Further Evidence for a Fourth Gene Causing X-Linked Pure Spastic Paraplegia

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X-linked hereditary spastic paraplegias (HSPs) present with two distinct phenotypes: pure and complicated. The pure form is characterized by slowly progressive weakness and spasticity of the lower limbs, whereas the complicated forms have additional features (optic neuropathy, retinopathy, extrapyramidal disturbance, dementia, epilepsy, ataxia, ichthyosis, mental retardation, and deafness). Three X-linked loci have been identified for the complicated HSP, while mutations in the proteolipid gene (PLP) (locus SPG2) were implicated in both pure and complicated forms. The absence of identified mutations in the PLP gene in families with both complicated and pure HSP, linked to the SPG2 locus, suggests the existence of another gene in close proximity. We had previously reported a large pedigree with an X-linked form of pure HSP affecting 24 males [Zatz et al., 1976: J Med Genet 13: 217–222]. Here, we present the results of linkage analysis in 19 members of this Brazilian family with markers in or near the PLP locus. Positive LOD scores were obtained with markers at the PLP locus (Zmax = 2.41 at Theta = 0); however, no mutation was found in the coding region of PLP, the intron-exon boundaries, or part of the promoter region. The possibility of a duplication of the PLP gene was also excluded. These results suggest either that there is another X-linked gene in close proximity to the PLP gene or that a novel mutation in the noncoding regions of the PLP gene may cause the disease in this family. © 2002 Wiley-Liss, Inc.

KEY WORDS: X-linked pure spastic paraplegia; new locus; genetic heterogeneity

INTRODUCTION

Hereditary spastic paraplegia (HSP) comprises a heterogeneous group of degenerative disorders of the central motor system, characterized by progressive spasticity of the lower limbs. The inheritance may be autosomal dominant, autosomal recessive, or X-linked. Clinically, two forms of HSP can be distinguished: a pure form, with leg spasticity and weakness, and complicated forms, with other manifestations such as optic neuropathy, movement disorders, dementia, epilepsy, ataxia, ichthyosis, mental retardation, and deafness [Johnston and McKusick, 1962; Zatz et al., 1976; Keppen et al., 1987; Gutman et al., 1989; Goldblatt et al., 1989; Cambi et al., 1995]. Both pure and complicated forms are heterogeneous, but pure HSP is usually autosomal dominant. The major neuropathological findings in the latter form is axonal degeneration involving the terminal ends of the longest fibers of the corticospinal tracts and dorsal columns [Reid, 1997].

Three loci have been identified to date for the complicated form (the most common form) of spastic paraplegia on the X chromosome. SPG1, at Xq28, was identified in a family with HSP associated with mental retardation and optic atrophy [Kenwick et al., 1986; Jouet et al., 1994]. SPG1 is caused by mutations in the gene that codes for L1-CAM, a cell surface glycoprotein that is expressed on the axons of postmitotic neurons and is involved in neuronal migration and neurite extension. Mutations in the LICAM gene cause clinical phenotypes that span a spectrum of disorders from the MASA syndrome, characterized by severe mental retardation, aphasia shuffling gait, and adducted thumbs...
[Jouet et al., 1994], to X-linked hydrocephalus. SPG2, at Xq22 [Saugier-Veber et al., 1994], is caused by mutations in the proteolipid protein (PLP) gene. PLP is a major protein component of the central nervous system myelin [Nave et al., 1987]. A mutation in the PLP gene was found by Cambi et al. [1996] in a family with pure X-linked spastic paraplegia, confirming that mutations in this gene may cause both pure and complicated forms. A third locus, more than 10 cM away from the PLP gene, was identified in a family with Pelizaeus-Merzbacher-like disease, but the gene product is still unknown [Lazzarini et al., 1997; Steinmüller et al., 1997; Claes et al., 2000].

We had previously reported a large pedigree with an X-linked form of pure HSP affecting 24 males [Zatz et al., 1976]. We were able to reassess part of the family (including 7 affected males) at least 20 years after the original study. Here we report results of linkage analysis based on 19 members of this large Brazilian genealogy, as well as mutational analysis of the PLP gene.

**SUBJECTS**

The complete pedigree, including 24 males affected by pure HSP, found in five generations, was published previously [Zatz et al., 1976]. In this study we are reanalyzing 17 members of this large Brazilian family, including 7 affected males and 3 obligate carriers who were reassessed at least 20 years from the original description (Fig. 1).

**Clinical Report**

The clinical diagnosis was based on the criteria of Fink et al. [1996]. Deceased subjects were classified as affected or unaffected based on relatives’ information.

The seven patients, currently aged 34–53 years old, had been diagnosed as affected (or probably affected) in the original pedigree published in 1976.

In all affected males the onset of clinical signs occurred in late childhood or adolescence (ranging from 10–19 years old). None of them had complaints of

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**Fig. 1.** Haplotype analysis in the family with pure X-linked spastic paraplegia. Arrows indicate the recombination events between markers. The at-risk haplotype is enclosed by boxes.
sphincter disturbance, sensory loss, or any additional abnormal neurological signs, confirming thus the diagnosis of pure spastic paraplegia.

The course of the disease was slowly progressive. With the exception of patients III-1 and III-2, aged 55 and 52, respectively, who walk with the aid of a cane, all others are able to walk unassisted.

Interestingly, affected patient III-8, who is currently 34 years old, had only brisk reflexes in the lower limbs when first ascertained at age 8, but his mother anticipated he would be affected.

On neurological examination, all affected members had lower limb hyperreflexia, a progressive spastic gait abnormality, bilateral extensor plantar reflex, bilateral sustained ankle and patellar clonus, and Babinski's sign.

Obligate carriers were asymptomatic.

**METHODS**

After informed consent, DNA was extracted from blood as described elsewhere [Miller et al., 1988].

Genotype analysis was carried out using microsatellite polymorphic markers flanking the SPG2 locus that were amplified by PCR, according to standard procedures. [α-32P]-dCTP was incorporated in the PCR. The PCR products were size-fractioned on a 6% denaturing gel electrophoresis. The gel was dried and exposed to an x-ray film at room temperature for 2–24 hr.

**Linkage Analysis**

Two-point linkage analysis was performed for each marker and the disease gene. The LOD scores were estimated through the MLINK subroutine of the LINKAGE program, version 5.1 [Lathrop et al., 1984], under the assumption of an X-linked gene with a frequency of 10⁻⁴ and penetrance of 100%. Alleles with equal frequencies were considered for this analysis. Map locations and genetic distances were obtained from a location database (http://cedar.genetics.soton.ac.uk/public_html/ldb.html).

**Mutational Analysis**

The seven exons of the PLP gene (and all their flanking intronic boundaries) were screened for mutations by use of single-strand conformation polymorphism (SSCP) analysis. A bidirectional sequencing of all PCR products, including part of the promoter region (which corresponds to a 422-bp fragment), of the PLP gene was carried out in an ABI 377 automated DNA sequencer. The Dye Deoxy Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems, Foster City, CA) was used, and the reaction was performed according to the ABI standard protocol, after purification of 100 ng of the PCR products with exonuclease I and shrimp alkaline phosphatase. The same primers were used for PCR amplification and sequencing.

For reverse transcription (RT)-PCR, 15 ml of Ethylenediaminetetraacetic Acid (EDTA)-anticoagulated blood was centrifuged at 1,000 g for 10 min. Cells from buffy coat were washed once in phosphate-buffered saline, and total RNA was extracted using TRIZOL (Gibco/BRL, Grand Island, NY). Primers for cDNA of the PLP gene and PCRs were described previously [Cambi et al., 1996].

For the analysis of a possible duplication of the PLP gene, one affected patient was tested through a quantitative PCR approach described previously [Hobson et al., 2000].

**RESULTS**

We analyzed 16 polymorphic markers (DXS996-17.3 cM-DXS999-40 cM-DXS993-31 cM-DXS453-20 cM-DXS454-2.5 cM-DXS1323-3.0 cM-DXS8020-1.7 cM-DXS8096-0.9 cM-DXS8063-1.2 cM-DXS1191-0.2 cM-DXS8112-1.6 cM-DXS1210-1.8 cM-DXS1059-0.8 cM-DXS424-1.2 cM-DXS8098-23 cM-DXS984), 10 of them located near the PLP gene (~15 cM) between DXS1191 and DXS8112. Results of linkage analysis are summarized in Table I and Figure 1. Positive LOD scores were found between markers DXS1323 and DXS424 throughout a recombination event that occurred in

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patients III-3 and III-5. The maximum LOD score was obtained for marker DXS1210 (Zmax = 2.41 at Theta = 0). The PLP gene is located within the region of positive LOD score.

Mutations in the PLP gene were screened through SSCP and sequencing of the exons and part of its promoter. No mutation was found in the coding region of PLP, the intron-exon boundaries, or part of the promoter region.

The possibility of a duplication of the PLP gene in the tested affected patient was excluded (data not shown).

**DISCUSSION**

X-linked HSPs present with two distinct phenotypes, pure and complicated. Mutations in the PLP gene were implicated in both pure and complicated forms (Pelizaeus-Merzbacher disease (PMD)) of X-linked HSP, confirming that the two conditions are allelic [Saugier-Veber et al., 1994; Cambi et al., 1996]. More recently, different mutations in the same codon of the PLP gene were found in three unrelated families with PMD disease [Hodes et al., 1999].

A family with X-linked pure HSP due to a missense mutation in the PLP gene was reported by Cambi et al. [1995]. However, sequencing of the PLP gene failed to reveal any pathological mutation in a second family with pure spastic paraplegia that was also linked to the PLP region (LOD score = 2.41). This finding, together with the observation that only two-thirds of the PMD families had mutations in the PLP coding region, led to the suggestion that another gene, close to the PLP locus, might cause X-linked spastic paraplegia [Cambi et al., 1995].

Indeed, three other families with complicated forms of X-linked HSP suggest the existence of other loci on the X chromosome. Lazzarini et al. [1997] excluded the PLP locus for a family with a phenotype that clinically resembles PMD but with a different degree of myelin involvement. They suggest the existence of a third locus at Xq21.2 for this PMD-like phenotype more than 10 cM away from the PLP gene.

Steinmüller et al. [1997] failed to find mutations in another family with a severe form of spastic paraplegia linked to the PLP region, suggesting again the existence of a third (or fourth) locus for X-linked HSP.

Also Claes et al. [2000] studied a family that appears to represent a previous undescribed X-linked complicated spastic paraplegia. Linkage analysis excluded the two known loci that code for L1-CAM and PLP proteins.

A duplication of the PLP gene as responsible for the disease probably causing hypomyelination has also been reported [Lazzarini et al., 1997]. However, it is expected that this mechanism would be associated with a severe phenotype [Hodes et al., 1999]. A duplication in the PLP gene was ruled out in the present family. The fact that our patients have a milder phenotype reinforces the hypothesis that duplications are associated with a severe course. It also gives further support to our hypothesis of another gene in close proximity. However, as pointed out previously [Cambi et al., 1996], the absence of mutation in the coding region of the PLP gene does not completely exclude it as the disease-causing gene.

It is important to point out that different genes in close proximity cause similar phenotypes in other conditions such as the limb-girdle muscular dystrophies (LGMD). Indeed, the gene causing autosomal dominant LGMD1A, which codes for the sarcromeric protein myotilin [Hauser et al., 2000], and the gene causing autosomal recessive LGMD2F, which codes for ε-sarcoglycan [Nigro et al., 1996; Passos Bueno et al., 1996], are both linked to the 5q33 region. Furthermore, the z-sarcoglycan gene, linked to autosomal recessive LGMD2D [Roberds et al., 1994; Passos Bueno et al., 1995], and the sarcromeric protein telethonin gene, linked to LGMD2G [Moreira et al., 1997, 2000], are located only 15 cM apart on chromosome 17 (in the 17q11 region). The absence of detectable mutations or duplication in the PLP gene in the present family may represent the strongest evidence for the existence of another nearby gene (since linkage analysis will show positive LOD scores for both loci).

In summary, the present family gives further support to the hypothesis of at least one more locus on the X chromosome responsible for spastic paraplegia. The present family, as well as the pedigree K101 reported previously [Cambi et al., 1995], has a mild form of pure X-linked spastic paraplegia. However, the other two families [Lazzarini et al., 1997; Steinmüller et al., 1997] have a severe complicated clinical picture. It will be interesting to know if these phenotypes may also represent allelic forms of the same gene(s) or if they are caused by nonallelic genetic heterogeneity.

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