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The impact of progesterone receptor isoforms expression to invasion capacity and SULT1E1 in endometrial cancer cells

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Summary

Aim: This study aimed to investigate the mechanism by which progesterone receptors (PRs) mediate in metastatic spread of endometrial cancer (EC). *Materials and Methods:* The expressions of human progesterone receptor isoforms A and B (hPRA and hPRB) in Ishikawa endometrial cancer cells transfected were examined by Western blot. Matrigel invasion assay was performed to illustrate the roles of hPRA and hPRB in EC metastasis.The underlying mechanism of PR regulating invasion was assessed by Western blot and RT-PCR. *Results:* In this study ,the authors found a significant increased expression of hPRA and hPRB in transfected cells, respectively, whereas hPRB exhibited a slightly decreased expression upon hPRA stimulation. Matrigel invasion assay demonstrated that hPRB decreased cell invasion, and this process could be largely restored by hPRA treatment. Further study of potential mechanism indicated that hPRB upregulated the expression of SULT1E1, which was connected with endometrial oncogenesis, and the suppression of hPRB via hPRA, exerted an inhibitory effect on the expression of SULT1E1. *Conclusions:* The above data showed that hPRB promoted the expression of SLUT1E1 to inhibite the invasion of EC cells. In contrast, hPRA played an opposite role in attenuating the effects of hPRB on the invasion of Ishikawa cells and the expression of SLUT1E1.

Key words: hPRA; hPRB; SULT1E1; Endometrial cancer; Invasion.

Introduction

Endometrial cancer (EC) is one of the most common malignant tumors of the female reproductive tract [1]. Most patients in an early stage could be diagnosed and treated successfully, whereas the treatment yields suboptimal results for women with advanced, recurrent, or metastatic endometrial cancer. Surgery combined with adjuvant radiation therapy or chemotherapy usually has a poor effect on progressive disease which accounts for mass mortality worldwide [2]. Therefore, a better understanding of carcinogenic mechanism and a new therapeutic strategy for this population have been a critical yet unrealized clinical need.

Progestin inhibits the growth of cancerous endometria via progesterone receptor (PR) and improves prognosis. However, response rates to progestin therapy go from 67–82% in atypical endometrial hyperplasia, to 50–70% in well-differentiated EC, and drops to 15–27% in the relapse type [3]. The declining responsiveness to progestin limits its use in clinic. Thus, it is essential to understand the functions of PR isoforms in endometrial pathologies.

Recently endometrial oncogenesis is connected with an altered expression of SULT1E1. SULT1E1, a member of sulfotransferases, is famous as a broad-specificity enzyme that detoxifies sorts of chemicals, including estrogens, by the transfer of sulfate. SULT1E1 exhibits the highest affinity for estrogens[4] and the most intense physiological activity to concentrations of estrogens [5]. It has been reported that SULT1E1 is related to the progress of uterine fibroid [6] and breast cancer [7]. The study by Falany [8] indicated that SULT1E1 activity in Ishikawa cells results in a lack of endometrial stimulation. However, its underlying mechanism remained unclear. Furthermore, this study highlighted to explore the effects of hPRA and hPRB on invasion capacity of Ishikawa cells and expression of SULT1E1.

Materials and Methods

Human EC cell line—Ishikawa cells were obtained from the Pathological Physiology Laboratory of Peking Universitynand maintained in Dulbecco's modified Eagle's medium with glucose content of 4.5 g/L, supplemented with 10% fetal calf serum, penicillin and streptomycin 100 u/mL each. The cells were incubated in 5% CO_2 at 37°C and saturated humidity. Cell cultures of 75% confluence were used for experiments.

After being transformed into Escherichia coli DH5α, the plasmids were inverted overnight at 37°C. Bacterial colonies with white-positive clone were chosen to grow in Luria-Bertani medium overnight. Plasmids were extracted according to the instruction of plasmid extraction kit and identified by restriction enzyme digestion.

Transfection was performed with lipofectamine 2000 according to the manufacturer's instructions. At the beginning, 1×10^6 Ishikawa cells/well were plated onto six-well plates and grown

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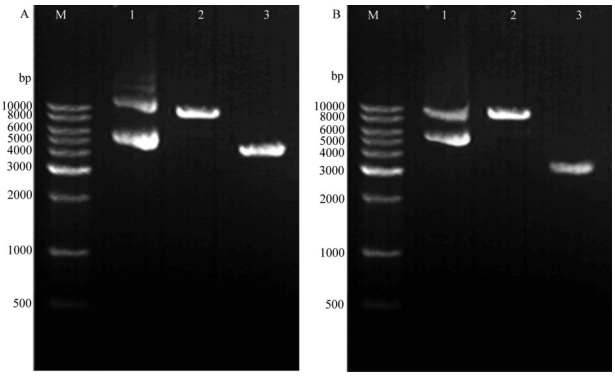

Figure 1. — Identification of pcDNA3.1-hPRA and pcDNA3.1-hPRB with restriction enzyme: (A) Identification of pcDNA3.1-hPRA with restriction enzyme. Lanes: 1, hPRA/pcDNA3.1 plasmid; 2, hPRA/pcDNA3.1 digested with EcoRI; 3, hPRA/pcDNA3.1 digested with XhoI. M, DNA KB ladder. (B) Identification of the recombinant plasmid pcDNA3.1-hPRB by restriction digestion. Lanes: 1, hPRB/pcDNA3.1 plasmid; 2, hPRB/pcDNA3.1 digested with EcoRI; 3, hPRB/pcDNA3.1 digested with XhoI. M, DNA KB ladder.

for 24 hours until the cells were 75% confluent. The cell samples were divided into five groups and each group had three parallel samples: group A: blank-control, group B: vector-control, group C: hPRA, group D: hPRB, and group E: hPRA+hPRB. Four μl of PBS, pcDNA3.1, hPRA, hPRB or hPRA+hPRB and 10 μl of lipofectamine 2000 were each diluted in 250 μl of serum-free DMEM, and incubated for five minutes at room temperature. Then, the diluted liquid and lipofectamine 2000 were combined at a 1:1 ratio. This combination was mixed gently, and incubated for 20 minutes at room temperature to form pcDNA3.1/lipofectamine 2000 composite; 500 μl of the combination was added to each well in a final volume of 2 ml/well. The cells were incubated for another 24 hours before the experiments were conducted.

To obtain the pure recombinant plasmids, the Ishikawa cells after transfection were selected by G418 with the best concentration 700 μg/ml.

A transwell plate (aperture size of 8 μm) was used for the invasion assay with the filter coated with pure Matrigel at 37°C for 5 minutes; 100 μl of 1×10^5/ml suspended Ishikawa cells of each group were added to the upper chambers, and the lower wells were filled with 2 ml of medium containing 10% fetal calf serum and 10 mmol/L of progesterone. The cells were allowed to invade for 48 h in 5% CO_2 at 37°C. The authors then removed the cells attached to the upper surface of the filter by scrubbing with a cotton swab. The cells on the lower surface were fixed in 95% methanol for 30 minutes at room temperature, and stained with Trypan Blue. For quantification, the cells that had migrated to the lower surface were counted under a light microscope in five predetermined fields at ×400 magnification. Each group had four repeat-wells and the assay was repeated three times. The results of invasion rate were expressed as a percentage of the ratio between the cells in experiment group and the cells in control group.

Total RNA was extracted using the TRIZOL reagent according to the supplier's instructions. The forward primer (5′-3′) of SULT1E1 Gene was TGAAGACTCATTTGCCACCTGA, and the reverse primer (5′-3′) was TGGATGACCAGCCACCATTAGA. Independent experiments were done in triplicate. House-keeping gene GAPDH was used as a loading control, and each total RNA sample was normalized to the content of GAPDH mRNA. For real-time PCR, the $2^{-\Delta\Delta Ct}$ value was used to evaluate expression level of SULT1E1 mRNA.

After indicating treatment, cells were harvested in radioimmune precipitation assay (RIPA) lysis buffer. Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to detect the target protein levels and beta-actin was used as the control.

Data from experiments were presented as mean ± SD. Analysis of variance (ANOVA) and the independent-samples *t*-test were performed by SPSS 15.0 software. Statistical significance was defined as $p < 0.05$.

Table 1. — *Effect of hPRA and hPRB on the inhibition of the invasion of Ishikawa cells.*

Group	Cell population	Rate of invasion
Blank-control	36.00±3.56	1.0
Vector -control	35.75±4.57	1.0±0.2
hPRA	34.00±3.65	0.9±0.2
hPRB	16.40±2.30	0.4±0.1
hPRA+hPRB	35.25±3.50	1.0±0.2

Table 2. — *The expression of SULT1E1 mRNA*

Group	Δ*ct*	ΔΔ*ct*	$2^{-\Delta\Delta ct}$
Blank-control	10.79±0.504	0.0±0.504	1(0.705-1.42)
vector -control	11.43±0.509	0.64±0.509	1.56(0.45-0.913)
hPRA	11.32±1.039	0.53±1.039	0.693(0.337-1.42)
hPRB	8.71±0.2	-2.08±0.2	4.23(3.68-4.86)
hPRA+hPRB	10.02±0.405	-0.77±0.405	1.71(1.29-2.25)

Results

After being transformed into Escherichia coli DH5α and selected by Ampicillin screening, the recombinant plasmids were confirmed by restriction enzyme digestion (Figure 1).

After transfection, the authors tested the expression of hPRA and hPRB by Western blot. The analysis indicated that transfection with hPRA resulted in a reproducible significant increase in hPRA expression compared with the blank-control and the vector-control ($p < 0.05$). The same trends were found in the group transfected with hPRB. Western blot results showed that transfection cells with the recombinant plasmid successfully expressed hPRA and hPRB protein, respectively (Figure 2). Moreover, the expression of hPRA was decreased after hPRB stimulation, and vice-versa, which indicated that hPRA and hPRB may have a mutual influence.

As shown in Table 1, hPRB exhibited a specific decrease in Ishikawa cell invasion instead of hPRA, compared with cells of the control group. More importantly, the suppression of Ishikawa cells invasion by hPRB was completely restored by hPRA stimulation ($t = -9.601$, $p < 0.001$). The invaded cells were readily apparent upon microscopic examination in five predetermined fields at ×400 magnification (Figure 3).

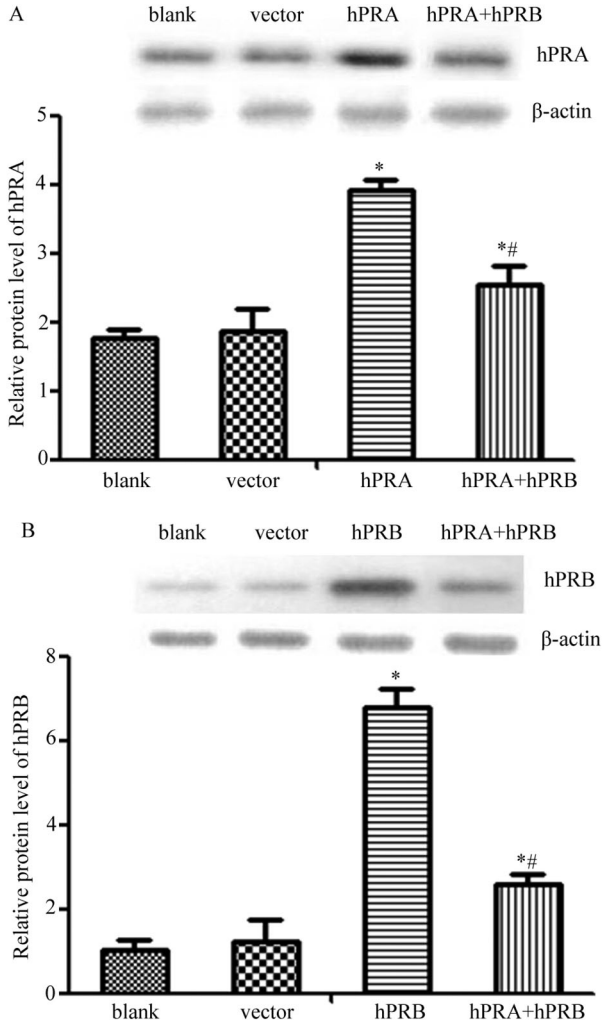

Figure 2. — Western blot analysis of hPRA and hPRB and quantification of corresponding protein level (A, B). Data are presented as means ± SD. *$p < 0.05$ *vs.* control group and vector group; #$p < 0.05$ *vs.* group transfected with hPRA or hPRB.

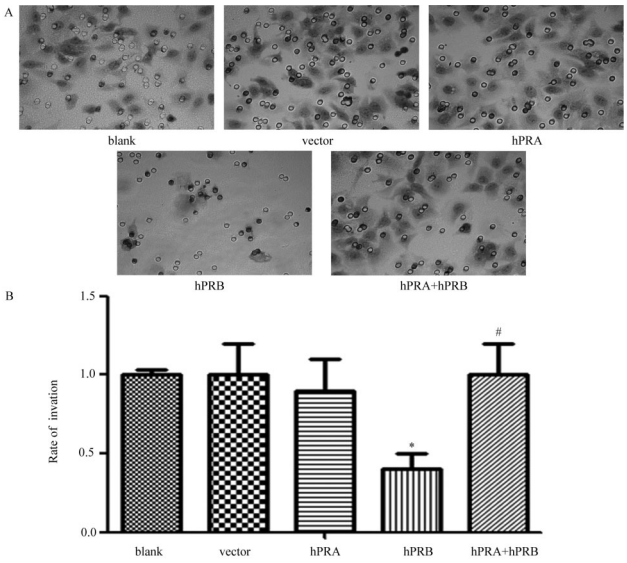

Figure 3. — Effect of hPRA and hPRB on the inhibition of the invasion of Ishikawa cells. (A, B) Invasion capacity of Ishikawa cells significantly decreased after transfection with hPRB and increased again as the same level as the control group after transfection with hPRA and hPRB. Data are presented as means ± SD. *$p < 0.05$ *vs.* control group and vector group; #$p < 0.05$ *vs.* group transfected with hPRB.

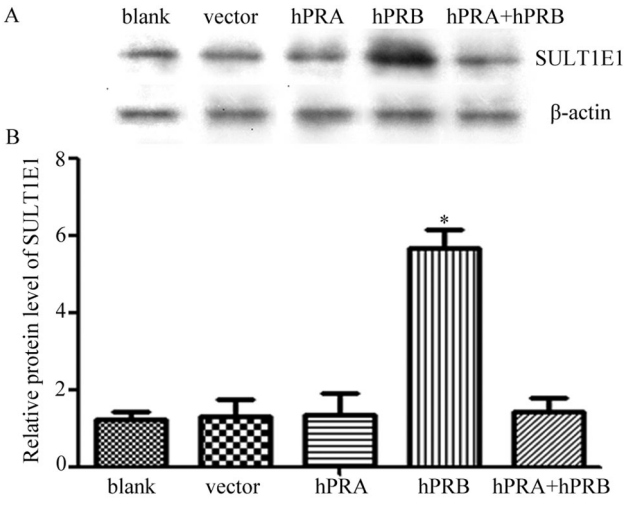

Figure 4. — Effect of hPRA and hPRB on the expression of SULT1E1 in Ishikawa cells. Western blot analysis of SULT1E1 (A) , and quantification of corresponding protein level (B). Data are presented as means ± SD. *$p < 0.05$ *vs.* control group and vector group.

The relationship between SULT1E1 and estrogen may suggest that downregulation of SULT1E1 expression could play an important role in generation and development of estrogen-dependent tumors, including EC. Progestin has been found to inhibit the growth of EC. However, the protect mechanism of progesterone on endometrium by influencing SULT1E1 is still uncertain.

To further explore the roles of hPRA and hPRB on SULT1E1 expression in Ishikawa cells, RT-PCR analysis was utilized in a further experiment. The expression of SULT1E1 mRNA in each group was determined, respectively, by RT-PCR in Ishikawa cells and drawn into standard and dissolution curves of target gene SULT1E1 and house-keeping gene GAPDH. Compared with the control cells, the great interference in SULT1E1 mRNA expression was achieved by transfection with hPRB ($t = 0.096$, $p < 0.05$), but the empty plasmid cells ($t = 0.888$, $p > 0.05$), cells transfected with hPRA ($t = -0.795$, $p > 0.05$) or with hPRA and hPRB ($t = 2.054$, $p > 0.05$) exhibited no obvious enhancement in SULT1E1 mRNA levels. Moreover, the level of SULT1E1 mRNA in hPRA and hPRB transfected group was significantly lower than that in hPRB

transfected group ($t = 0.257$, $p < 0.05$). Thus, the authors demonstrated that hPRB contributed to the upregulation of SULT1E1 expression in Ishikawa cells which can be inhibited by hPRA (Table 2).

In order to identify whether there was any effect on protein level, the authors detected the expression of SULT1E1 by Western blot. Compared with the control group, the authors found that SULT1E1 protein was significantly upregulated by hPRB in the group of transfection with hPRB, whereas a suppression of SULT1E1 protein expression in Ishikawa cells after transfection with hPRA and hPRB (Figure 4) showed the same tendency as that of mRNA detection.

Discussion

In this study, the authors uncovered a novel mechanism of hPRA and hPRB suppressing EC cell invasion. By using matrigel invasion assay, they showed that hPRB functioned as an inhibited regulator of EC cell invasion. Moreover, a positive correlation was found between hPRB and SULT1E1, whereas a non-correlation was found between hPRA and SULT1E1 in Ishikawa cells. However, hPRA apparently had an inverse correlation on the function of hPRB which led to down-regulation of SULT1E1.

Generally speaking, EC in the early stage only spreads in the uterus and patients always receive a series of effective treatments. However, the prognosis for advanced, recurrent, and metastatic EC still remains poor and the pathogenesis has not been fully elucidated. In order to improve therapy, it is necessary to explore the process which inhibits cancer progression.

Progesterone is a sexual steroid hormone that plays a critical role in women's reproductive system and its actions are mediated by the activation of PR. PR is a member of the nuclear receptor family of ligand-dependent transcription factors. PR has been associated with prognosis and treatment of endocrine-dependent diseases, such as breast [9] and gynecological cancer [10]. In progressive EC, the expression of PR is indeed lost [3]. The loss of PR downregulated the expression of CXCL14, DKK1, MMP7, and SFRP4, increased cancer cell metastasis [11-14]. Further mechanistic evidence showed that by DKK1 acting downstream of PR, progesterone could inhibit Wnt/B-catenin signaling pathway, thus counterbalancing endometrium proliferation and inhibiting neoplastic transformation [11]. Neubauer *et al.* [15] reported that hPRB induction of BIRC3 protected EC cells from AP1–59-mediated apoptosis. Resistance to progesterone therapy in breast cancer was shown to be associated with hPRA overexpression [16]. In this study, the authors investigated that hPRB overexpression decreased EC cell invasion, and this phenomenon was completely restored by hPRA. HPRA overexpression downregulated hPRB level when combined with each other, which may result in resistance to progesterone therapy in EC. So the further study of the mechanism of hPRA and hPRB expressions in Ishikawa cells will be helpful to guide the treatment of EC and the judgement of prognosis.

Ishikawa cells have a strong invasion capacity which is connected with molecular pathways leading to the development of EC [17]. It is still unclear whether the role of PR in cell invasion capacity. In the present study, hPRB significantly reduced invasion of Ishikawa cells through upregulating of SULT1E1 expression, and this suppression was completely restored by hPRA stimulation.

Sulfotransferase can mediate the cellular levels of estrogen by the metabolic deactivation pathway. Xu *et al.* [18] reported that the upregulation of SULT1E1 (estrogen sulfotransferase, EST) in the tumor tissue may sulfate active estrogen into an inactive form to protect the cells from mitogenicity by estrogen. The authors' previous study indicated the negative expression pattern of SULT1E1 in EC tissues and the positive one in normal tissues. Hirata *et al.* [19] supported that single nucleotide polymorphisms in the SULT1E1 gene led to the risk of EC. However, as far as is known, no studies on the association between progesterone and SULT1E1 in EC have reported to date. The present study suggested that hPRB overexpression induced a reproducible increase in the mRNA and protein expression levels of SULT1E1, whereas hPRA did not. Results also showed that transfection with hPRA and hPRB significantly reduced SULT1E1 compared with transfection with hPRB alone. SULT1E1 may serve as a regulator of progesterone during signal transduction process.

In conclusion, the present data on EC shows that hPRB suppresses the invasiveness of Ishikawa cells by simultaneously upregulating SULT1E1, and that there is a negative effect for hPRA on the function of hPRB. This indicates that PRB upregulating SULT1E1 may be an important mechanism of progesterone to treat EC, and by hPRA downregulating hPRB in recurrent EC, response rates to progestin therapy drops significantly. Further in-depth study of hPR subtypes and SULT1E1 will lead to a potential therapeutic strategy for cases of EC that are resistant to conventional treatment.

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