5S346 and D2S123).

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Supplementary Fig. 2

Representative images of MSI-H PCR results in MSI-H/pMMR discordant cases.

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