Frequency of single nucleotide platelet receptor gene polymorphism (*P2Y12-i744T>C*) in coronary artery disease patients among Tamilian population

R. Priyadharsini^{1,2} · G Umamaheswaran² · T. A. R. Raja³ · A. S. Arun Kumar² · K. Subraja⁴ · S. A. Dkhar² · S. Satheesh⁵ · C. Adithan^{2,6} · D. G. Shewade²

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Abstract Several factors contribute to the development of coronary artery disease (CAD). Adenosine diphosphate (ADP) activated P2Y12 receptor also plays a key role in platelet activation and aggregation. It has been found that common variation in the *P2Y12* gene was associated with increased platelet aggregation resulting in adverse cardiovascular outcomes. Thus, polymorphisms in the ADP receptor P2Y12 may contribute to the development of CAD. This study aims to determine the frequency distribution of platelet receptor polymorphism *P2Y12 (i744T>C)* in Tamilian population and to predict its possible role in CAD. Three hundred seventy-one subjects were recruited comprising of 221 healthy volunteers and 150 patients with CAD belonging to either sex, aged 18–60 years of Tamilian origin. Genomic DNA was extracted using phenol-chloroform method. Genotyping was done by PCR-RFLP (Polymerase chain reaction-restriction fragment

length polymorphism). The C allele frequency of *P2Y12* polymorphism in controls and cases was 8.4% and 17.7%, respectively. The TT, TC, and CC genotype frequencies in controls and cases were 83.7%, 15.8%, 0.5% and 66.7%, 31.3%, 2%, respectively. The genotype frequencies were in Hardy-Weinberg equilibrium. There was a significant association ($p < 0.05$) between the mutant genotypes of *P2Y12 (i744T>C)* polymorphism and risk of CAD. The odds ratio was found to be 2.6. The variant allele frequency of *P2Y12-i744T>C* was significantly different from other populations. There was a significant association between the mutant genotypes of *P2Y12 (i744T>C)* polymorphism and risk of developing CAD. Thus, the present study will emphasize on the relevance of pharmacogenetic testing of *P2Y12 (i744T>C)* receptor gene polymorphism in CAD patients.

✉ R. Priyadharsini
shalipriya85@gmail.com

- ¹ Department of Pharmacology, Indira Gandhi Medical College and Research Institute (IGMCRI), Puducherry 605009, India
- ² Pharmacogenomics Laboratory, Department of Pharmacology, Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Puducherry 605006, India
- ³ Department of Pharmacology, Melmaruvathur Adhiparasakthi Institute of Medical Sciences (MAPIMS), Melmaruvathur, Tamil Nadu, India
- ⁴ Pharma Consultancy, Chennai, India
- ⁵ Department of Cardiology, Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Puducherry 605006, India
- ⁶ Mahatma Gandhi Medical College and Research Institute (MGMC&RI), Pillaiyarkuppam, Pondicherry 607403, India

Introduction

Coronary artery disease (CAD) is a multifactorial disorder. Challenges to the pharmacotherapy of coronary artery disease have attracted the interest of researchers in recent years. Myocardial infarction and ischemic stroke are two of the leading causes of mortality and morbidity. To date, there are numerous genetic and environmental factors which contribute to atherosclerosis and thrombosis. One among them is the polymorphism in the platelet receptor gene *P2Y12*, which has been studied so far to find out its role in the development of CAD and also in the platelet response variability, leading to adverse outcomes like stroke, myocardial infarction etc. P2Y12 receptor, located on the platelet surface, plays an important role in the platelet activation as well as inhibition (Trenk et al. 2013) (Gurbel et al. 2012). This P2Y12 protein is a potential target for the treatment of thromboembolism and other clotting disorders. It is encoded by *P2Y12* gene (*rs2046934*), located on the chromosome 3q24-q25

(Zee et al. 2008). The *P2Y12* gene spans 47 kb and is made up of three exons and two introns. Initially, five *P2Y12* receptor gene polymorphisms were identified. Four of these (i-C139T, i-T744C, i-ins801A, G52T, C34T) were shown to be in complete linkage disequilibrium. They were designated as H1 and H2 haplotypes, frequencies being 86% and 14%, respectively. The H2 haplotype was found to have a strong association with cardiovascular risk (Fontana et al. 2003b).

Cavallari et al. have stated that gene sequence variation of the *P2Y12* receptor gene was associated with the presence of significant CAD (Cavallari et al. 2007). However, researches in various groups of patients proved almost a controversial result in the association between the common polymorphism of *P2Y12* and CAD patients. *P2Y12* receptor gene polymorphisms have been shown to be correlated, not only with CAD but also with peripheral arterial disease. There was a positive association between *P2Y12* receptor H2 haplotype and altered platelet aggregation in 184 cases with peripheral arterial disease (Fontana et al. 2003b). Therefore, the involvement of this particular type of polymorphism proves to be a major challenge in the field of cardiovascular therapy especially in patients with CAD. Henceforth, the exact role of *P2Y12* polymorphism in association with CAD has to be substantially evaluated in clinical settings. Moreover, there are studies reporting the allele and genotype frequency of *P2Y12* gene polymorphism in other populations (Azarpira et al. 2011) (Shalia et al. 2013) (Kar et al. 2013), but to our knowledge, there is no data available for South Indian Tamilian population. Hence, the present study was aimed to determine the genotype and allele frequency of *P2Y12* (*i744T>C*) gene polymorphism in the South Indian Tamilian population to predict its possible role in the development of CAD in this population.

Materials and methods

Subjects

The study was performed in 371 subjects aged between 18 and 60 years of age, both genders, comprising of 221 unrelated healthy volunteers and 150 proven cases of CAD patients recruited from the outpatient department of Cardiology, Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER) from January 2010 to July 2011. All participants were unrelated residents of Tamil Nadu and Pondicherry for three consecutive generations speaking Tamil as their native language. Institute Ethics Committee approval was obtained prior to the start of this study, and all subjects were encouraged to clarify their questions before signing the consent forms. Informed consent was obtained from all individual participants included in the study. Considering a power of 80%, the sample size for estimating the difference between cases and controls was calculated

based on frequency distribution of C allele (Martinelli et al. 2008) and was found to be a minimum of 129 subjects per group. Thus, the final sample size was estimated to be around 150 in each group which included the allowances for drop outs and sample loss during DNA extraction and genotyping.

Genotyping

Five milliliters of venous blood was collected using EDTA as an anticoagulant from all the subjects with sterile precautions. Genomic DNA was extracted using phenol-chloroform extraction procedure and genotyping was done by PCR-RFLP (Polymerase chain reaction-restriction fragment length polymorphism) targeting the *P2Y12-i744T>C* (*rs2046934*) single nucleotide polymorphism. The reaction was performed in a total volume of 25 μ l which includes 100 ng of DNA, 0.5 pmol of each primer (sense primer 5'-TCA CTT ATC TCT GGT GAA ATA AAA AGA TTA CGT A-3' and antisense primer 5'-GTC AGA AAT GGC CTG TGT ATA TAT GGT CAT GAG-3'), 100 μ mol/l of each dNTPs, 2.5 μ l of 10 $\times$ reaction buffer, 1.5 μ l of magnesium chloride, and 0.2 unit of Taq DNA polymerase. PCR parameters consisted of initial denaturation at 95 $^{\circ}$ C for 5 min, followed by 30 cycles at 95 $^{\circ}$ C for 30s, 60 $^{\circ}$ C for 60s, and 72 $^{\circ}$ C for 60s with the final extension for 10 min at 72 $^{\circ}$ C. The PCR product (230 bp amplicon) thus obtained was analyzed in 1% agarose gel followed by digestion with *RsaI* enzyme for 12 h at 37 $^{\circ}$ C and finally analyzed on 8% polyacrylamide gel electrophoresis with ethidium bromide staining. In the presence of C allele, the enzyme generates two fragments of 197 and 33 base pairs. It retains the single bond of 230 bp in the case of T allele (wild type). The genotyping method was confirmed by direct DNA sequencing.

Statistical analysis

Variables are expressed as mean $\pm$ standard deviation and frequencies in percentages. Direct gene counting method was used to determine the genotype and allele frequency. Chi-square test was used for assessing the Hardy-Weinberg Equilibrium. The genotype and allele frequency of the present study with the previous studies and between the cases and controls were compared using Chi-square or Fisher's exact test wherever it is appropriate. The statistical analysis was performed using SPSS for Windows version 16.0 software (SPSS, Chicago, USA). A *p* value <0.05 was considered statistically significant.

Results

There were no significant differences in the demographic profile between the cases and controls. Table 1 depicts the genotype and allele frequency distribution of *P2Y12* (*i744T>C*)

Table 1 *P2Y12 i744T>C* polymorphism and risk of coronary artery disease among cases and controls in Tamilian population

<i>P2Y12 i744T>C</i>	Controls (<i>n</i> = 221)	Cases (<i>n</i> = 150)	Odds ratio (95% CI)	<i>p</i> value
Allele				
T (%)	405(91.6)	247(82.3)	2.4 (1.5–3.7)	0.0002
C (%)	37(8.4)	53(17.7)		
Genotype				
TT (%)	185 (83.7)	100 (66.7)	2.6 (1.6–4.2)	0.0002
TC (%)	35 (15.8)	47 (31.3)		
CC (%)	1 (0.5)	3 (2)		

polymorphism among different cases and controls in South Indian Tamilian population. The observed frequency was found to be in Hardy-Weinberg equilibrium. Among the 221 controls, 185 (83.7%) belonged to TT genotype (wild), 35 (15.8%) were TC (heteromutant), and 1 (0.5%) belonged to CC genotype (homomutant). The T and C allele frequency was found to be 91.6% and 8.4%, respectively. On the contrary, among the 150 cases with CAD the TT, TC, and CC genotype frequencies were found to be 100 (66.7%), 47 (31.3%), and 3 (2%), respectively. The T and C allele frequency was found to be 82.3% and 17.7% with the cases. It was thus found that the *P2Y12* gene polymorphism is strongly associated with the development of CAD in the Tamilian population as well. The genotypic odds ratio (CT/CC) was estimated to be 2.6 ($P < 0.05$). The obtained genotype and allele frequency of our study was compared with that of the other

major ethnic groups and it was found that the frequency distribution among the Tamilian population was almost similar to that of the Maharashtrian, Gujarati, and Marwadi representing the Western region of India but different from the North Indian population. Similarly, the distribution of C allele frequency in Tamilian population was significantly different from Caucasians, Orientals, and other major population (Table 2). This implicates the role of *P2Y12 (i744T>C)* gene polymorphism being one of the risk factors for CAD especially in South Indian Tamilian population. The results of studies depicting the association between *P2Y12 (i744T>C)* polymorphism and the risk of CAD among other populations are shown in Table 3.

Discussion

The functional role of platelets in CAD has been investigated by many researchers in great detail in the past as well as in the present (Alexandru et al. 2012) (Ombrello et al. 2010). Platelets play a well-defined role in both hemostasis and atherothrombosis by releasing important mediators (Nesbitt et al. 2006). Adenosine diphosphate (ADP) is one of the mediators that plays an important role in hemostasis. Thus, the activation of *P2Y12* receptor by ADP results in arterial thrombosis by the formation of hemostatic plugs leading to the development of CAD (Jennings 2009). Association between *P2Y12* receptor polymorphism *i744T>C* and CAD has been studied both in healthy controls and in patients with atherothrombotic

Table 2 Comparison of distribution of genotype and allele frequencies (%) of *P2Y12 i744T>C* polymorphism with other ethnics

Population	Subjects (<i>n</i>)	<i>P2Y12 i744T>C</i>					Author	Year
		Genotype frequency			Allele frequency			
		TT	TC	CC	T	C		
South Indian Tamilian	221	185 (83.7)	35 (15.8)	1 (0.5)	405(91.6)	37(8.4)	Present	Present
Western Indian	102	87 (85.2)	12 (11.8)	3 (2.94)	186 (91.2)	18 (8.8)	(Shalia et al. 2013)	2013
American*	341	231 (68)	102 (30)	8 (2)	564 (82)	118 (18)	(Zee et al. 2008)	2008
	259	173 (66.8)	83 (32)	3 (1.2)	430 (83)	88 (17)		
	108	67 (62)	37 (34.3)	4 (3.7)	171 (79)	45 (21)		
American	415	249 (79)	60 (19.1)	6 (1.9)	558 (88.6)	72 (11.4)	(Martinelli et al. 2008)	2008
Egyptian*	40	39 (97.5)	1 (2.5)	0	79 (99)	1 (1)	(Zoheir et al. 2013)	2013
French	330	262 (79)	59 (18)	9 (3)	583 (88)	77 (12)	(Fontana et al. 2003b)	2003
French	98	74 (75.5)	21 (21.4)	3 (3.1)	169 (86.2)	27 (13.8)	(Fontana et al. 2003a)	2003
French*	29	14 (48.3)	11 (37.9)	4 (13.8)	39 (67.3)	19 (32.7)	(Bura et al. 2006)	2006
Iranian*	112	1 (1)	8 (7)	103 (92)	10 (4)	214 (96)	(Azarpira et al. 2011)	2010
Italian	387	309 (80)	69 (18)	9 (2)	687 (88.8)	87 (11.2)	(Cavallari et al. 2007)	2007
Korean*	385	258 (67)	115 (30)	12 (3)	631 (82)	139 (18)	(Lee et al. 2011)	2008
Korean*	415	283 (68.2)	125 (30.1)	7 (1.7)	691 (83)	139 (17)	(Jang et al. 2012)	2012

* $p < 0.05$ considered statistically significant compared to that of the Tamilians, values in parentheses indicate percentage

Table 3 Association of *P2Y12(i744T>C)* polymorphism among cases and controls with CAD or PVD in comparison with that of other ethnics

Population	Author (ref)	Year	Study type	Associated disease	Sample size cases/controls	C allele frequency cases/controls (%)	Association	Associated allele
South Indian	Present	Present	Case control	CAD	150/221	53 (17.7)/37 (8.4)	S	C
West Indian	(Shalia et al. 2013)	2013	Case control	AMI	26/102	9 (17.3)/18 (8.8)	S	C
North Indian	(Kar et al. 2013)	2013	Cross sectional	CAD	100	0	NS	NS
American	(Zee et al. 2008)	2008	Case control	AMI	341/341	107 (15.7)/118 (18)	NS	NS
American			Case control	IS	259/259	84 (16)/88 (17)	NS	NS
American			Case control	DVT/PE	108/108	27 (12.5)/45 (21)	S	T
American	(Lev et al. 2007)	2007	Cross sectional	ACS with PCI	120	36 (15)	NS	NS
American	(Martinelli et al. 2008)	2008	Case control	CAD	489/415	126 (13)/72 (11.4)	S*	C
American	(Smith et al. 2006)	2006	Cross sectional	ACS with PCI	Cohort 1 = 54 Cohort 2 = 55	— —	NS	NS
American	(Angiolillo et al. 2005)	2005	Cross sectional	CAD	Group A = 36 Group B = 83	17 (23.6) 28 (17)	NS	NS
Brazilian	(Schetttert et al. 2006b)	2006	Cross sectional	CAD	540	141 (13)	NS	NS
Egyptian	(Zoheir et al. 2013)	2013	Case control	CAD	40/40	12 (15)/1 (1)	S	C
French	(Cuisset et al. 2007)	2007	Cross sectional	CAD	597	151 (12.6)	NS	NS
French	(Fontana et al. 2003b)	2003	Case control	PAD	184/330	59 (16)/77 (12)	S	C
French	(Fontana et al. 2003a)	2003	Cross sectional	Healthy volunteers	98	27 (13.8)	S	C
French	(Bura et al. 2006)	2006	Cross sectional	Healthy volunteers	29	19 (32.7)	S	C
French	(Szymezak et al. 2011)	2011	Case report	CAD	1	—	S	C
Iranian	(Namazi et al. 2012)	2012	Cross sectional	CAD	112	—	NS	NS
Iranian	(Azarpira et al. 2011)	2010	Cross sectional	Healthy controls	112	214 (96)	S	C
Italian	(Cavallari et al. 2007)	2007	Case control	CAD	991/387	267 (13.5)/87 (11.2)	S	C
Italian	(Giusti et al. 2007)	2007	Cross sectional	ACS	1419	(10.4)	NS	NS
Korean	(Lee et al. 2011)	2008	Cross sectional	ACS with PCI	385	139 (18)	NS	NS
Korean	(Kim et al. 2013)	2013	Cross sectional	Healthy controls	158	(18)	NS	NS
Korean	(Jang et al. 2012)	2012	Case control	VTE	154/415	50 (16)/139 (17)	NS	NS
Mexican	(Isordia-Salas et al. 2012)	2012	Cross sectional	ACS with PCI	60	18 (15)	NS	NS
Polish	(Malek et al. 2008)	2008	Cross sectional	ACS with PCI	105	28 (14)	S*	C

CAD coronary artery disease, PVD peripheral vascular disease, AMI acute myocardial infarction, IS ischemic stroke, DVT deep vein thrombosis, PE pulmonary embolism, ACS acute coronary syndrome, PCI percutaneous coronary intervention, VTE venous thromboembolism, PAD peripheral arterial disease, S significant, NS not significant, *—in combination with other hemostatic gene polymorphisms, Group A—patients on 300mg loading dose of clopidogrel, Group B—patients with 75 mg maintenance dose of clopidogrel, Cohort 1—patients on 300 mg loading dose and 75 mg maintenance dose of clopidogrel, Cohort 2—patients on 600 mg loading dose of clopidogrel

disease. In Indian scenario, a study done in North Indian patients with CAD does not show any mutant genotypes for *P2Y12* (*i744T>C*) polymorphism (Kar et al. 2013). However, the study which was done on Maharashtrian, Gujarati, and Marwadi representing the western region of India showed a similar frequency distribution both in cases and controls as compared to that of the present study done in South Indian Tamilian population (Shalia et al. 2013). This shows that there is significant intraethnic and interethnic variation in the distribution of variant alleles among Indians.

Further, studies in other ethnics also showed potential conflicting associations. Few studies done on different population concluded that the carriers with H2 alleles have higher propensity for CAD with maximal platelet aggregation in response to ADP (Fontana et al. 2003a) (Bura et al. 2006) (Cavallari et al. 2007). However, on the contrary, similar studies concluded that the gene sequence variation in *P2Y12* did not contribute to the risk of development of CAD in Caucasian (Smith et al. 2006) (Lev et al. 2007) (Zee et al. 2008) (Malek et al. 2008), in French (Cuisset et al. 2007), in Brazilian (Schettert et al. 2006), in Mexican (Isordia-Salas et al. 2012), in Italian (Giusti et al. 2007), and Korean population (Lee et al. 2011) (Jang et al. 2012). Thus, the reasons for such conflicting results may be that a combination of hemostatic gene polymorphisms may be implicated in development of CAD rather than *P2Y12* as a single entity (Martinelli et al. 2008) (Tang et al. 2013).

It has also been found that this particular platelet receptor gene polymorphism may also play an important role in altered response to antiplatelet therapy especially with clopidogrel, thereby acting as a risk factor for CAD. In a study on Egyptian population, it was found that the *i744T>C* polymorphism is associated with an increased platelet activation response to ADP (Zoheir et al. 2013). A study done on 112 Iranian subjects concluded that the *P2Y12* polymorphisms may be implicated in clopidogrel response variability (Azarpira et al. 2011). Similarly, a cross-sectional study in 112 Iranian patients reported that 25.9% of them were non-responders to clopidogrel (Namazi et al. 2012). However, in a smaller study of 36 patients with 300 mg loading dose and 83 patients with 75 mg/d of maintenance dose of clopidogrel, the *P2Y12* receptor haplotypes were not associated with clopidogrel response variability (Angiolillo et al. 2005). Hence, further genotype-phenotype association studies are required to show the possibility of this particular polymorphism in the development of CAD, PVD, and clopidogrel response variability, which is considered as one of the limitations of our study.

Thus, the impact of platelet receptor gene polymorphism, acting as a risk factor for coronary artery thrombosis, remains much controversial. Interestingly, the presence of significantly higher prevalence of this particular polymorphism in cases in Tamilian population suggests a strong likelihood of this

particular genetic polymorphism being implicated in the pathogenesis of the disease progression in the CAD patients in Tamilian population.

Conclusion

The present case-control study done on 371 subjects of South Indian Tamilian population explores the possibility of the role of *P2Y12* (*i744T>C*) gene polymorphism being implicated for the risk of developing CAD. Hence, this platelet receptor gene polymorphism may also be taken into account while considering the risk assessment for CAD especially in Tamilian population since the frequency distribution is different from the other ethnic population. Thus, our study may emphasize on the relevance of pharmacogenetic testing of *P2Y12* (*i744T>C*) receptor gene polymorphism in Tamilian CAD patients.

Conflict of interest The authors declare that they have no conflict of interest.

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Compliance with ethical standards The study was performed in compliance with the ethical standard. Institute Ethics Committee approval was obtained prior to the start of this study, and all subjects were encouraged to clarify their questions before signing the consent forms. Written informed consent was obtained from all individual participants included in the study.

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