PROGNOSTIC SIGNIFICANCE OF KAI1/CD82 AND ITS RELATION TO D2-40 LABELED LYMPHATIC VESSEL INVASION (LVI) AND LYMPHATIC VESSEL DENSITY (LVD) IN ESOPHAGEAL SQUAMOUS CELL CARCINOMA (ESCC)

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ABSTRACT

Objectives: The KAI1/CD82 appears to inhibit multiple steps of cancer metastatic. D2-40 labeled LVI and LVD are also closely associated with cancer metastasis. We investigated the expression levels of KAI1/CD82, D2-40 labeled LVI and LVD and their correlation with clinicopathological factors in ESCC.

Methods: Immunohistochemistry and Western Blot were used to detect KAI1/CD82 expression levels in the peritumoral tissue and ESCC. LVI and LVD detected by D2-40 immunohistochemical staining. The relationships between the KAI1/CD82 expression levels, LVI and LVD were analyzed. The prognosis of ESCC was analyzed by Kaplan-Meier survival analysis and Cox’s proportional hazards model.

Results: KAI1/CD82 expression was markedly lower in ESCC than in the para-carcinoma tissue (P<0.05). Positive peritumoral LVI and high mean peritumoral LVD were positively correlated while KAI1/CD82 expression was negatively correlated with tumor invasion, lymph node metastasis, and clinical stage. Kaplan-Meier analysis revealed that high mean peritumoral LVD and positive LVI was negatively correlated with overall survival (OS) and disease-free survival (DFS) time, while KAI1/CD82 expression was positively correlated with OS and DFS time. Low KAI1/CD82 expression, high mean peritumoral LVD, positive LVI was associated with a poor prognosis in ESCC. Multivariate Cox regression analysis indicated that the positive LVI and KAI1/CD82 positive expression were independent predictors for OS in ESCC. Positive LVI and high mean peritumoral LVD were independent predictors for DFS in ESCC.

Conclusions: KAI1/CD82 expression, LVI and LVD were significantly correlated with some clinicopathological factors of ESCC including lymph node metastasis, differentiation, and clinical stage. Combined detection of these factors may be of significant value in predicting the prognosis and metastasis in ESCC patients.

Keywords: esophageal squamous cell carcinoma, KAI1/CD82, LVI; LVD, Metastasis.

DOI: 10.19193/0393-6384_2017_6_153

Introduction

In China, esophageal squamous cell carcinoma (ESCC) is the most common type of EC(1). The prognosis of ESCC patients remains poor, with a 5-year OS rate of less than 37%.(2) Lymph node metastasis was the principal factor contributed to the poor postoperative prognosis of ESCC. The 5-year OS rate after surgical resection is 18-47% for patients with LN metastasis(3). A significant proportion of ESCC already have micro-metastases even when the tumor showed clinically localized. Due to lack of effective biomarker, some advanced ESCC was treated as “early” ESCC, consequently poorer prognosis was achieved. Some biomarkers that detect cancer aggressiveness and lymph node metastasis are of great importance in order to assess the prognosis, predict metastasis, recurrence of can-
cer following radical esophagectomy, and even to design for treatment strategies.

The tumor suppressor gene CD82, which encodes the protein KAI1, is a member of the tetraspanin superfamily of glycoproteins. CD82 suppresses metastasis by multiple mechanisms including inhibition of cell motility and invasion, promotion of cell polarity as well as induction of senescence and apoptosis in response to extracellular stimuli\(^\text{4}\). The previous research results indicated that KAI1/CD82 can promote homotypic cell-cell adhesion which can be blocked with protein phosphatase 1 (inhibitor of Src kinase)\(^\text{5}\), and depend on E-cadherin\(^\text{6}\). In vitro studies show that over-expression of KAI1/CD82 inhibits cell motility and invasion\(^\text{7, 8}\). Abnormal of cell-cell adhesion and enhancement of cell motility and invasion result in tumor metastasis. So downregulated expression of KAI1/CD82 could improve tumor metastasis. The reversed relationship between KAI1/CD82 expression and clinical features of ESCC was firstly reported by Miyazaki and his colleagues\(^\text{9}\). However, the impact of KAI1/CD82 expression on OS and DFS of postoperative ESCC, and the interaction between KAI1/CD82 and LVI, LVD were not clarified yet.

LVI been demonstrated to be a negative prognostic factor for OS of ESCC\(^\text{10}\). For some “early ESCC patient” without regional lymph node metastasis, positive LVI has the ability to stratify those patients into risk groups may permit a more individualized approach to adjuvant treatment recommendations. D2-40 (podoplanin antigen) is expressed in lymphatic endothelial cells instead of endothelium of blood vessels, hence allowing lymphatic vessels (LVs) identification and assessment of their density (LVD) and LVI\(^\text{11}\).

In this study, LVI and LVD were evaluated in intra and peritumoral tissues in ESCC by immunohistochemical staining of D2-40. The relationship among the expression levels of KAI1/CD82, LVI, LVD and the various clinicopathologic characteristics of ESCC was analyzed.

**Material and methods**

**Clinical information**

81 cases of ESCC were collected from the Department of Cardiovascular and thoracic surgery, at the First people’s Hospital Affiliated to Nantong University from January 2008 to December 2010. No patients received chemotherapy or radiotherapy before surgery. All patients took routine examinations such as blood routine test, blood gas analysis, electrocardiogram, barium swallow, endoscopic ultrasonography for esophagus and stomach with biopsy (the pathological type in this cohort was all squamous cell carcinoma), chest and abdominal computed tomography. All patients were discussed at a multidisciplinary specialist team meeting. All patients underwent radical resection and regional Lymph node dissection. All hematoxylin and eosin (H&E)-stained slices were confirmed by two pathologists. The mean age of the 81 ESCC patients was 68.4 ± 10.1 (55-78) years. All patients were followed-up at 6-month intervals by phone, mail, or email. Survival time was calculated from surgery to death or 60 months (mean survival time: 45.2 months; range 6-60 months). Tumor differentiation grade was defined according to World Health Organization criteria.

There were 36 cases with well differentiation, 45 cases with poor differentiation. Clinical stages were according to International Union Against Cancer/American Joint Committee on Cancer TNM criteria. Furthermore, 16 cases were at stage I, 39 cases were at stage II, 26 cases were at stage III. Lymph node metastases were present in 55 cases and absent in 26 cases. Other clinicopathological characteristics are provided in Table 1.

**Table 1:** The relationship between KAI1/CD82 expression level, positive LVI, high LVD and clinicopathological characteristics of ESCC.
This study was approved by Ethics Committee of the First people’s Hospital Affiliated to Nantong University (the approved number 200606) and conducted in accordance with the ethical guidelines of the Declaration of Helsinki.

Methods

Immunohistochemistry test

Immunohistochemistry was used to determine KAI1 and Podoplanin (D2-40 labeled) protein expression levels and in the ESCC according to the manufacturer instructions. ESCC tissue specimens were continuously resected into 4-μm sections. Sections were deparaffinized in xylene and sequentially rehydrated in gradients of ethanol. Then, the sections were treated with 3% hydrogen peroxide and microwaved after pretreatment in 10 mM citric acid for antigen retrieval. The sections were incubated with blocking solution containing phosphate buffered solution (PBS) for 20 minutes at room temperature, and then incubated overnight at 4°C with and treated with KAI1 polyclonal antibody (1:200), anti-D2-40 monoclonal antibody (prediluted; Signet Laboratories Inc, Dedham, MA, USA). The slices were observed following paraffin dewaxing, microwave antigen repair, 3,3’-diaminobenzidine staining, hematoxylin re-dyeing, making transparent, and sealing. Phosphate-buffered saline was used instead of antibody as the negative control, and breast biopsy was adopted as a positive control. The relationship between KAI1 expression levels and LVI, LVD and ESCC invasion metastasis was analyzed.

Analysis of positive expression

The slices were reviewed by two pathologists using the double blind method. The positive cell number and cellular staining intensity were observed under high magnification (200-400X) for semi-quantitative analysis.

Cells that expressed KAI1 appeared as pale yellow or tan particles. Staining intensity was scored as follows: 0 represented no coloring, 1 represented dyeing to pale yellow, 2 represented dyeing to yellow, and 3 represented dyeing to tan. Five visions of each section were randomly selected for observation and the percentage of positive cells in 500 cells was evaluated for scoring: 0 represented less than 10% positive cells, 1 represented 10-40%, 2 represented 40-70%, and 3 represented 70% or higher. The two scores were added together to provide a measure of positive expression: positive (+), ≥ 2; negative (-), 0-1.

D2-40 immunostaining was assessed in primary tumor specimens, and peritumoral tissue lymphatics, not in node metastases. Any discrete D2-40 positive endothelial cell or cell cluster separate from adjacent structures, stained into brown yellow or chocolate brown was used as D2-40-positive expression, regardless of the presence of lumens, was counted as one lymphatic vessel. In the D2-40-positive lymphatic vessel cavity, ESCC cells were found, which defined as LVI. Thus, we preliminarily speculated that LVI was positive. This speculation was verified under a high-power lens (×400). For measuring the LVD, sections were scanned by light microscopy at low magnification (40×) to identify the areas of tissue with the greatest density of D2-40-positive endothelial cells (hot spots). Mean number of counting lymphatic vessels per field in 5 separate 200× fields was performed[10].

Statistical analysis

All statistical analyses were performed using SPSS18.0 software (Chicago, IL). The Pearson χ² test and the Fisher’s exact t-test were used to assess the relationship between protein expression and clinicopathological indices. Multivariate logistic regression analysis was used to clarify the relative factors for metastasis. The univariate survival analysis of OS and DFS was based on the Kaplan-Meier method with log rank tests. A multivariate Cox regression model was used to analyze the influence of various factors on OS and DFS. Covariates consisted of sex, age, tumor diameter, location, differentiation, depth of invasion, lymph node metastasis, distant metastasis, and expression of KAI1/CD82, LVI and LVD. Beta coefficients and 95 % confidence intervals (CI) were used for analysis. P values of less than 0.05 were considered significant.

Results

KAI1/CD82 expression levels and LVI, LVD in ESCC tissues

In immunohistochemistry test, Positive KAI1/CD82 expression was detected on the membrane of ESCC (Fig. 1A). KAI1/CD82 protein was expressed positively in 39.5% (32/81) of ESCC.

D2-40 positive labeled lymphatic vessels were present in all cases of ESCC. The vessels were found both in intratumoral (35.4% of cases) and
peritumoral (100% of cases) tissues. In the intratumoral area, the lymph vessels were mainly small compared to the more abundant wide lymphatics in peritumoral area (Fig. 1E). D2-40 labeled LVI was detected positively in 35.8% (29/81) peritumoral tissue of ESCC (Fig. 2C). LVD in intratumoral areas (mean 3.35±2.89) (Figure 1F) was lower than LVD in the peritumoral areas (mean 5.18±2.64) (Figure 1E).

Correlation between KAI1/CD82 expression levels, LVI, LVD and clinicopathological characteristics of ESCC

There was no relationship between KAI1/CD82 expression levels and sex, age, surgery mode, tumor location, and metastasis stage (P >0.05). The negative KAI1/CD82 expression showed a reversed correlation with TNM stage, depth of invasion (T stage), blood vessel and nerve invasion, differentiation, and lymph node metastasis (N stage) (P<0.05). No significant correlation was found between positive LVI and sex, age, surgery mode, tumor location, nerve invasion, however, positive LVI was positively correlated with TNM stage, depth of invasion (T stage), blood vessel invasion, differentiation, lymph node and distance metastasis (N and M stage) (P<0.05). LVD in intratumoral tissues was significantly decreased compared with peritumoral areas (P<0.001) (Table 1). LVD in intratumoral tissues had no significant relationship with clinicopathological characteristics of ESCC. Peritumoral LVD showed significant correlation only with lymph node metastasis (N stage), differentiation, and TNM stage (P<0.05) (Table 1).

Correlations among KAI1/CD82 expression levels and LVI, LVD in ESCC

There was a negative correlation between KAI1/CD82 positive expression and positive peritumoral LVI, peritumoral LVD (r=-0.326, p=0.003; r=-0.513, p<0.001; respectively) (Table 2). Positive peritumoral LVI showed positive correlation with peritumoral LVD (r=0.408, p<0.001) (Table 2).

Survival analysis

The positive lymph node metastasis, advanced TNM stage, negative expression of KAI1/CD82, positive LVI and high LVD had significant association with poor OS (Fig. 2A, B, C, D, E) and DFS (Fig. 3 A, B, C, D, E) (P<0.001, respectively). In addition, the combination of negative KAI1/CD82 expression, high LVD and positive LVI had a poorer OS and DFS compared with the contrary combination (P<0.001) (Fig. 2F, 3F). In the univariate analysis, OS and DFS time was significantly correlated with clinicopathological factors, including tumor differentiation, depth of invasion, positive lymph node metastasis, TNM stage, negative expression of KAI1/CD82, positive LVI and high LVD (Table 3, 4).

Multivariate analysis revealed that only TNM stage, positive KAI1/CD82 and positive LVI had significant relationship with OS, so they were independent prognostic factors for OS (P<0.05) (Table 3); however, only TNM stage, positive LVI, high LVD had significant relationship with DFS, so TNM stage, positive LVI, high LVD were independent prognostic factors for DFS (P<0.05) (Table 4).

Discussion

The progression and metastasis of ESCC are related to several factors. Among these factors, changes in intercellular adhesion molecules with...
loss of intercellular adhesion in tumor cells are an important step for tumor invasion and metastasis\(^{(13)}\). The metastasis suppressor gene KAI1 (Kangai1), was originally identified in a screen for genes on chromosome 11 that suppressed metastasis of rat AT6.1 prostate cancer cells. Re-expression of KAI1 in AT6.1 reduced the formation of metastases without affecting primary tumor growth\(^{(4)}\). KAI1/CD82 is mainly located on the cell membrane and is down-regulated in several digestive cancers such as colorectal\(^{(10)}\), hepatic\(^{(15)}\), gastric\(^{(16)}\), and ESCC\(^{(9)}\). It was reported that a decrease in KAI1/CD82 expression was associated with lymph node metastasis. The lymph node metastasis of ESCC is the major factor that leads to the poor prognosis. However, its relationship with D2-40 labeled LVI and LVD was unclear, what’s more, the impact of KAI1/CD82 on the postoperative OS and

**Figure 2:** Kaplan-Meier analysis of the overall survival rate of patients with ESCC. A: Overall survival of all patients in relation to positive LNM (\(P<0.001\)). B: Overall survival of all patients in relation to TNM stage (\(P<0.001\)). C: Overall survival of all patients in relation to the negative expression of KAI1/CD82 (\(P<0.001\)). D: Overall survival of all patients in relation to positive LVI (\(P<0.001\)). E: Overall survival of all patients in relation to high mean LVD (\(P<0.001\)). F: Overall survival of all patients in relation to the combination of negative expression of KAI1/CD82, positive LVI and high mean LVD (\(P<0.001\)).

**Figure 3:** Kaplan-Meier analysis of the Disease-free survival rate of patients with ESCC. A: Disease-free survival of all patients in relation to positive LNM (\(P<0.001\)). B: Disease-free survival of all patients in relation to TNM stage (\(P<0.001\)). C: Disease-free survival of all patients in relation to the negative expression of KAI1/CD82 (\(P<0.001\)). D: Disease-free survival of all patients in relation to positive LVI (\(P<0.001\)). E: Disease-free survival of all patients in relation to high mean LVD (\(P<0.001\)). F: Disease-free survival of all patients in relation to the combination of negative expression of KAI1/CD82, positive LVI and high mean LVD (\(P<0.001\)).

The metastasis suppressor gene KAI1 (Kangai1), was originally identified in a screen for genes on chromosome 11 that suppressed metastasis of rat AT6.1 prostate cancer cells. Re-expression of KAI1 in AT6.1 reduced the formation of metastases without affecting primary tumor growth\(^{(4)}\). KAI1/CD82 is mainly located on the cell membrane and is down-regulated in several digestive cancers such as colorectal\(^{(10)}\), hepatic\(^{(15)}\), gastric\(^{(16)}\), and ESCC\(^{(9)}\). It was reported that a decrease in KAI1/CD82 expression was associated with lymph node metastasis. The lymph node metastasis of ESCC is the major factor that leads to the poor prognosis. However, its relationship with D2-40 labeled LVI and LVD was unclear, what’s more, the impact of KAI1/CD82 on the postoperative OS and
DFS time of patients with ESCC have not been elucidated.

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**Table 3:** Results of Univariate and multivariate analyses of factors affecting overall survival (OS) time.

D2-40 has been utilized to detect LVI more accurately signaling that the tumor has already passed the initial step of lymph node metastasis (17). In this study, the evaluation of LVI and LVD through expression of D2-40 in ESCC was investigated in correlation with KAI1/CD82 expression and clinicopathological parameters.

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<td>LVD (30)</td>
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**Table 4:** Results of Univariate and multivariate analyses of factors affecting disease-free survival (DFS) time.

In the evaluation of the prognostic significance of KAI1/CD82 expression and its association with clinicopathological variables in ESCC, the results showed that KAI1/CD82 expression was negatively associated with TNM stage, depth of invasion (T stage), blood vessel and nerve invasion, differentiation, and lymph node metastasis (N stage). This suggests that KAI1/CD82 expression was downregulated in advanced ESCC. Our results showed that KAI1/CD82 expression was significantly associated with lymph node metastasis. Our results were in agreement with those of the results reported by Miyazaki (9).

There were few reports about the relationship between KAI1/CD82 expression and postoperative OS and DFS. We found that the lower expression of KAI1/CD82 had significant association with worse OS and DFS. The level of KAI1/CD82 protein was related to patient prognosis; in multivariate analysis KAI1/CD82 expression was an independent prognostic factor for postoperative OS but not for DFS. The KAI1/CD82 protein participates in reactions between cells or between the extracellular matrix and cells, and affects cancer cell invasion and metastasis. An important aspect of the function of KAI1/CD82 in inhibiting cell motility and invasion is by attenuating epidermal growth factor receptor (EGFR) signaling by promoting internalization of the activated receptor(10). KAI1/CD82 can regulate cell invasion by regulating the localization of extracellular proteases(10). The downregulated expression of KAI1/CD82 is related to esophageal cancer prognosis.

In this study, a positive rate of D2-40 labeled LVI was 35.8%. In previous studies, the results of D2-40 labeled LVI-positive rate were from 30% to 79%(20, 21). The difference was caused mainly by the heterogeneity of patients with different proportion of age, tumor location, TNM stage, and so on, especially the N stage (lymph node metastasis LNM). In more ESCC patients in this cohort were in advanced stage. LVI was found in close relation to lymph node metastasis of ESCC(10).

This result is consistent with the results reported by other studies (20, 21, 22). In addition, LVI was found in close relation to lesion depth, distance metastasis, differentiation, and clinical stage. In univariate and multivariate analysis of the relationship between D2-40-labeled LVI and prognosis showed a close relation with OS and DFS time, and LVI was an independent risk factor for both OS and DFS time. The rate of positive LVI in patients with positive LNM was higher than patients with negative LNM; however, 5 patients with negative LNM (19.2%) were detected with LVI positive.

The results indicated that LVI occurs before lymph node metastasis, and can be used as a predictive factor for lymph node metastasis. Some studies reported that D2-40 labeled LVI is closely related to the clinical prognosis of ESCC(20, 21). However, some studies have drawn an opposite conclusion(22, 23). This difference may be caused by follow-up time, post-operative therapy, and so on. The relationship between D2-40-labeled LVI and prognosis of ESCC need to be further studied with more clinical data. Identification of LVI provides a very good way for predicting lymph node metastasis.
sis, especially for the patients with ESCC in “early” stage (negative LNM but positive LVI).

The intratumoral and peritumoral LVD represent the lymphangiogenesis. In our study of 81 cases, the intratumoral LVD was significantly lower than peritumoral LVD ($P<0.001$). Inoue et al(23) reported that intratumoral lymphangiogenesis correlates with LNM, the depth of invasion and a worse patient prognosis in their study; however, no significant associations were found between intratumoral LVD and clinicopathological factors of ESCC in our study. Peritumoral LVD had significant associations with some clinicopathological factors including LNM, differentiation, TNM stage of ESCC, and also with prognosis in univariate analysis for OS and DFS time, but it was not a significant prognostic factor in multivariate analysis for OS time.

In this study, we analyzed the relationship between clinicopathological factors of ESCC and expression level of KAI1/CD82, D2-40 labeled LVI and LVD. The results suggested that negative expression KAI1/CD82 was significantly correlated with lymph node metastasis, differentiation, clinical stage and OS. It was an independent prognostic factor for OS time. Positive peritumoral LVI and High peritumoral LVD were also significantly correlated with some clinicopathological factors of ESCC such as lymph node metastasis, differentiation, and clinical stage and so on. In addition, positive peritumoral LVI was an independent prognostic factor both for OS and DFS time. High peritumoral LVD was an independent prognostic factor both for DFS time. Combined detection of these factors may provide a new direction for investigating the metastasis and prognosis of ESCC.

Conclusions

In this study, we analyzed the relationship between clinicopathological factors of ESCC and expression level of KAI1/CD82, D2-40 labeled LVI and LVD. The results suggested that negative expression KAI1/CD82 was significantly correlated with lymph node metastasis, differentiation, clinical stage and OS. It was an independent prognostic factor for OS time. Positive peritumoral LVI and High peritumoral LVD were also significantly correlated with some clinicopathological factors of ESCC such as lymph node metastasis, differentiation, and clinical stage and so on. In addition, positive peritumoral LVI was an independent prognostic factor both for OS and DFS time. High peritumoral LVD was an independent prognostic factor both for DFS time. Combined detection of these factors may provide a new direction for investigating the metastasis and prognosis of ESCC.

References


Foundation: supported by the Youth Fund Project of Nantong Health Authority (project number: WQ2014020)

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