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Frequencies of phenylalanine hydroxylase mutations I65T, R252W, R261Q, R261X, IVS10nt11, V388M, R408W, Y414C, and IVS12nt1 in Minas Gerais, Brazil

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ABSTRACT. In order to determine the phenylketonuria (PKU) mutation spectrum in the population of Minas Gerais State, Brazil, 78 unrelated PKU patients found by the neonatal screening program from 1993 to 2003 were tested for nine phenylalanine hydroxylase mutations. These mutations were selected due to their high frequencies in other Brazilian populations and in Portugal, where the largest contingent of the Caucasian component of the Brazilian population originated from. The most frequent mutations were V388M (21%), R261Q (16%), IVS10nt11 (13.4%), I65T (5.7%), and R252W (5%). The frequencies of the other four mutations (R261X, R408W, Y414C, and IVS12nt1) did not reach 2%. By testing these nine mutations, we were able to identify 64% of the PKU alleles in our sample. V388M frequency was higher than in any other known population and almost three times larger than that observed in Portugal, probably reflecting genetic drift. The mutation profile, as well as the relative frequency of the different mutations, suggest

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that the Minas Gerais population more closely resembles that of Portugal than do the other Brazilian populations that have already been tested.

Key words: Phenylalanine hydroxylase, Phenylketonuria, Mutation screening

INTRODUCTION

Phenylketonuria (PKU; OMIM #261600) is an autosomal recessive disorder caused by a deficiency of phenylalanine hydroxylase (PAH, EC 1.14.16.1). PAH catalyses the hydroxylation of phenylalanine to tyrosine. If it is precociously introduced, a low-phenylalanine diet prevents the mental retardation associated with PKU (MacDonald, 2000; Fisch, 2000; Scriver and Kaufman, 2001). Consequently, neonatal screening programs have constantly targeted PKU (McCabe et al., 2002; Carreiro-Lewandowski, 2002).

Currently, more than 490 PAH mutations have been described (PAH Mutation Analysis Consortium; http://www.pahdb.mcgill.ca, Scriver et al., 2003). PAH mutation frequencies show strong variation among populations. These differences are attributable to the history of the populations (Hofman et al., 1991; Perez et al., 1993, 1999; Guldberg et al., 1998; Yang et al., 2001; Zschocke, 2003).

Due to the large number of mutations, most of the patients are compound heterozygotes, which explains at least partially the considerable phenotypic variability observed in this disease. Determination of the PAH mutations in each patient can help predict the severity of the disease (Rivera et al., 1998; Benit et al., 1999; Pey and Martinez, 2005). Depending on the mutation, some patients are more efficiently treated with BH_4, the PAH co-factor (Bernegger and Blau, 2002; Blau and Erlandsen, 2004; Steinfeld et al., 2004; Cerone et al., 2004).

Minas Gerais is a state with 16 million inhabitants in southeastern Brazil. A neonatal screening program has been conducted in Minas Gerais since 1993 by the Núcleo de Pesquisa em Apoio ao Diagnóstico (NUPAD) of the Universidade Federal de Minas Gerais. Monthly, approximately 20,000 newborns are tested for PKU, hypothyroidism and sickle cell disease. The mean coverage is approximately 94% of the births in this state. PKU incidence in Minas Gerais has been estimated to be about 1:20,000 (Serjeant, 2000). This frequency is somewhat lower than previously reported figures for Caucasians (1:10,000) and Orientals (1:16,500; Scriver and Kaufman, 2001).

We sought to develop a cost-efficient strategy for PAH mutation screening in Minas Gerais. We selected for evaluation those mutations that were highly prevalent in Western Europe, particularly in Portugal, historically known to be the main source of the Caucasian component of the Brazilian population. Also, mutations found to be frequent in two other Brazilian subpopulations, those from São Paulo (Acosta et al., 2001) and from the two southernmost Brazilian states, Santa Catarina and Rio Grande do Sul (Santana da Silva et al., 2003), were added to the project. Initially, we selected a set of nine mutations.

PATIENTS AND METHODS

We examined 78 unrelated patients sequentially diagnosed by a neonatal screening

program. Prior to the beginning of the investigation, the project was approved by the Ethics Committee on Research Board of the Universidade Federal de Minas Gerais.

Patients were screened for the presence of the PAH mutations I65T, R252W, R261Q, R261X, IVS10nt11, V388M, R408W, Y414C, and IVS12nt1. A blood sample (5 mL) was collected from each patient, after obtaining informed consent from his or her parents or from a legally designated guardian. Genomic DNA was isolated, as previously described (Miller et al., 1988). PCR amplification was performed for the fragments of the PAH gene in which these mutations are located. Primers, annealing temperatures, and restriction enzymes used for digesting the PCR products are shown in Table 1. Restriction fragments were separated by polyacrylamide gel electrophoresis (Figure 1). Gels were stained with silver nitrate or with ethidium bromide.

Table 1. Primers, annealing temperatures, and restriction enzymes used for identifying phenylalanine hydroxylase mutations.

Mutations (restriction enzyme)	Annealing temperatures	Primers (5'-3')
I65T (*Taq* I)	55°C	3A (F): GTTAGGTTTTCCTGTTCTGG 3B (R): AACGAGAAGGTCTAGATTCG
R252W (*Ava*I) R261Q (*Hinf*I) R261X (*Dde*I)	56°C	7A (F): ACTACCAAAGGTCTCCTAGTGC 7B (R): CAAACCTCATTCTTGCAGCAGG
V388M (*Bsa*I) IVS10nt11 (*Dde*I)	65°C	11A (F): AAGGAACGGGGTAGATGAGAGAAGGGGC 11B (R): GGTACAAAGTTGCTGTAGACATTGGAGTCC
R408W (*Sty*I) IVS12nt1 (*Rsa*I)	50°C	12A (F): CCAAATGGTGCCCTTCACTCAAGCC 12BIVS12NT1Ra (R): CTCGTAAGGTGTAAATTACGTA
Y414C (*Rsa*I)	53°C	12A414Ra (F): CTCGGCCCTTCTCAGTTCGGT 12B (R): AGTCTTCGATTACTGAGAAA

(Adapted from Eiken et al., 1991, 1993; Zschocke et al., 1995; Perez et al., 1996; Acosta et al., 2001).

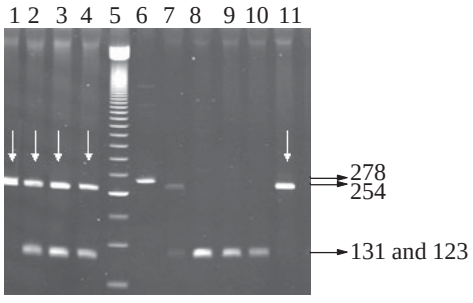

Figure 1. Detection of R261Q mutation: exon 7 was amplified and the 278-bp PCR products were digested with *Hinf*I. The R261Q mutation inactivates the restriction site. The fragment bearing the mutation is 254 bp long, whereas the normal allele produces 131- and 123-bp fragments, which do not separate in this gel concentration. The arrows point to the mutated allele. *Lanes 2*, *3*, *4*, and *7* have one mutant allele; *lanes 1* and *11* have the R261Q mutation in homozygosis. *Lanes 8*, *9*, and *10* do not have R261Q mutation. *Lane 6* - undigested PCR product. *Lane 5* - 50-bp DNA ladder (Amersham Pharmacia Biotech).

RESULTS

The most common mutations among the newborns were V388M (21%), R261Q (16%), IVS10nt11 (13.4%), I65T (5.7%), and R252W (5%). Individually, the other four mutations (R261X, R408W, Y414C, and IVS12nt1) had frequencies lower than 2%. By screening these nine mutations we were able to identify 100/156 alleles, corresponding to 64% of the PKU alleles in this sample (Table 2).

Table 2. Relative frequencies of phenylalanine hydroxylase mutations in Minas Gerais, Brazil.

Mutation	N	%
V388M	33/156	21
R261Q	25/156	16
IVS10nt11	21/156	13.4
I65T	9/156	5.7
R252W	8/156	5
R261X	2/140	1.4
R408W	1/102	0.98
IVS12nt1	1/140	0.7
Y414C	0/156	0

Among these 100 alleles, 38 were found in homozygosis and 62 in heterozygosis. Complete PAH locus genotyping was established for 39 of 78 PKU patients (50%). A further 22 PKU patients (28%) had one of the PKU alleles identified.

Consanguinity between the patient's parents was reported in 16/72 (22%) of the unrelated patients for whom information was available. Consanguineous marriages were reported by 54% of the parents of the true homozygous *vs* 3% of the parents of the compound heterozygous patients. This difference is significant ($\chi^2 = 13.84$; $P < 0.005$). Despite the high consanguinity values, we could not determine PAH allele frequencies for this sample, as we did not have an estimation of F (Sokal and Rohlf, 1969).

Maps of the geographical distribution of the PKU patients and of PAH alleles in the State of Minas Gerais were created with Tabwin programs (developed by SUS - Sistema Único de Saúde, Brazil, freely available at http://www.datasus.gov.br). There was no apparent geographic aggregation within the state, both for PKU patients and for the mutations (data not shown).

DISCUSSION

Three main groups have contributed to the Brazilian population: Amerindian, African, and Caucasian, the latter being predominantly Portuguese. However, there is a considerable variation in the contribution of each group to the ethnic admixture in the different regions of the country. Moreover, the three components are heterogeneous. At the time of the arrival of Europeans in Brazil, in 1500 A.D., Tupi-Guarani Amerindians occupied the region that nowadays constitutes the State of Minas Gerais. Although there were occasional incursions, the first Por-

tuguese settlements in the region were established only after 1693, when gold mines were discovered. During the next 150 years, succeeding economic cycles based on diamonds, milk, coffee, and iron brought African slaves to Minas Gerais, mainly from Guinea-Bissau and Angola. The influx of African slaves diminished during the first fifty years of the 19th century and was followed by migration from Europe, North Africa, and East Asia. When compared to Minas Gerais, São Paulo, and particularly the southern Brazilian states, received a proportionately smaller African and a larger and more diversified European contingent (Bortolini et al., 1997; Alves-Silva et al., 2000; Carvalho-Silva et al., 2001; Bortolini et al., 2003; Callegari-Jacques et al., 2003). The lower PKU incidence ascertained by the neonatal screening program in Minas Gerais, approximately 1:20,000, probably reflects the higher contribution of African ancestry to this population.

Differences in peopling among Brazilian regions after 1500 A.D. are strong enough to bring variations in allele frequencies, and consequently, each Brazilian state must establish its own strategy for a mutation-screening program.

In order to begin PKU mutation screening in Minas Gerais State we chose a set of nine mutations that can be easily screened by PCR amplification, which are prevalent in São Paulo, in the southern Brazilian states or in Portugal, followed by a restriction digest. We compared the frequencies of the mutations detected in Minas Gerais to those observed in São Paulo and southern Brazil (Table 3).

Table 3. Comparison of phenylketonuria mutations detected in three Brazilian subpopulations.

Mutation	Minas Gerais (%)	São Paulo (%)	Southern Brazil (%)
V388M	21	9.1	9.8
R261Q	16	12.2	9.8
IVS10nt11	13.4	17.4	2.4
I65T	5.7	3.5	20
R252W	5	6.5	0
R261X	1.4	3	9.8
R408W	0.98	3.5	9.8
Y414C	0	1.3	0
IVS12nt1	0.7	1.3	4.9

The frequencies shown correspond to the following references: Acosta et al. (2001) and Santana da Silva et al. (2003).

Phenylketonuria mutations in Minas Gerais *vs* Portugal and other European populations

The frequency found for V388M in Minas Gerais is the highest described so far in the world. This mutation accounts for 8.6% of the PKU alleles in Portugal and 6.2% in Spain (Figure 2). V388M is virtually absent in other European populations, and consequently it has been referred to as a Spanish or Iberian mutation (Desviat et al., 1995; Perez et al., 1996, 1997; Rivera et al., 1998). Coincidentally, the frequency of V388M in Chile (13%) is also higher than that observed in Spain (Perez et al., 1999). There is a large variation in the frequency of the R261Q mutation in Caucasian populations, varying from 1.2% in Ireland to 32% in Switzerland, its probable place of origin, with a mean European frequency of 4%. The frequency of this

mutation was somewhat higher in Minas Gerais than in Portugal (10.4%; Eisensmith and Woo, 1992; Zschocke et al., 1995; Rivera et al., 1998; Desviat et al., 1999; Zschocke, 2003). The other mutation, IVS10nt11, is also distributed throughout Europe with frequencies varying from 1 to 25% (Zschocke, 2003). Its frequency in Minas Gerais (13.4%) is similar to those described in Portugal (10.8%) and Spain (14.7%; Perez et al., 1997; Rivera et al., 1998). The I65T mutation, one of the three most common mutations in the Iberian Peninsula, also has a similar frequency in Minas Gerais (5.7%) and in other Latin American populations, as with the other PKU mutations detected in this study (Perez et al., 1997; Rivera et al., 1998; Desviat et al., 1999, 2001).

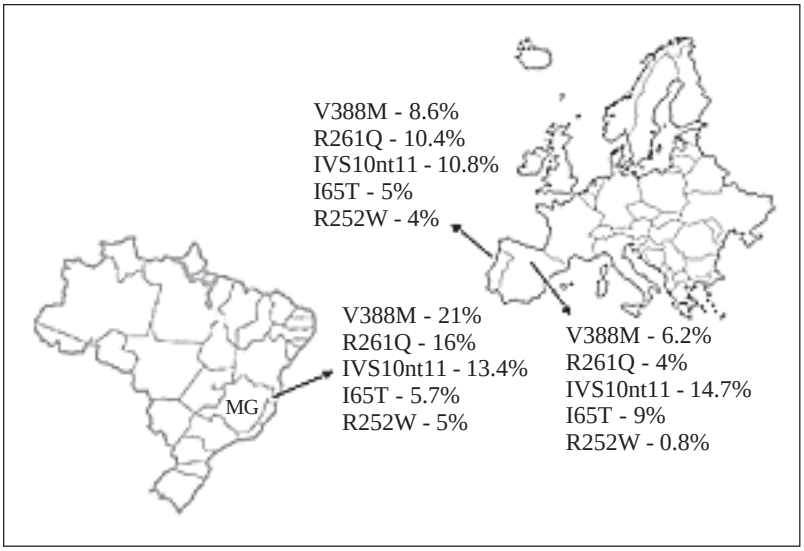

Figure 2. Map of the relative frequency distribution of five phenylalanine hydroxylase mutations in Portugal, Spain and Minas Gerais (MG) Brazil (not drawn to scale).

Based on our results, the first step of mutation screening for the population of Minas Gerais State should include the five most common mutations (V388M, R261Q, IVS10nt11, I65T, and R252W). This approach alone would allow the detection of 61% of the alleles and complete genotyping of 46% of the patients, with only five PCR and restriction digestion reactions/patient.

Taken together, these results suggest that the mutation spectrum at the PAH locus of the population from Minas Gerais resembles more closely that from Portugal than those from São Paulo and southern Brazil states do. The PAH locus mutation spectrum in São Paulo and in southern Brazilian states clearly reflects the larger contingent of immigrants from middle and Eastern Europe. However, this hypothesis, which is in good agreement with historical information, needs to be confirmed by haplotyping studies.

Most studies about the origins of Brazilian subpopulations are based on mitochondrial DNA or on Y-chromosome polymorphisms (Alves-Silva et al., 2000; Carvalho-Silva et al., 2001; Bortolini et al., 2003; Callegari-Jacques et al., 2003). The PAH gene offers a good alternative

for evaluating the evolution of the autosomal genome. This gene apparently has no mutational or recombination hotspots, and it exhibits considerable haplotypic diversity. Both the haplotype origins and the extension of linkage disequilibrium of the locus are well characterized (Eisensmith and Woo, 1994; Kidd et al., 2000; Zschocke, 2003). Further haplotyping will allow us to establish PKU allele origins, as well as to make inferences on regional immigration events in this state.

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