Gene polymorphisms in RANKL/RANK/OPG pathway are associated with ages at menarche and natural menopause in Chinese women

Peng Duan, Zhi-Ming Wang, Jiang Liu, Li-Na Wang, Zhi Yang and Ping Tu*

Abstract

Background: Age at menarche (AAM) and age at natural menopause (AANM) have been shown intimately associated with woman's health later in life. Previous studies have indicated that AAM and AANM are highly heritable. RANKL/RANK/OPG signaling pathway is essential for mammary gland development, which is also found associated with post-menopausal and hormone-related diseases. The aim of this study was to evaluate associations between the polymorphisms in the TNFSF11, TNFRSF11A and TNFRSF11B genes in the RANKL/RANK/OPG pathway with AAM and AANM in Chinese women.

Methods: Post-menopausal Chinese women (n = 845) aged from 42 to 89 years were recruited in the study. Information about AAM and AANM were obtained through questionnaires and the genomic DNA was isolated from peripheral blood from the participants. Total 21 tagging single nucleotide polymorphisms (SNPs) of TNFSF11, TNFRSF11A and TNFRSF11B were genotyped.

Results: Three SNPs of TNFRSF11A (rs4500848, rs6567270 and rs1805034) showed significant association with AAM (P < 0.01, P = 0.02 and P = 0.01, respectively), and one SNP (rs9962159) was significantly associated with AANM (P = 0.03). Haplotypes TC and AT (rs6567270-rs1805034) of TNFRSF11A were found to be significantly associated with AAM (P = 0.01 and P = 0.02, respectively), and haplotypes GC and AC (rs9962159-rs4603673) of TNFRSF11A showed significant association with AANM (P = 0.03 and P < 0.01, respectively). No significant association between TNFSF11 or TNFRSF11B gene with AAM or AANM was found.

Conclusions: The present study suggests that TNFRSF11A but not TNFSF11 and TNFRSF11B genetic polymorphisms are associated with AAM and AANM in Chinese women. The findings provide evidence that genetic variations in RANKL/RANK/OPG pathway may be associated with the onset and cessation of the menstruation cycle.

Keywords: RANKL, RANK, OPG, TNFSF11, TNFRSF11A, TNFRSF11B, Polymorphism, Age at menarche, Age at natural menopause

Background

Age at menarche (AAM) and age at natural menopause (AANM) have been shown intimately associated with woman's health later in life. Women with early menarche have high risks of breast cancer [1], ovarian cancer [2], type 2 diabetes [3] or metabolic syndrome [4], whereas late menarche can increase the risk of osteoporosis [5]. On the other hand, early AANM is associated with increased risk of cardiovascular diseases [6] and osteoporosis [7]. Recent data have shown that AAM and AANM were associated with all-cause mortality [8]. However, the factors that affect AAM and AANM are not entirely clear. AAM and AANM are complex traits which are influenced by both genetic and environmental factors and their interactions [9]. Twin and familial studies have indicated that AAM and AANM are highly heritable, ranging from 45% to 74% for AAM [10,11] and from 49% to 87% for AANM [12,13]. Genes involved in hormone biosynthesis and metabolic pathways were found to be associated with AAM and AANM [14,15], however, no specific genes have been identified yet.
The receptor activator of nuclear factor-kappa B ligand (RANKL), its receptor RANK and the decoy receptor osteoprotegerin (OPG) belong to the tumor necrosis factor superfamily and are encoded by genes \textit{TNFSF11}, \textit{TNFRSF11A} and \textit{TNFRSF11B}, respectively. RANKL/RANK/OPG signaling pathway plays important roles in bone modeling and remodeling [16], cell death and proliferation, inflammation, and immunity [17,18]. RANKL/RANK/OPG pathway is also found associated with post-menopausal and hormone-related diseases, such as osteoporosis [19] and reproductive cancer [20]. Furthermore, RANKL is found to be essential for mammary gland development in mice by promoting proliferation and maintaining survival of mammary epithelial cells [21]. Mammary gland changes are one of the hallmarks during menarche and menopause [22,23]. Therefore, RANKL/RANK/OPG pathway may involve in modulating the onset and cessation of the menstrual cycle. The present study investigated the associations of single nucleotide polymorphisms and haplotypes in \textit{TNFSF11}, \textit{TNFRSF11A} and \textit{TNFRSF11B} genes in RANKL/RANK/OPG pathway with AAM and AANM in Chinese females.

\textbf{Methods}

\textbf{Participants}

A total 1026 post-menopausal women from ten community centers in Nanchang from December 2011 to December 2012 were enrolled in the study. All the participants were from Han Chinese ethnic group. Age at interview, AAM, AANM, detailed medical history, birth history (number of live delivery), and abortion information (number of abortions) were obtained through a self-designed questionnaire, all the information collected in the study was self-reported. AAM was defined as the age at the first menstrual period. AANM was defined as one year without menstruation after the age at the last menstrual period. BMI, age at interview, number of deliveries and abortion information (number of abortions) were considered as covariates and were adjusted during analysis. The stepwise multiple regression analysis was used to analyze the relationships between the SNPs and AAM and AANM, subsequently, each SNP with different genotype was analyzed independently using one-way univariate analysis of variance (ANOVA), BMI, age at interview, number of deliveries and abortions were considered as covariates and were adjusted during analysis. Bonferroni correction was used to adjust the \( p \) values for multiple comparisons. The statistical analyses were performed using SPSS version 13.0 for Windows (SPSS Inc., Chicago, IL, USA). The linkage disequilibrium structure and allele frequencies were calculated as weight/height\(^2\).

All of the participants were subjected to blood counts, liver and kidney function tests, fasting plasma glucose tests. Subjects included in the study had normal blood counts, normal liver and kidney functions and blood glucose levels. Subjects were excluded from the study if they suffered from diseases and surgeries that could affect menstruation, such as severe chronic diseases, rheumatic diseases (e.g. systemic lupus erythematosus, rheumatoid arthritis), severe endocrine and metabolic diseases (e.g. diabetes, hyperparathyroidism, pituitary or adrenal diseases), malabsorption diseases (e.g. chronic diarrhea, anorexia nervosa), cancer, and uterine or ovarian resection. Participants who had taken glucocorticosteroid or sex hormone within the past 3 months were also excluded. Finally, 845 subjects were included in the study.

The study was approved by the Ethics Committee of The Third Hospital of Nanchang. Written informed consent was obtained from every participant.

\textbf{TagSNP selection}

Tagging SNPs of the three genes were selected from the software program Haplovview version 4.2 [24] (http://www.broad.mit.edu/mpg/haplovview/) with minor allele frequencies (MAF) > 10% in the Chinese Han population in HapMap (http://www.hapmap.org/), and the pairwise linkage disequilibrium (LD) was greater than a threshold of \( r^2 \) (\( r^2 = 0.8 \)). In addition to, SNPs reported in previous studies or potentially functional SNPs in three candidate genes were forced into the SNP selection process. Finally, a total of 21 SNPs were selected in three genes (9 in \textit{TNFRSF11A} gene, 6 in \textit{TNFSF11} gene, and 6 in \textit{TNFRSF11B} gene). Of these, 18 SNPs are located in the introns of the three genes, one in 5'UTR, two in the exonic region. All of these SNPs were authenticated using the NCBI (http://www.ncbi.nlm.nih.gov/SNP/) and HapMap databases.

\textbf{Genotyping}

Approximately 5 mL of venous blood was collected from all of the participants after a minimum of 10 h fasting and stored in tubes containing 100 μL of 10% ethylene diaminetetraacetic acid (EDTA). Genomic DNA was extracted from whole blood samples using the QIAamp DNA Mini Kit (Qiagen Inc., Hilden, Germany). DNA samples concentration and quality were detected spectrophotometrically at 260/280 nm and stored at –80°C until analysed. Genotyping was performed using the high-throughput Sequenom genotyping platform (MassARRAY MALDI-TOF MS system, Sequenom Inc., San Diego, CA). For quality control, 5% of the samples were repeatedly genotyped, and the results were found to be 100% concordant.
examined using Haplovlew 4.2 software [24]. The significance of each haplotype within the defined blocks was analyzed by PLINK software [25] (http://pngu.mgh.harvard.edu/~purcell/plink/). All analyses were two-tailed, and $P$-value < 0.05 was considered statistically significant.

**Results**

**Characteristics of the study participants**

The basic characteristics of the 845 participants aged from 42 to 89 years were shown in Table 1. The mean age at interview was 60.88 ± 8.72 years, the mean AAM was 14.97 ± 2.00 years and AANM was 48.77 ± 4.16 years. No statistically significant association was observed between AAM and AANM ($P = 0.15$).

**SNP genotyping and linkage disequilibrium**

The basic characteristics of the SNPs are listed in Table 2. All study SNPs had a minor allele frequency of at least 0.1 and were in agreement with Hardy-Weinberg equilibrium ($P > 0.05$). Linkage disequilibrium between alleles at polymorphic loci was shown in Figure 1. Four haplotype blocks and seventeen of the most common haplotypes (frequency > 5%) were further analyzed for the association of haplotype with AAM and AANM.

**Association analyses of the SNP and haplotypes with AAM and AANM**

Three SNPs in TNFRSF11A, i.e. rs4500848, rs6567270 and rs1805034, showed significant association with AAM ($P < 0.01$, $P = 0.02$ and $P = 0.01$, respectively), whereas only rs9962159 in TNFRSF11A was significantly associated with AANM ($P = 0.03$) (Table 2). After correction of age at interview, BMI, number of deliveries and abortions, the associations between those SNPs and AAM or AANM were found significant. After the Bonferroni correction, the rs4500848 was still significantly associated with AAM ($P = 0.04$), however, the associations between the others SNPs with AAM or AANM were no longer statistically significant (Table 2).

Individuals with the T/T genotype of SNP rs4500848 had an earlier onset of menarche by 0.59 years and those with the T/T genotype. Likewise, women with the C/G genotype of SNP rs9962159 had an earlier menopause by 0.79 years than those with the A/A genotype (Table 3).

Two haplotypes (TC and AT) of block rs6567270-rs1805034 of TNFRSF11A were found significantly associated with AAM ($P = 0.01$ and $P = 0.02$, respectively) (Table 4). Haplotypes GC and AC of block rs9962159-rs4603673 of TNFRSF11A were significantly associated with AANM ($P = 0.03$ and $P < 0.01$, respectively). Haplotype TAGCGT of block rs9525641-rs2277439-rs2324851-rs2875459-rs2200287-rs9533166 of TNFSF11 showed marginally significant association with AAM ($P = 0.06$). Notably, all the significantly associated SNPs and haplotypes were observed in TNFRSF11A. SNPs and haplotypes in TNFSF11 and TNFRSF11B genes did not show significant association with either AAM or AANM.

**Discussion**

According to the previous studies, there was a direct relationship between AAM and AANM, women with earlier menarche had earlier menopause in Poland [26]. However, other studies had reported no association between AAM and AANM [27]. In this study, no statistically significant association was observed between AAM and AANM. The present study revealed that three SNPs (rs4500848, rs6567270 and rs1805034) and two haplotypes of TNFRSF11A showed significant association with AAM in Chinese women. These findings are in line to a previous report by Pan et al. [28], the authors found five SNPs (rs7239261, rs8094884, rs3826620, rs8098929, and rs9956850) and seven haplotypes of TNFRSF11A significantly associated with AAM in Chinese women. Thus, polymorphisms in TNFRSF11A are highly associated with AAM in Chinese women. In contrast to TNFRSF11A, SNPs of TNFSF11 did not show association with AAM in our study. Noticeably, two SNPs (rs9525641 and rs2200287) of TNFSF11 displayed a strong association with AAM in white women [29], but no significant association was observed between the two SNPs and AAM in our study. The inconsistency between the results of the present study and the other [29] may due to the different ethnic populations used, different sample sizes and statistical approaches. The inconsistent results were also observed in the association of TNFSF11 gene polymorphisms and AANM. In the present study, no significant association between polymorphisms of TNFSF11 and AANM was found in Chinese women, however, such relationship was reported in white women, two SNPs (rs346578 and rs9525641) of TNFSF11 showed association with AANM [29]. Regardless of the discrepancy in TNFSF11, we and the others [29] both...
### Table 2: Associations for the SNPs of TNFSF11, TNFRSF11A and TNFRSF11B genes with AAM and AANM

| Gene     | SNP               | Allele | Position | Function        | HWE    | MAF        | AAM Beta | P       | AAM P-Bonf | AANM Beta | P       | AANM P-Bonf |
|----------|-------------------|--------|----------|-----------------|--------|------------|----------|---------|------------|-----------|---------|------------|
| TNFSF11  | rs1485286         | C/T    | Intron   |                 | 0.4237 | T = 0.406  | 0.0697   | 0.49    | 1.00       | 0.2428    | 0.25    | 1.00       |
| TNFSF11  | rs11573869        | A/G    | Intron   |                 | 0.8783 | G = 0.165  | −0.0529  | 0.69    | 1.00       | 0.1109    | 0.68    | 1.00       |
| TNFSF11  | rs3102728         | T/C    | Intron   |                 | 0.2913 | C = 0.138  | 0.0726   | 0.61    | 1.00       | −0.2352   | 0.43    | 1.00       |
| TNFSF11  | rs11573819        | G/A    | Intron   |                 | 0.9402 | A = 0.157  | 0.0501   | 0.71    | 1.00       | −0.1709   | 0.54    | 1.00       |
| TNFSF11  | rs2073618         | C/G    | Asn by Lys |               | 0.5148 | G = 0.258  | −0.0065  | 0.95    | 1.00       | 0.2687    | 0.25    | 1.00       |
| TNFSF11  | rs2073617         | A/G    | UTR-5    |                 | 0.7887 | G = 0.382  | −0.1137  | 0.26    | 1.00       | 0.1741    | 0.41    | 1.00       |
| TNFSF11A | rs9525641         | T/C    | Intron   |                 | 0.1546 | C = 0.472  | 0.0810   | 0.42    | 1.00       | −0.0656   | 0.75    | 1.00       |
| TNFSF11  | rs2277439         | A/G    | Intron   |                 | 0.5905 | G = 0.295  | −0.0611  | 0.56    | 1.00       | 0.2430    | 0.27    | 1.00       |
| TNFSF11  | rs2324851         | G/A    | Intron   |                 | 0.6728 | A = 0.294  | −0.0699  | 0.51    | 1.00       | 0.2351    | 0.29    | 1.00       |
| TNFSF11  | rs2875459         | C/T    | Intron   |                 | 0.8542 | T = 0.220  | −0.1036  | 0.38    | 1.00       | −0.0189   | 0.94    | 1.00       |
| TNFSF11  | rs2200287         | G/A    | G/A      |                 | 0.8891 | A = 0.220  | −0.0898  | 0.44    | 1.00       | −0.0215   | 0.94    | 1.00       |
| TNFSF11  | rs9531166         | T/C    | Intron   |                 | 0.5125 | C = 0.131  | −0.1694  | 0.23    | 1.00       | −0.2154   | 0.47    | 1.00       |
| TNFSF11A | rs9962159         | A/G    | Intron   |                 | 0.4172 | G = 0.435  | 0.1338   | 0.17    | 1.00       | −0.4434   | 0.03    | 0.57       |
| TNFSF11A | rs4603673         | C/G    | Intron   |                 | 0.7190 | G = 0.162  | −0.1363  | 0.31    | 1.00       | −0.2551   | 0.36    | 1.00       |
| TNFRSF11A| rs7239261         | C/A    | Intron   |                 | 0.2342 | A = 0.239  | −0.1656  | 0.14    | 1.00       | 0.3123    | 0.18    | 1.00       |
| TNFRSF11A| rs4500848         | C/T    | Intron   |                 | 0.7267 | T = 0.262  | −0.3395  | <0.01   | 0.04       | −0.0378   | 0.87    | 1.00       |
| TNFRSF11A| rs6567270         | T/A    | Intron   |                 | 0.1116 | A = 0.408  | 0.2265   | 0.02    | 0.39       | 0.1197    | 0.55    | 1.00       |
| TNFRSF11A| rs1805034         | T/C    | Ala by Val|               | 0.3758 | C = 0.288  | −0.2790  | 0.01    | 0.22       | 0.0069    | 0.98    | 1.00       |
| TNFRSF11A| rs4303637         | C/T    | Intron   |                 | 0.9818 | C = 0.471  | 0.1618   | 0.10    | 1.00       | 0.0701    | 0.73    | 1.00       |
| TNFRSF11A| rs4941131         | C/A    | Intron   |                 | 0.8166 | C = 0.330  | 0.0437   | 0.67    | 1.00       | −0.1892   | 0.38    | 1.00       |
| TNFRSF11A| rs9646629         | G/C    | Intron   |                 | 0.2416 | C = 0.460  | 0.1461   | 0.14    | 1.00       | −0.2256   | 0.28    | 1.00       |

HWE, P values for Hardy-Weinberg equilibrium. MAF, minor allele frequency. Beta, the regression coefficient. P-Bonf, P-value by Bonferroni correction. AAM, age at menarche. AANM, age at natural menopause.

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**Figure 1** Linkage disequilibrium (LD) patterns for TNFSF11, TNFRSF11A and TNFRSF11B genes. LD plots with $r^2$ values were generated by Haploview software. $r^2$ values are indicated by the degree of darkness, increasing from white to black. The $D'$ values multiplied by 100 are shown as numbers in the diamonds. There are one in TNFSF11 (block 1), two blocks in TNFRSF11A (block 2 and block 3), and one in TNFRSF11B (block 4), respectively.
found a strong association between polymorphisms of *TNFRSF11A* and AANM. The associations between polymorphisms of *TNFRSF11A* with AAM and AANM found in the present study can be explained by its possible roles in

### Table 3 Significant associations for the single SNPs of *TNFSF11*, *TNFRSF11A* and *TNFRSF11B* genes with AAM and AANM

| Genotype  | n AAM | Genotype  | n AAM |
|-----------|-------|-----------|-------|
| rs4500848 |       | rs1805304 |       |
| C/C       | 471   | 15.16 ± 1.95 | 64 | 14.53 ± 2.17 |
| C/T       | 313  | 14.76 ± 2.07 | 358 | 14.86 ± 2.04 |
| T/T       | 61   | 14.57 ± 1.85 | 423 | 15.12 ± 1.93 |
| P-value   | <0.01 | P-value    | 0.04 |

### Table 4 The associations of haplotypes of *TNFSF11*, *TNFRSF11A* and *TNFRSF11B* genes with AAM and AANM

| Gene          | Haplotype | Frequency | AAM | AANM |
|---------------|-----------|-----------|-----|------|
| *TNFSF11*     |           |           |     |      |
| TAGTAGT       | 0.131     | 0.00784   | 0.011 | 0.0761 |
| TAGACGT       | 0.292     | 0.00373   | 0.0195 | 0.038 |
| CAGCGT        | 0.469     | 0.00773   | 0.0195 | 0.038 |
| TAGCGT        | 0.017     | 0.07854   | 0.0113 | 0.0764 |
| *TNFRSF11A*  | rs9962159 | rs6567270 |     |      |
| A/G          | 0.161     | 0.1455    | 0.0243 | 0.38 |
| G/C          | 0.434     | 0.1294    | 0.0438 | 0.03 |
| A/C          | 0.405     | 0.0578    | 0.5574 | <0.01 |
| *TNFRSF11B*  |           |           |     |      |
| T/C          | 0.288     | 0.2782    | 0.01 | 0.0069 | 0.98 |
| A/T          | 0.408     | 0.2263    | 0.02 | 0.1197 | 0.55 |
| T/T          | 0.305     | 0.0113    | 0.0148 | 0.51 |
| *TNFRSF11B*  |            |           |     |      |
| TAGGG         | 0.250     | 0.0311    | 0.78 | 0.2791 | 0.24 |
| CATAC         | 0.155     | 0.0770    | 0.57 | -0.1808 | 0.52 |
| CACGC         | 0.139     | 0.0726    | 0.61 | -0.2352 | 0.43 |
| CAGGG         | 0.164     | 0.0407    | 0.76 | 0.1067 | 0.70 |
| TAGGG         | 0.154     | 0.0955    | 0.48 | 0.0410 | 0.88 |
| CAGGG         | 0.130     | -0.2041   | 0.17 | -0.1828 | 0.56 |

The analyses were performed under an additive model adjusted for age at interview and BMI. Beta, regression coefficient.

The present study, for the first time, demonstrated that *TNFRSF11A* but not *TNFSF11* and *TNFRSF11B* genetic

mammary gland development and menstruation. First, *TNFRSF11A* belongs to RANKL/RANK/OPG signaling pathway. RANKL plays an important role in mammary gland development, indicating its potential role in regulating or responding to sex hormone fluctuation and subsequently influencing menstrual cycles. Studies have shown that gonadotropin-releasing hormone (GnRH) can modulate RANKL expression in breast cancer cells [30], and expressions of RANK and RANKL in different cell lines are controlled by estrogen [31], follicle-stimulating hormone [32], and dehydroepiandrosterone [33]. Estrogen is also found to regulate gene expression and ratio of the RANKL/OPG [34]. Second, RANKL signaling pathway can stimulate ductal side-branching and alveologenesis in the mammary gland in mouse [35]. RANKL can be induced in mammary epithelium and can regulate the proliferation of cells [36]. Therefore, RANK signaling pathway may influence onset of puberty and menstrual cycle by regulating mammary gland development. Third, genome-wide association (GWA) studies have identified some novel genetic loci associated with AAM and AANM [37,38]. Gene set enrichment pathway analyses using the GWA dataset found that nuclear factor-kappa B (NF-κB) signaling pathway may be associated with timing of menopause [39]. Recent studies have revealed that NF-κB pathway plays an important role in mammary ducal morphogenesis [40], and ovarian cell function in animals [41]. It was well established that the RANKL/RANK/OPG pathway can activate NF-κB and its downstream players [42]. Thus, genes (e.g. *TNFRSF11A*) in the RANKL/RANK/OPG pathway may have role in the onset and cessation of the menstruation cycle.

The present study has some limitations. First, beside genetic other factors can influence timing of menarche and menopause, e.g. environment and socioeconomic status. We studied only the relationship between genetic variations and AAM and AANM. Furthermore, gene-environment interactions may also play a role in causing variation in the AAM and AANM. Second, The data of AAM and AANM were collected through retrospective self-report, which may cause recall bias. The participants in the study were aged from 42 to 89 years, with long interval periods, which might potentially incur recall error. It is reported that the accuracy of long-term recall of AAM and AANM varied from 70% to 84% [43,44]. In fact, it was found that some participants could not remember the exact age at the first menstrual period, and those subjects were excluded from the study. Large-scale studies are needed to confirm current findings, and the precise mechanisms underlying the observed associations in our study remain to be determined.

**Conclusions**

The present study, for the first time, demonstrated that *TNFRSF11A* but not *TNFSF11* and *TNFRSF11B* genetic
polymorphisms are associated with AAM and AANM in Chinese women. The findings provide evidence that genetic variations in RANKL/RANK/OPG pathway may be associated with the onset and cessation of the menstruation cycle.

Abbreviations
AAM: Age at menarche; AANM: Age at natural menopause; OPG: Osteoprotegerin; RANK: Receptor activator of nuclear factor-kappa B; RANKL: Receptor activator of nuclear factor-kappa B ligand; SNP: Single nucleotide polymorphisms; BMI: Body mass index; HWE: Hardy-Weinberg equilibrium; GnRH: Gonadotropin-releasing hormone; GWA: Genome-wide association; NF-κB: Nuclear factor-kappa B.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
PD participated in the molecular genetic studies, conducted statistical analyses and drafted the manuscript. ZMW assisted in genetic studies and drafted the manuscript. JL and LNW collected all samples and questionnaire information. ZY helped in recruiting subjects and statistical analyses. PT conceived of the study, participated in its design, recruited participants, revised the manuscript. All authors read and approved the final manuscript.

Acknowledgements
The authors would like to thank Prof. Gao Xin Yuan and Dr. Zhang Zeng for technical assistance. We thank all the participants in this study. This study was supported by grants from the National Natural Science Foundation of China (no. 81260133) and Key Projects of Health Department of Jiangxi province, China (no. 20114030).

Received: 5 December 2014 Accepted: 1 April 2015
Published online: 13 April 2015

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