

Original Article

Reduced CRHR-2 in placental tissues is associated with the high DNA methylation of CRHR-2 promoter in idiopathic preterm birth

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Abstract: The present study was to explore the expression of CRHR-1 and CRHR-2 in the placentas of idiopathic preterm birth (PTB-I), and to identify the *CRHR-1* and *CRHR-2* methylation with the deregulated CRHR-1 and CRHR-2. Immunohistochemical staining, real-time quantitative PCR and western blot analysis were performed to examine the expression of CRHR-1 and CRHR-2. The methylation in promoter regions of the *CRHR-1* and *CRHR-2* genes were specifically analyzed using MethyLight method. Results demonstrated that the CRHR-2 expression was reduced in both mRNA and protein levels in PTB-I placental tissues, associating with a high level methylation of *CRHR-2* gene in PTB-I placental specimens. *in vitro* experiments demonstrated that both CRHR-1 and CRHR-2 were downregulated, whereas the methylation of both *CRHR-1* and *CRHR-2* was upregulated in the hypoxia-treated human trophoblast-like JEG3 cells. In conclusion, CRHR-2 was downregulated in PTB-I in placenta tissues, in association with a high DNA methylation of *CRHR-2* promoter. Methylation inhibitor might be a promising agent for PTB-I treatment.

Keywords: Corticotrophin releasing hormone receptor (CRHR) 2, methylation, idiopathic preterm birth (PTB-I), hypoxia

Introduction

Preterm birth (PTB), a delivery before 37 weeks of gestation, still leads directly the cause of neonatal death, particularly in un-developed countries, accounting for approximately 30% of neonatal deaths worldwide annually [1] and 40% of childhood neurological morbidities [2]. Idiopathic PTB (PTB-I) accounts for two-thirds of all cases [3]. The molecular mechanism underlining PTB is currently considered as a complex result from environmental and genetic factors [4], such as a history of a prior PTB [5] and other factors [6]. Multiple gestations, uterine anomalies, infection and blood loss were conceived to associate with PTB-I.

Corticotrophin releasing hormone (CRH)-CRH receptor (CRHR) signaling has been implicated in the response to infection and in activating the inflammatory pathways leading to PTB [7]. CRH or its homologous peptides, urocortins (Ucns) can bind to CRHR and activate CRH-CRHR signaling [8]. CRH-CRHR signaling may

involve in the onset of PTB. Indeed, both CRH and Ucns stimulate ACTH [9] or prostaglandin [10] release by placental cells in culture, and also exert different effects on myometrial contractility. Therefore, the deregulated CRH-CRHR signaling might involve in the PTB-I.

This study was to investigate the expression of CRHR-1 and CRHR-2 in the placental tissues in PTB-I in subjects. Then the methylation of *CRHR-1* and *CRHR-2* was analyzed. In addition, we examined the regulation by hypoxia on the expression of both CRHR-1 and CRHR-2 in the human trophoblast-like JEC3 cells. Our results demonstrated that the CRHR-2 was reduced in placental tissues in PTB-I subjects, in association with the high DNA methylation of CRHR-2 promoter in PTB-I.

Materials and methods

Subjects and samples

22 puerperal with idiopathic preterm birth (PTB-I) and 23 puerpera with normal term were

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Table 1. Maternal and fetal characteristics in this study

Parameter	Normal term (N=35)	PTB-I (N=35)	P value
Mean maternal age (years)*	28.33±0.59	29.27±0.69	0.3116 [#]
Nulliparous	16 (69.57%)	18 (81.82%)	0.339 ^{##}
Smoking during pregnancy	2 (8.70%)	3 (13.64%)	0.598 ^{##}
Pregnancy BMI (kg/m ²)*	22.52±2.67	23.45±2.92	0.178 [#]
Mode of delivery			0.300 ^{##}
C-section	9	12	
Vaginal	14	10	
Gestational age at delivery (weeks)*	38.6±1.7	34.7±1.3	0.006 [#]
Neonatal weight (kg)	3.425±0.870	1.860±0.540	0.002 [#]
Neonatal gender			0.555 ^{##}
Male	10	12	
Female	13	10	

*: Mean±SD. Abbreviations: PTB-I, idiopathic preterm birth. Statistical analysis were performed by unpaired student's T-test[#] and Chi-square test^{##} as applicable. Statistical significance was considered when a P-value was less than 0.05.

recruited at the moment of admission to the Department of Obstetrics and Gynecology, the Affiliated Yantai Yuhuangding Hospital of Qingdao University. The study was formally approved by the ethics committee of our hospital. The written consent was signed by each subject. Placental tissue samples were collected post the delivery of live born neonates and the spontaneous membrane rupture of placentas, and were immediately stored at -80°C or were fixed with 4% formaldehyde.

Cell culture and reagents

Human trophoblast-like cell line JEG3 was purchased from the American Type Culture Collection (ATCC) (Rockville, MD, USA) and was cultured in the cultured in EMEM (Invitrogen, Carlsbad, CA, USA), which was supplemented with 10% (v/v) FBS (Hyclone, Pittsburgh, PA, USA) under 37°C in a humidified incubator with 5% CO₂. For the hypoxia treatment, cells were cultured in a hypoxic (1% O₂, 5% CO₂ and 94% N₂) incubator (Thermo Fisher Scientific, Waltham, MA, USA). 5-Aza-2'-deoxycytidine (DAC) (Sigma-Aldrich, St. Louis, MO, USA) was utilized to inhibit DNA methylation, and was dissolved in acetic acid: water solution (1:1) for 200 μM before use.

Immunohistochemical staining

Immunohistochemical staining was performed to examine the expression of CRHR-1 or CRHR-2 in placental tissue samples from both the

puerperal with idiopathic preterm birth and with normal term. Specimen slides were successively subject to antigen retrieving, the incubation with primary antibody against CRHR-1 or CRHR-2 (either 1:300, Abcam, Cambridge, UK) and to the incubation with the HRP-linked secondary antibodies (Sinobio, Beijing, China). Specific binding to the CRHR-1 or CRHR-2 antigen was counterstained with Mayer's hematoxylin. The intensity and extent of staining were independently evaluated and quantified according to optical density.

RNA extraction and quantitative real-time polymerase chain reaction (RT-qPCR)

Total mRNA was isolated with Oligotex mRNA Mini Kit (Qiagen, Valencia, CA, USA). The relative mRNA level of CRHR-1 or CRHR-2 was quantified by RT-qPCR method with QuantiTect SYBR Green PCR Kits (Qiagen, GmbH, Hilden, Germany) and with the CRHR-1- or CRHR-2-specific primers and probes. The mRNA of house-keeping Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was taken as an internal control. The mRNA level of CRHR-1 or CRHR-2 level was relatively quantified with 2^{(-Delta Delta C(T))} Method [11].

DNA methylation analysis

Genomic DNA samples from the placental tissues were isolated using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, USA). The methylation in promoter regions of the CRHR-1 and CRHR-2 genes were specifically was analyzed using MethyLight method [12], with CRHR-1- or CRHR-2-specific primers and the methylation-specific fluorescent probe. Two CpG sites were analyzed in the upstream of the transcription start site (TSS) in the promoter region of CRHR-1 or CRHR-2.

Western blot analysis

Protein samples were isolated with NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Pierce, Rockford, IL, USA), were separated

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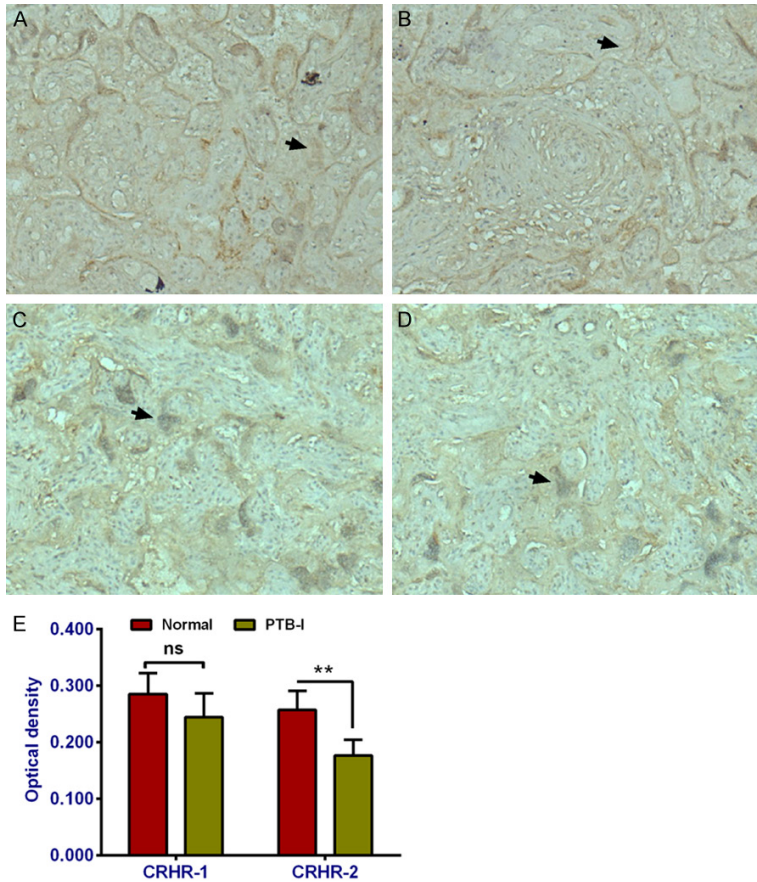


Figure 1. Typical immunohistochemical staining for CRHR-1 and CRHR-2 in the placental tissues with idiopathic preterm birth (PTB-I). (A-D) Representative images for CRHR-1 (A and B) or CRHR-2 (C and D) staining in normal (A and C) (N=23) or PTB-I (B and D) (N=22) groups. (E) Quantitative analysis of the CRHR-1 or CRHR-2 in both groups. Statistical significance was drawn with unpaired student *t* test, and was indicated as ***P*<0.01 or ns, no significance.

by electrophoresis using a 12% SDS-PAGE gel and were transferred to a nitrocellulose membrane (Millipore, Bedford, MA, USA), which was blocked overnight at 4°C with 2% BSA (Sigma-Aldrich, St. Louis, MO, USA). The membrane was successively incubated at 4°C for 2 hours with rabbit polyclonal antibody against human CRHR-1, CRHR-2 or GAPDH, with HRP-conjugated secondary antibody against rabbit IgG, and then with electrochemiluminescence (Amersham, Uppsala, Sweden) at room temperature for 30 minutes. The levels of CRHR-1 or CRHR-2 were presented as a percent gray value normalized to GAPDH.

Statistical analysis

Quantitative data were presented as means±SEM. ANOVA test and *t* test were performed for data analysis. Linear-regression analysis was

performed to evaluate the association of CRHR-1/2 mRNA level with CRHR-1/2 methylation. statistical significance was considered when *P*<0.05 or less.

Results

Reduced CRHR-2 expression in PTB-I placental specimens

As indicated in **Table 1**, there was no significant difference in average maternal age, multipara, smoking during pregnancy, pregnancy BMI, mode of delivery, neonatal gender between the two groups of subjects. The difference was significant in gestational age at delivery and neonatal weight. There was a high level of CRHR-1-positive cells in both normal and PTB-I placenta without significance (**Figure 1A, 1B**). However, The CRHR-2-positivity was markedly less in PTB-I than in normal group (0.176±0.028 vs 0.257±0.034, *P*<0.01, **Figure 1C, 1D**). RT-qPCR results demonstrated no significant difference in the CRHR-1 mRNA level between PBT-I and normal groups (**Figure 2A**). How-

ever, the CRHR-2 mRNA level was markedly lower in the PBT-I group than in the normal group (*P*=0.0047, **Figure 2B**).

Correlation of the reduced CRHR-2 expression with high methylation of CRHR-2 gene in PTB-I placental specimens

The methylation of CRHR-2 gene has recently been recognized to regulate the CRHR-2 expression in obesity and obesity-related chronic diseases [13]. The methylation was also an important regulation type on CRHR-1 expression [14]. The global methylation in the promoter of CRHR-1 and CRHR-2 was indicated in **Figure 2C**. The relative methylation level of CRHR-1 gene was 1.2580±0.1181 in the PTB-I group, with 1.0000±0.0817 as a baseline methylation level in normal group (*P*=0.0709). Moreover, the relative methylation level of CRHR-2 gene

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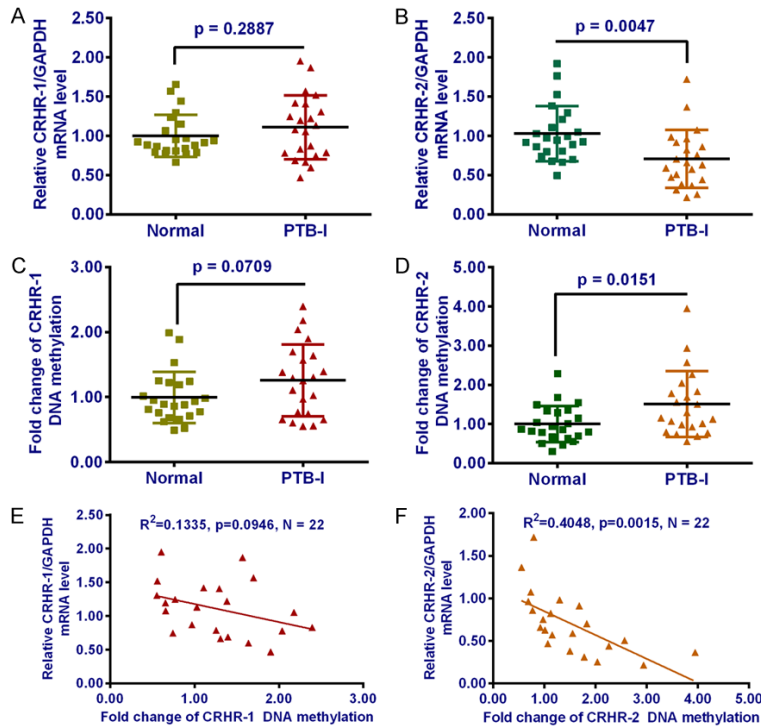


Figure 2. Reduced mRNA levels of CRHR-1 and CRHR-2 associated with increased methylation levels of *CRHR-1* and *CRHR-2* in the placental tissues with idiopathic preterm birth (PTB-I). (A and B) Relative mRNA level of CRHR-1 (A) or CRHR-2 (B) in the normal (N=23) or PTB-I (N=22) groups was quantified by real-time RT-PCR, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as control. (C and D) Total methylation level in the promoter of *CRHR-1* (C) or *CRHR-2* (D) in the placental tissues with idiopathic preterm birth (PTB-I). (E and F) Association of mRNA levels of CRHR-1 (E) or CRHR-2 (F) respectively with the methylation levels in the promoter of *CRHR-1* (E) or *CRHR-2* (F). Statistical significance was presented in each figure and was considered when $P < 0.05$ or less.

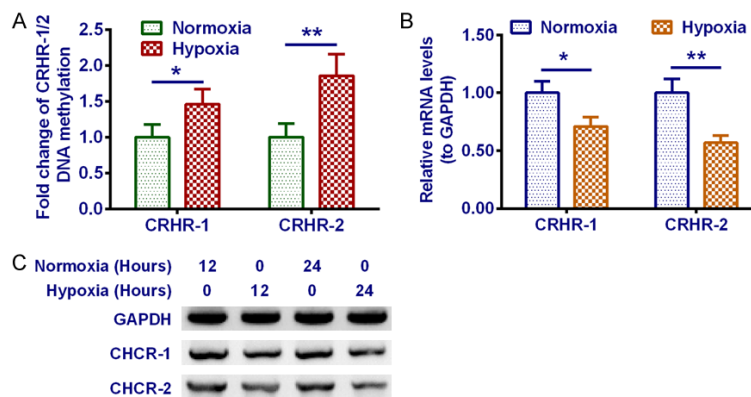


Figure 3. Methylation levels of *CRHR-1* and *CRHR-2* and mRNA levels of CRHR-1 and CRHR-2 in the JEG3 cells under hypoxia. (A and B) The DNA methylation levels of *CRHR-1* and *CRHR-2* (A) or the Relative mRNA level of CRHR-1 and CRHR-2 (B) in the JEG3 cells under normoxia or hypoxia for 12 hours; (C) Western blotting assay for CRHR-1 or CRHR-2 in the JEG3 cells under normoxia or hypoxia for 12 or 48 hours. Experiments were independently performed in triplicate. Statistical significance was indicated as * $P < 0.05$, ** $P < 0.01$ or ns, no significance.

was significantly higher in the PTB-I group (1.5090 ± 0.1790 vs 1.000 ± 0.0966) than in normal group ($P = 0.0709$, **Figure 2D**).

Then the Pearson correlation analysis was performed to examine the association between the CRHR-2 reduction and the high methylation of *CRHR-2* gene. The correlation between the CRHR-1 mRNA level and the *CRHR-1* methylation level was not significant ($R^2 = 0.1335$, $P = 0.0946$, $N = 22$, **Figure 2E**). However, the correlation between the CRHR-2 mRNA level and the *CRHR-2* methylation level was significant ($R^2 = 0.4048$, $P = 0.0015$, $N = 22$).

Hypoxia upregulates CRHR-2 methylation and reduces CRHR-2 expression in human trophoblast-like JEG3 cells

To further identify the signaling pathway underlining the reduced CRHR-2 expression and increased *CRHR-2* methylation in PTB-I. We then treated JEG3 cells under hypoxia, and then evaluated the CRHR-2 expression and increased *CRHR-2* methylation in JEG3 cells. We found that the methylation of both *CRHR-1* and *CRHR-2* was upregulated by hypoxia for 12 hours in the JEG3 cells ($P < 0.05$ or $P < 0.01$, **Figure 3A**). And the mRNA levels of CRHR-1 and CRHR-2 were conversely downregulated in the hypoxia-treated JEG3 cells ($P < 0.05$ or $P < 0.01$, **Figure 3B**). In addition, western blotting assay confirmed the downregulation of both CRHR-1 and CRHR-2 in the hypoxia-treated JEG3 cells (**Figure 3C**).

As indicated in **Figure 4A** that the methylation of either CR-

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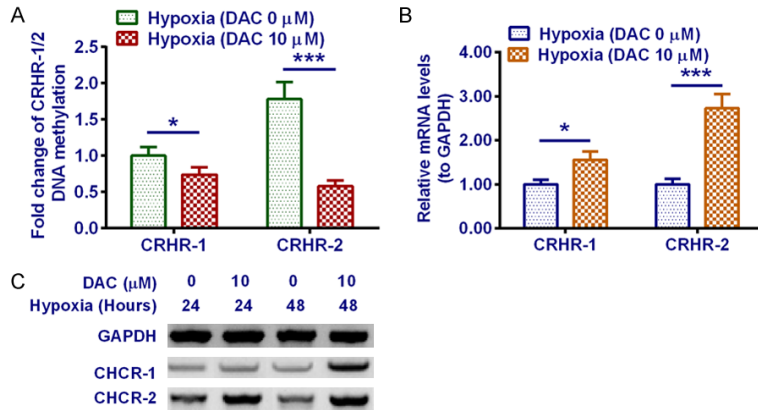


Figure 4. Influence by methylation inhibitor, DAC on mRNA levels of CRHR-1 and CRHR-2 in the JEG3 cells under hypoxia. (A and B) The DNA methylation levels of CRHR-1 and CRHR-2 (A) or the Relative mRNA level of CRHR-1 and CRHR-2 (B) in the 5-Aza-2'-deoxycytidine (DAC)-treated (10 μM) JEG3 cells under hypoxia for 12 hours; (C) Western blotting assay for CRHR-1 or CRHR-2 in the DAC-treated (10 μM) JEG3 cells under normoxia or hypoxia for 12 or 48 hours. Experiments were independently performed in triplicate. Statistical significance was indicated as * $P < 0.05$, *** $P < 0.001$ or ns, no significance.

CRHR-1 or CRHR-2 was markedly inhibited by methylation inhibitor, DAC (10 μM) ($P < 0.05$ or $P < 0.001$). Accordingly, the mRNA levels of both CRHR-1 and CRHR-2 were significantly reversed by the DAC treatment ($P < 0.05$ or $P < 0.001$, **Figure 4B**). And such reversion of CRHR-1 and CRHR-2 was also presented in protein level by western blotting assay (**Figure 4C**). Thus, we found the promotion by hypoxia to the methylation of both CRHR-1 and CRHR-2 genes in human trophoblast-like JEG3 cells.

Discussion

The placenta and fetal membranes might be key tissues for PTB-I or pPROM, in which chemokines, cytokines, and CRH might be upregulated [15]. CRH-CRHR signaling may exert some biological functions during pregnancy [15] via regulating immune and placental endocrine function [16]. We here found both CRHR-1 and CRHR-2 were reduced in the PTB-I placental tissues; particularly, the CRHR-2 reduction was significant. The RT-qPCR results also confirmed the reduction of CRHR-2 in PTB-I placental tissues.

Epigenetics is crucial for genomic imprinting, development, and disease, via regulating gene activity [17], with a relative stability throughout life [18]. Recently, the DNA methylation of some genes has been observed to be associated

with PTB-I [19]. Our study demonstrated different methylation in the promoter of CRHR-1 and CRHR-2. The methylation of CRHR-1 was significantly higher in the PTB-I placental tissues. And the Pearson correlation analysis confirmed the correlation between the CRHR-2 mRNA level and the CRHR-2 methylation level.

Deregulated gene methylation in placental trophoblasts is a common response to hypoxia, and contributes to cell differentiation and preeclampsia [20]. Gestational hypoxia could induce CRHR-1 methylation, which is linked to anxiety-like behavior [21]. We found that the methylation of both CRHR-

1 and CRHR-2 was upregulated by hypoxia in the JEG3 cells. CRHR-1 and CRHR-2 were conversely downregulated in the hypoxia-treated JEG3 cells. Moreover, the downregulation of CRHR-1 and CRHR-2 could be reversed by methylation inhibitor. Thus, we found the promotion by hypoxia to the methylation of both CRHR-1 and CRHR-2 genes in human trophoblast-like JEG3 cells.

In conclusion, CRHR-2 was downregulated in PTB-I in placenta tissues, in association with a high DNA methylation of CRHR-2 promoter. Methylation inhibitor might be a promising agent for PTB-I treatment.

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Disclosure of conflict of interest

None.

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