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Studies of the candidate genes in X-linked congenital cerebellar hypoplasia

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Abstract A gene for X-linked congenital cerebellar hypoplasia was recently localized to chromosome Xp11.21-q24. This region comprises several brain-specific genes responsible for various neurological disorders, including the *proteolipid protein* (*PLP*), *doublecortin*, and *PAK3* genes. We screened these genes for mutations in patients with X-linked congenital cerebellar hypoplasia and found no pathogenic nucleotide changes or gene dose alterations. These findings allow the ruling out of *PLP*, *doublecortin*, and *PAK3* as the disease-causing genes in this hereditary neurological syndrome.

Key words Congenital cerebellar hypoplasia · X-chromosome · Genetic locus · Candidate genes

Introduction

We recently described a novel syndrome of X-linked congenital cerebellar hypoplasia which segregated as an X-linked recessive trait in a large family from eastern Russia [6]. In brief, the disease is characterized by markedly delayed early developmental motor milestones, cerebellar ataxia, dysarthria, external ophthalmoplegia, pyramidal signs, and nonprogressive course. Neuroimaging studies reveal marked hypoplasia of the cerebellar vermis and hemispheres. Using linkage analysis we assigned the mutant gene to a 38-cM interval on chromosome Xp11.21-q24 flanked by the marker loci DXS991 and DXS1001 [6]. These findings allowed the application of a candidate gene approach in the chromosomal region of interest (Fig. 1).

One attractive gene, *proteolipid protein* (*PLP*), is located on chromosome Xq22 [8] and encodes two major CNS myelin proteins, PLP and DM 20 [9]. It has recently been shown that various mutations within the PLP gene result in at least three allelic X-linked disorders, namely Pelizaeus-Merzbacher disease [3], X-linked pure [2], and

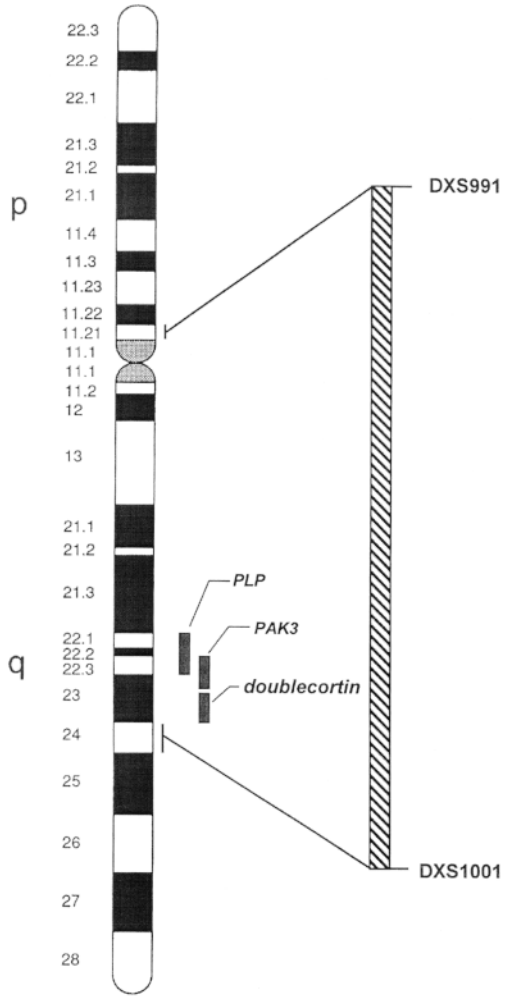

Fig. 1 Genetic map of the critical region on chromosome X. *Striped bar* genetic locus for X-linked congenital cerebellar hypoplasia flanked by marker loci DXS991 and DXS 1001; *gray bars* locations of the candidate genes

complicated [11] hereditary spastic paraplegia. Although these conditions are characterized by distinct clinical phenotypes, they have in common some neurological symptoms such as pyramidal spasticity and cerebellar ataxia; both signs are invariably observed in our patients with X-linked congenital cerebellar hypoplasia. Because the genetic interval determined in our study encompasses the *PLP* gene, it was reasonable to examine the *PLP* gene as a candidate disease gene for this newly described syndrome.

Two additional genes of interest are *doublecortin* and *PAK3*. Mutations in *doublecortin* were recently identified as causing double cortex/X-linked lissencephaly, a neuronal migration disorder in which affected patients have varying degrees of epilepsy and mental retardation [4, 10]. Although this disorder is clinically distinct from congenital cerebellar hypoplasia, both the chromosomal localization to Xq22.3–23 and its exclusive expression in the brain make it a possible candidate gene. *PAK3* is also expressed in the brain and is localized to Xq22.3–23 [4]. This gene, presumably participating in the control of neuronal shape and growth, was recently shown to be mutated in just one pedigree with X-linked nonsyndromic mental retardation [1], and its exact role in the brain development remains to be elucidated. Both *doublecortin* and *PAK3* are potential candidate genes and were screened for mutations in patients with X-linked congenital cerebellar hypoplasia.

Materials and methods

Family studies

A simplified pedigree of the family under study is shown in Fig. 2. The disease was manifested in seven affected males from three generations, and six patients were examined clinically in detail [6]. For the purpose of mutation screening, genomic DNA samples from the following individuals have been examined: single-strand

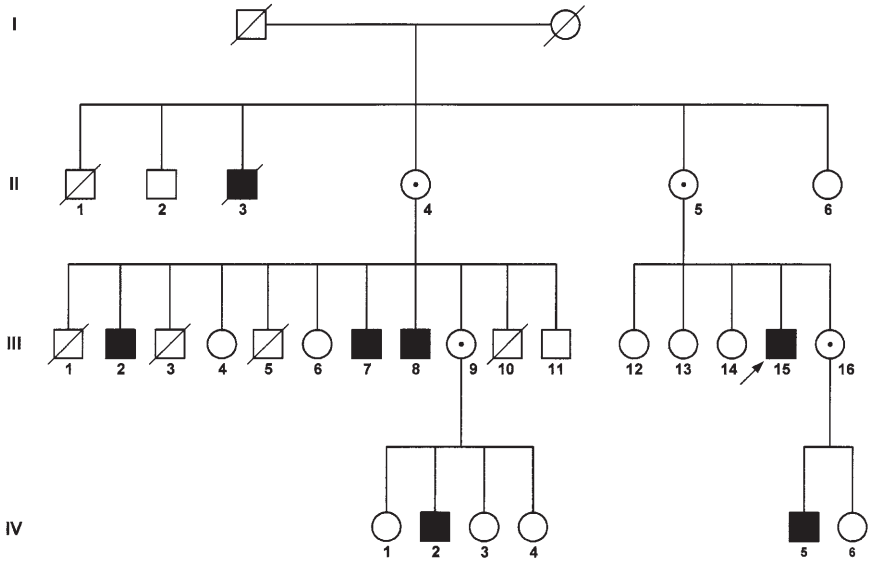

Fig. 2 A simplified pedigree of the family. *Squares* males; *circles* females; *open symbols* unaffected individuals; *solid symbols* affected individuals; *dotted circles*, obligate heterozygous carrier females; *slashed symbols* deceased; *arrow* proband

conformation polymorphism (SSCP) analysis of the *PLP* gene, individuals II-4, II-5, III-2, III-7, III-8, III-9, and III-15; sequencing of the *PLP* gene, individual III-7; sequencing of the *doublecortin* and *PAK3* genes, individual IV-2; gene dose studies of the *PLP* gene, individuals II-4, II-5, III-2, III-7, III-8, III-9, III-15, III-16, and IV-2.

SSCP analysis

The seven *PLP* exons were amplified from genomic DNA with the primers described by Strautnieks et al. [13]. SSCP analysis was performed as described [13] with minor modifications.

Sequence analysis

For systematic sequencing of the *PLP* gene exons and exon-intron junctions, polymerase chain reaction (PCR) reactions were set in a 50-µl volume containing 400 ng genomic DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 1.5 mM $MgCl_2$, 200 µm of each dNTPs, 8 pmol of each primer (one primer being biotinylated), and 2.5 U AmpliTaq polymerase. The cycling profile was was 94 °C for 5 min followed by 30 cycles of denaturation (94 °C, 1 min), annealing (52 °C, 30 s), and extension (72 °C, 1 min), with a final extension at 72 °C for 10 min. PCR products were purified and concentrated with SUPREC-02 (Takara). Single-stranded DNA was produced using magnetic Dynabeads M-280 streptavidin (Dynal AS). The two strands were sequenced directly with the same primers as for amplification; the CA strand was sequenced using an automated ALF DNA Sequencer II and AutoRead Sequencing kit (Pharmacia), and the GT strand using conventional autoradiography and Sequenase version 2.0 kit (U.S.B.).

For systematic sequencing of the coding exons and exon-intron junctions of *doublecortin* and *PAK3*, PCR was performed using QiagenTaq polymerase according to the manufacturer's recommendations. The cycling profile was 94 °C for 10 min followed by 35 cycles of denaturation (94 °C, 30 s), annealing (60 °C, 1 min), and extension (72 °C, 2 min), with a final extension at 72 °C for 10 min. Purified PCR products (GeneClean, Bio101) were sequenced independently with the forward and reverse primers by standard protocols and analyzed on an ABI377 automated sequencer (Perkin Elmer).

Gene dose analysis

Gene dose studies of the *PLP* gene were performed by a real-time quantitative PCR method [5] using ABI Prism 7700 Sequence Detector (Perkin Elmer Japan). Exon 7 of the *PLP* gene was amplified using the primers 5′-TGATGCCTCAGAGACATCGG-3′ (forward) and 5′-ACGCAGCATTGTAGGCTGTG-3′ (reverse). A TaqMan probe for the *PLP* gene exon 7 was designed, and the sequence for the probe was 5′-(FAM)AGCTCACCTTCATGATTGCTGCCAC(TAMRA)-3′. The internal control was constructed by adding a TaqMan β-actin probe and the primers to a sequence of the human β-actin gene [5]. Amplification conditions were as described [5]. DNA samples from five patients and four obligate carrier females were analyzed, and the results were compared to those obtained in the control group comprising three normal males and four normal females.

Results

On SSCP analysis of exons 1–7 of the *PLP* gene, we found no abnormally migrating variant bands in affected patients compared to control individuals. We repeatedly observed an altered SSCP band of exon 2 in one obligate carrier female (II-5, not shown). This abnormal band pattern was not observed in the affected males and therefore did not reflect a disease-causing change and was thus considered to be a result of a benign polymorphism. In patient III-7 we directly sequenced the entire coding region, at least 15 bp of intron at each intron/exon boundary, and 50 bp of 3′ untranslated region of the *PLP* gene. We found no difference from the normal sequence; this result was confirmed by sequencing of the two DNA strands.

Duplications in the *PLP* gene resulting in the disease phenotype have been described in a number of patients with Pelizaeus-Merzbacher disease [7, 12]. To exclude this type of mutation in X-linked congenital cerebellar hypoplasia we performed *PLP* gene dose analyses and found no statistically significant changes in the gene dose in the affected males and obligate carrier females compared to control individuals (data not shown).

Mutation analysis of the *doublecortin* and *PAK3* genes was performed in one affected male (IV-2) by sequencing the entire coding region and exon/intron boundaries of both genes. No mutations between the affected patients and normal individuals were identified.

Discussion

Molecular genetic analysis in the present family revealed no pathogenic nucleotide changes within the coding region and splice sites of the *PLP* gene and no evidence of gene dose abnormalities of the gene. Interestingly, in a number of patients with Pelizaeus-Merzbacher disease and Xq22-linked pure spastic paraplegia no mutations in the *PLP* gene were identified, which raised a possibility of genetic heterogeneity even within these seemingly homogeneous clinical groups [2, 3, 7, 13]. The fact that the clinical findings in our syndrome (in particular, nonprogressive course and cerebellar hypoplasia on magnetic resonance imaging) are remarkably different from those in the *PLP*-associated conditions together with the present molecular data provide, in our opinion, convincing grounds to rule out the *PLP* gene as the disease-causing gene in X-linked congenital cerebellar hypoplasia.

Mutation analysis of the two recently identified brain-specific genes, *doublecortin* and *PAK3*, associated, respectively, with double cortex/X-linked lissencephaly and nonsyndromic mental retardation (both conditions resulting from impairment of the brain development) also revealed no sequence alterations within the coding region and splicing junctions in patients with X-linked congenital cerebellar hypoplasia. It should be noted that some sequence alterations within the regulatory noncoding regions of the studied genes may theoretically account for the disease phenotype in our patients. However, this hypothesis seems very unlikely as these types of mutations, extremely rare in practice, have never been described be-

fore in the *PLP*-, *doublecortin*-, or *PAK3*-associated conditions. To rule out this possibility definitely, further molecular studies with the gene expression analysis are needed.

The large size of the genetic locus comprising the gene for X-linked congenital cerebellar hypoplasia precludes at present the direct application of conventional positional cloning strategies. The growing number of new transcripts in the critical chromosomal region will lead in the near future to the discovery of other appropriate candidate genes, and eventually identification of the causative gene for X-linked congenital cerebellar hypoplasia.

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