Association of a Notch 3 gene polymorphism with migraine susceptibility

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Abstract

Cerebral Autosomal Dominant Arteriopathy with Sub cortical infarcts and leucoencephalopathy (CADASIL) shares common symptoms with migraine. Most CADASIL causative mutations occur in exons 3 and 4 of the Notch 3 gene. This study investigated the role of C381T (rs 3815188) and G684A (rs 1043994) single nucleotide polymorphisms (SNP) in exons 3 and 4 respectively of the Notch 3 gene in migraine. The first study, in a population of 275 migraineurs and 275 control individuals, found a significant association between the C381T variant and migraine, specifically in migraine without aura (MO) sufferers. The G684A variant was also found to be significantly associated with migraine, specifically in migraine with aura (MA) sufferers. The follow-up study in 300 migraineurs and 300 control individuals did not show replicated association of the C381T variant with migraineurs. However the G684A variant was again shown to be significantly associated with migraine, specifically with MA.

Keywords:

Migraine, CADASIL, Notch 3

List of Tables:

Table 1: Distribution of the C381T polymorphism (SNP ref rs 3815188) in Notch 3 gene in migraineurs and controls in the two studied populations.

Table 2: Distribution of the G684A polymorphism (SNP ref rs 1043994) in Notch 3 gene in migraineurs and controls in the two studied populations.

Table 3: Chi-squared (X^2) analysis of the allelic and genotypic frequencies in all migraine groups against controls for the C381T polymorphism in the two studied populations.

Table 4: Chi-squared (X^2) analysis of the allelic and genotypic frequencies in all migraine groups against controls for the G684A polymorphism in the two studied populations.

Introduction

The etiology of migraine is continuously being explored in order to facilitate an understanding of the mechanism underlying the pain and accompanying symptoms of this disorder [1]. Although not all migraine genes have been identified, a number of causative mutations and susceptibility variants have been discovered and are already of significant clinical relevance [2, 3].

Cerebral Autosomal Dominant Arteriopathy with Sub cortical Infarcts and Leucoencephalopathy (CADASIL) is clinically typified by recurrent sub cortical ischemic strokes and migraine with aura (MA) [4]. CADASIL is a rare inherited autosomal dominant disease caused by mutations in the Notch 3 gene. The Notch 3 gene is located on chromosome 19p and encodes a large single-pass transmembrane protein expressed in the arterial vascular smooth muscle cells. More than 65% of all CADASIL mutations occur within exons 3 and 4 of the Notch 3 gene, with these exons routinely analysed when diagnosing patients with presenting symptoms related to CADASIL [5, 6]. In addition, a number of synonymous polymorphisms have also been identified in these exons.

Although synonymous polymorphisms have been commonly assumed to be functionally insignificant, recently, an increasing number of studies have reported non-functional polymorphisms in both coding and non-coding sequences that affect the splicing process as well as *in vivo* protein folding and consequently, gene function [7]. Here, we investigated whether two Notch 3 polymorphisms, specifically the C381T and G684A variants in exons 3 and 4, respectively of the Notch 3 gene contribute to the risk of migraine in an Australian Caucasian population.

Materials and methods

Subjects

Written informed consent was obtained from all 1,150 participants of the study and the study was approved by the Griffith University Ethics Committee for Experimentation on Human Subjects. All participants were interviewed and completed a detailed questionnaire that was administered through Griffith University's Genomics Research Centre (GRC). The questionnaire included demographic characteristics, family history for migraine, cerebrovascular, cardiovascular and neurological diseases, migraine symptoms, age of onset, frequency, severity and treatment as previously described [8, 9]. Migraine diagnosis was performed by an experienced clinical neurologist from responses provided on the questionnaire in accordance with the International Headache Society (IHS) criteria, also as described previously [10, 11]. Questions used to define migraineurs included length and frequency of attack; pain location, type and intensity; associated symptoms such as nausea, vomiting, phonophobia, photophobia and other visual disturbances and neurological symptoms. Those participants who experienced both subtypes of migraine were classed as being affected with MA. The control group consisted of individuals with no family history of migraine and individuals who did not meet these criteria were excluded from the study [11, 12].

All participants were Caucasians of European descent living in Australia, having emigrating ancestors within the last 160 years from various locations within the British Isles and other parts of Europe and were matched for sex and age and were recruited in parallel at a similar time from the East Coast of Australia as previously described [2]. In total ~600 cases and an equivalent number of controls were collected over a spread of several years. The first study population collected comprised of 275

migraineurs and 275 matched controls. The follow-up second study population consisting of 300 migraineurs and 300 controls were collected later and the DNA prepared as a second independent population [11]. Samples used for the genotyping studies were all individuals, not families, with care taken not to include any related individuals in the case control population. All participants of both independent cohorts were recruited from in an around the South Eastern Australia region, with collections undertaken in the Genomics Research Centre Clinic at the Gold Coast, Queensland, Australia. To minimize potential bias the control group was matched for sex, age (+/- 5 years) and ethnicity. Migraine patients were clinically defined and suitably matched with the control population [11, 13].

Notch 3 genotyping

Genomic DNA was obtained from leucocytes following a salting out method as previously described by Miller et al [14]. Exons 3 and 4 of the *Notch 3* gene were polymerase chain reaction (PCR) amplified using oligonucleotide primer pairs previously described by Wang et al. [4], Not3X3F (5'TGT GCT GCC CAA CCA AGC CA 3') and Not3-X3R (5' ACT GAC CAC ACC CCC GAC 3') specific for Exon 3 and Not3-X4F (5' TAG TCG GGG GTG TGG TCA GT 3') and Not3-X4R (5'CCT CTG ACT CTC CTG AGT AG 3') specific for exon 4. The PCR conditions used were an initial denaturation at 95°C for 4 minutes, followed by 35 cycles of 94°C for 1 minute, 62°C for 1minute, 72°C for 1 minute, with a final extension of 72°C for 10 minutes [9]. Polymerase chain reaction (PCR) products of exon 3 and 4 were determined to be 224 base pairs (bp) and 420 bp respectively. Genotyping of the Notch exon 3 for the C381T polymorphism was performed by digesting PCR products with the *Aci I* restriction enzyme and genotyping of the exon-4 G684A polymorphism was performed by digesting PCR products with the *Mwo I* restriction enzyme. The

Notch 3 exon 3*C allele resulted in digested fragments of 95bp and 7bp, while Notch 3 exon 3*T allele was characterized by an uncut fragment of 166bp. The G to A substitution at nucleotide 684 in exon 4 gene was distinguished by the uncut fragment of 168kb, while the wild type G allele was characterized by digested fragments of 107bp and 61bp [4]. Products were resolved using a 3% agarose gel, stained with ethidium bromide and visualised under ultra-violet light. To confirm results of the restriction enzyme digest, the DNA samples were also genotyped using an ABI-3130 Genetic Analyser. Sequence electrophoretograms were examined visually using the ABI Sequencing Analysis software 5.0 to determine the alleles of the two polymorphisms C381T and G684A. The human Notch 3 cDNA and protein sequences were obtained from GenBank (accession no. U97669).

Statistical Analysis

Allele and genotype frequencies were first analysed using standard contingency tables, incorporating the chi-squared test. χ^2 analysis for migraineurs MA, MO and combined migraine groups versus control subjects for both C381T and G684A polymorphisms were performed. CLUMP analysis was used if one allele or genotype occurred less than 5 times. Hardy-Weinberg equilibrium was verified for observed genotype frequencies of C381T and G684A to detect deviation from the normal genotype distribution in the population. Odds ratios (OR) with their associated 95% confidence intervals (95% CI) were calculated using logistic regression analysis. Due to multiple testing, Bonferroni correction was performed [15]. This set the level of statistical significance at 0.025 (i.e., 0.05/2). Linkage disequilibrium between the two SNPs was calculated using the program Haploview 4.1 [16].

Results

SNP identification and genotyping

The locations of the two known SNPs, with the dbSNP accession numbers rs3815188 (C381T) and rs1043994 (G684A), were annotated in bp using the NCBI Build 36.3 genomic assembly. The positional location of C381T and G684A on chromosome 19 based on build 36.3 are 14,871,514 and 14, 87,133 respectively. Genotype and allele frequencies along with statistical measures of association for C381T and G684A in exons 3 and 4 of the Notch 3 gene in the two independent populations studied are summarised in Table 1 and 2. The distribution of genotypes for both the variants in both populations did not deviate significantly from Hardy Weinberg equilibrium ($P>0.05$). Internal controls consisting of repeat samples and negative controls were used in the confirmation of the genotypes and to account for potential genotyping errors. A matched pair was only included in the analysis when the results for the repeat samples and the negative controls were obtained. The genotype and allele frequencies of both C381T and G684A from both populations did not differ significantly from those previously reported [17]. Haploview analysis did not provide any significant evidence of linkage disequilibrium between C381T and G684A in the first and the follow up second independent population studied ($D'=0.050$, 95% CI - 0.01, 0.12, $R^2 = 0.00$ and $D'=0.041$, 95% CI -0.01, 0.15, $R^2 = 0.001$, respectively).

C381T

The distributions of the genotype and allele frequencies of C381T in the two independent populations studied are summarised in Table 1. 252 migraineurs and 247 controls were successfully genotyped for this variant in the first population. There were 192 females and 60 males in the migraine group. Results demonstrated a significant association of the C381T variant with migraine in the first population studied for both allelic ($\chi^2=6.64$, $P=0.005$) and genotypic frequencies ($\chi^2=6.59$,

$P=0.02$). When analysed by subtype of migraine, the C381T variant was observed to be significantly associated with MO for both allelic ($\chi^2=8.36$, $P=0.002$) and genotypic ($\chi^2=8.16$, $P=0.007$) frequency distribution (Table 3).

In order to replicate the findings of a significant link between the C381T variant and migraine, a second independent population of 260 migraineurs and 229 controls were successfully genotyped for C381T. There were 221 females and 39 males in the migraine group. The frequency distribution of the alleles and genotypes obtained for C381T in the second independent populations are summarised in Table 1. Although initial observations demonstrated significant association with MO for C381T, this was not replicated in the second population studied. There was no significant association between either allelic ($\chi^2=0.46$, $P=0.245$) or genotypic ($\chi^2=3.03$, $P=0.115$) frequencies for C381T variant and migraine in the second population (Table 1). However a trend towards significance was observed in the MO group for both allelic ($\chi^2=2.43$, $P=0.06$) and genotypic ($\chi^2=4.42$, $P=0.051$) frequencies for the C381T variant (Table 3).

G684A

258 migraineurs and 247 controls were successfully genotyped for G684A in the first population studied. There were 189 females and 69 males in the migraine group. A significant association of the G684A alleles with the migraine group was observed in the first population studied ($\chi^2=7.21$, $P=0.015$). Interestingly, when we divided the migraine population into the two subtypes, MA and MO, we found the G684A variant to be significantly associated with MA for both allelic ($\chi^2=10.43$, $P=0.001$) and genotypic ($\chi^2=9.73$, $P=0.004$) frequencies (Table 4). The distribution and allele and

genotype frequencies for the migraine and the control groups are summarised in Table 2.

G684A was then genotyped in a second independent population consisting of 254 migraineurs and 229 matched controls. There 214 females and 40 males in the migraine group. As observed in the first population studied the G684A alleles were observed to be significantly associated with migraine ($\chi^2=7.18$, $P= 0.004$). Similarly, significant association was demonstrated between genotypic frequencies for the G684A variant and migraine ($\chi^2=7.21$, $P= 0.015$)(Table 2). Further analyses on the migraine subtypes demonstrated the G684A variant to be significantly associated with the MA group again for both allelic ($\chi^2=7.97$, $P= 0.003$) and genotypic frequencies ($\chi^2=8.03$, $P= 0.005$) in the second population (Table 4). Taken together, the positive results from both populations strongly suggest the G684A variant to be associated with migraine, specifically suffers of MA as compared to MO sufferers. The distribution and allele and genotype frequencies are summarised in Table 2.

Discussion

CADASIL, a common hereditary form of stroke, is caused by missense mutations leading to the loss or gain of a cysteine residue [5]. Migraine is one of the clinical hallmarks of CADASIL. The Notch 3 gene on C19p13 is implicated in CADASIL and also is also postulated to be linked to migraine. Studies undertaken by Hutchinson et al (1995) and Verin et al (1995) have suggested that hemiplegic migraine may be an allelic disorder of CADASIL [18, 19]. Oberstein et al reported that an increase in white matter hyperintensities was observed in brain MRI among 6 individuals who carried mutations in the Notch 3 gene and experienced a higher frequency of MA when compared to controls [20]. Population based magnetic resonance imaging

(MRI) CAMERA studies have reported that migraineurs had a significantly higher prevalence of cerebellar border zone infarct like lesions and white matter hyperintense lesions. Notably, these observations were more prevalent in MA sufferers than MO sufferers [21, 22]. Taken together these findings suggest a common genetic background underlying migraine, particularly MA and CADASIL [23].

Exons 3 and 4 of the Notch 3 gene are hotspots for most of the CADASIL causative mutations [6]. Apart from the well known mutations identified in the Notch 3 gene, some functional and non functional SNPs that do not cause CADASIL have also been identified. Although the role these SNPs play remains unclear, there are a growing number of studies investigating their possible association in different diseases. A recent study investigating the interaction of eight different variants in five candidate genes in unrelated stroke patients and non stroke controls found the interaction between one of the known Notch 3 SNPs, C381T with two other SNPs (MTHFR C677T and ALOX5AP T2354A) to be a significant contributor to thrombotic stroke [24]. The present study analysed the role of C381T and G684A in exons 3 and 4 respectively of the Notch 3 gene to determine their role in migraine pathogenesis. These results demonstrated significant association of the G684A SNP with migraine.

Previously, a genetic association study by Schwaag et al [25] that included 97 migraineurs, also reported significant association of genotypes, as well as alleles, of G684A with migraine. A weak association was also found between C381T and migraine. Interestingly, both SNPs were associated with MO rather than MA. Although it is unknown why an association was found between the SNPs examined and MO when MA is one of the clinical features that characterises CADASIL, these results support a correlation between the Notch 3 gene, CADASIL and migraine [25].

However not all mutations in Notch 3 may be implicated in migraine. An association study on an Italian population by Boroni et al investigated whether the functional Notch 3 polymorphism T6746C, which is not causative for CADASIL, might be linked to migraine. They examined 156 migraineurs, and reported no significant association between the functional polymorphism and migraine [26].

We have previously analysed the Notch 3 gene by sequencing all exons with known CADASIL mutations in a family previously linked to C19p13 and failed to find common migraine to be affected by any of the known CADASIL mutations analysed [23]. The present study tested two independent matched case-control populations for the C381T and G684A variants in exons 3 and 4 of the Notch 3 gene. Results of the study initially revealed an association between the allelic ($\chi^2=6.64$, $P= 0.005$) and genotypic ($\chi^2=6.59$, $P= 0.02$) frequency distribution of C381T variant and migraine, in particular the MO ($P< 0.05$) subgroup in the first population. However, this association was not replicated in the follow-up independent study group, although a trend towards significance was observed in MO samples for both allelic ($\chi^2=2.43$, $P= 0.06$) and genotypic ($\chi^2=4.42$, $P=0.051$) frequencies. The weak association between C381T and MO reported by Schwaag et al taken together with the inconsistent results observed in the current study, warrant further research into this variant and its role in migraine.

Interestingly, in the current study a significant association between the G684A variant and migraine in the first ($P=0.015$) and a second ($P= 0.004$) independent population was shown. The percentage of participants with the A allele was significantly increased in both migraine (14% and 13.2%, respectively) and the MA subtype (17.2% and 13.7%, respectively) when compared to controls (9.5% and 7.9%,

respectively) in both populations studied. Further, frequency data of both populations indicated a linear trend in the proportion of MA sufferers as the number of the A allele increased. This suggests a gene dosage effect, however with G684A being a synonymous polymorphism; it is not clear how, if at all, it may affect the activity of the Notch 3 gene.

G684A is a synonymous polymorphism, that does not alter coding sequences and therefore is expected to be non functional or silent. However recent studies have provided evidence that these seemingly non functional polymorphisms could still affect transcription, splicing, mRNA transport or translation, any of which could influence the resultant phenotype [7, 27]. Kimchi-Sarfaty et al. investigated the phenotypic effects of a synonymous polymorphism in a common haplotype in the multidrug resistance 1 (MDR1) gene and identified the variant responsible for the resultant altered effectiveness of inhibitors of the P-glycoprotein. The authors found the conformation-sensitive antibody binding and trypsin susceptibility of the variant to be different from that of the wild-type protein, suggesting that the need for the tRNAs to translate rare codons slows down translation and causes the protein to fold into a different conformation [7]. Whether this is the case for G684A is yet to be determined.

The results of this study indicate a correlation between the Notch 3 gene and migraine. Migraine is a multifactorial disorder and as such, the genetic aetiology is likely to encompass a variety of modest-effect susceptibility genes. It is possible that variants in the Notch 3 gene may be playing a role in susceptibility that influences both the severity and subtype of migraine. It is possible that the G684A variant is acting in combination with other functional variants in Notch 3 or an adjacent gene

yet to be identified to influence MA. Given the association between MA and G684A in the current study, further investigation into this SNP and the Notch 3 gene is warranted to further elucidate the exact role of this gene in migraine.

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