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ORIGINAL ARTICLE

Exploring the Molecular Mechanism and Biomarker of Recurrent Spontaneous Abortion Based on RNA Sequencing Analysis

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SUMMARY

Background: **Recurrent spontaneous abortion (RSA) is defined as the failure of two or more consecutive clinical pregnancies before 20 weeks of gestation. It is a hot issue in contemporary obstetrics. The etiology of RSA is complicated. Exploring the molecular mechanisms of RSA will be helpful for the prevention and precise therapy at the molecular level. This study aimed to provide novel insights into the biological characteristics and related pathways of differentially expressed genes (DEGs) in RSA.**

Methods: **The data set GSE121950 was obtained from GEO data sets. We identified the DEGs using the affy package in R programming software. Gene set enrichment analysis (GESA) and GenePattern tools were performed to examine the gene expression differences between RSA and control group. Protein-protein interaction (PPI) analysis was performed using STRING online tool (https://string-db.org/). qRT-PCR was carried out to validate the expression levels of DEGs in 16 villus tissue samples from patients with induced abortion and 16 villus tissue samples from RSA patients.**

Results: **A total of 628 DEGs with adjPval < 0.05 and |logFC| > 1 were obtained, including 155 up-regulated genes and 473 down-regulated genes.**

Ten gene ontology (GO) terms and 10 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were screened out by comparing the genome-wide gene set expression patterns of normal and RSA tissues. Eight genes involved in RSA were identified from the hippo signaling pathway, cytokine-cytokine receptor interaction pathway, and allograft rejection pathway.

Conclusions: **Present findings demonstrated that several cytokine regulation processes have a deep impact on RSA. A number of genes involved in the hippo signaling pathway, cytokine-cytokine receptor interaction pathway, and allograft rejection pathway may be critical mediators or participators in the pathogenesis of RSA. Although further *in vivo* and *in vitro* validations are required, our data may provide an important theoretical basis to elucidate the pathogenesis of RSA.**

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KEY WORDS

recurrent spontaneous abortion, biomarker, DEGs, pathway

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LIST OF ABBREVIATIONS

RSA - recurrent spontaneous abortion
GO - gene ontology
KEGG - Kyoto encyclopedia of genes and genomes
DEGs - differentially expressed genes
GSEA - gene set expression analysis
PPI - protein-protein interaction

INTRODUCTION

Recurrent spontaneous abortion (RSA) is a hot issue in contemporary obstetrics. It is estimated that approximately 1% of couples attempting to conceive experience three or more miscarriages and even 5% of couples experience two pregnancy losses [1]. However, the underlying etiology and pathogenesis are still not fully understood. Historically, RSA has been attributed to either environmental risk factors, immune, age, fetal chromosomal abnormalities, structural abnormalities, genital tract infections, thrombophilic disorders, genetic, smoking, endocrine, stress, or unexplained causes [1-3]. These factors may be identified in up to 50% of women with RSA. These factors may overlap to contribute to the pathological process. With the development of medical science, comprehensive intervention therapy has been recommended to reduce the frequency of recurrence or prevent new miscarriage, but no effective means is available to heal the disease.

Decidua is considered to play a critical role in the establishment and maintenance of pregnancy. The processes of embryo breaching the luminal endometrial epithelium and embedding in the stroma is called endometrial decidualization. In the process of decidualization, a large number of blood vessels are established or remodeled to provide sufficient blood supply and nutrition for the development and growth of embryos. This progress is essential for pregnancy in all species [4]. Recent work has revealed that the function of the immune cells that reside at the interface between the placenta and uterus are locally controlled by the decidua [5,6]. Abnormal immune tolerance, abnormal vessel remodeling in the developing decidua, and failure of cell invasion during early pregnancy may lead to RSA.

Genetic information variation was found in patients with RSA. Aberrant methylation variable positions and differentially methylated regions found in villus tissue of RSA patients with normal karyotype [7-9]. The chromosome microstructures, genome single-nucleotide polymorphism, gene expression, non-coding RNA expression variation in products of conception are associated with RSA [10-13].

In this study, we investigated bioinformatics to explore the DEGs in villus tissue of RSA patients by analyzing the results of high-throughput sequencing. We aimed to provide novel insights into the biological characteristics and related pathways of DEGs in RSA.

MATERIALS AND METHODS

RNA sequencing data

The RNA sequencing data in this study was obtained from the public database (www.ncbi.nlm.nih.gov/geo/), National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database, with the accession number GSE121950 which contains 3 control samples and 3 case samples. The case samples were composed of 3 villus tissue samples from RSA individuals. The control group included 3 villus tissue samples from induced abortion of normal pregnancy. Intact RNA isolated from 6 human decidua tissues was fragmented, end repaired, adapter ligation, and PCR amplified following Illumina protocol. Libraries were sequenced by Illumins Hiseq 2000. After quality control, sequence data were processed with STAR to generate read alignments with hg19. Raw read counts for annotated genes were obtained with feature counts with default settings, normalized, and analyzed using DEseq2.

Identification of DEGs

The raw data was imported and analyzed in the affy package in R programming software. The background noise was removed by Robust Multichip Average algorithm. The average scores in those reads which detected the same gene was considered as the expression value of the gene. The DEGs were identified by Limma package in R. The *t*-test was carried out and Bonferroni method was used for the adjustment of the p-values. The genes with adjPval < 0.05 and |logFC| > 1 were selected.

GSEA

GSEA software (www.broadinstitute.org/gsea) and Gene Pattern tools were used to analyze the gene expression differences. GSEA was used for GO and KEGG pathway expression analysis. Eight hundred twenty-five GO terms were included in GSEA GO biology process collection 5.1 and 186 KEGG pathways were included in KEGG gene sets collection 5.1. Statistical p-value was calculated by GSEA software for each gene set. $p < 0.05$ was considered statistically significant.

Real-time quantitative PCR (qRT-PCR) experiment

Total RNA was extracted from biopsies using RNAqueous® Total RNA Isolation Kit (#AM1912, Thermo Fisher Scientific) following the manufacturer's protocol. Reverse transcription PCR were performed after total RNA extraction. Then target genes and ACTB mRNA levels were detected using real-time quantitative PCR on the Roche Combas Z480. Three assays were carried out for each sample. Data were analyzed with the $2^{-\Delta\Delta Ct}$ method using ACTB as internal control.

Patients

We obtained decidua tissues from 16 RSA patients (mean age, 27.7 years; range, 23 to 34 years). Sixteen decidua tissue samples were obtained during 16 selected abortions (mean age, 25.3 years; range, 20 to 32 years) as healthy controls, without pathology in the control samples confirmed by clinical and histologic examination. The biopsies we obtained in this study were approved by the subjects. We excluded patients receiving systemic or topical treatments.

Statistics

Statistical analyses were calculated in GraphPad Prism 7.0 using the test appropriate for each comparison. $p < 0.05$ was considered statistically significant.

RESULTS

DEGs between villus tissue from RSA and normal controls

To gain mechanistic insight into the role of genes in RSA, we assessed the transcriptional profile (GSE 121950) of the genes involved (Figure 1A). After normalization of the raw RNA-seq data and *t*-test of gene expression between RSA and normal controls, a total of 628 DEGs with adjPval < 0.05 and |logFC| > 1, from which 155 up-regulated genes and 473 down-regulated genes were obtained (Figure 1B).

Functional annotation based on expression analysis

GSEA software (www.broadinstitute.org/gsea/index.jsp) was used to analyze the functions of DEGs between RSA villus tissues and normal villus controls according to the GO categories and KEGG pathways unit. Based on the analysis of biological functions of the identified GO terms, the top 10 GO terms which are involved in the process of RSA (Figure 2A) were shown. As illustrated in Figure 2A, T cell activation, leukocyte migration, positive regulation of cytokine production, response to molecule of bacterial origin, and other biologic processes were significantly involved in the pathogenesis of RSA. The data of KEGG pathways analysis showed that signaling pathways related to immune regulation function process were significantly screened out, including cytokine-cytokine receptor interaction, antigen processing, presentation allograft rejection, and so on (Figure 2B).

A number of genes are validated by qRT-PCR as being involved in the pathological process of RSA

Functional enrichment analysis for DEGs revealed that the screened out gene ontology terms for the biological processes category were exclusively immune activity related. The cytokine-cytokine receptor interaction pathway was significantly enriched. In this study, 628 genes were comprehensively analyzed. According to PPI analysis of DEGs, OSM, IL8, IL1RL1, IL1A, JUN, IL6, CCL20, CXCL9, TLR4, TNF, CXCL8, CCR5, CCL4, CD80, IL10, IL1A, and AFP were identified as hub genes (Figure 3).

To confirm these genes are involved in the pathogenesis of RSA, 16 villus tissue samples from patients with induced abortion and 16 villus tissue samples from RSA patients were subjected to qRT-PCR. We identified that 8 genes may be significant in the RSA pathological process. Four genes (IL8, IL1RL1, CCL20, CXCL9) in the cytokine-cytokine receptor interaction pathway were lowly expressed (Figure 4A - D), 3 genes (CD80, IL10, IL1A) in the allograft rejection pathway were lowly expressed (Figure 4E - G), and AFP involved in the hippo signaling pathway was highly expressed (Figure 4H). The prepared total RNA was used in qRT-PCR analysis. ACTB was used as an internal label gene. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method. All experiments were performed with three independent replicates. CTR, n = 16; RSA, n = 16. p-value as indicated.

DISCUSSION

The occurrence and development of RSA are a complex process that has been attributed to either environmental risk factors, immune, age, fetal chromosomal abnormalities, structural abnormalities, genital tract infections, thrombophilic disorders, genetic, smoking, endocrine, stress, or unexplained causes. In this study, we analyzed DEGs in GSE121950 data set of decidua villa between RSA and induced abortion of normal pregnancy. A total of 628 DEGs with adjPval < 0.05 and |logFC| > 1 were obtained, including 155 up-regulated genes and 473 down-regulated genes. The most significantly enriched biological processes were T cell activation, leukocyte migration, positive regulation of cytokine production, response to molecule of bacterial origin, regulation of T cell activation. According to PPI analysis of DEGs, 17 genes were identified as hub genes. After validation by qRT-PCR, IL8, IL1RL1, CCL20, CXCL9, CD80, IL10, IL1A, and AFP were considered significant in RSA pathogenesis.

GSEA analysis revealed several GO terms including T cell activation, leukocyte migration, positive regulation of cytokine production, and response to molecule of bacterial origin by comparing the genome-wide gene set expression patterns of normal and RSA tissues. Previous studies indicate that a variety of factors involved in immune regulation play a key role in the induction of immunological maternal-fetal tolerance [14]. Immunological factor dysfunction plays an important role in RSA pathogenesis. Cellular imbalances and disorders of immune cell functions may contribute to RSA [15]. Regulatory T lymphocytes and helper T lymphocytes are central in the human immune regulation system and play an important role in pregnancy. Immune imbalance between NK cells and T-lymphocyte subsets is critical for pregnancy. Natural killer cells (NK cells) exist in the endometrium and cooperate with T lymphocytes to create immune tolerance at

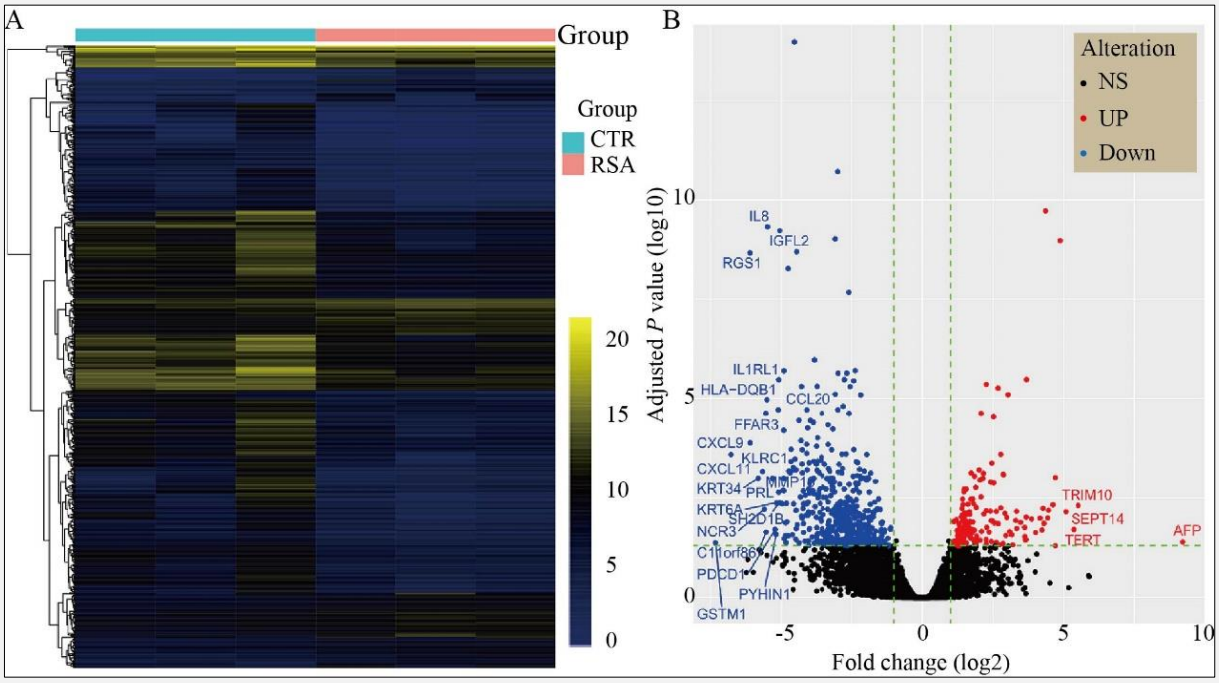

Figure 1. DEGs between villus tissue from RSA and normal controls.

(A) Functional profiling of genes differentially expressed in villus tissue from RSA and normal controls. Blue bar represented control villus tissues and pink bar represented RSA villus tissues. (B) Volcano plot of gene expression in normal villus tissues (n = 3) relative to RSA villus tissues (n = 3) with adjPval < 0.05 and |logFC| > 1.

the maternal-fetal interface, which is essential for successful pregnancy [16,17]. Human predecidual stromal cells are considered a therapeutic effect in an immune-based therapeutic strategy [18]. Immune cells and cytokine signaling pathways participate as mediators of these communications to promote healthy pregnancy. Understanding the contributions of the immune system in pregnancy provides important insights into the pathogenesis underlying RSA and sheds insights on possible targets for therapy. Several recent studies suggest that vitamin D can improve the internal environment for pregnancy through regulation of immune cell differentiation, enhancement of the shift toward Th2 cells and cytokine secretion [19,20]. In addition, there are adjuvant treatments for nutrition metabolism. A recent study showed that folic acid metabolism activity is significantly correlated with RSA. The examination of folate pathway gene polymorphisms (the methionine synthase reductase, MTRR A66G and the 5-10-methylenetetrahydrofolatereductase, MTHTR C677T, MTHFR A1298C) helps to assess folic acid metabolism and further individualized folate supplementation. Methyl folate, vitamins B6 and B12 supplementation in woman with MTHFR mutations has a beneficial effect on pregnancy outcome [21]. Multiple studies have shown that folic acid supplementation based on metabolic enzyme activity is beneficial to improve pregnancy outcome [21,22].

KEGG pathway enrichment analysis was also applied by GSEA. Cytokine-cytokine receptor interaction and the allograft rejection pathway were found to be significantly screened out in RSA. Cytokine-cytokine receptor interaction was also enriched in the embryo of mink uterus and pigs [23,24]. The changes in cytokine and cytokine receptor expression indicated the presence of abnormal immune cell regulation in women with reproductive failures [25]. Rejection of fetal "semi-allograft" by maternal allograft rejection pathway could cause pregnancy loss. New therapies are being developed based on the molecular mechanism of lymphocytes in RSA. In this work, AFP expression was significantly high in the RSA group. As is known to all, mid-trimester maternal serum AFP levels > 2.5 MoM are considered to reflect a defect in placentation associated with an increased risk for pregnancy complications [26,27]. Our finding suggests that AFP expression in villus tissue at early stage of pregnancy is predictive of miscarriage.

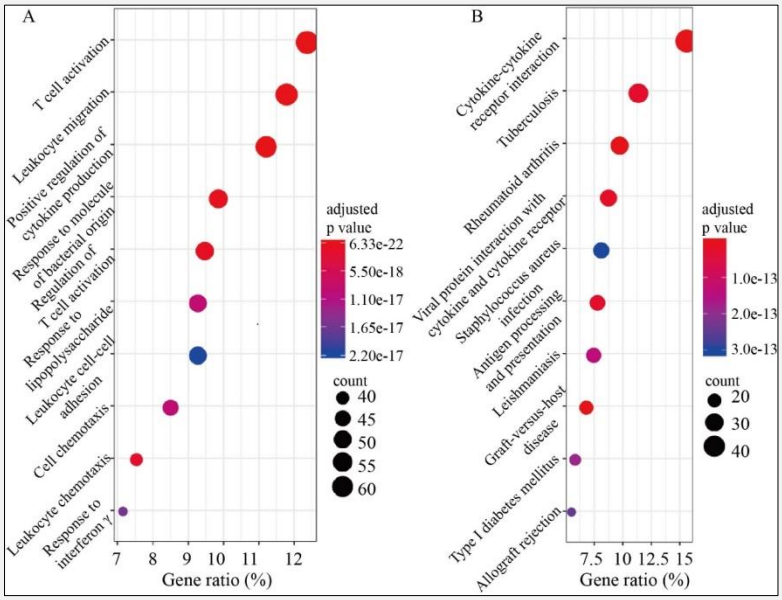

Figure 2. Functional annotation based on expression analysis.

GSEA enrichment plots for (A) GO terms and (B) KEGG pathways. Adjusted p value and gene count as indicated.

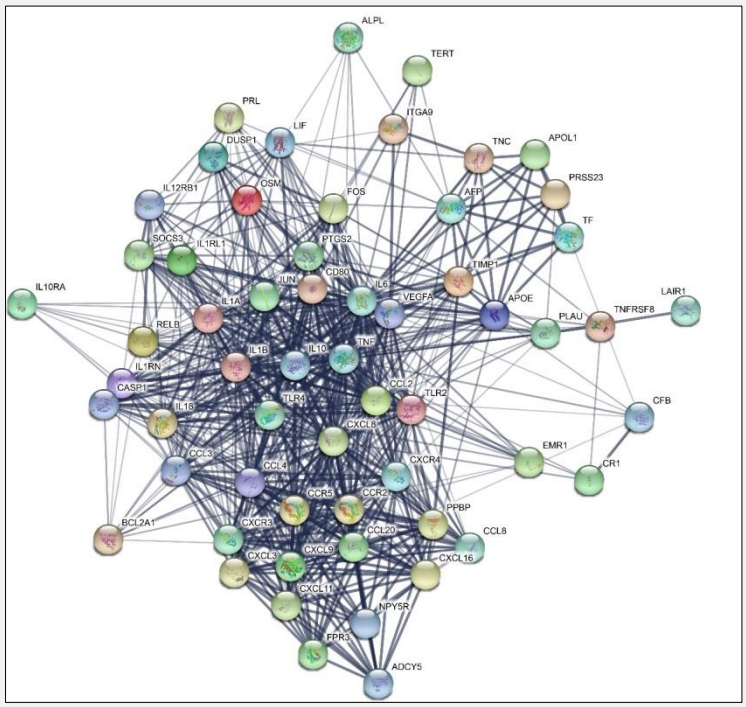

Figure 3. PPI analysis. PPI network of DEGs in GSE121950 data set. Disconnected nodes were hidden in the network.

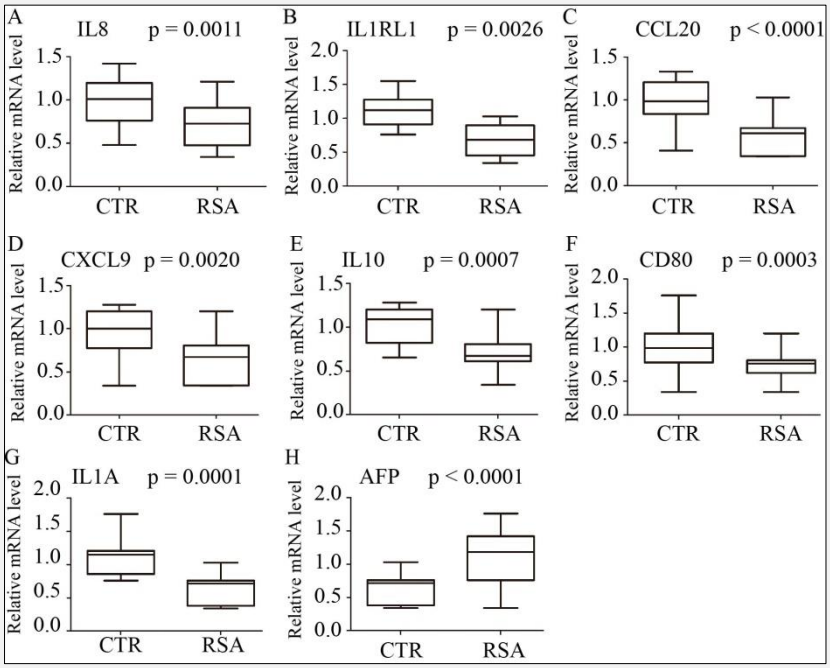

Figure 4. The expression levels of hub genes in RSA patients.

In this work, several genes involved in the cytokine-cytokine receptor interaction pathway and allograft rejection pathway were screened out from RSA patients. Detection these genes may help to predict the risk of miscarriage. However, gene expression was controlled by multiple factors, such as transcription factor, RNA splicing, methylation of promoter region, histone modification and so on. Therefore, exploring the underlying molecular mechanism of the differently expressed genes is interesting work in the future, which may contribute to a molecular therapeutic strategy for RSA. All in all, by the identification of DEGs, GO terms analysis, KEGG pathway analysis, and qRT-PCR experiments, 8 DEGs were screened out. Our results suggest a subset of novel marker genes that may be useful candidate genes in the evaluation and prediction of RSA risk. Although further *in vivo* and *in vitro* validations are required, our data provide an important theoretical basis to elucidate the pathogenesis of RSA and may provide important information for future studies.

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Declaration of Interest:
The authors declared that they have no conflicts of interest in regard to this work. We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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