Biotechnol Lett (2016) 38:409–416
DOI 10.1007/s10529-015-2007-x

ORIGINAL RESEARCH PAPER

MicroRNA-144-3p inhibits proliferation and induces apoptosis of human salivary adenoid carcinoma cells via targeting of mTOR

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Received: 9 October 2015 / Accepted: 17 November 2015 / Published online: 19 December 2015
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Abstract
Objectives To investigate the biological functions of microRNA-144-3p with respect to proliferation and apoptosis of human salivary adenoid carcinoma cell lines via mTOR.
Results After transfection of microRNA-144-3p agomir, cell viability assays confirmed that the salivary adenoid carcinoma cell (SACC) proliferation was inhibited and apoptosis was induced. Dual luciferase reporter assay validated that the mammalian target of rapamycin (mTOR) was a direct target of miR-144-3p. Western blot, immunofluorescent analysis and a xenograft mouse model of adenoid cystic carcinoma indicated that miR-144-3p was a tumor suppressor and repressed mTOR expression and signaling in SACCs.
Conclusions MicroRNA-144-3p inhibits proliferation and induces apoptosis of human salivary adenoid carcinoma cells by downregulating mTOR expression in vitro and in vivo.

Keywords Adenoid cystic carcinoma · Apoptosis · MTOR · MicroRNA-144-3p · Proliferation

Fangyi Huo and Chen Zhang contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s10529-015-2007-x) contains supplementary material, which is available to authorized users.

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Introduction

Adenoid cystic carcinoma (AdCC) is one of the most common malignancies of the major and minor salivary glands, accounting for approx. 15–25 % of all carcinomas at these locations. AdCCs have a prolonged clinical course and these carcinomas are characterized by an infiltrative nature, a high incidence of distant metastasis, and poor long-term patient survival (Spiro 1997).

MicroRNAs (miRNAs) are about 19–22 nt non-coding RNAs that are involved in multiple cellular processes as post-transcriptional regulators (Li et al. 2015b) of proliferation, differentiation and apoptosis. MiR-144 is expressed aberrantly in hepatocellular carcinoma, osteosarcoma and bladder and colorectal cancers (Cao et al. 2014; Guo et al. 2013; Kalimutho et al. 2011; Zhao et al. 2014). MicroRNA array data indicate that miR-144 is less abundant in salivary

adenoid cystic carcinoma tumor tissues compared to adjacent normal tissues (Mitani et al. 2013), but the underlying mechanism is unclear. We report that miR-144-3p is connected to proliferation and apoptosis of salivary adenoid carcinoma cell lines (SACC).

The mammalian target of rapamycin (mTOR) is an oncogene and controls many cellular processes via target substrate phosphorylation including STAT3 phosphorylation (p-STAT3) and phospho-S6 (p-S6). Activation of mTOR signaling transduction pathways may inhibit cell apoptosis, accelerate cell survival and proliferation, and are crucial to tumorigenesis (Li et al. 2015a). Thus, we studied the relevance of mTOR in SACC by assessing how miR-144-3p, an miRNA associated with adenoid carcinoma, interacts with the tumor promoter mTOR.

Materials and methods

Cell culture and transient transfection

Human salivary adenoid carcinoma cell lines SACC-83 (low metastasis and invasion rate) and SACC-LM (high metastasis and invasion rate) were gifts from School and Hospital of Stomatology, Peking University, and were cultured in RPMI-1640 (Hyclone) supplemented with 10 % (v/v) fetal bovine serum (FBS). The cells were incubated in a humidified atmosphere of 95 % air and 5 % CO_2 at 37 °C. For transient transfection, SACC cells were seeded in a 6-well plate at 2.5×10^5/ml. 75 nM miR-144-3p agomir or an equal concentration of scrambled sequence microRNA (GenePharma, Shanghai, China) was transfected into SACC cells using the Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Mock control (cells with Lipofectamine 2000) was also included.

Protein extraction and western blot analysis

A total protein lysis buffer (Thermo-Scientific) supplied with a protease inhibitor (Roche) was used for protein extraction. The BCA method was used for protein quantification. The protein samples were separated by 10 % (w/v) SDS-PAGE electrophoresis and then transferred to a PVDF membrane. The membrane was blocked and then incubated overnight with primary antibody for Bcl-2, C-PARP, mTOR, phospho- mTOR, phospho-STAT3, phospho-S6 and β-actin (CST) followed by incubation with horseradish peroxidase–conjugated anti-rabbit and anti-mouse secondary antibodies. The protein expression levels were normalized to β-actin.

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

After 24 h transfection with miR-144-3p agomir, microRNA was extracted, and real-time PCR was conducted. For detection of miR-144-3p expression, microRNA extraction was performed using miRNeasy Mini Kit (Qiagen); reverse transcription of RNA and the detection of mature miR-144-3p were performed using an All-in-One miRNA quantitative real-time PCR detection kit (GeneCopoeia, Guangzhou, China) on Bio-RAD CFX Connect system. The miR-144-3p expression level was normalized to U6 snRNA and the miR-144-3p relative expression level of each group was calculated using the $2^{\Delta\Delta Ct}$ method. All primers were synthesized by GeneCopoeia.

Dual luciferase assay

Dual luciferase assay was used to test whether miR-144-3p bound to the 3′-untranslated regions (3′-UTR) of mTOR mRNA directly. Fragments of mTOR mRNA containing wild type (WT) or mutant (MUT) miR-144-3p binding sites were cloned respectively into the pmiR-RB-REPORT miRNA expression reporter vector system (Ribobio, Guangzhou, China). Cells were co-transfected in 96-well plates with miR-144-3p (75 nM) or scramble control, together with reporter plasmid or mutant plasmid (100 ng/well)and Lipofectamine 2000. After 48 h of transfection, firefly and renilla luciferase activities were measured by Dual Luciferase Assay System (Promega). Firefly luciferase (hLuc) was used as internal reference.

Cell viability assay

Cell viability assay was performed to test the effects of miR-144-3p on the proliferation of SACC cells. After treatment with transfection, 2×10^3 cells were seeded in 96-well culture plates, cells were tested for proliferation every 24 h using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) and the A_{450} values were measured with a microplate reader. The experiments were independently performed in triplicate.

Immunofluorescence analysis

SACC-LM cells were seeded onto coverslips and transfected with microRNA-144-3p agomir for 2 days and then fixed in 4 % (v/v) paraformaldehyde for 30 min. Cells were blocked in 3 % (w/v) BSA for 1 h at room temperature. Cells were then incubated at 4 °C overnight with respective primary antibodies at dilutions recommended by the manufacturer. After the PBS washout, the cells were treated with dylight 594-labeled anti-rabbit antibody (diluted 1:200) for 1 h. The coverlips were then mounted on microscope slides with anti-fade fluorescence mounting medium with DAPI (ZSGB-BIO, Beijing,China). Cells immunofluorescence was photographed by a fluorescent microscope.

Flow cytometry

After being transfected with miR-144-3p agomir at 75 nM or scramble control, SACC-83 and SACC-LM cells were collected after 24 h, washed, resuspended and stained using the Annexin V-fluorescein isothiocyanate/propidium iodide apoptosis detection kit (BD Pharmingen) following manufacture' s instructions. Cells were analyzed using a flow cytometer.

Tumor formation in nude mice

Female BALB/c nude mice, 4–5 weeks, were used. All animal studies were carried out in compliance with the NIH guidelines for the use of laboratory animals in the Specific Pathogen Free (SPF) Animal Laboratory of Wuhan University and approved by the Animal Care and Use Committee of Wuhan University. Briefly, ten mice were injected subcutaneously with 2×10^6 SACC-LM cells in 0.2 ml PBS. After 2 weeks, the mice were randomly divided into miR-144-3p agomir or scramble group, which were injected into the tumors of mice twice a week at 6–8 sites. Tumor volumes and body weights were calculated with electronic caliper and balance every other day. Animals were sacrificed 4 weeks after the first injection and tumors were dissected.

Statistical analysis

For quantitative data,all experiments were performed independently at least three times. The data was presented as mean $\pm$ SD. Statistically significant difference between groups was determined using one-way analysis of variance (ANOVA) or Student's

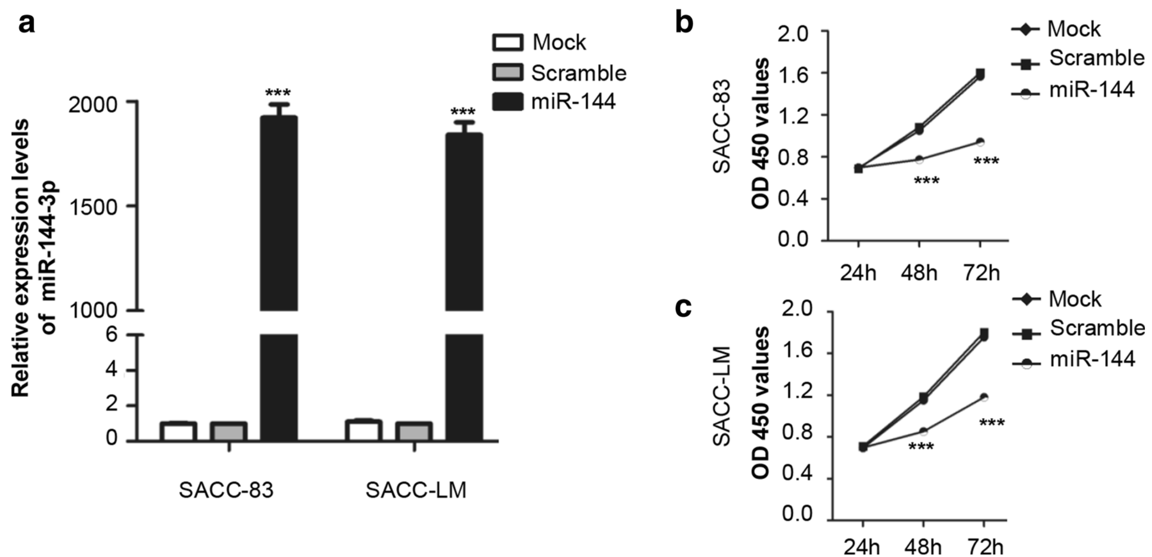


Fig. 1 MiR-144-3p inhibits proliferation of human salivary adenoid carcinoma cells (SACC). **a** qRT-PCR detected the expression levels of miR-144-3p in SACC-83 and SACC-LM transfected with miR-144-3p agomir compared with those transfected with scramble control. The results were presented as fold changes of expression level, $^{***}P < 0.001$ miR-144-3p compared with mock or scramble control. **b**, **c** Cell proliferation (A_{450}) detected by CCK-8 after transfection with miR-144-3p or scramble control, $^{***}P < 0.001$ miR-144-3p compared with mock or scramble control

t test using GraphPad Prism 5.01 (GraphPad Software, USA). $P < 0.05$ was considered to be significant.

Results

MiR-144-3p agomir inhibits proliferation and induces apoptosis in vitro

We used a cell viability assay to measure the effect of miR-144-3p on proliferation of human salivary adenoid carcinoma cells (SACC), and miR-144-3p agomir was used to increase miR-144-3p expression. MiR-144-3p agomir or scramble control was transfected into SACC-83 and SACC-LM, and miR-144-3p expression was measured using qRT-PCR. After transfection with miR-144-3p agomir, miR-144-3p expression increased in excess of 1800-fold in SACC-83 and 1900-fold in SACCC-LM compared with scramble or mock control (Fig. 1a). When transfected with miR-144-3p agomir, cell proliferation significantly decreased compared scramble or mock treated SACC-83 and SACC-LM, indicating overexpression of miR-144-3p could inhibit cell survival (Fig. 1b, c).

The proportion of apoptotic cells transfected with miR-144-3p or scramble control was assessed with flow cytometry (Fig. 2a, b) and 27 % of SACC-83 and 37 % of SACC-LM were apoptotic. Thus apoptosis in the miR-144-3p group was greater than the scramble or mock controls. With a DAPI staining assay, similar results in both cell groups were observed under a fluorescent microscopy (Fig. 2c, d). We next measured apoptosis-related protein expression by western blot (Fig. 2e, f). The expression of C-PARP, (the miR-144-3p-treated SACC-LM cells,) was greater than in scramble or mock control cells and Bcl-2 expression was decreased.

MiR-144-3p targets mTOR signaling in SACC cells

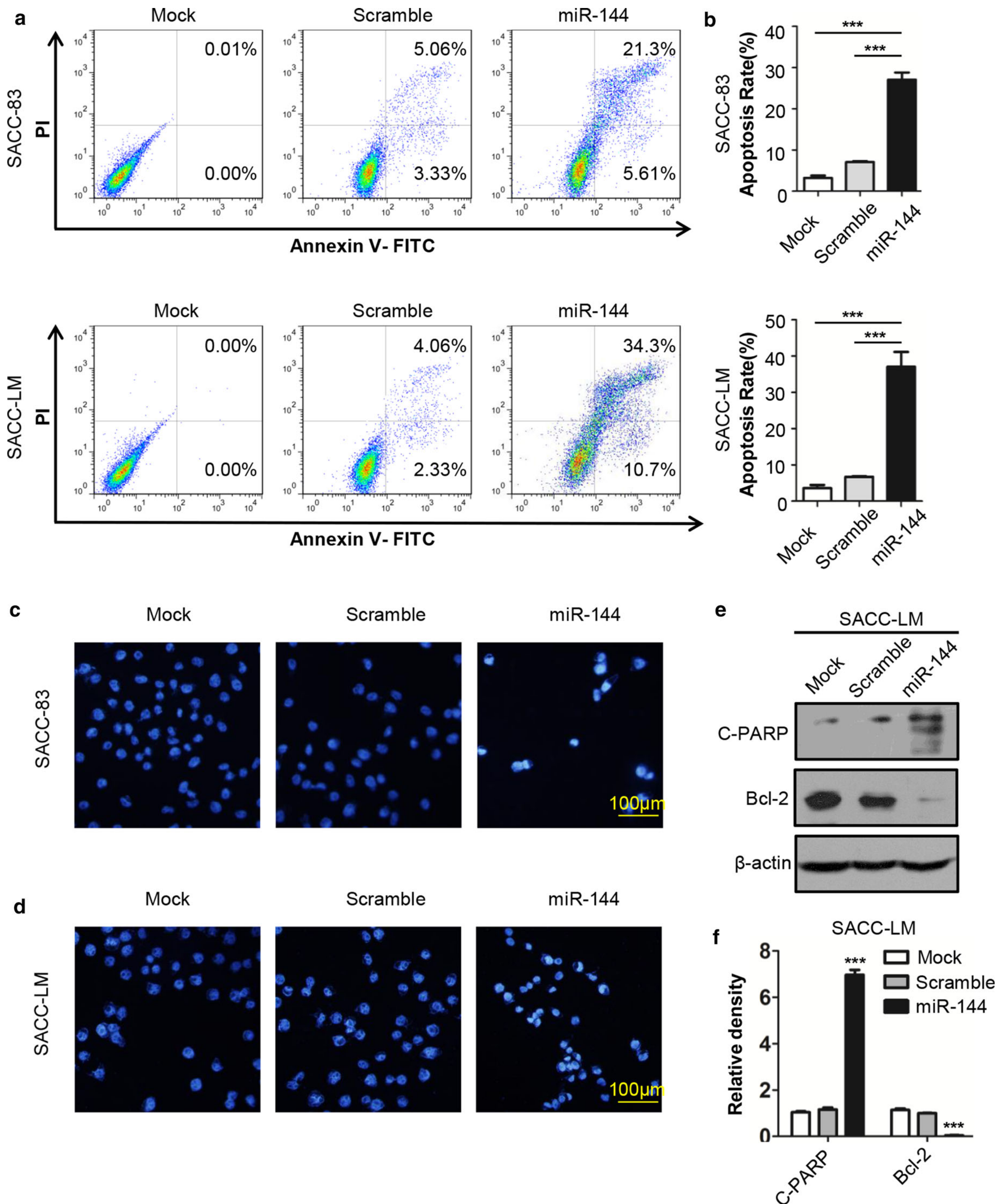
We hypothesized that miR-144-3p regulates the proliferation and apoptosis via mTOR and we therefore studied these using two bioinformatics algorithms, TargetScan (http://www.targetscan.org/) and miRanda (http://mirdb.org/miRDB/). Western blotting was used to measure miR-144-3p on the mTOR/STAT3 signaling pathway. After transfection with miR-144, mTOR, phospho-mTOR, phospho-$S6^{S235/236}$ (a downstream molecule of mTOR), and phospho-$STAT3^{T705}$ protein levels decreased significantly compared with scramble and mock controls (Fig. 3a–c). Immunofluorescence revealed that miR-144-3p significantly decreased p-STAT3 nuclear expression and mTOR cytoplasmic expression in SACC-LM (Fig. 3d). Thus, miR-144-3p targeted mTOR and negatively regulated its expression in SACCs.

Fig. 2 MiR-144-3p induces apoptosis of SACCs. **a** The representative flow chart of SACC-83 and SACC-LM transfected with scramble control or miR-144-3p agomir. Quadrant statistics: early apoptosis cells in lower right (LR) and late apoptosis cells in upper right (UR), viable cells and in necrosis cells separately in lower left (LL) and upper left (UL). **b** Apoptotic cells (early and late apoptosis) of SACC-83 and SACC-LM transfected with scramble control or miR-144-3p agomir. **c**, **d** The morphologic changes of SACC-83 and SACC-LM transfected with scramble control or miR-144-3p agomir were observed by fluorescence microscopy with DAPI staining. *Scale bar* 100 μm. **e**, **f** Western blot showed the protein levels of c-PARP and the Bcl-2 after transfection with scramble control or miR-144-3p agomir in SACC-LM, $^{***}P < 0.001$ miR-144-3p versus mock or miR-144-3p versus scramble control ▶

To investigate whether mTOR is a direct target of miR-144-3p, we used a luciferase reporter assay and deduced the presence of two predicted miR-144-3p binding sites on the 3′-UTR of mTOR mRNA. So WT and a MUT luciferase reporter plasmid were constructed (Supplementary Fig. 1a). Relative luciferase activity of cells transfected with WT luciferase reporter plasmid plus miR-144-3p agomir decreased to 68 % in SACC-83 and 72 % in SACC-LM compared with cells transfected with WT luciferase reporter plasmid plus scramble control (Supplementary Fig. 1b). However, relative luciferase activity in cells co-transfected with MUT luciferase reporter plasmid plus either miR-144-3p agomir or scramble control were not different. Thus miR-144-3p directly binds to the 3′-UTR of mTOR mRNA.

MiR-144-3p agomir suppresses tumor growth in a xenograft mouse model of adenoid cystic carcinoma in vivo

To investigate miR-144-3p agomir functions in vivo, we established a mouse tumor model by subcutaneously transplanting SACC-LM cells into nude mice. After two weeks of transplantation, mice were injected twice a week with 50 μl PBS containing 1 nmol miR-144-3p agomir or scramble control by multipoint intratumoral injection four times. Then,



28 days after transplantation, tumor masses were excised and analyzed (Fig. 4a). Transfection efficiency was confirmed by real-time PCR and Fig. 4b shows that miR-144-3p expression in tumors transfected with miR-144-3p agomir was 14.6 times higher than that in scramble control-transfected tissues (Fig. 4b). Figure 4c–e shows that tumor volume was reduced after administration of miR-

144-3p agomir compared with scramble control (Fig. 4c–e). Figure 4f indicates that animal weight did not change in any group, so microRNA agomir or scramble control was not toxic. Western blot confirmed our previous data, revealing decreased expression of mTOR, p- mTOR and Bcl-2 in tumors that had been treated with miR-144-3p agomir (Fig. 4g).

Discussion

Emerging evidence indicates that miRNAs are aberrantly expressed in diverse human cancers, acting as oncogenes or putative tumor suppressor genes (Chen et al. 2011; Yan et al. 2012). In many cancers, such as colorectal cancer, hepatocellular carcinoma, and osteosarcoma, miR-144 inhibits proliferation but contradicting reports exist (Cao et al. 2014; Kalimutho et al. 2011; Zhao et al. 2014). Zhang et al. (2013) suggested that miR-144 promoted proliferation, migration, and invasion of nasopharyngeal carcinoma by repressing PTEN. Thus the function of miR-144 in carcinogenesis is complex. According to Mitani et al. (2013), miR-144 was downregulated in adenoid cystic carcinoma tumor (AdCC) tissues compared with adjacent normal tissues according to microRNA array

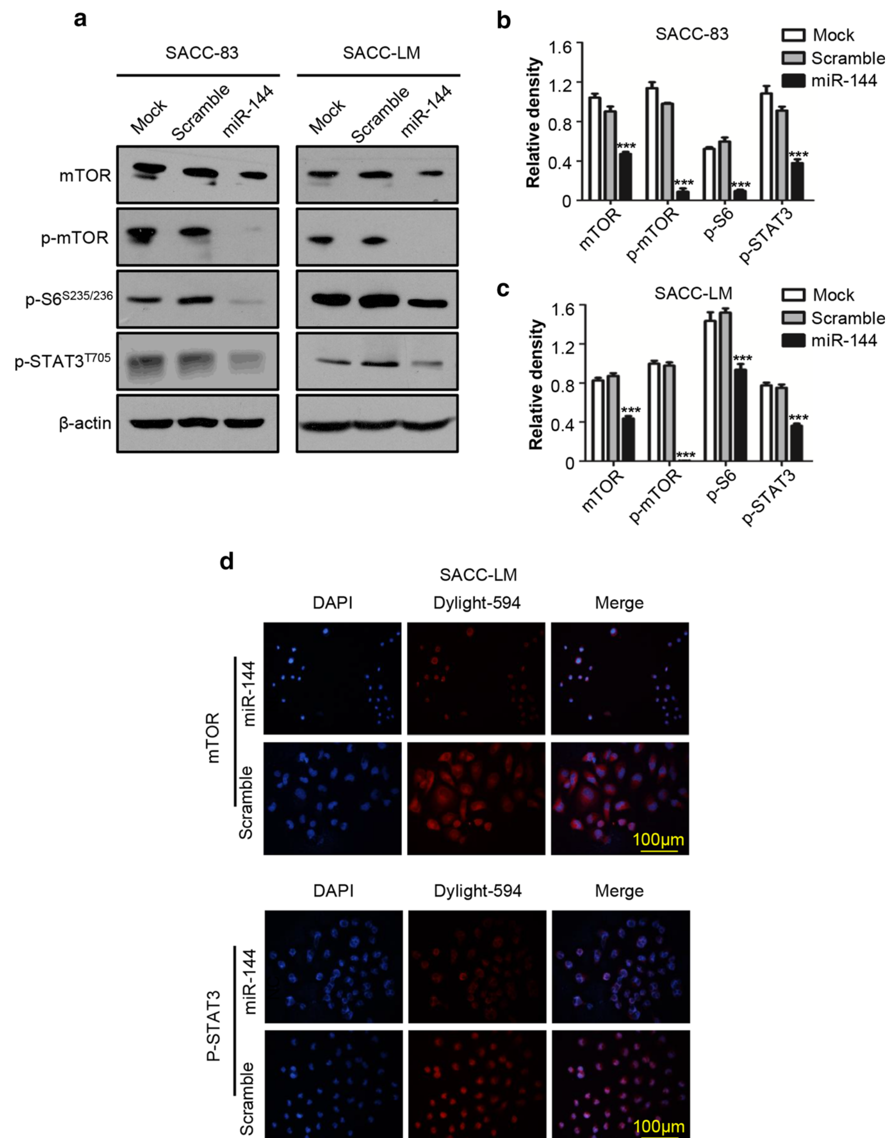


Fig. 3 MiR-144-3p targets mTOR/STAT3 signaling in SACCs. **a–c** Western blot analysis of SACC-83 and SACC-LM for mTOR signaling protein expression after transfection, expression levels of mTOR, phospho-mTOR, phospho-S6 and phospho-STAT3 were normalized to β-actin, ***$P < 0.001$ miR-144-3p versus mock or miR-144-3p versus scramble control. **d** Immunofluorescence revealed miR-144-3p decreased p-STAT3 nuclear expression and mTOR cytoplasmic expression in SACC-LM, *Scale bar* 100 μm

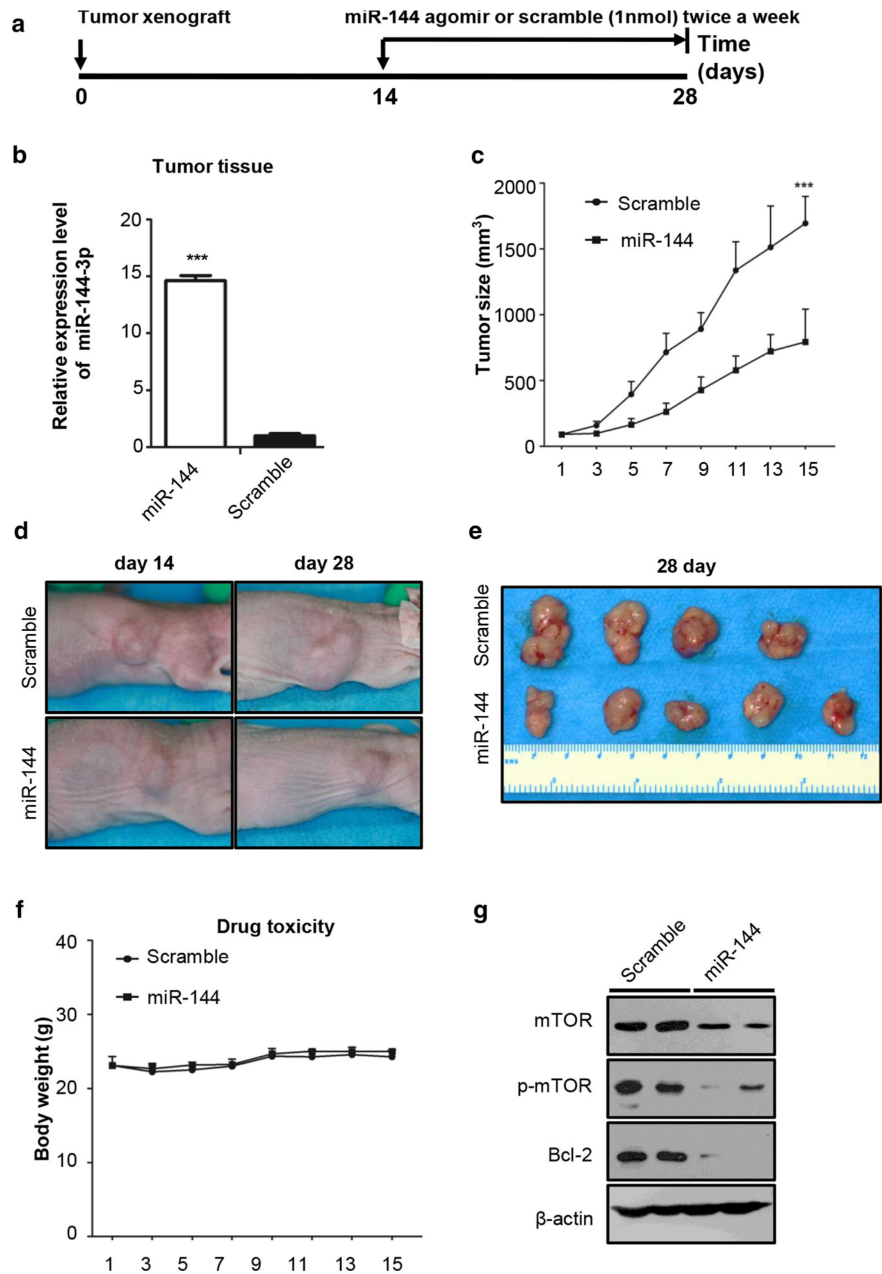


Fig. 4 MiR-144-3p agomir suppresses tumor growth in a xenograft mouse model of adenoid cystic carcinoma tumor (AdCC) in vivo. **a** Schematic diagram represented miR-144-3p agomir delivery strategy in SACC mouse model. **b** qRT-PCR detected the expression levels of miR-144-3p in AdCC mouse model transfected with miR-144-3p agomir compared with scramble control, $^{***}P < 0.001$. **c** Tumor volume curve showed miR-144-3p aogmir treatment delayed tumor growth, $^{***}P < 0.001$. **d** Representative photos showed the xenograft mouse model was delayed after miR-144-3p agomir treatment compared with scramble group. **e** Isolated tumors were presented after 14 days treatment. **f** Drug toxicity as indicated by gain of weight of miR-144-3p agomir or scramble control treated mice. **g** Western blot shows the protein levels of mTOR, p-mTOR and Bcl-2 in the xenograft mouse model

data. We found that introduction of miR-144 reduced salivary adenoid carcinoma cells (SACC) proliferation and induced apoptosis and in an in vivo animal study miR-144 agomir significantly decreased the tumor burden. Thus, miR-144 may be a tumor suppressor gene and its down-regulation contributes to progression of AdCC metastasis.

The mTOR/STAT3 pathway is a master intracellular signaling pathway important to tumor cell growth, differentiation, proliferation, apoptosis, and migration (He et al. 2015). Studies indicated that aberrant expression of mTOR alters cell growth and apoptosis in several cancers, such as hepatocellular carcinoma, gastric cancer, and osteosarcoma (Gobin et al. 2014;

Grabinski et al. 2012; Li et al. 2012). AdCC targeting mTOR/STAT3 signaling may decrease proliferation and induce apoptosis in vitro and in vivo (Bu et al. 2015; Yu et al. 2014). We report that overexpression of miR-144 decreased mTOR protein expression in AdCC cell lines and immunofluorescence confirmed that mTOR expression can be downregulated by miR-144 in SACCs. Luciferase assay confirmed that mTOR was a direct target of miR-144-3p. Therefore, miR-144-3p could decrease proliferation and induce apoptosis by targeting mTOR/STAT3 signaling.

In conclusion, miR-144 may be a tumor suppressor via targeting mTOR in SACC and miR-144 may serve as a potential therapeutic target for treating AdCC patients.

Acknowledgments This work was supported by the National Natural Science Foundation of China 81400545 for C.Z and 81170991 for H.H.

Supporting information Supplementary Fig. 1—MTOR is a direct target of miR-144-3p.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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