

Original Article

MicroRNA-29b regulates osteopontin dependent proliferation function and cell cycle in esophageal cancer lines

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Abstract: Bioinformatics analysis showed that osteopontin could be important target genes of miR-29b. The expression of miR-29b was down regulated in esophageal cancer tissues and esophageal cancer cell lines compared to the normal tissues and normal esophageal cell line respectively. MTT assay, colony formation assay and cell cycle analysis were performed to assess the effect of miR-29b on biological behavior of esophageal cancer cells. The gain-of-function studies that raised miR-29b expression showed reductions in cell proliferation, arrest of cell cycle in G0/G1 phase. Silencing target gene expression was used to investigate whether the effect of downregulation of target protein has the similar effect as miR-29b overexpression in esophageal cancer cell. Furthermore, the dual-luciferase reporter assay demonstrated that miR-29b inhibited the expression of the luciferase gene containing the 3'-UTRs of osteopontin mRNA. Western blotting and Q-PCR indicated that miR-29b down-regulated the expression of osteopontin at the protein and mRNA levels. Taken together, our results demonstrate that miR-29b serves as a tumor proliferation suppressor, which suppresses esophageal cancer cell proliferation and arrest cell cycle by directly inhibiting osteopontin expression. The results show that miR-29b may be a novel therapeutic candidate target to slow esophageal cancer proliferation.

Keywords: miR-29b, esophageal cancer, metastasis, osteopontin

Introduction

Esophageal cancer (EC) is one of the most common upper gastrointestinal tract cancers worldwide. Over the past 20 years, the incidence and the morbidity of esophageal cancer have been increasing rapidly all over the world [1]. In China, Esophageal squamous cell carcinoma is the 5th leading cause of cancer morbidities and 4th leading cause of cancer mortalities [2, 3]. Although some advances in the exploration of its possible etiological mechanism were made recently including behaviors and environmental risk factors as well as gene alterations, the complicated mechanism which the Kazakh ethnics abnormally suffered the highest is still largely unknown. Despite the improved thera-

peutic in the treatment for EC, the overall survival for ESCC is still low [4]. Thus it is desperately needed to understand the mechanism of EC.

MicroRNAs (miRNAs) are a class of small non-coding RNAs with 18-25 nucleotides in length that regulate gene expression by imperfect or perfect pairing with target mRNAs, thereby inhibiting the translation and/or degrading the mRNAs [5]. Recent literatures support the concept that miRNAs can function as tumor suppressors or oncogenes by regulating downstream target genes [6, 7]. MiRNAs play an important role in biological and pathologic processes including cell differentiation, proliferation, apoptosis and metabolism [8]. Aberrant

Role of miR-29b in esophageal cancer

miRNA expression may contribute to many types of human disease and they have been associated with every aspect of tumorigenesis [9]. ESCC is a multifactorial disease caused by the complicated interaction between multiple genes and environmental features. Several studies have shown that loss or gain of miRNAs may contribute to ESCC progression and the occurrence of malignant phenotypes [10]. In particular, miR-29b expression enhances the survival of patients with hepatocellular carcinoma (HCC) by repressing matrix metalloproteinase 2 (MMP-2) expression and activity [11]. Furthermore, forced miR-29b expression has been shown to abrogate myeloid cell leukemia-1 (MCL1) protein expression in human cholangiocarcinoma cells.

We explored the oncogenic role of miR-29b in EC and identified osteopontin as a direct target gene of miR-29b. Therefore, the findings of this study indicated the oncogenic role of miR-29b in EC tumorigenesis by targeting osteopontin, particularly in the apoptosis and proliferation processes.

Materials and methods

Specimen collection and ethics statement

30 pairs of fresh primary ESCC and adjacent normal tissues were obtained from the xxx hospital between 2012~2014, China. The present study was approved by the local Medical Ethics Committee and signed informed consent was obtained. All of the recruited patients were not subjected to preoperative radiotherapy or chemotherapy and were confirmed to have primary cancer by pathology. All tissues were stored properly for pathological diagnosis.

Cell culture and transfection

Two human EC cell lines (EC109) were maintained in RPMI-1640 medium (HyClone, USA) supplement with 10% fetal bovine serum (HyClone, USA) and cultivated at 37°C in 5% CO₂. And a human esophageal epithelial cell line (HEEC) was cultured in Dulbecco's modified Eagle's medium (HyClone, USA). The miR-29b mimic (miR-29b-mi) and inhibitor (miR-29b-in) were purchased by HanBio Corporation (China), and cells were transfected with miR-29b mimics, miR-29b inhibitor, and scramble sequence (NC) by lipofectamine RNAi Max (Life Technologies, USA) according to the instruction.

Transfection efficiency was detected by qPCR after 48 h.

Quantitative real-time PCR analysis

Total RNA was extracted from isolated tissues and cells by Trizol method (Invitrogen, USA) according to the manufacturer's protocol. The first-strand of cDNA was synthesized with M-MLV Reverse Transcriptase (Promega, USA). Total RNA was reverse-transcribed with a miR-29b-specific RT primer and amplified with PCR primers on the ABI 7500 RT-PCR System (Life Technologies, USA). The QRT-PCR was performed as follows: 20 µl PCR mix was initially incubated at 95°C for 45 s, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. RT-PCR for osteopontin was performed with primers specific for osteopontin. The primer sequences are as follows: miR-29b RT: 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAG ACCCCTAT-3'; miR-29b F: 5'-ACA CTCCAGCTGGGTTAATGCTAATCGTGAT-3', R: 5'-TGGTGTCTGGGAG TC G-3'. U6: F: 5'-CTCGCTTCGGCAGCACA-3', R: 5'-AACGCTT CACGA ATTTGCGT-3'. osteopontin F: 5'-CCA CGTACAATGACTCT TCT-3', R: 5'-TTC TTGGTTGGAGGAAGAAC-3'; For Osteopontin depletion, small interfering RNA (siRNA-Osteopontin) was synthesized and purified by GeneCopoeia Co. (Guangzhou, China). Transfection of siRNAs were performed using lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol.

EdU cell proliferation assay

Proliferating EC109 cells were detected by using cell-light 5-ethynyl-20-deoxyuridine (EdU) Apollo Imaging Kit (Ribo bio-tech, China) according to the manufacturer's protocol. Cells were incubated with 50 µM EdU for 3 h after transfection. Subsequently, fixation, permeabilization, and EdU staining were performed. Nucleic acids in all cells were stained with Hoechst 33342, resulting in blue fluorescence. Proliferating cells were stained by conjugated reaction of Apollo dye and EdU, resulting in red fluorescence. All images were obtained by a fluorescence microscope (Olympus, Japan).

Cell cycle analysis

Cells were harvested by trypsinization, washed in ice-cold phosphate-buffered saline (PBS) and fixed in 80% ice-cold ethanol in PBS operated as the manufacturer's protocol of cell

Role of microrna-29b in esophageal cancer

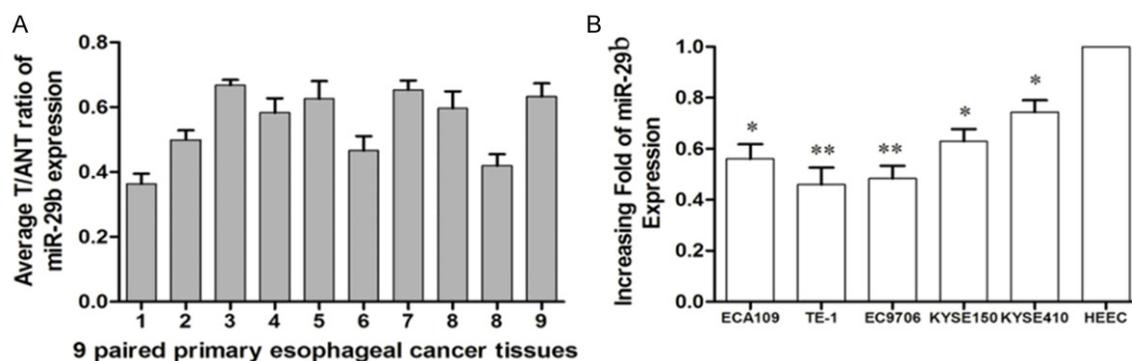


Figure 1. Expression of miR-29b was elevated in esophageal cancer tissues and cell lines. A. Relative miR-29b expression levels in 9 of 30 paired primary esophageal cancer tissues and matched tumor adjacent normal tissues (ANT) from the same patient were detected using qPCR analysis. B. Real-time PCR analysis of miR-29b expression in normal esophageal cell lines (HEEC) and esophageal cancer cell lines, including ECA109, ECA9706, TE-1, KYSE150 and KYSE410. Experiments were repeated at least three times. Each bar represents the mean of three independent experiments. β -actin was used as internal control. * $P < 0.05$, ** $P < 0.01$.

cycle reagent kit (Millipore, USA). Before staining, cells were sedimented in a chilled centrifuge and resuspended in cold PBS. 100 μ l RNase A was added and then the solution was heated at 37°C (water bath). After staining with PI and keeping in the dark at room temperature for 30 min, Cell cycle profiles of 5×10^4 cells were analyzed using a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA).

Luciferase reporter assay

Cells were transfected with luciferase reporter vector using Lipofectamine RNAiMAX which contains full length of pre-miR-29b sequence, and osteopontin-3'UTR were constructed into psiCHECK2 report plasmid (Progma, USA). 3 mutant nucleic acids were introduced at seed region of miRNA-29b. Luciferase reporter vector with Osteopontin mutant seed sequence was transfected in parallel as control. Luciferase activities in the cells were assayed using a luciferase assay kit (Promega, USA)

Colony formation assay

A total of 3×10^2 cells were plated into 6-cm plates. Cells were fixed with methanol and stained with 0.1% crystal violet after 10 days. The number of colonies with ≥ 50 cells/colony was counted. All experiments were performed in triplicate. Cell cycle assay and apoptosis by flow cytometry low cytometric analysis of cell cycle was performed using propidium iodide DNA staining (Invitrogen). Cellular apoptosis

was measured using Annexin-V-PE/7-AAD apoptosis detection kit (BDbiosciences) based on the manufacturer's instructions. Cells undergoing apoptosis were Annexin V-PE positive and 7-AAD negative. All experiments were performed in triplicate.

MTT assay

For cell proliferation analysis, cell proliferation was examined by the MTT assay (Sigma-Aldrich) according to the standard protocol. Cells were dispensed in a 96-well plate with 1500 cells per well. Each group consisted of three wells. The cells were incubated for 24 h, 48 h, 72 h, 96 h and 120 h after transfection respectively. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed by adding 20 μ l of MTT (5 mg/ml; Sigma-Aldrich, USA) for 4 h. When MTT incubation was completed, the supernatants were removed. Then, 150 μ l of dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA) was added to each well. After 15 min, the absorbance (OD) of each well was measured and the value was recorded using a microplate reader set at a wavelength of 490 nm. The number of colonies, defined as > 50 cells/colony were counted. The experiments were performed in triplicate.

Western blot

The protein level of OP was determined in EC109 cells using western blot after treated with miR-29b mimics. Total proteins (20 ng)

Role of microRNA-29b in esophageal cancer

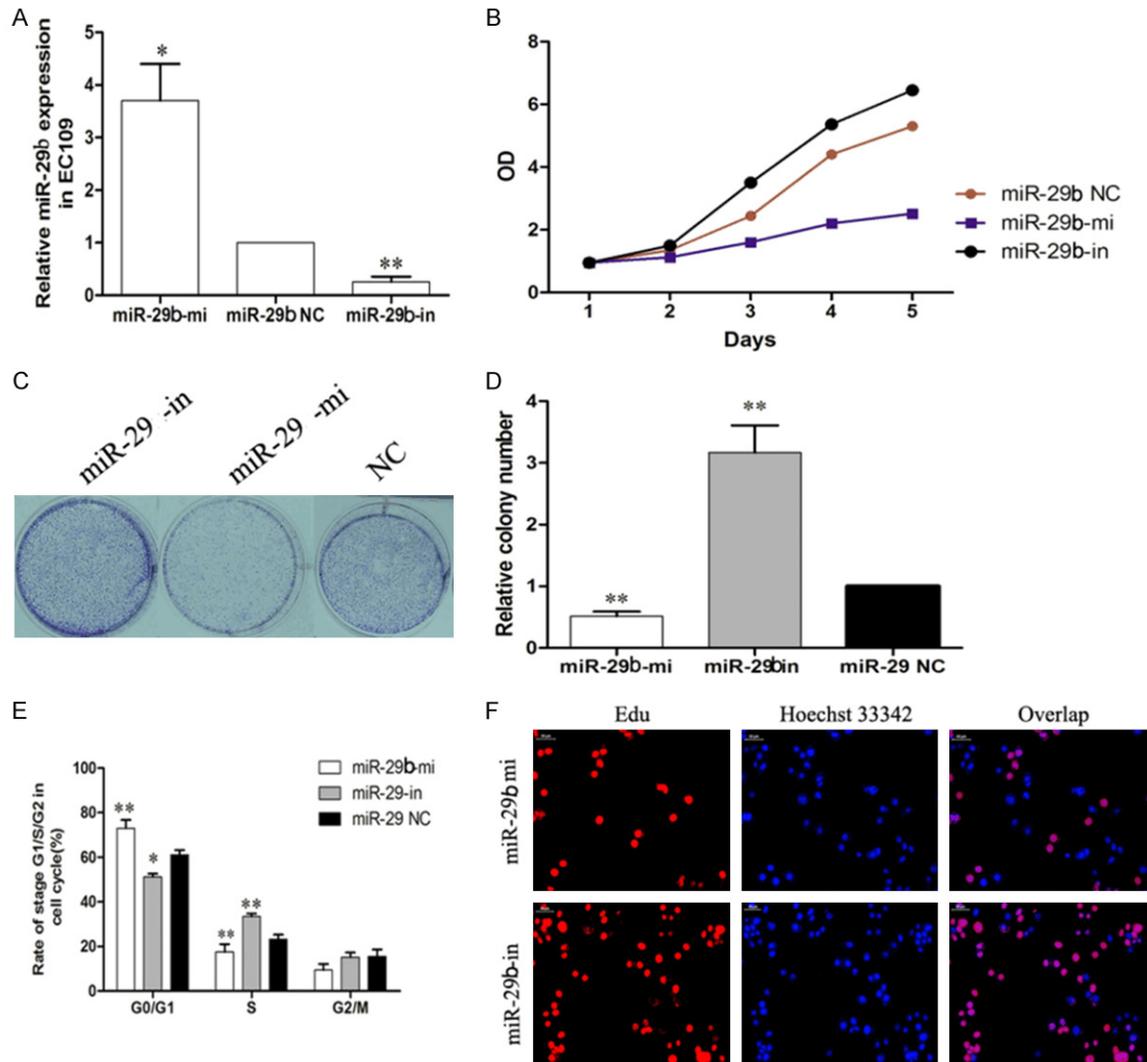


Figure 2. MiR-29b overexpression inhibited cell proliferation and arrest cell cycle in G0/G1 phase of esophageal cancer. A. Validation of miR-29b expression levels after transfection by qPCR analysis. B. MTT assays revealed that overexpression of miR-29b inhibited growth of ECA109 cell line. C. Representative micrographs of crystal violet-stained cell colonies. D. Representative micrographs of quantification of crystal violet-stained cell colonies. E. Cell cycle analysis of the indicated ECA109 cells transfected with NC, miR-29b-in or miR-29b-mi using Flow cytometric assay. Each bar represents the mean of three independent experiments. * $P < 0.05$, ** $P < 0.01$. F. Edu stain of cell proliferation assay. ECA109 cells were transfected with miR-29b-in or miR-29b-mi respectively. Following by observation using fluorescence microscope. Red color means cells stayed in S phase. Blue color means the total cells in digital figure. Pink color means the distribution and proportion of cells stayed in S phase in visual field after overlapping.

were mixed with 1×6 loading buffer and boiled for 5 min. Osteopontin (60 KD) were isolated by SDS-PAGE with 10% separation gel and 5% spacer gel concentrations, GAPDH was included as internal control. Subsequently, proteins were transferred to polyvinylidene fluoride membrane at 250 mA for 1.5 h, and the membrane was blocked with 5% nonfat milk overnight at 4 C. Primary mouse anti-human -actin IgG (Boster, China) and rabbit anti-human

osteopontin IgG (Cell Signaling, USA) were optimized at 1:600 and 1:1,000, respectively, and incubated with the membrane at 4 C overnight. The membrane was incubated with the secondary antibody (dilution 1:5,000 and 1:3,000, respectively) (Santa Cruz, USA and Cell Signaling, USA) for 1 h at room temperature after washing with TBST buffer. Signals were visualized with chemiluminescent substrate (Pierce, USA) by exposure to films.

Role of microrna-29b in esophageal cancer

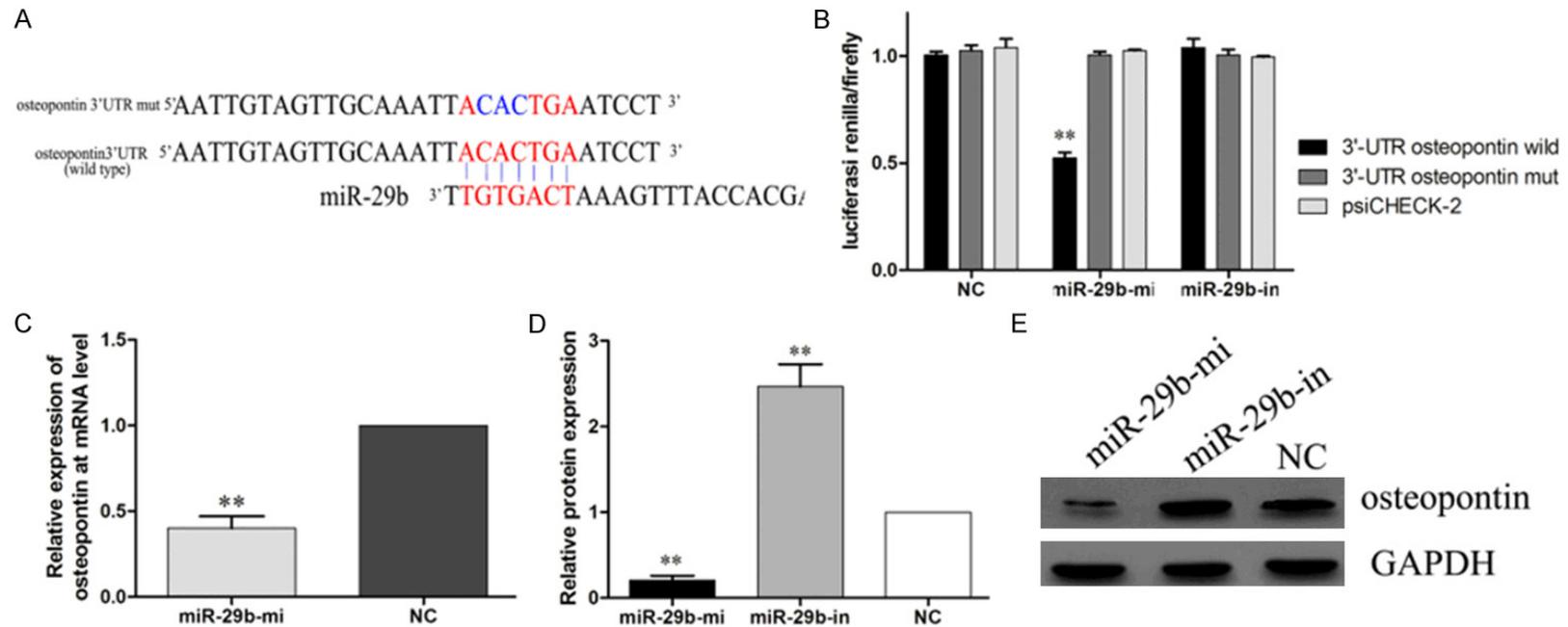


Figure 3. MiR-29b decreased osteopontin expression by directly targeting the osteopontin 3'-UTR and altered levels of protein and mRNA in ECA109 cells. A. Predicted miR-29b target sequence in the 3'-UTR of osteopontin (osteopontin-3'-UTR) and positions of three mutated nucleotides (blue) in the 3'-UTR of osteopontin (osteopontin-3'-UTR-mut). Binding state marked in red color. B. Luciferase reporter assay of the indicated cells transfected with the psiCHECK2-osteopontin-3'-UTR reporter or psiCHECK2-osteopontin-3'-UTR-mut reporter and miR-29b-mi or miR-29b-in with 40 nM oligonucleotides. C. Real-time PCR analysis of expression of osteopontin in indicated ECA109 cells. D. Quantification of western blotting analysis in E. ** $P < 0.01$, * $P < 0.05$. E. Western blotting analysis of osteopontin expression in cells transfected with miR-29b-mi or the miR-29b-in. GAPDH served as the loading control.

Role of microrna-29b in esophageal cancer

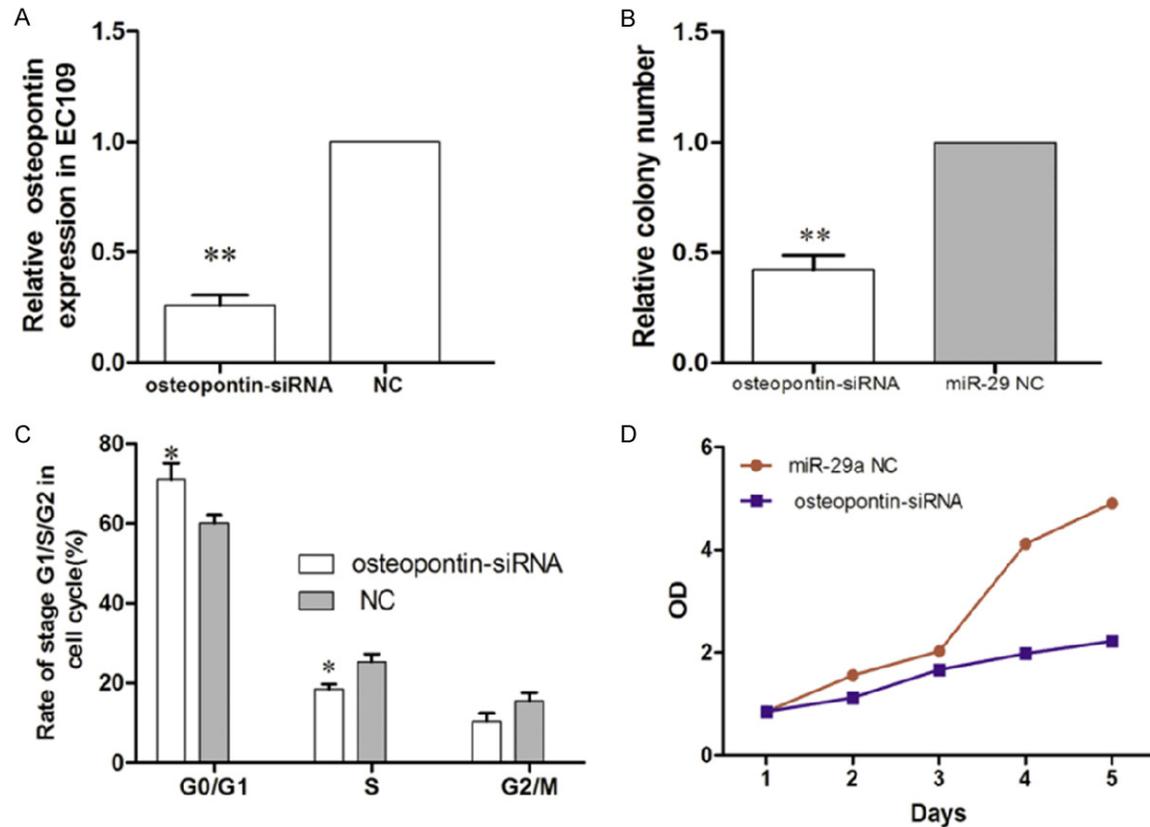


Figure 4. Osteopontin downregulation is required for miR-29b-induced proliferation of esophageal cancer cells. A. Validation of miR-29b expression levels after transfection of siRNA specific for osteopontin by qPCR analysis. $*P < 0.05$. B. Cell colony formation assay in ECA109 cells after transfected with osteopontin-siRNA. $*P < 0.05$. C. Cell cycle analysis in ECA109 cells after transfected with osteopontin-siRNA to verify the effect of direct of miR-29b on cell biological behaviors. D. MTT analysis verified that silencing osteopontin effectively inhibited cell proliferation. Each bar represents the mean of three independent experiments.

Statistical analysis

miRNA target was predicted by Target Scan (release 6.2, <http://www.targetscan.org/>), miR-base (<http://mirdb.org/miRDB/>) and RNAhybrid 2.2. The SPSS19.0 was used for conducting the statistical analyses. All results were expressed as means SD. Data was analysis using Student's t-test, One-way ANOVA and Pearson correlation coefficient. $*P < 0.05$ was considered statistically significant.

Results

MiR-29b expression was downregulated in esophageal cancer cell lines and esophageal cancer tissues

miR-29b has been reported to be down in various types of tumors; however, its expression in EC tumors has not been reported. As shown in

Figure 1A, using adjacent non-tumor tissues as a control, miR-29b expression in EC tissues was found to be significantly reduced ($P < 0.05$). It was also shown that the expression of miR-29b was significantly down-regulated in esophageal cancer cell lines compared with normal EC cell line HEEC (**Figure 1B**). In conclusion, miR-498 was abnormal down-regulated in esophageal cancer tissues and esophageal cancer cell lines.

Overexpression of miR-29b inhibited cell proliferation of esophageal cancer cell

We examined whether miR-29b could modulate proliferation in EC109 cells. Edu incorporation experiments and cell cycle analysis were performed in EC109 cells transfected with miR-29b mimic and NC. We expressed miR-29b in EC109 cells. The expression of miR-29b in EC109 cell line inhibited cell proliferation

Role of microrna-29b in esophageal cancer

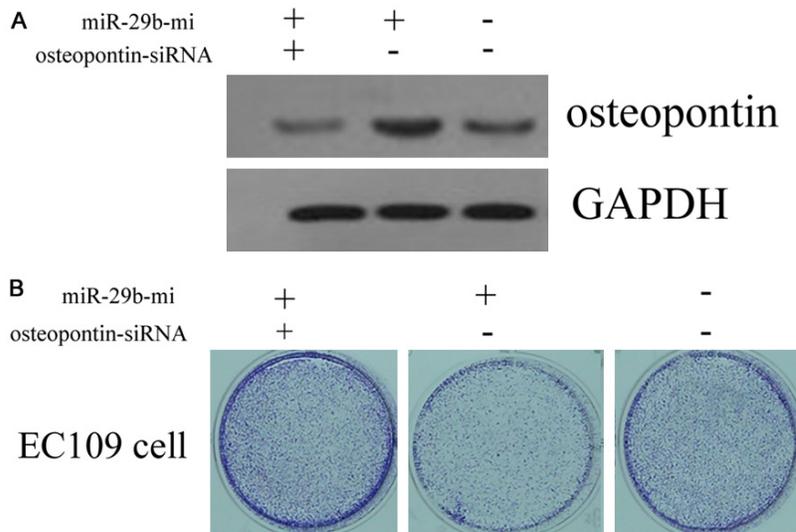


Figure 5. Osteopontin-siRNA reversed the effect of miR-29b-induced proliferation of esophageal cancer cells. (A) Validation of osteopontin expression levels after transfection of osteopontin-siRNA in miR-29c-transfected EC109 cells. GAPDH served as the loading control. (B) Representative micrographs of crystal violet-stained cell colonies after transfection of osteopontin-siRNA in miR-29c-transfected EC109 cell line.

(**Figure 2A**) as described. Results indicated that the over-expression of miR-29b significantly decelerated the cell proliferation rate in EC109 cells ($P < 0.05$). Using flow cytometry assays, we found that miR-29b overexpression decreased the percentage of cells in S phase and significantly increased the percentage of cells in G1/G0 (**Figure 2D**), the opposite result was obtained when the cells were treated with miR-29b-in (**Figure 3D**). Thus over-expression of miR-29b decelerated cell cycle progression.

MiR-29b was conversely correlated with osteopontin and altered levels of proteins related to cell proliferation and cycle in esophageal cancer cells

To identify miR-29b target genes that act as tumor suppressors, we questioned oncogenic genes highly expressed in tumor patients using miRNA site prediction (www.microrna.org). And the results showed Osteopontin was an miR-29b target gene. Therefore, we focused our investigations on Osteopontin in order to determine miR-29b function.

EC109 cells were transiently transfected with miR-29b mimics, miR-29b-in or the respective controls. The expression of Osteopontin was determined using Western blotting with necessary controls in the same panel of FFPE blocks

as detected for miR-29b. Interestingly, we observed that miR-29b was capable of down-regulating Osteopontin protein expression, while miR-29b-in clearly promoted its protein expression (**Figure 4B**).

We next used luciferase assays to further elucidate the relationship between miR-29b and Osteopontin. The results showed that the co-transfection of miR-29b markedly increased the firefly luciferase activity of psi-CHECK2-Osteopontin but failed to influence the luciferase activity of psi-CHECK2-Osteopontin-mut in EC109 cells (**Figure 4C**). Meanwhile, EC109 cells

transfected with miR-29b-in resulted in suppressing firefly luciferase activity of the wild-type reporter but unaffected the mutant reporter (**Figure 4C**). In summary, our data indicate that miR-29b directly attenuated the expression of osteopontin by targeting of its mRNA 3'UTR in ovarian cancer cell lines. Collectively, these results indicated that the expression of miR-29b could regulate expression of Osteopontin, and cell proliferation and cell cycle.

Osteopontin is involved in miR-29b-induced proliferation of esophageal cancer cells

To further understand the role of Osteopontin in miR-29b-mediated pro-proliferation, miR-29b-transfected EC109 cells were transfected with Osteopontin-siRNA. As showed in **Figure 5A**, results of Western blot analysis revealed that the Osteopontin expression in miR-29b-transfected EC106 cells was decreased after transfected with Osteopontin-siRNA. Colony formation assays showed that the treatment with Osteopontin-siRNA was able to reverse the miR-29b-decreased proliferation (**Figure 5B**), suggesting that miR-29b suppresses the proliferation of ovarian cancer cells by upregulating Osteopontin. Therefore, our results demonstrate that miR-29b was able to inhibit the proliferation of ovarian cancer cells through direct targeting Osteopontin.

Discussion

An extensive amount of research has led to the identification of miRNA as important regulators of gene expression in cancer biology [12]. Depending on their mRNA targets, miRNAs can function as either tumor-suppressors or oncogenes in various tumor microenvironments. The expression of miRNAs can vary by cancer types and plays a critical role in cell survival, apoptosis, proliferation, cell death, and tumorigenesis. MiR-29b, which was reported to function as a tumor suppressor in a variety of cancers is controversial in esophageal cancer [13, 14]. In this paper, we investigated the functions of miR-29b as tumor suppressor in esophageal cancer. According to our results, ectopic expression of miR-29b could decrease the cell proliferation of esophageal cancer, while miR-29b-in enhanced this effect. We also found Osteopontin was a direct target of miR-29b, and its expression is negatively related with the level of miR29b. Taken together, these findings demonstrated that miR-498 may play an important role in carcinogenesis and progression of esophageal cancer.

Osteopontin is an integrin-binding secreted adhesive glycoprotein involved in a variety of physiologic cellular functions, and it has been shown to play an important role in tumorigenesis, tumor invasion, and metastasis in variety of cancers [15, 16]. The function of osteopontin in tumor pathophysiology is complex and not fully investigated. Researchers have identified several important downstream osteopontin signals that regulate tumor progression and invasive behavior [17]. Recent researches revealed that osteopontin was identified as a leading marker of colon cancer progression, and its expression was shown to have the most significant association with the metastatic potential of hepatocellular carcinoma [18]. Although osteopontin overexpression is associated with tumor progression in various cancers [19], the role of osteopontin was not shown in esophageal cancer previously. In esophageal cancer, some researchers reported that osteopontin protein overexpression was observed in ESCC tumors [20]. Although a lot of reports showed that osteopontin overexpression was frequent in ESCC tumors, the relationship between osteopontin expression and miR-29b has not been shown in these studies [21]. In our report, we found that high osteopontin expression in 23

ESCC tumors by microarray analysis. Western blotting and Q-PCR indicated that miR-29b down-regulated the expression of osteopontin at the protein and mRNA levels

In our study, we found that the over-expression of miR-29b can suppress the proliferation of esophageal cancer cell line, EC109, and the repression of proliferation could be rescued when miR-183 expression was reduced via a miR-29b inhibitor. In addition, miR-29b accelerated cell progression in G0/G1 phase transition and inhibited cell proliferation. miRNA target prediction programs (TargetScan and miRDB) and mRNA microarray profiling were employed to identify the bona fide targets of miR-29b. Osteopontin was markedly identified as one of the targets of miR-183. The binding sites of miR-29b were in Osteopontin 3'UTR. The Osteopontin gene was originally considered to be up-regulated in esophageal cancer cells. Furthermore, results of previous studies suggested that Osteopontin is an important tumor suppressor gene. The present study confirmed that over-expression of miR-29b could inhibit Osteopontin protein expression. Moreover, several researches reported the expression level of Osteopontin was related to the expression of miR-29b, which was confirmed in esophageal cancer cell lines. In our study, the role of miR-29b in esophageal cancer was explored for the first time. We verified the negative correlation between miR-29b expression and Osteopontin expression and confirming target association.

In summary, the present study demonstrated an important link between miR-29b-mediated proliferation of esophageal cancer cells and upregulation of osteopontin. Our findings suggested that miR-29b inhibited proliferation, migration of EC, playing an important role in the suppressor gene of EC. Therefore, all the results indicated that miR-29b maybe serves as promising target for EC therapy.

Disclosure of conflict of interest

None.

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Role of microrna-29b in esophageal cancer

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