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CYP7B1 mutations in pure and complex forms of hereditary spastic paraplegia type 5

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Thirty-four different loci for hereditary spastic paraplegias have been mapped, and 16 responsible genes have been identified. Autosomal recessive forms of spastic paraplegias usually have clinically complex phenotypes but the *SPG5*, *SPG24* and *SPG28* loci are considered to be associated with 'pure' forms of the disease. Very recently, five mutations in the *CYP7B1* gene, encoding a cytochrome P450 oxysterol 7-α hydroxylase and expressed in brain and liver, have been found in SPG5 families. We analysed the coding region and exon–intron boundaries of the *CYP7B1* gene by direct sequencing in a series of 82 unrelated autosomal recessive hereditary spastic paraplegia index patients, manifesting either a pure ($n = 52$) or a complex form ($n = 30$) of the disease, and in 90 unrelated index patients with sporadic pure hereditary spastic paraplegia. We identified eight, including six novel, mutations in *CYP7B1* segregating in nine families. Three of these mutations were nonsense (p.R63X, p.R112X, p.Y275X) and five were missense

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mutations (p.T297A, p.R417H, p.R417C, p.F470I, p.R486C), the last four clustering in exon 6 at the C-terminal end of the protein. Residue R417 appeared as a mutational hot-spot. The mean age at onset in 16 patients was 16.4 ± 12.1 years (range 4–47 years). After a mean disease duration of 28.3 ± 13.4 years (10–58), spasticity and functional handicap were moderate to severe in all cases. Interestingly, hereditary spastic paraplegia was pure in seven SPG5 families but complex in two. In addition, white matter hyperintensities were observed on brain magnetic resonance imaging in three patients issued from two of the seven pure families. Lastly, the index case of one family had a chronic autoimmune hepatitis while his eldest brother died from cirrhosis and liver failure. Whether this association is fortuitous remains unsolved, however. The frequency of *CYP7B1* mutations were 7.3% ($n = 6/82$) in our series of autosomal recessive hereditary spastic paraplegia families and 3.3% ($n = 3/90$) in our series of sporadic pure spastic paraplegia. The recent identification of *CYP7B1* as the gene responsible for SPG5 highlights a novel molecular mechanism involved in hereditary spastic paraplegia determinism.

Keywords: CYP7B1; SPG5; hereditary spastic paraplegia; autosomal recessive spastic paraplegia; cholesterol metabolism

Abbreviations: AR-HSP = autosomal recessive hereditary spastic paraplegias; CTX = cerebrotendinous xanthomatosis; CYP7B1 = cytochrome P450 7α-hydroxylase B1; DHEA = dehydroepiandrosterone; ENMG = electromyography; ESE = exonic splicing enhancers; HSP = hereditary spastic paraplegias; MRI = magnetic resonance imaging; PNP = peripheral neuropathy; SPG = spastic paraplegia gene; WMH = white matter hyperintensities

Introduction

Hereditary spastic paraplegias (HSP) constitute a clinically and genetically heterogeneous group of neurodegenerative diseases characterized by a progressive 'dying back' degeneration of the cortico-spinal tracts (Fink, 2006; Depienne *et al*., 2007). The cardinal features consist of progressive spasticity of the lower limbs with brisk reflexes, extensor plantar reflexes (Babinski sign) and muscle weakness, frequently associated with deep sensory loss and urinary urgency. These features define 'pure' or 'uncomplicated' HSP while 'complex' or 'complicated' HSP are accompanied by other neurological signs, including ataxia, mental retardation, dementia, extrapyramidal signs, visual dysfunction, epilepsy or thin *corpus callosum*, or by extraneurological signs (Harding, 1983; Durr and Brice, 2000; Tallaksen *et al*., 2001).

HSP are transmitted according to all modes of inheritance and >34 loci have been described (Stevanin *et al*., 2008*b*). Sixteen different loci and six genes are known to be involved in autosomal recessive forms of HSP, mainly associated with a complex phenotype (SPG7/*paraplegin*, SPG11/*spatacsin*, SPG15/*spastizin*, SPG20/*spartin*, SPG21/*maspardin*, ARSACS/*sacsin*) (Casari *et al*., 1998; Engert *et al*., 2000; Patel *et al*., 2002; Simpson *et al*., 2003; Stevanin *et al*., 2007; Hanein *et al*., 2008). However, only one gene (SPG5/*CYP7B1*) is known among the three loci associated with pure autosomal recessive-HSP (SPG5, SPG24, SPG28). Mutations in *CYP7B1* (MIM# 603711) were very recently identified in six SPG5 families (MIM# 270800) (Tsaousidou *et al*., 2008). The protein product of *CYP7B1* is a cytochrome P450 7α-hydroxylase implicated in cholesterol metabolism (Fig. 1). In the liver, CYP7B1 is part of the alternate/acidic pathway for primary bile acid production, although the classic neutral pathway involves another 7α-hydroxylase, CYP7A1. Unlike CYP7A1, specifically expressed in the liver, CYP7B1 is also widely expressed in extra-hepatic tissues, including the brain, underlying its functions in the metabolism of neurosteroids and oxysterols (Wu *et al*., 1999). Indeed, CYP7B1 provides the primary metabolic route for cholesterol derivatives dehydroepiandrosterone (DHEA) and related hydroxysteroids (Rose *et al*., 1997).

We report six families with HSP and three sporadic patients for whom analysis of the *SPG5/CYP7B1* gene led to the identification of six novel mutations and two already known mutations. We describe detailed clinical and paraclinical data in a total of 16 affected relatives, which represents the largest series of SPG5-mutated patients so far reported.

Material and Methods

Subjects

We selected 82 kindreds compatible with autosomal recessive-HSP diagnosed according to Harding's criteria (Harding, 1983). This series included two SPG5-linked families, AAR-004 and FSP-790, which had enabled us to refine the genetic interval (Klebe *et al*., 2007). A family was classified as having complex HSP if at least one affected member had, in addition to the lower limb pyramidal syndrome, other features including mental impairment, cerebellar ataxia, clinical signs of peripheral neuropathy, extrapyramidal signs, epilepsy, optic atrophy, retinal degeneration and/or ichthyosis (Coutinho *et al*., 1999; Fink, 2003). Additional signs found in patients from the 30 complex families included in this study were: cerebellar signs ($n = 15$), clinical peripheral neuropathy (PNP) ($n = 12$) or an association of both of them ($n = 3$). Mental retardation and thin *corpus callosum* on brain magnetic resonance imaging (MRI) were exclusion criteria for this study. Parental consanguinity was noted in 16 kindreds. Most patients were French ($n = 43$), others originated from North Africa ($n = 20$), including seven Tunisian families, from other West European countries ($n = 14$) or from the Middle East ($n = 5$).

We also included, in this study, 90 patients with pure sporadic HSP. A great majority of these patients were French ($n = 68$), others originated from North Africa or the Middle-east ($n = 15$) and from other countries ($n = 7$).

This study was approved by the local Bioethics committee (approval No. 03-12-07 of the Comité Consultatif pour la Protection des Personnes et la Recherche Biomédicale Paris-Necker to Drs Durr and Brice). Informed written consent was obtained from all participating members of the families before blood samples were collected for DNA extraction. All clinical evaluations were performed according to a

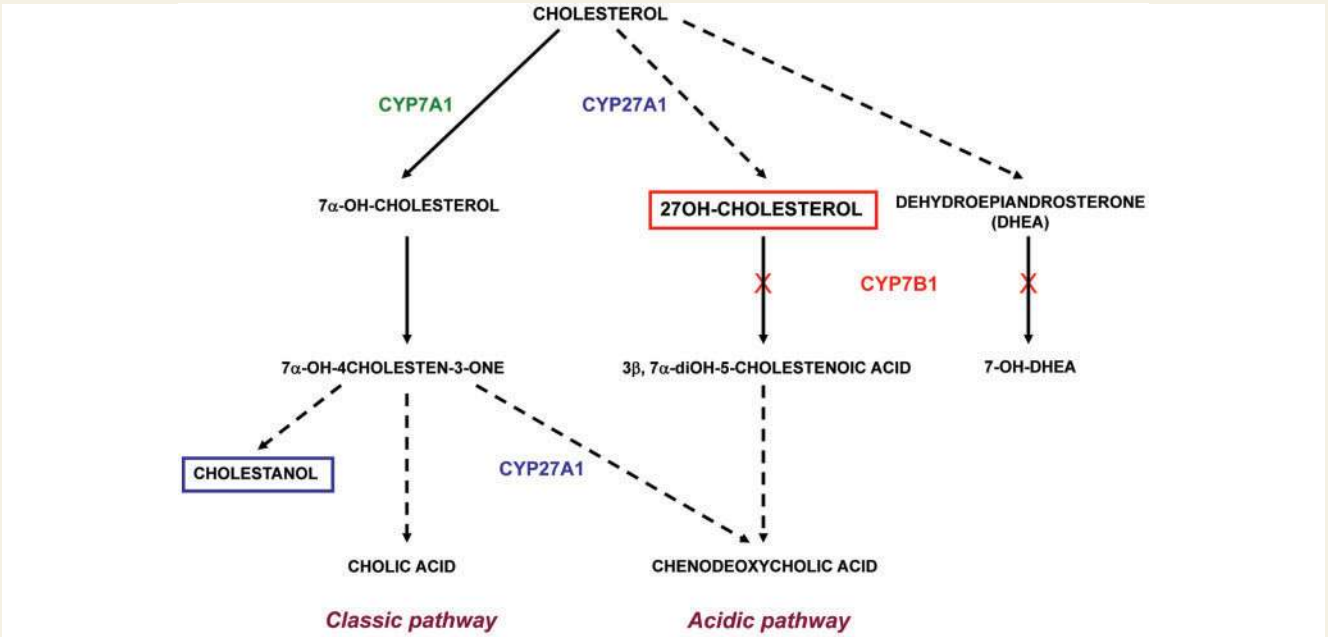

Figure 1 Major bile-acid and neurosteroid biosynthetic pathways. A simplified overview adapted from Tsaousidou *et al*., 2008.

protocol established by the European and Mediterranean network for spinocerebellar degenerations (SPATAX, coordinator: Dr A Durr) that included: full medical history and examination, estimation of the age at onset by the patient, presence or absence of additional neurological symptoms/signs, electroneuromyographic (ENMG) studies and brain MRI when possible. Disability was assessed on a seven-point scale: 1, minimal disability (slight stiffness of the legs); 2, mild disability (unable to run, but full autonomy); 3, moderate disability when walking (reduced perimeter, frequent falls); 4, severe disability (unilateral support required to walk); 5, bilateral support required to walk; 6, wheelchair bound; 7, bedridden.

Mutations in the paraplegin (MIM# 602783) gene responsible for SPG7 (MIM# 182601) were excluded in a subset of 39 index autosomal recessive-HSP patients by direct sequencing (Elleuch *et al*., 2006).

Point mutation detection

The coding sequence and exon–intron boundaries of the six exons of the *SPG5* gene *CYP7B1* (Genbank accession number NM_000008) were amplified by polymerase chain reaction (PCR) using the Fast Amp Mix kit (Applied Biosystems, Foster City, CA, USA) on a Fast Amp Thermocycler 3800. PCR primers and annealing temperatures were those used in the initial report (Tsaousidou *et al*., 2008) except for exon 3 (catgtagtgtaccttcgaatg/gtcacaaacaataactttcc and tgtatc cattctgcagctcaa/ttcaaggtcgccattttgtc, annealing temperature of 55°C). In cases carrying a single variant, 2000 bp upstream of the first start codon (ATG) of the *CYP7B1* cDNA sequence and 1500 bp downstream of the last termination codon of the *CYP7B1* gene were amplified with a classical protocol and sequenced using eight primers pairs (available upon request) in addition to the coding sequence. The amplicons were purified and sequenced in both directions using the Big Dye Terminator Cycle Sequencing Kit v2 (ABI Prism, Applied Biosystems) in an ABI Prism 3730 automated sequencer. The electrophoretic profiles were analysed with Seqscape 2.5 (Applied Biosystems).

Nucleotides were numbered relative to the A of the start codon (ATG) of the *CYP7B1* cDNA sequence. Segregation of the mutations/polymorphisms with the disease was verified by direct sequencing in all family members for whom DNA samples were available; 26 additional subjects were used in this study. In addition, 247 unrelated healthy subjects (168 French Caucasians, 79 North Africans) were screened to evaluate the frequency of missense, synonymous and intronic/UTR changes. These variations were systematically tested for an effect on the binding of exonic splicing enhancers (ESE) with ESEfinder software (http://www.rulai.cshl.edu/cgi-bin/tools/ESE/esefinder.cgi), for modification/creation of splicing consensus sequences (http://rulai.cshl.edu/new_alt_exon_db2/HTML/score.html and http://www.fruitfly.org/seq_tools/splice.html) and/or for binding of transcription factors (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3). Multiple alignments of orthologs of *CYP7B1* in various species were performed using ClustalW software (http://www.ebi.ac.uk/clustalw/) for the analysis of the conservation of affected amino-acids.

Detection of gene dosage anomalies

A search for intragenic deletions/duplications was performed using a previously described *CYP7B1*-specific multiplex ligation-dependent probe amplification (MLPA) kit (Schule *et al*., 2008). For seven patients with single heterozygous variants detected by sequencing, such analyses were used to exclude large genomic rearrangements.

Results

SPG5 mutation screening in autosomal recessive-HSP patients

Direct sequencing of *CYP7B1* was performed in 82 unrelated autosomal recessive-HSP index patients with either pure ($n = 52$)

Table 1 *CYP7B1* **mutations identified in six autosomal recessive-HSP families and in three sporadic pure HSP cases**

Family/ Patient	Family history	Consanguinity	Exon	Genomic change	Protein change	Genetic status	Familial segregation	ESE changes	Splicing changes	AA alignments
TUN-29	Yes	Yes	2	c.334C>T	p.R112X	hm	Yes	NA	NA	NA
FSP-945	Yes	Yes	3	c.825T>A	p.Y275X	hm	Yes	NA	NA	NA
FSP-834	Yes	Yes	6	c.1249C>T	p.R417C	hm	Yes	No	No	Highly conserved (7/7)
FSP-790	Yes	Yes	6	c.1250G>A	p.R417H	hm	Yes	Yes	No	Highly conserved (7/7)
AAR-004	Yes	No	6	c.1250G>A	p.R417H	ht	Yes	Yes	No	Highly conserved (7/7)
AAR-004	Yes	No	6	c.1408T>A	p.F470I	ht	Yes	No	No	Highly conserved (5/7)
FSP-563	Yes	Yes	6	c.1456C>T	p.R486C	hm	ND	Yes	No	Highly conserved (6/7)
FSP-279	No	No	2	c.187C>T	p.R63X	ht	ND	NA	NA	NA
FSP-279	No	No	4	c.889A>G	p.T297A	ht	ND	Yes	No	Highly conserved (7/7)
FSP-198	No	No	4	c.889A>G	p.T297A	hm	ND	Yes	No	Highly conserved (7/7)
FSP-293	No	No	6	c.1250G>A	p.R417H	hm	ND	Yes	No	Highly conserved (7/7)

Patients carrying a single heterozygous mutation are not indicated.
Hm = homozygous; ht = heterozygous; NA = not applicable; ND = no data.

or complex presentation ($n = 30$). We identified mutations in *CYP7B1* in six index patients (Table 1). Four mutations were missense (p.R417H, p.R417C, p.F470I, p.R486C) and two were nonsense (p.R112X, p.Y275X). Arginine residue at position 417 at the protein level was substituted in three families. All mutations were novel except p.R417H (Tsaousidou *et al*., 2008). Missense mutations affected highly conserved residues (Fig. 2) located in exon 6 at the C-terminal end of the protein, and were not found in 336 Caucasian and 158 North African control chromosomes, respectively. In five patients, mutations were found at the homozygous state whereas the last patient was compound heterozygote. The co-segregation of mutations with the disease was demonstrated in all families but one (FSP-563, samples not available) and none of the unaffected family members ($n = 19$) carried two mutations in the *SPG5* gene (Fig. 2A).

SPG5 mutation screening in sporadic pure HSP patients

Among the 90 patients with sporadic pure HSP analysed, we identified three carriers of two *CYP7B1* mutations (Fig. 2B and Table 1), all originating from France ($n = 3/90$, 3.3%). Two patients were homozygous for a missense mutation (p.T297A and p.R417H in patients FSP-198-19 and FSP-293-1, respectively) and the third (patient FSP-279-14) was compound heterozygous for mutations p.R63X and p.T297A (Table 1).

Heterozygous polymorphic variants

Three of the identified mutations were also detected in the heterozygous state, with no other pathological mutations or gene dosage anomalies detected by MLPA, in three additional index cases. The missense mutation c.1450C>T/p.R486C was present in the heterozygous state in patient FSP-932-001 (Great Britain) with pure HSP. This latter patient also carries the c.-1808C>T variant (Fig. 3) upstream the first codon, predicted *in silico* to alter the binding of various transcription factors, although this region was not necessary for gene expression in previous experiments (Wu and Chiang, 2001). In addition, the missense mutation c.889A>G/p.T297A was shown to segregate in the heterozygous state in three affected patients of family FSP-506 (France) with complex HSP, in the absence of any other detected mutation/rearrangement. Finally, mutation p.R63X was present in the heterozygous state in patient TUN-33-305. A heterozygous c.1133C>A variant in the 3′UTR was also identified in this patient but without predictable effects on miRNA binding (Fig. 3).

We also identified additional heterozygous nucleotide variations (Fig. 3) which are probably polymorphisms because they did not segregate with the disease in families, and/or because of their positions, low conservation and/or frequencies in controls: c.-308C>G, c.56T>C/p.L19P, c.64C>T/p.L22L, c.122 + 5G>A, c.860T>C/p.L287S, c.316C>T/p.H106Y and c.971G>A/p.R324H.

Clinical characteristics of SPG5 families

The clinical and paraclinical features of 16 patients from the nine SPG5 families with two *CYP7B1* mutations are shown in Table 2. Seven mutated families were European (five from France, two from Portugal) and two were North African (one Tunisian and one Algerian). The two families previously linked to the *SPG5* locus (FSP-790 from Algeria and AAR-004 from France) have been described in detail elsewhere (Klebe *et al*., 2007). At examination, all of the mutation carriers were symptomatic and had abnormal neurological signs, suggesting complete penetrance in SPG5.

Age at onset ranged from 4 to 47 years with a mean of 16.4 ± 12.1 years. The presenting symptoms were gait difficulties in all cases ($n = 16$). After a mean disease duration of 28.3 ± 13.4 years (range 10–58), all patients had a moderate to severe HSP presentation (Table 2). Most had severe handicap ($n = 11/16$, 69%), being wheelchair bound ($n = 6/16$, 38%) or requiring a walking aid ($n = 5/16$, 31%). Only five patients (31%) were still able to walk without help. Lower limb spasticity was severe in 63% ($n = 10/16$) and associated with severe weakness in three cases. Other signs were occasionally observed such as bladder dysfunction ($n = 10/16$, 63%) and pes cavus ($n = 7/16$, 44%). Distal or diffuse wasting was observed in six patients.

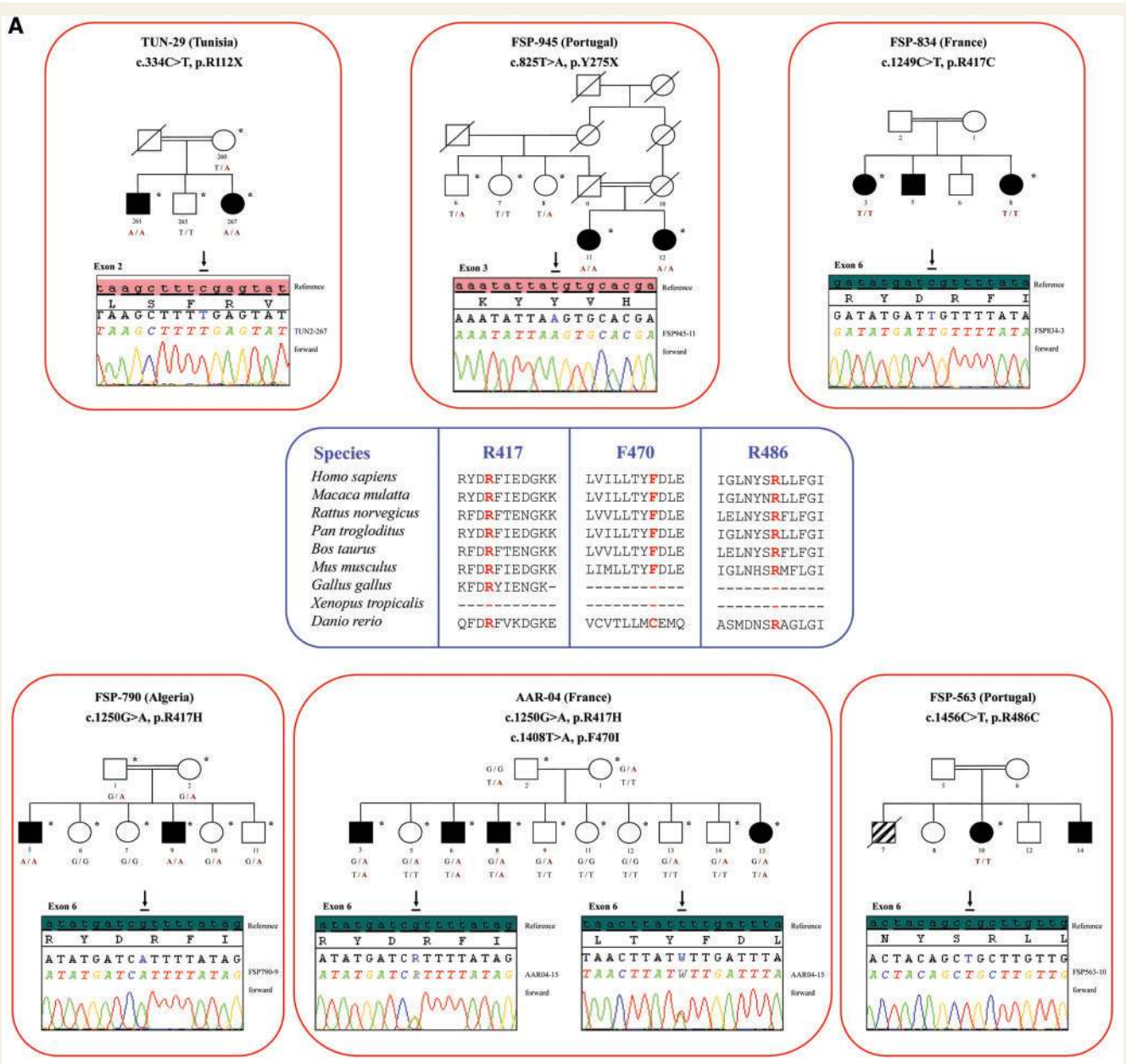

Figure 2 Pedigrees and *CYP7B1* mutations identified in nine SPG5 families. (**A**) Mutations identified in autosomal recessive-HSP pedigrees. (**B**) Mutations identified in sporadic cases. Conservation of the mutated residues is indicated for missense variants. The HSP-affected members of the families are indicated by black symbols. The hatched symbol corresponds to a patient who died of hepatic failure but with no known pyramidal signs. The asterisk indicates that DNA was available for genetic analyses. The genotype is indicated for each analysed family member.

In seven families (78%), the clinical presentation was suggestive of a pure form of HSP (Table 2). Cerebellar signs including mild upper limb dysmetria and saccadic pursuit were observed after long disease durations (>20 years) in four patients from two complex families (FSP-945 and AAR-004). The AAR-004 index patient (AAR-004-6) also had severe lower limb distal amyotrophy and axonal peripheral neuropathy evidenced on electroneuromyography after a disease duration of 24 years (Table 2) that occurred, however, in a context of severe chronic alcoholism. Serum cholesterol levels were normal in the nine patients for whom data was available (Table 2).

Brain MRI showed white matter hyperintensities (WMH) in three patients from two families with 'apparently' pure HSP (FSP-834 and TUN-29) and diffuse cerebral atrophy in one patient with complex HSP (AAR-004-6) (Table 2). Patchy periventricular hyperintense T_2-weighted lesions were observed in two individuals (patients TUN-29-267 and FSP-834-008), while white matter hyperintensities appeared confluent and diffuse in patient FSP-834-003 (Fig. 4). No abnormalities were found in the eight remaining patients with available brain MRI (Table 2).

Finally, the index patient FSP-563-010, a woman aged 60 years, had chronic liver disease. Family history was positive for liver

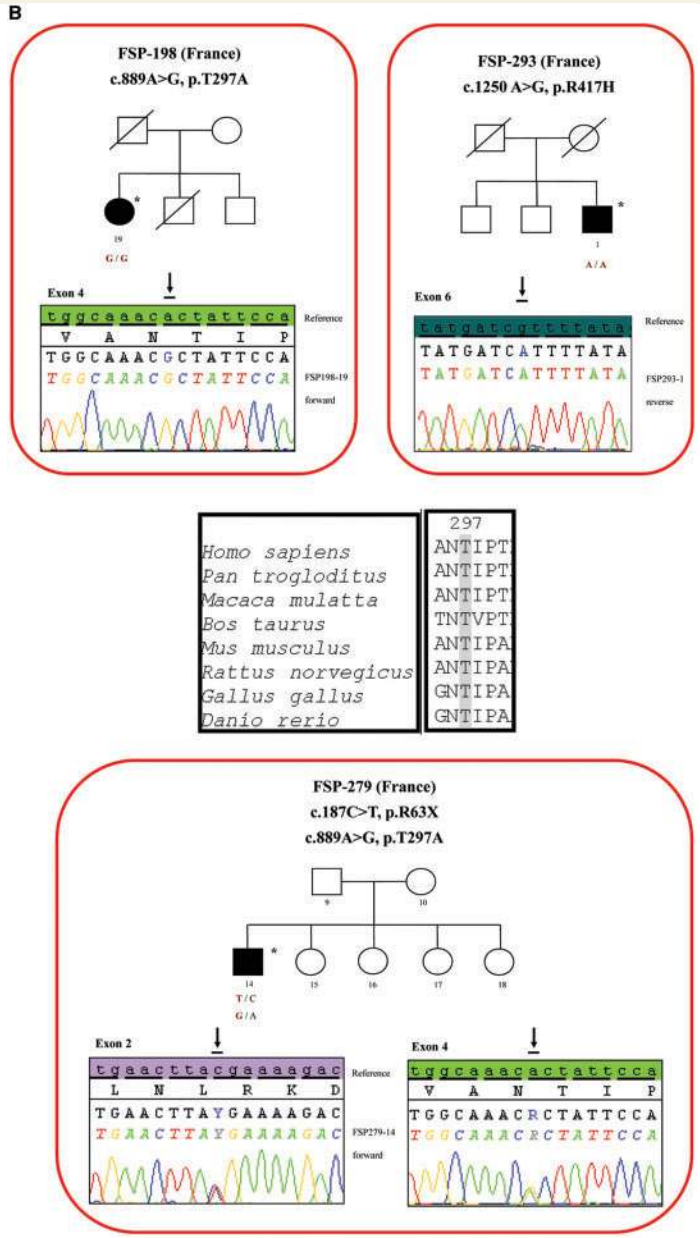

Figure 2 Continued.

disease (her elder brother died from cirrhosis of unknown cause at age 40 years) and for HSP (her younger brother had spastic paraplegia) (Fig. 2). Her medical history was marked by transitory jaundice after surgical tonsillectomy at age 35 years. This clinical episode resolved spontaneously but chronic mild elevation of aminotransferases (less than five times the upper limit of normal values) persisted. Serum bilirubin, gamma-GT and alkaline phosphatase levels always remained within the normal range. Hepatitis B and C serologies were negative and the patient denied exposure to hepatotoxic drugs or alcohol. Abdominal ultrasonography was regularly controlled, showing stable moderate hepatomegaly and gall bladder lithiasis. At age 51 years, concomitantly with a cholecystectomy, liver biopsy showed mild portal and peri-portal fibrosis with moderate inflammatory infiltration and piecemeal necrosis consistent with unspecific chronic hepatitis. Bile ducts were normal and there was no cholestasis. At age 60, she was found to have an antinuclear antibody (ANA) titre of 1:1280 together with an anti-soluble liver antigen (SLA) antibody. Anti-smooth muscle and anti-liver–kidney microsomal antibodies were absent. Her liver disease was classified as 'definite' autoimmune hepatitis according to the international autoimmune hepatitis scoring system (Alvarez *et al*., 1999) after a second liver biopsy showing the same histopathological pattern.

Discussion

We have identified *CYP7B1* mutations in nine SPG5 families, six with AR-HSP and three with sporadic HSP.

Exon/Intron	Variant	Heterozygous HSP cases	Cosegregation	Conservation	ESE changes	Splicing changes	Frequency in controls	
							Caucacians	North-Africans
promotor	c.-1808C>T	FSP-932-001	no (2 patients)	NA	no	no	0%	not examined
promotor	c.-308C>G	FSP-240-14 FSP-643-021	no additional samples no (2 patients)	NA	no	no	5%	not examined
Exon 1	c.56T>C,p.L19P	FSP-370-013	yes (2 patients)					
		FSP-399-022	no (2 patients)	no (3/8)	yes	no	0%	0%
		FSP-632-001	no additional samples					
Exon1	c.64C>T,p.L22L	FSP-255-15	no (3 patients)	NA	no	no	0%	0%
Intron 1	c.122+5G>A	FSP-097-006	no additional samples	NA	NA	no	0%	0%
Exon 3	c.316C>T,p.H106Y	FSP-643-021	no (2 patients)	no (3/8)	no	no	0%	0%
Exon 4	c.860T>C p.L287S	FSP-240-14	no additional samples	mild (5/8)	yes	no	0%	0%
Exon 4	c.971G>A,p.R324H	16 cases	not examined	no (3/8)	yes	no	8%	5%
3'UTR	c.1133C>A	TUN-33-305	no additional samples	NA	no	no	0.5%	not examined

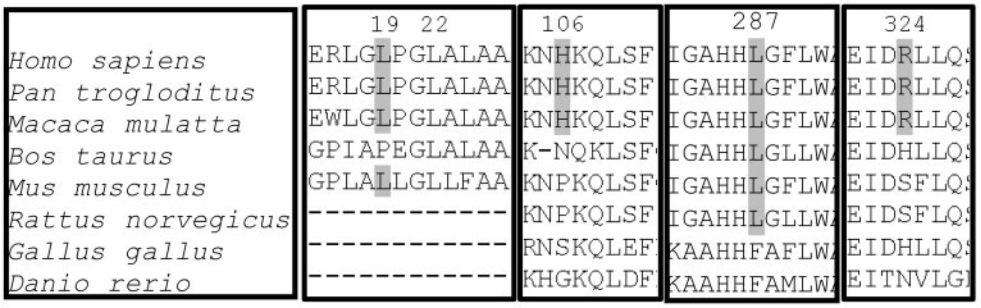

Figure 3 Additional variants found in patients and their conservation in species. NA = not applicable.

Autosomal recessive-HSP is usually associated with clinically complex phenotypes, although SPG5, SPG24 and SPG28 are considered to be pure forms of the disease (Hentati *et al*., 1994; Hodgkinson *et al*., 2002; Bouslam *et al*., 2005). In the literature, a total of 12 families of different origins were previously linked to the SPG5 locus on chromosome 8q12 with an interval of interest progressively reduced to 3.8 cM (Hentati *et al*., 1994; Coutinho *et al*., 1999; Wilkinson *et al*., 2003; Muglia *et al*., 2004; Klebe *et al*., 2007). Very recently, the causative gene was discovered by identifying five homozygous mutations—four missense (p.G57R, p.F216S, p.S363F, p.R417H) and one nonsense (p.R388X)—in six families with a pure form of HSP (Tsaousidou *et al*., 2008). The four residues substituted by missense mutations were conserved throughout evolution and were predicted to be highly deleterious for CYP7B1 function. The p.R388X mutation was identified in a sporadic patient with pure HSP. The presence of p.R388X was highly remarkable since this mutation had previously been detected in a neonate affected by severe liver disease who died at age 6 months (Setchell *et al*., 1998). The phenotypic discrepancy between the two patients carrying p.R388X may be explained by a combined biochemical defect in cholesterol metabolism that would have affected the neonate with liver disease (Tsaousidou *et al*., 2008). In this latter case, a complete absence of 7α-hydroxycholesterol was observed in serum and urine, suggesting a loss of function of both α-hydroxylating cholesterol classic and alternate pathways for bile acid synthesis in the liver.

Here, we show eight *CYP7B1* mutations, including six novel mutations. Six of them segregated in autosomal recessive-HSP families including those linked to the SPG5 locus in a previous study. Our results confirm that *CYP7B1* is the gene responsible for SPG5 (Tsaousidou *et al*., 2008). Three mutations, p.R63X, p.R112X and p.Y275X, were nonsense, suggesting a total loss of function in two homozygous patients (FSP-945 and TUN-29), as previously demonstrated for p.R388X (Setchell *et al*., 1998). The deleterious effects of the missense mutations are sustained by the high conservation throughout evolution of the residues involved (T297, R417, F470, R486), their absence from 494 control chromosomes and the familial co-segregation demonstrated for three of them (p.R417C, p.R417H, p.F470I). This is also supported by the previous linkage of autosomal recessive-HSP in families FSP-790 and AAR-004 to the *SPG5* locus and by exclusion of conventional mutations in 14 genes of the candidate region in patients from both linked families [*RAB2*, *Q8NB32*, *ASPH*, *ENSF00000000857*, *TTPA*, *BHLHB5*, *COPS5*, *BIG1*, *DEPDC2*, *MTFR1*, *YTHDF3*, *C8orf34*, *FAM77D* and *PRDM14*, (Klebe *et al*., 2007) and unpublished data]. Of the 14 mutations

Table 2 Clinical and paraclinical findings in the 13 autosomal recessive-HSP and three sporadic patients with *CYP7B1* mutations

Family/cases	FSP-945		FSP-563	FSP-834		TUN-29		AAR-004				FSP-790		FSP-198	FSP-279	FSP-293
Origin	Portugal		Portugal	France		Tunisia		France				Algeria		France	France	France
CYP7B1 Mutation	p.Y275X		p.R486C	p.R417C		p.R112X		p.R417H, p.F470I				p.R417H		p.T297A	p.R63X, p.T297A	p.R417H
Phenotype	**Complex**		**Pure**	**Pure**		**Pure**		**Complex**				**Pure**		**Pure**	**Pure**	**Pure**
Individual No. (sex)	11[a] (F)	12 (F)	10[a] (F)	8[a] (F)	3 (F)	267[a] (F)	261 (M)	3 (M)	8 (M)	6[a] (M)	15 (F)	5[a] (M)	9 (F)	19 (F)	14 (M)	1 (M)
Age at examination (years)	42	40	60	53	70	34	53	55	52	50	41	22	14	45	42	48
Age at onset (years)	5	5	47	10	12	9	10	20	30	30	7	6	4	33	17	21
Disease duration (years)	37	35	13	43	58	25	43	35	22	20	34	16	10	12	25	27
Disability score	5/7	3/7	4/7	6/7	6/7	3/7	6/7	6/7	4/7	6/7	6/7	3/7	4/7	4/7	3/7	3/7
LL spasticity	S	S	Mo	S	S	Mo	S	Mo	S	Mo	S	Mo	Mo	S	S	S
LL reflexes	+++	+++	+++	+	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+
LL weakness	Mo	No	Mi	No	S	Mo	S	Mo	Mo	Mo	S	Mi	Mi	Mo	Mi	Mi
LL amyotrophy	No	No	No	S	Mi	No	No	Mo	No	S	No	Mo	Mi	No	No	No
Babinski sign	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
UL spasticity	No	No	No	No	Mi	No	Mi	No	No	No	No	No	No	No	No	No
UL reflexes	+++	+++	N	N	N	N	+	N	N	N	N	+	+	+++	+++	+
UL weakness	No	No	No	No	Mi	No	No	Mi	No	No	No	Mi	No	No	No	Yes
UL amyotrophy	No	No	No	No	Mi	No	No	Mi	No	No	No	No	No	No	No	No
Sensory disturbance	DVS	DVS	DVS	DVS, distal HE	DVS, distal HE	No	DVS	DVS, distal HE	DVS, distal HE	DVS	DVS	DVS	DVS	DVS	DVS	DVS
Pes cavus/Scoliosis	Yes/No	No/No	No/No	Yes/No	Yes/No	No/No	No/No	No/No	No/No	No/No	No/No	Yes/No	Yes/No	Yes/Yes	Yes/No	No/Yes
Urinary symptoms	Yes	No	Yes	Yes	Yes	No	No	Yes	No	Yes	Yes	No	No	Yes	Yes	Yes
Others signs	Mild cerebellar signs	None	None	None	None	None	None	Mild cerebellar signs	Mild cerebellar signs	Mild cerebellar signs	None	None	None	Mild postural tremor	Nystagmus	None
Cerebral MRI	Normal	Normal	Normal	WMH	WMH	WMH	ND	ND	ND	Cortical and cerebellar atrophy[b]	Normal	Normal	Normal	Normal	ND	Normal
ENMG	Normal	ND	ND	Normal	ND	ND	ND	ND	ND	Axonal PNP[b]	Normal	Normal	Normal	Normal	ND	Normal
Serum cholesterol levels (g/l) ($N = 1.3–2.9$)	2.04	1.83	2.15	1.87	2.74	ND	ND	1.50	1.76	1.36	2.24	ND	ND	ND	ND	ND

ENMG = electromyography; M = men; F = women; Mi = mild; Mo = moderate; S = severe; N = normal reflexes; + = brisk reflexes; +++ = very brisk reflexes; UL = upper limbs; LL = lower limbs; DVS = decreased vibration sense; HE = hypoesthesia; Sc = subclinical; WMH = white matter hyperintensities; PNP = peripheral polyneuropathy; SM = sensory motor; MR = mental retardation; ND = not done.

a Index patient in each family.

b Chronic alcoholism may explain this abnormality.

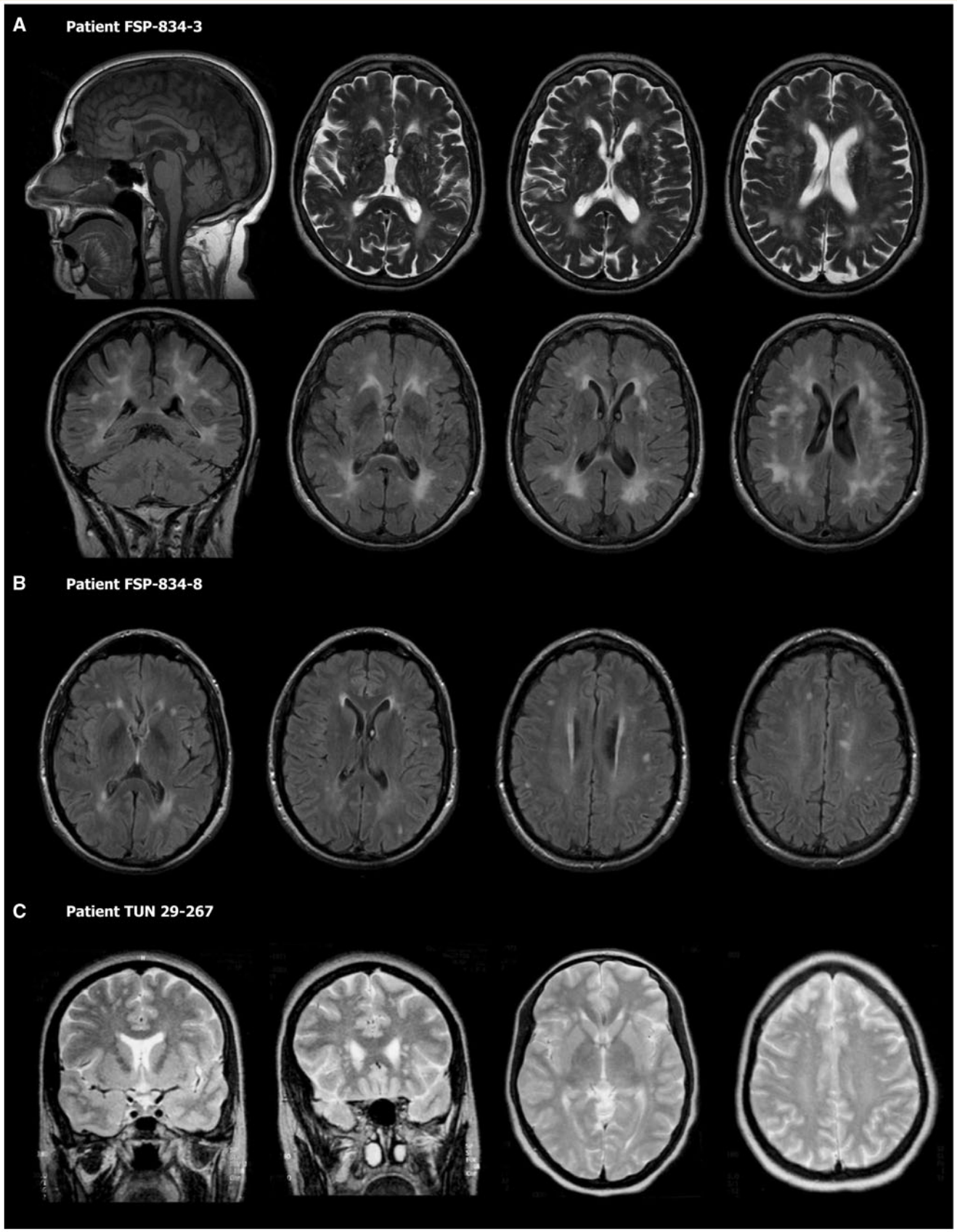

Figure 4 Brain magnetic resonance imaging (MRI) data from three patients with white matter hyperintensities (WMH). (**A**) T_2-weighted images (upper panel) and fluid-attenuated inversion recovery (FLAIR) images (lower panel) showing confluent and diffuse WMH in patient FSP-834-003. (**B**) FLAIR images showing patchy WMH in periventricular and subcortical regions in patient FSP-834-008. (**C**) T_2-weighted images showing patchy WMH in periventricular and subcortical regions in patient TUN-29-267.

now known [(Schule *et al*., 2008; Tsaousidou *et al*., 2008) and this study], four are nonsense, one is frameshift and nine are missense. Four of the missense mutations are clustered in exon 6 with an apparent mutational hot-spot on the arginine residue in position 417 at the protein level. R417 was substituted in a total of five different families [this study and (Tsaousidou *et al*., 2008)]. The 10 other mutations are distributed throughout the gene.

We also found nine polymorphisms in the heterozygous state, four of which caused the substitution of an amino acid residue. Additional studies will be required to determine the potentially deleterious effects of the missense variants (Fig. 3) which would then be classified as mutations. Mutation p.L19P is interesting; it was never found in controls and concerns a partially conserved residue, but did not segregate with the disease in one family. This is reminiscent of SPG7 where numerous missense variations have been identified, complicating routine diagnosis in clinical practice (Elleuch *et al*., 2006). In addition, the p.R324H variant was recently considered to be a possible modifier polymorphism associated with complex presentations of HSP (Schule *et al*., 2008). In our series of HSP patients, it was also more often associated with complex forms ($n = 6/30$, 20%), whereas the frequency in pure forms ($n = 10/142$, 7%) was similar to controls (5–8%). Whether they are associated with other mutations in other genes is still unknown.

We have described here the SPG5 phenotype in a total of 16 patients from nine families, the largest series investigated so far. The clinical features of most SPG5 patients were similar to those mentioned in previous reports and consisted of slowly progressive spastic paraplegia, sometimes associated with variable urinary symptoms and sensory disturbances (loss of proprioception and vibration sense) (Hentati *et al*., 1994; Wilkinson *et al*., 2003; Muglia *et al*., 2004; Klebe *et al*., 2007). Age at onset showed both interfamilial and intrafamilial variations, with a range of 4–47 years. After a long disease duration (28.3 ± 13.4), all SPG5 patients had a moderate to severe HSP presentation. Most of them needed walking aid (38%) or were wheelchair bound (31%), and only a few patients were able to walk without help. However, the disease course and functional handicap were less severe than that observed in SPG11 patients, who were wheelchair bound after a mean disease duration of 17.6 years (Stevanin *et al*., 2008*a*).

Clinical examination was suggestive of a pure HSP in seven families although a complex HSP was diagnosed in the last two kindreds (AAR-004 and FSP-945) (Table 2). In the latter two families, several patients showed mild cerebellar signs after a disease duration >20 years. Cortical and cerebellar atrophy was also observed on brain MRI in one patient (AAR-004-006), as well as severe axonal neuropathy caused at least partially by chronic alcoholism. Thus, a complex presentation of HSP in association with mild cerebellar features may be possible in SPG5, but only after a long disease duration. Notably, brain MRI revealed white matter hyperintensities on T_2-weighted sequences (Fig. 4) in two of the seven 'pure' families (FSP-834, TUN-29), expanding the phenotypic spectrum of SPG5. White matter hyperintensities appeared patchy in two patients (FSP-834-008, TUN-29-267) and confluent and diffuse in the last one (FSP-834-003). The origin of these lesions remains uncertain; vascular, inflammatory or degenerative processes, or a combination of them, might be involved. Interestingly, chronic liver disease was noted in patient FSP-563-010 carrying the homozygous p.R486C mutation. This liver disease should be considered in light of a previous neonatal case of severe liver failure reported in 1998 (Setchell *et al*., 1998). However, autoimmune hepatitis was suspected due to the presence of auto-antibodies (ANA and SLA) and confirmed using the international autoimmune hepatitis scoring system. In addition, the lack of biological and histological cholestasis was not compatible with a diagnosis of liver disease due to an error in bile acid synthesis. This suggests a fortuitous association rather than a direct relationship with *CYP7B1* mutations.

In our study, the relative frequency of SPG5 was 7.3% in autosomal recessive-HSP probands ($n = 6/82$) regardless of whether they had a pure or complex presentation including cerebellar signs and/or clinical signs of peripheral neuropathy, and regardless of their previous screening for other HSP genes (SPG7, etc.). The frequency was 7.7% ($n = 4/52$) in the pure autosomal recessive-HSP families. As the frequency of SPG7 is considered to be <5% in autosomal recessive-HSP (Elleuch *et al*., 2006), and as SPG24 and SPG28 have each been described in only one family (Hodgkinson *et al*., 2002; Bouslam *et al*., 2005), our results suggest that mutations in SPG5/*CYP7B1* gene represent the major cause of known pure autosomal recessive-HSP. The frequency among complex families is more difficult to establish since patients with mental retardation and thinning of the *corpus callosum* were excluded from this study. A frequency of 6.6% ($n = 2/30$) would likely represent the maximum frequency among complex cases. Finally, we also identified SPG5/*CYP7B1* mutations in sporadic pure HSP patients (3.3%, $n = 3/90$), suggesting that *CYP7B1* mutations should be evoked when faced with such presentations. To date, only the screening of SPG4 was shown to be valuable in routine diagnoses of sporadic HSP, with a success rate of 10% (Depienne *et al*., 2006).

Mutations in spastic paraplegia (SPG) genes mostly result in axonal trafficking impairment and mitochondrial metabolism dysfunction (Reid, 2003; Fink, 2006; Depienne *et al*., 2007; Stevanin *et al*., 2008*b*). However, SPG5 highlights a novel mechanism leading to neurodegeneration. Neurons may be particularly susceptible to abnormalities in cholesterol homeostasis. In SPG5, the neurodegeneration caused by *CYP7B1* mutations may be related to possible neurotoxicity caused by aberrant oxysterol levels as well as to a loss of neuroprotective function of DHEA-related neurosteroids (Pettersson *et al*., 2008). High expression of CYP7B1 was detected in pyramidal cells of human hippocampal sections with the use of monoclonal antibodies, arguing for an important role in motor neurons that specifically degenerate in HSP (Trap *et al*., 2005). Another rare progressive sterol storage disorder, cerebrotendinous xanthomatosis (CTX), is clinically characterized by the presence of juvenile cataracts, tendon xanthomas and progressive neurological manifestations (Moghadasian *et al*., 2002). The neurological manifestations are present in most patients and include dementia, spastic paraplegia progressing to tetraplegia, bulbar palsy and ataxia (Berginer *et al*., 1989). Brain MRI may show various changes including diffuse cerebral atrophy and white matter hyperintensities, reflecting diffuse leukodystrophy (Berginer *et al*., 1994). The occurrence of neurological

features in cerebrotendinous xanthomatosis is related to the accumulation of cholestanol and cholesterol in the CNS, triggered by mutations in *CYP27A1*, a gene acting prior to *CYP7B1* in the acidic pathway of bile acid synthesis (Fig. 1). Replacement therapy with chenodeoxycholic acid in cerebrotendinous xanthomatosis inhibits abnormal bile acid synthesis and is most effective in reducing elevated plasma cholestanol concentrations, and eliminating bile alcohols (Moghadasian *et al*., 2002). In addition, Niemann-Pick type C disease, a fatal autosomal recessive childhood-onset neurodegenerative disorder, is caused by mutations in *NPC1* and *NPC2*, resulting in lysosomal accumulation of unesterified cholesterol and glycolipids (Vanier and Millat, 2003). Neurological features appear generally between 3 and 15 years, and include cerebellar ataxia, dysarthria, seizures, dystonia, progressive dementia, vertical supranuclear ophthalmoplegia and white matter hyperintensities. Treatments using allopregnanolone and miglustat have recently shown some benefits in animal models and in human for miglustat (Patterson *et al*., 2007; Mellon *et al*., 2008). Likewise, biochemical analyses are required in SPG5 patients in order to identify the metabolic targets potentially amenable to therapeutic intervention.

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References

Alvarez F, Berg PA, Bianchi FB, Bianchi L, Burroughs AK, Cancado EL, et al. International Autoimmune Hepatitis Group Report: review of criteria for diagnosis of autoimmune hepatitis. J Hepatol 1999; 31: 929–38.

Berginer VM, Berginer J, Korczyn AD, Tadmor R. Magnetic resonance imaging in cerebrotendinous xanthomatosis: a prospective clinical and neuroradiological study. J Neurol Sci 1994; 122: 102–8.

Berginer VM, Salen G, Shefer S. Cerebrotendinous xanthomatosis. Neurol Clin 1989; 7: 55–74.

Bouslam N, Benomar A, Azzedine H, Bouhouche A, Namekawa M, Klebe S, et al. Mapping of a new form of pure autosomal recessive spastic paraplegia (SPG28). Ann Neurol 2005; 57: 567–71.

Casari G, De Fusco M, Ciarmatori S, Zeviani M, Mora M, Fernandez P, et al. Spastic paraplegia and OXPHOS impairment caused by mutations in paraplegin, a nuclear-encoded mitochondrial metalloprotease. Cell 1998; 93: 973–83.

Coutinho P, Barros J, Zemmouri R, Guimaraes J, Alves C, Chorao R, et al. Clinical heterogeneity of autosomal recessive spastic paraplegias: analysis of 106 patients in 46 families. Arch Neurol 1999; 56: 943–9.

Depienne C, Stevanin G, Brice A, Durr A. Hereditary spastic paraplegias: an update. Curr Opin Neurol 2007; 20: 674–80.

Depienne C, Tallaksen C, Lephay JY, Bricka B, Poea-Guyon S, Fontaine B, et al. Spastin mutations are frequent in sporadic spastic paraparesis and their spectrum is different from that observed in familial cases. J. Med Genet 2006; 43: 259–65.

Durr A, Brice A. Clinical and genetic aspects of spinocerebellar degeneration. Curr Opin Neurol 2000; 13: 407–13.

Elleuch N, Depienne C, Benomar A, Hernandez AM, Ferrer X, Fontaine B, et al. Mutation analysis of the paraplegin gene (SPG7) in patients with hereditary spastic paraplegia. Neurology 2006; 66: 654–9.

Engert JC, Berube P, Mercier J, Dore C, Lepage P, Ge B, et al. ARSACS, a spastic ataxia common in northeastern Quebec, is caused by mutations in a new gene encoding an 11.5-kb ORF. Nat Genet 2000; 24: 120–5.

Fink JK. The hereditary spastic paraplegias: nine genes and counting. Arch Neurol 2003; 60: 1045–9.

Fink JK. Hereditary spastic paraplegia. Curr Neurol Neurosci Rep 2006; 6: 65–76.

Hanein S, Martin E, Boukhris A, Byrne P, Goizet C, Hamri A, et al. Identification of the SPG15 gene, encoding spastizin, as a frequent cause of complicated autosomal-recessive spastic paraplegia, including Kjellin syndrome. Am J Hum Genet 2008; 82: 992–1002.

Harding AE. Classification of the hereditary ataxias and paraplegias. Lancet 1983; 1: 1151–5.

Hentati A, Pericak-Vance MA, Hung WY, Belal S, Laing N, Boustany RM, et al. Linkage of 'pure' autosomal recessive familial spastic paraplegia to chromosome 8 markers and evidence of genetic locus heterogeneity. Hum Mol Genet 1994; 3: 1263–7.

Hodgkinson CA, Bohlega S, Abu-Amero SN, Cupler E, Kambouris M, Meyer BF, et al. A novel form of autosomal recessive pure hereditary spastic paraplegia maps to chromosome 13q14. Neurology 2002; 59: 1905–9.

Klebe S, Durr A, Bouslam N, Grid D, Paternotte C, Depienne C, et al. Spastic paraplegia 5: locus refinement, candidate gene analysis and clinical description. Am J Med Genet B Neuropsychiatr Genet 2007; 144: 854–61.

Mellon SH, Gong W, Schonemann MD. Endogenous and synthetic neurosteroids in treatment of Niemann-Pick Type C disease. Brain Res Rev 2008; 57: 410–20.

Moghadasian MH, Salen G, Frohlich JJ, Scudamore CH. Cerebrotendinous xanthomatosis: a rare disease with diverse manifestations. Arch Neurol 2002; 59: 527–9.

Muglia M, Criscuolo C, Magariello A, De Michele G, Scarano V, D'Adamo P, et al. Narrowing of the critical region in autosomal recessive spastic paraplegia linked to the SPG5 locus. Neurogenetics 2004; 5: 49–54.

Patel H, Cross H, Proukakis C, Hershberger R, Bork P, Ciccarelli FD, et al. SPG20 is mutated in Troyer syndrome, an hereditary spastic paraplegia. Nat Genet 2002; 31: 347–8.

Patterson MC, Vecchio D, Prady H, Abel L, Wraith JE. Miglustat for treatment of Niemann-Pick C disease: a randomised controlled study. Lancet Neurol 2007; 6: 765–72.

Pettersson H, Holmberg L, Axelson M, Norlin M. CYP7B1-mediated metabolism of dehydroepiandrosterone and 5alpha-androstane-3beta,17beta-diol—potential role(s) for estrogen signaling. Febs J 2008; 275: 1778–89.

Reid E. Many pathways lead to hereditary spastic paraplegia. Lancet Neurol 2003; 2: 210.

Rose KA, Stapleton G, Dott K, Kieny MP, Best R, Schwarz M, et al. Cyp7b, a novel brain cytochrome P450, catalyzes the synthesis of neurosteroids 7alpha-hydroxy dehydroepiandrosterone and 7alpha-hydroxy pregnenolone. Proc Natl Acad Sci USA 1997; 94: 4925–30.

Schule R, Brandt E, Karle KN, Tsaousidou M, Klebe S, Klimpe S, et al. Analysis of CYP7B1 in non-consanguineous cases of hereditary spastic paraplegia. Neurogenetics 2008; DOI 10.1007/s10048-008-0158-9 (online publication date 15 October 2008).

Setchell KD, Schwarz M, O'Connell NC, Lund EG, Davis DL, Lathe R, et al. Identification of a new inborn error in bile acid synthesis: mutation of the oxysterol 7alpha-hydroxylase gene causes severe neonatal liver disease. J Clin Invest 1998; 102: 1690–703.

Simpson MA, Cross H, Proukakis C, Pryde A, Hershberger R, Chatonnet A, et al. Maspardin is mutated in mast syndrome, a complicated form of hereditary spastic paraplegia associated with dementia. Am J Hum Genet 2003; 73: 1147–56.

Stevanin G, Azzedine H, Denora P, Boukhris A, Tazir M, Lossos A, et al. Mutations in SPG11 are frequent in autosomal recessive spastic paraplegia with thin corpus callosum, cognitive decline and lower motor neuron degeneration. Brain 2008a; 131: 772–84.

Stevanin G, Ruberg M, Brice A. Recent advances in the genetics of spastic paraplegias. Curr Neurol Neurosci Rep 2008b; 8: 198–210.

Stevanin G, Santorelli FM, Azzedine H, Coutinho P, Chomilier J, Denora PS, et al. Mutations in SPG11, encoding spatacsin, are a major cause of spastic paraplegia with thin corpus callosum. Nat Genet 2007; 39: 366–72.

Tallaksen CM, Durr A, Brice A. Recent advances in hereditary spastic paraplegia. Curr Opin Neurol 2001; 14: 457–63.

Trap C, Nato F, Chalbot S, Kim SB, Lafaye P, Morfin R. Immunohistochemical detection of the human cytochrome P4507B1: production of a monoclonal antibody after cDNA immunization. J Neuroimmunol 2005; 159: 41–7.

Tsaousidou MK, Ouahchi K, Warner TT, Yang Y, Simpson MA, Laing NG, et al. Sequence alterations within CYP7B1 implicate defective cholesterol homeostasis in motor-neuron degeneration. Am J Hum Genet 2008; 82: 510–5.

Vanier MT, Millat G. Niemann-Pick disease type C. Clin Genet 2003; 64: 269–81.

Wilkinson PA, Crosby AH, Turner C, Patel H, Wood NW, Schapira AH, et al. A clinical and genetic study of SPG5A linked autosomal recessive hereditary spastic paraplegia. Neurology 2003; 61: 235–8.

Wu Z, Chiang JY. Transcriptional regulation of human oxysterol 7 alpha-hydroxylase gene (CYP7B1) by Sp1. Gene 2001; 272: 191–7.

Wu Z, Martin KO, Javitt NB, Chiang JY. Structure and functions of human oxysterol 7alpha-hydroxylase cDNAs and gene CYP7B1. J Lipid Res 1999; 40: 2195–203.