JIMD Reports
DOI 10.1007/8904_2013_284

RESEARCH REPORT

The Molecular Bases of Phenylketonuria (PKU) in New South Wales, Australia: Mutation Profile and Correlation with Tetrahydrobiopterin (BH_4) Responsiveness

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Received: 30 September 2013 / Revised: 20 November 2013 / Accepted: 22 November 2013 / Published online: 25 December 2013

Abstract Phenylketonuria (PKU) is an autosomal recessive inborn error of phenylalanine metabolism predominantly caused by mutations in the phenylalanine hydroxylase (*PAH*) gene. Mutation screening was carried out in a large cohort of PKU patients from New South Wales, Australia. Pathogenic mutations were identified in 99% of the alleles screened, with the two most common mutations (p.R408W and IVS12+1G>A) accounting for 30.7% of alleles. Most individuals were compound heterozygotes for previously reported mutations, but four novel mutations (c.163+1G>T, c.164-2A>G, c.461A>T [p.Y154F], and c.510-1G>A) and a novel polymorphism (c.60+62C>T) were also identified. A number of patients have been previously tested for their response to dietary supplementation of tetrahydrobiopterin (BH_4), the cofactor of PAH. Correlation between genotype and the responses revealed that although genotype is a major determinant of BH_4 responsiveness, patients with the same genotype may also show disparate responses to this treatment. A clinical and biochemical evaluation should be undertaken to determine the effectiveness of PKU treatment by supplementation of BH_4.

Communicated by: Nenad Blau, PhD

Competing interests: None declared

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Introduction

Phenylketonuria (PKU, OMIM 261600) is an inborn error of metabolism of phenylalanine (Phe), with an autosomal recessive mode of inheritance. The severity of the disorder varies between patients and is classified as 'classical', 'moderate', 'mild' or hyperphenylalaninaemia (HPA), depending on the blood Phe level at the time of diagnosis or dietary Phe tolerance (Blau et al. 2010). The causative gene in the majority of PKU patients is phenylalanine hydroxylase (*PAH*), located on chromosome 12 (Woo et al. 1983). To date, more than 800 variants have been reported in *PAH* (http://www.biopku.org, last accessed 21 November 2013) and the majority of patients are compound heterozygotes. Mutations in *PAH* lead to impaired function of the hepatic enzyme, PAH (EC 1.14.16.1), which catalyses the conversion of the essential amino acid L-phenylalanine (L-Phe) to L-tyrosine (L-Tyr), a precursor of the neurotransmitters dopamine, noradrenaline and adrenaline.

A study in Victoria, Australia (Boneh et al. 2005), reported the incidence of PKU to be 1 in 11,226, while in the state of New South Wales (NSW), Australia, the incidence is 1 in 8,900, mirroring the figures found in

other Caucasian populations (Scriver and Kaufman 2001). However, the pathogenic mutations in New South Wales have not been previously studied. Phenotype–genotype correlations in PKU have shown clear associations between some mutations and the severity of disease (Kayaalp et al. 1997; Bénit et al. 1999; Güttler et al. 1999; Zschocke 2003; Bercovich et al. 2008). Due to the large number of mutations and the low population frequency of some of these mutations, it is often difficult to ascertain the phenotypic consequences of a given mutation and correlations may also give rise to conflicting results (Bercovich et al. 2008). Genotyping more cohorts of PKU patients may therefore be useful to clarify the relationship between genotype and phenotype in these patients.

Mutation screening is also of value in deciding potential treatments for patients. Dietary supplementation of sapropterin, a synthetic form of BH_4, the cofactor of PAH, is efficacious in a subset of *PAH* mutations in lowering blood Phe levels (Kure et al. 1999; Bernegger and Blau 2002). More recently, a novel set of compounds have been developed specifically targeting nonsense mutations and *in vitro* studies have shown that these compounds may be of therapeutic benefit in PKU patients carrying nonsense mutations (Howard et al. 1996; Barton-Davis et al. 1999; Welch et al. 2007; Du et al. 2009; Nudelman et al. 2010; Ho et al. 2013). Mutation screening would identify those patients for whom these approaches, including other mutation-specific therapies such as anti-aggregation compounds for p.G46S (Leandro et al. 2011), may be applicable.

The aim of this study is to screen a cohort of patients at the PKU Clinic at the Children's Hospital at Westmead to identify the mutations prevalent in NSW and to determine which patients would benefit from sapropterin treatment and potentially nonsense-related therapy.

Materials and Methods

One hundred and eleven patients, including seven families with two or more affected individuals, were recruited from the records of the NSW Newborn Screening Programme or from the PKU Clinic at the Children's Hospital at Westmead, Sydney Australia. This research was approved by the Human Ethics Committee of the Children's Hospital at Westmead. Blood samples were collected and genomic DNA was extracted using a salting out extraction protocol (Miller et al. 1988).

The 13 coding exons and the intron–exon boundaries of *PAH* were amplified by polymerase chain reaction (PCR) using a combination of primers previously published (Bräutigam et al. 2003), and new primers designed using Primer3 software (Rozen and Skaletsky 2000). To reduce the cost of screening, a tiered sequencing approach was adopted, whereby patients were screened first for mutations in exons 7 and 12. Screening of exons 2, 3, 5 and 6 were then carried out if less than two pathogenic mutations were identified, and subsequently the remainder of the exons if required.

PCR products were sequenced in both the forward and reverse directions using ABI3730XL (Life Technologies, Carlsbad, CA) by Macrogen Inc. (Seoul, Korea), and the traces were analysed using MutationExplorer™ (SoftGenetics, State College, PA). The genomic DNA reference sequence was NC_000012.11 and the cDNA reference sequence was NM_000277.1, with the A from the ATG translation initiation start site numbered +1. Mutation nomenclature is in accordance with the guidelines from Human Genome Variation Society (den Dunnen and Antonarakis 2000) and also as reported in the *PAH*db Knowledgebase (http://www.pahdb.mcgill.ca).

Novel unreported mutations were first confirmed by an independent PCR and bi-directional sequencing. In addition, a minimum of 300 control alleles were screened for each of the novel mutations to provide additional support for pathogenicity. Detection of the novel mutations was carried by restriction digests: *Bgl* II for c.60+62C>T, *Mnl* I for c.168+1G>T, *Bfa* I for c.169-2A>G, *Rsa* I for c.461A>T (p.Y154F) (all enzymes from New England Biolabs, Ipswich, MA). Control screening for c.510-1G>A was carried out using Custom TaqMan® SNP Genotyping Assay (Life Technologies) according to manufacturer's instructions.

After primary sequence analysis, there were six patients in whom only one heterozygous pathogenic mutation had been identified. There were also 14 samples with apparently homozygous mutations. All 20 samples were then screened for large deletions and duplications using multiplex ligation-dependent probe amplification (MLPA, SALSA® MLPA® kit P055, MRC-Holland, Amsterdam, The Netherlands) according to the manufacturer's protocol. Fragments were resolved on ABI PRISM® 3100 Genetic Analyzer (Life Technologies). Control samples ($n \geq 4$) were included with each run, and the average of the peak areas of each amplified fragment was calculated using these controls. The ratio of the peak areas of patient samples to the average was used to determine if a deletion or insertion was present. A value of 0.6 or lower was indicative of a deletion and a value of 1.5 or higher was indicative of duplication, as per the manufacturer's recommendations. Samples with an apparent deletion or duplication were confirmed by a second independent MLPA reaction and real-time quantitative polymerase chain reaction (QPCR). QPCR conditions are available upon request.

Results

The coding region of *PAH* (including exon-intron boundaries) was analysed in 111 PKU patients, including seven families with multiple affected sibs (six sibling pairs and one trio) and five consanguineous families, equating to 201 independent chromosomes. The genotypes are summarized in Table 1. Sixty-one different pathogenic mutations, including five previously unreported variations and two large deletions (spanning an entire exon), were identified in 199 out of the 201 independent chromosomes, representing a mutation detection rate of 99%. The majority of patients (excluding consanguineous families and counting only one individual from each family, 93/99, 93.9%) were compound heterozygotes. In all patients with apparently homozygous mutations, the possibility of hemizygosity due to the presence of a large deletion was excluded using MLPA.

The two most common mutations [c.1222C>T (p.R408W) and c.1315+1G>A (IVS12+1G>A)] accounted for 18.6% and 12.1% respectively of the alleles. In all, the nine most common mutations accounted for 57.2% of alleles (Table 2). The most common type of mutations was missense, with 38 distinct mutations in 66.8% of alleles screened. Large deletions, encompassing an entire exon, were identified in four patients using MLPA and confirmed by quantitative PCR. Two patients had a heterozygous deletion of exon 6, while another two had a heterozygous deletion of exon 3. The percentage of alleles with large deletions in this study is 2.0%, a proportion similar to other mutation studies (Mallolas et al. 1999; Kozak et al. 2006; Birk Møller et al. 2007).

Four novel pathogenic mutations were identified: c.168 +1G>T (IVS2+1G>T), c.164-2A>G (IVS2-2A>G), c.461A>T (p.Y154F) and c.510-1G>A (IVS5-1G>A). For each of these patients, all 13 exons of *PAH* were sequenced to exclude the possibility of other pathogenic mutations. In addition, a minimum of 300 chromosomes from a normal (Caucasian) population was screened using the methods described above and none of the variants in question were detected in the normal population, or in dbSNP or 1000 Genomes database. The effect of mRNA splicing for the mutations c.168+1G>T, c.169-2A>G and c.510-1G>A was analysed *in silico* using Splice Site Prediction by Neural Network provided by Berkeley Drosophila Genome Project (http://www.fruitfly.org/seq_tools/splice.html, Reese et al. 1997). All three mutations were predicted to affect the splice site signal, leading to abnormal splicing. Attempts to extract and amplify *PAH* mRNA from peripheral blood from patients carrying the c.168+1G>T and c.510-1G>A mutations were unsuccessful. The levels of *PAH* transcripts were too low to be detected (data not shown). The classical phenotype in all three patients in whom the mutations were identified was in accordance with the prediction of these mutations affecting splicing of the *PAH* transcript and leading to a null allele.

A previously unreported missense mutation was identified in one patient with moderate PKU (p.Y154F) and a second patient with mild PKU (p.Y154F and p.G103C). The former patient with moderate PKU had the c.1315 +1G>A mutation as the second allele, which results in low amounts of protein and protein activity detected in in vitro systems, and is generally assumed to be a null allele (Waters et al. 1998). The phenotypes of these two patients suggest that the missense mutation p.Y154F retains some levels of residual PAH enzymatic activity.

Ten polymorphisms were identified in the cohort, with nine having been previously reported: c.168G>A (p.E56E, in *cis* with IVS2+1G>A), c.163+19T>C (IVS2+19T>C), c.353-22C>T (IVS3-22C>T), c.441+47C>T (IVS4 +47C>T), c.510-54A>G (IVS5-54A>G), c.696A>G (p.Q232Q), c.735G>A (p.V245V), c.969+43G>T (IVS9 +43G>T), c.1155G>C (p.L385L) and c.1242C>T (p.Y414Y). A novel variant (c.60+62C>T or IVS1+62C>T) in intron 1 was designated a silent polymorphism, as it is not predicted to affect transcript splicing and is also found at a high allele frequency in the controls screened (31% of 300 normal chromosomes) and in dbSNP (rs1522296, minor allele frequency 0.35).

There were seven families in the cohort with more than one affected child. For six of the families, the siblings shared the same two pathogenic mutations. In the last family, only one allele was shared (p.R408W), with the second allele being p.A345S in one sibling and p.S87R in the other two siblings. Parental screening showed that the father was heterozygous for p.R408W and the mother heterozygous for both p.S87R and p.A345S, presumed compound heterozygous. The mother was not initially suspected of having PKU, but Phe testing carried out after genotype was confirmed revealed persistent elevated levels of blood Phe (mean 184 micromol/L), indicative of non-PKU hyperphenylalaninaemia. In addition, the patient with the genotype p.[S87R];[R408W] was classified as having classical PKU compared to his two siblings with p.[A345S];[R408W] who had HPA. Therefore, it would appear that the p.S87R allele is a more severe mutation compared to p.A345S.

A number of patients in this cohort have been previously tested for response to a BH_4-load (Mitchell et al. 2005). These patients were given BH_4 over a period of 7 days, and blood Phe levels were measured at 8 h, 32 h and the 7th day. A decrease of Phe of 30 %, compared to pre-BH_4 level, was deemed to be clinically significant (Mitchell et al. 2005). Patients are classed as 'positive' if they reached a decrease of 30% after 8 h, 'intermediate' if a decrease of 30 % was observed at 32 h or at the 7th day and 'negative' if no decrease of 30% or greater was observed at any point

Table 1 Genotypes of the 111 PKU patients screened

	Allele 1		Allele 2		
ID	cDNA[a]	Protein	cDNA[a]	Protein	Comments[b]
1	c.842C>T	p.P281L	c.1241A>G	p.Y414C	
2	c.194T>C	p.I65T	*(Not detected)*		
3	c.60+5G>T (IVS1+5G>T)		c.1315+1G>A (IVS12+1G>A)		
4	c.331C>T	p.R111*	c.1162G>A	p.V388M	
5	c.473G>A	p.R158Q	c.1315+1G>A (IVS12+1G>A)		
6	c.117C>G	p.F39L	c.1222C>T	p.R408W	
7	c.500A>T	p.N167I	c.1222C>T	p.R408W	
8	c.194T>C	p.I65T	c.434A>T	p.D145V	
9	c.727C>T	p.R243*	c.782G>A	p.R261Q	
10	c.896T>G	p.F299C	c.1222C>T	p.R408W	
11	c.117C>G	p.F39L	*(not detected)*		
12	c.838G>A	p.E280K	c.1042C>G	p.L348V	
13	c.1222C>T	p.R408W	c.1222C>T	p.R408W	
14	c.117C>G	p.F39L	c.1222C>T	p.R408W	
15	c.896T>G	p.F299C	c.1222C>T	p.R408W	
16	c.581T>C	p.L194P	c.1222C>T	p.R408W	
17	c.461A>T	p.Y154F	c.1315+1G>A (IVS12+1G>A)		p.Y154F novel
18	c.1222C>T	p.R408W	c.1315+1G>A (IVS12+1G>A)		
19	c.169-?_352+?del (exon 3 deletion)		c.842C>T	p.P281L	
20	c.1222C>T	p.R408W	c.1222C>T	p.R408W	
21	c.782G>A	p.R261Q	c.1315+1G>A (IVS12+1G>A)		
22	c.782G>A	p.R261Q	c.1208C>T	p.A403V	
23	c.1222C>T	p.R408W	c.1222C>T	p.R408W	
24	c.754C>T	p.R252W	c.912+1G>A (IVS8+1G>A)		
25	c.143T>C	p.L48S	c.1222C>T	p.R408W	
26	c.969+5G>A (IVS9+5G>A)		c.969+5G>A (IVS9+5G>A)		Consanguinity
27	c.117C>G	p.F39L	c.896T>G	p.F299C	
28	c.561G>A	p.W187*	c.1315+1G>A (IVS12+1G>A)		
29	c.117C>G	p.F39L	c.1222C>T	p.R408W	
30	c.1222C>T	p.R408W	c.1241A>G	p.Y414C	
31	c.782G>A	p.R261Q	c.842C>T	p.P281L	
32	c.194T>C	p.I65T	c.818C>T	p.S273F	
33	c.266_267insG	p.A90fs	c.266_267insG	p.A90fs	Consanguinity
34	c.1222C>T	p.R408W	c.1315+1G>A (IVS12+1G>A)		
35	c.510-?_706+?del (exon 6 deletion)		c.896T>G	p.F299C	
36	c.727C>T	p.R243*	c.912+1G>A (IVS8+1G>A)		Sibling of p37
37	c.727C>T	p.R243*	c.912+1G>A (IVS8+1G>A)		Sibling of p36
38	c.473G>A	p.R158Q	c.1315+1G>A (IVS12+1G>A)		
39	c.1222C>T	p.R408W	c.1315+1G>A (IVS12+1G>A)		
40	c.1238G>C	p.R413P	c.1238G>C	p.R413P	
41	c.194T>C	p.I65T	c.782G>A	p.R261Q	
42	c.728G>A	p.R243Q	c.728G>A	p.R243Q	Consanguinity, sibling of p43
43	c.728G>A	p.R243Q	c.728G>A	p.R243Q	Consanguinity, sibling of p42
44	c.163+1G>A (IVS2+1G>A)		c.163+1G>A (IVS2+1G>A)		Consanguinity
45	c.194T>C	p.I65T	c.1222C>T	p.R408W	
46	c.1208C>T	p.A403V	c.1222C>T	p.R408W	

(continued)

Table 1 (continued)

ID	Allele 1		Allele 2		
	cDNA[a]	Protein	cDNA[a]	Protein	Comments[b]
47	c.1222C>T	p.R408W	c.1222C>T	p.R408W	
48	c.721C>T	p.R241C	c.782G>A	p.R261Q	Sibling of p49
49	c.721C>T	p.R241C	c.782G>A	p.R261Q	Sibling of p48
50	c.194T>C	p.I65T	c.912+1G>A (IVS8+1G>A)		
51	c.1066-11G>A (IVS10-11G>A)		c.1241A>G	p.Y414C	Sibling of p110
52	c.1222C>T	p.R408W	c.1315+1G>A (IVS12+1G>A)		
53	c.1222C>T	p.R408W	c.1315+1G>A (IVS12+1G>A)		
54	c.169-?_352+?del (exon 3 deletion)		c.1241A>G	p.Y414C	
55	c.733G>C	p.V245L	c.1223G>A	p.R408Q	
56	c.896T>G	p.F299C	c.1315+1G>A (IVS12+1G>A)		Sibling of p57
57	c.896T>G	p.F299C	c.1315+1G>A (IVS12+1G>A)		Sibling of p56
58	c.117C>G	p.F39L	c.1222C>T	p.R408W	
59	c.754C>T	p.R252W	c.1315+1G>A (IVS12+1G>A)		
60	c.143T>C	p.L48S	c.1066-3C>T (IVS10-3C>T)		
61	c.782G>A	p.R261Q	c.782G>A	p.R261Q	Consanguinity
62	c.1222C>T	p.R408W	c.1241A>G	p.Y414C	
63	c.653G>T	p.G218V	c.842C>T	p.P281L	
64	c.510-1G>A (IVS5-1G>A)		c.1066-11G>A (IVS10-11G>A)		c.510-1G>A novel
65	c.1066-11G>A (IVS10-11G>A)		c.1222C>T	p.R408W	Sibling of p66
66	c.1066-11G>A (IVS10-11G>A)		c.1222C>T	p.R408W	Sibling of p65
67	c.168+1G>T (IVS2+1G>T)		c.331C>T	p.R111*	c.168+1G>T novel
68	c.194T>C	p.I65T	c.1315+1G>A (IVS12+1G>A)		
69	c.168+1G>A (IVS2+1G>A)		c.745C>T	p.L249F	
70	c.926C>T	p.A309V	c.1222C>T	p.R408W	
71	c.1139C>T	p.T380M	c.1315+1G>A (IVS12+1G>A)		
72	c.311C>T	p.A104D	c.733G>C	p.V245L	
73	1243G>A	p.D415N	c.1315+1G>A (IVS12+1G>A)		
74	c.311C>T	p.A104D	c.806delT	p.I269fs	
75	c.473G>A	p.R158Q	c.842C>T	p.P281L	
76	c.1042C>G	p.L348V	c.1241A>G	p.Y414C	
77	c.441+5G>T (IVS4+5G>T)		c.1184C>G	p.A395G	
78	c.1157A>G	p.Y386C	c.1315+1G>A (IVS12+1G>A)		
79	c.143T>C	p.L48S	c.842+3G>C (IVS7+3G>C)		
80	c.510-?_706+?del (exon 6 deletion)		c.1241A>G	p.Y414C	
81	c.754C>T	p.R252W	c.1066-11G>A (IVS10-11G>A)		
82	c.169-2A>G (IVS2-2A>G)		c.814G>T	p.G272*	c.169-2A>G novel
83	c.1222C>T	p.R408W	c.1315+1G>A (IVS12+1G>A)		
84	c.194T>C	p.I65T	c.194T>C	p.I65T	
85	c.1033G>T	p.A345S	c.1222C>T	p.R408W	Sibling of p86 and p109
86	c.261C>A	p.S87R	c.1222C>T	p.R408W	Sibling of p85 and p109
87	c.727C>T	p.R243*	c.1315+1G>A (IVS12+1G>A)		
88	c.194T>C	p.I65T	c.1222C>T	p.R408W	
89	c.1241A>G	p.Y414C	c.1241A>G	p.Y414C	
90	c.1159A>G	p.E390G	c.1241A>G	p.Y414C	
91	c.140C>T	p.A47V	c.838G>A	p.E280K	
92	c.117C>G	p.F39L	c.1315+1G>A (IVS12+1G>A)		

(continued)

Table 1 (continued)

ID	Allele 1		Allele 2		
	cDNA[a]	Protein	cDNA[a]	Protein	Comments[b]
93	c.1222C>T	p.R408W	c.1222C>T	p.R408W	
94	c.1042C>G	p.L348V	c.1315+1G>A (IVS12+1G>A)		
95	c.782G>A	p.R261Q	c.1315+1G>A (IVS12+1G>A)		
96	c.503delA	p.Y168fs	c.842+3G>C (IVS7+3G>C)		
97	c.307G>T	p.G103C	c.461A>T	p.Y154F	p.Y154F novel
98	c.473G>A	p.R158Q	c.781C>T	p.R261*	
99	c.782G>A	p.R261Q	c.1045T>C	p.S349P	
100	c.331C>T	p.R111*	c.1208C>T	p.A403V	
101	c.727C>T	p.R243*	c.1222C>T	p.R408W	
102	c.727C>T	p.R243*	c.1222C>T	p.R408W	
103	c.912+1G>A (IVS8+1G>A)		c.1222C>T	p.R408W	
104	c.194T>C	p.I65T	c.814G>T	p.G272*	
105	c.284_286delTCA	p.I95del	c.1045T>C	p.S349P	
106	c.838G>A	p.E280K	c.1315+1G>A (IVS12+1G>A)		
107	c.117C>G	p.F39L	c.1315+1G>A (IVS12+1G>A)		
108	c.194T>C	p.I65T	c.441+5G>T (IVS4+5G>T)		
109	c.261C>A	p.S87R	c.1222C>T	p.R408W	Sibling of p85 and p86
110	c.1066-11G>A (IVS10-11G>A)		c.1241A>G	p.Y414C	Sibling of p51
111	c.818C>T	p.S273F	c.1222C>T	p.R408W	

[a] Numbering based upon reference sequence NM_000277.1, with the A from the ATG translation initiation start site numbered +1. Protein nomenclature based upon NP_000268.1

[b] 'Novel' indicates mutations not reported in *PAH*db (last accessed 07 August 2013, Scriver et al. 2003)

of the study. The genotypes of these patients were determined as part of this study and are summarized in Table 3. In four patients with a positive BH_4 response, the BH_4-responsive alleles were likely to be p.F39L, p.L48S and p.Y414C since the second alleles were null alleles. The fifth patient with a positive BH_4 response had two missense alleles (p.I65T and p.S273F), both of which may potentially be responsive alleles. From similar deductions in the patients with intermediate responses, other possible BH_4-responsive alleles were p.A104D, p.Y154F and p.R261Q. The mutation p.R261Q has been reported in BH_4-responsive patients (including a homozygous patient reported in Hennerman et al. 2005), whereas the mutation p.A104D has been identified in responsive patients compound heterozygous with a null second allele (Wang et al. 2007). Two of the alleles, p.D145V and p.V245L, have not been reported in BH_4-responsive patients previously. In addition, a nonresponsive patient from our cohort also had the p.V245L mutation, indicating it is less likely to be a BH_4-responsive allele. However, predictions based on genotype may not always give the correct BH_4 phenotype. The identical genotype p.[F39L];[R408W] was observed in three patients (#6, #14, #29), all three of which showed different responses to the BH_4 treatment. Of note, it has been previously reported that, contrary to what one might expect, a patient homozygous for the p.R408W mutation was BH_4 responsive (Leuzzi et al. 2006), but one of our patient's homozygous for this mutation was not responsive (data not shown). The other discrepant results were observed (patients #50 and #55). The former was heterozygous for p.I65T, which has been previously associated with BH_4 responsiveness (www.biopku.org/home/pah.asp). Similarly the p.R408Q allele carried by patient #55 is also considered a BH_4-responsive allele (www.biopku.org/home/pah.asp).

Discussion

The mutation analysis of 111 PKU patients in NSW, Australia, showed a wide spectrum of *PAH* mutations, with 61 distinct mutations present in 201 independent alleles. The two most common mutations, p.R408W and c.1315+1G>A, account for over 30% of the alleles and the nine most common mutations account for 57.2% of alleles. A comparison between the mutation spectra from NSW and Victoria (VIC), Australia (and between other world regions), is shown in Table 2. Interestingly, p.I65T was

Table 2 Allele frequencies (%) of the nine most common mutations from this study (bold) and selected others compared with those in other populations (minimum number of alleles = 100, with the exception of VIC, Australia) and their frequencies as reported in *PAH*db (http://www.pahdb.mcgill.ca)

Population	Number of alleles	Mutations (allele frequency %)														
		p. R408W	**c.1315 +1G>A**	**p. I65T**	**p. Y414C**	**p. R261Q**	**p. F39L**	**p. F299C**	**p. P281L**	**p. R243***	c.1066-11G>A	p. L48S	p. R243Q	Ex6-96A>G	p. R111*	p. R413P
NSW, Australia (this study)	201	18	12	5.5	5.0	5.0	3.5	3.0	2.5	2.5	2.0	1.5	1.0	0.0	1.5	1.0
VIC, Australia (Ramus et al. 1995)	83	19	16	18	0.0	4.8	3.6	2.4	1.2	2.4	6.0	1.2	0.0	0.0	0.0	0.0
England (Zschocke 2003)	124	10	27	8.0	2.0	0.0	3.0	3.0	2.0	0.0	4.0	7.0	0.0	0.0	0.0	0.0
Germany (Aulehla-Scholz and Heilbronner 2003)	438	25	10	0.0	4.6	3.9	0.7	0.9	3.7	2.1	3.0	2.1	0.0	0.0	0.0	1.0
Ireland (O'Donnell et al. 2002)	558	41	2.0	10	1.0	0.0	12	4.0	0.0	3.0	1.0	0.0	0.0	0.0	0.0	0.0
Lithuania (Kasnauskiene et al. 2003)	184	73	0.0	0.0	0.5	0.5	0.0	0.0	1.0	0.0	0.5	0.0	0.0	0.0	0.5	0.0
Denmark (Guldberg et al. 1993)	308	18	37	0.0	10	2.0	1.0	0.0	1.0	0.0	5.0	0.0	0.0	0.0	0.0	0.0
Catalonia, Spain (Mallolas et al. 1999)	198	2.9	0.5	5.6	1.2	5.6	0.0	0.0	0.0	2.2	13	0.5	3.4	0.0	1.1	0.0
Italy (Giannattasio et al. 2001)	289	0.7	1.0	0.0	2.1	14	0.0	0.0	3.1	0.7	19	9.7	0.0	0.0	0.0	0.0
Turkey (Dobrowolski et al. 2011)[a]	1176	5.9	0.0	0.2	0.0	7.7	0.0	0.0	7.7	1.4	23	6.6	0.7	0.0	0.3	0.3
Brazil (Acosta et al. 2001)	230	3.5	1.3	3.5	0.0	12	0.0	0.0	2.1	0.0	17	0.8	1.3	0.0	0.0	0.0
USA (Guldberg et al. 1996)	294	19	7.8	4.1	5.4	2.4	4.1	1.0	2.7	1.0	4.4	1.7	0.0	0.3	0.3	0.0
USA (Enns et al. 1999)	267	15	8.2	4.5	2.6	1.5	2.2	2.2	3.4	0.8	7.2	2.2	1.1	0.4	0.4	0.0
China, northern (Song et al. 2005)	370	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	22	11	8.7	7.0
*PAH*db (Scriver et al. 2003)	3206	6.7	2.8	4.1	3.6	2.1	1.3	1.4	2.9	1.1	5.2	2.0	0.9	0.5	0.6	0.4

[a] The number of independent alleles was not clear from the data provided. Allele frequencies are calculated using total number of chromosomes tested

Table 3 Genotypes and BH_4 responsiveness of patients tested in Mitchell et al. (2005)

BH_4 response[a]	ID	Allele 1	Allele 2	Putative BH_4-responsive allele[b]
Positive	#14	p.F39L	p.R408W	p.F39L
	#25	p.L48S	p.R408W	p.L48S
	#30	p.R408W	p.Y414C	p.Y414C
	#32	p.I65T	p.S273F	p.I65T or p.S273F
	#80	Exon 6 deletion	p.Y414C	p.Y414C
Intermediate	#8	p.I65T	p.D145V	p.I65T or p.D145V
	#17	p.Y154F	IVS12+1G>A	p.Y154F
	#29	p.F39L	p.R408W	p.F39L
	#31	p.R261Q	p.P281L	p.R261Q
	#54	Exon 3 deletion	p.Y414C	p.Y414C
	#72	p.A104D	p.V245L	p.A104D
Negative	#6	p.F39L	p.R408W	(p.F39L)
	#33	p.A90fs	p.A90fs	
	#44	p.R243Q	p.R243Q	
	#50	p.I65T	IVS8+1G>A	(p.I65T)
	#53	p.R408W	IVS12+1G>A	
	#55	p.V245L	p.R408Q	(p.R408Q)
	#69	IVS2+1G>A	p.L249F	
	#70	p.A309V	p.R408W	
	#96	p.Y168fs	IVS7+3G>C	

[a] BH_4 response determined by changes in blood Phe levels after BH_4 supplementation (20 mg/kg/day). Positive = more than 30 % decrease in blood Phe after 8 h; intermediate = some decrease in blood Phe during the duration of study (7 days); negative = no decrease in blood Phe

[b] Putative BH_4-responsive allele determined by presence of null allele or homozygosity or hemizygosity in previously reported BH_4-responsive patients; some of these alleles are also found in nonresponsive patients (*in brackets*). Full details of BH_4 loading test can be found in Mitchell et al. (2005)

more common in VIC than in NSW (Ramus et al. 1995), but otherwise the distribution of mutations in the two populations was similar. The p.R408W mutation was the predominant mutation in Ireland, Latvia and Lithuania (O'Donnell et al. 2002; Kasnauskiene et al. 2003; Pronina et al. 2003), whereas c.1315+1G>A had the highest frequency in England, Germany and Denmark (Guldberg et al. 1993; Aulehla-Scholz and Heilbronner 2003; Zschocke 2003). Other examples of region-specific mutations include c.1066-11G>A from Mediterranean regions and p.R243Q and p.R413P from Asia (Chien et al. 2004; Lee et al. 2004; Song et al. 2005). The low incidence of these mutations in our cohort reflects the high cultural diversity in NSW, Australia (Australian Bureau of Statistics 2012).

Pathogenic mutations were not found in only two of the alleles tested. Polymorphisms in the location of the PCR primers may lead to the drop-out of an allele during amplification, as well as complex tertiary structures in the DNA and poor template quality or quantity (Tvedebrink et al. 2009; Saunders et al. 2010). Alternate primer sets or whole exome/genome sequencing may be of use in the identification of the second alleles in these patients, as mutations further into the intron or in the promoter or untranslated regions were not investigated in this study. Also, large complex chromosomal arrangements not affecting copy number, such as gene inversions and balanced translocations, could not be ruled out as a cause of PAH deficiency.

Tetrahydrobiopterin (BH_4) is the natural cofactor of the PAH enzyme, and its synthetic form, sapropterin, has been approved for use as treatment of PKU (Cajigal 2008), although it is currently not widely available in Australia. The treatment is not suitable for all patients, and there is a general observation that patients with mild PKU or HPA are more likely to respond to BH_4 (Bernegger and Blau 2002). Indeed, screening of BH_4-responsive patients found that there are certain mutations associated with BH_4 responsiveness and that these mutations are generally ones previously classed as leading to a mild phenotype (Muntau et al. 2002). Following the assumption (Muntau et al. 2002) that patients with at least one BH_4-responsive mutation would be responsive to BH_4, as many as 47 % of patients in our cohort may benefit from this treatment.

A number of the patients in the NSW cohort have been previously tested for BH_4 responsiveness (Mitchell et al. 2005). The correlation between genotypes and BH_4 responses in these patients is uncertain (Table 3), supporting previous findings that genotype is not an absolute predictor of BH_4 responsiveness (Lindner et al. 2001; Karačić et al. 2009). There are various reasons proposed for the difficulty of correlating BH_4 responsiveness with genotype. First, the methods of ascertaining BH_4 responsiveness have changed with time, and there may also be differences in the interpretation of responsiveness between the centers at which the tests were carried out (Mitchell et al. 2005; Fiege and Blau 2007; Anjema et al. 2011). Individuals may differ in their rate of BH_4-absorption, protein catabolic rate and Phe intake during the test. There is also a suggestion that the combination of the genotypes may be of greater significance than the presence of the individual alleles due to the multimeric nature of the PAH enzyme and the majority of PKU patients being compound heterozygotes for two different mutations (Scriver and Kaufman 2001). On the other hand, improvement in enzymatic activity in certain mutant PAH proteins expressed in BH_4 supplemented media supports the notion that genotype indeed plays a role in determining BH_4 response (Kim et al. 2006). Regardless, genotypic information may allow patients with BH_4-responsive mutations to be prioritized for BH_4 trials ahead of those with only known nonresponsive mutations.

In summary, we have undertaken *PAH* mutation screening in a large clinic-based cohort of PKU patients from NSW and have examined phenotype–genotype correlations, including potential BH_4 responsiveness. Mutation analysis of PKU patients in NSW, Australia, revealed a wide spectrum of mutations present similar to what has been reported in other Caucasian populations. The decreasing costs of direct sequencing or the use of next generation sequencing technology will facilitate widespread genotyping of all PKU patients, as well as carrier testing for family members. These methods are not appropriate for the detection of large exonic deletions, although these remain rare causes of PKU. Mutation screening may assist with decisions relating to disease severity and management, especially with regard to the detection of alleles associated with BH_4 responsiveness and the identification of patients who may benefit from being given access to this treatment and other novel therapies.

Acknowledgment This research was supported by donations from the NSW PKU Association and from the Rotary Club of Pennant Hills.

Conflict of Interest

The authors declare no conflict of interest.

Synopsis

Mutation screening of a large cohort of phenylketonuria patients in Australia found a wide spectrum of mutations in this population, with a high proportion of patients likely to benefit from sapropterin treatment.

Compliance with Ethics Guidelines

Gladys Ho, Ian Alexander, Kaustuv Bhattacharya, Barbara Dennison, Carolyn Ellaway, Sue Thompson, Bridget Wilcken and John Christodoulou declare that they have no conflict of interest.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients for being included in the study.

Details of Contributions of Individual Authors

GH

Design of experiments, implementation of experiments, data analysis and manuscript preparation

IA, KB, BD, CE, ST, BW, JC

Design of experiments, clinical information and manuscript preparation

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