APOLIPOPROTEIN E (APOE) GENE POLYMORPHISMS AND THEIR ASSOCIATION WITH CARDIOVASCULAR DISEASE (CVD) IN TYPE 2 DIABETES MELLITUS (T2DM)

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Abstract

Apolipoprotein E (ApoE) is well known for its crucial role in lipid metabolism and is associated with an increased risk of cardiovascular disease (CVD) among subjects with type 2 diabetes mellitus (T2DM). Various apolipoprotein gene families have been determined, including APO (A-I), APO (A-II), APO (A-IV), APO (C-1), APO (C-II), APO (C-III), and APOE. A total of 3,597 nucleotides are encoded by the APOE gene, with four exons and three introns, constituting a polypeptide with 299 amino acids. The current study embarks on linking APOE gene polymorphisms with CVD among patients diagnosed with T2DM. This cross-sectional study involved 101 subjects with specific inclusion and exclusion criteria. The participants were separated into two groups, T2DM (n = 59) and T2DM with CVD (n = 42). Comparative analyses of clinical and biochemical characteristics were performed using student's t-test and Pearson's chi-square test (x^2). Univariate and multivariate analyses were applied to establish the relationship between APOE gene polymorphisms with ischemic heart disease. The ε3/ε3 genotype was the most prevalent among both groups. The ε3/ε3 genotype (AOR= 0.052; 95% CI = 0.003-0.792; p = 0.033), ε3 allele (AOR = 34.83; 95% CI = 1.118-1085.134; p = 0.043), systolic blood pressure (SBP) (AOR = 1.046; 95% CI = 1.002-1.091; p = 0.042), and HbA1c (AOR = 2.286; 95% CI = 1.577-3.314; p < 0.001) were significantly associated with CVD. The ε3/ε3 genotype was also significantly associated with the lipid parameter, low density lipoprotein cholesterol (LDLc) (p = 0.011). Most T2DM patients presented with ε3 allele which may affect lipid profiles and the risk of CVD disease. This highlights the need to establish APOE as a likely predictive gene for CVD disease in T2DM subjects.

Keywords: Apolipoprotein E, Polymorphism, Type 2 Diabetes Mellitus, Cardiovascular Disease

Introduction

The most prevalent metabolic disease, diabetes mellitus (DM) is specified as a state of hyperglycemia attributable to abnormalities in insulin secretion or insulin processing, or both (1). It is an incurable, progressive and injurious disease affecting individuals of all ages, but it is preventable (2). The two categories of diabetes are insulin-dependent diabetes (type 1 diabetes mellitus) and non-insulin-dependent diabetes (type 2 diabetes mellitus). Type 1 diabetes mellitus (T1DM) manifests expeditiously as insulin production from the pancreas is destroyed while type 2 diabetes mellitus (T2DM) develops insidiously as insufficient insulin causes blood glucose levels to rise (3). T2DM is due to insufficient secretion as well as resistance to the action of insulin. In addition, T2DM is an essential independent predictor for CVD such as coronary artery and ischemic heart disease.

APOE is a glycoprotein found in very-low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), high-density lipoprotein (HDL), chylomicron, and chylomicron remnants. There are several apolipoprotein gene families, including APOA-I, APOA-II, APOA-IV, APOC-1, APOC-II, APOC-III and APOE (4). A total of 3,597 nucleotides are encoded by four exons and three introns of the APOE gene, which constitute a 299-amino acid polypeptide (5). The ε2, ε3 and ε4 alleles are the most prevalent alleles. The ε3 allele consists of cysteine and arginine at position 112 and 158, respectively. The ε2 has cysteine at both positions, while ε4 has arginine (6). The ε3 allele is the most common allele and can be found in more than 80% of the general population, followed by ε4 and ε2. APOE has three major isoforms (ApoE2, ApoE3, and ApoE4) and various genotypes, ε2/ε2, ε4/ε2, ε3/ε2, ε3/ε3, ε4/ε3, and

ε4/ε4 (7). Each APOE isoform interacts differently with various receptors (8).

The ε3 is the most common isoform, encoded by three alleles, and it binds to its receptors well. Meanwhile, the ε2 and ε4 forms have lower and higher binding affinities, respectively (7). As a consequence, ε2 carriers have been observed to have low E-containing lipoprotein metabolism, which may lead to familial dysbetalipoproteinemia (9). APOE ε4 carriers have been linked to elevated cholesterol absorption, decreased sensitivity to statin treatment, and an increased possibility of atherosclerosis. Although it is mostly secreted by the liver and intestine, certain tissues and cells, such as the brain and macrophages, can synthesize it locally (9). This protein interacts with fats in the body to form lipoproteins. Lipoproteins play an important role in transporting cholesterol along with other fats via the bloodstream (10). Thus, the APOE gene assumes a significant role in lipoprotein metabolism and lipid transport (11).

Cardiovascular diseases, the top-tier cause of death in patients with diabetes, have greatly increased. In Malaysia, about 44.7% patients admitted for acute coronary syndrome were diabetic (12). Cardiovascular system harm in diabetic individuals has been linked to lipoprotein-related processes (13). Variations in APOE, which affects lipoprotein metabolism in the body, is one of the most studied molecular variations in diabetes and heart disease over the past ten years (14). A significant amount of research has been done to determine the link between APOE genotypes and lipid profiles.

Over recent decades, there has been a steady rise in the number of studies supporting an association between APOE polymorphisms in T2DM and CVD. Zheng et al. (15) initially investigated the relationship between T2DM complications with CVD and APOE polymorphisms among the Chinese population. Their findings demonstrated that the APOE ε4 allele enhanced the incidence of CVD in T2DM (15). This was also supported by other studies (16, 17). APOE ε2 allele was also found to be associated with CVD risk in T2DM (18). However, there are also several studies that found no association between APOE ε2/ε3/ε4 polymorphisms and the incidence of CVD in T2DM (19-21). Since research is limited and ambiguous, more research is required to ascertain the role of APOE allele subtypes with CVD. Thus, this study aimed to explore the link between APOE gene polymorphisms and heart disease among diabetic patients.

Materials and Methods

Subjects

A total of 101 T2DM patients from the Clinical Specialist Clinic, Universiti Sains Malaysia Bertam Medical Centre, Penang, Malaysia was recruited in this cross-sectional study. Patients aged between 18 to 80 years old that received a diagnosis of T2DM were divided into two groups, T2DM with CVD (n = 42) and T2DM without CVD (n = 59). These patients were referred from several local health clinics in the northern area of Peninsular Malaysia. The majority of patients were ethnically Malay. Meanwhile, the exclusion criteria were individuals who were smokers, pregnant, had underactive thyroid function, had kidney disorder(s) or with history of alcohol abuse. Informed consent and patient information sheets were obtained from all participants. The study protocol was endorsed by the Research and Ethics Committee of Universiti Sains Malaysia (Ethics Approval No. USM/JEPeM/18050242). Participation was strictly voluntary, and anonymity of the participants was assured.

Measurement of clinical and biochemical parameters

Clinical data such as body mass index (BMI), systolic blood pressure (SBP), diastolic blood pressure (DBP) and pulse rate were obtained from the patients' records. Isolated plasma samples (5 mL) were collected in a plain tube stored at 4°C and analyzed at the IPPT Chemical Pathology Laboratory (Universiti Sains Malaysia) using standardized in-house methods to measure biochemical parameters including total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDLc), and low-density lipoprotein cholesterol (LDLc).

APOE genotyping

Genomic DNA was isolated from 2 mL of whole peripheral blood using the QIAamp DNA extraction kit (Qiagen Hilden, Germany), in accordance with the manufacturer's instructions. Polymerase chain reaction (PCR) was performed with an initial denaturation at 95°C for 5 min (30 cycles), annealing at 60°C for 1 min, extension at 70°C for 2 min and final denaturation at 95°C for 1 min to complete all cycles. The primer sequences were as follows: F6 upstream primer 5'-TAAGCTTGGCACGGCTGTCCAAGGA-3', F4 downstream primer 5'-ACAGAATTCGCCCCGGCCTGGTACAC-3'. Restriction fragment length polymorphism (RFLP) analysis was subsequently used to determine the exact order of the APOE gene base pairs. APOE genotyping and allele frequency were performed via allele counting, using Thermo Scientific™ GeneRuler DNA Ladder, Ultra Low Range as a reference.

TaqMan® SNP Genotyping Assay was used to genotype the APOE gene. The encoded alleles 2 (rs429358-T + rs7412-T), 4 (rs429358-C + rs7412-C), and 3 (rs429358-T + rs7412-C) were determined by SNPs at locations 112 (rs429358) and 158 (rs7412). The ABI 7500 Real Time PCR (Applied Biosystems, USA) was used for genotyping analysis in accordance with the manufacturer's instructions. All primers and probes were developed by Applied Biosystems (Foster City, CA, USA). Negative controls were included for genotyping quality control. Ten percent of samples were randomly chosen and re-examined twice, with a 100% concordance rate.

Statistical analysis

Statistical package for Social Science (SPSS), IBM, Chicago, IL, USA version 27.0 was used for statistical analysis. Continuous data was expressed as mean value and standard deviation (SD), and categorical data as percentages (%). Comparative analysis of clinical parameters between the two groups (T2DM without CVD and T2DM with CVD) was performed using Student's t-test. Differences in allele frequency and genotypes were analyzed using the Pearson's chi-square test (x^2). Multiple logistic regression was applied to determine the associated factors for CVD in T2DM patients.

Results

Clinical attributes of study subjects

Overall, 101 participants who met the set criteria were recruited. Participants were divided into two groups; patients diagnosed with T2DM only (58.4%) and patients with T2DM and CVD (41.6%). Their clinical and biochemical data, including age, BMI, sex, blood pressure (SBP and DBP), glucose, HbA1c, and lipid parameters (TG, TC, LDLC, and HDLC) are listed in Table 1.

Table 1: Clinical attributes of the study subjects

Demographics	T2DM	T2DM + CVD	p-value#
	n = 59 (%)	n = 42 (%)	
Age (years)	52.47	59.43	0.003#
Sex			
Male	20 (47.6%)	37 (62.7%)	0.157
Female	22 (52.4%)	22 (37.3%)	0.096
BMI (kg/m²)	31.35 (7.55)	29.61 (6.49)	0.230
Blood pressure (mmHg)	137.83 (14.62)	146.17 (15.92)	0.008#
SBP	81.29 (10.91)	74.14 (18.01)	0.015#
DBP			
Pulse (Bpm)	79.58 (10.93)	83.17 (12.69)	0.131
Diabetic studies			
Glucose (mmol/L)	6.29 (1.72)	8.48 (4.73)	0.001#
HbA1c (%)	6.64 (1.13)	8.34 (1.79)	< 0.001#
Lipid profile (mmol/L)	1.59 (0.69)	1.62 (0.72)	0.830
TG	4.87 (1.17)	4.64 (0.94)	0.285
TC	2.86 (1.18)	2.54 (0.78)	0.128
LDLC	1.28 (0.42)	1.35 (0.48)	0.438
HDLC			

BMI = body mass index, *SBP* = systolic blood pressure, *DBP* = diastolic blood pressure, *HbA1c* = haemoglobin A1C, *TG* = triglyceride, *TC* = total cholesterol, *LDLC* = low-density lipoprotein cholesterol, *HDLC* = high-density lipoprotein cholesterol

*Student's t test
**Pearson's Chi-square test (x^2) of contingencies
#p-value < 0.05 is statistically significant

The prevalence of APOE genotypes and alleles

RFLP analysis of the most frequent genotype (ε3/ε3) is presented in Figure 1. The ε3/ε3 genotype was the prevalent genotype across both study groups (Table 2); T2DM (72.9%) and T2DM with CVD (71.4%). The ε2/ε4 and ε2/ε2 genotype were more common in the T2DM only group (16.9% and 3.4%, respectively). Meanwhile, the ε3/ε4 and ε2/ε3 genotype were prevalent in T2DM with CVD group (9.5% and 7.2%, respectively). The ε4/ε4 genotype was not detected in either group. Our analysis revealed that patients with the ε3/ε3 genotype had significantly different LDLc values (p-value = 0.011). Patients with ε3/ε3 genotype also had the highest TG (1.638 ± 0.736 mmol/L), TC (4.655 ± 0.878 mmol/L), and HDLC (1.395 ± 0.543 mmol/l) levels.

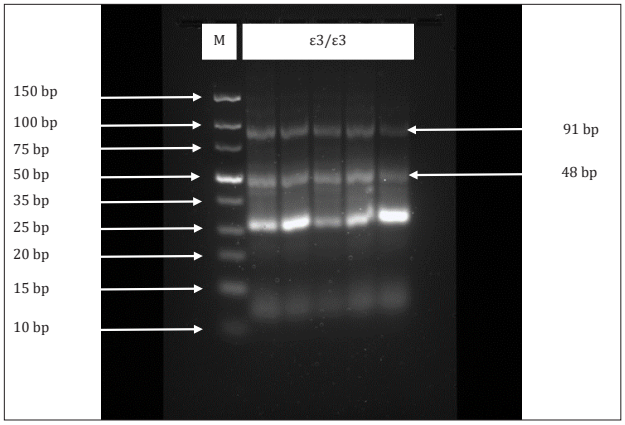

Figure 1: Electrophoresis Hhal fragments for isoform ε3/ε3. The fragments sizes (bp) were determined using DNA standards (marked as M on the left of the gel).

Table 2: APOE genotypes and alleles

Parameters	T2DM, n = 59 (%)	T2DM + CVD, n = 42 (%)	x^2
APOE genotypes	2 (3.4)	1 (2.4)	
ε2/ε2	43 (72.9)	30 (71.4)	
ε3/ε3	1 (1.7)	3 (7.2)	0.316
ε2/ε3	10 (16.9)	4 (9.5)	
ε2/ε4	3 (5.1)	4 (9.5)	
ε3/ε4			
APOE alleles			
ε2	3 (5.1)	4 (9.5)	
ε3	46 (78.0)	34 (81.0)	0.000
ε4	10 (16.9)	4 (9.5)	

Values are expressed as number and frequency. Analysis was performed using the Pearson's chi-square test (x^2)

Associated factors for CVD in T2DM patients

Multivariate logistic regression analysis after adjustment for other established risk factors revealed the ε3/ε3 genotype (AOR = 0.052; 95% CI = 0.003-0.792; p = 0.033), ε3 allele (AOR = 34.83; 95% CI = 1.118-1085.134; p = 0.043), SBP (AOR = 1.046; 95% CI = 1.002-1.091; p = 0.042) and HbA1c (AOR = 2.286; 95% CI = 1.577-3.314; p < 0.001) have a significant association with CVD as shown in Table 3.

Table 3: Associated factors for CVD in T2DM patients

Variables	T2DM + CVD		
	Regression coefficient (b)	Adjusted Odds Ratio (OR)[a] (95% CI)	p-value
APOE gene polymorphism			
ε3/3	-2.97	0.05 (0.00, 0.79)	0.03*
ε3	3.55	34.83 (1.12, 1085.13)	0.04*
SBP	0.05	1.05 (1.00, 1.09)	0.04*
HbA1c	0.83	2.29 (1.58, 3.31)	< 0.00*

[a] Multiple Logistic Regression, Backward LR method

*p-value < 0.05 is statistically significant

Assumption testing did not indicate any violation of interaction and multicollinearity.

Hosmer Lemeshow test confirmed that the model was a good fit for the data, with 76.2% accuracy in its prediction of CVD risk in T2DM.

Area under the ROC curve (0.819) was applied to check for model fitness.

Association between APOE genotypes and lipid profile in T2DM with CVD

Our study revealed that the ε3/ε3 genotype had a significant association with LDLc (p = 0.011) as shown in Table 4.

Table 4: Association between APOE genotypes and lipid profile in T2DM with CVD

	T2DM + CVD			
Parameters (mmol/L)	**ε3/ε3**	**p-value**	**Non ε3/ε3**	**p-value**
TG	1.638 ± 0.736	0.821	1.571 ± 0.706	0.314
TC	4.655 ± 0.878	0.115	4.600 ± 1.129	0.820
LDLc	2.488 ± 0.709	0.011**	2.653 ± 0.965	0.864
HDLc	1.395 ± 0.543	0.339	1.235 ± 0.227	0.583

Non ε3/ε3 = ε2/ε2, ε4/ε4, ε2/ε3, ε2/ε4 and ε3/ε4

*Student's t test

**p-value < 0.05 is statistically significant

Discussion

Apolipoprotein E is a 299 amino acid glycoprotein found in blood. APOE functions to transport cholesterol and other fats through the bloodstream (22). APOE typically binds to many hepatic lipoprotein receptors, including LDL-R and LDL-related protein (LRP1), and is engaged in various metabolic processes including transportation and degradation of lipoproteins (8, 23). Some pathophysiological conditions related to APOE may be associated with isoform-dependent modifications (13, 22). Previous studies found that APOE gene polymorphisms is an emerging risk factor for developing CVD among diabetic patients (24-29). Our research focused on identifying the relationship between APOE gene polymorphisms and heart diseases among T2DM patients and its ramification on plasma lipid profile.

It is interesting to note that the ε3 allele and ε3/ε3 genotype were the dominant APOE gene polymorphism. The ε3 allele was found to be a robust independent predictor for the development of CVD in patients with T2DM. According to previous studies, varying outcomes have been found across different ethnic groups (24-29). This disparity may possibly be due to the differences in genetics of various populations (29).

In agreement with our study, the ε3 allele was found to be the most frequent isoform in Asia and the most likely to be implicated in CVD (27). This is contrast with a study by Chaudhary et al., who reported that those with the ε4 allele have a higher possibility of developing CVD, as compared to those with the ε2 allele (30). Similarly, a study conducted within the Hakka population showed that the ε3/ε3 genotype was the most common genotype, and the ε4 allele may potentially be a major predictor of T2DM and CVD (13). Diabetic individuals with the ε3/ε4 genotype and ε4 allele had increased risk of developing CVD (31). However, Rahman et al. (25) and Marrzoq et al. (26) reported there were no statistically significant associations between APOE polymorphisms with T2DM and CVD in the Malaysian and Saudi population, respectively.

APOE alleles have been shown to have opposing effects on regulating the levels of differential lipids, as influenced by genetic variables (32). These variations can possibly be explained by the diversity of APOE genotypes found in the study population and their ethnic origins (32-34). Various outcomes associated with APOE gene polymorphism have been reported across different study populations. There are studies that report a strong relationship between T2DM, CVD and its effect on lipid profiles (22, 29, 30). This could be related to the interrelationship between APOE alleles and genetic-environmental interactions in differential lipid studies (1). In a Thai population, ε4 allele carriers have higher VLDLc and TG levels, and lower HDLc levels compared to ε3/ε3 carriers (30). Individuals with ε3/ε4 genotype were found to have lower HDLc and higher LDLc concentrations among both Indian and Chinese Han populations (35-37).

Since T2DM and CVD are both classified as multifactorial diseases caused by interactions between genetic and environmental factors, researchers should conduct further investigations using a larger sample size, incorporating genetic and environmental influences to definitively explore the interaction between APOE polymorphisms and

the risk of CVD among patients with T2DM (32). El-Lebedy et al. (22) proposed that genotype is linked with both the environment and population, thus highlighting the impact of APOE polymorphisms across various populations of T2DM patients. Moreover, the same study also showed a positive association between plasma lipid levels and APOE genotypes in both T2DM and CVD patients.

Our study demonstrated that SBP and HbA1c was significantly associated with increased risk for CVD in T2DM patients. It is worth noting that our study found that SBP and HbA1c are potential dominant predictors for developing CVD in T2DM patients. This finding was contrary with previous studies, where SBP and HbA1c showed no such associations (13, 22, 30). Fluctuations in blood glucose levels are closely linked to the incidence and progression of CVD (38). The International Expert Committee from the American Diabetes Association (ADA) issued a statement in 2009, recommending that a HbA1c value of 6.5% (48 mmol/mol) be used as a diagnostic level for diabetes diagnosis (39), since it was shown to be substantial, following which there was an increase in the incidence of retinopathy, a typical consequence that frequently manifests prior to the definitive identification of diabetes (39, 40). HbA1c levels exceeding 6.5% may cause cardiovascular damaged. Due to its continuous, slow, and irreversible non-enzymatic reaction, this can lead to the formation of glycosylated hemoglobin. The HbA1c concentration is influenced by long-term blood glucose levels rather than fluctuations that are triggered by immediate causes such as exogenous insulin therapy or acute glucose ingestion (41). HbA1c is released into the bloodstream as red blood cells are destroyed by the spleen. Free HbA1c can increase the level of C-reactive protein (42), oxidative stress (43), and blood viscosity (44). These processes collectively contribute to the development of CVD by causing damage to the endothelial cells lining blood vessels (45).

Intensive diabetes therapy reduces the risk of long-term CVD (46). Lipid-lowering therapy and blood pressure reduction also reduces all-cause mortality, emphasizing the critical role of these treatments in reducing CVD and all-cause mortality in T2DM patients (47). Previous hyperglycemia is highly associated with the likelihood of diabetic complications in T2DM patients. Any reduction in HbA1c is likely to minimize the risk of complications, with lower risk among individuals with HbA1c values within the normal range (48).

A study among the Hakka community reported that age and smoking to be independent risk factors for T2DM and CVD (13). This study also revealed that the ε3/ε3 genotype had a significant association with LDLc values. It may also explain the reason why APOE genotype is strongly related with CVD development. Consistent with findings reported by Elmadbouh et al. (32), our study showed that TC and LDLc levels are strongly related with the ε3 allele. However, a significant difference between the ε2 and ε4 allele in HDLC was not found. On the other hand, low HDLc levels have been linked to higher risk of CVD, mortality, atherosclerosis, and cognitive decline (33, 34).

This study contributes to the growing number of studies on APOE gene polymorphisms and their association with CVD risk in patients with T2DM. We also recommend the need to improve the monitoring and screening of T2DM patients with CVD as most studies have reported that patients carrying the ε4 allele have a greater likelihood to develop CVD complications. This is a significant concern for diabetic patients of all ages due to the corresponding increase in health-care costs. Another cross-sectional study of T2DM Malaysian patients who are diagnosed earlier should be conducted to recognize risk genotypes, which would aid in early prediction and identification of persons at risk. As a result of long-term DM and inadequate treatment, changes in lipoprotein metabolism can contribute to atherosclerosis-related morbidity and death.

Conclusion

Our findings show that APOE genotypes and the e3 allele is significantly associated with lipid panels and development of CVD. APOE gene polymorphisms were also associated with selected risk factors (SBP, HbA1c) among T2DM patients with CVD. This is in agreement with other studies that have substantiated a link between APOE gene polymorphisms with a greater risk of CVD development among T2DM patients, albeit with varying APOE alleles in different populations. APOE polymorphisms have the potential to be recognized as a predictor for CVD development in diabetic patients.

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Competing interests

The authors declare that they have no competing interests.

Ethical Clearance

This study was approved by the Research and Ethics Committee of Universiti Sains Malaysia. Ethics Approval No. USM/JEPeM/18050242.

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