

## Basic research

Common rs5918 (PIA1/A2) polymorphism in the *ITGB3* gene and risk of coronary artery diseaseMehri Khatami<sup>1</sup>, Mohammad Mehdi Heidari<sup>1</sup>, Sorour Soheilyfar<sup>2</sup><sup>1</sup>Department of Biology, Faculty of Science, Yazd University, Yazd, Iran<sup>2</sup>Department of Biology, Ashkezar Islamic Azad University, Ashkezar, Yazd, Iran

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## Abstract

**Introduction:** The T to C transition at nucleotide 1565 of the human glycoprotein IIIa (*ITGB3*) gene represents a genetic polymorphism (PIA1/A2) that can influence both platelet activation and aggregation and that has been associated with many types of disease. Here, we present a newly designed multiplex tetra-primer amplification refractory mutation system – polymerase chain reaction (T-ARMS-PCR) for genotyping a single nucleotide polymorphism (SNP) (dbSNP ID: rs5918) in the human *ITGB3* gene.

**Material and methods:** We set up T-ARMS-PCR for the rs5918 SNP in a single-step PCR and the results were validated by the PCR-RFLP method in 132 coronary artery disease (CAD) patients and 122 unrelated healthy individuals.

**Results:** Full accordance was found for genotype determination by the PCR-RFLP method. The multiple logistic regression analysis showed a significant association of the rs5918 polymorphism and CAD according to dominant and recessive models (dominant model OR: 2.40, 95% CI: 1.33–4.35;  $p = 0.003$ , recessive model OR: 4.71, 95% CI: 1.32–16.80;  $p = 0.0067$ ).

**Conclusions:** Our T-ARMS-PCR in comparison with RFLP and allele-specific PCR is more advantageous because this PCR method allows the evaluation of both the wild type and the mutant allele in the same tube. Our results suggest that the rs5918 (PIA1/A2) polymorphism in the *ITGB3* gene may contribute to the susceptibility of sporadic Iranian coronary artery disease (CAD) patients.

**Key words:** glycoprotein IIIa PIA1/A2 polymorphism, rs5918, T-ARMS-PCR, coronary artery disease.

## Introduction

Coronary artery disease (CAD) is most commonly due to atherosclerotic occlusion of the coronary arteries and is the leading cause of death worldwide for both men and women [1]. Coronary artery disease occurs when plaque builds up inside the coronary arteries and can involve many blood vessels with a variety of presentations [2]. This can lead to coronary artery disease or heart failure and arrhythmias. The conventional risk factors for CAD such as elevated cholesterol, hypertension, obesity, and smoking were well associated with 30–40% increasing in mortality and morbidity [3]. Multiple epidemiological, family, and other factors have documented a genetic predisposition for CAD [4]. Most polygenic diseases such as CAD are due to several common genes and have their genetic predisposition transmitted by multiple genes [5]. In polygenic disorders many DNA markers and single nucleotide polymorphisms (SNPs) of unrelated individuals need to be analyzed by different methods. A greater

frequency of a SNP in patients indicates the SNP is in close proximity to a genetic risk variant for the disease. These variants strongly show the importance of a genetic predisposition for CAD and also confirm that these SNPs are even more common than expected [6]. However, it is well recognized that certain genes will relate to plaque rupture and/or thrombosis, and the beginning of the process of atherosclerosis. Glycoprotein IIIa (GPIIIa) or the beta subunit of the platelet membrane adhesive protein receptor complex GP IIb/IIIa is coded by the *ITGB3* gene, and is a surface protein found in various tissues, participating in cell-surface mediated signaling and cell adhesion [7]. The exons and introns of the entire *ITGB3* gene have been demonstrated to contain many polymorphic regions, one of which was found to be associated with cardiovascular diseases. This common *ITGB3* polymorphism at codon 33 of exon 2 (T1565C, dbSNP ID: rs5918) corresponds to the polymorphism of amino acid residues (leucine/proline) at position 33 (PIA1/A2) of the polypeptide chain. It has been reported that this SNP is a risk factor of many types of disease, such as myocardial infarction [8], coronary heart disease, type 2 diabetes, asthma [9, 10], many cancers including colon, non-Hodgkin lymphoma [11], breast [12–14] ovarian cancer [15] and kidney cancer [16]. It has also been documented that the platelets bearing the  $\beta 3$  subunit of integrin  $\alpha \text{IIb}\beta 3$  with a proline at position 33 are characterized by an increased risk for aggregation and immunogenic properties of platelets [3, 4]. Since the amount of platelet aggregates is increased in carriers of the PIA2 allele with CAD, it is of interest to determine whether this polymorphic variant is associated with this disease. The previous data on the association of the polymorphic marker PIA1/A2 of the *ITGB3* gene with both arterial and venous thrombosis in various ethnic groups [5, 6] are contradictory [17, 18]. The presence of the PIA2 allele is associated with an increased binding affinity to fibrinogen as well as with platelet aggregability in response to epinephrine, adenosine diphosphate and collagen *in vitro* [19]. Several studies have suggested that the PIA2 allelic variant causes an altered sensitivity to aspirin and an increased sensitivity to platelet aggregation by various agonists [20, 21].

Single nucleotide polymorphism (SNP) genotyping has been done by PCR-RFLP, real time-PCR and direct sequencing methods [22–24]. These techniques have certain limitations and are relatively slow and very expensive, and fewer reactions are catalyzed per kit in comparison to the tetra primer-amplification refractory mutation system-PCR (T-ARMS-PCR). With this method, the genotyping could be done using only a thermocycler machine at the least time, and can be used to genotyping of essential SNPs in the entire genome.

In conventional ARMS PCR, the amplification of the normal and mutant allele is done in two separate reactions, but in T-ARMS-PCR, we can amplify both the normal and mutant allele with a control fragment in a single reaction. In the T-ARMS-PCR method two outer, non-allele-specific primers and two inner, allele-specific primers in opposite orientation to each other are used.

The outer primers amplify a large fragment of the target gene containing a variant nucleotide as a control fragment and smaller allele-specific amplicons with different sizes that can easily be discriminated in gel electrophoresis either as homozygous or heterozygous. A deliberate mismatch at position 2<sup>nd</sup> or 3<sup>rd</sup> nucleotide from the 3' terminal end of the inner primers can improve allele specificity [25].

The results of a meta-analysis of research showed that the PIA2 variant was associated with an increased risk of coronary heart disease [26, 27]. Some reports suggest that PIA1/A2 heterozygotes are prone to thrombotic disease, whereas PIA1/A1 homozygotes may be prone to early atherosclerosis and more rapid progression of stable coronary artery disease [10]. Presently, we are developing a rapid single-step method using T-ARMS-PCR for detection of Leu33Pro (PIA1/A2), which is a possible SNP in cardiovascular diseases studies.

## Material and methods

### Patients and DNA extraction

This study was performed on 132 patients selected from 412 patients who referred to cardiac centers in Afshar Hospital (Yazd, Iran) due to symptoms of myocardial infarction between 2012 and 2015. Patients had major lesions (> 50% narrowing of luminal diameter) in one, two, or three vessels (LAD, LCX, and RCA) that were candidate vessels for coronary artery bypass graft surgery. CAD patients were identified according to the coronary angiography guidelines [28].

When up to 50% blockage was observed in the major epicardial coronaries and their branches associated with stenotic lesions, coronary arterial disease was considered to be present. We also chose 122 unrelated healthy individuals matched for age, sex, and ethnicity with normal or near-normal angiography reports (no lesion greater than 30%) as a control group (Table I). All of the patients and the control group were informed of the aims of the study and gave their informed consent for the genetic analysis. Genomic DNA was isolated from the peripheral blood samples using a DNA isolation kit (DNAfast Kit-Genfanavar, Tehran, Iran).

### Primer design and bioinformatics analysis

A common missense rs5918 (PIA1/A2) polymorphism in the *ITGB3* gene was selected (<http://>

**Table I.** Clinical characteristics of coronary atherosclerosis patients

Parameter	Patients (n = 132)	Controls (n = 122)	P-value
Male gender (%)	75	71	0.670
Age [years]	52.6 ±6.8	52.6 ±6.9	0.56
Smokers (%)	28	23	0.517
Body mass index [kg/m <sup>2</sup> ]	26.1 ±2.4	25.3 ±1.9	0.0012
Cholesterol [mg/dl]	214.9 ±52.8	181.1 ±36.8	0.001
LDL-C [mg/dl]	128.7 ±43.4	118.2 ±44.1	0.145
HDL-C [mg/dl]	45.4 ±8.5	46.9 ±11.2	0.456
TGs [mg/dl]	205.7 ±102.1	153.4 ±96.1	0.014

**Table II.** PCR primers and conditions

ITGB3 (SNP ID: rs5918)	Primer sequence	Temperature [°C]	Amplicon size
	Fo: 5'-GGATTATCCCAGGAAGACCAC	66	T allele (284 bp)
	Ro: 5'-GACTTCCTCCTCAGACCTCCAC-3'	70	C allele (179 bp)
	Fi: 5'-TGTCTTACAGGCCCTGCCGCT-3'	68	Control band (424 bp)
	Ri: 5'-GGTCACAGCGAGGTGAGCACG-3'	70	

www.ncbi.nlm.nih.gov/books/SNP). The four primers used in this research were designed by the on-line website Primer1: <http://primer1.soton.ac.uk/primer1.html>. The specificity of the primers was checked by the 'BLAST' program at <http://www.ncbi.nlm.nih.gov/blast> (Table II). To enhance the specificity of inner primers, at the 3<sup>rd</sup> nucleotide from the 3'-terminus changes to destabilizing mismatch.

SWISS-MODEL (<http://swissmodel.expasy.org>) was used for protein modeling of mutant protein. SWISS-MODEL is a fully automated protein structure homology-modeling server. The PyMol software was used for visualizing the effect of the altered residue on the protein structure.

#### T-ARMS-PCR and PCR-RFLP analysis

Polymerase chain reaction was performed in a total volume of 12.5 µl containing 50 ng of template DNA, 250 nM of each primer, 250 µM dNTPs, 1.5 mM MgCl<sub>2</sub>, 1X buffer and 0.5 U of *Taq* polymerase (Yekta Tajhiz Azma, Tehran, Iran). PCR amplification (touchdown) was carried out at 95°C for 2 min, followed by denaturation at 95°C for 20 s, first annealing at 68°C (10 cycles), further (25 cycles) annealing at 69°C for 1 min and extension at 72°C for 50 s, followed by a final extension for 5 min. Non-denaturation polyacrylamide gel electrophoresis (6%) and silver staining were used for detection of PCR products.

This procedure rendered 2 bands in homozygotes (PIA1/A1 resulting in 424 and 285 bp, and

PIA1/A1 resulting in 424 and 180 bp) and 3 bands in heterozygotes (424, 285 and 180 bp). Figure 1 shows the products of tetra-primer ARMS-PCR for 5 different genomic DNA samples.

In this study, the concentration of PCR components such as primers, magnesium chloride, dNTP and *Taq* DNA polymerase were optimized. Beside PCR components, the PCR conditions were also optimized.

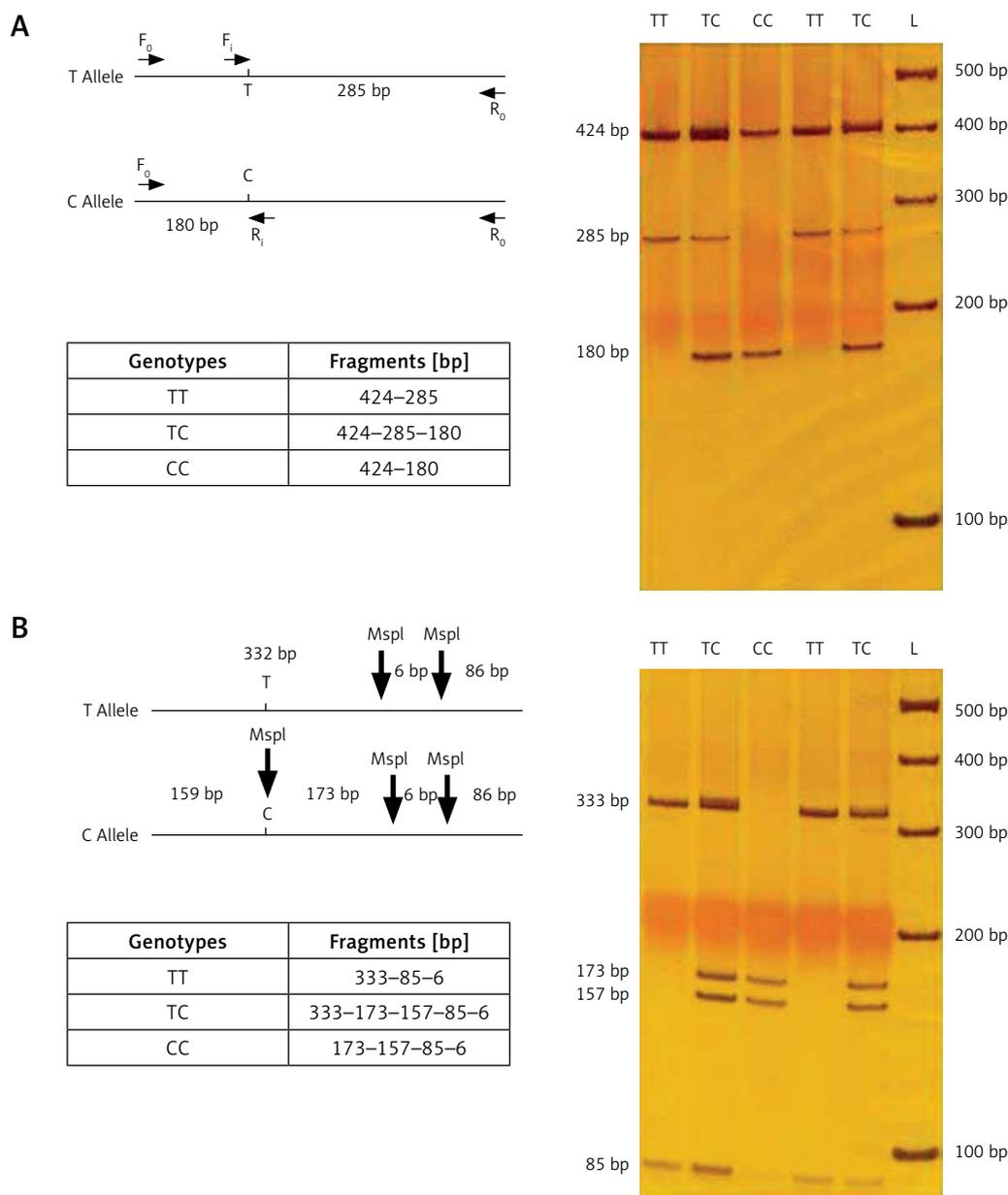
The forward (Fo) and reverse (Ro) primers were used to amplify the 424-bp fragment. The 424-bp product was digested with 5U *MspI* restriction enzyme for 16 h. Fragments were separated by electrophoresis using 6% polyacrylamide gel electrophoresis. The resulting fragments were 332, 86 and 6 bp for the PIA1 allele and 173, 159, 86 and 6 bp for the PIA2 allele (Figure 1).

#### Statistical analysis

The SPSS software (IBM SPSS 22, SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The association between two groups was examined by the  $\chi^2$  goodness-of-fit test. Multiple logistic regression models (co-dominant, dominant and recessive) were employed to analysis the genetic data. Values of  $p < 0.05$  were regarded as statistically significant.

#### Results

Total DNA samples analyzed with T-ARMS-PCR were re-evaluated by PCR-RFLP analysis using the restriction enzyme *MspI*. We obtained 100% accor-



**Figure 1. A** – Results of T-ARMS-PCR of *ITGB3* polymorphism (rs5918); **B** – validation using PCR-RFLP by *MspI* restriction endonuclease

dance between both methods for genotype determination. Mean age (mean  $\pm$  SD) was  $53.9 \pm 6.8$  and  $52.6 \pm 6.9$  years for patients and controls, respectively. Coronary angiography revealed 132 patients (CAD<sup>+</sup> group) with one vessel (LAD) ( $n = 31$ ), two vessels (LCX) ( $n = 51$ ), or three vessels (RCA) ( $n = 49$ ) that were candidate vessels for coronary artery bypass grafting (CABG) and 122 patients (CAD<sup>-</sup> group) with no angiographically identified narrowing.

Genotype distributions and allelic frequencies of the *ITGB3* polymorphism among CAD patients and controls are shown in Table III. The frequency of the *ITGB3* C allele was significantly higher in the CAD patients than in the control group ( $p < 0.001$ ).

The multiple logistic regression analysis showed a significant association of the rs5918 polymorphism and CAD according to dominant and recessive models (dominant model OR: 2.40, 95% CI: 1.33–4.35;  $p = 0.003$ , recessive model OR: 4.71, 95% CI: 1.32–16.80;  $p = 0.0067$ ) (Table III).

### Discussion

So far, several variants of the *ITGB3* gene have been described [27]. The correlation of the rs5918 polymorphism with the incidence of CAD was first reported by Marian *et al.* [29]. In the present study, the allele and genotype frequencies of the polymorphic marker P1A1/A2 of the *ITGB3* gene were determined in a group of patients with CAD and

**Table III.** Genotype and allele variant frequencies in patients and controls

<i>ITGB3</i> (rs5918)	Patients (n = 132)	Controls (n = 122)	OR (95% CI)	P-value
Codominant model:				
TT	88 (66.67%)	101 (85.2%)	1	
TC	30 (22.7%)	18 (14.8%)	0.523 (0.273–1.002)	0.051
CC	14 (10.6%)	3 (0.0%)	0.187 (0.052–0.671)	0.010
Dominant model:				
TT	88 (66.7%)	101 (82.8%)		
TC + CC	44 (33.3%)	21 (17.2%)	2.40 (1.33–4.35)	0.003
Recessive model:				
CC	14 (10.6%)	3 (2.5%)		
TC + TT	118 (89.4%)	119 (97.5%)	4.71 (1.32–1.608)	0.0067
Allele frequency:				
T	206 (78.0%)	220 (90.2%)		
C	58 (22.00%)	24 (9.8%)	0.387 (0.232–0.644)	< 0.001

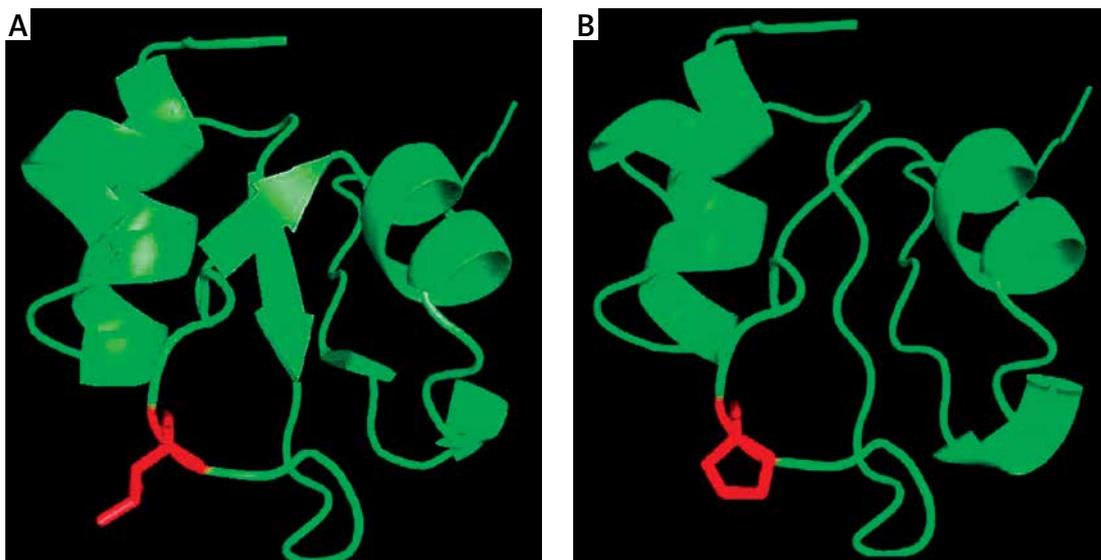
OR – odds ratio, CI – confidence interval.

control subjects. Weiss *et al.* demonstrated the association of the PIA2 allele with the risk of cardiovascular diseases [30]. PIA2 allele frequency was significantly higher in CAD patients than in control subjects. The frequency distributions of different genotypes and alleles using techniques for detection of the *ITGB3* gene in other population studies are described in Table IV [22, 24, 31–33].

Protein modeling based on human *ITGB3* protein structure (pdb id: 4g1m) shows that L33 is located on the beta strand of the extracellular domain. *ITGB3* is a heterodimer composed of noncovalently associated a and b subunits. These sub-

units have a large extracellular domain, a single transmembrane region, and a short cytoplasmic domain [12, 33]. The L33P change lies in the N-terminal of *ITGB3*. An analysis of the *ITGB3* wild-type and L33P mutant-derived protein structure using the SWISS-MODEL server indicates that three-dimensional structure of the protein is disrupted in the mutant (Figure 2).

In general, the allele and genotype frequency distributions in the Iranian population were similar to those in other populations. Thus, the results of our study suggest that the polymorphic variant PIA1/A2 is associated with CAD in the Iranian population.



**Figure 2.** Molecular model of human *ITGB3* protein base in PDB file 4g1m showing close-up of the L33 (A) and P 33 residues (B) using PyMol

**Table IV.** Distribution of *ITGB3* polymorphism in current study in comparison with previously published data

Study	Population	N	Genotype (%)			Allele (%)		Genotyping platform
			TT	TC	CC	T	C	
(rs5918 SNP)								
Our study	Iranian	254	74.4	18.9	6.7	83.9	16.1	T-ARMS-PCR
Torabi <i>et al.</i>	Iranian	200	70	14	16	77	23	PCR-RFLP
Nikolajevic-Starcevic <i>et al.</i>	Slovenia	342	47	36.8	16.2	65.5	34.5	PCR-RFLP
Zhang <i>et al.</i>	Chinese	622	96	4	0	98	2	High-resolution melting analysis (HRM)
Yilmaz <i>et al.</i>	Turkish	184	83.7	13	3.3	90.2	9.8	PCR-RFLP
Bianconi <i>et al.</i>	Austrian	109	79.8	17.4	2.8	88.5	11.5	PCR-RFLP

The common *ITGB3* studies have utilized *MspI* digestion to identify the rs5918 polymorphism. Because this polymorphism is a risk factor in many diseases including cardiovascular diseases and cancers, here we developed a rapid, sensitive and one-step tetra-primer PCR-ARMS method for detection of the rs5918 polymorphism.

The most critical step for successful development of the new multiplex tetra-primer amplification refractory mutation system-PCR method is the primer design. In this study, the primers for amplification of *ITGB3* rs5918 were designed using the online website Primer1: <http://primer1.soton.ac.uk/primer1.html>. Both the outer primers were designed with 21 base pairs (bp) in length and the inner primers were designed with 22 bp with a guanine and adenine located at the 3<sup>rd</sup> nucleotide from the 3'-terminus for forward (wild type allele) and reverse (mutant allele) primers to increase their specificity for the template DNA.

Our T-ARMS-PCR in comparison with RFLP and allele-specific PCR is more advantageous because this PCR method allows the evaluation of both the wild type and mutant allele in the same tube. This assay is a reproducible and stable single-tube reaction.

In conclusion, our T-ARMS-PCR needs only a small amount of traditional PCR reagents, without special equipment. Our results suggest that the rs5918 (PIA1/A2) polymorphism in the *ITGB3* gene may contribute to the susceptibility to sporadic CAD. Therefore, this procedure could be used in most clinical diagnostic laboratories.

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### Conflict of interest

The authors declare no conflict of interest.

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