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Original Article

Expression of KAI1/CD82 and CD44v6 in laryngeal squamous cell carcinoma and its significance

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Abstract: Objective: This study aimed to investigate the expression and distribution of KAI1/CD82 and CD44v6 in laryngeal squamous cell carcinoma (LSCC), to analyze their relationship with the clinicopathological factors of LSCC and the mutual relations of the two factors in LSCC, to explore their roles in the carcinogenesis and development of LSCC and their effect on prognosis. Methods: The expression levels of the mRNA and protein of KAI1/CD82 and CD44v6 were analyzed in 86 cases of LSCC tissues and 30 cases of adjacent normal mucosa tissues by polymerase chain reaction and western blot analysis, respectively. Immunohistochemistry was used to detect the expression and distribution of KAI1/CD82 and CD44v6 in 86 LSCC tissues and corresponding adjacent normal mucosa tissues, and the relationship between the expression of KAI1/CD82 and CD44v6 and the clinical parameters was analyzed. Statistical analyses and creation were carried out using Graphpad Prism 6 software. Results: It was revealed that expression of KAI1/CD82 mRNA and protein was significantly lower in LSCC tissues than in adjacent normal mucosa tissues ($P<0.05$), and expression of CD44v6 mRNA and protein was significantly higher in LSCC tissues than in adjacent normal mucosa tissues ($P<0.05$). In addition, both KAI1/CD82 and CD44v6 were mostly located in the cytoplasm and membrane, and KAI1/CD82 expression was negatively correlated with CD44v6 expression in these tissues. The expression of KAI1/CD82 was significantly correlated with clinical stages, tumor differentiation and tumor recurrence ($P<0.05$), and there was no significant correlation with sex, age and lymph node metast0asis ($P>0.05$). The expression of CD44v6 was significantly correlated with clinical stages, lymph node metastasis and tumor recurrence ($P<0.05$), and there was no significant correlation with sex, age and tumor differentiation ($P>0.05$). Patients with positive expression of KAI1/CD82 had an obvious higher survival rate and shorter survival time than patients with low expression ($P<0.05$), and patients with positive expression of CD44v6 had an obvious lower survival rate and shorter survival time than patients with low expression ($P<0.05$). Cox regression analysis indicated that age, lymph node metastasis, clinical stages and tumor recurrence were independent prognostic factors for LSCC. Conclusions: This study illuminated that the abnormal expression of KAI1/CD82 and CD44v6 was closely related with the carcinogenesis and development of LSCC, and that downregulation of CD44v6 along with the upregulation of KAI1/CD82 in LSCC tissues resulted in the inhibition of LSCC migration and invasion. Thus, our results suggested that combined detection of KAI1/CD82 and CD44v6 can be used as a valid reference index for the prognosis of LSCC patients.

Keywords: Laryngeal neoplasm, squamous cell carcinoma, KAI1/CD82, CD44v6

Introduction

The occurrence, development, metastasis and prognosis of cancer are a complicated process involving multiple genes and multiple steps, which is as a result of cooperation and interaction of multiple factors. This is not an exception for laryngeal squamous cell carcinoma (LSCC). However, despite advance in surgical and therapeutic strategies, the majority of LSCC patients have not yet achieved good curative effects. Therefore, many scholars have recently proposed that the combined detection of multiple genes may obtain a satisfactory result for prognostic judgment. Alone with tissue microarray technology and proteomics development, more and more molecular markers have been discovered, including growth factors, proteolytic enzymes penetrating the extracellular matrix, adhesion molecules, and angiogenesis factors which vascularize the tumor in several steps throughout metastasis [1]. Thus, to explore the relationship among genes exerting inhibitory effects on cancer metastasis is clinically important for the evaluation of prognosis and treatment of cancer. Molecular markers of LSCC for

Table 1. The clincopathological parameters of the patients with LSCC

Clincopathological parameters	Case (n)	Rate (%)
Sex		
Male	81	94.2
Female	5	5.8
Age (years)		
≤60	46	53.5
>60	40	46.5
Tumor differentiation		
Well	42	48.9
Moderately	27	31.4
Poorly	17	19.8
Lymph node metastasis		
No	61	70.9
Yes	25	29.1
Clinical stage		
I-II	56	65.1
III-IV	30	34.9
Recurrence		
No	67	77.9
Yes	19	22.1

prognostic prediction and therapeutic guidance remain urgent in clinical practice.

Both KAI1/CD82 and CD44v6 genes were mapped to the short arm of human chromosome 11, which belong to the adhesion molecules and play an important role in tumor growth, progression and metastasis. Many studies indicate that KAI1/CD82 and CD44v6 in relation to tumor metastasis are involved in the process of tumor progression through regulating the intercellular adhesion [2, 3]. However, there are few studies on the interaction between them. In this study, we detected the expression of KAI1/CD82 and CD44v6 in LSCC tissues and normal laryngeal mucosa by using quantitative real-time PCR, western blot assay and immunohistological technique, and observed the relationship between their expression and clinical pathological features, especially metastasis. The expression dependability of KAI1/CD82 and CD44v6 in LSCC, and their functions and interactions during the stages of initiation, development, and metastasis of LSCC were also discussed. The study aimed to investigate whether KAI1/CD82 and CD44v6 may serve as novel molecular prognostic markers in human LSCC and whether they could serve as new predictive factors for the diagnosis, treatment, and prognostic judgment of patients with LSCC.

Materials and methods

Cases and specimens

All subjects were Chinese. Tumor tissue and corresponding adjacent normal mucosa specimens were obtained from the 86 patients with LSCC undergoing total or partial laryngeal resection at the Department of Otolaryngology-Head and Neck Surgery, the People's Hospital of Guizhou Province, China, between 2009 and 2011. These tumor tissue samples and corresponding adjacent normal mucosa tissues that were used in quantitative real-time PCR, western blot analysis and immunohistochemical analysis were randomly collected from LSCC tissues in 2012. None of the patients had undergone prior treatment for cancer and all had complete clinicopathologic and follow-up data. The laryngeal cancer was classified according to the World Health Organization (WHO) tumor classification system. Six out of the 86 patients lost follow-up, and the follow-up time ranged from 60 months to 84 months. Written informed consent was obtained from all patients before surgery. The clinicopathologic characteristics of these patients are summarized in **Table 1**.

The study was approved by the Human Research Ethics Committee at the People's Hospital of Guizhou Province, and the work was supported by a grant from the Science and Technology Research Program of Guizhou Province (No.[2012]031).

The obtained specimens were divided into three parts: Those for mRNA determination were removed from different tissues, another part for protein determination, whereas the third part specimens for immunohistochemistry were fixed, dehydrated, and embedded routinely.

RT-PCR and real-time PCR for mRNA expression

Four groups which had various expressions of KAI1/CD82 and CD44v6 were selected for PCR assay. Briefly, total RNAs were extracted using Trizol (Invitrogen, US) in accordance with the manufacturer's instructions. cDNA was synthesized using Access RT-PCR System (Promega,

Table 2. Primer Sequences of KAI1/CD82, CD44v6, and β-actin (internal reference)

Gene	Primer	Primer sequence	Product size (bp)
KAI1/CD82	Forward	5'-CGTGGGTGTGGCCATCAT-3'	83
	Reverse	5'-TTGCTGTAGTCTTCGGAATGG-3'	
CD44v6	Forward	5'-CCAGGCAACTCCTAGTAGTACAAC-3'	107
	Reverse	5'- GGGAGTCTTCTCTGGGTGTTTG-3'	
β-actin	Forward	5'-GCTACGAGCTGCCTGACG-3'	112
	Reverse	5'-TCGTGGATGCCACAGGAC-3'	

America). Real-time PCR was performed using SYBR Green ER quantitative PCR (qPCR) uperMix (Thomero) according to the manufacturer's instructions. The relative mRNA levels of KAI1/CD82 and CD44v6 were respectively calculated using comparative cycle thresholds, according to the equation: $2^{-\hat{I}Ct}$[ÎCt=Ct(DNA-PKcs)-Ct(β-actin)]. All experiments were performed in triplicate. The relative expression levels of the target genes were the same. Only the results using β-actin as the reference gene were shown. Experimental results were analyzed using a multiimage analyzer (Bio-Rad, Hercules, CA). The specific primer sequences are shown in **Table 2**.

Western blot for protein expression

The protein expression levels of KAI1/CD82 and CD44v6 were detected with western blotting by using the same tissue specimens as quantitative real-time PCR. Proteins were extracted according to the manufacturer's protocol (Keygen, China). Proteins (20 μg per lane) were electrophoresed on 6% SDS-polyacrylamide gels. After electrophoresis, the separated proteins were transferred to polyvinylidene fluoride membranes (Bio-Rad). The membranes were blocked for 2 h in 5% milk dissolved Tris-buffered saline with 0.1% Tween and incubated overnight with the primary antibody at 4°C. The filters were incubated with the primary antibody (KAI1/CD82 and CD44v6, 1:200 dilution, Abcam, English, respectively). β-actin (1:200 dilution, Abcam, English) acted as the internal control. Antigen and primary antibody complexes were detected by the secondary antibody, horseradish peroxidase-conjugated antimouse IgG (cell signaling) (1:1000), and enhanced chemilumescence (Amersham Pharmacia Biotech). The signal was measured using Image Lab™ software (BIO-RAD, America). The western blot analyses were done in triplicate.

Immunohistochemistry for protein expression

Paraffin-embedded slides of the 86 pairs specimens of tumor tissues and corresponding adjacent normal mucosa tissues were dried, deparaffinized, and dehydrated in a graded series of ethanol. The slides were treated with 3% H_2O_2 for 10 minutes, followed by treatment with goat serum for 30 min at room temperature. The slides were incubated with monoclonal mouse anti-human KAI1/CD82/FITC and CD44v6/FITC (1:100 dilution, Abcam, English), and monoclonal mouse β-actin (1:200 dilution, Abcam, English) antibodies diluted in blocking buffer at 4°C for 8-12 hour or overnight. Immunohistochemistry was performed by using the EnVision method and based on the instructions of kits (Beijing ZSGB-BIO, China). The primary antibodies were replaced with PBC in the negative control. The above samples were incubated with the secondary antibody (EnVision™ two anti goat anti pika universal reagent Poly-HRP) for 15-30 min at room temperature. After washing with PBS (3×2 min), the samples were stained with 0.01% DAB hydrogen peroxide for about 3-10 min, thoroughly washed with tap water and then counterstained by hematoxylin for immunohistochemistry analysis using a light microscope.

Immunohistochemical staining was assessed blindly by two senior pathologists without knowledge of patient characteristics. Any discrepancies were resolved by reaching a consensus. The expression of KAI1/CD82 and CD44v6 was present on the membrane and in the cytoplasm, with brown or tan particles was defined as positive. The following scoring system was used to evaluate KAI1/CD82 and CD44v6 expression according to the intensity and percentage of positive cells: strong staining (scored as 3) indicated dark brown staining obscuring on the membrane and in the cytoplasm of tumor cells; yellow staining (scored as 2); primrose yellow staining (scored as 1); absence (scored as 0) indicated no appreciable staining in tumor cells. At the same time, staining was also scored according to the percentage of positive cells: negative, 0, positive cell rate ≤10%,

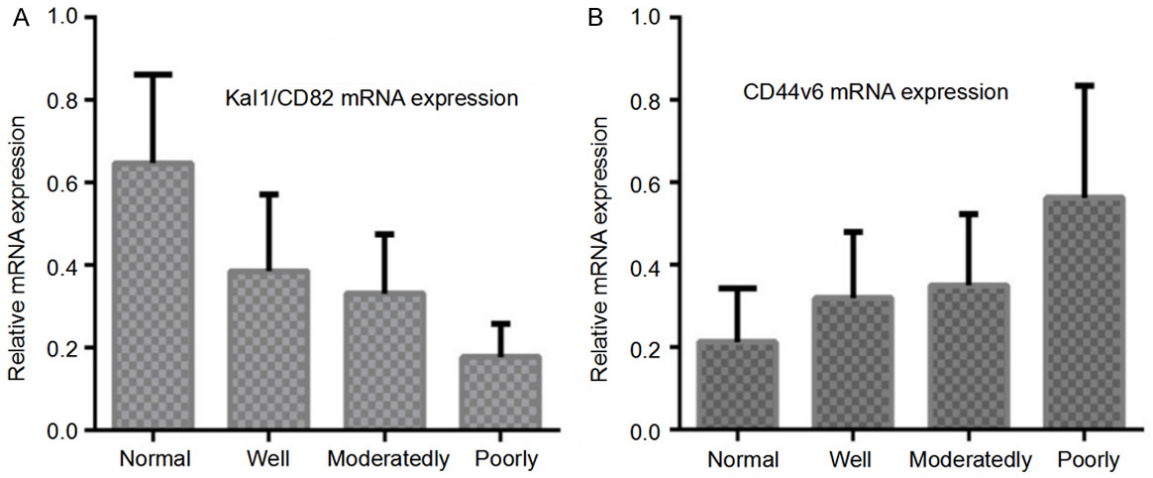

Figure 1. The quantitative RT-PCR results of KAI1/CD82 (A) and CD44v6 (B) and β-actin mRNA expression as the internal reference in four groups who had various expressions of KAI1/CD82 and CD44v6. Relative mRNA levels of KAI1/CD82 and CD44v6. Histograms represent the scanning densitometric analysis of PCR results shown in it. Each value was normalized for β-actin mRNA levels and expressed as a value relative to that of β-actin, which was set to a value of 1. The comparison of KAI1/CD82 and CD44v6 expression levels in normal mucosa tissues (normal) and different differentiated LSCC (well, moderately, poorly).

Table 3. The expression of KAI1/CD82 and CD44v6 mRNA levels in normal tissues and tumor tissues

Classification	KAI1/CD82 mRNA level	CD44v6 mRNA level
Normal tissues	0.798 ± 0.495	0.305 ± 0.121
Well differentiated LSCC	0.516 ± 0.253	0.433 ± 0.206
Moderately differentiated LSCC	0.432 ± 0.228	0.473 ± 0.228
Poorly differentiated LSCC	0.234 ± 0.121	0.755 ± 0.371

KAI1/CD82 and CD44v6 mRNA levels are expressed as values relative to those of β-actin. Mean ± SD, n=86. $P<0.05$.

1; 11%~50%, 2; 51%~75%, 3; ≥75%, 4. The two scores were multiplied, and the result was the final score. For the final score: 0~1 indicated negative (-); 2~3 indicated weak positive (+); 4~6 meant moderately positive (++); more than 6 indicated strong positive (+++). 2~12 positive results were statistically analyzed.

Statistical analysis

Statistical analyses and creation were carried out using Graphpad Prism 6 software. The bands were measured for integral optical density (IOD) and the results were described with mean ± standard errors. Mean values of the two groups were compared using *t*-test. The relationship of KAI1/CD82 and CD44v6 expression with clinicopathologic features was analyzed using the x^2 or the Fisher exact test. Survival curves were plotted using the *Kaplan-Meier* method and compared using the *Log-rank* test. Cox regression models were used for multiple factor analysis. We first evaluated all prognostic factors individually in univariate analyses and then in combination as multivariate models. The main end point in this study was percent survival. A significant relationship between KAI1/CD82 and CD44v6 expression and percent survival (defined as $P<0.05$) in univariate analyses was used as the criterion for including KAI1/CD82 and CD44v6 expression in the multivariate backward stepwise elimination procedure. The final multivariate model retained some prognostic factors. Difference with $P<0.05$ was considered statistically significant.

Results

RT-PCR and real-time PCR analysis

PCR results for the relative expression levels of KAI1/CD82 and CD44C6 mRNA are shown in **Figure 1**. Quantitative RT-PCR analysis was performed for KAI1/CD82 and CD44v6 in four selected subjects, i.e., three tumor tissues and one adjacent normal mucosa tissue. In all the patients, KAI1/CD82 mRNA levels were significantly higher in adjacent normal mucosa tissues compared with tumor tissues ($P<0.01$,

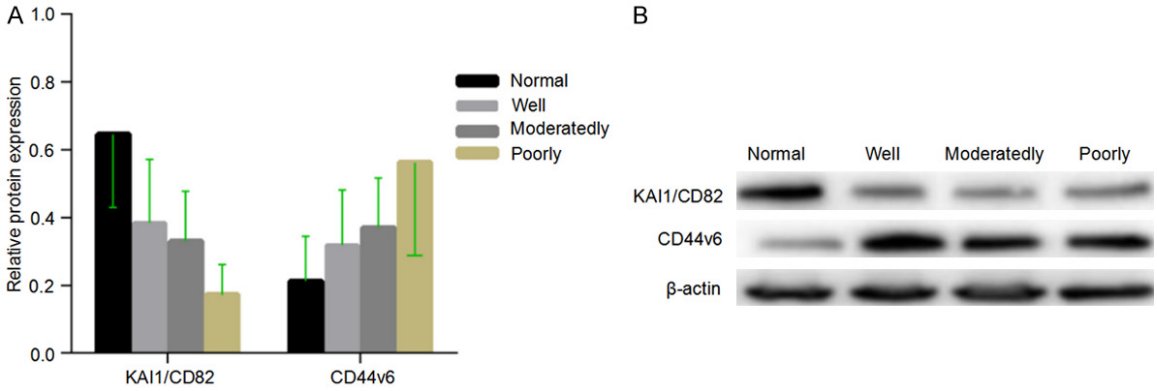

Figure 2. Western blot analysis of KAI1/CD82 and CD44v6 and β-actin in whole-cell extracts prepared from four individuals who had various expressions of KAI1/CD82 and CD44v6 (shown below the blots). A: The expression of KAI1/CD82 and CD44v6 protein in adjacent normal mucosa tissues (normal), well differentiated LSCC (well), moderately differentiated LSCC (moderately), and poorly differentiated LSCC (poorly). B: Relative protein levels of KAI1/CD82 and CD44v6. Histograms represent the scanning densitometric analysis of Western blots shown in B. Each value was normalized for β-actin protein levels and expressed as a value relative to that of β-actin, which was set to a value of 1. The comparison of KAI1/CD82 and CD44v6 expression levels in adjacent normal mucosa tissues (normal) and differently differentiated LSCC (well, moderately, poorly).

Table 4. The expression levels of KAI1/CD82 and CD44v6 protein in normal tissues and tumor tissues

Classification	KAI1/CD82 protein level	CD44v6 protein level
Normal tissues	0.799 ± 0.493	0.306 ± 0.119
Well differentiated LSCC	0.519 ± 0.251	0.434 ± 0.203
Moderately differentiated LSCC	0.434 ± 0.226	0.474 ± 0.266
Poorly differentiated LSCC	0.235 ± 0.109	0.756 ± 0.369

KAI1/CD82 and CD44v6 protein level is expressed as values relative to that of β-actin. Mean ± SD, n=86. $P<0.05$.

Figure 1), and average values of KAI1/CD82 mRNA levels of the 30 adjacent normal mucosa tissues were significantly higher than those of 86 tumor tissues (**Table 3**). CD44v6 mRNA levels were lower in adjacent normal mucosa tissues compared with tumor tissues ($P<0.01$, **Figure 1**). Average values of CD44v6 mRNA levels of the 30 adjacent normal mucosa tissues were significantly higher than those of 86 tumor tissues (**Table 3**). The mRNA levels of KAI1/CD82 in the well differentiated LSCC and the moderately differentiated LSCC were similar. The mRNA levels of KAI1/CD82 in poorly differentiated LSCC were lower than in the well differentiated LSCC or the moderately differentiated LSCC ($P<0.05$, **Figure 1**). The mRNA levels of CD44v6 in the well differentiated LSCC and the moderately differentiated LSCC were similar. The mRNA levels of CD44v6 in poorly differentiated LSCC were higher than those in the well differentiated LSCC or the moderately differentiated LSCC ($P<0.05$, **Figure 1**). mRNA levels of KAI1/CD82 and CD44v6 correlated to tumor differentiation. β-actin was used as the internal reference.

Western blotting analysis

As shown in **Figure 2**, protein levels of KAI1/CD82 and CD44v6 in tumor and normal tissues were also examined by western blot analysis using the same specimen as PCR. In all the patients, KAI1/CD82 protein levels were higher in adjacent normal mucosa tissues compared with tumor tissues (**Figure 2**), and average values of CD44v6 protein levels of the 30 adjacent normal mucosa tissues were significantly higher than those of 86 tumor tissues (**Table 4**). CD44v6 protein levels were lower in adjacent normal mucosa tissues compared with tumor tissues (**Figure 2**), and average values of CD44v6 protein levels of the 30 adjacent normal mucosa tissues were significantly higher than those of 86 tumor tissues (**Table 4**). The protein levels of KAI1/CD82 in the well differentiated LSCC and the moderately differentiated LSCC were similar. The protein levels of KAI1/CD82 in poorly differentiated LSCC were lower than those in the well differentiated LSCC and the moderately differentiated LSCC. The protein levels of CD44v6 in the well differentiated

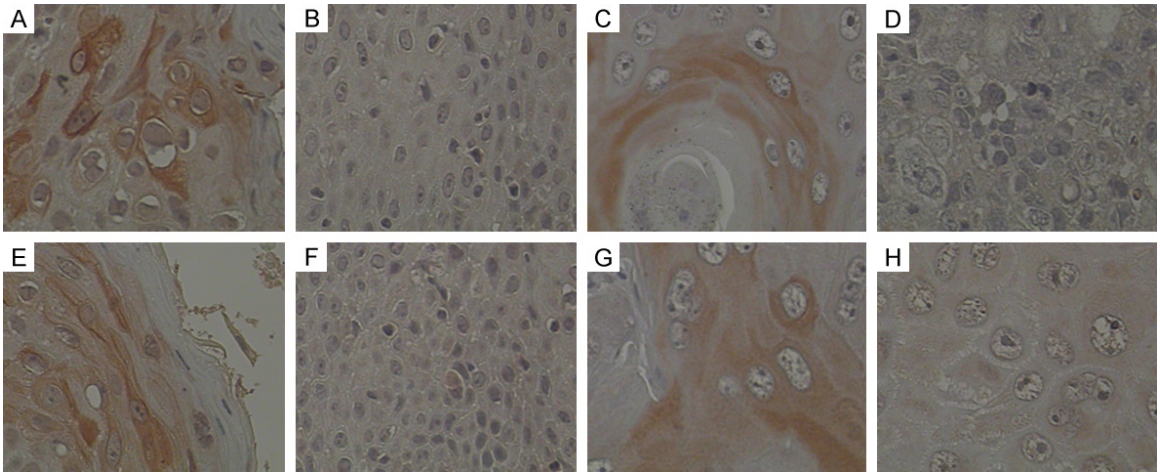

Figure 3. Expression of KAI1/CD82 and CD44v6 in the LSCC tissues and adjacent normal mucosa tissues revealed by immunohistochemical analyses (EnVision). KAI1/CD82 expression in the adjacent normal mucosa tissues, A (positive) and B (negative). KAI1/CD82 expression in the LSCC tissues, C (positive) and D (negative). CD44v6 expression in the adjacent normal mucosa tissues, E (positive) and F (negative). CD44V6 expression in the LSCC tissues, G (positive) and H (negative). Magnification ×400.

Table 5. The expression of KAI1/CD82 and CD44v6 in normal tissues and LSCC (positive rate %)

Classification	KAI1/CD82			CD44v6		
	Positive n (%)	x^2 value	*P* value	Positive n (%)	x^2 value	*P* value
Normal mucosa tissues						
Negative	14 (16.2)	4.350	0.011	75 (87.2)	5.015	0.009
Positive	72 (83.8)			11 (12.8)		
LSCC tissues						
Egative	56 (65.1)	3.043	0.023	28 (32.6)	2.039	0.032
Positive	30 (34.9)			58 (67.4)		

P<0.05 was considered statistically significant.

LSCC and the moderately differentiated LSCC were similar. The protein levels of CD44v6 in poorly differentiated LSCC were higher than those in the well differentiated LSCC and the moderately differentiated LSCC. Protein levels of KAI1/CD82 and CD44v6 correlated to tumor differentiation. β-actin was used as the internal reference.

Immunohistochemistry analysis

As shown in **Figure 3**, EnVision immunohistochemistry analysis was performed to examine KAI1/CD82 and CD44v6 expression by using 86 cases of tumor tissues and corresponding adjacent normal mucosa tissues. The positive reaction of KAI1/CD82 and CD44v6 staining assumed brown or tan particles in different tissues, and KAI1/CD82 and CD44v6 were primarily located on cell membranes and in cytoplasm, which were stained positive in different tissues. The expression of KAI1/CD82 was weakly positive or negative in tumor tissues, with strong positive in adjacent normal mucosa tissues. The expression of CD44v6 was strong positive in tumor tissues, which was weakly positive or negative in adjacent normal mucosa tissues. There was significant difference in KAI1/CD82 and CD44v6 expression levels between LSCC tissues and adjacent normal mucosa tissues ($P<0.05$), as shown in **Table 5**.

Correlation between the expression of KAI1/CD82 and CD44v6 and the pathological parameters of LSCC

The positive expression rates of KAI1/CD82 and CD44v6 also varied according to different pathological parameters of LSCC. The positive expression rates of KAI1/CD82 in patients with poorly differentiated, clinical stage III-IV, and recurrence LSCC were noticeably lower than those in patients with well differentiated, clinical stage I-II, and non-recurrence LSCC ($P<0.01$ or 0.05), whereas no significant difference was observed with regard to sex, age, and lymph node metastasis ($P>0.05$). The positive expres-

Table 6. Correlation between the expression of KAI1/CD82 and CD44v6 and the clincopathological parameters of LSCC/Case number (positive rate %)

Clincopathological parameters	n	KAI1/CD82			CD44v6		
		Positive n (%)	x^2 value	*P* value	Positive n (%)	x^2 value	*P* value
Sex							
Male	81	29 (35.8)	0.135	1.000	54 (66.7)	0.368	0.778
Female	5	1 (25.0)			4 (75.0)		
Age (years)							
≤60	46	14 (30.4)	0.601	0.520	32 (69.6)	0.579	0.895
>60	40	16 (40.0)			26 (65.0)		
Tumor differentiation							
Well	69	16 (23.2)	7.532	0.003	46 (66.7)	2.898	0.237
Poorly	17	14 (82.4)			12 (70.6)		
Lymph node metastasis							
No	61	21 (34.4)	2.719	0.221	35 (57.4)	9.794	0.003
Yes	25	9 (36.0)			23 (92.0)		
Clinical stage							
I-II	56	23 (41.1)	4.213	0.015	33 (58.9)	6.015	0.008
III-IV	30	7 (23.3)			25 (83.3)		
Recurrence							
No	67	26 (38.8)	4.045	0.023	41 (63.0)	3.456	0.028
Yes	19	4 (21.1)			17 (89.5)		

P<0.05 was considered statistically significant.

Table 7. Spearman correlation between the expression of KAI1/CD82 and CD44v6 and the clincopathological parameters

Clincopathological parameters	KAI1/CD82		CD44v6	
	Spearman CC	*P* value	Spearman CC	*P* value
Sex	-0.038	1.000	-0.066	0.675
Age (years)	-0.082	0.512	-0.810	0.502
Tumor differentiation	0.456	0.000	-0.168	0.137
Lymph node metastasis	-0.294	0.007	0.364	0.001
Clinical stage	0.367	0.001	0.429	0.001
Recurrence	0.338	0.003	0.267	0.013

Abbreviations: CC, correlation coefficient.

sion rates of CD44v6 in patients with lymph node metastasis, clinical stage III-V, and recurrence LSCC were noticeably higher than those in patients with non-lymph node metastasis, clinical stage I-II, and non-recurrence LSCC (*P*<0.01 or 0.05), whereas no significant difference was observed with regard to sex, age, and tumor differentiation (*P*>0.05). The results are summarized in **Table 6**.

The spearman correlation results showed KAI1/CD82 expression was positively related to tumor differentiation, clinical stage and tumor recurrence (*P*<0.05). The spearman correlation results showed that CD44v6 expression was positively correlated with clinical stage, lymph node metastasis and tumor recurrence (*P*< 0.05), and negatively correlated with tumor differentiation. The results are summarized in **Table 7**.

Correlation analysis on the expression of KAI1/CD82 and CD44v6 in LSCC

The expressions of KAI1/CD82 and CD44v6 in LSCC patients were divided into 4 groups, and the correlation between KAI1/CD82 expression and CD44v6 expression was evaluated. Results showed the KAI1/CD82 expression was negatively related to the CD44v6 expression (γ_s=-0.584, *P*<0.05) (**Table 8**).

Correlation between the expression of KAI1/CD82 and CD44v6 in LSCC and prognosis

Follow-up began from the operation, and the patients were followed up every half a year or one year. The 3-year survival rate and 5-year survival rate of the patients were 77.9% and 47.7%, respectively. Among 45 cases of death,

Table 8. Correlation between the expression of KAI1/CD82 and CD44v6 in LSCC

KAI1/CD82	CD44V6		Total (n)	γ_s *value*	*P value*
	Positive (n)	Negative (n)			
Positive (n)	22	8	30	-0.584	0.006
Negative (n)	36	20	56		
Total (n)	58	28	86		

P<0.05 were considered statistically significant.

23 cases died of tumor recurrence or metastasis, and 15 cases died of non tumor factors.

To investigate whether KAI1/CD82 expression in LSCC was associated with patient survival time, the percent survival curves were plotted with the Kaplan-Meier method and compared using the log-rank test. The results suggested that patients with high KAI1/CD82 expression had longer percent survival than patients with low expression, and the 3-year survival rate and 5-year survival rate of patients with high KAI1/CD82 expression were 86.7% and 63.3%, respectively, compared to 73.2% and 39.3% of patients with low KAI1/CD82 expression. Moreover, percentage survival of patients with high KAI1/CD82 expression was longer than that of patients with low KAI1/CD82 expression, and the significant difference was observed in the percent survival curves between high and low KAI1/CD82 group (**Figure 4A**, log-rank test, x^2=5.871, *P*=0.015).

To investigate whether CD44v6 expression in LSCC was associated with patient survival time, the percent survival curves were plotted with the Kaplan-Meier method and compared using the log-rank test. Patients with high CD44v6 expression had shorter percent survival than those with low expression, and 3-year survival rate and 5-year survival rate in patients with high CD44v6 expression were 78.6% and 39.7%, respectively, compared to 82.1% and 64.3% of patients with low CD44v6 expression . Moreover, percentage survival of patients with high CD44v6 expression was shorter than that in the low expression group, and the significant difference was observed in the percent survival curves between high and low CD44v6 group (**Figure 4B**, log-rank test, x^2=7.556, *P*=0.006).

Analysis on Cox proportional risk model related factors for prognosis of LSCC

Univariate *Cox* model was used to analyze sex, age, tumor differentiation, lymph node metastasis, clinical stage, tumor recurrence, and the expression of KAI1/CD82 and CD44v6, and the results showed that the patient survival rate was associated with the expression of KAI1/CD82 and CD44v6, tumor differentiation, lymph node metastasis, clinical stage and tumor recurrence. Furthermore, multivariate *Cox* regression analyses demonstrated that such factors affected the regression equation after stepwise regression analysis, as age (*P*=0.001), lymph node metastasis (*P*=0.043), clinical stage (*P*=0.002) and tumor recurrence (P=0.001) (**Table 9**).

Discussion

KAI1/CD82 is a member of the tetraspan transmembrane superfamily (TM4SF) and is a gene located on human chromosome 11p11.2 [4], which mediates the adhesion function between cells as well as between cells and the extracellular matrix (ECM) [2]. KAI1/CD82 plays an important role in cell fusion, adhesion, migration, signaling, fertilization, differentiation, and invasion [5-7]. Cell adhesion to the ECM is the first step of metastasis, required for cell migration and invasion of primary tumor. Moreover, adhesion of cancer cells to the ECM in microvessels and migration into the other organs are critical in the multistep metastatic process [8]. Cell adhesion to cells or ECM was altered by KAI1/CD82 expression. Specifically, higher levels of KAI1/CD82 were correlated with lower cell adhesion to cells or ECM. Consistent with these observations, previous reports have shown that KAI1/CD82 expression inhibits adhesion of cancer cells to cells or ECM, while KAI1/CD82 silencing enhanced the adhesion of cancer cells. In addition to cell adhesion, KAI1/CD82 expression levels also affected cell detachment from cells or ECM, further supporting the role of KAI1/CD82 in cell-to-ECM interactions. It has been shown that KAI1/CD82 suppresses metastasis by various mechanisms involving inhibition of invasion and motility, induction of apoptosis and senescence in response to extracellular stimuli [9].

In normal tissues, KAI1/CD82 is ubiquitously expressed. In tumor tissues, the presence of KAI1/CD82 expression predicts a better prognosis for cancer patients [10-12], whereas the downregulation or loss of KAI1/CD82 expression is constantly found in the clinically ad-

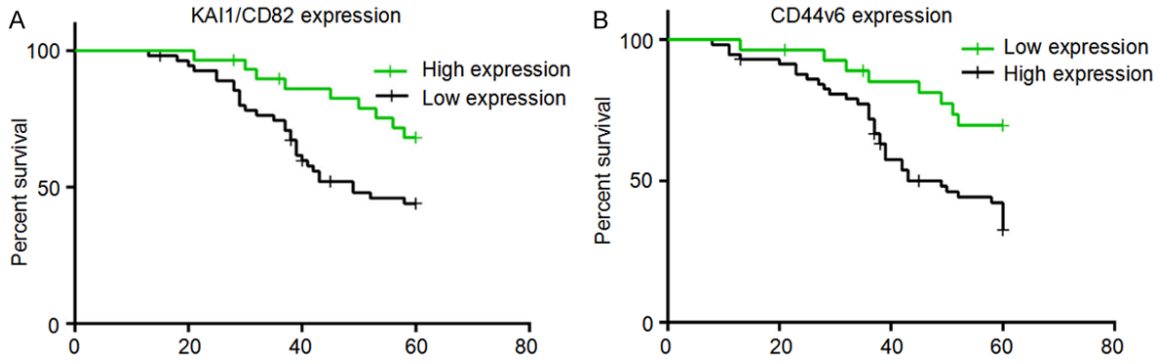

Figure 4. A: Kaplan-Meier analysis: Correlation between KAI1/CD82 expression and patients' survival time (Log-rank test: x^2=5.871, P=0.015). B: Kaplan-Meier analysis: Correlation between CD44v6 expression and patients' survival rates (Log-rank test: x^2=7.556, P=0.006).

Table 9. Analysis on Cox proportional risk model related factors for prognosis of LSCC

Influencing factors	B	*P* value	Exp (*B*)	95.0% CI for Exp (*B*)	
				Lower	Upper
Age	1.386	0.001	4.012	1.826	8.793
Lymph node metastasis	0.872	0.043	2.392	1.038	5.048
Clinical stage		0.002			
I+II	-1.863	0.039	0.141	0.212	0.925
III+IV	0.583	0.266	1.801	0.643	5.048
Tumor recurrence	1.504	0.001	4.505	1.872	9.846

Abbreviations: CI, confidence interval.

vanced cancer [13-15]. In addition, several studies have revealed that there are an association between reduced expression of KAI1/CD82 and increased metastatic ability in human malignant tumor such as bladder, cervical, ovarian, breast, prostate and hepatocellular carcinoma [16]. Many studies indicate that KAI1/CD82 expression is downregulated or abolished in a variety of malignant tumor [17]. Decreased KAI1/CD82 expression has been observed to correlate with metastasis and poor prognosis in many types of human solid tumor, such as prostate cancer [18], breast cancer [19], gastric cancer [20], basal cell carcinoma [21] and melanoma angiogenesis [22]. Although the mechanism underlying these pathological phenomena remains unknown, CD44v6, a variant of CD44, is located on chromosome 11p13 and encoded by a single gene [23] and is a member of the CD44 family which belongs to cell adhesion molecules. CD44 mediates cell-cell and cell-matrix interactions [24] by binding to its main ligand, hyaluronan, a glycosaminoglycan that is highly concentrated in the endosteal region [25]. Like other isoforms, CD44v6 plays an important role in tumor invasion and metastasis by regulating the ECM, promoting cell motility and suppressing tumor apoptosis [26]. Previous studies have shown that CD44v6 may enhance the invasion and metastasis of tumor cells by changing the composition and function of cell surface adhesion molecules. In recent years, abnormal expression of CD44v6, which is closely correlated with the progression, metastasis and prognosis of tumor, has been demonstrated in various malignant neoplasms [27].

The expression of CD44v6 in normal tissues is restricted to squamous and transitional epithelia [28, 29]. It has been reported that CD44v6 can regulate the extracellular matrix, promote cell motility, and suppress tumor apoptosis. In fact, CD44v6 has been implicated in promoting tumor progression [26]. CD44v6 overexpression has been shown in a variety of epithelial malignant tumor, such as head and neck, colon, endometrium and ovarian cancer, which is probably capable of promoting cancer cells to adhere to the vascular endothelium and base membranes, as well as enhancing the motility of cancer cells [30-32]. In addition, studies indicated that increased CD44v6 expression induced a high metastatic potential, which could serve as a prognostic marker in various malignant tumor, such as nonsmall cell lung cancer [33], gastric cancer [34], pharyngolaryngeal cancer [35], and esophageal squamous

cell carcinoma [36]. Currently, several studies have indicated a relationship between CD44v6 and the prognosis in laryngeal cancer. However, the results of these studies are inconsistent.

Many studies confirmed that the expression abnormalities presented by cell adhesion molecules were closely correlated with the tumor invasion and metastasis, and might be one of the determinants of tumor cells to gain the ability of invasion and distant metastasis. Changes in adhesion molecules that participate in cell-cell boundaries and cell-matrix interactions are a prerequisite for tumor invasion and dissemination. Adhesion molecule KAI1/CD82 is integrin-mediated cell adhesion to ECM [37], and CD44v6 changes the structure and function of adhesion molecules. Both KAI1/CD82 and CD44v6 are located on the short arm of human chromosome 11, which belong to the adhesion molecules. These proteins have a role in adhesion, and KAI1/CD82 inhibits the adhesion of tumor cells and metastasis, while CD44v6 promotes adhesion of tumor cells and invasion as well as metastasis. However, the specific mechanism of this protein in human LSCC is not clear.

It has been well established that combined markers promote clinical prediction when compared with a single factor [38, 39]. In this study, both mRNA and protein expression levels of KAI1/CD82 and CD44v6 were examined by the quantitative RT-PCR and western blot assay, respectively. Our data showed that KAI1/CD82 and CD44v6 expression levels of samples were exactly tested, which provided the correlation between the two markers (KAI1/CD82 and CD44v6) and the development of LSCC. KAI1/CD82 expression in the LSCC tissue specimens was significantly lower than that in the normal mucosa tissues, while CD44v6 expression in LSCC was noticeably higher than that in normal mucosa tissues. It was concluded that KAI1/CD82 and CD44v6 showed basically reverse changes in both mRNA and protein expression.

In this study, expression levels of KAI1/CD82 and CD44v6 in 86 patients with LSCC and 86 adjacent normal mucosa tissues were detected by immunohistochemistry. The results indicated that both KAI1/CD82 and CD44v6 proteins were located on cell membranes and in cytoplasm, and their expressions negatively correlated with each other. KAI1/CD82 expression in the LSCC tissue specimens was noticeably lower than that in the normal mucosa tissues, and their expression in patients with poorly differentiated, clinical stage III-IV, and metastatic LSCC was significantly lower than that in patients with well differentiated, clinical stage I-II, and non-metastatic LSCC. Muneyuki et al. and Maurer et al. reported that KAI1/CD82 expression decreased progressively with the advance of the tumor stage and was absent in lymph nodes [40, 41], consistent with our results (**Table 7**). However, CD44v6 expression in the LSCC tissue specimens was noticeably higher than that in the normal mucosa tissues, and its expression in patients with poorly differentiated, clinical stage III-IV, and metastatic LSCC was significantly higher than that in patients with well differentiated, clinical stage I-II, and non-metastatic LSCC. KAI1/CD82 and CD44v6 expression varied according to different tumor differentiation degrees, clinical stages, and lymphatic metastasis conditions. These findings indicate that the more powerful the invasiveness and metastasis potential of tumor cells are, the more serious the expression loss of KAI1/CD82 will be. The expression of CD44v6 is opposite to that. The expression of KAI1/CD82 and CD44v6 did not show the correlation with age and sex.

Nevertheless, we conclude that KAI1/CD82 and CD44v6 expression are related to metastasis of LSCC. Moreover, increasing evidence demonstrates that cell surface adhesion and ECM components are crucial for tumor metastasis [42]. In particular, KAI1/CD82 and CD0 44v6 are cell membrane proteins that bind to ECM or adhesion proteins [43, 44]. In addition, based on our analysis, KAI1/CD82 expression was negatively correlated with CD44v6 expression (**Table 8**). Similarly, Wei et al. demonstrated that the ablation of KAI1/CD82 increased CD44v6 expression and enhanced migration and invasion in endothelial cells [45]. Thus, we speculate that KAI1/CD82 may play a role in mediating the expression of CD44v6. Overall, these results indicate that there is a complex relationship between the KAI1/CD82 and CD44v6 in tumor progression. Combined with the results of the present study, it is reasonable to believe that the interaction of these factors is related to metastasis in LSCC. From our present study, it was found that the tumor metasta-

sis was closely related to the prognosis (**Table 9**).

In the present study, univariate and multivariate analyses indicated that KAI1/CD82 and CD44v6 mRNA and protein expression can serve as independent prognostic factors for survival in patients with LSCC, respectively. The downregulation of KAI1/CD82 and the upregulation of CD44v6 were markedly correlated with the worse survival rate, and patients with high expression of KAI1/CD82 had significantly longer percentage survival than those with low expression, while the expression of CD44v6 was opposite to that. Both by univariate and multivariate analysis, in accordance with other reports, our survival analysis showed that the reduction in KAI1/CD82 expression [46] and increased CD44v6 [47] expression were indicators of poor prognosis in LSCC patients (**Figures 3** and **4**). KAI1/CD82 expression and CD44v6 expression were identified as independent factors, consistent with the results of previous studies [48, 49] (**Table 9**), indicating that these molecules played important roles in LSCC prognosis.

In summary, this study illuminated that the abnormal expressions of KAI1/CD82 and CD44v6 were closely related to the carcinogenesis and development of LSCC, and that downregulation of CD44v6 along with the upregulation of KAI1/CD82 in LSCC tissues resulted in the inhibition of LSCC migration and invasion. Both KAI1/CD82 and CD44v6 expressions were negatively correlated with each other. Based on these findings, it is predictable that their inhibitory effect on tumor metastasis is related to their regulatory effect on cell adhesion. Therefore, the combined detection of these two genes is promising to provide an important index for the prognostic prediction of LSCC and to open up a new channel for the diagnosis and treatment of LSCC.

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Disclosure of conflict of interest

None.

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References

[1] Karaman S and Detmar M. Mechanisms of lymphatic metastasis. J Clin Invest 2014; 124: 922-928.

[2] Bienstock RJ and Barrett JC. KAI1, a prostate metastasis suppressor: prediction of solvated structure and interactions with binding partners; integrins, cadherins, and cell-surface receptor proteins. Mol Carcinog 2001; 32: 139-153.

[3] Kim HR, Wheeler MA, Wilson CM, Iida J, Eng D, Simpson MA, McCarthy JB and Bullard KM. Hyaluronan facilitates invasion of colon carcinoma cells in vitro via interaction with CD44. Cancer Res 2004; 64: 4569-4576.

[4] Dong JT, Lamb PW, Rinker-Schaeffer CW, Vukanovic J, Ichikawa T, Isaacs JT and Barrett JC. KAI1, a metastasis suppressor gene for prostate cancer on human chromosome 11p11.2. Science (Washington DC) 1955; 268: 884-886.

[5] Malik FA, Sanders AJ and Jiang WG. KAI1/CD82, the molecule and clinical implication in cancer and cancer metastasis. Histol Histopathol 2009; 24: 519-530.

[6] Miranti CK. Controlling cell surface dynamics and signaling: how KAI1/CD82 suppresses metastasis. Cell Signal 2009; 21: 196-211.

[7] Malik FA, Sanders AJ, Kayani MA and Jiang WG. Effect of expressional alteration of KAI1 on breast cancer cell growth, adhesion, migration and invasion. Cancer Genomics Proteomics 2009; 6: 205-213.

[8] Lim S, Lee HY and Lee H. Inhibition of colonization and cell-matrix adhesion after nm23-h1 transfection of human prostate carcinoma cells. Cancer Lett 1998; 133: 143-149.

[9] Waning DL and Guise TA. Molecular mechanisms of bone metastasis and associated muscle weakness. Clin Cancer Res 2014; 20: 3071-3077.

[10] Dong JT, Suzuki H, Pin SS, Bova GS, Schalken JA, Isaacs WB, Barrett JC and Isaacs JT. Downregulation of the KAI1 metastasis suppressor gene during the progression of human prostatic cancer infrequently involves gene mutation or allelic loss. Cancer Res 1996; 56: 4387-4390.

[11] Ow K, Delprado W, Fisher R, Barrett J, Yu Y, Jackson P and Russell PJ. Relationship be-

tween expression of the KAI1 metastasis suppressor and other markers of advanced bladder cancer. J Pathol 2000; 191: 39-47.

[12] Higashiyama M, Kodama K, Yokouchi H, Takami K, Adachi M, Taki T, Ishiguro S, Nakamori S, Yoshie O and Miyake M. KAI1/CD82 expression in nonsmall cell lung carcinoma is a novel, favorable prognostic factor: an immunohistochemical analysis. Cancer 1998; 83: 466-474.

[13] Kawana Y, Komiya A, Ueda T, Nihei N, Kuramochi H, Suzuki H, Yatani R, Imai T, Dong JT, Imai T, Yoshie O, Barrett JC, Isaacs JT, Shimazaki J, Ito H and Ichikawa T. Location of KAI1 on the short arm of human chromosome 11 and frequency of allelic loss in advanced human prostate cancer. Prostate 1997; 32: 205-213.

[14] Uchida S, Shimada Y, Watanabe G, Li ZG, Hong T, Miyake M and Imamura M. Motility-related protein (MRP-1/CD9) and KAI1/CD82 expression inversely correlate with lymph node metastasis in oesophageal squamous cell carcinoma. Br J Cancer 1999; 79: 1168-1173.

[15] Yu Y, Yang JL, Markovic B, Jackson P, Yardley G, Barrett J and Russell PJ. Loss of KAI1 messenger RNA expression in both high-grade and invasive human bladder cancers. Clin Cancer Res 1997; 3: 1045-1049.

[16] Odintsova E, van Niel G, Conjeaud H, Raposo G, Iwamoto R, Mekada E and Berditchevski F. Metastasis suppressor tetraspanin KAI1/CD82 regulates ubiquitylation of epidermal growth factor receptor. J Boil Chem 2013; 288: 26323-26334.

[17] Malik FA, Sanders AJ and Jiang WG. KAI-1/CD82, The molecule and clinical implication in cancer and cancer metastasis. Histol Histopathol 2009; 24: 519-530.

[18] Liu W, Iiizumi-Gairani M, Okuda H, Kobayashi A, Watabe M, Pai SK, Pandey PR, Xing F, Fukuda K, Modur V, Hirota S, Suzuki K, Chiba T, Endo M, Sugai T and Watabe K. KAI1 gene is engaged in NDRG1 gene-mediated metastasis suppression through the ATF3-NFkappaB complex in human prostate cancer. J Biol Chem 2011; 286: 18948-18959.

[19] Mooez S, Malik FA, Kayani MA, Rashid R, Zahid A and Khan A. Expressional alterations and transcript isoforms of metastasis suppressor genes (KAI1 and KiSS1) in breast cancer patients. Asian Pac J Cancer Prev 2011; 12: 2785-2791.

[20] Chen Z, Gu S, Trojanowicz B, Liu N, Zhu G, Dralle H and Hoang-Vu C. Down-regulation of TM4SF is associated with the metastatic potential of gastric carcinoma TM4SF members in gastric carcinoma. World J Surg Oncol 2011; 9: 43.

[21] Bozdogan O, Yulug IG, Vargel I, Cavusoglu T, Karabulut AA, Karahan G and Sayar N. Differential expression pattern of metastasis suppressor proteins in basal cell carcinoma. Int J Dermatol 2015; 54: 905-915.

[22] Tang Y, Bhandaru M, Cheng Y, Lu J, Li G and Ong CJ. The role of the metastasis suppressor gene KAI1 in melanoma angiogenesis. Pigment Cell Melanoma Res 2015; 28: 696-706.

[23] Shi J, Zhou Z and Di W. Correlation of CD44v6 expression with ovarian cancer progression and recurrence. BMC Cancer 2013; 13: 182-192.

[24] Ponta H, Sherman L and Herrlich PA. CD44: from adhesion molecules to signalling regulators. Nat Rev Mol Cell Biol 2003; 4: 33-45.

[25] Jin L, Hope KJ, Zhai Q, Smadja-Joffe F and Dick JE. Targeting of CD44 eradicates human acute myeloid leukemic stem cells. Nat Med 2006; 12: 1167-1174.

[26] Jung T, Gross W and Zöller M. CD44v6 coordinates tumor matrix-triggered motility and apoptosis resistance. J Biol Chem 2011; 286: 15862-15874.

[27] Deng Z, Niu G, Cai L, Wei R and Zhao X. The prognostic significance of CD44v6, CDH1, and β-Catenin expression in patients with osteosarcoma. Biomed Res Int 2013; 2013: 496193.

[28] Fox SB, Fawcett J, Jackson DG, Collins I, Gatter KC, Harris AL, Gearing A and Simmons DL. Normal human tissues, in addition to some tumors, express multiple different CD44 isoforms. Cancer Res 1994; 54: 4539-4546.

[29] Heider KH, Mulder JW, Ostermann E, Susani S, Patzelt E, Pals ST and Adolf GR. Splice variants of the cell surface glycoprotein CD44 associated with metastatic tumour cells are expressed in normal tissues of humans and cynomolgus monkeys. Eur J Cancer 1995; 31A: 2385-2391.

[30] Wang SJ, Wong G, de Heer AM, Xia W and Bourguignon LY. CD44 variant isoforms in head and neck squamous cell carcinoma progression. Laryngoscope 2009; 119: 1518-1530.

[31] Afify AM, Craig S, Paulino AF and Stern R. Expression of hyaluronic acid and its receptors, CD44s and CD44v6, in normal, hyperplastic, and neoplastic endometrium. Ann Diagn Patho 2005; 9: 312-318.

[32] Dong WG, Sun XM, Yu BP, Luo HS and Yu JP. Role of VEGF and CD44v6 in differentiating benign from malignant ascites. World J Gastroenterol 2003; 9: 2596-2600.

[33] Jiang H, Zhao W and Shao W. Prognostic value of CD44 and CD44v6 expression in patients with non-small cell lung cancer: meta-analysis. Tumour Biol 2014; 35: 7383-7389.

[34] Chen Y, Fu Z, Xu S, Xu Y and Xu P. The prognostic value of CD44 expression in gastric cancer: a meta-analysis. Biomed Pharmacother 2014; 68: 693-697.
[35] CChai L, Liu H, Zhang Z, Wang F, Wang Q, Zhou S and Wang S. CD44 expression is predictive of poor prognosis in pharyngolaryngeal cancer: systematic review and meta-analysis. Tohoku J Exp Med 2014; 232: 9-19.
[36] Luo WY, Cheng Y and Hu AY. Expression and biological significance of DMBT1, CD44v6 in esophageal squamous cell carcinoma. Hebei Med J 2015; 37: 1290-1292.
[37] Ruseva Z, Geiger PX, Hutzler P, Kotzsch M, Luber B, Schmitt M, Gross E and Reuning U. Tumor suppressor KAI1 affects integrin alphavbeta3-mediated ovarian cancer cell adhesion, motility, and proliferation. Exp Cell Res 2009; 315: 1759-1771.
[38] Newton KF, Newman W and Hill J. Review of biomarkers in colorectal cancer. Colorectal Dis 2012; 14: 3-17.
[39] Bohanes P, LaBonte MJ, Winder T and Lenz HJ. Predictive molecular classifiers in colorectal cancer. Semin Oncol 2011; 38: 576-587.
[40] Muneyuki T, Watanabe M, Yamanaka M, Shiraishi T and Isaji S. KAI1/CD82 Expression as a prognosic factor in sporadic colorectal cancer. Anticancer Res 2001; 21: 3581-3587.
[41] Maurer CA, Graber HU, Friess H, Beyermann B, Willi D, Netzer P, Zimmermann A and Büchler MW. Reduced expression of the metastasis suppressor gene KAI1 in advanced colon cancer and its metastases. Surgery 1999; 126: 869-880.
[42] Liotta LA and Stetler-Stevenson WG. Tumor Invasion and Metastasis: An Imbalance of Positive and Negative Regulation. Cancer Res 1991; 51 Suppl: 5054S-5059S.
[43] Aberle H, Schwartz H and Kemler R. Cadherin-catenin complex: protein interactions and their implications for cadherin function. J Cell Biochem 1996; 61: 514-523.
[44] Kauffman EC, Robinson VL, Stadler WM, Sokoloff MH and Rinker-Schaeffer CW. Metastasis suppression: the evolving role of metastasis suppressor genes for regulation cancer cell growth at the secondary site. J Urol 2003; 169: 1122-1133.
[45] Wei Q, Zhang F, Richardson MM, Roy NH, Rodgers W, Liu Y, Zhao W, Fu C, Ding Y, Huang C, Chen Y, Sun Y, Ding L, Hu Y, Ma JX, Boulton ME, Pasula S, Wren JD, Tanaka S, Huang X, Thali M, Hämmerling GJ and Zhang XA. CD82 restrains pathological angiogenesis by altering lipid raft clustering and CD44 trafficking in endothelial cells. Circulation 2014; 130: 1493-1504.
[46] Hashida H, Takabayashi A, Tokuhara T, Hattori N, Taki T, Hasegawa H, Satoh S, Kobayashi N, Yamaoka Y and Miyake M. Clinical significance of transmembrane 4 superfamily in colon cancer. Br J Cancer 2003; 89: 158-167.
[47] Huh JW, Kim HR, Kim YJ, Lee JH, Park YS, Cho SH and Joo JK. Expression of standard CD44 in human colorectal carcinoma: Association with prognosis. Pathol Int 2009; 59: 241-246.
[48] Muneyuki T, Watanabe M, Yamanaka M, Shiraishi T and Isaji S. KAI1/CD82 Expression as a prognosic factor in sporadic colorectal cancer. Anticancer Res 2001; 21: 3581-3587.
[49] Wu Q, Yang Y, Wu S, Li W, Zhang N, Dong X and Ou Y. Evaluation of the correlation of KAI1/CD82, CD44, MMP7 and β-catenin in the prediction of prognosis and metastasis in colorectal carcinoma. Diagn Pathol 2015; 10: 176.