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The relationship between *Multidrug Resistance Protein 1(rs1045642)* and *Cholesterol 24-hydroxylase (rs754203)* genes polymorphism with type 2 diabetes mellitus

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ABSTRACT

Background: The involvement of genetic factors like gene polymorphisms has been found to contribute significantly to the development and progression of type 2 diabetes (T2DM). Thousands of single nucleotide polymorphisms in various genes have been found to be associated with risk of T2DM. The present study was aimed to investigate association of Multidrug resistance 1 (*MDR1*) (rs1045642) and *CYP46A1* (rs754203) genes polymorphism with T2DM.

Subjects & Methods: Study includes 333 subjects, 183 T2DM cases and 150 healthy controls. Single nucleotide polymorphism was evaluated by PCR-PFLP. Alleles and genotype frequencies between cases and controls were compared using $\chi 2$ and Student's t-tests. Odds ratios and 95% confidence intervals were calculated by logistic regression to assess the relative association between disease and genotypes.

Results: In case of *CYP46A1* gene, CC ($p < 0.001$) and CT ($p = 0.001$) genotypes and C allele ($p < 0.001$) were found to be a positive risk factor and TT genotype ($p < 0.001$) and T allele ($p < 0.001$) as negative risk factor for T2DM whereas, no association of *MDR1* gene was found with T2DM (*P* values of all genotypes and alleles were greater than 0.001). *MDR1* (rs1045642) and *CYP46A1* (rs754203) genes polymorphism was not found associated with Fasting Blood Sugar (FBS), Diastolic Blood Pressure (DBP) and Systolic Blood Pressure (SBP).

Conclusion: *CYP46A1* gene polymorphism is associated with the risk of T2DM whereas, *MDR1* gene polymorphism was not found to confer any risk of T2DM in North Indian Ethnic group.

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MDR1; CYP46A1; gene polymorphism; type 2 diabetes mellitus

Introduction

Diabetes is posing a major disease epidemic throughout the globe. The number of people with diabetes has risen from 108 million in 1980 to 422 million in 2014.[1] The global prevalence of diabetes is rising and there is a predicted 60–70% increase in diabetic cases by the year 2030. India, as said to be the diabetic capital of the world, is alone harbouring around 40 million diabetes patients more than any other country of the world. This figure is expected to increase to 100 million by 2030.[2] Among the various forms of diabetes, type 2 diabetes mellitus (T2DM) is alone accounting for around 90% of the total diabetes cases. It is a complex disorder caused by interaction of multiple genetic and environmental factors. Increase in the genome-wide association studies along with the recent advancements in the field of genotyping had lead to identification of more than 65 genetic variants that increase the risk of T2DM by 10–30%.[3,4] A number of risk alleles for T2DM had been identified and mutations in several genes may add up and then predispose an individual to increased risk of T2DM.

Multidrug resistance 1 (*MDR1*) gene is a member of ATP-binding cassette transporter (ABC) family which is involved in many physiological processes such as lipid metabolism, ion homeostasis and in immune functions of pancreatic beta-cells. Mutations in genes encoding these transporters result in drug resistance in human pancreatic beta cells and also alter the pharmacokinetic properties of various antidiabetic drugs. Amongst the various ABC family transporter protein genes, *MDR1* encodes a 170 kDa ATP-dependent transmembrane efflux transporter called P-glycoprotein (P-gp) which confers drug resistance in cells. More than 50 single nucleotide gene polymorphisms have been found in *MDR1*, amongst which C3435T polymorphism, located in exon 26 have been identified to be a risk factor for numerous diseases by affecting posttranscriptional modifications of P-gp mRNA.[5] C3435T polymorphism leads to decrease in the expression of P-gp in individuals carrying the mutant TT genotype.[6–9] To date 15 ABC transporters have been found in pancreatic beta cells in humans and among them multidrug

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resistance protein 1 (MRP1/ABCC1), have been found to be associated with multidrug resistance (MDR) in diabetes mellitus.

Cytochrome P450 (CYP) is a phase I monooxygenase encoded by *CYP*, and which relies on haem cofactors to catalyse various reactions related to drug metabolism. Genetic variability in *CYP* may result in change in enzyme activity of the translated enzymes thus affecting the rate of drug metabolism. Polymorphisms in various *CYP* species, such as *CYP3A4* and *CYP2J2*, have been associated with the genetic risk for T2DM in Japanese and Chinese populations, respectively. [10,11] Apart from this, a number of studies have been conducted on *CYP46A1* polymorphisms in context to neurological disorders such as Alzheimer's disease and dementia.[12] An intronic T to C substitution in *CYP46* (rs754203) was found to be associated with increased risk of Alzheimer's disease in a Chinese population,[13] whereas there was a lack of association between this polymorphism encoding an enzyme acting on brain cholesterol turnover and Alzheimer's disease in Italian [14] and Hungarian populations [12]. Janson and colleagues reported an increased risk of T2DM in Alzheimer patients from south-east Minnesota.[15] Their study suggested a strong link between the neurodegenerative processes that lead to loss of cortical brain cells in Alzheimer disease and the loss of β-cells in T2DM. In support, pathology studies have also shown an increased islet amyloid in patients with Alzheimer disease this evidence suggests a link between Alzheimer's disease and T2DM. Thus, we hypothesised that polymorphism in *MDR1* (rs1045642) and *CYP46A1* (rs754203) are associated with risk of T2DM.

Materials and methods

Subjects:

Blood samples of 333 subjects including 183 T2DM cases and 150 controls were collected from Diabetic clinic of the Department of Medicine at Era's Lucknow Medical College & Hospital, Lucknow. Data collection included age, sex, blood pressure, body mass index, (BMI: Kg/m^2) height, weight, Hb A1C (%), lipid profile, etc.

T2DM cases were defined as patients having a fasting blood sugar (FBS) level of more than 6.99 mmol/l. Control samples were defined as those with FBS level below 6.105 mmol/l without family history of diabetes and none of them were receiving any medications at the time of participation. Patients suffering from type 1 diabetes, gestational diabetes, maturity-onset diabetes of the young or having secondary diabetic complications were excluded from the study. Protocol and procedures employed were reviewed and approved by the Institutional Ethical/ Review Committee. A written informed consent was taken from all participants.

Biochemical estimations:

Serum creatinine levels were measured using kinetic Jaffe method. FBS (glucose oxidase–peroxidase method), serum cholesterol (cholesterol oxidase–peroxidase), serum triglyceride (glycerol phosphate oxidase–peroxidase amidopyrine method), high-density lipoprotein (HDL) and cholesterol (immunoinhibition) were assessed by XL-300 Transasia Fully Auto Analyzer Transasia, Mannheim, Germany. Low-density lipoprotein (LDL) and total cholesterol levels were calculated using Friedewald's formula. HbA1C was measured using semiautoanalyzer (Transasia, Mannheim, Germany). For HbA1c estimation, we used Gen X hemoglobin A1c-Direct kit of Gen X special live series provided by Proton Biologicals, India Pvt. Ltd. The kit for calculation of results applies IFCC calibrated values using the following equation NGSP = (0.0915 X IFCC) + 2.15 expected values. (NGSP units in % while IFCC units were in mmol/mol Hb). All the assays were performed following the standard manufacturer's protocols. All experiments were performed in accordance with the ethical standards of the Helsinki Declaration. Genomic DNA was isolated from whole blood using DNA extraction kit (MACHEREY-NAGE, Germany) following manufacturers protocol. The DNA concentration was determined by a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific) and samples were stored at –20 °C.

Analysis of polymorphisms:

Genotyping for *MDR1 (C3435T) polymorphism* (rs1045642) was performed using PCR-RFLP with following primers,

Forward: 5′-GAT CTG TGA ACT CTT GTT TTC A-3′

Reverse: 5′-GAA GAG AGA CTT ACA TTA GGC-3′The 20 µl PCR reaction mixture had approximately 100–150 ng of genomic DNA, 10 pmol/l of each primer, 200 µmol/l of dNTPs, 20 mmol/l of TrisHCl, 50 mM of KCl, 2.5 mmol/l of $MgCl_2$, 1 U of Taq DNA polymerase and nuclease free water. The PCR Cycling Conditions include initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min and final extension at 72 °C for 5 min. After amplification, PCR products were subjected to restriction digestion using *DpnII* enzyme (New England Biolabs, USA) for 3 h at 55 °C. The fragment amplification and digestion results were revealed by 3% Agarose gel electrophoresis and visualised on a UV transilluminator after ethidium bromide staining. For *3435-C* allele, two fragments of sizes 172 and 72 bp were obtained whereas a fragment of size 244 was observed for *3435-T* allele (Figure 1).

Genotyping of CYP46A1 Polymorphism (rs754203):

Genotyping was performed using PCR-RFLP method using following primers, Forward: 5′-TGAAAACGA

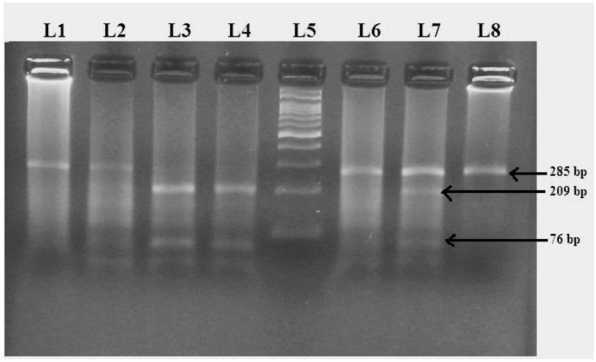

Figure 1. The 2% Agarose gel picture of *MspI* digested products of *CYP46A1* gene. Lane 1, 2, 6 and 8 shows the TT genotype corresponding to a single band size of 285 bp. Lane 3 and 4 shows CC genotype corresponding to two bands of 209 and 76 bp. Lane 7 shows CT genotype corresponding to three bands of 285, 209 and 76 bp whereas Lane 5 shows a 100 bp ladder.

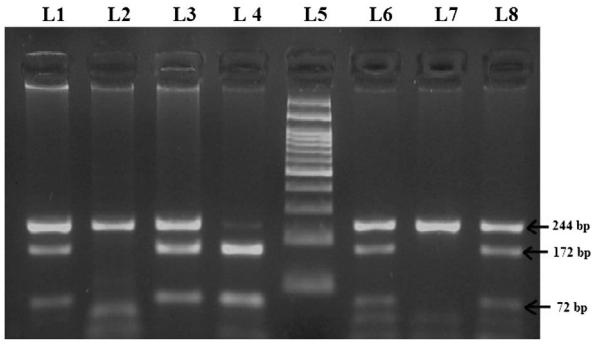

Figure 2. The 3% Agarose gel picture of *DpnII* digested products of *MDR1* gene. Lane 1, 3, 6 and 8 shows the CT genotype corresponding to band sizes of 244, 172 and 72 bp. Lane 2 and 7 shows TT genotype corresponding to a single band of 244 bp. Lane 4 shows CC genotype corresponding to two bands of 172 and 72 bp whereas Lane 5 shows a 100bp ladder.

GTTTCCCGTCC-3'Reverse:5'-GTGTGACCAGGTAACAGTCA-3' The 20 µl PCR reaction mixture had approximately 100–150 ng of genomic DNA, 10 pmol/l of each primer, 200 µmol/l of dNTPs, 20 mmol/l of TrisHCl, 50 mM of KCl, 1.5 mmol/l of $MgCl_2$, 1.25 U of Taq DNA polymerase and nuclease free water. The PCR Cycling Conditions include, initial denaturation at 95 °C for 8 min, after which the reaction mixture was subjected to 50 cycles of 1-min denaturation at 95°C, 1-min annealing at 53 °C, and a 2-min extension at 72 °C, followed by a final extension at 72 °C for 5 min. After amplification, PCR products were subjected for restriction digestion using *MspI* enzyme (New England Biolabs USA) over night. The fragment amplification and digestion results were revealed by 1.8% agarose gel electrophoresis and visualised on a UV transilluminator after ethidium bromide staining. The *CYP46A1*T* allele corresponded to the uncut 285-bp fragment, whereas the *CYP46A1*C* allele was characterised by two fragments of 209 and 76 bp (Figure 2).

Statistical analysis:

The sample size calculations were based on the proportion of *CYP46A1* genotypes among the cases and controls using the following formula:

$$N = (Z\alpha + Z\beta)2/\{\ln(1-e)\} \times \left[\{(1-p1)/p1\} + (1-p2)/p2\}\right]$$

where N is the required sample size for one group (when the groups are equal), Z is the level of significance, $p1 = 0.626$ and $p2 = 0.5$ (proportion of *MDR1 C3435T* polymorphism in cases and control) [16] Based on a 95% level of significance and 80% expected power (20% type 2 error), the minimum sample size was 136 in each group. Subject to the availability of cases, this was then increased so that the power would increase correspondingly. All the statistical analyses were performed with SPSS (Statistical Package for the Social Sciences) version 12 software. Clinical data are expressed as mean ± SD. The genotyping data were compared between cases and controls using χ^2 test. *P*-values ≤ 0.05 were considered as significant. Odds ratios (OR) and 95% confidence intervals (CI) were calculated to test the relative risk for association. Other variables were compared using Student's *t*-test for normally-distributed variables.

Results

Our study includes 333 individuals, 183 T2DM cases and 150 ethnicity matched healthy controls. Clinical and biochemical parameters of T2DM cases and controls are shown in Table 1. *MDR1 (C3435T)* and *CYP46A1* genotype distributions in all cases and controls were in line with Hardy–Weinberg equilibrium (all $p > 0.05$, data not shown).

No Significant differences were observed in the genotypes and alleles frequencies of *MDR1* on comparing T2DM cases with healthy control group. (Table 2)

Significant association was found between *CYP46A1* polymorphism with T2DM. CT, CC genotypes and C allele were found to increase the risk of T2DM whereas TT genotype and T allele conferred a low risk (Table 3).

There was no major difference in the frequencies of *MDR1* CC and TT genotypes in patients with a high FBS of more than 6.99 mmol/l. However, the frequency of occurrence of CT genotype was comparatively less when compared with CC and TT genotypes but overall, these genotypes were not found to be significantly associated with a high FBS. On comparing CC, CT and TT genotypes with high Systolic (SBP > 120 mm Hg) and Diastolic blood pressure (DBP > 80 mm Hg) no significant differences were observed.

There was no major difference in the frequencies of CC, CT and TT genotypes of *CYP46A1* in patients with a high FBS. Similarly, no significant difference was observed on comparing these three genotypes with high SBP and DBP. (Table 4).

Table 1. Biochemical and clinical characteristics of study subjects.

Variable	T2DM Cases ($N = 183$)	Controls ($N = 150$)	*P*-value
Sex (M/F) (*n*/%)	98 (53.55)/85 (46.45)	73 (48.67)/77 (51.33)	0.38
Age (years)	49.9 ± 10.2	42.6 ± 11.2	<0.0001
BMI (kg/m2)	27.0 ± 1.1	20.5 ± 8.6	<0.0001
SBP (mmHg)	137 ± 17	119 ± 3	<0.0001
DBP (mmHg)	86 ± 9	80 ± 5	<0.0001
FBS (mmol/l)	10.5 ± 4.6	5.04 ± 0.9	<0.0001
RBS (mmol/l)	12.8 ± 5.1	6.6 ± 0.9	<0.0001
Serum creatinine (micromol/l)	1260 ± 470	830 ± 970	<0.0001
Hb A1C (%)	8.6 ± 0.03	5.7 ± 0.2	<0.0001
Serum cholesterol (mmol/l)	10.8 ± 2.4	9 ± 1.1	<0.0001
Triglyceride (mmol/l)	1.7 (1.2–2.39)	1.2 (0.86–1.45)	<0.0001
HDL (mmol/l)	3.3 ± 0.8	3.2 ± 0.6	0.2
LDL (mmol/l)	5.6 ± 1.8	5.2 ± 0.9	0.03
VLDL (mmol/l)	2.8 ± 1.4	1.8 ± 0.7	<0.0001
WHR	0.95 ± 0.12	0.84 ± 0.8	0.1

Student's unpaired *t*-test was applied except in the case of gender comparison where the χ^2 test was applied and in Triglyceride comparison where Mann–Whitney test was applied. All values are mean ± standard deviation *n*(%), or median (IQR). BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; FBS: fasting blood sugar; RBS: random blood sugar; Hb A1C: glycated hemoglobin; HDL: high-density lipoprotein; LDL: low-density lipoprotein; VLDL: very low-density lipoprotein; WHR: waist to hip ratio.

Discussion

T2DM is a chronic, mutifactorial and progressive disease which has now become a topic of global concern. Untreated hyperglycemia may result in various secondary complications of T2DM resulting in long-term dysfunction and ultimately failure of organs including eyes, kidneys, nerves, the heart and blood vessels.[17]

MDR1 or *ABCB1* or cluster of differentiation 243 (*CD243*) gene encodes for P-gp protein, an ATP-dependent efflux pump, transporting foreign substances out of the cells. Researches till date have found around 50 SNPs and 3 insertion/deletion polymorphisms in the *MDR1* gene.[18] *C3435T* (rs1045642) is the most widely investigated SNP of *MDR1* which is implicated to be a risk factor for various diseases like diabetes, cancer,[19] Parkinson disease,[20] HIV-1 infection and progression,[21] hypertension,[22] rheumatoid arthritis [23] etc. Most of the effects of *MDR1 C3435T* polymorphism are said to occur as a result of decrease in the expression of P-gp protein which occurs due to the presence of polymorphic *C3435T* allele resulting in decreased stability of P-gp m-RNA.[24] Apart from decreased P-gp expression there was also a reduction in the expression of mRNA of few endogenous transcription inducers such as cytokines and growth factors in presence of *C3435T* genotype.[25]

An Asian study on *MDR1 C3435T* polymorphism reported that the presence of CC, TT and CT genotype was 18, 43 and 39% in Indians.[26] Another study reported these frequencies to be 12, 52 and 36% in the Indian population [27]. We found that the frequency of CT genotype in our T2DM cases was 45.9%, quite similar to the frequency of CT genotype in a Turkish population suffering from T2DM where it was reported to be 41.6%.[16] The frequency of TT genotype in T2DM cases in our study was 42.08% which was slightly higher than Turkish population (35.1%).[16] Our results showed that CT and TT genotypes were not associated with T2DM which is in agreement to the results from a similar Turkish study. On comparing the frequency of the CC genotype in our T2DM cases with the Turkish T2DM cases, we found that its frequency was lower in our population (12.02%) as compared to the Turkish population (23.4%).[16] The CC genotype showed no association with T2DM in our population ($p = 0.846$) which is similar to the results from Turkish population. The frequencies of C allele in our population (34.97%) was lower whereas the frequency of T allele was higher (65.03%) as compared to the Turkish population (44.15 and 55.85%) [16], respectively, and no association was found between T2DM and these alleles in either of the two populations. All the above-discussed genotyping results from our study population are shown in Table 2. On further studying the association of *MDR1 C3453T* polymorphism with biochemical parameters, we found no significant results similar to the results from Turkish population. The individuals having CT, TT, CC genotypes in our population were found to have nearly same mean systolic (139, 137 ± 14.904, 122 ± 15) and DBP (87, 88, 79) as the patients from Turkish population (138, 139, 127 and 86, 87, 79), respectively.[16] The above results from our population are shown in Table 3.

Cholesterol oxidation is mediated by two enzymes: cholesterol 24-hydroxylase encoded by *CYP46A1* and cholesterol 27-hydroxylase encoded by *CYP27A1* gene. Besseling et al. reported that high cholesterol in patients suffering from familial hypercholesterolemia lowers the risk of type 2 diabetes due to poor cholesterol uptake by cell.[28] A study on *CYP2D6, CYP2C9* and *CYP2C19* polymorphisms showed no relationship with diabetes pathogenesis in Bosnian population. Furthermore, *CYP* polymorphisms such as *CYP3A4*, have been associated with the genetic risk of T2DM in Japanese [10] and *CYP2J2 G-50T* polymorphism in Chinese populations. [11] In our study on *CYP46A1* polymorphisms, we found that the frequency of occurrence of CT and CC genotypes was higher in our T2DM cases (45.9 and 24%) as compared to healthy disease-free controls (29 and 4%) whereas the frequency of TT genotype was lower in cases (30.1%) as compared to controls (67.3%), respectively. A significant association was observed between all the three genotypes of *CYP46A1* polymorphism in our population with the risk of T2DM ($P \leq 0.001$) where CC and CT genotypes were found to increase the risk and TT genotype was decreasing the risk of T2DM. On studying the allele frequencies between cases and controls, we found that the frequency of carrying C allele was significantly higher in T2DM cases (47%, $p < 0.001$) as compared to controls (18%) suggesting C allele to be a positive risk factor for T2DM. On the other hand, the

Table 2. Genotypes & alleles frequencies of MDR1 in cases and controls.

	Controls ($N = 150$)		T2DM Cases ($N = 183$)							
Genotype/ Allele	*N*	Frequency (%)	*N*	Frequency (%)	Reference Genotype/Allele	OR	95% CI	χ^2	*P* values	Bonferroni corrected *p* values
CT	63	42	84	45.9	TT/CC	1.17	0.76–1.81	0.51	0.476	1.000
TT	70	46.67	77	42.08	CT/CC	0.83	0.54–1.28	0.70	0.401	1.000
CC	17	11.33	22	12.02	CT/TT	1.07	0.55–2.10	0.04	0.846	1.000
C	97	32.33	128	34.97	T	1.13	0.81–1.56	0.51	0.474	0.947
T	203	67.67	238	65.03	C	0.89	0.64–1.23	0.51	0.474	0.947

Note: Average power of study of this analysis = 0.813.

Table 3. Genotypes & alleles frequencies of CYP46A1 in cases and controls.

	Controls ($N = 150$)		T2DM Cases ($N = 183$)							
Genotype/ Allele	*N*	Frequency (%)	*N*	Frequency (%)	Reference Genotype/Allele	OR	95% CI	χ^2	*P* values	Bonferroni corrected *p* values
CT	43	28.67	84	45.9	TT/CC	2.11	1.33–3.34	10.38	0.001	0.003
TT	101	67.33	55	30.06	CT/CC	0.21	0.13–0.33	46	<0.001	<0.001
CC	6	4	44	24.04	CT/TT	7.6	3.14–18.40	25.95	<0.001	<0.001
C	55	18.33	172	46.99	T	3.95	2.76–5.65	60.28	<0.001	<0.001
T	245	81.67	194	53.01	C	0.25	0.18–0.36	60.28	<0.001	<0.001

Note: Average power of study of this analysis = 0.992.

Table 4. Association between FBS and blood pressure with CYP46A1 and MDR1 polymorphic and wild type genotypes.

		Genotypes				
Variables		CC	CT	TT	*F*-Value	*P* value
CYP46A1						
FBS (mmol/l)	Mean	11	11.5	10.4	0.251	0.779
	SD	3	5.5	5		
SBP (mm Hg)	Mean	137	136	134	0.167	0.847
	SD	19	17	15		
DBP (mm Hg)	Mean	85	84	88	1.197	0.307
	SD	11	9	8		
MDR1						
FBS (mmol/l)	Mean	11.7	6	10.4	0.036	0.964
	SD	4.8	4.7	4.7		
SBP (mm Hg)	Mean	122	139	137	1.517	0.228
	SD	15	20	15		
DBP (mm Hg)	Mean	79	87	88	1.576	0.216
	SD	7	10	9		

frequency of the T allele was significantly lower in T2DM cases (53%, $p < 0.001$) as compared to controls (82%) suggesting the T allele to be a negative risk factor for T2DM. The above results are shown in Table 2. In context to our study on biochemical parameters, we found no significant association between FBS, SBP and DBP and *CYP46A1* polymorphisms in our population. Individuals carrying CT, CC and TT genotypes were not found to significantly differ in their blood pressure and FBS levels ($p > 0.05$) as shown in Table 3.

The results generated by this study are novel, being the first association study of *CYP46A1* with T2DM and second global study after a Turkish study on *MDR* in T2DM. This study adds data beneficial for researcher working in various areas of diabetes research and ultimately give them a better prospective of biochemical and molecular mechanism of disease as insufficient information is available in relation to proposed polymorphisms with T2DM. As a preliminary finding this study should be replicated in individuals of non-Indian ethnicity and on a larger sample size so as to validate the results in a wider setting.

Summary table

What is known about this subject?

- The involvement of genetic factors such as gene polymorphisms contributes Significantly to the development and progression of type 2 diabetes (T2DM).
- A number of risk alleles for T2DM and mutations in several genes may add up and predispose an individual to increased risk of T2DM.

What this paper adds:

- CT, CC genotypes and C allele of *CYP46A1* increase the risk of T2DM whereas TT genotype and T allele conferred a low risk.
- *MDR1 (C3435T)* polymorphism was not found to be associated with T2DM.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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