Abstract. Cutaneous squamous cell carcinoma is a common malignant tumor. The aim of the present study was to examine the biological function of microRNA (miR)-27b-3p in cutaneous squamous cell carcinoma (CSCC) and its underlying mechanism. The relative expression levels of miR-27b-3p were determined in A-431, Colo-16 and NHEK/SVTERT3-5 cell lines. The regulatory effects of miR-27b-3p on the proliferation of CSCC cells were evaluated using MTT and colony formation assays. Transwell assays were conducted to examine the role of miR-27b-3p in the migratory and invasive abilities of CSCC cells. The levels of EGFR, MMP-13, Akt, phosphorylated (p)-Akt, cyclin D1, N-cadherin (CAD) and E-CAD were detected in CSCC cells using reverse transcription-quantitative PCR and western blot analysis. Binding between miR-27b-3p and the 3'-untranslated region (UTR) of EGFR or MMP-13 was assessed using a dual-luciferase reporter assay. miR-27b-3p was significantly downregulated in CSCC cell lines, compared with the skin keratinocyte cell line. Transfection with a miR-27b-3p mimic significantly reduced the proliferative, migratory and invasive abilities of CSCC cells in vitro. Moreover, miR-27b-3p mimic transfection downregulated the mRNA and protein levels of EGFR, MMP-13, cyclin D1, p-Akt and N-CAD, whilst upregulating E-CAD levels in CSCC cells. The inhibition of CSCC proliferation by miR-27b-3p was effectively reversed by EGFR overexpression. Moreover, the inhibitory effect of miR-27b-3p on the migratory and invasive abilities of CSCC cells was abolished by MMP-13 overexpression. In conclusion, miR-27b-3p inhibits the proliferation, migration and invasion of CSCC cells by downregulating the expression of EGFR and MMP-13 and may represent a potential diagnostic marker and therapeutic option for CSCC.

Introduction
Cutaneous squamous cell carcinoma (CSCC) is a malignant tumor originating from the epidermis or keratinocytes of skin appendages (1). CSCC ranks second to basal cell cancer as the most common type of skin cancer in the USA (2). This global incidence continues to increase annually with an estimated 50-200% increase in the last three decades (3), CSCC severely endangers human health and lives (4). Surgery is the preferred treatment option for patients with CSCC (5). Although surgical resection can eliminate small superficial tumor lesions, lymphadenectomy of the removal of the regional draining lymph node is necessary for cancer cases with high risks of lymph node invasion (6,7). The risk of nodal metastasis (NM) in cohort and tumor registry studies has ranged from 2.0 to 5.8%. A single cohort study reported a risk of disease-specific death (DSD) of 1.5% (8), which is mainly attributed to cancer cell infiltration and metastasis (9).

MicroRNA (miRNA/miR) is a type of endogenous small non-coding RNA (10). miRNA molecules can induce mRNA degradation, inhibit gene transcription or lead to mRNA deadenylation by binding the 3'-untranslated region (UTR) of target mRNA transcripts (11). A previous study have revealed the crucial functions of miRNA in cancer development (12). Miao et al (13) demonstrated that miR-27b-3p played an important role in the development of glioma and that Yes-associated protein 1 (YAP1) was the downstream target of this miRNA. Moreover, they also suggested that miR-27b-3p controlled the proliferation, migration and apoptosis of glioma cells by regulating YAP1 (13). Yang et al (14) also reported overexpression of miR-27b-3p in colorectal cancer and suggested that miR-27b-3p could significantly promote the migration and invasion of colorectal cancer by targeting homeobox A10.

EGFR is a transmembrane receptor tyrosine kinase that initiates multiple intracellular signaling pathways (15).
EGFR regulates the proliferation, invasion, metastasis and apoptosis of cancer cells (15). MMP-13 is a matrix metalloproteinase that participates in the degradation of the extracellular matrix and is involved in tumor cell metastasis (16,17). Our previous studies found that EGFR and MMP-13 are highly expressed in CSCC (18,19). However, the potential functions of EGFR and MMP-13 in the proliferation and metastasis of CSCC remain largely unclear. The aim of the present study was to examine the biological function of miR-27b-3p in CSCC and its underlying mechanism to find potential molecular markers and therapeutic targets for clinical treatment of CSCC.

Materials and methods

Cell culture. The human CSCC cell lines A-431 (cat. no. MZ-0014; Ningbo Mingzhou Biotechnology Co., Ltd.), Colo-16 (cat. no. MZ-1591; Ningbo Mingzhou Biotechnology Co., Ltd.), HSC-1 (cat. no. MZ-1501; Ningbo Mingzhou Biotechnology Co., Ltd.) and SCL-1 (cat. no. MZ-1504; Ningbo Mingzhou Biotechnology Co., Ltd.), human skin keratinocyte cell line NHEK/SVRTERT3-5 (cat. no. CE22072; Beijing Crespo Biotechnology, Co., Ltd.) and the 293T cell line (cat. no. MZ-0005; Ningbo Mingzhou Biotechnology Co., Ltd.) were provided by Ningbo Mingzhou Biotechnology Co., Ltd. and Beijing Crespo Biotechnology, Co., Ltd. NHEK/SVRTERT3-5 cells were used as a control.

The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco; Thermo Fisher Scientific Inc.) containing 10% fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific Inc.) and 1% penicillin-streptomycin. After 24-h cell culture, reverse transcription-quantitative PCR was carried out using SYBR Premix Ex Taq™ II with Tli RNaseH (Takara Bio, Inc.). The thermocycling conditions follows: 25˚C for 5 min, 42˚C for 15 min and 85˚C for 5 sec.

Total RNA was extracted from cell lines using TRIzol® (Invitrogen; Thermo Fisher Scientific Inc.) and purified with a gDNA eraser (Takara Primescript™ RT Reagent Kit (Takara Bio, Inc.), 1 µg/µl of the RNA sample was purified with a gDNA eraser and reverse transcribed to cDNA. The RT steps were as follows: 25˚C for 5 min, 42˚C for 15 min and 85˚C for 5 sec. qPCR was carried out using SYBR Premix Ex Taq™ II with Tli RNaseH (Takara Bio, Inc.). The thermocycling conditions consisted of an initial denaturation at 95˚C for 15 min, followed by 40 cycles at 95˚C for 5 sec, 60˚C for 30 sec and 72˚C for 40 sec, then a final extension at 72˚C for 10 min. The results were analyzed following the 2^(-ΔΔCq) method (20). GAPDH and U6 served as the internal references. Primer sequences are listed in Table I.

Western blot analysis. Total protein from CSCC cells was collected, and first isolated by RIPA lysis buffer (Beiytime Institute of Biotechnology), and protein samples was determined by BCA method at 1 µg/µl. The protein samples were then subjected to 5% (for EGFR, N-CAD and E-CAD) or 10% (for MMP-13, AKT, p-AKT, cyclin D1 and GAPDH) gel electrophoresis (50 µg/lane), then transferred to PVDF membranes. Non-specific antigen binding was blocked using 5% skimmed milk in room temperature for 1 h. Membranes were incubated with primary antibodies (1:1,000) at 4˚C overnight and then incubated with secondary antibodies (1:1,000) at room temperature for 1 h. Finally, the membrane was treated with chemiluminescent horse radish peroxidase (HRP) Substrate (cat. no. WBKLS0500; MilliporeSigma) to visualize the protein. Antibodies used in western blot assay were purchased from ABclonal Biotech Co., Ltd., and the catalog numbers were as follows: EGFR (cat. no. A4929); MMP-13 (cat. no. A11148); AKT (cat. no. A17909); p-AKT (cat. no. AP0637); cyclin D1 (cat. no. A11022); N-CAD (cat. no. A19083); E-CAD (A3044); GAPDH (cat. no. AC001) and the secondary antibody HRP goat anti-rabbit (cat. no. AS014). Band exposure was achieved using the enhanced chemiluminescence method and visualized by Quantity One software v.4.6.9 (Bio-Rad Laboratories, Inc.).

Colony formation assay. CSCC cells were seeded in a 6-well plate at a density of 2x10^5 cells/well, then cultured in DMEM containing 10% FBS for 14 days until the formation of visible colonies. The colonies were washed with PBS, fixed in 4% paraformaldehyde for 30 min at room temperature and stained in 1% crystal violet for 15 min at room temperature. Visible colonies were captured under an inverted microscope and the number of colonies were counted that had >50 cells.

MTT assay. MTT assay was conducted according to the manufacturer’s instructions of MTT Assay kit (ABclonal Biotech Co., Ltd.; cat. no. ab21109). CSCC cells were seeded in a 96-well plate at a density of 6x10^4 cells/well. At the 12, 24, 36, 48 and 60-h time points, 20 µl MTT (5 mg/l) was added to each well at 37˚C for 4 h. The supernatant was then discarded, and 150 µl DMSO was added to each well. After gentle shaking for 10 min, the optical density at 570 nm was measured using an ultraviolet spectrophotometer.

Wound healing assay. At the bottom of a 24-well plate, an auxiliary line (perpendicular to the cell scratch) was drawn every 0.5 cm to ensure the consistency of each observation site. Cells were seeded at 5x10^4 cells/well and cultured into a monolayer. An artificial wound was created using a pipette tip, and the scratched cells were washed in serum-free medium (21). After 24 h culture, cell migration was observed using an inverted microscope. The scratch area was calculated by ImageJ v.1.8.0 software (National Institutes of Health).
overexpression plasmids were generated by cloning the
used to the package of lentivirus.
Lentivirus transduction.
activity.
The ratio of firefly luciferase activity to
Corporation). Relative luciferase activity was expressed as
using a dual-luciferase reporter assay system (Promega
5% CO₂ atmosphere containing 5% CO₂
were added to the top and bottom chambers, respectively. After
24-h culture, the inner side cells that did not pass through the
membrane were discharged and stained with crystal violet
for 5 min. Photomicrographs were captured with an inverted fluorescence microscope (magnification, x100).

Dual-luciferase reporter assay. Target prediction for
miR-27b-3p was carried out using TargetScan v.7.2 (http://www.TargetScan.org/vert_72/). The 3'-UTRs of
EGFR and MMP-13 were cloned into pmir-GLO vectors (Promega Corporation) to generate EGFR wild-type (wt) and
MMP-13 wt vectors. The EGFR mutant (mut) and MMP-13
mut vectors were generated using the GeneTailer site-directed
mutagenesis kit (Invitrogen; Thermo Fisher Scientific Inc.).
The aforementioned vectors were mixed with the miR-27b-3p
mimic or mimic-NC and Lipofectamine 2000 (Invitrogen; Thermofisher Scientific Inc.) for another 20 min. The mixture
was then incubated with Lipofectamine® 2000 (Invitrogen; Thermofisher Scientific Inc.) for another 20 min. Finally, the mixture was incubated with 293T cells for 6 h at 37°C (2.5x10⁴ cells/plate in a 10-cm plate), which were previously incubated in Opti-MEM for 4 h. Then, the medium was then replaced with DMEM containing 10% FBS and
1% penicillin-streptomycin on the following day. At the 72-h
time point, the supernatant of the transfected 293T cells was
collected and centrifuged via 4.5 μm filter, then concentrated
by ultracentrifugation at 70,000 x g at 4°C for 2 h. The suspension
was filtered to determine the viral titers. The A-431 and
Colo-16 cells were cultured at 80% confluence, then infected
with lentivirus at a multiplicity of infection of 5 and with polybrene (Sigma-Aldrich; Merck KGaA) for 24 h. Fresh culture
medium was then used to replace the old medium. After 3
days, the fluorescence intensity was subsequently observed
to screen stable cell lines and the transfection efficiency was
determined by RT-qPCR.

Statistical analysis. Statistical analysis was performed using
GraphPad Prism 8 (GraphPad Software, Inc.). Data were
normally distributed and are presented as the mean ± SD.
Comparisons between multiple groups were analyzed using
one-way ANOVA followed by Tukey's post hoc test or
Bonferroni correction. P<0.05 was considered to indicate a
statistically significant difference. All experiments were
performed in triplicate and repeated three times.

Results

miR-27b-3p is downregulated in CSCC cells. The relative
expression levels of miR-27b-3p, EGFR and MMP-13 in
human CSCC cell lines and human normal skin fibroblasts
were detected (Fig. 1). miR-27b-3p was downregulated in
CSCC cell lines compared with the normal skin keratinocyte
cell line NHEK/SVRTERT3-5.

miR-27b-3p inhibits the proliferation, migration and invasion of CSCC cells. Compared with untransfected cells and
cells transfected with mimic-NC, transfection of A-431 and
Colo-16 cells with miR-27b-3p mimic upregulated the expres-
sion of miR-27b-3p, but downregulated the mRNA expression
levels of EGFR and MMP-13 (Fig. 2A). The results of colony

Table I. Primer sequences.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence, 5'-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-27b-3p-F</td>
<td>AGTGGCTAAGTTCTGCGTCG</td>
</tr>
<tr>
<td>miR-27b-3p-R</td>
<td>GATCCAGTGGCTGTCGG</td>
</tr>
<tr>
<td>Akt-F</td>
<td>TCTATGGCAGCTAGATGTG</td>
</tr>
<tr>
<td>Akt-R</td>
<td>CTTCATTGCGCCCTTCCTT</td>
</tr>
<tr>
<td>Cyclin D1-F</td>
<td>CAGTGGCAACTCTCCTCAACGA</td>
</tr>
<tr>
<td>Cyclin D1-R</td>
<td>TCCGACAGCCTCAGCAGTC</td>
</tr>
<tr>
<td>N-CAD-F</td>
<td>CCAACAGCACTGGAAGCCTAC</td>
</tr>
<tr>
<td>N-CAD-R</td>
<td>CACCTGATTCTGATGGCGGAC</td>
</tr>
<tr>
<td>E-CAD-F</td>
<td>GATCTTGTAATGACACATC</td>
</tr>
<tr>
<td>E-CAD-R</td>
<td>TGCCAGTTTCTGCATCTTG</td>
</tr>
<tr>
<td>GAPDH-F</td>
<td>GAAGGTGAAGTGGAGATG</td>
</tr>
<tr>
<td>GAPDH-R</td>
<td>GAAGATGGTGGAGGATTTC</td>
</tr>
<tr>
<td>U6-F</td>
<td>CTCCGTTCCGGCGACACA</td>
</tr>
<tr>
<td>U6-R</td>
<td>AACGCCTCCAGAATTTGCG</td>
</tr>
</tbody>
</table>

F, forward; R, reverse; miR, microRNA; CAD, cadherin.
formation and MTT assays indicated that overexpression of miR-27b-3p significantly inhibited the proliferative ability of the CSCC cells (Fig. 2B and C). Moreover, wound healing and Transwell assays demonstrated that miR-27b-3p could attenuate the migratory and invasive abilities of CSCC cells (Fig. 2D and E). The protein expression levels of p-Akt/total AKT, cyclin D1 and N-CAD were downregulated in CSCC cells overexpressing miR-27b-3p, whereas E-CAD was upregulated (Fig. 2F).

miR-27b-3p binds to the 3'-UTRs of EGFR and MMP-13. Target prediction with TargetScan indicated that miR-27b-3p contained sequences that could interact with the 3'-UTRs of EGFR and MMP-13 (Fig. 3A). The dual-luciferase reporter assay revealed that co-transfection with the miR-27b-3p mimic significantly decreased luciferase activity in EGFR wt and MMP-13 wt, but not EGFR mut and MMP-13 mut (Fig. 3B). This finding confirmed that miR-27b-3p targeted EGFR and MMP13.

Overexpression of EGFR or MMP-13 reverses the effects of miR-27b-3p on CSCC cells. A-431 and Colo-16 cells were co-transfected with the miR-27b-3p mimic and lentivirus infection. Transfection efficiency is shown in Fig. 4A and relevant RNA expression was shown as Fig. 4B. Notably, overexpression of EGFR significantly reversed the inhibitory effect of miR-27b-3p on CSCC proliferation (Fig. 4C and D). Overexpression of MMP-13 and the miR-27b-3p mimic increased the migration and invasion of CSCC cells, compared with cells transfected with the mimic alone (Fig. 4E and F). Co-overexpression of EGFR markedly promoted the viability and proliferation of CSCC cell lines, while overexpression of MMP-13 markedly enhanced the migration and invasion of CSCC cells (Fig. 4C-F). Moreover, overexpression of EGFR upregulated p-Akt/total AKT expression and cyclin D1. Overexpression of MMP-13 downregulated N-CAD and upregulated E-CAD levels compared with untreated or mimic-group (Fig. 4G). Therefore, overexpression of EGFR and MMP-13 reversed the inhibitory effect of miR-27b-3p on CSCC cells.

Discussion

The prognosis of CSCC is closely associated with clinical manifestations and histopathology, including tumor size, infiltration depth, nerve involvement, previous therapeutic effects, histological differentiation and immune status (22). The American Joint Committee on Cancer uses criteria including tumor diameter larger than 2 cm, poor cellular differentiation, depth of invasion more than 2 mm or to the reticular dermis (Clark level IV), perineural invasion, or ear or mucosal lip location to classify high-risk tumors (8). Gore et al (9) analyzed 57 patients with CSCC and found that patients with lymphatic metastasis experienced poor prognosis and high mortality. Among them, 8 patients presented lymphatic metastasis, with infiltration of nerves and lymphatic vessels as the main reasons affecting their prognosis. During the follow-up period of 19.4 months, 9 patients experienced recurrence, including 6 deaths. Thus, infiltration and metastasis may be the leading causes of deterioration and poor prognosis in CSCC.

miR-27b-3p, which exhibits anticancer effects, is downregulated in several types of cancer, such as breast cancer, lung carcinoma, esophageal carcinoma and colorectal carcinoma (14,23-25). Han et al (24) determined that miR-27b-3p was downregulated in esophageal squamous cell carcinoma (ESCC) tissue samples and cell lines and was associated with poor cell differentiation, TNM staging and lymphatic metastasis. The transcription factor nuclear-related factor 2 is the direct target of miR-27b-3p, as evidenced by dual-luciferase reporter assays (24). In a study conducted by Zeng et al (26), IL-10 induced the upregulation of miR-27b-3p and reduced the mRNA stability of proliferating cell nuclear antigen, which inhibited the development of hemangioma cavernosum. As the target of the long non-coding RNA HLA complex P5, miR-27b-3p drives the malignant development of gastric cancer, including proliferation and epithelial-to-mesenchymal transition (EMT) (27). However, the potential functions of miR-27b-3p in the development of CSCC have rarely been studied. The results of the present study revealed that miR-27b-3p was significantly downregulated in CSCC cell
Figure 2. miR-27b-3p inhibits the proliferation, migration and invasion of CSCC cells. (A) miR-27b-3p mimic transfection regulates the mRNA expression levels of EGFR and MMP-13 in CSCC cells. (B) Colony formation, (C) cell viability, (D) migration and (E) invasion of CSCC cells following transfection with the miR-27b-3p mimic (magnification, x100). (F) mRNA and protein expression levels of key molecules associated with proliferation and invasion in CSCC cells following transfection. Data were presented as the mean ± SD, and performed in triplicate. *P<0.05, **P<0.01 and ***P<0.001. CSCC, cutaneous squamous cell carcinoma; miR, microRNA; NS, not significant; NC, negative control; OD, optical density; CAD, cadherin.
Overexpression of miR-27b-3p attenuated the proliferative, migratory and invasive abilities of A-431 and Colo-16 cells compared with untreated group. In addition, the relative expression levels of EGFR, MMP-13, Akt, p-Akt and cyclin D1 were downregulated by miR-27b-3p overexpression, while E-CAD was upregulated compared with untreated group. These findings suggested that miR-27b-3p exerted an inhibitory role on the growth of CSCC cells.

EGFR is a transmembrane glycoprotein encoded by the proto-oncogene C-erbB-1 (28). Binding of ligands to the extracellular domain of EGFR triggers conformational changes in its transmembrane region and activates the intracellular region to bind to ATPase, leading to autophosphorylation and transphosphorylation. Consequently, multiple cellular signaling pathways are induced (29). For instance, EGFR can affect tumor development, metastasis and drug resistance mainly by activating the RAS-RAF-MAPK, the PI3K-PTEN-Akt and the JAK/STAT pathways (30). Diego Carrillo-Beltrán et al (31) analyzed relevant signaling pathways that mediate the carcinogenesis of oral cancer induced by high-risk human papillomavirus infection and demonstrated that HPV16 E7 activated the EGFR/PI3K/Akt1/NRF2, which in turn induced the activation of pirin/NF-κB signaling in oral cancer. In addition, Tang et al (32) demonstrated that knockdown of circ_0081143 suppressed hypoxia-induced migration, invasion and EMT in gastric cancer cells via the miR-497-5p/EGFR axis. Xiong et al (33) reported that WAP four-disulfide core domain 2 (WFDC2) levels negatively correlated to the Gleason score and incidence of metastasis in patients with prostate cancer. WFDC2 binds to the extracellular domain of EGFR and inhibits the EGFR/Akt/GSK3β/Snail signaling pathway, thus blocking the metastasis of prostate cancer (33).

In conclusion, miR-27b-3p reduces the proliferation, migration and invasion of CSCC cells by binding to the 3'-UTRs of EGFR and MMP-13. Thus, this miRNA may represent a potential diagnostic marker and therapeutic option for CSCC.
Figure 4. Overexpression of EGFR or MMP-13 reverses the effects of miR-27b-3p on CSCC cells. (A) Transfection efficiency of EGFR and MMP-13. (B) Co-transfection of miR-27b-3p mimic and EGFR or MMP-13 overexpression vectors. (C) Colony formation and (D) viability of CSCC cells after co-transfection with the miR-27b-3p mimic and EGFR. (E) Migration and (F) invasion of CSCC after co-transfection with the miR-27b-3p mimic and MMP-13 (magnification, x100). (G) mRNA and protein expression levels of key molecules associated with proliferation and invasion in CSCC cells following co-transfection with the miR-27b-3p mimic and EGFR or MMP-13 overexpression vectors. Data were presented as the mean ± SD, and performed in triplicate. *P<0.05, **P<0.01 and ***P<0.001. CSCC, cutaneous squamous cell carcinoma; miR, microRNA; NS, not significant; OD, optical density; CAD, cadherin.
Acknowledgements

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DL and ZZ made substantial contributions to conception and design, acquisition, analysis and interpretation of data. DL and ZZ performed the experiments. ZZ drafted the manuscript and revised it critically for important intellectual content. DL and ZZ confirmed the authenticity of the raw data. Both authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


