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**RESEARCH ARTICLE**

# The relationship between CD40 gene polymorphism and acute coronary disease in diabetic and non diabetic patients

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## *Abstract*

**Background:**

Genetic background of type 2 diabetes and CAD is still under investigations as an important player of differences in the clinical course of the disease. This study aimed to examine the association between CD40 gene polymorphism and coronary artery disease (CAD) in diabetic and non diabetic patients groups .

**Objective:**

To our current knowledge, no research had been conducted in Egypt to discuss the association between the single nucleotide polymorphism(SNP) and genetic susceptibility to ACS in the Egyptian population. In our study, we investigated the possible role of the polymorphism in the pathogenesis of ACS as a major complication of diabetes mellitus in the Egyptian population and the potential of using this polymorphism as a marker to identify high risk patients who would have adverse out comes and cardiac events.

**Methods:**

We randomly selected 135 myocardial infarction (MI) patients aged 35 to 65 years and 50 healthy age-matched controls from Mansoura University hospitals. Cases were further subdivided into two groups according to the presence or absence of DM. The gene polymorphism was detected by the polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) .

**Results:**

Analysis of the distribution of the CD40 gene polymorphism between patients and controls showed that *C* allele frequency was significantly higher than the control group. The *C* allele increased the risk of CAD about 1.8 times compared with the control group($p<0.05$).There was no statistically significant difference of genotype or allele type distribution in non-diabetic coronary heart disease patients ($p=0.502$) and diabetic coronary heart disease patients ($p=0.415$).

**Conclusion:**

Our results suggests that there is an association between SNP (−1C/T) and acute myocardial syndromes that is independent of the presence of diabetes. We found that C allele frequency increased the risk of disruption of CAD .



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## INTRODUCTION

Coronary artery disease (CAD) has become a major cause of death and disability, responsible for up to 40% of all lethal events[ Franchini et al.,2008]. CAD is a multi-factorial disease that results from the interaction between genetic and environmental risk factors[ Khoury et al.,2007].

Diabetic cardiovascular complications (DCC ) are attributed to genetic factors, environmental factors and interactions between them with more evidence for the role of genetic influence [ Xu et al., 2014].

CAD caused by vascular endothelial dysfunction in diabetes mellitus is a result of several underlying processes including inflammation . DM is a metabolic disorder characterized by increased mortality rates and importantly implicated in the atherogenetic process through pathophysiological components including hyperglycaemia ,insulin resistance, hyperinsulinemia, hyperlipidemia, and hyperhomocysteinemia **[**Tousoulis et al.,2013**]**.

Inflammation plays a major role in pathogenesis of atherosclerosis, and subsequently the pathogenesis of CAD **[**Belay et al.,2004**]**. Atherosclerosis can be considered as chronic inflammatory disease that turns to be acute event by the process of plaque rupture, leading to thrombosis [Fuster et al.,1992].

CD40, a 50-kDa cell surface transmembrane glycoprotein receptor of the tumour necrosis factor receptor (TNFR) super family [Yun et al.,2014]. A C/T single nucleotide polymorphism (SNP) (rs1883832) in the 5′ un translated region of CD40 gene located at the -1 position within the Kozak sequence may influence the CD40 production [Wang et al.,2011] .

CD40-CD40L interaction is suggested to be involved in the development and progression of atherosclerosis and has been recently associated with susceptibility to atherosclerotic diseases, CAD and acute coronary syndrome (ACS) in Chinese population [Ma et al.,2013, Yun et al.,2014].

## Subjects and methods:

### Subjects

The study was conducted on 135 myocardial infarction ( MI ) patients age range between 35 -65 years. They were randomly selected from Mansoura university hospitals, Egypt. The diagnosis of MI was based on; medical history, clinical examination, ECG, cardiac enzymes and coronary angiography in some cases.

Cases were further classified into 2 groups. The first group consists of 94 non-diabetic MI patients. The second one consists of 41 diabetic patients with myocardial infarction. The cases were compared to 50 healthy age-matched individuals serving as controls. The controls were proven healthy and euglycemic by clinical and laboratory tests.

**Exclusion criteria**

Cancer patients, those having other endocrinal diseases, severe infections, liver and kidney diseases were excluded from the study.

We obtained verbal and written consent from all participants. In addition, an approval was obtained from the ethical committee of Mansoura University, Egypt.

**Methods:**

**Sample collection and storage:**

After an overnight fast , 7 mL of venous blood were collected from each subject and were divided into aliquots; 3 mL were transferred to a vacutainer plain test tube and were left for twenty minutes then clear serum was separated and kept at -70˚c to be used for analysis of glucose, lipid profile, liver function, kidney function tests. Two mL were delivered to an EDTA tube and kept at -70˚c for DNA extraction and 2 mL were delivered to another EDTA tube for complete blood count and HbA1c estimation.

**Estimation of lipid profiles:**

Total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C) were estimated using HITACHI 902 automatic analyzer. Low-density lipoprotein cholesterol (LDL-C) was calculated by applying Freidewald's formula: LDL-C(mg/dL) = TC(mg/dL) - (TG(mg/dL)/5+ HDL-C(mg/dL)).

**DNA isolation and genotyping**

CD40 rs1883832 (-1C>T) polymorphism was studied using PCR-RFLP technique. Genomic DNA was isolated from white blood cells (WBCs) of peripheral blood using Whole Blood Genomic DNA Extraction Kit (Fermentas, USA, #K0781).

Enzymatic amplification was performed by PCR using Master Taq polymerase enzyme and thermal cycler (T personal thermo cycler, Biometra, analytical Jena Company).

The PCR reaction mixture (25 µ) contained 12.5 µ PCR Master Mix {10· PCR buffer, 4 mM MgCl2, 0.5 Taq DNA polymerase, 0.4 mM dNTPs (dATP, dCTP, dGTP, dTTP)}, 0.5 µ of each primer (25 pmol), 5 µ of genomic DNA and 6.5 µ sterilized nuclease - free water. The thermo cycling was performed in (T personal thermo cycler, Biometra, analytical Jena Company).

The amplification of CD40 gene was performed under conditions described by Wang et al.,2010 .The primers used were as follow: forward: 5_-CCTCTTCCCCGAAGTCTTCC-3_;reserve, 5_-GAAACTCCTGCGCGGTGAAT-3_,

The PCR product length was 302 base pair. The product was digested by NCO I which cut this product into 2 unequal products (169 and 133 bp). The uncut PCR product (302 bp) representing the T allele while the digested product resulted from digestion of C allele. Both products of digestion and PCR product was identified by electrophoresis on agarose gel in a 2.5% concentration.

**Statistical analysis**

Percentage distribution of the corresponding genotypes of CD 40 gene polymorphism was determined and the differences in genotype and allele frequencies between the specified groups were compared using the Pearson's chi square ($\chi 2$). The risk estimates for genotype contrasts were obtained by computing odds ratio (OR) and respective

95% confidence interval (CI). The genotype distribution patterns among the control and total patients groups were compared using Hardy-Weinberg system .

We used Student's t-test to compare the means of the two groups when both of them are normally distributed. Mann Whitney test was used to compare the variables across two groups when one or both of them were abnormally distributed.
P-value of less than 0.05 was considered to be statistically significant.
All these statistical analyses were performed using SPSS software (Chicago, USA) and graph pad program software (San Diego, CA).

## Results

Table (1): Clinical characteristics of the studied groups

| Parameter | Cases (No= 135) | Controls (N0=62 ) | P value |
|---|---|---|---|
| Age (years) | 63.7±10.1 | 62.1±11.0 | 0.1 |
| BMI ($Kg/m^2$) | 24.4±3.4 | 24.1±4.5 | 0.75 |
| Gender | No. (%) | No. (%) | |
| Male | 98 ( 72.5) | 50 ( 80.6) | |
| Female | 37 ( 27.5) | 12 ( 19.4) | 0.2 |
| Smoking | 38 (28.1) | 17 (26.1) | 0.66 |
| HTN (mmHg) | 75 (55.4) | 13 (20.6) | <0.001 |
| DM | 41 (30.4) | 11 (17.6) | <0.001 |
| Total cholesterol (mmol/L) | 5.13±2.53 | 4.58±1.14 | <0.001 |
| Triglycerides (mmol/L) | 1.75±1.07 | 1.74±0.95 | 0.77 |

Table (1) shows the characteristics of patient and control groups. There was no statistical difference between both groups as regard to age, BMI, sex and smoking ($p>0.05$). The clinical and laboratory results showed significant statistical difference between both groups as regard to HTN, DM, Cholesterol level ($p< 0.05$) while TG level was not statistically different between both groups ($p>0.05$).

Table (2): Distribution of CD40 polymorphism Genotypes and alleles in patient and control groups:

| Genotype/allele | Cases N=135 No.(%) | Controls N=62 No.(%) | OR | (95% CI) | P value |
|---|---|---|---|---|---|
| CC | 53 (39.3) | 18(29) | 2.9 | 1.3-6.5 | 0.007* |
| TC | 60(44.4) | 22(35.5) | 2.7 | 1.27-5.9 | 0.001* |
| CC+TC | 113(83.7) | 40(64.5) | 2.8 | 1.41-5.6 | 0.003* |
| TT (r) | 22(16.3) | 22(35.5) | | | 1 |
| C | 166(61.4) | 58 (46.7) | 1.8 | 1.18-2.79 | 0.0065* |
| T (r) | 104(38.6) | 66 (53.3) | | | 1 |

r=reference group. OR=Odds Ratio. CI=Confidence Interval.

Analysis of the distribution of the CD40 gene polymorphism between patients and controls showed that C allele was higher in patients while the T allele was lower and there was statistical significant differences when compared to control groups ($p<0.05$). When analyzing the distribution of genotypes we observed that the C containing genotypes (CC and CT) were higher in patients than controls when compared to TT as reference. To confirm this findings, we compared these genotypes (CC + CT) to the frequency of TT genotype in both groups and also the differences were statistically significant ($p<0.05$).

Table (3) : Allelle and Genotype frequencies of CD40 gene polymorphism among the patients groups:

| | Non diabetic Coronary heart disease patients (n=94) | | Diabetic Coronary heart disease patients (n=41) | |
|---|---|---|---|---|
| Genotype | N | % | N | % |
| CC | 38 | 40.4 | 15 | 36.60 |
| TC | 43 | 45.7 | 17 | 41.50 |
| TT | 13 | 13.8 | 9 | 21.95 |
| P value | 0.502 | | | |
| Allele type | N | % | N | % |
| T | 69 | 36.7 | 35 | 42.7 |
| C | 119 | 63.3 | 47 | 57.3 |
| P value<br>OR<br>RR | 0.415<br>0.779(0.459-1.321)<br>0.926(0.783-1.094) | | | |

Analysis of the distribution of the CD40 gene polymorphism between CAD patients with and without diabetes showed that there was no significant difference of genotype or allele type distribution in Non diabetic Coronary heart disease patients and Diabetic Coronary heart disease patients (p=0.502, 0.415 respectively).

## Discussion

Atherosclerosis, the key feature of macrovascular complications in T2DM, is the result of chronic inflammation of the large- and mid-sized arteries . The immune system plays an important role in all stages of atherosclerosis starting from plaques formation, then plaque rupture and subsequently vascular occlusion **[**Hansson and Hermansson ,2011**]**.

increasing evidence shows that the *CD40-CD40L* system has vital role in progression of atherosclerotic disease and increased coexpression of the *CD40-CD40L* system, may create a proinflammatory and prothrombotic milieu for aggravating the development of atherosclerosis and the instability of atherosclerotic plaques, and may be a valuable marker [Wang et al.,2010]**.**

CD40 is a type I transmembrane protein receptor and a member of the tumor necrosis factor superfamily. CD40 gene is located in chromosome 20 (q12-q13.2). CD40 expression is stimulated by proinflammatory signals, such as interleukin (IL)-1, -3, and -4, tumor necrosis factor-alpha, and interferon-γ **[**Antoniades et al.,2009**].**

Both CD40 and CD40L are expressed on immune cells , non-immune cells and present on cells in the atherosclerotic plaque. Initially, CD40–CD40L interactions stimulate leukocyte recruitment by the expression of adhesion molecules as a result of activation of CD40 on endothelial cells [Wagner et al.,2004]. second, macrophages present in initial plaques secrete pro-inflammatory chemokines upon CD40L exposure [Mach et al.,1997].Third, platelet CD40L assist the platelet–leukocyte aggregates formation and enhances the adhesion of leukocytes to the activated endothelium **[**Lievens et al.,2010**]** . In addition, CD40- CD40L interaction stimulates the release of adipokines from adipocytes, enhancing atherogenesis [Antoniades et al.,2009].

In advanced plaques, CD40–CD40L interactions further enhance the inflammatory process by stimulating macrophage cytokine production . Furthermore,CD40-induced production of matrix metalloproteinases leads to destabilization of the atherosclerotic plaque[Seijkens et al.,2012]**.**

Further to atheroma formation and plaque weakening The CD40/CD40L interactions induce tissue factor expression on macrophages and ECs and diminish thrombomodulin expression, favoring a local procoagulant and prothrombotic status [Aukrust et al.,2004, Antoniades et al.,2009]**.**

In this study, we investigated the relationship between the single nucleotide polymorphism (SNP; −1C/T) and acute myocardial syndromes in diabetic and non diabetic patients . The SNP analyzed in this work is located in the functional region of the *CD40* gene, the Kozak consensus sequence. This can cause major alterations in the initiation of gene transcription[Kozak ,1999,2002,Wang et al.,2011].

To our knowledge until now, no research had discussed the relation between the SNP and susceptibility to ACS in the Egyptian population.

Our data suggest that SNP is associated with acute myocardiac syndrome. This relationship was independent of the presence of diabetes as an important traditional risk factors as the patient group was further subdivided into two groups one diabetic and another non diabetic.

In patients group, the *C* allele frequency was significantly higher than the control group. The *C* allele increased the risk of disease about 1.8 times compared with the control group. Moreover, there was no significant difference in the *C* allele frequency between the diabetic and non diabetic patients so this mutation is likely to be related to the progression of ACS as a complication rather being implicated in the pathogenesis of diabetes itself. We can conclude that , this polymorphism may be a potential marker to identify patients at high risk for developing cardiac diseases.

This result is concordant with experimental and clinical studies that demonstrated the role of the CD40–CD40L axis in the progression of T2DM and its vascular complications [Hummasti and Hotamisligil,2010, Lumeng and Saltiel ,2011] , and to be associated with coronary artery disease (CAD)[Yan et al.,2010,Tian et al.,2010].

Also in agreement with **(wang et al., 2010)** who imply that the −1C/T gene polymorphism is associated with instability of coronary plaque with higher risk for *C* allele carriers. Also **(wang et al., 2011)** who suggest that the single-nucleotide polymorphism of CD40 gene is associated with susceptibility to ACS in Chinese population.

yun et al., 2014 In a recent meta-analysis, analyzing the data from 7 published case-control studies reported a significant association between rs1883832 in CD40 gene and atherosclerosis in Chinese population .

**Study Limitations**

This study showed an association between the rs1883832 polymorphism in CD40 gene and ACS as a complication of diabetes mellitus but not implicated in the diabetes mellitus itself in Egyptian population. A single case–control study may not be convenient enough because of the relatively small number of patients included . Cardiovascular diseases are caused by interactive effects of multiple genetic and environmental factors. Further studies are recommended in larger populations .

## Conclusion

Our study results showed an association between the rs1883832 polymorphism in the Kozak sequence of CD40 gene and ACS as a complication of DM but not implicated in the DM susceptibility in Egyptian population. We are aware that we cannot extrapolate from a single case–control study and our results may not be sufficient because of the relatively small sample size. Interactive effects of multiple genetic and environmental factors characterize complex processes involved in cardiovascular diseases. The possibility that a neighboring co-inherited gene is responsible for such an association cannot be excluded. Further studies are required to corroborate these findings in larger populations and to demonstrate their functional substrate.

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