Clinica Chimica Acta 398 (2008) 15–20

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

*CYP3A5*3A* allele is associated with reduced lowering-lipid response to atorvastatin in individuals with hypercholesterolemia

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ARTICLE INFO

Article history:
Received 7 April 2008
Received in revised form 29 July 2008
Accepted 31 July 2008
Available online 5 August 2008

Keywords:
CYP3A5
Hypercholesterolemia
Lipid-lowering response
Pharmacogenomics
Statins

ABSTRACT

Background: The cytochrome *P450* isoenzyme 3A5 (CYP3A5) has an important role on biotransformation of xenobiotics. *CYP3A5* SNPs have been associated with variations on enzyme activity that can modify the metabolism of several drugs.
Methods: In order to evaluate the influence of *CYP3A5* variants on response to lowering-cholesterol drugs, 139 individuals with hypercholesterolemia were selected. After a wash-out period of 4 weeks, individuals were treated with atorvastatin (10 mg/day/4 weeks). Genomic DNA was extracted by a salting-out procedure. *CYP3A5*3C*, *CYP3A5*6* and *CYP3A5*1D* were analyzed by PCR-RFLP and DNA sequencing.
Results: >Frequencies of the *CYP3A5*3C* and *CYP3A5*1D* alleles were lower in individuals of African descent (**3C*: 47.8% and **1D*: 55.2%) than in non-Africans (**3C*: 84.9% and **1D* 84.8%, $p<0.01$). Non-Africans carrying **3A* allele (**3C* and **1D* combined alleles) had lower total and LDL-cholesterol response to atorvastatin than non-**3A* allele carriers ($p<0.05$).
*Conclusion: CYP3A5*3A* allele is associated with reduced cholesterol-lowering response to atorvastatin in non-African individuals.

1. Introduction

Statins are potent inhibitors of the microsomal 3-hydroxy-3-methyl-glutaryl coenzyme A reductase (HMGCR), which is a critical step in the endogenous cholesterol synthesis [1]. Reduction of intracellular cholesterol subsequently induces the expression of the low-density lipoprotein (LDL) receptor that increases the LDL uptake by hepatic and peripheral cells reducing dramatically the cholesterolemia [2].

Atorvastatin is one of the most potent HMGCR inhibitors [3]. Although atorvastatin is regarded safe, approximately 1 to 5% of patients will have adverse drug reactions such as muscle damage (weakness, myopathy, serum CK increase up to 10 times, rabdomyolysis) [4]. Several factors have been related to variability in response to atorvastatin including drug pharmacokinetics.

Atorvastatin is metabolized primarily by CYP3A4 and CYP3A5 in the gut and liver, and it is further conjugated by glucuronidases UGT1A1 and 1A3, resulting in acyl-glucuronide intermediates [5]. Membrane transporters, such as P-glycoprotein also mediate the cellular efflux of atorvastatin contributing to the overall drug bioavailability [6].

CYP3A4 and CYP3A5 are important drug metabolizing enzymes due to their abundance in the intestinal epithelium and liver, and its ability to metabolize a large number of chemically unrelated drugs [7]. Inter-individual variability in CYP3A expression in liver is very high (20–40 fold). This variability has been suggested to be associated with variants in CYP3A4 and CYP3A5 encoding genes that may contribute to the differences found in response to several drugs, including atorvastatin [7–9].

Several single nucleotide polymorphisms (SNP) have been described in the *CYP3A5* [10–13]. The most common SNP appears to be A6986G (*CYP3A5*3C*), a genomic transition within intron 3 that creates a cryptic consensus splice site in the pre-mRNA, resulting in the incorporation of 131 bp of the intron 3 sequence in the mature mRNA (originating the so called pseudoexon 3B). This insertion causes a frameshift and may involve subsequent deletions or insertions of other intronic sequences. The abnormally processed RNAs (variant RNAs) contain several premature termination codons (PTC), so that the encoded protein is truncated, with loss of the enzyme activity [14]. Furthermore, wild-type *CYP3A5*1A* is differentially distributed among populations ranging from 10% in European individuals to 65% in Africans [15].

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doi:10.1016/j.cca.2008.07.032

Table 1
PCR primers sequences and thermal cycling conditions

SNP	Method of analysis	Primer sequence	Cycles	Annealing temperature	Size of the PCR product (bp)
A6986G (*CYP3A5*3C*) rs776746	PCR-RFLP	5′-CTTTAAAGAGCTCTTTTGTCTC*TCA-3′ 5′-CCAGGAAGCCAGACTTTGAT-3′	30	58	200
A6986G (*CYP3A5*3C*) rs776746	PCR sequencing	5′-TTGTGAGCACTTGATGATTTACC-3′ 5′-CCAGGAAGCCAGACTTTGAT-3′	35	60	370
G14690A (*CYP3A5*6*) rs not available	PCR-RFLP	5′-GTGGGTTTCTTGCTGCATGT-3′- 5′-GC**GAAACCAAATTTTAGGA ACT-3′	30	60	204
G14690A (*CYP3A5*6*) rs not available	PCR sequencing	5′-GTGGGTTTCTTGCTGCATGT-3′ 5′-GGCTCTAGATTGACAAAAACATTTT-3′	35	63	403
C31611T (*CYP3A5*1D*) rs17161788	PCR sequencing	5′-TGCTTTTACTATCCAGTATTTACCC-3′ 5′-GCCCATCTTTATTTCAAGGTTT-3′	35	59	378

Reference: www.ncbi.nlm.nih.gov. GenBank access number: AC 005020.
(*) Primer degenerated base.
(**) Non-complementary region.

*CYP3A5*3C* allele has been associated with higher statin response in a study with 46 hypercholesterolemic patients after 1 y treatment with several statins [16]. Homozygous *CYP3A5*3C* has been also associated with serum CK elevation in patients experiencing myalgia [17]. On the other hand, this SNP was not associated with response to simvastatin in Brazilian individuals of the European descent [18]. In addition, Chasman et al. evaluated 148 SNPs in hypercholesterolemic patients under pravastatin treatment and concluded that none of the 6 *CYP3A5* SNPs studied was associated with variation on the lowering-lipid response after 24 weeks of treatment [19].

Another *CYP3A5* SNP is G14690A (*CYP3A5*6*) that causes a partial deletion on exon 7 and results in a truncated protein with no functional activity [11]. This variant is found in African-derived individuals with frequency of 12% and in Caucasian individuals in frequency <1% [15,20].

SNPs located in 5′ and 3′ untranslated regions (UTR) of *CYP3A5* gene have also been described [15]. It is noteworthy that *CYP3A5*1D* SNP, located in the 3′UTR, is in linkage disequilibrium with *CYP3A5*3C* [10,14]. Both alleles present in the same individual originate *CYP3A5*3A* allele [11]. SNPs on UTRs tend to cause lower stability on mRNAs, affecting the expression rate and the catalytic activity of the enzyme [15]. In this study we evaluated the influence of the *CYP3A5*3C*, *CYP3A5*6* and *CYP3A5*1D* variants and their effects on lipid-lowering response to atorvastatin in Brazilian hypercholesterolemic individuals.

2. Materials and methods

2.1. Subjects and study protocol

The characteristics of the study design have been previously reported [21–23]. Briefly, 139 hypercholesterolemic (HC) individuals were selected at 2 Clinical Research Centers of Sao Paulo City, Brazil. Individuals with hypertriglyceridemia (triglycerides ≥4.52 mmol/l [approximately 400 mg/dl]), hypothyroidism, diabetes mellitus, liver or kidney diseases, and other forms of secondary dyslipidemia were excluded from this study.

All patients were informed about the study protocol and then agreed to participate as volunteers by signing the consent. The study protocol was approved by the Ethics Committees of the Dante Pazzanese Institute of Cardiology, University Hospital of the Sao Paulo University and School of Pharmaceutical Sciences of the Sao Paulo University, Sao Paulo, Brazil.

Information on age, body mass index (BMI), blood pressure, menopause status, tobacco smoking, alcohol consumption, hypertension, obesity, family history of coronary artery disease (CAD), physical activity and medication in use were recorded. Current tobacco smoking was defined as a daily intake of one or more cigarettes. We considered as "alcohol consumption" a daily intake of beer, wine, and distilled spirits of >1 g/day. Physical activity was considered the practice of sports, for example walking, running or swimming for at least 2 h/week. Each individual declared his ethnic group during the interview, as recommended by the most recent Brazilian Census [38].

The patients went through a wash-out period of four weeks with a low-fat diet (according to American Heart Association step one diet) [24] followed by 4 weeks of treatment with atorvastatin 10 mg orally once daily. Compliance to the low-fat diet was monitored through measurement of serum lipids after 4 weeks. Before and after atorvastatin therapy, all patients were evaluated for serum concentrations of lipids, alanine aminotransferase (ALT) and creatine kinase (CK). Patients continued taking other medications throughout the study that include diuretics, ACE inhibitors, β-blockers and calcium channel blockers.

2.2. Biochemical analyses

Blood samples were collected after a 12 hour fast, before and after the 4 week treatment with atorvastatin 10 mg/day. Serum total cholesterol, high-density lipoprotein (HDL) cholesterol and triglycerides were measured using routine enzymatic methods. LDL and very low-density lipoprotein (VLDL) cholesterol were calculated using the Friedewald formula [25]. Plasma apolipoprotein AI (apoAI) and apolipoprotein B (apoB) were determined by nephelometry. Serum ALT and CK were determined to detect liver and muscle damage during treatment with atorvastatin.

2.3. CYP3A5 genotyping

Genomic DNA was isolated from 1 ml EDTA-anticoagulated whole blood samples using a salting-out method described by our group [26]. *CYP3A5*3C* and *CYP3A5*6* SNPs were detected by polymerase chain reaction (PCR) followed by restriction fragment analysis (RFLP). *CYP3A5*1D* was detected by DNA sequencing. Primer sequences and PCR thermal cycling conditions used in this study are displayed in Table 1.

PCR assays contained 100 ng genomic DNA, 200 nmol/l primers (Integrated DNA Technologies, Coralville, USA), 200 mmol/l dNTPs (GE Healthcare, Amersham Bioscience, Sao Paulo, Brazil), 1 U DNA polymerase and PCR buffer [50 mmol/l KCl, 20 mmol/l $(NH_4)_2SO_4$, 2 mmol/l $MgCl_2$, 75 mmol/l Tris–HCl (pH 9.0)], (Biotools B&M Labs., Madrid, Spain) in 50 μl total volume. PCR thermal cycling conditions consisted of initial incubation at 98 °C for 3 min, followed by 30–35 cycles at 95 °C for 1 min, 58 °C–63 °C for 1 min and 72 °C for 1 min, and a final extension period of 72 °C for 10 min. Amplification was carried out in a PTC-200 Thermal Cycler (MJ Research Inc., Walthan, MA). PCR products were analyzed by 1% agarose gel electrophoresis after ethidium bromide staining.

PCR products (5 μl) were digested with 5 U of the restriction endonuclease DdeI (Promega Corporation, Madison, WI) for 4 h at 37 °C. The fragments were analyzed by 8% polyacrylamide gel electrophoresis after silver or SYBR Gold® (Molecular Probes, Invitrogen Detection Technologies, Eugene, OR) staining. Every restriction product had, in their sequence, not only a polymorphic site, but also a constitutive site (which allows monitoring of enzyme activity during the reaction). Thirty percent of the samples were re-tested randomly by RFLP in order to guarantee that the genotype once obtained is strictly correct. Genotype controls were used in every PCR-RFLP assay.

Genotyping accuracy was confirmed by DNA sequencing using the capillary electrophoresis system Mega BACE 1000 (Amersham Pharmacia Biotech, Upsala, Sweden). Five percent of all samples were amplified by PCR using sets of primers (Table 1) designed with Primer Premier® v. 5.0 (Premier Biosoft International, Palo Alto, CA), based on the available gene sequence (Access number AC005020) on GenBank from National Institute of Health (NIH) (www.ncbi.nlm.nih.gov/entrez/). Purified PCR products (370 bp) were sequenced in two directions using standard protocols of the Termo Sequenase II dye terminator cycle sequencing kit (Amersham Pharmacia Biotech, Uppsala, Sweden).

2.4. Statistical analysis

Categorical variables were compared by chi-square test or Exact Fisher test. The agreement of *CYP3A5*3C* genotypes frequencies with Hardy–Weinberg equilibrium expectations was tested using the MS-DOS QBasic V. 1.1 (Microsoft Informatica Ltd, Sao Paulo, Brazil). Continuous variables were presented as means ± standard deviation (SD) and compared by *t*-test. Variables without normal distribution were log transformed for analysis. The effects of the SNPs on serum lipids and apolipoproteins before and after treatment with atorvastatin were evaluated by Paired *t*-test or Mann Whitney Rank Sum Test. Differences in serum