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A STUDY ON THE ASSOCIATION OF SERUM ADIPONECTIN WITH METFORMIN LEVELS IN OCT1 GENETIC VARIANTS OF TYPE 2 DIABETES MELLITUS.

Biochemistry

Manju Koshy* Division of Biochemistry, Believers Church Medical College Hospital, St. Thomas Nagar, Kuttapuzha P.O Thiruvalla-689103, Kerala, India. *Corresponding Author

Jeeji Palocaren Division of Biochemistry, Malankara Orthodox Syrian Church Medical College (MOSC), Kolenchery, Kerala, India.

S. Sethupathy Division of Biochemistry, Rajah Muthiah Medical College and Hospital, Annamalai University, Annamalai Nagar, Tamil Nadu, India

ABSTRACT

Purpose: Metformin is a widely used drug for treatment of type 2 diabetes mellitus (T2DM) which functions mainly by stimulating AMP-activated protein kinase (AMPK), a major cellular regulator of lipid and glucose metabolism. AMPK acts as a master sensor of cellular energy balance in mammalian cells by regulating glucose and lipid metabolism. Hepatic metformin uptake depends on the expression of organic cation transporters (OCTs). Adiponectin also functions like Metformin to bring forth similar effects. The study was designed to investigate the role of Metformin in improving adiponectin levels in T2DM. So we tried to associate levels of serum adiponectin with Metformin in OCT1 snp genetic variants of T2DM.

Methods: The study subjects were all T2DM patients on metformin monotherapy (500 mg, bd) who were grouped into two on the basis of their HbA1c values and snp on SLC22A1 rs122083571 as Group I (C allele) and Group II (T allele). Genotyping for SLC22A1 rs122083571 C/T polymorphism using PCR assay was done along with other anthropometric and biochemical parameters.

Results: The results suggested that Group II patients with T allele of OCT1 rs122083571 had better therapeutic efficacy to metformin and improved adiponectin levels when compared to patients with C allele genetic polymorphism. Serum adiponectin and metformin levels were significantly correlated.

Conclusion: Serum metformin levels are correlated to adioponectin values. SLC22A1 rs122083571 T allele was associated with low serum metformin levels and good glycemic status with improved adiponectin levels.

KEYWORDS

Adiponectin, metformin, organic cation transporter 1, SLC22A1 gene PCR, single nucleotide polymorphism, type 2 diabetes mellitus

INTRODUCTION:

Metformin, often the first drug used to treat newly diagnosed type 2 Diabetes Mellitus (T2DM) seem to be related to activation (phosphorylation) of AMP activated protein kinase (AMPK), which suppresses glucagon stimulated glucose production and causes an increase in glucose uptake in muscle and hepatic cells (1, 2). Adipose tissue, considered as an important endocrine organ, in addition to its role in fuel storage, thermal insulation, and mechanical protection, releases biologically active and diverse cytokines termed adipokines (3, 4) that produces numerous factors affecting food intake, metabolism of lipids, carbohydrates and numerous other processes in human body. Adiponectin, an endocrine factor first identified in 1995 is synthesized and released from adipose tissue (5). Adiponectin is a 30 kDa multimeric protein secreted mainly by white adipose tissue and has antidiabetic, anti-inflammatory, anti-atherosclerotic properties (6, 7) due to insulin mimetic and insulin-sensitizing actions (8). Adiponectin plays a significant role in metabolic disorders, such as obesity, T2DM, coronary heart disease, and metabolic syndrome (6, 7) and exerts its action through its receptors AdipoR1, AdipoR2 and T-cadherin (9).

Adiponectin lowers blood glucose acutely through a reduction in hepatic glucose output with little or no effect on glucose disposal (10, 11, 12). The glucose-lowering functions of adiponectin have been attributed to the hepatic activation of AMPK, providing a mechanistic link to a signal transduction pathway, already established as an antagonist of hepatic glucose output and lipogenesis (13) by increasing β-oxidation and thereby lowering the fat content of these tissues and preventing gluconeogenesis in the liver, plying. Since these primary functions are precisely the same as the functions of AMPK, it was reasonable to speculate that AMPK may be regulated by adiponectin. Indeed, treatment of cells with adiponectin potently activates AMPK activity (14, 15). Furthermore, low levels of adiponectin are associated with a predisposition towards T2DM (16). Metformin activates AMPK in hepatocytes; as a result, acetyl-CoA carboxylase (ACC) activity is reduced, fatty acid oxidation is induced, and expression of lipogenic enzymes are suppressed (2). The molecular mechanisms underlying metformin action seem to be related to its activation (phosphorylation) of AMP activated protein kinase (AMPK), which suppresses glucagon stimulated glucose production and causes an increase in glucose uptake in muscle and hepatic cells (2).

So the activation of AMPK can be by metformin / adiponectin or by a combined effect of both. Genetic variants of SLC22A1 have been shown to modulate the pharmacokinetics of metformin after oral administration and reduces the therapeutic response, presumably by decreasing the hepatic uptake of the drug (17, 18). Previous studies by Shu et al., (19) suggests OCT1 snp at R61C shows elevated serum metformin values. OCT1-R61C was also associated with decreased hepatic uptake of metformin and thereby decreased metformin action i.e., phosphorylation of AMP- activated protein kinase (18). Genetic variants could modulate transport activity by altering the expression and activity of a variant protein.

MATERIALS AND METHODS:

The study was carried out in sixty, T2DM unrelated patients of South Indian Tamilian origin, aged between 35 and 55 years, of both gender, attending diabetic out-patient department of Rajah Muthiah Medical College Hospital, Annamalai Nagar, Tamil Nadu, India. 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. Genomic DNA was extracted from peripheral blood leucocytes using the standard phenol–chloroform method, and the samples were stored at -20°c (20). The subjects were divided into 2 groups: Group I (C allele) and Group II (T allele). Both groups consisted of patients who had HbA1c (glycated hemoglobin) values above 8%. Anthropometry, biochemical parameters like fasting plasma glucose (FPG), HbA1c, serum lipids, insulin, urea and creatinine were measured. Insulin resistance score value (HOMA) was calculated by the formula; [FPG (mg/dl) × insulin (mIU/L)]/ 405 (21).

Table I: Anthropometric and Biochemical parameters of Group I and II

Variables	GROUP I *CC* *(n=54) (90%)*	GROUP II *CT/TT* *(n=6)(10%)*
Age	46 ± 6.5	44 ± 6.7
Gender (M/F)	19/35	0/6
Waist Hip Ratio	0.92±0.07	0.90 ± 0.04
Body Mass Index	26.8 ± 4.4	24 ± 2.1*
Family History	25	2
Fasting plasma glucose (mg/dl)	136 ± 13.7	122 ± 11.8*

HbA1c	12.4 ± 1.08	10.2± 0.13*
Serum Cholesterol (mg/dl)	173.5± 11.8	145 ± 14.2*
Serum Triglycerides (mg/dl)	126.5± 37.2	121.2 ± 39.8
HDL Cholesterol (mg/dl)	42.05± 2.4	44.25 ± 3.09
LDL Cholesterol (mg/dl)	115± 14.2	86.61 ± 12.8*

Student's t test between Group I and Group II *p<0.05.

Biochemical parameters were done using commercially available kits, Human Humastar 300 chemistry analyser - Human GmbH Germany. Serum insulin levels were measured by ELISA using DIA source Ins-Easia kit from DIAsource immunoassays (S.A.-Rue de I'Industrie, 8-B-400 Nivelles-Belgium) on CHEMWELL analyser.

Table II: Hormonal Markers and Serum Metformin levels in Group I and II

Variables	GROUP I *CC* *(n=54)* (90%)	GROUP II *CT/TT* *(n=6)*(10%)
Serum Insulin (U/ml)	20.38 ± 2.7	16 ± 3.9*
HOMA-IR	7.6 ± 1.1	5.6 ± 1.0*
Adiponectin (µg/ml)	6.5 ± 0.34	9.42 ± 0.56*
Serum Metformin (µg/ml)	0.16 ± 0.005	0.08 ± 0.005*

Student's t test between Group I and Group II *p<0.05.

Serum Metformin levels were measured with a high-performance liquid chromatography – *HPLC* (22). 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. Serum adiponectin were measured using *ELISA* (DIA source Belgium) sandwich assay using two specific and high affinity antibodies. The colour reaction was measured within 30 min at 450 and values expressed in µg/ml.

SNP determination in the SLC22A1 gene with rs 12208357(C/T allele, 61 Arg>Cys or R61C (C>T)) using PCR method

(Taq Man SNP genotyping assay):

Genomic DNA was extracted from whole blood using genomic DNA extraction kit (Axygen Bioscience USA) and OCT 1 gene was amplified by polymerase chain reaction (Eppendorf 5331 Gradient Master Cycler). PCR primers were designed on the basis of published sequences of OCT1 (18) with forward primer:

5'- GCCCTGCGGAGGAGCTGAACTATA-3' and reverse primer: 5'-CCTGTCCCAGGAACTCCCATGTTAC-3'. The master mix (17.3 µl distilled water, 0.2µl Taq polymerase (IU/L), 0.5µL (10Mm), 2.5µl MgCl2 (25Mm), 0.5 µl forward primer, 0.5 µl reverse primer and DNA template 1µl) final volume is 25µl. Steps involved initial denaturation (94ºC for 5 min), denaturation (94ºC for 30 sec), annealing (66ºC for 30 sec) and final extension (72ºC for 5 min). Repeat second step upto 30 cycles. The resulting fragment was 257 bp in length which was digested with the restriction enzyme BsaJ1 at 30ºC for 2-3 hrs. The digested samples were separated by electrophoresis on 2% Agarose gel stained with ethidium bromide and visualized on Gel Documentation System. This resulted in the identification of 3 genotypes- wild type CC genotype, heterozygous CT genotype and mutant homozygous TT base pair length.

Figure- 1: AGAROSE GEL ELECTROPHORESIS SHOWING THE PCR-RFLP OF OCT1 R61C SNP

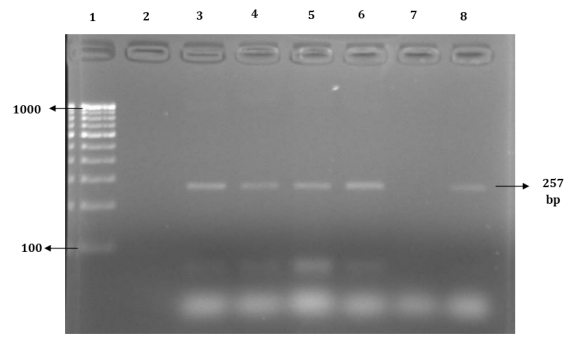
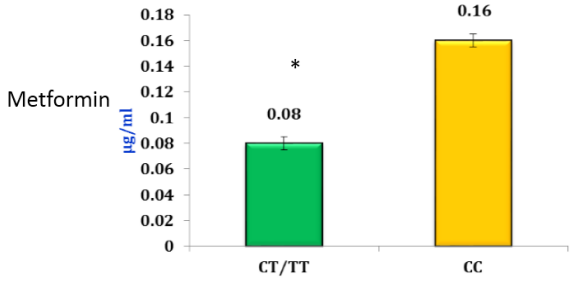


Figure shows OCT1 R61C polymorphism with three genotypes
Lane 1: 1000bp DNA Ladder
Lane 3, 4, 5 and 6: CT (Heterozygous)
Lane 7: CC Genotype (Wild type)
Lane 8: TT (Homozygous mutant)

RESULTS:

OCT1 gene R61C polymorphism possess three genotypes- Wild type CC; Heterozygous CT and Homozygous mutant TT. Figure-1 presents the genotype of OCT1 gene. Lane 3,4,5,6 and 7 of gel picture shows CT genotype, Lane 7 shows CC genotype. PCR product length was 257 base pairs. Band obtained at 257 base pair, was considered positive for C allele. Baseline parameters of OCT1 R61C SNP polymorphism in Group I and Group II patients are shown in Table-1. There was significant difference in the values of biochemical parameters of CT/TT genotype subjects compared to that of CC genotype groups. BMI, FPG, HbA1c, serum cholesterol and LDL-cholesterol were significantly decreased (p<0.001) in the T allele Group II subjects. Table II shows hormonal markers, adiponectin and serum metformin of study subjects. Serum metformin values were increased in Group I (CC) compared to Group II(CT/TT) (p<0.001) with subsequent decrease in adiponectin values (Figure 2).

Figure- 2: Serum Metformin Activity in OCT1 SNP R61C Genotypes



Comparison of serum metformin levels between CC genotype and TT genotype subjects (CT/TT). * p<0.05.

All anthropometric and biochemical parameters were normally distributed and therefore expressed 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.

DISCUSSION:

The fasting blood glucose, HbA1c and serum Insulin levels of Group I subjects were increased compared to Group II. The higher levels of total cholesterol and LDL-cholesterol in Group I compared to Group II can be possibly explained due to beneficiary effects of metformin, which effectively controlled blood sugar and lipid levels in Group II. Metformin efficacy is affected by gene polymorphism as seen in Group I subjects. Studies by Shu et al., (18) pointed out that R61C variant has a significant effect on metformin response. We analysed the association of OCT1 R61C variant with T2 DM and serum metformin levels and our results were supporting the study by Shu et al. In this study, Group I subjects with OCT1 polymorphism in the C allele had elevated serum metformin levels, FBS, HbA1c, total cholesterol and LDL-cholesterol values as compared to the T allele subjects of the same group. Studies have shown the effects of a combination of metformin and adiponectin on blood glucose more effective than the effects of adiponectin itself (23). Metformin treatment is also associated with a significant increase in serum adiponectin (24), which plays an important role in the suppression of the metabolic derangements that cause insulin resistance and T2DM (25). In Group I subjects, serum metformin levels were elevated with subsequent decrease in serum adiponectin, owing a strong co-relation between serum adiponectin and metformin levels with glycemic status. Administration of adiponectin is known to increase fatty acid oxidation in metabolic tissues, decrease hepatic glucose production, increase glucose uptake in muscle cell culture, and alter food intake and energy expenditure through central actions (26,27,28,29). Low levels of adiponectin are associated with a predisposition for T2DM. Adiponectin plays an important role in the suppression of the metabolic disarrangement that may result in type 2 diabetes. The levels of adiponectin was reduced in C allele subjects compared to T allele

subjects. There was significant relationship between R61C OCT1 polymorphism and serum metformin levels, which is invariably associated with serum glycemic status. When looking for more frequent SNP in uncontrolled T2DM in the South Indian Tamilian population, it was identified that C allele is more common than T allele. Patients with T allele of OCT1 rs122083571 seem to be more sensitive to metformin treatment compared to individuals with the CC genotype.

CONCLUSION:

Adiponectin is an important adipokine, with a key role in many metabolic activities and its levels are reduced in genetic variants (C allele) of OCT1 rs122083571 snp subjects, who are on treatment with Metformin. This increases the risk of developing cardiovascular disease. On the basis of our study, we assume that T allele of OCT1 rs122083571 polymorphism may be beneficial for T2DM patients, both in case of efficacy to Metformin and adiponectin levels. A combination of adiponectin with a common anti diabetic drug like metformin could be potentially beneficial for the treatment for T2DM.

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

DECLARATIONS:

a) Institutional ethical committee of Rajah Muthiah Medical College Hospital, Annamalai Nagar have approved the study
b) Informed consent was obtained from the patients who participated in this study.
c) No competing interests: There is no conflict of interest among the authors or any other organizations or funding agencies.
d) Funding: The study was not supported by any grant

AUTHORS CONTRIBUTIONS:

Dr.Manju Koshy designed and conducted the study, performed the statistical analysis and wrote the manuscript. Dr. Jeeji Palocaren and Dr. Sethupathy conceived and designed the study and gave valuable comments. All authors read and approved the final manuscript.

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