Association Studies of Common Variants in 10 Hypogonadotropic Hypogonadism Genes with Age at Menarche

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Context: Although the timing of puberty is a highly heritable trait, little is known about the genes that regulate pubertal timing in the general population. Several genes have been identified that, when mutated, cause disorders of delayed or absent puberty such as hypogonadotropic hypogonadism (HH).

Objective: Because severe variants in HH-related genes cause a severe puberty phenotype, we hypothesized that common subtle variation in these genes could contribute to the population variation in pubertal timing.

Design: We assessed common genetic variation in 10 HH-related genes in 1801 women from the Hawaii and Los Angeles Multiethnic Cohort with either early (age $<$ 11 yr) or late (age $>$ 14 yr) menarche and in other replication samples. In addition to these common variants, we also studied the most frequently reported HH mutations to assess their role in the population variation in pubertal timing.

Setting and Patients/Other Participants: Within the general community, 1801 women from the Hawaii and Los Angeles Multiethnic Cohort participated.

Main Outcome Measures: We assessed the association of genetic variation with age at menarche.

Results: We found no significant association between any of the variants tested and age at menarche, although we cannot rule out modest effects of these variants or of other variants at long distances from the coding region. In several self-reported racial/ethnic groups represented in our study, we observed an association between estimated genetic ancestry and age at menarche.

Conclusions: Our results suggest that common variants near 10 HH-related loci do not play a substantial role in the regulation of age at menarche in the general population. **(*J Clin Endocrinol Metab* 93: 4290–4298, 2008)**

Puberty is an important developmental milestone that marks the onset of reproductive capacity. The timing of puberty varies among individuals and among populations, with genetic factors contributing approximately 50–75% of the variance within a population (reviewed in Refs. 1–4). Twin studies indicate a high degree of heritability for age at menarche and other

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Abbreviations: AOM, Age of menarche; BMI, body mass index; HH, hypogonadotropic hypogonadism; MEC, Hawaii and Los Angeles Multiethnic Cohort; SNP, single-nucleotide polymorphism.

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aspects of pubertal development (5, 6); a recent large twin study estimated the heritability of age at menarche to be 0.7 (7). Despite the evidence for genetic control of pubertal timing, the genes involved remain largely unknown. Age at menarche is the most concrete and well-defined marker of puberty in girls and is correlated with thelarche (breast development) (8), suggesting that it can be used as a reasonable proxy for the onset of puberty in girls. Estimating the timing of puberty is more challenging in males, but boys seen in endocrine clinics for constitutional delay of puberty tend to have mothers with late age at menarche (9), suggesting that some genes for pubertal timing can affect both genders.

At one extreme of pubertal development are patients with hypogonadotropic hypogonadism (HH), who have either absent or severely delayed puberty, occasionally accompanied by additional phenotypes such as anosmia or obesity. Rare, severe mutations that lead to HH have been identified in several genes (reviewed in Refs. 10–12), the most common being *KAL1*, *FGFR1*, *GNRHR*, *GPR54*, *LEP*, *LEPR*, *PROK2*, and *PROKR2*. The identification of these genes represents substantial progress, but HH cases with extreme phenotypes account for only a small fraction of the total population variation in pubertal timing. Most people seen in endocrine clinics with the milder phenotype of late pubertal development have not been shown to carry severe mutations in the genes known to be responsible for HH (13–15).

However, the genes underlying severe syndromes may provide clues to the genes influencing phenotypic variation in the general population. Specifically, if severe mutations in a gene can cause severe phenotypes, mild mutations in the same gene may cause milder, yet related, phenotypes. Examples exist for diabetes (*KCNJ11*) and height (*HMGA2* and *GDF5*), among other phenotypes (16–23).

In light of these examples, we hypothesized that common genetic variants in some of the same genes responsible for HH may influence the timing of menarche in the general population. In a smaller, separate sample of women, we had previously examined common genetic variation in *GNRH* and *GNRHR* but did not identify any variants that had a large effect on pubertal timing (14). Here, we extended these studies to include additional HH genes and used data from the human haplotype map (www.hapmap.org) (24) to improve our ability to capture common genetic variation in these genes within a more robust study population. Specifically, we studied a panel of 1801 women from the Hawaii and Los Angeles Multiethnic Cohort (MEC) (25) with early (before 11 yr of age) or late (at 15 yr of age or later) menarche. We performed tests of association to investigate whether common genetic variation in 10 genes that underlie HH or are ligands for receptors encoded by these genes (*FGFR1*, *GNRH*, *GNRHR*, *GPR54*, *KAL1*, *KISS1*, *LEP*, *LEPR*, *PROK2*, and *PROKR*) influence age at menarche. We compared our results to results from a genome-wide association study performed in a panel of 2287 women from the Nurses' Health Study (He, C., and D. J. Hunter, unpublished data) as our primary replication and also attempted to replicate any nominally significant associations in a panel of parent-offspring trios (80% boys) from the United States and United Kingdom (13, 14). Additionally, to minimize the possibility of false-positive associations due to population stratification, we used ancestry informative markers to obtain estimates of genetic ancestry within our panel.

Subjects and Methods

Study panel

DNA from 1815 women from the MEC (25) was genotyped. The women in the panel represent five different self-reported racial/ethnic groups: non-Latina White, African-American, Japanese-American, Native Hawaiian, and Latina. Age at menarche was self-reported by mailed questionnaire. Categories of age at menarche on the questionnaire were less than 11 yr of age, 11–12 yr of age, 13–14 yr of age, 15–16 yr of age, and 17 yr or older. Women with early age at menarche (less than 11 yr) and late age at menarche (the categories 15–16 yr and 17 yr and older) were selected for analysis. We verified that all women in the panel with early menarche had regular periods before age 13 yr and that all women with late menarche eventually had regular periods. A current MEC follow-up questionnaire contained the same age at menarche question. Less than 1% of women reported an age at menarche in the second questionnaire that was at the extreme of their original category (*e.g.* first reported menarche at <11 yr and subsequently reported an age at menarche of 15 or older, and vice versa). Age at menarche in the MEC is consistent with published data on age at menarche in different self-described racial/ethnic groups. Age at menarche in the MEC also shows the expected correlations with body mass index (BMI) at age 21 and risk of breast cancer (26). Data on oral contraceptive use was obtained for the women selected for this age at menarche panel, and 14 women who may have started oral contraceptives before their first period were excluded from analysis, leaving a panel of 1801 women for analysis (Table 1).

This study was approved by the Institutional Review Boards at Children's Hospital in Boston, University Hospitals of Cleveland, the Salford and Trafford Local Research Ethics Committee (Manchester, UK), the University of Hawaii, the University of Southern California School of Medicine, and the Hospital for Sick Children (Toronto, Canada). Informed consent was obtained from all study participants.

Replication panels

As our main replication panel, we compared our findings to results from a genome-wide association study performed in a panel of 2287 women from the Nurses' Health Study. The scan included DNA from 2287 registered nurses of European ancestry: 1145 postmenopausal women with invasive breast cancer and 1142 matched controls (27). Information on age at menarche (in years) was collected in a baseline questionnaire in 1976. Women whose age at menarche was 18 yr or greater were excluded for suspected primary amenorrhea. We used an additional panel of 125 parent-offspring trios from the United States and United Kingdom where the offspring (80% boys) had delayed puberty [previously described (9, 13, 14)] to attempt replication of preliminary findings. The U.S. study group consisted of 63 boys and 18 girls and the United Kingdom study group consisted of 37 boys and seven girls. All subjects had documented spontaneous pubertal development. Ninety-four percent of subjects were non-Hispanic Whites.

Single-nucleotide polymorphism (SNP) selection

We selected tag SNPs based on data from the human haplotype map (24) and genotyped them in our age at menarche panel (see supplemental Methods for more detail, published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). Percent coverage [as the percentage of common (minor allele frequency >5%) variants with a high correlation ($r^2 > 0.8$) to the tag SNPs genotyped) for these 10 genes is presented in supplemental Table 2.

Screening for additional mutations

Because they represent relatively common causes of HH, some genes [*GNRHR* (and its ligand *GNRH*) and *FGFR1*] were screened by a com-

TABLE 1. Composition of the AOM panel

	No. of subjects		Average BMI (at age 21)[b]		Average age at questionnaire	
	Menarche < 11 yr	Menarche ≥ 15 yr[a]	Early	Late	Early	Late
African-American	208	183 (165, 18)	22.4 ± 4.0 (193)	20.5 ± 3.0 (169)	56.1 ± 7.8	59.6 ± 8.2
Japanese-American	118	146 (121, 25)	21.3 ± 2.9 (113)	20.2 ± 2.6 (142)	57.0 ± 7.8	62.6 ± 7.6
Native Hawaiian	156	142 (130, 12)	23.7 ± 4.9 (151)	21.2 ± 3.4 (140)	53.2 ± 7.6	56.1 ± 8.4
Latina	182	181 (160, 21)	22.4 ± 3.4 (171)	21.0 ± 3.0 (174)	56.3 ± 7.4	61.3 ± 8.0
Non-Latina White	245	240 (230, 10)	22.1 ± 4.0 (240)	20.3 ± 2.6 (247)	55.5 ± 7.3	56.1 ± 8.2
Total	909	892 (806, 86)				

Numbers of women with early and late menarche are shown by racial/ethnic group along with average BMI at age 21 (height reported at time of questionnaire; weight at age 21 by recall) and average age at questionnaire. The differences in recalled BMI at age 21 between the early and late menarche groups are significant with $P < 0.01$ for all racial/ethnic groups. The differences in average age at questionnaire are significant for all racial/ethnic groups (except for the whites) at $P < 0.001$.

[a] Numbers in parentheses represent the women in two subgroups of the late menarche group. The first number is women with menarche at 15–16 yr of age, and the second number is women with menarche at age 17 or later.

[b] Numbers in parentheses represent the number of women in the group with available data for BMI calculation.

bination of denaturing HPLC and resequencing in 40 children with constitutional delay of puberty to detect additional mutations to analyze in our menarche panel, as described previously (13, 14). For *FGFR1*, eight additional individuals with family history of hyposmia/anosmia and late pubertal development were also screened. Data from resequencing in *GNRH* and *GNRHR* have been previously described (14). Although no obviously deleterious mutations were identified, several polymorphisms were observed in *FGFR1*, and four polymorphisms were successfully genotyped in the menarche panel (supplemental Table 1). To ensure that our analysis also included the most common HH mutations, we searched the literature for HH mutations reported more than twice in unrelated HH patients. From this literature search, we added five mutations, three in *GNRHR* and two in *KAL1*, to our genotyping efforts (supplemental Table 1).

Genotyping

Genotyping was performed as described elsewhere (28), using the Sequenom MassARRAY platform (29) with both hME and iPLEX protocols (see supplemental Methods for more details).

Estimation of ancestry

We genotyped a small panel of markers that are highly informative for the geographic source populations that are thought to contribute genetic ancestry to the five populations in our panel (supplemental Table 3). These potential source populations were represented by the three populations genotyped in the human HapMap (CEU, JPT/HCB, and YRI) as well as Native American and Native Hawaiian populations. Markers were selected on the basis of known differences in allele frequencies between populations (see supplemental Methods for more details).

We estimated the contribution of the source populations to genetic ancestry of the women in our menarche panel using the program Structure (30, 31). For each self-reported racial/ethnic group, we identified the major ancestry, the ancestry with the highest estimated contribution to that group. We then tested for association between estimated major ancestry and age at menarche using a Wilcoxon rank-sum test.

We defined genetic outliers (individuals whose self-reported racial/ethnic group did not match their estimated genetic ancestry) as those whose estimated ancestry met one commonly used mathematical definition of an outlier. Specifically, we excluded individuals with a probability of major ancestry less than the 25th percentile minus 1.5 times the interquartile range. For Japanese-Americans, we considered all individuals with a probability of East Asian ancestry less than 0.9 to be outliers, to be consistent with ancestry estimates in the HapMap JPT population. We identified a total of 70 genetic outliers: nine African-Americans (four early, five late), four Japanese-Americans (one early, three late), two Latinas (zero early, two late), and 55 Whites (28 early, 27 late).

Association analysis

The software package PLINK (32) was used to test for association between genetic variants and age at menarche. Because the women in our panel represent five different self-reported racial/ethnic groups, we analyzed the whole panel by performing a stratified analysis by ethnicity using the Cochran-Mantel-Haenszel test. Both single SNPs and multimarker haplotypes that serve as proxies for untyped alleles (33) were tested for association. We also used PLINK to test for pairwise epistatic interactions between SNPs and/or multimarker haplotypes within and across genes. To further correct for the effects of ancestry, we performed a logistic regression analysis in PLINK using estimated genetic ancestry as a covariate. Regression analyses were performed for each ethnic group separately and then combined in a metaanalysis. For each racial/ethnic group, major ancestry was used as a covariate (see supplemental Methods for more details). The *P* values reported here are nominal *P* values and have not been corrected for multiple testing unless specifically indicated. We also performed an epistasis analysis in each racial/ethnic group separately and combined the results using standard metaanalytic techniques. We corrected epistasis *P* values using both the Bonferroni and the Benjamini-Hochberg (34) methods.

Replication samples

SNPs with nominally significant or suggestive *P* values ($P < 0.10$) were genotyped in the delayed-puberty trios. A transmission disequilibrium test was performed in PLINK to test for an association with late puberty. Additionally, results for SNPs with $P < 0.10$ in our panel were compared with results from the same SNPs in a panel of women from the Nurses' Health Study (He, C., and D. J. Hunter, unpublished data). Genotyping of the initial Nurses' Health Study genome-wide association study has been described elsewhere (27). In addition to the 528,173 SNPs that were genotyped using the Illumina HumanHap550 array, SNPs polymorphic in the HapMap CEU samples were imputed using a hidden Markov model as implemented in MACH (Li, Y., and G. R. Abecasis, unpublished data). Linear regression for age at menarche on the number of SNP minor alleles was performed in the software package PLINK to analyze the association between each SNP and age at menarche.

Results

Age at menarche panel characteristics

We selected women from the MEC who had early or late recalled age at menarche (Table 1). Women from the extremes (the top and bottom 10% of age at menarche) were chosen to reduce the degree of misclassification of age at menarche in our panel. We studied a

panel of women from among the five racial/ethnic groups represented in the MEC: non-Latina Whites, African-Americans, Native Hawaiians, Japanese-Americans, and Latinas. When examining BMI data (recalled at age 21 yr) in our panel, we observed the expected weak correlation between BMI and age at menarche; the women with early menarche have higher average BMI than those with late menarche ($P < 0.01$ for each racial/ethnic group).

Estimation of ancestry

Using samples from admixed populations is advantageous because it provides us with the rare opportunity to look at the relationship between age at menarche and genetic ancestry within these populations; however, it also introduces possible confounding due to population substructure. To estimate recent genetic ancestry corresponding to major ancestral populations (35), we genotyped additional ancestry informative markers in our panel and estimated for each individual the fractional contribution of the major ancestry for their self-reported racial/ethnic group (see *Subjects and Methods*).

Association of menarche with ancestry

To test for an association between genetic ancestry and menarche status, we compared the distribution of estimated ancestry for the early and late menarche groups, after removing 70 genetic outliers (see *Subjects and Methods*) (Fig. 1). We observed correlation between ancestry and age at menarche in some but not all of the self-reported racial/ethnic groups. For the Whites and African-Americans, average estimated ancestry did not significantly differ between the early and late menarche groups. For the Native Hawaiians, there was a suggestive association between Native Hawaiian ancestry and menarche status, with more estimated Native Hawaiian ancestry in the early menarche group than in the late menarche group ($P = 0.06$). For the Japanese-Americans, we found an association between increased East Asian ancestry (and decreased European ancestry) and late menarche ($P = 0.01$) that was driven by individuals with estimated East Asian ancestry between 0.9 and 0.95 (data not shown). When individuals with East Asian ancestry estimates less than 0.95 were removed from the analysis, the association became weaker but did not disappear entirely ($P = 0.03$). Most interestingly, for the Latinas, we observed a significant association between European ancestry and menarche status: the women in the early group had lower estimated European ancestry (and higher estimated Native American ancestry) than those in the late group ($P = 0.004$). Thus, ancestry is correlated with age at menarche in certain populations, suggesting that genetic factors that vary in frequency between ethnicities may influence pubertal timing. Furthermore, these results indicate that, in some populations, ancestry needs to be taken into account in association studies of age at menarche.

Association of HH gene variants and age at menarche

We then proceeded to test common variants in HH genes for association with age at menarche. We first selected tag SNPs designed to capture common genetic variation in the 10 HH genes of interest and genotyped them in our menarche panel (described in more detail in Supplemental *Methods*). The percentage of common (minor allele frequency greater than 5%) variation captured at strong levels of correlation ($r^2 > 0.8$) by the genotyped SNPs was more than 90% for nearly all genes tested in each of the three HapMap populations (CEU, combined East Asian JPT+HCB, and YRI) (supplemental Table 2). The mean maximal r^2 (see supplemental Table 2 footnotes) for all alleles, including those not captured at r^2 greater than 0.8, was approximately 0.9 or greater for all genes except *GPR54* in each HapMap population (supplemental Table 2), indicating that these SNPs capture the vast majority of common variation in these genes. *GPR54* has very few SNPs genotyped in HapMap, so we genotyped additional SNPs in that gene, meaning that our coverage is likely better than the coverage in HapMap.

We then tested the selected tag SNPs for association with age at menarche. Because we observed association between ancestry and age at menarche, and because population stratification can lead to false positive associations (35), we analyzed our data by using estimated genetic ancestry as a covariate. To obtain overall estimates of significance for the entire panel, we combined results from each self-reported racial/ethnic group using standard meta-analytic techniques (see *Subjects and Methods*).

We did not observe any strongly significant associations. Several SNPs showed nominally significant associations ($P < 0.05$) in the combined sample with age at menarche, but these do not survive correction for multiple hypothesis testing (Table 2). Nominally significant associations ($P < 0.05$) were also observed in single self-reported racial/ethnic groups (supplemental Table 4), but again, these associations do not survive correction for multiple hypothesis testing. A few SNPs showed nominal significance in multiple ethnic groups, but most SNPs were nominally significant in only one self-reported racial/ethnic group. To test whether the nominally associated SNPs from the combined sample could represent real associations with modest effects, we examined these SNPs in additional samples.

Replication samples

SNPs with nominally significant and suggestive associations ($P < 0.10$) in the age of menarche (AOM) panel were examined where possible for *in silico* replication using data from a genome-wide association study in the Nurses' Health Study (Table 2). Even though delayed puberty may represent a slightly different phenotype, we also genotyped these SNPs in an additional panel of 125 children (80% boys) with delayed puberty and their parents. We successfully genotyped 18 of 23 SNPs in the trios and were able to compare 15 of 23 SNPs *in silico* with the Nurses' Health Study results. Most SNPs did not show nominal evidence of replication in either panel. One SNP (FGFR1_rs3925) had a *P* value of 0.04 in the trios with an effect in the same direction as that observed in from the AOM panel. In the Nurses' Health Study data, however, the *P* value was not significant, and the effect was in the opposite direction. It is possible that this signal could represent a real, although modest, effect, but additional samples would be needed to conclusively support or rule out an effect of rs3925. One SNP (LEPR_rs1475397) had a *P* value of 0.07 in the Nurses' Health Study, but it had the opposite direction of effect on age at menarche as in the AOM panel, so this does not represent evidence of association. Therefore, we were unable to conclusively replicate any nominal associations iden-

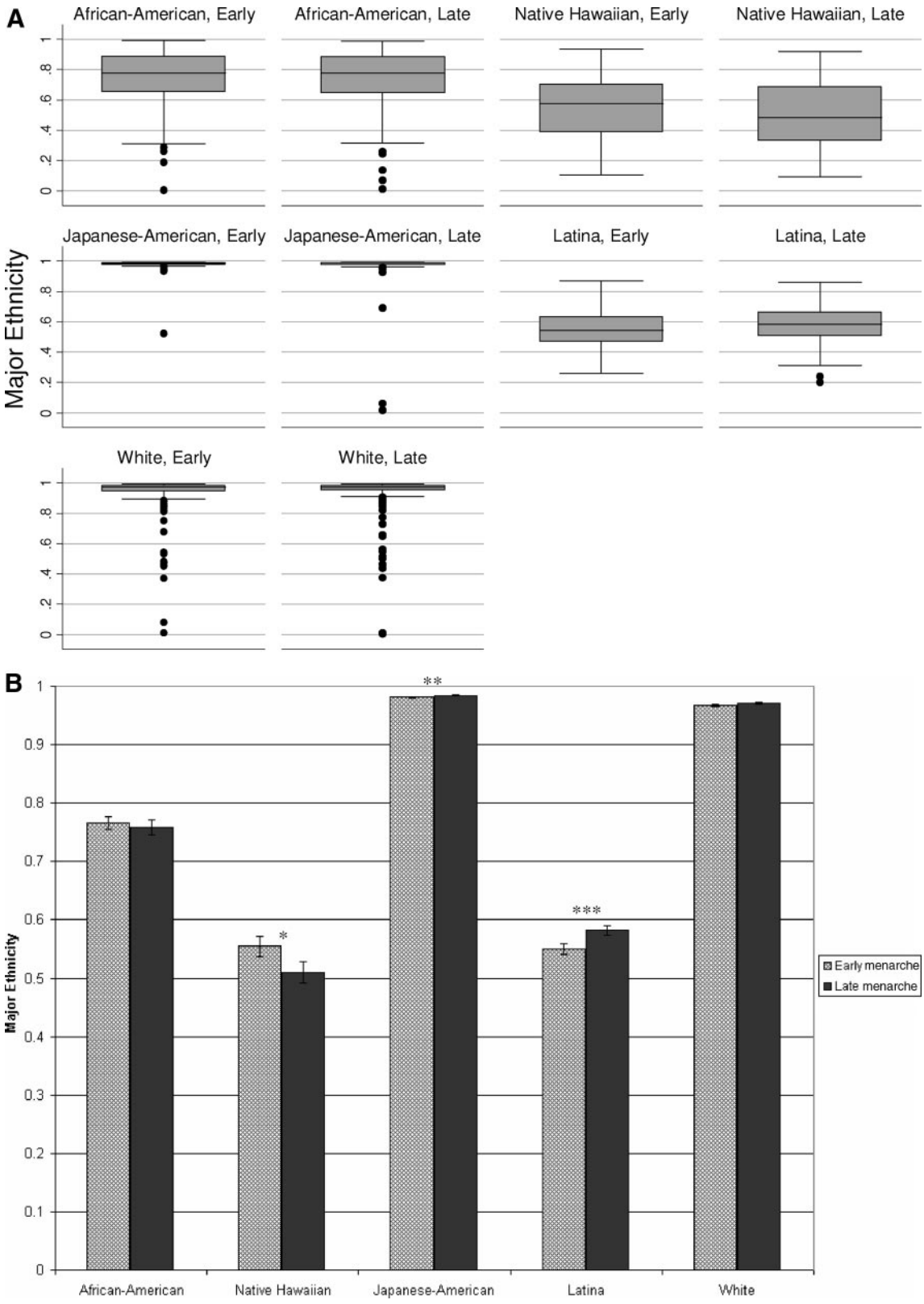

FIG. 1. Estimated ancestry and age at menarche by racial/ethnic group. A, Box plots for estimated genetic ancestry by self-reported racial/ethnic group. The y-axis represents the estimated proportion of major ancestry, or the estimated contribution of the ancestry that has the largest contribution to a given self-reported ethnic group. The *line within the box* is the median of the estimated ancestry for women with early or late menarche; the *upper boundary of the box* is the 75th percentile; the *lower boundary of the box* is the 25th percentile. The *lines extending from the box* represent the adjacent values (the largest and smallest non-outlying values observed in the dataset). *Dots* represent genetic outliers. For African-Americans, major ethnicity is estimated West African ancestry; for Native Hawaiians, major ethnicity is estimated Native Hawaiian ancestry; for Japanese-Americans, major ethnicity is estimated East Asian ancestry; for Latinas, major ethnicity is estimated European ancestry; and for Whites, major ethnicity is estimated European ancestry. B, Mean ancestry estimates for each self-reported racial/ethnic group separated by early (*light grey bars*) and late menarche (*dark grey bars*). Genetic outliers were removed before calculating the means. Major ethnicities are the same as in A. ***, $P < 0.01$; **, $P < 0.05$; *, $P < 0.1$.

TABLE 2. Association results, AOM, and replication panels

		AOM			Delayed-puberty trios			Nurses' Health Study				
SNP	Effect allele	n	*P* value	Direction	No. Transmissions	*P* value	Direction	n	β	*P* value	Direction	r^2 for imputation[a]
LEPR_rs9436740	T	1561	0.009	L	47	0.522	L	2270	0.016	0.780	L	0.42
PROK2_rs13094138	C	1587	0.01	E	24	0.884	L	2270	−0.020	0.728	E	0.96
GPR54_rs7969	G	1593	0.017	L	20	0.157	E	NA	NA	NA	NA	NA
LEPR_rs1938485[b]	G	1590	0.019	E	42	0.351	E	2270	0.003	0.940	L	0.96
LEPR_rs6678033	A	1587	0.023	E	46	0.424	E	2270	−0.003	0.940	E	0.96
KAL1_rs5934469	T	1552	0.027	E	NA	NA	NA	NA	NA	NA	NA	NA
FGFR1_rs3925	A	1579	0.035	L	36	0.047	L	2270	−0.031	0.509	E	0.96
LEPR_rs2154384[c]	T	1535	0.041	E	33	0.808	E	2270	0.011	0.792	L	1.00
GNRHR_rs17088591	A	1570	0.056	E	5	0.405	E	2270	−0.161	0.292	E	0.96
LEPR_rs1475397[d]	A	1530	0.059	L	32	0.714	E	2270	−0.084	0.073	E	1.00
FGFR1_rs4647905[e]	G	1582	0.064	L	28	0.264	E	2270	0.040	0.406	L	0.99
LEPR_rs10889557	A	1581	0.065	L	NA	NA	NA	2270	−0.052	0.254	E	0.91
KAL1_rs12710616	G	1557	0.068	E	NA	NA	NA	NA	NA	NA	NA	NA
PROK2_rs4677048	G	1575	0.072	L	40	0.453	E	2270	0.024	0.578	L	1.00
KAL1_rs5933676	A	1579	0.075	E	18	0.286	E	NA	NA	NA	NA	NA
GNRHR_rs17082306	T	1571	0.079	E	5	0.405	E	2270	−0.161	0.292	E	0.96
KISS1_rs12998	T	1552	0.083	L	7	0.467	E	NA	NA	NA	NA	NA
LEPR_rs7555955[f]	A	1530	0.087	L	NA	NA	NA	2270	−0.038	0.386	E	0.99
LEP_rs791602	T	1552	0.091	E	27	0.132	L	NA	NA	NA	NA	NA
GNRH_rs17201112	G	1539	0.092	L	NA	NA	NA	2270	−0.056	0.433	E	0.980
KISS1_rs12760824	T	1582	0.095	L	25	0.586	E	NA	NA	NA	NA	NA
FGFR1_rs3758101	T	1465	0.099	L	17	0.043	E	NA	NA	NA	NA	NA

Results in the delayed-puberty trios and the Nurses' Health Study for SNPs with $P < 0.10$ in the AOM panel. All results are given with respect to the same effect allele. The effect allele is the allele that produces the indicated effect on age at menarche or puberty. E means the effect is on early menarche; L means the effect is on late menarche. One SNP (FGFR1_rs3925) had $P < 0.05$ in the AOM panel and the trios, although it was not significant in ($P = 0.5$) and had an effect in the opposite direction in the Nurses' Health Study. NA, Not available.

[a] r^2 for imputation is a measure of the correlation between the SNP directly genotyped in the AOM panel and the SNP genotyped in the Nurses' Health Study.

[b] Nurses' Health Study data given for rs6678033, $r^2 = 0.956$ with rs1938485.

[c] Nurses' Health Study data given for rs1892534, $r^2 = 0.954$ with rs2154384.

[d] SNP predicted by the multimarker test LEPR_rs7537093, rs1177681, rs10749753.

[e] Nurses' Health Study data given for rs2016875, $r^2 = 0.951$ with rs4647905.

[f] SNP predicted by the multimarker test LEPR_rs9436300, rs10889557, and rs12065099.

tified in the AOM panel, although we also cannot rule out the possibility of modest effects of these SNPs.

Epistasis analysis

Interactions between loci that have weak or nonexistent effects on their own may play a role in human disease (36). To test for the possibility of epistatic interactions between SNPs in the 10 HH genes studied here, we tested all 41,024 pairwise combinations (257 SNPs and 29 multimarker tests) for an effect on age at menarche (supplemental Table 5). No interaction reached statistical significance after correcting for multiple-hypothesis testing using either the Bonferroni method or the Benjamini-Hochberg method.

Rarer variants

To begin to assess a possible role for rarer, more severe variants in these genes, we also genotyped five rare mutations identified in unrelated HH patients and reported in the literature more than twice, as well as four variants in *FGFR1* identified through screening of patients with delayed puberty (supplemental Table 1). We saw no association between the rare variants identified in clinical populations and the timing of puberty/menarche in our samples. We did not observe two of the mutations identified in HH patients in any of our samples, and observed the other three only very infrequently, in both women with early and late menarche (Table 3). We observed the *FGFR1* variants only at very low frequencies and in similar numbers of women with early and late menarche (Table 3). Thus, we did not observe strong association between age at menarche and either common or known rarer variants in these 10 genes.

Discussion

Here we report association testing with age at menarche and common genetic variation in 10 genes either known to cause HH or that encode ligands for receptors encoded by HH genes. There were several nominally significant associations between SNPs in these genes and age at menarche, but none of these is robust enough to withstand correction for multiple hypotheses. Although these genes all play critical roles in the reproductive endocrine axis, our results suggest that common genetic variation in these loci does not play a substantial role in influencing the normal spectrum of pubertal timing. This finding is important because it suggests that additional genes (and perhaps pathways) need to be examined (and perhaps identified) to understand the genetic regulation of pubertal timing in the general population.

This study extends our previous work by examining 10 genes encoding proteins (or their ligands) that underlie HH in a large panel of women from five different ethnic groups. The multiethnic nature of our cohort allows the possibility of detecting both modest effects shared across groups and large effects specific to one self-described racial/ethnic group. To our knowledge,

TABLE 3. Published HH mutations and FGFR1 variants

Gene	SNP	Amino acid change	Times observed, early menarche	Times observed, late menarche
KAL1	KAL1_Exon5_R191X	R191X	0	0
KAL1	KAL1_Exon10_R457X	R457X	1	0
GNRHR	GNRHR_Exon1_Q106R	Q106R	2	5
GNRHR	GNRHR_Exon3_R262Q	R262Q	1	1
GNRHR	GNRHR_Exon1_R139H	R139H	0	0
FGFR1	FGFR1_Exon3; rs2915665	S115S	7	6
FGFR1	FGFR1_Exon6_silent	Y236Y	1	1
FGFR1	FGFR1_Exon14_splice	NA	2	0
FGFR1	FGFR1_Exon17_silent	L754 L	17	15

This table lists the most commonly reported HH mutations from the literature as well as the *FGFR1* sequence variants found through resequencing, along with the number of times they were observed in the early and late menarche groups. No appreciable differences in frequency were observed between the two groups, with perhaps the exception of GNRHR_Exon1_Q106R, which was seen slightly more frequently in the late menarche group than in the early menarche group. This difference is not significant ($P = 0.28$). No individual carried more than one mutation/variant. NA, Not applicable.

this is the largest association study conducted to date examining age at menarche (and by extension pubertal timing). Previous studies have focused on fewer variants in smaller numbers of genes, have mostly been conducted in Caucasian samples, and have examined menarche [*IGF1* (37), *CCR3* (38), *CYP17* and *CYP19* (39), *ESR1* (40), *LEP* and *LEPR* (13), and *GNRH* and *GNRHR* (14)] as well as related phenotypes such as early pubertal development [*COMT* (41)]. In addition to SNPs from the 10 HH-related genes, we also genotyped 13 SNPs in other genes with previously reported associations with age at menarche and found no association in our samples for any of these SNPs (supplemental Table 6). We did see a consistent trend for association of age at menarche with the *ESR1 Pvu*II polymorphism (rs2234693), but neither the result from our study nor the original report of association (40) reached nominal significance. This trend was also seen in the Nurses' Health Study panel (data not shown).

Although we found no significant effects of common variants in these genes on age at menarche, our findings do not completely rule out variation in these genes as regulating puberty in the general population. Our study surveys mostly common variants (minor allele frequency >5%) and may not capture the effects of rarer variants on the timing of menarche or the effects of the small fraction of common variants not captured by our linkage disequilibrium-based approach. Furthermore, if the genes studied have very modest effects on pubertal timing, our study would lack the power to detect such effects. For example, although our study has approximately 90% power to detect variants that explain 0.5% of the variation in pubertal timing, we have only 50% power to detect variants that explain 0.25% of the variation in timing of puberty. In addition, there are variants that could influence HH gene expression but were not assessed using our strategy. These include variants at long distances from the HH genes and structural or other polymorphisms that are not in strong linkage disequilibrium with the variants tested in this study.

Our strategy began by searching for associations in females; thus, if there are male-specific effects of these genes on the timing of puberty, we would likely not have detected those effects. Previous nominally significant associations between SNPs in *GNRH* and *GNRHR* and delayed puberty, both those identified in the delayed puberty trios and in women from a breast cancer study in the MEC (14), were not observed in our menarche panel. These data indicate that those nominal associations were likely due to chance.

Although it is not known whether they contribute to clinical phenotypes, we also tested several variants identified in delayed-puberty and HH patients for association with age at menarche. Because some of these variants were identified in male patients with delayed puberty, if they have an effect on the timing of puberty, it may be related to male gender and/or their effect may be limited to cases where they combine with other mutations to produce an impact on phenotype. However, the data from our study are not supportive of an effect in females drawn from the general population.

Any observed differences in associations between racial/ethnic groups could be due to statistical fluctuations, a true difference of the effect across populations, or different linkage disequilibrium patterns in different populations. We guarded against the possibility of heterogeneity of effect due to differing linkage disequilibrium patterns by tagging variation in each of the different populations represented by the HapMap. Although the sample sizes of the five racial/ethnic groups in our studies are sufficiently large to allow the detection of large racial/ethnic-specific effects, it is possible we failed to detect modest racial/ethnic-specific effects. For example, our study has 80–90% power (depending on allele frequency) to detect racial/ethnic-specific variants that explain 2% of the variation in pubertal timing but only 35–40% power to detect variants explaining 1% of the variation and virtually no power to detect variants that explain 0.25% of the variation in any one ethnic group. Good power (80% or more) to detect all possible racial/ethnic-specific associations, however, would require sample sizes of many thousands of individuals in each racial/ethnic group.

As part of this study, we examined the correlation between genetic ancestry and age at menarche. Interestingly, we found a significant association between Native American ancestry and age at menarche (increased Native American ancestry is associated with early menarche) and a suggestive association between Native Hawaiian ancestry and age at menarche (increased Native Hawaiian ancestry is associated with early menarche). Particularly in the case of Native American ancestry, this association is not unexpected, given that U.S.-born Mexican-Americans (who would tend to have some Native American ancestry) have been reported to have earlier menarche than Whites in the United States

(42, 43). The association between East Asian ancestry and age at menarche (more East Asian ancestry is associated with late menarche), is equivocal, and additional large population genetics studies of self-described Japanese-Americans would be required to determine whether the women with slightly less East Asian ancestry correspond to a distinct subgroup within Japanese populations.

Our subjects were well past the onset of menarche when questionnaire data were collected, but studies have shown that recalled age at menarche is highly correlated with actual age at menarche (44, 45). Must *et al.* (45) found a high correlation between actual and recalled age at menarche even 30 yr later. Furthermore, in an effort to reduce the incidence of misclassification, the women in our sample were drawn from the extremes of menarcheal age. Both Must *et al.* (45) and Cooper *et al.* (46) found that the accuracy of recall was improved by categorizing age at menarche and that recall at the extremes appears more accurate. Additional evidence of accuracy of recall in our sample is that we observed correlation of age at menarche with BMI at age 21, with women with early menarche having a higher average BMI than women with later menarche (Table 1), and a correlation of age at menarche with birthplace in the Japanese subjects consistent with known effects of immigration on age at menarche (3). Thus, misclassification of women with menarche under age 11 *vs.* 15 yr or older (and vice versa) is likely to be quite low. Age at menarche is only one of several events that comprise puberty. Should large cohorts become available with phenotype data for other puberty-related events, they may provide additional insights into the genetics of the timing of puberty.

Age at menarche is a highly heritable trait, but so far there has been little success at conclusively identifying genes that contribute to the variation in age at menarche (and by extension the age of pubertal onset). If the HH genes studied here do not contribute substantially to population variation in menarcheal timing, what genes do contribute? Mutations in known HH genes are only responsible for approximately 30% of cases of HH (47), so there are likely other HH genes (and pathways) that have not yet been identified that may prove to contribute substantially to variation in pubertal timing in the general population as well. Variants in these or other genes may prove to influence the timing of puberty. Other attempts to identify genes that modulate pubertal timing have included linkage studies (48, 49), but the studies show little overlap in their linkage peaks, and no positional candidate has yet been convincingly confirmed as playing a role in pubertal timing. Additional candidate genes may also emerge from animal studies (50, 51). For example, the timing of vaginal opening, a marker of puberty in the mouse, varies between inbred mouse strains, and a quantitative trait locus has been identified on the distal portion of chromosome 6 (52, 53). These mouse genes, once identified, would be candidates to modulate pubertal timing in humans. Finally, genome-wide association studies where hundreds of thousands of SNPs are assayed provide an unbiased approach to the search for common genetic variants that influence complex traits such as pubertal timing (54). Well-powered genome scans will be able to address more fully which variants might influence age at menarche in the general population. The eventual identification of these variants will increase our understanding of the physiology of pubertal development and may inform our understanding of disorders such as precocious and delayed puberty and hypothalamic amenorrhea.

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