Biomarkers, 2012; 17(7): 610–617

ISSN 1354-750X print/ISSN 1366-5804 online
DOI: 10.3109/1354750X.2012.706642

informa
healthcare

RESEARCH ARTICLE

Role of thrombomodulin gene in Indian population with coronary artery disease

Swarup A. Shah[1], Tester F. Ashavaid[2], Ranjit Mankeshwar[3], Chandrashekhar K. Ponde[4], and Rajesh Rajani[4]

[1]*Research Laboratories, P. D. Hinduja National Hospital & Medical Research Centre, Mumbai, India,*
[2]*Department of Biochemistry, P. D. Hinduja National Hospital & Medical Research Centre, Mumbai, India,*
[3]*Department of Research, P. D. Hinduja National Hospital & Medical Research Centre, Mumbai, India, and*
[4]*Department of Cardiology, P. D. Hinduja National Hospital & Medical Research Centre, Mumbai, India*

Abstract

Context: Thrombomodulin (TM), a natural anticoagulant have been implicated in the pathogenesis of coronary artery disease (CAD) thus emphasizing its potential role as a biomarker.

Objectives: To investigate the role of the TM genetic variants and soluble TM (sTM) plasma levels in Indian population with CAD.

Materials and methods: This case–control study involved genotyping of the entire TM gene and sTM levels estimation in 266 subjects.

Results: None of the four TM genetic variants identified significantly increased CAD risk in the study population. However, further subgroup analysis revealed that in subjects ≤49 years, C1418T variant (Ala455Val substitution) was significantly associated with CAD.

Conclusion: The increased CAD risk in subjects ≤49 years due to TM Ala455Val substitution is a promising finding. Further validation on large Indian cohorts is required in order to screen asymptomatic young subjects for CAD risk and to establish the clinical utility of Ala455Val substitution.

Keywords: Atherosclerosis, coagulation, fibrinolysis, thrombin receptor, thrombomodulin variants, thrombosis

Introduction

Coronary artery disease (CAD) is one of the major causes of mortality worldwide. It has been reported that the cases of CAD in India may increase from 36 million in 2005 to approximately 61 million in 2015 (National Commission on Macroeconomics and Health report 2005).

Atherosclerosis, the underlying cause for CAD involves progressive narrowing of coronary arteries due to the formation of atherosclerotic plaque. However rupture of an atherosclerotic plaque activates the coagulation cascade thereby leading to thrombin generation which eventually causes thrombosis. Formation of thrombus completely occludes the coronary arteries leading to myocardial infarction (MI) and sudden ischemic death (Furie & Furie 2008). Under physiological conditions, procoagulant and natural anticoagulant mechanisms ensure that the production and inhibition of thrombin is in equilibrium. An important pathway required for down-regulation of excess thrombin is the activated protein C (APC) pathway. A prerequisite for activation of protein C (PC) is the formation of complex between thrombin and its endothelial surface receptor, thrombomodulin (TM) (Van de Wouwer et al. 2004). Upon interaction,

Address for Correspondence: Tester F. Ashavaid, PhD, FACB, CSci, Head, Department of Laboratory Medicine, Department of Biochemistry / Research Laboratories, P.D. Hinduja National Hospital and Medical Research Centre, Veer Savarkar Marg, Mahim, Mumbai – 400 016. India. Tel: +91 022 24447935. Fax: +91 022 24442318. E-mail: dr_tashavaid@hindujahospital.com; tashavaid@gmail.com

(Received 26 April 2012; revised 18 June 2012; accepted 22 June 2012)

the rate of PC activation is accelerated by >1000-fold (Esmon et al. 1982). Activated PC catalyzes the proteolytic degradation of coagulation factors FVa and FVIIIa, required for thrombin generation. Thus the formation of thrombin–TM complex is important for effective regulation of thrombin. On interaction with TM, thrombin undergoes a conformational change which leads to a reduced procoagulant activity as seen by its inability to form thrombus and allows thrombin to activate protein C and thrombin-activatable fibrinolysis inhibitor (TAFI) (Bajzar et al. 1996). Thus TM entirely switches the thrombin substrate specificity from fibrinogen to protein C and TAFI which inhibits coagulation and fibrinolysis respectively and hence given the name thrombomodulin. TM thus forms an important link between coagulation and fibrinolysis (Wu & Matijevic-Aleksic 2000).

The intronless TM gene is located on human chromosome number 20p11.2 and codes for a 60.3 kda protein comprising of 575 amino acids. TM is a transmembrane glycoprotein expressed constitutively on the vascular endothelium and consists of 5 domains, N-terminal lectin-like domain, an epidermal growth factor (EGF)-like domain, a highly glycosylated serine and threonine-rich domain, a transmembrane domain and a cytoplasmic domain. The EGF-like domain is the thrombin binding site that is critical for PC activation (Suzuki et al. 1987). Apart from the anti coagulant properties of TM, the antiinflammatory properties of TM have also been greatly studied in recent years (Li et al. 2009). TM also exits in a soluble form (sTM) in circulation in contrast to the intact membrane TM (Ishii & Majerus 1985). The physiological function of sTM is not known, however, it is thought to indicate endothelial damage (Ishii et al. 1991). Therefore there is great interest in knowing whether sTM could serve as a marker of atherosclerotic arterial disease.

Several genetic variants of the TM gene have been identified that are significantly associated with coronary atherosclerosis (Ireland et al. 1997; Konstantoulas et al. 2004; Doggen et al. 1998). Wu et al showed that the TM genetic variant (C1418T–Ala455Val) predicts the risk of developing CAD in blacks but not in whites, suggesting that the ethnic background plays an important role in analysis of the genetic risk factors for CAD (Wu et al. 2001). So far to the best of our knowledge there has been no data from India about the TM gene variants and its association with CAD, though the available literature suggests that it would be worthwhile studying them in Indian population (Ireland et al. 1997; Li et al. 2000; Park et al. 2002). Therefore in absence of any prior data from India on TM gene with respect to CAD, we intend to determine the TM genetic variants and sTM plasma levels in Indian population and its association with CAD, which might be used as a genetic marker for screening subjects at young age for future CAD risk.

Materials and methods

Subject selection

We performed a case–control study. The study design was approved by the Hospital's Ethics Review Board (ERB) committee. Subjects visiting the hospital's Cardiac catheterization laboratory were included for this study. Subjects selected were 18 years and above age. There was no upper age limit. Subjects with previous history of vascular disease (CAD, cerebrovascular disease) were excluded from the study. A total of 133 angiographically proven CAD cases and 133 angiographically negative (≤30% stenosis) age and gender matched controls were sequentially recruited for the study. Presence of CAD was defined by ≥70% diameter narrowing in at least 1 out of the 3 major coronary artery [Left Anterior Descending (LAD), Circumflex (Circ) and Right Coronary Artery (RCA)]. The percent stenosis after angiography was decided by the cardiologist who ascertained the cases and controls selection. As the selection of the cases and controls were from the same pool, selection bias was not a factor. Informed consent was obtained from each subject prior to inclusion in the study. Information about the comorbidity, risk factors and lipid profile from all subjects were recorded in a detailed proforma. Misclassification bias was addressed by stringent training of medical residents and proforma used was same for both cases and controls.

Subjects were considered,

- Hypertensive, if the documented blood pressure is greater than 140/90 mmHg and/or are been treated with antihypertensive treatment.
- Diabetic, if the fasting blood glucose level is >115 mg/dL and/or are receiving oral hypoglycemic agents or insulin treatment (American Diabetes Association 2006).
- Dyslipidemic, if the total cholesterol is >200 mg/dL; HDL cholesterol–males ≤40/female ≤45 mg/dL; Total triglycerides >150 mg/dL and LDL cholesterol >100 mg/dL (NCEP-ATP 2002)
- Overweight/Obese, if their Body Mass Index (BMI) is greater than ≥23.0 or ≥ 25 kg/m^2, respectively (Misra et al. 2009).
- All subjects were classified as smokers (current or ex-smokers) and non-smokers. Similar classification was considered for alcohol consumption.

Blood collection and DNA extraction

Venous blood sample was drawn in EDTA tube from subject by well-trained medical residents. The samples collected were then centrifuged at 2500 rpm for 15 min and the plasma supernatant was aliquoted. Modified Miller et al. salting-out method was used for extraction of DNA from blood leukocytes (Miller et al. 1988). The extracted DNA samples along with the respective plasma samples were stored at –80°C until further use.

Amplification of the TM gene

The entire TM gene including the promoter region was amplified in 12 different overlapping regions (A, B - & L) by polymerase chain reaction (PCR). Amplification was performed in a MJ Research PTC-200 thermal cycler (Bio-rad laboratories, Hercules, CA, USA) using primers synthesized by Sigma life sciences (Sigma-Aldrich, Bangalore, India). Primers used for the amplification along with the cycling conditions have been mentioned in the Supplementary Table S1. The PCR products were then electrophoresed on a 3% Agarose gel and visualized under UV light after ethidium bromide staining.

Single stranded conformation polymorphism for genotyping TM gene

Single stranded conformation polymorphism (SSCP) analysis is the one of the most widely used gel-based mutation detection technique which exploits the differences in mobility between the wild type and mutant strands of DNA. SSCP analysis was carried out individually for each of the 12 regions amplified to determine the TM genetic variants. The SSCP banding patterns was detected by silver staining technique (Hwang et al. 2006).

Positive controls for SSCP

In order to standardize the SSCP, a mutant primer was designed for each of the 12 regions of TM gene whose sequence was exactly similar to the corresponding wild type forward primer except for a single nucleotide change towards the 5′ end of the primer. The mutant primer sequences have been mentioned in the supplementary Table S1. Separate PCR reactions were carried out with wild and mutant primers, thus producing PCR products which differ only by a single nucleotide that was introduced in the mutant primer. This single nucleotide change between the 2 PCR products was used for SSCP standardization. The standardized SSCP electrophoresis conditions have been mentioned in the supplementary Table S2.

TM gene sequencing

DNA samples showing abnormal electrophoretic mobility pattern on SSCP analysis were sent for commercial automated DNA sequencing. The chromatogram data was then matched with the TM gene sequence (GenBank accession no: AF495471.1) to determine the exact nucleotide change in the sample.

Estimation of plasma sTM levels by enzyme linked immunosorbent assay

IMUBIND Thrombomodulin ELISA kit (American Diagnostica, Cat No. 837) was used for the estimation of sTM from plasma samples. The sTM levels were determined by measuring solution absorbance at 450 nm and comparing the values with those of a standard curve.

Statistical analysis

Stata SE 10.1 software was used to analyze data. Independent t test was used to assess differences in quantitative variables (age, gender, total cholesterol, triglycerides and sTM levels) between cases and controls. The TM genotype and risk factors were presented as number (%) of subjects with the condition and the differences in frequencies were analyzed by Pearson's Chi-square test. Subgroup analysis of age and other risk factors (e.g. smoking) was performed using Chi-square test. Frequency matching approach was used to match age and gender of controls with cases. To examine the relationship of each independent variable with the dependent variable (Cases vs. Controls) and to rule out confounding and interaction, Binary Logistic Regression was performed. To assess mean sTM by CAD severity (cases only), one way Analysis of Variance (ANOVA) was used. Odds ratio (OR) was calculated to study association between the variables and CAD. p value as well as 95% confidence interval (CI) were calculated for each OR. A p value (significance) of <0.05 is deemed statistically significant.

Results

In the present study, we have analyzed the TM gene including the promoter region by using PCR-SSCP technique in 133 CAD cases and 133 controls. The sTM levels were estimated by ELISA.

Clinical characteristic of subjects

It was observed that amongst all the non-modifiable risk factors (age, gender, family history, diabetes and hypertension) the family history of CAD ($p = 0.031$, OR = 1.73, 95% CI = 1.01–2.96) as well as personal history of diabetes mellitus ($p < 0.0001$, OR = 2.69, 95% CI = 1.57–4.62) was significantly associated with CAD. However, prevalence of hypertension in both the group was almost similar. Amongst the modifiable risk factors, it was seen that both body mass index ($p = 0.034$) and hypertriglyceridemia were significantly higher ($p = 0.0068$) in cases when compared with controls (Table 1).

Binary logistic regression analysis was used to evaluate the effects of the variables after adjusting for interaction and confounding. The risk factors that appeared to be potentially significant predictors ($p < 0.1$) in single-variable analyses were included in the multiple logistic regression analysis. Risk factors including family history of CAD, diabetes mellitus, BMI and hypertriglyceridemia were put into the multiple logistic regression model. In multivariate analysis only diabetes mellitus ($p = 0.001$; OR = 2.817; 95% CI = 1.558–5.096) and hypertriglyceridemia ($p = 0.005$; OR = 1.005; 95% CI = 1.001–1.009) were found to be independent risk factor for CAD (Supplementary Figure S1).

Table 1. Clinical characteristics of the coronary artery disease (CAD) cases and controls.

Characteristics	CAD (n = 133)	Controls (n = 133)	p value	OR (95% CI)
Age (years)	53.39 ± 9.60	52.55 ± 9.64	0.476	–
Gender				
Male	92 (69.1 %)	89 (66.9 %)	0.693	–
Female	41 (30.9 %)	44 (33.1 %)		
Family history of CAD	58 (43.6 %)	41 (30.8 %)	**0.031***	**1.73** (1.01–2.96)
Diabetes Mellitus (DM)	69 (51.8 %)	38 (28.5 %)	**<0.0001***	**2.69** (1.57–4.62)
Hypertension (HTN)	79 (59.3 %)	81 (60.9 %)	0.802	0.93 (0.55–1.58)
Smoking	32 (24.1 %)	23 (17.3 %)	0.173	1.51 (0.79–2.90)
Alcohol consumption	40 (30.1 %)	37 (27.8 %)	0.685	1.11 (0.63–1.96)
Body Mass Index (kg/m^2)				
Normal (18–22.9)	29 (21.8 %)	19 (14.3 %)	**0.034***	–
Overweight (23.0–24.9)	63 (47.4 %)	84 (63.1 %)		
Obese (≥25)	41(30.8 %)	30 (22.6 %)		
Total serum cholesterol[a] (>200 mg/dL)	183 ± 51.1(n–109)	179 ± 42.5(n–111)	0.549	–
Total Triglycerides[a] (>150 mg/dL)	183.07 ± 116.4(n–108)	147.43 ± 71.1(n–110)	**0.0068***	–

*Statistically significant.

[a]Data for total cholesterol and total triglycerides levels were missing for few subjects. Most of these subjects did not carry out the lipid estimation whereas others had carried the test in other local laboratory outside Hinduja hospital and hence their lipid levels were not included in the final analysis.

Genetic variants identified in the TM gene by SSCP

The designing of positive controls for each of the 12 regions of TM gene clearly showed a different mobility pattern compared to the wild type. This result was further validated by DNA sequencing (Supplementary Figure S2). A total of four TM genetic variants G-33A (rs13306848), Ala25Thr (rs1800576), Ala455Val (rs1042579) and Asp468Tyr (rs41348347) were identified (Table 2). Two genetic variants (G127A and G1456T) were observed in only one subject each (both cases) whereas the C1418T (Ala455Val substitution) genetic variant (Figure 1) was identified in almost 30% of the cases as compared to almost 24% in controls, however it failed to show any statistical significance with the risk of CAD. Similar observation was seen with the promoter variant (G-33A) identified in this study.

Association of Ala455Val substitution in younger subjects

Out of the four TM genetic variant identified, only Ala455Val substitution was seen in considerable number of study subjects (40 cases and 32 controls) which prompted us to further determine its role in younger subjects. Therefore in an attempt to minimize the effect of long term environmental influences on CAD aetiology and to determine the true role of Ala455Val substitution in CAD, the entire subject group was classified into 2 age groups; ≤49 and ≥ 50 years. The distribution of the Ala455Val substitution was determined in the 2 age groups formed in both cases and controls. Interestingly, in group I (≤49 years), the mutant allele (Val) distribution was significantly (p = 0.006; OR = 3.41; 95% CI = 1.29–9.23) higher in the cases (53.2 %) when compared with the controls (25.0%) (Table 3). It was further observed that in same age group, alcohol consumption (p = 0.024; OR = 4.4; 95% CI = 0.98–20.86) and BMI ≥ 23 kg/m^2 (p = 0.006; OR = 3.7; 95% CI = 1.28–10.9) were significantly associated with Ala455Val substitution (Figure 2). More striking is the observation that the number of hypertriglyceridemic subjects (>150 mg/dL) were significantly higher (p = 0.003) in group I cases (n = 12) as compared to the controls (n = 2), thus increasing the risk of CAD to more than 11-fold (OR = 11.25; 95% CI = 1.7–119.8) in Ala455val carriers.

Table 2. Distribution of the Thrombomodulin genetic variants identified.

TM Genetic variants (rs number)	Amino acid change	No. of cases (%)	No. of controls (%)	p value
G-33A (rs13306848)	–	03 (2.3%)	05 (3.8%)	0.473
G127A (rs1800576)	Ala25Thr	01 (0.7%)	–	0.316
C1418T (rs1042579)	Ala455Val	40 (30.1%)	32 (24.1%)	0.27
G1456T (rs41348347)	Asp468Tyr	01 (0.7%)	–	0.316

Estimation of sTM levels by ELISA

The plasma sTM levels were estimated in both the groups by ELISA. In all, sTM levels were marginally higher (p = 0.588) in controls (3.90 ± 1.12 ng/mL) than the cases (3.82 ± 1.22 ng/mL). The number of cases with single, double and triple vessel artery disease were 47 (35.3%), 47 (35.3%) and 39 (29.4%), respectively. However, there was no significant difference between the sTM levels in CAD cases with single (3.92 ± 1.1 ng/mL), double (3.62 ± 0.9 ng/mL) and triple vessel disease (3.93 ± 3.9 ng/mL). Thus the sTM levels showed no association with the increasing severity of coronary atherosclerosis (p = 0.399). sTm levels across the four identified TM

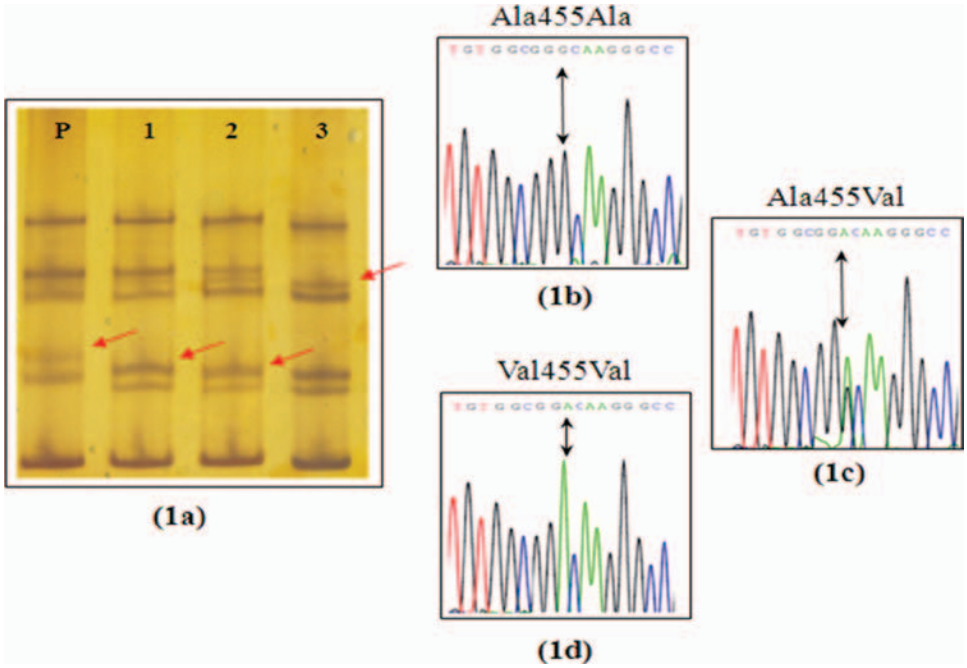

Figure 1. Gel picture showing C1418T (Ala455Val) genetic variant along with the chromatogram. (a) SSCP gel showing different migration pattern for C1418T variant [Lane 1 – Sample showing a wild type (C1418C – Ala455Ala) pattern; Lane 2 – Sample showing a heterozygous (C1418T – Ala455Val) pattern; Lane 3 – Sample showing a mutant (T1418T – Val 455 Val) pattern; P - Positive control)], (b–d) DNA chromatogram for the Ala455Ala, Ala455Val and Val455Val genotypes, respectively.

Table 3. Distribution of the Ala455Val genetic variant in two age groups (≤49 and ≥50 years).

Genotype	No. of Cases (%)	No. of controls (%)	*p* value	Odds ratio (95% CI)
Overall				
Ala455	93 (69.9%)	101 (75.9%)		
Ala455Val	40 (30.1%)	32 (24.1%)	0.27	–
Total	133	133		
Group I (Age ≤ 49 years)				
Ala455	22 (46.8%)	33 (75.0%)		
Ala455Val	25 (53.2%)	11 (25.0%)	**0.006***	**3.41** (1.29–9.23)
Total	47	44		
Group II (Age ≥ 50 years)				
Ala455	71 (82.5%)	68 (76.4%)		
Ala455Val	15 (17.5%)	21 (23.6%)	0.316	–
Total	86	89		

*Statistically significant.

genotypes were also analyzed to determine a genotype–phenotype association. The G127A and G1456T genetic variants were observed in an individual subject (both cases) and their sTM levels were found to be 3.72 and 4.07 ng/mL, respectively which did not deviate significantly from the mean sTM levels (3.82 ng/mL) observed in cases. Mean sTM levels in subjects with G-33A and C1418T variant was determined in both the group but it failed to show any association ($p = 0.473$ and 0.27, respectively).

Discussion

TM is an effective natural anticoagulant present on the endothelium (Suzuki et al. 1987). Regulation of thrombin production by TM is important in preventing the progression of atherosclerosis and arterial thrombus formation (Esmon 1997). In this study, the entire TM gene was screened to determine the role of TM gene in Indian population with CAD.

Amongst all the risk factors, diabetes mellitus ($p = 0.001$) and hypertriglyceridemia ($p = 0.005$) were found to be independent risk factors for CAD. Genotyping of TM gene in Indian subjects revealed the identification of four genetic variants: G-33A (rs13306848), G127A (rs1800576), C1418T (rs1042579) and G1456A (rs41348347). The G-33A promoter variant did not show any statistical significance with CAD in Indian population (Table 2). These findings were in contrast to those observed by Ireland et al. who found 3 mutations (C-133A, G-33A & GG-9/-10AT) in the promoter region of TM gene. Interestingly, the G-33A mutation was observed only in Asians suggesting a polymorphic site in this population (Ireland et al. 1997). This observation was later confirmed by Li et al who found a 23.8% frequency of this mutation in Han Chinese subjects of Mongolian origin. Moreover, Li et al. (2000) found evidence that G-33A mutation was significantly associated with CAD. Similar association was also observed by Park et al. (2002) in Korean population. Further, we identified three variants: G127A, C1418T and G1456T, all were identified in only one subject except the C1418T variant. The G127A was first reported by Norlund et al. (1997) and later Doggen et al. (1998) reported that

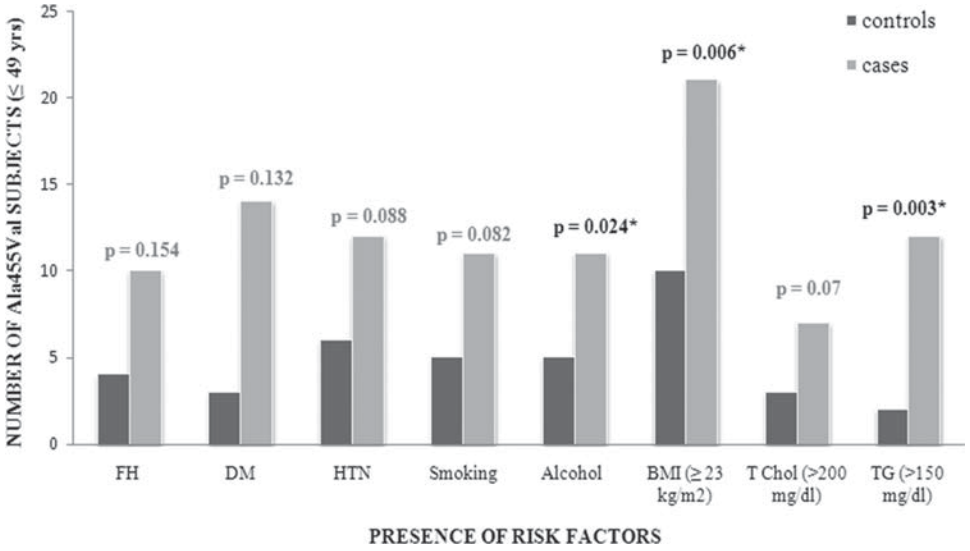

Figure 2. Association of risk factors in Group I subjects (≤ 49 years) with Ala455Val genetic variant.

the presence of G127A mutation increased the risk of coronary thrombosis by two-fold (Doggen et al. 1998). However in the present study, due to low prevalence of G-33A, G127A and G1456T variant, it can be concluded that either a large scale study would be required to find a true association with CAD or these genetic variants might not be prevalent in ethnically diverse Indian population.

Out the total 6 epidermal growth factor (EGF) – like repeat domain, the fourth to sixth EGF like repeats plays an important role in anticoagulant function of TM (Zushi et al. 1989). Since the Ala455Val substitution is present in the 6th EGF-like repeat domain which is required for the optimal binding of TM to thrombin (Nagashima et al. 1993), the crucial thrombin binding and protein C activation function of TM might be affected. Various studies carried out in different populations have identified C1418T (Ala455Val) TM genetic variant. However its true clinical significance with the CAD is quite varied. van der Velden et al. (1991) first identified this Ala455Val substitution and observed that the mutant Valine allele frequency (18%) was similar in both the controls as well as cases group, thus failed to show any association. Similar findings were also observed by Ohlin and Marlar (1995) and Ireland et al. (1997). Interestingly, a study by Norlund et al. (1997) in Swedish population observed that the mutant Valine allele was significantly higher in controls (26%) as compared to patients (18%), suggesting a protective role of Valine allele in controls, thus prompting further studies on this TM genetic variant. Later Wu et al. (2001) analyzed the Ala455Val substitution in black and white population residing in USA. The study showed that the presence of the valine allele increases the risk of CAD by 6.1-fold only in blacks but not in whites underscoring the importance of investigating the genetic risk factors by ethnic origins. The second Northwick Park Heart study (NPHSII) on prospective UK middle age men, revealed strong linkage disequilibrium between the Ala455Val and -1208/-1209TTdelTT variant. The Val/delTT haplotype had an overall small but non-significant increase in the risk for coronary heart disease (Konstantoulas et al. 2004). In the present study, the Ala455Val substitution failed to show any statistical significance with CAD in the entire study population. As the clinical manifestations of CAD are mainly evident in the fifth decade of the life (Sotirios et al. 2004), studies have been conducted to determine the association between TM genetic variants and CAD in subjects <50 years (Doggen et al. 1998; Chao et al. 2004). In the present study, categorizing the study subjects in different age groups (≤49 and ≥50 years) revealed interesting finding. It was observed that in subjects ≤49 years, the Ala455val substitution was significantly higher in cases and increases the CAD risk by more than three-fold (Table 3). This finding has for the first time shown the strong association of Ala455Val substitution with CAD in Indian subjects ≤49 years. Further it was seen that in subjects ≤49 years with Ala455Val substitution, the presence of risk factors like alcohol consumption and higher BMI increased the CAD risk by 4.4-fold and 3.7-fold, respectively. It has been observed that both alcohol consumption and higher BMI are associated with elevated C-reactive protein (CRP) levels in the circulation (Nan et al. 2005; Austin 2000). CRP is a known marker of inflammation which itself is associated with atherosclerosis and coronary thrombosis (Devaraj et al. 2009). It has been demonstrated that CRP significantly decreases the expression of TM in human endothelial cells, thereby promoting thrombogenic conditions (Nan et al. 2005). Thus alcohol consumption and high BMI can indirectly play a very important role in modulating the antithrombotic properties of endothelium by significantly decreasing the TM expression.

Also in the same group of subjects, the total number of hypertriglyceridemic subjects were significantly higher in cases, increasing the CAD risk by 11.25-fold. A possible explanation could be that increased triglycerides levels have been shown to be associated with

smaller LDL (low-density lipoprotein) size which is further associated with formation of oxidized LDL (Austin 2000). It has been suggested that the oxidized-LDL releases oxidized phospholipids which down-regulates TM expression (Ishii et al. 2003). A consequence of this would be reduced TM on the endothelium which may significantly contribute to the thrombotic properties of the cells in an atherosclerotic lesions.

With great interest in sTM as a marker of endothelial damage, various studies have investigated the role of sTM in atherosclerosis. Studies have shown that sTM levels are significantly associated with the severity of coronary atherosclerosis (Li et al. 2000; Nakagawa et al. 2001; Seigneur et al. 1993). Interestingly studies have also shown that individuals with high levels of sTM in plasma are associated with significant reduction in the relative risk of CAD (Salomaa et al. 1999; Wu 2003). Therefore the direct association of TM with coronary atherosclerosis is controversial. The present study finding's of marginally higher sTM levels in controls than cases was also observed by Salomaa et al. (1999) and Wu (2003), who showed that higher concentration of sTM levels in plasma is significantly associated with decreased risk of atherosclerosis. It has been suggested that the higher sTM levels seen in controls may be contributed primarily by the constitutive cleavage of endothelial TM and thus may be correlated with the levels of TM expression on endothelium (Wu 2003). Therefore a high concentration of sTM in plasma may indicate a high vasoprotective action with a low prothrombotic state and thus associated with a low risk of coronary atherosclerosis. But with no statistically significant difference seen in the sTM levels between the CAD cases and control group in the present study, the sTM levels might have a little role as a marker for the progression of coronary atherosclerosis. Furthermore the sTM levels did not shown any association with increasing severity of coronary atherosclerosis. The levels of sTM across the TM genetic variants also failed to show any significant association which suggests that sTM levels might be clinically insignificant in Indian population.

The limitation of the study is the small number of subjects in the subgroup analysis and therefore the observed association between the Ala455Val substitution and subject ≤49 years should be read with caution. However, based on the strong association, it would indeed be worthwhile to further validate this finding in order to screen young asymptomatic subjects for future CAD risk.

In conclusion, TM genetic variants did not increase the CAD risk in general Indian population. However, TM Ala455Val substitution significantly increases the CAD risk by three-fold in subjects ≤49 years and the risk is more apparent in presence of risk factors. This findings needs to be further substantiated in a large prospective Indian cohort of young subjects in order to establish the clinical utility of the TM Ala455Val substitution.

Declaration of interest

The study was funded by the National Health Education Society (NHES) of the P. D Hinduja National Hospital and Medical Research Centre. There is no actual or potential conflict of interest capable of influencing the judgment on the part of any authors.

References

American Diabetes Association. (2006). Diagnosis and classification of diabetes mellitus. *Diabetes Care* 29:s43–s48.

Austin MA. (2000). Triglyceride, small, dense low-density lipoprotein, and the atherogenic lipoprotein phenotype. *Curr Atheroscler Rep* 2:200–207.

Bajzar L, Morser J, Nesheim M. (1996). TAFI, or plasma procarboxypeptidase B, couples the coagulation and fibrinolytic cascades through the thrombin-thrombomodulin complex. *J Biol Chem* 271:16603–16608.

Chao TH, Li YH, Chen JH, Wu HL, Shi GY, Tsai WC, Chen PS, Liu PY. (2004). Relation of thrombomodulin gene polymorphisms to acute myocardial infarction in patients <or =50 years of age. *Am J Cardiol* 93:204–207.

Devaraj S, Singh U, Jialal I. (2009). The evolving role of c-reactive protein in atherothrombosis. *Clin Chem* 55, 229–238

Doggen CJ, Kunz G, Rosendaal FR, Lane DA, Vos HL, Stubbs PJ, Manger Cats V, Ireland H. (1998). A mutation in the thrombomodulin gene, 127G to A coding for Ala25Thr, and the risk of myocardial infarction in men. *Thromb Haemost* 80:743–748.

Esmon NL, Owen WG, Esmon CT. (1982). Isolation of a membrane-bound cofactor for thrombin-catalyzed activation of protein C. *J Biol Chem* 257:859–864.

Esmon CT. (1997). Defects in natural anticoagulant pathways as potential risk factors for myocardial infarction. Circulation 96:9–11.

Furie B, Furie BC. (2008). Mechanisms of thrombus formation. *N Engl J Med* 359:938–949.

Hwang SY, Jin LT, Yoo GS, Choi JK. (2006). Silver staining method for DNA in polyacrylamide gels using eriochrome black T as a silver-ion sensitizer. *Electrophoresis* 27:1744–1748.

Imhof A, Froehlich M, Brenner H, Boeing H, Pepys MB, Koenig W. (2001). Effect of alcohol consumption on systemic markers of inflammation. *Lancet* 357:763–767.

Ireland H, Kunz G, Kyriakoulis K, Stubbs PJ, Lane DA. (1997). Thrombomodulin gene mutations associated with myocardial infarction. *Circulation* 96:15–18.

Ishii H, Majerus PW. (1985). Thrombomodulin is present in human plasma and urine. *J Clin Invest* 76:2178–2181.

Ishii H, Tezuka T, Ishikawa H, Takada K, Oida K, Horie S. (2003). Oxidized phospholipids in oxidized low-density lipoprotein down-regulate thrombomodulin transcription in vascular endothelial cells through a decrease in the binding of RARbeta-RXRalpha heterodimers and Sp1 and Sp3 to their binding sequences in the TM promoter. *Blood* 101:4765–4774.

Ishii H, Uchiyama H, Kazama M. (1991). Soluble thrombomodulin antigen in conditioned medium is increased by damage of endothelial cells. *Thromb Haemost* 65:618–623.

Konstantoulas CJ, Cooper J, Warnock G, Miller GJ, Humphries SE, Ireland H. (2004). A combination of two common thrombomodulin gene variants (-1208-1209TTdelTT and A455V) influence risk of coronary heart disease: a prospective study in men. *Atherosclerosis* 177:97–104.

Li YH, Chen JH, Wu HL, Shi GY, Huang HC, Chao TH, Tsai WC, Tsai LM, Guo HR, Wu WS, Chen ZC. (2000). G-33A mutation in the promoter region of thrombomodulin gene and its association with coronary artery disease and plasma soluble thrombomodulin levels. *Am J Cardiol* 85:8–12.

Li YH, Shi GY, Wu HL. (2009). Thrombomodulin in the treatment of atherothrombotic diseases. *Front Biosci (Schol Ed)* 1:33–38.

Miller SA, Dykes DD, Polesky HF. (1988). A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 16:1215.

Misra A, Chowbey P, Makkar BM, Vikram NK, Wasir JS, Chadha D, Joshi SR, Sadikot S, Gupta R, Gulati S, Munjal YP; Concensus Group. (2009). Consensus statement for diagnosis of obesity, abdominal obesity and the metabolic syndrome for Asian Indians and recommendations for physical activity, medical and surgical management. *J Assoc Physicians India* 57:163–170.

Nagashima M, Lundh E, Leonard JC, Morser J, Parkinson JF. (1993). Alanine-scanning mutagenesis of the epidermal growth factor-like domains of human thrombomodulin identifies critical residues for its cofactor activity. *J Biol Chem* 268:2888–2892.

Nakagawa I, Matsubara T, Hori T, Imai S, Ozaki K, Mezaki T, Nasuno A, Kubota K, Nakano M, Yamazoe M, Aizawa Y. (2001). [Significance of soluble thrombomodulin in the coronary circulation of patients with coronary artery disease]. *J Cardiol* 38:145–152.

Nan B, Yang H, Yan S, Lin PH, Lumsden AB, Yao Q, Chen C. (2005). C-reactive protein decreases expression of thrombomodulin and endothelial protein C receptor in human endothelial cells. *Surgery* 138:212–222.

National Cholesterol Education Program (NCEP - III). Expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (adult treatment panel III) Final report (2002). *Circulation* 106:3143–3421.

National Commission on Macroeconomics And Health Report, Ministry Of Health And Family Welfare, Government Of India, New Delhi, August 2005: Available At http://www.who.int/macrohealth/action/report%20of%20the%20national%20commission.pdf.

Norlund L, Holm J, Zöller B, Ohlin AK. (1997). A common thrombomodulin amino acid dimorphism is associated with myocardial infarction. *Thromb Haemost* 77:248–251.

Norlund L, Zöller B, Ohlin AK. (1997). A novel thrombomodulin gene mutation in a patient suffering from sagittal sinus thrombosis. *Thromb Haemost* 78:1164–1166.

Ohlin AK, Marlar RA. (1995). The first mutation identified in the thrombomodulin gene in a 45-year-old man presenting with thromboembolic disease. *Blood* 85:330–336.

Park HY, Nabika T, Jang Y, Kwon HM, Cho SY, Masuda J. (2002). Association of G-33A polymorphism in the thrombomodulin gene with myocardial infarction in Koreans. *Hypertens Res* 25:389–394.

Salomaa V, Matei C, Aleksic N, Sansores-Garcia L, Folsom AR, Juneja H, Chambless LE, Wu KK. (1999). Soluble thrombomodulin as a predictor of incident coronary heart disease and symptomless carotid artery atherosclerosis in the Atherosclerosis Risk in Communities (ARIC) Study: a case-cohort study. *Lancet* 353:1729–1734.

Seigneur M, Dufourcq P, Conri C, Constans J, Mercié P, Pruvost A, Amiral J, Midy D, Baste JC, Boisseau MR. (1993). Levels of plasma thrombomodulin are increased in atheromatous arterial disease. *Thromb Res* 71:423–431.

Sotirios T, Glass CK, Steinberg D, Witztum JL. (2004). Lipoprotein oxidation, macrophages, immunity, and atherogenesis. In: Chien, KR. (second edition) *Molecular Basis Of Cardiovascular Disease*. Philadelphia: Elsevier Inc, pp. 385–413.

Suzuki K, Kusumoto H, Deyashiki Y, Nishioka J, Maruyama I, Zushi M, Kawahara S, Honda G, Yamamoto S, Horiguchi S. (1987). Structure and expression of human thrombomodulin, a thrombin receptor on endothelium acting as a cofactor for protein C activation. *EMBO J* 6:1891–1897.

Van de Wouwer M, Collen D, Conway EM. (2004). Thrombomodulin-protein c-epcr system: integrated to regulate coagulation and inflammation. *Arterioscler Thromb Vasc Biol* 24:1374–1383.

van der Velden PA, Krommenhoek-Van Es T, Allaart CF, Bertina RM, Reitsma PH. (1991). A frequent thrombomodulin amino acid dimorphism is not associated with thrombophilia. *Thromb Haemost* 65:511–513.

Visser M, Bouter LM, McQuillan GM, Wener MH, Harris TB. (1999). Elevated C-reactive protein levels in overweight and obese adults. *JAMA* 282:2131–2135.

Wu KK. (2003). Soluble thrombomodulin and coronary heart disease. *Curr Opin Lipidol* 14:373–375.

Wu KK, Aleksic N, Ahn C, Boerwinkle E, Folsom AR, Juneja H; Atherosclerosis Risk in Communities Study (ARIC) Investigators. (2001). Thrombomodulin Ala455Val polymorphism and risk of coronary heart disease. *Circulation* 103:1386–1389.

Wu KK, Matijevic-Aleksic N. (2000). Thrombomodulin: a linker of coagulation and fibrinolysis and predictor of risk of arterial thrombosis. *Ann Med* 32 Suppl 1:73–77.

Zushi M, Gomi K, Yamamoto S, Maruyama I, Hayashi T, Suzuki K. (1989). The last three consecutive epidermal growth factor-like structures of human thrombomodulin comprise the minimum functional domain for protein C-activating cofactor activity and anticoagulant activity. *J Biol Chem* 264:10351–10353.