A polymorphism in the AMH type II receptor gene is associated with age at menopause in interaction with parity

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BACKGROUND: Anti-Müllerian hormone (AMH) inhibits primordial follicle recruitment in the mouse ovary. We hypothesize that in women AMH signaling also regulates the usage of the primordial follicle pool and hence influences the onset of menopause. Since age at menopause has a strong genetic component, we investigated the role of AMH signaling using a candidate gene approach. METHODS: In two large population-based cohorts of Dutch post-menopausal women (n=2381 and n=248), we examined the association between two polymorphisms, one in the AMH gene and one in the AMH type II receptor (AMHR2) gene, and natural age at menopause. RESULTS: The AMH Ile⁴⁻⁹⁴Ser polymorphism (rs10407022) was not associated with age at menopause in either cohort. In the Rotterdam cohort, the AMHR2 –482 A>G polymorphism (rs2002555) was associated with age at menopause in interaction with the number of offspring (P=0.001). Nulliparous women homozygous for the G-allele entered menopause 2.6 years earlier compared with nulliparous women homozygous for the A-allele (P=0.005). In the LASA cohort, women with the G/G genotype tended to enter menopause 2.8 years earlier compared with the A/A genotype (P=0.063). CONCLUSIONS: The observed association of the AMHR2 –482 A>G polymorphism with natural age at menopause suggests a role for AMH signaling in the usage of the primordial follicle pool in women.

Keywords: anti-Müllerian hormone; menopause; follicle recruitment; polymorphism

Introduction

Menopause marks a dramatic change in the endocrine and reproductive status of women. In women, the onset of menopause is determined by the exhaustion of the ovarian follicle pool (te Velde et al., 1998b). From the establishment of the primordial follicle pool onwards, just before (for primates) or directly after (for mice) birth, dormant primordial follicles are continuously recruited into the growing follicle pool, a process called initial recruitment. After pubertal onset, a cohort of antral follicles is selected from this growing follicle pool as a result of the increase in circulating FSH levels during each reproductive cycle (McGee and Hsueh, 2000). From this rescued cohort, only one (for primates) or several (for rodents) follicle(s) will ovulate during each cycle, whereas most growing follicles will die as a result of atresia.

Primordial follicle recruitment is predominantly regulated by intra-ovarian factors. One of the factors known to regulate initial recruitment in mice is anti-Müllerian hormone (AMH), also known as Müllerian inhibiting substance (MIS). AMH, a member of the transforming growth factor-β (TGF-β) family, is expressed in the ovary from the onset of primordial recruitment onwards in a similar pattern in women and mice (Weenen et al., 2004). AMH expression starts in the granulosa cells of primary follicles, is highest in granulosa cells of pre-antral and small antral follicles and gradually diminishes in the subsequent stages of follicle development (Durlinger et al., 2002b). Studies of the AMH knockout (AMHKO) mice revealed that primordial follicles are recruited at a faster rate in the absence of AMH, illustrating that AMH plays an inhibitory role in the recruitment of primordial follicles. The absence of AMH results in a prematurely exhausted follicle pool and, subsequently, an earlier cessation of the estrus cycle (Durlinger et al., 1999). AMH inhibits mouse, bovine and human primordial follicle growth in vitro (Durlinger et al., 2002a; Gigli et al., 2005; Carlsson et al., 2006), although conflicting results have been reported (Schmidt et al., 2005). In addition to recruitment,
AMH attenuates FSH sensitivity in mice (Durlinger et al., 2001; Visser et al., 2007), albeit also for this role of AMH contrary results have been found (McGee et al., 2001). On the basis of the similar expression pattern of AMH in women and in mice, we hypothesize that also in women AMH inhibits primordial follicle recruitment and thus might influence the onset of menopause.

In Western countries, the average age at menopause is 50–51 years, but ranges from 40 to 60 years (te Velde et al., 1998a). Environmental factors and personal history (e.g. smoking and parity) explain only a minor part of the variety in natural age at menopause, whereas the main part is explained by genetic factors (Kok et al., 2005b). This conclusion is mainly based on the strong correlation of age at menopause in monozygotic twins, in whom heritability estimates range from 0.63 to 0.72 (Sniider et al., 1998; de Bruin et al., 2001). In addition, a genetic component of age at menopause was suggested by several candidate gene studies. For example, polymorphisms in genes involved in estrogen metabolism have been associated with age at menopause, e.g. ERα (Weel et al., 1999), CYP 17 (Gorai et al., 2003) and CYP1B1 (Heffler et al., 2005), although these findings have not been replicated (Gorai et al., 2003; Heffler et al., 2005; Kok et al., 2005a).

In a recent study, we have shown that in premenopausal women, genetic variants in AMH and its specific AMH type II receptor (AMHR2) gene are associated with estradiol levels, suggesting modulation of intra-ovarian FSH sensitivity by these variants (Kevenaar et al., 2007). In the present study, we have evaluated whether the AMH Ile49Ser (rs10407022) and the AMHR2 –482 A>G (rs2002555) polymorphisms are associated, independently and in interaction with environmental factors, with age at menopause in two large cohorts of Dutch postmenopausal women.

Materials and Methods

Subjects

The first study cohort was derived from women from the Rotterdam Study (n=7983, 61.6% women), a prospective population-based study of determinants of chronic disabling diseases in the elderly. The design and rationale of this study have been described earlier (Hofman et al., 1991). Written informed consent was obtained from each participant and the Rotterdam study was approved by the Medical Ethics Review board of Erasmus MC. During the home interview, each woman provided information on her reproductive and gynecological history, including the use of sex steroids at any time (Weel et al., 1999). Confounding factors, such as height, weight, smoking and socio-economic status, were defined as described previously (Weel et al., 1999). For this study, only women with a natural age at menopause were selected (n=3256). Natural age at menopause was defined as the age at the last menstrual period, which can only be defined retrospectively after at least 12 consecutive months of amenorrhoe. This last menstrual period should not be induced by surgery or other obvious causes, such as irradiation or hormone therapy (WHO Scientific Group, 1996). Women who reported hormone use during the onset of menopause were excluded to avoid uncertainty on menopausal age. DNA was available for 2564 eligible women, of whom 92.9% was successfully genotyped for the AMH and AMHR2 polymorphism, resulting in a final study cohort of 2381 women.

The second study cohort was derived from the Longitudinal Aging Study Amsterdam (LASA), an ongoing interdisciplinary cohort study on predictors and consequences of changes in autonomy and well-being in an aging population in The Netherlands (Deeg et al., 1993). The design of this study has been described previously (Pluijm et al., 2004; Schaap et al., 2005). Informed consent was obtained from all respondents and the study was approved by the Medical Ethics Review board of the VUMC. Information on oral contraceptive use and age at menarche was provided in the main interview of the first examination (1992/1993). At the medical interview during the second data collection (1995/1996), other gynecological and reproductive information was provided, including age at menopause, number of children and sex steroid use, along with the confounding factors height, weight, smoking (ever versus never smoking) and socio-economic status. DNA was available from 966 of the 1509 participants of the medical interview (471 men and 495 women) (Pluijm et al., 2004). In 461 of the women, the AMH and AMHR2 polymorphism were successfully genotyped. For the present study, only women with a natural menopause were selected. Furthermore, women who had ever used HRT or oral contraceptives were excluded, resulting in a final study cohort of 248 women.

Genotyping

Genomic DNA was extracted from peripheral blood using standard DNA extraction methods. The AMH Ile49Ser and AMHR2 –482 A>G genotypes were determined using Taqman allelic discrimination assays. For the AMH Ile49Ser polymorphism, an Assay-by-Design with the following probes was used: 5′-CTCCGGACCCACCAAA-3′ and 5′-CCAGGCAAGCCCAAAA-3′. For the AMHR2 –482 A>G promoter SNP, we used an Assay-on-Demand, Assay ID C_1673084_10 (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands). Reactions were performed as described previously (Kevenaar et al., 2007). A random selection of 5% of samples was independently repeated to confirm genotyping results. In the Rotterdam Study, the disagreement rate for the AMH Ile49Ser SNP was 0.4%, whereas it was 0.0% in the LASA study. The disagreement rate for the AMHR2 –482 A>G SNP was 0.0% in both study cohorts.

Statistical analysis

In both populations, genotype frequencies were tested for Hardy–Weinberg equilibrium proportions using the ARLEQUIN package (Schneider et al., 2000). Differences between the cohorts and differences between genotype groups within each cohort were tested using one-way analysis of variance (ANOVA) for continuous variables and the chi-squared test for categorical variables. Differences in age at menopause between genotype groups were adjusted for potential confounders (age, BMI, smoking, socio-economic status, age at menarche, parity and use of oral contraceptives and hormone replacement therapy) using ANCOVA. Possible interactions between genotypes and covariates were explored in plots and tested using the general linear model procedure of ANCOVA including product terms of main effects. In the Rotterdam cohort, stratified analysis for the number of offspring was performed. Because of the relatively small sample size, this stratified analysis was not performed in the LASA cohort. Subsequently, to increase statistical power, both cohorts were combined and differences in age at menopause between the AMHR2 genotype groups were analyzed using ANCOVA. All analyses were performed using Statistical Package for Social Sciences, SPSS, version 11.0.1 (SPSS Inc., Chicago, IL, USA). P-value ≤ 0.05 was considered to be significant.
Results

Characteristics of the two study cohorts

Women in the Rotterdam cohort had on average a lower age at the time of the interview and a lower BMI compared with women in the LASA cohort, although these differences were only minor. The mean age at natural menopause was similar in both cohorts. Possible confounding factors for age at menopause, such as smoking and age at menarche, were not different between both cohorts, whereas the average number of offspring and socio-economic status were different between the cohorts (Table 1). In addition, age at natural menopause (49.6 ± 4.4 year, mean ± SD) in our study subset of the Rotterdam cohort was nearly identical to the mean age at natural menopause (49.6 ± 4.5 year) in the total Rotterdam cohort.

Genotype distributions in the study populations

The allele and genotype frequencies of the AMH Ile49Ser and the AMHR2 2482 A>G polymorphism were similar in the Rotterdam study and the LASA study and did not differ from the frequencies in premenopausal women (Kevenaar et al., 2007) or in Caucasians in the NCBI database (www.ncbi.nlm.nih.gov) and in the HapMap database (www.hapmap.org) (The International HapMap Project, 2003). In both study cohorts, the genotype frequencies were in Hardy–Weinberg equilibrium proportions (Tables 2 and 3).

Analysis of the AMH Ile49Ser polymorphism

No differences were observed in the basal characteristics between the genotype groups of the AMH Ile49Ser polymorphism in both cohorts (Table 2). Age at natural menopause was similar between the genotype groups of the AMH Ile49Ser polymorphism, as were age at menarche, number of offspring (Table 2), smoking, socio-economic status and sex steroid use, including hormone replacement therapy and oral contraceptive use (results not shown). Adjustment of age at menopause for possible confounders did not affect the results.

Analysis of the AMHR2 2482 A>G polymorphism

Basal characteristics were similar between the genotype groups of the AMHR2 2482 A>G polymorphism in both cohorts (Table 3). In the Rotterdam cohort, crude age at menopause was not different between the AMHR2 genotypes, as were age at menarche and hormone use, whereas for the number of offspring, a significant difference was observed (P = 0.01). Homozygous carriers of the 2482G allele were more frequently nulliparous (31.6%) compared with women with

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**Table 1: Characteristics of the two study cohorts**

<table>
<thead>
<tr>
<th></th>
<th>Rotterdam cohort</th>
<th>LASA cohort</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>2381</td>
<td>248</td>
<td></td>
</tr>
<tr>
<td>Age at interview (year) (range)</td>
<td>70.1 ± 9.3 (55.0–98.7)</td>
<td>76.9 ± 6.4 (65.6–88.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.8 ± 4.1</td>
<td>27.4 ± 4.5</td>
<td>0.03</td>
</tr>
<tr>
<td>Ever smoked (%)</td>
<td>1081 (45.6)</td>
<td>103 (41.5)</td>
<td>0.23</td>
</tr>
<tr>
<td>SES education level I–II (%)</td>
<td>1523 (64.2)</td>
<td>175 (70.6)</td>
<td>0.046</td>
</tr>
<tr>
<td>Education level III–IV (%)</td>
<td>849 (35.8)</td>
<td>73 (29.4)</td>
<td></td>
</tr>
<tr>
<td>Age at menopause</td>
<td>49.6 ± 4.4</td>
<td>49.2 ± 4.8</td>
<td>0.18</td>
</tr>
<tr>
<td>Median</td>
<td>50</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Age at menarche</td>
<td>13.7 ± 1.8</td>
<td>13.8 ± 1.8</td>
<td>0.48</td>
</tr>
<tr>
<td>Median</td>
<td>14</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Offspring 0 (%)</td>
<td>511 (21.5)</td>
<td>45 (18.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>1 or 2 (%)</td>
<td>1023 (43.0)</td>
<td>80 (32.3)</td>
<td></td>
</tr>
<tr>
<td>&gt;2 (%)</td>
<td>847 (35.6)</td>
<td>123 (49.6)</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD.

* Information available for 2372 women.

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**Table 2: Characteristics of both cohorts by AMH Ile49Ser genotype**

<table>
<thead>
<tr>
<th>AMH</th>
<th>Rotterdam cohort</th>
<th>LASA cohort</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ile/Ile</td>
<td>Ile/Ser</td>
<td>Ser/Ser</td>
</tr>
<tr>
<td>N (%)</td>
<td>1631 (68.5)</td>
<td>682 (28.6)</td>
<td>68 (2.9)</td>
</tr>
<tr>
<td>Age (year)</td>
<td>70.0 ± 0.2</td>
<td>70.1 ± 0.4</td>
<td>71.8 ± 1.1</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.7 ± 0.1</td>
<td>27.0 ± 0.2</td>
<td>26.7 ± 0.4</td>
</tr>
<tr>
<td>Age at menopause</td>
<td>49.6 ± 0.1</td>
<td>49.5 ± 0.2</td>
<td>49.6 ± 0.6</td>
</tr>
<tr>
<td>Median</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Age at menarche</td>
<td>13.7 ± 0.05</td>
<td>13.6 ± 0.07</td>
<td>13.5 ± 0.20</td>
</tr>
<tr>
<td>Median</td>
<td>14</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>Offspring 0 (%)</td>
<td>352 (21.6)</td>
<td>148 (21.7)</td>
<td>11 (16.2)</td>
</tr>
<tr>
<td>1 or 2 (%)</td>
<td>699 (42.9)</td>
<td>291 (42.7)</td>
<td>33 (48.5)</td>
</tr>
<tr>
<td>&gt;2 (%)</td>
<td>580 (35.6)</td>
<td>243 (35.6)</td>
<td>24 (35.3)</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM.

*P-value for Hardy–Weinberg Equilibrium.
the AMHR2 −482 A/A genotype (22.5%) or AMHR2 −482 A/G genotype (18.2%) (Table 3). Since the number of offspring was different between the AMHR2 genotypes and the number of offspring is associated with age at menopause, we stratified the association analysis of age at menopause for this parameter. We observed a significant influence of the number of children on age at menopause in the AMHR2 482 G/G homozygous group (n=79). Nulliparous women with the G/G genotype had a 2.6 years earlier onset of menopause (46.6 ± 0.9 year, mean ± SEM) compared with nulliparous women with the AMHR2 A/A genotype (49.2 ± 0.2 year, P = 0.005) (Fig. 1). Women with one or two children and the G/G genotype had a similar onset of menopause compared with the other AMHR2 genotypes (P=0.51), whereas women with the G/G genotype and more than two children tended to have a 1.5 years later onset of menopause (51.4 ± 0.8) compared with the A/A genotype (49.9 ± 0.2), although this does not reach significance (P=0.072) (Fig. 1). When differences in age at menopause among genotype groups were tested in an univariate regression model, adjusted for all possible confounders, a strong synergistic interaction (P=0.001) between the AMHR2 G/G genotype and the number of offspring was observed.

In the LASA cohort, women homozygous for the AMHR2 −482G allele tended to enter menopause 2.8 years earlier compared with women homozygous for the 482A allele (P=0.054) (Table 3). After adjustment of age at menopause for possible confounders, this difference remained borderline.
significant (P=0.063). In the LASA cohort, no differences were observed between the AMHR2 genotype groups in age at menarche, number of offspring (Table 3), smoking and socio-economic status (results not shown).

When the Rotterdam cohort and the LASA cohort were analyzed together with adjustment for possible confounders, the AMHR2 $-482\ A>G$ polymorphism tended to be associated with age at menopause (A/A 49.7 ± 0.1, A/G 49.4 ± 0.2, G/G 48.9 ± 0.5, mean ± SEM, P=0.068). Combined analysis of the AMH Ile$^{49}$Ser polymorphism and the AMHR2 $-482\ A>G$ polymorphism revealed no additional associations with age at menopause (results not shown).

Discussion
In the present study, we investigated for the first time whether genetic variants in the AMH signaling pathway influence the onset of natural menopause. In two Dutch cohorts of post-menopausal women, the association of two polymorphisms in the AMH and AMHR2 gene, which both capture the common genetic variation in the gene (Kevenaar et al., 2007), with age at menopause was studied. In the Rotterdam study, the AMHR2 $-482\ A>G$ polymorphism was associated with age at menopause in interaction with parity. Also in the LASA cohort and when both cohorts were combined, the AMHR2 $-482\ A>G$ polymorphism tended to be associated with age at menopause.

Women with the AMHR2 $-482\ G/G$ genotype tended to have an earlier onset of menopause compared with women with the A/A genotype, which is indicative of less inhibition of primordial follicle recruitment. Hence, the AMHR2 $-482\ G/G$ genotype could result in diminished AMH signaling, which is in concordance with a previous study (Kevenaar et al., 2007), in which the $-482G$ allele was associated with higher estradiol levels in premenopausal women, correlating with less inhibition of FSH sensitivity by AMH. Indeed, the $-482\ A>G$ polymorphism is located at a potential c-Myb and c-Myc transcription factor-binding site (www.cbil.upenn.edu/tess) (Schug and Overton, 1997), and therefore may modify promoter activity.

Besides the subtle differences in age at menopause between the AMHR2 genotypes in the combined cohort, we observed a strong synergistic interaction between the AMHR2 $G/G$ genotype and the number of children in the Rotterdam cohort. This interaction suggests that the $-482\ A>G$ polymorphism influences the relation between age at menopause and parity. The relation between age at menopause and parity has been demonstrated in many epidemiological studies (Stanford et al., 1987; Whelan et al., 1990; Cramer et al., 1995; Cassou et al., 1997; van Noord et al., 1997). Nulliparous women enter menopause 0.5 (Whelan et al., 1990) to 1.5 years (Stanford et al., 1987) earlier compared with parous women, as is also observed in the Rotterdam study (0.6 years difference) (results not shown). Nevertheless, little is known about the underlying mechanism of this relation between age at menopause and parity. Two possible explanations have been proposed. First, it has been suggested that age at menopause and parity are not causally related but are both reflecting the process of ovarian aging (Kok et al., 2003). The second explanation is that during pregnancy, less primordial follicles are recruited, resulting in a delayed onset of menopause (Stanford et al., 1987; Whelan et al., 1990; McGee and Hsueh, 2000). The latter explanation is supported by rodent studies. In mice, the number of follicles that start growing is reduced during pregnancy (Pedersen and Peters, 1971), and rats allowed to undergo multiple pregnancies show a delay in reproductive aging (Matt et al., 1987). Furthermore, prolonged elevation of circulating progesterone in rats suppresses initial follicle recruitment, thus maintaining a larger primordial follicle pool (Lapolt et al., 1988; Lapolt et al., 1998). During pregnancy in women, AMH serum levels, which reflect the size of the growing and, indirectly, the primordial follicle pool (van Rooij et al., 2002; Visser et al., 2006), apparently do not change (La Marca et al., 2005), suggesting that during pregnancy initial recruitment continues. Alternatively, initial recruitment might halt but growing follicles might be rescued from atresia during pregnancy.

In view of the effects of AMH and possibly also parity on primordial follicle recruitment, it is intriguing that the relation between parity and age at menopause appears to be influenced by the AMHR2 $-482\ A>G$ polymorphism. The $-482\ A>G$ SNP, located in the promoter region of the gene, is in linkage disequilibrium with several other SNPs (Kevenaar et al., 2007), and therefore also other variants can drive the observed association. However, it is possible that changes in hormone levels during pregnancy, such as progesterone, prolactin and estradiol, alter the expression or function of the receptor. Although signaling of the G-allele derived AMHRII in regularly cycling women is probably less compared with the A-allele derived AMHRII, altered hormone levels during pregnancy might have a stronger effect on the G-allele AMHRII than on the A-allele AMHRII. This may result in increased expression and/or activity of the G-allele derived AMHRII and thereby a stronger inhibition of primordial follicle recruitment during pregnancy. However, functional studies and additional replication studies are necessary to obtain definite conclusions regarding the effect of the AMHR2 $-482\ A>G$ polymorphism on age at menopause.

For the AMH Ile$^{49}$Ser polymorphism, no association with age at menopause is observed in both cohorts, suggesting that this polymorphism does not affect AMH function in follicle recruitment. In contrast, in our previous study (Kevenaar et al., 2007), we observed that the AMH Ile$^{49}$Ser polymorphism is associated with altered FSH sensitivity. It is possible that the effect of this polymorphism on primordial follicle recruitment is masked or compensated by other factors.

In the Rotterdam and the LASA cohort, age at menopause was determined retrospectively, which has been shown to be susceptible to bias (den Tonkelaar, 1997; Hahn et al., 1997). Nevertheless, it seems unlikely that misclassification due to recall bias is different across genotypes.

In conclusion, the observed association of genetic variation in the AMHR2 gene with age at menopause suggests a role for AMH signaling in the complex process of human ovarian aging. Although the potential consequences of the AMHR2 $-482\ A>G$ polymorphism on receptor function still need to
be elucidated, our results suggest that the AMHR2 polymorphism contributes to the wide range in onset of menopause. Furthermore, our results may provide more insight into the mechanism that drives the relationship between age at menopause and parity. It will be interesting to determine whether the AMHR2 polymorphism also influences the risk of menopause-related diseases, such as osteoporosis and breast cancer.

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