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The chemokine (C-C-motif) receptor 3 (CCR3) gene is linked and associated with age at menarche in Caucasian females

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Abstract

Chemokine (C-C-motif) receptor 3 (CCR3), playing an important role in endometrium related metabolic pathways, may influence the onset of menarche. To test linkage and/or association between CCR3 polymorphisms with the variation of age at menarche (AAM) in Caucasian females, we recruited a sample of 1,048 females from 354 Caucasian nuclear families and genotyped 16 SNPs spanning the entire CCR3 gene. Linkage disequilibrium and haplotype blocks were inferred by Haploview. Both single-SNP markers and haplotypes were tested for linkage and/or association with AAM using QTDT (quantitative transmission disequilibrium test). We also tested associations between CCR3 polymorphisms and AAM in a selected random sample of daughters using ANOVA (analysis of variance). We identified two haplotype blocks. Only block two showed significant results. After correction for multiple testing, significant total associations of SNP7, SNP9 with AAM were detected (P = 0.009 and 0.006, respectively). We also detected significant within-family association of SNP9 (P = 0.01). SNP14 was linked to AAM (P = 0.02) at the nominal level. In addition, there was evidence of significant total association and nominal significant linkage (P = 0.008 and 0.03, respectively) with AAM for the haplotype AGA reconstructed by SNP7, SNP9 and SNP13. ANOVA confirmed the results by QTDT. For the first time we reported that CCR3 is linked and associated with AAM variation in Caucasian women. However, further studies are necessary to substantiate our conclusions.

Introduction

Menarche is the hallmark maturational event in a female's life. Early menarche is a risk factor for breast cancer, endometrial cancer (Rees 1995) and cardiovascular disease (Remsberg et al. 2005). Delayed menarche is associated with irregular menstrual cycles (Anai et al. 2001; Kjaer et al. 1992) and increases the risk of osteoporosis (Anai et al. 2001; Ulrich et al. 1995). Therefore, from a clinical point of view, it is of interest to identify factors that may influence the variation of age at menarche (AAM).

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Age at menarche is a complex trait determined by multiple environmental and genetic factors (Chie et al. 1997; Kaprio et al. 1995; Meyer et al. 1991; Treloar and Martin 1990). Previous investigations based on twin and family studies demonstrated that genetic factors can explain about 53–74% of the variation of AAM (Chie et al. 1997; Kaprio et al. 1995; Sharma 2002). Linkage and association studies have suggested either quantitative trait loci (QTLs) or candidate genes underlying the variation of AAM, such as genomic region of 22q13 (Guo et al. 2006) and the genes like cytochrome P450, subfamily XVII (CYP17) (Gorai et al. 2003; Lai et al. 2001), estrogen receptor α (ER-α) (Long et al. 2005; Stavrou et al. 2002) and sex hormone-binding globulin (SHBG) (Xita et al. 2005). However, the genetic studies in this field are still lacking and the results have been inconsistent.

During the immediate premenstrual phase, there is an influx of macrophages, neutrophils, and eosinophils into the endometrium. Circulating leukocytes into endometrium tissues is a complex process requiring the production of chemokines by leukocytes. Of these chemokines, eotaxin, belonging to the CC group of chemokines and acting through its receptor CCR3, is the most selective for eosinophils. Zhang et al. (2000) identified that eotaxin and CCR3 were strongly expressed by human eosinophils and endometrial epithelials using immunohistochemical analyses. It has been confirmed that eotaxin acted as a paracrine growth factor and regulates eosinophils into endometrium through CCR3 (Zhang et al. 2000). Eosinophils are likely to be critical for the focal inflammatory endometrial tissue breakdown, which characterizes menstruation (Jones et al. 2004; Salamonsen and Lathbury 2000). As menarche is the first natural endometrium destruction in a women's life, it is possible that CCR3 may influence the onset of menarche given the role of CCR3 in endometrium related metabolic pathways. This study represents our effort to test such hypothesis and is the first association study aiming at investigating the relationship between CCR3 and AAM. We investigated the LD (linkage disequilibrium) patterns and haplotype structures using high density SNPs of CCR3, and tested for linkage and/or association with AAM variation by quantitative transmission disequilibrium test (QTDT) in a large sample of Caucasian nuclear families.

Subjects and methods

Subjects

This study was approved by the Creighton University Institutional Review Board. The design and sampling procedures were published before (Xiong et al. 2005). Signed informed-consent documents were obtained from all participants before entering the study. Initially, all of the 1,873 participants from 405 nuclear families were US Caucasians of European origin recruited from Omaha and its surrounding areas for bone mineral density (BMD) research. For menarche study, we selected families that contain at least two informative female subjects (≥1 daughters) with AAM data. At first, 50 families having only male offsprings and 1 family without enough AAM data for daughters were excluded. To select "healthy" subjects for AAM study from the remained 354 families, females with diseases (such as gynecopathia, eating disease, and kidney disease) relevant to AAM before menarche were excluded. At last, 1,048 female subjects from 354 nuclear families were studied, aging from 19 to 85 years old.

AAM data

For each study subject, a detailed medical history, including menstrual history was recorded by nurse-administered questionnaire. AAM was collected using a retrospective method. The correlation between the self-recalled menarche age and observed menarche age is significantly high because menarche is an important change both physiologically and psychologically in a female's life (Kirchengast et al. 1998). AAM was calculated as the date of menarche following the onset of menses minus the date of birth (in years rounded to the tenth decimal). After

excluding three outliers whose AAM were 18 year or older (diagnosed as primary amenorrhea), our AAM data followed normal distribution as verified by the Kolmogorov–Smirnov test implemented in the software Minitab (Minitab Inc., State College, PA, USA). AAM of the final sample ranged from 8.5 to 17.0 years with a mean of 13.0 (SD = 1.4). As environmental factors, such as date of birth, may affect AAM, we performed correlation analysis between birth year and AAM but found no significant association ($P = 0.874$) in the present sample. So we did not include birth year as a covariate in the subsequent association analyses.

SNP selection and genotyping

A total of 16 SNPs in and around CCR3 gene were selected on the basis of the following criteria: (1) validation status, especially in Caucasians, (2) an average density of 1 SNP per 3 kb, (3) degree of heterozygosity, i.e., minor allele frequencies (MAF) >0.05, (4) functional relevance and importance, (5) reported to dbSNP by various source. Genomic DNA was extracted from whole blood using a commercial isolation kit (Gentra Systems, Minneapolis, MN, USA) following the procedure detailed in the kit. DNA concentration was assessed by a DU530 UV/Vis Spectrophotometer (Beckman Coulter, Inc, Fullerton, CA, USA). All the subjects were genotyped, yield a large set of genotype data that are further subject to Mendelian inheritance checking, allele frequency estimation and haplotype reconstruction. About 16 SNPs were successfully genotyped using the high-throughput BeadArray SNP genotyping technology of Illumina Inc (San Diego, CA, USA). The average rate of missing genotype data was reported by Illumina to be ~0.05%. The average genotyping error rate estimated through blind duplicating was reported to be less than ~0.01%. The 16 SNPs were spaced ~2.7 kb apart on average and covered the full transcript length of the CCR3 gene.

Statistical analysis

Using PedCheck (O'Connell and Weeks), we checked the Mendelian consistency of SNP genotype data and removed any inconsistent genotypes. Then the error checking option embedded in Merlin (Abecasis et al. 2002) was run to identify and disregard the genotypes flanking excessive recombinants, thus further reduced genotyping errors. Allele frequencies for each SNP were calculated by allele counting, and the Hardy-Weinberg equilibrium (HWE) was tested using the PEDSTATS procedure embedded in Merlin. Linkage disequilibrium block structure was examined by the program Haploview (Barrett et al. 2005) using 703 unrelated parents. The $|D'|$ values for all pairs of SNPs were calculated and the haplotype blocks were estimated using the confidence-interval method (Gabriel et al. 2002). SNPs with low MAF may inflate estimates of $|D'|$ and the use of confidence-bound estimates for $|D'|$ reduces this bias. We used the default settings which invoked a one-sided upper 95% confidence bound of $|D'| > 0.98$ and a lower bound of $|D'| > 0.7$ to define SNP pairs in strong LD. When at least 95% of SNP pairs in a region meet these criteria for strong LD, a block is identified. Haplotype tag SNPs (htSNPs) were selected by Haploview on a block-by-block basis. Haplotypes defined by the tagging SNPs within each block of CCR3 gene for all of the studied subjects were inferred by PedPhase V2.0 (http://www.cs.ucr.edu/~jili/haplotyping.html).

The QTDT software (http://www.sph.umich.edu/csg/abecasis/QTDT/), a powerful family based test for nuclear families of any size (Abecasis et al. 2000), was used to test each SNP and haplotype with estimated frequencies greater than 5% for linkage and association with AAM variation. It was based on the variance component framework and can test population stratification, total association, within-family association, linkage and linkage while modeling association simultaneously. We adopted the orthogonal model (Abecasis et al. 2000), which decomposes the total association into orthogonal between (β_b) and within-family (β_w) components. The between-family component is sensitive to population admixture, while the within-family component is significant only in the presence of LD and robust to population stratification/admixture. If significant associations were observed, the phenotypic variation

due to the detected marker is calculated as $2p(1-p)a^2$, where p is the allele frequency of the marker and a is the estimated additive variance. Population stratification is tested according to whether $\beta_b = \beta_w$ (Fulker et al. 1999). If there is no population stratification ($\beta_b = \beta_w$), the test result of total association should be more powerful (Abecasis et al. 2000).

Linkage tests are based on the standard variance component method and identity by decent (IBD) sharing among relatives. The program SimWalk2 (http://www.genetics.ucla.edu/software/simwalk2) was used to calculate IBD probabilities. A test for linkage while modeling association (Fulker et al. 1999) is also implemented in QTDT to evaluate whether a study marker is a functional variant. If the significant linkage is still detected after modeling association, we can conclude that the candidate marker is only in linkage disequilibrium with the lurking causal variant instead of being the causal one itself.

Since multiple markers were analyzed, a correction for multiple testing is required. The commonly used method is Bonferroni correction. However, the Bonferroni correction seems to be too conservative and potentially result in loss of statistical power (Nyholt 2001). This is because that some statistical tests here are highly correlated as some SNPs in the CCR3 gene are in significant strong LD. Therefore, Monte-Carlo permutation test (McIntyre et al. 2000) were performed 1,000 times to establish an empirical threshold, which was $P \leq 0.01$ for an individual test to achieve a global significance level of 0.05 for our analyses in the present study.

One daughter from each of the 354 nuclear families was randomly selected for population-based association analysis, which was performed by SPSS 11.5 (Language systems Corp, Chicago). The final random sample included 351 daughters after excluding 3 daughters without menarche data.

Results

Allele frequencies, LD and haplotype reconstruction

The information of all the CCR3 SNPs was summarized in Table 1. There is no potential functional mis-sense polymorphism in the dbSNP for CCR3. The MAFs of these 16 SNPs ranged from 0.07 to 0.45. All the SNPs were in HWE ($P > 0.01$). We identified two blocks with high LD, with the sizes of 10 and 31 kb, respectively. Block one ranged from 5′ UTR (untranslated region) to intron1 with SNP1 and SNP2 selected as htSNPs. Block 2 extended from intron1 to 3′ UTR with SNP7, SNP9 and SNP13 selected as htSNPs (Fig. 1). Therefore, four block one haplotypes and eight block two haplotypes were reconstructed accordingly with their frequencies presented in Table 2.

QTDT analyses

Table 3 summarizes the linkage and association results of CCR3 gene with AAM in nuclear families by QTDT. As block one polymorphisms showed no significant results, here we only included the results of block two polymorphisms. We did not find any significant evidence of population stratification for either single-SNP markers or haplotypes. This finding might rule out the confounding effect of population stratification on the tests of total association. For single-SNP marker analyses, after correcting for multiple tests, we detected significant total associations for SNP7 ($P = 0.009$) and SNP9 ($P = 0.006$) and significant within-family association for SNP9 ($P = 0.01$). The contributions of SNP7, SNP9 to AAM variation were 1.45 and 1.93%, respectively, calculated by total association test. At the nominal significant threshold of $P \leq 0.05$, significant total association for SNP10 ($P = 0.03$) and within-family associations for SNP7 ($P = 0.04$) and SNP10 ($P = 0.02$) were also observed. Within-family associations were in well consistent with total associations for these polymorphisms.

Interestingly, for SNP13, we found nominal significant within-family association ($P = 0.03$), but no significant signal of total association due to borderline significant population stratification ($P = 0.09$). In addition, the linkage signal to AAM was nominal significant for SNP14 ($P = 0.02$). After modeling association, there was still nominal significant linkage signal ($P = 0.02$), suggesting that SNP14 could not be a causal variant underlying AAM.

Four major haplotypes with frequencies above 5% (AAA, AGA, GGA and GGT) in block two were analyzed by QTDT. We detected further evidence of nominal linkage ($P = 0.04$) and significant total association ($P = 0.008$) for haplotype AGA. The contribution of haplotype AGA to the variation of AAM was 2.01%. Linkage signal was still nominal significant after modeling association ($P = 0.03$), implying that it may only be a locus in linkage disequilibrium with AAM variation. However, we did not find any evidence of within-family association for the four haplotypes. On the whole, the total associations with AAM variation were consistent between haplotype AGA and its component alleles of single-SNPs. In our sample, females carrying haplotype AGA (AAM 13.20 ± 1.55) were, on average, 4 months later ($P = 0.01$) in terms of the onset of menarche compared with the non-carriers (AAM 12.84 ± 1.38).

Population association analyses

We further investigated the associations of CCR3 polymorphisms with AAM variation in a random female sample by population-based association analysis. The significant single-SNP markers were SNP6, SNP7, SNP9, SNP10, SNP13 and SNP15 ($P = 0.04$, 0.008, 0.0008, 0.005, 0.02 and 0.01, respectively), with the contributions to AAM variation in our sample of 1.27, 1.98, 3.46, 2.23, 1.50 and 1.67%, respectively, as determined by the ANOVA r^2 values (Table 4). We also observed a significant association with AAM variation for the haplotype AGA in block two ($P = 0.001$), which could explain about 3.04% AAM variation in the population (Table 4). These findings supported our QTDT results.

Discussion

Previous data have established the genetic control of AAM variation (Chie et al. 1997; Guo et al. 2006; Kaprio et al. 1995; Long et al. 2005; Meyer et al. 1991; Treloar and Martin 1990). In recent years, much effort has been made to find and confirm genes or genomic regions underlying AAM variation. Motivated by the important role of CCR3 gene in the normal function of endometrium, we tested linkage and/or association between CCR3 and AAM variation in a large Caucasian sample of 354 nuclear families. The family based analysis, QTDT can directly test LD between a quantitative trait and a marker locus and is robust against population stratification. It is a powerful tool in testing linkage and association of candidate genes with complex traits (Allison 1997). To our best knowledge, this is the first family based linkage and association study between CCR3 gene and AAM.

A recent genome-wide scan for AAM by our group (Guo et al. 2006) also revealed certain linkage evidence to AAM variation in the CCR3 harboring region in Caucasian women (LOD = 0.84, $P = 0.024$, in another set of pedigrees that are different from pedigrees used in this study, but sampled from the same US Caucasion population). In our study, significant total associations were found for SNP7, SNP9 and the haplotype AGA reconstructed by the htSNPs —SNP7, SNP9 and SNP13 in block two. It was shown that females carrying haplotype AGA were 4 months later with respect to the onset of menarche than the non-carriers. Our population-based analyses confirmed the family based analysis as well. In addition, the significant within-family associations were found for SNP9. Taken together, our results strongly suggested that CCR3 polymorphisms may influence AAM variation in Caucasian women.

It is not unexpected to detect the associations between CCR3 gene polymorphisms with the AAM variation. In endometrium, leukocytes, especially eosinophils rise dramatically before

the focal inflammatory endometrial destruction. Leoukocytes produce a series of chemokins and cytokins including CCR3 that in concert result in focal production and activation of matrix metalloproteinases by endometrial cells and the subsequent breakdown of tissue characterizing menstruation (Salamonsen and Lathbury 2000). Other chemokins, such as MCP-3, and MCP-4 also bind to CCR3 and are involved in the regulation of endometrial cell function (Zhang et al. 2000). All those molecular and cell studies suggested that CCR3 play an important role in menstruation (Jones et al. 2004; Salamonsen and Lathbury 2000).

Furthermore, we previously reported the significant genetic correlation between AAM and BMD in Caucasians (Guo et al. 2005). We also detected a significant genetic correlation between AAM and covariates-adjusted (age, weight, menopause status) spine BMD ($r = -0.1462$, $P < 0.001$) in the present study, using the same analysis method as in our previous study (Guo et al. 2005). Other studies demonstrated that CCR3 polymorphisms were associated with bone resorption and the expression of CCR3 gene was up-regulated in subjects with low BMD (Day et al. 2004; Graves et al. 1999; Lisignoli et al. 2002; Liu et al. 2005; Murdoch and Finn 2000). Together, it may be suggested that CCR3 gene has pleiotropic effects on both AAM and BMD, which are two closely correlated phenotypes (Kroger et al. 1993; Stoll 1998; Theintz et al. 1992; Vihko and Apter 1984).

In summary, our study firstly provided evidence for the linkage and association between CCR3 gene polymorphisms and AAM in Caucasian females. These results should be valuable in terms of initiating the follow-up research aiming at replicating our findings and identifying the associated causal functional variants influencing AAM variation. Our results, though, are limited within Caucasian women. So, the validity of them in other ethnic groups awaits further investigation. Eventually, unraveling the hidden mechanism driving the linkage and association observed by us will definitely contribute to the understanding of the genetic control of AAM variation that is highly correlated with the bone health of women.

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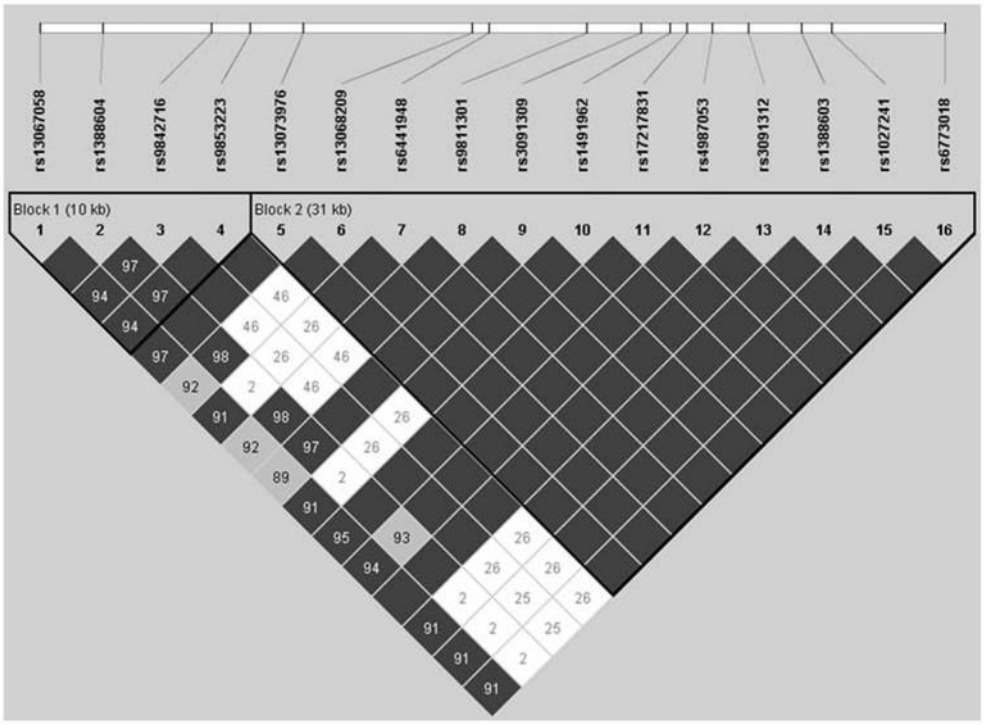

Fig. 1.

Linkage disequilibrium pattern of CCR3 gene. *Block 1* ranged from SNP1 to SNP4, with size of ~10 kb, *block 2* ranged from SNP5 to SNP16, with size of ~31 kb

Table 1

SNP marker information

SNP	Marker[a]	Minor allele frequence	Role[c]	Allels	Position[b]
1	rs13067058	0.0738(A)	5′ UTR	A/G	46248770
2	rs1388604	0.3654(T)	5′ UTR	A/T	46251882
3	rs9842716	0.4581(A)	5′ UTR	A/G	46257178
4	rs9853223	0.4576(A)	Intron1	A/G	46259097
5	rs13073976	0.0719(A)	Intron1	A/G	46261667
6	rs13068209	0.3251(A)	Intron1	A/G	46269961
7	rs6441948	0.4434(A)	Intron1	A/G	46270718
8	rs9811301	0.324(A)	Intron1	A/C	46275552
9	rs3091309	0.1907(A)	Intron1	A/G	46278188
10	rs1491962	0.4447(G)	Intron2	A/G	46279630
11	rs17217831	0.0729(A)	Intron2	A/C	46280445
12	rs4987053	0.0746(G)	Exon3	A/G	46281704
13	rs3091312	0.2492(T)	3′ UTR	A/T	46283476
14	rs1388603	0.4452(A)	3′ UTR	A/G	46286070
15	rs1027241	0.4455(A)	3′ UTR	A/G	46287543
16	rs6773018	0.4453(A)	3′ UTR	A/G	46293055

[a] SNP ID in the dbSNP (http://www.ncbi.nlm.nih.gov/SNP)

[b] Chromosome position in dbSNP (http://www.ncbi.nlm.nih.gov/SNP)

[c] Only one exonic SNP (rs4987053) is included; two other non-synonymous SNPs (rs4987125 and rs5742906) are listed in the db-SNP but they are rare (<1%)

Table 2

Haplotype information

Haplotype	Frequency
Block 1[a]	
AA	0.565
AT	0.356
GA	0.078
GT	0.001
Block 2[b]	
AAA	0.184
AAT	<0.001
AGA	0.261
AGT	0.001
GAA	<0.001
GAT	<0.001
GGA	0.306
GGT	0.248

[a] Haplotypes were reconstructed with SNP1 and SNP2

[b] Haplotypes were reconstructed with SNP7, SNP9 and SNP13

Table 3

QTDT analyses for the linkage and association between block 2 polymorphisms of CCR3 and age at menarche (AAM) variation

SNP	Marker	Stratification	Within-family association	Total association	Linkage	Linkage modeling association
5	rs13073976	—	—	—	—	—
6	rs13068209	—	0.07	—	—	—
7	rs6441948	—	0.04*	0.009**	—	—
8	rs9811301	—	0.07	—	—	—
9	rs3091309	—	0.01**	0.006**	—	—
10	rs1491962	—	0.02*	0.03*	—	—
11	rs17217831	—	—	—	—	—
12	rs4987053	—	—	—	—	—
13	rs3091312	0.09	0.03*	—	—	—
14	rs1388603	—	—	0.08	0.02*	0.02*
15	rs1027241	—	0.09	0.06	—	—
16	rs6773018	—	0.09	0.06	—	—
Block 2	Haplotype				0.04*	
	AAA	—	—	—		0.03*
	AGA	0.08	0.09	0.008**		0.03*
	GGA	—	—	—		0.05*
	GGT	—	—	—		0.03*

Data not shown for block one polymorphisms, for which there is no significant evidence of either linkage or association

– $P > 0.10$

* Significant results at the nominal threshold $P \leq 0.05$

** Global significant results at the empirical threshold $P \leq 0.01$ after 1,000 times Monte Carlo permutation

Table 4

Population association analyses for CCR3 gene polymorphisms with AAM variation using randomly selected daughters (including only the polymorphisms with significant results)

Polymorphisms	ANOVA *P* value	r^2 (%)
SNP6	0.04	1.27
SNP7	0.008	1.98
SNP9	0.0008	3.46
SNP10	0.005	2.23
SNP13	0.02	1.50
SNP15	0.01	1.67
Haplotype AGA	0.001	3.04