Association of Cholesteryl Ester Transfer Protein (CETP) Taq1B Polymorphism with Response to Simvastatin Treatment in Hypercholesterolemic Filipino Patients

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ABSTRACT

Although statins reduced cardiovascular mortality, these drugs did not prevent myocardial infarction in some patients. Previous studies showed that genetic variation in *cholesteryl ester transfer protein (CETP)* gene was linked to this response. The identified gene is characterized by two different variants: *B1* and *B2* alleles identified by the presence and absence, respectively, of a restriction site for the enzyme *Taq1* in intron 1. The present study identified the variation in *Taq1B* of the gene using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) in 130 patients. An association study of *Taq1B* with the response of 24 middle-aged dyslipidemic patients to simvastatin treatment for 8 weeks was also done. The overall allele frequencies of *B1* and *B2* alleles were 0.548 and 0.462, respectively. The genotype frequencies were in Hardy-Weinberg equilibrium. The distinguishing feature of individuals with *B1B1* genotype when treated with simvastatin was their rapid increase in high density lipoprotein (HDL) observed after 2 weeks which continued till the 8th week treatment. The expected HDL elevation among individuals with *B1B2* genotype was observed only after the 8th week simvastatin treatment.

Key Words: cholesteryl ester transfer gene variation, intron, Polymerase Chain Reaction-Restriction Fragment Length Polymoprhism (PCR-RFLP), simvastatin, Filipinos

Introduction

Lipid transfer proteins regulate the concentrations of cholesteryl esters and triglycerides in the blood. Cholesteryl ester transfer protein (CETP), a lipid transfer protein, catalyzes the net mass transfer of cholesteryl ester from high density lipoprotein (HDL) and low density lipoprotein (LDL) to very low density lipoprotein (VLDL).[1] Excess cholesterol from the peripheral tissues, on the other hand is transported back to the liver for excretion into the bile.[2] Increased activity of CETP lowers plasma HDL concentration and increases formation of small-sized LDL. The plasma CETP concentration in normolipidemic individuals vary over a 3-fold range and is influenced both by genetic and environmental factors. Several studies had been done to observe the effect of *CETP* gene polymorphism on CETP concentrations and plasma lipid parameters.[1-3]

The *CETP* gene (Genbank Accession No. M83573) encodes a mature polypeptide of *Mr* 74,000.[4] It has 16 exons, spans 25 kb and resides in chromosome 16q2l. There are several reported polymorphisms in the human *CETP* gene located in exons, introns and the promoter sequences.[5] The best-studied polymorphism is *Taq1B* located in intron 1. *CETP Taq1B* includes two different variants, *B1* and *B2* characterized by the presence and absence, respectively, of a recognition site for the restriction enzyme *Taq1*. This polymorphism involves a G to A transition in the 177th nucleotide of intron 1.[6]

CETP Taq1 B2B2 is associated with low CETP activity and high HDL concentration.[7] In the Framingham cohort, men with a *B2* allele had a lower coronary heart risk.[8] It has also been reported that male patients with type 2 diabetes and homozygous *Taq1 B2B2* genotype may have a decreased risk for coronary artery disease progression.[9] In the Regression Growth Evaluation Statin Study (REGRESS), Dutch men with established coronary artery disease and with homozygous *Taq1 B1B1* genotype was associated with increased progression of coronary atherosclerosis but not in *Taq1 B2B2* genotype.[10] In response to treatment with pravastatin, individuals with *B1B1* genotype had decreased significantly their progression to coronary atherosclerosis as evidenced by less focal atherosclerosis.

Pravastatin and simvastatin belong to statins, a class of cholesterol lowering drugs known as 3-hydroxy-3-methylglutaryl CoA (HMG CoA) reductase inhibitors. Since the enzyme has more affinity to statins, endogenous synthesis of cholesterol is blocked and reduces LDL levels as

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well. If given to asymptomatic subjects with hypercholesterolemia, pravastatin reduced the risk of coronary artery events.[11] Simvastatin treatment, on the other hand, reduced cardiovascular and coronary mortality by 36% and 43%, respectively.[12] In other trials with pravastatin[13] and simvastatin,[14] it was observed that these drugs did not prevent myocardial infarctions in a substantial percentage of patients. In the REGRESS DNA substudy, Kuivenhoven, et al.[10] cited that genetic factors were thought to contribute to this lack of response to cholesterol-lowering strategies. This was confirmed by the association of the greater response among patients with *CETP Taq1 B1B1* genotype during pravastatin treatment than among patients with *CETP Taq1 B1B2* and *B2B2 genotypes.* It was also noted that *CETP* genotype was predictive of the outcome.

The present study aims to determine the allele and genotype frequencies of *CETP Taq1B* polymorphism using PCR-RFLP in a cross-section of asymptomatic Filipino volunteers, and to determine whether response to simvastatin treatment is associated with *CETP Taq1B* polymorphism among middle-aged Filipinos with mild to moderate hypercholesterolemia.

Methods

Patients

The *CETP Taq1B* genotyping was done in two groups of study sample: in a cross-section of 106 Filipino asymptomatic volunteers and 24 hypercholesterolemic patients treated with simvastatin. The study participants were recruited sequentially. All the volunteers are residents of Metro Manila, Philippines for not less than 5 years. The levels of total cholesterol (TC), HDL, LDL and triglyceride (TG) of these participants were determined.

The association of response to simvastatin treatment was carried out among 24 hypercholesterolemic patients who participated in the previous study entitled "An Open, Non-Comparative Study on the Lipid Lowering Effect of Simvastatin among Filipino Patients with Hypercholesterolemia".[15] Out of the 28 subjects who participated in the previous study, only 24 patients volunteered to be genotyped and were included in the present study.

The brief background of the simvastatin treatment study of hypercholesterolemic volunteers is described as follows: The study design and method was an open-label, non-comparative, Phase III study of mild and to moderate hypercholesterolemic adult Filipinos who received 20 mg daily of simvastatin until completion of the study (8 weeks) if LDL level <130 mg/dl. The dose was increased to 40 mg daily at week 4 until completion of the study if the LDL level >130 mg/dl. The results obtained served as the basis of response of the hypercholesterolemic volunteers to simvastatin treatment.

The study was approved by a local Independent Ethics Committee upon compliance of the research protocol with the principles enunciated in the Declaration of Helsinki. An informed consent was taken from the volunteers after the orientation of the nature of the study.

Determination of Total Cholesterol (TC), High density lipoproteins (HDL), Low density lipoproteins (LDL) and Triglyceride (TG)

Serum total cholesterol, HDL, LDL and TG concentrations were determined using colorimetric endpoint by an automated Alize Blood Chemistry Analyzer (Tokyo, Boeki).

Genotyping by PCR-RFLP

Genomic deoxyribonucleic acid (DNA) was extracted from blood leukocytes using phenol-chloroform method. *CETP Taq1B* genotyping was performed as described by Fumeron, et al. [16] and modified in the present study. A DNA fragment of 535 base pairs (bp) in intron 1 of the *CETP* gene was amplified by polymerase chain reaction (PCR) in an MJ Cycler using oligonucleotide primers (forward 5'-CACTAGCCCAGAGAGAGGAGTGCC-3', reverse 5'-CTGAGCCCAGCCGCACACTAAC-3'). DNA template was denatured at 95°C for 30 seconds and then each PCR reaction was subjected to 30 cycles with a temperature cycle of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 45 seconds and, finally an extension at 72°C for 5 minutes. The PCR product was restricted with *Taq1B* restriction enzyme at 65°C for 16 hours. The fragments were separated by gel electrophoresis on 1.5% agarose gel. The DNA fragments were visualized by ethidium bromide.

Statistical Analysis

The observed genotype distributions of *CETP Taq1B* among the volunteers were compared with the expected genotype frequencies obtained from the Hardy-Weinberg equilibrium equation and was tested for significance using chi-square.

The TC, HDL, LDL and TG levels of the volunteers were analyzed according to determined genotype. For the analysis of the effect of genotype among the dyslipidemic volunteers reflected on their responses to simvastatin treatment, percentage (%) reduction of TC, LDL and TG, and percentage (%) increase in HDL were calculated. Paired and unpaired observations were analyzed by t-test and analysis of variance (ANOVA).

Results

A representative gel obtained in genotyping of the volunteers is shown in Figure 1. The expected 535 bp amplicon was generated using *CETP Taq1B* specific oligonucleotide forward and reverse primers. Restriction of the PCR product with *Taq1* resulted in specific migration patterns observed in the agarose gel (Figure 1). The

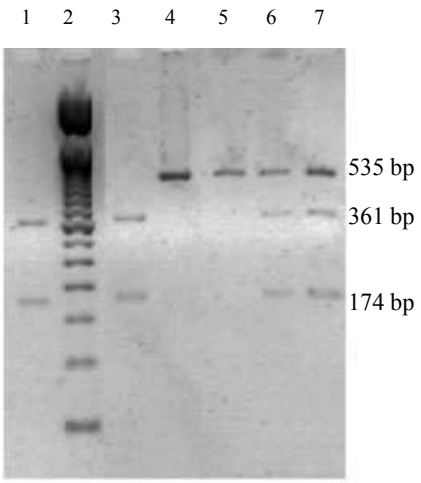

Figure 1. Restriction digestion of the PCR products with *Taq1*. Lane 1, a volunteer with 361 base pairs (bp) and 174 bp products; lane 2, 50bp DNA marker; lane 3, a volunteer with 361 bp and 174 bp products; lane 4, the unrestricted 535bp PCR product (control); lane 5, a volunteer with undigested 535 bp product after restriction, and lanes 6 and 7, volunteers with products 535bp, 361 and 174 bp

restriction products of *B1B1* genotype consisted of 2 bands: 361 bp and 174 bp (lanes 1 and 3); and *B1B2* genotype consisted of 3 bands: 535bp, 361bp and 174 bp (lanes 6 and 7). The *B2B2* genotype is shown in lanes 4 and 5, a single band corresponding to 535 bp.

The genotype frequencies obtained among 106 asymptomatic volunteers were 22.6% for *B1B1*, 58.6% for *B1B2* and 18.9% for *B2B2*. The frequencies for *B1* and *B2* alleles were 0.519 and 0.481, respectively.

The baseline characteristics of the asymptomatic volunteers when classified according to *CETP Taq1B* genotype is shown in Table 1. It was observed that no significant statistical differences were found with respect to gender, age, and levels of TC, LDL, HDL and TG when the volunteers were classified according to their *Taq1*B genotype.

Among the patients in the simvastatin treatment group, the genotype frequencies obtained were 29.2% for *B1B1*, 66.6% for *B1B2* and 4.2% for *B2B2*. The frequencies of *B1* and *B2* alleles were 0.625 and 0.375, respectively. There was no significant statistical difference observed between the genotype frequencies of the symptomatic volunteers and hypercholesterolemic patients ($p>0.05$).

Combining the results from the two groups, the overall genotype frequencies were 23.8% for *B1B1*, 60.0% for *B1B2* and 16.2% for *B2B2*. The overall frequencies of *B1* and *B2* were 0.548 and 0.462, respectively.

The medical histories of the patients who were treated with simvastatin are summarized in Table 2. There were 15 patients who reported other ailments. All the four

Table 1. Baseline Characteristics of 106 asymptomatic individuals when classified according to *cholesteryl ester transfer protein (CETP) Taq1B* genotype

Variable	CETP Taq1B Genotype *B1B1* (N=24)	*B1B2* (N=62)	*B2B2* (N=20)	p value
Gender				0.4911
Female	10	43	12	
Male	14	19	8	
Mean age, year	39	44	42	0.2642
Blood Chemistry				
Total Cholesterol (TC), mg/dl	200.3 ± 46.7	201.9 ± 44.5	222.6 ± 47.8	0.2560
High density Lipoprotein (HDL),mg/dl	53.4 ± 73.3	33.7 ± 7.9	43.0 ± 49.2	0.4632
Low density Lipoprotein (LDL),mg/dl	138.5 ± 45.0	145.8 ± 38.4	159.3 ± 25.8	0.2879
Triglyceride (TG), mg/dl	118.9 ± 90.3	108.8 ± 54.9	127.8 ± 50.4	0.8727

* p value, significant, $\alpha = 0.05$

Table 2. Other ailments observed among hypercholesterolemic Filipino patients when classified according to *cholesteryl ester transfer protein (CETP) Taq1B* genotype

Medical History	*B1B1*	*B1B2*	*B2B2*
Hypertension	0	4	0
Bronchial Asthma	1	1	0
Allergies (foods, drugs)	3	5	0
Musculoskeletal diseases (scoliosis)	1	0	0

Note: only 15 patients reported other ailments cited above; no other ailments reported by the remaining 9 patients

hypertensive patients were found to have a *B1B2* genotype. Moreover, out of the two patients found to have bronchial asthma, one patient had a *B1B1* genotype and the other one had a *B1B2* genotype. Out of eight patients with food and drug allergies, three patients were found to have a *B1B1* genotype and five patients had a *B1B2* genotype. Only one patient was found to have scoliosis and had a *B1B1* genotype.

The baseline characteristics of the 24 patients treated with simvastatin when classified according to *CETP Taq1B* genotype are shown in Table 3. The statistical analysis of the variables was limited to *B1B1* and *B1B2* genotypes since there was only one patient with a determined *B2B2* genotype. Among individuals with *B1B1* and *B1B2* genotypes, no statistical significant differences were found with respect to gender, age, TC, HDL and LDL levels ($p>0.05$). On the other hand, the difference in TG levels between these two genotypes was statistically significant (205.9 mg/dl ± 22.0 vs 139.6 ± 49.5 mg/dl, $p<0.05$).

Table 3. Baseline characteristics of 24 dyslipidemic individuals with *cholesteryl ester transfer protein* (*CETP) Taq1B* genotype

Variable	*CETP Taq1B* Genotype			*B1B1* vs *B1B2*
	B1B1 (N=7)	*B1B2* (N=16)	*B2B2* (N=1)	p value
Gender :				
Female	3	5	0	
Male	4	11	1	0.6219
Mean age, year	47	45	41	0.4377
Blood chemistry				
Total cholesterol (TC), mg/dl	284.5 ± 18.2	289.8 ± 28.6	329.2	0.6599
High Density lipoprotein (HDL), mg/dl	50.0 ± 11.1	54.9 ± 12.6	46.3	0.3912
LDL, mg/dl	193.3 ± 12.0	205.3 ± 21.2	262.3	0.1771
(TG), mg/dl	205.9± 22.0	139.6± 49.5	103.0	0.0437*

*p value, significant, $\alpha = 0.05$

The 2nd and 8th weeks of treatment outcome observed in the 24 hypercholesterolemic patients categorized by CETP *Taq1B* is shown in Figure 2. Individuals with *B1B1* genotype, 2 weeks of treatment resulted in a 31.6% reduction in TC, 42.4% reduction in LDL-C and a 13.1% reduction in triglyceride. The reductions were statistically significant ($p<0.05$). However, the 7.5% increase in HDL was not statistically significant ($p>0.05$). After 8 weeks, the treatment resulted in a 35.3% TC reduction, 45.4% reduction in LDL and 18.7% reduction in TG. The reductions observed in these variables were statistically significant ($p>0.05$). At this time, 11.1% increase in HDL is statistically significant.

Similarly, in individuals with *B1B2* genotype, 2 weeks of treatment resulted in a 32.0% reduction in TC and a 41.0% reduction in LDL. The TC and LDL reductions were statistically significant ($p<0.05$). A reduction of 4.5% in HDL and a 3.6% TG increase were observed, both of which were not statistically significant. After 8 weeks of treatment, a 33.9% reduction in TC, 44.1% reduction in LDL and a 14.8% reduction in TG were found. The observed reductions were statistically significant ($p<0.05$). The 4.6% HDL increase from the baseline, on the other hand, was not statistically significant ($p>0.05$).

In individuals with homozygous *B1* genotype,

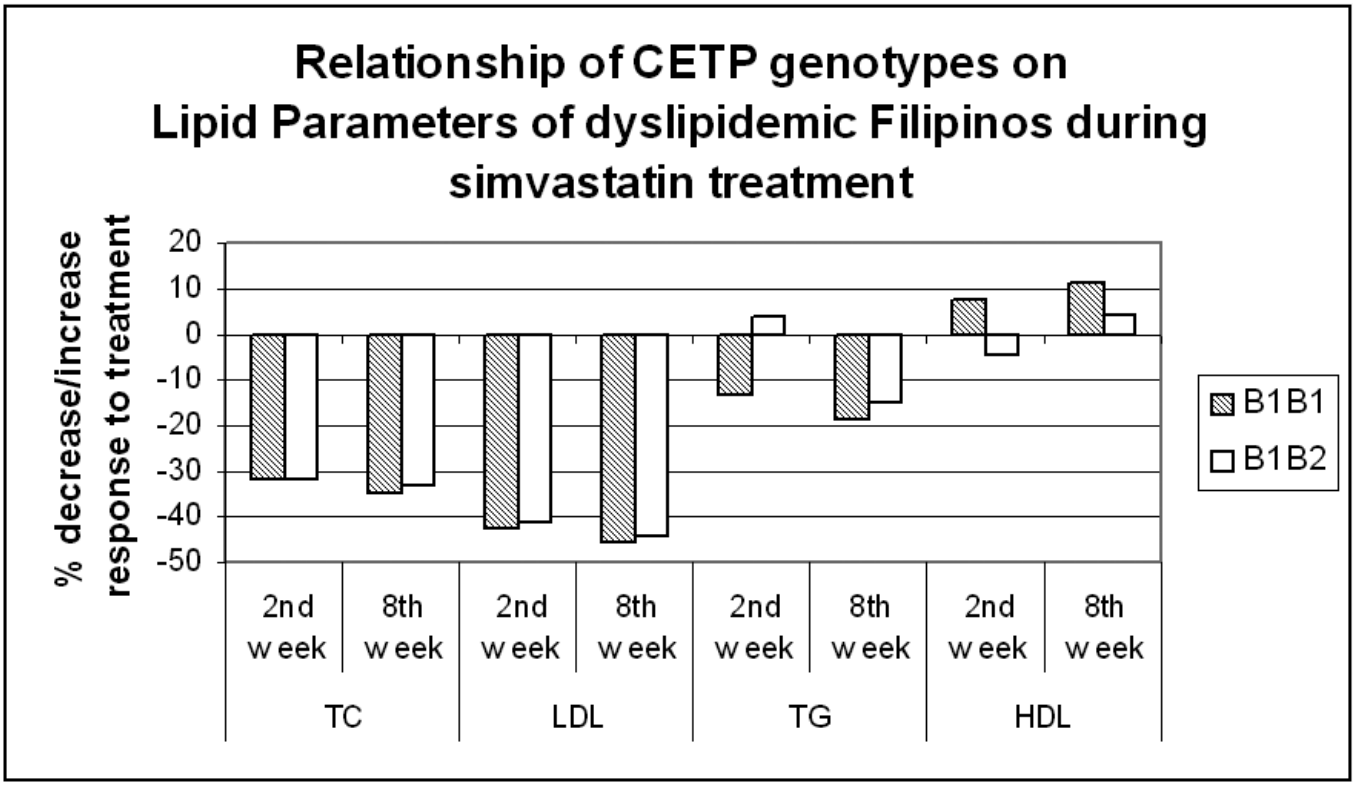

Figure 2. The effect of simvastatin treatment on % change in total cholesterol (TC), low density lipoprotein (LDL), triglyceride (TG) and high density lipoprotein (HDL) among dyslipidemic patients with varying *CETP Taq1B* genotypes after 2nd and 8th week of treatment with simvastatin.

significant responses in TC, LDL and TG were seen after 2 weeks of treatment, whereas individuals in the heterozygous group, significant responses were seen in TC and LDL. After 8 weeks of treatment, significant responses in all parameters (TC, HLD, LDL, TG) were seen in individuals with homozygous *B1* group, whereas in heterozygous genotype, increase in HDL had not been significantly improved.

Discussion

CETP Taq1B genotyping of 106 asymptomatic and 24 Filipinos with mild to moderate hypercholesterolemia showed that the heterozygous *B1B2* genotype was more frequent than the homozygous *B1B1* and *B2B2* genotypes as summarized in Table 4. It is also observed that *B1* allele is more frequent than *B2* allele. This observed pattern of genotype and allele frequencies is similarly reported in other population studies: in the Japanese,[17] Americans,[2] Dutch[10] and French.[2] Based on the initial data shown in Table 4, there seems to be no racial or ethnic differences in the frequency distribution of *CETP Taq 1B*. No statistical difference in genotype frequencies was observed between the asymptomatic volunteers and hypercholesterolemic patients.

When the hypercholesterolemic patients were treated for 2 weeks with 20 mg simvastatin, an early rapid response in HDL-C was found among patients with *B1B1* genotype, while a catch-up rise was observed among *B1B2* genotypes after 8 weeks. The 7.5% increment among the patients with *B1B1* genotype was consistent with the Adult Treatment Panel III (ATP-III) guidelines.[18] An approximate 5% to 12% increment suggested by ATP-III is referred to as a moderate HDL increase and described as a distinct characteristic of a statin response. The upper limit set by the guideline was attained after 8 weeks by the patients with *B1B1* genotype, wherein a significant 11.1% increment was found. These findings were similarly observed in other reported studies showing the effect of *B1B1* genotypes on HDL upon treatment of pravastatin[10] and atorvastatin.[19] These responses of hypercholesterolemic patients with *B1B1* genotypes also suggest that 20 mg simvastatin was adequate to achieve another favorable treatment outcome; specifically, the rapid HDL increase and a progressive increment upon continuous treatment. The results of the analyses also indicate that the treatment is independent of baseline HDL levels. Likewise, the triglyceride reduction of *B1B1* genotypes also started on the 2nd week and reduced further up to the 8th treatment.

The changes in HDL among patients with *B1B2* genotypes were characterized by mean drop of 4.5% in HDL after the 2nd week of treatment, and a 4.6% catch-up rise from baseline in HDL after the 8th week of treatment. However, the observed increase in HDL after the 8th week was not at par with the guidelines set by ATP-III.[20] A similar drop in HDL after a 30-week statin treatment was previously reported among dyslipidemic patients with type 2 diabetes carrying the *B2B2* genotype.[19] The response of individuals with *B1B2* genotypes was also characterized by a slow triglyceride reduction in response to simvastatin treatment and a catch-up reduction after the 8th week of treatment.

The distinguishing feature of individuals with *B1B1* genotype in response to simvastatin treatment was characterized by an early rapid HDL response with an accompanying triglyceride reduction. This was followed by a progressive HDL increase and TG reduction upon treatment maintenance. A catch-up response was observed among patients with *B1B2* genotypes only after the 8th treatment. This similar pattern of slower response of the *B1* heterozygotes to elevate their HDL levels and to lower their triglyceride levels suggests that indeed *CETP* gene polymorphism affected all these observations.

In summary, significant responses in TC, LDL and TG were seen after 2 weeks of treatment in individuals with homozygous *B1* genotype, whereas significant responses were seen only in TC and LDL in heterozygous individuals. After 8 weeks of treatment, significant responses in all parameters (TC, HLD, LDL, TG) were seen in homozygous *B1* individuals, whereas in those individuals with heterozygous genotype, no significant HDL improvement was noted.

The results of this present study among the hypercholesterolemic Filipino patients with *B1B1* genotype showing a greater response to increase their HDL levels upon simvastatin treatment is consistent with the results observed among Dutch men [10] during pravastatin treatment of patients with *B1B1* genotype than among patients with *B1B2* and *B2B2* genotypes. Since this present study used dyslipidemia as clinical outcome within the 8-week

Table 4. *Cholesteryl ester transfer protein (CETP) Taq1B* genotype and allele frequencies among Filipinos compared with other races

		Genotype frequency (%)			Allele frequency (%)		
Population	N	*B1B1*	*B1B2*	*B2B2*	*B1*	*B2*	Reference
Filipino, normal	106	22.6	58.5	18.9	0.519	0.481	Present study
Filipino, hypercholesterolemic	24	29.2	66.6	4.2	0.625	0.375	Present study
Japanese, type 2 diabetic	443	34.1	47.9	18.0	0.580	0.420	Kawasaki et al, 2002
Americans with CHD*	852	34.7	51.3	13.9	0.604	0.396	Brousseau et al, 2002
Dutch, with CAD+	807	35.0	49.0	16.0	0.594	0.406	Kuivenhoven et al, 1998
French, type 2 diabetic	176	38.1	43.8	18.1	0.600	0.400	Bernard et al, 1998

*CHD - coronary heart disease; +CAD - coronary artery disease

simvastatin treatment, it is interesting to observe the association of *CETP Taq1 B1B1* genotype among Filipinos with their decreased progression to myocardial infarction and mortality as clinical outcomes during simvastatin treatment in a large-scale, long-term clinical trial.

Recommendations

Since the distinguishing feature of individuals with *B1B1* genotype was their rapid increase in HDL levels which continued till the 8th week of simvastatin treatment and that of the *B1B2* individuals was for HDL levels to catch up only on the 8th week, it is recommended that a large-scale long-term prospective study be carried out to compare the translated benefits of treatment on individuals with *B1B1* genotype in comparison with patients with *B1B2* genotype in terms of clinical outcome, specifically for example, on how it affects patients in terms of myocardial events and mortality.

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