Mutational analysis of SAL-Like 4 (SALL4) in Han Chinese women with premature ovarian failure

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ABSTRACT: Pluripotency associated transcription factor, SAL-Like 4 (SALL4), might play an important role in conferring totipotency on oocytes. In the present study, we screened SALL4 coding regions for mutations in 100 Han Chinese women with non-syndromic ovarian failure and discovered two novel non-synonymous variants in the SALL4 gene: c.541G>A (p.Val181Met) and c.2449A>G (p.Thr817Ala). The former variant was located in an evolutionary conserved region of SALL4 protein and might affect its function. This is the first report to suggest that SALL4 might be a potential candidate gene of premature ovarian failure.

Key words: premature ovarian failure / SAL-Like 4 / variant

Introduction

Premature ovarian failure (POF) was defined as a primary ovarian defect characterized by secondary amenorrhea, infertility, hypogonadism, and elevated gonadotrophin level of over 40 IU/l before the age of 40 years (Coulam et al., 1986). POF affects 0.1% of women by the age of 30 and 1–2% of women by the age of 40 (Coulam et al., 1986). POF can be heritable in 30% of patients and is genetically heterogeneous (Vegetti et al., 1996).

Mutational analysis has identified several genes as candidates for developing POF, including FOXL2 (Gersak et al., 2004; Nallathambi et al., 2007), FSHR (Aittomaeki et al., 1995; Touraine et al., 1999; Jiang et al., 1998; Rannikko et al., 2002), INHA (Marozzi et al., 2002; Harris et al., 2005; Dixit et al., 2006), GDF9 (Dixit et al., 2005; Chand et al., 2006; Laisse et al., 2006; Kovanci et al., 2007; Zhao et al., 2007), BMP15 (Chand et al., 2006; Di Pasquale et al., 2006; Laisse et al., 2006), FOXO3 (Watkins et al., 2006), FIGL1 (Zhao et al., 2008) and NOBOX (Qin et al., 2007). Mutations in other genes required for oogenesis and folliculogenesis are also likely to be the causes of POF.

SALL4 encodes a zinc-finger transcription factor named Sal-like 4 and belongs to the Spalt (sal) gene family, which has important roles in regulating the developmental processes of many organisms (Ma et al., 2001, 2002; Nishinakamura et al., 2001). The human SALL4 gene is located at 20q 13.13–13.2 and is encoded by four exons. Several studies have proposed that heterozygous mutations in human SALL4 are the cause of Duane Radial Ray Syndrome (Okihiro syndrome), which is characterized by eye retraction in association with limb and multiple developmental defects in other organs (Al-Baradie et al., 2002; Kohlhase et al., 2002; Miertsits et al., 2006). The murine Sall4 gene is expressed in oocytes (Su et al., 2002) and binds to the highly conserved regulatory region of the Pou5f1 (also known as Oct4) distal enhancer activating Pou5f1 expression in vivo and in vitro (Zhang et al., 2006). In human ES cells, it was observed that the depletion of SALL4 could down-regulate Pou5f1 (Zhang et al., 2006).

Qin et al. (2007) reported two novel missense variations in the NOBOX homeodomain in women with POF and showed that one mutation disrupted the binding of the NOBOX homeodomain to DNA, which could be the cause of POF in these women. In a murine study, pluripotency associated transcription factors, Sal4 and Pou5f1, were drastically down-regulated by more than 20- and 30-fold, respectively, using microarray analysis of newborn mouse ovaries lacking Nbox (Choi et al., 2007). These two transcription factors, Sall4 and Pou5f1, play a crucial role in pluripotency and cell fate determination in early development.

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factors are expressed early during oogenesis and the 8-mer sequences to which Nobox homeodomain preferentially binds are found in Sall4 and Pou5f1 (Choi et al., 2007). Thus, the expression and down-regulation of Sall4 and Pou5f1 in newborn oocytes (Choi et al., 2007), and in the Nobox deficient ovaries (Rajkovic et al., 2004), signifies that their networks lie downstream of Nobox. Also, Choi and Rajkovic (2006) showed that Nobox can bind these elements in the promoters of Gdf9 and Pou5f1, and might therefore regulate transcription of Gdf9 and Pou5f1, which are specifically expressed in germ cells.

In summary, Gdf9, Sall4, Pou5f1 might all lie downstream of Nobox. Among the four genes, POF-associated mutations in the Gdf9 gene (Dixit et al., 2005; Chand et al., 2006; Laissue et al., 2006; Kovanci et al., 2007; Zhao et al., 2007) and NOBOX gene (Qin et al., 2007) have been identified. Although the role of Sall4 in folliculogenesis in mice is unknown, human SALL4 might play important roles in conferring oocytes the ability to reprogram, i.e. totipotency (Choi et al., 2007). It is possible that the SALL4 gene might contribute to the development of POF. In the present study, we performed the mutational analysis of the SALL4 gene in Han Chinese women with POF.

### Materials and Methods

#### Study population

A total of 100 Han Chinese POF patients were recruited in this study from the First Affiliated Hospital, Anhui Medical University, China. The study was approved by the Ethics Committee of the National Research Institute for Family Planning and informed consent was obtained from all participants. Diagnostic criteria were: menopause occurring before the age of 40 for at least 4 months, with at least two different serum samples showing FSH concentrations of >40 IU/l. The ages of the patients ranged from 19 to 39 years. The percentage of women with primary amenorrhea was 15%. Patients with abnormal chromosomes, associated endocrinopathies or autoimmune disorders, iatrogenic agents, such as chemotherapy or radiotherapy, and infections were excluded. None of the patients in the POF group presented with Duane Radial Ray Syndrome (Okihiro syndrome). Our control group comprised 300 women with no evidence of POF, who displayed normal menarche, normal menstrual cycles and no clinical evidence of infertility.

#### DNA analysis

Genomic DNA was extracted from peripheral blood leukocytes using standard methods. The four exons and associated introns of the SALL4 gene were amplified by polymerase chain reaction (PCR) using LA Taq (Takara, Dalian, China) and four pairs of SALL4 gene-specific primers (Supplementary Table S1).

Each PCR product was resolved on a 1.5% agarose gel with Golden-view and sequenced using primers (Supplementary Table S1) and a BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). The sequencing reactions were run on an ABI 3730XL automated sequencer (Applied Biosystems, Foster City, CA, USA) to perform the mutational analysis.

### Results

We found five variants by analyzing mutations in the SALL4 gene in the POF subjects. Three known single nucleotide polymorphisms were found in the POF and control groups (Table I). Two novel non-synonymous variants: c.541G>A (p.Val181Met) and c.2449A>G. (p.Thr817Ala) were found only in the POF group and not in the controls (Table I and Fig. 1). To avoid DNA amplification errors, we have repeated the sequencing on different PCR products from the woman with the novel non-synonymous variant (c.2449A>G).

The variant c.541G>A resulted in a substitution of a Valine residue with Methionine at position 181. Val181 is evolutionarily conserved among many species [human, dog, Norway rat, house mouse, platypus, chicken, African clawed frog and zebrafish, (Fig. 2)]. This variation was only presented in one 32-year-old married woman who had elevated FSH and LH level (105 and 92 U/l, respectively). The value of E2 was 83.84 pmol/l. This woman has never been pregnant and has secondary amenorrhea. Her mother had had normal cycles; however, menopause occurred at the age of 42 after oophorectomy of both ovaries due to an ovarian neoplasm. However, upon investigating the DNA of the patient’s parents, we discovered that the patient’s father had the same variant (c.541G>A (p.Val181Met)) in his SALL4 gene, but that the woman’s mother did not (Fig. 3).

The other novel variant, c.2449A>G, resulted in a substitution of a Threonine residue with Alanine at position 817. Thr817 is not particularly conserved among species (Fig. 2). The variant was only present in one 36-year-old married woman who had elevated FSH level (53.50 IU/l). Her E2 value was 85.69 pmol/l. She had associated secondary amenorrhea and has been pregnant using in vitro fertilization with donor oocytes. The women’s parents had died, so we were unable to obtain blood samples to genotype her parents.

#### Table I Variations of SALL4 in 100 Han Chinese women with POF

<table>
<thead>
<tr>
<th>Variants</th>
<th>Exon</th>
<th>Sequence variation</th>
<th>Amino acid variation</th>
<th>No. of cases (n = 100)</th>
<th>No. of controls (n = 300)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Novel</td>
<td>2</td>
<td>c.541G&gt;A</td>
<td>p.Val181Met</td>
<td>1/100; 0/100</td>
<td>0/300; 0/300</td>
</tr>
<tr>
<td>rs1303899</td>
<td>2</td>
<td>c.1056G&gt;A</td>
<td>Silent</td>
<td>3/100; 0/100</td>
<td>16/300; 0/300</td>
</tr>
<tr>
<td>rs6126344</td>
<td>2</td>
<td>c.1520T&gt;G</td>
<td>Leu507Arg</td>
<td>53/100; 23/100</td>
<td>136/300; 104/300</td>
</tr>
<tr>
<td>rs6021437</td>
<td>2</td>
<td>c.1860A&gt;G</td>
<td>Silent</td>
<td>53/100; 23/100</td>
<td>136/300; 104/300</td>
</tr>
<tr>
<td>Novel</td>
<td>2</td>
<td>c.2449A&gt;G</td>
<td>Thr817Ala</td>
<td>1/100; 0/100</td>
<td>0/300; 0/300</td>
</tr>
</tbody>
</table>
Discussion

It has been reported that SALL4 is significantly down-regulated in Nbox deficient mouse ovaries and 8-mer Nbox homeodomain binding sequences can be found in SALL4 gene which suggests that the expression of SALL4 is activated by NOBOX (Choi et al., 2007). Additionally, NOBOX is an oocyte-specific gene and is a candidate gene for non-syndromic ovarian failure (Qin et al., 2007). It has also been suggested that these two genes might regulate totipotency in oocytes (Rajkovic, 2007). Consequently, SALL4 might be a candidate gene for POF.

The two novel variants we found in the SALL4 gene result in non-synonymous substitutions. The substitution of a Valine residue with Methionine occurs at position 181, which is evolutionarily conserved. Although both Valine and Methionine residues are hydrophobic, they are different. Valine is a branched-chain amino acid, although Methionine contains a non-polar methyl thioether group in its side-chain. Amino acid substitutions are more likely to affect protein function when the amino acids are located at residues that are highly conserved among species. The c.541G>A (p.Val181Met) variant was also found in the patient’s father but not her mother, which demonstrated that the patient inherited the variant from her father and was consistent with our observation that her mother did not display POF. The other substitution, of a Threonine residue with Alanine occurs at position 817, which is not evolutionarily conserved. These residues are also different: Threonine has an uncharged polar side-chains containing β-hydroxyl groups, which render the aliphatic side-chain hydrophilic, whereas Alanine has a saturated aliphatic side-chain comprising a methyl group.

Although the two substitutions are not located in the functional domain of the zinc finger, we can hypothesize that the c.541G>A (p.Val181Met) and/or c.2449A>G. (p.Thr817Ala) variants may potentially affect the structure and DNA binding of adjacent SALL4 zinc finger domains. A previous study showed that Sall4 binds to the highly conserved regulatory region of the Pou5f1 distal enhancer and activates Pou5f1 expression in vivo and in vitro (Zhang et al., 2006). Also POU5F1 is expressed in primordial oogonia (Rajpert-De Meyts et al., 2004). The pathogenesis of the potential mutation might be a SALL4 dominant negative effect, which might be explained thus: SALL4 is a transcriptional activator of POU5F1, and the SALL4 variant might compete for binding to the highly conserved regulatory region of POU5F1 distal enhancer leading to no activation of...
POUSF1 expression. This might affect the development of the primordial germ cells, which could result in an early cessation of ovarian function and trigger POF.

In conclusion, mutational analysis of the SALL4 gene in 100 Han Chinese POF patients revealed two novel variants: c.541G>A (p.Val181Met) and c.2449A>G (p.Thr817Ala), which might be POF-associated gene variants.

To our knowledge, this is the first study to suggest that the SALL4 gene might be involved in the etiology of POF. Further studies are necessary to determine the functional significance of the non-synonymous variants of SALL4.

**Supplementary data**

Supplementary data are available at http://molehr.oxfordjournals.org/.

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