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Experimental research

Screening for *GFAP* rearrangements in a cohort of Alexander disease and undetermined leukoencephalopathy patients

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ABSTRACT

Alexander disease (AxD), a fatal degenerative leukoencephalopathy, is caused by *de novo* heterozygous missense mutations in the Glial Fibrillary Acidic Protein (GFAP) gene. The pathological hallmark of the disease is the presence of Rosenthal fibers, cytoplasmic inclusions in astrocytes, composed mainly of GFAP, αB-crystallin and HSP27.

To date, several patients with a typical presentation of the disease or displaying characteristic Rosenthal fibers in brain material have been reported with no GFAP mutation. Recently, several studies have demonstrated a correlation between Rosenthal fiber formation and wild-type GFAP overexpression, despite the absence of mutations.

We tested the hypothesis that a GFAP gene rearrangement could modulate AxD severity or promote GFAP overexpression and aggregation, resulting in leukoencephalopathy. A QMPSF assay was validated for 11 exonic fragments: 3 in control genes (*CFTR*, *DSCR1*, *F9*) and 8 corresponding to *GFAP* exons. A total of 97 patients suspected of AxD were analyzed: 28 with a GFAP mutation; 69 with clinical and magnetic resonance imaging criteria compatible with the disease. Neither duplications nor deletions of *GFAP* were found, suggesting that *GFAP* coding-region rearrangements may not be involved in AxD or Alexander-related leukoencephalopathies.

In addition, 80 patients with undetermined leukodystrophies, and negative for *PLP1*, *GJA12*, *Sox10 and MCT8* mutations and *PLP1* and *Lamin B1* rearrangements, were tested. These patients were also negative for *GFAP* rearrangements.

Other hypotheses should be investigated for a molecular diagnosis in patients with undetermined leukoencephalopathy: mutations in *GFAP* isoforms, splicing sites or regulatory regions, or defaults in genes encoding molecular partners of GFAP.

1. Introduction

Alexander disease (AxD, OMIM 203450) is a rare, progressive and usually fatal neurological disorder affecting the cerebral white matter (WM). Three clinical subtypes based on age of onset have been defined. The infantile form, the most common and clinically homogenous subtype, has an onset before 2 years, and a rapid progression; this form is usually fatal within the first decade. The juvenile form is less common and the clinical features are more variable; onset occurs between 2 and 12 years of age and progression of the disease is variable, with patients surviving several decades. Patients with the adult form (onset after 12 years) show a wide clinical spectrum, often with very slow neurological deterioration or in some cases a complete absence of symptoms or WM abnormalities in magnetic resonance imaging (MRI).

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De novo heterozygous missense mutations have been identified in the Glial Fibrillary Acidic Protein (GFAP) gene in neuropathologically-diagnosed (Brenner et al., 2001) and MRI-diagnosed (Rodriguez et al., 2001) AxD patients. Indeed, the majority (>90%) of infantile AxD patients show a non-conservative mutation in the coding region of *GFAP* (Brenner et al., 2001; Gorospe et al., 2002; Li et al., 2005; Rodriguez et al., 2001). However, juvenile and to a greater extent adult forms present a lower mutation detection frequency, probably related to clinical heterogeneity (van der Knaap et al., 2006). Moreover, the absence of GFAP mutations has been reported in neuropathologically-proven AxD patients with different onset forms (Brenner et al., 2001; Huttner et al., 2007; Li et al., 2005; Rodriguez et al., 2001), suggesting that sequencing of the *GFAP* coding region may be inconclusive for a final diagnosis. A phenotype–genotype correlation was attempted (Li et al., 2005; Rodriguez et al., 2001); however, in several instances, the same mutation produces distinctly different outcomes (Gorospe et al., 2002; Kyllerman et al., 2005; Li et al., 2005; Rodriguez et al., 2001) and intrafamilial clinical heterogeneity exists in the dominant forms (Messing et al., 2012; Stumpf et al., 2003; Thyagarajan et al., 2004; van der Knaap et al., 2006). This suggests that other genetic or environmental factors could influence the phenotype.

In AxD, mutated GFAP aggregates in Rosenthal fibers (RF), intracellular inclusions in pathological astrocytes (Herndon et al., 1970). Recently published murine models of AxD have shown a correlation between phenotypic severity and overexpression of total GFAP, suggesting that RF formation also depends on astrocytic GFAP levels (Hagemann et al., 2006; Tanaka et al., 2007).

This study was based on the hypothesis that GFAP overexpression could promote aggregation and subsequent RF formation independently of the presence of mutations, or contribute to modulate the phenotypic severity of AxD. Therefore *GFAP* copy number changes were investigated by a QMPSF (Quantitative Multiplex PCR of Short Fragments) assay in a large cohort of patients with AxD or with other types of undetermined leukoencephalopathies.

2. Materials and methods

2.1. Patients

A total of 177 unrelated patients and 53 control individuals were investigated. For each patient, a blood sample was collected after obtaining written informed consent. All patients were examined by a clinical geneticist and a neurologist. Ninety-seven patients were genetically confirmed or clinically suspected of AxD: 28 had an identified GFAP mutation; 69 had clinical and MRI criteria compatible with infantile, juvenile or adult AxD (Pareyson et al., 2008). A group of 80 patients affected with undetermined leukoencephalopathy was also investigated. Forty-seven had a diffuse “hypomyelination” pattern in the WM on T1 and T2 brain MRI (normal T1, hyper T2): 30 had a Pelizaeus-Merzbacher Like Disease (PMLD) phenotype and 17 a form of spastic paraplegia (Boespflug-Tanguy et al., 2008). Mutations in the coding sequence for *PLP1*, *Sox10*, *GJA12 and MCT8* genes and *PLP1* genomic rearrangements were ruled out. Thirty-three had a non-diffuse hypomyelinating or demyelinating pattern without specific features; in this last group, *Lamin B1* duplication was also ruled out. A series of 50 normal controls (25 males and 25 females) and three patients with trisomy 21 was used to determine the conditions of analysis and as references for the interpretation of patient results.

2.2. QMPSF analysis of the GFAP gene

Eleven short exonic fragments (85–251 bp) from the nine *GFAP* exons (chromosome 17) and 3 internal control genes, *CFTR* (chromosome 7), *DSCR1* (chromosome 21) and *F9* (chromosome X), were simultaneously amplified by PCR using dye-labeled primers (Table 1).

Using Genescan software (Applied Biosystems), electropherograms from patients were superposed to those generated from a normal control individual by adjusting *CFTR* peaks to the same level. This adjustment was confirmed by the superposed peaks from both *DSCR1* and *F9* amplifications. Thereafter, the levels of the eight *GFAP* peaks were compared. The first graphical interpretation was then confirmed by an arithmetic validation of the results using peak areas (A) and the following formula: (A $GFAP_{patient}$/A $CFTR_{patient}$) X (A $CFTR_{control}$/A $GFAP_{control}$). The expected ratios for one, two and three copies of a gene are 0.5, 1 and 1.5 respectively.

3. Results

3.1. Design and validation of QMPSF for GFAP rearrangement screening

In order to detect *GFAP* duplications/deletions, a molecular assay based on the QMPSF method was developed. The assay was first validated using 53 control DNAs correctly amplified and superposable for *CFTR* and *GFAP* amplicons. Then, DNA samples from male/female and trisomy 21 patients were used to validate the quantification of *F9* and *DSCR1* respectively. Samples were categorized as harboring a normal copy number, duplication or deletion, considering a threshold of 2 SD. Mean *F9/CFTR* ratios for female and male patients confirmed the detection of the expected 2 copies and 1 copy of *F9*, respectively (Table 2, Fig. 1A). Similarly mean *DSCR1/CFTR* ratios for normal controls and trisomy 21 patients distinguished between individuals carrying 2 and 3 *DSCR1* copies (Table 2, Fig. 1B).

3.2. QMPSF analysis of patients affected by AxD or undetermined leukodystrophies

Using this protocol, a cohort of 177 leukoencephalopathic patients was analyzed (Table 2). The quantitative assay was first validated graphically and then by comparing control *DSCR1/CFTR* and *F9/CFTR* values: ratios from patients were very similar to those obtained from the healthy control population, validating the normalization of the results. The eight *GFAP/CFTR* ratios were then interpreted and no abnormal *GFAP* quantification was observed.

4. Discussion

GFAP copy number changes were considered in AxD as several recent studies have suggested that in addition to point mutations, *GFAP* overexpression could promote or worsen disease features. Transgenic mice carrying additional copies of the human wild-type GFAP gene show increased levels of total GFAP and small HSP, and display inclusion bodies identical to RF in hypertrophic astrocytes (Messing et al., 1998). Cultured astrocytes derived from these animals show increased levels of both human and murine GFAP, as well as αB-crystallin, HSP27 and vimentin, replicating some features of human AxD (Eng et al., 1998). These studies confirm that the formation of RF could be due to an excess of GFAP despite the absence of mutation. The intensity of these features was correlated with the expression level of total GFAP (mRNA and protein) and increased with time, confirming a GFAP dose effect. In the same way, the formation of cytoplasmic inclusions, disruption of the