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RFLP DISCORDANCE IN A PKU FAMILY DUE TO A DELETION IN THE PAH GENE*

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SUMMARY: Bosco P, Ceratto N, Cali F, Goltsov AA, Eisensmith RC, Novelli G, Piccola BD, Romano V. (Istituto per la Ricerca sul Ritardo Mentale e l'Involuzione Cerebrale (IRCCS), Troina, and the Università degli studi di Roma Tor Vergata, Rome, and the Casa Sollievo della Sofferenza, S. Giovanni Rotando, Italy, and the Department of Cellular Biology, Baylor College of Medicine, Houston, Texas, USA). RFLP discordance in a PKU family due to a deletion in the PAH gene. Turk J Pediatr 1996; 38: 497-504.

We have previously reported preliminary data on a PKU family showing a discordant segregation of Pvu II (b) alleles at the PAH locus. A combination of several restriction enzymes and probe C2.6 (intron 2) as well as STR typing were used to dissect the molecular structure of the PAH gene around exon 3. In this family, the results of this analysis and a re-examination of the physical map of the 5'-end of the gene provided strong evidence for the occurrence of a deletion removing exon 3. The "Sicilian" (approximately 2.5 kb) and "Yemenite Jew" (6.7 kb) deletions, the latter one also deleting exon 3, are different in terms of both the 5'-end breakpoint and apparent length. This study, besides adding a new member to the long and increasing list of nearly 200 PAH gene mutations, also proposes to undertake a careful evaluation of RFLP discordances incidentally detected at the PAH locus. *Key words: classical PKU, PAH gene mutation, RFLP discordance.*

In Sicily the study of the mutant alleles causing phenylalanine hydroxylase (PAH) deficiency has recently led to the identification of 40 mutations altering the structure and function of the PAH enzyme[1]. These mutations account for approximately 98 percent of all mutant alleles in this population, and 22 of these defects are individually represented at a low frequency (0.9%). This observation is consistent with the presence of many founding populations for phenylketonuria (PKU) in Sicily, as is also documented by historical and archeological records. According to a consultation of the PKU Consortium Database[2], 24 deletions of the PAH gene have been reported to date. Large deletions are rare. One of the largest deletions reported so far has been well characterized at the molecular

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level and removes 6.7 kb of genomic DNA containing exon 3 and intronic flanking regions. It is the only mutation responsible for classical PKU in Yemenite Jews, probably because of founder and genetic drift effects[3]. We have previously reported preliminary data on the identification of a new mutation, also deleting exon 3 of the PAH gene, found in a single mutant chromosome borne by a Sicilian, classical PKU patient[4]. In this study we extend the molecular analysis of this mutation and provide strong evidence that the deletion removes approximately 2.5 kb of genomic DNA. This molecular lesion is responsible for the discordant segregation of alleles of the Pvu II (b) restriction fragment length polymorphism (RFLP) in the affected family. Although this mutation partially overlaps the deletion described by Avigad et al.,[3] in Yemenite Jews it could be demonstrated that the two deletions have different 5' breakpoints.

Material and Methods

Patients and Diagnosis

The index case in family F2 is a 22-year-old, mentally retarded boy who was sent to the OASI Institute (Troina) for diagnosis. Determination of his blood phenylalanine level showed that the had classical PKU, untreated since birth. His younger sister (16 years old) was also affected by classical PKU but had been enrolled in the regional neonatal screening program for PKU and had been successfully treated since birth. The occurrence of defects involving homeostasis or synthesis of the phenylalanine hydroxylase cofactor (BH4) was ruled out by urinary pterins testing.

Polymorphism and Mutation Analyses

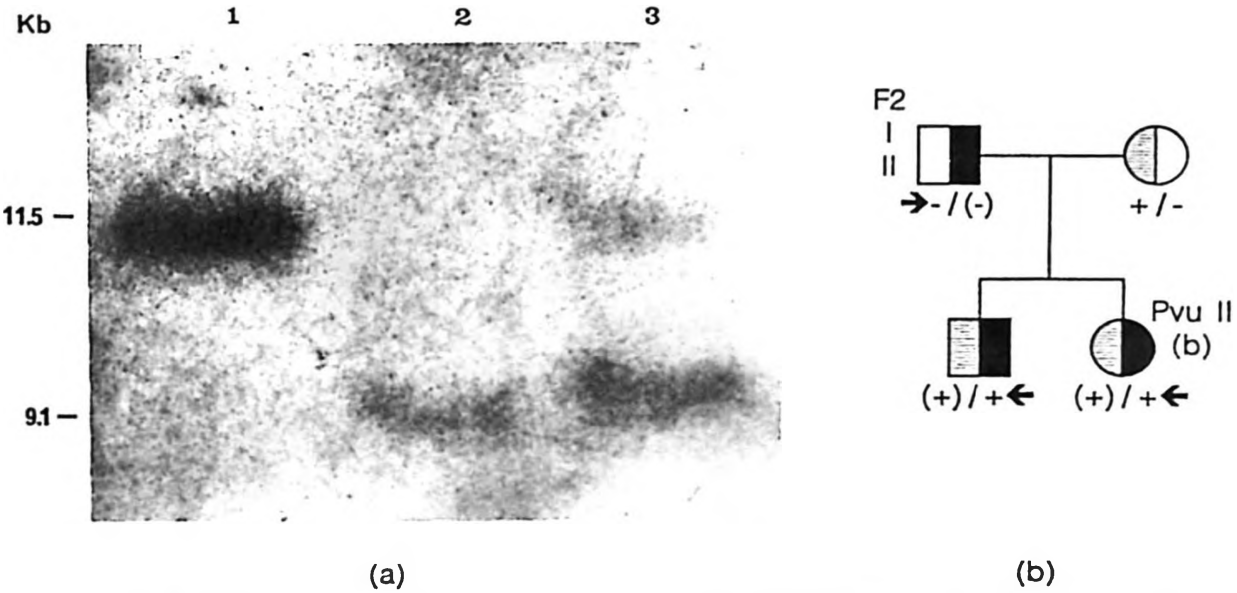
RFLP analysis at the PAH locus was performed as described by Lidsky et al.[5]. The haplotypes identified in this family (F2) have been previously reported[4]. A previous mutation analysis performed in this family had shown that the two affected siblings and the mother were heterozygous for the IVS X nt 546 mutation[1-4]. Probe C2.6 (kindly provided by Dr. Shiloh, Tel Aviv, Israel) was hybridized to genomic DNAs digested with one of the following enzymes: Eco RI, Pvu II or Hind III (biolabs, USA). Probe C2.6 is a genomic fragment, 2.6 kb long, derived from intron 2 of the PAH gene. It was generated by digesting with Hind III and Eco RI probe C, which is 4 kb long. The 3' end of probe C2.6 maps in intron 2, 0.7 kb away from exon 3. Further details about probes C2.6 and C have been reported by Avigad et al.[3]. Analysis of the intragenic short tandem repeats (STR) polymorphism was performed as described by Goltsov et al.[6] STR allele sizes determined according to the latter authors were two base pairs shorter than the true size[7]. DNA fingerprinting analysis was carried out by typing D1S80[8], ApoB[9] and HLA DQ[10] polymorphic loci. Mendelian inheritance was demonstrated for each allele of the latter three polymorphisms in family F2. Statistical analysis was performed according to Balazs et al.[11] and Wong et al.[12].

Illegitimate Transcription

This analysis was performed according to Abadie et al.[13]. Two oligonucleotides were used for primer extension on RNA (primer 2) and PCR amplification (primers 1 and 2). Primer 1: 5'CTT TGG ACA GGA AAC AAG CT 3'; primer 2: 5' GAT CTT TAA AAC GAG GGT GGT CA 3'. Both primers were synthetized according to the published sequence of the PAH cDNA[14] with an automated oligo-synthetizer (Applied Biosystem, USA). They span the junctions between exons 1 and 2 (primer 1) and exons 4 and 5 (primer 2), respectively.

Results

Fig. 1a shows the results of Pvu II (b) analysis performed in family F2. The father (lane 1) carries a single 11.5 kb band, the mother (lane 3) carries both the 11.5 and 9.0 kb bands, and the affected boy (lane 2) bears only the maternal 9.0 kb allele. Identical results were obtained for the affected sister (II-2 in Fig. 1b). Apparently the paternal 11.5 kb allele was not transmitted to the two affected sibs. All other RFLPs showed concordant segregation[4]. DNA fingerprinting analysis was undertaken to exclude the occurrence of false parenthood. In particular, three mini-satellites corresponding to the highly polymorphic variable number of tandem repeats ApoB, D1S80 and HLA DQ loci were used to fingerprint this family. Mendelian segregation analysis of these three polymorphisms showed identical haplotypes between parents and the putative siblings (data not shown). Haplo-identity could be ascertained with a probability of 99.99 percent.



(a) (b)

Fig. 1: Autoradiograph (a) and pedigree (b) of a Sicilian PKU family showing discordant segregation of Pvu II (b) RFLP alleles. The Pvu II (b) 11.5 allele was not transmitted by the father to the affected boy. The mother transmitted the Pvu II (b) 9.0 kb allele. 1, 2, and 3 refer to the father, the affected son and the mother, respectively.

To further analyze the region surrounding exon 3, a combination of probe C2.6 with several enzymatic digestions (Eco RI, Pvu II and Hind III) of genomic DNAs was used. Hind III digestion showed the presence of a normal 3.3 kb fragment in the father and in the two affected siblings (Fig. 2, lane 1 ⇨ 3, lanes 4 ⇨ 7 refer to normal individuals). Eco RI analysis (Fig. 3) allowed the identification of a new 6 kb fragment in the father[1-2], in the two affected siblings (II-3 and II-4) and in the two sisters fathered by 1-2 in a previous marriage (II-1 and II-2). The abnormal Eco RI 6 kb fragment showed concordant Mendelian segregation only with the mutated chromosome, thus suggesting that II-1 and II-2 were PKU carriers. Also, the father's sister[1-4] was known to be a PKU carrier because of the presence of the Eco RI 6 kb fragment. Pvu II-digests of genomic DNAs also probed with C2.6 in this family uncovered a new 8.8 kb fragment that was only found in all individuals who also bore the new Eco RI 6 kb band (data not shown). Consistent with Eco RI results, segregation studies also showed, as expected, that the new Pvu II fragment (8.8 kb) was transmitted by the father to the affected daughter (Fig. 4). STR analysis showed that the father (224/240), the mother (244/252), the affected son (224/252) and the affected daughter (224/252) were all heterozygous for this intragenic microsatellite polymorphism. STR allele no. 224 is associated with the paternal mutant PAH gene and has not been previously described. A tentative map of exon 3 and flanking intronic regions based on the results of this study and on published[15-3] and unpublished (Eisensmith and Goltsov: personal communication) data is displayed in Fig. 5. Based on Hind III, EcoRI and Pvu II data, the map shows a deletion of approximately 2.5 kb which removes exon 3. Finally, illegitimate transcription analysis carried out in RNA isolated from peripheral blood lymphocytes of the affected boy led to the identification of a normal (375 bp) cDNA fragment.

Fig. 2: Autoradiograph obtained by hybridization of probe C2.6 to Hind III digested genomic DNA from the father and the two affected siblings (asterisks) or from other normal individuals. Only the normal 3.3 kb fragment is seen. The Hind III 4.2 kb fragment which is diagnostic for the deletion observed in Yemenite Jewish patients is missing.

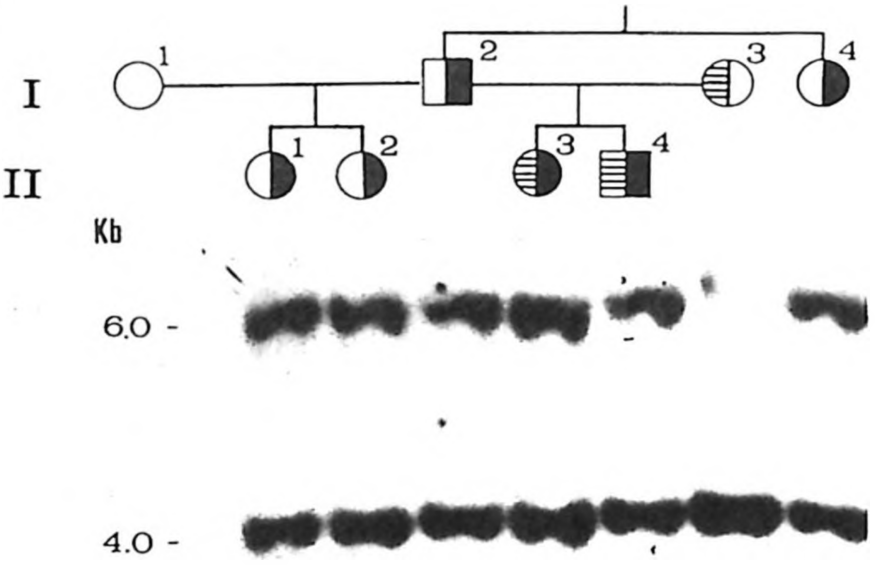


Fig. 3: Segregation analysis of the abnormal Eco RI 6 kb fragment detected with probe C2.6 in family F2. Individuals indicated with half-filled squares or circles bear the EcoRI 6 kb fragment diagnostic for the deletion of exon 3 in the PAH gene. Half-striped squares or circles indicate that the mother and the two affected siblings are heterozygous for the IVS X nt546 mutation. Only the mother (I-3) did not carry the abnormal fragment.

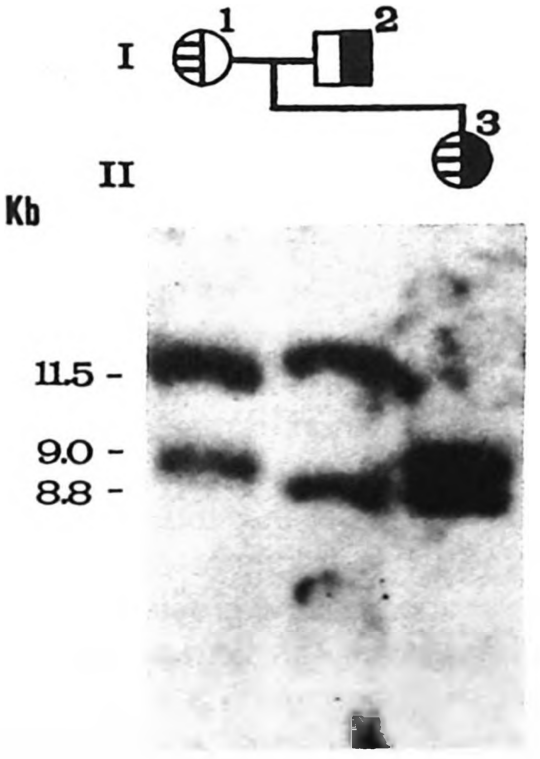


Fig. 4: Segregation analysis of the abnormal Pvu II 8.8 kb fragment hybridizing to probe C2.6. This fragment was transmitted from the father to the daughter, who was affected by classical PKU. Other PKU carriers and the affected brother also bear this fragment (data not shown).

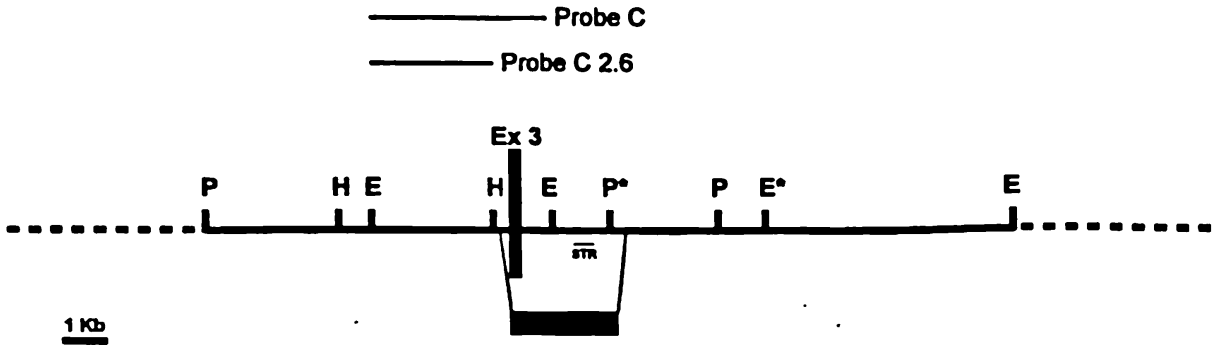


Fig. 5: Restriction map of the PAH gene in the exon 3 region, showing the deletion of approximately 2.5 kb (filled rectangle below the restriction map) removing exon 3 in PKU family F2. The position of Hind III (H) and EcoRI (E) sites were located according to Avigad et al.,[3] Di Lella et al.,[15] and Eisensmith and Goltsov (personal communication), respectively. P* indicates the Pvu II (b), and polymorphic site E* refers to an EcoRI site not reported in the map published by Di Lella et al.[15] The STR locus map 0.7 kb downstream to exon 3[6], however its relative position to the polymorphic Pvu II site is tentative.

Discussion

The results of DNA fingerprinting analysis make unlikely the possibility that the Pvu II (b) RFLP discordance was due to false parenthood. An alternative explanation would assume that the father is hemizygous for the Pvu II (b) 11.5 kb allele, and the mother transmits to the two affected siblings the IVS X nt 546 mutation. Such a hypothesis implies that exon 3 in the PAH gene inherited from the father is deleted, since this is the only coding region contained in the Pvu II (b) polymorphic fragments recognized by the phPAH247 probe[15]. The deletion is probably masked by the presence of the other normal 11.5 kb allele in the father and by the presence of the maternal PKU chromosome Pvu II (b) 9.0 kb allele in the affected son and daughter. The new fragments seen with Eco RI and Pvu II using probe C2.6 are likely to reflect the occurrence of a gene rearrangement around exon 3, rather than the presence of restriction fragment-length polymorphisms. For instance, the occurrence of a new 6 kb fragment could be due to a new Eco RI polymorphism. However, this possibility is unlikely because, according to the map published by Di Lella et al.,[15], neither the appearance of a new Eco RI site nor the abolition of a preexisting one, would lead to a 6 kb fragment. Such a fragment could only be generated by the contemporary (and unlikely) abolition and creation of two Eco RI restriction sites. The Eco RI data are rather consistent with the deletion of a DNA fragment including exon 3 and the Eco RI site neighboring the 3' end of exon 3. Consequently, the Eco RI 6 kb band could be generated as a junction fragment linking the two deletion breakpoints. In our preliminary report on this family[4], a deletion of approximately 9-10 kb was suspected, based on the restriction map published by Di Lella et al.[15] However, a recent reexamination of the Eco RI restriction map

of the original cPAH 10 lambda clone (Eisensmith and Goltsov: personal communication) allowed the identification of one additional Eco RI site (see E* in Fig. 5) not reported in the map of the PAH gene published by Di Lella et al.[15] Accordingly, the length of DNA removed by the deletion would be approximately 2.5 kb long. The latter estimate fits well now with the reduction of the Pvu II (b) fragment size from 11.5 kb to 8.8 kb (Fig. 4). On the other hand, by taking into account the STR data only, the deletion could be shorter (i.e., less than 1.4 kb). The discrepancy between STR and restriction enzyme analysis clearly prevents a precise estimate of the deletion width, and it is likely to be due to the still incomplete characterization of the physical map in this specific region of the PAH gene. Furthermore, no other mutations altering the coding sequence were detected by DGGE and sequence analyses carried out on the paternally inherited, mutant PAH gene[1]. On the other hand, our attempts to identify a shorter transcript due to the deletion of exon 3 by illegitimate transcription analysis were unsuccessful. This result suggests that the deletion probably impairs the correct splicing of exons 2 and 4. Since probe C2.6 identifies in these patients a normal Hind III 3.3 kb fragment (Fig. 2) 3, the deletion must have its 5' breakpoint further downstream compared to the Yemenite Jews' mutation (the closest Hind III site upstream to exon 3 is conserved). It is possible that intronic regions flanking exon 3 contain hot spots for recombination. According to previous reports[1-4], the frequency of this mutation in Sicily is approximately one percent. The results of this study also emphasize the importance of undertaking a detailed molecular analysis of RFLP discordances incidentally detected at the PAH locus, since they may underlie the presence of PKU-causing mutations.

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