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Original Article

A Pilot Study of FOXP3 Gene (rs2232365, rs3761547, and rs3761548) Mutations in Migraine Patients

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Abstract

Background: The role of immune dysfunction in migraine pathogenesis is currently debated. Migraines are common among family members, yet no significant gene polymorphisms have been identified to date. However, our earlier clinical study findings led us to hypothesize that migraine initiation could be influenced by genes that regulate immune function, specifically Forkhead box P3 (FOXP3). To test this hypothesis, we conducted a pilot study targeting FOXP3 Single Nucleotide Polymorphisms (SNPs), commonly associated with other autoimmune conditions in patients with migraine.

Materials and Methods: The minimum sample size required for this study was determined by considering the mean difference in Regulatory T cells (Tregs) reduction reported in previous studies. Twenty participants (10 patients and 10 controls) were recruited for this study. Familial clinical history was collected for pedigree analysis and the samples were tested for the targeted SNPs.

Results: The prevalence of the AG genotype for rs2232365 was 50% among patients and 20% among controls (OR: 4.0, CI: 0.54-29.09, p=0.17). Regarding rs3761547, the AG genotype was observed in 60% of patients and 10% of controls (OR: 13.5, CI: 1.19-152.2, p=0.03), whereas the AC genotype was found in 70% of patients and only 10% of controls (OR: 21.0, CI: 1.77-248.1, p=0.01).

Conclusion: Our preliminary analysis suggested a possible association between FOXP3 variance and migraine predisposition, particularly with the AG and AC genotypes at the rs3761547 and rs3761548 SNPs, respectively. These findings highlight the importance of conducting a more extensive FOXP3 mutation study in a larger cohort of migraine patients.

Keywords: Migraine; FOXP3 Gene; Immune Dysregulation; Regulatory T Cells; Autoimmune Disorders; Genetic Mutation

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Introduction

Although the exact cause of migraine remains elusive, it is widely believed that a combination of genetic, environmental, and lifestyle factors plays a significant role in migraine development (1). Migraine is a highly prevalent neurological disorder affecting approximately 12% of the global population, with a disproportionately higher incidence in women. Numerous studies have indicated a strong familial predisposition to migraine, with up to 80% of individuals experiencing migraine having a family history of the condition (2). In the southern regions of India, approximately 25.2% of the population experiences migraine symptoms annually (3,4). To date, approximately 40 genetic mutations, including mutations in MTHFR, TRPM8, and CACNA1A, have been linked to migraine (5). These genes are involved in critical biological processes such as blood vessel regulation, neurotransmitter release, and pain signal processing in the brain (6,7). Despite significant progress in identifying genetic associations with migraine, no consistent correlation has been established between specific gene mutations and migraine (8). This lack of consistency may be attributed to the investigation of target genes that may not directly cause migraine.

Recent studies have highlighted the involvement of immune cells in migraine pathogenesis (9); however, the role of genes controlling immune cell function in this context has been largely overlooked. One such gene of interest is Forkhead box-P3 (FOXP3), which plays a crucial role in the function and development of regulatory T cells (Tregs) that are essential for immune system control (10). FOXP3, located on the X chromosome (Xq11.23), serves as a transcription factor crucial for Treg development and function, thus playing a vital role in immune system regulation. Several clinical studies have suggested that mutations in FOXP3, particularly in the promoter region, may disrupt Treg cell regulation and lead to autoimmune disorders such as immune dysregulation, polyendocrinopathy, and enteropathy X-linked (IPEX) syndrome, multiple sclerosis (MS), asthma, allergic rhinitis (AR), and Hashimoto's thyroiditis (HT). An earlier study by Faraji and colleagues (11) revealed a connection between the rs3761548 polymorphism in FOXP3 and migraine in an Iranian population. Based on these findings, we recently hypothesized that key genes are involved in migraine pathophysiology (12). With this as our starting point, our study aimed to examine three SNPs - rs2232365, rs3761547, and rs3761548 - that have been strongly correlated with FOXP3 dysregulation and may be implicated in other autoimmune disorders. Consequently, in this preliminary study, we analyzed these SNPs within the South Indian population to gain insight into the genetic variations associated with migraine in this specific ethnic group.

Materials and Methods

Ethical Approval and Data Collection

The study was approved by the Human Institutional Ethics Committee of Sri Ramachandra Institute of Higher Education and Research and registered with the Clinical Trials Registry India-Indian Council for Medical Research (CTRI-ICMR) under Registration Number: CTRI/2022/08/044663. Volunteers were recruited based on Proforma (**Supplementary File 1**) feedback, and data were collected with their consent in the presence of a physician. The study adhered to the National Ethical Guidelines for Biomedical and Health Research Involving Human Participants, ICMR, 2017. Collected data included demographic details (name, age, sex), duration and frequency of migraine attacks, previous migraine medications, family history, lifestyle, and any other associated diseases.

Sample Size Calculation

The sample size for the study was determined based on the mean and standard deviation observed in our previous study on the reduction in Treg cells in patients with migraine (13). The sample size for the study was calculated using a power of 5% and 95% confidence interval (14,15). Twenty participants were deemed necessary to achieve the desired statistical significance and reliability; 20 participants were deemed necessary for this study. The participants were divided into two groups: 10 individuals in the "Cases" group and 10 individuals in the "Controls" group.

Inclusion and Exclusion Criteria

Patients with migraine were selected based on headache frequency, incidence, presence of aura, symptoms such as nausea or vomiting, and famil-

ial prevalence. The control group comprised individuals with no history of migraine, headaches, or related complications and no family history of migraine or autoimmune disorders. Pedigree charts for both groups were obtained to assess the familial migraine prevalence and ensure accuracy.

Blood Sample Collection and DNA Isolation

After obtaining informed consent, blood samples from patients and controls were collected under the supervision of a physician at the Department of Neurology, Sri Ramachandra Hospital. DNA was isolated using the Qiagen spin column method, and its purity was evaluated using the nanodrop method. The integrity and base-pair fragments of the purified DNA were assessed using gel electrophoresis.

Genotyping

Selected SNPs were genotyped via Polymerase Chain Reaction (PCR) with primers and required fragment sizes were identified. The study examined three SNPs: rs3761548 (-3279A/C), rs2232365 (-924A/G), and rs3761547 (-3499A/G) **(Table 1)**. PCR was conducted in a 20µl volume with the following cycling parameters: initial denaturation at 95^{o}C for 3 min, 32 cycles of 95^{o}C for 30 s, annealing at 55^{o}C for 30 s, extension at 72^{o}C for 45 s, and a final extension at 72^{o}C for 5 min. DNA was extracted from the gel using column purification, and DNA sequencing by applying sanger sequencing method and PCR was performed with a 10µL reaction mixture. Sequencing data were analyzed using SnackVAR Software.

Statistical Analysis

Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) version 2.5. The chi-square test was used to compare the cases and controls. Quantitative data were presented as means and standard deviations, whereas qualitative data were presented as frequencies. The odds ratio and 95% confidence interval (CI) were calculated, and a p-value of less than 0.05 was considered statistically significant.

Result

Descriptive Statistics

All cases and controls were randomly selected from the Sri Ramachandra Hospital, Sri Ramachandra Medical College, India. The median age of the migraine patients was 24 years, with an Interquartile Range (IQR) of 21–26 years. In contrast, healthy volunteers serving as controls had a slightly lower median age of 23 years, and their IQR ranged from 22 to 24 years. No significant differences in age or sex were observed between the two groups.

Association of FOXP3 Polymorphisms between Migraine Patients and Healthy Controls

The prevalence of alleles and genotypes, along with their observed variations, for the three specific SNPs in both migraine patients and healthy controls are presented in **Table 2**, and shown in **Figure 1.**

Allelic and Genotypic distribution of rs2232365

The frequencies of the A and G alleles, as well as the AG genotype, were assessed in both the case and control groups. The A allele prevalence (OR: 0.06, 95% CI: 0.002-1.36) and G allele prevalence (OR: 1.50, 95% CI: 0.25-8.81) were similar between groups. Similarly, the AG genotype distribution was consistent across cases and controls (OR: 4.0, 95% CI: 0.54-29.09). Notably, the difference in the AG genotype distribution between patients and controls was not statistically significant (p=0.17).

Allelic and Genotypic distribution of rs3761547

The frequencies of the A and G alleles and the AG genotype were calculated for both the case and control groups. The A allele (OR: 0.18, 95% CI: 0.02-1.24) and G alleles (OR: 0.44, 95% CI: 0.03-5.88) showed similar prevalence in both cohorts. The AG genotype distribution was also similar (OR: 13.5; 95% CI: 1.19-152.2). Notably, 60% of patients had the AG genotype compared to 10% of controls, a statistically significant difference (p=0.03).

Allelic and Genotypic distribution of rs3761548

Allele frequencies for A and C, as well as for the AC genotype, were calculated for both the case and control groups. The A allele (OR: 0.04, 95% CI: 0.004-0.56) and C alleles (OR: 1.0, 95% CI: 0.11-8.9) were uniformly distributed in both groups. The AC genotype frequency was similarly

distributed (OR: 21.0, 95% CI: 1.77-248.1). Notably, 70% of the patients had the AC genotype compared to 10% of the controls. The distribution of AC genotypes between cases and controls showed significant variation (p=0.01).

Discussion

Table 1. Position, primer's sequence, and fragment size of selected SNPs

Position	Primer sequence	PCR product
rs2232365 (-924A/G)	Forward: 5′-GTGGAGGGCTTTCAAGGT-3′ Reverse: 5′-TCCTCGGAGTCCTATTTTGCC-3′	420bp
rs3761547 (-3499A/G)	Forward: 5′-CTCTGGCTCTCCATGCATGT-3′ Reverse: 5′-TGCAGGGCTTCAAGTTGACAG-3′	390bp
rs3761548 (-3279A/C)	Forward: 5′-CTTAACCAGACAGCGTAGAAGG-3′ Reverse: 5′-CATCATCACCACGCTCTG-3′	200bp

Table 2. Distribution of the FOXP3 alleles and genotypes in migraine patients and healthy controls

FOXP3 polymorphism	Case (N = 10) N (%)	Control (N = 10) N (%)	OR (95% CI)	P-value
rs2232365 A/G				
AG	5 (50)	2 (20)	4.0 (0.54-29.09)	0.17
A	0	4 (40)	0.06 (0.002-1.36)	0.07
G	5 (50)	4 (40)	1.50 (0.25-8.81)	0.6
rs3761547 A/G				
AG	6 (60)	1 (10)	13.5 (1.19-152.2)	0.03
A	3 (30)	7 (70)	0.18 (0.02-1.24)	0.08
G	1 (10)	2 (20)	0.44 (0.03-5.88)	0.53
rs3761548 A/C				
AC	7 (70)	1 (10)	21.0 (1.77-248.1)	0.01
A	1 (10)	7 (70)	0.04 (0.004-0.56)	0.01
C	2 (20)	2 (20)	1.0 (0.11-8.9)	1

In our previous clinical study, we observed a significant difference in Treg levels among migraine patients in the South Indian population (13). Additionally, independent clinical studies from various ethnic backgrounds have reported substantial differences in Treg levels between patients with migraine and healthy volunteers (16,17). To ensure validity and statistical significance, we used data from the same South Indian population to calculate the sample size. Thus, the sample size was determined by assessing the mean difference in Treg levels between patients with migraine and healthy volunteers, as indicated by our previous studies within the South Indian population. As mentioned earlier, in the context of sample size determination for preliminary studies (14), we calculated a total requirement of 20 samples (10 for each case and control group) with a power of 5% and a confidence interval of 95%.

Volunteers were recruited in accordance with the International Classification of Headache Disorders (ICHD) - Fourth Edition Guidelines for Migraine Studies (18). The study design and population size were carefully considered, and samples were selected based on stringent criteria. Inclusion criteria were a diagnosis of high-frequency and chronic migraine for at least five years. Both the volunteers and their families were screened to exclude any comorbid conditions, particularly autoimmune-related clinical conditions.

Based on the feedback in Proforma **(Supplementary File 2)**, we created pedigree charts for

selected migraine patients and healthy volunteers. Notably, 80% of patients with migraine reported a family history of the condition, indicating a strong genetic predisposition **(Supplementary File 3).** This association prompts questions about the lack of correlation in earlier genetic mutation studies and suggests the need for further investigation of specific genetic regions, especially in immunology.

The study revealed a higher occurrence of

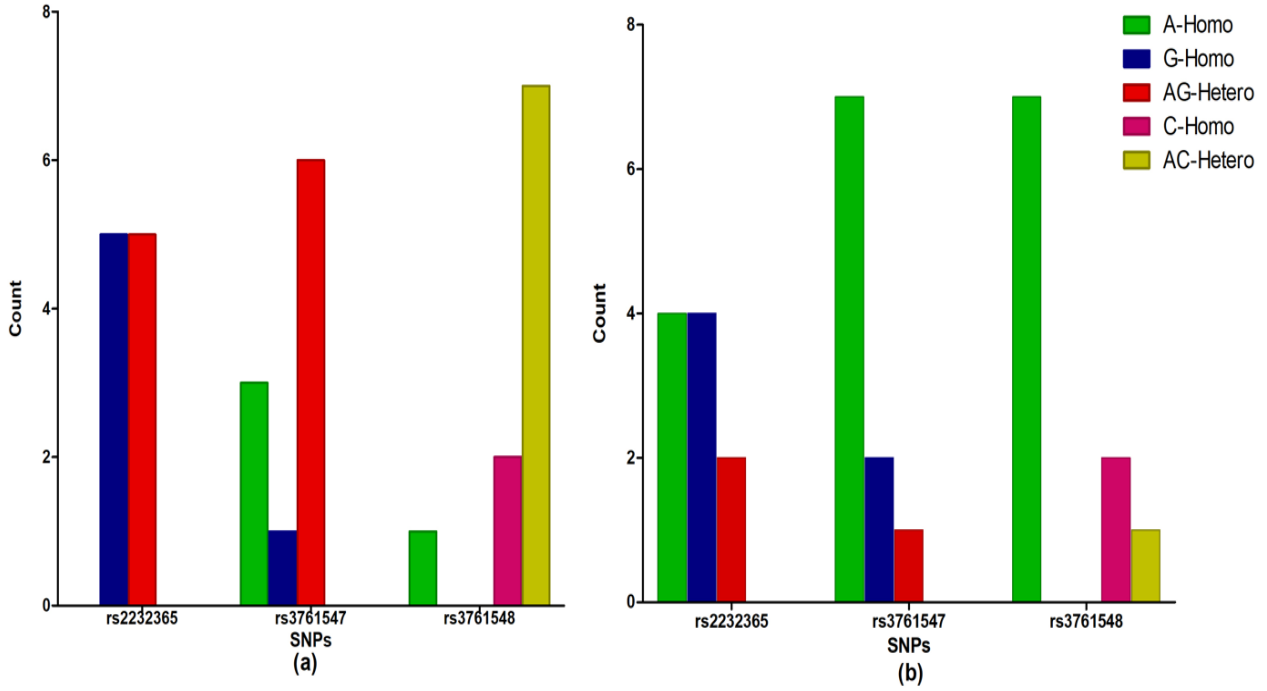

Figure 1. This figure presents the distribution of Single Nucleotide Polymorphisms (SNPs) across different genotypes.
(a) and **(b)** represent two different datasets or groups being compared.
The x-axis indicates the SNP identifiers (rs2232365, rs3761547, rs3761548).
The y-axis represents the count of individuals for each genotype.
This comparison highlights the frequency variations of different SNP genotypes within the analyzed groups.

heterozygous genotypes for two specific FOXP3 SNPs, AG at rs3761547 and AC at rs3761548, among migraine patients compared to healthy controls. Conversely, rs2232365 SNP showed no significant correlation, indicating a lower frequency of heterozygous genotypes related to migraine susceptibility within FOXP3. Pedigree analysis combined with genetic data revealed that many patients with migraine-affected relatives had either the AG, AC, or both genotypes (p=0.001), suggesting a possible link with compound heterozygous mutations in the South Indian population.

Extensive documentation supports the crucial role of FOXP3 in the development and function of Tregs, which are vital for maintaining immune balance and preventing excessive immune responses (19). Numerous studies have explored potential SNPs within FOXP3 that may be associated with specific autoimmune disorders (20). Therefore, our findings from this preliminary study provide a rationale for conducting a large cohort study on FOXP3 mutations in the migraine population.

As expected, variations in all three SNPs were observed in the migraine population compared with healthy controls. However, despite these observations in a small sample, we were unable to establish convincing evidence for a significant correlation between genetic variation and migraine prevalence. Furthermore, there is currently a lack of clear pathophysiological evidence demonstrating the cause of FOXP3 polymorphisms in migraine patients. Thus, our study suggests that future research focusing on FOXP3 could potentially contribute to advancements in the understanding of migraine pathophysiology.

This study has some limitations. The main limitation of this study was the restricted size of the population. At the same time, it is essential to acknowledge that the effectiveness of Hardy-Wein-

berg Equilibrium (HWE) analysis is compromised by the small sample size, as the study primarily relied on a trial-and-error approach. It is important to note that the samples were collected in the South Indian region.

Therefore, this study elucidates our hypothesis regarding the involvement of immune-regulatory gene mutations in patients with migraine (12). However, it is important to conduct extensive genetic analyses, such as genome-wide association studies (GWAS), across diverse ethnic backgrounds to gain a more comprehensive understanding of the genetic underpinnings of migraine progression. This approach may be essential to explicate the relationship between immune regulation and migraine.

Conclusion

The results of our study suggest a possible association between variance in FOXP3, specifically the AG and AC genotypes at the rs3761547 and rs3761548 SNPs, respectively, and a predisposition to migraine. However, further research involving larger population samples is required to obtain a more comprehensive understanding of how FOXP3 gene mutations contribute to migraine susceptibility, and to identify additional genetic factors involved in this complex disorder.

Conflict of interest

The authors declare no conflict of interest.

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Authorship contribution statement

Subalakshmi Sugumar: Investigation, sample analysis, genetic counseling, data collection, pedigree chart formulation, statistical analysis, writing – original draft, review, and editing. Murugesan Arumugam: Conceptualization, study design, investigation, statistical analysis, interpretation, writing – original draft, review, and editing. Jerad A Suresh: Interpretation, writing – original draft, writing – review, and editing. Deepa Avadhani: Study volunteer recruitment, genetic counseling, interpretation, writing – original draft, writing – review, and editing. Philo Hazeena: Study volunteer recruitment, genetic counseling, interpretation, writing – original draft, writing – review, and editing.

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