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The Polymorphism of Regulatory Region of NOS3 Gene (rs2070744, Genotype CC) Protect Patients from Chronic Migraine

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Authors' contributions

This work was carried out in collaboration between all authors. Authors EK, KS, JA, AS, AR and GT designed the study. Authors EK, NK and KS managed the literature searches. Authors KS, JA and AS supplied the patients' samples. Authors NK, ZK and EN carried out all laboratories work and performed the statistical analysis. Authors EK and NK wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: There is evidence that endothelial nitric oxide synthase has a role in migraine pathophysiology. In our research, the role of SNP rs2070744 (c.-813C>T) in promoter region of NOS3 gene in the episodic and chronic forms of migraine is considered.

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Place and Duration of Study: University Headache Clinic between June 2012 and November 2014 and Department of Genetics, Faculty of Biology of Lomonosov Moscow State University between October 2013 and March 2016.
Methodology: The study included 138 patients with migraine (44 with chronic and 96 with episodic migraine). The control group included 348 unexamined subjects. Genotypes were determined using real-time PCR with allelic discrimination test. Statistical processing was performed using Fisher test and Pearson's chi-squared test.
Results: Our study evaluated the link of CC genotype of rs2070744 with migraine (Fisher's p=0.026) and episodic migraine (Fisher's p=0.022).
Conclusion: Genotype CC of SNP rs2070744 in the regulatory region of *NOS3* gene is more specific for episodic migraine and may prevents the chronification of migraine.

Keywords: NOS3; rs2070744; migraine; chronic migraine; episodic migraine.

1. INTRODUCTION

Migraine affects 14.7% of people worldwide causes more years lost to disability than any other neurologic disorder [1]. A nationwide population-based survey of headache disorders in Russia revealed a considerably higher 1-year prevalence of migraine (20.3%) and an unusually high prevalence (10.5%) of headache occurring on ≥15 days/month [2].

Genetic predisposition to migraine is well-known and proven by epidemiological genetic studies [3]. The study of the genome in families of patients with hemiplegic migraine revealed five types of migraine with monogenic inheritance (familial hemiplegic migraine I, II, III, IV, V). However, these forms are extremely rare and are not involved in the development of common migraine with aura or without aura [4].

Vascular hypothesis, specifically endothelial dysfunction, is currently discussed as a shared mechanism for migraine and neurovascular disorders [5,6].

Nitric oxide (NO) is an important mediator of vasodilation in the intra- and extracranial vessels. This small molecule easily penetrates the membranes of vascular smooth muscle cell, and activates soluble guanylate cyclase and cGMP (cyclic guanosine monophosphate) synthesis, leading to vasodilation. NO also promotes the release of CALCA (calcitonin related polypeptide alpha, also known as CGRP – calcitonin gene-related peptide), which is a powerful vasodilator. The SNPs (single nucleotide polymorphism) in gene coding endothelial NO synthase (*NOS3*) may influence the pathogenesis of migraine. Some investigators suggest the role of the polymorphisms in *NOS3* gene in the pathogenesis of migraine or in the formation of clinical characteristics of migraine (aura) [7-9]. Other investigators have noted the absence of such associations for single SNP [10-14]. Haplotype association analysis of multiple SNPs in *NOS3* gene shows positive results [11,13]. These studies examined the association of the polymorphisms in *NOS3* gene with migraine, migraine with or without aura. But search of association of polymorphism in the *NOS3* gene with chronic and episodic migraine was not carried out earlier.

The objective of our study was to determine the effect of a single nucleotide polymorphism rs2070744 (c.-813C>T), which is located in the promoter region of *NOS3* gene, in the episodic and chronic forms of migraine.

2. PATIENTS AND METHODS

2.1 Patients

The retrospective study included 138 patients with migraine living in Moscow and Moscow region, with the average age of 41.6 ± 12.5 years old (44 with chronic and 94 with episodic migraine). All those patients primarily applied to specialized University Headache Clinic (Moscow) for headache in 2012-2015 years.

The inclusion criteria were:

- Migraine (chronic migraine according to the International Classification of Headaches III (ICHD-III), 2013) [15];
- The age of 18–69 years.

The exclusion criteria were:

- Probable migraine, according to the ICHD-III;

- Familial or sporadic hemiplegic migraine, according to the ICHD-III;
- Other significant medical conditions except migraine;
- Actual chronic migraine risk factor (current evidence of depression based on a Beck Depression Inventory (BDI) total score > 19, sleep disorders, stressful life events, caffeine overuse);
- Any prophylactic treatment for migraine for at least 3 months prior to the study.

Table 1. Baseline characteristics of patients with migraine and controls

Migraine (all), n	138 (100%)
Episodic migraine, n	94 (68.2%)
Chronic migraine, n	44 (31.8%)
Control, n	348

The control group included 348 unexamined subjects (population control), inclusion criteria - place of residence in Moscow and Moscow region. Patients and controls were age-matched. Migraine was diagnosed by experienced neurologists based on the criteria of the ICHD-III. The patients were questioned regarding the disease duration, the type of migraine, the frequency of migraine attacks (for the last 3 months), drugs used, underwent a clinical neurological examination and blood sampling for genotyping The study was approved by the local ethics committee and all subjects gave informed consent to participate in the study. The study was conducted according to the Declaration of Helsinki Principles.

2.2 Molecular Genetic Testing and Data Analysis

The DNA extraction was performed according to the protocol for a commercial set DNA Magna™ DNA Prep 200 (Isogene Laboratory LLC, Moscow, Russia). The assessment of allelic states of SNP studied was performed using real-time polymerase chain reaction PCR. The primers, fluorescent probes to rs2070744, and PCR conditions were selected by DNA Synthesis, LLC (Moscow, Russia): F: 5'-ACCAGGGCATCAAGCTCTTC-3', R: 5'-GCAGGTCAGCAGAGAGACTAG-3', rs2070744-C: 5'-VIC-AGGGTCAGCCGGCCAG-BHQ1-3', rs2070744-T: 5'-FAM-AGGGTCAGCCAGCCAG-BHQ1-3'. For real-time PCR a commercial kit qPCR mix (Evrogen JSC, Moscow, Russia) was used. PCR was performed on CFX96 thermocycler (BioRad, USA) using an allelic discrimination test. PCR conditions: 95℃ – 3', 40 cycles of 95℃ – 30'', 55℃ – 60'', 72℃ – 30''.

Statistical processing was performed using Fisher test and Pearson's chi-squared test available in software WinPePi.

For reconstruction of signaling pathways the program PathwayStudio 10.0 with ResNet12 data base (Elsevier) was used.

3. RESULTS

The frequencies of genotypes and alleles of rs2070744 substitution in patients with migraine and in the control sample are presented in Table 2.

The significant differences between patients with migraine and control subjects are obtained. For the study SNP the significance of recessive model of inheritance is observed (Table 3).

The significant differences between patients with chronic migraine and control subjects not found (Two-tailed Fisher's P = 0.447). No significant differences between patients with chronic and episodic migraine found (Two-tailed Fisher's P = 0.478). Significant association found with episodic migraine, recessive model of inheritance (Table 4).

Table 2. The frequencies of genotypes of rs2070744 in studied samples.

Genotypes / alleles	Control	Migraine (all)	Chronic migraine	Episodic migraine
CC	0.103	0.178	0,136	0.198
CT	0.894	0.815	0.864	0.792
TT	0.003	0.007	0.000	0.010
C	0.550	0.586	0.568	0.594
T	0.450	0.414	0.432	0.406

Table 3. The associations between rs2070744 and migraine

Genotypes	Migraine n = 146	Control n = 348	Two-tailed Fisher's P	Pearson's chi-square (P)	OR (95% CI)
CC	0.178	0.103	0.026	5.220 (0.022)	1.88 (1.04 – 3.35)
CT+TT	0.822	0.897			0.53 (0.33 – 0.96)

Table 4. The associations between rs2070744 and episodic migraine (EpMigr)

Genotypes	EpMigr n = 96	Control n = 348	Two-tailed Fisher's P	Pearson's chi-square (P)	OR (95% CI)
CC	0.198	0.103	0.022	6.187 (0.013)	2.14 (1.09 – 4.07)
CT+TT	0.802	0.897			0.53 (0.25 – 0.91)

4. DISCUSSION

The polymorphism of *NOS3* gene encoding an enzyme, that synthesizes NO in endothelial cells, considered as a possible genetic risk factor for migraine [16]. Early studies showed that migraine patients more often had a headache after the administration of nitroglycerin than patients without migraine [17]. NO initiates a slow pathological reaction that leads to headaches; it is also due to the fact that migraine patients are hypersensitive to exogenous and endogenous NO [18].

Also, the artificial increase in NO concentration can increase the activity of NO synthase in nociceptive trigeminal neurons [19,20]. At the level of the trigeminal system, neuronal NOS controls production of NO, as a result of the activation of the ejection of CGRP from the trigeminal fibers, this in turn, causes vasodilation. At the level of the blood vessels, CGRP appears to activate endothelial NO-synthase, as a result, this leads to production of NO and relaxes of vascular smooth muscle [21-24].

The *NOS3* gene (nitric oxide synthase 3 (endothelial cell)) is located on chromosome 7q36, it consists of 29 exons (Gene ID: 4846). The NOS3 protein constitutively synthesize nitric oxide by the reaction of converting L-arginine to L-citrulline, resulting in a five-electron transfer, carried out using nicotinamide adenine dinucleotide phosphate (NADP) [25]. The enzyme acts as a homodimer, which may be functionally divided into two main areas: the C-terminal reductase domain and the N-terminal oxygenase domain [22]. Catalytic activity of NOS3 requires the presence of heme and cofactors: tetrahydrobiopterin, flavinadeninedinucleotide, flavinmononucleotide and calmodulin [26]. Production of nitric oxide is regulated by changes in the expression or activity of the enzyme *NOS3*, or by changing in the suitability of activating endogenous cofactors or inhibitory molecules [27,28].

In several studies the associations of *NOS3* gene polymorphisms with migraine have shown. A group of researchers from Italy showed that the genotype AspAsp (rs1799983, p.Glu298Asp) met 3 times more frequently in patients with migraine with aura, compared with migraine without aura, and 2 times more often than the control [29]. The increase of the frequency of the minor allele A (rs3918166) in patients with migraine with aura, compared with migraine without aura, was shown by Investigators from USA [9]. When studying 5 polymorphic sites (rs2070744, rs1799983, 27 bp VNTR in intron 4, rs3918226 and rs743506) in patients from Brazil was found that genotype GA (rs743506) is more common in controls than in patients while haplotype "CCa-Glu-G" and "CCb-Glu-G" (rs2070744 - rs1799983 - 27 bp VNTR - rs3918226 - rs743506) predominant in patients with migraine with aura compared with migraine without aura [13].

But there were no the studies dedicated to the association of the polymorphism of the *NOS3* gene with chronic or episodic migraine. Allele C decreases the promoter activity of *NOS3* gene [30]. What is role of allele C of *NOS3* gene in the pathogenesis of migraine? To evaluate the effect of allele C in chronic or episodic migraine, we have reconstructed, according to the literature, the signaling pathways, describing the activation of transcription of endothelial nitric oxide synthase and its role in the development of major migraine symptoms: vasodilation and pain (Fig. 1).

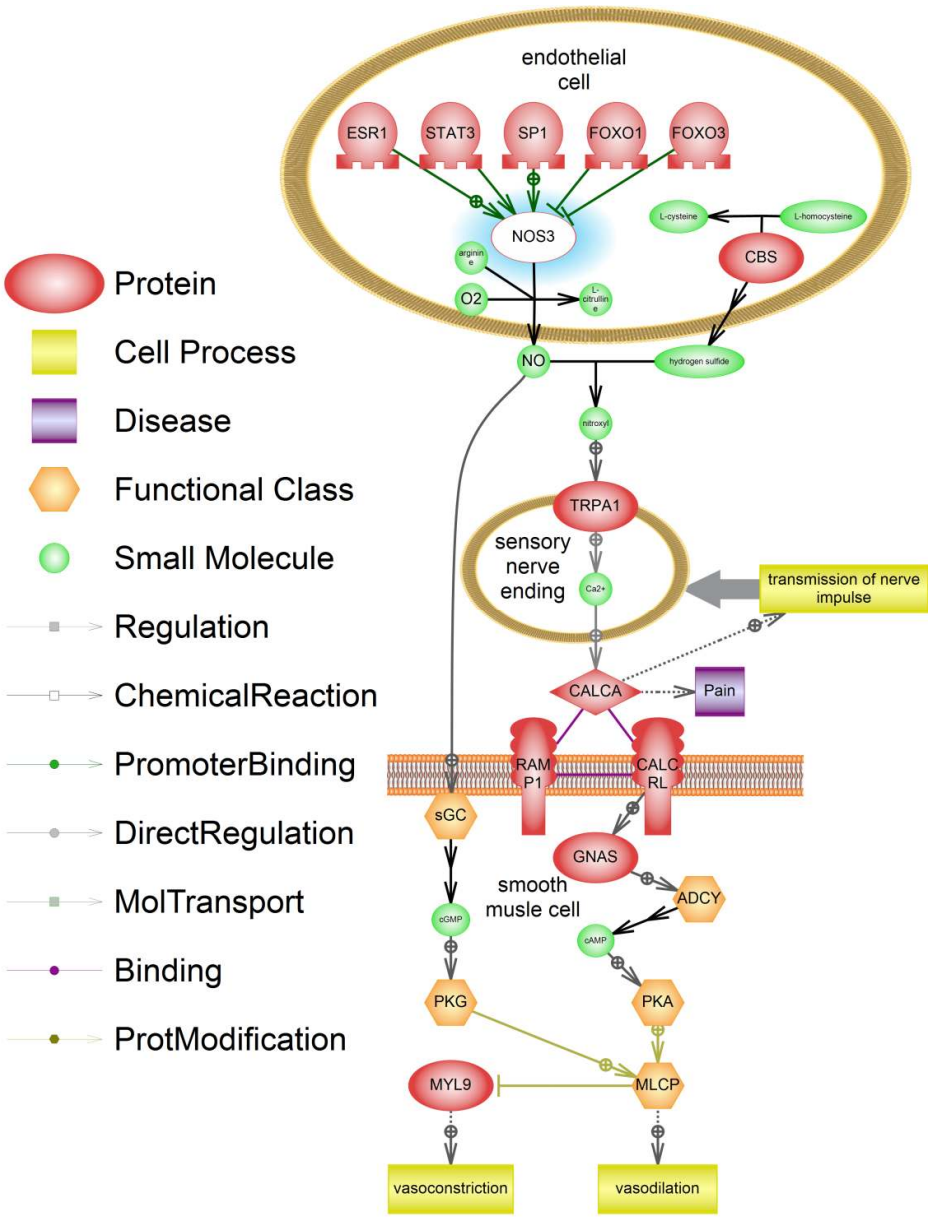

Fig. 1. Production of NO lead to release of CGRP (CALCA) and activation of Pain and Vasodilation. The main activators of *NOS3* transcription activity are ESR1, STAT3, SP1. The FOXO1 and FOXO3 transcription factors are repressors of NOS3 mRNA expression. In the vascular endothelial cells together with the eNOS (NOS3) the cystathionine-beta-synthase (CBS) are active. One of the products of CBS is hydrogen sulfide. This molecule reacting with nitric oxide (NO), which leads to the formation of nitroxyl. Nitroxyl activates TRPA1 (transient receptor potential cation channel, subfamily A, member 1), leading to an influx of calcium into the cell and release of CGRP (CALCA). The action of CGRP on vascular smooth muscle cells leads to vasodilation. Also CGRP activates trigeminal afferent nerve fibers, which leads to more of its release and activation of trigeminal pain signal

Reconstructed signaling pathways show that presence of allele C lead to decrease of production of NO. This in turn leads to stop of CGRP (CALCA) release cycle and stop of activation of pain and vasodilation. Thus, allele C is preventing the disease to become chronic.

5. CONCLUSION

Thus, genotype CC of SNP rs2070744 in the regulatory region of *NOS3* gene decreases the promoter activity of *NOS3* gene. This genotype is more specific for episodic migraine and may prevent the chronification of migraine.

CONSENT

This research used de-identified administrative data obtained from University Headache Clinic; informed consent was not required.

ETHICAL APPROVAL

The study was conducted in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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