RESEARCH NOTE

Genomewide association study identifies no major founder variant in Caucasian moyamoya disease

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Introduction

Moyamoya disease (MMD) is an idiopathic cerebrovascular occlusive-stenosis disorder at the terminal portion of internal carotid arteries and its main branches, accompanied by collateral vascular networks at the base of the circle of Willis (Takeuchi and Shimizu 1957; Suzuki and Takaku 1969). MMD has the highest prevalence in East Asian countries and a low prevalence in European countries (Goto and Yonekawa 1992; Kuroda and Houkin 2008). We have found that the p.R4810K variant in the ring finger protein 213 (RNF213) is a major founder susceptibility gene for East Asian MMD (Liu *et al.* 2010, 2011). In this study, we aimed to test whether there is a major founder susceptibility gene for Caucasian MMD using a genomewide association study (GWAS). We demonstrated that there was no major founder variant in Caucasian MMD as it is in East Asian MMD. We identified several suggestive association regions for Caucasian MMD.

Materials and methods

Study subjects

Ethical approval was given by the Institutional Review Boards and Ethics Committees of Kyoto University School of Medicine, Japan; Medical Faculty of the University of Tübingen, Germany; and the Faculty of Medicine and Dentistry of Palacký University, Czech Republic, respectively. Written informed consent was obtained from all participants. We calculated the required size of the study population to obtain enough statistical power in Caucasian population by assuming the following conditions as was reported in East Asian population (Liu *et al.* 2011): the frequency of the high risk allele is 0.01, the prevalence of MMD is $<10^{-4}$; the relative risk of risk allele is 100 by assuming an autosomal dominant mode of inheritance; the marker D' is 0.99 and its frequency >0.05; type I error is 0.05 and statistical power ≥ 0.8 (http://pngu.mgh.harvard.edu/~purcell/gpc/). The calculation indicated that 40 for cases and controls can assure the statistical power of 0.8. Cases are composed of 38 unrelated subjects: seven were Czech and 31 were German. Those cases were case-series patients between February 2008 and November 2009 in participating hospitals. Controls were all unrelated Germans, in whom, absence of MMD were confirmed by magnetic resonance imaging (MRI) in the same period.

DNA isolation, genotyping and quality control

Genomic DNA was extracted from the blood samples using a QIAamp DNA Blood Mini Kit (Qiagen, Germantown, USA) according to the manufacturer's protocol. Genomewide genotyping was performed using an Illumina Human610-Quad BeadChip Kit (Illumina, San Diego, USA). Samples with overall call rates <95% were excluded. Single nucleotide polymorphisms (SNPs) that met the following criteria were excluded: had call rates <0.99 in all GWAS samples and genotype distributions deviated from those expected by Hardy–Weinberg equilibrium (HWE). Principal component analysis showed no outliers or population stratification, and all subjects were confirmed to be of Caucasian ethnicity (see figure 1 in electronic supplementary material at http://www.ias.ac.in/jgenet/). A quantile–quantile plot revealed that the distribution of observed *P* values followed the expected distribution (see figure 2 in electronic supplementary material). After quality control, 565,294 out of 620,901 SNPs for the 38 cases and 41 controls were retained for GWAS.

Candidate genes sequencing by the Sanger method

Five cases and one control were randomly selected for sequencing of all coding exons and at least 100-bp exon–intronic boundaries in eight candidate genes from within five suggestive association regions for the case–control study. The primers that were used for direct sequencing are shown in table 1 in electronic supplementary material. The sequencing products were separated on an ABI Prism 3100 Avant DNA sequencer (Applied Biosystems, Kyoto, Japan). In addition, we investigated the association of SNPs in known two genes, actin, alpha 2, smooth muscle, aorta (*ACTA2*) (Guo *et al.* 2009) and *RNF213* (Liu *et al.* 2011), with MMD by GWAS.

Statistical analysis

GWAS was conducted using the SNP and Variation Suite V7 (Golden Helix, Bozeman, USA). Thresholds of 5×10^{-8} and 1×10^{-5} were set for genomewide significance and suggestive association, respectively. We assumed that a minor allele frequency of the SNP (i.e., risk marker allele), which is postulated to be the closest marker of the putative moyamoya susceptibility gene, was 0.5 in cases and 0.05 in controls as it does in Japanese population (Liu *et al.* 2011). Under this condition, with the current population sizes for cases and controls, simulation (http://pngu.mgh.harvard.edu/~purcell/gpc/) predicts that 29 out of 38 cases are homozygous or heterozygous for the risk marker allele while four controls were heterozygous for the risk marker allele and 37 controls were homozygous for the wild marker allele. Given an autosomal dominant in HWE, we can expect the odds ratio of the risk marker allele to be 29.8 with $P = 9.53 \times 10^{-10}$. When we assume a more modest condition for an odds ratio of 5, the statistical power was 2×10^{-3} and 215 cases and controls are needed to obtain the SNPs with the genomewide significant P of 5×10^{-8}.

Results

Demographic and clinical profiles of the participants

The participants comprised 38 cases and 41 unrelated controls (see table 2 in electronic supplementary material). The mean age for cases and controls was 31.37 ± 14.83 and 27.34 ± 11.55 years, respectively. The sex ratio of male:female was 14:24 and 8:33 for cases and controls, respectively. Age of onset for the cases was 22.93 ± 16.95 years which indicates that the adult onset was dominant. Cerebral infarction was the major clinical symptom, followed by transient ischemic attack and others. Thirty-five of the cases were diagnosed as bilateral MMD; the other three were unilateral MMD.

Genomewide association study

GWAS was performed under two conditions. One was case–control comparison and the other was cases with and without cerebral infarction comparison. No SNPs reached genomewide significant association under both conditions ($P < 5 \times 10^{-8}$, figure 1). However, GWAS revealed multiple suggestive associations under case–control comparison ($P < 1 \times 10^{-5}$, figures 1a and 2a–d). The top association SNPs were rs1023115 (odds ratio, OR = 6.63, $P = 2.60 \times 10^{-6}$) on 1q23.3, rs11681583 (OR = 6.51, $P = 2.93 \times 10^{-6}$) on 2p22.1, rs3742257 (OR = 0.20, $P = 5.73 \times 10^{-6}$) on 13q14.11, rs2058364 (OR = 5.57, $P = 5.90 \times 10^{-6}$) on 17p13.3 and rs720607 (OR = 0.17, $P = 5.59 \times 10^{-6}$) on 20q13.33 (see table 3 in electronic supplementary material; figure 2, a–d). Under case-only analysis, two SNPs reached genomewide suggestive associations (rs17078141 on 3p22.1, OR = 0.04, $P = 6.10 \times 10^{-6}$; rs2178763 on 4q22.3, OR = 21.81, $P = 6.88 \times 10^{-6}$, table 3 in electronic supplementary material; figures 1b and 2e).

Mutation analysis of candidate genes

Candidate regions covering top suggestive association SNPs were further investigated by direct sequencing (figure 2). There was no annotated gene on 17p13.3 and 4q22.3 that covered rs2058364 and rs2178763, respectively. A summary of the variants detected by direct sequencing is available in table 4 in electronic supplementary material for the case–control study. We identified a total of 79 variants among which five were missense and eight were synonymous; the remaining variants were located in noncoding regions. We considered that synonymous and noncoding variants were unlikely to cause the MMD, even when they have low frequencies in general population. All of the missense variants detected were found in dbSNPs database (http://www.ncbi.nlm.nih.gov/projects/SNP/, NCBI build 36.3). Their allele frequencies in general Caucasian populations were greater than 0.1, suggesting that these missense SNPs were unlikely to be associated with the development of MMD in the Caucasian population. In terms of case-only analysis, four genes (*MYRIP*, *FLJ33065*, *ENTPD3* and *FLJ36665*) were found but no sequence determinations were done.
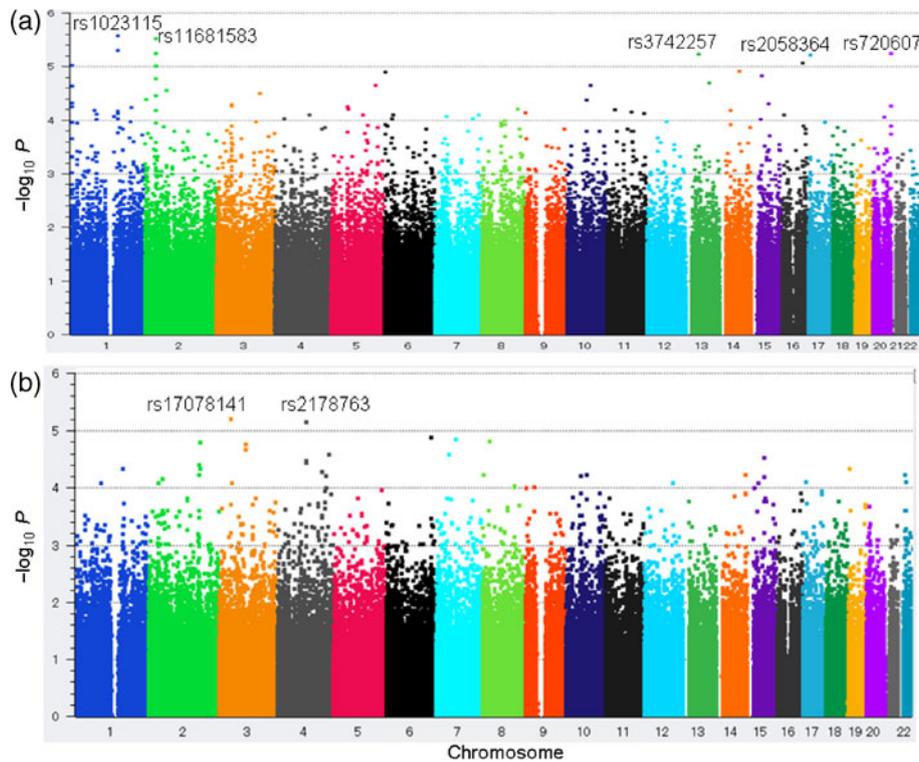
Figure 1. Manhattan plot of the genomewide *P* values of association. The $-\log_{10} P$ values (*y*-axis) are presented against their chromosomal positions (*x*-axis). The top significant associated SNPs are showed. A *P*-value threshold of 1×10^{-5} was taken to represent suggestive association. (a) Analysis for case–control comparison and (b) analysis for case with and without cerebral infarction comparison.

Association with ACTA2 and RNF213

We investigated the association of SNPs in *ACTA2* and *RNF213* with MMD (table 5 in electronic supplementary material). Neither association of SNPs in *ACTA2* nor *RNF213* with MMD was confirmed. However, a cluster of 10 of 25 SNPs in *RNF213* gene region had *P* values less than 0.05, suggesting a possible association with *RNF213* with MMD.

Discussion

We conducted a GWAS to identify the major founder variant for Caucasian MMD but could not find any major founder variant in Caucasian MMD as we did in East Asian MMD. However, we found several suggestive association regions that might be responsible for the Caucasian MMD.

A major founder mutation of p.R4810K in *RNF213* within 17q25.3 was first rigorously identified in East Asian MMD (Liu *et al.* 2011) and perfectly replicated in different cohorts (Miyatake *et al.* 2012; Wu *et al.* 2012). The number of patients with MMD was estimated to be at least 53,800 in East Asian populations (Liu *et al.* 2012). In contrast, we could not detect any founder variants in *RNF213* for Caucasian MMD, suggesting the different genetic background between Caucasian and East Asian MMD. The low

prevalence of Caucasian MMD might be due to the lack of the predominant founder variant. However, it should be addressed that there was a cluster of SNPs in *RNF213* region weakly associated with MMD, suggesting *RNF213* as a potential susceptibility gene for MMD even in Caucasian.

Mutations in *ACTA2* were first identified in Caucasian MMD (Guo *et al.* 2009), but they have not been replicated in Japanese and another Caucasian MMD (Shimojima and Yamamoto 2009; Liu *et al.* 2011; Roder *et al.* 2011). We confirmed the absence of association. Importantly, case-only association analysis revealed that *MYRIP* was located around the suggestive association region for cerebral infarction (figures 1b and 2e). *MYRIP* has the highest expression in brain. Fukuda and Kuroda (2002) showed that the C-terminal domain of *MYRIP* directly bound actin, which was encoded by *ACTA2*. The above features suggest that *MYRIP* might be responsible for the progression of MMD in Caucasian population. This observation warrants further investigation in future. The present study, on the other hand, failed to demonstrate the association of SNPs in *RNF213* (see table 5 in electronic supplementary material) with MMD.

This study had several limitations. First, the small number of available samples caused by the rareness of MMD might be the most critical limitation. In this relation, the current study suggests that our study assumption for calculation of statistical power is not realistic. If so, there may be no
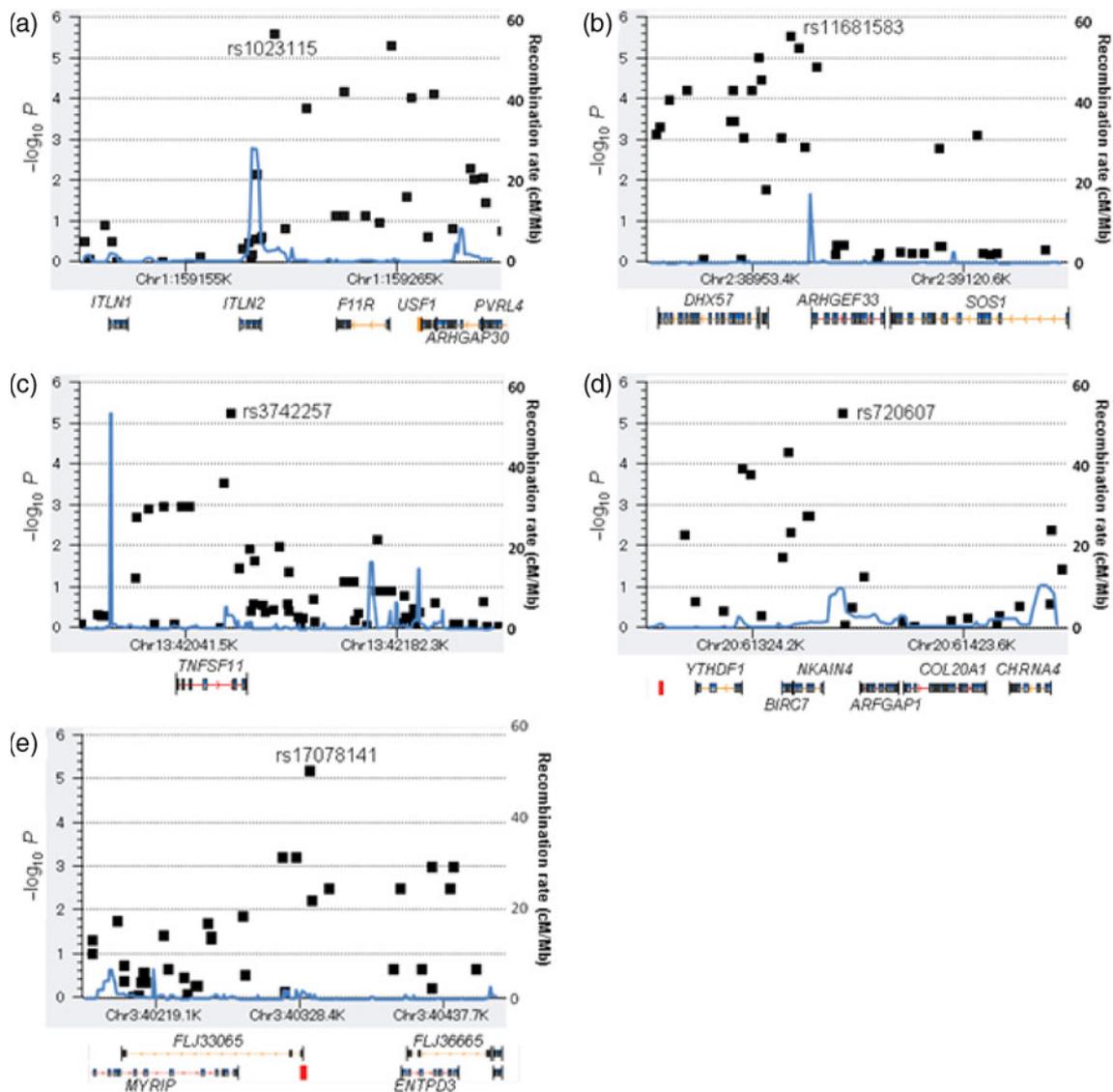
Figure 2. Regional association plots for the suggestive association regions. (a) Association plot for loci at rs1023115 on 1q23.3. (b) Association plot for loci at rs11681583 on 2p22.1. (c) Association plot for loci at rs720607 on 13q14.11. (d) Association plot for loci at rs2058364 on 20q13.33. (e) Association plot for loci at rs17078141 on 3p22.1. The results ($-\log_{10} P$) are for SNPs in the regions flanking the top SNPs. The recombination rate (blue line) was estimated based on the CEU, YRI and JPT+CHB populations from the Phase 2 HapMap (Release 22, NCBI 36). The annotated genes within the suggestive association regions are shown beneath the plots.

such predominant susceptible or causative rare variant in Caucasian cases as it is in East Asian MMD. Second, the rare variants cannot be discovered through GWAS. Next-generation sequencing will be of help in identifying rare variants. Third, several missense variants identified in candidate genes were unlikely to be causative genes because they are common in general Caucasian population. Searching for mutations by exome in a large number of Caucasian patients in these candidate genes is needed in future to find causative genes.

In summary, we were unsuccessful in our attempt to identify predominant founder variants in Caucasian MMD. The absence of the founder variant in Caucasians is in strong contrast to the major founder variant that has been identified in East Asians. Taken together, the present result probably indicates that locus heterogeneity as well as disease heterogeneity may be much greater in Caucasian MMD than in Asian MMD. Further study is warranted to investigate the genetic basis for global MMD.

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