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Single Nucleotide Polymorphisms in PON1 Gene and their Relation with PON1 Activity in Vokkaliga Population of Mysore, South India

Jyothi Mallaiah, Rajashekara Reddy Ravuri[*], Elizabeth A. Frank and Cletus J. M. D'Souza

Department of Studies in Biochemistry, Manasagangotri, University of Mysore, Mysore 570 006, Karnataka, India
Telephone: +91- 0821 2419621, E-mail: jyothm@gmail.com
[*]Anthropoligical Survey of India, Southern Regional Centre, Bogadi II Stage North, Mysore 570 026, Karnataka, India

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ABSTRACT The cardio protective functions of HDL are attributed to the HDL-associated proteins like Paraoxonase 1 (PON1). PON1 is associated with HDL through a N-terminal signal peptide. HDL-associated PON1 alone is cardio protective. Indians have normal HDL levels and yet a high risk of cardiovascular diseases. Hence, the presence of polymorphisms in the promoter region and first exon of PON1 were investigated in 256 individuals to find out whether the polymorphisms could be the cause of the high risk. Out of these 256 individuals, 195 were from Vokkaliga community and the rest were from other communities. Whole blood was collected, plasma and cells were separated. Plasma was used for determination of PON1 activity and for lipid analysis. Cells were used for DNA extraction.DNA was sequenced from -1246 to +203 of the PON1 gene. Nine single nucleotide polymorphisms(SNPs) were identified of which three were new SNPs, two of which were in the 5' flanking region of the putative mRNA for the protein but none in the first exon. Our results show that the low active PON1 phenotype was predominant in the Vokkaliga population.

INTRODUCTION

Cardiovascular diseases (CVD) are the leading cause of death for men and women among all racial and ethnic groups (Pyrola et al. 1987). Diverse studies have established the role of many risk factors for atherosclerosis and the only negative risk factor was found to be High Density Lipoprotein (HDL) measured as HDL-cholesterol (Tan 1980). It is now established that the cardioprotective function of HDL is due to the presence of various enzymes and proteins associated with the HDL particle. The antioxidant enzyme of HDL extensively characterized in recent years is paraoxonase.

Paraoxonase (EC 3.1.8.1) is a 43 KDa protein coded by the PON1 gene containing 9 exons and encoding a 354 aminoacid glycosylated peptide. It is located on the long arm of choromosome 7 (q 21.3 - q22.1) along with the other members of the gene family, namely, PON2 and PON3 (Primo-Parmo et al. 1996 ; Durrington et al. 2001). Polymorphic forms of PON1 protein were reported with widely differing substrate specificities and specific activities. The two important polymorphic forms are L55M and Q192R. The Q192R polymorphism causes substrate dependent differences in the kinetics of hydrolysis. Arginine at 192 specifies high PON activity whereas glutamine specifies low PON activity. In L55M polymorphism, Leucine at 55 is correlated with higher PON1 activity and mRNA levels than Methionine (Brophy et al. 2001).

In addition to the polymorphisms in the coding region, PON1 has six single nucleotide polymorphisms (SNPs) in the promoter region. They are– -108 , -126, -162, -832 , -909 and -1076 (Brophy et al. 2001a ; Brophy et al. 2001b). Since promoters are important cis acting elements, promoter variants may also alter gene expression. (Hoogendoorn et al. 2003). In our previous studies we found that the distribution of PON1 activity in south Indians was negatively skewed and showed a large standard deviation (Mahadeshprasad et al 2010). We also found that PON1

Address for correspondence:
Jyothi Mallaiah
Department of Studies in Biochemistry,
Manasagangotri, University of Mysore,
Mysore 570006, Karnataka, India.,
Telephone:+91 821 2419621,
E-mail: jyothm@gmail.com

was not completely associated with HDL (Jaichander 2006). Since PON1-HDL association is essential for its cardioprotective function, and since PON1-HDL association is through the N-terminal hydrophobic peptide of PON1 (Sorenson et al. 1999), in this study we investigated possible SNPs in the promoter region and the first exon which would affect the amount of PON and its association with HDL.

We have chosen members of the Vokkaliga community for our study since they represent an endogamous group where cross cousin marriages are allowed. They are mostly non-vegetarians. Cultivation is their main occupation as indicated by their name '*vokku*', meaning 'to thresh grain' They are found scattered in almost all parts of the Karnataka State, India (Prabhakar and Gangadhar 2009) and account for about 15% of the total population.

In our study sample, we report the presence of nine SNPs in the promoter region, with three novel SNPs. Out of these, two were found exclusively in Vokkaliga population and one exclusively in the others.

MATERIALS AND METHODS

Chemicals and reagents used were from Sigma Aldrich, USA. The sequencing chemicals were from Applied Biosystems. The primers 5'agctgaccac-tttccctctc3'; 5'agcactcactttgtgccatc3' and 5'aggaa-atgaatgagctgtgtg3'; 5'agagtgtgca-tctagca-cctg 3' were from Sigma Genosys.

Sample Collection: Whole blood was taken from subjects from Gollanabeedu and Saragur villages of HD Kote Taluk , Mysore District, Karnataka, India. The study sample comprised of a total of 256 individuals (195 belonged to Vokkaliga community and 61 belonged to other communities). Ethical clearance for this study was obtained from the Institutional Human Ethical committee, University of Mysore, Mysore. Informed consent was obtained from each subject before taking the blood sample.

Enzyme Activity: PON1 enzyme activity was determined spectrophotometrically using phenyl acetate as substrate (Eckerson et al.1983). One unit of PON1 activity was defined as 1 micromole of phenyl acetate hydrolyzed per min.

Lipid Analysis: Total cholesterol and triglyceride were estimated by enzymatic methods using diagnostic kits (Accurex Biomedical) (Goswami and Bandyopadhyay 2003).

DNA Extraction and Sequencing: DNA was extracted using phenol: chloroform method (Sambrook et al. 1989).

DNA Extraction: Briefly the cells are lysed in an alkaline lysis buffer. The lysate was incubated at 56°C in the presence of Proteinase K and SDS to partially digest cellular proteins and loosen the association between proteins and chromosomal DNA. The cell lysate was then treated with buffer-saturated phenol:chloroform . The DNA remains in the aqueous phase while the cellular proteins are extracted into the organic phase. The aqueous phase was extracted a second time with phenol: chloroform to ensure complete removal of the proteins. The aqueous phase, containing the DNA was then treated with isopropanol to precipitate the DNA. The DNA was washed with chilled ethanol and then dissolved in TE Buffer (10 mM Tris-HCl, 1mM EDTA, pH 8.0). DNA extracted this way was generated as high molecular weight (average fragment size>20kb) and double stranded. The DNA quality was assessed by the ratio of absorption at 260nm and 280nm . Ratio of 1.6 to 2.0 was used as an indicator of DNA free from contamination.

Prior to PCR amplification, DNA samples were checked for purity and integrity. Electrophoretic analysis of DNA using agarose gels was used to confirm integrity of DNA. DNA fragments were visualized under UV light by staining with ethidium bromide. The final concentration of DNA used in PCR amplification was decided based on the preliminary gel electrophoretic scan. Contaminated or sheared samples were discarded.

PCR and DNA Sequencing: The DNA sequence from -1246 to +203 was amplified using primers which cover both promoter region and first exon of PON1 gene. Cycling conditions were as follows: 94°C for 3 minutes for initial de-naturation followed by 35 cycles of following conditions. 94°C for one minute, 56°C for 45 seconds and 72°C for 2.5 minutes and final extension for 7 minutes at 72°C. PCR product was used to perform cycle sequencing reaction with ABI Prism BigDye™ v 3.1 as per the instructions provided by the manufactures of the kits. After cleanup, the cycle sequencing product was run on an ABI 3730 DNA Analyzer to get the sequence of the amplified region.

DNA Sequencing: Sequencing was carried out using Applied Biosystems 3730 genetic analyzer with a 48 capillary system. During the sequencing run, the labeled DNA fragments migrate through

the gel according to their size and a laser is used to excite the fluorescently labeled fragments as they pass the laser window. Fluorescence is detected, data collected, processed and interpreted by a computer. The final output is a colour profile (chromatogram), which displays peaks and corresponding base calls. All the samples were sequenced and data analysed statistically

Statistical Analysis: Statistical analysis was carried out using SNP associated R package-software and SPSS v12.

RESULTS AND DISCUSSION

The main objective of this study was to look for the presence of novel polymorphisms in the promoter region and first exon of PON1 gene and hence, a non- random sample was selected for the study. In this sample we also found members of the other communities and they constitute the accidental sample. A total of 16 communities are represented in this accidental sample. The number of individuals in each of these groups varied from 1 to a maximum of 18. Biochemical characteristics of the subjects are presented in Table 1. All the parameters studied had similar distribution in the two study groups namely the Vokkaligas and Others (non-Vokkaligas) with no significant differences between them except PON1 activity showed a significant difference ($P<0.05$). PON1 activity was lower in Vokkaliga population compared with others. Since the sample was a non- probability sample and one of the groups was an accidental sample, the difference in PON1 activity between Vokkaligas and others may be an artifact. However, the large sample variations both in Vokkaligas and others was seen which was consistent with our earlier observation.

Table 1: Biochemical Characteristics of Vokkaliga and Non-Vokkaliga population

S. No.	Parameters	Vokkaliga (Mean±SD)	Others (Mean ±SD)
1	Cystolic blood pressure	121.89 ± 9.48	127.16 ± 15.74
2	Diastolic blood pressure	79.85 ± 5.16	80.69 ± 7.54
3	BMI	21.63 ± 3.66	23.59 ± 3.68
4	WHR	0.91± 0.06	0.94 ± 0.11
5	PON1 activity	19279.91 ±18782.67	27500.15 ± 20743.30
6	Total cholesterol	170.67 ± 27.22	172.65 ± 34.01
7	Triglycerides	151.35 ± 77.74	157.26 ± 65.10
8	HDL	44.09 ± 4.08	43.17 ± 4.73
9	LDL	96.28 ± 21.07	97.94 ± 28.69

BMI – Body Mass Index; WHR – Waist Hip Ratio; HDL – High Density Lipoprotein;
LDL – Low Density Lipoprotein

The results of genotype and allele frequencies are presented in Table 2. A total of nine SNPs were detected. Six of these were already known polymorphisms. Three SNPs, namely, -725, -283 and -165 were novel Out of the three novel polymorphisms two (-725 and -283) were found exclusively in Vokkaliga population and one (-165) exclusively in non-Vokkaligas. It is possible that there are other polymorphisms but with lesser frequency. In order to study this, a larger sample would be required for the study. Novel polymorphism (-165) found exclusively in the other group is possibly an accidental finding. There was only one representative from Vishwakarma community and he had this polymorphism. To know the frequency of these polymorphisms larger sample size is required. However, the three novel polymorphisms identified in our study are completely new and have not been reported in literature so far. Also, the chances of accumulating polymorphism, if any would be high in Vokkaliga community compared with others in this region, since Vokkaligas are an endogamous group.

In our study, the PON1 activity was found to be lower in Vokkaliga population when compared with others. This was consistent with the observation that in the exclusive polymorphism of the -725 SNP, the AA genotype was associated with low PON1 activity whereas the AG genotype was associated with high PON1 activity. However, the frequency of the high PON1 active AG genotype was very small (0.0044) suggesting that the genotype for low PON1 activity is highly prevalent among Vokkaligas.

A study of the human promoter polymorphisms of a variety of genes has shown that they may modify gene expression by as much as 50% or more (Voetsch et al. 2007). Since the -725 SNP is in the 5' flanking region, whether it is the cause of low level of transcription, is not known.

In our study, polymorphism beyond -108 upto +203 was not observed. This region would have the N-terminal signal sequence. Since no polymorphism was observed in this region, it is unlikely that lack of association of PON1 with HDL may be the cause of low cardio protection.

Table 2: Genotype and PON activity

SNP	*rs Number*	*Alleles*	*Minor allele freq%*	*HWE (p value)*	*PON activity*		*% Sample having polymorphism*	
					Vokkaliga	*Others*	*Vokkaliga*	*Others*
1076	rs 854573	a/g	26.2	1	17865.59 ± 15919.77	28041.2 ± 20799.22	35.9	49.8
-909	rs 854572	g/c	44.5	0.311	17028.7 ± 171117.33	25614.06± 21511.41	44.1	52.5
-832	rs 854571	g/a	33.2	0.887	17033.31 ± 15319.32	23846 ± 16180.65	44.1	13.3
-725	Novel	a/g	0.2	-	58779	-	0.5	0
-283	Novel	t/c	0.2	-	10382	-	0.5	0
-165	Novel	g/c	0.2	-	-	16794	0	1.6
-162	rs 705381	g/a	26.4	0.333	17901.05 ± 15943.45	28767.61± 21250.5	33.3	14.4
-126	rs 705380	g/c	11.5	0.756	23804.68 ± 18959.68	21657.31± 14040.43	19.5	21.3
-108	rs 705379	c/t	36.9	0.893	17757.42 ± 19876.67	25665.54± 21206.07	44.1	42.6

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