Published in final edited form as:

Climacteric. 2012 February ; 15(1): 75–81. doi:10.3109/13697137.2011.587556.

Association analyses suggest the effects of *RANK* and *RANKL* on age at menarche in Chinese women

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Abstract

Objectives—Age at menarche (AAM), the time of the first menstrual bleeding, is an important developmental milestone in the female life. It marks the beginning of the reproductive period. AAM is implicated in the risk of many health complications in later life. In this study, we conducted an analysis for association of single nucleotide polymorphisms (SNPs) and common haplotypes of two candidate genes, *RANK* (receptor activator of the NF- κ B) and *RANKL* (receptor activator of the NF- κ B ligand), with AAM in 825 unrelated Chinese women.

Methods—In total, 73 SNPs of *RANKL* and 23 SNPs of *RANK* were genotyped. The SNPs and common haplotypes were then analyzed for their association with AAM. Age and age² were used as covariates.

Results—We found five individual SNPs (rs7239261, rs8094884, rs3826620, rs8089829, and rs9956850) of *RANK* significantly associated with AAM ($p < 0.05$). Although no significant association was identified for the *RANKL* gene, three polymorphisms showed nearly significant ($0.05 < p < 0.08$) association with AAM. Seven haplotypes of *RANK* were significantly associated with AAM ($p < 0.05$); the most significant association of the AT haplotype composed by rs1805034 and rs4524034 ($p = 9.4 \times 10^{-4}$) remained significant ($p = 0.0235$) after the Bonferroni correction for multiple testing. Three haplotypes of *RANKL* were significantly associated with AAM ($p < 0.05$). Importantly, the association of rs3826620 replicated our previous findings for Caucasian females.

Conclusions—The results of the present study suggest that the *RANK* and *RANKL* are two candidate genes for AAM in Chinese women.

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Conflict of interest The authors report no conflict of interest. The authors alone are responsible for the content and writing of this paper.

Supplementary material available online

Table S1 to be found online at <http://www.informahhealthcare.com/doi/10.3109/13697137.2011.587556>.

Keywords

AGE AT MENARCHE; SINGLE NUCLEOTIDE POLYMORPHISMS; ASSOCIATION; *RANK*; *RANKL*; HAPLOTYPES

INTRODUCTION

Menarche is the first menstruation and normally occurs between the ages of 11 and 16 years¹. Age at menarche (AAM) is a complex trait and often used in epidemiological studies as a surrogate indicator of puberty since it is difficult to accurately measure the stages of puberty using other phenotypic characters². Age of pubertal events is important individually, socially and culturally, and has a great impact on health later in life. In particular, later AAM may increase the risk of osteoporosis^{3,4} and pre-eclampsia⁵, whereas early AAM is associated with a higher risk for endometrial⁶, breast^{7,8} and ovarian cancers^{9,10}, psychological problems¹¹, and obesity^{12,13}. Therefore, the identification of the factors underlying AAM may potentially help in preventing these health complications.

Since AAM is a complex trait, it is determined by multiple environmental and genetic factors as well as by their interactions^{14,15}. Various studies estimate a contribution of genetic factors to the variation of this trait of approximately 45–74%^{16,17}. Previous investigations, including the recent genome-wide association studies^{18,19}, have discovered several genomic regions, genes and single nucleotide polymorphisms (SNPs) that are the candidates for AAM.

Sex hormones play a vital role in pubertal life and possibly AAM, which is the milestone of girls' sexual development^{20,21}. Previous studies showed the contribution of estrogen and estrogen metabolizing genes to AAM^{21–24}. The receptor activator of the NF- κ B (*RANK*), located at chromosome 18, and its cognate ligand, receptor activator of the NF- κ B ligand (*RANKL*), located at chromosome 13, together with diverse cytokines and hormones, have a great impact on various cellular processes, including bone remodeling and mammary gland development^{25,26}. *RANKL* and *RANK* are widely expressed in osteoclasts, dendritic cells, fibroblasts, B and T lymphocytes^{27–29}. They regulate T cell/dendritic cell communication, dendritic cell survival and activity, and lymph node formation³⁰. *RANK*/*RANKL* system activation, combined with various cytokines and chemokines, stimulates the inflammatory state of immune response³¹. Furthermore, the product of the *RANK* and *RANKL* gene expression was found in mammary gland cells and was implicated in their development during lactation³⁰. During menarche, the development of the mammary gland is followed by sexual maturation. All the above data suggest that *RANK* and *RANKL* may potentially contribute to AAM in various ways. Recently, we identified several *RANK* and *RANKL* polymorphisms and their interactions, which were associated with AAM in Caucasian females³². However, no such association studies have been conducted on the other ethnicities, including Chinese. In the present study, we attempted to replicate the above-mentioned findings about the association of *RANK* and *RANKL* with AAM in Caucasians in a large cohort of unrelated Chinese females.

METHODS

Study subjects

The study was approved by the local institutional review boards or the office of research administration of all participating institutions, Hunan Normal University and Xi'an Jiaotong University. After signing an informed consent, all subjects were assisted in completing a structured questionnaire that included questions about anthropometric variables, lifestyle

factors, and medical history. The information about AAM was collected using a questionnaire. To minimize the effect of the potential non-genetic confounding factors, a comprehensive set of exclusion criteria³³ was applied. Briefly, we exclude subjects with severe chronic diseases and conditions involving vital organs (heart, lung, liver, kidney, and brain) and severe endocrinological, systemic metabolic diseases (diabetes, hypo- and hyperparathyroidism, etc.), and nutritional diseases (chronic diarrhea, chronic ulcerative colitis, etc.) that might affect regular menstrual cycles. The assessment of the exclusion criteria was conducted through the nurse-administered questionnaire and/or medical records (when available). The final study sample consisted of 825 unrelated Chinese women of Han ethnicity.

AAM was defined as the age at the first menstrual bleeding less the birth date (in years rounded to the first decimal). The general characteristics of the study subjects are given in Table 1.

Genotyping

Genomic DNA was isolated from leukocytes of peripheral blood using a commercially supplied kit (Gentra Systems, Inc., Minneapolis, MN, USA) and according to the manufacturer's protocol. The SNPs were genotyped using the Affymetrix SNP Array 6.0 Set (Affymetrix, Santa Clara, CA, USA). In total, 23 SNPs located in or closely to *RANK* and 73 SNPs for *RANKL* were genotyped.

Statistical analyses

The χ^2 test was applied to check the Hardy–Weinberg equilibrium (HWE) of all SNPs. SNPs that did not follow HWE were excluded from the further analysis. The effects of the SNPs and haplotypes of the *RANK* and *RANKL* genes on AAM were estimated by linear regression models. In our Chinese sample, parameters such as age, age² were tested for their association with AAM using stepwise regression³⁴. Significant ($p < 0.05$) terms were included as covariates to adjust the raw AAM values for subsequent analyses. The residuals from a linear model after adjusting for the significant covariates were used as traits in the follow-up data analyses.

The association of the SNPs with AAM was analyzed by the univariate analysis of variance. In the univariate analysis, each marker was investigated independently. The obtained values were adjusted for multiple testing by applying the Bonferroni correction. The LD patterns of the *RANK* and *RANKL* genes were analyzed using the Haploview program³⁵ (www.broad.mit.edu/mpg/haploview/). Haplotype blocks were initially determined using the procedure implemented in Haploview and then analyzed for their association with the traits under study. These analyses were conducted using the PLINK software³⁶ available at <http://pngu.mgh.harvard.edu/~purcell/plink/>.

RESULTS

Study participants' characteristics

The total sample used in the study consisted of 825 Chinese women. The AAM (mean $\pm$ standard error) of the participants was 13.9 ± 0.1 years. The basic characteristics of the participating females, including age, weight, height and AAM are summarized in Table 1.

SNP association analyses

Age and age² were included as covariates to adjust the raw AAM values for the subsequent analyses. All studied polymorphisms indicated no deviation from the HWE (Table S1 to be found online at <http://www.informahhealthcare.com/doi/10.3109/13697137.2011.587556>).

Five *RANK* polymorphisms showed significant association with AAM: rs7239261 ($p = 0.006$), rs8094884 ($p = 0.009$), rs3826620 ($p = 0.018$), rs8089829 ($p = 0.034$), and rs9956850 ($p = 0.046$) (Table 2). The result for one of these SNPs, rs3826620, replicated our previous findings for its association with AAM in independent, healthy, Caucasian females of European descent ($p = 0.022$)³². The effects of the *RANK* polymorphisms on AAM are modest (Table 2). For example, homozygotes at the minor allele of rs7239261 have about 0.5 year later menarche (14.5 ± 0.31 years) than the average for the sample (13.9 ± 0.1 years). The contributions of rs7239261, rs8094884, rs3826620, rs8089829, and rs9956850 to the variation of AAM in Chinese were 0.5%, 0.7%, 0.2%, 0.3% and 0.1%, respectively. Interestingly, homozygotes at the minor allele of all five polymorphisms have later AAM than homozygotes at the major allele. However, no individual SNP association remained significant after the correction for multiple testing. Although the analyses did not reveal any association between the individual SNPs of the *RANKL* gene and AAM, three SNPs showed nearly significant association: rs7334307 ($p = 0.069$), rs17063218 ($p = 0.070$), rs7983721 ($p = 0.078$).

Seven haplotypes of the *RANK* gene were found to be significantly associated with AAM (Table 3). The association of the AT haplotype of polymorphisms rs1805034 and rs4524034 ($p = 9.43 \times 10^{-4}$) remained significant even after the Bonferroni correction ($p = 0.024$). Three haplotype blocks of *RANKL* were associated with AAM (Table 3). Similar to the SNPs, the effect of the haplotypes on AAM is modest. The average contribution of each haplotype of the *RANK* and *RANKL* genes to the variation of AAM in Chinese females is approximately 0.8% and 0.56%, respectively. The five haplotypes (H1, H2, H3, H5, and H6) of the *RANK* gene and one haplotype (h2) of the *RANKL* gene confer later AAM; the rest of them (H4 and H7 in *RANK* gene; h1 and h3 in *RANKL* gene) confer earlier AAM (Table 3).

DISCUSSION

This study is the first to report a possible association of the *RANK* and *RANKL* genes with AAM in Chinese women of Han ethnicity. The high-affinity interaction exists between the *RANK* and *RANKL* genes, which are functionally linked, so that they are frequently considered together as to their effect on various traits³². There are several polymorphisms and haplotypes of *RANK* or *RANKL* associated with AAM in Chinese and Caucasian female subjects. This study suggests that *RANK* and *RANKL* genes are possible contributors to AAM. However, only one SNP, rs3826620, appeared to be significantly associated with AAM in both ethnicities. It may be explained by the genetic variance between different ethnic groups. Previously, we hypothesized that different prevalence of some complex traits in different ethnicities may have ethnogenetic background³². Indeed, the minor allele frequencies (MAFs) of some SNPs in *RANK* and *RANKL* in the Chinese population under present study are significantly different from those reported for Caucasians³². For example, the MAF of rs3826620 is 0.406 in Chinese and 0.266 in Caucasians; the MAF of rs12959396 is 0.143 in Chinese and 0.482 in Caucasians. In addition, the effect of rs3826620 on AAM in the Chinese population, although it has the same direction, is weaker than in Caucasians. Specifically, homozygotes at the minor allele of rs3826620 have about 0.1 years later menarche (14.1 ± 0.2 years) than heterozygotes at the major allele (14.0 ± 0.1 years), and 0.3 years later menarche than homozygotes at the major allele (13.8 ± 0.1 years) in the Chinese population; while the homozygotes at the minor allele of rs3826620 have about 0.7 years later menarche (13.7 ± 0.3 years) than heterozygotes at the major allele (13.0 ± 0.1 years), and 0.8 years later menarche than homozygotes at the major allele (12.9 ± 0.1 years) in the Caucasian population³². Overall, the minor alleles of the same *RANKL* polymorphisms seem to confer later timing of menarche in the Chinese population, except rs9956850 heterozygotes at the major allele

(Table 2). As mentioned above, later or earlier AAM may be associated with several health complications. However, there are no data that would help to define how exactly late or early AAM will contribute to these complications. Therefore, although there is only less than 1 year's difference between the rs3826620 genotypes, it may potentially be important for female health in later life.

The H1 haplotype of the *RANK* gene, composed by polymorphisms rs1805034 and rs4524034, showed a strong association with AAM. However, no association was determined for each polymorphism separately. This can be explained by the individual weak effect. The rs1805034 polymorphism is a missense variant (Ala192Val) located in exon 6. However, the potential mechanisms for the association between the H1 haplotype and AAM are yet to be elucidated.

The tumor necrosis factor (TNF) receptor superfamily, member 11a (*TNFRSF11A*), also known as receptor activator of nuclear factor- κ B (*RANK*), and its cognate ligand (*TNFSF11* or *RANKL*) have been recognized as key players in many cellular and physiological processes, including bone metabolism^{14,37,38}, lymph node organogenesis³⁹, mammary gland development during pregnancy²⁶, immune response²⁸ and vascular calcification⁴⁰.

At the molecular level, RANK interacts with RANKL, together with signaling molecules of the TNF receptor-associated factor (TRAF) family, to activate transcription factor NF- κ B⁴¹. The transcription factor NF- κ B signaling pathway plays a role in various processes of cell development and death⁴². Several studies have illustrated that this pathway affects some age-dependent traits, and is also involved in cell senescence^{43,44}. The activation of NF- κ B correlates with age-dependent responses in different animal models⁴⁵. The role of the NF- κ B signaling pathway in cell senescence was supported by results from the integrated microarray study of nine tissue types⁴⁶. All these data suggest that the *RANK* and *RANKL* genes may contribute to the timing of menarche and puberty by stimulating the age-dependent mechanisms through the activation of the transcription factor NF- κ B signaling pathway. Along with our previous results on Caucasians³², this study may further support that the NF- κ B signaling pathway is a possible contributor to the timing of menarche in various ethnicities. However, the detailed mechanism of this contribution is still unknown.

The effect of *RANK* and *RANKL* genes on AAM is probably sex hormone-dependent. There is direct evidence that estrogen, prostaglandin and parathyroid hormone control the expression of *RANK* and *RANKL*^{47,48}. Maturation of ovaries during puberty increases estrogen production, which, in turn, may affect *RANK* and *RANKL* expression. It is also known that estrogen, while being a major contributor to pubertal development⁴⁹, is important for the timing of menarche and the menstrual period^{23,50}. Sex hormone therapy, e.g. estrogenic and/or antiandrogenic actions, interferes with the normal onset of puberty⁵¹. It was hypothesized that the widespread presence of endocrine-disrupting chemicals, which have a predominantly estrogenic effect, may contribute to the decrease of AAM⁵¹.

The premature activation of the hypothalamic–pituitary–gonadal (HPG) axis is one of the major causes of menstrual cycle disorders⁵² and may result in gonadotropin-dependent precocious puberty (GDPP)⁵³. Experiments on human breast cancer cell lines indicated that activation of tumor gonadotropin-releasing hormone (GnRH) receptors attenuated RANKL expression⁵⁴. Long-acting GnRH analogs are the treatment of choice in GDPP⁵⁵. With the onset of puberty, the pulsatility of output of the gonadal sex steroidal is increased markedly⁵⁶. These increasing changes in circulating gonadotropin secretion levels reflect increased pulsatile secretion of GnRH and stimulate follicle maturation and estrogen synthesis in the ovaries^{57,58}. The RANK/RANKL system seems to contribute via the HPG axis and interaction with hormones not only to AAM but to sexual maturity and puberty in

general. Specifically, various female sex hormones were shown to influence the *RANKL*/*RANK* involved in proliferation of the mammalian gland⁵⁹. However, the exact mechanism by which the *RANK* and *RANKL* genes interact with hormones to affect AAM is still not completely understood and needs further investigation.

Similarly to many other studies involving characteristics of reproductive history, the data of AAM were collected by questionnaire, which might potentially cause a recall error. According to the various studies, the accuracy of long-term recall of AAM ranges from 70% to 84%^{60,61}. In this study, it is expected to be smaller than in our previous study on Caucasians³² and closer to the upper bound, because the average age of the subjects was about 42 years vs. 61 years.

Although several single polymorphism associations identified in this research became non-significant after the correction for multiple testing, they should not be ignored. The Bonferroni correction is conservative and tends to accept the null hypothesis in most cases, thus masking a potential association, especially when the effect of the given SNP is weak, which is common for pleiotropic genes. For example, our estimates of the effects of some other genes on AAM yielded similar figures, ranged between 0.1 and 2.0%^{34,62}.

Together with our previous finding in Caucasians³², the results of the present study suggest that *RANK* and *RANKL* are associated with AAM in different ethnicities.

CONCLUSIONS

This is the first study on *RANK* and *RANKL*, two important genes for various complex traits, as possible candidate genes for the timing of menarche in Chinese women. This contribution incorporates direct SNP association and haplotype association. In the present study, we found several SNPs of *RANK* significantly associated with AAM. The association of one SNP, rs3826620, was a successful replication of the previous finding in the independent Caucasian sample³². We also found nearly significant association between the *RANKL* gene and AAM. In addition, several haplotypes of the *RANK* and *RANKL* genes were significantly associated with AAM. Our results suggested that the *RANK* and *RANKL* genes likely contribute to AAM in different ethnic populations, albeit with different strength. However, the currently available data on these genes are limited and do not allow for making any definite conclusions about an exact mechanism of this contribution. This warrants the necessity of further more extensive studies on other cohorts of women of various ethnicities.

Acknowledgments

Source of funding Investigators in this work were partially supported by grants from NIH (R01 AR050496-01, R21 AG027110, R01 AG026564, and P50 AR055081). The study also benefited from 211 State Key Research Fund by Hunan Normal University and the University of Hong Kong start-up fund (to V.D.).

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Table 1

Characteristics of the study participants. Data are given as mean $\pm$ standard error, unless otherwise indicated

Characteristics	Average	Minimum	Maximum
<i>n</i>	825	–	–
Age (years)	42.0 $\pm$ 0.4	22	77
Age at menarche (years)	13.9 $\pm$ 0.1	10	20
Height (cm)	158.4 $\pm$ 0.2	143	174
Weight (kg)	54.6 $\pm$ 0.3	35	91

Significant associations for the single nucleotide polymorphisms (SNPs) of the *RANK* gene with age at menarche (mean $\pm$ standard error) in Chinese women. 11, 12, 22 denote homozygote at the minor allele, heterozygote and homozygote at the major allele, respectively; numbers in brackets indicate percentage of the studied sample

Table 2

SNP identity	11	12	22	p	p-Bonf.
rs9956850	14.2 $\pm$ 0.5 (1.4)	13.8 $\pm$ 0.1 (22.7)	14.0 $\pm$ 0.1 (75.9)	0.046	1.000
rs7239261	14.5 $\pm$ 0.3 (5.1)	14.0 $\pm$ 0.1 (37.2)	13.8 $\pm$ 0.1 (57.7)	0.006	0.145
rs3826620	14.1 $\pm$ 0.2 (16.5)	14.0 $\pm$ 0.1 (48.7)	13.8 $\pm$ 0.1 (34.8)	0.018	0.436
rs8094884	15.0 $\pm$ 0.8 (0.5)	14.2 $\pm$ 0.1 (19.5)	13.9 $\pm$ 0.1 (80.0)	0.009	0.216
rs8089829	14.2 $\pm$ 0.3 (4.9)	14.0 $\pm$ 0.1 (41.9)	13.8 $\pm$ 0.1 (53.2)	0.034	0.815

p, unadjusted p value; p-Bonf., p value after Bonferroni correction for multiple testing

Common haplotypes of the *RANK* and *RANKL* genes showing significant association with age at menopause in Chinese women

Table 3

Gene	Haplotype identity	Haplotype block	Haplotype	Frequency	β	R ²	p	p-Bonf.
<i>RANK</i>	H1	rs1805034 rs4524034	AT	0.100	0.479	0.014	9.43×10^{-4}	0.024
	H2	rs7239261 rs8086340	AG	0.233	0.266	0.009	0.009	0.231
	H3	rs8094884 rs4524035	AA	0.098	0.372	0.008	0.011	0.291
	H4	rs7239261 rs8086340	CG	0.139	-0.27	0.006	0.029	0.301
	H5	rs12455775 rs3826620	AT	0.405	0.204	0.007	0.019	0.479
	H6	rs8089829 rs17069904 rs12959396	CCA	0.118	0.333	0.008	0.012	0.740
	H7	rs2981003 rs2981004 rs6567263 rs7233197 rs9956850 rs4941125	CATCTA	0.129	-0.256	0.005	0.046	1.145
<i>RANKL</i>	h1	rs12874142 rs7326472 rs11147871	GTT	0.158	-0.237	0.005	0.043	0.838
	h2	rs9590697 rs727243 rs12864265 rs7316953 rs1324005 rs9525625 rs720824	GGCCCTA	0.604	0.202	0.007	0.019	1.924
	h3	rs9525610 rs238281 rs9525613 rs430586 rs417768 rs912100 rs17063218 rs17522044 rs238270	TCAGTTTCC	0.151	-0.238	0.005	0.049	2.240

p, unadjusted p value; p-Bonf., p value after Bonferroni correction for multiple testing