Acta Biomed 2021; Vol. 92, N. 6: e2021282 DOI: 10.23750/abm.v92i6.9622

Original article

Association study of polymorphism in Thrombomodulin gene (rs1042579) with cardiovascular disease

Elham Khosravi[1], Ladan Sadeghian[2], Parisa Mohamadynejad[3], Minoo Dianatkhah[4], Mahsa Hajizadeh[5], Mojgan Gharipour[6]

[1]Hypertension Research Center, Cardiovascular Research Institute, Isfahan University of Medical Sciences, Isfahan, Iran; [2]Isfahan Cardiovascular Research Center, Cardiovascular Research Institute, Isfahan University of Medical Sciences, Isfahan, Iran; [3]Department of Biology, Faculty of Basic Science, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran; [4]Interventional Cardiology Research Center, Cardiovascular Research Institute, Isfahan University of Medical Sciences, Isfahan, Iran; [5]Isfahan Research Committee of Pathology, School of medicine, Isfahan University of Medical Sciences, Isfahan, Iran; [6]Isfahan Cardiovascular Research Center, Cardiovascular Research Institute, Isfahan University of Medical Sciences, Isfahan, Iran.

Abstract. *Background and aim:* Thrombomodulin (*THBD*) gene plays an important role in activation and control of protein C. Regulation protein C levels as an important risk factor for cardiovascular disease. Mutations in this gene can affect thrombomodulin levels. In this study, we aimed to investigate the role of rs1042579 SNP within *THBD* gene in patients with cardiovascular disease. *Methods:* The samples of this case-control study consisted of 105 Iranian patients with cardiovascular disease and 95 controls who were free of CVD events enrolled from March 2017 to December 2018 in this study. Demographic data, medical history, and para-clinical were measured. Genotyping was carried out using ARMS-PCR technique and Sanger sequencing was used. Twenty percent of the samples were sequenced to confirm the result of the genotyping. *Results:* Data analysis revealed that the rs1042579 within *THBD* gene was associated with a risk of cardiovascular disease. Molecular assay confirmed that TT genotype is a factor associated with CVD in patients compared to healthy controls. *Conclusion:* The results of this study showed that the rs1042579 was associated with an increased risk of cardiovascular disease. (www.actabiomedica.it)

Key words: THBD, cardiovascular disease, rs1042579, Iran

Introduction

Cardiovascular disease (CVD) is currently one of the most common diseases in Iran (1, 2), and its number has been increasing in recent decades (3). CVD is caused by injury and obstruction of coronary arteries (4).

Thrombomodulin is an integral glycoprotein of the endothelial cell membrane that binds to thrombin in the presence of calcium ion and reduces its specificity to fibrinogen. In addition, the thrombin-Thrombomodulin Ca^{++} complex activates protein C. Protein C is a vitamin K-dependent anticoagulant proenzyme that is made in the liver and has a half-life of 2-5 hours. Protein C is activated in the presence of the endothelial cell cofactor (Thrombomodulin) and activates protein C that has enzymatic activity. Activated protein C as an anticoagulant disables the active forms of factor V and VIII. It also increases fibrinolysis by deactivating the plasminogen inhibitor (5, 6). S protein is a plasma glycoprotein dependent on vitamin K, which is synthesized in the liver and acts as a protein C cofactor (7). It is supposed that the presence of common polymorphisms in the *THBD* gene. The rs1042579 single nucleotide

polymorphism (SNP) is related to a non-synonymous amino acid (Ala473Val) and is in the sixth EGF-like domain within this gene. The region responsible for thrombin binding and activation of protein C (5). This polymorphism has previously been linked to plasma soluble Thrombomodulin levels and this has made it functionally important in Cardiovascular patients (8). Based on previous studies and what seems to be the desired role of Thrombomodulin in our study, there was no study in cardiovascular disease in the Iranian population. We aimed to investigate this variant in the Iranian population.

The aim of this study was to determine the frequency of rs1042579 in the Iranian population, which could determine the genetic factors affecting the development of cardiovascular disease in Iran, as well as provide epidemiological information for future studies.

Materials and Methods

Study Population: The data used in this investigation was collected through the Selenegene study(9). All subjects in this study were residents of the Isfahan Province, Iran. Patients were recruited sequentially during their angiography, myocardial revascularization or coronary artery bypass grafting (CABG) in the Chamran and Nour hospitals, which are tertiary university hospitals in Isfahan. An intervention was undertaken for recruitment which ran from March 2017 until the following December in 2018. Both case and control study population were selected from patients who had cardiac risk factors and were candidate for angiography. Subjects with confirmed stenosis in one, two or three vessels with angiographical documentation or who had history of invasive and interventional cardiology such as Percutaneous Coronary Intervention (PCI) or CABG consider as case group, and control who were free of CVD events enrolled in the study.

Details of inclusion and exclusion criteria were published elsewhere (9). The patients were interviewed to obtain their medical histories and then underwent laboratory assessments. Initial interviews and laboratory assessments included a questionnaire to collect demographic data, medical history and detailed information for a nutritional profile including diet, selenium intake, and biochemical laboratory measurements. Information about age, sex, smoking habits, nutritional habits, history of CVD and related risk factors, along with the medication were collected based on interview questionnaires. The body mass index (in kg/m2) was calculated. Diabetes mellitus was defined as a plasma glucose ≥ 126 mg/dL, a self-report of a physician diagnosis of diabetes, or as the current medication use.

Sample collection: Fresh blood (5 mL) was collected from the antecubital vein of all subjects in the fasting state. The blood samples were used for isolation of DNA and extracted DNA was frozen and stored at -70°C.

Genotyping analysis: DNA was isolated from peripheral blood lymphocytes using the standard salting out method (10). Genotyping was carried out using ARMS-PCR technique. The primer sequencing was forward for C allele:5'- GCCCGACTCGGCCCTTGC-3', for T allele: 5'- GCCCGACTCGGCCCTTGT-3', reverse outer: 5'- GCCAAAAGCGCCACCACCAG-3'.

The reaction details are as follows: total valume of 20 ul, containing 10ul PCR master mix 2X, 0.5 pmol for each primer, 1.5 ug genomic DNA and 7.5ul H2O. PCR amplification was carried out by denaturation at 95°C for 5 min, followed by 32 cycles of 95°C for 30 secs, 67°C for 30 secs and 72°C for 15 secs with a final extension at 72°C for 1 min. PCR products were analyzed by 2% agarose gel electrophoresis. Then 20% of the patients were sequenced bidirectionally using ABI 3130XL automated sequencer (Applied Biosystems, Foster City, California, USA).

Biochemical Analysis: Total cholesterol, triglyceride, and HDL cholesterol were measured with the use of a Hitachi 902 Analyzer and using standard enzymatic kits (Parsazmun, Tehran, Iran). LDL-cholesterol concentrations were calculated using the Friedewald formula (11).

Statistical analysis: Test of normality for distribution of variables was performed using a Kolmogorov–Smirnov test. Data were presented as mean ± SD. Differences between the groups were tested using the one-way ANOVA test or the Kruskal-Wallis test for continuous variables. The strength of association was presented as odds ratio (OR (95% confidence interval))

by using a logistic regression model. $P < 0.05$ was considered statistically significant.

Results

In this case-control study After analyzing the data, it was found that the rs1042579 SNP within *THBD* gene is associated with the risk of coronary heart disease. Table 1. displays the demographic characteristics of CAD positive and negative patients. No significant differences were observed between either group with regard to age (57.3 ± 7.85 vs. 55.6 ± 8.01 P = 0.14), but a significant difference has been found with regards to gender prevalence (P = 0.006). Fasting blood sugar was higher among subjects with CAD (P=0.002). Triglyceride level was higher among subjects with CAD (175.7±83.2 vs. 1145.7±57.339.1 ± 86.5, P = 0.007). Also, Systolic blood pressure was higher among subjects with CAD (P = 0.053). In CAD positive subjects heart disease familial history was higher than CAD negative subjects (P=0.002).

Table 1. Demographic and para clinical characteristics of study participants.

Variable parameter	CAD Positive (total no.=92)	CAD Negative (total no.=92)	p-value
Age	57.3±7.85	55.6±8.01	0.14
Sex(female)	34(36.9)	48(52.2)	0.006
Level of Education			
Illiterate	24(26.8)	18(19.5)	0.10
Primary school	54(58.7)	42(45.6)	
Secondary school	9(9.8)	19(20.6)	
University education	18(19.5)	12(13)	
Body Max Index(Kg/m2)	28.3±3.39	28.8±4.94	0.38
Round abdominal size	102.1±10.9	99.2±11.2	0.07
Abdominal to hip ratio	0.98±0.08	0.96±0.07	0.08
TG (mg/dL)	175.7±83.2	145.7±57.3	0.007
HDL_C (mg/dL)	42.9±11.9	44.8±9.42	0.24
FBS (mg/dL)	129.9±60.4	111.8±30.4	0.01
Chol (mg/dL)	172.9±41.6	175.1±39.0	0.71
LDL (mg/dL)	94.9±36.6	101.2±35.6	0.25
Systolic blood pressure	131.4v18.5	125.5±18.6	0.03
Diastolic blood pressure	77.4±11.7	76.5±9.93	0.53
Metabolic syndrome	56(60.8)	38(41.3)	0.11
Diabetes	36(39.1)	28(30.4)	0.88
blood pressure	71(77.1)	50(54.3)	0.07
Residential area	91(98.9)	83(90.2)	0.29
Heart disease familial history	43(46.7)	18(19.6)	0.002
Life style			
Smoking	14(15.2)	16(17.4)	0.43
Using statin	67 (72.8)	61 (66.3)	0.35

Continuous variables are reported as mean ± SD. Classification variables are reported as absolute numbers (percentages).

In figure 1. Genotypes Frequency was demonstrated based on CAD positive and negative patients, and sex. Sequencing results confirmed the presence of CC homozygous, heterozygous CT and homozygous TT genotypes. Figure 2. represented the chromatogram of three types of genotypes identified in samples

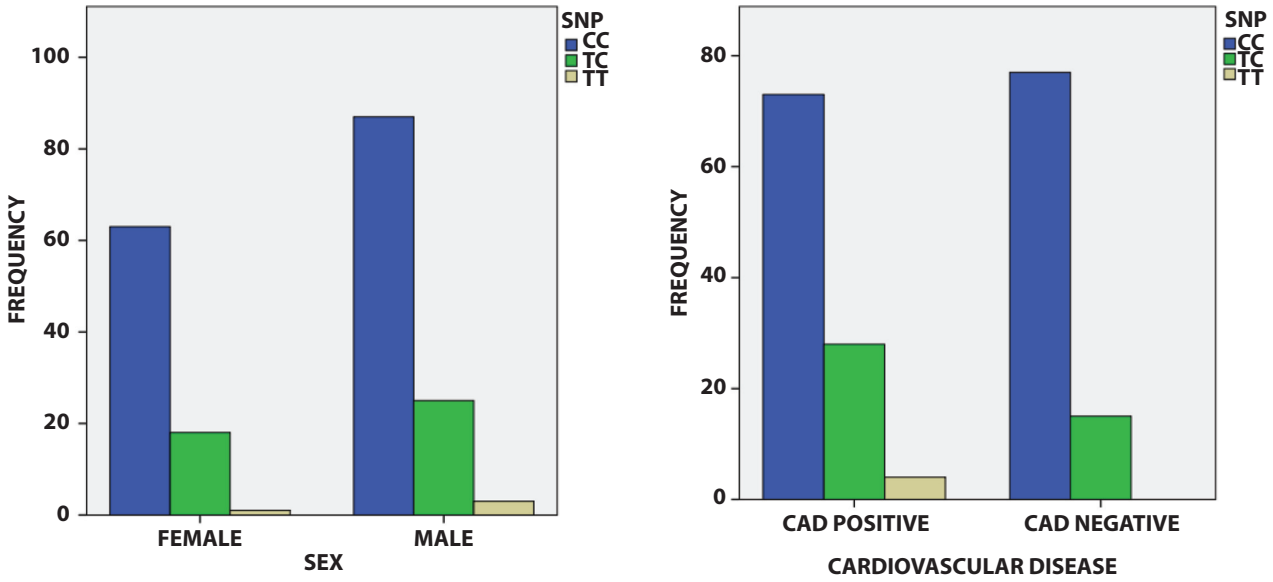

Figure 1. A.CC, TC and TT genotypes in CAD positive and negative patients. **B.** CC, TC and TT Genotypes Frequency by sex (Male and Female).

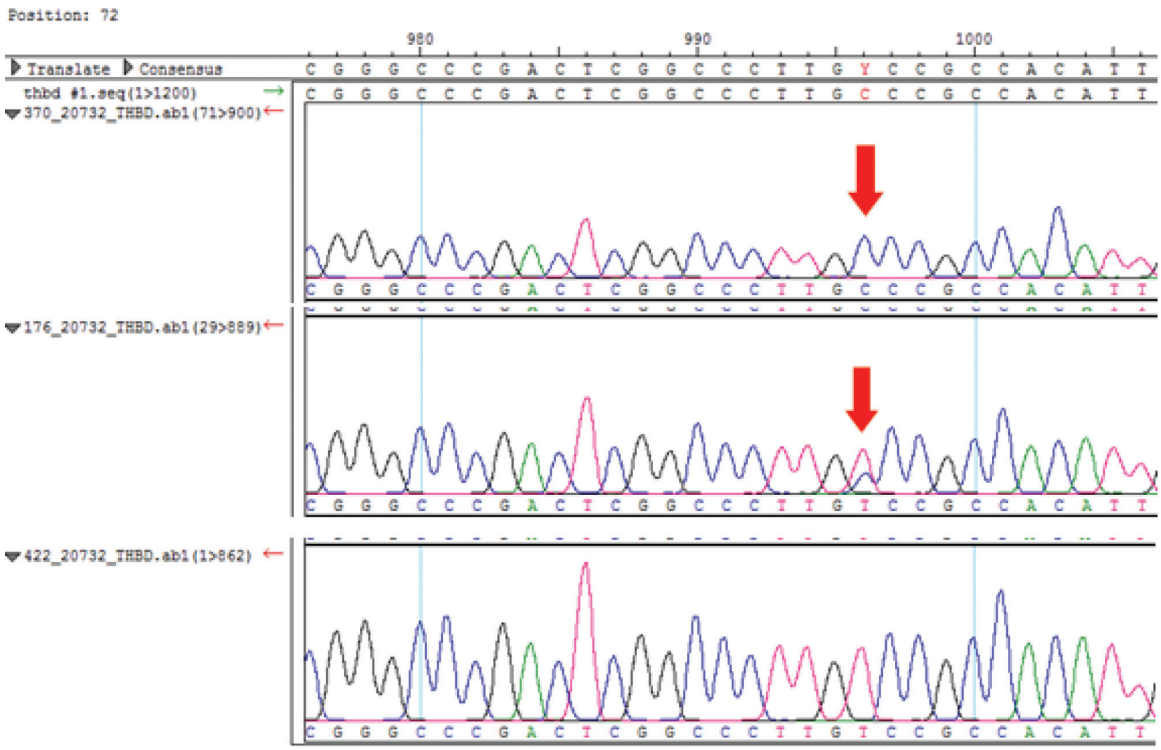

Figure 2. Chromatograms of variants represented CC, CT, TT genotype, respectively.

Table 2. The rs1042579 SNP Frequency in CAD positive and CAD negative subjects.

Total	Allele/genotype frequency	CAD positive	CAD negative	P-value
	Genotype frequency			
	CC	72(69.5)	77(82.7)	0.027
	TC	28(26.7)	15(16.3)	
	TT	4(2.8)	0(0)	
	Allele frequency			
	C allele	174(82.9)	169(91.8)	-
	T allele	26(17.1)	15(0.08)	
Female				
	Genotype frequency			
	CC	24(70.6)	29(81.2)	0.32
	TC	9(26.4)	9(18.8)	
	TT	1(2.9)	0(0.0)	
	Allele frequency			
	C allele	57(82.8)	87(82.8)	-
	T allele	11(16.2)	18(17.2)	
Male				
	Genotype frequency			
	CC	49(69.0)	28(86.4)	0.07
	TC	19(26.8)	6(12.6)	
	TT	2(4.2)	0(0)	
	Allele frequency			
	C allele	117(82.3)	82(92.0)	-
	T allele	**25(17.6)**	**6(0.07)**	

Table 3. Risk ratio in patients compared to healthy individuals based on CT genotype compared to CC genotype.

Model	OR (95% CI)	p-value
Crude	1.97 (1.66-2.32)	<0.001
Model 1	2.01 (1.74-2.46)	<0.001
Model 2	2.28 (1.86-2.78)	<0.001
Model 3	2.40 (1.96-2.94)	<0.001

Model 1: Adjusted based on Age & Sex; Model 1: Further Adjusted based on Waist to Hip Ratio, Hypertension & Family History of CVD; Model 3: Further Adjusted based on TG & FBS

that were sequenced to confirm the result. There was also a significant difference in genotypic frequency between two groups of cardiovascular and control patients (p-value <0.027) and variance also appeared (Table 2). TT has a risk associated with patients compared to healthy controls. According to Table 3, the variance of CT seems to be a risk factor in patients compared to healthy individuals (OR: 1.97 (1.66-2.32)). This significance is modified in models based on 1- age and sex, 2- blood pressure and family history. Triglycerides and fasting blood glucose were also observed (p-value <0.001), indicating a risk ratio in patients with T allele in healthy subjects compared to the C allele (Table 4). According to the table1, there is a significant correlation between the independent and dependent variables (OR: 1.97 (1.66-2.32)). In other words, the T allele can be considered as a factor associated with coronary artery disease (p-value <0.001). This significance is also observed in models adjusted for 1- age and sex, 2- hypertension and family history 3- triglyceride and fasting blood sugar (p-value <0.001).

Table 4. Risk ratio in sick people compared to healthy individuals based on T allele to C allele.

Model	OR (95% CI)	p-value
Crude	1.09 (1.07-1.10)	<0.001
Model 1	1.09 (1.07-1.11)	<0.001
Model 2	1.10 (1.08-1.12)	<0.001
Model 3	1.11 (1.01-1.13)	<0.001

Model 1: Adjusted based on Age & Sex; Model 1: Further Adjusted based on Waist to Hip Ratio, Hypertension & Family History of CVD; Model 3: Further Adjusted based on TG & FBS

Discussion

To the best of our knowledge, this is the first study to investigate the role of rs1042579 SNP in *THBD* gene in patients with cardiovascular disease in Iranian population. An important finding from this prospective case-control study is that having CT mutation in *THBD* gene. The transition variant c.1418C>T (NM_000361.2; NP_000352.1) which convert Alanine 473 to Valine. This mutation increases the risk of CVD by 2.4-fold in Iranian population. Thrombomodulin is an integral glycoprotein of the endothelial cell membrane that binds to thrombin and inhibits the function of blood coagulation factors. This same function has made thrombomodulin an important physiological anticoagulant (5). Okura et al. (1996) suggested that the *THBD* gene acts as a blood clotting inhibitor gene (12). The function of this gene was first reported in 1992 by Siang et al. (13). Other studies have suggested that the thrombomodulin plays an important role in the risk of cardiovascular disease along with other coagulation markers (14).

The rs1042579 and rs3176123 SNPs were identified and analyzed in the study by Aero et al. These two SNPs have previously been reported as imbalances in polymorphisms (15). In this study, they stated that neither mono-nucleotide polymorphisms nor *THBD* gene haplotypes show any association with the risk of cardiovascular disease or mortality. Contrary to the results of the study by Aero et al., The results of our study confirmed the association between rs1042579 and susceptibility to coronary heart disease.

Results by Nan et al. showed rs1042579 polymorphism of the *THBD* gene increased the risk of hypertension. They evaluated 95 hypertensive patients and found the significant relationship between this SNP and the prognosis of the cardiovascular disease (16). But in our study the level of systolic and diastolic blood pressure was normal because all patients were candidate for angiography and used antihypertensive drugs such as thiazide diuretics, calcium channel blockers, ACE inhibitors, angiotensin II receptor antagonists (ARBs), and beta blockers.

Limitations

This study is limited to the small sample size and coronary heart disease, so caution should be exercised when generalizing the results to other diseases.

Conclusion

The results of this study showed that the rs1042579 SNP was associated with an increased risk of cardiovascular disease. It is recommended that future research use other methods, such as protein and docking studies, to complement the data and increase their validity. To generalize the results, repeat the study in other cities in Iran, as well as groups other than coronary heart disease. It is recommended that further studies increase the volume of healthy and patient specimens to help us validate the results and analyze the results more accurately. Further research should be done on other cardiac patients.

Conflict of interest: Each author declares that he or she has no commercial associations (e.g. consultancies, stock ownership, equity interest, patent/licensing arrangement etc.) that might pose a conflict of interest in connection with the submitted article.

Acknowledgment: The authors would like to thank the patients for participating in this research and also the staff of the Genetics Studies unit, Isfahan Cardiovascular research institute due to their unassuming contributions.

References

1. Forouzanfar MH, Sepanlou SG, Shahraz S, et al. Evaluating causes of death and morbidity in Iran, global burden of diseases, injuries, and risk factors study 2010. Arch Iran Med. 2014;17(5):304.
2. Naghavi MJTTP. Features of death in 18 province of Iran in 2000. Tandis Publications 2002.
3. Bosch J, Yusuf S, Pogue J, et al. Use of ramipril in preventing stroke: double blind randomised trial. Bmj. 2002;324(7339):699.
4. Bovet P, Burnier M, Madeleine G, Waeber B, Paccaud FJBotwho. Monitoring one-year compliance to antihypertension medication in the Seychelles. ull World Health Organ. 2002;80:33-9.
5. Sadler JEJT, haemostasis. Thrombomodulin structure and function. Thromb. Haemost. 1997;78(07):392-5.
6. Esmon CTJTFJ. Thrombomodulin as a model of molecular mechanisms that modulate protease specificity and function at the vessel surface. FASEB J. 1995;9(10):946-55.
7. Walker FJ. Regulation of activated protein C by protein S. The role of phospholipid in factor Va inactivation. Journal of Biological Chemistry. 1981 Nov 10;256(21):11128-31.
8. Lobato RL, White WD, Mathew JP, et al. Thrombomodulin gene variants are associated with increased mortality after coronary artery bypass surgery in replicated analyses. Circulation. 2011;124(11_suppl_1):S143-S8.
9. Gharipour M, Sadeghi M, Salehi M, et al. Association of expression of selenoprotein P in mRNA and protein levels with metabolic syndrome in subjects with cardiovascular disease: Results of the Selenegene study. J. Gene Med. 2017;19(3): e2945.
10. MWer S, Dykes D, Polesky H. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 1988; 16(3):1215.
11. Kannan S, Mahadevan S, Ramji B, Jayapaul M, Kumaravel V. LDL-cholesterol: Friedewald calculated versus direct measurement-study from a large Indian laboratory database. Indian journal of endocrinology and metabolism. Indian J Endocr Metab. 2014;18(4):502.
12. Okura Y, Kato K, Hanawa H, et al. Pericardial mesothelioma secreting thrombomodulin. Am Heart J. 1996;132(6): 1309-11.
13. Tsiang M, Lentz SR, Sadler JE. Functional domains of membrane-bound human thrombomodulin. EGF-like domains four to six and the serine/threonine-rich domain are required for cofactor activity. J Biol Chem. 1992;267(9):6164-70.
14. Conway EM, Rosenberg RD. Tumor necrosis factor suppresses transcription of the thrombomodulin gene in endothelial cells. Mol Cell Biol. 1988;8(12):5588-92.
15. Auer PL, Stitziel NO. Genetic association studies in cardiovascular diseases: do we have enough power?. Trends Cardiovas Med 2017;27(6):397-404.
16. Nan B, Lin P, Lumsden AB, Yao Q, Chen CJTr. Effects of TNF-α and curcumin on the expression of thrombomodulin and endothelial protein C receptor in human endothelial cells. Thromb. Res. 2005;115(5):417-26.

Correspondence:
Received: 26 April 2020
Accepted: 2 June 2020
Dr. Mojgan Gharipour,
Isfahan Cardiovascular Research Center, Isfahan Cardiovascular Research Institute, Isfahan University of Medical Sciences, Isfahan, Iran
Tel number: +983136115116.
E-mail: mojgharipour@yahoo.com