# Competing endogenous RNA network analysis of CD274, IL-10 and FOXP3 co-expression in laryngeal squamous cell carcinoma

JUAN SUN[1,2], MENG LIAN[1], HONGZHI MA[1], RU WANG[1], ZHIHONG MA[1], HAIZHOU WANG[1], JIE ZHAI[1], LINGZHAO MENG[3], LING FENG[1], YUNFEI BAI[2], XIAOBO CUI[2] and JUGAO FANG[1]

[1]Department of Otorhinolaryngology Head and Neck Surgery, Beijing Tongren Hospital, Capital Medical University, Beijing 100730; [2]Department of Otorhinolaryngology, Inner Mongolia Medical University Affiliated Hospital, Hohhot, Inner Mongolia 010050; [3]Department of Otorhinolaryngology Head and Neck Surgery, Beijing Anzhen Hospital, Capital Medical University, Beijing 100730, P.R. China

Received August 7, 2016; Accepted June 15, 2017

DOI: 10.3892/mmr.2017.8307

**Abstract.** Laryngeal squamous cell carcinoma (LSCC) is one of the most common types of head and neck malignant tumor; however, there is a lack of effective molecular targets for therapy. The present study detected the expression of three immunity-associated molecules [forkhead box (FOX)3, interleukin (IL)-10 and cluster of differentiation (CD)274] in 133 LSCC samples using immunohistochemistry (IHC); subsequently, the association between their expression and the clinical characteristics of LSCC were analyzed. Spearman's rank correlation method, Kaplan-Meier and Cox regression model were used to analyze the correlations of the three proteins and their clinical significance. StarBase and miRTarBase databases were used to establish the competitive endogenous (ce)RNA network of the three molecules. IHC demonstrated that the positive expression rates of FOXP3, IL-10 and CD274 were 68.4, 73.7 and 58.6% in 133 LSCC samples, respectively. In addition, it was identified that the expression of the three proteins was closely correlated with the clinical characteristics of LSCC, including lymph node metastasis and prognosis (P<0.05). There was also a significant association of co-expression between any two proteins (P<0.001). Furthermore, the expression levels of FOXP3, IL-10 and CD274 were negatively associated with the survival rate of patients with LSCC (P<0.05). The results of a Cox regression model suggested that the three proteins were prognostic risk factors for LSCC (P<0.05). The ceRNA network revealed that 10 microRNAs (miRs; including miR-16-5p and miR-214-3p), 123 long non-coding RNAs (including X-inactive specific transcript, H19 and metastasis associated lung adenocarcinoma transcript 1) and 408 circular RNAs (including ATP-binding cassette subfamily C member 1 hsa_circ_001569 and ISY1 splicing factor homolog hsa_circ_001859) may regulate the expression of FOXP3, IL-10 and CD274. The data generated from the present study may increase the understanding of the immune escape mechanisms of LSCC and may be beneficial for the development of a specific immunotherapy.

*Correspondence to:* Professor Jugao Fang, Department of Otorhinolaryngology Head and Neck Surgery, Beijing Tongren Hospital, Capital Medical University, 88 Majiabao Street East, Beijing 100730, P.R. China
E-mail: jugaofang@outlook.com

*Key words:* laryngeal squamous cell carcinoma, forkhead box P3$^+$ regulatory T cells, cluster of differentiation 274, interleukin-10, co-expression, competitive endogenous RNA network

## Introduction

Laryngeal squamous cell carcinoma (LSCC) is one of the most common types of head and neck squamous cell carcinoma, the incidence of which continues to rise. Despite the continuous improvement in surgical, radiotherapeutic and chemotherapeutic techniques, an efficient prolongation of the survival rate of patients with LSCC has yet to be achieved (1,2). The 5-year survival rate of patients with LSCC is ~30% (3). Due to the specificity of the anatomic location of the throat, patients with LSCC not only have to endure painful surgery, but they also experience postoperative impairment and loss of throat function. Subsequent physical and psychological afflictions have a serious impact on the outcome of postoperative rehabilitation and the quality of life of these patients (4). In recent years, cancer immunotherapy has gradually become a subject undergoing intense study in cancer research and has achieved satisfactory therapeutic efficacy in the clinical treatment of cancers (5). However, compared with other types of cancer treatment, progress in the development of LSCC-related immunotherapy has been fairly slow. One of the main reasons behind this slow progress is the lack of effective molecular targets for immunotherapy. Therefore, it is necessary to determine the immunoregulatory mechanisms underlying LSCC and to identify factors that are critical for the success of immunotherapy.

Regulatory T cells (Tregs) comprise a subpopulation of T cells with unique immunomodulatory functions. Tregs are able to suppress immune responses and therefore serve important roles in the maintenance of immune homeostasis, tumor immunity and transplant tolerance (6). Forkhead

box P3 (FOXP3) is a characteristic Treg surface marker, and a key factor for Treg development and maintenance of function (7). Franco *et al* (8) silenced the expression of FOXP3, to reduce the infiltration of $FOXP3^+$ Tregs, and identified that melanoma growth was significantly inhibited, and that the immunosuppressive microenvironment was restored. Treg-secreted cytokines, including interleukin (IL)-10, serve an important role in the function of Tregs. These cytokines may initiate inhibitory signals and suppress the activity of functional T lymphocytes, which inhibits the tumor immune response (9). In addition, it has previously been demonstrated that certain costimulatory molecules exhibit an important influence on the role of Tregs in cancer immune evasion. For example, the costimulatory molecule cluster of differentiation (CD)274 (also known as programmed death-ligand 1) is abnormally expressed in various tumor tissues. CD274 is an important molecule that mediates cancer immune evasion (10). The expression status of CD274 is closely associated with the prognosis of various tumors, including ovarian cancer, bladder cancer and pancreatic cancer (11-13). These previous findings suggest that Tregs positive for the tumor immunity-associated molecules IL-10, CD274 and FOXP3 serve an important role in tumor development and progression. However, correlations among the expression of these three molecules, and the association between these three molecules and the prognosis of LSCC remain to be elucidated.

Numerous studies have demonstrated that the coding and non-coding gene regulatory associations are extensive in tumor immunity, and that they serve essential roles in tumor development and progression. Liang *et al* (14) indicated that microRNA (miR)-22 was able to impair antitumor immunity through targeting p38. In addition, various regulatory associations exist between coding and non-coding genes in complex gene regulatory networks. For instance, miRNAs bind to sites in the 3'-termini of their target genes to regulate gene expression, and long non-coding RNAs (lncRNAs) or circular RNAs (circRNAs) act as competitive endogenous RNAs (ceRNAs) to regulate target gene expression (15). Therefore, it is useful to identify the non-coding genes that regulate IL-10, FOXP3 and CD274 in order to understand the molecular mechanism underlying tumor immunity in LSCC.

In the present study, immunohistochemistry (IHC) was employed to examine the expression of IL-10, CD274 and FOXP3 in tumor samples derived from 133 patients with LSCC. Subsequently, associations between the expression of IL-10, CD274 and FOXP3 and the clinical characteristics of LSCC were analyzed. In addition, Spearman's rank correlation method was used to analyze the correlations among IL-10, CD274 and FOXP3 protein expression. The Kaplan-Meier (KM) method and the Cox regression model were employed in combination with the clinical data to analyze the effects of the expression of these three proteins on the survival rate of patients with LSCC. The present study aimed to increase understanding regarding the immune evasion mechanisms of LSCC and to aid the development of LSCC immunotherapy.

## Materials and methods

*Collection of LSCC samples*. The present study was approved by the Clinical Research Ethics Committee of Beijing Tongren Hospital, Capital Medical University (Beijing, China), and written informed consent was obtained from the patients. This investigation was conducted according to the Declaration of Helsinki. A total of 133 LSCC samples were collected for use in the present study; these samples consisted of paraffin-embedded tissue blocks obtained from patients with primary LSCC that underwent surgical treatment between January 2012 and July 2013 at Beijing Tongren Hospital affiliated with Capital Medical University. The patients received a definite pathological diagnosis, and complete clinical information was available. In addition, none of the patients received preoperative radiotherapy or chemotherapy. The inclusion criteria were established based on tumor size and location, invasion status of the primary tumors and the status of lymph node metastasis. The LSCC samples were subjected to histopathological classification and tumor stage evaluation, which were conducted according to the Koppel standards and the tumor-node-metastasis (TNM) staging system, respectively. All pathological diagnoses were verified by senior pathologists through a review of the tissue sections (Table I). The normal laryngeal mucosa tissue and vocal cord polyp tissue were derived from the patients with recurrent cysts and vocal cord polyps who had undergone surgery between January 2012 and July 2013 at Beijing Tongren Hospital affiliated with Capital Medical University (Beijing, China).

*Hematoxylin and eosin (H&E) staining*. Phosphate-buffered saline (PBS) was used to wash the sections of the normal and tumor tissues for 5 min. The nucleus was stained with hematoxylin for 5 min and was differentiated with hydrochloric acid and ethanol for 30 sec. Tap water and distilled water was used to wash the sections for 1 min. Eosin staining was performed for 30 sec. The slices were washed with a 70% ethanol for 30 sec, a 90% ethanol rinse for 30 sec, a 95% ethanol rinse for 30 sec, two 100% ethanol rinses for 2 min each time and xylene clearing twice for 5 min each time. Finally, an inverted light microscope was used to observe the stained sections.

*IHC staining*. Paraffin-embedded tissue sections (4 $\mu$m) were deparaffinized and rehydrated according to conventional methods. The tissue sections were washed in PBS and were subjected to antigen retrieval. EDTA buffer (pH 9.0) was used to retrieve antigen as a repair solution. The antigen was placed in an autoclave for 2 min. Once cool, the tissue sections were rinsed with distilled water and then stored in Tris Buffered Saline (pH 7.4). Following antigen retrieval, each section was incubated with 50 $\mu$l 3% $H_2O_2$ at room temperature for 10 min to block endogenous peroxidase activity. Anti-CD274 antibody (NBP1-76769; 1:200) and anti-FOXP3 antibody (NB100-39002; 1:200) were purchased from Novus Biologicals, LLC (Littleton, CO, USA), and the anti-IL-10 antibody (BA1201-1; 1:200) was purchased from Wuhan Boster Biological Technology, Ltd. (Wuhan, China). Following washing with PBS, the primary antibodies (1:200 dilution) were added dropwise to the sections, followed by incubation at 4˚C overnight. Subsequently, the sections were washed again with PBS. According to the protocol of an IHC two-step detection kit (PV-6001; OriGene Technologies, Beijing, China), the section was incubated with 100 $\mu$l

Table I. General information and clinical characteristics of patients with LCSS.

| Variable | Cases | % |
|---|---|---|
| Gender | | |
| Male | 128 | 96.2 |
| Female | 5 | 3.8 |
| Age | | |
| <60 years | 48 | 36.1 |
| ≥60 years | 85 | 63.9 |
| Tumor location | | |
| Supraglottic | 30 | 22.6 |
| Glottic | 101 | 75.9 |
| Subglottic | 2 | 1.5 |
| TNM | | |
| T1/T2 | 91 | 68.4 |
| T3/T4 | 42 | 31.6 |
| LNM | | |
| No | 98 | 73.7 |
| Yes | 35 | 26.3 |
| Clinical stage | | |
| I | 48 | 36.1 |
| II | 38 | 28.6 |
| III | 26 | 19.5 |
| IV | 21 | 15.8 |
| Course of disease | | |
| ≤6 months | 53 | 39.8 |
| 6-12 months | 28 | 21.1 |
| ≥12 months | 52 | 39.1 |
| Pathological stage (differentiation) | | |
| Well | 52 | 39.1 |
| Moderate | 53 | 39.8 |
| Poor | 28 | 21.1 |

LNM, lymph node metastasis; LSCC, laryngeal squamous cell carcinoma; TNM, tumor-node-metastasis classification.

antibodies at room temperature for 30 min. Subsequently, the sections were exposed to the chromogenic substrate 3,3'-diaminobenzidine (OriGene Technologies) and observed under a light microscope for 3-10 min. The appearance of brownish-yellow particles indicated positive staining. Following washing with distilled water, the sections were counterstained with hematoxylin at room temperature for 10 min, differentiated in 0.1% hydrochloric acid and rinsed with tap water. The sections were then dehydrated in a graded series of alcohol, cleared in xylene and mounted in neutral gum. Negative controls were established by replacing the primary antibodies with PBS. Gastric cancer tissues from the tissue bank of Beijing Tongren Hospital were used as positive controls. Uniformly stained sections that exhibited clear tissue structure were subjected to semi-quantitative analysis. The staining results were evaluated by two pathologists using the double-blind method.

*Assessment of the IHC results*. The IHC results were scored based on the semi-quantitative immunoreactivity scoring system developed by Remmele and Stegnerde (16). For each tissue section, 5 areas within the tumor were randomly selected under x200 magnification. The percentage of cells in the selected areas that exhibited positive cytoplasmic/plasma membrane staining was calculated and assigned a score between 0 and 4, as follows: 0, No cells exhibited positive staining; 1, 1-10% of the cells exhibited positive staining; 2, 11-50% of the cells exhibited positive staining; 3, 51-75% of the cells exhibited positive staining; and 4, >75% of the cells exhibited positive staining. In addition, the staining intensity was scored as follows: 0, No staining; 1, weak intensity (light yellow); 2, moderate intensity (brownish yellow); and 3, strong intensity (dark brown). The two scores were multiplied together to generate IHC scores, and the samples were graded according to their IHC scores as follows: Negative (-), 0; weakly positive (+), 1, positive (++), 2-3; and strongly positive (+++), >3. This semi-quantitative scoring system was used to determine staining in tumor-infiltrating lymphocytes. Initially, the distribution characteristics of the positive cells in whole tissue sections were examined under low magnification (x40), which allowed cancer foci to be distinguished from tumor stroma. To quantify the tumor-infiltrating lymphocytes, the 5 areas richest in tumor-infiltrating lymphocytes were selected from each tissue section under low magnification (x40), and the tumor-infiltrating lymphocytes were counted under a high-power field (x400). The number of tumor-infiltrating lymphocytes was expressed as the average number of cells in the five fields of view. The distribution characteristics of the CD274-, FOXP3- and IL-10-positive and -negative cells in whole tissue sections were examined under low magnification. The appearance of brownish-yellow precipitate in the cytoplasm and/or the cell membrane indicated positive staining. The staining results were evaluated using a semi-quantitative scoring system as described above. The infiltrating lymphocytes were classified into the following four categories based on the IHC staining scores for CD274, FOXP3 and IL-10: (-), <1; (+), 1-5; (++), 6-19; and (+++), >20. In addition, (-) and (+) indicated low expression, whereas (++) and (+++) indicated high expression. These scores were determined independently by two pathologists and were averaged and subjected to statistical analysis.

*KM survival rate analysis and Cox regression model*. The KM model was used to assess the effects of FOXP3, IL-10 and CD274 expression on the survival rate of patients with LSCC. Due to the advantages associated with the KM model, including the accurate assessment of the survival rates and the median survival time, and the pairwise comparison of survival time distributions between numerous groups, this model has been widely adopted. In addition, the Cox regression model was employed to analyze and compare the effects of various factors on the survival time of patients with LSCC (17). The Cox regression model is commonly used to analyze the prognosis of tumors and other chronic diseases. The equation used in the Cox regression analysis is as follows: $h(t/X)=h0(t)\ \exp(\beta1\ X1 + \beta2\ X2 + ... + \beta p\ Xp)$, where $h(t/X)$ is the baseline hazard function, namely, the hazard function at time t when all independent variables assume a value of zero. The influencing

factors (variables) are denoted by X1, X2, ..., Xp, whereas the regression coefficients are represented by β1, β2, ..., βp.

*Identification of miRNA, lncRNA and circRNA that regulate FOXP3, IL-10 and CD274*. To identify the potential mechanism underlying the correlations among the three genes, the miRTarBase database (http://mirtarbase.mbc.nctu.edu.tw/) was used to predict the miRNAs of FOXP3, IL-10 and CD274. Based on the miRNAs, StarBase version 2.0 database (http://starbase.sysu.edu.cn/) was used to identify the lncRNAs and circRNAs that regulate the miRNAs. These interactions were confirmed by experiments including western blot analysis and PCR. Based on these interactions, Cytoscape software (http://www.cytoscape.org/) was used to establish the target gene-miRNA-lncRNA and the target gene-miRNA-circRNA networks.

*Statistical analysis*. The data were statistically analyzed using SPSS software version 21.0 (IBM Corp., Armonk, NY, USA). Comparisons between the two groups were performed using the $\chi^2$ test. A correlation analysis was conducted using Spearman's rank correlation test. The KM method and log-rank test were used to analyze survival rates. The association between the expression levels of CD274, Foxp3 and IL-10 and various clinicopathological characteristics was subjected to a rank transformation and entered into Spearman rank correlation test tests. P<0.05 was considered to indicate a statistically significant difference.

## Results

*CD274, FOXP3 and IL-10 expression in normal laryngeal mucosa, vocal cord polyp and LSCC*. The hematoxylin and eosin staining of LSCC tissues is presented in Fig. 1A. The results of IHC demonstrated that the positive expression rate of CD274 was 58.6% (Fig. 1B), the positive expression rate of IL-10 was 73.7% (Fig. 1C) and the positive expression rate of FOXP3 was 68.4% (Fig. 1D) in the 133 LSCC tissues. However, their expression in normal laryngeal mucosa and vocal cord polyp tissues was negative or at a low level (Table II).

*Analysis of the correlations between the clinicopathological features and CD274, FOXP3 and IL-10 expression*. In patients with LSCC, CD274 expression demonstrated no correlation with tumor location, TNM stage, clinical stage or pathologic grade (P>0.05). However, CD274 expression was significantly correlated with lymph node metastasis, disease course prior to hospital admission, the age of the patients and prognosis (P<0.05). FOXP3 expression appeared to exhibit no correlation with the age of the patients, tumor location, T stage, clinical stage or pathological grade (P>0.05). However, FOXP3 expression exhibited clear correlations with disease course prior to hospital admission, lymph node metastasis and prognosis (P<0.05). IL-10 expression demonstrated correlations with all factors, with the exception of patient age and tumor location (P<0.05; Table III).

*Correlation of CD274, FOXP3 and IL-10 expression in LSCC*. The results of a Spearman analysis demonstrated that there was a positive expression correlation between CD274 and FOXP3 ($r_s$=0.767, P<0.001), between CD274 and IL-10 ($r_s$=0.640, P<0.001), and between IL-10 and FOXP3 ($r_s$=0.637, P<0.001; Fig. 2).

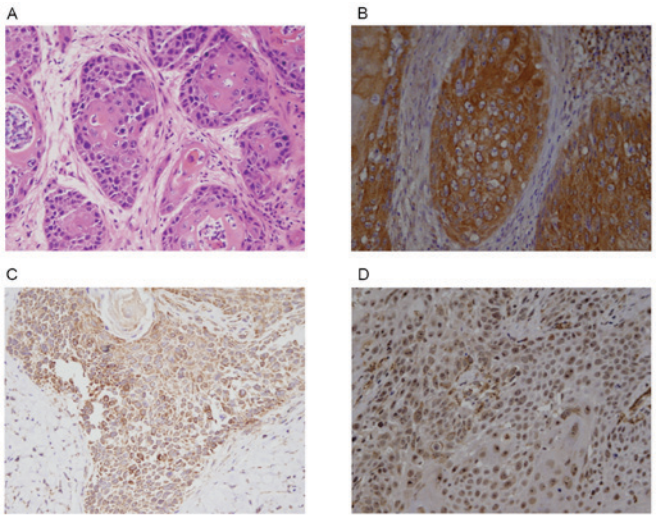


Figure 1. CD274, FOXP3 and IL-10 expression in LSCC. (A) Hematoxylin and eosin staining of LSCC tissue. Immunohistochemical staining of LSCC tissue: (B) CD274; (C) FOXP3; and (D) IL-10 expression. Magnification, x40. CD, cluster of differentiation; FOX, forkhead box; IL, interleukin; LSCC, laryngeal squamous cell carcinoma.

*Correlation between CD274, FOXP3 and IL-10 expression and the prognosis of patients with LSCC*. The KM analysis revealed that the CD274-, FOXP3- and IL-10-negative patients with LSCC experienced a significantly prolonged mean survival time compared with the CD274-, FOXP3- and IL-10-positive patients with LSCC (P<0.05; Fig. 3). A multivariate Cox regression analysis of the effects of the expression of CD274, FOXP3 and IL-10 on the prognosis of LSCC yielded hazard ratios >1 and P<0.05, which indicated that the expression of these three proteins was a significant risk factor for LSCC (Table IV).

*CD274, FOXP3 and IL-10-associated ceRNA networks*. Using the miRTarBase and StarBase v2.0 databases, it was established that lncRNA- and circRNA-associated ceRNA networks regulated CD274, FOXP3 and IL-10 (Figs. 4 and 5). The two networks revealed that the three genes can be regulated by specific miRNAs including hsa-miR-106a-5p, which regulated IL-10; hsa-miR-31-5p and hsa-miR-210-3p, which regulated FOXP3; and hsa-miR-124-3p, hsa-miR-103a-3p, hsa-miR-214-3p, hsa-miR-16-5p, hsa-miR-326, hsa-miR-708-5p and hsa-miR-29c-3p, which regulated CD274 (Fig. 4). The number of neighboring nodes directly connected to the node indicates the importance of the node in the network. The degree values of the network were then analyzed using Cytoscape software. The results demonstrated that miRNA nodes, including hsa-miR-16-5p, hsa-miR-106a-5p and hsa-miR-103a-3p in the network, exhibited larger degree values (Figs. 4B and 5B). Based on the network, 35 IL-10-associated lncRNAs [including long intergenic non-protein coding RNA 116, X-inactive specific transcript (XIST) and zinc ribbon domain containing 1-AS1], 12 FOXP3-associated lncRNAs (including membrane-associated guanylate kinase, WW, and PDZ domain-containing protein 2-AS3, small nucleolar RNA

Table II. Expression of CD274, FOXP3 and IL-10 in three tissue types.

| | | CD274 | | | | FOXP3 | | | | IL-10 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Tissue type | Cases | + | - | $\chi^2$ | P-value | + | - | $\chi^2$ | P-value | + | - | $\chi^2$ | P-value |
| NLM | 15 | 0 | 0 | / | / | 3 | 12 | 31.10 | <0.001 | 2 | 13 | 38.07 | <0.001 |
| VCP | 15 | 0 | 0 | | | 1 | 14 | | | 2 | 13 | | |
| LCSS | 133 | 78 | 55 | | | 91 | 42 | | | 98 | 35 | | |

CD, cluster of differentiation; FOX, forkhead box; IL, interleukin; LCSS, laryngeal squamous cell carcinoma; NLM, normal laryngeal mucosa; VCP, vocal cord polyp.

Table III. CD274, FOXP3 and IL-10 expression in LCSS.

| | CD274 | | | FOXP3 | | | IL-10 | | |
|---|---|---|---|---|---|---|---|---|---|
| Variable | - | + | P-value | - | + | P-value | - | + | P-value |
| Age | | | | | | | | | |
| ≤60 years | 14 | 34 | 0.032 | 16 | 32 | 0.744 | 14 | 34 | 0.575 |
| >60 years | 41 | 44 | | 26 | 59 | | 21 | 64 | |
| Tumor location | | | | | | | | | |
| Supraglottic | 11 | 19 | 0.497 | 9 | 21 | 1.000 | 6 | 24 | 0.453 |
| Glottic | 44 | 57 | | 33 | 68 | | 29 | 72 | |
| Subglottic | 0 | 2 | | 0 | 2 | | 0 | 2 | |
| TNM | | | | | | | | | |
| T1/T2 | 41 | 50 | 0.202 | 33 | 58 | 0.087 | 31 | 60 | 0.003 |
| T3/T4 | 14 | 28 | | 9 | 33 | | 4 | 38 | |
| LNM | | | | | | | | | |
| No | 48 | 50 | 0.003 | 37 | 61 | 0.010 | 32 | 66 | 0.005 |
| Yes | 7 | 28 | | 5 | 30 | | 3 | 32 | |
| Clinical stage | | | | | | | | | |
| I | 24 | 25 | 0.570 | 17 | 32 | 0.579 | 20 | 29 | 0.008 |
| II | 14 | 23 | | 13 | 24 | | 10 | 27 | |
| III | 10 | 16 | | 8 | 18 | | 4 | 22 | |
| IV | 7 | 14 | | 4 | 17 | | 1 | 20 | |
| Course of disease | | | | | | | | | |
| ≤6 months | 13 | 40 | 0.004 | 7 | 46 | <0.001 | 8 | 45 | 0.024 |
| 6-12 months | 13 | 15 | | 9 | 19 | | 7 | 21 | |
| ≥12 months | 29 | 23 | | 26 | 26 | | 20 | 32 | |
| Pathological stage (differentiation) | | | | | | | | | |
| Well | 26 | 26 | 0.169 | 20 | 32 | 0.076 | 18 | 34 | 0.026 |
| Moderate | 21 | 32 | | 18 | 35 | | 15 | 38 | |
| Poor | 8 | 20 | | 4 | 24 | | 2 | 26 | |
| Prognosis | | | | | | | | | |
| Recurrence (-) | 45 | 25 | <0.001 | 34 | 36 | <0.001 | 30 | 40 | <0.001 |
| Recurrence (+) | 10 | 53 | | 8 | 55 | | 5 | 58 | |

CD, cluster of differentiation; FOX, forkhead box; IL, interleukin; LNM, lymph node metastasis; LSCC, laryngeal squamous cell carcinoma; TNM, tumor-node-metastasis classification.

host gene 16 and DNA polymerase δ-interacting protein 2) and 134 CD274-associated lncRNAs (including long intergenic non-protein coding RNA 662, H19 and HOX transcript antisense RNA) were identified. These lncRNAs may regulate the

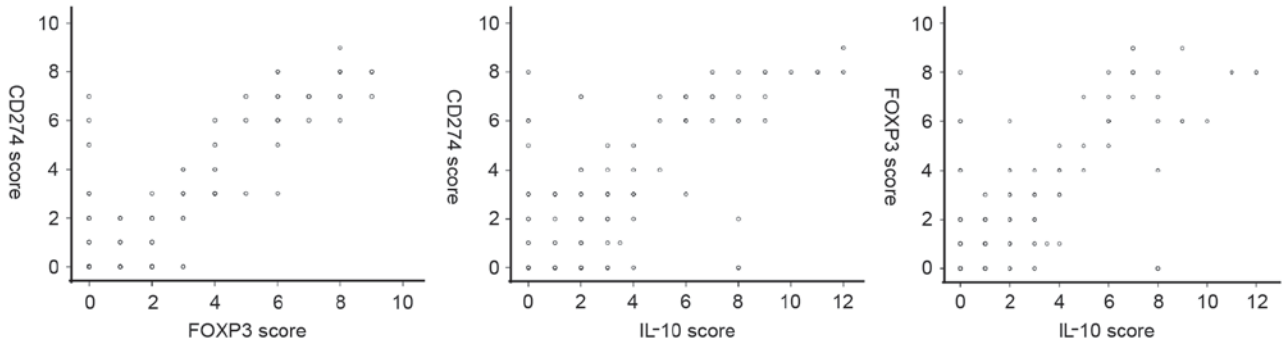


Figure 2. Correlation of CD274, FOXP3 and IL-10 expression in LSCC. Spearman analysis revealed that there was a positive expression correlation between CD274 and FOXP3 ($r_s$=0.767, P<0.001), between CD274 and IL-10 ($r_s$=0.640, P<0.001), and between IL-10 and FOXP3 ($r_s$=0.637, P<0.001). CD, cluster of differentiation; FOX, forkhead box; IL, interleukin; LSCC, laryngeal squamous cell carcinoma.

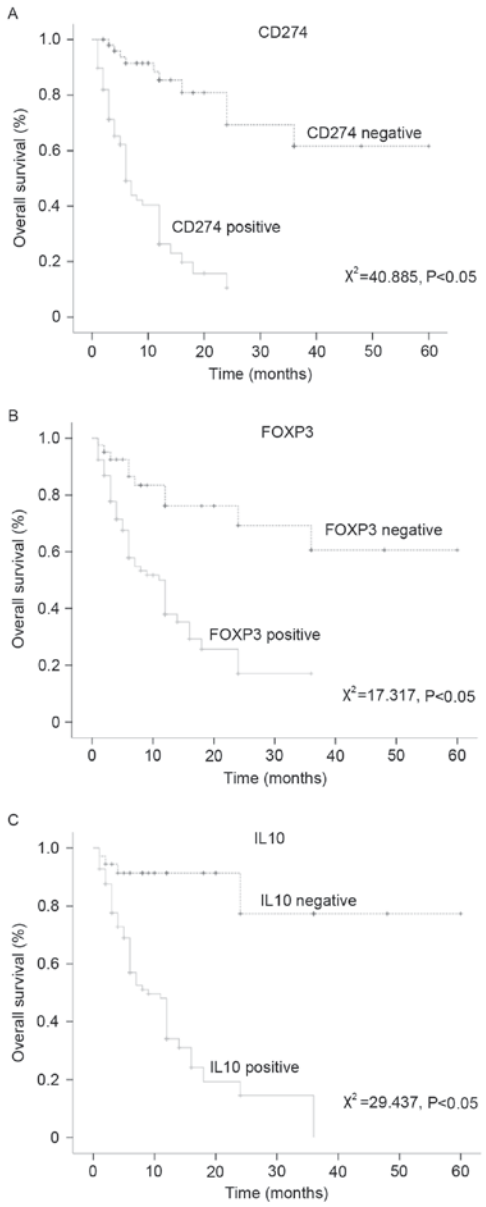


Figure 3. Correlation between the expression of CD274, FOXP3 and IL-10 and the prognosis of patients with LSCC. The Kaplan-Meier analysis revealed that (A) CD274-, (B) FOXP3- and (C) IL-10-negative patients with LSCC experienced a significantly prolonged mean survival time compared with the CD274-, FOXP3- and IL-10-positive patients with LSCC (P<0.05). CD, cluster of differentiation; FOX, forkhead box; IL, interleukin; LSCC, laryngeal squamous cell carcinoma.

three genes through their ceRNA effects. The results demonstrated that lncRNA nodes in the network, including XIST, RP11-467L20.9 and AC084219.4, exhibited larger degree values. (Fig. 4C). In addition to lncRNAs, 408 circRNAs that regulate the three genes in LSCC were identified (Fig. 5A). Among them, it was identified that ISY1 splicing factor homolog hsa_circ_001859, tubulin γ complex associated protein 3 hsa-circ_001373 and tubulin α 1b hsa_circ_002179 in the network exhibited larger degree values (Fig. 5C).

## Discussion

As an important mechanism of tumorigenesis, immune evasion has been a subject undergoing intense study in cancer research. Tumor development and progression depend on the tumor microenvironment. The infiltration and function of T cells in the tumor microenvironment determine the outcome of tumor immunosurveillance. The recently identified Tregs are a subpopulation of T cells that possess immunoregulatory functions. Tregs possess two major functions: Induction of immune incompetence and immunosuppression (18,19). FOXP3 is a characteristic surface marker of Tregs. It has previously been identified that the proportion of $FOXP3^+$ Tregs is increased in the peripheral blood of patients with cancer, including lung, breast, pancreatic and colon cancer (20-23). These findings indicated that $FOXP3^+$ Tregs are closely associated with tumor development. The present study identified that FOXP3 expression was not only increased in LSCC but was also negatively correlated with the survival rate of patients with LSCC. Such a finding indicated that $FOXP3^+$ Tregs may promote the malignant progression of LSCC. The correlation analysis of clinical factors revealed that the expression levels of FOXP3 were significantly elevated in patients with a long disease course and in those who developed lymph node metastasis. These results demonstrated that FOXP3 was closely associated with the degree of malignancy of LSCC.

The promoter effect of Tregs on cancer immune evasion depends on the expression of inhibitory molecules, including IL-10, which are secreted by Tregs themselves (24). Previous studies have demonstrated that Tregs initiate inhibitory signals through the secretion of IL-10 and other cytokines, which inhibits the activity of functional T lymphocytes and suppresses tumor immune responses (9,25). IL-10 not only induces immune tolerance through the inhibition of the dendritic cell-mediated

Table IV. Analysis of prognostic factors in LSCC.

| Factor | B | SE | Wald | P-value | HR | 95% CI | |
|---|---|---|---|---|---|---|---|
| CD274 | 0.109 | 0.045 | 5.986 | 0.014 | 1.115 | 1.022 | 1.217 |
| FOXP3 | 0.132 | 0.042 | 9.777 | 0.002 | 1.141 | 1.050 | 1.239 |
| IL-10 | 0.076 | 0.037 | 4.239 | 0.040 | 1.079 | 1.004 | 1.160 |

B, regression coefficients; CD, cluster of differentiation; CI, confidence interval; FOX, forkhead box; HR, hazard ratio; IL, interleukin; LSCC, laryngeal squamous cell carcinoma; SE, standard error; Wald, Wald test $\chi^2$ value.

transformation of T cells into cytotoxic T lymphocytes, but also induces immunosuppression through the inhibition of the antigen-presenting function of antigen-presenting cells (26-28). In certain patients with cancer, IL-10 expression may serve as an independent negative prognostic factor (29,30). The present study identified that IL-10 was highly expressed in LSCC. In addition, the expression levels of IL-10 exhibited a significant negative correlation with the survival rate of patients with LSCC. These findings indicated that IL-10 may be closely associated with the malignant progression of LSCC. Analysis of the clinicopathological factors demonstrated that the expression levels of IL-10 were significantly positively correlated with the TNM stage of cancer, lymph node metastasis, course of the disease and the pathological grade of cancer in patients with LSCC. The results further demonstrated that IL-10 may be involved in the malignant progression of LSCC. In addition, a significant correlation between IL-10 and FOXP3 expression was identified, which indicated that $FOXP3^+$ Tregs may affect the malignant progression of LSCC through the secretion of IL-10.

It has previously been demonstrated that the expression of negative costimulatory molecules in the tumor microenvironment is involved in tumor escape from the immune system (10). Negative costimulatory molecules are important factors that constitute the tumor microenvironment. CD274 is one of the primary members of the B7 family of negative costimulatory molecules, which is closely associated with the progression of various tumors, including melanoma and glioma (11,31). Previous studies have reported that CD274 exerts its effects on tumors through the regulation of tumor immunity. For example, CD274 suppresses type 1 T helper cell-based immune responses and induces T cell apoptosis *in vitro* via the inhibitory receptor programmed cell death protein 1, which aids the escape of tumor cells from the immune system (32,33). The present study demonstrated that the expression levels of CD274 in LSCC were positively correlated with the TNM stage of cancer, lymph node metastasis and the pathological stage of cancer. In addition, the survival rate was significantly reduced in patients with high CD274 expression compared with those with low CD274 expression. These findings indicated that CD274 may be involved in the malignant progression of LSCC. In addition, CD274 and FOXP3 co-expression was detected in LSCC tissues, thus indicating that CD274 not only inhibits the proliferation of activated T cells but also exerts an immunosuppressive effect through an increase in the number of $FOXP3^+$ Tregs, which promotes the progression of LSCC. Therefore, CD274 may serve as a potential target for LSCC immunotherapy, whereas CD274 expression may serve as an important prognostic indicator of LSCC. Furthermore, in addition to the finding that CD274 was co-expressed along with FOXP3, CD274 expression was also positively correlated with that of IL-10. Based on the aforementioned results, the following hypothesis was proposed: A high level of CD274 expression increases the number of $FOXP3^+$ Tregs, and, as a result, the expression of the immunosuppressive molecule IL-10 is increased, ultimately promoting LSCC immune evasion and enhancing the degree of malignancy of LSCC. This hypothesis is likely to represent an important mechanism underlying LSCC immune evasion; however, this hypothesis needs to be verified in the future through large-scale scientific studies.

To understand the potential molecular mechanism underlying the correlation among these three genes in LSCC, miRTarBase and StarBase databases were used to identify the miRNAs and lncRNAs that regulated the three genes. The results demonstrated that certain miRNAs in the network exhibited larger degree values, including hsa-miR-16-5p and hsa-miR-103a-3p. Previous studies have demonstrated that miRNAs may serve important roles in cancer progression. Hudcova *et al* (34) identified that reduced expression of miR-29c-3p in tumor tissue was associated with worse relapse-free survival rate in head and neck cancer. Ye *et al* (35) indicated that miR-106a-5p was associated with the expression of circulating exosomes and immune escape in human nasopharyngeal carcinoma. In addition to miR-29c-3p and miR-106a-5p, the association between other miRNAs and head and neck cancer is not clear; however, their roles in other cancers have been reported. Li *et al* (36) reported that miR-16-5p is able to control the malignant development of osteoarthritis in chondrocytes. Wang *et al* (37) demonstrated that miR-326 can regulate cell proliferation and migration in lung cancer. In addition, miR-106a-5p can inhibit the proliferation and migration of astrocytoma cells and promote apoptosis (38). Based on these findings, it may be hypothesized that these miRNAs exhibit the potential to serve important roles in LSCC.

Salmena *et al* (15) introduced the concept of ceRNAs in 2011, which states that all types of RNA molecules (e.g., lncRNA and circRNA) are able to mutually regulate one another by competitively binding to miRNAs as long as they share common miRNA-binding sites. In the present study, the effects of ceRNAs in the lncRNA-miRNA-mRNA regulatory network in LSCC were evaluated. The results demonstrated that certain lncRNAs in the network exhibited larger degree values, including XIST, H19 and metastasis associated lung

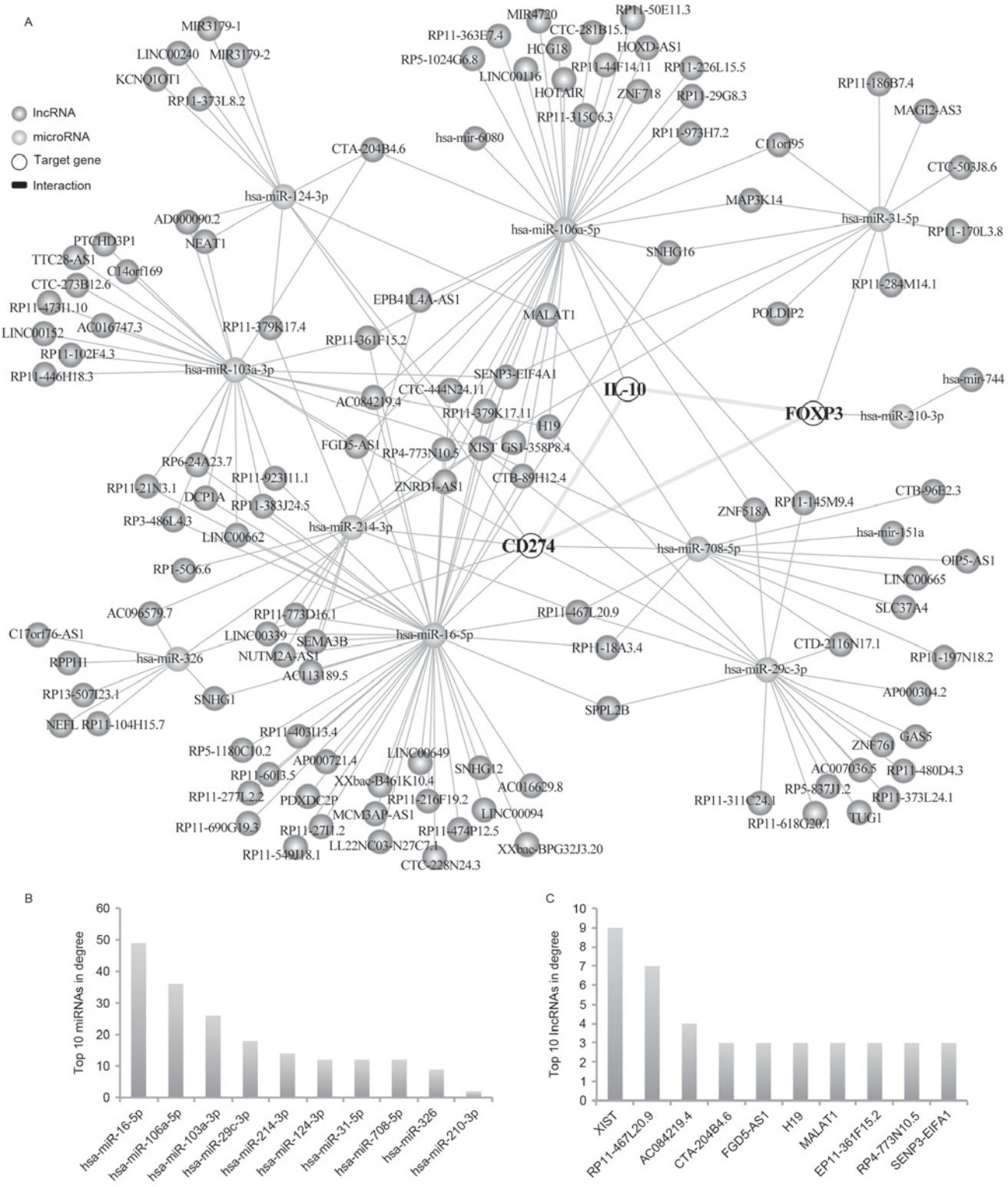


Figure 4. lncRNA-associated ceRNA network of CD274, FOXP3 and IL-10 in LSCC. (A) Based on the association among lncRNA, miRNA and mRNA, databases were used to establish the ceRNA network. The number of neighboring nodes directly connected to the node indicates the importance of the node in the network. The degree values of the network were then analyzed using Cytoscape software. (B) Top 10 miRNAs, according to degree values. (C) Top 10 lncRNAs, according to degree values. CD, cluster of differentiation; ceRNA, competitive endogenous RNA; FOX, forkhead box; IL, interleukin; LSCC, laryngeal squamous cell carcinoma; lncRNA, long non-coding RNA; miRNA, microRNA.

adenocarcinoma transcript 1 (MALAT1). Numerous studies have identified that these lncRNAs are associated with the process of cancer. Huang *et al* (39) demonstrated that a reduction in XIST expression in breast tissue can upregulate Akt phosphorylation and tumor cell viability. Li *et al* (40) reported that the lncRNA H19 can promote cell invasion in nasopharyngeal carcinoma. In addition, it has been identified that MALAT1 can serve roles in numerous types of cancer, including breast and pancreatic cancer (41,42). Chen *et al* (43) revealed that MALAT1 is an important target of cisplatin and

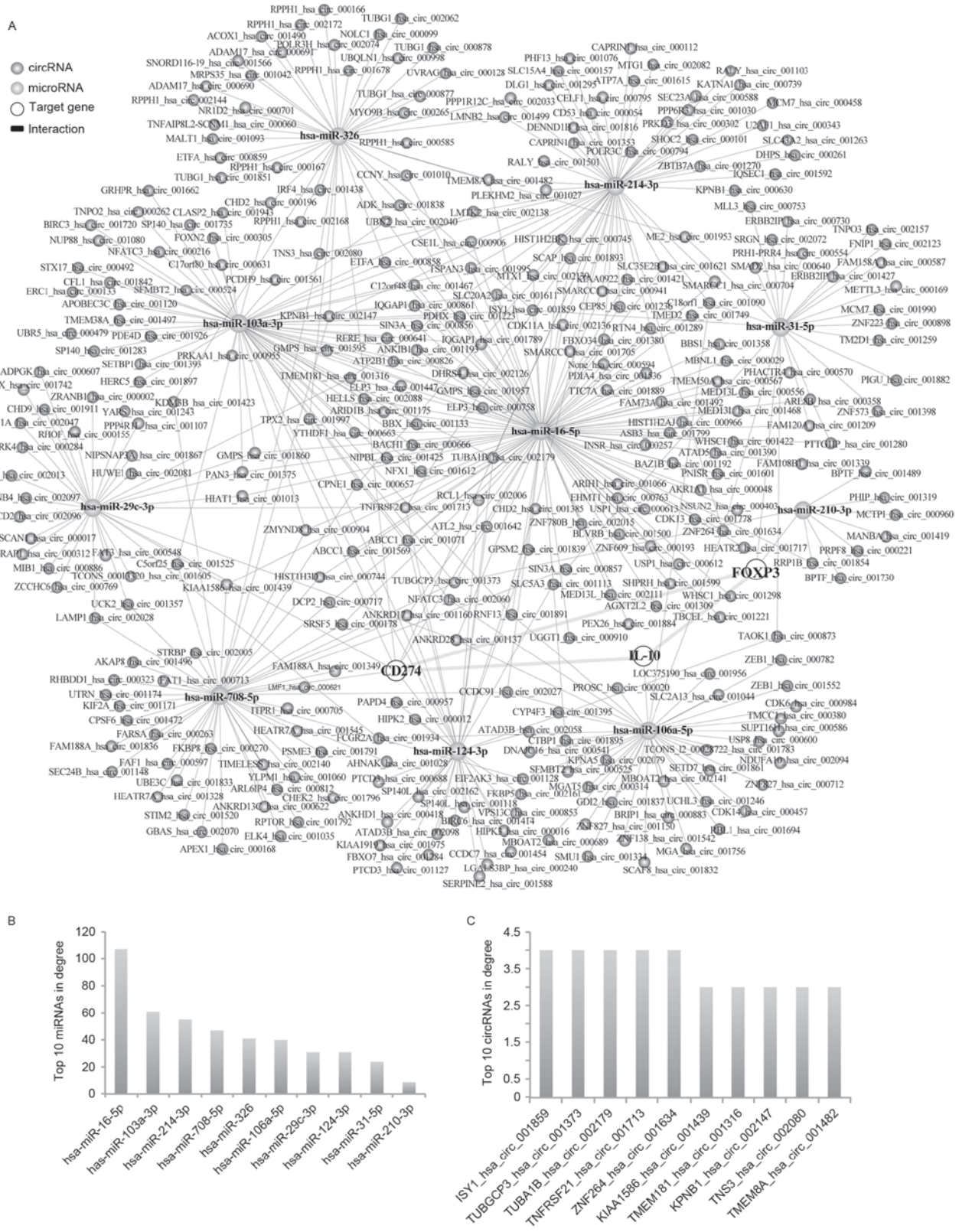


Figure 5. circRNA-associated ceRNA network of CD274, FOXP3 and IL-10 in LSCC. (A) Based on the association among circRNA, miRNA and mRNA, databases were used to establish the ceRNA network. The number of neighboring nodes directly connected to the node indicates the importance of the node in the network. The degree values of the network were then analyzed using Cytoscape software. (B) Top 10 miRNAs, according to degree values. (C) Top 10 circRNAs, according to degree values. CD, cluster of differentiation; circRNA, circular RNA; FOX, forkhead box; IL, interleukin; LSCC, laryngeal squamous cell carcinoma; miRNA, microRNA.

paclitaxel, and may have the potential as a novel molecular target for the treatment of patients with LSCC. Based on these findings, it may be hypothesized that these lncRNAs exercise their ceRNA effects to regulate LSCC through upregulating IL-10, FOXP3 or CD274. The lncRNAs predicted in the present study may serve as a basis for new studies regarding

the mechanism of tumor immunity in LSCC. In addition, the circRNA-related ceRNA network identified 408 circRNAs that regulated the three genes. It has been identified that some of these genes bind with miRNAs and act as natural miRNA sponges to inhibit the activities of associated miRNAs. For instance, Xie *et al* (44) demonstrated that circ_001569 can promote the proliferation and invasion of colorectal cancer via its ceRNA effect. The present study identified circ_001569 in the ceRNA network, thus suggesting that circ_001569 may serve roles in LSCC. As research on circRNA remains in the early stages, the effects of more circRNAs on cancer are yet to be elucidated. However, the present study indicated that these circRNAs may bind with the miRNAs that regulate CD274, FOXP3 and IL-10. Based on these findings, it may be hypothesized that these circRNAs exhibit the potential to be ceRNAs of the three genes that regulate the progression of LSCC.

In conclusion, the present study reported that the expression levels of CD274, FOXP3 and IL-10 were higher in LSCC tissue compared with their expression levels in normal tissues. Notably, there was a strong positive correlation among the expression of these three proteins. In addition, it was identified that the expression of the three proteins was closely correlated with the clinical characteristics of LSCC, and the expression levels of FOXP3, IL-10 and CD274 had a negative association with the survival rate of patients with LSCC. The results of the Cox regression model suggested that the three proteins were prognostic risk factors for LSCC. In addition, the ceRNA network of the three genes was established. Data generated from the present study provided novel information, which may increase understanding regarding the immune evasion mechanisms of LSCC, and aid the development of LSCC immunotherapy.

## Acknowledgements

The present study was supported by the Inner Mongolia Natural Science Foundation (grant nos. 2014MS0856 and 2017MS0838), the Natural Science Foundation of China (grant no. 81473499) and the General Research Projects of the Affiliated Hospital of Inner Mongolia Medical University (grant no. NYFY YB 027).

## References

1. Seiferlein E, Haderlein T, Schuster M, Gräßel E and Bohr C: Correlation between coping strategies and subjective assessment of the voice-related quality of life of patients after resection of T1 and T2 laryngeal tumours. Eur Arch Otorhinolaryngol 269: 2091-2096, 2012.
2. Marur S and Forastiere AA: Head and neck squamous cell carcinoma: Update on epidemiology, diagnosis, and treatment. Mayo Clin Proc 91: 386-396, 2016.
3. Olsen KD: Reexamining the treatment of advanced laryngeal cancer. Head Neck 32: 1-7, 2010.
4. Moser SE: Laryngeal problems. Prim Care 41: 99-107, 2014.
5. Naldini L: Gene therapy returns to centre stage. Nature 526: 351-360, 2015.
6. Arce-Sillas A, Álvarez-Luquín DD, Tamaya-Domínguez B, Gomez-Fuentes S, Trejo-García A, Melo-Salas M, Cárdenas G, Rodríguez-Ramírez J and Adalid-Peralta L: Regulatory T cells: Molecular actions on effector cells in immune regulation. J Immunol Res 2016: 1720827, 2016.
7. Takeuchi Y and Nishikawa H: Roles of regulatory T cells in cancer immunity. Int Immunol 28: 401-409, 2016.
8. Franco A, Almanza G, Burns JC, Wheeler M and Zanetti M: Endoplasmic reticulum stress drives a regulatory phenotype in human T-cell clones. Cell Immunol 266: 1-6, 2010.
9. Chaudhry A, Samstein RM, Treuting P, Liang Y, Pils MC, Heinrich JM, Jack RS, Wunderlich FT, Brüning JC, Müller W and Rudensky AY: Interleukin-10 signaling in regulatory T cells is required for suppression of Th17 cell-mediated inflammation. Immunity 34: 566-578, 2011.
10. Chen N, Fang W, Zhan J, Hong S, Tang Y, Kang S, Zhang Y, He X, Zhou T, Qin T, *et al*: Upregulation of PD-L1 by EGFR activation mediates the immune escape in EGFR-driven NSCLC: Implication for optional immune targeted therapy for NSCLC patients with EGFR mutation. J Thorac Oncol 10: 910-923, 2015.
11. Budczies J, Bockmayr M, Denkert C, Klauschen F, Gröschel S, Darb-Esfahani S, Pfarr N, Leichsenring J, Onozato ML, Lennerz JK, *et al*: Pan-cancer analysis of copy number changes in programmed death-ligand 1 (PD-L1, CD274)-associations with gene expression, mutational load, and survival. Genes Chromosomes Cancer 55: 626-639, 2016.
12. Qian Y, Deng J, Geng L, Xie H, Jiang G, Zhou L, Wang Y, Yin S, Feng X, Liu J, *et al*: TLR4 signaling induces B7-H1 expression through MAPK pathways in bladder cancer cells. Cancer Invest 26: 816-821, 2008.
13. Winograd R, Byrne KT, Evans RA, Odorizzi PM, Meyer AR, Bajor DL, Clendenin C, Stanger BZ, Furth EE, Wherry EJ and Vonderheide RH: Induction of T-cell immunity overcomes complete resistance to PD-1 and CTLA-4 blockade and improves survival in pancreatic carcinoma. Cancer Immunol Res 3: 399-411, 2015.
14. Liang X, Liu Y, Mei S, Zhang M, Xin J, Zhang Y and Yang R: MicroRNA-22 impairs anti-tumor ability of dendritic cells by targeting p38. PLoS One 10: e0121510, 2015.
15. Salmena L, Poliseno L, Tay Y, Kats L and Pandolfi PP: A ceRNA hypothesis: The Rosetta Stone of a hidden RNA language? Cell 146: 353-358, 2011.
16. Remmele W and Stegner HE: Recommendation for uniform definition of an immunoreactive score (IRS) for immunohistochemical estrogen receptor detection (ER-ICA) in breast cancer tissue. Pathologe 8: 138-140, 1987 (In German).
17. Abd ElHafeez S, Torino C, D'Arrigo G, Bolignano D, Provenzano F, Mattace-Raso F, Zoccali C and Tripepi G: An overview on standard statistical methods for assessing exposure-outcome link in survival analysis (Part II): The Kaplan-Meier analysis and the Cox regression method. Aging Clin Exp Res 24: 203-206, 2012.
18. Wang YM, Ghali J, Zhang GY, Hu M, Wang Y, Sawyer A, Zhou JJ, Hapudeniya DA, Wang Y, Cao Q, *et al*: Development and function of Foxp3(+) regulatory T cells. Nephrology (Carlton) 21: 81-85, 2016.
19. Paust S and Cantor H: Regulatory T cells and autoimmune disease. Immunol Rev 204: 195-207, 2005.
20. Kryczek I, Liu R, Wang G, Wu K, Shu X, Szeliga W, Vatan L, Finlayson E, Huang E, Simeone D, *et al*: FOXP3 defines regulatory T cells in human tumor and autoimmune disease. Cancer Res 69: 3995-4000, 2009.
21. Yamamoto T, Yanagimoto H, Satoi S, Toyokawa H, Hirooka S, Yamaki S, Yui R, Yamao J, Kim S and Kwon AH: Circulating CD4+CD25+ regulatory T cells in patients with pancreatic cancer. Pancreas 41: 409-415, 2012.
22. Watanabe MA, Oda JM, Amarante MK and Cesar Voltarelli J: Regulatory T cells and breast cancer: Implications for immunopathogenesis. Cancer Metastasis Rev 29: 569-579, 2010.
23. Yan F, Du R, Wei F, Zhao H, Yu J, Wang C, Zhan Z, Ding T, Ren X, Chen X and Li H: Expression of TNFR2 by regulatory T cells in peripheral blood is correlated with clinical pathology of lung cancer patients. Cancer Immunol Immunother 64: 1475-1485, 2015.
24. Sakaguchi S, Sakaguchi N, Shimizu J, Yamazaki S, Sakihama T, Itoh M, Kuniyasu Y, Nomura T, Toda M and Takahashi T: Immunologic tolerance maintained by CD25+ CD4+ regulatory T cells: Their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. Immunol Rev 182: 18-32, 2001.
25. Maynard CL, Hatton RD, Helms WS, Oliver JR, Stephensen CB and Weaver CT: Contrasting roles for all-trans retinoic acid in TGF-beta-mediated induction of Foxp3 and Il10 genes in developing regulatory T cells. J Exp Med 206: 343-357, 2009.
26. Dennis KL, Blatner NR, Gounari F and Khazaie K: Current status of interleukin-10 and regulatory T-cells in cancer. Curr Opin Oncol 25: 637-645, 2013.
27. Tucci M, Stucci S, Passarelli A, Giudice G, Dammacco F and Silvestris F: The immune escape in melanoma: Role of the impaired dendritic cell function. Expert Rev Clin Immunol 10: 1395-1404, 2014.

28. Raker VK, Domogalla MP and Steinbrink K: Tolerogenic dendritic cells for regulatory T cell induction in man. Front Immunol 6: 569, 2015.
29. Cai T, Mazzoli S, Meacci F, Tinacci G, Nesi G, Zini E and Bartoletti R: Interleukin-6/10 ratio as a prognostic marker of recurrence in patients with intermediate risk urothelial bladder carcinoma. J Urol 178: 1906-1912, 2007.
30. Gupta M, Han JJ, Stenson M, Maurer M, Wellik L, Hu G, Ziesmer S, Dogan A and Witzig TE: Elevated serum IL-10 levels in diffuse large B-cell lymphoma: A mechanism of aberrant JAK2 activation. Blood 119: 2844-2853, 2012.
31. Huang BY, Zhan YP, Zong WJ, Yu CJ, Li JF, Qu YM and Han S: The PD-1/B7-H1 pathway modulates the natural killer cells versus mouse glioma stem cells. PLoS One 10: e0134715, 2015.
32. Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, Roche PC, Lu J, Zhu G, Tamada K, *et al*: Tumor-associated B7-H1 promotes T-cell apoptosis: A potential mechanism of immune evasion. Nat Med 8: 793-800, 2002.
33. Kubo S, Yamada T, Osawa Y, Ito Y, Narita N and Fujieda S: Cytosine-phosphate-guanosine-DNA induces CD274 expression in human B cells and suppresses T helper type 2 cytokine production in pollen antigen-stimulated CD4-positive cells. Clin Exp Immunol 169: 1-9, 2012.
34. Hudcova K, Raudenska M, Gumulec J, Binkova H, Horakova Z, Kostrica R, Babula P, Adam V and Masarik M: Expression profiles of miR-29c, miR-200b and miR-375 in tumour and tumour-adjacent tissues of head and neck cancers. Tumour Biol 37: 12627-12633, 2016.
35. Ye SB, Li ZL, Luo DH, Huang BJ, Chen YS, Zhang XS, Cui J, Zeng YX and Li J: Tumor-derived exosomes promote tumor progression and T-cell dysfunction through the regulation of enriched exosomal microRNAs in human nasopharyngeal carcinoma. Oncotarget 5: 5439-5452, 2014.
36. Li L, Jia J, Liu X, Yang S, Ye S, Yang W and Zhang Y: MicroRNA-16-5p controls development of osteoarthritis by targeting SMAD3 in chondrocytes. Curr Pharm Des 21: 5160-5167, 2015.
37. Wang R, Chen X, Xu T, Xia R, Han L, Chen W, De W and Shu Y: MiR-326 regulates cell proliferation and migration in lung cancer by targeting phox2a and is regulated by HOTAIR. Am J Cancer Res 6: 173-186, 2016.
38. Zhi F, Zhou G, Shao N, Xia X, Shi Y, Wang Q, Zhang Y, Wang R, Xue L, Wang S, *et al*: miR-106a-5p inhibits the proliferation and migration of astrocytoma cells and promotes apoptosis by targeting FASTK. PLoS One 8: e72390, 2013.
39. Huang YS, Chang CC, Lee SS, Jou YS and Shih HM: Xist reduction in breast cancer upregulates AKT phosphorylation via HDAC3-mediated repression of PHLPP1 expression. Oncotarget 7: 43256-43266, 2016.
40. Li X, Lin Y, Yang X, Wu X and He X: Long noncoding RNA H19 regulates EZH2 expression by interacting with miR-630 and promotes cell invasion in nasopharyngeal carcinoma. Biochem Biophys Res Commun 473: 913-919, 2016.
41. Huang NS, Chi YY, Xue JY, Liu MY, Huang S, Mo M, Zhou SL and Wu J: Long non-coding RNA metastasis associated in lung adenocarcinoma transcript 1 (MALAT1) interacts with estrogen receptor and predicted poor survival in breast cancer. Oncotarget 7: 37957-37965, 2016.
42. Jiao F, Hu H, Yuan C, Wang L, Jiang W, Jin Z, Guo Z and Wang L: Elevated expression level of long noncoding RNA MALAT-1 facilitates cell growth, migration and invasion in pancreatic cancer. Oncol Rep 32: 2485-2492, 2014.
43. Chen H, Xin Y, Zhou L, Huang JM, Tao L, Cheng L and Tian J: Cisplatin and paclitaxel target significant long noncoding RNAs in laryngeal squamous cell carcinoma. Med Oncol 31: 246, 2014.
44. Xie H, Ren X, Xin S, Lan X, Lu G, Lin Y, Yang S, Zeng Z, Liao W, Ding YQ and Liang L: Emerging roles of circRNA_001569 targeting miR-145 in the proliferation and invasion of colorectal cancer. Oncotarget 7: 26680-26691, 2016.