Association study of anti-Müllerian hormone and anti-Müllerian hormone type II receptor polymorphisms with idiopathic primary ovarian insufficiency

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STUDY QUESTION: Are the genetic polymorphisms of the anti-Müllerian hormone (AMH) and anti-Müllerian hormone type II receptor (AMHR2) genes associated with idiopathic primary ovarian insufficiency (POI) in a Korean population?

SUMMARY ANSWER: The distribution of the AMH and the AMHR2 polymorphisms in a Korean POI population was not significantly different from controls.

WHAT IS KNOWN ALREADY: AMH plays an important role in regulating both the primordial follicle recruitment and the cyclic selection of the antral follicles. The AMHR2 -482A>G polymorphism was associated with an earlier menopause and nulliparous women with the GG genotype had a 2.6 years earlier onset of menopause compared with the AA genotype women. Therefore, genetic variants in the AMH signal transduction pathway might affect the ovarian function of women.

STUDY DESIGN, SIZE, DURATION: Case–control study. The subjects consisted of 211 idiopathic POI patients and 233 post-menopausal controls.

PARTICIPANTS/MATERIALS, SETTING, METHODS: The frequency of the AMH Ile49Ser and AMHR2 -482A>G polymorphisms was analyzed in 211 patients with idiopathic POI and in 233 post-menopausal controls, and we also analyzed clinical characteristics, such as age at the time of POI and LH, FSH as well as estradiol levels according to the specific genotype. Genotyping for the AMH Ile49Ser and the AMHR2 -482A>G polymorphisms was performed by a minor groove binder primer/probe Taqman assay.

MAIN RESULTS AND THE ROLE OF CHANCE: The genotype distributions and allele frequencies for the AMH Ile49Ser and the AMHR2 -482A>G polymorphisms were similar between the POI patients and the controls. Within POI population, the AMH Ile49Ser and the AMHR2 -482A>G polymorphisms were not associated with age at the time of POI and LH, FSH as well as estradiol levels. Haplotype analysis also showed no significant difference between groups.

LIMITATIONS, REASONS FOR CAUTION: Study is limited to a Korean population.

WIDER IMPLICATIONS OF THE FINDINGS: Our findings suggest that genetic variants in the AMH signal transduction pathway may not influence the susceptibility of idiopathic POI. This is the first report on the association between the AMH and AMHR2 polymorphisms and idiopathic POI.

STUDY FUNDING/COMPETING INTEREST(S): No conflict of interest exists. This study was supported by a grant of Seoul National University Hospital Research Fund (04-2011-0870).

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Key words: AMH / AMHR2 / polymorphism / primary ovarian insufficiency
Introduction

Primary ovarian insufficiency (POI), also known as premature ovarian failure (POF), is a devastating disease in women and is considered to be present when a woman has a disrupted ovarian function, namely amenorrhea for 4 months or more in association with menopausal FSH levels before or at the age of 40 (Nelson, 2009). The occurrence of POI is ~1% in women of reproductive age (Coulam et al., 1986), and affected women have severely compromised fertility and need long-term hormone therapy to relieve symptoms of a hypo-estrogenic condition (Anasti, 1998; Goswami and Conway, 2005). A number of chromosomal and genetic studies have been carried out to find the major determinant factor for the etiology and pathogenesis of ovarian insufficiency; however, the etiology of POI is still poorly understood in a large proportion of cases.

Anti-Müllerian hormone (AMH) is a member of the transforming growth factor-β superfamily and is produced by the granulosa cells of growing follicles in the ovary. AMH has been identified as an useful marker for ovarian reserve based on results that serum AMH levels decrease progressively with age and strongly correlate with the size of the pool of primordial follicles and the number of antral follicles (van Rooij et al., 2005; Visser et al., 2006; Lee et al., 2012). Other studies showed that AMH is also associated with follicular recruitment and selection of the dominant follicle. In a mouse study, AMH null mice showed a relatively early depletion of their pool of primordial follicles (Durlinger et al., 1999) and increased sensitivity of follicles to FSH (Durlinger et al., 2001). This suggests that AMH has an inhibitory role in the recruitment of primordial follicles and their progression to the primary follicle stage and that it decreases FSH sensitivity. Considering that the rate of follicular depletion is associated with ovarian aging and the major role of FSH in follicular recruitment (Visser et al., 2007a,b; Voorhuis et al., 2010), menopausal age related to parity (Kevenaar et al., 2010) and infertility (Rigon et al., 2011) and infertility (Rigon et al., 2011). They suggested that the genetic polymorphisms in the AMH signaling pathway may modulate the FSH sensitivity in the ovary, and have a role in the usage of the primordial follicle pool, namely the process of human ovarian aging, and seem to be associated with unexplained infertility. In other pathophysiologic conditions, AMH inhibits aromatase activity as well as follicular recruitment (Visser et al., 2006) and carriers of the AMH-49Ser allele in polycystic ovary syndrome (PCOS) women were less likely to exhibit polycystic ovaries and low androgen levels (Kevenaar et al., 2007a;b; Voorhuis et al., 2011) and infertility (Rigon et al., 2010). They suggested that the genetic polymorphisms in the AMH signaling pathway may modulate the FSH sensitivity in the ovary, and have a role in the usage of the primordial follicle pool, namely the process of human ovarian aging, and seem to be associated with unexplained infertility. In other pathophysiologic conditions, AMH inhibits aromatase activity as well as follicular recruitment (Visser et al., 2006) and carriers of the AMH-49Ser allele in polycystic ovary syndrome (PCOS) women were less likely to exhibit polycystic ovaries and low androgen levels (Kevenaar et al., 2008), suggesting a contribution of AMH polymorphism to the severity of the PCOS phenotype.

Considering these roles of AMH with reference to the regulation of ovarian function, it could be postulated that the genetic variants in the AMH signaling pathway could lead to an impaired reproductive function of women, and may also serve as a risk factor for POI. In this study, we therefore investigated whether polymorphisms of the AMH and its type II receptor (AMHR2) genes are associated with susceptibility to idiopathic POI in our Korean population. For this, we analyzed the frequency of the AMH Ile49Ser and AMHR2 -482A > G polymorphisms in patients with idiopathic POI and in controls, and also analyzed clinical characteristics, such as age at the time of POI and LH, FSH as well as estradiol levels according to the specific genotype.

Materials and Methods

Subjects

We recruited POI patient irrespective of their etiology from four university hospitals in Korea between 1999 and 2012. All of the patients underwent gynecological examination and complete POI workup, including karyotype analysis as determined by conventional GTG banding in their centers as described earlier (Yoon et al., 2010). Subjects who had known causes of POI (i.e. chromosomal abnormalities and chemo, radiation or surgery induced POI) were excluded from this study. In the end, we enrolled 211 patients with idiopathic POI, in which 25 cases were affected with primary amenorrhea (primary POI; 11.8%) and 186 cases with secondary amenorrhea (secondary POI; 88.2%). A total of 233 women served as controls. They all visited the healthcare center in Seoul National University Hospital for an annual comprehensive medical checkup and had no specific health problems. They experienced their menopause at age >45 years. Recruiting controls from this age group maximizes the probability that these subjects were not affected by POI. The review board for human research of Seoul National University Hospital approved this study, and written informed consent was obtained from all participants.

Genotyping of AMH Ile49Ser & AMHR2 -482A > G polymorphisms

Genomic DNA was isolated and extracted from peripheral blood leukocytes by the Wizard DNA purification kit (Promega, Madison, WI, USA). The Ile49Ser polymorphism in the AMH gene and -482A > G polymorphism in the AMHR2 gene were genotyped by a minor groove binder primer/probe Taqman assay on the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The following primers and probes were used: for the AMH Ile49Ser, forward primer, 5′-GTGCCCC TCATCTTCGAGAAA-3′, reverse primer, 5′-CACCAAGCACAGAGGC T-3′, reporter ‘G (Serine)’ 5′-(VIC)-TCCAGGGACCCCAAA-(NFQ)-3′, reporter ‘T (Isoleucine)’ 5′-(FAM)-CTCCAGGATCCCAA-(NFQ)-3′ and for the AMHR2 -482A > G, forward primer, 5′-GGCTGTGAGGCTC TCCATGC-3′, reverse primer, 5′-AGGCTTAAGATCTCTTTATTACG CTTCA-3′, reporter ‘A’ 5′-(FITC)-CCCCAACGTTGGAC-(NFQ)-3′, reporter ‘G’ 5′-(FAM)-CCAAAGGGTTGGAC-(NFQ)-3′. The PCR mixture consisted of 10 μl of TaqMan Universal PCR Master Mix 2x (Applied Biosystems) and 25 ng DNA. The PCR cycling conditions consisted of one 2-min cycle at 95°C, and one 10-min cycle at 95°C, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. We used distilled water as a negative PCR control in each amplification.

Statistical analysis

Genotype distributions of the AMH and AMHR2 polymorphisms were examined for significant departure from Hardy–Weinberg equilibrium by a goodness-of-fit χ² test. Data were analyzed by the χ² test or one-way analysis of variance (ANOVA) as appropriate. All data analyses were performed by using the Statistical Package for the Social Sciences software (version 19.0, IBM SPSS Inc., NY, USA). Haplotypic frequencies were estimated by utilizing the Haploview version 4.1 (available at http://www.broad.mit.edu/mpg/haplovie) to ensure consistency in type I error probability caused by multiple testing (two single nucleotide polymorphisms, haplotype and four clinical variables per polymorphism), the statistical significance was specified.
by means of Bonferroni correction and therefore \( P < 0.0045 \) was considered significant.

**Results**

We have investigated the polymorphisms of the AMH and AMHR2 genes from 211 patients with idiopathic POI and 233 post-menopausal women. The median age (interquartile range) of onset of POI was 30.7 (23.5–37.8) years and the median values (interquartile range) of LH, FSH and estradiol in the POI group were 29.8 (18.7–40.0) mIU/ml, 67.9 (47.1–90.2) mIU/ml and 20.0 (11.2–31.0) pg/ml, respectively.

Genotypes of the two polymorphic loci were successfully determined in all subjects, and the genotype distributions in both groups were compatible with Hardy–Weinberg equilibrium. The results of subgroup analyses were not shown in the main outcome for the purpose of maintaining consistency in the interpretation of the results after Bonferroni correction. There were no significant differences in the genotype distributions or allele frequencies of the AMH Ile49Ser and the AMHR2 -482A>G polymorphisms between the POI and the control group (AMH Ile/Ile/IleSer/Ser/Ser rates: 47.4/41.7/10.9% in the POI versus 47.6/40.8/11.6% in the control group, \( P = 0.965 \), Table I; AMHR2 AA/AG/GG rates: 62.1/34.6/3.3% in the POI versus 60.9/36.5/2.6% in the control group, \( P = 0.843 \), Table II). Haplotype analysis for the AMH Ile49Ser and the AMHR2 -482A>G polymorphisms showed that four haplotypes were estimated to be present and there was also no significant difference in the haplotype frequencies between the POI and the control group (Table III).

**Discussion**

The aim of the present study was to evaluate the association of the genetic variants of the AMH signaling pathway susceptibility to idiopathic POI in Korean women. The genotype distributions and allele frequencies for the AMH Ile49Ser and the AMHR2 -482A>G polymorphisms were similar between the POI and the control group, suggesting that the polymorphisms in genes of the AMH signaling pathway are not major determinants to the risk of POI. To the best of our knowledge, this is the first report on the association between the AMH and AMHR2 polymorphisms and idiopathic POI.

The genotype distributions for both polymorphisms in our general population were consistent with other reports; e.g. for the AMH Ile49Ser, the genotype distributions were similar to those of Asians in the HapMap database; for the A/A genotype in the AMHR2 -482A>G polymorphism, 60.9% in ours versus 62.5% in Dutch, 66.0 in Dutch plus German and 63.0 in Italian (Kevenaar et al., 2007a,b; Rigon et al., 2010).

**Table I** The distribution of the AMH gene Ile49Ser polymorphism in the POI patients and controls.

<table>
<thead>
<tr>
<th>Group</th>
<th>( n )</th>
<th>Genotypes</th>
<th>Ile/Ser</th>
<th>Ser/Ser</th>
<th>( P )-value(^a)</th>
<th>Allele frequency</th>
<th>( P )-value(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ile/Ile</td>
<td></td>
<td></td>
<td></td>
<td>Ile</td>
<td>Ser</td>
</tr>
<tr>
<td>Total POI</td>
<td>211</td>
<td>100 (47.4)</td>
<td>88 (41.7)</td>
<td>23 (10.9)</td>
<td>0.965</td>
<td>288 (68.2)</td>
<td>134 (31.8)</td>
</tr>
<tr>
<td>Primary</td>
<td>25</td>
<td>7 (28.0)</td>
<td>15 (60.0)</td>
<td>3 (12.0)</td>
<td></td>
<td>29 (58.0)</td>
<td>21 (42.0)</td>
</tr>
<tr>
<td>Secondary</td>
<td>186</td>
<td>93 (50.0)</td>
<td>73 (39.2)</td>
<td>20 (10.8)</td>
<td></td>
<td>259 (69.6)</td>
<td>113 (30.4)</td>
</tr>
<tr>
<td>Control</td>
<td>233</td>
<td>111 (47.6)</td>
<td>95 (40.8)</td>
<td>27 (11.6)</td>
<td></td>
<td>317 (68.0)</td>
<td>149 (32.0)</td>
</tr>
</tbody>
</table>

POI, primary ovarian insufficiency; AMH, anti-Müllerian hormone.

Data are \( n \) (%).

\(^{a}\)Evaluated by the \( \chi^{2} \) test in comparison with the control group.

**Table II** The distribution of the AMHR2 gene -482A>G polymorphism in the POI patients and controls.

<table>
<thead>
<tr>
<th>Group</th>
<th>( n )</th>
<th>Genotypes</th>
<th>AA</th>
<th>AG</th>
<th>GG</th>
<th>( P )-value(^a)</th>
<th>Allele frequency</th>
<th>( P )-value(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ile</td>
<td>Ser</td>
</tr>
<tr>
<td>Total POI</td>
<td>211</td>
<td>131 (62.1)</td>
<td>73 (34.6)</td>
<td>7 (3.3)</td>
<td>0.843</td>
<td>335 (79.4)</td>
<td>87 (20.6)</td>
<td>0.942</td>
</tr>
<tr>
<td>Primary</td>
<td>25</td>
<td>18 (72.0)</td>
<td>6 (24.0)</td>
<td>1 (4.0)</td>
<td></td>
<td>42 (84.0)</td>
<td>8 (16.0)</td>
<td></td>
</tr>
<tr>
<td>Secondary</td>
<td>186</td>
<td>113 (60.8)</td>
<td>67 (36.0)</td>
<td>6 (3.2)</td>
<td></td>
<td>293 (78.8)</td>
<td>79 (21.2)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>233</td>
<td>142 (60.9)</td>
<td>85 (36.5)</td>
<td>6 (2.6)</td>
<td></td>
<td>369 (79.2)</td>
<td>97 (20.8)</td>
<td></td>
</tr>
</tbody>
</table>

POI, primary ovarian insufficiency; AMHR2, anti-Müllerian hormone type II receptor.

Data are \( n \) (%).

\(^{a}\)Evaluated by the \( \chi^{2} \) test in comparison with the control group.
Considering the menopausal transition (Richardson et al., 1987), the main characteristic of POI is an early or accelerated depletion of follicular pool, which results in hypergonadotropic hypogonadism before or at the age of 40. There were reports that the AMHR2 -482A>G polymorphism was associated with age at menopause in which nulliparous women with the GG genotype had a 1.9–2.6 years earlier onset of menopause compared with the AA genotype women (Kevenaar et al., 2007a,b). An in vitro study also showed that the bioactivity of AMH is diminished in the AMHR2 49Ser protein compared with the AMH 49Ile protein (Kevenaar et al., 2008). However, in our POI cohort, genotypes of both polymorphisms were not associated with clinical characteristics. Age at the time of POI was similar between the carriers of the AMH 49Ser and the AMHR2 -482G allele and the non-carriers, as were levels of LH, FSH and estradiol between groups (Table III). Our results indicate that they might not be the true candidate genetic variation for POI or be insufficient to cause POI although the AMH 49Ser polymorphism could modulate the AMH function.

In addition, we anticipated that primary POI group would have more significant variations than secondary POI group in the present study. In subgroup analyses, there was a tendency that the frequency of the AMH 49Ile allele was lower in primary POI group compared with the secondary POI group, however, there were also no significant differences between genotype groups (data not shown).

Although this study does not demonstrate an association between the AMH and AMHR2 polymorphisms with idiopathic POI, the results must be interpreted with caution. First, the exact functional role of AMH signaling pathway on the pathogenesis of POI has not been elucidated yet. More functional studies about the influence of this polymorphism on the ovarian ageing are needed. Secondly, as the POI has a complex trait like menopause, genetic interaction with other factors or another genetic variants of the AMH signaling pathway that were not assessed in this study might influence to the development of POI. Finally, ethnic variations might influence the pattern of the polymorphisms of the AMH signaling pathway in the POI patients. Pathophysiology of POI in a Korean population might be different from that in other ethnicities. Other studies from different populations are needed to replicate and confirm these results in the idiopathic POI.

It has been proposed that variation of menopausal age is largely influenced by genetic factors (Voorhuis et al., 2010), and genes involved in primary follicle recruitment are associated with timing of menopause in a genetic association study with a large menopausal cohort (Voorhuis et al., 2011). Even though it is unclear whether POI and menopause have the same genetic mechanisms or not, there may be a chance that involved genes overlap each other. POI may be also caused by a
heterogeneous set of genetic factors, and it has been reported that genetic aberrations of POI involve not only X chromosome but also autosomal abnormalities (Lami et al., 2002; Goswami and Conway, 2005). Despite the inconsistent results of replication studies, it may be still expected that underlying genetic aberrations involved in maintaining ovarian function might cause ovarian dysfunction. AMH plays an important role in regulating both the primordial follicle recruitment and the cyclic selection (Durlinger et al., 1999; McGee and Hsueh, 2000). Therefore, the role of the AMH signaling pathway in the pathophysiology of POI still remains to be investigated despite the negative results of our study. Elucidation of factors involved in this genetic pathway could help to find a susceptible factor to POI and provide more insight into the underlying mechanisms of early ovarian dysfunction.

In conclusion, the polymorphisms in the AMH and AMHR2 genes did not show any association with idiopathic POI in Korean women, implying that they might not influence the POI susceptibility. Further studies from diverse ethnicities are needed to identify a role of the AMH signaling pathway in idiopathic POI.

Authors’ roles
S.H.Y. contributed to the conception and design, and participated in the analysis and interpretation of data and in the drafting of the paper; Y.M.C. contributed to the conception and design, and participated in the final approval of the paper to be published; M.A.H. performed genomic DNA analysis; J.J.K. and S.Y.M. contributed to the revision of the article; K.H.L. and K.R.H. contributed to the acquisition of data.

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Conflict of interest
None declared.

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