PON1 (Paraoxonase 1) Q192R Gene Polymorphism in Ischemic Stroke among North Indian Population

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Abstract

Background: PON1 is an High Density Lipoprotein (HDL)-associated esterase. Two common polymorphisms in the PON1 gene, Q192R and L55M substitutions, determine the inter-individual variation in PON1 activity. The association of these polymorphisms with the risk of ischemic stroke remains controversial. In the present study, the role of PON1 Q192R gene polymorphism in ischemic stroke was studied in the Indian population. **Design and Methods:** In the present case-control study, the PON1 Q192R gene polymorphism was screened in ischemic stroke patients (n: 63) and age, sex-matched controls (n: 63) using the Polymerase Chain Reaction-Restriction Segment Length Polymorphism (PCR-RFLP) method. **Results:** The mean age of stroke presentation was 58.11 ± 15.4 years. A total of 17.4% cases presented with young stroke (<45 years age) and 9.52% cases were seen to have a recurrent stroke. The distribution of -192Q/R PON1 gene polymorphism was not seen to differ between cases and controls. The traditional stroke risk factors did not have any effect on the PON1 genotype expression. A multivariate logistic regression analysis was done in order to assess an independent association of age, gender, traditional stroke risk factors, and PON1 polymorphism with acute ischemic stroke. However, neither the RR genotype nor the presence of the R allele was associated with an increase in the risk of acute ischemic stroke (OR [RR genotype]-4.76, P value: 0.24, 95% CI: 0.3497–64.8531; OR [R allele]-0.94, P value: 0.90, 95% CI: 0.3516–2.4989). **Conclusion:** PON1 Q192R gene polymorphism is not associated with an increased risk of acute ischemic stroke in the North Indian population. Further studies with a larger sample size are needed before PON1 Q192R gene polymorphism can be considered as a genetic risk factor for ischemic stroke.

Keywords: Genetic polymorphism, ischemic stroke, paraoxonase 1, PON1

INTRODUCTION

The global burden of disease demonstrates a hallmark of 'epidemiological transition,' i.e., a shift in mortality and morbidity from infectious to non-infectious diseases.[1] Stroke is the second leading cause of death and disability worldwide, with a particularly large impact in developing countries like India.[2] Stroke is a heterogeneous multifactorial condition with a wide spectrum of underlying causes that encompass any or a combination of the traditional risk factors such as hypertension, diabetes mellitus, smoking, dyslipidemia, etc., along with evidence of a strong genetic influence.[3] Studies suggest that genetic factors play a crucial role in stroke etiology through their interactions with various environmental components.[4] Therefore, newer risk factors and genetic markers are being extensively looked for in order to detect stroke early and institute primary as well as secondary prevention strategies.

Mutations in the genes encoding MTHFR (methylenetetrahydrofolate reductase), haptoglobin, Apolipoprotein-E (Apo-E), ACE (angiotensin-converting enzyme), AGT (angiotensinogen), eNOS (endothelial nitric oxide synthase), ICAM-1 (intercellular adhesion molecule-1), MIF (macrophage migratory inhibitory factor), PON1 (paraoxonase 1), MCP-1 (monocyte chemoattractant protein-1), factor V, prothrombin, PDE4D (phosphodiesterase 4D), C-reactive protein (CRP), etc., have largely been associated with a high risk of developing ischemic stroke.[5]

PON1 is a calcium-dependent esterase synthesized by the liver and bound exclusively to the HDL particles (apolipoprotein A1 and clusterin) in the plasma. PON1 has been shown to prevent atherosclerosis by inhibiting cellular uptake and oxidative modification of Low-Density Lipoprotein (LDL). It preserves the HDL function and adds to the antioxidant properties of HDL by multiple mechanisms such as hydrolysis of lactones or peroxides in the LDL and HDL, protection of macrophages from oxidation, thus, preventing foam cell formation, maintaining endothelial homeostasis, and acting as a homocysteine-thiolactonase.[6-9] Polymorphisms in the gene

encoding PON1 may result in a decreased enzyme activity and an increased susceptibility to atherosclerosis. Thus, PON1 polymorphism may confer an increased risk of developing ischemic stroke. There are multiple single nucleotide polymorphisms in the coding region of the PON1 gene that are known to affect the PON1 enzyme activity.[10] The Q192R and L55M polymorphisms are the two major polymorphisms in the PON1 gene that have been implicated in various atherosclerotic diseases.[11] However, most of the data on this genetic polymorphism is derived from the Western literature (mostly in the Caucasian and East Asian populations) and conflicting results have been reported in various case-control studies with respect to the association between the R allele of PON1 Q192R gene polymorphism and the risk of developing acute ischemic stroke.[12-14] Furthermore, no such study has been conducted in the North Indian population. Hence, the present study aimed to investigate the association of PON1 Q192R gene polymorphism in acute ischemic stroke among the North Indian population.

METHODS

Study population

The study was a hospital-based case-control study conducted over a period of 1½ years at a tertiary care center in New Delhi and predominantly catering to the North Indian population. The study was approved by the institutional ethics board before commencing enrolment of the participants. Before the recruitment, all the participants or their legally authorized representatives gave written and informed bilingual consent. A total of 126 participants comprising 63 cases of acute ischemic stroke and 63 controls were recruited in the study. The patients aged >18 years, presenting with clinical signs and symptoms of acute ischemic stroke as per the WHO definition of stroke,[15] and radiological evidence of acute ischemic stroke on Computed Tomography(CT)/Magnetic Resonance Imaging(MRI) (CT/MRI) brain presenting to the emergency department were recruited as the cases. Age- and sex-matched unrelated apparently healthy volunteers were recruited as controls. The cases with a history of traumatic brain injury, transient ischemic attacks (TIAs), hemorrhagic stroke, and pre-existing neurodegenerative disorder, and controls with any major illnesses or infections were excluded from the study.

All the participants were subjected to a stepwise evaluation via a structured proforma which included a detailed history and general physical and neurological evaluation. In addition, all the participants had a routine hemogram, biochemistry analysis (including fasting lipid profile), fasting blood sugar analysis, urine routine analysis, and had a 12-lead electrocardiogram (ECG) done.

Sample collection and processing

Seven milliliters (7 mL) of venous blood was collected within 48 h of the symptom onset (among the cases with acute ischemic stroke), in an ethylene diamine-tetra acetic acid (EDTA) vial, from all the participants under sterile conditions. Four milliliters were used for hematological and biochemical analyses while 3 mL of the blood sample was transported within 24 h of collection to the genetic lab in an ice-pack for the extraction of Deoxyribonucleic acid (DNA).

DNA extraction and genotyping

Genomic DNA was extracted from the whole blood by using the modified salting-out method.[16] A polymerase chain reaction followed by the restriction fragment length polymorphism was used to genotype the Q192R polymorphism of the PON1 gene. The PCR was carried out in a 10 µL reaction mixture comprising 0.05 µg genomic DNA, 10 µM each primer and 3 µL PCR master mix containing 2.5 mM each dNTP (dATP, dCTP, dGTP, and dTTP), 1.0 mM MgCl2, 1X Taq buffer, and 0.33 U/µL Taq DNA polymerase. The primer sequences used for the amplification of the gene were 5'-TAT TGT TGC TGT GGG ACC TGA G-3' (forward) and 5'-CCT GAG AAT CTG AGT AAA TCC ACT-3' (reverse). The pre-denaturation step was carried out for 3 min at 94°C, followed by a denaturation step at 93°C for 1 min, annealing at 61°C for 30 s, and polymerization at 72°C for 2 min with a final extension at 72°C for 10 min. Thirty-five PCR amplification cycles were carried out in an Applied Biosystems thermal cycler (Darmstadt, Germany).

The PCR product obtained was 238bp which was digested using the *AlwI* restriction enzyme at 37°C for 16 h. The digested product sizes for the genotypes were QQ = 238bp, QR = 238bp, 175bp, and 63bp, RR = 175bp and 63bp, which were visualized on 3% agarose gel.
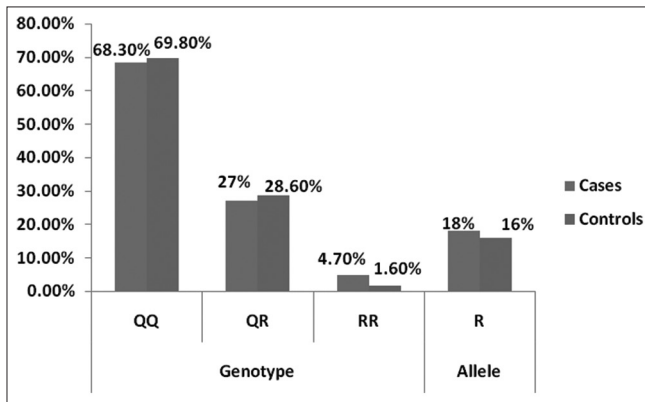
Statistical analysis

Statistical analyses of the data were performed using SPSS version 20.0. The continuous variables were expressed in terms of means ± standard deviations wherever applicable. The categorical data were expressed in terms of percentage of proportions. The Student's *t*-test was used for continuous data while the Chi-square analysis was used for categorical data. Binary regression analysis was done to find out the association of various genotypes with acute ischemic stroke. Furthermore, adjusted analysis was done in order to assess the association between the RR genotype and the R allele independent of traditional stroke risk factors. The genotypic and allele frequencies were calculated using the gene-counting method. Hardy-Weinberg equilibrium (HWE) was tested for the selected genetic marker. A *P* value < 0.05 was considered statistically significant.

RESULTS

The mean age among the cases was 58.11 ± 15.4 years while the mean age among the controls was 57.57 ± 14.5 (*P* value: 0.84). The occurrence of smoking, hypertension, diabetes mellitus, and dyslipidemia was found to be significantly higher among the cases compared to the controls (*P* < 0.05). In the present study, a total of 11 (17.4%) cases also presented with young stroke, i.e., <45 years of age. The demographic and clinical characteristics of the study population are presented in Table 1.

Figure 1 shows the genotypic distribution and allele frequencies of the PON1 Q192R gene polymorphism among cases and controls. PON1 Q192R was seen to follow the Hardy-Weinberg law in both cases and controls. The frequency of the individuals with mutant homozygote (RR) genotype was found to be higher among the cases (4.8%) compared to the controls (1.6%), whereas the frequency of individuals with QQ and QR was slightly lower among the cases (QQ: 68.3%, QR: 27%) compared to the controls (QQ: 69.8%, QR: 28.6%). However, no differences were seen between the cases and controls with respect to the distribution of the PON1 Q192R genotype distribution (*P*-0.88). Minor

allele frequency (*R* allele) was found to be slightly higher among the cases (0.18) compared to the controls (0.16), with no statistical difference.

In order to rule out any confounding effects of age, gender, and various traditional stroke risk factors (hypertension, diabetes, dyslipidemia, smoking, and alcoholism) on PON1 polymorphism, a multivariate regression analysis was performed. We found that PON1 polymorphism (QR/RR genotype) was not significantly associated with any of the above factors [Table 2].

In addition, a multivariate logistic regression analysis was done in order to assess an independent association of age, gender, traditional stroke risk factors, and PON1 polymorphism with acute ischemic stroke. However, neither the RR genotype nor the presence of the R allele was associated with an increase in the risk of acute ischemic stroke (OR [RR genotype]-4.76, *P* value: 0.24, 95% CI: 0.3497–64.8531; OR [R allele]-0.94, *P* value: 0.90, 95% CI: 0.3516–2.4989) [Table 3].

DISCUSSION

The aim of the present study was to evaluate the role of PON1 Q192R polymorphism in the predisposition to acute ischemic stroke (if any). PON1 is an important HDL-associated enzyme which is involved in preventing atherosclerosis via multiple mechanisms. Various case-control studies among different ethnical groups have shown that genetic polymorphisms in the PON1 gene are associated with an increased risk of coronary artery disease (CAD), acute coronary syndrome (ACS), carotid stenosis, and diabetes mellitus with microangiopathy.[17-20] However, the role of this genetic polymorphism in acute ischemic stroke is still uncertain particularly in the Indian population. In fact, the only Genome Wide Association Study (GWAS) study on ischemic stroke in the Indian population did not include any study on PON1 in acute ischemic stroke, reflecting the dearth of literature regarding the role of this polymorphism in ischemic stroke particularly in the Indian population.[21] In our study, we found that the R allele and the RR genotype were not significantly associated with an increased risk of acute ischemic stroke in the North Indian population. This finding is in line with the other studies conducted in various populations across the globe.[12,14,22] However, a recent meta-analysis comprising 29 studies (5,253

Figure 1: Genotypic and minor allele frequency distribution of PON1 Q192R gene polymorphism among ischemic stroke cases and controls

Table 1: Distribution of demographic and clinical characteristics among cases and controls

Variables	Cases N (%)	Controls N (%)	χ^2 P
Age (years) (mean±SD)	58.11±15.4	57.57±14.54	0.84
Male	34 (53.98%)	34 (53.98%)	1
Smoking	13 (20.6%)	5 (7.9%)	0.04*
Alcoholism	11 (17.5%)	4 (6.4%)	0.07
Hypertension	32 (50.8%)	6 (9.5%)	<0.001*
Diabetes mellitus	13 (20.6%)	3 (4.8%)	0.01*
Dyslipidemia	23 (36.5%)	6 (9.5%)	<0.001*
Young stroke	11 (17.4%)	-	NA
Recurrent stroke	6 (9.52%)	-	NA

*-Significant

Table 2: Multivariate logistic regression analysis for the association between the presence of PON1 polymorphism (QR/RR genotypes) and various confounding factors

Variable	Coefficient	Standard error	Odd's ratio (OR)	P	95% Confidence Intervals (CI)
Age	0.0033	0.0145	1.0033	0.8214	0.9752-1.0321
Gender	0.8613	0.4435	2.3662	0.0521	0.9921-5.6439
Hypertension	−0.0402	0.4666	0.9606	0.9313	0.3849-2.3969
Diabetes	0.1213	0.6313	1.1290	0.8476	0.3276-3.8910
Smoking	−0.2891	0.6074	0.7490	0.6341	0.2278-2.4630
Alcoholism	−0.0842	0.6478	0.9193	0.8966	0.2583-3.2720
Dyslipidemia	−0.1705	0.5147	0.8433	0.7405	0.3075-2.3127

Table 3: Multivariate logistic regression analysis for independent predictors of acute ischemic stroke					
Variable	Coefficient	Standard error	Odd's ratio (OR)	*P*	95% Confidence Intervals (CI)
Age	−0.0193	0.0161	0.9809	0.2305	0.9505-1.0123
Gender	−0.3029	0.4926	0.7386	0.5386	0.2813-1.9397
Hypertension	2.0548	0.5470	7.8053	0.0002*	2.6714-22.8050
Diabetes	1.3580	0.8100	3.8884	0.0936	0.7948-19.0230
Smoking	1.4011	0.7125	4.0596	0.0493*	1.0046-16.4050
Alcoholism	0.8890	0.7594	2.4328	0.2417	0.5492-10.7772
Dyslipidemia	1.5984	0.6148	4.9449	0.0093*	1.4821-16.4987
PON1 polymorphism (RR genotype)	1.5607	1.3324	4.7623	0.2414	0.3497-64.8531
PON1 polymorphism (single R allele)	−0.0647	0.5003	0.9373	0.8971	0.3516-2.4989

*-Denotes significant

ischemic stroke cases and 10,398 controls) showed a statistically significant association of Q129R polymorphism via the dominant, recessive, and additive models in Asians.[23] In a recent systematic review of 40 original case-control studies and three meta-analyses, it was noted that ethnicity was a major factor contributing to the distribution of PON1 polymorphism. In this review, there were more significant reports compared to non-significant reports regarding the PON1 Q192R polymorphism among the Asians (particularly the Chinese population) whereas the European Caucasian ethnicity showed a greater number of non-significant reports.[24] Hence, our findings may be due to an overall different genetic pool among the North Indians as compared to the other ethnicities. Moreover, only limited sampling of the population was possible and may have impacted our results. Thus, larger population-based case-control studies may be required in the North Indian population before any conclusions can be drawn.

In the Indian population, there has only been one other study which has attempted to find an association between PON1 polymorphism and acute ischemic stroke. This study by Murugun *et al.*[25] was conducted in the South Indian population and found that the risk of acute ischemic stroke portended by the QR + RR genotype was significantly greater compared to the QQ genotype (OR: 1.67, 95% CI: 1.218–2.281, *P* value: 0.00178). Hence, this study showed that Q192R polymorphism is associated with an increased risk of acute ischemic stroke via the dominant model. This finding is in contrast to the results of our study and may be explained by the different genetic pool among the South Indians compared to the North Indian population recruited in our study.[26] Hence, larger studies across different Indian regions may be required before substantiating this polymorphism as a risk for acute ischemic stroke in the Indian population.

Apart from ethnicity, there are other environmental determinants which influence the activity of the PON1 enzyme and can result in an increased risk of ischemic stroke in a genetically predisposed individual. Studies have shown that dietary vegetable/fruit intake, diabetes, inflammation, and lifestyle factors are major environmental regulators of PON1 gene expression.[27,28] In a family-based case-control study conducted among the Chinese population, it was found that people with

the rs662_A allele were protected from acute ischemic stroke if they had a higher intake of vegetables.[29] Various animal and some human studies have shown that the consumption of an atherogenic diet can result in a decreased expression of PON1 mRNA and decreased PON1 enzyme activity.[30,31] Some studies have shown that the consumption of oleic acid (from olive oil) resulted in an increase in serum PON1 activity among those with 192RR genotype only.[32] Hence, PON1 gene expression (upregulation/repression) may be affected by these environmental factors. Thus, these environmental factors may play a confounding role in the studies aimed at assessing the role of PON1 polymorphism with acute ischemic stroke. This may partly explain the conflicting results noted in the studies conducted across different regions in India as well as across the globe. Hence, there is a need for future studies from India on investigating the relationship between the environmental confounders, serum PON1 enzyme activity, and PON1 gene polymorphisms.

Genes as risk factors for stroke may be more important in the younger subset of the population which lack the traditional confounding risk factors.[33] Hence, targeting young strokes in genetic studies could yield more conclusive results. In our study, there were 11 (17.4%) cases of young strokes (age <45 years). Thus, larger studies with a greater number of young strokes could possibly clarify the role of PON1 polymorphism in acute ischemic stroke in the Indian population.

PON1 Q192R gene polymorphism has been reported to be associated with CAD, which presents with similar conventional and prothrombotic risk factors, among North Indian patients.[34-36] The increased (but not statistically significant) odds of the RR genotype (OR: 4.76) of PON1 Q192R polymorphism among the acute ischemic stroke cases in the present study hints toward the plausible role of this mutation in stroke among the North Indian population. Although polymorphism was not a significant independent risk factor for stroke, it might still predispose to the development of acute ischemic stroke in the presence of various traditional stroke risk factors which can influence PON1 gene expression and PON1 activity. It is also known that serum PON1 enzyme levels can vary widely among individuals (up to 40-folds) regardless of the PON1 genetic polymorphism.[37] Moreover,

our sample size was relatively small. Hence, the role of this mutation in the stroke in the presence of traditional risk factors may not be ignored and larger studies with measurement of PON1 activity in addition to genetic polymorphism are needed in the North Indian population before any definite conclusions can be drawn.

A prior episode of stroke is associated with an increased risk of recurrence and studies have found a higher risk of recurrence in the first year post-stroke.[38,39] PON1 gene has been implicated in the recurrence of stroke among the Chinese population.[40] In the present study, a total of six (9.52%) cases presented with a prior history of stroke but a significant association between PON1 Q192R gene polymorphism and a history of a prior stroke was not observed. Surprisingly, all the cases presenting with a prior history of stroke ($N = 6$) were found to have a QQ genotype. The absence of QR and RR genotype among the cases with a prior history of stroke may be because of chance or it maybe possible that individuals possessing QR/RR genotype were not able to survive for long (after the first stroke) to present with a recurrence. However, larger prospective follow-up studies with a larger number of recurrent ischemic strokes are required to validate this finding.

Our study had limitations such as a small sample size, selection, ascertainment bias (due to the hospital-based nature of the study), lack of data on PON1 enzyme activity, no analysis regarding the association of lipid profile parameters with Q192R polymorphisms, and a small number of young strokes recruited in the study. This study primarily aimed at investigating any association between PON1 (Q192R) polymorphism and acute ischemic stroke among the North Indian population and we did not specifically study any association between PON1 (Q192R) polymorphism and the various mechanisms of stroke. However, some prior studies have found that PON1 (Q192R) polymorphism may confer an increased risk of large artery ischemic strokes among the Chinese Han population due to an increased risk of atherosclerosis.[41] Thus, further large population-based genetic studies aimed at assessing the association between PON1 polymorphism, serum PON1 levels among acute ischemic stroke caused by various mechanisms may be required before considering it as a definite genetic stroke risk factor, particularly among the Indian population.

Conclusions

To the best of our knowledge, this is the first study from North India which has attempted at finding an association between PON1 Q192R polymorphism and acute ischemic stroke. The results of the present study did not find an independent association between PON1 Q192R gene polymorphism and the risk of acute ischemic stroke in the North Indian population. However, its role in predisposing individuals to acute stroke in the presence of other traditional stroke risk factors cannot be ruled out. Further studies with larger sample size and concurrent measurement of serum PON1 enzyme levels among

strokes caused by varied mechanisms are required before the PON1 Q192R gene polymorphism can be considered as an independent genetic risk factor for ischemic stroke in the North Indian population.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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