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Whole exome sequencing revealed a heterozygous elongation factor Tu GTP-binding domain containing 2 (*EFTUD2*) mutation in a couple experiencing recurrent pregnancy loss

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To the Editor: Recurrent pregnancy loss (RPL) is defined as the failure of two or more clinically recognized pregnancies.[1] Both parental and embryonic/fetal factors are associated with RPL. Parental factors include balanced chromosome rearrangements, maternal antiphospholipid syndrome, uterine anomalies, and hormonal or metabolic disorders.[2] Of the examined products of conception (POC), approximately 60% of early pregnancy losses result from sporadic chromosomal abnormalities in embryos, specifically numeric chromosome errors. However, up to 50% of RPL cases remain unexplainable by known causes. Here, we report a couple who have experienced four consecutive clinical pregnancy losses within 10 weeks of gestation, and describe a novel (elongation factor Tu GTP-binding domain containing 2 [*EFTUD2*], Online Mendelian Inheritance in Man [MIM] #603892) nonsense mutation (c.1012G>T, p.E338*) found in embryonic tissues from each of the last three miscarriages by whole exome sequencing (WES) analysis. In addition, we generated a zebrafish line with a mutation homologous to the human *EFTUD2* disruption to investigate the relationship between this mutation and RPL. We hypothesized that the mutation was associated with embryonic lethality, which may induce RPL in patients. The study was approved by the Medical Ethics Committee of the West China Second University Hospital, Sichuan University, China (No. 2016 [029]). Written informed consent was obtained from the couple before genetic testing.

A non-consanguineous Chinese couple presented with a history of four consecutive clinical pregnancy losses within 10 weeks of gestation. Peripheral karyotypic analyses of the 33-year-old female and 36-year-old male were normal.

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Assessment of antiphospholipid syndrome in the female, including screening for lupus anticoagulant, anti-cardiolipin antibodies, and anti-β2 glycoprotein I, yielded normal results. Maternal anatomic abnormalities and endocrine disorders, including diabetes, thyroid dysfunction, and hyperprolactinemia, were excluded. Sperm quality and sperm DNA fragmentation in the male partner were normal. Based on these findings, further genetic examination was performed. We obtained peripheral blood samples from the couple, semen samples from the male partner, and the last three miscarried embryonic tissues (POC-2, POC-3, and POC-4).

To detect embryonic genetic factors of RPL, chromosomal microarray analyses (CMA) were successively performed using the three miscarried embryonic tissues. These analyses yielded negative results. Furthermore, trio-WES was performed on POC-4 and on peripheral blood samples from the partner. A novel *de novo* heterozygous nonsense mutation was identified (c.1012G>T) in exon 12 of the *EFTUD2* gene (NM_004247.4), which could result in structural changes in the EFTUD2 protein (p. E338*). WES was subsequently performed on POC-3, and the same mutation was detected. The presence of the mutation in embryonic tissues from all three miscarriages was confirmed by Sanger sequencing [Figure 1A (a-c)]. The mutation was not detected in peripheral blood samples of the partner. Gonadal mosaicism was suspected, as all POC detected the same mutation. Exome sequencing identified the same mutation (c.1012G>T) in the *EFTUD2* gene in 13.5% of the male partner's sperm cells (ref [bias-field method]/[alternative method] alt:

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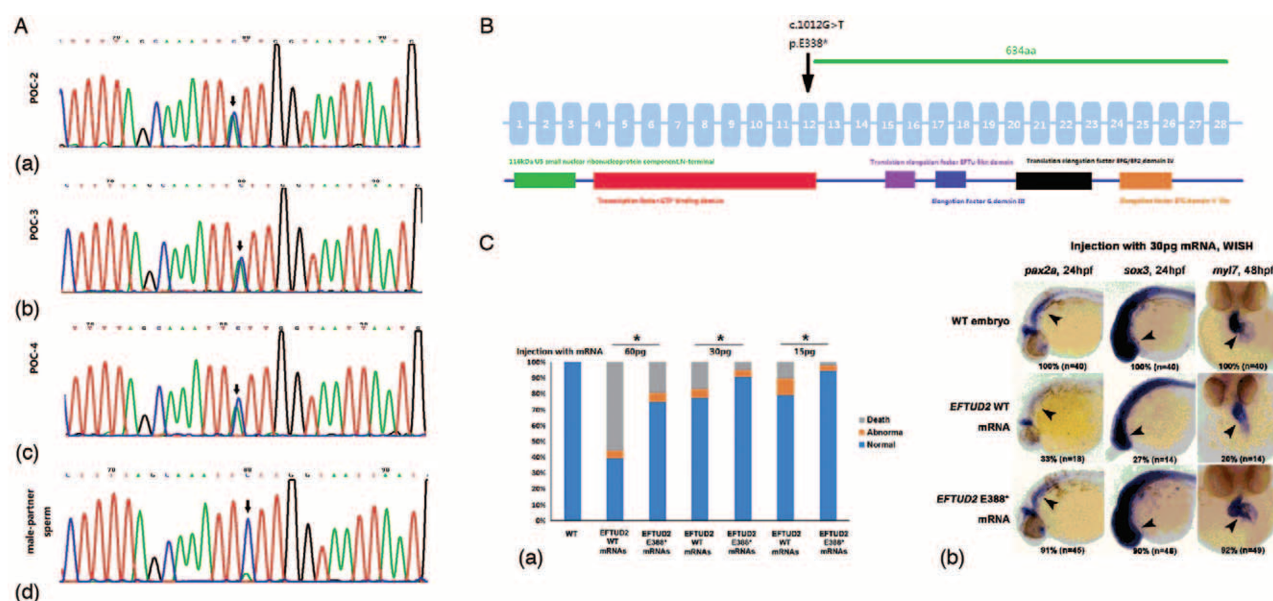

Figure 1: (A) Sanger sequences of the novel *EFTUD2* nonsense mutation. (a-c) WES Identified a novel *EFTUD2* nonsense mutation (c.1012G>T, p.E338*) In a couple with RPL. The mutation was detected in all the obtained miscarried embryonic tissues (POC-2, POC-3, POC-4). (d) The mosaic mutation was detected in sperm. The black arrows indicate the point of mutation (G>T). (B) Protein domains and mutation location of *EFTUD2* gene. The nonsense mutation (c.1012G>T) in *EFTUD2* gene results in premature termination of transcription (p.E338*) and produces a truncated EFTUD2 protein lacking the last 634 amino acids at its C-terminal. The black arrows indicate the point of mutation. (C) p.E338* mutation leads to loss of function of *EFTUD2* gene in zebrafish model. (a) *EFTUD2* WT mRNAs, but not p.E338* mutant mRNAs, produced high rate of embryonic death/deformities. (b) p.E338* mutation caused a loss of gene function of *EFTUD2* during zebrafish embryogenesis. *EFTUD2* p.E338* mutant mRNA failed to impair embryonic neurodevelopment and cardiac development. pax2a and sox3 expression at 24 hpf stage, *myl7* expression at 48 hpf, and embryos at 24 hpf shown are lateral views with anterior to the left; embryos at 48 hpf shown are ventral view with the anterior at the top. Arrow heads point to the signal generated by detected marker genes. The percentage and numbers indicated in each picture are the ratio for the number of affected embryos with phenotype similar to what is shown in the picture. *EFTUD2*: Elongation factor Tu GTP-binding domain containing 2; hpf: Hours per fertilization; POC: Products of conception; RPL: Recurrent pregnancy loss; WES: Whole exome sequencing.

564/88). The mosaic mutation was confirmed by Sanger sequencing of the sperm [Figure 1A (d)] but was not found in the male partner's saliva.

To understand the potential impact of the c.1012G>T mutation on *EFTUD2* function, *in silica* analysis was performed using Protein Variation Effect Analyzer (PROVEAN, http://provean.jcvi.org/index.php), version 1.1.3. The nonsense mutation (c.1012G>T) results in a change in glutamic acid to a premature stop codon (UAA) at amino acid position 338 (p.E338*), producing a truncated *EFTUD2* protein that lacks its C-terminal 634 amino acids. Wild type *EFTUD2* protein (NP_004238.3), composed of 972 amino acids, contains six vital domains (the Protein Families [Pfam] database [http://pfam.xfam.org]): 116 kDa U5 small nuclear ribonucleoprotein component N-terminus (amino acids 4–110); elongation factor Tu GTP-binding domain (amino acids 129–377); elongation factor Tu domain 2 (amino acids 491–566); elongation factor G, domain III (amino acids 586–648); elongation factor G, domain IV (amino acids 707–823); and elongation factor G, domain V-like (amino acids 826–914). Amino acid 338 is located within the elongation factor Tu GTP-binding domain, before translation elongation factor Tu-like domain 2. The mutated protein contains the 116 kDa U5 small nuclear ribonucleoprotein component N-terminus and an aberrant elongation factor Tu GTP-binding domain [Figure 1B]. PROVEAN predicted the *EFTUD2* mutation (p.E338*) to be pathogenic (PROVEAN: deleterious, with a score of –2333.598).

The *EFTUD2* gene is located on chromosome 17q21.31. It encodes U5-116 kDa, a highly conserved GTPase component of the major spliceosome.[3] Several studies have reported that *EFTUD2* haploinsufficiency is linked to mandibulofacial dysostosis, Guion-Almeida type (MIM # 610536),[4,5] a rare syndrome with a wide spectrum of congenital anomalies, characterized by malar and mandibular hypoplasia, microcephaly, micrognathia, dysplastic ears with hearing loss, cleft palate, choanal atresia, and facial asymmetry.[6] However, there is a paucity of studies that have evaluated the function of *EFTUD2* in spontaneous miscarriage.[7,8]

Since the couple has experienced recurrent abortions, a possible explanation for the difference between outcomes for our patients and previously reported patients is that the truncating protein (p.E338*) may induce embryonic lethality, which can be considered the most severe phenotype caused by *EFTUD2* mutations in humans. We used a zebrafish model to confirm the effects of loss of *EFTUD2* gene function *in vivo*. Messenger RNAs (mRNAs) encoding wild type (wt) and mutant human *EFTUD2* were synthesized and injected into zebrafish embryos. The human *EFTUD2* coding region sequence was obtained from Sino Biological Inc. (Catalog Number HG14427-G, Beijing, China). When zebrafish embryos

were injected with several doses of mRNA, wt *EFTUD2* mRNAs produced significantly higher rates of embryonic death/deformities at 24 h per fertilization (hpf) than *EFTUD2* p.E338* mutant mRNAs (60 pg, 60.5% [26/43] *vs.* 25.0% [22/88], $P < 0.001$; 30 pg, 22.3% [21/94] *vs.* 9.3% [9/97], $P = 0.013$; 15 pg, 20.6% [20/97] *vs.* 5.4% [5/93], $P = 0.002$) [Figure 1C (a)]. These results suggest that the detected *EFTUD2* c.1012G>T mutation in the human *EFTUD2* gene causes loss of function. Based on clinical symptoms, we investigated brain and heart development during embryogenesis by marker analyses using whole-mount *in situ* hybridization. The hindbrain neuron marker *pax2a* decreased significantly with injection of ~30 pg of wt *EFTUD2* mRNA at the indicated stages. Heart development was also affected by wt *EFTUD2*. Cardiac marker *myl7* showed that wt *EFTUD2* mRNAs induced looping defects. A small head phenotype was also observed in embryos injected with wt *EFTUD2* mRNAs. Embryos injected with the same doses of *EFTUD2* p.E338* mutant mRNAs displayed little alteration in brain and heart development [Figure 1C (b)]. Taken together, these results demonstrate that the *EFTUD2* c.1012G>T mutation causes loss of gene function in *EFTUD2*.

Because the novel *EFTUD2* c.1012G>T mutation potentially correlates with RPL, the couple received *in vitro* fertilization (IVF) with pre-implantation genetic testing (PGT) to prevent the mutation. One embryo sample without the mutation (c.1012G>T) in the *EFTUD2* gene was selected for transfer, and a successful pregnancy was confirmed by human chorionic gonadotropin and ultrasound examination. In the second trimester, amniocentesis was performed at 18 gestational weeks with negative results by CMA for chromosome abnormality and by Sanger sequencing for the *EFTUD2* gene mutation (c.1012G>T). At present, the fetus is at 24 gestational weeks, and ultrasound abnormalities have been excluded by detailed second trimester fetal anomaly scans.

In conclusion, we have identified a novel *EFTUD2* c.1012G>T mutation in a non-consanguineous Chinese couple, which potentially correlates with RPL. WES is useful for determining the etiology of unsolved RPL cases. This *EFTUD2* gene proved useful information for genetic counseling, leading the couple to receive IVF with PGT to prevent the mutation. As our data are based on only one family, further functional tests and investigations are needed to determine *EFTUD2* function in human embryonic/fetal development.

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Conflicts of interest

None.

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