Gynecologic Oncology 131 (2013) 191–197

Contents lists available at ScienceDirect

Gynecologic Oncology

journal homepage: www.elsevier.com/locate/ygyno

Down-regulation of focal adhesion signaling in response to cyclophilin A knockdown in human endometrial cancer cells, implicated by cDNA microarray analysis

Zhengyu Li *, Jinhai Gou, Jing Xu

Department of Gynecology and Obstetrics, West China Second University Hospital, Sichuan University, Chengdu, People's Republic of China

HIGHLIGHTS

- CypA knockdown leads to suppression of migratory/invasive capacity of HEC-1-B cells.
- CypA knockdown leads to down-regulation of focal adhesion signaling, implicated by microarray analysis.
- Suppression of migratory/invasive capacity is likely associated with down-regulation of focal adhesion signaling.

ARTICLE INFO

Article history:
Received 23 May 2013
Available online 27 July 2013

Keywords:
Endometrial cancer
Cyclophilin A
Microarray
Focal adhesion

ABSTRACT

Objective. CypA had been identified as a potential therapeutic target to endometrial cancer in our previous research. Herein, we aimed to further elucidate the underlying comprehensive mechanisms of CypA knockdown-associated anticancer effects by cDNA microarray-based approach.

Methods. LV-shCypA was constructed and transfected into HEC-1-B cells. The efficiency of CypA knockdown was determined by qRT-PCR and Western blotting. The migratory/invasive capacity was examined by transwell assay. CypA knockdown-induced comprehensive gene expression alterations were analyzed using NimbleGen Human Gene Expression Microarray consisting of 45,033 probes for human genes. Functional analysis of the microarray data was performed using KEGG and Gene Ontology analyses. The selected differentially expressed genes were validated by qRT-PCR.

Results. Knockdown of CypA by LV-shCypA led to a significant decrease of migratory/invasive cell proportions in HEC-1-B cells. Microarray analysis showed 3533 and 2772 genes to be up-regulated and down-regulated in CypA-knockdown cells, respectively. Functional analysis showed 50 up-regulated pathways and 14 down-regulated pathways in CypA-knockdown cells, and focal adhesion signaling was one of the most enriched down-regulated pathways. The expression patterns of 16 genes in focal adhesion signaling, which encoded MAPK kinases, focal adhesion kinase (FAK), integrin subunits and laminin subunits, were validated by qRT-PCR and the consistency percentage with microarray data reached 100%.

Conclusions. Suppression of migratory/invasive capacity by CypA knockdown is likely associated with the down-regulation of focal adhesion signaling, which may contribute to the understanding of the role of CypA as a potential therapeutic target for endometrial cancer.

Introduction

Endometrial cancer is one of the most common malignancies of the female genital tract with an approximately 80% 5-year survival for all stages taken together. Presently, surgery combined with adjuvant or postsurgical chemotherapy is the predominant treatment strategy for endometrial cancer. However, although the case fatality rate is lower than that of other female genital malignancies, a group of patients also suffered cancer recurrence, metastasis or drug resistance, making the prognosis unoptimistic. Hence, there is a persistent need for the discovery of novel therapeutic target and optimization of treatment regimens for endometrial cancer. In a previous proteomic study [1], we identified cyclophilin A (CypA) as one of the most significantly altered proteins in endometrial cancer. Multivariate analysis indicated that it was an independent prognostic factor for survival of endometrial cancer patients. Knockdown of CypA by RNA interference led to the suppression of cell proliferation, the induction of apoptosis in endometrial cancer HEC-1-

* Corresponding author at: Department of Gynecology and Obstetrics, West China Second University Hospital, Sichuan University, Chengdu 610041, People's Republic of China. Fax: +86 28 85502391.
E-mail address: qingshanxiagu@tom.com (Z. Li).

0090-8258/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ygyno.2013.07.095

B cells in vitro, and the inhibition of xenograft tumor growth in vivo. These results suggested the possibility of CypA serving as a novel therapeutic target for endometrial cancer.

CypA is a peptidylprolyl cis-trans isomerase (PPIase), which plays important roles in protein folding, trafficking, assembly, immune-modulator and cell signaling. Recently some studies demonstrate that it displays an elevated expression in various types of cancer, promotes cancer cell proliferation and cell migration/invasion and inhibits apoptosis [2]. There have been some reports being proposed to explain the underlying mechanism of CypA-associated effects, but most of them have focused on a few specific genes or pathways. CD147 is a widely expressed plasma membrane protein, serving as a receptor for CypA. CypA interacts with a proline-containing peptide in the transmembrane domain and initiates a signaling cascade that leads to the activation of MAPK kinase pathways, including ERK1/2 and p38 MAPK [2–4], the most common signal pathways shared by other growth factors. Exogenous CypA stimulated cell proliferation through CD147 by activating MAPK kinase pathways in diverse cancer types, including pancreatic cancer, lung cancer, and cholangiocarcinoma [5–8].

As a natural continuation of our proteomic discovery, we aimed to clarify the mechanism of CypA knockdown-induced anticancer effects in a genome-wide extent, for the first time to our knowledge, using cDNA microarray technology. Alternatively, this approach makes no assumptions about known or unknown genes, allowing the process to be independent of any presupposed hypotheses. Additionally, strict validation processes are also necessary to the unbiased target selection. Our findings should contribute to the better understanding of the role of CypA as a potential therapeutic target for human endometrial cancer.

Materials and methods

Lentiviral short hairpin RNA construction

Four siRNA sequences were designed against the human cyclophilin A sequence in GenBank (Accession NM_021130) using the on-line siRNA software (http://ihome.ust.hk/~bokcmho/siRNA/siRNA.html). The short hairpin RNA (shRNA) expression cassettes were designed according to the siRNA sequences, and the synthesized sequences were listed in Table 1. A randomly computer-generated nonsense sequence was selected as negative control (NC). The shRNA vectors were generated by inserting annealed oligo sequences into the digested pGCSIL-GFP vectors (GeneChem Co., Ltd, Shanghai, China) between *Hapl* and *XhoI* sites. The recombinant plasmids were transformed into competent *Escherichia coli* DH5α cells, and positive clones were identified by PCR. Insertion at the intended site was confirmed by DNA sequencing. For lentiviral vector production, the transfer vector pGCSIL-GFP-CypA, the packaging vector pCMV-dR8.74 and the VSV-G expression plasmid pMD2G were amplified in *E. coli* DH5α cells and purified using a plasmid purification kit (Qiagen, Hilden, Germany). Lentivirus producer HEK-293T cells were transfected with pGCSIL-GFP-CypA (20 μg), pCMV-dR8.74 (15 μg) and pMD2G (10 μg) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) overnight in the 5% CO_2 incubator. The next day, the medium was replaced, and the supernatants were collected, clarified, and filtered through polyvinylidene difluoride filters (Millipore, MA, USA) at 48 h post-transfection. The supernatants were then concentrated by adding 10% of PEG-8000, incubated at 4 °C overnight, centrifuged at 1500 *g* for 10 min, and named as LV-shCypA-1, -2, -3 and -4. Lentiviral titers were determined by fluorescence microscopic analysis of GFP fluorescence in HEK-293T cells. The lentivirus pellets were resuspended in a small volume of Opti-MEM and stored at −80 °C.

Cell lines and transfection

The human endometrial cancer cell line HEC-1-B was purchased from American Type Culture Collection (ATCC, Manassas, VA). This cell line has a karyotype of diploid to tetraploid with large submetacentric marker, and can form moderately well differentiated adenocarcinomas consistent with endometrial carcinoma (grade II) (https://www.atcc.org/). Cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FCS) in a 5% CO_2 atmosphere at 37 °C. Cells were plated in 6-well plates and transfection of lentiviral vectors was conducted at a 5 multiplicity of infection (MOI) when the cell confluence reached about 50%. Interference efficiency was determined by qRT-PCR and Western blotting.

Quantitative RT-PCR

Total RNA was extracted using the RNeasy Kit (Qiagen, Valencia, CA), and reverse transcription to cDNA was conducted using the Superscript First Strand synthesis system (Invitrogen, Carlsbad, CA). All reactions were carried out on an ABI 7500 Real-Time PCR instrument (Applied Biosystems, Foster City, CA) using the SYBR Green Real-Time PCR Master Mix kit (Toyobo, Osaka, Japan) according to the manufacturer's instruction. The 18s rRNA primer was included in each plate to account for sample variations, and the mRNA level of each sample was normalized to that of 18s rRNA. Each sample was run independently in triplicate, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control to normalize reactions. After completion of the PCR amplification, the relative fold change was calculated based on the 2-ΔΔ Ct method. The qRT-PCR primer sequences for human CypA were 5′-CCTAAAGCATACGGGTCCTG-3′ (sense) and 5′-TTTCACTTTGCCAAACACCA-3′ (antisense), and primer sequences for the differentially expressed genes were listed in Table 3.

Western blotting

The cells were collected and lysed in RIPA lysis buffer. After being quantified by a Bradford assay kit (Bio-Rad, Hercules, CA), the lysates were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. After blocking with 5% dry milk in TBS-Tween 20, the membranes were incubated with rabbit anti-CypA antibody (1:5000, Upstate Biotechnology, VA, USA) overnight at 4 °C. The blots were labeled with horseradish peroxidase-conjugated secondary antibodies, visualized by chemiluminescent detection. The equivalent

Table 1
The sequences of synthesized oligonucleotides.

No.		5′	STEM	Loop	STEM	3′
pGCSIL-GFP-CYPA-1	Oligo 1	Ccgg	aaGTGAAAGAAGGCATGAATA	TTCAAGAGA	TATTCATGCCTTCTTTCACtt	TTTTTg
	Oligo 2	aattcaaaaa	aaGTGAAAGAAGGCATGAATA	TCTCTTGAA	TATTCATGCCTTCTTTCACtt	
pGCSIL-GFP-CYPA-2	Oligo 1	Ccgg	CTGACTGTGGACAACTCGAAT	TTCAAGAGA	ATTCGAGTTGTCCACAGTCAG	TTTTTg
	Oligo 2	aattcaaaaa	CTGACTGTGGACAACTCGAAT	TCTCTTGAA	ATTCGAGTTGTCCACAGTCAG	
pGCSIL-GFP-CYPA-3	Oligo 1	Ccgg	GAATGGCAAGACCAGCAAGAA	TTCAAGAGA	TTCTTGCTGGTCTTGCCATTC	TTTTTg
	Oligo 2	aattcaaaaa	GAATGGCAAGACCAGCAAGAA	TCTCTTGAA	TTCTTGCTGGTCTTGCCATTC	
pGCSIL-GFP-CYPA-4	Oligo 1	Ccgg	GTTTGCAGACAAGGTCCCAAA	TTCAAGAGA	TTTGGGACCTTGTCTGCAAAC	TTTTTg
	Oligo 2	aattcaaaaa	GTTTGCAGACAAGGTCCCAAA	TCTCTTGAA	TTTGGGACCTTGTCTGCAAAC	