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Original Article

Expression of LGR5 in oral squamous cell carcinoma and its correlation to vasculogenic mimicry

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Abstract: Background: LGR5, also named as GPR49, is considered as a biomarker of cancer stem cells which have been responsible for the initiation, progression, metastasis, and recurrence of cancers. Vasculogenic mimicry (VM) which defines the formation of fluid-conducting tubes by highly progressive and genetically dysregulated cancer cells has been considered as useful biomarker for metastasis and prognosis in various cancers. In this study, we analyzed associations between LGR5 and VM in oral squamous cell carcinoma (OSCC), and their association with clinicopathological characters in OSCC. Methods: Positive rates of LGR5 and VM in 190 OSCC tissue samples and correspondence normal tissues were detected by immunohistochemical and histochemical staining. Patients' clinical data were also collected. Results: Positive rates of LGR5 and VM were significantly higher in OSCC tissues than those in normal tissues. Positive rates of LGR5 and VM were positively related to tumor size, grades, lymph node metastasis, and TNM stages, and inversely with patients overall survival time. And there was a positive association between the expression of LGR5 and positive rate of VM. In multivariate analysis, high expression of LGR5 and positive VM and lymph node metastasis, as well as TNM stages were to be considered as independent prognosis factors for overall survival time in patients with OSCC. Conclusions: The expression of LGR5 and VM represent potential biomarkers for metastasis and prognosis, as well as therapeutic targets for OSCC.

Keywords: OSCC, LGR5, VM, cancer stem cells, prognosis

Introduction

New oral cancer cases (including lip cancer) were estimated at 300,000 and death cases were estimated at approximately 145,000 in 2012 worldwide [1]. Oral suqamous cell carcinoma accounts for approximately 90% of all diagnosed oral cancer. The major risk factors for oral cancer are Smoking, alcohol, betel quid, and HPV infection in China [2, 3].

Recurrence and metastasis are the main reasons for cancer treatment failure. This may link to a small population of cancer cells, named as cancer stem cells (CSCs). CSCs are considered as having the capacity of self-renewal and differentiation ability [4]. CSCs have been isolated and indentified from cancer tissues by using various methods and some biomarkers [5, 6], such as CD133, CD44, ALDH1, ABCG2, and LGR5. Leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5), also named as GPR49, is a seven transmembrane receptor encode by the LGR5 gene in various tissues [7]. LGR5 is important for cancer development and a regulated target of Wnt signaling pathway [8]. LGR5 is also considered as a stem cell marker in diverse tissues and organs, such as intestine, stomach, and hair follicle [9-11]. Recently, studies have indicated that LGR5 is overexpressed in diverse types of human cancers, including colorectal cancer, gastric cancer, esophageal cancer, hepatocellular carcinoma, and pancreatic adenocarcinoma [7, 8, 12-14].

Angiogenesis is also a critical process for cancer metastasis and recurrence. However, the benefit of anti-angiogenic therapy in cancers is still unsatisfactory [15]. Vasculogenic mimicry, a new cancer blood supply, is defined the formation of fluid-conducting tubes by highly progressive and genetically dysregulated cancer cells [16]. VM plays an important role in the process of tumor cells proliferation, invasiveness,

Table 1. Clinicopathological characteristics of patients with oral squamous cell carcinoma

Patients characteristics	Frequency (n)	Percentage (%)
Age (years)		
<60	72	37.9
≥60	118	62.1
Gender		
Male	124	65.3
Female	66	34.7
Size (cm)		
≤2.0	105	55.3
>2.0, ≤4.0	79	41.6
>4.0	6	3.2
Location		
Tongue	104	54.7
Gingival	59	31.1
Palate	20	10.5
Tonsil	7	3.7
Smoking		
No	90	47.4
Yes	100	52.6
Alcohol		
No	85	44.7
Yes	105	55.3
Differentiation		
Well	141	74.2
Moderately	24	12.6
Poor	25	13.2
Lymph node metastasis		
N0	123	64.7
N1	51	26.8
N2	16	8.4
TNM stage		
I+II	126	66.3
III+IV	64	33.7

N0: regional lymph node metastasis; N1: the number of regional lymph node metastasis is no more than 3; N: the number of regional lymph node metastasis is more than 3.

and metastasis by its special structure which is composed of highly progressive cancer cells and remodeling of the extracellular-rich matrix. VM, as a new blood supply pattern, provided tubes structure by cancer cells which can connect to the host microcirculation system [17-19]. VM can directly nourish cancer cells and take cancer cells with blood into the circulation system which causes cancer cells metastasis [17-20]. VM maybe be able to explain the failure of anti-angiogenic therapy [21]. It has been revealed that patients with VM have a poorer overall survival and are easier to metastasize than patients without VM [17-20].

However, associations between LGR5 and VM in OSCC have not yet been far-ranging reported. In this study, we performed an immunohistochemical and special histochemical investigation to explore the role of LGR5 and VM in metastasis and prognosis in 190 samples of OSCC.

Patients and methods

Patients and tissue samples

We collected samples from all 190 patients (median age: 61.0 years, range: 26-86 years) who were treated for OSCC at the Department of Pathology of the First Affiliated Hospital of Bengbu Medical College, from January 2007 to December 2010, along with 190 samples of the corresponding adjacent normal tissues. Patients who had received any preoperative anti-cancer therapy (chemo- or radio-therapy or other therapies) were excluded. All samples were obtained with patients writing consent. The study was approved by the ethics committee of Bengbu Medical College and conducted in accordance with the guidelines of the Declaration of Helsinki. We collected the entirely clinicopathological and follow-up data (at 6-month intervals by phone, e-mail, or social application). Tumor-node-metastasis stages were evaluated according to the 7th edition of the American Committee on Cancer. Tumor grades were according to the World Health Organization standards. Other characteristics were see **Table 1**.

Immunohistochemistry and histochemistry

Immunohistochemistry was carried according to the Elivision^{TM} Plus detection kit instructions (Lab Vision, USA). OSCC and corresponding normal oral cavity mucosa tissues were all fixed in 10% buffered formalin and embedded in paraffin. Tissue sections (4 μm) were deparaffinized and dehydrated using xylene and a graded ethanol solutions as standard procedures, then were stained with double staining of CD34 and PAS. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide at room temperature for 10 min; then all sections were

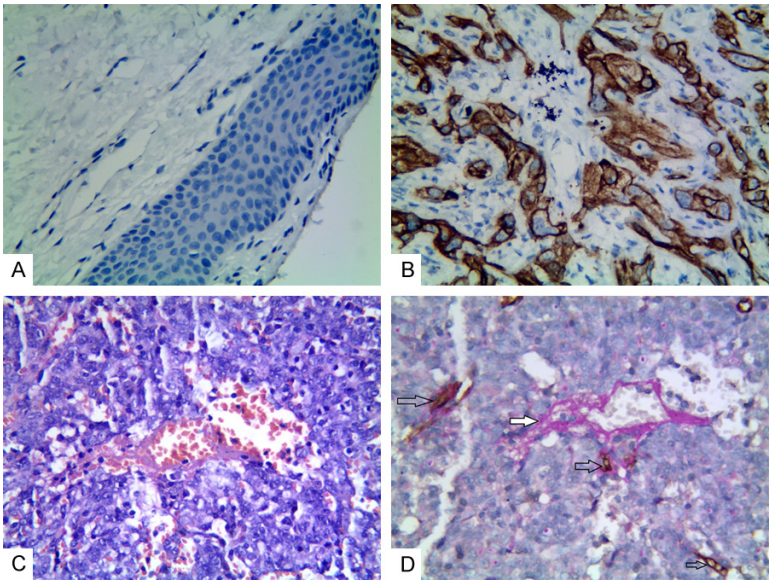

Figure 1. Positive staining of LGR5 and VM in oral squamous cell carcinoma or the control tissue. (A) Negative staining of LGR5 in the control tissues (100 magnification); (B) Positive staining of LGR5 in the cytoplasm and membrane of OSCC tissue (400 magnification); (C) Positive staining of VM in OSCC tissues (H&E staining, 100 magnification); (D) Positive staining of VM in OSCC tissues (CD34-PAS dual staining, black arrow is microvessels, white arrow is VM; C and D are serial sections).

soaked in citrate buffer (pH 6.0) and placed in an autoclave at 121°C for antigen repair for 2 min. After several washings with Phosphate buffered saline (PBS), all sections were blocked with goat serum at room temperature for 20 min, and then incubated with mouse monoclonal antibody against human CD34 (Abcam, Cambridge, MA, USA) or LGR5 (Abcam, Cambridge, MA, USA) at 37°C for 1 h. After washing with PBS again, all sections were incubated with DAB (DAKO, Glostrup, Denmark) for 5 min, then washing distilled water and incubated with PAS for 10 min, washing with distilled water again. Finally, all sections were counterstained with hematoxylin. Negative controls were stained by omitting primary antibody from staining procedure.

Assessment of staining

Two independent pathologists who were blind to all patients' clinical and follow-up data assessed semi-quantitatively immunostaining results. Ten high-power-field (HPF) representative fields from different areas of every OSCC's section were analyzed to prevent any intratumoral heterogeneity of biomarker expression. Immunostaining scores were graded using intensity (no staining: 0; weak staining: 1; moderate staining: 2; strong staining: 3) and extent (positive cells <11%: 1; 10%< positive cells <51%: 2; 50%< positive cells <76%: 3; positive cells >75%: 4). The intensity and extent scores were multiplied to reach final scores which ranged 0-12. Score >2 was determined positive.

Statistical analysis

Spearman coefficient analysis was used to analyze the association between LGR5 expression and VM. Survival analysis was assessed using Kaplan-Meier method and compared by log-rank test. Associations between positive rate of biomarkers and clinicopathological characteristics were analyzed using Fisher's exact test or Chi-square test. Independent prognostic factors were defined using Cox regression model for multivariate analysis. All statistical analyses were conducted using SPSS 19.0 software for Windows (Chicago, IL, USA). $P<0.05$ was defined statistically significant.

Results

Associations between positive rate of LGR5 or VM and clinicopathological characteristics

To assess the contributions of LGR and VM to OSCC, the results thereof were immunohistochemically assessed for both OSCC and corresponding normal oral cavity mucosa tissue samples. All data were compared to patients clinicopathological characteristics. LGR5 positive staining was mainly located in the cell cytoplasm and membrane. The positive rate of LGR5 results in the OSCC samples (52.1%, 99/190) was significantly higher that that in the control tissues (10.5%, 20/190; $P<0.001$; **Figure 1A** and **1B**). The positive rate of LGR5 in OSCC was positively associated with tumor size, grade, lymph node metastasis (LNM), and TNM stages, but not with patients' gender, ages, smoking, alcohol, or location (**Table 2**).

Table 2. Correlation between the expression of LGR5 and VM and clinicopathological characteristics in OSCC

Variable	LGR5		*P* value	VM		*P* value
	Negative	Positive		Negative	Positive	
Age (years)			0.885			0.229
<60	34	38		53	19	
≥60	57	61		77	41	
Gender			0.301			0.208
Male	56	68		81	43	
Female	35	31		49	17	
Size			<0.001			<0.001
≤2.0 cm	66	39		85	20	
2.0 cm < size ≤4.0 cm	24	55		44	35	
>4.0 cm	1	5		1	5	
Location			0.545			0.158
Tongue	54	50		74	30	
Gingival	25	34		36	23	
Palate	8	12		13	7	
Tonsil	4	3		7	0	
Smoking			0.400			0.090
No	46	44		67	23	
Yes	45	55		63	37	
Alcohol			0.429			0.228
No	38	47		62	23	
Yes	53	52		68	37	
Grade			<0.001			<0.001
Well	86	55		111	30	
Moderately	4	20		9	15	
Poor	1	24		10	15	
Lymph node metastasis			<0.001			<0.001
No	78	45		102	21	
≤3	11	40		25	26	
>3	2	14		3	13	
TNM stages			<0.001			<0.001
I+II	84	42		103	23	
III+IVa	7	57		27	37	
VM*			<0.001			
Negative	82	48				
Positive	9	51				

*: positive correlation, r = 0.447, P<0.001.

Small vessel, which is like a tube in OSCC, was PAS-positive but CD34-negative is considered as VM structures. Moreover, we found that there was no necrosis and hemorrhage near the VM structures in tumors. A modified method was used to assess VM in the OSCC tissues and control tissues [22]. The rate of VM+ results in the OSCC samples (31.6%, 60/190) was significantly higher that that in the control tissues (0%, 0/190; P<0.001; **Figure 1C** and **1D**). VM in OSCC was positively associated with tumor size, grade, LNM, and TNM stages, but not with patients' gender, ages, smoking, alcohol, and location (**Table 2**).

Association between expression of LGR5 and VM in OSCC

Spearman coefficient analysis showed that positive association between LGR5 positive ex-

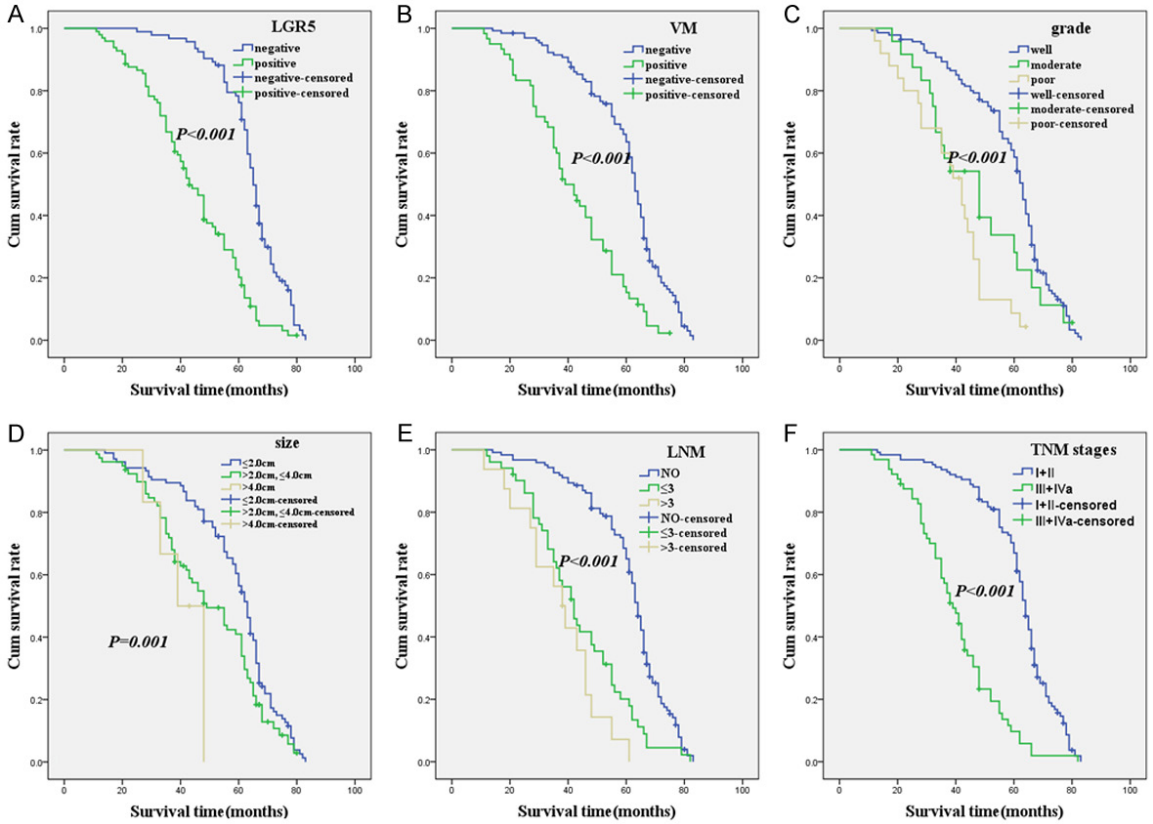

Figure 2. Kaplan-Meier analysis of the survival rate of patients with OSCC. The y-axis represents the percentage of patients; the x-axis, their survival in months. (A) Overall survival of all patients in relation to LGR5 (log-rank = 53.077, P<0.001); (B) Overall survival of all patients in relation to VM (log-rank = 46.847, P<0.001); In (A, B) analyses, the green line represents patients with positive LGR5, or VM and the blue line representing the negative LGR5, or VM group. In (C) analyses, the blue line represents patients with tumor grades: well group, the green line represents moderate group, the brown line represents poor group. In (D) analyses, the blue line represents patients with tumor size: ≤2.0 cm group; the green line represents 2.0 cm < S ≤4.0 cm group; the brown line represents >4.0 cm group. In (E) analyses, the blue line represents patients with N0 group, the green line represents patients with N1 group, the brown line represents patients with N2 group. In (F) analyses, the blue line represents patient with I+II stage group, the green line represents patients with III+IV stage group.

pression and positive rate of VM (rs = 0.447, P<0.001; **Table 2**).

Univariate and multivariate analyzes

Follow-up data showed that OS was significantly shorter in OSCC patients with LGR5-positive samples (43.6±16.1 months) compared with those with LGR5-negative (63.7±10.7 months; log-rank = 53.077, P<0.001, **Figure 2A**). Similarly, OS of VM-positive patients (41.1±16.0 months) compared with those with VM-negative (58.9±14.5 months; log-rank = 46.847, P< 0.001; **Figure 2B**). In univariate analysis, OS was significantly associated with clinicopathological characteristics, including tumor grade (log-rank = 40.501, P<0.001, **Figure 2C**), size (log-rank = 14.003, P = 0.001, **Figure 2D**), LNM (log-rank = 61.017, P<0.001, **Figure 2E**), and TNM stages (log-rank = 75.575, P<0.001, **Figure 2F**; **Table 3**).

Multivariate analysis showed that LGR5-positive, VM-positive, and LNM, as well as TNM stages, were independent prognostic factors for OSCC (**Table 4**).

Discussion

Cancer stem cells (CSCs) were originally identified in acute myeloid leukemia. At later, CSCs were found in various other malignancies, such as lung, colon, breast, ovary, stomach, and liver cancers [5, 7, 12, 20, 23, 24]. CSCs play an important role in the process of initiation, development, metastasis, immune evasion and

Table 3. Results of univariate analyses of overall survival (OS) time

Variable	n	Mean OS (months)	Log-rank	*P* value
LGR5			53.077	<0.001
Negative	93	63.7±10.7		
Positive	97	43.6±16.1		
VM			46.847	<0.001
Negative	130	58.9±14.5		
Positive	60	41.1±16.0		
Age (years)			2.112	0.146
<60	72	53.7±14.8		
≥60	118	53.0±18.4		
Gender			2.024	0.155
Male	124	52.6±16.6		
Female	66	54.6±17.9		
Size			14.003	0.001
≤2.0 cm	105	57.9±15.6		
2.0 cm < size ≤4.0 cm	79	48.1±17.6		
>4.0 cm	6	39.7±8.4		
Location			0.882	0.830
Tongue	104	54.2±16.8		
Gingival	59	52.2±16.6		
Palate	20	50.5±19.6		
Tonsil	7	55.1±19.9		
Smoking			0.782	0.377
No	90	54.0±17.0		
Yes	100	52.6±17.2		
Alcohol			0.738	0.390
No	85	53.8±16.8		
Yes	105	52.8±17.4		
Tumor grade			40.501	<0.001
Well	141	57.5±15.4		
Moderate	24	44.5±17.1		
Poor	25	37.9±14.2		
LNM			61.017	<0.001
N0	123	60.0±13.8		
N1	51	42.6±16.5		
N2	16	36.4±13.6		
TNM stage			75.575	<0.001
I+II	126	60.6±13.4		
III+IV	64	38.9±14.2		

recurrence of cancers [8, 25]. OSCC is a highly heterogeneous cancer. For avoiding any intratumoral heterogeneity of biomarker expression, we chose ten HPF representative fields from different areas of every OSCC's section to analyze immunostaining results. LGR5 is a common biomarker of CSCs and was expressed at the base of crypt stem cells. In this study, we analyzed LGR5 protein expression in OSCC and corresponding normal oral cavity mucosa tissues from 190 patients and compared to clinicopathological characteristics. We found that LGR5 expression was significantly higher in OSCC tissues than that in the normal tissues. Moreover, it was positively associated with tumor size, grade, LNM, and TNM stages. Furthermore, Kaplan-Meier survival analysis suggested that OSCC patients with LGR5-positive expression had significantly shorter survival time than did LGR5-negative patients. Our findings are similar to the other studies demonstrating that LGR5 should be effective as clinical biomarker for OSCC [8, 26, 27].

Angiogenesis supports the rapid growth of tumor by its functions of transporting nutrient and oxygen. The traditional angiogenesis theory was focused on the endothelial cells forming the neovasculature from preexisting vascular. However, the clinical benefits of anti-angiogenesis for cancer therapy is still unsatisfactory [15, 28]. This may indicate that there is another mechanism of tumor blood supply. In 1999, Maniotis and his coworker found a new blood supply which directly interconnected to form channel-like structures by tumor cells----vasculogenic mimicry (VM) [16]. Accumulating evidence suggested that VM plays an important role in promoting blood supply for tumors. Results in this study demonstrated that positive rate of VM was significantly higher in OSCC samples than that in the control samples. And its positive rate was positively associated with tumor size, grade, LNM, and TNM stages. Moreover, we found that patients with positive VM had significantly lower survival time than did VM-negative patients. The above findings suggested that VM should be involved in the progression and metastasis of OSCC, and could be an effective biomarker in conducting this disease. Our results are similar to previous studies, including those of OSCC and other malignancies [20, 28-31].

TNM stages can provide guidelines therapeutic tactics for patients with OSCC, however, it can't provide entire information about OSCC's bio-

Table 4. Multivariate survival analysis of 190 patients with OSCC

Covariate	B	SE	Sig	Exp (B)	95% CI
LGR5	0.569	0.209	0.007	1.766	1.172-2.662
VM	0.515	0.200	0.010	1.674	1.131-2.476
TNM	0.697	0.254	0.006	2.009	1.221-3.304
LNM	0.439	0.178	0.014	1.552	1.095-2.199

logical behavior. Therefore, it is urgent to find novel and efficient biomarker to predict OSCC's patient biological behavior. In this study, multivariate analysis suggested that LGR5 expression, positive VM, LNM, as well as TNM stages are independent prognostic biomarkers for OSCC patients. This finding demonstrates that LGR5 and VM should be considered as credible biomarkers for OSCC, especially in predicting prognosis.

The niche where CSCs reside is composed of microvessels and microlymphatic vessels. Vascular niche can regulate CSCs self-renewal. CSCs can promote angiogenesis to meet rapid tumor growth [32]. CSCs can differentiate various differentiation tumor cells and stromal cells, including endothelial cells [33]. So CSCs can mimic endothelial cells to form tube structures---VM in the tumor tissues. In this study, there was a positive association between the positive expression of LGR5 and VM in OSCC. This indicated that CSCs and VM should promote OSCC's proliferation, progression, and metastasis.

Conclusions

Our results imply that LGR5 affect OSCC metastasis and prognosis, and combined detection of LGR5 and VM, to some extent, should reflect OSCC's cell biological behavior, thus considering as valuable biomarkers of metastasis and prognosis in OSCC.

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Disclosure of conflict of interest

None.

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