Original Paper

miR-10b Downregulated by DNA Methylation Acts as a Tumor Suppressor in HPV-Positive Cervical Cancer via Targeting Tiam1

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Key Words

miR-10b • Methylation • HPV • Cervical cancer • Tiam1

Abstract

Background/Aims: microRNAs (miRNAs) are known to act as oncogenes or tumor suppressors in diverse cancers. Although miR-10b is an oncogene implicated in many tumors, its role in cervical cancer (CC) remains largely unclear. Here, we investigated the function and underlying mechanisms of miR-10b in human CC. **Methods:** Quantitative RT-PCR was used to measure miR-10b expression in CC and normal tissues, and its association with clinicopathologic features was analyzed. Methylation of CpG sites in the miR-10b promoter was analyzed by methylation sequencing. Cell proliferation, apoptosis, migration, and invasion assays were used to elucidate the biological effects of miR-10b and expression of the target gene was assayed with Western blot. **Results:** miR-10b was downregulated in CC tissues compared with normal tissues, and less miR-10b expression was associated with larger tumors, vascular invasion and HPV-type 16 positivity. miR-10b expression decreased in HeLa (HPV18-positive) and SiHa (HPV16-positive) cells compared with C-33A (HPV-negative), but increased after treatment with 5-Aza-CdR. Methylation ratio of site -797 in the miR-10b promoter in C-33A was lower than that in HeLa and SiHa. Further analysis indicates that site -797 is located within a transcription factor AP-2A (TFAP2A) binding element. Functionally, overexpression of miR-10b in HeLa and SiHa suppressed cell proliferation, migration and invasion, and induced apoptosis and miR-10b downregulation had opposite effects. Mechanistically, T-cell lymphoma invasion and metastasis 1 (Tiam1) was identified as a direct and functional target of miR-10b. **Conclusion:** miR-10b acts as a tumor suppressor in CC by suppressing oncogenic Tiam1, and its expression may be downregulated through methylation of TFAP2A binding element by HPV.

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Introduction

Cervical cancer (CC) is the second most common malignant tumor in women [1, 2] and data show that persistent infection of high-risk human papillomavirus (HR-HPV) is associated with cervical carcinogenesis. HPV is thought to be necessary but not sufficient for developing CC [3, 4]. Like other tumor types, CC is metastatic and potentially lethal. Many CC cases are diagnosed at late stages, which worsens patient prognosis [5]. Thus, we need better biomarkers for earlier CC diagnosis and individualized therapies.

MicroRNAs (miRNAs), typically 18-25 nucleotides long, are small non-coding small RNAs highly conserved and endogenously expressed across many species. Most mature miRNAs suppress gene expression either by cleaving their target messenger RNAs (mRNAs) or by inhibiting their translation upon imperfect base pairing to the 3'-untranslated regions (UTRs) of mRNAs [6]. miRNAs can be beneficial or detrimental to developing tumors, serving as tumor suppressors or initiators [7]. Although the full extent of miRNA biological functions have not been elucidated, they may be intrinsic regulators of many biological processes, including cell proliferation, differentiation, apoptosis, invasion and metastasis. Among these miRNAs, miR-10b may be best described as it influences invasion and metastasis of cancers by targeting multiple genes. miR-10b is overexpressed in hepatocellular carcinoma and promotes cell proliferation, migration and invasion through RhoC, uPAR and MMPs [8]. miR-10b induces invasion and metastasis of breast cancer cells [9], which is also found to be associated with tumor invasiveness in gastric [10], bladder [11], pancreatic cancers [12] and nasopharyngeal carcinoma [13]. miR-10b is reported to be significantly downregulated in advanced versus early stage small-cell carcinoma of the cervix [14]. Our previous study showed that miR-10b expression in HPV-positive HeLa and CaSki cells was significantly lower than that in HPV-negative C-33A cells [15]. However, aberrant expression of miR-10b and its role and underlying mechanism in CC is not clear.

Here, we measured miR-10b expression in CC tissues and cell lines and miR-10b was downregulated in cancerous tissues. Less miR-10b expression was associated with larger tumors, vascular invasion and HPV type-16 positivity. Methylation analysis of the miR-10b promoter indicated that its expression was regulated by methylation of correlated CpG sites to some extent. In addition, miR-10b overexpression inhibited proliferation, migration and invasion of CC cells and promoted apoptosis; miR-10b downregulation had opposite effects. Our results suggest that miR-10b may act as a suppressor by targeting T-lymphoma invasion and metastasis 1 (Tiam1) in CC and it may be a potential therapeutic target for diagnosis and treatment of CC.

Materials and Methods

Ethics statement

This study was approved by the medical ethics committee of the Third Affiliated Hospital of Soochow University, and all patients gave written informed consent before enrolment.

Tissue sample collection

A total of 70 cervical tissues were collected from the Gynecology Department of the Third Affiliated Hospital of Soochow University between July 2014 and September 2015. Patients received no preoperative radiotherapy and/or chemotherapy. All patients were staged according to the International Federation of Gynecology and Obstetrics (FIGO) staging criteria for CC. After sample excision, samples were frozen in liquid nitrogen and stored at -80 °C until use.

HPV-DNA detection and genotyping

All CC tissues were assayed with a HPV subtype test. DNA extraction was performed using Ezup Column Animal Genomic DNA Purification Kit (Sangon Biotech, China) according to the manufacturer's instructions. Genotyping of 27 HPV types included 19 high-risk types and 8 low-risk types was carried out

using Nucleic Acid genotyping Kit for Human Papillomavirus (Tellgen, China) based on high throughput luminex 200 technology.

Cell lines and cell culture

Human C-33A (HPV-negative), HeLa (HPV18-positive) and SiHa (HPV16-positive) CC cell lines were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Minimum Essential Medium (MEM) containing 10% fetal bovine serum (FBS) (Gibco, Life Technologies, Australia), 10% non-essential amino acids solution (NEAA), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco, Life Technologies, USA), and incubated at 37 °C in a humidified atmosphere with 5% CO₂.

Lentivirus infection and siRNA interference

Recombined LV-GV369-miR-10b-EGFP and LV-GV280-miR-10b-inhibition-EGFP, and their respective negative control vectors were synthesized by GeneChem Co, Ltd (Shanghai, China). Lentiviral vectors were transduced into HeLa and SiHa cells according to the manufacturer's instructions. Cells were seeded (2×10⁵ cells/ml) in 6-well plates and incubated for 24 h to reach 50% confluence, and then replaced with infection medium containing lentiviral vectors at a multiplicity of infection (MOI) of 20 plaque-forming units/cell. Successfully infected cells were GFP positive and observed under a fluorescent microscope after 72 h, and efficiency of miR-10b overexpression and inhibition was evaluated using quantitative real-time PCR (qPCR).

To inhibit Tiam1 expression, a small-interference RNA (siRNA) sequence (5'-GCGA AGGAGCAGGUUUUCUTT-3') of the human Tiam1 was synthesized by GenePharma (Shanghai, China), and a scramble sequence (5'-UUCUCCGAACGUGUCACGUTT-3') was synthesized as a negative control. HeLa and SiHa cells from miR-10b inhibition group were seeded (2×10⁵ cells/ml) in 6-well plates and transfected with siRNA oligo using siRNA-Mate reagent (GenePharma, China) 24 h later. Cells were harvested for protein extraction 48 h post-transfection, and the efficiency of Tiam1 knockdown was quantified using Western blot.

5-Aza-CdR treatment

5-Aza-CdR (Sigma-Aldrich, St Louis, MO, USA) was dissolved in water to a final concentration of 0.25 mg/ml and stored in aliquots at -20 °C. Cells were plated in 6-well plates (2×10⁵ cells/ml) and treated 24 h later with 5-Aza-CdR (5, 10 and 20 µM) for 72 h. Culture medium was changed daily for control and treated cells under the same conditions to maintain the drug stability during treatment. Three days later, cells from each group were harvested for miRNA isolation and qPCR analysis.

Methylation analysis

Genomic DNA of the human C-33A, HeLa, SiHa CC cell lines and CC tissues (5 cases for HPV16-positive and 6 cases for HPV18-positive) were extracted with Ezup Column Animal Genomic DNA Purification Kit (Sangon Biotech, China). DNA amounts of 2 µg in a volume of up to 20 µl was modified with bisulfite and purified using the Epitect Bisulfite Kit (Qiagen, Germany) according to the manufacturer's instructions. Bisulfite sequencing PCR (BSP) was used to analyze methylation status in the 3123 bp region upstream of the miR-10b promoter. Sequences of methylation primers are presented in Table 1. PCR products were gel extracted to confirm that a single band had been obtained and were then sequenced by Sangon Biotech (Shanghai, China).

Table 1. Sequences of the primers used for methylation analysis

Name	Primers	Sequences (5'→3')	Product length
Primer 1	Fp 1	TTTAGGGTGGGAGAGATAGGAAGTAG	346 bp
	Rp 1	ACCCAAACCTCCCTACTTCAATAC	
Primer 2	Fp 2	GTGGTTTGTGTTTGTGAGGATG	335 bp
	Rp 2	AAAAAACAACCTTCTCTATCC	
Primer 3	Fp 3	GAAGTGGTGGGTAAAGGGGT	306 bp
	Rp 3	ACAAACTCTTAACAAAAATAAACACCTT	
Primer 4	Fp 4	GGTTTTTTTGTGTTTGTATAGGTGTAG	324 bp
	Rp 4	ATCCCAACCCCAACCCAC	
Primer 5	Fp 5	TGGGTGTGTTGAGTTGTTTGT	388 bp
	Rp 5	TCTATAACTCTCCCTCAATACCAT	
Primer 6	Fp 6	TTGGATTTTTTGTAGTTGTTGTG	357 bp
	Rp 6	ACCCCTTCACTTAAACCAACCT	
Primer 7	Fp 7	TTGGAAAAGTTGGGGTTGGT	338 bp
	Rp 7	TTCACTCCCACTCAACTACCC	
Primer 8	Fp 8	TGGTTTTGGGTGTTTATTTTGTAG	351 bp
	Rp 8	TTCCCTAAATACCAAAACCTCC	
Primer 9	Fp 9	TTGGGAGTTTTTGGTATTTAGGG	339 bp
	Rp 9	TTCAAAACACAAAAATACCCCTAATC	
Primer 10	Fp 10	TATGTGGGAGGTAGGAAGGTAAT	320 bp
	Rp 10	ACTAATAATAAAAAACAAAAAATCAT	
Primer 11	Fp 11	GAATTTTTGTATTTTGTGTGTTGTG	353 bp
	Rp 11	CCAAAAAACAACAAATACCTCAAC	
Primer 12	Fp 12	GGTAGTAGGAAAAGGAGTAGATATAGGA	312 bp
	Rp 12	AAACAATTCAAATAACCTAATAAACTT	
Primer 13	Fp 13	TTGATTTTGGTAGAAGATGAGGG	271 bp
	Rp 13	TTTTTACATCAACCATATATCCCCT	

RNA isolation and qPCR

miRNA was isolated from CC tissues and cells using miRcute miRNA Isolation Kit (Tiangen, China), whereas total RNA was extracted by the Total RNA Purification Kit (Biocolor, China). Concentration and purity of RNA was spectrophotometrically assessed, measuring absorbance at 260/280 nm by BioPhotometer (Eppendorf, Germany). For the detection of mature miR-10b, RNA was reverse-transcribed by miRcute miRNA first-strand cDNA synthesis kit (Tiangen). cDNA from total RNA was synthesized by the RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, Lithuania). Expression of miR-10b was quantified by qPCR using miRcute miRNA qPCR detection kit (Tiangen), while the quantification of Tiam1 mRNA was performed using Immolase™ DNA Polymerase (Bioline, USA) and analyzed by LightCycler 480 II PCR system (Roche, Germany). qPCR results were analyzed using the comparative threshold cycle method ($2^{-\Delta C_t}$), with U6 small nuclear RNA (U6 snRNA) or GAPDH as an endogenous reference gene for quantification and normalization. Sequences of primers and probes are presented in Table 2.

Table 2. Sequences of primers and probes

Name	Primer/Probe	Sequences (5'→3')
hsa-miR-10b U6 snRNA	Forward primer	GGATACCCTGTAGAACCAGAA
	Forward primer	CTCGCTTCGGCAGCACA
	Forward primer	TTCCCTGGGTGATGCCTTC
Tiam1	Reverse primer	CTTCCTTGGTGGTGCCTC
	Probe	FAM-TTTTTCAGACCACTAGCCAGACGGAGC-BHQ1
	Forward primer	CAGGGCTGCTTTAACTCTGGT
GAPDH	Reverse primer	CATGGGTGAATCATATTGGAAC
	Probe	CY5-TGGATATTGTCATCAATGACCCT-BHQ2

Cell proliferation assay

Cell proliferation was measured using a Cell Counting Kit-8 (CCK-8) (Dojindo, Japan) according to the manufacturer's instructions. Briefly, cells were seeded into a 96-well plate (5×10^3 cells/well), with six replicate wells per group. CCK-8 was added to wells at 24, 48 and 72 h post-infection (10 μ l/well), and examined 1 h later. Optical density (OD) values were measured at 450 nm to estimate viable cells. Data represent three independent experiments in triplicate.

Apoptosis assay

Cells from each group were harvested at 72 h post-transfection by trypsinization. Cells pellets were resuspended at a density of 1×10^6 cells/ml in $1 \times$ binding buffer solution. After double staining with Annexin V/Alexa Fluor 647 and propidium iodide (PI) using the Annexin V-Alexa Fluor 647 Apoptosis Detection Kit (Fcmacs, China) for 15 min at room temperature, samples were analyzed on a FACScan flow cytometer equipped with Cell Quest software (BD Biosciences). Annexin V- and PI- cells were used as controls, and Annexin V+ and PI+ cells were designated as necrotic.

Wound healing assay

After 72 h of lentivirus infection, infected cells were harvested and subjected to a wound healing assay. Cells from each group were seeded in 6-well plates at 5×10^5 cells/ml and cultured until confluent. Then, cells were washed twice with PBS and replaced with culture medium containing 5% FBS for 24 h. Scratch wounds were created across the center of each well using a sterile 200 μ l pipette tip, and any non-adherent cells were washed off. Wound images were captured with an inverted microscope (Olympus CKX41, Japan) and a digital camera (Olympus, Japan) at 0, 24 and 48 h after wounding.

Invasion assay

Cell density was adjusted to 4×10^5 cells/ml at 72 h post-infection. Before seeding, 24-well Transwell chambers with 8 μ m pores (Corning, USA) were coated with Matrigel (BD Biosciences, San Jose, CA). The upper chamber was loaded with 150 μ l of cell suspension and the lower chamber was supplied with 800 μ l of the culture medium containing 10% FBS. After 48 h of incubation, medium was removed from the upper chamber and cells were scraped off with a cotton swab. Cells that invaded to the other side of the membrane were fixed with methanol, followed by staining with 0.1% crystal violet (Sigma-Aldrich, USA). Invaded cells were counted under an inverted microscope in at least three randomly selected fields.

Tumor formation in nude mice

For establishing stable miR-10b overexpressing cells, SiHa cells were infected with LV-GV369-miR-10b-EGFP lentiviral vectors (Genechem, China) and were selected in cell culture medium containing 4 μ g/ml puromycin for 48 h. Four week-old female BALB/c nude mice (Slac, China) were injected subcutaneously

in the right armpit with 5×10^6 cells. Tumors were measured two or three times each week (1, 3, 5, 7, 10, 12, 14, 17 and 18 days) and tumor volumes (TV) were calculated using the formula: $\pi/6 \times \text{length} \times \text{width}^2$. All mice were sacrificed after the last measurement, and tumors were separated, weighed and immediately frozen in liquid nitrogen for further Western blot analysis. To correct TV and tumor weight (TW), ratios of TV to body weight (BW) and TW to BW of each mouse were calculated respectively. All animal procedures were conducted in accordance with the approved guidelines.

Luciferase reporter assay

The binding sites between miR-10b and the 3'UTR region of Tiam1 were predicted by searching in TargetScan (<http://www.targetscan.org/>). For luciferase reporter assay, the wild type (WT) 3'UTR of Tiam1 and mutant type (Mut) including putative miR-10b binding sites were amplified by PCR and cloned into GV272 vector (Genechem, China). WT and Mut inserts were further confirmed by sequencing. 293T cells were (1×10^5 cells/well) were seeded into a 24-well plate and cotransfected with 0.1 μg of the firefly luciferase reporter construct, 0.02 μg of Renilla luciferase construct (for normalization) and 0.4 μg of miRNA expression plasmid. At 48h after transfection, cells were lysed to measure the luciferase activity of the lysates using the Dual-Luciferase Reporter Assay System (Promega, Inc., Madison, USA) according to the manufacturer's instructions. Firefly luciferase values were normalized to Renilla and the ratio of Firefly/Renilla values was reported.

Western blot

Experimental cells were harvested and washed twice with ice-cold PBS, and then lysed using a total protein extraction kit (Bestbio, China). Cervical tissues and nude mice tumors were also lysed and subjected to protein extraction as described above. Protein was measured using a BCA protein quantitative kit (Bestbio, China). Protein samples were mixed with 5 $\times$ SDS-PAGE loading buffer (Beyotime, China) and boiled for 5 min. A total of 50 μg of protein was separated using 8% SDS-PAGE, then transferred onto polyvinylidene fluoride (PVDF) membranes (Merck Millipore, Billerica, MA, USA). Subsequently, membranes were blocked in 3% BSA for 2 h at room temperature, and then incubated with anti-Tiam1 antibody (ab211518, Abcam) overnight at 4 °C. After washing three times with TBST, membranes were incubated for 2 h at room temperature with horseradish peroxidase-conjugated affinitypure anti-rabbit IgG (SA00001-2, Proteintech). Protein bands were visualized with a Pierce ECL Western Blotting Substrate kit (Thermo Scientific, Rockford, IL, USA) after washing with TBST. GAPDH or β -actin was used as the internal control to normalize protein loading.

Statistical analysis

Statistical testing was performed using GraphPad Prism 5.0 software (Inc, San Diego, CA). Data are presented as means $\pm$ SEM. A Student's *t* test (two-tailed) was used to determine statistical differences between two groups. One-way analysis of variance (ANOVA) followed by Dunn's multiple comparison test for multiple comparisons. Results were considered significant when *p*-values < 0.05.

Results

miR-10b was significantly downregulated in CC patients

miR-10b expression was measured in 51 CC cases and 19 normal controls using qRT-PCR and data showed that miR-10b in tumor tissues was significantly lower than in normal tissues ($p < 0.0001$; Fig. 1A), suggesting that miR-10b downregulation might be related to cervical carcinogenesis. The analysis between miR-10b expression and clinicopathological parameters confirmed that less expression of miR-10b in CC was associated with larger tumors, vascular invasion and HPV type-16 positivity ($p < 0.05$). There was no significant difference in age, pathological type, FIGO stage, tumor grade, lymph node metastasis or depth of invasion (Table 3).

Fig. 1. miR-10b expression in CC tissues and cell lines. (A) miR-10b expression in 51 cases CC and 19 normal controls was quantified with qRT-PCR, normalized to U6. Fold-change was calculated using $2^{-\Delta\Delta C_t}$. Relative miR-10b expression of normal controls was 1, and $p < 0.0001$ compared to controls. All data were compared using a Student's t test and are shown as means $\pm$ SEM. (B) miR-10b expression in three CC cell lines was measured using qRT-PCR. Asterisks indicate significant differences compared with C-33A. miR-10b expression increased in a dose-dependent manner after 72 h of 5-Aza-CdR exposure in C-33A (C), HeLa (D) and SiHa (E) cells. Asterisks indicate significant differences compared with controls. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data are means $\pm$ SEM.

Expression of miR-10b and methylation of its promoter region in CC cell lines

miR-10b expression was measured in C-33A, HeLa and SiHa cells and data showed that miR-10b expression in HPV-positive HeLa ($p < 0.01$) and SiHa ($p < 0.05$) cells was less than in HPV-negative C-33A cells (Fig. 1B). 5-Aza-CdR treatment reactivated expression of miR-10b in HeLa and SiHa cells (Fig. 1C-E). The PCR product amplified by 13 pairs of methylation primers contained 3123 bp region upstream of the miR-10b promoter. Methylation sequencing revealed that all 111 CpG sites in the promoter region of miR-10b were fully or partially methylated in three CC cell lines. Comparing methylation status of all CpG sites among three CC cell lines confirmed an inconsistent methylation status of site -797

Table 3. Association of miR-10b expression with clinicopathological parameters. SCC: squamous cell carcinoma; AC: adenocarcinoma; NA: not applicable; NS (No significance, $p > 0.05$); * Significant difference, $p < 0.05$

Cases (n=51)		Expression level of miR-10b Ratio of miR-10b/U6 [Median (P ₂₅ , P ₇₅)]	p-value*
Age (years)			
< 45	18 (35.29%)	0.0771 (0.0276, 0.4559)	0.9921 (NS)
≥ 45	33 (64.71%)	0.0925 (0.0347, 0.3259)	
Pathological type			
SCC	47 (92.16%)	0.0763 (0.0317, 0.4074)	0.3907 (NS)
AC	4 (7.84%)	0.2195 (0.0614, 0.3669)	
Tumor size			
< 4.0 cm	34 (66.67%)	0.1028 (0.0450, 0.4149)	0.0350 (*)
≥ 4.0 cm	17 (33.33%)	0.0347 (0.0163, 0.1921)	
FIGO stage			
I (Ib1/b2)	30 (58.82%)	0.0604 (0.0339, 0.2870)	0.5723 (NS)
II (IIa1/a2)	21 (41.18%)	0.1042 (0.0314, 0.4833)	
Tumor grade			
G1	0 (0.00%)	NA	0.0809 (NS)
G2	24 (47.06%)	0.0441 (0.0272, 0.2345)	
G3	27 (52.94%)	0.0940 (0.0429, 0.5539)	
Lymph node metastasis			
Absent	36 (70.59%)	0.0889 (0.0412, 0.4003)	0.3909 (NS)
Present	15 (29.41%)	0.0778 (0.0217, 0.3310)	
Vascular invasion			
Negative	32 (62.75%)	0.1082 (0.0424, 0.5434)	0.0380 (*)
Positive	19 (37.25%)	0.0763 (0.0257, 0.1042)	
Depth of invasion			
< 1/2 layer	12 (23.53%)	0.1074 (0.0349, 0.4297)	0.5268 (NS)
≥ 1/2 layer	39 (76.47%)	0.0763 (0.0317, 0.3310)	
HPV type 16			
Negative	13 (25.49%)	0.3310 (0.0427, 0.7871)	0.0283 (*)
Positive	38 (74.51%)	0.0604 (0.0279, 0.1749)	

in the miR-10b promoter, where methylation ratio of HeLa and SiHa were higher than HPV-negative cell, C-33A (Fig. 2). Further clone sequencing showed that methylation ratio of this site in C-33A, HeLa and SiHa was 10%, 30% and 100%, respectively (Table 4). Subsequent

Fig. 2. Schematic of miR-10b promoter location in the genome. Transcription start site of miR-10b is indicated as +1 and number indicates relative distance from miR-10b transcription start site. Pink sequence indicates bases from the -827 to -769 region upstream of the miR-10b transcription start site; blue sequence is after bisulfite treatment. Yellow box marks inconsistent methylation status of the CpG site -797 in the miR-10b promoter. Green box: TFAP2A binding element (5'-GCCCACTC-3') was noted at -805 to -797 region upstream of the miR-10b transcription start site.

sequencing results of CC tissues with HPV-16 or 18 positive indicated that site -797 was methylated in varying degrees. As shown in Fig. 2, a TFAP2A binding element (5'-GCCCACTC-3') predicted online (<http://jaspar.genereg.net>) was found to be existing at the -805 to -797 region upstream of the miR-10b transcription start site.

Table 4. Methylation status of site -797 in the miR-10b promoter. Methylation status was analyzed by clone sequencing and ten clones were selected for each cell line

Cell line	No. of methylated sites	No. of unmethylated sites	Methylation ratio (%)
C-33A	1	9	10
HeLa	3	7	30
SiHa	10	0	100

miR-10b inhibits HPV-positive CC cells proliferation in vitro and in vivo

The biological role of miR-10b in CC was studied in HeLa and SiHa cells with ectopically expressed and silenced miR-10b. Transfection was efficient at (>90%) 72 h according to EGFP expression data (Fig. 3A-D). qRT-PCR to measure expression of miR-10b in cells showed that miR-10b in overexpressing HeLa and SiHa cells was greater than in negative controls and in silenced cells, expression was less than controls.

To investigate whether miR-10b expression affected cell proliferation and tumor formation, CCK-8 assays were used and data showed that miR-10b overexpression inhibited HeLa (Fig. 3E) and SiHa growth (Fig. 3F) but miR-10b downregulation had opposite effects. In nude mice models we investigated whether miR-10b inhibited CC tumor formation and after injection of SiHa cells, tumors were observed in all animals, suggesting that overexpression of miR-10b decreased the ratio of TV to WB, especially on day 14 (Fig. 3G). After 18 days, mice were sacrificed and tumors were excised and weighed. Data showed that tumors were smaller after miR-10b overexpression but these differences were not statistically significant (Fig. 3H and I). So, miR-10b inhibited CC cell proliferation and tumor formation.

miR-10b promoted HeLa and SiHa apoptosis

Flow cytometry was used to measure apoptosis after successful overexpression of miR-10b and inhibition. Overexpression of miR-10b increased apoptosis compared with negative controls in HeLa and SiHa cells, whereas miR-10b knockdown decreased apoptosis (Fig. 4A-D).

Fig. 3. miR-10b inhibits HPV-positive CC cells proliferation in vitro and in vivo. (A) Fluorescence micrographs of HeLa cells and SiHa cells (C) were taken 72 h after infection. HeLa, Scale bar = 100 μ m. SiHa, Scale bar = 200 μ m. Infection efficiency of miR-10b was determined by qRT-PCR in HeLa (B) and SiHa (D) cells, and was normalized to U6. Up: overexpression of miR-10b; Up-NC: negative control for overexpression of miR-10b; Down: downregulation of miR-10b; Down-NC: negative control for downregulation of miR-10b. Asterisks indicate significant differences compared with respective negative control. (E) Effect of miR-10b on HPV-18 positive HeLa cell proliferation. (F) Effect of miR-10b on HPV-16 positive SiHa cell proliferation. (G) Effect of miR-10b on tumor volume during the observational period. TV (tumor volume), BW (body weight). (H) Effect of miR-10b on tumor weight (TW). (I) Representative photos of the mice and gross morphology of the tumors. 1-8 represents the negative control (NC) group and 9-16 represents the miR-10b overexpression (miR-10b-Up) group. Data are means $\pm$ SEM. Student's t test was used to analyze significant differences, * $p < 0.05$, ** $p < 0.01$ versus respective negative control.

miR-10b inhibited migration and invasion of HeLa and SiHa cells

CC metastasis and vascular invasion are key to patient survival, so a wound healing and a Transwell invasion assay was used to assess miR-10b on the regulation of cell migration and invasion. Fig. 4E-L showed that miR-10b overexpression inhibited migration (Fig. 4E-H) and invasion (Fig. 4I-L) of both cell lines compared with negative controls and migration and invasion increased after transfection with miR-10b inhibition lentivirus compared to controls.

Fig. 4. miR-10b promotes apoptosis and inhibits migration and invasion of HeLa and SiHa cells. (A, B) HeLa and (C, D) SiHa apoptosis was measured using flow cytometry. Q1 represents necrotic cells, Q2 represents late apoptosis, Q3 represents early apoptosis and Q4 represents viable cells. Apoptosis is designated Q2+Q4. (E, F) HeLa and (G, H) SiHa migration was measured using a wound-healing assay. Wound images were captured using a digital camera and an inverted microscope at 0, 24 and 48 h after wounding. (I, J) HeLa and (K, L) SiHa invasion was measured with Transwell assays. Cells that invaded were stained with crystal violet and imaged and counted under a microscope. Scale bar = 100 μ m. Data are means $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus respective negative control.

Tiam1 is a direct and functional target of miR-10b in CC

Our data suggested that miR-10b might be a tumor suppressor in CC by inhibiting proliferation, migration and invasion, and promoting apoptosis of HeLa and SiHa cells. Bioinformatics analysis by TargetScan showed that one of the potential targets of miR-10b was Tiam1, which had a possible 3'UTR putative binding site with miR-10b. To validate this prediction, we cloned a sequence including the predicted Tiam1 3'UTR target site or a mutated sequence with 100 bp upstream and downstream of the binding elements for miR-10b into the luciferase reporter constructs (Fig. 5A). For the luciferase reporter assays, the luciferase reporter constructs were co-transfected with miR-10b or its negative control (miR-NC) into 293T cells. The results indicated that expression of miR-10b only significantly decreased the relative luciferase activity of reporter gene with the wild-type (WT), but not mutant Tiam1 3'UTR (Mut), compared with miR-NC control (Fig. 5B). Subsequently, Tiam1 mRNA and protein were measured in cervical tissue, CC cell lines and tumors in nude mice. We found that Tiam1 mRNA and protein were increased in tumor tissues compared with normal controls (Fig. 5C and D). Moreover, overexpression of miR-10b significantly decreased Tiam1 protein in tumors in nude mice (Fig. 5E).

In HeLa and SiHa cells, qRT-PCR and Western blot showed that miR-10b overexpression induced a significant decrease of Tiam1 mRNA and protein, whereas miR-10b downregulation increased Tiam1 expression (Fig. 6A-D). Thus, we hypothesized that miR-10b might regulate the biological role through targeting Tiam1 in HPV-positive CC cells. Fig. 6E and F showed that expression of Tiam1 was successfully knocked down cell lines relative to control siRNA, which was confirmed by Western blot ($p < 0.01$). CCK-8 assays confirmed that miR-10b downregulation promoted proliferation of HeLa and SiHa cells and knockdown of Tiam1 attenuated this increased proliferation compared with negative control (Fig. 6G and H). Thus, Tiam1 as an oncogene is a direct and functional target of miR-10b in CC.

Fig. 5. miR-10b could bind to the 3'UTR of Tiam1 increasing in CC tissues. (A) Binding sites of miR-10b in the Tiam1 3'UTR predicted by TargetScan. WT: wild type; Mut: mutated type. The mutant nucleotides are underlined in red. (B) Luciferase activity in 293T cells co-transfected with miR-10b (or miR-NC) and Tiam1 3'UTR Reporter (WT or Mut). Luciferase activity was detected at 48 hours after transfection and normalized to Rellina. ** $p < 0.01$. (C) Tiam1 mRNA levels in 51 cases CC and 19 normal controls were quantified with qRT-PCR. * $p < 0.05$. (D) Tiam1 protein levels were analyzed using Western blot in CC tissues (C1–C4) and normal cervical tissues (N1–N4). β -actin was a loading control. * $p < 0.05$. (E) Tiam1 protein levels were analyzed using Western blot in tumors in nude mice. 1–8 represents the NC group and 9–16 represents the miR-10b-Up group. GAPDH was a loading control. ** $p < 0.01$.

Discussion

miRNAs have been implicated in the development and progression of various tumors including human CC, which is one of the most common malignant tumors. Although several studies suggest that miR-10b acts as either oncogene or tumor suppressor in tumorigenesis, how it contributes to CC remain largely unclear. Zou et al. [16] reported that miR-10b was significantly downregulated during cervical cancer progression, but the mechanism of downregulation has not been clarified. In this study, the results also demonstrated that miR-10b was significantly downregulated in CC tissues compared with the controls. Our data further indicated that less expression of miR-10b was associated with HPV positivity (Table 3). Moreover, miR-10b expression was downregulated in HeLa and SiHa compared with C-33A cells which are HPV-negative. In consideration of relation between HPV and CC, we speculated that downregulation of miR-10b might be implicated with HPV. In order to verify this assumption, 5-Aza-CdR, a demethylating agent, was used to treat three CC cell lines. The results indicated that demethylation could significantly increase miR-10b expression in HeLa and SiHa cells with HPV-positive, but not in C-33A cells with HPV-negative, which suggesting HPV might decrease miR-10b expression via methylation of a CpG island in its promoter. Following, we performed methylation analysis of the miR-10b promoter and found methylation ratio of site -797 in the miR-10b promoter in C-33A was lower than that in HeLa and SiHa (Table 4). This result suggested that site -797

Fig. 6. Validation of Tiam1 as a direct and functional target of miR-10b. (A, B) Tiam1 mRNA was quantified by qRT-PCR in HeLa and SiHa cells. (C, D) Tiam1 protein was assayed with Western blot in HeLa and SiHa cells. (E, F) Quantification of Tiam1 protein expression with Western blot presented as a ratio relative to expression of NC in HeLa and SiHa cells transfected with NC or Tiam1 siRNAs. GAPDH was a loading control. (G, H) CCK-8 proliferation data at 0, 24, 48 and 72 h post-transfection of HeLa and SiHa cells from the miR-10b inhibition group. Data are means $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ versus respective negative control.

Fig. 7. Diagram of signaling pathway involved in miR-10b-mediated CC progression.

might be a key CpG dinucleotide and its methylation induced by HPV could influence miR-10b expression. Subsequently, as shown in Fig. 2, we found this specific CpG site was just located within a TFAP2A binding element (5'-GCCCCACTC-3') at position -805 relative to the transcription start site of miR-10b, and this element was ever named AP-2 like element shared by the mouse and human perforin genes [17]. According to reports, mutations within the AP-2 binding site could result in a 4- to 6-fold reduction in cAMP and TPA induced transcription and caused a several-fold reduction in basal transcription [18, 19]. Moreover, DNA methylation within the AP-2 binding site caused similar inhibitory effects on *in vivo* activity of human proenkephalin cAMP and phorbol ester inducible enhancer [19, 20]. Jimenez-Wences's group [21] reported a relationship among HPV infection, cellular DNA methylation, and miRNA expression. E6 and E7 oncoproteins of HR-HPV increased expression and activity of DNA methyltransferase 1 (DNMT1), which could catalyze aberrant methylation of miRNA genes. Therefore, as shown in Fig. 7, we hypothesized that E6 and E7 oncoproteins of HR-HPV may increase expression and activity of DNMT1, which could catalyze methylation of CpG site of TFAP2A binding element in the miR-10b promoter. TFAP2A could not bind to the methylated element, which inhibited transcription of the miR-10b gene. The details of this mechanism remain to be explored.

Table 3 showed that less expression of miR-10b was associated with larger tumors and vascular invasion, which implied miR-10b was involved in growth and invasion of CC cells. However, this study also presented contradict results that higher miR-10b expression was associated with lower differentiation (G3), although the difference was not significant. The possible reason is that miR-10b might have feedback regulation mechanisms. According to previous report, when responding to stress, cells either restore homeostasis or enforce a program to adapt to the new environment until conditions are again favorable. This decision is partially mediated by miRNA functions, specifically modulation of miRNAs biogenesis, expression of mRNA targets, and miRNA-protein complex activity [22]. DNA methylation, a mechanism modifying gene expression, is mediated in part by the enzyme DNA methyltransferase. Yang J et al. demonstrated that the inhibition of DNA methylation caused an increase in the DNA methyltransferase activity and a corresponding mRNA transcription [23]. It suggested that DNA methyltransferase was itself regulated in part by DNA methylation status, possibly representing a feedback mechanism. Slack A et al. also found that DNA methylation was an attractive system for feedback regulation of DNA methyltransferase [24]. Thus, we proposed the hypothesis that the activity of DNA methyltransferase in low differentiated tissues was decreased via negative feedback regulation, which led to an increase of miR-10b expression to restore homeostasis of CC cells and prevent growth and invasion of tumor.

In order to examine whether miR-10b influence the biological function of CC cells, we carried out a series of experiments including cell proliferation, apoptosis, wound healing and invasion assay. The results demonstrated that miR-10b overexpression significantly inhibited CC proliferation, migration and invasion of HPV-positive CC cells and enhanced apoptosis. Conversely, miR-10b downregulation had opposite effects. Furthermore, Fig. 3G showed that overexpression of miR-10b decreased the ratio of TV to BW. Thus, miR-10b might act as a tumor suppressor gene whose downregulation contributes to the progression of CC.

To clarify mechanisms underlying the effects of miR-10b on CC, bioinformatics software was used to analyze and predict possible target genes of miR-10b. Our previous study [15] presented some genes including HOXD10, KLF4, HOXA1, Tiam1, MMP9 and etc. Zou et al. [16] reported that miR-10b inhibited the cell proliferation and invasion through targeting HOXA1. In this study, dual-luciferase reporter assays identified Tiam1 as a critical downstream target of miR-10b. Tiam1, a member of the Dbl family of guanine nucleotide exchange factors (GEFs) that regulate small G proteins of the Rho family, was first identified in 1994 by proviral tagging in combination with *in vitro* selection for invasiveness from murine lymphoma cells [25, 26]. According to the literature, Tiam1 was involved in regulation of Rac1-mediated cellular processes including cytoskeletal activities, cell polarity, endocytosis and membrane trafficking, cell growth and survival, cell adhesion, migration, invasion and metastasis of various cancers, such as breast cancer [27], gastric cancer [28, 29], hepatocellular carcinoma [30] and colorectal cancer [31], and so on. In addition to its role as a GEF, Tiam1 can also participate in the regulation of apoptosis, suggesting that Tiam1 plays an important role in tumorigenesis and carcinoma progression [32]. Our study indicated that Tiam1 was upregulated in CC compared with normal tissues, and exogenous miR-10b could downregulate the expression of Tiam1 mRNA and protein (Fig. 6). Moreover, we found that knockdown of Tiam1 attenuated cell proliferation induced by miR-10b downregulation. These results suggest that Tiam1 as an oncogene is a direct and functional target of miR-10b in CC. However, the downstream mechanism of miR-10b targeting Tiam1 remains to be explored, and we hypothesize that abundant protein interaction domains of Tiam1 allow it to affect diverse cellular processes by interactions with different signaling proteins. Moreover, the ability of miR-10b to target Tiam1 and its influence on the function of Tiam1 might depend on multiple factors, such as cell type, miR-10b expression pattern and signals regulating Tiam1 expression and function (Fig. 7).

Conclusion

HPV may decrease miR-10b expression via TFAP2A influenced by methylation of site -797 in miR-10b promoter. Meanwhile, miR-10b acts as a tumor suppressor in CC by suppressing oncogenic Tiam1 at the post-transcriptional level. Thus, miR-10b could be served as a potential molecular biomarker for assessing CC risk and it might be a therapeutic target for diagnosis and treatment of CC.

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Disclosure Statement

The authors declare that they have no competing interests.

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