RESEARCH ARTICLE

Aberrant Expression of Markers of Cancer Stem Cells in Gastric Adenocarcinoma and their Relationship to Vasculogenic Mimicry

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Abstract

Background: Gastric cancer is the second leading cause of cancer-related death in Asia, and the majority type is gastric adenocarcinoma (GAC). Most GAC patients die of recurrence and metastasis. Cancer stem cells (CSCs) have been thought to be responsible for the initiation, development, metastasis, and ultimately recurrence of cancer. In this study, we aimed to investigate expression and clinical significance of CSCs markers, CD133 and Lgr5, and vasculogenic mimicry (VM) in primary GAC. Materials and Methods: Specimens from 261 Chinese patients with follow-up were analyzed for CD133, Lgr5 protein expression and VM by immunohistochemical and histochemical staining. The Pearson Chi's square test was used to assess the associations among the positive staining of these markers and clinicopathological characteristics. Postoperative overall survival time was were studied by univariate and multivariate analyses. Results: In GAC tissues, positive rates of 49.0%, 38.7%, and 26.8% were obtained for CD133, Lgr5, and VM, respectively. The mean score of microvessel density (MVD) was 21.7±11.1 in GAC tissues. There was a significantly difference between the positive and negative groups. There was a positive relationship between the VM, the expression of CD133 and Lgr5, and the score of MVD and the grades of tumor, lymph node metastasis, TNM stages (all $p<0.05$). The overall mean survival time of the patients with CD133, Lgr5, VM, and MVD (≥22) positive expression was lower than that of patients with negative expression. The score of MVD, positive expression of CD133 and VM were independent prognostic factors of GAC ($p<0.05$). Conclusions: VM, and expression of CD133, Lgr5, and the score of MVD are related to grades of tumor, lymph node metastasis, TNM stages, and overall mean survival time. It is suggested that CSCs and VM could play an important role in the evolution of GAC.

Keywords: Gastric neoplasm - CD133 - Lgr5 - VM - MVD - CSCs

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Introduction

Gastric cancer is the most common cancer of the digestive system with an estimated incidence of approximately 952,000 cases in the worldwide (Torre et al., 2015), China is one of the highest incidence area. When the patient is diagnosed, the cancer is often unsuitable for curative resection. It is urgent to raise new early diagnostic tools and therapeutic methods about gastric cancer. Cancer stem cells (CSCs), which play an important role in cancer initial and development, are small population of cancer cells which possess self-renewing capacity, resistance to chemo- or radio-therapy, and tumorigenic capacity. CD133, which is a common CSCs marker, is a transmembrane glycoprotein and was initially considered as a marker of hematopoietic stem and progenitor cells (Miraglia et al., 1997; Yin et al., 1997) CD133 is expressed in various normal and stem cells. Recently, more and more studies indicate that CD133 has been described as a CSCs marker in many types of solid tumors (O'Brien et al., 2007; Zhu et al., 2009; Yan et al., 2011; Flesken-Nikitin et al., 2013).

Leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5), also known as GPR49, is a seven transmembrane receptor that is identified as a stem cell marker in intestine, stomach, and hair follicle (Lin and Andersen, 2008; Barker et al., 2013; Sato and Clevers, 2013). Recently, rapidly accumulated evidence indicates that Lgr5 had been described as a new CSCs marker to be involved in many human cancers, such as colon cancer, gastric carcinoma, and pancreatic adenocarcinoma (Amsterdam et al., 2013; Yang et al., 2013; Liu et al., 2014). LGR5, which was found to be expressed in multiple organs, may be a global marker of stem cells.

The traditional theory of vascularization in gastric tumors is a complex process that involves angioblast vasculogenesis and intussusceptive microvascular growth. In 1999, Maniotis (Maniostis et al., 1999) described

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a new phenomenon in aggressive human melanoma which melanoma cell mimicked endothelial cell and formed a fluid-conducing channel to convey blood was called vasculogenic mimicry (VM). VM is associated with increased tumor aggressive biology and tumor-related patient mortality. The channel of VM consists of plasticity of tumor cells in the external wall, remodeling of the extracellular matrix (ECM) on the inner wall, and connection to the host microcirculation system (Sun et al., 2011; Yao et al., 2013; Zhang et al., 2014) with genetically dysregulated tumor cells having a pluripotent embryonic-like gene type (Hendrix et al., 2003), suggesting the participation of CSCs. We and others have demonstrated that vascular niche may regulate CSCs fate (Hilbe et al., 2004; EI Hallani et al., 2010; Wu et al., 2012) and promote metastasis through the down-regulation of E-cadherin.

In addition, CSCs may play an important role in the formation of VM (Scully et al., 2012; Wu et al., 2012), which is significant for aggressive tumors to obtain adequate nutrition and oxygen during the stage of progression. Although previous reports showed that CSCs and VM played an important role in many cancers, there is little evidence about the relationship between CSCs and VM and clinicopathological characteristics of GAC. In this study, we performed an immunohistochemical detection to explore the role of CSCs and VM in 261 cases of GAC to reveal their clinicopathology and prognosis.

Materials and Methods

Patients and clinical samples

Two hundred and sixty-one gastric adenocarcinoma (GAC) patients and matched GAC adjacent normal gastric tissues were obtained from the Department of Pathology, the First Hospital Affiliated to Bengbu Medical College between January, 2004 and December, 2006 (Patients who had been administered by chemo- or radio-therapy prior to operation were excluded). None of them had synchronous cancers. Pathological tumor-node-metastasis stages of GAC were assessed on the basis of classification system recommended by the American Joint Committee on Cancer stage (7th edition) and JGCA guidelines. This study was approved by the ethical committee of Bengbu Medical College before its start. The ages of the patients ranged from 23 to 88 years, the mean age was 59.6±7.8 years. Of the patients, 172 were men and 89 were women. According to histological grading, 36 were grade 1, 165 were grade 2, 60 were grade 3. 14 were stage I, 108 were stage II, 124 were stage III, 15 were stage IV. All patients were followed up for survival. By December 2012 (the time of data analysis), patients had died and patients were alive. The mean survival time was 43.9 months.

Immunohistochemical analysis

Immunohistochemical staining of CD133, Lgr5, E-cadherin, and CD34 was carried out as previously described [18]. 4 μm thick tissue sections were cut from embedded in paraffin. The sections were deparaffinized in xylene and dehydrated in a graded series of alcohol. The endogenous peroxidase activity was quenched by incubation in 3% hydrogen peroxidase-methanol for 10 min at room temperature. Antigen retrieval was performed by heated for 5 min in a pressure cooker and then cooled to room temperature. After several washes in phosphate-buffered saline (PBS), the sections were blocked with 10% goat blood serum to prevent non-specific binding for 20 min at room temperature. And then purified primary rabbit monoclonal antibody Lgr5 (Abcam, USA) and mouse monoclonal antibody CD133 (Abcam, USA), CD34 (LabVision), and E-cadherin (LabVision) were incubated with sections overnight at 4°C in a humidified chamber. After washed in PBS, the sections were treated with polymer enhancer (Reagent A) for 20 min at room temperature. And wash in PBS, the sections were treated with goat anti-mouse antibody (Reagent B) for 30 min at room temperature. Then a complete wash in PBS, the sections were develop in freshly prepared diaminobenzedine solution (DAB) for 8 min, and then counterstained with hematoxylin, dehydrated, airdried, and mounted. Negative controls were staining with the same procedure without primary antibody. Repeatedly validated colon adenocarcinoma specimens with Lgr5 positive served as positive controls.

All samples were submitted for PAS-CD34 dual staining to characterize endothelial cells glycosylated basement membranes of vessels as well as vessel-like (VM) channels (Ahmadi et al., 2010).

Evaluation of immunostaining

Sections were examined and scored independently by two pathologists who were blind to the clinical data of patients. The evaluation was analyzed according to both the percentage of positive cells and intensity immunohistochemical staining. The intensity of positivity was scored on a scale of 0 to 3, 0 records as negative, 1 as weak staining, 2 as moderately staining, 3 as strong staining. Percentage of positive cells was also scored on a scale of 1 to 4, <10% records as 1, 11%-50% as 2, 51%-75% as 3, >75% as 4. The final score was determined by multiplying the intensity of positivity and the scores of percentage of positive cells, yielding a range from 0 to 12. The expression of CD133, Lgr5, E-cadherin and CD34 was considered positive when the scores were >2.

Statistical analysis

SPSS 19.0 software package (Chicago, USA) was used for statistical analysis. The Pearson Chi's square test and Spearman's correlation coefficient was used to assess the associations among the positive staining of CD133, Lgr5, and E-cadherin and VM and clinicopathological characteristics. The Kaplan-Meier method was used to assess univariate survival analysis, and the difference the survival curves was analyzed with log-rank test. Cox regression method was used to evaluate multivariate survival analysis. A value of $p<0.05$ was recognized as statistically significant.

Results

The relationship between expression of CD133, Lgr5, E-cadherin and VM and clinicopathological factors

CD133 protein was expressed positively in 49.0%

(128/261) of GAC and 5.0% (13/261) of normal gastric tissues. The positive staining for CD133 was confined to the cells membrane and cytoplasm. There was a significant difference between the GAC group and the control group (p<0.005) (Figure 1A and 1B). There was a significant difference between the expression level of CD133 and histological grade, depth of invasion, lymph node metastasis and pTNM stages (p<0.05). The positive expression of Lgr5 protein was in 41.4% (108/261) of GAC and 24.9% (65/265) of normal gastric tissues. The positive staining for Lgr5 was predominantly localized in the cell cytoplasm and on cell membrane. The Lgr5 protein expression between GAC group and control group was significantly different (p<0.001) (Figure 1C and 1D). There was a significant difference between the positive expression of Lgr5 and histological grade, lymph node metastasis, depth of invasion, and pTNM stage (p<0.001). In the normal gastric tissues, the positive staining for Lgr5 protein was present at the base of gastric crypts. The positive expression of E-cadherin protein was in 38.7% (101/261) of GAC and 100% (261/261) of normal tissues. The positive staining for E-cadherin was in the cell cytoplasm and on the cell membrane. There was a significant difference between the expression of E-cadherin in GAC group and control group (p<0.05) (Figure 1E and 1F). There was a negative association between E-cadherin protein expression and GAC histological grades, lymph node metastasis, depth of invasion, and pTNM stage (p<0.05). Small vessel-like structures in the tumors that were PAS-positive but CD34-negative were to be VM channels. The cells external to the lumen of the VM channel were positive for tumor cells, which indicated the vessel-like structures were formed by GAC cells (Figure 1G and 1H). The presence as well as pattern (linear, tubular, and network) of VM channel were recorded in each specimen. VM channel was identified in GAC group in 26.8% (70/261) GAC tissues. None of the control tissues contained VM. There was a significant difference between the GAC group and the control group (p<0.05) (Table 1). The positive rates of VM channel were found to be significantly associated to GAC histological grade, lymph node metastasis, depth of invasion and pTNM stage (p<0.05). The score of MVD was positive related to the grade of tumor, lymph node metastasis, depth of invasion, and pTNM stages. However, the positive rates of CD133, Lgr5, and E-cadherin expression, VM channel, and the score of MVD were no significant association with gender, ages, tumor location, and diameter of tumors (p>0.05) (Table 1).

Prognosis and multivariate analysis

Follow-up data showed that there was a significantly decreasing trend in the overall mean survival time between the carcinomas with the expression of CD133 (32.6±27.6 months) and those without (54.9±30.5 months)(Log rank=32.504, p<0.001) (Figure 2A). The survival time of Lgr5 positive expression was significantly shorter than that of negative expression (p<0.001) (Figure 2B). The survival time of E-cadherin positive expression was significantly longer than that of negative expression (p<0.001) (Figure 2C). The survival time of the VM group was significantly shorter than the non-VM group (p<0.001) (Figure 2D). The survival time of MVD score≥22' group was significantly shorter than that of MVD score <22' group (p<0.001) (Figure 2E). CD133, VM, the score of MVD, and pTNM stages were independent prognostic factors by Cox regression multivariate analysis (p<0.05) (Table 2).

Correlation of CD133, Lgr5, E-cadherin, and VM in GAC

In positive expression of CD133 group, the positive expression of E-cadherin was 14.1% (18/128); in negative expression of CD133 group, E-cadherin positive expression was 67.7% (90/133). There was a negative correlation between CD133 expression and E-cadherin

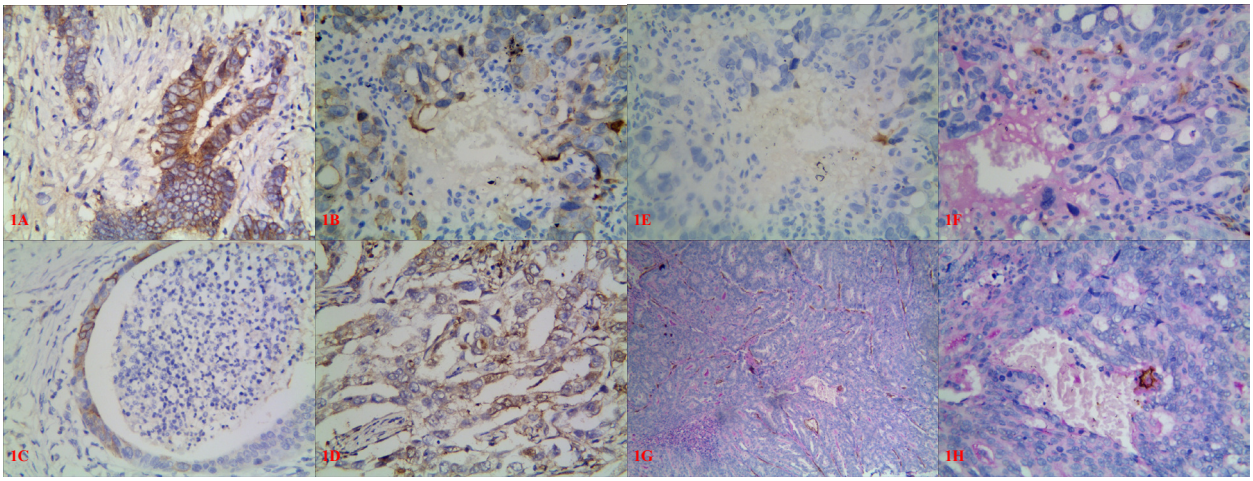

Figure 1. Representative Results of CD133, Lgr5, E-cadherin, and VM in Gastric Adenocarcinoma Group and Control Group. A) Gastric adenocarcinoma cells expressed CD133 in the membrane and cytoplasm. B. CD133 predominantly localized in the membrane and cytoplasm in poorly differentiated GAC (grade 3) (CD133×400). C. A few scattered cells displaying Lgr5 staining. Lgr5-positive cells exhibiting focal (C) and patchy (D) distribution patterns in some cases of GAC. E. Control gastric epithelial cells expressed E-cadherin in the cytoplasm and membrane. F. GAC cells did not express a detectable level of E-cadherin. G. H&E staining in moderately differentiated squamous cell carcinoma, red arrow is VM (Figure 1B, Figure 1C, Figure 1F, and Figure 1G are serial sections) (grade 3). H. Endothelial cells are detected with anti-CD34 immunhistochemical staining (dark brown) and vascular basement membrane with PAS staining (purple magenta) (red arrow: vessel stained positively with PAS but negatively with CD34; black arrow: vessel stained positively with CD34) (×100)

Table 1. Correlation of CD133, Lgr5, and E-cadherin Expression, VM, and MVD to Clinicopathological Characteristics in GAC

Variable	CD133		*P*	Lgr5		*P*	E-cadherin		*P*	VM		*P*	MVD mean	F	*P*
	-	+		-	+		-	+		-	+				
Age (years)			0.203			0.589			0.129			0.755		1.021	0.313
<60	54	62		69	47		74	42		86	30		20.0±11.3		
≥60	79	66		91	54		79	66		105	40		21.4±10.9		
Gender			0.029			0.222			0.122			0.036		0.137	0.711
Male	96	76		110	62		95	77		133	39		20.9±11.3		
Female	37	52		50	39		58	31		58	31		20.4±10.7		
Location			0.519			0.598			0.763			0.248		1.256	0.286
Antrum	69	63		84	48		75	57		93	39		20.2±9.9		
Cardia	45	51		55	41		57	39		70	26		20.6±11.4		
pylorus	19	14		21	12		21	12		28	5		23.6±14.1		
Gross type			0.205			0.735			0.785			0.222		3.099	0.047
Polypoid	19	12		21	10		17	14		25	6		16.3±8.8		
Ulcerative	81	91		104	68		100	72		120	52		21.6±11.2		
Invasive	33	25		35	23		36	22		46	12		20.6±11.5		
Diameter of tumors						0.009			0.346			0.37		1.049	0.352
<4.0cm	33	27		43	17		32	28		48	12		19.4±11.3		
≥4.0cm, <8.0cm	87	79		103	63		97	69		119	47		20.9±11.3		
≥8.0cm	13	22		14	21		24	11		24	11		22.7±9.6		
Depth of invasion			<0.001			<0.001			<0.001			0.027			
Submucosa	15	2		13	4		6	11		15	2		12.2±6.4	15.087	<0.001
Muscularis	48	33		64	17		35	46		67	14		17.7±9.5		
Subserosa	67	80		75	72		98	49		99	48		22.1±11.0		
Adjacent structures	3	13		8	8		14	2		10	6		33.4±10.5		
Grades of tumor			0.04			0.001			0.023			0.001		0.105	0.9
Well	25	11		31	5		14	22		30	6		20.3±10.8		
Moderately	82	83		100	65		99	66		128	37		21.0±11.2		
Poorly	26	34		29	31		40	20		33	27		20.4±11.2		
Lymph node metastasis			<0.001			<0.001			0.002			<0.001		30.567	<0.001
No	88	54		103	39		71	71		119	23		17.5±10.3		
Yes	45	74		57	62		82	37		72	47		24.7±10.8		
pTNM stages			<0.001			<0.001			<0.001			<0.001		116.615	<0.001
Iand II	84	38		100	22		51	71		110	12		14.2±7.7		
IIIand IV	49	90		60	79		102	37		81	58		26.5±10.4		

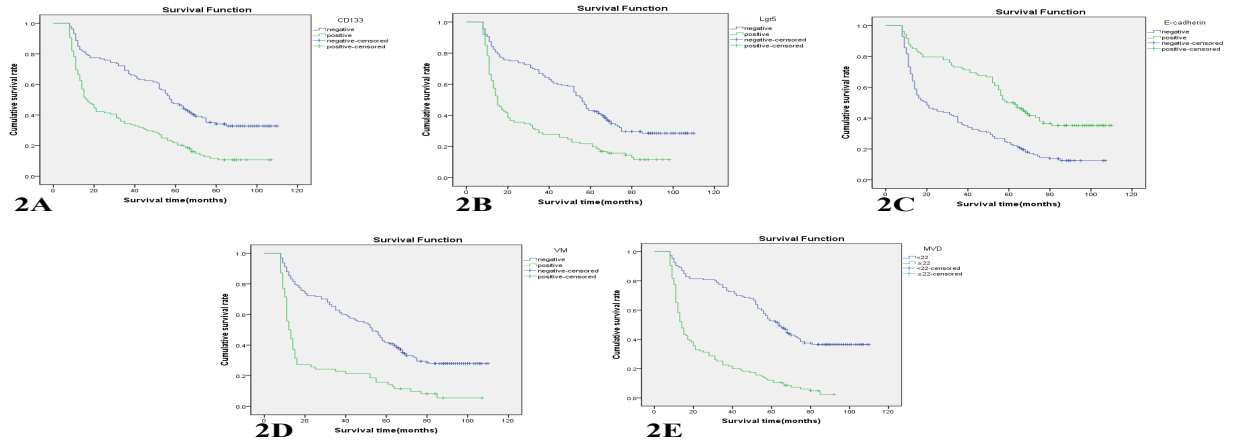

Figure 2. Kaplan-Meier Survival Analysis by CD133, Lgr5, E-cadherin, VM, and MVD Status (n=261). The y-axis represents the percentage of patients; the x-axis, their survival in months. A: the green line represents CD133 positive patients with a worse trend survival than the blue line representing CD133 negative gastric adenocarcinoma patients (p<0.001). Mean survival time was 32.6 months for the CD133 positive group and 54.9 months for the CD133 negative group. B: the green line represents Lgr5 positive patients with a worse trend survival than the blue line representing Lgr5 negative GAC patients (p<0.001). Mean survival time was 30.2 months for the Lgr5 positive group and 52.6 months for the Lgr5 negative group. C: the green line represents E-cadherin positive patients with a better trend survival than the blue line representing E-cadherin negative GAC patients (p<0.001). Mean survival time was 57.5 months for the E-cadherin positive group and 34.4 months for the E-cadherin negative group. D: the green line represents VM positive patients with a worse trend survival than the blue line representing VM negative gastric adenocarcinoma patients (p<0.001). Mean survival time was 25.1 month for the VM positive group and 50.8 months for the VM negative group. F: the green line represents MVD score≥22's patients with a worse trend survival than the blue line representing MVD score<22's GAC patients (p<0.001). Mean survival time was 25.3 months for MVD score≥22's group and 58.6 months for MVD score<22's group

Table 3. Correlation among CD133, Lgr5, and E-cadherin Expression, VM and MVD

Variable	CD133		r	*P*	Lgr5		r	*P*	VM		r	*P*
MVD			0.21	0.001			0.309	<0.001			0.177	0.004
<22	88	58			109	37			117	29		
≥22	45	70			51	64			74	41		
E-cadherin			-0.544	<0.001			-0.316	<0.001			-0.368	<0.001
-	43	110			74	79			91	62		
+	90	18			86	22			100	8		
Lgr5			0.354	<0.001							0.318	<0.001
-	104	56							135	25		
+	29	72							56	45		
VM			0.271	<0.001			0.318	<0.001				
-	113	78			135	56						
+	20	50			25	45						

Table 2. Multivariate Survival Analysis of 261 Patients with GAC

Covariable	B	SE	P	RR	95%CI
CD133	0.539	0.18	0.003	1.714	1.205-2.438
VM	0.538	0.176	0.002	1.713	1.214-2.418
MVD	0.713	0.183	<0.001	2.04	1.424-2.921
pTNM	0.982	0.226	<0.001	2.67	1.713-4.160

expression (r=-0.544, p<0.001); and the same correlation between Lgr5 expression and E-cadherin expression (r=-0.316, p<0.001). The positive correlation between CD133 or Lgr5 expression and the positive rate of VM was found (r=0.271, p<0.001 or r=0.318, p<0.001). The negative correlation between E-cadherin expression and the positive rate of VM (r=-0.368, p<0.001) (Table 3).

Discussion

CSCs are defined as a subset population of tumor cells with the ability to be self-renewal and potentially promote proliferation and formation of tumors (Singh et al., 2004; O'Brien et al., 2007; Rosen and Jordan, 2009; Malanchi et al., 2012; Long et al., 2012). Conventional cancer chemo- or radio-therapy indiscriminately kills proliferating cells and is often unsuccessful because of the survival of quiescent CSCs. Therefore, anti-CSCs target-drug conjugates may induce CSCs differentiation or eliminate CSCs by inhibiting the maintenance of the stem cell state (Economopoulou, et al., 2012; Malanchi et al., 2012). CD133 is a common marker of CSCs and its expression in cell surface down-regulates quickly when cell differentiated (Peichev et al., 2000). CD133 has often been used as a marker to identify CSCs in gastric, lung, brain, and ovarian (O'Brien et al., 2007; Zhao et al., Curley et al., 2009; 2010;Wu et al., 2012; Steg et al., 2012; Zarkoob et al., 2013). In this study, we use immunohistochemical staining to detect the expression of CD133 protein in 128 (49.0%) and it is significantly associated with the poor prognosis of GAC patients. Further study shows that CD133 protein expression was significantly associated with the poor differentiation, lymph node metastasis, and high pTNM stage. These results suggest that over expression of CD133 should be involved in the initiation, development, and metastasis of GAC. And patients with CD133 protein positive expression indicated a shorter survival time. This study was consistent with previous studies in gastric carcinoma (Zhao et al., 2010; Hashimoto et al., 2014). However, there was a controversy whether CD133 was a marker of CSCs or not. Many studies (Immervoll et al., 2008; Schneider et al., 2012; Wu et al., 2012) showed that CD133 protein expression was not only restricted to CSCs or tumor initiating cells (TIC), but also expressed in normal tissues. Some researchers thought that CD133 might play an important role in tumorigenesis (Schneider et al., 2012). Our study also showed that CD133 protein expression was broadly distributed in GAC cell, and only a part of CD133 positive expression possesses the capacity of stem cells (Long et al., 2012).

Lgr5, a WNT target gene, is a member of the G-protein-coupled receptor (GPR) family of proteins. Some researchers thought that GPRs have been closely linked to CSCs during tumorigenesis (McClanahan et al., 2006; Huang et al., 2008). Lgr5 was found to be expression at the base of crypt stem cells. In our study, we found that Lgr5 was significantly overexpressed in the majority of GAC when compared with the controls. The expression level of Lgr5 was significantly associated with depth of invasion, grades of tumor, lymph node metastasis, and pTNM stage. The above results indicate that the abnormal expression of Lgr5 should be associated with more malignant, invasive, and metastatic tumors. And patients with Lgr5 protein positive expression showed a worse prognosis of GAC. This result was consistent with previous studies (Wu et al., 2012; Bu et al., 2013; Femia et al., 2013). And these Lgr5-positive expression cells may contain more CSCs. These residual CSCs after routine therapy may lead to metastasis and recurrence of GAC through the self-renewal and multilineage differentiation in agreement with CSCs theory hypothesis (Vermeulen et al., 2008; Long et al., 2015).

VM formed by highly aggressive GAC cells is a new formation of tumor microcirculation pattern, which is different from classically defined endothelium-dependent angiogenesis. The VM can provide a perfusion pathway for rapidly growing tumors, transport nutrition and oxygen from leaky vessels, and/or connect with endothelial-dependent vasculature. Since the introduction of VM, a lot of highly aggressive tumors have described the VM phenomenon, including melanoma, sarcomas (osteosarcoma and alveolar rhabdomyosarcoma)(Sun et

al., 2004; Fu et al., 2011), carcinoma of breast, bladder, lung, kidney, ovary, liver, and stomach (Hilbe et al., 2004; Tang et al., 2009; Sun et al., 2011; Jiang et al., 2011; Wu et al., 2012; Liu et al., 2013; Zhang et al., 2013; Sun et al., 2013), and glioma (Yao et al., 2013) and glioblastoma (EI Hallani et al., 2010). In some GAC cases, we find that VM channels are composed of a basement membrane with CD34-negative and PAS-positive staining in the absence of endothelial cells. There are some red blood cells in the VM channels. Based on dual staining (PAS and CD34), we can observe some morphologic patterns of VM, such as straight lines, loops, arcs, networks, and patterns. In our study, we found that VM was significantly associated with grade of tumor, lymph node metastasis, depth of invasion, and pTNM stages. And further research, we found that the VM-positive-group patients correlated with a shorter survival time. These results indicate that VM promotes the likelihood of development, invasion and hematogenous metastases and is a worse prognostic factor of patients. Our study is consistent with previous researches (Shirakawa et al., 2002; Sun et al., 2004; Sun et al., 2008; Li et al., 2010; Wu et al., 2012; Zhang et al., 2013).

We found that the positive-VM is formed by GAC cells with a much higher level in CD133-positive /Lgr5-positive cells, where high-density microvessels also observe. This means a link between VM and CSCs. Indeed, it has been demonstrated that CSCs should promote the formation of VM. Our previous study reported that CD133-positive tumors fractions preferentially promote VM formation (Wu et al., 2012). Furthermore, more and more evidence supports the conception that the markers of CSCs expression are not only significantly associated with VM formation and transdifferentiation into endothelial cells, but also associated with more rapid tumor growth and metastasis. Our study is consistent with these researches (Valyi-Nagy et al., 2012; Liu et al., 2013; Yao et al., 2013). We speculated that the VM might provide the sufficient nutrition and oxygen to promote the tumor growth and metastasis after the nutrition and oxygen supplied by neovascularization could not meet the needs of tumor growth. The function of epithelial cadherin (E-cad) protein is to inhibit aberrant cell proliferation and migration by maintaining stable cell-cell contact. In this study, Down-expression of E-cad was observed and further promoted development, invasion, and metastasis of GAC. The expression of CD133, Lgr5, E-cad, and VM can reflect the biological behavior of GAC, so giving a choice of molecular targeting therapy for GAC in the future.

In conclusion, it is suggested that CSCs may play an important role in evolution of GAC. Additionally, CD133, Lgr5, E-cad, and VM appear to be relevant candidate prognostic factors of GAC and enable targeted treatment of GAC to prevent recurrence or metastasis..

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