Cephalalgia

Candidate-gene association study searching for genetic factors involved in migraine chronification

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Abstract

Introduction: Chronic migraine (CM) is at the severe end of the clinical migraine spectrum, but its genetic background is unknown. Our study searched for evidence that genetic factors are involved in the chronification process.

Methods: We initially selected 144 single-nucleotide polymorphisms (SNPs) from 48 candidate genes, which we tested for association in two stages: The first stage encompassed 262 CM patients, the second investigated 226 patients with high-frequency migraine (HFM). Subsequently, SNPs with p values < 0.05 were forwarded to the replication stage containing 531 patients with CM or HFM.

Results: Eight SNPs were significantly associated with CM and HFM in the two-stage phase. None survived replication in the third stage.

Discussion: We present the first comprehensive genetic association study for migraine chronification. There were no significant findings. Future studies may benefit from larger, genome-wide data sets or should use other genetic approaches to identify genetic factors involved in migraine chronification.

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Introduction

According to the International Classification of Headache Disorders, third edition beta (ICHD-III beta) classification criteria, a clinical diagnosis of chronic migraine (CM) is made when a patient has 15 or more days with headache per month of which at least eight days have features of migraine headache (or that are described by the patient as migraine and are relieved by migraine-specific medication) (1). CM is at the severe end of the clinical migraine spectrum with a substantially decreased quality of life and increased disability, and is strongly associated with depression, medication overuse, and/or cutaneous allodynia (2,3). The reported prevalence of CM is estimated to be around 0.5% to 2.0% (4,5). Recently, several genome-wide association studies (GWAS) have identified a dozen susceptibility gene variants and loci for episodic migraine (6–8), but until now no studies have focused on identifying genetic risk factors for CM.

It is debatable whether it is meaningful to make a strict distinction between episodic migraine and CM because headache frequency in patients varies from month to month and the thresholds of 15 headache days and eight migraine days, while practical, are arbitrary (9). Genetic studies in rarer complex disease subtypes, such as CM, are particularly challenging as collecting sufficiently large numbers of well-characterized patients is difficult. Therefore, we decided to also include a group of patients with high-frequency migraine (HFM) who suffer from headache 10 to 14 days per month, with half or more days meeting the criteria for migraine.

The aim of this study was to obtain evidence for association of variants in genes, acting in pathways possibly implicated in the chronification process of migraine as well as relevant secondary hits from GWAS, with chronification of migraine. In total, 144 single-nucleotide polymorphisms (SNPs) selected based on literature and previous studies were tested in a three-stage design.

Methods

Participants and design of the genetic association study

Participants included in our study were patients diagnosed with either CM or HFM, and healthy control individuals. Migraine diagnoses were based on ICHD criteria. A three-stage genetic association study was performed (Figure 1). The discovery stage included 262 CM patients and 2879 control individuals (all patients came from the CHROMIG study (Spain), or the Leiden University Migraine Neuro Analysis (LUMINA) study (the Netherlands)). In this stage, all 144 SNPs (in 48 genes) were tested. The selected markers fulfilled one or more of the following criteria: i) SNPs had been positively associated with migraine and not replicated in other migraine candidate-gene association studies; ii) the corresponding genes had already been implicated in mechanisms relevant to the chronification of migraine; or iii) SNPs were identified as secondary findings in previous migraine GWAS. In the second stage, all SNPs of the first stage that showed a p value < 0.05 were tested in a further 226 patients diagnosed with HFM vs. the same 2879 controls (patients again came from Spain or the Netherlands). In the third stage, SNPs with p values < 0.05 in the first two stages were tested for replication in 531 patients with CM or HFM (all patients came from the CHROMIG study (Spain), the LUMINA study (the Netherlands), or the Nord-Trøndelag Health Study (HUNT) (Norway)). In this stage, 2491 different control individuals from the three countries were tested.

Gene and SNP selection

We designed a candidate-gene association study focusing on genes that are likely associated with migraine or migraine comorbidities and may act as risk factors for migraine progression. To date, many association studies have been performed to identify genetic factors that confer susceptibility to common migraine (10,11). We selected genetic variants that had been studied in Caucasian populations, especially those that were studied only once. According to these criteria, a total number of 42 SNPs in 26 genes were selected. These genes were related to: i) ion metabolism transport (calcium channel, voltage-dependent, beta 2 subunit (*CACNB2*) and potassium voltage-gated channel, Shab-related subfamily, member 2 (*KCNB2*) (12), syntaxin 1A (*STX1A*) (13), endothelin 1 (*EDN1*), endothelin receptor type A (*EDNRA*) and endothelin receptor type B (*EDNRB*)) (14–16); ii) dopamine (*DBH*) (17) and serotonin metabolism (*HTR2B*) (18); iii) hormonal metabolism (*ESR1*) (19–23); iv) vascular disease (interleukin (*IL*)-9, potassium channel, subfamily K, member 17 (*KCNK17*), low-density lipoprotein receptor-related protein 1 (*LRP1*), matrix metallopeptidase 12 (macrophage elastase) (*MMP12*), methylenetetrahydrofolate dehydrogenase (nicotinamide adenine dinucleotide phosphate (NADP) + dependent) 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase (*MTHFD1*), nitric oxide synthase 3 (endothelial cell) (*NOS3*), sodium channel, non-voltage-gated 1 alpha subunit (*SCNN1A*), transforming growth factor, beta 1 (*TGFB1*) and tumor necrosis factor (*TNF*)) (24–26); v) autonomous nervous system dysfunction (GNAS complex locus (*GNAS1*)
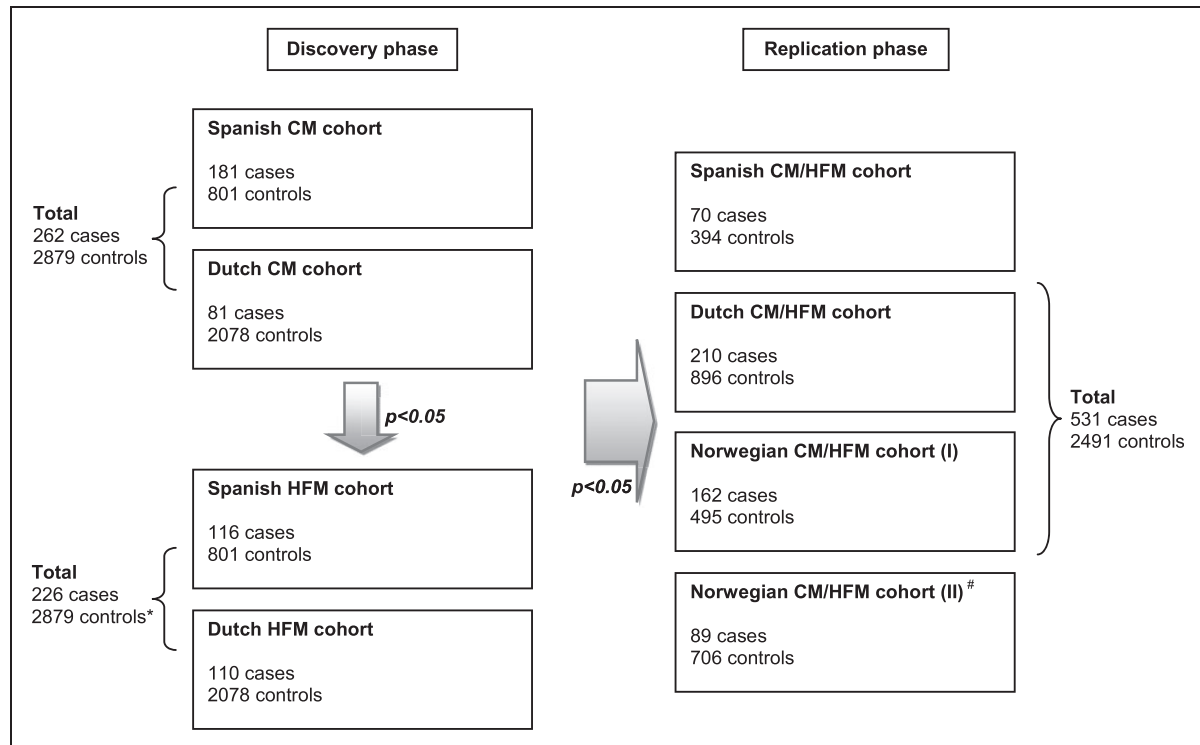
Figure 1. Three-stage gene association cohort study design.
CM: chronic migraine; HFM: high-frequency migraine; GWAS: genome-wide association study.
*In stages 1 and 2, the same group of 2879 controls was used. #The second Norwegian cohort was previously genotyped as part of a GWAS (5). This cohort was used for in silico replication in the present study. The other cohorts in stage 3 were genotyped using a TaqMan genotyping assay.

and potassium inwardly-rectifying channel, subfamily J, member 1 (*KCNJ1*)) (27,28); vi) stress-response (brain-derived neurotrophic factor (*BDNF*)) (29); vii) and psychiatric disorder-related genes, specially associated with anxiety and depression syndrome (catechol-O-methyltransferase (*COMT*), cryptochrome circadian clock 1 (*CRY1*), vasoactive intestinal peptide receptor 2 (*VIPR2*), regulator of G-protein signaling 2 (*RGS2*), sodium channel, voltage-gated, type IX, alpha subunit (*SCN9A*) and Wolfram syndrome 1 (wolframin) (*WFS1*)) (30–33). In addition, we selected candidate genes that encode molecules known to play an important role in migraine pathophysiology but that had not been studied before in candidate-gene association studies for migraine. In more detail, 37 TagSNPs were selected from CEU Hapmap data using a tagger pairwise tool with $r^2 > 0.8$ (Haploview tool) that codes for calcitonin-gene related peptide (*CALCA*) and its CGRP-receptor subunits (calcitonin receptor-like (*CALCRL*) and receptor (G protein-coupled) activity modifying protein 1 (*RAMP1*)). The pituitary adenylatecyclase-activating polypeptide (*PACAP*), a neuroexcitatory peptide released to periaqueductal gray matter during neurogenic inflammation, encoded by the adenylate cyclase activating polypeptide 1 (pituitary) (*ADCYAP1*) gene, and its receptor, encoded by adenylate cyclase activating polypeptide 1 (pituitary) receptor type I (*ADCYAP1R1*), were also investigated with 17 tagSNPs. *ESR1* and *ESR2,* but not G protein-coupled estrogen receptor 1 (*GPR30*) estrogen receptors, have been previously studied in relation to migraine, so we included *GPR30*, which encodes a multi-pass membrane protein that binds estrogen. Fractalkine, a chemokine that has been associated with neuroprotection (chemokine (C-X3-C motif) ligand 1 (*CX3CL1*) gene), and its receptor (*CX3CR1*) were chosen as candidate genes with six tagSNPs (34). We also focused on two molecules that had been previously reported in a microarray study as probable migraine with aura biomarkers, namely alpha-phodrin (*SPTAN1*) and hippocalcin-like protein (*HPCAL1*) (35). The former is a cytoskeletal protein of the spectrins family and the latter is a member of the neuron-specific calcium-binding protein family and is involved in neuronal signaling in the central nervous system. Two tagSNPs in both genes were genotyped. A tagSNP in peripherin (*PRPH*), a cytoskeletal protein localized in neurons of the peripheral nervous system, the expression of which has been

associated with *GPR30*, was also studied (36). Finally, eight tagSNPs in a gene involved in circadian rhythm and metabolism regulation (*CLOCK*) were added to the panel. Overall, 77 non-previously studied gene variants in 12 genes were selected.

Finally, 25 polymorphisms extracted from the list of secondary top hits in the analysis of the first migraine GWAS (6) that was carried out by our International Headache Genetics Consortium (IHGC) were included. The list included 15 intergenic SNPs and 10 variations that were located in gene-coding regions (acyl-CoA synthetase long-chain family member 5 (*ACSL5*), chromosome 4 open reading frame 22 (*C4Orf22*), DCC netrin 1 receptor (*DCC*), insulin-induced gene 2 (*INSIG2*), opioid binding protein/cell adhesion molecule-like (*OPCML*), olfactory receptor, family 9, subfamily Q, member 1 (*OR9Q1*), reelin (*RELN*), SET and MYND domain containing 3 (*SMYD3*), STAM binding protein-like 1 (*STAMBPL1*) and transient receptor potential cation channel, subfamily M, member 8 (*TRPM8*)). In summary, 119 SNPs were genotyped in 38 candidate genes, as well as 25 additional SNPs from GWAS data.

Cohorts

Spanish CM and HFM patients were recruited at the Headache Unit of the Vall d'Hebron University Hospital (Barcelona). Patients with CM were diagnosed by a clinical interview and physical examination by a headache-specializing neurologist, according to the ICHD-III beta classification (1). HFM was diagnosed when patients suffered from headache 10 to 14 days per month, of which half or more days fulfilled the criteria for migraine. Healthy controls were blood donors. Exclusion criteria for this control population were migraine, a positive family history for migraine and any type of severe or recurrent headache in first-degree relatives.

Dutch CM and HFM patients were available from the well-defined, web-based LUMINA population (Leiden University Migraine Neuro Analysis program) (www.lumc.nl/hoofdpijn). Details of this study are described elsewhere (37). Migraine was diagnosed according to the ICHD-III beta criteria (1). CM was diagnosed when patients suffered from migraine and indicated that they experienced severe headache 15 or more days per month. HFM was diagnosed when patients suffered from migraine and indicated that they experienced severe headache 10–14 days per month. Control samples for the discovery phase were part of the population-based Rotterdam Study (38). Control samples for the replication phase were collected via a Dutch blood bank.

The Norwegian patients were recruited from the population-based HUNT-2 (1995–1997) and HUNT-3 (2006–2008) studies, in which all inhabitants (age ≥ 20 years) of the Nord-Trøndelag county of Norway were invited to participate (39,40). Migraine was diagnosed based on a modified version of the most recent ICHD criteria at the time of each study, and this questionnaire-based headache classification has been validated by interview diagnoses (39,40). Migraineurs reporting headache seven or more days per month were classified as HFM, and those reporting headache 15 or more days per month were classified as CM. Controls were recruited from the same two studies, and participants fulfilling criteria for migraine were excluded from the control population.

Genotyping

Spanish cohorts. Venous blood samples of individuals who fulfilled inclusion criteria were collected in ethylenediaminetetraacetic acid (EDTA) tubes and conserved at −80°C until DNA extraction. DNA was extracted from blood lymphocytes at the Centre de Regulació Genòmica (CRG, Barcelona, Spain) with the Chemagen® extraction kit (Perkin Elmer, Germany) and at the Departament de Genètica (Facultat de Biologia, Universitat de Barcelona, Barcelona, Spain) by a standard salting-out procedure (41). Quantity and quality of DNA samples were controlled spectrophotometrically with NanoDrop ND1000 (NanoDrop, Wilmington, DE, USA). Genotyping of SNPs in the discovery sample set was performed with VeraCode® GoldenGate® technology (Illumina, CRG, Barcelona, Spain). For the replication phase, an additional 70 CM and HFM patients and 394 controls were recruited under the same criteria and procedures that were used for the discovery sample. Blood sampling and DNA extraction were performed in the same way. Genotyping was performed with a TaqMan® SNP Genotyping Assay (Applied Biosystems, Foster City, CA, USA) using the 7900HT Sequence Detection System (SDS, Applied Biosystems) in 384-well plates and following the manufacturer's protocol.

Dutch cohorts. Peripheral blood samples were collected in EDTA tubes. Subsequently, DNA was isolated using a standard salting-out method. Genotyping of the samples had been previously performed as part of two GWAS for common migraine (6,7). Genotyping of the replication cohort was performed with a TaqMan® SNP Genotyping Assay (Applied Biosystems, Foster City, CA, USA). A standard polymerase chain reaction (PCR) was carried out using the TaqMan Universal PCR Master Mix. Genotyping clusters were analyzed using the LightCycler LC-480

machine and LightCycler® 480 1.5.0 software, version 1.5.0.39 (Roche Applied Science, Penzberg, Upper Bavaria, Germany) in 384-well plates following the manufacturer's protocol.

Norwegian cohorts. DNA from all Norwegian samples was extracted from blood using two kits: Autopure Kit (Qiagen, Duesseldorf, Germany) and Masterpure Kit (Medinor, Oslo, Norway), both based on a salting-out procedure. Quantity and quality of DNA samples were controlled regularly by monitoring every eighth sample spectrophotometrically with NanoDrop ND1000 and ND8000 (NanoDrop). Genotyping of the replication cohort was performed with a TaqMan® SNP Genotyping Assay (Applied Biosystems). A standard PCR reaction was carried out using the TaqMan Universal PCR Master Mix. Genotyping clusters were analyzed using the LightCycler LC-480 machine and LightCycler® 480 1.5.0 software, version 1.5.0.39 (Roche Applied Science, Penzberg, Germany) in 384-well plates following the manufacturer's protocol. A part of the Norwegian sample for replication had previously been genotyped with the Illumina 670 k platform, as part of a GWAS of migraine (8), and was used for in silico replication for the current study. We used the Illuminus calling algorithm, with the following filters for genotyped SNPs: minimum call rate per SNP and per individual (0.97), Hardy-Weinberg equilibrium (HWE) p value higher than 1.00 E–06 and minor allele frequency (MAF) >0.01. For those SNPs that were not directly genotyped, imputation was performed with Impute v.2.1.2 in a standardized pipeline, using HapMap2 data from a CEU population as the reference panel.

Statistical analyses

We performed power calculations for all three steps of our design, assuming an additive model, an effect allele frequency of 0.20 and effect sizes ranging from 1.2 to 1.4. We added the outcome of these power calculations to the online Supplementary Material. Statistical analyses were performed using PLINK v1.07 (42) and SNPTEST v2.2.0 (43). GTOOL v0.7.5 was used to combine different cohorts. First, the entire panel of SNPs was tested for the HWE for each cohort considering $p < 0.05$ as the threshold. Then, both allele and genotype frequencies were compared between cases and controls, considering additive, genotypic (co-dominance), dominant and recessive models. Subsequently, a meta-analysis was performed using GWAMA v2.1. For all analyses, the threshold for statistical significance was defined as a p value below 0.05. Approval was obtained from local medical ethics committees and written informed consent was obtained from all participants.

Results

For this study, 144 SNPs in genes already implicated in migraine or that had surfaced as interesting secondary hits in GWAS (see online Supplementary Material) were used in a three-stage association design (Figure 2). In the first stage, SNPs were tested in 262 patients with CM vs. 2879 control individuals. Nominal significant associations (p value < 0.05) were obtained for 30 SNPs (see also online Supplemental Material, Table 2). These 30 SNPs were taken forward to the second stage with 226 patients with HFM and the same control data set, where eight SNPs showed a nominally significant association; rs5742912 (in *SCNN1A*), rs3792603 (in *CLOCK*), rs2956 (in *CALCA*), rs858745 (in *CALCRL*), rs302680 (in *RAMP1*), rs2267730 and rs2299908 (in *ADCYAP1R1*), and rs217693, which is an intergenic SNP (see Table 1 for detailed information on these 8 SNPs). These eight SNPs were taken forward to the replication stage and were genotyped in three replication cohorts from Spain (70 patients with CM or HFM and 394 controls), the Netherlands (210 patients with CM or HFM and 896 controls), and Norway (162 patients with CM or HFM and 495 controls). The availability of GWA data allowed testing of seven of the eight SNPs in 89 additional Norwegian patients with CM or HFM and 706 controls.
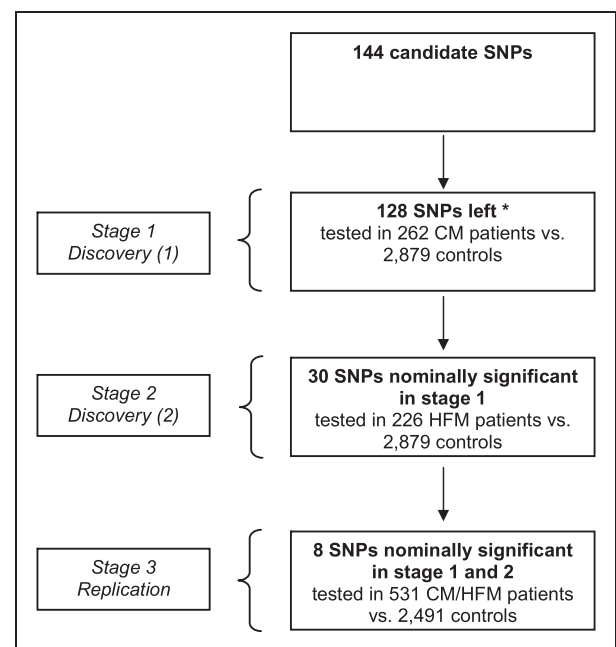

Figure 2. SNP selection study flow.
SNP: single-nucleotide polymorphism; CM: chronic migraine; HFM: high-frequency migraine; HWE: Hardy-Weinberg equilibrium. *16 SNPs excluded due to genotyping failure, low HWE, or low rate of successful genotypes. Nominally significant: $p < 0.05$.

Subsequently, a combined meta-analysis of the association results from these replication cohorts with 531 patients with CM or HFM and 2491 controls was performed but showed no statistically significant associations.

Discussion

Here we present the first comprehensive genetic association study in CM and HFM migraineurs testing 144 SNPs from 48 genes in 1019 patients with CM or HFM, without significant associations. Patient numbers in each cohort were relatively small, largely because of the rarity of CM, which makes it difficult to collect large enough patient samples. As CM is a complex genetic disorder, it is likely that multiple genetic variants, each with relatively small effect, contribute to disease susceptibility, suggesting that large numbers of patients and controls are needed to reach sufficient power to detect a genetic association. We attempted to address this challenge in two ways. First, to increase overall numbers, we decided to include not only CM patients, but also HFM individuals, as we consider the cutoff values for a diagnosis of CM to be rather arbitrary and instead favor the idea that migraine chronification has a broader spectrum with respect to the number of headache days (9). Second, by selecting only candidate genes (and SNPs therein) we reduced the massive correction for multiple testing that is needed for unbiased GWA approaches. Considering the negative results, our approach may still have had insufficient statistical power or we may have selected SNPs irrelevant to migraine chronification. As even large international collaborations, such as the IHGC, have difficulties collecting large enough cohorts of well-characterized patients with CM and HFM, we feel that studies like ours will probably remain underpowered in the immediate future. We are working with the IHGC on unifying the criteria to select patients so that future studies will be able to count on larger and better phenotyped cohorts.

CM is severely disabling and difficult to manage, as affected patients experience substantially more-frequent headaches, comorbid pain and affective disorders, and fewer pain-free intervals, than do those with episodic migraine (4). Furthermore, the relationship of CM with cutaneous allodynia has been investigated, indicating that cutaneous allodynia is a clear risk factor for migraine chronification (3). Different models have been proposed to explain this relationship. Further investigations into the basic mechanisms of cutaneous allodynia, and its relationship with migraine chronification, could lead to new potential genes that should be studied in future designs.

Clinical and genetic studies have shown that migraine is a multifactorial disorder with complex interaction between multiple predisposing genetic and modulating non-genetic factors. GWAS have identified 13 gene variants pointing, among others, at pathways involved in glutamatergic neurotransmission and synaptic function (8). Translating results from GWAS to pathophysiological mechanisms, however, remains one of the biggest challenges in molecular biology as gene effect sizes are small and their interactions are complex.

Table 1. Results of the replication phase (stage 3).

General SNP information					Stage 1	Stage 2	Stage 3				
					Discovery phase (CM)	Discovery phase (HFM)	Replication phase (CM/HFM)				
SNP	Chr. (position)	Gene	Ref. allele	Alt. allele	*p* value	*p* value	*p* value	OR (95% CI)	Cohorts (N)	Samples (N)	Effects
rs5742912	12 (6458350)	SCNN1A	G	A	0.035	0.003	0.31	0.872 (0.672–1.132)	3	2108	–/?/–/–
rs3792603	4 (56302058)	CLOCK	G	A	0.006	0.004	0.69	1.024 (0.911–1.151)	4	2943	–/+/–/+
rs217693	14 (62402801)	Intergenic	G	A	0.045	0.019	0.10	0.911 (0.816–1.018)	4	2930	–/–/–/–
rs2956	11 (14989121)	CALCA	T	A	<0.001	0.029	0.90	0.993 (0.895–1.102)	4	2967	–/+/+/+
rs858745	2 (188216807)	CALCRL	T	C	<0.001	0.004	0.12	1.129 (0.971–1.313)	4	2925	+/+/+/+
rs302680	2 (238791396)	RAMP1	G	A	0.040	0.010	0.89	1.009 (0.887–1.147)	4	2937	+/–/–/+
rs2267730	7 (31122630)	ADCYAP1R1	T	C	0.043	0.036	0.36	0.956 (0.868–1.052)	4	2845	–/+/+/–
rs2299908	7 (31138096)	ADCYAP1R1	G	A	0.026	0.046	0.34	0.946 (0.843–1.061)	4	2871	–/–/+/–

SNP: single-nucleotide polymorphism; CM: chronic migraine; HFM: high-frequency migraine; Ref. allele: Reference allele; Alt. allele: alternative allele; OR: odds ratio; CI: confidence interval. Genomic position in basepairs according to Build 37. Stage 1 included 262 CM cases and 2879 controls, stage 2 included 226 HFM cases and 2879 controls (same controls as stage 1), and stage 3 included 531 CM/HFM cases and 2491 controls. Effects: Direction of individual effects in the four replication cohorts, depicted in the following order: Spanish CHROMIG (TaqMan)/Norwegian Nord-Trøndelag Health Study (HUNT) (in silico)/Norwegian HUNT (TaqMan)/Dutch Leiden University *Migraine* Neuro *Analysis* (LUMINA) (TaqMan). (+) risk addition; (–) risk reduction; (?) not calculated (because of missing data).

We suggest that for future designs it is relevant to consider the outcome of withdrawal from medication, as the vast majority of CM patients are (over)using acute headache medication. In this study, we did not have sufficient data to include this aspect in the analysis. However, future studies would benefit from subdividing CM individuals into patients responsive to withdrawal therapy and returning to episodic migraine after withdrawal of their medication, and patients in whom such withdrawal has no or less effect on attack frequency. Lastly, although the problem of statistical power will remain problematic in association studies for CM and HFM, we would like to put forward that perhaps other genetic approaches are more fruitful in detecting genes and pathways involved in CM, such as gene-expression studies, epigenetic studies or the analysis of rare variants.

Clinical implications

- No genetic variants were detected, indicating that genetic testing to identify patients at increased risk for migraine chronification is not warranted so far.
- Medication overuse and the success of reverting to episodic migraine after withdrawal of acute medication in individual patients should be considered when studying chronic migraine.
- Future studies may benefit from larger data sets or should use other genetic approaches to identify genetic factors involved in migraine chronification.

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