G protein-coupled estrogen receptor/miR-148a/human leukocyte antigen-G signaling pathway mediates cell apoptosis of ovarian endometriosis

SHUN ZHI HE 1,2, JING LI 3, HONG CHU BAO 2, MEI MEI WANG 2, XIN RONG WANG 2, XIN HUANG 2, FENG HUA LI 2, WEI ZHANG 2, AN LI XU 4, HAO CUI FANG 2 and YANG XING SHENG 1

1 Department of Gynecology and Obstetrics, Qilu Hospital of Shandong University, Jinan, Shandong 250012; 2 Reproductive Medicine Center; 3 Electrocardiogram Room; 4 Department of Gynecology, Affiliated Yantai Yuhuangding Hospital of Qingdao University, Yantai, Shandong 264000, P.R. China

Received January 23, 2017; Accepted August 3, 2017

 DOI: 10.3892/mmr.2018.9039

Abstract. The focus of the current study was a G protein-coupled estrogen receptor (GPER)/microRNA (miR)-148a/human leukocyte antigen-G (HLA-G) signaling pathway in ovarian endometriosis. Reverse transcription-quantitative polymerase chain reaction was performed to analyze the changes in miR-148a expression. A MTT assay, flow cytometry and caspase-3/9 activity assays were performed to analyze cell proliferation, apoptosis and caspase-3/9 activity levels, respectively. Protein expression was measured using western blot analysis. In tissue samples from healthy controls, and patients with endometriosis and endometriosis-associated ovarian cancer, the expression of miR-148a was lower in in in endometriosis and EAOC samples compared with healthy controls. Overexpression of miR-148a using miR mimics significantly decreased proliferation, promoted apoptosis, increased the Bax/Bcl-2 ratio and caspase3 activity, and suppressed HLA-G protein expression in Hs 832(C).T cells. The findings indicate that GPER/miR-148a/HLA-G signaling pathway may mediates the development of ovarian endometriosis and may become a potential therapeutic target for the treatment of endometriosis.

Introduction

Endometriosis refers to a heterogeneous disease that presents as endometrial tissue with growth activity (including the glands and stroma) outside the corpus uteri and engraftment growth. Regarding the pathomorphology, it is benign (1); however, it presents malignant biological properties, such as infiltration and metastasis. The incidence rate of endometriosis is 10-15% of women (2). The epidemiological data of endometriosis reported in existing literature is incomplete since the disease can only be definitively diagnosed with laparoscopic and pathological diagnosis; however, many women do not receive laparoscopic examination if disease presents no symptoms or they are not willing to be subject to examination (3). Endometriosis can seriously affect patient quality of life, and physical and psychological health due to pelvic pain, infertility and high recurrence rate (4).

In recent years, researchers have detected that a novel form of estrogen receptor in a variety of human tissues, namely G protein-coupled receptor 30, which is also termed G protein-coupled estrogen receptor (GPER) (5). It rapidly activates signal cascade pathways in cells following binding to estrogen, generating second messengers, such as Ca2+, cyclic adenosine 3’,5’-monophosphate and nitric oxide, activate a variety of protein kinase, and regulate gene transcription. The process is termed a ‘rapid nongenomic effect’ since it only lasts for several sec or min. GPER belongs to the G protein coupled receptor protein family (6). It has no structural homology with the estrogen receptor. GPER is extensively expressed in the reproductive system, mammary glands, heart and cerebral vessels, bone and other tissues acting with estrogen (7). GPER indirectly regulates the biological functions of estrogen through a variety of signal pathways, and also can has rapid
effect through directly combining with endogenous estrogen, tamoxifen and its metabolite 4-hydroxytamoxifen, environmental estrogen, specific G-1 agonists and other ligands. At present, the researches on ER in endometriosis have become quite developed (7). However, there are extremely few studies regarding GPER (7). The detection of GPER has motivated re-evaluation of the effect of estrogen in pathogenic mechanism of endometriosis. Such studies may be conducive to further reveal the physiological roles of estrogen, and also provide new avenues for developing a novel generation of drugs for the treatment of estrogen-associated diseases (8).

MicroRNAs (miRNAs) are endogenous RNA molecules (~22 nucleotides) with regulatory function discovered in eukaryotes in recent decades. Numerous miRNAs are expressed in plants, animals and viruses (9). miRNAs negatively regulate gene expression by complementary base pairing with mRNA, which leads to mRNA degradation or translation inhibition. miRNAs, as important regulatory molecules, participate in a series of important cellular, including viral defenses, hematopoietic processes, organ development, cell proliferation and apoptosis, fat metabolism and tumorigenesis (10,11).

Human leukocyte antigen-G (HLA-G) is a non-classical major histocompatibility complex-β antigen molecule detected in 1997 (11). Under physiological conditions, HLA-G is restrictively expressed among certain tissues (11). Specific expression participates in maternal-fetal immune tolerance in extravillous trophoblasts to protect the fetus from maternal immune surveillance and immune rejection. Recent research has reported that HLA-G can restrain the activity of cytotoxic T cells, natural killer cells and other effector cells (11,12). Certain other studies have indicated that serum HLA-G levels in patients with endometriosis is higher than in individuals without endometriosis, suggesting that HLA-G may enhance the ability of ectopic endometrial cells to escape from immune surveillance, which facilitates implanting and proliferation at ectopic sites (11,13). In the current study, the role of miRNA (miR)-148a in ovarian endometriosis was explored and may be involved in the signaling pathway that induces apoptosis.

Materials and methods

Human tissue samples and ethical approval. All healthy controls (aged 52-68; n=6), patients with endometriosis (aged 55-63; n=7) and patients with endometriosis-associated ovarian cancer (EAO; aged 58-69; n=7) were recruited from the Reproductive Medicine Center and Department of Gynecology, Affiliated Yantai Yuhuangding Hospital of Qingdao University (Yantai, China) from June-July 2015. Endometriosis and endometriosis-associated ovarian cancer was diagnosed and confirmed by histological examination of laparoscopic biopsies. The research design was approved by the ethics committee of the Affiliated Yantai Yuhuangding Hospital of Qingdao University.

Written informed consent was obtained from each participant prior to the study. Healthy participants were diagnosed no evidence of ovarian pathology using laparoscopic biopsy due to a suspicious ovarian cyst. Endometriosis and EAO were diagnosed, with the American Society for Reproductive Medicine stage II/III for endometriosis (14) for inclusion in this study. Endometrium tissue was collected from patients with endometriosis, tumor tissue was collected from patients with endometriosis-associated ovarian cancer and healthy tissue was collected from healthy controls. All tissue was collected during the laparoscopic surgical examination or resection. Peripheral blood was centrifuged at 1,000 x g for 10 min at 4°C and the serum was subsequently collected and stored at -80°C until further use.

RNA isolation, reverse transcription (RT) and quantitative polymerase chain reaction (qPCR). Total RNA was isolated from serum or cells using TRIzol reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA). cDNAs were synthesized using a Thermoscript RT kit (Thermo Fisher Scientific, Inc.) for mRNA transcripts, at 37°C for 60 min and 85°C for 5 min. qPCR was performed using a Roche LightCycler 480II Real-Time PCR System (Roche Molecular Diagnostics, Pleasanton, CA, USA) using the SensiFAST SYBR No-Rox PCR kits (Bioline Reagents Ltd, London, UK). The 2-ΔΔCq method was used to quantify mRNA expression (15).

Cell culture and cell transfection. Endometriosis cell line Hs 832(C).T from a benign ovarian cyst was purchased from Chinese Academy of Sciences, Shanghai Cell Bank (Shanghai, China) and cultured in RPMI-1640 medium (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich; Merck KGaA) at 5% CO2 at 37°C. miR-148a mimics and negative mimics were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). miR-148a mimics and negative mimics (50-100 nM) was transfected to the cells using Oligofectamine (Invitrogen; Thermo Fisher Scientific, Inc.). GPER inhibitor, G15 (100 nM, Cayman Chemical Company, Ann Arbor, MI, USA) was added to Hs 832(C).T cells transfected with miR-148a mimics after 42 h and cultured for a further 6 h.

MTT assay of cell viability. Following transfection, cells were plated at 5x10^5 cells/well in 96-well plates for 0, 12, 48 and 72 h. Cell viability at each time point was measured using MTT (5 mg/ml; Sigma-Aldrich; Merck KGaA) assay for 4 h at 37°C. Subsequently, dimethyl sulfoxide (5 mg/ml; Sigma-Aldrich; Merck KGaA) was added to every well after 4 h and dissolved for 20 min. Absorbance of the solution at 490 nm was read using a spectrophotometric plate reader.

Flow cytometry analysis of apoptosis. Cells were plated at 1x10^6 cells/well in 6-well plates. At 48 h after transfection, cells were stained with 5 ml Annexin-V and 5 ml propidium iodide (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) for 30 min at 37°C in the dark. Apoptosis was detected by flow cytometry (BD FACSCanto; BD Biosciences, Franklin Lakes, NJ, USA).

Activity assay of caspases. Cells were plated at 1x10^6 cells/well in 6-well plates. At 48 h after transfection, lysesates were prepared using radioimmunoprecipitation assay (RIPA) buffer (Beyotime Institute of Biotechnology, Haimen, China) and protein concentration was determined by the Coomassie blue method (Beyotime Institute of Biotechnology). The proteins
(5-10 mg) were incubated with 2 mM Ac-DEV-D-pNA (cat. no. C1116) or Ac-LEHD-pNA (cat. no. C1158, Beyotime Institute of Biotechnology) for 1 h at 37°C in the dark. Absorbance of the solution at 405 nm was read using a spectrophotometric plate reader.

Western blot analysis. Cells were plated at 1x10^6 cells/well in 6-well plates. At 48 h after transfection, lysates were prepared using RIPA buffer and protein concentration was determined by the Coomassie blue method (Beyotime Institute of Biotechnology). The proteins (30-50 mg) were separated in 8-10% SDS-PAGE gels and then transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membrane was blocked with 5% nonfat milk at 37°C for 1 h and then incubated with anti-Bcl-2 associated X apoptosis regulator (Bax; cat. no. sc-6236; 1:500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), Bcl-2 apoptosis regulator (Bcl-2; cat. no. sc-56015; 1:500; Santa Cruz Biotechnology, Inc.), HLA-G (cat. no. sc-53825; 1:500; Santa Cruz Biotechnology, Inc.), GPER (cat. no. ab154069; 1:1,000; Abcam) and GAPDH (cat. no. sc-25778; 1:5,000; Santa Cruz Biotechnology, Inc.) at 4°C overnight. The membrane was washed with Tris-buffered saline Tween and incubated with horseradish peroxidase conjugated anti-rabbit antibody (1:10,000; cat. no. ab97064; Abcam, Cambridge, UK) for 1 h at room temperature. The membrane was visualized using ECL reagents (Beyotime Institute of Biotechnology) and analyzed using Image-Pro Plus software (version 6.0; Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis. Data are presented as the mean ± standard deviation of three independent experiments and was analyzed using SPSS 21.0 software (IBM Corp., Armonk, NY, USA). The Student’s t-test or one-way analysis of variance followed by Tukey’s post-hoc test was performed to compare experimental groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of miR-148a. Healthy controls, and patients with endometriosis and EAOC were recruited in the current study. As presented in Fig. 1A, miR-148a expression of in patients with endometriosis and EAOC were significantly lower than that of healthy volunteers. Subsequently, miR-148a mimics were used to increase miR-148a expression in Hs 832(C).T cells (Fig. 1B), compared with a negative control group.

Effect of miR-148a overexpression on cell proliferation and apoptosis of Hs 832(C).T cells. Whether overexpression of miR-148a affects cell proliferation and apoptosis of Hs 832(C).T cells was analyzed using MTT and flow cytometry, respectively. Fig. 2A indicates that overexpression of miR-148a reduced cell proliferation of Hs 832(C).T cells following transfection at 12, 24 and 48 h. Overexpression of miR-148a significantly reduced proliferation of Hs 832(C).T cells compared with negative group after transfection for 48 h.

Effect of miR-148a overexpression on caspase-3 and caspase-9 activity, and Bax/Bcl-2 ratio in Hs 832(C).T cells. To further explore whether overexpression of miR-148a affects HLA-G in Hs 832(C).T cells, HLA-G protein expression was measured using western blot analysis. The results from western blot analysis demonstrated that overexpression of miR-148a significantly inhibited HLA-G protein expression in Hs 832(C).T cells after transfection for 48 h, compared with the negative control group (Fig. 3).

Effect of miR-148a overexpression on HLA-G in Hs 832(C).T cells. To further explore whether overexpression of miR-148a affects HLA-G in Hs 832(C).T cells, HLA-G protein expression was measured using western blot analysis. The results from western blot analysis demonstrated that overexpression of miR-148a significantly inhibited HLA-G protein expression in Hs 832(C).T cells after transfection for 48 h, compared with the negative control group (Fig. 3).

Effect of miR-148a inhibition on proliferation in Hs 832(C).T cells. Subsequently, an MTT assay was used to analyze the effects of miR-148a inhibition on cell viability in Hs 832(C).T cells. As demonstrated in Fig. 5, anti-miR-148a reduced miR-148a expression, and increased the proliferation of Hs 832(C).T cells.

Effect of miR-148a inhibition on caspase-3 and caspase-9 activity, and Bax/Bcl-2 ratio in Hs 832(C).T cells. To further explore the apoptotic mechanisms of miR-148a in Hs 832(C).T cells, caspase-3 and caspase-9 activity, and Bax/Bcl-2 protein expression were measured using caspase-3 and caspase-9 activity kits and western blot analysis. Inhibition of miR-148a significantly also decreased the caspase-3 and caspase-9 activities, and reduced the Bax/Bcl-2 ratio in Hs 832(C).T cells after transfection for 48 h, compared with the negative control group (Fig. 6).

Effect of miR-148a inhibition on HLA-G in Hs 832(C).T cells. Western blot analysis was used to measure HLA-G protein expression in Hs 832(C).T cells following miR-148a inhibition. The results from western blot analysis demonstrated that inhibition of miR-148a significantly increased HLA-G protein expression of Hs 832(C).T cells after transfection for 48 h compared with the negative group (Fig. 7).

Effect of GPER inhibition on proliferation of Hs 832(C).T cells. The role of GPER expression on the effects of miR-148a in Hs 832(C).T cells was examined. GPER inhibitor was used to reduce the expression of GPER protein. A GPER inhibitor, G15 (100 nM) was added to Hs 832 (C). As presented in Fig. 8, GPER inhibition significantly suppressed GPER and HLA-G protein expression of Hs 832(C).T cell after transfection with miR-148a, compared with transfected cells that were not treated with G15 (Fig. 8A-C). However, the inhibition of GPER significantly increased miR-148a
miR-148a expression in Hs 832(C).T cells transfected with miR-148a for 48 h, compared with miR-148a transfection only group (Fig. 8).

Effect of GPER inhibition on miR-148a-induced changes to cell viability and apoptosis of Hs 832(C).T cells. It was ascertained whether the suppression of GPER alters the
effect of miR-148a on cell viability and apoptosis in Hs 832(C).T cells. Overexpression of miR-148a reduced cell viability and induced apoptosis of Hs 832(C).T cells; these effects were significantly intensified by the suppression of GPER using G15 in Hs 832(C).T cells, compared with the miR-148a only group (Fig. 9).

Effect of GPER inhibition on miR-148a-induced changes to caspase-3 and caspase-9 activity, and Bax/Bcl-2 ratio of Hs 832(C).T cells. Whether the suppression of GPER alters the effect of miR-148a on caspase-3 and caspase-9 activity, and the Bax/Bcl-2 ratio in Hs 832(C).T cells was determined. The suppression of GPER significantly enhanced the effect
HE et al.: miR-148a IN OVARIAN ENDOMETRIOSIS

1146

of miR-148a overexpression on caspase-3, caspase-9 and Bax/Bcl-2 ratio in Hs 832(C).T cells (Fig. 10).

Discussion
Endometriosis is a benign pathomorphological feature. However, it is an estrogen-dependent disease exhibiting malignant biological behaviors. Endometriosis is prone to occur during the childbearing period (3). The formation of endometriosis is a quite complex process involving numerous molecular events, including propagation and apoptosis of endometrial cells, migration, invasion and attaching, local hypoxia injury, inflammatory cell aggregation, formation of new vessels and remodeling of the cytoskeleton (16). Therefore, in-depth studies specific to estrogen signaling pathways and functional proteins associated with cell invasion, migration and cell apoptosis may offer novel insight into the pathogenic mechanism of endometriosis (17). These results suggest that miR-148a expression was lower in endometriosis tissue and patients with EAOC compared with healthy volunteers.

The incidence rate of endometriosis is increasing year upon year (18). However, the pathogenic mechanism is not clear. miRNAs are endogenous RNAs with regulatory function (19). Existing research indicates that miRNAs are closely associated with the development of various diseases. In addition, miRNAs have an important role in the initiation and development of endometriosis (9). In the current study, overexpression of miR-148a significantly decreased cell proliferation, promoted apoptosis, increased the Bax/Bcl-2 ratio and caspase-3/9 activity in Hs 832(C).T cells.

Analysis of estrogen-dependent tumors, such as breast and endometrial cancer, has verified that GPER can promote the invasion and migration of tumor cells through the downstream signaling pathway (5). Combining with biological functions of GPER and the estrogen dependency characteristics of endometriosis, it can be speculated that the expression of GPER is associated with the formation of ectopic foci (20). The current
study reported that overexpression of miR-148a significantly inhibited HLA-G protein expression in Hs 832(C).T cells and the inhibition of GPER expression significantly increased miR-148a expression in Hs 832(C).T cells. Together, the results of these studies suggest that miR-148a may have different regulatory mechanisms of angiogenesis in endometriosis.

HLA-G is specifically expressed in extravillous trophoblasts to avoid sertoli cells being damaged by natural killer immune cells, thus to induce maternal-fetal interface to generate immune tolerance, protecting the fetus from maternal immune surveillance and immune rejection (21). It is reported in the literature that the pathological characteristics of endometriosis exhibit biological behavior similar to tumor cells, which may indicate how HLA-G protein expression in endometriotic cells promotes ectopic endometrium growth on ectopia and other areas may be facilitated through the high expression of HLA-G protein, which promotes escape from immune surveillance (24). The results of the current study demonstrated that overexpression of miR-148a significantly reduced HLA-G protein expression in Hs 832(C).T cells; the inhibition of GPER activity using G15 significantly decreased cell viability, promoted apoptosis, increased Bax/Bcl-2 ratio and caspase-3 activity, and suppressed HLA-G protein expression in Hs 832(C).T cells. Together, the results demonstrate that miR-148a may negatively regulate the GPER signaling pathway in endometriosis.

In conclusion, it was identified that GPER/miR-148a/HLA-G signaling may be involved in mediating ovarian endometriosis. However, further in vivo studies of GPER/miR-148a/HLA-G are necessary in the future in order to develop novel ovarian endometriosis treatments.

Acknowledgements

Not applicable.
Funding
No funding was received.

Availability of data and materials
The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Author's contributions
YXS designed the study. SZH, JL, HCB, MMW, XRW, XH, FHL, WL, ALX and CFH performed the experiments. SZH and YXS analyzed the data. YXS wrote the manuscript.

Ethics approval and consent to participate
The research design was approved by the ethics committee of the Affiliated Yantai Yuhuangding Hospital of Qingdao University. Written informed consent was obtained from each participant prior to the study.

Consent for publication
Not applicable.

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

References