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Variation Analysis of *TMEM150B* Gene in Chinese Women with Premature Ovarian Insufficiency

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TMEM150B gene plays a role in autophagy and is closely associated with age at natural menopause, early menopause and POI. No plausible causative variants of *TMEM150B* gene contributing to POI were identified, indicating that mutations in *TMEM150B* gene may not be a common cause for POI in Han Chinese women.

Abstract

Research Question: *TMEM150B* gene, which promotes cell survival under stress condition by modulating autophagy, is closely associated with age at natural menopause, early menopause and premature ovarian insufficiency (POI) in European

women. However, whether gene variants of *TMEM150B* contribute to pathogenesis of POI needs to be determined.

Design: A case-control genetic study was performed in which all exons and exon-intron boundaries of *TMEM150B* gene were screened in 408 Han Chinese women with non-syndromic POI by Sanger sequencing and the results were analyzed by statistics and bioinformatics.

Result: Two novel variants located in 3' untranslated region of the *TMEM150B* gene were identified, but neither was potentially disease-causing indicated by bioinformatic analyses. Besides, six known single-nucleotide polymorphisms (SNPs) were found, and they were not potentially causative for POI.

Conclusion: The results suggest that the perturbations in *TMEM150B* gene are not a common explanation for POI in Chinese women. The role of autophagy played in pathogenic mechanism of POI needs further exploration.

Key message:

TMEM150B gene plays a role in autophagy and is closely associated with age at natural menopause, early menopause and POI. No plausible causative variants of *TMEM150B* gene contributing to POI were identified, indicating that mutations in *TMEM150B* gene may not be a common cause for POI in Han Chinese women.

Keywords:

Premature ovarian insufficiency, Early menopause, *TMEM150B*, Autophagy, Variant screening

Introduction

Premature ovarian insufficiency (POI) is a clinical syndrome defined by loss of ovarian activity before the age of 40. POI is characterized by menstrual disturbance (amenorrhea or oligomenorrhea) with raised gonadotropins and low estradiol (De Vos et al., 2010; Laven, 2016). The POI prevalence was reported to be 2.8% in Chinese women (Wu et al., 2014). POI not only causes infertility, but also is prone to result in chronic diseases such as cardiovascular diseases and osteoporosis, which seriously jeopardize women's long-term health.

The etiology of POI is complicated and highly heterogeneous. Genetic factors, iatrogenic factors, autoimmunity dysfunction, metabolic factors, infections and environmental factors have been reported to cause the occurrence and development of POI (Beck-Peccoz and Persani, 2006). Chromosomal abnormality is one of the main causes of POI, accounting for 10% to 15% of POI patients (Jiao et al., 2012). Causative genes of POI have been discovered, such as *FSHR*, *BMP15*, *NOBOX*, *FIGLA*, *NR5A1*, *STAG3*, *MCM8*, *CSB-PGBD3*, *MSH5*, *e*tc., related with follicular development, meiosis and DNA repair, respectively (Qin et al., 2015a; Qin et al., 2015b; Guo et al., 2017). However, their mutation frequency in POI patients is usually no more than 1-2%. Overall, genetic factors can explain about 20-25% of all cases (Qin et al., 2015b). However, the etiology in over 50% of POI patients is still confusing and needs to be illustrated.

The ovarian reserve is exhausted with increasing age, leading to menopause which represents the loss of female reproductive potency. It has been reported that the mean age at natural menopause (ANM) was 48.8 years with considerable variation (Davis et

al., 2015). The menopause occurring before the age of 40 is diagnosed as POI, whereas women experiencing menopause under the age of 45 are defined as early menopause (EM) (Shelling, 2010). Recent studies have suggested that common genetic susceptibility loci are shared by ANM, EM and POI (Day et al., 2015; Laisk-Podar et al., 2016; Murray et al., 2011; Perry et al., 2013; Qin et al., 2012; Stolk et al., 2012), and further studies have indicated that some genes related to ANM, such as *MCM8* and *MSH5*, also play an important role in POI (AlAsiri et al., 2015; Guo et al., 2017). *TMEM150B* gene is another candidate gene which is significantly associated with ANM and EM (Perry et al., 2013; Stolk et al., 2012), with the SNP rs11668344 located in intron 2 of *TMEM150B* gene showing the most significant association with POI (Perry et al., 2013).

TMEM150B gene encodes the protein called DRAM-related/associated member 3 (DRAM-3), which has been reported to promote cell survival in stress condition by prompting autophagy (Mrschtik et al., 2015). Autophagy is a self-degradation of intracellular components, which is highly conserved between species. It participates in many biological processes, including adaption to stress, development, and cell death, and the autophagy dysregulation is involved in pathogenesis of human diseases, such as aging, cancer and neurodegenerative diseases (Wirawan et al., 2012). Accumulated evidence also shows that maintenance of autophagy homeostasis is crucial for normal ovarian function (Choi et al., 2014; Gawriluk et al., 2011; Gawriluk et al., 2014; Shen et al., 2017; Song et al., 2015). Given the pivotal role of *TMEM150B* played in autophagy, it might be a potential candidate gene for POI. In this study, we sequenced the

TMEM150B gene in a large cohort of Han Chinese women with idiopathic POI to determine whether variants in this gene contribute to human POI.

Materials and methods

Patients

A total of 408 Han Chinese women suffering from idiopathic POI were recruited from the Hospital for Reproductive Medicine Affiliated to Shandong University. Inclusion criteria were defined as primary or secondary amenorrhea before 40 years old, at least two measurements of serum FSH concentrations > 40 IU/L, without abnormal karyotype and family history of POI. Furthermore, patients who were affected by autoimmune disorders or had undergone radiotherapy, chemotherapy or ovarian surgery were excluded. The clinical characteristics of all the patients are summarized in Table 1. Informed consent was signed by each participate. We obtained the data of controls' genotype and allele frequencies from East Asian population in 1000 Genomes Browser (http://www.internationalgenome.org/). The study was approved by the Institutional Review Board of Reproductive Medicine of Shandong University on 30 May 2016 (reference number 24).

Variants screening and bioinformatic analysis

Genomic DNA was extracted from peripheral blood samples of the patients using QIAamp DNA Blood Mini Kits (QIAGEN, Hilden, Germany). All eight exons, intron-exon boundaries and rs11668344 of the *TMEM150B* gene (Reference sequence NC_000019.10) were amplified by polymerase chain reaction (PCR) through 7 pairs of

primers (Table 2). Following analyzed by agarose gel electrophoresis, the PCR products were purified with the method of polyethylene glycol precipitation and sequenced on ABI 3730XL DNA analyzer (Applied Biosystems, Forster City, CA) using the ABI-Prism big-dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). All variants were confirmed by forward and reverse sequencing from three independent experiments. The online tool RegRNA (http://regrna.mbc.nctu.edu.tw/) was utilized to predict the binding of microRNAs to the 3'untranslated region (UTR) of *TMEM150B* mRNA.

Statistics

The sequencing results were analyzed with Sequencer 4.9 software. Statistical analysis was conducted to comparing frequencies of genotype and allele between patients and controls using the chi-squared test or Fisher's exact test where appropriate with the Statistical Package for Social Sciences version 17.0 (IBM Inc., USA). Statistical significance was considered when $P < 0.05$.

Results

As shown in Table 3, eight variants of *TMEM150B* were identified in 408 Chinese women with POI. Two novel variations were located in 3'UTR of exon 8, which did not change microRNA binding as predicted by RegRNA software. The remaining six variants were known single-nucleotide polymorphisms (SNPs) in the *TMEM150B* gene. Two of the six SNPs were synonymous and localized in exon 7 (rs375066257) and exon 8 (rs369826666) respectively; three of them were missense SNPs located in exon

4 (rs774183872), exon 7 (rs199806152) and exon 8 (rs7246479), respectively; the rest one was an intronic SNP (rs75629207). For allele frequencies of these six SNPs, there was no significant difference between POI cases and controls. Comparison of the genotype frequencies for five SNPs located in exonic regions showed no significant difference between cases and controls, but the genotype frequency of the intronic SNP (rs75629207) was different between cases and controls. However, this intronic variant may not affect splicing because of its long distance from the exon-intron boundary. Besides, we detected the intronic SNP rs11668344 in our POI patients and found no significant differences in both genotype and allele frequencies compared with the control population.

Discussion

About 10% of women experience their final menses before age of 45 years (early menopause), and about 1% develop POI by the age of 40 years (Coulam CB et al., 1986). It was reported that idiopathic POI and early menopause shared similar genetic pattern and were considered as variable expressions of a same genetic disease (M.G.Tibiletti, 1999), which has been supported by recent genetic studies and some common susceptibility genes identified in POI (Qin et al., 2012; Perry et al., 2013). Mutations in some genes associated with ANM, such as *MCM8* and *MSH5*, resulted in POI by disrupting DNA repair process, which were verified by animal studies (AlAsiri et al., 2015; Guo et al., 2017). Indicated by the genome-wide association studies, not only was *TMEM150B* found closely correlated with ANM (Stolk et al.,

2012; Day et al., 2015), but also was identified as a gene significantly associated with EM and POI (Perry et al., 2013).

TMEM150B encodes a 233-aa protein with five putative transmembrane domains and a predicted N-terminal signal peptide. TMEM150B protein is also named DRAM-3, a member of the protein family of DRAM (damage regulated autophagy modulator). DRAM-3 mainly localizes to endosomes, autolysosomes/lysosomes, and the plasma membrane (Mrschtik et al., 2015). There are 5 members of DRAM family, including DRAM-1, DRAM-2, TMEM150A/B/C. The expression of DRAM-1 can be induced by p53, and it links the tumor suppressor p53 and autophagy. In addition to autophagy, various roles of DRAM1 have been described in several processes, including cell death, immune response and cellular differentiation (Crighton et al., 2006; Mrschtik and Ryan, 2016). The high similarity between TMEM150B and DRAM-1 implies that TMEM150B may play a significant role in autophagy and cell death. Under the condition of sufficient nutrition, TMEM150B was involved in the regulation of cellular autophagy. But, when cells were deprived of glucose, TMEM150B enhanced colony formation and reduced cell death independent of autophagy (Mrschtik et al., 2015). Therefore, TMEM150B is a new modulator for cell autophagy and death. Furthermore, it has been reported that the dysfunction of autophagy could compromise DNA damage repair, causing deleterious effects on integrity maintenance of the genome (Gomes et al., 2017). Due to the positive regulatory role of TMEM150B plays in autophagy, it is possible that TMEM150B has a direct role in DNA repair or maintenance, or there are some up- or downstream regulatory effects of the major SNPs in *TMEM150B* in

autophagy-related DNA repair process.

Autophagy is a highly conserved process of self-degradation in eukaryotic cells. A series of substrates, such as proteins and organelles, are degraded and recycled by lysozyme in autophagic process (Mizushima and Komatsu, 2011). This well-regulated intracellular degradation system plays roles in self-renewal, homeostasis maintenance, metabolism adaptation regulation and genomes integrity control. A series of studies revealed that autophagy abnormality can lead to a variety of diseases such as cancer and neurodegenerative diseases (Green and Levine, 2014; Wirawan et al., 2012). Furthermore, it has been reported that the defects of autophagy could compromise the formation of primordial follicles (Gawriluk et al., 2011; Song et al., 2015). Therefore, it is possible that *TMEM150B* gene variants or protein disorders may cause the POI disease by inhibiting autophagy and thus the establishment of ovarian reserve. In this study, we firstly sequenced *TMEM150B* gene in 408 Chinese women with POI but no potentially causative variants were found. Although difference in the genotype frequency was shown in the intronic SNP (rs75629207), the long distance of this variation site from the exon-intron boundary made it not a potential disease-causing variation for POI. Meanwhile, the intronic SNP rs11668344, which was previously reported significantly correlated with POI in European women (Perry et al., 2013), was not verified in Chinese population. The inconsistent results may be ascribed to ethnic differences.

This study indicated that mutations in *TMEM150B* gene may not be pathogenic for POI in Chinese women. Considering ethnic diversity, *TMEM150B* could not be ruled

out as a candidate gene for human POI. Further studies are required to elucidate the role of autophagy played in pathogenic mechanism of POI.

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Table 1 Clinical characteristics of 408 patients with premature ovarian insufficiency

Characteristic	**Primary Amenorrhea**	**Secondary Amenorrhea**
Number	47	361
Age (years)	26.4±3.3	28.1±4.3
Age at menarche (years)	-	14.5±2.0
Age at irregular menstruation (years)	-	21.0±5.2
Age at amenorrhea (years)	-	21.8±4.74
Serum FSH (IU/L)	70.9±19.2	77.2±24.9

Table 2 Polymerase chain reaction primers used for amplification of human *TMEM150B* gene

Primer	Sequence 5'-3'	Product size (bp)
Exon1-F	CACAAGCGGTTTCCTCAATATG	236
Exon1-R	ATGTGACCTGCTTGGACCCT	
Exon2-F	GAGACAGGACACGGTGGTTTG	425
Exon2-R	CAGGTGCATGAGATTGTGGC	
Exon3-5-F	AGGAGAATGCGTGAGATAGGG	962
Exon3-5-R	GGTTTGAGAAAGGAGCGGTT	
Exon6-F	CCTCTTTCCCCAAAATCCG	940
Exon6-R	ATCCACCTACCTTGGCTTCC	
Exon7-F	CCCAACTTCTCCCTTCTCCT	460
Exon7-R	GGATGACGAAACCATTGACA	
Exon8-F	CGTCTGTGAGGAGCTACGG	435
Exon8-R	TCTCCAGTGGCTCATCTTGC	
Intron2- F	GAGGACAGCTGGCCGATTAG	414
Intron2-R	GGCTTGGTATTTAGGGATTTGG	

The annealing temperature of all reactions is 59°C.

Table 3 Variations of the *TMEM150B* gene identified in Chinese women with premature ovarian insufficiency

Location	dbSNP ID	Sequence variation	Amino acid variation	n	Genotype	Genotype frequency			Allele	Allele frequency		
						POI, %(n)	Control, %(n)[c]	P		POI, %(n)	Control, %(n)[c]	P
Intron2	rs11668344	c.-58+352T>C	-	340	AA	85.59 (291)	80 (208)	0.100[a]	A	91.47 (622)	89.81 (467)	0.325[b]
					AG	13.53 (46)	19.62 (51)		G	8.53 (58)	10.19 (53)	
					GG	0.88 (3)	0.38 (1)					
Exon8	Novel mutation	c.*22T>A	3'UTR	408	TT	99.75 (407)	-	-	T	99.88 (815)	-	-
					TA	0.25 (1)	-		A	0.12 (1)	-	
					AA	0 (0)	-					
Exon8	Novel mutation	c.*52G>T	3'UTR	408	GG	99.75 (407)	-	-	G	99.88 (815)	-	-
					TG	0.25 (1)	-		T	0.12 (1)	-	
					TT	0 (0)	-					
Exon4	rs774183872	c.77T>C	p.Ile26Thr	408	TT	99.75 (407)	-	-	T	99.88 (815)	-	-
					TC	0.25 (1)	-		C	0.12 (1)	-	
					CC	0 (0)	-					
Intron4	rs75629207	c.129-20G>A	-	408	GG	82.84 (338)	88.08 (229)	0.044[a]	G	91.42 (746)	93.86 (488)	0.104[b]
					GA	17.16 (70)	11.54 (30)		A	8.57 (70)	6.15 (32)	
					AA	0 (0)	0.38 (1)					
Exon7	rs375066257 cosm1241833	c.384C>T	Asn128Asn	408	CC	99.51 (406)	100 (260)	0.523[a]	C	99.75 (814)	100 (520)	0.524[a]
					TC	0.49 (2)	0 (0)		T	00.25 (2)	0 (0)	
					TT	0 (0)	0 (0)					
Exon7	rs199806152	c.397C>G	Leu133Val	408	CC	99.75 (407)	100 (260)	1.000[a]	C	99.88 (815)	100 (520)	1.000[a]
					CG	0.25 (1)	0 (0)		G	0.12 (1)	0 (0)	

					GG	0 (0)	0 (0)				
Exon8	rs7246479	c.597A>C	Leu199Phe	408	AA	67.15 (274)	62.69 (163)	0.131[b]	A	81.74 (667)	80.38 (418)	0.536[b]
					AC	29.17 (119)	35.39 (92)		C	18.26 (149)	19.62 (102)	
					CC	3.68 (15)	1.92 (5)					
Exon8	rs369826666	c.624C>T	Ser208Ser	408	CC	99.75 (407)	100 (260)	1.000[a]	C	99.88 (815)	100 (520)	1.000[a]
					TC	0.25 (1)	0 (0)		T	0.12 (1)	0 (0)	
					TT	0 (0)	0 (0)					

[a] Fisher's exact test.

[b] Chi-square test.

[c] The genotype and allele frequencies of control group were obtained from East Asian population in 1000 Genomes Browser (http://www.internationalgenome.org/).

POI, premature ovarian insufficiency.