Pathology - Research and Practice 215 (2019) 152675

Contents lists available at ScienceDirect

Pathology - Research and Practice

journal homepage: www.elsevier.com/locate/prp

Differential expression of HER2 and downstream proteins in prediction of advanced tumor phenotypes and overall survival of patients with Epstein-Barr virus-positive vs. negative gastric cancers

Yi-wang Zhang[a,1], Dan He[a,1], Cui Tan[b], Min Dong[a], Lu Zhou[a], Chun-kui Shao[a,*]

[a] Department of Pathology, The Third Affiliated Hospital, Sun Yat-sen University, No. 600 Tianhe Road, Guangzhou 510630, Guangdong Province, China
[b] Department of Pathology, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, No. 107 Yangtze River Road, Guangzhou 510120, Guangdong Province, China

ARTICLE INFO

Keywords:
Gastric cancer
HER2
Epstein-Barr virus
Biomarker
Prognosis

ABSTRACT

This study evaluated the associations of HER2 protein, *HER2* gene amplification, and positivity for p-AKT, p-ERK, and p-PLCγ proteins with clinicopathological status and overall survival (OS) of patients who had Epstein-Barr virus-associated gastric cancer (EBVaGC; n = 58) or EBV-negative GC (EBVnGC; n = 329). Tissue samples were subjected to immunohistochemistry and fluorescence *in situ* hybridization (FISH). Results showed that EBVaGC less expressed HER2 and amplified *HER2* gene. p-AKT ($p = 0.035$) and p-ERK ($p = 0.001$) were inhibited in EBVaGC than in EBVnGC, while p-PLCγ ($p = 0.034$) was upregulated. Among EBVaGC patients, p-ERK positivity was associated with Lauren classification ($p = 0.023$), and p-PLCγ positivity was inversely associated with TNM stage ($p = 0.041$) and lymph node metastasis ($p = 0.041$). In contrast, among EBVnGC patients, HER2 expression was associated with distant metastasis ($p = 0.043$) and p-AKT positivity was associated with intestinal subtype ($p < 0.004$), lymph node metastasis ($p = 0.031$), distant metastasis ($p < 0.001$), and elder age (> 60y, $p < 0.004$). Overall analysis showed that EBVaGC patients presented better OS than EBVnGC patients ($p = 0.044$). Among EBVaGC patients, p-AKT positivity ($p = 0.008$) was associated with worse OS; as well as, HER2 high expression ($p < 0.001$), p-AKT positivity ($p = 0.010$), and p-PLCγ ($p < 0.001$) were associated with worse OS in EBVnGC patients. Multivariate analysis showed that distant metastasis (95% CI: 1.559 to 4.028, $p < 0.001$), HER2 high expression (95% CI: 1.058 to 2.454, $p = 0.026$), and p-PLCγ positivity (95% CI: 1.056 to 2.435, $p = 0.027$) were independent prognostic predictors of OS in EBVnGC patients. Our results indicated that p-AKT positive patients presented worse OS than p-AKT negative ones in EBVaGC, as well as, HER2, p-AKT, and p-PLCγ are prognostic biomarkers for OS in EBVnGC patients.

1. Introduction

Gastric cancer (GC) is the fifth most-common malignancy worldwide, with 951,600 new cases and 723,100 cancer-related deaths each year [1]. GC is associated with diet, methods of food storage, and *Helicobacter pylori* infection [2,3]. Recent studies demonstrated that Epstein-Barr virus (EBV) infection correlated with the occurrence and development of GC. EBV is not only a ubiquitous human herpesvirus and the main cause of infectious mononucleosis [4] but is also associated with development of Burkitt lymphoma, Hodgkin's lymphoma, gastric cancer, and nasopharyngeal carcinoma (NPC). EBV-associated gastric cancer (EBVaGC) [5,6] is defined by the presence of EBV in GC cells, as detected by EBV-encoded RNA (EBER) *in situ* hybridization [7,8]. EBVaGC occurs in approximately 10% of all GCs diagnosed worldwide, with a range of 1.3–20.1% depending on the population [9,10]. Our previous study showed that EBVaGC had an incidence of 6.7% (45/676) in Guangzhou, Southern China, a region where NPC is endemic [11]. Clinically, EBVaGC has several distinct clinicopathological features, such as preferential diffuse-type GC and frequent occurrence in the gastric cardia and body [7]. The Cancer Genome Atlas (TCGA) Research Network performed a comprehensive molecular evaluation of 295 primary gastric adenocarcinoma patients in 2014 and classified EBVaGC as a molecular subtype of gastric cancer [12].

Like the most of other human cancers, the pathogenesis of GC is involved in risk factors-induced oncogene activation and silencing of tumor suppressor genes [12,13]. Human epidermal growth factor receptor 2 (HER2), which belongs to the epidermal growth factor receptor

* Corresponding author.
E-mail address: chunkuishao2011@163.com (C.-k. Shao).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.prp.2019.152675
Received 17 June 2019; Received in revised form 17 September 2019; Accepted 27 September 2019

(EGFR) superfamily, is recognized as an oncogene. In gastric cancer, HER2 is overexpressed in 7–34% of patients [14–18] and is related to poor tumor prognosis and aggressive behavior [14,17]. In 2010, the Trastuzumab for Gastric Cancer (ToGA) trial demonstrated that trastuzumab (a humanized monoclonal anti-HER2 receptor antibody) improved the survival of patients with HER2-positive GC [18]. Our own study identified a lower prevalence of HER2 positivity in EBVaGC cases than EBVnGC cases (5.1% *vs.* 23.7%) [19]. In agreement, Lee et al. reported that 1 of 63 (1.6%) EBVaGC patients had HER2 overexpression, whereas 38 of 281 patients (13.5%) of EBV-negative GC (EBVnGC) cases had HER2 overexpression [20]; Sukawa et al. reported that HER2 overexpression occurred in only 1 of 18 (5.6%) EBVaGC cases, but in 19 of 213 (8.9%) EBVnGC cases [21]; Song HJ et al. presented that HER2 overexpression (strong intensity, score 3) by IHC was less in 123 EBVaGC than in 405 EBVnGC (0.8% *versus* 9.4%, $p < 0.02$) [22].

What is the reason for the reduced prevalence of HER2 overexpression in EBVaGC? Our previous study firstly certified that Latent membrane protein 2A(LMP2A), an EBV latency protein, might inhibit *HER2* transcription through a pathway involving TWIST and YB-1. However, patients with LMP2A-negative EBVaGC also had a lower prevalence of HER2 overexpression than those with EBVnGC (9.1% *vs.* 23.7%; $p < 0.001$) [19]. Differences in *HER2* gene amplification might possibly explain the differences in HER2 expression in these two groups [23]. We wondered condition of *HER2* amplification in EBVaGC.

Moreover, as a member of the erythroblastosis oncogene B (ErbB) receptor tyrosine kinase (RTK) family, HER2 activates many downstream second messenger pathways, and crosstalk with other transmembrane signaling pathways can lead to diverse biological effects [24]. Previous studies have suggested that HER2 can initiate or interact with the phosphoinositide-3-kinase (PI3K)/AKT pathway, the mitogen activated protein kinase (MAPK) pathway, and the phospholipase C (PLC)-γ pathway, and thereby promote growth, survival, proliferation, and migration of cancer cells [25–27]. Because HER2 levels are lower in EBVaGC patients, we speculated that EBV might affect the activation of one or more of these key pathways. Sukawa et al. showed that there was no significant difference in p-AKT expression between patients with EBVaGC and EBVnGC [21,28], but no studies have yet measured p-ERK or p-PLCγ expression in patients with EBVaGC. The associations of p-AKT, p-ERK, and p-PLCγ proteins with clinicopathological status and overall survival (OS) of patients with EBVaGC had not been reported.

In this study, we collected 58 EBVaGC and pTNM-classification matched 329 EBVnGC to evaluate HER2 protein expression and gene amplification in EBVaGC and EBVnGC. We also assessed differential expression of HER2 downstream proteins to predict advanced tumor phenotypes and survival of patients with EBVaGC and EBVnGC.

2. Materials and methods

2.1. Ethics statement

The use of human subjects was approved by the Clinical Research Ethics Committee of the Third Affiliated Hospital, Sun Yat-sen University, Guangzhou, China. All adult subjects provided informed consent, and a patient or guardian of any children participant provided informed consent on their behalf.

2.2. Tissue specimens

A total of 1013 surgical resection gastric adenocarcinoma cases were collected at the Second and Third Affiliated Hospitals of Sun Yat-sen University, Guangzhou, southern China from January 1, 2004 to December 31, 2012. Fifty-eight cases (5.7%) were identified as EBVaGC by EBER1 *in situ* hybridization (ISH). Among the 955 EBVnGC cases, 329 EBVnGC cases had pTNM classification that matched the 58 EBVaGC cases.

Table 1
IHC and FISH results of HER2 in 58 EBVaGC and 50 EBVnGC.

	EBVaGC (N = 58)		EBVnGC (N = 50)	
	FISH positive	FISH negative	FISH positive	FISH negative
IHC 0/1+	–	55	–	36
IHC 2+	–	2	2	6
IHC 3+	1	–	6	–

Clinicopathologic features and outcome data were obtained from the archives of the four hospital. Histology of the gastric carcinomas was classified as intestinal- and diffuse-type, according to the Lauren classification [29]. Cancer staging was classified according to the TNM cancer staging system of the American Joint Committee of Cancer [30]. The clinical outcome was followed up from the date of primary surgery to the date of death or Dec.31, 2017. The median follow-up period was 26 months (range, 1–75 months).

2.3. Tumor tissue microarrays

Twenty-one tissue microarray (TMA) blocks that contained samples of all 1013 cases were prepared as described by Chen et al [9]. Each TMA block contained up to 76 tissue cores from 38 cases, and each case had 2 tissue cores. An adequate case was defined by tumor occupancy of more than 30% of the core area. The 58 EBVaGC samples and the 329 EBVnGC samples were in 11 TMA blocks.

2.4. EBER1 in situ hybridization (ISH)

An ISH assay was performed on the TMA block sections with an EBV oligonucleotide probe complementary to the EBV-encoded small RNA-1 (EBER-1; PanPath, Amsterdam, Netherlands), according to the manufacturer's instructions. Sections (4-μm thick) were cut from each tissue array block to detect EBV using an *in situ* hybridization assay. The hybridization signals were visualized with 3,3 0 -diaminobenzidine (DAB; Vector Laboratories, Inc., Burlingame, CA), and positive signals were recognized as dark brown nuclear staining under light microscopy. Known EBER-1-positive NPC tissues were used as the positive control, and a sense probe for EBER-1 was used as the negative control. EBER-1 (+) and EBER-1 (-) cases were defined as EBVaGC and EBVnGC, respectively. Among all 1013 cases, 58 cases (5.7%) were EBVaGC.

2.5. HER2 fluorescent in situ hybridization (FISH)

Dual-probe hybridization was performed using the PathVysion HER-2 DNA Probe Kit (Vysis, Shanghai, China). The HER2 probe and the centromere 17 (CEP17) probe were directly labeled with SpectrumOrange and SpectrumGreen, respectively. Using an Olympus microscope equipped with a double-band pass filter, two investigators visually counted FISH data for at least 100 tumor cell nuclei in two or more separate regions of the tissue section. Averages of HER2 gene and chromosome 17 copy number counts were rounded off to the nearest whole number. The number of HER2 signals and CEP 17 signals were counted for each nucleus and an overall mean HER2: CEP 17 ratio was calculated for each case. As proposed by the ASCO/CAP guideline [31], an absolute HER2 gene copy number lower than four or an HER2: CEP 17 ratio of less than 1.8 was considered HER2 negative; an absolute HER2 copy number between four and six or an HER2: CEP 17 ratio between 1.8 and 2.2 was considered HER2 equivocal, and an absolute HER2 gene copy number greater than six or an HER2: CEP 17 ratio higher than 2.2 was considered HER2 positive. Lymphocytes, (myo)fibroblasts, and normal epithelial cells served as internal controls.

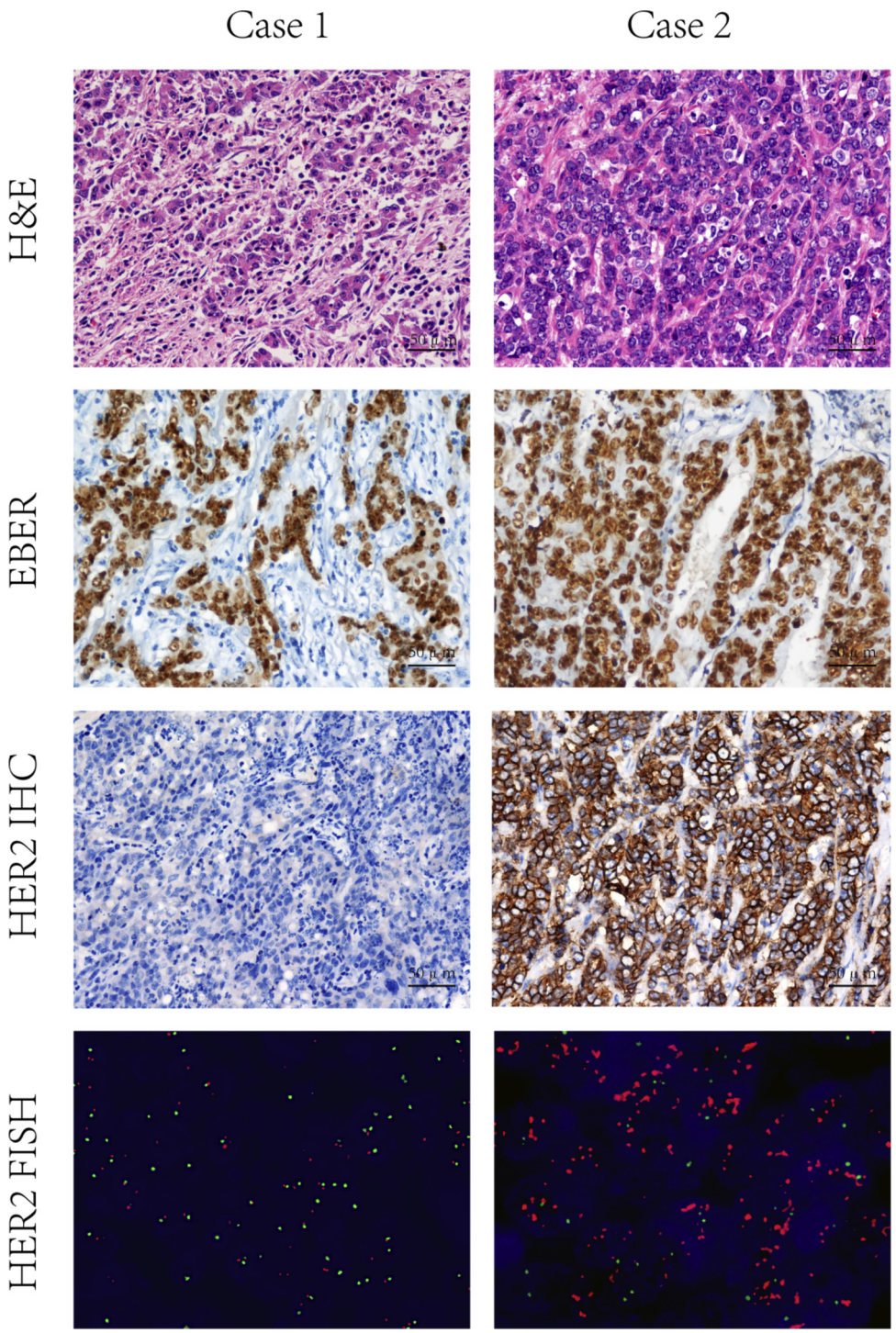

Fig. 1. H&E staining, ISH analysis of the EBV gene *EBER1*, IHC of HER2, and FISH of *HER2* in two representative cases with EBVaGC. Both cases were positive for *EBER1*. Case #1 had no HER2 expression (ICH = 0) and no *HER2* gene amplification, whereas Case #2 had HER2 overexpression (ICH = 3+) and *HER2* gene amplification.

2.6. Immunohistochemical staining

The Envision Immunohistochemistry (IHC) system was used to analyze the expression of HER2, p-AKT, p-ERK and p-PLCγ [32]. The slides were incubated with rabbit-derived polyclonal antibody against HER2 (A0485, 1:200 dilution; Dako, Copenhagen, Denmark), rabbit-derived monoclonal antibodies against p-AKT(#4060, 1:50 dilution), p-ERK (#4370, 1:400 dilution) or p-PLCγ (#8713, 1:200 dilution; Cell Signaling Technology, Beverly, MA, USA) as the primary antibody, and HRP-labeled secondary antibody (Dako Envision). A HER2-positive breast carcinoma case was used as positive controls and substitution of the primary antibodies with PBS was used as a negative control.

The HER2 IHC staining results were scored into 0, 1+, 2+ and 3+ according to the criteria recommended by the ToGA [33]. Staining of P-AKT [34], p-ERK [35] and p-PLCγ [36] were localized in the cytoplasm and nucleus of the tumor cells. The level of p-AKT, p-ERK and p-PLCγ expression were scored according to the intensity of staining. Five high power fields (x200, magnification) in each section were randomly

Table 2
Association of HER2 with p-AKT, p-ERK, and p-PLCγ in EBVnGC tissue specimens.

	No.	p-AKT +		p-ERK +		p-PLCγ +	
		N (%)	[a]p	N (%)	p	N (%)	p
HER2							
IHC 0/1 +	243	141 (58.0)	0.002	108 (44.4)	0.48	47 (19.3)	< 0.001
IHC 2/3 +	86	66 (76.7)		42 (48.8)		34 (39.5)	

[a] analyzed by using the Pearson Chi-Square test.

Table 3
Differences expression of p-AKT, p-ERK, and p-PLCγ between EBVaGC and EBVnGC tissue specimens.

	EBVaGC N (%)	EBVnGC N (%)	[a]p
p-AKT			
positive	28(48.3)	207(62.9)	0.035
negative	30(51.7)	122(37.1)	
p-ERK			
positive	14(24.1)	150(45.6)	0.001
negative	44(75.9)	179(54.4)	
p-PLCγ			
positive	22(37.9)	81(24.6)	0.034
negative	36(62.1)	248(75.4)	

[a] analyzed by using Pearson Chi-Square test.

selected for analysis according to the percentage of cells stained and the intensity of the staining. The percentage of positive cells per section was scored as 0 (0–5%), 1 (6–25%), 2 (26–50%), 3 (51–75%), or 4 (> 75%). The staining intensity was scored as: 0 (negative); 1 (weak); 2 (moderate); 3 (strong). The percentage and intensity scores were added to calculate a total score (staining score = percentage score + intensity score). The total score was used to classify each sample as positive (≥2) or negative (< 2) [37]. All of the slides were evaluated by two experienced pathologists without knowledge of the clinicopathological features or clinical outcome.

2.7. Statistical analysis

A chi-square test, Fisher's exact test, or the Kruskal-Wallis test was used for statistical analysis of the IHC results and other variables. OS analyses were performed using the Kaplan-Meier method, the log-rank test, and Cox multi-factor analysis. The OS time was defined as the time from the date of surgery until Dec. 31, 2017 or death, irrespective of the cause of death. A two-sided *p*-value below 0.05 was considered statistically significant. All statistical analyses were performed using SPSS 13.0 for Windows (SPSS Inc., Chicago, Illinois, USA).

3. Results

3.1. HER2 positivity and HER2 gene amplification in EBVaGC and EBVnGC patients

We measured HER2 expression by IHC in 58 EBVaGC and 329 EBVnGC cases that were matched for pTNM classification. Gastric carcinoma samples were defined as high (2+, 3+) and low (0, 1+) expression of HER2 based on IHC results [33]. Only 3 (5.1%) EBVaGC cases showed high expression of HER2. In EBVnGC, HER2 was highly expressed in 86 (26.2%) cases, with 48 cases (14.6%) having an IHC score of 2+ and 38 cases (11.6%) having an IHC score of 3 + . had IHC scores of 2+ or 3+(Table 1). EBVaGC cases exhibited a significantly less expression of HER2 than EBVnGC cases (26.2% *vs*. 5.1%, $\chi^2 = 15.0$, $p = 0.01$).

To analyze *HER2* amplification in EBVaGC and EBVnGC, 50 EBVnGC cases were selected from 329 EBVnGC for further *HER2* FISH study. Clinicopathological features of the 50 EBVnGC cases were statistically matched with that of the 58 EBVaGC cases. This match of clinicopathological features could eliminate the effect of these features on univariate analyzing the relationship between EBV and HER2 amplification. FISH study showed that only 1 EBVaGC case had *HER2* amplification, and 8/50 EBVnGC cases presented *HER2* amplification (1.7% *vs*. 16%, $\chi^2 = 5.417$, $p = 0.02$).

According to ToGA test, IHC2+ and FISH positive or IHC3+ would be defined as HER2 positive [33]. In our cohort, 1/58 EBVaGC and 8/50 EBVnGC were HER2 positive (1.7% *vs*. 16%, $\chi^2 = 5.417$, $p = 0.02$). These results demonstrated that both HER2 expression and *HER2* amplification were inhibited in EBVaGC.

Represented IHC and FISH images of EBER, HER2 expression and *HER2* amplification in cancer cells were presented in Fig. 1.

3.2. p-AKT, p-ERK, and p-PLCγ levels in EBVaGC and EBVnGC

We next measured positivity for p-AKT, p-ERK, and p-PLCγ in EBVaGC and EBVnGC samples. Among the 58 EBVaGC cases, 28 were p-AKT-positive (48.3%), 14 were p-ERK-positive (24.1%), and 22 were p-PLCγ-positive (37.9%). In the 329 EBVnGC cases, these percentages were 62.9%, 45.6%, and 24.6%, respectively. Comparisons indicated that EBVaGC cases had significantly lower positivity for p-AKT ($p = 0.035$) and p-ERK ($p = 0.001$), and a significantly greater positivity for p-PLCγ ($p = 0.034$, Table 3).

The HER2 positive EBVaGC was positive for p-AKT and p-PLCγ, and negative for p-ERK. Two of the three HER2 high expressing EBVaGC(IHC 2+, 3+) were positive for p-AKT and p-PLCγ, and negative for p-ERK. In EBVnGC, p-AKT levels were increased (76.7% *vs*. 58%, $\chi^2 = 9.54$, $p = 0.002$), and p-PLCγ levels were elevated (19.3% *vs*. 39.5%, $\chi^2 = 13.95$, $p < 0.001$) in HER2 high expressing cases comparing with the HER2 low expressing cases. There was no association between HER2 expression and p-ERK levels (Table 2).

Represented IHC images of p-AKT, p-ERK, and p-PLCγ in cancer cells were presented in Fig. 2.

3.3. Association of HER2, p-AKT, p-ERK, and p-PLCγ with clinicopathological data

We next assessed associations of altered protein expression with clinicopathological features. In EBVaGC, p-ERK levels were associated with Lauren subtype ($\chi^2 = 7.89$, $p = 0.023$), and those with the Lauren mixed subtype were more likely to have p-ERK positivity than those with the intestinal subtype ($p = 0.014$) or diffuse subtype ($p = 0.013$; Table 4). Moreover, p-PLCγ positivity as associated with early TNM stage ($\chi^2 = 4.15$, $p = 0.041$) and the absence of tumor lymph node metastasis ($\chi^2 = 4.18$, $p = 0.041$; Table 4). However, p-AKT positivity was not associated with any clinicopathological feature among EBVaGC cases.

In EBVnGC, distant metastasis was associated with HER2 high expression ($\chi^2 = 4.09$, $p = 0.043$). p-AKT positivity was associated with distant metastasis ($p < 0.001$), lymph node metastasis ($p = 0.031$), Lauren subtype ($p = 0.004$), and elder age (age > 60 years old; $p = 0.004$; Table 5). However, p-ERK positivity and p-PLCγ positivity were not associated with any clinicopathological features among the 329 EBVnGC cases (Table 5).

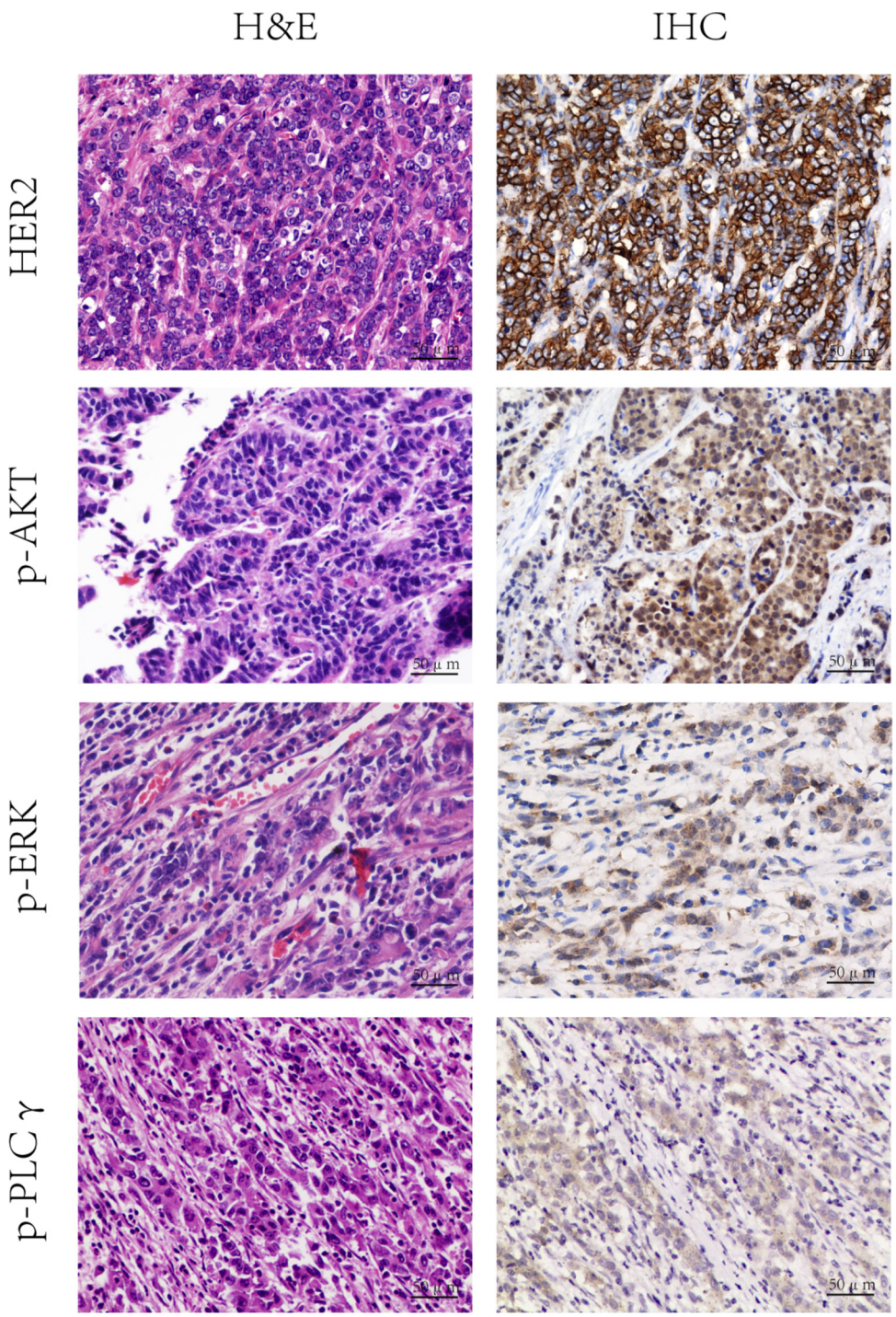

Fig. 2. IHC staining of representative EBVaGC tissue specimens. Each gastric carcinoma tissue was stained with H&E and antibodies against HER2, p-AKT, p-ERK, and p-PLCγ.

3.4. Association of HER2, p-AKT, p-ERK, and p-PLCγ with patient overall survival (OS)

We next determined the association of HER2, p-AKT, p-ERK, and p-PLCγ positivity with OS. We were only able to collect data from 44 of 58 EBVaGC patients and 240 of 329 EBVnGC patients due to loss of contact for some patients. The Kaplan-Meier curves and the log-rank test results showed a significantly better OS in EBVaGC patients than EBVnGC patients ($p = 0.044$; Fig. 3A). Furthermore, analysis of EBVaGC patients indicated p-AKT positivity was associated with significantly worse OS (45.8 ± 9.4 *vs.* 100.9 ± 8.1 months, $p = 0.008$; Fig. 3B), but p-ERK and p-PLCγ positivity had no effect on OS (Fig. 3C-D).

Analysis of EBVnGC patients indicated that poor OS was associated with HER2 positivity (31.4 ± 3.3 *vs.* 63.6 ± 4.1 months, $p < 0.001$; Fig. 4A) p-AKT positivity (40.9 ± 2.7 *vs.* 70.7 ± 5.3 months, $p = 0.010$; Fig. 4B), and p-PLCγ positivity (30.1 ± 3 .1 *vs.* 63.1 ± 4.1 months, $p < 0.001$; Fig. 4D). However, p-ERK positivity had no effect on OS in EBVnGC patients (Fig. 4C).

Our univariate analysis indicated that tumor size ($p = 0.042$), Lauren classification ($p = 0.011$), tumor invasion ($p = 0.024$), lymph node status ($p = 0.018$), distant metastasis ($p < 0.001$), and TNM stage ($p < 0.001$) were all predictors of OS in EBVnGC patients,

Table 4
Association of clinicopathological data from 58 EBVaGC patients with p-AKT, p-ERK, and p-PLCγ.

	No.	p-AKT		p-ERK		p-PLCγ	
		positive N(%)	[a]p	positive N(%)	p	positive N(%)	p
Gender							
Male	53	25(47.2)	0.936	14(26.4)	0.440	22(41.5)	0.145
Female	5	3(60.0)		0		0	
Age (years)							
0～39	9	3(33.3)	0.675	1(11.1)	0.340	5(55.6)	0.504
40～60	30	15(50.0)		6(20.0)		11(36.7)	
61～99	19	10(52.6)		7(36.8)		6(31.6)	
Lauren type							
Intestinal	10	4(40.0)	0.268	1(10.0)	0.023[b]	3(30.0)	0.714
Diffuse	40	18(45.0)		8(20.0)		15(37.5)	
Mix	8	6(75.0)		5(62.5)		4(50.0)	
[c]Tumor size							
0～3cm	14	8(57.1)	0.399	5(35.7)	0.338	6(42.9)	0.706
＞3cm	43	19(44.2)		8(18.6)		16(37.2)	
pTNM							
I + II	22	3(59.1)	0.198	7(31.8)	0.285	12(54.5)	0.041[b]
III + IV	36	15(41.7)		7(19.4)		10(27.8)	
Invasion							
T1＋T2	7	6(85.7)	0.087	2(28.6)	1.000	2(28.6)	0.897
T3＋T4	51	22(43.1)		12(23.5)		20(39.2)	
Lymph node metastasis							
Absent	15	9(60.0)	0.291	4(26.7)	1.000	9(60.0)	0.041[b]
Present	43	19(44.2)		10(23.3)		13(30.2)	
Distant metastasis							
Absent	49	26(53.1)	0.181	12(24.5)	1.000	6(42.9)	0.706
Present	9	2(22.2)		2(22.2)		16(37.2)	

[a] analyzed by using Pearson Chi-Square test, Continuity Correction, or Fisher's exact test.
[b] Considered to be statistically significant.
[c] One EBVaGC case without data on tumor size.

Table 5
Association of clinicopathological data from 329 EBVnGC patients with p-AKT, p-ERK or p-PLCγ.

	No.	HER2 IHC2/3＋ N (%)	p-AKT positive N(%)	p-ERK positive N(%)	p-PLCγ positive N (%)
Gender					
Male	223	58(26.0)	142(63.7)	102(45.7)	50(22.4)
Female	106	28(26.4)	65(61.3)	48(45.3)	31(29.2)
[a]p		0.938	0.679	0.938	0.179
Age (years)					
0～39	22	5(22.7)	14(63.6)	14(63.6)	7(31.8)
40～60	137	34(24.8)	72(52.6)	67(48.9)	33(24.1)
61～99	170	47(27.6)	121(71.2)	69(40.6)	41(24.1)
p		0.796	0.004[b,c]	0.074	0.720
Lauren type					
Intestinal	220	57(25.9)	149(67.7)	93(42.3)	51(23.2)
Diffuse	105	26(24.8)	54(51.4)	54(51.4)	28(26.7)
Mix	4	3(75.0)	4(100)	3(75.0)	2(50.0)
p		0.099	0.004[b]	0.152	0.291
[c]Tumor size					
0～3cm	106	24(22.6)	69(65.1)	48(45.3)	27(25.5)
＞3cm	218	62(28.4)	135(61.9)	98(45.0)	52(23.9)
p		0.267	0.580	0.955	0.750
pTNM					
I + II	137	35(25.5)	80(58.4)	56(40.9)	35(25.5)
III + IV	192	51(26.6)	127(66.1)	94(49.0)	46(24.0)
p		0.836	0.151	0.147	0.742
Invasion					
T1＋T2	82	21(25.6)	47(57.3)	31(37.8)	21(25.6)
T3＋T4	247	65(26.3)	160(64.8)	119(48.2)	60(24.3)
p		0.900	0.226	0.102	0.810
Lymph node metastasis					
Absent	98	22(22.4)	53(54.1)	43(43.9)	27(27.4)
Present	231	64(27.7)	154(66.7)	107(46.3)	54(23.4)
p		0.321	0.031[b,c]	0.684	0.422
Distant metastasis					
Absent	272	65(23.9)	159(58.5)	122(44.9)	62(22.8)
Present	57	21(36.8)	48(84.2)	28(49.1)	19(33.3)
p		0.043[b,c]	＜0.001[b,c]	0.556	0.093

[a] Analyzed by using Pearson Chi-Square test, Continuity Correction, or Fisher's exact test.
[b] Five EBVnGC cases without data on tumor size.
[c] Considered to be statistically significant.

although other clinicopathological data, such as gender and age, had no impact (Table 6). The multivariate analysis showed that distant metastasis (hazard ratio [HR]: 2.506; 95% confidence interval [CI]: 1.559 to 4.028, $p < 0.001$), HER2 positivity (HR: 1.611; 95% CI: 1.058 to 2.454, $p = 0.026$), and p-PLCγ positivity (HR: 1.603; 95% CI: 1.056 to 2.435, $p = 0.027$) were independent prognostic predictors of OS in EBVnGC patients (Table 6).

4. Discussion

Based on a cohort containing 58 EBVaGC and pTNM classification-matched 329 EBVnGC (p = 0.596), we suggested that both HER2 protein expression and gene amplification in EBVaGC were reduced, compared with EBVnGC. pTNM-matching was crucial for eliminating the influence of tumor stage when detecting the relevance among EBV positive, HER2 expression and HER2 downstream pathway activity.

Previous studies also demonstrated that EBVaGC less expressed HER2 [38]. To date, only few studies have reported *HER2* amplification in EBVaGC. In particular, the Cancer Genome Atlas (TCGA) Research Network observed 17% of patients with *HER2* amplification among 295 GC patients using high-throughput DNA sequencing, and 12% of 27 EBVaGC patients had *HER2* amplification [12]. Our current study showed that only 1 of 58 EBCaGC patients (1.7%) had *HER2* amplification, much less than 12% observed in the TCGA project. This inconsistency might be due to differences in the study populations and/or the use of different detection technologies. Additionally, the rarity of *HER2* amplification in EBVaGC patients implies that EBV might inhibit *HER2* amplification, although the potential mechanisms remain to be elucidated. Our previous study demonstrated that LMP2A, an EBV latency protein, inhibited the expression of *HER2* through downregulation of the TWIST and YB-1 pathways [19]. This finding may partly explain why EBVaGC patients had lower HER2 expression than EBVnGC patients.

In present study, we observed that EBVaGC patients with *HER2* amplification had increased positivity for p-AKT and p-PLCγ, but lower positivity for p-ERK. Among EBVnGC cases positivity for p-AKT and p-PLCγ were associated with HER2 positivity. This finding suggests that the HER2 protein may preferentially induce the PI3K/AKT or PLCγ/PKC pathway, rather than the RAS/MAPK pathway in GC. Previous studies reported that different phosphorylation sites within the cytoplasmic domain of HER2 led to its activation [24,27], which in turn activated the RAS/MAPK pathway. Indeed, the most common HER2 phosphorylation sites (Tyrosine 1139, 1222, and 1248) are associated with activation of the RAS/MAPK pathway, while phosphorylation at Tyr1196 is associated with activation of the PI3K/AKT pathway [17]. These results should be cautiously considered when treating GC patients with lapatinib, an anti-HER2 dual tyrosine kinase inhibitor [39].

We also identified a trend in EBVaGC patients of presenting lower p-AKT positivity (48.3% *vs.* 62.9%) and p-ERK positivity (24.1% *vs.* 45.6%), but higher p-PLCγ positivity (37.9% *vs.* 24.6%) than EBVnGC patients. No previous studies have systematically analyzed the association between EBV and HER2 downstream pathways in GC. Only Sukawa et al. reported no statistically significant difference in p-AKT expression among 18 EBVaGC cases and 204 EBVnGC cases [21]. Expression of p-ERK and p-PLCγ in patients with EBVaGC has not been reported previously. We hypothesis that the high rate of p-PLCγ

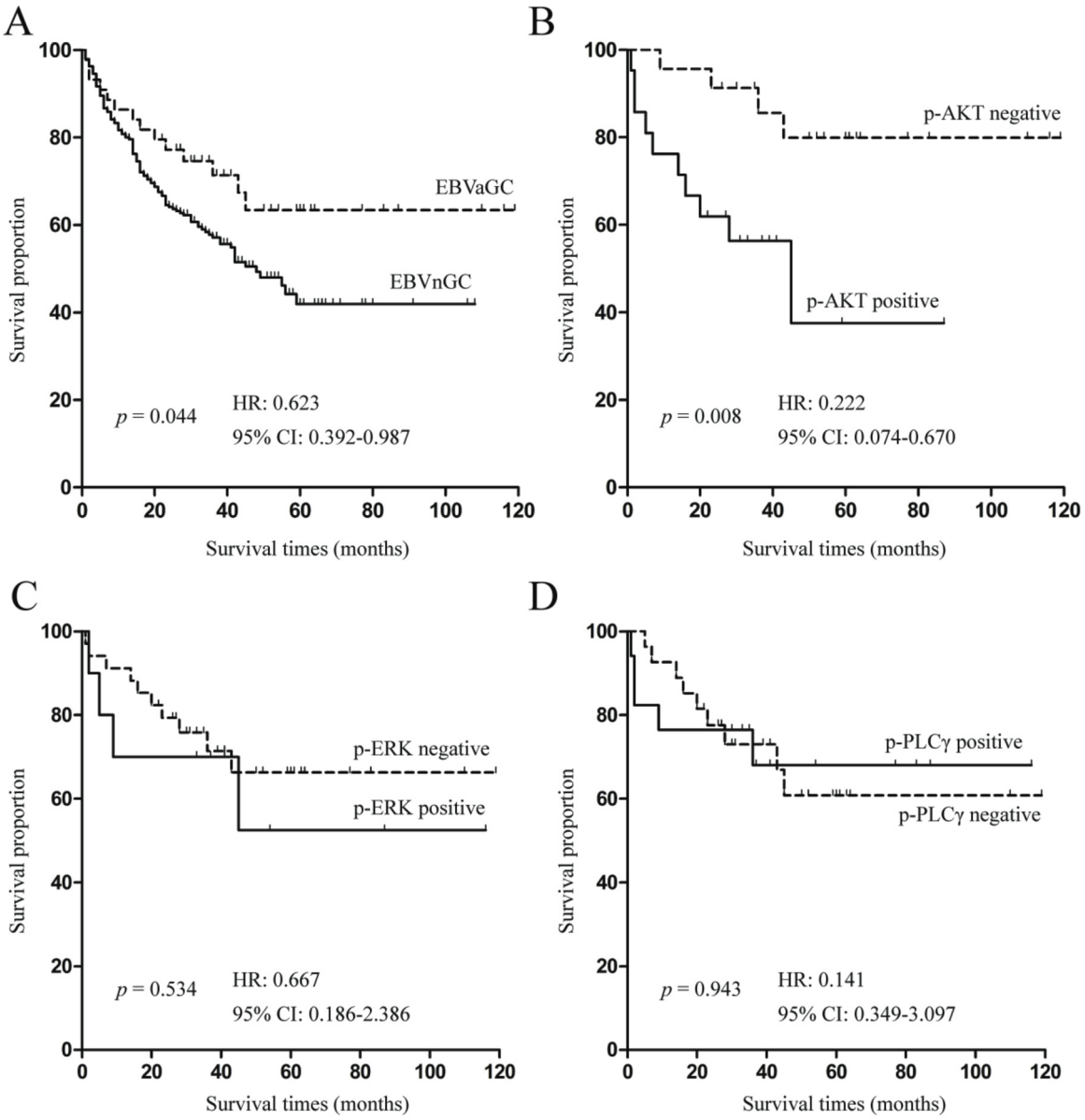

Fig. 3. Kaplan-Meier curves and the log-rank test of overall survival (OS) stratified by positivity for different markers in patients with EBVaGC (n = 44) and EBVnGC (n = 240). (A) OS in EBVnGC *vs.* EBVaGC cases. (B) OS in EBVaGC patients stratified by p-AKT positivity. (C) OS in EBVaGC patients stratified by p-ERK positivity. (D) OS in EBVaGC patients stratified by p-PLCγ positivity. Here and below: HR, hazard ratio; CI, confidence interval.

positivity in our EBVaGC cases may be due to activated PLCγ in EBV-transformed human B cell lines [40], consistent with the report that LMP1 activated the PLC/PKC pathway in NPC [41,42].

None studies had reported the relationship between p-AKT, p-ERK or pPLCγ expression with clinicopathological features or prognosis in EBVaGC. We observed no associations between p-AKT positivity and the clinicopathological features of EBVaGC patients, even though p-AKT-positive EBVaGC cases had worse OS. In EBVnGC, Cinti et al. showed that high p-AKT expression was associated with depth of infiltration, positive lymph nodes, and poor clinical outcome [43]. Other research reported that activated AKT signaling predicted worse clinical outcome of GC patients [21,28]. Meanwhile, Murakami et al. reported p-AKT positivity in 81 (58%) of 140 GC patients, but found no associations of p-AKT positivity with clinicopathological features of these patients [44]. Our present study identified an association of p-AKT positivity with worse OS in both EBVaGC and EBVnGC.

Moreover, activation of PLCγ pathway was considered to promote tumor cell growth and migration. In breast cancer, studies showed that PLCγ activation contributed to metastasis [36,45,46] and a study of oral squamous cell carcinoma also showed that PLCγ activation contributed to the development of this cancer [47]. In EBVnGC, we found that patients with p-PLCγ expression presented worse OS, and the Cox multivariate analysis confirmed p-PLCγ as an independent prognostic predictor for these patients. However, in EBVaGC, p-PLCγ positivity was inversely associated with pTNM stage and lymph node metastasis, but not with OS. These EBVaGC data are novel, and may indicate a special influence from EBV on PLCγ pathway. Further study is needed to further clarify the role of PLCγ in EBVaGC.

In conclusion, our current study validated that EBV inhibit HER2 expression and gene amplification in GC. HER2 preferentially activates the AKT and PLC pathways over the MAPK pathway in EBVnGC. Relative to EBVnGC patients, EBVaGC patients had a lower rate of positivity for HER2 downstream signaling molecules (p-AKT and p-ERK), but a higher rate of positivity for p-PLCγ. Moreover, p-AKT positivity is associated with worse clinical outcome in EBVaGC patients, and HER2, p-AKT, and p-PLCγ are prognostic biomarkers for OS in

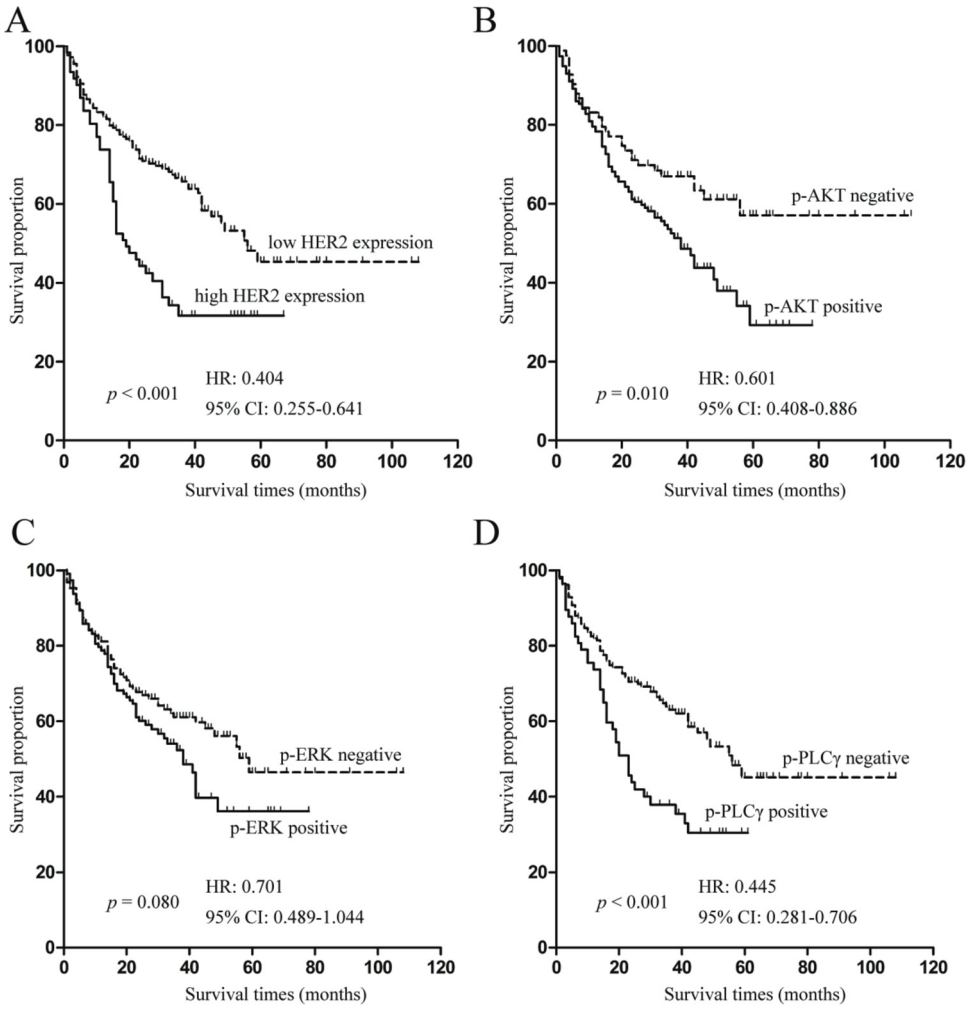

Fig. 4. Kaplan-Meier curves and the log-rank test of overall survival (OS) stratified by positivity for different markers in patients with EBVnGC (n = 240). (A) OS stratified by HER2 positivity. (B) OS stratified by p-AKT positivity. (C) OS stratified by p-ERK positivity. (D) OS stratified by p-PLCγ positivity.

Table 6
Univariate and Multivariate analyses of overall survival in EBVaGC and EBVnGC.

	Univariate analysis		Multivariate Cox regression survival analysis in EBVnGC			
	EBVaGC (N = 44)	EBVnGC (N = 240)	p-values	HR	95% CI	
					Lower	Upper
Gender	p = 0.971	p = 0.905				
Age (years)	p = 0.146	p = 0.064				
Lauren type	p = 0.032	p = 0.011[a]	0.919	0.981	0.674	1.426
Tumor size	p = 0.674	p = 0.042[a]	0.573	1.154	0.701	1.900
pTNM	p = 0.078	p < 0.001[a]				
Invasion	p = 0.527	p = 0.024[a]	0.199	1.473	0.816	2.659
Lymph node metastasis	p = 0.054	p = 0.018[a]	0.502	1.185	0.721	1.948
Distant metastasis	p = 0.737	p < 0.001[a]	< 0.001[a]	2.506	1.559	4.028
HER2 expression	NA	p < 0.001[a]	0.026[a]	1.611	1.058	2.454
p-AKT expression	p = 0.008[a]	p = 0.010[a]	0.104	1.438	0.928	2.228
p-ERK expression	p = 0.534	p = 0.080				
p-PLCγ expression	p = 0.948	p < 0.001[a]	0.027[a]	1.603	1.056	2.435

Abbreviations: NA, not available.
[a] Considered to be statistically significant.

EBVnGC patients. Further investigation is needed to determine the exact roles of EBV, HER2, AKT, ERK, and PLCγ in the pathogenesis of GC, and to validate their use as biomarkers for the early detection and prognosis of GC.

Funding support

This work was supported in part by grants from the Guangzhou Science and Technology Project (#201707010119), Guangdong Province, China, the Natural Science Foundation of Guangdong Province (#2017A030313502 and #2014A030313034) and the National Natural Science Foundation of China (#81572309).

Authors' contributions

Chun-kui Shao conceived and designed the experiments; Yi-wang Zhang and Dan He prepared the manuscript; Yi-wang Zhang, Dan He, Cui Tan, and Lu Zhou performed the experiments; Yi-wang Zhang and Min Dong analyzed the data.

Declaration of Competing Interest

The authors declare that there is no conflict of interest in this work.

Acknowledgements

All work was completed at Sun Yat-sen University, China. We thank our study team for their support during the writing of this manuscript.

References

[1] L.A. Torre, F. Bray, R.L. Siegel, J. Ferlay, J. Lortet-Tieulent, A. Jemal, Global cancer statistics, 2012, CA Cancer J. Clin. 65 (2015) 87–108.
[2] D.M. Parkin, The global health burden of infection-associated cancers in the year 2002, Int. J. Cancer 118 (2006) 3030–3044.
[3] M. Plummer, S. Franceschi, J. Vignat, D. Forman, C. de Martel, Global burden of gastric cancer attributable to Helicobacter pylori, Int. J. Cancer 136 (2015) 487–490.
[4] M.H. Ebell, JAMA PATIENT PAGE. Infectious mononucleosis, JAMA 315 (2016) 1532.
[5] M.P. Thompson, R. Kurzrock, Epstein-Barr virus and cancer, Clin. Cancer Res. 10 (2004) 803–821.
[6] L.S. Young, A.B. Rickinson, Epstein-Barr virus: 40 years on. Nature reviews, Cancer 4 (2004) 757–768.
[7] J.H. Lee, S.H. Kim, S.H. Han, J.S. An, E.S. Lee, Y.S. Kim, Clinicopathological and molecular characteristics of Epstein-Barr virus-associated gastric carcinoma: a meta-analysis, J. Gastroenterol. Hepatol. 24 (2009) 354–365.
[8] M. Fukayama, T. Ushiku, Epstein-Barr virus-associated gastric carcinoma, Pathol. Res. Pract. 207 (2011) 529–537.
[9] J.N. Chen, Y.G. Ding, Z.Y. Feng, H.G. Li, D. He, H. Du, B. Wu, C.K. Shao, Association of distinctive Epstein-Barr virus variants with gastric carcinoma in Guangzhou, southern China, J. Med. Virol. 82 (2010) 658–667.
[10] D. Shibata, L.M. Weiss, Epstein-Barr virus-associated gastric adenocarcinoma, Am. J. Pathol. 140 (1992) 769–774.
[11] J. Han, J.N. Chen, Z.G. Zhang, H.G. Li, Y.G. Ding, H. Du, C.K. Shao, Sequence variations of latent membrane protein 2A in Epstein-Barr virus-associated gastric carcinomas from Guangzhou, southern China, PLoS One 7 (2012) e34276.
[12] N. Cancer Genome Atlas Research, Comprehensive molecular characterization of gastric adenocarcinoma, Nature 513 (2014) 202–209.
[13] M. Uhlen, C. Zhang, S. Lee, E. Sjostedt, L. Fagerberg, G. Bidkhori, R. Benfeitas, M. Arif, Z. Liu, F. Edfors, K. Sanli, K. von Feilitzen, P. Oksvold, E. Lundberg, S. Hober, P. Nilsson, J. Mattsson, J.M. Schwenk, H. Brunnstrom, B. Glimelius, T. Sjoblom, P.H. Edqvist, D. Djureinovic, P. Micke, C. Lindskog, A. Mardinoglu, F. Ponten, A pathology atlas of the human cancer transcriptome, Science 357 (2017).
[14] C. Gravalos, A. Jimeno, HER2 in gastric cancer: a new prognostic factor and a novel therapeutic target, Ann. Oncol. 19 (2008) 1523–1529.
[15] J. Meza-Junco, H.J. Au, M.B. Sawyer, Critical appraisal of trastuzumab in treatment of advanced stomach cancer, Cancer Manag. Res. 3 (2011) 57–64.
[16] J. Ruschoff, W. Hanna, M. Bilous, M. Hofmann, R.Y. Osamura, F. Penault-Llorca, M. van de Vijver, G. Viale, HER2 testing in gastric cancer: a practical approach, Mod. Pathol. 25 (2012) 637–650.
[17] J.W. Liang, J.J. Zhang, T. Zhang, Z.C. Zheng, Clinicopathological and prognostic significance of HER2 overexpression in gastric cancer: a meta-analysis of the literature, Tumour Biol. 35 (2014) 4849–4858.
[18] C.Y. Ock, K.W. Lee, J.W. Kim, J.S. Kim, T.Y. Kim, K.H. Lee, S.W. Han, S.A. Im, T.Y. Kim, W.H. Kim, Y.J. Bang, D.Y. Oh, Optimal patient selection for Trastuzumab treatment in HER2-positive advanced gastric cancer, Clin. Cancer Res. 21 (2015) 2520–2529.
[19] Y.W. Zhang, X.X. Zhao, C. Tan, Z.G. Zhang, Y. Jiang, J.N. Chen, H.B. Wei, L. Xue, H.G. Li, H. Du, C.K. Shao, Epstein-Barr virus latent membrane protein 2A suppresses the expression of HER2 via a pathway involving TWIST and YB-1 in Epstein-Barr virus-associated gastric carcinomas, Oncotarget 6 (2015) 207–220.
[20] H.S. Lee, M.S. Chang, H.K. Yang, B.L. Lee, W.H. Kim, Epstein-barr virus-positive gastric carcinoma has a distinct protein expression profile in comparison with epstein-barr virus-negative carcinoma, Clin. Cancer Res. 10 (2004) 1698–1705.
[21] Y. Sukawa, H. Yamamoto, K. Nosho, H. Kunimoto, H. Suzuki, Y. Adachi, M. Nakazawa, T. Nobuoka, M. Kawayama, M. Mikami, T. Matsuno, T. Hasegawa, K. Hirata, K. Imai, Y. Shinomura, Alterations in the human epidermal growth factor receptor 2-phosphatidylinositol 3-kinase-v-Akt pathway in gastric cancer, World J. Gastroenterol. 18 (2012) 6577–6586.
[22] H.J. Song, A. Srivastava, J. Lee, Y.S. Kim, K.M. Kim, W. Ki Kang, M. Kim, S. Kim, C.K. Park, S. Kim, Host inflammatory response predicts survival of patients with Epstein-Barr virus-associated gastric carcinoma, Gastroenterology 139 (2010) 84–92 e82.
[23] D.J. Slamon, G.M. Clark, S.G. Wong, W.J. Levin, A. Ullrich, W.L. McGuire, Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene, Science 235 (1987) 177–182.
[24] R. Roskoski, The ErbB/HER family of protein-tyrosine kinases and cancer, Pharmacol. Res. 79 (2014) 34–74.
[25] S. Kaptain, L.K. Tan, B.Y. Chen, Her-2/neu and breast cancer, Diagn. Mol. Pathol. 10 (2001) 139–152.
[26] C. Lohrisch, M. Piccart, An overview of HER2, Semin. Oncol. 28 (2001) 3–11.
[27] A. Dittrich, H. Gautrey, D. Browell, A. Tyson-Capper, The HER2 signaling network in breast cancer-like a spider in its web, J. Mammary Gland Biol. 19 (2014) 253–270.
[28] Y. Sukawa, H. Yamamoto, K. Nosho, M. Ito, H. Igarashi, T. Naito, K. Mitsuhashi, Y. Matsunaga, T. Takahashi, M. Mikami, Y. Adachi, H. Suzuki, Y. Shinomura, HER2 expression and PI3K-Akt pathway alterations in gastric cancer, Digestion 89 (2014) 12–17.
[29] P. Lauren, The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. An attempt at a histo-clinical classification, Acta Pathol. Microbiol. Scand. 64 (1965) 31–49.
[30] K. Washington, 7th edition of the AJCC cancer staging manual: stomach, Ann. Surg. Oncol. 17 (2010) 3077–3079.
[31] M. Kimura, H. Tsuda, D. Morita, T. Ichikura, S. Ogata, S. Aida, Y. Yoshizumi, T. Maehara, H. Mochizuki, O. Matsubara, A proposal for diagnostically meaningful criteria to classify increased epidermal growth factor receptor and c-erbB-2 gene copy numbers in gastric carcinoma, based on correlation of fluorescence in situ hybridization and immunohistochemical measurements, Virchows Arch. 445 (2004) 255–262.
[32] H.M. Lourenco, T.P. Pereira, R.R. Fonseca, P.M. Cardoso, HER2/Neu detection by immunohistochemistry optimization of in-house protocols, Appl. Immunohistochem. Mol. Morphol. 17 (2009) 151–157.
[33] Y.J. Bang, E. Van Cutsem, A. Feyereislova, H.C. Chung, L. Shen, A. Sawaki, F. Lordick, A. Ohtsu, Y. Omuro, T. Satoh, G. Aprile, E. Kulikov, J. Hill, M. Lehle, J. Ruschoff, Y.K. Kang, G.A.T.I. To, Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial, Lancet 376 (2010) 687–697.
[34] O. Tapia, I. Riquelme, P. Leal, A. Sandoval, S. Aedo, H. Weber, P. Letelier, E. Bellolio, M. Villaseca, P. Garcia, J.C. Roa, The PI3K/AKT/mTOR pathway is activated in gastric cancer with potential prognostic and predictive significance, Virchows Arch. 465 (2014) 25–33.
[35] Y.Y. Shao, C.L. Chen, M.C. Ho, C.C. Huang, H.C. Tu, C.H. Hsu, A.L. Cheng, Dissimilar immunohistochemical expression of ERK and AKT between paired biopsy and hepatectomy tissues of hepatocellular carcinoma, Anticancer Res. 32 (2012) 4865–4870.
[36] R. Lattanzio, M. Marchisio, R. La Sorda, N. Tinari, M. Falasca, S. Alberti, S. Miscia, C. Ercolani, A. Di Benedetto, L. Perracchio, E. Melucci, S. Iacobelli, M. Mottolese, P.G. Natali, M. Piantelli, Cinbo, overexpression of activated phospholipase C gamma 1 is a risk factor for distant metastases in T1-T2, N0 breast cancer patients undergoing adjuvant chemotherapy, Int. J. Cancer 132 (2013) 1022–1031.
[37] A. Reiner, B. Neumeister, J. Spona, G. Reiner, M. Schemper, R. Jakesz, Immunocytochemical localization of estrogen and progesterone receptor and prognosis in human primary breast cancer, Cancer Res. 50 (1990) 7057–7061.
[38] S.I. Hay, S.P. Jayaraman, T. Truelsen, R.J.D. Sorensen, A. Millear, G. Giussani, E. Beghi, GBD 2015 disease and injury incidence and prevalence collaborators. Global, regional, and national incidence, prevalence, and years lived with disability for 310 diseases and injuries, 1990–2015: a systematic analysis for the Global Burden of Disease Study 2015 (vol 388, pg 1545, 2016), Lancet 389 (2017) E1.
[39] K. Imami, N. Sugiyama, H. Imamura, M. Wakabayashi, M. Tomita, M. Taniguchi, T. Ueno, M. Toi, Y. Ishihama, Temporal profiling of lapatinib-suppressed phosphorylation signals in EGFR/HER2 pathways, Mol. Cell Proteomics 11 (2012) 1741–1757.
[40] A. Kuruvilla, G. Putcha, E. Poulos, W.T. Shearer, Tyrosine phosphorylation of phospholipase C concomitant with its activation by platelet-activating factor in a human B cell line, J. Immunol. 151 (1993) 637–648.
[41] W. Luo, G. Yan, L. Li, Z. Wang, H. Liu, S. Zhou, S. Liu, M. Tang, W. Yi, Z. Dong, Y. Cao, Epstein-Barr virus latent membrane protein 1 mediates serine 25 phosphorylation and nuclear entry of annexin A2 via PI-PLC-PKCalpha/PKCbeta pathway, Mol. Carcinog. 47 (2008) 934–946.
[42] H. Zheng, L.L. Li, D.S. Hu, X.Y. Deng, Y. Cao, Role of Epstein-Barr virus encoded latent membrane protein 1 in the carcinogenesis of nasopharyngeal carcinoma, Cell. Mol. Immunol. 4 (2007) 185–196.
[43] C. Cinti, C. Vindigni, A. Zamparelli, D. La Sala, M.C. Epistolato, D. Marrelli, G. Cevenini, P. Tosi, Activated Akt as an indicator of prognosis in gastric cancer, Virchows Arch. 453 (2008) 449–455.
[44] D. Murakami, S. Tsujitani, T. Osaki, H. Saito, K. Katano, S. Tatebe, M. Ikeguchi, Expression of phosphorylated Akt (pAkt) in gastric carcinoma predicts prognosis and efficacy of chemotherapy, Gastric Cancer 10 (2007) 45–51.
[45] H.J. Nam, J.K. Kang, J.S. Chang, M.S. Lee, S.T. Nam, H.W. Jung, S.K. Kim, E.M. Ha, H. Seok, S.W. Son, Y.J. Park, H. Kim, Cells transformed by PLC-Gamma 1 overexpression are highly sensitive to clostridium difficile toxin A-induced apoptosis and mitotic inhibition, J Microbiol Biotechn 22 (2012) 50–57.
[46] E. Charafe-Jauffret, C. Ginestier, F. Iovino, J. Wicinski, N. Cervera, P. Finetti, M.H. Hur, M.E. Diebel, F. Monville, J. Dutcher, M. Brown, P. Viens, L. Xerri, F. Bertucci, G. Stassi, G. Dontu, D. Birnbaum, M.S. Wicha, Breast cancer cell lines contain functional cancer stem cells with metastatic capacity and a distinct molecular signature, Cancer Res. 69 (2009) 1302–1313.
[47] L.W. Ma, Z.T. Zhou, Q.B. He, W.W. Jiang, Phospholipase C-gamma 1 expression correlated with cancer progression of potentially malignant oral lesions, J. Oral Pathol. Med. 42 (2013) 47–52.