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Research Article

OCT1 GENETIC POLYMORPHISM AND ITS ASSOCIATION WITH ELEVATED SERUM METFORMIN LEVELS AND LIPID PEROXIDATION STATUS IN TYPE II DIABETES MELLITUS

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ABSTRACT

To investigate the possible association of OCT1 rs122083571polymorphism(C/T allele) with serum metformin levels and lipid peroxidation status in type II Diabetes mellitus.
Sixty, already diagnosed T2DM patients on metformin monotherapy (500 mg ,bd) were recruited for the study and the genotypes for SLC22A1 rs122083571 C/T polymorphism using PCR assay were done. Serum fasting plasma glucose (FPG), Postprandial Plasma Glucose (PPG), Glycated Hemoglobin (HbA1c), Fasting Serum Insulin (FINS), Triacylglycerol (TG), Cholesterol (CHO), Low-Density Lipoprotein (LDL-c), High-Density Lipoprotein Cholesterol (HDL-c), and Homeostasis Model Assessment for Insulin Resistance (HOMA-IR), Body Mass Index (BMI), blood pressure (BP) serum metformin levels,Thio barbituric acid reactive substances (TBARS) , Ferric ion reducing antioxidant power (FRAP) assay values were determined .
In patients with C allele, the BMI,BP ,FPG ,PPG Insulin levels and TBARS were significantly higher with diminished antioxidant status when compared to that of the T allele subjects.The values of TGL and HDL in both groups of T2DM patients were not significantly different. But the total cholesterol level and LDL level were significantly increased in T2DM patients with C allele.
SLC22A1 rs122083571 C allele is associated with poor glycemic status in spite of high blood metformin levels and the BMI, fasting plasma glucose, insulin ,total and LDL cholesterol levels were also significantly higher. The increased lipid peroxidation rate and decreased concentration of antioxidants could be attributed to persistent hyperglycemia and poor glycemic status.

Key words: metformin ;organic cation transporter 1; PCR; single nucleotide polymorphism; SLC22A1 gene ,type 2 diabetes mellitus.

Abbreviations:

SNPs: Single Nucleotide Polymorphisms; PCR: Polymerase Chain Reaction; T2DM: Type 2 Diabetes Mellitus; T2DM: Type 2 Diabetes Mellitus; OCTs: Organic Cation Transporters;BMI: Body Mass Index; FPG: Fasting Plasma Glucose; PPG: Postprandial PlasmaGlucose; HbAlc: Glycated Hemoglobin; FINS: Fasting Serum Insulin;PINS: Postprandial Serum Insulin; HOMA-IR: Homeostasis Model Assessment for Insulin Resistance; TC: Total Cholesterol; LDL-c: Low density Lipoprotein-cholesterol; HDL-c: High-density Lipoprotein cholesterol.

INTRODUCTION:

Oxidative stress and oxidative damage to tissues are common end points of chronic diseases, such as atherosclerosis, diabetes, cancer and rheumatoid arthritis [(1)]. Reactive oxygen species are generated by environmental factors, such as ionizing radiation and chemical carcinogens, and also by endogenous processes, including energy metabolism in mitochondria. ROS produced

either endogenously or exogenously can attack lipids, proteins and nucleic acids simultaneously in living cells [2].They are potentially dangerous and are commonly referred to as pro-oxidants.

Diabetes mellitus is characterised by hyperglycaemia together with biochemical alterations of glucose and lipid peroxidation[3].This persistent hyperglycemia in Diabetes increases the production of ROS through glucose autoxidation[4]. The superoxide generated in this process of glucose autoxidation is associated with the formation of glycated proteins in the plasma of diabetic patients. Polyol pathway, prostanoid synthesis and protein glycation are associated factors which disturbs the antioxidant defence system, increasing the amount of reactive oxygen species[5].

Diabetes mellitus propagates its complications with increased free radical formation [6,7]. The increased oxidative stress in diabetes mellitus is owing to the increase in the production of oxygen free radicals and a deficiency in antioxidant defence mechanisms [4, 8]. Increased concentration of oxygen free radicals causes lipid peroxidation of cellular structures, which is thought to play an important role in atherosclerosis and micro-vascular complications of diabetes mellitus [9]. Hyperlipidemia has also been reported as one of the causative factors for increased lipid peroxidation in diabetes mellitus [10,11]. Hyperglycemia increases oxygen-reactive species generation and reduces the protective capabilities of antioxidant defence systems.

Metformin is used as a first line drug for the management of Type 2 Diabetes as it reduces liver gluconeogenesis, increases the peripheral utilization of glucose and lowers lipid levels [12,13]. Metformin is regarded as an anti hyperglycemic agent because it lowers blood glucose concentrations in T2D without causing overt hypoglycemia and is also frequently described as an insulin sensitizer leading to reduction in insulin resistance and significant reduction of plasma fasting insulin level.

Polyspecific organic cation transporters (OCTs) belonging to the family of solute carrier SLC22 superfamily of transporters translocate a wide variety of endogenous and exogenous substances of cationic nature. Three isoforms of OCTs, namely *OCT1*, *OCT2* and *OCT3*, with similar membrane topology consisting of 12 transmembrane domains are studied so far[14].Of these, OCT1 is one of the most abundantly expressed transporters in the liver and plays a major role in the hepatic uptake and renal transport of the biguanide agent, metformin[15].

Polymorphisms of OCT1 have been described with variable effects on metformin response [16].A number of rare and relatively common SLC22A1 polymorphisms which has shown reduced efficacy to metformin in lowering blood glucose levels have been identified. The gene encoding human OCT1, SLC22A1, is located on chromosome 6q25.3 and consists of 11 exons spanning 37 kb. Human OCT1 gene is highly polymorphic, leading to differences in transporter function which very well explains a possible mechanism accounting for variation in drug response [15,18] .

The objective of the present study is to evaluate the lipid peroxidation status in the C allele group and T allele group of T2 DM patients who are being treated with Metformin.

MATERIALS AND METHODS:

The study was carried out in 60 Type II diabetic unrelated patients of South Indian

Tamilian origin, aged between 35 and 55 years, of both sex, attending diabetic out-patient department of Rajah Muthiah Medical College Hospital, Annamalai Nagar. Subjects of the study were randomly selected and were on Metformin (500 mg bd) therapy during the time of study. Patients on insulin, smokers ,alcoholics, tobacco chewers, hypertension, and other systemic illness were excluded from this study. Institutional ethical committee of Rajah Muthiah Medical College Hospital, Annamalai Nagar have approved the study and informed consent was obtained from the patients. Genomic DNA was extracted from peripheral blood leucocytes using the standard phenol–chloroform method, and the samples were stored at -20 °C [(24)].

Anthropometric measurement

Anthropometric data including height, weight, blood pressure and BMI were measured. Body mass index (BMI) was calculated by dividing the weight in kilograms by height in meters squared. BP was measured with a standard mercury sphygmomanometer.

Biochemical analysis

Fasting venous blood was collected immediately after enrolment in tubes containing EDTA. Blood samples were centrifuged at 2000 x g for 10 min. Samples were analyzed for Lipid Profile (Total Cholesterol, HDL-c, LDL-c, Triglycerides), Renal function Tests (Urea, Creatinine) by auto analyzer using kits. Serum insulin levels were determined by ELISA kit. The fasting venous plasma glucose (FPG), was determined with the glucose oxidase method. HbA1c was measured with a high-performance liquid chromatography[(17)].Insulin levels (FINS was measured using standard Insulin ELISA kits).Body mass index (BMI) was calculated by dividing body weight in kg divided by the square of the height in meters. Estimation of lipid peroxidation was done by TBARS and antioxidant status was studied by FRAP assay.

Hormones assay

Serum insulin levels were determined by using Immunoenzymometric assay[(19)].
The homeostasis model assessment for insulin resistance (HOMA-IR) was calculated as fasting Insulin (mg/dl) x Fasting glucose (mg/dl) divided by 405 [(20)].

Thiobarbituric acid (TBARS)

TBARS levels were measured as an index of lipid peroxidation using the colorimetric method described by Satoh [(21)]. After reaction of thiobarbituric acid with malondialdehyde (MDA), the reaction product was extracted in butanol. Separation of the organic phase was facilitated by centrifugation at 3000 rpm for 10 mins. and its absorbance was determined spectrometrically at 530 nm.

Total antioxidant activity-FRAP Assay

FRAP assay [(22)] uses antioxidants as reductants in a redox linked colorimetric method, employing an easily reduced oxidant system present in stoichiometric excess.

Serum Metformin Analysis

Serum Metformin levels were measured with a high-performance liquid chromatography (HPLC) (23).Chromatograms were recorded at 241 nm using a detector SPD-20AVShimadzu UV visible detector.The retention time for Metformin was 3.78 minute. The optimum wavelength selected for determination of Metformin was 241nm.

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SNP determination in the SLC22A1 gene with rs 12208357(C/T allele)using PCR method
(TaqMan SNP genotyping assay):

Genomic DNA was extracted from peripheral blood leucocytes using the standard phenol–chloroform method, and the samples were stored at 4°C [(24)] .PCR primers were designed on the basis of published sequences of OCT1.The primer set(forward primer: 5'-GCCCTGCGGAGGAGCTGAACTATA-3'; reverse primer for: 5'-CCTGTCCCAGGAACTCCCATGTTAC-3';was designed to amplify the portion of the SLC22A1 gene encoding the SNP. Master mix contained Taq polymerases, dNTP's , $MgCl_2$,forward primer, reverse primer and template DNA. The polymerase chain reaction (PCR) reaction was carried out in duplicates using 25 µl final volume that contained 13 µl of Taq Man Universal PCR master mix, 1 µl of forward and reverse primers each , 1 µl of $MgCl_2$and 4 µl of genomic DNA diluted in DNase-free water and 5 µl of deionised water. The PCR reaction was placed in the Master cycler (Eppendorf thermal cycler) that was programmed to improve the specificity of PCR amplification. There were 35 PCR cycles at the annealing temperature of 60 °C with a final extension at 72 °C for 10 min.PCR products were analyzed by electrophoresis on 3% agarose gels followed by ethidium bromide staining and inspection under UV light[(25).]

PCR product length was 257 base pairs. If band was obtained at 257 bp, it was considered positive for C allele.

Statistical analysis
All results were shows as mean ± SD. Results were evaluated using Student's t-test. P-value <0.05 was considered statistically significant. Statistical analysis was performed using SPSS software.

RESULTS AND DISCUSSION:

Hyperglycemia by diabetes causes oxidative stress which leads to enhanced production of mitochondrial ROS[(26,27)] which leads to the activation of stress-sensitive signalling pathways, which worsen both insulin secretion and action, and promote the development of type 2 diabetic mellitus (T2DM) [(28)].

Increased lipid peroxidation may be due to the increased glycation of proteins in diabetes mellitus. These glycated proteins might themselves act as a source of free radicals and it may be also thought to play a role in increased lipid peroxidation in diabetes mellitus [(11,27,28,29,30)] .

The maximum benefit of a drug is obtained when there is maximum efficacy. Metformin is used as a first line drug for the management of Type 2 Diabetes as it reduces liver gluconeogenesis, increases the peripheral utilization of glucose and lowers lipid levels[(13,14)].OCT1 was shown to be responsible for transporting metformin into hepatocytes, and for the hepatic glucose lowering effect of metformin[(33)].The major action of metformin is exerted in the liver, by the activation of adenosine monophosphate-activated protein kinase (AMPK[(34,35,36)].AMPK is a phylogenetically conserved serine/threonine protein kinase viewed as a master regulator of systemic and cellular energy status .It plays a crucial role in protecting cellular functions under energy-restricted conditions.

Polymorphisms of OCT1 have been described with variable effects on metformin response [(16,37)]. Many studies have proved that SNPs in OCT1 gene leads to decreased metformin uptake. In our study we analysed

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a SNP in OCT1 gene with rs12208357(C/T) allele.

In the present study, geneotyping of rs122083571, the PCR method was performed to detect the C>T SNP in the SLC22A1 gene, which encodes OCT1, using *TaqMan* SNP genotyping assay method.

The primer design and polymerase chain reaction conditions are described in Shu et .al [16].By applying PCR conditions, better specificity was achieved, and the results were reliable. In this study, out of 60 patients 18 had SNP OCT1(C allele).The C allele frequency was 11% compared to the T allele frequency of 89%.

Table 1: General Characteristics:

PARAMETER	C ALLELE	T ALLELE	SIGNIFICANCE
AGE	46 ± 6.5	44 ± 6.7	NS
BMI(wt/m^2)	26.8 ± 4.4	24 ± 2.1	0.05
Systolic B.P(mmHg)	126.91± 12.91	124.77±10.95	NS
FPG (mg/dl)	136 ± 13.7	122 ± 11.8	0.05
PPG(mg/dl)	246± 11.3	208 ± 10.8	0.05
BLOOD UREA(mg/dl)	25.83 ± 5.6	25.5 ± 5.8	NS
SERUM CREATININE(mg/dl)	0.81 ± 0.10	0.80 ± 0.00	NS
FINS(fasting insulin)(µIu/ml)	20.38 ± 2.7	16 ± 3.9	0.05
HbA1c(%)	12.4 ± 1.08	10.2 ± 0.13	< 0.05
HOMA-IR	7.6 ± 1.1	5.6 ± 1.0	< 0.05

No significant difference in age, blood urea and serum creatinine values between the C allele group and the T allele group. Data are expressed as mean ± SD, P<0.05 was considered statistically significant. According to Table 1, BMI, FPG(Fasting plasma glucose,),PPG(post prandial glucose)Insulin levels,HbA1c,HOMA-IR and B.P values(both systolic and diastolic)were elevated in C allele.

In C allele patients, HbA1c levels were significantly elevated due to constant liver gluconeogenesis, since metformin transport to the liver is defective in these patients. Correspondingly, there is increased post prandial blood sugar which elevates HbA1c, showing the possible association of OCT1 polymorphism with poor metformin action and glycemic control.

Table 2: Lipid Profile

LIPID PROFILE	C ALLELE	T ALLELE	SIGNIFICANCE
Total cholesterol(mg/dl)	173.5 ± 11.8	145± 14.2	0.05
TGL(mg/dl)	126.5 ± 37.2	121.2 ± 39.8	NS
HDL-c(mg/dl)	42.05 ± 2.4	44.25 ± 3.09	NS
LDL-c(mg/dl)	115 ± 14.2	86.61 ± 12.8	0.05

The Lipid profile values mentioned in the table 2, the total cholesterol and LDL-c shows elevation in the C allele while that of the HDL-c and TGL had no relevant significance.

In diabetic patients, the LDL and total cholesterol levels are increased because of insulin deficiency or insulin resistance as insulin acts to lower total and LDL cholesterol by stimulating insulin dependent lipase in the adipocytes. The total cholesterol and LDL values are significantly higher in C allele. The BMI of C allele patients is significantly increased($p<0.05$).This study shows that since metformin efficacy is reduced in C allele patients, the insulin release increases and the peripheral resistance to insulin increases, leading to reduced hypolipidemic activity of insulin. This could explain for the raised total cholesterol and LDL-c fractions seen in the C allele subjects.

TABLE 3: Antioxidant status and serum metformin levels of C allele subjects and T allele subjects:

PARAMETERS	C ALLELE	T ALLELE	SIGNIFICANCE P value
METFORMIN(µg/ml)	0.16 ± 0.013	0.08 ± 0.017	< 0.05
TBARS(nmol/ml)	15.7 ± 2.6	13.8 ± 3.6	< 0.05
FRAP(µM)	3.00 ± 0.62	4.46 ± 0.84	< 0.05

Data are expressed as mean ± SD, $P<0.05$ was considered statistically significant. In patients with C allele polymorphism, serum metformin,lipid peroxidation levels(TBARS) are slightly increased and the antioxidant capacity(FRAP) is significantly decreased compared to the T allele population.

The serum metformin levels in C allele patients were significantly increased. Fasting plasma glucose, post prandial glucose and serum insulin levels were also increased in those patients($p<0.05$).This explains that OCT1 transporter is required for the uptake of metformin into the liver. So in patients with OCT1 polymorphism, metformin is not taken into the liver and hence its levels remained elevated in the blood stream [(37)].

Free radical activity is an important cause of vascular complication in type II D.M [(31, 32)]. It plays an important role in both microvascular and macrovascular complication in D.M. Long term complications of D.M are supposed to be ,at least in part, mediated by, and increased free radical generation and subsequent oxidative stress[(38)].Increased concentration of oxygen derived free radicals are implicated in the pathogenesis of vascular complications in D.M. In hyperglycaemic state, the

production of superoxide is stimulated ,and the enzyme superoxide dismutase is inhibited by non enzymatic glycosylation known as Millard reaction[39].Glycation was shown to affect the C-terminal end of the enzyme, reducing its heparin binding affinity. Thus protection against extracellular radicals by cell surface attached SOD may be impaired in D.M leaving the endothelial cell susceptible to damage by superoxide anion. Thus in diabetic conditions, normal levels of antioxidant enzymes may be insufficient or may be functionally impaired, so as to preserve a physiological contractile response[40].Many previous studies have shown an increase in the free radical concentration with subsequent decrease in the antioxidant enzymes in the hyperglycaemic state[31,32].

TBARS (thiobarbituric acid reducing substances) assay is an indirect measure of lipid peroxidation .Here in this study the TBARS level of C allele patients are relatively higher than in T allele patients. It might be due to poor action of metformin, hyperglycemia , glucose oxidation and increased free radicals as a consequence of OCT1 SNP.

FRAP (ferric ion reducing antioxidant power) assay is a measure of antioxidant capacity of an individual. In this study, the FRAP levels of C allele patients were relatively lower than the T allele group.

The results of the TBARS and FRAP of the C allele patients revealed the possible association of OCT1 polymorphism with reduced metformin action , hyperglycemia, and increased lipid peroxidation.

Among 60 subjects 18 had OCT1 polymorphism in the C allele (11%).ie, 18 subjects who had C allele, showed decreased absorption of Metformin by liver from blood .So this group had elevated blood metformin levels. In the present study, the serum Metformin levels in C allele patients were significantly increased. Plasma glucose, serum insulin ,lipid peroxidation levels were also found to have increased in these subjects ($p < 0.05$) and there is marked decrease in the antioxidant status in the C allele subjects compared to that of the T allele subjects. In the present study, the serum Metformin levels in C allele patients were significantly increased.

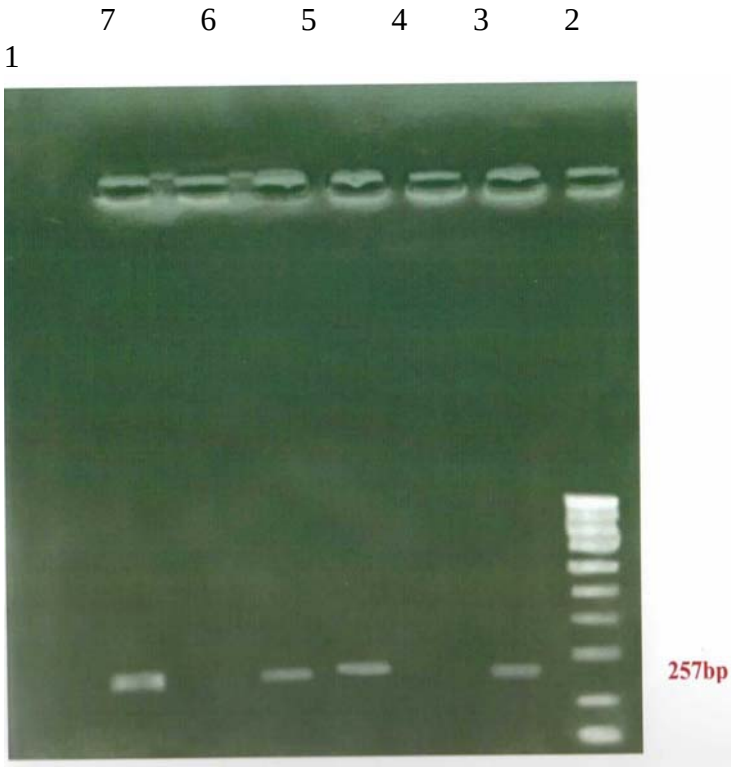

Fig :1 OCT1 : PCR product result in gel documentation

Representative polyacrylamide gel electrophoresis picture of *OCT1* rs122083571 C/T polymorphism . Lane 1 is

the 100 base pair DNA ladder.The PCR product length was 257 bp.

OCT 1	NUMBERS	%
C ALLELE	18	11
T ALLELE	42	89

TABLE 4: Frequency of Expression of polymorphism

OCT1 transporter is required for the uptake and transport of metformin from the blood to the liver. In patients with OCT1 polymorphism, metformin was not taken from the bloodstream to the liver, and hence its level remained elevated in the blood causing persistent hyperglycemia, elevated oxidative stress and reduced antioxidant properties.

CONCLUSION:

In summary, we demonstrated that the common SLC22A1 rs122083571 C/T (Arginine 61 Cys variant) significantly alters uptake of endogenous compounds and drugs. Those subjects with T allele had better response to Metformin therapy and had better antioxidant status when compared to that of the C allele. In those subjects with C allele, the Metformin levels were increased, due to poor transport of metformin to its sites, resulting in elevated fasting plasma glucose, insulin, HbA1c, total cholesterol, LDL levels. This persistant hyperglycemia increased oxidative stress, producing more free radicals with a marked reduction in the antioxidant property. Metformin could not exert any of its antidiabetic properties in those subjects probably due to its poor transport into the cells, which might have occurred due to the genetic polymorphism in the OCT1 transporter protein. This study provides evidence to the fact that snp in the SLC22A1 rs122083571 C allele is associated with the poor glycemic status which in turn is responsible for the, increased lipid peroxidation and decreased concentration of antioxidants.

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