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Characterization of Phenylketonuria Alleles in the Italian Population

Irma Dianzani[a]
Sergio Giannattasio[c]
Luisa de Sanctis[a]
Carla Alliaudi[a]
Paolo Lattanzio[c]
Carlo Dionisi Vici[d]
Alberto Burlina[e]
Massimo Burroni[f]
Gianfranco Sebastio[g]
Franco Carnevale[h]
Vito Guzzetta[g]
Ersilia Marra[c]
Clara Camaschella[a,b]
Alberto Ponzone[a]

[a] Clinica Pediatrica e Clinica Medica, University of Torino, and
[b] CNR-CIOS, Torino, and
[c] CNR-Centro di Studio sui Mitocondri e Metabolismo Energetico, Bari and Trani, and
[d] Ospedale Bambin Gesù, Rome, and
[e] Dipartimento di Pediatria, University of Padua, and
[f] Servizio Autonomo di Neuropsichiatria Infantile, Fano, and
[g] Cattedra di Pediatria, II Facoltà di Medicina, University of Naples, and
[h] Divisione Malattie Metaboliche, Ospedale Pediatrico Giovanni XXIII, Bari, Italy

Key Words
Phenylketonuria · Phenylalanine hydroxylase · Genotype/phenotype correlation

Abstract
In order to identify the molecular basis of phenylketonuria (PKU) in Italy, we screened the entire coding sequence of the phenylalanine hydroxylase gene in 20 Italian PKU patients, whose origins are scattered throughout Italy. The frequency of each identified mutation and of 5 other European mutations was determined within a panel of 92 Italian PKU patients. This approach allowed us to identify 20 different PKU mutations and characterize 64% of the Italian PKU chromosomes. Eleven mutations (IVS10nt546, L48S, R158Q, R261Q, P281L, R261X, R252W, ΔT55, IVS7nt1, IVS12nt1, Y414C) represent 55.4% of the Italian PKU alleles, the most common mutations being IVS10nt546 (12.4%) and L48S (9%). All the other mutations are very rare. These data confirm the great heterogeneity expected from previous RFLP haplotype studies. Genotype/phenotype correlation allowed for assessment of the clinical impact of the 20 identified mutations.

Introduction

Cloning of the gene encoding phenylalanine hydroxylase (PAH) in 1985 [1] has prompted the definition of the molecular basis of phenylketonuria (PKU), the disease caused by loss of function of this gene [2]. To date, more than 160 candidate mutations have been identified [3], many of which have proven to be causal by analysis of the expression of the mutated gene in vitro [4].

A novel approach to diagnosis, treatment, prognosis and also prevention of the disease has been implemented by these studies. Pre-

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Dr. Irma Dianzani
Istituto di Clinica Pediatrica
Piazza Polonia 94
I–10126 Torino (Italy)

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natal diagnosis and carrier detection are easily performed by tracing the inheritance of causal mutations in characterized families. On the other hand, several polymorphic markers within the gene (RFLPs, VNTR, STR) [5, 6] can be used to identify the mutated uncharacterized alleles by comparison with an affected sibling [7]. The elucidation of genotype/phenotype correlation may allow for early, optimal treatment based on the mutation [8].

However, the possibility of using molecular information in clinics is dependent on both precise characterization of mutated alleles as well as mutation frequency in the examined population. Great heterogeneity in PKU mutations was shown by the earliest studies focussed on the distribution of RFLP haplotypes at the PAH locus. However, some populations were shown to be relatively homogeneous for PKU mutations, with a low number of the represented haplotypes, whereas others showed a large number of different haplotypes and were expected to carry multiple mutations. Generally, greater homogeneity was observed in populations from northern as compared to southern Europe [4]. Thirty-five mutations account for 99% of mutant alleles in Denmark, of which only 4 account for 70% of the total [9]. On the other hand, the great number of different haplotypes identified in the Italian population suggested great heterogeneity of PKU mutations in Italy [10].

The aim of this work was to define the molecular defects responsible for PKU in the Italian population.

Materials and Methods

Patients

Ninety-two Italian PKU patients, corresponding to 177 unrelated PKU chromosomes, were included in this study. Patients were followed up by six clinical departments, two located in northern Italy (Turin and Padua), two in central Italy (Rome and Fano), and two in southern Italy (Naples and Bari). The panel of patients is representative of the Italian population, since their geographic origin is uniformly spread throughout Italy. The origin of the patients was assessed as far back as the four grandparents of the probands: 67 chromosomes were original to the northern and central Italian regions, 110 to the southern regions. The division of Italy into a northern/central and a southern region was obtained by tracing a line below Lazio and Marche.

PKU diagnosis was assessed after exclusion of defects in tetrahydrobiopterin [11]. PKU classification was obtained by analysing the following parameters in each patient: (a) pretreatment serum phenylalanine, (b) time (days) necessary to normalize serum phenylalanine values after introduction of a phenylalanine-free diet, and (c) phenylalanine tolerance (maximal daily phenylalanine intake as required to maintain serum phenylalanine below 360 μmol/l).

Using the criteria mentioned above, 50 patients were assigned to classic PKU, because they showed pretreatment plasma phenylalanine higher than 1,500 μmol/l, dietary tolerance lower than 300 mg/day and required more than 4 days for normalization of plasma phenylalanine. Twenty-two patients, assigned to the mild phenotype, showed pretreatment plasma phenylalanine between 900 and 1,500 μmol/l, dietary tolerance between 300 and 700 mg/day and required less than 4 days for normalization of plasma phenylalanine. Six patients were assigned to the benign phenotype, because they showed pretreatment plasma phenylalanine lower than 600 μmol/l, dietary tolerance higher than 700 mg/day. Moderate PKU was assigned to the 14 patients who showed a pattern midway between classic and mild phenotypes (for example: pretreatment phenylalanine values >1,500 μmol/l, but tolerance >300 mg/day and days for normalization <4).

Genomic DNA was extracted from peripheral blood leucocytes by standard methods. RFLP haplotype was determined for 27 patients, 12 of whom were part of a previous analysis [10]. For the other 15 haplotyped PKU patients, RFLPs were detected by either Southern blot analysis or multiplex PCR at the PAH locus followed by restriction digestion [12].

Mutation Detection

Twenty patients, whose origins were spread uniformly throughout Italy, were selected from the panel of 92 patients and their DNA was submitted to mutation detection techniques. Selection was made on the basis of the geographical origin of the patients' chro-

mosomes: 14 chromosomes were original to the northern and central regions (representing 20.9% of northern/central chromosomes), 26 chromosomes were original to southern regions (representing 23.6% of southern chromosomes). Ten patients showed a classic, 5 a moderate and 5 a mild phenotype.

The 13 PAH exons were amplified by PCR using the appropriate primers complementary to intronic regions. Exons 7 to 11 were analysed by chemical cleavage method (CCM) [13, 14], while exons 3, 4, 5, 8, 9, 10 and 11 were analysed by the combination of single-strand conformation polymorphism (SSCP) and heteroduplex methods. All other exons were sequenced directly (exons 1, 2, 6, 12 and 13). Since in previous reports [13, 14], exon-intron boundaries of some exons had not been screened, these exons were amplified again using primers, allowing us to obtain products encompassing the unscreened regions, after which they were subjected to direct sequencing or the combination of SSCP and heteroduplex methods. In this way, several exons were studied with two different methods, but using different PCR primers.

The combination of SSCP and heteroduplex methods allows a greater sensitivity in mutation detection as compared to using a single method [15]. Heteroduplexes are clearly distinguished from SSCP when a non-denatured control is included in electrophoresis: heteroduplexes migrate at the same level as the non-denatured sample in the bottom portion of the gel, whereas single strands have a lower migration velocity. The following SSCP protocol was used. To obtain labelled products, to one tenth of each PCR reaction were added 1 μl [^{35}S]dATP (3,000 Ci/mmol) and 0.5 U of Taq polymerase, then five more PCR cycles were performed. 20 μl of 0.1% SDS, 10 m*M* EDTA pH 8, were added to half of the labelled PCR reactions. A fifth of the solution was mixed with an identical volume of stop buffer (95% formamide, 20 m*M* EDTA pH 8, 0.05% xylene cyanol, 0.05% bromophenol blue). The samples were heated to 95 °C for 5 min, allowed to reanneal at room temperature and finally loaded onto 40-cm long, 5–8% polyacrylamide gels in the presence of 5% glycerol in 0.5 × TBE buffer. Gels were run at 26 W for 3 h at 4 °C, dried on Whatman 3MM paper and exposed on Kodack AR films for 2–6 days. Exons for which a shift in band migration was detected were sequenced using the Sequenase Version 2.0 kit (United States Biochemical Corporation) after purification of relevant PCR amplification products (Qiaex kit, Qiagen).

The frequency of each newly identified mutation as well as five known mutations described in Europeans (IVS12nt1, R408W, R252W, P281L and A300S) was evaluated by restriction digestion [16] within the panel of the 177 unrelated alleles (table 1). If no restriction site was either created or abolished in the presence of the mutation, mutations were introduced in the PCR amplification products using mutagenic amplification primers to create a new restriction site in the normal or the mutant allele. The experimental approach used to search for each PKU mutation is reported in table 1. S359X and S231P mutations were searched by allele-specific oligonucleotide (ASO) analysis [17] using ASOs complementary to the mutated sequences [14, 18].

Results

Mutation Detection

Mutation detection analysis of the entire PAH coding sequence allowed for complete characterization of 17 out of 20 patients, with only one mutation identified in the remaining 3 patients. The 3 uncharacterized mutations might be located in unscreened regions of PAH, such as introns (cryptic intronic splice sites) or promoter regions.

We were able to characterize a total of 64% (113/177) of the Italian PKU chromosomes. Twenty mutations have been identified (table 2). Mutations S231P, S359X and IVS7nt1 have been previously reported by our group. All mutations are reported in the PAH Mutation Analysis Consortium Database [3].

Eleven mutations (IVS10nt546, L48S, R158Q, R261Q, P281L, R261X, R252W, ΔT55, IVS7nt1, IVS12nt1 and Y414C) represent 55.4% of the Italian PKU alleles, the most common mutations being IVS10nt546 (12.4%) and L48S (9%). All the other mutations are very rare. The A300S mutation, which has a frequency of 5.7% in Sicily and seems to be associated with a non-severe phenotype, has not been found in our panel of patients.

The RFLP haplotye association has been assessed for several characterized chromosomes (table 2).

Table 1. PKU mutations screened in 92 Italian patients

Mutation	Localization	Oligonucleotide (5′–3′)	Restriction enzyme	Origin of restriction site
L48S(TTG→TCG)	exon 2		*Bal*I	NA
ΔT55 (TTT→TT)	exon 2	f GGCCAAAGTATTGCGCTTAC	*Dde*I	ACMA
S67P (TCT→CCT)	exon 2		*Xba*I	NA
R158Q (CGG→TGG)	exon 5	f ATCCTGTGTACCGTGCAAGC	*Msp*I	ACNA
L194P (CTG→CCG)	exon 6	f GGGGCACAGTGTTCAAGGCT	*Nla*IV	ACMA
S231P (TCT→CCT)	exon 6	ASO		
R252W (CGG→TGG)	exon 7		*Ava*I	NA
A259V (GCC→GTC)	exon 7	f CTCGGGATTTCTTGGGTAGC	*Pal*I	NA[a]
R261Q (CGA→CAA)	exon 7		*Hin*fI	NA
R261X (CGA→TGA)	exon 7		*Dde*I	MA
P281L (CCg→CTg)	exon 7	r TAGCTGGAGGACAGTACTCC	*Msp*I	ACNA
IVS7nt 1 (gt→at)	intron 7		*Nla*III	MA
A300S (GCC→TCC)	exon 8	r CTTACCTGGGAAAACTGCG	*Avi*II	NA
IVS10nt546 (gg→ag)	intron 10		*Dde*I	MA
ΔG353 (AAG→AA)	exon 11		*Hin*dIII	NA
S359X (TCA→TGA)	exon 11	ASO		
Y386C (TAT→TGT)	exon 11		*Mae*III	MA
R408W (CGG→TGG)	exon 12		*Mnl*I	NA
IVS12nt1 (gt→at)	intron 12	r TCGTAAGGTGTAAATTACGTA	*Rsa*I	ACNA
A403V (GCT→GTT)	exon 12		*Bbv*I	NA
Y414C (TAC→TGC)	exon 12		*Bbv*I	MA

f = Forward mutagenic primer; r = reverse mutagenic primer; NA = normal allele; MA = mutant allele; AC = amplification created; ASO = allele specific oligonucleotide.

[a] The mutagenic primer abolishes a further *Pal*I site 1 bp upstream from the relevant *Pal*I site, allowing for resolution of the digestion products.

The following five known polymorphisms have also been identified: −71A/C, IVS2nt19T/C, 696A/G (Q232Q), 735G/A (V245V) and 1155G/C (L385L) (PAH cDNA numbered according to the PAH Mutation Analysis Consortium Database version [3], which represents the combined and revised nucleotide sequence from Kwok et al. [1] and Konecki et al. [19]). The frequencies for these polymorphisms were found to be 27.2, 22.2, 38, 26.6 and 17%, respectively.

A previously unreported polymorphism 22 bp upstream from exon 4 identified in intron 3 (C/T) showed a frequency of 25%.

Genotype/Phenotype Correlation

71 patients (77%) were completely (43) or partially (28) characterized: 58 (81.7%) are compound heterozygotes for different mutations and 13 (18.3%) are homozygotes. Genotype/phenotype correlation has been assessed for the 43 fully characterized subjects, with the results summarized in table 3.

A tentative clinical classification of PKU mutations was deduced from our data using the following criteria: the amount of residual activity in vitro (if data available), the phenotype shown by patients homozygous for each mutation and the phenotype shown

Table 2. Distribution of PKU mutations in 177 unrelated Italian PKU chromosomes

Mutation	Numer of positive chromosomes	Frequency %	RFLP haplotype
IVS10nt546	22	12.4	6, 9[a]
L48S	16	9.0	4
R158Q	10	5.6	1
R261Q	9	5.1	1
P281L	9	5.1	ND
R261X	8	4.5	1
R252W	6	3.4	7
ΔT55	5	2.8	1
IVS12nt1	5	2.8	ND
IVS7nt1	4	2.3	1
Y414C	4	2.3	ND
A403V	3	1.7	1
A259V	3	1.7	ND
ΔG363	2	1.1	ND
R408W	2	1.1	2
S67P	1	0.6	4
L194P	1	0.6	4
S231P	1	0.6	2
S359X	1	0.6	6
Y386C	1	0.6	ND
Uncharacterized	64	36.1	
Total	177	100.0	

ND = Not determined.

[a] IVS10nt546 has been found in association with H9 in a single patient.

when in compound heterozygosity with a severe or a non-severe mutation. A mutation was considered severe either when residual activity lower than 15% was shown in in vitro expression experiments, or when it conferred a severe phenotype either in homozygosity or heterozygosity with another severe mutation. A mutation was considered non-severe when a residual activity higher than 15% was shown in in vitro expression experiments, or when it conferred a non-classic phenotype either in homozygosity or heterozygosity with another non-severe mutation. On the other hand, we observed that the association of a non-severe with a severe mutation can result in all possible phenotypes, i.e. mild, moderate or classic.

R261X, IVS10nt546, R252W, R158Q, P281L and ΔG363 can be seen as severe mutations, since they confer a classic phenotype in homozygosity in our series (table 3). Moreover, they confer a severe phenotype in compound heterozygosity with other mutations, such as IVS12nt1 [8], R408W [8] and ΔT55 [20], classified as severe by data from the literature.

Mutations R403V and S67P were considered non-severe, because the first confers a mild or a benign phenotype in association with severe mutations, such as P281L, R261X and R158Q, while the second confers

mild PKU when associated with other mild mutations, such as L48S. IVS7nt1 and S359X are probably severe mutations, since a disruption of the protein could be expected from the nature of these defects. In our limited series they give a classic phenotype when associated with a severe mutation, such as IVS10nt546 and ΔT55. A further mutation, S231P, was shown to have no residual activity when expressed in vitro [18].

It is interesting to note that the R158Q mutation presents a severe phenotype in homozygosity both in our series and in that of Okano et al. [8], but gives a moderate phenotype in compound heterozygosity with a severe mutation, such as IVS12nt1 or a non-severe mutation such as R261Q (in 2 patients) or L48S (in 1 patient). A residual activity of 10% shown by the mutated enzyme in expression experiments might explain this peculiar pattern [8].

L48S, R261Q and Y414C mutations could be classified as non-severe, as found in other series [8, 21, 22, 23, 24]. A mild phenotype was shown by the single patient homozygous for R261Q and by the 2 patients homozygous for Y414C. A mild phenotype was shown by compound heterozygotes between L48S and other non-severe mutations (R261Q, S67P). All but Y414C (which was never found associated with another mutation) were shown to give either a classic or mild or moderate phenotype when in compound heterozygosity with a severe mutation. This pattern was also observed when analysing incompletely characterized patients. The 7 patients who carried L48S in association with an undetected mutation showed all varieties of phenotypes: 2 classic, 3 mild, 1 moderate and 1 benign.

Four prenatal diagnoses [7, 25] and 6 carrier diagnoses were requested during the period of the study. In all cases, diagnosis was proven to be easily assignable with either RFLPs, VNTR, STR or mutation analysis.

Table 3. Genotype/phenotype association in 43 completely characterized PKU patients, 13 homozygotes and 30 compound heterozygotes for different PKU mutations (one patient identified for each combination, unless otherwise indicated)

Genotype	Phenotype
Homozygotes	
IVS10nt546:IVS10nt546	classic (n = 3)
R261X:R261X	classic (n = 2)
P281L:P281L	classic (n = 2)
Y414C:Y414C	mild (n = 2)
R158Q:R158Q	classic
R252W:R252W	classic
ΔG363:ΔG363	classic
R261Q:R261Q	mild
Compound heterozygotes	
IVS7nt1:R261Q	classic
IVS10nt546:A259V	classic
IVS10nt546:IVS7nt1	classic (n = 2)
IVS10nt546:P281L	classic
IVS10nt546:ΔT55	classic
IVS10nt546:R261Q	classic
IVS12nt1:P281L	classic
L194P:R261X	classic
R252W:L48S	classic
R408W:L48S	classic
S231P:R261Q	classic
S359X:ΔT55	classic
R158Q:Y386C	classic
L48S:IVS10nt546	moderate; mild (n = 2)
L48S:IVS7nt1	moderate
L48S:R158Q	moderate
R158Q:R261Q	moderate (n = 2)
R158Q:IVS12nt1	moderate
R261Q:ΔT55	moderate
R261Q:L48S	mild
L48S:ΔT55	mild
L48S:S67P	mild
A403V:P281L	mild
A403V:R261X	mild
A403V:R158Q	benign
R261Q:ΔG363	moderate

Discussion

The present work attempted to evaluate the molecular basis of PKU in Italy. The main result was the identification of extreme heterogeneity, much higher than in other European populations. Our approach allowed us to characterize 64% of the Italian PKU chromosomes. By analogy with a recent study focussed on the inhabitants of Sicily [26], it can be argued that the remaining 36% of uncharacterized chromosomes represent rare or private muations.

To date, the extreme heterogeneity of PKU mutations in Italy precludes the introduction of programs for carrier detection at a population level, thus restricting family counselling to families with an affected sibling. In fact, to be effective in terms of costs/benefits, a carrier screening program should only be carried out in populations where at least 95% of mutations are characterized and where the number of mutant alleles is not excessively large.

The cause for the extreme heterogeneity of PKU in Italy might be ascribed to the different ethnic origins of the many populations which inhabited ancient Italy to become the progenitors of the modern Italian population [27]. Three mutations show a non-random geographic distribution and different relative frequencies in northern/central and southern Italian regions [27]. Two of them, IVS10nt546 and L48S, are more frequent in southern Italy, whereas IVS12nt1 is more common in northern Italy. We have proposed that IVS10nt546 and L48S might have their origin in the Middle East. From there they might have been carried to western and northern regions by the migration of Neolithic farmers [27]. On the other hand, the distribution of IVS12nt1 fits the hypothesis of an origin within Uralic-speaking populations [28].

The extension of molecular analysis to a larger number of patients original to each Italian region might allow identification of a panel of PKU mutants specific for each region. This might suggest new hypotheses regarding the origin and spread of other PKU mutations in Italy. Moreover, this analysis might be useful for the introduction of regional programs for carrier screening or for prenatal diagnosis using methods able to screen simultaneously for the most frequent mutations [29], including the 10–15 mutations which occur more frequently in each Italian region. Use of polymorphic markers could be the method of choice for rare or uncharacterized DNA changes.

As a consequence of heterogeneity, most of the patients were found to be compound heterozygotes for different mutations, thus making the definition of the role of each mutation in the pathogenesis of PKU in Italy difficult to obtain. To circumvent this problem, we used a tentative approach by taking into account patients' clinical parameters, biochemical parameters obtained with in vitro expression studies, and association with other mutations. The introduction of a fourth phenotype, named 'moderate PKU' [30], allowed us to resolve discrepancies found by Okano et al. [8], who observed an overlap between classic and mild phenotypes.

We observed that the association of a mild with a severe mutation could result in all possible phenotypes. This has been observed by other authors regarding R261Q, which in heterozygosity has generally been associated with a less severe phenotype, but also with classic PKU [8, 24, 31]. In other studies, this mutation had been found to be associated with both a benign and mild PKU when homozygous, but in our series the single homozygote shows a mild PKU.

Although the causes for this variability are unknown, it is probable that other factors may influence the phenotype. Negative interaction between different mutant subunits might oc-

cur in the mature oligomeric protein. This might be the case for mutations associated with some residual activity, such as R158Q. In addition, the hydroxylating system might be modulated by modifier genes. It is also important to note that expression analyses are usually performed in monkey-kidney cell or *Escherichia coli* systems, which are markedly different from the human liver environment in vivo.

Finally, the association of mutations with specific phenotypes might account for the different clinical presentation of PKU among populations. Seen in this light, the higher frequency of non-severe mutatioMol such as L48S and R261Q, could well be the cause of the lower severity of PKU in Italy compared to northern Europe, where severe mutations (such as IVS12nt1 and R408W) are prevalent.

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