

MicroRNA-92a regulates the development of cutaneous malignant melanoma by mediating FOXP1

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Abstract. – **OBJECTIVE:** MicroRNAs are non-coding RNAs which are involved in the occurrence and progression of tumors. This study aims to explore the role of microRNA-92a in cutaneous malignant melanoma (CMM) and its underlying mechanism.

PATIENTS AND METHODS: The expression level of microRNA-92a in 75 pairs of CMM tissues and paracancerous tissues was determined using quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The correlation between microRNA-92a expression and clinical data of CMM patients was analyzed. Besides, microRNA-92a expression in CMM cells and primary epidermal melanocytes (PEMs) was determined by qRT-PCR. After transfection of si-microRNA-92a in CMM cells, biological performance of CMM were determined using cell counting kit (CCK-8), colony formation and transwell assay, respectively. FOXP1 expression in CMM cells and tissues was determined using Western blot. Kaplan-Meier survival curves were drawn to explore the correlation between the FOXP1 expression and prognosis of CMM patients.

RESULTS: MicroRNA-92a was highly expressed in CMM tissues compared with that in paracancerous tissues. Compared with CMM patients with lower expression of microRNA-92a, those with higher expression of microRNA-92a presented higher tumor stage, higher incidences of lymph node metastasis and distant metastasis, as well as lower overall survival. The knockdown of microRNA-92a remarkably decreased proliferative, invasive and metastatic capacities of CMM cells. Western blot results elucidated that microRNA-92a knockdown in CMM cells upregulates FOXP1 expression. Additionally, rescue experiments

showed that mi-croRNA-92a regulates biological performances of CMM cells by regulating FOXP1.

CONCLUSION: MicroRNA-92a is highly expressed in CMM, which is remarkably correlated to tumor stage and poor prognosis of CMM patients. We found that microRNA-92a promotes the malignant progression of CMM by regulating FOXP1.

Key Words:

MicroRNA-92a, FOXP1, CMM, Proliferation.

Introduction

Cutaneous malignant melanoma (CMM) is one of the most common surface malignancies originating from melanocytes, which is the fastest growing skin malignancy^{1,2}. CMM frequently occurs in the areas where are rarely exposed to ultraviolet light. About 70-80% of CMM cases in the colored race primarily occurs in the skin, especially in extremities. By comparison, up to 90% of CMM cases occur in the skin of Caucasians^{3,4}. The malignant level of CMM is much higher than that of skin squamous cell carcinoma and basal cell tumor⁵. CMM is mostly progressed from sputum or pigmented plaque. Once CMM enters the rapid growth phase, the prognosis is extremely poor with a very high mortality rate. The average survival of CMM is only 30.3 months, and the disease onset becomes younger⁶. In recent years, there has been an increase of about 3-5% every year in the incidence of CMM.

Among 80% of patients with skin malignancies die from CMM⁶. The complex pathogenesis of CMM involves multiple factors, finally leading to the uncontrolled cancer cell growth^{7,8}. Gene therapy for tumors based on oncogenes or tumor-suppressor genes have been well concerned⁸. Differentially expressed genes in CMM may be involved in the malignant progression of CMM, which may serve as therapeutic targets for CMM.

MicroRNAs are small noncoding RNAs that are involved in multiple biological activities^{9,10}. Abnormally expressed microRNAs would lead to uncontrolled proliferation or differentiation of tumor cells, finally aggravating tumor development¹¹. Researches^{12,13} have found some certain microRNAs that are related to CMM. They seriously affect malignancy, proliferation and metastasis of CMM.

FOXP1 is a member of the FOX family. Studies^{14,15} have shown that FOXP1 is closely related to the occurrence and development of tumors. However, the relationship between microRNA-92a and FOXP1 in the development of CMM has not been explored. The FOX family can affect the proliferation of CMM cells, but its specific mechanism is still unclear. Our previous study¹⁶ reported that microRNA-92a is highly expressed in tumor tissues and serum of CMM patients, suggesting the potential role of microRNA-92a in the occurrence and progression of CMM. It is reported that microRNA-92a is differentially expressed in various tumors, including nasopharyngeal cancer and oral squamous cell carcinoma. Hence, microRNA-92a is considered to be an evaluable hallmark of tumor development. This research aims to explore the regulatory effect of microRNA-92a on malignant progression of CMM, which provides therapeutic targets for clinical treatment.

Materials and Methods

Patients

55 pairs of tumors and paracancerous tissues were surgically resected from CMM patients after surgical resection. All patients were pathologically diagnosed as OSCC according to the 8th edition of UICC/AJCC. Enrolled patients did not receive preoperative anti-tumor treatments. This investigation has been approved by the Ethics Committee of Shenzhen Hospital of Southern Medical University. Patients and their families have signed informed consent.

Cell Lines

7 human CMM cell lines (A375.S2, A7, MeWo, RPMI-7951, SK-MEL-5, SK-MEL-24, and SK-MEL-28) and primary epidermal melanocytes (PEM) were provided by the American Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and maintained at 37°C, 5% CO₂.

Transfection

Si-RNA NC and microRNA-92a were provided by GenePharma Biotechnology Co., Ltd. (Shanghai, China). The cells were seeded in 6-well plates and cell transfection was performed until 70% of confluence using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Counting Kit-8 (CCK-8) Assay

Transfected cells were plated into 96-well plates at 2000 cells per well. After cell culture for 24 h, 48 h, and 72 h, respectively, 10 μ L of CCK-8 reagent (Dojindo Laboratories, Kumamoto, Japan) was added in each well. 2 hours later, optical density (OD) value at the wavelength of 490 nm was measured using a microplate reader.

5-Ethynyl-2'-Deoxyuridine (EdU) Proliferation Assay

CMM cells were seeded in 6-well plates and 50 μ M EdU reagent was added in each well. 2 hours later, cells were stained with AdoLo and 4',6-diamidino-2-phenylindole (DAPI) in the dark, followed by detection of EdU-positive cells under a microscope.

Transwell Assay

CMM cells were digested and resuspended in serum-free medium. Cell density was adjusted to 2.0×10^5 /mL. Transwell chamber containing Matrigel or not was placed in a 24-well plate. 200 μ L of the cell suspension and 500 μ L of medium containing 10% FBS were added to the upper and lower chamber, respectively. After 48 hours of cell culture, the chamber was removed. Cells were fixed with 4% paraformaldehyde for 30 min and stained with crystal violet for 15 min. The inner layer cells were carefully removed. Penetrating cells were captured in 5 randomly selected fields of each sample.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from CMM cell lines and tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and RNA was reverse transcribed into cDNA using PrimeScript RT Reagent (TaKaRa, Otsu, Shiga, Japan). QRT-PCR was performed using SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan), and StepOne Plus Real-time PCR System (Applied Biosystems, Foster City, CA, USA). Primers used in this study were as follows: MicroRNA-92a: forward: 5'-ACAG-GCCGGACAAGTGCAATA-3', reverse: 5'-GCTGTCACAGATACGCCATGAGTACG-3'; U6: forward: 5'-CTCGCTTGCGCAGCACA-3', reverse: 5'-AACGCTTCACGAATTGGGT-3'; FOXP1: forward: 5'-CCCCAAACCACTGATA-ACTCG-3', reverse: 5'-AGACACCATACCCAA-CATTCC-3'; β -actin: forward: 5'-CCTGGCAC-CCAGCACAAT-3', reverse: 5'-TGCCGTAGGT-GTCCCTTTG-3'.

Western Blot

Total protein was extracted using a cell lysis reagent (RIPA; Beyotime, Shanghai, China). Protein samples were separated in a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Membranes were then blocked with the blocking solution for 1 h. Primary antibody was used for incubation at room temperature for 2 h. After being washed with Tris-Buffered Saline and Tween-20 (TBST), the corresponding secondary antibody was used for incubation for 2 h at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL; Pierce and Warriner, Rockford, IL, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (IBM, Armonk, NY, USA) was used for data analyses. Data were expressed as mean \pm standard deviation. The continuous variables were analyzed using the *t*-test, and the categorical variables were analyzed using the χ^2 test or Fisher's exact test. The Kaplan-Meier method was used to evaluate the prognosis of CMM patients, and the difference between different curves was compared by the Log-rank test. $p < 0.05$ was considered to be statistically significant.

Results

MicroRNA-92a Was Highly Expressed in CMM Tissues and Cell Lines

We examined the expression level of microRNA-92a in 75 pairs of CMM tissues and paracancerous tissues by qRT-PCR. The results showed that microRNA-92a is highly expressed in CMM tissues than that of paracancerous tissues (Figure 1A, 1B). MicroRNA-92a expression in CMM cells and PEM was detected as well. Similarly, microRNA-92a was highly expressed in CMM cells compared with PEM (Figure 1C). Specifically, A375 and SK-MEL-28 cells presented relatively high expression of microRNA-92a and were selected for the subsequent experiments.

MicroRNA-92a Expression Was Correlated With Clinical Stage and Overall Survival in CMM Patients

Based on the expression level of microRNA-92a, CMM patients were divided into the high microRNA-92a level group and the low microRNA-92a level group, respectively. The relationship between microRNA-92a expression and age, sex, tumor stage, lymph node metastasis, and distant metastasis of CMM patients was analyzed. It is shown that high expression of microRNA-92a is positively correlated to tumor stage, lymph node metastasis and distant metastasis, whereas not correlated to age and sex of CMM patients (Table I). To further analyze the correlation between microRNA-92a expression and prognosis of CMM patients, their follow-up data were collected. The Kaplan-Meier survival curves were conducted based on the follow-up data, and the results elucidated that high expression of microRNA-92a is correlated to the poor prognosis of CMM (Figure 1D). The higher the expression of microRNA-92a, the worse the prognosis of CMM patients. We suggested that microRNA-92a may serve as a new prognostic hallmark for CMM.

Knockdown of MicroRNA-92a Inhibited Proliferation of CMM Cells

To explore the effect of microRNA-92a on the proliferation of CMM cells, we first established microRNA-92a knockdown model by constructing si-microRNA-92a and siRNA NC (Figure 2A, 2B). The proliferative capacity of CMM cells was

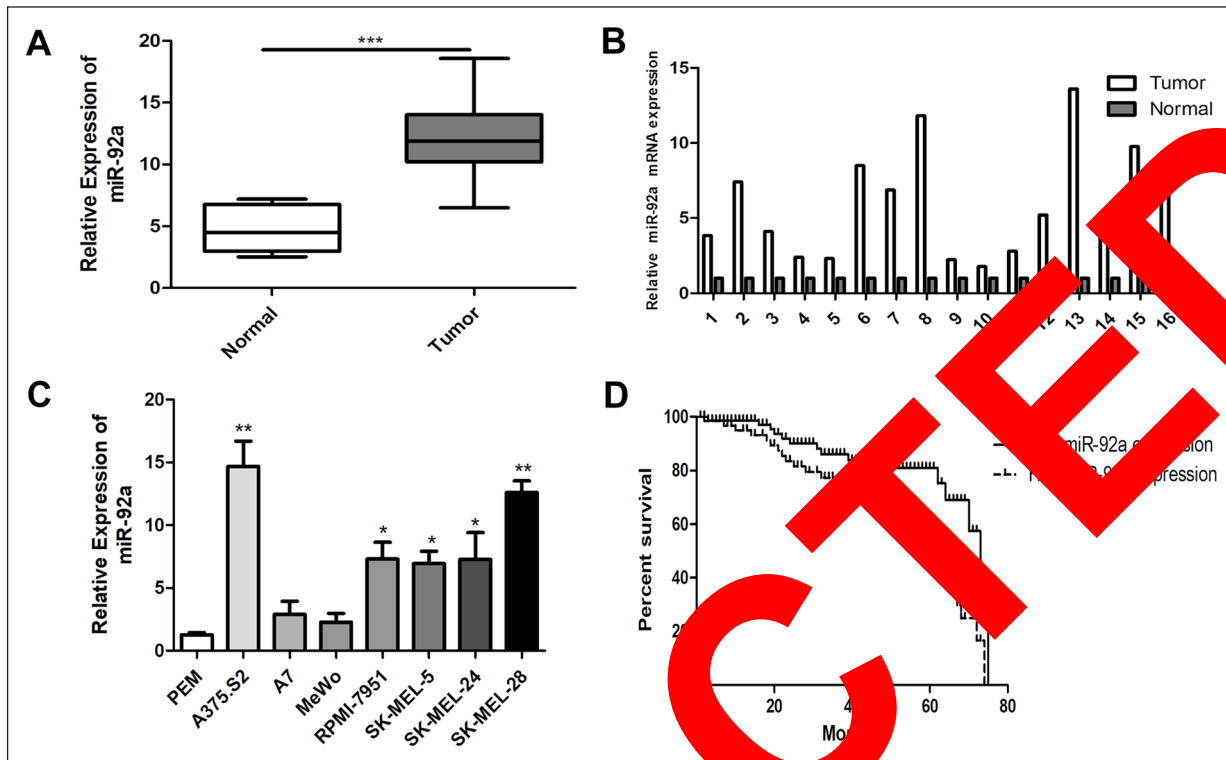


Figure 1. A-B, The expression of microRNA-92a in cutaneous malignant melanoma tissues and paracancerous tissues. C, Expression levels of microRNA-92a in 7 cutaneous malignant melanoma cell lines (A375.S2, A7, MeWo, RPMI-7951, SK-MEL-5, SK-MEL-24, SK-MEL-28) and normal primary cultured melanocyte cell (PEM). D, Kaplan-Meier survival curves of patients with cutaneous malignant melanoma based on miR-92a expression. Patients in the high expression group had a significantly more favorable prognosis than those in the low expression group. A representative data set was displayed as mean \pm SD values. * $p < 0.05$.

determined by CCK-8 and Transwell assay, respectively. Both experiments revealed that miR-NA-92a knockdown reduces the proliferative rate of CMM cells (Fig. 2C-2E).

Knockdown of MicroRNA-92a Inhibited Migration and Invasion of CMM Cells

Transwell assay was conducted to determine the migratory and invasive abilities of CMM

Table I. Association of miR-92a expression with clinicopathologic characteristics of malignant melanoma.

Parameter	Number of cases	miR-92a expression		p-value
		Low (%)	High (%)	
Age				0.435
< 60	32	20	12	
≥ 60	43	23	20	
Gender				0.294
Male	27	22	15	
Female	38	18	20	
Stage				0.021
T1-T2	42	29	13	
T3-T4	33	14	19	
Lymph node metastasis				0.032
No	45	30	15	
Yes	30	12	17	
Distance metastasis				0.036
No	60	38	22	
Yes	15	5	10	

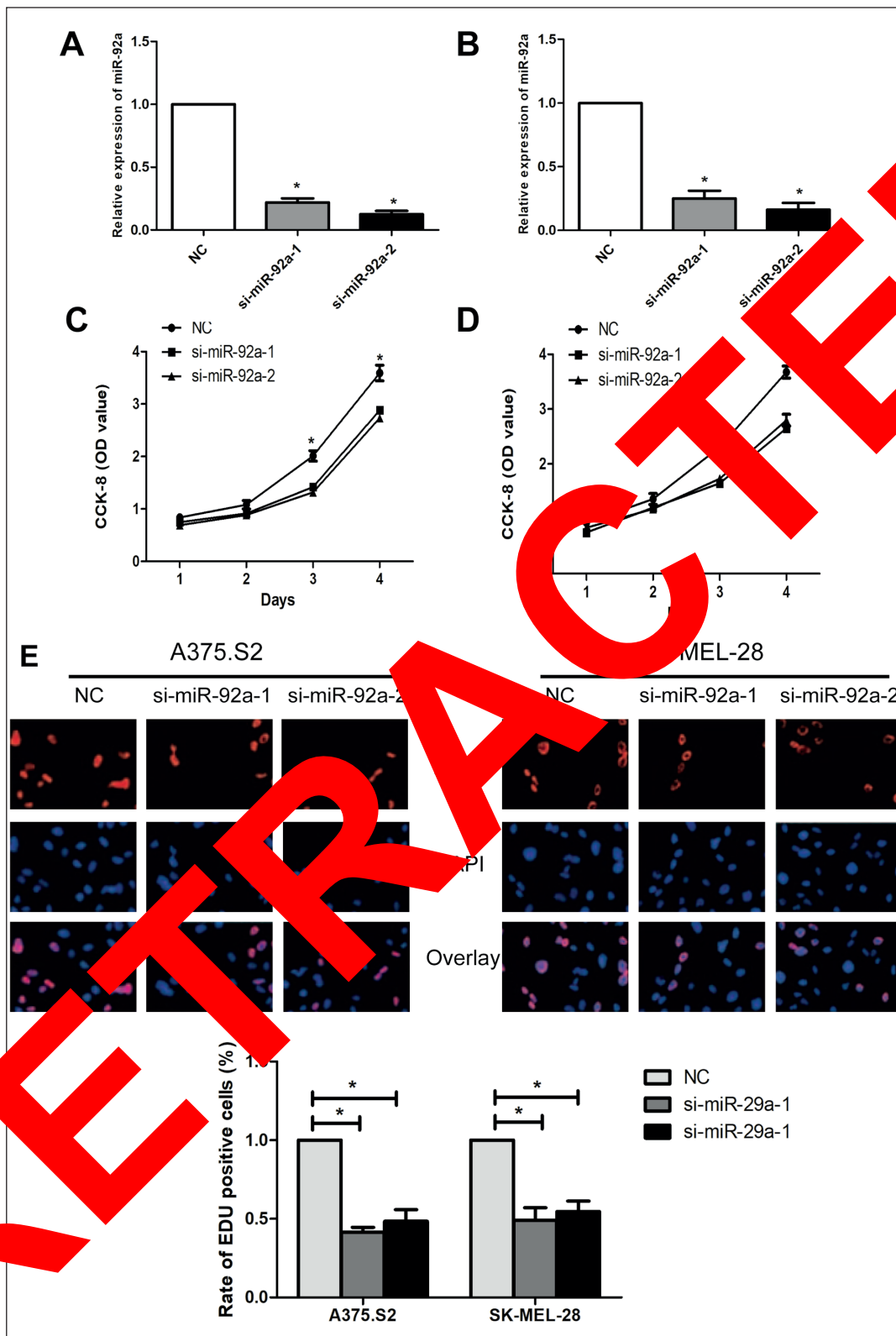


Figure 2. A-B, QRT-PCR was used to verify the efficiency of microRNA-92a knockdown in A375.S2 and SK-MEL-28 cell lines. C-D, Growth curve analysis showed the cell growth of A375.S2 and SK-MEL-28 cells with microRNA-92a knockdown. E, EdU-positive A375.S2 and SK-MEL-28 cells with microRNA-92a knockdown (magnification: 40×). A representative data set was displayed as mean ± SD values. * $p < 0.05$, ** $p < 0.01$.

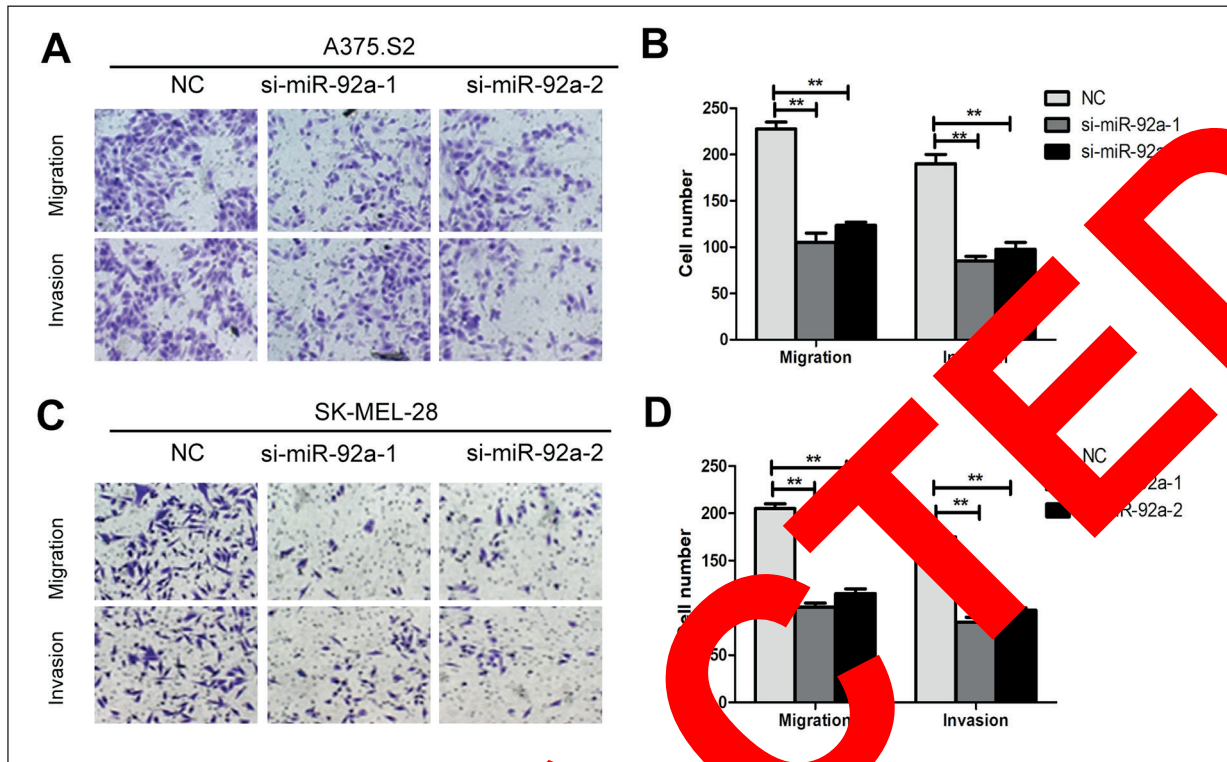


Figure 3. A-B, A375.S2 cells transfected with si-miR-92a displayed significantly lower migratory and invasive capacities (magnification: 40 \times). C-D, SK-MEL-28 cells transfected with si-miR-92a displayed significantly lower migratory and invasive capacities (magnification: 40 \times). The presented data represent the mean \pm SD values. * p <0.05, ** p <0.01.

cells. After microRNA-92a knockdown in A375.S2 cells, the amount of penetrating cells markedly decreased, suggesting that the migration and invasion of CMM cells (Figure 3A, 3B). Similarly, the knockdown of microRNA-92a also decreased the migration and invasive capacities of SK-MEL-28 cells (Figure 3C, 3D).

Knockdown of MicroRNA-92a Changed the Expression of FOXP1

Through literature review, we speculated that FOXP1 may exert a function in the malignant progression of CMM. Western blot results showed up-regulated FOXP1 after microRNA-92a knockdown in CMM cells (Figure 4A). Therefore, we believed that there may be an interaction between microRNA-92a and FOXP1 in the CMM development.

FOXP1 Modulated MicroRNA-92a Expression in CMM Cells

As previously described, FOXP1 expression was regulated by microRNA-92a. Subsequently, bioinformatics showed that FOXP1

may be a target gene of microRNA-92a (data not shown). Here, we found that the FOXP1 expression is lower in CMM tissues than that of paracancerous tissues (Figure 4B). Additionally, FOXP1 was also lowly expressed in CMM cells than that of PEM (Figure 4C). We thereafter selected 16 pairs of CMM tissues and paracancerous tissues from the 75 enrolled patients to perform correlation analyses. Both mRNA and protein levels of FOXP1 were negatively correlated to microRNA-92a (Figure 4D). Survival curves showed worse prognosis in the CMM patients with lower expression of FOXP1 (Figure 4E).

Overexpression plasmid of FOXP1 was conducted to verify its role in malignant progression of CMM. The transfection efficacy of overexpression plasmid of FOXP1 in CMM cells was determined at first (Figure 5A, 5B). CMM cells were co-transfected with si-microRNA-92a and overexpression plasmid of FOXP1. Rescue experiments indicated that the inhibited migration and invasion by microRNA-92a knockdown are reversed after FOXP1 overexpression (Figure 5C,

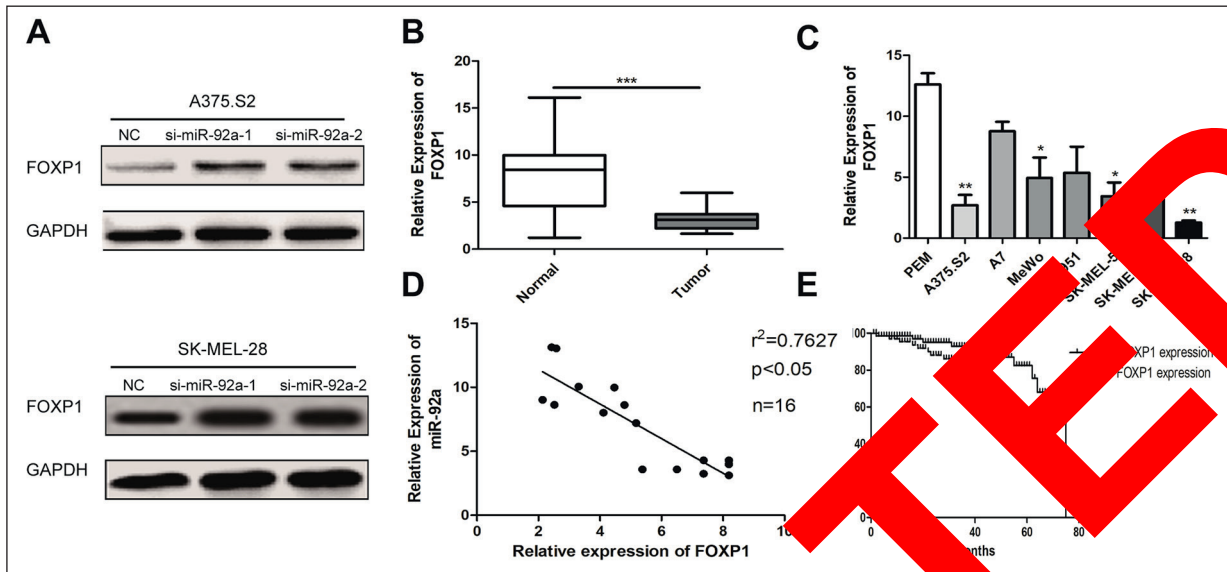


Figure 4. A, Knockdown of microRNA-92a expression significantly upregulates the expression of FOXP1. B-C, The mRNA expression level of FOXP1 relative to GAPDH in human cutaneous malignant melanoma tissues and paracancerous tissues and cell lines were detected using qRT-PCR. D, A negative correlation was found between microRNA-92a and FOXP1 in tumor samples. E, Kaplan-Meier survival curves of patients with cutaneous malignant melanoma based on FOXP1 expression. Patients in the high expression group had a significantly more unfavorable prognosis than those in the low expression group. A representative data set was displayed as mean \pm SD values. * $p < 0.05$, ** $p < 0.01$.

5D). As a result, we proved that microRNA-92a promotes the malignant progression of CMM by inhibiting FOXP1.

Discussion

Malignant melanoma (MM) frequently occurs in the skin and has become one of the common malignant tumors of the skin. Although the incidence rate of MM is lower than that of cutaneous basal cell carcinoma and squamous cell carcinoma of the skin, its malignant degree is significantly higher than the others. The incidence rate of MM increases year by year, with a young disease onset¹⁻⁴. MM is characterized by rapid development, high invasion, frequent metastasis and recurrence. CMM is commonly progressed from benign nevus⁵. CMM patients are often diagnosed at advanced stage due to the lack of attention of the underlying symptoms, which is explained as one of the reasons for its poor prognosis and high mortality⁷. Current treatments for CMM include surgery, chemotherapy, radiotherapy, biological therapy, etc. However, the therapeutic efficacy of these treatments is not satisfactory¹⁶⁻¹⁸. Searching for new treatments are of great significance for CMM patients.

Recent studies¹⁰⁻¹² showed crucial roles of microRNAs as oncogenes or tumor-suppressor genes in tumor development. Those microRNAs that are related to the pathogenesis of CMM may be utilized as therapeutic targets for CMM^{19,20}. This research explored the specific role of microRNA-92a in the malignant progression of CMM. Our data showed a higher expression of microRNA-92a in CMM tissues than that of paracancerous tissues, suggesting that microRNA-92a may be an oncogene. Subsequently, microRNA-92a knockdown remarkably inhibited proliferation, migration and invasion of CMM cells. However, the specific mechanism remains unclear.

FOXP1 is reported to be closely related to the occurrence and progression of tumors²¹. It exerts multiple biological functions, such as anti-apoptosis, transcription activation, DNA repair and embryonic development regulation. FOXP1 could affect the survival of tumor cells and chemotherapeutic outcomes^{14,15}. Relative studies have pointed out the differential expression of FOXP1 in prostate and breast cancer^{22,23}. Since FOXP1 is related to tumor angiogenesis and tumor cell growth, it is served as a hallmark of tumor development. In addition, FOXP1 is involved in the regulation

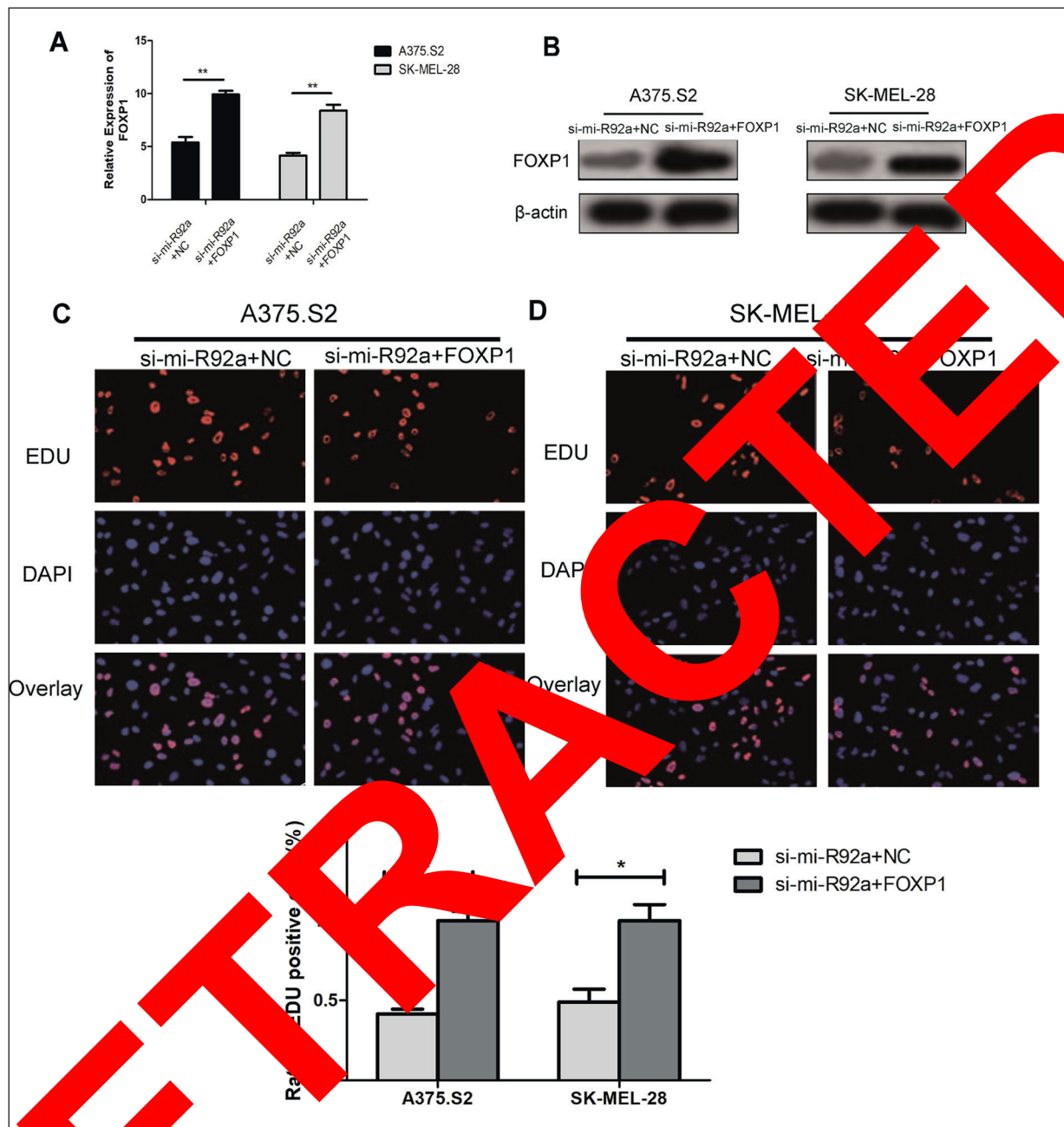


Figure 3. The expression of FOXP1 was verified by qRT-PCR in co-transfected cell lines. **B**, Western blot was used to verify the expression of FOXP1. **C-D**, The roles of microRNA-92a and FOXP1 in the regulation of cutaneous malignant melanoma cell proliferation and invasion were examined by EdU. A representative data set was displayed as mean \pm SD values (significance: $*p < 0.05$, $**p < 0.01$).

of tumor metastasis and apoptosis^{24,25}. In the present investigation, we proved that FOXP1 is regulated by microRNA-92a in CMM. MicroRNA-92 promoted the malignant progression of CMM by targeting FOXP1. We considered that FOXP1 may be applied in the clinical treatment of CMM.

Conclusions

MicroRNA-92a is highly expressed in CMM, which is remarkably correlated to tumor stage and poor prognosis of CMM patients. We found that microRNA-92a promotes the malignant progression of CMM by regulating FOXP1.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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