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Correlation analysis between COX-2 gene polymorphism and eclampsia

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Abstract. – OBJECTIVE: The aim of this study was to investigate the expression of cyclooxygenase-2 (COX-2) in eclampsia patients, and to explore the correlation between COX-2 polymorphism and incidence of eclampsia.

PATIENTS AND METHODS: From January 2016 to January 2018, a total of 280 pregnant women diagnosed in the Obstetrics and Gynecology Department of our hospital were selected for this study. All patients were divided into two groups, including normal pregnancy control group (n=120) and eclampsia group (n=160). The expression of COX-2 in placenta and umbilical cord tissues of eclampsia group and normal group was detected *via* Reverse Transcription-Polymerase Chain Reaction (RT-PCR), Western blotting and immunohistochemical staining. The single-nucleotide polymorphisms (rs1526172, rs1245231 and rs2198532) in the promoter region of the COX-2 gene were typed *via* conformation difference gel electrophoresis. Whether the distribution frequency of COX-2 genotypes met Hardy-Weinberg equilibrium law was detected *via* the Chi-square test. Meanwhile, the correlation between COX-2 alleles and gene polymorphisms and the incidence of eclampsia was analyzed.

RESULTS: The messenger ribonucleic acid (mRNA) and protein expression levels of COX-2 in the eclampsia group were significantly higher than those of the normal group ($p<0.05$). According to the analysis, three polymorphisms of COX-2 gene were all in line with Hardy-Weinberg equilibrium distribution ($p>0.05$). Gene association analysis revealed that only polymorphisms (rs1526172 and rs1245231) and alleles were correlated with the incidence of eclampsia ($p<0.05$). However, polymorphism rs2198532 and alleles were not correlated with the incidence of eclampsia ($p>0.05$).

CONCLUSIONS: Rs1526172 and rs1245231 in the promoter region of COX-2 are correlated with the incidence of eclampsia, while rs2198532 has no correlation with eclampsia.

Key Words:
COX-2, Polymorphism, Eclampsia.

Introduction

Eclampsia is common fatal acute complication in females during pregnancy. The main symptoms of eclampsia are hypertension and proteinuria. Meanwhile, it can also involve many other important organs, such as brain, heart, kidney, liver and placenta[1,2]. However, the pathogenesis of eclampsia has not been elucidated so far. Current researches have demonstrated that the incidence of eclampsia is mainly due to a series of pathophysiological changes in the placenta. This may lead to systemic vascular endothelial cell inflammation and injury[3]. On the one hand, vascular endothelial cells act as an important barrier for substance exchange between blood and other organs[4]. On the other hand, vascular endothelial cells also have important endocrine functions. They can release a variety of vasoactive factors *via* autocrine, including endothelin, nitric oxide and prostacyclin. These vasoactive factors regulate blood coagulation function, vasomotion and exchange of substance. Therefore, inhibiting vascular endothelial cell inflammation and injury is a key target for the treatment of eclampsia[5,6].

Cyclooxygenase-2 (COX-2) is an important member of the COX family. It is the primary rate-limiting enzyme for the synthesis of prostaglandins with arachidonic acid as a substrate[7]. Studies have demonstrated that COX-2 cannot only inhibit cell apoptosis, but also promote the proliferation of vascular endothelial cells. Therefore, it is of great importance in angiogenesis[8,9]. In addition, COX-2 can produce 15ΔPGJ2 binding to a variety of pro-inflammatory transcription factors, thereby inhibiting the occurrence and development of the inflammatory response[10]. The above findings all indicate a potential correlation between COX-2 and incidence of eclampsia.

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Therefore, the objetive of this study was to detect the expression level of COX-2 in placenta and umbilical cord tissues of eclampsia patients and normal pregnant women. Meanwhile, we aimed to analyze the correlation between single-nucleotide polymorphisms (rs1526172, rs1245231 and rs2198532) in the promoter region of the COX-2 gene and eclampsia. Our study might provide a certain basis for clinical prevention and treatment of eclampsia in the future.

Patients and Methods

Patients

From January 2016 to January 2018, a total of 280 pregnant women in the Obstetrics and Gynecology Department of our hospital were selected for this study. In the normal pregnancy control group, there were 120 pregnant women aged (24.21±12.34) years. In the eclampsia group, there were 160 pregnant women aged (25.12±11.49) years. 4 mL of venous blood was collected from each subject. Subsequently, it was anti-coagulated with sodium citrate and stored in a refrigerator at -20°C. The umbilical cord and placenta tissue specimens were retained at the time of delivery. This study was approved by the Ethics Committee of our hospital. Informed consent was obtained from each subject before the study.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

(1) Total ribonucleic acid (RNA) was extracted from placenta and umbilical cord tissues according to the instructions of the TRIzol method (Invitrogen, Carlsbad, CA, USA). The concentration and purity of extracted RNA were detected using an ultraviolet spectrophotometer (Mettler-Toledo, Columbus, OH, USA). RNA with absorbance $(A)_{260}/A_{280}$ of 1.8-2.0 could be used. (2) Messenger RNA (mRNA) was synthesized into complementary deoxyribonucleic acid (cDNA) through RT and stored at -80°C for subsequent use. (3) RT-PCR system was as follows: 2.5 μL 10× Buffer, 2 μL cDNA, 0.25 μL forward primer (20 μmol/L), 0.25 μL reverse primer (20 μmol/L), 0.5 μL dNTPs (10 mmol/L), 0.5 μL Taq enzyme (2×10^6 U/L) and 19 μL double-distilled H_2O (ddH_2O). The amplification system of RT-PCR was the same as above.

Western Blotting

Placenta and umbilical cord tissues in both groups were fully ground in the lysis buffer, followed by ultrasonic pyrolysis and lysate centrifugation. Then, the supernatant was taken and transferred into an Eppendorf (EP) tube. The concentration of extracted protein was detected *via* bicinchoninic acid (BCA) method (Pierce, Waltham, MA, USA) and ultraviolet spectrometry. All the protein samples were quantified to be the same concentration before use. Subsequently, the extracted protein was sub-packaged and placed in the refrigerator at -80°C. After separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). Then, the membranes were incubated with primary antibody at 4°C overnight. On the next day, the membranes were incubated again with goat anti-rabbit secondary antibody in the dark for 1 h. The protein band was scanned and quantified using the Odyssey scanner. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference.

Immunohistochemical Staining

Placenta and umbilical cord tissue sections were baked in an oven at 60°C for 30 min. The sections were dewaxed with xylene (5 min × 3 times), followed by dehydration with 100% ethanol, 95% ethanol and 70% ethanol 3 times, respectively. The activity of endogenous peroxidase was inhibited using 3% hydrogen peroxide-methanol. The tissue sections were then sealed with goat serum for 1 h and incubated with anti-COX-2 antibody (diluted at 1:200) at 4°C overnight. After washing with Phosphate-Buffered Saline (PBS) on a shaking table 4 times, the sections were incubated again with secondary antibody. The color was developed using diaminobenzidine. After that, 6 samples were randomly selected from each group, and 5 visual fields were randomly selected in each sample. Finally, photography was performed under a light microscope (400×).

DNA Extraction

4 mL blood was first collected and anti-coagulated with Ethylene Diamine Tetraacetic Acid (EDTA). Subsequently, genomic DNA was extracted according to instructions of DNA extraction kit (Solarbio, Beijing, China). 2 μmL DNA was taken to detect its mass *via* 1.5% agarose gel electrophoresis. The concentration of extracted DNA was detected using an ultraviolet spectrophotometer.

Table I. Primer sequences and product sizes of different sites in the COX-2 gene promoter region.

Site	Primer sequence (5'-3')	Product (bp)
rs1526172	Forward: GCTAGGCTCTATCGTAGCGACC Reverse: TGGGAACTCTAGCTAGCTAGT	289
rs1245231	Forward: ACGTGTGCAATCGTTAGGGCAA Reverse: GGCATTGACTTAGGCCAATG	305
rs2198532	Forward: ACCTGTCATCGATCGTAGCTAG Reverse: ACGATGCTAGTCGATGCACGT	286

PCR Amplification

Primers were designed for the amplification of rs1526172, rs1245231 and rs2198532 in COX-2 gene. Specific primer sequences of each site were shown in Table I. The PCR system (20 μL) was as follows: 2.0 μL DNA template, 10.0 μL 2× MIX, 0.4 μL forward primer, 0.4 μL reverse primer and 7.2 μL ddH_2O (Thermo Fisher Scientific, Waltham, MA, USA). The PCR amplification conditions were as follows: 95°C for 120 s, 94°C for 30 s, 57°C for 90 s, 72°C for 60 s, for a total of 35 cycles, and extension at 72°C for 10 min. Finally, the amplification of gene segments was detected *via* agarose gel electrophoresis.

Ligase Detection Reaction

Forward and reverse probes used in this reaction were designed and synthesized by BGI (Table II). All the forward probes were prepared into probe mixture at a concentration of 12.5 pmol/μL after 5'-terminal phosphorylation modification. The ligase detection reaction system (3.05 μL) was as follows: 0.05 μL ligase, 1 μL buffer, 1 μL PCR product and 1 μL probe mixture. The PCR amplification conditions were as follows: 95°C for 120 s, 94°C for 15 s, 50°C for 25 s, for a total of 30 cycles. After that, the concentration was measured by the ultraviolet spectrophotometer. Subsequently, the target gene was sequenced and the fragment was analyzed by BGI. All data were analyzed using GeneMapper.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA) was used for all statistical analysis. Enumeration data were expressed as frequency and percentage. Measurement data were expressed as mean ± standard deviation. After calculating the genotype frequency in the samples, the Hardy-Weinberg equilibrium formula was performed. Chi-square test was used to compare the difference among enumeration data. The *t*-test and analysis of variance were adopted for measurement data. $p<0.05$ was considered statistically significant.

Results

COX-2 mRNA Expression in Umbilical Cord and Placenta Tissues of Eclampsia Group and Normal Group

The mRNA expression of COX-2 in the umbilical cord and placenta tissues of both groups was first detected. The RT-PCR results showed that the mRNA expression level of COX-2 in the umbilical cord tissues of the eclampsia group was significantly higher than that of the normal group ($p<0.05$) (Figure 1A). At the same time, the mRNA expression level of COX-2 in placenta tissues of the eclampsia group was also significantly higher than that of the normal group ($p<0.05$) (Figure 1B). The above results indicated that the

Table II. Ligase reaction probe sequences and product sizes of different sites in COX-2-10.

Site	Probe	Probe sequence (5'-3')	Product (bp)
rs1526172	rs1526172	TTTTTACGACGAYTTTTTTTTTTTTTTTT-FAM	114
	rs1526172-A	TTTTTTTTTTTTTTTGGTCGTAGCTAGTCGAT	
	rs1526172-T	TTTTTTTTTTTTTTTCGCTAGTCGATGCTA	
rs1245231	rs1245231	P-CGCTTGACTAGGGTTTTTTTTTTTT-FAM	100
	rs1245231-C	TTTTTTTTTTTTTTTTTTTTTTTTTTTAGCGATTCG	
	rs1245231-G	TTTTTTTTTTTTTTTTTTTTTTTTTTTTAGCGCGA	
rs2198532	rs2198532	P-CGCATTCGATTTTTTTTTTTTTTTTTTT-FAM	128
	rs2198532-C	TTTTTTTTTTTTTTTTTTTTTTTTTTACGTTACGTGAC	
	rs2198532-T	TTTTTTTTTTTTTTTTTTTTTTTTTATCGGATCGTTACC	

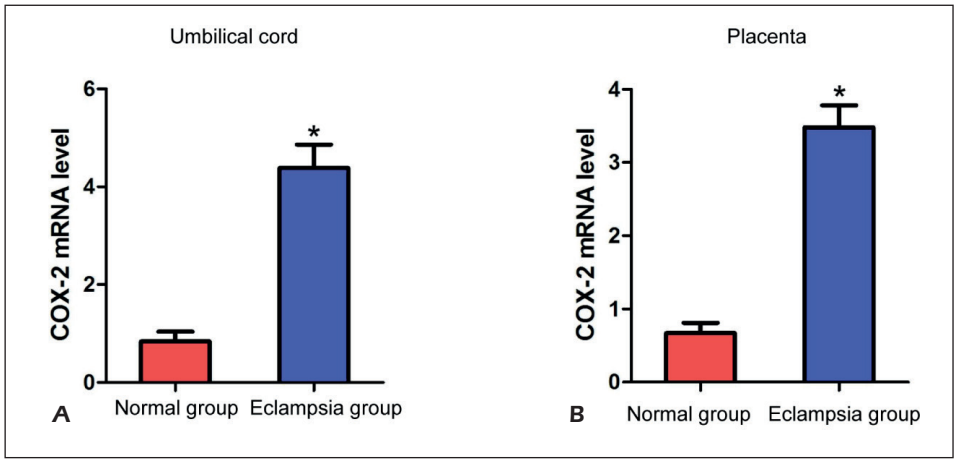

Figure 1. ***A***, The mRNA expression of COX-2 in umbilical cord was remarkably increased in the eclampsia group compared with the normal group. ***B***, The mRNA expression of COX-2 in placenta tissues was also significantly increased in eclampsia group. *$p<0.05$ *vs.* normal group.

mRNA expression levels of COX-2 in the umbilical cord and placenta tissues of eclampsia patients were remarkably elevated when compared with normal pregnant women.

COX-2 Protein Expression in Umbilical Cord and Placenta Tissues of Eclampsia Group and Normal Group

To further evaluate the protein expression of COX-2 in the umbilical cord and placenta tissues of the eclampsia and normal groups, Western blotting was performed. The results revealed that the protein expression of COX-2 in the umbilical cord (Figure 2A, 2B) and placenta tissues (Figure 2C, 2D) of the eclampsia group were significantly higher than those of the normal group ($p<0.05$). The above results further confirmed that the protein level of COX-2 in the umbilical cord and placenta tissues of eclampsia patients were markedly higher than those of normal pregnant women.

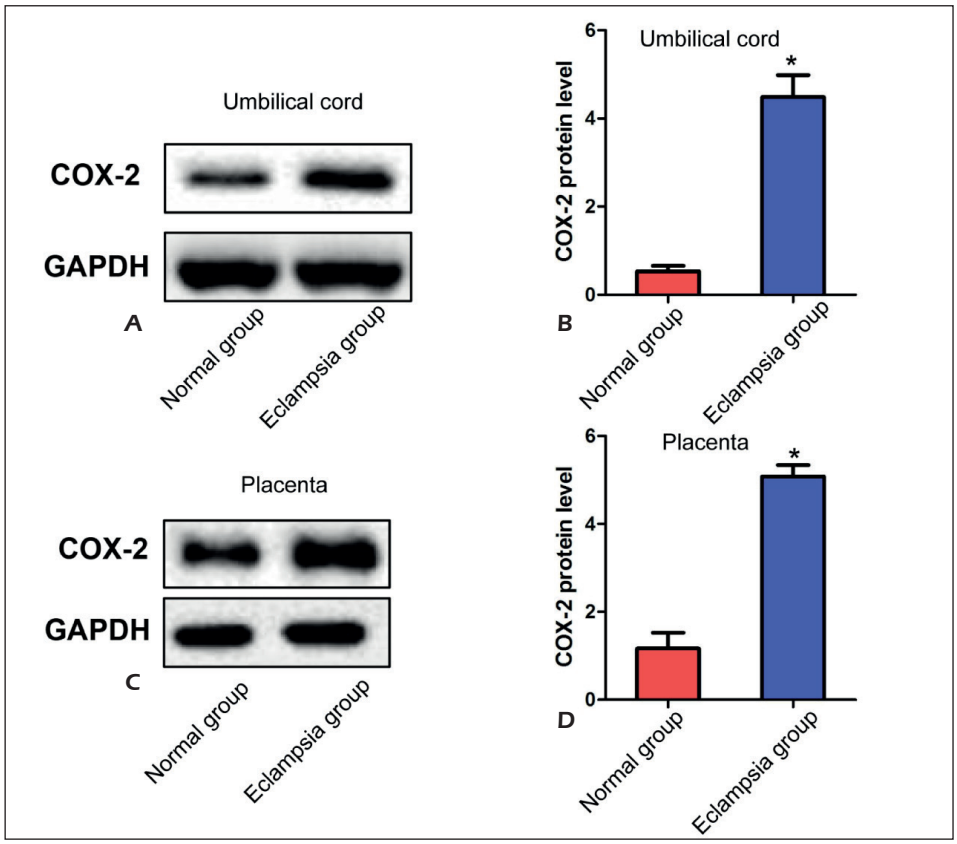

Figure 2. A-B, The protein expression of COX-2 in umbilical cord was remarkably elevated in eclampsia group. **C-D**, The protein expression of COX-2 in placenta tissues was markedly increased in eclampsia group. *$p<0.05$ *vs.* normal group.

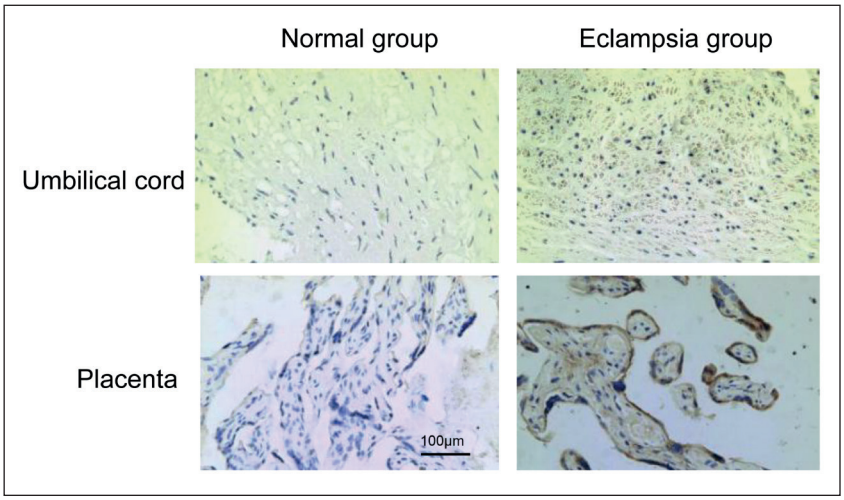

Figure 3. Immunohistochemical staining of COX-2 in umbilical cord and placenta tissues of eclampsia group and normal group. Brown: positive index. (Magnification: 200×).

Immunohistochemical Staining of COX-2 in Umbilical Cord and Placenta Tissues of Eclampsia Group and Normal Group

To understand the expression of COX-2 in the umbilical cord and placenta tissues more intuitively, immunohistochemical staining was performed. The results showed that the expressions of COX-2 in the umbilical cord and placenta tissues of eclampsia patients were significantly higher than those of normal pregnant women (p<0.05) (Figure 3).

Hardy-Weinberg Equilibrium Test

Linkage disequilibrium of different sites of COX-2 gene was detected using Hardy-Weinberg equilibrium formula. As shown in Table III, r^2 of sites in each group was less than 0.33. This suggested that the sites in each group were in line with Hardy-Weinberg equilibrium.

Table III. Linkage disequilibrium test results of sites of COX-2 gene in each group.

Site	r^2		
	rs1526172	rs1245231	rs2198532
rs1526172	-	0.009	0.015
rs1245231	0.009	-	0.202
rs2198532	0.015	0.202	-

Correlation Between COX-2 Gene Polymorphism and Eclampsia

The genotype frequency of gene polymorphism in the two groups was shown in Table IV. It was found that the polymorphisms rs1526172 and rs1245231 had significant correlations with the incidence of eclampsia (p<0.05). However, the polymorphism rs2198532 was not correlated with the incidence of eclampsia (p>0.05).

Correlation Between COX-2 Alleles and Eclampsia

The allele frequency of the three sites in the eclampsia and normal group was shown in Table V. The results demonstrated that the alleles of rs1526172 and rs1245231 were significantly correlated with the incidence of eclampsia (p<0.05). However, there was no significant correlation between the alleles of rs2198532 and eclampsia (p>0.05).

Table IV. Distribution of different genotypes in COX-2 gene in eclampsia patients and normal pregnant women.

Group	rs1526172			rs1245231			rs2198532		
	AA	AT	TT	CC	CG	GG	CC	CT	TT
Eclampsia group	25.2%	60.0%	14.8%	10.1%	50.9%	39.0%	19.9%	51.0%	29.1%
Normal group	41.2%	43.2%	15.5%	25.0%	52.2%	22.8%	10.3%	50.5%	29.2%
C^2	0.461			0.309			0.901		
p	0.021			0.005			0.727		

Table V. Correlation between COX-2 alleles and eclampsia.

Group	rs1526172		rs1245231		rs2198532	
	A	T	C	G	C	T
Eclampsia group	80.00%	20.00%	30.00%	70.00%	45.23%	54.77%
Normal group	32.42%	67.58%	82.11%	17.89%	42.08%	57.92%
C^2	0.723	1.432	1.642			
p	0.004	0.001	0.781			

Discussion

Pregnancy-induced hypertension is an idiopathic hypertension syndrome occurring in the late trimester of pregnancy[11]. As the most important stage of pregnancy-induced hypertension, eclampsia is also one of the important causes of maternal and perinatal death[12]. Currently, it is believed that the pathogenesis of eclampsia is mainly as follows: 1) The superficial implantation of placenta reduces placental perfusion, leads to placental ischemia and hypoxia, and produces a variety of toxic and harmful substances. 2) The disorders of the maternal immune system break the immune tolerance balance between mother and fetus. 3) Vascular endothelial cells are damaged by various stresses, including oxidative stress, endoplasmic reticulum stress, mitochondrial stress, etc. 4) Genetic susceptibility, such as individual differences in blood pressure-regulating genes, thrombosis factors, vascular endothelial injury-related genes and immune-related genes[13-15]. In this work, the mRNA and protein expression levels of COX-2 in placenta and umbilical cord tissues of eclampsia patients and normal pregnant women were detected. The genotypes of rs1526172, rs1245231 and rs2198532 in the target gene COX-2 were classified. Meanwhile, the genotype and allele frequency distribution of each site in different groups was recorded. It was found that the expression levels of COX-2 in placenta and umbilical cord tissues of eclampsia patients were significantly higher than those of normal pregnant women. Rs1526172 and rs1245231 polymorphisms and alleles in COX-2 gene had significant correlations with eclampsia. However, rs2198532 polymorphism and alleles in COX-2 gene were not correlated with eclampsia.

COX-2 possesses both catalase activity and COX activity[16,17]. Under normal conditions, the expression level of COX-2 is extremely low in normal tissues. However, it increases dramatically after various physical and chemical stimuli in the body. This is involved in the body's inflammatory response by catalyzing arachidonic acid into prostaglandin[18,19]. Certain physiological inflammatory responses can occur in the mother during pregnancy. Studies have also demonstrated that the inflammatory response in eclampsia patients is significantly higher than that of normal pregnant women. This can ultimately lead to disorders of anti-inflammatory and anti-oxidative system in eclampsia patients. The overexpression of COX-2 in eclampsia patients accelerates the synthesis and release of inflammatory factors, promotes the migration and adhesion of immune cells and increases the vascular permeability. Ultimately, it may result in endothelial cell injury and excessive systemic inflammatory response[20]. Therefore, COX-2 inhibitors are expected to become potential therapeutic drugs for eclampsia in the future.

Moreover, COX-2 gene polymorphism is closely related to the occurrence and development of multiple diseases. For example, the C allele of COX-2-899G>C can significantly increase the risk of gastric cancer. Meanwhile, it has a synergistic effect with *Helicobacter pylori* infection in gastric cancer occurrence and development[21]. In addition, the risk of colorectal cancer increases significantly in smokers with COX-2-8473TT genotype or high body mass index (BMI) people with COX-2-765GG genotype[22]. Besides, there is a certain interaction between COX-2-8473T>C site and gender, as well as -1195G>A and age. It may eventually affect the risk of hepatocellular cancer in human[23]. In this study, the correlations of rs1526172 and rs1245231 polymorphisms and allele of the COX-2 gene with eclampsia were confirmed. However, there were some limitations in this work: 1) The clinical sample size was relatively small, and 2) the objects of this study were Han people, and the results might be different in other ethnic groups.

Conclusions

Rs1526172 and rs1245231 in the promoter region of COX-2 have a certain correlation with the incidence of eclampsia. Eclampsia is less likely to

occur in Han people with rs1526172 TT genotype than those with CC genotype. In addition, it is less likely to occur in Han people with rs1245231 CC genotype than those with GG genotype.

Conflict of Interests

The authors declare that they have no conflict of interest.

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