



PARAOXONASE ACTIVITY AND PON1 (Q/R192) GENE POLYMORPHISM IN ISCHEMIC STROKE PATIENTS

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ABSTRACT

Background: Paraoxonase (PON1) has two polymorphisms Q/R192 and L/M 55 associated with ischemic stroke. The effect of Q/R192 PON1 variants, its activity, and lipid profile in ischemic stroke were studied in South Indians. Design and Methods: The PON1-Q192R polymorphism, serum PON1-ARE activity and lipid profile were analyzed in 308 ischemic stroke patients and in 330 age and sex matched controls. Results: R allele frequency of the codon 192 polymorphism was higher in ischemic stroke cases when compared with controls. Odds ratio of the cases with RR + QR genotype versus QQ genotype was 1.67 ($P < 0.01$). Patients with ischemic stroke cases with QQ, QR, RR genotypes had highly significant LDL ($P < 0.01$) and HDL cholesterol ($P < 0.01$). Conclusion: Our data suggested that there was a significant association between PON1 activity, and PON1-Q192R genotypes in ischemic stroke in South Indians.

Keywords: Paraoxonase, allele, ischemic stroke, polymorphism.

1. INTRODUCTION

Stroke is the third-leading cause of death and leads to severe neurological disability worldwide. It is a heterogeneous disease with multiple risk factors like age, hypertension, transient ischemic attack, diabetes, smoking, atrial fibrillation, etc. The increasing incidence of stroke in Indian patients is possibly due to industrialization, stress, lack of exercise, smoking, hypertension, sedentary habits, and other factors. The underlying pathogenesis may be atherosclerosis. HDL plays a major role in prevention of atherosclerosis by virtue of its association with PON1. The name of the enzyme derives from one of its most commonly used *in vitro* substrate paraoxon. In the 1980s, Mackness and co-workers first suggested the role for serum PON1 in the metabolism of phospholipids based on similarities in the structures of

organophosphates and phospholipids (Mackness, 1989). PON1 also hydrolyzes toxic metabolites of a number of other insecticides such as diazinon, chlorpyrifos, nerve agents (sarin and soman), aromatic esters (phenylacetate, lactones and cyclic carbonates) (Draganov and La Du, 2004). Purified human PON1 was shown to be highly effective in preventing lipid peroxidation of LDL (Mackness *et al.*, 1991; Mackness and Durrington, 1995). HDL may also be oxidized but is prevented by PON1-mediated hydrolysis of lipid peroxides and of cholesteryl linoleate hydroperoxides (Rosenblat *et al.* 2006). PON1 was also found to be substantially hydrolyze hydrogen peroxide (H_2O_2), a major reactive oxygen species produced under oxidative stress during atherogenesis. Thus, PON1 preserves antiatherogenic functions of HDL in reverse

cholesterol transport and protects oxidation of LDL (Aviram *et al.* 1998). Atherosclerosis in the carotid arteries represents a risk factor for ischemic stroke. PON1 might have a protective role against the development of ischemic stroke as its activity and the serum levels of PON1 are variable between individuals (Davies *et al.* 1996; Richter and Furlong, 1999). PON1 exhibits single nucleotide polymorphisms (SNPs) in the coding (192Q/R and 55L/M) region of the gene. (Brophy *et al.*, 2002). In the PON1 gene, a glutamine to arginine substitution at amino acid 192 (Q192R) affects PON1 activity (Li *et al.*, 2003; Humbert *et al.* 1993). The 192Q isoform prevents LDL oxidation by cupric *in vitro* more effectively than does the 192R form (Li *et al.*, 2003; Ayub *et al.* 1999). Thus, the 192R allele might increase the risk of cardio or cerebrovascular disease (Herrmann *et al.* 1996; Ko *et al.* 1998; Heijmans *et al.* 2000; Imai *et al.* 2000; Sen-Banerjee *et al.* 2000; Voetsch *et al.* 2002; Zuliani *et al.* 2002; Wang *et al.* 2003; Lawlor *et al.* 2004). The documented information on the role of PON1 activities in stroke has been very limited. Hence it was proposed to analyze the association of PON1 activities (arylesterase) and its gene polymorphism in stroke cases along with lipid profile. The present study was aimed to determine the effect of Q/R192 PON1 variants and its activity in ischemic stroke in this part of the country. The correlation between PON1 genotypes and lipid profile was also determined in 308 patients with documented ischemic stroke and was compared with 330 age- and sex-matched controls in our population.

2. MATERIALS AND METHODS

2.1 Subjects

Patients included in this study were those admitted in the Medical wards of Rajiv Gandhi Government General Hospital, Chennai. The study groups were between the age group of 25–80 years. A total of 308 ischemic stroke patients and 330 controls were recruited for the study. Patients having other major illnesses, including renal or hepatic diseases and those who had the serum triglyceride levels above 400 mg/dl were excluded from the study. The age sex, height,

weight, smoking habits, and history of previous illness were recorded at the time of admission. Age- and sex-matched healthy volunteers ($n = 330$) who were free of any illness were included as controls. Written consent was obtained from all subjects as per our Institute's human research ethical guidelines. A medical history was obtained using a questionnaire.

2.2 Biochemical Measurements

Blood was drawn after 12 h fast and collected in EDTA-coated tubes and plain tubes without anticoagulant. The EDTA tubes were centrifuged at 3000 rpm for 20 min and the buffy coat is harvested for DNA extraction. Blood collected in plain tubes without anticoagulant is used for PON1-ARE activity and serum lipid profile determinations. Serum was separated immediately and stored at -20°C for the estimation of PON1-ARE activity and IgG antibody. Total cholesterol, triglycerides, and HDL-cholesterol were measured by standard methods. LDL-cholesterol was calculated using Friedewald formula (Friedewald *et al.* 1972). Glucose, urea and creatinine were also measured in these samples. PON1-ARE activity toward phenylacetate was measured at 270 nm by using a spectrophotometer. Typically, 5 μl of serum was added to a total volume of 1 ml containing 10 nM phenylacetate in 20 mM Tris-HCl, pH 8.0 and 1 mM CaCl_2 . The increase in optical density was monitored every 1 min for 4 min of interval. One unit of arylesterase activity was equal to 1 μM of phenylacetate hydrolyzed per ml per minute (Gan *et al.* 1991).

2.3 DNA Extraction

DNA isolation was carried out according to the modified Miller *et al.* (1988) protocol. Red blood cells (RBC) were lysed using the buffer NH_4Cl (0.155 M) and Tris base (0.17 M). White blood cell (WBC) lysis was carried out using Tris-HCl (1 M), Disodium EDTA (0.5 M), NaCl (1 M) and double distilled water. A total of 500 μl of proteinase K and 200 μl of sodium dodecyl sulfate were added to the tube. It was incubated for 16 h in a water bath at 37°C . A total of 1 ml of 6 M NaCl was added and vigorously shaken for 30 s. The tube was centrifuged at 3000 rpm

for 20 min and 4 ml of supernatant was transferred to another fresh tube. Then, double the volume of ice-cold ethanol was added and tilted once or twice. The DNA fibers were transferred to a 1.5 ml centrifuge tube and the DNA was dissolved in 200 μ l of TE buffer. It was incubated at room temperature for 1 h and the sample was stored at -20°C .

2.4 Polymerase Chain Reaction and Restriction Endonuclease Digestion

Q/R192 PON1 polymorphisms were assessed by polymerase chain reaction (PCR) amplification and digestion. The sequences of PCR primers were 5'-TATTGTTGCTGTGGGACCTGAG-3' and 5'-GACATACTTGCCATCGGGTGAA-3' which amplifies a PCR product size of 199 bp. PCR was carried out in a total volume of 20 μ l containing 2 μ l of template DNA, a pre-mixed 12.5 μ l of 2 \times concentration Red Dye PCR Master Mix buffer (GeNeiTM), and forward and reverse primers each of 1 μ l. The remaining 3.5 μ l were autoclaved with double distilled water. The amplification conditions consisted of 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 61°C for 35 s and 72°C for 30 s. The run was terminated by final elongation at 72°C for 5 min. Amplification was performed in an Eppendorf Thermocycler. The products were digested with 5 U of *Alw1* restriction enzyme at 37°C for 4 h and obtained 199 + 135 bp DNA products for allele Q and 199 + 135 + 64 bp for allele R. The run was performed in a 3% agarose gel electrophoresis and visualized in UV transilluminator (Fig. 1).

2.5 Statistical Analysis

Statistical Package for Social Sciences (SPSS) Windows, version 15.0, was used for statistical analysis. Allele frequencies were estimated by gene counting. Agreement with Hardy–Weinberg expectations was tested using a Chi-square goodness-of-fit test. Chi-square test or Fisher's exact *t* test as appropriate was used to compare the proportions of genotypes or alleles. One-way ANOVA or Student's *t* test as appropriate was used to compare groups for continuous variables. Logistic regression analysis was performed to compare frequencies after adjustment for age,

BMI and sex differences between groups of comparison. $P < 0.05$ was considered statistically significant. Power was estimated using an online *post-hoc* power computation tool http://www.dssresearch.com/toolkit/spcalc/power_p2.asp) and Genetic Power Calculator (S. Purcell & P. Sham, 2001–2009).

3. RESULTS

The clinical history of ischemic stroke patients and control subjects are presented in Table 1. The mean age and sex ratio of males and females in the ischemic stroke cases were matched to controls. Among 308 ischemic stroke patients, 243 were males and 65 were females. In 330 controls 258 were males and 72 were females. There was a positive family history of hypertension (51.94%) and diabetes (49.35%) in stroke patients compared with controls with hypertension (32.7%) and diabetes (27.87%). The smoking (50.32%), BMI (25.1 ± 3.92) and alcoholism (47.92%) are significantly high in ischemic stroke patients compared with controls ($P < 0.05$). The routine biochemical parameters of the ischemic stroke patients and controls are compared and are documented in Table 2. Stroke patients had significantly increased levels of serum total cholesterol (202.84 ± 86.48 mg/dl), LDL (129.30 ± 88.40 mg/dl) and Triglyceride (171.91 ± 42.49 mg/dl) concentrations whereas the control subjects had significantly lower Total Cholesterol (172.90 ± 19.34 mg/dl), lower LDL (101.44 ± 19.50 mg/dl) and low triglyceride (157.45 ± 34.95 mg/dl) ($P < 0.01$) respectively. The stroke cases had significantly lower serum levels of HDL ($P < 0.01$) and PON1-ARE ($P < 0.05$) when compared with control. The genotypic distribution of PON1192 was in Hardy–Weinberg equilibrium. Patients had a higher frequency of QR and RR genotype than QQ when compared with controls. The odds ratio of developing the stroke with RR genotype versus QQ genotype was 1.49 (0.872–2.533, $P = 0.1856$) and with QR genotype versus QQ genotype is 1.72 (95% CI 1.231–2.391, $P = 0.00184$). When the stroke patients with RR + QR genotype compared with QQ genotype, the RR + QR was significantly higher with an odd ratio (95% CI) of 1.67 (1.218–2.281, $P = 0.00178$). R allele frequency of ischemic

stroke is significantly higher with odd ratio (95% CI) of 1.01 (0.777–1.250, $P = 0.0082$) compared with Q allele of controls. The mean PON1-ARE activities were significantly higher in cases (137.40 ± 28.46) and controls

(147.38 ± 28.15) with QQ genotype and lower in cases (68.01 ± 25.86) and controls (57.33 ± 6.25) with RR genotype. This is highly statistically significant.

Table 1
Baseline characteristics of study subjects

| Particulars | Controls (n = 330) | Ischemic stroke (n = 308) |
|------------------------------------|--------------------|---------------------------|
| Age | 50.42 \pm 9.23 | 51.54 \pm 9.20 |
| Sex (M/F) | 258/72 | 243/65 |
| BMI (kg/m ²) | 23.9 \pm 1.99 | 25.1 \pm 3.92* |
| Smoking (%) | 43.33 | 50.32 |
| Alcoholism (%) | 36.6 | 47.92** |
| Family history of diabetes (%) | 27.87 | 49.35** |
| Family history of hypertension (%) | 32.7 | 51.94* |
| Mixed diet (%) | 89.9 | 87.6 |
| SBP (mmHg) | 116.61 \pm 7.59 | 136.50 \pm 20.93** |
| DBP (mmHg) | 77.00 \pm 6.89 | 85.59 \pm 12.28** |

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure.

Values are M \pm SD.

*** $P < 0.05$, compared with control.**

**** $P < 0.01$, compared with control.**

Table 2
Clinical and biochemical characteristics of patients

| Particulars | Control (n = 330) | Ischemic stroke (n = 320) |
|---------------------------|--------------------|---------------------------|
| Total cholesterol (mg/dl) | 172.90 \pm 19.34 | 202.84 \pm 86.48** |
| Triglycerides (mg/dl) | 157.45 \pm 34.95 | 171.91 \pm 42.49** |
| HDL (mg/dl) | 39.96 \pm 4.93 | 36.70 \pm 5.95** |
| LDL (mg/dl) | 101.44 \pm 19.50 | 129.30 \pm 88.40** |
| Urea (mg/dl) | 22.26 \pm 3.69 | 25.10 \pm 6.31* |
| Creatinine (mg/dl) | 0.85 \pm 0.18 | 0.90 \pm 0.34 |
| Glucose (mg/dl) | 90.91 \pm 17.94 | 113.97 \pm 44.11** |
| PON1-ARE activity (kU/l) | 118.18 \pm 39.92 | 107.26 \pm 34.99* |

HDL, high density lipoprotein; LDL, low density lipoprotein; PON1-ARE, aryl esterase.

Values are M \pm SD.

*** $P < 0.05$, compared with control.**

**** $P < 0.01$, compared with control.**

4. DISCUSSION

The present study correlates the PON1-ARE activity, PON1 genetic polymorphism and lipid profile in ischemic stroke patients in this part of the country. The importance of determination of both genotypes and serum PON1-ARE activity for correlation with disease had been emphasized by several authors (Jarvik *et al.* 2000; Richter and Furlong, 1999; Mackness *et al.*, 2001; Draganov and La Du, 2004; Brophy *et al.*, 2002). According to the World Health Organization Global Burden of Diseases Study, it is estimated

that the annual stroke incidence is projected to increase in 2015 to 91 per 100,000 and in 2030 to 98 per 100,000 particularly among Asian populations in developing countries (Ezzati M, Rodgers A, Lopez AD *et al.* 2004). In India, the incidence of ischemic stroke was high as 80% (Bhatnagar D, Anand IS *et al.* 1995) and the prevalence of stroke was estimated as 203 per 100,000 population (Sethi, 2002). In addition Saha *et al* (2003) reported that the prevalence of stroke in urban India appears to be higher than in

rural areas where the prevalence rate was only 92 per 100,000. The incidence in India might have been on the increase due to the change in the life style, urbanization, etc. Hypertension ($p < 0.01$) and alcoholism ($P < 0.01$) were found to be significantly high in our study group as observed in various other studies (Uhl and Farell, 1983; Chen *et al.* 1995; Zimmermann *et al.* 1995), which might also contribute for the increased incidence of stroke cases.

Frequency of PON1 alleles widely varies across human population. The frequency distribution and significance of PON1 Q192R polymorphism in the present study is shown in Table 3. The QQ genotype is documented to have more PON1-ARE activity and has lower risk for ischemic stroke. The QQ genotype that developed stroke is (41.55%) and is significantly lower than controls (54.2%). However the patients with QQ genotype who had developed ischemic stroke had decreased HDL and increased LDL. The low socioeconomic status, life style, and nutritional factors would have been the cause for the development of stroke in spite of the protective nature of QQ in our patients. We documented a significantly low serum PON1-ARE activity in ischemic stroke patients ($P < 0.05$) when compared with controls. Similar observations were noticed in other studies also (Jarvik *et al.* 2000; Jarvik *et al.* 2003; Ayub *et al.* 1999; Mackness *et al.* 2003; Graner *et al.* 2006; Aydin *et al.* 2006; Kim *et al.* 2007). The fact that the PON1 activity is responsible for the antioxidant role of HDL explains the decreased PON1-ARE activity and the low HDL in the stroke cases in our study. Liu *et al.* showed increased lipid peroxidation in patients with stroke (Aydin *et al.* 2006). They also demonstrated the presence of an LDL, which was more susceptible to oxidation *in vitro*. It may be due to decreased PON1 activity associated with decreased HDL, which is responsible to prevent lipid peroxidation.

In our study ischemic stroke patients have markedly decreased HDL concentration and PON1-ARE activity. Under most circumstances, the serum PON1-ARE activity is therefore likely to be dependent on the number of PON1 molecules in HDL rather than the serum HDL concentration. The PON1 molecule in HDL also depends on the polymorphism as shown in our

results (Table 4). When genotype analysis was performed the distributions of R+ (QR, RR) were significantly higher among cases (58.4%) when compared with controls (45.8%). R+ genotype is an independent risk factor for atherosclerosis. R+ has included both QR and RR genotypes. However when QR and RR were analyzed separately both genotypes were high among ischemic stroke cases (QR 47.4% and RR 11%) than controls (QR 36.1% and RR 9.7%) (Table 3). Similar studies by other workers showed (Liu and Cuddy, 1992; Imai *et al.* 2000; Voetsch *et al.* 2002; Ranade *et al.* 2005; Baum *et al.* 2006) the presence of even a single R allele is an independent risk factor for ischemic stroke. Identification of 192R allele as risk factor for ischemic stroke is apparently reasonable since 192R isozyme has a lower potential to decrease the lipid peroxides in both coronary and carotid lesion homogenates than 192Q isozymes. The significant decrease in HDL level (36.70 ± 5.95) and increase in LDL level (129.30 ± 88.40) in ischemic stroke cases shows that HDLs protective mechanism is lower in the patients as seen by the genotype namely QR and RR. The QQ genotype shows significant higher activity (137.40 ± 28.46) compared with QR (89.97 ± 16.32) and RR genotypes (68.01 ± 25.86). This clearly shows the QQ is a protective genotype for ischemic stroke.

5. CONCLUSION

We have documented a highly significant association between the ischemic stroke, the Q/R192 PON1 polymorphism, PON1 activity, and altered lipid profile (increased total cholesterol, LDL, TGL, and decreased HDL) in this study. The low PON1-ARE activity is associated with the PON1 QR and RR genotypes and altered lipid profile especially decreased HDL level. Further population studies may be undertaken to evaluate the incidence of PON1 Q192R polymorphism in our population. However the awareness of having healthy quality life among our people may decrease the incidence of stroke in an already risky population. Serum PON1-ARE activity may be included as another index while screening for ischemic vascular disease, namely coronary artery disease and ischemic stroke.

Table 3
Genotype and allele frequencies in cases and controls

| Groups | PON1 codon 192 polymorphism | | | Allele frequency | |
|----------|-----------------------------|--------------|-------------|------------------|-------|
| | QQ, n (%) | QR, n (%) | RR, n (%) | Q | R |
| Cases | 128 (41.55%) | 146 (47.40%) | 34 (11.05%) | 0.653 | 0.347 |
| Controls | 179 (54.2%) | 119 (36.1%) | 32 (9.7%) | 0.723 | 0.277 |

Chi square test: P = 0.0082.

Table 4
Biochemical characteristics according to PON1 192Q/R genotypes in ischemic stroke patients and controls

| Group | Control | | | | Ischemic stroke | | | |
|---------------------------|----------------|----------------|----------------|---------|-----------------|----------------|----------------|---------|
| | QQ (n = 179) | QR (n = 119) | RR (n = 32) | P value | QQ (n = 128) | QR (n = 146) | RR (n = 34) | P value |
| Total cholesterol (mg/dl) | 172.60 ± 18.86 | 173.18 ± 19.91 | 169.00 ± 16.48 | <0.01 | 204.16 ± 55.63 | 187.11 ± 48.82 | 265.44 ± 20.5 | <0.01 |
| Triglycerides (mg/dl) | 161.40 ± 32.74 | 149.54 ± 37.57 | 164.00 ± 34.96 | <0.01 | 172.26 ± 43.12 | 171.99 ± 35.15 | 170.26 ± 64.87 | <0.01 |
| HDL (mg/dl) | 39.57 ± 4.73 | 40.63 ± 5.22 | 39.56 ± 4.98 | <0.01 | 36.52 ± 5.69 | 37.25 ± 6.07 | 34.96 ± 6.15 | <0.01 |
| LDL (mg/dl) | 100.74 ± 19.61 | 102.63 ± 19.31 | 96.63 ± 16.25 | <0.01 | 130.04 ± 54.56 | 113.03 ± 48.67 | 128.67 ± 46.22 | <0.01 |
| PON 1-ARE activity (kU/l) | 147.38 ± 28.15 | 91.22 ± 15.41 | 57.33 ± 6.25 | <0.01 | 137.40 ± 28.46 | 89.97 ± 16.32 | 68.01 ± 25.86 | <0.01 |

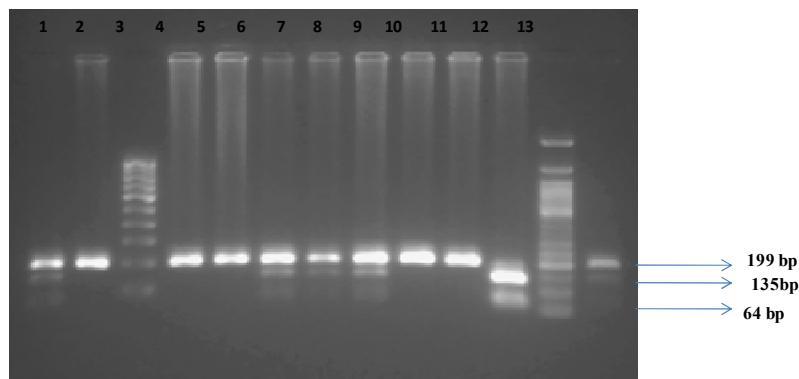
HDL, high density lipoprotein; LDL, low density lipoprotein; PON1-ARE, aryl esterase.

Values are M ± SD.

**P < 0.05, compared with control.*

***P < 0.01, compared with control.*

Figure 1: 3% agarose gel electropherogram showing restriction digestion pattern of Q192R polymorphism of PON1 gene region using of Alw I restriction enzyme



8 µl of products + 2 µl of dye electrophoresed at 50V for 2 hours.

- Lanes 2,4,5,9 and 10 - Amplicons showing the QQ genotype (Homozygous normal) with a single uncut band corresponding 199 bp.
- Lanes 1,6-8 and 13 - Amplicons showing the QR genotype (heterozygous condition) with three bands corresponding to 199 bp, 135 bp, 64bp respectively.
- Lane 11 - Amplicon showing the RR genotype (homozygous mutant) with two bands corresponding to 135bp and 64bp respectively.
- Lane 3 - 100 bp DNA marker
- Lane 12 - 50 bp DNA marker

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