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miR-29b upregulates miR-195 by targeting *DNMT3B* in tongue squamous cell carcinoma

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Abstract MicroRNAs play important roles in the development and progression of various cancers, including tongue squamous cell carcinoma (TSCC). miR-29b and miR-195 have been reported to be tumor suppressors in TSCC. Here, we investigated the expression of miR-29b and miR-195 and their relationship in TSCC. Our data showed that miR-29b and miR-195 were significantly downregulated in TSCC compared with their matched nonmalignant tissues in 60 paired samples. The level of miR-29b was positively correlated with that of miR-195 in TSCC and the matched nonmalignant tissues. Moreover, miR-29b overexpression induced the demethylation of CpG islands upstream of miR-195 via targeting *DNMT3B*, leading to the upregulation of miR-195 in TSCC cell lines. Following *DNMT3B* silencing, the expression of miR-195 was increased and the methylation of CpG islands upstream of miR-195 was reduced. Although overexpression of miR-29b alone significantly increased miR-195 expression, co-transfection of miR-29b with DNMT3B resulted in no change in miR-195 expression. Taken together, our results demonstrated that miR-29b could upregulate miR-195 by directly targeting DNMT3B in TSCC. The interaction between miR-29b and miR-195 might provide new insights in developing novel therapeutic approaches of TSCC.

Keywords miRNA · miR-29b · miR-195 · DNMT3B · Tongue squamous cell carcinoma

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1 Introduction

Tongue squamous cell carcinoma (TSCC) is the most common type of oral cancer and has a particularly poor prognosis due to its invasive nature [1]. Although the etiology of TSCC is not fully understood, aberrant epigenetic regulation contributes substantially to the onset and progression of TSCC as well as many other cancers [2, 3]. Among the mechanisms of epigenetic regulation, noncoding RNAs [4] and DNA methylation [5] are key determinants in the pathogenesis of human cancer.

MicroRNAs (miRNAs) are endogenous small noncoding RNAs that control target gene expression by messenger RNA (mRNA) degradation or translation inhibition [6]. Aberrant expression of certain miRNAs is associated with squamous cell carcinoma; they can function as oncogenes or tumor suppressor genes depending on the mRNAs targets they regulate [7–9]. For instance, miR-7/184/138/21

have been shown to play critical roles in the development and progression of TSCC [10–13]. We also previously showed that miR-29b and miR-195 are significantly reduced in TSCC [14, 15]. miR-29b functions as a tumor suppressor in many malignant tissues by targeting genes including *MMP2* [16], *CX3CL1* [17], *Sp1* [14, 18], and *c-FOS* [19]. miR-29b represents an example of "epi-miRNAs" since it targets epigenetic effectors like DNMT3A/3B, leading to the re-activation of oncosuppressive genes [20–24]. Meanwhile, aberrant miR-195 expression is observed in multiple types of cancer [25–27]. Aberrant expression of miRNA could, in part, be caused by epigenetic regulators that control their expression [28, 29]. Particularly, the expression of miR-195 is silenced by hypermethylation of the upstream cytosine-phosphate-guanine (CpG) island [30]. Considering that mir-29b targets DNMT3B and the expression of miR-195 is influenced by DNA methylation, we postulated that miR-29b could modulate miR-195 expression by regulating DNMT3B activity in TSCC. Informed consents was received from each patient.

In the present study, we demonstrated that miR-29b upregulates miR-195 by directly targeting DNMT3B in TSCC. Our findings present a novel modulatory mechanism of miRNA biogenesis and provide insight into the treatment of TSCC.

2 Materials and methods

2.1 Human tissue specimens

Paired primary TSCC samples and adjacent histologically normal tissues were obtained from 60 patients after obtaining informed consent. None of the patients received treatment prior to radical surgical treatment. Tumor tissues and matched nonmalignant tissues at least 1.5 cm distal to the tumor margins were snap-frozen in liquid nitrogen and stored at −80 °C until use; part of these specimens were also used in our previous studies [14, 15]. This study was approved by the Institutional Ethics Committee of Peking University School of Stomatology (Beijing, China).

2.2 RNA isolation and quantitative stem-loop reverse transcription PCR (qRT-PCR)

Total RNAs were isolated from the tumor and normal tissue samples using TRIzol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Oligo-dT-primed cDNA was obtained using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, USA). Quantitative PCR was conducted at 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s in an ABI 7500 real-time PCR machine (Applied Biosystems). The relative expression of miR-29b and miR-195 was calculated by the $2^{-\triangle\triangle Ct}$ method. The qRT-PCR primers used to amplify miR-29b, miR-195, *DNMT3B*, *U6* (internal control for miRNAs), and *β-actin* (internal control for mRNAs) are listed in Table 1.

2.3 Vector construction and luciferase reporter assay

The expression vector pcDNA3.1-miR-29, harboring miR-29b precursor, was generated as described previously [14]. A firefly luciferase reporter plasmid including wild-type and the 3′-untranslated region (UTR) of mutant *DNMT3B* was also created [31]. TSCC cells grown in 48-well plates were co-transfected with 400 ng of pcDNA3.1-miR-29 and 40 ng of the firefly luciferase reporter plasmid including either the wild-type or mutant 3′-UTR of *DNMT3B*, and 4 ng of pRL-TK, a plasmid expressing *Renilla* luciferase (Promega, Madison, USA). Luciferase activity was measured 24 h after transfection using the Dual-Luciferase Reporter Assay System (Promega).

2.4 Cell culture and transfection

The human tongue cancer cell lines SCC-15 and CAL27 were purchased from the American Type Culture Collection (Manassas, USA). All tongue cancer cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10 % fetal bovine serum containing 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were cultured at 37 °C in a humidified atmosphere with 5 % CO_2. SCC-15 and CAL27 cells were seeded onto 6-well plates the day before transfection to ensure 80 % confluency at the time of transfection.

2.5 5-Aza-2-deoxycytidine (5-Aza-dC) treatment

Cells were seeded 24 h prior to treatment with 5 mmol/L 5-Aza-dC (Sigma-Aldrich, St. Louis, USA). After 5 d of treatment, cells were harvested and total RNAs were prepared for qRT-PCR analysis.

2.6 Bisulfite sequencing for DNA methylation analysis

The University of California Santa Cruz database was used to identify CpG islands (CGI) spanning miR-195 genes. Bisulfite sequencing for miR-195 promoter DNA methylation analysis was performed as previously described [30]. DNA was extracted with phenol–chloroform and treated with bisulfite using a CpGenome^{TM} DNA modification kit (Millipore, Billerica, USA) according to the manufacturer's instructions. Bisulfite-converted genomic DNA, in which unmethylated cytosines were converted to uracils, was amplified with specific primers as previously described

[30]. Purified PCR fragments were cloned into pGM-T (TIANGEN, Beijing, China), and individual clones were sequenced. The primers used for the amplification of CGI on the miR-195 gene are listed in Table 1.

2.7 Western blot analysis

Western blotting was performed as described previously [17]. Primary antibodies against DNMT3B (Abgent, San Diego, USA) and β-actin (Santa Cruz Biotechnology, Santa Cruz, USA) were diluted 1:1,000. Horseradish peroxidase-conjugated second antibodies (Zhongshan Goldenbridge, Beijing, China) were diluted 1:10,000.

2.8 RNA oligoribonucleotides

Small interfering RNAs (siRNAs) targeting the human *DNMT3B* transcript, termed siDNMT3B-1, siDNMT3B-2, and siRNA control, were purchased from Integrated Biotech Solutions Co. (Shanghai, China) as described previously [31]. The sequences of these siRNAs are listed in Table 1.

2.9 Statistical analysis

All statistical analyses were performed using SPSS for Windows version 16.0. Differences between groups were analyzed by Student's *t* test. Correlations between the expression of miR-29b and miR-195 were analyzed using Pearson's rank correlation coefficient analysis. All cell culture experiments were performed (at least) in triplicate. All data are expressed as the mean ± standard deviation (SD). A two-tailed *P* value <0.05 was considered to indicate statistical significance.

3 Results

3.1 miR-29b and miR-195 were reduced in TSCC

The expression of miR-29b and miR-195 was evaluated in 60 pairs of TSCC and matched nonmalignant tissue samples by qRT-PCR. The average expression of miR-29b and miR-195 in tumor tissues was significantly lower than that in matched nonmalignant tissues (Fig. 1), suggesting a correlation between miR-29b and miR-195 in TSCC.

3.2 miR-29b and miR-195 were positively correlated in TSCC specimens

Since miR-29b and miR-195 were both reduced in TSCC, we next investigated the correlation between their expression levels. Pearson's rank correlation coefficient analysis revealed

Table 1 Primers used in this study

Name	Sense strand/sense primer (5′–3′)	Antisense strand/antisense primer (5′–3′)
Primers for RT-PCR		
miR-29b RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAACACT	
miR-195 RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGCCAAT	
Primers for real-time quantitative PCR		
miR-29b	GTGCAGGGTCCGAGGT	GCATAGCACCATTTGAAATCAGT
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
miR-195	CGTAGCAGCACAGAAAT	GTGCAGGGTCCGAGGT
DNMT3B	CGGGAAATCGTGCGTGAC	CAGGCAGCTCGTAGCTCTT
DNMT3B	AATGTGAATCCAGCCAGGAAAGGC	ACTGGATTACACTCCAGGAACCGT
Primers used to clone miR-29b fragment into the vector named as pcDNA3.1-miR-29b		
miR-29b	TCTGACTTCTCCTGCCTTTACC	AGACCTGACTGCCATTTGTGAT
siRNA duplexes		
siDNMT3B-1	AGAUGACGGAUGCCUAGAGTT	CUCUAGGCAUCCGUCAUCUTT
siDNMT3B-2	CAGCUCUUACCUUACCAUCGATT	UCGAUGGUAAGGUAAGAGCUGTT
siRNA control	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT
Primers for cloning wild-type or mutant 3′-UTR of DNMT3B		
wild-type 3′UTR	AGGGAATTCTCTTCCTCAGCTG	TAACTCGAGCCCTTGGGTTTGT
mutant 3′UTR	GGTGCAACGATGGATGAACAACAA AATTTCAAAATCC	GGATTTTGAAATTTTGTTGTTCATCCAT CGTTGCACC
Primers used for miR-195 gene bisulfite sequencing		
miR-195-CG-BSF1	GTGTTTATTTGTAGTGATTT	
miR-195-CG-BSR1	TAACTCCCTCAATCTCTTATTCTT	

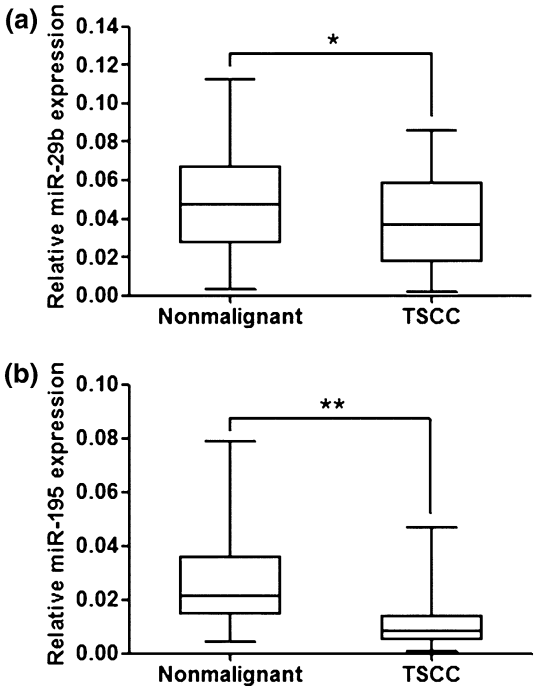

Fig. 1 miR-29b and miR-195 were reduced in TSCC samples compared with matched adjacent nonmalignant tissues. All data are expressed as the mean ± standard deviation (SD). $*P < 0.05$, $**P < 0.01$

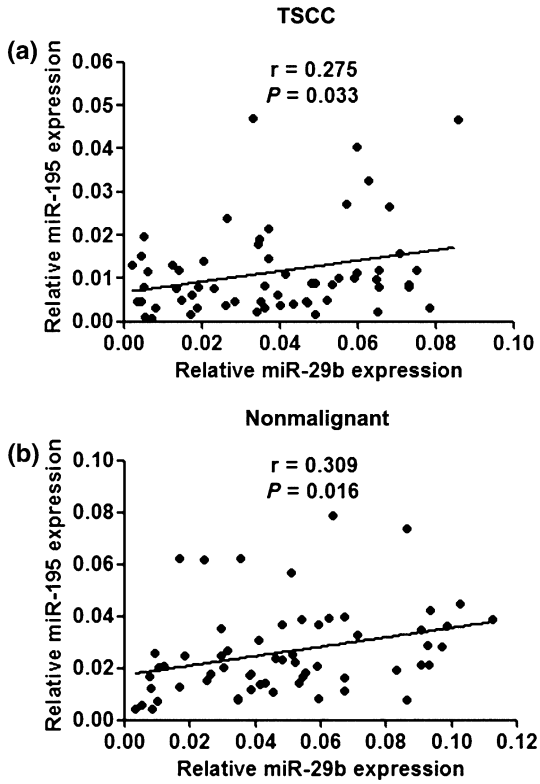

Fig. 2 miR-195 expression was positively correlated with miR-29b expression in TSCC and matched nonmalignant tissues. Statistical analysis was performed using Pearson's correlation coefficient analysis, with r and P values as indicated

a positive correlation between miR-29b and miR-195 expression in TSCC ($r = 0.275$, $P = 0.033$) and matched nonmalignant tissues ($r = 0.309$, $P = 0.016$; Fig. 2).

3.3 Overexpression of miR-29b upregulated miR-195 expression via a reduction in promoter DNA methylation

Transfection of miR-29b into SCC15 or CAL 27 cells resulted in more than five folds increase in its expression (data not shown) and also resulted in significant increase in miR-195 expression in SCC15 and CAL27 cells compared with those transfected with the control vectors (Fig. 3a). The methylation status of CpG islands in the nucleotide sequence from −5424 to −5142 bp upstream of miR-195 gene was then evaluated through bisulfite sequencing. Upon the overexpression of miR-29b, lower frequency of methylated CpG sites was observed in SCC-15 (84.6 % vs. 57.8 %) and CAL27 (76.5 % vs. 58.8 %; Fig. 3b). To determine whether CpGs methylation affects miR-195 expression, cancer cells were treated with the DNA demethylating agent 5-Aza-dC. Cells treated with 5-Aza-dC exhibited higher expression of miR-195 (Fig. 3c) and lower frequency of methylated CpG sites located in the upstream of miR-195, compared with that of the control (Fig. 3d).

3.4 Overexpression of miR-29b decreased DNMT3B expression by targeting its 3′-UTR

DNMT3B mRNA contains one conserved putative miR-29b target site in its 3′-UTR according to two of the most commonly used software algorithms (TargetScan and MiRanda; Fig. 4a). We observed that overexpression of miR-29b suppressed the luciferase activity of the reporter containing the wild-type 3′-UTR (Fig. 4b), but not that containing the mutant 3′-UTR of DNMT3B. Both the mRNA and protein levels of DNMT3B were significantly decreased in CAL27 and SCC15 cells upon miR-29b overexpression compared with those transfected with the control vectors (Fig. 4c, d). These results indicated that DNMT3B was a direct target of miR-29b in TSCC cell lines.

3.5 DNMT3B mediated miR-29b-induced upregulation of miR-195 expression

To investigate whether the downregulation of *DNMT3B* by miR-29b overexpression contributed to the upregulation of miR-195, we knocked down the expression of endogenous

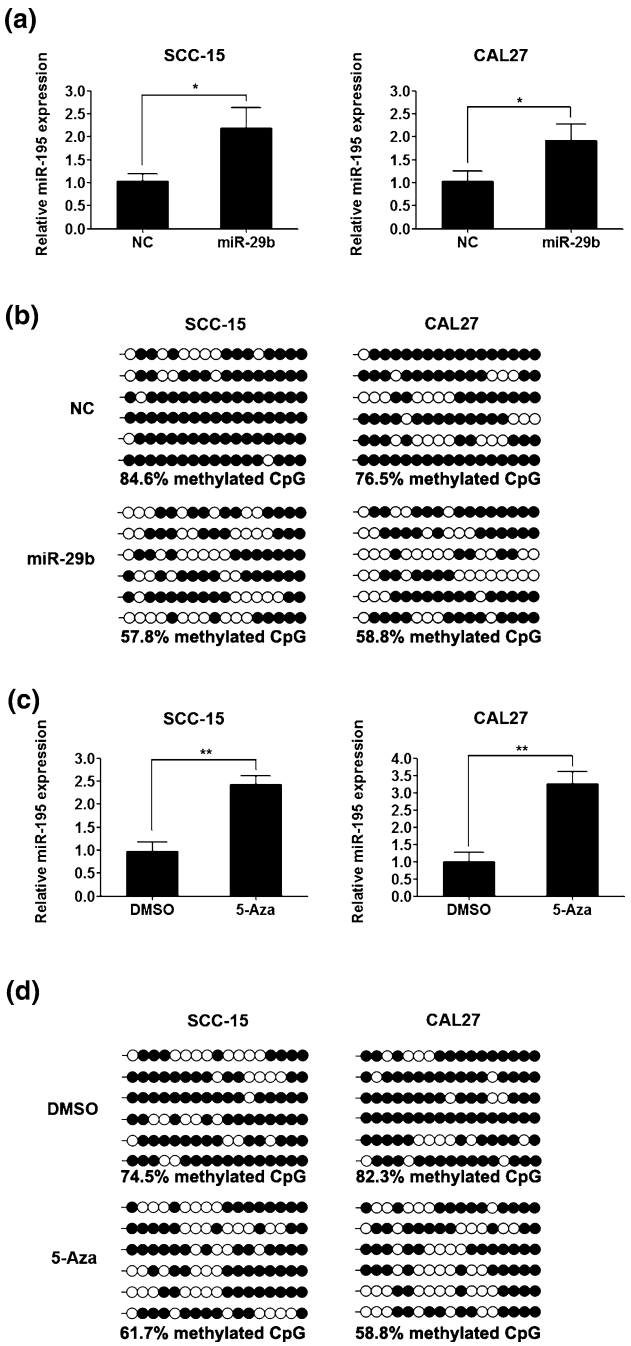

Fig. 3 Overexpression of miR-29b upregulated miR-195 expression via a reduction in promoter DNA methylation. **a** Overexpression of miR-29b upregulated the expression of miR-195. Data are expressed as the mean ± standard deviation (SD). **b** Bisulfite sequencing results are represented by lollipop showing the reduced percentage of methylated CpG in SCC-15 and CAL27 cells by miR-29b overexpression. **c** miR-195 was significantly increased in SCC15 and CAL27 cells treated with 5-Aza-dC. Data are expressed as the mean ± standard deviation (SD). **d** Lollipop diagrams showed 5-Aza-dC treatment reduced the percentage of methylated CpG compared with DMSO as revealed by bisulfite sequencing. Filled circles indicate methylated CpG sites, while open circles indicate unmethylated CpG sites. Horizontal rows represent individual clones, and vertical columns show the position of each CpG. The percentage of methylated sites is indicated below the sequence data. $^{*}P < 0.05$, $^{**}P < 0.01$

DNMT3B to mimic the effect of miR-29b overexpression. The knockdown of DNMT3B by siRNAs in TSCC lines promoted miR-195 expression (Fig. 5a) and reduced the percentage of methylated CpGs sites located in the region upstream of miR-195 (Fig. 5b). Moreover, a vector containing the full-length cDNA of *DNMT3B* was constructed and co-transfected into CAL27 and SCC15 cells with miR-29b. Co-transfection of miR-29b with *DNMT3B* resulted in no change in miR-195 expression, whereas transfection of miR-29b alone resulted in a significant increase in miR-195 expression (Fig. S1 online), suggesting that DNMT3B mediated miR-29b-induced upregulation of miR-195.

4 Discussion

The present study showed that both miR-29b and miR-195 were significantly reduced in TSCC and that the expression of miR-29b was positively correlated with miR-195 expression in the tumor tissues and matched nonmalignant tissues, indicating their biological relevance. As tumor-suppressive miRNAs, aberrant expression of miR-29b and miR-195 have been detected in various types of tumor tissues [27–29, 32], suggesting the potential interaction between miR-29b and miR-195 related to tumor development and progression. Mechanistically, miR-29b and miR-195 are involved with the same targets or signaling pathway. Both miR-29b and miR-195 attenuate cell cycle progression and suppress tumor cell proliferation by targeting *CDK6* [33, 34]. Additionally, either miR-29b or miR-195 inhibits Wnt signaling pathway which contributes to tumor promotion [25, 35]. Consistent with their compatible biological function, our results showed that overexpression of miR-29b resulted in increased expression of miR-195 and demethylation of its promoter. miR-195 can inhibit cell cycle progression and promote cell apoptosis in TSCC cell lines [15]. Hence, miR-29b-induced demethylation of miR-195 promoter and its reexpression may account for the suppressive role of miR-29b during tumorigenesis of TSCC.

Actually, the mechanisms that control miRNAs expression are largely unknown. Recent studies have also shown that the promoters of certain miRNAs, including miR-143, miR-145, and miR-497, contain CpGs that are susceptible to DNA methylation [36]. Notably, miR-195 expression was also shown to be regulated by a common methylation mechanism of CpGs upstream of the miR-195 promoters in breast cancer [30]. However, since DNA methylation occurs in a tissue-specific or developmental stage-specific manner, whether CpGs methylation was responsible for the downregulation of miR-195 in TSCC is unclear. Here, we observed that demethylating agent treatment resulted in the demethylation of miR-195 promoter and corresponding increase in its expression, which were in accordance with the effects caused by miR-29b overexpression.

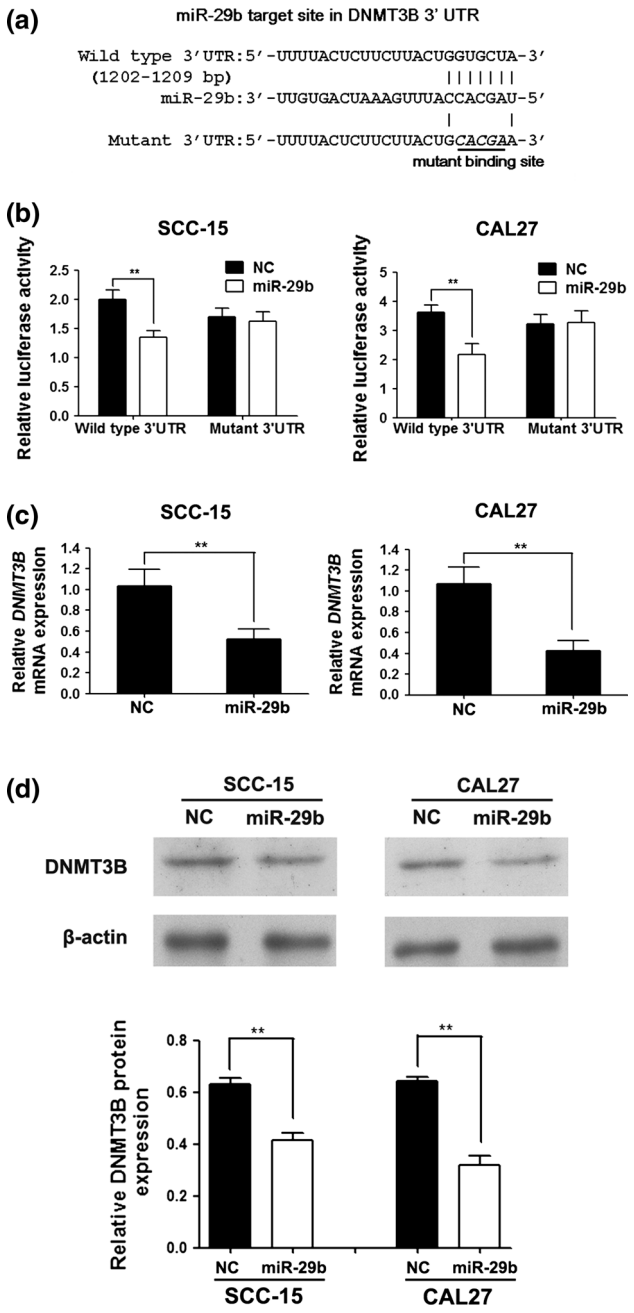

Fig. 4 Overexpression of miR-29b decreased DNMT3B expression by targeting its 3′-UTR. **a** The seed sequence of miR-29b (middle) matches the 3′-UTR of *DNMT3B* (top). Mutations were introduced into the 3′-UTR of *DNMT3B* (bottom). **b** miR-29b inhibited 3′-UTR luciferase reporter activity of wild-type, but not mutant *DNMT3B* in SCC-15 and CAL27 cells. **c** The overexpression of miR-29b decreased *DNMT3B* mRNA expression in CAL27 and SCC15. **d** DNMT3B protein levels were significantly decreased in CAL27 and SCC15 cells transfected with pcDNA3.1-miR-29b compared with those transfected with control vectors. Histogram shows the quantification of band intensities. All data are expressed as the mean ± standard deviation (SD). $^{**}P < 0.01$

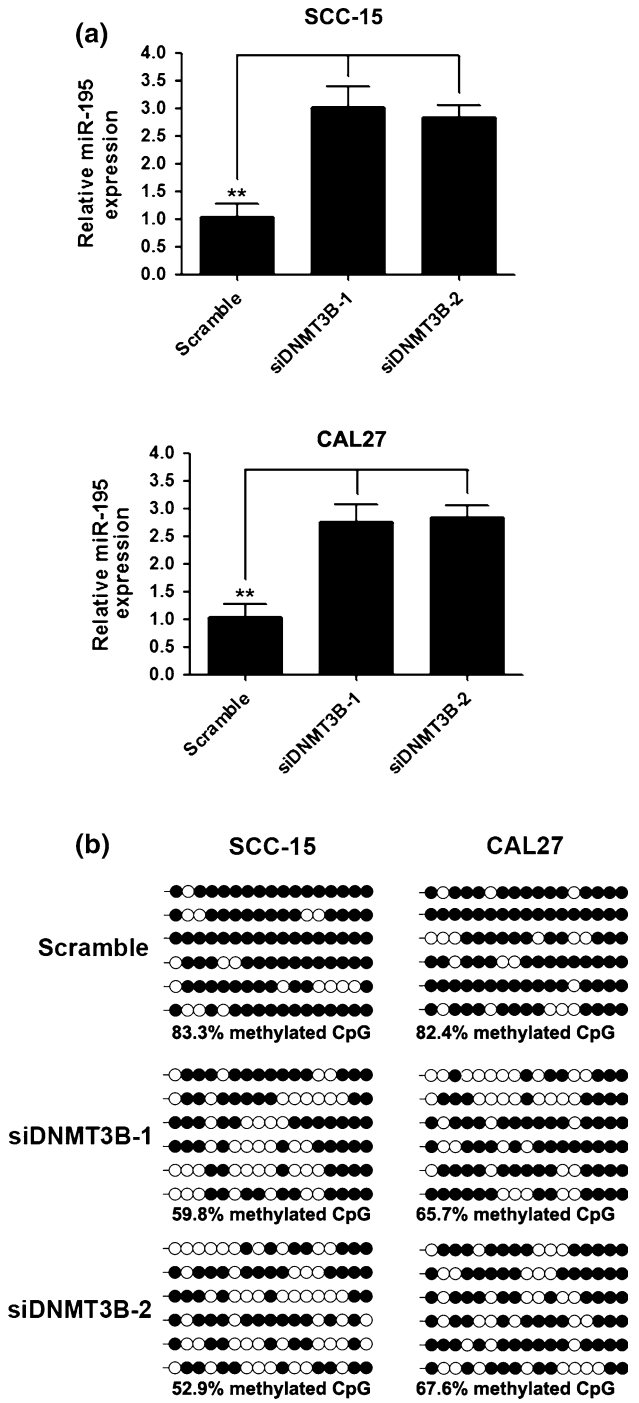

Fig. 5 DNA methylation is responsible for miR-195 downregulation. **a** The inhibition of DNMT3B induced miR-195 upregulation. All data are expressed as the mean ± SD. $^{**}P < 0.01$. **b** Bisulfite sequencing results are represented by lollipop diagrams. SCC-15 and CAL-27 cells were transfected with siRNA control, siDNMT3B-1 or siDNMT3B-2, as indicated. Filled circles indicate methylated CpG sites, while open circles indicate unmethylated CpG sites. Horizontal rows represent individual clones, and vertical columns show the position of each CpG. The percentage of methylated sites is indicated below the sequence data

Generally, a certain miRNA would act as a modulator of other miRNAs biogenesis via direct or indirect pathways [37, 38]. For example, miR-107 binds directly to let-7, forming a hybrid, through unusual patterns of miRNA complementarity and affecting let-7 stability [39], while miR-26a indirectly enhances let-7 expression by targeting the repressors of its maturation [40]. Regarding miRNAs which are susceptible to DNA methylation, the miRNA-induced DNA methylation revealed in this study may serve as a conserved mechanism for the regulation of them.

We also confirmed the role of miR-29b in the regulation of DNMT3B in TSCC cell lines in the current study, consistent with the previous study [20–23]. Various miRNAs participate in human cancers by regulating DNA methylation via DNMTs, resulting in the silencing or activation of many methylation-sensitive tumor suppressor genes. Specifically, DNMT3B, which is responsible for establishing de novo DNA methylation, is regulated through multiple mechanisms by miRNAs apart from miR-29b [31, 41]. miR-29b also targets the 3′-untranslated region of the Sp1 transcript, which is a transactivator of the *DNMT1* and *DNMT3A* genes [14, 42, 43]. Therefore, although miR-29b was shown to directly target DNMT3B in the present study, our analysis does not exclude the possibility that other indirect regulatory mechanisms are also involved in regulating DNA methylation of miR-195 promoter region. Moreover, miR-29b also targets *DNMT3A* and reduces global DNA methylation [20–24]. However, in the present study we only explored whether DNMT3B mediates the miR-29b-induced upregulation of miR-195 due to the reason that we previously observed that DNMT3B is upregulated in TSCC [31]. Since DNMT3A is also targeted by miR-29b, future studies are needed to explore whether DNMT3A is also involved in the miR-29b-induced upregulation of miR-195.

In conclusion, our results demonstrated that downregulation of DNMT3B by miR-29b resulted in upregulation of miR-195, which acts as a mechanism partially accounting for miR-29b's function in TSCC. These results extend our understanding of the miRNA-mediated tumor suppression, providing implications to develop novel strategies for cancer therapy via targeting the miRNA biogenesis pathway.

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Conflict of interest The authors declare that they have no conflict of interest.

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