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The Role of Monogenic Factors in Recurrent Pregnancy Loss in Iraqi Consanguineous Family with Variant Identifications by Couple whole Exome Sequencing Test

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Abstract

Miscarriages occur in 15% of clinically identified pregnancies. At least two pregnancy losses constitute recurrent miscarriage (RM), which affects 1% to 5% of couples seeking to conceive. Both the genetic makeup and clinical presentation of RM are extremely varied. This research included 10 RM couples. Samples were taken from October 2021 to November 2022. Whole Exome Sequencing was used to identify the unknown genetic factors responsible for RM in tenth couples. The analysis of the carrier test determined a rare recurrent pregnancy loss associated with the autosomal recessive mutation. In addition, most of these cases were associated with close relative marriage. This type of pregnancy loss occurs in women, whose hormone level, ultrasound, and biochemical test reports were normal. This study identified thirteen pathogenic variants (PAH, ETHE1, BTD, GJB2, FOX3, HBA2, CYP21A2, NAGA, TBCE, PKHD1, FKBP10, ASPA, PLA2G6), seven likely pathogenic variants (FRAS1, NAGLU, PIEZO, DDHD2, TRIP4, CTU2, AGRN), and one VUS in FUT8 gene, in the autosomal recessive genes using WES. However, both patients and partners have altered variants in 20% **ETHE1** genes; 15% **FOX3;** 10% of **ASPA**, **DDHD2**, **PIEZO1**, **PKHD1**, **FUT8**, and **PAH**. The detected mutations linked to multisystem abnormalities, neurodevelopmental disorders, cardiac malformations, skeletal dysplasia, metabolic disorders and renal diseases, fetal development during pregnancy, and recurrent miscarriage.

Keywords

RM: Recurrent Miscarriage, ACMG: American College of Medical Genetics, NGS: Next-Generation Sequencing, PKD: Polycystic kidney disease

Miscarriage occurs in 15% of clinically recognized pregnancies (Warburton and Fraser, 1964). Recurrent miscarriage (RM) happens in 1%-2% of attempted couples, which is greater than chance (0.34%). As a result, both clinically and emotionally, RM affects patients and clinicians. Three miscarriages increase the risk of having another one by 45%, according to many studies. RM can develop after a live birth (Royal College of Obstetricians & Gynaecologists, 2011). RM can be caused by anatomical anomalies, endocrine dysfunctions, immunological issues, genetic and acquired thrombophilia, and environmental factors (Rai and Regan, 2006). RM can be caused by smoking, drinking, uterine defects, and blood type incompatibility (Holly et al., 2009). Idiopathic RMs account for 50% of all RMs (Daher et al., 2003).RM is characterized by single-gene disorders. Examples include hemoglobinopathies, metabolic abnormalities, and X-linked illnesses. Fetal hydrops and stillbirth are caused by

untreated alpha thalassemia major (Bart syndrome). Loss is uncommon in conceptus hemoglobinopathies. Lysosomal or amino acid problems, mucolipidoses, key enzyme deficits, mitochondrial dysfunction, glycogen storage, and fatty acid oxidation are all autosomal recessive metabolic illnesses. Until recently, karyotypes and microarrays could not detect single-gene anomalies during pregnancy or infancy. With whole exome and genome sequencing, single gene abnormalities may enhance pregnancy loss detection.Single gene polymorphisms may increase the probability of loss. These polymorphisms may be common and difficult to discover if they slightly increase the risk of miscarriage while allowing normal pregnancies. Polymorphisms in complex disorders like hypertension must be combined with other exposures or events that cause loss.RM gene mutation research is made possible by molecular technologies, and this has been discovered hundreds of miscarriage-related genes. Potential mechanisms and routes for genes of interest include aneuploidy, autoimmune disorders, angiogenesis, human leukocyte antigen problems, maternal immunologic dysregulation and hyper-immunity, placental development anomalies, and thrombophilia. In more significant study cohorts, more detailed analyses of numerous potential gene abnormalities may identify their linkages as well as management and treatment alternatives. Future studies of single gene polymorphisms should include analyses of genomic material of parent and embryo (Pereza et al., 2016).

Method

Specimen collection was carried out from Al-kadhimiya Teaching Hospital and Al-Yarmouk Teaching Hospital in Baghdad. In the current study, 5 ml venous blood specimens were collected from the RM patients.Ten couples with RM and previously assessed miscarriages were considered for whole exome sequencing due to the availability of substantial DNA from the couple and the absence of clearly pathogenic Genetic variants at the time of exome analysis. The obstetric histories and clinical findings for miscarriages of the couples were reported and summarized in Table SI of the Supplemental data. In the study population, there were no multiple miscarriages (twins or triplets). The study was approved by the University of Baghdad's Committee for Ethical Review of Research Involving Human Subjects in the Biotechnology department. All subjects gave informed written consent.

DNA extraction

According to the manufacturer's instructions, DNA was extracted from the peripheral blood of parents using ReliaPrep™ Blood gDNA Miniprep System (Promega-USA). Following extraction, all DNA samples were stored at − 20 °C. Before the analysis, the DNA quality and concentration were determined photometrically (OD260/OD280 1.8–2.0). DNA samples were sent to the Centogene GMBH for Whole Exome Sequencing Analysis.

Illumina WES workflow

Exome capture was performed on 20 samples using Illumina's Nextera Rapid Capture Exome Kit (Illumina). It encompasses 214405 exons and is approximately 37 MB in size. Using HiSeq4000 sequencers (Illumina), 2150 bp reads were generated by pooling up to 9 WES per lane. Raw sequencing reads were converted to basic fastq format utilizing bcl2fastq software 2.17.1.14 (Illumina) and supplied into an in-house established pipeline for the assessment of the WES database based on the 1,000 Genomes Project (1000G) data analysis pipeline and GATK best management suggestions, which include commonly used open-source software projects. Using bwa software's mem protocol, the short reads were aligned to the GRCh37 (hg19) build of the human reference genome. The alignments were converted to the binary bam file format, resolved, and de-duplicated on the fly. The primary alignment files for each sample were improved and accompanied with additional details in accordance with GATK best practices. The secondary alignment files were then subjected to variant calling using three distinct variant callers (GATK Haplotype Caller, freebayes, and samtools).

Variant annotation and filtering

Statistics on average coverage and the proportion of bases with minimal coverage were obtained by coverage analyses, which assessed coverage at the single-base level over the whole design in a two-step process. The splice junctions and coding bases in RefSeq that were judged to be confidently callable have to have at least 10 coverage and no more than 10% MAPQ0 (ambiguously

mapped) reads. All samples were targeted to have a mean depth of coverage of 100 or greater. Annovar12 and custom-built bioinformatics methods were used to annotate the variants. The Integrative Genomics Viewer v.2.313 and Alamut v.2.4.5 were used to visually verify the alignments (Interactive Biosoftware, Rouen, France). A series of filters were used in a hierarchical fashion to prioritize variants, as is common practice. Centogene's own internal mutation database (CentoMD), HGMD, and ClinVar15 were all queried for all discovered variations to immediately identify changes previously documented in the literature as absolutely or certainly harmful, uncertain, and benign. Next, think about all of the potential variations that were found on both of the sequenced DNA strands and that make up at least 20% of the total readings at that position with a minimum depth of coverage of 10. Common benign variants (1% in the general population) and recurrent artifact variant calls were eliminated through comparisons to databases such as 1000G (January 2016, http://www.1000genomes.org), the Exome Variant Server (January 2016, http://evs.gs.washington.edu), the Exome Aggregation Consortium (January 2016, http://exac.broadinstitute.org), and CentoMD (January 2016, http://centomd.com).

Evaluation of the pathogenicity of the variants and reporting

Prior to analysis, all detected variants were regarded as variants of unknown significance (VUS). All variants that probably lead in a prematurely terminated protein (nonsense, frameshifts, altering initiation codon, single exon, or multiexon deletions), and all other major genomic rearrangements, as well as canonical splice site variants (2 bps), were assigned a high priority. The biophysical and biochemical difference between the wild-type and altered amino acid, the evolutionary conservation of the nucleotide and altered amino acid residue in orthologs,16 a variety of in silico predictors (SIFT, Polyphen-2, Mutationtaster, and others), and population frequency data were all taken into account when assessing missense variants and in-frame deletions. Alamut version 2.4.5 (Interactive Biosoftware) was used to examine putative splicing variants by comparing normal and variant sequences for differences in potential regulatory signals. This software package employs multiple splice site prediction methods. Then, using resources like the Online Mendelian Inheritance in Man (OMIM, January 2016, http://omim.org/), HGMD, CentoMD, and PubMed (http://www.ncbi.nlm.nih.gov/), we analyzed the variants that had been prioritized to determine whether or not they were consistent with the clinical phenotype provided for the index and whether or not they were associated with the suspected disease mode of inheritance. The HPO ontology was implemented to identify patient phenotypes, and all specified clinical features were used for each case. At least one skilled and one senior human geneticist reevaluated the chosen variants to determine those relevant to the patient's phenotype. Pathogenic, likely pathogenic, and VUS variants were determined for each selected candidate using the criteria reported by Richards et al,17, Variations were ranked in two primary levels according to phenotype compatibility as variants entirely or partially explaining the clinical phenotype of the index.

Result

The whole exome was investigated by NGS to identify how mutations and gene ablation affect miscarriage. Ten couples (20 individuals) with repeated miscarriages provided DNA samples. Quantus Fluorometer assessed sample purity and quality for effective sequencing. Centogene, a commercial service provider, performed the WES on Illumina HiSeq 2000 NGS platform. During the creation of the Centogene library, additional DNA sample quality and quantity evaluations were performed.

After exome sequencing, various filtering processes were utilized by CentoMD (centogene's bioinformatics tool) to minimize the number of variations. Under the recessive mode of transmission, numerous mutations were identified in the compound heterozygous situation; for variants inherited from the mother's side, twenty-one variants were identified; however, only nine of these variants are present in the father and could be problematic for the offspring. Using Sanger sequencing, Centogene validated the variations in the indicated genes that were revealed by Whole Exome Sequencing, and their segregation in accessible and informative family members was established.

The findings revealed that the couples were found to be carriers of many variations in several genes. Because all identified genes have an autosomal recessive mode of inheritance, only the variant that is present in both partners can affect their offspring.

According to the results of whole-exome sequencing

(WES) performed on the couples, only the following genes FUT8, PKHD1, PIEZO1, FOXE3, DDHD2, ASPA, ETHE1, and PAH were carried by both partners and could potentially have an effect on their offspring and/or be the cause of recurrent miscarriage (Figure 2).

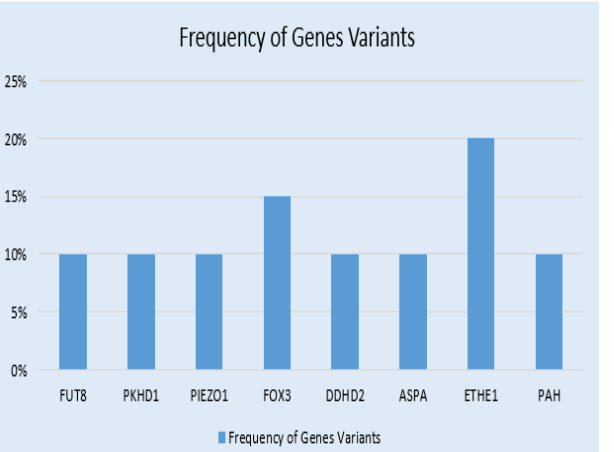

Figure 1: presents the frequency of genes FUT8, PKHD1, PIEZO1, FOXE3, DDHD2, ASPA, ETHE1, and PAH that were carried by both partners and could potentially have an effect on their offspring.

Table1: this table include all genes variants that detected in the studied group, all detected genes were autosomal recessive genes, the highlighted genes include the genes that carried by both couples and have possibility effectiveness in their offspring and may associated with recurrent miscarriage.

Gene carried	Variant coordinates	Amino Acid change	SNP Identifier	Zygosity	Type and Classification (ACMG)
PAH	NM_000277.1: c.898G>T	p. (Ala300Ser)	rs5030853	homozygous	Missense Pathogenic (class 1)
ETHE1					Copy number variant (4 exome) Loss Pathogenic (class 1)
BTD	NM_001281723.2: c.1336G>C	p. (Asp446His)	rs13078881	heterozygous	Missense Pathogenic (class 1)
GJB2	NM_004004.5: c.35del	p. (Gly12Valfs*2)	rs80338939	heterozygous	Frameshift Pathogenic (class 1)
FOXE3	NM_012186.2: c.720C>A	p. (Cys240*)	rs80358194	heterozygous	Nonsense Pathogenic (class 1)
FRAS1	NM_025074.6: c.1372_1375dup	p. (Tyr459Phefs*58)	N/A	heterozygous	Frameshift Likely Pathogenic (class 2)
HBA2	NM_000517.4: c.*92A>G		rs63750067	heterozygous	Effect unknown Pathogenic (class 1)
NAGLU	NM_000263.3: c.2045T>G	p. (Leu682Arg)	N/A	heterozygous	Missense Likely Pathogenic (Class 2)
FUT8	NM_178155.2: c.166C>T	p. (Gln56*)	N/A	heterozygous	Nonsense Uncertain significance (class 3)
CYP21A2	NM_000500.5: c.1360C>T	p. (Pro454Ser)	rs6445	heterozygous	Missense Pathogenic (class 1)
NAGA	NM_000262.2: c.973G>A	p. (Glu325Lys)	rs121434529	heterozygous	Missense Pathogenic (class 1)
TBCE	NM_001287801.1: c.155_166del	p. (Ser52_Gly55del)	rs767004810	heterozygous	In-frame Pathogenic (class 1)
PKHD1	NM_138694.3: c.10174C>T	p. (Gln3392*)	rs201082169	heterozygous	Nonsense Pathogenic (class 1)
PIEZO1	NM_001142864.2: c.1846C>T	p. (Gln616*)	N/A	heterozygous	Nonsense Likely Pathogenic (Class 2)
AGRN	NM_001305275.1: c.3003dup	p. (Gly1002Argfs*58)	rs776252707	heterozygous	Frameshift Likely Pathogenic (Class 2)
FKBP10	NM_021939.3: c.831dup	p. (Gly278Argfs*95)	rs781896189	heterozygous	Frameshift Pathogenic (class 1)

DDHD2	NM_001164232.1: c.306C>G	p. (Tyr102*)		heterozygous	Nonsense Likely Pathogenic (Class 2)
TRIP4	NM_016213.4: c.136C>T	p. (Arg46*)	rs553667435	heterozygous	Nonsense Likely Pathogenic (Class 2)
ASPA	NM_000049.2: c.79G>A	p. (Gly27Arg)	rs766328537	heterozygous	Missense Pathogenic (class 1)
CTU2	NM_001318507.1: c.1086G>A	p. (=)	rs769481947	heterozygous	Silent Likely Pathogenic (class 2)
PLA2G6	NM_003560.2: c.2070_2072del	p. (Val691del)	rs587784344	heterozygous	In-frame Pathogenic (class 1)

The FUT8 mutation c.166C>T p. (Gln56*) results in an early stop codon. Only a few disease-causing variations, including several loss-of-function variants, have been documented in the scientific literature to yet. However, Yu and his colleagues in 2019 proposed that this gene encodes a glycoprotein that is required for trophoblastic cell migration and control. These cells are necessary for embryo implantation and placentation (Yu et al., 2019). It is classified as a variation of undetermined significance by ACMG standards (class 3).

The FUT8 gene mutation is linked to Congenital disease of glycosylation with deficient fucosylation, an autosomal recessive multisystem condition that manifests itself at birth. Affected newborns have delayed psychomotor development, growth retardation, failure to thrive, hypotonia, skeletal deformities, and intellectual impairment. Other extremely varied congenital abnormalities may be observed (Ng et al., 2018).

The PKHD1 mutation c.10174C>T p. (Gln3392*) results in an early stop codon. According to HGMD Professional 2020.3, Ward et al., 2002 (PMID: 11919560), and Shuster et al., 2019 identified this variant as pathogenic for Polycystic kidney disease (PMID: 30650191). This mutation has been found in several individuals and families with autosomal recessive polycystic kidney disease in the compound heterozygous state (Schueler M et al., 2015). ClinVar classifies this variation as pathogenic (clinical testing, Variation ID: 488579). It is classed as pathogenic under the ACMG standards (class 1).

Pathogenic mutations in the PKHD1 gene are related to polycystic kidney disease type 4, with or without hepatic illness (OMIM®: 263200 - PKD4).

The PIEZO1 mutation c.1846C>T p. (Gln616*) causes premature stop codon. According to ACMG standards, it is categorized as class 2 (likely pathogenic). Pathogenic mutations in the PIEZO1 gene are associated with lymphatic malformation type 6 (LMPHM6), which is inherited in an autosomal recessive inheritance pattern (Andolfo et al., 2019). Large transmembrane ion channel proteins called PIEZOs have recently been related to hydrops fetalis through mutations in this gene. PIEZO1 is involved in the regulation of urine osmolarity, regulation of blood pressure, and growth of blood vessels.

The FOXE3 variant c.720C>A p. (Cys240*) results in a premature stop codon. According to HGMD Professional 2021.3, this variant has been described as pathogenic for Aphakia, congenital, primary by Valleix et al., 2006 (PMID: 16826526), Reis et al., 2010 (PMID: 20140963), and Chassaing et al., 2014. This variant is included in ClinVar's database (Interpretation: Pathogenic; Variation ID: 8448). This variant was found in a heterozygous state in the partner. It is classified as pathogenic under ACMG's criteria (class 1).

The DDHD2 Variant c.306C>G causes Hereditary spastic paraplegia (HSP), a series of neurological diseases characterized by stiffness and weakness of the lower limbs. These symptoms are caused by length-dependent axonopathy of corticospinal motor neurons, which is occasionally accompanied by a loss of cortical neurons and anterior horn cells (Maruyama et al., 2018).

The ASPA variant c.79G>A p. (Gly27Arg) results in a Gly to Arg amino acid substitution at position 27. According to HGMD Professional 2022.1, this variant was previously described as the cause of Canavan disease by Kaul et al., 1996 and Sreevishnupriya et al., 2012. This variant is listed in ClinVar (Interpretation: Pathogenic/Likely pathogenic; Variation ID: 188888). According to ACMG standards, it is classified as pathogenic (class 1).

Canavan disease (CD) is a neurological disorder with severe versions defined by leukodystrophy, macrocephaly, and severe developmental delay, as well as a very rare mild/juvenile type with modest developmental delay. Clinically, there are two types of

CD: severe Canavan disease beginning in the neonatal period or infancy, and moderate Canavan disease detected in children. Patients with the severe type exhibit significant hypotonia, developmental delay, and other neurologic abnormalities, as well as very high NAA concentrations in urine, blood, and cerebrospinal fluid. Mild Canavan disease can cause mild developmental delay, speech or academic difficulties, and urine NAA levels that are slightly increased (Bley et al., 2021).

ETHE1 mutations result in autosomal recessive ethylmalonic encephalopathy (Tiranti et al, 2004). The identified variant has been classified as pathogenic according to ACMG standards (class1). Ethylmalonic encephalopathy (EE) is a severe autosomal recessive metabolic disorder in babies that affects the brain, gastrointestinal tract, and peripheral blood vessels. The condition is defined by neurodevelopmental delay and regression, substantial pyramidal and extrapyramidal symptoms, repeated petechiae, orthostatic acrocyanosis, and persistent diarrhea. Necrotic lesions in the deep gray matter are seen by an MRI of the brain. Death usually occurs throughout the first decade of life (Drousiotou et al, 2011).

The PAH variant c.898G>T causes PKU, or phenylketonuria, is a metabolic condition that causes mental retardation and is treatable. In the absence of the enzyme that converts phenylalanine to tyrosine, phenylalanine accumulates. The accumulation of phenylalanine causes detrimental blood and brain levels (Zurfluh et al, 2008). Severe intellectual disability, failure to thrive, motor deficits, hypopigmentation, microcephaly, cognitive impairments, ataxia, and seizures ensue from untreated PKU (Kawashima et al, 1988). The fetus exhibited 100% phenylketonuria based on the genetic pattern of both parents. The carrier mother and infected fetus with phenylketonuria may increase the level of phenylalanine in the mother's blood, slow the fetus's growth and development pace, and in most cases result in spontaneous miscarriages (ókowska et al., 2017).

Conclusion

Recurrent miscarriage is a condition which has both psychological and economical adverse effects on both for the couples and scientific experts dealing with these patients. Emphasizing the real reason behind these cases will be beneficial for both patients and the experts. This is very important to emphasize that the consanguineous marriages in the Iraqi population are so much responsible for pregnancy loss and recurrent miscarriage or to give birth with congenital deformities. The WES analysis detected several autosomal recessives with a frequency of 55.5 % in the studied group, including thirteen pathogenic variants, seven likely pathogenic variants, and one variant of uncertain significance (VUS) from this research. However, the gene variants reported in patients and their partners included ASPA, FOX3, DDHD2, PIEZO1, PKHD1, FUT8, PAH, and ETHE1 genes. They were associated with multi-system abnormalities, neurodevelopmental disorders, cardiac anomalies, skeletal dysplasia, metabolic disorders, renal diseases, fetal development during pregnancy, and recurrent miscarriages. The ETHE1 gene was reported with a higher frequency of 20% of the studied group. The FOX3 gene was identified in 15% of the studied population, while the ASPA, DDHD2, PIEZO1, PKHD1, FUT8, and PAH genes were identified in 10%, and the remaining genes were identified in frequency of 5%. In the whole exome sequencing testing group, we also detect an increased frequency of the heterozygous genotype in autosomal recessive genes in these RM women.

Supplementary material

Table SI: The clinical presentation of studied couples.

Couple Number	Clinical presentation of the couple
Couple 1	Recurrent Miscarriage; Daughter 1: Nystagmus; Daughter 2: Chronic diarrhea, Death in infancy
Couple 2	1 stillbirth, Recurrent spontaneous abortion
Couple 3	Recurrent Miscarriage; Child 1: Brain atrophy; Child 2: Brain atrophy; Child 3: Abnormality of vision.
Couple 4	Recurrent spontaneous abortion
Couple 5	Recurrent Miscarriage; Child: Brain imaging abnormality, Cerebellar hypoplasia, Death in infancy, Leukoencephalopathy, Pachygyria

Couple 6	Recurrent Miscarriage; 1 stillbirth with Blindness and Microphthalmia
Couple 7	Recurrent Miscarriage; Son 1: Brain atrophy, Death in infancy, Recurrent infections; Son 2: Arteriovenous fistula, Death in infancy, Diarrhea, Fever, Seizure.
Couple 8	Hypertension; Proteinuria; Recurrent Miscarriage;
Couple 9	Recurrent Miscarriage;
Couple10	Recurrent Miscarriage; Daughter: Abnormal cerebral white matter morphology, Abnormal CNS myelination, Abnormality of eye movement, Dysphagia, Hyperreflexia, Leukodystrophy, Macrocephaly, Progressive spasticity, Sleep disturbance, Spasticity

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