

Original Article

Full-length spleen tyrosine kinase inhibits the invasion and metastasis of human laryngeal squamous cell carcinoma

Zhihai Li¹, Zhiyi Cai^{1,2}, Baohong Tao¹, Qiaozhi Jin¹

¹Department of Otolaryngology, Taizhou Municipal Hospital, Taizhou 318000, Zhejiang, China; ²The First Clinical College of Wenzhou Medical University, Wenzhou, China

Received October 15, 2015; Accepted November 28, 2015; Epub December 1, 2015; Published December 15, 2015

Abstract: Objective: This study aimed to investigate correlation between full-length spleen tyrosine kinase [SYK (L)] expression and clinical characteristics of laryngeal squamous cell carcinoma (LSCC), and explore effects of SYK (L) on invasion and metastasis of LSCC. Methods: The human laryngeal cancer Hep-2 cells with low SYK (L) expression were transfected with pIRES2-EGFP-SYK (L) vector and empty vector pIRES2-EGFP to generate Hep-2-SYK (L) cells and Hep-2-neo cells. The cell invasion and migration abilities were determined. Results: The SYK (L) positive expression rate in LSCC tissues was significantly lower than in vocal cord dysplasia tissues and normal laryngeal tissues ($P < 0.05$). There was a significant correlation between SYK (L) expression and LSCC T stage, histopathological grade and lymph node metastasis ($P < 0.05$). mRNA expression of SYK (L) in Hep-2-SYK (L) cells was significantly higher than in Hep-2-neo cells and Hep-2 cells ($P < 0.01$). The protein expression of SYK (L) in Hep-2-SYK (L) cells was markedly higher than in Hep-2-neo cells and Hep-2 cells ($P < 0.01$). The number of invasive cells was significantly lower in Hep-2-SYK (L) group than in Hep-2-neo group and Hep-2 group ($P < 0.01$). The average number of migrating cells in Hep-2-SYK (L) group also markedly reduced as compared to Hep-2-neo group and Hep-2 group ($P < 0.01$). Conclusion: The SYK (L) expression was down-regulated in LSCC, which was closely correlated with cancer growth and lymph node metastasis. SYK (L) up-regulation was able to inhibit the invasion and metastasis of LSCC, therefore suppressing tumor development. Thus, SYK (L) may be a potential target for the LSCC treatment.

Keywords: Full-length spleen tyrosine kinase, laryngeal cancer, invasion, metastasis

Introduction

Laryngeal cancer ranks the second most common malignancy among the head and neck cancers [1]. A variety of studies have shown that the onset and development of laryngeal cancer is a long-term, multi-step process of cell transformation mediated by various factors. The molecular mechanism underlying the cell transformation is the activation of oncogenes and inactivation of tumor suppressor genes. As such, gene therapy is promising to treat laryngeal cancer by changing or modifying the related genes and their expressions. Gene therapy has the advantage of removing cancer while maintaining the laryngeal function and improving the quality of life, and has become a focus in the biological treatment for laryngeal cancer [2]. It has been reported that about 30-40% laryngeal cancer patients die from its recurrence and metastasis. Therefore, finding new

targets related to tumor invasion and metastasis is a key to the success of gene therapy [3].

Spleen tyrosine kinase (SYK) is a type of non-receptor tyrosine kinase. Studies have shown that SYK is down-regulated in many malignant tumors. SYK plays important roles in the tumor invasion and metastasis, suggesting that SYK is an important target for the gene therapy of malignancies [4-6]. However, the SYK expression in laryngeal cancer and the roles of SYK in the invasion and metastasis of laryngeal cancer have never been reported. SYK has two isoforms, the full-length form [SYK (L)] and the truncated form [SYK (S)] that lacks 23 amino acids in the IDB region.

Studies have shown that the re-expression of SYK (L) but not SYK (S) in breast cancer and liver cancer can inhibit the invasion of these

SYK (L) inhibits laryngeal carcinoma

Table 1. Correlation between SYK (L) protein expression and clinicopathological characteristics of laryngeal cancer

Characteristics	Number	-	+	++	+++	Positive (n)	Positive rates (%)	χ^2
Types								
Supraglottis	17	8	3	3	3	9	0.52	11.79
Glottis	16	14	6	3	3	12	46.20	
Subglottis	5	3	1	1	0	2	40.00	
T stages								
T1	17	6	3	4	4	11	0.64	0.30
T2	25	15	6	2	2	10	0.40	
T3	6	4	1	1	0	2	0.33	
Histopathological grades								
High	19	7	5	4	3	12	0.63	13.89
Intermediate	16	9	3	2	2	7	0.44	
Low	13	9	2	2	1	1	0.30	
Lymph node metastasis								
Yes	11	8	2	1	0	3	0.27	9.02
No	37	17	8	6	6	20	0.54	

cancers, suggesting that SYK (L) might be a potential tumor suppressor gene [7]. To determine the role of SYK (L) in the invasion and metastasis of laryngeal cancer, the SYK (L) protein expression was detected in LSCC tissues by immunohistochemistry and the correlation of SYK (L) protein expression with the histopathological characteristics of LSCC was also evaluated. In addition, LSCC Hep-2 cells were transfected with SYK (L) gene to explore the effects of SYK (L) on invasion and migration of laryngeal cancer cells *in vitro*.

Materials and methods

Sample collection

Paraffin embedded tissues were collected from patients who underwent surgery in our department from January 2002 to May 2013. Patients had complete preoperative clinical data and did not receive prior treatment. There were 48 LSCC tissues, 38 vocal cord dysplasia tissues and 40 adjacent normal laryngeal tissues. All the samples were pathologically confirmed. The clinical characteristics of LSCC patients are shown in **Table 1** (clinical stage, T stage, histopathological grade and lymph node status).

Immunohistochemistry and grading

Rabbit anti-human SYK (L) antibody (Abcam; 1:100), goat anti-rabbit SP immunohistochemi-

cal staining kit, and DAB chromogenic kit (Fuzhou Maxim New Biotechnology Development Co., Ltd) were used in the present study. Immunohistochemistry was conducted according to the manufacturer's instructions. Cells with yellow or brown nuclei were counted as SYK (L)-positive cells. Three fields were randomly selected ($\times 400$) from each section and the staining intensity and positive rate of SYK (L) expression were determined. The staining intensity was determined as follows: no obvious staining, 0; light or faint yellow,

1; dark yellow or brown, 2; tan or dark brown, 3; The percentage of positive cells was expressed as the number of positive cells per 100 cells in each field. The positive rate score was determined as follows: positive cells < 10%, 0; 10-45%, 1; 45-70%, 2; > 70%, 3; The product of staining intensity score and positive rate score was calculated as the final score according to which tissues were divided as follows: 0-1, negative (-); 2, weakly positive (+); 3-4, positive (+); 5-6, strongly positive (+ + +). Scores were determined independently by two pathologists.

Effects of SYK (L) on the invasion and migration of Hep-2 cells

Human laryngeal carcinoma Hep-2 cells were purchased from Chinese Academy of Sciences Shanghai Cell Bank. pIRES2-EGFP-SYK (L) vector was constructed and verified as described in our previous studies [8]. Empty vector pIRES2-EGFP was purchased from Shanghai Generay Biotech. G418 was purchased from Gibco and Lipofectamine® 2000 from Invitrogen. High-purity total RNA extraction kit was purchased from Generay. RevertAid First Strand cDNA synthesis Kit was purchased from Fermentas and IQ SYBR Green Supermix was from Bio-Rad. GAPDH antibody was purchased from Abcam, Transwell invasion chambers from Costar and Matrigel from BD.

SYK (L) inhibits laryngeal carcinoma

Cell culture and SYK (L) gene transfection

Hep-2 cells were maintained in RPMI 1640 containing 10 ml/L heat-inactivated fetal bovine serum (FBS) at 37°C in an environment with 5% CO₂. One day before transfection, 1.0 × 10⁶ Hep-2 cells were seeded into six-well plates and cultured under antibiotic-free condition. Cells were 80-90% confluent at the time of transfection. Cells were divided into three groups: pIRES2-EGFP-SYK (L) transfection group, empty vector pIRES2-EGFP group and non-transfection group. Cells were transfected with pIRES2-EGFP-SYK (L) or pIRES2-EGFP in the presence of Lipofectamine® 2000. Two days after transfection, cells in six-well plates were passaged and maintained in T25 flasks. Three clones were obtained by G418 selection and expanded for 2 months. Cells of pIRES2-EGFP-SYK (L) group and pIRES2-EGFP group were named Hep-2-SYK (L) cells and Hep-2-neo cells, respectively. Cells of non-transfection group were still called Hep-2 cells.

Detection of mRNA and protein SYK (L) expression

Quantitative real time fluorescence polymerase chain reaction (Q-RT-PCR) was used to detect the mRNA expression of SYK (L). Briefly, total RNA was extracted from cells using Trizol and reversely transcribed into cDNA using the reverse transcription kit according to the manufacturer's instructions. Q-RT-PCR was carried out in a 25- μ l mixture. The forward and reverse primers for SYK (L) were 5'-TACCCAACATTACGCCAAGAT-3', and 5'-AGAATGCTCCACATCAACA-3', respectively. GAPDH gene was used as an internal control. The forward and reverse primers for GAPDH were 5'-AGAAGGCTGGGGCTCATTG-3' and 5'-AGGGCCATCCACAGTCTTC-3', respectively. RT-PCR was conducted as follows: 50.0°C, 3 min; 95.0°C, 3 min; followed by 40 cycles of 95.0°C 10 s, 60.0°C 20 s and 72.0°C 30 s. Non-transfection group was used as a control and housekeeping gene GAPDH as an internal control. Detection of each sample was repeated five times and the average CT value was obtained. The relative mRNA expression of SYK (L) was calculated with 2^{- Δ ACT} method [9].

Detection of SYK (L) protein expression was performed by Western blot assay. Proteins were extracted from cells using ActiveMotif kit

and the protein concentration was measured using PIERCE BCA protein assay kit. Then, 50 μ g of protein sample was mixed with 5 × SDS buffer, separated by polyacrylamide gel electrophoresis and then transferred onto PVDF membrane. The membrane was blocked and incubated with anti-SYK (L) antibody at room temperature for 2 h, followed by incubation with secondary antibody for 1 h at room temperature. Proteins were detected using the chemiluminescent kit. GAPDH was used as an internal reference, and the relative expression of SYK (L) was determined using Image J. The experiment was repeated thrice.

Determination of cell invasion and migration abilities

Cells in logarithmic growth phase were maintained in RPMI-1640 containing 1% FBS for 24 h for synchronization. Then, 100 μ l of diluted Matrigel (25 μ g) was added to the upper chamber of a 24-well transwell plate, which was then incubated at 37°C. The upper chamber was rinsed with serum-free RPMI-1640. Cells were collected using 0.25% trypsin after 24-h synchronization and diluted to 5 × 10⁶ cells/ml. Then, 100 μ l of cell suspension was added to the upper chamber and 600 μ l of culture medium to the lower chamber. Cells were grown at 37°C in an environment with 5% CO₂ for 24 h. Non-migratory cells were then removed from the upper chamber using a Q-tip. Transwell inserts were inverted, air-dried, and fixed with 4% paraformaldehyde for 30 min. Then, 500 μ l of 0.1% crystal violet solution was added to a 24-well plate into which the transwell inserts were transferred. After incubation at room temperature for 10 min, inserts were removed and rinsed with PBS. Four fields were chosen and imaged. Cells were counted to determine the invasion ability of cancer cells. The migration ability of cells was performed similarly as the invasion assay, except that there was no Matrigel coating. Experiments were repeated thrice.

Statistical analysis

Data were tested for normality using SPSS version 12.0. Data with abnormal distribution were subjected to normalization. All data were expressed as means \pm standard deviation ($\bar{x} \pm$ SD). ANOVA analysis was used for comparisons among groups. A value of $P < 0.05$ was considered statistically significant.

SYK (L) inhibits laryngeal carcinoma

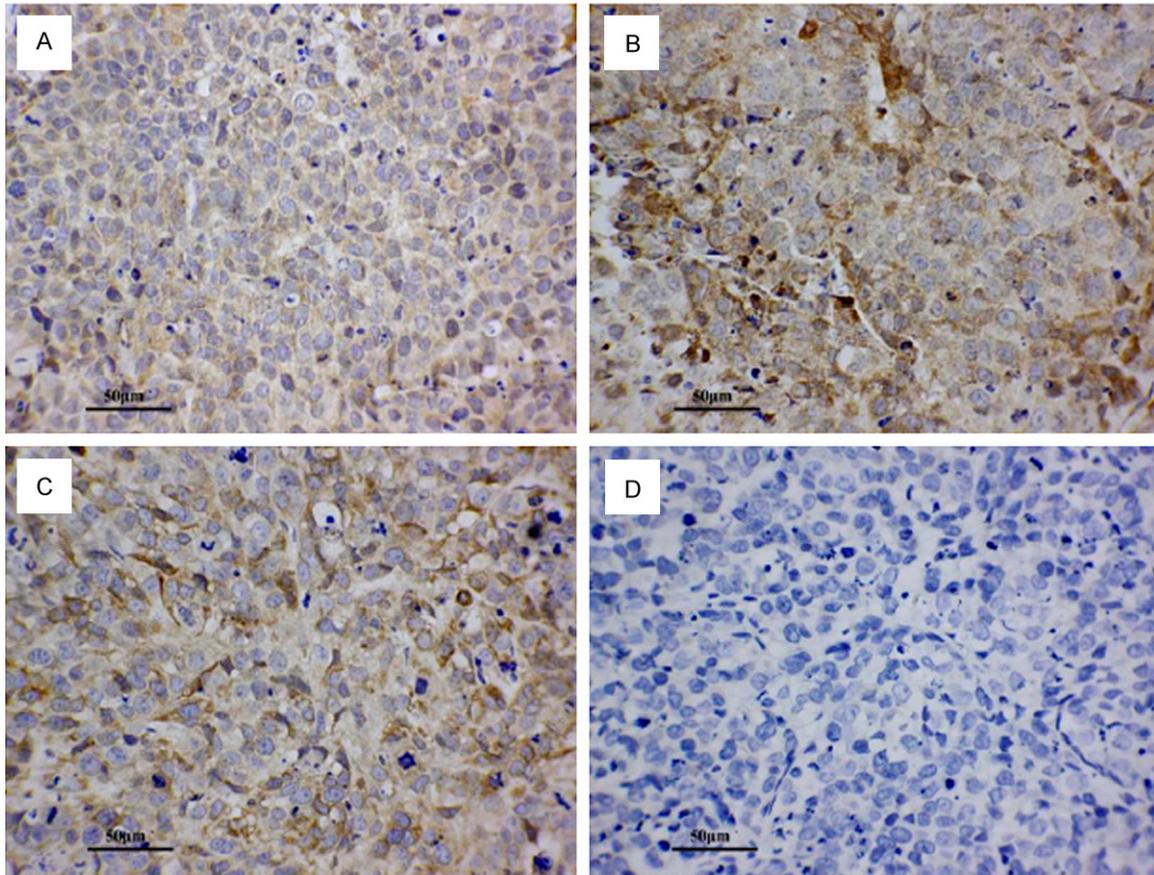


Figure 1. SYK (L) expression in various laryngeal tissues (SP \times 400). (A) LSCC tissues; (B) Vocal cord dysplasia tissues; and (C) Adjacent normal laryngeal tissues. (D) There was no SYK (L) positive expression in negative control.

Table 2. SYK (L) protein expression in different laryngeal tissues (immunohistochemistry)

Tissues	Number	-	+	++	+++	Positive (n)	Positive rate (%)
Laryngeal cancer	48	25	10	7	6	23	47.90
Dysplasia	38	9	13	9	7	29	76.30
Normal mucosal tissue	40	6	15	11	8	34	85.00

reduced in LSCC tissues as compared to vocal cord dysplasia tissues and adjacent normal laryngeal tissues ($\chi^2 = 14.89$, $P < 0.05$).

Correlation between SYK (L) protein expression and clinicopathological characteristics of LSCC

Results

Correlation between SYK (L) protein expression and clinicopathological characteristics of LSCC

Immunohistochemistry showed that both nuclear and cytosol staining (light yellow to brown) was found in LSCC tissues, vocal cord dysplasia tissues and adjacent normal laryngeal tissues. SYK (L) staining was not observed in the negative control samples. Only cells with nuclear staining were counted as SYK (L)-positive cells (**Figure 1**). The SYK (L) protein expression in various tissues is listed in **Table 2**. SYK (L) protein expression significantly

As shown in **Table 1**, the SYK (L) protein expression was significantly correlated with the T stage, histopathological grade and lymph node metastasis ($\chi^2 = 14.35$, 13.89 and 9.02 , respectively; all $P < 0.05$).

Effect of SYK (L) on the invasion and migration abilities of Hep-2 cells

Relative mRNA expression was expressed as $2^{-\Delta\Delta CT}$. The SYK (L) mRNA expression was 30.197 ± 0.075 , 3.092 ± 0.023 , and 1.005 ± 0.021 in Hep-2-SYK (L) cells, Hep-2-neo cells, and Hep-2 cells, respectively. Statistical analysis showed the mRNA expression of SYK (L) in

SYK (L) inhibits laryngeal carcinoma

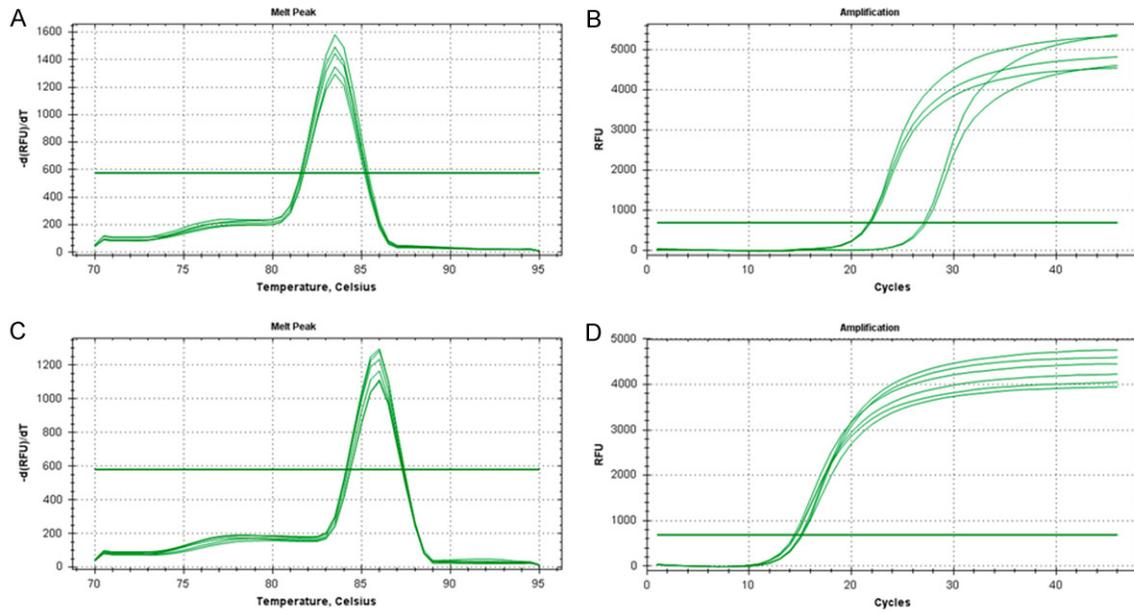


Figure 2. mRNA expressions of SYK (L) and GAPDH in Hep-2 cells. A. Melting curves for SYK (L); B. Amplification curve for SYK (L); C. Melting curve for GAPDH; D. Amplification curve for GAPDH.

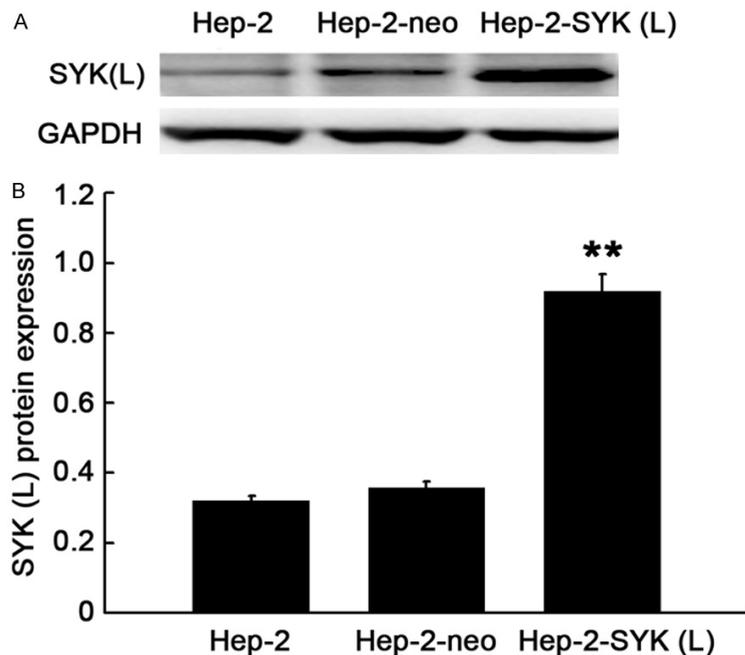


Figure 3. SYK (L) protein expression in cells (Western blot assay). A. SYK (L) protein expression was detected by Western blot assay using GAPDH as an internal control. Lane 1, Hep-2 cells; Lane 2, Hep-2-neo cells; Lane 3, Hep-2-SYK (L) cells. B. Relative protein expression of SYK (L) protein in each group. SYK (L) expression in Hep-2-SYK (L) group was significantly higher than in Hep-2-neo and Hep-2 groups, ** $P < 0.01$.

Hep-2-Syk (L) cells was significantly lower than in Hep-2-neo cells and Hep-2 cells ($F = 7.85$, P

< 0.01). There was no significant difference in SYK (L) mRNA expression between Hep-2-neo cells and Hep-2 cells ($F = 2.39$, $P > 0.05$) (Figure 2).

As determined by Western blot assay, the relative expression of SYK (L) protein in Hep-2-SYK (L), Hep-2-neo and Hep-2 cells was 0.921 ± 0.038 , 0.358 ± 0.034 and 0.319 ± 0.037 , respectively (Figure 3). Statistical analysis showed that the relative expression of SYK (L) protein in Hep-2-SYK (L) cells was significantly higher than in Hep-2-neo cells and Hep-2 cells ($F = 7.01$, $P < 0.01$, Figure 3). However, there was no significant difference in SYK (L) protein expression between Hep-2-neo cells and Hep-2 cells ($F = 3.01$, $P > 0.05$).

As shown in Figure 4, the average number of invasive cells was 156.02 ± 24.01 in Hep-2-SYK (L) group, which was significantly smaller than in

SYK (L) inhibits laryngeal carcinoma

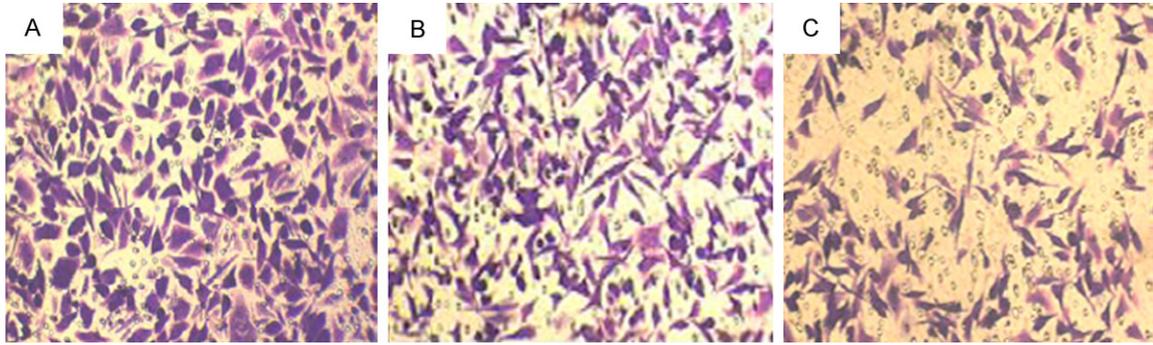


Figure 4. Invasion ability of different laryngeal carcinoma cells ($\times 400$). A. Hep-2 cells; B. Hep-2-neo cells; C. Hep-2-SYK (L) cells.

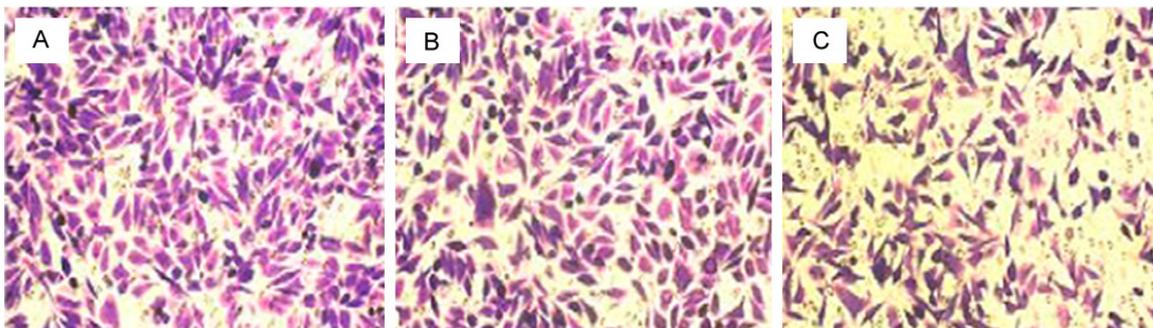


Figure 5. Migration ability of different laryngeal carcinoma cells ($\times 400$). A. Hep-2 cells; B. Hep-2-neo cells; C. Hep-2-SYK (L) cells.

Hep-2-neo group (331.04 ± 19.45) and Hep-2 group (328.06 ± 18.27) ($F = 8.24$, $P < 0.01$), but there was no marked difference between Hep-2-neo group and Hep-2 group ($F = 3.20$, $P > 0.05$). As shown in **Figure 5**, the average number of migrating cells was 236.04 ± 23.02 in Hep-2-SYK (L) group, which was significantly lower than in Hep-2-neo group (536.04 ± 25.65) and Hep-2 group (506.04 ± 28.13) ($F = 9.02$, $P < 0.01$), but there was no significant difference between Hep-2-neo group and Hep-2 group ($F = 2.92$, $P > 0.05$). These results indicated that up-regulation of SYK (L) expression inhibited the invasion and migration of Hep-2 cells.

Discussion

Tumor invasion and metastasis plays an important role in the long-term survival of patients with laryngeal cancer. To understand the mechanism underlying the tumor invasion and metastasis is crucial for the clinical therapy of laryngeal cancer [10]. Non-receptor tyrosine kinases are a class of protein kinases that cat-

alyze the transfer of 7-phosphate from ATP to a tyrosine residue in a protein. They can catalyze the phosphorylation of multiple tyrosine residues in proteins and play critical roles in the cell growth, proliferation and differentiation [11]. SYK is a non-receptor tyrosine kinase that can activate signal transduction pathways through binding its N-terminal SH2 domains with the tyrosine activation motif on the immune receptors. SYK particularly activates the signal transduction in lymphocytes and immune cells and plays an important role in the tyrosine phosphorylation regulation of multiple proteins in cells [12]. Recent studies show that SYK expression is down-regulated in breast cancer, liver cancer, nasopharyngeal cancer, stomach cancer, colon cancer, pancreatic cancer, prostate cancer and lung cancer, and SYK is able to inhibit the cell division, proliferation and tumor formation in a variety of solid malignant tumors. SYK down-regulation plays an important role in the development of a variety of malignancies and is especially associated with tumor invasion and metastasis [13]. These findings strongly indicate that SYK is a potential

SYK (L) inhibits laryngeal carcinoma

tumor suppressor gene related to the tumor invasion and metastasis, and may become an important target in the therapy of cancers [14, 15].

SYK protein has two isomers, including SYK (L) and SYK (S). SYK (L) has a nuclear localization signal that regulates the translocation of SYK (L) from the cytoplasm to the nucleus. Nuclear SYK (L) may act as a tumor suppressor. SYK (L) translocation into the nucleus is necessary for its tumor inhibition effect [7]. Therefore, the expression of SYK, particularly the SYK (L), should be detected to determine the role of SYK in the occurrence and development of cancers [13]. Hence, in this study, to investigate whether SYK (L) is involved in the occurrence and development of laryngeal cancer, SYK (L)-specific antibody was used to detect the nuclear expression of SYK (L) protein in LSCC tissues. Immunohistochemistry indicated that SYK (L) protein expression was significantly down-regulated in LSCC as compared to vocal dysplasia tissues and adjacent normal laryngeal tissues, suggesting that SYK (L) may be involved in the occurrence and development of LSCC. Further studies indicated that SYK (L) protein expression was not significantly correlated with clinical pathology parameters in LSCC, but was markedly associated with clinical stage, histopathological grade and lymph node metastasis, suggesting the role of SYK (L) in the invasion and metastasis of laryngeal cancer.

To further clarify the role of SYK (L) in the invasion and metastasis of laryngeal cancer, laryngeal carcinoma Hep-2 cells with a low SYK (L) expression were used. Cells were transfected with EGFP-SYK (L) vector [8] or pIRES2-EGFP empty vector and G418 was employed selected positive cells: Hep-2-SYK (L) cells with high SYK (L) expression and Hep-2-neo cells with low SYK (L) expression. Results showed the SYK (L) mRNA and protein expressions in Hep-2-SYK (L) cells were significantly higher than in Hep-2-neo cells and Hep-2 cells. To determine cell invasion and migration abilities in these groups, Transwell chamber and Matrigel were used to establish an *in vitro* invasion and migration model. Transwell chamber is similar to Boyden chamber. It contains two chambers separated by a permeable polycarbonate membrane that allows the migration of tumor cells. Matrigel is an extracellular matrix from rat sarcoma and is

very similar to the basement membrane. It gels at 37°C and mimics the basement membrane. The upper Transwell chamber was coated with Matrigel and the number of cells migrated through the membrane can be counted to reflect the invasiveness of tumor cells. In the absence of Matrigel, the migration ability of tumor cells can be determined [16]. *In vitro* cell invasion and migration assays showed that Hep-2-SYK (L) cells had significantly lower invasion and migration abilities than did Hep-2-neo cells and Hep-2 cells. These suggest that SYK (L) may act as a negative regulator to inhibit the invasion and metastasis of LSCC. Up-regulation of SYK (L) expression in LSCC can inhibit tumor invasion and metastasis, thereby inhibiting tumor occurrence and development.

It's still unclear how SYK (L) inhibits the invasion and metastasis of cancer cells. During the invasion and metastasis, degradation of extracellular matrix (ECM) triggers the tumor invasion into normal tissues and subsequent metastasis. Matrix metalloproteinases (MMPs) are the most important enzymes responsible for the degradation of ECM, especially type IV collagen [17]. Studies have shown that up-regulation of SYK (L) expression decrease the expression of MMPs (such as MMP2 and MMP9) in breast cancer and liver cancer, and reduce the degradation of ECM and basement membrane, thereby inhibiting tumor invasion and metastasis [18-20]. In-depth investigation is needed to determine whether SYK (L) inhibits the invasion and metastasis of LSCC by inhibiting MMP or via other mechanisms.

Taken together, our results indicate that the SYK (L) expression was down-regulated in LSCC, which was closely correlated with cancer growth and lymph node metastasis. SYK (L) up-regulation was able to inhibit the invasion and metastasis of LSCC, therefore suppressing tumor development. Thus, SYK (L) may be a potential target for the LSCC treatment. However, further studies are required to investigate the effects of SYK (L) on the LSCC invasion and metastasis and the underlying mechanism, which may help to identify new markers for the prediction of LSCC prognosis as well as new targets for the gene therapy of LSCC.

Acknowledgements

This study was supported by Taizhou Science and Technology Project (14SF05) and Zhejiang

SYK (L) inhibits laryngeal carcinoma

Province Chinese Medicine Research Foundation (2013ZA132).

Disclosure of conflict of interest

None.

Address correspondence to: Zhiyi Cai, Department of Otolaryngology, The First Clinical College of Wenzhou Medical University, Wenzhou, China. E-mail: caizy008@tom.com

References

- [1] Dawson MA and Kouzarides T. Cancer epigenetics: from mechanism to therapy. *Cell* 2012; 150: 12-27.
- [2] Zhang HL, Wang XF, Wen SX, Gao W and Wang BQ. The role of metastasis-associated gene 1 in laryngeal squamous cell carcinoma invasion and metastasis. *Chin J Exp Sur* 2011; 28: 301-303.
- [3] Yao XB, Wang XX and Zhang SQ. The effect of COX-2 antisense oligodeoxynucleotide on the inhibition of malignancy in laryngeal cancer. *Xi'an Jiaotong Univ J (Med Sci)* 2009; 4: 233-236.
- [4] Prinos P, Garneau D, Lucier JF, Gendron D, Couture S, Boivin M, Brosseau JP, Lapointe E, Thibault P, Durand M, Tremblay K, Gervais-Bird J, Nwilati H, Klinck R, Chabot B, Perreault JP, Wellinger RJ and Elela SA. Alternative splicing of SYK regulates mitosis and cell survival. *Nat Struct Mol Biol* 2011; 18: 673-679.
- [5] Mocsai A, Ruland J and Tybulewicz VL. The SYK tyrosine kinase: a crucial player in diverse biological functions. *Nat Rev Immunol* 2010; 10: 387-402.
- [6] Jin QZ, Yan C, Tao BH, Li ZH and Cai ZY. 5-Aza-2-deoxycytidine induces the demethylation of SYK promoter in nasopharyngeal carcinoma cells. *Chin J Cell Biol* 2013; 34: 865-871.
- [7] Wang L, Wang JP, Song XM and Lan P. The distribution of two SYK isoforms in breast cancer cells. *Chin J Exp Sur* 2006; 23: 1186-1188.
- [8] Li ZH, Cai ZY, Tao BH and Jin QZ. Construction of PIRES2-EGFP-SyK (L) vector and establishment of SYK (L)-expressing stable laryngeal cancer Hep-2 cell line. *Chin J Health Lab Tech* 2013.
- [9] Lee JJ, Ko E, Cho J, Park HY, Lee JE, Nam SJ, Kim DH and Cho EY. Methylation and Immunoexpression of p16 (INK4a) Tumor Suppressor Gene in Primary Breast Cancer Tissue and Their Quantitative p16 (INK4a) Hypermethylation in Plasma by Real-Time PCR. *Korean J Pathol* 2012; 46: 554-561.
- [10] Banno K, Kisu I, Yanokura M, Masuda K, Ueki A, Kobayashi Y, Susumu N and Aoki D. Epigenetics and genetics in endometrial cancer: new carcinogenic mechanisms and relationship with clinical practice. *Epigenomics* 2012; 4: 147-162.
- [11] Orth P, Weimer A, Kaul G, Kohn D, Cucchiaroni M and Madry H. Analysis of novel nonviral gene transfer systems for gene delivery to cells of the musculoskeletal system. *Mol Biotechnol* 2008; 38: 137-144.
- [12] Song XT, Dan BE and Hu J. Construction of eukaryotic expression vector for SYK gene and study on the effect of SYK gene on breast cancer cell invasion. *Chin J Immunol* 2008; 24: 698-702.
- [13] Yang JL, Hong J and Liao YD. The effect of full-length spleen tyrosine kinase on the inhibition of liver cancer cell invasion and metastasis. *Sun Yat-sen Univ J (Med Sci Ed)* 2012; 33: 23-27.
- [14] Schmitte R, Tipold A, Stein VM, Schenk H, Flieshardt C, Grothe C and Haastert K. Genetically modified canine Schwann cells—In vitro and in vivo evaluation of their suitability for peripheral nerve tissue engineering. *J Neurosci Methods* 2010; 186: 202-208.
- [15] Koo TY, Kim YJ, Yang WS, Park JS, Han NJ, Lee JM and Park SK. Mycophenolic acid regulates spleen tyrosine kinase to repress tumour necrosis factor-alpha-induced monocyte chemotactic protein-1 production in cultured human aortic endothelial cells. *Cell Biol Int* 2013; 37: 19-28.
- [16] Xiao YH, Li XH, Tan T, Liang T, Yi H, Li MY, Zeng GQ, Wan XX, Qu JQ, He QY, Li JH, Chen Y and Xiao ZQ. Identification of GLIPR1 tumor suppressor as methylation-silenced gene in acute myeloid leukemia by microarray analysis. *J Cancer Res Clin Oncol* 2011; 137: 1831-1840.
- [17] Cherblanc F, Chapman-Rothe N, Brown R and Fuchter MJ. Current limitations and future opportunities for epigenetic therapies. *Future Med Chem* 2012; 4: 425-446.
- [18] Liu M, Sun YN, Jiao H, Tian LL and Guo YL. [Role of siRNA mediated matrix metalloproteinase-2 gene silencing in the inhibition of invasion and growth of laryngeal cancer cells]. *Zhonghua Er Bi Yan Hou Tou Jing Wai Ke Za Zhi* 2008; 43: 612-616.
- [19] Choe JY, Yun JY, Nam SJ and Kim JE. Expression of c-Met Is Different along the Location and Associated with Lymph Node Metastasis of Head and Neck Carcinoma. *Korean J Pathol* 2012; 46: 515-522.
- [20] Martinez EC, Wang J, Gan SU, Singh R, Lee CN and Kofidis T. Ascorbic acid improves embryonic cardiomyoblast cell survival and promotes vascularization in potential myocardial grafts in vivo. *Tissue Eng Part A* 2010; 16: 1349-1361.