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Maternal plasma cell-free DNA nucleosome footprints can reveal changes in gene expression profiles during pregnancy and pre-eclampsia

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Abstract

Background Differential gene expression analysis is important to understand pregnancy processes and disease development. However, no non-invasive and comprehensive methods exist to identify differentially expressed genes (DEGs) in the fetus and placenta during pregnancy or pregnancy complications. Nucleosome footprints in maternal peripheral blood plasma cell-free DNA (cfDNA) reflect the gene expression profile of the cell of origin, mainly immune cells in the maternal blood and placenta. This study aimed to validate the feasibility of detecting changes in gene expression profiles as differentially depth gene (DDGs) based on plasma nucleosome footprints as a potential biomarker for pregnancy and pre-eclampsia.

Methods Deep sequencing was performed on separated plasma cfDNA collected from 34 women, including eight non-pregnant women, 14 healthy pregnant women, and 12 pre-eclamptic pregnant women. The number of reads in the promoter region of each gene was extracted and normalized. Normalized depths of genes were compared between healthy pregnant vs. non-pregnant women, all pregnant women vs. non-pregnant women, and healthy pregnant women vs. pre-eclamptic pregnant women using the Wilcoxon rank-sum test to identify statistically significant DDGs. The roles of these genes were identified by functional enrichment analysis using gene ontology.

Results Plasma cfDNA revealed different nucleosome footprints in preeclampsia pregnant, healthy pregnant, and non-pregnant women. Gene annotation revealed that the functions of 629 DDGs in pregnant and non-pregnant women mainly involved immune regulation, regardless of pre-eclampsia. 1978 DDGs between healthy pregnant and pre-eclamptic pregnant women displayed differences in immune regulation, cell cycle regulation, and sensory perception. These results are consistent with prior microarray and RNA-sequencing data.

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Conclusions The depth of the cfDNA nucleosome footprint in maternal plasma can be used to reflect changes in the gene expression profile during pregnancy and pre-eclampsia. The plasma cfDNA nucleosome footprint is a potential non-invasive biomarker for pregnancy and placental-origin pregnancy complications.

Keywords Deep sequencing, Pre-eclampsia, Differential analysis, CfDNA, Nucleosome footprint

Background

Differential expression and functional enrichment analyses of differentially expressed gene sets to infer biological functions have been widely applied in various biological fields [1], including pregnancy and pregnancy complication research. Classical differential expression analysis involves gene expression profiling obtained by microarray or RNA-sequencing (RNA-seq) [2], followed by a comparison between groups. Original tissue materials are necessary for this comparison. However, this approach is invasive or inadequate for pregnant women. A non-invasive approach that can comprehensively reflect maternal and fetal gene expression changes is needed.

Plasma cell-free DNA (cfDNA) primarily originates from rapidly proliferating and metabolically active cells within the body. In contrast, in pregnant women, cfDNA mainly originates from immune cells in maternal blood and placenta. Analysis of the nucleosome footprint in cfDNA is becoming a common genomic research tool in studying tumors and pregnancy diseases. Gene promoter regions with varying expression levels exhibit different degrees of nucleosome encapsulation. Therefore, gene promoter regions undergo varying degrees of hydrolysis as cells enter the death process. Consequently, the number of DNA fragments released into the circulatory system may be biased. Ulz et al. proposed that the nucleosome footprint of plasma cfDNA reflects gene expression in blood cells [3]. Briefly, highly expressed genes exhibit low transcriptional start site (TSS) depth, while low-expression genes had high TSS depth in plasma cfDNA. Recently, it has been reported that the nucleosome footprint could be used to predict the occurrence of pregnancy complications and the sensitivity of neoadjuvant diagnosis [4] and breast cancer treatment [5]. Another study demonstrated the difference in nucleosome footprint of cfDNA between trisomy pregnancy and normal pregnancy [6], which was associated with phenotype. Researchers have increasingly focused on the traceability of cfDNA, wherein the nucleosome footprint plays a pivotal role. Thus, differences in the cfDNA nucleosome footprint may mirror differences in gene expression in pregnant women.

During pregnancy, the presence of the placenta and fetus is an exogenous "foreign" body for the mother [7]. Maintaining the presence of the placenta means that the mother's immune system is finely regulated to protect the mother and fetus from environmental influences, while tolerating the presence of the placenta and fetus [8]. The presence of a fetus alters the mother's response to the environment, providing a developing, active immune system. Pre-eclampsia, a leading pregnancy complication, affects an estimated 2–8% of pregnancies worldwide [9]. Placental-derived diseases involve multiple genetic, angiogenic, structural, and metabolic pathways, cause a wide range of maternal endothelial dysfunctions, and incur a large burden of maternal and fetal morbidity and mortality [10, 11]. However, due to ethical limitations and the risk of puncture sampling, previous studies have been based mostly on peripheral blood mononuclear cells (PBMC) from the mother and the placenta from abortion or cesarean section. Since gene expression changes in PBMC and the placenta may lead to an increase in cfDNA levels, to a certain extent, the cfDNA nucleosome footprint in maternal plasma may reflect the gene expression profiles of maternal immune cells in placental.

This study aimed to validate the feasibility of detecting changes in gene expression profiles based on plasma nucleosome footprints as a potential biomarker for pregnancy and pre-eclampsia. In this study, we sequenced the whole genome of peripheral plasma cfDNA from 34 women, including non-pregnant women, healthy pregnant women, and pre-eclamptic pregnant women. We captured 100 million reads per sample, which was a higher depth than previous study [6]. The differentially depth genes (DDGs) during pregnancy were determined between normal and pre-eclamptic pregnancies. DDGs were further analyzed to identify their biological roles through gene ontology (GO) functional annotation, and the results were compared with those obtained from microarray and RNA-seq.

Methods

Collection of samples and study performance

The 34 enrolled women included 8 non-pregnant, 14 healthy pregnant, and 12 pre-eclamptic pregnant women (Supplementary Table S1). Pregnant women were recruited from Nanfang Hospital of Southern Medical University in Guangzhou, China, between March 2021 and March 2022. Pre-eclampsia was diagnosed according to the criteria of the International Society for the Study of Hypertension in Pregnancy. Non-pregnant women were volunteers from Guangzhou Darui Biotechnology Company. All individuals

provided written informed consent for this study; the ethical approval of this study was obtained from the Institutional Review Board of Southern Medical University (No: NFEC 2021–261).

Whole genome sequencing for plasma cfDNA

A total of 6 mL of peripheral blood was collected in EDTA (Ethylene Diamine Tetraacetic Acid) anticoagulation tubes from each woman in the three groups. Plasma was separated using a two-step centrifugation procedure. In detail, whole blood was centrifuged (Heraeus Multifuge X1R Centrifuge; Thermo Fisher Scientific, Waltham, MA, USA) at 1,600 g for 10 min at 4 °C. The supernatant was collected and then centrifuged at 16,000 g for 10 min at 4 °C. The plasma was collected in new low-adsorption centrifuge tubes and stored at − 20 °C before use. Plasma cfDNA was captured using magnetic beads and released into microcentrifuge tubes after washing using the QIAamp DNA Mini kit (QIAGEN, Hilden, Germany).

Purified DNA fragments were used to construct whole genome sequencing (WGS) libraries through end preparation, adapter ligation, and amplification using the Vazyme VAHTS® Universal DNA Library Prep Kit for Illumina (ND607) (Vazyme, Jiangsu, China). A Qubit 3 Fluorometer (Thermo Fisher Scientific) was used to quantitatively evaluate DNA and libraries, and an Qsep100 Bio-Fragment Analyzer (BiOptic, New Taipei City, Taiwan) was used for fragment size control. Quantified libraries were sequenced on an Illumina NovaSeq 6000 system with a PE150 read length, and 100 million reads were obtained per library. We omitted the fragmentation and length selection steps because of the degraded nature of plasma DNA.

Pipeline for WGS data analysis

Low-quality bases and sequencing adapter queries were removed to obtain high-quality queries using fastp [12, 13] (version 0.12.4). All clean reads were mapped to the human reference genome hg19 using the BWA-MEM algorithm [14, 15] (version 0.7.17-r1188). PCR (Polymerase Chain Reaction) duplications were removed using Picard (version 2.18.29-SNAPSHOT), and GCbias was computed and corrected using deeptools [16] (version 3.5.1). The read counts of regions ranging from − 1000 bp to + 1000 bp around the TSS were calculated using bedtools [17] coverage (version 2.17.0). All gene information and TSS positions were obtained from RefSeq [18]. Read counts were normalized using the reads per kilobase per million mapped reads (RPKM) method.

$$RPKM = \frac{Number\ of\ Reads}{Numberof\ Total\ Mapped\ Reads\ (millions)\ *\ Length\ of\ Region\ (2kb)}$$

Volcano plots were generated using the ggplot2 [19] package, and heat maps were plotted using pheatmap. All data preparation and screening were performed using R version 4.1.1.

Differential analysis of nucleosomes between the different groups

The fold change in the depth of the TSS region between the two groups was calculated, and the Wilcoxon rank-sum test (two-sided) was used to test for significance. The false discovery rate (FDR) method described by Benjamini and Hochberg was used for multiple testing corrections. Finally, TSSs with p-values < 0.05, |log2(fold change) | ≥ log2(1.5), and FDR < 0.2 were determined as differential TSSs.

Gene function analysis

To explore the function of the genes corresponding to the differential TSSs, GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed using the R package clusterProfiler [20] (Version 4.2.0). GO terms and KEGG pathways were obtained from the QuickGo [21] and KEGG [22] websites. All gene aliases and IDs in different databases were downloaded from GeneCards [23].

Results

Plasma cfDNA of pregnant women in different pregnancy statuses has different nucleosome footprints

The fragmentation profile showed that the peripheral blood fragments of women in different pregnancy states had roughly similar trends and characteristics (Fig. 1A). To verify the hypothesis that highly expressed genes have low TSS depth and low-expression genes have high TSS depth in cfDNA, the promoter regions (− 1 K to + 1 K) of the top 500 genes with the highest expression and the bottom 500 genes with the lowest expression in maternal whole blood (Fig. 1B) and the depth of each base around the TSS region (Fig. 1C) were determined. The results confirmed the previously proposed hypothesis that highly expressed genes have fewer promoter reads and lower TSS depth. We explored whether this difference in cfDNA could be used to distinguish the three groups of samples. Therefore, we used the all TSS regions for principal component analysis (PCA) after dimension reduction. The three groups of samples occupied different positions on the PCA plot, suggesting that this difference could be used to distinguish women with different pregnancy states.

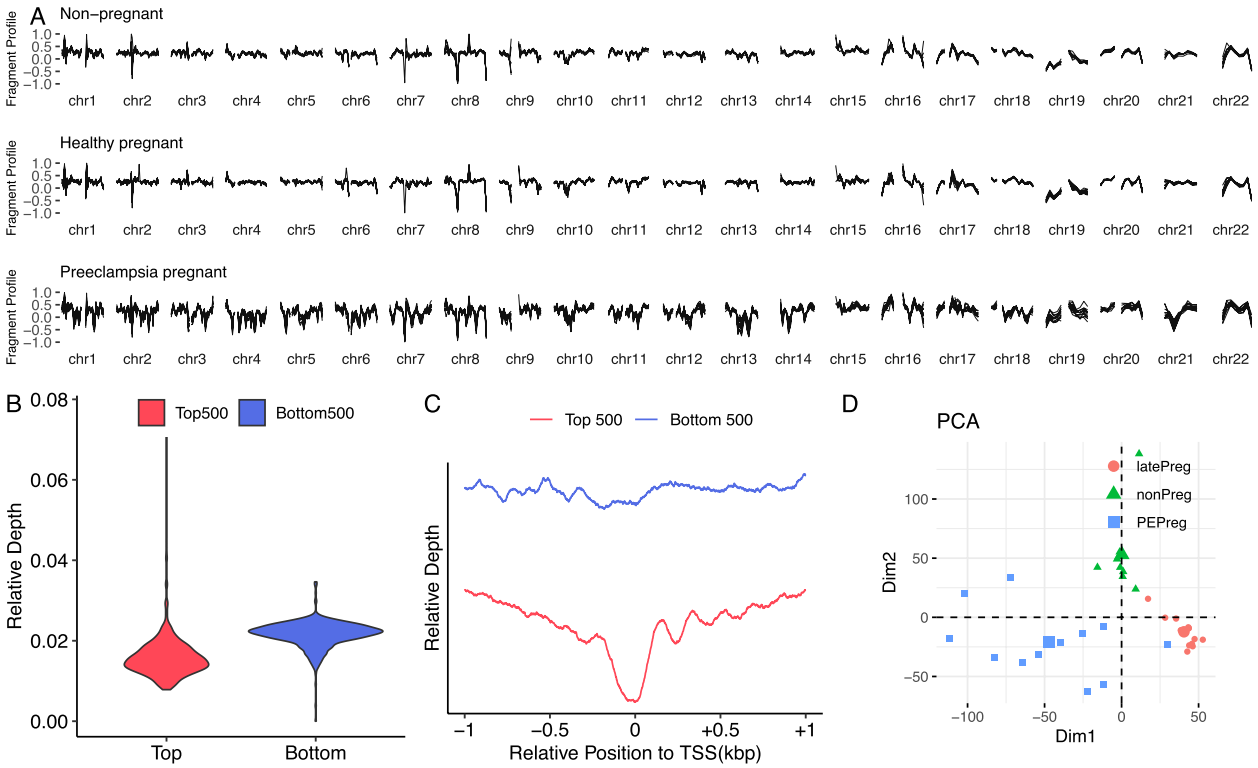

Fig. 1 Plasma cfDNA nucleosome footprints reflect gene expression in the placenta. **A** Fragment profiles of cfDNA in the three groups on 22 chromosomes. The y-axis is the standardized z-score unit for depth. **B** Violin plot of the distribution of depth from + 1000 to – 1000 bp around transcriptional start sites of non-constitutive (Bottom500, blue violin, means 500 genes with lowest expression level) and housekeeping (Top500, red violin, means 500 genes with highest expression level) genes. The non-constitutive and housekeeping genes were obtained [4] from the additional materials of the previous study (GSE85307 [24]). **C** Base-depth patterns for non-constitutive (blue line) and housekeeping (red line) genes. **D** Principal component analysis (PCA) results derived from TSS coverage profiles imputed by pregnant and non-pregnant women. "LatePreg" means healthy pregnancy, "nonPreg" means women without pregnancy, and "PEPreg" means pre-eclampsia pregnancy

Immune regulation is the main function of different genes between healthy pregnant and non-pregnant women

Differences in the promoter regions between healthy pregnant and non-pregnant women were compared. Among the 26,654 unique promoter regions, 908 showed significant differences ($p < 0.05$, log2FC > log2 (1.2)). The 908 regions included 569 with lower depth in healthy pregnant and 339 with higher depth (Fig. 2A, B). To explore the functions of these differential genes, a gene function enrichment analysis was performed. GO functional enrichment analysis showed that the functions of these DDGs were mainly involved in immune regulation (GO:0042110, GO:0001906, GO:0032736, GO:0001909, GO:0050727, GO:0045088, GO:0006909, GO:0002228) and nervous system development (GO:0060998, GO:0016358) (Fig. 2C). The findings are consistent with our understanding that pregnancy poses an immunological challenge, because a genetically distinct fetus must be supported within the pregnant female for the required gestational period. Nervous system development-related GO terms suggest that nervous system development starting at 3 weeks of pregnancy is still ongoing.

We compared all pregnant women, including 14 healthy pregnant and 12 pre-eclamptic, with non-pregnant women and identified 629 DDGs. These genes included 300 with higher depth in PE pregnant and 329 with lower depth. Among the 629 DDGs (Fig. 3A), 342 were differentially expressed between healthy pregnant and non-pregnant women and showed the same trend of changes in the two comparisons (Fig. 3B, C). Functional enrichment analysis of GO suggested that immune regulation occurred in all pregnant women, regardless of pre-eclampsia (Fig. 3D).

Cell cycle regulation and sensory related are the main functions of different genes between healthy pregnant and pre-eclamptic women

Finally, we directly compared the nucleosome footprint of cfDNA in the peripheral blood of pregnant women with healthy pregnancies and those with pre-eclampsia. Among 1978 promoters,, 742 with lower depth and

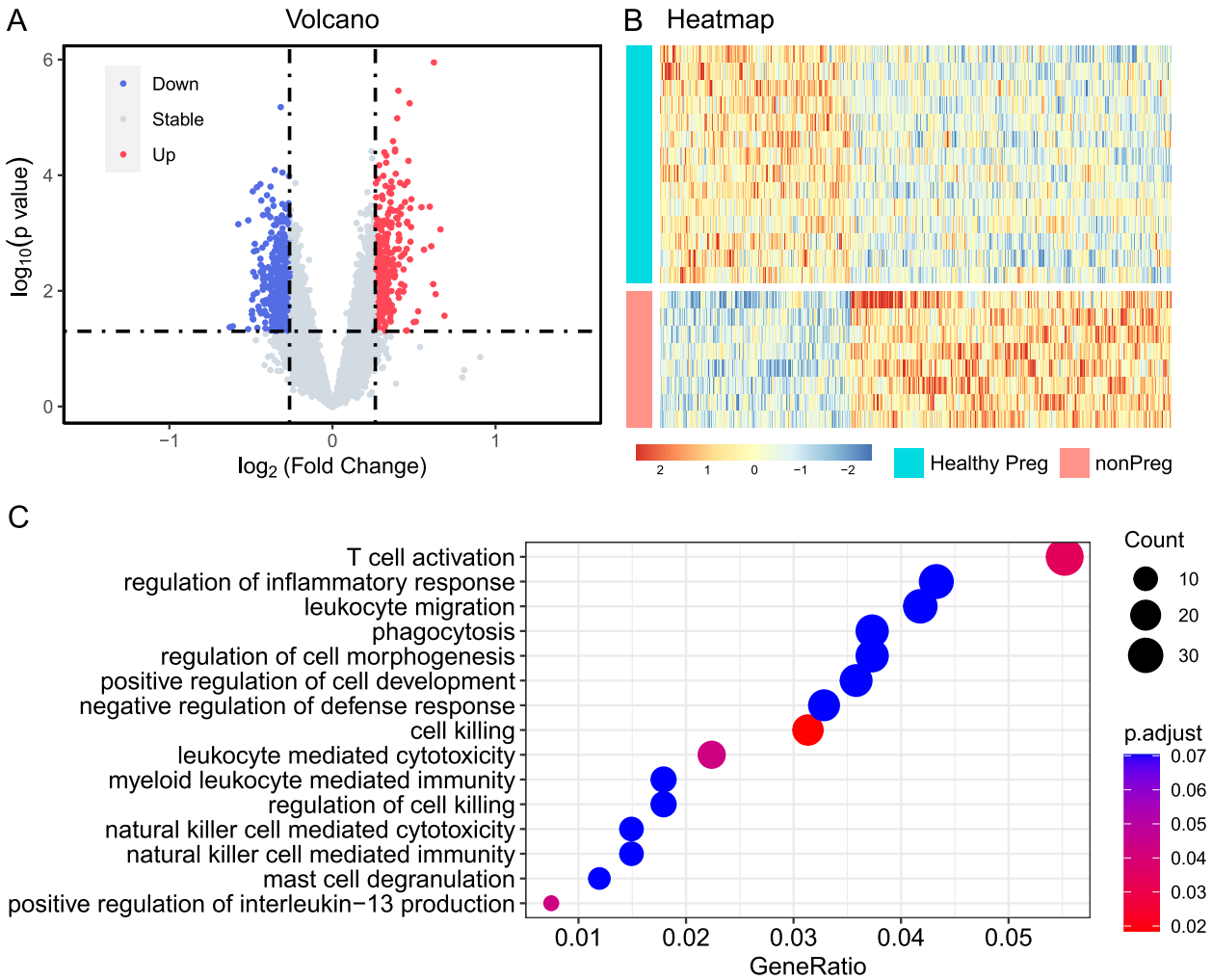

Fig. 2 Different analysis between healthy pregnant and non-pregnant women. **A** Volcano plot of coverages in the region around the transcript start site between healthy pregnant and non-pregnant women. The red dots indicate that counts of reads in the region around TSS in healthy pregnant women were significantly higher than those in non-pregnant women, and the blue dots indicate that they were significantly lower. **B** Heatmap for gene coverage of healthy pregnant women and non-pregnant women. **C** Bubble chart of GO enrichment analysis of different depth genes between healthy pregnant and non-pregnant women

1236 with higher depth were observed, showing significant differences (Fig. 4A, B). They were mainly enriched in processes, such as immune regulation (GO:0032611, GO:0032651, GO:0032612, GO:0031349), cell cycle regulation (GO:0006913, GO:0051169, GO:0033314, GO:0044818, GO:1,902,750), and sensory related functions (GO:0007608, GO:0050907, GO:0050911) (Fig. 4C). In contrast to the immunosuppression that occurs in normal pregnancies, pre-eclamptic pregnancy is characterized by excessive immune activation [25], which is consistent with our results that the function of genes with lower depth (highly expressed genes) is closely related to immune response (Figure S1F),. Reports of abnormal expression of cell cycle-related genes in pre-eclamptic pregnancy are available [26].

Discussion

Currently, there is no direct evidence to proving the relationship between nucleosome footprint and gene expression. A series of studies have shown that nucleosome imprinting can reflect the level of gene expression to a certain extent, especially in newly active tissues such as tumors and placentas. Previous studies have used the cfDNA nucleosome footprint to predict the occurrence of pregnancy syndromes with a prediction accuracy of 80% [4]. The cfDNA nucleosome footprint

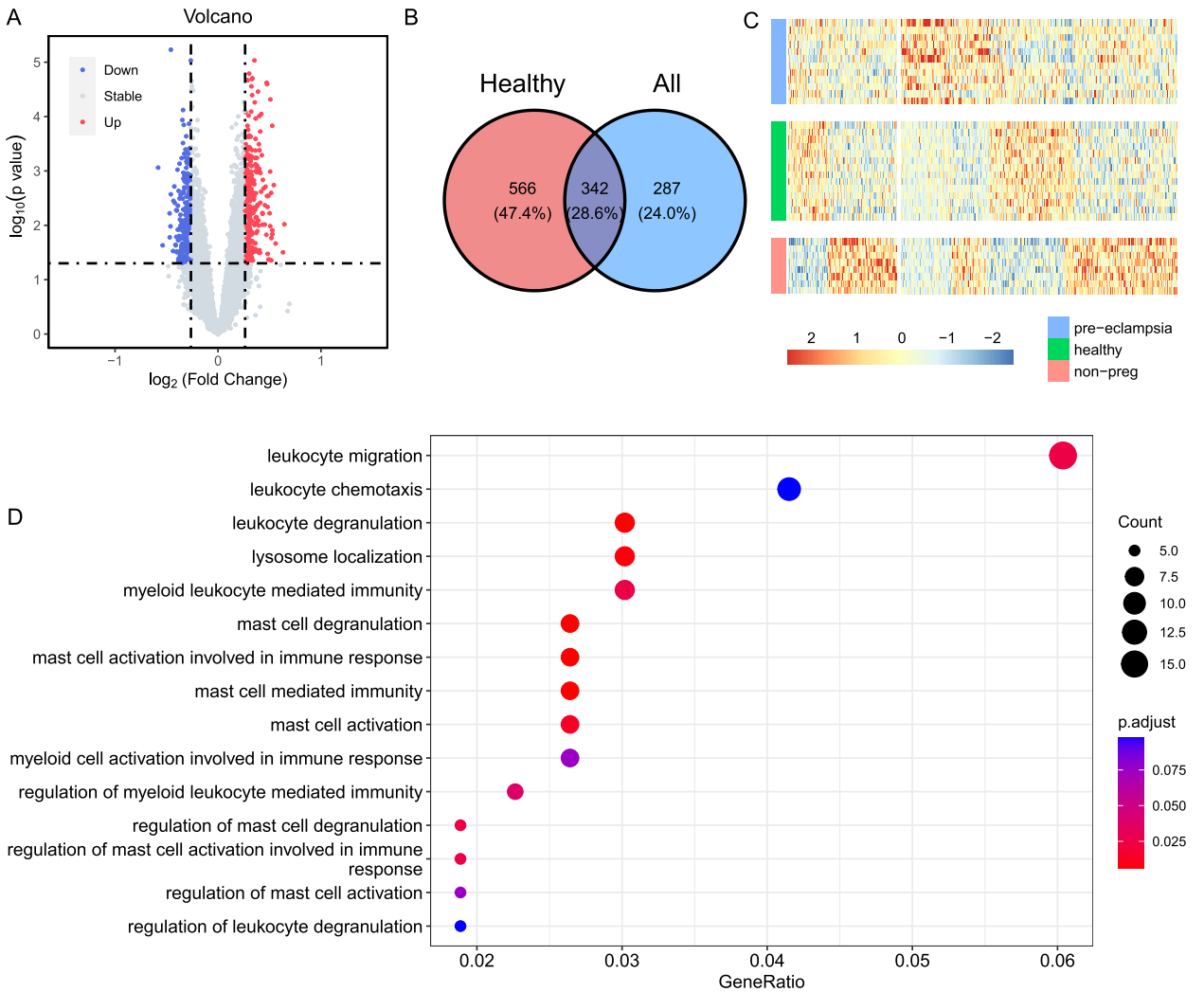

Fig. 3 Differential analysis between all pregnant and non-pregnant women. **A** Volcano plot of coverages in the region around the transcript start site between pregnant and non-pregnant women. **B** Venn plot for different genes in healthy pregnant vs. non-pregnant women (Healthy) and all pregnant vs. non-pregnant women (All). **C** Heatmap for gene coverages of all pregnant women and non-pregnant women. The left area indicates 342 different genes in B, and the right area indicates the other 853 genes. **D** Bubble chart of GO enrichment analysis of different depth genes in all pregnant vs. non-pregnant women

has been used to predict the sensitivity of patients with colorectal cancer to neoadjuvant chemotherapy and radiotherapy [27]. In addition, a few researchers have used the cfDNA nucleosome footprint to analyze aneuploid pregnancies. However, in these studies, the sequencing depth of cfDNA was often less than 20 million reads, making it difficult to determine whether the loss of reads in the region was due to the capture process of sequencing or hydrolysis during cell death. In this study, the sequencing depth of each sample was up to 100 million, which reduced the contingency of the analysis results and more accurately reflected the distribution of cfDNA in the body.

For a long time, researchers have relied on transcriptome sequencing to study organ development and disease occurrence and development. Transcriptome sequencing requires primary tissue materials. Therefore, tissue research on certain special parts, such as the placenta and embryo, is hindered by the lack of materials and the feasibility of specimen collection techniques. Currently, alternative methods include in vitro culture techniques [28, 29] and animal models [30]. However, the cultivation of zygotes that have been fertilized for more than 14 days is prohibited in many countries. Therefore, in vitro culture technology is limited to early pregnancy. Mice are commonly used

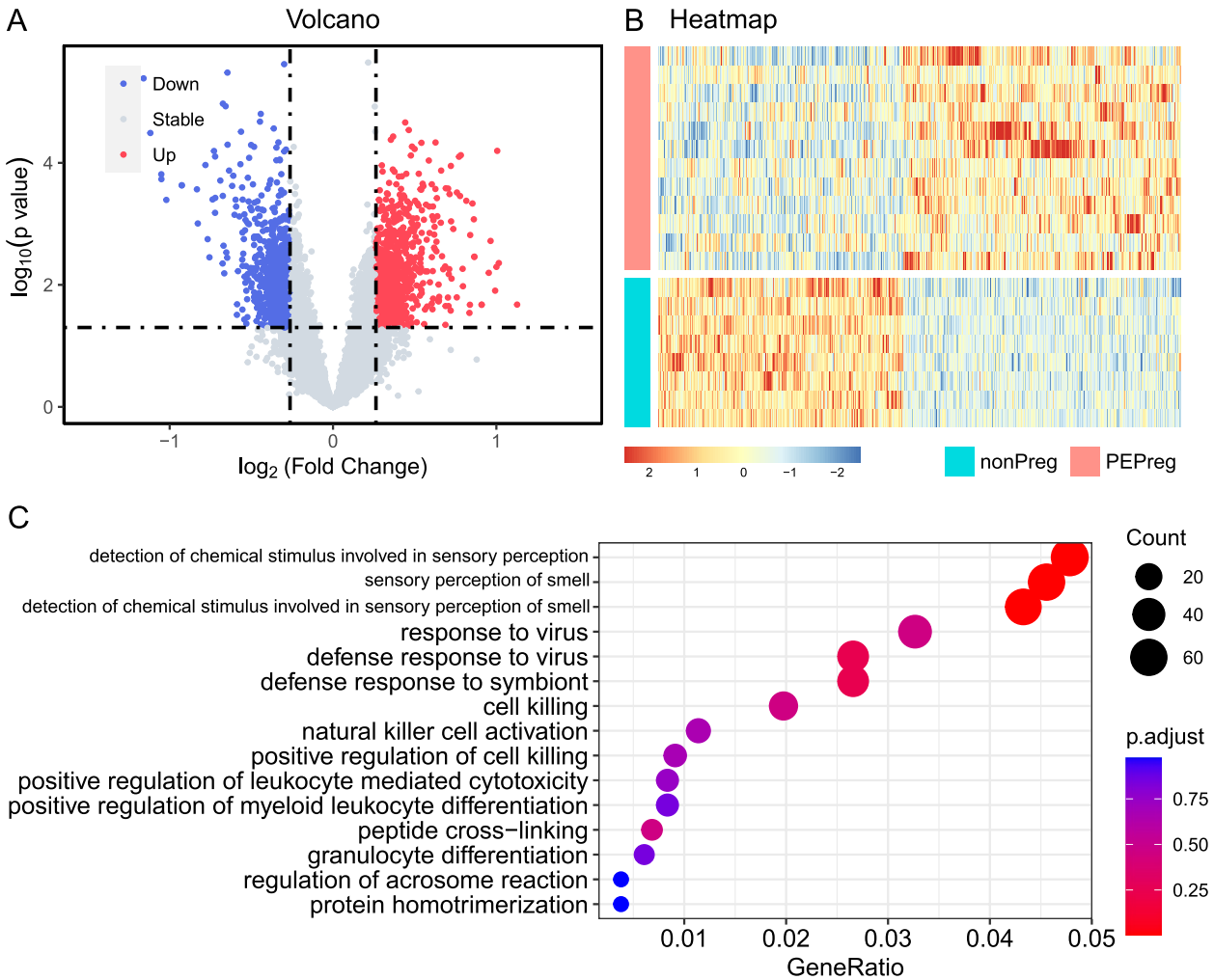

Fig. 4 Different analysis between pre-eclamptic pregnant and healthy pregnant women. **A** Volcano plot of coverage in the region around the TSS between both groups of women. The red dots indicate that counts of reads in the region around TSS in healthy pregnant women were significantly higher than those in pre-eclamptic pregnant women. The blue dots indicate significantly lower counts. **B** Heatmap for gene coverage of healthy pregnant and pre-eclamptic pregnant women. **C** Bubble chart of GO enrichment analysis of different depth genes between healthy pregnant and pre-eclamptic pregnant women

to study the maternal–fetal interface in the immune environment during pregnancy. However, there are differences in placental structure, pregnancy, and human maternal tolerance mechanisms between mice and humans. In particular, the timeframe and establishment of the maternal–fetal interface vary among species. The pregnancy time in humans exceeds 40 weeks, whereas that in mice is approximately three weeks. Therefore, these two methods cannot effectively and repeatedly obtain gene expression profiles or differential gene profiles of the developing placenta. The method based on maternal plasma cfDNA nucleosome imprinting described in this study can be used to obtain the gene expression differences between pre-eclampsia and normal pregnancy placentas to a certain extent and to correlate with the phenotype in other studies.

In this study, we comprehensively studied the differences in the cfDNA nucleosome footprint between 14 healthy pregnant, 12 pre-eclamptic pregnant, and 8 non-pregnant women. We identified 908 differential depth genes between healthy pregnant and non-pregnant women, 629 differential depth genes between all pregnant and non-pregnant women, and 1708 differential depth genes between healthy pregnant and pre-eclamptic pregnant women. Functional enrichment analysis showed that the differential genes were mainly involved in immune regulation (GO:0042110, GO:0001906, GO:0032736, GO:0001909, GO:0050727, GO:0045088, GO:0006909,

and GO:0002228). This finding is consistent with our current understanding of pregnancy. During pregnancy, the mother regulates the strength of the corresponding immune system to maintain the positive development of the fetus. Compared to healthy pregnant women, there are differences in the regulation of the immune system in pre-eclamptic pregnant women. The current mainstream view is that the systemic inflammatory response is characteristic of all pregnancies and reaches extreme intensity in pregnancies complicated by p inflammation [25]. This is expressed by abnormally upregulated immune reactions that activate the innate immune system and other pro-inflammatory factors [31]. In particular, there were functions related to sensory perception (GO:0050907, GO:0007608, and GO:0050911). This may be a branch of nervous system development, and similar GO terms have been observed in previous studies [32–34].

While this study offers novel insights into cfDNA and the nucleosome footprint, several limitations should be acknowledged. First, hg38 was released in 2013, however hg19 was still used as the reference genome in this study because of its widespread compatibility with existing tools. Compared with hg19, hg38 exhibits significant enhancements in complex regions, such as those in the vicinity of centromeres, and contains more gene variation information. Although these improvements have little impact on our method, using hg38 for analysis in the future may improve the performance in some gene regions. Second, sample collection in this study was limited by the pandemic. However, as an exploratory study with lower statistical power requirements, we successfully demonstrated the potential of nucleosome footprints in reflecting gene expression. Future studies will expand the sample size to enhance statistical power and validate the generalizability of our findings.

Overall, the results of this study based on the nucleosome footprint are highly consistent with those of previous studies based on microarrays and RNA-seq. This means that the maternal plasma cell-free DNA nucleosome footprint can reveal changes in gene expression profiles during pregnancy and pre-eclampsia. This indicates that the plasma cfDNA nucleosome footprint is a potential non-invasive biomarker for pregnancy and placental-origin pregnancy complications. In the future, cfDNA has the potential to be used as the biomarker for non-invasive prediction of gene expression profiles in specific tissues such as placenta.

Abbreviations

cfDNA	Cell-free DNA
DEGs	Differentially expressed genes
DDGs	Differentially depth genes
FDR	False discovery rate
GO	Gene ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
PBMC	Peripheral blood mononuclear cells
PCA	Principal component analysis
RNA-seq	RNA-sequencing
RPKM	Reads per kilobase per million mapped reads
TSS	Transcriptional start site
WGS	Whole genome sequencing
EDTA	Ethylenediaminetetraacetic acid
PCR	Polymerase chain reaction

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12884-025-07453-y.

Supplementary Material 1.

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Authors' contributions
Xuexi Yang, Chao Sheng, and Wenbo Hao designed the research; Kun Li, Zhiwei Guo analyzed data; Kun Li, Min Zhang, Xingyu Wei, and Shijing Lu performed the experiment; and Shijing Lu, Fenxia Li, and Chao Sheng provided patient samples. Kun Li and Xuexi Yang wrote the manuscript. All authors read and approved the final manuscript.

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Data availability
The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive (Genomics, Proteomics & Bioinformatics 2021) in National Genomics Data Center (Nucleic Acids Res 2022), China National Center for Bioinformation / Beijing Institute of Genomics, Chinese Academy of Sciences (GSA-Human: PRJCA019177) that are publicly accessible at https://ngdc.cncb.ac.cn/gsa-human. And the normalized depths of nucleosome footprint were available in the supplementary material.

Declarations

Ethics approval and consent to participate
This study was performed in accordance with the Declaration of Helsinki. The ethical approval was obtained from the Institutional Review Board of Southern Medical University (No: NFEC 2021–261). Informed consent was obtained from all participants.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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