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Original article

Genomic answers for recurrent spontaneous abortion in Saudi Arabia: An array comparative genomic hybridization approach☆

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ABSTRACT

To study the genomics/genetic factors associated with recurrent spontaneous abortion (RSA), as ~50% of RSA are unexplained. However, chromosome abnormalities have been reported to play major role in RSA. We performed whole genome array-CGH based genomic analysis of forty four Saudi RSA patients to identify potential molecular and chromosomal abnormalities. We identified a total of 845 alterations, usually not detected by classic cytogenetic methods, in different genomic regions using a cut off value of −0.25 and 0.25 for structural loss and gain, whereas −1.0 and 0.58 were used for single copy number deletion and duplication respectively. We identified frequent (present at least in 10% of patients) alterations including three macro-alteration at 8p23.1, 10q11.21-q11.22 and 15q11.2 as well as large numbers of micro-deletions/amplifications with affected genes including 22q11.23 (GSTT1), 3p22.2 (CTDSPL), 6p21.32 (HLA), and 8p22 (MSR1). Pathway analysis of genes located in detected CNVs regions revealed the allograft rejection signaling, IL-4 signaling, and autoimmune thyroid disease signaling as the most significant canonical pathways associated with RSA. Whole genome array CGH technique can be used to identify potential genes, biofunctions and chromosomal abnormalities associated with RSA which is supported by our findings of a number of novel CNVs/genes (22q11.23/GSTT1, 3p22.2/CTDSPL, 6p21.32/HLA, 8p22/MSR1, and 14q32.33/AKT1) and pathways in patients affected with RSA. To improve diagnosis and treatment of RSA, a comprehensive procedure is needed for identification and validation of causative genes.

1. Introduction

Human reproduction is a remarkably inefficient process and more than 50% of all pregnancies get aborted before their clinical recognition [1,2] and up to 3% of couples face recurrent spontaneous abortion (RSA) (≥3 subsequent pregnancy losses before 22nd weeks of gestation) leading to psychical stress, disappointments, and inadequacy of affected couple [3–8]. It has been reported that about 15% of clinically recognized pregnancies terminate spontaneously in their first trimester [9–11]. RSA might be associated with early arrest of cell division, or early embryo implantation failure before its establishment [12–14]. Although a

Abbreviations: RSA, recurrent spontaneous abortion; CGH, comparative genomic hybridization; CNVs, copy number variations; IL, interleukin; SNP, single nucleotide polymorphism; bp, base pair; GO, gene ontology; HLA, human leukocyte antigen; Th1, T-helper1; Th2, T-helper2; GSTT1, glutathione S-transferase theta 1; CTDSPL, CTD small phosphatase-like protein; MSR1, macrophage scavenger receptor; DGV, database of genomics variation.

☆ Whole genome array-CGH technique has potential to identify molecular and chromosomal abnormalities associated with recurrent spontaneous abortion complications and improve the diagnoses and treatment after validation of causative genes.

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number of RSA risk factors like uterine anatomic abnormalities, immunologic factors, endocrine abnormalities, maternal age, hormonal disorders, infections, environmental factors, sperm quality and thrombophilic disorders are known, still in around 50% of patients etiology of RSA remains unidentified [4,15–17]. In view of this, detection of genetic abnormalities involved in molecular pathogenesis of RSA is mandatory to help explaining unknown causes of recurrent pregnancy loss.

Contribution of the genetic predisposition and chromosome abnormalities to RSA is well reported and classical cytogenetics technique is routinely used to detect chromosomal abnormalities. However, it suffers from limitations such as low resolution, frequent failure of cell culture, contamination of maternal cell, poor chromosome morphology, and is a time consuming procedures [18–21]. Fluorescence *in situ* hybridization (FISH) is another technique that can be used to confirm chromosomal abnormalities that are clinically suspected, however, theses can not be used for hitherto unknown anomalies. Additionally, single nucleotide polymorphisms (SNP) based studies have also reported more than hundred RSA candidate genes though with conflicting results [22–24]. More recently, genetic, epigenetic and gene expression profiling have been applied to identify chromosome abnormalities associated with RSA [25–28]. Array-comparative genomic hybridization (array-CGH) technique can detect submicroscopic (microdeletions and microduplications) chromosomal aberrations, without the need to initiate cell cultures [29,30].

In the current genome-wide study, we performed high-density array CGH analysis to define genomic alteration patterns and to understand the role of CNVs in predisposing to RSA among couples suffering from unexplained RSA residing in Saudi Arabia. Our major finding was identification of 845 genomic alterations, including frequent CNV in 3p22.2 (CTDSPL), 6p21.23 (HLA), 8p22 (MSR1) and 22q11.23 (GSTT1) that in the unaffected status might be associated with healthy pregnancy maintenance.

2. Materials and methods

2.1. Patients and samples

Forty four cases of RSA (16 couples and 12 mothers only) were included in present cytogenetic and array CGH based study. The study was approved by King Abdulaziz University local Bioethics Committee and by the CEGMR ethical committee (No. 09-CEGMR-ETH-01). Informed consent was taken from all subjects included in the present study.

2.2. Cytogenetic methods

A standard 72-h lymphocyte culture and GTG banding (G banding by Trypsin and Giemsa) was carried out in metaphase chromosomes of RSA cases from King Abdulaziz University Hospital, Jeddah. Microscopic examinations were done in at least 20 metaphases for each patient. In case with suspected mosaicism, this number was expanded to one hundred metaphases. Chromosomes were analyzed by using a semi-automatic applied imaging karyotyper and karyotyping software (Applied Imaging, Santa Clara, CA) and International System for Human Cytogenomic Nomenclature (ISCN, 2013) was used to describe karyotypes [31].

2.3. Array comparative genomic hybridization profiling

The array-CGH analysis was performed as per manufacturer's protocol using Agilent sure print G3 Human CGH 2×400 K arrays, Agilent labeling kit (Agilent Technologies, USA).

(i) *DNA Preparation*: Genomic DNA was extracted from peripheral blood using QIAamp DNA blood mini kit (Qiagen, USA), and was quantified by using a NanoDrop ND-1000 Spectrophotometer. (ii) *Genomic DNA Fragmentation:* Patients DNA (500 ng) and reference DNA (Promega, USA) from the same sex were digested at 37 °C by *RsaI* and *AluI* (Promega, USA) for 2 h. The reference DNA was heat-fragmented for 10 min at 95 °C. (iii) *Fluorescent Labeling, Purification and Hybridization:* Patient and reference DNA were labeled with Cy5-dUTP and Cy3-dUTP respectively. Labeled samples were purified by using Microcon YM-30 filter units (Millipore, Billerica, Massachusetts, USA). Cot-1 DNA (Invitrogen, Carlsbad, California, USA), hybridization buffer and blocking agent were mixed with labeled DNA and denaturation was performed at 95 °C before hybridization at 65 °C for 40 h at 20 rpm. (iv) *Microarray Washing, Scanning and Feature Extraction*: Firstly microarray slides and gaskets were disassembled and washed for 10 min in wash buffer 1 (Agilent, cat# 5188–5221), then were shifted to wash buffer 2 (Agilent, cat# 5188–5222) and agitated at 37 °C for 2–3 min. Slides were washed with anhydrous acetonitrile. Chip scanning, image analysis and data extraction were performed on an Agilent Scanner (G2505C), and Agilent's Feature Extraction software (V.1.5.1.0) respectively. (v) *Data Analysis*: Array CGH profiling was done using Agilent CytoGenomics v2.7 software to visualize, detect and analyze aberrations. All detected alterations were identified by using log2 ratio for diploid cases- test(cy5)/reference(cy3) ratio (2n:2n) is equal to 1 on linear scale but 0.0 on $\log_2$ scale respectively, however for multiple cases the standard deviation of $\log_2$ ratios in an oligonucleotide array is on the order of 0.25, i.e anything beyond 0.0 ± 0.25 is considered gain (+0.25) or loss (-0.25). Thus, quantitative loss or gain in copy number were detected by shift values in the $\log_2$ ratio from zero. One set copy number gain (3n:2n) or loss (1n:2n) was measured by both linear scale (1.5 and 0.5) and $\log_2$ scale (+0.58 and -1) respectively.

2.4. Functional enrichment analysis

We derived the genes symbol, p-value and fold change values from altered chromosomal locus and uploaded these data into the Ingenuity Pathways Analysis software (Ingenuity Systems, Redwood City, CA, USA; IPA http://www.ingenuity.com/). The Ingenuity Pathways Knowledge Base, which is a large network database of curated molecular interactions and pathways, was used to generate gene maps of interest in order to identify significant biological associations including, interaction and functional framework. Networks or sub-networks were presented graphically using direct and indirect molecular relationships. The gene ontology method predicted the significance of overrepresentation of biological processes, by assesing the number of significantly associated gene in relation to the curated background genes. The impact of the link between the aberration/expression data and canonical pathways were computed by using Benjamini-corrected modified Fisher's exact test and estimated by p values (significance <0.05).

3. Results

3.1. Cytogenetic study

We report the chromosomal analysis of the 44 recurrent spontaneous abortion patients. The age of the subjects ranged from 24 to 48 years (mean age 32.17) and the number of abortions per couple varied from 3 to 15 (mean 4.22). Most of the documented pregnancies in this cohort, got terminated in their first trimester (79.50%) followed by second (16%) and third (4.5%) trimester. Fifty seven percent of RSA patients were able to achieve viable pregnancies with at least one live births whereas remaining 43% had two or multiple pregnancy termination experiences without