

Original Article

Downregulation of miR-10a inhibits cutaneous squamous cell carcinoma cell proliferation, migration, and invasion by targeting Syndecan-1

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Abstract: Background: Cutaneous squamous cell carcinoma (cSCC) is a malignancy of epidermal keratinocytes which accounts for approximately one-third of skin cancer-related death yearly. In this study, we aim to investigate the mechanism of miR-10a in regulating cellular function of cSCC cells and its possible role in prognosis of cSCC. Methods: The expression of miR-10a was detected by qRT-PCR. Target mRNA candidates were detected by bioinformatic analysis. Proliferative and migration capability of cSCC cell were examined by MTT assay, wound healing assay, and invasion assay, respectively. miR-10a expression was monitored in cSCC patients to elucidate the relationship between miR-10a expression and outcomes of cSCC. Results: In our study, we found that expression of miR-10a was significantly down-regulated in cSCC cell in vitro and in vivo. Moreover, our results revealed that SDC-1 was a likely target of miR-10a in regulating biologic function of cSCC cell. Additionally, miR-10a expression level was inversely correlated with cSCC cell differentiation and tumor progression. Conclusion: These findings in this study indicate the importance of miR-10a in cSCC cell hallmarks and its use as a novel target for cSCC treatment.

Keywords: Cutaneous squamous cell carcinoma, miR-10a, proliferation, migration

Introduction

Cutaneous squamous cell carcinoma (cSCC) is a form of nonmelanoma skin cancer, which is the second most common human skin cancer. cSCC originates from epithelial keratinocytes or their appendages. Unlike almost all basal cell carcinomas, cutaneous squamous cell carcinomas are associated with a substantial risk of metastasis, posing a significant challenge for public health. It is reported that the incidence of cSCC has been steadily increasing annually in the past decades [1]. Patients with primary cSCC have a favorable prognosis; however, the long-term prognosis of metastatic cSCC is poor even after receiving intensive therapy. Despite the advance in treatment for cSCC during last several years, the 1-year survival rate is less than 56% [2]. The mechanism of metastasis in cSCC remains unclear and there is a need for effective therapeutic strategies.

MicroRNAs (miRNAs) are a class of short, endogenous single-stranded RNA, that play impor-

tant roles in regulating gene expression, controlling physiologic and pathologic processes [3]. Since single miRNAs may target up to hundreds of mRNAs that participate in cell functions, aberrant miRNA expression will affect key process of proliferation, cell cycle control, apoptosis, differentiation, and migration. It is estimated that more than 60% of all mRNAs are predicted to be affected by miRNA mediated regulation [4]. Thus, even a deregulation of a small subset of miRNA may profoundly alter the expression pattern and trigger disease-like transformation of cells. Previous studies revealing the effect of miRNAs on cancer are numerous. Examples include that miR-200c acts as a tumor-suppressor in breast cancer [5]. Likewise, miR-126 is associated with the long-term prognosis of metastatic colorectal cancer [6]. Similar roles have been recapitulated in many human cancers, as for instance exemplified by downregulation of miR-150 in pancreatic cancer [7] or miR-21 in colorectal cancer [8]. miR-10a has aberrant expression in many cancer

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Table 1. Clinical characteristics of cSCC patients and healthy individuals

Clinical data	cSCC patients (n=21)	Healthy controls (n=18)	P value
Age (Mean \pm SD)	55.7 \pm 8.9	53.3 \pm 10.2	0.16
Gender (Men, %)	15	12	0.45
Smoking (Number, %)	9	10	0.33
Skin inflammation (Number, %)	2	3	0.14
Radiation exposure (Number, %)	2	1	0.09

types including colorectal [9], breast [10], and non-small cell lung cancer [11].

Syndecans are heparan sulfate cell surface proteoglycans which participate in binding growth factors and function in cell-to-cell and cell to extracellular matrix (ECM) interactions [12]. Syndecans also act as tumor suppressors by inhibiting tumor cell proliferation. For instance, the syndecan-1 (SDC-1) ectodomain suppresses the growth of epithelial-derived tumor cell without affecting the growth of normal epithelial cells [13]. However, little is known about the function of SDC-1 and the interaction of miR-10a cutaneous squamous cell carcinoma cells.

In this study, we identified the miRNA expression profile in cSCC and found miR-10a as one of the most abundant miRNAs in cSCC. We also observed that down-regulation of miR-10a inhibits cutaneous squamous cell carcinoma cell proliferation, migration, and invasion. Moreover, we showed miR-10a-mediated regulation of SDC-1 may account for alteration in the biologic function of cSCC cells.

Materials and methods

Clinical tumor sample

Cutaneous SCC samples (n=21) or healthy skin tissue (n=18) were obtained from patients diagnosed with cutaneous SCC or adult healthy donors respectively at our institute. All samples were collected with patients' informed consent and confirmed by the pathological examination. This study was approved by the Institutional Review Board of our hospital. Tumors grade were evaluated according to the SCC Broders Pathological Classification: stage I (well differentiated) with 0-25% undifferentiated cells; stages II-III (moderately differentiated) with 25-75% undifferentiated cells; stage IV (poorly

differentiated) with 75-100% undifferentiated cells. Patients and healthy controls were matched by age, gender, and other risk factors (**Table 1**).

Cell lines

Three cutaneous SCC lines, A431, SCC13, and HSC-5, were obtained from China Center for Type Culture Collection and Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and human benign epidermal keratinocyte cell line HaCaT was obtained from China Center for Type Culture Collection (Wuhan, China). All cell lines were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin and streptomycin (Invitrogen, Carlsbad, CA) 37°C with 5% CO₂ incubator.

RNA isolation and quantitative real-time-PCR

Total RNA was extracted from cell lines and tissue samples using a mirVana™ miRNA Isolation Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Serum miRNA was isolated with miRNeasy RNA isolation kit (Qiagen, Valencia, CA). The reverse-transcription reaction and quantitative real-time PCR (qRT-PCR) were performed with a TaqMan qRT-PCR kit (Takara Bio, Shiga, JP) and TaqMan microRNA assay (Ambion, Austin, TX). The GAPDH mRNA and U6 levels were used for normalization. The relative expression of SDC-1 mRNA compared with GAPDH mRNA was calculated using the 2^{- $\Delta\Delta$ Ct} method.

MiRNA expression profiles

MiRNA expression profiling was performed using a miRNA microarray analysis and miRNA array probes (LC Sciences) according to previous methods [14]. Each sample was investigated and significant differences between cSCC and healthy controls for a given detectable miRNA signal were recorded. The ratio of the two sets of detected signals (log₂ transformed, balanced) and the P values for the t-test were calculated.

Cell transfection

For in vitro assay, A431 cells were transfected with miR-10a mimics (GenePharma, Shanghai)

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using Lipofectmine 2000 (Invitrogen, Carlsbad, CA) to overexpress miR-10a. In contrast, to decrease the expression of miR-10a in A431 cells, miR-10a inhibitor was transfected into A431 cells. Overexpression and inhibition of the mature, biologically active form of miR-10a was confirmed by real-time PCR at 48 h after transfection.

MTT assay

A431 cells (10×10^4 cells/per well) were plated in 96 well plates (Corning Costar, NY). MTT solution (20 μ l, 5 mg/ml) was added into each well for different time periods of incubation. The absorbance was measured in dual wave mode (570 nm). The percentage survival of the cells after transfection was calculated.

Wound healing assay

Cultured cells were grown to reach a confluence of 80-90%. A scratch was made with a sterile 200 ml pipette tip. A431 cells were kept in DMEM medium. Three fields were randomly taken at 0 hour and 12 hours after scratch. The area of the wound was analyzed with Image J software. The experiments were performed in triplicate.

Invasion assay

24 well transwell plate (Corning Costar, NY) coated with Matrigel (BD Biosciences, San Jose, CA) were used to determine cell invasion in vitro. Transfected cells (2×10^5 cells in 200 μ l DMEM medium) were resuspended and seeded into the upper chamber. 500 μ l medium with 10% FBS was added to the lower chamber as chemoattractant. After 24 h incubation, non-invaded cells on the upper surface of the membrane were rinsed with PBS. The invasive cells, which penetrated to the lower surface, were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet (Beyotime, Shanghai). The number of cells invading the membrane was counted from 3 randomly fields with an inverted microscope at 100 \times magnification. Data were obtained from three independent experiments.

Luciferase assay

The pMIR-SDC-1-3'UTR plasmid containing the putative binding site of the SDC-1 3'UTR down-

stream of the firefly luciferase gene was generated by cloning and inserting of a sequence into the SpeI and HindIII sites of the pMIR-REPORT Luciferase vector (Ambion, TX). Then HEK293 T cells were plated in 24-well plates and reached 60-70% confluence. After that, the cells were transfected with Luciferase plasmid and Renilla plasmid, as well as miR-10a mimics or inhibitor or negative control. The activity of Luciferase and Renilla was assessed after 48 h with the Dual Luciferase Reporter 1000 Assay System (Promega, WI).

Western blot analysis

Cultured cells were lysed using RIPA reagent (Pierce, Appleton, WI). The protein concentration of the cell lysates was quantified using a BCA Protein Assay Kit (Pierce, Appleton, WI). Labeled bands were detected using the ECL chemiluminescent kit (Pierce, Appleton, WI). SDC-1 antibody (ThermoFisher Scientific, Waltham, MA) were used to detect the expression level. GAPDH antibody (ThermoFisher Scientific, Waltham, MA) was used for loading control.

Statistical analysis

Data are presented as mean \pm SD. Student's t-test was applied to compare differences between two groups. One-way ANOVA test was used to compare the differences among the groups. Univariate comparisons of two independent groups were done using the Mann-Whitney-U test. Comparisons of multiple groups were performed with the Kruskal-Wallis test. Probability of survival was determined by Kaplan-Meier analysis, and the significance of differences between groups was analyzed by the log-rank test. $P < 0.05$ was considered significant. All statistical analyses were carried out using SPSS v21 (SPSS, Chicago, IL).

Results

The expression of miR-10a is up-regulated in cutaneous SCC

The expression of miRNA in cSCC and healthy control samples was determined using a microarray assay (**Figure 1A**). Four miRNAs (up-regulated miR-10a and let-7e and down-regulated miR-21 and miR-125) showed significant fold

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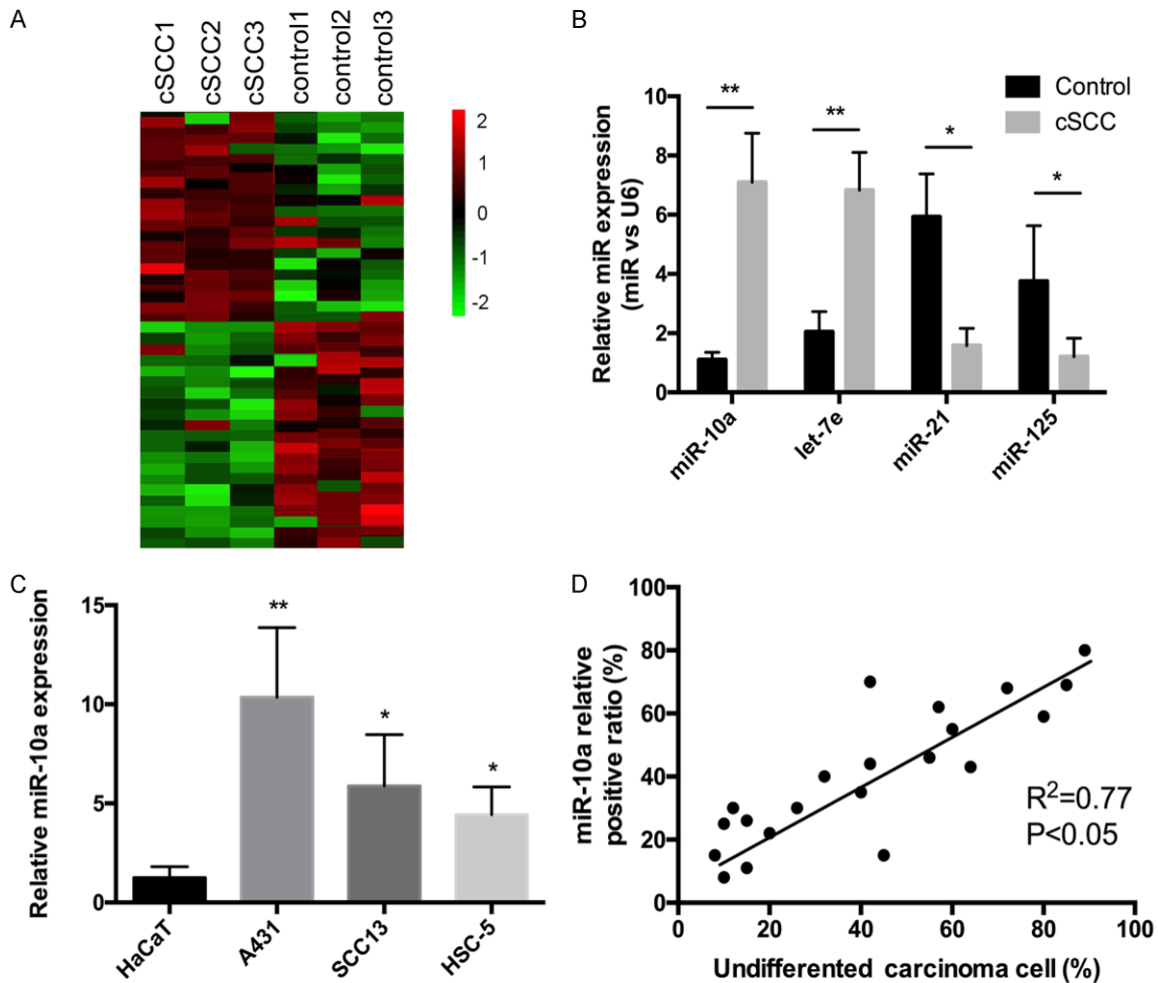


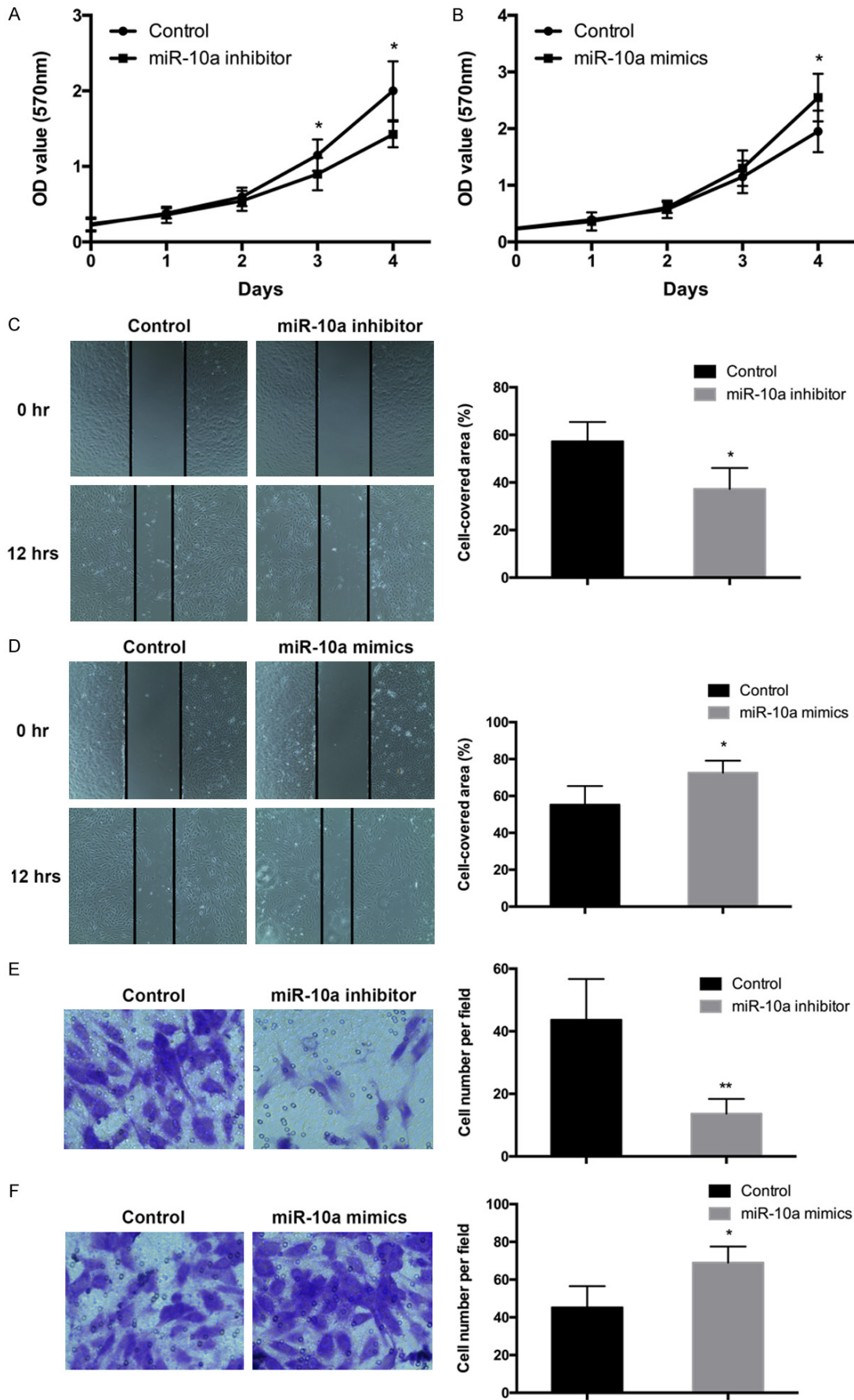
Figure 1. Relative miRNA expression in cSCC tissue. A. Differently expressed miRNAs from cSCC tissue and healthy controls. B. Four aberrantly expressed miRNAs were identified by qRT-PCR. * $P < 0.05$ and ** $P < 0.01$ vs. healthy control. C. miR-10a expression was evaluated in different cSCC cell lines by qRT-PCR. * $P < 0.05$ and ** $P < 0.01$ vs. HaCaT cell line. D. Correlation of miR-10a positive expression with the cutaneous SCC histologic differentiation.

changes (**Figure 1B**). To further determine the different expression of these miRNAs, we tested by using qRT-PCR in cutaneous SCC cell lines including A431, SCC13 and HSC-5. Our results showed that compared with control cell line HaCaT, the expression of miR-10a was significant increased in all three cell lines, with a maximal increase of 10.2-fold in A431 and a minimal increase of 4.7-fold in HSC-5 (**Figure 1C**). Moreover, we analyzed the correlation between miR-10a expression level and differentiation of cutaneous SCC. The results revealed an inverse correlation between miR-10a and cSCC differentiation (**Figure 1D**). Taken together, these results provide strong evidence that miR-10a was markedly up-regulated in cutaneous SCC.

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Given that miR-10a is markedly up-regulated in cSCC cells, loss of miR-10a expression may inhibit the biologic function of cSCC cells. To investigate our hypothesis, we down-regulated the expression of miR-10a in cSCC cells with miR-10a inhibitor and examined the cell proliferation via MTT assay. Moreover, we also determined the capability of migration and invasion of cSCC cells with wound healing assay and invasion assay. The results revealed that reduced miR-10a expression inhibited cSCC proliferation, migration and invasion (**Figure 2A, 2C and 2E**). To further confirm the role of miR-10a in cSCC cell, we employed miR-

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Figure 2. Ectopic expression of miR-10a affects cSCC cell function. A. Proliferation was examined by MTT assay in cSCC cells transfected with miR-10a inhibitor or negative control, respectively. * $P < 0.05$ vs. NC. B. Proliferation was examined by MTT assay in cSCC cells transfected with miR-10a mimics or negative control, respectively. * $P < 0.05$ vs. NC. C. Wound healing assay was performed to test the migration in cSCC cells transfected with miR-10a inhibitor or negative control. * $P < 0.05$ vs. NC. D. Migration was evaluated in cSCC cells transfected with miR-10a mimics or negative control. * $P < 0.05$ vs. NC. E. Transwell assay was used to determine the invasion in cSCC cells transfected with miR-10a inhibitor or negative control. ** $P < 0.01$ vs. NC. F. Invasion was evaluated in cSCC cells transfected with miR-10a mimics or negative control. * $P < 0.05$ vs. NC.

10a mimics to overexpress miR-10a. cSCC cell transfected with miR-10a exhibited an opposite behavior on cell proliferation, migration, and invasion (**Figure 2B, 2D and 2F**).

SDC-1 is the target gene of miR-10a in cSCC cells

To clarify how miR-10a exerts its effect on cSCC cells, we searched multiple database (TargetsScan, miRanda and miRDB) to predict the potential target gene (**Figure 3A**). Among all genes, SDC-1, function in binding growth factors and other soluble molecules and participate in cell-to-cell and cell to extracellular matrix (ECM) interactions [15], was predicted to have a putative binding site of miR-10a in 3'UTR. To further confirm that SDC-1 is a direct target of miR-10a, we performed luciferase report assay. Luciferase reporters were constructed containing either a wild-type SDC-1 3'UTR sequence containing the miR-10a binding site (pMIR/SDC-1), or a mutated SDC-1 3'UTR (pMIR/SDC-1/mut) (**Figure 3B**). The results showed that the relative luciferase activity was markedly decreased in luciferase vectors with a region of SDC-1 3'UTR (**Figure 3C**).

Next, we investigated the ability of miR-10a to inhibit SDC-1 expression by transfecting cSCC cells with miR-10a mimics or miR-10a inhibitors. We found that cSCC cells transfected with miR-10a mimics exhibited markedly reduced SDC-1 protein level while cSCC transfected with miR-10a inhibitors presented increased SDC-1 protein level (**Figure 3D**). Besides, we also revealed that SDC-1 protein level was decreased in cSCC cells transfected with SDC-1 siRNA (**Figure 3E**).

miR-10a regulates cSCC cell function by targeting SDC-1

To further identify the role of SDC-1 in regulating cSCC cell function, we examined the proliferation,

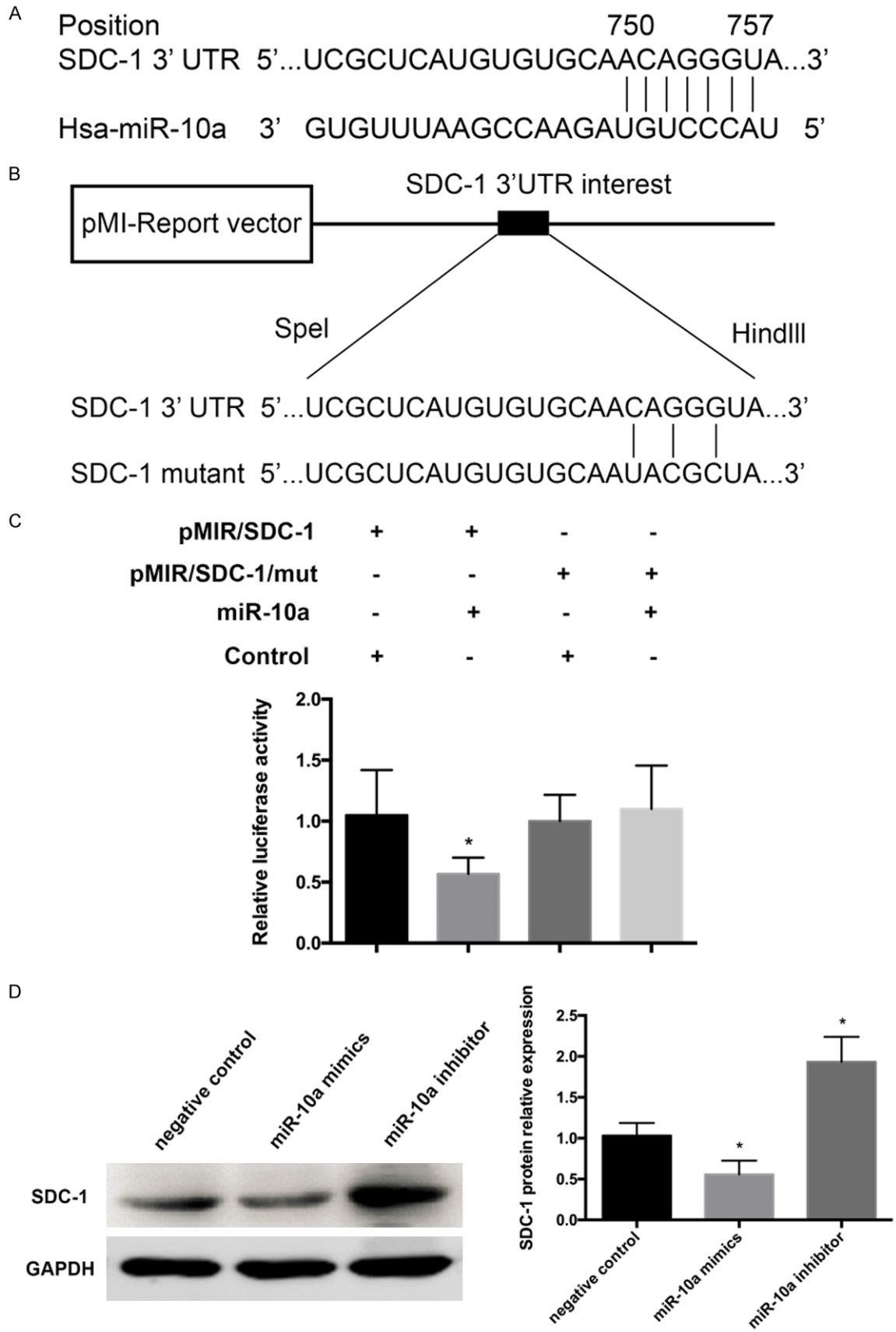
migration, and invasion ability of cSCC cells transfected with SDC-1 siRNA. We observed that cSCC cells transfected with SDC-1 siRNA exhibited increased cell proliferation (**Figure 4A**), migration (**Figure 4B**), and invasion ability (**Figure 4C**). We also conducted rescue experiments to confirm that miR-10a regulated cSCC cells by targeting SDC-1. As expected, cSCC cells co-transfected with miR-10a and SDC-1 exhibited a similar trend of cell proliferation, migration, and invasion (**Figure 4A-C**).

Serum concentration of miR-10a in cSCC patients

We tested the serum concentrations of miR-10a in cSCC patients and healthy individuals, respectively. As shown in **Figure 5A**, serum miR-10a levels in cSCC patients were higher than those in healthy individuals. Furthermore, serum miR-10a in stage III and stage IV showed a significant higher expression compared to those in stage I and stage II patients (**Figure 5B**). In addition, we investigated the expression of miR-10a and its role in predicting prognosis of cSCC. 21 patients were divided into two groups with low or high serum miR-10a using the median expression level of all cases as the cut-off point. Disease-specific death was regarded as the primary endpoint. During the 3-year follow-up period, 4 cases died because of cSCC. Kaplan-Meier analysis revealed that patients with high level of serum miR-10a had unfavorable outcomes (**Figure 5C**). Taken together, our data showed that the expression level of miR-10a is associated with the prognosis of cSCC.

Discussion

The role of miRNAs in the development and progression of cutaneous squamous cell carcinoma has not been well examined. Yamane et al [16] reported that miR-124 and miR-214 inhibited cSCC cell proliferation by targeting ERK.



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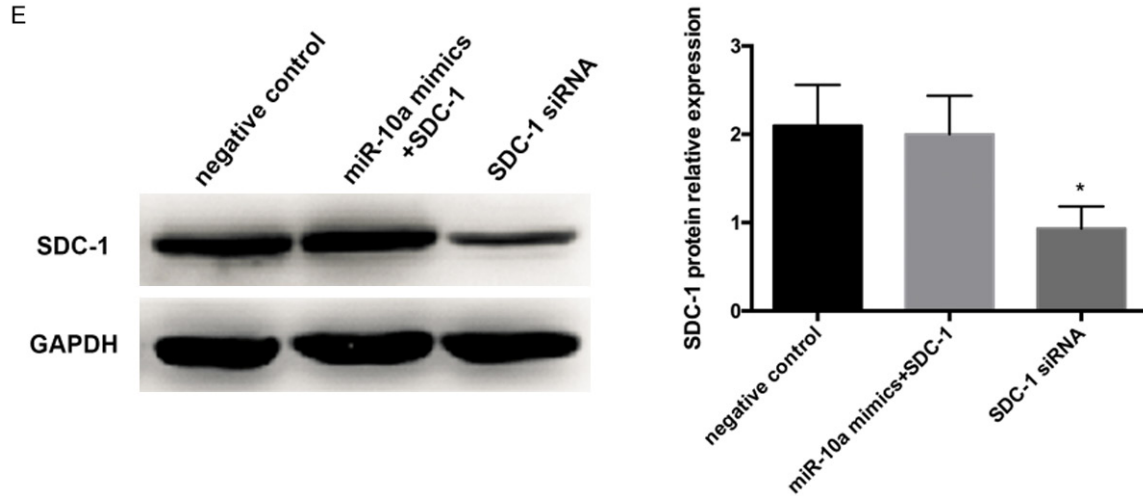


Figure 3. SDC-1 is a target gene of miR-10a in cSCC cell. A. The potential binding site in SDC-1 3'UTR region was predicted by bioinformatic analysis. B. Luciferase reporters were constructed containing either a wild-type SDC-1 3'UTR sequence containing the miR-10a binding site or a mutated SDC-1 3'UTR. C. Luciferase report assays were performed on HEK 293 T cells. * $P < 0.05$. D. Western blot was used to test SDC-1 protein expression in cSCC cells transfected with negative control, miR-10a mimics or miR-10a inhibitor, respectively. * $P < 0.05$ vs. NC. E. SDC-1 expression in protein level was examined in cSCCs transfected with negative control, miR-10a mimics plus SDC-1 or SDC-1 siRNA, respectively. * $P < 0.05$ vs. NC.

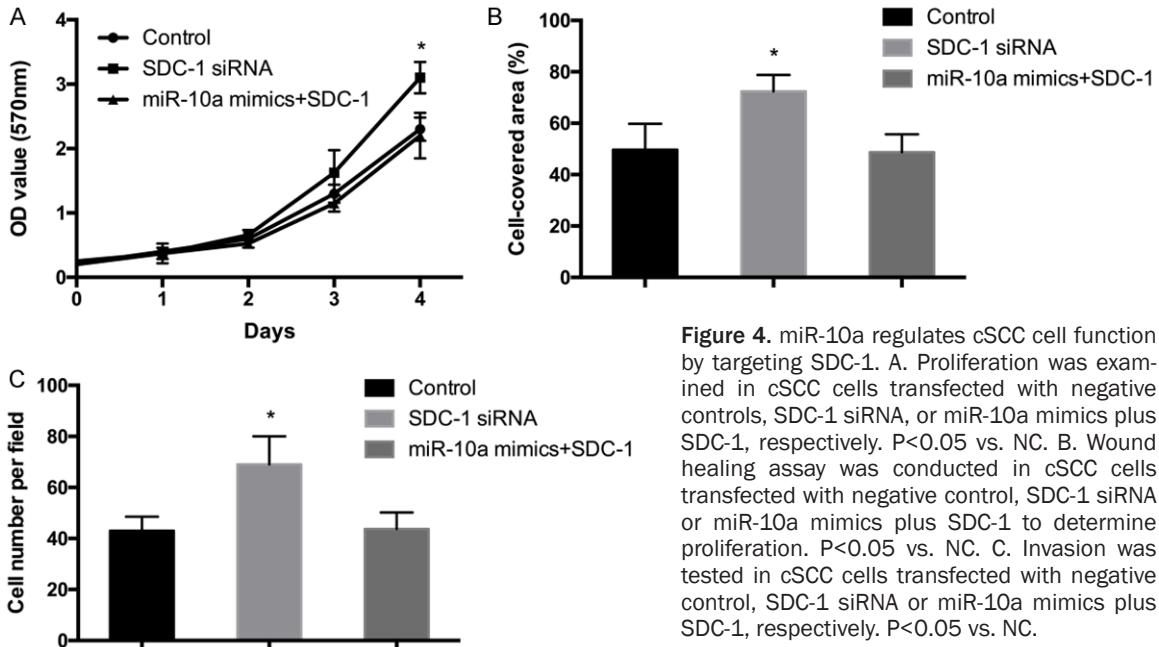


Figure 4. miR-10a regulates cSCC cell function by targeting SDC-1. A. Proliferation was examined in cSCC cells transfected with negative controls, SDC-1 siRNA, or miR-10a mimics plus SDC-1, respectively. $P < 0.05$ vs. NC. B. Wound healing assay was conducted in cSCC cells transfected with negative control, SDC-1 siRNA or miR-10a mimics plus SDC-1 to determine proliferation. $P < 0.05$ vs. NC. C. Invasion was tested in cSCC cells transfected with negative control, SDC-1 siRNA or miR-10a mimics plus SDC-1, respectively. $P < 0.05$ vs. NC.

Zhou et al [17] found that miR-365 may act as an onco-miRNA in cutaneous SCC. Similarly, in this study we found that miR-10 was up-regulated in cutaneous squamous cell carcinoma. miR-10 microRNA precursor is a short non-coding RNA gene involved in gene regulation. Previous studies have studied the abnormal levels of expression of miR-10a in cancer.

Increased levels of miR-10a have been found in glioblastoma [18], pancreatic carcinomas [19] and colon cancer [20]. It is reported that miR-10a was up-regulated in human cervical cancer and contributed to colony formation activity, migration, and invasion of cervical cancer cell lines [21]. Other miR-10 precursors also play an important role in cancer progression and prog-

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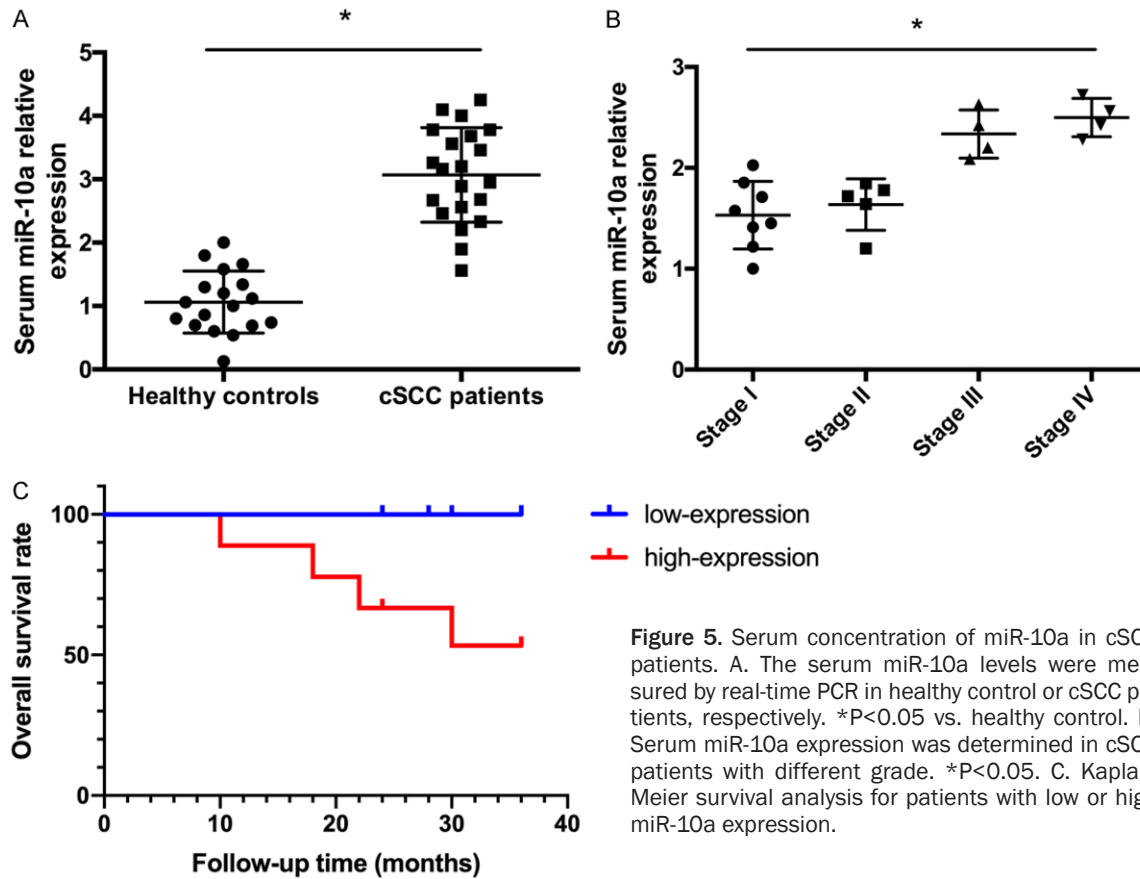


Figure 5. Serum concentration of miR-10a in cSCC patients. A. The serum miR-10a levels were measured by real-time PCR in healthy control or cSCC patients, respectively. * $P < 0.05$ vs. healthy control. B. Serum miR-10a expression was determined in cSCC patients with different grade. * $P < 0.05$. C. Kaplan-Meier survival analysis for patients with low or high miR-10a expression.

nosis. Ma et al [22] reported that miR-10b was highly expressed in metastatic breast cancer cells and positively regulates cell migration and invasion. Besides, overexpression of miR-10b in non-metastatic breast induced tumors-initiated invasion and metastasis. However, down-regulation of miR-10 was detected in some other cancers. For example, miR-10a was found down-regulated in CD34+ cells from patients with chronic myeloid leukemia. Furthermore, down-regulation of miR-10a may increase USF2 and contribute to the increase in cell proliferation of CML implicating a miRNA in the abnormal behavior of CML [23].

We also found that miR-10a regulated cSCC cell function by targeting SDC-1. Syndecans (SDC) are heparan sulfate cell surface proteoglycans which participating in binding growth factors and other molecules. Syndecans are also involved in cell-to-cell and cell to extracellular matrix (ECM) interactions [24]. Previous studies demonstrated that Syndecans played an important role in cell adhesion and cohesion, especially for maintaining normal cell

architecture, differentiation, migration, and growth [12]. Inki et al [25] have showed that SDC-1, as the most studied membrane protein of the Syndecans family of cell surface proteoglycans, is widely expressed in normal stratified squamous epithelium. Furthermore, SDC-1 is capable of inhibiting invasion of cell type I collagen gels [26]. Decreased expression of SDC-1 is considered as an important step towards metastatic phenotype for carcinoma cells. Parimon et al [27] showed that loss of SDC-1 expression in lung cancer cells was associated with higher-grade cancer and worse clinical prognosis. Similarly, results from another study revealed that ionizing radiation (IR)-induced MMP-9 enhances SDC1 shedding, corroborating the tube-inducing ability of medulloblastoma (MB) cells [28]. In our study, we found SDC-1 as an anti-tumor molecule in cSCC. Similar to our findings, the role of SDC-1 in repression cSCC migration and invasion was previously reported by Mukunyadzi et al [15]. Our results showed that SDC-1 is a likely target of miR-10a. Thus, it is not surprising that miR-10a possessed the fundamental role in regulating the

invasion-metastasis cascade of cSCC. Besides, increasing evidence has recently shown that serum miRNAs can be used as biomarkers, especially for various malignant tumors [29]. Clinically, the identification of tumor marker sensitive for early stage SCC is urgently needed for early diagnosis. Thus, in our study we determined the potential correlation of serum miR-10a expression and cancer grade. Our results revealed that serum miR-10a expression level was correlated with the cSCC grade.

In conclusion, our results demonstrate a high endogenous level miR-10a in cSCC, and that miR-10a expression inversely correlated with cSCC differentiation. Furthermore, inhibition of miR-10a can repress cSCC cell proliferation, migration, and invasion by the downregulation of SDC-1. These findings indicate the importance of miR-10a in cSCC hallmarks and its potential as a novel target for cSCC treatment.

Disclosure of conflict of interest

None.

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