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ORIGINAL ARTICLE

Clinicopathological characteristics of Epstein–Barr virus and microsatellite instability subtypes of early gastric neoplasms classified by the Japanese and the World Health Organization criteria

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Abstract

Gastric cancer is a heterogenous disease with different phenotypes, genotypes, and clinical outcomes, including sensitivity to treatments and prognoses. Recent medical advances have enabled the classification of this heterogenous disease into several groups and the consequent analysis of their clinicopathological characteristics. Gastric cancer associated with Epstein–Barr virus (EBV) and microsatellite-unstable tumors are considered to be the two major subtypes as they are clearly defined by well-established methodologies, such as *in situ* hybridization and polymerase chain reaction-based analyses, respectively. However, discrepancies in the histological diagnosis of gastric neoplasms remain problematic, and international harmonization should be performed to improve our understanding of gastric carcinogenesis. We re-evaluated Japanese cases of early gastric cancer according to the current World Health Organization (WHO) criteria and classified them into genomic subtypes based on microsatellite instability (MSI) and EBV positivity to determine the initial genetic events in gastric carcinogenesis. A total of 113 Japanese early gastric cancers (including low- and high-grade dysplasias) treated with endoscopic resection over 5 years were archived in our hospital. A histological re-evaluation according to the WHO criteria revealed 54 adenocarcinomas, which were divided into 6 EBV-positive (11.1%), 7 MSI-high (MSI-H, 13.0%), and 41 microsatellite stable cases (75.9%). MSI-H adenocarcinoma was confirmed by an immunohistochemistry assay of mismatch repair proteins. Programmed death-ligand 1 immunostaining with two antibodies (E1L3N and SP263) was positive in tumor cells of one MSI-H adenocarcinoma case (1/7, 14.3%). The proportion of stained cells was higher with clone SP263 than with E1L3N. Histologically, EBV-positive carcinomas were poorly differentiated (83.8%), and MSI-H cancers were frequent in well to moderately differentiated adenocarcinoma (85.7%), indicating that the EBV-positive subtype presented with high-grade morphology even when an early lesion. Our study indicates that the WHO criteria are useful for subdividing Japanese early gastric cancers, and this subdivision may be useful for comparative analysis of precursor lesions and early carcinoma.

Keywords: early gastric cancer; gastric dysplasia; carcinogenesis

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Introduction

Gastric cancer is a leading cause of cancer-related death worldwide. Significant advances in the understanding of this tumor have been achieved as a result of its comprehensive molecular characterization. The Cancer Genome Atlas (TCGA) classification established four genomic subtypes of gastric cancer: tumors positive for Epstein–Barr virus (EBV), microsatellite unstable tumors, genomically stable tumors, and tumors with chromosome instability [1]. Although the TCGA classification indeed provides molecular distinction, the natural history and treatment sensitivity of the subtypes remain to be elucidated.

The Asian Cancer Research Group analyzed the gene expression data of clinically relevant gastric cancers and identified two major categories: tumors with microsatellite instability (MSI) and microsatellite stable (MSS) tumors. MSS tumors were further divided into the following three types: the MSS/epithelial-to-mesenchymal transition (EMT), MSS/TP53−, and MSS/TP53+ types [2]. These subtypes were shown to be associated with disease progression and prognosis. MSS/EMT gastric cancers were diagnosed at advanced stage and correlated with a poor prognosis. Although the above-mentioned classifications may be utilized for personalized medicine in the near future, such molecular classification requires complex and high-cost genome analyses.

MSI is characterized by mismatch repair (MMR) deficiency causing an inability to repair alterations in the microsatellite regions. MSI can be seen in inherited genetic disorders caused by germline MMR gene mutations, such as Lynch syndrome [3]. Inherited gastric cancers are very rare, and most of the MSI gastric cancers are sporadic and caused by methylated silencing of the mut1 homolog 1 (*MLH1*) gene [4–6]. Hypermethylation of the CpG islands in the *MLH1* promoter region has been observed in most sporadic MSI-high (MSI-H) gastric cancers. Previous studies have shown that the MSI-H type is found in 15–33% of gastric cancers and is more commonly seen in elderly women and is associated with intestinal histology and a relatively good prognosis [7,8]. As MSI-H tumors are known to be associated with response to immune checkpoint blockade therapy, examination of MSI status is recommended in patients with advanced gastric cancer [9].

EBV-positive gastric cancer, accounting for approximately 8% of gastric cancers, has clinically distinct characteristics and predicts favorable prognosis [10,11]. It is associated with male gender, proximal location, and poorly differentiated histology characteristically with lymphocytic infiltration. Furthermore, several studies have shown that EBV-positive tumors show robust programmed death-ligand 1 (PD-L1) expression in both cancer and immune cells [12]. EBV positivity may be a promising biomarker that predicts the efficacy of immune checkpoint inhibitors, as seen in MSI-H tumors [13]. Previous studies have also suggested possible endoscopic treatment of this gastric cancer subtype, even if poorly differentiated, given the low frequency of lymph node metastasis reported in its early stage [14,15].

A number of studies have examined the clinicopathological and molecular features of gastric cancers, focusing on advanced tumors. In Japan, many early gastric cancers are treated endoscopically and diagnosed histologically according to the Japanese criteria. Early gastric cancers diagnosed in Japan are well known worldwide to include a certain percentage of tumors that would be diagnosed as high-grade, even low-grade, dysplasia/adenoma in Western countries [16,17]. Based on the most recent World Health Organization (WHO) diagnostic criteria, early gastric cancers diagnosed in Japan would likely be divided into low-grade dysplasia/intraepithelial neoplasia (IEN), high-grade dysplasia/IEN, and adenocarcinoma [18].

The present study reclassified our cases of early gastric cancer in Japan based on the WHO criteria and investigated their clinicopathological and etiologic characteristics to determine their initial gastric carcinogenesis.

Materials and methods

Sample recruitment

Gastric cancer patients who were treated with endoscopic mucosal dissection (ESD) at Asahikawa Medical University Hospital between 2014 and 2018 were studied (Figure 1). Patient age, gender, history of *Helicobacter pylori* eradication treatment, and *H. pylori* infection status were retrieved from medical records. From the surgical pathology archives, 113 tumors (from 111 patients) diagnosed as early gastric carcinoma according to the classification of the Japanese Gastric Cancer Association were retrieved. Two cases with synchronous multiple gastric tumors were included in this study. The endoscopic diagnosis of gastric mucosal atrophy border, judged using the Kimura–Takemoto classification, was obtained from endoscopic records. The degree was classified into three categories: mild (C-1, C-2), moderate (C-3, O-1), and severe (O-2, O-3). These patients underwent annual examinations with endoscopy and/or computed tomography. The overall survival and gastric cancer-specific survival were included in this assessment. The resected specimens were fixed with 10% formalin, embedded in paraffin (FFPE), and

examined histologically. The tumor diameter, histological types, depth of submucosal invasion, and presence or absence of lymphatic/vascular invasion of the tumor cells were obtained from the pathology reports. Ten patients were ineligible for the study because of a lack of written informed consent and thus were excluded. The subtype analysis of EBV, MSI-H, and MSS was performed on the residual 100 tumors (98 patients). This study was performed in accordance with the Declaration of Helsinki, and the protocol was approved by the Asahikawa Medical University Research Ethics Committee (approval #18261).

Histological evaluation

According to the Japanese diagnostic criteria, gastric cancers in our cases were histologically divided into three subtypes: well-differentiated tubular adenocarcinoma (tub1), moderately differentiated tubular adenocarcinoma (tub2), and poorly differentiated adenocarcinoma (por), based on the most predominant histology if two or more subtypes were present. Recut sections of the tumors were evaluated for adequacy for the molecular analyses, and two cases were excluded because the small tumors had been cut through with no tissue left on the slides.

According to the Japanese criteria, a total of 100 tumors (98 patients) were diagnostically confirmed as early gastric cancers. All of these tumors were then histologically re-evaluated according to the current WHO classification (Western criteria) [18] by a both United States- and Japan-trained, experienced pathologist (HTak) who was blinded to the molecular or genetic data. After this, the tumors were classified as low-grade dysplasia/IEN, high-grade dysplasia/IEN, or adenocarcinoma. Based on the Western criteria, 7 low-grade dysplasia/IEN, 39 high-grade dysplasia/IEN, and 54 adenocarcinomas were identified (Table 1). A comparative analysis between high-grade dysplasia/IEN and adenocarcinoma was subsequently conducted. The depth of submucosal invasion was measured from the deepest portion of the muscularis mucosae and the tumors were subsequently subclassified into SM1 (submucosal invasion of less than 500 μm) and SM2 (500 μm or more).

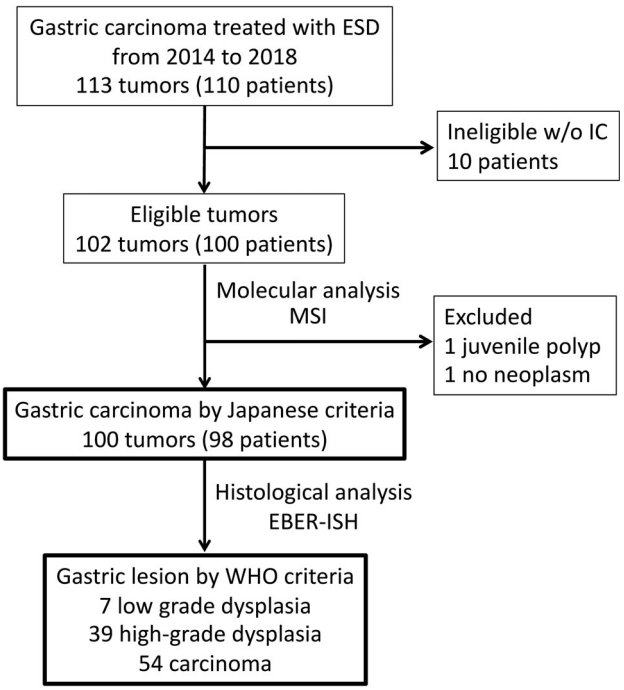

Figure 1. Flow chart of case enrollment in the study. EBV infection and MSI were determined using molecular analysis. w/o IC, without informed consent.

MSI assay

Genetic analysis was performed on matched paired gastric tumor and non-neoplastic gastric tissues. Under the microscope, tumor lesions were manually macrodissected from unstained 10-μm-thick FFPE sections. The genomic DNA of each case was then extracted and purified, using the GeneRead DNA FFPE kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and finally eluted in 20 μl of elution buffer. The sample of extracted DNA was quantified using a Qubit dsDNA HS Assay Kit with a Qubit2.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Polymerase chain reactions (PCRs) were performed with an MSI Analysis System, version 1.2 (Promega, Madison, WI, USA). Reactions were

Table 1. Histological correlation in gastric neoplasia between Japanese and WHO criteria.

		WHO criteria			
				Adenocarcinoma	
		Low-grade dysplasia/IEN	High-grade dysplasia/IEN	Well to moderately	Poor
Japanese criteria	tub1	7	39	35	0
	tub2	0	0	9	0
	por	0	0	0	10

carried out in 10-µl reaction volumes containing 1 µl of Gold STAR buffer, 0.15 µl of GoTaq MDx Hot Start Polymerase (Promega) containing primer pair mix (BAT-26, NR-21, BAT-25, MONO-27, and NR-24), and 2 ng of the DNA sample. The thermal cycling profile followed the manufacturer's instruction. Capillary electrophoresis of the amplicons with the Internal Lane Standard 600 was performed in a DS3000 Compact CE Sequencer (Hitachi High-Tech, Tokyo, Japan), and the DNA fragment analysis was conducted with the Gene Marker version 3.0 software program (SoftGenetics, State College, PA, USA). A tumor was considered to be MSI-H when two or more of the five markers showed instability.

EBV-encoded RNA *in situ* hybridization

FFPE sections (4 µm) of the tumor were prepared for each case. EBV-encoded RNA *in situ* hybridization (EBER-ISH) was performed with BOND EBER Probe (Leica Microsystems, Wetzlar, Germany). The reaction was detected with BOND Polymer Refine Detection using a BOND-III fully automated stainer (Leica Biosystems, Nussloch, Germany). Tumors with diffuse and strong nuclear staining were interpreted as EBV-positive gastric cancers.

Immunohistochemistry

FFPE sections were used for the histological and immunohistochemical examinations. MMR proteins were assessed in the tumor cells with antibodies against MLH1, mutS homolog 2 (MSH2), mutS homolog 6 (MSH6), and postmeiotic segregation increase 2 (PMS2). The following clones were used: M1 (Roche Diagnostics, Basel, Switzerland), G210-1129 (Roche Diagnostics), PU29 (Leica Microsystems), and EP52 (Agilent Technology, Santa Clara, CA, USA) for MLH1, MSH2, MSH6, and PMS2, respectively. Non-neoplastic epithelial cells adjacent to the tumor served as a positive control.

Two PD-L1 antibody clones – E1L3N (Cell Signaling Technology, Denver, MA, USA) and SP263 (Ventana Medical Systems, Tucson, AZ, USA) – were also tested, and tumor cells and immune cells for which more than 1% of the tumor cells were immunoreactive for this antibody were considered PD-L1-positive.

Statistical analyses

Continuous variables were identified as a quantitative variable and are expressed as the mean ± standard deviation. Categorical variables are presented in number and proportions. Clinicopathological data were analyzed using the SPSS software program, version 25 (IBM, New York, NY, USA). Variables were assessed by the Pearson's chi-square test or Fisher's exact test for categorical variables and by the Kruskal–Wallis test for continuous variables. A P value of <0.05 was regard as statistically significant. The survival time was calculated from the endoscopic treatment to the date of death, surgical resection, or last follow-up examination. A Kaplan–Meier curve and log-rank test were used to compare the survival.

Results

Clinicopathological features of gastric neoplasms

Regarding subtyping, the 100 early gastric cancers diagnosed using the Japanese criteria included 6 EBV-positive (6.0%), 10 MSI-H (10.0%), and 84 MSS (84.0%) cases (Figure 2). The clinicopathological characteristics of these gastric carcinomas are shown in supplementary material, Table S1. The Japanese gastric cancer cases were primarily male, but MSI-H cases were primarily female. The *H. pylori* status was not determined in all cases, but gastric atrophy evaluated by endoscopy was well recorded. Cases with severe gastric atrophy were frequent among Japanese gastric cancer patients with MSI-H tumors. According to the WHO criteria, 39 high-grade dysplasia/IEN and 54 carcinomas were subjected to comparative analyses (Table 2). Both of these categories consisted of elderly patients with an average age >70 years and male dominance. No significant differences were found in the tumor location, size, or macroscopic features. The

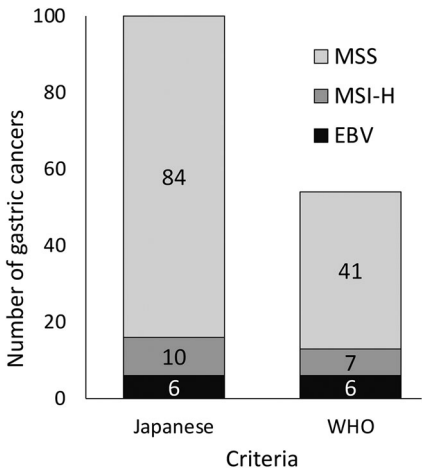

Figure 2. The number of gastric cancer subtypes classified by the Japanese and WHO criteria.

adenocarcinoma cases comprised 44 cases of well to moderately differentiated tubular carcinoma and 10 cases of poorly differentiated tubular carcinoma according to the WHO criteria, and the agreement rate of carcinoma with the Japanese criteria was 54.0% (54 versus 100; Table 1). Of these, submucosal invasion was found in 21 cases (38.9.%), including 12 cases (22.2%) of SM2 invasion. Cases with a histological evaluation of noncurative resection according to the Japanese Gastric Cancer Association guidelines version 4 [19] were recommended to receive gastrectomy. Twelve cases of submucosal invasive adenocarcinoma agreed to undergo additional gastrectomy with lymph node dissection, and one of these cases had histological lymph node metastasis. The other patients who refused gastrectomy were examined annually with both endoscopy and computed tomography.

The follow-up time of all cases diagnosed with gastric carcinoma according to the Japanese criteria was 2.9 ± 1.8 years, and the 5-year overall survival rate was 95.2% (see supplementary material, Figure S1). A total of four patients have died, and the causes were renal failure, pneumonia, cardiovascular disease, and malignancy (one each). As no metastatic recurrence of gastric carcinoma was observed in any patients, the cancer-specific survival rate was 100%. In a comparative analysis between high-grade dysplasia and carcinoma, the 5-year overall survival rates were 93.4 and 95.6%, respectively, showing no significant difference ($p = 0.647$; Figure 3).

Regarding subtyping, the 54 cases of adenocarcinoma were subclassified into 6 EBV-positive (11.1%), 7 MSI-H (13.0%), and 41 MSS (75.9%) cases, and their clinicopathological comparisons are shown in Table 3. The macroscopic features and histological subtypes were significantly different among these groups ($p = 0.007$ and <0.001, respectively). Three of six EBV-positive gastric carcinomas were characterized by a poorly differentiated histology with deep submucosal invasion (see supplementary material, Figure S2 and Table S2). Ten MSI-H tumors were found, including one case of low-grade dysplasia/IEN, two cases of high-grade dysplasia/IEN, and seven carcinomas. The carcinomas included six well to moderately and one poorly differentiated adenocarcinoma (Tables 3 and 4). Macroscopically, the MSI-H

Table 2. Clinicopathological characteristics of cases with high-grade dysplasia and carcinoma according to the WHO criteria.

	High-grade dysplasia (n = 39)	Carcinoma (n = 54)	P value
Age (years)	71.9 ± 9.5	73.1 ± 7.2	0.873
Sex			0.319
Male	28 (71.8)	44 (81.5)	
Female	11 (28.2)	10 (18.5)	
Location			0.535
Esophagogastric	1 (2.6)	2 (3.7)	
Upper third	9 (23.1)	13 (24.1)	
Middle third	12 (30.8)	23 (42.6)	
Lower third	17 (43.6)	16 (29.6)	
Size (mm)	21.8 ± 12.2	19.2 ± 11.1	0.2
Macroscopic feature			0.679
Depressed	20 (51.2)	28 (51.9)	
Elevated	12 (30.8)	13 (24.1)	
Mixed	7 (17.9)	13 (24.1)	
Depth of invasion			
Mucosa	39 (100)	33 (61.1)	
Submucosa	0 (0)	21 (38.9)	
Lymphatic invasion			
Positive	0 (0)	6 (11.1)	
Negative	39 (100)	48 (88.9)	
Vascular invasion			
Positive	0 (0)	3 (5.6)	
Negative	39 (100)	51 (94.4)	
EBV			0.038
Positive	0 (0)	6 (11.1)	
Negative	39 (100)	48 (99.9)	
MSI			0.295
MSI-H	2 (5.1)	7 (13.0)	
MSS	37 (94.9)	47 (87.0)	

Age and size are expressed as mean ± standard deviation. Others are number of cases (%).

carcinomas in Table 3 were elevated in five cases (71.4%) and mixed in two (28.6%), while none were depressed only. The endoscopic appearances of the tumors are shown in supplementary material, Figure S3. The gastric mucosa surrounding the tumors showed severe atrophic gastritis with vascular translucency. In this analysis, older age and female gender were frequent among MSI-H carcinoma cases, but without statistical significance.

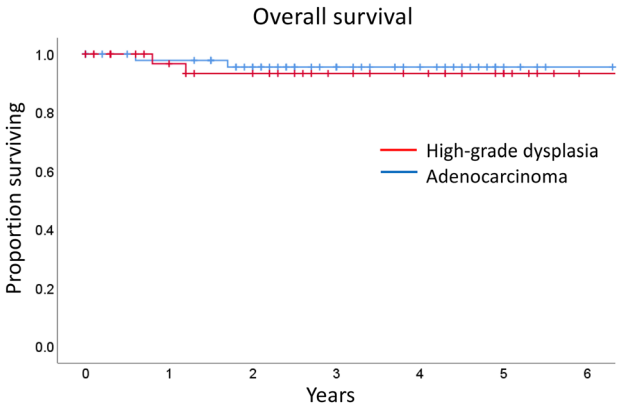

Figure 3. Overall survival in cases with gastric high-grade dysplasia and carcinoma according to the WHO criteria. The 5-year overall survival rates of cases with high-grade dysplasia and carcinoma were 93.4 and 95.6%, respectively. There is no statistical difference (p = 0.647, log-rank test).

Immunohistochemical findings of MSI-H tumors

MSI was determined by PCR-based analysis (Figure 4). Ten tumors, including seven carcinomas, two high-grade dysplasia/IEN, and one low-grade dysplasia/IEN, were found to be MSI-H. MMR protein expression in these MSI-H neoplasms was examined by immunohistochemistry (Table 4 and Figure 5). Of the 10 MSI-H tumors, 9 (90.0%) showed loss of MMR proteins: both MLH1 and PMS2 loss was seen in 8 cases, while isolated PMS2 loss was seen in 1 case. The loss of both MLH1 and PMS2 expression was consistent with MLH1 deficiency, while isolated PMS2 loss was due to PMS2 deficiency.

As PD-L1 helps tumor cells escape from immune attack, the inhibition of ligand–receptor binding is now applied clinically. There seems to be a relationship between PD-L1 expression and neoantigens produced by MSI-H tumors [20,21]. Among our seven

Table 3. EBV and MSI status of the gastric carcinomas according to the WHO criteria.

	EBV (n = 6)	MSI-H (n = 7)	MSS (n = 41)	P value
Age (years)	69.7 ± 7.8	76.3 ± 8.0	73.0 ± 7.2	0.471
Sex				0.205
Male	5 (83.3)	4 (57.1)	35 (85.4)	
Female	1 (16.7)	3 (42.9)	6 (14.6)	
Location				0.635
Esophagogastric	0 (0)	0 (0)	2 (4.9)	
Upper third	0 (0)	1 (14.3)	12 (29.3)	
Middle third	4 (66.7)	3 (42.9)	16 (39.0)	
Lower third	2 (33.3)	3 (42.9)	11 (26.8)	
Size (mm)	24.5 ± 10.2	18.9 ± 5.9	18.6 ± 11.9	0.192
Macroscopic features				0.007
Depressed	4 (66.7)	0 (0)	24 (58.5)	
Elevated	2 (33.3)	5 (71.4)	6 (14.6)	
Mixed	0 (0)	2 (28.6)	11 (26.8)	
Histology (WHO criteria)				<0.001
Well to moderately	1 (16.7)	6 (85.7)	37 (90.2)	
Poorly	5 (83.3)	1 (14.3)	4 (9.8)	
Depth of invasion				0.393
Mucosa	3 (50.0)	4 (57.1)	26 (63.4)	
SM1	0 (0)	1 (14.3)	8 (19.5)	
SM2	3 (50.0)	2 (28.6)	7 (17.0)	
Lymphatic invasion				0.846
Positive	1 (16.7)	1 (14.3)	4 (9.8)	
Negative	5 (83.3)	6 (85.7)	37 (90.2)	
Vascular invasion				0.495
Positive	0 (0)	1 (14.3)	2 (4.9)	
Negative	6 (100)	6 (85.7)	39 (95.1)	

Age and size are expressed as mean ± standard deviation. Others are number of cases (%).

Table 4. MMR protein expression/loss in MSI-H gastric tumors.

Tumor #	WHO criteria	Japanese criteria	MLH1	PMS2	MSH2	MSH6
3	Well to moderately	tub1	–	–	+	+
7	Well to moderately	tub2	–	–	+	+
8	Low-grade dysplasia	tub1	–	–	+	+
22	Well to moderately	tub2	–	–	+	+
23	Well to moderately	tub1	+	–	+	+
59	High-grade dysplasia	tub1	+	+	+	+
62	Well to moderately	tub1	–	–	+	+
63	Well to moderately	tub1	–	–	+	+
87	Poorly	por	–	–	+	+
105	High-grade dysplasia	tub1	–	–	+	+

+, Expressed; –, loss.

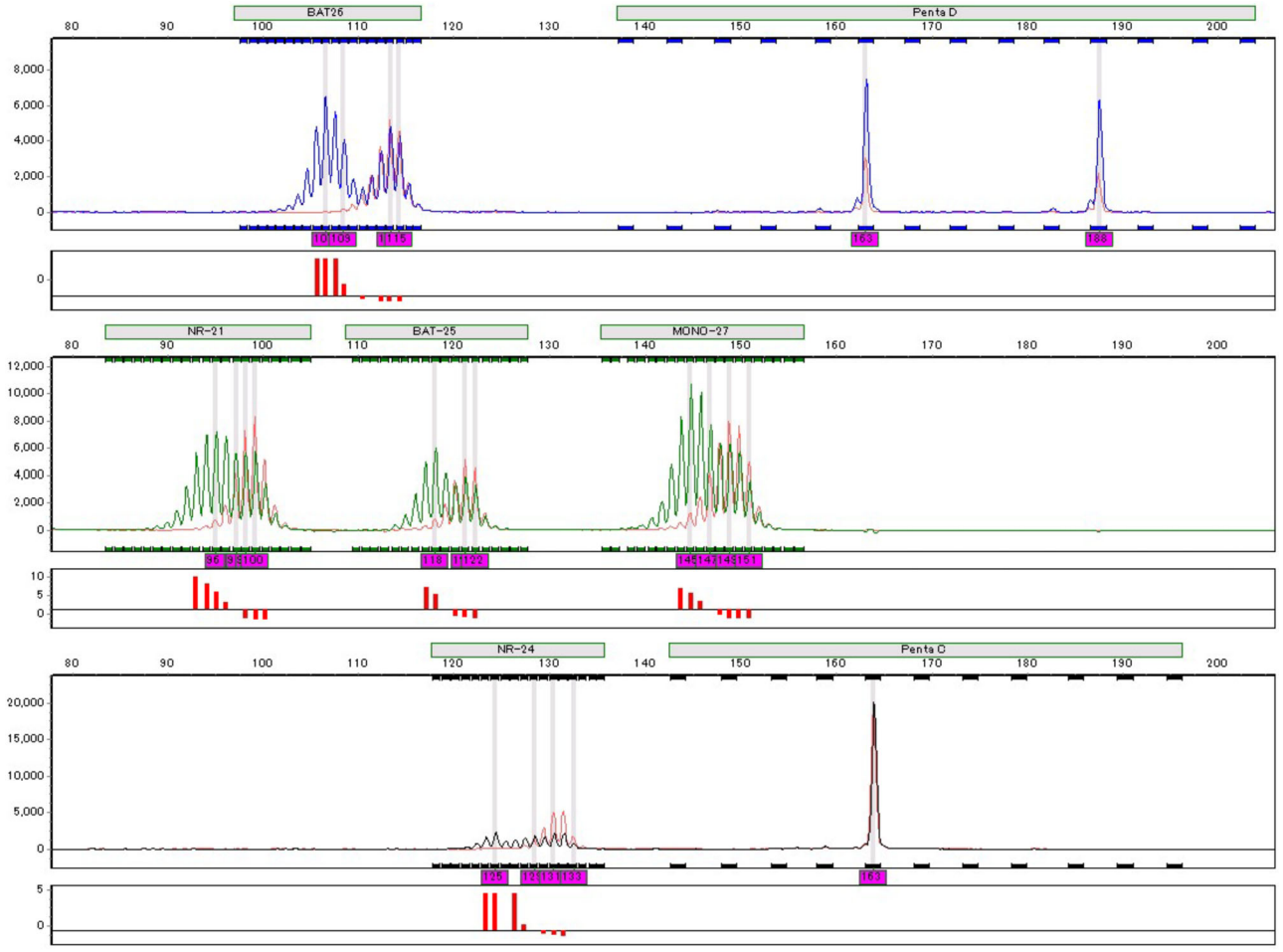

Figure 4. MSI analysis. PCR was conducted with the following markers: BAT-26, NR-21, BAT-25, MONO-27, and NR-27. A DNA fragment analysis was conducted with the Gene Marker software program. A representative electropherogram of a case (#59) is shown. There are shifts in five peaks of amplicons of the microsatellite markers between the cancerous (blue, green, and black) and non-neoplastic tissue (red), indicating high MSI. The peak shifts are clearly indicated by red bars below.

MSI-H carcinomas, PD-L1 immunohistochemical expression on the surface of tumor cells was found in one case (14.3%) with E1L3N and two (28.6%) with SP263 (Table 5 and Figure 6). PD-L1 expression in immune cells was 42.9% (3/7) and 85.7% (6/7) using E1L3N and SP263, respectively.

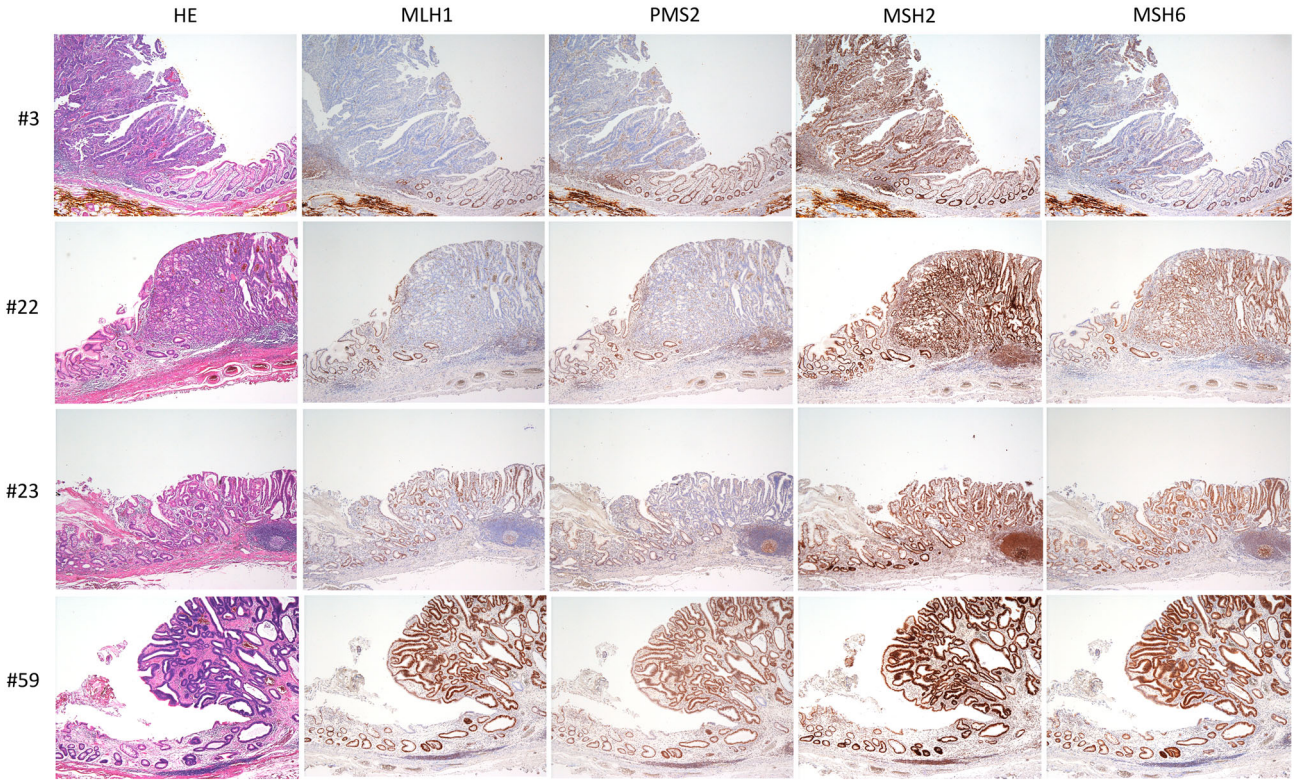

Figure 5. Histological features of MSI-H gastric neoplasms. The indicated numbers of the tumors correspond to the tumor numbers in Table 4. Negativity is defined when the tumor is stained more weakly than the surrounding non-neoplastic tissue. Negative immunostaining for MLH1 and PMS2 is observed in tumors #3 and #22. HE, hematoxylin and eosin.

Table 5. PD-L1 expression in MSI-H gastric tumors examined with two antibodies.

Tumor #	Histology WHO criteria	E1L3N		SP263	
		Tumor cells	Immune cells	Tumor cells	Immune cells
3	Well to moderately differentiated AC	1–10%	+	10%	+
7	Well to moderately differentiated AC	–	+	–	+
8	Low-grade dysplasia	–	–	1–10%	–
22	Well to moderately differentiated AC	–	–	–	+
23	Well to moderately differentiated AC	–	+	–	+
59	High-grade dysplasia	–	–	–	+
62	Well to moderately differentiated AC	–	–	–	–
63	Well to moderately differentiated AC	–	–	–	+
87	Poorly differentiated AC	–	–	–	+
105	High-grade dysplasia	–	+	–	–

+, Positive; –, negative; AC, adenocarcinoma.

Clinicopathological features of EBV-associated gastric carcinoma

Intense nuclear staining with EBER-ISH, defined as EBV positivity, was found in six cases. These were submucosally infiltrating poorly differentiated adenocarcinomas with prominent lymphocytic infiltration (see supplementary material, Table S2 and Figure S2). In the submucosa, a pattern of gastric carcinoma with lymphoid stroma (GCLS) was observed with SM2 invasion in half of the cases. Gastrectomy was subsequently performed in three cases, and no lymph node metastasis was found. In two cases, the histological diagnosis of the endoscopically biopsied specimens was challenging as most cancer cells were located more deeply than the biopsy forceps could reach. However, ESD successfully resected the entire cancerous lesion, including the deep submucosal

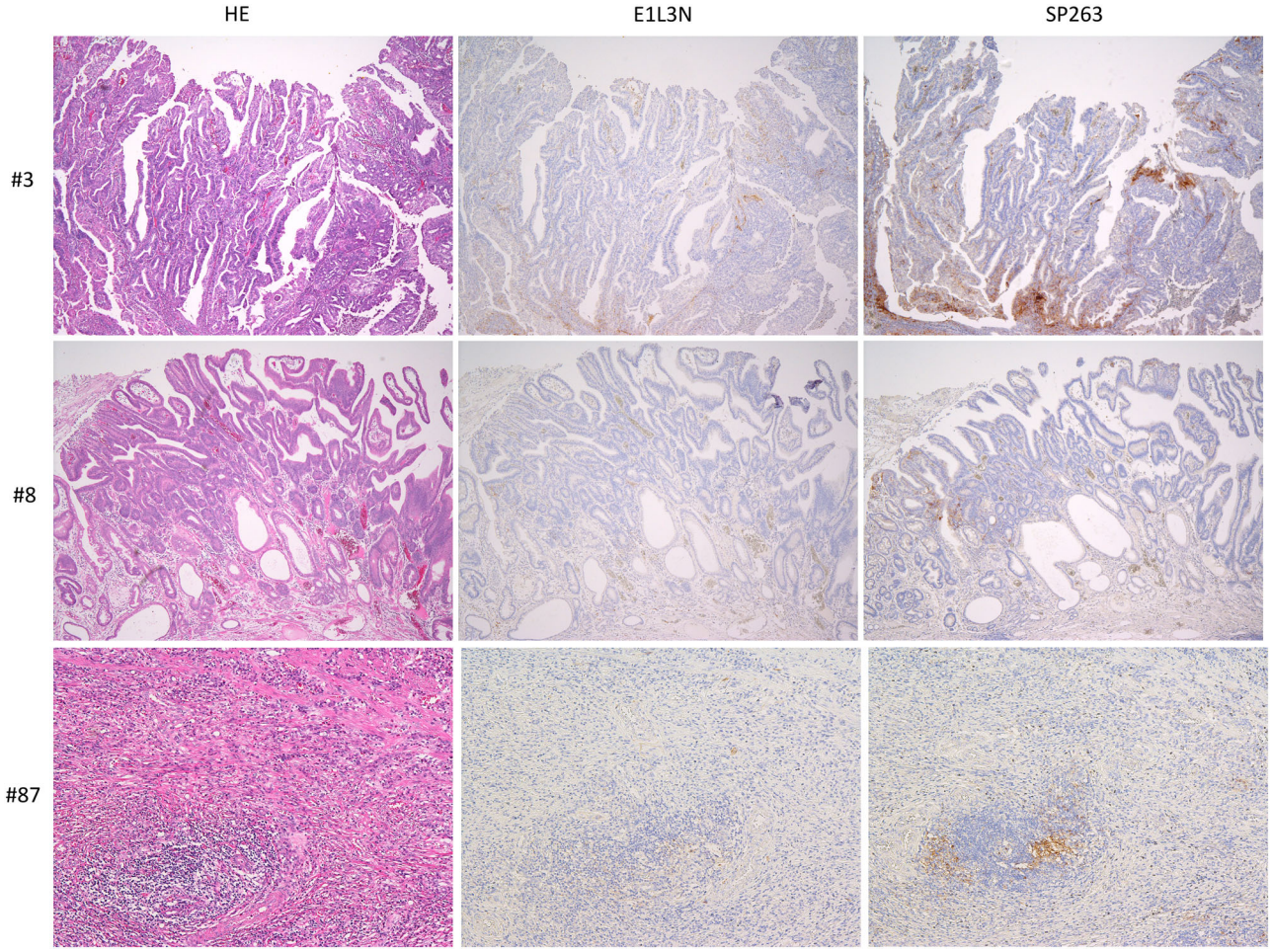

Figure 6. Immunohistochemical study of PD-L1 expression using two antibodies. An immunohistochemical analysis performed with two specific antibodies for PD-L1 (clones E1L3N and SP263) was used to determine the expression on tumor cells and immune cells. The case numbers correspond to the numbers in Table 5. The membrane of gastric tumor cells is positively stained with clones E1L3N and SP263 in case #3 (magnification: ×40). The number of stained tumor cells was higher with clone SP263 than with E1L3N. Negative staining is observed in case #8 with E1L3N, but more than 1% of the tumor cells are stained with SP263 (×40). Immune cells are positively stained with SP263, but not with E1L3N (×100). HE, hematoxylin and eosin.

GCLS component. *In situ* hybridization revealed extensive EBER positivity in the cancer cells, but not in the tumor-associated lymphocytes. In contrast, in some cases of EBER-negative gastric carcinoma, EBER positivity was found in scattered intramural lymphocytes, but not in neoplastic cells (tumor #27, see supplementary material, Figure S4). No cases with EBER positivity in both cancer cells and tumor-associated lymphocytes were present in our study.

Discussion

Specific gastric subtypes (i.e. EBV-positive and MSI-H) were assessed in early gastric neoplasms to determine the initial genetic events in gastric carcinogenesis. The incidence of gastric carcinoma is reported to be much higher in Japan than in Western countries, partly because of a higher prevalence of *H. pylori* infection. Another probable reason is that the Japanese gastric cancer criteria place much more emphasis on cytologic atypia for a cancer diagnosis, resulting in a certain percentage of cases of high-grade dysplasia/IEN diagnosed by the WHO criteria being classified as intramucosal adenocarcinoma. Given its frequent progression to invasive cancer and high likelihood of coexisting carcinoma, gastric high-grade dysplasia/IEN requires endoscopic treatment [22]. Such lesions may be considered an initial change of well-differentiated adenocarcinoma. The subjects of the present study were a series of ESD-treated

gastric carcinomas diagnosed by the Japanese criteria, all of which were reclassified according to the current WHO criteria. The reclassified groups of gastric adenocarcinoma and high-grade dysplasia/IEN were clinicopathologically compared. Although well-differentiated adenocarcinoma (tub1) according to the Japanese criteria corresponded to high-grade dysplasia/IEN by the WHO criteria, moderately (tub2) and poorly differentiated adenocarcinomas (por) according to the Japanese criteria were all classified as adenocarcinoma by the WHO criteria (Table 1). These histological discrepancies were previously described, while comparing the differences in the diagnostic criteria for gastric carcinoma [18], and our findings are in line with those of that previous report. As a particularly important finding in this study, there was no significant difference in the survival rate between high-grade dysplasia and carcinoma according to the WHO criteria. In addition, a high survival rate was observed among cases with gastric carcinoma according to the Japanese criteria. This trend is considered to be due to these neoplasms being curatively resected endoscopically or surgically.

In this analysis, the number (rate) of MSI-H gastric tumors was 10 (10.0%) among all gastric carcinomas diagnosed according to the Japanese criteria. While the number was 7 (13.0%) among gastric carcinomas diagnosed according to the WHO criteria: the latter proportion was close to that reported by TCGA (21.6%), in which advanced gastric carcinomas were included [1]. In high-grade dysplasia/IEN according to the WHO criteria (Table 2), 2 (5.1%) of 39 neoplasms were MSI-H tumors. A Korean group that examined the incidence of MSI-H tumors in early gastric neoplasms reported 3.2% in the dysplastic group, 10.3% in the early gastric carcinomas, and 19.7% in the advanced gastric carcinoma [23]. The rates of MSI-H tumors in early gastric neoplasia gradually increased from adenoma to carcinoma. Given these results, there may be a process pathway whereby MSI-H gastric carcinoma arises through the gastric adenoma–carcinoma sequence. MSI causes genetic instability and may induce histological cellular and structural abnormalities from the initiation of carcinogenesis [24]. The pathway may be associated with the functional loss of MMR (by loss of MMR protein), resulting in acquired genetic instability, during the progression from dysplasia to invasive cancer. Alternatively, MMR deficiency due to germline genetic mutations, as seen in Lynch syndrome, may be involved. Small percentages of colorectal cancers arising from apparently normal mucosa with a genetic mutational background are diagnosed as Lynch syndrome. Such families are recommended to undergo genetic counseling and germline testing to check for familial tumors in many organs. Gastric cancer is not included in the Amsterdam criteria, which are used to help identify Lynch syndrome families [25,26]. MMR gene promoters are frequently methylated in gastric cancer cells, and the loss of these functions eventually causes MSI-H [27]. This methylation pathway is thus considered likely to underlie MSI-H gastric carcinogenesis. The most recent hot topic of interest is that MSI-H tumors are a good target of immune checkpoint inhibitors [28,29]. MSI-H and a high tumor mutation burden are considered biomarkers for assessing the efficacy of immune checkpoint inhibitors [30]. One of the biomarkers for sensitivity to immune checkpoint inhibitors is PD-L1 expression by tumor and immune cells. Our immunohistochemical analysis using two PD-L1 antibodies indicated slight differences in the staining proportion between clones, with SP-263 being higher than E1L3N. Clones of PD-L1 antibody should therefore be carefully chosen [31].

We focused on MSI-H and EBV-associated carcinomas because these subtypes portend a better prognosis than the other subtypes. MSI-H correlates with increased neoantigens and may induce lymphocytic infiltration of the tumors. Such histological lymphocyte infiltration is frequently seen in EBV-associated carcinoma as well [32]. EBV-associated gastric carcinoma was recently reported to have a low rate of lymph node metastasis, making it associated with a better prognosis than other cancer types, especially in its early stages. The indication of endoscopic resection may be expanded to include EBV-associated submucosal cancers. EBV-associated submucosal gastric carcinoma is reported to have a low prevalence of lymph node metastasis, so these lesions can be considered candidates for endoscopic resection [14,15]. Submucosal invasive carcinomas with <500 μm of submucosal invasion are usually treated by endoscopic resection, and nine tumors were included in our SM1 group (Table 3). For SM2 invasion, 12 tumors showed ≥500 μm of submucosal invasion and thus were indicated for standard gastrectomy with lymph node dissection [19]. Our three cases of EBV-associated carcinoma had submucosal GCLS components that were characterized by prominent tumor-associated lymphocytic infiltration. Of these, subsequent gastrectomy was performed for two cases, and surgery was refused in the other case. A treatment strategy involving endoscopic resection for histological evaluation of submucosal EBV-associated gastric carcinoma could be considered. Previous cases of EBV-positive mucosal carcinoma were moderately differentiated adenocarcinomas forming a lace pattern [33]. In our analysis, EBV-associated carcinoma was detected in six (6.0%) of all carcinoma cases diagnosed according to the

Japanese criteria and in six (11.1%) among gastric carcinomas diagnosed according to the WHO criteria. The EBV-associated carcinoma rate reported by TCGA was 8.8% [1]. There have been no reported cases of gastric adenoma with EBV positivity. Therefore, given the lack of EBV-positive gastric adenoma in our cases and the reported cases, EBV-associated gastric carcinomas most likely arise *de novo* in a different manner from MSI-H gastric cancers mentioned above.

MSS carcinomas in this study were mostly well to moderately differentiated adenocarcinomas according to the WHO criteria, as the object of this study was ESD-treated gastric carcinoma cases. Most of the poorly differentiated adenocarcinomas are indicated for surgical treatment. The Japanese Gastric Cancer Association treatment guidelines recommend that intramucosal and small (<2 cm) gastric cancers, even those of poorly differentiated type, be treated endoscopically [19]. In the TCGA classification, MSS carcinomas were classified into two subgroups: genomically stable of diffuse-type and chromosomally unstable intestinal-type gastric cancer [1,18]. MSS carcinomas in this study can be classified as the chromosomally unstable type. The present comparison study among three groups (i.e. EBV, MSI, and MSS) was reasonable and adequate. The differences in macroscopic and histological features among the groups were statistically significant (p = 0.007 and <0.001, respectively). The characteristics of MSS carcinoma were depressed and well-differentiated carcinoma, although the genetic pathway of MSS tumors was not determined in this study. Whether MSS cancer cells arise through the gastric adenoma–carcinoma sequence or *de novo* should be investigated in a future study.

One limitation of this study is that it was conducted at a single institution with few pathologists. A previous study described differences in the histological diagnostic criteria between Japanese and Western criteria demonstrated by several pathologists from multiple countries [16]. Two concerns were raised in this Japanese single institutional study. First, the sample size of this study was relatively small. A multicenter study should therefore be conducted to increase the number of cases for evaluation. Second, the histological diagnostic criteria are still unclear, and the differences in the evaluation criteria between Asian and Western countries should be resolved. The simultaneous application of the WHO and Japanese classification systems is associated with some limitations. Indeed, interobserver variation in the diagnosis of dysplasia and carcinoma was found between two pathologists from Japan and Korea [34]. Artificial intelligence with deep learning is also expected to improve the diagnostic accuracy for cancer research. The automated histological classification of gastric biopsy specimens has been reported, and a comparison between human pathologist and the e-pathologist showed a 55.6% concordance rate, but the kappa coefficient remained low at 0.28 [35]. In particular, a strict diagnosis to define malignancy is expected in the analysis of early carcinogenesis. Our understanding of genetic alterations in early-stage tumor development is expected to improve from analyses such as those conducted in the present study.

In summary, we reclassified gastric cancer cases according to the Japanese and WHO criteria, and revealed the clinicopathological characteristics of the subgroups of gastric carcinoma. The WHO criteria enabled the classification of early gastric cancers diagnosed using the Japanese criteria into more detailed groups, dysplasia and early carcinoma, and their clinicopathological characteristics were compared. The frequency of MSI-H tumors gradually increased from adenoma to carcinoma in the gastric adenoma–carcinoma sequence. EBV-associated tumors were found to be poorly differentiated and submucosally invasive carcinomas. Our findings highlight the fact that EBV-associated subtypes may arise via a *de novo* mutational pathway.

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Author contributions statement

HTan planned the research and wrote the manuscript. YMi carried out data analysis and management. HTak performed pathology review and reviewed the manuscript. NT and YO performed MSI analysis. YK, YMu, TK, TS and KT performed endoscopic treatment and sample recruitment. KA, NU and SK analyzed data and performed statistical analysis. SY and MT performed histological analysis and immunohistochemical analysis. KH and YS performed surgical treatment and sample recruitment. MF and TO supervised the research and reviewed the manuscript. All authors approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL ONLINE

Figure S1. Overall survival in cases (n = 100) with gastric carcinoma according to the Japanese criteria

Figure S2. Histological findings of EBV-associated gastric carcinoma

Figure S3. Endoscopic findings of MSI-H gastric tumors

Figure S4. Pathological findings of EBER-ISH

Table S1. Clinicopathological characteristics of gastric carcinoma according to the Japanese criteria

Table S2. Pathological findings of Epstein–Barr-positive gastric carcinoma