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***ALOX12* gene is associated with the onset of natural menopause in white women**

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Abstract

Objective—Natural menopause is a key physiological event in a woman's life. Timing of menopause affects risk for many postmenopausal systemic disorders and may thus influence life expectancy. Age at natural menopause (ANM) is largely determined genetically, but a list of candidate genes is far from complete. This study investigated the *ALOX12* gene for its possible association with ANM.

Methods—Six single-nucleotide polymorphisms (SNPs) of the gene (rs9904779, rs2073438, rs11571340, rs434473, rs2307214, and rs312462) were genotyped in a random sample of 210 unrelated white women. The SNPs and common haplotypes were then analyzed for their association with ANM. Smoking, alcohol consumption, and duration of breast-feeding were used as covariates.

Results—Two SNPs, rs9904779 and rs434473 (encodes a replacement of asparagine by serine in the protein), were significantly associated with ANM ($P = 0.022$ and 0.033 , respectively). The minor alleles of both SNPs seem to promote about 1.3- to 1.5-year earlier menopause and confer a 1.6 to 1.8 times higher risk for early menopause. All SNPs indicated significant or nearly significant interactions with alcohol use and duration of breast-feeding. Five common haplotypes were also associated with ANM.

Conclusions—The *ALOX12* gene seems to be associated with the timing of natural menopause in white women.

Keywords

Age at natural menopause; Association; *ALOX12*; Polymorphisms; Haplotypes

Natural menopause is one of the most important physiological events: it is related to the cessation of ovarian function and the end of the reproductive cence. Average age at natural menopause (ANM) in white women is about 50 years.^{1,2} Entering the menopausal transition is often associated with various psychological problems,^{3,4} which are especially pronounced

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under premature menopause.⁵ Early onset of natural menopause increases risk for many postmenopausal health complications, such as osteoporosis,^{6–8} ischemic disease,⁹ and ovarian cancer,^{10,11} whereas later menopause is a risk factor for endometrial^{12,13} and breast¹⁴ cancer. In such a way, timing of menopause may affect life expectancy.¹⁵ Therefore, knowing the factors that underlie ANM may potentially help to forecast onset of menopause, contribute to the prophylaxis of postmenopausal health problems, and thus increase overall longevity.

According to various estimates, genetic factors may account for up to 63% to 74% of ANM variation.^{16,17} Recent studies identified several genes and genomic regions that may contribute to ANM,^{18–21} but they constitute only a small portion of quantitative trait loci underlying ANM.

Arachidonate 12-lipoxygenase (ALOX12; EC 1.13.11.31) is an important enzyme of lipid metabolism. It catalyzes the transformation of arachidonic acid into 12(S)-hydroperoxyeicosatetraenoic acid by oxygenating its 12-position. Several considerations are in favor of the *ALOX12* gene being a possible contributor to the onset of menopause. First, several studies provided evidence that menopause may increase risk for obesity and affect body fat distribution.^{22–25} This suggests that genes involved in fat metabolism (including *ALOX12*) may be associated with menopause. Second, the reaction catalyzed by ALOX12 is a step in the biosynthesis of leukotriene, a hormone playing an important role in inflammation and immunity.²⁶ ALOX12 and its metabolites stimulate cell proliferation and were therefore implicated in the etiology of various tumors,^{27–29} including breast cancer.^{30–33} Because breast cancer and some other cancers are associated with ANM,^{12–14} it is suggested that ALOX12 may also contribute to ANM. Third, in addition to its role in cancers, the gene was reported as a candidate for other primarily postmenopausal disorders, osteoporosis^{34,35} and Alzheimer's disease.³⁶ Finally, more evidence for a possible association of ALOX12 with menopause comes from the data about its expression in female reproductive organs and tissues, uterus,³⁷ and thecal and granulosa layers of preovulatory follicles.³⁸

Here, we present the results of the analysis of six ALOX12 single-nucleotide polymorphisms (SNPs) for their possible association with ANM in white women.

METHODS

Participants

The female participants in this study were recruited as part of our large-scale project on the genetic basis of osteoporosis. This study was approved by the institutional review board of Creighton University, and each participant signed an informed consent document before entering the study. The sample was composed of 210 randomly selected white women. All participants identified themselves as being of European origin. The exclusion criteria that were used for the participant recruitment have been described previously.³⁹ Based on these exclusion criteria, women with systemic metabolic diseases (eg, diabetes, hypoparathyroidism and hyper-parathyroidism, hyperthyroidism), chronic diseases of vital organs (brain, lung, heart, liver, kidney), malnutrition conditions (eg, chronic diarrhea, chronic ulcerative colitis), and surgical menopause (hysterectomy or oophorectomy) in their medical history were not included in the study. The participants also provided data about their age at menarche and menopause, smoking and alcohol consumption status, parity, breast-feeding status, and oral contraceptive use before menopause by answering a questionnaire.

ANM was determined as the age at the last menses (years) followed by one calendar year with no menses. To eliminate the probability of including women with premature ovarian failure, only data collected from women who experienced ANM after 40 years of age were analyzed. Early menopause was defined as ANM lower than the mean for the studied population. Data on the participants are given in Table 1.

Genotyping

Genomic DNA was isolated from peripheral blood using a Puregene kit (Gentra Systems, Inc, Minneapolis, MN) according to the manufacturer's protocol. The SNPs were selected using dbSNP (<http://www.ncbi.nlm.nih.gov/SNP/>). The following criteria were adopted for the SNP selection: (1) position in or around the gene, (2) minor allele frequencies greater than 0.05, (3) validation status in white women, and (4) being reported to dbSNP by various sources. The polymorphisms were genotyped using the Integrated Bead-Array System (Illumina, Inc).

Statistical analyses

All genotypes were tested for their correspondence to the Hardy-Weinberg equilibrium using PLINK software,⁴⁰ which is available online at <http://pngu.mgh.harvard.edu/~purcell/plink/>. PedCheck⁴¹ was used to verify whether genotype data are consistent with the Mendelian segregation.

Because of the very low frequencies of their minor allele homozygotes, the genotypes of three SNPs (rs11571340, rs2307214, and rs312462) were divided into two groups (with or without the minor allele, respectively) to increase the power of the analyses. The participants were also categorized according to the number of pregnancies and months of breast-feeding (Table 1).

Multiple linear and univariate regression analyses were applied to estimate the effects of the lifestyle factors and the studied SNPs on ANM. Each marker was investigated independently in the univariate analysis. This analysis was applied also to estimate pairwise SNP-SNP and SNP-environment interactions on ANM. To exhaustively exploit haplotype information, we subjected alleles (contiguous sets of markers) from sliding windows of all sizes to haplotype association tests. The haplotype-specific analysis that tests each haplotype at one time (each vs all others) was performed with the χ^2 test, with 1 degree of freedom. All statistical analyses were conducted using PLINK⁴⁰ and SPSS, version 16.0.1 (SPSS Inc, Chicago, IL).

RESULTS

Study participants' characteristics

The study participants were, on average, 63.7 ± 0.7 years old. Their mean $\pm$ SE age at menarche was 13.0 ± 0.1 years, and the mean $\pm$ SE ANM was 49.4 ± 0.3 years (Table 1). The skewness and kurtosis of the ANM values were -0.081 and 0.789 , respectively, that is, slightly deviated from the normal distribution parameters. According to the multiple regression analysis, cigarette smoking ($P = 0.037$), alcohol consumption ($P = 0.021$), and duration of breast-feeding ($P = 0.014$) were determined to influence ANM significantly in this sample and were thus used as covariates in the analyses.

SNP association analyses

All studied polymorphisms were in concordance with Hardy-Weinberg equilibrium (Table 2). Two SNPs, rs9904779 (SNP1) and rs434473 (SNP4), were significantly associated with ANM ($P = 0.022$ and 0.033 , respectively; Table 3). The minor alleles of these SNPs seem to confer a higher risk for early menopause. Women who carry a minor allele C of SNP1 have,

on average, 1.3 years earlier menopause (48.9 ± 0.5 y) than do the major allele homozygotes (50.2 ± 0.5 y) and 1.6 times higher relative odds for entering menopause before the mean ANM for the studied population, that is, 49.4 years (95% CI, 0.914–2.770). Likewise, carriers of the SNP4 minor allele (*G*) experience menopause about 1.5 years earlier (48.8 ± 0.5 y) than do homozygotes for the major allele (50.3 ± 0.5 y) and have 1.8 times higher relative odds for earlier menopause than the average ANM (95% CI, 1.032–3.213).

In addition to the individual SNPs, five haplotypes were associated with ANM (Table 4). Four of them include either allele of SNP4. Importantly, haplotypes B and D, carrying a major allele *A* of SNP4, seem to confer later ANM (variable β ; Table 4), whereas haplotypes C and E, carrying a minor allele *G* of this SNP, confer earlier ANM. Haplotype A, although including a minor allele of SNP1 (which supposedly confers lower ANM), is nevertheless associated with later ANM. This is due to the combined effects of alleles at SNP1 and SNP2.

All studied SNPs showed a significant or nearly significant interaction effect with alcohol consumption and duration of breast-feeding on ANM (Table 5). In addition, SNP1 and SNP4 indicated significant interaction with parity ($P = 0.05$ and 0.02 , respectively). On the other hand, no significant interactions between the polymorphisms were detected.

DISCUSSION

Despite the apparent importance of timing of menopause for health in the later part of a woman's life and, respectively, for longevity,⁴² only a few studies have been done to determine the genetic basis of this physiological event. The present study adds *ALOX12* to the list of candidate genes for ANM.^{18–20,43–45}

The exact role of *ALOX12* in the onset of menopause is not clear. Flatman et al³⁷ were among the first who reported a lower activity of *ALOX12* in the uterine cervix of postmenopausal women as compared with premenopausal women. This suggests that *ALOX12* expression level is related to the menopause status of a woman, and thus, the respective gene may be associated with menopause. Some more assumptions may be drawn from the available data about the relationship between menopause and arachidonic acid status. Several studies reported an effect of natural menopause on the content of arachidonic acid in female tissues, although the data were controversial. Specifically, postmenopausal women were reported to have either a decreased⁴⁶ or an increased^{47,48} level of arachidonic acid as compared with premenopausal women. On the other hand, surgical menopause (ovariectomy) did not affect the fatty acid composition of platelets but significantly increased the serum concentration of thromboxane B2, a metabolite of arachidonic acid.⁴⁹

In a quite recent study of induced ovulation using a murine model, Kurusu et al³⁸ provided evidence that inhibition of *ALOX12* resulted in impaired ovulation. This suggests that *ALOX12* is likely to have an important role in ovary function and, respectively, a potential contribution to the cessation of this function, that is, menopause.

One of the most pronounced effects of menopause is estrogen depletion, which is considered a key cause of post-menopausal adverse health consequences.^{50–52} Because catechol estrogens have a strong inhibitory effect on arachidonic acid metabolism, their lower concentration after menopause may promote increased synthesis of leukotriene and higher risk for associated disorders.⁵³ However, this assumption still needs to be examined further: the other studies either support⁵⁴ or reject⁵⁵ it.

Interestingly, all genetic variants (SNPs and haplotypes) identified here as those promoting earlier menopause have a lower population frequency as compared with the variants

associated with later ANM. If earlier menopause indeed increases mortality,⁴² then this may be a reason for the lower population frequency of the slightly deleterious genetic variants, which confer early menopause.

Importantly, the above studies, are essentially in favor of this effect per se although the exact mode of the ALOX12 effect on menopause is controversial. The body of evidence suggests that ALOX12 probably affects ANM by regulating the level of arachidonic acid and respective metabolites. With reference to this, two SNPs that are significantly associated with ANM may be functionally important. SNP1 (rs9904779) is located in the 5'-flanking region of the gene, 822 bp upstream the first codon, and may be within a regulatory area. SNP4 (rs434473) is a missense mutation resulting in the substitution of asparagine by serine at position 322 of the ALOX12 protein. Although asparagine and serine are similar in their physiochemical properties, the substitution may nonetheless affect the protein activity. For example, replacement of arginine by similar glutamine at position 261 results in a significant increase in ALOX12 enzyme activity in platelets and a higher risk for esophageal squamous cell carcinoma.⁵⁶

The difference in menopause onset of only 1.3 to 1.5 years between the genotypes (Table 3) may look clinically irrelevant. However, it should be noted that ANM is a complex trait determined by many genes and environmental factors. As such, the contribution of any single candidate gene is expected to be modest. However, a cumulative effect of several such genes, including possible gene-gene interactions, may be quite noticeable.

There are data suggesting a potential role of ALOX12 in aging processes. Specifically, the higher expression of the gene was associated with the diagnosed Alzheimer disease^{36,57} and cardiovascular problems.^{58–60} As the proportion of older people in the human population steadily increases, proper managing of healthy aging becomes more and more important. In these terms, identifying lifestyle and genetic factors that contribute to ANM helps to accomplish this task and improve overall quality of life.

The present study has several limitations that should be acknowledged. The observed significant interactions should be treated with certain caution because the sample size is small and, respectively, statistical power to detect interactions is limited. However, because several SNPs and lifestyle factors were analyzed, this might bring about a problem of multiple testing. We did not adjust for multiple testing for several reasons. First, this is a candidate gene study with previous knowledge, and the number of analyzed SNPs was small. Second, all six SNPs in our study were in strong linkage disequilibrium (data not shown), and therefore, the tests were highly correlated. Therefore, although a risk for false-positives still exists, it seems to be low. In addition, because simple correction for multiple testing is conservative, it may result in a further decrease in power to detect real effects.

CONCLUSIONS

As an important component of the key biochemical process (metabolism of arachidonic acid and, more generally, lipids), ALOX12 may potentially contribute to various post-menopausal metabolic disorders, which is indeed evidenced by many studies^{34–36,57–60}. However, the exact role of the gene in these health complications is largely unknown. It may be associated with the ALOX12 contribution to the onset of menopause. Despite the previously mentioned limitations, our study, along with the other independent observations mentioned here, provides support for this statement. However, further studies on larger samples and various ethnicities are needed to corroborate or reject this assumption.

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TABLE 1

Characteristics of the participants in this study

Participant characteristics	Value
Age, y	63.7 ± 0.7
Age at menarche, y	13.0 ± 0.1
Age at menopause, y	49.4 ± 0.3
Height, cm	162.0 ± 0.0
Weight, kg	73.8 ± 1.2
Use of oral contraceptive	54.7
Smoking	15.2
Alcohol consumption	65.7
Breast-feeding	54.3
Months of breast-feeding	7.9 ± 0.9
None	46.6
1–6	23.0
7–12	11.3
13–24	7.4
≥25	11.8
No. of pregnancies	4.3 ± 0.2
Pregnancies	
None	1.4
1 or 2	24.3
3 or 4	38.1
≥5	36.2

Data are presented as mean ± SD or percentage of the sample.

TABLE 2

Summary information about the studied SNPs in the total sample

SNP	SNP ID	Common name	Allele variants ^a	Position in the gene	MAF	P, HWE
SNP1	rs9904779		G/C	5'-flanking region	0.357	0.449
SNP2	rs2073438		C/T	Intron1	0.264	0.475
SNP3	rs11571340		G/A	Intron8	0.081	0.147
SNP4	rs434473	N322S	A/G	Exon8	0.388	0.463
SNP5	rs2307214		A/G	Exon11	0.081	0.147
SNP6	rs312462		C/T	Exon14	0.088	0.215

SNP, single-nucleotide polymorphism; ID, identification code; MAF, minor allele frequency; HWE, Hardy-Weinberg equilibrium.

^aThe minor allele is in bold.

TABLE 3

Results of the association analyses for the SNPs of the ALOX12 gene with age at natural menopause (mean $\pm$ SE) in white women

SNP	11	12	22	P
SNP1	49.0 $\pm$ 0.6 (13.8)	48.7 $\pm$ 0.4 (43.8)	50.2 $\pm$ 0.5 (42.4)	0.033
SNP2	49.3 $\pm$ 0.8 (7.7)	48.8 $\pm$ 0.4 (37.5)	49.8 $\pm$ 0.4 (54.8)	0.330
SNP3		50.2 $\pm$ 0.8 (14.8)	49.2 $\pm$ 0.3 (85.2)	0.369
SNP4	48.8 $\pm$ 0.6 (16.2)	48.8 $\pm$ 0.4 (45.2)	50.3 $\pm$ 0.5 (38.6)	0.022
SNP5		50.2 $\pm$ 0.8 (14.8)	49.2 $\pm$ 0.3 (85.2)	0.369
SNP6		48.5 $\pm$ 0.7 (16.2)	49.6 $\pm$ 0.3 (83.8)	0.395

11, 12, and 22 denote homozygote at minor allele and heterozygote and homozygote at major allele, respectively (the missed values for homozygotes at minor allele indicate that they were combined with heterozygotes into a single group). Numbers in parentheses indicate percentage of the studied sample.

SNP, single-nucleotide polymorphism.

Haplotypes of the ALOX12 gene showing significant or nearly significant association with ANM

TABLE 4

Haplotype	SNP1	SNP2	SNP3	SNP4	SNP5	Haplotype frequency	β	P
A	C	T				0.619	0.72	0.018
B		T		A		0.609	0.84	0.016
C			G	G		0.392	-1.37	0.016
D	C	T		A		0.605	0.68	0.021
E			G	G	A	0.392	-1.37	0.016

ANM, age at natural menopause; SNP, single-nucleotide polymorphism.

TABLE 5

Results of the univariate analysis (P values) for interaction effect between the SNPs of the ALOX12 gene and lifestyle factors on ANM

SNP	Factor	
	Alcohol consumption	Duration of breast-feeding
SNP1	0.038	<i>0.083</i>
SNP2	0.027	0.067
SNP3	0.018	0.005
SNP4	0.034	<i>0.071</i>
SNP5	0.018	0.005
SNP6	<i>0.064</i>	0.035

Nearly significant *P* values (> 0.05) are italicized.

SNP, single-nucleotide polymorphism; ANM, age at natural menopause.