

Evaluation of circulating placenta-related long noncoding RNAs as potential biomarkers for preeclampsia

XIN WANG^{1,2}, YANHONG CHEN¹, LILI DU¹, XIAOMEI LI¹, XIUYING LI¹ and DUNJIN CHEN¹

¹Department of Obstetrics and Gynecology, Third Affiliated Hospital of Guangzhou Medical University, Guangzhou, Guangdong 510150; ²Department of Obstetrics and Gynecology, Affiliated Hangzhou Hospital (Hangzhou First People's Hospital), Nanjing Medical University, Hangzhou, Zhejiang 310006, P.R. China

Received May 5, 2017; Accepted October 23, 2017

DOI: 10.3892/etm.2018.5968

Abstract. Increasing evidence has indicated that circulating placental RNAs may reflect the pathophysiology of the placenta. In the current study, circulating placenta-related long noncoding RNAs (lncRNAs) were evaluated as potential biomarkers for preeclampsia (PE). Two parts of the case-control study were simultaneously executed, including the following sets: 52 patients with late-onset PE (LOPE) (diagnosed after 34 weeks) and 52 gestational age (GA)-matched controls; 58 patients with early-onset PE (EOPE) (diagnosed before 34 weeks) and 58 GA-matched controls. LncRNA expression was detected in the placenta analysis part, and the participants were delivered by caesarean sections. The levels of circulating placenta-associated lncRNAs were measured in the plasma analysis part and all pregnant women were included. Using microarray analysis, 163 differentially expressed lncRNAs were identified in placental samples from patients with LOPE, some of which were also detected in plasma samples from pregnant women. There were significant positive correlations between plasma, and placental expression levels of *NONHSAT116812* and *NONHSAT145880*, which in plasma provided high diagnostic efficiencies for LOPE and EOPE. The present study demonstrated that circulating placenta-associated lncRNAs, particularly *NONHSAT116812* and *NONHSAT145880* have potential as biomarkers for PE.

Introduction

Preeclampsia (PE) is a pregnancy-specific multisystem disorder that contributes substantially to maternal, fetal, and neonatal mortality and morbidity rates worldwide (1). Despite extensive research, causes of PE remain unclear, and removal

of the placenta is the only effective cure for the disease (2). Researchers have speculated that placental defects, especially shallow placental implantation, can induce the clinical disease (3,4). Others have suggested that long noncoding RNAs (lncRNAs) in human placenta may play roles in regulating trophoblast cell invasion and inducing PE (5,6).

lncRNAs comprise a diverse class of RNA transcripts that exceed 200 nucleotides in length with limited protein-coding potential, which were believed to have surprisingly complex and diverse functions (7). Misregulation of lncRNAs have been identified to be involved in various disorders such as cancer, cardiovascular disease and PE (8). In addition to their possible role in the biological functions, circulating lncRNAs have been characterized as a new class of potential biomarkers in the diagnosis and prognosis of different types of malignant tumors and for possibly predicting and monitoring treatment response (9,10). Recently, two papers described the differentially expressed lncRNAs in PE placenta using microarray, and revealed that many of these lncRNAs may be involved in the pathological mechanisms of PE (11,12). Therefore, We postulated that placenta-related lncRNAs might be also released into the maternal circulation in pregnancy and could be utilized to detect and monitor PE.

Materials and methods

Participant enrollment. All study participants were pregnant and non-pregnant women of the Department of Obstetrics and Gynecology of the Affiliated Third Hospital of Guangzhou Medical University (Guangzhou, China) between February 2015 and February 2017. This case-control study involved the following sets of cases and controls: late-onset PE (LOPE) patients (diagnosed after 34 weeks, n=52) and gestational age (GA)-matched healthy pregnant women (control group 1, n=52); early-onset PE (EOPE) patients (diagnosed before 34 weeks, n=58) and GA-matched healthy pregnant women who underwent routine outpatient antenatal examination and did not develop hypertension during their pregnancies (control group 2, n=58). Of these, 21 LOPE patients and all EOPE patients received anti-hypertensive drugs and/or magnesium sulfate prior to and following the termination of pregnancy. In addition, 30 healthy never-pregnant women were also recruited in this study. All pregnant women had

Correspondence to: Professor Dunjin Chen, Department of Obstetrics and Gynecology, Third Affiliated Hospital of Guangzhou Medical University, 63 Duobao Road, Guangzhou, Guangdong 510150, P.R. China
E-mail: chendunjin@hotmail.com

Key words: biomarkers, lncRNA, microarray, PCR, preeclampsia

singleton pregnancies and none of the PE patients had any other maternal complications. PE is defined according to the criteria of the American College of Obstetricians and Gynecologists (1). Demographic and clinical characteristics of all participants are summarized in Table I. This study was approved by the ethics committee of the Third Affiliated Hospital of Guangzhou Medical University. Informed consent was obtained from all individual participants included in the study.

Sample collection and preparation. Peripheral venous blood samples were collected from all of the participants, particularly blood samples from women who had delivered were obtained within 48 h before termination of pregnancy, without spontaneous or nonspontaneous labor. Up to 2.5 ml of blood was drawn from each nonfasting subject into an anticoagulation tube containing K₂EDTA. Blood was processed within 30 min of collection. Plasma was collected via a two-step centrifugation protocol of 1,800 g for 10 min at 4°C and 12,000 g for 10 min at 4°C. This process ensured the thorough elimination of peripheral blood cells and platelets. Separated plasma samples were transferred to an RNase/DNase-free tube and stored at -80°C until total RNA extraction. Blood samples with hemolysis were excluded from analysis.

Placental tissues were obtained during elective caesarean sections (C/S) from women who delivered in the absence of labor. Of the 117 placental tissue samples, 40 were from the LOPE group, 35 were from control group 1, and 42 were from the EOPE group. Placental tissue that was about 2 cm away from the attachment site of the umbilical cord in the placenta was randomly dissected within 5 min of delivery, including a full-thickness of placenta. Harvested samples were washed extensively in cold saline immediately after resection and stored in sufficient quantities of RNAlater (AM7020; Ambion, Thermo Fisher Scientific, Inc., Waltham, MA, USA) to protect RNA from degradation. Liquid was removed after 24 h of storage, and placental samples were stored at -80°C until use.

lncRNA microarray analysis. For microarray analysis, 8 randomly and blindly selected placental samples from LOPE patients and matched controls (4 samples per group) were used to extract total RNA. The expression profiles of the placental lncRNAs were detected using the Agilent Human lncRNA Microarray V4.0 (OE Biotech, Shanghai, China). The threshold for a dysregulated lncRNA was set as a fold-change (FC) value of 2.0 or greater. Gene Ontology (GO; <ftp://ftp.ncbi.nlm.nih.gov/gene/DATA/gene2go.gz>, October 2015) and Kyoto Encyclopedia of Genes and Genomes (KEGG; <http://www.genome.jp/kegg/>, Release 76.0) analyses were applied to explore the roles of the differentially expressed lncRNAs that exhibited a significance value of $P < 0.05$.

RNA extraction from placenta and plasma. Total RNA was extracted from placenta tissue using Trizol Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The RNA integrity was evaluated using agarose gel electrophoresis stained with GelRed. For plasma, total RNA was extracted from 800 μ l of each sample using the mirVana PARIS kit (1556; Ambion, Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions.

The average recovery volume was 45 μ l, and the RNA yield calculated using spectrophotometry was approximately 11.3 ng/ μ l.

Quantitative polymerase chain reaction (qPCR). A total of 1 μ g or 7 μ l of RNA from each placental tissue or plasma was reverse transcribed to cDNA using the PrimeScript™ RT reagent kit with gDNA Eraser (RR047A; Takara Biotechnology Co., Ltd., Dalian, China) in a 20- μ l reaction volume. All primers used in this study were designed and synthesized by Shanghai Generay Biotech Co., Ltd. (Shanghai, China), according to the sequences obtained from the National Center for Biotechnology Information Database. Then, qPCR was performed on a StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, Inc.) using SYBR® Premix EX Tag™ II (RR820A; Takara Biotechnology Co., Ltd.) in a 20- μ l reaction volume, and each sample was analyzed in triplicate and the specificity of each PCR reaction was confirmed by melt curve analysis. The qPCR reaction conditions were as follows: A denaturation step at 95°C for 30 sec, followed by 40 PCR cycles at 95°C for 5 sec and 60°C for 30 sec. Relative expression levels of lncRNAs were quantified based on the C_{quantification} cycle values and normalized to the internal control housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), which has been shown to be the optimal endogenous control for lncRNA expression in tissues and plasma (10,13). Expression levels were calculated by using the Δ C_q method. A smaller Δ C_q value indicates higher expression, and relative lncRNA expression was calculated by the $2^{-\Delta\Delta C_q}$ method normalized to *GAPDH*.

Statistical analyses. All statistical analyses were performed with the Statistical Package for Social Sciences software version 22.0 (SPSS, Inc., Chicago, IL, USA). Comparisons between two subgroups were estimated by using the independent samples t-test or Mann-Whitney U-test for continuous data and Fisher's exact test for categorical data. Comparisons between multiple subgroups were performed by one-way analysis of variance (ANOVA). Spearman's rank analysis was used for correlation analysis. All reported P-values are two-sided, and $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Microarray analysis and functional prediction. We identified 163 lncRNAs that were differentially expressed between LOPE placentas and healthy controls, with 38 up- and 125 downregulated lncRNAs. *NONHSAT084322* (CUST_4347_PI429545410) was the most upregulated (FC=9.202518) and *NONHSAT028367* (CUST_33686_PI429545402) was the most downregulated lncRNA (FC=16.553854). The lncRNA microarray expression results have been uploaded to NCBI Gene Expression Omnibus (GEO) and are accessible with the GEO series accession number GSE97898 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE97898>).

Using the same criteria as the lncRNAs, we found 14 upregulated and 67 downregulated mRNA transcripts. The most upregulated and downregulated mRNA transcripts were

Table I. Demographic and clinical characteristics of the study groups.

Variable	LOPE (n=52)	EOPE (n=58)	Control group 1 (n=52)	Control group 2 (n=58)	Non-pregnant (n=30)
Age (years)	31.1±5.5	30.9±6.1	30.8±5.7	30.4±4.3	31.0±2.3
Prepregnancy BMI (kg/m ²)	21.6±2.5	21.7±3.1	21.4±2.3	21.5±2.9	21.5±2.6
Nulliparity n (%)	26 (50.0%)	30 (51.7%)	25 (48.1%)	29 (50.0%)	30 (100.0%)
SBP (mmHg)	162.8±18.3 ^a	171.1±22.1 ^a	119.1±7.2	118.8±7.7	117.5±6.2
DBP (mmHg)	103.9±13.1 ^a	108±17.4 ^a	78.4±6.7	77.9±6.5	77.8±5.8
24-h urine protein (g)	3.1±2.2	4.9±3.9	ND	ND	ND
GA at blood collection (weeks)	38.2±2.4	31.9±3.0	38.8±1.6	32.0±1.9	0
Birth weight (g)	3,090.7±509.1 ^a	1,575.2±364.7	3,456.1±474.4	ND	0

Data are presented as mean ± standard deviation or number (%) and compared by independent samples t-tests for continuous variables or chi-square test for categorical variables. LOPE, late-onset preeclampsia; EOPE, early-onset preeclampsia; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; GA, gestational age; ND, not determined. Control group 1, LOPE-matched normal pregnant women; Control group 2, EOPE-matched healthy pregnant women who underwent routine outpatient antenatal examination and did not develop hypertension during their pregnancies; Non-pregnant: healthy never-pregnant women. ^aP<0.05 vs. control group 1 or 2.

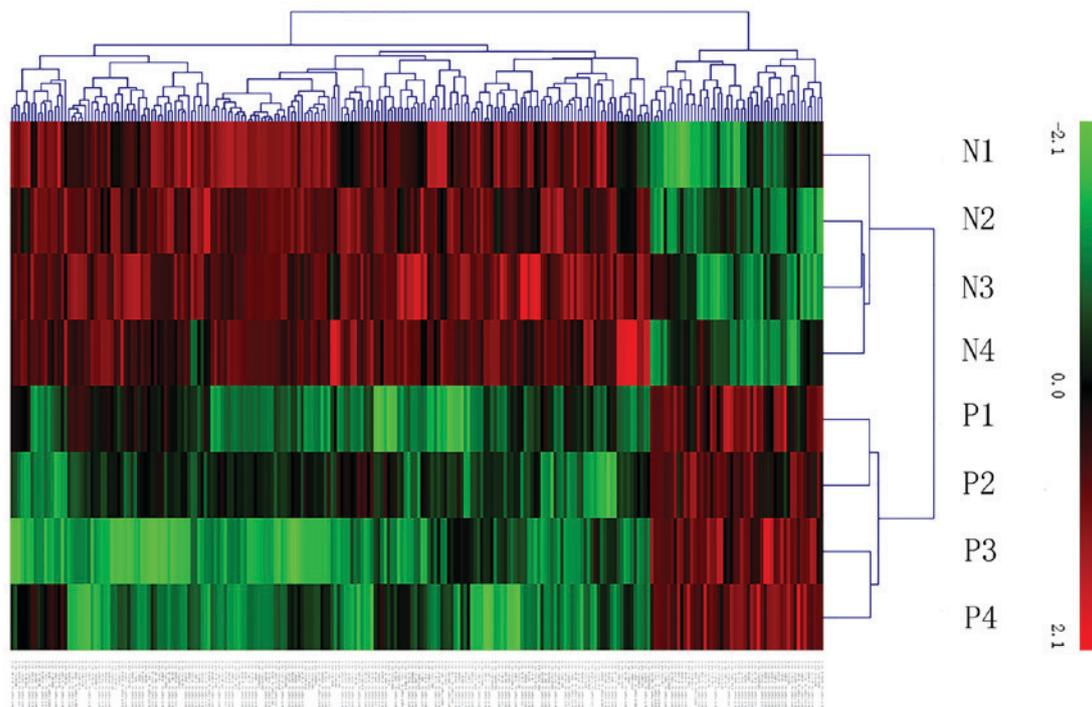


Figure 1. Heat map and hierarchical clustering of lncRNA and mRNA profile comparison between the LOPE and healthy placenta samples. Each row represents one tissue sample, and each column represents one lncRNA or mRNA. The relative lncRNA expression or mRNA is depicted according to the color scale. Red indicates upregulation; green indicates down regulation. 2.1, 0 and -2.1 are folds-changes in the corresponding spectrum, whereas N represents normal placenta samples and P represents LOPE tissue. The differentially expressed lncRNAs and mRNAs clearly self-segregated into N and P clusters. lncRNA, long noncoding RNAs; LOPE, late-onset preeclampsia.

GSTT1 (A_23_P254944) and KRT6A (A_33_P3292886), with FCs of 9.89536 and 22.835077, respectively. Hierarchical clustering of the expression of those lncRNAs and mRNAs based on centered Pearson correlation clearly separated LOPE from normal tissues (Fig. 1). Differentially expressed lncRNAs were clustered into hundreds of GO and KEGG pathway annotations. According to enrichment counting, the significantly enriched GO terms corresponded to biological processes, involving musculoskeletal movement, immune responses, and metabolic

processes, and the most enriched KEGG pathways included oxidative phosphorylation and metabolic pathways (Fig. 2).

Confirmation of lncRNAs by qPCR. For practical purposes, to validate the microarray results and to increase the detection probability of plasma lncRNAs, we first selected differentially expressed lncRNAs that have high normalized probe signal values on the array platform, and then subjected to qPCR validation in placental tissues from the LOPE patients (n=40) and

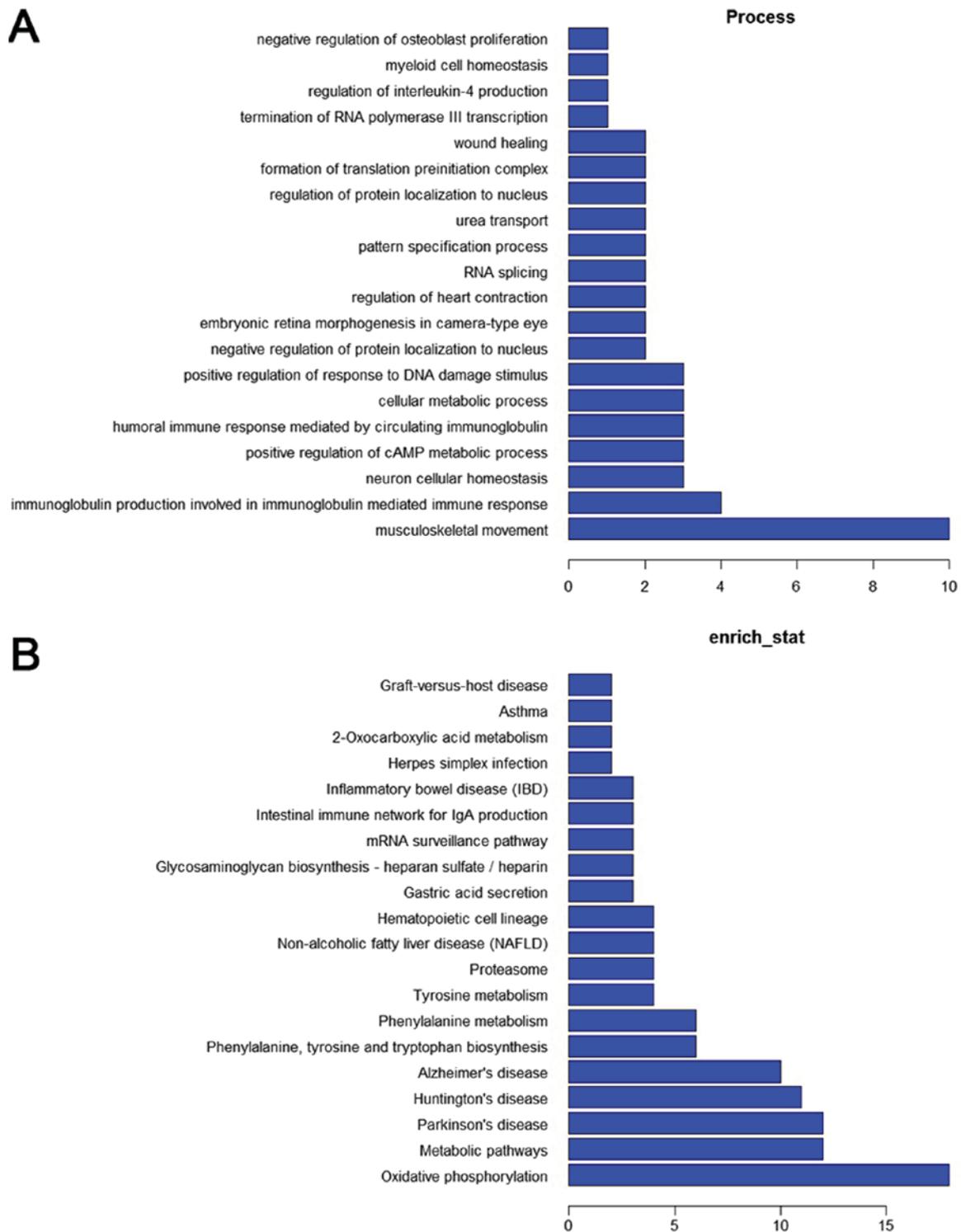


Figure 2. Top 200 hits of GO and KEGG annotations of aberrantly expressed lncRNAs. (A) The top 200 enriched GO terms (biological processes) are listed. The x axis indicates the number of lncRNAs annotated, and the y axis indicates the GO annotations; (B) The top 200 significantly enriched pathways are identified by KEGG. The x axis indicates the number of lncRNAs annotated, and the y axis indicates the KEGG annotations. lncRNAs, long noncoding RNAs; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

controls (n=35) delivered by caesarean section. Demographic and clinical characteristics of all of the pregnant women are summarized in Table II. Finally, 9 highest expressed lncRNAs in placental tissues were selected as candidate targets for subsequent circulating lncRNA assay, and their primers used are shown in Table III. The qPCR results showed the same tendencies (upregulation or downregulation) as the microarray data (Fig. 3).

Presence of placental lncRNAs in human plasma. To explore whether the nine placenta-related lncRNAs were present at detectable levels in maternal plasma, we used qPCR to examine lncRNA expression levels in plasma samples from the LOPE group and control group 1 (52 samples per group, 104 samples total) and from the EOPE group and control group 2 (58 samples per group, 116 samples total). As shown

Table II. Demographic and clinical characteristics of LOPE patients and controls delivered by caesarean section.

Variable	LOPE (n=40)	Control (n=35)	P-value
Age (years)	31.0±5.8	30.9±5.7	0.757
Prepregnancy BMI (kg/m ²)	21.5±2.9	21.3±2.4	0.533
Nulliparity n (%)	21 (52.5%)	18 (51.4%)	0.425
SBP (mmHg)	163.2±18.4	120.1±7.8	<0.001
DBP (mmHg)	103.8±13.7	79.3±6.5	<0.001
24-h urine protein (g)	3.3±2.4	ND	<0.001
Gestational age at delivery (weeks)	38.3±2.3	38.9±1.3	0.086
Birth weight (g)	3,068±625.7	3,422±437.6	<0.001

Data are presented as mean ± standard deviation or numbers (%), and compared by independent samples t-tests for continuous variables or chi-square test for categorical variable. LOPE, late-onset preeclampsia; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; ND, not determined.

Table III. The nine lncRNAs and their primers used in this study.

Target ID	FC(abs)	Primer sequence (5' to 3')	Product (bp)
<i>NONHSAT145880</i>	2.5↑	Forward TGTCAAGAAATACCCTGAGCC Reverse AGCCTGGGCGACAAAGTGA	165
<i>ENST00000587240</i>	2.2↑	Forward TTTACCCCTCAACATACACC Reverse TACGGCTTCCAACAATGA	144
<i>NONHSAT116812</i>	2.6↓	Forward GAGGCGGATGGCTGTGAC Reverse CCCTTGGGGCCTGAGTAG	87
<i>NONHSAT104536</i>	2.6↓	Forward CTTGGGTTCAAGGCTTTGTT Reverse TCTCCTCTGCACTGGCTGTT	143
<i>FR339600</i>	2.5↓	Forward GCAGAGCCAACATACACC Reverse AGGCAAGGCACATACAAA	59
<i>NONHSAG018907</i>	2.1↓	Forward ACCTGAAAAGTGGAATGATAG Reverse AGTAAGAGCAGCAACACACA	96
<i>TCONS_12_00014782</i>	2.1↓	Forward CCTCCTGACAGCCCCATTA Reverse GAGGCATCACCCAGTTGTTT	184
<i>NONHSAT134432</i>	2.0↓	Forward AGTGGAGAGGGTGAGGAG Reverse GGAGGAAGGAACAGAGGA	146
<i>NONHSAG024318</i>	2.0↓	Forward GGGTGCAGTTAGGTCACCTT Reverse CAGCACAAATTCCTCCGTCT	113
<i>GAPDH</i>	ND	Forward AAGAAGGTGGTGAAGCAGG Reverse GTCAAAGGTGGAGGAGTGG	144

FC(abs), fold-change(absolute); ND, not determined; ↑, upregulation; ↓, downregulation.

in Fig. 4, the expression level of *NONHSAT116812* and *NONHSAT145880* was significantly lower or higher in PE patients than in healthy controls with a 100% detection rate, respectively. The other seven lncRNAs had detection rates below 30% in both PE plasma samples and healthy controls and, therefore, were excluded from subsequent analyses. To verify that these two lncRNAs were present in plasma samples which were obtained from PE patients and healthy controls, the qPCR products (165 bp of *NONHSAT145880* and 87 bp of *NONHSAT116812*) were followed by Sanger sequencing with the primers used for qPCR, using BigDye™ Terminator v3.1 Cycle Sequencing kit (Applied Biosystems:

4337455) by Guangzhou Ige Biotech Co., Ltd. (Guangzhou, China). As expected, their sequences were identical to those derived from *NONHSAT116812* (http://noncode.org/show_rna.php?id=NONHSAT116812) and *NONHSAT145880* (http://noncode.org/show_rna.php?id=NONHSAT145880) (Fig. 5). However, these two lncRNAs were also detected in plasma samples from non-pregnant individuals at a rate of 100%, whereas other lncRNAs were not detectable. Therefore, we compared expression levels of *NONHSAT116812* and *NONHSAT145880* in plasma samples from pregnant women (n=52 from the LOPE group, n=52 from control group 1, n=58 from the EOPE group, and n=58 from control group 2) and

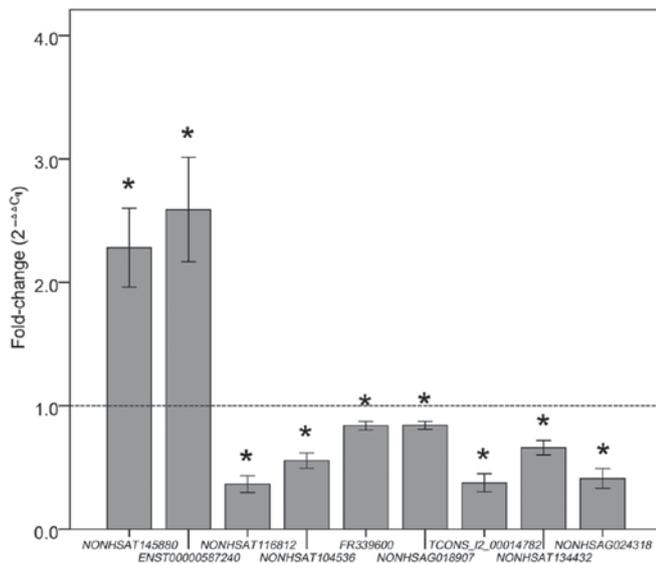


Figure 3. Validation of placenta-related lncRNAs expression in placental tissues. The differentially expressed lncRNAs were subjected to qPCR validation in placental tissues from late-onset PE patients (n=40) and healthy controls (n=35) delivered by caesarean section. $2^{-\Delta\Delta C_t}$ method was used to calculate lncRNA expression normalized to *GAPDH* and the expression levels of each lncRNA are presented relative to the healthy control group, as indicated by the horizontal dotted line at $y=1$. Data were compared by independent samples t-test or Mann-Whitney *U*-test. The bars indicate the standard deviation. * $P < 0.05$ vs. healthy control group. lncRNAs, long noncoding RNAs; PE, preeclampsia.

non-pregnant women (n=30) by measuring the ΔC_t value in the plasma. As expected, we found significantly larger ΔC_t values of these two lncRNAs in never-pregnant women than in pregnant women, and significantly smaller ΔC_t values in PE patients than in healthy pregnant women (Table IV).

Correlation of lncRNA levels between plasma and placenta. To determine whether expression levels of lncRNAs in plasma and placentas were related, we compared expression levels of *NONHSAT116812* and *NONHSAT145880* in paired plasma and placental samples from 117 pregnant women delivered by C/S (n=42 from EOPE group, n=40 from LOPE group, and n=35 from control group 1). The data showed that plasma and placental measurements were strongly correlated for *NONHSAT116812* ($r=0.841$, $P < 0.001$) and *NONHSAT145880* ($r=0.739$, $P < 0.001$) (Fig. 6).

Evaluation of plasma lncRNAs as biomarkers for detecting PE. We evaluated the ability of these two placenta-related lncRNAs to be used as plasma biomarkers for PE. Receiver operating characteristic (ROC) curves and areas under the ROC curves (AUCs) were obtained from the data of all pregnant women. Strong separation was found between ROC curves from the LOPE patients (n=52) and the healthy controls (n=52). *NONHSAT116812* showed an AUC of 0.828 (95% CI: 0.750-0.907, $P < 0.001$), sensitivity of 86.5%, and specificity of 63.5%. *NONHSAT145880* showed an AUC of 0.815 (95% CI: 0.734-0.896, $P < 0.001$), sensitivity of 82.7%, and specificity of 67.3%. ROC curves also discriminated the EOPE patients (n=58) from the healthy controls (n=58), with an AUC of 0.864 (95% CI: 0.797-0.930, $P < 0.001$), 72.4% sensitivity, and 91.4%

specificity for *NONHSAT116812*, and with an AUC of 0.859 (95% CI: 0.792-0.925, $P < 0.001$), 79.3% sensitivity, and 75.9% specificity for *NONHSAT145880* (Fig. 7).

Discussion

PE is a serious disease that cannot be cured except removal of the placenta. Early identification of women at high risk of developing PE would enable surveillance and intervention, with the potential for drastically improving pregnancy outcomes. To date, although numerous protein biomarkers of placental origin have been strongly associated with PE, these lack adequate accuracy to be used clinically (14,15). The circulating placental RNAs biomarkers have obvious advantage over protein biomarkers, changes of which in gene expression might be detectable before changes in protein levels occur and reflect alterations in placental function (16). However, little is known about the value of circulating placenta-related lncRNAs as potential biomarkers for PE.

In this study, we first selected LOPE placental tissues and healthy controls for lncRNA microarray analysis and a total of 163 differentially expressed lncRNAs ($FC \geq 2.0$) were identified. The results are consistent with a previous study that described the expression patterns of 738 differentially expressed lncRNAs ($FC \geq 1.5$) in placentas from patients with LOPE (11). However, a recent study evaluated the differential expression of lncRNAs in placental samples from EOPE patients and preterm controls and identified 28,824 dysregulated lncRNAs ($FC \geq 2.0$) (12). The distinct expression profiles of lncRNAs may be because EOPE is more serious than LOPE and placental defects are more prevalent (17,18). However, these studies have shown that aberrantly expressed lncRNAs in placenta may function in the pathogenesis and development of PE.

Further, 9 highest expressed lncRNAs in placental tissues were evaluated by qPCR to measure their expression levels in plasma. We found that the expression levels of *NONHSAT116812* and *NONHSAT145880* were significantly lower or higher in plasma from LOPE patients compared to healthy controls with a 100% detection rate, and the trends were consistent with the results in placental samples. Interestingly, the expression levels of the two lncRNAs in plasma from EOPE patients have the same trends with those of LOPE patients. However, our results are inconsistent with a previous study that revealed higher expression levels of circulating placental RNAs in EOPE compared with LOPE (19). This inconsistencies may be due to the existence of a wide range of heterogeneity between the studied populations, with samples collected across various gestations and processing procedures (20).

In addition, the expression levels of these two lncRNAs in placenta and plasma from the same individual correlated strongly. While other lncRNAs were also detectable in plasma, the detection rates were less than 30% in plasma samples from PE patients and corresponding controls. Moreover, to explore whether the nine lncRNAs were specific to pregnancy, we measured their expression levels in plasma samples from non-pregnant women and the results showed that *NONHSAT116812* and *NONHSAT145880* were detectable with a detection rate of 100%, but their expression levels were significantly lower than those in pregnant women, and the other lncRNAs were not detectable in non-pregnant women. Therefore, We hypothesized

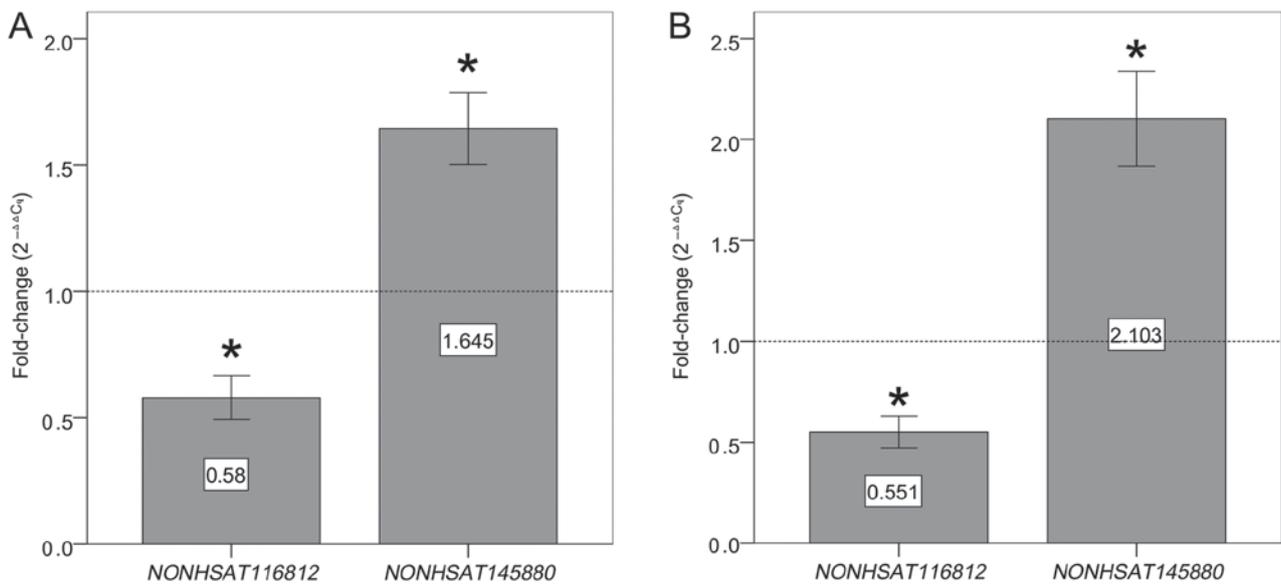


Figure 4. Differential expression patterns of placenta-related lncRNAs in PE plasma. Relative expression levels of *NONHSAT116812* and *NONHSAT145880* in late-onset PE group and control group 1 (n=52 per group) (A) and in early-onset PE group and control group 2 (n=58 per group) (B). $2^{-\Delta\Delta C_t}$ method was used to calculate lncRNA expression and the expression levels of each lncRNA are presented relative to the healthy control group, as indicated by the horizontal dotted line at y=1, respectively. Statistical differences were analyzed using independent samples *t*-test. Bars indicate standard deviation. *P<0.05 vs. healthy control group. lncRNAs, long noncoding RNAs; PE, preeclampsia.

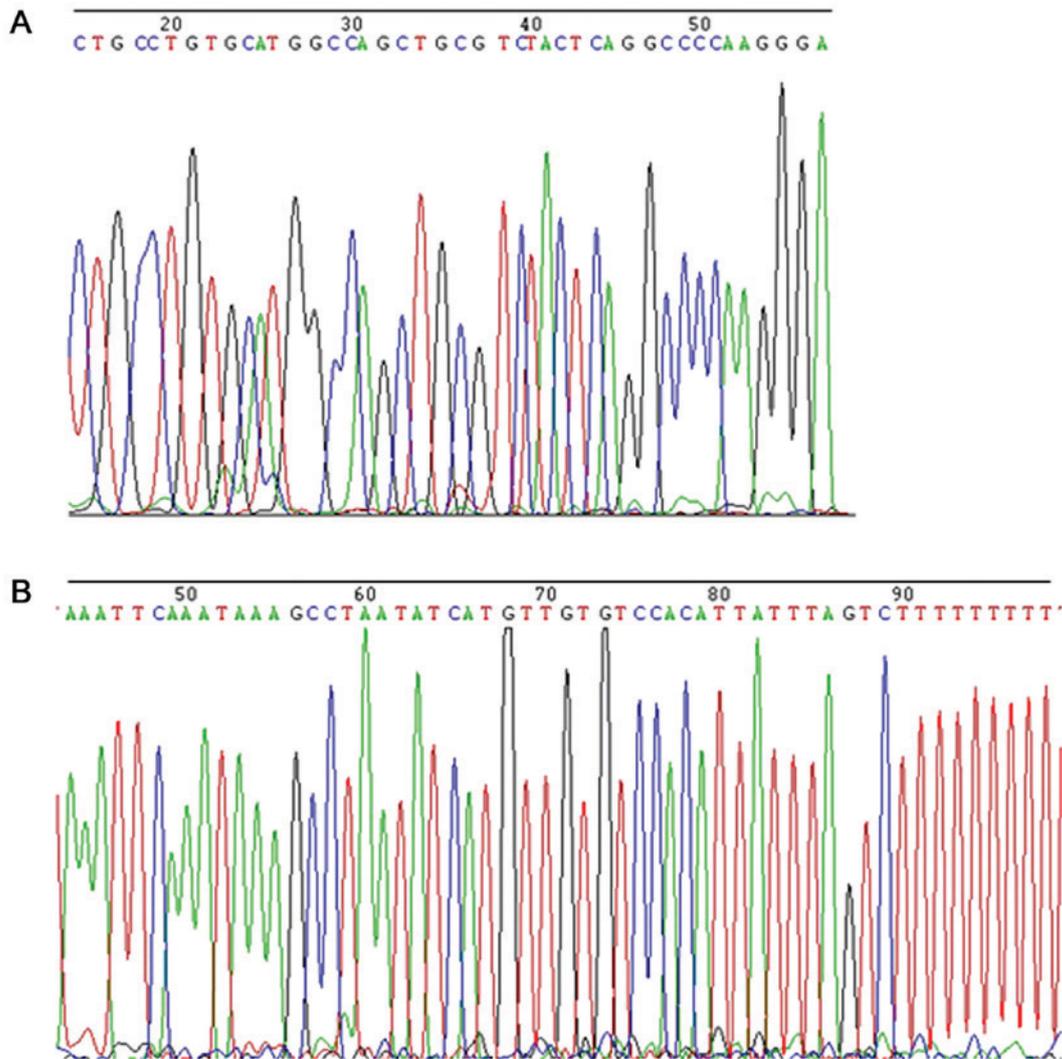


Figure 5. Sequencing results of plasma qPCR products of *NONHSAT116812* (A) and *NONHSAT145880* (B).

Table IV. Comparison of plasma ΔC_q value in each group.

LncRNA	LOPE (n=52)	EOPE (n=58)	Control group 1 (n=52)	Control group 2 (n=58)	Non-pregnant (n=30)
<i>NONHSAT116812</i>	5.308±1.509 ^a	5.419±1.736 ^a	4.295±1.210	4.336±1.427	7.319±1.385 ^b
<i>NONHSAT145880</i>	5.131±1.308 ^a	5.071±1.565 ^a	5.993±1.450	6.018±1.603	7.208±1.267 ^b

Data are presented as mean±standard deviation and compared by independent samples t-tests for continuous variables. LOPE, late-onset preeclampsia; EOPE, early-onset preeclampsia. Control group 1, LOPE-matched normal pregnant women; Control group 2, EOPE-matched healthy pregnant women who underwent routine outpatient antenatal examination and did not develop hypertension during their pregnancies; Non-pregnant: healthy never-pregnant women. ^aP<0.05 vs. control group 1 or 2; ^bP<0.05 vs. LOPE or EOPE or control group 1 or 2.

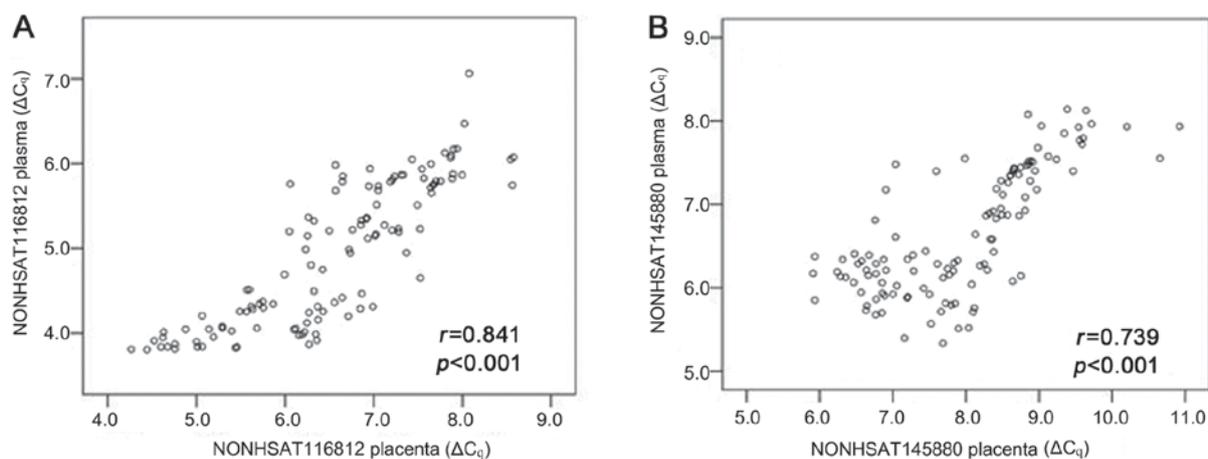


Figure 6. Correlation of placenta-related lncRNAs expression between placenta and plasma. Spearman's rank correlation scatter plot of (A) *NONHSAT116812* and (B) *NONHSAT145880* levels in placental samples and plasma, which were obtained from 117 pregnant women delivered by C/S (n=42 from early-onset PE group, n=40 from late-onset PE group, and n=35 from control group 1). Data were presented as ΔC_q values normalized to *GAPDH*. lncRNAs, long noncoding RNAs; PE, preeclampsia.

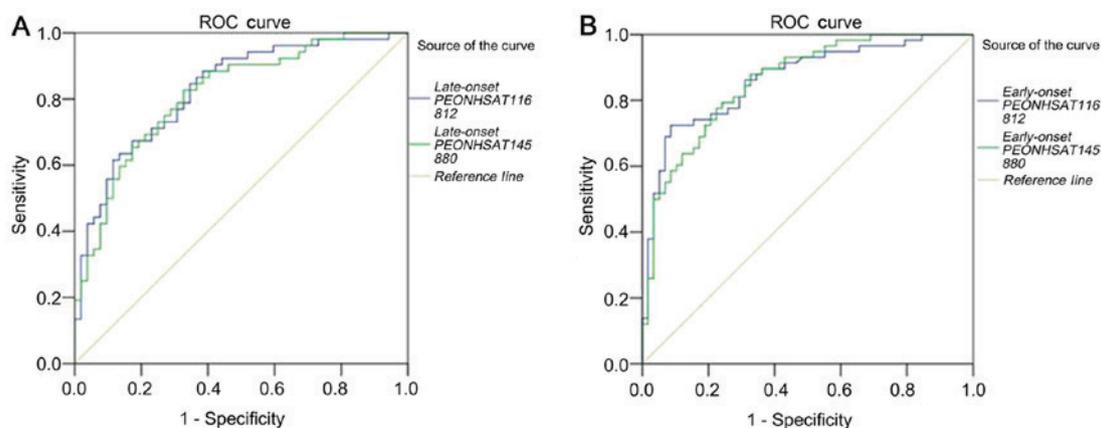


Figure 7. Evaluation of plasma lncRNAs for detection of PE. ROC curves were drawn with the data of plasma lncRNAs from 52 patients with late-onset PE and 52 healthy controls, and from 58 patients with early-onset PE and 58 healthy controls, respectively. (A) ROC-AUC for late-onset PE (*NONHSAT116812*, 0.828, P<0.001; *NONHSAT145880*, 0.815, P<0.001); (B) ROC-AUC for early-onset PE (*NONHSAT116812*, 0.864, P<0.001; *NONHSAT145880*, 0.859, P<0.001). lncRNAs, long noncoding RNAs; PE, preeclampsia; ROC, receiver operating characteristic.

that these two lncRNAs are expressed not only in the placenta but also in other tissues of the body and, for the first time, *NONHSAT116812* was detected in the placenta (http://noncode.org/show_rna.php?id=NONHSAT145880; http://noncode.org/show_rna.php?id=NONHSAT116812). To further verify that these two lncRNAs were present in plasma, the qPCR

products were validated by a traditional Sanger-based method. As expected, their sequences were identical to those derived from *NONHSAT145880* and *NONHSAT116812*. Taken together, these data provided strong evidence that placenta-related lncRNAs could be released into the circulation and their different expression patterns in plasma could be utilized as

biomarkers for detecting and monitoring PE. Recently, several studies have suggested that cell-free lncRNAs are detectable in human plasma and serum and may be utilized as minimally invasive biomarkers for disease prediction, diagnosis, and prognosis (21-23). Although the precise mechanism of lncRNA release into the extracellular environment is not completely understood, recent studies have shown that circulating lncRNAs are packaged into microparticles or other secondary structures that are protected from endogenous RNases (24,25).

Finally, the diagnostic power of the two circulating placenta-related lncRNAs was evaluated for PE. ROC curves illustrated a strong separation between PE patients and corresponding controls indicating that *NONHSAT116812* and *NONHSAT145880* have high diagnostic power for the detection of LOPE and EOPE. Thus, these two lncRNAs could be worthy of further research when seeking novel biomarkers for predicting and monitoring onset of PE. However, our study has some limitations, such as a modest sample size, use of a qPCR method with relatively low-sensitivity, and lack of an in-depth functional investigation of placenta-related lncRNAs. Therefore, prospective cohort studies are required to determine whether these findings are the consequence or cause of PE, and to determine the clinical applicability of using these molecules as the early markers of PE.

In conclusion, aberrantly expressed placenta-related lncRNAs might play a key or partial role in the pathogenesis of PE. More importantly, differentially expressed lncRNAs can be released into the maternal circulation, particularly *NONHSAT116812* and *NONHSAT145880* had high diagnostic power for PE. Further research is required to measure circulating placenta-related lncRNAs as potential tools for PE prediction and management.

Acknowledgements

We gratefully thank Professor Bolan Yu and Dr Hao Yan for providing statistical support and conducting the experiments. We appreciate all of the nurses who collected blood samples. We thank the staff in the medical records room for supplying case information and the biobank of the Third Affiliated Hospital of Guangzhou Medical University for sample support. This study was supported by the National Natural Science Foundation (no. 81671533) and National Key R&D Program of China (no. 2017YFC1001402).

References

- American College of Obstetricians and Gynecologists; Task Force on Hypertension in Pregnancy: Hypertension in pregnancy. Report of the American college of obstetricians and Gynecologists' task force on hypertension in pregnancy. *Obstet Gynecol* 122: 1122-1131, 2013.
- Baumann MU, Bersinger NA, Mohaupt MG, Raio L, Gerber S and Surbek DV: First-trimester serum levels of soluble endoglin and soluble fms-like tyrosine kinase-1 as first-trimester markers for late-onset preeclampsia. *Am J Obstet Gynecol* 199: 266.e1-6, 2008.
- Fu G, Ye G, Nadeem L, Ji L, Manchanda T, Wang Y, Zhao Y, Qiao J, Wang YL, Lye S, *et al*: MicroRNA-376c impairs transforming growth factor- β and nodal signaling to promote trophoblast cell proliferation and invasion. *Hypertension* 61: 864-872, 2013.
- He G, Xu W, Chen Y, Liu X and Xi M: Abhealthy apoptosis of trophoblastic cells is related to the up-regulation of CYP11A gene in placenta of preeclampsia patients. *PLoS One* 8: e59609, 2013.
- Zhang Y, Zou Y, Wang W, Zuo Q, Jiang Z, Sun M, De W and Sun L: Down-regulated long non-coding RNA MEG3 and its effect on promoting apoptosis and suppressing migration of trophoblast cells. *J Cell Biochem* 116: 542-550, 2015.
- Chen H, Meng T, Liu X, Sun M, Tong C, Liu J, Wang H and Du J: Long non-coding RNA MALAT-1 is downregulated in preeclampsia and regulates proliferation, apoptosis, migration and invasion of JEG-3 trophoblast cells. *Int J Clin Exp Pathol* 8: 12718-12727, 2015.
- Nagano T and Fraser P: No-nonsense functions for long noncoding RNAs. *Cell* 145: 178-181, 2011.
- Schmitz SU, Grote P and Herrmann BG: Mechanisms of long noncoding RNA function in development and disease. *Cell Mol Life Sci* 73: 2491-2509, 2016.
- Arita T, Ichikawa D, Konishi H, Komatsu S, Shiozaki A, Shoda K, Kawaguchi T, Hirajima S, Nagata H, Kubota T, *et al*: Circulating long non-coding RNAs in plasma of patients with gastric cancer. *Anticancer Res* 33: 3185-3193, 2013.
- Tong YS, Wang XW, Zhou XL, Liu ZH, Yang TX, Shi WH, Xie HW, Lv J, Wu QQ and Cao XF: Identification of the long non-coding RNA POU3F3 in plasma as a novel biomarker for diagnosis of esophageal squamous cell carcinoma. *Mol Cancer* 14: 3, 2015.
- He X, He Y, Xi B, Zheng J, Zeng X, Cai Q, Ouyang Y, Wang C, Zhou X, Huang H, *et al*: LncRNAs expression in preeclampsia placenta reveals the potential role of LncRNAs contributing to preeclampsia pathogenesis. *PLoS One* 8: e81437, 2013.
- Tong W, Rui C, Song X, Dai X, Xue X, Lu Y, Shen R, Li J, Li J and Ding H: Distinct expression profiles of lncRNAs between early-onset preeclampsia and preterm controls. *Clin Chim Acta* 463: 193-199, 2016.
- Yang Z, Guo X, Li G, Shi Y and Li L: Long noncoding RNAs as potential biomarkers in gastric cancer: Opportunities and challenges. *Cancer Lett* 371: 62-70, 2016.
- Wu P, van den Berg C, Alfirevic Z, O'Brien S, Röthlisberger M, Baker PN, Kenny LC, Kublickiene K and Duvetot JJ: Early pregnancy biomarkers in Pre-eclampsia: A systematic review and meta-analysis. *Int J Mol Sci* 16: 23035-23056, 2015.
- Kleinrouweler CE, Cheong-See FM, Collins GS, Kwee A, Thangaratnam S, Khan KS, Mol BW, Pajkrt E, Moons KG and Schuit E: Prognostic models in obstetrics: Available, but far from applicable. *Am J Obstet Gynecol* 214: 79-90 e36, 2016.
- Tsui NB, Jiang P, Wong YF, Leung TY, Chan KC, Chiu RW, Sun H and Lo YM: Maternal plasma RNA sequencing for genome-wide transcriptomic profiling and identification of pregnancy-associated transcripts. *Clin Chem* 60: 954-962, 2014.
- Ogge G, Chaiworapongsa T, Romero R, Hussein Y, Kusanovic JP, Yeo L, Kim CJ and Hassan SS: Placental lesions associated with maternal underperfusion are more frequent in early-onset than in late-onset preeclampsia. *J Perinat Med* 39: 641-652, 2011.
- Raymond D and Peterson E: A critical review of early-onset and late-onset preeclampsia. *Obstet Gynecol Surv* 66: 497-506, 2011.
- Kodama M, Miyoshi H, Fujito N, Samura O and Kudo Y: Plasma mRNA concentrations of placenta-specific 1 (PLAC1) and pregnancy associated plasma protein A (PAPP-A) are higher in early-onset than late-onset pre-eclampsia. *J Obstet Gynaecol Res* 37: 313-318, 2011.
- Farina A: The Role of RNAs and microRNAs in non-invasive prenatal diagnosis. *J Clin Med* 3: 440-452, 2014.
- Amorim M, Salta S, Henrique R and Jerónimo C: Decoding the usefulness of non-coding RNAs as breast cancer markers. *J Transl Med* 14: 265, 2016.
- Wang J, Sun J, Wang J, Song Y, Gao P, Shi J, Chen P and Wang Z: Long noncoding RNAs in gastric cancer: Functions and clinical applications. *Onco Targets Ther* 9: 681-697, 2016.
- Chauhan R and Lahiri N: Tissue- and serum-associated biomarkers of hepatocellular carcinoma. *Biomark Cancer* 8 (Suppl 1): S37-S55, 2016.
- Redman CW, Tannetta DS, Dragovic RA, Gardiner C, Southcombe JH, Collett GP and Sargent IL: Review: Does size matter? Placental debris and the pathophysiology of pre-eclampsia. *Placenta* 33 (Suppl): S48-S54, 2012.
- Li Q, Shao Y, Zhang X, Zheng T, Miao M, Qin L, Wang B, Ye G, Xiao B and Guo J: Plasma long noncoding RNA protected by exosomes as a potential stable biomarker for gastric cancer. *Tumour Biol* 36: 2007-2012, 2015.

