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Original article

The relation of thrombomodulin G33A and C1418T gene polymorphisms to the risk of acute myocardial infarction in Egyptians

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ABSTRACT

Background: Acute myocardial infarction (AMI) is one of the major causes of morbidity and mortality worldwide. There is an increased interest in the genetic risk factors in the pathogenesis of ischemic heart disease. Thrombomodulin (TM), a natural anticoagulant, may play a critical role in the pathogenesis of AMI.

Aim of the study: To assess whether Thrombomodulin (TM) G33A and C1418T gene polymorphisms are related to the risk of AMI in Egyptians or not.

Subjects and methods: 102 AMI patients were recruited vs 110 healthy controls. For every subject, measurement of plasma soluble Thrombomodulin level was done by enzyme-linked immunosorbent assay (ELISA). Further, DNA samples were genotyped by PCR-RFLP method for the TM G33A polymorphism and by PCR-ASO for the TM C1418T polymorphism.

Results: Our results revealed that the C1418T gene polymorphism was significantly associated with increased risk of AMI (**CT**: OR = 2.34, 95% CI = 1.28–4.29, P = 0.006; **TT**: OR = 8.03, 95% CI = 0.97–66.47, P = 0.026; **CT** + **TT**: OR = 2.65, 95% CI = 1.47–4.78, P = 0.001; **T** allele: OR = 2.51, 95% CI = 1.51–4.18, P < 0.001). On the other side, the TM G33A polymorphism was not detectable in any of patients or controls. Further, plasma soluble TM concentrations were higher in AMI patients, compared to the control group (P < 0.001).

Conclusions: TM 1418 C > T gene polymorphism, but not TM 33-G > A, may be associated with an increased risk of AMI in the Egyptian population. These results may have clinical implication in the management of AMI in the future.

1. Introduction

Acute myocardial infarction (AMI) is one of the major causes of mortality worldwide. Atherosclerosis, the underlying cause for AMI, involves progressive narrowing of coronary arteries due the development of atherosclerotic plaques [1]. Although the traditional risk factors for ischemic heart disease were extensively studied, there is an increased awareness of the contribution of polymorphic variants of genes as risk factors [2].

Thrombomodulin (TM) is a vascular endothelial cell-bound glycoprotein, which has been identified as a natural anticoagulant, and its major function is thrombin binding. The association of thrombin with TM significantly inhibits thrombin's procoagulant activity and decreases the production of thrombin itself. TM also plays an important role in the protein C anticoagulation pathway [3,4]. TM may play a critical role in the pathogenesis of AMI, as a high level of soluble thrombomodulin (sTM) signified protection and a lower risk of AMI [5].

Thrombomodulin gene is located on human chromosome number 20p11.2. Several polymorphisms have been found in the TM gene, of which G33A, and C1418T have gained much importance [6–9]. Some studies have investigated the correlation between these polymorphisms and AMI risk [6–15]. However, the results were largely inconclusive, limited mainly to Chinese, Thai, Indian and Bahraini populations, and controversial. Moreover, these polymorphisms have not been investigated in Egyptian patients yet.

2. Aim of the study

To investigate plasma levels of soluble Thrombomodulin (sTM) as an endothelial injury marker, and TM G33A and C1418T

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polymorphisms in Egyptian patients with acute myocardial infarction (AMI).

3. Subjects and methods

This study included two groups; group (A) that comprised 102 AMI patients and group (B) that included 110 apparently healthy, age and sex matched subjects from the blood bank donors as a control group. Patients were recruited from the cardiology department, Tanta University Hospital from September 2014 to January 2016. Both patients and controls originated from Delta region in Egypt and were recruited into the study randomly with no consanguinity between any of the participants. All subjects were subjected to full history taking, clinical examination, electrocardiogram (E.C.G), Echocardiography, and laboratory tests in the form of lipid profile plasma levels of soluble Thrombomodulin (sTM), and screening for TM G33A and C1418T polymorphisms.

3.1. Inclusion criteria

AMI patients included in group A were diagnosed as evidenced by ischemic ECG changes and elevated cardiac enzyme troponin I.

3.2. Exclusion criteria

Patients who had old myocardial infarction, left ventricular hypertrophy (LVH), severe aortic stenosis, and patients with heart failure on digitalis.

The study was approved by the local ethical committee and consents from all patients and controls were taken to approve sharing in the study after full description of the steps and the aim of the study. In addition, the study has been carried out in accordance with the code of Ethics of the World Medical Association (Declaration of Helsinki for experiments involving humans and laboratory tests).

Blood sample for every subject in this study was tested for lipid profile, plasma soluble thrombomodulin level (ELISA), and polymorphisms for thrombomodulin by polymerase chain reaction and restriction fragment length polymorphism (PCR–RFLP) for the TM G33-A polymorphism and by polymerase chain reaction and restriction allele specific amplification (PCR-ASO) for the TM C1418T polymorphism.

3.3. Measurement of plasma soluble thrombomodulin levels

Plasma levels of sTM were measured using enzyme linked immunosorbent assay (ELISA) (Human Thrombomodulin set, catalogue number 851.690.001) supplied by Diaclone (Besancon Cedex, France), according to the manufacturer's protocol.

Peripheral blood specimens were obtained in ethylenediaminetetra acetic acid (EDTA) tubes (Vaccutest, Italy) (two tubes per subject) and centrifuged at 1500*g* for 15 min at room temperature. The supernatants were stored at −80 °C until assayed. The results were expressed as nanograms per milliliter (ng/ml).

3.4. Genotyping methods

3.4.1. TM G33-A polymorphism

Genomic DNA was extracted using a blood mini kit (Thermo Scientific GeneJET) from the whole peripheral blood buffy-coat white blood cells were collected into ethylenediaminetetra acetic acid (EDTA) tubes (vaccutest, Italy) and stored at −20 °C. Genotyping for the TM G33-A polymorphism was carried out by polymerase chain reaction and restriction fragment length polymorphism (PCR–RFLP). A 259-bp DNA fragment, containing the G33-A polymorphism site for TM, was amplified by PCR using the following primers: forward, (5′-GGC CAG GGC TCG AGT TTA TAA AGG C-3′); reverse, (5′-CGG GGA CAG TCG TCT GTT ACA G- 3′) [16].

Table 1
Primers used for genotyping of THBD Polymorphisms genotypes.

Polymorphism	Primers		Assay
TM G-33A	Forward	5′-GGC CAG GGC TCG AGT TTA TAA AGG C-3′	PCR-RFLP [16]
	Reverse	5′-CGG GGA CAG TCG TCT GTT ACA G- 3′	
TM C1418T	TMC+	5′-GCCCGACTCGGCCCTTGC-3′	PCR-ASO [17].
	TMC−	5′-GGCACAGGCTCGCGATGG-3′	
	TMT+	5′-TCCCCACGAGCCGCACAG-3′	
	TMT−	5′-CGGTGCCAATGTGGCGGA-3′	

Polymerase chain reaction (PCR) conditions were carried out in thermocycler PTC-100 (Bio-Rad, Hercules, CA, USA). Complete PCR was run under standard conditions, using Taq DNA polymerase. Hard initial denaturation was done at 94 °C for five minutes, then 32 cycles of 94 °C for 40 s, annealing at 64 °C for 30 s, then extension at 72 °C for 40 s, and a final step at 72 °C for 8 min. Digests (10 μl) containing one unit of restriction enzyme *StuI* (Thermo Scientific FastDigest) were incubated for 12 h at 37 °C for the PCR products. The restriction site for StuI is 5′-AGG*CCT-3′, which represents the mutant type A allele and produces two fragments for the AA genotype (24 and 235 bp). The wild-type G allele cannot be recognized by the StuI enzyme, and only shows one 259 bp band (for the GG genotype). The GA heterozygote has three bands, with sizes of 259, 235, and 24 bp. The digests were separated on 3% agarose gel and then visualized using ethidium bromide staining.

3.4.2. TM C1418T polymorphism

The C → T substitution at nucleotide 1418 in codon 455 (Ala455Val) of the thrombomodulin gene was detected by allele specific amplification (PCR-ASO) with the primer sets as in Table 1 [17]. Homozygous results generated by allele specific amplification were repeated to exclude the possibility of non-amplification of one allele.

3.5. Statistical analysis

All variables were tested for normality with the Kolmogorov-Smirnov test. Normally distributed continuous variables are expressed as mean ± standard deviation. Non-normally distributed continuous variables are summarized as medians with interquartile range (25th and 75th percentiles). Categorical variables are expressed as numbers (percentages). Genotype and allele frequencies were estimated by gene counting and expressed as percentages of the totals. The distribution of genotypes for TM G33A polymorphism was checked for Hardy-Weinberg Equilibrium by the Chi-square test. The frequencies of genotypes and alleles for the polymorphism in cases and controls were compared using Fisher's Exact test or the Chi-square test. The odds ratios (OR) and 95% CI for each SNP were calculated by logistic regression models. Univariate and multivariate linear regression analysis was performed to determine the independent correlations of studied parameters. P value < 0.05 was considered statistically significant. The statistical analysis was performed with SPSS for Windows version 18.0 (SPSS, Chicago, IL, USA).

4. Results

This study consisted of two groups of patients; group (A) that included 102 AMI patients group (B) of 110 subjects as a control

Table 2
Demographic, clinical and laboratory data of the studied groups.

Parameter		Control (n = 110)	AMI (n = 102)	P value
Sex	M/F (numbers)	93/17	87/15	NS
Age (years)	Mean ± SD	53.7 ± 2.3	53.4 ± 2.6	NS
BMI (kg/m^2)	Mean ± SD	26.06 ± 0.9	26.51 ± 0.7	NS
Diabetes mellitus N (%)	Diabetic	41 (37.3%)	39 (38.2%)	NS
	Non Diabetic	69 (62.7%)	63 (61.8%)	NS
Smokers N (%)	Current	79 (71.8%)	74 (72.5%)	NS
	Never	31 (28.2%)	28 (27.5%)	NS
Hypertension	Systolic blood pressure (mmHg)	126.5 ± 3.9	128.1 ± 3.3	NS
	Diastolic blood pressure (mmHg)	91.0 ± 2.1	93.2 ± 1.6	NS
Lipid Profile	Total Cholesterol (mg/dl)	160.2 ± 8.8	163.8 ± 6.5	NS
	HDL cholesterol (mg/dL)	40.4 ± 1.4	39.2 ± 1.5	NS
	LDL cholesterol (mg/dL)	114.9 ± 7.8	117.1 ± 5.3	NS
	Triglycerides (mg/dL)	132.1 ± 4.1	134.9 ± 3.4	NS
Plasma sTM (ng/mL)	Median, quartiles	15.6 (10.3–23.1)	34.2 (22.9–41.5)	<0.001*
E.C.G.	Normal N (%)	110 (100%)	0 (0%)	<0.0001*
	ST, T-wave (%)	0 (0%)	73(71%)	
	ST-elevation	0(0%)	29 (27%)	
Echocardiography	EDD (mm ± SD)	52 ± 4	52 ± 6	NS
	ESD (mm ± SD)	35 ± 3	37 ± 2	
	EF (%± SD)	65 ± 4%	62 ± 6%	

* Significant, BMI (body mass index), NS (Non-Significant), sTM = Soluble Thrombomodulin, E.C.G.(electrocardiogram), EDD (end diastolic dimension), ESD (end systolic dimension), EF (ejection fraction), SD (standard deviation).

Table 3
Genotype and allele frequencies of *THBD* -33G > A, 1418C > T single nucleotide polymorphisms in AMI cases compared with controls.

Polymorphism	Genotype & Allele Frequency	Control (n = 110)		AMI (n = 102)		(OR) (95%CI)	*P* value
THBD	(GG)	110 (100%)		102 (100%)		R	–
33-G > A	(GA)	0 (0)		0 (0)		–	–
	(AA)	0 (0)		0 (0)		–	–
	(GA)+ (AA)	0 (0)		0 (0)		–	–
	G	220	1.0	204	1.0	–	–
	A	0	0.0	0	0.0	–	–
THBD	(CC)	84 (76.36%)		56 (54.9)		R	–
1418C > T	(CT)	25 (22.73%)		39 (39.24)		2.34 [1.28–4.29]	0.006*
	(TT)	1 (0.91)		7 (6.86)		8.03 [0.97–66.47]	0.026*
	(CT)+(TT)	26 (23.6%)		46 (45.09)		2.65 [1.47–4.78]	0.001*
	C	193	0.8773	151	0.7402	R	–
	T	27	0.1227	53	0.2598	2.51 [1.51–4.18]	<0.001*

r = Reference category (OR = 1.0).
* Significant.

group. The demographic, clinical and laboratory data of both groups are shown in Table 2 which showed that the age, sex, and BMI were similar in both groups. Moreover, there were no differences between both groups, regarding the risk factors of hypertension, smoking, and lipid profiles. Soluble TM median levels were significantly higher in AMI group, compared with the controls (34.2 ng/ml versus 15.6 ng/ml, $p < 0.001$). (Table 2). Table 3 showed the genotype and allele frequencies of THBD (Thrombomodulin gene) 33-G > A and THBD 1418C > T single nucleotide polymorphism in AMI cases compared to control group. Our results revealed that the C1418T gene polymorphism was significantly associated with increased risk of AMI (**CT**: OR = 2.34, 95% CI = 1.28–4.29, P = 0.006; **TT**: OR = 8.03, 95% CI = 0.97–66.47, P = 0.026; **CT+TT**: OR = 2.65, 95% CI = 1.47–4.78, P = 0.001; **T** allele: OR = 2.51, 95% CI = 1.51–4.18, P < 0.001). On the other side, the TM G33A polymorphism was not detectable in any of patients or controls.

5. Discussion

Thrombomodulin is an effective natural anticoagulant expressed on the endothelium [4]. Conflicting data have been reported regarding the possible contributions of the TM -33G > A and 1418C > T polymorphisms to AMI. In this study, we investigated the relation of THBD 33-G > A and 1418C > T polymorphisms to the risk of AMI in Egyptians. We found that the polymorphism of THBD 1418C > T but not THBD 33-G > A was associated with increased risk of AMI in Egyptian population. Additionally, the increased level of soluble TM was associated with AMI.

Zhao et al [13] conducted a study for the association of the TM -33G > A polymorphism with coronary artery disease and myocardial infarction in Chinese Han population. They concluded that their results seemed not to support a significant association of the TM -33G > A polymorphism with myocardial infarction (MI) or premature MI in the study population. These findings were replicated in Thai population as no associations were found between TM -33GA polymorphism with AMI, ischemic heart disease risk factors and the severity of AMI in Thai [14]. On the other side, the findings of the study by Li et al [6] in Chinese suggested that there is a significant association of the -33G > A mutation in thrombomodulin gene with myocardial infarction, and this mutation could influence soluble TM levels in those patients. Moreover, in two meta-analyses of a total of 13 and 14 case-control studies, there were significant associations between both of TM -33G/A Ala455Val polymorphisms and AMI risk [11,12]. However, most

of the included individual studies in these meta-analyses were carried on Chinese. Further, in Arabian population, there was no relation of the TM -33 GA polymorphism with AMI and both soluble and monocyte TM levels were not related to the cardiovascular events [15]. In contrast to our results, a meta-analysis provided robust evidence of an association between the -33G/A polymorphism in the TM gene and the risk of AMI in Asians, however, the TM 1418C polymorphism was not associated with myocardial infarction risk [18]. In Indians, the mean plasma sTM levels are associated with early-onset AMI. Moreover, TM 1418C/T polymorphism is an independent predictor of AMI and synergies with smoking [19]. Moreover, Norlund et al [20] reported the association of TM 1418C (Ala455) allele with premature MI in 97 Swedish MI survivors and 159 healthy controls. Further, in a large cohort study by Wu et al on 376 AMI cases showed that TM 1418C increased the risk of AMI by 6.1-fold and it is an independent risk factor for AMI in blacks [7].

A study by Ranjith et al [10] on 195 patients with AMI <45 years of Asian Indian origin in South Africa revealed that TMAla455Val variant allele was a significant risk factor for ischemic heart disease. In contrast, no difference was observed between patients and control participants for the allelic frequency of TM 1418C/T (Ala455Val) with 33 of 104 individuals homozygous for 1418T (455Val) within both the patient and the control groups in a study by Ireland et al [21]. Moreover, Park et al [9] showed that TM 1418C/T was not an independent risk factor for one-, two, or three vessels disease. Furthermore, the TM 1418C/T polymorphism (CT + TT genotypes) was not associated with AMI onset in a study by Chao et al. [22].

Protein C is converted to the anticoagulant serine protease by a complex interaction between thrombin and TM on the endothelial cells surfaces through the augmentation of endothelial cell protein C/activated protein C receptor (EPCR). Activated protein C (APC) then binds to protein S on the endothelium and this complex inactivates the activated coagulation factor V (Factor Va) and the activated coagulation factor VIII (Factor VIIIa), thus preventing further thrombin generation [23]. The Thrombomodulin residue 455 is located in the last epidermal growth factor (EGF)-like repeat which is functionally important for protein C activation and thrombin binding. The Alanine 455 to Valine replacement at the 455 residue of TM molecule might alter the functional activity of the TM and the activation of the protein C anticoagulant pathway [24].

Soluble TM in plasma is a degradation product of endothelial TM. The plasma concentrations of soluble TM may also be affected by increased proteolytic activity from activated white blood cells that cleaves soluble TM from endothelial TM. Thus, a high concentration of soluble TM may be related to inflammation. In atherosclerotic patients, soluble TM concentration may reflect the degree of endothelial damage and associated inflammation, rather than the expression of TM. This clearly explains our results of higher plasma soluble TM levels in patients with AMI as compared to the controls [25].

6. Conclusion

Plasma soluble TM concentrations are higher in AMI patients, compared to the control group. Further, Thrombomodulin 1418C > T gene polymorphism, but not TM 33-G **> A,** may be associated with an increased risk of AMI in the Egyptian population. The results of this study may have clinical implication in the management of acute coronary syndrome in the near future.

Declaration of conflicting interests

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