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Mutations in the X-linked filamin 1 gene cause periventricular nodular heterotopia in males as well as in females

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Periventricular heterotopia (PH) is a human neuronal migration disorder in which many neurons destined for the cerebral cortex fail to migrate. Previous analysis showed heterozygous mutations in the X-linked gene filamin 1 (*FLN1*), but examined only the first six (of 48) coding exons of the gene and hence did not assess the incidence and functional consequences of *FLN1* mutations. Here we perform single-strand conformation polymorphism (SSCP) analysis of *FLN1* throughout its entire coding region in six PH pedigrees, 31 sporadic female PH patients and 24 sporadic male PH patients. We detected *FLN1* mutations by SSCP in 83% of PH pedigrees and 19% of sporadic females with PH. Moreover, no PH females (0/7 tested) with atypical radiographic features showed *FLN1* mutations, suggesting that other genes may cause atypical PH. Surprisingly, 2/24 males analyzed with PH (9%) also carried *FLN1* mutations. Whereas *FLN1* mutations in PH pedigrees caused severe predicted loss of FLN1 protein function, both male *FLN1* mutations were consistent with partial loss of function of the protein. Moreover, sporadic female *FLN1* mutations associated with PH appear to cause either severe or partial loss of function. Neither male could be shown to be mosaic for the *FLN1* mutation in peripheral blood lymphocytes, suggesting that some neurons in the intact cortex of PH males may be mutant for *FLN1* but migrate adequately. These results demonstrate the sensitivity and specificity of DNA testing for *FLN1* mutations and have important functional implications for models of FLN1 protein function in neuronal migration.

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INTRODUCTION

Disorders of neuronal migration in the human cerebral cortex arise from a disruption of normal cellular and molecular mechanisms that guide cortical development. During corticogenesis, immature neurons undergo a directed migration from the ventricular and subventricular zones to the cerebral cortex. They are guided through the intermediate zone on radial glial cells and ultimately form the respective laminae of the mature cortex (1). Disturbances of neuronal migration, including periventricular heterotopia (PH) (2–5), are increasingly commonly recognized as a cause of human epilepsy. PH is characterized by a failure of a subset of neurons to migrate from the periventricular region during corticogenesis, resulting in nodules of neurons, referred to as heterotopia, abnormally located along the ventricle. Individuals with PH usually present with seizures, though the overlying cortex is often well preserved and intelligence is often normal (4–8). In this context, the underlying neuropathology of PH will provide insight into normal cortical development as well as epilepsy.

Pedigree analysis has previously suggested that the migratory defect seen in PH may reflect a mosaic phenotype created by random X-chromosome inactivation (4,5,9). Within PH pedigrees, the disorder segregates as an X-linked dominant disorder, presenting in females and associated with a high incidence of spontaneous miscarriages, predominantly of male fetuses (4,5). These observations would imply hemizygous male lethality with the observed cortical malformations occurring only in heterozygous females. It would be expected that neurons residing in the heterotopia would express the mutant PH allele and thereby fail to migrate into the cortical laminae. Conversely, neurons residing in the cortex would be expected to express the alternate normal allele. Thus, both the pedigree studies and the neuroanatomical phenotype of PH have led to the prevailing view that patterns of X-inactivation give rise to the normal cortex and the aberrant nodules, though this model has not been directly tested.

Recent studies have demonstrated that PH can result from mutations in the X-linked gene, filamin 1 (*FLN1*) (3). *FLN1* encodes a large (280 kDa) cytoplasmic phosphoprotein with demonstrated binding domains for multiple membrane receptors and for actin, thereby providing potentially crucial links between signal transduction and the cytoskeleton (10–15). Structurally, the protein consists of an actin-binding domain at the N-terminus, 23 repeats that resemble Ig-like domains and form a rod-like structure interrupted by two hinge regions, and a C-terminal repeat which undergoes dimerization and facilitates binding to membrane receptors. The FLN1 protein is involved in many fundamental processes surrounding cellular protrusion and motility (16–19). Although a similar influence on neuroblast migration during cortical development would not be unexpected, the mechanism of action of FLN1 and the other proteins with which it interacts in migrating neurons are unknown. Since the only published reports of *FLN1* mutations in PH analyzed only six of the 48 coding exons (numbered as exons 2–7) of the *FLN1* gene, no information is available about the sensitivity of DNA-based diagnostic testing, nor about the functional consequences of *FLN1* mutations to the predicted protein.

The current studies sought to further characterize *FLN1* mutations in the PH population on two levels: (i) clinically, to determine the incidence of *FLN1* mutations within spontaneous and familial PH and (ii) functionally, to determine potential domains of FLN1 required for proper neuronal migration and to explore potential mechanisms giving rise to the heterotopia, based on these mutations.

RESULTS

Detection rate of *FLN1* mutations in females with PH

To address the incidence of *FLN1* mutations in females with PH, single-strand conformation polymorphism (SSCP) mutational analysis was performed on all 48 coding exons of the *FLN1* gene in six pedigrees (all of which showed PH inherited from female to female) and 31 PH females with no evidence of inherited PH by family history. The primers used to amplify the entire coding region of the *FLN1* gene are presented in Table 1. In some cases, larger exons were divided into multiple PCR primers for SSCP analysis. In order to provide a statistically accurate estimate of the sensitivity of DNA mutational analysis of *FLN1* in PH, mutations that were identified in previously published analyses of the first six coding exons (3,20) were included in this analysis. These previously identified alleles correspond to pedigrees P1, P2 and P3, and sporadic cases F1, F2, F3 and F5.

SSCP analysis of the entire *FLN1* coding region revealed two new pedigrees with *FLN1* mutations, so that overall, *FLN1* mutations were identified in five of six pedigrees that were adequately screened (83%). In contrast, only six of 31 sporadic female PH patients (19%) showed *FLN1* mutations. The probability of identifying an *FLN1* mutation was significantly higher in pedigrees with PH compared with sporadic patients ($P < 0.006$, two-tailed Fisher's exact test). All of the females with identified *FLN1* mutations showed typical radiographic features of PH, which are described elsewhere in detail (20) and are illustrated briefly in Figure 1. Moreover, 0/7 females with atypical radiographic features showed *FLN1* mutations. Thus there is a high probable association of an *FLN1* mutation presenting with a typical magnetic resonance imaging (MRI) scan in females ($P < 0.0001$, two-tailed Fisher's exact test).

Familial PH is often associated with severe truncating *FLN1* mutations within females

Segregation analysis of the two pedigrees (P4 and P5) with *FLN1* mutations discovered in this study confirm the findings of the previously described pedigrees (P1–P3) in suggesting that PH and *FLN1* gene mutations are inherited in an X-linked pattern from female to female, with no surviving affected male offspring. Each pedigree shows a history of multiple male miscarriages (3,20). The apparent prenatal lethality of hemizygous males with *FLN1* mutations presumably reflects a severe loss of function within the FLN1 protein. Mutations in the male-lethal pedigrees represent nonsense mutations near the N-terminus of the predicted protein (P1) or else splicing mutations that are likely to destabilize the entire mRNA as well as truncate any protein product that is actually translated (P2–P5; Fig. 2 and Table 2). Thus, familial PH in females is very likely to be associated with *FLN1* gene mutations, and these mutations are usually associated with abnormalities of

Table 1. PCR primer sequence used to amplify filamin 1 exons

Exon (F)	Filamin 1 SSCP primers		Exon (R)
2.1F	CCGCATTTAAAGGGCTCGCT	TCCAGCAGAACACTTTCACGC	2.1R
2.2F	ACCGAGAAGGACCTGGCG	CACAACCAGCGGCCCACT	2.2R
2.3F	GGCTTATCGCGCTGTTGGAG	CGTGCGTCCTTCCATCTCC	2.3R
3.1F	GTGCTGCCAGACCCTGACC	GGATGAGGAGGCCAAGAAGC	3.1R
3.2F	ACCCTGATCCTGCACTACTCC	GAGACTGGGCTGGGGTCAC	3.2R
4F	GGCGCAGAGGCAGGAGAG	GTAGGGGACCGGATCGGC	4R
5F	GGATCGGCAGTTGGGAGAG	CCGTGTGCCAACGTCTTCC	5R
6F	AAGGGTGGAGGGGTGAGG	CTTTCAGTGGGGCTGCTCTT	6R
7F	CTTTCAGTGGGGCTGCTCTT	GACCTCAGGCAGTGGCTGG	7R
8F	AGGCTTGTGACCTCAGGCAG	CTGGTCTGTGGGGAGAGCC	8R
9F	CTGAGCAGGTGCCTCGTG	CCTGACTGCCCTCTGCTGT	9R
10F	CTCTGAGGGACCCACCAATC	GGATGGGTGGCGGCAGC	10R
11F	CCGGGTTCACTGCTGGGC	GCATCCCTCTCCCAGCTCT	11R
12F	GTGGTGATCCTCGGTGTTCC	GACAGACGATGGCAAGGACG	12R
13F	GGGCACTGAGGGGACTGGT	CCTAAGTCTCGCCCTGCTGC	13R
14F	GGTGGGGATGGCACTCTGT	ACTGACCAGCAGGCCACC	14R
15F	TTCGGGTCCAAGTCCAGGTA	AACCTCCACCGGCCTTTAGT	15R
16F	TTGTGTGCCTGCCAGTGTAG	TCTCAGCCTCCGCTCCTC	16R
17F	GGTGTCCCTGCGAGGTCT	TCTGCCCTCCTCTAAGGCC	17R
18F	GCTCCTGGCCGCTAAGATG	GCATCAAGGGTAGGAGGGCT	18R
19F	GGGGCATCAAGGGTAGGA	TACAGTCACCGAGTCCCCAG	19R
20F	GGGAGCAATTCTGGTGTCTCTAA	GGATTGGCTGAGCTGGGTGT	20R
21.1F	GGATTGGCTGAGCTGGGTGT	GTGACCTATGACGGCGTGC	21.1R
21.2F	GGGGGCTGACAACAGTGTG	CTTCTCCAGACCTGCCCTAAA	21.2R
22.1F	TCTGGGATCGGGGCATAGTG	ACAACATCAACATCCTCTTCGCT	22.1R
22.2F	TGCTTGGACAATGGGGATGG	ATCTGCTCGGAGGCGGG	22.2R
22.3F	GCGCGGAGCTGACCATTGA	GTATGTGACGGGGGACAGGG	22.3R
23F	ACGGGGGACAGGGACCAG	GACTCCCACAGGCTGGCAG	23R
24F	CCTCCTGACCTGGCCTGC	GGAACCCCAGGAATGACCG	24R
25F	GCTGGTGGGCCTGGGAAC	TCTGCTCGTCCCCACCCT	25R
26F	CGTCCCCACCCTGTCTCAT	GCAGTGTCCTGTTTGAGGGC	26R
27F	GAATGGAGGCCCAGGAGACTA	ACTGCTCACAACACCAATCCCT	27R
28F	GGCTGGATGCTGAGAACCTG	AGTGTGGGCTGCGGGTGG	28R
29F	TGCCTCCCCTGCCTCTGC	TGTGCCTGTGTGCAAGGGC	29R
30F	GCTTGGGTTGTGCCTGTGTG	AATGCCCCTCCTGCCCAGA	30R
31.1F	AGGCGGACGGGAACAACC	CGACATCTTCTACACGGCCC	31.1R
31.2F	GATGTGGACGTGGTGGAGAAT	TGACAACAGACTCTCCAGCAGC	31.2R
32F	CAACAGACTCTCCAGCAGCTC	TACCATGTGTGAGGAGGGACC	32R
33F	GTGAGGAGGGACCCCAGAT	AGACTGTGCCAATGAGCTGC	33R
34F	GCCAGACTGTGCCAATGAGC	ACTCCACCGCCACCACCTC	34R
35F	CACACGAGGCTGCCATTCC	TCTTGACCTGCTCTGTGCCC	35R
36F	GAGTGTCCCCAGCATAGTTCC	ACAGAAACGGGTGGCAGGGT	36R
37F	TGGCTGGACGCACACTGAT	GTTCAGAAAGGAGGCAGCCT	37R
38F	CATGCCCTCCCTGACTGACA	GCATAGCACCGAGGCTCAGG	38R
39F	CCGAGGCTCAGGGGTATCC	ATGTGACTGGAGGGGCGTG	39R
40F	GCGTGGGCCGTGCTTTCT	TCAAGCAGCCCCAAGAGGAG	40R
41.1F	CCCAGGGCTGCTGCTCAC	TTCCAGTTCACCGTGGGGC	41.1R
41.2F	CCGCTTTGTTCCCGCTGA	AGTGCTCGCTCTCCTGCGT	41.2R
42F	AGCAGCCTTCAGTGAGGACA	GTATTGGACCCAGGGCTGG	42R
43F	GCTGATGAGCCGGTCTTACAC	GGACAGACCAGAGCCACCG	43R
44F	CAAGCACCCCCATCTAACCAT	TGTAAGCAGCCAAGACGGG	44R
45F	CCCAGGCCCACAGCATGA	GCTCTTGGTGACAACAGGAGG	45R
46F	AGTCTGGCTCTGCCTGACCT	CACCACAGCCACCTCTTAGC	46R
47F	CCTCTTAGCCCCACCCACTC	CTTCCCACCAAAATGAGGCCA	47R
48F	GGCTGGGACCTGGGACTGA	CTACCCAAGCAGCCCCGC	48R

Primers used for amplification of *FLN1* gene for mutation detection. Given the length of exons 2, 21, 22, 31 and 41, additional primer pairs were used to amplify each of these exons. The primers are complementary to exonic or intronic DNA surrounding each exon, to amplify the entire coding region and the splice donor/acceptor sites.

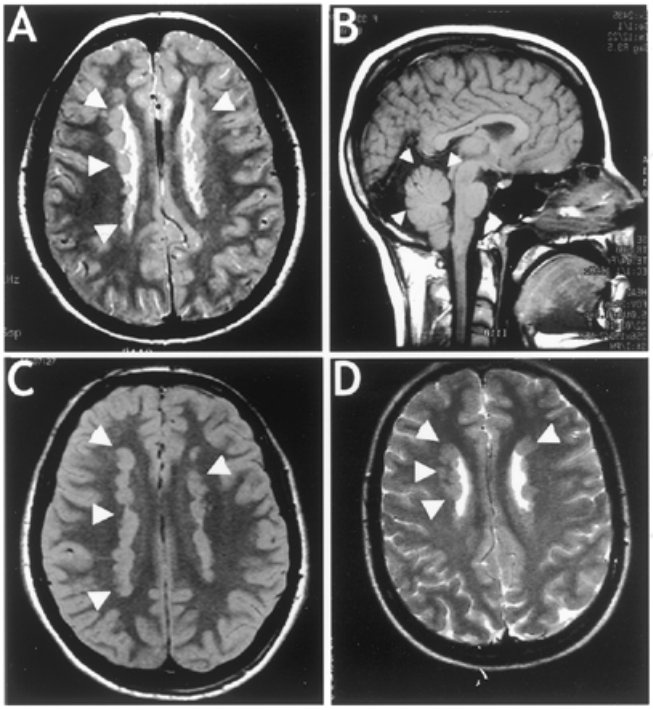

Figure 1. Brain MRI appearance of female patients with PH from *FLN1* mutations. (**A**) Axial T2-weighted image from sporadic female 5 (F5) demonstrates typical bilateral PH (arrowheads). (**B**) Sagittal T1-weighted image of the same patient as in (A) shows a subtle displacement of the cerebellum (arrowheads) by an enlarged cisterna magna, a feature commonly associated with PH. (**C** and **D**) As with the sporadic cases, proton density and T2-weighted images, respectively, from a PH pedigree (P3) similarly display the characteristic PH feature of the bilateral periventricular nodules (arrowheads).

splicing and/or severe truncations and presumed loss of function of the FLN1 protein.

Sporadic PH in males can arise from *FLN1* mutations that might retain some residual function

Given the prominent absence of surviving males in the PH pedigrees, we considered it unlikely that *FLN1* gene mutations would be observed in males with PH. We were surprised to find that two of the 24 males with PH (9%) that we screened for *FLN1* mutations did indeed show mutations (Fig. 2 and Table 2). One male (M1) harbors a C→G substitution at 6915 bp of the cDNA which causes a nonsense mutation and truncates the receptor-binding region of the FLN1 protein at amino acid 2305 of 2648. MRI studies of this patient revealed an MRI pattern that was remarkably indistinguishable from that of females with *FLN1* mutations (compare Fig. 1 and Fig. 3A–C). This male showed characteristically symmetrical nodules within the ventricular region of the cortex, and an enlargement of the cisterna magna behind the cerebellum (Fig. 3) that is commonly seen in affected females as well. The clinical features of this patient were also comparable to the clinical features of females with *FLN1* mutations; the patient was not mentally retarded and suffered only from mild to moderate seizures.

The second male PH patient with an *FLN1* mutation (M2) showed a C→T substitution that creates a Leu→Phe substitution in the fifth Ig-like domain of the FLN1 protein (Table 2 and Fig. 2). This second male displayed an even milder radiographic phenotype than any of the females with *FLN1* mutations, as the heterotopic nodules were very few in number and could be appreciated only in the right hemisphere (Fig. 3D). In this patient there were no other associated radiographic features. This male patient was also cognitively normal, with his only complaint being seizures that were not intractable. Although in neither case was the mRNA confirmed to be stable or the protein shown to be expressed, both of the male *FLN1* mutations would be consistent with retention of some limited function of the mutated FLN1 protein. A partial loss of FLN1 function in these males might explain their survival and their relatively mild neurological picture.

Sporadic *FLN1* mutations in females appear to represent a range of mutation types

While some females with sporadic mutations of the *FLN1* gene show severe predicted loss of FLN1 function, other alleles in sporadic females may be milder. Four previously reported sporadic mutations (F1, F2, F3 and F5) in PH females result in severe truncation of the protein or abnormal splicing, similar to the male-lethal mutations observed in the familial cases. Two additional sporadic female cases identified in this study (F4 and F6), however, suggest that female *FLN1* mutations might also lead to partial loss of function. One female (F4) has a G→A substitution, changing an Ala to Thr at amino acid 1764, and the other female (F6) possesses a 4 bp deletion (CAGC) within exon 41, causing a translational frameshift and premature termination at amino acid 2212. The mutation in the first individual (F4) results in a single amino acid substitution and would not be inconsistent with retention of some FLN1 function, provided that the amino acid substitution does not destabilize the entire protein. Furthermore, this particular female had five children (none available for further evaluation), all sons, and no history of spontaneous miscarriages. Thus, this pedigree shows a prominent lack of the excess of daughters that characterize the offspring of most PH females. Two of the five sons of this patient show mental retardation or other learning disorders and so might be affected. Finally, the mutation in the last individual (F6) represents a 4 bp deletion that causes a translational frameshift and early termination at amino acid 2212, but (assuming the mRNA and protein are stable) could preserve >80% of the FLN1 protein.

Mosaic analysis of males with *FLN1* mutations

We hypothesized that the two male *FLN1* mutations could have arisen *de novo* after fertilization, giving rise to somatic mosaicism. Such a mechanism has been reported for doublecortin (*DCX*) mutations in some, but not all, males (21–24), and has been reported in other congenital neurological conditions such as tuberous sclerosis (TSC) (25,26) and neurofibromatosis (NF) as well (27). In order to get an initial indication as to whether the two affected males were mosaic for *FLN1* mutations, SSCP was used to determine whether both normal and abnormal *FLN1* sequence conformers were present, which would imply that the peripheral blood lymphocytes were mosaic for *FLN1* mutations. In the one patient whose DNA was available for further study (M2), only the mutant *FLN1* conformer could be detected by SSCP, while

Table 2. Summary of *FLN1* mutations

	FLN1 DNA mutation	Predicted protein defect
Pedigree		
P1	Point mutation C→T at 544 bp	Truncation at amino acid 182
P2	Point mutation T→C at exon 4 + 2	Missplice exon 4, probable truncation
P3	Point mutation A→G at exon 7 – 2	Missplice intron 6, probable truncation
P4	Point mutation G→A at exon 20 – 1	Missplice intron 20, probable truncation
P5	Deletion AGGAGGTG at exon 25 + 4	Missplice intron 25, probable truncation
Sporadic females		
F1	Deletion GGCCC at 287–291 bp	Frameshift and truncation after amino acid 96
F2	Point mutation C→G at exon 4 – 3	Missplice intron 3, probable truncation
F3	Point mutation G→A at exon 2 + 1	Missplice intron 2, probable truncation
F4	Point mutation G→A at 5290 bp	Ala→Thr at amino acid 1764
F5	Point mutation C→T at 688 bp	Truncation at amino acid 230
F6	Deletion CAGC at 6636–6639 bp	Frameshift and truncation after amino acid 2212
Sporadic males		
M1	Point mutation C→G at 6915 bp	Truncation at amino acid 2305
M2	Point mutation C→T at 1966 bp	Leu→Phe at amino acid 656

Each *FLN1* DNA mutation is numbered with reference to the ATG site or the exon site ± bp. The predicted filamin protein alteration is numbered with reference to the amino acid residue number.

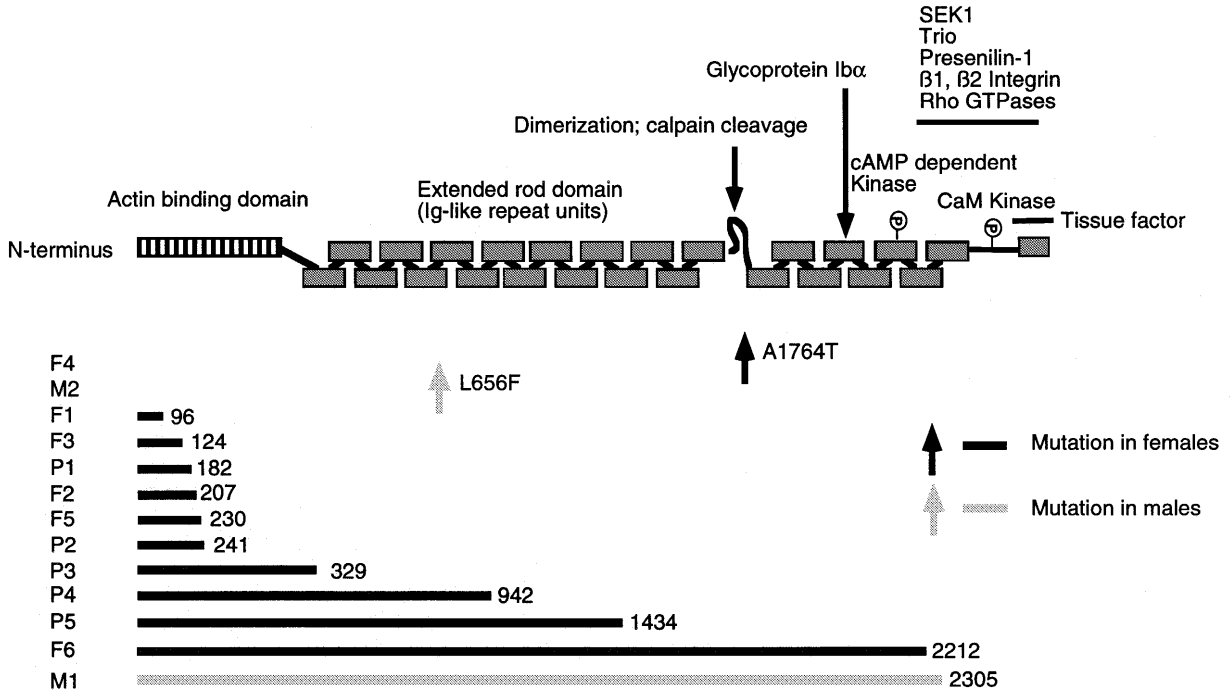

Figure 2. Schematic representation of *FLN1* mutations. Summary is taken from patients presented in this study and previously published sporadic female cases and pedigrees (F1–F3 and F5, and P1–P3; refs 1 and 20). Filamin 1 protein (FLN1) is represented as a solid bar extending from the start codon (N-terminus) to the stop codon at amino acid 2648. Indicated are the actin-binding domain, extended Ig-like repeat units, a calpain cleavage site within the first hinge, and multiple demonstrated protein interactions within the receptor binding region. Arrows correspond to amino acid substitutions (L656F, Leu→Phe substitution at amino acid 656; A1764T, Ala→Thr substitution at amino acid 1764) and bars signify truncation or splicing mutations (F, females; M, males; P, pedigrees with affected females).

none of the conformer corresponding to the normal DNA sequence was observed (Fig. 4A). Repeat DNA sequencing of the sample corresponding to the exon containing the mutation (Fig. 4B) also confirmed the presence of the expected mutant sequence (GC**T**TCA) and the absence of any detectable normal DNA sequence (GC**C**TCA). Thus, the vast majority, if not all, peripheral blood lymphocytes carry the mutation in this patient. Of course, we cannot rule out a small number of non-mutant cells among peripheral blood lymphocytes, or a more complicated type of somatic mosaicism with some cells restricted to the brain carrying a non-mutated *FLN1* gene. However, the contrast between the very mild heterotopia in

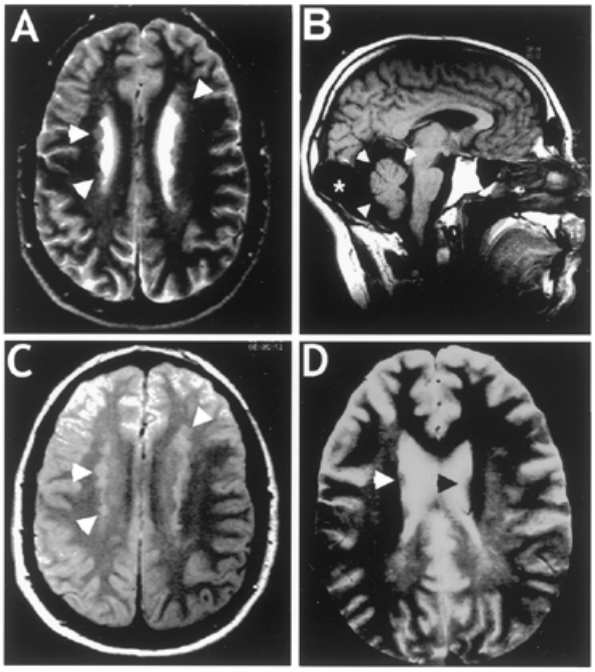

Figure 3. MRI appearance of male patients with PH from *FLN1* mutations. (**A**–**C**) Axial T2-weighted (A) and proton density images (C) from sporadic male 1 (M1) demonstrate typical bilateral PH (arrowheads) indistinguishable radiographically from sporadic female PH patients, or female PH patients in pedigrees (Fig. 1). (B) Sagittal T1-weighted image of the same patient as in (A) shows an enlarged cisterna magna (asterisk) posterior to the hypoplastic cerebellum (arrowheads), another typical feature of PH. (**D**) Axial T2-weighted image from sporadic male 2 (M2) illustrates atypical, unilateral, right-sided PH consisting of just two nodules (white arrowhead). The head of the caudate (black arrowhead) extends into the left lateral ventricle in normal fashion and resembles the heterotopia but is clearly distinguishable from the unilateral right-sided heterotopia in other sections.

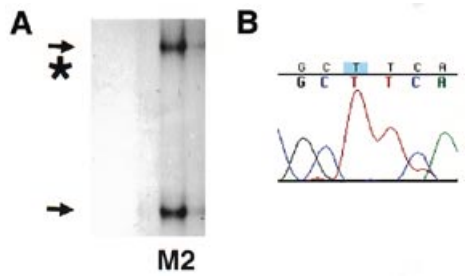

Figure 4. Mosaic analysis of a male with PH. (**A**) SSCP analysis of exon 13 of patient M2. The SSCP gel shows only two conformers (arrows) representing mutant sequence. In other experiments when control DNA is analyzed in parallel, a wild-type conformer migrates slightly faster than the upper mutant band, at the approximate position indicated by the asterisk (not shown). (**B**) Sequence analysis of the PCR product corresponding to exon 13 from patient M2 also shows only the mutant sequence (GC**T**TCA) and none of the normal sequence (GC**C**TCA).

this patient (Fig. 3D) and the apparent absence of normal *FLN1* sequence in the peripheral blood cells is striking. No tissue or DNA of patient M1 was available for repeat study. Review of the original SSCP gel in this patient showed a vast predominance of the mutant *FLN1* sequence in peripheral blood lymphocytes (data not shown), although whether the non-mutated sequence was completely absent or present at very low levels could not be determined. These observations in general argue against somatic mosaicism as a major cause of the PH in males with *FLN1* mutations, though more extensive study of a larger number of patients would be needed to determine this definitively.

DISCUSSION

This study presents the first systematic analysis of the *FLN1* gene in a large cohort of patients with PH. SSCP analysis identified *FLN1* mutations in six of 31 sporadic female patients (19%) and in five of six pedigrees (83%). All of the mutations in PH pedigrees were associated with typical radiographic findings of bilateral PH (20) and male-lethality. The corresponding mutations in *FLN1* created severe disruptions of the predicted protein, resulting in an absent protein in affected cells, or a truncated protein that would contain only a portion of the actin-binding domain. In contrast, sporadic PH can occur occasionally (2/24 tested males) in males due to *FLN1* mutations, but these mutations appear to suggest some partially retained function of the protein. Finally, sporadic female PH patients can apparently present with either severe or partial loss-of-function mutations.

The detection rate for *FLN1* mutations is higher in patients from PH pedigrees than from sporadic cases. This discrepancy implies that PH is etiologically heterogeneous. For example, whereas inherited PH obviously suggests a genetic disorder such as *FLN1*, sporadic PH may arise from multiple environmental or genetic causes. This interpretation is also consistent with the observation that the neuronal heterotopia caused by inherited *FLN1* mutations in females are always associated with stereotypical radiographic findings. This suggests the possibility that non-*FLN1* mutation cases have a range of features and may reflect a variety of causes. Alternatively, sporadic PH may have a higher proportion of non-coding region mutations, as seen in conditions such as Unverricht–Lundborg disease (28), thereby accounting for differences in detection rate. Although SSCP screening is probably not sufficiently sensitive to identify all mutations, with reported detection rates of 80–95% (29), the limitations of SSCP analysis are unlikely to account for the stark differences in mutation detection rate between pedigrees and sporadic cases.

Although the numbers of mutations are still fairly small, there appear to be differences in *FLN1* mutation type between familial and sporadic female PH patients, and male PH patients. In 'classical' PH pedigrees with affected females and male lethality, protein truncation or splicing mutations tend to cluster at the N-terminal of the protein, leaving only a small translated portion of the actin-binding region. Sporadic females with PH and no family history seem to show either severe loss-of-function alleles or alleles that could be consistent with partial loss of function. Since mutations can occur along the entire length of the *FLN1* gene, the predilection of truncation mutations for the actin-binding region may merely reflect the incidence of clinical presentations for severe mutations in females. Mild to moderate mutations in *FLN1* may have fewer clinical phenotypes in females and thus avoid detection. Finally, in males, severe to moderate defects in FLN1 function appear to lead to fetal death and consequently only partial loss-of-function mutations are found in viable

males with PH. Thus, although our sample size is small, there appear to be systematic differences in mutation between males, females and pedigrees.

Our current observations, as well as some prior evidence, suggest that *FLN1* mutations can give rise to male PH in the absence of somatic mosaicism (at least in some cases). First, recent studies have documented maternal transmission of Xq28-linked PH from female to male, suggesting that a mild germ-line defect of *FLN1* might not necessarily be male-lethal (30). Secondly, one PH female (F4) from our studies has a mild *FLN1* mutation with a single amino acid substitution, consistent with a partial loss-of-function mutation. She has five male offspring, and two of them have mental retardation or other learning disorders that could represent *FLN1* mutations inherited from female to male. However, since the children were not available for MRI or DNA testing this remains unproven. Thirdly, one of the male patients with *FLN1* mutations described here (M2) had no detectable wild-type bands seen on SSCP analysis of the peripheral blood lymphocytes, and the other male patient had little or no wild-type sequence. Finally, a male with a duplication of the *FLN1* gene and PH has been reported (31), and there is no evidence of somatic mosaicism of that duplication. Samples from the peripheral lymphocytes cannot definitively prove lack of mosaicism within the central nervous system, since even if the normal allele is not detected in lymphocytes, some cells with the normal allele may be present in brain. However, these studies do strongly suggest that a pattern similar to what is seen in the peripheral blood also exists in the cortex, with all or nearly all the neurons expressing the mutated FLN1 protein but showing two distinct migratory behaviors. Taken together, the current and prior observations would indicate that the developmental abnormality that gives rise to PH need not be solely due to X-inactivation or mosaicism of the *FLN1* allele. Rather, temporally and spatially dependent interactions of the abnormal FLN1 protein with other cell intrinsic or extrinsic signals may prevent a subset of neurons from migrating out of the ventricular zone. On the other hand, as more male patients with PH are studied, somatic mosaicism is likely to be uncovered in some cases, given the precedent of somatic mosaicism for other genes with similar phenotypes such as *TSC*, *NF* and *DCX*.

Given the absence of obvious mosaicism, the male mutations give insight into possible functions of the specific regions within the filamin protein. One point mutation (M2) leads to a missense mutation, replacing a Leu for Phe within the Ig-like domains. Presumably, this effects a conformational change on the predicted β-pleated sheets and alters the signal transduction cascade following receptor/ligand binding by affecting protein stability (10). The nonsense mutation (M1) causes a truncation of the FLN1 protein within the receptor-binding region, also suggesting that the entirety of FLN1 may be needed for the protein to fulfill its role in migrating or pre-migratory neurons. Alternatively, the critical region for FLN1 function in the cortex may lie downstream of amino acid 2305, within the receptor-binding region. The critical domain of the FLN1 protein that determines male lethality or viability may be the Glycoprotein 1bα binding site. Unlike many cell surface receptors that bind FLN1 at its extreme C-terminus, Glycoprotein 1bα binds to FLN1 about three-quarters of the way through the FLN1 protein, and this interaction appears to be critical for many of the roles of FLN1 in vascular development and hemostasis (14). Mutations that leave this domain intact (such as the nonsense mutation in M1) may allow male viability, whereas disruption of this interaction may determine male lethality. These functional interpretations, however, also presume that the particular genetic mutations lead to the expected protein truncation or stable protein product. It remains possible that any such mutations could lead to mRNA instability and complete absence of a translated protein.

The causal association of the *FLN1* gene with PH provides the opportunity to study the genetic mechanisms in a human disorder of neuronal migration. *FLN1* is known to regulate cell stability, protrusion and motility across various biologic systems. As it can be embryonic lethal, the *FLN1* gene will likely represent a fundamental gene important for cell migration as well as cortical development. The current studies show that diagnostic tests are fairly sensitive for familial cases with typical radiographic findings of PH, and that most of these patients will reflect *FLN1* mutations. However, the sporadic male cases provide the most information with regard to *FLN1* function. Further mutational analysis in patients with typical PH and the *FLN1* mutations will better define preferential mutations, and give insight into the function of the different domains of the *FLN1* gene.

MATERIALS AND METHODS

Patients

Six pedigrees and 55 sporadic patients (24 males and 31 females) with PH were evaluated in this study. All patients had documented contiguous or nodular gray matter lining the ventricles (PH) on neuroimaging. Further subgrouping was performed with respect to typical or atypical features noted on these studies. Criteria for a typical MRI or computerized tomography scan included bilateral gray matter predominantly lining the lateral ventricles with otherwise normal appearing white and cortical gray matter, since these radiographic features have previously been seen in patients with *FLN1* mutations (3,5,20). Associated findings of mild cerebellar hypoplasia, enlarged cisterna magna and thinning or agenesis of the corpus callosum were also accepted features of typical PH patients (Fig. 1). Atypical features were characterized by any other abnormal findings, including hydrocephalus, unilateral heterotopia, highly asymmetric or focally located nodules, and nodules which extended from the surface of the cortex down to the ventricles, or disorders of the overlying cortex. Patients were enrolled in accordance with clinical protocols approved by the institution review boards at the Beth Israel Deaconess Medical Center, as well as several other participating medical centers. The mutations for three pedigrees (P1–P3) and four sporadic cases (F1–F3 and F5) have been reported previously (3,20).

Mutational analysis

SSCP analysis was performed using 55 primer pairs designed against the sequences of the 48 coding exons of *FLN1* (Table 1). The *FLN1* gene of patients included in this study was subjected to SSCP analysis until either a convincing mutation was discovered, or >90% of 55 PCR products were

adequately screened. Patients in whom <90% of the gene was successfully analyzed by SSCP are not included in this study so that a first estimate of the sensitivity of DNA-based diagnosis could be provided.

Mutational analysis followed slight modifications of previously described analysis and procedures (3,21). In brief, genomic DNA was extracted from lymphocytes of participating individuals. Exons were amplified by PCR with *Taq* polymerase (Qiagen, Northridge, CA), using the genomic DNA of patients and controls. The PCR products were diluted in SSCP loading buffer (1:1 v/v; 95% formamide, 20 mM NaOH, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol), heated and flash cooled. Samples were then loaded, run on a non-denaturing polyacrylamide gel (0.5× MDE, FMC Bioproducts, Rockland ME), and visualized by silver staining (Promega, Madison, WI). Normal and aberrantly migrating bands from the stained SSCP gels were covered with polyacrylamide gel elution buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA pH 8.0, 0.1% SDS), excised, mixed in additional elution buffer and incubated at 37°C for >3 h. The supernatant was used as a template for PCR reactions to reamplify products from the excised bands. These products, along with the original heterozygous products run on the SSCP gels, were gel-purified (Geneclean kit, Bio 101, Carlsbad, CA) and sequenced (Big Dye Cycle Sequencing kit, Applied Biosystems, Foster City, CA) using the same primers used for the initial PCR reactions. Sequencing products were run on an ABI 377 automated sequencer and analyzed for the suspected mutations (Sequencher program, Gene Codes, Ann Arbor, MI). All mutations were verified by reamplifying products from genomic DNA and repeating the SSCP analysis and sequencing.

Statistical analysis

The significance of the data was assessed using a two-tailed Fisher's exact test for two-by-two comparisons of the type of patient (sporadic versus familial) versus the detection of a mutation (*FLN1* mutation versus no *FLN1* mutation). Further subgrouping of the radiographic findings of the PH (typical versus atypical) was also examined with regard to presence or absence of the *FLN1* mutation.

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