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Genetic Polymorphism of Cytochrome P450 2D6*4 and 2D6*5 in an Adult Population Sample from Costa Rica

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ABSTRACT Currently, there is a defined group of drugs, previously established by the Food and Drug Administration (FDA), that requires pharmacogenomic tests, due to possible polymorphisms in the cytochromes that metabolizes them, leading to a potential modification of their pharmacokinetical and/or pharmacodynamical properties, and therefore their dosage. The main objective of this study was to determine the frequency of two single-nucleotide polymorphism (SNP) for CYP2D6 cytochrome, related to a poor metabolizer phenotype: CYP2D6*4 and CYP2D6*5 in a group of adults from Costa Rica. CYP2D6*4 allele was determined by PCR RFLP methodology and CYP2D6*5 allele by multiplex PCR in 389 adult blood samples from Costa Rica. The allelic frequency determined for CYP2D6*4 was of 12.2 percent, and of 2.8 percent for CYP2D6*5. There were 23 subjects with a homozygote polymorphic genotype (4*/4*) and 3 with a double heterozygote mutation (wt/*4 and wt/*5); that corresponds to a 6.7 percent of the sample. The relevance of this finding is due to the fact that a slower metabolism for selective substrates has been demonstrated in subjects from Costa Rican origin, due to SNP in alleles for CYP2D6; nevertheless, other CYP2D6 polymorphisms implied in this phenotype should be analyzed, in order to give a definitive characterization.

INTRODUCTION

After the Human Genome Project in 2003, genomic medicine expanded fast and vastly, developing innovative techniques for high performance DNA sequencing, genetic mapping and bioinformatics; these lead to the development of pharmacogenomics, a pharmaceutical science that has provided individualized pharmacological strategies for patients (Kirchheinet et al. 2007; Crew 2012; Gustafson-Brown 2012; Relling and Evans 2015).

Frequently, at clinical practice, there are patients treated with same dosages that show variability in therapeutical responses and adverse drug reactions patterns. This phenomenon can be attributed to pharmacokinetic and pharmacodynamic issues that cause changes in those drugs' plasmatic concentrations and could affect their effectiveness and security (Ingelman-Sundberg 2005; Ingelman-Sundberg et al. 2007; Ingelman-Sundberg 2008; Ingelman et al. 2010; Wang et al. 2011; Garay 2012; CYP2D6 2014). Metabolism is one of the most determinant pharmacokinetic processes in control of the biological activity and elimination of a drug. These metabolic reactions are categorized in phase I (attach or expose the primal drug to a functional group) and phase II (drug conjugation, mainly with glucuronide acids); these are essential for the elimination of several drugs, therefore variations in enzymatic activity on this system can influence interindividual differences in drug responses, due to this pharmacogenetic variability (Ingelman-Sundberg 2005; Ingelman et al. 2007; Ingelman-Sundberg 2008; Wang et al. 2011; Garay 2012; CYP2D6 2014).

Cytochrome P450 (CYP450) is a system of hemoproteins that composes the 70-80 percent of all phase II reduction-oxidation reactions. They are localized in smooth endoplasmic reticules in several tissues, mainly liver, testicles and adrenal glands; CYP450 is essential in xenobiotics and endogenous substances degradation and several single nucleotide polymorphisms (SNP) can modify their function, like those found in CYP2D6 (Ingelman-Sundberg 2005; Ingelman-Sundberg 2008; Redlich et al. 2008; Ingelman et al. 2010; Garay 2012; Machin-García 2012; CYP2D6 2014).

The codifying gene for CYP2D6 is localized in chromosome 22q13.1, accompanied by two

pseudo genes (CYP2D7 and CYP2D8), this phenomenon increases the possibility of allelic mutations due to cross reaction that produces polymorphic alleles. Actually, more than 70 polymorphic alleles have been identified and their phenotypical expression is categorized as poor, intermediate, extensive and ultraextensive metabolizers (Ingelman-Sundberg 2005; González et al. 2006; Laika et al. 2009; Ingelman et al. 2010; Zachrisson et al. 2010; Crews et al. 2012;, Garay 2012; Valdes et al. 2011; Lyon et al. 2012; Machin García 2012; McCarthy et al 2013; CYP2D6 2014; Human Cjytochrome P450 (CYP) Allele Nomenclature Database 2014; Relling et al. 2015).

These phenotypical expressions are translated in sub therapeutical or toxic effect, such as Poor metabolizers (PM), which are patients with a lesser drug elimination or a lack of therapeutical effects, intermediate metabolizers (IM) that includes patients that need therapy monitoring in case of polymedication, because they could need a dosage reduction, extensive metabolizers (EM) that are patients that have a normal, complete and total metabolism capacity for drugs. and ultraextensive metabolizers (UM) which includes patients that could need an increase in their drug's usual dosage (Kirchheiner 2007; Redlich et al. 2008; Scott 2011; Crews et al. 2012; Lyon et al. 2012).

Therefore, pharmacogenomics' objective is to develop individualized therapeutical strategies, thus enhancing drugs' effectiveness and safety through acknowledging the genetic variations in humans and how those affect pharmacotherapy (Kirchheiner 2007; Redlich et al. 2008; Johnson 2012; Crews et al. 2012).

Currently, genes that account for individual sensibility, resistance and toxicity towards a specific drug have been identified, as well as the causes of inter individual variations in their expression, function and clinical incidence, including mRNA roles, DNA methylation, SNP and copy-number variations (CNVs) (variations related to the number of copies of a gene or genes). It has been estimated that human beings possess nearly 7 million of SNPs, of which the less frequent allele is present at 5 percent; nevertheless, the importance of uncommon variations is more recognized nowadays (Kirchheiner 2007; Redlich et al. 2008; Crews et al. 2012; McCarthy et al. 2013).

The pharmacogenomics' long term objective is to translate information from genetic responses to drugs into a more effective and less toxic response for individualized therapies (Crews et al. 2012).

The FDA provides a list of drugs to consider in pharmacogenetic studies to predict drug's responses related to their metabolism. For CYP2D6 there are few drugs for which there has been identified the polymorphisms that could affect their metabolism (Flochkart et al. 2009).

In Costa Rica very little is known about the genetic characteristics of the population and its effect over the therapeutical answer. There are few pharmacogenomic tests that might be used to optimize medical therapy; nevertheless they are quite expensive.

The aim of this study was to identify the frequency of CYP2D6*4 and CYP2D6*5 alleles in a sample of adult population from Costa Rica, using methodologies that include the polymerase chain reaction (PCR) and PCR with restriction enzymes (PCR-RFLP). These alleles are known to influence a lower metabolic activity or produce a complete absence of it, therefore their importance for this research (Zackrisson et al. 2010; Scott 2011; Garay 2012; Johnson 2012; Machin García 2012; McCarthy 2013; CYP2D6 2014).

MATERIAL AND METHODS

The study was developed at the Pharmacology Department of Pharmacy Faculty and the Food Microbiology Department of Microbiology Faculty, at the University of Costa Rica (UCR).

The study included 389 anonymous EDTA supplemented blood samples obtained during 2014 from adult population (over 18 years old) of the country's central zone (known as Gran Área Metropolitana or GAM). The only information gathered from patients included sex and age, thus 64.6 percent of the samples belonged to women and 25.4 percent to men. The average age was 51.9 for women and 54 for men. No other personal or demographic information, like race, ethnic background or otherwise, about the patients of these samples is known. The anonymity and ethical use of the samples is warranted under fundamental bioethical principles.

Samples were stored at 2°C until DNA extraction. DNA was kept at -70°C until genotyping CYP450 2D6*4 and 2D6*5 alleles.

DNA Extraction

For DNA extraction from blood samples a DNA, RNA and protein purification MACHERY-NAGEL® Kit was used.

CYP2D6*4 Allele Detection by PCR-RFLP

This procedure was accomplished using the method described by Schur et al. (2001) which uses the Mval restriction enzyme. Briefly, two primers (5'-GCCTTCGCCAACCACTCCG-3') and the reverse (5'-AAATCCTGCTCTTCCGAG-GC-3') were used. For each reaction, a total mixture volume of 25 µL was used, composed of 5 µL of DNA, 0.8 µM of each primer, 0,2 mM of dNTPs, 2 U of DNA Taq-polymerase in Tris-HCl 20 mM pH 8.4, KCl 50 mM y MgCl2 2 mM. Amplification was performed on a thermal cycler using the following program: denaturation at 94ºC for 5 minutes, followed by 30 cycles, each one composed of three phases: denaturalization, (94ºC for 60 seconds); annealing, (67ºC for 30 seconds), and extension (72ºC for 90 seconds). The reaction was completed with a final step at 72ºC for 5 minutes (Schut et al 2001; Garay 2012).

The PCR's final products were observed by a horizontal electrophoresis mean, using a 2 percent agarose gel dyed with Fast Red. A mass ruler of 100- 3000 pb was used. The process was done at 100 volts for 45 minutes in a TBE 1X buffer, where an amplicon of approximately 355 pb is observed with UV light, which indicates the gen presence. (Schut et al. 2001; Garay 2012).

Restriction Analysis

The amplicon obtained was digested with the Mval restriction enzyme for 5 minutes at 37ºC in a R 1X buffer [Tris-HCl 10 mM (pH 8.5), magnesium chloride 10 mM, potassium chloride 100 mM y BSA 0,1 mg/mL]. The volume reaction was 16 µL, of which 8 µL came from PCR, 10 U from the MvaI enzyme, 1 ìL of Tango buffer and 5 µL of water. This mixture went under a horizontal electrophoresis in a 2 percent agarose gel dyed with Fast Red, and the products were visualized under UV light (Schut et al 2001; Garay 2012).

The detection of a 355 pb band indicates the presence of the polymorphic homozygote genotype *4/*4, as well as the presence of two bands at 105 and 205 pb indicates the wild homozygote genotype (wt/wt) and the presence of three bands at 104, 205 and 355 pb, is categorized as the polymorphic heterozygote genotype (wt/4*).

CYP2D6*5 Allele Detection by PCR Multiplex

PCR multiplex reaction was done according to the methodology described by Hersberger et al. Primers used are described on Table 1. Briefly, a 52 µL mixture, composed of 34.2 µL of water, 5.0 µL of buffer 3 (2.25 mmol/L MgCl2), 0.75 µL of Enzyme Mix (3.5 U/ µL), 1.75 µL of dNTP mixture (10 mmol/L), 0.8 µL of Dup primer, 0.8 µL of Dlow primer, 1.6 µL of DPKup primer, 1.6 µL of DPKlow primer and 5.5 µL of genomic ADN (50 ng/µL), was prepared. The cycling conditions used include 1 minute at 94 °C for denaturation, 35 cycles at 94°C for 60 seconds, 65°c for 30 seconds, 68°C for 5 minutes and a final cycle at 68°C for 7 minutes. The resulting products from this PCR were observed also in a 2 percent agarose gel dyed with Fast Red, electrophoresis conditions included a 2 hour run at t 100 volts . A mass ruler of 100-3000 pb was used.

The expected results for this allele included the formation of a 5000 pb band associated with the wild type and a band close to 3000 pb, which corresponds to the heterozygote allele for CYP2D6*5.

RESULTS

The results obtained for CYP2D6*4 genotype frequency are detailed in Table 2. A 5.91 percent of the population evaluated presented the A/A

Table 1: Oligonucleotides used for CYP2D6*5 polymorphism detection

Primer	*Position 5´*	*Sequence*	*Position 3´*
Dlow	7846	CAGGATGAGCTAAGGCACCCAGAC	7822
Dup	43	CACACCGGGCACCTGTACTCCTCA	66
DPKlow	4844	GCCGACTGAGCCCTGGGAGGTAGGTA	4819
DPKup	-259	GTTATCCCAGAAGGCTTTGCAGGCTTCA	-232

Modified from: Hersberger M, Marti-Jaun J, Rentsch K, Hänseler E. Rapid Detection of the CYP2D6*3, CYP2D6*4, and CYP2D6*6 Alleles by Tetra-Primer PCR and of the CYP2D6*5 Allele by Multiplex Long PCR. Clinical Chemistry. 2000. 1072-107.

mutation of the *4/*4 allele. There were 26 women and 23 men in the sample with the 1*/4* allele as well as 14 women and 9 men with the 4*/4* allele.

Table 2: Genotypic frequency for CYP2D6*4 (G1-846A)

Genotype	*Mutation*	*n*	*%*
CYP2D6*1/*1	G/G	317	81.49
CYP2D6*1/*4	G/A	49	12.60
CYP2D6*4/*4	A/A	23	5.91

*Total number of subjects for this procedure was 389.

Differing from the genotype frequency, which is calculated with the total number in the sample, the allelic frequency is calculated with the total number of alleles in the sample (788); this value is the most reported in the majority of pharmacogenetic studies and is detailed in Table 3.

Table 3: Allelic frequency for CYP2D6*4

Alleles	*n*	*%*
*1	683	87.79
*4	95	12.21

Results obtained for CYP2D6*5 allele showed that 11 samples presented a band at 3000 pb, therefore a 2 percent of the sample had the mutation in the CYP2D6*5 allele; of those samples 9 belonged to women and 2 to men.

For the present investigation there were 3 subjects with a double heterozygote mutation (wt/*4 and wt/*5); that correspond to a 0.8 percent of the sample.

DISCUSSION

Pharmacogenomics links the knowledge of genetic and metabolic aspects that have an effect over the efficacy of a medicine and how these factors interact in order to produce phenotypes related to answers to drugs. It also offers the opportunity of leading to a secure and efficient pharmacological therapy for each individual patient, based on his genetic profile (Hersberger et al. 2000; Crews et al. 2012; McCarthy et al. 2013; Relling et al. 2015).

This genetic profile is determined using pharmacogenetic studies, that identify the polimorphisms that might have an influence over the response to a drug, a fact of vital importance in order to establish clinical correlations. Nevertheless, in order to have fully accepted and useful pharmacogenetics tests, its validation and utility have to be demonstrated (Sachse et al. 1997; Bernal et al. 1999; CYP2D6 2014).

This genetic profile is established by pharmacogenetic studies that can determine the genetic polymorphism responsible for the variability in these responses, which is extremely relevant for the consequent clinical decisions that should be taken (Garay 2012; Machin Garcia 2012; Human Cytochrome P450 Allele Nomenclature Database 2014).

Despite this relevance, there are a wide variety in alleles that can express mutations that affect the drug metabolism done by CYP2D6, being the *3, *4, *5 and *6 the most common, and those are associated with around a 95 percent of enzyme inactivation, causing a PM phenotype (Hersberger et al. 2000; McElroy et al. 2000; Scott 2011; Johnson 2012; McCarthy et al. 2013).

The present paper achieves the characterization of two of these polymorphisms related to a PM phenotype: the CYP2D6*4 allele (G1846A) corresponding to a G1846 A transition that causes a premature codon, which at its presence produces a dysfunctional protein, removing completely the catalytic active enzyme. Also, the presence of the CYP2D6*5 allele (A2549del) expresses a deletion of it that is responsible for the same effect (Hersberger 2000; Garay 2012).

The results obtained in this paper showed a 12.2 percent frequency for CYP2D6*4 in the population studied. This result agrees with the findings reported by Machín, in 2012, where he gathered the results of the pharmacogenetic studies available currently in America, finding that the CYP2D6*4 allele is the most frequent one, reaching a frequency up to 18 percent in European descendants. It also agrees with the frequencies reported in other Latin countries, including Mexico, Colombia, Nicaragua and Ecuador, being all above 10 percent (Ingelman-Sundberg et al. 1999; McElroy et al. 2000; Hicks et al.2012; Machin Garcia 2012).

In regard of CYP2D6*5 tests, a frequency of 2.8 percent (11 subjects) was obtained. These individuals might present a deficiency in the metabolism of some drugs. When comparing with other studies from America and Spain, this percentage is close to those found in Mexican descendants in USA and mestizos in Mexico, as well as in Caucasian population (Sachse et al.1997; Bernal et al. 1999; Schur et al. 2001).

Although the results obtained are comparable with those reported in the region, it is important to clarify that they only represent a small sample of the Costa Rican population, and can't be extrapolated to the whole country; nevertheless they represent an initial knowledge of the possibility of therapeutical failure that could arise.

CONCLUSION

The clinical consequences of this genotype can vary depending of the phenotype of the patient. The subjects with homozygote polymorphic alleles ($4^*/4^*$) or a heterozygote combination (wt/*4, wt/*5) can be related to a PM activity. For the present investigation there were found 23 subjects with a homozygote polymorphic genotype ($4^*/4^*$) and 3 with a double heterozygote mutation (wt/*4 and wt/*5); that corresponds to a 6.7 percent of the sample. This could count toward a PM activity in that enzyme for those people; nevertheless further investigation shall be performed in order to have a more complete metabolic profile of the individuals tested.

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