miR-215 promotes epithelial to mesenchymal transition and proliferation by regulating LEFTY2 in endometrial cancer

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Abstract. Endometrial cancer (EC) is the most common gynecological tumor in developed countries with an increasing incidence. Left-right determination factor 2 (LEFTY2), a suppressor of cell proliferation and tumor growth, is a negative regulator of EC progression. The roles of LEFTY2 are emerging; however, the regulatory mechanisms of its expression have not been well understood. MicroRNA (miR)-215 as an oncogene serves an important role in tumorigenesis by regulating target genes. In the present study, it was demonstrated that overexpression of miR-215 promoted epithelial to mesenchymal transition (EMT), colony formation and DNA synthesis in EC HEc-1A cells and its expression was upregulated in EC tissues. Using online miR target prediction software, it was revealed that LEFTY2 is predicted as a target of miR-215. Using western blot analysis and immunofluorescence assays, it was demonstrated that overexpression of miR-215 markedly downregulated LEFTY2 protein expression levels in HEc-1A cells and LEFTY2 protein expression was downregulated in EC tissues, which was inversely correlated with miR-215 expression. Furthermore, the present study indicated that overexpression of LEFTY2 protein promoted mesenchymal to epithelial transition and sensitized HEC-1A cells to cisplatin treatment. In addition, it was revealed that the overexpression of LEFTY2 inhibited colony formation and DNA synthesis in HEC-1A cells. Thus, miR-215 may promote EMT and proliferation by regulating LEFTY2 in EC.

Introduction

Endometrial cancer (EC) is the most common gynecological tumor in developed countries, and its prevalence is increasing (1). Two clinicopathological subtypes of EC exist: The estrogen-related (type I) endometrioid adenocarcinoma and the non-estrogen related (type II) non-endometrioid adenocarcinoma (2). Patients with EC are often diagnosed when the disease is still confined to the uterus (2). Despite the overall prognosis for EC being good, relapse will eventually occur in some patients who may then ultimately succumb to the disease (2). Therefore, a thorough understanding of the molecular basis of EC progression may contribute to the identification of novel therapeutic targets to improve patient outcome.

Epithelial to mesenchymal transition (EMT) is an essential process that drives plasticity during development; however, it is also an unintentional behavior of cells during the progression of malignant tumors (3-5). As a result of EMT, epithelial cells lose their defined cell-cell/cell-substratum contacts and their structural/functional polarity, and they become spindle-like (5). The alteration of several layers of regulation, including the transcription and translation machinery, expression of non-coding RNA, alternative splicing and protein stability, result in disturbance of a controlled epithelial balance (5-7).

MicroRNA (miR), a class of small non-coding RNA, regulate gene expression by facilitating mRNA degradation or translational inhibition (8). They serve important roles in development, cellular differentiation, proliferation, cell cycle control and cell death (8-12), and have been demonstrated to be involved in various human diseases, such as cancer (9,13). Aberrant miR-215 expression has been implicated in cancer progression by regulating target genes (14-16).

Left-right determination factor 2 (LEFTY2), also known as endometrial bleeding-associated factor, is secreted as a 42-kda precursor susceptible to proteolytic cleavage (17). It is a member of the transforming growth factor (TGF)-β family (18,19) and its active forms induce mitogen-activated protein kinase activity and inhibit TGF-β signaling (17). LEFTY2 expression is low in healthy endometrium; however, its expression levels are increased prior to or during menstrual bleeding (20). Recently, it has been reported that LEFTY2 is able to downregulate marker of proliferation Ki-67 expression and focal adhesion kinase activity (21). It may upregulate E-cadherin expression and inhibit proliferation and migration in EC cells (21). The present study demonstrated that miR-215 promotes epithelial to mesenchymal transition and proliferation by regulating LEFTY2 in EC.
promoted EMT, colony formation and DNA synthesis by regulating LEFTY2 in EC.

Materials and methods

Clinical specimens. The Ethics Committee of The Fourth Affiliated Hospital of Harbin Medical University (Harbin, China) approved the present study and patient consent was obtained prior to tissue collection. A total of 58 samples were obtained from 58 patients at The Fourth Affiliated Hospital of Harbin Medical University between January 2014 and January 2016. The patients diagnosed as EC ranged in age from 35-70 years-old. Snap-frozen EC samples were obtained for type (type I endometrioid Ec). Normal endometrial chemotherapy or radiation, and were histologically validated from patients undergoing hysterectomy, without preoperative resection and stored at -80˚C until use.

Samples were frozen in liquid nitrogen immediately after samples were obtained from adjacent normal tissues. All precursor (pre-miR-215; sequence: 5'-AUG ACC UAU GAA UUG ACA UAU CGC A-3') were purchased from Ambion (Thermo Fisher Scientific, Inc.). For transfection experiments, cells were cultured in serum-free medium without antibiotics) for 24 h. Subsequently, MTT, reverse transcription-quantitative polymerase chain reaction (RT-qPCR), western blotting, immunocytochemistry and colony formation assays were performed as described below.

Western blot analysis. Western blot analysis was performed as previously described (23-28). Total protein was prepared using extraction buffer comprising NaCl/P, containing 0.5% Triton X-100, 1 mM EDTA, 1 mM PMSF, and complete protease inhibitors (Roche Diagnostics, Basel, Switzerland). The concentration of each protein lysate was determined using a BCA™ protein assay kit (Thermo Fisher Scientific, Inc.). Equal quantities (20 µg) of total protein were subjected to 12% SDS-PAGE. The samples were then transferred onto nitrocellulose membranes and blocked for 60 min at room temperature in 5% skim milk powder in NaCl/Pi. The membranes were immunoblotted using antibodies against human LEFTY2 (ab204283; 1:500; Abcam, Cambridge, MA, USA), E-cadherin (ab40772; 1:500) and vimentin (ab92547; 1:500; all from Abcam), snail (ab82846; 1:500 Abcam) and β-actin (ab8227 1:500; Abcam) overnight at 4˚C. IRDye®-800 conjugated anti-rabbit secondary antibody (1:10,000; ab191866, Abcam) was used for incubation at room temperature for 30 min. The specific proteins were visualized using an Odyssey™ infrared imaging system (Gene Company, Ltd., Lincoln, NE, USA). The expression of β-actin was used as an internal control to ensure equal loading of the protein samples.

5-bromo-2-deoxyuridine (BrdU) incorporation assay. A BrdU assay was performed as previously described. For BrdU labeling, BrdU was added into cell culture medium at a concentration of 50 µM for 4 h. Cells were then fixed with 70% of ethanol for 1 h at 37°C in an atmosphere containing 5% CO₂, permeabilized and incubated with the BrdU antibody (1:500; ab152095, Abcam, Cambridge) for 1 h at 37°C followed by a Alexa Fluor®-conjugated secondary antibody (1:500; ab150077, Abcam) for 30 min at 37°C in an atmosphere containing 5% CO₂. Finally, the cells were stained with propidium iodide for 30 min at 37°C. Microscopic analysis was performed with a confocal laser-scanning microscope (Leica Microsystems GmbH, Wetzlar, Germany). A total of 200 cells were counted and percentages of positive cells presented.

Colony formation assay. A colony formation assay was performed as previously described (29,30). Each well (100 mm) of a culture dish was coated with 5 ml bottom agar mixture [DMEM, 10% (v/v) Fetal calf serum (FCS, R92157; Thermo Fisher Scientific, Inc.), 0.6% (w/v) agar]. Following solidification of the bottom layer, 5 ml top agar medium mixture [DMEM, 10% (v/v) FCS, 0.3% (w/v) agar] containing 5x10⁴ cells was added, and the dishes were incubated at 37°C for 4 weeks. Plates were stained with 2 ml 0.005% crystal violet for 1 h at room temperature and then a light microscope (Olympus Corporation, Tokyo, Japan) was used to count the number of colonies. Discrete colonies containing 50 or more cells were counted with the microscope.

RT-qPCR. Total RNA was extracted from the cells by homogenizing cells in TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer’s protocol. Total
RNA (500 ng) was quantitated at 260 nm and reverse-transcribed into cDNA using the PrimeScript RT reagent kit (Takara Biotechnology, Co., Ltd., Dalian, China) according to the manufacturer’s protocol, at 37˚C for 15 min and 85˚C for 30 sec.

The cDNA then served as the template for SYBR qPCR using Power SYBR-Green PCR Master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). All reactions were run in triplicate on an iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using miR-215/429-specific primers (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following primers were used: miR-215, forward 5'-GGGTCCGAGGTATTCCGACT-3', and reverse, 5'-CGATGACCTATGAATTGACAGC-3'; miR-429, forward 5'-UAUACUGUCCGUAACCGU-3', and reverse 5'-UUCUCGCGAACGUGACCGUT-3' and U6, forward 5'-GCTTCGCGACGACATATAA-3' and reverse, 5'-AACGGTCACGATTTCGCT-3. The amplification profile was as follows: Denaturation at 95˚C for 10 min, followed by 40 cycles of denaturation at 95˚C for 15 sec, annealing at 60˚C for 30 sec and extension at 72˚C for 1 min. The comparative cycle threshold method was applied to quantify the miR expression levels. The relative amount of miR-215/miR-429 to small nuclear U6 RNA was calculated using the equation 2^-ΔCq, where ΔCq=ΔCq miR-215/miR-429-ΔCq U6 RNA). The fold change of gene expression was calculated using the 2^-ΔΔCq method (31). U6 small nuclear RNA was used as the internal standard.

Bioinformatics analysis. The analysis of potential miR target sites was performed using a commonly used prediction algorithm, miRanda (mircrorna.org/microrna/home.do) (32). The miR search function of the website was utilized to search for target genes of miR-215 for the species of Homo sapiens.

Immunofluorescence staining. Immunofluorescence was performed as described previously (33). HEc-1A cells were plated on glass coverslips in six-well plates and transfected with pre-miR-215 and control miR (mock). At 48 h after transfection, the cells were fixed in 4% paraformaldehyde for 15 min at room temperature, and then blocked with goat serum type to a spindle-like phenotype. No marked morphological changes were observed in ESCs (Fig. 1B).

To determine whether miR-215 regulates colony formation of HEc-1A cells, a colony formation assay was performed. The results revealed that overexpression of miR-215 significantly promoted colony formation in HEc-1A cells compared with that observed in the mock group (Fig. 1C).

Overexpression of miR-215 promotes EMT in HEc-1A cells. To identify the effects of pre-miR-215 and pre-miR-429 in HEc-1A cells and ESCs, HEc-1A and ESCs were transfected with pre-miR-215 and pre-miR-429. RT-qPCR was subsequently performed to detect their expression. The results demonstrated that miR-215 and miR-429 expression was evidently upregulated in the cells transfected with pre-miR-215 or pre-miR-429, respectively, compared with the respective mock (Fig. 1A). Additionally, the present results indicated that overexpression of miR-215 induced notable morphological changes in HEc-1A cells (EMT; Fig. 1B). For example, cells morphologies were altered from a cobblestone-like phenotype to a spindle-like phenotype. No marked morphological changes were observed in ESCs (Fig. 1B).

Overexpression of miR-215 promotes colony formation and DNA synthesis in HEc-1A cells. To determine whether miR-215 regulates colony formation of HEc-1A cells, a colony formation assay was performed. The results revealed that overexpression of miR-215 significantly promoted colony formation in HEc-1A cells compared with that observed in the mock group (Fig. 2A). In addition, DNA synthesis was detecte in HEc-1A cells transfected with pre-miR-215 or control miR by a BrdU incorporation assay. The results demonstrated that pre-miR-215 significantly promoted DNA synthesis in HEc-1A cells compared with the levels observed in the mock group (Fig. 2B).
miR-215 expression is upregulated in EC tissues. RT-qPCR was performed to detect miR-215 expression in EC tissues and adjacent normal tissues. The results demonstrated that miR-215 expression was significantly upregulated in EC tissues compared with the levels in adjacent normal tissues (Fig. 3).

miR-215 inhibits LEFTY2 protein expression in HEC-1A cells. miR are a class of small non-coding RNA (~22 nucleotides in length) that negatively regulate protein-coding gene expression by targeting mRNA degradation or translation inhibition (38-42). Therefore, it was hypothesized that miR-215 may regulate EMT and colony formation by regulating target gene expression. miRanda was utilized to screen target genes of miR-215. The website identified multiple target genes; the present study focused on LEFTY2 as it has been proposed as a tumor suppressor gene (18,19). Sequences of target sites on the 3’-untranslated region (UTR) of LEFTY2 are demonstrated in Fig. 4A (partial match, not exact match). Identical sequences were found in the human (H. sapiens), mouse (Mus musculus) and rat (Rattus norvegicus) mRNA orthologues (data now shown).

Western blotting and immunofluorescence assays were performed to detect LEFTY2 expression in HEC-1A cells.
transfected with pre-miR-215 and control miR. The results demonstrated that LEFTY2 protein expression levels were notably downregulated in HEC-1A cells transfected with pre-miR-215 compared with the levels in the mock group (Fig. 4B and C).

LEFTY2 is downregulated in EC tissues. Western blotting was performed to analyze LEFTY2 protein expression in EC tissues and adjacent normal tissues. The results demonstrated that LEFTY2 protein was significantly downregulated in EC tissues compared with the levels in adjacent normal tissues (Fig. 5).

LEFTY2 is inversely correlated with miR-215 expression in EC tissues. Spearman's correlation was used to analyze the correlation between miR-215 and LEFTY2 expression levels.
Linear correlation analysis indicated a significant inverse correlation between miR-204 and LEFTY2 protein expression in EC (P=-0.56; P<0.01; data not shown).

**LEFTY2 promotes mesenchymal to epithelial transition (MET) and upregulates sensitivity to cisplatin in HEC-1A cells.** To identify the effects of LEFTY2 in regulating EMT, cells were transfected with LEFTY2-expressing plasmids or empty vectors and western blotting was then performed to detect LEFTY2 protein expression. The results revealed that LEFTY2 protein expression was upregulated by LEFTY2-expressing plasmids compared with the level in the mock group (Fig. 6A). Although overexpression of LEFTY2 did not notably promote morphological changes in HEC-1A cells (Fig. 6B), it was revealed that its overexpression downregulated vimentin expression (Fig. 6C).

In order to determine whether LEFTY2 affects cisplatin efficacy in HEC-1A cells, cells were transfected with LEFTY2-expressing plasmids and empty vectors and then treated with various concentration of cisplatin. An MTT assay was performed in HEC-1A cells treated as indicated (Fig. 6D). The results demonstrated that overexpression of LEFTY2 sensitized HEC-1A cells to cisplatin (Fig. 6D).

**Overexpression of LEFTY2 inhibits colony formation and DNA synthesis in HEC-1A cells.** In order to identify whether LEFTY2 expression affects colony formation in HEC-1A cells, colony formation assays were performed. The results demonstrated that overexpression of LEFTY2 significantly inhibited colony formation in HEC-1A cells compared with the level observed in the mock group (Fig. 7A).

To evaluate whether DNA synthesis was affected by LEFTY2 in HEC-1A cells, a BrdU incorporation assay was performed to detect DNA synthesis in HEC-1A cells transfected with LEFTY2-expressing plasmids and empty vectors. The results demonstrated that LEFTY2 significantly inhibited DNA synthesis in HEC-1A cells compared with the level in the mock group (Fig. 7B).

**Discussion**

EMT is one of the key processes discussed in regards to progression and metastasis of a wide range of cancer types, including EC (43-46). miR-429 has been suggested as a member of the miR-200 family, and four members of this family have been identified to serve crucial roles in the modulation of EMT in a number of tumor types (47-51). However, the present study demonstrated that overexpression of miR-429 could not affect the epithelial or mesenchymal status in HEC-1A cells and ESCs, which indicated that miR-429 was not a regulator of EMT in these cell types.

miR-215 may promote malignant progression in gastric cancer (16,52,53). However, to the best of our knowledge, there have been no previous studies regarding the role of miR-215 in EC. The present study revealed that overexpression of miR-215 promoted EMT and colony formation in HEC-1A cells. Increased DNA synthesis is able to promote colony formation (54). The present study demonstrated that overexpression of miR-215 induced DNA synthesis when it promoted EMT and colony formation in HEC-1A cells. Additionally, the present study indicated that miR-215 was upregulated in EC tissues compared with the levels in adjacent normal tissues. However, Myatt et al (10) did not detect any significant difference between tumor tissues and corresponding normal samples. The present study only used type I endometrioid EC samples, in contrast with the previous report (10), and so the different results observed may be due to the different sample types.
LEFTY2, a regulator of cell proliferation, tumor growth, embryonic differentiation and stemness, is a negative regulator of cancer progression (21). The present study identified that LEFTY2 is a target of miR-215 by bioinformatics and revealed that miR-215 inhibits LEFTY2 protein expression in HEC-1A cells. In addition, LEFTY2 is inversely correlated with miR-215 expression, which further demonstrated that miR-215 may regulate LEFTY2 protein expression in EC. Unfortunately, the present study failed to confirm whether miR-215 directly targets LEFTY2 in EC as a luciferase reporter assay using plasmids of the 3'-UTR of LEFTY2 was not performed. In addition, the present study identified that overexpression of LEFTY2 inhibited colony formation and DNA synthesis, and its overexpression promoted MET in HEC-1A cells. This is in agreement with previous studies that observed the overexpression of LEFTY2 to upregulate E-cadherin expression (21,55). Epithelial status and E-cadherin expression have been proposed as markers of chemotherapy sensitivity (56). The present study demonstrated that overexpression of LEFTY2 sensitized HEC-1A cells to cisplatin treatment.

In conclusion, elucidating the mechanism by which miR-215 promotes EMT and proliferation by regulating LEFTY2 in EC will aid in further understanding the molecular basis of EMT and proliferation of the disease. Thus, suppression of miR-215 represents a novel therapeutic strategy to reverse EMT. However, the roles of miR-215 and LEFTY2 should be further investigated in vivo.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions
XG, YC and RA made substantial contributions to the concept and design of the present study; XG and RA drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The present study was approved by the Ethics committee of the Fourth Affiliated Hospital of Harbin Medical University (Harbin, China). Written informed consent form at the time of enrollment was obtained from each patient.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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