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CNS demyelination in fibrodysplasia ossificans progressiva

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Abstract

Fibrodysplasia ossificans progressiva (FOP) is a rare genetic disorder of progressive heterotopic ossification (HO) caused by a recurrent activating mutation of ACVR1/ALK2, a bone morphogenetic protein (BMP) type I receptor. FOP is characterized by progressive HO, which is associated with inflammation in the setting of dysregulated BMP signaling, however, a variety of atypical neurologic symptoms are also reported by FOP patients. The main objective of this study is to investigate the potential underlying mechanism that is responsible for the observed atypical neurologic symptoms. We evaluated two mouse models of dysregulated BMP signaling for potential CNS pathology through noninvasive magnetic resonance imaging (MRI) studies and histological and immunohistochemical approaches. In one model, BMP4 is over-expressed under the control of the neuron-specific enolase promoter; the second model is a knock-in of a recurrent FOP mutation of ACVR1/ALK2. We also retrospectively examined MRI scans of four FOP patients. We consistently observed demyelinated lesions and focal inflammatory changes of the CNS in both mouse models but not in wild-type controls, and also found CNS white matter lesions in each of the four FOP patients examined. These findings suggest that dysregulated BMP signaling disturbs normal homeostasis of target tissues, including CNS where focal demyelination may manifest as the neurologic symptoms frequently observed in FOP.

Keywords

Fibrodysplasia ossificans progressiva (FOP); Animal model; Demyelination; Bone morphogenetic protein (BMP); ACVR1/ALK2; Magnetic resonance imaging (MRI)

Introduction

Fibrodysplasia ossificans progressiva (FOP; OMIM 135100) is a rare genetic disorder of progressive heterotopic ossification (HO) in which individuals form a second skeleton of heterotopic bone [1]. Classic FOP, which is seen in 98 % of affected individuals worldwide, is caused by a recurrent heterozygous activating mutation (R206H) in the glycine–serine activation domain of Activin A receptor type I/Activin-like kinase 2 (ACVR1/ALK2), a bone morphogenetic protein (BMP) type I receptor [1–3]. The FOP mutation leads to both ligand-independent and ligand-enhanced dysregulated BMP signaling [4–6]. Disease

progression is episodic and associated with inflammation [1, 7], and disability is cumulative. A recent worldwide survey of members of the International FOP Association identified an increased prevalence of certain neurological symptoms, such as allodynia, neuropathic pain, myoclonus, and severe headache, among individuals with classic FOP ([8], companion article submitted with this manuscript). Other neurological problems, such as mild cognitive delay, have been reported in occasional patients with variants of FOP [3]. The pathogenesis of these neurologic problems is unknown.

Bone morphogenic protein signaling is a potent inhibitor of oligodendroglial differentiation and remyelination [9, 10], and gain-of-function mutations in ACVR1/ALK2 would predictably enhance this potent inhibition. These observations, in conjunction with the atypical neurologic phenotypes seen in FOP patients, led to the hypothesis that dysregulated BMP signaling causes CNS demyelination, and CNS demyelination is an underlying mechanism for the observed atypical neurologic phenotypes in FOP patients. To examine this hypothesis, we evaluated two independent mouse models of FOP through non-invasive MRI studies and histological and immunohistochemical approaches. We consistently found demyelinated lesions in the CNS in mice with dysregulated BMP signaling, but not in wild-type controls. Notably, focal inflammatory markers were up-regulated in all CNS demyelinated lesions. A review of MRI studies of four FOP patients identified focal white matter lesions in the CNS in all patients. These findings are not only important for understanding the effects of the FOP ACVR1 mutation and FOP disease progression but also more generally for understanding how CNS inflammation and the highly conserved but dysregulated BMP signaling pathway interact to produce pathological changes leading to neurological problems.

Materials and methods

Animal study procedures

Transgenic and knock-in mouse models—The Nse-BMP4 mouse is a transgenic mouse model with features of both sporadic HO and FOP in which BMP4 is driven by neuron-specific enolase (Nse) promoter [11]. Homologous recombination was used to generate ES cells heterozygous for the ACVR1 R206H mutation; these cells were injected into host blastocysts to generate mice that are cellular chimeras for mutant and wild-type cells [12] (a detailed description will be reported elsewhere). Sex- and age-matched wild-type mice were used as controls. Animal experiments in this study were approved by the Animal Care and Use Committee at The University of Pennsylvania and Northwestern University.

Small animal magnetic resonance imaging (MRI) and MRI scans for FOP patients—Magnetic resonance imaging was performed on mice anesthetized using inhalant isoflurane mixed with medical air in a 7T preclinical MR imaging system (Clinscan, Bruker). A high-performance and high signal-to-noise surface coil (I.D. = 2 cm) was used to acquire the data sets for each subject across the whole length of the spinal cord. Respiration triggering was used when possible to reduce motion artifacts due to breathing. Physiological body temperature (~ 37 °C) was maintained throughout the session and animals were revived at the end of the procedure. Post-acquisition image processing was performed visually by an experienced MR physicist (DP) together with experienced neurologist (LK). Magnetic resonance imaging hyperintense regions of interest (ROI) were visually detected and manually selected and identified as lesions if the corresponding signal intensity was higher than that from the rest of the spinal cord. In some subjects, corresponding MR slice images were taken for comparison with histological slices.

For FOP patients, in most cases, both standard T2-weighted scan and specialized MRI scans, such as fluid attenuated inversion recovery (FLAIR) scan were used. T2-weighted scan is a basic type of MR clinical scan. It differentiates fat from water (fat is darker and water brighter). Fluid attenuated inversion recovery is an inversion-recovery pulse sequence used to null signal from fluids, so that it can be used in brain imaging to suppress cerebrospinal fluid (CSF) and highlight the periventricular hyperintense lesions.

Histology and immunohistochemistry

Luxol fast blue (LFB) staining was performed according to standard protocol (http://www.library.med.utah.edu/WebPath/HISTHTML/MANUALS/LFB.PDF). Immunostaining was performed as previously reported [11]. Briefly, sections were fixed with 4 % paraformaldehyde in PBS. Non-specific binding was blocked with 10 % normal serum diluted in 1 % bovine serum albumin (BSA, Jackson Lab, USA) and 0.25 % Triton X-100 for 1 h at room temperature. The sections were then incubated with primary antibodies diluted with 1 % BSA + 0.25 % Triton X-100 at 4 °C overnight. The sections were then incubated with appropriate secondary antibodies (Cy3 or Cy2 conjugated antibodies (Jackson Lab) diluted with 1 % BSA + 0.25 % Triton X-100, or Alexa Fluor 488, Alexa Fluor 594, Alexa 647 (1:1,000, Invitrogen)) in the dark at room temperature for 2 h. Counterstaining was then performed with DAPI (1:5,000). Antibodies against MBP (Covance), CNPase (Covance), β-III Tubulin (Sigma), GFAP (Sigma), IBA1 (Wako), F4/80 (eBioscience) and Neomycin Phosphotransferase II (Millipore) were used in this study.

Patients and clinical summaries

We examined CNS MRIs from four individuals with FOP. For patients 1 and 2, the MRI was done as part of the investigation of their severe neurological symptoms, including headaches and myoclonus. The MRIs for patients 3 and 4 were conducted to evaluate the masses on their head and neck that occurred prior to a diagnosis of FOP. Parental and/or patient consent was obtained to include the MRIs in this report for all four patients.

Patient 1 was previously described by Kaplan et al. [3]. She was a healthful, active, athletic child until 11 years of age, when she spontaneously developed a painful flexion contracture of the left hip. An excisional biopsy indicated “aggressive fibromatosis”. She developed heterotopic ossification and ankylosis of the left hip within 2 weeks and ankylosis of all major joints of the axial and appendicular skeleton including the jaw, within 6 months. The great toes were clinically and radiographically normal, and she was classified as an FOP variant [3]. Sequence analysis of genomic DNA revealed a unique in-frame 3-bp heterozygous mutation (c.590_592delCTT; P197_F198delinsL) in the GS domain of ACVR1/ALK2 that replaces amino acids proline (codon 197) and phenylalanine (codon 198) with leucine.

At 23 years of age (in 2006), in association with FOP flare-up, she developed transient dizziness and ataxia, vertical diplopia, right-sided facial numbness, decreased taste, and paresthesias of the right side of the tongue in association with transient and migratory paresthesias of the upper and lower limbs and abdominal wall. One brain MRI was preformed at this time point (see Results). At 28 years of age (in 2011), in association with an FOP flare-up of the right shoulder, she developed recurrent, transient, right-sided vertical diplopia and right-sided numbness of the face, mouth, and tongue. Another brain MRI was preformed at this time point (see Results).

Patient 2 was a 17-year-old female and was diagnosed with FOP at age 3 years based on malformed great toes and progressive heterotopic ossification in characteristic anatomic patterns for FOP progression [13, 14]. She had a history of seizures at age 2 months that

were treated successfully with phenobarbital and a brachial plexus neuropathy at age 10 years that lasted 8 weeks. At age 16, she developed severe headaches, hyperesthesias, allodynia, and myoclonus. Patient 2 received four MRIs of her brain and seven of her spinal cord in different hospitals over a period of 1.5 years (see Results). The classic FOP mutation (ACVR1/ALK2 c.617G > A; R206H) was confirmed by DNA sequence analysis.

Patient 3 was a 27-month-old male and presented with malformed great toes and evanescent soft tissue masses on his neck, trunk, and back. Although no obvious neurological symptoms were present, a neoplastic process was suspected and MR imaging of the head and neck was performed (see Results). Subsequently, FOP was diagnosed clinically on the basis of malformed great toes and soft tissue swelling that progressed in characteristic anatomic patterns for FOP. The classic FOP mutation (ACVRl/ALK2c.617G > A;R206H) was confirmed by DNA sequence analysis.

Patient 4 was a 33-month-old female with malformed great toes who presented with firm swelling of the sternocleidomastoid muscles and progressive limitation of neck movement. Fibrodysplasia ossificans progressiva was diagnosed. Magnetic resonance imaging of the neck was performed (see Results). At present (in 2012), the child has no obvious neurological symptoms. The classic FOP mutation (ACVR1/ALK2 C.617G > A; R206H) was confirmed by DNA sequence analysis.

Results

Multiple hyperintense lesions are detected by magnetic resonance imaging of the CNS in Nse-BMP4 mice

To determine if dysregulated BMP signaling leads to CNS demyelination, we first studied a well-established transgenic mouse model that over-expresses BMP4 under the control of the neuron-specific enolase (Nse) promoter [11]. Previous studies showed that this mouse model recapitulated many features of both sporadic HO and FOP, and that overexpression of BMP4 in this model increased astrocytes and decreased oligodendrocytes in the CNS of neonatal mice [15]. However, no detailed histologic or MRI imaging studies had been performed on the CNS of adult Nse-BMP4 mice.

Magnetic resonance imaging examination of brain and spinal cord of a 6-month-old Nse-BMP4 mouse with progressive HO identified multiple hyperintense lesions in the brain and spinal cord (Fig. 1). Additional Nse-BMP4 transgenic mice (with HO, age range 3–5 months old, $n = 5$) were compared with wild-type mice, and we found that all BMP4 over-expressing mice with HO had hyperintense lesions ($n = 4–6$) across the spinal cord and in the brain. Younger Nse-BMP4 mice (at 2 months) without HO did not exhibit clear evidence of hyperintense lesions in MR images ($n = 5$, data not shown). No hyperintense lesions were identified in the MR images of wild-type mice at any age ($n = 5$, data not shown).

Multiple demyelinated lesions identified by histologic examination of the CNS in Nse-BMP4 mice

To further characterize alterations in the CNS, we conducted histological analyses using luxol fast blue (LFB) staining to detect myelin directly. Multiple weakly LFB-stained demyelinated lesions in various regions of the brain and spinal cord (Fig. 2) were identified, even in young adult (2 months old) mice without obvious HO formation. Immunohistochemical staining with myelin basic protein (MBP) specific antibody confirmed the decreased presence of myelin. The potential association of this demyelination phenotype with markers of inflammation was examined by additional immunohistochemical studies and we found that up-regulation of markers of inflammation, such as MHC, IBA1

(Fig. 2) and F4/80 (data not shown), were consistently detected in local areas of demyelination. Calcium-binding adaptor molecule-1 (IBA1) is a marker that is constitutively expressed by microglia but its expression is dramatically up-regulated in activated microglia. In contrast, major histocompatibility complex-II (MHC-II) often only expressed by activated microglia, and in brain, F4/80 is selectively expressed in activated microglia/macrophages. In contrast, GFAP, an astrocytic marker, did not show obvious changes within or in close proximity to the lesions. Interestingly, MHC staining indicated that increasing lesion sizes and numbers in the CNS correlate with increasing age.

Hyperintense MRI lesions correlate with histologically detected areas in the CNS of Nse-BMP4 mice

To determine if hyperintense lesions in the CNS detected on MRI studies represent regions of histological demyelination, we first imaged and located hyperintense MRI lesions in the spinal cords of Nse-BMP4 mice. We then removed and immediately fixed the spinal cords for histological and immunohistochemical studies. We found that the hyperintense MRI lesions correlated well with areas of demyelination (Suppl Fig. 1). By contrast, areas of the spinal cord where no obvious hyperintense lesions were found on MRI contained no areas of frank demyelination and only a few small areas of mildly decreased myelin staining (data not shown). Thus the findings of the MR imaging and the histologic analyses correlated well with each other. The histological/immunohistochemical approach was understandably more sensitive than MRI in detecting microscopic lesions (less than 100 μm across), likely due to the resolution and sensitivity limitations of MRI.

Areas of CNS demyelination are consistently present in mice with the classic FOP mutation (ACVR1 R206H)

Even though the phenotype of Nse-BMP4 mice closely mimics many important features of FOP, this BMP4 over-expression model does not recapitulate the genetic defect that causes the disease nor does it recapitulate all phenotypic abnormalities seen in FOP. For this reason, we examined mice with heterozygous knock-in of the FOP mutation (ACVR1 R206H). Mice that are cellular chimeras of mutant and wild-type cells survive to adulthood and were used for histological and immunohistochemical studies to determine if demyelination lesions occur in the adult brain and spinal cord. Although myelinated structures were grossly intact in chimeric animals (Suppl Fig. 2), areas of demyelination were consistently found in the cerebellum, spinal cord, and other brain regions (Fig. 3 and data not shown).

In the ACVR1 R206H chimeric mice, mutant cells express neomycin and therefore can be identified by immunohistochemistry. Double staining for mutant cells (neo+) and oligodendrocyte markers (CNPase) determined that mutant cells are highly incorporated in the cerebellum (Suppl Fig. 3), correlating well with regions of demyelination. Double staining also indicated that mutant cells (neo+) and oligodendrocytes are mutually exclusive. By contrast, many mutant cells express GFAP, a marker of astrocytes, and some mutant cells also express the neuronal marker β-III tubulin (Suppl Fig. 4). Importantly, consistent with results from Nse-BMP4 mice, inflammation markers (IBA1 and F4/80) were up-regulated in the lesions (Fig. 4). We chose to use these markers to explore the potential correlation of demyelination with local residential inflammation markers, since profound lymphocyte infiltration was not consistently found in all of the lesions (data not shown). In addition, astrocytes were abnormally distributed in the spinal cords of chimeric FOP mice, although the number of GFAP+ cells was not significantly altered (Suppl Fig. 5).

MR imaging shows hyperintense lesions in the CNS of FOP patients

We retrospectively examined MRI analyses of the head and neck in four FOP patients who had these studies performed as part of evaluations for intercurrent neurologic symptoms (patients 1 and 2) or as part of their evaluations prior to the definitive diagnosis of FOP (patients 3 and 4).

Two MRIs were preformed on patient 1. The earlier MRI (in 2006) showed isolated lesions with pathologic signals (hyperintense on T2 and FLAIR) in the right and left frontal lobes and right middle cerebellar peduncle (Fig. 5, Suppl Fig. 6; data not shown). The second MRI preformed in 2011 showed that all her original lesions remained present at this time point, but the sizes of all lesions were increased. In addition, numerous new lesions were detected in multiple brain regions bilaterally, especially in the peri-ventricular white matter, subcortical white matter, the pons, and the cerebellum. Many lesions were partially confluent around the posterior horns at the later time point (Fig. 5 and Suppl Fig. 6). These data strongly suggest that demyelination lesions can progress significantly within a 5-year period.

Patient 2 received four MRIs of her brain and seven of her spinal cord in different hospitals over a period of 1.5 years. Only the last two MRI scans at age 17 showed clear hyperintense lesions on T2-weighted imaging in the left frontal periventricular white matter, and spinal cord (T2–T3) (Fig. 6). This patient had refractory propriospinal myoclonus.

Patient 3 and 4 are young children (a 27-month-old male and 33-month-old female, respectively) without obvious neurological symptoms. In one individual (patient 3) hyperintense lesions of the dorsal pons and the dentate nuclei were noted bilaterally on T2-weighted images. In the other individual (patient 4), hyperintense lesions were noted in the dentate nuclei and surrounding the fourth ventricle on T2-weighted and FLAIR images (Fig. 6).

Discussion

We consistently found areas of demyelination in the CNS in two mouse models of dysregulated BMP signaling, including knock-in mice with the recurrent FOP mutation ACVR1 R206H and the Nse-NMP4 over-expression model. Nse-BMP4 mice were used to show that demyelinated regions of the CNS correlated with hyperintense areas observed by MRI. Hyperintense white matter lesions in the CNS were detected by MRI in four FOP patients with established ACVR1 mutations, suggesting that demyelination occurs in response to ACVR1 mutation and dysregulated BMP signaling, and this causes the neurologic symptoms experienced by many FOP patients.

Pervious in vitro and in vivo studies have consistently shown that BMPs promote production of astrocytes and inhibit production and maturation of oligodendroglia developmentally [9, 15]. However, the actions of the BMPs are still not well characterized in adults, even though our previous study indicated that BMPs may play key roles in regulating injury response in adults [7]. Studies suggested that the underlying mechanisms of demyelination in adults and neonates are likely different [15, 16]. Accumulating data further suggested that the major difference is whether injury/inflammation is involved, i.e., the demyelination in adults is closely associated with injury/inflammation, while the demyelination in neonates is not.

Since it is hard to categorically distinguish loss of myelination vs. lack of formation, it is currently unclear whether the demyelination observed in neonates was actually dysmyelination, nevertheless, based on previous reports and our observations, the lesions can be categorized as two types (Suppl Fig. 7). The type II lesions (in patients 3 and 4) were

found in young children mainly in pons and cerebellum, and the type I lesions (in patients 1 and 2) were found in older teenagers or young adults mainly in other brain regions.

We reason that the type I lesions found in patients 1 and 2 probably represent adult-onset disease reflecting abnormal injury responses in the context of high BMP signaling. Such lesions could cause lasting, progressive, and potentially severe functional deficits, since a prominent feature of such lesions in the animal models was inflammation. Interestingly, the demyelination lesions in adult Nse-BMP4 mice closely mimic this type of lesion. These unexpected findings in the CNS are highly reminiscent of the focal inflammation observed in skeletal muscle, connective tissues and other affected peripheral tissues in FOP and raise the intriguing possibility that demyelination occurs through a similar pathologic process.

In contrast, type II lesions (in patients 3 and 4) closely resemble a lesion previously reported in a 3-year-old boy with FOP [17]. These lesions may reflect the delayed oligodendroglial lineage commitment in the developing brain caused by a high level of BMP signaling, thus, likely represent dysmyelination [15]. The similar phenotypes in neonatal Nse-BMP4 mice that over-express BMP4 mimic this type of lesion and support this possibility.

Interestingly, type I (adult-onset) lesions show histological and functional similarity with other demyelinating disorders, such as multiple sclerosis. In fact, the symptoms of patient 1 led her physicians to perform stringent diagnostic tests to exclude a suspected diagnosis of multiple sclerosis. However, it is worth mentioning that even though our data strongly suggest the involvement of inflammation in the adult-onset demyelination lesions, our current data seem to suggest the involvement of the innate immune system, not the acquired immune system, which is opposite to what we have seen in multiple sclerosis. Future studies are needed to further clarify whether or not the acquired immune system is also involved in our model.

Individuals with FOP report a bewildering array of atypical neurological symptoms including motor and sensory complaints [3], but the etiology of these symptoms has been unclear. A recent study demonstrated a critical role for neuroinflammatory factors in HO initiation and progression in soft tissues in FOP both in animal models and patients [7]. These findings in conjunction with the present study suggest that the nervous system may be more intimately involved in FOP than previously expected.

Results of this study strongly suggest that demyelinated lesions may be a common finding in the FOP and a thorough evaluation of both neurologically symptomatic and asymptomatic individuals with FOP appears warranted. Further detailed studies are needed to determine the precise pathophysiology of atypical neurological problems of FOP patients. Specifically, it would be helpful to determine the frequency of occurrence of progressive demyelination in FOP patients, and whether neurological problems are secondary to progressive and disabling heterotopic ossification or are directly due to focal inflammatory demyelinating lesions of the CNS.

The findings in this study correlate well with the many reported cases of neurological symptoms in the FOP patient population ([8], companion article submitted with this manuscript). It is also noteworthy that individuals with ultra-rare phenotypic and genotypic variants of FOP have been identified with structural malformations of the cerebellum and corpus callosum of unknown clinical significance [3]. A large proportion of these individuals also have neurologic impairment, but comprehensive MRI studies have, to our knowledge, not been performed in this patient population.

Our study of both animal models and FOP patients indicates that (1) demyelinated lesions in the CNS of FOP patients are likely underestimated, (2) two types of lesions can be

identified, and (3) not all demyelination leads to detectable neurological symptoms. Although these findings provide a likely pathological basis for the neurological symptoms reported by patients with FOP, currently, we cannot exclude the possibility that atypical neurologic symptoms in FOP patients are not directly related to the observed demyelinated lesions. Nevertheless, the present study provides a potential common mechanism through which tissues such as skeletal muscle, soft connective tissue, brain and spinal cord may be susceptible to inflammatory changes in the setting of dysregulated BMP signaling.

The current study not only provides insights into this underappreciated aspect of FOP but also raises many intriguing questions: since mutated ACVR1 is widely expressed, why is only focal demyelination seen in FOP and in animal models of the condition rather than global demyelination or dysmyelination? Is inflammation a necessary prelude to demyelination in the setting of dysregulated BMP signaling, and does low-grade, clinically unapparent inflammation play an inductive role in the pathophysiology of demyelination FOP? Why are certain cell populations in the CNS more sensitive than others to the effects of dysregulated BMP signaling? Might inhibition of the BMP signaling pathway abrogate demyelination lesions in animal models of FOP or MS?

Overall, our study suggests that inflammation and dysregulated BMP signaling is associated with focal CNS demyelination and may represent a common pathological mechanism by which normal tissue repair is disturbed in susceptible organs in patients with FOP. These CNS lesions may be a common underlying reason for the neurologic symptoms frequently observed in FOP.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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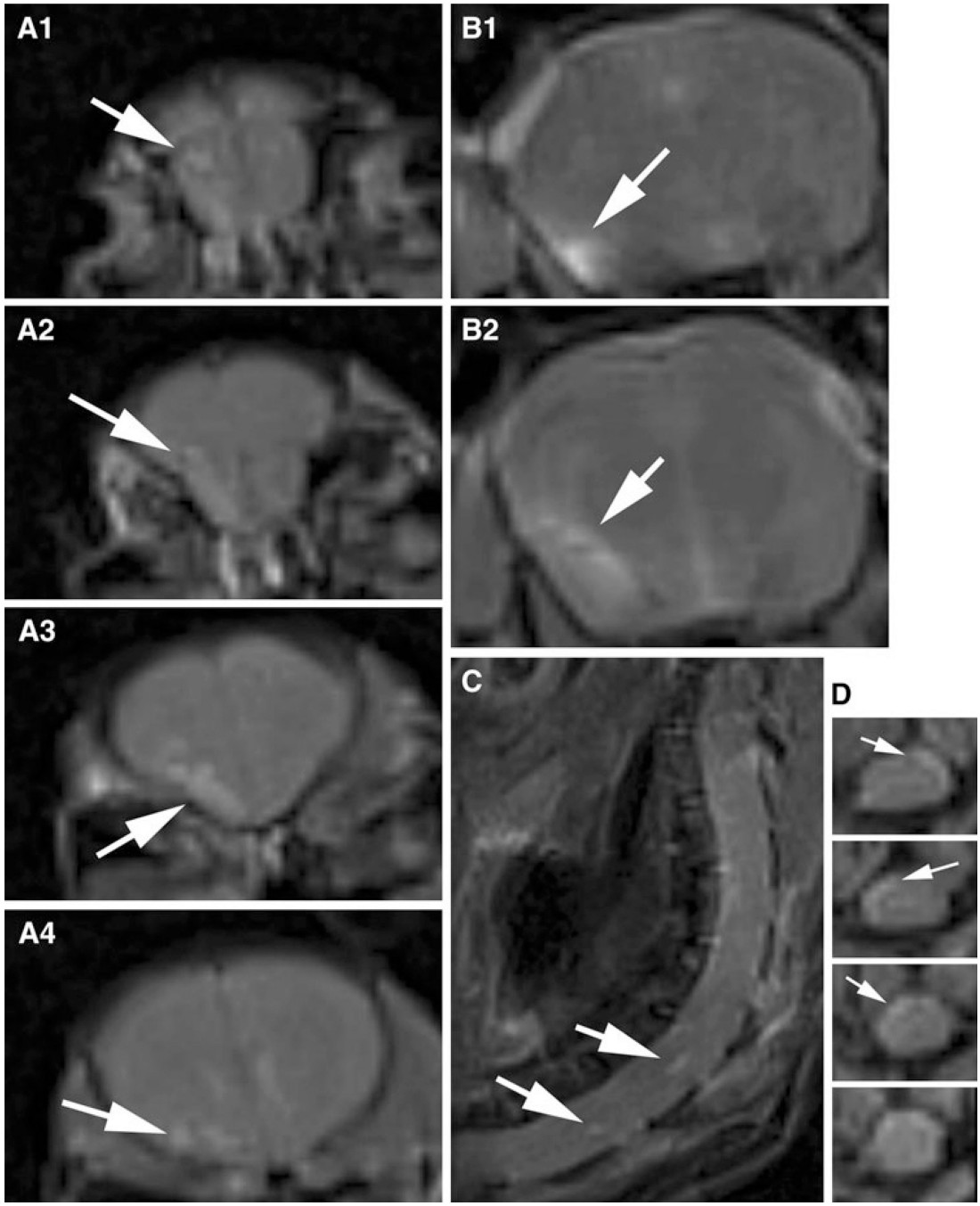

Fig. 1.

T2-weighted MRI detects hyperintense lesions in the CNS of Nse-BMP4 mice. **a** T2-weighted imaging (*continuous sections*) revealed large irregular hyperintense lesions in left anterior olfactory nucleus (A1), piriform area (A2 and A3), and substantia innominata and nucleus accumbens (A4). **b** Images show a large hyperintense area in the left piriform and amygdalar nucleus (B1) and a relatively small area of hyperintensity in the adjacent region (B2). **c** A sagittal section shows multiple hyperintense lesions in the lumbar spinal cord. **d** Serial cross sections of thoracic spinal cord show asymmetrical signal intensity in the white

matter of the top 3 sections but not the bottom panel. *White arrows* in all panels point to the hyperintense regions

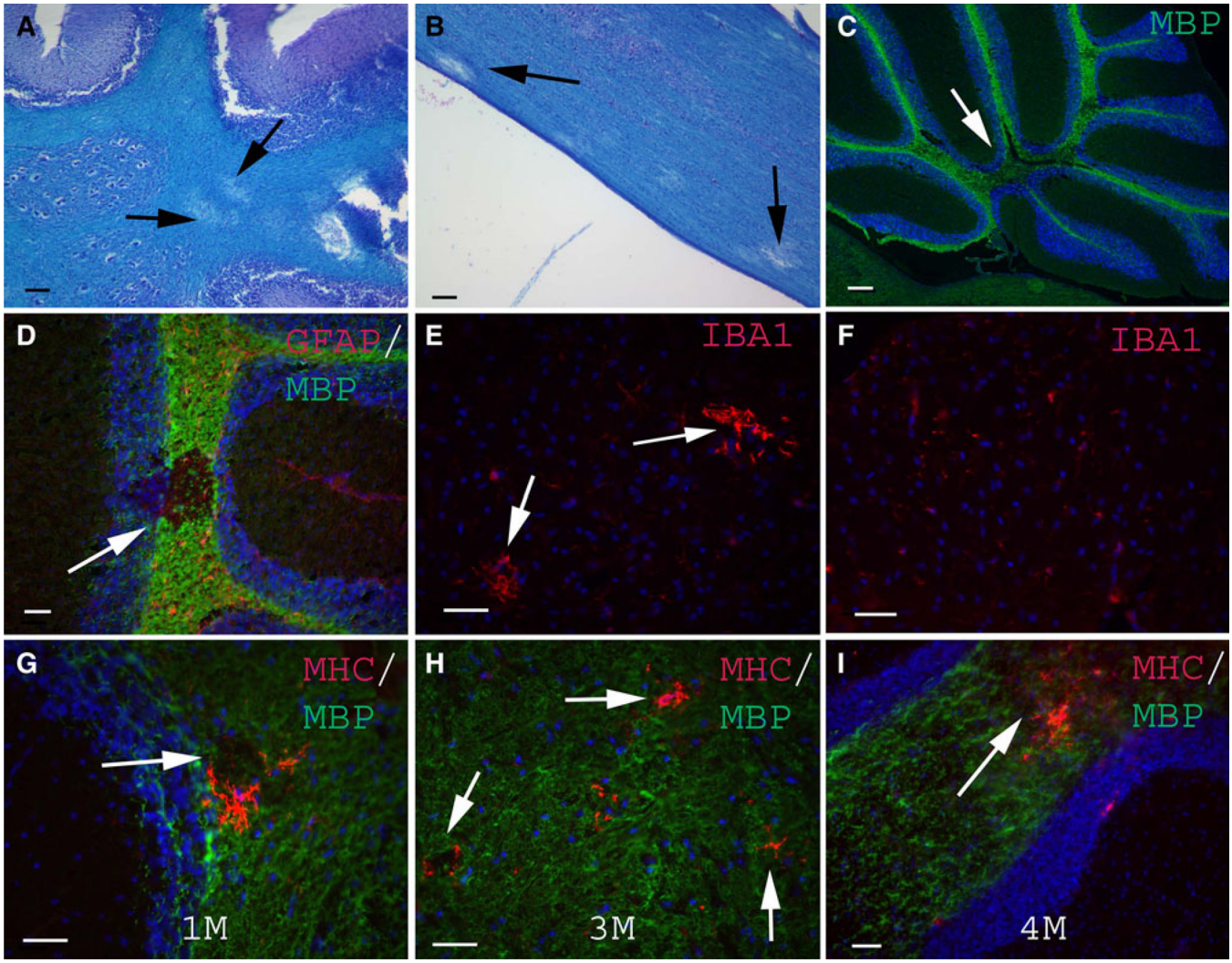

Fig. 2.

Histologic evaluation identifies multiple demyelinated areas which co-localize with activated microglia in the CNS of Nse-BMP4 mice, **a, b** Multiple areas of demyelination in the cerebellum (**a**, *sagittal section*) and spinal cord (**b**, *longitudinal section*) detected by luxol fast blue staining in adult Nse-BMP4 mice. *Black arrows* in (**a** and **b**) point to areas of demyelination. **c** Typical low power image of myelin basic protein (MBP) staining (*green*) confirmed the multiple areas of demyelination in the cerebellum of adult Nse-BMP4 mice. *White arrow* points to areas of demyelination. **d** MBP (*green*)/GFAP (*red*) double staining showed that GFAP, an astrocyte marker, is detected inside and in adjacent areas to the lesion (absence of MBP). *White arrow* points to area of demyelination. (**e, f**) IBA1 (*red*) staining shows local accumulation of $IBA1^+$ activated microglia in young (1 month old) transgenic mice (**e**), but not in WT (**f**) controls. Note that the accumulated activated microglia have short and wide processes in (**e**), compared to control mice (**f**). *White arrows* in e point to areas of lesions, where clusters of multiple activated microglia are located, **g–i** MBP (*Green*)*/MHC* (activated microglial marker, *red*) double staining shows that MBP and MHC are almost always mutually exclusive, and the lesion sizes and numbers increase with age. **g** MHC (*green*)*/MBP* (*red*) double staining showed that MHC^+ cells with multiple processes surrounding an area of demyelination in cerebellum of a 1-month-old (1 M) Nse-BMP4 mouse. **h** MHC (*green*)/MBP (*red*) double staining showed that at least three small lesion

sites with MHC^+ cells are present in a 3-month old (3 M) Nse-BMP4 mouse, **i** MHC (*green*)/MBP (*red*) double staining showed that a larger lesion with profound accumulation of MHC^+ cells and severe demyelination in a 4-month old (4 M) Nse-BMP4 mouse. *White arrows* in **g–i** point to areas of lesions, where clusters of multiple activated microglia are located. DAPI counterstaining (*blue*) was performed in **c–i**, *Bar* = 200 μm in **c**, *bar* = 40 μm in all other panels

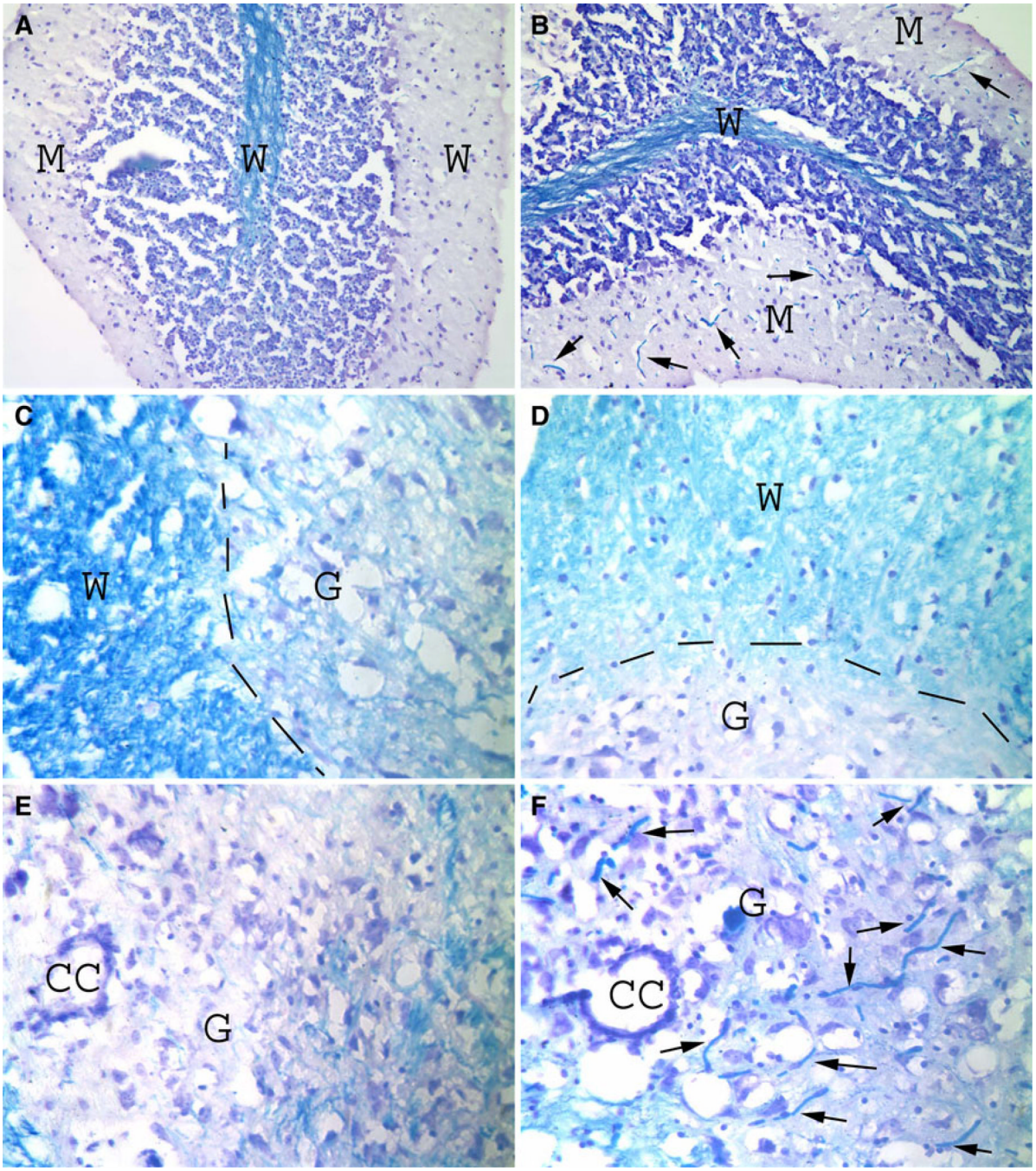

Fig. 3.

Focal demyelination is consistently found in the cerebellum and spinal cord of FOP knock-in (ACVR1/ALK2 (R206H)) chimeric mice. Luxol fast blue staining of tissues from ACVR1 R206H mice (**a, c, d**, and e) and control tissues (**b** and **f**) identified demyelination only in chimeric mice (**a, d** and **e**). **a, b** Luxol fast blue staining identified demyelination in white matter and molecular layer of cerebellum. *Black arrows* in (**b**) point to myelinated fibers in the molecular layer in control tissue; these myelinated fibers are absent in ACVR1 R206H mice (**a**). Note also that the white matter is under-myelinated in chimeric mice. **c–e** Demyelination in the spinal cord of an ACVR1 R206H mouse. **c** Typical image of normal

Luxol fast blue staining of white matter in a region of spinal cord from an ACVR1 R206H mouse. **d** Typical image of local demyelination of white matter in spinal cord of an ACVR1 R206H mouse. *Broken lines* in **c** and **d** indicate the border between white matter and gray matter. **e** Typical image of local demyelination of gray matter in the spinal cord of an ACVR1 R206H mouse. **f** Typical image of normal Luxol fast blue staining of gray matter in the spinal cord of control mouse. *Black arrows* in **f** point to myelinated fibers in gray matter; these myelinated fibers are absent in chimeric mice (**e**). *CC* central canal, *M* molecular layer, *W* white matter, *G* gray matter

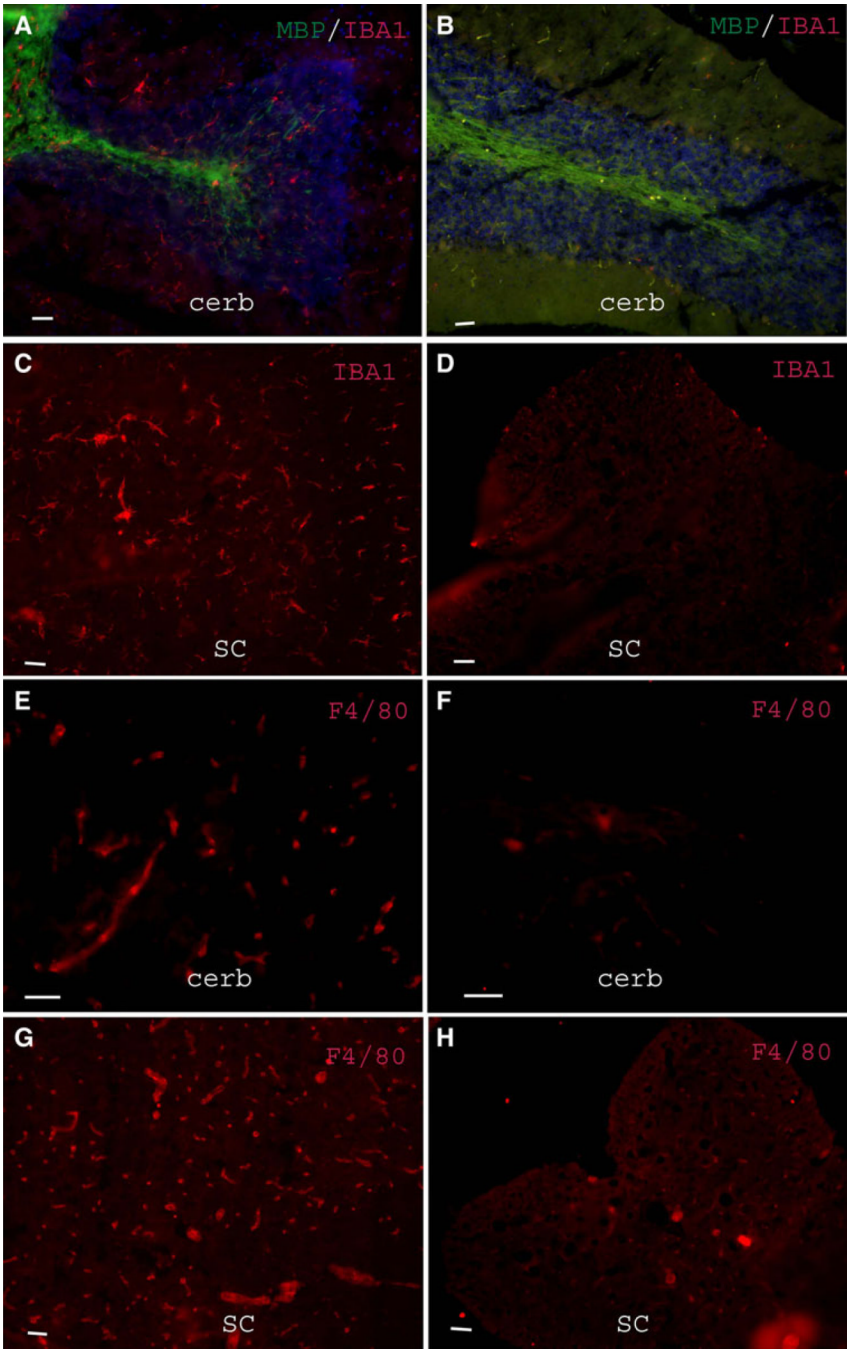

Fig. 4.

Inflammation markers (IBA1 and F4/80) are up-regulated in areas of demyelination in chimeric ACVR1 R206H FOP knock-in mice. **a–d** In cerebellum (**a** and **b**) and spinal cord (**c** and **d**), IBA1 is up-regulated in ACVR1 R206H mice (**a** and **c**) compared to controls (**b** and **d**). **e–h** In spinal cord (**g** and **h**) and cerebellum (**e** and **f**), F4/80 is up-regulated in ACVR1 R206H mice (**e** and **g**) compared to controls (**f** and **h**). In both spinal cord and cerebellum, F4/80 staining is closely associated with small blood vessels. *Bar* = 40 μm in all panels

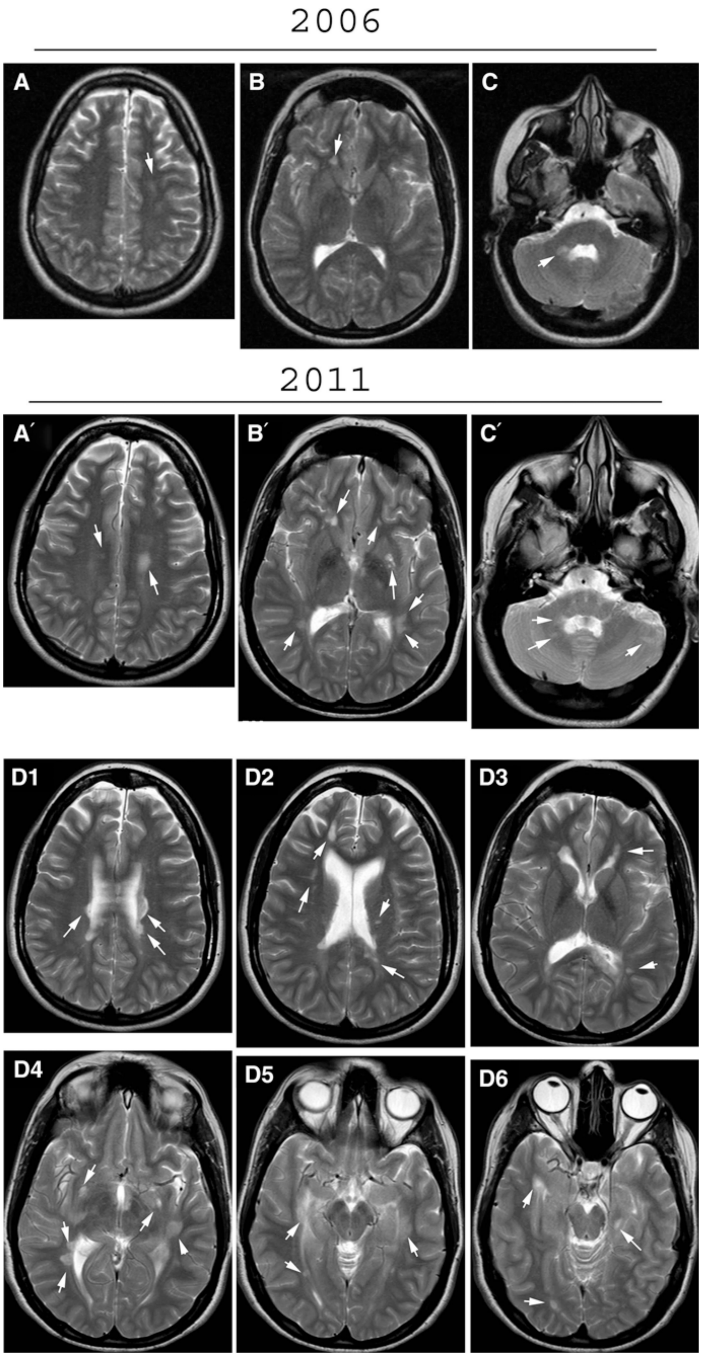

Fig. 5.

MRI detects progression of hyperintense lesions in the CNS of a FOP patient. **a–c** T2-weighted brain images of patient 1 in 2006 showed isolated hyperintense lesions in the *left* (**a**), and *right* (**b**) frontal lobe, and cerebellar peduncle (**c**) (*axial views*). (**a′–c′**) showed the lesions in the similar locations 5 years later (in 2011), however the sizes and numbers of the detected lesions increased. At the later time point, some of the lesions were merging with each other. **d1–d6** show additional hyperintense lesions in additional brain regions (at 2011, *axial views*). *White arrows* in all panels indicate hyperintense regions

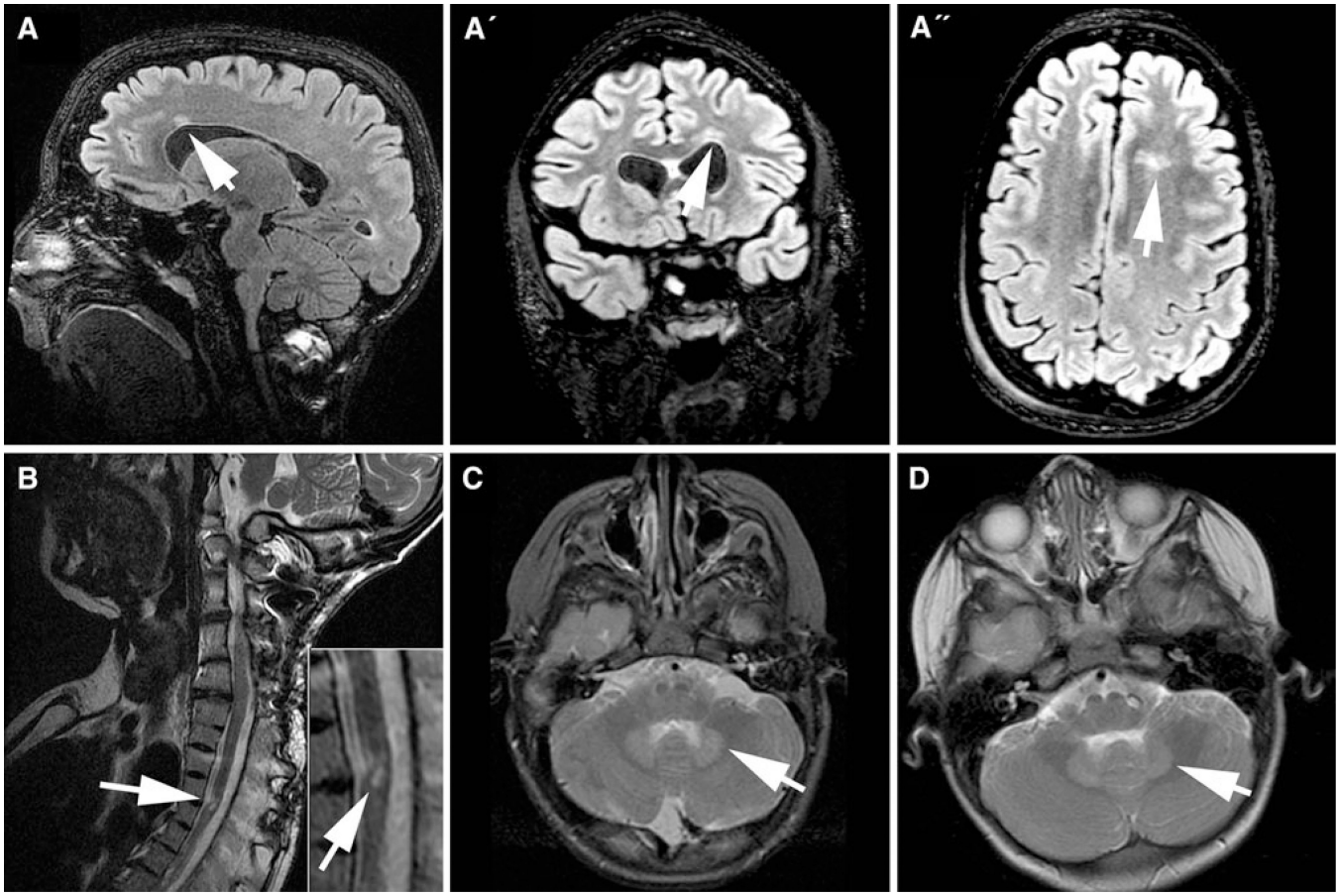

Fig. 6.

MRI detects hyperintense lesions in the CNS of FOP patients. (**a–a″**) T2-weighted brain images of patient 2. An extensive hyperintense lesion in the left frontal periventricular white matter is shown from *sagittal* (**a**), *coronal* (**a′**), and *axial* (**a″**) views. **b** T2-weighted image of the spinal cord of patient 2. Hyperintense lesions are shown from a *sagittal view*. *Insert* in **b** shows a higher-power image surrounding the lesion. **c** T2-weighted image shows hyperintensity of the dorsal pons and the dentate nuclei bilaterally in patient 3 from an *axial view*. **d** T2-weighted image shows hyperintense lesions in the dentate nuclei and surrounding the fourth ventricle in patient 4 from an *axial view*. *White arrows* in all panels indicate hyperintense regions