

miR-30a-5p inhibits the proliferation, migration and invasion of melanoma cells by targeting SOX4

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Abstract. MicroRNA (miR)-30a-5p has been reported to suppress the progression of hepatocellular cancer, renal cell carcinoma, oral cancer and gastric cancer. However, whether miR-30a-5p is involved in the regulation of melanoma remains unclear. The present study revealed that miR-30a-5p was downregulated in melanoma tissues and cell lines. Overexpression of miR-30a-5p significantly inhibited the proliferation, migration and invasion of melanoma cells *in vitro*. In addition, ectopic expression of miR-30a-5p delayed tumor growth *in vivo*. In terms of mechanism, miR-30a-5p targeted sex determining region Y-box 4 (SOX4) and impeded the expression of SOX4 in melanoma cells. In addition, SOX4 was upregulated in melanoma tissues and cell lines when compared with normal tissues or cells. Furthermore, overexpression of SOX4 significantly rescued the proliferation, migration and invasion of melanoma cells transfected with miR-30a-5p mimics. Taken together, the results of the present study demonstrated that miR-30a-5p suppressed the proliferation, migration and invasion of melanoma cells in SOX4-dependent manner.

Introduction

Originated from the transformation of melanocytes, malignant melanoma is the most aggressive skin cancer and contributes to most of skin cancer-related deaths (1). The incidence and recurrence of melanoma is rapidly rising worldwide every year (2). Surgery and therapeutic agents are a favorable therapeutic method for melanoma of early stage. However, there is no effective approach for the intervention of advanced or metastatic melanoma (3), which contributes to a large

proportion among melanoma. The 5-year survival rate is quite low. Therefore, a better understanding of the molecular mechanism of melanoma progression and metastasis will benefit the treatment of melanoma patients.

MicroRNAs (miRNAs) are small and endogenous noncoding RNAs with a length of approximately 18-22 nucleotides (4). miRNAs regulate the expression of target genes via association with the complementary sites of 3'-UTR region of mRNAs (5). Large numbers of studies indicate that miRNAs serve as important regulators in diverse biological processes, including cell survival, apoptosis and metastasis (6,7). Therefore, miRNA expression is closely correlated with tumor development. More and more evidence shows that miRNAs are dysregulated in various tumors, including melanoma. For instance, Peng *et al* (8) showed that miR-155 promotes uveal melanoma cell proliferation and invasion by regulating NDFIP1 expression. Liu *et al* (9) reported that miRNA-675 inhibits cell proliferation and invasion in melanoma by directly targeting metadherin. Kang *et al* indicated that miRNA-326 inhibits melanoma progression by targeting KRAS and suppressing the AKT and ERK signalling pathways (10). Therefore, investigation of the function and mechanism of miRNAs in the progression of melanoma is very important for melanoma intervention.

Previous research shows that miR-30a-5p acts as a tumor suppressor in a kind of cancers, such as gastric cancer (11) and hepatocellular carcinoma (12). Other reports also showed that miR-30 family exerts an important roles in breast cancer (13), papillary thyroid cancer (14) and bladder cancer (15). However, whether miR-30a-5p plays a role or not in melanoma remains largely unknown. In this study, we found that miR-30a-5p was downregulated in melanoma tissues and cell lines. Moreover, overexpression of miR-30a-5p significantly inhibited the proliferation, migration and invasion of melanoma cells. Concerning the mechanism, we found that sex determining region Y-box 4 (SOX4) is a direct target of miR-30a-5p in melanoma cells. Through functional experiments, we found that overexpression of SOX4 overrides the effects of miR-30a-5p on melanoma cells. Taken together, our findings demonstrated the key role of miR-30a-5p/SOX4 axis in melanoma progression.

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Materials and methods

Clinical specimens and cell lines. Twenty two pairs of malignant melanoma and the tumor adjacent normal tissues

were acquired from patients undergoing a surgical procedure and histopathologically diagnosed at Shanxian Central Hospital (Shandong, China). Correlation between miR-30a-5p expression and clinical pathological characteristic was listed in Table I. For all samples, written informed consent was obtained and the present study was approved by the Independent Ethical Committees of Shanxian Central Hospital. Tissue samples were stored at -80°C .

Human melanoma cell lines, including A375, SK-HEP-1, SK-MEL-1, and MV3, were purchased from the American Type Culture Collection (Manassas, VA, USA). Human primary melanocytes (HPM) were obtained from PromoCell (Beijing, China). Melanoma cell lines were cultured in a Roswell Park Memorial Institute-1640 medium containing 10% fetal bovine serum (FBS; both Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1% penicillin-streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.). HPM cells were grown in serum-free and PMA-free melanocyte growth medium M2 (PromoCell) per the manufacturer's instructions. Cells were maintained at 37°C and placed in a humidified incubator containing 5% CO_2 .

Oligonucleotide and transfection. The miR-30a-5p mimics (5'-UGUAAACAUCUCCGACUGGAAG-3'), inhibitors (5'-CTTCCAGTCGAGGATGTTTACA-3') and corresponding negative controls (NC, 5'-UCACAACCUCCUAGAAAGAGUAGA-3') were synthesized by GenePharma Co., Ltd (Shanghai, China) and transfected into cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.).

Cell proliferation assay. Cell proliferation was detected by Cell Counting kit (7 Sea Biotech, Shanghai, China). Cells were grown in 96-well plate with 2×10^3 per well and incubated in 37°C with 5% CO_2 until cell confluent rate reached 70%. After transfected with plasmid for 48 h, cells were still incubated for 24, 48 and 72 h. $10 \mu\text{l}$ CCK8 solution was seed into each well. The absorbance at 450 nm was measured with SUNRISE Microplate Reader (Tecan, Group, Ltd., Mannedorf, Switzerland).

Transwell migration and invasion assay. A total of 1×10^4 cells were transfected with miR-30a-5p mimics or inhibitor for 48 h. The transfected cells were then suspended in a $500 \mu\text{l}$ serum-free medium and seeded onto a Transwell membrane (Corning Inc., Corning, NY, USA) precoated with Matrigel (BD Bioscience; San Jose, CA, USA). The lower chamber was filled with a $500 \mu\text{l}$ growth medium containing 10% FBS, which served as a chemoattractant. The cells were cultured at 37°C for 24 h. The non-invading cells on the top well were gently scraped off. Subsequently, 0.4% crystal violet (Sigma, St. Louis, MO, USA) was used to stain the invaded cells on the lower filter side. Cell invasion was evaluated under the microscope (Olympus Corp., Tokyo, Japan). For migration assay, Transwell membranes were not precoated with Matrigel. Other steps are the same as invasion assay.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cultured cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol and cDNA

Table I. Correlation between microRNA-30a-5p expression and clinical pathological characteristic.

Clinical characteristics	Low expression (n=11)	High expression (n=11)	P-value
Age, years			0.659
<50	3	5	
≥ 50	8	6	
Sex			0.670
Male	5	7	
Female	6	4	
TNM stage			0.030
I-II	2	8	
III	9	3	

TNM, tumor-node-metastasis staging system.

was synthesized from $1 \mu\text{g}$ of total RNAs by a PrimerScript RT Reagent kit (Takara Bio, Inc., Otsu Japan). MiRNA from total RNA was reverse transcribed using the Prime-Script miRNA cDNA Synthesis kit (Takara). RT-qPCR was performed with the SYBR-Green Premix Ex Taq II (Takara) on Applied Biosystems Step One Plus Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). The procedure was as follows: 95°C -3 min; $39 \times (95^{\circ}\text{C}$ -15 sec, 60°C -60 sec, 72°C -30 sec, for mRNA; 95°C -15 sec, 60°C -60 sec for miRNA); 95°C -10 sec, followed by a melt curve analysis (60 - 95°C , increment 0.5°C for 20 sec) to confirm specificity of the PCR primers. GAPDH was used as the endogenous control for detection of mRNA expression level, while U6 was used as endogenous control for miRNA expression analysis. The $2^{-\Delta\Delta\text{C}_q}$ method was used to analyze the data (16). The RT-qPCR primer sequences are as follows: miR-30a-5p forward, 5'-AACGAGACGACAGAC-3' and reverse, 5'-TGTAACATCCTCGACTGGAAG-3', U6 forward, 5'-AACGAGACGACGACAGAC-3' and reverse, 5'-GCAAATTCGTGAAGCGTCCATA-3', SOX4 forward, 5'-GCACTAGGACGTCTGCCTTT-3' and reverse, 5'-ACACGGCATATTGCACAGGA-3') and GAPDH forward, 5'-ATGTTGCAACCGGGAAGGAA-3' and reverse, 5'-AGGAAAAGCATCACCCGGAG-3').

Tumor xenograft model. The protocol of animal experiments was reviewed and approved by the Medical Ethics Committee of Shanxian Central Hospital. For tumor growth assay, BALB/c nude mice of four-week-old were used for the tumor growth xenograft models ($n=8$ per group). 1×10^7 A375 cells transfected with control vector or miR-30a-5p mimic construct were suspended in $100 \mu\text{l}$ of medium and injected subcutaneously into the lower left flank regions of mice model. The tumor volume and weight were measured.

Western blot analysis. Cells were collected and lysed with radio immunoprecipitation assay buffer according to the manufacturer's instruction. The protein concentration was measured by BCA protein assay kit (Boster, Wuhan, China). Twenty micrograms of proteins was separated on

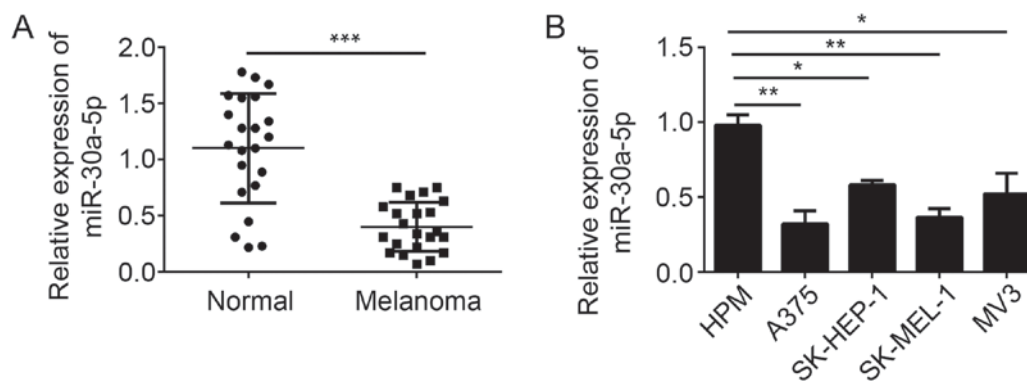


Figure 1. miR-30a-5p is downregulated in melanoma tissues. (A) RT-qPCR analysis indicated that miR-30a-5p was downregulated in melanoma tissues compared with normal tissues. (B) The expression patterns of miR-30a-5p in melanoma cell lines were determined by RT-qPCR. All data are representative of three independent experiments and expressed as the mean \pm standard deviation. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, as indicated. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; miR, microRNA.

12% SDS-PAGE and electrophoretically transferred onto polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% nonfat skim milk for 1 h at the room temperature, and then incubated with primary antibodies overnight at 4°C. After rinsing, the membranes were incubated with the corresponding secondary horseradish peroxidase-conjugated secondary antibodies (Abcam, Cambridge, UK) for 2 h at 37°C. The protein bands were visualized with ECL chemiluminescent kit (Thermo Fisher Scientific, Inc.). GAPDH was used for normalization.

Luciferase reporter assay. The wild-type or mutant 3'-UTR of SOX4 were designed and prepared by GenePharma Co., Ltd. and then cotransfected into the cells with miR-30a-5p. Relative luciferase activity was calculated 48 h post-transfection by the Dual Luciferase Reporter Assay (Promega, Madison, WI, USA).

Statistical analysis. SPSS 19 statistical software for Windows (IBM Corp., Armonk, NY, USA) and GraphPad Prism 5.01 (GraphPad Software, Inc., La Jolla, CA, USA) software were used for statistical analysis. All data are presented as the mean \pm standard deviation from at least three independent experiments. Student's t-test and one-way analysis of variance followed by Tukey's post hoc test were used to analyze 2 or multiple groups, respectively, for statistical significance. Chi-square test was used for analysis of correlation between miR-30a-5p expression and clinicopathological characteristics. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

miR-30a-5p was downregulated in melanoma tissues. To explore the potential effect of miR-30a-5p in melanoma progression, we examined the expression of miR-30a-5p in melanoma tissues and cell lines by RT-qPCR. We found that the expression of miR-30a-5p was significantly downregulated in melanoma tissues compared with adjacent normal tissues (Fig. 1A). Moreover, miR-30a-5p expression was also downregulated in melanoma cell lines, including A375, SK-HEP-1, SK-MEL-1 and MV3, compared to HPM

cells (Fig. 1B). These data indicated that miR-30a-5p may play a role in the development of melanoma.

MiR-30a-5p suppressed the proliferation, migration and invasion of melanoma cells in vitro. To further investigate the function of miR-30a-5p on melanoma cells, we overexpressed or knocked down miR-30a-5p in A375 cells (a malignant melanoma cell line) via transfection with miR-30a-5p mimics or inhibitors. Through RT-qPCR, we found that the expression of miR-30a-5p was significantly upregulated in A375 cells transfected with miR-30a-5p mimics and downregulated in A375 cells transfected miR-30a-5p inhibitors (Fig. 2A). Through CCK8 assay, we found that overexpression of miR-30a-5p significantly inhibited the proliferation of A375 cells, and vice versa (Fig. 2B). Moreover, we checked the cell-cycle by FACS, and found that overexpression of miR-30a-5p remarkably inhibited the cell numbers in S and G2/M phases, and vice versa (Fig. 2C). Tumor metastasis contributes to the malignance of melanoma. We then conducted Transwell assay to determine the effect of miR-30a-5p on cell migration and invasion. As shown, we found that overexpression of miR-30a-5p markedly inhibited the migration and invasion of A375 cells, and vice versa (Fig. 2D and E).

MiR-30a-5p overexpression inhibited tumor growth in vivo. To evaluate the effect of miR-30a-5p on tumor growth *in vivo*, we performed a xenograft experiment. Consistent with the results *in vitro*, we found that overexpression of miR-30a-5p significantly inhibited the tumorigenesis by A375 cells. As shown, miR-30a-5p overexpression reduced the tumor volume and weight (Fig. 3A and B). Moreover, we analyzed the proliferation of formed tumor tissues by western blot. The result indicated that overexpression of miR-30a-5p suppressed the protein level of Cyclin D1 and SOX4 (Fig. 3C), which suggested that miR-30a-5p was a negative regulator of cell cycle by regulating SOX4 *in vivo*.

SOX4 was a target of miR-30a-5p. That miRNAs target the 3'-UTR of mRNAs to exert functions is widely demonstrated. Therefore, we search the target gene of miR-30a-5p by a TargetScan tool. We found that SOX4 was a potential target of miR-30a-5p. There was a potential binding site of miR-30a-5p in the 3'-UTR region

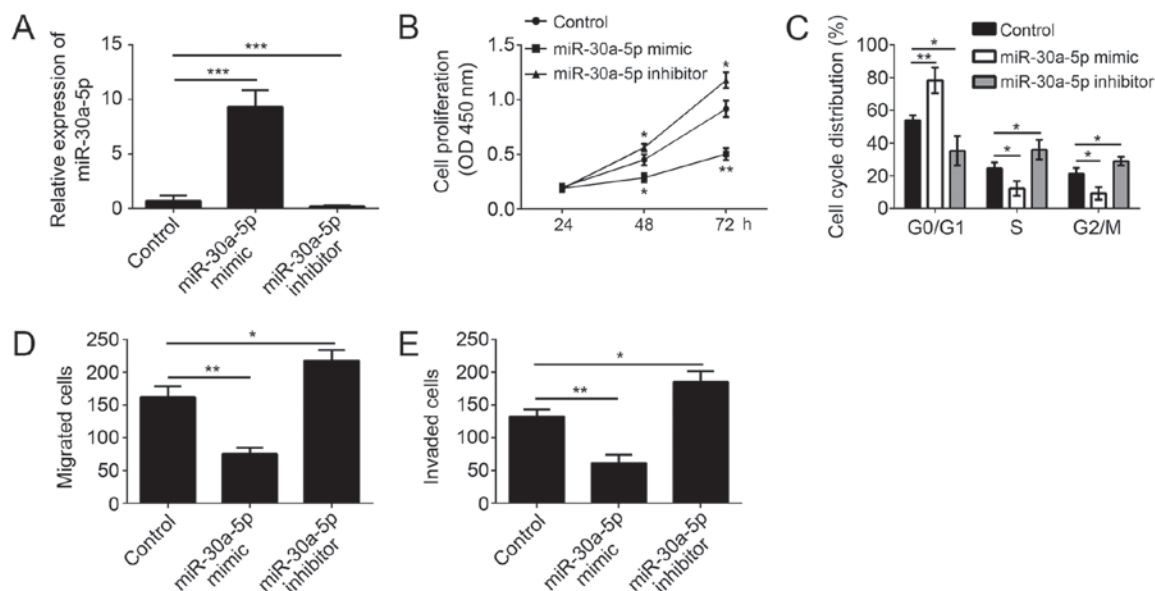


Figure 2. MiR-30a-5p suppresses the proliferation, migration and invasion of melanoma cells *in vitro*. (A) MiR-30a-5p expression was determined by reverse transcription-quantitative polymerase chain reaction in A375 cells transfected with miR-30a-5p mimics, inhibitors or controls. (B) Cell Counting kit-8 assay was used to analyze the proliferation of A375 cells transfected with miR-30a-5p mimics, inhibitors or controls. (C) Cell cycle distribution of A375 cells was examined by flow cytometric analysis. (D and E) Transwell assay was used to detect the (D) migration and (E) invasion of A375 cells transfected with miR-30a-5p mimics, inhibitors or controls. All data are representative of three independent experiments and expressed as the mean \pm standard deviation. * P <0.05, ** P <0.01 and *** P <0.001, as indicated. miR, microRNA.

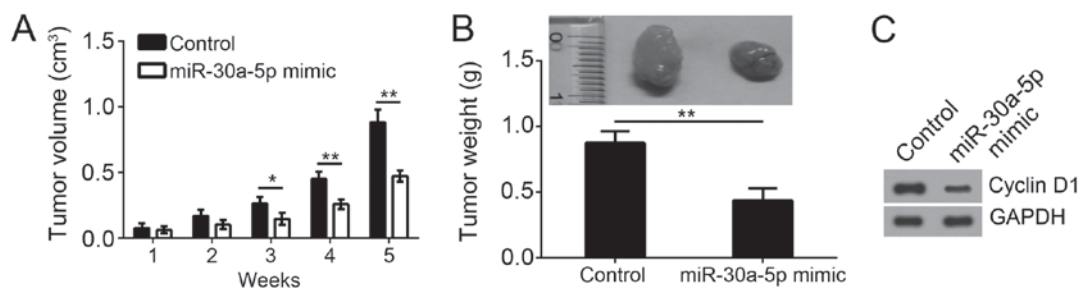


Figure 3. miR-30a-5p overexpression inhibits tumor growth *in vivo*. (A) Tumor volume was determined at the indicated time points. (B) Tumor weight was measured at the endpoint of the xenograft experiment. (C) The protein expression of Cyclin D1 in the tumor tissues was measured by western blotting. All data are representative of three independent experiments and expressed as the mean \pm standard deviation. * P <0.05 and ** P <0.01, as indicated. miR, microRNA.

of SOX4 mRNA (Fig. 4A). We then constructed luciferase reporter plasmid with SOX4-WT-3'-UTR or SOX4-Mut-3'-UTR. By luciferase reporter assay, we found that overexpression of miR-30a-5p significantly inhibited the luciferase activity in A375 cells transfected with SOX4-WT-3'-UTR but not SOX4-Mut-3'-UTR (Fig. 4B), which proved the direct interaction of miR-30a-5p with SOX4 mRNA. Moreover, we analyzed the mRNA and protein levels of SOX4 in A375 cells transfected with miR-30a-5p mimics or inhibitors. We found that overexpression of miR-30a-5p significantly inhibited SOX4 expression in A375 cells, at both the mRNA and protein levels, and vice versa (Fig. 4C and D). Furthermore, RT-qPCR analysis indicated that SOX4 was highly expressed in melanoma cell lines and tissues compared to normal cells or tissues (Fig. 4E and F). Consistently, the protein levels of SOX4 were also upregulated in melanoma cell lines (Fig. 4G) and tissues (Fig. 4H).

Overexpression of SOX4 rescued the proliferation, migration and invasion suppressed by miR-30a-5p. To determine

whether SOX4 was involved in miR-30a-5p-mediated regulation on melanoma progression, we restored SOX4 expression in A375 cells transfected with miR-30a-5p (Fig. 5A). Then we performed CCK8 assay and found that restoration significantly increased the proliferation of A375 cells transfected with miR-30a-5p (Fig. 5B). Moreover, Transwell assay also showed that restoration of SOX4 rescued the migrated and invaded cell numbers (Fig. 5C and D). Additionally, in the xenograft experiment, SOX4 expression was also downregulated in miR-30a-5p overexpressing group (Fig. 5E). Taken together, our results demonstrated that miR-30a-5p suppressed the proliferation, migration and invasion of melanoma cells through targeting SOX4.

Discussion

Accumulating evidence has demonstrated that miRNAs have vital functions in the development and progression of melanoma (17). Also, some studies indicate that miRNAs

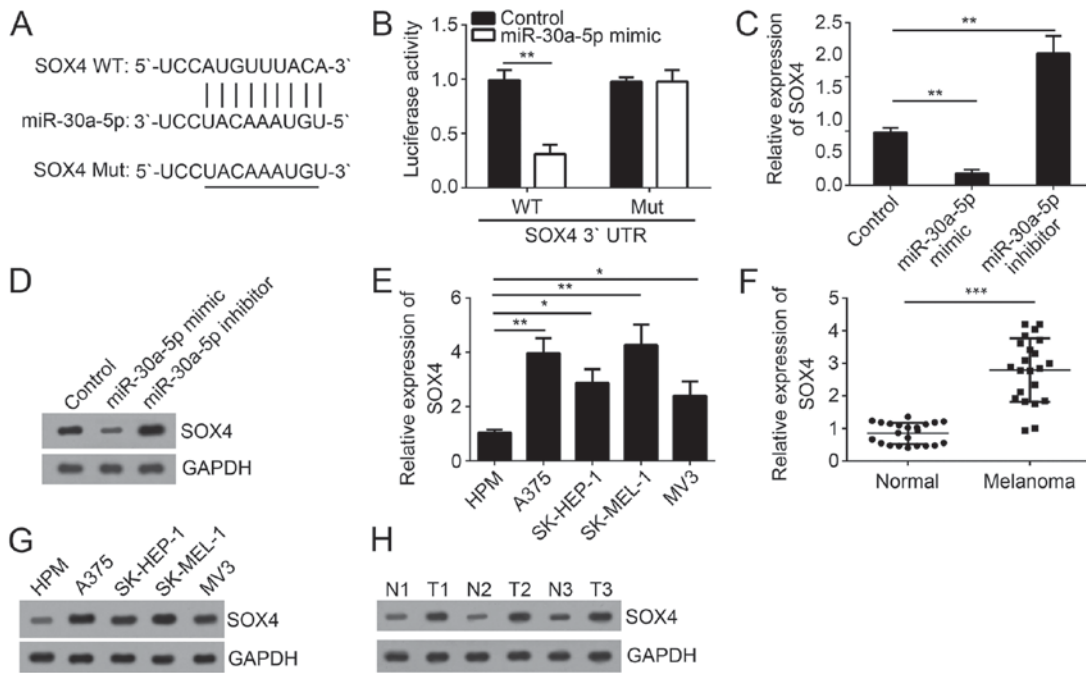


Figure 4. SOX4 is a target of miR-30a-5p. (A) The binding site of miR-30a-5p in the 3'-UTR region of SOX4 mRNA was predicted by bioinformatics analysis. (B) Luciferase activity reporter assay indicated that overexpression of miR-30a-5p inhibited the luciferase activity in A375 cells transfected with SOX4-WT-3'-UTR. (C) The mRNA and (D) protein levels of SOX4 in A375 cells transfected with miR-30a-5p mimic, inhibitor or control were measured by RT-qPCR and western blotting. (E) RT-qPCR was used to measure the expression of SOX4 in melanoma cell lines. (F) RT-qPCR was used to measure the expression of SOX4 in melanoma tissues and adjacent normal tissues. (G and H) The protein levels of SOX4 in (G) melanoma cell lines, and (H) pairs of tissues were measured by western blotting. All data are representative of three independent experiments and expressed as the mean \pm standard deviation. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, as indicated. N, normal tissue; T, melanoma tissues; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; miR, microRNA; UTR, untranslated region; SOX4, sex determining region Y-box 4; WT, wild-type; Mut, mutant.

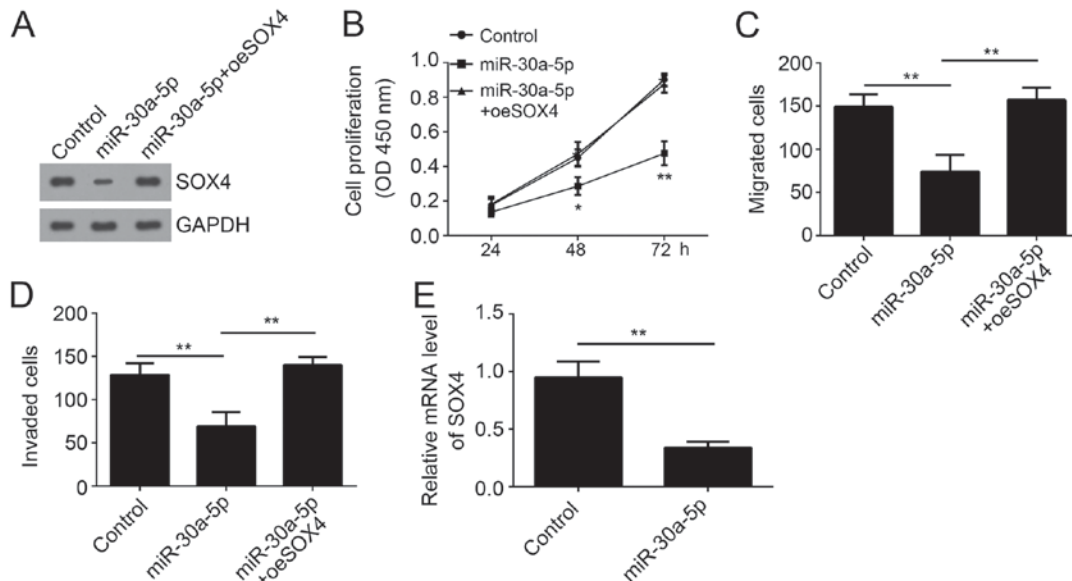


Figure 5. Overexpression of SOX4 rescues the proliferation, migration and invasion that was suppressed by miR-30a-5p. (A) Western blotting was used to determine the expression of SOX4 in A375 cells transfected with miR-30a-5p, as well as SOX4 ectopic expressing plasmid or not. (B) Cell Counting kit-8 assay was used to evaluate the proliferation of A375 cells transfected with miR-30a-5p as well as SOX4 ectopic expressing plasmid or not. * $P < 0.05$ and ** $P < 0.01$ vs. control. (C and D) Transwell assay was used for analysis of (C) migration and (D) invasion in A375 cells transfected with miR-30a-5p as well as SOX4 ectopic expressing plasmid or not. (E) Reverse transcription-quantitative polymerase chain reaction analysis indicated that SOX4 expression was downregulated in formed tumor tissues derived from miR-30a-5p-overexpressing melanoma cells. All data are representative of three independent experiments and expressed as the mean \pm standard deviation. ** $P < 0.01$, as indicated. SOX4, sex determining region Y-box 4; miR, microRNA; oe, overexpression.

could serve as promising biomarkers and therapeutic targets for melanoma diagnosis, prognosis and treatment (18,19). Therefore, it is extremely important to reveal the molecular

mechanism of miRNA-mediated progression of melanoma. In the present study, we observed that the expression of miR-30a-5p was significantly downregulated in melanoma

tissues and cell lines. And overexpression of melanoma suppressed the proliferation, migration and invasion of melanoma cells through targeting SOX4. Our findings identified miR-30a-5p as a novel key regulator in melanoma development.

In the past decades, a large number of miRNAs has been identified as important regulators in the regulation of melanoma progression. For example, miR-26b inhibits melanoma cell proliferation and enhances apoptosis by suppressing TRAF5-mediated MAPK activation (20). MiR-211 is epigenetically regulated by DNMT1 mediated methylation and inhibits EMT of melanoma cells by targeting RAB22A (21). In addition, miR-579-3p controls melanoma progression and resistance to target therapy (22). In this study, we demonstrated miR-30a-5p was a novel miRNA involved in the regulation of melanoma progression. The function of miR-30a-5p in other cancers has been widely investigated. MiR-30a-5p has been identified as a tumor suppressor in hepatocellular cancer (23), renal cell carcinoma (24), small cell lung cancer (25), upper tract urothelial carcinoma (26), gastric cancer (27) and breast tumor (28). Moreover, Wei *et al* (29) indicated that miR-30a-5p suppresses tumor metastasis of human colorectal cancer by targeting ITGB3. Meng *et al* (30) reported that overexpression of miR-30a-5p significantly reduced the expression of the PI3 K regulatory subunit (PIK3R2) to further induce cell apoptosis, and inhibit cell invasion and migration properties. These evidences indicate miR-30a-5p play a tumor suppressive function. Additionally, Li *et al* (31) showed that miR-30a-5p confers cisplatin resistance by regulating IGF1R expression in melanoma cells. However, the role of miR-30a-5p in melanoma progression remains elusive. In our study, through CCK8 and Transwell assay, we demonstrated that overexpression of miR-30a-5p significantly inhibited the proliferation, migration and invasion *in vitro*. Moreover, *in vivo* assay also showed that miR-30a-5p overexpression led to decreased tumor size and reduced Cyclin D1 expression, which indicated that miR-30a-5p inhibited melanoma development.

SOX4 is a member of SOX family of transcription factors, and has been acknowledged as a tumor promotor. Abnormal overexpression of SOX4 has been observed in various human cancers, including prostate cancer (32), breast cancer (33) and esophageal squamous cell carcinoma (34). Additionally, Wang *et al* (35) reported that increased expression of SOX4 is a biomarker for malignant status and poor prognosis in patients with non-small cell lung cancer. Hur *et al* (36) showed that SOX4 overexpression regulates the p53-mediated apoptosis in hepatocellular carcinoma. Besides, many studies show that SOX4 regulates tumor growth and metastasis. In melanoma, previous study also indicated that SOX4 promotes proliferative signals through AKT activation (37). Another study indicated that SOX4 promotes melanoma cell migration and invasion though the activation of the NF- κ B signaling pathway (38). Above evidences demonstrated an oncogenic role of SOX4. In our study, we also demonstrated SOX4 acted as an oncogene. We found that SOX4 was a target of miR-30a-5p in melanoma. We showed that overexpression of miR-30a-5p significantly inhibited the mRNA and protein levels of SOX4 in melanoma cells. Moreover, through CCK8 and Transwell assay, we found overexpression

of SOX4 rescued the proliferation, migration and invasion of melanoma cells suppressed by miR-30a-5p.

In conclusion, our results demonstrated that miR-30a-5p suppressed the development and progression of melanoma through targeting SOX4, which provide a new insight on the pathogenesis of melanoma.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

EL, XS and CZ conceived and designed the present study, analyzed and interpreted the results, and wrote the manuscript. JL performed the experiments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

For the use of human samples, the protocol for the present study was approved by the Institutional Ethics Committee of Shanxian Central Hospital and all enrolled patients signed a written informed consent document. In addition, all procedures involving animals conformed to the national guidelines of, and were approved by, the Animal Care Ethics Committee of Shanxian Central Hospital.

Patient consent for publication

All patients recruited to the present study provided written informed consent for the publication of their data.

Competing interests

The authors declare that they have no competing interests.

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