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Original Article

Value of CagA, HER2, ALDH1, and KiSS-1 in predicting metastasis and prognosis for gastric adenocarcinoma

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Abstract: Background: Cytotoxin-associated gene A (CagA), is able to translocate into gastric epithelial cells. Human epidermal growth factor receptor 2 (also named as HER2, is a proto-oncogene which can encode a transmembrane receptor), Aldehyde dehydrogenase 1 (ALDH1, a biomarker of cancer stem cells), and KiSS-1 (a suppressor gene of cancer metastasis) are all valuably predictive biomarkers for various human cancers. The purpose of this study is to investigate the associations among CagA, HER2, ALDH1, and KiSS-1 in gastric adenocarcinoma (GAC), and their respective associations with clinical characteristics and survival in GAC. Methods: The expression of CagA, HER2, ALDH1, and KiSS-1 in 232 cases of whole GAC tissues were detected by immunohistochemical staining. Patient clinical and survival data were also collected. Results: Positive expression of CagA, HER2, and ALDH1 is significantly higher, and positive expression of KiSS-1 is significantly lower, in GAC tissues than in the corresponding normal tissues. Furthermore, the positive expression of CagA, HER2, ALDH1, and KiSS-1 were significantly associated with tumor grade, tumor stage, lymph node metastasis (LNM) stage, and tumor node metastasis (TNM) stages, and with patients' overall survival (OS); whereas the KiSS-1 positive group had longer OS than did the KiSS-1 negative group. In logistic analysis, positive expression of CagA, HER2, ALDH1, and KiSS-1 are significantly associated with LNM of patients with GAC. COX regression analysis indicated that positive expression of CagA, HER2, ALDH1, and KiSS-1, and tumor stages, LNM stages, and TNM stages were independent prognostic factors for patients with GAC. Conclusions: Expression of CagA, HER2, ALDH1, and KiSS-1 should be considered as promising biomarkers for metastasis and prognosis, as well as potential therapeutic targets for GAC.

Keywords: Gastric adenocarcinoma, CagA, HER2, ALDH1, KiSS-1, metastasis, prognosis

Introduction

Gastric cancer is the third most lethal cancer worldwide and the second most lethal cancer in China [1, 2]. Gastric adenocarcinoma (GAC) is the most common type of gastric cancer, and accounts for approximate 90% of all diagnosed cancers. Cancer recurrence and metastasis are the main reasons for cancer treatment failure.

Cytotoxin-associated gene A (CagA) is able to translocate into gastric epithelial cells. CagA is able to promote tyrosine phosphorylation on the specific EPIYA sequence by Src family tyrosine kinases in the host cells [3-5]. CagA is the most common virulence factor of *H. pylori* and plays an important role in *H. pylori* mediated carcinogenesis in the stomach [3, 6]. CagA also promotes aberrant gastric epithelial cell proliferation, cytoskeletal abnormalities, and suppresses apoptosis [5, 6]. The latest study has suggested that CagA has prognostic and therapeutic significance [3].

Human epidermal growth factor receptor 2, also known as HER2, is a transmembrane protein with tyrosine kinase activity implicated in cell growth and differentiation [7]. Overexpression of HER2 in GAC is closely related to poor prognosis [8]. In metastatic or recurrent GAC, the standard treatment is chemotherapy. Despite advances in chemotherapy treatment, the outcome of GAC is still poor. Recently, the ToGA study indicated that targeting HER2 combined with a chemotherapy regimen significantly prolonged OS of patients with advanced GAC when compared with the same chemotherapy alone [9]. Now, targeting HER2 regimen is considered a standard treatment for patients with HER2+GAC.

Table 1. Patient characteristics

Characteristic	Frequency (n)	Percentage (%)
Gender		
Male	148	63.8
Female	84	36.2
Ages		
≤60	121	52.2
>60	111	47.8
Size		
≤2.0 cm	53	22.8
>2.0 cm, ≤5.0 cm	147	63.4
>5.0 cm	32	13.8
Location		
Antrum	119	51.3
Cardis	85	36.6
Pylorus	28	12.1
Gross type		
Polypoid	21	9.1
Ulcerative	164	70.7
Infiltrating	47	20.3
Alcohol		
No	108	46.6
Yes	124	53.4
Smoking		
No	123	53.0
Yes	109	47.0
Tumor stages		
Submucosa	25	10.8
Muscularis	64	27.6
Serosa	131	56.5
Visceral peritoneum	12	5.2
Grade		
Well	34	14.7
Moderate	139	59.9
Poor	59	25.4
Lymph node metastasis		
N0	112	48.3
N1	57	24.6
N2	55	23.7
N3	8	3.4
TNM stage		
I	78	33.6
II	99	42.7
III	55	23.7

Cancer recurrence and metastasis are also closely associated with a subpopulation of tumor cells that are defined as cancer stem cells (CSCs). The capabilities of CSCs are self-renewal, multi-directional differentiation potential, and natural resistance to chemo- or radio-therapy [11, 12]. CSCs also initiate heterogeneous tumor cells that compose tumors [13]. Aldehyde dehydrogenase 1 (ALDH1) is an important member of ALDHs family that are located in the cytoplasm, mitochondria, and nucleus [14]. ALDH1 is involved in tumor cell proliferation, differentiation, invasion, metastasis, survival, and oxidative stress [12, 15, 16]. Accumulating studies have demonstrated that ALDH1 should be considered a valuable biomarker for predicting cancer metastasis and prognosis in various cancers [12, 14-18].

Inactivation of metastasis suppressor genes promotes cancer cell invasiveness and metastasis. KiSS-1, a suppressor gene of tumor metastasis, is located on chromosome 1q32 and was originally identified in melanoma by analysis of subtractive hybridization [19]. KiSS-1 gene consists of 6151 base pairs in length and encodes a 145-amino-acid peptide. KiSS-1 is involved in cell proliferation, motility, invasion, and metastasis through binding GPR54 or KiSS-1R [20]. KiSS-1 inhibits tumor metastasis by promoting E-cadherin expression and suppressing MMP expression [21, 22]. Therefore, aberrant expression of KiSS-1 should promote tumor invasion and metastasis [12, 21-23].

Overall, studies of CagA, HER2, ALDH1, and KiSS-1 in relation to metastasis and prognosis indicated that these biomarkers should influence tumor development. However, associations among CagA, HER2, ALDH1, and KiSS-1 in GAC have not yet been extensively reported. The purpose of this study is to explore the hypothesis that these biomarkers are mutually associated and are associated with metastasis and prognosis in GAC.

Material and methods

Specimens

All 232 GAC tissues and surrounding "normal" gastric mucosa tissues were collected from the Department of Pathology of the First Affiliated Hospital of Bengbu Medical College from January 2011 to December 2012. All patients underwent radical resection and lymph node dissection (patients who underwent any preoperative anti-cancer therapy were excluded). The "normal" gastric mucosa tissues were removed from the same patients and from surrounding gastric mucosa tissues at least 5 cm away from

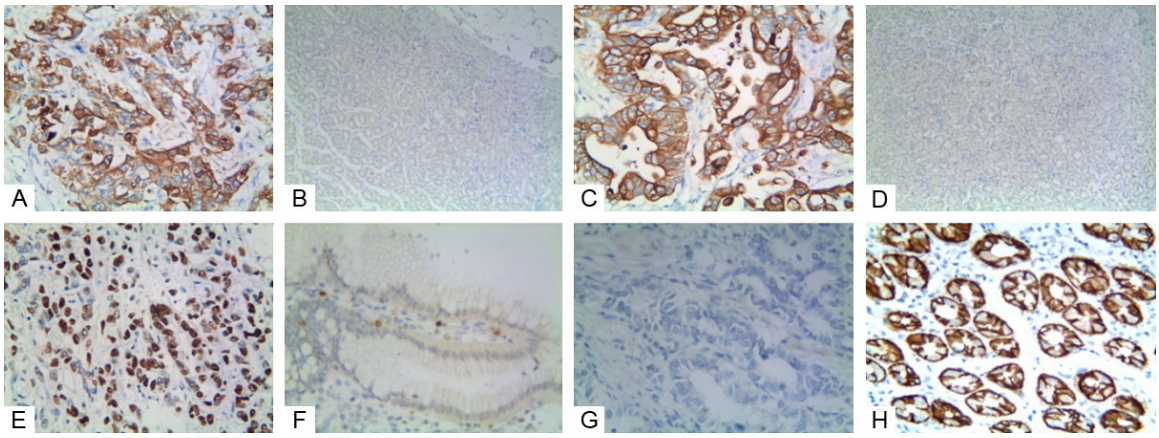

Figure 1. Expression of markers in gastric adenocarcinoma. A. Positive CagA expression in the cytoplasm of cancer cells (×400 magnification). B. Negative CagA expression in the "normal" gastric mucosa epithelial cells (×100 magnification). C. Positive HER2 expression in the membrane of cancer cells (×400 magnification). D. Negative HER2 expression in the "normal" gastric mucosa epithelial cells (×100 magnification). E. Positive ALDH1 expression in the cytoplasm and nucleus of cancer cells (×400 magnification). F. Negative ALDH1 expression in the "normal" gastric mucosa epithelial cells (×400 magnification). G. Negative expression of KiSS-1 in the cancer cells (×400 magnification). H. Positive expression of KiSS-1 in the cytoplasm of "normal" gastric mucosa epithelial cells (×400 magnification).

the cancer edge. All specimens were obtained with patients writing consent. All patients had complete demographic, pathological, and follow-up data (at 6 months intervals by mobile phone and social applications). Overall survival (OS) was calculated from surgery date to death date or December 2017 (mean OS: 48.7 months, range 10-83 months). This study was authorized by the ethics committee of Bengbu Medical College and carried out in accordance with the guidelines of the Declaration of Helsinki. Tumor stages and TNM stages were evaluated in accordance with the 7th edition of the American Joint Committee on Cancer (AJCC). Differentiation of tumor was assessed in accordance with World Health Organization (WHO) standards. Patient characteristics are shown in **Table 1**.

Immunohistochemistry

Immunohistochemical staining was carried out in accordance with the Elivision™ Plus detection kit instructions (LabVision, USA). All GAC and corresponding "normal" gastric mucosal tissues were fixed in 10% buffered formalin, then embedded in paraffin and continuous 4-μm-thick sections were cut. Subsequently, all slices were deparaffinized and dehydrated in xylene and graded alcohol. All sections were washed with phosphate buffer saline (PBS, pH 7.2) and incubated in methanol containing 3% H_2O_2 at room temperature for 10 min for quenching endogenous peroxidase activity. Following, all sections were washed with PBS and placed in citrate buffer (pH 6.0) and heated to 95°C for 30 min to repair antigen. Then, all sections were washed with PBS several times and quenched with goat serum at room temperature for 30 min. After washing with PBS, all sections were incubated with mouse monoclonal antibody against human CagA (Abcam, USA), HER2 (Roche, Swiss), ALDH1 (Abcam, USA), and KiSS-1 (Santa Cruz Biochnology, Santa Cruz, CA, USA) at 37°C for 1 h. Lastly, all sections were counterstained with hematoxylin, dehydrated, air-dried, and mounted.

Estimation of immunostaining

Immunostaining results were estimated semi-quantitatively by two independent and experienced pathologists who were blind to patients' clinical, pathological, demographic, and follow-up data. From different areas, random ten representative fields at high-power-fields (HPF) of per GAC slice were evaluated to control for any intratumoral heterogeneity of biomarker expression. Immunostain results were assessed by staining intensity and staining extent. Grades of staining intensity were as follows: none staining = 0, weak staining = 1, moderate staining = 2, strong staining = 3. Grades of staining extent were as follows: <11% positive cells = 1, 11-50% positive cells = 2, 51-75% positive cells = 3, >75% positive cells = 4. Intensity and

Table 2. Correlation between CagA, HER2, ALDH1, or KiSS-1 and clinicopathological characteristics in gastric adenocarcinoma

Variable	CagA		P	HER2		P	ALDH1		P	KiSS-1		P
	Negative	Positive		Negative	Positive		Negative	Positive		Negative	Positive	
Gender			0.787			0.077			0.950			0.486
Male	52	96		125	23		57	91		88	60	
Female	31	53		63	21		32	52		46	38	
Ages			0.724			0.097			0.485			0.408
≤60	42	79		103	18		49	72		73	48	
>60	41	70		85	26		40	71		61	50	
Size			0.071			0.855			0.866			0.652
≤2.0 cm	26	27		44	9		22	31		28	25	
>2.0 cm, ≤5.0 cm	47	100		119	28		55	92		86	61	
>5.0 cm	10	22		25	7		12	20		20	12	
Location			0.647			0.203			0.256			0.943
Antrum	40	79		96	23		47	72		70	49	
Cardia	31	54		66	19		28	57		48	37	
Pylorus	12	16		26	2		14	14		16	12	
Gross type			0.098			0.256			0.329			0.531
Polypoid	11	10		17	4		11	10		10	11	
Ulcerative	52	112		129	35		59	105		98	66	
Infiltrating	20	27		42	5		19	28		26	21	
Alcohol			0.708			0.398			0.878			0.049
No	40	68		85	23		42	66		55	53	
Yes	43	81		103	21		47	77		79	45	
Smoking			0.170			0.435			0.826			0.799
No	49	74		102	21		48	75		72	51	
Yes	34	75		86	23		41	68		62	47	
Tumor stages			<0.001			<0.001			<0.001			<0.001
Submucosa	20	5		25	0		22	3		5	20	
Muscularis	40	24		56	8		39	25		20	44	
Serosa	23	108		105	26		28	103		97	34	
Visceral peritoneum	0	12		2	10		0	12		12	0	
Grade			<0.001			<0.001			<0.001			<0.001
Well	30	4		32	2		29	5		4	30	
Moderate	47	92		119	20		56	83		78	61	
Poor	6	53		37	22		4	55		5	7	
Lymph node metastasis			<0.001			<0.001			<0.001			<0.001
N0	61	51		109	3		65	47		39	73	
N1	19	38		49	8		18	39		38	19	
N2	3	52		30	25		6	49		49	6	
N3	0	8		0	8		0	8		8	0	
TNM stage			<0.001			<0.001			<0.001			<0.001
I	60	18		76	2		58	20		16	62	
II	20	79		87	12		28	71		67	32	
III	3	52		25	30		3	52		51	4	

extent were multiplied to reach final scores that ranged 0-12. Scores >2 were defined as positive. For slices that were positive for all four of: CagA, HER2, ALDH1, and KiSS-1, an average of the final scores of per slice was taken.

Statistical analysis

Associations between expression of CagA, HER2, ALDH1, or KiSS-1 and clinicopathological characteristics and demographic data were compared using Fisher's exact test or Pearson Chi-square test. Associations among expression of CagA, HER2, ALDH1, or KiSS-1 were compared using Spearman's coefficient test. Multivariate logistic regression analysis was used to clarify the relative factors for metastasis. Univariate analysis between OS and expression of CagA, HER2, ALDH1, or KiSS-1 was

Table 3. Correlation among CagA, HER2, ALDH1 and KiSS-1 in GAC

Variable	CagA		r	P	HER2		r	P	KiSS-1		r	P
	Negative	Positive			Negative	Positive			Negative	Positive		
CagA			0.269	<0.001#			0.269	<0.001#			-0.636	<0.001*
Negative	79	109			79	4			13	70		
Positive	4	40			109	40			121	28		
HER2											-0.347	<0.001*
Negative									93	95		
Positive									41	3		
ALDH1			0.613	<0.001#			0.291	0.029#			-0.599	<0.001*
Negative	65	24			85	4			18	71		
Positive	18	125			103	40			116	27		

#: positive correlation; *: negative correlation.

Table 4. Univariate analysis and multivariate analysis of factors affecting lymph node metastasis

Variable	Categories	Univariate analysis	Multivariate analysis		
		P	HR	95% CI	P
Tumor stage	Submucosa+Mucluaris+Subserosa/Visceral peritoneum	0.024	0.463	0.069-3.114	0.428
Grades	Well+Moderate/Poor	0.038	0.456	0.169-1.234	0.122
HER2	Negative/Positive	<0.001	11.381	2.944-43.992	<0.001
KiSS-1	Negative/Positive	<0.001	0.327	0.146-0.733	0.007
CagA	Negative/Positive	<0.001	1.876	0.764-4.610	0.170
ALDH1	Negative/Positive	<0.001	2.056	0.915-4.617	0.081

compared using Kaplan-Meier method with log-rank test. Multivariate analysis for OS was done using Cox regression model test. Statistical analysis was carried out using SPSS 19.0 software for Windows (Chicago, IL, USA). A value of $P<0.05$ was considered significant.

Results

Associations between CagA, HER2, ALDH1, or KiSS-1 and clinical, pathological and demographic data

CagA, ALDH1, and KiSS-1 positive staining were mainly confined to the cytoplasm of cancer cells; HER2 positive staining was mainly confined at the membrane of cancer cells. The positive expression of CagA in the control tissues (47.4%, 110/232) was significantly lower than that in the GAC tissues (64.2%, 149/232; P<0.001; **Figure 1A** and **1B**). CagA positive expression in GAC was positively associated with tumor grades, tumor stages, LNM stages, and TNM stages, but not with patient age, gender, tumor location, gross type, smoking, alcohol, and tumor size (**Table 2**).

Similar to CagA, HER2 positive expression was significantly higher in GAC tissues (19.0%, 44/232) than in the control tissues (0%, 0/232; P<0.001; **Figure 1C** and **1D**). Moreover, the positive expression of HER2 in GAC was significantly associated with tumor grade, tumor stage, LNM stage, and TNM stage, but not with patient age, gender, tumor location, gross type, smoking, alcohol, and tumor size (**Table 2**).

ALDH1 positive expression was significantly higher in GAC tissues (61.6%, 143/232) than in the control tissues (17.2%, 40/232; P<0.001; **Figure 1E** and **1F**). In addition, the ALDH1 positive expression was significantly associated with tumor grade, tumor stage, LNM stage, and TNM stage, but not with patient age, gender, tumor location, gross type, smoking, alcohol, and tumor size (**Table 2**).

Positive expression of KiSS-1 was significantly lower in GAC tissues (42.2%, 98/232) than in the control tissues (90.5%, 210/232; P<0.001; **Figure 1G** and **1H**). The positive expression of KiSS-1 was inversely associated with alcohol, tumor grade, tumor stage, LNM stage, and TNM stage. No association was found between positive expression of KiSS-1 and patient age, gender, tumor location, gross type, smoking, and tumor size (**Table 2**).

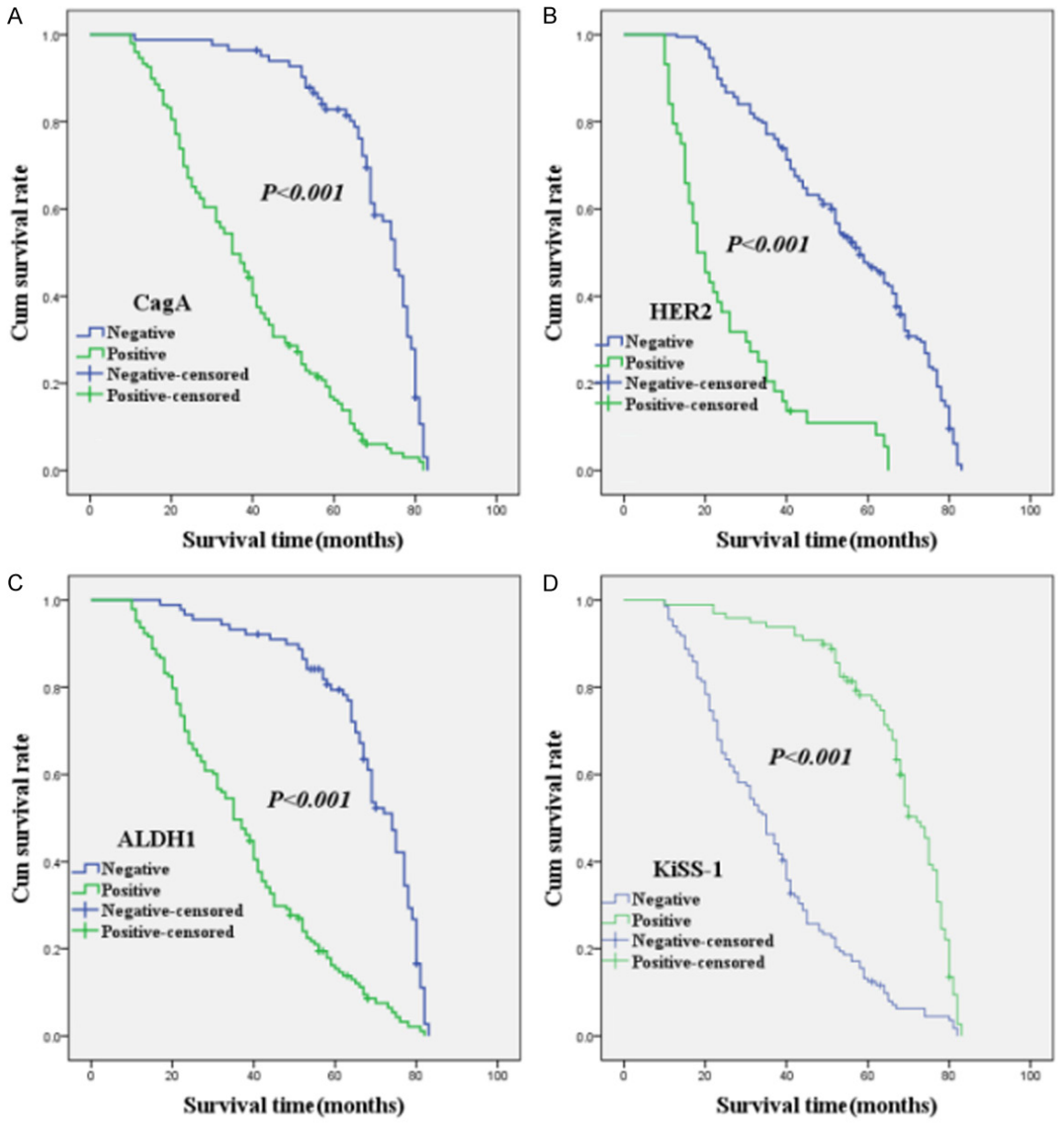

Figure 2. Kaplan-Meier analysis of overall survival (OS) time of patients with gastric adenocarcinoma. A. OS time of all patients in relation to CagA expression (log-rank =101.358, P<0.001). B. OS time of all patients in relation to HER2 expression (log-rank =100.355, P<0.001). C. OS time of all patients in relation to ALDH1 expression (log-rank =93.105, P<0.001). D. OS time of all patients in relation to KiSS-1 expression (log-rank =93.699, P<0.001). The green line represents positive expression of CagA, HER2, ALDH1, and KiSS-1; the blue line represents negative expression of CagA, HER2, ALDH1, and KiSS-1.

Associations among CagA, HER2, ALDH1, and KiSS-1 in GAC

There was a negative association between KiSS-1 expression and expression of CagA, HER2, and ALDH1 (r=-0.636; r=-0.347; r=-0.599; respectively; P<0.001) (**Table 3**) in GAC. The expression of CagA showed a positive association with HER2 and ALDH1 expression (r=0.269, P<0.001; r=0.613, P<0.001) in GAC. The expression of HER2 and ALDH1 showed a positive association (r=0.291, P<0.001) (**Table 3**) in GAC.

Metastasis analysis

Univariate analysis indicated that tumor grade and stage were positively associated with lymph node metastasis (P<0.05) in GAC. In multivariate logistic regression analysis, HER2

Table 5. Results of univariate analyses of overall survival (OS) time

Variable	n	Mean OS (months)	Log-rank	*P* value
CagA			101.358	<0.001
Negative	83	68.5±13.7		
Positive	149	37.6±18.3		
HER2			100.355	<0.001
Negative	188	54.2±20.0		
Positive	44	25.2±15.7		
ALDH1			93.105	<0.001
Negative	89	66.6±15.5		
Positive	143	37.5±18.4		
KiSS-1			93.699	<0.001
Negative	134	36.2±18.2		
Positive	198	65.7±15.2		

Table 6. Results of multivariate analyses of overall survival (OS) time

Variable	B	SE	P	RR	95% CI
CagA	0.508	0.222	0.022	1.661	1.075-2.567
HER2	0.641	0.215	0.003	1.898	1.244-2.896
ALDH1	0.542	0.203	0.008	1.720	1.156-2.561
KiSS-1	-0.448	0.198	0.024	0.639	0.433-0.942
Tumor stages	0.450	0.189	0.017	1.568	1.083-2.270
LNM stages	0.424	0.157	0.007	1.529	1.123-2.081
TNM stages	0.520	0.254	0.041	1.682	1.022-2.769

and KiSS-1 positive expression were significantly associated with lymph node metastasis (**Table 4**) in GAC.

Survival analysis

By univariate analysis, follow-up data showed that OS was significantly lower in GAC patients with CagA positive expression (37.6±18.3 months) compared with those with CagA negative specimens (68.5±13.7 months; log-rank=101.358, P<0.001; **Figure 2A**). Similarly, OS of HER2 positive expression (25.2±15.7 months) was significantly shorter than in those-with HER2 negative expression (54.2±20.0 months; log-rank =100.355, P<0.001; **Figure 2B**). OS of ALDH1 positive expression (37.5± 18.4 months) was significantly shorter than in those with ALDH1 negative patients (66.6±15.5 months; log-rank =93.105, P<0.001; **Figure 2C**). Conversely, OS with KiSS-1 positive expression (65.7±15.2 months) was significantly longer than in those who were KiSS-1 negative (36.2±18.2 months; log-rank =93.699, P<0.001; **Figure 2D**; **Table 5**).

Multivariate analysis showed that CagA+, HER2+, ALDH1+, and KiSS-1+, and Tumor stages, LNM stages, and TNM stages, were independent prognostic factors for GAC (**Table 6**).

Discussion

Gastric cancer is a multifactorial disease which includes environmental, genetic, and dietary factors. It is well known that Helicobacter pylori (*H. pylori*) infection is the most common risk factor for gastric cancer. Cytotoxin-associated gene A (CagA), a biomarker for the cag pathogenicity island, which is a most significant virulence factor of *H. pylori* [3, 6]. Patients infected with CagA+ *H. pylori* strains have a much higher risk of developing gastric cancer [24-26]. In this study, we found that CagA expression was positively associated with tumor grade, tumor stage, LNM stage, and TNM stage. Furthermore, overall survival analysis indicated that CagA-positive patients had significantly lower survival time than did CagA-patients. These above findings suggested that CagA is involved in GAC progression and metastasis, and should be considered a valuable biomarker in managing this disease. Our findings are similar to previous studies [3, 25, 26].

HER2, which is an important member of the HER family, is a transmembrane protein. HER2 is able to promote cell growth, differentiation, motility, and apoptosis [27]. Accumulating studies have demonstrated that HER2 plays an important role in the tumorigenesis of various human cancers, such as breast, lung, and gastric cancer [28]. In this study, we found that HER2 expression was positively associated tumor grade, stages, LNM stage, and TNM stage. The further OS analysis indicated that HER2+ patients had a significantly longer survival time than did HER2- patients. These results supported that HER2 plays a key role in GAC progression and metastasis, and was associated with poor prognosis. Other studies had similar results [7-10, 29].

ALDH1, a common biomarker of CSCs, is able to metabolize and detoxify many endogenous

and exogenous wastes by its intracellular enzyme activity [12, 14-18]. ALDH1 overexpression is closely related to alcohol-related carcinogenesis and poor prognosis [30]. In this study, we also confirmed that ALDH1 positive expression was significantly higher when compared with corresponding normal gastric mucosa tissues. ALDH1 expression is also associated with tumor grade, stage, LNM stage, and TNM stage. In addition, positive expression of ALDH1 was related to poor prognosis in GAC patients. The current results demonstrated that ALDH1 overexpression could be involved in GAC development, invasion, and metastasis and have a worse prognosis. These findings are consistent with many other studies [12, 14-18, 31].

KiSS-1, known as a suppressor of tumor metastasis, is able to suppress cancer metastasis but not inhibit carcinogenesis [32]. KiSS-1 can also regulate cell adhesion and cytoskeleton reorganization [33]. In this study, we also demonstrated that the positive rate of KiSS-1 expression was significantly lower in GAC tissues when compared with the control tissues, and its positive expression was negatively associated with alcohol, tumor grade, stage, LNM stage, and TNM stage. OS analysis showed that KiSS-1 positive expression patients had a longer survival time compared to negative patients. Our findings suggested that lower expression of KiSS-1 further promotes GAC metastasis and also predicts metastasis and prognosis, like prior studies [12, 21-23, 32].

GAC is the most common type of gastric cancer. *H. pylori* infection may lead to gastric cancer and is a common risk factor for gastric cancer [34]. CagA is the most common virulence factor of *H. pylori* and translocates into gastric epithelial cells which can lead to tumorigenesis in GAC. ALDH1 is a biomarker of CSCs, so its overexpression should be involved in the initiation, progression, and metastasis of GAC. CagA and ALDH1 may play a synergistic role in the tumorigenesis of GAC, and together promote progression. Also, HER2 overexpression can further promote tumor cell growth and differentiation. HER2 promotes tumor cells motility and invasion through inhibition of β-catenin from the E-cad complex [7]. Normal KiSS-1 suppresses tumor metastasis through inhibition of MMP expression and induction of E-cad expression [21, 22]. Downregulation of KiSS-1 should lower or lose its inhibition of metastasis, leading to metastasis.

Conclusions

Our findings suggested that CagA and ALDH1 play a synergistic role in the GAC evolution; and that combined investigation of CagA, HER2, ALDH1, and KiSS-1 are useful indicators of metastasis and prognosis in GAC.

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Disclosure of conflict of interest

None.

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