XPR1 mutations are a rare cause of primary familial brain calcification

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ORIGINAL COMMUNICATION

XPR1 mutations are a rare cause of primary familial brain calcification

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Abstract Mutations in *XPR1*, a gene encoding an inorganic phosphate exporter, have recently been identified in patients with primary familial brain calcification (PFBC). Using Sanger sequencing, we screened *XPR1* in 18 unrelated patients with PFBC and no *SLC20A2*, *PDGFB*, or *PDGFRB* mutation. *XPR1* variants were tested in an in vitro physiological complementation assay and patient blood cells were assessed ex vivo for phosphate export. We identified a novel c.260T > C, p.(Leu87Pro) *XPR1* variant in a 41-year-old man complaining of micrographia and dysarthria and demonstrating mild parkinsonism, cerebellar ataxia and executive dysfunction. Brain ^{123}I-Ioflupane scintigraphy showed marked dopaminergic neuron loss. Peripheral blood cells from the patient exhibited decreased phosphate export. XPR1 in which we introduced the mutation was not detectable at the cell surface and did not lead to phosphate export. These results confirm that loss of XPR1-mediated phosphate export function causes PFBC, occurring in less than 8 % of cases negative for the other genes, and may be responsible for parkinsonism.

Keywords Primary familial brain calcification · Idiopathic basal ganglia calcification · Phosphate export · XPR1 · Fahr disease

M. Anheim, U. López-Sánchez contributed equally to the work.

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Introduction

Primary familial brain calcification (PFBC) is a rare condition defined by calcification affecting at least both lenticular nuclei more severely than during uncomplicated aging [1], with no cause identified following accurate etiological assessment [2]. Calcification may also affect caudate nuclei, thalami, dentate nuclei, cerebellum, supratentorial white matter and cortical sulci. Clinical signs associated with PFBC are numerous and optional. In a French-Brazilian case series, 58 % patients exhibited at least one neuropsychiatric sign [3], of which the most frequent were psychiatric signs (76 % of symptomatic patients), movement disorders (MD) (61 %) and cognitive impairment (58 %), followed by speech, gait, cerebellar syndrome, pyramidal signs, and seizures. Akinetic-hypertonic syndrome, with or without tremor, was the most common MD. Inheritance of PFBC is autosomal dominant. Four causative genes have been identified: *SLC20A2* [4], *PDGFRB* [5], *PDGFB* [6] and, most recently, *XPR1* [7]. *SLC20A2* encodes PiT2, an inorganic phosphate importer and we have recently shown that XPR1 [8] is the only known human phosphate exporter [9, 10]. *SLC20A2* loss of function variants have been identified in a majority of cases [11], and all 4 *XPR1* missense variants identified in 5 unrelated families were located in the SPX putative regulatory domain of XPR1, causing significantly altered XPR1-mediated phosphate export [7]. In this study, we aimed at extending these recent clinical, molecular and functional data by screening new patients and functional characterization of the variants.

Methods

Patients

Patients were included following previously described criteria [1]. Briefly, probands exhibited (1) at least bilateral lenticular calcification, (2) a total calcification score (TCS) above the age-specific threshold using our visual rating scale [1], and (3) a negative etiological assessment.

The objective of the etiological assessment was to search for other causes of brain calcification. For review see Ref. [12]. The assessment was based on clinical examination, brain MRI when available, and the exclusion of causes of non-syndromic brain calcification by the following minimal biological assessment: Phosphate and calcium levels in blood and urine, parathormone and 25-OH-vitamine D levels, and blood lactates and pyruvates. Depending on the clinical and MRI presentation, other assessments may be required to look for other causes; however, this was not the case for any of the patients included in this study.

The patients gave written consent and local ethics committee approved the study. All patients were negatively screened for *SLC20A2*, *PDGFB*, and *PDGFRB* variants by Sanger sequencing and Quantitative Multiplex PCR of Short Fluorescent fragments (QMPSF) (*SLC20A2*, *PDGFB*). *XPR1* sequencing was performed by Sanger sequencing as previously described [7]. The reference transcript NM_004736.3 was used for variant nomenclature.

Functional analyses

Cells

HEK293T cells were cultured in DMEM supplemented with 10 % FBS (PAN-Biotech) and non-essential amino acids. Cells were incubated at 37 °C in a 5 % CO_2 and humid atmosphere. For phosphate-free experiments, cells were cultured in phosphate-free DMEM supplemented with 10 % dialyzed FBS [7, 10].

Plasmids and siRNAs

The c.260T > C, p.(Leu87Pro) mutation, thereafter referred to as L87P, was generated by recombinant PCR site-direct mutagenesis. The HA-tagged human wild type (WT) and L87P XPR1 variant were introduced in the pCHIX [13] and pLXSN-retroviral vectors [14]. Small interfering RNAs (siRNA, Integrated DNA Technologies) targeting the human *XPR1* 3′UTR and the *firefly luciferase* gene were described previously [10]). For the XPR1 complementation assay, HEK293T cells grown on poly-D-lysine-coated 6-well plates were transfected with 50 pmol siRNA per well using the calcium phosphate method along with either empty or *XPR1* expression vectors.

Phosphate fluxes

Phosphate uptake and efflux assays were performed in HEK293T cells as previously described [10]. Percentage of phosphate uptake was calculated as the ratio of cellular [^{33}P]phosphate to total [^{33}P]phosphate supplemented. Percentage of phosphate efflux was calculated as the ratio of released [^{33}P]phosphate to total cellular [^{33}P]phosphate. Although synchronization was arduous, we were able to perform phosphate efflux assays in peripheral blood mononuclear cells (PBMC) from some patients as previously described [7].

Immunoblotting

Cell extracts were separated by 12 % SDS-PAGE under reducing conditions and transferred to PVDF membranes. Protein expression and transfer were monitored with anti-HA tag antibodies (3F10, Roche; 1:5000) or anti-β-actin (A5441, Sigma-Aldrich; 1:5000), followed by horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rat antibodies (Southern Biotech; 1:5000), and visualization with Pierce ECL western blotting substrate (Thermo Scientific).

Flow cytometry

Cell surface expression of phosphate transporters was monitored with soluble ligands derived from the receptor-binding domain (RBD) of different retroviral envelope glycoproteins. Production of and binding with RBD from X-MLV (XRBD), and koala retrovirus (KoRBD), or with a soluble ligand derived from the surface unit (SU) of amphotropic-MLV (ASU), used to detect XPR1, PiT1, and PiT2, respectively were performed as previously described [10, 15]. Briefly, 5×10^5 cells were resuspended in PBA (PBS with 2 % FBS) containing the adequate RBD and incubated for 30 min at 37 °C, followed by two washes with PBA and incubation with an Alexa Fluor 488-conjugated anti-mouse IgG1 antibody (Life Technologies; 1:5000) for 20 min at 4 °C. Cells were promptly analyzed on FACSCalibur instrument (Becton–Dickinson) and data were analyzed with the FlowJo package.

Statistical analysis

Student's *t* test was applied using GraphPad Prism 6 software; *P* values were as follows: $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$.

Results

Eighteen patients with PFBC were included: 6 probands with a pedigree suggestive of an autosomal dominant inheritance and 12 sporadic cases. In one patient, we identified a novel *XPR1* missense variant, c.260T > C, p.(Leu87Pro), thereafter referred as L87P, not reported in exome databases, including the ExAC 60,706 exomes (http://exac.broadinstitute.org/). The change, located on a highly conserved residue, is predicted to affect protein function by at least three programs (Mutation Taster, SIFT, Polyphen2). DNA of relatives was not available. This variant was absent from 126 French control exomes (not shown).

The patient carrying the L87P variant is a right-handed 41-year-old man, with neither previous medical history nor treatment. He was referred to our tertiary MD center because he was complaining of insidious, slowly progressive writing difficulties, slight dysarthria and concentration deficiency for 10 years. His father experienced parkinsonism as well as dysarthria and concentration deficiency, associated with calcification on CT scan before dying from cancer at age 64. The patient's paternal aunt was diagnosed with Parkinson's disease but subsequently lost to follow-up (Fig. 1). Examination of the proband revealed mild dysarthria suggestive of both parkinsonism and cerebellar dysfunction, micrography (which was not the case several years before), mild hypermetria during the finger chase test and mildly abnormal fast alternative hand movement. There was mild rigidity of right wrist (1/4) but no tremor, dystonia, chorea, or imbalance. SARA score was 3/40 [16] and UPDRS motor score was 3/108. Anxiety was noticed and neuropsychological tests showed mild executive dysfunction with mild disturbances of initiation, planification flexibility and encoding episodic memory. Brain CT scan revealed bilateral, symmetrical calcifications of lenticular and caudate nuclei, thalami, cerebellar hemispheres and vermis as well as mild sub-cortical and cortical calcifications (TCS = 46/80) (Fig. 1) [1]. Brain scintigraphy with ^{123}I-Ioflupane showed marked, bilateral and symmetrical dopaminergic neuron loss (Fig. 2) with a mean specific striatum uptake of 2.3 (right) and 2.2 (left) (normal values >3.5) whereas the putamen/caudate uptake ratio was 0.72 (right) and 0.71 (left) (normal values >0.85). A new examination at age 42 revealed no particular change although the patient felt better following speech and writing therapy.

In a complementation assay, we found that WT XPR1 fully complemented phosphate export inhibited by a siRNA directed against *XPR1* 3′UTR and that the L87P-

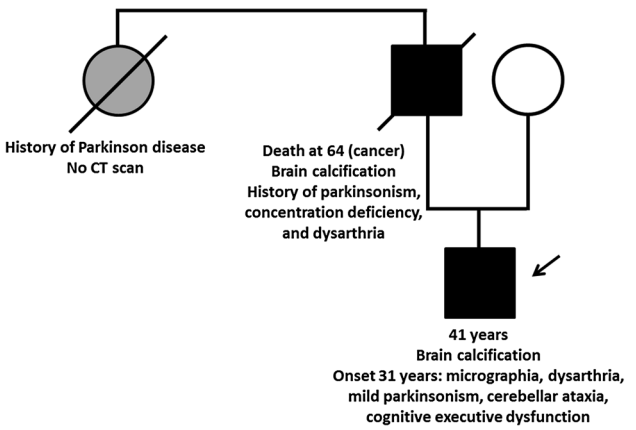

Fig. 1 Reduced pedigree of the patient carrying the novel p.(Leu87-Pro) *XPR1* variant. *Filled symbols* represent patients carrying brain calcification and the *grey circle* represents the paternal aunt with a history of parkinsonism but no CT scan

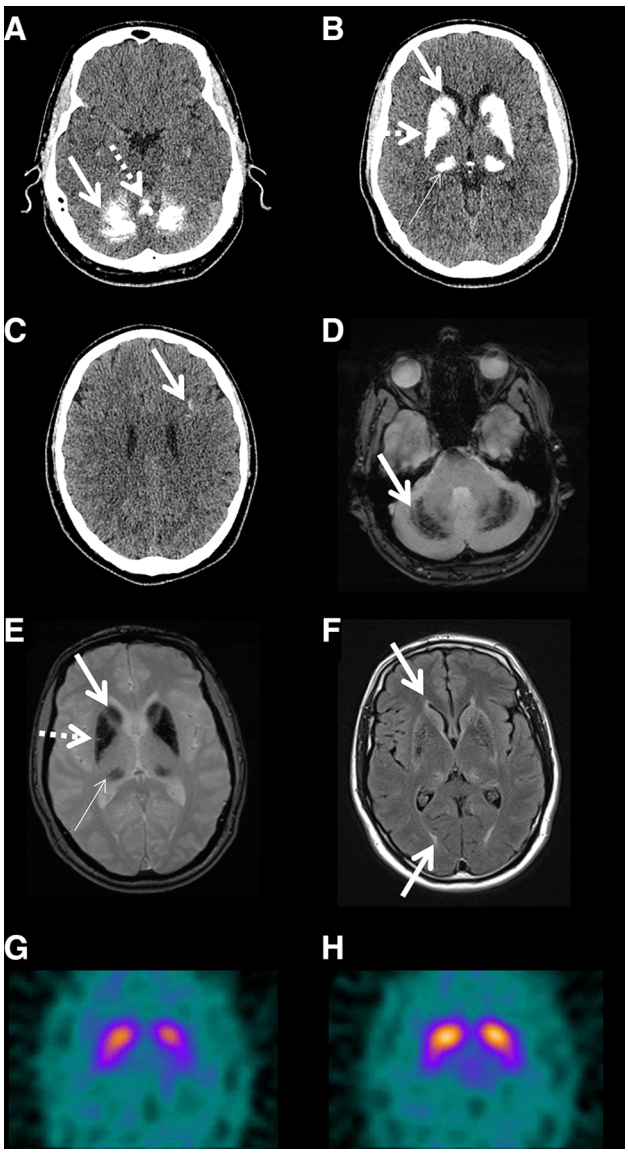

Fig. 2 Brain imaging of the patient carrying the novel p.(Leu87Pro) *XPR1* variant. Calcifications appear hyperdense on CT scan (**a**, **b**, **c**) and hypointense on T2*-weighted MRI (**d**, **e**). They affect both cerebellar hemispheres (**a**, **d**, *full arrows*), the vermis (**a**, *dotted arrow*), the caudate nuclei (**b**, **e**, *full arrows*), the lenticular nuclei (**b**, **e**, *dotted arrows*), the thalami (*thin arrows*), cerebral white matter (not shown) and the cortex in the depth of one sulcus (**c**). On FLAIR-weighted images, white matter hyperintensities are observed in periventricular regions and posterior cerebral white matter (**f**). 123Ioflupane scintigraphy axial slides (**g**, **h**): marked, bilateral loss of nigro-striatal dopaminergic neurons with left predominance

mutated XPR1 did not (Fig. 3a), despite efficient expression, at the expected size of 82Kda albeit at a slightly lower level than WT XPR1 (Fig. 3b). However, the mutant was not detected at the cell surface, as monitored by flow cytometry with XRBD, the XPR1 ligand (Fig. 3c), while phosphate uptake and cell surface expression of the PiT1 and PiT2 phosphate importers remained unchanged (Supplementary Figure). Decreased phosphate export was also observed in PBMC obtained from the L87P PFBC patient (Fig. 3d).

Discussion

This is the first report of an *XPR1* pathogenic variant in a PFBC patient since our primary report [7], confirming that *XPR1* is involved in the genetic determinism of PFBC and may be responsible for parkinsonism. *XPR1* mutations are scarce since within the group of 65 patients negatively screened for *SLC20A2*, *PDGFB*, and *PDGFRB*, including the 18 patients reported here and the 47 patients previously reported with the same inclusion criteria [7], 4 different *XPR1* pathogenic variants (i.e. class 5 following ACMG recommendations [17]) in 5 families were found (7.7 %, namely p.Ser136Asn, p.Leu140Pro, p.Leu145Pro, and p.Leu218Ser). Two other rare missense variants (p.Lys53Arg and p.Ile575Val) were detected in the primary report but were predicted benign or tolerated by SIFT and Polyphen2 programs with probably no effect on XPR1 function, as already shown for the p.Lys53Arg variant [7]. These two variants are therefore considered as likely non-pathogenic (class 2 variants).

Although global correlation between calcification severity and symptomatic status has been observed in case series [1, 3, 18], no individual correlation could be inferred, as illustrated by the apparent severity of the calcifications on CT scan in our patient and the absence of marked disability even after 10 years of disease duration. In the same way, parkinsonism was mild whereas the loss of dopaminergic neurons was marked. Of note, although relatives could not be examined and DNA was not available, clinical presentation was quite homogeneous in this family since all experienced parkinsonism and that, according to the patient, his clinical picture was comparable to his father's. Brain calcification involved both lenticular, caudate nuclei, thalami, cerebellar hemispheres as well as vermis and the depth of one cortical sulcus, as observed in several but not all *XPR1* mutation carriers [7]. In PFBC, calcification leads to the diffuse involvement of the brain which causes potential combination of numerous unspecific signs including, beside parkinsonism, cerebellar ataxia, executive dysfunction and anxiety such as in our case, and, in other cases, dystonia, chorea, various psychiatric disturbances, pyramidal signs, seizures or migraine.

Herein, we showed that the L87P variant causes dramatic drop of both XPR1 cell surface expression and phosphate export, similar to what we previously reported in other patients harboring the p.(Leu145Pro) variant [7]. As L87P, all previously reported pathogenic variants (c.407G > A, p.(Ser136Asn); c.420T > C, p.(Leu140Pro);

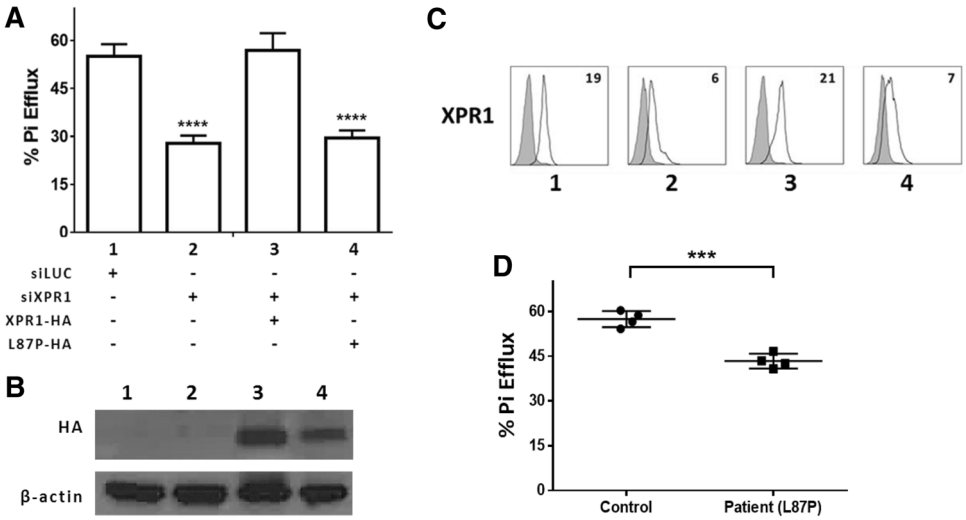

Fig. 3 Effect of the L87P mutation on XPR1 expression and phosphate efflux. **a** Efflux of inorganic ^{33}P (Pi) assay performed in HEK293T cells transfected either with siRNA directed against luciferase (siLUC), or anti *XPR1* siRNA (siXPR1), or siXPR1 and either an expression vector coding for HA-tagged WT XPR1 (XPR1-HA) or the L87P mutant (L87P-HA) human *XPR1*. Results are shown as mean ± SEM in a representative experiment ($n = 3$); ***$P \leq 0.001$, ****$P \leq 0.0001$, in comparison to the control (siLUC). **b** Immunoblot of HA-tagged XPR1-containing cell lysates probed with an anti-HA antibody (*upper panel*), or with an anti β-actin antibody, used as loading control (*lower panel*). **c** Cell surface detection of XPR1 with the X-MLV Env receptor-binding domain (*open* histograms), in HEK293T transfected cells as in **a** and **b**. Non-specific staining with the secondary IgG antibody alone is shown (*grey* histograms). *Numbers* indicate the specific mean fluorescence intensity of a representative experiment ($n = 3$). **d** Efflux of inorganic ^{33}P (Pi) assay in PBMC isolated either from a healthy donor (*circles*) or from a PFBC patient carrying the p.(Leu87Pro) alteration (*squares*). *Bars* represent SEM ***$P \leq 0.001$

and c.434T > C, p.(Leu145Pro) in exon 4, and c.653T > C, p.(Leu218Ser) in exon 6) map to the SPX domain [7], whose exact role is not yet understood [10, 19]. We found that deletion of SPX in WT XPR1 does not alter phosphate export [10]. This and the facts that *XPR1* mutations found in PFBC altered XPR1-mediated phosphate efflux, including two mutations that altered presence of XPR1 at the cell surface (L87P and p.(Leu145Pro) [7]), suggests that SPX plays a regulatory role and that mutations are likely to alter interactions with factors involved in cell surface trafficking and phosphate export. *XPR1* mutations found in PFBC that altered phosphate export could be distinguished as forms that were not expressed at the cell surface, p.(Leu145Pro) [7] and L87P, and forms that could be efficiently detected at the cell surface. However, no obvious clinical difference could be noted between these two groups of patients, or with the carriers of *SLC20A2*, *PDGFB*, or *PDGFRB* variants [3, 7].

PFBC-associated *SLC20A2* and *XPR1* genes encode phosphate importer and exporter, respectively. The mechanisms underlying brain calcification with molecules transporting phosphate in opposite directions remains hypothetical. Two recent studies showed that mice with *Slc20a2* haploinsufficiency presented high phosphate level in CSF, predisposing to vascular brain calcification [20, 21]. Therefore, it is tempting to propose that PiT2 and XPR1 participate in phosphate directional transport from CSF to the blood in epithelial cells of the choroid plexus or ependyma, known for regulating ion concentrations in CSF. Finally, our study demonstrates that *XPR1* mutations are a scarce cause of PFBC and may be responsible for parkinsonism.

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Compliance with ethical standards

Conflicts of interest J-L.B. and M.S. are inventors on provisional a patent describing the use of ligands, including XRBD, for the analysis of human cells (PCT/EP2010/050139); M.S. is a co-founder of METAFORA-biosystems, a start-up company that focuses on metabolite transporters under physiological and pathological conditions.

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