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Phosphodiesterase 4 D gene polymorphism in relation to intracranial and extracranial atherosclerosis in ischemic stroke

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Abstract. In ischemic stroke, extracranial MR angiography (ECMRA) is more frequently abnormal in Caucasians and intracranial (ICMRA) in Asians which may have a genetic basis. We report phosphodiesterase (PDE4D) gene polymorphism and its correlation with MRA findings in patients with ischemic stroke.

Consecutive patients with MRI proven ischemic stroke undergoing MRA were included in this study. The severity of atherosclerotic stenosis on MRA was categorized into moderate 50%–80%, severe 80%–99%, and total occlusion 100% using NASCET criteria. The polymorphism in SNP 32, SNP 83 and SNP 87 of PDE4D gene was analyzed by PCR both in the patients and in 188 controls.

Among the 148 patients, MRA was abnormal in 77% patients; ECMRA in 53.8%, ICMRA in 66% and both were abnormal in 42% patients. The frequency of CC genotype of PDE4D83 was significantly higher in the patients with ischemic stroke compared to controls (OR 3.38, 95% CI 1.61–7.11, $P = 0.001$). The frequency of TT genotype of PDE4D87 was significantly higher ICMRA abnormalities (20%) compared to normal ICMRA (2%). The genotype and allele frequency of PDE4D83 and PDE4D32 were not significantly related to MRA abnormalities. The role of PDE4D87 in atherosclerosis needs confirmation in larger studies.

Keywords: Ischemic stroke, atherosclerosis, gene polymorphism

1. Introduction

Ischemic stroke is the major cause of mortality and morbidity in the world and constitutes 80% of all strokes. It is mostly due to atherosclerosis of extracranial (carotid or vertebrobasilar) or intracranial arteries. The distribution of carotid atherosclerosis has a racial and/or geographical trend, which has been confirmed in autopsy and angiographic studies. In Caucasians, extracranial atherosclerosis accounts for the majority of cases whereas in Asians and blacks, intracranial atherosclerosis is commoner [1–3]. In a study on MR angiography (MRA), extracranial abnormality was present in 56.3% and intracranial in 63.3% highlighting the distribution of atherosclerosis in Indians is midway between Asians and Caucasians [4]. Diabetes is more commonly associated with intracranial atherosclerosis whereas hypertension, hypercholesterolemia, ischemic heart disease and peripheral vascular disease with extra cranial atherosclerosis [5–7].

Phosphodiesterase (PDE4D) genotype has been reported to be associated with ischemic stroke, carotid atherosclerosis and coronary artery disease [8–10]. The carotid atherosclerosis in these studies was evaluated

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on the basis of Doppler sonography in normal population. The action of PDE4D is poorly understood though these enzymes have been reported to involve in systematic degradation of messenger cyclic AMP and has a key role in signal transduction and regulation of physiological responses. In the vascular wall, low cyclic AMP leads to inflammation, proliferation and migration of smooth muscles which may contribute to the atherosclerosis and occurrence of stroke. Hydrolysis of cAMP is mediated partly by PDE4D which may in turn results in atherosclerosis [11]. The distribution of atherosclerosis (intrcranial or extracranial) may have a genetic basis but the role of atherosclerosis related genes have not been studied in this context. MR angiography provides a non invasive and objective method for documenting intracranial and extracranial atherosclerosis. In the present study, we report for the first time the PDE4D gene polymorphism in patients with ischemic stroke and correlate with MR angiographic findings.

2. Subjects and methods

MRI proven ischemic stroke patients attending the neurology service of Sanjay Gandhi Post Graduate Institute of Medical Sciences were included. For the assessment of stroke subtypes, the classification of the trial of ORG 10172 in acute stroke treatment (TOAST) investigators was used [12]. The patients with cardioembolic and arterial dissection related stroke were excluded. The study was duly approved by Institute Ethics Committee. Informed consent was taken from all the patients or their first degree relatives and healthy controls who were included in the study. The demographic and personal history included age, gender, area of residence, ethnicity, dietary habits, life style, smoking (current tobacco smoker or till 1 year back), and alcohol intake ($>$ 100 ml/day). The clinical risk factors of stroke include systemic hypertension (SBP $\geqslant$ 140 mm Hg and DBP $\geqslant$ 90 mm Hg, history of hypertension or on antihypertensive drugs), diabetes (fasting blood sugar $\geqslant$ 106 mg/dl, 2 h post prandial blood sugar $\geqslant$ 200 mg/dl or on oral hypoglycemic drug or insulin therapy), dyslipidemia (abnormal lipid profile, history of statin therapy, coronary artery disease or peripheral vascular disease) [13], history of stroke in the first degree relatives and past history of stroke were noted. The patients with cardio embolic stroke, arterial dissection, aortoarteritis, Moyamoya disease, liver and kidney failure, thyroid disorder and cerebral venous sinus thrombosis were excluded.

2.1. Investigations

Blood counts, hemoglobin, serum chemistry including fasting lipid profile were carried out in all the patients. Radiograph of chest and electrocardiogram were done.

MR angiography: The MR studies were carried out within 1 week of admission on a 1.5 T MR scanner (Signa echospeed plus, Milwaukee, US) with a quadrature head coil for the intracranial MR angiography (MRA) and NV array or spinal array coil for the extracranial MR angiography in the region of the neck. The sequences used were multislab (3 slab for intracranial and 6 slab for extracranial regions) 3D SPGR sequences based on time of flight principle. The repetition time, echo time and FLIP angle were 26 ms, 6.9 ms and 20 degrees respectively for these sequences. MRAs were reviewed by a neuroradiologist (S.K) who was blinded to the clinical data. Stenotic abnormalities were assessed systematically on both extra cranial (EC) and intra cranial (IC) MRA including carotid and vertebral arteries. Any narrowing in vessel diameter of 50% or more was regarded as significant stenosis on both EC and IC MRA. The measurement of stenosis was computed directly on maximum intensity projection view of MRA. The collapse view of the propcher was also taken into measurement in the evaluation of MRA. Collapse view was also taken for evaluation of stenosis. Percent stenosis was computed by measuring the residual lumen diameter comparing it with normal segment above or below (NASCET) [14]. The vessel in which signal void was regained distal to occlusion was considered patent and defined as 80%–99% stenosis. The results were categorized as clinically insignificant if the stenosis on MRA was $<$ 50%, and clinically significant if the atherosclerotic stenosis was $\geqslant$ 50%; moderate 50–80%, severe 80–99%, and total occlusion 100%. Carotid siphon stenosis was not included because of possibility of artifacts arising from anterior clinoid process, sphenoid sinus and intra voxel dephasing.

2.2. Genetic studies

5 ml of venous blood was collected in EDTA tubes. Genomic DNA was extracted using salting out method [15]. Polymerase chain reaction (PCR) was performed in a standardized way using the primers for the specific regions containing these SNPs (32, 83 and 87) as described previously [16]. These SNPs were selected due to its relationship with ischemic stroke [17].

Aliquots of 5 μL PCR product were subjected to restriction endonuclease digestion for genotyping using the restriction enzymes. The polymorphism in SNP 32, SNP 83 and SNP 87 was detected by digesting the PCR product with restriction enzymes MSPA1I (New England Biolabs), TaiI fast digest (New England Biolabs) and SSP1 (New England Biolabs) respectively. The digested bands were visualized on 10% polyacrylamide gel electrophoresed for 5 hours at 200 V. Positive and negative controls were included on each run.

2.3. Statistical analysis

We have included 148 patients and 188 controls belonging to the same geographic and ethnic group. Hardy Weinberg equilibrium was first tested for PDE4D gene in healthy controls and was found to be $p > 0.05$. Clinical variables were compared between patients with and without MRA abnormality using chi square tests for categorical variables. All the statistical tests were two sided, and were considered significant if the two tailed p value was < 0.05. Chi square test was applied for the analysis of genotypic and allelic distribution. Association of each studied polymorphism with ischemic stroke was assessed by the logistic regression using odds ratio (OR) with 95% confidence interval (CI) after adjusting for confounding variables including age and gender when compared with the controls. Bonferroni correction was done for multiple comparisons. 3 patients in PDE4D 83, and 2 patients in PDE4D32 were excluded in logistic regression analysis due to genotyping difficulties leaving a total of 145 and 146 cases respectively.

3. Results

148 patients with ischemic stroke were included whose median age was 61 (21–85) years and 40 (27%) were females. 80 (54.1%) patients were vegetarians, 39 (26.4%) were tobacco smokers, 23 (15.5%) took alcohol and 44 (29.7%) chewed tobacco. 111 (75%) patients were hypertensive, 50 (33.8%) were diabetic, 44 (30%) had high LDL, 15 (10.2%) had hypercholesterolemia, 52 (35.3%) had hypertriglyceridemia and 68 (59.6%) had hyperhomocysteinemia. More than 2 risk factors were present in 74 (50%) patients and family history of stroke in the first degree relatives was present in 35 (23.6%) patients.

Stroke was located in middle cerebral arterial territory in 82 (55.4%), anterior cerebral artery in 1 (0.7%), posterior cerebral in 31 (20.9%), vertebrobasilar arterial territory in 19 (12.8%) and the remaining 15 (10.2%) patients had multiple territory infarctions. Cortical infarction (large vessel disease) was present in 48 (32.4%), subcortical (small vessel) in 42 (28.3%) and remaining had both cortical and subcortical infarctions.

3.1. MRA

MRA was carried out in 148 patients; extracranial MRA was carried out in 130, intracranial in 144 and both in 126 patients. MRA abnormalities were extracranial in 70 (53.8%), intracranial in 95 (66%), both in 53 (42%) and any MRA (ICMRA/ECMRA/both) was abnormal in 114 (77%) patients. The extracranial abnormalities were present in common carotid artery (CCA) in 3, internal carotid artery (ICA) in 42 and vertebral artery in 6, CCA+ICA in 17 and both carotid and vertebral arteries in 2 patients. Total occlusion was present in 13, severe stenosis in 9, moderate stenosis in 18 and mild stenosis in 30 patients. The locations of intracranial MRA abnormality were in internal carotid artery in 43, middle cerebral artery in 51, anterior cerebral artery in 21, posterior cerebral in 31, basilar artery in 14 and vertebral artery in 11 patients. Evaluation of various risk factors such as age, gender, blood pressure, diabetes, ischemic heart disease, family history of stroke, smoking, alcohol, tobacco, cholesterol, triglycerides, HDL, LDL, homocystein and number of risk factors with extracranial, intracranial and combined MRA abnormalities revealed significant association of family history of stroke with intracranial MRA abnormality ($P = 0.007$) and number of risk factors (> 2) with extracranial MRA abnormalities ($P = 0.01$). 29 out of 95 (30.5%) patients with family history of stroke had ICMRA abnormality whereas only 5 out of 49 (10.2%) without family history of stroke had ICMRA abnormality. The remaining variables were not associated with MRA abnormalities (Table 1).

3.2. Genetic association study:

The frequency of CC genotype of PDE4D83 was significantly higher in patients with ischemic stroke compared to controls (OR 3.38, 95% CI 1.61–7.11, $P = 0.001$). The frequency of TT genotype of PDE4D87 (OR 1.28, 95%CI 0.56–2.91, $P = 0.35$) and AA genotype of PDE4D32 (OR 0.70, 95%CI 0.32–1.57, $P = 0.36$) however did not differ between patients and controls. None of the allele frequency of PDE4D in stroke

Table 1
Distribution of clinical variables among ischemic stroke patients with normal MRA and abnormal MRA

Variables	Abnormal MRA $N = 114$	Normal MRA $N = 34$	P^* value
MeanAge ± SD(years)	59.4 ± 12.5	55.8 ± 12.5	0.28
Females	25.4%	32.3%	0.51
Hypertension	75.4%	73.5%	0.82
Diabetes	36.0%	9.3%	0.41
FHO stroke	27.2%	11.8%	0.07
Smoking	29.8%	14.7%	0.12
Tobacco	32.4%	20.6%	0.21
Sedentary	47.4%	39.4%	0.44
BMI	24.5 ± 4.1	24.3 ± 5.1	0.86
↑LDL	31.0%	27.3%	0.83
↑Triglyceride	33.6%	41.2%	0.42
↑Cholesterol	9.0%	14.7%	0.34
↑Homocystein	60.5%	57.1%	0.83
No.of stroke risk factors	85.7%	84.6%	0.63

MRA = magnetic resonance angiography, FHO = family history of, BMI = body mass index, LDL = Low density lipoprotein, $P^* = p$ value calculated between normal MRA and abnormal MRA for each clinical variable.

Table 2
Frequency distribution of *PDE4D* genotypes and alleles and its association with patients and healthy controls

Genotype/allele	Healthy controls	Ischemic stroke	OR*	95% CI#	$P^\wedge$
PDE4D83(T/C)	$N = 188$ (%)	$N = 145$ (%)			
TT[a]	58 (30.9)	27 (18.6)			
CT	92 (48.2)	64 (44.1)			
CC	38 (20.2)	54 (37.2)	3.38	1.61–7.11	0.001
T	208 (55.3)	118 (40.7)			
C	168 (44.7)	172 (59.3)	1.07	0.78–1.48	0.63
PDE4 D87(C/T)	$N = 188$ (%)	$N = 148$ (%)			
CC[b]	72 (38.3)	51 (34.5)			
CT	92 (48.9)	77 (52.0)			
TT	24 (12.8)	20 (13.5)	1.28	0.56–2.91	0.35
C	236 (62.8)	179 (60.5)			
T	140 (37.2)	117 (39.5)	1.08	0.78–1.50	0.61
PDE4D32(G/A)	$N = 188$ (%)	$N = 146$ (%)			
GG[c]	49 (25.4)	53 (36.3)			
GA	94 (51.8)	70 (47.9)			
AA	44 (22.8)	23 (15.8)	0.70	0.32–1.51	0.36
G	211 (56.1)	176 (60.3)			
A	265 (43.9)	116 (39.7)	0.79	0.57–1.08	0.15

*OR = age gender adjusted Odds ratio, # CI = Confidence interval, $P^\wedge = p$ value, [a,b,c] = reference genotype in each marker.

was significantly different from controls (Table 2). Comparing the MRA abnormalities with those with normal MRA revealed significantly higher frequency of TT genotype of PDE4D87 in patients with ICMRA abnormalities. The frequency of TT genotype was 20% in patients with abnormal ICMRA but it was only 2% in those with normal ICMRA. After adjustment for age, gender, smoking, alcohol, tobacco, cholesterol, LDL, family history of stroke, hypertension and diabetes PDE4D87 however was not significantly related to any form of MRA abnormality. The genotype and allele frequency of PDE4D83 and PDE4D32 were also not significantly associated with any form of MRA abnormalities. The details are summarized in Table 3.

4. Discussion

In our study PDE4D83 polymorphism was significantly associated with ischemic stroke compared to controls and the frequency of TT genotype of PDE4D87 was significantly higher in patients with ICMRA abnor-

Table 3

Logistic regression analysis of ischemic stroke with and without MR angiographic abnormalities

	EC/ICMRA ($N = 145$)			ICMRA ($N = 141$)			ECMRA ($N = 127$)		
	Abnormal	Normal	OR,CI,P	Abnormal	Normal	OR,CI,P	Abnormal	Normal	OR,CI,P
PDE4D83 T/C		($N = 145$)			($N = 141$)			($N = 127$)	
TT[b]	19 (16.8)	8 (25)		14 (14.9)	11 (23.4)		10 (14.5)	14 (24.1)	
CT	51 (45.1)	13 (40.6)	0.94,(0.29–3.0)0.92	43 (45.7)	21 (44.7)	0.75(0.26–2.13)0.59	32 (46.4)	24 (41.4)	0.66(0.22–1.98)0.47
CC	43 (38.1)	11 (34.4)	0.77(0.25–2.59)0.68	37 (39.4)	15 (31.9)	0.61(0.22–1.80)0.37	27 (39.1)	20 (34.4)	0.54(0.17–1.66)0.28
T	89 (39.4)	29 (45.3)		71 (37.8)	(43) 45.7		52 (37.7)	52 (44.8)	
C	137 (60.6)	35 (54.7)	0.80(0.45–1.41)0.45	117 (62.2)	51 (54.3)	0.72(0.43–1.19)0.20	86 (62.3)	64 (55.2)	0.76(0.45–1.20)0.28
PDE4 D87 C/T		($N = 148$)			($N = 144$)			($N = 130$)	
CC[b]	38 (33.3)	13 (38.2)		30 (31.6)	20 (40.8)		29 (41.4)	18 (30)	
CT	57 (50)	20 (58.8)	1.15(0.48–2.80)0.75	46 (48.4)	28 (57.1)	0.96(0.42–2.15)0.92	33 (47.1)	33 (55)	0.38(0.10–1.34)0.13
TT	19 (16.7)	1 (2.9)	0.14(0.02–1.20)0.07	19 (20)	1 (2)	0.53(0.01–0.45)0.01	8 (11.4)	9 (15)	0.84(0.26–2.71)0.77
C	133 (58.3)	46 (67.6)		106 (55.8)	68 (69.4)		91 (65)	69 (57.5)	
T	95 (41.7)	22 (32.4)	0.96(0.42–2.15)0.92	84 (44.2)	30 (30.6)	0.57(0.32–0.90)0.03	49 (35)	51 (42.5)	1.37(0.83–2.26)0.21
PDE4D32G/A		($N = 146$)			($N = 142$)			($N = 128$)	
GG[b]	41 (36.6)	12 (35.3)		34 (36.2)	19 (39.6)		26 (38.4)	19 (31.7)	
AG	54 (48.2)	16 (47.1)	0.92(0.35–2.53)0.95	45 (47.9)	22 (45.8)	1.08(0.45–2.59)0.87	33 (48.5)	29 (48.3)	0.73(0.23–2.29)0.59
AA	17 (15.2)	6 (17.6)	1.10(0.33–3.69)0.87	15 (16)	7 (14.6)	0.84(0.27–2.52)0.75	9 (13.2)	12 (20)	0.64(0.21–1.93)0.43
G	136 (60.7)	40 (58.8)		113 (60.1)	60 (62.5)		85 (62.5)	67 (55.8)	
A	88 (39.3)	28 (41.2)	1.1(0.63–1.92)0.73	75 (39.9)	36 (37.5)	0.90(0.54–1.50)0.70	51 (37.5)	53 (44.2)	1.33(0.80–2.20)0.26

[a]OR, odds ratio; CI, confidence interval, $P = P$ value. [b] = reference genotype.

malities compared to those with normal ICMRA. After adjustment for other risk factors of stroke, none of the PDE4D genotype and allele was however significantly associated with ICMRA, ECMRA or any MRA abnormalities. Our results are in agreement with Asian and Icelandic study in which no association of PDE4D83 gene was found with ischemic stroke [16–18]. In a study from USA, PDE4D87 was associated with cardioembolic stroke both in whites and blacks [8]; however, other studies have not found any association of PDE4D87 [19–21]. In the present study, we have excluded cardioembolic stroke and stroke due to arterial dissection. Different studies evaluating PDE4D32 in different populations also revealed no association with ischemic stroke [16,22,23]. In a study from south India, SNP 83 was found to be significantly associated with the intracranial large artery atherosclerosis and small artery occlusion [24]. Staton et al. have found a positive association of SNP 83 with ischemic stroke in an Australian population [25].

In our study, the frequency of TT genotype of PDE4D87 was significantly higher in ICMRA abnormalities compared to those patients with normal ICMRA but its significance was lost after adjustment of confounding variables such as hypertension, diabetes, age, smoking, hyperlipidemia and family history of stroke. This lack of association on PDE4D polymorphism with atherosclerosis after adjustment of other stroke risk factors may be due to multifactorial nature of atherosclerosis. Atherosclerosis and stroke are interlinked and both are influenced by various nonmodifiable and modifiable risk factors. PDE4D83 and PDE4D32 were not significantly associated with increased risk of ICMRA, ECMRA or any MRA abnormalities.

Although many studies on PDE4D gene focused on replicating the Icelandic findings but the results are inconsistent. The lack of association may be due to relatively small sample size which might have missed true association of modest effect. Moreover PDE4D is a very large gene spanning greater than 1.5 Mb with several hundreds of SNPs which may also account for variation in the results. We have selected SNPs 32, 83 and 87 of PDE4D gene based on the report of Gretarsdottir et al. in which these SNPs are shown to have maximum risk of ischemic stroke [17]. The other possible reason for discordant results may be due to difference in allele frequency and haplotypes in different ethnic populations which reflect different patterns of linkage disequilibrium for non causal markers. The role of PDE4D is still unclear even though it has been associated with stroke in certain populations. These genes are postulated to involve in the signaling pathway of cyclic AMP and cyclic GMP. Cyclic GMP regulates the level of these phosphodiesterases which in turn links with NO production by vascular endothelium [26]. This is important because the NO-cGMP pathway is believed to be dysfunctional in stroke [27]. There are reports that the phosphodiesterase 4 inhibitor activity was greatly reduced if cGMP synthesis was inhibited. Phosphodiesterase 4 inhibitors have been reported useful in the prevention of ischemic stroke [26,27]. The analysis of PDE4D83 in our ischemic stroke patients with and without MRA abnormality did not reveal any significant difference. However the frequency of TT genotype of PDE4D87 was significantly higher in patients with intracranial atherosclerosis compared to normal ICMRA. Presence of more than 2 risk factors in our patients was associated with ECMRA abnormality and family history of stroke in first degree relative with ICMRA abnormality.

The results of this study suggest significant association of *PDE4D83* with ischemic stroke compared to controls as well as significantly higher frequency of TT genotype of PDE4D87 in patients with ICMRA abnormality compared to those with normal ICMRA but its significance was lost after adjustment of other stroke risk factors. This may be due to multifactorial nature of stoke.

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