Placenta 34 (2013) 133–140

Contents lists available at SciVerse ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

Transcriptional profiling with a pathway-oriented analysis in the placental villi of unexplained miscarriage

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ARTICLE INFO

Article history:
Accepted 5 December 2012

Keywords:
Placental villi
Unexplained miscarriage
Genome-wide expression profiles
Gene set enrichment analysis (GSEA)
Oxidative stress

ABSTRACT

Introduction: Miscarriage is the most common placental-related complication of pregnancy. It has been extensively investigated to discover the underlying mechanism(s) by which miscarriage occurs, but in many cases the etiology still remains unclear. The aim of this study was to analyze genome-wide expression profiles of placental villi (PV) from unexplained miscarriage with a pathway-oriented method for identifying underlying mechanism(s) of unexplained miscarriage.
Methods: We investigated PV of 18 women with unexplained miscarriage and 11 women underwent normal pregnancy. Each PV was obtained through dilatation & evacuation and chorionic villous sampling, respectively. Genome-wide expression profiles of PV were analyzed by Gene Set Enrichment Analysis (GSEA) to find dysregulated signaling pathways in PV of unexplained miscarriage.
Results: Unsupervised hierarchical clustering showed heterogeneity of expression profiles between PV of normal developing pregnancy and unexplained miscarriage. GSEA, a supervised analysis, with KEGG pathways revealed that several gene sets associated with mitochondrial function including glutathione metabolism and oxidative phosphorylation are dysregulated in PV from unexplained miscarriage. RT-PCR, real-time RT-PCR and/or immunohistochemistry reinforced that expression of genes constituting these gene sets enriched in normal pregnancy and Cu/Zn-superoxide dismutase was down-regulated in PV of unexplained miscarriage.
Discussion: Structural vulnerability of placental villi for reactive oxygen species (ROS), which is caused by systemic down-regulation of mitochondrial pathways involved in mitochondrial redox balance and functions, aggravates oxidative stress with increased ROS production in PV of unexplained miscarriage.
Conclusion: Systemic vulnerability for ROS in PV could be a major cause of unexplained miscarriage.

1. Introduction

Miscarriage or spontaneous abortion is defined as the involuntary termination of pregnancy before 20 weeks of gestation or below a fetal weight of 500 g. Miscarriage is the most common complication of pregnancy and complicates up to 50% of all conceptions. Approximately 12–15% of all clinically recognized pregnancies end in miscarriage between 4 and 20 weeks of gestation. The causes of miscarriage include chromosomal anomalies of embryos and early fetuses, structural abnormalities of the female genital tract, maternal immune dysfunction and endocrine disorders, various infections and psychological factors. Fetal aneuploidy is the most common cause of miscarriage before 10 weeks' gestation [1]. At least 50–60% of all miscarriages are associated with

Abbreviations: ROS, reactive oxygen species; D&E, dilatation & evacuation; CVS, chorionic villous sampling; GSEA, gene set enrichment analysis; SOD, superoxide dismutase; GST, glutathione s-transferase; GPX, glutathione peroxidase; IMM, inner mitochondrial membrane; GSH, glutathione; PISD, phosphatidylserine decarboxylase.

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http://dx.doi.org/10.1016/j.placenta.2012.12.003

cytogenetic abnormalities [2]. However, in many cases the etiology still remains unknown.

Because normal placental development is essential for the maintenance of pregnancy, miscarriage is considered as one of the placental-related diseases of pregnancy. Normal placental development is under the control of differentiation and invasion of the trophoblasts, specialized cells of the placenta. Villous trophoblasts are able to differentiate to form the overlying syncytiotrophoblast layer of floating villi in maternal-fetal interface, while extravillous trophoblasts invade into the maternal deciduas to modify the maternal spiral arteries to sustain pregnancy. The early stages of placental development take place in a relatively hypoxic environment which protects the developing fetus against the deleterious and teratogenic effects of reactive oxygen species (ROS) and plays an important role in the regulation of trophoblast differentiation [3]. Pregnancy complications such as miscarriage, preeclampsia, and intrauterine growth restriction are related to abnormal development and function of the placenta [4,5]. The factors which affect intrauterine environment for normal placental development can be main causes of unexplained miscarriage. However, causes and underlying mechanisms of unexplained miscarriage still remain undiscovered.

Global gene expression profiling using an oligonucleotide microarray is a useful method to understand underlying pathophysiology of complex and heterogeneous diseases such as miscarriage. Although several causes of unexplained miscarriage have been identified so far, genome-wide understanding of altered pathways in the placental villi of unexplained miscarriage has not been investigated. Thus, we have performed microarray experiments to identify aberrantly regulated signaling pathways between the placental villi of normal developing pregnancy and unexplained miscarriage. In this study, application of gene set enrichment analysis (GSEA), a knowledge-based supervised computational analysis tool, has provided that unexplained miscarriage could be caused by aberrant responses due to down-regulation of genes associated with mitochondrial pathways contributing to the balance of mitochondrial redox system and function.

2. Methods

2.1. Patient characteristics and chorionic villus sampling (CVS) tissue collection

This study was approved by the Institution Review Board (IRB) at CHA Gangnam Medical Center before sample collection, and all women signed an informed consent form before participating in the study. From March 2010 to March 2011, samples were obtained from women with singleton gestation. The CVS procedure was performed at gestational age 10–12 weeks through transcervical approach. The indication for the CVS included history of previous fetal anomaly, increased nuchal translucency or other abnormal ultrasound findings and advanced maternal age. A physician specialized in CVS obtained placental villi for clinical cytogenetics by aspiration of tissue into a 20 cc syringe containing Aminomax solution for cytogenetics cell culture (Invitrogen, Carlsbad, CA). After sufficient amount of aspirated placental villi was taken for clinical cytogenetics, remaining placental villi grossly free of deciduas and blood were placed in an Eppendorf tube, snap-frozen in less than 10 min of CVS aspiration and stored at −80 °C for analyses. Among collected samples, the only CVS specimens which were identified as having a normal karyotype and obtained from women having normal fetal development until 25 gestational weeks were recruited as control for this study. Four of 11 control specimens enrolled in this study were randomly selected for microarray analysis.

2.2. Dilatation & evacuation (D&E) tissue collection

Placental villi were collected from women with a diagnosis of miscarriage at gestational age 10–12 weeks and singleton gestation. The diagnosis of miscarriage was based on clinical history, clinical examination and ultrasonography. Samples were obtained by D&E and collected in sterile containers containing 10 ml of RPMI-1640 with L-glutamine (Invitrogen, CA, USA). The placental chorionic villi were dissected, selected and snap-frozen by trained technicians in the genetics laboratory in less than 10 min after D&E. Each sample was divided into two groups, one for testing karyotype and the other for being stored at −80 °C for the study. All procedures for karyotype, including cell harvesting, slide preparation and staining, were conducted following standard protocols [6]. At least 20 GTG-banded metaphases were analyzed in each case. The only placental villi which were identified as having a normal karyotype and obtained from women having no definitive causes of miscarriage were recruited as unexplained miscarriage for this study. Four of 18 miscarriage specimens were randomly selected for microarray analysis.

2.3. RNA isolation

Total RNA was isolated from each specimen using Trizol reagent (Life Technologies, Carlsbad, CA) following the manufacturer's protocol. The purity and concentration of all RNA samples were assessed by using a microspectrophotometer (T & I, Seoul, Korea). Four CVS and 4 D& E RNA samples were randomly selected from both groups and used to synthesize probes for microarray hybridization. The integrity of RNA samples for hybridization probes were analyzed by running an aliquot of the RNA samples on an Agilent bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). Other RNA samples were used in RT-PCR and/or real-time RT-PCR to validate the results of data analysis.

2.4. Microarray hybridization and data analysis of expression profiling

Agilent Human whole genome 4 × 44 K array (Agilent Technologies, Santa Clara, CA) containing approximately 43,400 human transcripts were hybridized with cRNA probes at the core facility of GenoCheck (Ansan, Kyunggi, Korea). The expression value and detection calls were computed from the raw data and GSEA version 3.7 (Broad Institute, Cambridge, MA) was applied to interpret expression profiles from microarrays [7]. GSEA was originally developed to identify cohorts of genes whose functions are integrated into a certain biological process and/or signaling pathways. Pathways were ranked according to the significance of enrichment, and the validation mode measure of significance was used to identify pathways of greatest enrichment. Significance was tested by comparing the observed enrichment with the enrichment seen in data sets in which sample labels were randomly permutated ($n = 1000$). KEGG gene sets were used in the analyses and gene sets consisting of less than 20 or more than 500 genes were filtered out by gene set size filters.

2.5. RT-PCR and real-time RT-PCR

To validate the results of microarrays, selected genes were analyzed by RT-PCR and/or real-time RT-PCR. One microgram placental RNA was subjected to reverse transcription using M-MLV reverse transcriptase (Roche Applied Science, Indianapolis, Indiana, USA) for cDNA synthesis. Synthesized cDNA was utilized for PCR with specific primers at optimized cycles (Table 1). Appropriate sequences for PCR primers were provided from PrimerBank (http://pga.mgh.harvard.edu/primerbank). Real-time RT-PCR was performed using IQ5 icycler (Bio-Rad, Hercules, California, USA) with QuantiTectSYBRGreen PCR kit (Qiagen, Hilden, Germany). Values were then normalized to the relative amounts of rPL19 cDNA by using a −ΔΔCT method. All PCRs were performed induplicate.

Table 1
Primers used for RT-PCR and real-time PCR in this study.

Gene symbol	Primer sequence (5′→3′)	GenBank accession	Expected fragment size (bp)
GSTA4	F: CCGGATGGAGTCCGTGAGAT R: GGGCACTTGTTGGAACAGC	NM_001512	127
GSTA3	F: TATTTCCCTGCCTTCGAAAA R: CTTCAGCAGAGGGAAGTTGG	NM_000847.4	143
GSTZ1	F: GCCAGCGTGCGTATGATTTC R: GGGCGTTAAAGCCACAAGTG	NM_145870	136
MGST3	F: CCACCTAGCCATCAATGTTTC R: CGCTGAATGCAGTTGAAGATG	NM_004528	108
GPX1	F: CAGTCGGTGTATGCCTTCTCG R: GAGGGACGCCACATTCTCG	NM_201397	297
ATP6V1F	F: CAAGAACCGCCATCCCAATTTCC R: ACTGGTTGATGAGGATGATGCC	NM_004231.3	116
NDUFB1	F: TCCCTGTTGCCCTTGGTCTC R: GCAGTTAGCCGTTCATCACTC	NM_004545.3	162
UQCRB	F: GGTAAGCAGGCCGTTTCAG R: AGGTCCAGTGCCCTCTTAATG	NM_006294	200
ATP5G1	F: CCAGACGGGAGTTCCAGAC R: GACGGGTTCCTGGCATAGC	NM_005175	157
ATP5G3	F: ATGGTGTGTCTCAGCTAATCCA R: CCAAAGACTGTTCCAATACCAGC	NM_001689	142
Cu/Zn SOD	F: AGGGCATCATCAATTTCGAGC R: GCCCACCGTGTTTTCTGGA	NM_000454	177
rPL19	F: TGAGACCAATGAAATCGCCAATGC R: ATGGACCGTCACAGGCTTGC	NM_000981.3	94