Pathogenic variants of meiotic double strand break (DSB) formation genes PRDM9 and ANKRD31 in premature ovarian insufficiency

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PURPOSE: The etiology of premature ovarian insufficiency (POI) is heterogeneous, and genetic factors account for 20–25% of the patients. The primordial follicle pool is determined by the meiosis process, which is initiated by programmed DNA double strand breaks (DSB) and homologous recombination. The objective of the study is to explore the role of DSB formation genes in POI pathogenesis.

METHODS: Variants in DSB formation genes were analyzed from a database of exome sequencing in 1,030 patients with POI. The pathogenic effects of the potentially causative variants were verified by further functional studies.

RESULTS: Three pathogenic heterozygous variants in PRDM9 and two in ANKRD31 were identified in seven patients. Functional studies showed the variants in PRDM9 impaired its methyltransferase activity, and the ANKRD31 variations disturbed its interaction with another DSB formation factor REC114 by haploinsufficiency effect, indicating the pathogenic effects of the two genes on ovarian function were dosage dependent.

CONCLUSION: Our study identified pathogenic variants of PRDM9 and ANKRD31 in POI patients, shedding new light on the contribution of meiotic DSB formation genes in ovarian development, further expanding the genetic architecture of POI.

INTRODUCTION

Premature ovarian insufficiency (POI) is characterized by amenorrhea, infertility, and elevated level of follicle stimulation hormone (FSH > 25 IU/L) in women under the age of 40 [1]. According to the recent meta-analyses of Golezar et al., 3.7% of women are affected by POI [2]. The etiology of POI is heterogeneous, and known causes include genetic, autoimmune, iatrogenic, and infectious factors [3]. Approximately 20–25% of cases have genetic defects [4]. Until now, dozens of genes have been reported to be responsible for POI. However, over 50% of patients are still idiopathic.

Meiosis is initiated by programmed DNA double strand break (DSB) and homologous recombination (HR). Oocytes arresting at the diplotene stage of meiosis I determine the primordial follicles pool and reproductive lifespan of women [5]. In the recent years, with the widespread application of exome sequencing, the contribution of meiotic DSB formation genes in POI etiology, such as PRDM9 and ANKRD31, and the pathogenic effects of variants were further explored.

MATERIALS AND METHODS

Participants

The 1,030 POI patients recruited in this exome sequencing project were women with (1) primary or secondary amenorrhea before 40 years of age and (2) at least twice serum follicle-stimulating hormone (FSH) > 25 IU/L at an interval of 4 to 6 weeks. Most of the patients (91.75%) were recruited from Shandong province, China; others were from the north (4.88%) and south (1.86%) of China. Patients with chromosomal abnormalities, ovarian surgery, chemo/radiotherapy, and known autoimmune disease (such as systemic lupus erythematosus, Sjogren syndrome, rheumatoid arthritis, and autoimmune thyroiditis) were excluded. Written informed consents were obtained from all participants.

Exome sequencing

Peripheral blood was collected from the patients, and the DNeasy Blood & Tissue Kit (Qiagen) was used to isolate the genomic DNA from leukocytes. Exome capture was carried out with SureSelect Target Enrichment System, and sequencing was performed on the Illumina platform (Illumina HiSeq). Reads were aligned against the National Center for Biotechnology Information (NCBI) hg19 reference human genome. Variants were called using ANNOVAR and Genome Analysis Toolkit. The variations in DSB formation genes, including SPO11, PRDM9, EWSR1, HELLS, MEI1, ME4, IHO1, ANKRD31, REC114, and TOPOVIBL, were selected and classified according to American College of Medical Genetics and Genomics/Accociation for Reproductive Medicine, Cheeulo College of Medicine, Shandong University, Jinan, Shandong, China. 4National Research Center for Assisted Reproductive Technology and Reproductive Genetics, Shandong University, Jinan, Shandong, China. 5Key laboratory of Reproductive Endocrinology of Ministry of Education, Shandong University, Jinan, Shandong, China. 6Shandong Provincial Clinical Medicine Research Center for Reproductive Health, Shandong University, Jinan, Shandong, China. 7These authors contributed equally: Yiyang Wang, Ting Guo. 8email: qinyingying1006@163.com

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Plasmid construction and expression in HEK293 cells

The ANKR3D1 functional fragment (NM_001164443.1: 351-1299aa deletion) was synthesized by Changsha Zeqiong Biotechnology Limited Company and cloned into the pcDNA3.1 vector with 3X FLAG tag at the N-terminus. REC114 (NM_001042367) plasmid with HA tag at C-terminus and PRDM9 plasmid (NM_020227.3) with 3X FLAG tag at N-terminus were constructed in the same way. The point variants were generated using the QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies).

HEK293 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) with 10% fetal bovine serum and 1% penicillin-streptomycin (Gibco) at 37°C. The plasmids were transfected into HEK293 cells using Lipofectamine 3000 Transfection Reagent (Invitrogen) according to the manufacturer’s protocol.

Immunofluorescence microscopy

HEK293 cells were cultured and transiently transfected with wild-type or mutant plasmids for 48 hours. Then, the cells were fixed in 4% paraformaldehyde. After permeabilizing with 0.3% Triton X-100 for 20 minutes and blocking with 10% bovine serum albumin for 1 hour, the cells were incubated with FLAG antibody (rabbit, Cell Signaling) at 4°C overnight and the secondary antibody conjugated with Alexa Fluor 488 (Invitrogen) for 2 hours. DAPI (Beyotime) was used to label DNA. The fluorescent images were captured with the fluorescence microscopes (Olympus, Japan).

Western blotting

HEK293 cells were transiently transfected with wild-type or mutant PRDM9 plasmids for 48 hours, then cells were harvested using SDS lysis buffer (Beyotime) supplemented with 1% protease inhibitor cocktail (Sigma). Equal amounts of protein were separated on polyvinylidene fluoride membranes, blocked with 5% nonfat milk diluted in Tris-buffered saline and Tween 20, followed by incubating with antibodies against FLAG (rabbit, Immunoway), H3K4me3 (rabbit, EpiGenTek), histone H3 (rabbit, MilliPore). After incubating with the secondary antibodies, the blots were subjected to chemiluminescent detection with Chemidoc MP System (Bio-Rad). The grayscale of bands was quantified by Image J software.

In vitro ovarian culture

To elucidate the effect of PRDM9 heterozygous loss-of-function variations on follicle survival, we obtained Pdrm9 heterozygous knockout mice from Professor Dianying Li at Shanghai University [15]. Ovaries were dissected from the wild-type or heterozygous Pdrm9 knockout mice at postnatal 5 days (PDS) and washed three times in Leibovitz’s L-15 medium (Gibco) containing 10% fetal bovine serum and 1% penicillin-streptomycin. Then, the ovaries were transferred into the culture inserts (Millipore) in a 6-well culture plate. The medium containing DMEM/Nutrient Mixture F-12 (DMEM/F12) (Gibco) with 5% insulin–transferrin–selenium–(Sigma), 1 mg/mL BSA (Sigma), 1 mg/mL Albumax II (Gibco), 100 μM L-ascorbic (Sigma), and 1% penicillin–streptomycin was used for the in vitro culture. A drop of medium from the well was placed to cover the top of the ovary to prevent drying. Then, the ovaries were cultured at 37°C and 5% CO2 for 8 days, and the medium was changed every 2 days. The 4-vinylcyclohexene diepoxide (VCD) (30 μM), which destroys primordial and primary follicles by accelerating the apoptosis of oocytes [16], was used to induce exogenous stress to challenge follicle survival.

Histology and immunostaining

Ovaries cultured in vitro were fixed in 4% PFA, dehydrated, embedded in paraffin, and sectioned at 5-μm thickness. For Immunostaining, sections were incubated at 4°C overnight with DDX4 antibody (Goat, Bio Techne) and cleaved PARP antibody (Rabbit, CST), then the sections were incubated with specific secondary antibodies for 1 hour. DAPI was used to mark nucleus. Images were captured with fluorescence microscopes (Olympus, Japan).
| Gene name | Gene function | Patient number | Variants identified | Clinical characteristics |
|-----------|---------------|----------------|--------------------|-------------------------|
| PRDM9    | Determine the location of DSB recombination hotspots, where genetic recombination occurs | 27              | Het                | p.Arg77* |
|          | Act as a scaffold to anchor REC114 and MEI4, thereby regulating DSB formation | 33              | Het                | p.Gln329* |
| ANKRD31  | SI conversion factor to convert FSH to IUL, multiply by 1.0, to convert E2 to pmol/L, multiply by 3.671, H3K4me3 level was significantly lower in the three mutant groups than the wild-type group (Fig. 1e, f), indicating the H3K4me3 modification was more susceptible to exogenous stress. | 13              | Het                |
|          |                        | 16              | Het                |
|          |                        | 15              | Het                |
|          |                        | 15              | Het                |
|          |                        | 14              | Het                |
|          |                        | 13              | Het                |

**Table 1.** Pathogenic variants of DSB formation genes identified in POI patients and their clinical characteristics.

**Clinical characteristics**

- **Age at menarche**
- **Menopause onset, years**
- **Stature, months**
- **BMI ng/mL**
- **AMH, mIU/mL**
- **FSH, ng/mL**
- **E2, pg/mL**

**ACMG/AMP genotype classification**

- **Pathogenic**
- **Variants identified**
- **Patient number**

**Pathogenic**

- **PRDM9**
  - p.Arg77*
  - p.Lys226Met
  - p.Ile213Ser

- **ANKRD31**
  - p.Gln329*

- **SI conversion factor:** To convert FSH to IU/L, multiply by 1.0; to convert E2 to pmol/L, multiply by 3.671.

**DISCUSSION**

In the prophase of meiosis I, accurate DSB localization and formation are crucial for homologous chromosome recognition and pairing.

Plasmids were overexpressed in HEK293 cells. Immunofluorescence against FLAG showed that wild-type PRDM9, mutant p.Lys226Met and p.Ile213Ser were expressed in the nucleus. However, significant reduced nuclear staining of FLAG was observed in cells overexpressing mutant p.Arg77* (Fig. 1d), indicating the truncated protein was resided in cytoplasm due to loss of NLS sequence.

Three **PRDM9** mutants adversely affected their histone methylation activity.

The PRDM9-dependent H3K4 trimethylation (H3K4me3) is essential for determination of recombination hotspots. To illustrate whether the three variants of PRDM9 affected its methylation activity, the level of H3K4me3 was tested among the cells overexpressing wild-type or mutant PRDM9. Results showed that H3K4me3 level was significantly lower in the three mutant groups than in the wild-type group (Fig. 1e, f), indicating the H3K4 methylation transferase activity of PRDM9 was impaired by the three variants.

**Pdmd9**/− oocytes were more vulnerable to exogenous stress.

To elucidate the impact of heterozygous loss-of-function variants on oocytes survival, VCD was used to induce exogenous stress. The oocytes were dissected from Pdmd9+/− and Pdmd9+/− mice at PDS and cultured for 8 days with VCD. The ovary sections stained with cleaved PARP showed more apoptotic oocytes in Pdmd9+/− ovaries (Fig. 51), indicating the heterozygous knockout oocytes were more susceptible to exogenous stress.

ANKRD31 p.Gln329* affected ANKRD31–REC114 interaction

As a meiosis-specific protein, ANKRD31 interacts with REC114 to stabilize and regulate the binding of DSB formation factors (MEI4 and IHO1) onto chromatin [13, 18]. It has been shown that the CR5 region of ANKRD31 directly bonds to the N-terminus of REC114, facilitating the generation of ANKRD31–REC114 heterodimers [13]. Therefore, Co-IP was performed to observe whether the nonsense variant p.Gln329* locating before the CR5 domain impaired the REC114 interaction. The result showed that, compared to the wild-type ANKRD31, the truncated protein p.Gln329* had significantly weaker binding with REC114 (Fig. 2d). Furthermore, to illustrate the dominant or haploinsufficiency effect of the heterozygous variant, p.Gln329* and wild-type plasmids were cotransfected into cells with the ratio of 1:1. The level of REC114 pulled down by the cotransfected ANKRD31 was slightly higher than mutant, while lower than wild type, indicating the heterozygous mutant p.Gln329* affected its interaction with REC114 by haploinsufficiency effect.

ANKRD31 c.1565-2A>G resulted in truncated transcripts.

To clarify whether the variant c.1565-2A>G disrupted RNA splicing, minigene assay was performed. The pcMINI vector with exon a–intron 10 (245 bp)–exon 11 (143 bp)–intron 11 (332 bp)–exon B (exon A and exon B were the existing exons in pcMINI vector) produced two transcripts both in HEK293 and MCF-7 cells: (1) the longer belt (belt b) contained 16 bp of intron 11 and full length of intron B without exon 11; (2) the shorter belt (belt c) lacked exon 11 (Fig. 2e, f). Both two splicing modes would cause frameshift and premature termination, producing two transcripts without the CR5 domain. Therefore, the splice site variant c.1565-2A>G would exert similar pathogenic effect as nonsense variant p.Gln329* with an impaired interaction with REC114.
Recent studies have found biallelic variants of MEI1 and SPO11 in males with familial nonobstructive azoospermia [20, 21], and variants of TOROVIBL, MEI1, and REC114 in females with recurrent miscarriage and hydatidiform moles [22, 23]. These studies revealed the essential role of DSB formation genes in human gametogenesis.

In the present study, pathogenic variants of PRDM9 and ANKRD31 were identified in POI patients, giving the evidence of roles of meiotic DSB formation genes in the maintenance of human follicle pool and ovarian function.

Meiotic DSBs are generated by SPO11 at the hotspots marked by PRDM9-catalyzed H3K4me3 on open chromatin [24]. In Pdmd9<sup>−/−</sup> mice, DSBs were initiated at PRDM9-independent H3K4me3 sites, such as promoters and enhancers, resulting in aberrant synapsis and recombination that led to germ cell apoptosis [25, 26]. The Pdmd9<sup>−/−</sup> mice showed accelerated oocyte loss from embryonic day 17.5 and infertility, which was similar to the ovarian phenotype of human POI [10]. In the present study, three novel heterozygous variants of PRDM9 were identified in four POI patients. Among them, the variant p.Lys226Met and p.Ile213Ser, which localized in the PR/SET domain, adversely affected the methyltransferase activity of PRDM9. Whereas the nonsense variant p.Arg77*, which resulted in the loss of NLS, PR/SET, and zinc finger domain, not only disturbed the nuclear localization of PRDM9 but also abolished its methyltransferase activity.

Fig. 1 Three pathogenic variants of PRDM9 identified in premature ovarian insufficiency (POI) patients affected its methyltransferase activity. (a) Chromatograms of the three heterozygous variants. (b) The mutant amino acids were highly conserved in mammals. (c) PRDM9 c.229C>T (p.Arg77*) localized in the KRAB domain, before the nuclear localization signal (NLS) sequence; both PRDM9 c.638T>G (p.Ile213Ser) and PRDM9 c.677A>T (p.Lys226Met) localized on PR/SET domain (residues 195–415), which determined the methyltransferase activity of PRDM9. (d) HEK293 cells were transiently transfected with wild-type (WT) or mutant PRDM9 expression vectors, the subcellular location of PRDM9 protein were indicated by FLAG (green). Scale bar: 20 μm. (e) H3K4me3 was detected by western blot in HEK293 cells overexpressing empty vector (EV), wild-type (WT) or mutant PRDM9-FLAG. (f) The relative grayscale of H3K4me3 was calculated with the use of ImageJ, and compared between subgroups.

[19] Recent studies have found biallelic variants of MEI1 and SPO11 in males with familial nonobstructive azoospermia [20, 21], and variants of TOROVIBL, MEI1, and REC114 in females with recurrent miscarriage and hydatidiform moles [22, 23]. These studies revealed the essential role of DSB formation genes in human gametogenesis. In the present study, pathogenic variants of PRDM9 and ANKRD31 were identified in POI patients, giving the evidence of roles of meiotic DSB formation genes in the maintenance of human follicle pool and ovarian function.
Fig. 2  **ANKRD31 p.Gln329* impaired ANKRD31–REC114 interaction and c.1565-2A>G affected RNA splicing.** (a) Chromatograms of the two heterozygous variants in ANKRD31. (b) The variants were highly conserved among species. (c) **ANKRD31 c.985C>T (p.Gln329*)** localized in exon 7, before conserved region 5 (CR5), which was responsible for interaction with REC114. Splice site variant c.1565-2A>G localized at the donor splice site of intron 10. (d) Coimmunoprecipitation (Co-IP) analysis showed **ANKRD31 p.Gln329* generated a truncated protein and impaired ANKRD31–REC114 interaction.** (e) After 48 hours of transfection in two human cell lines (HEK293T and MCF-7 cell), agarose gel electrophoresis showed two belts of c.1565-2A>G transcripts in contrast with wild type. (f) Sequence analysis demonstrated that c.1565-2A>G variant caused two transcripts: belt b lacked exon 11 while contained 16 bp of intro11 and intron B; belt c skipped exon 11.
localization of PRDM9, but also suppressed the recognition and methylation of target DNA motifs. Therefore, due to the reduced PRDM9-dependent H3K4me3 sites and thereby defective meiosis HR, women carrying the three variants might experience accelerated oocyte apoptosis. Interestingly, the function of PRDM9 has been proved to be dosage sensitive. Prdm9+/− mice were sub fertile, and showed increased percentage of germ cells at abnormal pachytene stage with decreased number of PRDM9-dependent DSBs and insufficient recombination [27, 28]. Moreover, in response to VCD induced exogenous stress, the Prdm9+/− oocytes demonstrated with increased level of apoptosis, which might explain the reduced number of follicle in Prdm9+/− ovaries [29], and the patients carrying heterozygous PRDM9 variants presented with secondary amennorrea in this study. Intriguingly, the age of POI onset was much earlier in the two patients carrying variant p.Arg77* (23 years and 27 years, respectively) compared with women carrying p.Lys226Met and p.Ile213Ser (31 years and 33 years, respectively). The former variant lost the NLS and following functional domains of PRDM9, whereas the latter only affected its methyltransferase activity, suggesting the different variation sites might be responsible for the heterogeneous phenotypes of women with POI.

As an essential component of DSB-targeting and control machinery, ANKRD31 interacted with REC114, which stabilized and regulated the localization of DSB formation machinery onto the chromosome axis [13, 18]. ANKRD31 deficiency led to elevated germ cell apoptosis due to altered DSB number, timing, and location. Although Ankrd31−/− female mice were fertile, they were observed to have 4.95-fold lower median oocyte numbers than that in wild-type mice at 6–7 weeks of age, which lost fecundity much earlier [18]. In our study, two heterozygous variants in ANKRD31 were identified in three patients. Mutant p.Gln329* generated a preterminated protein lacking CRS region, which impaired the ANKRD31–REC114 heterodimer formation, while the truncated protein induced by splice site variation identified in ANKRD31 were dosage sensitive. ANKRD31 interacted with REC114, which stabilized and regulated the localization of DSB formation machinery onto the chromosome axis [13, 18]. ANKRD31 deficiency led to elevated germ cell apoptosis due to altered DSB number, timing, and location. Although Ankrd31−/− female mice were fertile, they were observed to have 4.95-fold lower median oocyte numbers than that in wild-type mice at 6–7 weeks of age, which lost fecundity much earlier [18]. In our study, two heterozygous variants in ANKRD31 were identified in three patients. Mutant p.Gln329* generated a preterminated protein lacking CRS region, which impaired the ANKRD31–REC114 heterodimer formation, while the truncated protein induced by splice site variation c.1565-2A>G also lost the CRS domain, which would cause dysfunctional DSB formation and meiosis due to the similar effect of variant p.Gln329*. Furthermore, Ankrd31−/− mice had increased default DSBs and delayed RAD51 recruitment, suggesting ANKRD31 had a dosage-dependent effect during DSB formation and meiosis [13]. Combined with the observation that the level of REC114 pulled down by cotransfected ANKRD31 (wild-type and mutant) was lower than that pulled down by wild-type protein, the heterozygous variants in ANKRD31 were suggested to cause POI by the haploinsufficiency effect.

The mechanism that most genes causing ovarian failure by biallelic defects in animal models are heterozygous pathogenic in human has long been discussed. This could be explained partially by a dominant-negative effect, such as the Nbox heterozygous variation identified in POI patients [30]. Some other genes presented a dosage-dependent effect on ovarian function both in animal models and patients; for example, heterozygous Bnc1 and Fanca knockout mice showed impaired meiosis or compromised DNA repair [31, 32]. Similarly, the women carrying heterozygous variants were predisposed to POI. Furthermore, some genes without POI phenotypes in heterozygous knockout mice, such as STAG3, MCM8, and FANCL, were found responsible for human POI in heterozygous condition. Haploinsufficiency effect has also been suggested by functional studies [33–36]. The finding that heterozygous pathogenic variants of DNA repair and meiosis genes are related to the POI phenotypes raises the possibility that functional integrity of these genes may be not requisite to maintain ovarian function along the reproductive lifespan but their partial deficiency could lead to POI. In the present study, considering the dosage-dependent effect of PRDM9 and ANKRD31 on meiosis and ovarian function in mice, female heterozygotes might be predisposed to POI by haploinsufficiency effect as well.

The follicle pool is determined by the number of primordial germ cells migrating to the genital ridge, followed by germ cell proliferation and functional meiosis, established as the number of primordial follicles at puberty, which will be activated by FSH and recruited to be antral follicles for ovulation [37]. Defects and dysfunction during these processes would cause insufficient follicle formation or accelerated follicle depletion, resulting in POI. The genes involved in follicle activation, development, and steroid hormone synthesis, such as FIGLA, NOBOX, and FSHR [6], were identified to be causative for POI. With the rapid development of next-generation sequencing, the spectrum of POI candidate genes has been greatly expanded. Intriguingly, an increasing number of genes involved in meiotic processes have been found, such as DSB end processing genes EXO1, MND1, and MEIOB; homologous recombination genes RAD51, BRCAA2, MSH4, and MSH5 [38–44]; and synaptonemal complex genes SYCE1, SYCP3, and STAG3 [45–47]. The findings in DSB formation genes PRDM9 and ANKRD31 further enriched the knowledge of meiotic genes in POI pathogenesis.

In conclusion, we identified pathogenic variants in PRDM9 and ANKRD31; these findings expanded the genetic spectrum of POI, further highlighting the essential role of meiotic DSB formation genes in maintenance of ovarian function.

DATA AVAILABILITY

The exome sequencing data sets supporting the study have not been deposited in a public repository because of privacy and ethical restrictions but are available from the corresponding authors on request.

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