MicroRNA-424 suppresses estradiol-induced cell proliferation via targeting GPER in endometrial cancer cells

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Abstract  
Endometrial carcinoma (EC) is the most common gynecologic malignancy with increasing morbidity in recent years. MicroRNAs (miRNAs), a type of non-coding RNA, have been proven to be critical in the process of tumorigenesis. miR-424 has been reported to play a protective role in various type of cancer including endometrial carcinoma. It has been reported that high levels of estrogen increase morbidity of EC by promoting cell growth ability. The current research was designed to delineate the mechanism of miR-424 in regulating E2 (17β-estradiol)-induced cell proliferation in endometrial cancer. In this study, we confirmed that cell proliferation is increased significantly in E2-treated endometrial carcinoma cell lines. Moreover, miR-424 overexpression dramatically decreased E2-induced cell proliferation, indicating a pivotal role in endometrial cancer cell growth. In addition, the results suggest that miR-424 up-regulation inactivated the PI3K/AKT signaling, which was mediated by G-protein-coupled estrogen receptor-1 (GPER) in endometrial cancer. Furthermore, the luciferase report confirmed the targeting reaction between miR-424 and GPER. After transfection with the GPER overexpression vector into E2-induced endometrial cancer cells, we found that GPER significantly attenuated the inhibition effect of miR-424 in E2-induced cell growth in EC. Taken together, our study suggests that increased miR-424 suppresses E2-induced cell growth, and providing a potential therapeutic target for estrogen-associated endometrial carcinoma.

Key words: Endometrial carcinoma, miR-424, PI3K/AKT, GPER.

Introduction

Endometrial carcinoma (EC) is one of the most common genital tract malignancy cancers diagnosed in females with increasing morbidity reported worldwide in recent years (1). In spite of advances in diagnostics and new therapeutic strategies that have been applied in the treatment of endometrial cancer, the death rate of EC has still increased in the last 20 years (2). Therefore, the identification of molecular targets would be useful to improve the outcome of patients with endometrial carcinoma.

MicroRNAs (miRNA) are a class of small RNAs (size of 21-24 nucleotides) that function as major important regulators in gene expression by interacting with the 3'-untranslated region (3'-UTR) of their target mRNA (3). Since their discovery, research has demonstrated that miRNAs play an important role in regulating the key process in cellular life cycle including stem cell division, developmental timing, senescence and apoptosis (4). Therefore, these are believed to participate in the development of various human diseases, such as Alzheimer's disease (AD), and cardiovascular and autoimmune disease (5). It has been reported that more than half of human miRNA genes are located at sites or regions that are easily dysregulated in cancer (6). In addition, the abnormal expression of miRNAs has been detected in several cancers, including colon, breast and lung cancer (7). Gynecologic malignancies such as endometrial cancer are usually characterized by both genetic and epigenetic anomalies, including the abnormal expression of miRNAs, leading to altered gene expression and regulating the survival and proliferation of cells (8).

It has been reported that miR-424 is involved in several human malignancies, such as hepatocarcinoma, pancreatic cancer and cervical cancer, and plays various roles in tumor progression. Recently, miR-424 has been reported to be a protective factor in endometrial carcinoma (9). However, its roles and the underlying mechanism involved in endometrial carcinoma remain poorly undefined. Using bioinformatics predictions, we identified G-protein-coupled estrogen receptor-1 (GPER) to be the potential target gene of miR-424. GPER is a seven-transmembrane receptor that binds estrogen (17β-estradiol, E2) and elicits non-genomic, rapid signaling as well as genomic transcriptional events of estrogen. It is well-known that high estrogen levels elevate the risk of endometrial cancer in patients through promoting the cell growth ability of endometrial cancer cells (10), which is associated with GPER expression (11).

In the present study, the cell proliferation in E2-induced endometrial cancer cells was determined. Importantly, we investigated the role of miR-424 in E2-induced EC cells, and further inspected the potential regulation and underlying mechanism of miR-424 on GPER.

Materials and methods

Cell lines and cell culture  
Human endometrial carcinoma cell lines Ishikawa and HEC-1A were obtained from the American Type Culture collection (ATCC, Manassas, VA). The two cell lines were cultured in Dulbecco Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco, Rockville, MD), 100 μg/ml streptomycin and penicillin. For E2 treatment, Ishikawa and HEC-1A cells were cultured with 1 μM of E2 (Sigma, St. Louis, MO, USA) in DMEM medium containing...
10% FBS. Cells were incubated in a humidified atmosphere with 5% CO₂ at 37°C.

**Oligonucleotide transfection**

miRNAs were synthesized by RiboBio (Guangzhou, Guangdong, China). For transfection, the miR-424 mimic or miR-424 mimic controls were delivered at a final concentration of 100 nM using Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA, USA).

**RNA extraction and qRT-PCR detection**

Total RNA and miRNA was extracted from cultured endometrial carcinoma cells using miRVana miRNA Isolation Kit (Ambion, Austin, Texas, USA) according to the manufacturer's instructions. The purity and concentration of total RNA were determined by an Ultraviolet Spectrophotometer (Eppendorf, German). cDNAs were synthesized using the One Step PrimeScript™ miRNA cDNA Synthesis Kit (Takara Biotechnology, Dalian, China). miRNA was analyzed by real-time PCR using primers synthesized by Shanghai Sangon Biological Engineering and Technology Service (Shanghai, China). Briefly, 20 μl reactions containing 50 ng of total RNA, 10 μl of 2× SYBR Green PCR Master Mix, 6.25 U of AMV reverse transcriptase, 10 U of RNase inhibitor and 0.1 mM of primers were subjected to one cycle of 95°C for 10 min and then 40 cycles of 95°C for 15 s, 56°C for 30 s and 72°C for 45 s. miR-424 expression was normalized to the U6 RNA. Gene mRNA expression was normalized to β-actin. All results were calculated according to the ΔCt method and expressed as fold-changes.

**Protein extraction and western blot**

Total protein from the endometrial carcinoma cells was extracted using RIPA lysis buffer (Beyotime, Nan-tong, China) and quantified by BCA assay (Pierce, Rockford, IL). Equal amounts of protein were separated by 10% sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) and transferred to nitrocellulose membranes. Membranes were blocked in Tris-buffered saline-Tween buffer containing 5% nonfat dry milk for 30 min; the target proteins were incubated overnight at 4°C with GPER, p-AKT, AKT, and β-actin primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were washed and incubated with the secondary antibodies for 1 h at room temperature. A fluorescent Western blotting detection system was used. The band density of each gene was normalized to the corresponding density of β-actin.

**MTT assay**

Cell viability was detected by using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, cells were seeded in 96-well culture plates with the same starting cell number per well (1 × 10⁴ cells/well) and cultured for 2, 3, 4, 5, or 6 days (medium was refreshed, and treatment were renewed every 2 days). Thereafter, the culture medium in each well was discarded and fresh medium containing MTT (5 mg/ml MTT in PBS; Sangon, Shanghai, China) was added and incubated for an additional 5 h. After removing the remaining supernatant, 100 μl of dimethyl sulfoxide (DMSO) was used to dissolve the formed crystal formazan. Then, cell viability was analyzed by measuring the absorbance of each well at 490 nm. Relative cell viability was shown as the absorbance percentage of the treatment group to the control group.

**In vitro luciferase reporter assay**

After target gene prediction using an open access database (miRBase, http://www.mirbase.org/) and online bioinformatics software program (RegRNA, http://regna2.mbc.nctu.edu.tw/), GPER was considered a potential target gene for miR-424.

For luciferase reporter experiments, the 3'-UTR sequence of GPER was predicted to interact with miR-424 or a mutated sequence within the predicted target sites was synthesized and inserted into the Mlu I and Hind III sites of the pG3L3 vector (Promega, Madison, WI). The recombinant plasmids were confirmed by DNA sequencing. For the reporter assay, Ishikawa cells were plated onto 12-well plates, then co-transfected with the above recombinant plasmids and 100 nM miR-424 mimic or mimic control using Lipofectamine™ 2000 for 48 h. The firefly luciferase and renilla luciferase activity were detected by a fluorescence detector (Promega, Madison, Wisconsin, USA); the relative luciferase activity was normalized to renilla luciferase activity for each transfected well. The experiment was replicated three times for data calculate.

**The construction and transfection of GPER overexpression vector**

Recombinant GPER vectors were constructed using pFLAG plasmid (Invitrogen, Carlsbad, CA, USA). Briefly, the expression sequences encoding GPER were amplified from cDNA, which was synthesized from total RNA extracted from the Ishikawa cells. Then, these were subcloned into pFLAG plasmids which were then co-transfected with the miR-424 mimic into Ishikawa cells. The negative control group was transfected with empty pFLAG plasmid and miR-424 mimic control.

**Statistical Analysis**

All data were processed with SPSS13.0. Measurement data were presented as mean ± standard deviation. Differences between groups were compared with standard deviation followed by independent-samples T test. Repeated analysis of variance (ANOVA) was used for comparison between groups. A difference was considered significant at p < 0.05.

**Results**

**E2 induces the level of cell proliferation in Ishikawa and HEC-1A cells**

To investigate the effect of E2 on cell growth in endometrial cancer, we evaluated cell proliferation in E2-treated and normal control endometrial cancer cell lines. MTT analysis revealed that E2 treatment obviously increased cell proliferation of Ishikawa (Fig. 1A) and HEC-1A (Fig. 1B) cells compared with control groups.

**MiR-424 inhibits E2-induced cell proliferation increase in Ishikawa and HEC-1A cells**

To explore the function of miR-424 on the development of endometrial cancer, we evaluated the effect of
miR-424 overexpression on E2-induced cell growth in Ishikawa and HEC-1A cells. To quantify the direct contribution of miR-424 in endometrial cancer cell growth, we successfully induced the expression levels of miR-424 in Ishikawa and HEC-1A cells by the transfection with miR-424 mimic as detecting by RT-PCR (Fig. 2A). Importantly, MTT analysis demonstrated that the cell viability was significantly decreased in the miR-214 mimic transfection group compared with the mimic control group (Fig. 2B). Therefore, these results showed that miR-424 transfection inhibited E2-induced cell viability in endometrial cancer cells.

miR-424 exhibited negative effect on E2-induced cell growth by regulating the activation of PI3K/AKT pathway

It has been reported that the PI3K/AKT signaling pathway plays a critical role in the development of cancer progression. To assess the functional significance of miR-424 in Ishikawa and HEC-1A cells, we investigated whether overexpression of miR-424 could alter the PI3K/AKT pathway.

The expression levels of p-AKT and AKT were measured by western blot (Fig. 3A). The results showed that transfection with miR-424 mimics significantly decreased the expression of p-AKT (Fig. 3B), but not the total expression level of AKT (Fig. 3C) compared with the negative control group. Therefore, overexpression of miR-424 inactivated the PI3K/AKT pathway.

Prediction and identification of the target gene GPER of miR-424

It has been confirmed that GPER can positively regulate the PI3K/AKT signaling in E2-induced endometrial cancer cells. To further clarify the mechanism involved in the miR-424-mediated inactivation of PI3K/AKT, we analyzed the expression of GPER in E2 treated Ishikawa cells. As shown in Fig. 4A and B, the mRNA and protein expression level of GPER was significantly decreased with miR-424 mimic transfection compared with the negative control group. It has been demonstrated that miRNAs can negatively regulate the expression level of their target gene primarily by interacting with the 3'-UTR of mRNA, which ultimately leads to mRNA...
degradation or translational inhibition. According to the sequence analysis with RegRNA, the potential binding target sequence of miR-424 was found in the 3'-UTR of the GPER gene (Fig. 4C). To further clarify the mechanism involved in the miR-424-mediated inactivation of PI3K/AKT, we investigated the target reaction between GPER and miR-424. The luciferase reporter vector of GPER wild-type (wt) and GPER mutant type (mut), which contained the wt and mut of the predicted binding sequence in the 3'-UTR of GPER, respectively, were constructed and co-transfected with miR-424 mimic or miR-424 mimic control into Ishikawa cells to determine whether GPER is a target of miR-424. The results showed that co-transfection of miR-424 mimic with GPER wt in Ishikawa cells significantly suppressed luciferase activity, but not in GPER mut transfection groups (Fig. 4D).

Overexpression of GPER abrogates the inhibition effect of miR-424 in E2-induced cell growth Ishikawa and HEC-1A cells

To further analyze the effect of miR-424-triggered E2-induced cell growth inhibition by regulating GPER in Ishikawa and HEC-1A cells, the E2 treated endometrial cancer cells were co-transfected with miR-424 mimic and the GPER overexpression vector that only contains the coding region of GPER, which should attenuate miR-424 function by escaping the targeting regulation of miR-424. After transfection with the overexpressed GPER vector into Ishikawa and HEC-1A cells, miR-424-inhibited GPER expression was significantly increased (Fig. 5A). Moreover, the overexpression of GPER significantly attenuated the inhibition effect of miR-424 in E2-induced cell growth increase (Fig. 5B). Simultaneously, the decrease of p-AKT expression level induced by miR-424 was abrogated by GPER overexpression (Fig. 5C). Additionally, there was no significant difference in the expression level of AKT.

In conclusion, the data further confirmed that miR-424 mediated cell growth in E2-induced endometrial cancer cells through targeting GPER.

Figure 4. Identification of the targeting reaction between GPER and miR-424. (A and B) The protein expression level of GPER was measured by western blotting. Relative protein expression level was quantified using Image-Pro Plus 6.0 software and normalized to β-actin. The target gene was predicted by bioinformatics software programs, identified by luciferase activity report. (C) The wild type miR-424 binding sequence and the mutant miR-424 binding sequence in the 3'-UTR of the GPER gene; (D) Relative luciferase activity in Ishikawa cells co-transfected with miR-424 mimic or negative control together with luciferase reporter vector wt-GPER or mut-GPER. Luciferase activity was represented as firefly luciferase normalized to renilla luciferase. *P<0.05 versus the negative control group.

Figure 5. GPER overexpression abrogates the inhibition effect of miR-424 in E2-induced endometrial cancer cell lines. Ishikawa and HEC-1A cells were co-transfected with miR-424 mimic and GPER overexpression vector or empty control vector. (A and C) The protein expression level was measured by western blotting. Relative protein expression of GPER (B), p-AKT (E) and AKT (F) were quantified using Image-Pro Plus 6.0 software and normalized to β-actin. (D) The cell proliferation level was detected by MTT method. *P<0.05 versus the control group.
Discussion

miRNAs are of high interest as potential endometrial cancer therapeutics. However, their function and underlying mechanism in endometrial cancer remain to be elucidated. It has been reported that estrogen signaling is important in the development and progression of endometrial cancer (12). Estrogen is a remarkable regulator of physiologic processes, ranging from regulation of the menstrual cycle and reproduction to the modulation of brain function, bone density and so on (13). In endometrial cancer, high estrogen levels exhibit a growth-promoting property (10). Although estrogen is now considered a classic etiologic factor for endometrial cancer, the modulation mechanisms of estrogen in EC are still poorly understood. In the present study, we found that miR-424 inhibits E2-induced cell proliferation increase in endometrial cancer cells. Furthermore, miR-424 was involved in modulation of the PI3K/AKT signaling by directly targeting its receptor GPER. Together, these results suggest that miR-424 may play a critical role in the development and progression of endometrial cancer.

miRNAs are known to be noncoding RNAs that can suppress the expression of protein-coding genes by binding to the target sequence at the 3′-UTR of the target gene, and consequently involve in the regulation of cell apoptosis, differentiation, proliferation and other different cellular processes (14). It has been reported that miRNAs play an important role in certain types of cancer, including endometrial cancer. Among them, miR-424 has been demonstrated to act as a potential protective miRNA (15). For example, miR-424 was down-regulated in cervical cancer tissues, and inhibited cell proliferation, migration and invasion in cervical cancer (16). Moreover, miR-424 expression was significantly decreased and accompanied by potent suppression of the oncogene PLAG1 in chronic lymphocytic leukemia (17). In lines with these results, miR-424 was reported to be a protective factor in endometrial cancer in a previous study (9). In the present study, we observed that cell proliferation was significantly increased in E2-treated endometrial cancer cell lines, which was consistent with the former research (10). Importantly, we detected miR-424 overexpression to dramatically decrease the E2-induced cell proliferation increase, indicating a pivotal role in endometrial cancer cell growth. Consequently, these data suggested that miR-424 might act as a suppressor and regulate the progression in estrogen-associated endometrial cancer.

It has been reported that one of the main roles of the PI3K/AKT pathway was the promotion of cell proliferation and the inhibition of apoptosis (18). Increased PI3K/AKT pathway activity is diagnosed in many human cancers (19). Recently, the PI3K/AKT signaling pathway has been proven to be a targeting pathway in endometrial cancer due to its contribution to the pathology development of EC (20). A previous study has shown that E2 can active PI3K/AKT signaling in endometrial carcinoma (21). To investigate the underlying mechanism involved in miR-424-mediated cell growth resistance in E2-induced endometrial cancer cells, we linked the PI3K/AKT signaling together. In accordance with our hypothesis, miR-424 overexpression obviously inactivated the PI3K/AKT signaling pathway. However, the direct target for mir-424 in regulating the PI3K/AKT pathway is still undefined.

After target gene prediction and filtration using bioinformatics software programs, we selected GPER to be the target gene for miR-424. GPER is a receptor for estrogen, and has been proven to be involved in the non-transcriptional effect of estrogen on activation of the PI3K/AKT pathway in endometrial cancer cells (22). To verify the targeting reaction between miR-424 and GPER, the luciferase reporter vectors of wild type and mutant GPER were constructed. The results showed that the overexpression of miR-424 inhibits luciferase expression when cells were transfected with the wt-GPER luciferase reporter system. These results demonstrate that GPER is a target gene for miR-424. Further mechanism analysis validated that overexpression of GPER blocked the inhibition effect of miR-424 on E2-induced cell growth in endometrial cancer cells. Simultaneously, the inactive effect of mi-424 on the PI3K/AKT pathway was remarkably attenuated. Together, these results suggested that miR-424 inhibits E2-induced cell growth by directly regulating GPER-PI3K/AKT signaling in EC.

In conclusion, this study confirmed that estrogen elevates cell proliferation in endometrial cancer cell lines. Moreover, miR-424 inhibits the E2-induced cell proliferation increase by directly targeting GPER-PI3K/AKT signaling, which will ultimately facilitate the development of endometrial carcinoma. Consequently, this study provides an important clue to help to elucidate the pathogenesis of endometrial carcinoma and implicates miR-424 as a potential therapeutic target for estrogen-associated endometrial cancer.

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References


