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ORIGINAL RESEARCH

The Identification of FN1 as an Early Diagnostic Marker for Recurrent Abortion by Single-Exosome Profiling

Chenlu Wang[1,*], Zhaojin Lu[1,*], Guangpeng She[2], Kaining Chen[1], Huazhong Zhou[1], Xueli Zhan[3], Hongyan Yu[1], Lei Pi[1], Liandong Zuo[4], Di Che[1]

[1]Department of Clinical Biological Resource Bank, Guangzhou Institute of Pediatrics, Guangdong Provincial Key Laboratory of Research in Structural Birth Defect Disease, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, Guangzhou, 510620, People's Republic of China; [2]Department of Laboratory Medicine, The Second Affiliated Hospital, School of Medicine, South China University of Technology, Guangzhou, Guangdong, 510180, People's Republic of China; [3]Department of Pediatric Surgery, Guangzhou Institute of Pediatrics, Guangdong Provincial Key Laboratory of Research in Structural Birth Defect Disease, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, Guangzhou, Guangdong, 510620, People's Republic of China; [4]Department of Andrology, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, Guangzhou, 510620, People's Republic of China

*These authors contributed equally to this work

Correspondence: Di Che, Department of Clinical Biological Resource Bank, Guangzhou Institute of Pediatrics, Guangdong Provincial Key Laboratory of Research in Structural Birth Defect Disease, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, No. 9 Jinsui Road, Guangzhou, Guangdong, 510620, People's Republic of China, Email chedi@gwcmc.org; Liandong Zuo, Department of Andrology, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, 9 Jinsui Road, Guangzhou, Guangdong, 510620, People's Republic of China, Email zuold@163.com

Purpose: Recurrent abortion(RA) is a prevalent adverse pregnancy event. Exosomes, secreted by various body fluids, are known to play a role in disease diagnosis and serve as biomarkers through intercellular communication. This study aims to analyze single exosomes in patients with recurrent abortion to identify new biomarkers that may significantly contribute to recurrent abortion, providing new directions for its treatment.

Patients and Methods: A total of 244 serum exosomes were collected, including 216 patients with recurrent abortion of varying outcomes and 28 normal pregnancies. We performed the proximity barcoding assay (PBA) to analyze single exosome surface proteins, which allowed us to identify individual exosomes related to the development of RA as well as the major subpopulations of exosomes. After PBA treatment, samples were analyzed for single exosomes, and exosomes from each group were compared using volcano plots, dot plots, and ROC curves.

Results: By intersecting all significantly differentially expressed genes obtained from comparisons between the normal pregnancy control group and the recurrent abortion group, including the RA before abortion, RA after abortion, and RA non-pregnancy groups, we identified seven shared differential genes: FN1, APIPOQ, CDH13, DSG1, CLDN4, CD36, and ULBP3. Among these, FN1 was the most significantly differentially expressed gene in exosomes, with FN1 | log2 (fold change) |>1.5 and an AUC of 0.7414. In addition, exosome subpopulation analyses showed that cluster 11 accounted for the largest proportion of the total 16 subpopulations, and FN1 was the marker with the highest concentration of cluster 11.

Conclusion: Single-exosome profiling and exosome subpopulations of RA by PBA yielded significant differential gene FN1, which provides new possibilities for diagnostic screening of RA.

Keywords: recurrent abortion, exosomes, FN1, PBA

Introduction

Recurrent abortion (RA) is a common pathological pregnancy that occurs in 1–5% of all pregnancies and is defined as two or more clinically recognized pregnancy losses, including embryonic and fetal loss, before 20–24 weeks of gestation.[1,2] Its etiology is extremely complex, and its pathogenesis remains unclear. Chromosomal abnormalities,

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anatomical defects, pathogenic infections,[3–6] endometrial dysfunction, and autoimmune diseases can lead to recurrent abortion.[7,8] 75% of cases are still difficult to explain, and the likelihood of achieving a live birth decreases with each subsequent pregnancy loss.[2,9] Furthermore, due to RA patients often lack specific clinical manifestations, at least half of the couples clearly have no underlying pathological changes,[10] which complicates efforts in predicting and preventing. Investigating the cause of recurrent abortion, finding new treatment directions, and providing guidance for clinical personalized treatment and prevention are still urgent issues in RA research.

Recently, the pathological functions of exosomes and their potential for diagnostic and therapeutic applications have been increasingly recognized, drawing widespread attention to exosomes in biomedical research. Exosomes are a type of membrane vesicle with a diameter of 50 to 150 nm (mainly around 100 nm) secreted by cells into the surrounding environment.[11] They can circulate in all body fluids to transmit biological information between cells and maintain cellular homeostasis.[12] This intercellular vesicular transport pathway plays a crucial role in various aspects of human health and disease,[13] indicating the potential value of exosomes in clinical settings for early disease diagnosis and prognosis assessment. For example, the analysis of urinary proteins via mass spectrometry and the elevated levels of exosomes in the blood at the late stages of tumors can validate the significant role of exosomes in diagnostic and biomarker research within the fields of renal disease, urology, and oncology.[6,14]

Existing studies indicate that exosomes, as major regulators of cellular communication, play an important role in the functioning of the female reproductive system and successful conception. Exosomes are released through the umbilical cord, placenta, amniotic fluid, and amniotic membrane and they are also involved in angiogenesis, endothelial cell migration and embryo implantation.[15] At the same time, intercellular communication serves as an essential mechanism for the maintenance and development of various organs, including the female reproductive system. Various pregnancy diseases such as gestational hypertension, premature birth, and fetal growth restriction may be related to the content of placental exosomes during pregnancy.[16] Based on this, it is believed that exosomes contain genetic and proteomic information and can be used as biomarkers or therapeutic targets for pregnancy-related diseases or placental function.[17] Previous studies have demonstrated that exosomes derived from M1 macrophages[18] and decidual macrophages[19] can inhibit trophoblast migration and invasion through different pathways, thereby participating in the pathogenesis of RA. In summary, we attempted to analyze the variation in exosome content in RA to find if significantly different exosomes could be used as diagnostic screening markers for RA.

The molecular composition of exosomes is highly heterogeneous due to different stimuli from the microenvironment and cellular origin. Exosome membrane proteins reflect the donor cell type and help specifically target exosome vesicles to exert physiological or pathological effects on recipient cells.[20,21] Therefore, it is meaningful to study exosomes separately. Here, we utilized proximity barcoding assay(PBA), an innovative and rapid high-throughput single-exosome analysis method using a combination of antibody-DNA conjugates and unique tag sequences to simultaneously analyze more than a hundred surface proteins on a single exosome, enabling the analysis of highly heterogeneous surface protein composition to differentiate exosomes and analyze exosome subpopulations in human serum for identification and quantification of the large number of exosomes that may be released into the blood from specific tissues in health and disease[20,22].

This study selected serum samples from normal pregnant women and patients with RA of different statuses and outcomes for PBA single exosome assay. The RA group includes those pregnancy with history of RA, those with a history of RA but not currently pregnant, those after recurrent miscarriage, and those with RA not expelled. We will perform single exosome analyses between normal pregnancy controls and four groups of RA samples in different states, with adequate comparisons to find new potential markers of RA.

Materials and Method

Research Population

This study enrolled 244 normal pregnancies and recurrent abortion patients who in the Guangzhou Women and Children's Medical Center, including 30 recurrent abortion patients before abortion (embryo arrest), 25 recurrent abortion patients after abortion, 90 non-pregnant patients with history of recurrentabortion, 71 pregnant women with recurrentabortion, and 28 pregnant women with normal pregnancy were included in the healthy control group. The selected sample

groups were designed to comprehensively compare RA patients with those with normal pregnancy, and the inclusion of non-pregnant subjects with a history of RA was intended to confirm possible changes in exosomes that were unrelated to pregnancy status after the occurrence of recurrent abortion. Inclusion criteria: Meet the current clinical consensus on recurrent abortion, including before abortion, after abortion, before uterine curettage, and after uterine curettage. Exclusion criteria: Those who conceived under artificial assisted reproductive technology and those with other organic uterine diseases, infectious diseases, or clotting disorders.

Human Blood Samples Preparation

The intravenous blood from each sample contributor was stored in blood collection tubes in vacuum-loaded blood collection tubes. Serum samples were prepared by centrifugation of whole blood at 3000 rpm for 10 minutes.

Proximity Barcoding Assay

Biomarkers were detected at the single-exosome level with PBA. Mix 2ul of serum with 1 μL of PBA buffer and incubate with 1 μL of mixed antibodies (2 μg/mL for each antibody) for 2 hours at room temperature. To prepare exosome capture plate, biotinylated cholera toxin subunit B (biotin-CTB, 2.5 μg/mL in PBS, C34799, Thermo-Fisher Scientific, USA) was added to each well of a 96-well plate with streptavidin coating (PCR0STF-SA5/100, Biomat, Italy). The wells were incubated for 20 min at room temperature and then washed three times with PBS containing Tween 20 (PBST). The serum/antibody mixture was diluted to 20 μL with PBA buffer and transferred to each well of a CTB-coated plate to affinity capture exosomes by interaction between CTB and GM1 enriched in the exosome lipid membrane. Captured exosomes were fixed with paraformaldehyde solution (4%). Oligonucleotides on the same exosome acquire a unique exosome label in an extension reaction following binding of an exosome label template.[20] The PBA experiment were conducted in SecreTech (Shenzhen,China) according to the standard operational procedure provided by Vesicode (Solna,Sweden).[22] The working scheme of PBA is drawing with FigDraw, material licensing ID: YSSYW35c4e).

Statistical Analyses

The protein expression dataset was obtained by summing the protein counts in all exosomes in each sample. TMM normalization was performed to compare protein expression levels. The receiver operating characteristic (ROC) curve was used to evaluate the ability of different biomarkers to predict RA based on indicators such as AUC, sensitivity, and specificity. All data was analyzed using the IBM SPSS Statistic 26.0. We used the two independent sample Student's *t*-test to compare the differences between the two groups. Continuous variables that conform to a normal distribution are expressed as median ± standard and mean ± standard.

Results

Exosome Proteomic Biomarker of Single Exosome Analyzed by Proximity Barcoding Assay

A total of 244 serum cases from normal pregnancy and RA groups were enrolled in our study, which included 30 recurrent abortion patients before miscarriage, 25 recurrent abortion patients after miscarriage, 90 non-pregnant recurrent abortion patients, 71 RA pregnancies, and 28 women with normal pregnancies (Sample information is given in Table 1). From Table 1, we can see that the gestational weeks of the four groups of subjects in different pregnancy status ranged from 3.2 weeks to 12.7 weeks, all under 14 weeks, indicating that our pregnant subjects were all in first trimester.

After serum collection and exosome purification, a PBA assay was conducted to analyses proteins expressed on exosome. (The workflow of PBA is shown in Figure 1) In PBA, exosomes are initially mixed with PBA probes and then sparsely captured in 96-well microtiter wells coated with cholera toxin subunit B (CTB), followed by enzymatic elongation through hybridization of oligonucleotides on the PBA probe to specific RCA products by binding the same exosomes. The complexTag present in the RCA product was amplified in combination with the standard sequence, and prehybridization with the RCA product prevented the DNA polymerase from extending to nearby monomers. PCR primers 1 and 2 amplified DNA molecules on the PBA probe by PCR, while other products could not be amplified by this primer. DNA sequencing of

Table 1 General Characteristics of the Study Population

Group	Normal Pregnancies (n=28)	RA Pregnancies (n=71)	Recurrent Abortion Patients Before Abortion(n=30)	Recurrent Abortion Patients After Abortion(n=25)	Non-Pregnant Patients with History of Recurrent Abortion (n=90)
Age	28±4.2018	33±4.3190	33±5.3286	32±4.7153	31±4.4180
Gestation week(s)	8.14±2.76	7.85±4.6484	8.93±3.7705	8.88±2.2710	/
No. of pregnancies	1	4.24±1.1767	3.23±1.3047	2.96±1.0385	3.39±1.9354
No. of pregnancy losses	0	2.75±1.1048	2±1.2318	2.35±0.5616	2.84±1.7668
No. of live births	0	0.61±0.5727	0.37±0.4901	0.42±0.5038	0.6±0.6837

Notes: The age information of each group of samples is presented as median ± SD; other information is presented as mean ± SD; all participants selected for the normal pregnancy control group were first-time pregnancies.

PCR products ultimately identifies and quantifies all protein molecules detected by a single exosome in a sample by counting the total number of different molecular tags that protein tags with the same complex tag have.[22]

From our serum samples, the median number of exosomes per sample detected by PBA that were captured on plates was 1.88×10^5 with a mean square deviation of 1.36×10^{11} (Figure 2A). The median number of proteins counted by label assay reads was 1.30×10^6 (Figure 2B), with an average of 2.9 proteins detected per exosome (Figure 2C). There were significant differences in the number of exosomes and proteins between the normal pregnancy control group and the RA non-pregnant group (defined as having a history of recurrent abortion but not currently pregnant), the RA pregnant group, the recurrent abortion patients after miscarriage and the recurrent abortion patients before miscarriage group. A total of 198 biomarkers were available for our study. The PBA assay compared the normal pregnancy group with the other RA group separately, and the volcano plots illustrated the differentially expressed proteins analyzed in the comparison of the two groups, normalized to the trimmed mean of M-values (TMM) criteria for protein expression data.

Firstly, the normal pregnancy control group was compared to the RA pregnancy group, reflecting the differences in exosomes expression between RA and healthy pregnancy controls. The volcano plots showed that there were seven

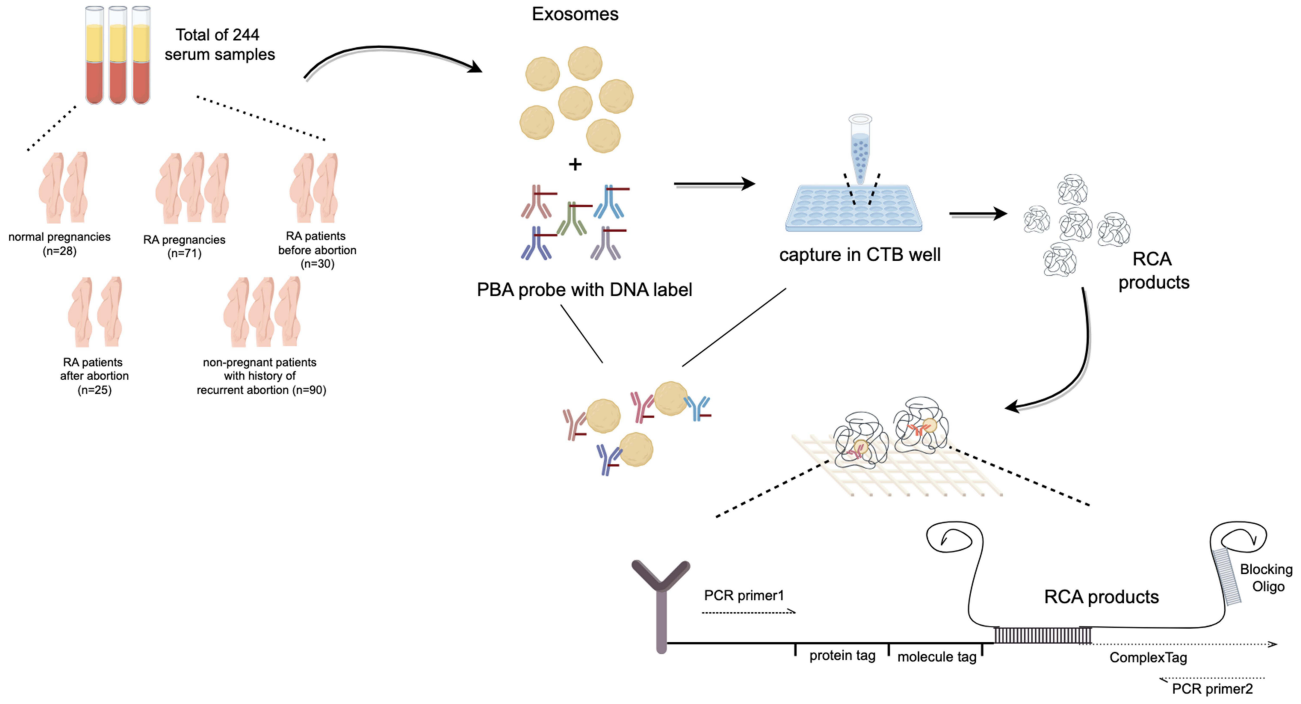

Figure 1 The working scheme of PBA.

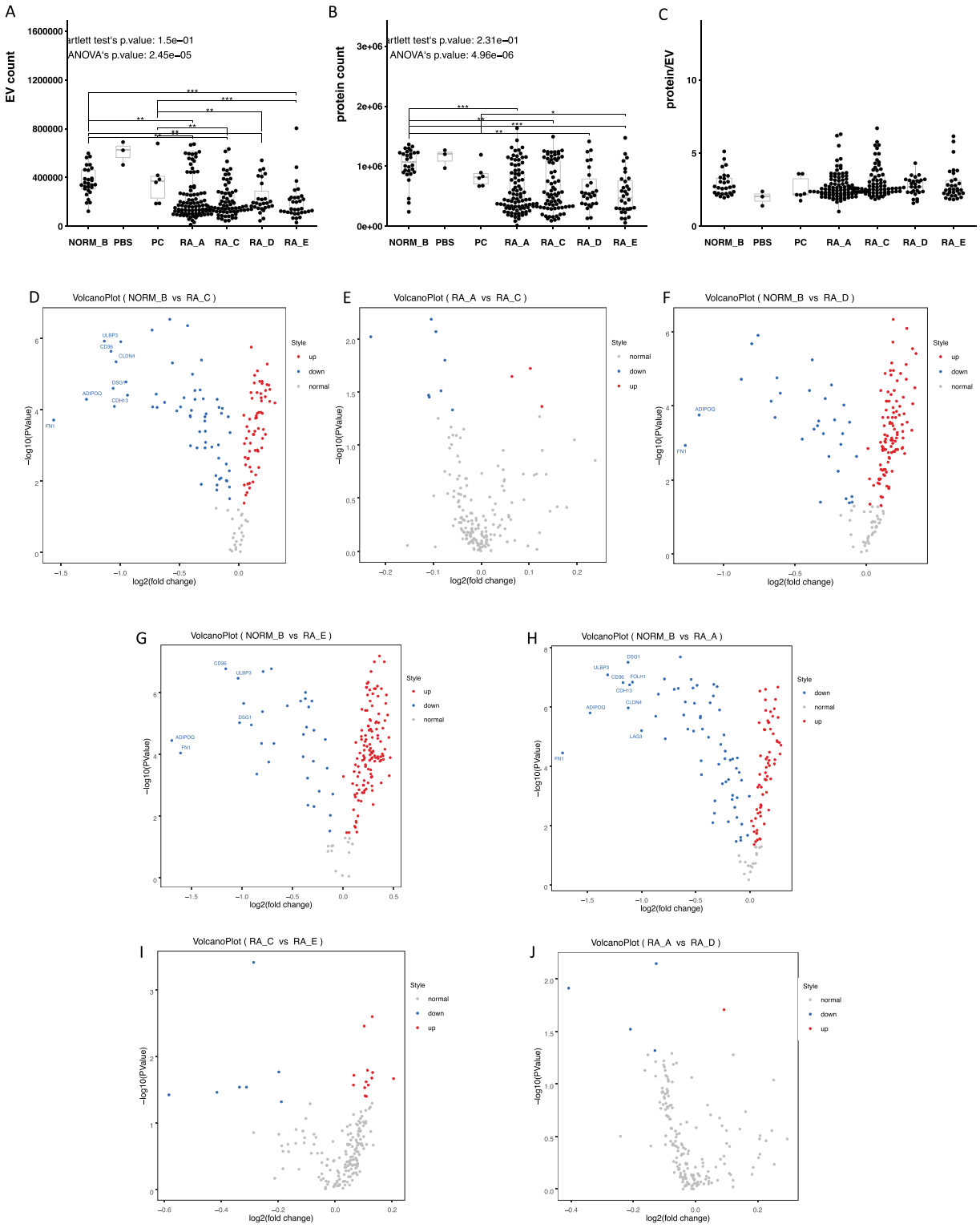

Figure 2 (**A**) The number of exosomes detected in each group of serum samples. ***P* < 0.01; ****P* < 0.001 (**B**) The number of proteins detected in each group of serum samples. **P* < 0.05; ***P* < 0.01; ****P* < 0.001 (**C**) Detected proteins per exosome in each group of serum samples. (**D**) Differential protein expression between normal pregnancy control (group B) and RA pregnancy group (group C) shown in the volcano plot. (**E**) Differential protein expression between RA non-pregnant group (group A) and RA pregnancy group (group C) shown in the volcano plot. (**F**) Differential protein expression between normal pregnancy control (group B) and RA after miscarriage (group D) shown in the volcano plot. (**G**) Differential protein expression between normal pregnancy control (group B) and RA before miscarriage group (group E) shown in the volcano plot. (**H**) Differential protein expression between normal pregnancy control (group B) and RA non-pregnant group (group A) shown in the volcano plot. (**I**) Differential protein expression between RA pregnancy group (group C) and RA before miscarriage group (group E) shown in the volcano plot. (**J**) Differential protein expression between RA non-pregnant group (group A) and RA after miscarriage (group D) shown in the volcano plot.

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significantly down-regulated differentially expressed genes, namely FN1, APIPOQ, CDH13, DSG1, CLDN4, CD36, and ULBP3 (Figure 2D). We also compared the RA non-pregnant group with the RA pregnant group, attempting to find differences in exosomes when not pregnant in RA states. From the volcano plot, we can see that there are no significantly differentially expressed genes between the two groups, with the log2 (fold change) value is less than 0.25, and only one-half of the differentially expressed ploidy (Figure 2E). In contrast, the comparison between the RA non-pregnant group and the control group of a normal pregnancy yielded nine significantly down-regulated genes (Figure 2H), indicating that differences in exosome expression still exist between RA and healthy controls in a non-pregnant state. The significantly differentially expressed genes in the normal pregnancy control group compared with the RA non-pregnant group and the RA pregnant group were compared and intersected to identify the common differentially expressed genes FN1, ADIPOQ, ULNBP3, CD36, CLDN4, DSG1, and CDH13. When comparing the normal pregnancy group with the post-abortion group of RA, the volcano plot revealed two down-regulated genes, FN1andADIPOQ (Figure 2F), reflecting the differences in exosomes between normal controls and RA when they showed abortion symptoms. The comparison of the normal pregnancy group to the aborted group resulted in significant down-regulation of the genes ADIPOQ, FN1, DSG 1, ULBP3, CD36 (Figure 2G). Finally, we also performed within-group comparisons of several different groups of RA, which showed that there were no significantly different genes in the RA pregnancy group compared to the pre-abortion group, with a log2 (fold change) value of less than 0.5 (Figure 2I). The RA non-pregnant group also showed no significant genetic differences when compared to the RA post-abortion group (Figure 2J), with log2 (fold change) values around 0.25.

Overall, for the RA groups with different outcomes, within-group comparisons illustrated that exosome expression was not significantly different before and after abortion. In summary, we took the intersection of the differentially expressed genes obtained from the comparison of each group and chose the gene with the highest number of repetitive occurrences and the largest and most significant multiplicity of differentially expressed genes as FN1.

To further evaluate the diagnostic biomarkers, we show the Receiver Operating Characteristic (ROC) curves in Figure 3A–3G. The common differential genes derived from the comparison of normal pregnancy controls with each group of RA pregnancies were selected and the ROC curves were plotted. The area under the curve (AUC) for FN1 was 0.7414 (95% confidence interval [CI], 0.6348 to 0.8481); for ADIPOQ, 0.7626 (95% CI, 0.6549 to 0.8703); for CD36, 0.8063 (95% CI, 0.7062 to 0.9065); AUC for CDH13 was 0.7555 (95% CI, 0.6531 to 0.8580); for CLDN4 was 0.7973 (95% CI, 0.6627 to 0.8695); for DSG1 was 0.7731 (95% CI, 0.6980 to 0.8796); and for ULBP3 was 0.7626 (95% CI, 0.6549 to 0.8703).); AUC for ULBP3 was 0.8149 (95% CI, 0.7369 to 0.9119).

Altered Exosome Subpopulations in Patients With Recurrent Abortion

To classify exosomes based on proteomic features, FlowSOM, a visual clustering tool utilizing self-organizing maps, is discussed here. This method visualizes exosome subgroups in a t-distributed stochastic neighbor embedding (t- SNE) plot. Exosomes from all samples are clustered as shown in the t-SNE plot (Figure 4A). Among these, 16 clusters were identified by FlowSOM, with the biomarkers of each cluster are shown in the heat map (Figure 4B).

Among all the subpopulations, cluster 11 exhibited the highest percentage (Figure 4A). By analyzing the t-SNE maps of each group individually (Figure 4C–4G), we assessed the similarities and differences in the distribution of subclusters across groups. As shown in Figure 4C, cluster 11 had the highest percentage in five distinct subgroups: normal pregnancy control (group B), RA pregnancy group (group C), RA non-pregnant group (group A), RA after miscarriage (group D), and RA before miscarriage (group E). Compared to the normal control, the percentage of cluster 11 gradually increased from 11.26% to 14.28%. The proteomic profiles of cluster 11 are presented in Figure 5A and B. After marking the positions of exosomes subpopulations in different groups with colors, it is evident that cluster 11 has the highest frequency in group C and group A (Figure 5A). By comparing the expression frequency of each detected protein in cluster 11, we can determine the relative frequency of protein expression.

For the biomarkers with relatively high expression in cluster 11, their expression levels are illustrated in Figure 5B. Notably, FN1 is the most highly expressed protein in cluster 11, accounting for the largest proportion of proteins.

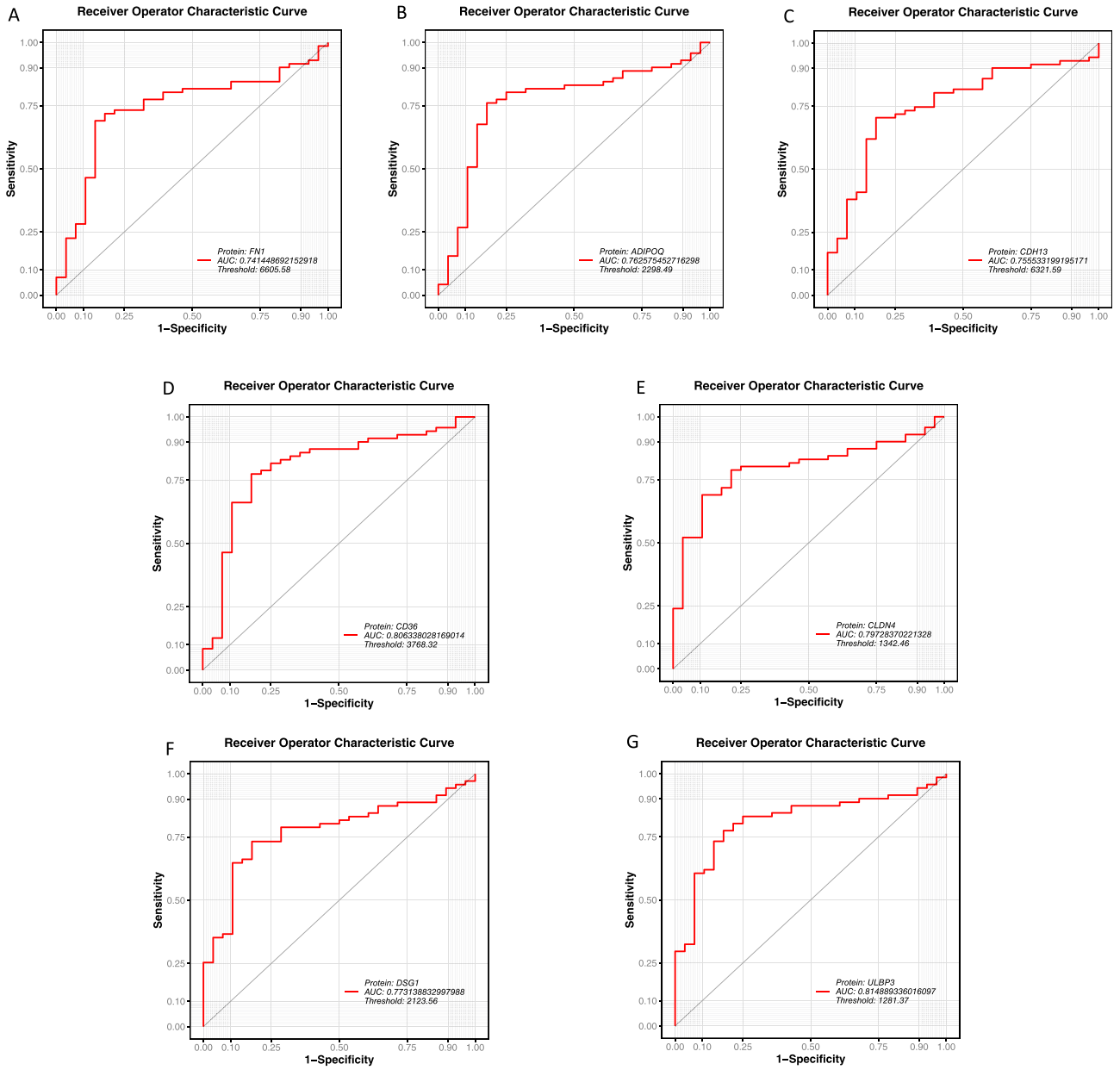

Figure 3 (**A**–**G**) Receiver operating characteristic (ROC) curve of exosomes diagnostic biomarkers in RA pregnancy from HC.

Discussion

In the past, the importance of exosomes in the fundamental mechanisms of intercellular communication, signaling and regulation has been extensively studied. The use of exosomes as innovative dynamic markers in assessing human diseases represents a cutting-edge approach, and the potential for future diagnostics based on exosome analysis has garnered considerable interest.[23,24] Disease-associated exosomes in body fluid samples may hide their diagnostic potential due to their low abundance compared to other exosomes. We investigated the heterogeneous surface proteins of individual exosomes using PBA, a novel exosome analysis method that identifies a large number of exosomes directly from human serum while analyzing the protein assemblies on individual exosomes.[20] In this study, we identified FN1 as a potential diagnostic marker from serum samples of patients with recurrent abortion screened by the PBA technique, providing new evidence for the role of FN1 in pregnancy-associated diseases.

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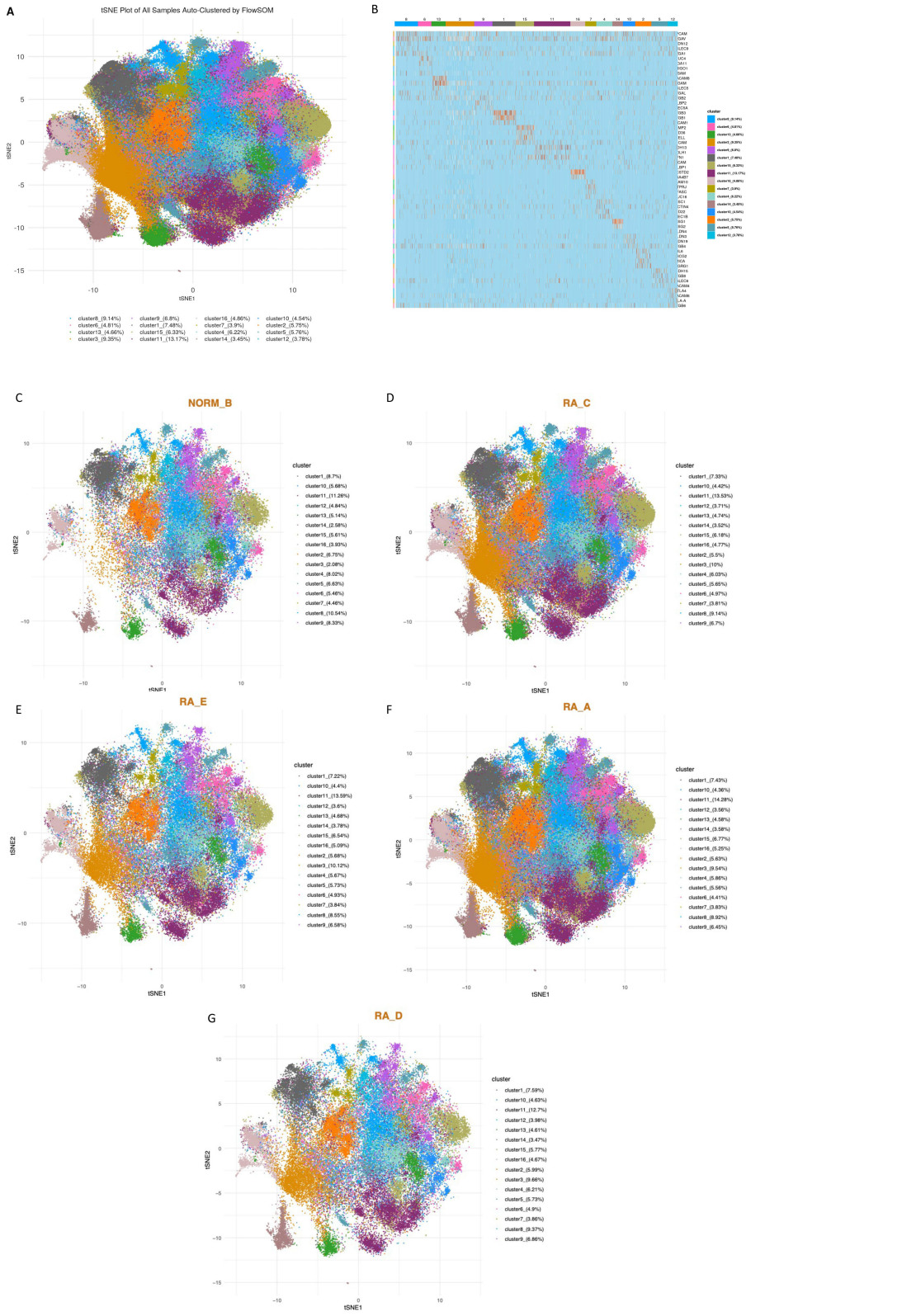

Figure 4 Exosome subpopulation alteration in RA patients. (**A**)The FlowSOM algorithm was used to identify exosome subpopulations in serum samples from all normal pregnancy controls and RA patients. Sixteen subpopulations are shown in the t-distributed random neighbor embedding (t-SNE) plot. (**B**)Proteomic biomarkers of each subpopulation were shown in heatmap. (**C**) Distribution of Exosome Subpopulations in the Normal Pregnancy Control Group. (**D**) Distribution of Exosome Subpopulations in the RA pregnancy group. (**E**) Distribution of Exosome Subpopulations in the RA before miscarriage. (**F**) Distribution of Exosome Subpopulations in the RA non-pregnant group. (**G**) Distribution of Exosome Subpopulations in the RA after miscarriage.

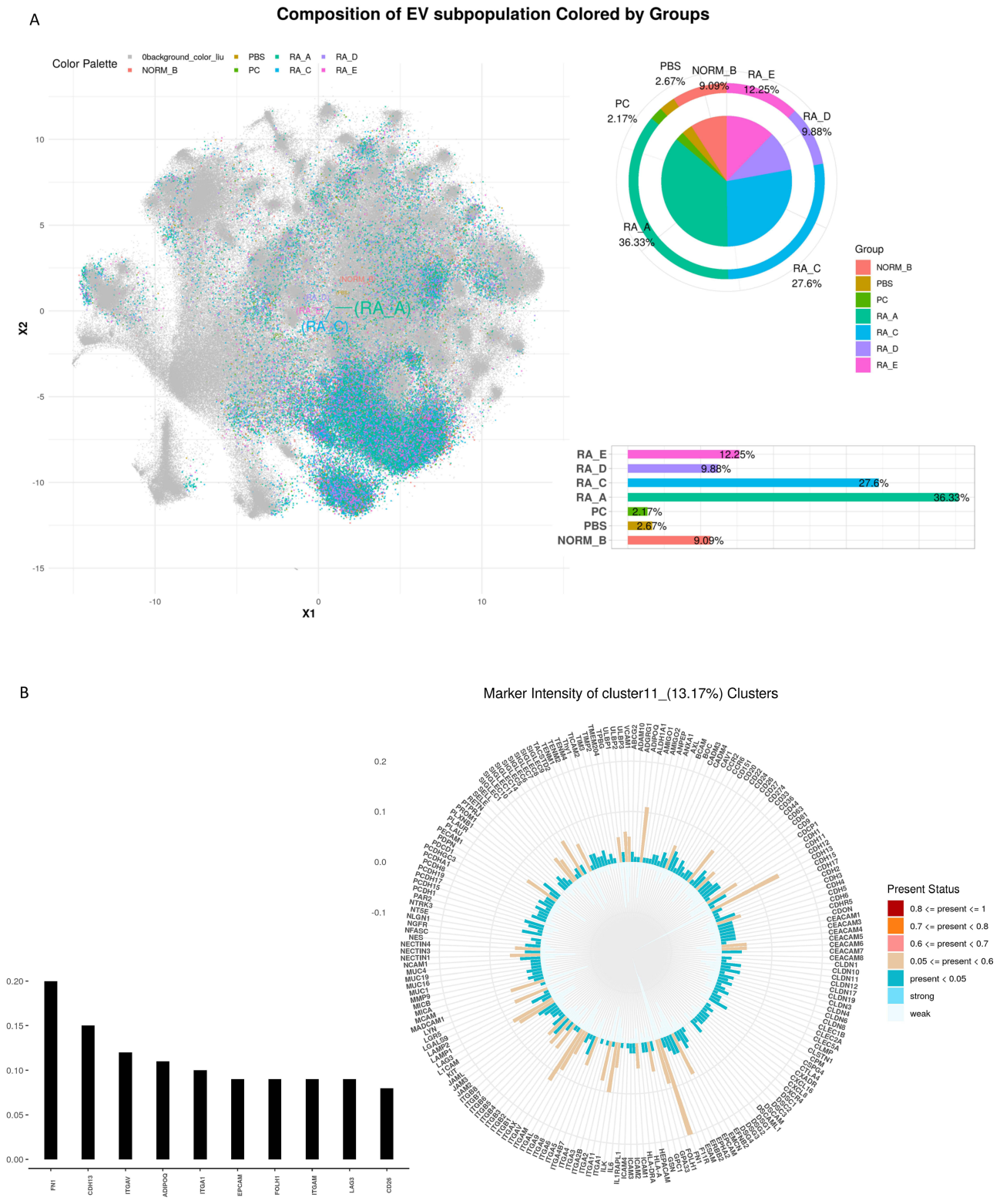

Figure 5 (**A**) The expression of cluster 11 increased significantly in RA groups. (**B**) FN1 was significantly and specifically overexpressed in cluster 11.

FN1 (Fibronectin 1) is an extracellular matrix glycoprotein that exists as a soluble dimer in serum and is also found in connective tissues and on cell surfaces.[25,26] Fibronectin is synthesized by many differentiated cell types and is involved in cell adhesion and migration processes, including embryogenesis, wound healing, blood coagulation, host defense and metastasis.[27,28] The role of FN1 in tumors has been adequately studied, and fibronectin can play an important role in the

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migration,[29] invasion and metastasis of tumor cells, serving as a prognostic marker for tumors by affecting the immune microenvironment and associating with tumor infiltration.[26] With relation to FN1 in pregnancy, it has been reported that FN1 regulates differentiation, apoptosis and invasion of human outer trophoblast cells through bioinformatics methods based on pathway enrichment analysis and functional interactions with human gene expression microarray data from pre-eclamptic placenta.[30] It has also been associated with oocyte maturation, significantly enhanced in the follicular fluid milieu, contributing to its role in embryonic development.[31]

The causes of RA are extremely complex, including embryonic chromosomal abnormalities, anatomical and structural malformations, endocrine disorders, immunological abnormalities, infections, and more.[32] Currently, 60%-70% of RA cases have an unknown cause, which is known as unexplained RA.[33] The establishment, maintenance, and timely termination of a normal pregnancy depend on the functions of trophoblast cells, such as proliferation, migration, and invasion; dysfunction in these processes can lead to a series of pregnancy-associated disorders.[34,35] Reduced trophoblast invasive capacity and insufficient invasive migration can result in shallow placental attachment, potentially causing miscarriage, gestational hypertension, and other pregnancy-related disorders, making it a significant cause of RA.[36] We are able to analyze approximately 200,000 individual exosomes for each sample with the help of the PBA method, enabling the screening for biomarkers of RA at the single exosome level. This was followed by the identification of exosome subpopulations by clustering of single exosomes, which allowed us to find exosome-derived cells by analyzing clusters of protein aggregates for subsequent functional validation of the biomarker. The drawbacks include that the PBA assay kit can be further expanded to detect more protein types, and our sample size must continue to be expanded as well as our classification of RA samples can be further refined to validate the value of FN1 in RA screening and early detection.

In conclusion we used a new method to analyses single exosomes in human body fluid samples to show the exosome profiles associated with various conditions of RA. Ultimately, we concluded that FN1 might be able to serve as a new biomarker for RA through cross-comparison among different groups. Combined with the current research status, we present a fresh perspective on FN1's potential role in RA, offering a new avenue for screening marker research in this area. However, the limitation of our study is that we have not been able to verify the important role of FN1 in the occurrence of RA through in vivo and in vitro experiments, and the specific mechanism of FN1's action remains to be clarified.

Conclusion

Our study demonstrated that analysis of serum exosomes from RA patients by PBA showed that FN1 was the most significantly differentially expressed and most recurrent protein. The ROC curve showed that AUC for FN1 is 0.7414. Exosome subpopulation analysis also showed that FN1 was the most recurrent gene in cluster 11, which accounted for the largest proportion. Accordingly, we suggest a new role for FN1 in the early diagnosis and prediction of RA patients.

Abbreviations

RA, recurrent abortion; PBA, Proximity Barcoding Assay; ROC, Receiver Operating Characteristic; AUC, area under the curve; t- SNE, t-distributed neighbor embedding; TMM, trimmed mean of M-values.

Ethics Approval and Informed Consent

The study was conducted according to the principles expressed in the Declaration of Helsinki. This study was approved by the Guangzhou Women and Children's Medical Center Medical Ethics Committee (ethics number 2021074B00,2018022202). All participants are adults and have provided written informed consent.

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Disclosure

The authors report no conflicts of interest in this work.

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