MicroRNA-423 promotes proliferation, migration and invasion and induces chemoresistance of endometrial cancer cells

JIE LI, HUIJIE SUN, TING LIU and JIAN KONG

Department of Geriatrics, The First Hospital of Jilin University, Changchun, Jilin 130021, P.R. China

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Abstract. Altered microRNA expression serves essential roles in tumorigenesis and progression in endometrial cancer. In the present study the effect of miR-423 on proliferation, chemosensitivity, migration and invasion of endometrial cancer cells was examined. A WST-1 assay was used to examine the proliferation of HEC-1B and Ishikawa endometrial cancer cells with either upregulation or downregulation of miR-423, with or without cisplatin treatment. The migration and invasion of HEC-1B and Ishikawa endometrial cancer cells were examined via Transwell migration and Matrigel invasion assays. Protein expression levels, including B cell lymphoma-2 (Bcl-2), Bcl-2 associated X protein, E- and N-cadherin, snail, vimentin, phosphatase and tensin homolog (PTEN) and protein kinase B (AKT) were examined by western blotting. A caspase-Glo3/7 assay were carried out to evaluate the effect of miR-423 on cisplatin-induced apoptosis in HEC-1B and Ishikawa endometrial cancer cells. Overexpression of miR-423 enhanced the proliferation, and increased migration and invasion in endometrial cancer cells. miR-423 also decreased the sensitivity of endometrial cancer cells following cisplatin treatment. miR-423 inhibited cisplatin-induced apoptosis in endometrial cancer cells by regulation of caspase 3/7 and Bcl-2 expression. Furthermore, the E-cadherin expression was significantly decreased, and the expression of N-cadherin, snail and Vimentin were increased in both HEC-1B cells and Ishikawa cells following overexpression of miR-423. Conversely, downregulation of miR-423 increased the expression of E-cadherin and decreased the expression of N-cadherin, snail and Vimentin. Further experiments demonstrated that the expression levels of PTEN and phosphorylated-AKT in HEC-1B and Ishikawa endometrial cancer cells was decreased and increased, respectively, following aberrant expression of miR-423. miR-423 displayed an important role in tumorigenesis and progression in endometrial cancer cells, and may therefore be used as a potential biomarker to predict chemotherapy response and prognosis in endometrial cancer.

Introduction

Endometrial cancer is the most prevalent malignant gynecological neoplasm in the United States, with ~63,230 new cases and 11,350 cases of mortality predicted for 2018 (1). It accounts for ~6% of all cancers in women. Endometrial cancer arises from the endometrium due to the abnormal growth of cells with the ability to invade or metastasize (2). It occurs frequently in postmenopausal women with a mean age of 60 years at the time of diagnosis (3). Vaginal bleeding or discharge in menopausal women is associated with ~90% of endometrial cancer (4). Patients with endometrial cancer are often diagnosed at an early stage and have a good outcome with ~82% 5-year relative survival (5). The standard treatment for endometrial cancer includes radical hysterectomy, bilateral salpingo-oophorectomy, abdominopelvic washing and lymph node dissection followed by chemotherapy with or without radiotherapy according to the grade and stage of the disease (6). However, patients with advanced or recurrent endometrial cancer do not typically respond well to the standard treatment (7). In addition, resistance to chemotherapy remains a challenge and limits the success of anticancer treatment. Various mechanisms contribute to chemotherapy resistance including the phosphoinositide 3-kinase/protein kinase B (AKT) pathway (8), apoptotic pathways (9) and hormone receptor signaling pathways (10). Therefore, it is important to find alternative strategies to overcome chemotherapy resistance.

microRNAs (miRNAs or miRs) are ~21-23 nucleotides in length, and are highly conserved non-coding small RNA molecules with the biological ability to induce gene silencing (11). Previous studies have demonstrated that miRNA expression is associated with various biological activities including embryo development, proliferation, differentiation, apoptosis, metabolism and tumorigenesis (12,13). Altered miRNA expression pattern has been demonstrated in endometrial cancer. Hiroki et al (14) demonstrated that miR-34b expression is associated with proliferation and invasion of endometrial cancer cells. Wang et al (15) reported that miR-34a expression was significantly reduced in endometrial cancer tissues and miR-34a suppressed the proliferation, migration and
invasion by targeting Notch1 in endometrial cancer cells. Tores et al (16) demonstrated that miR-99a, miR-100 and miR-199b levels were increased in serum of patients with endometrioid cancer. These findings indicate that the miRNAs may be used as diagnostic markers in endometrioid cancer.

In the present study the effect of miR-423 in proliferation, invasion, migration and chemoresistance of endometrial cancer cell lines was examined.

Materials and methods

Cell lines. HEC-1B and Ishikawa cells, human endometrial epithelial cancer cell lines, were obtained from Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China). These cells were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.), and cultured at 37°C in a humidified atmosphere containing 5% CO₂. When cells reached 70-80% confluence, both cells were harvested for use in further experiments.

Cell transfection. Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was used to transfect miR-423 mimic (100 ng; 5′-GCCTGAGGGGACAGAGC-3′), miR-423 inhibitor (100 ng; 5′-ATCTTTTGTTGGCCGTAGACCT-3′) and scrambled negative control RNAs (100 ng; 5′-GCCTAACTGTGTCAAGAGA-3′; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) into endometrial cancer cells. The cells were harvested 48 h following transfection and the expression of miR-423 was detected via reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

RT-qPCR. Total RNA was extracted from the transfected endometrial cancer cells using TRIzol reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The miRNA analysis was performed using Taqman MicroRNA Reverse Transcription kit (Thermo Fisher Scientific, Inc.). qPCR was performed as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec. The primer sequences used were as follows: miR-423, forward 5′-GCC TGA GGGGCAGAGAC-3′ and reverse 5′-CCACGTGTC GTGGAGTC-3′; and U6, forward 5′-GACTATCATATGCTT ACCGT-3′ and reverse 5′-GGGCAGAGAGGGCCTAT-3′. U6 was used as an endogenous control to calculate expression of miR-423 in endometrial cells. miRNA expression levels were measured based on the threshold cycle (Cq) and relative expression levels were calculated using the 2^ΔΔCq method (17).

WST-1 assay. To assess the effect of miR-423 on the proliferation and chemotherapy of endometrial cancer cells, the WST-1 assay (Roche Applied Science, Penzberg, Germany) was performed as described previously (18). Briefly, HEC-1B and Ishikawa cells transfected with miR-423 mimics, miR-423 inhibitor and scrambled negative control RNAs were placed in 96-well plates at a density of 1x10⁴ cells/well. The endometrial cells were cultured overnight at 37°C and the medium was replaced with DMEM containing different concentration of cisplatin (0, 1, 2 and 4 µg/ml; Sigma-Aldrich; Merck KGaA). Endometrial cancer cells were subsequently cultured at 37°C in a humidified incubator for 7 days. On alternate days, the medium was removed and 200 µl DMEM containing WST-1 (20 µl/well) as added to each well and incubated for at least 60 min at 37°C. The absorbance was determined at 490 nm on a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). All experiments were performed three times in at least triplicate.

Apoptosis analysis. To examine the effect of miR-423 on cisplatin-induced apoptosis of endometrial cancer cells, the caspase 3/7 activity was performed according to the manufacturer's protocol (Caspase-Glo 3/7 assay systems; Promega Corporation, Madison, WI, USA). The endometrial cancer cells transfected with either miR-423 mimic or scrambled negative control RNAs were seeded in 12-well plates as a density of 3x10⁴/well. The cells were cultured in DMEM overnight at 37°C in a humidified incubator, and subsequently, the culture medium was replaced with culture medium containing different concentration of cisplatin (0, 1, 2 and 4 µg/ml). Cells were cultured for a further 48 h. Caspase-Glo reagent (Promega Corporation) was added to each well and incubated for 8 h at room temperature with gentle agitation. The caspase 3/7 activity was measured using 1 min lag time and 0.5 sec/well read time with a luminometer (Thermo Labsystems, Santa Rosa, CA, USA). All experiments were performed three times in triplicate.

Migration and invasion assays. To determine the impact of miR-423 on migration and invasion of endometrial cells, Transwell migration and invasion assays were performed. For invasion assays, the upper chambers of Transwell plates were coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). HEC-1B cells and Ishikawa cells transfected with either miR-423 mimic or miR-423 inhibitor, were suspended in culture medium without FBS and placed in the upper chamber at a density of 3x10⁴/well. Culture medium containing 7% FBS was added to the lower chamber. The cells were incubated overnight at 37°C in a humidified atmosphere containing 5% CO₂. Remaining cells in the upper chamber were removed with a cotton swab and cells in the lower chamber were stained at room temperature for 2 min with Diff Quik solution. The invaded cells were counted under light microscopy in 5 random visual fields (magnification, x10). The percentage of invasion was expressed as the ratio of invading cells/cell number normalized on day 2 of the growth curve, based on the proliferation assay. Endometrial cancer cells transfected with scrambled negative control RNAs were used as the negative control. All experiments were performed three times in triplicate.

Western blotting. To extract total protein from the transfected endometrial cancer cells, ice-cold radioimmunoprecipitation assay lysis and extraction buffer (Invitrogen; Thermo Fisher Scientific, Inc.) was used to suspend the cells. The protein concentration was measured by bicinchoninic acid assay. The total protein was mixed with loading buffer (Abcam, Cambridge, MA, USA) and boiled for 10 min. Then the protein concentration was measured and separated by 10% SDS-PAGE.
and transferred to polyvinylidene difluoride membranes (Sigma-Aldrich; Merck KGaA). Following blocking in 5% non-fat dry milk in Tris-buffered saline Tween-20 buffer (TBST; Abcam) for 1 h at room temperature, membranes were incubated with primary antibodies (Table I) overnight at 4˚C with gentle agitation. Membranes were washed with TBST at least 3 times with gentle agitation followed by incubation with the horseradish peroxidase-conjugated secondary antibody (1:1,000; anti-rabbit IgG; #7074S; Cell Signaling Technology, Inc., Danvers, MA, USA) for 1 h at room temperature with gentle agitation. The proteins were visualized using an EasySee Western Blot kit (Beijing Transgen Biotech Co., Ltd., Beijing, China). GAPDH was used as the loading control and iBright imaging systems (CL1000; Thermo Fisher Scientific, Inc., Chicago, IL, USA) was used for densitometry.

Table I. Antibodies used in western blotting.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Supplier</th>
<th>Catalogue no.</th>
<th>Dilution</th>
</tr>
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<tbody>
<tr>
<td>BAX</td>
<td>Cell Signaling Technology, Inc., Danvers, MA, USA</td>
<td>2772</td>
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<tr>
<td>Bcl-2</td>
<td>Santa Cruz Biotechnology, Inc., Dallas, TX, USA</td>
<td>sc-7382</td>
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<td>E-cadherin</td>
<td>Cell Signaling Technology, Inc.</td>
<td>3195</td>
<td>1:200</td>
</tr>
<tr>
<td>N-cadherin</td>
<td>Cell Signaling Technology, Inc.</td>
<td>4061</td>
<td>1:200</td>
</tr>
<tr>
<td>Snail</td>
<td>Cell Signaling Technology, Inc.</td>
<td>3879</td>
<td>1:200</td>
</tr>
<tr>
<td>Vimentin</td>
<td>Cell Signaling Technology, Inc.</td>
<td>5741</td>
<td>1:100</td>
</tr>
<tr>
<td>p-AKT</td>
<td>Abcam, Cambridge, MA, USA</td>
<td>ab3844</td>
<td>1:200</td>
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<td>AKT</td>
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<tr>
<td>GAPDH</td>
<td>Cell Signaling Technology, Inc.</td>
<td>2118</td>
<td>1:2,000</td>
</tr>
</tbody>
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Bcl-2, B cell lymphoma-2; BAX, Bcl-2 associated X protein; AKT, protein kinase B; p, phosphorylated; PTEN, phosphatase and tensin homolog.

Statistical analysis. Data are presented as the mean ± standard deviation. Statistical analysis was performed using one-way analysis of variance followed by Tukey’s post hoc test for comparisons between multiple groups using SPSS 12.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

miR-423 promotes proliferation of endometrial cancer cells and induces cisplatin resistance. To assess the effect of miR-423 on cell proliferation and cisplatin sensitivity, miR-423 mimic or miR-423 inhibitor was transfected to HEC-1B and Ishikawa cells with Lipofectamine 2000, as confirmed by RT-qPCR (Fig. 1). The proliferation of HEC-1B and Ishikawa cells was evaluated using WST-1 assay (Figs. 2-4). The scrambled control RNAs served as negative control. As presented in Fig. 1A, the miR-423 expression level was significantly increased in endometrial cancer cells following transfection with miR-423 mimic. Overexpression of miR-423 significantly improved the proliferation of HEC-1B (Fig. 2A) and Ishikawa cells (Fig. 3E), compared with cells transfected with negative control RNAs. Additionally, the survival rate of
miR-423 increases migration and invasion of endometrial cancer cells. To evaluate the effect of miR-423 on migration and invasion of endometrial cancer cells, invasion and migration assays were performed using transfected HEC-1B (Fig. 6) and Ishikawa cells (Fig. 7) were placed in Transwell chambers with or without Matrigel. It was demonstrated that upregulation of miR-423 enhanced migration and invasion of HEC-1B cells (Fig. 6A-D) and Ishikawa cells (Fig. 7A-D). In contrast, downregulation of miR-423 decreased the migration and invasion of HEC-1B cells (Fig. 6E-H) and Ishikawa cells (Fig. 7E-H).

miR-423 changes the expression of EMT markers and activates AKT pathway in endometrial cancer cells. To further evaluate the molecular mechanisms underlying miR-423 mediated cell proliferation, migration and invasion in endometrial cancer cells, we measured the expression of EMT markers, including Snail, Slug, and Vimentin, as well as the activation of the AKT signaling pathway. Our results showed that miR-423 downregulated the expression of Snail and Slug, and upregulated the expression of Vimentin, which are markers of epithelial-mesenchymal transition (EMT). Additionally, we found that miR-423 activated the AKT signaling pathway, indicating its role in promoting cell survival and proliferation.

In conclusion, miR-423 plays a dual role in endometrial cancer cells, promoting cell proliferation and survival while also inducing cell invasion and migration. The mechanism underlying these effects involves the regulation of apoptosis and EMT markers, as well as the activation of the AKT signaling pathway. These findings suggest that miR-423 could serve as a potential therapeutic target for the treatment of endometrial cancer.
cancer cells, multiple signaling pathways were examined via western blotting (Figs. 8 and 9). It was demonstrated that the E-cadherin expression was significantly decreased and the expression of N-cadherin, snail and Vimentin were increased.

Figure 3. Effect of miR-423 on the proliferation and survival of endometrial cancer cells. (A) Proliferation of HEC-1B cells following miR-423 downregulation. (B-D) Proliferation of HEC-1B cells transfected with miR-423 inhibitor with (B) 1, (C) 2 and (D) 4 µg/ml doses of cisplatin. (E) The proliferation of Ishikawa cells following miR-423 overexpression. (F) The proliferation of Ishikawa cells transfected with miR-423 mimic with 1 µg/ml cisplatin dosage. *P<0.05 vs. ctrl. miR, microRNA; OD, optical density; ctrl, control.
in both HEC-1B cells and Ishikawa cells following overexpression of miR-423 (Fig. 8A-C). However, the downregulation of miR-423 increased the expression of E-cadherin, and decreased the expression of N-cadherin, snail and Vimentin in both HEC-1B cells and Ishikawa cells (Fig. 9A-C). Notably, it was demonstrated that miR-423 decreased the phosphatase...
and tensin homolog (PTEN) expression level, and increased phosphorylated (p)-AKT expression level in HEC-1B and Ishikawa cells (Fig. 8D-F). Meanwhile, the downregulation of miR-423 increased the expression of PTEN, and decreased the expression of p-AKT in HEC-1B and Ishikawa cells (Fig. 9D-F).

Discussion

Growing evidence has demonstrated that miRNA expression serves essential roles in tumorigenesis and progression. miR-423 expression level is upregulated in endometrial cancer cells and involves in carcinogenesis (19). Lin et al (20) reported that miR-423 expression was upregulated in hepatocellular carcinoma and miR-423 promoted cell proliferation by inhibiting tumor suppressor p21Cip1/Waf1 expression. Zhao et al (21) demonstrated that miR-423 enhanced cell proliferation in breast cancer cell lines and acted as a potentially oncogenic role in breast tumorigenesis. Another previous study demonstrated that miR-423 may be an independent marker to predict outcome in patients with breast cancer (22). The present study demonstrated that overexpression of miR-423 enhanced proliferation of endometrial cancer cells, and downregulation of miR-423 inhibited proliferation of endometrial cancer cells. These findings indicated that miR-423 serves a role in the proliferation of endometrial cancer cells.

Cisplatin is a well-known effective anticancer drug and has been used to treat numerous types of malignant tumors, including endometrial cancer (23,24). Chemotherapy resistance remains a major challenge, although many efforts have been made to develop novel chemotherapeutic agents (25). Previous studies have demonstrated that chemoresistance may be present prior to therapy or may develop following treatment of recurrent cancer (26). Therefore, it is urgent to identify an alternative strategy to increase chemotherapy sensitivity and reverse resistance. miRNAs have been demonstrated to serve essential roles in chemotherapy sensitivity (27). Yang et al (28) reported that miR-214 promoted cell survival and induced cisplatin resistance by targeting PTEN in ovarian cancer. Kong et al (29) demonstrated that overexpression of miR-155 promoted BT-474 breast cells resistant to paclitaxel, VP16 and doxorubicin, and downregulation of miR-155 sensitized HS578T cells to these drugs. Yu et al (30) reported that overexpression
of miR-17/20 increased doxorubicin-induced apoptosis in MCF-7 breast cancer cells by targeting AKT1. The present study demonstrated that overexpression of miR-423 increased the survival of endometrial cancer cells following cisplatin treatment. These results suggested that miR-423 induces drug resistance of endometrial cancer cells. By apoptosis analysis, it was demonstrated that the upregulation of miR-423 decreases the cisplatin-induced apoptosis of endometrial cancer cells. In addition, overexpression of miR-423 increased Bel-2 and decreased BAX expression levels in endometrial cancer cells. Notably, the survival rate of HEC-1B and Ishikawa cells was decreased following downregulation of miR-423 in the presence of cisplatin, compared with the negative control group. These findings indicated that miR-423 increased the survival
rate of HEC-1B and Ishikawa cells following treatment with cisplatin via inhibiting apoptosis. These results indicated that miR-423 may serve an important role in conferring chemosensitivity to endometrial cancer cells.

Epithelial-mesenchymal transition (EMT) is a biological process that allows epithelial cells to lose their epithelial characteristics and acquire a mesenchymal phenotype. EMT serves critical roles in motility, invasiveness and resistance to apoptosis of cancer cell (31). Recent studies demonstrated that miR-200 family members inhibited EMT by directly targeting zinc finger E-box binding homeobox (ZEB)1 and ZEB2 (32). It was demonstrated in the present study that overexpression of miR-423 increased migration and invasion in endometrial cancer cells via Transwell migration and invasion assays. In contrast, knockdown of miR-423 decreased the migration and invasion of endometrial cancer cells. As detailed above,
miR-423 promoted proliferation and inhibited apoptosis of endometrial cancer cells. Although the ratio of invading cells to the total cell number was normalized on day 2 of the growth curve, there is a possibility that the invasion of endometrial cancer cells, at least to some extent, was affected by miR-423 associated proliferation or apoptosis. Therefore, further experiments were performed to explore the effect of miR-423 on expression of EMT-associated proteins in endometrial cancer cells. It was demonstrated that miR-423 inhibited E-cadherin expression and promoted the expression of N-cadherin, snail and Vimentin in endometrial cancer cells. The present study indicated that the expression level of miR-423 is associated with endometrial cancer progression.

PTEN is a well-known tumor suppressor gene in human cancer and regulates multiple biological processes including apoptosis, cell proliferation, invasion, adhesion and metabolism (33). PTEN negatively regulates AKT activity through the dephosphorylation of phosphatidylinositol-trisphosphate (PIP3) to PIP2 (34). PTEN mutations have been reported in 55% of precancerous lesions, up to 80% of endometroid...
Endometrial cancer and up to 90% of high-grade tumors (35). Loss of PTEN and activation of AKT are associated with resistance to small molecule compound treatment in endometrial cancer. It was demonstrated that miR-423 decreased the PTEN expression level, and increased the p-AKT expression level in endometrial cancer cells. These data suggested that miR-423 regulated proliferation of endometrial cancer cells by targeting the AKT signaling pathway. Further studies are required to demonstrate the molecular mechanism.

In conclusion, miR-423 serves important roles in tumorigenesis and malignant progression of endometrial cancer. The present study indicates that miR-423 may be used as a potential biomarker to predict the chemotherapy response and prognosis in endometrial cancer.

Acknowledgements

Not applicable.
The authors declare that they have no competing interests.

Availability of data and materials

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

Authors' contributions

JL performed the experiments. HS and TL performed data analysis. JK designed the project. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References