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Three Common Variants of *LEP*, *NPY1R* and *GPR54* Show No Association with Age at Menarche

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Key Words

Age at menarche · Genetics · Candidate genes · Association study

Abstract

Context: Twin and family studies indicate a significant heritability of pubertal timing and more specifically of age at menarche (AAM). **Objective:** Test the association of AAM with common variants of three candidate genes suspected to have a prominent role in reproductive physiology: leptin (*LEP*), neuropeptide Y receptor 1 (*NPY1R*) and *GPR54*. **Design and Methods:** We selected the -2459 *LEP*, the rs7687423 *NPY1R* and the rs350132 *GPR54* variants as the more common coding or regulatory variants (minor allelic frequency >0.10) in these gene regions. To avoid stratification problems that can impair association studies, we used the Q-TDT method based on allele transmission to evaluate the relationship of these variants with AAM in 245 healthy women from 107 families of European ancestry. **Results:** We found no association of AAM with any of the studied variants. **Conclusions:** Keeping in mind that common variants do not recapitulate the whole genetic variation in a given gene region, this study indicates that the studied *LEP*, *NPY1R* and *GPR54* variants do not have a major influence upon pubertal timing in Caucasian women. Effects of these genetic loci on age at menarche can definitively be excluded only through determination of extended haplotypes in a larger cohort.

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Introduction

The timing of menarche is influenced both by environmental and by genetic factors, each group of factors accounting for approximately half the AAM variation in developed countries [1]. Genetic determinants of AAM remain unknown, but several association studies have started to unravel positional candidates across associated genomic regions [2–10].

Another approach is a direct test of association of AAM with suspected candidate gene polymorphisms. In this respect, the current study tested if the individual variability of AAM is associated with major variants of *LEP*, *NPY1R* or *GPR54*, genes that are known for their participation to the hypothalamic pathways that regulate the gonadotropic function. In each of these three gene regions, we selected a variant based on two criteria: the variant has to be common in Caucasians (mean allele frequency >0.10) and should possibly have a functional effect.

Studies in animal models and in humans support a role of leptin in the physiology of sexual maturation [11–13]. Mutations in the *LEP* [14] and *LEPR* [15] genes are associated with hypogonadotropic hypogonadism. The *LEP* gene is located in 7q31.3, spans 20 kb and contains 3 exons separated by two introns. The -2548 SNP selected for the current study is frequent, is located in the promoter region and was suggested to be regulatory [16, 17]. Our hypothesis was that through the control of circulat-

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ing leptin concentration, the *LEP* gene variant could modulate the variability of pubertal timing independent of fat mass.

Although there are no data implicating NPYR in humans, animal models suggest that NPY1R mediates the hypothalamic effects of NPY, an orexigenic neurotransmitter which influences gonadotropic function [18] and interferes with leptin effects in NPY neurons [19, 20]. The *NPY1R* gene, located in 4q31.3-q32, is subject to alternative splicing, which makes the functional location of the common variant that we selected difficult to infer (intron, exon 1, or promoter of the *NPY1R* gene). *NPY1R* and *NPY5R* genes are transcribed in opposite directions from a common promoter region suggesting that their transcriptional regulation is common [21]. If the studied variant of the *NPY1R* gene is a functional SNP which influences the transcriptional activity, it could also regulate the expression of NPY5R. NPY5R is known to be implicated in the NPY control of GnRH secretion in prepubertal rats [22].

The third common non synonymous SNP that we tested (rs350132, His-Leu 364) is located in exon 5 of *GPR54*, a gene that is a key regulator of gonadotropic function [23–25]. While hypogonadotropic hypogonadism has been associated with deletions encompassing exon 5 in *GPR54* [23], there are no data, however, indicating that this SNP has a functional role in GPR54 signal transduction.

Materials and Methods

Patients

The study was approved by the local Institutional Review Board and informed written consent was obtained for each participant. The study population, based on a sister-pair approach, included 245 healthy women of recent European ancestry from 106 families; 79 pedigrees of two, 23 pedigrees of three, two pedigrees of four and two pedigrees of five sisters volunteered for the study. Growth, body mass index and puberty data were collected from growth charts ('Carnet de Santé') and medical interviews. Age at menarche was recalled from women using both status quo and direct recall methods validated in previous studies [26, 27].

Genotyping

Genomic DNA was extracted from whole blood with the Genra Extraction Kit. Amplification (35 cycles) was carried out in 50 μ l containing 200 ng of genomic DNA, 1 μ M of each primer, 1 \times PCR buffer (Invitrogen), 0.2 mM of each dNTP, 1.5 mM MgCl₂ and 1.25 units of Taq polymerase (Invitrogen). The following primers and annealing temperatures were used: *LEP* forward: TTTCCTGTAATTTCCCGTGA, *LEP* reverse: AAAGCAAA-GACAGGCATAAAAA, annealing temperature 50°C; *NPY1R* forward: AAGGCTCTACAGTCTGTACC, *NPY1R* reverse: GTG-

GAGCACACCTGTAGTCC, annealing temperature 58°C and *GPR54* forward: GCCTTTCGTCTAACCACCTTC, *GPR54* reverse: GGAGCCGCTCGGATTCAC, annealing temperature 62°C. PCR products, 242 bp for *LEP*, 432 bp for *NPY1R* and 524 bp for *GPR54*, were digested respectively with *CfoI* restriction enzyme (Roche) for *LEP*, *PstI* restriction enzyme (Biolabs) for *NPY1R* and *HpyCH4V* restriction enzyme (Biolabs) for *GPR54*, then analyzed by gel electrophoresis. For the -2459 *LEP* variant, two fragments of 181 and 61 bp identified homozygote CC subjects; three fragments of 242, 181 and 61 bp identified CA heterozygotes; and a single 242-bp fragment identified AA homozygotes. For the rs7687423 *NPY1R* variant, two fragments of 256 and 176 bp identified homozygote TT subjects; three fragments of 432, 256 and 176 bp identified CT heterozygotes, and a single 432 bp fragment identified CC homozygotes. For the rs350132 *GPR54* variant, four fragments of 160, 153, 137 and 74 bp identified AA homozygotes; five fragments of 297, 160, 153, 137 and 74 bp identified AT heterozygotes, and three fragments of 297, 153 and 74 bp identified TT homozygotes.

Statistical Analysis

AAM showed a continuous distribution in the cohort. The studied variants followed Hardy-Weinberg equilibrium. To test for both linkage and association with the variation of AAM we used the Q-TDT method implemented in Merlin [28]. The orthogonal model of Abecasis et al. was used in our analyses, where the total association is partitioned into orthogonal within and between family components (β_b and β_w , respectively). The between family component is specific for each nuclear family and could be confounded by population stratification. However, the within family component is significant only in the presence of linkage disequilibrium caused by close linkage. Thus, a Q-TDT test of the significance of β_w based on allelic transmission is robust to population stratification. The null model was 'means = $\mu + \beta_b$ ' and the full model was 'means = $\mu + \beta_b + \beta_w$ '. The analysis was adjusted for BMI and BMI Z score at age at menarche for reasons explained in a previous analysis [10]. Results are expressed as means \pm SD (standard deviation).

Results

Table 1 describes the genotypic and phenotypic characteristics of the 245 women. Mean age of participants was 34.4 \pm 13 years. Age at menarche averaged 13.1 \pm 1.5 years as reported in comparable Caucasian women modern populations.

None of the studied polymorphisms of the *LEP*, *NPY1R* and *GPR54* genes were found to be associated with AAM when each SNP was considered individually. When Q-TDT analyses were corrected for BMI and BMI Z score at age at menarche, results were still nonsignificant. All p values were above 0.2. The p values for the studied variants of *LEP*, *NPY1R* and *GPR54* were, respectively, 0.9, 0.8 and 0.6 when nonadjusted; 0.2, 0.6 and 0.7 when adjusted to BMI at age at menarche, and 0.7 for the three

Table 1. Age at menarche for the whole sample and for each genotypic group of the studied variants of *LEP*, *NPY1R* and *GPR54*

	Whole sample	LEP			NPY1R			GPR54		
		CC	CA	AA	TT	CT	CC	AA	AT	TT
Genotype										
Number	245	67	145	33	95	117	32	150	81	8
Frequency		0.27	0.59	0.14	0.39	0.48	0.13	0.63	0.34	0.03
Age at menarche (years, mean ± SD)	13.1 ± 1.5	13.4 ± 1.5	13.2 ± 1.6	13.2 ± 1.4	13.1 ± 1.4	13.2 ± 1.6	13.5 ± 1.5	13.2 ± 1.5	13.3 ± 1.5	13.4 ± 1.6
Range	9–18	9.5–17	9–18	10–16	9–16	9.5–18	10–18	9.5–18	9–17	12–17

variants when adjusted to BMI Z score at age at menarche.

Due to the limited size of the sample, our study was underpowered to evaluate potential effects of gene-gene interactions using the combinations of genotypes at the three studied loci. In an attempt to combine information at two loci, we could only compare 11 women with *LEP* CC and *NPY* CC genotypes in whom median AAM was 13.2 compared with 13.1 years in the rest of the sample (n = 193).

Discussion

Since AAM averaged 13.1 years with a continuous distribution of values in the studied population sample, we considered that it was representative of modern Caucasian women of Western Europe [29].

Association studies are a priori a powerful means for identifying the common variants that underly complex traits [30–32]. Our study, however, has several major weaknesses. (1) One is the size of the studied sample which is insufficient for quantitative traits like AAM that are likely to involve polygenic determinants of limited effect size [33]. (2) Another one is the limited number and arbitrary choice of genes that we tested (not based on positional information from genomewide studies) versus the multiple ones that could be involved in the control of puberty timing. (3) The study is based on the belief that the selected variants could be functional, which was proven for none of them, nor were they in linkage disequilibrium with known functional variants. (4) Our strategy of analysis is entirely based on the search for common variants and nothing indicates yet that common variants are the basis for the genotypic variation of evolutionary traits, like AAM.

Several studies that searched for associations of candidate genes with AAM in Caucasian women of North America or Europe, and in Japanese, ‘Hispanic Americans’ and Asian women have remained negative overall [2–9].

In an extensive haplotype-based association study of *GnRH1* and *GnRHR* in 125 white men and women from North America, three intronic SNPs of *GnRHR* (rs3822196, hCV1960913 and rs3796718) were weakly associated with late pubertal development (p < 0.05). In 506 American women of European, Asian or African descent, another *GnRHR* SNP (hCV3145733) was marginally associated with late AAM and a rare *GnRHR* haplotype with early AAM. None of the studied *GnRH1* variants showed any relationship with the phenotypes [2].

SNPs in the estrogen α receptor (A/C 351, T/C 397) or β receptor (A/G 1730) were reported to be possibly associated with AAM in 145 adolescents of a closed rural community in Greece [3], in which none of the studied genotypes, however, reached Hardy-Weinberg equilibrium.

Among 38 SNPs covering the full transcript length of *CYP17* and *CYP19* genes, Guo et al. [4] found that five SNPs of the *CYP19* gene were significantly associated with AAM in 1,048 white American females, while none of the coding *CYP17* variants showed any association. However, a functional variant in the promoter of the *CYP17* gene was associated with AAM in 317 Japanese women, and in 583 Caucasian, African and Asian women living in North America [5, 7]. The polymorphic variants of the *CYP3A4*, *CYP1B1* (exon 3 Leu to Val) and *CYP1A2* (intron 1) genes showed no association either with AAM in 583 Caucasian, African or Asian women living in North America [5], while an association was found between the *CYP3A4*1B* functional promoter variant and early puberty in 137 African-American, Hispanic and Caucasian girls [6].

The functional (TAAAA)_n polymorphism located in the promoter of the sex hormone-binding globulin (SHBG) gene was found to have a genetic contribution to AAM. Late menarche was more frequently associated with long alleles (>8 repeats) of this variant in 130 adolescents of a closed Greek community [8]. The Glu298Asp missense variant and T-786C gene polymorphisms located in the promoter region of the endothelial nitric oxide synthase (NOS1) gene showed no association with AAM in 87 Caucasian women [9]. A recent study showed no association of specific *LEP* (3'CTTT repeat) and *LEPR* (Gln>Arg substitution, exon 6) polymorphisms with constitutional delay of growth and puberty [34].

The current study examined the possibility that yet untested common variants of *LEP*, *NPY1R* and *GPR54* genes could be major genetic predicting markers or determinants of pubertal timing in humans. The study was designed to detect only a relatively large effect size of the

studied polymorphisms considered separately. The negativity of our findings does not exclude that genetic variation in the considered genes could influence AAM, because other SNP combinations at the *LEP*, *NPY1R* or *GPR54* gene loci that we have not tested could be involved. In such case, however, the genomic variability would have to involve variants that are not in LD with the studied SNPs. Our hypothesis was tested with the robust Q-TDT approach which excludes potential stratification effects in the studied women cohort [29].

Since the level of influence of a given allele on the individual variability of a continuous trait is a direct function of both frequency and magnitude of the effect of this variant, the current association analysis suggests that none of the studied variant is involved at a major level (>10% of the trait variance) in the genetic determination of pubertal maturation in humans.

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