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Expression of miR-195 in laryngeal squamous cell carcinoma and its effect on proliferation and apoptosis of Hep-2

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Abstract. – OBJECTIVE: To investigate the expression of miR-195 and its relationship with clinicopathological characteristics in laryngeal squamous cell carcinoma (LSCC), and to explore its effect and possible mechanism on proliferation and apoptosis of Hep-2.

PATIENTS AND METHODS: Real-time fluorescence quantitative PCR was used to detect the expression of miR-195 in laryngeal carcinoma tissues and adjacent normal tissues from 98 cases. Dual-luciferase reporter plasmid with Bcl-2 wild type and mutant type 3' untranslated region was created to verify the target of miR-195 by luciferase assay. After Hep-2 cells were transfected with miR-195/Bcl-2, miR-195, Bcl-2 siRNA and negative control by lipofectamine, the protein expression of Bcl-2 was detected by Western blot analysis. The proliferation and apoptosis of Hep-2 were detected by MTS method and flow cytometry, respectively.

RESULTS: Compared with adjacent normal tissues, the expression of miR-195 was lower in laryngeal carcinoma tissues ($p < 0.01$). The low expression of miR-195 was positively correlated with distant metastasis and clinical stage ($p < 0.05$). The average survival time of patients with low expression was shorter than those with high expression by Kaplan-Meier method ($p < 0.01$). Multivariate Cox analysis showed that miR-195 expression and lymph node metastases were independent prognostic factors ($p < 0.05$).

CONCLUSIONS: The expression of miR-195 was significantly decreased in laryngeal carcinoma tissues, which was closely related to the clinicopathological characteristics of LSCC. miR-195 may inhibit the proliferation and promote the apoptosis of Hep-2 by regulating Bcl-2 expression, which as an anti-oncogene could have the potential to be a therapeutic strategy in the treatment of LSCC.

Key Words:
Laryngeal squamous cell carcinoma, miR-195, Lymph node metastases, Proliferation and apoptosis, Bcl-2.

Introduction

Laryngeal squamous cell carcinoma (LSCC) is one of the most common types of tumor of the head and neck which accounts for approximately 25% of it. In 2013, it resulted in 88,000 deaths up from 76,000 deaths in 1990[1]. And five-year survival rates in the United States are 60% in 2013, and there is no increase in the past 30 years[2]. Thus, exploring the molecular biological mechanism of the occurrence and development of LSCC has important clinical significance in the diagnosis, treatment, and prognosis of the disease.

Studies[3] showed that miR-195 was down regulated in many tumors, such as liver cancer, LSCC, breast cancer, bladder cancer, chronic lymphocytic leukemia and other tumors. A recent study[4] has obtained the differential expression profile of miR-195 from laryngeal squamous tissues, and proved that miR-195 has the same low expression in LSCC. However, it has not been reported about the effect of miR-195 on LSCC. In the present study, we investigated the expression of miR-195 in laryngeal carcinoma tissues (LCT) and adjacent normal tissues and tried to explore its relationship with clinicopathological characteristics and the possible mechanism on proliferation and apoptosis in LSCC.

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Patients and Methods

Specimen Source

LSCC patients with complete data in The Second Hospital of Tianjin Medical University and Tianjin Medical University Cancer Institute & Hospital were retrospectively analyzed from January 2007 to August 2015, and 98 patients were included in this research. The LCT and adjacent normal tissues following surgical resection were taken into liquid nitrogen and kept at -80°C. All patients had not been treated with radiotherapy and chemotherapy before the surgery and postoperative pathological diagnosis was LSCC. The data of the patients were showed in Table I.

This study was approved by the Ethics Committee at our hospital and was conducted in accordance with the provisions of the Declaration of Helsinki, Good Clinical Practice guidelines, and local laws and regulations.

Cell Culture and Transfections

Hep-2 cells were obtained from ATCC (USA) and maintained in RPMI supplemented with 10% fetal bovine serum (Hyclone, GE Healthcare Life Sciences, HyClone Laboratories, South Logan, UT, USA). All the cell lines were cultured in a humidified atmosphere with 5% CO_2 at 37°C. Transfections were done using Lipofectamine 2000/Lipofectamine LTX-Plus (Invitrogen, Carlsbad, CA, USA) reagent according to the manufacturer's instructions. miR-195 mimics (forward: 5'-UAGCAGCACAGAAAUAUUGGC-3', reverse: 5'-CAAUAUUUCUGUGCUGCUAUU-3'), Bcl-2 (forward: 5'-GGCACCTGCACACCTGGAT-3', reverse: 5'-GGCCGTACAGTTCCACAAAGG-3'), Bcl-2 siRNA (forward: 5'-GGGAGAUAGUGAUGAAGUATT-3', reverse: 5'-UACUUCAUCACUAUCUCCCTT-3'), control siRNA (forward: 5'-UUCUCCGAACGUGUCACGUTT-3', reverse: 5'-ACGUGACACGUUCGGAGAATT-3').

Quantitative RT-PCR Detection of miR-195

Total RNA was extracted from tissues by using Trizol Kit (Invitrogen, Carlsbad, CA, USA). Agarose gel electrophoresis was used to identify the integrity of the total cellular RNA. Total cellular RNA was reverse transcribed to cD-

Table I. Relationship between miR-195 expression and clinicopathological characteristics of laryngeal squamous cell carcinoma.

Parameter	n	Expression of miR-195	*p*-value
Age (year-old)			
≤ 60	48	0.5394 ± 0.4974	0.595
> 60	50	0.4872 ± 0.4703	
Gender			
Male	63	0.5080 ± 0.4966	0.896
Female	35	0.5214 ± 0.4615	
Primary site			
Glottis	48	0.5170 ± 0.4911	0.933
Supraglottis	33	0.5266 ± 0.5003	
Subglottis	17	0.4738 ± 0.4440	
Differentiation level			
High	61	0.4607 ± 0.4412	0.171
Medium-low	37	0.5987 ± 0.5378	
T staging			
T1+T2	25	0.8263 ± 0.5353	0.000
T3+T4	73	0.4054 ± 0.4143	
Lymph node metastases			
N0	70	0.5404 ± 0.4758	0.373
N+	28	0.4438 ± 0.4990	
Distant metastasis			
M0	95	0.5234 ± 0.4837	0.222
M1	3	0.1766 ± 0.3050	
Clinical stage			
I+II	17	0.9697 ± 0.5373	0.000
III+IV	81	0.4168 ± 0.4126	
Preoperative smoking			
No	20	0.5065 ± 0.5634	0.948
Yes	78	0.5144 ± 0.4629	

NA by primers of miR-195 and U6. miR-195 mimics (forward: 5'-UAGCAGCACAGAAAUAUUGGC-3', reverse: 5'-CAAUAUUUCUGUGCUGCUAUU-3'), U6 (forward: 5'-CTCGCTTCGGCAGCACA-3', reverse: 5'-AACGCTTCACGAATTTGCGT-3'), Bcl-2 (forward: 5'-GGCACCTGCACACCTGGAT-3', reverse: 5'-GGCCGTACAGTTCCACAAAGG-3'), Using cDNA as the template, U6 as the internal reference, real-time PCR was done with Opticon 2 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Reaction conditions: 95°C for 10 min, 40 cycles (95°C for 10s and 60°C for 30s). $2^{-\Delta\Delta Ct}$ was adapted to analyze relative quantification. The groups include the control group, miR-195/Bcl-2 group, miR-195 group, and Bcl-2 siRNA group.

Dual Luciferase Reporter Gene Method

Human embryonic kidney cells (HEK293T) were cultured in DMEM medium containing 10% fetal bovine serum (FBS), 5% CO_2 at 37°C. Through point mutation method, the cDNA fragment containing the miR-195 binding site with wild type Bcl-2 and mutant type 3' untranslated region (3'-UTR) were inserted into pmirGLO (Promega, Madison, WI, USA) and the sequence was verified by sequencing. Recombinant vector pmirGLO-BCL-2 (Invitrogen, Carlsbad, CA, USA) or pmirGLO-mut BCL-2 and miR-195 mimics or miR-negative control (miR-NC) were transfected into HEK293T by liposome transfection method and incubated for 48 h collecting lysate. 100 μL renilla luciferase detection liquid (Invitrogen, Carlsbad, CA, USA) was taken into 100 μL supernatant, and renilla luciferase activity was detected. In addition, 100 μL firefly luciferase detection reagent (Invitrogen, Carlsbad, CA, USA) was taken into 100 μL supernatant and firefly luciferase activity was detected after mixing. The ratio of the absolute value of firefly luciferase with the absolute value of renilla luciferase was set as relative luciferase activity, and this experiment was repeated 3 times.

Western-blot Detection

Protein extraction was performed with Protein Extraction Kit (Beyotime Biotechnology, Shanghai, China), according to the manufacturer's protocol. Protein concentration was measured using Bradford method. The protein samples were boiled for 5 min with buffer, and 40 μg was got to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis (Invitrogen, Carlsbad, CA, USA). The membrane was blocked with TBST (NaCl 500 Mm, Tris 20 mM, pH7.5) containing 5% skim milk for 60 min, and then probed with the primary antibodies (Mouse anti human Bcl-2 1:300, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. The secondary antibody (Sheep anti mouse, 1:2000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was applied for 1 h. After each incubation, the membrane was thoroughly washed with TBST; then, the membrane was treated with ECL color solution (Thermo Fisher Scientific, Waltham, MA, USA) by Western blot and exposed to the GE-ImageQuant-LAS-4000 system (Invitrogen, Carlsbad, CA, USA).

Detection of Proliferation and Apoptosis of Hep-2

The proliferation of Hep-2 was detected by MTS cell proliferation and toxicity detection Kit (Beyotime Biotechnology, Shanghai, China) and the apoptosis of Hep-2 was detected by Annexin V-FITC apoptosis detection Kit (Nanjing KeyGen Biotech. Inc., Nanjing, China), according to the manufacturer's protocol. Absorbance was measured at 490 nm with an enzyme marker, and flow cytometry was used to detect the apoptosis.

Statistical Analysis

For the statistical analysis, a commercially available software package was used (SPSS 22.0, SPSS Inc., Chicago, IL, USA). Data are shown as mean ± SD. Kaplan-Meier method was used in survival analysis. Statistical significance was determined by Student's *t*-test and one-way variance (ANOVA), with a p-value of < 0.05 considered to be statistically significant.

Results

Average expression of miR-195 in LCT was 0.513 ± 0.482 times than that in adjacent normal tissues ($p < 0.01$, Figure 1). Relative expression ≤ 0.436 was set as low expression and > 0.436 as high expression. In this study, high expression and low expression was each for 49 cases. The result showed the low expression proportion of miR-195 was increased in T staging and clinical stage (III+IV) ($p < 0.05$), whereas it is the reverse in distant metastasis (M0) and early clinical stage (I+II) ($p < 0.05$). The expression of miR-195 had no statistically significant difference with

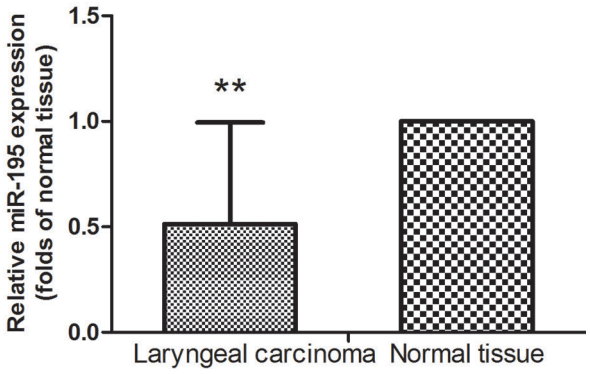

Figure 1. Relative expression of miR-195 in laryngeal carcinoma tissues and adjacent normal tissues.

age, gender, primary site, differentiation level, T staging, lymph node metastases, and preoperative smoking ($p > 0.05$, Table I).

Median follow-up time was 67.0 (15-104) months, 7 patients were lost to follow-up, and the follow-up rate was 92.9% combined active review with passive follow-up. Survival analysis by Kaplan-Meier method and log-rank test found average survival time with low expression was 68.152±5.859 months, which is shorter than that with high expression (90.329 ± 3.758 months, χ^2= 10.050, p = 0.002, Figure 2). Multivariate survival analysis by Cox regression model with

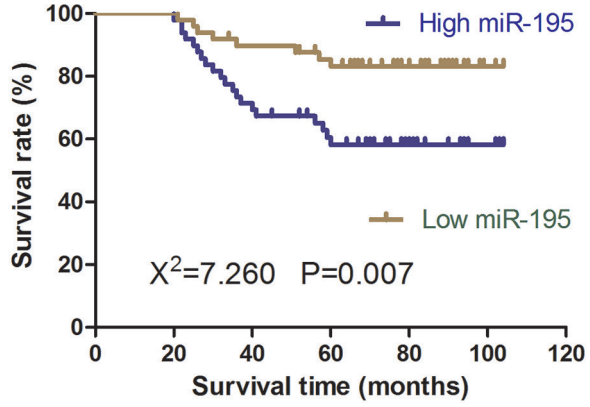

Figure 2. Survival curves of patients with different expression of miR-195.

age, gender, primary site, differentiation level, T staging, lymph node metastases, distant metastasis, clinical stage and preoperative smoking found miR-195 expression (RR = 0.126, 95%CI 0.045-0.350, p = 0.000) and lymph node metastases (RR = 11.319, 95%CI 4.286-29.894, p = 0.000) were independent prognostic factors for patients (Table II).

The change times of fluorescence activity in Bcl-2 WT 3'UTR group and the control group was a significant difference ($p < 0.01$), while which is no difference in Bcl-2 MUT 3'UTR group and the control group ($p > 0.05$). This means miR-195 has a direct regulatory relationship with its target gene Bcl-2 (Figure 3).

The results of Western-blot detection were showed in Figure 4. After 72 h with miR-195 or Bcl-2 siRNA, protein expression of Bcl-2 both increased ($p < 0.05$), but there was no increase in miR-195/Bcl-2 group ($p > 0.05$.) The same results also appeared in the detection experiment of proliferation and apoptosis of Hep-2 (Figure 5).

Discussion

LSCC is one of the common malignant tumors in the head and neck, which accounts for 25% of it and is a serious threat to the life of patients. The present studies found that the abnormal expression of multiple genes is associated with the prognosis of LSCC[5]. Looking for effective gene therapy target for laryngeal cancer research has been a hot and key point[6]. In recent years, microRNA (miRNA) has become the focus in the field of molecular biology and other fields. miRNA is a small non-coding RNA molecule (containing about 22 nucleotides) found in plants, animals, and some viruses that function in RNA silencing and post-transcriptional regulation of gene expression[7]. miRNA is either completely or partially combined with 3' untranslated region (UTR) of the target gene mRNA untranslated mediating cleavage or inhibiting translation of target

Table II. Multivariate Cox analysis of prognostic factors in patients with laryngeal squamous carcinoma.

Prognostic factors	Regression coefficient	Standard error	Wald	*p*-value	Relative risk	95% CI
miR-195 expression	-1.559	0.631	6.109	0.013	0.210	0.061-0.724
Lymph node metastases	1.174	0.456	6.621	0.010	3.234	1.323-7.906

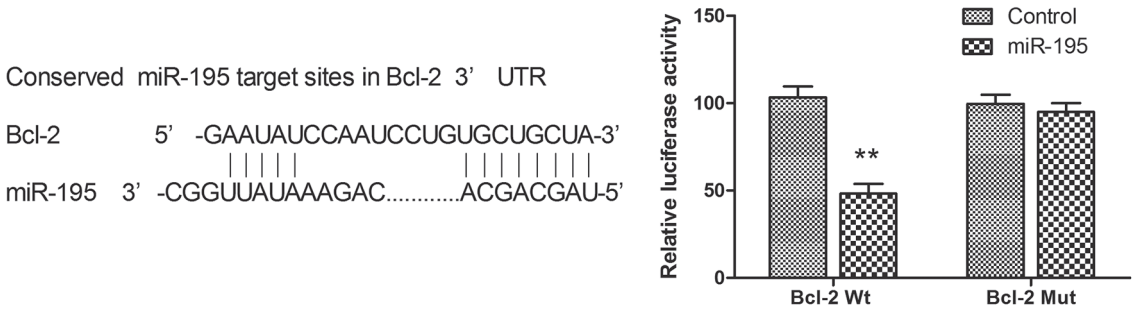

Figure 3. Relationship of miR-195-5p with target gene Bcl-2.

gene mRNA, which mainly regulate gene expression at the post-transcript level. miRNA was involved in regulating biological development and growth, cell differentiation, proliferation and apoptosis, and closely related to the occurrence and development of human tumors[8].

Recent studies[9] have indicated that there is a close relationship between miRNA and LSCC, which provides a new way for the study of the development and diagnosis of LSCC. The study found that the specific miRNA plays an important role in the abnormal expression of LSCC. Many up-regulated miRNA have the function of oncogenes, while other down-regulated miRNA may have the function of anti-oncogene. These abnormal expressions of miRNA are involved in the occurrence of LSCC. miR-195 is an important member of the microRNA-15/16/195/424/497 family, which was proved to maybe an important tumor suppressor. A large number of studies[10-13] have indicated that miR-195 is related to the mechanisms such as cell cycle, apoptosis, proliferation, and so on, which can promote cell division, apoptosis and inhibit cell proliferation at the same time. Bai et al[14] showed that miR-195 negatively regulated HIF-1α by targeting its 3'-untranslated region. Moreover, the founding indicated miR-195 greatly increased apoptosis and downregulated HIF-1α mRNA occurred simultaneously in hypoxic chondrocytes. MiR-195 still could enhance cardiomyocyte apoptosis induced by hypoxia/reoxygenation injury via downregulating c-myb[15].

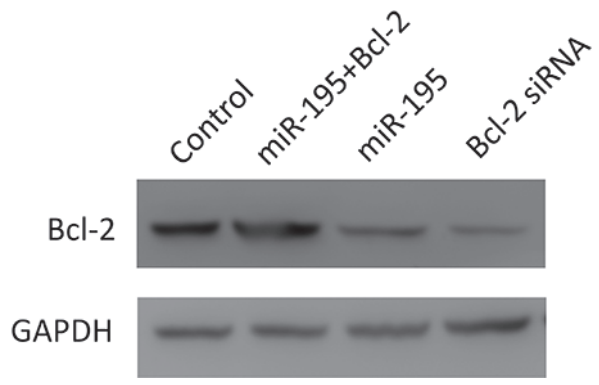

Figure 4. Protein expression of Bcl-2 by Western-blot.

Through quantitative RT-PCR of miR-195 expression in LCT and adjacent normal tissues from 98 cases, we found miR-195 expression is low in LCT indicating miR-195 may act as a tumor suppressor in LSCC. Statistical results showed that the expression of miR-195 was significantly associated with distance metastasis and clinical stage in patients with laryngeal squamous cell carcinoma ($p < 0.05$). Average survival time of patients with low expression was shorter than that with high expression by Kaplan-Meier method ($p < 0.01$). Multivariate Cox regression model showed miR-195 expression and lymph node metastases were independent prognostic factors for patients ($p < 0.05$). This suggests that miR-195 may play an important role in the occurrence and development of LSCC.

Cell apoptosis is a natural death process controlled by many genes. Bcl-2 is found to be one of the proto-oncogenes which are closely related to the apoptosis at present. In mitochondria, Bcl-2 family proteins regulate the stability of the structure and function of the mitochondrial membrane by a synergistic effect with other apoptotic proteins playing the role of "the main switch" of apoptosis, which plays an important role in the occurrence and development of tumor[16,17]. The Bcl-2 family proteins regulate apoptosis via mitochondrial maintenance. These proteins consist of anti- and pro-apoptotic members, and interactions of them decide whether the mitochondria should initiate the programmed death by releasing pro-apoptotic factors[18]. Liu et al[19] revealed

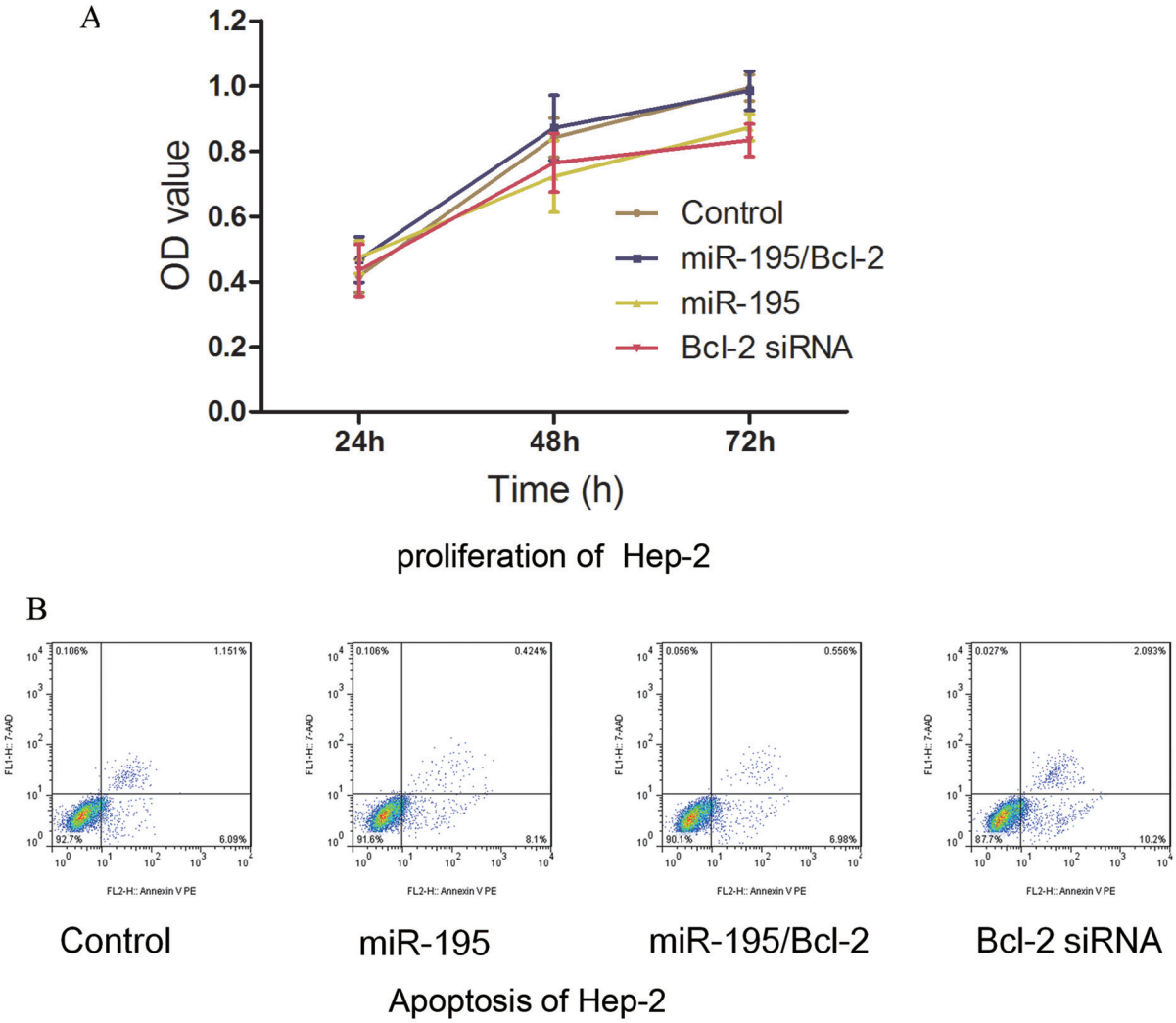

Figure 5. Proliferation and apoptosis of Hep-2.

that the expression of Bcl-2 is lower in atherosclerotic plaque due to its inhibition by miR-181a, suggesting inhibition of miR-181a might contribute to anti-atherosclerosis therapy. The high expression of Bcl-2 can regulate and enhance the expression of vascular endothelial growth factor, thereby favoring tumor angiogenesis[20]. In this study, dual-luciferase reporter plasmid with Bcl-2 wild type and mutant type 3' UTR was created to detect the fluorescence ratio changes after transfection. The results proved that miR-195 can be combined with the Bcl-2 3' UTR, which plays a regulation role of Bcl-2 after transcription.

We introduced miR-195, miR-195/Bcl-2 and Bcl-2 siRNA into Hep-2 for 72 h by liposome transfection technique in this study, and the results present miR-195 and Bcl-2 siRNA had successfully reduced the expression of the Bcl-2 protein, inhibited the proliferation of Hep-2 and induced the apoptosis of Hep-2 ($p < 0.01$). However, in miR-195/Bcl-2, there was no significant difference in the expression of the Bcl-2 protein, Hep-2 proliferation and Hep-2 apoptosis in LSCC ($p > 0.05$). The experiment proved that exogenous Bcl-2 could rescue the change of Bcl-2 protein, the proliferation and apoptosis of Hep-2 caused by overexpression of miR-195 in LSCC.

Conclusions

The expression of miR-195 was significantly decreased in LCT, which was closely related to the clinicopathological characteristics of LSCC. miR-195 may inhibit the proliferation and pro-

mote the apoptosis of Hep-2 by downregulating Bcl-2 expression *in vitro*. Thus, miR-195 as an anti-oncogene could be a potential target to diagnose and treat LSCC.

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Conflict of Interest
The Authors declare that they have no conflict of interests.

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