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A Simple Allele-Specific Polymerase Chain Reaction Method to Detect the Gly143Glu Polymorphism in the Human Carboxylesterase 1 Gene: Importance of Genotyping for Pharmacogenetic Treatment

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Human carboxylesterases 1 and 2 (*CES1* and *CES2*) catalyze the hydrolysis of many exogenous compounds. Alterations in *CES* sequences could lead to variability in both the inactivation of drugs and the activation of prodrugs. The human *CES1* gene encodes for the enzyme carboxylesterase 1, a serine esterase governing both metabolic deactivation and activation of numerous therapeutic agents. Some of theses drugs are the antiviral oseltamivir used to treat some types of influenza infections and the methylphenidate employed in the treatment of patients with attention deficit. The Gly143Glu polymorphism in *CES1* gene has been shown to reduce enzyme activity. The aim of the present study was to develop an easy and cheap method to detect this polymorphism. For this, we studied a group of people from Córdoba, a Mediterranean area from Argentina. Our results show that our methodology could detect the presence of this polymorphism with a frequency around 1.8%, only in the heterozygote form. These results could be relevant to patients before the treatment with some drugs where the *CES1* enzyme is involved.

Introduction

Carboxylesterases (CESs) are members of the αβ hydrolases expressed in many tissues, especially in the liver, small intestine, and lung (Satoh and Hosokawa, 2006; Ross and Crow, 2007). Three CES genes are well characterized in the human genome: CES1, CES2, and CES3. All three are expressed in the liver. CES3 is more highly expressed in brain endothelial cells than in liver and has been suggested to function at the blood–brain barrier (Yamada *et al.*, 2010). The major human carboxylesterases include *CES1* and *CES2*, which are largely distinguished from one another by their substrate specificity and tissue distribution (Imai *et al.*, 2005; Satoh and Hosokawa, 2006). *CES1* more readily catalyzes substrates with a relatively large acyl group and small alcohol group, such as methylphenidate, temocapril, and oseltamivir (Sun *et al.*, 2004; Imai *et al.*, 2005; Shi *et al.*, 2006). In contrast, *CES2* preferentially hydrolyzes compounds bearing a small acyl moiety and bulky alcohol group, which includes agents such as cocaine and irinotecan. *CES1* predominates in human liver, whereas *CES2* is the major carboxylesterase expressed in the intestine (Imai *et al.*, 2005). Hepatic *CES1* is the major esterase governing the metabolism of numerous and structurally diverse therapeutic agents formulated as carboxylic acid esters, carbamates, thioesters, and amide compounds including those prodrugs formulated as esters. In addition, a large number of endogenous substrates are recognized.

Pharmacogenetic studies would hopefully lead to individualized treatment protocols in the near future by providing a panel of informative genetic markers to check before starting pharmacotherapy. Pharmacogenetic studies explore how individual genetic variations influence the pharmacokinetic and pharmacodynamic properties of the medicine, such as drug metabolism, efficiency and side effects.

In a recent study, Zhu *et al.* (2008) identified two *CES1* mutations, p.Gly143Glu [exon 4, codon 143 (GGG>GAG)] and p.Asp260fs in a subject who displayed profound alteration of the pharmacokinetics of racemic (dl)-methylphenidate, one of the first-line treatments in attention deficit hyperactivity disorder and a selective *CES1* substrate, during

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a single-dose pharmacokinetic study (Patrick *et al.*, 2007). The first variant was a nonsynonymous amino acid substitution of glycine 143 to glutamic acid (Gly143Glu). The second variant identified (p.Asp260fs) was a deletion resulting in a premature stop codon. The minor allele frequency of Gly143Glu was estimated to be 3.7%, 4.3%, 2.0%, and 0% in white, black, Hispanic, and Asian populations, respectively, by a genotyping study that contained a total of 925 subjects with varied racial and ethnic backgrounds.

Moreover, the two newly discovered *CES1* mutations were determined to be dysfunctional enzymes in terms of hydrolyzing methylphenidate to its inactive metabolite ritalinic acid.

On the other hand, oseltamivir is widely used in the treatment and prophylaxis of both influenza virus A and B infections. For instance, oseltamivir has been approved by the Food and Drug Administration for the treatment or prevention of 2009 influenza A (H1N1). Oseltamivir has recently been shown to be a selective substrate of *CES1* (Shi *et al.*, 2006), making it an excellent candidate compound to assess the effects of the identified *CES1* mutations on prodrug activation. The active metabolite exerts its antiviral effects via selective inhibition of neuraminidase. Zhu's findings suggested that the genetic variants of *CES1* that result in dysfunctional enzyme activity could likewise play an important role in therapeutic efficacy as well as tolerability or toxicity during oseltamivir therapy.

In the present study, we develop a new method to detect and investigate the influence of the Gly143Glu polymorphism in the *CES1* gene in a group of Argentinean healthy blood donors from Córdoba.

Materials and Methods

A total of 509 apparently healthy adult blood donors, randomly selected from Córdoba (Argentina), were assayed for Gly143Glu polymorphism (rs71647871) in the *CES1* gene (accession no. NG_012057). There were 213 men and 296 women (mean age, 41.8 years; age range, 18–84 years). The study was approved by the Committee of Ethics and Research from the Hospital de Niños Santísima Trinidad de Córdoba and Ministerio de Salud de Córdoba. In all cases, the subjects were informed and their consent was obtained.

Total DNA was extracted from peripheral blood using standard procedures (Stewart and Via, 1993). DNA fragment amplifications of the *CES1* gene were carried out by polymerase chain reaction (PCR) using our allele-specific primers (Table 1). Then, 80 ng of patient's DNA was amplified in two tubes for each specific allele. The cycle parameters used were as follows: 94°C for 5 min, then 40 cycles of 94°C for 40 s, 61°C for 40 s, and 72°C for 40s, and a final extension of 72°C for 7 min. We used 10 μM of a common reverse primer and 10 μM of two forward primers (Table 1). The first forward primer is specific for the mutant allele, giving a 200-bp product, whereas the second one amplifies a 368-bp product from both mutant and wild-type alleles, acting as an internal PCR control.

The amplified products were separated by electrophoresis in agarose gel (1.2%, p/v) and visualized by UV light after ethidium bromide staining.

The accuracy of the method was confirmed by sequencing six selected samples (three wild-type/wild-type and three Gly143Glu/wild-type) using the forward internal control primer and the common reverse primer. Gly143Glu/Gly143Glu was not present in the analyzed population.

Allelic frequencies were determined and statistical analysis was performed using the chi-square (χ^2) test.

Table 1. Primers Sequences Used for Carboxylesterase 1 Genotyping in the Studied Population

Allele-specific primers	
Forward (internal control):	5′ TGCAGGAGAGAGATTGCCTT 3′
Forward (specific A):	5′ TGTGGATCCACGGAGGGGA 3′
Forward (specific G):	5′ GTGGATCCACGGAGGGGG 3′
Reverse (common):	5′ ATGACCAGAGCTGGGTCCTA 3′

Results

The frequency of Gly143Glu polymorphism in the *CES1* gene was evaluated in a sample of 509 healthy Argentinean subjects, representative of the population of the central area of the country. The genotypes found in this group are summarized in Table 2. The *CES1* genotype distribution for Gly143Glu polymorphism was in Hardy–Weinberg equilibrium. We have found nine Gly143Glu/wild-type (1.8%) and 499 wild-type/wild-type (98.2%). Double wild-type homozygote was the most common genotype and the Gly143Glu/Gly143Glu was not detected.

Finally, the method described here is fast, quick, and rapid and could be employed as a simple and cost-effective determination of *CES1* polymorphism in patients under treatment with drugs activated or inactivated by the *CES1* enzyme.

Discussion

CES1 is the predominant hydrolase in the liver and plays an important role in the biotransformation of drugs and prodrugs that contain ester bonds. *CES1* genetic variants and their potential for having therapeutic implications have been increasingly reported recently (Sun *et al.*, 2004; Shi *et al.*, 2006; Zhu *et al.*, 2009). The *CES1* is the hydrolase that, among others, transforms oseltamivir to an active metabolite with antiviral effect in the treatment of H1N1 influenza.

According to official data from the government agencies of Argentina, the 2009 influenza H1N1 epidemic was responsible for more than 95% of total flu cases analyzed.

In this work, we determined the frequency of one genetic polymorphism in the CES1 gene in a healthy volunteer

Table 2. Distribution of Carboxylesterase 1 Genotypes in the Studied Population According to Sex

Genotype		*Sex*	
		n *(% of total population)*	
Gly143Glu	*Total (%)*	*Male*	*Female*
−/−	500 (98.2)	212 (41.7)	288 (56.5)
+/−	9 (1.8)	1 (0.2)	8 (1.6)
Total	509 (100)	213 (41.9)	296 (58.1)

Plus sign, mutated allele; minus sign, wild-type allele; *n*, number of subjects tested.

population from Córdoba, Argentina. We selected a population that included 509 volunteers of both sexes, none of them younger than 18 years.

To analyze the Gly143Glu polymorphism in *CES1* gene, we developed a simple allele-specific PCR amplification and separation of fragments by agarose gel electrophoresis, which allowed us to obtain the results in <24 h. We found that only a 1.8% of the studied population showed the presence of Gly143Glu variant. The values found are in agreement with those mentioned in the study in United States by Zhu *et al.* (2008).

As the activation of many ester prodrugs depends on a great degree upon functional *CES1* enzyme to produce the therapeutic moiety, dysfunctional *CES1* variants could hinder prodrug activation and lead to the alteration of therapeutic effects and accumulation of the parent prodrug with continued dosing. Taking this into account, it is important to remark that the presence of this mutation was found only in the heterozygote form. This suggests the possibility that *CES1* activity could be affected only partially. Nevertheless, several publications show that the levels of active *CES1* enzyme vary considerably between adults versus children, showing enzyme activity between 15% and 25% in children related to adults (Yang *et al.*, 2009). Even, within the same group of age, there are very important differences. On the other hand, the administration of other drugs, such as clopidogrel, could be inhibited by the enzyme activation (Shi *et al.*, 2006). For all these reasons, the levels of activity of this enzyme and whether they affect the therapeutic efficacy must be evaluated by *in vivo* studies.

The application of this methodology is rapid and simple to detect this polymorphism before administration of several drugs, such as the oseltamivir for the treatment of H1N1 pandemic flu, especially for people considered at risk.

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Disclosure Statement

No competing financial interests exist.

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