Clinic-pathological Features of Epstein-barr Virus Infection, Microsatellite Instability, Tumor Mutation Burden and PD-L1 Status in 2504 Chinese Gastric Cancer Patients

Li Zhang

Department of Pathology, Peking University Cancer Hospital, No.52 Fucheng Road Haidian District, Beijing 100142, P.R.China

Aiwen Wu (✉ wuaw@sina.com)

Department of Surgical Oncology, Peking University Cancer Hospital, No.52 Fucheng Road Haidian District, Beijing 100142, P.R.China

Zhongwu Li

Department of Pathology, Peking University Cancer Hospital, No.52 Fucheng Road Haidian District, Beijing 100142, P.R.China

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Research

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Abstract

Objectives: Gastric cancer is the 4th most common cancer worldwide. Different subtype showed unique molecular features that could potentially guide therapeutic decisions. The aim of this study was to investigate the Epstein-Barr virus infection, microsatellite instability status, PD-L1 expression and gene mutation in surgically treated gastric cancer patients.

Methods: We reviewed all GC patients who underwent potentially curative gastrectomy with lymphadenectomy at Peking University Cancer Hospital between 2013 and 2018 from a prospective collected medical database. We analyzed the clinic-pathological factors associated with immunohistochemistry profiles. We also analyzed gene changes through next-generation sequencing.

Results: d-MMR gastric cancer patients are more likely to expression programmed death-ligand 1 ($p<0.001$, programmed death-ligand 1 cut off value 1%). EBV-positive and d-MMR patients were identified in 4% and 7.5% of the 2504 gastric cancer patients, respectively. The MLH1/PMS2 negative case number was 126. The MSH2/MSH6 negative case number was 14. d-MMR status was related to diffuse/mixed group ($p<0.05$), but not related to tumor differentiation. In our study, the microsatellite instability results detected by next generation sequencing and d-MMR gastric cancer results detected by immunohistochemistry were in high consistency. d-MMR gastric cancer patients had more microsatellite instability core. Many pathogenic genes were detected in microsatellite instability gastric cancer patients, such as POLE, ETV6, TP53, BRCA, RNF43 and other genes.

Conclusion: Through immunohistochemistry and next generation sequencing, we got MSI status, protein expression, TMB and gene changes of GC, which provided a theoretical basis for future G clinical treatment.

Introduction

Gastric cancer (GC) is the 4th most common cancer and the 2nd leading cause of cancer-related death worldwide [1]. Comprehensive molecular characterization at the genomic and transcriptomic levels has led to the identification of distinct GC subtypes [2,3]. Different subtypes showed unique molecular features that could potentially guide therapeutic decisions, and have been shown to have prognostic significance, with EBV being associated with the best prognosis. Since the molecular classification has potential prognostic and therapeutic implications, it may identify possible biomarkers and therapeutic targets of each subtype, particularly through the stratification according to Epstein-Barr virus infection and microsatellite instability (MSI) [4-8]. Currently, programmed death-ligand 1 (PD-L1) expression in

tumor cells has been validated as a predictive marker for tumor response to anti programmed cell death-1 protein (anti-PD-1) or PD-L1 immunotherapy in different malignancies, including gastric cancer [9]. According to The Cancer Genome Atlas (TCGA) study and recent trials, EBV and MSI GC subgroups may benefit from therapy with PD-1/PD-L1 antibodies [10,11]. PD-1/PD-L1 inhibitors appear to improve the antitumor activity in advanced gastric cancer patients [12-15]. However, the frequency and prognostic value of PD-L1 expression in GC remain controversial. The proto-oncogene human epidermal growth factor receptor-2 (HER-2) is a 185-kDa trans-membrane tyrosine kinase receptor and a member of the epidermal growth factor receptor family. There is growing evidence that HER-2 is an important biomarker and key driver of tumor-genesis in gastric cancer. HER-2 is associated with cell proliferation, apoptosis and differentiation. Trastuzumab in association with systemic cytotoxic chemotherapy is a therapeutic option for patients with advanced or metastatic HER-2 positive gastric carcinoma. The status of the HER-2 overexpression or gene amplification is an important predictive marker in gastric cancer [16,17]. The aim of this study was to investigate the EBV infection by in situ hybridization (ISH), and mismatch repair (MMR) status and PD-L1 expression using immunohistochemistry (IHC) in surgically treated GC patients. Additionally, we analyzed the clinic-pathological and prognostic factors associated with IHC profiles. We also used the next-generation sequencing (NGS) technology to analyze the different gene changes of d-MMR/p-MMR gastric cancer patients, tumor mutation burden (TMB) data and MSI status.

Materials And Methods

2.1 Patients of General Information

We reviewed all gastric adenocarcinoma patients who underwent potentially curative gastrectomy with lymphadenectomy at Peking University Cancer Hospital between 2013 and 2018 from a prospective collected medical database. Inclusion criteria were: (1) gastric adenocarcinoma; (2) formalin-fixed paraffin-embedded tissue (FFPE) blocks available for analysis. Surgical specimens were fixed in 10% buffered formalin. The slices were evaluated according to the College of Chinese Pathologists protocol. IHC with cytokeratin were performed in some cases to detect lymph node micro-metastases. The 2014 edition of the WHO Digestive System Tumor Pathology and Genetics classification of gastric cancer TNM staging criteria was used. The study was approved by Peking University Cancer Hospital ethics committee.

All paraffin-embedded specimens were cut in 4µm sections using a conventional histological technique and transferred to a slide. IHC staining was performed using a hercept test kit (Dako, Carpinteria, CA, USA) according to the manufacturer's protocol using an automatic immunostainer (Dako). Staining intensity was evaluated using the 0 to 3+ scale according to the test scoring criteria.

2.2 Evaluation of PD-L1 IHC expression

All tissue cores were evaluated by two pathologists. Specimens were scored on the positive staining area of stained tumor cells (TCs) or tumor-infiltrating immune cells (TIICs) by immunohistochemistry (IHC): positive staining area less than 1 %; from 1 % to less than 10 %; from 10 % to less than 50 %; 50 % or more. The primary antibody against PD-L1 was SP142 (Spring Bioscience, Pleasanton, CA, USA).

2.3 Evaluation of MMR protein expression and EBV infection status by IHC/ISH

Tumors were considered loss for MLH1, MSH2, PMS2, or MSH6 expression only if there was a complete absence of nuclear staining in the tumor cells, and normal epithelial cells and lymphocytes were used as an internal control. Tumors lacking MLH1, MSH2, PMS2, or MSH6 expression were considered to be MMR deficiency, whereas tumors that maintained expression of all markers were considered to be MMR proficiency (as long as the tumor cell nucleus is positive, regardless of the positive percentage). MMR protein expression was tested by IHC using antibody clones (MLH1, M1 antibody; MSH2, G2191129 antibody; MSH6, 44antibody; PMS2, EPR3947 antibody (Ventana Medical Systems, Inc., Tucson, AZ, USA). The complete absence of protein expression (0+ in 100% of cells) was considered a loss of MMR and thus d-MMR. EBV-encoded RNA (EBER) ISH kit was used to detect EBV infection status (OriGene Technologies, Inc., Beijing, China).

2.4 TMB data and gene mutation through NGS

We also used the next generation sequencing technology to detect the MSS and MSI status of gastric cancer samples, including TMB and gene mutation.

2.5 HER-2 expression by IHC

HER-2 was located in the cell membrane and scored for stained tissue according to the HER-2 Detection Guide for Gastric Cancer. Response or < 10% tumor cell membrane staining for HER-2 (0); ≥ 10% tumor cell membrane weak or faintly visible membrane staining, or only partial membrane staining for HER-2 (1 +); ≥ 10% tumor cells are weak to moderate basal membrane, lateral membrane or complete membrane staining for HER-2 (2 +); ≥ 10% tumor cell basal membrane, lateral membrane or complete membrane strong staining for HER-2 (3 +).

2.6 Statistical analysis

Comparisons of categorical variables were done by a chi-square test or Fisher's exact test as appropriate. Differences with p -values < 0.05 were considered statistically significant.

Results

3.1 Relationship between PD-L1 expression and clinic-pathological features of gastric cancer

PD-L1-positive cases on TCs or TILCs were defined by the presence of at least 1 % of TCs or TILCs with membrane staining. According to this standard, the proportion of PD-L1 positive cases accounted for 20.2%. PD-L1 expression on TCs, were identified in 11.6%, 10.9%, 4% respectively, at different cut-off points (1%, 10%, and 50%, according to the positive staining area of cell membrane). d-MMR GC patients are more likely to expression PD-L1 ($p<0.001$, PD-L1 cut off value 1%) (Figure 1).

3.2 EBER ISH ratio in 2504 patients

EBV positivity was 4%. MSI ratio was 7.5%. EBER positive patients were usually male, diffused/mixed Lauren type and poorly differentiated ($p<0.001$) (Table 1, Figure 2). The number of EBER positive patients was 96 cases. The number of EBER negative patients was 2408. PD-L1 expression had no significant difference in EBER (+) and EBER- patients in our study ($p=0.524$).

3.3 MMR protein expression status and clinic-pathological features

EBV-positive and d-MMR patients were identified in 4% and 7.5% of the 2504 GCs, respectively. The MLH1/PMS2 protein deficiency case number was 126. The MSH2/MSH6 protein deficiency case number was 14. d-MMR status was related to diffuse/mixed group ($p<0.05$), but not related to tumor differentiation (Table 2).

3.4 HER-2 expression in 2504 gastric cancer patients

The number of HER-2 1+ patients was 628. The number of HER-2 2+ patients was 313. The number of HER-2 3+ patients was 102. HER-2 3+ cases accounted for 4.1%. HER-2 2+ cases accounted for 12.5%, and HER-2 1+ accounted for 25.1%. There were 1461 cases without HER-2 protein expression, and the rate was 58.3%. HER-2 expression was not related to EBER and MMR status ($p>0.05$, Table3, Table4).

3.5 Results of the next generation sequencing, comparison of NGS-MSI and IHC-MMR results in gastric cancer

The results of MSI detected by next generation sequencing and IHC results of d-MMR gastric cancer were in high consistency, patients with d-MMR status had higher MSI score, p-MMR patients' score was very low (Figure3). Many pathogenic genes were detected in MSI patients, such as ETV6, TP53, BRCA, POLE, RNF43 and other genes (Figure4). In our data, the most significantly mutated genes were LRP1B (79.07%), ARID1A (74.42%), RNF43(69.77%), ZFH3(65.12%), TP53(58.14%), GANS (51.16%), BRCA2(51.16%), PIK3CA (51.16%), NOTCH1 (51.16%), SMARCA4 (48.84%), ATR (46.51%), POLE (41.86%), ATM (39.53%). In TMB high and MSI tumors, we can see the deletion mutation of some genes, such as RNF43, BCORL1, ATR etc. Figure5.

Discussion

Gastric cancer is the fourth most common cancer worldwide. For lots of patients, their disease has become inoperable when they are diagnosed, or their disease often recurs after curative resection. For patients with advanced, unresectable cancer, systemic chemotherapy is generally prescribed as the primary therapy [18]. Traditionally, GC classification has been based on histopathological and morphological features, which was first described in 1965 [19,20]. Unfortunately, classifications based on morphology are unable to identify molecular targets. So next generation sequencing, largescale molecular profiling has led to different molecular-based classifications, which may be exploited for therapeutic interventions. HER-2 is associated with cell differentiation, proliferation and apoptosis. Trastuzumab in association with systemic cytotoxic chemotherapy is a therapeutic choice for patients with late or metastatic HER-2 positive gastric carcinoma. The status of the HER-2 overexpression or gene amplification is an important predictive marker in gastric cancer. So far, there is no large-scale study on HER-2 expression of gastric cancer in China. In Wang's data [21], The expression rate of HER-2 protein was 39.3%, but they only studied 135 cases of gastric cancer. In our study, there are 2504 gastric cancer patients, among these positive cases, patients with HER-2 protein (3+) accounted for 4.1%, patients with HER-2 protein (2+) accounted for 12.5%. HER-2 expression was not related to EBER and MMR status.

Some studies showed EBV infection GC comprises about 9% of all cases of GC and constitutes a distinct clinic-pathological and molecular entity [22]. In our data, the EBV positivity is 4%, and the EBV-positive patients were usually male, diffused/mixed Lauren type and poorly differentiated ($p < 0.001$). Less understood is the participation of EBV in chronic gastric inflammation, but some studies argue that EBV, similar to and together with *Helicobacter pylori*, is an early participant in the GC oncogenic process through promoting chronic inflammation and increased tissue damage. EBV infection might contribute to the malignant transformation of GC cells by involving various cellular processes and signaling pathways. EBV infection GC has shown the following distinct characteristics in contrast to other subtypes. In our study, PD-L1 expression had no difference in EBV positive and EBV negative patients ($p = 0.524$), according PD-L1 positivity was $> 1\%$. In a small cases' study [23], PD-L1 expression was significantly associated with EBV infection ($p < 0.001$). In our 2504 patients research, PD-L1 high expression was likely in d-MMR patients ($p < 0.001$) (PD-L1 cut off value was 1%). In Haron NH's study [24], a total of 60 gastric cancer cases were retrieved. Microsatellite analysis identified ten MSI positive cases (16.7%), out of which only six cases (10.3%) showed absence of MLH1 ($n = 3$) or MSH2 ($n = 3$) protein expression by IHC. In our study, the number of MLH1/PMS2 protein deficiency case was 126, MSH2/MSH6 protein deficiency number was 14. d-MMR GC patients are more likely to expression PD-L1 ($p < 0.001$). We also sequenced some GC cases, and obtained many differentially expressed genes through cluster analysis. In Cho J's study [25], they performed massive parallel sequencing of 381 cancer-related genes and compared the results with clinical and pathologic findings in 330 GC. The most significantly mutated genes were TP53 (54%), ARID1A (23%), CDH1 (22%), PIK3CA (12%), RNF43 (10%) and KRAS (9%). In Yoon K's study [26], they identified 18,377 MS mutations of five or more repeat nucleotides in gene coding sequences and untranslated regions, and discovered 139 individual genes whose expression was down-regulated in association with UTR MS mutation. In our research, many pathogenic genes were detected in d-MMR patients, such as ETV6, TP53, BRCA1, POLE, RNF43 and other genes. In our data, the most

significantly mutated genes were LRP1B (79.07%), ARID1A (74.42%), RNF43 (69.77%), ZFHX3 (65.12%), TP53 (58.14%), GANS (51.16%), BRCA2(51.16%), PIK3CA (51.16%), NOTCH1 (51.16%), SMARCA4 (48.84%), ATR (46.51%), POLE (41.86%), ATM (39.53%). RNF43 mutation is a frame shift mutation, which leads to the early truncation of the protein, which may inactivate the protein and predict the possible pathogenesis. Preclinical studies of gastric and colorectal cancer have shown that inactivation of RNF43 promotes cell proliferation and tumor growth [27]. BRCA2 is a frame shift mutation, which leads to the early truncation of the protein, which may inactivate the protein and predict the possible pathogenesis. The mutation has been reported in breast cancer. PIK3CA Y1021C is located in PI3K / PI4k domain of PIK3CA protein. In cell culture, mutation leads to an increase in the transformation ability of cell lines. GNAS R201C is located in the GTP binding region of GNAS protein. In the mouse model, R201C resulted in the loss of GTP enzyme activity, the continuous activation of downstream signals, cell proliferation and tumor formation. ATM mutation leads to premature truncation of ATM protein. Due to the deletion of all known functional domains, predictive mutations lead to the loss of protein function. C135F is located in the DNA binding domain of p53 protein. This mutation leads to the loss of trans activation ability of TP53, which is predicted to be inactivated mutation. The mutation has been reported in gastric cancer, lung cancer, breast cancer and other cancers. KRAS pathogenic mutation leads to the decrease of KRAS GTPase activity. ARID1A mutation often occurs in gastric cancer with poor prognosis. The AKT signaling pathway can be activated by the decreased expression or function of ARID1A. The levels of multiple immune markers and TMB in patients with ARID1A mutation were significantly higher than those in patients with ARID1A wild type. ARID1A promotes mismatch repair, so ARID1A defects are related to mismatch repair and microsatellite instability. The expression of PD-L1 in alimentary tract cancer patients with ARID1A mutation was significantly higher than that in wild-type patients [28-30]. AKT inhibitor GSK693 showed antitumor activity in ARID1A deficient gastric cancer cells. E157G is located in the phosphatase tensin type domain of PTEN. It is predicted that E175G mutation will lead to the loss of protein function. It is predicted that V158fs will lead to the inactivation of PTEN. In HER-2 positive GC patients, PTEN deletion mutations are associated with Trastuzumab resistance, and the loss of heterozygosis of this gene has been reported more frequently in gastric cancer [31]. In solid tumor patients receiving immunotherapy, the median OS of patients with POLE/POLD1 mutation was significantly better than that of non-carriers. 26% of patients with POLE/POLD1 gene mutation were combined with MSI-H status. After removing these patients, OS in mutant group still benefited. That is to say, patients with MSS who cannot benefit from immunotherapy can still judge whether they can benefit from immunotherapy by POLE/POLD1 gene mutation. Multivariate analysis confirmed that POLE/POLD1 mutation can be used as a new independent index to predict immunotherapy benefit [32]. To the best of our knowledge, our study is the largest research on the pathological characteristics of gastric cancer patients in China. Through immunohistochemistry, in situ hybridization and NGS, we have a deeper understanding of GC, including MSI status, HER-2 and PD-L1 expression, TMB and gene changes in GC patients, which provided a theoretical basis for future GC clinical treatment. In future studies, we will study the mechanism of these mutations in the development of gastric cancer.

Declarations

Authors' contributions

Li Zhang conceived the study and drafted the manuscript. Li Zhang, Aiwen Wu and Zhongwu Li acquired the data. All authors read and approved the manuscript.

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No funding was received.

Availability of data and materials

Not applicable.

Ethics approval and consent to participate

The study was approved by Peking University Cancer Hospital ethics committee. People who participated in this research had complete clinical data. Signed informed consents were obtained from the patients and/or the guardians.

Consent for publication

Not applicable.

Author details

¹Department of Pathology, Peking University Cancer Hospital, No.52 Fucheng Road Haidian District, Beijing 100142, P.R.China; ²Department of Surgical Oncology, Peking University Cancer Hospital, No.52 Fucheng Road Haidian District, Beijing 100142, P.R.China.

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Tables

Table1 PD-L1 expression, EBER ISH status withclinic-pathological features of GC patients

Variables	EBER ISH status		<i>p</i> value
	EBER positive (+)	EBER negative (-)	
Sex			0.003*
male	82	1717	
female	14	691	
Lauren type			0.000*
Diffuse/mixed	77	1503	
intestinal	19	905	
Differentiation			0.001*
poorly differentiated	79	1600	
well-moderately	17	808	
T stage			0.322
pT3+T4	55	1500	
pT1+T2	41	908	
Lymphnodemetastasis			0.326
LNM+	53	1450	
LNM-	43	958	
PD-L1 expression			0.524
PD-L1 -	52	1617	
PD-L1>=1%	25	664	

Table2 Clinicopathological features of 2504p-MMR and d-MMR GC patients

Variables	MMR status		<i>p</i> value
	p-MMR	d-MMR	
Sex			0.102
male	516	45	
female	1374	86	
Lauren type			0.012*
Diffuse/mixed	1201	74	
intestinal	690	66	
Differentiation			0.256
poorly	1264	87	
well-moderately	627	53	
T stage			0.038
pT3+T4	1193	88	
pT1+T2	698	52	
Lymphnode metastasis			0.246
LNM+	1121	76	
LNM-	770	64	
PD-L1 expression			0.000*
PD-L1 -	1337	69	
PD-L1 ≥1%	554	71	

Table 3 Correlation of HER-2 expression and EBER status in GC patients

Variables	EBER+	EBER-	<i>p</i> value
HER-2 3+	2	102	0.300
HER-2 0/1+/2+	94	3205	

Table 4 Correlation of HER-2 expression and MMR status in 2504 GC patients

Variables	p-MMR	d-MMR	<i>p</i> value
HER-2 3+	75	2	0.129
HER-2 0/1+/2+	1816	138	

Figures

Figure 1

PD-L1 strong staining ind-MMR gastric cancer patients. A.poorly differentiated gastric cancer, Hematoxylin and Eosin staining(H&E staining), 200x magnification; B.The positive staining area of PD-L1 on tumor cells was more than 90%, moderate to strong positive, 200x magnification; C.MLH1 IHCnegative staining, 200x magnification,stromal cells with positive staining as internal control; D. PMS2 IHCnegative staining, 200x magnification, stromal cells with positive staining as internal control.

Figure 2

EBERpositive patients were usually had diffused/mixed Lauren type and poor differentiation. A.poorly differentiated gastric cancer, diffuse type, H&Estaining, 200x magnification; B.poorly differentiated gastric cancer, EBER ISH positive staining, 200x magnification C. moderately differentiated gastric cancer, intestinal type, H&E staining, 200x magnification; D. moderately differentiated gastric cancer,EBER ISH negative staining, 200x magnification;

Figure 3

The results of MSI detected by NGS were highly consistent with IHC results of d-MMR GC. d-MMR patients had higher MSI scores.

Figure 4

NGS results of d-MMR gastric cancer patients. Many pathogenic genes were detected, such as POLE, ETV6, TMPRS52, BRCA1, BRCA2, TP53 and other genes.

Figure 5

TMB×MSI status, gene mutation frequency and mutation type detected by NGS. In high TMB and MSI tumors, we can see the deletion mutation of some genes,such as RNF43,BCORL1,ATR etc.