doi: 10.4149/gpb_2023021

Correlation of microsatellite status and EBV infection with clinical characteristics of patients with gastric cancer

Pengfei Lu[1,2], Kadeliya Yushan[3], Gulimilai Yusufu[3], Hui Wang[2] and Rui Mao[1]

[1] *Cancer Center, The First Affiliated Hospital of Xinjiang Medical University, Xinjiang, China*
[2] *State Key Laboratory of Pathogenesis, Prevention and Treatment of High Incidence Diseases in Central Asia, Clinical Medicine Institute, The First Affiliated Hospital of Xinjiang Medical University, Xinjiang, China*
[3] *Graduate School, Xinjiang Medical University, Xinjiang, China*

Abstract. This study was designed to investigate the correlation of microsatellite status (MS) and Epstein-Barr virus (EBV) infection with the clinical characteristics of gastric cancer (GC) patients. MS was detected by immunohistochemistry. EBV was detected by *in situ* hybridization. There were 31.3% cases showed mismatch repair-deficient (dMMR)/ microsatellite instability (MSI) and 68.7% cases showed mismatch repair-proficient (pMMR)/ microsatellite stability (MSS). The dMMR/MSI was more common in the elderly, in patients with cardia GC, smaller tumor diameter or non-poorly differentiated carcinoma. The survival in dMMR/MSI patients tended to be longer than that in pMMR/MSS patients. Total 7.6% cases showed EBV-positive (EBV(+)) among 198 GC patients. EBV(+) was more common in patients with advanced GC or poorly differentiated adenocarcinoma. MSI was more common in EBV-negative (EBV(−)) patients than in EBV(+) patients. The dMMR/MSI patients with stage II GC benefited from chemotherapy. The survival of EBV(+) patients tended to be longer than that of EBV(−) patients.

Key words: Gastric cancer — Microsatellite status — EBV — Clinicopathological parameters — Survival prognosis

Abbreviations. dMMR, mismatch repair-deficient; EBV, Epstein-Barr virus; EBVaGC, EBV-associated gastric cancer; GC, gastric cancer; ICIs, immune checkpoint inhibitors; MLH1, mutL homolog 1; MS, microsatellite status; MSI, microsatellite instability; MSS, microsatellite stability; MSH2, mutS homolog 2; MSH6, mutS homolog 6; pMMR, mismatch repair-proficient; PMS2, postmeiotic segregation increased 2; TMB, tumor mutational burden.

Introduction

Gastric cancer (GC), one of the most common malignant tumors in the world, causes great threat to global public health. The significant increase in GC patients is attributed to various reasons such as genetic factors, dietary habits, living environment, and gene mutations. According to the latest data from the International Agency for Research on Cancer (IARC) in

Electronic supplementary material. The online version of this article (doi: 10.4149/gpb_2023021) contains Supplementary material.

Correspondence to: Rui Mao, Cancer Center, The First Affiliated Hospital of Xinjiang Medical University, 137 Liyushan Road, Urumqi 830054, Xinjiang, China
E-mail: maorui44@sohu.com
Hui Wang, State Key Laboratory of Pathogenesis, Prevention and Treatment of High Incidence Diseases in Central Asia, Clinical Medicine Institute, The First Affiliated Hospital of Xinjiang Medical University, Xinjiang, China
E-mail: wangh0923@126.com

2020, it is the fourth leading cause of cancer-related death in humans and has the fifth highest morbidity among the most common cancers (Sung et al. 2021). In 2014, based on array-based somatic copy number analysis, whole-exome sequencing, array-based DNA methylation profiling, messenger RNA sequencing, microRNA (miRNA) sequencing and reverse-phase protein array (RPPA), researchers have classified GC into four subtypes, including tumors positive for Epstein-Barr virus (EBV), microsatellite instability (MSI) tumors, genomically stable (GS) tumors and tumors with chromosomal instability (CIN) (Petrelli et al. 2014). The identification of these subtypes contributes to targeted immunotherapy and long-term prognosis of GC patients.

To date, various methods, including radical surgical resection, systemic intravenous chemotherapy or oral drug chemotherapy, radiotherapy, immunotherapy, targeted therapy, traditional Chinese medicine etc., have been widely used for the treatment of GC, but it is still a challenge to achieve a high survival rate. The 5-year overall survival of patients with advanced GC received traditional treatments was only 5–20% (Kahraman and Yalcin 2021). Although immunotherapy has benefited a large number of cancer patients, immune checkpoint inhibitors (ICIs) will cause drug resistance and adverse reactions in patients (Flynn and Larkin 2017). Therefore, it is particularly urgent to find accurate biomarkers to identify the candidates for ICIs.

The currently known biomarkers associated with ICIs include high-level microsatellite instability (MSI-H), programmed death ligand 1 (PD-L1), tumor mutational burden (TMB) and EBV-positive (EBV(+)), which contributed to the screening of candidates for the different treatment options (Yoon et al. 2020). Among them, MSI and EBV can especially help clinicians formulate and optimize complete treatment plans for relevant patients (Kawazoe et al. 2017). Several studies have shown that there were more molecules for the immune activation and immunosuppression in the surrounding microenvironment of MSI-type tumors, which suggested that the diagnosis of microsatellite status (MS) may help predicting the efficacy of ICIs (Pino and Chung 2011). In addition, MSI-type tumors were often accompanied by TMB that happens to be one of the biomarkers sensitive to immunotherapy (Gjoerup et al. 2020). EBV-associated gastric cancer (EBVaGC) is a common malignancy with unique clinicopathological and molecular features. However, a controversy remains about the association of EBVaGC with better prognosis (Yang et al. 2020). Additionally, the information regarding the association of MSI and EBV with other clinicopathological parameters of patients has been limited.

In this study, we investigated the relationship between MS and EBV infection to the pathological characteristics and survival prognosis of patients with stage IB, stage II, and stage III GC who received radical gastrectomy. In addition, we also integrated data from MSI patients and EBV(+) patients for analysis. This study is expected to identify immunotherapy-sensitive populations and provide a reference for the precise treatment of GC.

Materials and Methods

Patients and tumor tissues derived from clinical trial

The GC patients who underwent D2 radical gastrectomy (a radical treatment of GC where the stomach was removed along with the first and second tier nodal stations) in the First Affiliated Hospital of Xinjiang Medical University from January 2012 to December 2016 were collected retrospectively. Patients with detailed postoperative clinicopathological, follow-up data and postoperative pathological type adenocarcinoma (including signet ring cell carcinoma) were candidates for inclusion. The exclusion criteria were as follows: patients who had undergone adjuvant radiotherapy or adjuvant chemotherapy before surgery; patients with cancer cells that have metastasized to the liver, lung or other distant sites found in preoperative examinations; patients with primary malignant tumors in other sites at the same time; and patients with liver disease, kidney disease or other diseases that prevent treatment and follow-up. Finally, 232 cases with stage I, II, and III GC who met the requirements were screened, of which 21 were lost to follow-up. Therefore, a total of 211 patients (male, n = 169; female, n = 42; mean age, 62.5 years) who accomplished the MSI test were included in this study, which were classified into stage I (n = 54, including 18 cases with stage IA GC and 36 cases with stage IB GC), stage II (n = 53) and stage III (n = 104). Among them, 198 cases (male, n = 158; female, n = 40; mean age, 61.4 years) completed the EBV test, which were classified into stage I (n = 51), stage II (n = 51) and stage III (n = 96). The postoperative pathology of the above patients was staged based on the GC staging system of the American Joint Committee on Cancer (AJCC). A total of 98 patients received not less than 3 cycles of 5-fluorouracil-based chemotherapy after radical gastrectomy. The tumor tissue sections were prepared in accordance with a previous report with some modification (Kuboki et al. 2016). Briefly, three representative tumor cores (2 mm in diameter) were selected from the same formalin-fixed, paraffin-embedded tissue block in each case. The 4-μm-thick sections were prepared for immunohistochemical staining and *in situ* hybridization (ISH). Baseline characteristics were reviewed from medical records.

The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Immunohistochemical staining and evaluation

The expressions of four mismatch repair (MMR) proteins, including mutL homolog 1 (MLH1), mutS homolog 2

(MSH2), mutS homolog 6 (MSH6) and postmeiotic segregation increased 2 (PMS2), were detected by immunohistochemistry. The primary antibodies used for immunohistochemistry were anti-MLH1 rabbit monoclonal antibody (ab92312; experimental concentration, 1:100), anti-MSH2 rabbit monoclonal antibody (ab227941; experimental concentration, 1:8000), anti-MSH6 rabbit monoclonal antibody (ab92471; 1:400), and anti-PMS2 rabbit monoclonal antibody (ab110638; 1:100), which were purchased from Abcam (Cambridge, MA, USA). The secondary antibody was goat anti-rabbit IgG (HRP polymer), which was included in the universal two-step assay kit (PV-6001) from Zhongshan Golden Bridge Biotechnology (Beijing, China). The whole process of immunohistochemical staining was performed cautiously according to the manufacturer's instructions.

MLH1, MSH2, MSH6 and PMS2 were all located in the nucleus, and a clear yellow or brown could be observed. The tumor tissues with lacked expression of one or more MMR proteins were considered to be mismatch repair-deficient (dMMR), whereas those held expressions of all MMR proteins were considered to be mismatch repair-proficient (pMMR). The sections with known positive expression or from normal gastric mucosal and interstitial lymphocytes of the same tissue were used as positive controls. The antibody diluent instead of the primary antibody was used as a negative control. The results were interpreted with a double-blind manner by two senior pathologists in the pathology department of our hospital. Firstly, specimens were scored on the basis of staining intensities of nucleus. 0, no yellow or brown; 1, light yellow; 2, yellow; and 3, brown. Secondly, three high-power microscope fields were selected randomly. Specimens were scored based on the percentage of positive (stained) nuclei in each field. 0, less than 5%; 1, 5–25%; 2, 26–50%; 3, 51–75%; and 4, 76% or more. For each tumor tissue, three tissue sections were observed, and the final score was expressed as an average. Finally, the result was judged according to the product of the above two scores. A score of 0–5 was considered to be dMMR, while that of 5–12 was considered to be pMMR.

ISH and evaluation

The EBV infection was detected by ISH method. In short, the deparaffinizing, pretreatment, and protease digestion procedures followed the instructions of EBV-encoded RNA (EBER) detection kit (ISH-7001) purchased from Zhongshan Golden Bridge Biotechnology (Beijing, China). Next, EBER probe (digoxigenin-labeled) or blank control reagent was added to the mixture and hybridized at 37°C for 2–4 h or overnight, then the coverslip was removed and rinsed with PBS buffer. Then the HRP-labeled anti-digoxigenin antibody from Zhongshan Golden Bridge Biotechnology (Beijing, China) was added and the mixture was incubated at 37°C for 5–20 min, and which rinsed with PBS buffer and deionized water. Afterwards the sections were incubated with 3,3'-diaminobenzidine (DAB) for 10 min, and counterstained with hematoxylin, dehydrated in alcohol, cleared with xylene and mounted. The results were interpreted by qualified pathologists in our hospital under an optical microscope. Positive staining was localized to the nucleus. The cases could be judged to be EBV(+) if there were brown granules in tumor cell nuclei, while those maintained completely unstained in cell nuclei or were stained in cell membranes, interstitial tissue, cytoplasm and fibrous tissue were considered EBV-negative (EBV(−)).

Follow up

All the patients were followed up after surgery until August 2019. During the follow up, we recorded the recurrence, metastasis, or death of each patient.

Statistical analysis

Statistical analyses were performed with IBM SPSS Statistics version 23.0 (IBM, Armonk, NY, USA). The non-normally distributed measurement data were analyzed by Z test, and the enumeration data were analyzed by chi-square test or Fisher's exact test as appropriate. Overall survival (OS) curves were described by the Kaplan-Meier method, OS rates were analyzed by the Log-rank method. Differences between the groups according to MMR status, clinicopathological characteristics and EBV status were identified by univariate and multivariate analyzes by Cox proportional hazards model. Differences were considered statistically significant when $p < 0.05$.

Results

Expression of four MMR proteins

All four MMR proteins were expressed in normal gastric mucosa and interstitial lymphocytes, whereas they were expressed defectively in GC tissues. Among the 211 GC tissues, 145 cases (68.7%) were categorized into pMMR group, and 66 cases (31.3%) were categorized in the dMMR group (Table S1 in Supplementary materials). The defective expressions of four proteins were shown in Figure 1, and the expression status of each MMR protein in GC specimens was summarized in Table S1. Obviously, the defect rates of MLH1, MSH2, MSH6 and PMS2 were 26.5% ($n = 56$), 7.6% ($n = 16$), 9.0% ($n = 19$) and 22.7% ($n = 48$), respectively. In the dMMR group, 16 (24.2%) cases were deficient in only 1 protein, 33 (50.0%) cases were deficient in 2 proteins, 11 (16.7%) cases were deficient in 3 proteins, and only 6 (9.1%) cases were deficient in 4 proteins. It should be noted that the

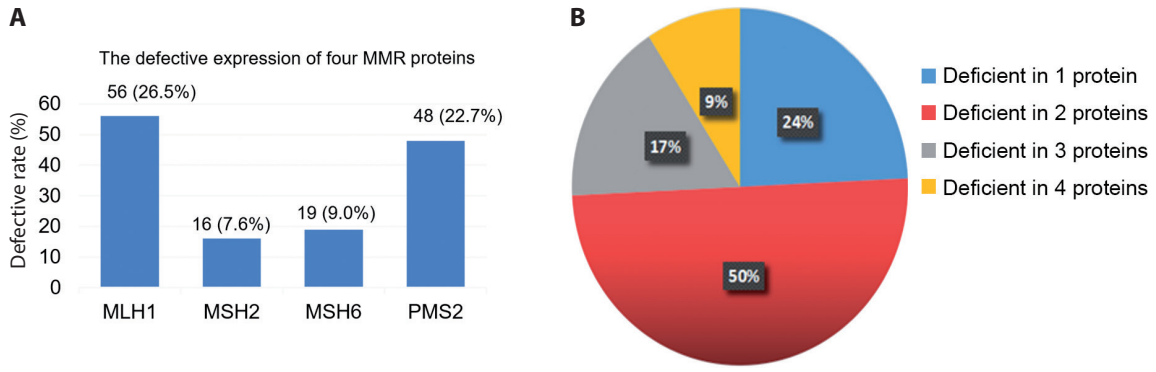

Figure 1. The defective expression of four MMR proteins in dMMR/MSI GC specimens. **A.** The defect rates of MLH1, MSH2, MSH6 and PMS2 were 26.5% (n = 56), 7.6% (n = 16), 9.0% (n = 19) and 22.7% (n = 48), respectively. **B.** A total of 16 (24.2%) cases were deficient in only 1 protein, 33 (50.0%) cases were deficient in 2 proteins, 11 (16.7%) cases were deficient in 3 proteins, and only 6 (9.1%) cases were deficient in 4 proteins.

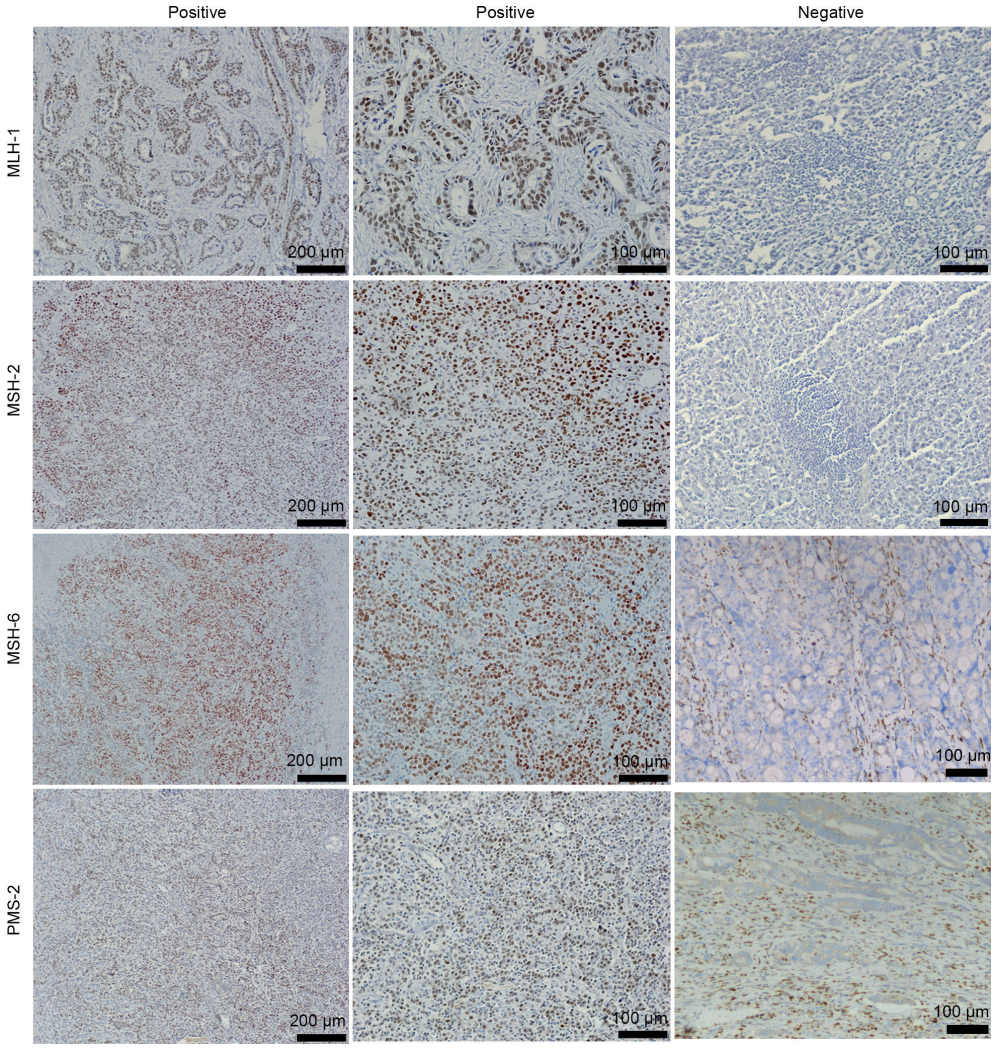

Figure 2. The representative immunohistochemical images of the positive and negative expression of MLH-1, MSH-2, MSH-6 and PMS-2 proteins in GC specimens.

deficiency of PMS2 was often accompanied by the deficiency of MLH1. A total of 43 cases exhibited the deficiency of PMS2 and MLH1, which accounting for a substantial part (65.2%) of all dMMR cases. In addition, MLH1, MSH2, MSH6 and PMS2 expressions were more obvious in the basal part of the alimentary canal glands (Fig. 2).

Onset age

The onset age comparison results of patients with dMMR/MSI and pMMR/MSS were shown in Table S2. The median age of the 211 patients was 63 years (23–86 years). The Z test was used for statistical comparison, and the results showed that the difference of onset age between the two groups was statistically significant. The onset age of GC patients with MSI showed a trend of older than those exhibited MSS.

Correlation of MS with the baseline characteristics of patients

The correlations of MS with other baseline characteristics were analyzed and the results were summarized in Table 1. The dMMR/MSI was more frequently observed in patients with cardia gastric cancer, in patients with smaller tumor diameter and in those with non-poorly differentiated carcinoma ($p < 0.05$). No statistical significance was

Table 1. Correlation of MS with baseline characteristics

Baseline characteristics	dMMR/MSI *n* (%)	pMMR/MSS *n* (%)	Chi-square test	*p* value
Sex				
Male	49 (74.2)	120 (82.8)	2.063	0.841
Female	17 (25.8)	25 (17.2)		
Nationality				
Han nationality	50 (75.7)	102 (70.3)		
Uighur	5 (7.5)	23 (15.9)	2.786	0.453
Others	11 (16.8)	20 (13.8)		
Depth of tumor invasion				
T1 and T2	22 (33.3)	45 (31.0)	0.111	0.119
T3 and T4	44 (66.7)	100 (69.0)		
Lymph node metastasis (N)				
N0	27 (40.9)	58 (40.0)	0.016	0.509
N+	39 (59.1)	87 (60.0)		
Total tumor stage				
Stage I	17 (25.8)	37 (25.5)		
Stage II	20 (30.3)	33 (22.8)	1.586	0.287
Stage III	29 (43.9)	75 (51.7)		
Differentiation degree of tumor				
Poor differentiation	32 (48.5)	93 (64.1)	4.602	0.023
Non-poor differentiation	34 (51.5)	52 (35.9)		
Tumor site				
Cardia	25 (37.9)	25 (17.2)	10.683	0.001
Others	41 (62.1)	120 (82.8)		
Tumor diameter				
≥ 5 cm	25 (37.9)	57 (39.3)	6.113	0.010
< 5 cm	41 (62.1)	88 (60.7)		
Vascular invasion				
Yes	24 (36.4)	52 (35.9)	0.005	0.531
No	42 (63.6)	93 (64.1)		
Nerve invasion				
Yes	29 (43.9)	58 (40.0)	0.290	0.348
No	37 (56.1)	87 (60.0)		

dMMR/MSI: total number of patients 66; pMMR/MSS: total number of patients 145; *n*, the number of patients in each item.

noted for the relationship between MS and other baseline characteristics.

Correlation of MS and baseline characteristics with survival prognosis

The longest follow up period in this study was 91 months. The patients with 1-year, 3-year and 5-year survival time were 79.1%, 61.9%, and 49.1%, respectively (Table 2). The Cox proportional hazards model was used to obtain independent prognostic factors for survival. The results showed that there was no correlation between MS and survival time of GC patients. However, the patient's ethnicity, tumor diameter, degree of tumor differentiation, depth of tumor invasion, lymph node metastasis, total tumor stage, vascular invasion and nerve invasion had statistically significant effects on the survival prognosis of patients ($p < 0.05$). The results of multivariate analyses

Table 2. Correlation of MS and baseline characteristics with survival prognosis

Baseline characteristics	*n* (%)	1-year survival (%)	3-year survival (%)	5-year survival (%)	*p* value
Age (years)					
≥70	62 (29.4)	74.1	59.1	40.1	0.062
<70	149 (70.6)	82.1	61.1	49.3	
Sex					
Male	169 (80.1)	79.3	63.4	48.6	0.841
Female	42 (19.9)	78.6	54.5	48.1	
Nationality					
Han nationality	152 (72.0)	80.3	65.4	51.5	
Uighur	28 (13.2)	67.5	33.8	20.3	0.007
Others	31 (14.8)	83.7	65.9	54.9	
Tumor site					
Cardia	50 (23.7)	73.9	54.2	41.3	0.180
Others	161 (76.3)	80.7	63.3	49.7	
Tumor diameter					
≥5 cm	95 (45.0)	73.7	48.5	35.6	0.001
<5 cm	116 (55.0)	83.6	72.9	56.9	
Differentiation degree of tumor					
Poor differentiation	125 (59.2)	75.9	55.8	39.1	0.003
Non-poor differentiation	86 (40.8)	83.7	69.9	60.4	
Depth of tumor invasion					
T1 and T2	67 (31.8)	88.1	82.3	76.2	<0.001
T3 and T4	144 (68.2)	74.9	51.5	34.1	
Lymph node metastasis (N)					
N0	85 (40.3)	88.9	83.5	78.6	<0.001
N+	126 (59.7)	72.1	46.0	28.7	
Total tumor stage					
Stage I	54 (25.6)	92.6	86.8	81.1	
Stage II	53 (25.1)	79.2	69.6	58.5	<0.001
Stage III	104 (49.3)	72.0	44.1	25.1	
Vascular invasion					
Yes	76 (36.0)	75.8	45.7	33.2	0.001
No	135 (64.0)	80.7	70.4	57.8	
Nerve invasion					
Yes	87 (41.2)	74.6	46.2	38.7	0.001
No	124 (58.8)	82.3	72.4	55.4	
Microsatellite status					
dMMR	66 (31.3)	81.8	63.4	51.1	0.458
pMMR	145 (68.7)	77.9	61.4	46.3	

Table 3. Analyses of independent prognostic factors for survival of patients received radical gastrectomy based on Cox proportional hazard model

Factor	B	Standard error	Wald	*p* value	HR	95% CI of HR	
						lower	upper
Differentiation degree of tumor	−0.493	0.220	5.043	0.025	0.611	0.844	2.342
Tumor diameter	−0.430	0.198	4.698	0.030	0.650	0.441	0.960
Depth of tumor invasion	1.241	0.260	22.782	<0.001	3.460	2.078	5.761
Lymph node metastasis (N)	0.849	0.362	5.499	0.019	2.337	1.150	4.753
Total tumor stage	1.452	0.322	20.272	<0.001	4.272	2.270	8.037
Vascular invasion	−0.471	0.210	5.026	0.025	0.624	0.413	0.942
Nerve invasion	−0.448	0.210	4.506	0.034	0.639	0.422	0.966

HR, hazard ratio; CI, confidence interval.

(Table 3) showed that the degree of tumor differentiation, tumor diameter, T stage, lymph node metastasis, total tumor stage, vascular invasion and nerve invasion were independent prognostic factors for survival of patients received radical gastrectomy ($p < 0.05$).

Correlation between MS and postoperative chemotherapy efficacy in patients with stage IB, II and III GC

There were 36 patients with stage pT2N0M0 IB, among which 15 patients received postoperative adjuvant chemotherapy. The median survival time could not be obtained in this study because of the number of deaths did not reach half of the total number of patients with early GC. As shown in Figure 3A, the 5-year survival rates of the patients in chemotherapy group and non-chemotherapy group were both higher than 70%, which were no statistical significance. Besides, the patients with stage IB GC were divided into dMMR/MSI group and pMMR/MSS group according to their MS. The results suggested that there was no statistically significant prolongation of survival time in chemotherapy group compared with non-chemotherapy group, whether in dMMR/MSI group or in pMMR/MSS group.

We found that there were 53 patients with stage II GC, of which 26 cases received chemotherapy. The dMMR/MSI patients accounted for 37.7% (20/53). It can be seen from the Kaplan-Meier plots (Fig. 3B) that the survival time of patients received chemotherapy had a tendency

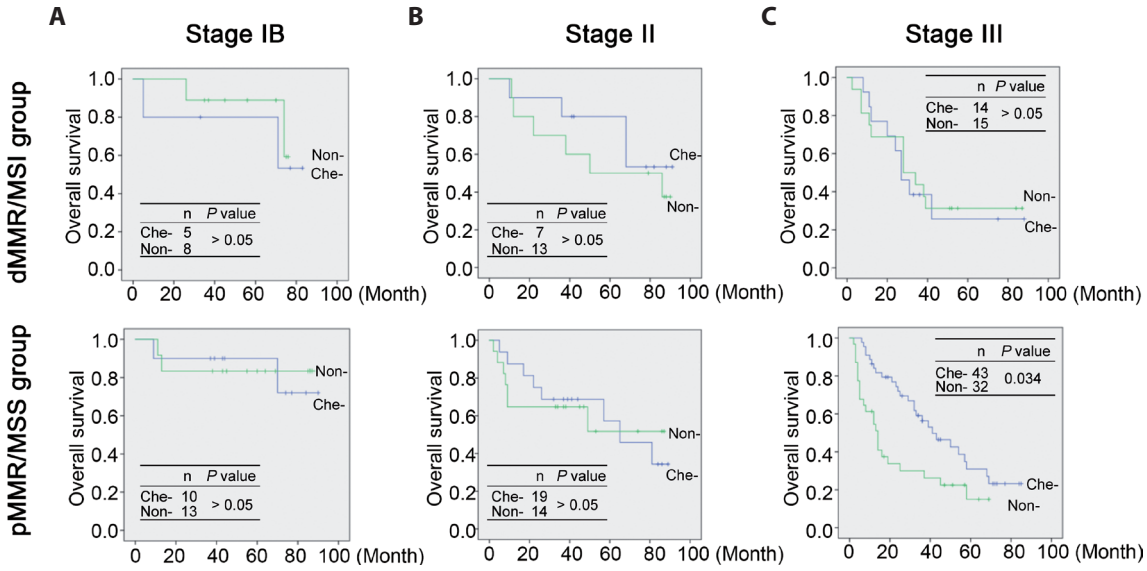

Figure 3. Kaplan-Meier plots of overall survival (OS) in stage IB (**A**), stage II (**B**) and stage III (**C**) GC patients who received chemotherapy (Che-) *versus* those didn't receive chemotherapy (Non-). In pMMR/MSS group, OS of the patients in stage III GC who received chemotherapy (Che-) was prolonged than those didn't receive chemotherapy (Non-) ($p = 0.034$). No statistical differences were found in other comparisons.

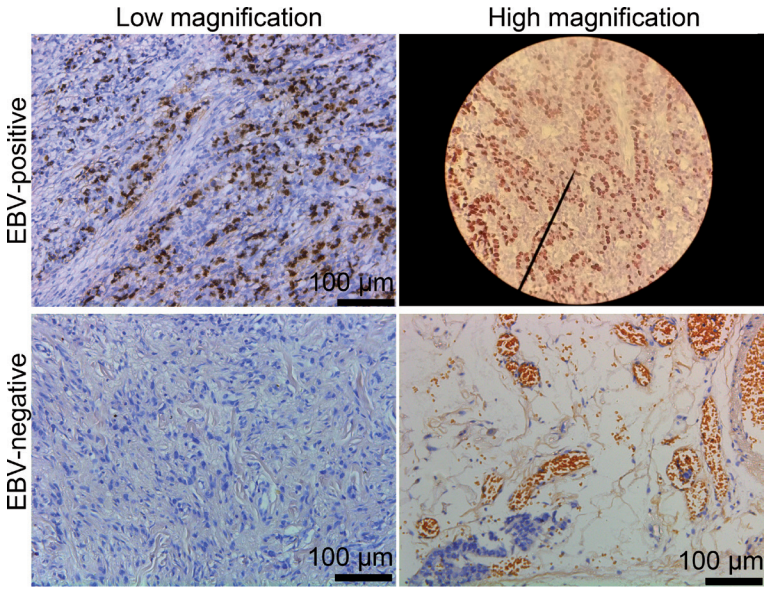

Figure 4. The representative *in situ* hybridization (ISH) images at low and high magnifications of EBV-positive and EBV-negative GC specimens.

to prolong, whether in dMMR/MSI group or in pMMR group. However, the difference was not statistically significant.

There were 104 patients with stage III GC in this study, which included 57 patients who received chemotherapy. The patients with dMMR/MSI accounted for 27.9% (29/104). The Kaplan-Meier plots (Fig. 3C) showed that the survival time of the patients received chemotherapy was prolonged than those received no chemotherapy in pMMR/MSS group (p = 0.034). However, this statistical significance was not found in dMMR group. The above results indicated that the patients with stage III GC in pMMR/MSS group had a more significant benefit than those in dMMR/MSI group.

Expression of EBV

The expression of EBV in GC tissues was shown in Figure 4. There were 15 cases with EBV(+) and 183 cases with EBV(−), and the positive rate was 7.6% (Table S3).

Correlation between EBV infection and baseline characteristics

The correlation between EBV infection and baseline characteristics in GC patients was analyzed based on chi-square test or Fisher's exact test using SPSS 23.0 software. The results were shown in Table 4. We found that EBV infection was significantly correlated with tumor differentiation (p = 0.013) and total stage (p = 0.004). However, there was no significant correlation between EBV infection and sex, age, nationality, tumor site, tumor diameter, vascular invasion, nerve invasion, depth of tumor invasion, and lymph node metastasis.

Among all patients, 63.6% (126/198) cases had poorly differentiated carcinoma and 36.4% (72/198) cases had non-poorly differentiated carcinoma. Among the patients with EBV(+), 93.3% (14/15) cases had poorly differentiated carcinoma and only 6.7% (1/15) cases had non-poorly differentiated carcinoma. Among the patients with EBV(−), 61.2% (112/183) cases had poorly differentiated carcinoma and 38.8% (71/183) cases had non-poorly differentiated carcinoma. The results suggested that poorly differentiated carcinoma was more common in patients with EBV(+) than in those with EBV(−) (p = 0.013).

Correlation between EBV infection and MS

The MS of all 211 patients was analyzed. Due to the unsuitability of some specimens, only 198 patients were tested for both MS and EBV infection. There were 30.3% (60/198) cases with MSI and 69.7% (138/198) cases with MSS. The patients from above two groups were divided into EBV-positive group and EBV-negative group. The results showed that there were 13.3% (n = 2) patients with MSI and 86.7% (n = 13) patients with MSS in EBV-positive group. In EBV-negative group, there were 31.7% (n = 58) patients with MSI and 68.3% (n = 125) patients with MSS. We analyzed the above results statistically (Table S4) and found that the differences were not statistically significant (p = 0.24).

Correlation of EBV infection status and baseline characteristics with survival prognosis

The longest follow up time of 198 patients was 91 months. The 1-year, 3-year, and 5-year survival rates of all patients

were 83.8%, 56.5%, and 26.7%, respectively. The survival curves of 198 GC patients were drawn by the Kaplan-Meier method. The survival rate was analyzed based on the Log-rank method. The multivariate analysis was performed based on the Cox proportional hazards model to obtain the independent prognostic factors for survival (Table 5). The results indicated that the overall survival of GC patients after radical gastrectomy was statistically related to nerve invasion and total tumor stage. No statistical significance was noted between the overall survival and sex, age, nationality, tumor site, differentiation degree of tumor, vascular invasion, depth of tumor invasion, lymph node metastasis, MS and EBV infection. The results of multivariate analysis (Table S5) showed that nerve invasion and total tumor stage were independent prognostic factors for survival of patients after radical gastrectomy ($p < 0.05$). In addition, we

Table 4. Correlation between EBV infection and baseline characteristics

Baseline characteristics	*n* (%)	EBV(+) *n* (%)	EBV(–) *n* (%)	Chi-square test	Fisher's exact test	*p* value
Sex						
Male	158 (79.8)	14 (93.3)	144 (78.7)		1.355	0.313
Female	40 (20.2)	1 (6.7)	39 (21.3)			
Age, years						
≥65	85 (42.9)	9 (60.0)	76 (41.5)	1.930		0.165
<65	113 (57.0)	6 (40.0)	107 (58.5)			
Nationality						
Han nationality	142 (71.7)	9 (60.0)	133 (72.7)		−1.045	0.37
Others	56 (28.3)	6 (40.0)	50 (27.3)			
Tumor site						
Cardia	51 (25.8)	2 (13.3)	49 (26.8)			
Stomach	104 (52.5)	12 (80.0)	92 (50.3)		4.360	0.111
Pylorus	43 (21.7)	1 (6.7)	42 (22.9)			
Tumor diameter						
≥5 cm	88 (44.4)	4 (26.7)	84 (45.9)	2.077		0.149
<5 cm	110 (55.6)	11 (73.3)	99 (54.1)			
Differentiation degree of tumor						
Poor differentiation	126 (63.6)	14 (93.3)	112 (61.2)	6.185		0.013
Non-poor differentiation	72 (36.4)	1 (6.7)	71 (38.8)			
Vascular invasion						
Yes	73 (36.9)	6 (40.0)	67 (36.6)	0.068		0.794
No	125 (63.1)	9 (60.0)	116 (63.4)			
Nerve invasion						
Yes	82 (41.4)	7 (46.7)	75 (40.9)	0.185		0.667
No	116 (58.6)	8 (53.3)	108 (59.1)			
Total tumor stage						
Stage I	51 (25.8)	7 (46.7)	44 (24.0)		7.909	0.018
Stage II	51 (25.8)	0	51 (27.9)			
Stage III	96 (48.4)	8 (53.3)	88 (48.1)			
Depth of tumor invasion						
T1	23 (11.6)	1 (6.7)	22 (12.0)			
T2	42 (21.2)	6 (40)	36 (19.7)		3.085	0.372
T3	44 (22.2)	2 (13.3)	42 (22.9)			
T4	89 (45.0)	6 (40.0)	83 (45.4)			
Lymph node metastasis (N)						
N0	84 (42.4)	8 (53.3)	76 (41.5)			
N1	33 (16.7)	2 (13.3)	31 (17.0)		1.059	0.845
N2	30 (15.1)	1 (6.7)	29 (15.8)			
N3	51 (25.8)	4 (26.7)	47 (25.7)			

divided 198 patients into EBV(+) group and EBV(−) group. The Kaplan-Meier plots (Fig. 5A) showed that the patients with EBV(+) tended to have longer overall survival than those with EBV(−), but the prolongation was not statistically significant.

Table 5. The univariate analysis of prognosis in 198 GC patients

Baseline characteristics	*n*	Chi-square test	*p* value
Sex			
Male	158	1.637	0.201
Female	40		
Age, years			
≥65	85	0.002	0.965
<65	113		
Nationality			
Han nationality	142	0.253	0.615
Others	56		
Tumor site			
Cardia	51		
Stomach	104	5.785	0.055
Pylorus	43		
Differentiation degree of tumor			
Poor differentiation	126	< 0.001	0.987
Non-poor differentiation	72		
Vascular invasion			
Yes	73	0.746	0.388
No	125		
Nerve invasion			
Yes	82	6.009	0.014
No	116		
Total tumor stage			
Stage I	51		
Stage II	51	12.352	0.006
Stage III	96		
Depth of tumor invasion			
T1	23		
T2	42	5.388	0.145
T3	44		
T4	89		
Lymph node metastasis (N)			
N0	84		
N1	33	3.195	0.363
N2	30		
N3	51		
Microsatellite status			
MSI	60	2.690	0.101
MSS	138		
EBV infection			
EBV-positive	15	0.108	0.743
EBV-negative	183		

Correlation between EBV infection status and survival prognosis in patients with stage I and stage III GC after gastrectomy

There were 51 patients with stage I GC (EBV(+), n = 7; EBV(−), n = 44) and 90 patients with stage III GC (EBV(+), n = 6; EBV(−), n = 84). The overall survival curves of the stage I and stage III GC patients in EBV(+) group and EBV(−) group were depicted based on the Kaplan-Meier method, and the results were shown in Figure 5B and C. We found that the difference in overall survival between the patients (with stage I or stage III GC) in EBV(+) group and the patients in EBV(−) group was not statistically significant.

Discussion

Microsatellites are short tandem repeats (1–6 nucleotides) scattered throughout the genome that are prone to mutation. MSI is defined as a hypervariable phenotype that occurs in genomic MS in the presence of dMMR mechanisms (Baretti and Le 2018). The MS status can be determined by detecting the expression of MMR by immunohistochemistry, and four antibodies including MLH1, MSH2, MSH6 and PMS2 are often used (Luchini et al. 2019). MS status was obtained by the above method in this study. The results showed that there were 66/211 cases had defective expression of MMR, and the defect rate was 31.3%, which was consistent with the 5–37% defect rate reported in the previous research (Choi et al. 2014; Polom et al. 2018). Besides, the defect rates of MLH1, MSH2, MSH6 and PMS2 were 26.5% (n = 56), 7.6% (n = 16), 9.0% (n = 19) and 22.7% (n = 48), respectively. Moreover, MSI had a high degree of DNA methylation, which often accompanied by MLH1 deficiency and abundant genetic mutations (Usui et al. 2021). The high defect rate of MLH1 in this study coincided with this notion. These results suggested that the defective expression of MLH1 may be the main oncogenic mechanism of GC exhibiting MSI/dMMR.

MSI was more likely to occur in the elderly in this study (p = 0.047), which was consistent with the result of Velho et al. (2014). In addition, this research concluded that MSI of patients with stage I, II, and III GC was significantly correlated with tumor site, tumor diameter, and differentiation degree of tumor. MSI was more frequently observed in patients with cardia gastric cancer, in patients with smaller tumor diameter and in patients with non-poorly differentiated carcinoma. MSI was not statistically related to the depth of tumor invasion, lymph node metastasis, total tumor stage, vascular invasion and nerve invasion in GC tumor tissues. The 1-year, 3-year and 5-year survival rates were slightly higher than the overall survival rate of dMMR/MSI-patients, which was consistent with the results of a convincing meta-analysis about MSI based on a large cohort (about 1500

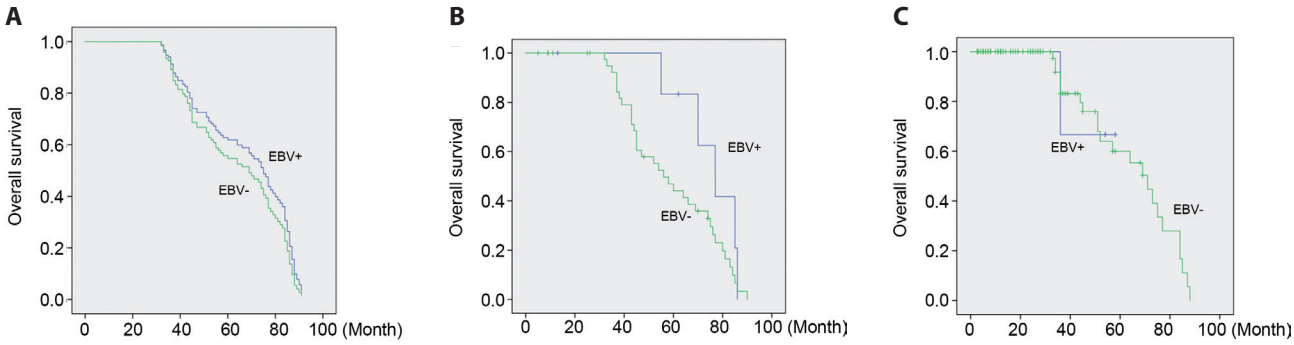

Figure 5. Kaplan-Meier plots of overall survival (OS) about all (**A**), stage I (**B**) and stage III (**C**) GC patients with EBV(+) *versus* with EBV(−). No statistical difference was found in OS between the EBV(+) and EBV(−) patients.

cases) tumor specimen. This meta-analysis revealed that the patients with dMMR/MSI-type GC had a longer overall survival than those with pMMR/MSS-type GC (Pietrantonio et al. 2019).

MSI has become a research hotspot, and abundant studies on its guiding role in chemotherapy have also appeared. Previous research showed that there was no significant difference in the effect of MS on the disease-free survival curve of patients with stage I, II, III and IV GC. Moreover, the patients with MSS showed better prognosis than those with MSI-H after chemotherapy (An et al. 2012). For the patients with stage IB and stage II GC after chemotherapy in this study, no significant difference was noted in the prolongation of overall survival between dMMR/MSI group and pMMR/MSS group. Among patients with stage III GC, the pMMR/MSS group had significant benefit compared with the dMMR/MSI group. Although there were some differences, the results of this study were basically consistent with previous studies, which may be related to the small sample size and the limitations of the retrospective study. The results may have guiding significance for the formulation of chemotherapy regimens for patients with stage III GC.

The infection of EBV can be monitored based on the characteristic that EBV can exist in the nucleus by binding to ribonucleoprotein. ISH technique was considered to be the gold standard for diagnosing EBV infection (Park et al. 2015). In this study, EBER ISH was used to detect the EBV infection. The results showed that there were 7.6% (15/198) cases with EBV(+) in GC tumor tissues. It has been reported that the proportion of EBVaGC varies widely around the world. Europe had the highest incidence (about 13.9%), while Asia had the lower incidence (7.5%) (Cheng et al. 2015). The proportion of EBVaGC in this study was consistent with previous reports.

In this study, EBV infection was significantly correlated with tumor differentiation and total stage. There was no significant correlation between EBV infection and sex, age, nationality, tumor site, tumor diameter, vascular invasion, nerve invasion, depth of tumor invasion and lymph node metastasis. However, the mechanism involved between EBV infection and clinicopathological parameters is still unclear. Therefore, elucidating the mechanism based on a large number of specimens will become our next important task.

Previous studies have shown that the patients with EBV and MSI may exhibit complex clinical responses in the immune system such as high levels of tumor-infiltrating lymphocytes (Chang et al. 2018). We explored the relationship between MS and EBV infection, the results showed that 13.3% patients had both MSI and EBV(+), and 68.3% patients had both MSS and EBV(−). It is worth noting that the patients with both MSI and EBV(+) may be immunotherapy sensitive.

No EBV(+) patients with stage II GC were observed in this study, so we only described the survival curves of patients with stage I and III GC used Kaplan-Meier method. The results showed that EBV infection had no statistical significance in prolonging the survival time of patients after chemotherapy. Further clarification of prognostic factors for EBVaGC is required.

In conclusion, we compared the baseline characteristics, MS, EBV infection, and survival prognosis of 211 patients with stage I, II, and III GC. We found that the patients in dMMR/MSI group and those in pMMR/MSS group had different clinicopathological parameters. The depth of tumor invasion, lymph node metastasis, total tumor stage, tumor diameter, differentiation degree of tumor and vascular/nerve invasion were independent prognostic factors for survival of patients who received radical gastrectomy. There was no significant difference in the overall survival time and chemotherapy response between the patients after who received radical gastrectomy in dMMR/MSI group and those in pMMR/MSS group. In addition, we found a low incidence of EBV(+) GC. The clinicopathological characteristics of EBV(+) GC were not significantly differ-

ent from those of EBV(−) GC. Besides, nerve invasion and total tumor stage were prognostic factors for GC patients who received radical gastrectomy. There was no significant difference in the effect of EBV infection on the overall survival time. The results of this study are still slightly different from existing reports, which may be attributed to geographical differences, single source and small number of research objects. There is no doubt that this study provides some ideas for the prognosis analysis of GC. However, the mechanism still needs to be analyzed in depth based on a large number of specimens.

Funding. This work was supported by the Xinjiang Uygur Autonomous Region "Tianshan Youth Plan" – Outstanding young scientific and technological talents training project (grant number 2020Q008); and the Beijing Science and Technology Innovation Medical Development Foundation (grant number KC2021-JX-0186-75).

Conflict of interest. The authors declare that they have no conflict of interest.

Author contribution. RM, HW contributed to the study conception and design. Material preparation, data collection and analysis were performed by PL, KY and GY. The first draft of the manuscript was written by PL and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data availability statement. The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval. This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the ethical committee of the First Affiliated Hospital of Xinjiang Medical University (No. K202210-06). Informed consent was obtained from all individual participants included in the study.

References

An JY, Kim H, Cheong JH, Hyung WJ, Kim H, Noh SH (2012): Microsatellite instability in sporadic gastric cancer: its prognostic role and guidance for 5-FU based chemotherapy after R0 resection. Int. J. Cancer **131,** 505-511 https://doi.org/10.1002/ijc.26399

Baretti M, Le DT (2018): DNA mismatch repair in cancer. Pharmacol. Ther. **189,** 45-62 https://doi.org/10.1016/j.pharmthera.2018.04.004

Chang YH, Heo YJ, Cho J, Song SY, Lee J, Kim KM (2018): Computational measurement of tumor immune microenvironment in gastric adenocarcinomas. Sci. Rep. **8,** 13887 https://doi.org/10.1038/s41598-018-32299-0

Cheng N, Hui DY, Liu Y, Zhang NN, Jiang Y, Han J, Li HG, Ding YG, Du H, Chen JN, et al. (2015): Is gastric lymphoepithelioma-like carcinoma a special subtype of EBV-associated gastric carcinoma? New insight based on clinicopathological features and EBV genome polymorphisms. Gastric Cancer **18,** 246-255 https://doi.org/10.1007/s10120-014-0376-9

Choi YY, Bae JM, An JY, Kwon IG, Cho I, Shin HB, Eiji T, Aburahmah M, Kim HI, Cheong JH, et al. (2014): Is microsatellite instability a prognostic marker in gastric cancer? A systematic review with meta-analysis. J. Surg. Oncol. **110,** 129-135 https://doi.org/10.1002/jso.23618

Flynn MJ, Larkin JMG (2017): Novel combination strategies for enhancing efficacy of immune checkpoint inhibitors in the treatment of metastatic solid malignancies. Expert. Opin. Pharmacother. **18,** 1477-1490 https://doi.org/10.1080/14656566.2017.1369956

Gjoerup O, Brown CA, Ross JS, Huang RSP, Schrock A, Creeden J, Fabrizio D, Tolba K (2020): Identification and utilization of biomarkers to predict response to immune checkpoint inhibitors. AAPS J. **22,** 132 https://doi.org/10.1208/s12248-020-00514-4

Kahraman S, Yalcin S (2021): Recent advances in systemic treatments for HER-2 positive advanced gastric cancer. Onco. Targets Ther. **14,** 4149-4162 https://doi.org/10.2147/OTT.S315252

Kawazoe A, Kuwata T, Kuboki Y, Shitara K, Nagatsuma AK, Aizawa M, Yoshino T, Doi T, Ohtsu A, Ochiai A (2017): Clinicopathological features of programmed death ligand 1 expression with tumor-infiltrating lymphocyte, mismatch repair, and Epstein-Barr virus status in a large cohort of gastric cancer patients. Gastric Cancer **20,** 407-415 https://doi.org/10.1007/s10120-016-0631-3

Kuboki Y, Yamashita S, Niwa T, Ushijima T, Nagatsuma A, Kuwata T, Yoshino T, Doi T, Ochiai A, Ohtsu A (2016): Comprehensive analyses using next-generation sequencing and immunohistochemistry enable precise treatment in advanced gastric cancer. Ann. Oncol. **27,** 127-133 https://doi.org/10.1093/annonc/mdv508

Luchini C, Bibeau F, Ligtenberg MJL, Singh N, Nottegar A, Bosse T, Miller R, Riaz N, Douillard JY, Andre F, et al. (2019): ESMO recommendations on microsatellite instability testing for immunotherapy in cancer, and its relationship with PD-1/PD-L1 expression and tumour mutational burden: a systematic review-based approach. Ann. Oncol. **30,** 1232-1243 https://doi.org/10.1093/annonc/mdz116

Park S, Choi MG, Kim KM, Kim HS, Jung SH, Lee JH, Noh JH, Sohn TS, Bae JM, Kim S (2015): Lymphoepithelioma-like carcinoma: a distinct type of gastric cancer. J. Surg. Res. **194,** 458-463 https://doi.org/10.1016/j.jss.2014.12.005

Petrelli F, Coinu A, Borgonovo K, Cabiddu M, Ghilardi M, Lonati V, Barni S (2014): Oxaliplatin-based chemotherapy: a new option in advanced hepatocellular carcinoma. a systematic review and pooled analysis. Clin. Oncol. (R. Coll. Radiol.) **26,** 488-496 https://doi.org/10.1016/j.clon.2014.04.031

Pietrantonio F, Miceli R, Raimondi A, Kim YW, Kang WK, Langley RE, Choi YY, Kim KM, Nankivell MG, Morano F, et al. (2019): Individual patient data meta-analysis of the value of microsatellite instability as a biomarker in gastric cancer. J. Clin. Oncol. **37,** 3392-3400 https://doi.org/10.1200/JCO.19.01124

Pino MS, Chung DC (2011): Microsatellite instability in the management of colorectal cancer. Expert. Rev. Gastroenterol. Hepatol. **5,** 385-399 https://doi.org/10.1586/egh.11.25

Polom K, Marano L, Marrelli D, De Luca R, Roviello G, Savelli V, Tan P, Roviello F (2018): Meta-analysis of microsatellite instability in relation to clinicopathological characteristics and overall survival in gastric cancer. Br. J. Surg. **105,** 159-167 https://doi.org/10.1002/bjs.10663

Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F (2021): Global Cancer Statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J. Clin. **71,** 209-249 https://doi.org/10.3322/caac.21660

Usui G, Matsusaka K, Mano Y, Urabe M, Funata S, Fukayama M, Ushiku T, Kaneda A (2021): DNA methylation and genetic aberrations in gastric cancer. Digestion **102,** 25-32 https://doi.org/10.1159/000511243

Velho S, Fernandes MS, Leite M, Figueiredo C, Seruca R (2014): Causes and consequences of microsatellite instability in gastric carcinogenesis. World J. Gastroenterol. **20,** 16433-16442 https://doi.org/10.3748/wjg.v20.i44.16433

Yang J, Liu Z, Zeng B, Hu G, Gan R (2020): Epstein-Barr virus-associated gastric cancer: A distinct subtype. Cancer Lett. **495,** 191-199 https://doi.org/10.1016/j.canlet.2020.09.019

Yoon H, Fuchs C, Özgüroğlu M, Bang Y, Di Bartolomeo M, Mandala M, Ryu M, Fornaro L, Olesinski T, Caglevic C (2020): O-12 KEYNOTE-061: response to subsequent therapy following second-line pembrolizumab or paclitaxel in patients with advanced gastric or gastroesophageal junction adenocarcinoma. Ann. Oncol. **31,** 236 https://doi.org/10.1016/j.annonc.2020.04.065

Received: February 23, 2023
Final version accepted: June 16, 2023

doi: 10.4149/gpb_2023021

Supplementary Material

Correlation of microsatellite status and EBV infection with clinical characteristics of patients with gastric cancer

Pengfei Lu[1,3], Kadeliya Yushan[2], Gulimilai Yusufu[2], Hui Wang[1,3] and Rui Mao[1]

[1] *Cancer Center, The First Affiliated Hospital of Xinjiang Medical University, Xinjiang, China*
[2] *Graduate School, Xinjiang Medical University, Xinjiang, China*
[3] *State Key Laboratory of Pathogenesis, Prevention and Treatment of High Incidence Diseases in Central Asia, Clinical Medicine Institute, The First Affiliated Hospital of Xinjiang Medical University, Xinjiang, China*

Supplementary Tables

Table S1. The expression status of MLH1, MSH2, MSH6, PMS2 proteins in pMMR and dMMR GC patients

Group	*n*	Percentage	MLH1	MSH2	MSH6	PMS2
pMMR	145	68.7%	+	+	+	+
	6		−	−	−	−
	7		−	+	+	+
	3		+	−	+	+
	2		+	+	−	+
	4		+	+	+	−
dMMR	3	31.3%	−	−	−	+
	4		−	+	−	−
	4		−	−	+	−
	29		−	+	+	−
	3		−	+	−	+
	1		+	+	−	−

GC, gastric cancer; MLH1, mutL homolog 1; MSH2, mutS homolog 2; MSH6, mutS homolog 6; PMS2, postmeiotic segregation increased 2; pMMR, mismatch repair-proficient; dMMR, mismatch repair-deficient. There were 68.7% (145/211) pMMR cases and 31.3% (66/211) dMMR cases among 211 GC patients.

Table S2. Onset age comparison of patients in dMMR group and pMMR group

Group	Median age, years (P_{25}, P_{75})	*p* value
dMMR/MSI	66 (54.75, 73)	0.047
pMMR/MSS	62 (54, 69)	

Table S3. Expression of EBV in GC tissues

EBV status	*n* (%)
EBV-positive	15 (7.6)
EBV-negative	183 (92.4)
Total	198 (100)

Table S4. Correlation between EBV infection and MS in GC tumor specimens

Microsatellite status	*n* (%)	EBV(+) *n* (%)	EBV(–) *n* (%)	Fisher's exact test	*p* value
MSI	60 (30.3)	2 (13.3)	58 (31.7)	–	0.24
MSS	138 (69.7)	13 (86.7)	125 (68.3)		

Table S5. Multivariate analysis of prognosis based on Cox proportional hazards model

Factor	B	Standard error	Wald	*p* value	HR	95% CI of HR	
						lower	upper
Nerve invasion	0.772	0.238	10.571	0.001	2.165	1.359	3.448
Total tumor stage	–1.249	0.609	15.749	0.001	0.287	0.061	1.05

HR, hazard ratio; CI, confidence interval.