RESEARCH ARTICLE

KAI1/CD82 and MRP1/CD9 Serve as Markers of Infiltration, Metastasis, and Prognosis in Laryngeal Squamous Cell Carcinomas

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Abstract

Objective: The current study explored the expression of KAI1/CD82 and MRP1/CD9 and its significance in laryngeal squamous cell carcinoma (LSCC). Methods: The expression levels of KAI1/CD82 and MRP1/CD9 in 100 LSCC tissue specimens, as well as in 30 para-LSCC non-carcinomatous tissue specimens randomly taken from the patients, were assessed using the quantitative polymerase chain reaction (Q-PCR) and immunohistochemistry and correlations with pathological parameters of LSCC and their influence on survival function were analyzed. Results: KAI1/CD82 and MRP1/CD9 showed basically consistent changes in both mRNA and protein expression. Their expression in the 30 LSCC specimens was significantly lower compared with that in the corresponding non-carcinous tissues ($P < 0.01$ or 0.05), notably correlating with TNM stage, differentiation degree, clinical stage, and lymphatic metastasis ($P < 0.01$ or 0.05), but not gender, age, and LSCC growth sites ($P > 0.05$). The median survival of patients with positive KAI1/CD82 and MRP1/CD9 protein expression was longer than that of patients with negative protein expression ($P < 0.01$ or 0.05). KAI1/CD82 protein expression negatively correlated with MRP1/CD9 protein expression in LSCC ($\chi^2 = 31.25$, $P < 0.01$). Conclusion: KAI1/CD82 and MRP1/CD9 may jointly participate in the development of LSCC. They may serve as the markers for judging the infiltration, metastasis, and prognosis of LSCC.

Keywords: Laryngeal neoplasms - KAI1/CD82 - MRP1/CD9 - metastasis marker

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Introduction

Laryngeal squamous cell carcinoma (LSCC) is one of the most common malignancies occurring in the head and neck area. In recent years, the incidence of LSCC is on the increase, and its infiltration and metastasis have become the primary factors greatly influencing patients' quality of life and causing deaths. Despite great advance in research, good curative effect on LSCC has not yet been achieved. Ideal markers of LSCC for prognostic judgment and therapeutic guidance remain urgent in clinical practice. The transmembrane 4 superfamily (TM4SF) embraces CD37, CD53, ME491/CD63, TAPA1/CD81, KAI1/CD82, and MRP1/CD9, and is correlated with the movement, proliferation, and metastasis of tumor cells (Woegerbauer et al., 2010; Chen et al., 2011). Among the members, KAI1/CD82 and MRP1/CD9 have been extensively studied. However, their expression in LSCC remains unclear.

To explore the roles of KAI1/CD82 and MRP1/CD9 in the development of LSCC, we utilized quantitative polymerase chain reaction (Q-PCR) and immunohistochemistry to detect their expression in LSCC. The aim of this study was to provide new indices for the diagnosis, treatment, and prognostic judgment of LSCC.

Materials and Methods

Cases and specimens

A total of 100 LSCC patients (78 males and 22 females) that received laryngeal cancer resection at the Second Affiliated Hospital of Harbin Medical University were enrolled in this study. Their ages ranged from 40 years to 81 years with an average of 58.7. All patients were confirmed pathologically with LSCC. They had not received chemotherapy or radiotherapy and had complete clinical, pathological, and follow-up data. Thirty para-LSCC non-carcinous tissue specimens were collected randomly (1 cm within the border of the tumor and pathologically excluded from cancer cell infiltration). Clinical classification and staging were carried out in line with the tumor, neck, and metastasis (TNM) classification

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Table 1. Primer Sequences of KAI1/CD82, MRP1/CD9, and β-actin (internal reference)

Gene	Primer sequence	Amplified fragment (bp)
KAI1/CD82	5' - CGT GGG TGT GGC CAT CAT -3'	83
	5' - TTG CTG TAG TCT TCG GAA TGG -3'	
MRP1/CD9	5' - GCA TTG CCG TGG TCA TGA T -3'	62
	5' - TGC GGA TAG CAC AGC ACA AG -3'	
β-actin	5' -CCC AGC ACA ATG AAG ATC AAG ATC AT -3'	101
	5' -ATC TGC TGG AAG GTG GAC AGC GA-3'	

scheme by the Italian-international Union for the Control of Cancer (1997). According to differentiation, 61 cases were well differentiated, 23 were moderately differentiated, and 16 were undifferentiated. According to clinical stages, 35 were in stage I, 23 in stage II, 25 in stage III, and 17 in stage IV. According to lymphatic metastasis, 33 were metastatic and 67 were not. According to tumor growth sites, 43 were glottic, 50 were supraglottic, and 7 were subglottic. Eleven out of the 100 patient were lost to follow up, and the follow-up time ranged from 5months to 84 months. This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of the Second Affiliated Hospital of Harbin Medical University. Written informed consent was obtained from all participants.

The obtained specimens were divided into two parts: Those for mRNA determination were removed from coagulated blood immediately after separation and then stored in liquid nitrogen, whereas those for immunohistochemistry were fixed, dehydrated, and embedded routinely.

Q-PCR for mRNA expression

Total RNA was extracted using Trizol (Invitrogen, US) according to the instructions. Its purity and content were detected using an ultraviolet spectrophotometer. About 1 μg of total RNA was reversely transcribed (the kit was the product of Shanghai Biowaston, China) and the reaction parameters in sequence were 16 °C for 30 min, 42 °C for 30 min, 85 °C for 5 min, and 4 °C for 5 min (TaQ enzyme was purchased from Shanghai Hikey, China). The primers were synthesized by Shanghai GeneCore Bio Technologies Co., Ltd., China. The reaction system for fluorescent Q-PCR was 20 μl and the amplification conditions consisted of 95 °C for 5 min and 40 cycles of 95 °C for 10 s, 60 °C (for ACTIN; the annealing temperatures for KAI1/CD82 and MRP1/CD9 were 58 °C and 56 °C, respectively) for 20 s, and 72 °C for 20 s. Three pores were set for each reaction and all experiments were repeated thrice. β-actin served as the internal reference, and the relative mRNA levels of KAI1/CD82 and MRP1/CD9 were respectively calculated using comparative cycle thresholds (2- CT).

Immunohistochemistry for protein expression

The tissue sections were routinely dewaxed, hydrated, and subjected to antigen repair with citric acid. KAI1/CD82 and MRP1/CD9 rabbit anti-human antibodies (Santa Cruz, US) were diluted according to 1:200 ratios. Immunohistochemistry was performed using the SP two-stage method and according to the instructions of kits (Beijing ZSGB-BIO, China). The primary antibodies were replaced with PBC in the negative control. Cells positive for KAI1/CD82 and NGX were those with yellow-stained cytoplasm and cell walls. At least five visual fields were observed for each section under high power lens and a total of 300-500 cells were counted for calculating the percentage of positive cells. Staining intensity was judged based on the following standards: 0 point was assigned to no staining, 1 to amber staining, 2 to yellow or deep yellow staining, and 3 to brown or sable staining. The accumulated points were calculated and those ≥ 80 were judged as positive.

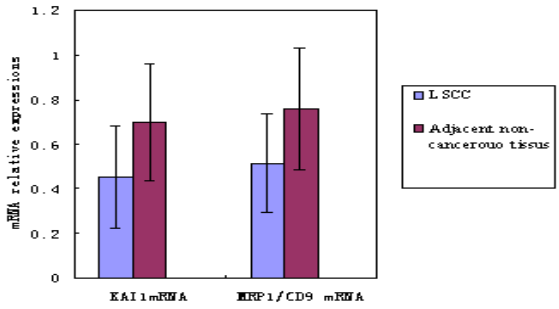

Figure 1. Expression of KAI1/CD82 and MRP1/CD9 mRNA in the LSCC and para-LSCC Non-carcinous Tissues

Statistical analysis

All data were analyzed using SPSS17.0 software. All measurement data are presented as x ± s. One-factor ANOVA and q tests were used to compare among groups and t tests to compare between groups. All enumeration data were compared using chi square tests for the differences in the expression of KAI1/CD82 and MRP1/CD9 between the LSCC tissues and the para-LSCC normal tissues. The correlations of the protein expression levels of the two genes with the pathological parameters of LSCC as well as the correlation between the expression levels of the two genes were analyzed using chi square tests. Survival curves were drawn using the Kapla-Meier method and log-rank tests were performed for life spans. Differences with $P < 0.05$ were considered statistically significant.

Results

Expression of KAI1/CD82 and MRP1/CD9 mRNA

The relative expression levels of KAI1/CD82 mRNA in the LSCCs and the non-carcinous tissues were 0.452 ± 0.230 and 0.699 ± 0.260, and those of MRP1/CD9 mRNA were 0.514 ± 0.222 and 0.756 ± 0.272, respectively. The mRNA expression of KAI1/CD82 and MRP1/CD9 in the 30 LSCCs was significantly lower than that in the non-carcinous tissues ($P < 0.01$; Figure 1).

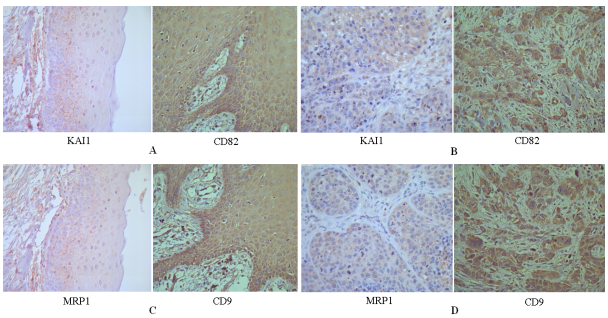

Figure 2. Expression of KAI1/CD82 and MRP1/CD9 Proteins in the LSCC and Para-LSCC Non-carcinous Tissues. A, KAI1/CD82 protein expression in the para-LSCC non-carcinous tissue. B, KAI1/CD82 protein expression in the LSCC tissue. C, MRP1/CD9 protein expression in the para-LSCC non-carcinous tissue. D, MRP1/CD9 protein expression in the LSCC tissue

Table 2. Correlations of the mRNA Expression of KAI1/CD82 and MRP1/CD9 with the Clinical Pathological Parameters of LSCC

Pathological characteristics	n	Relative expression amount of KAI1/CD82	*P*	Relative expression amount of MRP1/CD9	*P*
Gender					
Male	78	0.465±0.252	0.763	0.517±0.227	0.684
Female	22	0.447±0.233		0.493±0.237	
Age					
≥60 years	53	0.449±0.241	0.593	0.526±0.221	0.504
< 60 years	47	0.476±0.256		0.499±0.238	
TNM stage					
I-II	70	0.553±0.211	0	0.617±0.175	0.041
III-IV	30	0.249±0.188		0.464±0.122	
Differentiation					
Well	84	0.493±0.243	0.032	0.538±0.218	0
Poorly	16	0.396±0.198		0.374±0.242	
Clinical stage					
I-II	59	0.578±0.193	0.02	0.638±0.164	0
III-IV	41	0.394±0.219		0.329±0.181	
Lymphatic metastasis					
Yes	33	0.304±0.212	0	0.393±0.222	0
No	67	0.542±0.226		0.570±0.211	
Site					
Glottic	43	0.520±0.245	0.269	0.582±0.201	0.198
Supra- and sub-glottic	57	0.487±0.241		0.478±0.235	

Expression of KAI1/CD82 and MRP1/CD9 proteins

Positive KAI1/CD82 and MRP1/CD9 proteins were primarily located in cytoplasm and on the membranes (Figure 2). The respective positive expression rates of KAI1/CD82 and MRP1/CD9 in the 30 LSCC specimens were 56.7% (17/30) and 53.3% (16/30), which were significantly lower than those in the corresponding non-carcinous tissues (83.3% (25/30) and 80.0% (24/30), respectively; $P < 0.05$) (Table 1).

Correlations of the mRNA expression of KAI1/CD82 and MRP1/CD9 with the pathological parameters of LSCC

The relative mRNA expression levels of KAI1/CD82 and MRP1/CD9 varied according to different pathological stages of LSCC. The expression of both KAI1/CD82 and MRP1/CD9 in patients with TNM stage III-V, poorly differentiated, clinical stage III-IV, and metastatic LSCC was noticeably lower than that in patients with TNM stage I-II, well differentiated, clinical stage I-II, and non-metastatic LSCC ($P < 0.01$ or 0.05). No significant difference was observed with regard to gender, age, or growth sites ($P > 0.05$). The results are summarized in Table 2.

Table 3. Correlations of the Protein Expression of KAI1/CD82 and MRP1/CD9 with the Clinical Pathological Parameters of LSCC/Case Number (positive rate %)

Pathological characteristics	n	KAI1/CD82	χ^2	*P*	MRP1/CD9	χ^2	*P*
Gender							
Male	78	40(51.3)	0.23	>0.05	38(48.4)	0.07	>0.05
Female	22	12(54.5)			12(54.5)		
Age							
≥60 years	53	30(56.6)	1.97	>0.05	32(60.4)	0.88	>0.05
<60 years	47	20(42.6)			24(51.1)		
TNM stage							
I-II	70	43(61.4)	8.31	<0.01	40(57.1)	4.76	<0.05
III-IV	30	9(30.0)			10(33.3)		
Differentiation							
Well	84	48(57.1)	5.56	<0.05	49(58.3)	8.44	<0.01
Poorly	16	4 (25.0)			3(18.6)		
Clinical stage							
I-II	59	39(66.1)	13.13	<0.01	36(61.0)	5.78	<0.05
III-IV	41	12(29.3)			15(36.6)		
Lymphatic metastasis							
Yes	33	11(33.3)	5.47	<0.05	13(39.4)	4.23	<0.05
No	67	39(58.2)			41(61.2)		
Site							
Glottic	43	24(55.8)	0.44	>0.05	25(58.1)	0.8	>0.05
Supra- and sub-glottic	57	28(49.1)			28(49.1)		

Table 4. Correlation Between the Protein Expression of KAI1/CD82 and MRP1/CD9 in LSCC

KAI1/CD82	MRP1/CD9	
	+	-
+	42	10
-	11	37

Correlations of the protein expression of KAI1/CD82 and MRP1/CD9 with the pathological parameters of LSCC

The positive protein expression rates of KAI1/CD82 and MRP1/CD9 also varied according to different pathological stages of LSCC. The positive expression rates of both KAI1/CD82 and MRP1/CD9 in patients with TNM stage III-V, poorly differentiated, clinical stage III-IV, and metastatic LSCC was noticeably lower than that in patients with TNM stage I-II, well differentiated, clinical stage I-II, and non-metastatic LSCC ($P < 0.01$ or 0.05), whereas no significant differences were observed with regard to gender, age, and growth sites ($P > 0.05$). The results are summarized in Table 3.

Correlation analysis of the protein expression of KAI1/CD82 and MRP1/CD9 in LSCC

The expression of KAI1/CD82 protein was in a positive correlation with that of MRP1/CD9 in LSCC ($\chi^2 = 31.25$, $P < 0.01$; Table 4).

Correlation of the protein expression of KAI1/CD82 and MRP1/CD9 in LSCC with prognosis

The median survival of the LSCC patients with positive KAI1/CD82 protein expression was 78 months, whereas

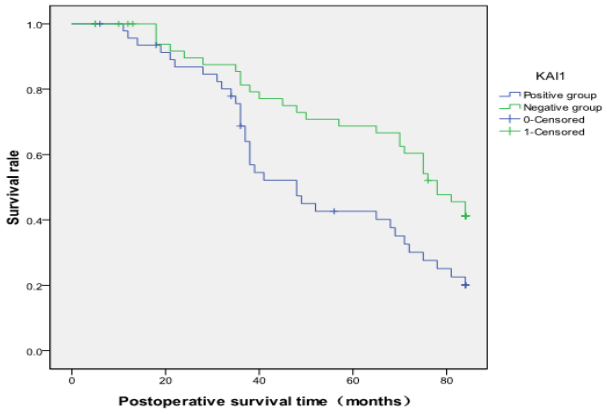

Figure 3. Correlation Between KAI1/CD82 Protein Expression and Patients' Survival Rate

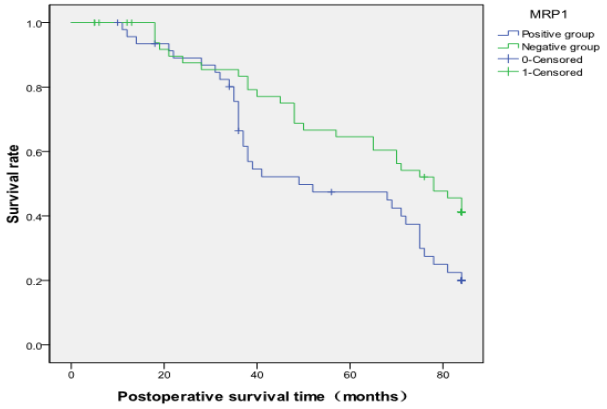

Figure 4. Correlation Between MRP1/CD9 Protein Expression and Patients' Survival Rate

that of the LSCC patients with negative KAI1/CD82 protein expression was 48 months ($\chi^2 = 6.98$, $P = 0.008$; Figure 3). The median survival of the LSCC patients with positive MRP1/CD9 protein expression was 78 months, whereas that of the LSCC patients with negative MRP1/CD9 protein expression was 49 months ($\chi^2 = 5.45$, $P = 0.02$; Figure 4).

Discussion

The development of tumors is a complicated process which involves multiple genes and steps, and they are the results of the intercoordination and combined action of numerous factors. This is no exception of LSCC. For LSCC patients, the recurrence and metastasis of LSCC constitute the primary life-threatening reasons. In recent years, scholars have proposed that the combined expression of multiple genes may serve as a satisfactory marker for prognostic prediction. As tissue microarray technology and proteomics develop, more and more metastasis suppressor genes have been discovered. Exploring the relations among anti-metastasis genes plays an important role in the prognostic prediction and treatment of LSCC.

TM4SF is a membrane protein family which has been recently studied and it is extensively expressed in human cells. The members of this family participate in the connection signal transduction between cell surface molecules, playing critical roles in regulating the proliferation, activation, and adhesion activity of cells. TM4SF plays important roles in the growth, migration, and angiogenesis of tumors (Yauch et al., 2000). To date, more than 30 member of this family have been found, most of which are considered subject to tumor suppressor genes, such as CD9 and CD82. The products of these members negatively correlate with the development and prognosis of tumors. However, the newly-discovered TM4SF member, CD151, is totally different from other members: It is positively correlated with the development and prognosis of tumors (Ang et al., 2004). Even more, it is the only oncogene in the family (Hong et al., 2006).

KAI1/CD82 is an anti-metastasis gene discovered by Dong et al in 1995. Its coding region encodes a 29.6-kDa protein of 267 amino acid residues which has the same structure as the CD82 protein (a leukocyte surface glycoprotein). KAI1/CD82 has an inhibitory effect on the metastasis of many tumors, thereby related to the invasiveness and prognosis of tumors. The loss or downregulation of its expression has been observed in a variety of tumors, such as lung carcinoma (Malik et al., 2009, Shiwu et al., 2012), gastric carcinoma (Chen et al., 2011), pancreatic carcinoma (Xu et al., 2008), breast carcinoma (Malik et al., 2009), bladder carcinoma (Jackson et al., 2007), and so on. MRP1 (motility related protein 1) is a cell movement-inhibiting transmembrane glycoprotein which shares the same gene sequence identity with the leukocyte differentiation antigen CD9. The transfection of MRP1/CD9 cDNA reduces the locomotivity and growth of tumor cells (Radford et al., 1995). MRP1/CD9 cDNA transfected melanoma cells in mice show a lower metastatic potential compared with their parent cells (Powner et al., 2011). These findings indicate that MRP1/CD9 directly regulates the movement of tumor cells and influences their growth. The inhibitory effect of MRP1/CD9 on the movement and proliferation of tumor cells is not only reflected on the level of cells. Such effect has also been evidenced by studies of a variety of solid tumors, such as gastric carcinoma (Soyuer et al., 2010), gastrointestinal mesenchymal neoplasm (Setoguchi et al., 2011), and oral squamous carcinoma (Buim et al., 2010). The expression levels of KAI1/CD82 and MRP1/CD9 decrease in invasive retinoblastoma (Mohan et al., 2007).

In this study, KAI1/CD82 and MRP1/CD9 showed basically consistent changes in both mRNA and protein expression: Their expression in the LSCC specimens was noticeably lower than that in the non-carcinous tissues; their expression varied according to different TNM stages, tumor differentiation degrees, clinical stages, and lymphatic metastasis conditions, or specifically, their expression in patients with TNM stage III-IV, poorly differentiated, clinical stage III-IV, and metastatic LSCC was significantly lower than that in patients with TNM stage I-II, well differentiated, clinical stage I-II, and non-metastatic LSCC. 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 and MRP1/CD9 will be. This study did not show the correlation of age, gender, or tumor growth sites with the expression of KAI1/CD82 and MRP1/CD9, which is in consistency with the results reported in the

studies of other tumors. However, a study has reported that KAI1/CD82 and MRP1/CD9 cDNA transfection inhibits the lymphatic metastasis of tumors rather than their growth at the primary sites (Takeda et al., 2007). Our result contradicts this claim. Different tumors studied may contribute to this disagreement. Therefore, further studies of LSCC remain necessary. Furthermore, the analysis of the survival curves in this study showed that the median survival of patients with positive protein expression of KAI1/CD82 and MRP1/CD9 was markedly longer than that of patients with negative protein expression. This finding is basically in line with the conclusions drawn in the studies of head and neck squamous cell carcinoma and breast carcinoma (Huang et al., 1998; Erovic et al., 2003; Buim et al., 2010). In addition, this study showed a significant positive correlation between the protein expression levels of KAI1/CD82 and MRP1/CD9 (χ^2 = 31.25, $P < 0.01$). This finding indicates that KAI1/CD82 and MRP1/CD9 may exert a certain synergetic effect on the development, infiltration, and metastasis of LSCC.

TM4SF may regulate the infiltration and metastasis of tumors through the following two mechanisms. First, it binds with integrins to form large complexes on cell surface and influences cell adhesion by regulating the functions of the integrins. Second, it directly regulates cell adhesion. KAI1 gene products interconnect with other TM4SF members (Zoller, 2006). CD9, CD63, and CD82 interlink with each other and bind with integrins to form complexes, whereby to regulate the adhesion function of the integrins and influence cell transfer medicated by them (Ono et al., 1999, Mazurov et al., 2013). KAI1/CD82 and MRP1/CD9 cDNA transfection greatly reduces the locomotivity of KAI1/CD82 or MRP1/CD9 expressed ovarian mutant cells and causes the necrosis of most of them, which indicates that the common glycosylation of KAI1/CD82 and MRP1/CD9 inhibits cell movement and necrosis (Shibagaki et al., 1999).

Although the complete mechanisms underlying the inhibitory effect of KAI1/CD82 and MRP1/CD9 on the infiltration and metastasis of LSCC remain unknown, immunohistochemistry in this study showed that both KAI1/CD82 and MRP1/CD9 proteins were located on cell membranes and in cytoplasm and that they positively correlated with each other. Based on these findings, it is predictable that their inhibitory effect on tumor metastasis is related with their regulatory effect on cell adhesion. Although the mechanisms underlying the effect of KAI1/CD82 and MRP1/CD9 on LSCC remain to be explored, 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 this condition.

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