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Original Article

Silencing microRNA-181a-5p suppresses malignant behavior of lacrimal adenoid cystic carcinoma cells by upregulating large tumor suppressor 2

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Abstract: To uncover the effect of miR-181a-5p regulating large tumor suppressor 2 (LATS2) in biologic processes of adenoid cystic carcinoma (ACC) cells, miR-181a-5p and LATS2 expression in lacrimal ACC (LACC) tissues were assessed. The ACC cell lines were respectively treated with altered miR-181a-5p or LATS2 to determine the biologic functions in ACC cells. Binding ability of miR-181a-5p and LATS2 was confirmed. Tumor growth *in vivo* was assessed as well. MiR-181a-5p was upregulated while LATS2 was downregulated in LACC tissues. Reduced miR-181a-5p restrained malignant phenotype of ACC cells and decelerated xenograft growth. Conversely, LATS2 reduction had opposite effects compared to miR-181a-5p knockdown on ACC cells. Furthermore, downregulated LATS2 could abolish the alterations in ACC cells induced by miR-181a-5p silencing. MiR-181a-5p inhibition upregulated LATS2 to suppress malignant behavior of ACC cells *in vivo* and *in vitro*.

Keywords: Lacrimal adenoid cystic carcinoma, microRNA-181a-5p, large tumor suppressor 2, proliferation, apoptosis, migration

Introduction

Adenoid cystic carcinoma (ACC) is a rare and slow-growing malignancy of the secretory glands with a prolonged clinical course. ACC occurring in the eye and made up for 1.8% of the total patients, among who the lacrimal gland was implicated in more than 80%, and was related to the poor survival [1]. It was reported that lacrimal ACC (LACC) accounts for about 1% of head and neck malignant tumors and 1.6% of all orbital tumors [2]. The main characteristics of LACC are multiple recurrences, including intracranial extension and potential distant metastases to the lung, bone, brain and liver [3]. The survival rate of LACC is low, with less than 50% survival at 5 years and 20% at 10 years [4]. Hence, it is necessary to uncover the underlying molecular mechanism modulating metastatic dissemination of LACC.

MicroRNAs (miRNAs) are known to degenerate mRNA or repress translation by targeting 3'UTR of target genes [5]. As previously reported, miR-24-3p repressed malignant behavior of LACC [2], and miR-93-5p enhanced LACC cell tumorigenesis [6]. MiR-181a-5p is a miRNA associated with development of several human tumors, such as hepatocellular carcinoma [7] and chondrosarcoma [8], and interestingly, miR-181a has been reported to suppress LACC metastasis [9]. However, the mechanisms of miR-181a-5p in LACC remain to be explored. Belonging to the large tumor suppressor (LATS) family, LATS2 is situated on chromosome 13q11-12 [10]. It has been demonstrated that LATS2 exerted an anti-tumor impact on liver cancer death [11] and facilitated lung cancer cell apoptosis [12]. However, effects of LATS2 on LACC development remain largely unknown. MiRNAs are known to regulate target genes to affect disease progression, and miR-181a-5p was verified to target SIRT1 in hypoxia-reoxygenation-induced cardiomyocyte apoptosis [13] and target SECISBP2 in osteoarthritis [14]. Nevertheless, binding of miR-181a-5p and LATS2 remains scarcely studied.

Table 1. Primer sequences

Gene	Primer sequence (5'-3')
miR-181a-5p	F: 5'-AGCGCTGAGTTTTGAGGTTGC-3'
	R: 5'-CGCAGGGTCCGAGGTATTC-3'
U6	F: 5'-CTCGCTTCGGCAGCACA-3'
	R: 5'-ACGCTTCACGAATTTGCGT-3'
LATS2	F: 5'-GCTTCATCCACCGAGACATCAA-3'
	R: 5'-CGACAGTTAGACACATCATCCCAGA-3'
GAPDH	F: 5'-GGTGGTCTCCTCTGACTTCAACA-3'
	R: 5'-GTTGCTGTAGCCAAATTCGTTGT-3'

Note: F, forward; R, reverse; miR-181a-5p, microRNA-181a-5p; LATS2, large tumor suppressor 2; GAPDH, glyceraldehyde phosphate dehydrogenase.

In view of the reported dysregulation of miR-181a-5p/LATS2 in disease progression, we designed the study to investigate roles of altered miR-181a-5p and LATS2 in biologic functions of ACC cells. We inferred that miR-181a-5p downregulation may repress ACC cell growth through upregulating LATS2.

Materials and methods

Ethics statement

Protocol of this study was confirmed by the Ethic Committee of the 4th People's Hospital of Shenyang, and protocol of animal experiments was approved by the Institutional Animal Care and Use Committee of the 4th People's Hospital of Shenyang.

Study subjects

Fifty-one LACC and adjacent non-tumor tissues were harvested from patients accepted resection in the 4th People's Hospital of Shenyang from May 2017 to August 2019. These patients had not accepted chemotherapy or radiotherapy before the surgery.

Cell culture, grouping and treatment

ACC cells ACC-2 and ACC-M were cultured in DMEM with 10% FBS. Cells were respectively transfected with inhibitor negative control (NC), miR-181a-5p inhibitor, small interfering RNA (si) NC, si-LATS2, miR-181a-5p inhibitor + si NC and miR-181a-5p inhibitor + si-LATS2 according to protocols of Lipofectamine 2000 (Invitrogen, CA, USA). The control group was set and subsequent experiments were performed after 48-h transfection.

CCK-8 assay

Cells were seeded and 3 duplicates were set at 0, 24, 48 and 72 h for the culture. The plates were cultured and 10 μL CCK-8 solution was added at relative time points. Optical density at 450 nm of each well was measured after 4-h culture.

Flow cytometry

The experiment was in line with instructions of FITC/PI double staining kits (BestBio, Shanghai, China) and a flow cytometer was used to evaluate the apoptosis.

Transwell assay

Cells were seeded and cultured for 48 h (5×10^5 cells/mL). The Transwell chambers had 500 μL DMEM (10% FBS) added and the apical chambers got 200 μL cell suspension for 24-h culture. With the medium discarded, chambers were fixed for 30 min and stained by crystal violet for 60 min. Five images were collected from each well for analysis.

Dual luciferase reporter gene assay

Binding site of miR-181a-5p and LATS2 3'UTR was predicted by Starbase. LATS2 3'UTR wild type (WT) plasmid (LATS2-WT) and LATS2 3'UTR mutant type (MUT) plasmid (LATS2-MUT) were constructed, and then were transfected with miR-181a-5p mimic and its NC into cells by Lipofectamine™ 2000 for 48 h. Luciferase activity was determined.

Subcutaneous tumorigenesis in nude mice

Fifteen 6-week-old nude mice (Weishi Testing Technology Service Co., Ltd., Jilin, China) were divided into 3 groups (n = 5): the control, inhibitor NC and miR-181a-5p inhibitor groups. The mice were given subcutaneous injection of 200 μL cell suspension according to the grouping. The tumor volume was measured at the 7th d and mice were euthanized at the 35th d. The xenografts were harvested and weighed.

RT-qPCR

Total RNA was extracted by Trizol kits and primers (**Table 1**) were synthesized by TaKaRa Biotechnology Co., Ltd. (Liaoning China). RNA was reversely transcribed to cDNA by Rever Tra

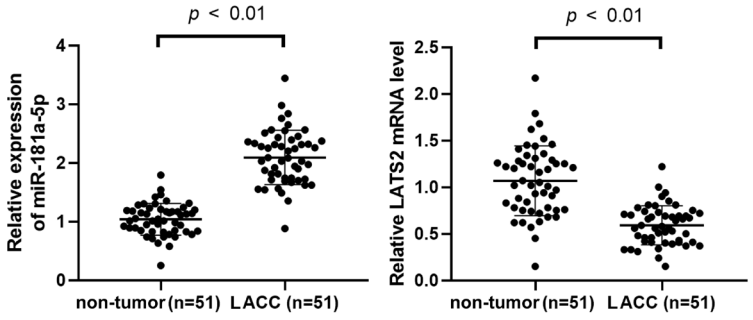

Figure 1. MiR-181a-5p is upregulated in LACC tissues. MiR-181a-5p and LATS2 expression in LACC tissues and non-tumor tissues were determined using RT-qPCR; the unpaired t-test was performed for comparisons between two groups; **, $P < 0.01$; N = 51.

Ace Qpcr RT Master Mix kits, and PCR was conducted with U6 and GAPDH as the loading controls of miR-181a-5p and LATS2. Data were analyzed by $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

Proteins were extracted and subjected to 10% SDS-PAGE, and transferred to membranes that were fixed with 5% skim milk. Afterwards, membranes were incubated with primary antibodies (Abcam, Cambridge, UK) LATS2 (1 μg/mL), Bax (1:1000), Bcl-2 (1 μg/mL) and β-actin (1 μg/mL) at 4°C, and then were incubated with relative secondary antibody for 1 h. The enhanced chemiluminescent reagent (Pierce, CO, USA) was used for development and the protein bands were analyzed by Image J2x V2.1.4.7 (Rawak Software, Inc., Germany).

Statistical analysis

Data analyses were performed by SPSS 21.0 software. Measurement data conforming to the normal distribution were expressed as mean ± standard deviation. Unpaired t-test was conducted for comparisons between two groups, one-way ANOVA was performed for comparisons among multiple groups, and Tukey's post hoc test was used for pairwise comparisons after one-way ANOVA. Pearson test was used for correlation analysis. *P* value < 0.05 indicated a significant difference.

Results

MiR-181a-5p is upregulated in LACC tissues

We used RT-qPCR to assess miR-181a-5p expression in LACC tissue. We found higher miR-181a-5p expression and lower LATS2 expression in LACC tissue versus non-tumor tissue ($P < 0.01$, **Figure 1**).

MiR-181a-5p knockdown suppresses malignant behavior of ACC cells

MiR-181a-5p was downregulated to observe its role in ACC cells ($P < 0.01$, **Figure 2A**). It was found that miR-181a-5p inhibitor repressed proliferation and migration, and promoted apoptosis of ACC cells ($P < 0.01$, **Figure 2B-E**).

MiR-181a-5p targets LATS2

A binding site between miR-181a-5p and LATS2 was found on Starbase (**Figure 3A**), and a negative relation was found between expression of miR-181a-5p and LATS2 ($P < 0.01$, **Figure 3B**, **3C**). Furthermore, miR-181a-5p inhibitor upregulated LATS2 in ACC cells ($P < 0.01$, **Figure 3D**, **3E**).

LATS2 silencing reverses inhibitory role of reduced miR-181a-5p in ACC cell growth

LATS2 was altered in cells, and a rescue experiment was conducted as well. We found that si-LATS2 downregulated LATS2, and transfection of si-LATS2 after miR-181a-5p inhibition reduced LATS2 expression ($P < 0.01$, **Figure 4A**, **4B**). Moreover, it was observed that treatment with si-LATS2 repressed ACC malignant behavior; transfection of si-LATS2 after miR-181a-5p inhibition reversed the impact of miR-181a-5p inhibitor on ACC cells ($P < 0.01$, **Figure 4C-F**). The above data showed that LATS2 silencing reversed inhibitory role of reduced miR-181a-5p in ACC cells.

MiR-181a-5p knockdown decelerates ACC cell growth in vivo

Subcutaneous tumorigenesis was studied and outcomes revealed that miR-181a-5p inhibitor restricted the growth rate and weight of ACC xenografts ($P < 0.01$, **Figure 5A-C**). It could be concluded that miR-181a-5p knockdown decelerated ACC cell growth *in vivo*.

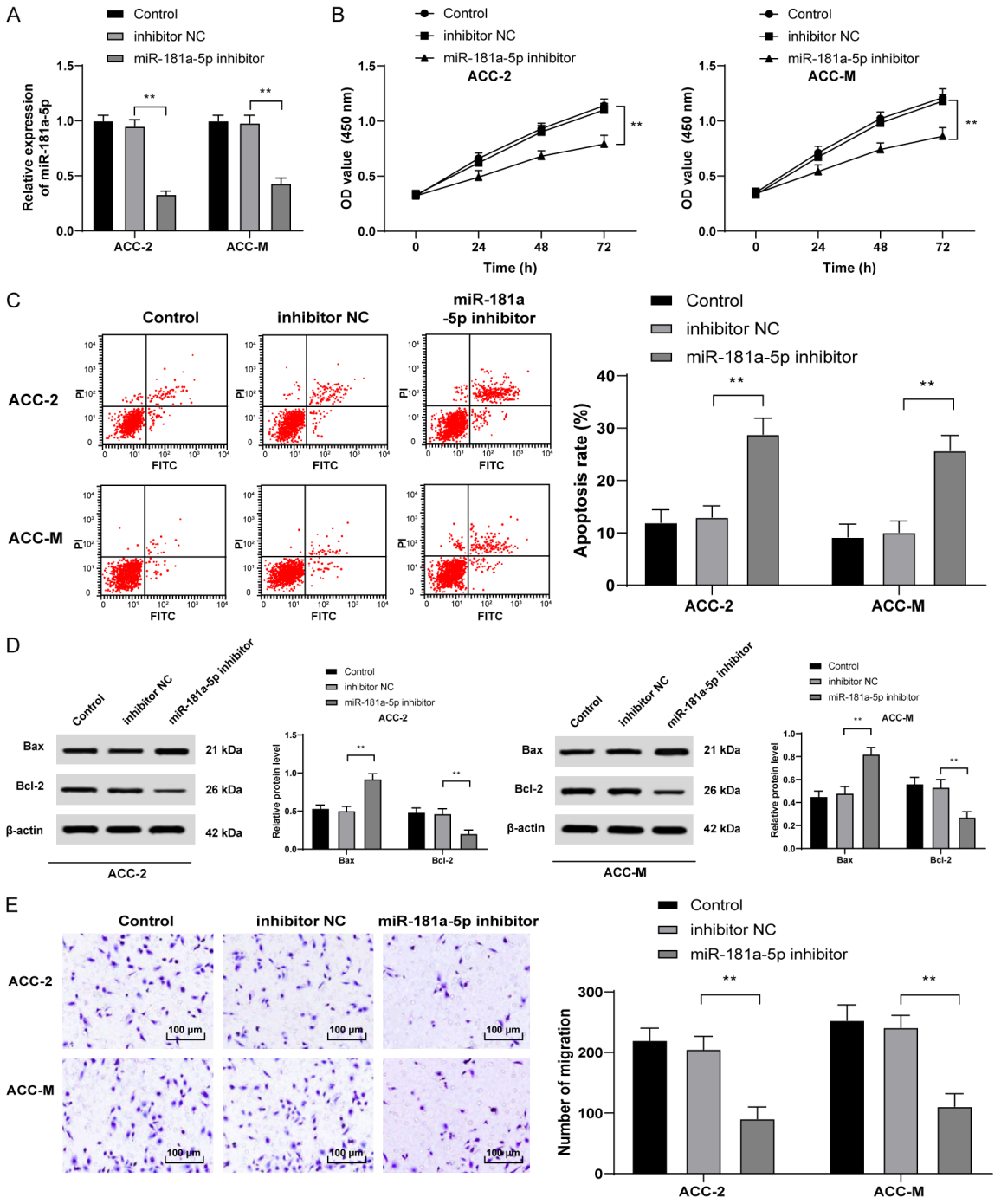

Figure 2. MiR-181a-5p knockdown suppresses malignant behavior of ACC cells. A. miR-181a-5p expression in ACC cells was determined using RT-qPCR; B. MTT assay was used to measure the proliferation of ACC cells; C. Flow cytometry was used to detect the apoptosis of ACC cells; D. Apoptosis protein expression in ACC cells was evaluated using western blot analysis; E. Transwell assay was performed to assess migration of ACC cells; one-way ANOVA was used for comparisons among multiple groups and Tukey's post hoc test was used for pairwise comparisons after one-way ANOVA; **, $P < 0.01$; N = 3.

Discussion

In China, ACC is one of the commonest epithelial tumors of the lacrimal gland, and LACC has an aggressive behavior and is associated with significant morbidity and mortality [15]. MiRNAs exert critical effects on biologic processes, including cellular proliferation, migration, and

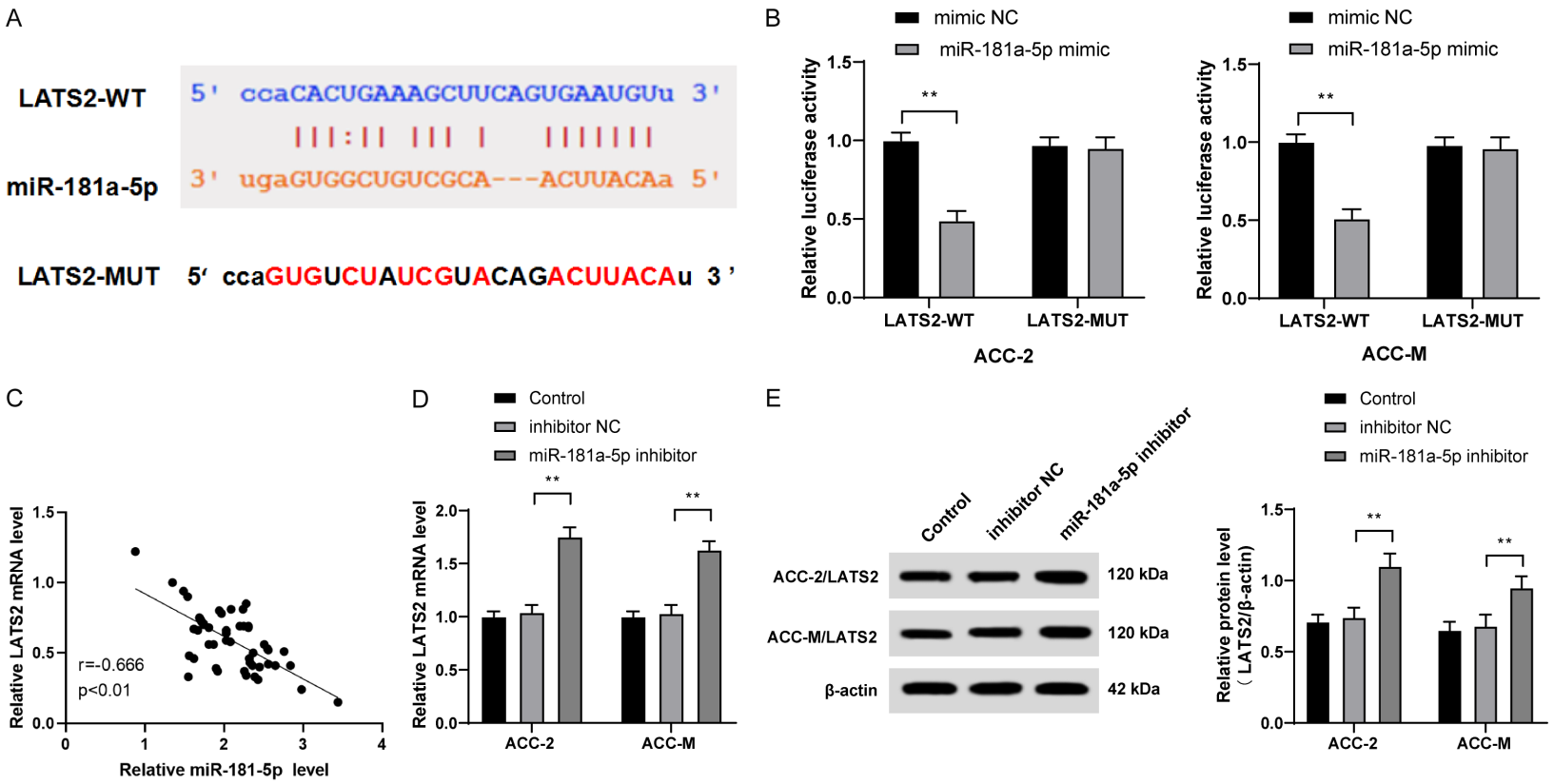

Figure 3. MiR-181a-5p targets LATS2. A. Binding site of miR-181a-5p and LATS2 was predicted by Starbase; B. Targeting relationship between miR-181a-5p and LATS2 was confirmed by dual luciferase reporter gene assay; C. Correlation analysis of expression of miR-181a-5p and LATS2; D. LATS2 mRNA expression in ACC cells was assessed by RT-qPCR; E. LATS2 protein expression in ACC cells was assessed by western blot analysis; Pearson test was used in C; one-way ANOVA was used for comparisons among multiple groups and Tukey's post hoc test was used for pairwise comparisons after one-way ANOVA; **, $P < 0.01$; N = 3.

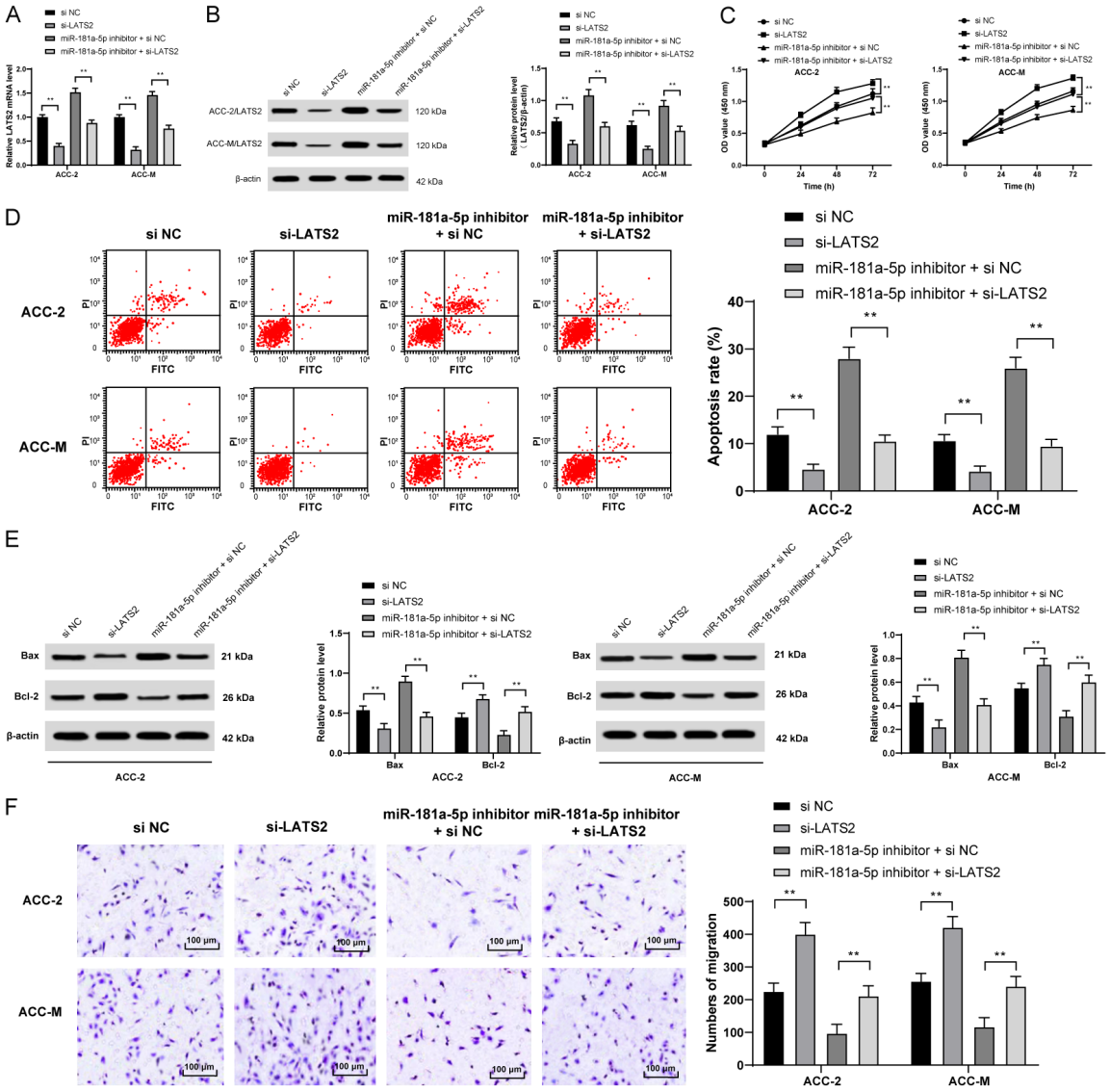

Figure 4. LATS2 silencing reverses inhibitory role of reduced miR-181a-5p in ACC cell growth. A. LATS2 mRNA expression in ACC cells was assessed by RT-qPCR; B. LATS2 protein expression in ACC cells was assessed by western blot analysis; C. MTT assay was used to measure the proliferation of ACC cells; D. Flow cytometry was used to detect the apoptosis of ACC cells; E. Apoptosis protein expression in ACC cells was evaluated using western blot analysis; F. Transwell assay was performed to assess migration of ACC cells; one-way ANOVA was used for comparisons among multiple groups and Tukey's post hoc test was used for pairwise comparisons after one-way ANOVA; **, $P < 0.01$; N = 3.

apoptosis [16]. This research investigated the function of miR-181a-5p targeting LATS2 on the biologic functions of ACC cells, and we found that miR-181a-5p downregulation suppressed proliferation and migration, and promoted apoptosis of ACC cells by promoting LATS2, while these effects triggered by inhibited miR-181a-5p were reversed by LATS2 silencing.

We assessed miR-181a-5p and LATS2 expression in LACC tissues. Our outcomes reflected that miR-181a-5p was enhanced and LATS2 was downregulated in LACC tissues compared to adjacent non-tumor tissues. Similarly, Yang *et al*. have validated that cervical cancer cell lines showed higher miR-181a-5p expression than the normal cervical epithelium cell line [17], and recent literature showed that miR-

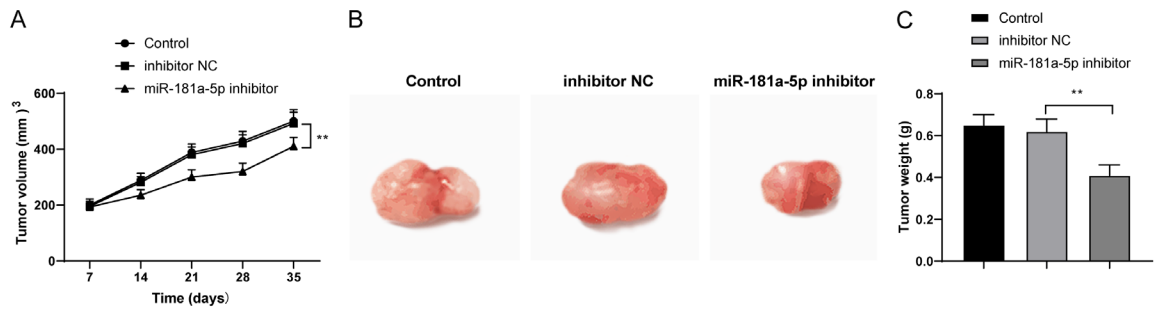

Figure 5. MiR-181a-5p knockdown decelerates ACC cell growth *in vivo*. A. Volume of ACC xenografts; B. Images of ACC xenografts; C. Weight of ACC xenografts; one-way ANOVA was used for comparisons among multiple groups and Tukey's post hoc test was used for pairwise comparisons after one-way ANOVA; **, $P < 0.01$; N = 5.

181a-5p was upregulated in gastric cancer (GC) tissues with distant metastasis compared to those without metastasis [18]. As for the aberrant expression of LATS2, it has been validated that LATS2 was downregulated in A549 lung cancer cells [12], and Guo *et al*. showed that LATS2 was upregulated in glioma [19], indicating that LATS2 usually serves as a tumor repressor. We further determined the relation between miR-181a-5p and LATS2, and it was discovered that miR-181a-5p and LATS2 expression were inversely correlated, and LATS2 was targeted by miR-181a-5p. The targeting relationship of miR-181a-5p and SIRT1 in cardiomyocyte apoptosis has been verified [13], and miR-181a-5p also targeted SECISBP2 in osteoarthritis [14]. Nevertheless, the relation between miR-181a-5p and LATS2 is still unclear.

The ACC cells were treated with altered miR-181a-5p or LATS2 to assess their roles in cell growth, and we found through MTT assay, Transwell assay and flow cytometry that miR-181a-5p inhibition facilitated apoptosis and restrained viability and migration of ACC cells. Likewise, Mi *et al*. have identified that reduced miR-181a-5p inhibited GC cell growth [18]. It has also been elucidated that miR-181a-5p facilitated malignant behavior of cervical cancer cells [17]. Moreover, the si-LATS2-transfected ACC cells showed enhanced migration and viability as well as inhibited apoptosis. A similar finding in a recent study implied that reduction of endogenous LATS2 promoted cell growth and the disrupted cell cycle in breast cancer [20]. The outcomes of the rescue experiment in our research indicated that the downregulated miR-181a-5p-induced effects on ACC cells were reversed by LATS2 silencing. Similarly, LATS2 upregulation has been validated to eliminate the promoting role of elevated miR-224-3p in development of retinoblastoma [21], and Sun *et al*. have reported that LATS2 silencing abolished the impact of miR-492 reduction on retinoblastoma cell proliferation and invasion [22]. In addition, our *in vivo* experiment reflected that miR-181a-5p knockdown decelerated growth of ACC xenografts. Similarly, Tian *et al*. showed that miR-181a inhibition decelerated osteosarcoma xenograft growth, while miR-181a elevation had a reverse effect on the tumor growth [23]. These studies provide a theoretical basis for revealing functions of miR-181a-5p and LATS2 in tumor progression.

Collectively, we validated that miR-181a-5p downregulation decelerated growth of ACC cells by promoting LATS2, and these effects induced by inhibited miR-181a-5p were reversed by LATS2 silencing. Our study may contribute to the treatment of LACC, while further investigations are still needed to reveal the underlying molecular mechanisms in LACC.

Disclosure of conflict of interest

None.

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References

[1] Noh JM, Lee E, Ahn YC, Oh D, Kim YD, Woo KI, Ko YH and Kim S. Clinical significance of post-surgical residual tumor burden and radiation

therapy in treating patients with lacrimal adenoid cystic carcinoma. Oncotarget 2016; 7: 60639-60646.
[2] Zhang MX, Zhang J, Zhang H and Tang H. miR-24-3p suppresses malignant behavior of lacrimal adenoid cystic carcinoma by targeting PRKCH to regulate p53/p21 pathway. PLoS One 2016; 11: e0158433.
[3] Zhou Q, Chang H, Han YD, Gao Y and Liu HG. High-grade transformation in adenoid cystic carcinoma: a clinicopathologic study. Zhonghua Bing Li Xue Za Zhi 2013; 42: 106-10.
[4] Esmaeli B, Ahmadi MA, Youssef A, Diba R, Amato M, Myers JN, Kies M and El-Naggar A. Outcomes in patients with adenoid cystic carcinoma of the lacrimal gland. Ophthalmic Plast Reconstr Surg 2004; 20: 22-6.
[5] Ye L, Wang Y, Nie L, Qian S and Xu M. MiR-130 exerts tumor suppressive function on the tumorigenesis of human non-small cell lung cancer by targeting PTEN. Am J Transl Res 2017; 9: 1856-1865.
[6] Hao J, Jin X, Shi Y and Zhang H. miR-93-5p enhance lacrimal gland adenoid cystic carcinoma cell tumorigenesis by targeting BRMS1L. Cancer Cell Int 2018, 18: 72.
[7] Korhan P, Erdal E and Atabey N. MiR-181a-5p is downregulated in hepatocellular carcinoma and suppresses motility, invasion and branching-morphogenesis by directly targeting c-Met. Biochem Biophys Res Commun 2014; 450: 1304-1312.
[8] Mutlu S, Mutlu H, Kirkbes S, Eroglu S, Kabukcuoglu YS, Kabukcuoglu F, Duymus TM, ISık M and Ulasli M. The expression of miR-181a-5p and miR-371b-5p in chondrosarcoma. Eur Rev Med Pharmacol Sci 2015; 19: 2384-2388.
[9] He Q, Zhou X, Li S, Jin Y, Chen Z, Chen D, Cai Y, Liu Z, Zhao T and Wang A. MicroRNA-181a suppresses salivary adenoid cystic carcinoma metastasis by targeting MAPK-Snai2 pathway. Biochim Biophys Acta 2013; 1830: 5258-5266.
[10] Takahashi Y, Miyoshi Y, Takahata C, Irahara N, Taguchi T, Tamaki Y and Noguchi S. Down-regulation of LATS1 and LATS2 mRNA expression by promoter hypermethylation and its association with biologically aggressive phenotype in human breast cancers. Clin Cancer Res 2005; 11: 1380-1385.
[11] Zhang L, Li S, Wang R, Chen C, Ma W and Cai H. Anti-tumor effect of LATS2 on liver cancer death: role of DRP1-mediated mitochondrial division and the Wnt/beta-catenin pathway. Biomed Pharmacother 2019; 114: 108825.
[12] Xie Y, Lv Y, Zhang Y, Liang Z, Han L and Xie Y. LATS2 promotes apoptosis in non-small cell lung cancer A549 cells via triggering Mff-dependent mitochondrial fission and activating the JNK signaling pathway. Biomed Pharmacother 2019; 109: 679-689.
[13] Qi M, He L, Ma X and Li Z. MiR-181a-5p is involved in the cardiomyocytes apoptosis induced by hypoxia-reoxygenation through regulating SIRT1. Biosci Biotechnol Biochem 2020; 1-9.
[14] Xue J, Min Z, Xia Z, Cheng B, Lan B, Zhang F, Han Y, Wang K and Sun J. The hsa-miR-181a-5p reduces oxidation resistance by controlling SECISBP2 in osteoarthritis. BMC Musculoskelet Disord 2018; 19: 355.
[15] Yang J, Zhou CD, Wang YF, Fan XQ and Jia RB. Multimodal therapy in the management of lacrimal gland adenoid cystic carcinoma. BMC Ophthalmol 2019; 19: 125.
[16] Feliciano A, Garcia-Mayea Y, Jubierre L, Mir C, Hummel M, Castellvi J, Hernandez-Losa J, Paciucci R, Sansano I, Sun Y, Ramon YCS, Kondon H, Soriano A, Segura M, Lyakhovich A and LLeonart ME. miR-99a reveals two novel oncogenic proteins E2F2 and EMR2 and represses stemness in lung cancer. Cell Death Dis 2017; 8: e3141.
[17] Yang M, Zhai X, Ge T, Yang C and Lou G. miR-181a-5p promotes proliferation and invasion and inhibits apoptosis of cervical cancer cells via regulating inositol Polyphosphate-5-Phosphatase A (INPP5A). Oncol Res 2018; 26: 703-712.
[18] Mi Y, Zhang D, Jiang W, Weng J, Zhou C, Huang K, Tang H, Yu Y, Liu X, Cui W, Zhang M, Sun X, Zhou Z, Peng Z, Zhao S and Wen Y. miR-181a-5p promotes the progression of gastric cancer via RASSF6-mediated MAPK signalling activation. Cancer Lett 2017; 389: 11-22.
[19] Guo C, Liang C, Yang J, Hu H, Fan B and Liu X. LATS2 inhibits cell proliferation and metastasis through the Hippo signaling pathway in glioma. Oncol Rep 2019; 41: 2753-2761.
[20] Hua K, Jin J, Zhao J, Song J, Song H, Li D, Maskey N, Zhao B, Wu C, Xu H and Fang L. miR-135b, upregulated in breast cancer, promotes cell growth and disrupts the cell cycle by regulating LATS2. Int J Oncol 2016; 48: 1997-2006.
[21] Song L, Huang Y, Zhang X, Han S, Hou M and Li H. Downregulation of microRNA-224-3p hampers retinoblastoma progression via activation of the Hippo-YAP signaling pathway by increasing LATS2. Invest Ophthalmol Vis Sci 2020; 61: 32.
[22] Sun Z, Zhang A and Zhang L. Inhibition of microRNA492 attenuates cell proliferation and invasion in retinoblastoma via directly targeting LATS2. Mol Med Rep 2019; 19: 1965-1971.
[23] Tian BG, Hua Z, Wang ZJ and Li J. Knockdown of microRNA-181a inhibits osteosarcoma cells growth and invasion through triggering NLRP3-dependent pyroptosis. Eur Rev Med Pharmacol Sci 2020; 24: 1030-1040.