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TESE DE DOUTORADO

AS BASES MOLECULARES DA FENILCETONÚRIA NO ESTADO DE MINAS GERAIS

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Luciana Lara dos Santos

AS BASES MOLECULARES DA FENILCETONÚRIA NO ESTADO DE MINAS GERAIS

Tese apresentada à Universidade Federal de Minas Gerais, como requisito parcial para a obtenção do grau de Doutor em Genética.
Área: Genética Humana
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LISTA DE ABREVIATURAS, SIGLAS E UNIDADES

°C	graus Celsius
µL	microlitros
AV	Valores arbitrários
BH_4	tetrahidrobiopterina
mg/kg/dia	miligramas, por quilo, por dia
HPA	hiperfenilalaninemia
IBGE	Instituto Brasileiro de Geografia Estatística
kb	quilobases
M	molar
mL	mililitros
mM	milimoles
ng/µL	nanograma por microlitro
NAGE	Nucleo de Analise de Genoma
NUPAD	Núcleo de Pesquisa em Apoio Diagnóstico, da Faculdade de Medicina da Universidade Federal de Minas Gerais
OMIM	O*nline* mendelian inheritance man
PAH	fenilalanina hidroxilase
pb	pares de bases
PCR	reação em cadeia da DNA polimerase
PKU	fenilcetonúria
PRA	atividade residual enzimática
RFLP	polimorfismos do comprimento dos fragmentos de restrição
STR	repetições *in tandem* curtas
SSCP	*Single strand conformation polimorphism*
UFMG	Universidade Federal de Minas Gerais
VNTR	número variável de repetições em *tandem*

RESUMO

A fenilcetonúria (PKU) é uma doença causada por mutações no gene fenilalanina hidroxilase (PAH). Mais de 500 mutações já foram identificadas neste gene e suas freqüências variam conforme a população estudada. Os objetivos do presente estudo foram: (1) determinar as bases moleculares da fenilcetonúria em Minas Gerais pela identificação das mutações e haplótipos presentes em 78 indivíduos com PKU do Estado; (2) investigar a correlação genótipo-fenótipo; (3) estimar o coeficiente de endogamia do Estado e corrigir a freqüência alélica da PKU; (4) revisar os tratamentos alternativos para a doença e determinar prováveis respondedores ao tratamento com o BH4. Os resultados encontrados foram: (1) 98% dos alelos causadores de PKU foram determinados, sendo que nove mutações correspondem a 80% dos alelos, V388M (21.2%), R261Q (16.0%), IVS10-11G>A (15.3%), I65T (5.7%), IVS2+5G>C (5.7%), R252W (5.1%), IVS2+5G>A (4.5%), P281L (3.8%) e L348V (3.2%). Uma mutação nova foi encontrada (Q267X). Os haplótipos encontrados são freqüentes na população caucasiana, entretanto encontramos alguns haplótipos raros que já foram descritos na população Africana e que foram encontrados em indivíduos pretos ou pardos desta amostra; (2) Nos estudos de correlação genótipo-fenótipo, foram utilizados dois sistemas diferentes. No sistema de valores arbitrários (AV) tivemos uma porcentagem de 49% de concordância entre o fenótipo esperado e observado, sendo que a maioria da concordância foi encontrada nos indivíduos que possuem duas mutações nulas. Nas análises estatísticas, o genótipo se mostrou um bom preditor do curso clínico da PKU estando significantemente correlacionado com a tolerância medida aos nove meses de vida. Uma forte correlação entre os valores de fenilalanina medidos na primeira consulta foi observado com o genótipo, atividade residual enzimática e soma dos AVs; (3) O coeficiente de endogamia em Minas Gerais foi estimado por microssatélites e o valor encontrado para o Estado (0.003) é de quase quinze vezes menor do encontrado para a amostra PKU (0.04). A freqüência do alelo PKU (q) em MG corrigido pelo coeficiente de endogamia do Estado é de 0.0057 e com esta estimativa podemos verificar que os casamentos consangüíneos são responsáveis por aproximadamente 35% da incidência da PKU no Estado. Quando a amostra da PKU é analisada o valor de q encontrado é de 0.001 mostrando que o impacto da endogamia na freqüência pode ser ainda mais forte; (4) Nas análises de responsividade ao BH4, 20 pacientes possuem genótipos que mutio provavelmente responderiam ao tratamento com o cofator. Os resultados encontrados neste estudo confirmaram a grande heterogeneidade alélica presente no gene PAH, possiblitaram a caracterização das mutações em uma população com alto contigente negróide e contribuíram para o conhecimento da doença de um modo geral no Estado.

ABSTRACT

Phenylketonuria (PKU) is an autossomal recessive disorder caused by mutations in phenylalanine hydroxilase gene. More than 500 mutations have already been described in this gene and their frequencies vary among populations. The aim of this study were: (1) to determine the molecular basis of phenylketonuria in Minas Gerais by identification of PAH mutations and haplotypes in 78 PKU patients from the state; (2) to investigate genotype-phenotype correlation; (3) to estimate inbreeding coefficient in te state and to correct the PKU allele frequency; (4) to review alternative treatments for PKU and to determined probable BH4-responsive patients. The results found were: (1) 98% of the PKU alleles were characterized and nine mutations correspond to 80% of the alleles in the state, V388M (21.2%), R261Q (16.0%), IVS10-11G>A (15.3%), I65T (5.7%), IVS2+5G>C (5.7%), R252W (5.1%), IVS2+5G>A (4.5%), P281L (3.8%) and L348V (3.2%). A new mutation was identified (Q267X). Most of the haplotypes identified are frequent in Europe. However, some rare haplotypes were found, which have already been described in África; (2) In the genotype-phenotype correlation studies, two different systems were used. In the arbitrary value system, 49% of the phenotype observed were in concordance with phenotype expected, and most of the concordances found was present in individuals with two null mutations. In the statistical analysis the genotype was found correlated with tolerance at age of 9 months and it was considered as a good predictor of the clinical course of the patient. A strong correlation was observed as weel among phenylalanine values at first interview, genotype, residual enzymatic activity and AV sum; (3) Based on microssatellites, inbreeding coefficient for Minas Gerais population was estimated (0.003). This value was almost fifteen times lower than that estimated in PKU sample (0.04). The frequency of PKU allele (q) in the state, once corrected by inbreeding coefficient, was estimated to be about 0.0057. Therefore, approximately 35% of the PKU incidence in the state would be due to consaguineous marriages. When q value is estimated taken in account inbreeding coefficient found in PKU patients, q frequency would be 0.001, suggesting that impact of the inbreeding in the PKU allele frequency may be even stronger; (4) In the BH4 responsiveness analysis, 20 patients have genotypes already described in the literature as responsive to cofator. The results found here confirmed the high allelic heterogeneity in the PAH gene, permited the characterization of the mutations in a population with high negroid content and contribuited to better understanding of this disease in the state.

APRESENTAÇÃO

Introdução

Desde a clonagem do gene PAH (gene da fenilalanina hidroxilase), a fenilcetonúria (PKU) tem sido alvo de uma série de estudos genéticos, bioquímicos, moleculares, de correlação genótipo-fenótipo e de haplotipagem. Apesar de todos estes estudos, ainda existem aspectos interessantes a serem investigados na fenilcetonúria. Em primeiro lugar, novas estratégias terapêuticas estão surgindo no mercado tendo como base funcional o espectro mutacional de cada paciente. A caracterização molecular do gene PAH além de fornecer informações sobre o perfil e freqüências das mutações em cada população nos permite prever como seria a responsividade destes pacientes a uma terapia deste tipo. Além disso, são pouquíssimos os estudos em populações Afro-descendentes, e não existe estudo de PKU em indígenas. O conhecimento das bases moleculares da PKU nestes grupos contribuiria para o melhor entendimento da evolução e distribuição da PKU no mundo.

Em 2001, logo após a aprovação pelo Comitê de Ética em Pesquisa da UFMG, foi iniciado em Minas Gerais um projeto de detecção de mutações no gene PAH, em sujeitos com PKU do Estado. A população mineira é a primeira a ser testada, que tem um componente indígena e negróide importante. Até então, os únicos estudos moleculares da PKU no Brasil haviam sido desenvolvidos no Rio Grande do Sul e em São Paulo (Acosta e cols., 2001, Santana da Silva e cols., 2003).

Em um primeiro momento, os pais ou responsáveis pelos pacientes fenilcetonúricos foram informados da pesquisa e assinaram um termo de consentimento livre e esclarecido, permitindo a coleta de sangue dos pacientes para posterior análise (ANEXO 1).

O material coletado de cada paciente totalizou uma amostra de 111 indivíduos, onde 78 são triados pelo programa de triagem neonatal de Minas Gerais e o restante é constituido por casos de diagnóstico tardio ou imigrantes de outros estados. O DNA foi extraído das amostras coletadas e inicialmente foram triadas, nos 78 pacientes, nove mutações no gene PAH, pela metodologia de PCR e digestão com enzimas de restrição.

Com esta estratégia foi possível determinar 100 alelos dos 156 testados, correspondendo a 64% da amostra triada pelo programa de triagem neonatal (Castro-Magalhães, 2003; Reis, 2003; Lara, 2004; Lara e cols., 2006). O artigo com os resultados obtidos neste trabalho se encontra em anexo por se tratar de produção bibliográfica referente ao trabalho de mestrado do autor e colaboradores (ANEXO 2).

Nesta primeira etapa da triagem de mutações em MG, foram triadas apenas mutações freqüentes na Europa, particularmente na Península Ibérica e algumas descritas com alta freqüência em outros estados brasileiros.

O presente trabalho é uma continuação dos estudos iniciados anteriormente com uma abordagem ampliada da PKU no Estado de Minas Gerais.

Objetivos

Objetivo geral

Investigar as bases moleculares da fenilcetonúria no Estado de Minas Gerais e aumentar os conhecimentos sobre a doença nos aspectos molecular, populacional e clínico.

Objetivos específicos

1. Averiguar, através de SSCP seguido de sequenciamento, a existência de mutações nos 13 éxons e na região promotora do locus PAH;
2. Averiguar a freqüência dos alelos PKU em MG;
3. Averiguar, por determinação dos haplótipos, a origem das mutações identificadas em MG;
4. Averiguar a existência de correlação genótipo-fenótipo para aqueles genótipos mais frequentes no Estado;
5. Estimar o coeficiente de endogamia da população de Minas Gerais e da amostra de pacientes com PKU através de marcadores microssatélites;
6. Estimar uma frequencia corrigida dos alelos causadores de PKU (q) no estado de Minas Gerais, com base no coeficiente de endogamia encontrado;
7. Revisar a literatura sobre estratégias terapêuticas para fenilcetonúria;
8. Averiguar quais seriam os pacientes com PKU que provavelmente responderiam ao tratamento com o cofator BH4, em função do genótipo.

Apresentação da tese

Este trabalho será apresentado na forma de artigos, distribuídos em capítulos. Alguns comentários e resultados, que não se adequam à publicação internacional, podem fazer parte dos capítulos para um maior detalhamento do trabalho. No final da tese será apresentada uma conclusão geral abrangendo todos os capítulos.

CAPÍTULO 1

REVISÃO DA LITERATURA

Aspectos gerais da Fenilcetonúria

O gene PAH é responsável pela codificação da enzima fenilalanina hidroxilase, que realiza a hidroxilação da fenilalanina em tirosina. Quando o gene se encontra mutado, observa-se um aumento nos níveis séricos de fenilalanina e seus metabólitos secundários, assim como excreção de ácido fenilpirúvico na urina (OMIM; Scriver e cols., 2001; Christ, 2003, Feillet, 2006). Estas alterações podem levar a uma série de manifestações fenotípicas, que variam desde uma hiperfenilalaninemia (HPA) transitória à uma doença grave e de fenótipo persistente: a fenilcetonúria clássica (TAB. 1). A fenilcetonúria (PKU) se caracteriza pelo aumento de fenilalanina sérica a níveis superiores a 600μmol/L, e o fenótipo pode variar entre PKU leve, moderada e clássica, dependendo das concentrações de fenilalanina encontradas (OMIM; Okano e cols., 1991; Eiken e cols., 1996; Scriver e cols., 2001; Chris, 2003; Feillet, 2006; Scriver 2007).

As HPAs transitórias podem ser semelhantes à PKU no período neonatal, entretanto, com o passar do tempo, estes pacientes apresentam aumento da tolerância à fenilalanina e os níveis séricos voltam ao normal (Güttler, 1980; Avigad e cols., 1991; Eiken e cols., 1996).

TABELA 1

Classificação das hiperfenilalaninemias de acordo com os níveis séricos e com a tolerância à fenilalanina

Tipos	Níveis séricos (mM)	Quantidade de fenilalanina tolerada na dieta (mg/Kg/dia)
PKU clássica (persistente)	> 1,2	< 20
PKU moderada (persistente)	0,9 - 1,2	20 -25
PKU leve (persistente)	0,6 – 0,9	25- 50
HPA não-PKU (persistente)	< 0,6	>50
HPA transitória	variável	variável

Nota: Em Minas Gerais, a classificação em PKU moderada não é usada. Todos os pacientes que apresentam níveis séricos entre 0,6 e 1,2 mM são classificados como tendo PKU leve.

Dentre as HPAs, 98% são decorrentes de defeitos no gene PAH e cerca de 2% correspondem a defeitos em outros genes, relacionados à biossíntese e regeneração da tetraidrobiopterina (BH_4), cofator essencial para a ação da PAH (Scriver e cols., 2001).

A PKU é herdada de forma autossômica recessiva, entretanto, nos últimos anos, uma série de fatores que agregam complexidade à doença vêem sendo bastante discutidos.

A heterogeneidade alélica encontrada no gene PAH é um dos fatores de grande interesse na PKU, pois o número de mutações encontradas já passa de 500, incluindo os causadores de PKU e polimorfismos não-patogênicos. A maioria dos indivíduos fenilcetonúricos apresenta duas mutações distintas (heterozigotos compostos) e o efeito das mutações na atividade da enzima é bastante diversificado. Além disso, a enzima PAH ativa é tetramérica e as possibilidades de interação entre as subunidades são ainda maiores (Guldberg e cols., 1998; Clark, 1998; Jennings e cols., 2000; Dipple e cols., 2000; Rivera e cols., 2000; Scriver e cols., 2001 e 2003; Kasnauskiene e cols., 2003; Perez e cols., 2005; Kim e cols., 2006. A combinação entre as mutações é um fator de grande importância na determinação das diferenças fenotípicas encontradas nos pacientes com PKU e até mesmo na forma e intensidade de tratamento a ser instituído.

Há bastante variação fenotípica entre indivíduos com um mesmo genótipo no *locus* PAH. Isto pode ser explicado por diferenças em outros *loci*, que estejam envolvidos em funções relacionadas, como por exemplo, em genes que participam do controle da absorção intestinal, de processos de degradação da fenilalanina, da tolerância à fenilalanina, da entrada e distribuição da fenilalanina na barreira hemato-encefálica etc. (Eisensmith e cols., 1996; Guldberg e cols., 1996; Kayaalp e cols., 1997; Scriver e cols., 1999; Jennings e cols., 2000; Scriver e cols., 2002; Weglage e cols., 2002; Pey e cols., 2003; Ghozlan e Munnich 2004).

Mesmo com todos estes aspectos influenciando a expressão fenotípica, o genótipo PAH ainda é o principal determinante do fenótipo metabólico. Determinadas mutações estão correlacionadas a uma classe específica de PKU. Desta forma, é possível antecipar para alguns pacientes, a partir do conhecimento do genótipo, as necessidades dietéticas e a resposta à interrupção da dieta (Guldberg e cols., 1998; Bénit e cols., 1999; Rivera e cols., 2000; Guttler & Guldberg, 2000; Greeves e cols., 2000; Gjetting e cols., 2001; Pey e cols., 2003; Bilginsoy e cols., 2005).

Apesar da doença ser rara, o diagnóstico da PKU é altamente relevante, pois o fenótipo pode ser modificado por terapia dietética iniciada nos primeiros dias de vida (MacDonald, 2000; Fisch, 2000; Przyrembel & Bremer, 2000; Walter e cols., 2002; Olsson e cols., 2007). Se o tratamento adequado não for instituído precocemente, os indivíduos com PKU podem apresentar atraso no desenvolvimento psicomotor, microcefalia, graus variáveis de retardo mental, irritabilidade, epilepsia, alterações na marcha e na postura. Além disto, podem apresentar hipopigmentação cutânea e eczema, e urina com odor característico. Catarata e calcificações cerebrais também podem ser encontradas na PKU clássica não-

tratada (Woo e cols., 1983; Surtees & Blau, 2000; Scriver e cols., 2001; OMIM). Em função disto, a PKU é investigada em quase todos os programas de triagem neonatal do mundo.

Uma revisão detalhada das estratégias terapêuticas que vêm aparecendo para tratamento da PKU é apresentada no capítulo 5.

A triagem neonatal para fenilcetonúria foi implantada em Minas Gerais em 1993. A triagem é realizada pelo Núcleo de Pesquisa em Apoio Diagnóstico (NUPAD) da Faculdade de Medicina da UFMG e atualmente o programa tem uma cobertura média de 98% dos nascimentos do Estado (Januário, 2002; Aguiar, 2004).

A triagem consiste em dosagem sérica de fenilalanina, que é realizada como parte do "teste do pezinho". A coleta de material é feita a partir do 5º dia de vida, e a criança deve ter ingerido quantidades adequadas de proteínas antes do exame (revisto por Scriver, 1998; Clague & Thomas, 2002).

Os neonatos, que apresentem níveis séricos de fenilalanina acima de 240 μmol/L, são encaminhados ao Ambulatório São Vicente do Hospital das Clínicas da Universidade Federal de Minas Gerais, onde são atendidos por uma equipe multidisciplinar. Outros testes são então realizados para classificação precisa da doença e para distinguir os pacientes com HPA por deficiência de PAH daqueles, cerca de 2%, que apresentam HPA por defeitos na síntese e reciclagem do cofator.

Aspectos Bioquímicos

Os níveis de fenilalanina no sangue ou em outros fluídos corporais necessitam estar no estado normal de equilíbrio dinâmico e para isso é necessário que as concentrações dos diversos componentes se mantenham dentro de intervalos específicos, apesar da dinâmica no organismo. Existem mecanismos regulatórios que mantêm o sistema em equilíbrio, permitindo pequenas e transitórias variações (Scriver e cols., 2001).

O metabolismo da fenilalanina, assim que transportada para o interior das células, pode se dar por incorporação protéica, conversão metabólica e hidroxilação pela PAH. Normalmente, cerca de 75% da fenilalanina é convertida em tirosina (hidroxilação), sendo esta a via mais importante para o estado de equilíbrio deste aminoácido (Scriver e cols., 2001).

Hidroxilação da fenilalanina

A hidroxilação da fenilalanina é uma reação catalizada pela fenilalanina hidroxilase (PAH). Esta reação acontece nos hepatócitos e depende da síntese e regeneração de um cofator, a tetra-hidrobiopterina (BH_4, FIG. 1; Thony e cols., 2000; Spaapen & Rubio-Gozalbo, 2003).

A via de biossíntese e reciclagem do BH_4 (FIG. 1) tem como precursor a guanosina trifosfato e envolve seis enzimas. Deficiência de qualquer uma destas enzimas leva a HPA (Güttler, 1980; Scriver e cols., 2001).

A PAH utiliza um átomo de oxigênio da molécula de O_2 para a reação de hidroxilação. Durante o evento catalítico, este oxigênio é transferido para o aminoácido e a tetraidrobiopterina (BH_4) é oxidada à 4α-carbinolamina. A 4α-carbinolamina por sua vez, é convertida em quinonóide diidrobiopterina (FIG. 2) e o outro átomo de oxigênio passará a fazer parte de uma molécula de água, que é então liberada (Thony e cols. 2000; Scriver e cols., 2001).

A forma quinonóide da diidrobiopterina produzida da hidroxilação da fenilalanina é reduzida de volta a tetraidrobiopterina (BH_4) pela enzima diidrobiopterina redutase (FIG. 1, acima), em uma reação que requer NADH (Teigen e cols.,1999; Scriver e cols., 2001).

Este cofator é também requerido por três outras enzimas, a tirosina hidroxilase, triptofano hidroxilase e a óxido nítrico sintase. Estas enzimas realizam a hidroxilação da tirosina e do triptofano em precursores dos neurotransmissores dopamina e serotonina, respectivamente, e síntese do óxido nítrico, usando a L-arginina como precursor. Em função disto, os pacientes com deficiência de BH_4 apresentam um quadro neurológico mais severo do que os pacientes que têm HPA por deficiência de PAH. (Teigen e cols., 1999; Thony e cols., 2000; Scriver e cols., 2001; Zorzi e cols., 2002; Spaapen & Rubio-Gozalbo, 2003).

Conversão metabólica da fenilalanina

O próximo passo, assim que a fenilalanina é convertida à tirosina, é a conversão da tirosina em p-hidroxifenilpiruvato que passa por outras reações até formar o fumarato e acetoacetato. A tirosina também participa da formação da melanina, e dos hormônios tiroidianos T3 e T4 (FIG. 3; Thony e cols., 2000).

Uma segunda via do metabolismo da fenilalanina, normalmente pouco usada, é a transaminação da fenilalanina na presença de piruvato, produzindo fenilpiruvato. A fenilalanina e o fenilpiruvato se acumulam no sangue e tecidos dos pacientes com PKU e são excretados na urina. Muito do fenilpiruvato antes de ser excretado na urina é decarboxilado em fenilacetato ou reduzido a fenilactato (FIG. 4). O fenilacetato é o metabólito que causa o odor característico na urina dos indivíduos com PKU (Scriver, 2001).

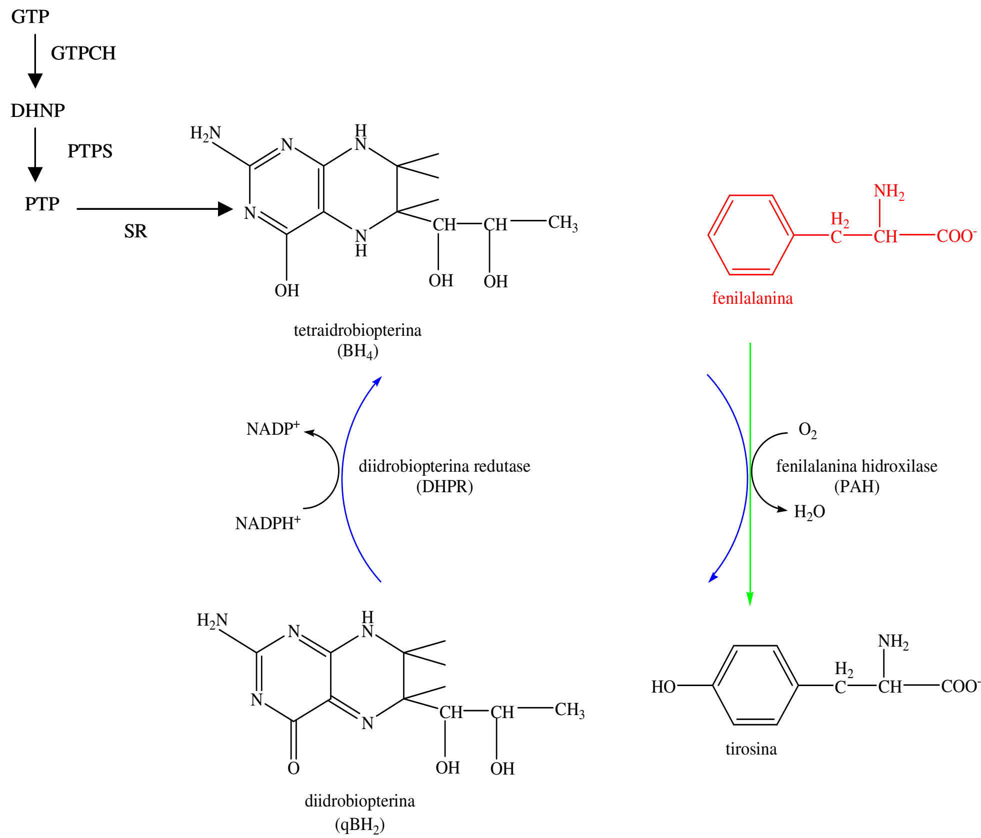

Figura 1 - Reação de hidroxilação da fenilalanina

Hidroxilação da fenilalanina em tirosina, assim como a síntese e regeneração do cofator tetraidrobiopterina (BH_4). GTP – Guanosina trifosfato; GTPCH – GTP cicloidrolase; DHNP – d-eritro-diidroneopterina trifosfato; PTPS – 6-piruvol-tetraidrobiopterina; SR – sepiapterina redutase.

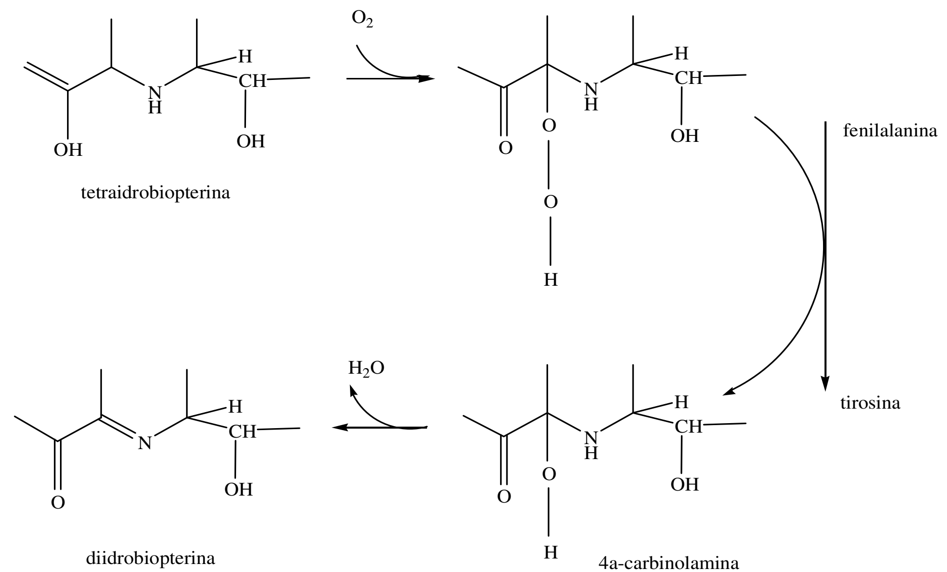

Figura 2 - Reação de regeneração do BH_4

A figura mostra de forma detalhada a reação de regeneração do cofator BH_4 destacando a utilização da molécula O_2 e liberação da molécula de H_20.

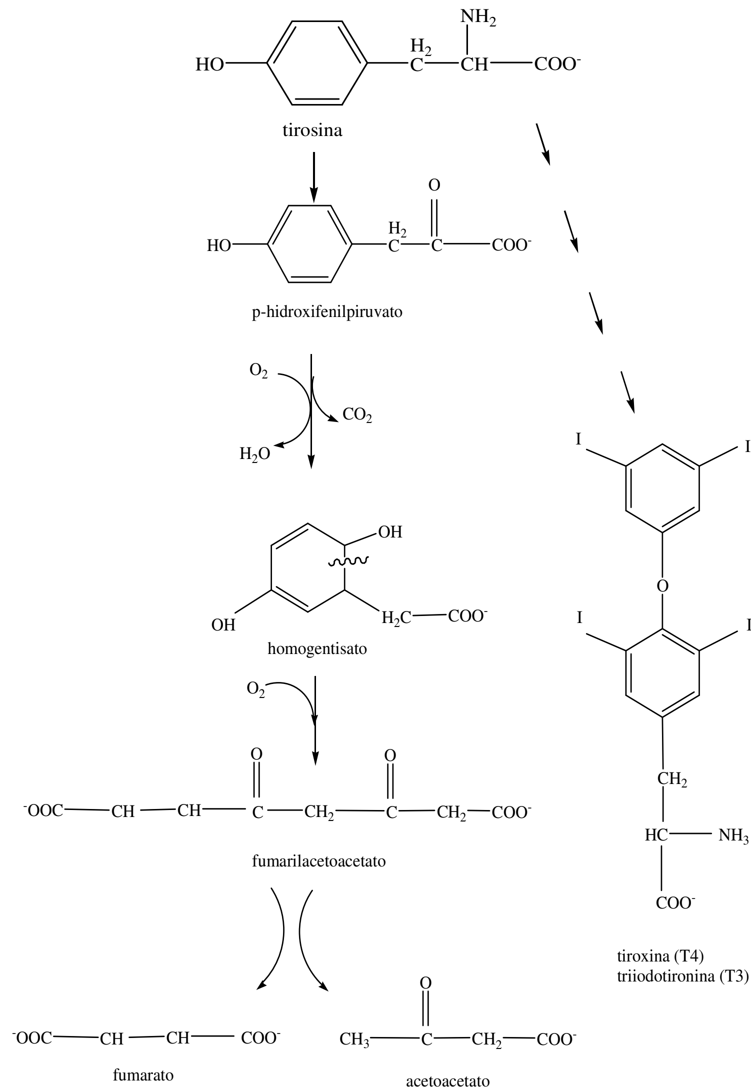

Figura 3 - Degradação da tirosina em fumarato e acetoacetato

A figura também representa a participação da tirosina na formação dos hormônios tireoideanos T3 e T4.

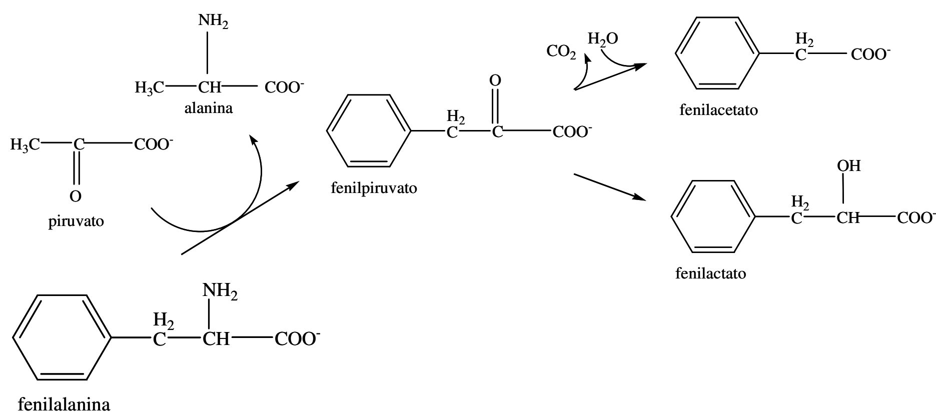

Figura 4 - Reação de transaminação da fenilalanina

Reação de transaminação da fenilalanina para produção de fenilpiruvato, seguida da sua decarboxilação em fenilacetato, e redução a fenillactato.

Aspectos Moleculares

O gene e a enzima

A enzima fenilalanina hidroxilase humana é codificada pelo gene PAH localizado no cromossomo 12, região 12q22-q24.1. O gene é composto por 13 éxons, distribuídos em um segmento genômico de ~ 90 kb (Lidksy e cols., 1984). O RNA mensageiro maduro possui aproximadamente 2,4 kb, a enzima é formada por 452 aminoácidos e se encontra organizada em um domínio regulatório N-terminal, um domínio catalítico e um domínio de oligomerização C-terminal (Teigen e cols., 1999).

A enzima é ativa na forma tetramérica e sua estrutura tridimensional pode ser visualizada no banco de dados do *PAH Mutation Analysis Consortium* (http://www.pahdb.mcgill.ca/; Scriver e cols., 2000).

As mutações

A freqüência das mutações encontradas no gene PAH varia consideravelmente entre populações (OMIM; Scriver e cols., 2003). A freqüência da PKU é de 1:10.000 em populações caucasianas e em Minas Gerais a freqüência encontrada é de 1:20.000 (Scriver e cols., 2001; Serjeant, 2000).

A maioria das mutações no gene PAH resulta na deficiência da atividade enzimática e causam algum tipo de hiperfenilalaninemias. Até o momento mais de 500 mutações já foram identificadas no gene e a freqüência relativa das mutações, conforme o tipo, é (http://www.pahdb.mcgill.ca/):

- 61,58 % - mutações de sentido trocado;
- 13,37 % - deleções
- 11,49 % - mutações em sítios de splicing
- 6,03 % - mutações silenciosas
- 4,90 % - são mutações sem sentido;
- 1,88 % - inserções
- 0,56% - Sil/Splice
- 0,19% - não conhecidas

Os haplótipos

Com o estudo do gene PAH, vários polimorfismos foram identificados e estes foram amplamente utilizados para estudos evolutivos (Daiger e cols., 1989). O sistema de haplotipagem desenvolvido permite a identificação da origem e distribuição das mutações que causam a doença e é baseado em:

- sete RFLPs (polimorfismos do comprimento dos fragmentos de restrição), bialélicos, reconhecidos pelos nomes das enzimas de restrição que os identificam (*Bgl*II, *Pvu*IIa, *Pvu*IIb, *Eco*RI, *Msp*1, *Xmn*1 e *Eco*RV);
- uma VNTR (repetições em tandem de número variável) com um monômero de 30 pb, com mais de 10 alelos diferentes;
- uma STR (repetição curta em tandem) do tetranucleotídeo TCTAn, com 9 alelos.

Este sistema permite o reconhecimento de pelo menos 87 haplótipos e as frequências destes haplótipos variam entre as populações (Scriver e cols., 2000).

PKU materna

Outra manifestação importante é a condição chamada “PKU materna”. É caracterizada por alterações do desenvolvimento fetal nos filhos de mulheres afetadas por PKU, sem restrição dietética durante a gestação. A sintomatologia inclui retardo mental, microcefalia, malformações congênitas múltiplas, e até morte intra-uterina. A freqüência de malformações congênitas se correlaciona com os níveis de fenilalanina no sangue materno. Estas alterações ocorrem independentemente do genótipo do feto. O controle rigoroso da dieta, desde antes da concepção, permite o desenvolvimento normal do feto (Woolf e cols., 1975). Entretanto, muitas vezes, quando a mulher com PKU percebe que está grávida, o período crítico da embriogênese já passou (Antshel & Waisbren, 2003; Koch e cols., 2003; e Matalon e cols., 2003; Gambol 2007).

Estudos têm mostrado que mulheres com HPA não-PKU que possuem concentrações plasmáticas de fenilalanina abaixo de 400µmol/L quando não tratadas podem ter filhos normais (Antshel & Waisbren, 2003).

CAPÍTULO 2

ANÁLISES MOLECULARES

PKU in Southeast Brazil: Genetic and population studies

Este artigo foi submetido à revista Human Mutation e está em processo de análise.

PKU in Southeast Brazil: Genetic and population studies

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Abstract

This work was undertaken to ascertain the molecular basis of phenylketonuria in Minas Gerais State, Brazil. This is a trihybrid population formed by miscegenation from Europeans, Africans and the native population. All 13 exons of the PAH gene from 78 PKU patients were analyzed, including splicing sites and promoter region. We identified 30 different mutations and 98% of the PAH alleles were established. A new mutation was identified (Q267X) as well. The most common mutations found were V388M (21.15%), R261Q (16%), IVS10-11G>A (15.3%), I65T (5.77%), IVS2+5G>C (5.77%), R252W (5.13%), IVS2+5G>A (4.48%), P281L (3.84%) and L348V (3.2%). These nine mutations correspond to 80% of the PKU alleles in the state. Haplotypes were determined to characterize the origin of the PAH alleles. The majority of the mutations, with respective haplotypes, found here are frequent in the Iberian Peninsula. However, we found some mutations that are rare in Europe and four previously unreported mutation-haplotype associations. I65T and Q267X were found in association with haplotype 38 and these associations were found only in black or brown PKU patients, suggesting their African origin.

Key words: mutations, PAH gene, phenylketonuria, Brazil

INTRODUCTION

Phenylketonuria (PKU) is an autosomal recessive disorder caused by a deficiency of the enzyme phenylalanine hydroxylase (PAH; OMIM 261600) and is included in the hyperphenylalaninaemia` group (HPA). Approximately 500 mutations have already been described in this gene leading to a defective PAH enzyme and to an accumulation of phenylalanine in plasma and tissues of the patients (Scriver et al., 2001; *PAHdb;*

http://www.pahdb.mcgill.ca/). Restriction of phenylalanine intake is required to ensure normal development in PKU patients, and for this reason this disorder is targeted by neonatal screening tests (Güttler, 1980; Scriver et al., 2001). Treatment must be neonatally established in order to prevent neurological damage. Other clinical manifestations are “mousy” odor, light skin pigmentation, peculiar sitting posture, eczema, epilepsy, etc (Scriver et al., 2001; Giovannini et al., 2007).

PAH deficiency is highly heterogeneous showing an enormous phenotypic variability. This variability is mainly due to allelic heterogeneity at the PAH locus. However, in the last years, other factors have been reported contributing to final phenotype determination (Kayaalp et al., 1997; Benit et al., 1999; Scriver and Waters, 1999).

PKU incidence has been estimated to be about 1:10,000 in Caucasians, and it varies among different populations (Scriver et al., 2001). There is no neonatal screening program for PKU in Sub-Saharan African countries and PKU mutations in Africa have never been systematically characterized. There are only few studies including African descendents living outside Africa, suggesting that the PKU prevalence may be much lower on the African continent than in Europe. An incidence of 1:50,000 has been estimated in African Americans (Hofman et al., 1991; Gjetting et al., 2001; Hardelid et al., 2007). In Minas Gerais State, the PKU incidence has been estimated to be about 1:20,000 (Aguiar, 2004).

Minas Gerais is the second most populous state in Brazil with approximately 21 million inhabitants (IBGE - Instituto Brasileiro de Geografia Estatística - http://www.ibge.gov.br/home/). This is a trihybrid population composed of Europeans (mainly from Portugal), Africans and Amerindians. Besides, this population differs from all others already tested due to its high proportion of African descendents, estimated to be approximately 50% (IBGE 2000). There are no reports of PKU mutations in South America aborigine populations.

For these reasons, the Minas Gerais population turns out to be an interesting target for mutation as well as population genetic studies in PKU patients. We screened this population with the aim of ascertaining PKU mutations in both African and South American aborigine descendents. The neonatal screening for PKU started in 1993 and currently has a coverage of 98% of births in the state (Aguiar, 2004). We initiated the mutation screening study by testing nine of the most frequent mutations in Portugal, or those detected with high frequencies in previous studies in Brazil (Acosta et al., 2001; Santana da Silva et al., 2003). Our first study led to the identification of 64% of the PKU alleles in the state (Santos et al., 2006). Here, we describe the results of the mutation screening and haplotype characterization over the whole PAH gene.

MATERIALS AND METHODS

Patients: Seventy-eight not related PKU patients ascertained through the Minas Gerais State neonatal screening program were analyzed in this study. Among them, 16 patients were classified by health workers as black or brown (intermediate), the rest of them were classified as white. No patient was classified as South American aborigine or was known to belong to any such group. Prior to the beginning of the investigation, the project was approved by the Ethics on Research Committee of the Universidade Federal de Minas Gerais, and parents signed an informed consent form.

Blood collection and DNA extraction: Blood samples (5 ml) were collected from the patients and their parents. Genomic DNA was isolated from peripheral blood samples according to standard procedures (Miller et al., 1988).

Mutation detection: Mutation analysis was performed by polymerase chain reaction (PCR) amplification of the 13 exons and the promoter region of the PAH gene using primers and conditions described elsewhere (Santana da Silva et al., 2003). PCR products were denatured at 99ºC for 5 min with running buffer (95% formamide, 20mM EDTA, 0.05%

bromophenol blue and 0.05% xylene cyanol). For SSCP analysis, samples were electrophoresed on non-denaturing polyacrylamide gels, ranging from 8 to 12% depending on the exon in the test. Electrophoresis was performed at 150 to 300 V at 4ºC, and thereafter gels were silver-stained. In those cases where abnormal SSCP migration patterns were observed, PCR products were purified with polyethylene glycol (PEG 8000) and sequenced using DYEnamic ET Dye Terminator Cycle Sequencing Kit in a MegaBACE DNA Analysis System (Amersham Biosciences) or Big Dye Terminator v3.1 Cycle Sequencing Kit in an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems). Phred-Phrap-Consed and PolyPhred Softwares were used to align the sequences for mutation detection (Nickerson et al., 1997; Ewing et al., 1998; Gordon et al., 1998). All mutations detected by PolyPhred were confirmed by visual inspection of the electropherograms.

Analysis of the RFLP/VNTR haplotypes: RFLP haplotype analysis for five sites was performed using specific restriction endonucleases (*Pvu*II(a), *Pvu*II(b), *Msp*I, *Bgl*II and *Xmn*I). VNTR at the 3´ end of the PAH gene was also analyzed (Dworniczak et al., 1991a; Dworniczak et al., 1991b; Wedemeyer et al., 1991, Goltsov et al., 1992a; Goltsov et al., 1992b; Eisensmith and Woo, 1992).

RESULTS

A total of 78 PKU patients from Minas Gerais were analyzed. Thirty different mutations were detected. Twenty-six of them are disease-associated mutations (one of them being a new mutation, Q267X), and the remaining four are known polymorphisms. The results are summarized in Tables 1 and 2. V388M mutation is the most frequent in Minas Gerais (21.15%), followed by R261Q (16%), IVS10-11G>A (15.3%), I65T (5.7%) and IVS2+5G>C (5.7%).

Using PCR-RFLP and SSCP followed by sequencing, it was possible to detect 98% of the mutant alleles in the 78 PKU patients from Minas Gerais. We found 27 homozygous and 50 compound heterozygous patients. In one individual, no mutation was identified, and in another patient, only one allele was found, in spite of the sequencing of the whole gene and its promoter. The highest number of mutations was found in exon 7.

In the SSCP analysis, 69 abnormal migration patterns were found, and every one of them corresponded to a mutation or a polymorphism identified with sequencing. Therefore, no false positive SSCP results were observed in the present study. However, five mutations (IVS10-11G>A, D84Y, R158Q, Q267X and F410C) did not show abnormal SSCP migration, leading to false negative results.

Haplotypes were determined in order to characterize the origin of PAH alleles. A total of 89.7% of the haplotypes were identified and 11 different haplotypes were found, one of them in the patient for whom no mutation was identified. The haplotype-mutation associations found are described in Table 1. The most common was haplotype 1 (52.5%) which was also the one associated with the largest number of different mutations (Table 3).

DISCUSSION

The Brazilian population was formed by miscegenation from three main groups: Europeans, manly from Portugal, arriving from 1500 on, Africans who were brought as slaves, and Amerindians, the native population. Peopling differed from region to region in the country. Minas Gerais State settlement was really effective only from 1693 on, due to a gold rush that attracted people from Portugal as well as from other Brazilian regions, who at that time represented the results of miscegenation that occurred over the preceding 200 years. During all the gold cycle, a large number of Africans were brought to this region as slaves. In the middle of the 18th century, gold mining was substituted by agriculture and cattle raising, and

the influx of African slaves diminished gradually (IBGE 2000; Alves-Silva et al., 2000; Carvalho-Silva, 2001; Salzano and Bortolini 2002).

Considering the high degree of miscegenation in the Minas Gerais population, differences in the type and frequency of the PKU alleles would be expected, both in comparison to samples from other countries as well as from other regions in Brazil. It is important to emphasize that racial classification in Brazil is not necessarily related to ancestry or origin but, more commonly, to appearance or phenotype as skin color, hair texture, and nose and lip width (Telles, 2002). The color classification system used in Brazilian censuses is black, white, brown (intermediate) or yellow. However, ancestry-informative markers (AIM) were already used to assign an African ancestry index in Brazilian populations, showing that physical evaluation is a poor predictor of genomic African ancestry (Parra et al., 2003). Classifying a person on the basis on his/her appearance as black or white, does not mean that this person has African or European ancestry, only. This is particularly true in Minas Gerais State, where genetic admixture is highly frequent.

In this study, 98% of the Minas Gerais PKU alleles were established, and a total of 26 pathogenic mutations were identified. Among them, nine mutations (V388M, R261Q, IVS10-11G>A, I65T, IVS2+5G>C, R252W, IVS2+5G>A, P281L and L348V) correspond to 80% of the PKU alleles in the state (Table 1). The three most common mutations found in our study (V388M, R261Q and IVS10-11G>A) are also the most frequent PKU alleles in Portugal. Mutation frequencies were different among the two populations, probably reflecting genetic drift. Haplotypes associated with these mutations were 1.7, 1.8 and 6.7, respectively, the same found in Europe (Zschocke, 2003). R261Q mutation was also found associated with haplotype 2.3 in two patients. This association has also already been described for other European populations (*PAHdb*).

Four mutations were detected at similar frequencies in the sample studied: R252W (5.13%), I65T (5.77%, see discussion below), IVS2+5G>C (5.77%) and IVS2+5G>A (4.48%). IVS2+5G>C has already been reported in some other populations including those in Brazil (Acosta et al., 2001; *PAHdb*), but has not been reported in Portuguese or Spanish populations (Perez et al., 1997; Rivera et al., 1998). In our sample, it is associated with haplotype 5.9, the same association found in São Paulo and Europe (*PAHdb*). IVS2+5G>A mutation, however, has not been found in other Brazilian populations and seems to be rare in Europe, where it has been reported only in Germany and Poland. Haplotypes associated with IVS2+5G>A in these populations were not described. In our sample, it was found associated with haplotype 1.8 (Guldberg et al., 1996; *PAHdb*).

P281L, and L348V frequencies ranged from 3 to 4%. They were associated with the same haplotypes reported in Portugal (Rivera et al., 1998). The other 17 mutations were found at frequencies lower than 2%.

One undescribed mutation was found in the present sample (Q267X). This mutation results in a stop codon in exon 7, which codes for the enzyme catalytic site. This mutation was found in heterozygosis with IVS10-11G>A, and the patient was classified as classical PKU according to phenylalanine levels. Considering that IVS10-11G>A has been described as a null mutation, the phenotype observed in this patient suggested that Q267X also produces complete loss of function. Q267X mutation was found associated with haplotype 38.12. Another mutation, leading to PKU, in the same nucleotide (Q267E) was already detected in a previous study (Song et al., 2005).

PKU mutations, as well as their respective haplotypes, suggest a predominantly European contribution to PKU in Brazil. Haplotype 1, for instance, is the most common one in Europe and particularly in Portugal (64%) and is the most frequent in Minas Gerais as well (52.5%). Due to its high frequency and the high number of mutations associated with it, it has

been proposed that haplotype 1 is the most ancestral one at the PAH locus (Daiger et al., 1989).

Several new mutation-haplotype associations have been found in the present sample, e. g., I65T associated with haplotype 38.12, D84Y with haplotypes 5.9 and 11.9, IVS7+1G>A with haplotype 11.9, and C217R with haplotype 44.3. D84Y mutation has been described in some European populations but associated with the haplotype 4. This haplotype differs from haplotype 5 and 11 by five and four sites, respectively, and at least two recombination events would be necessary to transform haplotype 4 into either of them. Therefore, the hypothesis of a recombinational origin for these two mutation-haplotype associations is improbable, and mutation recurrence should be considered. The same reasoning applies to IVS7+1G>A mutation, which has been described in association with haplotypes 1 and 4, both differing from haplotype 11 by four sites. Additional evidence for mutation recurrence in this case is that IVS7+1G>A occurs at a known mutational hot spot (*PAHdb*).

Haplotype 38 is rare in Europe and has been found only in some of the few studies published to date that include North African descendents. For instance, it was identified in a study conducted in France, where 10% of the sample came from Algeria, Tunisia and Morocco (Rey et al., 1988). In another study, it has been found in strong association with E280K mutation, in a Mediterranean sample including some North African families (Lyonnet et al., 1989). In the present study, haplotype 38 has been found associated with two mutations: I65T, also reported in Afro-Caribbean patients (*PAHdb*), and Q267X. Both mutations (I65T and Q267X) associated with haplotype 38 occurred only in patients classified as black or brown, suggesting an African origin for these alleles.

G352>Vfs mutation has been found in three patients in our sample associated with haplotype 2.3. It has been previously described in England, France and Italy, but also in North Africa and in Lebanese (*PAHdb*). This mutation has been found in São Paulo associated with

the same haplotype (Acosta et al., 2001). Other mutations already described in African descendents (IVS5-54A>G, P122Q, L255S and I318T) were not found in the present sample (Gjetting et al., 2001; *PAHdb*).

Another interesting point is related to C217R and haplotype 44.3. This mutation was previously reported only in the Wales population, but the haplotype was not reported (*PAHdb*). Haplotype 44.3 has been described in Polynesians. Thereafter, it was reported in a Chinese sample, in association with R480W mutation (Hertzberg et al., 1989; *PAHdb*). In recent years, most molecular genetic studies of Amerindians have described evidence of their Asian origins and it could be hypothesized that C217R mutation in association with haplotype 44 may be evidence of an indigenous contribution to PKU phenotype. However, there could be some other explanations for this association: a) haplotype 44 can be produced by recombinations involving haplotype 4 (Hertzberg et al., 1989); b) C217R and haplotype 44.3 association may be Asian in origin, although not yet reported, and may have been introduced in the Minas Gerais population by migrations over the last two centuries. Asian contribution to Minas Gerais population, although small, should not be overlooked.

In conclusion, the analysis of PAH mutations and haplotypes in the PKU patients from the Minas Gerais population suggests that most of PKU alleles can be traced to the Iberian Peninsula, reflecting our peopling history. Recently, it has been proposed that PKU would have appeared only in some particular populations and during or after the Out-of-Africa expansion of modern humans (Scriver, 2007). This hypothesis is founded on the low frequency of PKU in African descendents and lack of information about the disease in Sub-Saharan countries. However, in studying a highly miscegenated population, we found several PKU mutation/PAH haplotype associations that have not been reported in Europe so far. Besides, some of them have been described in samples including North African patients. Moreover, two of these associations (I65T and haplotype 38.12; Q267X and haplotype 38.12)

were identified in black and brown patients in the present sample. Based on these lines of evidence, we propose that these two mutations may be African in origin.

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REFERENCES

Acosta A, Silva W, Jr., Carvalho T, Gomes M, Zago M. 2001. Mutations of the phenylalanine hydroxylase (PAH) gene in Brazilian patients with phenylketonuria. Hum Mutat 17: 122-30.

Aguiar MJ. 2004. Genetic services and research in the state of Minas Gerais, Brazil. Community Genet 7: 117-20.

Alves-Silva J, da Silva Santos M, Guimaraes PE, Ferreira AC, Bandelt HJ, Pena SD, Prado VF. 2000. The ancestry of Brazilian mtDNA lineages. Am J Hum Genet 67: 444-61.

Benit P, Rey F, Blandin-Savoja F, Munnich A, Abadie V, Rey J. 1999. The mutant genotype is the main determinant of the metabolic phenotype in phenylalanine hydroxylase deficiency. Mol Genet Metab 68: 43-7.

Carvalho-Silva DR, Santos FR, Rocha J, Pena SD. 2001. The phylogeography of Brazilian Y-chromosome lineages. Am J Hum Genet 68: 281-6.

Daiger SP, Chakraborty R, Reed L, Fekete G, Schuler D, Berenssi G, Nasz I, Brdicka R, Kamaryt J, Pijackova A, Moore S, Sullivan S, Woo SLC. 1989. Polymorphic DNA haplotypes at the phenylalanine hydroxylase (PAH) locus in European families with phenylketonuria (PKU). Am J Hum Genet 45: 310-18.

Dworniczak B, Wedemeyer N, Eigel A, Horst J. 1991. PCR detection of the PvuII (Ea) RFLP at the human phenylalanine hydroxylase (PAH) locus. Nucleic Acids Res 19: 1958.

Dworniczak B, Wedemeyer N, Horst J. 1991. PCR detection of the Bg1II RFLP at the human phenylalanine hydroxylase (PAH) locus. Nucleic Acids Res 19: 1958.

Eisensmith RC, Woo SL. 1992. Molecular basis of phenylketonuria and related hyperphenylalaninemias: mutations and polymorphisms in the human phenylalanine hydroxylase gene. Hum Mutat 1: 13-23.

Eisensmith RC, Woo SL. 1994. Population genetics of phenylketonuria. Acta Paediatr Suppl 407: 19-26.

Ewing B, Hillier L, Wendl MC, Green P. 1998. Base-calling of automated sequencer traces using phred. I. Accuracy assessment. Genome Res 8: 175-85.

Giovannini M, Verduci E, Salvatici E, Fiori L, Riva E. 2007. Phenylketonuria: dietary and therapeutic challenges. J Inherit Metab Dis 30: 145-52.

Gjetting T, Romstad A, Haavik J, Knappskog PM, Acosta AX, Silva WA, Jr., Zago MA, Guldberg P, Guttler F. 2001. A phenylalanine hydroxylase amino acid polymorphism with implications for molecular diagnostics. Mol Genet Metab 73: 280-4.

Goltsov AA, Eisensmith RC, Konecki DS, Lichter-Konecki U, Woo SL. 1992a. Associations between mutations and a VNTR in the human phenylalanine hydroxylase gene. Am J Hum Genet 51: 627-36.

Goltsov AA, Eisensmith RC, Woo SL. 1992b. Detection of the XmnI RFLP at the human PAH locus by PCR. Nucleic Acids Res 20: 927.

Gordon D, Abajian C, Green P. 1998. Consed: a graphical tool for sequence finishing. Genome Res 8: 195-202.

Guldberg P, Mallmann R, Henriksen KF, Guttler F. 1996. Phenylalanine hydroxylase deficiency in a population in Germany: mutational profile and nine novel mutations. Hum Mutat 8: 276-9.

Guttler F. 1980. Hyperphenylalaninemia: diagnosis and classification of the various types of phenylalanine hydroxylase deficiency in childhood. Acta Paediatr Scand Suppl 280: 1-80.

Hardelid P, Cortina-Borja M, Munro A, Jones H, Cleary M, Champion MP, Foo Y, Scriver CR and Dezateux C. 2007. The Birth Prevalence of PKU in Populations of European, South Asian and Sub-Saharan African Ancestry Living in South East England. Ann Hum Genet 71:1–7.

Hertzberg M, Jahromi K, Ferguson V, Dahl HH, Mercer J, Mickleson KN, Trent RJ. 1989. Phenylalanine hydroxylase gene haplotypes in Polynesians: evolutionary origins and absence of alleles associated with severe phenylketonuria. Am J Hum Genet 44: 382-7.

Hofman KJ, Steel G, Kazazian HH, Valle D. 1991. Phenylketonuria in U.S. blacks: molecular analysis of the phenylalanine hydroxylase gene. Am J Hum Genet 48: 791-8.

IBGE – Instituto Brasileiro de Geografia Estatística (2000) Brasil: 500 Anos de Povoamento. Rio de Janeiro: IBGE.

Kayaalp E, Treacy E, Waters PJ, Byck S, Nowacki P, Scriver CR. 1997. Human phenylalanine hydroxylase mutations and hyperphenylalaninemia phenotypes: a metanalysis of genotype-phenotype correlations. Am J Hum Genet 61: 1309-17.

Lyonnet S, Caillaud C, Rey F, Berthelon M, Frezal J, Rey J, Munnich A. 1989. Molecular genetics of phenylketonuria in Mediterranean countries: a mutation associated with partial phenylalanine hydroxylase deficiency. Am J Hum Genet 44: 511-7.

Miller SA, Dykes DD, Polesky HF. 1988. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 16: 1215.

Nickerson DA, Tobe VO, Taylor SL. 1997. PolyPhred: automating the detection and genotyping of single nucleotide substitutions using fluorescence-based resequencing. Nucleic Acids Res 25: 2745-51.

Parra FC, Amado RC, Lambertucci JR, Rocha J, Antunes CM, Pena SD. 2003. Color and genomic ancestry in Brazilians. Proc Natl Acad Sci U S A 100: 177-82.

Perez B, Desviat LR, Ugarte M. 1997. Analysis of the phenylalanine hydroxylase gene in the Spanish population: mutation profile and association with intragenic polymorphic markers. Am J Hum Genet 60: 95-102.

Rey F, Berthelon M, Caillaud C, Lyonnet S, Abadie V, Blandin-Savoja F, Feingold J, Saudubray JM, Frezal J, Munnich A, et al. 1988. Clinical and molecular heterogeneity of phenylalanine hydroxylase deficiencies in France. Am J Hum Genet 43: 914-21.

Rivera I, Leandro P, Lichter-Konecki U, Tavares de Almeida I, Lechner MC. 1998. Population genetics of hyperphenylalaninaemia resulting from phenylalanine hydroxylase deficiency in Portugal. J Med Genet 35: 301-4.

Santana da Silva LC, Carvalho TS, da Silva FB, Morari L, Fachel AA, Pires R, Refosco LF, Desnick RJ, Giugliani R, Saraiva Pereira ML. 2003. Molecular characterization of phenylketonuria in South Brazil. Mol Genet Metab 79: 17-24.

Salzano FM and Bortolini MC. 2002. The evolution and genetics of Latin American populations. Cambridge: Cambridge University Press.

Santos LL, Magalhaes Mde C, Reis Ade O, Starling AL, Januario JN, Fonseca CG, Aguiar MJ, Carvalho MR. 2006. Frequencies of phenylalanine hydroxylase mutations I65T, R252W, R261Q, R261X, IVS10nt11, V388M, R408W, Y414C, and IVS12nt1 in Minas Gerais, Brazil. Genet Mol Res 5: 16-23.

Scriver CR, Kaufman S. 2001. The hyperphenylalaninemias. In: Scriver CR, Beaudet AL, Sly WS, Valle D, editors. The Metabolic and Molecular Bases of Inherited Disease 8th ed. New York: McGraw-Hill. p 1667–1724.

Scriver CR, Waters PJ. 1999. Monogenic traits are not simple: lessons from phenylketonuria. Trends Genet 15: 267-72.

Scriver CR. 2007. The PAH gene, phenylketonuria, and a paradigm shift. Hum Mutat. 28: 31-45.

Song F, Qu YJ, Zhang T, Jin YW, Wang H, Zheng XY. 2005. Phenylketonuria mutations in Northern China. Mol Genet Metab 86: 107-18.

Telles EE. 2002. Racial ambiguity among the Brazilian population. Ethnic and Racial Studies 25: 415–441.

Wedemeyer N, Dworniczak B, Horst J. 1991. PCR detection of the MspI (Aa) RFLP at the human phenylalanine hydroxylase (PAH) locus. Nucleic Acids Res 19: 1959.

Zschocke J. 2003. Phenylketonuria mutations in Europe. Hum Mutat 21: 345-56.

Table 1. PKU mutation frequencies and associated haplotypes in Minas Gerais patients

Location	Mutation	n	%	Haplotypes	N
Exon 2	L48S	1	0.64	4.3	1
Exon 2	F55>Lfs	1	0.64	1.8	1
Intron 2	IVS2+5G>C	9	5.77	5.9	9
Intron 2	IVS2+5G>A	7	4.48	1.8; ND	6; 1
Exon 3	I65T	9	5.77	9.8; 38.12	7; 2
Exon 3	D84Y	2	1.28	5.9;11.9	1;1
Exon 5	R158W	2	1.28	4.3	2
Exon 5	R158Q	1	0.64	4.3	1
Exon 6	R176X	1	0.64	1.8	1
Exon 6	C217R	1	0.64	44.3	1
Exon 7	R243Q	1	0.64	ND	1
Exon 7	L249F	3	1.92	1.7	3
Exon 7	R261X	3	1.92	1.8; ND	2; 1
Exon 7	R270K	3	1.92	1.7; ND	1; 2
Exon 7	P281L	6	3.84	1.8; ND	4; 2
Exon 7	R252W	8	5.13	1.8	8
Exon 7	R261Q	25	16	1.8; 2.3*; ND	21; 2; 2
Exon 7	Q267X	1	0.64	38.12	
Intron 7	IVS7+1G>A	1	0.64	11.9	1
Exon 10	L348V	5	3.2	9.8; ND	4; 1
Exon 10	G352>Vfs	3	1.92	2.3*	3
Exon 11	V388M	33	21.15	1.7; 1.8	32; 1
Intron 11	IVS10-11G>A	24	15.3	6.7; ND	20; 4
Exon 12	R408W	1	0.64	2.3*	1
Exon 12	F410C	1	0.64	ND	1
Intron 12	IVS12+1G>A	1	0.64	3.8	1
ND		3	1.92	1.8; 7.8	1; 2
		156	99.84		

*: haplotypes 2.3 and 24.3 are distinguishable only by testing EcoRI and EcoRV sites, which were not tested in the present study. Haplotype 2.3 was assumed, because its association with R261Q, R408W and G352>Vfs mutations is well established in the literature (www.pahdb.mcgill.ca/). ND: not determined.

Table 2. Polymorphisms detected in the present study

Location	Polymorphism
Intron 2	IVS2+19T>C
Exon 6	Q232Q
Exon 7	V245V
Exon 11	L385L

Table 3. Haplotypes associated with different mutations

Haplotype	Mutation
1.8	F55>Lfs
	IVS2+5G>A
	R261X
	P281L
	R261Q
	R252W
	R176X
	V388M
1.7	L249F
	R270K
	V388M
2.3 or 24.3	R261Q
	G352>Vfs
	R408W
4.3	L48S
	R158W
	R158Q
5.9	IVS2+5G>C
	D84Y
9.8	L348V
	I65T
11.9	D84Y
	IVS7+1G>A
38.12	I65T
	Q267X

Dados complementares

As informações que se seguem complementam o artigo apresentado.

Amostra

A amostra analisada nesta etapa do trabalho constava de 39 indivíduos com PKU, nascidos em MG e averiguados pelo programa de triagem neonatal. Estes indivíduos já haviam sido testados por PCR-restrição e em cada caso, nenhum ou apenas um dos alelos PKU havia sido identificado.

Alterações de migração detectadas pela técnica de SSCP

Na TAB. 1 são mostrados os resultados da análise por SSCP. Nenhuma alteração foi detectada nos éxons 1, 3, 4, 8, 9 e 12. Na FIG. 1 podemos visualizar a eletroforese no SSCP, com amostras que apresentaram migração alterada.

TABELA 1

Alterações de migração dos éxons do gene PAH encontradas na análise por SSCP

Éxons	Alterações de migração encontradas ao SSCP
1	0
2	22
3	0
4	0
5	1
6	6
7	21
8	0
9	0
10	8
11	7
12	0
13	4
Total	69

Figura 1 - Gel de SSCP do éxon 7 do gene PAH (poliacrilamida 8%, corado com nitrato de prata). As setas apontam para as amostras que tiveram uma mobilidade eletroforética diferente das demais, indicando a presença da mutação ou polimorfismo (canaletas 4,5,10,11,12,13 e 14). Canaleta 7: padrão de peso molecular de 50 pares de bases. Canaleta 18: controle negativo.

Genótipos encontrados

Todos os genótipos dos pacientes assim como os haplótipos associados estão descritos na TAB. 2.

TABELA 2

Genótipos determinados para os 78 pacientes com PKU de Minas Gerais

ID	Alelo 1	haplótipo	Alelo 2	haplótipo
62	P281L	ND	R261X	ND
63	R261Q	1.8	R261Q	1.8
64	V388M	1.7	R261Q	1.8
65	R261Q	2.3	G352>Vfs	2.3
66	R261Q	1.8	L348V	9.8
68	V388M	1.7	I65T	9.8
69	R261Q	ND	R261Q	ND
70	V388M	1.7	V388M	1.7
71	V388M	1.7	V388M	1.7
73	R270K	ND	R270K	ND
74	V388M	1.7	R252W	1.8
77	IVS10-11G>A	6.7	IVS12+1G>A	3.8
78	IVS10-11G>A	6.7	IVS10-11G>A	6.7
79	R261Q	1.8	IVS10-11G>A	6.7
80	V388M	ND	IVS10-11G>A	ND
81	V388M	1.7	I65T	9.8
82	V388M	1.7	I65T	9.8
84	IVS2+5G>A	1.8	IVS2+5G>A	1.8
85	R261Q	1.8	R261Q	1.8
86	R261X	1.8	R261X	1.8
88	R408W	2.3	R261Q	1.8
90	R252W	1.8	P281L	1.8
92	V388M	1.7	G352>Vfs	2.3
93	IVS10-11G>A	6.7	L348V	9.8
94	IVS10-11G>A	6.7	L249F	1.7
95	V388M	1.7	V388M	1.7
97	R261Q	1.8	L348V	9.8
98	V388M	1.7	R252W	1.8
99	V388M	1.7	G352>Vfs	2.3
100	R252W	1.8	R252W	1.8
101	V388M	1.7	V388M	1.7
103	IVS10-11G>A	ND	R243Q	ND
105	I65T	9.8	I65T	9.8
106	IVS10-11G>A	6.7	R261Q	1.8
109	V388M	1.7	V388M	1.7

110	IVS2+5G>A	1.8	IVS2+5G>A	1.8
111	IVS2+5G>A	1.8	IVS2+5G>A	1.8
112	V388M	1.8	R261Q	1.8
114	IVS10-11G>A	6.7	IVS10-11G>A	6.7
115	IVS10-11G>A	6.7	V388M	1.7
116	R261Q	1.8	R261Q	1.8
118	I65T	38.12	I65T	38.12
120	R261Q	1.8	I65T	9.8
124	V388M	1.7	I65T	9.8
126	P281L	1.8	P281L	1.8
130	L348V	9.8	IVS7+1G>A	11.9
131	IVS2+5G>C	5.9	C217R	44.3
132	P281L	1.7	D84Y	11.9
133	R176X	1.8	R158Q	4.3
134	IVS10-11G>A	6.7	IVS2+5G>C	5.9
136	R261Q	1.8	R252W	1.8
139	R261Q	1.8	R252W	1.8
141	IVS2+5G>C	5.9	D84Y	5.9
142	IVS2+5G>C	5.9	IVS2+5G>C	5.9
145	P281L	1.8	L249F	1.7
146	IVS10-11G>A	6.7	IVS2+5G>C	5.9
147	R261Q	1.8	L48S	4.3
148	R158W	4.3	R158W	4.3
149	IVS10-11G>A	6.7		1.8
151	V388M	1.7	R261Q	1.8
153	V388M	1.7	IVS2+5G>C	5.9
154	IVS10-11G>A	6.7	Q267X	38.12
155	IVS10-11G>A	ND	F410C	ND
157	V388M	1.7	R261Q	1.8
159	L348V	ND	IVS2+5G>A	ND
161	IVS10-11G>A	6.7	IVS2+5G>C	5.9
165	R261Q	1.8	R270K	1.7
215	R252W	1.8	IVS2+5G>C	5.9
217	V388M	1.7	F55>Lfs	1.8
218	IVS10-11G>A	5.7	V388M	1.7
224	R261Q	1.8	R261Q	2.3
226	V388M	1.7	V388M	1.7
228	IVS10-11G>A	6.7	IVS10-11G>A	6.7
229	V388M	1.7	L249F	1.7
230	V388M	1.7	V388M	1.7
231		7.8		7.8
232	IVS10-11G>A	6.7	IVS10-11G>A	6.7
233	IVS10-11G>A	ND	V388M	ND

ND = não definido

Detalhamento dos programas de análises utilizados

Phred-Phrap-Consed

Phred, Phrap e Consed são programas que possibilitam a análise de milhares de seqüências de DNA geradas pelo sequenciador automático (Ewing e cols., 1998, Gordon e cols., 1998). O Phred é uma ferramenta utilizada para leitura dos cromatogramas gerados pelo sequenciamento. O programa realiza o processo de "basecalling", agregando valores de qualidade, que indicam a probabilidade de identificação correta das bases. Portanto, valores de Q = 20, representam que a probabilidade da base estar errada é de 1:100, se Q = 30, a probabilidade é de 1:1000 e assim por diante. Em termos práticos, valores acima de 20, indicando probabilidade de erro menor que 1%, são considerados com qualidade suficiente para serem analisados.

O Phred, juntamente com o Phrap, faz o alinhamento de todas as seqüências, construindo uma seqüência contínua, ou contig. O Consed permite a visualização das seqüências alinhadas. A qualidade do sequenciamento é mostrada por base, em escala de cinza: quanto mais escura a base, menor a qualidade do seu sequenciamento. A edição da seqüência pode ser realizada e a análise restrita a um segmento de boa qualidade.

Poly-Phred

A detecção de mutações em homozigose é simples. Entretanto, nos heterozigotos ocorrem dois sinais na mesma posição e isto é avaliado pelos programas Phred-Phrap como baixa qualidade de seqüência. Para a detecção de heterozigotos é necessária a utilização de programas específicos, como o Poly-Phred (Nickerson e cols., 1997).

Com o PolyPhred, triam-se as seqüências montadas com o Phred-Phrap e os resultados são visualizados com o Consed (Ewing e cols., 1998, Gordon e cols., 1998).

A identificação dos sítios heterozigotos é baseada na presença de picos sobrepostos e na redução de aproximadamente metade na altura do pico quando comparado com picos homozigotos (Kwok e col., 1994 e Nickerson e cols., 1997). O PolyPhred faz a leitura da área dos picos e identifica os prováveis sítios heterozigotos por cores.

Existem parâmetros que o programa segue de acordo com o comando do pesquisador onde é possível usar valores de qualidade e "ranks" de detecção diferenciados, a taxa de verdadeiro positivo aumenta de acordo com o aumento dos ranks e para cada rank existe uma cor característica. Ex: rank 1 = 97% de verdadeiro positivo e é marcado de

vermelho, rank 2 = 75% de verdadeiro positivo e é marcado de laranja, etc. Todas as informações podem ser encontradas na documentação da versão 5.0 do programa Poly-Phred.

A eficiência do Poly-prhed na identificação dos sítios heterozigotos depende da qualidade e do número de seqüências fornecidas. Foram analisadas no mínimo quatro seqüências para cada éxon do gene PAH de cada indivíduo. Foram realizados dois tipos de análise através do programa. Na primeira delas, cada éxon foi analisado separadamente, partindo-se de ranks baixos (r = 4 ou 5) para a detecção de um maior número de sítios heterozigotos possíveis. Os sítios identificados foram analisados manualmente pela visualização dos cromatogramas e confirmação da alteração. Um sítio foi considerado heterozigoto, quando o mesmo par de picos esteve presente em todos os cromatogramas. Na dúvida, o sequenciamento foi repetido.

Numa segunda análise, todas as seqüências de cada éxon de todos os pacientes foram alinhadas, com um critério mais estringente (r= 2). Com um número grande de seqüências, o programa aponta com maior qualidade os prováveis sítios heterozigotos (FIG. 2). A análise comparativa dos dois sistemas de alinhamento, de cada paciente individualmente e do conjunto de pacientes, serviu de base para confirmação da alteração.

Ainda assim, houve um paciente, com alteração ao SSCP e cuja análise de seqüência pelos dois sistemas descritos acima não permitiu detecção de mutação. Neste caso, o sítio variante foi identificado com a inspeção visual dos cromatogramas.

Mega 3.1 (Molecular Evolutionary Genetics Analysis 3.1)

Este software é destinado à análise comparativa de seqüências. No projeto, ele foi utilizado para que as amostras seqüenciadas fossem alinhadas com a seqüência normal do fragmento disponibilizada pelo GeneBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi). O programa permite a visualização dos alinhamentos, apontando com cores diferentes os locais onde não existe correspondência entre as bases (FIG. 3). Este alinhamento é essencial, pois não é possível inserir uma seqüência do GenBank para análise nos programas Phred-Phrap.

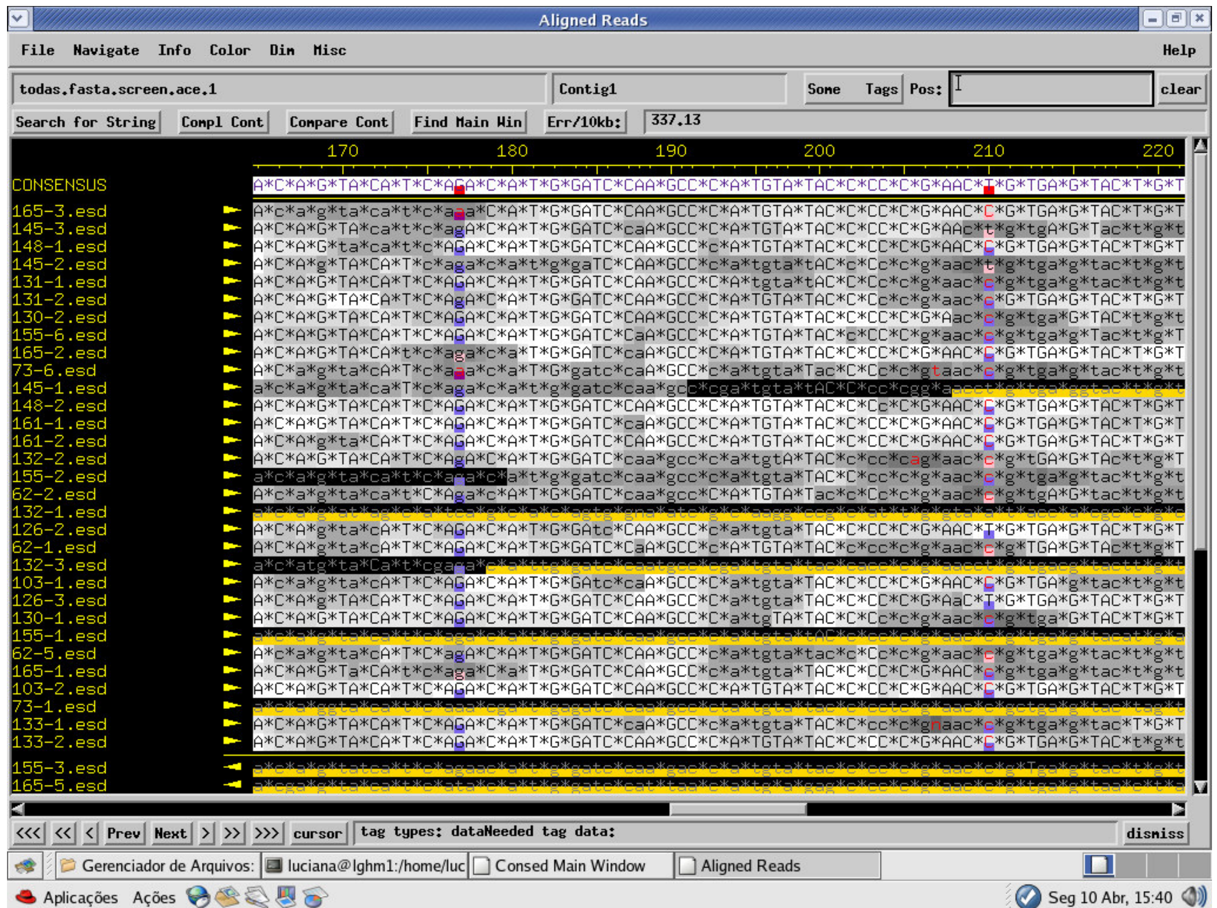

Figura 2 – Detecção das mutações R270K e P281L pelo programa PolyPhred. As seqüências do éxon 7 de todos os indivíduos analisados foram alinhadas e analisadas em conjunto.

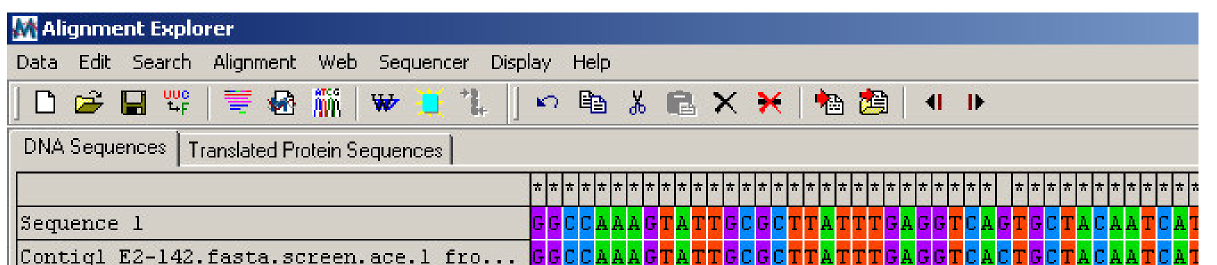

Figura 3 – Alinhamento da seqüência do éxon 2 de um individuo com PKU com a seqüência normal disponibilizada pelo *GenBank usando o programa MEGA 3.1. N*esta análise foi identificada a mutação IVS2nt5g>c em homozigose.

CAPÍTULO 3

CORRELAÇÃO GENÓTIPO-FENÓTIPO

Este artigo será submetido à revista Molecular Genetics and Metabolism.

Genotype-phenotype correlation's complexity in PKU patients

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Abstract

Phenylalanine hydroxylase deficiency is a trait inherited in an autossomal recessive pattern and the associated phenotype exhibits a large variation. This variation is mainly due to the high allelic heterogeneity in the PAH locus. The aim of this study was to establish the genotype-phenotype correlation in PKU patients from Minas Gerais, Brazil. Two systems were used. The first was a phenotype prediction system based on arbitrary values attributed for each mutation (AV), and the second a statistical correlation analysis. The observed phenotype for AV analysis was represented by the clinical diagnosis established by the overloading phenylalanine test. Among 51 PKU patients analyzed, 51% of the predicted phenotype did not match with the observed phenotype and most of concordance was found in patients with null/null genotypes. In the statistical analysis, the genotype was observed as a good predictor of the clinical course of the patients and significant correlation was found between phenylalanine values at first interview and PRA, genotype and AV sum.

Key words: Phenylketonuria, Minas Gerais, Brazil, genotype, phenotype, correlation

Introduction

Phenylketonuria (PKU, OMIM 261600) and other hyperphenylalaninemias (HPA) are caused by mutations in the gene encoding phenylalanine hydroxylase enzyme (PAH, EC 1.1.4.16.1). More than 500 mutations in PAH gene, resulting in any kind of HPA or non-patagenic polymorphisms, have already been described. Due to PAH high allelic heterogeneity, most PKU patients have two different mutations (compound heterozygotes). Interactions between two different mutant polypeptides in the tetrameric enzyme molecule increase disease complexity. The large number of possible allelic combinations difficults the

prediction of phenotype (Guldberg e cols., 1998; Dipple and McCabe., 2000; Scriver e cols., 2001; Kasnauskiene et al., 2003; Kim et al., 2006). Besides, *in vitro* expression analysis studies have shown a large range of residual activities among different mutations (null to 75%) (Waters et al., 1998; Jennings et al. 2000; Waters 2003).

Previous studies on genotype-phenotype correlations suggest that PAH genotype do not completely explain the phenotypic manifestations and that other factors exist influencing phenylalanine homeostasis (Kayaalp et al. 1997; Mallolas et al. 1999, Scriver and Waters, 1999; Benit et al., 1999). Correlation studies have been difficult to develop due both the high allelic heterogeneity and broad phenotypic variability. Recently, a system has been established for estimating genotype-phenotype correlations based on the prediction of the phenotypic impact of each mutation (Guldberg e cols, 1998; Guttler et al;. 1999).

In Minas Gerais State, Southeastern Brazil, nine mutations correspond to 80% of the PKU alleles (Santos et al., 2006 and manuscript submitted) what permit a better investigation of their phenotypic effect. This is particularly interesting because some of the mutations that are common in Minas Gerais, such as V388M, IVS2+5G>A, and IVS2+5G>C, have been only seldom described elsewhere. The aim of this study was to ascertain genotype-phenotype correlations for 28 different genotypes distributed in 54 PKU patients.

Materials and methods

Subjects: Fifty four PKU patients were analyzed, who were detected by Minas Gerais State neonatal screening program. For these patients, four different informations were available: phenylalanine levels at neonatal screening tests, established by fluriometry (Clague and Thomas 2002); phenylalanine levels at the first consultation (pretreatment phenylalanine values) established by fluriometry and HPLC (High performance liquid chromatography; Matteson 1995), phenylalanine values at overloading test (O'Flynn et al. 1980), that was done

in the sixth month of life; and dietary phenylalanine tolerance estimated at ages of 3, 6, and 9 months.

Based on the phenylalanine overloading tests, patients were assigned to one of the four phenotypic PKU subtypes (classical PKU, moderate PKU, mild PKU or HPA). Patients with serum phenylalanine values higher than 1200mM were classified as classical PKU, those with values between 900 and 1200µM, as moderate PKU, and those between 600 and 900µM as mild PKU. Patients with values lower than 600mM were classified as HPA (O'Flynn et al. 1980; Guttler 1980). No HPA patients were included in the present sample.

Informations on genotype and phenylalanine overloading test were available for all individuals analyzed. Phenylalanine values in the neonatal screening tests were available for 53 patients and dietary phenylalanine tolerance for 47 patients. Serum phenylalanine values at the first interview were available for 50 patients.

Genotyping: Genomic DNA isolation and mutation analysis were done previously by PCR and RFLP or single-strand conformation polymorphism (SSCP) and sequencing (Santos et al., 2006 and manuscript submitted).

Mutations present in PKU patients (Table 1) included six missense mutations (I65T, R261Q, R270K, V388M, L348V, and R158W) and nine null mutations, corresponding five splice-site mutations (IVS2nt5g>c, IVS2nt5g>a, IVS7nt1g>a, IVS10-11G>A, and IVS12nt1g>a), one stop condon (R261X), and three missense mutations (R252W, P281L, R408W) with null effect demonstrated by *in vitro* expression analysis studies. Predicted residual enzymatic activity (PRA) for each one of these mutations has been previously reported (Waters et al., 1998; Erlandsen and Stevens 1999; Rivera 2000; Pey et al., 2003; www.pahdb.mcgill.ca/), except for R158W. Mean PRA values were calculated for each genotype.

Phenotypic prediction system: Mutations were assigned to one of the four phenotype categories (classical, moderate, mild and HPA non-PKU) according to Guldberg et al., 1998.

An arbitrary value (AV) was assigned to each mutation: AV = 1 for classical PKU mutation; AV = 2 for moderate PKU mutation; AV = 4 for mild PKU mutation and AV = 8 for non-PKU HPA mutation. Phenotypes resulting from the combination of the two mutant alleles were expressed as the sum of the two mutations' AVs (Guldberg et al., 1998). For two mutations (R270K and R158W) present in three individuals (5.5%) there were no AV estimates in the literature, and therefore, these patients were excluded from those analysis depending on AV information.

Statistical analysis: Statistical analysis was implemented with the SAS/STAT® (SAS 2003). Spearman correlation was estimated among pretreatment phenylalanine levels (values from neonatal screening test and at the first interview), dietary phenylalanine tolerance at 3, 6 and 9 months of life, serum phenylalanine at the overloading phenylalanine test, diagnosis, established on basis on phenylalanine overloading test, mean PRA and AV sum. Genotypes were codified in two different ways. Initially, a simple sequencial order was used. Thereafter, codes based in each allele residual activity were constructed. For that, alleles classified as null or with some residual activity were distribiuted in three genotype classes: null/null; null/residual; residual/residual. Linear regression was tested between each variable listed above and genotype. The significance level of $p < 0.05$ was considered for all the analysis.

Results

Based on phenylalanine overloading test, 31 patients were classified as classical PKU, 17 individuals were classified as moderate PKU and only one patient was classified as mild PKU (supplementary data).

Twenty eight different genotypes formed by combinations of 15 PAH mutations were analyzed in this study (Table 1). In 54 individuals, 23 were homoallelic and 31 heteroallelic.

Among the last group 13 were functionally hemizygous (one null mutation and one mutation with some residual activity).

Phenotypic prediction system: The results of the phenotype prediction based on AVs are summarized in Table 1. In the present sample, 14 patients had null/null genotypes, 11 had functionally hemizygous genotypes and 25 individuals had their genotypes composed of two mutations with some enzymatic residual activity. From 51 patients for whom AV information was available, 25 (49%) showed a phenotype (clinical classification based on phenylalanine overloading tests) in concordance to the AV predicted for their genotype. In this group, it was observed that 11 patients had genotypes with two null mutations (involving mutations IVS2+5G>A, IVS2+5G>C, IVS10-11G>A, IVS10-11G>A, IVS12+1G>A, P281L, R252W). The rest of group was composed of four patients with functionally hemizygous genotypes and ten individuals had two missense mutations with residual enzymatic activity.

However, in 26 patients (51%) the observed phenotypes were different from the expected ones (Table 1 and supplementary material). There were three genotype-phenotype inconsistencies among the individuals bearing two null mutations (R252W/P281L, R261X/P281L and IVS10-11G>A/IVS10-11G>A) which were not associated to classical PKU, as it would be expected. Seven individuals had functionally hemizygous genotypes and 15 had genotypes composed of two mutations with some enzymatic residual activity.

All genotypes composed of two missense mutations (N = 23) conferred more than one phenotype, except for R261Q/I65T genotype that was observed in only one individual.

Table 1 - Predicted *vs*. observed phenotypes in 54 PKU individuals from Minas Gerais, Brazil

Genotypes	n	AV sum	Predicted phenotype	Observed phenotype	Inconsistences
V388M / V388M	6	4	Mo/Mi	5C/1Mo	5
R261Q / R261Q	5	4	Mo/Mi	3C/2Mo	3
R261Q / V388M	4	4	Mo/Mi	1C/2Mo/1Mi	1
V388M / I65T	4	4	Mo/Mi	3C/1Mo	3
V388M / IVS10-11G>A	4	3	Mo	2C/2Mo	2
IVS2+5G>A / IVS2+5G>A	3	2	C	C	
R261Q / R252W	2	3	Mo	2C	2
R261Q / L348V	2	4	Mo/Mi	1C/1Mo	1
V388M / R252W	2	3	Mo	2C	2
I65T / I65T	2	4	Mo/Mi	1C/1Mo	1
IVS2+5G>C / IVS10-11G>A	2	2	C	C	
IVS10-11G>A / IVS10-11G>A	2	2	C	1C/1Mo	1
R261Q / IVS10-11G>A	1	3	Mo	C	1
R261Q / R408W	1	3	Mo	Mo	
R261Q / R270K	1	?	?	Mo	?
R261Q / I65T	1	4	Mo/Mi	Mo	
IVS2+5G>C / IVS2+5G>C	1	2	C	C	
IVS10-11G>A / IVS12+1G>A	1	2	C	C	
P281L / R261X	1	2	C	Mo	1
P281L / R252W	1	2	C	Mo	1
P281L / P281L	1	2	C	C	
R252W / IVS2+5G>C	1	2	C	C	
R252W / R252W	1	2	C	C	
IVS2+5G>A / L348V	1	3	Mo	C	1
IVS10-11G>A /L348V	1	3	Mo	C	1
IVS7+1G>A / L348V	1	3	Mo	Mo	
R158W / R158W	1	?	?	C	?
R270K / R270K	1	?	?	C	?
Total	54				

Note: AV = arbitrary value; C= classic PKU; Mo= moderate PKU; Mi = mild PKU.

Statistical analysis: Spearman correlation analysis revealed a highly significant inverse correlation ($r = -0.55554$ $p < 0.0001$) between PRA values and phenylalanine values at the first consultation (pretreatment values). AV sums significantly correlated with PRA, phenylalanine values at the first consultation and genotype as well (both the three with $p < 0.0001$). However, no significant correlation was observed between PRA and phenylalanine values at the neonatal screening test. In the regression analysis, phenylalanine values at the first interview, dietary phenylalanine tolerance at age of 9 months, PRA and AV sum (dependent variables) were associated with the genotype (independent variable), $p = 0.0016$, 0.002, 0.0012, and 0.0003, respectively. Dietary phenylalanine tolerance measured at 3, 6 and 9 months of age correlated one with each another (tolerance at 3 months X tolerance at 6 months, $r = 0.38895$ and $p = 0.0069$; tolerance at 6 months X tolerance at 9 months, $r = 0.48951$ and $p = 0.0005$; tolerance at 3 months X tolerance at 9 months, $r = 0.32258$ and $p = 0.0270$. The diagnosis was correlated with the overloading test value as expected ($r = 0.82022$; $p < 0.0001$).

Discussion

It has been proposed that PAH genotype is not a rigorous predictor for clinical progression in phenylketonuria patients. Many factors can apport phenotypical variation to PKU, such as interindividual variations in the intestinal absorption, hepatic uptake of dietary phenylalanine, rate of incorporation of phenylalanine into proteins, rates of influx of phenylalanine across the blood brain barrier, mutations affecting tetrahydrobiopterin (which works as cofactor but also protecting PAH enzyme against proteolitc degradation), as well as interactions of PAH gene with other loci etc. (Kayaalp et al., 1997; Scriver 1999; Dipple et al., 2000; Dipple 2001; Scriver 2002, Scriver 2007).

Two different approaches for phenotype-genotype correlation studies have been found in the literature, one based an arbitrary values phenotypic prediction system, that usually do not implies a formal correlation test and another based on statistical pairwise correlations/regressions.

AV based phenotypic prediction system

The AV based method for phenotypic prediction was already applied in some studies (Gütter, 1999; Acosta et al., 2001; Kasnauskienė et al., 2003). In each study, metabolic phenotypes were predicted by observing functionally hemizygous individuals from each population. We have adopted the same AV categorization previously described (Guldberg et al. 1998). This systems was also adopted in some other studies (Zschocke 2003; Auleha-Scholz and Heilbronner 2003).

We have investigated 15 mutations distributed in 28 different genotypes. The most frequent homozygous genotypes were R261Q/R261Q (n=5) and V388M/V388M (n=6). R261Q mutation was classified as moderate (Guldberg et al., 1998). However, in the present sample three out five homozygous individuals for this mutation showed classical PKU. Besides, three out four patients with genotypes composed of R261Q and any known null allele were also associated with the classical PKU.

The same happened with V388M/V388M homozygous genotypes. This mutation was classified as moderate based in only one functional hemizygous patient (Guldberg et al., 1998). In the present sample, five out six patients with homozygous genotype had classical PKU and only one had moderate PKU. Moreover, four out six patients bearing V388M and any null allele presented also classical PKU, suggesting that the effect of this mutation is more severe than that previously suggested.

L348V mutation is another example of the same situation. Although reported as moderate, classical PKU was observed in association with two L348V/null genotypes and in

one out two R261Q/L348V genotypes. Therefore, this mutation is also more frequently associated with classical PKU in the present sample.

Another interesting effect observed in the present sample was that heterozygote genotypes constituted by two mutations with similar severities lead to a less severe phenotype than that observed if they were in homozygosis, as previously described in the literature (Guldberg et al., 1998). In the present sample, patients with R261Q/V388M genotype showed a milder phenotype than homozygous individuals R261Q/R261Q or V388M/V388M. However, the suggestion that severity of the illness in most cases is determined by the less severe allele (Guldberg et al., 1998; Kasnauskienė et al., 2003) was not confirmed in the present study. Ten out 13 genotypes of this type (R261Q/null, V388M/null, L348V/null) were associated to classical PKU, do not supporting the generalization.

Better aggreement to previous reports was observed for those genotypes composed by null/null mutations, but even among these, some genotypes (R252W/P281L, R261X/P281L and IVS10-11G>A/IVS10-11G>A) were not assorted to classical PKU, as expected. To the other hand, all genotypes constituted by two missense mutations were associated with both classical and moderat PKU in the present sample (Table 1).

AV estimatives for R270K and R158W mutations were not found in the literature. In spite of *in vitro* expression studies for R158W mutation are not available, this mutation distort the enzyme active-site structure and probably results in classical PKU, as R158Q mutation in the same site (Erlandsen et al. 2003). R270K mutation has been described with low enzyme residual activity (2.1%; Leandro et al. 2006). For both mutations, homozygote patients observed in the present study have classical PKU.

Taken together, these results point to an efficience for the AV system of 49% in our sample. The system was particularly useful as a predictor of clinical course for patients bearing null/null genotypes. The efficience of the method based on AVs estimates will vary

depending on the set of mutations present in a specific population. For some populations, the system proved to be high useful. For example, this system reachs 96% of concordance in a sample in which the most common mutations are null ones (Kasnauskienė et al., 2003).

Statistical analysis: Good predictors to clinical course of the patient would be important in the beginning of the treatment and with statistical analysis these predictors could be identified.

Phenylalanine values at the neonatal screening test did not significantly correlated to any other values tested. This result may be attributable to the moment when the test is done, by the 5th day of life, when some children may still not have estabilized feeding. This result suggests that this value would be a weak predictor.

Phenylalanine pretreatment values (at first interview) are significantly correlated with PRA and AV sum. Besides, highly significant results were found at regression analysis observed between AV sum and genotype, and between genotype and phenylalanine values at first interview. The genotype explain 20% of the phenylalanine values variation found at first interview, contrasting to the absence of correlation with phenylalanine values at overloading test. However, phenylalanine pretreatment values (at first interview) do not correlate with tolerance and phenylalanine overloanding test.

The correlations found among the tolerances at ages of 3, 6 and 9 months suggest a good consistency of these values. In this sample, genotype (codified as consecutive numbers) was significantly correlated with tolerance at age of 9 months, in spite the fact that genotype (codified according to residual activity of each alele), PRA and AV sum do not correlate with tolerance at 9 months. Phenylalanine values at neonatal screening test, first consultation and overloading test do not correlate with any measure of tolerance as well. These results suggest that genotype may be a good predictor of clinical progress in phenylketonuria patients. Amazingly, the predictor is the genotype itself, and not funcional values atribuited to it.

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References

C. Aulehla-Scholz and H. Heilbronner, Mutational Spectrum in German Patients With Phenylalanine Hydroxylase Deficiency. Hum Mutat, (2003) DOI: 10.1002/humu.9116

A. Acosta, W. Silva, Jr., T. Carvalho, M. Gomes, M. Zago, Mutations of the phenylalanine hydroxylase (PAH) gene in Brazilian patients with phenylketonuria. Hum Mutat, 17 (2001) 122-30.

P. Benit, F. Rey, F. Blandin-Savoja, A. Munnich, V. Abadie, J. Rey, The mutant genotype is the main determinant of the metabolic phenotype in phenylalanine hydroxylase deficiency. Mol Genet Metab, 68 (1999) 43-7.

A. Clague, A. Thomas, Neonatal biochemical screening for disease. Clin Chim Acta, 315 (2002) 99-110.

K.M. Dipple, E.R. McCabe, Phenotypes of patients with "simple" Mendelian disorders are complex traits: thresholds, modifiers, and systems dynamics. Am J Hum Genet, 66 (2000) 1729-35.

K.M. Dipple, J.K. Phelan, E.R. McCabe, Consequences of complexity within biological networks: robustness and health, or vulnerability and disease. Mol Genet Metab, 74 (2001) 45-50.

H. Erlandsen, R.C. Stevens, The structural basis of phenylketonuria. Mol Genet Metab, 68 (1999) 103-25.

H. Erlandsen, M.G. Patch, A. Gamez, M. Straub, R.C. Stevens, Structural studies on phenylalanine hydroxylase and implications toward understanding and treating phenylketonuria. Pediatrics, 112 (2003) 1557-65.

P. Guldberg, F. Rey, J. Zschocke, V. Romano, B. Francois, L. Michiels, K. Ullrich, G.F. Hoffmann, P. Burgard, H. Schmidt, C. Meli, E. Riva, I. Dianzani, A. Ponzone, J. Rey, F. Guttler, A European multicenter study of phenylalanine hydroxylase deficiency: classification of 105 mutations and a general system for genotype-based prediction of metabolic phenotype. Am J Hum Genet, 63 (1998) 71-9.

F. Guttler, Hyperphenylalaninemia: diagnosis and classification of the various types of phenylalanine hydroxylase deficiency in childhood. Acta Paediatr Scand Suppl, 280 (1980) 1-80.

F. Guttler, C. Azen, P. Guldberg, A. Romstad, W.B. Hanley, H.L. Levy, R. Matalon, B.M. Rouse, F. Trefz, F. de la Cruz, R. Koch, Relationship among genotype, biochemical phenotype, and cognitive performance in females with phenylalanine hydroxylase deficiency: report from the Maternal Phenylketonuria Collaborative Study. Pediatrics, 104 (1999) 258-62.

I.G. Jennings, R.G. Cotton, B. Kobe, Structural interpretation of mutations in phenylalanine hydroxylase protein aids in identifying genotype-phenotype correlations in phenylketonuria. Eur J Hum Genet, 8 (2000) 683-96.

J. Kasnauskiene, L. Cimbalistiene, V. Kucinskas, Validation of PAH genotype-based predictions of metabolic phenylalanine hydroxylase deficiency phenotype: investigation of PKU/MHP patients from Lithuania. Med Sci Monit, 9 (2003) 142-6.

E. Kayaalp, E. Treacy, P.J. Waters, S. Byck, P. Nowacki, C.R. Scriver, Human phenylalanine hydroxylase mutations and hyperphenylalaninemia phenotypes: a metanalysis of genotype-phenotype correlations. Am J Hum Genet, 61 (1997) 1309-17.

S.W. Kim, J. Jung, H.J. Oh, J. Kim, K.S. Lee, D.H. Lee, C. Park, K. Kimm, S.K. Koo, S.C. Jung, Structural and functional analyses of mutations of the human phenylalanine hydroxylase gene. Clin Chim Acta, 365 (2006) 279-87.

J. Leandro, C. Nascimento, I. T. de Almeida, P. Leandro, Co-expression of different subunits of human phenylalanine hydroxylase: evidence of negative interallelic complementation. Biochim Biophys Acta, 1762 (2006) 544-50.

J. Mallolas, M. Mila, N. Lambruschini, F.J. Cambra, J. Campistol, M.A. Vilaseca, Biochemical phenotype and its relationship with genotype in hyperphenylalaninemia heterozygotes. Mol Genet Metab, 67 (1999) 156-61.

M.E. O'Flynn, N.A. Holtzman, M. Blaskovics, C. Azen, M.L. Williamson, The diagnosis of phenylketonuria: a report from the Collaborative Study of Children Treated for Phenylketonuria. Am J Dis Child, 134 (1980) 769-74.

A.L. Pey, L.R. Desviat, A. Gamez, M. Ugarte, B. Perez, Phenylketonuria: genotype-phenotype correlations based on expression analysis of structural and functional mutations in PAH. Hum Mutat, 21 (2003) 370-8.

I. Rivera, A. Cabral, M. Almeida, P. Leandro, C. Carmona, F. Eusebio, T. Tasso, L. Vilarinho, E. Martins, M.C. Lechner, I.T. de Almeida, D.S. Konecki, U. Lichter-Konecki, The correlation of genotype and phenotype in Portuguese hyperphenylalaninemic patients. Mol Genet Metab, 69 (2000) 195-203.

L.L. Santos, C. Magalhaes Mde, O. Reis Ade, A.L. Starling, J.N. Januario, C.G. Fonseca, M. J. Aguiar, M.R. Carvalho, Frequencies of phenylalanine hydroxylase mutations I65T, R252W, R261Q, R261X, IVS10nt11, V388M, R408W, Y414C, and IVS12nt1 in Minas Gerais, Brazil. Genet Mol Res, 5 (2006) 16-23.

C.R. Scriver and S. Kaufman, The hyperphenylalaninemias, in C.R. Scriver, A.L. Beaudet, W.S. Sly, and D. Valle (Eds.), *The Metabolic and Molecular Bases of Inherited Disease*, McGraw-Hill, New York, 2001, pp. 1667–1724.

C.R. Scriver, P.J. Waters, Monogenic traits are not simple: lessons from phenylketonuria. Trends Genet, 15 (1999) 267-72.

SAS (2003). *SAS/STAT® Software: Changes and Enhacements through Release 9.1*, SAS Institute, Cary, USA.

C.R. Scriver, Why mutation analysis does not always predict clinical consequences: explanations in the era of genomics. J Pediatr, 140 (2002) 502-6.

C.R. Scriver, The PAH gene, phenylketonuria, and a paradigm shift. Hum Mutat, 28 (2007) 31-45.

C.R. Scriver, M. Hurtubise, D. Konecki, M. Phommarinh, L. Prevost, H. Erlandsen, R. Stevens, P.J. Waters, S. Ryan, D. McDonald, C. Sarkissian, PAHdb 2003: what a locus-specific knowledgebase can do. Hum Mutat, 21 (2003) 333-44.

P.J. Waters, M.A. Parniak, P.Nowacki, C.R. Scriver, In vitro expression analysis of mutations in phenylalanine hydroxylase: linking genotype to phenotype and structure to function. Hum Mutat, 11 (1998) 4-17.

P.J. Waters, How PAH gene mutations cause hyper-phenylalaninemia and why mechanism matters: insights from in vitro expression. Hum Mutat, 21 (2003) 357-69.

K.J. Matteson, HPLC analysis of amino acids in inborn errors of metabolism. Southeast Asian J Trop Med Public Health, 26 (1995) 120-2

J. Zschocke, Phenylketonuria mutations in Europe. Hum Mutat, 21 (2003) 345-56.

Supplementary data

Data of the PKU sample from Minas Gerais

ID	NST	First Consult	T3M	T6M	T9M	Overloading test	Diag.	allele 1	allele 2	PRA 1	PRA 2	mean PRA	AV sum
224	1340,00	976,00	59,90	61,30	34,40	1407,70	3	R261Q	R261Q	30	30	30	4
69	838,20	.	.	.	.	1083,60	2	R261Q	R261Q	30	30	30	4
63	625,80	1814,60	54,70	30,60	22,40	1081,80	2	R261Q	R261Q	30	30	30	4
85	1238,50	1181,90	50,50	50,00	55,85	1310,30	3	R261Q	R261Q	30	30	30	4
116	1265,40	936,40	262,66	.	41,50	1249,20	3	R261Q	R261Q	30	30	30	4
136	1132,30	2036,00	62,23	43,11	34,86	1887,20	3	R261Q	R252W	30	0	15	3
139	893,30	1510,60	48,90	56,70	44,99	1288,36	3	R261Q	R252W	30	0	15	3
106	1084,00	1021,60	70,80	46,18	41,80	1615,10	3	R261Q	IVS10-11G>A	30	0	15	3
88	1171,90	1699,60	40,90	38,20	40,00	1134,80	2	R261Q	R408W	30	0	15	3
97	1055,30	1219,60	72,70	42,38	38,30	964,00	2	R261Q	L348V	30	43	31,5	4
66	924,50	961,90	48,00	53,60	39,80	1344,70	3	R261Q	L348V	30	25	27,5	4
165	1806,00	1870,00	.	41,50	.	1125,25	2	R261Q	R270K	30	.	.	.
120	1936,70	1312,36	63,60	31,80	58,80	993,70	2	R261Q	I65T	30	23	26,5	4
64	792,90	776,85	38,00	52,10	41,90	877,10	1	V388M	R261Q	43	30	31,5	4
151	1059,30	710,00	47,43	31,70	36,36	1656,76	3	V388M	R261Q	43	30	31,5	4
112	1394,60	1649,30	51,00	52,00	63,00	1097,30	2	V388M	R261Q	43	30	31,5	4
157	935,70	1365,00	35,05	37,39	30,00	1183,00	2	V388M	R261Q	43	30	31,5	4
68	1295,00	1410,40	51,00	38,00	37,00	1482,70	3	V388M	I65T	43	23	34,5	4
81	1387,69	789,60	79,40	51,81	43,40	1180,43	2	V388M	I65T	43	23	34,5	4
124	1388,60	809,00	33,00	22,00	62,35	1956,70	3	V388M	I65T	43	23	34,5	4
82	1137,70	1200,00	42,56	57,49	49,00	1649,30	3	V388M	I65T	43	23	34,5	4
118	699,40	1753,20	72,00	62,35	57,00	2880,00	3	I65T	I65T	23	23	23	4
105	2355,00	.	.	.	.	1129,00	2	I65T	I65T	23	23	23	4
71	903,70	2029,20	29,60	27,90	35,79	1472,40	3	V388M	V388M	43	43	43	4
95	880,30	1141,40	45,60	48,33	36,00	1625,10	3	V388M	V388M	43	43	43	4
101	874,10	1277,20	57,80	28,38	.	1435,40	3	V388M	V388M	43	43	43	4
109	1103,50	1005,70	7,02	11,54	16,79	1009,00	2	V388M	V388M	43	43	43	4
226	1483,90	1321,20	57,90	52,95	53,26	1970,76	3	V388M	V388M	43	43	43	4
222	1113,10	558,70	.	.	.	1352,10	3	V388M	V388M	43	43	43	4

74	2880,00	2880,00	65,00	32,70	34,19	1953,40	3	V388M	R252W	43	0	21,5	3
98	848,70	1899,76	30,60	30,70	27,99	1848,30	3	V388M	R252W	43	0	21,5	3
115	1639,10	1989,40	40,75	62,96	55,22	1221,26	3	V388M	IVS10-11G>A	43	0	21,5	3
80	1194,70	2027,60	13,,5	14,55	29,00	982,30	2	V388M	IVS10-11G>A	43	0	21,5	3
233	2067,00	1565,00	34,15	33,67	45,60	1121,00	2	V388M	IVS10-11G>A	43	0	21,5	3
218	988,50	1093,10	48,00	34,58	27,40	1380,36	3	V388M	IVS10-11G>A	43	0	21,5	3
161	1820,60	2429,40	64,19	38,76	31,35	1324,40	3	IVS10-11G>A	IVS2+5G>C	0	0	0	2
134	1075,60	1629,40	84,90	58,00	34,80	2118,53	3	IVS10-11G>A	IVS2+5G>C	0	0	0	2
142	775,40	1624,50	107,60	54,32	48,01	2043,26	3	IVS2+5G>C	IVS2+5G>C	0	0	0	2
77	1721,70	.	62,60	38,00	34,76	1636,23	3	IVS10-11G>A	IVS12+1G>A	0	0	0	2
84	965,00	1250,90	35,00	45,32	17,30	2851,50	3	IVS2+5G>A	IVS2+5G>A	0	0	0	2
110	1571,80	2877,40	24,90	48,40	14,43	1993,80	3	IVS2+5G>A	IVS2+5G>A	0	0	0	2
111	1185,30	2880,00	62,34	41,99	34,64	1717,20	3	IVS2+5G>A	IVS2+5G>A	0	0	0	2
62	2474,00	2547,00	28,38	34,80	22,98	1029,50	2	P281L	R261X	0	0	0	2
90	952,00	2021,50	54,00	63,09	62,10	943,20	2	R252W	P281L	0	0	0	2
126	752,00	2346,80	9,70	23,00	14,20	1404,00	3	P281L	P281L	0	0	0	2
215	1510,00	1983,00	40,76	34,92	28,40	2880,00	3	R252W	IVS2+5G>C	0	0	0	2
100	2880,00	.	.	.	.	1265,92	3	R252W	R252W	0	0	0	2
78	1189,60	110,90	50,80	51,50	53,70	2188,20	3	IVS10-11G>A	IVS10-11G>A	0	0	0	2
232	1126,00	2200,30	39,00	31,80	37,84	1099,00	2	IVS10-11G>A	IVS10-11G>A	0	0	0	2
159	2406,80	2139,40	40,00	46,42	82,02	1653,80	3	IVS2+5G>A	L348V	0	25	12,5	3
93	722,20	1416,90	48,03	33,00	29,10	2191,40	3	IVS10-11G>A	L348V	0	25	12,5	3
130	.	1886,60	58,70	26,09	49,45	955,50	2	IVS7+1G>A	L348V	0	25	12,5	3
148	1271,50	2128,40	14,50	20,41	13,30	2344,20	3	R158W	R158W	.	.	.	
73	747,09	2819,20	31,60	44,30	43,30	1567,30	3	R270K	R270K	.	.	.	

Legend:

ID = identification number
NST = serum phenylalanine levels at the neonatal screening test (µM)
T3M = Phenylalanine tolerance at 3 months of life
T6M = Phenylalanine tolerance at 6 months of life
T9M = Phenylalanine tolerance at 9 months of life

Overloading test = serum phenylalanine levels found at overloading test (µM)
Diag = Diagnostic number: 1 = mild PKU; 2 = moderate PKU; 3 = classical PKU
PRA 1 = predicted residual activity of the allele 1
PRA 2 = predicted residual activity of the allele 2
. = missing values

CAPÍTULO 4

Coeficiente de endogamia e freqüência alélica da PKU no Estado de Minas Gerais

Este artigo será submetido à revista Annals of Human Genetics

Estimating inbreeding coefficients by microsatellite approaches: implications for PKU allele frequency

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Running head: Inbreeding and PKU allele frequency

Key Words: inbreeding, microsatellites, PKU, Minas Gerais, Brazil

Summary: Estimates of allele frequencies for recessive diseases are generally based on the frequency of affected individuals (q^2). However, these estimates can be strongly biased due to inbreeding in the population. The purpose of this study was to gain a better understanding of how inbreeding in the Minas Gerais State population affects PKU incidence in the state and to explore an alternative approach to determine the inbreeding coefficient based on microsatellites. *F*it, *F*st and *F*is were estimated for a sample of 76 PKU patients and for 104 controls matched by year of birth. No genetic differentiation was observed between the samples. However, the *F*is value found for PKU sample (0.042) was almost 15 times higher than that found among controls (0.003). When corrected by the inbreeding coefficient found among the controls, the PKU allele frequency decreased to 0.0057. Approximately 35% of the PKU patients from the Minas Gerais population were due to consanguineous marriages. Still lower values were obtained when allele frequency was corrected by the Fis value observed among PKU individuals (p=0.0011), suggesting a stronger impact on PKU allele frequency in the Minas Gerais population. The results suggest that microsatellites can be a useful approach to estimate inbreeding coefficients in human populations and can be consequently used to correct allele frequencies for recessive diseases.

INTRODUCTION

Phenylketonuria (PKU, OMIM 261600) is an inherited metabolic disorder resulting in progressive mental retardation if not treated (Güttler 1980; Scriver et al. 2001; Scriver 2007). Therefore, PKU has been included in most neonatal screening programs (Carreiro-Lewandowski 2002). The PKU neonatal screening program has been conducted in Minas Gerais, Brazil since 1993, and the PKU incidence in the state has been estimated to be 1:20,000 (Serjeant 2000; Aguiar 2004). Minas Gerais is the second most populous state from southeastern Brazil with 21 million inhabitants distributed in 853 municipalities (IBGE - Instituto Brasileiro de Geografia Estatística - http://www.ibge.gov.br/home/), and it has an area of 586,528 Km^2, similar to France. Minas Gerais State has the most efficient PKU neonatal screening

program in Brazil, with a mean coverage of approximately 98% of the births in the state, permitting a good estimate of the frequency of affected individuals. The relative frequency of different mutations for PKU in the state has been established (Santos et al. 2006 and manuscript in preparation).

The estimation of allele frequencies for recessive diseases is generally based on the frequencies of affected individuals. However, they may be strongly biased, depending on the inbreeding present in the population. This effect could be corrected by estimates of the allele frequency in heterozygotes. This approach requires the testing of large samples and is therefore seldom done. Estimating inbreeding coefficients is another option, but it is also not a trivial task, requiring time-consuming interviews or analysis of large civil or parish marriage records.

Recently, inbreeding coefficient estimates based on microsatellites have been published (Chambers & MacAvoy 2000; Balloux et al. 2004; Carothers et al. 2006). Due to their high polymorphic information content and ease of handling, microsatellites seem to be a good alternative to obtain these estimates.

The objective of this study was to analyze the impact of inbreeding on PKU allele frequency in Minas Gerais State. Inbreeding coefficients of control and phenylketonuria patients samples were estimated through a microsatellite approach. Besides, the amount and distribution of genetic variation within and among phenylketonuria patients and control sample were characterized.

MATERIALS AND METHODS

Sample: We studied two samples from the Minas Gerais population. The first one is composed of 76 PKU patients sequentially screened from 1993 to 2004. The second sample is composed of 104 unrelated individuals ascertained at a parentage test center and matched by birth date with the patients in the PKU sample. The study was approved by the Ethics in Research Committee of the Universidade Federal de Minas Gerais. Both patients and controls signed an informed consent form before being included in the study.

Microsatellite analysis: The microsatellite loci tested were CSF1PO, TPOX, TH01, vWA, D16S539, D7S820, D13S317, D3S1358, D14S1434, D22S1045 and D10S1248. None of these STR markers map to chromosome 12, where the phenylalanine hydroxylase gene (PAH) is located. PCR amplification was performed in a 12.5µl volume containing 50 mM KCl, 10 mM Tris – HCl, pH 8.4, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.1 mM of each dNTP, 10 ng of genomic DNA, 0.6 U of Taq DNA polymerase, and 0.1 mM of each specific primer. PCR amplifications entailed a 2 min initial denaturation step at 96 Cº, followed by 10 cycles of 1 min at 94 Cº, 1 min at 60 Cº, and 1.5 min at 70 Cº, and 20 cycles consisting of 1 min at 90 Cº, 1 min at 60 Cº, and 1.5 min at 70 Cº, with a final extension step at 72 Cº for 5 min. Primer sequences are described elsewhere (http://www.cstl.nist.gov/biotech/strbase/promega_primers.htm). D14S1434, D22S1045 and D10S1248 loci were amplified according to the literature and allele nomenclature was established as recommended elsewhere (Coble & Butler 2006; Butler & Coble 2007). STR alleles were electrophoretically separated on 6% denaturing polyacrylamide gels. Fragments were visualized with silver nitrate staining (Sambrook & Rousset 2002).

Statistical Analysis: GenePop Software (Raymond & Rousset 1995) was used to estimate basic population genetic descriptive statistics for each marker and sample: gene frequencies, observed number of alleles, observed and expected heterozygosities and P-values for Hardy-Weinberg tests under the alternative hypothesis of heterozygote deficit. Levene's correction for multiple testing was used in the heterozygosity estimates (Levene 1949; Guo & Thompson 1992). Population differentiation was estimated by Wright´s fixation indices *F*it, *F*st, and *F*is (Wright 1951) in the form of F, θ, and f for each locus across the samples (Weir & Cockerham 1984). Mean effective number of alleles and total inbreeding coefficient for each sample was estimated with the FSTAT program, version 9.3 (Goudet et al. 2002). The frequency of the q allele for PKU was estimated with the formula

$$i = qF + q2\ (1 - F) \qquad (1)$$

as described elsewhere (Hartl & Clark 2007).

RESULTS

Eighty-five microsatellite alleles were amplified at 11 loci in 180 individuals from both samples, phenylketonuria patients and controls. The observed number of alleles (*N*a) across the loci in the populations ranged from 77 in the phenylketonuria sample to 80 in the control sample. *N*a per locus per population ranged from 6 to 8 in the PKU sample and 6 to 9 in controls. Private alleles were observed in both samples, 6 of them among the controls and 5 in the PKU sample. Observed heterozygosity ranged from 0.49 (CSF1PO) to 0.64 (vWA) in the PKU sample and 0.62 (D22S1045) to 0.85 (D13S317) in the control sample (Table 1).

Table I - *Statistics for each one of 11 markers among phenylketonuria patients and controls samples from Minas Gerais, Brazil*

	PKU sample				Control sample					
Locus	Het Exp	Het Obs	Fis (W&C) (PKU)	P-value (HW)	Het Exp	Het Obs	Fis (W&C) (controls)	P-value (HW)	Fst	Fit
CSF1PO	54.68	49	0.105	0.2329	74.61	73	0.022	0.5409	0.0171	0.0729
TPOX	54.55	54	0.010	0.4508	73.79	72	0.024	0.6048	-0.0053	0.0132
TH01	59.90	62	-0.035	0.6859	83.28	83	0.003	0.4020	0.0013	-0.0115
VWA	62.15	64	-0.030	0.7948	80.97	82	-0.013	0.5659	-0.0034	-0.0237
D16S539	59.65	59	0.011	0.4817	82.11	77	0.063	0.1496	-0.0027	0.0383
D7S820	60.84	52	0.146	0.0010	79.74	81	-0.016	0.5496	-0.0051	0.0494
D13S317	60.92	50	0.180	0.0264	81.00	85	-0.050	0.8955	-0.0025	0.0467
D3S1358	59.27	54	0.089	0.0501	80.59	83	-0.030	0.7909	-0.0024	0.0182
D14S1434	52.01	50	0.039	0.1411	61.36	64	-0.043	0.8033	0.0120	0.0065
D22S1045	56.24	58	-0.031	0.5623	64.65	62	0.041	0.2277	0.0098	0.0172
D10S1248	57.28	59	-0.030	0.2155	67.68	66	0.025	0.2189	0.0044	0.0041
Global			**0.042**	**0.0049**			**0.003**	**0.6655**	**0.0018**	**0.0211**

No statistically significant deviations from Hardy-Weinberg expectations were found when each locus was tested separately in the control sample under the alternative hypothesis of heterozygote deficit. Contrastingly, in the PKU sample, three loci were not in Hardy-Weinberg equilibrium, D3S1358, D7S820 and D13S317 (P-values = 0.0501, 0.0010, and 0.0264, respectively). When the information on the 11 markers tested was used to test HW equilibrium *per* sample, only the PKU sample showed significant deviation from HW expectations (P-value = 0.0049). However, when both samples were considered together, deviations from HW equilibrium were not significant (P-value = 0.1101).

Results of *F*-statistics for each one of the 11 loci across the samples are presented in Table 1. *F*is estimates per locus per sample showed higher values in the PKU sample than in the controls for some loci. *F*is values for each locus were observed in the PKU sample ranging from -0.030 for D10S1248 to +0.18 for D13S317. In the control sample, *F*is varied from -0.050 for D13S317 to +0.063 for D16S539. Overall *F*is values for the PKU and control samples were 0.042 and 0.003, respectively, in spite of the low overall genetic differentiation among samples (*F*st = 0.0018). The *F*it estimate for both samples together amounted to 0.02.

Comparing the PKU and control samples, significant genic differentiation was observed at two loci, CSF1PO (P-value = 0.03456, SE = 0.00137) and D14S1434 (P-value = 0.02753, SE = 0.00120). However, when all loci were analyzed together no genic differentiation could be observed between the samples (chi squared: 27.82; df: 22; P-value = 0.18). Significant genotypic differentiation was observed only for the D14S1434 locus (P-value = 0.0244; SE = 0.0027). Again, genotypic differentiation among samples, when all the loci were considered together, was not significant (chi squared: 26.23; df: 22; P-value = 0.24).

Two different strategies were used in order to approximate the frequency of the PKU allele (q). In the first one, *F*is values estimated for the PKU sample (0.042) was used as *F* in the

formula (1), and in the second, the *F*is value of the control sample (0.003) was used. With these strategies, estimates of PKU allele frequencies were 0.0011 and 0.0057, respectively.

DISCUSSION

Estimation of q allele frequency for rare genetic diseases is limited by difficulties in collecting samples. Generally, patients are ascertained at genetic counseling centers and samples are not representative. This problem is solved when the samples are collected by means of neonatal screening programs with high coverage rates. In Minas Gerais State, the neonatal screening program covers 98% of newborns and has been useful in estimating frequencies of alleles leading to diseases. Diseases screened in the program are: hypothyroidism, phenylketonuria, sickle cell disease, cystic fibrosis, and congenital toxoplasmosis (Aguiar 2004). However, inbreeding and other aspects of the population structure may result in misleading q estimates for recessive disorders.

Inbreeding estimates in populations are usually based on interviews (family history), analysis of civil or religious marriage records, or consecutive birth series (Vogel & Motulsky 1996). Inbreeding coefficient estimations based on family history may be underestimated due to difficulties in obtaining information about more than 2 or 3 generations. The other methods may be limited to sociocultural strata (those who marry) or to fertile matings (Vogel & Motulsky 1996). In spite of some studies suggesting that multilocus heterozygosity is only weakly correlated with the individual inbreeding coefficient (Balloux et al. 2004; Carothers et al. 2006), methods to estimate the population inbreeding coefficient based on microsatellites have been extensively used in the last few years (Pariset et al. 2003; Luiz et al. 2007). There is no ideal approach to estimate the population inbreeding coefficient, and results may vary depending on the methods chosen. Perhaps, a better approximation would be obtained by comparing results from different strategies.

Besides, population structure may be difficult to ascertain. Some demes are easily identifiable, such as Ashkenazi Jews, gypsies and Rajpoot (Motulsky 1980; Wang et al. 2000; Martin & Gamella 2005). Demes generated by distance and cultural or socio-economic factors may be elusive, particularly when occurring within large populations. Therefore, populations considered as homogeneous may have some levels of substructure.

The frequency of consanguineous marriages varies greatly among human populations (Vogel & Motulsky 1996; Jaber et al. 1998). In some regions of Africa and Asia, consanguineous marriages represent 22 to 55% of total marriages (Modell 1991; el Hazmi et al. 1995). For Europe and North America, rates of consanguineous marriages of about 0.5% have been described (Bittles 1994; Jaber et al. 1998), and high inbreeding coefficients have been found only in some small communities or in religious, geographic and ethnic isolates (Vogel & Motulsky 1996). In South America, the frequency of consanguineous marriages based on a series of consecutive normal births (ECLAMC - Latin American Collaborative Study of Congenital Malformations) was estimated to be close to 1%, and Brazil has shown one of the highest values (1.6%) (Liascovich et al. 2001). Inbreeding coefficient estimated by marriage records for Brazilian population was 0.002, but with great heterogeneity among regions (Freire-Maia 1957). The South region of the country is characterized by the lowest frequency of consanguineous marriages and the Northeast region by the highest coefficients of inbreeding. Other regions show intermediate values.

For Minas Gerais State, the inbreeding coefficient was estimated to be 0.00305 (Freire-Maia 1957). This value is amazingly close to that obtained in our study (Fis= 0.003).

In the present study, *F*is values found for the PKU sample were almost 15 times higher than that found among controls (0.042 and 0.003, respectively). High consanguinity between the parents of the PKU patients was also ascertained through medical questionnaires, where it was reported for 16/72 (22%) of the patients for whom information was available (Santos et

al. 2006). The high *Fis* estimated corroborates the frequency of consanguineous marriage reported by the parents.

We also estimated some parameters for genetic differentiation between PKU patients and control samples, representing the Minas Gerais population. In spite of all the differences found between the two samples analyzed, such as genic and genotypic differentiation for some loci (CSF1PO and D14S1434), deviations from HW expectations for some loci in the PKU sample (D7S820, D13S317 and D3S1358), and *F*is values almost 15 times higher among PKU patients, differences found were not sufficient to establish a genetic differentiation between the samples (*F*st = 0.0018). This result was expected, since both samples were extracted from the same population and because PKU families do not constitute any kind of isolate.

Assuming random matings and no deviations from Hardy-Weinberg expectations, the q allele frequency (representing all alleles resulting in PKU) could be estimated from the PKU incidence. For the Minas Gerais State population, with a PKU incidence of 1:20,000, the frequency of q would be 0.0071 and 2pq would be 0.0141. When the inbreeding coefficient found for the control sample from Minas Gerais population is used in this estimate, the q value obtained is 0.0057 and 2qp is 0.011. Taking into account this q value, PKU incidence would be 1:30,487. This correction enables us to infer that approximately 35% of the PKU recessive homozygotes from the Minas Gerais population could be due to consanguineous marriages. However, if we estimate the frequency of q allele using the *Fis* value estimated for the PKU sample itself, the q frequency would be much lower (0.0011), and the impact of the consanguinity in the PKU incidence would be still stronger.

In spite of some evidence suggesting a low sensitivity for microsatellites in estimating the individual *f* coefficient (Carothers et al. 2006), the method offers a useful alternative in

determining inbreeding coefficients in human populations and, therefore, for approximating the effects of inbreeding on allele frequencies for recessive diseases.

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REFERENCES

Aguiar, M.J. (2004) Genetic services and research in the state of Minas Gerais, Brazil. *Community Genet.* 7, 117-20.

Balloux, F., Amos, W., Coulson, T. (2004) Does heterozygosity estimate inbreeding in real populations? *Mol Ecol.* 13, 3021-31.

Bittles, A.H. (1994) The role and significance of consanguinity as a demographic variable. *Pop Dev Rev*. 20, 561 - 584.

Butler, J.M. & Coble, M.D. (2007) Authors` Response. *J. Forensic Sci*. 52, 494.

Carothers, A.D., Rudan, I., Kolcic, I., Polasek, O., Hayward, C., Wright, A.F., Campbell, H., Teague, P., Hastie, N.D., Weber, J.L. (2006) Estimating human inbreeding coefficients: comparison of genealogical and marker heterozygosity approaches. *Ann Hum Genet.* 70, 666-76.

Carreiro-Lewandowski, E. (2002) Newborn screening: an overview. *Clin Lab Sci.* 15, 229-38.

Chambers, G.K., MacAvoy, E.S. (2000) Microsatellites: consensus and controversy. *Comp Biochem Physiol B Biochem Mol Biol.* 126, 455-76.

Coble, M.D. & Butler, J.M. (2005) Characterization of new miniSTR loci to aid analysis of degraded DNA. *J. Forensic. Sci*. 50, 43-53.

el-Hazmi, M.A., al-Swailem, A.R., Warsy, A.S., al-Swailem, A.M., Sulaimani, R., al-Meshari, A.A. (1995) Consanguinity among the Saudi Arabian population. *J Med Genet.* 32, 623-6.

Excoffier, L., Heckel, G. (2006) Computer programs for population genetics data analysis: a survival guide. *Nat Rev Genet.* 7, 745-58.

Freire-Maia, N. (1957) Inbreeding in Brazil. *Am J Hum Genet.* 9, 284-98.

Goudet, J., Perrin, N., Waser, P. (2002) Tests for sex-biased dispersal using bi-parentally inherited genetic markers. *Mol Ecol.* 11, 1103-14.

Guo, S.W., Thompson, E.A. (1992) Performing the exact test of Hardy-Weinberg proportion for multiple alleles. *Biometrics.* 48, 361-72.

Guttler, F. (1980) Hyperphenylalaninemia: diagnosis and classification of the various types of phenylalanine hydroxylase deficiency in childhood. *Acta Paediatr Scand Suppl.* 280, 1-80.

Hartl, D.L. & Clark, A.G. (2007) *Principles of Population Genetics, 4rd edition.* Sunderland, Massachusetts: Sinauer Associates, Inc. Publishers.

Jaber, L., Halpern, G.J., Shohat, M. (1998) The impact of consanguinity worldwide. *Community Genet.* 1, 12-7.

Levene, H. (1949) On a matching problem arising in genetics. *Annals of Mathematical Statistics*. 20, 91–94.

Liascovich, R., Rittler, M., Castilla, E.E. (2001) Consanguinity in South America: demographic aspects. *Hum Hered.* 51, 27-34.

Luis, C., Cothran, E.G., Oom Mdo, M. (2007) Inbreeding and genetic structure in the endangered Sorraia horse breed: implications for its conservation and management. *J Hered.* 98, 232-7.

Martin, E. & Gamella J.F. (2005) Marriage practices and ethnic differentiation: The case of Spanish Gypsies (1870–2000). *The History of the Family* 10, 45–63

Modell, B. (1991) Social and genetic implications of customary consanguineous marriage among British Pakistanis. Report of a meeting held at the Ciba Foundation on January. *J. Med. Genet.* 28, 720-723.

Motulsky, A. G. (1980) Ashkenazi Jewish gene pools: admixture, drift and selection. In: *Population structure and genetic disorders*. (eds. Eriksson, A.W., Forsius, H., Nevanlinna, H.R., Workman, P.L., Norio, R.K), pp 353-365. London: Academic.

Raymond, M. & Rousset, F. (1995) GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *J. Heredity*. 86, 248-249.

Sambrook, J. & Russell, D. W. (2002) *Molecular Cloning a Laboratory Manual, 3rd edition*. New York: Cold Spring Harbor Laboratory Press

Santos, L.L., Magalhaes M. C., Reis A. O., Starling, A.L., Januario, J.N., Fonseca, C.G., Aguiar, M.J., Carvalho, M.R. (2006) Frequencies of phenylalanine hydroxylase mutations I65T, R252W, R261Q, R261X, IVS10nt11, V388M, R408W, Y414C, and IVS12nt1 in Minas Gerais, Brazil. *Genet Mol Res.* 5, 16-23.

Scriver, C.R. (2007) The PAH gene, phenylketonuria, and a paradigm shift. *Hum Mutat.* 28, 831-45.

Scriver, C.R. & Kaufman, S. (2001) The hyperphenylalaninemias. In *The Metabolic and Molecular Bases of Inherited Disease* (eds. Scriver, C.R., Beaudet, A.L., Sly, W.S., and Valle, D.), pp. 1667–1724. New York: McGraw-Hill.

Serjeant, G.R. (2000) Screening for sickle-cell disease in Brazil. *Lancet.* 356, 168-9.

Vogel, F. & Motulsky G. W. (1996) *Human Genetics. Problems and Approaches*, *3rd edition*. Berlin: Springer.

Wang, W., Sullivan, S.G., Ahmed, S., Chandler, D., Zhivotovsky, L.A., Bittles, A.H. (2000) A genome-based study of consanguinity in three co-resident endogamous Pakistan communities. *Ann Hum Genet.* 64, 41-9.

Weir, B.S. & Cockerham, C.C. (1984). Estimating F-statistics for the analysis of population structure. *Evolution.* 38, 1358-70.

Wright, S. (1951) The genetical structure of populations. *Ann. Eugenics*. 15, 323–354.

Pariset, L., Savarese, M.C., Cappuccio, I. and Valentini, A. (2003) Use of microsatellites for genetic variation and inbreeding analysis in Sarda sheep flocks of central Italy. *J. Anim. Breed. Genet.* 120, 425–432.

Informações relevantes

Os marcadores D14S1434, D22S1045 e D10S1248, foram padronizados antes do início deste estudo, já que se tratavam de marcadores novos no laboratório onde foi realizada a genotipagem das amostras. Para estes marcadores, e para outros três (CD4, FABP2 e D12S391), foi realizado um estudo de freqüência na população de Minas Gerais, utilizando o banco de dados da Geneticenter - Centro de Genética e Reprodução. Todos os parâmetros estatísticos foram estimados. O artigo resultante deste trabalho foi submetido à Forensic Science International e se encontra como anexo desta tese porque escapa ao escopo do trabalho (ANEXO 3).

CAPÍTULO 5

TRATAMENTO PARA FENILCETONÚRIA E RESPONSIVIDADE AO BH4

Aspectos gerais

Os estudos relacionados à fenilcetonúria tiveram início em 1934 e mesmo nos dias atuais a doença é alvo freqüente de pesquisas no mundo todo. Hoje em dia, uma grande atenção é dispensada em se conhecer o perfil mutacional de várias populações e principalmente na busca por novas terapias menos rigorosas e que ofereçam uma qualidade de vida melhor para os pacientes.

Uma série de terapias estão sendo desenvolvidas e testadas, e provavelmente em um futuro próximo o tratamento de cada paciente será instituído através de uma combinação destas terapias de acordo com as suas necessidades. Diante de todas as alternativas de tratamento que surgiram nos últimos anos, foi realizada uma análise crítica da literatura, e escrito um artigo de revisão, que se segue.

The time has come: a new scene for PKU treatment

Dados complementares

Como discutido anteriormente, os pacientes com PKU têm a qualidade de vida bastante prejudicada pelas grandes restrições dietéticas, que são necessárias para o desenvolvimento normal. Com a expectativa de que estes pacientes possam ter uma vida melhor com a terapia do BH4, associada ou não a dieta pobre em fenilalanina, o conhecimento das mutações presentes no gene PAH já é considerado um passo crucial para um diagnóstico mais preciso.

Atualmente, conhecer quais seriam os indivíduos que responderiam a um determinado tratamento e oferecer a segurança de doses administradas em quantidades corretas é foco de pesquisa de várias instituições.

Responsividade ao BH4

Existe uma série de estudos no mundo todo relatando os testes de administração do cofator BH4 e a associação da resposta ao genótipo do paciente. De acordo com os genótipos encontrados nos indivíduos com PKU de Minas Gerais faremos uma análise da literatura para tentarmos estabelecer quais responderiam ao tratamento com o cofator.

Mutações associadas à responsividade ao cofator BH4

Para que um paciente responda ao tratamento com o BH4 parece ser necessário alguma atividade enzimática residual. Seria bastante improvável que pacientes portando dois alelos nulos (PKU clássica) respondessem ao tratamento, já que a enzima tem a atividade completamente abolida (Blau & Erlandsen 2004). Alguns pacientes com PKU clássica já se mostraram responsivos ao tratamento, entretanto em todos eles pelo menos um alelo era parcialmente ativo (Bernegger & Blau, 2002, Blau & Erlandsen, 2004, Hennermann e cols., 2005).

Dos 78 pacientes com PKU da amostra do presente estudo, 19 deles têm genótipos que não condizem com resposta ao BH4, ou seja, possuem dois alelos nulos (TAB. 1).

A PRA e a responsividade ao BH4 foram determinadas em vários estudos (Bernegger & Blau, 2002; Blau & Erlandsen 2004; Perez-Dueñas e cols., 2004; Desviat e cols., 2004, Fiege e cols., 2005).

TABELA 1

Genótipos dos pacientes com PKU que provavelmente não responderiam ao tratamento com o cofator BH4

Ident.	alelo1	PRA	alelo2	PRA	Ident.	alelo1	PRA	alelo2	PRA
126	P281L	<1	P281L	<1	228	IVS10-11G>A	0	IVS10-11G>A	0
62	P281L	<1	R261X	<1	114	IVS10-11G>A	0	IVS10-11G>A	0
90	P281L	<1	R252W	0	232	IVS10-11G>A	0	IVS10-11G>A	0
100	R252W	0	R252W	0	142	IVS2+5G>C	0	IVS2+5G>C	0
215	R252W	0	IVS2+5G>C	0	110	IVS2+5G>A	0	IVS2+5G>A	0
86	R261X	<1	R261X	<1	111	IVS2+5G>A	0	IVS2+5G>A	0
77	IVS10-11G>A	0	IVS12nt1g>a	0	84	IVS2+5G>A	0	IVS2+5G>A	0
146	IVS10-11G>A	0	IVS2+5G>C	0	134	IVS10-11G>A	0	IVS2+5G>C	0
154	IVS10-11G>A	0	Q267X	0	161	IVS10-11G>A	0	IVS2+5G>C	0
78	IVS10-11G>A	0	IVS10-11G>A	0					

Vinte pacientes apresentam genótipos que muito provavelmente responderiam ao tratamento (TAB. 2), pois possuem combinações de alelos com atividade enzimática residual prevista consideráveis e já foram descritos como responsivos em vários estudos (Desviat e cols., 2004, Fiege e cols., 2005; Leuzzi e cols., 2006).

Uma situação interessante envolve o alelo R261Q que tem uma atividade enzimática residual demontrada por estudos de expressão *in vitro* de 30% e em homozigose tem uma resposta bastante eficiente ao BH4 (Fiege e cols., 2005). Em heterozigose parece não responder ao tratamento dependendo do outro alelo. Por exemplo: R261Q/I65T não responde, R261Q/V388M responde. Estas diferenças podem ser devidas ao nível de atividade residual prevista (PRA) para o alelo, já que a I65T tem um PRA de ~23% e a V388M de 43%. Entretanto, mesmo com uma PRA de 23% esperava-se algum nível de resposta já que nenhum dos dois alelos é nulo.

TABELA 2

Genótipos dos pacientes com PKU que provavelmente respondem ao tratamento com o cofator BH4

Ident.	alelo1	PRA	alelo2	PRA	Ident.	alelo1	PRA	alelo2	PRA
63	R261Q	30	R261Q	30	68	V388M	43	I65T	23
69	R261Q	30	R261Q	30	81	V388M	43	I65T	23
85	R261Q	30	R261Q	30	124	V388M	43	I65T	23
116	R261Q	30	R261Q	30	70	V388M	43	V388M	43
224	R261Q	30	R261Q	30	71	V388M	43	V388M	43
64	R261Q	30	V388M	43	95	V388M	43	V388M	43
151	R261Q	30	V388M	43	101	V388M	43	V388M	43
157	R261Q	30	V388M	43	109	V388M	43	V388M	43
112	R261Q	30	V388M	43	226	V388M	43	V388M	43
82	V388M	43	I65T	23	230	V388M	43	V388M	43

Vários genótipos se demonstram inconsistentes, as vezes se associando à resposta, as vezes não (TAB. 3). A mutação IVS10-11G>A em heterozigose, por exemplo, já foi associada com resposta ao BH4 em um estudo recente (Fiege e cols., 2005), entretanto, em DESVIAT e cols., 2004, nenhum genótipo que tinha como alelo integrante a mutação IVS10-11G>A, respondeu ao tratamento.

As razões para esta variabilidade interindividual de resposta não são conhecidas. Estudos recentes têm sugerido que esta variabilidade possa ser devida a diferenças na absorção do BH4 e farmacocinética (Fiege e cols., 2003)

Na TAB. 3 estão descritos além dos genótipos que se apresentam inconsistentes aqueles que não foram encontrados em estudos de resposta ao cofator, para os quais, portanto, não temos informações.

TABELA 3

Genótipos dos pacientes com PKU associados à respostas inconsistentes ao BH4, ou para os quais não há informação disponível

Ident.	alelo1	PRA	alelo2	PRA	Ident.	alelo1	PRA	alelo2	PRA
66	R261Q	30	L348V	25	218	V388M	43	IVS10-11G>A	0
97	R261Q	30	L348V	25	233	V388M	43	IVS10-11G>A	0
120	R261Q	30	I65T	23	115	V388M	43	IVS10-11G>A	0
147	R261Q	30	L48S	39	93	IVS10-11G>A	0	L348V	25
136	R261Q	30	R252W	0	103	IVS10-11G>A	0	R243Q	10
139	R261Q	30	R252W	0	145	P281L	<1	L249F	?
165	R261Q	30	R270K	?	118	I65T	23	I65T	23
106	R261Q	30	IVS10-11G>A	0	105	I65T	23	I65T	23
79	R261Q	30	IVS10-11G>A	0	153	V388M	43	IVS2+5G>C	0
88	R261Q	30	R408W	0	159	IVS2+5G>A	0	L348V	25
74	V388M	43	R252W	0	73	R270K	?	R270K	?
98	V388M	43	R252W	0	130	L348V	25	IVS7nt1g>a	0
80	V388M	43	IVS10-11G>A	0	148	R158W	?	R158W	?
65	R261Q	30	G352>Vfs	0	229	V388M	43	L249F	?
92	V388M	43	G352>Vfs	0	155	IVS10-11G>A	0	F410C	?
99	V388M	43	G352>Vfs	0	94	IVS10-11G>A	0	L249F	?
217	V388M	43	F55>Lfs	0	141	IVS2+5G>C	0	D84Y	?
131	C217R	?	IVS2+5G>A	0	133	R176X	0	R158Q	?
132	P281L	<1	D84Y	?	149	IVS10-11G>A	0	?	?
231	?	?	?	?					

Um grande número de mutações encontradas em pacientes com resposta ao BH4 se encontram listadas na database BIOPKU (http://www.bh4.org/biopku.html).

CONCLUSÕES GERAIS

No presente estudo, nós investigamos as bases moleculares da PKU em Minas Gerais, com base em indivíduos fenilcetonúricos triados pelo programa de triagem neonatal do Estado. Este estudo finaliza a pesquisa iniciada em 2001, que teve por finalidade conhecer o perfil de mutações presentes nos pacientes com PKU de Minas Gerais. A pesquisa teve ainda uma abrangência maior, fornecendo informações sobre como o genótipo se correlaciona com os fenótipos apresentados pelos pacientes de Minas Gerias, sobre o coeficiente de endogamia desta população e nos pacientes com a doença, a freqüência do alelo causador da PKU no Estado e além disso, sobre os tratamentos alternativos que vêm surgindo nos últimos anos e os prováveis pacientes que responderiam ao tratamento com o cofator BH4.

Com base nos resultados encontrados podemos concluir que a população de Minas Gerais possui um perfil de mutações bastante heterogêneo. A maioria das mutações encontradas é de origem Européia, principalmente da Penísula Ibérica. Entretanto, a população do Estado, por ser fruto de um processo de miscigenação, possui também mutações que são raras na Europa e principalmente, haplótipos que já foram descritos em populações Africanas. A diversidade alélica observada no gene PAH assim como as grandes diferenças nas freqüências das mutações encontradas, quando comparadas a outras populações, ficaram evidentes neste estudo. A heterogeneidade encontrada reflete muito bem a complexa mistura étnica presente em nosso país juntamente com as particularidades nas formas de povoamento do Estado. A mutação nova encontrada será acrescentada no banco de dados *PAH*db.

Pela primeira vez, foi feita uma análise do efeito da endogamia na freqüência alélica da PKU através de microssatélites. A metodologia se mostrou eficiente, e nós podemos concluir que a endogamia tem um grande impacto na freqüência alélica da PKU em Minas Gerais sendo responsável por aproximadamente 35% da incidência da doença no Estado.

Os valores de fenilalanina pré-tratamento, medidos na primeira consulta dos pacientes, foi o valor que obteve maior correlação com os genótipos e com os valores de atividade enzimática residual. Além disso, o genótipo se mostrou um bom preditor do curso clínico da doença. O sistema de valores arbitrários pareceu ser bastante eficiente para os genótipos que possuíam dois alelos nulos, entretanto, para os genótipos com duas mutações com atividade enzimática residual, o sistema de previsão é falho, já que estes genótipos levaram em todos os casos a fenótipos diferentes.

De acordo com os genótipos encontrados nos pacientes com PKU, 20 deles provavelmente responderiam ao tratamento com o BH4. Este resultado pode ser útil para o delineamento de estratégias de tratamento futuros para os indivíduos com PKU em Minas Gerais.

Portanto, este trabalho contribuiu para o maior conhecimento da fenilcetonúria em Minas Gerais, em todos os aspectos, seja ele molecular, populacional ou clínico.

Referências

Acosta, A., Silva, W., Jr., Carvalho, T., Gomes, M. and Zago, M. (2001). Mutations of the phenylalanine hydroxylase (PAH) gene in Brazilian patients with phenylketonuria. *Hum Mutat* 17: 122-30.

Aguiar, M.J. (2004) Genetic services and research in the state of Minas Gerais, Brazil. *Community Genet.* 7: 117-20.

Antshel, K.M., Waisbren, S.E. (2003) Timing is everything: executive functions in children exposed to elevated levels of phenylalanine. *Neuropsychology.* 17: 458-68.

Avigad, S., Kleiman, S., Weinstein, M., Cohen, B.E., Schwartz, G., Woo, S.L., Shiloh, Y. (1991) Compound heterozygosity in nonphenylketonuria hyperphenylalanemia: the contribution of mutations for classical phenylketonuria. *Am J Hum Genet.* 49: 393-9.

Benit, P., Rey, F., Blandin-Savoja, F., Munnich, A., Abadie, V., Rey, J. (1999) The mutant genotype is the main determinant of the metabolic phenotype in phenylalanine hydroxylase deficiency. *Mol Genet Metab.* 68: 43-7.

Bernegger, C. and Blau, N. (2002). High frequency of tetrahydrobiopterin-responsiveness among hyperphenylalaninemias: a study of 1,919 patients observed from 1988 to 2002. *Mol Genet Metab* 77: 304-13.

Bilginsoy, C., Waitzman, N., Leonard, C.O., Ernst, S.L. (2005) Living with phenylketonuria: perspectives of patients and their families. *J Inherit Metab Dis.* 28: 639-49.

Blau, N. and Erlandsen, H. (2004). The metabolic and molecular bases of tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency. *Mol Genet Metab* 82: 101-11.

Castro-Magalhães, M. (2003). Freqüência das mutações V388M, R408W, IVS10nt1 e IVS12nt1 e em indivíduos com fenilcetonúria do Estado de Minas Gerais. Dissertação apresentada para obtenção do título de Mestre ao Departamento de Biologia Geral, UFMG, Belo Horizonte.

Christ, S.E. (2003) Asbjorn Folling and the discovery of phenylketonuria. *J Hist Neurosci.* 12: 44-54.

Clague, A., Thomas, A. (2002) Neonatal biochemical screening for disease. *Clin Chim Acta.* 315: 99-110.

Clark, A.G. (1998) Mutation-selection balance with multiple alleles. *Genetica.* 102-103: 41-7.

Daiger, S.P., Chakraborty, R., Reed, L., Fekete, G., Schuler, D., Berenssi, G., Nasz, I., Brdicka, R., Kamaryt, J., Pijackova, A., et al. (1989) Polymorphic DNA haplotypes at the

phenylalanine hydroxylase (PAH) locus in European families with phenylketonuria (PKU). *Am J Hum Genet.* 45: 310-8.

Desviat, L.R., Perez, B., Belanger-Quintana, A., Castro, M., Aguado, C., Sanchez, A., Garcia, M.J., Martinez-Pardo, M. and Ugarte, M. (2004). Tetrahydrobiopterin responsiveness: results of the BH4 loading test in 31 Spanish PKU patients and correlation with their genotype. *Mol Genet Metab* 83: 157-62.

Dipple, K.M., McCabe, E.R. (2000) Phenotypes of patients with "simple" Mendelian disorders are complex traits: thresholds, modifiers, and systems dynamics. *Am J Hum Genet.* 66: 1729-35.

Eiken, H.G., Knappskog, P.M., Boman, H., Thune, K.S., Kaada, G., Motzfeldt, K., Apold, J. (1996) Relative frequency, heterogeneity and geographic clustering of PKU mutations in Norway. *Eur J Hum Genet.* 4: 205-13.

Eisensmith, R.C., Martinez, D.R., Kuzmin, A.I., Goltsov, A.A., Brown, A., Singh, R., Elsas, L.J., II, Woo, S.L. (1996) Molecular basis of phenylketonuria and a correlation between genotype and phenotype in a heterogeneous southeastern US population. *Pediatrics.* 97: 512-6.

Ewing, B., Hillie, L., Wend,l M.C., Green, P. (1998). Base-calling of automated sequencer traces using phred. I. Accuracy assessment. *Genome Res* 8: 175-85.

Feillet, F. (2006) [Phenylketonuria]. *Presse Med.* 35: 502-8.

Fiege, B., Ballhausen D, Kiera L (2003) Plasma tetrahydrobiopterin and its pharmacokinetics following oral administration. *Mol Genet Metab* 81:45-51.

Fiege, B., Bonafe, L., Ballhausen, D., Baumgartner, M., Thony, B., Meili, D., Fiori, L., Giovannini, M. and Blau, N. (2005). Extended tetrahydrobiopterin loading test in the diagnosis of cofactor-responsive phenylketonuria: a pilot study. *Mol Genet Metab* 86 Suppl 1: S91-5.

Fisch, R.O. (2000) Comments on diet and compliance in phenylketonuria. *Eur J Pediatr.* 159 Suppl 2: S142-4.

Ghozlan, A., Munnich, A. (2004) [MAOB: a modifier gene in phenylketonuria?]. *Med Sci (Paris).* 20: 929-32.

Gjetting, T., Romstad, A., Haavik, J., Knappskog, P.M., Acosta, A.X., Silva, W.A., Jr., Zago, M.A., Guldberg, P., Guttler, F. (2001) A phenylalanine hydroxylase amino acid polymorphism with implications for molecular diagnostics. *Mol Genet Metab.* 73: 280-4.

Gordon, D., Abajian, C., Green, P. (1998). Consed: a graphical tool for sequence finishing. *Genome Res.* 8: 195-202.

Greeves, L.G., Patterson, C.C., Carson, D.J., Thom, R., Wolfenden, M.C., Zschocke, J., Graham, C.A., Nevin, N.C., Trimble, E.R. (2000) Effect of genotype on changes in

intelligence quotient after dietary relaxation in phenylketonuria and hyperphenylalaninaemia. *Arch Dis Child.* 82: 216-21.

Guldberg, P., Levy, H.L., Hanley, W.B., Koch, R., Matalon, R., Rouse, B.M., Trefz, F., de la Cruz, F., Henriksen, K.F., Guttler, F. (1996) Phenylalanine hydroxylase gene mutations in the United States: report from the Maternal PKU Collaborative Study. *Am J Hum Genet.* 59: 84-94.

Guldberg, P., Levy, H.L., Henriksen, K.F., Guttler, F. (1996) Three prevalent mutations in a patient with phenylalanine hydroxylase deficiency: implications for diagnosis and genetic counselling. *J Med Genet.* 33: 161-4.

Guldberg, P., Rey, F., Zschocke, J., Romano, V., Francois, B., Michiels, L., Ullrich, K., Hoffmann, G.F., Burgard, P., Schmidt, H., Meli, C., Riva, E., Dianzani, I., Ponzone, A., Rey, J., Guttler, F. (1998) A European multicenter study of phenylalanine hydroxylase deficiency: classification of 105 mutations and a general system for genotype-based prediction of metabolic phenotype. *Am J Hum Genet.* 63: 71-9.

Guttler, F. (1980) Hyperphenylalaninemia: diagnosis and classification of the various types of phenylalanine hydroxylase deficiency in childhood. *Acta Paediatr Scand Suppl.* 280: 1-80.

Guttler, F., Guldberg, P. (2000) Mutation analysis anticipates dietary requirements in phenylketonuria. *Eur J Pediatr.* 159 Suppl 2: S150-3.

Hennermann, J. B., Buhrer, C., Blau, N., Vetter, B., Monch, E. (2005). Long-term treatment with tetrahydrobiopterin increases phenylalanine tolerance in children with severe phenotype of phenylketonuria. *Mol Genet Metab.* Suppl 1:86-90.

Jennings, I.G., Cotton, R.G., Kobe, B. (2000) Structural interpretation of mutations in phenylalanine hydroxylase protein aids in identifying genotype-phenotype correlations in phenylketonuria. *Eur J Hum Genet.* 8: 683-96.

Kasnauskiene, J., Cimbalistiene, L., Kucinskas, V. (2003) Validation of PAH genotype-based predictions of metabolic phenylalanine hydroxylase deficiency phenotype: investigation of PKU/MHP patients from Lithuania. *Med Sci Monit.* 9: CR142-6.

Kayaalp, E., Treacy, E., Waters, P.J., Byck, S., Nowacki, P., Scriver, C.R. (1997) Human phenylalanine hydroxylase mutations and hyperphenylalaninemia phenotypes: a metanalysis of genotype-phenotype correlations. *Am J Hum Genet.* 61: 1309-17.

Kim, S.W., Jung, J., Oh, H.J., Kim, J., Lee, K.S., Lee, D.H., Park, C., Kimm, K., Koo, S.K., Jung, S.C. (2006) Structural and functional analyses of mutations of the human phenylalanine hydroxylase gene. *Clin Chim Acta.* 365: 279-87.

Koch, R., Moseley, K.D., Yano, S., Nelson, M., Jr., Moats, R.A. (2003) Large neutral amino acid therapy and phenylketonuria: a promising approach to treatment. *Mol Genet Metab.* 79: 110-3.

Lara, L. S. (2004). Freqüência das mutações I65T, R261Q, R252W e Y414C em indivíduos com fenilcetonúria do estado de Minas Gerais. Dissertação apresentada para obtenção do título de Mestre ao Departamento de Biologia Geral, UFMG, Belo Horizonte.

Lara, L. S., Castro-Magalhães, M., Reis, A. O., Starling, A. L. P., Januário, J. N., Fonseca, C. G., Aguiar, M. J. B. and Carvalho, M. R. S. (2006) Frequencies of phenylalanine hydroxylase mutations I65T, R252W, R261Q, R261X, IVS10nt-11, V388M, R408W, Y414C, and IVS12nt1 in Minas Gerais, Brazil. Genetics and Molecular Research 5 (1): 16-23.

Leuzzi, V., Carducci, C., Chiarotti, F., Artiola, C., Giovanniello, T. and Antonozzi, I. (2006). The spectrum of phenylalanine variations under tetrahydrobiopterin load in subjects affected by phenylalanine hydroxylase deficiency. *J Inherit Metab Dis* 29: 38-46.

MacDonald, A. (2000) Diet and compliance in phenylketonuria. *Eur J Pediatr.* 159 Suppl 2: S136-41.

Matalon, R., Surendran, S., Matalon, K.M., Tyring, S., Quast, M., Jinga, W., Ezell, E., Szucs, S. (2003) Future role of large neutral amino acids in transport of phenylalanine into the brain. *Pediatrics.* 112: 1570-4.

Nickerson, D.A, Tobe, V.O., Taylo, S.L. (1997). PolyPhred: automating the detection and genotyping of single nucleotide substitutions using fluorescence-based resequencing. *Nucleic Acids Res.* 25: 2745-51.

Okano, Y., Eisensmith, R. C., Guttler, F., Lichter-Konecki, U., Konecki, D. S., Trefz, F. K., Dasovich, M., Wang, T., Henriksen, K., Lou, H. et al. (1991). Molecular basis of phenotypic heterogeneity in phenylketonuria. *N Engl J Med. 324*: (18) 1232-8

Olsson, G.M., Montgomery, S.M., Alm, J. (2007) Family conditions and dietary control in phenylketonuria. *J Inherit Metab Dis.*

OMIM: http://www.ncbi.nlm.nih.gov/sites/entrez?db=OMIM

PAHdb: http://www.pahdb.mcgill.ca/

Perez, B., Desviat, L.R., Gomez-Puertas, P., Martinez, A., Stevens, R.C., Ugarte, M. (2005) Kinetic and stability analysis of PKU mutations identified in BH4-responsive patients. *Mol Genet Metab.* 86 Suppl 1: S11-6.

Perez, B., Desviat, L.R., Ugarte, M. (1997) Analysis of the phenylalanine hydroxylase gene in the Spanish population: mutation profile and association with intragenic polymorphic markers. *Am J Hum Genet.* 60: 95-102.

Perez-Duenas, B., Vilaseca, M.A., Mas, A., Lambruschini, N., Artuch, R., Gomez, L., Pineda, J., Gutierrez, A., Mila, M. and Campistol, J. (2004). Tetrahydrobiopterin responsiveness in patients with phenylketonuria. *Clin Biochem* 37: 1083-90.

Pey, A.L., Desviat, L.R., Gamez, A., Ugarte, M., Perez, B. (2003) Phenylketonuria: genotype-phenotype correlations based on expression analysis of structural and functional mutations in PAH. *Hum Mutat.* 21: 370-8.

Przyrembel, H., Bremer, H.J. (2000) Nutrition, physical growth, and bone density in treated phenylketonuria. *Eur J Pediatr.* 159 Suppl 2: S129-35.

Reis A. H., (2003). *Freqüência da mutação R261X em pacientes com fenilcetonúria do Estado de Minas Gerais.* Monografia apresentada para obtenção do título de Bacharel em Genética, ao Departamento de Biologia Geral, UFMG, Belo Horizonte

Rivera, I., Cabral, A., Almeida, M., Leandro, P., Carmona, C., Eusebio, F., Tasso, T., Vilarinho, L., Martins, E., Lechner, M.C., de Almeida, I.T., Konecki, D.S., Lichter-Konecki, U. (2000) The correlation of genotype and phenotype in Portuguese hyperphenylalaninemic patients. *Mol Genet Metab.* 69: 195-203.

Santana da Silva, L.C., Carvalho, T.S., da Silva, F.B., Morari, L., Fachel, A.A., Pires, R., Refosco, L.F., Desnick, R.J., Giugliani, R. and Saraiva Pereira, M.L. (2003). Molecular characterization of phenylketonuria in South Brazil. *Mol Genet Metab*. 79: 17-24.

Scriver, C.C. (1998) A simple phenylalanine method for detecting phenylketonuria in large populations of newborn infants, by Robert Guthrie and Ada Susi, Pediatrics, 1963;32:318-343. *Pediatrics.* 102: 236-7.

Scriver, C.R. (2002) Why mutation analysis does not always predict clinical consequences: explanations in the era of genomics. *J Pediatr.* 140: 502-6.

Scriver, C.R. (2007). The PAH gene, phenylketonuria, and a paradigm shift. *Hum Mutat*. 28: 31-45.

Scriver, C.R., Hurtubise, M., Konecki, D., Phommarinh, M., Prevost, L., Erlandsen, H., Stevens, R., Waters, P.J., Ryan, S., McDonald, D., Sarkissian, C. (2003) PAHdb 2003: what a locus-specific knowledgebase can do. *Hum Mutat.* 21: 333-44.

Scriver, C.R., Kaufman, S.. The hyperphenylalaninemias. In The Metabolic and Molecular Bases of Inherited Disease, 8th ed. (Scriver CR, Beaudet AL, Sly WS, and Valle D, eds.) New York: McGraw-Hill, pp. 1667–1724, 2001.

Scriver, C.R., Waters, P.J. (1999) Monogenic traits are not simple: lessons from phenylketonuria. *Trends Genet.* 15: 267-72.

Serjeant, G.R. (2000) Screening for sickle-cell disease in Brazil. *Lancet.* 356: 168-9.

Spaapen, L.J., Rubio-Gozalbo, M.E. (2003) Tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency, state of the art. *Mol Genet Metab.* 78: 93-9.

Surtees, R., Blau, N. (2000) The neurochemistry of phenylketonuria. *Eur J Pediatr.* 159 Suppl 2: S109-13.

Teigen, K., Froystein, N.A., Martinez, A. (1999) The structural basis of the recognition of phenylalanine and pterin cofactors by phenylalanine hydroxylase: implications for the catalytic mechanism. *J Mol Biol.* 294: 807-23.

Thony, B., Auerbach, G., Blau, N. (2000) Tetrahydrobiopterin biosynthesis, regeneration and functions. *Biochem J.* 347 Pt 1: 1-16.

Walter, J.H., White, F.J., Hall, S.K., MacDonald, A., Rylance, G., Boneh, A., Francis, D.E., Shortland, G.J., Schmidt, M., Vail, A. (2002) How practical are recommendations for dietary control in phenylketonuria? *Lancet.* 360: 55-7.

Weglage, J., Wiedermann, D., Denecke, J., Feldmann, R., Koch, H.G., Ullrich, K., Moller, H.E. (2002) Individual blood-brain barrier phenylalanine transport in siblings with classical phenylketonuria. *J Inherit Metab Dis.* 25: 431-6.

Woo, S.L., Lidsky, A.S., Guttler, F., Chandra, T., Robson, K.J. (1983) Cloned human phenylalanine hydroxylase gene allows prenatal diagnosis and carrier detection of classical phenylketonuria. *Nature.* 306: 151-5.

Woolf, L.I., McBean, M.S., Woolf, F.M., Cahalane, S.F. (1975) Phenylketonuria as a balanced polymorphism: the nature of the heterozygote advantage. *Ann Hum Genet.* 38: 461-9.

Zorzi, G., Thony, B., Blau, N. (2002) Reduced nitric oxide metabolites in CSF of patients with tetrahydrobiopterin deficiency. *J Neurochem.* 80: 362-4.

Zschocke, J. (2003) Phenylketonuria mutations in Europe. *Hum Mutat.* 21: 345-56.

ANEXO 1

Termo de consentimento livre e esclarecido

Projeto: ESPECTRO E FREQÜÊNCIA DAS MUTAÇÕES CAUSADORAS DE FENILCETONÚRIA NO ESTADO DE MINAS GERAIS

Este projeto está sendo proposto com o objetivo de esclarecer que mutações causam a fenilcetonúria no Estado de Minas Gerais.

A fenilcetonúria é uma doença genética. Os pacientes apresentam aumento de fenilalanina no sangue. A fenilalanina faz parte das proteínas dos alimentos. Dentro do nosso corpo, ela sofrerá modificações: através de uma série de transformações, a fenilalanina dará origem a muitos compostos importantes para o nosso organismo. Estas transformações são feitas por enzimas. A deficiência de uma destas enzimas, chamada fenilalanina hidroxilase, causa a fenilcetonúria.

Para uma pessoa apresentar fenilcetonúria, é preciso que ela receba uma cópia defeituosa (mutação) do gene que codifica a fenilalanina hidroxilase do pai e outra da mãe. Pessoas que têm uma cópia defeituosa e uma cópia normal, não têm a doença, mas podem transmiti-la a seus filhos. Estas pessoas são chamadas de heterozigotas.

Mais de 300 mutações diferentes já foram identificadas no gene da fenilalanina hidroxilase. A criança afetada pode ter duas mutações iguais ou diferentes. Algumas destas mutações já são bem conhecidas, outras não. Se uma criança com fenilcetonúria tem mutações bem conhecidas, é possível ter uma idéia mais clara sobre como a doença será nesta criança.

Para sabermos qual(ais) a(s) mutação(ções) presente(s) em uma criança precisamos colher uma pequena quantidade de sangue desta criança. Esta amostra de sangue será usada para identificarmos que mutações provocam a fenilcetonúria na criança. Quando soubermos o resultado do exame, os pais serão informados pelos médicos da equipe dos resultados.

Para a realização do exame precisamos colher uma pequena quantidade de sangue (3 a 10 ml) no braço do seu(sua) filho(a) através de agulha. O material usado é todo descartável, não causando riscos para seu(sua) filho(a). O único incômodo é a dor da picada, que é discreta e dura pouco (menos de um minuto). O exame não é obrigatório. Cabe aos pais ou responsáveis decidir se desejam autorizar a participação da criança na pesquisa ou não. Se a pessoa não desejar autorizar a participação da criança na pesquisa, não. tem problema, a criança continuará a ser atendida da mesma forma pelos membros da equipe.

Caso os pais autorizem a participação da criança na pesquisa, garantimos a todos o sigilo sobre os dados clínicos e laboratoriais, e a proteção de sua identidade, em caso de publicação na imprensa científica ou leiga.

O participante deve ter ciência de que, mesmo após a assinatura deste termo, ele pode se desligar do projeto, à qualquer momento, se isto lhe convier. No final do termo de consentimento há os telefones do pesquisador e do órgão da Universidade que controla as pesquisas. Qualquer dúvida, os pais ou responsáveis poderão ligar para um dos números. Uma cópia deste termo será entregue aos pais ou responsáveis.

Eu, ---, --------------- anos,
tendo lido o terno acima e esclarecidas eventuais dúvidas. declaro minha decisão em participar do projeto de pesquisa.

Belo Horizonte, -----1-----1-----.

Eu, --, -------------- anos,
abaixo assinado,----------- mãe,-------------- pai,------------- ou responsável por
--, ------------- anos, tendo lido o termo acima e esclarecido todas as minhas dúvidas, autorizo a participação de meu filho (ou minha filha) no projeto de pesquisa.

Belo Horizonte, -----1-----1------.

Assinatura da mãe, pai ou responsável:---.

Eu, -- abaixo assinado,
confirmo ter lido este termo de consentimento informado juntamente com o (a)
Sr.(a) --, respondido suas perguntas e explicado todas as possíveis implicações de sua participação no projeto.
Confirmo outrossim que deixei claro que caso não desejem participar na pesquisa isto não terá qualquer repercussão no atendimento que recebem neste Serviço.

Belo Horizonte, -----1-----1------.

Assinatura do profissional:---.

Pesquisador responsável: Marcos José B. Aguiar

Telefone: 3274-3453

Comitê de Ética em Pesquisa da Universidade Federal de Minas Gerais

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ANEXO 2

Artigo publicado na Genetics and Molecular Research

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

ANEXO 3

Validation of six STR/miniSTR loci (CD4, FABP2, D12S391, D14S1434, D22S1045, and D10S1248) for forensic purposes in Southeastern Brazil

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Abstract: Allele frequencies for six STR/miniSTR loci were determined in a sample of unrelated individuals from Southeastern Brazil. No significant deviations from Hardy-Weinberg equilibrium proportions were observed for the loci investigated (P values ≥ 0.2320). Statistical parameters of forensic interest as heterozygosity (H), power of discrimination (PD) and power of exclusion (PE) were estimated. Except for marker FABP2, all STR/miniSTRs tested showed observed heterozygosities over 0.66. Combined power of discrimination and power of exclusion were 0.9999993 and 0.9925, respectively. Due to their easiness of analysis and high informativity, these new STR multiplexes will be useful for extending current marker sets for forensic and paternity purposes.

Keywords: STR/miniSTR, Allele frequencies, Southeastern Brazil

Population: Blood samples or buccal swabs were taken from 500 unrelated individuals ascertained from paternity cases investigated at Geneticenter - Centro de Genética e Reprodução (Minas Gerais, Brazil). All individuals signed an informed consent. Individuals were mainly from Minas Gerais and São Paulo States (~ 80%) and the rest of them were from the two other states of the Southeastern Brazil (Espírito Santo and Rio de Janeiro States). The Southeastern Brazil is the most populous region with an extension of 924,511.292 Km^2 and 78.6 millions of inhabitants [1]. This is a highly miscegenated population composed of Amerindians, Europeans, and African descendents [2]. In complex kinship analysis, paternity testing in deficiency cases or within related individuals, current CODIS markers set does not always provide sufficiently high discriminatory power. Therefore, these STRs were evaluated in order to increase the number of markers available for forensic and paternity tests for this population. There are no other studies with these markers in the Brazilian population, except for CD4 locus [3].

Extraction: DNA samples were extracted by standard Chelex 100 (Promega) methods.

PCR: Amplifications were performed in 12.5 µl volume using 50 mM KCl, 10 mM Tris - HCl pH 8.4, 0.1% Triton X-100, 1.5 mM $MgCl_2$ buffer, 0.1 mM of dNTPs, 0.6 U of Taq DNA polymerase, 0.2 mM of each specific primers and 10 ng of genomic DNA. Primer sequences, motifs and alleles in reference DNA for the loci tested are described on table 1. In order to save time and costs, two multiplexes were established, one composed of CD4 and FABP2, and the other of D14S1434, D22S1045 STRs, and D10S1248 miniSTR. D12S391 STR was amplified alone. All PCR amplifications entailed a first denaturation step at 96Cº for 2 min., followed by 10 cycles of 1 min. at 94 Cº, 1 min. at 60 Cº and 1.5 min. at 70Cº, and by 20 cycles of 1 min at 90 Cº, 1 min at 60 Cº, 1.5 min at 70 Cº and a last extension step at 72 Cº for 5min.

Typing: STR/miniSTR alleles were separated on 6% denaturing polyacrylamide gels. Fragments were visualized with silver nitrate staining. STRs ladders were assembled by reamplification of PCR products from several individuals, in order to include all the different alleles observed. A commercially available reference DNA sample (K562) was genotyped as reference standard. The length of the amplified fragments was confirmed by analysis in GenBank through the web program BLAST [12]. Alleles were designed by comparison with the allelic ladders. FABP2 STR alleles were designed using as reference the allele present in the sequence deposited in GenBank (NT 016354, GI 88977422), which contains 10 repeats and generates a 205 bp PCR product. K562 DNA was typed for FABP2 STR in the laboratory (genotype 10/11; table 1). Allele designations for D10S1248, D14S1434, and D22S1045 STRs were changed as suggested elsewhere [8, 13].

Quality control: Proficiency testing of the GEP-ISFG working group.

Table 1 – Primer sequences, motifs, and alleles in reference DNA for the loci tested

Multiplex/ monoplex	Markers/refs	Motif	K562 DNA	Primer sequence 5´- 3´
1	CD4 [a] [4, 5]	(AAAAG)n	9/9	5'-TTGGAGTCGCAAGCTGAACTAGCG-3' 5'-CCAGGAAGTTGAGGCTGCAGTGAA-3'
	FABP2 [6]	(TTA)n	10/11 [b]	5'-AACTCAGAACAGTGCCTGAC-3' 5'-ATTTCCCTCAAGGCTCCAGGT-3'
2	D14S1434 [7, 8]	(CTRT)n	10/10	5'-ACAATTCCAGAAACTTCCCC-3' 5'-ATCAGTGAGCCAATTCCTTG-3'
	D22S1045 [7, 8]	$[ATT]_n$ ACT $[ATT]_2$	16/16	5'-GCTAGATTTTCCCCGATGAT-3' 5'-ATGTAAAGTGCTCTCAAGAGTGC-3'
	D10S1248 [7, 8]	(GGAA)n	12/12	5' TTAATGAATTGAACAAATGAGTGAG-3' 5'GCAACTCTGGTTGTATTGTCTTCAT-3'
3	D12S391 [9, 10, 11]	(AGAR)n	23/23	5'-AACAGGATCAATGGATGCAT-3' 5'-TGGCTTTTAGACCTGGACTG-3'

[a] the number of repeats results from using the bottom strand; [b] K562 typed in the Lab.

Results: Allele frequencies and forensic parameters for the six STR/miniSTR loci are shown in table 2.

Analysis of data: Observed heterozygosity (OH), polymorphic information content (PIC), power of discrimination (PD), and power of exclusion (PE) were estimated for each locus. Possible deviations from Hardy-Weinberg equilibrium were tested by Fisher´s exact test. Statistical analyses were carried out using the GENEPOP software version 3.4, Cervus version 2.0, and PowerStats Promega V12 [14, 15, 16]. Population differentiation tests were carried out with the GENEPOP software version 3.4 [17]. The level of significance adopted was 0.05 for these analyses.

Access to data: See supplementary data.

Table 2 - Observed frequencies and forensic parameters for the six STR/miniSTR loci (D12S391, CD4, FABP2, D14S1434, D22S1045, D10S1248) in Southeastern Brazil

Allele	D12S391	CD4	FABP2	D14S1434	D22S1045	D10S1248
3		0.001				
4		0.384				
5		0.198				
6		0.005				
7		0.044				
8		0.014	0.013			
9		0.248	0.057	0.004		
10		0.063	0.520	0.198	0.011	
11		0.023	0.082	0.037	0.112	0.022
12		0.013	0.030	0.034	0.019	0.049
13		0.007	0.214	0.317	0.004	0.276
14			0.081	0.388	0.052	0.265
15	0.065		0.003	0.019	0.302	0.243
16	0.026			0.004	0.351	0.101
17	0.108				0.142	0.041
17.3	0.007				-	-
18	0.243				0.004	0.004
18.3	0.017				-	-
19	0.149				0.004	
19.3	0.005					
20	0.106					
21	0.103					
22	0.091					
23	0.046					
24	0.023					
25	0.012					
n	302	500	500	134	134	134
OH	0.868	0.782	0.656	0.716	0.739	0.821
PD	0.968	0.890	0.853	0.853	0.899	0.902
PE	0.730	0.566	0.364	0.454	0.491	0.638
PIC	0.86	0.71	0.63	0.66	0.71	0.74
P values	0.2320	0.5621	0.9480	0.2224	0.9553	0.3993

N: sample size; OH: observed heterozygosity; PD: power of discrimination; PE: power of exclusion; PIC: polymorphic information content; p values: Hardy-Weinberg equilibrium.

Other remarks: STR systems chosen for this study have been recently described and there is a lack of published information about their allelic distributions in different populations, especially in Brazil. In order to ascertain the usefulness of these loci for forensic and paternity purposes in Brazilian population, frequencies and other statistical parameters were estimated. For all loci examined, no deviations from Hardy-Weinberg equilibrium were found (P values = 0.2320 – 0.9553). Observed heterozygosities ranged from 0.66 to 0.87. Combined power of discrimination and power of exclusion for the six studied STR loci were 0.9999993 and 0.9925, respectively.

Comparison of the allele frequencies found in this study with published data for other populations were done. No significant differences (P> 0.05) were found for D10S1248 system when our results were compared with those from Chinese, Spanish, and African American populations. On the other hand, significant differences in allele frequencies were observed when compared with those published for D14S1434 in the Chinese population (P=0.0001) and for D22S1045 in Chinese and African American populations (P=0.0286 and P=0.0406, respectively) [7, 18, 19].

CD4 locus showed significant differentiation for all populations compared: China, Portugal, African Americans (P<0.001), and interestingly, also for a study with a sample from São Paulo State, Brazil (P= 0.0374) [3, 20].

Allele frequencies in D12S391 locus revealed significant differences when compared with frequencies found in the populations from China and Mozambique (P = 0.0002 and P = 0.036, respectively). In contrast, the pattern of allele distribution was very similar to the Spanish population [21, 22, 23]. Three microvariant alleles for D12S391 (17.3, 18.3, and 19.3), previously described, were also detected in this study [10, 23, 24].

Data for FABP2 is available in the literature only for the Chinese population, and no significant differences were found (P= 0.1679) by comparison of allele frequencies distributions [25].

In conclusion, high informativity, easiness of handling and conformity with Hardy-Weinberg equilibrium expectations suggest that these systems are appropriated to be used for forensic and paternity purposes in Brazilian population. This paper follows the guidelines for publication of population data request by the journal [26].

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References

[1] IBGE - Instituto Brasileiro de Geografia e Estatística - http://www.ibge.gov.br

[2] F.M. Salzano and M.C. Bortolini, The evolution and genetics of Latin American populations, Cambridge University Press, Cambridge, 2002.

[3] L.B. Ferreira, C.T. Mendes-Junior, C.E.V. Wiezel, M.R. Luizon, A.L. Simões, Genomic ancestry of a sample population from the state of São Paulo, Brazil. Am. J. Hum. Biol. 18 (2006) 702–705.

[4] B. Glock, D.W.M. Schwartz, M. Elisabeth, Schwartz-Jungl, W.R. Mayr, Sequence determination of an allelic ladder for the STR polymorphism at the CD4 locus and application of the ladder in testing an Austrian Caucasian population sample. Forensic Sci. Int. 78 (1995) 125 - 130.

[5] M.C. Edwards, P.R. Clemens, M. Tristan, A. Pizzuti, R.A. Gibbs, Pentanucleotide repeat length polymorphism at the human CD4 locus. Nucleic Acids Res. 19 (1991) 4791.

[6] M.H. Polymeropoulos, D.S. Rath, H. Xiao, C.R. Merril, Trinucleotide repeat polymorphism at the human intestinal fatty acid binding protein gene (FABP2). Nucleic Acids Res. 18 (1990) 7198.

[7] M.D. Coble, J.M. Butler, Characterization of new miniSTR loci to aid analysis of degraded DNA. J. Forensic Sci. 50 (2005) 43-53.

[8] C.R. Hill, M.D. Coble, and J.M. Butler, Characterization of 26 New miniSTR Loci, National Institute of Standards and Technology, Biochemical Science Division, Gaithersburg, http://www.cstl.nist.gov/biotech/strbase/pub_pres/Promega2006_Hill.pdf

[9] Y. Shigeta, Y. Yamamoto, Y. Doi, S. Miyaishi, H. Ishizu, Evaluation of a method for typing the microsatellite D12S391 locus using a new primer pair and capillary electrophoresis. Acta Med. Okayama 56 (2002) 229-36.

[10] B. Glock, E.M. Dauber, D.W. Schwartz, W.R. Mayr, Additional variability at the D12S391 STR locus in an Austrian population sample: sequencing data and allele distribution. Forensic Sci. Int. 90 (1997) 197-203.

[11] M.V. Lareu, M.C. Pestoni, F. Barros, A. Salas, A. Carracedo, Sequence variation of a hypervariable short tandem repeat at the D12S391 locus. Gene 182 (1996) 151-153.

[12] S.F. Altschul, W. Gish, W. Miller, E.W. Myers, D.J. Lipman, Basic local alignment search tool. J. Mol. Biol. 215 (1990) 403-10.

[13] J.M. Butler, M.D. Coble, Authors` Response. J. Forensic Sci. 52 (2007) 494.

[14] http://wbiomed.curtin.edu.au/genepop/genepop

[15] http://helios.bto.ed.ac.uk/evolgen/cervus/cervus.html

[16] http://www.promega.com/geneticidtools/powerstats/Default.htm

[17] M. Raymond and F. Rousset, GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. J. Hered. 86 (1995) 248-249.

[18] R.Y.Y. Yong, L.S.H. Gan, M.D. Coble, E.P.H. Yap, Allele frequencies of six miniSTR loci of three ethnic populations in Singapore. Forensic Sci. Int. 166 (2007) 240-243.

[19] P. Martín, O. García, C. Albarrán, P. García, I. Yurrebaso, A. Alonso, Allele frequencies of six miniSTR loci (D10S1248, D14S1434, D22S1045, D4S2364, D2S441 and D1S1677) in a Spanish population. Forensic Sci. Int. 169 (2007) 252-254.

[20] http://www.uni-duesseldorf.de/WWW/MedFak/Serology/dna.html

[21] M. Shi, X. Yu, R. Bai, X. Shu, G. Zhu, J. Lv, Y. Tu, Genetic polymorphism of 14 non-CODIS STR loci for forensic use in southeast China population. Forensic Sci. Int. (2007), doi:10.1016/j.forsciint.2007.02.021.

[22] F. Corte-Real, L. Andrade, D.N. Vieira, A. Carracedo, M.C. Vide, Allele distribution of three STRs in a population from Mozambique. Int. J. Legal Med. 112 (1999) 345-347.

[23] M.J. Farfan, P. Sanz, M.V. Lareu, A. Carracedo, Population data on the D1S1656 and D12S391 STR loci in Andalusia (south Spain) and the Maghreb (north Africa). Forensic Sci. Int. 104 (1999) 33-6.

[24] T. Ribeiro, R.M. Brito, R. Espinheira, H. Geada, New alleles of D12S391 STR locus in a Portuguese population, in: B. Olaisen, B. Brinkmann, P.J. Lincoln (Eds.), Progress in Forensic Genetics, Vol. 7, Elsevier Science B., Amsterdam, 1998, pp. 275-277.

[25] J.C Lee, C. Chen, L. Tsai, A. Linacre, J. Chang, The screening of 13 short tandem repeat loci in the Chinese population. Forensic Sci. Int. 87 (1997) 137-144.

[26] P. Lincoln, A. Carracedo, Publication of population data of human polymorphisms. Forensic Sci. Int. 110 (2000) 3-5.

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