



A homozygous *NOP14* variant is likely to cause recurrent pregnancy loss

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Abstract

Recurrent pregnancy loss is newly defined as more than two consecutive miscarriages. Recurrent pregnancy loss occurs in <5% of total pregnancies. The cause in approximately 40–60% of recurrent pregnancy loss cases remains elusive and must be determined. We investigated two unrelated Iranian consanguineous families with recurrent pregnancy loss. We performed exome sequencing using DNA from a miscarriage tissue and identified a homozygous *NOP14* missense variant (c.[136C>G];[136C>G]) in both families. *NOP14* is an evolutionally conserved protein among eukaryotes and is required for 18S rRNA processing and 40S ribosome biogenesis. Interestingly, in zebrafish, homozygous mutation of *nop14* (possibly loss of function) resulting from retrovirus-mediated insertional mutagenesis led to embryonic lethality at 5 days after fertilization, mimicking early pregnancy loss in humans. Similarly, it is known that the *nop14*-null yeast is inviable. These data suggest that the homozygous *NOP14* mutation is likely to cause recurrent pregnancy loss. Furthermore, this study shows that exome sequencing is very useful to determine the etiology of unsolved recurrent pregnancy loss.

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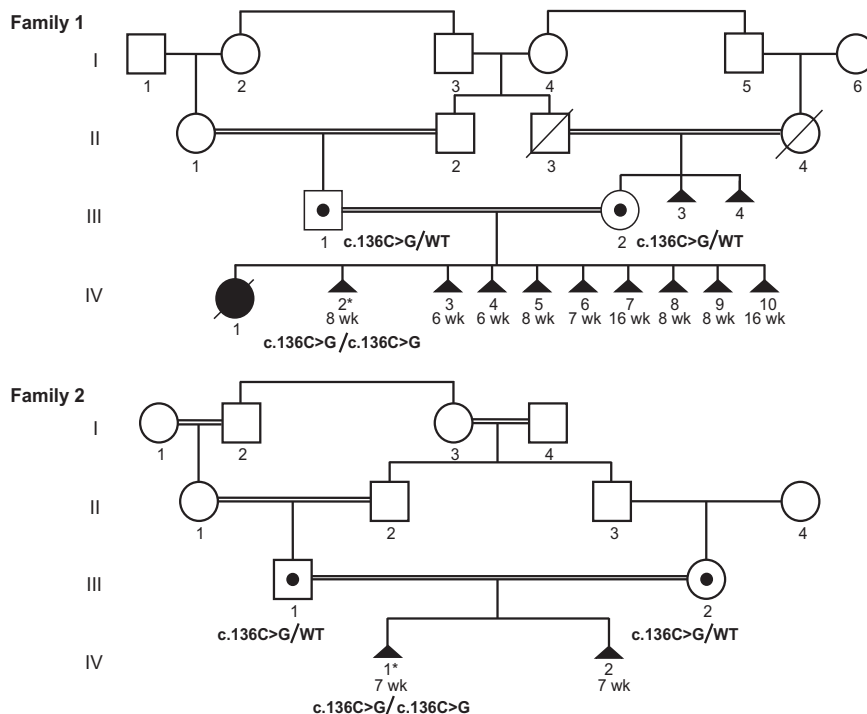
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Introduction

Recurrent pregnancy loss (RPL) is a painful and frustrating problem for reproductive-aged couples wishing to have children. Spontaneous abortion occurs in approximately 15–25% of clinically diagnosed pregnancies [1]. RPL was traditionally defined as three or more spontaneous consecutive pregnancy losses, but not including ectopic, molar and biochemical pregnancies [2]. In 2012, RPL was newly defined as two or more failed clinical pregnancies [1]. It has been estimated that rates of more than two and three failed clinical pregnancies are <5 and 1% of total pregnancies, respectively [1].

The risk factors of RPL include maternal factors (such as endocrine abnormalities, anatomical factors, immunological disorders, inherited thrombophilic disorders), parental genetic factors (such as balanced structural chromosome rearrangements), and fetal genetic disorders [3, 4]. Parental and fetal genetic factors were estimated to be approximately 3–5% of all RPL risk factors [5]. Recently, the causative mutation for RPL were first discovered by exome sequencing [6]. Subsequently, various causative variants have been found in genes essential for embryonic development, including genes related to ciliogenesis (*KIF14* and

Fig. 1 Family pedigrees and a *NOPI4* variant. Pedigrees of two families with RPL and a homozygous *NOPI4* mutation. *WES was performed. WT wild type



IFT122) [7, 8], and RNA export mediation (*GLE1*) [9]. However, the cause of approximately 40–60% of RPL remains unknown [3, 5].

Here we describe two consanguineous families with RPL and their possible causative variant is discussed.

Materials and methods

Subjects

In this study, we recruited two unrelated consanguineous Iranian families with RPL. Genomic DNA was extracted from miscarried fetal tissues and blood samples of parents and family members, collected after obtaining written informed consent. The institutional review board of Yokohama City University School of Medicine approved this study protocol.

Whole exome sequencing and confirmatory Sanger sequencing

Whole exome sequencing (WES) was performed using tissues from a miscarriage of each RPL family as previously described [10]. In brief, 3 µg genomic DNA was sheared using the Covaris model S2 system sonicator (Covaris, Woburn, MA, USA). The target size of sheared DNA was 200 bp. Genomic DNA was captured using the SureSelectXT Human All Exon v5 kit (Agilent Technologies, Santa Clara, CA, USA). WES was performed on a HiSeq

2500 (Illumina, San Diego, CA, USA) with 101 bp paired-end reads. Quality-controlled reads were mapped to the human reference genome (UCSC hg19, NCBI build 37.1) using NovoAlign (<http://www.novocraft.com/products/novoalign/>). After removal of PCR duplication by Picard (<https://broadinstitute.github.io/picard/>), variants were called and genotyped using Genome Analysis Toolkit (<https://software.broadinstitute.org/gatk/>). Called variants were annotated using ANNOVAR (<http://annovar.openbioinformatics.org/en/latest/>). Among total variants within coding exons and intronic regions ± 30 bp from exon–intron borders, synonymous variants and common variants with minor allele frequency (MAF) of more than 1% in our in-house exome database (Japanese population, $n = 575$) or the Exome Aggregation Consortium (ExAC: <http://exac.broadinstitute.org/>) [11] were removed. The missense mutation was assessed using in silico prediction tools: SIFT, PolyPhen2, MutationTaster, and pRec in ExAC [11]. The allele frequencies of candidate variants were checked using NHLBI GO Exome Sequencing Project (ESP: <http://evs.gs.washington.edu/EVS/>), our in-house 575 Japanese database, the Human Genetic Variation Browser (HGVD: <http://www.hgvd.genome.med.kyoto-u.ac.jp/>) [12], ExAC, and the Greater Middle East Variome Project (GME Variome: <http://igm.ucsd.edu/gme/index.php>) [13] which is a public database of the Middle Eastern population including 87 Iranian subjects. In addition, we checked the allele frequencies for candidate causative variants using 55 Iranian exome database and another set of 115 Iranian control samples by Sanger method. Finally, we checked the

Table 1 *NOP14* mutation identified in two Iranian families

Gene	Mutation ^a	Amino acid change	Grantham score	SIFT	PolyPhen2	Mutation Taster	pRec	dbSNP142 (MAF%)	ESP (MAF %)	HGVD (MAF%)	ExAC (MAF%)	GME Variome	In-house 55 Iranian exomes	In-house 575 exomes
<i>NOP14</i>	c.136C>G	p. Arg46Gly	125	Deleterious	Probably damaging	Disease causing	0.98	rs199574576 (0.006)	0	0	0.005906	0	0	0

ESP NHLBI GO Exome Sequencing Project, ExAC Exome Aggregation Consortium, HGVD Human Genetic Variation Database, MAF minor allele frequency

^a The mutation description is based on human genome reference build hg19

called variants against known disease genes registered in The Human Gene Mutation Database (HGMD: <http://www.hgmd.cf.ac.uk/ac/index.phpversion>). Segregation of variants was evaluated by Sanger sequencing.

Copy number variation analysis

Copy number analysis was performed using WES data with the modified eXome Hidden Markov Model (XHMM) [14]. The results were visualized with SignalMap (Roche NimbleGen, Madison, WI, USA). Among candidate copy number variations (CNVs), we focused only on those regions involving known disease-related genes registered in HGMD.

Homozygosity mapping

Homozygosity mapping was performed by HomozygosityMapper (<http://www.homozygositymapper.org/>) using WES genotype data. The exome read counts for each called homozygous variant were checked manually using the Integrative Genomics Viewer. The variants were defined as “confidential SNPs” if covered by 10 reads and more.

Results

Clinical information for two RPL families

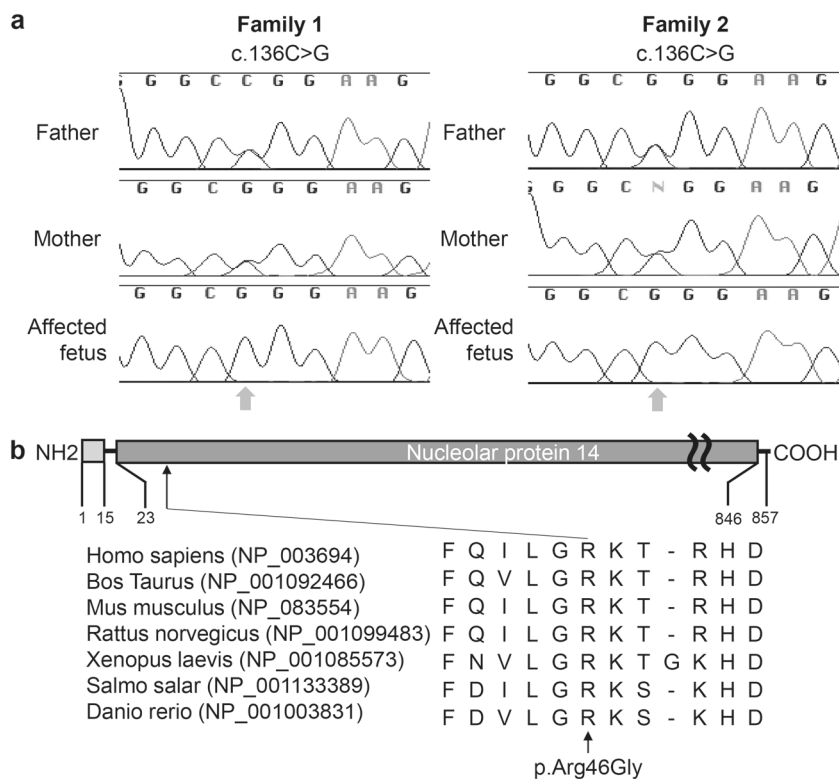
We analyzed miscarriage tissues from each Iranian family with RPL (Fig. 1). Families 1 and 2 had nine and two spontaneous abortions, respectively. All spontaneous abortions occurred in the first and second trimester of pregnancies (Fig. 1). Clinical information of parents in families 1 and 2 is summarized in Supplementary Information (Supplementary Table 1). In families 1 and 2, RPL risk factors (uterine malformation, thyroid abnormality, gestational diabetes, and abnormalities in blood coagulation) were all excluded (Supplementary Table 1). G-band karyotyping indicated that the mother in family 1 had 46,XX, inv(16)(p11.2q11.2), while the father had a normal karyotype. Karyotype tests were not performed on parents of family 2 (Supplementary Table 1). In family 1, IV-1 was

born at 30 weeks' gestation because of non-reassuring fetal status, and died at 13 days because of respiratory distress. After that, nine consecutive miscarriages occurred. IV-2, showing fetal swelling, miscarried at 8 weeks' gestation. IV-7 with oligohydramnios miscarried at 16 weeks' gestation. IV-10 with fetal growth restriction and oligohydramnios miscarried at 16 weeks' gestation. In IV-3, IV-4, IV5, IV-6, IV-8, and IV-9, fetal heart beats were undetected by ultrasound test and karyotyping of aborted samples was not done. As materials were available only from IV-2 in family 1 and IV-1 in family 2, no further genetic information was obtained in other aborted products. In family 2, fetal abnormality of IV-1 and IV-2 could not be detected by ultrasound examinations. We obtained parental samples and miscarriage tissue from only one fetus from each family (IV-2 in family 1 and IV-1 in family 2). No further clinical information was available.

A homozygous *NOP14* mutation was identified in two families

WES was performed using DNA from two miscarriages (IV-2 in family 1 and IV-1 in family 2). Read coverage of coding regions is summarized in Supplementary Information (Supplementary Table 2). Considering the recurrence of miscarriages and consanguineous marriage in both families, autosomal recessive inheritance of pathogenic variants was first considered (Supplementary Tables 3 and 4). We checked homozygous and compound heterozygous variants in known disease genes registered in HGMD. However, we could not find any candidate variants in registered genes associated with RPL. Next, we checked the candidate genes shared by both families, and found an identical homozygous variant in a gene encoding *NOP14* nucleolar protein (*NOP14*, MIM611526, NM_003703.2, c. [136C>G]; [136C>G], p.[Arg46Gly];[Arg46Gly]) in both families. Sanger sequencing confirmed that both miscarriages were homozygous for this variant and that their parents were heterozygous for the variant (Fig. 1). This variant had been unregistered in our in-house Japanese and Iranian exome data, ESP6500, HGVD, and GME Variome including 87 Iranian exome data [13], but was registered in ExAC with an extremely low MAF of 0.006% (7/118,514

Fig. 2 The *NOPI4* variant and its evolutionary conservation. **a** Electropherograms of the *NOPI4* variant (c.136C>G) in two families. **b** *NOPI4* protein structure and evolutionary conservation of the variant. p.Arg46Gly occurs within the nucleolar protein 14 domain, as determined by Pfam (<http://pfam.xfam.org/protein/P78316>). (Color figure online)



alleles, all as heterozygotes) (Table 1 and Supplementary Table 5). In addition, only one of 170 Iranian control individual had this variant as heterozygous state; thus, the MAF of this variant was calculated to be (1/340 alleles, 0.29%) in this ethnic population (Table 1). In addition, this amino acid change was predicted as pathogenic by SIFT, PolyPhen2, and MutationTaster (Table 1). This altered amino acid residue is highly evolutionarily conserved from zebrafish to human, and is located within the nucleolar protein 14 domain by Pfam (<http://pfam.xfam.org/>) (Fig. 2). The pRec score in ExAC, representing the probability of intolerance of homozygous loss of function variants, was 0.98 (pathogenic if the pRec score is more than 0.9) (Table 1) [11].

Because the two consanguineous families are of the same ethnic background and had an identical missense variant, we checked haplotype blocks that include the variant. As expected, the *NOPI4* variant was located within the same 526 kb haplotype block which was easily confirmed as a homozygously stretched region (chr4: 2,513,615–3,039,150) (Supplementary Table 6). No other pathogenic CNVs involving *NOPI4* or genes known to be associated with RPL or Mendelian disorders were detected.

Discussion

We enrolled two consanguineous families with RPL. In family 1, fetal hydrops, oligohydramnios, and fetal growth restriction occurred, but in family 2, no such anomalous condition was recognized. Consanguinity in families 1 and 2 prompted us to investigate genetic factors, regardless of clinically different presentation. Interestingly, we identified a homozygous *NOPI4* variant possibly contributing to RPL in two families. Identified variant (c.136C>G) with *NOPI4* was unregistered at least in 142 Iranian exome data (total 284 alleles) from both in-house and GME variome databases. Additionally, this mutation of MAF was also rare (0.29%) in Iranian controls. Therefore, this mutation was denied as a common variant in Iranian population. *NOPI4* is evolutionarily conserved among eukaryotes and is a nuclear protein required for processing 18S rRNA and for 40S ribosome biogenesis [15]. Ribosomes are the site of protein synthesis and consist of 79 ribosomal proteins that form two subunits, known as small (40S) and large (60S) subunits [16]. The 40S subunit contains the 18S rRNA and 33 ribosomal proteins [16]. *NOPI4* is essential for the embryonic development of different organs and structures. It was previously reported in zebrafish that homozygous *nop14* mutation (likely loss of function) using retroviral-mediated insertional mutagenesis led to a lethal phenotype with a slightly thinner body, a rudimentary liver and gut,

and pericardial edema at 5 days after fertilization, indicating that *nop14* is essential for early embryonic development [17–19]. Similarly, it is known that *nop14*-null yeast is inviable [20]. Therefore, NOP14 should be essential for early developments in various species.

In addition, it is known that interaction between NOP14 and EMG1 (essential for mitotic growth 1, MIM611531, NM_006331.7) is required for maturation of 18S rRNA and 40S ribosome biogenesis in yeast and human [15, 21, 22]. EMG1 is an essential nucleolar RNA methyltransferase in humans. One homozygous *EMG1* mutation (p.Asp86Gly) causes a lethal autosomal recessive disorder, Bowen–Conradi syndrome (MIM 211180), characterized by severe pre-natal and post-natal growth restriction, profound psychomotor restriction, bone marrow failure, and lethality in the early infantile period [22]. EMG1 is recruited to the nucleolus by a complex comprising NOP14, NOC4L, and UTP14A, and plays an essential function in the development of the 40S ribosomal subunit [21].

The mother of family 1 has a pericentric inversion, 46,XX,inv(16)(p11.2q11.2). This inversion has been reported as a polymorphism [23]. Karyotyping of II-3 and II-4 in family 1 was not examined because they were already deceased, and two pregnancy losses occurred between II-3 and II-4. Therefore, it is possible that 46,XX,inv(16)(p11.2q11.2) may potentially contribute to the RPL between III-1 and III-2 as well as between II-3 and II-4 in family 1, but we could not confirm the karyotype of II-3 and II-4. To our knowledge, four individuals with a pericentric inversion between 16p11.2 and 16q11.2 have been reported with the following phenotypes: a 13-year-old boy with hypogonadism and his phenotypically normal father in one family [24], and a girl with no abnormal phenotype and her healthy father in another family [25]. However, there is no clear evidence as to whether the female with this inversion has PRL or not. Pericentric inversion of chromosome 16 should be extremely rare [26]. In addition, recent studies suggest that parents with chromosomal polymorphisms experience higher rates of RPL compared with the normal population [27–29]. Therefore, we cannot rule out the possibility that the inversion might have additive effects on RPL in family 1. In family 1, nine consecutive miscarriages occurred. If the homozygous variant is a sole factor for these exceptional events, the incidence should be extremely rare, $[(1/4)^9 = 1/262,144]$. We should have examined other aborted samples, but unfortunately no other samples were available for further testing.

In conclusion, we identified an identical homozygous *NOP14* variant as a possible contributing factor for RPL in two Iranian families. In genetic counseling for both families, preimplantation genetic diagnosis might be an option to avoid the homozygous variant. As our data are based on

only two families, further investigations are needed to determine *NOP14* functions in human development.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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