G-protein coupled receptor 64 (GPR64) acts as a tumor suppressor in endometrial cancer

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Abstract

Background: Endometrial cancer is the most common gynecological cancer. G-protein coupled receptor 64 (GPR64) belongs to a family of adhesion GPCRs and plays an important role in male fertility. However, the function of GPR64 has not been studied in endometrial cancer. Our objective is to investigate the role of GPR64 in endometrial cancer.

Methods: We examined the levels of GPR64 in human endometrioid endometrial carcinoma by immunohistochemistry analysis. To determine a tumor suppressor role of GPR64 in endometrial cancer, we used a siRNA loss of function approach in human endometrial adenocarcinoma cell lines.

Results: GPR64 levels were remarkably lower in 10 of 21 (47.62%) of endometrial carcinoma samples compared to control. Depletion of GPR64 by siRNA transfection revealed an increase of colony formation ability, cell proliferation, cell migration, and invasion activity in Ishikawa and HEC1A cells. The expression of Connexin 43 (Cx43), a member of the large family of gap junction proteins, was reduced through activation of AMP-activated protein kinase (AMPK) in Ishikawa cells with GPR64-deficiency.

Conclusions: These results suggest that GPR64 plays an important tumor suppressor role in endometrial cancer.

Keywords: Endometrial cancer, Tumor suppressor, GPR64, Connexin 43

Background

Endometrial cancer is the most common gynecologic malignancy, with an estimated 63,230 new cases in 2018 [1]. The most common type of endometrial cancer is endometrioid adenocarcinoma, which originates from endometrial epithelial cells [2]. The development of endometrial hyperplasia, a proliferative process in the epithelium, is the abnormal thickening of the lining of the uterus due to an increase in the number of endometrial glands. It is a critical risk factor for endometrioid endometrial carcinoma [3]. Despite most cases being diagnosed in the early stages of endometrial cancer, a subset of these patients have poor outcomes and a high rate of recurrence and metastasis [4]. Recently, many studies have focused on targeted molecular therapies for controlling endometrial malignancies [5], however they are still insufficient. Therefore, it is important to identify molecular mechanisms involved in the development and progression of endometrial cancer.

G-protein coupled receptor 64 (GPR64) is a member of GPCR superfamily, which is crucial for male fertility [6–8]. GPR64 was expressed in the proximal epididymis and efferent ductule regions with are responsible for spermatozoa maturation and rete testis fluid reabsorption [6–8]. In addition, expression of GPR64 was found in fibroblast-like synovial cells in osteoarthritis [9]. The level of GPR64 was higher in ewing sarcoma than other mesenchymal neoplasms, and GPR64 induces placental growth factor (PGF) and metalloproteinase (MMP1) expression [10]. Loss of GPR64 in ewing sarcoma cell line leads to decreased PGF and MMP1 expression and reduced
cellular growth with induced TRAIL dependent apoptosis [10]. Also, GPR64 knock-down in an ewing sarcoma tumor model in immune deficient mice, reduced metastasis and invasiveness to the liver and lung [10]. GPR64 can activate G-proteins GS/Gq when over-expressed in xenopus melanophores [11]. Furthermore, GPR64 was identified as a target gene of β-catenin/T-cell factor (TCF) in ovarian endometrioid adenocarcinoma [12]. However, the role of GPR64 in endometrial cancer is unknown.

Connexin 43 (Cx43) is a member of the large family of gap junction proteins [13]. Gap junctions are intercellular plasma membrane proteins that provide for the exchange of ions and small molecules between adjacent cells [14]. Some studies have indicated that the Cx43 channel was localized at the plasma membrane, but not involved in Gap junction formation [15]. The Cx43 channel may regulate cell growth by transportation of calcium ions or other ions between intracellular cytoplasm and the extracellular environment [15, 16]. Other studies suggest that Cx43 can regulate cell growth and death by direct interaction with regulated cell cycle proteins including cyclin A, cyclin D1, p21, and p27 [17, 18]. Dysregulation of gap junction intercellular communication was to linked several human diseases such as cancer, cardiac ischemia, Charcot-Marie-Tooth (CMT), and Visceroatrial Heterotaxia Syndrom (VAH) [19, 20]. Cx43 is ubiquitously expressed in human tissues and controls cell growth and differentiation via multiple mechanisms. Attenuation of Cx43 is frequently observed in cancers, resulting in loss of gap junctional intercellular communication [21, 22]. Activation of Cx43 in cancer cells derived from various tissue types has been shown to result in restoration of normal cell growth and differentiation [23]. Small interfering RNA (siRNA)-mediated knockdown of Cx43 results in a more aggressive growth of breast cancer cells [24]. Moreover, knock-out of Cx43 in mice results in increased susceptibility to chemically induced lung adenomas [25]. There is an inverse correlation between Cx43 expression and tumor grade in endometrial cancer [26]. These observations suggest that Cx43 has a tumor suppression function and is a potential target in cancer therapy.

In this study, we examined the levels of GPR64 in human endometrioid endometrial carcinoma. To investigate the function of GPR64 in endometrial cancer, we used GPR64 siRNA in human endometrial cancer cell lines. Our results showed a new tumor suppressor role for GPR64 in endometrial cancer.

Methods

Human endometrium samples

The human endometrioid endometrial carcinoma samples were obtained from The University of Texas MD Anderson Cancer Center. The control endometrial samples were obtained from hysterectomies (e.g., due to leiomyoma or a uterus prolapse). All patients with endometrial carcinoma underwent surgery. Twenty four controls and 21 endometrial cancer samples (not paired) were fixed in 10% buffered formalin prior to embedding in paraffin wax.

Immunohistochemistry analysis

Immunohistochemistry analysis was performed as previously described [27]. Uterine cross sections from paraffin-embedded tissue were cut into 6µm sections, mounted on saline-coated slides, deparaffinized and rehydrated in a graded alcohol series. For antigen retrieval, heat-induced epitope retrieval was performed using a pressure cooker with antigen unmasking solution (H$^{−}$3300, Vector Laboratories, Burlingame, CA) and then sections were pre-incubated with 10% normal rabbit serum in phosphate-buffered saline (PBS; pH 7.5) then incubated with anti-GPR64 (Sc-69,492; Santa Cruz Biotechnology, Dallas, TX) antibody in PBS supplemented with 10% normal rabbit serum overnight at 4°C. The next day, sections were washed with PBS and incubated with secondary antibody conjugated to horseradish peroxidase (Vector Laboratories, Burlingame, CA) for 1 h at room temperature. Immunoreactivity was detected using diaminobenzidine (SK-4100; Vector Laboratories) then counterstained with hematoxylin and coverslipped with permount. Immunostaining was analyzed using microscopy software from NIS Elements, Inc. (Nikon Instruments Inc., Melville, NY). A semi-quantitative grading system (H-score) was used to compare the immunohistochemical staining intensities.

Cell culture and siRNA transfection

Human endometrial adenocarcinoma Ishikawa and HEC1A cells were maintained in phenol red–free DMEM/F12 medium (Gibco, Grand Island, NY) containing 0.1 mM sodium pyruvate (Gibco), 10% fetal bovine serum (FBS; Gibco), and 1% penicillin streptomycin (P/S; Gibco). Cells were cultured in monolayer at 37°C in 5% CO₂. GPR64 siRNA was obtained from Dharmacon (Lafayette, CO), RNAi Technologies. Human GPR64 siRNA was transfected using Lipofectamine 2000 reagent (Invitrogen Crop., Carlsbad, CA) prior to in vitro culture.

Colony forming assay

After siRNA treatment, Ishikawa and HEC1A cells were seeded into 6-well plates at a density of $2 \times 10^5$ cells per 2 ml cell culture medium and media was changed every 72 h for 14 days. Upon completion of culture, the plate wells were washed with PBS, fixed with 4% paraformaldehyde and permeabilized with 100% methanol. Colonies were stained with 1% crystal violet and images were
captured via microscopy (Nikon Instruments Inc.) using software from NIS Elements, Inc.

Cell proliferation assay
Cell proliferation was measured with a cell count kit-8 (Dojindo molecular technologies, Kumamoto, Japan) assay according to the manufacturer’s instructions. After siRNA treatment, 1 × 10⁵ cells were seeded into 24-well plate and cell proliferation was documented every 24 h for 10 days.

Wound healing assay
Cell migration was measured by a wound healing assay. 1 × 10⁵ Ishikawa and HEC1A cells were seeded into 12-well plate and human GPR64 siRNA was transfected into cells. After siRNA treatment, cells were incubated for 24 h. After 24 h, a pipette tip was used to create a scratch through the cell monolayer and cells were maintained in growth medium at 37 °C in 5% CO₂. Cell migration was measured after 48 h, via inverted microscopy (Nikon Instruments Inc.) and distance was determined by Image J software (National Institute of Health, USA). Cell migration rate was converted to a percentage that measured the area compared to directly after the scratch.

Invasion assay
For the transwell invasion assay, Ishikawa and HEC1A cells treated with siRNA were plated in the top chamber of a matrigel-coated membrane (24-well insert; pore size, 8 μm; BD Biosciences) at a density of 2.5 × 10⁵ per 200 μl serum-free culture medium and culture medium with 10% serum was used as a chemoattractant in the lower chamber. The cells were incubated at 37 °C in 5% CO₂ for 48 h and cells that did not invade through the pore were removed by a cotton swab. Cells on the lower surface of the membrane were fixed with 4% paraformaldehyde and permeabilized with 100% methanol. Cells were stained with 1% crystal violet, and images were captured via fluorescent microscopy (Nikon Instruments Inc.) using software from NIS Elements, Inc.

Annexin V/PI assay
Apoptosis was measured with a FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen, San Diego, CA) assay according to the manufacturer’s instructions. After siRNA treatment, 1 × 10⁶ cells were washed twice with cold PBS and resuspend cells in 1X binding buffer. 100 ul of the solution with 1 × 10⁵ cells was transfered to a 5 ml culture tube and added 5 ul of FITC Annexin V and 5 ul PI. The cells were gently vortexed and incubated at room temperature in the dark. After 15 min, 400 ul of 1X binding buffer was added to culture tube and analyzed by Flow cytometry (FACScalibur; Becton Dickinson, San Jose, CA). The data was analyzed using BD cell/Quest Pro software (Becton Dickinson).

RNA isolation and quantitative RT-qPCR
Total RNA was isolated using the RNeasy total RNA isolation kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. As a template for quantitative RT-qPCR, cDNAs were synthesized using quantitative PCR random hexamers and MMLV Reverse Transcriptase (Invitrogen Corp.). The expression of Cx43 was quantified by real-time PCR using a CFX96 Real-time Detection System (Bio-Rad Laboratories, Hercules, CA) and iQ™ SYBR Green Supermix (Bio-Rad Laboratories). RPL7 expression was included in each treatment group for normalization. The sequences of the primers used for GPR64 were 5’-CTGCGGATCCCATTCG-3’ and 5’-AGAACGGTTGAATCTCCC-3’, for Cx43 were 5’-ATGAGCAGTCTGCTTTGCTG-3’ and 5’-TCTGCTTCAAGTGATGTGTC-3’, and for RPL7 were 5’-AAGAGGCAATTGCGTTC-3’ and 5’-CAAATCCTCCACTAGAATG-3’.

Western blot analysis
Western blot analyses were performed as described previously [27]. Briefly, twenty-five micrograms of protein lysates were electrophoresed via SDS-PAGE and transferred onto polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA). The membrane was blocked with Casein (0.5% v/v) prior to exposure to anti-GPR64 (sc-69,492 Santa Cruz Biotechnology), anti-phospho-AMPK (Thr 172, #5235; Cell Signaling, Danvers, MA), anti-AMPK (#2532, Cell Signaling), anti-Cx43 (#3512, Cell Signaling), and anti-β-actin (SC-4778; Santa Cruz Biotechnology) antibodies. Immunoreactivity was visualized by incubation with a horseradish peroxidase-linked secondary antibody followed by exposure to electrochemiluminescence reagents (ECL) according to the manufacturer’s instructions (GE Healthcare Biosciences, Piscataway, NJ).

Immunofluorescence
Immunofluorescence was performed as described previously [27]. Ishikawa cells were grown on glass coverslips and transfected with GPR64 siRNA. Upon completion of growth, coverslips were washed with PBS, fixed with 4% paraformaldehyde and permeabilized with 0.1% of Triton X-100 (Sigma-Aldrich, St. Louis, MO). After further washing, Ishikawa cells were exposed to anti-GPR64 (sc-69,492; Santa Cruz Biotechnology) and anti-Cx43 (#3512; Cell Signaling) antibodies overnight at 4°C and secondary antibodies for 2 h at room temperature. Washed coverslips were then mounted onto microscope slides with a DAPI-impregnated mounting media (Vector Laboratories) to enable nuclear visualization, and
images were captured via a Zeiss LSM700 confocal microscope (Carl Zeiss microImaging GmbH, Jena, Germany) using ZEN 2009 software (Carl Zeiss microImaging).

Statistical analysis
Cell experiments were measured in triplicate and averaged to achieve a single value for each combination of treatment, time point, and cell line. Statistical analyses were performed using one-way ANOVA analysis, Tukey’s post hoc multiple range test or Student’s t-tests using the Instat package from GraphPad (San Diego, CA). p < 0.05 was considered statistically significant.

Results
The levels of GPR64 are altered in a subset of endometrial cancer
To determine the levels of GPR64 in endometrial cancer, we performed immunohistochemical analysis using tissue from 24 controls and 21 endometrioid endometrial carcinoma samples. In control endometrium, GPR64 proteins were strongly detected in the nucleus, cytosol and apical membranes of stromal and epithelial cells of endometrium from the proliferative phase and secretory phases in women and the levels of GPR64 proteins were not significantly changed during the menstrual cycle (Fig. 1a). Interestingly, the levels of GPR64 were significantly lower in 47.62% (10/21) of endometrial cancer tissue compared with controls (p < 0.001). However, levels of GPR64 were unchanged in 52.38% (11/21) of endometrial cancer samples (Fig. 1b; p = 0.0841). Epididymal tissue was included for the immunohistochemistry of GPR64 as a positive control [28, 29]. The expression of GPR64 was detected in apical membranes as well as in some nuclei of epididymal duct epithelial cells (Additional file 1: Figure S1). These results suggest that GPR64 may play a tumor suppressor role in certain cases of endometrial cancer.

Attenuation of GPR64 increases colony forming ability and cell proliferation
The continual unregulated proliferation of cells is essential to cancer development [30, 31]. To characterize the proliferative role of GPR64 in endometrial cancer, we performed a colony forming assay and a cell proliferation assay in Ishikawa and HEC1A cells transfected with GPR64 siRNA. First, we confirmed the decrease of GPR64 mRNA and protein levels by GPR64 siRNA transfection compared to non-targeting pool siRNA by RT-qPCR and western blot analysis, respectively (Fig. 2 a and b). The colony formation assay enables us to determine the survival and proliferation of cells [32]. Colony formation ability was significantly increased in Ishikawa and HEC1A cells transfected with GPR64 siRNA compared to control (Fig. 2 c and d). Furthermore, the
proliferation levels of Ishikawa cells were significantly increased by GPR64 siRNA transfection after 4 days (Fig. 2e). The proliferation of HEC1A cells was also significantly increased by GPR64 siRNA transfection after 3 days (Fig. 2f). Next, we examined whether GPR64 regulates cell apoptosis using an Annexin V/PI assay. The ratio of apoptotic cells was not different between GPR64 siRNA-transfected cells and non-targeting pool siRNA transfected cells of Ishikawa and HEC1A cells (Additional file 1: Figure S2). These results suggest that GPR64 suppresses epithelial proliferation of endometrial cancer cells.

**Attenuation of GPR64 increases cell migration and invasion**

We examined the effect of GPR64 attenuation in cell migration and invasion. Our wound healing assay revealed that reduction of GPR64 by RNA interference significantly increased the migration ability of Ishikawa and HEC1A cells (Fig. 3). The GPR64-deficient Ishikawa cells recovered the wound over 61% after 48 h, while control cells healed less than 44% of wound distance (Fig. 3a). The HEC1A cells with GPR64 knockdown recovered the wound over 71% but the control cells healed only less than 59% of wound distance (Fig. 3b).

Attenuation of GPR64 exhibited a significantly higher infiltration rate in the transwell invasion assay of Ishikawa and HEC1A cells compared to siRNA controls. Invasion ability of GPR64-deficient Ishikawa cells was increased by more than three-times compared to control cells (Fig. 4a), and invasion ability of GPR64 knockdown HEC1A cells was increased by more than two-times (Fig. 4b).

**Attenuation of GPR64 reduces the expression of Connexin 43 trough activation of AMPK**

Activation of AMP-activated protein kinase (AMPK) plays a critical role in induction of EMT in multiple cancer cell types [37]. To determine whether AMPK activity is regulated by depletion of GPR64, we examined phosphorylation level of AMPK (Thr 172) in Ishikawa cells transfected with GPR64 siRNA. The levels of phosphorylated AMPK were increased by GPR64 knockdown compared to control, however total AMPK levels...
were not different between control and GPR64-deficient cells (Fig. 5a).

Connexin proteins are frequently dysregulated in tumors, resulting in loss of gap junctional intercellular communication [21, 22]. Many tumors with decreased Cx43 expression exhibited dysfunctional gap junctional intercellular communication [19, 38, 39]. The expression and function of Cx43 have been correlated with carcinogenesis in endometrial cancer [26, 40]. Therefore, we examined the levels of Cx43 proteins and mRNA in Ishikawa cells with GPR64 knockdown using RT-qPCR and Western Blot analysis. Depletion of GPR64 significantly reduced the levels of Cx43 proteins on day 1 and day 6 compared with non-targeting pool siRNA by Western blot analysis (Fig. 5a and b). The expression of Cx43 mRNA was significantly reduced in the Ishikawa cells treated with GPR64 siRNA on day 3 and day 6 compared with non-targeting pool siRNA (Fig. 5c).

Next, we performed double immunofluorescence for GPR64 and Cx43 after GPR64 knock-down (Fig. 6). GPR64 is colocalized with Cx43 in Ishikawa cells. However, the expression of Cx43 protein was remarkably reduced in GPR64-deficient cells by GPR64 siRNA compared to non-targeting pool siRNA.

**Discussion**

G protein-coupled receptors (GPCRs) are transmembrane receptors and play important roles in multiple biological processes. Aberrant expression of these receptors has been linked to cancer development and progression [43–45]. However, the molecular function of GPCRs is not known in endometrial cancer. The present study shows that the expression of GPR64 was distinctly lower in a subset of endometrioid endometrial carcinoma. These results suggest a tumor suppressor role of GPR64 in endometrial cancer.

Recently, Richter et al. identified that GPR64 is specifically overexpressed in Ewing sarcoma (ES) but is also up-regulated in prostate, kidney, and lung carcinoma [10]. This study identified that suppression of GPR64 in ES by siRNA, led to impaired colony formation, cell growth, and metastasis in Rag2(−/−)γC(−/−) mice. Moreover,
suppression of GPR64 induced TRAIL mediated apoptosis and reduced PGF and MMP1 expression [10]. GPR64 is also involved in cell adhesion and migration through activation of serum response element (SRE) and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and its knockdown by siRNA in the highly motile breast cancer cell lines results in a reduction in cell adhesion and migration [46]. However, our results showed that depletion of GPR64 increases the cell proliferation, migration, and invasion of endometrial cancer cells. These results suggest the role of GPR64 as a tumor suppressor in endometrial cancer. It remains possible that GPR64 plays dual functions in different cancer conditions depending on the spatial and temporal distribution and abundance of different GPR64 downstream targets and factors that regulate GPR64. Our results suggest a new role of GPR64 as a tumor suppressor in endometrial cancer, but further studies are needed to provide a conclusive answer.

Unfortunately, we could not characterize the subtype of endometrial cancers where GPR64 could be a tumor suppressor.
suppressor. The levels of GPR64 were not correlated with grade or stage of endometrial endometrioid adenocarcinoma which is the most frequently occurring endometrial cancer cell type [47]. Cancer classification in the clinic is primarily based on histological analysis in the proper clinical context. Although highly informative, histopathology can be hampered by limitation in its ability to distinguish subtypes of cancers and molecular signatures. Although we could not characterize the subtype of endometrial tumors based on GPR64 levels in this study, composite molecular profiling of tumor specimens is increasingly becoming recognized as an adjunct to traditional histopathology. Therefore, the molecular characterization of tumor types using GPR64 expression may help to identify additional objective tools to enhance the classification of endometrial cancer. However, there is a need to investigate a tumor suppressor role of GPR64 in specific subtypes of endometrial cancer.

Several adhesion GPCRs have been shown to be involved in cell adhesion and migration, hereby influencing tumor progression [46]. GPR26 is a potent regulator of energy homeostasis through controlling hypothalamic AMP-activated protein kinase (AMPK) activation [48]. AMPK inhibits Cx43 expression in bladder smooth muscle cells [36]. While total AMPK levels were not different between control and GPR64-deficient cells, the levels of phosphorylated AMPK were increased by GPR64 knock-down compared to control. AMPK is associated with cancer development as an important mediator in maintaining cellular energy homeostasis [33, 34], and its activity is increased by extracellular changes such as depletion of ATP, low glucose, and changes of NADPH levels [35]. Additionally, AMPK inhibits Cx43 expression in bladder smooth muscle cells [36]. Cx43 expression has been associated with a wide variety of cancers, including liver tumor, colon cancer, breast cancer, ovarian carcinoma, and endometrial cancer [41]. Its role in controlling cell motility and polarity contributes to cancer development and metastasis. Especially, Cx43 decreased with poor differentiation of endometrial cancer [26], and Cx43 is considered to be weakened in progression of carcinogenesis [40, 42]. Thus, our results suggest that GPR64 is a tumor suppressor in endometrial cancer by regulating Cx43 expression through regulation of AMPK activity.

**Conclusions**

We found an attenuation of GPR64 expression in a subset of endometrial cancer. We demonstrated that depletion of GPR64 induced tumorigenic potentials by promoting cell proliferation, migration, and invasion in endometrial cancer cells. GPR64 regulates the expression of Cx43 and AMPK activity in endometrial cancer cells. These results suggest that GPR64 acts as a tumor suppressor in endometrial cancer.

**Additional file**

**Additional file 1: Figure S1.** Expression of GPR64 in mouse epididymis. The immunohistochemistry for GPR64 was performed in mouse epididymis as a positive control. Immunohistochemical staining of mouse epididymis shows membranous and nuclear positivity in epididymal duct epithelial cells. **Figure S2.** Effect of GPR64 on cell apoptosis in human endometrial cancer cells. Annexin V/PI assay was performed in Ishikawa (A) and HEC1A (B) cells transfected with non-targeting pool (NT) siRNA or GPR64 siRNA to determine the effect of GPR64 on cell apoptosis. The apoptotic cells were analyzed by flow cytometry. No difference was found between there were no significant difference between NT siRNA and GPR64 siRNA treatments. (PPTX 330 kb)
Abbreviations
CMT: Charcot-Marie-Tooth; Cx43: Connexin 43; DAG: Diaminobenzidine; ES: Ewing sarcoma; ESR1: Estrogen receptor α; GPCR: G-protein-coupled receptors; GPR64: G-protein coupled receptor 64; MMP: Metalloproteinase; PBS: Phosphate-buffered saline; PGF: Placental growth factor; PGR: Progesterone receptor; siRNA: Small interfering RNA; TCF: T-cell factor; VASH: Visceral atrophy Syndrome

Authors’ contributions
JIA and JYY conceived and designed the experimental approach, performed experiments and prepared the manuscript. THK, YIK and JYA performed the experiments and analyzed the results. RRB provided the human endometrial specimens and performed analysis and interpretation of data. JML and JWJ conceived and designed the experimental approach, performed data analysis and prepared the manuscript. All authors have read and approved the final version of manuscript.

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Availability of data and materials
The raw data available upon reasonable request from the corresponding authors.

Ethics approval and consent to participate
The study has been approved by Institutional Review Committee of University of Texas MD Anderson Cancer Center (Houston, TX), and written informed consent was obtained from all participants.

Consent for publication
Not applicable.

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

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