Abstract. Cervical cancer is the second most common gynecological malignancy. Accumulating evidence has suggested that microRNAs (miRNAs) are involved in the occurrence and development of cervical cancer. The present study aimed to investigate the function and underlying molecular mechanism of microRNA (miRNA/miR)-29a in cervical cancer. Reverse transcription-quantitative PCR and methylation-specific PCR were used to examine the expression of miR-29a and methylated status of p16 promoter, respectively. Cell Counting Kit-8 analysis and flow cytometry were performed to evaluate cell viability and cycle, respectively. Dual-luciferase reporter assay was performed to verify the interaction between miR-29a and its targets. Western blot analysis was performed to detect the protein levels of DNA methyltransferases (DNMT)3A and DNMT3B. The results demonstrated that miR-29a expression was downregulated in cervical cancer tissues and cells, and negatively correlated with p16 promoter hypermethylation. Furthermore, cell experiments confirmed that miR-29a suppressed cell proliferation and induced cell cycle arrest in HeLa and C-33A cells. Mechanically, miR-29a restored normal methylation pattern of the p16 gene by sponging DNMT3A and DNMT3B. Taken together, the results of the present study demonstrated the epigenetic regulation of tumor suppressor p16 by miR-29a as a unique mechanism, thus providing a rationale for the development of miRNA-based strategies in the treatment of cervical cancer.

Introduction

Cervical cancer is the second most common gynecological malignancy, with an estimated 570,000 cases and 311,000 deaths in 2018 worldwide (1). Although several studies have revealed that the human papillomavirus is the most important cause of cervical cancer, other factors are required for malignant transformation of cervical cell (2). Genetic factors are also involved in the development of cervical cancer (3). Thus, innovative biomarkers and related molecular mechanisms are essential for the diagnosis, prognosis and treatment of cervical cancer.

DNA methylation is one of the predominant epigenetic modifications in mammals, which performs a critical function in regulating gene expression (4). Aberrant DNA methylation, particularly methylation of CpG islands in gene promoter regions, often occurs in different types of cancer, including cervical cancer and is an early event of malignant transformation (5,6). P16 is a common studied tumor suppressor gene (7). The promoter regions of p16 are often methylated, which decreases the levels of p16 in cervical cancer (8). Previous studies have demonstrated that p16 promoter methylation is closely associated with the development and progression of cervical cancer, so it is considered a potential diagnostic and therapeutic target (9,10). The changes involved in DNA methylation are controlled by DNA methyltransferases (DNMTs) (11). A total of three catalytically active DNMTs (DNMT1, DNMT3A and DNMT3B) have been identified in mammals (4). DNMT1 maintains methylation pattern, while DNMT3A and DNMT3B are responsible for de novo DNA methylation (12). Previous studies have reported elevated levels of DNMT1, DNMT3A and DNMT3B in various tumors, including hepatic, prostate, colorectal and breast cancers (13-16). Recently, high DNMT1 protein expression was reported in cervical cancer, and is associated with poor survival outcome (17). Inhibition of DNMTs can reactivate the expression of methylation-silenced tumor suppressor genes in human cervical cancer cells (18-20).

MicroRNAs (miRNAs/miRs) are a class of short (20-24 nucleotides) non-coding RNA molecules that negatively regulate gene expression by translational inhibition or destabilization of targets through binding to the 3'-untranslated region (UTR) of mRNAs (21). miRNAs play...
important roles in several biological processes, such as cell proliferation, apoptosis, differentiation and cell cycle (22). Thus, abnormal expression or dysfunction of miRNAs are associated with the development of diseases, including cancer (23). miR-29a is a tumor suppressor gene that can inhibit the malignant proliferation, invasion and metastasis of several human cancer cells (24-26). Furthermore, miR-29a suppresses cell proliferation by targeting SIRT1 in cervical and hepatocellular carcinomas (27,28). miR-29a also inhibits cell proliferation, migration and invasion by directly targeting CDCA42 in cervical cancer, osteosarcoma, gliomas and breast cancer (29-32). In addition, miR-29a inhibits cancer cell migration and invasion by targeting HSP47 in cervical squamous cell carcinoma (33). Increasing evidence suggests that miR-29a regulates DNA methylation with the suppression of DNMTs in lung cancer (34), hepatocellular carcinoma (35,36), Burkitt lymphoma cells (37) and T-cell acute lymphoblastic leukemia (38).

Thus, the present study aimed to investigate the role of miR-29a and whether miR-29a regulates the methylated status of p16 promoter by modulation of DNMT3s in cervical cancer.

Materials and methods

Tissues samples. In the present study, 40 patients who underwent cervical cancer surgery at the Affiliated Hospital of Qinghai University between January 2017 and December 2018 were recruited. All participants were female, with a median age of 55 years (age range, 32-68 years). Cervical cancer tissues and paired adjacent normal tissues were collected in surgery, and the distance between adjacent normal and cancer tissue boundary was ~1-2 cm. The present study was approved by the Ethics Committee of the Affiliated Hospital of Qinghai University (Xining, China; approval no. SL-2018016) and written informed consent was provided by all patients prior to the study start. Tissue samples were obtained during surgical resection and immediately snap-frozen in liquid nitrogen, and stored at -80˚C until subsequent experimentation. Diagnosis was independently confirmed via two pathologists from the Affiliated Hospital of Qinghai University.

Cell culture. The human cervical cancer cell lines, HeLa and C-33A, were purchased from The Cell Bank of the Chinese Academy of Sciences, while the ectocervical epithelial cell line, ECT1/E6E7, was purchased from the American Type Culture Collection. The cervical cell lines were maintained in DMEM supplemented with 10% fetal bovine serum (both purchased from Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.), at 37˚C with 5% CO₂.

Reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted from cervical cancer cells and tissues using TRIzol® reagent (Invitrogen, Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. RT was performed using PrimeScript 1st Strand cDNA synthesis kit (cat. no. 6110A; Takara Bio, Inc.) at 37˚C for 15 min. qPCR was subsequently performed using the SYBR Green PCR Master Mix (Takara Bio, Inc.). The following conditions were used for all RT-PCR assays: 95˚C for 30 sec, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 35 sec. After the PCR run, a melting curve analysis was performed at a melting rate of 0.1˚C/sec, and data were collected every 0.23˚C from 6-95˚C (LineGene9600 version 1, Bioer Technology). Relative expression levels were calculated using the 2⁻ΔΔCq method (39) and all experiments were performed in triplicate. miR-29a expression was assessed via the stem-loop RT primer assay and U6 was used as the internal control. DNMT3A and DNMT3B mRNA expression was standardized to control values of GAPDH. The primers sequences used for qPCR are listed in Table I.

Methylation-specific PCR (MSP). Genomic DNA was bisulfite converted using the EZ DNA Methylation Gold bisulfite conversion kit (cat. no. D5008, Zymo Research Corp. Irvine, CA) and diluted to a final concentration of 20 ng/µl. MS-PCR primers [targeting methylated sequence (M) and unmethylated sequence (U)] were designed using MethPrimer 2.0 to span the CpG island of the p16 promoter region. The following thermocycling conditions were used: Initial denaturation at 95˚C for 2 min, followed by 35 cycles of denaturation at 94˚C for 30 sec, annealing at 55˚C for 30 sec, extension at 72˚C for 60 sec and a final extension at 72˚C for 4 min. The methylation-specific primers for p16 are presented in Table I. The PCR products were stained with ethidium bromide for 2 min at 37˚C, analyzed on 2% agarose gels and subsequently visualized via UV illumination. The presence of specific bands in (M) or both (M) and (U) were considered positive for methylation. However, the presence of specific bands only observed in (U) but not in (M) were considered unmethylated.

Western blotting. Total protein was extracted from cervical cancer cells using RIPA lysis buffer (Sigma-Aldrich; Merck KGaA) and qualified using the BCA detecting kit (cat. no. P0006, Beyotime Institute of Biotechnology), according to the manufacturer's instructions. A total of 50 µg protein/lane was separated by 10% SDS-PAGE, transferred onto PVDF membranes (EMD Millipore) and blocked with 5% dry milk blocking buffer for 2 h at room temperature. The membranes were incubated with primary antibodies against DNMT3A (1:1,000; cat. no. ab188470; Abcam), DNMT3B (1:1,000; cat. no. ab79822; Abcam) and tubulin (1:2,000; cat. no. ab7291; Abcam) overnight at 4˚C. Following the primary incubation, membranes were incubated with HRP-conjugated goat anti-rabbit IgG (H+L) (1:2,000; cat. no. ab205718; Abcam) at room temperature for 40 min. Protein bands were visualized using an enhanced chemiluminescence detection system (Pierce; Thermo Fisher Scientific, Inc.) and intensities of bands were quantified using Image Lab™ software (Bio-Rad Laboratories, Inc.).

Cell transfection. miR-29a mimics (miR-29a) and scrambled miRNA (Scrambled), siRNA for DNMT3A (si-DNMT3A), siRNA for DNMT3B (si-DNMT3B) and negative control (siRNA-NC) were purchased from Shanghai GenePharma Co., Ltd., and the sequences are presented in Table I. HeLa and C-33A cells were seeded into 6-well plates at a density of 2x10⁵ cells/well and cultured at 37˚C for 24 h, prior to transfection using the Lipofectamine® 2000 kit (cat. no. 11668027; Invitrogen, Thermo Fisher Scientific, Inc.) at 37˚C for 48 h, according to the manufacturer's protocol.
The final concentrations of miR-29a mimics and siRNAs were 50 nM and 80 nM, respectively. Cells were harvested 24 h post-transfection.

**Colony formation assay.** Hela and C-33A cells were seeded into 6-well plates, incubated at 37°C for 14 days and fixed with 4% paraformaldehyde for 30 min at room temperature. Cells were subsequently stained with 0.1% crystal violet for 2 h at room temperature. Colonies (>50 cells) were observed under a light microscope (magnification, x100). Colony forming efficiency = number of colonies/number of seeded cells.

**Cell cycle analysis.** Transfected cells were digested with trypsin and fixed with 70% ice-cold ethanol overnight at -20°C. Cells were subsequently stained with propidium iodide (50 µg/ml) and RNAse A (0.1 mg/ml) for 30 min at 37°C (both purchased from Sigma-Aldrich; Merck KGaA), and analyzed using the FACS Calibur flow cytometer (BD Biosciences). All experiments were performed in triplicate.

**miRNA target prediction.** Potential miR-29a binding sites in the 3'-UTR regions of DNMT3A and DNMT3B mRNA were predicted using the TargetScanHuman 7.2 database (www.targetscan.org). Position 862-868 for DNMT3A and position 1206-1213 for DNMT3B were identified as putative conserved binding sites for miR-29a.

**Dual-luciferase reporter assay.** The 3'-UTR regions of DNMT3A and DNMT3B mRNA harboring the predicted miR-29a binding sites [wild-type (wt)-DNMT3A and wt-DNMT3B] or the corresponding mutants [mut-DNMT3A and mut-DNMT3B] were synthesized by Beijing Genomics Institute (https://www.genomics.cn) and subsequently inserted into the pmiRGLO vector (Promega Corporation). HeLa and C-33A cells were transfected with wt-DNMT3A or mut-DNMT3A, as well as wt-DNMT3B or mut-DNMT3B, followed by transfection with miR-29a mimics or scrambled miRNA using the Lipofectamine® 2000 kit (cat. no. 11668027; Invitrogen, Thermo Fisher Scientific, Inc.) at 37°C for 48 h. Finally, luciferase activities were detected using a dual-luciferase reporter assay system (Promega Corporation), according to the manufacturer's protocol. The luciferase activity was expressed as fold change compared with the non-treated controls, both as normalized Firefly/Renilla readouts and single luciferase read-outs.

**LinkedOmics database.** The LinkedOmics database (http://www.linkedomics.org) contains multi-omics data and clinical data for 32 cancer types and a total of 11,158 patients from The Cancer Genome Atlas (TCGA) project (40). LinkedOmics has three data analysis modules: LinkFinder, LinkCompare and LinkInterpreter. The LinkFinder module was used to calculate the association between miR-29a expression and DNMT3A or DNMT3B mRNA expression, and association between p16 expression and DNMT3A or DNMT3B mRNA expression in the TCGA cervical and endocervical cancers (CESC) cohort (n=304), using Pearson's correlation coefficient.

---

**Table I. Primer sequences.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-29a</td>
<td>F: ACACTCCAGCTGGTTTGGAGTCT R: CTCAAATGGTGTCGTTGGA</td>
</tr>
<tr>
<td>U6</td>
<td>F: CTGCTCAGGCTCCCTATG R: AACGCTTCAGAATTTTCCTG</td>
</tr>
<tr>
<td>DNMT3A</td>
<td>F: GCTGCACCTGGCCTTATG R: GGCTTTCTCTCAGGCTGATC</td>
</tr>
<tr>
<td>DNMT3B</td>
<td>F: CCAATCTGGAGGCTATCGG R: CGCTTCAGGGACTTGTTG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: ATGACATCAAGAAGTGGT R: GCGTCAAAGGTTGAGGA</td>
</tr>
<tr>
<td>P16 [U]</td>
<td>TTATTAGAGGGTTGGGGTTGGTATTG R: CACCCCCAAAACACCCATAAA</td>
</tr>
<tr>
<td>P16 [M]</td>
<td>TTATTAGAGGGTTGGGGCTGACGC R: GACCCCCGACACCGGCCCTGA</td>
</tr>
<tr>
<td>DNMT3A siRNA</td>
<td>GGGUCACACAGAAGCAAUATT</td>
</tr>
<tr>
<td>DNMT3B siRNA</td>
<td>UGGUGUUGGGCAACACUGAA</td>
</tr>
<tr>
<td>siRNA-NC</td>
<td>CAGAUGUGGCACAAACAAGA</td>
</tr>
<tr>
<td>miR-29a mimics</td>
<td>ACCCCCTTAGAGATGACTGTATTCTTCTTGGTGTTGTCG AGTCGATAGTTTTTCTAGGACCA TCTGAAATCGGTATAATGATTGAGGA</td>
</tr>
</tbody>
</table>

miR, microRNA; DNMT, DNA methyltransferase; si, small interfering; NC, negative control; F, forward; R, reverse.
miR-29a INHIBITS CERVICAL CANCER CELL PROLIFERATION

Statistical analysis. Statistical analysis was performed using SPSS v21.0 software (SPSS, Inc.) and the Prism statistical v8.0 software package (GraphPad Software, Inc.). All experiments were performed in triplicate and data are presented as the mean ± standard deviation. Unpaired Student’s t-test was used to compare differences between two groups, while one-way analysis of variance and Tukey’s post hoc test were used to compare multiple groups. Pearson’s correlation coefficient was used to assess linear correlation between miR-29a and DNMT3A or DNMT3B, and the correlation between p16 and DNMT3A or DNMT3B. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-29a levels decrease in cervical cancer tissues and cells and are negatively correlated with p16 hypermethylation. RT-qPCR analysis was performed to detect miR-29a expression in 40 cervical cancer tissues and paired normal cervical tissues. The results demonstrated that miR-29a expression was significantly decreased in cervical cancer tissues compared with paired normal tissues (P<0.001; Fig. 1A). Consistently, miR-29a expression was lower in the cervical cancer cell lines (HeLa and C-33A) compared with the normal cervical cell line, ECT1/E6E7 (P<0.001; Fig. 1B). The methylation status of p16 promoter was assessed in 40 cervical cancer tissues and paracancerous tissues. As presented in Fig. 1C, hypermethylation of p16 occurred in 62.5% (25/40) of cervical cancer tissues and 15.0% (6/40) of paracancerous tissues. In addition, p16 was hypermethylated in HeLa and C-33A cells compared with ECT1/E6E7 cells (Fig. 1D). Notably, the levels of miR-29a in the unmethylated p16 group were higher than the methylated p16 group (P<0.05; Fig. 1E), suggesting that miR-29a may be associated with the methylation status of p16 in cervical cancer.

miR-29a suppresses cell proliferation and induces cell cycle arrest of HeLa and C-33A cells. To assess the biological
function of miR‑29a in cervical cancer cell lines, HeLa and C‑33A cells were transfected with miR‑29a mimics or negative control, and subjected to the colony formation assay and cell cycle analysis. The results demonstrated that compared with the control groups, miR‑29a mimics effectively increased the expression of miR‑29a in HeLa and C‑33A cells (P<0.001; Fig. S1). Overexpression of miR‑29a significantly decreased the colony formation capacity in both HeLa and C‑33A cells compared with the negative control groups (P<0.01; Fig. 2A). Furthermore, cell cycle analysis demonstrated that overexpression of miR‑29a significantly promoted cell cycle arrest at G<sub>0</sub>/G<sub>1</sub> phase in both HeLa and C‑33A cells (P<0.001; Fig. 2B). Taken together, these results suggest that miR‑29a exerts an antitumor effect in cervical cancer cells.

miR‑29a inhibits DNMT3A and DNMT3B expression by directly targeting their 3'-UTRs. To determine the association between miR‑29a expression and p16 methylation status, DNMT3A and DNMT3B were selected as potential miR‑29a targets for further experiments based on literatures and bioinformatics analysis. As predicted by the TargetScanHuman 7.2 database, miR‑29a had intriguing complementarity sites in the 3'-UTRs of the DNMT3A and DNMT3B genes (Fig. 3A). To validate the interaction between miR‑29a and targets, the 3'-UTRs of DNMT3A and DNMT3B were cloned into a modified pGLO3 plasmid downstream of the luciferase reporter gene. Corresponding mutant versions with the binding site mutagenesis were also constructed, and subsequently co‑transfected with miR‑29a mimics in HeLa cell. The results demonstrated that miR‑29a significantly decreased the luciferase activities in the wt‑DNMT3A and wt‑DNMT3B groups compared with the scrambled oligonucleotide (P<0.01; Fig. 3B). miR‑29a mimics was transfected into HeLa and C‑33A cells to assess whether miR‑29a regulates DNMT3A and DNMT3B expression. As presented in Fig. 3C, transfection with miR‑29a mimics significantly increased miR‑29a expression in HeLa and C‑33A (P<0.001). Furthermore, DNMT3A and DNMT3B expression in HeLa and C‑33A cells significantly decreased following overexpression of miR‑29a (P<0.01; Fig. 3D). The association between DNMT3A or DNMT3B and miR‑29a

Figure 2. Ectopic miR‑29a expression restrains cell proliferation and cell cycle of cervical cancer cells. (A) The colony formation efficiency decreased following overexpression of miR‑29a in HeLa and C‑33A cells. (B) Cell cycle analysis demonstrated that miR‑29a induced cell cycle arrest at G<sub>0</sub>/G<sub>1</sub> phase in HeLa and C‑33A cells. Data are presented as the mean ± standard deviation (n=3). **P<0.01, ***P<0.001 vs. control or scrambled group. miR, microRNA.
expression levels in cervical cancer tissues was determined using LinkedOmics (http://www.linkedomics.org). Pearson's correlation analysis demonstrated that miR-29a expression was negatively correlated with DNMT3A and DNMT3B expression (P<0.01; Fig. 3E). Collectively, these results suggest that miR-29a decreases mRNA DNMT3A and DNMT3B expression by directly targeting to their 3'‐UTRs.

miR-29a inhibits p16 gene methylation via modulation of DNMT3A and DNMT3B. To assess the potential effect and molecular mechanism of miR-29a on the methylation pattern of p16 gene, HeLa and C-33A cells were transfected with miR-29a mimics, or siRNAs for DNMT3A and DNMT3B. The levels of DNMT3A and DNMT3B significantly decreased in cells transfected with their specific siRNAs compared with their corresponding control groups (P<0.001; Fig. S2A and B). The MSP results demonstrated that miR-29a attenuated the methylation status of p16 in HeLa and C-33A cells (Fig. 4A). Furthermore, silencing of DNMT3A or DNMT3B, two key enzymes involved in DNA methylation (41), normalized aberrant methylation pattern of p16 in cervical cancer (Fig. 4B). In addition, LinkedOmics analysis demonstrated that p16 (also known as CDKN2A) mRNA expression was inversely correlated with DNMT3A or DNMT3B mRNA levels in cervical cancer tissues (P<0.001; Fig. 4C). Thus, miR-29a inhibits aberrant methylation of tumor suppressor gene p16 by regulating the levels of DNMT3A and DNMT3B.
miRNAs exhibit abnormal expression in different types of cancer, and exert tumor suppression or promotion effects by regulating the expression of target genes (42). For example, Chen et al (43) reported that miR-132 expression is significantly downregulated in thyroid cancer tissues and overexpression of miR-132 exerts tumor-suppressing functions through targeting FOXA1. Previous studies have demonstrated that miRNAs play a crucial regulatory role in cervical cancer. These studies contribute to a profound understanding of the molecular mechanism involved in the cervical cancer (44,45). miR-29a has been reported to exert an antitumor effect in different types of cancer, and the disorder of miR-29a is associated with the development and progression of cancer (26).

The results of the present study demonstrated that miR-29a expression was downregulated in cervical cancer tissues and cell lines compared with normal cervical tissues and cells. In addition, overexpression of miR-29a inhibited the proliferation and induced cell cycle arrest in cervical cancer cells, which was similarly observed in previous studies. It has been demonstrated that miR-29a expression is downregulated in papillary thyroid cancer (46), oral squamous cell carcinoma (47), lung cancer (48) and retinoblastoma (48), as well as cervical cancer (49,50), and ectopic miR-29a expression significantly inhibits proliferation and invasion. Taken together, these results confirm that miR-29a is a tumor suppressor (51).

The results of the present study demonstrated a significant correlation between miR-29a expression and methylation patterns of p16, and that overexpression of miR-29a normalized aberrant methylation status of the p16 gene. The p16 gene is a well-known tumor suppressor gene that blocks the G1-S phase of the cell cycle and inhibits abnormal proliferation of cancer cells (52). In addition, p16 protein inhibits the activation of cyclin-dependent kinase 4 and the phosphorylation of pRb, and further blocks the cell cycle (10). Mutation, deletion and abnormal methylation of the p16 gene are frequently observed, which inactivates the p16 protein in different types of cancer and is closely associated with the development and progression of cancers (53,54). Methylation of the p16 gene promoter inactivates p16, significantly decreasing its expression (55,56). This results in the loss of the tumor suppressor function of p16, which promotes the development of cervical cancer (8). However, the regulatory mechanism of p16 promoter methylation remains unknown. Thus, to investigate the molecular mechanism by which miR-29a regulates the methylation status of p16, which promotes the development of cervical cancer (8).

The results of the present study confirmed that miR-29a is involved in methylation modification of the tumor suppressor gene, p16, by directly targeting DNMT3s. The results demonstrated the underlying molecular mechanism by which miR-29a inhibits cell proliferation and arrests the cell cycle in cervical cancer. Taken together, these results provide a novel perspective for the biological significance of miR-29a in regulating methylation modification with potential diagnostic and therapeutic biomarkers for clinical cervical cancer management.
Acknowledgements

Not applicable.

Funding

The present study was supported by the Qinghai Basic Research Program of China (grant no. 2018-0301-ZJC-0101).

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

AW, QX and GG were involved in the study concept and design, analysis and interpretation of data and drafting the initial manuscript. RS, TB and XX were involved in the acquisition of data and analysis. All authors performed the experiments, revised and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Affiliated Hospital of Qinghai University (Xining, China; approval no. SL-2018016), and written informed consent was provided by all patients prior to the study start.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References