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Research Article

MiR-182 regulates cell proliferation and apoptosis in laryngeal squamous cell carcinoma by targeting the CRR9

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Background: The effect of miR-182 on the expressions of CRR9 in laryngeal squamous cell carcinoma (LSCC) cells, and the impact on invasion and metastasis of LSCC were investigated in the present paper.
Methods: The expressions of miR-182 in LSCC tissue and cell line were detected by RT-qPCR. MTT assay and Annexin V staining were used to detect the effects of miR-182 on tumor cells proliferation. Target gene prediction and screening, and luciferase reporter assay were designed to verify downstream target genes of miR-182. The mRNA and protein expressions of CRR9 were detected by qRT-PCR and Western blot. Finally, the expressions of CRR9 were measured by transfecting cells with miR-182 in mice.
Results: Compared with normal tissue and cell, the expressions of miR-182 in tumor tissues and cells were much lower. Over-expressions of miR-182 can increase apoptosis rate. Luciferase reporter assay revealed that CRR9 was a downstream gene of miR-182. Reintroduction of CRR9 abolished miR-182-induced LSCC cell growth inhibition. In animal models, over-expressions of miR-182 can reduce tumor weight and promote apoptosis.
Conclusion: miR-182 can inhibit the proliferation of LSCC cells by directly inhibiting the expressions of CRR9, thereby suppressing the occurrences and developments of LSCC.

Introduction

Laryngeal squamous cell carcinoma (LSCC) is the most common malignant tumor of the head and neck [1,2]. So far, its pathogenesis has not been studied clearly. Although patients with LSCC can be treated to a certain extent, the survival rate of patients has not improved [3,4]. In the past, researchers found that many factors promote the occurrence and malignant processes of LSCC, such as activation of oncogenes, inactivation of tumor suppressor genes, and abnormal expressions of growth factors [1,5,6]. A variety of kidney cancer-related genes and signaling pathways have been discovered, and many targeted therapeutic drugs have been introduced. However, their therapeutic effects on renal cancer are still limited, and there are still resistance problems [7–9]. Therefore, the majority of medical workers need to carry out further basic experiments to study the pathogenesis of renal cell carcinoma, and the changes of signal transduction pathways in cancer cells and the changes of oncogene or tumor suppressor genes. Comprehensive understanding of the biological characteristics of renal cell carcinoma is necessary so that we can find more targeted key targets and more effective clinical therapeutic drugs.

With the rapid development of modern molecular biology, more scholars are paying attention to the research of miRNA and malignant tumor occurrence and development process [10,11]. MiR-182 is one of the hotspots and critical points for medical research. It can regulate the expressions of different target genes, and a series of physiological processes such as growth, development, and cell differentiation. It plays the role of oncogene or tumor suppressor gene in the process of malignant tumor [12–14]. Studies have

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confirmed that miR-182 is abnormally expressed in a variety of solid malignancies, including lung cancer, pancreatic cancer, cholangiocarcinoma, colorectal cancer, prostate cancer, liver cancer, and breast cancer. These results suggest that it may play an oncogene-like role in these malignant tumors [15].

A large number of reports have confirmed the various life processes of CRR9 in living organisms' cells [16]. The current research on CRR9 has made some breakthroughs. It has shown that CRR9 is abnormally highly expressed in various human malignant tumor tissues and corresponding cell lines, especially in some malignant tumors with high invasiveness and metastasis [17]. CRR9 was first isolated by screening of the cisplatin (CDDP) resistance-related gene. CRR9 has a cancer-promoting property in lung cancer [18]. Notably, anti-CRR9 antibody robustly inhibited the growth of lung cancer xenografts [19]. Despite this progress, the exact biological properties of CRR9 are still obscure; thus, further studies are needed to elucidate the property of CRR9 in LSCC.

The present study aimed to explore the effects of the expressions of miR-182 on the proliferation, invasion, and metastasis of LSCC cells by detecting the expressions of miR-182 in tumor tissues and cells. We also hope to explore the regulation of the activation or inhibition of miR-182 signal on the expressions of CRR9 in LSCC cells and its biological effects on LSCC cells.

Materials and methods

Tissue specimens and cell culture

Thirty LSCC samples and their corresponding tumor-free samples were obtained from Ningbo Medical Center Li-huili Hospital during surgical resection. The study agreed with the Ethics Committee of Li huili hospital affiliated to Ningbo University. Experiments using human materials were performed in strict accordance with Declaration of Helsinki. Informed consent was signed for all patients. LSCC cells, Hep2 cells, and TU212 cells, normal human keratinocytes (HaCaT) and human embryonic kidney cell line (HEK293T) were cultured in RPMI-1640 containing 100 μg/ml streptomycin and 100 U/ml penicillin, (Gibco, Carlsbad, CA, U.S.A.). The cells were subcultured in a 5% CO_2 incubator at 37°C.

Oligonucleotides, lentiviral vectors, plasmid construction, and cell transfection

MiR-182 mimic, negative control oligonucleotide (miR-NC), miR-182 inhibitor (miR-182 inhibitor), scramble siRNA of CRR9 (si-NC) CRR9 pEGFP-N2 vector (ZFP36), negative control oligonucleotide (NC), small interfering RNA of CRR9 (siZFP36), control vector (Vector), were obtained from RiboBio (Guangzhou, China). MiRNA-182 mimics miRNA-182 were used to over-express miRNA-182. MiRNA-182 inhibitors were used to knockout miRNA-182. MiRNA-182-182 inhibitors and CRR9 inhibitors were used to knockout CC9. MiRNA-182 and CRR9 were knocked out using miRNA-182 inhibitor and CRR9, respectively. In strict accordance with the instructions, transfection was performed using Lipofectamine 2000 reagent (Invitrogen). After transfection, cells were cultured 48 h for further analysis, and finally, transfection efficiency was detected by qRT-PCR.

MTT method

The cells were seeded at a density of 5×10^3 per well in 96-well plates. Moreover, then, after transfection, the cells were cultured for 0, 24, 48, and 72 h. MTT was added into each pore cell. After cell adherence, the supernatant was then removed. Then, each pore cell added 150 μl of dimethyl sulfoxide. Microplate reader was used for analysis the absorbance at 490 nm (Thermo Fisher Scientific, Rockford, IL, U.S.A.).

Colony formation

The cells were seeded at a density of 5×10^3 per well in 96-well plates. Then, each pore cell added ADR for 24 h. Each well was washed, the culture was updated, and the cells were cultured in an incubator at 37°C for 8 days. Finally, colonies were counted after fixation for 10 min with 10% formaldehyde, and then colonies were combined with Crystal Violet for 10 min. GraphPad Prism 6 was used for analysis the cell viability (GraphPad Sofware, Inc., San Diego, CA, U.S.A.).

Annexin V-FITC-PI method

The cells were seeded at a density of 5×10^3 per well in six-well plates. In strict accordance with the instructions, each pore cell was collected. The cells were suspended in a binding buffer. In strict accordance with the instructions, the cells were combined with Annexin V-FITC-PI Assay Kit. FACS can flow cytometer was used for the analysis of the apoptotic rate (BD Biosciences, San Jose, CA, U.S.A.).

Tumor xenograft model

All the animals' experiments were done at the Animal Center of Li huili hospital affiliated to Ningbo University, and the ethical approval consent was obtained from the animal experiment ethics of Li huili hospital affiliated to Ningbo University. All procedures were performed when the mice were under anesthesia with sodium pentobarbital, and efforts were made to avoid animal suffering. Four to five weeks of female athymic nude mice were purchased from Shanghai SLAC Experimental Animal Co., Ltd. (Shanghai, China) and kept in animal resource facilities. Hep2 cells (5×10^6) or cells stably expressing miR-182 were subcutaneously injected into SCID mice (6–8 weeks old). The tumor was examined once a week, the length and width of the tumor were measured using a caliper, and the tumor volume was calculated using the equation $(L \times W^2)/2$. Six weeks later, the mice were killed, and the tumor was weighed.

Luciferase assay

The 3′-untranslated region (UTR) luciferase assay was used for the pmirGLO dual-luciferase miRNA target expressions vector (Hanheng, Shanghai, China). The cells were seeded at a density of 5×10^3 per well in six-well plates, and hsa-miR-182 mimics, and wild-type or mutant target sequences were co-transfected into each well using Lipofectamine 2000 (Invitrogen). Cells were then harvested at 48 h after transfection, and were cultured at 48 h. The activity of Freon was used by the dual-luciferase reporter system (Promega) was used for analysis. The activity of *Renilla* luciferase was used by the dual-luciferase reporter system (Promega). The average value of the results of the miR-control transfected cells was set to 1.0.

Real-time PCR analysis

Total RNA containing miRNA was collected extracted from the cells using by the RNeasy Mini Kit (Qiagen, Valencia, CA). According to the specifications of the instructions, Quantitec was used to reverse transcription kit (Shanghai, China) to synthesize the cDNA. cDNA was synthesized by the QuantiTect Reverse Transcription Kit (TransGen Biotech, Beijing, China) according to the manufacturer's recommended instructions. SYBR Green qRT-PCR master mix (TaKaRa, Otsu, Shiga, Japan) and GAPDH were used as internal controls. The expressions of miR-182 was measured using the mirVana qRT-PCR microRNA detection kit according to the manufacturer's protocol (Ambion Inc., Austin, TX).

Western blot

We extracted the proteins from cells, and centrifuged them for 5 min at 12000 r/min. The proteins were transferred to SDS and heated for denaturation; 1 μl cells were treated with lysate, and the proteins were electrophoresed by SDS/PAGE. Then, the proteins were sent to PVDF membranes, and were incubated with 1% BSA, and rabbit anti-polyclonal antibodies. After washing, 1:5000 anti-rabbit secondary antibody was used. ECL was utilized for signal visualization.

Statistical method

SPSS19.0 was used for data analysis, which were presented as mean $\pm$ SD. The comparison between two groups was performed by *t* test, and among three groups was carried out by one-way ANOVA. LSD was performed as well. $P<0.05$ indicated that the differences were statistically significant.

Results

MiR-182 was down-regulated in LSCC tissues and cell lines

In order to investigate the functional role of miR-182 in LSCC, 30 patients with LSCC were included, as shown in Figure 1A, compared with normal tissues, the expressions of miR-182 in LSCC tissue was significantly down-regulated. As shown in Figure 1B, miR-182 expression was significantly down-regulated in Hep2 cells and TU212 cells compared with the non-malignant laryngeal squamous cell line, HaCaT.

Effects of miR-182 on proliferation and apoptosis in LSCC cells

The expressions of miR-182 in Hep2 cells and TU212 cells after a scramble and miR-182 mimic were detected by qRT-PCR. The results in Figure 2A showed that compared with the scramble group, the expression level of miR-182 in the miR-182 mimic group was significantly higher ($P<0.05$). The proliferation activities of Hep2 cells and TU212 cells were measured by MTT method at 24, 48, and 72 h after transfection. The results showed that (Figure 2B), compared with the scramble group, the proliferation rate of Hep2 cells and TU212 cells transfected with miR-182

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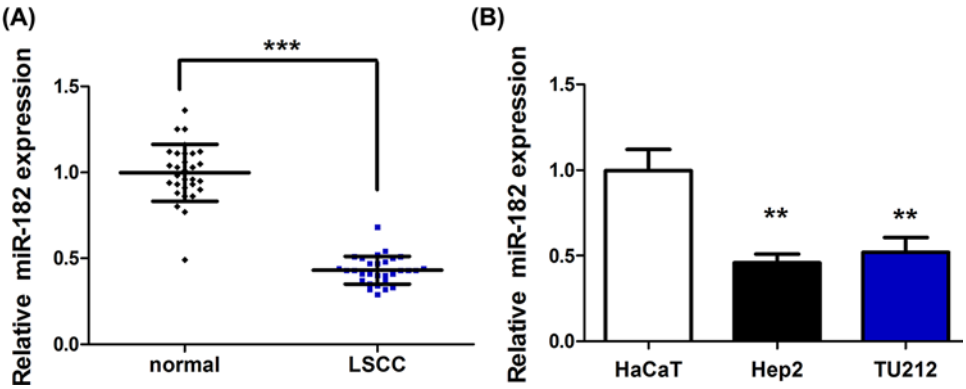

Figure 1. The expressions of miR-182 in LSCC samples and cell lines

(**A**) The level of miR-182 in 30 LSCCs in normal controls and LSCC samples. (**B**) Expression levels of miR-182 levels in Hep2 cells, TU212 cells, and HaCaT cells. $^{**}P<0.01$, $^{***}P<0.001$.

mimic at 72 h was significantly lower ($P<0.05$). These results suggested that miR-182 had an inhibitory effect on the proliferation of LSCC Hep2 cells and TU212 cells. The colony formation rate of Hep2 cells and TU212 cells was detected by colony formation experiments. The results showed that (Figure 2C) the colony formation rate of Hep2 cells and TU212 cells transfected with miR-182 mimic was lower than that of scramble group, and the difference was statistically significant ($P<0.05$). These results suggested that miR-182 had an inhibitory effect on the growth of LSCC Hep2 cells and TU212 cells. The apoptosis rate of Hep2 cells and TU212 cells was detected by Annexin V-PI. The results showed that (Figure 2D) the apoptosis rate of Hep2 cells and TU212 cells transfected with miR-182 mimic was significantly higher than that of the scramble group ($P<0.05$).

CRR9 was a direct target gene of miR-182

In order to determine the underlying mechanism of action of miR-182 in LSCC, CRR9 was identified as a potential target of miR-182 by bioinformatics prediction (Figure 3A). Subsequently, the miRNA-182 binding region was mutated in the CRR9 3′-UTR to verify the interaction between miRNA-182 and CRR9 3′-UTR. The wild-type CRR9 luciferase vector (CRR9-WT) and the mutant CRR9 luciferase vector (CRR9-MUT) were co-transfected into cells with miRNA-189 mimics. As shown in the results of Figure 3B, miRNA-182 mimics significantly reduced relative luciferase activity in cells transfected with the CRR9 (WT) vector, and mutations in the 3′-UTR matching site of CRR9 had no significant effect on luciferase activity, suggesting that the interaction between miR-182 and the binding site of the CRR9 3′-UTR can directly regulate the expressions of the luciferase reporter gene.

The difference in expressions of CRR9 between cells was analyzed by qRT-PCR and Western blot. The results of qRT-PCR as shown in Figure 3C showed that the expressions of CRR9 in Hep2 cells and TU212 cells transfected with miR-182 mimic were significantly lower than that in the miR-con group ($P<0.05$). Figure 3D found that compared with the scramble group, the expressions of CRR9 protein in Hep2 cells and TU212 cells transfected with miR-182 mimic was significantly lower ($P<0.05$).

Figure 3E showed that in the miR-182 mimic group and the miR-182 mimic + CRR9 group, the cell viability was significantly reduced compared with the control group. Compared with the miR-182 mimic group, the cell viability was significantly increased in the miR-182 mimic + CRR9 group ($P<0.05$). According to Figure 3F, in the miR-182 mimic group and the miR-182 mimic + CRR9 group, the apoptotic rate was significantly increased compared with that in the control group. In the miR-182 mimic + CRR9 group, the apoptotic rate was significantly reduced compared with that in the miR-182 mimic group ($P<0.05$). The results indicated that miR-182 inhibited the proliferation of LSCC by targeting CRR9.

As shown in Supplementary Figure S1, in the miR-182 mimic group, the level of expression of CRR9 and Bcl-xL was significantly increased compared with that in the control group, and co-transfection with CRR9 could partially eliminate the effect of miRNA-182 mimic on expression of CRR9 and Bcl-xL ($P<0.01$). The results indicated that miR-182 promoted the apoptosis of LSCC by targeting CRR9.

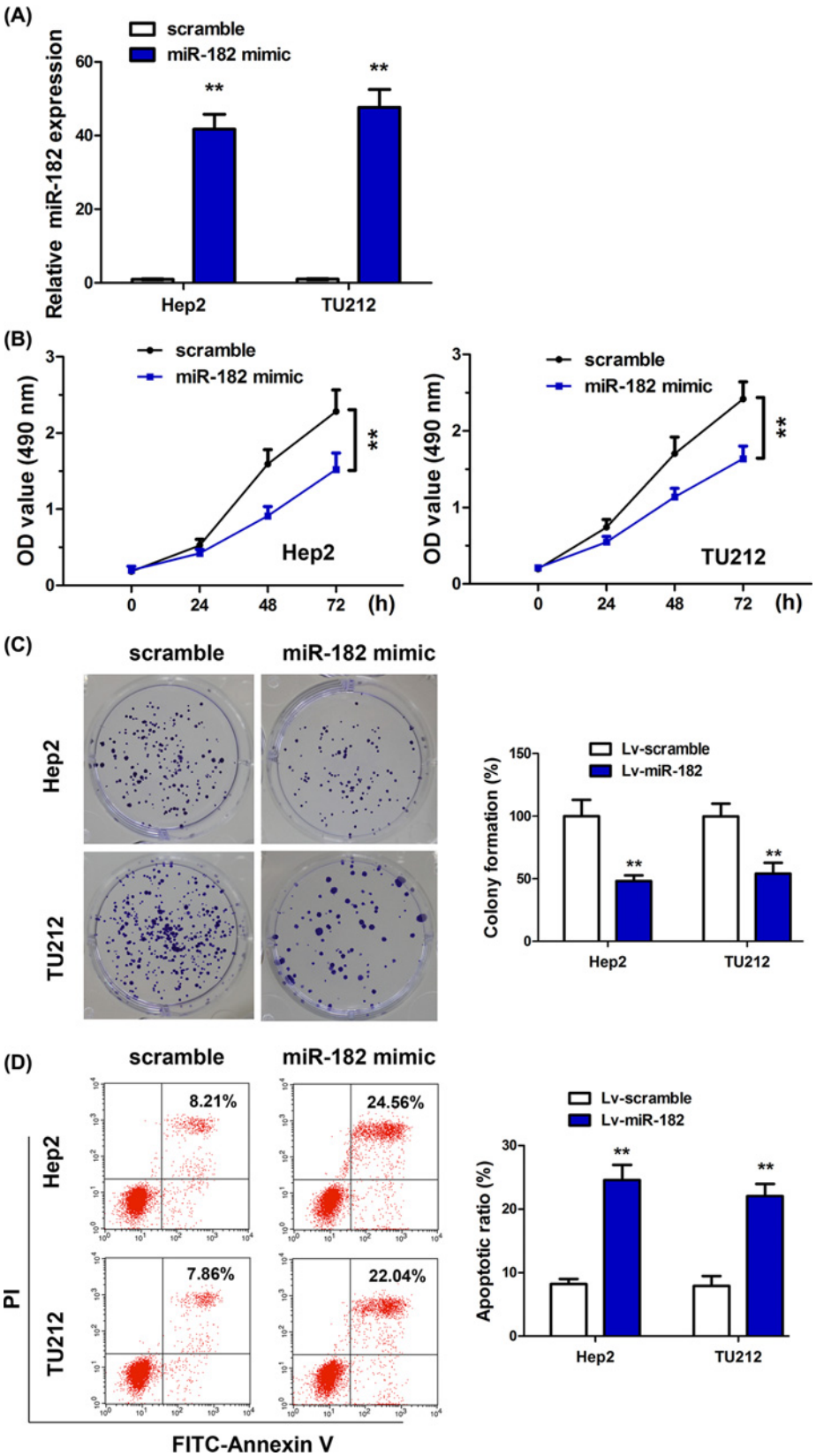

Figure 2. The expressions of miR-182 induced inhibition, proliferation and apoptosis in LSCC cells

(**A**) The expressions of miR-182 in LSCC cells transfected with miR-182 mimic or hybrid. (**B**) Cell viability. (**C**) Colony formation assay was examined by colony formation assay. (**D**) Apoptosis was assessed by Annexin V-PI staining. $^{**}P<0.01$.

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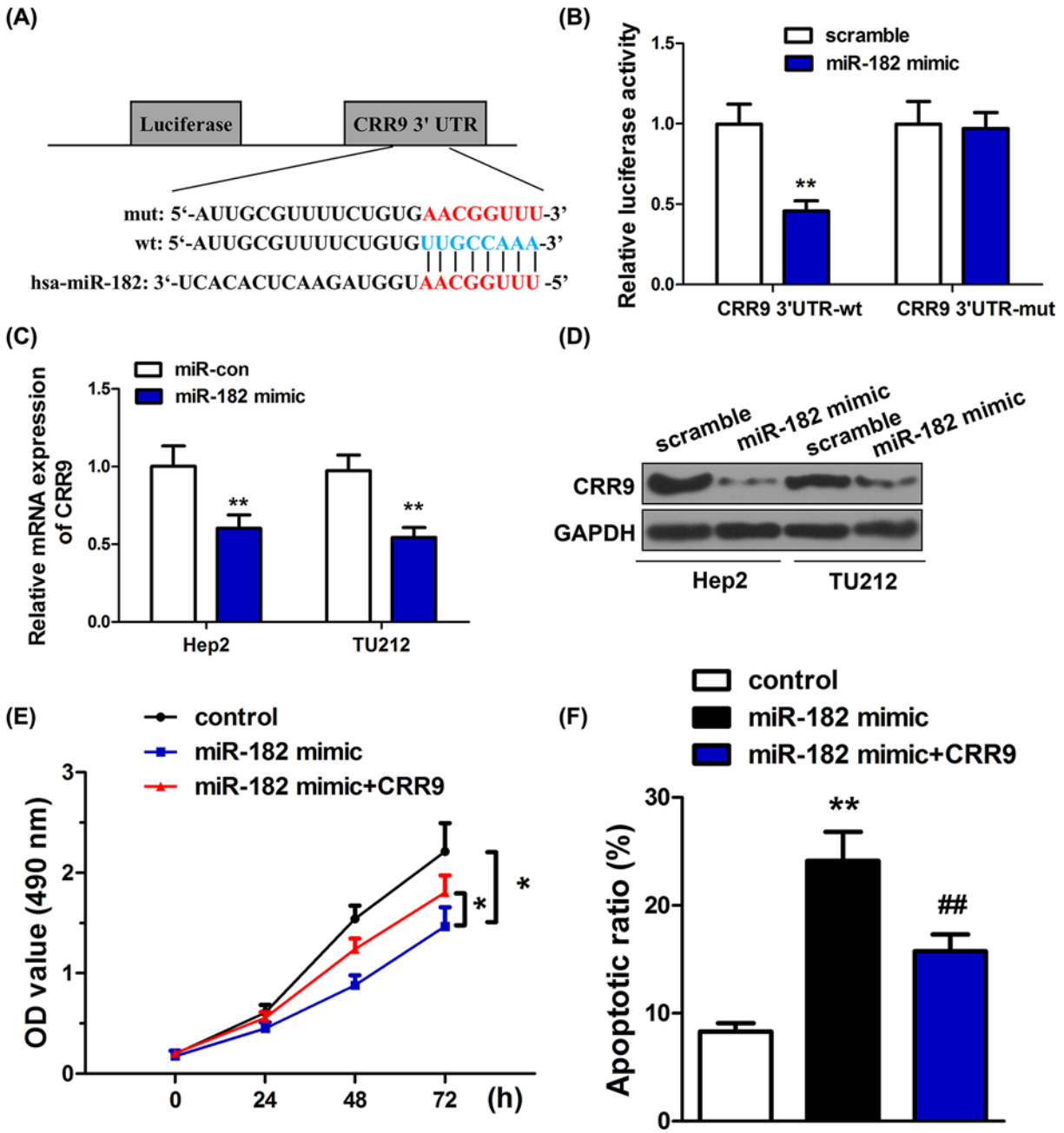

Figure 3. CRR9 was a direct target of miR-182

(**A**) The targeting site and corresponding mutant sequence in the 3′UTR of CRR9. (**B**) Relative luciferase activity of HEK293 cells after co-transfection with wild-type (WT) or mutant (MUT) CRR9 3′UTR luciferase reporter vector and miR-182 mimic or scramble. qRT-PCR (**C**) and Western blot (**D**) analysis of CRR9 expressions. Cell viability (**E**), cell viability (**F**) respectively. *$P<0.05$, **$P<0.01$.

MiR-182 regulated lesions in mice with LSCC

The effect of miR-182 on the pathological changes of LSCC mice was researched by establishing a mouse model of LSCC. Figure 4A,B revealed the volume of the xenograft tumor gradually increased with time. The volume of the xenograft tumor excised in the miR-182 group was significantly smaller than that of the scramble group. The volume difference of the xenograft tumors excised by the two groups of mice was statistically significant ($P<0.05$). As is shown in Figure 4C, the weight of the excised xenograft tumors in the miR-182 group was significantly smaller than that in the scramble group ($P<0.05$). The experiments *in vivo* were used to analyze whether miR-182 can regulate the

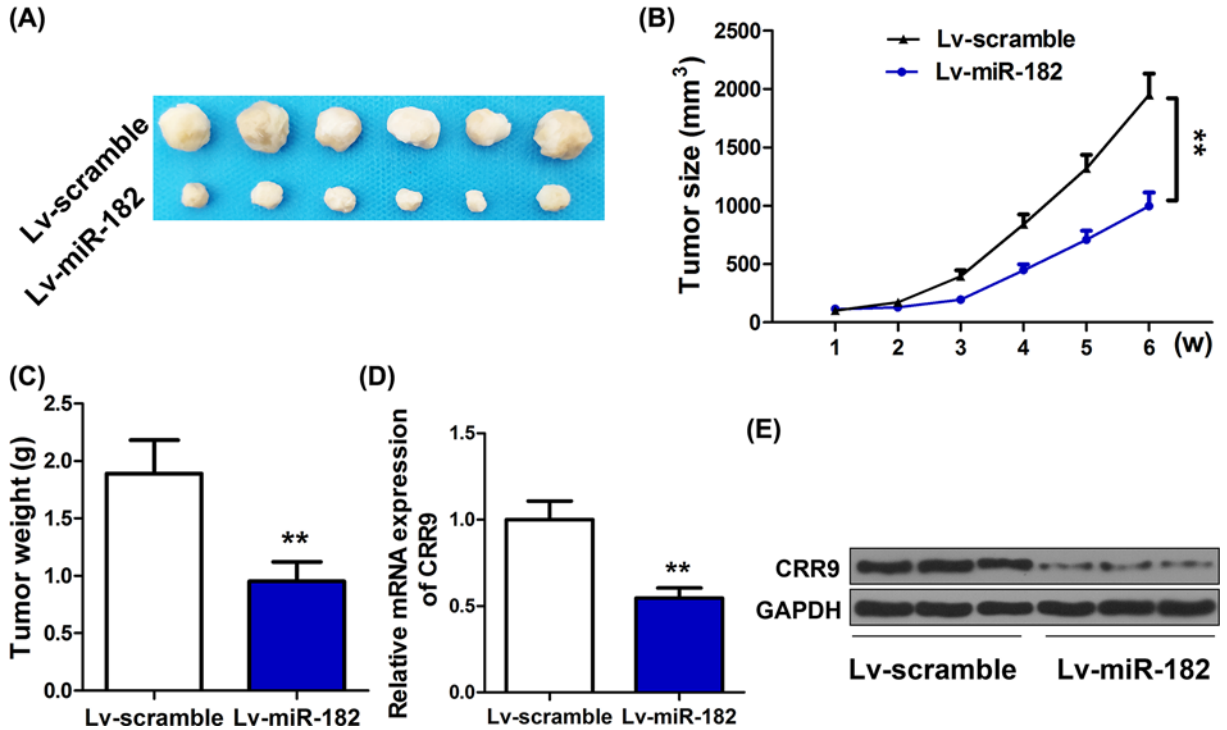

Figure 4. The expressions of miR-182 *in vivo*

(**A**) The images of tumor size (n=6). (**B**) Tumor growth curve. (**C**) Measurement of tumor weight. qRT-PCR (**D**) and Western blot (**E**) analyses of CRR9 expressions. $^{**}P<0.01$.

apoptosis of cells induced by CRR9. In Figure 4D,E, the expressions level of CRR9 in the miR-182 over-expressions group was significantly lower than that in the scramble group ($P<0.05$). In conclusion, *in vitro* and *in vivo* studies demonstrated that over-expressions of miR-182 could induce proliferation of LSCC cells by targeted regulation of CRR9.

Discussion

At present, laryngeal cancer is mainly treated by surgery. Although most patients' tumor development can be controlled through surgery, some patients still inevitably relapse and metastasize, which affects their survival rate [20,21]. Clinical observations have found that LSCC is characterized by rapid local invasion and early invasion and metastasis of cervical lymph nodes. Therefore, it is of great clinical significance to study the mechanism of growth, invasion, and metastasis of LSCC [22].

In recent years, miR-182 can regulate a series of physiological processes such as growth, development, and cell differentiation of biological organisms by regulating the expressions of different target genes. It also plays an oncogene in the process of malignant tumors [23,24]. The results of this study showed that the expressions of miR-182 in LSCC tissues and cells was significantly lower than that of normal adjacent normal tissues and normal cells ($P<0.05$). Studies also examined the expressions of miR-182 in breast cancer and its cell lines. The results indicated that the expression of miR-182 was significantly increased in breast cancer compared with adjacent normal tissues. In summary, miR-182 can be used as a molecular target for the treatment of LSCC, and can promote the development of tumor by promoting its expressions.

A series of *in vitro* cell experiments were performed to verify the function of miR-182 in the progression of malignant progression of LSCC and how it plays a role in tumor suppressor genes. Inhibition of miRNA expressions by over-expressions or silencing is used to study the mechanism of action of the corresponding miRNA in malignant tumors [25,26]. Mimics and inhibitors can more effectively up-regulate and down-regulate the expressions of miRNA [27,28]. Cell growth and proliferation are the most basic physiological characteristics of the organism. MiR-182 mimics were transfected into laryngeal squamous cells, and the results showed that when miR-182 was over-expressed in laryngeal squamous cells, the proliferation and growth ability are inhibited. It can be seen that miR-182 acted as a

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tumor suppressor gene in the development and progression of laryngeal squamous cells. The mechanism may be the inhibition of the growth, proliferation, invasion, and metastasis of laryngeal squamous cells through specific signaling pathways.

Studies have found that miR-182 can promote the metastasis of primary osteosarcoma; the mechanism was due to the direct action of miR-182 on its target genes *MSS1* and *Rsul* [29]. Therefore, miR-182 can play a different role in the malignant process, mainly dependent on its regulation of different downstream target genes. We finally screened the target gene of CRR9 to miR-182 through the database. The results showed that the luciferase expression was significantly decreased in the CRR9 3′UTR WT group, but not in the CRR9 3′UTR Mut group, suggesting that miR-182 regulated its expressions by targeting 3′UTR of CRR9 gene. In addition, the expressions levels of CRR9 mRNA and protein were significantly decreased when the expression of miR-182 was up-regulated in the cells compared, and the above experimental results were further confirmed by *in vivo* animal experiments. The above results further confirmed that miR-182 had a direct inhibitory effect on the expressions of CRR9. Moreover, miR-182 inhibited the proliferation of laryngeal squamous carcinoma cells by directly acting on CRR9.

Differentially expressed miRNAs have been found in tumor tissues of laryngeal cancer mice, suggesting that miRNA is involved in the development of laryngeal cancer. In the previous experiments, it was found that the expressions level of miR-182 was significantly decreased in squamous cancer cells. However, the role of mi RNAs in animal models of laryngeal cancer was controversial [30]. This study found that miR-182 can significantly reduce the weight and size of the excised xenograft tumors ($P<0.05$) in mice and the expressions of CRR9 in the miR-182 over-expressions group was significantly lower ($P<0.05$).

In summary, it was confirmed that miR-182 inhibited the proliferation of LSCC cells by directly inhibiting the expressions of CRR9. Further research will be conducted to identify the mechanism by which CRR9 affected the expressions of downstream genes. Therefore, further research is needed.

Conclusion

Over-expression of miR-182 significantly inhibited cell viability and induced apoptosis *in vitro*. CRR9 was identified as a novel and direct downstream target for miR-182. CRR9 was able to inhibit miR-182-induced cell growth inhibition *in vivo*. In summary, miR-182 inhibited cell proliferation and induced apoptosis of LSCC cells by directly targeting CRR9, providing experimental evidence for the clinical prognosis of the tumor and further targeted intervention therapy.

Author Contribution

Y.L.: study design, clinical studies, manuscript preparation and manuscript editing. D.Y.: clinical studies and statistical analysis. S.Q.: experimental studies. J.Z. and Z.S.: data acquisition and data analysis. Y.S.: statistical analysis. H.D.: guarantor of integrity of the entire study, study concepts, manuscript review and funding acquisition. All authors read and approved the final manuscript.

Ethics Approval and Informed Consent

The study agreed with the Ethics Committee of Li huili hospital affiliated to Ningbo University. Experiments using human materials were performed in strict accordance with Declaration of Helsinki. Informed consent was signed for all patients.

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Competing Interests

The authors declare there are no competing interests associated with the manuscript.

Abbreviations

Bcl-xL, B-cell lymphoma-extra large; CDDP, cisplatin; cDNA, complementary DNA; CRR9, cisplatin resistance-related protein 9; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LSCC, laryngeal squamous cell carcinoma; LSD, Least Significant Difference; PI, Propidium Iodide; qRT-PCR, real-time reverse transcription-PCR; SCID, Severe combined immunodeficient; UTR, untranslated region.

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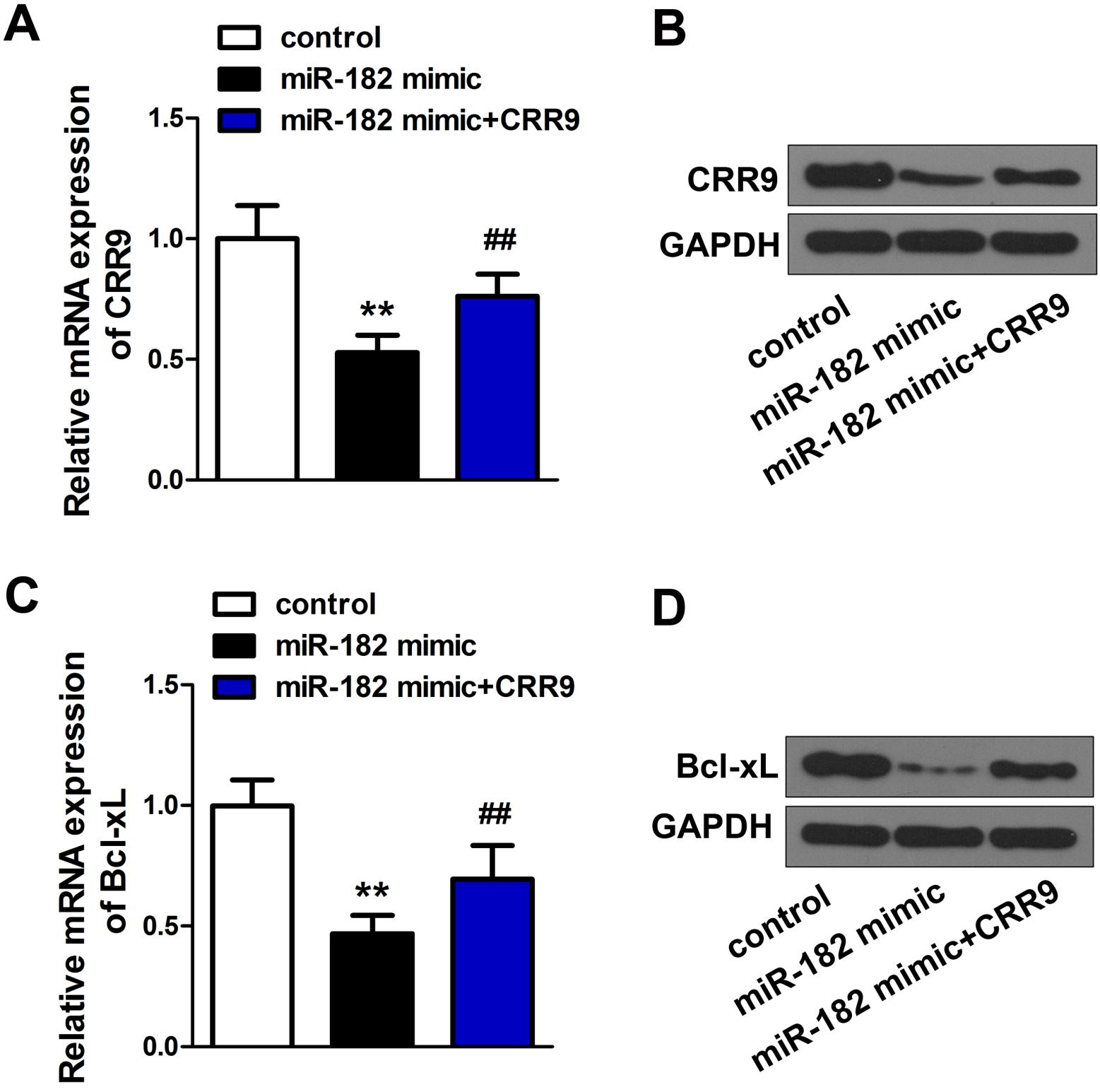
A
control
miR-182 mimic
miR-182 mimic+CRR9
Relative mRNA expression of CRR9
1.5
1.0
0.5
0.0
**
##
B
CRR9
GAPDH
control
miR-182 mimic
miR-182 mimic+CRR9
C
control
miR-182 mimic
miR-182 mimic+CRR9
Relative mRNA expression of Bcl-xL
1.5
1.0
0.5
0.0
**
##
D
Bcl-xL
GAPDH
control
miR-182 mimic
miR-182 mimic+CRR9

Supplementary figure 1. MiR-182 promoted the apoptosis of LSCC by targeting CRR9. qRT-PCR (A) and Western blot (B) analysis of CRR9 expressions. qRT-PCR (C) and Western blot (D) analysis of Bcl-xL expressions.