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ORIGINAL PAPER/OBSTETRICS

The relationship between hsa_circ_0051326 and HLA-G expression in the blood of patients with pre-eclampsia

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ABSTRACT

Objectives: To investigate the relationship between hsa_circ_0051326 and HLA-G expression in the blood of patients with pre-eclampsia.

Material and methods: Real-time PCR (qRT-PCR) was used to detect the hsa_circ_0051326 expression level. Enzyme-linked immunosorbent assay (ELISA), qRT-PCR, and western blotting were used to detect HLA-G expression in blood in 50 patients with pre-eclampsia and 50 normal pregnant women.

Results: HLA-G protein expression level was decreased significantly in the blood of patients with pre-eclampsia. hsa_circ_0051326 and HLA-G mRNA in blood were decreased significantly in the pre-eclampsia patients compared with normal pregnant

women. There was a positive correlation between the expression of serum hsa_circ_0051326 with HLA-G mRNA.

Conclusions: Serum hsa_circ_0051326 was a new diagnostic biomarker for pre-eclampsia with equivalent efficiency of HLA-G. Maternal serum hsa_circ_0051326 level may pre-diagnose potentially pre-eclampsia before delivery.

Key words: pre-eclampsia; Human leukocyte antigen-G; hsa_circ_0051326; serum

INTRODUCTION

Pre-eclampsia (PE) as the common obstetric syndrome affects more than 10% of pregnant women, which is the leading cause of death of the mother and fetus [1]. Symptomatic treatment is generally administrated in its clinical management since the exact pathogenesis of PE has not been elucidated [2]. At present, one theory indicated that the occurrence of PE is due to abnormal immune tolerance at the maternal-fetal interface [3]. Normal development of the placenta and adequate trophoblast functioning are prerequisites for a successful pregnancy. Therefore, the major challenge in establishing a proper maternal-fetal interface during pregnancy is that the embryo must evade the mother's immune system [4]. More pieces of evidence indicate that insufficient invasion of extraembryonic trophoblasts might contribute to changes in the placental micro-environment, which will affect the immune balance between the mother and the fetus, such as enhancing the mother's immune activity and weakening the immunosuppressive function and eventually cause immunopathological damage to mother and fetus [5]. However, it has been revealed that human leukocyte antigen-G (HLA-G) produced by trophoblasts seemed to play an important role in this process [6].

Human leukocyte antigen-G (HLA-G) is a non-classical major histocompatibility complex (MHC) class I gene that is concentrated at the maternal-fetal interface during pregnancy [7]. Two isoforms of HLA-Gs, membrane-bound HLA-G and soluble HLA-G (sHLA-G), are synthesized by trophoblast and fetal cells [8]. The sHLA-G isoform circulates in maternal blood. Its main function is to protect fetal tissues from the maternal NK cell activity and establish immune tolerance at the maternal-fetal interface [9]. It may lead to an enhanced immune rejection at the maternal-fetal interface when the expression of HLA-G is down-regulated, which can eventually contribute to the occurrence of PE [10]. In addition, PE was proved to be linked with low-to-undetectable HLA-G in plasma. Therefore, low HLA-G might be a potential risk marker for PE. Clinically, serum HLA-G expression has been used as a biomarker for early diagnosis of PE and the evaluation of therapeutic effects [11].

In recent years, more researchers have used microarray technology to explore the relationship between biological molecules and diseases [12]. Circular RNA (circRNA), a non-coding RNA, is a popular research subject recently. CircRNA is an endogenous non-coding RNA with decent conservation and high specificity, which can regulate key target genes. Screening circRNAs with a differential expression as disease-related biomarkers and functional research can better understand the molecular mechanism of disease occurrence, improving the prevention and diagnosis of related diseases [13]. It is proved that circRNAs in the blood act as the biomarker for early prediction of PE, such as circRNA-101222 [14]. More circRNAs have been detected in placenta tissue, including hsa_circ_0051326 [15]. However, up to now, serum hsa_circ_0051326 was not detected in patients with PE.

In this study, we first detected hsa_circ_0051326 expression in the blood of patients with PE. Moreover, we compared the expression of hsa_circ_0051326 in the blood of normal pregnant women with PE patients. The relationship between hsa_circ_0051326 and HLA-G was explored, which demonstrated serum hsa_circ_0051326 could act as a new biomarker for the early diagnosis and treatment of PE.

Objectives

This study aimed to identify the relationship between hsa_circ_0051326 and HLA-G expression in the blood of patients with PE.

MATERIAL AND METHODS

Clinical samples

All the specimens of 50 patients with PE from the Hainan General Hospital, Hainan Affiliated Hospital of Hainan Medical University were collected from April 2019 to December 2019, with 50 paired normal pregnant women as the control group. The including criteria for patients with PE were as follows: (1). systolic blood pressure (SBP) higher than 140mmHg or diastolic blood pressure (DBP) higher than 90 mmHg

after 20 weeks of pregnancy; (2). Twenty-four hour urinary protein more than 0.3 g/day. 5 mL of peripheral blood was collected during the period of waiting for delivery in the hospital. Total proteins and mRNAs from these patients' blood were immediately obtained when samples were still fresh. Informed consent was obtained from all patients, those patients' clinical data were recorded during hospitalization and the research program was approved by the Ethics Committee of the Hainan General Hospital, Hainan Affiliated Hospital of Hainan Medical University (Code#: XXXXXX).

Enzyme-linked immunosorbent assay (ELISA)

The collected blood samples were centrifuged at 3000g for 10min, and the serum was stored in liquid nitrogen immediately. The concentrations of serum HLA-G protein in serum samples were determined by a specific double monoclonal sandwich enzyme immunoassay ELISA technique (R&D Systems, American). The analytical sensitivity was 1 U/mL. The range of the calibration curve was 3.91–125 Units/mL. The intra-assay coefficient of variation was 6.0% and the inter-assay coefficient of variation was 5.7%. The anti-HLA G antibody was purchased from the Abcam company, USA.

Coomassie blue staining and western blot

According to ELISA results, serum samples were diluted 10 times with PBS. The mixtures were boiled for five minutes, 100 °C after adding 5X loading buffer. We took 30 μg protein for 10% SDS-PAGE. The protein glues after SDS-PAGE were divided into two equal parts. One part was stained by Coomassie blue as the control. The other part was transferred to the polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was blocked at room temperature for one hour by 5% skim milk. Anti-HLA-G antibody diluted 1: 1000 were added and reacted at 4 °C overnight. After rinsed three times by TBST solution, 1:10000 diluted goat anti-mouse IgG was added. After reaction for one hour at room temperature and rinsed three times by TBST solution, chemiluminescence reagent was added for development imaging. Image Lab software was used to analyze the gray value of protein bands.

Design primers

The HLA-G and β-actin primers were designed according to the gene sequence from Genebank (https://cipotato.org/genebankcip). The specific primers were as follows: HLA-G upstream primer 5'-GCTACGGAATGAAGACGCCA-3', downstream primer 5'-TACCTCATGGAGTGGGAGCC-3'; β-actin upstream primer 5'-CTCCATCCTGGCCTCGCTGT-3', downstream primer 5'-GCTGTCACCTTCACCGTTCC-3'. The hsa_circ_0051326 primers were designed according to a circRNA database, CircInteractome (https://circinteractome.nia.nih.gov/index.html) [16]. The specific primers were as follows: hsa_circ_0051326 upstream primer 5'-GACGCAGGATCCTACACCTT-3', downstream primer 5'-AGAACATCCTTCCCCTCGGA-3'.

qRT-PCR

The total RNA of serum samples was extracted with QIAzol Lysis Reagent provided in the miRNeasy Mini Kit (QIAGEN). For HLA-G, the total RNA was reverse-transcribed into cDNA using PrimeScript™ RT reagent Kit (MANUFACTURER) with gDNA Eraser. The reaction conditions: 37 °C 15 minutes, 85 ℃ 5 seconds. After 15 minutes, this cDNA was used as a template to apply SYBR® Premix Ex Taq™ (MANUFACTURER) for PCR amplification. PCR reaction conditions on ABI 7300 real-time fluorescence quantitative machine: 95 ℃ 30 seconds; 95 ℃ 5 seconds, 40 cycles at 60 ℃ for 30 seconds. The mRNA concentration of each gene was represented by a $2^{-\Delta\Delta CT}$ value relative expression level. Moreover, for hsa_circ_0051326, the qRT-PCR protocol was adapted from the previously published papers [17, 18]. For qRT-PCR, β-actin was used as the internal reference. All the experiments in this study were repeated three times.

Statistical analysis

Analysis data through SPSS 26.0 (MANUFACTURER) were expressed as mean and standard deviation. The comparison of data between the two groups was performed using a t-test. The comparison of data between multiple groups was performed using a single factor analysis of variance. Pearson's correlation analysis was used to analyze the correlation between hsa_circ_0051326 and HLA-G. $p < 0.05$ was considered

statistically significant.

RESULTS

General information of patients with PE

For patients with PE, their mean age was 30.6 years old. The average gestational weeks was 35.95 weeks. Among 50 patients with PE, the newborns included eight premature infants and 42 term infants, and the neonatal weight was 3254.28 g. On the other hand, for the control group where there were 50 normal women with physiological pregnancy, their mean age was 30.09 years old, and the average gestational weeks were 36.52 weeks. The newborns from normal pregnant women included three premature infants and 47 term infants, and the neonatal weight was 3328.46 g. Other than being diagnosed as PE, compared with the control group, there was no difference in the general information of patients with PE.

Serum HLA-G expression level was decreased in patients with PE

We detected serum HLA-G protein expression level by ELISA, found that the mean concentrations of serum HLA-G in patients with PE was 20.28 ng/mL, which was significantly lower than that in normal pregnant women (50.67 ng/mL). For total protein samples, 50 patients with PE and 50 normal pregnant women were randomly paired into 50 pairs. One-to-one paring label was ranked from No.1 to No.50. We performed western blotting after the proteins were diluted to the same concentration with PBS. Figure 1A showed that serum HLA-G protein level was decreased in patients with PE compared with the normal pregnant women. Moreover, we detected serum HLA-G mRNA level and found that HLA-G mRNA was down-regulated in patients with PE (Fig. 1B). The HLA-G mRNA of the 50 patients and paired controls were shown in Figure 1C. The overall HLA-G mRNAs were decreased in patients with PE compared with normal pregnant women.

Serum hsa_circ_0051326 expression level was lower in patients with PE

For total RNA samples, the one-to-one paring label was the same as that in western blotting. We detected serum hsa_circ_0051326 expression level and found that serum

hsa_circ_0051326 level in patients with PE was lower than that in normal pregnant women (Fig. 2A). All of 50 patients and 50 control group hsa_circ_0051326 expression results were shown in Figure 2B. The overall hsa_circ_0051326 were lower in patients with PE than that in normal pregnant women. In addition, serum hsa_circ_0051326 in all the patients with PE was lower than the mean hsa_circ_0051326 level in normal pregnant women.

Serum hsa_circ_0051326 expression level was positively correlated with HLA-G

In normal pregnant women, the correlation coefficient between serum hsa_circ_0051326 level and HLA-G mRNA level was 0.57, shown in Figure 3A. On the other hand, for patients with PE, the correlation coefficient between serum hsa_circ_0051326 level and HLA-G mRNA level was 0.78, shown in Figure 3B. Obviously, the correlation in patients with PE is higher than that in normal pregnant women. That is, for patients with PE, the levels between hsa_circ_0051326 level and HLA-G mRNA were more correlated, and both hsa_circ_0051326 level and HLA-G mRNA were reduced, suggesting that serum hsa_circ_0051326 could be a diagnostic biomarker for PE and serum hsa_circ_0051326 level changes were closely related to HLA-G.

DISCUSSION

Membrane-bound HLA-G and sHLA-G are synthesized by trophoblast cells and fetal cells. The sHLA-G subtype is present in maternal blood circulation. Its primary physiological function is to protect fetal tissues from the activity of maternal NK cells and to establish immune tolerance at the maternal-fetal interface [9]. Variable splicing of the HLA-G gene generated seven different transcription subtypes, of which four encode membrane-bound products and the other three encode soluble proteins [19]. HLA-G1, HLA-G2, HLA-G3, and HLA-G4 bind to the cell surfaces, while HLA-G5, HLA-G6, and HLA-G7 are soluble isotypes [20]. In our study, we observed two binds during western blotting of serum HLA-G protein, one bind located at ~35KDa and the other located at ~30KDa, which may be two subtypes of HLA-G in serum. The

sHLA-G protein derived from the mRNA splicing form takes part in the vascular remodeling of the maternal spiral artery during pregnancy by interacting with CD4 [21]. The main isoforms of HLA-G existing in serum are sHLA-G1 and sHLA-G5, which are produced by shedding or proteolytic cleavage of membrane-bound isoforms and secretion of soluble isoforms, respectively [20]. They exist not only on the cell surface but also in maternal serum and amniotic fluid.

PE is a special and severe clinical manifestation of pregnancy hypertension, which leads to an increased in maternal and infant mortality [22]. Therefore, early prediction and early intervention of PE are important to reduce the risk of pregnancy hypertension [23]. In patients with PE, HLA-G expression has been proved to decline and is often accompanied by an inadequate invasion of trophoblast cells to the spiral arteries of the uterine wall, which leads to poor blood transport to the placenta [24]. HLA-G is an immune tolerance molecule that maintains maternal-fetal tolerance, so that HLA-G expression decreases, which can easily lead to miscarriage and PE [25]. sHLA-G can not only establish an immune tolerance at the maternal-fetal interface but also spread throughout the body with the blood flow, affecting the entire immune system [26]. Peripheral blood sHLA-G reflects the expression level of HLA-G in the body, and its concentration is negatively correlated to the occurrence of PE. Many studies have shown that the serum HLA-G concentration in patients with PE is significantly lower than that of normal women in the late trimester of pregnancy [27]. Meanwhile, the decrease of sHLA-G in blood during mid-term pregnancy leads to the infiltration of extravillous trophoblasts into the uterine spiral artery, which may be related to the mechanism of PE [28]. Serum HLA-G can be used as a new detection method in prenatal diagnosis for PE in practice.

At present, circRNA has been shown involved in regulating the occurrence and development of gynecological cancers [29]. In recent years, many studies have reported that circRNA played a significant role in other diseases in women, such as habitual abortion and PE [30]. In the blood cells of early pregnant women, the expression of circ-101222 was significantly different between PE and normal

pregnant women [14]. In blood cells of PE and premature women, hsa_circ_0004904 and hsa_circ_0001855 were significantly different between the two groups, and they were significantly up-regulated in the PE group [31]. With the development of DNA and RNA sequencing technology, more circRNAs have been identified in placental tissue, including hsa_circ_0051326. However, circRNAs are stable and conserved. CircRNA is expressed in specific tissues and developmental stages. CircRNAs are promising diagnostic biomarkers for PE [32]. An increasing amount of studies have indicated that circRNAs expression in blood and placental tissues of patients with PE were different from that in normal pregnant women, suggesting that circRNAs have important value in the diagnosis of PE [33, 34]. For early diagnosis of diseases, serum biomarkers were better than biomarkers in tissues. Although hsa_circ_0051326 expression in placental tissues has been reported, its level in blood was still unclear. Our previous studies have shown that serum HLA-G could be used as a diagnostic biomarker for the early diagnosis and prognosis of PE. We would like to explore whether serum hsa_circ_0051326 is related to serum HLA-G level in further studies.

In our study, we used qRT-PCR and western blot to comprehensively study the expression level of HLA-G in the serum of PE and normal pregnant women, which shown HLA-G protein and mRNA levels were decreased in patients with PE. We also detected hsa_circ_0051326 expression in serum and found lower expression in patients with PE. Surprisingly, in normal pregnant women, the correlation between hsa_circ_0051326 and HLA-G expression was positive, while their correlation coefficient was higher in patients with PE than that in normal pregnant women. Like serum HLA-G, hsa_circ_0051326 is highly correlated with the occurrence of PE.

CONCLUSIONS

In summary, early diagnosis of PE is very important for decreasing mortality in pregnant women and fetuses. Our studies first confirmed that hsa_circ_0051326 was decreased in PE, which was positively related to HLA-G expression, suggesting that hsa_circ_0051326 can become a new biomarker to diagnose and evaluate the

prognosis of PE. However, the molecular mechanism between hsa_circ_0051326 and HLA-G is not clear, we need to explore further the mechanism in the following research. This study elaborated a new biomarker for PE and provided a new theoretical and experimental basis for the role of circRNA in obstetrics and gynecology-related diseases.

Ethics approval and informed consent to participate

The inclusion of human participants, as well as the use of human data and human samples in this study, was approved by the Ethics Committee of the Hainan General Hospital, Hainan Affiliated Hospital of Hainan Medical University**.**

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Conflict of interest

The authors declare that they have no conflicts of interest.

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Figure 1. Serum HLA-G expression in patients with pre-eclampsia and normal pregnant women.

A. After randomly pairing one-on-one for the total proteins samples between pre-eclampsia and normal pregnant women, western blotting results found that serum HLA-G protein level was decreased in patients with pre-eclampsia. The 9th and 34th pairs (No.09 and No.34) were shown as examples;

B. After randomly pairing one-on-one for the total RNA samples between pre-eclampsia and normal pregnant women, qRT-PCR results found that serum HLA-G mRNA level was also decreased in patients with pre-eclampsia. The 9th and 34th pairs (NO.09 and NO.34) were shown as examples, **p < 0.01;

C. The box-plot of serum HLA-G mRNA level for 50 patients with pre-eclampsia and 50 normal pregnant women, **p < 0.01

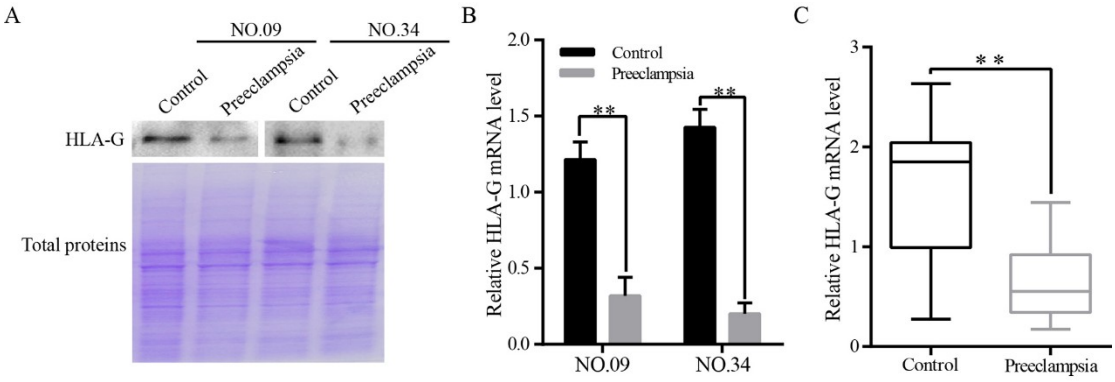

Figure 2. Serum hsa_circ_0051326 expression in patients with pre-eclampsia and normal pregnant women.

A. After randomly pairing one-on-one for the total RNA samples between pre-eclampsia and normal pregnant women, qRT-PCR results found that serum hsa_circ_0051326 level was also decreased in patients with pre-eclampsia. The 9th and 34th pairs (NO.09 and NO.34) were shown as examples, **p < 0.01;

B. The box-plot of serum hsa_circ_0051326 level for 50 patients with pre-

eclampsia and 50 normal pregnant women, **p < 0.01

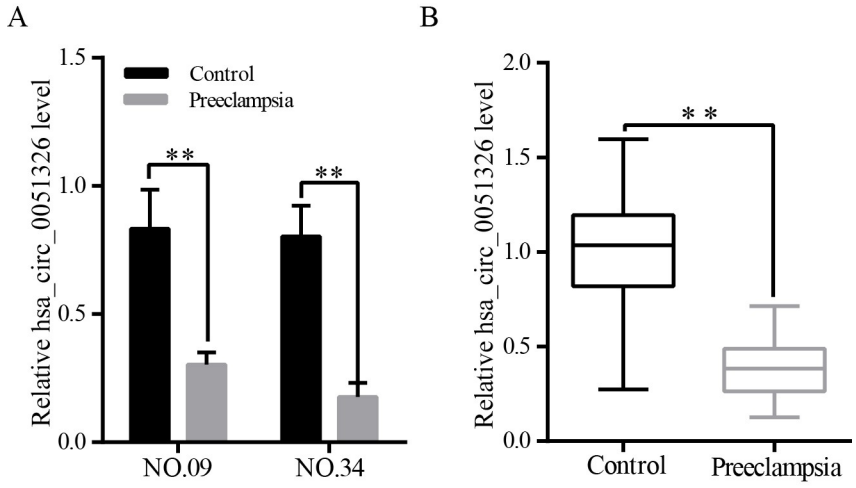

Figure 3. The relationship between hsa_circ_0051326 and HLA-G mRNA level.

A. Pearson correlation analysis between hsa_circ_0051326 and HLA-G mRNA level in normal pregnant women. “r” expressed the correlation coefficient;

B. Pearson correlation analysis between hsa_circ_0051326 and HLA-G mRNA level in patients with pre-eclampsia. “r” expressed the correlation coefficient

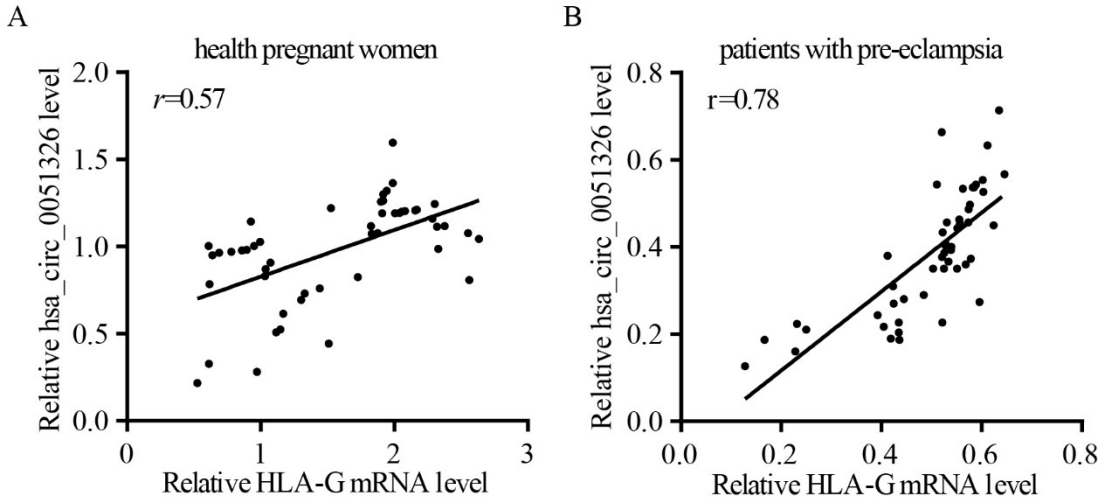

Table 1. Clinical characteristics of recruited populations

	Pre-eclampsia patients (N = 50)	Normal pregnant women (N = 50)
Age	30.60 ± 1.4	30.09 ± 2.6
Gestational weeks	35.95 ± 1.8	36.52 ± 2.2
Systolic pressure (mmHg)	145 ± 12.5	128 ± 19.1
Diastolic pressure (mmHg)	93 ± 8.6	85 ± 9.6
Urine protein (g/24 h)	0.18 ± 0.03	0.01 ± 0.00