

Original Article

MicroRNA-101 regulates the viability and invasion of cervical cancer cells

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Abstract: Background: Cervical cancer has the second highest morbidity and mortality rates of any malignancy in women worldwide, and it is one of the leading causes of death in Uygur women in Xinjiang China. MicroRNAs are involved in cancer development and progression. Previously, we found that miR-101 is significantly down-regulated in cervical cancer tissues from Uyghur women. The underlying pathophysiology and relevance to tumorigenesis of miR-101 is still largely unknown. The purpose of this study was to elucidate the molecular mechanisms of miR-101 regulation of cervical cancer cell viability and invasion. Materials and methods: The expression of miR-101 in cervical cancer cell line (SiHa) was detected by real-time PCR. A miR-101 mimic was overexpressed in SiHa cells, and MTT assays were performed to determine the impact on cell proliferation. Cell wound healing assays and flow cytometry were used to detect migratory ability and cellular apoptosis, respectively. Immunohistochemistry was performed to assess protein expression of the miR-101 target gene COX-2. Results: MiR-101 was endogenously expressed in SiHa cells, and alterations in its expression had profound effects on cellular migration and invasion efficiency. Overexpression of miR-101 decreased proliferation in the MTT assay (the mimics at 490 nm absorbance is lower 60% than normal, and decreased cellular motility in the cell wound healing assay (transfected: 37 ± 2 m, pre-transfected 184 ± 2 m). Apoptosis rate was significantly higher with overexpression of miR-101 relative to control (transfected: 76.6%, pre-transfected: 3.5%) ($P < 0.05$). The expression of Cox-2 was decreased in transfected cells. Conclusions: MiR-101 likely acts as a tumor suppressor in cervical cancer. Overexpression of miR-101 decreased expression of its target gene Cox-2 and inhibited proliferation and invasion, and promoted apoptosis to suppress tumorigenicity. MiR-101 is a promising new target for the development of therapeutic strategies for the clinical treatment of cervical cancer.

Keywords: MiR-101, cervical cancer, proliferation, invasion, Cox-2

Introduction

Cervical cancer is the second most common form of cancer in women worldwide. Cervical cancer is the disease threatened women's life and health seriously, with the increase of its high suffering and death rate annually. On a global scale, approximately 500,000 new cases of cervical cancer are reported annually and about 230,000 women die due to cervical cancer every year [1]. In China alone there are 131,500 new cases each year, and in Xinjiang area, especially in the southern border region where economic conditions are poor, cervical cancer is one of the leading causes of death in Uygur minority (one of the minority nationality

in Xinjiang) women. The prevalence rate is 527/100,000, and presents early with an average age of 45.04 years (50.85 years for Han nationalities people) [2]. Regarding the etiology of cervical cancer, the leading cause is infected with human papilloma virus (HPV) [3]. However not all women infected with high-risk HPV will develop to cervical carcinoma, and it is apparent that many genetic and epigenetic alterations occur during its tumorigenesis. Aberrant regulation of miRNA and its target genes is one of the significant carcinogenesis of cervical cancer. Although progress has been made for the diagnosis and treatment of cervical cancer, there are still many unexplored areas, and a large part percentage of patients with meta-

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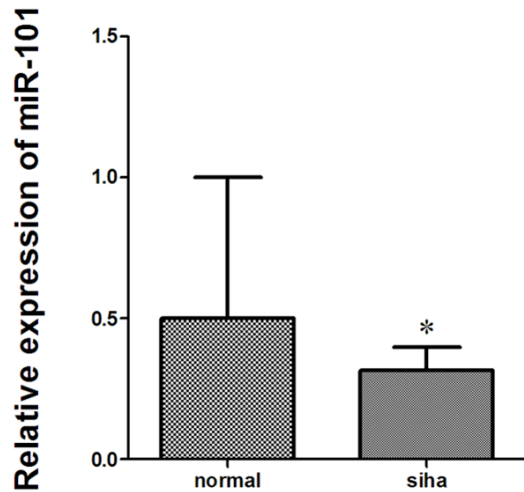


Figure 1. MiR-101 expression in human cervical epithelial cells of immortalized.

static or recurrent diseases have poor prognoses.

MicroRNAs (miRNA) are a class of approximately 22 nucleotide-long [4], endogenously expressed, highly conserved noncoding RNAs with important regulatory functions in tumor [5]. MiRNAs either act through imperfect base pairing with the untranslated region (UTR) of target genes or bind directly to their target mRNA through perfect base pairing [6]. It was found that at least 30% of protein-coding genes are regulated by miRNAs, and approximately 50% of miRNA genes are located in cancer-related genomes [7]. MiRNAs could regulate cancer development as tumor suppressors or oncogenes, and may be an ideal target for cancer prediction and treatment [8].

In our previous study, we identified via microRNA array a set of 12 miRNAs that were significantly decreased in cervical cancer tissues relative to control group [9]. MiR-101 was significantly down-regulated in cervical cancer tissues, implying that miR-101 may participate in the pathogenesis and development of cervical cancer. Our study is the first to identify an association between miR-101 and cervical cancer.

Material and methods

Cell culture and transfection

Human cervical epithelial cells of immortalized, Human cervical cancer cell lines (SiHa) were

cultured in 1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 50 U/mL of penicillin, and 50 mg/mL of streptomycin. SiHa cells were transfected with 10 μ L of miR-101 mimics (Invitrogen, Carlsbad, CA, USA) with Lipofectamine™2000 (Invitrogen). After 24 h, cells were collected for the following assays.

RNA isolation and real-time quantitative RT-PCR

Total RNA from frozen cultured cells was isolated using the TRIzol kit (Invitrogen) according to the manufacturer's instructions. RNA quantity was determined by UV measurement at OD 260/280 nm using NanoDrop 2000 (Thermo Scientific, Boston, MA, USA). To quantitate the expression level of mature miRNA-101, isolated RNA was reverse transcribed and amplified using the Thermo Scientific Revert Aid First Strand cDNA Synthesis kit according to the manufacturer's protocol. The U6 small nuclear RNA was amplified as a loading control. The primers for this U6 internal control were purchased from Invitrogen. Data are shown as fold change ($2^{-\Delta\Delta Ct}$) and analyzed initially using GraphPad Prism 5.0 software (La Jolla, CA, USA). Real-time PCR was performed using the QuantiFast SYBR Green PCR mixture (Invitrogen).

Cell wound healing assay

Migration ability of SiHa cells was determined using a cell-wound healing assay. For both normal cells and 24 h post-transfection, an area identified on the culture monolayer was monitored photographically for three consecutive days.

MTT proliferation assay

Cellular proliferation was measured with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Twenty-four h after transfection, SiHa cells (approximately 10^4) were seeded into 96-well culture plates for 24, 48, 72, and 96. The cells were incubated with 50 μ L of MTT (5 mg/mL) for 4 h at 37°C, and 150 μ L of DMSO was added for 20 min at room temperature to solubilize the crystals. The optical density was determined with a spectrophotometer at a wavelength of 490 nm.

miR-101 regulates the biological behavior of SiHa cells

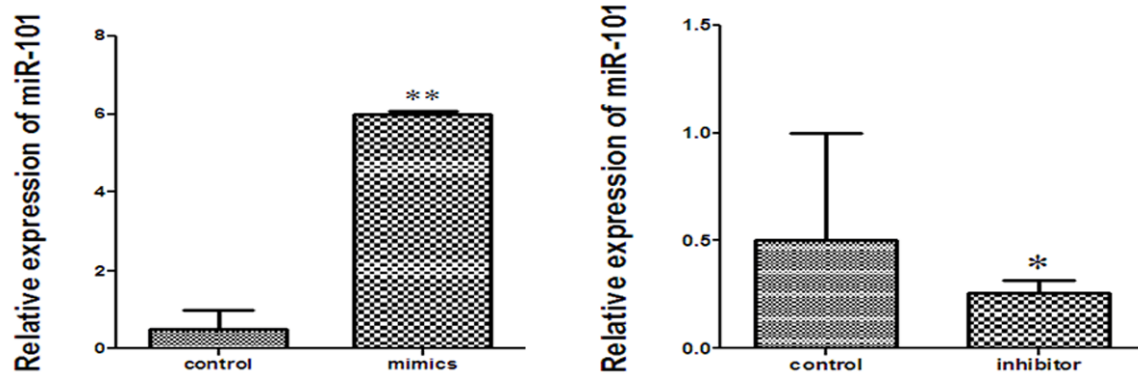


Figure 2. MiR-101 expression in cervical cancer cell line SiHa. MiR-101 expression was measured with RT-PCR. Transfection with miR-101 mimic increased levels of miR-101, and an inhibitor decreased these levels relative to untransfected control.

Apoptosis assays

The Annexin V-FITC Apoptosis Detection kit I (Abcam, Cambridge, UK) was used to detect and quantify apoptosis by flow cytometry. In brief, cells were harvested 24 h after transfection and collected by centrifugation for 5 min at $1000 \times g$. Cells were resuspended at a density of 1×10^6 cells/ml in $1 \times$ binding buffer, stained with FITC-labeled Annexin V for 5 min, and immediately analyzed by Beckman Coulter Epics Altra. The data obtained were analyzed using GraphPad Prism 5.0 software.

Immunohistochemistry

Immunohistochemistry was performed according to the kit instruction. Briefly, cells were washed twice with phospho buffered saline (PBS), and fixed with 4% paraformaldehyde for 15 min. The slides of cells were then incubated with 3% hydrogen peroxide for 15 min, and blocked with serum solution in 37°C for 20 min. The slides were incubated with Cyclooxygenase-2 (Cox-2) antibody (1:150, Boster, Fremont, CA, USA) overnight at 4°C . All sections were labeled using EnVision HRP (mouse) kit at room temperature for 30 min, incubated with 3, 3'-diaminobenzidine liquid substrate (Dako, Glostrup, Denmark), and counterstained with Mayer's hematoxylin (Dako). The number of positively stained cells was scored independently by two senior pathologists using the following criteria: 0 indicates that less than 5% of the cells were stained positively; 1 indicates 5% to 24%; 2 indicate 25% to 49%; 3 indicate 50% to 74%; and 4 indicates more than 74%.

Statistical analyses

All mapping were using GraphPad Prism 5.0 software. All statistical analyses were carried out using SPSS version 17.0 statistical software (SPSS Inc, Chicago, IL, USA). *P*-values less than 0.05 were considered to be statistically significant, and all of statistical tests were two-sided. Quantitative data were expressed as the mean \pm SD. χ^2 test was used to determine whether there were significant differences in the expression of Cox-2 protein. Student's *t* test and ANOVA were used to determine statistical significance, where differences were considered significant at $P < 0.01$.

Results

miR-101 expression in SiHa cervical cancer cell line

In order to confirm previous clinical observations that miR-101 was down-regulated in cervical cancer tissues, we investigated the expression of miR-101 in cervical cancer cell lines. MiR-101 expression was remarkably reduced in the SiHa cervical cancer cell. In addition, transfection with miR-101 mimics increased miR-101 levels as detected by RT-PCR 12 times of control, and miR-101 inhibitor decreased miR-101 levels by 50% (Figure 1).

miRNA-101 inhibits the proliferation of cervical cells in vitro

Because of the significant reduction of miR-101 expression in cervical cell lines, we next

miR-101 regulates the biological behavior of siHa cells

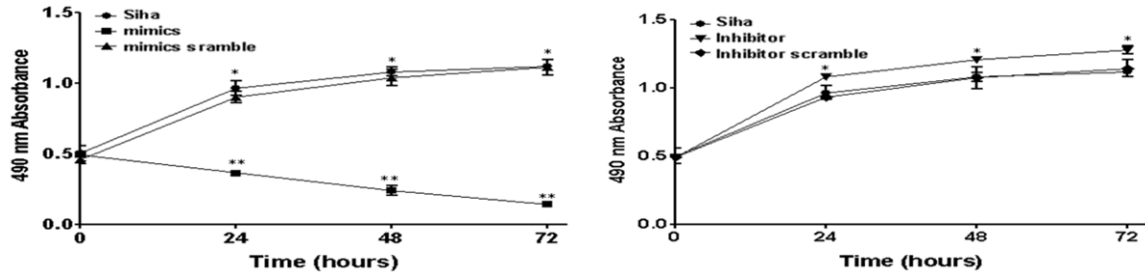


Figure 3. MiR-101 inhibits the proliferation of cervical cells in vitro. Proliferation of siHa cells was measured with MTT assay. MiR-101 mimic decreased, and miR-101 inhibitor increased cell proliferation at 24, 48, and 72 h.

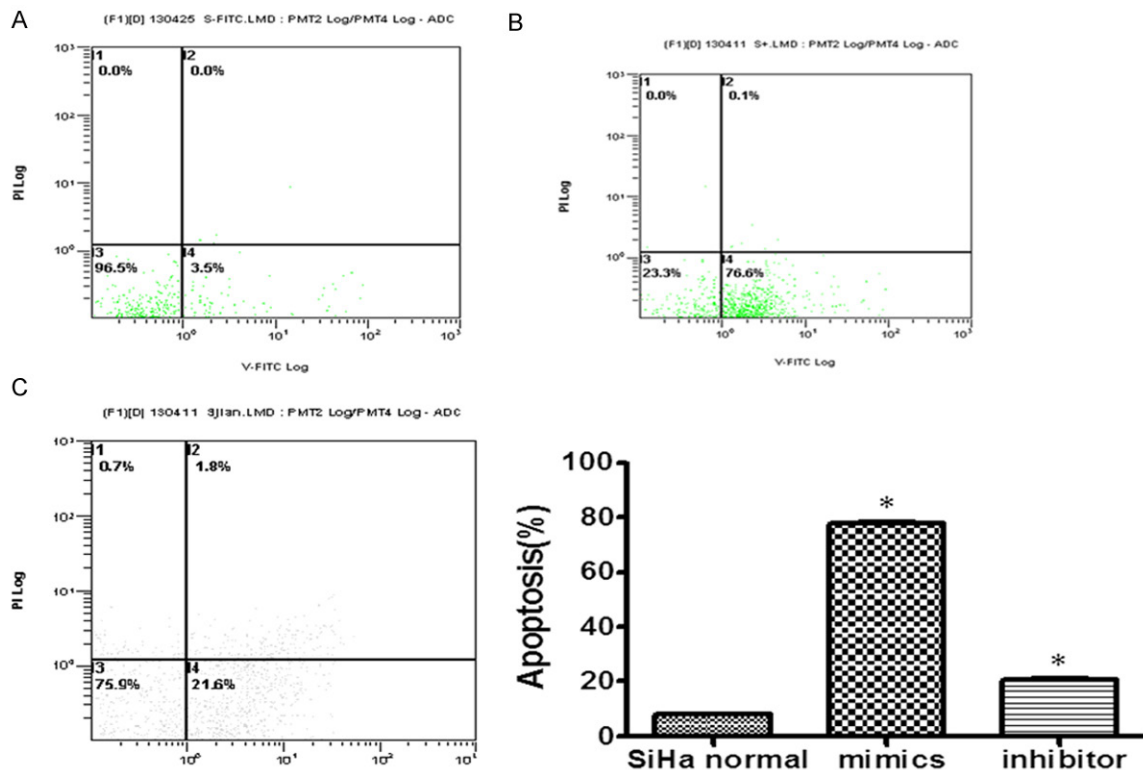


Figure 4. Ectopic expression of miRNA-101 induces apoptosis of siha cells. A: Flow cytometric analyses of propidium iodide-stained cells were performed in triplicate for each condition. B: Percentages of apoptotic cells are shown in the histogram. * $P < 0.01$ vs. control group. A: Normal siHa; B: Transfection miR-101 mimics; C: Transfection miR-101 inhibitor.

explored the possible biological significance of miR-101 in tumorigenesis. We measured cellular proliferation using the MTT assay in cells transfected with either miR-101 inhibitor or mimics for 24, 48, and 72 h. There was no significant difference in the proliferation rate at 24 h after transfection. However, SiHa cells treated with miR-101 mimics exhibited a 26% increase in growth rate compared with blank control ($P < 0.01$) at 48 h, and a 14% increase ($P < 0.01$) at 72 h (Figure 2). Conversely, at 48

and 72 h post transfection with the miR-101 inhibitor, cell proliferation was significantly decreased in SiHa cells. These results imply that MiR-101 might as a tumor suppressor in cervical cells in vitro.

miR-101 induces apoptosis in cervical cell lines

We also analyzed the effect of miRNA-101 on apoptosis of SiHa cells transfected with miR-

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Table 1. The positive cells of Cox-2 protein in different groups

Group	Positive	Negative	Positive rate (%)	P
SiHa	940	60	94.0	< 0.01
miR-101 mimics	710	290	71.0	
miR-101 inhibitor	980	20	98.0	
Total	2630	370		

101 inhibitor or mimics by conducting Annexin V and PI double staining assay. At 72 h after transfection, the early apoptosis rate in SiHa cells transfected with miR-101 mimic (76.6%) and inhibitor (21.6%) were significantly different ($P < 0.05$, **Figure 3**). Annexin-V-FITC/PI double staining assay also showed that miRNA-101 mimics induced apoptosis of SiHa cells.

MiRNA-101 depresses the invasion of cervical cells in vitro

Invasive growth is an important biological characteristic of cervical cancer cells. To evaluate the impact of miR-101 on the invasive ability and motility of SiHa cells, we employed a wound healing assay. In SiHa cells, miR-101 mimics inhibited cell migration, spread, and adhesion; and invasion of the matrigel layer was broader relative to both the control and inhibitor group. In the wound healing assay, the miR-101 mimics caused a marked restrained wound-healing rate in SiHa cells, whereas the miR-101 inhibitor moderately increased the wound-healing rate (**Figure 4**). Taken together, these findings demonstrate that miRNA-101 significantly reduces the invasion capability of cervical cancer cells.

COX-2 is a potential target of miR-101 in cervical cancer cells

MicroRNAs function by targeting specific, validated genes. To further understand the molecular mechanisms underlying miR-101 inhibition of cervical cancer cell growth and invasion, we searched for putative miR-101 targets through TargetScan, PicTar, and miRBase software. We identified COX-2 as a predictive target gene of miR-101. Since microRNA always regulates its target genes at the posttranscriptional level, we examined the level of Cox-2 protein by immunohistochemistry in SiHa cells. We found that Cox-2 protein expression in cells transfected

with the miR-101 inhibitor was significantly higher than both the mimic and control groups (**Table 1; Figure 5**). Therefore, expression of Cox-2 was elevated when miR-101 levels were decreased.

Discussion

MiR-101, belongs to a family of miRNAs that is involved in a series of cellular activities, e.g. cell proliferation, invasion, angiogenesis [10]. The expression pattern and targets of miR-101 vary in different types of tumors. Analysis of a series of cancer tissues and cells revealed widespread down expression of miR-101 in the majority of prostate cancers [11], hepatocellular carcinomas [12], and bladder cancers [13]. However, little information has been focused on the role of miR-101 in the regulation of its target gene expression in human cervical cancer [14]. In summary, miR-101 suppressed cell proliferation and impaired the invasive potential as a tumor suppressor.

In the present study, we examined miR-101 expression in cervical cancer cell line SiHa and explored the functional consequences of miR-101 regulation in human cervical cancer cells. In gain- and loss-of-function experiments, we demonstrated that miR-101 regulated cell proliferation, migration, and apoptosis in human cervical cancer cells. Furthermore, we identified a set of putative miR-101 target genes using a biochemical approach. Several of these candidate targets are functionally associated with cell proliferation and migration. We selected miR-101 as the microRNA most strongly correlated with COX-2 activation. Our findings provide an important lead for further investigations into the functional role of miR-101 in the development of cervical cancer. MiR-101 suppressed cell growth, inhibited cell migration, enhance cell apoptosis, and miR-101 negative regulating its target gene COX-2 in cervical cancer cell lines.

We confirmed that miR-101 in cervical cell line SiHa endogenously expressed was consistent with our previous findings in cervical cancer tissue [9].

To further evaluate the function of miR-101 as a tumor suppressor, we performed a series of in vitro studies. Functionally, miR-101 in SiHa cervical cells was either downregulated with an

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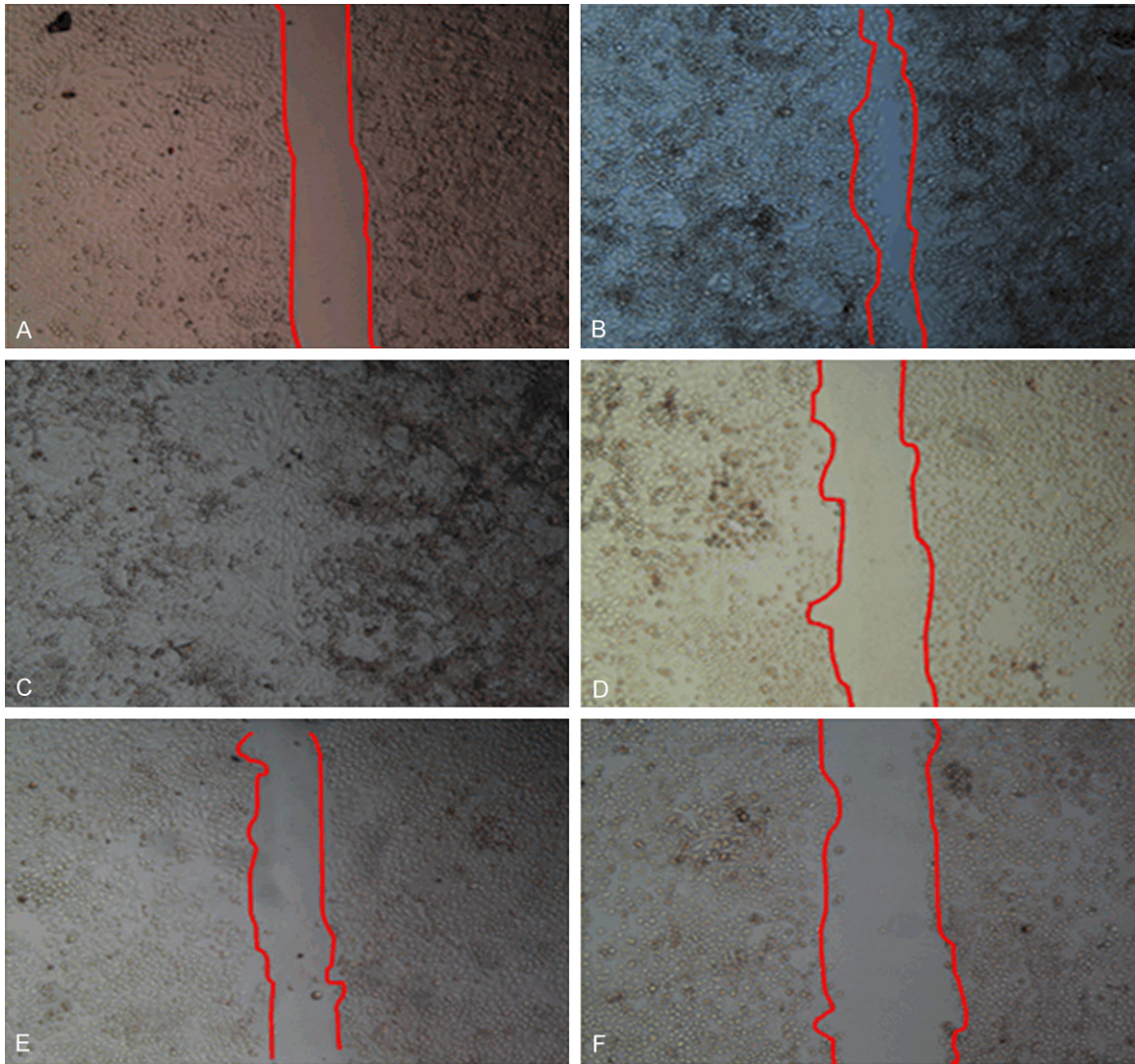


Figure 5. MiR-101 significantly inhibits tumor cell migration. Tumor cell (siHa) motility was examined by wound healing assay. Red dotted lines mark the edges of the scratch at the start of experiments, and green dotted lines mark the edges at the end of experiments. A: SiHa group 0 h; B: SiHa group 24 h; C: SiHa group 48 h. D: Transfection miR-101 mimics 0 h; E: Transfection miR-101 mimics 24 h; F: Transfection miR-101 mimics 48 h.

inhibitor or upregulated with a mimic. In miR-101-overexpressing cells, we observed a significant decrease in measures of cell proliferation, including decreased cell growth and increased cellular apoptosis. Following miR-101 inhibition in SiHa cells, proliferation was significantly decreased and apoptosis was increased. Our findings are consistent with reports observing similar effects on cell proliferation and apoptosis in other human cancer cells. For example, miR-101 inhibits cell proliferation and invasion and enhances paclitaxel-induced apoptosis in non-small-cell lung carcinoma (NSCLC) cells [15]. Our experiments indicate that miR-101 inhibits the motility and inva-

sion properties of SiHa cells. Taken together, these findings support our hypothesis that miR-101 functions as a tumor suppressor gene in cervical cancer.

The various effects of miR-101 in different tissues may be due to the specific targets repressed in each tissue. Indeed, different cancer tissues or cells have different target genes of miR-101, including EZH2 [16], EP4 receptor [17], COX-2 [18], and Fos [14]. Thus, miR-101 may be associated with a complex network of gene expression regulation that could be tissue and stage dependent. Predictions from PicTar and TargetScan DNA analysis software sug-

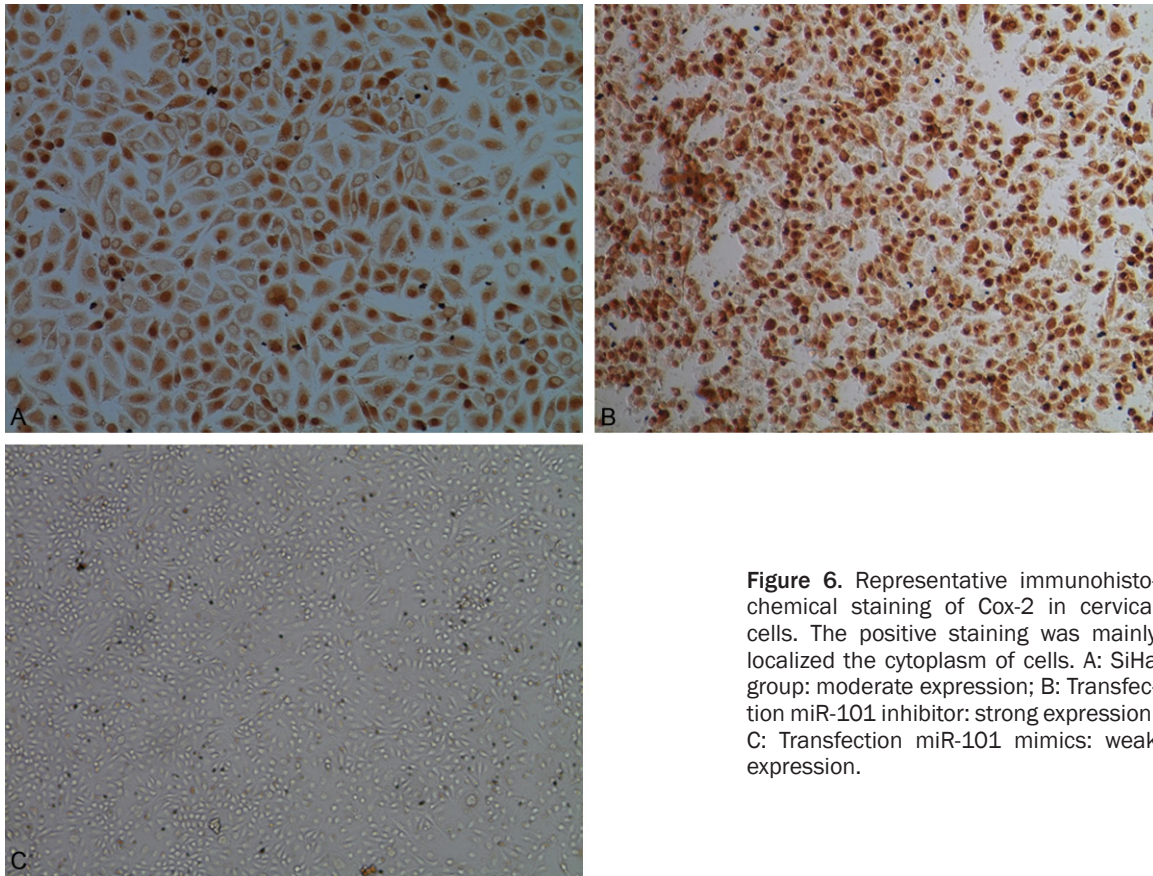


Figure 6. Representative immunohistochemical staining of Cox-2 in cervical cells. The positive staining was mainly localized the cytoplasm of cells. A: SiHa group: moderate expression; B: Transfection miR-101 inhibitor: strong expression; C: Transfection miR-101 mimics: weak expression.

gested that COX-2 could be one direct target for miR-101 because miR-101 has a seed region, which is able to bind to the COX-2 mRNA 3-UTR. Consistent with studies in other cancer cell lines, we observed an inverse relationship between miR-101 and Cox-2 expression (**Figure 6**). COX-2 is an attractive target for molecular imaging because it is an inducible enzyme that is expressed in response to inflammatory and proliferative stimuli. In addition, COX-2 is a proposed target for the prevention and treatment of cervical cancer. Based on our findings, we believe that miR-101 inhibits cell motility and migration partly through regulation of COX-2 expression and signaling.

Conclusions

In summary, we have demonstrated for the first time that miR-101 inhibits tumorigenesis in human cervical cancer by attenuating cell proliferation and migration and promoting apoptosis. Our findings suggest that miR-101 and its target gene Cox-2 may play important roles in the pathogenesis of cervical cancer, and miR-

101 (and its target genes) may be potential diagnostic or therapeutic targets for cervical cancer.

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Disclosure of conflict of interest

None.

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