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G protein-coupled estrogen receptor (GPER) expression in endometrial adenocarcinoma and effect of agonist G-1 on growth of endometrial adenocarcinoma cell lines

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ABSTRACT

The G protein-coupled estrogen receptor (GPER, GPR30) is suggested to be involved in non-nuclear estrogen signaling and is expressed in a variety of hormone dependent cancer entities. This study was performed to further elucidate the role of this receptor in endometrial adenocarcinoma. We first analyzed GPER expression at the mRNA level in 88 endometrial cancer or normal endometrial tissue samples and compared it to those of nuclear steroid hormone receptors. GPER transcript levels were found to be about 6-fold reduced, but still present in endometrial cancer. Expression of this receptor was decreased in all grading subgroups when compared to pre- or postmenopausal endometrium. GPER mRNA expression was associated with PR mRNA levels (Spearman's rho 0.4610, $p < 0.001$). We then tested the effect of the GPER ligand G-1 on growth of three endometrial cancer cell lines with different GPER expression. GPER protein levels were highest in RL95-2 cells, moderate in HEC-1A cells and not detectable in HEC-1B cells. The moderate expression level in HEC-1A cells was similar to average tumor tissue expression. Treatment with G-1 significantly inhibited growth of the GPER-positive cell lines RL95-2 and HEC-1A in a dose-dependent manner, whereas the GPER-negative line HEC-1B was not affected. Though GPER transcript levels were found to be reduced in endometrial cancer, our *in vitro* data suggest that moderate GPER expression might be sufficient to mediate growth-inhibitory effects triggered by its agonist G-1.

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1. Introduction

Endometrial carcinoma is the most common malignancy of the genital tract among women in western countries. Endometrial carcinoma is the third most common cause of gynecological cancer death behind ovarian and cervical cancer and affects mostly postmenopausal women [1]. Endometrial cancer is known to be hormone-dependent. Whereas the effect of female sex hormones classically has been thought to be mediated by nuclear receptors like estrogen- and progesterone receptors, recent evidence indicated that the non-nuclear receptor GPER (GPR30) plays an important role in steroid hormone signalling [2–6]. GPER, which belongs to the G-protein coupled receptor superfamily, localizes to the endoplasmic reticulum and the cell membrane and its activation initiates a signaling cascade which results in intracellular calcium mobilisation and the release of other second messengers and of epidermal growth factor [7–9]. In the last years, particularly activation of this protein has been a controversial issue. GPER expression in breast cancer cells lacking estrogen receptor (ER) α and β positively correlated with estrogen responsiveness [10]. GPER was suggested to be a third estrogen receptor activated by estrogens or G-1 [3–5,11], but other groups did not observe binding of 17-β estradiol (E2) to GPER [12–14]. However, recent studies supported the fact that this steroid is an important GPER ligand [15,16].

GPER gene expression has been detected in various hormone-dependent cancer entities including breast, endometrial and ovarian cancer [17–20]. GPER has been reported to be downregulated in breast cancer, and a recent study suggested that GPER expression might predict poor survival in ovarian cancer [18,20]

The role of GPER in endometrial carcinoma has been examined in a limited number of studies yielding contradictory results with regard to GPER expression in cancer tissue. Whereas in one study, GPER expression was claimed to be an indicator for poor survival, in a recent study GPER loss was associated with poor outcome [19,21].

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In this study, we first compared GPER expression in normal and malignant endometrial tissue specimens and then tested to what extent the non-steroidal GPER agonist G-1 would be able to affect growth of three endometrial cancer cell lines exhibiting different GPER expression.

2. Patients and methods

2.1. Patients

Tissue samples from normal and malign endometrium were collected between 2007 and 2011 by the Second Department of Gynecology of the Medical University of Lublin, Poland and by the Clinic of Obstetrics and Gynecology, Medical University of Regensburg, Germany. From the 11 premenopausal women, aged 43–53 years (median 47 years), six were in the proliferative phase and five in the secretory phase of the menstrual cycle. Furthermore, 17 endometrial samples from postmenopausal women, aged between 46 and 90 years (median 60 years), were collected. Normal endometrial tissue was obtained from women subjected to surgery for reasons other than pathology of the endometrium. Additionally, we collected 60 endometrial adenocarcinoma tissue samples during routine surgery of the patients. We included 20 patients per tumor grading subgroup (G1–G3), aged 54–82 years (median 61 years) (Table 1). All patients granted consent for the collection and use of biologic material. Tissue samples of patients were collected in accordance with Polish and German regulations and in agreement with the Ethical Committees of the University School of Medicine in Lublin, Poland and of the University of Regensburg, Germany. Complete clinical data were available for every patient. Immediately following surgery, tissues were stored in liquid nitrogen until RNA extraction.

2.2. Cell culture and growth assays

Endometrial cancer cells HEC-1A, HEC-1B and RL95-2 (ATCC, Manassas, USA) were maintained in phenol red-free DMEM/F12 medium supplemented with 10% FCS. Cells were cultured with 5% CO_2 at 37 °C in a humidified incubator. For determination of cell growth, cells were seeded in 96-well plates in medium containing 1% FCS and 1× serum replacement 2 (SR2, Sigma, Deisenhofen, Germany) (10^3 cells per well). The next day cells were treated with GPR30 agonist G-1 (10 nM, 100 nM, 250 nM, 500 nM, 1 μM). At days 0, 3, 4, 5 and 6 relative numbers of viable cells were measured in comparison to solvent controls by means of the fluorimetrical, resazurin-based Cell Titer Blue assay (Promega) according to the manufacturer's instructions at 560Ex/590Em nm in a Victor3 multilabel counter (PerkinElmer, Germany). Cell growth was expressed either as percentage of medium control or as percentage of the solvent control. For statistical analysis we performed a two-way ANOVA analysis and Bonferroni post-tests, using Graph Pad Prism Version 5.01 Software (Graph Pad, San Diego, USA). We considered data as significant at $p < 0.05$.

Table 1
Characteristics of the endometrial tumor samples ($n = 60$) tested in this study. *DMI = depth of myometrial invasion.*

FIGO	G1	G2	G3	DMI > 50%
IA	5	–	–	–
IB	10	12	–	–
IC	5	7	–	12
IIA	–	1	–	1
IIB	–	–	7	7
IIIA	–	–	13	13

2.3. RNA preparation and real-time RT-PCR

Total RNA was isolated from 30 to 80 mg frozen tissue or from cell lines (10^6 cells) by means of Trizol reagent (Invitrogen, Karlsruhe, Germany) according to manufacturer's protocol. RNA purity and concentration was analyzed by spectrophotometry. From each sample, 500 ng of total RNA was reverse transcribed to cDNA using 40 units of M-MLV Reverse Transcriptase and RNasin (Promega, Mannheim, Germany) with 80 ng/μl random hexamer primers (Invitrogen, Karlsruhe, Germany) and 10 mM dNTP mixture (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions. After reverse transcription, the transcript levels of GPER, ERα, PR and PTEN were determined by real-time PCR. For this purpose, 4 μl of cDNA were amplified using LightCycler® FastStart DNA Master^{PLUS} SYBR Green I (Roche Diagnostics GmbH, Mannheim, Germany) and 5 mM of each primer (Table 2). Oligonucleotides (Metabion, Planegg-Martinsried, Germany) were designed intron-spanning to avoid genomic contaminations.

Real-time PCRs were carried out in a LightCycler® 2.0 Instrument (Roche, Mannheim, Germany) under the following conditions: initial denaturation at 95 °C for 15 min, followed by 45 cycles with 10 s denaturation at 95 °C, 5 s annealing at 60 °C and 12 s extension at 72 °C. The PCR program was completed by a standard melting curve analysis. Negative controls were prepared by adding distilled water instead of cDNA. To verify the identity of the PCR products, they were initially analyzed by electrophoresis in 1.5% agarose gels and stained with ethidium bromide. After size check, each PCR product was then purified using the "QIAquick Gel Extraction Kit" (Qiagen, Hilden, Germany), following the manufacturer's protocol

Table 2
Primers used for qRT-PCR analysis of gene expression.

Gene	Primer (5′-3′)	Amplicon [bp]
GPER	TCCTCTCTCTAGCCCTGCTC CTCTCTGGGTACCTGGGTTG	148
ERα	CACATGAGTAACAAAGGCATGG ATGAAGTAGAGCCCGCAGTG	181
PR	CAACTACCTGAGGCCGGATT CATTGCCCTCTTAAAGAAGACCT	160
PTEN	CCAGAGACAAAAAGGGAGTAACTA TCACCTTTAGCTGGCAGACC	189

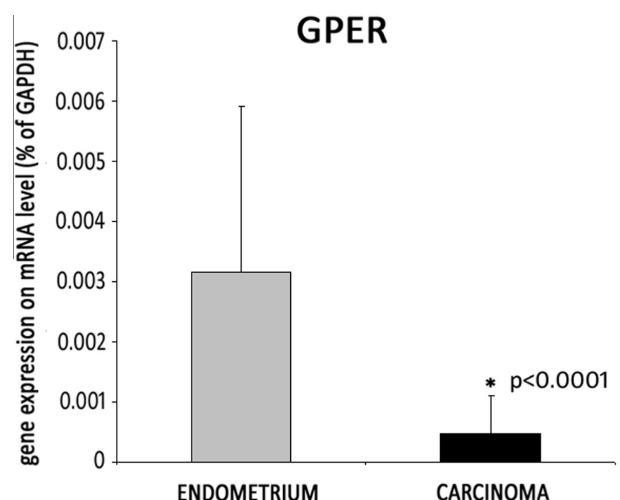

Fig. 1. GPER expression in normal endometrium and in endometrial carcinoma tissue. Gene expression was assessed at the mRNA level by means of RT-qPCR as described in the materials and methods section. Values are expressed in percent of GAPDH expression. * = $p < 0.001$ vs. endometrium.

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