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ASSOCIATION OF OCT1 GENE POLYMORPHISM WITH GLYCEMIC STATUS AND SERUM METFORMIN LEVELS IN TYPE II DIABETES MELLITUS PATIENTS

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ABSTRACT:

Aim: To investigate the possible association of OCT1 rs122083571 polymorphism (C/T allele) with glycemic status and serum metformin levels in type II Diabetes mellitus.

Methods: 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) were determined.

Results: In patients with C allele, the BMI,BP ,FPG ,PPG and Insulin levels were significantly higher 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.

Conclusion: 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.

INTRODUCTION: Single nucleotide poly-morphisms (snp) has gained considerable interest over the last decade, providing exciting and novel information regarding individual disease susceptibility, response to therapy and progression [1, 2]. These SNPs, at least partially decide on an individual's susceptibility to disease and response to therapy [2]. Novel discoveries on genetic variations in drug transporters, drug targets, effector proteins and metabolizing enzymes have provided better insights into the field of pharmacogenomics and its aims to achieve the best therapeutic outcome based on genomic insights, to ultimately lead to the development of "personalized medicine" [3].

Over the last many years, great efforts are made to understand the etiological mechanism by which T2D develops and inter individual variability for the same standard treatment therapy [4].A wide class of anti diabetic drugs are available for the treatment of T2D [5, 6]. Among them Metformin, a biguanide, considered

as a potent insulin sensitizer, is the most widely prescribed medicine for the treatment of T2DM [7]. Metformin is the first-line of therapy for the management of T2D as it reduces blood glucose levels without the risk of hypoglycemia and checks on the progression of micovascular and macrovascular complications. Metformin controls blood sugar by suppressing hepatic glucose by inhibiting gluconeogenesis and decreasing intestinal glucose absorption [7, 10, 11].

Zhou *et al*., observed considerable inter-individual variability in the clinical Efficacy of Metformin [12]. Metformin is transported across the plasma membrane by a group of transport proteins known as "organic cation transporters" (OCT's). They are cationic transporters which translocate a wide variety of endogenous and exogenous cations. Polymorphisms in OCT's are believed to be responsible for inter patient variability in metformin therapy [13]. Of this, OCT1 which is primarily present in the liver, is required for metformin uptake and pharmacological actions in the liver [14].

The SLC22A1 and SLC22A2 genes encode for OCT1 and OCT2, respectively. 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 [8, 15].

Many studies have been reported about single nucleotide polymorphisms in OCT1 gene among different population. The aim of the present study is to determine the genotype and allele frequency of OCT1 gene polymorphism(C/T allele) in diabetics and its association with glycemic status and serum metformin levels in the South Indian Tamilian T2M diabetes patients.

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 picked and were on Metformin (500 mg bd) therapy at the time of the 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 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.

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 [16].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.

Hormones assay: Serum insulin levels were determined by using Immunoenzymometric assay [17].

The homeostasis model assessment for insulin resistance (HOMA-IR) was calculated as fasting Insulin (mg/dl) x Fasting glucose (mg/dl) divided by 405 [18].

Serum Metformin Analysis: Serum Metformin levels were measured with a high-performance liquid chromatography (HPLC) [19]. Chromatograms were recorded at 241 nm using a detector SPD-20AV Shimadzu UV visible.The retention time for Metformin was 3.78 minute. The optimum wavelength selected for determination of Metformin was 241nm.

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 [31]. PCR primers were designed on the basis of published sequences of OCT1.The primer set(forward primer: 5'-GCCCTGC GGAGGAGCTGAACTATA-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 [20].

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: Diabetes Mellitus is a progressive polygenic disease which might arise either due to insulin resistance or due to inadequate secretion of insulin by the β-cells of pancreas ,contributing to hyperglycemia , dyslipedemia and artherosclerosis and coronary artery diseases [21, 22]. According to the International Diabetes Federation, the number of diabetic patients in India will increase from 50.8 million to 79.4 million by 2030 (Prevalence of Diabetes available at www.idf.org, assessed on December 23, 2010).

Obesity is always associated with insulin resistance. Most of the obese individuals with or without metabolic syndrome develops peripheral resistance to insulin.

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 [23, 24].

The present study was done to identify the patient group for whom metformin is potentially beneficial and for whom it is not. OCT1 was shown to be responsible for transporting metformin into hepatocytes, and for the hepatic glucose lowering effect of metformin [25]. Metformin is regarded as an antihyperglycemic 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.

The improvement in insulin sensitivity by metformin could be ascribed to its positive effects on insulin receptor expression and tyrosine kinase activity [26]. The major action of metformin is exerted in the liver, by the activation of adenosine monophosphate-activated protein kinase (AMPK) [6, 9, 10].

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. AMPK is a heterotrimeric protein consisting of a catalytic α-subunit and two regulatory subunits β and γ and each subunit has at least two isoforms. AMPK is activated by increase in the intracellular AMP/ADP-or-ATP ratio resulting from imbalance between ATP production and consumption.

AMP binds to regulatory sites on the γ subunits of AMPK causing its activation which brings about conformational changes that allosterically activate the enzyme to bring out its metabolic effects on glucose and lipid metabolism [27, 28, 29]. Activation

(phosphorylation) of AMPK suppresses hepatic gluconeogenesis, and reduces glucagon-mediated glucose output by the liver [6, 8].

OCT1 gene polymorphisms have gained considerable attention due to their role in the differential response of metformin. Metformin can act only in the presence of insulin and hence is beneficial only to those who have residual function of endocrine pancreas. The glycemic response to Metformin is variable, with some people having a marked response and others gaining no benefit. Polymorphisms of OCT1 have been described with variable effects on metformin response [14, 30].

A number of rare and relatively common SLC22A1 polymorphisms which has shown reduced efficacy to metformin in lowering blood glucose levels have been identified. Many studies have proved that SNPs in OCT1 gene leads to decreased metformin uptake. In our study we analysed 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*., [14]. 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%. The research further investigated the relatioinship between SLC22A1 gene, snp, serum Metformin levels and the glycemic status in these T2DM patients treated with metformin.

According to **Table 1**, BMI, FPG (Fasting plasma glucose,), PPG (post prandial glucose) Insulin levels were elevated in C allele. In the lipid profile values mentioned in the **table 2**, the total cholesterol and LDL-c shows significant increase in the C allele group.

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)	131 ± 12.91	124 ±10.95	NS
DiastolicB.P(mm/Hg)	79 ± 8.61	74 ± 6.79	NS
FPG (mg/dl)	136 ± 13.7	122 ± 11.8	0.05
PPG(mg/dl)	289 ± 11.3	228 ± 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

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.

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

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

PARAMETERS	C ALLELE	T ALLELE	SIGNIFICANCE (P value)
METFORMIN	0.16 ± 0.013	0.08 ± 0.017	< 0.05
HbA1c(%)	10.4 ± 1.08	9.2 ± 0.93	< 0.05
HOMA-IR	7.6 ± 1.1	5.6 ± 1.0	< 0.05

Data are expressed as mean ± SD, P<0.05 was considered statistically significant. In patients with C allele polymorphism, serum metformin, Hba1c levels and HOMA-IR are significantly increased 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 C allele, metformin could not taken into the liver and hence its levels remained elevated in the blood stream [25]. Since there is reduced uptake of metformin into the liver, the desired hypoglycaemic activity of the drug is not obtained and there is continuous hepatic gluconeogenesis, shown by the increased insulin levels and increased blood glucose levels.

HbA1c is a standard test for the measure of glycemic status in diabetic patients. As the blood level increases, HbA1c levels also increase. i.e, It directly correlates with the glycemic control of the patient for the last 2-3 months. 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.

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.

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.

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.

TABLE 4: FREQUENCY OF EXPRESSION OF POLYMORPHISM

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

In the present study, the serum Metformin levels in C allele patients were significantly increased. Fasting plasma glucose and serum Insulin were also found to have increased in these subjects ($p < 0.05$).

OCT1 transporter is required for the uptake and transport of metformin from the blood to the liver. In patients with OCT1 polymorphism C allele, metformin was not taken from the bloodstream to the liver, and hence its level remained elevated in the blood.

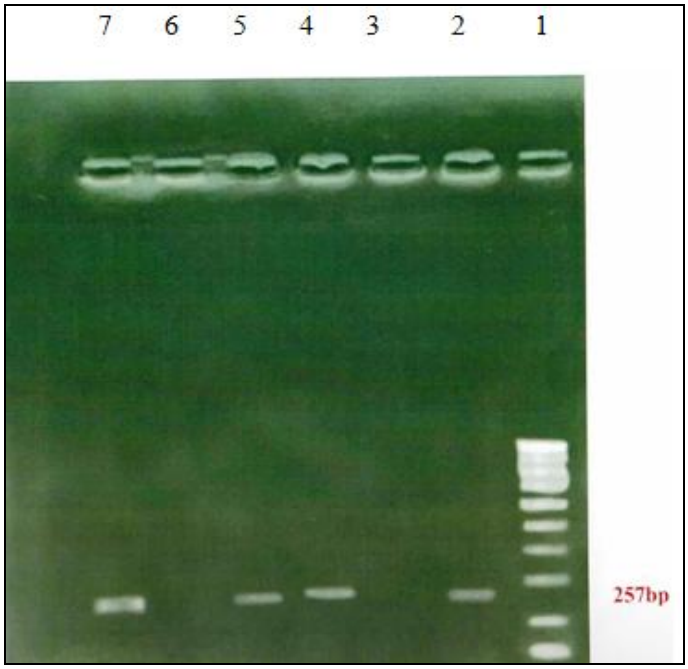

FIG 1: OCT1: PCR PRODUCT RESULT IN GEL DOCUMENTATION

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 glycemic status when compared to that of the C allele. In those subjects with C allele, the Metformin levels were increased probably due to poor transport of metformin to its sites, resulting in elevated fasting plasma glucose, insulin, HbA1c, total cholesterol, LDL levels.

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.

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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 Plasma Glucose; HbAlc: Glycated Haemoglobin; 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.

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