Normal variants of *Microcephalin* and *ASPM* do not account for brain size variability

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Normal human brain volume is heritable. The genes responsible for variation in brain volume are not known. *Microcephalin* (*MCPH1*) and *ASPM* (abnormal spindle-like microcephaly associated) have been proposed as candidate genes as mutations in both genes are associated with microcephaly, and common variants of each gene are apparently under strong positive selective pressure. In 120 normal subjects, we genotyped these variants and measured brain volumes using magnetic resonance imaging. We found no evidence that the selected alleles were associated with increases or decreases in brain volume. This result suggests that the selective pressure on these genes may be related to subtle neurobiological effects or to their expression outside the brain.

INTRODUCTION

Variations in normal human brain size are genetically mediated, with heritability estimates in larger studies ranging from 65 to 93% (1,2) after correcting for well-known sexual dimorphism (3). None of the genes contributing to this normal variability has yet been identified, although several genes accounting for pathologically abnormal brain size are known. It has been recently suggested that high-frequency normal variants of two genes associated with autosomal recessive microcephaly, *Microcephalin* (*MCPH1*) on chromosome 8p23 and *ASPM* (abnormal spindle-like microcephaly associated) on chromosome 1q31, have been under strong positive selective pressure in modern humans (4,5). Evidence also suggests strong selective pressure on these two genes in the evolutionary lineages leading from monkeys and apes to humans (6–10).

For *MCPH1*, a single nucleotide polymorphism (SNP) in exon 8 at position 37995, designated *G37995C*, is consistently found in the high-frequency haplotype reported to be under positive selective pressure and in genealogically closely related haplotypes, but is rarely found in the ancestral haplotypes (4). The *G37995C* SNP is estimated to have arisen 37 000 years ago; strong linkage disequilibrium with this SNP extends over a 29 kb region of the gene, so it is possible that positive selective pressure is acting on other variants sharing the same haplotype with *G37995C*. Similarly, for *ASPM*, an SNP in exon 18 at position 44871, designated *A44871G*, is associated with the selected haplotype, minor variants of the selected haplotype and recombinants derived from these haplotypes (5). The most recent common ancestor of individuals having this SNP has been estimated to have lived 5800 years ago, and linkage disequilibrium with *A44871G* extends over a 62.1 kb region (5). If the positive selective pressure associated with these two genes is related to increased brain size, the *G37995C* SNP of *MCPH1* and the *A44871G* SNP of *ASPM* should be associated with increased brain volume. To test this hypothesis, we genotyped these variants and measured brain volumes in a sample of normal adults who had undergone magnetic resonance imaging as part of an ongoing study conducted by the International Consortium for Brain Mapping (11). Our findings do not support a major role for normal variants of these genes in determining brain size.
RESULTS

We genotyped subjects for the MCPH1 G37995C and ASPM A44871G SNPs. Subjects who categorized themselves as African-American, Middle Eastern or ‘other’ were genotyped, but excluded a priori from statistical analysis because the numbers of subjects with distinct genotypes were insufficient to warrant inclusion; post hoc re-analysis including these groups did not alter the findings presented here. One hundred and twenty subjects were included for statistical analysis. The G37995C allele of MCPH1 had an overall frequency of 82% and was in Hardy–Weinberg equilibrium among the self-described Asian (P = 0.16), Caucasian (P = 0.67) and Hispanic (P = 1.0) sub-populations and in the three sub-populations combined using Fisher’s method (χ² = 4.4, df = 6, P = 0.62). The A44871G allele of ASPM had an overall frequency of 33% and was in Hardy–Weinberg equilibrium among self-described Asians (P = 0.56) and Hispanics (P = 1.0) but among self-described Caucasians was present more often in heterozygotes and less often in homozygotes than predicted (P = 0.014). However after Bonferroni correction for the six comparisons performed (two genes in each of three populations), this departure from Hardy–Weinberg equilibrium was no longer significant. Moreover, combining the three sub-populations using Fisher’s method showed no violation of Hardy–Weinberg equilibrium for ASPM (χ² = 9.8, df = 6, P = 0.13). These results are compatible with the interpretation that the apparent violation of Hardy–Weinberg equilibrium for ASPM in Caucasians may have been due to random chance rather than to other causes of heterozygote excess such as migration, negative assortative mating or heterozygote advantage (12). Genotyping error is unlikely because duplicate specimens from each subject gave identical results in all cases.

Figure 1 shows the relationship between brain volumes and the SNP genotypes of MCPH1 and ASPM as a function of sex and self-reported racial or ethnic category. When modeling the relationship between brain volume and genotype, two different approaches can be taken. The first approach, which corresponds to the definition of broad sense heritability (H²), includes a term for each of the three possible genotypes and has two associated degrees of freedom. Using this approach, which allows for dominance effects, no significant relationship was found between brain volume and ASPM genotype (df = 2, 114, P = 0.49) or MCPH1 genotype (df = 2, 112, P = 0.20) when correcting for racial or ethnic category and for sex. Residuals from the ANOVAs did not depart from normality by Shapiro–Wilks testing (ASPM: W = 0.9863, P = 0.27; MCPH1: W = 0.984, P = 0.17). The effect of sex on brain volume was highly significant (df = 1, 116, P = 1.4 × 10⁻¹⁰, with correction for race or ethnicity), and the corresponding residuals did not depart from normality by Shapiro–Wilks testing (W = 0.9851, P = 0.21). Age was considered as a potential covariate but was omitted from the other analyses because it was not significant (df = 1, 115, P = 0.23, correcting for sex and race or ethnicity). Because members of different racial and ethnic categories may have differed in socioeconomic status, these categories were not tested for significance but were included in the model based on a planned a priori data analysis strategy.

Including race or ethnicity and sex as covariates, estimated H² for brain volume related to the MCPH variant was 0.018, and 95% confidence intervals (with Tukey-Kramer correction) for differences in brain volumes in cubic centimeters (cc) between the three possible MCPH1 genotypes (where ‘+’ represents the ancestral nucleotide) were

\[
\text{G37995C/G37995C minus G37995C*/+} = -233 \text{ to } +35 \text{ cc}
\]

\[
\text{G37995C/G37995C minus G37995C*/+} = -54 \text{ to } +51 \text{ cc}
\]

\[
\text{G37995C*/+ minus G37995C*/+} = -235 \text{ to } +39 \text{ cc}
\]

Similarly, estimated H² for brain volume related to the ASPM variant was 0.008, and 95% confidence intervals for differences in brain volumes between the three possible ASPM genotypes were

\[
\text{A44871G/A44871G minus A44871G*/+} = -93 \text{ to } +106 \text{ cc}
\]

\[
\text{A44871G/A44871G minus A44871G*/+} = -69 \text{ to } +127 \text{ cc}
\]

\[
\text{A44871G*/+ minus A44871G*/+} = -72 \text{ to } +28 \text{ cc}
\]

Including race or ethnicity as a covariate, the 95% confidence interval for the difference in brain volume between males and females was 99–179 cc.

The second approach to modeling genetic effects is to use the model used in estimating narrow sense heritability (h²) in which heterozygotes are assumed to have a phenotype that is the average of that of the corresponding homozygotes. This approach is relevant because the effect of selection depends on additive genetic variance and not on dominance effects (13). Self-described race or ethnicity and sex were included as covariates and the number of A44871G or G37995C alleles (zero, one or two) was treated as the genetic covariate of interest. Residuals from the ANOVAs did not depart from normality by Shapiro–Wilks testing (ASPM: W = 0.9859, P = 0.25; MCPH1: W = 0.984, P = 0.16). For ASPM, each additional A44871G allele was associated with a non-significant (df = 1, 115, P = 0.55) 10.9 cc decrease in brain volume resulting in an estimated h² of 0.002. The 95% confidence interval for the change in brain volume per A44871G allele was −46.8 cc (h² = 0.04) to 25.0 cc (h² = 0.01). For MCPH1, each additional G37995C allele was associated with a non-significant (df = 1, 113, P = 0.30) 19.5 cc decrease in brain volume resulting in an estimated h² of 0.006. The 95% confidence interval for the change in brain volume per G37995C allele was −56.8 cc (h² = 0.05) to 17.8 cc (h² = 0.005).

DISCUSSION

Our findings suggest that the alleles of MCPH1 and ASPM reported to be under strong selective pressure in modern humans are not major contributors to normal variation in human brain volume. We cannot exclude the possibility that these alleles might nonetheless be associated with small differences in brain volume. However, we can place constraints on the heritability of brain volume associated with these variants. Assuming, as has been postulated or implied (4,5), that the selected genotypes are associated with increases in brain volume rather than decreases, our data are not compatible with a brain volume h² > 0.01 for the ASPM variant or with a brain volume h² > 0.005 for the MCPH1 variant. If the selected genotypes are associated with decreases in brain volume rather than increases, slightly larger heritabilities of...
0.04 or 0.05 remain consistent with our findings. Although selective pressure in favor of smaller brain volumes might seem counterintuitive, it is relevant to note that the fossil record suggests that brain size in humans has decreased over the past 50,000 years, with the trend continuing through the Neolithic, reversing more recently only at higher latitudes (14, 15). Interestingly, the selected variant of \textit{MCPH1} is thought to have arisen about 37,000 years ago (4), making it a candidate for a gene responsible for this general decline. The \textit{ASPM} variant is thought to have arisen only 5,800 years ago and does not show clear evidence of variation by latitude (5). These archaeological changes in brain size have been paralleled by changes in body size (14, 15), and it is possible that decreases in body size, driven by a variety of factors (15), may have exerted selective pressure for corresponding decreases in brain size. The size of a mother’s pelvis relative to the size of her child’s brain is believed to be an evolutionary constraint on brain size (16) and might provide a mechanism for such pressure.

Even an extremely low heritability would not exclude the possibility that associated phenotypic variations, though small, might nonetheless serve as the basis for selective pressure on these two genes. Moreover, a negative result from an extremely large sample size would still not disprove the hypothesis that the selective pressure is exerted through the phenotype of brain size. However, it is relevant to note that both \textit{ASPM} and \textit{MCPH1} are expressed outside the brain (17, 18), so it is certainly possible that the selection of these variants might have nothing to do with the brain. A recent scan for positively selected genes in humans and chimpanzees found that genes with maximal expression in the brain generally showed little evidence for positive selection, whereas genes involved in immune defenses, sensory perception, tumor suppression, apoptosis and spermatogenesis showed the strongest evidence (19), a result that emphasizes the importance of considering non-brain sources of positive selection.

The high heritability of brain volume as a trait, combined with the low (and possibly zero) heritability of brain volume that we observed in association with the selected variants of \textit{ASPM} and \textit{MCPH1}, indicates that considerable caution is warranted when attempting to explain positive selective pressure on these genes on the basis of their effects on brain volume. A complete and convincing picture is likely to emerge only after other genes that make substantial aggregate contributions to differences in brain volume both within and across species have been identified and evaluated for evidence of selective pressure, and these findings have been reconciled with the fossil record. To the extent that human brain volumes may already represent an optimal balance between opposing forces acting on brain size relative to body size, positive or negative selection acting on some other phenotype associated with any gene affecting brain volume might be sufficient to
drive compensatory selection of ASPM and MCPH1 to maintain this balance. Consequently, even if the selected variants of these two genes were known to be associated with a small increase (or decrease) in brain volume, this would not necessarily imply that brain volume as a phenotypic trait is under selective pressure to change.

Although the role of recessive mutations of both of these genes in producing microcephaly is undisputed, our findings suggest that it is potentially misleading to refer to either of these genes as controlling, regulating or determining human brain size outside the context of the microcephalic state. The titles of several recent papers notwithstanding (4–10), evidence of positive selective pressure acting on genes known to be associated with human microcephaly and mental retardation does not necessarily imply that such genes account for the variations in brain size or intelligence that are encountered in normal human populations. In future work, it would be informative to investigate the brain and non-brain phenotypes of relatives of patients with microcephaly who lack one normal copy of these genes because this could potentially unmask subtle phenotypic differences related to the remaining normal variant.

MATERIALS AND METHODS

The research protocol was approved by the UCLA IRB. Subjects, aged 18–39, were recruited through advertisements in nearby communities and on campus. After giving informed consent, subjects were screened to exclude those with a history or physical findings indicating neurological or psychiatric abnormality. Subjects self-reported their racial or ethnic background in a computerized questionnaire that offered the following categories: African-American, Asian, Caucasian, Hispanic, Middle Eastern or other. The questionnaire allowed a second categorization, but none of the subjects reported here responded with a second categorization.

Magnetic resonance imaging was performed using a 3.0 T General Electric Signa scanner using a 3D spoiled GRASS sequence. The repetition time was 24 ms and the echo time was 4 ms. One hundred and sixty contiguous sagittal slices were acquired with a slice thickness of 1.2 mm. The in-plane resolution was 0.98 mm. Initial segmentation of the brain from non-brain tissues used an automated, model-based level set algorithm developed in-house, and results were then corrected manually using anatomic criteria. Brain volumes were based on the manual corrections of a single observer, whose results agreed with those of a second observer within 1% in a 35-subject subset of the sample.

Genotyping of DNA from duplicate blood specimens for each subject utilized TaqMan 7900 HT assays from Applied Biosystems (ABI) designed using manufacturer’s Assays-by-Design service. Assays were based on the ASPM A44871G and MCPH1 G37995C polymorphisms and 100 bases of the 5’ and 3’ flanking sequences. Sample DNA concentrations were determined using the Quant-iT™ Broad-Range DNA Assay Kit (Invitrogen) according to manufacturer’s protocol, and sample aliquots were normalized to 10 ng/µl in Tris–EDTA buffer. Assays were set up in a 384-well plate (ABI) using 2.5 µl of TaqMan Universal PCR Mastermix, No AmpErase UNG (ABI), 0.125 µl of 40× assay primer mix and 2.375 µl of DNA for a total reaction volume of 5 µl. Plates were sealed using Optical adhesive covers (ABI). PCR was performed using a Perkin Elmer 9700 dual 384 PCR machine. An initial hold for 10 min at 95°C was followed by 40 cycles at 92°C for 15 s alternating with 60°C for 1 min. After a 4°C soak, end point reads were obtained on a TaqMan 7900 HT instrument (ABI). Clustering and allele calling were performed with the instrument software package. The sequences of primers and probes were as follows.

ASPM forward primer sequence: GCTGCCATTATTATCAGAGACATTG.
ASPM reverse primer sequence: GCACGAGATTAGTTTCTGTATCTT.
ASPM reporter 1 (VIC fluorescent reporter dye) primer sequence: CATTATCTCCACCTTAGAGC.
ASPM reporter 2 (FAM fluorescent reporter dye) primer sequence: ATTATCTCCACCTTAGAGC.
ASPM Quencher: non-fluorescent quencher.
MCPH1 forward primer sequence: CTTCAAGAAATATTGAGTAAAGTAGT.
MCPH1 reverse primer sequence: CTTCAACGTCCTCAGACATACC.
MCPH1 reporter 1 (VIC fluorescent reporter dye) primer sequence: CACCCCCGACAAAA.
MCPH1 reporter 2 (FAM fluorescent reporter dye) primer sequence: CACCCCTCACAAAA.
MCPH1 Quencher: non-fluorescent quencher.

Alleles were tested for Hardy–Weinberg equilibrium in the three sub-populations using the exact Hardy–Weinberg test (20) implemented in GENEPOP version 3.4 (available from http://ftp.cefe.cnrs.fr/PC/MSDOS/GENEPOP/). Results were combined across sub-populations using Fisher’s combined probability test (21) as implemented in GENEPOP. ANOVA’s, Shapiro–Wilk’s tests and confidence intervals were performed using the statistical package R (available from http://www.r-project.org/). Heritabilities of brain volume related to genotype were estimated by using the coefficients of the genetic terms in the full fitted ANOVA model to compute the variance attributable to genotype. This variance was then divided by total variance to estimate heritability. For narrow sense heritabilities estimated using an additive genetic model, the monotonic relationship between heritability and the absolute value of the coefficient of the genetic term allows confidence intervals on this coefficient to be used to generate confidence intervals on heritability. As two independent coefficients contribute to broad sense heritabilities, confidence intervals for broad sense heritability could not be estimated in this way.

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Conflict of Interest statement. The authors have no conflict of interest to declare.

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