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Original Article

Vasculogenic mimicry and expression of Twist1 and KAI1 correlate with metastasis and prognosis in lung squamous cell carcinoma

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Abstract: Background: Vasculogenic mimicry (VM) is a new blood supply development often seen in highly aggressive cancers and has been considered as a usefully metastatic and prognostic factor for many cancers. Twist1 (a biomarker of epithelial-mesenchymal transition), and KAI1 (a suppressor of tumor metastasis) are both usefully predictive factors for metastasis in many cancers. However, the metastatic and prognostic value of VM, Twist1, or KAI1 in lung squamous cell carcinoma (LSCC) is unclear. In this study, we analyzed associations among VM, Twist1, and KAI1 in LSCC, and their respective associations with clinicopathological parameters and survival in LSCC. Case presentation: Positive rates of VM, Twist1, and KAI1 in 157 whole LSCC tissue specimens were detected by immunohistochemistry and histochemical staining. Patient's clinical data were also collected. Levels of VM and Twist1 were significantly higher, and levels of KAI1 were significantly lower, in LSCC tissues than in normal lung tissues. Levels of VM and Twist1 were positively associated with tumor grade, lymph node metastasis (LNM), and tumor-node-metastasis (TNM) stage, and inversely with patients overall survival (OS) time; levels of KAI1 was negatively associated with tumor grade, LNM, and TNM stage, and the KAI1+ subgroup had significantly longer OS time than did the KAI1- subgroup. In multivariate analysis, high VM, or Twist1 levels, TNM stage, size of tumors, and low KAI1 levels were potential to be independent prognostic factors for OS time in patients with LSCC. Conclusions: VM, and the expression of Twist1 and KAI1 represent promising markers for metastasis and prognosis, and potential therapeutic targets for LSCC.

Keywords: Lung squamous cell carcinoma, VM, Twist1, KAI1, prognosis

Introduction

In 2012, lung cancer was reportedly found in 1.8 million newly diagnosed cases, caused about 1.6 million deaths [1], making it the leading lethal cancer. Non-small cell lung cancer accounts for about 85% of all diagnosed lung cancers [2]. As lung cancer is usually asymptomatic in its early stages, majority of patients diagnosed with lung cancer in China have advanced stage disease. Despite advances in treatment, 5-year survival rate was still less than 20% [2].

Cancer requires an adequate blood and nutrient supply for its rapid growth and metastasis. About cancer blood supply, it was long believed that the attention was focused on role of angiogenesis. However, the clinical benefit of anti-angiogenesis therapy for cancers was still unsatisfactory [3]. Later, some researchers found that when the endothelium-dependent vessels growth was insufficient to support the rapid growth of cancer tissues, some cancer cells could mimic endothelial cells and form vessel structures through a process called vasculogenic mimicry (VM) [4, 5]. VM which is a lumen-like structure is a method that provides the blood and nutrient supply and also promotes metastasis [6]. VM is composed of three structures: the stem-like cancer cells, remodeling of the extracellular matrix, and the lumen-like structure which can connect to the host microcirculation system [6, 7]. Accumulating researches have demonstrated that patients

Table 1. Patients characteristics

Patients characteristics	Frequency (n)	Percentage (%)
Age (years)		
<60	115	73.2
≥60	42	26.8
Location		
Left	82	52.2
Right	75	47.8
Size (cm)		
<3.0	23	14.6
≥3.0, <7.0	107	68.2
≥7.0	27	17.2
Gender		
Female	92	58.6
Male	65	41.4
Type		
Central	124	79.0
Peripheral	33	21.0
Grade		
Well	22	14.0
Moderate	97	61.8
Poor	38	24.2
LNM		
N0	81	51.6
N1	55	35.0
N2	21	13.4
TNM stage		
I	69	43.9
II	61	38.9
IIIA	27	17.2

N0: No regional lymph node metastasis; N1: Metastasis in ipsilateral peribronchial and/or ipsilateral hilar lymph nodes and intrapulmonary nodes, including involvement by direct extension; N2: Metastasis in ipsilateral mediastinal and/or subcarinal lymph node(s).

with cancer-associated VM had poor prognoses and were prone to metastasis [4-12].

It has been demonstrated that the epithelial-mesenchymal transition (EMT) plays an important role in invasion and metastasis in many cancers [13-15]. Twist1, which is a highly conserved basic helix-loop-helix (bHLH) transcriptional factor, is associated with initiating tumor EMT and promotes tumor invasion and metastasis [16]. Evidence indicated that Twist1 played an important role in regulating EMT process and overexpression of Twist1 could directly suppress E-cadherin expression and promote the synthesis of N-cadherin [16], thereby inducing dramatic cancer cells morphological changes and cell-matrix adhesion genes to increase cells migration and invasion ability [17, 18].

KAI1, also named as CD82, was the first identified a suppressor of metastasis gene of prostate cancer [19]. KAI1 gene which is located on chromosome 11p11.2 is an important member of tetraspanin superfamily which contains four transmembrane domains [20] with a variety of biological functions [21]. It has been demonstrated that KAI1 inhibits cancer invasion and metastasis in a number of ways, such as inhibiting cell motility, strengthening cell-cell adhesion, and reducing invasion [22, 23]. Overexpression of KAI1 by gene transfection significantly inhibited secondary metastases without affecting primary tumor growth [24]. KAI1 is also been considered to be a useful marker of metastasis and prognosis.

Overall, studies of VM, Twist1, and KAI1 in relation to tumor metastasis and prognosis demonstrate that these indicators affect tumor progression; however, the relationships among VM, Twist1, and KAI1 in LSCC have not been widely reported. In this study, we examined the hypothesis that these indicators are mutual correlated and related to metastasis and prognosis in LSCC.

Materials and methods

Specimens

We collected specimens from all 157 patients (median age: 57.2 years; range: 26-78 years) who were treated for LSCC at the First Affiliated Hospital of Bengbu Medical College, from January 2009 to December 2010, along with 157 samples of the corresponding adjacent normal tissues. Patients who had received preoperative chemo- or radio-therapy were excluded. All tissue cases were obtained with patients writing consent. The study was approved by the ethics committee of Bengbu Medical College and performed in accordance with the guidelines of the Declaration of Helsinki. We collected patients for whom we had completely clinicopathological and follow-up data (at 6-months intervals by phone, mail, or e-mail). Overall survival (OS) time was calculated from the patient's surgery date to his/her death date or December 2015 (mean OS: 43.0 months; range: 8-71

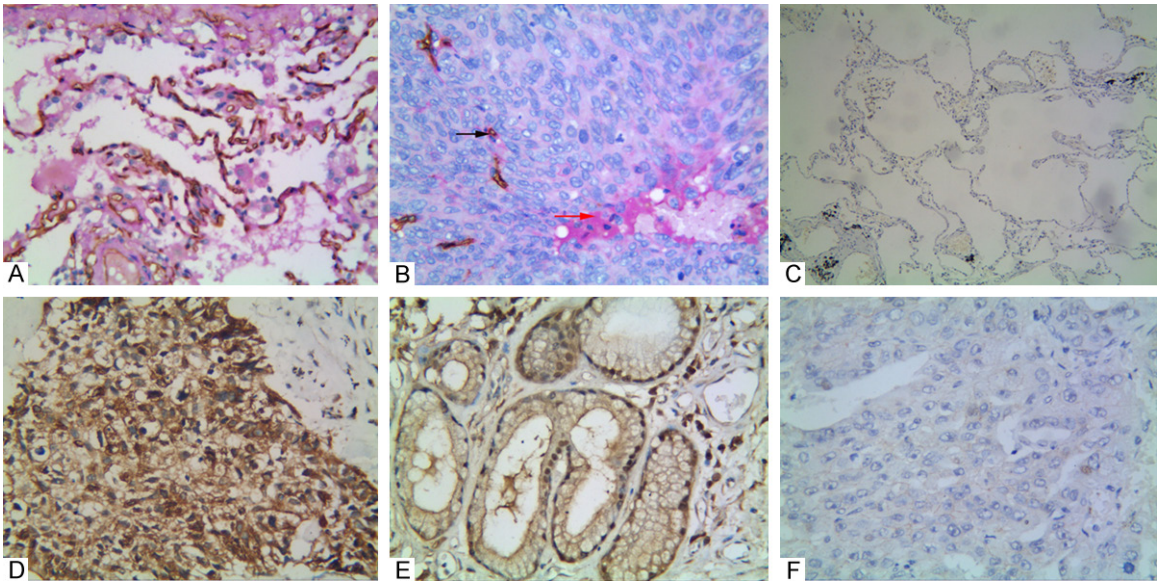

Figure 1. Immunostaining of VM, or Twist1, or KAI1 in LSCC or the control tissue. A: Negative staining of VM in the control tissue (100 magnification); B: Positive staining of VM in the LSCC tissue (400 magnification, red arrow is VM structure, black arrow is microvessel); C: Negative staining of Twist1 in the control tissues (4000 magnification); D: Positive staining of Twist1 in the cytoplasm and nuclei of cancer cells (100 magnification); E: Positive staining of KAI1 in the membrane and cytoplasm of the control tissue (100 magnification); F: Negative staining of KAI1 in the cancer cells (400 magnification).

months). Tumor-node-metastasis stage was assessed according to the 7th edition of the American Joint Committee on Cancer (AJCC). Tumors were graded according to World Health Organization (WHO) standards. Specific parameters see **Table 1**.

Immunohistochemistry

Immunohistochemistry was performed according to the Elivision™ Plus detection kit instructions (Lab Vision, USA). All LSCC and corresponding normal lung tissues were fixed in 10% buffered formalin and embedded in paraffin. Continuous 4 μm thick sections were cut. All sections were deparaffinized and dehydrated in xylene and graded alcohol, then washed with phosphate buffer saline (PBS, pH 7.2) for 10 min. Endogenous peroxidase activity was quenched by incubating sections in methanol containing 3% H_2O_2 for 10 min at room temperature (RT); then placed in citrate buffer (pH 6.0) and heated to 95°C for antigen repair for 30 min. After several washes with PBS, all sections were blocked with goat serum at RT for 30 min, then incubated with mouse monoclonal antibody against human CD34 (Abcam, USA), Twist1 (Abcam), and KAI1 (Abcam, USA) at 37°C for 1 h. All samples were conducted periodic acid-Schiff (PAS)-CD34 dual staining to determine endothelial cells in glycosylated basement membranes of vessels, as well as vessel-like (VM) structure [8].

Yue's method was used to evaluate VM in the LSCC tissues and the control tissues [32]. All sections were counterstained with hematoxylin, dehydrated, air-dried, and mounted. Twist1 stains were mainly seen in tumor cell cytoplasm and nuclei. KAI1 stains were mainly seen in tumor cell membrane and cytoplasm.

Evaluation of staining

Staining results were evaluated semi-quantitatively by two independent pathologists who were blind to patients' clinicopathological and follow-up data. Ten representative fields at high-power-fields (HPF) from different areas of per LSCC slide were analyzed to avoid any intratumoral heterogeneity of antibody expression. The results were scored according to intensity (none staining = 0; weak staining = 1; moderate staining = 2; strong staining = 3) and extent (<11% positive cells mean 1; 11-50% positive cells mean 2; 51-75% positive cells mean 3; >75% positive cells mean 4). The scores for the

Table 2. The associations between VM and expression of Twist1 and KAI1 and clinicopathological characteristics of lung squamous cell carcinoma (LSCC)

Variables	VM		P	Twist1		P	KAI1		P
	-	+		-	+		-	+	
Age			0.467			0.771			0.321
<60 years	65	50		44	71		73	42	
≥60 years	21	21		15	27		23	19	
Location			0.979			0.951			0.483
Left	45	37		31	51		48	34	
Right	41	34		28	47		48	27	
Size (cm)			0.201			0.044			0.339
<3.0	16	7		13	10		11	12	
≥3.0, <7.0	58	49		40	67		67	40	
≥7.0	12	15		6	21		18	9	
Gender			0.650			0.232			0.677
Female	37	28		28	37		41	24	
Male	49	43		31	61		55	37	
Type			0.716			0.331			0.464
Central	67	57		49	75		74	50	
Peripheral	19	14		10	23		22	11	
Grade			0.017			0.024			0.009
Well	17	5		14	8		7	15	
Moderate	54	43		33	64		63	34	
Poor	15	23		12	26		26	12	
LNM			<0.001			<0.001			0.001
N0	58	23		45	36		38	43	
N1	22	33		11	44		43	12	
N2	6	15		3	18		15	6	
TNM stages			<0.001			<0.001			0.001
I	53	16		41	28		31	38	
II	28	33		15	46		43	18	
IIIA	5	22		3	24		22	5	

Table 3. Correlation among VM, expression of Twist1 and KAI1 in LSCC

Variable	VM		r	P	KAI1		r	P
	-	+			-	+		
VM							-0.357	<0.001*
-					39	47		
+					57	14		
Twist1			0.388	<0.001@			-0.380	<0.001*
-	47	12			22	37		
+	39	59			74	24		

*Negative association; @Positive association.

intensity and extent were multiplied to yield final scores that ranged 0-12. Scores ≥3 were considered positive. For sections that were positive for both two of Twist1 and KAI1, an average of the final score of each section was taken.

Statistical analysis

Associations between clinicopathological characteristics and VM, Twist1, or KAI1 were compared using Fisher's exact test or Chi-square test. Association between VM, or Twist1, or KAI1 was compared using Spearman's coefficient test. Effects of VM, Twist1, or KAI1 on survival were determined by univariate and multivariate analyses. Independent prognostic factors were determined using the Cox regression model for multivariate analysis. The Kaplan-Meier method with log-rank test for univariate analysis was used to assess associations between OS time and VM+, Twist1+, or KAI1+ results or clinicopathological characteristics, using SPSS 19.0 software for Windows (Chicago, IL). A value of $P<0.05$ was considered as statistically significant.

Results

Associations between VM, Twist1, and KAI1 expressions and clinicopathological characteristics

To assess the contributions of VM, Twist1, and KAI1 to LSCC, the results thereof were immunohistochemically assessed for both LSCC and corresponding normal lung tissue specimens. These data were compared to patient's clinicopathological characteristics. The positive rate of VM findings (small vessel, which is like a lumen in LSCC, the lumen was PAS-positive but CD34-negative. The VM structure pattern included tubular, linear, and network, etc.) in the LSCC specimens (45.2%, 71/157) was significantly higher than that in the corresponding normal lung tissues (0%, 0/157; P<0.001; **Figure 1A** and **1B**). The posi-

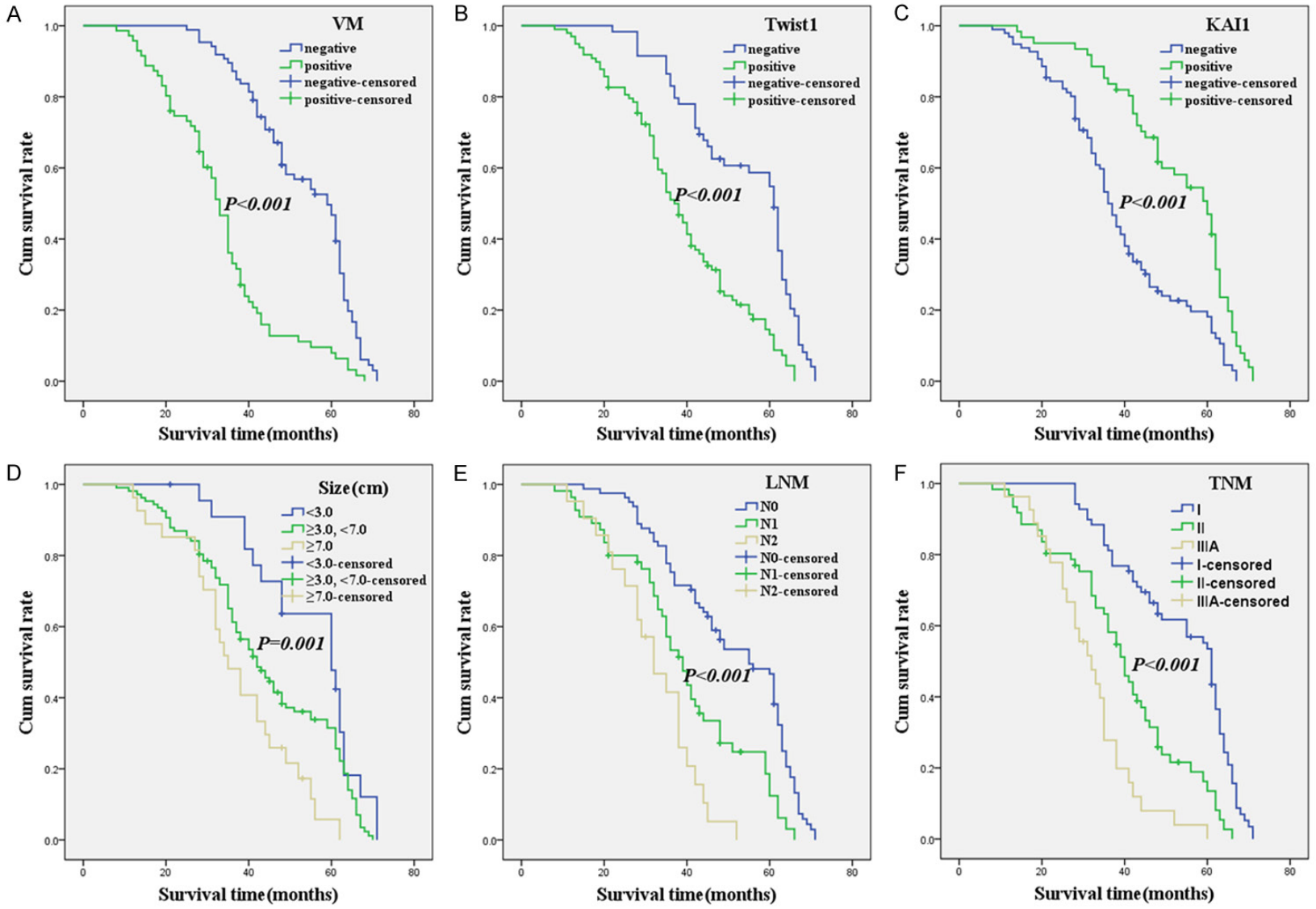

Figure 2. Kaplan-Meier analysis of the survival rate of patients with LSCC. The y-axis represents the percentage of patients; the x-axis, their survival in months. (A) Overall survival of all patients in relation to VM (log-rank = 53.783, P<0.001); (B) Overall survival of all patients in relation to Twist1 expression (log-rank = 31.156, P<0.001); (C) Overall survival of all patients in relation to KAI1 expression (log-rank = 23.906, P<0.001); In (A-C) analyses, the green line represents patients with

positive VM, or Twist1, or KAI1 and the blue line representing the negative VM, or Twist1, or KAI1 group. In (D) analyses, the blue line represents patients with tumor size <3.0 cm group, the green line represents patients with 3.0 cm≤ tumor size <7.0 cm group, the brown line represents patients with tumor size ≥7.0 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 stage group, the green line represents patients with II stage group, the brown line represents patients with IIIA stage group.

Table 4. Results of univariate analyses of overall survival (OS) time

Variable	n	Mean OS (months)	Log-rank	*P* value
VM			53.783	<0.001
Negative	86	51.6±12.1		
Positive	71	32.6±13.7		
Twist1			31.156	<0.001
Negative	59	52.3±13.7		
Positive	98	37.4±14.6		
KAI1			23.906	<0.001
Negative	96	37.5±14.5		
Positive	61	51.7±14.4		
Gender			0.259	0.611
Male	92	44.5±16.1		
Female	65	41.0±15.7		
Ages (years)			0.250	0.617
<60	115	43.5±16.3		
≥60	42	41.8±15.1		
Type			0.697	0.404
Central	124	43.1±16.7		
Peripheral	33	42.7±12.7		
Location			0.236	0.627
Left	82	42.2±16.5		
Right	75	43.9±15.3		
Size (cm)			14.642	0.001
D<3.0	23	52.0±14.0		
3.0≤D<7.0	107	42.7±16.2		
7.0≤D	27	36.7±13.4		
Tumor grade			2.030	0.362
Well	22	50.9±14.1		
Moderate	97	42.4±16.2		
Poor	38	40.2±15.2		
LNM			38.353	<0.001
N0	81	49.7±14.7		
N1	55	37.5±14.9		
N2	21	31.6±10.6		
TNM stage			55.604	<0.001
I	72	52.2±13.5		
II	60	37.7±14.7		
IIIA	25	31.4±10.8		

tive rate of VM in LSCC was positively related to tumor grade, LNM, and TNM stage, but not patient's age, gender, location, type, or size (**Table 2**).

Similar to VM, Twist1+ expression was significantly higher in LSCC tissues (62.4%, 98/157) than that in the control tissues (9.6%, 15/157; P<0.001; **Figure 1C** and **1D**). The positive rate of Twist1 expression in LSCC was related to tumor grade, LNM, TNM stage, and size, but not patient's age, gender, location, or type (**Table 2**).

The positive rate of KAI1 expression was significantly less in LSCC tissues (38.9%, 61/157) than that in the control tissues (96.8%, 152/157; P<0.001; **Figure 1E** and **1F**). The positive rate of KAI1 expression was inversely associated with tumor grade, LNM, and TNM stage. No correlation was found between KAI1 expression and patient's age, gender, size, location, or type (**Table 2**).

Correlations among VM, and expression of Twist1 and KAI1 in LSCC

Spearman correlation coefficient analysis indicated that negative correlations between KAI1+ expression and that of VM (r = -0.357, P<0.001), or Twist1 (r = -0.380, P<0.001). Expression of Twist1 was positive associated with the positive rate of VM (r = 0.388, P<0.001; **Table 3**).

Univariate and multivariate analyzes

Follow-up data showed that OST was significantly lower in LSCC patients with VM+ specimens (32.6±13.7 months) compared with those with VM- (51.6±12.1 months; log-rank = 53.783, P<0.001; **Figure 2A**). Similarly, OST of Twist1+ patients (37.4±14.6 months) was significantly lower than those of Twist1- patients (52.3±13.7 months; log-rank = 31.156, P<0.001; **Figure 2B**). The OST of KAI1+ patients (51.7±14.4 months) was significantly higher than those who were KAI1- (37.5±14.5 months; log-rank = 23.906, P<0.001; **Figure 2C**). In univariate analysis, OST was significantly related to clinicopathological characteristics, including tumor size (log-rank = 14.642, P = 0.001, **Figure 2D**), LNM (log-rank = 38.353, P<0.001,

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

Covariate	B	SE	P	HR	95% CI
VM	0.854	0.217	<0.001	2.348	1.535-3.592
Twist1	0.453	0.212	0.032	1.574	1.039-2.383
KAI1	-0.493	0.200	0.014	0.611	0.413-0.904
TNM stage	0.497	0.221	0.025	1.643	1.065-2.535
Size	0.382	0.159	0.016	1.466	1.074-2.000

Figure 2E), and TNM stage (log-rank = 55.604, P<0.001, **Figure 2F**; **Table 4**).

Multivariate analysis demonstrated that VM+, Twist1+, and KAI1+ samples, tumor size, and TNM stage, were independent prognostic factors for LSCC (**Table 5**).

Discussion

LSCC is a highly heterogeneous cancer, which can interfere with the reproducibility of biomarker assessment. Therefore, prognostic value of candidate biomarker should be thoroughly assessed to ensure their validity. In this research, we found that VM was positively correlated with tumor grade, LNM, and TNM stage. Furthermore, Kaplan-Meier survival analysis indicated that VM+ LSCC patients had significantly lower OST than did VM- patients. These findings suggested that VM should play an important role in LSCC progression and metastasis, and should be considered as a useful biomarker in managing this cancer. VM maybe be responsible for the failure of anti-angiogenesis therapy and should be considered as a potential therapeutic targets for LSCC [26, 27]. Some other researchers showed similar results [7-12].

Twist1 gene which contains two exons and on intron is located on human chromosome 7q21.2 [28]. Twist1 is a key transcription factor of EMT. Overexpression of Twist1 could promote tumor EMT. In LSCC, Twist1 expression has been positively associated with tumor size, tumor grade, LNM, and TNM stage. In addition, Kaplan-Meier survival analysis showed that Twist1+ LSCC patients had significantly lower OST than did Twist1- patients. These results demonstrated that overexpression of Twist1 should promote LSCC invasion and metastasis and mean a poor prognosis. Our findings are consistent with other studies, including those of lung cancers and other cancers [14-16, 29].

KAI1 is widely considered as a suppressor of tumor metastasis in various human cancers [19-24]. KAI1 can limit cancer cell motility, invasion, and metastasis and inhibit cancer cells metastatic potential [22, 23]. Findings in this study also showed that KAI1 expression was significantly lower in LSCC tissues than that in control tissues, and its expression was negatively correlated with tumor grade, LNM, and TNM stage. Moreover, Kaplan-Meier survival demonstrated that LSCC patients with KAI1+ samples had significantly longer survival time than did KAI1- patients. These findings suggested that down-regulation of KAI1 should promote LSCC progression and metastasis, which is similar to results of previous researches [20-24, 30].

TNM stages guide therapeutic strategies for patients with LSCC, but do not provide comprehensive information about LSCC's behavior. Therefore, it is urgent to find novel and effective biomarkers to predict LSCC behavior, metastasis, and patient's prognosis. In our study, multivariate analysis showed that VM, expression of Twist1 and KAI1 and tumor size, as well as TNM stages, are independent prognostic factors for LSCC patients. Our findings thus demonstrated VM, Twist1, and KAI1 as reliable biomarkers for LSCC, especially in predicting metastasis and prognosis.

Lung squamous cell carcinoma (LSCC) is the most common pathological type of NSCLC. It is characterized by local invasion and a high rate of regional lymph node metastasis. When tumor grows a certain size, tumor can stimulate angiogenesis. But when the blood supply by angiogenesis cannot meet the need of tumor rapid growth, some stem-like cancer cells can mimic endothelial cell and form vasculogenic mimicry [5, 8]. During cancer progression, cancer cells often undergoing an EMT process which converts epithelial cells into mesenchymal-like phenotype acquire mesenchymal cell characteristics [31]. Thus, EMT promotes cancer cell invasion and metastasis. Twist1 which is a transcription factor can regulate the EMT process [32]. Overexpression of Twist1 could induce cancer cell EMT process as well as promoted cell motility, migration, and invasion ability by regulating E-cadherin, N-cadherin, and MMP9 expression [17]. In the same time, over-

expression of Twist1 could increase cancer cell plasticity, metastasis, and VM [33]. KAI1 could inhibit the EMT process to prevent tumor angiogenesis [24, 34]. Down-regulation of KAI1 loses inhibiting the activation of angiogenesis and stabilization of E-cadherin-β-catenin complexes to further promote cancer cell invasion and metastasis [35].

Conclusions

Our findings imply that Twist1 and KAI1 affects LSCC evolution; and that combined detection of VM, Twist1, and KAI1 are valuable factors of metastasis and prognosis in LSCC.

Acknowledgements

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Disclosure of conflict of interest

None.

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