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Phenylalanine Hydroxylase Gene Mutation R408W Is Present on 84% of Estonian Phenylketonuria Chromosomes

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Key Words
Phenylalanine hydroxylase
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Abstract
Phenylalanine hydroxylase (PAH) is the enzyme which converts phenylalanine into tyrosine. In case of its deficiency, hyperphenylalaninemia is observed, which leads to phenylketonuria (PKU), a disease causing mental retardation, unless treated with a low-phenylalanine diet since early childhood. In Estonia, PKU is among the most common inherited metabolic diseases. The data from retrospective study and newborn screening show an approximate incidence of 1 in 6,000 newborns. Molecular analysis of 34 Estonian patients has revealed high genotypic homogeneity in this group, as 84% of the mutant alleles carry the R408W mutation. The high rate of this mutation in the Estonian population rises the speculation of Finno-Ugric contribution to the East European pool of mutant PAH alleles. Five more mutations – IVS12nt1, R261Q, R252W, R158Q, S349P – have been detected. The mutation detection rate was 92% among the studied patients.

Introduction

Phenylketonuria (PKU) is a disorder of amino acid metabolism which depends on hepatic enzyme phenylalanine hydroxylase (PAH). In the case of PAH deficiency, hyperphenylalaninemia (HPA) is observed, a condition that causes mental impairment unless treated with a low-phenylalanine diet since early childhood [1]. After cloning and sequencing the PAH cDNA in 1985 [2], evidence of different mutations in the same locus became available. Direct identification of the mutations that cause the lack of enzyme activity, silent polymorphisms and polymorphic hypervariable regions helps confirming the diagnosis. About 290 mutant/polymorphic sites in the PAH gene have been identified up to date according to the data of the PAH Gene Mutation Analysis Consortium [3]. This has led to high population heterogeneity [4] both at the molcular and phenotypical levels. The molecular cause of PKU can be analyzed directly using several techniques for mutation detection [5].

In Estonia (population 1.6 million; 13,000 newborns in 1995), PKU is one of the most frequent inherited metabolic diseases with an incidence of 1 in 5,236 newborns [6]. Newborn screening was started in 1993. One part of the diagnostic program is the detection of molecular lesions in Estonian PKU patients. Direct mutation detection [7] was chosen as the main strategy of the present study.

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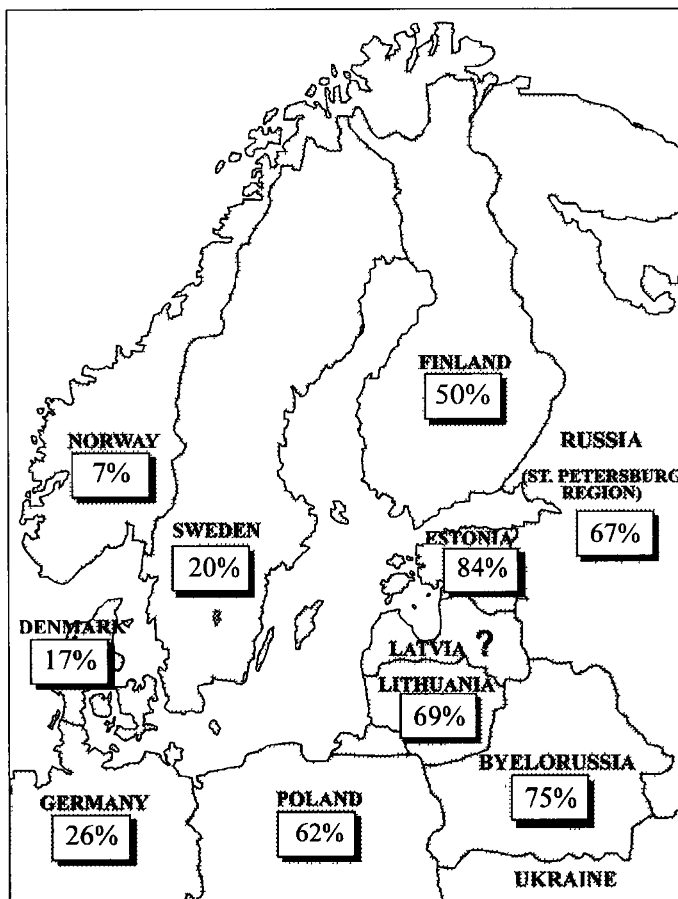

Fig. 1. Relative frequencies of PAH gene mutation R408W among patients with phenylketonuria (PKU) and milder HPA in North-Eastern Europe [14, 17–19, current study].

Table 1. Primers used in the current study for PCR amplification of phenylalanine hydroxylase gene exons

Primers used for amplification	Length of amplified product, bp
1F: 5′-TTAAACCTTCAGCCCCACG-3′ 1R: 5′-TGGAGGCCCAAATTCCCCTAACTG-3′	197
2F: 5′-GAGGTTTAACAGGAATGAATTGCT-3′ 2R: 5′-TCCTGTGTTCTTTTCATTGC-3′	264
3F: 5′-GCCTGCGTTAGTTCCTGTGA-3′ 3R: 5′-CTTATGTTGCAAAATTCCTC-3′	267
5F: 5′-TCATGGCTTTAGAGCCCCC-3′ 5R: 5′-AGGCTAGGGGTGTGTTTTTC-3′	213
7F: 5′-AAAGGTCTCCTAGTGCCTCTG-3′ 7R: 5′-GATGGCGCTCATTGTGCCTG-3′	253
8F: 5′-TGGCTTAAACCTCCTCCCCT-3′ 8R: 5′-CTGGGCTCAACTCATTTGAG-3′	189
9F: 5′-ATGGCCAAGTACTAGGTTGG-3′ 9R: 5′-GAGGGCCATAGACTATAGCA-3′	185
10F: 5′-TTAACGATCATAGAGTGTGC-3′ 10R: 5′-ACAAATAGGGTTTCAACAAT-3′	232
11F: 5′-TGCAGCAGGGAATACTGATC-3′ 11R: 5′-TAGACATTGGAGTCCACTCTC-3′	295
12F: 5′-ATGCCACTGAGAACTCTCTT-3′ 12R: 5′-AGTCTTCGATTACTGAGAAA-3′	245

Materials and Methods

Patients

Thirty-four available Estonian PKU patients born since 1980 were included into the current study, providing 68 independent affected chromosomes. This makes up 87% of the known patients born during this period.

Phenotypically, all patients express the 'classic' or severe PKU phenotype according to the pretreatment phenylalanine level or phenylalanine tolerance in diet [8]. In Estonia, the exact measurement of serum phenylalanine concentration was not possible until 1992, and therefore the earlier patients' type was determined by the physician only.

Out of the 34 analyzed families, 20 were of Estonian origin at least during 2 generations, 6 families were of Slavic origin (including Russian, Ukrainian, Polish), 8 families had at least 1 ancestor of non-Estonian origin, including Russian, Polish, Armenian, German.

Samples of peripheral blood were collected into tubes containing K_3EDTA or Na citrate; DNA was isolated using proteinase K treatment and phenol extraction.

DNA Amplification

Polymerase chain reaction (PCR) was used for amplifying PAH gene exons 5, 7, 11 and 12 as the regions of most common mutations [9]. Firstly, mutations were screened in these four exons. If no results were obtained, other exons were considered. PCR primers (table 1) were chosen using data from reported amplification systems [10, 11]. GoldStar *Taq* DNA polymerase (Eurogentec, Belgium) was used with an appropriate buffer system, 33 cycles (94 °C for 45 s; 56 °C for 1 min; 72 °C for 1 min 30 s) were performed.

Sequence Analysis

Solid-phase sequence analysis [12] was performed by the Sanger dideoxy chain termination method using the Sequenase™ Version 2.0 DNA Sequencing Kit (Amersham Life Science) and [^{35}S]α-ATP, according to the protocol provided by the manufacturer. One biotin-linked oligonucleotide PCR primer was used for preparing the single-stranded probes which were bound to streptavidine-coated magnetic beads (Dynabeads® M-280, Dynal A.S.) for strand separation. Exons 5, 7 and 12 were sequenced completely, if no R408W was found.

Table 2. Distribution of PAH gene mutations in Estonian population and among different ethnic groups

Mutation	Number of chromosomes			
	Estonian	Slavic	mixed	total
R408W	35	9	13	57
IVS12nt1	1	–	1	2
R252W	–	1	–	1
R261Q	1	–	–	1
R158Q	–	–	1	1
S349P	–	1	–	1
Not determined	3	1	1	5
Total	40	12	16	68
Relative frequency of R408W, %	87.5	75	81.3	83.8

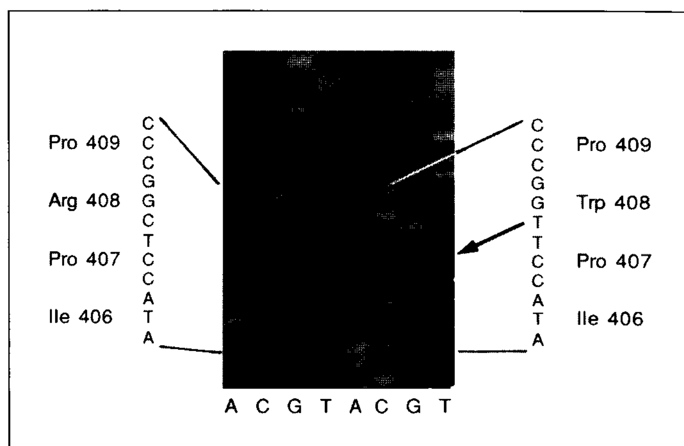

Fig. 2. Photograph of a sequencing gel of the PAH exon 12 region presenting a normal sequence (left) and a patient homozygous for the R408W mutation (right).

Restriction Analysis

Many of the proposed mutations in the DNA areas under research could be analysed by digesting the PCR product with restriction endonucleases [13]. Special attention was paid to the R408W mutation which is distributed with high frequency in areas geographically close to Estonia [14–16] (fig. 1) and can be effectively identified by *Sty*I digestion [20], as it creates a new restriction site in exon 12. *Hinf*I, *Ava*I and *Bam*HI restriction enzymes were used for digestion exon 7 to check for probable R261Q, R252W, G272X mutations accordingly and *Dde*I to detect IVS10nt546 in exon 11 (flanking regions) [13].

Restriction fragments were analysed by 2.5% agarose gel electrophoresis in Tris-borate-EDTA (TBE) buffer.

Single-Stranded Conformational Polymorphism Analysis

Denatured single-stranded PCR 185- to 295-bp products (table 1) were separated by electrophoresis on homogeneous 12.5% polyacrylamide PhastGel® gels using two different temperatures: +4 and +15 °C and developed by silver-staining. SSCP was used if previously described methods did not reveal the mutations.

Results

Using the above-described methods, mutations of 63 alleles out of the 68 affected ones were identified (table 2).

Out of 68 alleles under study, 57 (84%) were detected as defective due to R408W. Twenty-four patients were homozygotes for R408W, 9 were compound heterozygotes for R408W, and only 1 patient was a compound heterzygote with R158Q/IVS12nt1 mutations. These data correlate well with Hardy-Weinberg equilibrium, giving evidence that the Estonian PKU patient pool is balanced and it is probable that other (milder) forms have not been missed.

The chromosomes with R408W carry 3 VNTR repeats [21], as typical of Eastern Europe, not the 'Celtic' allele with 8 repeats [22]. The same is confirmed by STR analysis [23], which revealed the presence of a 240-bp allele on all chromosomes with R408W, except in 1 case (of slavic origin), where the STR repeat was longer.

Digestion of exon 7 with *Hinf*I, *Ava*I, *Bam*HI and exon 11 with *Dde*I led to detection of R261Q and R252W. IVS10nt546 and G272X were not found. Mutations were confirmed by sequencing (fig. 2).

All the samples that did not reveal their mutant sites by the described methods were sequenced for the exons 5, 7 and 12. This enabled us to identify the splicing-mutation IVS12nt1 in 2 cases and R158Q in 1 case. SSCP analysis revealed a polymorphims in exon 10, that was identified as S349P with *Van*91I restriction. Using the described methods, only 5 independent mutant alleles were not detected, i.e. 7.4% of the current sample. No patient had unidentified alleles in both chromosomes.

Discussion

This study describes the mutations of PAH gene in the Estonian population. Although the number of samples studied is not very large, it is clear that Estonia is one of the most homogeneous countries for the structure of

mutant PAH alleles. According to published data, no other population displays such a high frequency (84%) of R408W mutation [14].

After detection of a HPA child in the screening, several tests for cofactor synthesis and regeneration must be made to avoid the possible damage if imperfectly diagnosed and treated [24, 25]. Since the Estonian population is rather homogeneous for the R408W mutation, which is easily detectable by the PCR/*Sty*I restriction test, any new patient should be analyzed for it first. The presence of this affected allele confirms that the person is a PAH-deficient PKU patient without the need for further investigations of the BH_4 system. If absent, the BH_4 loading test should be performed as suggested [24].

Usually, dietary tolerance of phenylalanine varies widely among PKU and HPA patients [8]. Clinically, all our patients exhibit the classical PKU phenotype with low phenylalanine tolerance. Mutation R408W leaves no residual enzyme activity [26] and the fact that our patient group is balanced explains its clinical homogeneity.

Among the patients of Estonian origin, the incidence of R408W is even higher than in Slavic and mixed groups and in the whole population (87.5, 75 and 81.3%), respectively (table 2). The relative frequency of R408W in the Slavic group is close to other Slavic populations as described before [15–17]. Statistical χ^2 analysis did not reveal a significant difference in the distribution of the R408W allele among ethnically distinct groups of the Estonian population – the Estonians and Russians (approximately 30% of the Estonian population), but the main cause is the relatively small size of the latter group.

It has been suggested that R408W on the East-European haplotype is of Balto-Slavic origin [18, 27]. So far, the highest frequency of this mutation is described in Byelorussia, where it was present on 75% of mutant alleles [17]. Linguistically and ethnoculturally, the Estonians belong to the Finno-Ugric peoples, and the results of the current study indicate that R408W may originate from early Finno-Ugric tribes who inhabited large areas of Eastern Europe including northwest Russia and the Volga River region. They were largely assimilated by overcoming Slavic and Baltic tribes and could donate the mutant allele to them, later spreading widely.

Another possibility that can explain the very high frequency of one mutant allele is genetic drift. Periods with decreasing number of inhabitants clearly exist in the history of Estonia, most recently at the beginning of the 18th century. In this way, one allele can occasionally reach a higher frequency.

The other identified mutations were well known. IVS12nt1 was detected twice (3%), providing evidence of contacts with Scandinavian peoples. The mutation S349P originates from an Ukrainian person, and the rather high incidence of this allele (5.3%) in Ukraine has been mentioned earlier [16]. Alleles with R261Q, R252W, R158Q have a random distribution in several Caucasoid populations [9].

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