Original Article
Association of Kruppel-like factor 4 expression with the prognosis of esophageal squamous cell carcinoma patients

Ming-Quan Ma1,2, Hong-Dian Zhang1,2, Peng Tang1,2, Hong-Jing Jiang1,2, Chuan-Gui Chen1,2
1Department of Esophageal Tumor, Tianjin Medical University Cancer Institute and Hospital, National Clinical Research Center for Cancer, Tianjin 300060, China; 2Key Laboratory of Cancer Prevention and Therapy, Tianjin 300060, China

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Abstract: Objective: To investigate the association of Kruppel-like factor 4 (KLF4) expressions with the prognosis of esophageal squamous cell carcinoma (SCC) patients. Methods: Ninety-eight cases of esophageal carcinoma patients were enrolled. The expression of KLF4 in the esophageal SCC and normal esophageal mucosa tissues were examined by immunohistochemistry. The correlations between the expression of KLF4 protein and patients’ clinical characteristics and prognosis were analyzed. Results: We observed higher expressed KLF4 in normal esophageal mucosa tissues than esophageal SCC tissues, with positive rate of 82.7% (81/98) and 43.8% (43/98) respectively. In patients with lymphatic metastasis, the positive rate of KLF4 was 24.4% (10/41), whereas it was 57.9% (33/57) in patients without lymphatic metastasis, and the difference was significant (χ² = 10.871, P = 0.001). The positive rates of KLF4 were 62.5% (5/8), 53.1% (26/49) and 29.3% (12/41) in stage I, II and III patients, respectively. There were no correlations between the expression of KLF4 and gender, age, tumor size, location, differentiation grade and infiltration depth. The 5-year survival rates and median survival times were 48.8% and 25.5%, and 55 and 26 months for the patients with KLF4 positive and negative expression, respectively. There were significant differences between the patients with KLF4 positive expression and negative expression in the 5-year survival rates and median survival times (χ² = 5.747 and 4.493, P = 0.017 and 0.034). Conclusion: KLF4 might act as a tumor suppressor in esophageal SCC and the expression status of KLF4 could be considered as a prognosis predictor for esophageal SCC patients.

Keywords: Esophageal squamous cell carcinoma, Kruppel-like factor 4, prognosis

Introduction
Kruppel-like factor 4 (KLF4), a zinc-finger-type transcriptional factor that is widely expressed in many human tissues, plays an important role in regulating cell proliferation, differentiation, apoptosis and maintaining the activity of telomerase [1, 2]. Studies over the past several decades have also identified key physiologic and pathologic phenotypic modulators of KLF4, it could act as either a cell cycle suppressor or an oncogene, depending on the tissue type [3, 4]. KLF4 is able to arrest the G1/S cell cycle and thereby inhibits DNA synthesis, cell proliferation and differentiation. Knockout of KLF4 in gastrointestinal epithelial cells results in the abnormal proliferation, differentiation and cell infiltration [5]. It has been demonstrated that the down regulation of KLF4 in gastric cancer, colorectal cancer, bladder cancer and cervical cancer inhibits the occurrence and development of tumors, whereas KLF4 is up regulated in breast cancer, head and neck cancer, oral cancer and skin cancer, and subsequently promotes the survival and progression of tumors [6-8]. These results suggest that the expression of KLF4 plays a very important role in the occurrence, progression and metastasis of tumors. Research performed by Ghaleb et al indicated that under physiological conditions, KLF4 inhibits cell proliferation. Conversely, KLF4 mediates proinflammatory signaling in macrophages and its overexpression in the esophageal epithelium activates cytokines, leading to inflammation-mediated esophageal squamous cell can-
Prognosis of KLF 4 for esophageal squamous cell carcinoma patients

Figure 1. Immunohistochemistry of KLF4 protein expression; it was mainly expressed in cytoplasm and nucleus as yellow or brown-color staining. A. Normal esophageal mucosa tissues; B. Esophageal squamous cell carcinoma tissues; C. Negative expression of KLF4 protein in esophageal squamous cell carcinoma.

cer formation in mice [9]. However, the expression and the role of KLF4 in esophageal squamous cell carcinoma patients have not been clearly investigated.

In this study, we invested the expression of KLF4 in the esophageal squamous cell carcinoma tissues and the corresponding normal esophageal mucosa tissues, and analyzed the correlation with clinical characteristics and prognosis of these patients.

Patients and methods

Patients and ethnic consideration

This study was approved by an Institutional Review Board of Tianjin Medical University Cancer Institute and Hospital and was conducted in accordance with good clinical practice, all applicable regulatory requirements and the guiding principles of the Declaration of Helsinki. Written informed consent was obtained from all patients prior to admission to the study.

A total of 98 cases of esophageal carcinoma patients (64 men and 34 women; median age: 60 years old) were enrolled successfully from June 2006 to June 2007 in Tianjin Medical University Cancer Institute and Hospital.

Recruited patients were confirmed having no history of chemotherapy, radiotherapy and immunotherapy before the surgery. All the samples used in the present study were confirmed by pathological examination. All the 98 cases were squamous cell carcinoma, including 41 cases with lymph node metastasis and 57 cases without lymph node metastasis. There were 8 cases in I stage, 43 cases in IIa stage, 6 cases in IIb stage and 41 cases in III stage according to the 6th edition of esophageal cancer TNM staging criteria drafted by American Joint Committee on Cancer (AJCC) and Union for International Cancer Control (UICC) [10]. Their follow-up ended at August 2012 with a median follow-up period of 45 (3-72) months.

Sample preparation

The tumor tissue was taken from the tumor center with size about of 1 cm * 1 cm * 1 cm, normal esophageal mucosa was taken from operation resection specimens. The resected tissue pieces were put into the freezing tube and rapidly frozen in liquid nitrogen for analysis.

Immunohistochemistry

The expression of KLF4 was examined by immunohistochemical (IHC) staining methods. Esophageal carcinoma tissues were fixed with 4% paraformaldehyde and embedded with paraffin using standard methods. IHC was carried out using a specific mouse monoclonal anti-KLF4 antibody (1:50, Santa Cruz, CA, USA). Counterstaining was performed with hematoxylin. Control staining was conducted by omitting the primary antibody. The images were obtained with an Olympus DP70 optical microscope (Tokyo, JPN) and analyzed by the image analysis system (Beihang University, CM-2000B, Beijing, China).

The image results were scored by two independent pathologists. Briefly, the images were first scored according to the staining depth: negative staining, score 0; faint yellow, score 1; brown madder, score 2; dark brown, score 3. Then the images were scored according to the percentage of KLF4 positive cells in the total tumor cells: ≤ 10%, score 0; 11%-25%, score 1;
Prognosis of KLF 4 for esophageal squamous cell carcinoma patients

26%-50%, score 2; 51%-75%, score 3; > 75%, score 4. The scores of the same slide were summed to produce a final score (Final score = staining depth score × positive cell percentage score): 0-3 was considered as negative (-); 4-6, weakly positive (+); > 6, strong positive (++)

Western blot

Western blot was used to determine the protein level in the cancer tissues. Briefly, frozen tissues were homogenated with lysis buffer, then centrifuged at 4°C for 30 min (12000 r/min). The supernatant was collected; BCA method was used to determine protein concentration. A 10% polyacrylamide gel was prepared to load protein samples, 5% nonfat dry milk was added to block the non-specific antigen. The primary antibodies (anti-KLF4, catalog no.ab56542, Abcam, Cambridge, UK) and secondary antibodies were applied, β-actin served as control. Bio-Rad Gel Doc 2000 image processing system (Hercules, CA, USA) was used for image analysis.

Statistical analysis

SPSS 17.0 (SPSS Inc., Chicago, IL) was used to perform the statistical analysis. The Chi-square test was used to assess the association between patients’ clinical features and the expression of KLF4. OS and the 95% confidence intervals (CIs) were evaluated by the Kaplan-Meier method comparing the different groups by log-rank test. P-values were considered statistically significant if less than 0.05.

Results

Expression of KLF4 in the esophageal squamous cell carcinoma and normal esophageal mucosa tissues

Immunohistochemistry showed the KLF4 protein was mainly expressed in cytoplasm and nucleus as yellow or brown-color staining. As shown in Figure 1, the expression of KLF4 was strong in both the cytoplasm and the nucleus. KLF4 was overexpressed in the normal esophageal mucosa tissues (Figure 1A), with a positive expression rate of 82.7% (81/98). But in the esophageal squamous cell carcinoma tissues (Figure 1B), the expression of KLF4 was very low (43.8%, 43/98), at the same time, there was almost no positive brown staining of the negative expression of KLF4 protein in esophageal squamous cell carcinoma (Figure 1C). There was a significant difference between the esophageal squamous cell carcinoma and normal esophageal mucosa tissues ($x^2 = 31.701, P < 0.001$). The immunohistochemical staining scores (Figure 2) were $8.27 ± 0.35$ and $3.41 ± 0.33$ in the normal esophageal mucosa and esophageal squamous cell carcinoma tissues, respectively. The difference between the two groups was also significant ($P < 0.01$).

Correlation between the expression of KLF4 protein and patients’ clinical characteristics

For esophageal cancer patients with lymphatic metastasis, the positive expression rate of KLF4 was 24.4% (10/41), whereas it was 57.9% (33/57) in patients without lymphatic metastasis. The difference between the two groups was significant ($x^2 = 10.871, P = 0.001$). The positive expression rates of KLF4 were 62.5% (5/8), 53.1% (26/49) and 29.3% (12/41) in stage I, II and III patients, respectively. A significant difference was detected among the 3 groups ($x^2 = 6.482, P = 0.039$). However, there were no cor-
relations between the expression of KLF4 and gender, age, tumor size, location, differentiation grade and infiltration depth (Table 1).

**Western blot**

We compared 20 cases of normal esophageal mucosa and esophageal squamous cell carcinomas. Result showed that there were 16 cases of KLF4 protein expression level were lower in esophageal squamous cell carcinomas than that of normal esophageal mucosa (Figure 3A). After image scanning and analysis by using Bio-Rad Gel Doc 2000 imaging system, we measured gray values of KLF4 and β-actin products, the expression level of KLF4 protein was calculated as the ratio of KLF4 protein and β-actin. Results showed the average level of KLF4 expression in esophageal squamous cell carcinoma was 0.576 ± 0.050, and 0.684 ± 0.095 in normal esophageal mucosa tissues, the difference was statistically significant (t = 4.932, P < 0.01) (Figure 3B).

**Correlation between the expression of KLF4 and the prognosis of patients with esophageal squamous cell carcinoma**

All the patients were followed-up till August 2012, with a median follow-up period of 45 months (3~72 months). The 5-year survival rate was 35.7% for all the patients. The 5-year survival rates and median survival times were 48.8% and 25.5, and 55 and 26 months for the patients with KLF4 positive expression and negative expression, respectively (Figure 4). There were significant differences between the patients with KLF4 positive expression and

<table>
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<th>Pathological factors</th>
<th>Case (N)</th>
<th>expression of KLF4 protein</th>
<th>x² value</th>
<th>P value</th>
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<tr>
<td></td>
<td></td>
<td>Negative (55)</td>
<td>Positive (43)</td>
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<tr>
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<td>64</td>
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Prognosis of KLF 4 for esophageal squamous cell carcinoma patients

Discussion

KLF4, also known as gut-enriched Kruppel-like factor (GKLF) and epithelial zinc finger (EZF), is a member of the Kruppel-type zinc finger transcription factors [11-13]. The human KLF4 gene located on chromosome 9q31, which contains cDNA encodes a 55kD protein of 470 amino acids. Research indicated that KLF4 is an important regulator of cell proliferation and differentiation through regulating the transcription of target genes, blocking the G1/S conversion process by inhibiting DNA synthesis and cell proliferation and differentiation. In addition, knocking out of KLF4 in epithelial cells at the gastrointestinal tract could change the abnormal proliferation and differentiation, infiltration [5].

Compelling reports have indicated the transcriptional role of KLF4 in regulating inflammatory stimuli [14, 15], breast cancer [16, 17], stem cells [2] and esophageal cancer cell lines [18]. KLF4 has been found to be overexpressed in oral and skin squamous carcinoma cells as well [19], reduced expression of KLF4 decreased the capacities of cancer stem cells to resist the chemicals, migrate, invade, and generate tumors in vitro and in vivo [20]. Although these results are not consistent, they suggest that KLF4 plays an important role in the development and progression of these tumors [21].

Our results showed that the expression of KLF4 was lower than that in the normal esophageal mucosa tissues. The expression of KLF4 was negatively correlated with the lymphatic metastasis and the disease stage of esophageal SCC patients. In addition, our results also showed that positive KLF4 expression in the esophageal SCC tissues correlated with higher 5-year survival rate and longer median survival time compared with negative KLF4 expression.

In the current study, a large number of esophageal SCC patients (98 cases), who were followed-up for a long period (a mean follow-up duration of 45 months), were enrolled. The immunohistochemical staining showed the expression features of KLF4 expression and its clinical-pathological relationships. Our results showed that the expression of KLF4 protein in esophageal SCC was obviously lower than that of normal esophageal mucosa, indicating that KLF4 protein participates in the terminal differentiation of epithelial cells and plays an important role in regulating and maintaining the esophageal mucosal homeostasis and in the progression of esophageal SCC. Some studies have suggested that the downregulation or inactivation of KLF4 in many tumors may be due to the point mutation, loss of heterozygosity hypermethylation in the 5’ non-transcribed region of KLF4 gene.

Noti’s study also found that KLF4 could recruit histone deacetylases to the promoter region of the CD11d, and thereby inhibit gene expres-
sion, suggesting that KLF4 may also promote the occurrence of tumor through chromatin remodeling [22]. However, the mechanisms of the down regulation of KLF4 in esophageal SCC need to be further studied in the future. We also found that KLF4 was closely correlated with the lymphatic metastasis and the clinical stage of esophageal SCC patients.

The expression of KLF4 is low in the esophageal SCC patients with lymphatic metastasis or late stage disease, consistence with what was found in gastric and colorectal cancer [23, 24]. However, there was no difference in the KLF4 expression among tumor tissues with different differentiation degree, which was in consistence with the results observed in other gastrointestinal cancers [25]. Furthermore, we found that esophageal SCC patients with negative KLF4 expression showed a lower 5-year survival rate that patients with positive KLF4 expression, suggesting KLF4 may be used as a prognosis predictor of the esophageal SCC after radical resection.

In conclusion, our data demonstrated that KLF4 was highly expressed in the esophageal mucosa compared with esophageal SCC tissues. KLF4 expression is correlated with the lymphatic metastasis, the pathologic stage and prognosis of esophageal SCC patients. Our data indicate KLF4 may act as a tumor suppressor in esophageal SCC and the expression status of KLF4 may be considered as a prognosis predictor of the esophageal SCC patients.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Chuan-Gui Chen, Department of Esophageal Tumor, Tianjin Medical University Cancer Institute and Hospital, Huanhuixi Road, Ti-Yuan-Bei, Hexi District, Tianjin 300060, China. Tel: +86-22-23359930; Fax: +86-22-23359933; E-mail: chenchg6956@163.com

References

Prognosis of KLF 4 for esophageal squamous cell carcinoma patients


