

# CYPB upregulates and promotes cell proliferation in endometrial carcinoma

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## Research

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# Abstract

**BACKGROUND** The molecular pathogenesis of endometrial cancer is not yet completely understood, preventing the development of successful therapies. Here, we determine the effect of CYPB on the growth of endometrial cancer. **METHODS** In this study, we examined the expression of CYPB in endometrial cancer tissues using immunohistochemistry. CYPB silencing in the human cell line HEC-1-B was used to evaluate the role of CYPB in the malignant phenotype of endometrial tumor cells, while CCK-8 and colony formation assays were performed to assess its effect on tumor cell proliferation. Furthermore, microarray analysis was carried out to compare the global mRNA expression profile between the normal and CYPB-knockdown cell. Gene ontology and KEGG pathway enrichment analysis were performed to determine the potential function of different expressed genes related to CYPB. **RESULTS** We found that CYPB was upregulated in endometrial cancer, while cells with suppressed expression of CYPB exhibited markedly reduced migration. We identified 1536 differentially expressed genes (onefold change,  $p < 0.05$ ), among which 652 genes were upregulated and 884 genes were downregulated, and most of them were enriched in cell cycle, glycosphingolipid biosynthesis, adherens junctions, and metabolism pathways. **CONCLUSIONS** The results of our study suggest that CYPB may serve as a novel regulator of endometrial cell proliferation, thus representing a novel target for gene-targeted endometrial therapy.

## Introduction

Endometrial cancer represents a group of epithelial malignant tumors occurring in the endometrium and is one of the three most common malignant tumors of the female genital tract. Seventy-five percent of the patients are diagnosed in an early phase, and the 5-year survival rate was 65–92% [1, 2]. Molecular pathogenesis of endometrial cancer involves abnormalities in many genes and signaling pathways. For example, mutations in P53, increased microsatellite instability, mutations in PTEN, and abnormalities in the Notch signaling pathway all lead to uncontrolled cell proliferation and apoptosis, which in turn lead to the occurrence and development of endometrial cancer [3–6]. At present, the molecular pathogenesis of endometrial cancer is not entirely understood, and further research is warranted in order to develop successful therapies.

Cyclophilins are highly conserved proteins that are ubiquitously expressed intracellularly. They were first recognized as host cell receptors for the potent immunosuppressive drug cyclosporin A [7, 8]. They act as molecular chaperones that fold, translocate, and process newly synthesized proteins. Cyclophilin B (CYPB) is a 21 kDa peptidyl-prolyl cis-trans isomerase [9] that is expressed in the endoplasmic reticulum (ER) lumen [10] and nucleus [11]. It has been implicated in hepatitis virus replication [12], immunosuppression [13], chemotaxis [14], and prolactin signaling [15]. Moreover, its increased expression may significantly contribute to the pathogenesis of human breast cancer [16], myeloma [17], hepatic carcinoma [18], gastric cancer [19], head and neck squamous cell carcinoma [20], and glioblastoma [21]. Finally, it has also been used as a serum biomarker for early detection of pancreatic cancer [22]. However, to date, no study has investigated the role of CYPB in endometrial cancer.

Hence, to determine the effect of CYPB on the growth of endometrial cancer, we aimed to assess its expression in endometrial tissues and CYPB-silenced HEC-1-B cells and measure the relative gene expression with microarray analysis.

## Materials And Methods

### Cell lines and transient transfection

The HEC-1-B cells were purchased from the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in modified Eagle's medium (Gibco, Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA). And cell lines were maintained at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>.

The CYPB-siRNA and negative control siRNA (NC-siRNA) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA; sc-35145 and sc-36869, respectively). Cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

### Sample Collection

All the tissue samples were collected via biopsy of surgical resection between December 2017 and September 2018 in the department of Gynecology, Affiliated Yantai Yuhuangding Hospital (Yantai, Shandong, China). The study was approved by the Ethics Committee of Yantai Yuhuangding Hospital in November 27, 2017 (registration number: YLYLLS [2018] 008). All samples were collected after obtaining written informed consent. The samples were snap-frozen in liquid nitrogen and stored at - 80 °C prior to RNA extraction or generation of formalin-fixed, paraffin-embedded tissue sections for immunohistochemistry.

### Cell Proliferation And Clone Formation

Cell proliferation was determined using the Cell Counting Kit-8 assay (CCK-8; Beyotime Biotechnology, Shanghai, China) per the manufacturer's instructions. Cells in the logarithmic growth phase ( $1 \times 10^4$  cells/mL per well) were grown in 96-well plates in medium containing 10% FBS in an incubator with 5% CO<sub>2</sub> at 37 °C for 72 h. Afterwards, 10 mL of CCK-8 solution was added to each well, and the plates were incubated for an additional 4 h. The absorbance in each well was measured at a wavelength of 450 nm with a microplate reader.

For clone formation, HEC-1-B cells were transfected with CYPB-siRNA for 48 h and were collected and seeded in triplicate into 6-well plates at a density of 1,000 cells/mL per well. The cells were incubated for 10 days at 37 °C in a 5% CO<sub>2</sub> atmosphere. They were then fixed with 4% paraformaldehyde for 30 min

and stained with Giemsa (Beyotime Biotechnology) for 20 min. After washing with double-distilled H<sub>2</sub>O several times, images of the cell plates were taken (Canon, Inc., Tokyo, Japan).

## **RNA extraction, reverse transcription, qRT-PCR and microarray analysis**

Total RNA was extracted using TRIzol, and cDNA was synthesized with PrimeScript RT reagent Kit (TaKaRa, Dalian, China). Gene expression was assessed by qRT-PCR using SYBR Premix Dimer Eraser (Perfect Real Time, TaKaRa) assay kits. Relative fold changes in expression were calculated using the comparative Ct ( $2^{-\Delta\Delta Ct}$ ) method. Purified RNA samples were submitted to Phalanx Biotech (Hsinchu, Taiwan) for microarray analysis. We used the Phalanx Human OneArray Plus Gene Expression Profiling platform 6.1 to analyze the CYPB-mediated alterations of mRNA expression.

## **Immunohistochemistry (ihc)**

Sections (4 μm) were cut from the constructed TMA blocks, deparaffinized, and rehydrated. Heat-induced epitope retrieval was performed onboard of the Leica Bond RX platform at 100 °C using EDTA buffer (pH 9.0, Leica) for 20 minutes, followed by 15 min of incubation with anti-CYPB antibody (#43603, Cell Signaling Technology, Danvers, MA, USA) or anti-β-catenin antibody (#8480, Cell Signaling Technology) at room temperature and with Bond™ Polymer Refine Detection kit (Leica Biosystems, Buffalo Grove, IL, USA) for 8 min. The reaction was visualized using 3,3'-diaminobenzidine tetrahydrochloride for 10 min and with hematoxylin as a counterstain. Scoring was performed by pathologists (MK, PR) using Nikon Eclipse microscope on TMA glass slides at 20 × magnification. Tissues were scored for CYPB expression, and the scoring system reflected the extent and intensity of staining: the intensity was assigned a score of 0, 1, 2, or 3, representing negative, weak, moderate, or strong expression, respectively; while the extent was assigned a score of 0, 1, 2, 3 or 4, representing < 5%, 6–25%, 26–50%, 51–75% and > 75% of cells stained. The overall quantitation of the score was obtained by multiplying the average intensity and score of five different high-power fields (at 400 × magnification). The samples were divided into two groups based on final staining scores, which ranged from 0 to 7: the high expression group (scores of ≥ 4) and the low expression group (scores of < 4)[23].

## **Gene Ontology Functional And Pathway Enrichment Analysis**

GO and KEGG pathway enrichment analysis were used for differentially expressed genes (DEGs) using the DAVID database. FDR values of < 0.05 were set as the cut-off criterion for the two analyses.

## **Statistical analysis**

Statistical analysis was performed using SPSS software, version 18.0 (SPSS, Chicago, IL, USA). The chi-square test was used to determine the differences in age and tumor grades between high and low expressed CYPB groups. Differences between two groups were analyzed using Student's t-test for comparison of two groups or by one-way analysis of variance for comparison of more than two groups. P values of < 0.05 were considered statistically significant.

## Results

### CYPB is over expressed in endometrial cancer

Seventy-four control tissue samples, consisting of 24 normal endometrium tissue and 50 of atypical hyperplastic endometria, and 96 endometrial cancer tissues were used to validate protein expression by immunohistochemistry. CYPB expression was significantly higher in endometrial cancer tissues compared with non-cancer tissues, and it was higher in intrauterine dysplasia than in the normal endometrium (Fig. 1), suggesting that higher expression of CYPB is associated with the incidence of endometrial cancer. Furthermore, no significant association was observed between CYPB expression and patient age or tumor grades (Table 1).

Table 1  
The CypB expression between age and tumor grades.

		CypB expression levels			
		High	low	Ratio (high/low)	P*
Characteristic	N(%)				
Ages					
<55	39	16	23		0.916
≥ 56	57	24	33		
Grade					
Tis + I	68	40	28	1.43	0.864
II + III	28	17	11	1.55	
Low (I + II)	85	50	35	1.43	
High (III)	8	7	1	7	
* chi-square test was used					

# Downregulation of CYPB inhibits HEC-1-B cell proliferation and colony formation

In order to investigate the role of CYPB in endometrial cancer, we treated the HEC-1-B cell line with *CYPB*-siRNA. *CYPB*-siRNA significantly reduced *CYPB* mRNA expression by over 70% (Figure 2), indicating that a highly efficient knockdown of *CYPB* expression was achieved.

Microscopic observation of the HEC-1-B cells were transfected with *CYPB*-siRNA or NC-siRNA showed a decrease in cell proliferation after downregulation of *CYPB* (Figure 3a). The results of the CCK-8 assay indicated that, compared control cells, the proliferation of *CYPB*-knockdown HEC-1-B cells decreased 72h after transfection (Figure 3b). Finally, *CYPB* silencing in HEC-1-B cells substantially reduced colony formation as well (Figure 3c).

## Identification of DEGs in endometrial cancer cell with downregulated CYPB expression

We performed a microarray analysis, comparing the global mRNA expression profile between the normal and *CYPB*-knockdown HEC-1-B cells. A volcano plot of the identified quality-controlled genes ( $p < 0.05$ ; fold change,  $> 1$ ) is presented in Figure 4a. The microarray identified 1536 differentially expressed mRNAs in total, of which 652 were upregulated and 884 downregulated in the *CYPB*-knockdown group. A heat map was generated to show genes that were previously identified as significantly upregulated in HEC-1-B cells (Figure 4b).

## GO functional and pathway enrichment analysis

GO and pathway enrichment analysis showed that the DEGs were significantly enriched in cell cycle, glycosphingolipid biosynthesis, adherens junctions, and metabolism pathways. Important genes and pathways involved in this process are shown in Figure 5.

## Validation of differentially expressed RNA by qRT-PCR

To evaluate the reliability of microarray data, we verified the expression of five differentially expressed mRNA in HEC-1-B cells by qRT-PCR assay. As shown in Figure 6, the mRNA expression of SREBF1, SPP1, PPIB and PLAT were significantly downregulation in the CypB-knockdown cells. While the CDKN1A mRNA shown a much higher expression in the CypB-knockdown cells. All these results were consistent with the microarray data.

## Discussion

Endometrial carcinoma remains one of the leading causes of death among women, and, therefore, the discovery of novel molecular targets for its diagnosis, prognosis, and treatment is required for improving the clinical strategy and outcome for this disease. Cyclophilins have been implicated in a variety of cancers; however, their expression has not been studied in endometrial carcinoma. There are 16 types of human cyclophilins, cyclophilins A (CYPA) and CYPB being the two most abundant and the most studied ones.

Recent studies have found that CYPA expression is implicated in several cancers, including lung cancer [24, 25], pancreatic cancer [26, 27], hepatocellular cancer [28], and buccal squamous cell carcinoma [29], and that it might play a role in apoptosis through the activation of caspases and apoptosis-inducing factor. A study with a two-dimensional gel electrophoresis and MALDI-Q-TOF MS/MS-based proteomics approach found that overexpression of CYPA is significantly correlated with a low degree of cancer differentiation, and its overexpression was associated with decreased survival in endometrial carcinoma[30].

On the other hand, the structurally similar CYPB, which is found in the endoplasmic reticulum, has been implicated in STAT3 activation and in the generation of reactive oxygen species in cancer cells [21]. CYPB facilitates the transcriptional activity of STAT5 by inducing the release of the repressor PIAS3, resulting in significantly enhanced STAT5-mediated gene expression [11, 15]. At the cell surface, CYPB also serves as a ligand for the CD147 receptor [31], which regulates MAPK activation, motility, calcium transport [31–33], and the expression of the pro-apoptotic protein BIM [34]. Gene expression studies revealed that CYPB is highly upregulated in malignancies. Ablation of CYPB expression in glioblastoma multiforme cells suppresses several canonical oncogenic signaling pathways, including mutant P53, MYC, and CHK1.

## Conclusions

In this study, we demonstrated that patients with endometrial cancer exhibit a significantly higher expression of CYPB, suggesting that CYPB expression can be considered an effective indicator for the clinical outcome of endometrial cancer. Furthermore, our results demonstrate that CYPB acts as an oncogene in endometrial cancer and that silencing its expression affects the expression of many genes involved in the cell cycle, glycosphingolipid biosynthesis, adherens junctions, and metabolism pathways.

## Declarations

### Acknowledgments

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### Authors' contributions

JL, YZ and HKW contributed to the conception and design. GMQ, XS contributed to the acquisition, analysis, and interpretation of data. XS and ZHL contributed to drafting the work. TGZ, ZHZ and HKW contributed to revising it critically. All authors approved the final version to be published and agreed to be accountable for all aspects of the work.

### **Ethics approval and consent to participate**

The study was approved by the Ethics Committee of Yantai Yuhuangding Hospital in November 27, 2017 (registration number: YLYLLS [2018] 008). Written informed consent was obtained from all participants.

### **Funding**

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### **Availability of data and materials**

The datasets generated and/or analyzed during the current study is available from the corresponding author on reasonable request.

### **Consent for publication**

The consent for publication is not applicable because this study does not include any individual details, images, or videos.

### **Competing interests**

The authors have no conflicts of interest to declare.

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## Figures

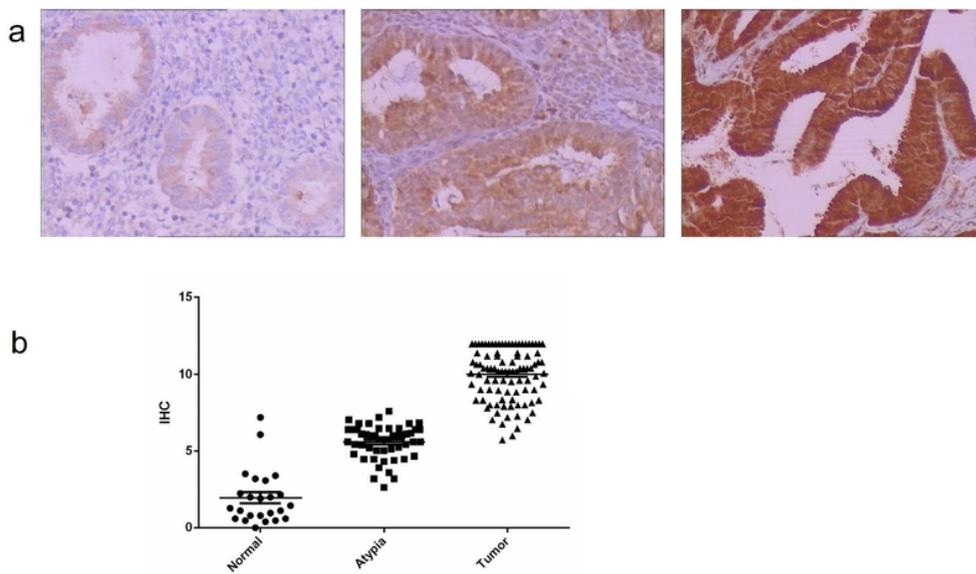


Figure 1

## Figure 2

CYPB is overexpressed in endometrial cancer. (a) Representative photomicrographs of immunohistochemical staining for CYPB among endometrial tissue samples are presented. (b) Statistical analysis of relative CYPB expression levels in normal tissues, atypic tissues, and endometrial tissues. These findings indicate that CYPB was significantly upregulated in tumor tissues.

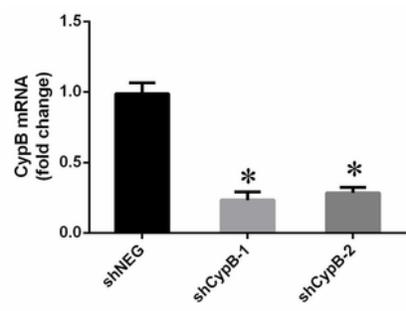


Figure 2

#### Figure 4

siRNA inhibited CYPB expression efficiently in HEC-1-B cells. shCYPB-1 and shCYPB-2 significantly downregulated the expression of CYPB.

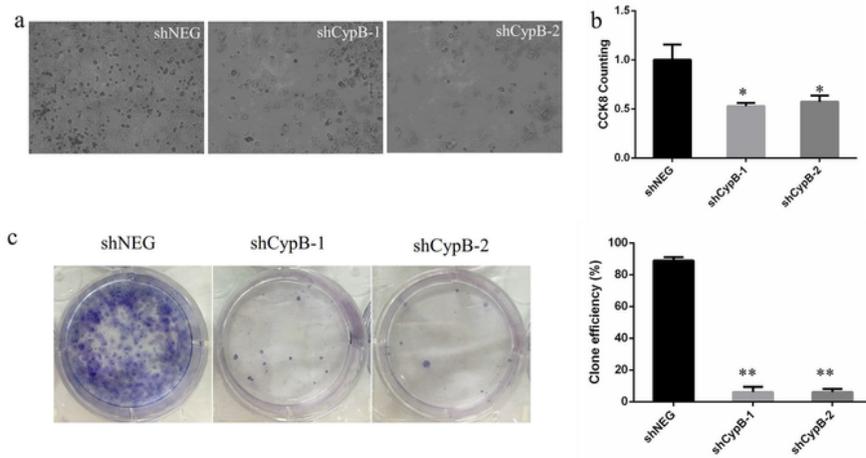


Figure 3

## Figure 6

CYPB knockdown suppressed proliferation in HEC-1-B cells. (a) The conventional microscope pictures of HEC-1-B cells treated with shNEG and siRNAs s (-1 and -2). (b) CCK-8 assay and colony formation (c) assay show that CYPB silencing significantly inhibited HEC-1-B cell proliferation and colony formation. (\* $p < 0.05$ , \*\* $p < 0.005$ ).

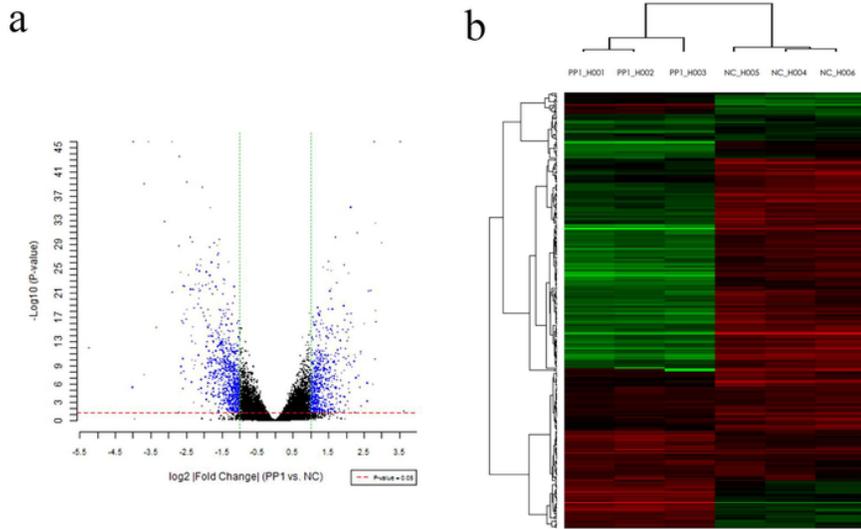


Figure 4

## Figure 8

Identification of DEGs in endometrial cancer cell. (a) The vertical lines correspond to 1-fold up and down, respectively, and the horizontal line represents a p value of 0.05. (b) Heatmap plot of differentially expressed genes in human endometrial cancer.



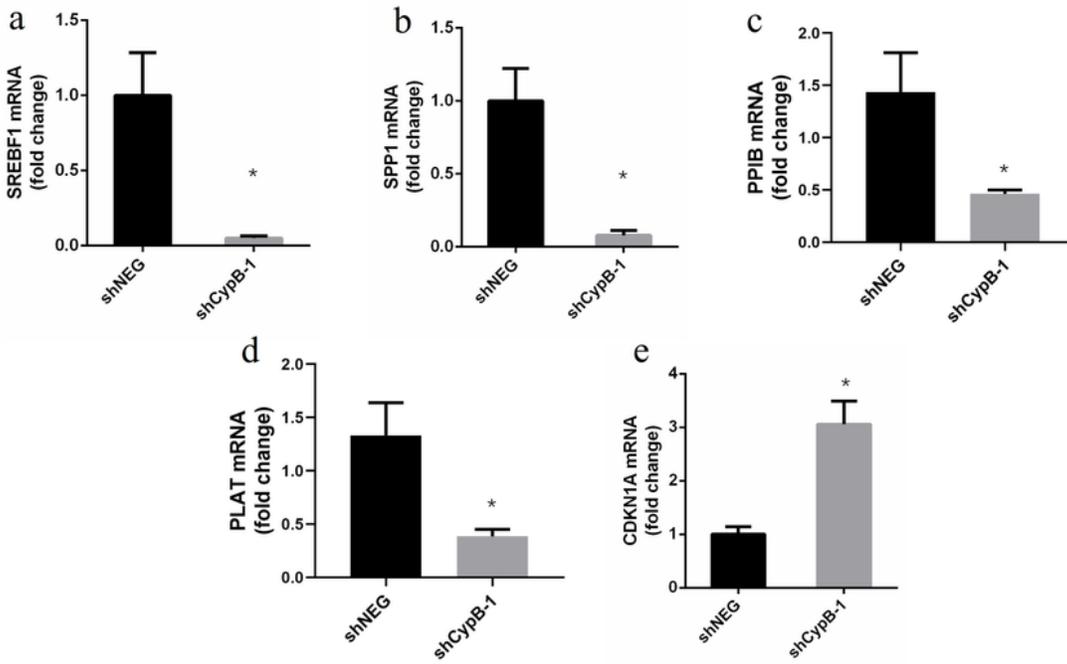


Figure 6

## Figure 12

The expression of real-time qPCR was in accordance with microarray data