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Original article

ACE (rs4646994) and MTHFR (rs1801133) single nucleotide polymorphisms in type 2 Diabetes Mellitus patients with dyslipidemia

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Abstract

Dyslipidemia associated with diabetes mellitus (DM) is the most common cause of coronary artery disease mortality. Both genetic and environmental factors have a role in the development of type 2 DM and dyslipidemia. The current study aims to find the role of angiotensin-converting enzyme (ACE) (rs4646994) and methyl-tetra-hydro-folate reductase (MTHFR) (rs1801133) polymorphisms with dyslipidemia in type 2 DM patients. One hundred and fifty (150) type 2 DM patients with dyslipidemia were included in this study, in addition to 150 type 2 DM patients without dyslipidemia and 150 healthy age and sex-matched control group. Venous blood samples were collected for the different laboratory workups and the gene polymorphism testing using the polymerase chain reaction-restriction enzyme fragment polymorphism (PCR-RFLP) technique. Both demographic and clinical parameters were collected from the study population. The results showed significant associations between ACE and MTHFR polymorphisms among the two patient groups (type 2 DM with dyslipidemia, with p-value<0.001 and 0.011, respectively), (type 2 DM without dyslipidemia with p-value=0.007 and 0.016, respectively). This study supports the notion that these commonly found ACE and MTHFR polymorphisms could be incriminated in the pathogenesis of dyslipidemia associated with type 2 DM.

Keywords: ACE, MTHFR, Single Nucleotide Polymorphisms, Diabetes mellitus.

Introduction

Type 2 DM is the most common type of diabetes, characterized by hyperglycemia as a result of defects in insulin hormone secretion and/or its physiological function. DM is associated with many health problems and complications affecting almost all body organs, including heart, kidney, nerves, eyes and blood vessels. Lipid abnormalities are one of the most common sequelae of uncontrolled DM. Dyslipidemia is the term used to describe high plasma triglyceride level, decreased high-density lipoprotein (HDL) cholesterol level and high level of low-density lipoprotein (LDL) cholesterol [1].

In recent decades, the prevalence of DM has risen dramatically in many countries, including the Middle East region and is expected to continue to increase dramatically. According to the International Diabetes Federation's (IDF) Middle East and North Africa (MENA), this is due to a range of factors, including rapid economic development and urbanization; lifestyle changes led to reduced levels of physical activity, increased intake of refined carbohydrates, and a rise in obesity. The current prevalence of diabetes in adults in the region is estimated to be around 9.2%. Of the 34 million people affected by diabetes, nearly 17 million were undiagnosed and therefore, at considerable risk of disease complications and poor health outcomes.

The annual health cost caused by diabetes and its complications accounts for around 6–12% of all healthcare expenditures [2]. Regarding the Egyptian population, DM is widespread in many families; nearly 15.6% of Egyptians (aged 20–79 years) have type 2 DM. Moreover, Egyptians' unhealthy diet may also contribute to the high prevalence of diabetes [3].

Patients with type 2 DM frequently are prone to cardiovascular diseases (CVD) and predisposed to dyslipidemia [1]. Dyslipidemia is a multi-factorial disorder resulting from the interaction of environmental and genetic factors. Genetic studies have focused on identifying target genes contributing to the risk of common complex diseases and their predisposing factors. Candidate genes increasing the risk of dyslipidemia were identified, such as apolipoprotein E, interleukin-6 and lipoprotein lipase dyslipidemia [4, 5]. The involvement of the Renin-angiotensin system (RAS) in cardiovascular hemodynamics is known and also in the development of CVD.

Angiotensin-converting enzyme (ACE) has a key role in RAS. It functions to convert angiotensin-1 to angiotensin-2 in the liver and inactivate bradykinin in many tissues. Insulin resistance activates the RAS, which could promote the development of dyslipidemia and diabetes [6]. MTHFR catalyzes the conversion of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a co-substrate for homocysteine re-methylation to methionine. Variations in this gene are quite common in healthy people. Some of these variants were associated with disease susceptibilities such as neural tube defects, dementia, colon cancer, and acute leukemia. MTHFR C677T allele was found to be associated with metabolic syndrome and its components, including central obesity, dyslipidemia, hypertension and hyperglycemia [7]. This study aimed to study the association of ACE (rs4646994) and MTHFR (rs1801133) polymorphisms in the development of dyslipidemia in type 2 DM patients.

Material and methods

This case-control study was conducted at the Suez Canal University hospital. The proposal was approved by the Ethics Committee of the Suez Canal University and carried out in accordance with the principles of the Helsinki Declaration. The study was carried out from October 2014 to July 2015. The control group was recruited from apparently healthy university staff volunteers.

In this study, the minimum sample size to identify the outcome was calculated to be 300, based on the prevalence of dyslipidemia was ~25.5% [8]. One hundred and fifty type 2 DM patients with dyslipidemia were included in this study, additionally, 150 type 2 DM patients without dyslipidemia and 150 healthy age and sex-matched control group. All study subjects were of Egyptian origin without any known other ethnicity. Informed consent was obtained from patients and healthy controls. All subjects underwent a complete physical examination and routine biochemical blood testing, including fasting blood glucose and lipid profile. Height and weight were taken to calculate body mass index (BMI).

Dyslipidemia was considered present when one or more lipid values (total cholesterol, LDL and triglycerides) increased or decreased HDL alone or in combination, using as cutoff values, those recommended by the ATP III guidelines on dyslipidemia and atherosclerosis prevention [9]. BMI was calculated using weight in kilograms/height in meter square. Serum creatinine concentration, HbA1c, random blood sugar, total cholesterol, triglyceride, and HDL- cholesterol were assessed by a fully automated auto-analyzer Cobas 6000 (Roche Diagnostics, Mannheim, Germany). LDL cholesterol levels were calculated using the Friedewald formula. Genomic DNA was extracted using the commercially available Spin-column technique kit for DNA extraction from whole human blood (QIAamp®DNA Blood Mini Kit, QIAGEN, 28159 Avenue Stanford, Valencia, CA 91355, USA). Extracted DNA samples were stored at -20°C for subsequent use.

ACE polymorphism

PCR for determination of ACE gene polymorphism was done using the following primers' sequence [10]:

Forward primer:

5'-CTGGAGACCACTCCCATCCTTTCT-3'

Reverse primer:

5'-GATGTGGCCATCTTCGTCAGAT-3'

PCR was carried out using a ready-to-use PCR buffer (Gen-Taq Master Mix, BIORON, Germany). The PCR condition was an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of melting at 94°C for 45 seconds, annealing at 60°C for 60 seconds, and extension at 72°C for 120 seconds, with a final extension step of 5 minutes at 72°C, using a thermal cycler (ThermoHybaid PX2, UK). The amplified product of 490 bp for allele I and 190 bp for allele D were separated using a 2% agarose gel.

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MTHFR polymorphism

It was carried out using PCR-Restriction enzyme Fragmentation Length Polymorphism (PCR- RFLP). It was done using the following primers' sequence [11]:

Forward primer:

5'-TGAAGGAGAAGGTGTCTGCGGGA-3'

Reverse primer:

5'-AGGACGGTGCGGTGAGAGTG-3'

PCR was carried out using a ready-to-use PCR buffer (Gen-Taq Master Mix, BIORON, Germany). The PCR condition was an initial denaturation at 94°C for 4 minutes, followed by 35 cycles of melting at 94°C for 30 seconds, annealing at 61°C for 30 seconds, and extension at 72°C for 30 seconds, with a final extension step of 7 minutes at 72°C, using a thermal cycler (ThermoHybaid PX2, UK). The amplified product was digested by HinfI for 10 hours at 37°C, producing fragments of 198 bp for the allele C and 175 bp for the allele T.

Statistical analysis

Statistical analyses were performed using SPSS/PC Statistical Package v.16.0 (SPSS, Inc., Chicago, IL). All p-values were two-tailed; $p<0.05$ was considered statistically significant. Clinical data are expressed as mean±SD. The Chi-square test was used to compare genotyping data between cases and controls. Odds ratios (OR) and 95% confidence intervals (CI) were calculated to test the relative risk for the association. Other variables were compared using Student's t-test for normally distributed variables.

Results

This study included 150 type 2 DM patients with dyslipidemia, 150 type 2 DM patients without dyslipidemia and 150 healthy age and sex-matched control group. Demographic, clinical and biochemical variables of study cases and controls are presented in Table 1.

Type 2 DM patients with dyslipidemia showed a significant association of BMI, serum triglyceride level and HDL-cholesterol level compared to both type 2 DM patients without dyslipidemia and control with p-value<0.001. Genotype distribution and allele frequencies of ACE and MTHFR genes among the different study groups are presented in Table 2. Regarding the ACE gene, there were significant differences for both genotypes II and ID among type 2 DM patients without dyslipidemia in comparison to healthy control (p-value<0.001 and p-value=0.011, respectively), as well as among type 2 DM patients with dyslipidemia in comparison to healthy control (p-value<0.001 and <0.001 respectively). Additionally, the patient who carries the ID genotype has approximately 3 times more to develop dyslipidemia than other genotypes (OR=2.62; 95% CI: 1.52–4.53; p-value<0.001), as shown in Table 3.

As for the MTHFR gene, when comparing genotype frequencies, a significantly higher frequency of genotype CT and a significantly lower frequency of genotype CC were noticed among type 2 DM patients with and without dyslipidemia in comparison to healthy control (p-value<0.001).

Discussion

Type 2 DM is one of the major and widely spread health problems around the globe. As with complex diseases, it is caused by environmental and genetic factors and their interaction. Researchers studied many genes to assess their incrimination in the causation and complications of this widespread health problem. To date, various gene mutations and polymorphisms have been linked to the risk for type 2 DM [12, 13]. In the current study, ACE (rs4646994) and MTHFR (rs1801133) polymorphisms were studied for their association with type 2 DM patients with and without dyslipidemia among Egyptians. The distribution of both single nucleotide polymorphisms among all cases and controls was in line with the Hardy-Weinberg equilibrium.

Table 1: Demographic, clinical and biochemical variables of study populations.

Item	Healthy control	Type 2 DM without dyslipidemia	Type 2 DM with dyslipidemia
Age (years)	49.1±8.1	50.2±7.4	52.3±6.1
BMI (kg/m²)	23.6±3.2	24.3±2.9	28.3±3.9
RBS (mg/dl)	116±37	216±55	238±72
S. creatinine	0.91±0.17	1.02±0.21	1.24±0.32

Table 1: Continued.

Item	Healthy control	Type 2 DM without dyslipidemia	Type 2 DM with dyslipidemia
HbA1c (%)	5.5±0.5	7.9±0.7	8.4±0.8
T. chol (mg/dl)	145±33	245±31	294±41
TG (mg/dl)	126±19	218±27	315±37
HDL-C (mg/dl)	55.2±9.4	44.3±8.4	37.3±8.3
LDL-C (mg/dl)	98±13	128±12	193±16

Note: BMI – Body mass index; RBS – Random blood sugar; S. – Serum; T. chol. – Total cholesterol; TG – Triglycerides; HDL-C – High density lipoprotein-cholesterol; LDL-C – Low density lipoprotein-cholesterol.

Several previous studies were done to assess the association between ACE polymorphism with the risk for type 2 DM among different ethnic groups. The results revealed an inconsistency with the risk of type 2 diabetes and related complications. Studies done among Indian, Iranian and Tunisian populations noticed that allele D of the ACE gene was highly associated with the risk of type 2 DM and its related complications [14, 15]. However, studies done among Indonesian and Malays populations did not find an association of either allele with type 2 DM nor with its renal or cardiovascular complications [16, 17].

This study showed that among type 2 DM patients without dyslipidemia, about 35% were genotype II. This figure is high compared to Brazilians, who had a prevalence of about 19%, and the UAE population, which showed a prevalence of about 11% [18, 19]. Also, among type 2 DM patients with dyslipidemia, about 65% of them carried the genotype ID. This figure is also higher compared to Kuwaiti populations with type 2 DM, which showed a prevalence of about 44% [19]. Our results presented a significant association between the genotype ID of the ACE gene and the development of type 2 DM, regardless of the presence or absence of

Table 2: Genotype distribution and allele frequency of ACE and MTHFR genes among study populations.

Item	Type 2 DM without dyslipidemia [a]	Type 2 DM with dyslipidemia [b]	Healthy control [c]	P-value [a] *versus* [b]	P-value [b] *versus* [c]	P-value [a] *versus* [c]
ACE gene						
Genotypes						
II	53 (35.3%)	30 (20%)	21 (14%)	<0.001	<0.001	0.116
ID	66 (44%)	98 (65.3)	87 (58%)	<0.001	0.067	0.171
DD	31 (20.7%)	22 (14.7%)	42 (28%)	0.137	0.139	0.011
Allele						
I	172 (57.3%)	158 (52.7%)	129 (43%)	0.201	<0.001	0.011
D	128 (42.7%)	142 (47.3%)	171 (57%)	0.202	<0.001	0.011
MTHFR gene						
Genotypes						
CC	55 (36.7%)	64 (42.6%)	85 (56.7%)	0.311	<0.001	0.003
CT	70 (46.7%)	60 (40%)	43 (28.7%)	0.196	<0.001	0.018
TT	25 (16.6%)	26 (17.4%)	22 (14.6%)	0.812	0.598	0.413
Allele						
C	180 (60%)	188 (62.7%)	213 (71%)	0.451	0.091	0.071
T	120 (40%)	112 (37.3%)	87 (29%)	0.399	0.102	0.061

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Table 3: The univariate logistic regression analysis of the distribution of the genotypes and allele frequency of ACE and MTHFR genes between Type 2 DM without and with dyslipidemia.

Item	Type 2 DM without dyslipidemia	Type 2 DM with dyslipidemia	OR (95% CI)	P-value
ACE gene				
Genotypes				
II	53 (35.3%)	30 (20%)	1.00	
ID	66 (44%)	98 (65.3)	2.62 (1.52-4.53)	<0.001
DD	31 (20.7%)	22 (14.7%)	1.25 (0.62–2.54)	
Allele				
I	172 (57.3%)	158 (52.7%)	1.00	0.202
D	128 (42.7%)	142 (47.3%)	1.207 (0.875–1.666)	
MTHFR gene				
Genotypes				
CC	55 (36.7%)	64 (42.6%)	1.00	
CT	70 (46.7%)	60 (40%)	0.74 (0.45–1.21)	0.485
TT	25 (16.6%)	26 (17.4%)	0.89 (0.46–1.72)	
Allele				
C	180 (60%)	188 (62.7%)	1.00	0.421
T	120 (40%)	112 (37.3%)	0.893 (0.643–1.241)	

dyslipidemia. This finding was in agreement with a study done among the Chinese population [20].

The distribution of the different genotypes of the MTHFR gene was found to be highly variable between different ethnic populations and even within the same ethnicity [21]. However, several previous studies reported that carriers for the genotype 677TT were at high risk for the development of dyslipidemia and high blood pressure, which are key components of metabolic syndrome [22, 23]. The current study showed that type 2 DM patients with and without dyslipidemia had a lower significant prevalence of genotype CC compared to healthy controls. In contrast, the genotype CT was significantly higher among type 2 DM patients with and without dyslipidemia compared to the healthy group. Carriers of genotype CC for the MTHFR gene among type2 DM populations with dyslipidemia comprised about 42%, a figure higher in comparison to hypertensive Chinese patients with dyslipidemia (about 25%) [24].

Among the study populations, about 61% of the type 2 DM carried the allele C and about 39% of them carried the allele T. Brazilian populations with type 2 DM showed a nearly similar prevalence of both allele frequencies (68% and 32 respectively) [25]. Also, among type 2 DM patients without dyslipidemia, about 36% were carriers for the genotype CC, which is considered higher in comparison to Turkish type 2 DM patients (about 29%) [26].

Conclusion

In conclusion, ACE and MTHFR genes appear to be implicated in the risk of dyslipidemia with T2DM cases. The complex interplay between genetic and environmental factors should be considered in order to evaluate the etiological role of ACE and MTHFR polymorphisms in type 2 DM and dyslipidemia. This result adds to the pool of genetic pre-distortion to complex diseases such as T2DM and its complications in different ethnic groups. Further analysis of these polymorphisms in different ethnic groups is required.

Conflict of interest

The authors declare no conflict of interest.

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