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Frequency of Common Variants in Genes Involved in Lipid-Lowering Response to Statins in Chilean Subjects with Hypercholesterolemia

Frecuencia de Polimorfismos en Genes Relacionados a la Respuesta Terapéutica a Estatinas en Individuos Chilenos con Hipercolesterolemia

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SUMMARY: Interindividual differences in activity and expression of the metabolizing enzymes cytochrome P450 (CYP) 3A4 and 3A5 and the multidrug efflux pump P-glycoprotein (P-gp, encoded by ABCB1 gene) contribute considerably to lipid-lowering efficacy of statin treatment in subjects with hypercholesterolemia. Variability in the activity of CYP3A4, CYP3A5 and P-gp could be considered to result from genetic polymorphisms encoding their genes. However, the available data indicate that the frequencies of ABCB1, CYP3A4 and CYP3A5 gene polymorphisms differ significantly across populations. Thus, the aim of the present study was to determine the allelic frequency of three common variants of these genes in Chilean individuals with primary hypercholesterolemia (HC) and controls. A total of 135 unrelated patients (44 ± 7 years old) with diagnosis of hypercholesterolemia (Total cholesterol ≥ 240 mg/dL) and 120 normolipidemic healthy controls (40 ± 10 years old; total cholesterol ≤ 200 mg/dL) were included in this study. The 3435C>T (MDR1), -290A>G (CYP3A4) and 6986A>G (CYP3A5) gene polymorphisms were analyzed by PCR-RFLP. The genotype distribution for 3435C>T variant of ABCB1 in HC patients (CC: 49%, CT: 37%, TT: 14%) and controls (CC: 41%, CT: 48%, TT: 11%) was comparable (P=0.186). Similarly, the genotype distribution for -290A>G polymorphism of CYP3A4 in HC subjects (AA: 73%, AG: 27%, GG: 0%) and controls (AA: 71%, AG: 29%, GG: 0%) was equivalent (P = 0.863). Finally, the genotype distribution for 6986A>G variant of CYP3A5 in HC individuals (AA: 4%, AG: 41%, GG: 55%) and controls (AA: 4%, AG: 47%, GG: 49%) was similar (P=0.594). The allelic frequencies of 3435C>T (ABCB1), -290A>G (CYP3A4) and 6986A>G (CYP3A5) polymorphisms are similar between Chilean HC patients and controls, and comparable to frequencies found in Asian populations.

KEY WORDS: Polymorphisms; Statins; Hypercholesterolemia; ABCB1.

INTRODUCTION

There is abundant evidence that, for prevention and treatment of cardiovascular diseases (CVD), an important reduction in common risk factors must be achieved, promoting changes in lifestyle, which accompanied with specific pharmacological treatment of lipid disorders, can reduce in a significant manner a future cardiovascular event (Lanas *et al*., 2007; Medina & Kaempffer, 2007).

Different types of drugs are co-administered to CVD patients, many of which have become a mainstay in the primary (Pignone *et al*., 2000) and secondary prevention (Shepherd *et al*., 1995) of coronary events, reducing cardiovascular mortality and morbidity. However, the backbone of lipid lowering medication is accomplished mainly by 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-Co A) reductase inhibitors, or statins (Vaughan *et al.*, 2000; Newman *et al*., 2003).

Statins are among the most used drugs worldwide. They are usually very well tolerated. Nevertheless, they can cause severe adverse reactions, such as myopathy and rhabdomyolysis, the risk of which is increased by certain

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interactions as a rare, plasma concentration–dependent adverse reaction (Niemi, 2010). Alternatively, the benefits of statins are very well documented. Experimental studies have recognized evidence of different positive effects such as an increment in the nitric oxide (NO) expression, anti-inflammatory, immunomodulatory, anti-thrombotic, anti-proliferative, and anti-oxidant effects (Echeverri *et al*., 2005; Kirmizis *et al*., 2009).

The mechanism of action of statins is to inhibit in a competitive and reversible way an enzyme named HMG-CoA reductas, which catalyzes biosynthesis of hepatic cholesterol by blocking the formation of mevalonic acid and reducing intracellular cholesterol synthesis, resulting in a compensatory increase in expression of LDL receptors in liver cells, reducing LDL-C levels of circulating total cholesterol (20% - 50% approximately, respectively) subsequently increases the removal of triglyceride-rich lipoproteins from plasma and reduces synthesis of apoB-containing lipoproteins, such as VLDL. It also give rise to a modest decrease in triglycerides (TG, 10% to 40%) and a small increased levels of high density lipoprotein (HDL) cholesterol (5% to 15%) levels (Vaughan *et al.*; Marhuenda, 2002). Several studies have shown that with 1% reduction in LDL cholesterol level, there is an associated 1% reduction in risk of clinical cardiovascular events (LaRosa, 2007).

To date, there are more than 40 genes investigated that could affect clinical response to statins (Thompson *et al*., 2005), which are related to both, pharmacokinetics (metabolizing enzymes and transport proteins) and pharmacodynamics (receptors and signal transduction pathways) (Neuvonen *et al*., 2006). Some inter-individual drug effects are based on pathophysiological factors and environmental interactions, but also genetic characteristics. Large interindividual pharmacokinetic differences observed among humans are partially due to genetic polymorphisms in drug-metabolizing enzymes such as cytochrome P450, or transporter proteins such a ABCB1 (Evans *et al.*, 2001). These variations include genes involved in intestinal absorption of cholesterol and apolipoprotein E (APOE), ATP transporter (ATP binding cassette) the production of cholesterol, including HMG-CoA reductase, the metabolism of lipoproteins and apolipoprotein B and LDL receptor, also including the cytochrome P450 (Salazar *et al.*, 2000; Kajinami *et al.*, 2005; Wang *et al*., 2005; Kerola *et al*., 2010).

Statins are metabolized in hepatic and intestinal tissue by the cytochrome P450-system (CYP450), mainly by CYP3A sub-family (CYP3A4 and CYP3A5 isoforms included). CYP3A plays an important role in human metabolism (Igel *et al.*, 2001). On the other hand, simvastatin, lovastatin, and atorvastatin are metabolized by cytochrome P450 (CYP) 3A4 (Malinowski, 1998; Neuvonen *et al*.). In the case of atorvastatin, there are differences in the clinical response, which could be explained by genetic polymorphisms (Cascorbi *et al*., 2001). An example of this is the case of CYP3A sub-family, variations in genes involved in uptake, distribution, and metabolism of statins may also significantly modulate response. In the same way, P-glycoprotein, the gene product of ABCB1 (MDR1), is an integral membrane protein of 170 kd, belonging to the adenosine triphosphate-binding cassette superfamily of membrane transporters, conferring multidrug resistance and playing a fundamental role in the bioavailability (absorption, distribution and elimination) of common drugs used in medical care.

The level of protein expression and functional integrity of P-glycoprotein directly affects its pharmacokinetic interaction with therapeutically administered drugs and therefore plays an important role in efficacy and toxicity of drug treatment (Eichelbaum *et al*., 2004; Ieiri *et al*., 2004). ABCB1 transports a wide range of drugs, including atorvastatin (Dietrich *et al.*, 2003). The latter undergoes hepatic metabolism mediated by phase I and phase II enzymes, or may be excreted without any further transformation. ABCB1 is located in the canalicular membrane of hepatocytes, contributing to statin removal and their metabolites via bile (Lennernäs, 2003; Rodrigues *et al*., 2006). Genetic variants of MDR1 can naturally affect the pharmacokinetic and pharmacodynamic profile of many drugs, generating interindividual difference and accounting for a variation in bioavailability of various P-gp substrates.

Various studies have analyzed single nucleotide polymorphisms (SNPs) in candidate genes as an approach for understanding partially the subjacent reason of drug response variability. This includes genes relevant to metabolizing enzymes such a CYP3A4 and CYP3A5 gene (Lee *et al*., 2002), transport protein MDR1 (ABCB1) gene (Ayrton & Morgan, 2001), cholesterol biosynthesis (HMGCR). In these studies, different common variants were identified -260A>G (CYP3A4) (Kajinami *et al*., 2004a), 6986A>G (CYP3A5) (Kim *et al*., 2007) and 3435C>T (ABCB1 or MDR1) (Rodrigues *et al*., 2005) which were associated to a possible response variation and with efficacy of lipid-lowering therapy. CYP3A5*3 allele, was found to cause alternative splicing and protein truncation, resulting in the absence of functional CYP3A5 from liver tissue (Kuehl *et al.*, 2001).

Futhermore, SNPs are considered to be a genetic contributor to inter-individual differences in response to statin therapy. Nevertheless, polymorphisms in drug

metabolizing enzymes and transporters and their relationship to efficacy and adverse effects of statin therapy necessitate further research. Thus, the objective of this study was to determine the allelic frequency of three common genetic variants related to lipid-lowering response to statins in Chilean individuals with hypercholesterolemia and controls.

MATERIAL AND METHOD

Subjects. We analyzed 135 unrelated individuals with diagnosis of primary hypercholesterolemia (Total cholesterol ≥ 240 mg/dL) and 120 normolipidemic controls (Total cholesterol ≤ 200 mg/dL), in according to Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (2001) criteria, selected from Hernán Henríquez Aravena Hospital (Temuco, Chile). None of the subjects had diabetes, hepatic disease, kidney disease, endocrinological disorders, malignant disease or was receiving concomitant lipid-lowering therapy. Patients with clinical diagnosis of familial hypercholesterolemia (FH) were also excluded. The study protocol was approved by the Ethics Committee of Universidad de La Frontera and all participants included voluntarily signed an informed consent.

Blood samples were obtained after 12 hours fast. Biochemical measurements were determined by enzymatic methods previously described (Jaramillo *et al*., 2006) and low-density lipoprotein cholesterol was calculated by Friedewald formula, when plasma triglycerides did not exceed 400 mg/dL. The accuracy of biochemical determinations was controlled using normal and pathological commercial serums (Human, Germany).

Molecular analysis. Genomic DNA was extracted from blood leukocytes using a method previously described (Salazar *et al.*, 1998). The integrity of the genomic DNA was evaluated by electrophoresis in a 1.5% agarose gel. The 3535C>T (ABCB1), -290A>G (CYP3A4) and 6986A>G (CYP3A5) gene polymorphisms were detected using polymerase chain reaction followed by restriction fragment length polymorphism (PCR-RFLP) in according to conditions previously described (Cavalli *et al*., 2001; Rodrigues *et al*., 2005; Kim *et al*.). As positive control a homozygous sample for each variant was included. In addition, all gels were reread blindly by two persons and 20% of the analyses were randomly repeated. The digestion fragments were separated by electrophoresis (3.0% agarose gel), stained with ethidium bromide and visualized on a UV transilluminator.

Statistical analysis. The collected data was analyzed using the SigmaStat software, version 2.0 (Jandel Sci., San Rafael, CA, USA). The association between the different analyzed variables was verified by Student t test. For comparison of proportions and to evaluate the achievement of the Hardy-Weinberg equilibrium Chi-square test (c^2) was used. Statistical significance was considered as $P < 0.05$.

RESULTS

Clinical variables. Table I summarizes the anthropometric, clinical and laboratory characteristics of 255 Chilean individuals enrolled in the study. The serum lipids concentrations (mean ± SD) of hypercholesterolemic (HC) subjects and controls are also presented. The baseline total cholesterol, LDL-cholesterol and triglycerides were higher in patients with HC ($p<0.001$). The individuals belonging to control group showed higher HDL-cholesterol levels ($p<0.001$) than HC group. No differences were achieved in the mean value of diastolic ($p =0.066$) and systolic ($p= 0.049$) blood pressures between the studied groups.

Allelic frequencies. The genotype distribution and relative allele frequencies for the 3435C>T (ABCB1), -290A>G (CYP3A4), and 6986A>G (CYP3A5) gene polymorphisms are shown in Table II. In both groups, the observed genotype distribution was consistent with Hardy-Weinberg equilibrium (Hypercholesterolemic subjects: $c2 = 3.33$, $c^2 = 2.40$ and $c2= 1.76$ and controls: $c^2 = 0.46$, $c^2 = 3.49$ and $c^2= 3.48$, for 3435C>T, -290A>G and 6986A>G polymorphisms, respectively). The genotype distribution for 3435C>T variant of ABCB1 gene in HC patients (CC: 49%, CT: 37%, TT: 14%) and controls (CC: 41%, CT: 48%, TT: 11 %) was comparable ($p=0.186$). The mutated allele (3435T) in control and HC subjects was also similar (0.35 vs. 0.33, $p=0.631$).

Similarly, the genotype distribution for -290A>G polymorphism of CYP3A4 gene in HC subjects (AA: 73%, AG: 27%, GG: 0%) and controls (AA: 71%, AG: 29%, GG: 0%) was equivalent ($p=0.863$). The GG homozygous genotype was not observed in our study. The allelic frequency of mutated allele variant (-290G) in HC and control subjects was also comparable. Finally, the genotype for 6986 A>G variant of CYP3A5 in HC individuals (AA: 4%, AG: 41%, GG: 55%) and controls (AA: 4%, AG: 47%, GG: 49%) was similar ($p=0.594$). The frequency for the wild-type (CYP3A5*1) and variant (CYP3A5*3) alleles were 0.27 and 0.73 for control subjects and 0.24 and 0.76 for HC individuals.

Table I. Clinical characteristics of Chilean subjects with hypercholesterolemia (HC) and controls.

	HC (n=135)	Controls (n=120)	P*
Age, years	44 ± 7	40 ± 10	0.161
Male, %	60	56	0.598
BMI, Kg/m_	27.6 ± 4,9	25.5 ± 3.3	<0.001
SBP, mmHg	118 ± 10	110 ± 11	0.049
DBP, mmHg	76 ± 10	70 ± 10	0.066
Total cholesterol, mg/dL	267 ± 25	163 ± 23	<0.001
LDL-C, mg/dL	185 ± 23	94 ± 24	<0.001
HDL-C, mg/dL	39 ± 10	50 ± 12	<0.001
Triglycerides, mg/dL	192 ± 64	98 ± 45	<0.001

Results are expressed as mean ± SD. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL-C, low density lipoprotein-cholesterol; HDL-C, high density lipoprotein-cholesterol. *Student's t-test.

Table II. Genotype distribution and relative allele frequencies for 3435C>T (ABCB1), -290A>G (CYP3A4) and 6986A>G (CYP3A5) polymorphisms in Chilean individuals with hypercolesterolemia (HC) and controls.

Polymorphism	Group	Genotypes (%)			Alleles	
		CC	CT	TT	C	T
3435C>T (ABCB1)	HC	49 (66)	37 (50)	14 (19)	0.67	0.33
	Controls	41 (49)	48 (58)	11 (13)	0.65	0.35
		χ^2 =3.36; d.f.=2 ; p=0.186			χ^2=0.23; d.f.=1 ; p=0.631	
		AA	AG	GG	A	G
-290 A>G (CYP3A4)	HC	73 (98)	27 (37)	0 (0)	0.86	0.14
	Controls	71 (85)	29 (35)	0 (0)	0.85	0.15
		χ^2 =0.29; d.f.=1 ; p=0.863			χ^2=0.24; d.f.=1 ; p=0.875	
		AA	AG	GG	A	G
6986 A>G (CYP3A5)	HC	4 (5)	41 (55)	55 (75)	0.24	0.76
	Controls	4 (5)	47 (56)	49 (59)	0.27	0.73
		χ^2 =1.04; d.f.=2 ; p=0.594			χ^2=0.61; d.f.=1 ; p=0.434	

Number of subjects in parenthesis; d.f., degree of freedom

DISCUSSION

There is a large interest in the connection between lipid-lowering drug response and the relationship with genetic variants. Therefore, many investigations have been focusing on SNPs identification, and recognition of polymorphism frequency associated with lipid-lowering response in our populations has gained much importance. Given this scenery, the aim of the present study was to determine the frequency of three common variants in genes involved in statin response in Chilean HC individuals.

Our data shows that the frequency of the G allele was 0.35 in control subjects and 0.33 in HC for 3435C>T polymorphism in the studied subjects. The results are similar to the frequencies observed by Wielandt *et al*. (2004) in Mapuche and Mestizo ethnicities of our country, having allelic frequencies of 0.35 and 0.33, respectively. Similarly, in Brazilian hypercholesterolemic subjects, the frequency was 0.47 for T allele (Rodrigues *et al*., 2005). In addition, the frequency found in our study, for the T allele of the variant 3435C> T is similar to that observed in Asian population such as Koreans (0.37) (Yi *et al*., 2004), higher in Rapanui populations (0.75) (Wielandt *et al*.), and differs significantly from that observed in Afro-Americans (0.21) (Kajinami *et al.*, 2004b), Africans (0.17), European Caucasian (0.52) (Ameyaw *et al.*, 2001) and Oceania population (0.53) (Roberts *et al.*, 2002).

As for the polymorphism -290A>G of CYP3A4 gene, the G allele was found in 15% of control and 14% of HC

individuals studied. There are no previous reports of this polymorphism in our population, thus, this becoming the first study of variants of CYP3A4 in Chilean population. Comparing the frequency of the G allele (0.15) with those reported by other studies, we see a similarity with Brazilian population (0.13) (Cavalli *et al*.), Asian (0.16) (Thompson *et al*.) and Hispanic (0.09) (Ball *et al.*, 1999), and the frequency of the mutant variant CYP3A4*1B was significantly higher than that observed in Europeans (0.05) (Sata *et al.*, 2000) and American population, both white (0.04) and Black (0.54) (Ball *et al*.) and lower in Africans (0.82) (Zanger *et al.*, 2008) and Afro-American (0.67) (Sata *et al*.). Gao *et al*. (2008) demonstrated an association between the presence of G allele in hypercholesterolemic individuals who had a greater reduction of total cholesterol levels, when treated with atorvastatin 20 mg/day for 4 weeks in Asiatic population. Other investigators associated the presence of this variant (allele G) to higher levels of LDL cholesterol after treatment, in 340 HC individuals undergoing atorvastatin treatment 10 mg/day (Kajinami *et al.*, 2004a).

In relation to the genetic polymorphism 6986 A>G, the G allele frequency was 0.73 in control subjects and 0.76 in hypercholesterolemic subjects. The results are very similar to the respective frequencies observed in some Asian populations such as Chinese (Hu *et al.*, 2005) and Japanese (Horinouchi *et al*., 2002) for this variant, 0.77 and 0.76, respectively, different from the reported in American population (0.93) (Mega *et al*., 2009), Africans (0.15) (Kudzi *et al.*, 2010), European Caucasians (0.93) (Dally *et al.*, 2004) and very different in Afro-American population (0.36) (Kajinami *et al.*, 2004b).

It has been well known that interindividual differences in the metabolic profile of many drugs are mainly due to sequence variants in gene encoding drug metabolizing enzymes. Therefore, knowledge of the prevalence of SNPs in a population is essential for the estimatation of the likelihood of interindividual differences of drug efficacy and other side effects. The influence of ethnicity on pharmacogenetic response must be taken into account by performing and comparing clinical trials in various ethnic groups.

Pharmacogenetic testing increases the impact on the individualization of drug treatment and could therefore contribute significantly to enhanced drug safety and efficacy (Pfost *et al.*, 2000). In the near future, pharmacogenomics may allow the identification of patient subgroups which most likely profit from lipid-lowering therapy.

In summary, our study shows that the allelic frequencies of 3435C>T (ABCB1), -290A>G (CYP3A4) and 6986A>G (CYP3A5) polymorphisms are similar between Chilean HC patients and controls, and comparable to frequencies described in Asian populations. However, the influence of these genetic variants on lipid-lowering response to statins in our population needs to be investigated in a further study.

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RESUMEN: Polimorfismos de los genes CYP3A4, CYP3A5 y ABCB1 se han asociado a variaciones en la respuesta a fármacos hipolipemiantes, como las estatinas; principales medicamentos utilizados para disminuir los niveles plasmáticos de colesterol (CT). Sin embargo, la frecuencia de estas variantes genéticas puede variar entre las poblaciones. Así, el objetivo de este trabajo fue evaluar la frecuencia de tres polimorfismos de los genes CYP3A4, CYP3A5 y ABCB1, relacionados previamente a la respuesta a estatinas, en individuos chilenos hipercolesterolémicos (HC) y controles. Se analizaron 135 sujetos con diagnóstico de hipercolesterolemia primaria (CT ≥ 240 mg/dL) y 120 controles (CT≤ 200 mg/dL) pertenecientes a la Región de La Araucanía (Chile). La genotipificación de las variantes genéticas se efectuó mediante la técnica de reacción en cadena de la polimerasa seguido de restricción enzimática (PCR-RFLP). La distribución de genotipos para la variante 3435C>T del gen ABCB1 en los individuos HC (CC: 49%, CT: 37%, TT: 14%) y controles (CC: 41%, CT: 48%, TT: 11%) fue semejante (P = 0,186). De forma similar, la distribución de genotipos para el polimorfismo -290A>G del gen CYP3A4 en los pacientes HC (AA: 73%, AG: 27%, GG: 0%) y controles (AA: 71%, AG: 29%, GG: 0%) fue equivalente (P = 0,863). Del mismo modo, la distribución de genotipos para la variante 6986A>G del gen CYP3A5 en el grupo HC (AA: 4%, AG: 41%, GG: 55%) y grupo control (AA: 4%, AG: 47%, GG: 49%) fue similar (P = 0,594). En resumen, nuestro estudio demuestra que las frecuencias de los polimorfismos 3435C>T (ABCB1), -290A>G (CYP3A4) y 6986A>G (CYP3A5) no difieren entre individuos HC y controles, y son comparables a las frecuencias encontradas en poblaciones asiáticas. Su efecto sobre el tratamiento con estatinas en la población chilena debe ser investigado.

PALABRAS CLAVE: Polimorfismos; Estatinas; Hipercolesterolemia; ABCB1.

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