

Original Article

miR-98 suppresses tumor cell growth and metastasis by targeting IGF1R in oral squamous cell carcinoma

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Abstract: Increasing evidences indicate that dysregulation of miRNAs contributes to the pathogenesis of oral squamous cell carcinoma (OSCC). However, little is known about the potential role of miR-98 in OSCC. Here, we found that miR-98 was downregulated in OSCC tissues and cell lines. Overexpression of miR-98 inhibited proliferation, colony formation, migration, and invasion of OSCC cells. IGF1R was identified as the potential target of miR-98 using dual luciferase assay, qRT-PCR and western blot. Furthermore, restoration of IGF1R remarkably reversed the tumor-suppressive effects of miR-98 on OSCC cells. Moreover, miR-98 expression was inversely correlated with IGF1R expression in 19 cases of OSCC. These findings suggest that miR-98 inhibits cancer cell growth and metastasis by direct targeting IGF1R, implicating miR-98 as a novel potential therapeutic target for OSCC.

Keywords: oral squamous cell carcinoma, miR-98, IGF1R, proliferation, migration, invasion

Introduction

Oral cancer is frequently the most common type of head and neck cancers in terms of incidence and mortality, and oral squamous cell carcinoma (OSCC) accounts for 95% of all oral cancer cases. Despite recent advances in diagnosis, surgery and chemotherapy strategy, the 5-year survival rate for OSCC is still quite low [1-3]. Therefore, it is urgent to elucidate the molecular mechanisms underlying tumorigenesis and progression of OSCC to identify novel markers for the diagnosis and treatment for OSCC. Recently, emerging evidences demonstrate that deregulation of microRNAs (miRNAs) can also contribute to OSCC development by influencing cell growth, apoptosis, migration, or invasion except for the multiple genetic and epigenetic changes to protein coding genes in OSCC [4].

miRNAs are a class of small noncoding RNAs that are approximately 20 nucleotides in length, which could regulate gene expression post-transcriptionally through base pairing with the 3'-untranslated region (3'-UTR) of target mRNAs [5]. Accumulating evidence shows that abnor-

mal miRNAs can function as oncogenes or tumor suppressors in the initiation and progression of various cancers, including OSCC [4, 5]. For instance miR-21 [6], let-7b [7], and miR-31 [8], have been proved to contribute to the growth and metastasis of OSCC. miR-98 functions as a tumor suppressor in some cancers [9, 10], but the detailed role of miR-98 in OSCC is unclear.

In this study, we investigated the expression level of miR-98 in OSCC tissues and cell lines. Furthermore, we explored the role and underlying molecular mechanism of miR-98 on the carcinogenesis of OSCC by targeting the Insulin-like growth factor 1 receptor (IGF1R).

Materials and methods

OSCC tissues and cell culture

19 samples of primary OSCC tissues and paired normal tissues were obtained from our hospital. Fresh samples were snap frozen in liquid nitrogen immediately after resection and stored at -80°C. The present study was approved by the ethics committee of School and Hospital of

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Stomatology, Wenzhou Medical University. Written informed consent was obtained from each patient.

OSCC cell lines SCC-25 and Tca-8113 were obtained from ATCC and Ninth People's Hospital, Shanghai Second Medical University, respectively. HEK 293 cell line was obtained from the Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences. All cells were cultured in Dulbecco's MEM (Invitrogen, Carlsbad, CA, USA). All the media were supplemented with 10% fetal bovine serum (FBS, PAA) at 37°C under 5% CO₂.

Plasmid construction and transfection

The oligonucleotides of pre-miR-98 sense (5'-AATTCAGATTCTGCTCATGCCAGGGTGGAGGTAGTAAGTTGTATTGTTGTGGGGTAGGGATATTAGGCCCAATAGAAGATAACTATACAACCTACTACTTCCCTGGTGTGGCATATTCAA-3') and anti-sense (5'-AGCTTTGAATATGCCACACACCAGGGAAGTAGTAAGTTGTATAGTTATCTTCTAATTGGGGCCTAATATCCCTACCCCACTTCAATACAACCTACTACTCACCCCTGGCATGAGCAGAATCCTG-3') were synthesized (Sangon Biotech, Shanghai, China) and cloned into pcDNA6.2-GW plasmid. IGF1R cDNA was purchased from Sino Biological Inc. (Beijing, China). The complimentary sites in 3'UTR of IGF-1R (wild-type IGF1R/IGF1R-wt, sense: 5'-CTTACAAGCCTCCTGTACCTCAGC-3', anti-sense: 5'-TCGAGCTGAGGTACAGGAGGCTTGTGAAGAGCT-3') and mutation (mutation of IGF1R/IGF1R-mt, sense: 5'-CTTACAAGCCTCCTGTAGGAGAGC-3'; antisense: 5'-TCGAGCTCCTACAGGAGGCTTGTGAAGAGCT-3') for miR-98 were synthesized and cloned into pmirGLO plasmid at Sac 1 and Xho 1 sites (Promega). Cell transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

RNA preparation and quantitative real time PCR (qRT-PCR)

Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA). miR-98 was reversely transcribed into cDNA by special RT-primer (5'-GTCGTATCCAGTGCCTGTCGTGGAGTCGGCAATTGCACTGGATACGACAACAATA-3'). The quantitative real-time PCR (qRT-PCR) was performed using a SYBR Green mix (Takara, Tokyo, Japan) on ABI 7900 (ABI, Foster City, CA, USA). The following primers were used: miR-98 forward: 5'-ATCCAGTGCCTGTCGTG-3', reverse: 5'-TGCT-

TGAGGTAGTAAGTTG-3'. IGF1R forward: 5'-TTAAATGGCCAGAACCTG-3', reverse: 5'-ATTATAACCAAGCCTCCAC-3'. U6 and β -actin were used as control. Data were normalized using the 2^{- $\Delta\Delta$ CT} method relative to U6 and β -actin. Each assay was repeated in triplicate.

Cell proliferation assay

The cells were plated in 96-well plates at 3000 cells/well. The (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was performed at 24, 48 and 72h, respectively. A 20 μ l of MTT solution was added and incubated for 4 h at 37°C, then the supernatant was discarded, and 150 μ l Dimethyl sulfoxide (DMSO) was added to each well. Optical density (OD) was measured at 492 nm in a microplate spectrophotometer.

Colony formation assay

2000 transfected cells were seeded into 12-well plate, 2 weeks later, Colonies were fixed and stained with 0.5% crystal violet for 15 min. Then the clones were counted and normalized to the control group under a microscope.

Migration and invasion assays

1 \times 10⁵ transfected cells were plated into cell culture inserts with microporous filters (BD, U.S.) coated with (invasion) or without (migration) Matrigel. After 24 h incubation at 37°C, Cells remained on the upper surfaces of the membranes were removed using cotton swabs, cells adhering to the lower membrane were fixed and stained with 0.1% crystal violet and imaged using microscope. All experiments were repeated for three times.

Luciferase reporter assay

The IGF1R-wt or IGF1R-mt and pre-miR-98 were co-transfected into HEK-293 cells. Forty-eight hours after transfection, cells were harvested and luciferase activity was measured using Dual-Luciferase Reporter Assay System (Promega). Experiments were repeated three times independently.

Western blot

Cells were washed twice with cold PBS and lysed on ice in RIPA buffer (Beyotime, Shanghai, China). Protein was loaded and separated by 10% SDS-PAGE gel and transferred onto PVDF membrane. The membranes were blocked with

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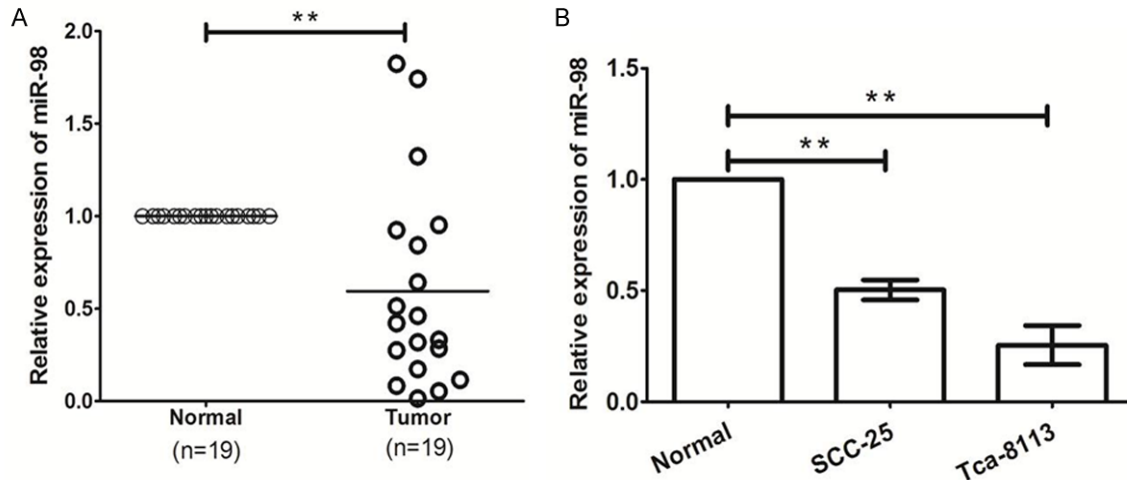


Figure 1. miR-98 is downregulated in OSCC tissues and cell lines. A. Relative expression level of miR-98 in 19 pairs of OSCC tissue samples and paired normal tissues was analyzed using qRT-PCR. B. Relative expression of miR-98 in two OSCC cell lines (Tca-8113 and SCC-25 cells) and the normal epithelial cells. U6 was used as an endogenous control. Data are mean \pm SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$.

5% non-fat milk in Tris-buffered saline Tween-20 (TBST) for 0.5 h at room temperature and incubated with specific primary antibodies overnight at 4°C. Then with secondary antibodies labeled with HRP and detected by ECL.

Statistical analysis

Data are expressed as mean \pm SD, and analyzed using SPSS 13.0. The differences between groups were analyzed using one-way ANOVA or non-paired *t* test. The correlation between miR-98 expression and IGF1R mRNA expression was measured by Pearson's correlation. $P < 0.05$ was considered as statistically significant.

Results

miR-98 is downregulated in OSCC tissue samples and cell lines

To study the expression of miR-98 in OSCC, we measured the expression of miR-98 in 19 pairs of OSCC and matched normal tissues using qRT-PCR. As shown in **Figure 1A**, the miR-98 was significantly decreased in OSCC tissues compared with the adjacent normal tissues ($P = 0.005$). Furthermore, the expression of miR-98 was also downregulated in Tca-8113 and SCC-25 cell lines compared with the normal epithelial cells (**Figure 1B**, $P = 0.003$, $P = 0.005$).

miR-98 suppresses the growth of OSCC cells

To ascertain the functional effect of miR-98 on growth. We firstly transfected OSCC cells with pre-miR-98. The expression of miR-98 was measured by qRT-PCR, 24 h after transfection. We found that the miR-98 was significantly increased by in transfected Tca-8113 and SCC-25 cells (**Figure 2A**, $P = 0.001$, $P = 0.003$). MTT assay was used to examine the proliferation of OSCC cells, overexpression of miR-98 significantly suppressed proliferation of Tca-8113 and SCC-25 cells compared with the control group (miR-Ctrl) (**Figure 2B**, $P < 0.05$). Similarly, miR-98 overexpression substantially suppressed colony formation of Tca-8113 and SCC-25 cells (**Figure 2C**, $P = 0.008$, $P = 0.004$).

miR-98 suppresses OSCC cell migration and invasion

To explore the function of miR-98 in the regulation of cell migration and invasion. Tca-8113 cells were transfected with pre-miR-98 or miR-Ctrl, after 24 h, as shown in **Figure 3**, overexpression of miR-98 dramatically suppressed tumor cell migration in Tca-8113 cells compared with miR-Ctrl group ($P = 0.011$, $P = 0.009$).

IGF1R is a target of miR-98 in OSCC cells

To uncover the mechanism by which miR-98 suppresses OSCC cell growth and invasion,

miR-98 suppresses OSCC progression by targeting IGF1R

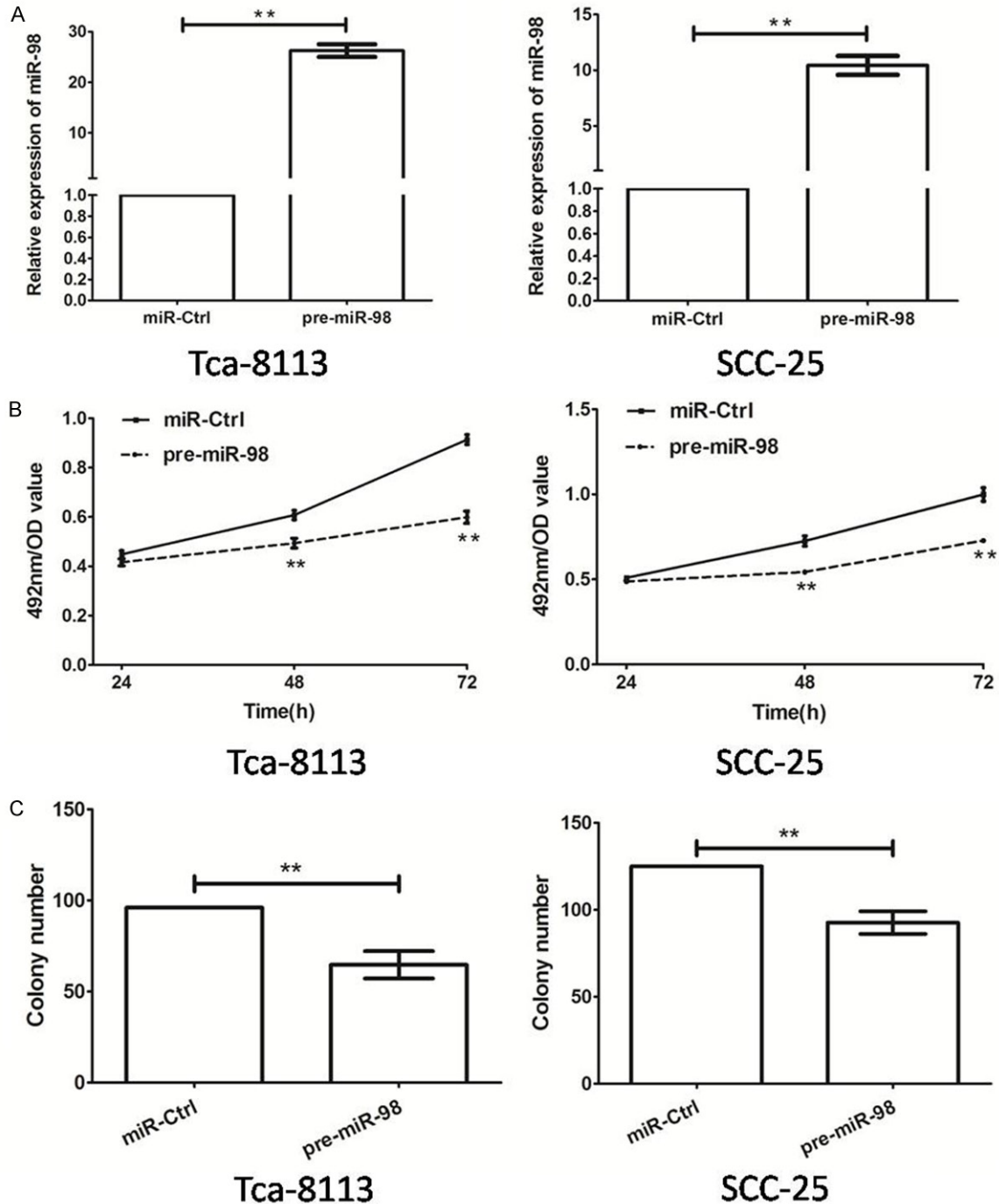


Figure 2. Ectopic expression of miR-98 inhibits growth of OSCC cells. A. The relative expression level of miR-98 in Tca-8113 and SCC-25 cells were determined by qRT-PCR after transfection with pre-miR-98 or miR-Ctrl. U6 was used as an endogenous control. B. The proliferation was determined by MTT assay after transfection with pre-miR-98 or miR-Ctrl. C. The capacity of colony formation was determined by colony formation assay after transfection with pre-miR-98 or miR-Ctrl. Data are mean \pm SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$.

TargetScan 6.2 was used. IGF1R was predicted to be a target of miR-98. Wild type or the mutated IGF1R 3'-UTR sequences (IGF1R-wt/IGF1R-mt) were amplified and sub-cloned into the

luciferase reporter vector (Figure 4A). Luciferase activity assay showed that miR-98 significantly inhibited the WT but not the Mt luciferase activity of IGF1R in HEK-293 cells (Figure

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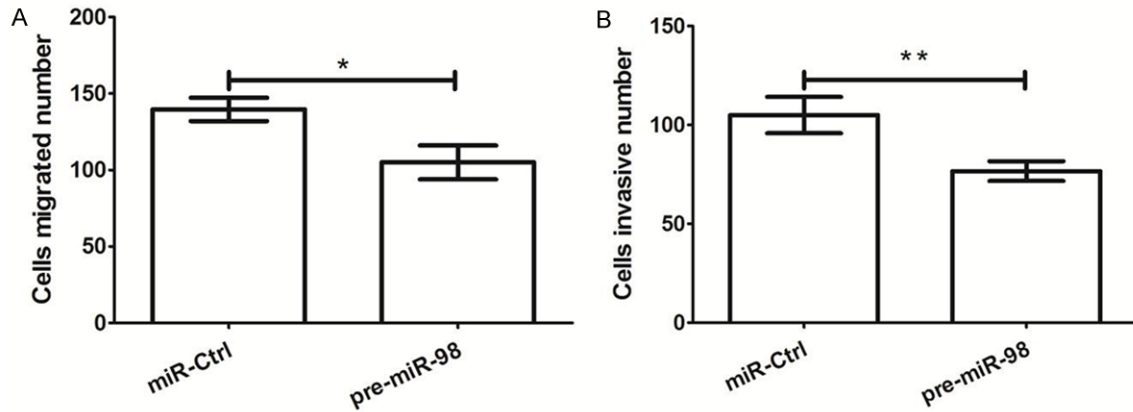


Figure 3. Ectopic expression of miR-98 inhibits migration and invasion of OSCC cells. The migration (A) and invasion assays (B) of Tca-8113 cells transfected with pre-miR-98 or miR-Ctrl. Experiments were performed in triplicate. *P<0.05, **P<0.01 compared with control.

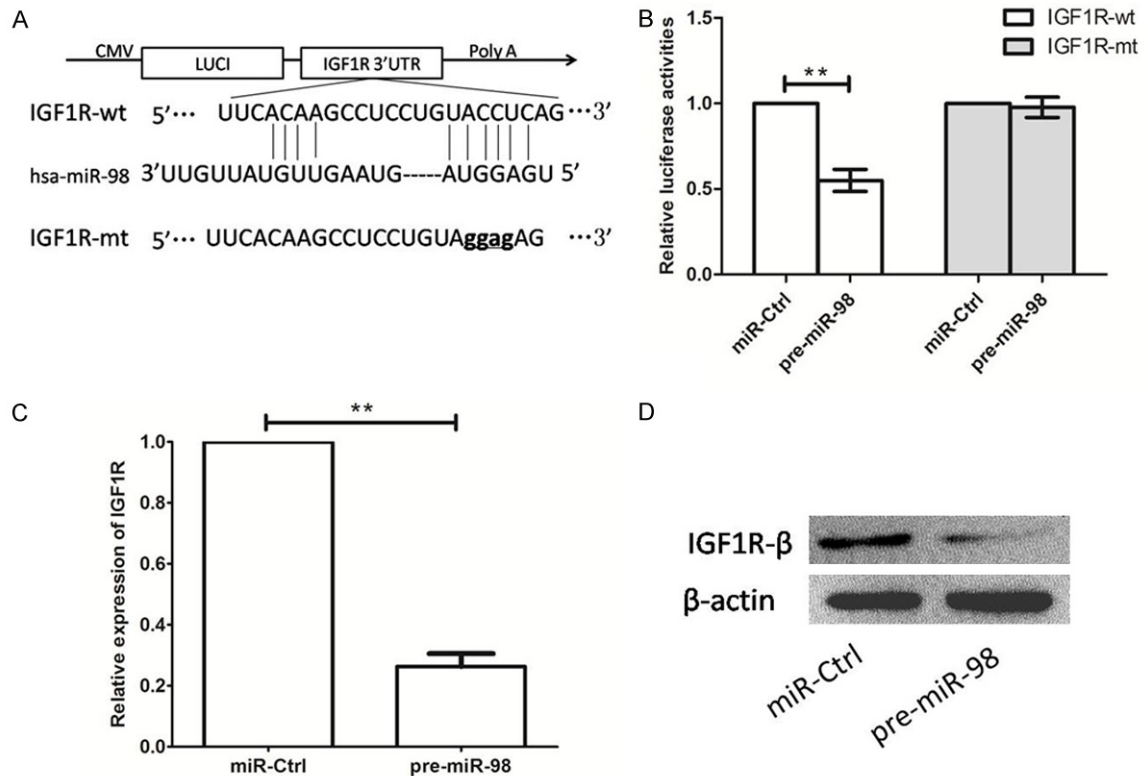


Figure 4. IGF1R is a target of miR-98 in OSCC cells. A. Bioinformatics analysis of the predicted interactions of miR-98 and its binding sites within the 3'-UTR of IGF1R. B. HEK-293 cells were co-transfected with pre-miR-98/miR-Ctrl with wt/mt 3'-UTR of IGF1R. Relative luciferase activity was assayed. C. The relative expression level of IGF1R in Tca-8113 cells were determined by qRT-PCR after transfection with pre-miR-98 or miR-Ctrl. U6 was used as an endogenous control. D. The protein level of IGF1R in Tca-8113 cells were determined by western blot after transfection with pre-miR-98 or miR-Ctrl. Data represents the mean \pm SD of three independent experiments. *P<0.05, **P<0.01.

4B, P=0.007). Furthermore, the mRNA level of IGF1R was decreased after transfection with pre-miR-98 using qRT-PCR (Figure 4C, P<

0.001). Consistent with this result, overexpression of miR-98 significantly inhibited IGF1R expression using western blot (Figure 4D).

miR-98 suppresses OSCC progression by targeting IGF1R

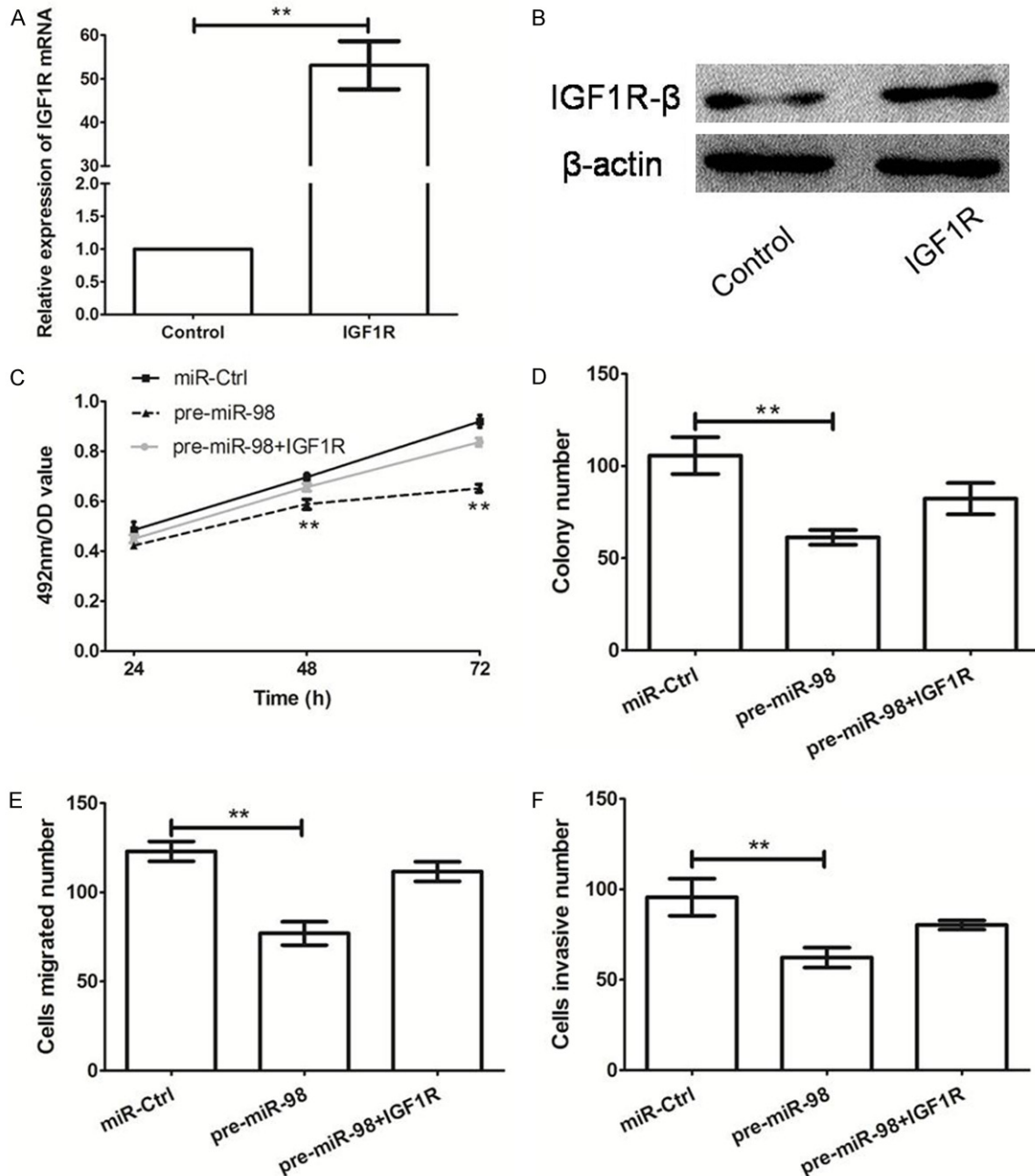


Figure 5. miR-98 suppresses OSCC progression partially by targeting IGF1R. The relative expression level of miR-98 in Tca-8113 cells were determined by qRT-PCR (A) and western blot (B) after transfection with IGF1R plasmid. U6 and β-actin was used as an endogenous control, respectively. Tca-8113 cells were co-transfected with pre-miR-98 or miR-Ctrl with IGF1R plasmid. MTT (C), colony formation (D), migration (E), and invasion (F) assays were performed. Data represents the mean ± SD of three independent experiments. *P<0.05, **P<0.01.

IGF1R is involved in miR-98 mediated suppression of OSCC growth and metastasis

We further investigated whether restoration of IGF1R could reverse the tumor-suppressive effects of miR-98. Firstly, the effect of IGF1R

overexpression was confirmed by qRT-PCR (Figure 5A, P<0.001) and western blot (Figure 5B). Then, our results demonstrated that IGF1R overexpression significantly attenuated the tumor suppressive effects of miR-98 using MTT assay, colony formation, migration and invasion

miR-98 suppresses OSCC progression by targeting IGF1R

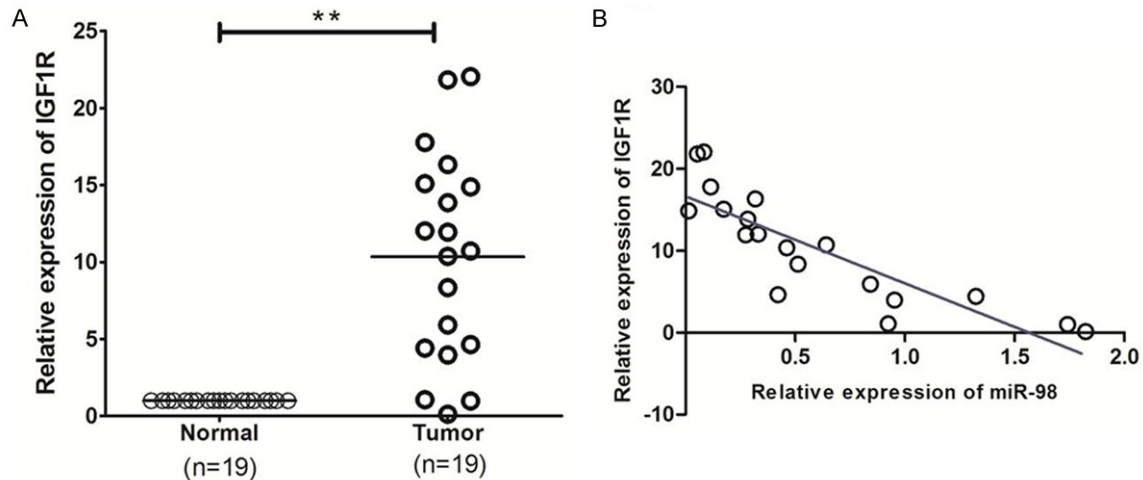


Figure 6. IGF1R is inversely correlated with miR-98 in OSCC. A. Relative expression level of IGF1R in 19 pairs of OSCC tissue samples and paired normal tissues was analyzed using qRT-PCR. β -actin was used as an endogenous control. Data represents the mean \pm SD of three independent experiments. B. IGF1R mRNA level was inversely correlated with miR-98 level in OSCC tissues. * $P < 0.05$, ** $P < 0.01$.

(Figure 5C-F, $P < 0.01$, $P = 0.002$, $P = 0.001$, $P = 0.008$).

miR-98 is inversely correlated with IGF1R expression in OSCC tissues

Expression of IGF1R in 19 OSCC tissues was examined by qRT-PCR. As shown in **Figure 6A**, the expression of IGF1R was significantly increased in OSCC tissues compared with the adjacent normal samples ($P < 0.001$). In addition, the level of IGF1R mRNA expression was inversely correlated with that of miR-98 OSCC tissues (**Figure 6B**, $r = -0.921$, $P < 0.001$).

Discussion

Emerging data have documented that miRNAs are frequently deregulated in various types of cancers, which indicates that miRNAs may play an essential role in OSCC tumorigenesis and progression. miR-98 was a tumor suppressive miRNA and decreased in different human cancers [9, 10]. Aberrant expression of miR-98 could contribute to the initiation and progression of tumors. Ting HJ *et al.* [11] found that miR-98 was transcriptionally induced by $1\alpha,25$ -dihydroxyvitamin D(3) ($1,25$ -VD) in prostate cancer cells, knockdown of miR-98 suppressed $1,25$ -VD anti-growth effect and overexpression of miR-98 suppressed the prostate cancer cells growth via inducing G2/M arrest. In addition, in glioma tissues, miR-98 was sig-

nificantly reported to lower than that in normal brain tissues, forced expression of miR-98 accelerated the inhibition of glioma cell invasion by targeting gene HMG2 [12]. In this present study, we demonstrated that miR-98 was significantly downregulated in OSCC samples and human OSCC cell lines. Furthermore, overexpression of miR-98 inhibited cell proliferation, migration, and invasion of OSCC cells, which provides important evidence in support of miR-98 functioning as a tumor suppressor in OSCC.

In addition, we identified that an oncogenic gene, IGF1R, was a target of miR-98. IGF-1R is a transmembrane receptor tyrosine kinase, which could bind to adaptor molecules and then trigger phosphatidylinositol 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) signaling pathways to regulate oncogenic transformation, growth and survival of cancer cells [13, 14]. Accumulating evidence suggests that IGF1R is frequently overexpressed in many cancers, and plays a positive role in tumorigenesis [7, 15, 16]. Gao *et al.* indicated that overexpression of IGF-1R was negatively associated with histological differentiation of OSCC patients, and triggered the PI3K and MAPK pathways to accelerate OSCC cells proliferation both *in vivo* and *in vitro* [7]. Furthermore, IGF1 was recombined to magnetic iron oxide nanoparticles (IONPs) to develop the theranostic IGF1-IONP-Dox for targeted

delivery of therapeutic agents into IGF1R expressing drug resistant tumor cells and tumor associated stromal cells [17]. Here, we found that miR-98 suppressed IGF1R expression, and overexpression of IGF1R could dramatically attenuated the tumor suppressive effects of miR-98 in OSCC cells. Furthermore, IGF1R was inversely correlated with miR-98 in OSCC samples, these findings suggest that oncogenic IGF1R may be involved in progression of OSCC.

In conclusion, the present study showed that miR-98 was significantly downregulated in OSCC tissues and cell lines. Overexpression of miR-98 suppressed growth and metastasis of OSCC cells by targeting IGF1R. These findings suggest that miR-98 may serve as a predictor for prognosis and a therapeutic target for OSCC patients.

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Disclosure of conflict of interest

None.

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References

- [1] Lambert R, Sauvaget C, de Camargo Cancela M and Sankaranarayanan R. Epidemiology of cancer from the oral cavity and oropharynx. *Eur J Gastroenterol Hepatol* 2011; 23: 633-641.
- [2] Adami GR and Adami AJ. Looking in the mouth for noninvasive gene expression-based methods to detect oral, oropharyngeal, and systemic cancer. *ISRN Oncol* 2012; 2012: 931301.
- [3] Mehrotra R, Sharma N, Umudum H, Ceyhan K and Rezanko T. The role of cytopathology in diagnosing HPV induced oropharyngeal lesions. *Diagn Cytopathol* 2012; 40: 839-843.
- [4] Gomes CC and Gomez RS. MicroRNA and oral cancer: future perspectives. *Oral Oncol* 2008; 44: 910-914.
- [5] Jansson MD and Lund AH. MicroRNA and cancer. *Mol Oncol* 2012; 6: 590-610.
- [6] Hedback N, Jensen DH, Specht L, Fiehn AM, Therkildsen MH, Friis-Hansen L, Dabelsteen E and von Buchwald C. MiR-21 expression in the tumor stroma of oral squamous cell carcinoma: an independent biomarker of disease free survival. *PLoS One* 2014; 9: e95193.
- [7] Gao L, Wang X, Wang X, Zhang L, Qiang C, Chang S, Ren W, Li S, Yang Y, Tong D, Chen C, Li Z, Song T, Zhi K and Huang C. IGF-1R, a target of let-7b, mediates crosstalk between IRS-2/Akt and MAPK pathways to promote proliferation of oral squamous cell carcinoma. *Oncotarget* 2014; 5: 2562-2574.
- [8] Lu WC, Kao SY, Yang CC, Tu HF, Wu CH, Chang KW and Lin SC. EGF up-regulates miR-31 through the C/EBPbeta signal cascade in oral carcinoma. *PLoS One* 2014; 9: e108049.
- [9] Siragam V, Rutnam ZJ, Yang W, Fang L, Luo L, Yang X, Li M, Deng Z, Qian J, Peng C and Yang BB. MicroRNA miR-98 inhibits tumor angiogenesis and invasion by targeting activin receptor-like kinase-4 and matrix metalloproteinase-11. *Oncotarget* 2012; 3: 1370-1385.
- [10] Li F, Li XJ, Qiao L, Shi F, Liu W, Li Y, Dang YP, Gu WJ, Wang XG and Liu W. miR-98 suppresses melanoma metastasis through a negative feedback loop with its target gene IL-6. *Exp Mol Med* 2014; 46: e116.
- [11] Ting HJ, Messing J, Yasmin-Karim S and Lee YF. Identification of microRNA-98 as a therapeutic target inhibiting prostate cancer growth and a biomarker induced by vitamin D. *J Biol Chem* 2013; 288: 1-9.
- [12] Chen Z, Cheng Q, Ma Z, Xi H, Peng R and Jiang B. Overexpression of RKIP inhibits cell invasion in glioma cell lines through upregulation of miR-98. *Biomed Res Int* 2013; 2013: 695179.
- [13] Chitnis MM, Yuen JS, Protheroe AS, Pollak M and Macaulay VM. The type 1 insulin-like growth factor receptor pathway. *Clin Cancer Res* 2008; 14: 6364-6370.
- [14] Johnson GL and Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* 2002; 298: 1911-1912.
- [15] Xu W, Hang M, Yuan CY, Wu FL, Chen SB and Xue K. MicroRNA-139-5p inhibits cell proliferation and invasion by targeting insulin-like growth factor 1 receptor in human non-small cell lung cancer. *Int J Clin Exp Pathol* 2015; 8: 3864-3870.
- [16] Flashner-Abramson E, Klein S, Mullin G, Shoshan E, Song R, Shir A, Langut Y, Bar-Eli M, Reuveni H and Levitzki A. Targeting melanoma with NT157 by blocking Stat3 and IGF1R signaling. *Oncogene* 2015; [Epub ahead of print].
- [17] Zhou H, Qian W, Uckun FM, Wang L, Wang YA, Chen H, Kooby D, Yu Q, Lipowska M, Staley CA, Mao H and Yang L. IGF1 Receptor Targeted Theranostic Nanoparticles for Targeted and Image-Guided Therapy of Pancreatic Cancer. *ACS Nano* 2015; 9: 7976-91.