No Association of Genetic Markers with Carotid Intimal Medial Thickness in β -Thalassemia Major Patients

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J Pediatr Genet 2018;7:19–22.

Abstract

Regular transfusion leads to cardiac siderosis resulting in cardiac complications that account for more than 71% of the total mortality in thalassemia patients. We aimed to study the variants of matrix metalloproteinase-9 (MMP9), matrix Gla protein (MGP), and estrogen receptor α (ER α), which might be contributing to atherosclerosis, leading to heart failure in thalassemia major. One hundred and five thalassemia patients on regular transfusion and iron chelation therapy were enrolled for the study. Carotid artery intimal medial thickness (CIMT) measurement was done to check for atherosclerosis. *MMP9* (C1562T), *MGP* (T138C), and *ER α* gene (*PvuII* (rs2234693T > C) and *XbaI* (rs9340799A > G) polymorphism were analyzed using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. CIMT values were within the normal range (<0.90 mm) in all patients. There was no difference in mean CIMT values between males and females (0.56 ± 0.11 versus 0.56 ± 0.12 , $p = 0.928$). There was no correlation of CIMT with age, body surface area, and body mass index as well as with serum ferritin levels. No statistically significant difference in frequency of *MMP9*, *MGP*, and *ER α* genotypes was seen in two dichotomized groups of CIMT (CIMT < 0.56 and CIMT $\geq$ 0.56). Variants of *MMP9*, *MGP*, and *ER α* have a reserved influence on cardiac disease pathogenesis, and the disease phenotype in thalassemia patients may be more strongly impacted by other factors.

Keywords

- β -thalassemia major
- MMP9
- MGP
- ER α
- CIMT

Introduction

Cardiac complication continues to be leading cause of mortality in humans, having significant impacts on morbidity and mortality. Cardiomyopathies (which account for 71%) in β -thalassemia major (β -TM) include atherosclerosis, arrhythmias, pulmonary hypertension, and systolic and diastolic heart failure. Data from studies done previously in diseased coronary artery from patients with coronary artery disease found ferritin to be present in human atherosclerotic lesions. Thus, increased

serum ferritin levels is considered to be a contributing factor for atherosclerosis.^{1,2} Atherosclerosis occurs with the structural change in arterial wall, characterized by gradual thickening of the intima.³ Increased serum ferritin and thickening of the carotid intimal medial thickness (CIMT) are the conditions prevalent in β -TM patients.^{4–6}

Matrix metalloproteinases (MMPs) are inflammatory mediators which belong to a family of structurally related zinc-binding proteolytic enzymes found to be widely distributed in human tissues. MMP9 plays role in the

received

August 10, 2017

accepted after revision

October 11, 2017

published online

December 6, 2017

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Verlag KG, Stuttgart · New York

DOI <https://doi.org/10.1055/s-0037-1608796>.
ISSN 2146-4596.

breakdown of extracellular matrix in normal physiological processes and are found to be involved in all states of atherosclerosis process by the activation of migration and proliferation of smooth muscle cells and by induction and destabilization of atherosclerotic plaques.^{7,8} Several single nucleotide variants have been revealed by sequence analysis with some being functionally important. *MMP9* C1562T polymorphism at promoter region controls the *MMP9* activity by abolishing the DNA–protein interaction, showing its involvement in susceptibility to atherosclerosis.^{9,10}

Gla-containing proteins play a crucial role in atherosclerotic arteries. Gla is formed posttranslationally from glutamic acid through gamma-carboxylation by the vitamin K-dependent γ -glutamate carboxylase. Matrix Gla protein (MGP) is a 10-kDa circulating protein consisting of five Gla residues secreted by chondrocytes and vascular smooth muscle cells of the arterial media. MGP is the strongest known inhibitor of tissue calcification. Loss of functional mutations of MGP may result in abnormal cartilage calcification.^{11–13} T138C variant in *MGP* at promoter region has been shown as a contributing factor for the binding of calcium with resultant deposition in arterial wall.^{14,15}

Estrogen has pleiotropic effects on the cardiovascular system which includes vascular function. The two nuclear estrogen receptors (ERs) are ER α and ER β . ER α is known for cardioprotective effects, a knockout model of ER α showed reduced production of estrogen thereby increasing formation of atherosclerotic plaques. There are numerous studies supporting not only the expression of ER in cardiomyocytes, but also their functional activity at genomic levels. Variants in ER α rs2234693 c.454–397T > C (*PvuII*) and rs9340799 c.454–351A > G (*XbaI*) have been studied and documented to contribute in heart disease progression.^{16,17}

Atherosclerosis, later, manifests as cardiac complication in β -TM. Thus, considering the role of *MMP9*, *MGP*, and ER α in atherosclerosis, this study was aimed to assess the association of their variants with the atherosclerosis (measured by CIMT) in TM patients for causing cardiac damage.

Materials and Methods

Subjects

A total of 105 β TM patients visiting to Department of Genetics, of a tertiary care center of North India for regular blood transfusion, were recruited. Patients below the age of 10 years were excluded. Patients having disorders such as diabetes, hypertension, and hyperlipidaemia which may cause vascular damage were also excluded from the study. The study protocol was approved by the institutional ethics committee and written informed consent was obtained from all the patients and parents of children under 18 years. Patient’s clinical details were recorded in a pro forma.

Carotid Artery Measurements

Patients were examined supine with the head rotated 45 degrees toward the left side. Imaging was done in the plane perpendicular to the neck with the jugular vein lying immediately above the common carotid artery (or at 45 degrees from

the vertical if the internal jugular vein was not present). CIMT was measured 2 cm proximal to the carotid bulb as the distance from the leading edge of the first echogenic line to that of the second echogenic line. The first line represented the lumen–intima interface, and the second line, the collagen-containing upper layer of tunica adventitia.

Laboratory Analysis

Five milliliters of blood was drawn from peripheral veins under aseptic conditions. The serum ferritin levels were measured using enzyme-linked immunosorbent- based serum ferritin assay kit (Omega, Ferritin, Pathozyyme). Genomic DNA was isolated using the standard phenol–chloroform method. *MMP9* (C1562T), *MGP* (T138C), and ER α gene *PvuII* (rs2234693T > C) and *XbaI* (rs9340799A > G) polymorphisms were identified using the polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method (–Supplementary Table S1, available in the online version).

Statistical Analyses

Data were analyzed for mean and standard deviation. Chi-square test was used to compare the groups based on CIMT values in different genotypes. Pearson’s correlation test was applied to see the correlation of CIMT with age and other anthropological variables. A *p*-value of < 0.05 was considered to be statistically significant. All statistical analysis was performed using SPSS 20.0 for windows.

Results

Our study included 105 patients with mean age of 15.4 $\pm$ 5.6 years. The mean CIMT was 0.56 $\pm$ 0.11 mm (–Table 1). CIMT was within the normal limits (CIMT < 0.90 mm) in all the patients. There was no difference in mean CIMT values in between male and females (0.56 $\pm$ 0.11 versus 0.56 $\pm$ 0.12, *p* = 0.928). We did not find any correlation of CIMT with age, anthropological variables, and serum ferritin (–Table 2). Patients were dichotomized according to the CIMT values (Group A: CIMT < 0.56 mm, and Group B: CIMT $\geq$ 0.56 mm). There was no significant difference in frequency of *MMP9* (C1562T) and *MGP* (T138C) genotypes across the two groups.

The association study showed no statistical significance between the ER α gene *PvuII* (rs2234693T > C) and *XbaI* (rs9340799A > G) polymorphisms and the increased CIMT.

Table 1 Demographic and clinical profile of patients

Variables	Particulars
Age (in y)	15.4 $\pm$ 5.6
Weight (kg)	30.6 $\pm$ 10.8
Height (cm)	148.7 $\pm$ 6.7
Hemoglobin level (g/dL)	8.4 $\pm$ 1.5
Serum ferritin (ng/mL)	2686 $\pm$ 1313
CIMT (mm)	0.56 $\pm$ 0.11

Abbreviation: CIMT, carotid intimal medial thickness.

Table 2 Correlation of CIMT with age, anthropological parameter, and serum ferritin

Parameter	Pearson's coefficient	p-Value
Age	0.123	0.211
Weight	0.207	0.088
Height	0.173	0.078
BMI	0.102	0.412
BSA	0.206	0.095
Serum ferritin	−0.076	0.441

Abbreviations: BMI, body mass index; BSA, body surface area; CIMT, carotid intimal medial thickness.

We also tested for dominant and recessive model but found no significance (→ **Table 3**).

Discussion

This study was taken up to evaluate the association of genetic markers (MMP9, MGP, and ERα (PvuII and XbaI)) with CIMT

Table 3 Genotype frequency of SNPs according to CIMT

MMP9	CIMT (<0.56)	CIMT (≥0.56)	p-Value
CC (18) ^a	7 (21.9)	11 (15.1)	0.144
CT (39)	15 (46.9)	24 (32.9)	
TT (48) ^b	10 (31.2)	38 (52.1)	
CC + CT (57) ^b	22 (68.8)	35 (47.9)	0.058
TT + CT (87) ^a	25 (78.1)	62 (84.9)	0.409
MGP			
CC (18) ^a	7 (21.9)	11 (15.1)	0.665
CT (44)	12 (37.5)	32 (43.8)	
TT (43) ^b	13 (40.6)	30 (41.1)	
CC + CT (62) ^b	19 (59.4)	43 (58.9)	1.000
TT + CT (87) ^a	25 (78.1)	62 (84.9)	0.394
ERα (PvuII)			
TT (75) ^a	24 (75.0)	51 (69.9)	0.324
TC (20)	7 (21.9)	13 (17.8)	
CC (10) ^b	1 (3.1)	9 (12.3)	
CC + CT (30) ^a	31 (96.9)	64 (87.7)	0.277
TT + CT (95) ^b	8 (25.0)	22 (30.1)	0.646
ERα (XbaI)			
AA (70) ^a	20 (62.5)	50 (68.5)	0.622
AG (26)	8 (25.0)	18 (24.7)	
GG (9) ^b	4 (12.5)	5 (6.8)	
AA + AG (96) ^b	28 (87.5)	68 (93.2)	0.450
GG + AG (35) ^a	12 (37.5)	23 (31.5)	0.654

Abbreviations: CIMT, carotid intimal medial thickness; ERα, estrogen receptor α; MGP, matrix Gla protein; MMP9, matrix metalloproteinase-9.
^{a,b} The p-value was calculated by comparing the frequency of genotype for dominant and recessive models.

as a structural marker of atherosclerosis. These markers have been studied previously and reported to have associations with the structural markers of atherosclerosis.^{16,18,19} CIMT is a noninvasive method to detect early subclinical atherosclerosis and correlates well with overall vascular injury, and thus is used for the detection of early atherosclerosis in children with β-TM.

The human *MMP9* gene is located on chromosome 20 and has functional implications in infarct healing.²⁰ The C1562T variant is the most studied, and functional studies reveal its allele-specific effects on MMP9 expression. MMP9 is reported to be present in early stages of atherosclerosis having a protective role against the rupture. Variant T allele has been associated with an increase expression and high MMP9 levels. Overexpression of MMP9 was found in atherosclerosis.^{21,22} Although previous studies show a positive correlation with the studied variant with the progression of cardiac diseases, a recent meta-analysis could not establish a positive correlation of the studied variant with cardiac diseases in Asian population.^{23,24} This study also found no association of the studied variant with CIMT in the β-TM patients.

The influence of T-138C polymorphism on various pathological processes can be realized through the changes in the *MGP* promoter activity. An assumed molecular mechanism of T-138C polymorphism is that it affects calcification and confers an increased risk of plaque and myocardial infarction.²⁵ An analysis of the *MGP* promoter activity revealed that the T-138C allele reduced the promoter activity by 20% in the rat vascular smooth muscle cells and by up to 50% in the human fibroblast cell line. The study also showed that a nuclear protein binds to the T-138C polymorphic site and that the binding is enhanced in the presence of the T allele. Thus, they showed that the difference in the promoter activity is due to differential binding of a nuclear protein that is important in *MGP* transcription.¹⁴ Taken together with previous studies^{15,18} along with our results, it is shown that T-138C polymorphism may not be a crucial factor contributing to atherosclerosis. Furthermore, may be because of numerous other factors that are known to independently and strongly affect formation of atherosclerosis, the contribution of this promoter polymorphism at T-138 within the *MGP* gene may not be an independent factor in our study of β-TM patients.

ERα is a ligand-activated transcription factor that plays an important role in vascular physiology and is involved in regulating multiple cellular pathways. ERα protein is expressed in human vascular endothelial and smooth muscle cells, and transgenic mice experiments have shown that ERα accelerates re-endothelialization, stimulates endothelial nitric oxide production, and inhibits the vascular injury response.^{26–29} ERs can affect the estrogen action at the tissue level, and thus play an essential role in the development of severe cardiac diseases.³⁰ Our study showed no association between the ERα gene variants (*PvuII* (rs2234693T > C) and *XbaI* (rs9340799A > G)) and the risk of atherosclerosis. Thus, we can say that these variants have a reserved influence on cardiac disease pathogenesis and the disease phenotype in our patient may be more strongly impacted by other factors.

Limitations

We have not followed and evaluated the patients for long term to see the association of these SNPs with the life-time risk of atherosclerosis.

Funding

This study was funded by Uttar Pradesh Council of Science and Technology, Uttar Pradesh, India.

Conflict of Interest

None.

Acknowledgment

The authors express gratitude to Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow, Uttar Pradesh, for providing the infrastructure for the work.

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