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MiR-1193 was sponged by LINC00963 and inhibited cutaneous squamous cell carcinoma progression by targeting SOX4

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ABSTRACT

Cutaneous squamous cell carcinoma (CSCC), a class of skin tumor derived from epidermal keratinocyte, is reputed as one of the most malignant tumors globally. MicroRNAs (miRNAs) are increasingly identified as essential players in CSCC. Current study aimed to uncover the impact and mechanism of miR-1193 in CSCC. We identified the low expression of miR-1193 in CSCC cell lines. Gain- and loss-of-function assays showed that miR-1193 acted as an inhibitor of proliferation and migration in CSCC cells. Furthermore, we illustrated that miR-1193 targeted and inhibited SRY-box 4 (SOX4), and that long intergenic non-protein coding RNA 963 (LINC00963) sponged miR-1193 to upregulate SOX4 expression. Rescue assays showed that LINC00963 regulated CSCC progression through miR-1193/SOX4 axis. In conclusion, our study firstly revealed the LINC00963/miR-1193/SOX4 axis in CSCC, indicating miR-1193 as a promising biological target in CSCC progression.

1. Introduction

Cutaneous squamous cell carcinoma (CSCC) ranks the second as the most common skin cancer types, and takes up approximately 20% of the skin cancer-caused mortalities around the world [13,22,23]. Although majorities of CSCC patients can be cured by surgical resection or radiotherapy, a proportion of cases may present aggressive clinical behaviors, with the increased risk of local recurrence and metastasis [2]. Therefore, mechanism of CSCC progression is in requirement of better understanding.

MicroRNAs (miRNAs) are a group of single strand, short, non-coding RNA transcripts, able to post-transcriptionally modulate gene expression by targeting the 3′ untranslated region (3′UTR) of mRNAs [1]. Through regulating the expression of certain target genes, miRNAs participate in myriads of cellular activities, including tumorigenesis, cell growth, apoptosis, invasion, and migration [9,14,17,25]. Several miRNAs have been revealed to participate in CSCC. For example, miR-221 facilitated CSCC progression through targeting PTEN [10]. MiR-365 aggravated CSCC through inhibiting Nuclear Factor I/B (NFIB) [29]. MiR-1193 has been identified to have anti-tumor functions by inhibiting proliferation, migration, and invasion in multiple cancers, such as breast cancer and T-cell leukemia [15,21]. However, the role of miR-1193 in CSCC remains covered.

Long non-coding RNAs are a group of non-protein coding transcripts longer than 200 bases [6]. Studies have shown that lncRNAs could sponge miRNAs to prevent miRNAs from inducing mRNA degradation of target genes in cancer progression [7,8]. The participation of lncRNAs in CSCC has been unveiled by studies [20]. For instance, lncRNA PICSAR prompts CSCC growth via modulating ERK1/2 activity [19]. Long intergenic non-protein coding RNA 963 (LINC00963) has been illustrated to play carcinogenic roles in a number of cancers, such as non-small cell lung cancer [26], hepatocellular carcinoma [24], and melanoma [12]. Nevertheless, its performance in CSCC is never been explored.

SRY-box 4 (SOX4) is known to be a transcription factor belonging to the SOX (Sry-related high-mobility group box) family which is involved in diverse developmental processes, such as stem cell maintenance and terminal differentiation in a wide range of cell types. Mounting studies have suggested that SOX4 is a carcinogene in cancers, such as in osteosarcoma [3], lung cancer [11], and clear cell renal cell carcinoma [16]. However, its role and interplay with miR-1193 in CSCC remain elusive.

Present study aimed to uncover the bioloical function and mechasim of miR-1193 in CSCC.

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2. Materials and methods

2.1. Cell culture

Human immortalized epidermal cells (HaCaT), human renal epithelial cells (293 T) and human CSCC cells (SCC13, Tca8113, SCL-1, A431) were all acquired from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cells were maintained in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and 1×10^5 U/L penicillin G and 1×10^5 U/L streptomycin (Invitrogen, Carlsbad, CA, USA) in humidified atmosphere containing 5% CO_2 at 37 °C. Media were changed every three days.

2.2. Cell transfection

A431 and SCC13 cells were put in 96-well plates when cell confluence achieved 40–50%. Cell transfection was performed in light of the protocol of Lipofectamine2000 Reagent (Invitrogen, Carlsbad, CA, USA). Design and synthesis of miR-1193 mimic, miR-1193 inhibitor, NC mimic and NC inhibitor in this research was accomplished by Genepharma (Shanghai, China). The LINC00963-specific shRNAs (sh-LINC00963#1/2/3) was obtained commercially from Genechem Co., Ltd. (Shanghai, China), along with the non-specific shRNA as control (named as sh-NC). To overexpress LINC00963 or SOX4 expression, cells were transfected with the pcDNA3.1 vector targeting LINC00963 or SOX4 and the empty vector as control (Genechem Co., Ltd). Transfection was terminated after 48 h.

2.3. Quantitative real-time PCR

Total RNAs were extracted from A431 and SCC13 cells by TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.). Using reverse transcriptase kit (Takara, Tokyo, Japan), total RNAs were reverse-transcribed into cDNA. The cDNA was subsequently amplified by SYBR Premix Ex Taq™ II (TaKaRa,) under the following conditions: at 95 °C for 30 s, 40 cycles of amplification at 95 °C for 5 s, at 59 °C for 30 s, ended with at 72 °C for 30 s. The relative expression of RNA was measured by $2^{-\Delta\Delta Ct}$. The miR-1193 expression was normalized to U6, while LINC00963 and SOX4 expressions were normalized to GAPDH.

2.4. Cell viability assay

After transfection, A431 and SCC13 cells were harvested and put in 96-well plates (100 μL, 10^4/well). Cells were reaped at 24, 48, 72 and 96 h and subjected to Cell Counting Kit-8 solution (CCK-8; Dojindo, Tokyo, Japan) without FBS in line with the user guide. Absorbance was measured at 450 nm by a microplate reader.

2.5. Colony formation assay

Transfected A431 and SCC13 cells were seeded in 6-well plates. After 15 days, cells grew into visible colonies. Cells were then washed with PBS for three times and fixed with 4% PFA (Sigma-Aldrich, St. Louis, USA). After staining with 0.1% Crystal Violet (Sigma-Aldrich), the number of the colonies more than 50 cells were counted manually.

2.6. Transwell assay

When the cell concentration reached 1×10^5 cells per well, transfected A431 and SCC13 cells were seeded into the upper chambers (Corning, NY, USA) culturing in serum-free medium. The medium with 10% FBS was added to the lower chambers. During the 12 h's incubation, cells were allowed to migrate across the filter. Cells on the top side of the filter were scrapped. Migrated cells were then fixed with methanol and stained with crystal violet. Stained cells were observed and counted under a microscope.

2.7. Luciferase reporter assay

The wild-type and mutant sequences of miR-1193 in 3′UTR of SOX4 (SOX4 WT and SOX4 Mut; Genepharma, Shanghai, China) were separately cloned into the pmirGLO luciferase reporter vector. 293 T cells were co-transfected with SOX4 WT or SOX4 Mut and miR-1193 mimic or NC mimic. The reporter plasmids of LINC00963 (LINC00963 WT and LINC00963 Mut) were constructed by Genepharma and co-transfected with miR-1193 mimic or NC mimic into 293 T cells using Lipofectamine2000. After 48 h, the relative luciferase activity was detected via the dual-luciferase reporter assay system (Promega, Madison, WI, USA).

2.8. Ago2-RIP assay

Ago2-RIP assay was conducted to evaluate the combination of miR-1193 with SOX4 and LINC00963. Imprint RNA immunoprecipitation kit (Sigma-Aldrich, Burlington, Massachusett, USA) was utilized to conduct Ago2-RIP assay in the light of the protocol. A431 or SCC13 cells were lysed in RIP lysis buffer (Solarbio, Beijing, China) and incubated in media containing proteinase K (Absin, Shanghai, China) together with anti-Ago2 or anti-IgG overnight at 4 °C. Immunoprecipitated RNA was purified and assessed by RT-qPCR.

2.9. Western blotting analysis

Protein was extracted by RIPA lysis buffer and quantified via BCA protein assay (Beyotime, Shanghai, China). Protein was isolated on 8–15% SDS-PAGE gels and transferred to PVDF membranes (Millipore, Bedford, MA, USA). 5% non-fat milk was used to block the membranes in TBST at room temperature for 1 h. Subsequently, membranes were treated with the primary antibody, including anti-SOX4 (1 μg/ml, ab80261, Abcam, Cambridge, USA) and anti-GAPDH (1/1000, ab8245, Abcam) at 4 °C overnight. The membranes were rinsed with TBST for three times and incubated with secondary antibody at room temperature for 1 h. Finally, protein bands were observed with the ECL system (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.10. Bioinformatics analysis

Starbase v3.0 (http://starbase.sysu.edu.cn/) predicted that SOX4 and LINC00963 containing the putative binding sites of miR-1193.

2.11. Statistical analysis

Each experiment was conducted for at least three times in this study. SPSS 23.0 (SPSS Inc, Chicago, IL, US) was applied for statistical analysis. All data in this study were presented as mean ± standard deviation (SD). Differences analysis was performed via Student's *t*-test or one-way analysis of variance (ANOVA). $P < 0.05$ was seen to be statistically significant.

3. Results

3.1. MiR-1193 was downregulated in CSCC and inhibited proliferation and migration in CSCC cells

First of all, we probed the participation of miR-1193 in CSCC by investigating its expression in cell lines. We observed that miR-1193 presented overt downregulation in CSCC cell lines versus normal cell line (Fig. 1A). To figure out the biological role of miR-1193, we implemented gain- and loss-of-function assays. We overexpressed miR-1193 in A431 cells expressing low miR-1193 level, and silenced miR-

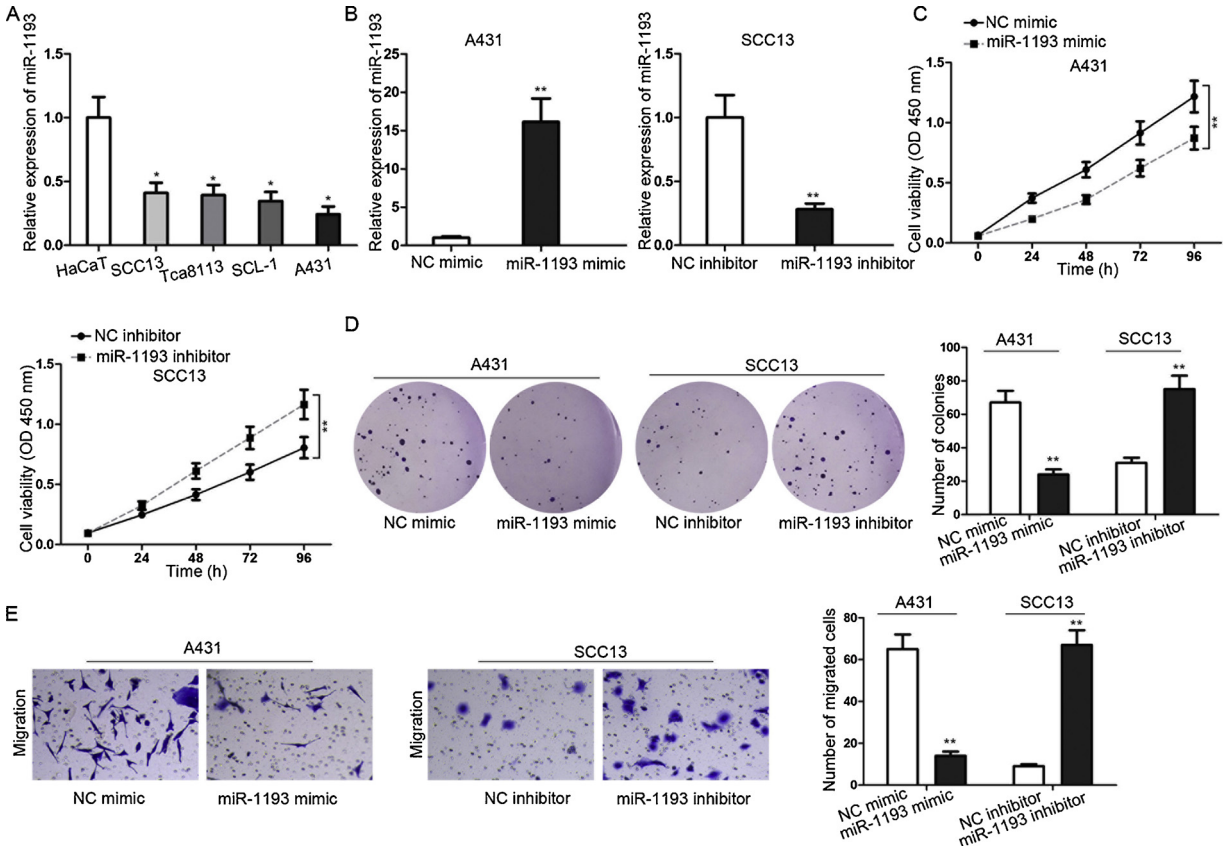

Fig. 1. MiR-1193 was downregulated in CSCC and inhibited proliferation and migration in CSCC cells. (A) RT-qPCR analysis was used to detect the level of miR-1193 in CSCC cell lines and normal cell line. (B) RT-qPCR analysis was used to confirm the overexpression and silence of miR-1193 in A431 and SCC13 cells. (C–D) CCK-8 and colony formation assays were used to assess the proliferation of A431 and SCC13 cells upon the overexpression and silence of miR-1193. (E) Transwell migration assay was used to detect the migration of A431 and SCC13 cells upon the overexpression and silence of miR-1193. Each assay was performed in triplication. $^{*}P < 0.05$, $^{**}P < 0.01$.

1193 expression in SCC13 cells expressing relatively high miR-1193 level (Fig. 1B). Results of CCK-8 and colony formation assays depicted that proliferation of A431 cells was attenuated upon miR-1193 overexpression, whereas that of SCC13 cells was prompted upon miR-1193 inhibition (Fig. 1C-D). Besides, overexpression of miR-1193 retarded migration of A431 cells, whereas silence of miR-1193 exerted opposite effects in SCC13 cells (Fig. 1E). In collection, these data indicated that MiR-1193 was downregulated in CSCC and inhibited proliferation and migration in CSCC cells.

3.2. MiR-1193 targeted and inhibited SOX4 in CSCC

Next, we tried to investigate the downstream mechanism of miR-1193 in CSCC. It is axiomatically acknowledged that miRNAs could repress gene expressions by binding to the 3′UTR of mRNAs, so as to realize their functions in cancer progression [10,29]. Therefore, we tried to find the target gene for miR-1193. Through searching Starbase 3.0 (http://starbase.sysu.edu.cn/index.php), we found that SOX4 was a putative target for miR-1193. SOX4 has been demonstrated to facilitate proliferation and migration in a number of cancers, such as osteosarcoma [3], lung cancer [11], and clear cell renal cell carcinoma [16]. Therefore, we speculated that miR-1193 regulated SOX4 in CSCC. First, we confirmed that SOX4 was highly expressed in CSCC cell lines at mRNA and protein levels (Fig. 2A). To detect the interaction between miR-1193 and SOX4, we obtained the binding sites on SOX4 mRNA for miR-11193 and mutated the sites for luciferase reporter assay (Fig. 2B). It turned out that in 293 T cells, overexpression of miR-1193 reduced luciferase activity of SOX WT rather than SOX Mut (Fig. 2B). RT-qPCR analysis following the RIP assay validated that miR-1193 and SOX4 mRNA were both precipitated by the Ago2 antibody (Fig. 2C), further confirmed the interplay between miR-1193 and SOX4. Thereafter, we detected the impact of miR-1193 on SOX4 expression. The mRNA and protein levels of SOX4 were reduced in A431 cells responding to the overexpression of miR-1193, whereas were increased in SCC13 cells responding to the silence of miR-1193 (Fig. 2D). Therefore, the results above confirmed that miR-1193 targeted and inhibited SOX4 in CSCC.

3.3. LINC00963 sponged miR-1193 to upregulate SOX4 in CSCC

Thereafter, we explored the upstream mechanism of miR-1193 in CSCC. It has been demonstrated by numerous studies that miRNAs could be sponged by lncRNAs in cancer progression, which prevented miRNAs from targeting downstream genes [4,5]. Therefore, we tried to identify the lncRNA regulating miR-1193 in CSCC. Through browsing Starbase 3.0, we found that LINC00963 potentially targeted miR-1193. LINC00963 has been revealed to facilitate the tumor progression in multiple cancers, such as non-small cell lung cancer [26], hepatocellular carcinoma [24], and melanoma [12]. Hence, we speculated that LINC00963 may also play a part in CSCC. We identified the elevation of LINC00963 level in CSCC cell lines compared with the normal cell line (Fig. 3A). To investigate the interaction between LINC00963 and miR-1193, we identified the binding sites on LINC00963 for miR-1193, and the sites were mutated for luciferase reporter assay (Fig. 3B). Consequently, overexpression of miR-1193 weakened the luciferase activity of LINC00963 WT instead of LINC00963 Mut in 293 T cells (Fig. 3B). RIP analysis followed by RT-qPCR assay confirmed the co-immunoprecipitation of miR-1193 and LINC00963 by anti-Ago2 (Fig. 3C), verifying that miR-1193 interacted with LINC00963. Moreover, we

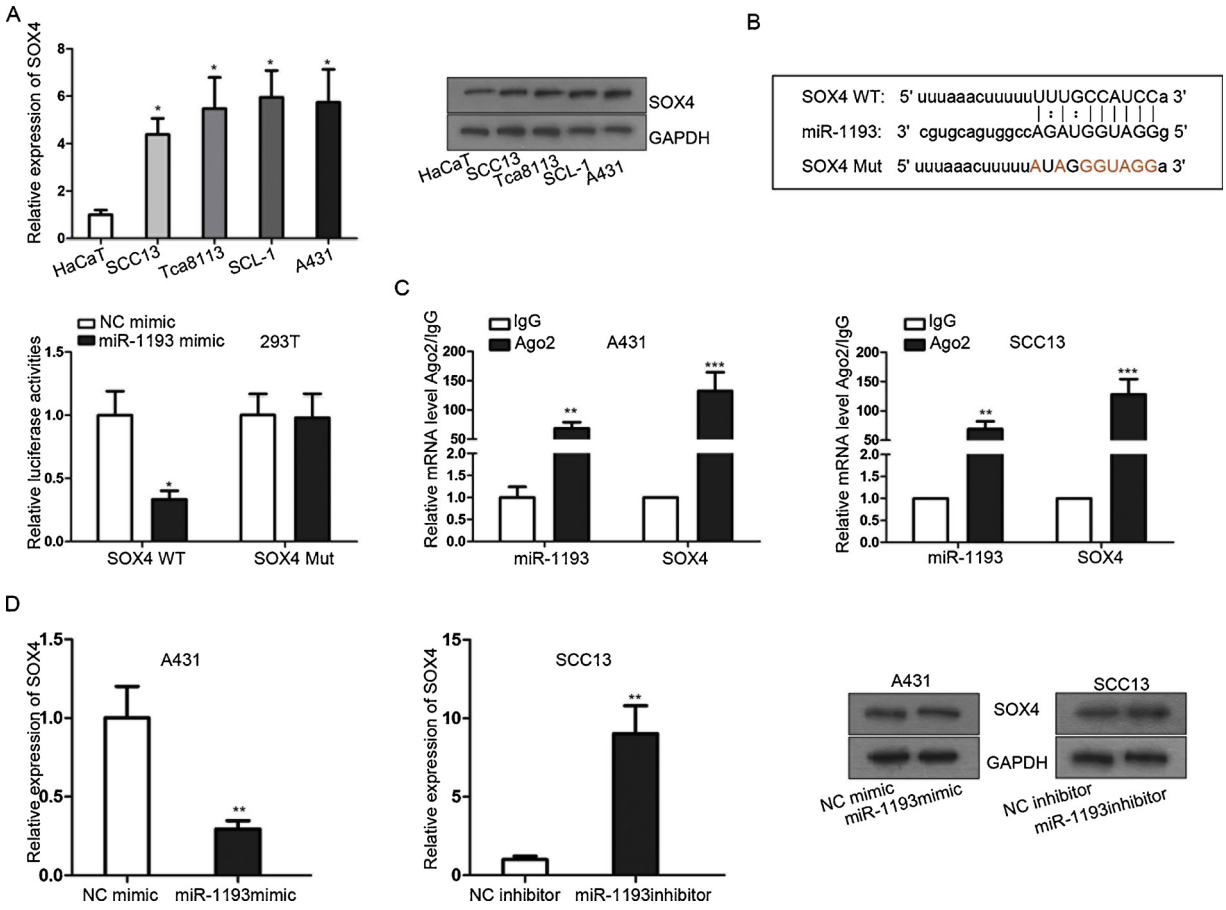

Fig. 2. MiR-1193 targeted and inhibited SOX4 in CSCC. (A) RT-qPCR and western blot analyses were used to detect the mRNA and protein levels of SOX4 in CSCC cell lines and normal cell line. (B) The binding sequences on SOX4 mRNA for miR-1193 were obtained from Starbase 3.0 and the mutated sites were designed. Luciferase reporter assay was used to assess the binding of miR-1193 on SOX4 mRNA. (C) RIP assay followed by RT-qPCR analysis was used to detect the interaction between SOX4 and miR-1193. (D) RT-qPCR and western blot analyses were used to detect the mRNA and protein levels of SOX4 in A431 and SCC13 cells upon the overexpression and silence of miR-1193. Each assay was performed in triplication. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

found that miR-1193 mimic resulted in the decrease of LINC00963 level in A431 cells, while miR-1193 inhibitor led to the increase of LINC00963 level in SCC13 cells (Fig. 3D). Additionally, we also probed the impact of LINC00963 on miR-1193 expression. We silenced LINC00963 in A431 cells and overexpressed LINC00963 in SCC13 cells (Fig. 3E). Since RT-qPCR data confirmed that sh-LINC00963#1/2 knocked down LINC00963 more efficiently in A431 cells, we used them for subsequent experiments. As a result, silenced expression of LINC00963 induced miR-1193 expression in A431 cells, whereas ectopic expression of LINC00963 reduced miR-1193 in SCC13 cells (Fig. 3F). Furthermore, we detected the effect of LINC00963/miR-1193 axis on SOX4 expression. We found that miR-1193 inhibitor counteracted the inhibitory effect of LINC00963 silence on SOX4 mRNA and protein levels in A431 cells (Fig. 3G). Also, mutating the miR-1193 sites on LINC00963 or overexpressing miR-1193 could impair the inductive effect of LINC00963 overexpression on SOX4 mRNA and protein levels (Fig. 3G). Therefore, these data suggested that LINC00963 sponged miR-1193 to upregulate SOX4 in CSCC.

3.4. The role of LINC00963/miR-1193/SOX4 axis in CSCC

Finally, we assessed the role of LINC00963/miR-1193/SOX4 axis in CSCC. RT-qPCR analysis confirmed that miR-1193 mimic abrogated the inductive effect of LINC00963 overexpression on SOX4 level, and co-transfection of pcDNA3.1/SOX4 restored the SOX4 level in SCC13 cells (Fig. 4A). CCK-8 and colony formation assays demonstrated that overexpression of miR-1193 reversed the facilitative effect of LINC00963 overexpression on SCC13 cell proliferation, and co-transfection of pcDNA3.1/SOX4 could recover the proliferation of SCC13 cells (Fig. 4B-C). Also, the migration of SCC13 cells induced by LINC00963 overexpression was impaired by miR-1193 mimic, and could be recovered by the overexpression of SOX4 (Fig. 4D). In sum, we confirmed that LINC00963 regulated proliferation and migration in CSCC cells by miR-1193/SOX4 axis.

4. Discussion

Statistics have shown that the patients with CSCC are still faced with awful prognosis despite the advancing therapeutic regimes [18,27,28], which indicates that better understanding of the molecular mechanism of CSCC progression is necessitated for the prognosis improvement of CSCC patients.

Past decades have seen the rising roles of miRNAs in cancer progression by modulating cellular activities, including tumorigenesis, cell growth, apoptosis, invasion, and migration [9,14,17,25]. In CSCC, the significant role of miRNAs has been revealed as well. For example, miR-221 facilitated CSCC progression through targeting PTEN [10]. MiR-365 aggravated CSCC through inhibiting Nuclear Factor I/B (NFIB) [29]. Herein, present study firstly revealed that miR-1193 was downregulated in CSCC cell lines, indicating that miR-1193 might be implicated in CSCC. Previously, studies have proved that miR-1193 served as an anti-tumor gene in breast cancer and T-cell leukemia, inhibiting proliferation, migration, and invasion [15,21]. In concordance, we validated through gain- and loss-of-function assays that miR-1193

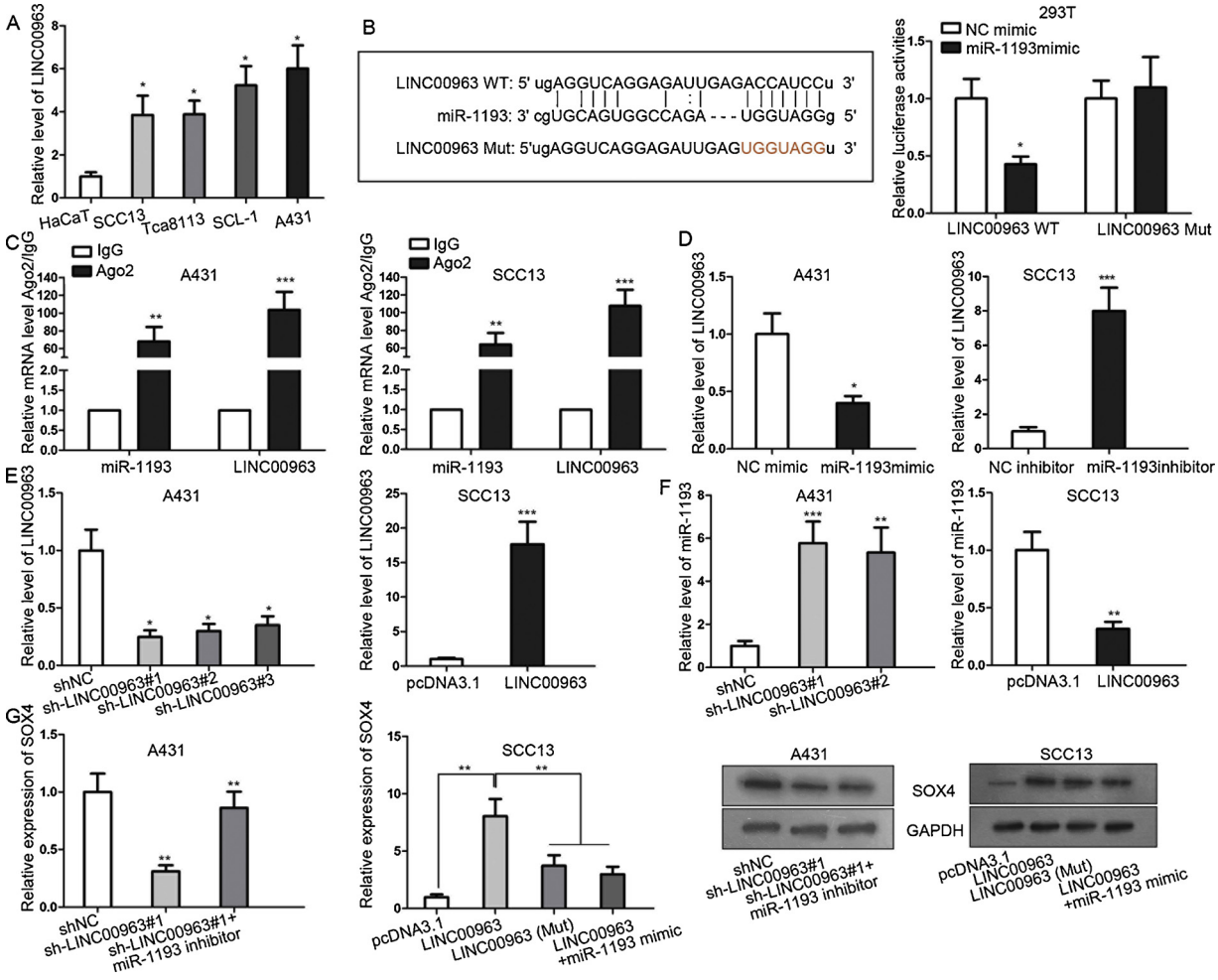

Fig. 3. LINC00963 sponged miR-1193 to upregulate SOX4 in CSCC. (A) RT-qPCR analysis was used to detect the level of LINC00963 in CSCC cell lines and normal cell line. (B) The binding sequences on LINC00963 for miR-1193 were obtained from Starbase 3.0 and the mutated sites were designed. Luciferase reporter assay was used to assess the binding of miR-1193 on LINC00963. (C) RIP assay followed by RT-qPCR analysis was used to detect the interaction between LINC00963 and miR-1193. (D) RT-qPCR analysis was used to detect the level of LINC00963 in A431 and SCC13 cells upon the overexpression and silence of miR-1193. (E) LINC00963 was silenced in A431 cells by sh-LINC00963#1/2/3, and overexpressed in SCC13 cells by pcDNA3.1/LINC00963 as confirmed by RT-qPCR analysis. (F) RT-qPCR analysis was used to detect the level of miR-1193 in A431 and SCC13 cells upon the silence and overexpression of LINC00963. (G) A431 cells were transfected with sh-NC, sh-LINC00963#1, or sh-LINC00963#1 + miR-1193 inhibitor respectively, SCC13 cells were transfected with pcDNA3.1, pcDNA3.1/LINC00963, pcDNA3.1/LINC00963 (Mut) (with mutant miR1193 sites), or pcDNA3.1/LINC00963 + miR-1193 mimic respectively. RT-qPCR and western blot analyses were used to detect the mRNA and protein levels of SOX4 in A431 and SCC13 cells of each group. Each assay was performed in triplication. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

inhibited proliferation and migration in CSCC cells, suggesting that miR-1193 as a tumor-suppressive gene in CSCC.

Furthermore, we found through Starbase that SOX4 was a downstream target for miR-1193. SOX4 is one of the transcription factors in the SOX family, and has been reported by a number of studies to promote the progression of cancers. For example, SOX4 downregulation suppressed the cell proliferation and migration, and induced apoptosis in osteosarcoma [3]; LncRNA CCAT1 upregulated SOX4 by targeting miR-130a-3p to increase cisplatin resistance in non-small-cell lung cancer cell line [11]; and miR-138 inhibited epithelial-to-mesenchymal transition (EMT) by targeting SOX4 in clear cell renal cell carcinoma [16]. Our study was the first to unveal the interaction between SOX4 and miR-1193 in CSCC. We also uncovered that SOX4 was upregulated in CSCC cells and that miR-1193 negatively regulated SOX4 expression in CSCC, suggesting that SOX4 was a positive regulator in CSCC.

Moreover, we interrogated the upstream mechanism of miR-1193 in CSCC. It has been largely reported by former studies that miRNAs could be sponged by lncRNAs, so that they could be prevented from inducing mRNA degradation of target genes in cancer progression [7,8]. By searching Starbase 3.0, we firstly found that LINC00963 potently targeted miR-1193. We selected LINC00963 for further investigation because LINC00963 has been previously illustrated to facilitate proliferation and migration in a number of cancers, such as non-small cell lung cancer [26], hepatocellular carcinoma [24], and melanoma [12], but never has it been explored in CSCC. In accordance, we firstly revealed that LINC00963 was upregulated in CSCC cell lines, and confirmed that LINC00963 interacted with miR-1193. We also demonstrated that LINC00963 and miR-1193 formed a negative feedback loop by inhibiting each other's expression in CSCC cells. In addition, we mutated the miR-1193 sites on LINC00963 and found that the effect of LINC00963 on inducing SOX4 level, suggesting that LINC00963 regulated SOX4 expression through miR-1193. Finally, rescue assay suggested that LINC00963 regulated miR-1193/SOX4 to promote proliferation and migration in CSCC cells.

Recent years, the association between lncRNAs and clinicopathological features of cancer patients have been analyzed and reported. Moreover, high expression of lncRNAs in tumor samples implicated poor prognosis for cancer patients. Lacking of clinical samples is a deficient of our current study. Although LINC00963/miR-1193/SOX4 axis was not detected in clinical samples, finding of this pathway

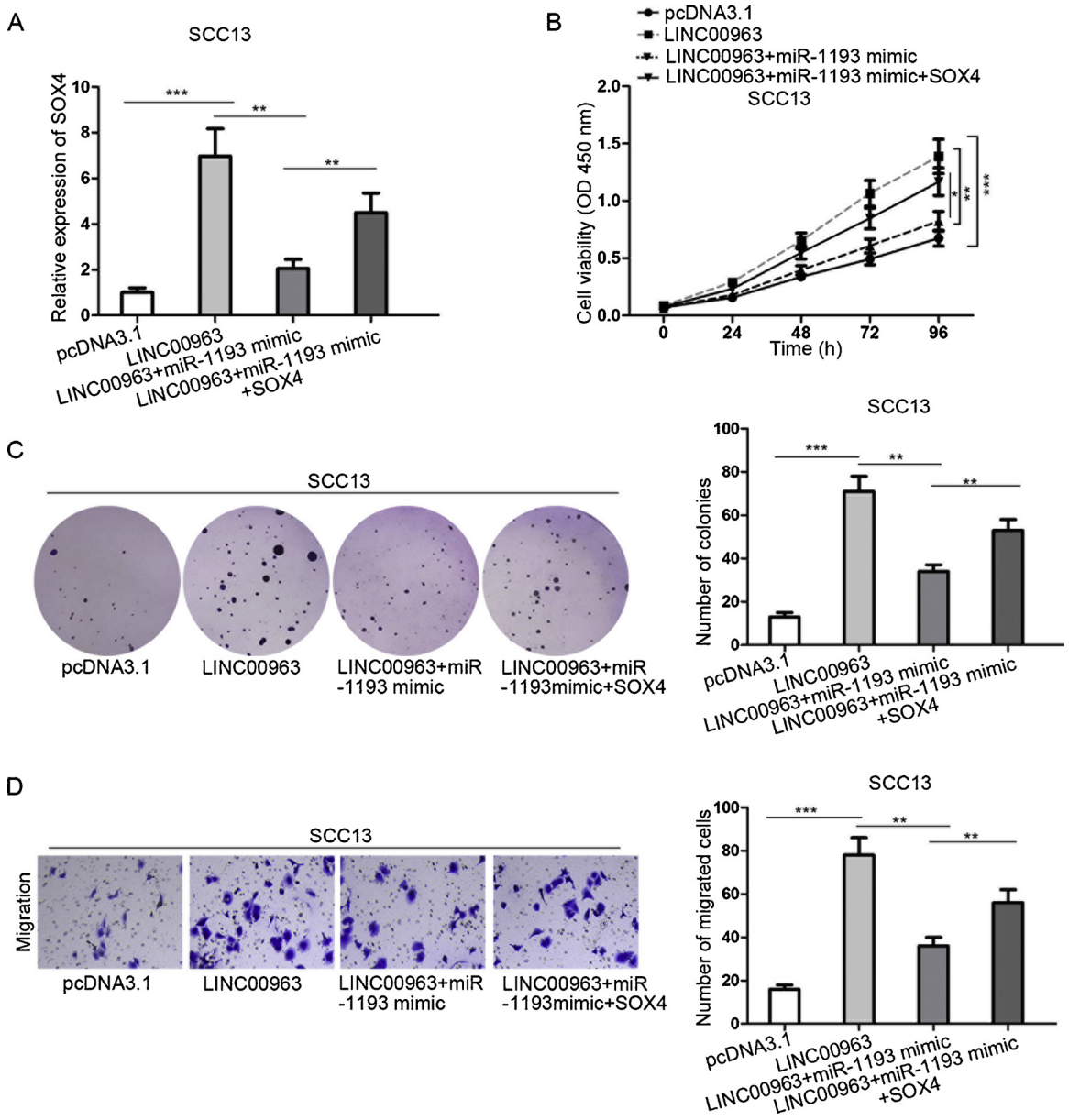

Fig. 4. The role of LINC00963/miR-1193/SOX4 axis in CSCC. SCC13 cells were transfected with pcDNA3.1, pcDNA3.1/LINC00963, pcDNA3.1/LINC00963 + miR-1193 mimic, or pcDNA3.1/LINC00963 + miR-1193 mimic + pcDNA3.1/SOX4 respectively. (A) RT-qPCR analysis was used to detect the mRNA level of SOX4 in SCC13 cells of each group. (B–C) CCK-8 and colony formation assays were used to assess the proliferation of SCC13 cells of each group. (D) Transwell migration assay was used to detect the migration of SCC13 cells of each group. Each assay was performed in triplication. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

was still considered to be significant in accordance with the experimental data. We will make further investigation to identify the clinical significance of LINC00963/miR-1193/SOX4 axis in our future study.

In conclusion, present study firstly showed that miR-1193 was sponged by LINC00963 and inhibited cutaneous squamous cell carcinoma progression by targeting SOX4, indicating miR-1193 as a novel biological marker in CSCC. Besides, more experiments on animal models are required in the future to confirm the therapeutic significance of miR-1193 in CSCC.

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Declaration of Competing Interest

The authors state that there are no conflicts of interest.

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