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ORIGINAL

Polymorphism in apolipoprotein E among migraineurs and tension-type headache subjects

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Abstract Nitric oxide plays an important role in the pathogenesis of migraine as well as tension-type headache. Studies suggest that the expression of molecules involved in the pathogenesis of headache, i.e., nitric oxide and interleukin, is influenced by apolipoprotein E (APOE) and is gene specific. Hence, we hypothesized that APOE polymorphism may be associated with migraine as well as tension-type headache.The study sample comprised of three groups: migraineurs, tension-type headache subjects as well as a healthy control group. A total of 50 subjects in each group were included after screening for the inclusion and exclusion criteria. None of the subjects was a blood relative of any other subject included in the present study. Their venous blood was drawn and stored at −20°C. Genomic DNA extraction was performed with a commercial kit and simple sequence-specific primer PCR was performed to assess the APOE polymorphism. Data were analyzed with the help of SPSS V11.0 for Windows. χ^2 test and logistic regression analysis were run. The results of the study showed that APOE ε2 gene increases the risk of migraine as compared to the control group and the tension-type headache group (OR = 4.85; 95% CI = 1.92–12.72; $P < 0.001$ and OR = 2.31; 95% CI = 1.08–4.94; $P = 0.01$, respectively). Interestingly, APOE ε4 gene was protective against migraine as well as tension-type headache. This study shows that APOE ε2 gene increases the risk of migraine, while APOE ε4 gene is protective against migraine and tension-type headache. Further research is required to confirm the findings of the present study in a larger sample and to elucidate the role of APOE polymorphism in headache.

Keywords Migraine · Tension-type headache · APOE polymorphism

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Introduction

Nitric oxide (NO) is thought to play a central role in the pathogenesis of migraine as well as tension-type headache. Up-regulation of the endogenous L-arginine/NO pathway and increased NOS expression (perhaps the constitutive form) have been hypothesized in migraineurs during spontaneous migraine attacks [1]. Whether this up-regulation is expressed only at the endothelial level or also occurs at the neural level, especially in the pain transmission pathways, has been a matter of controversy among headache researchers [2]. There is evidence that trigeminal neurons contain nitric oxide and its activity is regulated by nNOS, as suggested by the increase in c-fos immunoreactivity of trigeminal nucleus caudalis following administration of nitrate donor [3]. Furthermore L-NAME, a nitric oxide synthatase inhibitor, can reduce this activity [4].

Nitric oxide has its role not only in the development of migraine, but also in the pathogenesis of tension-type headache [5]. It is suggested that nitroglycerin increases the

pre-existing central sensitization in chronic tension-type subjects, and nitric oxide synthetase inhibitors are helpful in the management of chronic tension-type headache by reducing the central sensitization [5].

Besides nitric oxide synthetase, nitric oxide production is also dependent on apolipoprotein E (APOE) polymorphism and this production is gene specific [6]. Available literature suggests that APOE ε4 increases the uptake of arginine in microglia as compared to APOE ε3 and thus may regulate production of nitric oxide [6]. Relatively increased nitric oxide production has been reported by APOE ε4 containing monocytes as compared to the monocytes harbouring APOE ε3 gene [7]. This production is independent of the expression of iNOS gene and may be largely dependent on the arginine uptake [6]. The increased production of NO is consistent with the increased nitrative/oxidative stress observed with APOE ε4 and may underlie the greater neuronal damage seen after closed head injury and stroke [7]. The production of nitric oxide may be gender dependent, as monocytes from APOE ε4/ε4 male transgenic mice have been shown to produce more nitric oxide as compared to APOE ε3/ε3 mice. However, this difference was not observed in female APOE transgenic mice [8]. Not only the gender, but also the amount and isoform of APOE might influence the development of inflammation. Higher inflammation activity was associated with the APOE ε4 gene as compared to the APOE ε3 gene [9].

Similarly, APOE polymorphism also influences the expression of the cytokines that are commonly involved in migraine and tension-type headache [10, 11]. APOE ε3 gene containing cells have lower expression of cytokines TNF-α and IL-6 as compared to cells expressing APOE ε2 and APOE ε4 genes [11]. Moreover, circulating IL-10 levels are also dependent on the presence of APOE ε4 gene [12].

Based on these evidences, we hypothesized that APOE gene polymorphism should be associated with migraine and TTH. The present study was planned to assess the role of APOE gene polymorphism in migraine as well as TTH patients.

Method

A total of 50 subjects suffering from migraine, 50 subjects with tension-type headache and 50 controls were recruited from the headache clinic of a teaching hospital according to convenient sampling. The study was approved by the institutional ethics committee and informed consent was obtained from all the study subjects. Migraine and TTH were diagnosed according to the International Classification of Headache Disorders (ICHD-2) criteria [13]. The control group consisted of subjects who never had recurrent primary headaches and in whom family history was negative for primary headaches. All the participants belonged to the same ethnic group and had comparable socio-economic status. None of the subjects were genetically related to the other subject included in the study.

All the subjects were screened for the exclusion criteria and if present, then that subject was excluded from the study. Subjects with the following were excluded from the study:.major neurological disorders, e.g., epilepsy, space occupying lesions , neurodegenerative disorders, chronic daily headache (undiagnosed or mixed type), substance use disorders (except tobacco), those taking prophylactic drugs for migraine or tension-type headache for more than 3 weeks, those with co-morbid other primary headache or co-morbid psychiatric disorder, and those consuming antioxidants or multivitamins for more than 1 week.

Patient's history of headache was taken in detail, followed by the clinical examination and, wherever required, appropriate laboratory investigation to rule out secondary headache. Parallel information was also collected from a reliable family member regarding headaches of the sufferer. All the information was recorded on a semi-structured performa.

Genotyping

In a sterile EDTA vacutainer, 3 ml venous blood was collected, at least 3 days after the attack of headache and immediately stored at −20°C. Genomic DNA was extracted with the help of Himedia Pura$^{(R)}$ kit, based on the spin-column technique to provide 4–20 μg of genomic DNA from 200 μL of human blood.

DNA samples were subjected to polymerase chain reaction according to the method described by Pantelidis et al. [14] with slight modifications. This method involved use of four primers, which were combined to form three sets, each set containing two primers to detect the presence of a given allele of the APOE gene. Because this genotyping system is based on the presence or absence of PCR amplification by allele-specific primers, it is imperative to ensure PCR amplification for those reactions that do not produce allele-specific amplicons. For this reason, each APOE-specific primer mix also contained a pair of "control primers" (primers 8 and 9), which amplified two regions of chromosome 6 in the HLA-DR locus, to verify PCR amplification in the absence of haplotype-specific amplification in each PCR reaction.

Amplification was performed in a thermal cycler (Appendorf) using a high stringency PCR protocol with high annealing temperature to ensure specificity of amplification. The conditions were as follows: initial denaturation for 1 min at 96°C, followed by 5 cycles of 20 s at 96°C, 45 s at 70°C and 25 s at 72°C; 21 cycles of

25 s at 96°C, 50 s at 65°C and 30 s at 72°C; 4 cycles of 30 s at 96°C, 60 s at 55°C and 120 s at 72°C.

The PCR products were analyzed by electrophoresis on a 1.5% Tris–borate-EDTA/editium bromide agarose gel with 1 μL of loading dye at 6–8 V/cm. For all PCR reactions (APOE ε2, APOE ε3, and APOE ε4), the presence of a 173-bp band indicated the presence of the specific APOE gene and a band at 785 bp depicted the product of the control gene.

Statistical analysis

Statistical analysis was done with the help of SPSS v 11.0 for Windows. For the categorical variables, χ^2 test was run. Binary logistic regression was applied to calculate the odds. The results were considered to be significant when P value was less than 0.05. The power of the study was calculated using Ca-TS software (http://csg.sph.umich.edu/) and deviation from Hardy–Weinberg equilibrium (HWE) was calculated manually.

Results

Composition of study sample

All the three study groups were identical with respect to average age (27.66 years among migraineurs; TTH group 27.6 years; control group 25.18 years). Both the headache groups had preponderance of females (82% among migraineurs; 100% in TTH group; 25% in control group $\chi^2 = 70.59$, $P < 0.001$).

When illness-related factors were analyzed, it was found that the migraine and tension-type headache groups did not differ with regard to the total duration of illness, duration since the illness became disabling and the duration of the individual headache episodes.

In the migraine subgroup, 76% of the subjects had migraine without aura; 12% had migraine with aura and the rest had headache of both types. In the tension-type headache group, 56% suffered from chronic headache, while in the remaining group, headache was episodic. Family history of primary headache was reported by 26% of migraineurs versus 14% of TTH subjects ($\chi^2 = 2.11$; $P = 0.34$). However, due to recall bias the exact diagnosis was not made. None of the subjects in the control group had a family history of primary headache, as such cases were excluded from the study.

Distribution of genes and genotypes

Distribution of APOE genes in the sample is shown in Table 1. Genotypes APOE ε2/ε2 and APOEε2/ε3 were most frequent among migraineurs, whereas APOE ε3/ε3 was the most prevalent genotype in tension-type headache followed by APOE ε3/ε4 and APOE ε2/ε4. The control group had the highest frequency of APOE ε3/ε4 genotype followed by APOE ε3/ε3. The detailed distribution is depicted in Table 2.

Table 1 APOE allelic frequencies (%) in the study subjects ($N = 50$ in each group)

Group	APOE ε2	APOE ε3	APOE ε4
Migraine	24 (16)	53 (35)	23 (16)
TTH	12 (11)	65 (38)	23 (20)
Controls	6 (6)	58 (40)	36 (32)

The number of subjects is given in parentheses

Table 2 APOE genotype distribution in the study subjects

APOE genotype	Migraine ($N = 50$)	TTH ($N = 50$)	Control ($N = 50$)
APOE ε2/ε2	8 (0.16)	1 (0.02)	–
APOE ε2/ε3	8 (0.16)	2 (0.04)	–
APOE ε2/ε4	–	8 (0.16)	6 (0.12)
APOE ε3/ε3	18 (0.36)	27 (0.54)	18 (0.36)
APOE ε3/ε4	9 (0.18)	9 (0.18)	22 (0.44)
APOE ε4/ε4	7 (0.14)	3 (0.06)	4 (0.08)

The percentage of subjects is given in parentheses

Odds to develop headache

Results show that APOE ε2 gene increases the odds of developing migraine to nearly five times (95% CI = 1.92–12.72; $P < 0.001$) as compared to controls. However, APOE ε4 gene was protective for migraine as well as TTH (OR = 0.53; 95% CI = 0.28–0.98; $P = 0.01$ for both groups). Other genes did not have any effect on the migraine or the tension-type headache.

When tension-type headache group is taken as a reference category, APOE ε2 gene was found to increase the odds of migraine by 2.31 (95% CI = 1.08–4.94; $P = 0.01$). The other gene did not show any effect.

Testing of the power of the study and deviation from Hardy–Weinberg equilibrium

Power analysis shows that considering the prevalence of migraine close to 15%, $\alpha = 0.05$ and other data from Table 1, the power of the present study is 85% in the single-stage additive model.

Testing for Hardy–Weinberg equilibrium showed that all the three groups deviated significantly from the equilibrium (migraine $\chi^2 = 25.52$, $df = 3$, $P < 0.001$; TTH

$\chi^2 = 18.44$, $df = 3$, $P < 0.001$; and control group $\chi^2 = 11.68$, $df = 3$, $P < 0.01$).

Discussion

In short, the present study revealed that APOE ε2 gene increased the odds in favor of migraine. A very interesting and surprising finding of the study was the protective effect offered by APOE ε4 gene. At least one study in the past attempted to find out the effect of APOE polymorphism on the risk of migraine, but they did not find any particular association [15]. However, it must be noted that subjects in that study had mixed headache, i.e., migraine as well as tension-type headache.

It was surprising to find the protective effects of APOE ε4 gene, since it increases the microglial nitric oxide production. Though direct studies addressing the issue of microglial involvement in migraine are not available, we could find two studies that hypothesized that microglial nitric oxide production might be important in migraine [16, 17]. Contrary to this hypothesis, results of the present study suggest that nitric oxide generated during migraine and tension-type headache is probably independent of the microglial nitric oxide production, which is increased by the presence of APOE ε4 in the microglia as reported by Colton et al. [7].

The microglia also comes into light when we see that cytokines also play their role in primary headaches [10]. Pro-inflammatory and anti-inflammatory cytokines show different relationships with the migraine attack. Perini et al. [11] found that TNF-α levels were increased during migraine attacks and they quantitatively correlated with the time elapsed after the headache onset. Similarly, levels of IL-1β also elevate during migraine, but that of other pro-inflammatory cytokines IL-2 and IL-6 remain unchanged. Not only pro-inflammatory, but also the anti-inflammatory cytokine, e.g., IL-10, increases during headache, especially soon after attack; however, the level of IL-4 remains unchanged. Increase in IL-6 in serum was demonstrated in two studies within 1 h of initiation of migraine attack [18, 19]. Furthermore, microglial cytokines are also influenced by APOE polymorphism and, together, these reports deduce that APOE ε3 gene could be protective and APOE ε4 might predispose to migraine [11, 12, 20–22], which was not seen in the present study.

It is known that APOE ε2 lessens the amount of pro-inflammatory cytokine production from microglia, while APOE ε3 and APOE ε4 increase it linearly [20]. APOE ε4 increases the release of IL-1β, PGE2 as well as TNF-α and IL-6 [21, 22]. Hence, APOE ε4 predisposes the person to suffer a sustained inflammatory response both in the central nervous system as well as in the rest of the body [22, 23]. This inflammatory response usually manifests as gliosis in the central nervous system. However, neuro-imaging studies of the brain in subjects suffering from migraine failed to show any such findings [24], and this further supports our results. This also suggests that the increase in pro-inflammatory cytokines (TNF-α and IL-1β) in migraineurs may not be related to the APOE genotypes, and this increase probably represents an epi-phenomenon secondary to the increased CGRP [10]. Another possibility remains that similar to the case of nitric oxide discussed above, these cytokines are perhaps not generated from the microglia during these headaches. As seen in the migraine cases, structural neuroimaging studies of tension-type headache patients also do not show any evidence of gliosis, and neither was increased cytokines reported in their blood during headache [18]. The observed increment of IL-6 in TTH patients was ascribed to the psychological stress rather than to the pathology itself [19].

Akin to this study, previous studies also failed to find the association linkage of polymorphisms of various genes including neuronal NOS [25], inducible NOS [26], dopamine transporter gene [27] and interleukin-6 gene [28] with the migraine or its subtypes, probably owing to the heterogenicity of the migraine.

Like any other study, this study also had some limitations. Migraine and TTH are frequently co-existent with other illnesses, e.g., depression, anxiety, allergies, etc., and also with each other [29]. Hence, it was difficult to identify and recruit subjects with pure migraine as well as isolated tension-type headache. The rigorous exclusion increases the validity of our findings, but at the cost of limiting the sample size. Moreover, as already mentioned, the power of the present study is 85% in the single-stage additive model. Hence, the results of the study are important and valid. Secondly, in this study, we excluded subjects with active coronary artery disease, hypertension and focal neurological deficit, which could be a bias against our finding. However, reports suggest that the prevalence of APOE ε4 gene is otherwise low in India [30, 31]. A recent review suggests that the absolute risk for ischemic vascular events in migraineurs, thought to be predisposed by the presence of APOE ε4 gene, is also low [32–34]. Thirdly, the effect of gene doses was not taken into consideration during the present study owing to the small number of genotypes analyzed in each group. This has shown associations with other disease markers, particularly brain atrophy in a few, but not all, studies [35–37]. Hence, its significance is still debatable, but it is an interesting area and may be explored in future studies. Fourth, the genotype distribution in all groups, especially control group, was deviant from the Hardy–Weinberg equilibrium. Deviation from the Hardy–Weinberg equilibrium is considered to be a measure of genotyping error, stratification or inbreeding. However, a recent study finds that deviation from HWE is not

consistent with genotyping error [38]. In addition, another study suggested that any data set showing deviation from the HWE should not be discarded as it may mask an important and causal polymorphism [39]. Moreover, our data is consistent with the frequencies of APOE genes described in previous studies [40–42]. The deviation in the present study is due to the absence of gene in the control group that has been attributed as being causative. Though we cannot rule out the errors due to small sample size, looking at the power of the study, the present findings appear true.

In conclusion, the present study shows that APOE ε2 gene increases the risk of migraine. This is theoretically unexpected and a new finding. This finding requires confirmation in a population-based study. Also, further research is required to elucidate the pathophysiological role of APOE gene polymorphism and microglia in migraine.

Conflict of interest Sun Pharmaceutical Industries Limited, India provided financial support for procuring the kits and chemicals used in this study.

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