VOLUME-8, ISSUE-11, NOVEMBER-2019 • PRINT ISSN No. 2277 - 8160 • DOI : 10.36106/gjra

Original Research Paper

Diabetology

STUDY OF SERUM PARAOXONASE 1 LEVELS AND PARAOXONASE 1 GENE (192A/G) POLYMORPHISM IN TYPE 2 DIABETES MELLITUS.

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ABSTRACT Paraoxonase1 is a HDL associate antioxidant enzyme helping in evaluating oxidative stress status and complications in diabetes mellitus. Paraoxonase gene polymorphism influence Paraoxonase 1 levels and may be the molecular basis for inter individual variability seen in Diabetes Mellitus. Hence, this study was taken up to analyze the concentration of serum Paraoxonase 1 levels and Paraoxonase 1 gene (192A/G) polymorphism in type 2 diabetes.The study included 40 patients (≥ 30 years) with diabetes mellitus and 40 ages and sex matched healthy controls. Paraoxonase 1 level were determined ELISA and PCR assay targeting Paraoxonase 192(A/G) gene was done. Restriction enzyme Alw1 is used to digest PCR product.There was a significant reduction in Paraoxonase1 levels (p value =0.005) among the Type 2 D M patients compared to healthy controls along with higher Paraoxonase levels in AA genotype (251.47±299.07 pg/ml) as compared to AG genotype (120.73±17.88pg/ml) with p value = 0.024 showing the G variant to be more prone to oxidative damage caused by hyperglycemia thereby lowering the Paraoxonase 1 levels. The results were analyzed using 20th version of SPSS.T2 DM patients have increased oxidative stress as indicated by decreased levels of enzyme Paraoxonase 1. This reduction in PON1 activity has been suggested as a reason for the increased risk of cardiac disease in these patients. In this study the G variant is more prone to oxidative damage caused by hyperglycemia thereby lowering the Paraoxonase 1 levels.

KEYWORDS : Paraoxonase 1, Paraoxonase 1 Gene (192A/G) Polymorphism, Type 2 Diabetes Mellitus.

INTRODUCTION:

Diabetes mellitus is hyperglycemia resulting from defects in insulin secretion, insulin action or both. It is associated with long-term dysfunction, damage and failure of normal functioning of various organs, especially the eyes, nerves, kidneys, heart and blood vessels. Diabetes associated micro vascular disease is a leading cause of blindness, renal failure and nerve damage. [1]Diabetes has become a pandemic and it is expected to reach 552 million by 2030. India has the second highest prevalence of diabetes in world with 61.3 million people affected. [2]

Hyperglycaemia generates reactive oxygen species (ROS), which cause damage to the cells and ultimately results in secondary complications of diabetes mellitus. Oxidative stress has received attention as one of the most important mechanism leading to biomolecular damage and cellular dysfunction in Diabetes. Mechanisms for this increased risk may be attributed to the imbalance between pro-oxidants (free radicals) and antioxidants resulting in increased oxidative stress and oxidative damage. [3, 4] The increased oxidative stress in patients of diabetes mellitus is associated with auto-oxidation of glucose, protein glycation, and activation of protein kinase C. It could also lead to peroxidation of cellular membrane and also oxidative modification of amino acids and DNA. [5]These abnormalities would lead to long term complications of diabetes (retinopathy, nephropathy and neuropathy) and increases morbidity and mortality in these patients. [6] Paraoxonase 1 activity may help in evaluating oxidative stress status and therefore complications in diabetes Mellitus.

Paraoxonase1 has 354 amino acids with a molecular mass of 43 kDa. These enzymes are secreted from liver cells and are associated with HDL in the circulation.[7] It is Ca^{2+} dependent ester hydrolase with antioxidant properties and it has a preventive role in oxidative stress due to hyperglycaemia.[8] PON1 protects lipoproteins and arterial cells against oxidation, probably by hydrolyzing lipid peroxides such as specific oxidized cholesteryl esters and phospholipids and it contribute to the antioxidant protection conferred by HDL on LDL oxidation.[9]

Plasma lipids modify composition, function and concentration of the HDL. As a result, both the conformation and function of HDL may be altered. This glycation of HDL or directly of PON1 in HDL in diabetes may result in detachment of PON1 itself from the HDL and PON1 inactivation.[10,11] PON1 is bound by HDL to a lesser extent in diabetic patients as compared to healthy persons and its activity is poorly stabilized. [12] PON1 has recently been implicated as protecting against other diseases including type 2 diabetes mellitus (T2D).[13,14] However relationship of PON1 toT2D is still poorly understood. Limited evidence suggests that the activity of PON1 in serum is negatively associated with established T2D. [15, 16] The lower PON1 activity is thought to be due to the lowering of its specific activity by non –enzymatic glycation as a result of elevated blood glucose.[13,17] This reduction in PON1 activity has been suggested as a reason for the increased risk of cardiac disease in these patients.[18] There is also evidence indicating that T2D's origins lie in inflammation.[19]

Two common polymorphism are (192A/G) in which glutamine (A) to arginine (G) substitution at amino acid 192 and other (55T/A) polymorphism at amino acid 55, in which leucine to methionine substitution have been said to affects Paraoxonase1 activity. In (192A/G) polymorphism the exchange of codon CAA to CGA in exon 6 of paraoxonase1 gene determines isoforms of the enzyme which differ greatly in the rate of hydrolysis a number of substrates.[20]

The PON1 gene has two common polymorphisms:

- First glutamine-to-arginine substitution at position in coding region c.575A > G giving rise to amino acid substitutions at positions 192 (c.575A > G).
- Second a leucine - to-methionine substitution in coding region c.163T > A giving rise to amino acid substitutions at positions 55 (c.163T>A).

Both independently influence PON1 activity and have been referred to as the molecular basis for this inter individual variability and major determinant of the PON1 activity against organophosphates.[21] The PON1 coding region contains two common polymorphisms, glutamine (Q) to arginine (R) substitution at codon 192 (Q192R). Another polymorphism at amino acid 55, leucine to methionine substitution also affects PON1 activity. These polymorphisms are only 8.6 Kb apart from each other (Gen Bank accession number AC004022).[22] The genetic basis of the inter-individual variability of PON1 activity has been attributed to the presence of an A/G polymorphism in the coding region of the gene coding for this enzyme. [23, 24] The A/G polymorphism corresponds to glutamine/arginine polymorphism at amino acid position 192. In (192A/G) polymorphism the exchange of codon CAA to CGA in exon 6 of paraoxonase1 gene determines isoforms of the enzyme which differ greatly in the rate of hydrolysis a number of substrates .[20] Effect of the Human serum paraoxonase 55 and 192 genetic polymorphism on the protection by high density lipoprotein against low density lipoprotein oxidative modification. Serum PON1 levels and activity varies widely among individuals and populations of different ethnic groups and seems to be related to the Q192R and L55M polymorphisms.[4]

METHODS:

Hospital based observational cross-sectional study to find out association of Paraoxonase1 levels with Paraoxonase 1 gene (192A/G) polymorphism in type 2 diabetes mellitus cases and apparently healthy controls. 40 diagnosed patients of T2 DM disease of either sex and 40 age and sex matched controls with no evidence of Diabetes mellitus were taken. Study was conducted in the Department of Biochemistry and in collaboration with Department of medicine in a Medical College, New Delhi. The study was approved by institutional Ethical committee. 5ml of fasting venous blood sample was collected for routine biochemical investigations. Tests were performed on AU-480 fully automated analyser. Human paraoxonase 1 levels in serum was done by Using Elisa kit from BOSTER Immunoleade based on standard sandwich enzyme-linked immune-sorbent assay technology.

For gene polymorphism detection the DNA Extraction from whole blood was done using QIAamp DNA mini kit from QIAGEN. The extracted genomic DNA was amplified by PCR using flanking polymorphic region of Paraoxonase 192(A/G) gene. Primer pair used was as Forward Primer: 5'-TATTGTTGCTGTGGGACCTGAG-3' (22bp), Reverse Primer: 5'-CCTGAGAATCTGAGTAAATCCACT-3' (24bp). PCR assay targeting Paraoxonase 192(A/G) Gene was done in a total volume of 25 l of reaction mixture.Preparation of reaction mixture (Table 1) was done taking all necessary precaution to avoid any contamination. Paraoxonase 1 (192A/G) gene amplification was done on Peltier Thermal Cycler (PTC-100 BIO- RAD) and programming was done as following:

1. Initial Denaturation 95℃ X 3 min.
2. Cycle Denaturation 94℃ X 30 sec
3. Cycle Annealing 60℃ X 40 sec
4. Cycle Extension 72℃ X 35 sec
5. Final Extension 72℃ X 7 min.
6. Hold 4℃ X 8 min

RFLP was done by use of restriction enzyme AlW1 to digest PCR product by the following procedure: 5 μl of PCR product was mixed with 0.5 μl of AlW1 Restriction enzyme containing 10X buffer 5.0 μl and nuclease free water 14.5 μl. This mixture was incubated overnight for 16 hour at 37 C. Digested product was run on 2% agarose gel. Gel was loaded with 5 l of digested PCR product which was mixed with 3 l of DNA loading dye. Then gel was run on Genetix electrophoretic equipment at 60 mV for 60 minutes. After completion of the run gel was visualized under UV rays in geldoc (AlphaDigiDoc™). Study and analysis of restriction fragments by geldoc (AlphaDigiDoc™) In AA there is no restriction site and single band of 238 bp product was obtained, In homozygous GG genotype, restriction site is preserved and two band of 172 bp and 66 bp were obtained and in heterozygous AG three bands of 238bp, 173 bp and 66 bp were obtained.(figure 1)

Gln_{192} ----TTACAATCC---- AATGTTAGG----
No cut with AlwI

Arg_{192} ----TTACGATCC--- ----AATGCTAGG----
Cut with AlwI

Data were analyzed using 20th version of SPSS (statistical package for social sciences). All the data were expressed as mean ±SD /±SE .The p value of < 0.05 was considered as significant. The data obtained were compared between two groups by student t-test. Pearson's correlation coefficient was applied for correlation when appropriate. Observation of allelic frequencies of the genotype was compared with biochemical parameters using appropriate statistical tools.

RESULTS:

The mean age of patients in the study group was 49.12±9.2 years and in control group was 48.12±6.1 years and p value was 0.53.The difference between the two was not significant. Case and control were age matched. Sex difference was not statistically significant. (Table 1).

Table 1: Components of the reaction mixture

PCR Constituents	Volume
DNA	7.5 μl
Taq Polymerase (5 U/μl)	0.25 μl
10X Taq buffer with 1.5 mM MgCl2	2.5 μl
dNTP mix (100 mM)	0.2 μl
Forward Primer (100 μM)	0.1 μl
Reverse Primer (100 μM)	0.1 μl
NFW	14.35 μl
Total	25 μl

The cases and controls were sex matched. The mean BMI in the cases was 24.37±4.77 kg/mt^2 while in the control was 22.90±3.040 kg/mt^2. The difference between the two was not statistically significant (p value = 0.448) (Table 2).

Table 2: Demographic profile

Parameters	Cases with T2DM		Controls		P value
Mean age of presentation	48.12±6.1 yr		49.12±9.2 yr		0.535
BMI (Kg/m2)	24.37±4.77		22.90±3.04		0.448
SEX	MALE	FEMALE	MALE	FEMALE	
	58 %	42 %	56 %	46 %	

The mean Serum Paraoxonase 1 activity in the cases were 110.77±17.3361 nmol/min/ml and in the control group was 252.20±46.16 61 nmol/min/ml . The difference between the two was statistically significant (p value=0.005*). (Table 3) p value=0.005 is considered statistically significant

Table 3: Serum Paraoxonase activity in cases and controls (n=40 each) nmol/min/ml

Study group	**Mean ± SEM**
Case	110.77±17.33
Control	252.20±46.16
P value	**0.005***

In our study population mean serum Paraoxonase activity in AA genotype was higher (251.47±299.07 nmol/min/ml) as compared to AG genotype (120.73±17.88 nmol/min/ml). Difference was statistically significant with p value = 0.024. (Table 4)

Table 4: Intergenotypic distribution of paraoxonase 1 in cases and controls

Genotype	Mean±SD
AA	251.47±29.91
AG	120.73±17.88
p value	0.024

AA genotype was found in 14 individuals (35%) of cases and 18 individuals (45%) of control group. AG genotype was found in 23 individuals (52.5%) of cases and 21 individuals (52.5%) of control group. GG genotype was found in 3 subjects individuals (7.5%) of cases and 1 individual (2.5%) of control group. The frequency of genotypic distribution with $2 = 1.591$ and (p value=0.45) not significant. (Table 5)

Table 5: Distribution of Paraoxonase 1 gene (192A/G) polymorphism in cases and controls:

GENOTYPE	CASE	Frequency%	CONTROL	Frequency%	Total
AA	14	35%	18	45%	32
AG	23	57.5%	21	52.5%	44
GG	3	7.5%	1	2.5%	4

The plasma fasting glucose levels in cases was (202.85±60.91mg/dl) and in controls was (86.80±9.91 mg/dl). p value=0.000 is statistically significant.The mean plasma post prandial glucose level in cases was (270.92 mg/dl) and in controls was (102.70 mg/dl). p value=0.000 is (statistically significant). Serum HbA1C levels in cases was (8.91±2.42 mg/dl) and in controls (5.17±0.62 mg/dl). p value=0.000 is statistically significant. (Table 6)

Table 6 . Glycemic profile in cases and controls

Parameter	Study group	Mean±S.D	p value
FBS (mg/dl)	case control	202.85±60.91 86.80±9.91	0.00*
PPBS(mg/dl)	case control	270.92±74.69 102.70±9.95	0.00*
HBA1C (%)	case control	8.91±2.42 5.17± 0.62	0.00*

Paraoxonase has significant negative correlation with FBS, PPBS, and with HbA_{1C} with r = -0.217(p value =0.05), -0.234(p value =0.037), and -0.238(p value =0.033).(Table 7)

Table 7: Correlation of Paraoxonase 1 with glycemic profile markers using pearson's co-eficient

Parameter	r value	p value
FBS	-0.22	0.05
PPBS	-0.23	0.04
Hb_{A1C}	-0.24	0.03

DISCUSSION:

The mean plasma paraoxonase 1 level in the study group (cases) was 109.61±109.61 (pg/ml) and in the control group was 252.20±291.98 (pg/ml). The decrease in the level was statistically significant (p value=0.005*) (Table 3). Our findings are in accordance with study conducted at South Karnataka, Suvarna et al report that the levels of HDL-C and PON1 activity are decreased significantly in diabetic patients with complications in comparison to diabetics without complications.[25] Further, in a study among North West Indian Punjabi diabetic population also there is decreased PON1 activity in comparison to healthy population.[26] Paraoxonase is an antioxidant that protects LDLs from lipid peroxidation. It has been suggested that diabetic patients are at an increased risk for oxidative stress. [27] This may be due, in part, to hyperglycemia and advanced glycosylation end products. Mechanisms have been proposed by which both could initiate and accentuate lipid oxidation. [28,29]

Reduced Paraoxonase 1 levels in cases can be due to increased glycation of proteins and other biomolecules including Paraoxonase. Prolonged hyperglycemia caused increased free radical synthesis depleting the total antioxidant stores along with Paraoxonase. Furthermore, an increased prooxidative status in turn leads to reduced Paraoxonase-1 levels due to the inhibition of Paraoxonase-1 by its substrates, i.e. lipid peroxides.[30]

In our study population, mean serum Paraoxonase levels in AA genotype was higher (251.47±299.07 pg/ml) as compared to AG genotype (120.73±17.88 pg/ml). Difference was statistically significant with p value = 0.024. That was supported by finding of Mackness el al. [31,4] The polymorphism is associated with significant differences in the expression of a reporter gene in cell transfection studies.[32] In our study 192G allele is consistently associated with lower Paraoxonase expression, lower serum concentrations, and lower activities of the enzyme. The data are consistent with the hypothesis that low expression of the enzyme represents a risk factor in type 2 diabetes. The higher-risk 192(G) allele has modified levels toward exogenous substrates, although the physiological relevance of the levels polymorphism has not been clarified.[33] In the study, a statistically significant negative correlation was found between Paraoxonase 1 and $HbA_{1C,}$ showing that poorly control diabetes may increase oxidative stress which further deplete Paraoxonase 1 levels.

Our study highlights the role of paraoxonase 1 as an important novel marker of oxidative stress in diabetes mellitus. It has shown that G variant may be associated with decrease in Paraoxonase 1 levels. A change in polymorphism may be contributory factor for lowering the Paraoxonase 1 levels independent of hyperglycemia. However as T2 DM is a multi factorial disease with many confounding factors so whether this effect of PON 1 gene (192A/G) polymorphism and lowered Paroxonase 1 activity is an additional risk factor in the pathogenesis of type 2 diabetes or not to determine this fact further investigations using larger sample sizes in matched case-control studies of various ethnic groups are required. This Study has limitations of being small in relation to the data collected.

ACKNOWLEDGEMENT: "No funding received"

Disclosure: "None declared"

Conflicts of interest: There is no conflict of intrest.

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