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Pelizaeus-Merzbacher Disease: Detection of Mutations Thr[181]→Pro and Leu[223]→Pro in the Proteolipid Protein Gene, and Prenatal Diagnosis

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Summary

A family with an apparent history of X-linked Pelizaeus-Merzbacher disease presented for genetic counseling, requesting carrier detection and prenatal diagnosis. RFLP analysis using the proteolipid protein (PLP) gene probe was uninformative in this family. A prenatal diagnosis on a chorionic villus sample (CVS) was carried out using single-strand conformation polymorphism (SSCP) analysis of a variant in exon 4 of the PLP gene. The fetus was predicted to be unaffected. Sequencing of the exon from the CVS, the predicted-carrier mother, and the obligate-carrier grandmother revealed an A-to-C change at nucleotide 541 in the two women but not in the fetus. As this change results in a Thr-to-Pro change at amino acid 181 in a region of the gene predicted to be part of a transmembrane segment, it was concluded that this was the mutation causing the disease in this family. In addition, in a second family, an exon 5 variant band pattern on SSCP analysis was shown by sequencing to be due to a T-to-C change at nucleotide 668. This results in a Leu-to-Pro change in a carrier mother and in her two affected sons. These results provide further examples of mutations in PLP that cause Pelizaeus-Merzbacher disease and illustrate the value of SSCP in genetic analysis.

Introduction

Pelizaeus-Merzbacher disease (PMD) causes loss of myelinating cells and myelin in the central nervous system. It is genetically and pathologically heterogeneous (Scheffer et al. 1991), but molecular genetic studies have recently demonstrated the role of the proteolipid protein (PLP) gene in the classical X-linked form of the disease. Five independent point mutations of the PLP gene have been reported (Gencic et al. 1989; Hudson et al. 1989; Trofatter et al. 1989; Pham-Dinh et al. 1991; Pratt et al. 1991), but an additional 14 cases reported by Pham-Dinh et al. (1991), 11 with an apparent X-linked pedigree, failed to reveal any sequence changes except for one silent change. This leaves the possibility that there may be another X-linked gene involved. Evidence for autosomal inheritance in some families has also been presented (Begleiter and Harris 1989), particularly in the connatal form of the disease.

Carrier detection is especially difficult in PMD, as female carriers are asymptomatic and no biochemical tests exist. Magnetic imaging has been used but is inaccurate in young females (Boltshauser et al. 1988). As with any X-linked lethal disorder, there is the complication of new mutations, and in addition, in PMD there is the possibility of genetic heterogeneity.

If a PLP mutation is assumed in an apparently X-linked pedigree, then an RFLP for the gene can be followed through the family. The PLP gene is highly conserved. Two polymorphisms have been reported. An *Msp*I polymorphism has an allele frequency of .92/.08 (Wu et al. 1987) and is relatively uninformative. A second polymorphism arising from a single-base silent substitution in aspartate codon 202 has allele frequencies of .74/.26 (Trofatter et al. 1991).

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As the PLP gene has been assigned to Xq22 by somatic cell hybrids and linkage (Willard et al. 1987), closely linked polymorphic markers can also be useful, although this introduces the possibility of error due to recombination.

In view of these difficulties it is clear that direct mutation detection by DNA sequencing is highly desirable in families requiring carrier detection or prenatal diagnosis. As messenger RNA is not readily available and as the gene has seven exons, a preliminary exon screen to identify the most likely site of mutation will simplify this task.

Single-strand conformation polymorphism (SSCP) is a convenient method of screening coding regions for changes (Hayashi 1991) and has been used to identify mutations in several genes, including those involved in familial polyposis coli (Groden et al. 1991) and DRASH syndrome (Pelletier et al. 1991). It is based on the principle that single-stranded DNA under nondenaturing conditions will adopt a unique sequence-dependent conformation and that this will determine its mobility on electrophoresis. In practice, radiolabeled PCR products are denatured by heating and are rapidly cooled and run on 6% nondenaturing polyacrylamide gels. We have applied this technology to two families with PMD.

Subjects and Methods

Clinical Details

Family 1. — III-1 was born with normal birth weight at 37 wk gestation after a normal pregnancy. In his first few weeks of life his parents noticed that he was a "snuffly" baby. At the age of 5 wk laryngeal stridor was noted by the general practitioner. Over the next few weeks there were problems with abdominal colic, and the child did not gain weight properly. He was admitted to hospital at age 12 wk. Laryngosocopy at that stage showed a "floppy" larynx. He was also noted to have pale optic discs and was thought to have nystagmus. From the age of 12 mo there was no social or motor development. He remained profoundly handicapped. A maternal uncle (II-2) died at the age of 3 years, having had a very similar illness, with laryngeal stridor and failure of development. Autopsy was performed in Bristol and confirmed the diagnosis of PMD. Carrier detection and prenatal diagnosis was requested by II-3.

Family 2. — II-1 was born to nonconsanguineous parents after an uneventful pregnancy which went to term. He was their firstborn child, and it was of note in the family tree that the mother had a brother who died at the age of 9 mo and who was said to been mentally and physically handicapped. On further inquiry there was evidence from the hospital notes that this male was hydrocephalic, had pale optic disks, and was quadriplegic; and it must remain uncertain whether he had the same condition as his two nephews. II-1 weighed 8 lb 6 oz at birth, and his early development seemed normal in that he smiled at 6 weeks and fed well.

At 10 wk of age he was admitted to the local hospital because of inspiratory stridor, and at that time his mother noted his jerky eye movements. He was referred to the Hospital for Sick Children, Great Ormand Street, where, on examination, he was found to be floppy and to have nystagmus, and a diagnosis of a mild laryngomalacia was made to explain the stridor. After discharge, his stridor settled, but he made poor developmental progress. At the age of 13 mo he was still unable to sit, and he was readmitted for further investigation. At that stage he had truncal hypotonia but had brisk reflexes with extensor plantar responses. A computed-tomography brain scan showed minor cerebral atrophy, but his brain-stem auditory evoked responses revealed only wave I, which is often found where central myelination is poor. All other investigations, including those of chromosomes and a variety of metabolic tests were normal.

By the age of 3 years II-1 was still very developmentally behind and, an MRI scan was undertaken. This showed gross undermyelination throughout the white matter of both hemispheres and the posterior fossa. In fact, there was no evidence of any normal myelin formation, and a diagnosis of PMD was made.

II-2 is the brother of II-1, and he was born when II-1 was 21 mo of age. At that age a diagnosis in his brother had not been arrived at.

The pregnancy was again uneventful, and II-2 had a good birthweight. It had been noted antenatally that the fetus had a left renal tract abnormality, and postnatally this was shown to be a duplex kidney on one side. He was jittery at birth, and, like his brother, he developed stridor. His progress was poor, and nystagmus was noted early in life. The optic disks were pale, and further investigations revealed abnormal brain-stem evoked responses identical to those of his brother. At 22 mo he is still floppy but has a few words. Clinically, II-2 is identical to II-1, and further investigations were not undertaken.

Chorionic Villus Sampling (CVS)

DNA was prepared from a CVS sample by using standard procedures. Fetal sex was established by direct karyotyping culture and by DNA analysis.

Restriction-Enzyme Analysis

DNA was prepared from the nuclei of peripheral leukocytes by guanidinium extraction (Jeanpierre 1987). *Taq*I, *Msp*I, and *Pst*I digestions were carried out according to the manufacturer's (NBL) instructions. The resulting fragments were separated by electrophoresis on 0.8% agarose gels, either for 24 h at 95 mA or for 35 h at 95 mA, to separate closely sized fragments for hybridization with CX52.5 (DXS101), 19-2 (DXS3), S21 (DXS17), and PLP cDNA probes. Gels were denatured with 0.4 M NaOH, 1.5 M NaCl, were blotted directly onto Hybond N^+ (Amersham International), and were fixed by being rinsed with 0.4 M NaOH, followed by two washes with 2 × SSC.

Hybridization

The probes were labeled by random hexanucleotide primer extension with ^{32}P dCTP. Prehybridization and hybridization each were carried out in 10 × Denhardt's solution, 4 × SSC, 50 µg sonicated salmon sperm DNA/ml, 0.1% SDS for 16 h at 65°C in a rotating hybridization oven (HYBAID). Filters were washed for 3 × 20 min in 3 × SSC, 0.1% SDS at room temperature. Final washes for all probes except CX52.5 were carried out at 65°C for 10 min in 0.5 × SSC, 0.1% SDS, followed by a 0.2 × SSC, 0.1% SDS wash if background persisted. CX52.5 filters were washed in 3 × SSC, 0.1% SDS for 10 min at 65°C.

Oligonucleotide Primers

Primers, from the introns of the PLP gene, for the PCR were provided by Dr. L. Hudson (Gencic et al. 1989). Further syntheses were carried out on an Applied Biosystems 381A DNA synthesisor. 5′ Biotinylation was carried out during oligonucleotide syntheses using DMT-biotin-C-6-PA (CRB) according to the manufacturer's instructions. Primer sequences, including rested primers for sequencing and PCR product sizes, are given in table 1.

SSCP Analysis

Exons 1–7 of the PLP gene were individually amplified by PCR. Reactions were carried out in a 50-µl volume by using 100–200 ng of genomic DNA; 25 pmol of each primer; 20 nM dCTP, 200 nM each of dATP, dCTP, and dGTP; 0.1 µl of P^{32}-dCTP, reaction buffer (PROMEGA), and 10^{-5} M TEMAC.

Reactions were overlaid with approximately 50 µl of mineral oil and denatured at 94°C for 10 min and were cooled to 55°C (annealing temperature), and 1.5–2 units of *Taq* polymerase (Promega) were added. Twenty-five cycles, each comprising 72°C (1 min), 94°C (1 min), and 55°C (30 s) were carried out, followed by 10 min at 72°C on a Techne PHC-2 machine. PCR products were checked by running 10 µl on a 1.5% agarose gel, followed by ethidium bromide staining.

For SSCP analysis 4 µl of each PCR product were mixed with an equal volume of stop solution (U.S.B. Sequenase kit). Samples were denatured at 94°C for 10 min, placed in a dry-ice/ethanol bath, microfuged briefly, and placed on dry ice again. One to one and one-half microliters of each sample were loaded onto a 6% 37.5:1 acrylamide: bis nondenaturing polyacrylamide (Protogel, Lablogic) gel, and electrophoresis carried out at 4°C for 24 h at 380–400 V, on a BRL model 2 sequencing system. The gel was blotted onto Whatman paper, dried under vacuum at 80°C, and exposed to X-ray film (Kodak) for 24–120 h at −70°C.

Sequencing of PCR Products

The PCR reaction was performed using 5 pmol of each primer, one of which (5′ side) was biotinylated at the 5′ terminus, 200 nM of each dNTP, 10^{-5} M TEMAC, reaction buffer (Promega), and 200–500 ng of genomic DNA in a total volume of 100 µl. Reaction conditions and product analysis were as described previously for SSCP, except that the annealing temperature was 52°C and 30 cycles were performed.

To produce single-stranded DNA, 50 µl of PCR product (avoiding oil) and 30 µl of magnetic Dynal M-280 streptavidin (Dynal UK) beads were mixed and incubated for 5 min at 20°C with gentle agitation, and the reaction tube was placed in a magnet (Promega). The beads with bound double-stranded PCR product collected to one side, and the supernatant containing unincorporated nonbiotinylated primer and nucleotides was removed. The reaction was removed from the magnet, incubated with 0.15 M NaOH for 5 min at 20°C to denature the double-stranded product, and replaced in the magnet, and the supernatant containing the unbiotinylated PCR strand was removed.

The single-stranded product was washed in turn

Table I

Positions and Sequences of PCR and Sequencing Priemrs

Exon and Position	Sequence	Size (bp)
1:		
Intronic 3'.............	5' CCC GAA CCC CAA AGT TGG AA 3'	511
Intronic 5'.............	5' AAG TTT CCA GGA CAT CTTCA 3'	
2:		
Intronic 3'.............	5' TGC TAG TTA CTA AGG TAC TG 3'	362
Intronic 5'	6' TGA GCA CAG GGC CTG GCA GA 3'	
3:		
Intronic 3'.............	5' ACG AGG CCA CAG ACT CGC GC 3'	412
Intronic 5'.............	5' ACC TCA CTT ATG TCG GGA AA 3'	
4:		
Intronic 3'	5' ACC ACC CTC CTT ACA CTA AG 3'	335
Intronic 5'	5' ACT CCA GGA TCT CCC AGT TT 3'	
Intronic 3'	5' CTT CCA TAG TGG GTA GGA GA 3'	Nested sequencing primers
Intronic 5'	5' GAT CTG CAG GCT GAT GCT GA 3'	
5:		
Intronic 3'	5' ATT GAA GGC CAT GGG TGT AA 3'	236
Intronic 5'	5' AGA GAT GGA AGA AGG GCT CT 3'	
Intronic 3'	5' TGG TAT TAG CTA CTC CCT TG 3'	Nested sequencing primers
Intronic 5'	5' CCC CGT AAC TCC ATA AAG CT 3'	
6:		
Intronic 3'	5' TTG CCT TTC AGA ATA GCT GT 3'	239
Intronic 5'	5' AAA GAT ATC AAC ACA TTC AG 3'	
7:		
Intronic 3'	5' GGT GGC AAA GGC AAA GAG TT 3'	261
Intronic 5'	5' TTT TAT TTC TAC CCT TCC TC 3'	

with 100 µl TES ([10 mM Tris pH 8.0, 1 mM EDTA, 0.1 M NaCl] × 2) and 100 µl of water by adding the solution and mixing, placing it in the magnet, and removing the supernatant before resuspending it in 7 µl of water. The sequencing reaction was carried out using the 3' side sequencing primer according to the Sequenase kit (U.S.B.) protocol. The labeled strands were then separated from the beads by denaturing at 65°C for 5 min, placing them in the magnet, and removing the supernatant. The supernatant was heated to 85°C for 2 min, 2 µl were loaded onto a 6% polyacrylamide (Acugel; Lablogic) denaturing gel (8.3 M urea), and electrophoresis was carried out at 55–60 W on a BRL model 2 sequencing apparatus for 2–2.5 h. The gel was fixed in 10% methanol, 10% acetic acid for 30 min before the procedure was continued in the manner used for SSCP gels.

Results

Pedigree 1 (fig. 1) shows the characteristic pattern of X-linked recessive inheritance. II-3, whose brother (II-2) and nephew (III-1) have died of PMD, presented for genetic counseling, requesting carrier detection and prenatal diagnosis. Her mother (I-2) and sister (II-1) are obligate carriers, having had an affected son and grandson and affected son and brother, respectively. No DNA from either affected male was available, and at this stage II-3 had a 50% risk of being a carrier. Pedigree 2 (fig. 2) shows that the mother (I-1) is an obligate carrier, having had two affected sons (II-1 and II-2).

RFLP Analysis

RFLP analysis of the *Msp*I polymorphism, with alleles of 4 and 4.5 kb, proved uninformative in families 1 and 2. Linked markers from the Xq22 region were used, and three of these S21 (*Taq*I), 19-2 (*Taq*I), and CX52.5 (*Msp*I) proved partially informative in family 1. In each case (fig. 1), I-2, the obligate carrier mother, was heterozygous, which enabled her mutant and normal chromosomes to be distinguished. The mutant chromosome is the one she has passed on to her obligate carrier daughter (II-1), in this case identified by

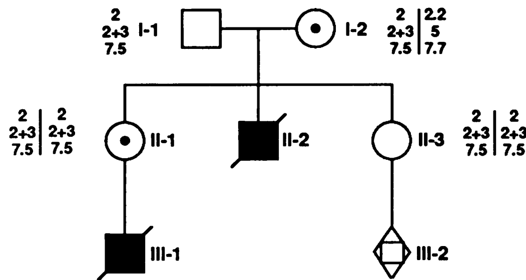

Figure 1 Partial pedigree of family 1, showing RFLP results with S21(*Taq*I), 19-2(*Taq*I), and CX52.5(*Msp*I) probes. ■ = Affected males; □ = normal males; ⊙ = carrier females; ◇ = male fetus; and ○ = females of normal or undetermined carrier status.

the 2.0-, 2 + 3-, and 7.5-kb alleles detected with probes S21, 19-2, and CX52.5, respectively.

As II-3 had also inherited these alleles from her mother, she was predicted to be a carrier. On the basis of S21 and 19-2 probe results, and with account taken of the possibility of recombination when recombination fractions of .08 between DXS3 and PMD and .04 between DXS17 and PMD are used, II-3 was given a 98.7% carrier risk.

The grandfather (I-1) is hemizygous for the 7.5-, 2.0-, and 2 + 3-kb alleles. Thus, II-1 and II-3 were both homozygous for these probes, and prenatal diagnosis was not possible. At this stage II-3 had a 50% risk of having an affected boy, and her only options were to terminate all male pregnancies or accept this risk.

Subsequent to the prenatal diagnosis carried out in family 1 its members were tested for the exon 4 RFLP, detected by *Aha*II, reported by Trofatter et al. (1991). The polymorphism is visualized by digesting a 227-bp PCR product, from exon 4, with *Aha*II; this product digests to two fragments—of 183 and 44 bp—when the cutting site is present. I-2 was heterozygous for the presence of the *Aha*II site but passed on the *Aha*II$^-$ form to both daughters, who were, therefore, uninformative for the polymorphism.

In the case of family 2, the mode of inheritance could not be determined from the pedigree, leaving the possibility of autosomal inheritance, and thus prenatal diagnosis could not be offered in this family, even though the mother (I-1) was heterozygous for S21 (*Taq*I) and pXG12 (*Pst*I). Only direct detection of a deleterious mutation would establish the mode of inheritance and enable counseling to be given to this family.

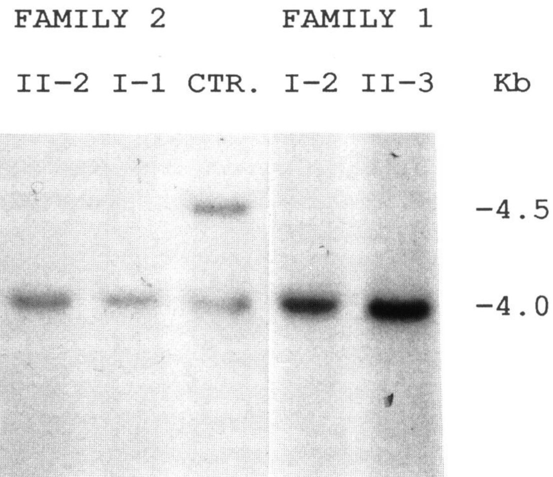

Figure 2 Partial pedigree of family 2, showing RFLP results with S21(*Taq*I) and PGX-12(*Pst*I) probes. Symbols are as in fig. 1.

SSCP Analysis

SSCP analysis of exons 1–7 of the PLP gene showed a variant band pattern in exon 4, which segregated with carrier status in family 1 (fig. 3). The arrow in figure 3 represents the variant band, which II-3 and II-1 have inherited from their obligate-carrier mother. The mutant and normal chromosomes could now be distinguished, and prenatal diagnosis was now possible in this family. The variant band was not observed in 51 unrelated chromosomes used as controls.

Under the conditions used, only one strand of the mutant chromosome shows a conformation suffi-

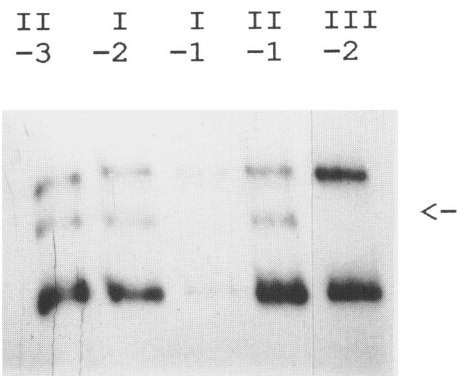

Figure 3 SSCP analysis of exon 4 in family 1. Radiolabeled PCR product was mixed with stop solution, denatured, and electrophoresed at 4°C on a 6% polyacrylamide gel at 380–400 V. The arrow indicates the variant band, which is present in all the carrier women (II-1, II-3, and I-2) but not in the normal (I-1) or CVS (III-2) samples.

ciently altered to modify mobility on electrophoresis. On fetal sexing of CVS material, the fetus of II-3 was shown to be a male. SSCP analysis of exon 4 (fig. 3) showed that it did not have the variant band pattern, and a 95% prediction of normality was given. The 5% risk allowed for both 2.5% based on the possibility of genetic heterogeneity and another 2.5% for the use of an as yet novel technique.

SSCP analysis of exons 1–7 of family 2 showed a variant band pattern in exon 5 (fig. 4). The mother (I-1) had both the variant band and the normal band, and the affected boys (II-1 and II-2) had only the variant. The variant band was not seen in 51 unrelated chromosomes used as controls.

The PCR products from three of the larger exons were digested with suitable restriction enzymes which cut within the exon-coding sequence, in order to increase the chance of observing a shift when SSCP was used. Exon 1 products were digested with *Msp*I, exon 3 products with *Msp*I, and exon 4 products with *Rsa*I. No additional shifts were seen.

Sequencing

Sequencing in I-1, I-2, II-1, II-3, and the CVS sample (III-2) from family 1 showed an A-to-C change at nucleotide 541 in the three carrier women (I-2, II-1, and II-3) (fig. 5). This results in a threonine (ACC)-to-proline(CCC) change at amino acid 181. In each case, because of heterozygosity, both the A and C bands were present. The CVS and I-1 (grandfather) had only the normal A band, supporting the SSCP analysis result. The pregnancy is continuing. The risk to the fetus is now considered to be reduced below the 5% quoted to the family after SSCP analysis and is probably less than 1%.

Sequencing of exon 5 in family 2 showed a T-to-C change at nucleotide 668 (fig. 6). The mother (I-1) had both C and T bands, while her affected sons (II-1 and II-2) had only the mutant C band. This results in a leucine(CTT)-to-proline(CCT) change at amino acid 223.

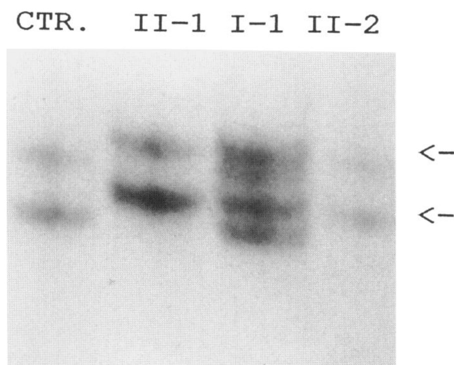

Figure 4 SSCP analysis of exon 5 in family 2. Conditions were as in fig. 3. The arrows indicate the variant bands. The carrier mother (I-1) is heterozygous, while both affected boys (II-1 and II-2) are hemizygous for the variant bands. CTR = unaffected control.

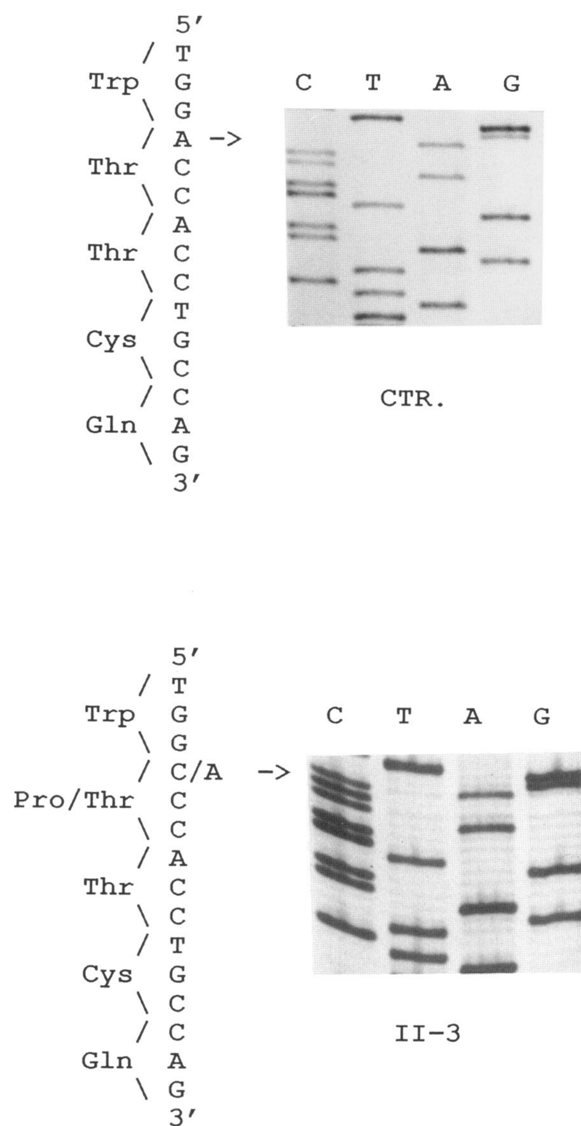

Figure 5 DNA sequence of exon 4 PCR product from a normal control (CTR.) and a carrier female (II-3) in family 1. The arrow indicates the A-to-C change which substitutes a proline for a threonine at amino acid 181.

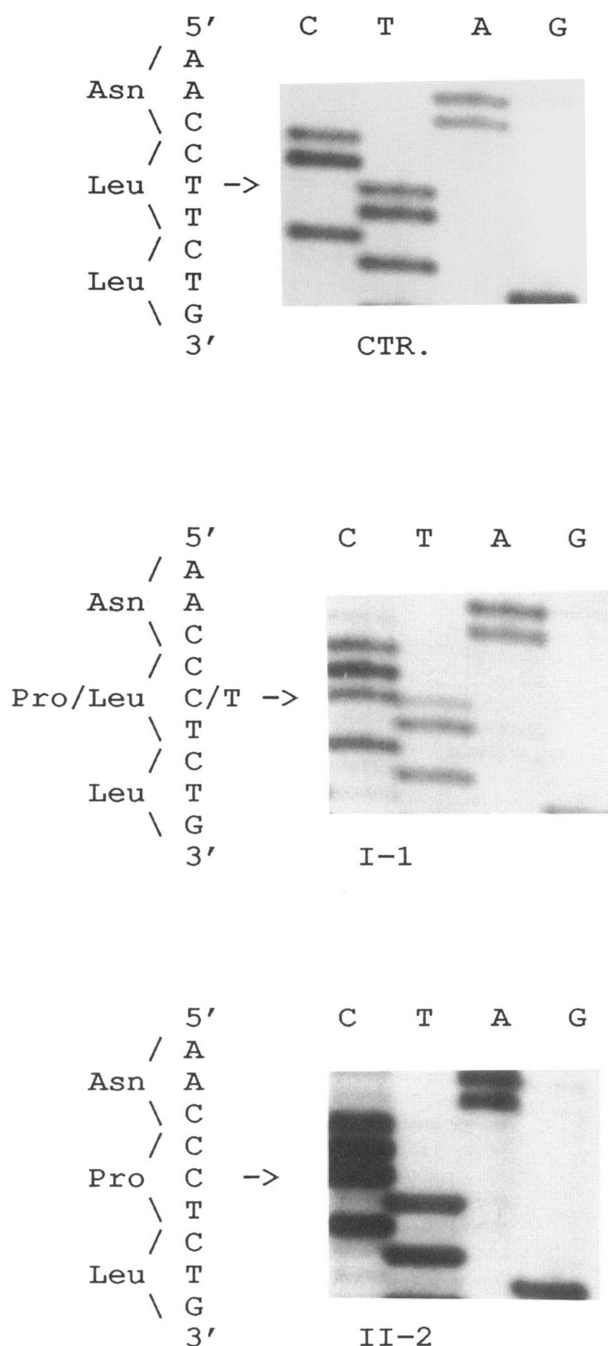

Figure 6 DNA sequence of exon 5 PCR product from a normal control (CTR.), the carrier mother (I-1), and an affected (II-2) boy in family 2. The arrow indicates the T-to-C change which substitutes a proline for a leucine at amino acid 223.

Discussion

Mutations in the PLP gene clearly result in classical X-linked PMD. However, as the PLP gene has been sequenced, in a number of cases, without evidence of a mutation, the possibility of further genes causing PMD, either X-linked or autosomal, is still unresolved. This complicates carrier detection and prenatal diagnosis using genetic markers in or around the PLP gene. The examples presented here illustrate the importance and usefulness of direct sequencing of the exons. The risk to a male fetus was initially raised from the pedigree risk of 25% to around 50% when it was shown, on the basis of linked probes, that the mother was likely to be a carrier. This risk was reduced substantially after SSCP analysis—and even further after sequencing. The finding of a Thr181-to-Pro change in a membrane-spanning segment established that the mutation causing PMD in this family is almost certainly in the PLP gene (see below) and that the fetus did not carry it.

SSCP analysis failed to detect the *Aha*II polymorphism present in the mother. The altered band found could not be a result of this polymorphism, as the mother alone was heterozygous, whereas the altered SSCP band was shared by the mother and both daughters.

As DNA sequencing becomes more straightforward, particularly as automated sequencing is introduced, it may be more efficient to sequence directly. However, at the present time many clinical molecular genetic laboratories will find that SSCP provides a straightforward and fast method of screening for sequence changes. It involves no special primers or equipment and is likely to be equally effective for small deletions or point mutations.

SSCP will also pick up changes which are silent or polymorphic. A silent change within the PLP gene has been described. Pham-Dinh et al. (1991) and Trofatter et al. (1991) found a frequent (.26) silent substitution (GAT to GAC) in aspartate codon 202. This falls within exon 4, but we failed to observe it by SSCP analysis. The exon 4 PCR product was 335 bp, which is slightly longer than the optimum reported for SSCP detection. We digested the products with *Rsa*I, which cuts at amino acids 30 and 67 to give three fragments closer to the optimal size of 200 bp or less (Hayashi 1991). The *Aha*II polymorphism falls within the central portion of 110 bp. However, an SSCP change was still not observed.

Other easy screening methods are increasingly being reported. For example, Hydrolink gels appear to be efficient in detecting heteroduplexes (Tassabehji et al. 1992) and may in future be used in conjunction with SSCP.

As polymorphisms detected by SSCP may not be

disease-causing changes, sequencing of the altered exon is necessary. However, in family 1 the grandmother is an obligate carrier in the pedigree, and new mutation is not an issue, so the SSCP change could be used as a variant to follow the mutant PLP gene through the family, even if it has not been established that it reflects the disease-causing mutation. This is particularly useful for genes, such as PLP, where there are very few polymorphisms. SSCP polymorphisms are easy to detect and use PCR technology. They are likely to be widely applicable, as illustrated here for the CVS sample, but may be replaced as sequencing becomes more automated.

We report here two previously unreported mutations of the PLP gene in cases of PMD. A model of the protein structure and function has been proposed (Stoffel et al. 1984). In this model the mutation we find in exon 4 would arise inside an alpha-helical transmembrane segment, and the presence of a proline residue which has been shown to be an alpha-helix breaker makes it very likely that this is the disease-causing mutation. The mutation found in exon 5 also involves a nonconservative amino acid change, in this case Leu to Pro at amino acid 223. Amino acid 223 is predicted to be in an extracytosolic loop. We cannot predict how the altered amino acid would change function, but PLP gene structure is very highly conserved across species. In addition, as we have not seen either of these changes in 51 other X chromosomes studied, we feel that it is very likely that they are the disease-causing mutations.

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