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Clinicopathologic Characteristics of Microsatellite Instable Gastric Carcinomas Revisited: Urgent Need for Standardization

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Abstract: Microsatellite instable gastric cancer (MSI-GC) is a specific molecular subtype of GC. We studied the phenotypes, genotypes, and clinicopathologic characteristics of MSI-GC in a white GC cohort and compared our findings with an extended literature review. The study cohort consisted of 482 patients. Specimens were available from 452 cases and were used for immunostaining (MLH1, PMS2, MSH2, MSH6) and molecular biological analyses (BAT-25, BAT-26, NR-21, NR-24, NR-27; Epstein-Barr virus in situ hybridization). Thirty-four (7.5%) GCs were MSI. Loss of MLH1 and/or PMS2 was found in 30 (88%) MSI-GC, 3 (9%) showed loss of MSH2 and/or MSH6. One (3%) MSI-GC was identified only by molecular biological testing. A single case was heterogeneous and contained microsatellite-stable and instable tumor areas. Twenty-one (62%) MSI-GCs showed unusual histologic features. MSI-GC was not found in diffuse-type or Epstein-Barr virus-positive GC. MSI-GC was significantly more prevalent in elderly patients, distal stomach, and was associated with a significantly lower number of lymph node metastases and a significantly better overall and tumor-specific survival. MSI-GC constitutes a small but relevant subgroup of GC with distinct clinicopathologic characteristics. Our literature review illustrates the shortcomings of missing standardized testing algorithms with prevalences of MSI-GC ranging from 0% to 44.5%. Future studies should test the hypothesis that patients with MSI-GCs may not need adjuvant/perioperative chemotherapy. However, this will require a standardized, quality-controlled diagnostic algorithm of MSI for GC.

Key Words: gastric cancer, EBV, MSI, MSH2, MSH6, MLH1, PMS2

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In recent decades, we witnessed major advancements in the understanding of the epidemiology, pathology and pathogenesis of gastric cancer (GC). Infection with *Helicobacter pylori* or Epstein-Barr virus (EBV), dietary, and lifestyle factors contribute to the risk of developing GC. Recently, whole-genome sequencing and comprehensive molecular profiling of GCs identified subtype-specific genetic and epigenetic alterations with unique mutational signatures.[1,2] Two different molecular subtypes were found which share the CpG island methylator phenotype, that is, EBV-associated GC and the microsatellite instable GC (MSI-GC).[2] Additional analyses demonstrated new insights in the genomic profile of diffuse GC.[3] Evidence is accumulating that patient prognosis and treatment response do not only depend on tumor stage but also on specific genotypic and phenotypic tumor characteristics. The advancements of targeted therapy provide compelling evidence that cancers of the same anatomic origin, for example, lung or colon, show great variability in their response rates to chemotherapies necessitating a more in depth phenotypic/genotypic classification before treatment. In colorectal cancer, examination of the MSI status is now used to classify the tumors as low (MSI-H) or high risk [microsatellite stable (MSS)].[4] Furthermore, phase II studies currently explore novel therapeutic options for MSI cancers. Thus, recognition and verification of MSI-GC in surgical pathology specimens may have clinical implications. In this retrospective study, we sought to answer the following questions: what is the prevalence of MSI-GC in a white GC cohort? Harbor MSI-GCs a specific phenotype that could guide MSI testing? Should MSI status be considered in patient treatment? Finally, we combined our findings with a literature review in order to identify demands for future translational research agendas and patient care.

MATERIALS AND METHODS

Ethics Statement

This project was approved by the local ethics committee of the University Hospital in Kiel, Germany

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M.M. and C.R.: designed the study. C.R., V.S.W., and C,B,: assembled the cohort and collected the clinicopathologic patient characteristics. J.H. and S.K.: carried out the immunostaining and molecularbiological analyses. M.M. and C.R.: reviewed all cases histologically, assessed immunostaining, and drafted the manuscript. H.M.B.: performed the statistical analysis. All authors read and approved the final manuscript.
The authors declare no conflict of interest.
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(reference number D 453/10). All patient data were pseudonymized before study inclusion.

Study Population

From the archive of the Institute of Pathology, University Hospital Kiel, we identified all patients who had undergone either total or partial gastrectomy for adenocarcinomas of the stomach or esophagogastric junction between 1997 and 2009 (GC cohort). The following patient characteristics were retrieved: type of surgery, age at diagnosis, sex, tumor localization and tumor size, tumor type, tumor grade, depth of invasion, number of lymph nodes resected, and number of lymph nodes with metastases. Date and cause of patient death was obtained from the *Epidemiological Cancer Registry* of the state of Schleswig-Holstein, Germany. Follow-up data of patients still alive were retrieved from hospital records and general practitioners.

Study Inclusion and Exclusion Criteria

Inclusion and exclusion criteria for the GC cohort were defined as follows: patients were included when (1) histology confirmed an adenocarcinoma of the stomach or esophagogastric junction; and (2) the date of death or survival data were available. Patients were excluded when (1) histology identified a tumor type other than adenocarcinoma; (2) histopathologic data were incomplete; (3) patients had previously undergone a resection of a Billroth II stomach with cancer in the gastric remnant; and (4) date of patient death or survival data had not been recorded. (5) Patients who received perioperative chemotherapy were also excluded.

Histology and TNM Classification

Tissue specimens were fixed in formalin and embedded in paraffin. Deparaffinized sections were stained with hematoxylin and eosin (H&E). Tumors were classified according to the Laurén classification.[5] All cases included in this study were reexamined by 3 surgical pathologists (M.M., V.S.W., and C.R.). pTNM stage of all study patients was determined according to the seventh edition of the Union for International Cancer Control guidelines[6] and was based solely on surgical pathologic examination including classification of distant metastases (pM category). In the seventh edition, all tumors of the esophagogastric junction and tumors of the proximal 5 cm of the stomach with extension into the esophagus are classified as esophageal tumors.[6] Patients were recategorized accordingly.

After the microsatellite status was assessed by immunohistochemistry and molecular biology (see below), the histology of the tumors was reexamined by 2 experienced surgical pathologists (M.M., C.R.) addressing potential distinct morphologic features of MSI compared with microsatellite-stable GCs: the main focus was on cell morphology, pushing versus infiltrative borders, lymphocytic infiltrate, mixed differentiation, and desmoplastic stromal reaction.

Tissue Micro Array (TMA) Construction

Formalin-fixed and paraffin-embedded tissue samples were used to generate TMA as described previously.[7] Three morphologically representative regions of the paraffin "donor" blocks were chosen. Tissue cylinders of 1.5 mm diameter were punched from these areas and precisely arrayed into a new "recipient" paraffin block. Sections of 2 μm of the TMA blocks were cut for further analysis.

Immunohistochemistry

Immunohistochemistry was carried out with monoclonal antibodies directed against MLH1 (clone G168-15, dilution 1:50; BD Biosciences, Heidelberg, Germany), PMS2 (clone MRQ-28, 1:20; Cell Marque Corporation, Rocklin), MSH2 (clone FE11, 1:30; Calbiochem, Merck KGaA, Darmstadt, Germany), and MSH6 (clone 44, 1:30; BD Biosciences).

Antigen retrieval was performed in TEC buffer (Tris-EDTA-citrate pH 7.8) using the DakoCytomation Pascal pressure chamber (Dako, Hamburg, Germany) at 125°C for 1 minute (MLH1, MSH2, MSH6), respectively. Automated antigen retrieval was performed in ER2 (EDTA-buffer Bond pH 8.9; PMS2). Immunostaining was done with the Bond Max-System (PMS2) using the Bond Polymer Refine Detection Kit (Menarini Diagnostics, Berlin, Germany). Immunostaining of MSH6, MSH2, and MLH1 was performed manually. After a blocking step with Hydrogen Peroxide Block (Thermo Scientific, Waltham; MSH6, MSH2, MLH1) the samples were incubated with the respective antibodies at 4°C overnight and the immunoreaction was visualized with the Histofine simple stain MAX PO multidetection reagent (Nichirei Biosciences Inc., Tokyo, Japan) in combination with the DAB Peroxidase Substrate Kit (Vector Laboratories Inc., Burlingame; MSH6, MSH2, MLH1) according to the manufacturer's instructions. Counterstaining was done with hematoxylin (Dr K. Hollborn & Söhne GmbH & Co KG, Leipzig, Germany).

The staining was regarded as present when the tumor nuclei stained positively with the same intensity as the control tissue (non-neoplastic gastric mucosa, intratumoral lymphocytes, fibroblasts). To achieve a high sensitivity for detecting MSI-GC, any case with loss (absence) of nuclear immunostaining or reduced protein expression, when compared with normal tissue, was submitted to molecular analysis of microsatellite status. Nuclear negativity and no convincing immunostaining of the internal positive control tissue were classified as "missing value" (nonimmunoreactivity of single antigens) and also analyzed by molecular pathology. Finally, after molecular biological analysis, all cases were reviewed by a trained surgical pathologist with a special expertise in MSI analysis (M.M.).

EBV Testing

EBV-encoded RNA was detected using the EBER-probe (Novocastra, Leica Microsystems GmbH, Nussloch, Germany) and the BondMax-detection system

according to the manufacturer's instructions (Leica Microsystems GmbH).

Virtual Microscopy With Area Analysis for Tumor Heterogeneity

If necessary, tissue slides were scanned using a Leica SCN400 microscopic whole-slide scanner (Leica Biosystems) at its maximum, nominally 40 times magnification. In the scanned images, pixel-to-pixel distance represents 0.26 μm. Images were exported from the scanner system into files of Leica SCN format. The images (Leica SCN file format) were displayed and analyzed by computer assisted technique as described previously.[8] The viewer program was extended by a polygon line drawing function. This was used by the pathologist to separately trace the outlines of mismatch repair (MMR) protein negative tumor tissue and MMR protein positive tumor tissue. Finally, a homogeneously color-filled sketch of the outlines was analyzed for total area size (in %).

DNA Isolation

Genomic DNA was extracted from formalin-fixed and paraffin-embedded tissue using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The integrity and amplifiability of the isolated DNA was evaluated by a qualitative size range polymerase chain reaction (PCR) assay.[9] Tissue sections were manually microdissected before DNA isolation to enrich tumor cells (> 80%).

Microsatellite Instability (MSI) Assay

MSI was determined by comparison of the allelic profiles of the mononucleotide repeat markers BAT-25, BAT-26, NR-21, NR-24, and NR-27 in tumor and corresponding normal tissue.[10] All markers were coamplified in a pentaplex PCR assay with the Qiagen Multiplex PCR Master Mix (Qiagen) following the manufacturer's recommendations for amplification of microsatellite loci. The amplified loci were analyzed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Darmstadt, Germany). Samples were judged as microsatellite unstable (MSI-H) when the tumor showed instability in at least 2 of the 5 (40%) microsatellites analyzed.

External Quality Assurance

The immunohistochemical evaluation of DNA-MMR proteins (MSH2, MSH6, MLH1, and PMS2) and the molecular biological MSI assay were certified successfully by the quality assurance program of the German Society of Pathology and the *Bundesverband Deutscher Pathologen e.V.*

Statistics

Statistical analyses were performed using SPSS 20.0 (IBM Corporation). For continuous variables, cases were divided into 2 groups by splitting at the median value. Median overall and tumor-specific survival was determined using the Kaplan-Meier method, and the log-rank test was used to determine significance. For comparison purposes, the median survival time, its SD, and 95% confidence interval (CI) were calculated. To investigate prognostic relevance, we included all variables having $P < 0.10$ into a Cox regression model and used the backward LR method ($P_{in} = 0.05$ and $P_{out} = 0.10$) to reduce the model to the independent variables. The significance of correlation between clinicopathologic parameters and biomarker expressions was tested using Fisher exact test. For parameters of ordinal scale (T-category, N-category, tumor stage) we applied Kendall τ test instead. *R* version 3.2.0 was used to calculate 95% CIs of proportions with continuity correction. A $P \leq 0.05$ was considered statistically significant. To account for the effects of multiple testing, we applied the explorative Simes (Benjamini-Hochberg) procedure within each group of tests (correlations and log-rank tests). The *P*-values are given unadjusted but are marked where they lose significance under the explorative Simes procedure.

RESULTS

Study Population

The clinicopathologic patient characteristics are summarized in Table 1. A total of 482 patients fulfilled all study criteria. MSI was assessed by immunohistochemistry using antibodies directed against MLH1, PMS2, MSH2, and MSH6 (Fig. 1) and subsequent molecular pathologic MSI analysis in 452 patients using mononucleotide repeat markers, that is, BAT-25, BAT-26, NR-21, NR-24, and NR-27. A total of 158 GCs showed either a decreased (compared with the internal positive control), missing, or not evaluable immunostaining of any of the 4 DNA-MMR proteins and were selected for subsequent molecular pathologic analysis, which identified 34 (7.5%; 95% CI, 5.3%-10.5%) highly MSI-GCs. All these cases showed instability of all 5 mononucleotide repeat markers. Instability of < five, that is, 1 to 4, respectively, was not found in any of the 158 cases.

Subsequently, all 34 MSI-H carcinomas were reevaluated on the immunostained whole-tissue sections for the expression of the MMR proteins by an experienced pathologist (M.M.). This reassessment identified a loss of MLH1 and/or PMS2 in 30 (88%; 95% CI, 72%-96%) cases (including 1 where MLH1 was not evaluable for technical reasons but a loss of PMS2 could be demonstrated), and 3 (9%; 95% CI, 2%-25%) showed complete loss of expression of MSH2 and/or MSH6. In 1 (3%; 95% CI, 0%-17%) case the tumor was MSI-GC; however, immunostaining of MLH1 and PMS2 was compromised by missing or insufficient staining of the internal positive controls (ie, non-neoplastic cells), carrying the risk of a false-negative test result, whereas MSH2 and MSH6 were expressed in this case.

Tumor Heterogeneity

One particular case of a 76-year-old male patient attracted our attention due to a biphasic MSH2 expression pattern. It was the only MSI-GC carcinoma that showed absence of MSH2 and was MSI. A staining of the whole slide unexpectedly demonstrated a loss of MSH2 in

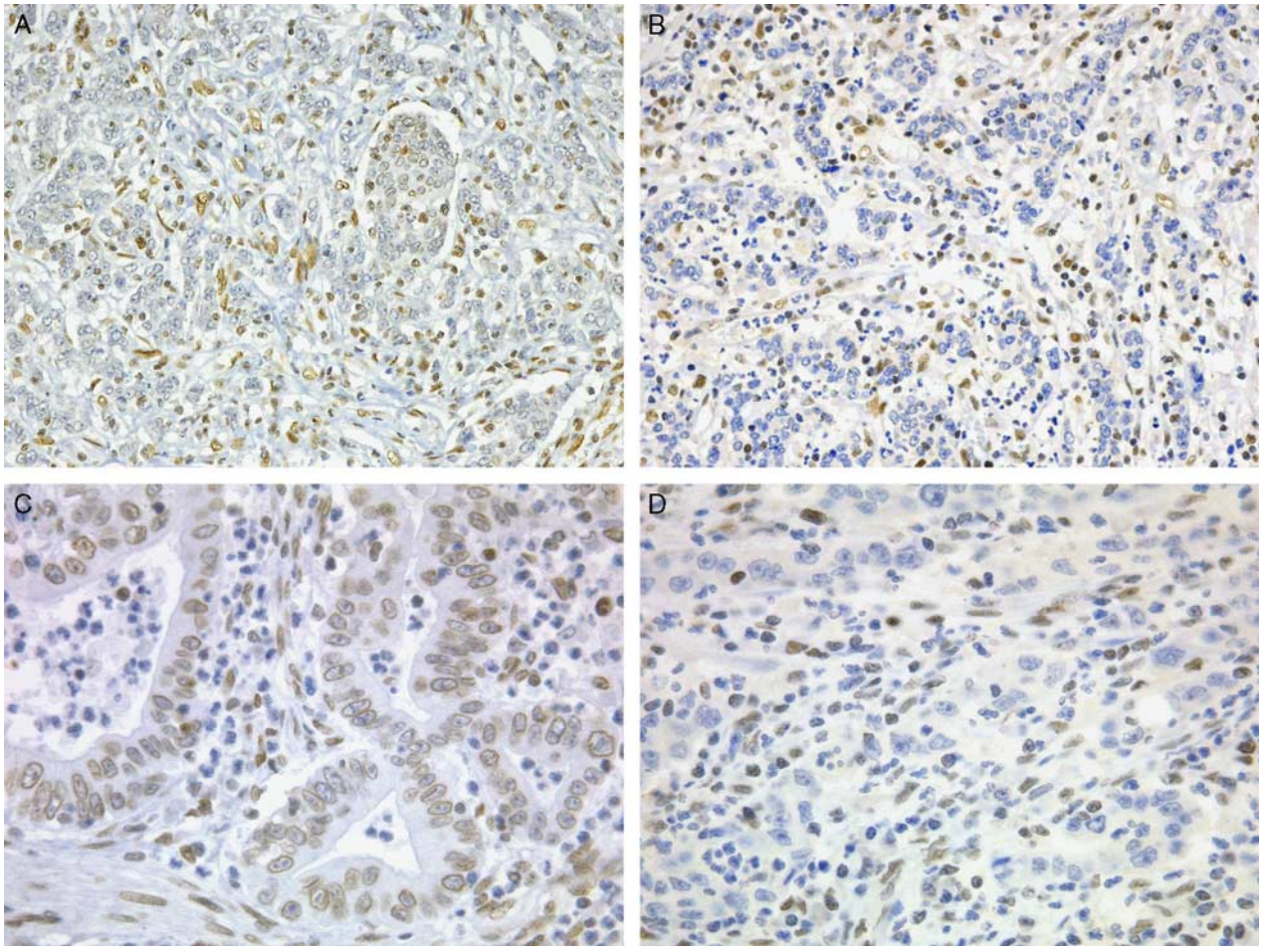

FIGURE 1. Immunohistochemistry for detection of mismatch repair (MMR) proteins in gastric cancer: MLH1 (A and C) and PMS2 (B and D). Typical staining pattern with combined loss of the complex forming MMR proteins. Lymphocytes and stromal cells serve as internal positive control. Especially in tumors with small tumor cells it is indispensable to differentiate these from the internal control to avoid misinterpretation (A, B, and D). Another potential source of error is the MLH1-deficient tumor that depicts a faint perinuclear staining pattern (C). In these, immunonegativity for PMS2 leads to the correct diagnosis. Original magnifications: (A and B) ×200; (C and D) ×400.

the major part (between 77% and 95%) of the tumor located at the gastroesophageal junction, whereas a small part (minimum 5%, maximum 23%) expressed MSH2 (Fig. 2). The exact amount of positive to negative tissue was analyzed by computer using virtual microscopy. Immunohistochemically, positive and negative stained areas intermingled (Fig. 3), which were indistinguishable by conventional H&E morphology. Subsequent molecular biological analysis of the microdissected tumor areas confirmed MSS in the MSH2-intact tumor area and MSI in the MSH2-lost area. As the GC had metastasized to 10 lymph nodes, we then analyzed all 10 lymph node metastases for their MSH2 expression profile immunohistochemically: all were positive for MSH2 (Fig. 2F). MLH1 and PMS2 were expressed evenly in the tumor. MSH6 was classified as "missing value," because neither the internal positive nor the tumor showed any staining in this case. No other case showed a clonal loss of any of the MMR proteins.

Correlation With Phenotypes of GCs

In a previous study, conducted in a blinded manner, we noticed that MSI was the only genotypic characteristic of GC, which correlated highly significantly with the phenotype according to Laurén.[11] As MSI colorectal carcinomas often exhibit certain histologic features uncommon in microsatellite stable carcinomas, which raise suspicion of either hereditary or sporadic MSI cancer,[12] we wished to scrutinize whether this also applies to MSI-GC. H&E-stained whole mount tissue sections of all MSI-GCs were reexamined with a special focus on their histologic appearance. Interestingly, 21 of 34 (62%; 95% CI, 43%-77%) MSI-GCs showed unusual histologic features: They consisted predominantly of highly pleomorphic tumor cells with large vesicular nuclei in a trabecular, nested, microalveolar, or solid growth pattern (Fig. 4). Tumor cell size was variable, sometimes exhibiting tumor cells that had lymphocytoid or blastoid appearance. An abundant tumor-associated inflammatory stroma

TABLE 1. Clinicopathologic Patient Characteristics of the Gastric Cancer Cohort and Correlation With MSI Status

				MSS		MSI		
Characteristics	**Valid n**		**Total [n (%)]**	**n (%)**	**95% CI**	**n (%)**	**95% CI**	***P***
Sex	452	Female	173 (38.3)	158 (91.3)	85.8-94.9	15 (8.7)	5.1-14.2	0.469
		Male	279 (61.7)	260 (93.2)	89.4-95.7	19 (6.8)	4.3-10.6	
Age (y)	439	< 68	219 (49.9)	209 (95.4)	91.5-97.7	10 (4.6)	2.3-8.5	0.042
		≥ 68	220 (50.1)	198 (90.0)	85.1-93.5	22 (10.0)	6.5-14.9	
Laurén phenotype	452	Intestinal	233 (51.5)	211 (90.6)	85.9-93.9	22 (9.4)	6.1-14.1	< 0.001
		Diffuse	141 (31.2)	141 (100)	96.7-100	0 (0.0)	0.0-3.3	
		Mixed	29 (6.4)	28 (96.6)	80.4-99.8	1 (3.4)	0.2-19.6	
		Unclassified	49 (10.8)	38 (77.6)	63.0-87.8	11 (22.4)	12.2-37.0	
Mucin phenotype	417	Intestinal	121 (29.0)	114 (94.2)	88.0-97.4	7 (5.8)	2.6-12.0	0.105
		Gastric	62 (14.9)	56 (90.3)	79.5-96.0	6 (9.7)	4.0-20.5	
		Mixed	167 (40.0)	151 (90.4)	84.7-94.2	16 (9.6)	5.8-15.3	
		Unclassified	67 (16.1)	66 (98.5)	90.9-99.9	1 (1.5)	0.0-9.1	
Localization	452	Proximal	142 (31.4)	134 (94.4)	88.8-97.4	8 (5.6)	2.6-11.2	0.343
		Distal	310 (68.6)	284 (91.6)	87.8-94.3	26 (8.4)	5.7–12.2	
T-category	452	T1a	8 (1.8)	8 (100)	60.0-100	0 (0.0)	0.0-40.2	0.351
		T1b	42 (9.3)	39 (92.9)	79.4-98.1	3 (7.1)	1.9-20.6	
		T2	53 (11.7)	45 (84.9)	71.9-92.8	8 (15.1)	7.2-28.1	
		T3	186 (41.2)	174 (93.5)	88.7-96.5	12 (6.5)	3.5-11.3	
		T4a	126 (27.9)	116 (92.1)	85.5-95.9	10 (7.9)	4.1-14.5	
		T4b	37 (8.2)	36 (97.3)	84.2-99.9	1 (2.7)	0.1-15.8	
N-category	449	N0	123 (27.4)	107 (87.0)	79.4-92.2	16 (13.0)	7.8-20.6	0.014
		N1	65 (14.5)	61 (93.8)	84.2-98.0	4 (6.2)	2.0-15.8	
		N2	76 (16.9)	71 (93.4)	84.7-97.6	5 (6.6)	2.4-15.3	
		N3/a/b	185 (41.2)	176 (95.1)	90.7-97.6	9 (4.9)	2.4-9.3	
Stage (seventh edition)	443	IA	39 (8.8)	37 (94.9)	81.4-99.1	2 (5.1)	0.9-18.6	0.369
		IB	29 (6.5)	25 (86.2)	67.4-95.5	4 (13.8)	4.5-32.6	
		IIA	55 (12.4)	48 (87.3)	74.9-94.3	7 (12.7)	5.7-25.1	
		IIB	47 (10.6)	46 (97.9)	87.3-99.9	1 (2.1)	0.1-12.7	
		IIIA	51 (11.5)	47 (92.2)	80.3-97.5	4 (7.8)	2.5-19.7	
		IIIB	81 (18.3)	77 (95.1)	87.2-98.4	4 (4.9)	1.6-12.8	
		IIIC	66 (14.9)	62 (93.9)	84.4-98.0	4 (6.1)	2.0-15.6	
		IV	75 (16.9)	71 (94.7)	86.2-98.3	4 (5.3)	1.7-13.8	
Lymph node ratio (median = 0.22)	438	< 0.22	219 (50.0)	195 (89.0)	84.0-92.7	24 (11.0)	7.3-16.0	0.010
		≥ 0.22	219 (50.0)	210 (95.9)	92.1-98.0	9 (4.1)	2.0-7.9	
Tumor grade	439	G1/G2	101 (23.0)	94 (93.1)	85.8-96.9	7 (6.9)	3.1-14.2	1.000
		G3/G4	338 (77.0)	312 (92.3)	88.8-94.8	26 (7.7)	5.2-11.2	
Resection margin	429	R0	375 (87.4)	346 (92.3)	89.0-94.7	29 (7.7)	5.3-11.0	0.783
		R1/R2	54 (12.6)	51 (94.4)	83.7-98.6	3 (5.6)	1.4-16.3	
Helicobacter pylori infection	385	Negative	325 (84.4)	300 (92.3)	88.7-94.9	25 (7.7)	5.1-11.3	1.000
		Positive	60 (15.6)	56 (93.3)	83.0-97.8	4 (6.7)	2.2-17.0	
EBV infection	447	Negative	431 (96.4)	397 (92.1)	89.0-94.4	34 (7.9)	5.6-11.0	0.623
		Positive	16 (3.6)	16 (100)	75.9-100	0 (0.0)	0.0-24.1	
Overall survival (mo)	439	Events (dead)	348 (79.3)	329	80.8	19	59.4	0.010
		Alive	91 (20.7)	78	19.2	13	40.6	
		Median survival		14.2 ± 1.1		35.8 ± 18.1		
		95% CI		12.0-16.4		0.3-71.4		
Tumor-specific survival (mo)	409	Events	288 (70.4)	276	72.8	12	40.0	0.002
		Censored	121 (29.6)	103	27.2	18	60.0	
		Median survival		15.4 ± 1.3		51.7 ± NC		
		95% CI		12.9-18.0		NC		

CI indicates confidence interval; EBV, Epstein-Barr virus; MSI, microsatellite instable; MSS, microsatellite stable; NC, not calculable.

consisting of either polymorphs and/or lymphocytes was frequently observed in these cases, usually with little or no desmoplastic stroma. Thirteen patients demonstrated several of these histologic features, whereas 8 had only few discriminating features mainly consisting of pushing margins and/or a lymphocytes or polymorphs. In 3 cases the inflammatory component was the only unusual feature.

The MSI-GC of 13 (38%; 95% CI, 23%-56%) patients showed no distinctive histologic features and were indistinguishable from any of the microsatellite stable GCs. Interestingly enough, there were no pure poorly cohesive, signet-cell carcinomas in the MSH-GC group even though signet cells can be part of a mixed pattern in MSI-GC.

Correlation With Clinicopathologic Patient Characteristics

Next, we compared the MSI status of GC with clinicopathologic patient characteristics (Table 1). On average, patients with MSI-GCs were older (older than

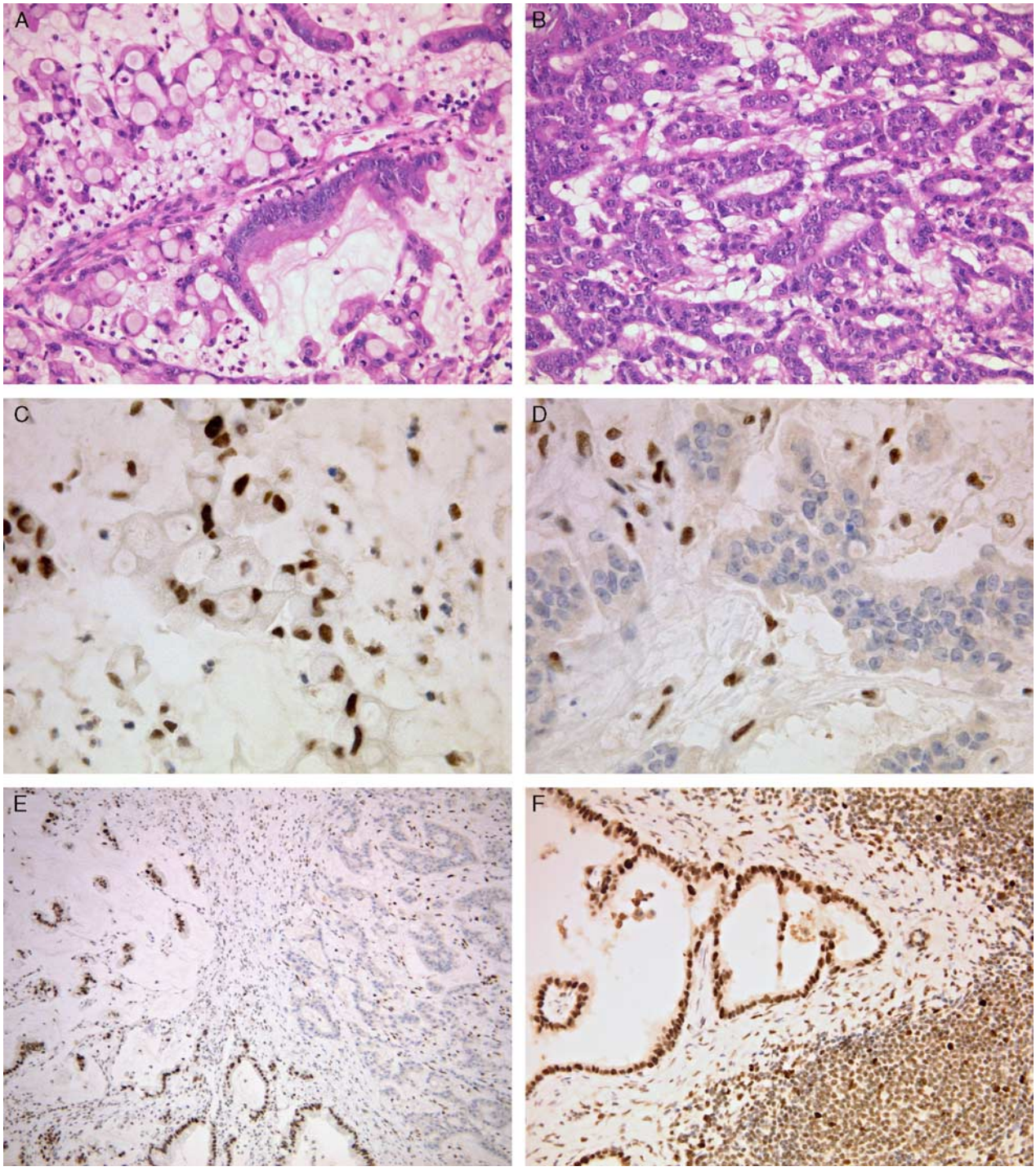

FIGURE 2. Heterogeneous expression of MSH2 with metastasizing microsatellite stable component. An adenocarcinoma of the proximal stomach—pT4a pN3a (10/19)—with mixed differentiation patterns: tubular, poorly cohesive, mucinous (A and B). No histologic evidence of a collision tumor. Focal expression of MSH2 [overall <15%, (C) after microdissection and molecular pathologic analysis: microsatellite stable] in otherwise complete loss of MSH2 [(D and E) after microdissection and molecular pathologic analysis: microsatellite instable]. All lymph node metastases were positive for MSH2 (F) and microsatellite stable. Original magnifications: (A–D) ×400; (E and F) ×200.

68 y; the youngest patient in our cohort with MSI-GC was 58 y old), had less lymph node metastases, thereby often were in a lower Union for International Cancer Control stage and had a longer median overall survival (35.8 vs. 14.2 mo in MSS-GC) as well as a longer median tumor-specific survival (51.7 vs. 15.5 mo).

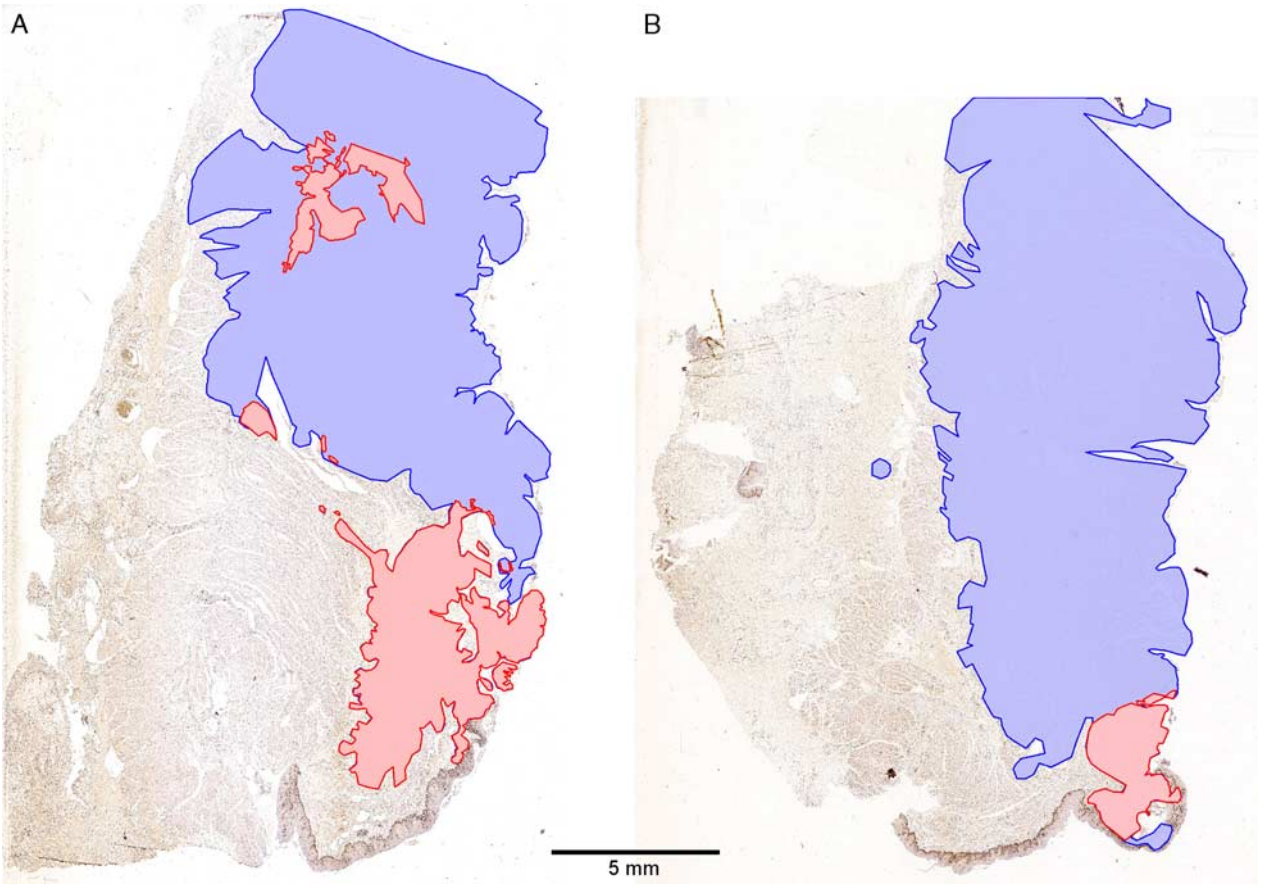

FIGURE 3. Tumor heterogeneity illustrated by virtual microscopy. The tumor of the proximal stomach showed a biphasic expression of MSH2, intermingling positive, microsatellite stable areas (red, positive nuclear staining) with larger areas that were MSH2 immunonegative and MSI (blue) (A and B). To illustrate the percent distribution, 2 large tissue sections were immunostained with MSH2, scanned, and using a viewer program with a polygon line drawing function, marked. The computed microsatellite stable tumor area was 23% (A) and 5% (B) in 2 separate tissue blocks from the same primary tumor.

EBV Infection and MSI-H Status Mutually Exclude One Another

EBV infection was found by EBER in situ hybridization in 16 (3.6%; 95% CI, 2.1%-5.9%) cases. None of these cases was MSI.

DISCUSSION

Recently, whole-genome sequencing and comprehensive molecular profiling of 100 tumor-normal pairs of GC unraveled distinct genotypes, 2 of which share the CpG island methylator phenotype, that is, EBV-associated GC and MSI-GC.[2] In view of these novel findings, also supported by another recent comprehensive molecular characterization of GC,[1] we explored the prevalence, phenotype, and clinicopathologic characteristics of MSI in a large consecutive cohort of 482 white GC patients and combined our findings with an extensive literature review (Table 2).

Assessment of MSI in GC

In colon cancer, usage of at least 5 different molecular markers in combination with immunohistochemistry is still considered state-of-the-art for testing MSI yielding satisfactory specificity and sensitivity.[50] However, there is no consensus for testing MSI in GC. Our literature review shows that 19 (51%) of 37 studies explored MSI in GC by immunohistochemistry and molecular biology (Table 2). Only 5 (14%) of these tested all 4 DNA-MMR proteins by immunohistochemistry. The size of the study populations and patient selection varied significantly between different studies ranging from as little as 17 to up to 1990 patients.[14,39] Accordingly, the prevalence of MSI-GC ranged from 0% to 44.5% (Table 2). The prevalence in our cohort (7.5%; 95% CI, 5.3%-10.5%) is slightly below the prevalence reported by a few other groups.[32,37,41,46] The vast difference in the overall prevalence of MSI is related to patient selection and methodological issues. For molecular testing, we compared the allelic profiles of 5 mononucleotide repeat markers, which have been shown to be highly reliable for colon cancer.[10] Several investigators used a 5-marker panel, which was established for colon cancer.[13,16,23,25,27] Only 2 groups previously used our 5 markers.[24,37] Others used only 1 or 2 markers[15,29,36,40] mixed microsatellite markers established for colon cancer

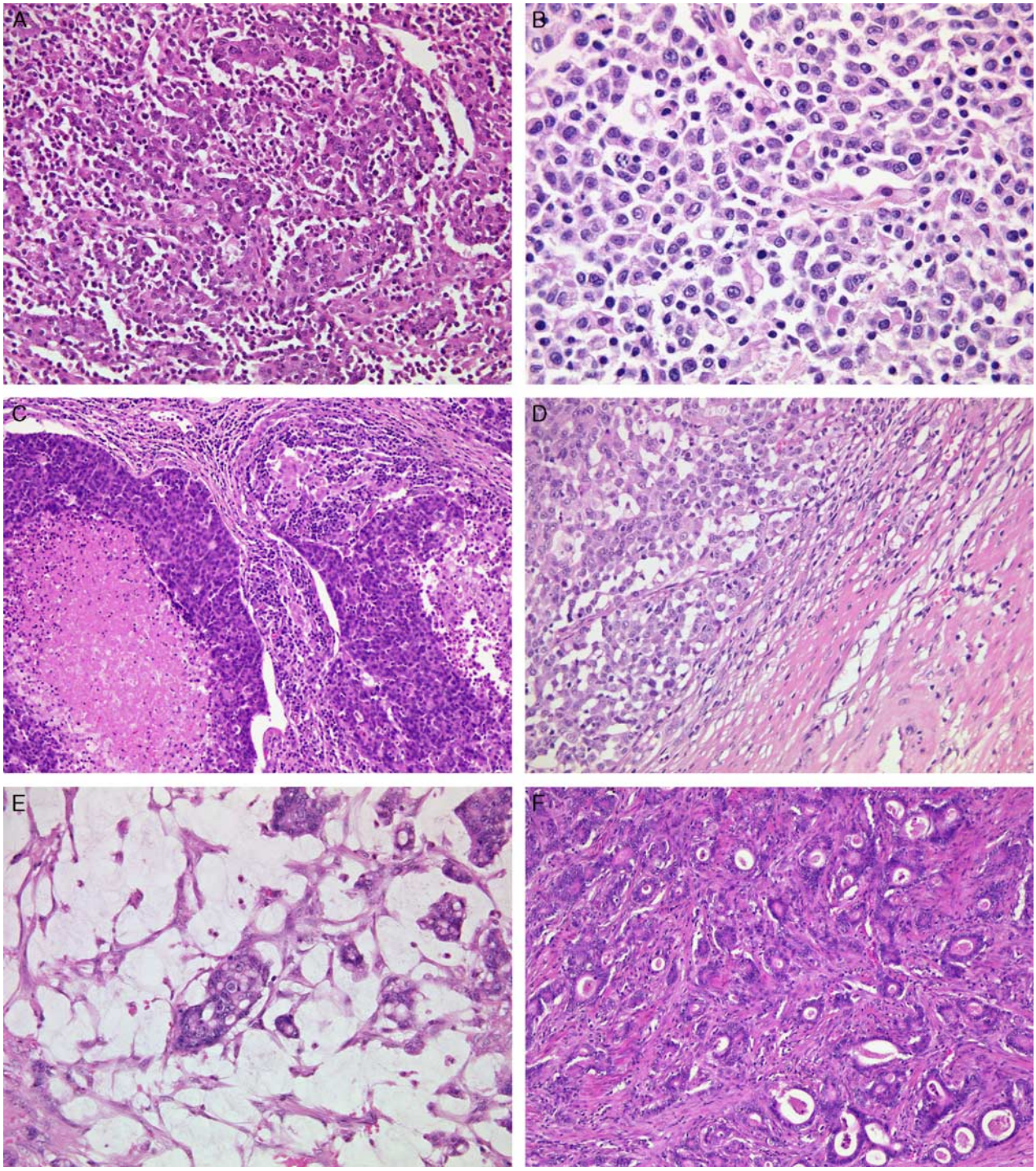

FIGURE 4. Histomorphology in MSI gastric cancers. Diffuse and solid sheets of tumor cells (A and D) with large amounts of tumor-infiltrating or surrounding lymphocytes (A, C, and D). Solid, nested growth with comedo necrosis (C), pushing margins with lymphocytes aggregating peritumorally (C and D). Some tumors demonstrated a more conventional morphology but then combined >1 pattern in 1 tumor, such as glandular and mucinous (E and F). Hematoxylin and eosin stained. Original magnification: ×200.

with other microsatellite markers, or even used a panel of 40 different markers[33] (Table 2). Different marker panels probably lead to different test results. Especially study groups, which use only 1 marker, are unable to determine the MSI status as high MSI is defined as a rate of 40% instable markers or instability in 2 of 5 markers. Interestingly, all our MSI-GC patients showed MSI of all 5 markers, that is, BAT-25, BAT-26, NR-21, NR-24, and

TABLE 2. Literature Review

					Molecular Biology		Immunohistochemistry										
No.	References	Tumor	White/ Asian	No. Patients	No. Markers	MSI Markers	MLH1	PMS2	MSH2	MSH6	Tested for EBV	Methylation Status	MSI (%)	MSI-L (%)	MSI (%)	MSS (%)	Positive Correlation MSI to Patient Survival
1	An et al[13]	GC	Mixed	83	5	BAT-25, BAT-26, D2S123, D5S346, D17S250	–	–	–	–	–	+	19.0	17.0	—	64.0	1
2	An et al[14]	GC	Asian	1990	5	BAT-25, BAT-26, D5S346, D2S123, D17S250	+	+	+	+	–	–	8.5	—	—	—	0
3	Arai et al[15]	GC	Asian	420	2	BAT-25, BAT-26	–	–	–	–	–	–	—	—	17.7	76.8	0
3	Bacani et al[16]	GC	White	139	8	BAT-25, BAT-26, BAT-40, D5S346, D17S250	+	–	+	+	–	+	5.0	24.5	—	70.5	0
5	Beghelli et al[17]	GC	White	510	2	BAT-25, BAT-26	+	–	+	–	–	–	—	—	16.0	84.0	2
6	Carvalho et al[18]	Early GC	White	40	3	BAT-25, BAT-26, BAT-40	+	–	+	+	+	–	—	—	—	100.0	NE
7	Chang et al[19]	GC	Asian	387	1	BAT-26	–	–	–	–	+	–	—	—	9.7	90.3	NE
8	Chang et al[20]	GC	Asian	549	1	BAT-26	–	–	–	–	+	–	—	—	9.7	90.3	NE
9	Chiaravalli et al[21]	GC	White	185	3	BAT-25, BAT-26, BAT-40	+	–	+	–	–	–	—	—	20.0	80.0	2
10	Chiaravalli et al[22]	GC	White	200	5	BAT-25, BAT-26, BAT-40, D5S346, D2S123	+	+	+	+	+	–	—	—	—	—	–
11	Choe et al[23]	GC	Asian	28	5	BAT-25, BAT-26, D2S123, D5S346, D17S250	+	–	–	–	–	–	50.0	—	—	50.0	1
12	Corso et al[24]	GC	White	250	5	BAT-25, BAT-26, NR-24, NR-21, NR-27	–	–	–	–	–	–	—	—	25.2	74.8	2
13	Corso et al[25]	MSI-GCs	White	63	5	BAT-25, BAT-26, D10S219, D10S541, D10S551	–	–	–	–	–	–	100.0	0.0	—	0.0	NE
14	dos Santos et al[26]	GC	White	61	6	D1S158, D2S104, D5S82, D5S346, D6S252, ARG1	–	–	–	–	–	–	—	—	34.4	65.6	2
15	Falchetti et al[27]	GC	White	159	8	BAT-25, BAT-26, D1S104, D3S1611, D2S123, D5S107, D17S261, D18S342	+	–	+	–	–	–	17.0	—	—	83.0	2
16	Fang et al[28]	Familial and sporadic GC	Asian	326	5	BAT-25, BAT-26, D2S123, D5S346, D17S250	+	+	+	+	+	+	16.3/ 14.6	—	—	83.7/ 85.4	2
17	Ferrasi et al[29]	GC	White	89	1	BAT-26	–	–	–	–	–	–	—	—	17.2	82.8	NE
18	Grogg et al[30]	GC	unknown	110	0	—	+	–	+	+	+		16.0	—	—	84.0	2
19	Kim et al[31]	GC	Asian	1786	5	BAT-25, BAT-26, D2S123, D5S346, D17S250	–	–	–	–	–	–	9.0	4.9	13.9	86.0	2
20	Kim et al[32]	GC	Asian	1178	5		+	+	+	+	–	+	9.1				1

						BAT-25, BAT-26, D2S123, D5S346, D17S250											
21	Kim et al[33]	GC	Asian	116	40	Panel	–	–	–	–	–	–	—	—	14.0	86.0	NE
22	Kulke et al[34]	Barrett Carcino-ma	White	80	5	BAT-25, BAT-26, D10S219, D10S541, D10S551	+	–	+	–	–	–	0.0	16.0	—	84.0	0
23	Lee et al[35]	GC	Asian	156	0	—	+	–	+	–	–	–	—	—	10.9	—	NE
24	Lee et al[36]	GC	Asian	327	2	BAT-25, BAT-26	+	–	+	–	–	–			9.5	90.5	1
25	Leite et al[37]	GC	White	410	5	BAT-25, BAT-26, NR-21, NR-24, NR-27	+	+	+	+	–	–	—	—	23.1	76.9	NE
26	Leung et al[38]	GC	Asian	35	8	Tp53, D18S58, D18S57, D2S123, D5S346, BAT-26, BAT-40, BAT-RII	+	–	+	–	+	+	8.5	6.5	—	85.0	NE
27	Lu et al[39]	Medullary GC	Asian	17	4	BAT-26, D1S548, D5S346, TP3	–	–	–	–	–	–	—	—	41.0	59.0	NE
28	Mizoshita et al[40]	GC	Asian	100	1	BAT-26	+	–	–	–	–	–	—	—	18.6	81.4	1
29	Oki et al[41]	GC	Asian	240	8	D2S123, D5S107, D10S197, D11S904, D13S175	–	–	–	–	–	–	9.4	10.7	—	79.9	0
30	Ottini et al[42]	GC	White	108	6	ACTC, D2S123, D3S1611, D5S107, D17S250, D18S34	–	–	–	–	–	–	15.7	14.8	—	51.8	0
31	Sakurai et al[43]	GC	Asian	167	5	D2S123, D5S107, D10S197, D11S904, D13S175	–	–	–	–	–	–	—	—	21.0	89.0	0
32	Seo et al[44]	GC	Asian	328	5	BAT-25, BAT-26, D2S123, D5S346, D17S250	+	–	+	–	–	–	8.2	—		91.8	1
33	Solcia et al[45]	GC	White	294	5	BAT-25, BAT-26, BAT-40, D5S346, D2S123	+	–	+	–	+	–	14.0	—	14.0	86.0	1
34	Sugai et al[46]	Early GC	Asian	62	5	BAT-25, BAT-26, D2S123, D5S346, D17S250	–	–	–	–	–	+	—	—	9.7	90.3	NE
35	Tay et al[47]	GC	Asian	58	5	BAT-25, BAT-26, D5S346, D2S123, D17S250	–	–	–	–	–	–	12.0	8.6	—	79.4	2
36	Wirtz et al[48]	GC	White	126	10	BAT-25, BAT-26, D2S119, D2S123, D5S107, D5S346, D10S197, D11S904, D17S261, D18S34	–	–	–	–	–	–	9.8	31.7	44.5	55.5	0
37	Wu et al[49]	GC	Asian	62	5	BAT-25, BAT-26, D1S191, D5S346, D17S250	–	–	–	–	–	+	23.0	14.0	37.0	63.0	NE

"+" denotes the marker investigated in the study; "–" denotes marker was not studied in this study.
"NE" denotes not examined, "0" no correlation, "1" insignificant correlation, "2" significant correlation.
GC indicates gastric cancer; MSI, microsatellite instable.

NR-27 and were hence interpreted as MSI-high. We were unable to detect any MSI-low, although this classification was frequently used by others (Table 2). In addition, we are the first who validated their immunohistochemical and molecularbiological methods for MSI analysis in GC by an external quality assurance program. Collectively, these data show that standardization and external quality control of MSI testing in GC is urgently needed and should include immunohistochemistry as well as molecular biological analyses to achieve a high sensitivity and specificity of the testing. This may lead to a more reliable estimation of the prevalence of MSI-GC.

Compatible with previous observations, MSI in sporadic GC was associated most commonly with a loss of MLH1/PMS2. Only 2 of our patients harbored a loss of MSH2. Germline mutations of *MSH2* are frequently found in Lynch syndrome. However, none of our patients had a history of cancer from that cancer syndrome spectrum. Because of German regulations for genetic testing of germline mutations requiring informed consent, a germline mutation analysis of the MMR pathway was not performed in this retrospective analysis and cannot be ruled out entirely. However, the youngest patient of our cohort with MSI-GC was 58 years old, making GC as first presenting tumor of a Lynch syndrome less likely. GC as first and only presenting tumor of a Lynch syndrome occurs in $<1\%$ of Lynch syndrome patients.[51]

MSI, GC Phenotype, and Clinical Patient Characteristics

In clinical practice, tumor tissue, obtained by biopsy or resection, is primarily forwarded to conventional histologic examination by a surgical pathologist. In view of the probably overall low prevalence of MSI in GC (see above), it is not justified to test every GC by immunohistochemistry and/or molecular biology. Thus, preselection based on a phenotype or specific patient characteristics is highly appreciated. Sixty-two percent (95% CI, 44%-77%) of our MSI-GCs showed a unique phenotype sharing similarities with a "medullary"-type cancer harboring highly pleomorphic tumors cells or lymphoid-like tumor cells arranged in a trabecular, nested, alveolar, or solid growth pattern, surrounded by an inflammatory tumor stroma and showing pushing tumor borders. This is in accordance to the description by Watanabe et al.[52] In our series, no poorly cohesive carcinoma was MSI, even though poorly cohesive features could be observed as a part of the tumor in mixed phenotypes and was previously misinterpreted by our own study group as diffuse-type GC.[11] This finding is in line with recent deep sequencing data: poorly cohesive or diffuse-type GCs belong to the stable genotype and not to the CpG island methylator phenotype, like MSI-GC.[2]

The characteristic phenotype found in MSI-GC is in accordance with a large part of published data on MSI or hereditary cancers, particularly of the colon and breast, and was supported by a publication on GC,[39] and can be reliably used as a deviance indicative of possible MSI. It might be worthwhile considering a distinct tumor group (medullary carcinoma) for MSI-GC in the World Health Organization classification of gastrointestinal tumors, as was already formerly proposed by Lu et al.[39] So far, carcinomas with medullary differentiation and prominent lymphoid infiltration are categorized as a poorly differentiated version of the tubular carcinoma,[4] which they most likely are not. And like in colon cancer, MSI testing may also be applied to categorize MSI-GCs as low-risk GCs despite a morphology suggesting a poorly differentiated cancer.

Many other publications linked MSI to the intestinal type[15,16,25,32,35] and could not show MSI in poorly cohesive carcinomas. Another study performed in Japan[40] did not find an association with the tumor phenotype. Interestingly, 38% (95% CI, 23%-56%) of our patients failed to show evidence of a specific phenotype. Here, clinical characteristics such as patients' age might aid to select cases for MSI analysis. In addition, an intestinal tubular morphology was mostly accompanied by other differentiation features and/or a lymphocytic and eosinophilic infiltrate. The tumor cells invaded the surrounding tissue not typically destructively but with pushing margins. Thus, phenotype is only partially indicative and further clinicopathologic patient characteristics might be helpful.

In correlating patient characteristics with MSI we noticed that MSI-GC was more common in patients aged 68 years and older (10.0% vs. 4.6%; 95% CI, 6.5%-14.9% vs. 2.3%-8.5%) and more prevalent in the distal stomach (8.4% vs. 5.6%; 95% CI, 5.7%-12.2% vs. 2.6%-11.2%; Table 1). However, due to the low patient numbers, only patients' age was significantly different. Thus, although MSI-GCs have some distinctive histologic and clinical features, the specificity remains low to tailor MSI testing, except for the presence of a so-called medullary phenotype, probably leading to a false-negative rate of approximately 38% (95% CI, 23%-56%). Tumor localization and patients' age might be used as a selective criterion for MSI testing, which also carries the risk of false-negative test results. Thus, the highest sensitivity of detecting MSI-GC is obtained by screening all non–diffuse-type GCs, that is, intestinal, mixed, and unclassified GCs (Table 1). Using this criterion, 11% (95% CI, 8%-15%) of our patients were MSI-GC.

MSI and EBV Infection

The whole genome project provided evidence that MSI and EBV are distinct molecular subtypes of GC. EBV infection has been shown to correlate with better survival in GC patients.[53] As possible explanation, extensive infiltration of tumor nests with cytotoxic T cells, has been proposed. A distinct lymphoid infiltrate can also be observed in MSI-GCs. Nonetheless, of our 34 MSI-GCs studied in our cohort, none harbored an EBV infection. These results confirm the whole cancer genome data as well as previous observations.[19,20]

Clinical Significance of MSI

On clinical grounds, MSI testing may be justified if it has prognostic or therapeutic implications. In our cohort,

MSI was associated with a significant longer patient survival (overall survival 35.8 ± 16.6 vs. 14.2 ± 1.1 mo, $P = 0.010$; tumor-specific survival 51.7 vs. 15.5 ± 1.3 mo, $P = 0.002$), which may have been related to the significantly lower rate of lymph node metastases in MSI-GCs (Table 1). Similar observations were among others made by An et al,[13] Beghelli et al,[17] and others.[24,27,28] To the contrary, no correlation or even an inverse correlation with patient survival was also observed and further underscores the necessity of standardized, quality-controlled MSI analysis.[15,16,31,40,41,48] As for other tumor types, the reasons for better survival remain ill defined. It has been suggested that MSI leads to the attraction of tumor-infiltrating lymphocytes, and a tumor-suppressive immune response.[22,30] With regard to therapeutic implications, none of our patients received perioperative or adjuvant chemotherapy, and at present we cannot comment on whether MSI-GCs are more or less sensitive to chemotherapy. Meta-analyses of colorectal cancer provided evidence that patients with stage II highly MSI colorectal cancer may not benefit from chemotherapy. However, adjuvant chemotherapy is not the standard of care for stage II CRC in Germany, whereas stage III MSI CRCs clearly highly benefit from adjuvant chemotherapy. We could only find a single study investigating the effect of MSI on therapeutic response in GC.[54] Herein, 23 patients in a randomized phase III trial were evaluated concerning their response to 5-fluoruracil and cisplatin. The results suggest that MSI directly contributes to a cisplatin resistance, as was shown in vitro, possibly by promoting the survival of cancer cells with damaged DNA after chemotherapy. Currently, there is not enough evidence to recommend MSI testing in GC on a routine basis to tailor chemotherapy. However, with regard to general clinical management, these tumors seem to have a favorable prognosis in an elderly patient population.

MSI, Tumor Heterogeneity, and Survival

Fascinatingly, we found a GC with a biphasic MSH2 expression pattern: Approximately 85% of the primary tumor area was MSI and between 5% and 23% MSS. More interestingly, all 10 lymph node metastases expressed MSH2 and were MSS. MSS encompassed 5% and 23% of the primary tumor area, depending on the paraffin block studied and only this tumor fraction spread to the lymph nodes. This observation, albeit casuistic, further supports the notion that MSI carcinomas are less aggressive and have a better survival. However, it also shows that heterogeneity is a specific issue in GC biology and also applies to MSS/MSI: a minor fraction of the primary tumor can be more aggressive and clinically important. The etiology of a heterogeneous microsatellite status remains obscure. Currently, we have no evidence to suggest a collision tumor. Whether de novo mutations of genes involved in the regulation and maintenance of DNA methylation in tumor cell subclones may contribute to this phenomenon, necessitates further investigations.

In summary, there is ample evidence now that MSI hallmarks a distinct genotype and phenotype of GC. It is associated with particular clinicopathologic patient characteristics, that is, medullary phenotype, more prevalent in elderly patients, and the distal stomach, with a significant lower number of lymph node metastases and a significant better overall and tumor-specific survival. Our literature review illustrates the major shortcoming of missing standardized testing algorithms and low patient numbers. Future studies should test the hypothesis that patients with MSI-GCs may not need adjuvant/perioperative chemotherapy. However, this will require a standardized, quality-controlled diagnostic algorithm also for GC, which should be developed based on large studies with adequate power.

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