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LETTER

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The RNA fusion landscape of human placentae and pre-eclampsia

Songfa Zhang,[1,10] Mengqi Yang,[2,3,10] Yuqi Pan,[2,5,6,10] Hai Zhang,[4,10] Yunyun An,[2] Xiaoyi Liu,[2,5,6] Huizhen Lin,[2,5,6] Bianbian Tang,[2,7] Fanglei Gong,[2,5,6] Wanqiu Wang,[2] Yunxia Bai,[2] Dingxue Hu,[2] Hetong Li,[1] Yu Zhao,[8] Xiaojing Pan,[5] Yang Zhang,[9] Qiong Luo,[1] and Kun Sun[2,5,*]

[1]Women's Hospital, School of Medicine, Zhejiang University, Hangzhou 310006, China
[2]Institute of Cancer Research, Shenzhen Bay Laboratory, Shenzhen 518132, China
[3]Division of Life Science, Department of Chemical and Biological Engineering, and State Key Laboratory of Nervous System Disorders, Hong Kong University of Science and Technology, Hong Kong SAR 999077, China
[4]Shenzhen Maternity & Child Healthcare Hospital, Shenzhen 518028, China
[5]Shenzhen Medical Academy of Research and Translation, Shenzhen 518107, China
[6]School of Life Sciences, Westlake University, Hangzhou 310030, China
[7]School of Science, Shenzhen Campus of Sun Yat-sen University, Sun Yat-sen University, Shenzhen 518107, China
[8]Molecular Cancer Research Center, School of Medicine, Shenzhen Campus of Sun Yat-sen University, Sun Yat-sen University, Shenzhen 518107, China
[9]Institute of Molecular Physiology, Shenzhen Bay Laboratory, Shenzhen 518132, China
[10]These authors contribute equally
*Correspondence: sunkun@szbl.ac.cn

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Dear Editor,

The placenta is a critical organ in human reproduction mediating the exchange of oxygen and nutrients between mother and fetus while secreting various hormones and growth factors to regulate pregnancy, fetal development, and parturition. Dysfunction of placental tissues can lead to various pregnancy complications, including miscarriage, intrauterine growth restriction, hyperglycemia (gestational diabetes mellitus), and pre-eclampsia. Pre-eclampsia is a complex, multifactorial gestational hypertensive disorder characterized by new-onset high blood pressure and proteinuria after 20 weeks of gestation. Clinically, pre-eclampsia and related hypertensive disorders rank as the second most severe complications, contributing to 14% of maternal deaths annually.[1] Current treatments are limited and often ineffective, with delivery being the only definitive cure. Recent studies have suggested that low-dose aspirin may have preventive effects for early-stage pre-eclampsia patients prior to symptom onset, underscoring the importance of early diagnosis. However, current diagnostic standards primarily rely on clinical features, which limits applicability in early-stage pre-eclampsia patients without obvious symptoms. Besides plasma or serum biomarkers (e.g., sFLT1, PlGF, sENG),[2-4] recent studies have proposed several promising biomarkers based on placentae released circulating cell-free DNA and RNA (cfDNA and cfRNA), while novel biomarkers and assays are still under active development to meet urgent clinical needs.

In pathology, placental tissues are non-malignant yet share numerous similarities with cancerous tumors in aspects such as cell behavior, microenvironment, and metabolites. This leads us to hypothesize that hallmarks typically associated with tumors, such as gene or RNA fusion, which refers to hybrid RNA formed by the joining of two or more originally independent genes/transcripts, may also be present in placentae. Gene or RNA fusions can produce aberrant RNA or proteins distinct from their original components, often considered deleterious to normal cells and potentially contributing to diseases.[5] Interestingly, several pan-tissue studies have suggested the presence of RNA fusions in placentae;[6] however, a comprehensive profiling and validation of the entire catalog is lacking. Furthermore, the involvement of RNA fusions in gestational diseases, particularly pre-eclampsia, has yet to be explored. Therefore, in this study, we investigated RNA fusions in placentae and their potential as noninvasive diagnostic biomarkers for pre-eclampsia.

CATALOGUE OF RNA FUSIONS IN HUMAN PLACENTAE

We recruited 61 pregnant women with singleton pregnancies, including 18 with late-onset pre-eclampsia (the major subtype), 9 with hyperglycemia, 3 with both late-onset pre-eclampsia and hyperglycemia, 5 with other conditions, and 26 age- and gestational age-matched controls without known diseases (https://github.com/hellosunking/placental-fusion/blob/main/Supplementary.materials/Suppl.Tables.xlsx). Pre-eclampsia and hyperglycemia patients were diagnosed according to the guidelines of Chinese Medical Association. Placental tissues were collected after delivery, and whole transcriptome sequencing (RNA-seq) was performed on these samples. Through bioinformatic analyses, rigorous filtering, and manual curations (see Methods at https://github.com/hellosunking/placental-fusion/blob/main/Supplementary.materials/Suppl.Methods.pdf), we identified a total of 15 RNA fusion transcripts, which are illustrated in Figure 1A. Sequences surrounding the fusion junctions are provided in https://github.com/hellosunking/placental-fusion/blob/main/Supplementary.materials/Suppl.Tables.xlsx. One example, the DEPDC1B-PDE4D fusion formed by DEPDC1B and PDE4D genes, is depicted in Figure 1B. To strengthen our findings, we analyzed three publicly available placental RNA-seq datasets and found that all fusion events were readily detectable in the external datasets (https://github.com/hellosunking/placental-fusion/blob/main/Supplementary.materials/Suppl.Tables.xlsx), suggesting high fidelity of our RNA fusion list. Moreover, among the 15 RNA fusion transcripts, 7 had been previously documented in cancer-related studies or databases, while 8 had not been reported prior to this study. Notably, different from the fusions found in tumors, all RNA fusion transcripts originate from nearby genes, suggesting that these fusion events may be read-through transcripts attributed to the spatial proximity of the transcribed RNA.

To validate the RNA fusion transcripts identified in RNA-seq experiments, we designed primers based on the sequences surrounding the fusion junctions and performed reverse transcription PCR (RT-PCR) on two randomly selected placental tissues, one from a pre-eclampsia patient and the other from a control subject. RT-PCR products of the expected sizes were obtained from both samples for all fusion events (Figure 1A). These PCR products were then subjected to Sanger sequencing to verify their sequences, confirming that they matched the designed sequences and contained the fusion junctions. Figure 1B presents the Sanger sequencing results for the DEPDC1B-PDE4D fusion, while results for the rest 14 RNA fusion transcripts can be found in https://github.com/hellosunking/placental-fusion/blob/main/Supplementary.materials/Suppl.Figures.pdf. Additionally, we explored the presence of RNA fusion transcripts in BeWo, a trophoblast-derived choriocarcinoma cell line, using the same RT-PCR primers. This analysis revealed the stable expression of 14 out of the 15 fusion events in BeWo cells (https://github.com/hellosunking/placental-fusion/blob/main/Supplementary.materials/Suppl.Tables.xlsx and https://github.com/hellosunking/placental-fusion/blob/main/Supplementary.materials/Suppl.Figures.pdf). Moreover, to explore the detection limit, we investigated two RNA fusion transcripts (DEPDC1B-PDE4D and CHSY1-AC090907) using a serial of diluted RNA from BeWo cells and found that both fusion events are readily detectable with as low as 50 ng RNA input (equivalent to roughly 2,000 cells; https://github.com/hellosunking/placental-fusion/blob/main/Supplementary.materials/Suppl.Figures.pdf).

DIFFERENTIALLY EXPRESSED RNA FUSION TRANSCRIPTS IN GESTATIONAL DISEASES

Quantifying RNA fusion transcript expression from RNA-seq data is chal-

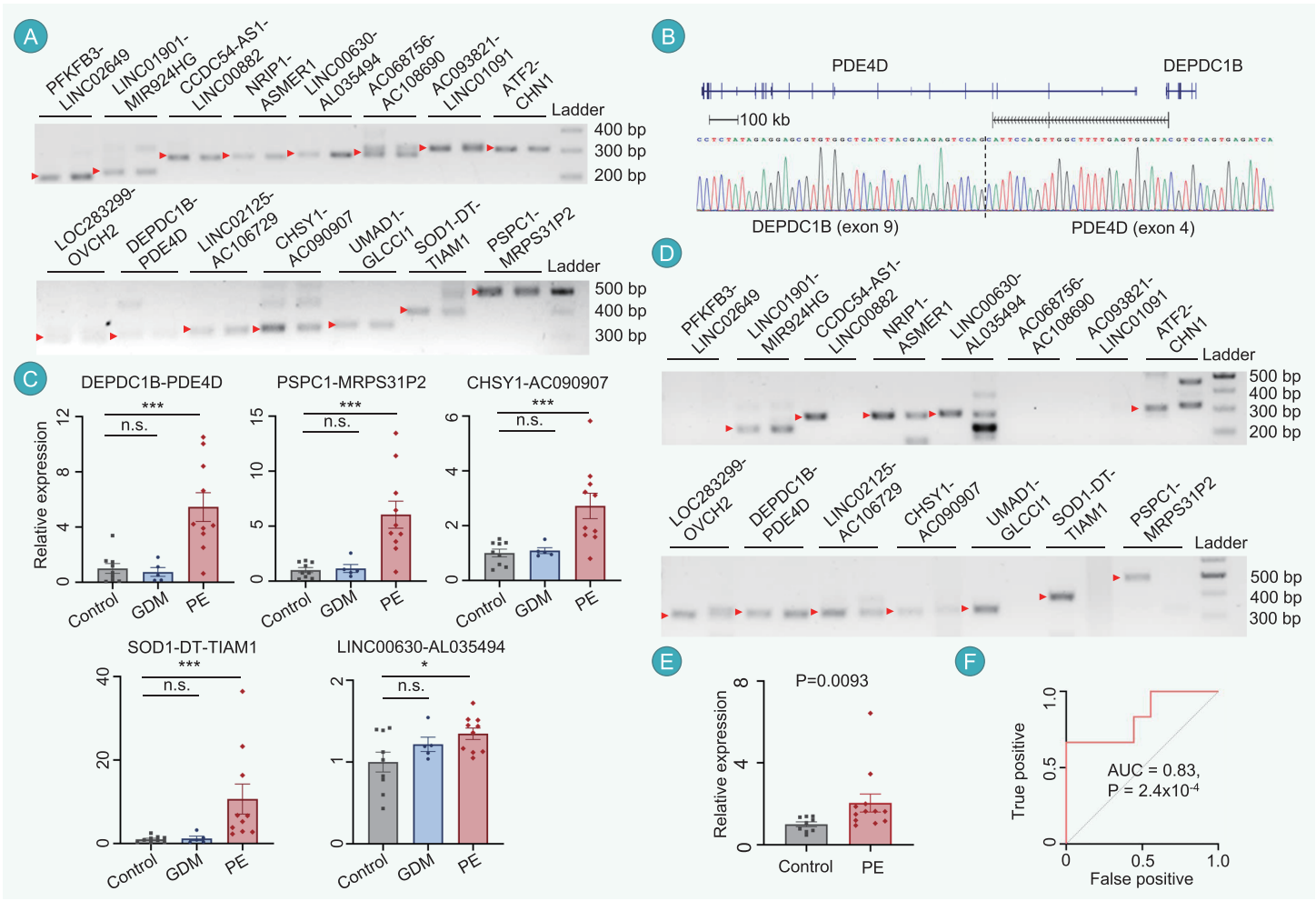

Figure 1. Landscape of RNA fusions in placentae and gestational diseases (A) RT-PCR assay results for 15 RNA fusion transcripts identified in this study in 2 placental tissues from 1 pre-eclampsia patient and 1 control. Red arrows point to the bands with expected sizes. (B) Structure of DEPDC1B-PDE4D in relative to its paternal genes, and Sanger sequencing result around the fusion junctions (indicated by the dashed line). (C) Differentially expressed RNA fusion transcripts in placental tissues with gestational diseases determined by RT-qPCR. (D) RT-PCR validations of RNA fusion transcripts in cfRNA from maternal plasma samples. Red arrows point to the bands with expected sizes. (E) Expression quantifications of LINC00630-AL035494 in cfRNA of control pregnancies and pre-eclampsia patients. Note that reportable results were obtained from 21 out of 41 (51.2%) samples. (F) ROC curve in differentiating pre-eclampsia patients from controls using LINC00630-AL035494 expression. GDM: gestational diabetes mellitus, or hyperglycemia; PE: pre-eclampsia. In (C,E), data are plotted as mean ± standard error of mean; n.s.: p≥0.05; *: p<0.05; ***: p<0.001, calculated using Mann-Whitney U tests. In (F), p-value for AUC was calculated using Z-test.

lenging due to their low expression levels. To address this, we redesigned primers and conducted quantitative real-time PCR (RT-qPCR) experiments on placental tissues with sufficient samples remaining (10 from pre-eclampsia patients, 5 from hyperglycemia patients, and 9 controls, for a total of 24 samples). We found that 5 RNA fusion transcripts, DEPDC1B-PDE4D, PSPC1-MRPS31P2, CHSY1-AC090907, SOD1-DT-TIAM1, and LINC00630-AL035494, were significantly upregulated in pre-eclampsia, whereas no dysregulated RNA fusion transcripts were identified in hyperglycemia (Figure 1C).

Given that DEPDC1B is known to play key roles in placental tissues and associated with pre-eclampsia,[7] we further investigated the expression of DEPDC1B-PDE4D in pre-eclampsia. RT-qPCR experiments showed that DEPDC1B, while not PDE4D, was significantly over-expressed in pre-eclampsia. Interestingly, the ratio of DEPDC1B-PDE4D expression to DEPDC1B (i.e., fraction of DEPDC1B RNA getting fused) did not show significant differences in hyperglycemia nor pre-eclampsia; however, the ratio of DEPDC1B-PDE4D expression to PDE4D was significantly elevated in pre-eclampsia, which was validated in two sets of primers targeting different exons of PDE4D gene (https://github.com/hellosunking/placental-fusion/blob/main/Supplementary.materials/Suppl.Figures.pdf). The results suggested that over-expression of DEPDC1B-PDE4D might lead to an elevated ratio of PDE4D transcripts getting fused, resulting in a decrease of normal PDE4D gene in pre-eclampsia.

RNA FUSION TRANSCRIPTS AS POTENTIAL NONINVASIVE DIAGNOSTIC BIOMARKERS

Given that placental tissues can release cfRNA into maternal circulation,[8] we investigated whether the RNA fusion transcripts were detectable in maternal plasma and their potential as noninvasive diagnostic biomarkers for gestational diseases. We collected 41 pre-delivery plasma samples (22 controls, 3 pre-eclampsia patients without hyperglycemia, and 16 patients with hyperglycemia), extracted and reverse-transcribed cfRNA from these samples, and then performed RT-PCR and RT-qPCR for all RNA fusion transcripts using the primer sets utilized for placental tissues. The RT-PCR results showed that 12 out of 15 RNA fusion transcripts were readily detectable in maternal plasma (Figure 1D). In the RT-qPCR experiments, most RNA fusion transcripts (11 out of 12) did not show significant differences between plasma samples from pre-eclampsia patients and controls. However, for LINC00630-AL035494, which yielded 21 reportable results from 12 pre-eclampsia patients and 9 controls after quality control (e.g., filtering out non-specific amplifications), we observed significantly elevated expression in pre-eclampsia patients compared to controls (P=0.0093, Mann-Whitney U test; Figure 1E). Notably, this gene also showed significant upregulation in placental samples from pre-eclampsia patients (Figure 1C). Further receiver operating characteristic (ROC) analysis revealed that the expression of LINC00630-AL035494 could differentiate pre-eclampsia patients from controls, achieving an area under the ROC curve (AUC) of 0.83 (95% confi-

dence interval: 0.66-1.00, P=2.4x10^{-4}, Z-test; Figure 1F). At 100% specificity, 7 out of 12 pre-eclampsia samples were correctly identified, yielding a sensitivity of 63.6% (95% confidence interval: 30.8-89.1%). These results demonstrated the translational potential of this placental RNA fusion transcript as a noninvasive diagnostic and monitoring tool for pre-eclampsia.

DISCUSSIONS AND PERSPECTIVES

In this study, we profiled the landscape of RNA fusions in human placentae, a non-cancerous tissue. Our catalogue contained 15 RNA fusion transcripts (Figure 1A), all of which were supported by external placental RNA-seq datasets. Importantly, the existence of these RNA fusion transcripts was experimentally validated using RT-PCR and Sanger sequencing (Figures 1A-B). Of note, all RNA fusion transcripts identified in this study originated from nearby genes. As most placental tissues are unlikely to harbor chromosomal rearrangements, the formation of fusion transcripts in placentae may differ from those in tumors. Previous studies have suggested that fusion events in normal cells could be deleterious,[9] suggesting important roles of gene or RNA fusions in human diseases. However, to date, functions of most gene or RNA fusions have not been comprehensively investigated, neither in tumors nor placental tissues. Interestingly, 11 out of 15 fusions (73.3%) identified in this study were generated from at least one non-coding RNA (ncRNA). In fact, ncRNA-involved fusions are abundant and diverse, yet the vast majority of these fusions remain poorly characterized. As most of the RNA fusion transcripts identified in this study are expressed in BeWo cells, it would be feasible to perform functional investigations with this commonly used cell line in the following studies.

Besides validating the existence, we further designed RT-qPCR assays to measure the expression of the RNA fusion transcripts quantitatively. As a result, we identified 5 RNA fusion transcripts with significantly elevated expression levels in pre-eclampsia (Figure 1C). Quantitative expression measurements of the fusion transcripts and their parental genes might provide clues to infer their potential implications. For DEPDC1B-PDE4D, one of its parental genes, DEPDC1B, which is up-regulated in pre-eclampsia, is known to play a significant role in placental development and is associated with pre-eclampsia; the other parental gene, PDE4D, a cAMP-specific phosphodiesterase, has been linked to inflammation-induced preterm delivery. Here we showed that over-expression of DEPDC1B-PDE4D would lead to a decrease of normal PDE4D gene in pre-eclampsia. In cell biology and development, precise spatiotemporal control of gene expression is essential; the results suggested that the formation of RNA fusion transcripts might adversely affect the expression of the normal genes (and interfere with regulatory networks) in placental tissues. Moreover, the formation of fusion transcripts can also affect the activity of the splicing machinery and alter RNA stability by modifying regulatory elements such as 3′ UTRs, resulting in novel splice sites or disrupting existing ones and inappropriate accumulation or degradation of various genes. Together, the abnormal expression, splicing, or stability of fusion transcripts during critical windows of development may contribute to developmental defects. Since most of the fusion transcripts are expressed in BeWo cells, functional assays using this cell line would be feasible and of value to investigate the functions of these RNA fusion transcripts in placentae and pre-eclampsia.

Clinically, early diagnosis and intervention are essential for managing the large population of pre-eclampsia patients. Recent studies showed that the AUCs for sFLT1/PlGF ratio, one of the most promising biomarkers, in differentiating/predicting pre-eclampsia ranged between 0.73 to 0.84 in China,[10] which performances are apparently unsatisfactory. In this study, we demonstrated that LINC00630-AL035494 was significantly elevated in the plasma of pre-eclampsia patients, consistent with its increased expression in placental tissues from these patients. More importantly, LINC00630-AL035494 could serve as a promising noninvasive diagnostic biomarker as its expression in maternal plasma could effectively differentiated pre-eclampsia patients from normotensive controls, suggesting comparable performance (e.g., AUC values and sensitivity) to previous cfRNA and cfDNA methylation assays. One advantage of this biomarker is that quantitative analysis could be achieved using a simple, cost-effective, and straightforward RT-qPCR assay. On the other hand, considering the limited sample size and the absence of early-stage samples, the results should be considered as a proof-of-principle. In future studies, it would be of clinical significance to validate the performance of our LINC00630-AL035494 fusion as well as comparisons/combinations of the current biomarkers (e.g., sFLT1/PlGF ratio) using large-scale, multi-center cohorts with time-serial samples towards building high-performance models for early noninvasive diagnosis of pre-eclampsia.

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AUTHOR CONTRIBUTIONS

Study conception and design: K.S.; Study supervision: S.Z., K.S.; Development of methodology: M.Y., Y.P., K.S.; Patient recruitment and clinical data analysis: S.Z., H.Z., H.L., Q.L.; Acquisition of data: all authors; Analysis and interpretation of data: all authors; Writing the manuscript: S.Z., M.Y., K.S. All authors contributed to the manuscript and approved the final version.

DECLARATION OF INTERESTS

M.Y. and K.S. had filed patent applications based on this work; the remaining authors declare no conflict of interests.

ETHICAL STATEMENT AND PATIENT CONSENT

This study had been approved by the Ethics Committee of Women's Hospital of Zhejiang University (IRB-20230011-R), Ethics Committee of Shenzhen Bay Laboratory (YL2023-009), and Ethics Committee of Shenzhen Maternity & Child Healthcare Hospital (SFYLS[2024]106). Participants were recruited from Women's Hospital of Zhejiang University and Shenzhen Maternity & Child Healthcare Hospital with written informed consents. All participants were fully informed about the study's purpose and voluntarily agreed to participate.

DATA AND CODE AVAILABILITY

Raw sequencing data generated in this study had been deposited in the Genome Sequence Archive in National Genomics Data Center (GSA-Human) under accession number HRA008081. Public bulk RNA-seq datasets were downloaded from Gene Expression Omnibus (GEO) with accession numbers GSE77085, GSE234729, and GSE186257. Computational scripts used in this work, methods, supplementary figures and tables had been deposited at github (https://github.com/hellosunking/placental-fusion).

LEAD CONTACT WEBSITE

Kun Sun: https://www.szbl.ac.cn/research/group/Kun-Sun.htm