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UPDATE IN CLINICAL NEUROGENETICS

Primary familial brain calcification: update on molecular genetics

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Abstract Primary familial brain calcification is a neuropsychiatric disorder with calcium deposits in the brain, especially in basal ganglia, cerebellum and subcortical white matter. The disease is characterized by a clinical heterogeneity, with a various combination of symptoms that include movement disorders and psychiatric disturbances; asymptomatic patients have been also reported. To date, three causative genes have been found: *SLC20A2, PDGFRB* and *PDGFB*. *SLC20A2* gene codes for the 'sodium-dependent phosphate transporter 2' (PiT-2), a cell membrane transporters of inorganic phosphate, involved in Pi uptake by cells and maintenance of Pi body levels. Over 40 pathogenic variants of *SLC20A2* have been reported, affecting the regulation of Pi homeostasis. It was hypothesized that *SLC20A2* mutations cause brain calcification most likely through haploinsufficiency. *PDGFRB* encodes for the platelet-derived growth factor receptor-β (PDGFRβ), a cell-surface tyrosine-kinase (RTK) receptor that regulates cell proliferation, migration, survival and differentiation. *PDGFB* encodes for the 'platelet-derived growth factor beta' (PDGFβ), the ligand of PDGFRβ. The loss of function of PDGFRβ and PDGFβ could lead to the impairment of the pericytes function and blood brain barrier integrity, causing vascular and perivascular calcium accumulation. *SLC20A2* accounts for about 40 % of familial form and 14 % of sporadic cases, while *PDGFRB* and *PDGFB* mutations are likely rare. However, approximately 50 % of patients are not genetically defined and there should be at least another causative gene.

Keywords Primary familial brain calcification · Idiopathic basal ganglia calcification · Fahr disease · SLC20A2 gene · PDGFRB gene · PDGFB gene

Introduction

Primary familial brain calcification (PFBC), also called idiopathic basal ganglia calcification (IBGC), or Fahr's disease, is a neuropsychiatric disorder characterized by bilateral brain calcification, usually transmitted as an autosomal dominant trait with incomplete penetrance [1–5]. The disease was first described by Delacour in 1850 [1] and recently, different genetic causes of PFBC have been discovered [2–4]. PFBC patients show bilateral calcium depositions commonly in basal ganglia, but other brain regions may be involved, such as the cerebellum, thalamus and subcortical white matter (Fig. 1) [5]. Furthermore, a mild frontal lobe atrophy with enlarged lateral ventricles and cerebellar atrophy might be present [6]. Neuropathological examinations showed accumulation of granular material, mostly calcium salts, around the walls of capillaries, small arteries and veins of the affected brain regions [5, 6]. PFBC patients display normal serum levels of calcium, phosphate, alkaline phosphatase and parathyroid hormone features which distinguish 'primary' brain calcification from those 'secondary' to parathyroid dysfunctions. Indeed, a common cause of basal ganglia calcification is hypoparathyroidism (HP): low serum levels of parathyroid hormone (PTH) could give rise to

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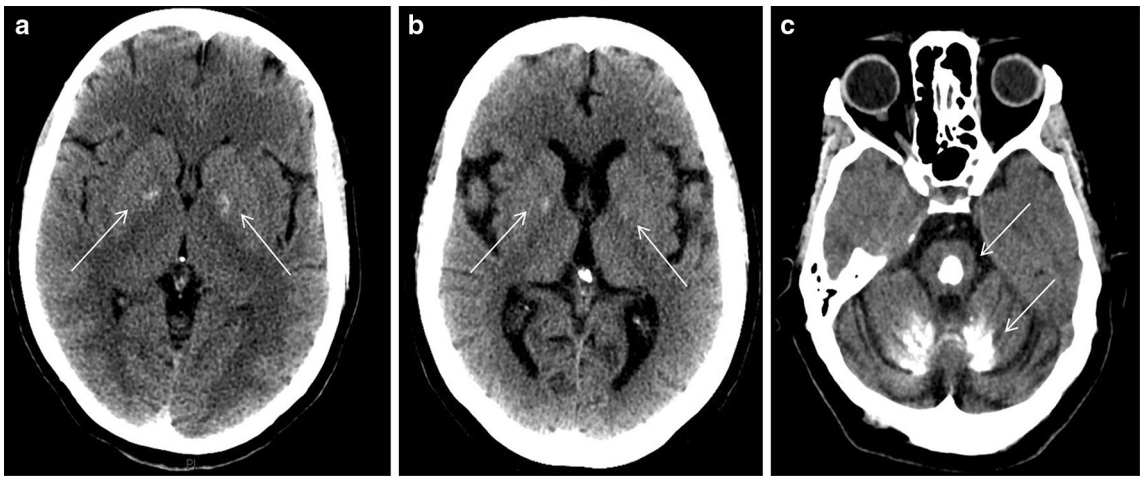

Fig. 1 Brain calcifications detected at CT scans of different patients. *Arrows* indicate calcifications in basal ganglia (**a**, **b**), brainstem and cerebellum (**c**)

hypocalcaemia and hyperphosphatemia, causing an ectopic calcification in brain tissue [7]. Secondary brain calcification might occur also in several conditions, such as mitochondrial disorders (mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes—MELAS; myoclonic epilepsy associated with ragged red fibres—MERRF), autoimmune diseases (systemic lupus erythematosus), infectious and inflammation disorders. Furthermore, calcium deposition in the basal ganglia may be encountered in some neurodegenerative disorders (pantothenate kinase-associated neurodegeneration, PKAN; polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy, PLOSL) and congenital syndromes (Aicardi–Goutieres syndrome) [5, 8]. Last, brain calcifications of undefined pathogenicity are an incidental finding in about 1–20 % of brain CT scans of healthy people, especially elderly, as a consequence of the ageing process [9]. PFBC is clinically characterized by a wide spectrum of symptoms: movement disorders, cognitive impairment and psychiatric signs [5–10]. Movement disorders include akinetic-hypertonic syndrome with or without tremor, chorea, dystonia and oro-facial dyskinesia. Among cognitive impairment, the most common sign is memory loss; furthermore, a large number of patients present a dysexecutive syndrome. The main psychiatric signs are mood disorders, including depression and bipolar disorder, followed by psychosis. Moreover, seizures and chronic headache have been also reported [5, 10]. Patients can show a variable combination of these symptoms. The age of onset of the disease is typically between 30 and 60 years, but both the severity of symptoms and the age of onset are very variable. Furthermore, a large number of patients with brain calcification, about 30 %, can remain asymptomatic throughout life [5, 10]; therefore, sometimes the diagnosis of PFBC is based only on CT scan findings [5]. In addition, no correlation between the location, extent of calcification and the severity of symptoms has been observed [10]. The PFBC is usually inherited in an autosomal dominant fashion with incomplete clinical penetrance. Recent studies have shown that PFBC is genetically heterogeneous; so far, mutations in three genes (*SLC20A2, PDGFRB, PDGFB*) have been discovered as a cause of autosomal dominant forms of PFBC. Yet, mutations in these three genes account for only approximately 50 % of the cases [2–4, 11], suggesting that additional disease-causing genes remain to be identified. Very recently, a first autosomal recessive form of brain calcification has been reported [12]. The spectrum of PFBC-causing mutations is very wide, with a uniform and widespread distribution worldwide. So far, genotype–phenotype correlations have not been observed in patients with *SLC20A2* and *PDGFRB* mutations [10].

Molecular genetics

SLC20A2

The *SLC20A2* gene located on chromosome 8 (8p11.21) codes for the PiT-2 protein (652 amino acids), the 'sodium-dependent phosphate transporter 2'. This protein belongs to the 'SLC20-type III Na^+ co-transporter family', that also includes PiT-1 (sodium-dependent phosphate transporter 1), encoded by *SLC20A1* [11]. PiT-1 and PiT-2 are cell membrane transporters of inorganic phosphate (Pi), consisting of 12 transmembrane domains, a large intracellular domain and extracellular N- and C- terminal tails [13, 14]. These transporters are widely expressed at

various levels in human tissues and play a housekeeping role in Pi uptake by the cell and phosphate tissue homeostasis [13]. In 2012, Wang and colleagues [2] associated PFBC with PiT-2 by the discovery of seven mutations in the *SLC20A2* gene (p.Gly498Arg, p.Ser601Gln, p.Ser601Leu, p.Glu575Lys, p.Thr595Met, p.Val42del, p.Pro470Leufs*37) in patients from seven PFBC families of various ancestry. All the missense variants and the deletion were used to perform $^{32}P_i$ transport assays in *Xenopus laevis* oocytes. By co-expressing mutated and wild-type PiT-2 transporters, these Authors suggested that mutations in *SLC20A2* most likely have an effect through haploinsufficiency [2]. In the mammalian brain, PiT-2 is ubiquitously expressed; at cellular level, PiT-2 has been mostly detected in neurons, astrocytes and endothelial cells and also in vascular smooth muscle cells (VSMCs) [15, 16]. Experiments with knockout mice for *SLC20A2* gene confirmed the previously suggested hypothesis that calcification starts around the walls of brain vessels [2, 16]. *SLC20A2* mutations might impair the Pi uptake, leading to a local increase in extracellular phosphate [16]. Afterwards, high levels of extracellular phosphate might result in a passive precipitation of calcium-phosphate products, triggering an active cell-mineralization process, probably via PiT-1 [2, 16]. Indeed, PiT-1 transporter is required for the normal bone cell differentiation and mineralization and is also involved in pathological smooth muscle cell (SMC) calcification. Interestingly, inducers of calcification, as the bone morphogenetic protein-2 (BMP-2), calcium and platelet-derived growth factor PDGF, upregulated PiT-1, but not PiT-2 expression [17, 18]. In vitro studies demonstrated that elevated phosphate levels result in the loss of smooth muscle markers (SM alpha actin, SM22 alpha) and expression of osteochondrogenic markers (Runx2/Cbfa1, osterix, alkaline phosphatase, osteopontin). Indeed, elevated phosphate concentrations may enhance the expression of the transcription factor Cbfa-1 (core-binding factor-1), that regulates the expression of osteogenic genes, such as osteopontin and osteocalcin [19]. Moreover, in vitro experiments showed that the phosphonoformic acid (PFA), an inhibitor of Pi transporters, abolishes the Pi uptake and prevents the mineralization of human aortic smooth muscle cells [20]. Although the involvement of PiT-1 in bone differentiation and cell mineralization has been deeply investigated, the molecular mechanism of calcification caused by PiT-2 transport dysfunctions is still to be elucidated and further studies are needed. So far, over 40 pathogenic variants in *SLC20A2* gene have been reported in patients with PFBC, including missense, frameshift and non-sense mutations, but also deletions and one splice-site mutation; among them, one de novo variant has been found (Table 1) [21]. Missense mutations in PiT-2 protein could impair transport function; for instance, His502 and Glu575, substituted by a glutamine and a lysine, respectively, are critical for Pi transport [2, 14, 22]. Some frameshift and non-sense mutations, such as p.Leu170*, p.Val195Leufs*61, p.Pro470Leufs*37 and p.R172fs*19, are predicted to generate a premature termination codon (PTC) leading to the "non-sense mediated decay" (NMD) process, a surveillance mechanism that degrades aberrant mRNA, causing the loss of the transcript [22, 23]. Particularly, the frameshift mutation p.R172fs*19 leads to a 30 % reduction of the *SLC20A2* mRNA expression, revealing that NMD process takes place and confirming haploinsufficiency as the most likely disease mechanism [23]. Furthermore, a genomic deletion of 563 kb in the chromosome 8 (g.42275321_42329908del), including *SLC20A2*, has been described in one large family. This deletion comprised seven genes (*VDAC3, SLC20A2, C8ORF40, CHRNB3, CHRNA6, THAP1, RNF170*) and partial deletion of *HOOK3*; interestingly, *THAP1* is the causative gene of a familial form of dystonia (DYT16), indeed the patients displayed dystonia as main symptom [24]. In two studies, mutations in the *SLC20A2* gene have been found in 41 % of the familial PFBC cases and 14 % of sporadic patients, showing that PiT-2 impairment is a frequent and widespread cause of primary brain calcification [22, 25].

PDGFRB

The *PDGFRB* gene is located on chromosome 5 (5q33.1) and encodes the platelet-derived growth factor receptor-β (PDGFRβ, 1106 amino acids), recognized by growth factor homodimers PDGF-BB and PDGF-DD [26]. PDGFRβ is a cell-surface tyrosine-kinase receptor, consisting of five extracellular immunoglobulin (Ig) loops and an intracellular tyrosine-kinase domain [26]. In the brain, it is expressed in neurons, VSMCs and pericytes [3, 26]. The binding of the ligand triggers the dimerization, autophosphorylation and activation of the PDGFRβ receptor, which, in turn, initiates the downstream signalling leading to cell proliferation, migration, survival and differentiation [3, 26]. Recently, four missense mutations in the *PDGFRB* gene have been reported in one PFBC family and three sporadic cases (Table 2). All mutations, p.Leu658Pro, p.Arg695Cys, p.Arg987Trp and p.Glu1071Val, cause the substitution of conserved amino acids and were predicted to be pathogenic. Cell culture experiments showed that variants in the tyrosine-kinase domain (from 562 to 953 aa) reduce the receptor levels and the autophosphorylation [27]. It has been demonstrated that the missense mutation p.Leu658Pro reduces the kinase activity, while p.Arg987Trp mutation causes a rapid degradation of the receptor and impairs the activation of STAT3, a

Table 1 *SLC20A2* mutations found in patients with PFBC

Nucleotide change	Amino acid change	Location	References
c.82G>A	p.Asp28Asn	Exon 2	[10, 25]
c.124_126delGTG	p.Val42del	Exon 2	[2]
c.152C>T	p.Ala51Val	Exon 2	[37]
c.185T>C	P.Leu62Pro	Exon 2	[25]
c.212G>A	p.Arg71His	Exon 2	[37]
c.260_261delTC	p.Leu87Hisfs*6	Exon 2	[37]
c.323T>C	p.Leu108Pro	Exon 3	[38]
c.338C>G	p.Ser113*	Exon 3	[24]
c.344C>T	p.Thr115Met	Exon 3	[37]
c.431-1G>T	p. Val144Glyfs*85	IVS 3	[3]
c.509delT	p.Leu170*	Exon 4	[22]
c.514A>T	p.Lys172*	Exon 4	[22]
c.515delA	p.Leu172Arg172fs*20	Exon 4	[23]
c.551C>T	p.Pro184Leu	Exon 5	[3]
c.581A>G	p.Asn194Ser	Exon 5	[10]
c.583_584delGT	p.Val195Leufs*61	Exon 5	[22]
c.760C>T	p.Arg254*	Exon 7	[22]
c.935-1G>A	p.Gly312Valfs*8	IVS 7	[25]
c.1086delC	p.His362Glnfs*54	Exon 8	[39]
c.1101_1102delCG	p.Glu368Glyfs*46	Exon 8	[40]
c.1145G>A	p.Arg382Gln	Exon 8	[22]
c.1158C>G	p.Tyr386*	Exon 8	[20]
c.1301C>G	p.Ser434Trp	Exon 8	[40]
c.1399C>T	p.Arg467*	Exon 8	[37]
c.1409delC	p.Pro470Leufs*37	Exon 8	[2]
c.1470_1478delGCAGGTCCT	p.Gln491_Leu493del	Exon 8	[25]
c.1483G>A	p.Ala495Thr	Exon 8	[41]
c.1492G>A	p.Gly498Arg	Exon 8	[2]
c.1506C>A	p.His502Gln	Exon 8	[22]
c.1520_1521delTG	p.Val507Glufs*2	Exon 8	[42]
c.1523+1G>A	p.Gly312Valfs*8	IVS 8	[22]
c.1527delT	p.Asn509LysFs*7	Exon 9	[10]
c.1618G>A	p. Gly540Arg	Exon 9	[43]
c.1652G>A	p.Trp551*	Exon 9	[22]
c.1703C>T	p.Pro568Leu	Exon 9	[22]
c.1711G>A	p.Glu571Ser	Exon 10	[10]
c.1723G>A	p.Glu575Lys	Exon 10	[2]
c.1784C>T	p.Thr595Met	Exon 10	[2, 40]
c.1794+1G>A	p.Ser570Argfs*30	IVS 10	[22]
c.1794+1G>C	p.Ser570Argfs*30	IVS 10	[22]
c.1802C>G	p.Ser601Trp	Exon 11	[2]
c.1802C>T	p.Ser601Leu	Exon 11	[2, 22]
c.1828_1831delTCC	p.Ser610Alafs*17	Exon 11	[22]
c.1909A>C	p.Ser637Arg	Exon 11	[37]
g.42275321_42329908del		Whole gene	[24]

transcription activator, blocking the downstream signalling. On the other hand, the mutation p.Glu1071Val does not affect the phosphorylation and the signalling and probably it might be a polymorphism [28]. It has been hypothesized that the loss of function of PDGFRβ could lead to the impairment of the blood brain barrier (BBB) integrity,

Table 2 *PDGFRB* mutations found in patients with PFBC

Nucleotide change	Amino acid change	Location	References
c.1973T>C	p.Leu658Pro	Exon 14	[3]
c.2083C>T	p. Arg695Cys	Exon 15	[27]
c.2959C>T	p.Arg987Trp	Exon 22	[3]
c.3212A>T	p.Glu1071Val	Exon 23	[10]

causing vascular and perivascular calcium accumulation [3]. Moreover, a deficient PDGF-β signalling is highly damaging to VSMCs and pericytes, resulting in complete lack of pericytes or pericyte hypoplasia, endothelial hyperplasia, increased vessel diameter, increased vascular permeability and vessel instability [4, 27]. Alternatively, it has been suggested that mutations in *PDGFRB* gene might be activating mutations, impairing the PDGFRβ-PiT-1 signalling and inducing VSMCs mineralization. In VSMCs, the PDGFRβ pathway enhances the expression of PiT-1, increasing the abundance of the receptor in the endoplasmic reticulum membranes and stimulating the Pi uptake [29]. To date, few functional analyses have been carried out to clarify the molecular mechanism of PFBC due to *PDGFRB* mutations. Recently, *PDGFRB* mutations have been also found in patients with autosomal dominant infantile myofibromatosis, a disorder of mesenchymal proliferation, characterized by benign tumour of soft tissue in infancy and childhood [30, 31]. Two germinal mutations, c.1681C>T (p.Arg561Cys) and c.1978C>A (p.Pro660Thr), and one somatic mutation c.1998C>A (p.Asn666Lys) have been reported. The variant p.Arg561Cys is located outside the kinase domain and probably compromises the auto-inhibition of the receptor. While the amino acids Asn666 and Pro660 are located in the kinase domain, and probably the variant p.Asn666Lys may abolish the interaction with inhibitors, deregulating the kinase activity [30, 31].

PDGFB

PDGFB gene is located on chromosome 22 (22q13.1) and encodes for the 'platelet-derived growth factor beta' (PDGFβ-241 amino acids), the ligand of the PDGFRβ receptor. PDGFβ is an antiparallel disulphide-linked dimer, a paracrine factor synthesized and secreted by angiogenic endothelial cells, which acts on pericytes and VSMCs which in turn have PDGFRβ in the cell-surface membrane [26]. In these cells, the PDGF signalling promotes the proliferation and migration along the newly developing blood vessels [32]. So far, eight *PDGFB* mutations, including one de novo variant, have been found in eight PFBC patients [33] (Table 3). Three are missense mutations: p.Leu9Arg inserts a charged amino acid in the signal

Table 3 *PDGFB* mutations found in patients with PFBC

Nucleotide change	Amino acid change	Location	References
c.3G>A	p.Met1?	Exon 1	[4]
c.26T>G	p.Leu9Arg	Exon 1	[4]
c.356T>C	p.Leu119pro	Exon 4	[4]
c.433C>T	p.Gln145*	Exon 4	[4]
c.445C>T	p.Arg149*	Exon 4	[4, 44]
c.439C>T	p.Gln147*	Exon 4	[33]
c.726G>C>C	p.*242Tyrext*89	Exon 6	[4]
7.2-kb Intragenic deletion		Exons 3–5	[34]

peptide that is essential for the protein export; p.Leu119Pro occurs in the receptor-binding loop and p.*242>Tyrext*89 substitutes the stop codon with a tyrosine, leading to an extension of 89 codons in the transcript [4]. Three nonsense mutations, p.Gln145*, p.Gln147* and p.Arg149*, are predicted to remove part of the protein [4, 33]. The mutation P.Met1? may replace the start methionine, but the consequences of this variant have not yet been clarified [4]. Finally, a large intragenic deletion (7.2-kb) within *PDGFB* has been found in a patient with brain calcification and leukoencephalopathy [34]. This deletion comprises exons 3, 4 and 5, which encode for receptor-binding sites and dimerization domains, and might result in a truncated, not functional protein [34]. Mutations in *PDGFB* are predicted to be 'loss of function' and the discovery of a partial *PDGFB* gene deletion confirms this hypothesis [34]. Furthermore, Keller and colleagues showed that mice deficient in PDGFβ develop age-related calcified nodules in the thalamus and midbrain, which are similar to the lesions observed in the PFBC patients. Moreover, a correlation between endothelial PDGFβ, but not neuronal PDGFβ, and brain calcification in mice has been described [4]. Mice expressing PDGFβ, that lacks the retention motif which is essential for the diffusion of the protein in the tissues interstitium, showed an alteration of the local concentration and bioavailability of PDGFβ and a reduction of pericyte recruitment. These data strongly support a correlation between brain calcification and BBB impairment, caused by pericyte deficiencies [4].

Brain calcification and ISG15 gene

Recently, homozygous mutations in *ISG15* gene have been found in six young patients with brain calcification, from three families from China, Iran and Turkey [12, 35]. *ISG15* gene (1p36.33) encodes for an interferon (IFN)-α/β—inducible-ubiquitin-like modifier involved in the innate immune response to viral infection. It acts by conjugation to a target protein (ISGylation) or as a free and unconjugated protein and it is a negative regulator of IFN α/β immunity

[12, 35]. Three mutations have been discovered in this gene: c.379G>T (p.Glu127*), c.336_337insG (p.Leu114fs) and c. 163 C>T (p.Gln55*). All mutations are in homozygous state and lead to the lack of ISG15 protein and a subsequent increase of IFN-α/β immunity. Also the Aicardi–Goutieres syndrome and spondyloenchondromatosis (SPENCD), in which brain calcification is a common feature, have been associated with up-regulation of IFN-α/β immunity [12, 35, 36]. *ISG15* mutations are also linked to Mendelian susceptibility to mycobacterial disease (MSMD), in which severe clinical disease occurs following infection with weakly virulent mycobacteria, due to an insufficient production of ISG15-dependent IFN-γ [35]. However, brain calcification disease caused by mutation in *ISG15* gene is quite different from PFBC, since the involvement of the IFN-α/β immunity and the autosomal recessive inheritance.

Conclusion

The recent genetic discoveries point to abnormalities of PiT-2 transport and the PDGFβ/PDGFRβ pathway, leading to the accumulation of calcium salts in the brain. PiT-2, PDGFβ and PDGFRβ are widely expressed in human tissues, but calcifications occur only in the brain. Future investigations are warranted to understand the detailed molecular mechanism leading to PFBC. PFBC is a clinically heterogeneous disease and the wide spectrum of symptoms could make the diagnosis challenging. The recent findings of disease-causing mutations in three genes confirm the previously suggested genetic heterogeneity of PFBC [11], and it allows a molecular diagnosis to be made in several patients. However, the genetic defect remains currently unknown in about 50 % of the autosomal dominant PFBC cases, suggesting the existence of at least another genetic form. Future work will be focused on the identification of mutations in additional genes for PFBC.

Conflict of interest The authors declare that they have no conflict of interest.

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