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Original Article

miR-590-5P inhibits the progression of tongue squamous cell carcinoma by targeting FasL

Yanxue Xu*, Tongtong Han*, Youming Zhu, Qiaoer Chen

*Stomatologic Hospital & College, Anhui Medical University, Key Laboratory of Oral Diseases Research of Anhui Province, Hefei 230032, China. *Equal contributors.*

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Abstract: Objective: This study evaluated microRNA-590-5P (miR-590-5P), which functions as an anti-onco-miRNA in TSCC by downregulating FasL expression. Methods: In this study, immunohistochemistry was used to detect FasL protein expression in 30 OSCC samples and 8 normal oral mucosa tissue samples. Target Scan was used to predict miRNAs that target FasL. Luciferase reporter assays were used to confirm the effects of miRNA on FasL. Subsequently, the SCC3 tongue cancer cell line was transfected with a miR-590-5P mimic or miR-590-5P inhibitor. qPCR and Western blots were used to detect the expression levels of miR-590-5P and FasL. SCC3 cell viability, apoptosis and growth were assayed by MTT assays, colony formation assays, and a xenograft model. Results: FasL expression was significantly higher in OSCC tissue samples than in normal oral mucosa tissue samples. miR-590-5P could downregulate the expression of FasL in vitro via direct binding to its 3'-untranslated region (3'-UTR). Overexpression of miR-590-5P inhibited the proliferation of SCC3 cells. Moreover, miR-590-5P increased the sensitivity of SCC3 cells to the chemotherapeutic agent cisplatin (DDP) and led to a significant decrease in colony formation ability. The xenograft experiment confirmed that miR-590-5P can suppress the development of TSCC. Conclusions: These results suggest that miR-590-5P targets FasL to inhibit the development of tongue cancer and that miR-590-5P may be a novel therapeutic target for TSCC.

Keywords: FasL, miRNA-590-5P, SCC3, tongue squamous cell carcinoma

Introduction

Oral squamous cell carcinoma (OSCC) is a complex and devastating disease. Tongue squamous cell carcinoma (TSCC) is one of the major subtypes of OSCC and remains a highly prevalent and lethal malignancy. Despite modern multidisciplinary treatments, including surgery, chemotherapy and radiotherapy, the 5-year survival rate of TSCC patients remains poor [1-3]. Therefore, it is imperative to find the underlying molecular mechanisms of TSCC and identify promising therapeutic targets to improve treatment strategies.

Fas is a type I transmembrane receptor that belongs to the tumour necrosis factor receptor superfamily. It is widely expressed in a variety of cell types, including activated B and T cells and neutrophils [4, 5]. FasL is a type II transmembrane protein that induces cells to send an apoptotic signal to Fas-positive cells [6]. FasL is expressed on activated T cells, natural killer (NK) cells, epithelial cells in the anterior chamber of the eye and other immune-privileged cells [7-9]. The induction of apoptosis is triggered by the interaction of Fas with FasL, which enables the recruitment of the adaptor protein Fas-associated death domain (FADD) and the binding of procaspase-8. Activated caspase-8 subsequently initiates the apoptosis-executing caspase cascade [10, 11]. Under normal circumstances, the Fas/FasL system is involved in maintaining T cell and NK cell cytotoxicity and regulating immune responses [6, 12]. Recent research has demonstrated that FasL functions in various solid tumours and can induce cancer cells to attack T cells [13-15]. Therefore, a better understanding of the mechanisms that regulate FasL expression may provide novel targets for therapeutic intervention.

miRNAs are a class of non-coding RNA molecules that are 18-24 nucleotides (nt) in length

Table 1. FasL expression in normal oral mucosa and OSCC

Group	Cases	FasL expression				Positive cases (n, %)	*P*
		(-)	(+)	(++)	(+++)		
Normal mucosa	8	8	0	0	0	0 (0.0)	
OSCC	30	12	5	8	5	18 (60.0)	<0.05*
Well differentiated	14	6	2	4	2	8 (57.1)	
Moderately differentiated	9	3	2	2	2	6 (66.6)	
Poorly differentiated	7	3	1	2	1	4 (57.1)	

OSCC, oral squamous cell carcinoma; FasL, Fas ligand; *Compared to the normal mucosal group.

and play critical roles in various biological processes, including cell cycle regulation, cell differentiation, apoptosis, growth and angiogenesis [16, 17]. miRNAs regulate specific mRNA targets by directly binding to miRNA recognition elements to suppress mRNA translation and degradation [18]. Studies have shown that targeting specific genes with miRNAs is a promising method for inhibiting cancer cell proliferation [19-21]. In this study, we observed that FasL was upregulated in OSCC patients. Then, we used bioinformatics tools to identify a potential miRNA (miR-590-5P) targeting FasL, and this miRNA bound to the FasL mRNA 3'-UTR to downregulate its expression. Our results showed that SCC3 cell proliferation was suppressed by the upregulation of miR-590-5P and induced by the inhibition of miR-590-5P. In addition, this research examined the inhibitory effect of miR-590-5P on TSCC in nude mice. miR-590-5P may be suitable for development as a therapeutic option for treating TSCC.

Materials and methods

Specimens

A total of 30 patients with OSCC from our hospital (Stomatologic Hospital of Anhui Medical University) were enrolled in the study. All patients provided informed consent, and this study was approved by the Ethics Committee of the Stomatologic Hospital of Anhui Medical University. Of the obtained specimens, 14 were well differentiated OSCC, 9 were moderately differentiated OSCC, and 7 were poorly differentiated OSCC. The patients did not receive radiotherapy or chemotherapy before surgery. Eight normal oral mucosa tissue samples were also obtained from our hospital. All specimens were fixed in 10% neutral formalin and embedded in paraffin.

Immunohistochemistry

Specimens were cut into 4-μm-thick sections. FasL expression was determined by the streptavidin-peroxidase (S-P) immunohistochemical technique. The ultraSensitive TMS-P kit, primary antibody and second antibody were purchased from Maixin-Biotechnology Company (Fuzhou, China). Tissue sections were incubated with a rabbit polyclonal anti-human FasL antibody at 4°C overnight and then washed with PBS. The sections were then incubated with a biotinylated secondary antibody for 10 min at room temperature and washed again with PBS. The stained sections were developed with 3, 3'-diaminobenzidine (DAB) for 5 min and counterstained with haematoxylin. PBS was used instead of primary antibody as a negative control following the manufacturer's instructions. Staining intensity was scored as 0, negative; 1, light yellow; 2, brown-yellow; and 3, brown. Sections with scores of 2 or 3 were defined as FasL (+). Ten random high-power fields were observed, and the percentage of positive cells was recorded. For the extent of staining, 1%-5% was defined as weak staining (+), 26%-50% as moderate staining (++), >51% as strong staining (+++), and 0% as negative staining (-).

Cell culture and transfection

SCC3 cells were purchased from the Institute of Materia Medica (Jiangsu, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum at 37°C and 5% CO_2. The miR-590-5P mimic, miR-590-5P inhibitor, negative control (NC) and NC inhibitor were synthesized by Shanghai GenePharma Company. After the cells reached 60% confluence, these agents were transfected into SCC3 cells by Oligofectamine (Invitrogen), according to the manufacturer's instructions.

Luciferase reporter assay

The FasL 3'-UTR with a predicted miR-590-5P binding site and the FasL 3'-UTR mutant were each cloned into the luciferase reporter vector.

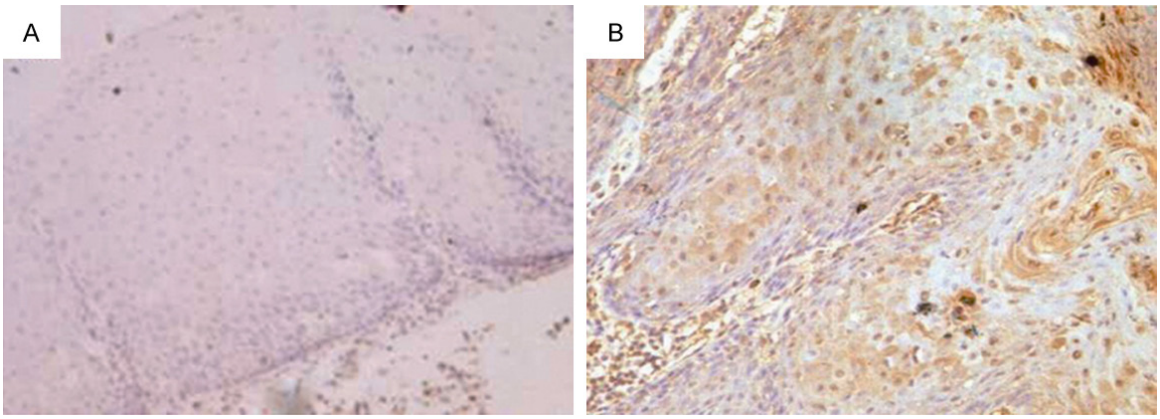

Figure 1. FasL expression by immunohistochemistry analysis of normal oral mucosa (A. magnification: × 100) and OSCC (B. magnification: × 200). FasL, Fas ligand; OSCC, oral squamous cell carcinoma.

SCC3 cells were grown to 60% confluence in 24-well plates and transfected with the luciferase reporter vectors and miR-590-5P or the NC. At 48 h after transfection, luciferase activity was measured by luciferase assays, according to the manufacturer's instructions. Renilla luciferase was used for normalization.

Real-time PCR assay

Total RNA was extracted from cells using TRIzol (Sigma-Aldrich, USA). RNA was reverse-transcribed into complementary DNA (cDNA) by using a PrimeScript RT kit according to the manufacturer's instructions (Takara, Japan). qPCR was performed with SYBR Premix Ex Taq (Takara, Japan), and ROX was then analysed using a Stratagene Mx3000p real-time PCR system (Agilent Technologies, USA). Small nuclear U6 and β-actin were used as internal controls. The primer sequences were as follows: U6 snRNA: 5'-GTGCTCGCTTCGGCAGCACAT-3' and 5'-TACCTTGCGAAGTGCTTAAAC-3'; hsa-miR-590-5P: 5'-GAGCTTATTCATAAAAGT-3' and 5'-TCCACGACACGCACTGGATACGAC-3'; FasL: 5'-AACTCAAGGTCCATGCCTCTG-3' and 5'-GGTGAGTTGAGGAGCTACAGACA-3'; and β-actin: 5'-TCCTGACCCTGAAGTACCCCATTG-3' and 5'-GGAACCGCTCATTGCCGATAGT-3'.

Western blot assays

Briefly, cells were harvested and boiled in sample loading buffer for 10 min. Cell proteins were harvested, separated by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto PVDF membranes (Millipore, USA). The proteins were probed using a primary antibody for anti-FasL (Santa Cruz Biotechnology, USA; 1:1000) and the appropriate secondary antibody. The bands were visualized by using an enhanced chemiluminescence method. GAPDH (Santa Cruz Biotechnology, USA; 1:1000) was used as a loading control.

MTT assay

Cells were seeded in 96-well plates and treated with DDP for the indicated periods of time. Twenty microliters of 5 mg/ml MTT stock solution was added to each well and incubated at 37°C for 4 h. The cells were then incubated with DMSO for 10 min at 37°C. The absorbance at 570 nm was recorded using a microplate reader.

Colony formation assay

Cells were plated in a 6-well plate at a density of 300 cells/well and cultured for 10 days. After fixation with 4% paraformaldehyde for 15 min, the colonies were stained with crystal violet for 4 h. Finally, the cells were washed with TBST.

Tumour formation assay

The xenograft experiment was conducted according to the NIH Guide for the Care and Use of Laboratory Animals, and all experimental procedures involving animals were approved by the Animal Care and Use Committee of Anhui Medical University. Six-week-old male nude mice (BALB/c) were used to examine tumourigenicity. A total of 1×10^6 SCC3 cells express-

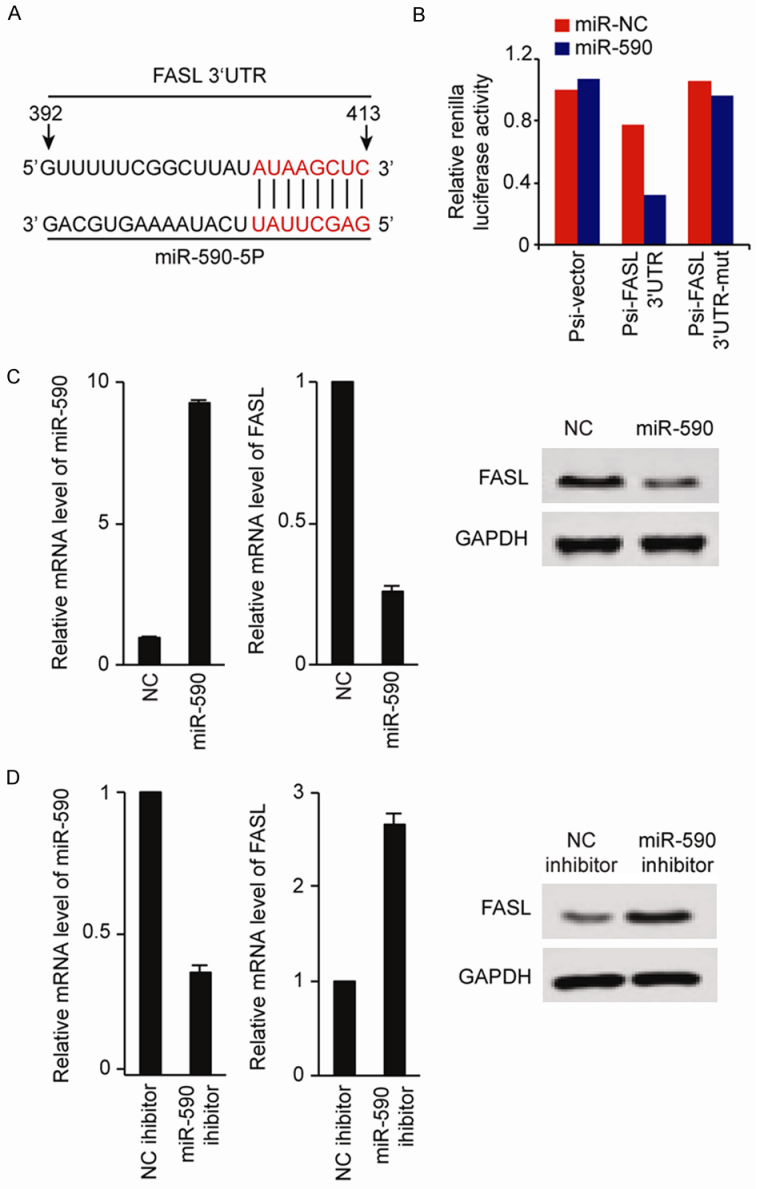

Figure 2. miR-590-5P suppresses FasL expression via direct binding to the FasL 3'-UTR. A. Predicted binding site of miR-590-5P to the FasL 3'-UTR. B. Relative luciferase activities measured in SCC3 cells following co-transfection with the luciferase reporter vectors and miR-590-5P mimic or NC. C. The expression of miR-590-5P mRNA and FasL protein was detected by qPCR and Western blot in SCC3 cells transfected with the miR-590-5P mimic. D. The expression of miR-590-5P mRNA and FasL protein was detected by qPCR and Western blot in SCC3 cells transfected with the miR-590-5P inhibitor. *P<0.05 vs. NC; #P<0.05 vs. NC inhibitor. miR-590, microRNA-590-5P; NC, negative control; FasL, Fas ligand; 3'-UTR, 3'-untranslated region; mut, mutant; mRNA, messenger RNA.

ing different genes were separately injected subcutaneously into the nude mice. The mice were euthanized after 2 weeks. The tumours were isolated for histology, and their sizes were compared.

Statistical analysis

Data are presented as the mean ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance; P<0.05 was considered statistically significant.

Results

FasL is upregulated in OSCC tissues

We investigated FasL expression in OSCC tissues and normal oral mucosa tissues using immunohistochemistry. The data showed that FasL expression was significantly upregulated in 18/30 (60.0%) of OSCC tissue specimens compared to normal oral mucosa tissues (P<0.05, **Table 1** and **Figure 1**). These results indicate that abnormal FasL expression may be related to OSCC progression.

miR-590-5P directly targets FasL and decreases FasL expression

In this study, we used the TargetScan program to predict miRNAs that target FasL, and we found one putative binding site for miR590-5P in the nucleotide sequence of FasL. FasL contains a miR-590-5P seed match at position 392-413 of its 3'-UTR (**Figure 2A**). Furthermore, luciferase reporter assays demonstrated that miR-590-5P significantly inhibi-ted the luciferase activity of the FasL 3'-UTR but not that of the FasL 3'-UTR mut in SCC3 cells (**Figure 2B**). To further determine whether FasL was the target of miR-590-5P, we transfected the miR-590-5P mimic, miR-590-5P inhibitor, NC and NC inhibitor into SCC3 cells. qPCR and Western blot assays showed that the mRNA and protein levels of

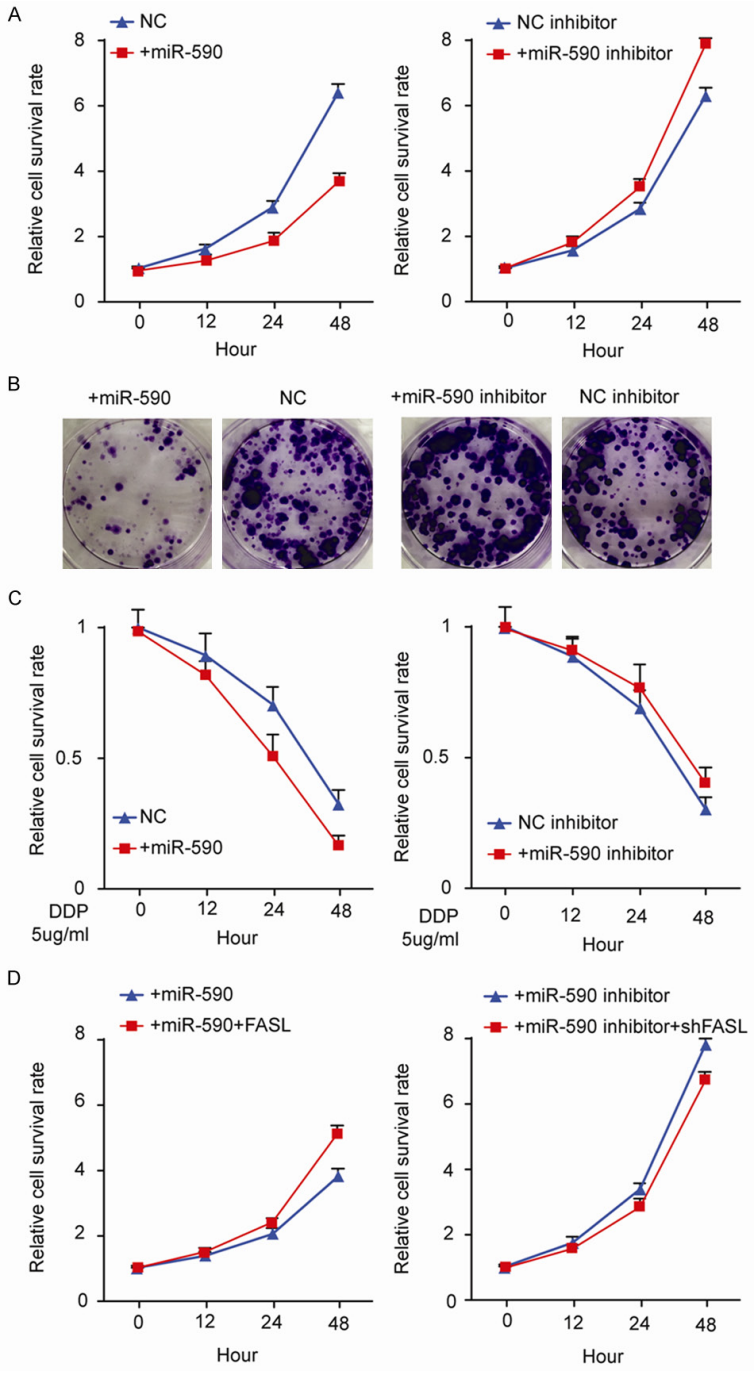

Figure 3. Effects of miR-590-5P on proliferation and apoptosis in SCC3 cells. A. MTT assays revealed that SCC3 cell proliferation was inhibited by the upregulation of miR-590-5P expression and promoted by the downregulation of miR-590-5P. B. Representative images of colony formation assays show that the colony formation ability of SCC3 cells was decreased by the overexpression of miR-590-5P and increased by the inhibition of miR-590-5P. C. MTT assays demonstrated that apoptosis in DDP-treated SCC3 cells was promoted by miR-590-5P and prevented by the miR-590-5P inhibitor. D. MTT assays indicating the proliferation ability of SCC3 cells transfected with miR-590-5P, miR-590-5P plus FasL, miR-590-5P inhibitor, and miR-590-5P inhibitor plus shFasL. *P<0.05 vs. NC; #P<0.05 vs. NC inhibitor. miR-590, microRNA-590-5P; NC, negative control; FasL, Fas ligand; DDP, cisplatin; shRNA, short hairpin RNA.

FasL were significantly suppressed when miR-590-5P was increased but were increased when miR-590-5P was silenced via inhibitor (**Figure 2C** and **2D**). These results implied that miR-590-5P directly binds to the 3'-UTR of FasL to downregulate its expression at the post-transcriptional level.

Upregulation of miR-590-5P inhibits SCC3 cell proliferation and induces apoptosis

To assess the effect of miR-590-5P on SCC3 cell proliferation, MTT and colony formation assays were performed. Cell proliferation was significantly inhibited by the overexpression of miR-590-5P but was upregulated by the knockdown of miR-590-5P (**Figure 3A**). Consistent with these results, colony formation assays showed that the ectopic expression of miR-590-5P markedly decreased the number of colonies, whereas inhibiting miR-590-5P expression increased the number of colonies (**Figure 3B**). Moreover, MTT assays showed that overexpressing miR-590-5P sensitized SCC3 cells to DDP treatment, but miR-590-5P suppression augmented the apoptosis resistance of DDP-treated SCC3 cells (**Figure 3C**).

miR-590-5P-induced cell growth arrest and apoptosis are mediated by FasL

In addition, to understand whether miR-590-5P affects cell proliferation via the FasL pathway, we performed MTT assays. The data showed that miR-590-5P-induced cell growth arrest was abolished by the upregulation of FasL. Likewise, cell growth induced by

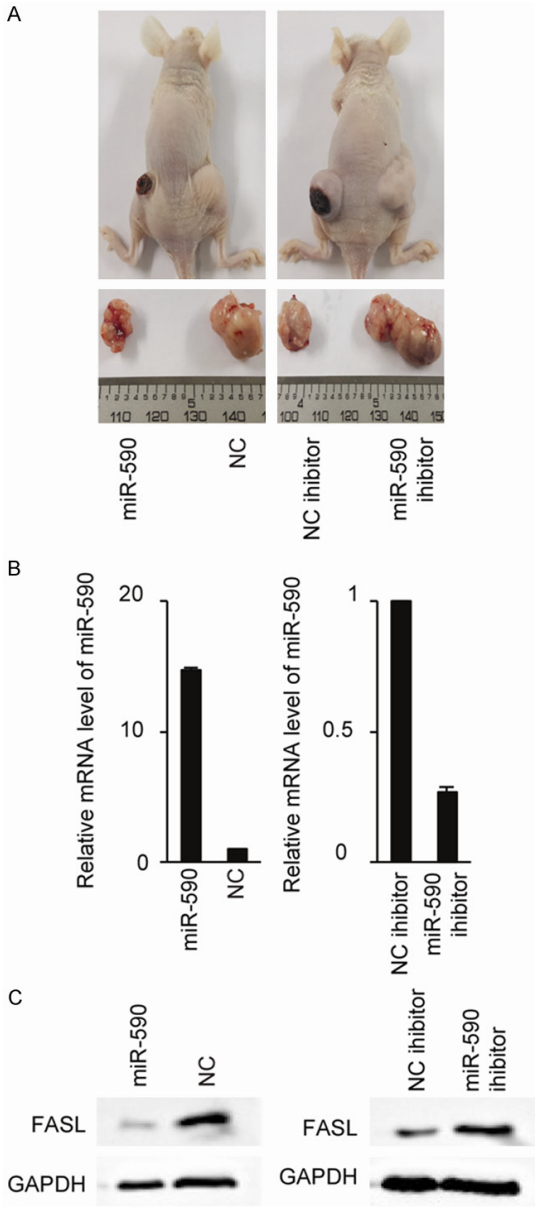

Figure 4. Effects of miR-590-5P on the growth of TSCC in vivo. A. Transfected SCC3 cells from different groups were subcutaneously injected into nude mice. Tumours were harvested after 2 weeks. Representative tumours from the different groups show that tumour growth in vivo was inhibited by miR-590-5P and promoted by the miR-590-5P inhibitor. B. qPCR showing that miR-590-5P mRNA expression in tumours was significantly upregulated in the miR-590-5P transfection group and downregulated in the miR-590-5P inhibitor group. C. Western blot showing that FasL protein expression in tumours was considerably downregulated in the miR-590-5P group and upregulated in the miR-590-5P inhibitor group. **P*<0.05 vs. NC; #*P*<0.05 vs. NC inhibitor. miR-590, microRNA-590-5P; NC, negative control; FasL, Fas ligand; mRNA, messenger RNA.

miR-590-5P inhibition was reversed by the downregulation of FasL (**Figure 3D**). Collectively, these results indicate that the effect of miR-590-5P on SCC3 cell proliferation is mediated by FasL.

miR-590-5P overexpression prevents TSCC growth in vivo

In the following experiments, we further examined the effect of miR-590-5P on TSCC progression in vivo. The administration of miR-590-5P caused a significant reduction in tumour volume compared with that of the NC group. The miR-590-5P inhibitor considerably increased the size of tumours compared with that in the NC inhibitor group (**Figure 4A**). Upon further exploration, we determined the relative expression of miR-590-5P and FasL in tumours. The level of miR-590-5P mRNA in the tumours of the miR-590-5P transfection group was markedly upregulated. In contrast, the expression of miR-590-5P in the miR-590-5P inhibitor group was considerably attenuated (**Figure 4B**). Western blots revealed that treatment with miR-590-5P caused a decrease in the FasL expression. However, the expression of FasL was dramatically increased in the tumours of the miR-590-5P inhibitor group (**Figure 4C**). In conclusion, miR-590-5P overexpression inhibits the growth of TSCC in vivo.

Discussion

TSCC, an aggressive type of cancer, is well-known for its high rate of proliferation and recurrence. Despite advancements in therapeutic strategies, the mortality rate of TSCC patients has not changed. Genetic alterations are considered the leading cause of cancer-related mortality. Identifying the involved genes and understanding the molecular genetics of TSCC are important for providing a theoretical basis for future treatments.

Accumulating evidence has suggested that FasL is overexpressed in multiple cancers, including cardia cancer, colon cancer, oesophageal cancer and other malignant tumours [22-24]. Therefore, several studies have focused on reducing the expression of FasL in tumours. Ji *et al.* stated that efficient downregulation of FasL expression could be achieved in basal cell carcinoma (BCC) and that this downregulation may prime BCC for the attack by immune ef-

fector cells [25]. In addition, Wu *et al.* found that suppressing FasL expression in breast cancer cells notably abrogated FasL-mediated apoptosis in T lymphocytes and that this effect prevented tumour immune privilege [26]. Thus, FasL can be considered a promising gene target for anti-tumour therapies.

miRNAs, a type of endogenous short non-coding RNA, act as negative gene regulators at the post-transcriptional level. Each miRNA can regulate many target genes, and an individual mRNA may also be regulated by multiple miRNAs; these processes give rise to an extensive regulatory network [27]. It has been well established that miRNAs play essential roles in tumourigenesis and may thus function as promising targets for the treatment of cancers. miR-590-5P has been proposed to function as an oncogene or tumour suppressor gene. Previously, Chu *et al.* demonstrated that miR-590-5P contributes to cervical cancer cell growth and invasion by targeting CHL1 [28]. Xiao *et al.* showed that miR-590-5P promotes clear cell renal carcinoma progression by targeting PBRM1 [29]. It has also been reported that miR-590-5P inhibits colorectal cancer angiogenesis and metastasis by regulating the nuclear factor 90/vascular endothelial growth factor A axis [30]. Nevertheless, there is little known about the role of miR-590-5P in TSCC. In this study, TargetScan online software predicted that the FasL 3'-UTR contained a miR-590-5P seed match. Next, luciferase reporter assays confirmed that miR-590-5P targeted the FasL 3'-UTR. Finally, the results demonstrated significant suppression of FasL expression, as well as decreased SCC3 cell proliferation and tumour formation in vitro and in vivo, after transfection with miR-590-5P. In contrast, transfection of the miR-590-5P inhibitor into SCC3 cells upregulated FasL levels and promoted cell growth and cancer development.

Taken together, the findings of the present study reveal that miR-590-5P could inhibit TSCC cell growth by downregulating FasL. Consequently, targeting the miR-590-5P/FasL axis may represent a potential therapeutic strategy for treating TSCC. Furthermore, it is important to understand the underlying mechanism in greater detail, and the clinical application of miR-590-5P requires thorough investigation. Solutions to these issues might lead to the development of novel gene therapy strategies in the future.

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Disclosure of conflict of interest

None.

Address correspondence to: Qiaoer Chen, Stomatologic Hospital & College, Anhui Medical University, Key Laboratory of Oral Diseases Research of Anhui Province, 69 Meishan Rd, Hefei 230032, China. Tel: +86-13955135875; Fax: +86-551-65111538; E-mail: chqe0111@163.com

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