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Original Article

KAI1/CD82 and CyclinD1 as biomarkers of invasion, metastasis and prognosis of laryngeal squamous cell carcinoma

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Abstract: Objective: This study aimed to investigate the expressions and significance of KAI1/CD82 and CyclinD1 in laryngeal squamous cell carcinoma (LSCC). Methods: Real-time quantitative PCR (Q-PCR) and Western blot assay were employed to detect the expressions of KAI1/CD82 and CyclinD1 in the laryngeal tissues of 86 LSCC patients, 32 patients with laryngeal polyp and 38 patients with laryngeal leukoplakia, and the influence of both proteins on the clinicopathological features and survival of LSCC patients. Results: The changes in mRNA and protein expressions of KAI1/CD82 and CyclinD1 were consistent in three groups, and the expressions of KAI1/CD82 and CyclinD1 were significantly different among three groups ($P<0.01$ or <0.05). The KAI1/CD82 expression in patients with TNM stage III-IV LSCC, poorly differentiated LSCC, clinical stage III-IV LSCC or lymph node metastasis was markedly lower than that in those with TNM stage I-II LSCC, well differentiated LSCC, clinical stage I-II LSCC or no lymph node metastasis ($P<0.01$ or <0.05). However, there was no marked difference in KAI1/CD82 expression between males and females and among patients in different age groups ($P>0.05$). In LSCC patients positive for KAI1/CD82 protein expression, the median survival time was 76 months, which was significantly longer than that in LSCC patients negative for KAI1/CD82 protein expression (48 months; $X^2=16.293$, $P=0.000$). The CyclinD1 expression in patients with TNM stage III-IV LSCC, poorly differentiated LSCC, or clinical stage III-IV LSCC was dramatically higher than that in patients with TNM stage I-II LSCC, well differentiated LSCC, or clinical stage I-II LSCC ($P<0.01$ or <0.05). However, no marked difference was noted in CyclinD1 expression between males and females, among patients in different age groups and between patients with and without lymph node metastasis ($P>0.05$). In LSCC patients positive for CyclinD1 protein expression, the median survival time was 40 months, which was markedly shorter than that in LSCC patients negative for CyclinD1 protein expression ($X^2=9.517$, $P=0.02$). In LSCC patients, there was a negative correlation between KAI1/CD82 expression and CyclinD1 expression ($X^2=7.86$, $P<0.01$). Conclusion: KAI1/CD82 affects cell cycle. Both KAI1/CD82 and CyclinD1 are involved in the occurrence and development of LSCC, and may provide clinical information for evaluation of invasiveness, metastasis and prognosis of LSCC. Thus, KAI1/CD82 and CyclinD1 may serve as markers for determination of invasiveness, metastasis and prognosis of LSCC.

Keywords: Laryngeal squamous cell carcinoma, KAI1/CD82, CyclinD1

Introduction

Laryngeal cancer is one of the most common malignancies of the head and neck, and its incidence is increasing over time. Invasion and metastasis of laryngeal cancer significantly influence the quality of life and are major causes of death in these patients. Although great progress has been achieved in the studies on laryngeal cancer, there are no ideal markers for the determination of prognosis and the guidance of treatment in laryngeal cancer patients. Members in transmembrane 4 superfamily (TM4SF) include CD37, CD53, ME491/CD63, TAPA1/CD81, KAI1/CD82 and CD9, and they are closely related to the movement, proliferation and metastasis of cancer cells [1, 2]. KAI1/CD82 is a common member in TM4SF family, but the mechanisms underlying the inhibitory effect of KAI1/CD82 on the invasion and metastasis of cancers are still poorly understood. Some investigators proposed [3]

Table 1. Primers for KAI1/CD82, CyclinD1 and β-actin

Gene	Sequence	Length (bp)
KAI1/CD82	5′-CGT GGG TGT GGC CAT CAT-3′ 5′-TTG CTG TAG TCT TCG GAA TGG-3′	83
CyclinD1	5′-AGGAGAACAAACAGATCA-3′ 5′-TAGGACAGGAAGTTGTTG-3′	163
β-actin	5′-CCC AGC ACA ATG AAG ATC AAG ATC AT-3′ 5′-ATC TGC TGG AAG GTG GAC AGC GA-3′	101

that KAI1/CD82 may interfere with cell cycle progression via inhibiting

EGFR/K-ras/JNK/c-Jun/CyclinD1 pathway, which delays the progression from G0/G1 phase to S phase exerting anti-tumor effect. CyclinD1 is a positive regulator of cell cycle progression, and CyclinD1 over-expression may promote the progression from G1 phase into S phase leading to cell proliferation. In the present study, real-time fluorescence qualitative polymerase chain reaction (PCR) and Western blot assay were employed to detect the expressions of KAI1/CD82 and CyclinD1 in laryngeal tissues of patients with different laryngeal lesions. This study aimed to investigate the role, significance of KAI1/CD82 and CyclinD1 expressions in the occurrence and development of laryngeal squamous cell carcinoma (LSCC), and to explore the potential mechanisms of inhibitory effect of KAI1/CD82 on LSCC. Our findings may provide markers for the diagnosis, treatment and prognosis of LSCC, which is beneficial to develop strategies to improve the diagnosis, treatment and prognosis of LSCC.

Materials and methods

Sample collection

Between January 2004 to December 2004, 86 patients with LSCC were recruited from the Department of Otolaryngology Head and Neck Surgery in the Second Affiliated Hospital of Harbin Medical University. These patients received surgical intervention, and LSCC was pathologically proven. They had no radiotherapy and chemotherapy before study, had completed clinical, pathological and follow up data. There were 57 males and 29 females with a median age of 59.6 years (range: 43-81 years). Pathological staging was done according to the TNM staging system developed by UICC (1997). LSCC was classified as well differentiated LSCC (n=52), moderately differentiated LSCC (n=8), poorly differentiated LSCC (n=19) and undifferentiated LSCC (n=7). In addition, clinical staging was done as stage I LSCC (n=16), stage II LSCC (n=27), stage III (n=32) and stage IV LSCC (n=11). Additionally, 36 patients were diagnosed with lymph node metastasis, and 50 patients had no lymph node metastasis. Among 86 patients, 9 were lost to follow up. The follow up period ranged from 5 months to 84 months. Moreover, 32 patients with laryngeal polyp and 38 patients with laryngeal leukoplakia were recruited as controls in the same period. These patients also received surgical intervention, and cancer cells were not observed in the laryngeal tissues. Atypical hyperplasia was noted in the laryngeal leukoplakia tissues. The laryngeal tissues were collected, divided into two and stored in liquid nitrogen.

Materials and reagent

TRIZOL (Invitrogen), TaQ polymerase (Shanghai Haiji Company), reverse transcription kit (Shanghai Huashun Biotech Co., Ltd), antibodies against KAI1/CD82 and CyclinD1 (Santa Crnz, USA) and primers (**Table 1**; Shanghai Jikang Biotech Co., Ltd) were used in the present study. Other detection kits were purchased from Beijing Zhongshan Biotech Co., Ltd (**Table 1**).

Detection of mRNA expressions of KAI1/CD82 and CyclinD1 by qPCR

Total RNA was extracted with Trizol from laryngeal tissues. The quality and concentration of RNA were determined with a UV spectrophotometer. Then, 1 μg of total RNA was used for reverse transcription into cDNA at 16°C for 30 min, 42°C for 30 min, 85°C for 5 min and 4°C for 5 min. Fluorescence qualitative PCR was done in 20-μl mixture, and the conditions were as follows: 40 cycles of denaturation at 95°C for 5 min, 95°C for 10 s, annealing at 60°C (58°C for ACTIN; 56°C for KAI1/CD82 and CyclinD1) and extension at 72°C for 20 s. Experiment was done three times and three wells were included in each group. β-actin served as an internal reference. $2^{-\Delta\Delta}$CT method was employed to calculate the relative expressions of KAI1/CD82 and CyclinD1.

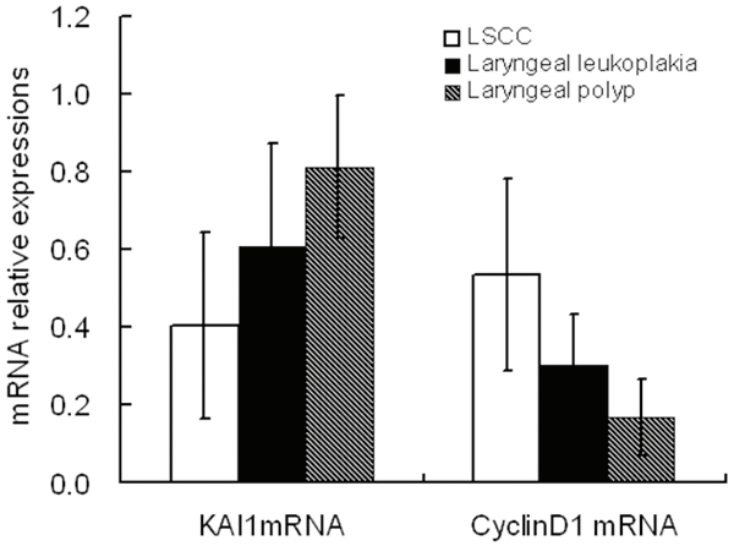

Figure 1. mRNA expressions of KAI1/CD82 and CyclinD1 in laryngeal polyp, laryngeal leukoplakia and LSCC.

Detection of protein expressions of KAI1/CD82 and CyclinD1

Tissues were grounded in liquid nitrogen and total protein was extracted with RIPA lysis buffer. BCA method was employed to determine the protein concentration. The protein solution was mixed with loading buffer and then boiled for use. Then, 50 μg of total protein were loaded for electrophoresis at 20 mA for 3 h. Proteins were transferred onto PVDF membrane at constant current for 2 h. The membrane was blocked in 5% non-fat milk at room temperature for 1 h and then treated with primary antibody at 1:200 overnight at 4°C. After washing in TBST thrice (5 min for each), the membrane was treated with HRP conjugated secondary antibody at 1:5000 for 1 h at room temperature. Following washing in TBST thrice (5 min for each), visualization was done with ECL kit. β-actin served as an internal reference.

Statistical analysis

Statistical analysis was done with SPSS version 17.0 for Windows. Comparisons between two groups were done with t test. Qualitative data (expressions of KAI1/CD82 and CyclinD1) were tested with chi square test. Survival curve was delineated with Kapla-Meier method. Survival time was evaluated with log-rank test. A value of $P<0.05$ was considered statistically significant.

Results

mRNA expressions of KAI1/CD82 and CyclinD1 in laryngeal polyps, laryngeal leukoplakia and LSCC

At mRNA level, the relative expression of KAI1/CD82 was 0.406±0.240, 0.708±0.265 and 0.812±0.184 in LSCC, laryngeal leukoplakia and laryngeal polyp, respectively; the relative expression of CyclinD1 was 0.437±0.247, 0.204±0.131 and 0.198±0.098 in LSCC, laryngeal leukoplakia and laryngeal polyp, respectively. Significant differences in the expressions of KAI1/CD82 and CyclinD1 were observed among three groups ($P<0.01$ or 0.05) (**Figure 1**).

Protein expressions of KAI1/CD82 and CyclinD1 in laryngeal polyps, laryngeal leukoplakia and LSCC

At protein level, the relative expression of KAI1/CD82 was 0.414±0.190, 0.785±0.215 and 0.841±0.128 in LSCC, laryngeal leukoplakia and laryngeal polyp, respectively; the relative expression of CyclinD1 was 0.457±0.262, 0.267±0.189 and 0.186±0.109 in LSCC, laryngeal leukoplakia and laryngeal polyp, respectively. Significant differences in the expressions of KAI1/CD82 and CyclinD1 were observed among three groups ($P<0.01$ or 0.05) (**Figure 2**).

Correlation of mRNA expressions of KAI1/CD82 and CyclinD1 with clinicopathological features of LSCC

Significant differences were observed in the mRNA expressions of KAI1/CD82 and CyclinD1 among patients with different TNM stages, different degrees of differentiation and different clinical stages ($P<0.01$ or 0.05). In addition, patients with lymph node metastasis had different mRNA expression of KAI1/CD82 from those without lymph node metastasis, but the mRNA expression of CyclinD1 was comparable between them. Moreover, there were no significant differences in the mRNA expressions of KAI1/CD82 and CyclinD1 between males and females and among patients in different age groups ($P>0.05$) (**Table 2**).

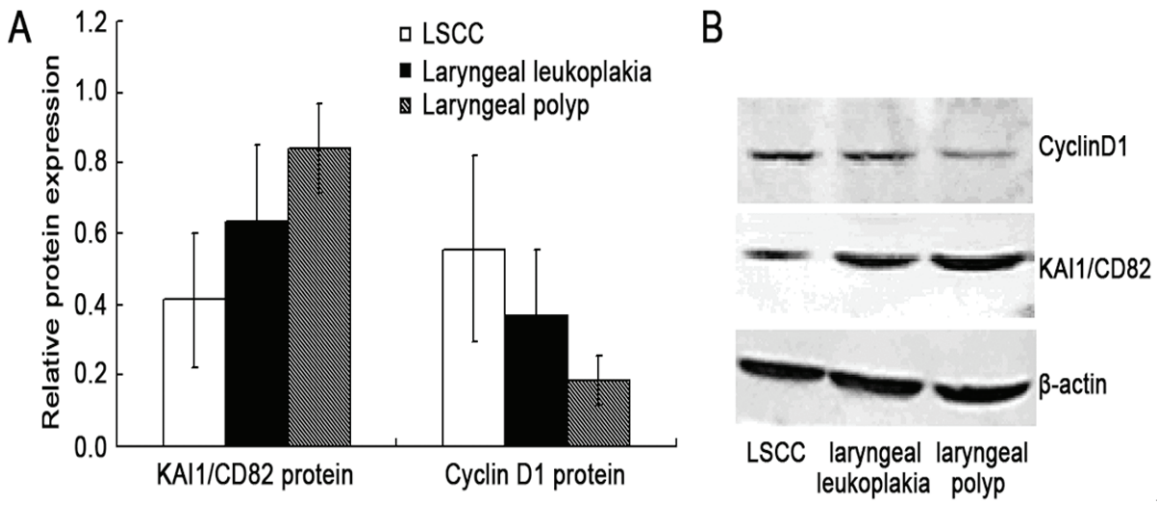

Figure 2. Protein expressions of KAI1/CD82 and CyclinD1 in laryngeal polyp, laryngeal leukoplakia and LSCC.

Table 2. Correlation of mRNA expressions of KAI1/CD82 and CyclinD1 with clinicopathological features of LSCC

Clinicopathological features	n	Relative expression of KAI1/CD82	P	Relative expression of CyclinD1	P
Male/female					
Male	57	0.421±0.258	0.407	0.416±0.243	0.303
Female	29	0.377±0.203		0.478±0.255	
Age (year)					
≥60	39	0.395±0.221	0.720	0.413±0.246	0.432
<60	47	0.415±0.257		0.457±0.232	
Invasive depth (T grade)					
T1-T2	58	0.473±0.224	0.000	0.400±0.263	0.048
T3-T4	28	0.267±0.214		0.514±0.194	
Pathological grade differentiated					
Well	60	0.469±0.240	0.000	0.373±0.228	0.000
Poorly	26	0.260±0.169		0.585±0.228	
Clinical stage					
I-II	43	0.546±0.189	0.000	0.279±0.164	0.000
III-IV	43	0.266±0.203		0.595±0.213	
Lymph node metastasis					
Positive	36	0.277±0.187	0.000	0.464±0.222	0.206
Negative	50	0.499±0.233		0.418±0.211	

Correlation of protein expressions of KAI1/CD82 and CyclinD1 with clinicopathological features of LSCC

Significant differences were observed in the protein expressions of KAI1/CD82 and CyclinD1 among patients with different TNM stages, different degrees of differentiation and different clinical stages (P<0.01 or 0.05). In addition, patients with lymph node metastasis had different protein expression of KAI1/CD82 from those without lymph node metastasis (P<0.01), but the protein expression of CyclinD1 was comparable between them. Moreover, there were no significant differences in the mprotein expressions of KAI1/CD82 and CyclinD1 between males and females and among patients in different age groups (P>0.05) (**Table 3**).

Correlation between KAI1/CD82 protein expression and CyclinD1 protein expression in LSCC

On the basis of median expressions of KAI1/CD82 and CyclinD1 in LSCC, patients were

Table 3. Correlation of protein expressions of KAI1/CD82 and CyclinD1 with clinicopathological features of LSCC

Clinicopathological features	n	Relative expression of KAI1/CD82	P	Relative expression of CyclinD1	P
Male/female					
Male	57	0.406±0.193	0.561	0.456±0.261	0.944
Female	29	0.430±0.186		0.459±0.267	
Age (year)					
≥60	39	0.378±0.195	0.096	0.468±0.227	0.739
<60	47	0.444±0.182		0.448±0.290	
Invasive depth (T grade)					
T1-T2	58	0.510±0.141	0.000	0.409±0.272	0.013
T3-T4	28	0.215±0.103		0.556±0.212	
Pathological grade differentiated					
Well	60	0.455±0.206	0.043	0.373±0.148	0.000
Poorly	26	0.319±0.148		0.651±0.197	
Clinical stage					
I-II	43	0.530±0.158	0.000	0.291±0.185	0.000
III-IV	43	0.298±0.144		0.621±0.219	
Lymph node metastasis					
Positive	36	0.287±0.182	0.016	0.663±0.238	0.091
Negative	50	0.505±0.195		0.508±0.157	

Table 4. Correlation between KAI1/CD82 protein expression and CyclinD1 protein expression in LSCC

KAI1/CD82	CyclinD1	
	+	-
+	15	28
-	28	15

divided into 4 groups, and the correlation between KAI1/CD82 protein expression and CyclinD1 protein expression was evaluated. Results showed the KAI1/CD82 protein expression was negatively related to the CyclinD1 protein expression (X^2=7.86, P<0.01) (**Table 4**).

Correlation of KAI1/CD82 protein expression and CyclinD1 protein expression with prognosis of LSCC

On the basis of median expressions of KAI1/CD82 and CyclinD1 in LSCC, patients were divided into 4 groups. In LSCC patients, the median survival time was 76 months in those positive for KAI1/CD82 protein expression, which was longer than that in patients negative for KAI1/CD82 protein expression (48 months; X^2=16.293, P=0.000) (**Figure 3**); the median survival time was 40 months in those positive for CyclinD1 protein expression, which was shorter than that in patients negative for CyclinD1 protein expression (70 months; X^2=9.517, P=0.002) (**Figure 4**).

Discussion

The occurrence and development of cancers are a complex process involving multiple genes and multiple steps, which is as a result of cooperation and interaction of multiple factors. That is true for LSCC. The recurrence and metastasis are major factors threatening the human health. In recent years, some investigators propose that combined markers might be superior to single marker in predicting the prognosis of cancers. With the development of tissue microarray technique and proteomics, increasing genes that may inhibit the cancer metastasis are identified. Thus, to explore the relationship among genes exerting inhibitory effect on cancer metastasis is clinically important for the evaluation of prognosis and treatment of cancers.

KAI1/CD82 is a tumor metastasis suppressor and was first found by Dong et al 1995 [4]. The KAI1/CD82 gene encodes 267 amino acids with a molecular weight of 29.6 kDa. The KAI1/CD82 protein has similar structure to CD82. CD82 is a glycoprotein on the leukocytes and belongs to TM4SF [5]. KAI1/CD82 may exert inhibitory effect on the metastasis of numerous cancers and is closely related to the invasiveness and prognosis of cancers. Dong et al [4] transfected melanoma cells with high metastatic capability, and their results showed the

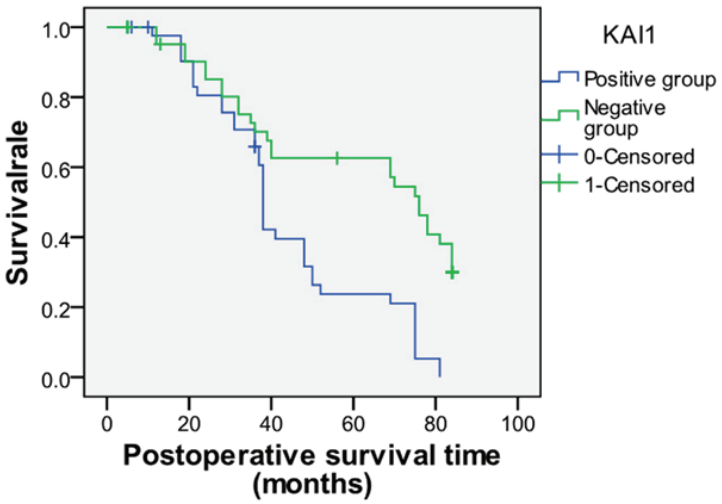

Figure 3. Correlation between KAI1/CD82 protein expression and survival time.

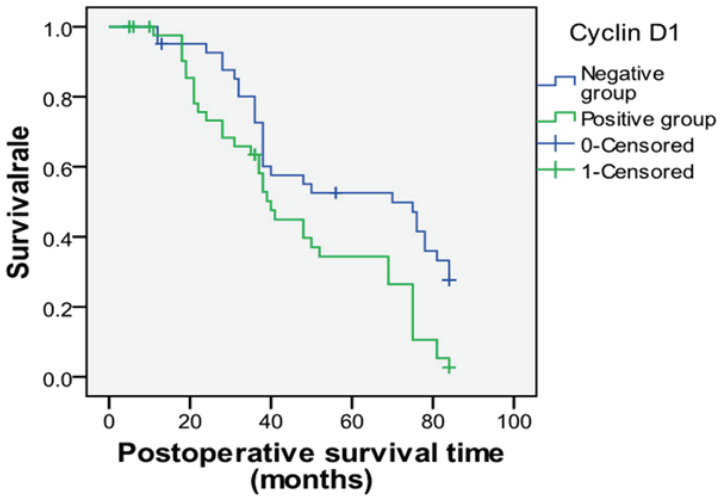

Figure 4. Correlation between CyclinD1 protein expression and survival time.

phagocytosis and invasiveness of these cancer cells reduced significantly in a dose dependent manner. In mice with inoculation of these cancer cells, the colonies of these cancer cells also dramatically reduced in the lung when compared with their counterparts. These demonstrated the tumor suppressive effect. The deficiency or down-regulation of KAI1/CD82/CD82 expression have been identified in numerous cancers including lung cancer [5], gastric cancer [2], pancreatic cancer [6], breast cancer, bladder cancer, et al. Our results showed the mRNA and protein expressions of KAI1/CD82 were consistent. The KAI1/CD82 expression in LSCC was markedly lower than that in laryngeal leukoplakia and laryngeal polyp. In addition, the KAI1/CD82 expression was different in patients with different TNM stages, different degrees of differentiation and different clinical stages and those with and without metastasis. That is, the KAI1/CD82 expression in patients with TNM stage III-IV LSCC, poorly differentiated LSCC, clinical stage III-IV LSCC and LSCC with lymph node metastasis was significantly lower than that in patients with TNM stage I-II LSCC, well differentiated LSCC, clinical stage I-II LSCC and LSCC without lymph node metastasis. These findings suggest that the more potent the metastatic and invasive capabilities, the lower the KAI1/CD82 expression is. However, there was no marked difference in KAI1/CD82 expression between males and females and patients in different age groups. Survival curve analysis showed patients positive for KAI1/CD82 protein expression had a median survival time of 76 months, which was longer than that (48 months) in patients negative for KAI1/CD82 protein expression (X^2=16.293, P=0.000).

CyclinD1 gene is mapped to 11q13 and possesses 5 exons and 4 introns. It is about 15 kb in length and encodes 295 amino acids with the molecular weight of 34 kD. Under physiological conditions, CyclinD1 expression undergoes periodic alteration with the progression of cell cycle. Generally, CyclinD1 is initially synthesized in G0 phase, and its synthesis reaches a maximal level in G1 phase and then reduces in S phase. In other phases of cell cycle, CyclinD1 expression remains at a low level. CyclinD1 has been found to promote the progression of cell cycle and is related to cell proliferation. There is evidence [7] showing that cyclinD1 actions in a retinoblastoma (Rb) dependent manner. The inactivation, compromised Rb function or over-expression of cyclinD1 may influence the normal regulation of cell cycle. This may cause transition across the G1/S checkpoint resulting in rapid repair of DNA injury of any cause and the rapid cell proliferation, which finally causes malignant trans-

formation of cells and formation of cancers. Cyclin D family has 3 members: CyclinD1, CyclinD2 and CyclinD3. Breast cancer and head and neck squamous cell carcinoma mainly express CyclinD1 [8]. The carcinogenic effect of CyclinD1 is attributed to the promotion of cell cycle progression. In normal breast tissues, CyclinD1 has a very low expression. Some clinical studies indicate that 10%-25% (mean: 20%) of cancers have increased CyclinD1 expression. In breast cancer, the mRNA and protein expression of CyclinD1 are as high as 25%-81% and 45%-83%, respectively [9, 10]. Umekita et al [11] followed up 173 patients with invasive ductal carcinoma of the breast after surgery, and results showed CyclinD1 expression was at a high level, and could be used as an independent factor determining the prognosis of ER negative breast cancer. Wang et al [12] investigated the role of CyclinD1 in the metastasis and migration of breast cancer. Their results showed the CyclinD1 expression was closely related to the metastasis of breast cancer, and breast cancer with low CyclinD1 expression had a poor capability of metastasis when compared with its parental cells. These suggest that CyclinD1 may regulate the migration of cancer cells and affect their growth. CyclinD1 exerts inhibitory effect on cell proliferation not only at cellular level but in vivo (such as LSCC animal model [13]). In LSCC, increased CyclinD1 expression was found to be related to a poor prognosis [14]. Cattani et al [15] found CyclinD1 expression was associated with HPV infection in laryngeal cancer. CyclinD1 might exert effect with HPVE6/7 to interfere with cell cycle resulting in the occurrence of laryngeal cancer. Our results showed the mRNA and protein expression of CyclinD1 were consistent in LSCC. The CyclinD1 expression in LSCC was markedly higher than that in laryngeal leukoplakia and laryngeal polyp. In addition, the CyclinD1 expression was different in patients with different TNM stages, different degrees of differentiation and different clinical stages and those with and without metastasis. That is, the CyclinD1 expression in patients with TNM stage III-IV LSCC, poorly differentiated LSCC, and clinical stage III-IV LSCC was significantly higher than that in patients with TNM stage I-II LSCC, well differentiated LSCC and clinical stage I-II LSCC. These findings suggest that the more potent the metastatic and invasive capability, the higher the CyclinD1 expression is. However, there was no marked difference in CyclinD1 expression between males and females, among patients in different age groups and between patients with and without lymph node metastasis. Survival curve analysis showed patients negative for CyclinD1 protein expression had a median survival time of 70 months, which was longer than that (40 months) in patients positive for CyclinD1 protein expression (X^2=9.517, P=0.02).

In the present study, we also evaluated the relationship between KAI1/CD82 protein expression and CyclinD1 protein expression. Results showed the KAI1/CD82 protein expression was negatively related to CyclinD1 protein expression (X^2=5.66, P<0.05). In laryngeal polyp, laryngeal leukoplakia and LSCC, the expressions of KAI1/CD82 and CyclinD1 were significant different, which implies that both proteins involve in the malignant transformation of benign laryngeal lesions, and both might exert synergist effect in this process. KAI1/CD82 may inhibit the activity of EGFR/K-ras/JNK/c-Jun/CyclinD1 pathway to delay the progression from G0/G1 phase to S phase exerting anti-tumor effect. This also confirms the previous hypothesis [3]. Of course, this should be validated in numerous studies.

The interaction between two proteins is required to elucidate in future studies. Our findings indicate that both KAI1/CD82 and CyclinD1 may become promising markers for predicting the prognosis of LSCC, which is beneficial for the diagnosis and treatment of LSCC.

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