Published in final edited form as:

Hum Mutat. 2014 August ; 35(8): 964–971. doi:10.1002/humu.22582.

Genetic screening and functional characterization of *PDGFRB* mutations associated with Basal Ganglia Calcification of Unknown Etiology

Monica Sanchez-Contreras¹, Matthew C. Baker¹, NiCole A. Finch¹, Alexandra Nicholson¹, Aleksandra Wojtas¹, Zbigniew K. Wszolek², Owen A. Ross¹, Dennis W. Dickson¹, and Rosa Rademakers^{1,*}

¹Department of Neuroscience, Mayo Clinic, 4500 San Pablo Road, Jacksonville, FL 32224, USA

²Department of Neurology, Mayo Clinic, USA, 4500 San Pablo Road, Jacksonville, FL 32224, USA

Abstract

Three causal genes for Idiopathic Basal Ganglia Calcification (IBGC) have been identified. Most recently, mutations in *PDGFRB*, encoding a member of the platelet-derived growth factor receptor family type β , and *PDGFB*, encoding PDGF-B, the specific ligand of PDGFR β , were found implicating the PDGF-B/PDGFR β pathway in abnormal brain calcification. In this study we aimed to identify and study mutations in *PDGFRB* and *PDGFB* in a series of 26 patients from the Mayo Clinic Florida Brain Bank with moderate to severe basal ganglia calcification (BCG) of unknown etiology. No mutations in *PDGFB* were found. However, we identified one mutation in *PDGFRB*, p.R695C located in the tyrosine kinase domain, in one BGC patient. We further studied the function of p.R695C mutant PDGFR β and two previously reported mutants, p.L658P and p.R987W PDGFR β in cell culture. We show that, in response to PDGF-BB stimulation, the p.L658P mutation completely suppresses PDGFR β autophosphorylation whereas the p.R695C mutation results in partial loss of autophosphorylation. For the p.R987W mutation, our data suggest a different mechanism involving reduced protein levels. These genetic and functional studies provide the first insight into the pathogenic mechanisms associated with *PDGFRB* mutations and provide further support for a pathogenic role of *PDGFRB* mutations in BGC.

Keywords

Basal Ganglia Calcification; BGC; IBGC; Fahr disease; *PDGFRB*; *PDGFB*

INTRODUCTION

Idiopathic Basal Ganglia Calcification (IBGC), also known as Fahr disease, is pathologically characterized by bilateral calcification of the basal ganglia with or without involvement of

*Corresponding author: Rosa Rademakers, Ph.D., Department of Neuroscience, Mayo Clinic, Jacksonville, 4500 San Pablo Road, Jacksonville, FL 32224, Phone: (904) 953-6279, Fax: (904) 953-7370, rademakers.rosa@mayo.edu.

The authors declare that they do not have conflicts of interest.

other brain regions, and clinically associated with a spectrum of neuropsychiatric and motor syndromes including dystonia, parkinsonism, tremor and chorea. Although calcification is a normal finding in the aging brain, the affected brain regions of IBGC patients present with abnormally severe, bilateral calcification predominantly of small vessels and perivascular spaces (Miklossy, et al., 2005). The study of IBGC families presenting with an autosomal dominant inheritance has recently led to the identification of three causal IBGC genes: solute carrier family 20 (phosphate transporter) member 2 (*SLC20A2*; MIM# 158378) (Wang, et al., 2012), platelet-derived growth factor receptor type β (*PDGFRB*; MIM# 173410) (Nicolas, et al., 2013) and platelet-derived growth factor type B (*PDGFB*; MIM# 190040) (Keller, et al., 2013). More than 30 pathogenic mutations have been identified in *SLC20A2* in IBGC families of varied ancestry (Hsu, et al., 2013; Kasuga, et al., 2014; Lemos, et al., 2013; Yamada, et al., 2014; Zhang, et al., 2013) establishing this gene as the most common genetic cause of IBGC. The reported loss-of-function *SLC20A2* mutations and our recent study of a complete deletion of *SLC20A2* as the cause of IBGC in a large Canadian family (Baker, et al., 2013) support that *SLC20A2* haploinsufficiency is the causal factor in IBGC. *SLC20A2* encodes the type III sodium-dependent phosphate transporter 2 (PiT2) and it is suggested that the loss-of-function mutations affect the phosphate transport capacity of PiT2, which in turn affects phosphate-dependent cell processes such as calcification (Wang, et al., 2012). However, PiT2 is widely expressed in the body and it remains unclear how PiT2 defects specifically result in brain calcification (Collins, et al., 2004). The second IBGC gene is *PDGFRB*, encoding the cell surface tyrosine kinase receptor PDGFR β (also called PDGFR β) (Nicolas, et al., 2013). In particular, one mutation in the PDGFR β tyrosine kinase domain, rs397509381:p.L658P, showed segregation with IBGC in a large French family (Nicolas, et al., 2013). The same group identified a second novel mutation, rs397509382:p.R987W, in a sporadic IBGC patient (Nicolas, et al., 2013). This mutation is located in a highly conserved region but is outside of the tyrosine kinase domain, suggesting multiple possible disease mechanisms may be associated with *PDGFRB* mutations in IBGC. Shortly after *PDGFRB* mutations were identified, mutations and a partial deletion in *PDGFB*, PDGFR β 's specific ligand, were also associated with IBGC (Keller, et al., 2013; Nicolas, et al., 2014), highlighting the importance of the PDGF-B/ PDGFR β signaling pathway in the development of brain calcifications. PDGF-B is a growth, proliferation and motility factor for a variety of cells including mesenchymal cells, endothelial cells, pericytes and neurons (Andrae, et al., 2008). Homo- or heterodimeric forms of PDGF-B bind to PDGFR β to induce receptor dimerization at the cell surface, cross-phosphorylation at multiple sites of the tyrosine kinase domain and activation of downstream pathways involved in cell proliferation, differentiation, survival and migration (Heldin, et al., 1998). The pathogenicity of mutations in *PDGFRB* and *PDGFB* has been inferred based on computational predictions but no functional studies have been reported to date. In this study we aimed to identify and characterize *PDGFRB* and *PDGFB* mutations in a brain bank series of patients with moderate to severe basal ganglia calcification (BGC) of unknown etiology and we evaluated the pathogenicity of reported and newly identified *PDGFRB* mutations using an *in vitro* system.

MATERIALS AND METHODS

Patient cohort

Twenty-six patients (15 males, 11 females) with BGC were selected from the Mayo Clinic Florida (MCF) Brain Bank. Demographic, clinical and imaging findings were reviewed and are summarized in Supp. Table S1. Clinically, patients manifested as Alzheimer's disease (AD), frontotemporal dementia (FTD), Parkinson's disease (PD), progressive supranuclear palsy (PSP), dementia with Lewy bodies (DLB) and dystonia. All patients were Caucasian, except one African-American patient and one American-Asian patient. The average age at death was 81 years (range, 62–94 years). Upon pathological examination all cases presented moderate to severe basal ganglia calcification with in almost all cases involvement of other brain regions (Supp. Table S2). Additional staining and assessment of TDP-43 pathology, Braak neurofibrillary tangle (NFT) stage, Thalamus senile plaques (SP) phase, Cerebral amyloid angiopathy (CAA), Arteriosclerotic cerebrovascular disease (ASCVD), atherosclerosis and infarcts was performed and summarized on Supp. Table S2. In most patients, pathological findings of calcification were accompanied by AD, Lewy body disease (LBD), PSP or frontotemporal lobar degeneration (FTLD) pathology. In two patients (cases #21 and 23 in Supp. Tables S1 and S2), loss-of-function mutations in *SLC20A2* were recently identified (Baker, et al., 2013).

Sanger sequencing analyses

Genomic DNA was extracted from brain tissue using the relevant Gentra Puregene kit (Qiagen). All coding regions of *PDGFRB* (exons 2–23 of NG_023367.1) and *PDGFB* (exons 1–6 of NG_012111.1) were sequenced. *PDGFRB* exons were amplified by PCR using primers designed to flank intronic sequence in both directions and labeled with one M13 sequence to facilitate sequencing. *PDGFB* exons were amplified using the primers used by Keller et al. (Keller, et al., 2013). Prior to sequencing, PCR products were purified using the AMPure system (Agencourt Biosciences). Sequence reactions were purified with CleanSEQ (Agencourt Biosciences) and then sequenced on an ABI3730xl Genetic Analyzer. Sequences were analyzed using Sequencer 4.5 software (Gene Codes). The DNA mutation numbering was based on cDNA sequence where +1 corresponds to the A of the ATG translation initiation codon in the reference sequence, with the initiation codon as codon 1. The pathogenicity of genetic variations was tested using SIFT, PolyPhen and MutationTaster protein prediction programs. Minor allele frequencies were obtained from dbSNP and NHLBI GO Exome Sequencing Project. Additionally, a custom-designed Taqman genotyping assay was used to screen 863 controls for the presence of the c. 2083C>T (p.R695C) mutation in *PDGFRB*.

Mutagenesis of *PDGFRB* cDNA clones

A cDNA construct encoding human PDGFR β on a pCMV vector was purchased from Sino Biological Inc. (HG10514-G-N). The p.L658P, p.R695C, p.R987W and p.A1096V mutations were introduced independently using the QuickChange site-directed mutagenesis kit (Stratagene) and mutagenesis primers. Mutation-positive clones were identified by sequencing analysis and transformed into One Shot TOP10-competent Escherichia coli cells, after which DNA was extracted using the Nucleobond Xtra Maxi Plus EF kit (Clontech).

The cDNA sequence of wild-type (WT) and p.R987W pCMV constructs was subcloned into a pAAV vector by using compatible restriction sites.

Cell culture transfection, mRNA expression and western blotting

HeLa cells were maintained in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C and 5% CO₂. One day before transfection, cells were plated at 180,000 cells per well in six-well culture dishes. The next day, cells were transiently transfected with 2 µg of PDGFRβ wild-type (PDGFRβ WT) or mutant (PDGFRβ L658P, PDGFRβ R695C, PDGFRβ R987W and PDGFRβ A1096V) plasmid DNA using X-tremeGENE HP DNA transfection reagent (Roche).

For mRNA expression analysis, transfected HeLa cells were harvested after 3 days. Total RNA was extracted from cells using the RNeasy Plus Mini Kit (Qiagen), and its quality was assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies). RNA samples were normalized to 50 ng/µL and 300 ng was used as template. The reverse transcription reaction was performed using the Superscript III system (Life Technologies). Real-time quantitative PCR using an ABI7900 was performed in triplicate for each sample using gene expression probes for *PDGFRB* (Hs01019589_m1), *GAPDH* (Hs00266705_g1) and *RPLP0* (Hs00420895_gh). Results were analyzed using SDS software version 2.2 (Life Technologies) and relative quantities of *PDGFRB* mRNA were determined.

To induce the activation of PDGFRβ, we treated the HeLa cell cultures with PDGF-BB, a recombinant human PDGF homodimer of two B chains (PeproTech). The culture medium was changed to serum-free medium 16h before treatment with 30 ng/mL of PDGF-BB. PDGF-BB treatments followed by harvesting of the cells were performed 3 days after transfection. PDGF-BB remained in the medium for 5, 15, 30 and 60 minutes before the cells were harvested in RIPA buffer (Boston BioProducts) supplemented with protease inhibitors. HeLa cells without PDGF-BB treatment were collected as controls. To inhibit proteasomal degradation, we treated transfected HeLa cells with 10µM MG-132 (Sigma-Aldrich) for 8 hours. MG-132 treated and untreated cells were harvested as described for PDGF-BB treatment. For western blot, cell lysates were diluted in an equivalent volume of 2x Novex Tris-glycine SDS sample buffer (Invitrogen) and protein was denatured at 95°C for 10 minutes. Equal volumes were run on 8–16% Tris-Glycine gels (Invitrogen). Samples were then transferred to Immobilon membranes (Millipore) and immunoblotted. Total PDGFRβ levels were detected using PDGFRβ antibody (clone 28E1,1:10,000) from Cell Signaling Technology. PDGFRβ autophosphorylation was detected using PDGFRβ phospho-tyrosine primary antibodies against p-Tyr751 (1:3,000), p-Tyr1009 (1:1,000) and p-Tyr1021 (1:2,000) from Cell Signaling Technology. Anti-alpha-tubulin (clone DM1A, 1:50,000) primary antibody from Sigma was used to ensure equal protein loading. Blots were then incubated with anti-mouse and anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Promega), and bands were detected by enhanced chemiluminescence using Western Lightning Plus-ECL reagents (Perkin Elmer). Quantitative values from each blot were obtained using ImageJ 1.44 (National Institutes of Health, USA). Numerical values of optical intensity were obtained by measuring the mean number of pixels per area for each band and normalizing to the values obtained from a linker sample with an

intermediate optical intensity. The total PDGFR β protein levels were calculated by adding the intensity of ~180, ~160 and ~130 kDa bands. Phosphorylated PDGFR β was obtained by calculating the percentage of the band obtained for each anti-phospho-tyrosine antibody as compared to the total PDGFR β ~180 kDa band.

The significance of the comparisons between experimental groups was measured by two-way ANOVA followed by Bonferroni post-test analysis using the GraphPad Prism software.

RESULTS

PDGFRB and *PDGFB* mutation screening in BGC patients

We sequenced 26 BGC patients for mutations in the coding exons of *PDGFRB* and *PDGFB*. No mutations were found in *PDGFB*. In *PDGFRB*, one patient carried a potentially pathogenic mutation c.2083C>T inducing the p.R695C change in the tyrosine kinase domain of PDGFRB predicted to be damaging by Polyphen2, MutationTaster and SIFT. This variant was reported on ClinVar Database (SCV000120650) and can be accessed on this URL address <http://www.ncbi.nlm.nih.gov/clinvar/?term=SCV000120650>. We did not detect this variant in 863 healthy controls and this specific variant was not reported in any public database. Of note, dbSNP and the exome variant databases report two variants in the same codon position that are also reported to be damaging: rs138008832 consists of a c.2083C>A change that induces a p.R695S change and rs375978065 consists of a G>A change in a different base position (c.2084) and induces a p.R695H change. Each one of these variants has an associated frequency in the database of 0.0002 in African Americans and they were not found in European Americans.

The patient who was found to carry the p.R695C mutation had PSP as primary neuropathology diagnosis consisting of Braak NFT stage II and prominent calcification at globus pallidus, putamen and cerebellum (case #5 in Supp. Table S2). Mutational analysis of the microtubule-associated protein tau gene (*MAPT*; MIM# 157140) was negative. Retrospective evaluation of her medical records revealed that she died at the age of 83 and was diagnosed with an atypical parkinsonian disorder most consistent with PSP parkinsonism (PSP-P) (Williams, et al., 2005) (case #5 in Supp. Table S1). She was treated with Levodopa with little or no response. Her disease course was four years, with first signs being balance difficulties and unexplained falls. She also had bradykinesia, flexed posture, shuffling gait, and right hand tremor. On examination, she had axial rigidity, postural instability, photosensitivity, and vertical supranuclear gaze palsy. One year before she died, she had Hoehn-Yahr stage 3–4 parkinsonism, with worsening dysarthria and dysphagia. She had mild cognitive impairment (28/30 on the Mini-Mental State Exam) characterized by word finding difficulties. There was no family history of neurologic disease. Her past medical history was notable for hypothyroidism on replacement therapy, hypertension, cataracts, and osteoporosis with severe thoracic kyphosis and hip fracture. She never had neuroimaging studies.

In addition to this pathogenic variant, we also identified one patient carrying the known missense variant c.3287C>T (p.A1096V), previously reported in dbSNP (rs114435947) with

a frequency of 0.0018 and predicted to be a benign variant. This mutation was included as a control in all cell culture experiments.

Effect of *PDGFRB* mutations on PDGFR β stability

As an initial functional assessment, we evaluated the effect of *PDGFRB* mutations p.R695C and p.A1096V (identified in this study), as well as p.L658P and p.R987W (identified previously), on PDGFR β protein stability in transiently transfected HeLa cells. Typically, PDGFR β is detected as 3 different electrophoretic bands depending on its processing stage (Keating and Williams, 1987). An early PDGFR β precursor of ~130 kDa probably results from core glycosylation. ER processing and further N-glycosylation results in an EndoH-susceptible PGFRB precursor of ~160 kDa and finally Golgi processing of high mannose to complex mannose residues results in a ~180 kDa PDGFR β . This 180 kDa form is expressed in the extracellular membrane and corresponds to the mature form of PDGFR β that is susceptible to autophosphorylation (Inui, et al., 1993). In our studies, the 160 and 180 kDa bands were consistently detected in our assays, whereas the 130 kDa band had low intensity (Figure 1A).

Compared to WT PDGFR β , all mutants showed a decrease in the total levels of PDGFR β which may be attributed to decreased transfection efficiency; however, this decrease was only significant for p.R987W (80.4% less total PDGFR β compared to WT $p < 0.001$, Figure 1B and D). Additionally, p.L658P, p.R695C and p.R987W mutants all showed significantly less expression of the mature 180 kDa form as compared to WT (21.9 %, $p < 0.0001$, 10.7% $p < 0.01$ and 11.9%, $p < 0.001$ less respectively), while normal expression of this band was seen for p.A1096V (Figure 1C and D). To quantitatively assess changes in the differential expression of the two most prominent processing forms of PDGFR β , we calculated a ratio of the 180 kDa form to the 160 kDa form (180/160 ratio) for each mutation. In WT transfected cells we obtained a 180/160 ratio of 1.0. Similarly, p.A1096V transfected cells expressed PDGFR β at the same 180/160 ratio as WT (Figure 1C and D), suggesting that PDGFR β p.A1096V does not affect the receptor stability despite somewhat lower level of total PDGFR β expression. The decreased amount of the 180 kDa species in the p.L658P, p.R695C and p.R987W mutants on the other hand, decreased the 180/160 ratio to 0.4, 0.6 and 0.7 respectively (Figure 1C and D) suggesting that these mutations may affect the final protein processing of PDGFR β reducing the availability of mature PDGFR β .

Different from the p.L658P and p.R695C mutations, the p.R987W mutation decreased the expression of total PDGFR β to levels that potentially interfere with physiological receptor function. To further explore potential factors affecting the expression of p.R987W PDGFR β in our experimental setting, we first evaluated whether the reduced levels of total p.R987W PDGFR β were due to low expression from the particular expression vector used. However, the same low levels of total PDGFR β were observed when p.R987W PDGFR β was expressed from a second independently generated pAAV vector (Figure 2A). We next evaluated mRNA levels of WT and p.R987W PDGFR β since low PDGFR β protein levels of p.R987W in the presence of reduced mRNA expression may indicate deficient gene expression. However, mRNA levels of p.R987W PDGFR β were not decreased. In fact, we observed a significant increase in p.R987W PDGFR β mRNA levels compared to WT

regardless of the expression vector (19.7 % increase in the pCMV vector $p < 0.01$ and 30.6 % increase in the pAAV vector $p < 0.001$, Figure 2B). Together these results suggested instability of p.R987W PDGFR β at the protein level. Since the protein degradation of PDGFRs has been previously reported to predominantly occur by the proteasome (Keating and Williams, 1987), we next evaluated whether inhibition of proteasomal degradation of PDGFR β using the proteasomal degradation inhibitor MG-132, could restore levels of p.R987W PDGFR β to WT PDGFR β levels. Vehicle-treated cells transfected with p.R987W PDGFR β had 49.9% less total PDGFR β than WT but after treatment with 10 μ M MG-132 for 8 hours the levels of total R987W PDGFR β increased 19.4% higher than WT accounting for a 69.5% change from non-treated ($p < 0.0001$) (Figure 2C and D) and suggesting that the p.R987W mutation may increase the degradation of PDGFR β .

Effect of *PDGFRB* mutations on the PDGF-BB-induced PDGFR β autophosphorylation

To further test the functional significance of the p.L658P and p.R695C mutations located within the protein tyrosine kinase domain and already shown to affect PDGFR β processing, we studied the effects of these proteins on the autophosphorylation of PDGFR β . Findings for p.L658P and p.R695C were compared to the autophosphorylation of WT PDGFR β and the p.A1096V benign variant which is located outside the tyrosine kinase domain and therefore presumed to not affect receptor phosphorylation. Three days after transient transfection WT, p.L658P, p.R695C and p.A1096V PDGFR β expressing cells were stimulated with 30 ng/mL PDGF-BB for 5, 15, 30 and 60 minutes. Upon stimulation, we observed phosphorylation on multiple tyrosine residues (positions 751, 1009 and 1021) for PDGFR β WT, p.R695C, and p.A1096V. However, cells transfected with p.L658P PDGFR β showed no detectable receptor phosphorylation at any time point, suggesting a complete loss of function (Figure 3A). Further quantification revealed that even though some phosphorylation was detected for the p.R695C mutant, a significant decrease in the level of phosphorylation was observed at multiple time points as compared to WT PDGFR β suggesting a partial deficiency in PDGFR β function for p.R695C (Figure 3A and B and Supp. Table S3).

Effect of *PDGFRB* mutations in the activation of downstream signaling molecules in the PDGF-B/PDGFR β pathway

Following receptor binding of PDGF-BB, the autophosphorylation of PDGFR β receptor at specific tyrosine residues generates binding sites for signal transduction molecules leading to the initiation of intracellular signaling pathways including Akt/PKB, phosphatidylcholine-specific phospholipase D PLD, PKC, MAP kinase and Src pathways (Heldin, et al., 1998). To test whether the defective receptor phosphorylation associated with p.L658P and p.R695C mutations had deleterious effects on the activation of major signaling pathways downstream of PDGFR β , we analyzed the phosphorylation of downstream signaling molecules ERK and AKT in HeLa cells transiently transfected with mutant and WT PDGFR β . Upon stimulation with PDGF-BB, a modest decrease in the phosphorylation of ERK in the p.L658P mutant-transfected cells at 15 min was observed compared to WT (Figure 3A, 3B and Supp. Table S3) and no effects were seen for the other mutants. Of note, the ERK phosphorylation in p.L658P transfected cells was comparable to ERK phosphorylation observed in non-transfected cells exposed to PDGF-BB (Supp. Figure S1) suggesting that, in response to high levels of PDGF-BB, ERK is activated by alternative

PDGFR β -independent survival pathways and/or by the stimulation of endogenous PDGFR β receptors in HeLa cells. For AKT, phosphorylation followed a time-dependent curve pattern with peak phosphorylation levels at 15 min of PDGF-BB exposure in the WT and the p.A1096V benign variant; however, AKT phosphorylation in p.R695C transfected cells had a peak at 15 min exposure that was higher than WT suggesting AKT overstimulation (47% increase compared to WT, $P < 0.01$. Figure 3A, 3B and Supp. Table S3). In contrast, PDGFR β mutant p.L685P showed significantly reduced levels of AKT phosphorylation at this time point and also at the 30 min time point (95.2% decrease compared to WT, $p < 0.0001$, and 99.5% decrease compared to WT, $p < 0.0001$, respectively. Figure 3A, 3B and Supp. Table S3). Similar to what was observed for pERK, AKT phosphorylation in p.L658P transfected cells was comparable to non-transfected cells exposed to PDGF-BB but in this case the phosphorylation of AKT is dramatically different from WT at 15 and 30 min (Supp. Figure S1) and therefore suggests a complete ablation of PDGFR β -related activation of the AKT pathway by the p.L658P mutant.

DISCUSSION

Recent advances in the genetics of IBGC have started to elucidate the causes of this disease and are providing clues to the pathogenic mechanism leading to abnormal brain calcification. *SLC20A2* mutations were identified as a major cause of familial and sporadic IBGC cases (Hsu, et al., 2013; Wang, et al., 2012). The subsequent identification of mutations in *PDGFB* (Keller, et al., 2013) and its receptor *PDGFRB* (Nicolas, et al., 2013) strongly suggest that the loss of function of the PDGF-B/PDGFR β mitogenic and survival pathway is also involved in the IBGC pathogenic process. As inclusion criteria for our study we selected patients with moderate to severe basal ganglia calcification upon pathological examination from our Mayo Clinic brain bank series; therefore, frequency of mutations in our cohort cannot be extrapolated to clinical IBGC populations. We previously performed mutation analyses of *SLC20A2* in this series and described pathogenic mutations in two of our patients (Baker, et al., 2013). The current study focused on the mutation screening of *PDGFRB* and *PDGFB*. No mutations in *PDGFB* were found; however, we identified one patient with a p.R695C mutation in *PDGFRB*. This mutation is located within the tyrosine kinase domain of PDGFR β and is predicted to be damaging. A first assessment of PDGFR β protein stability in cell culture suggested that p.R695C and also the previously described p.R987W and p.L658P mutations affected the levels of the mature 180 kDa PDGFR β form. While the p.L658P and p.R695C mutants seemed to alter the relative levels of the PDGFR β forms, the p.R987W mutant showed severely reduced total levels of PDGFR β , which was at least in part due to degradation by the proteasome.

Cell signaling via the PDGF-B/PDGFR β pathway requires the activation of PDGFR β receptor at the cell surface. Binding of PDGF ligands to PDGFR β receptor facilitates dimerization and phosphorylation in trans of the two PDGFR β monomers in the complex which result in receptor activation (Heldin, et al., 1998). The intrinsic functions of activated PDGFR β receptor are mediated by the protein tyrosine kinase domain, an intracellular domain spanning amino acid residues 562 to 953. Phosphorylation of multiple tyrosine residues at the tyrosine kinase domain creates docking sites for downstream signaling molecules such as SH2 domain containing proteins and regulates receptor autoactivation

(Heldin, et al., 1998); therefore, altered phosphorylation at the tyrosine kinase domain may potentially induce impaired signaling via the PDGF-B/PDGFR β pathway. Using phosphorylation-specific antibodies, we first analyzed how the mutations p.R695C and p.L658P located in the tyrosine kinase domain affect the phosphorylation of PDGFR β . PDGFR β carrying the p.R695C mutation was only partially phosphorylated compared to WT; in contrast, the p.L658P mutation completely suppressed PDGFR β phosphorylation. Since the p.A1096V benign variant (included as a control) was phosphorylated in a manner comparable to WT, we conclude that missense mutations in the tyrosine kinase domain of PDGFR β directly interfere with the normal autophosphorylation of the PDGFR β receptor. By exploring potential downstream effectors in the PDGFB/PDGFR β pathway we further found that the p.L658P mutant receptor has a defective activation of ERK and AKT downstream pathways. Although modest, the reduction of ERK phosphorylation in the absence of phosphorylation at Tyr1009 of the p.L658P mutant suggests deficient activation of the Ras/Gap pathway in response to PDGF-BB. Additionally, the p.L658P mutation significantly inhibited the AKT pathway at the 15 min and 30 time points. This deficient phosphorylation of AKT observed in the p.L658P mutant correlates with the lack of phosphorylation at Tyr751, which is one of the sites located in the PDGFR β kinase domain responsible for AKT activation via PI3-kinase (Fantl, et al., 1992; Franke, et al., 1995). Moreover, this defective AKT phosphorylation resembles the lack of sustainability of AKT phosphorylation after 5 min induced by inhibition of ERK5 in cells stimulated with PDGF-BB described by Lennartsson et al. (Lennartsson, et al., 2010). Since ERK phosphorylation was reduced in the p.L658P mutant our results support that, at least partially, the MAPK pathway contributes to the lack of sustained AKT phosphorylation after 5 min. In contrast, AKT was found to be overstimulated by the p.R695C mutant PDGFR β receptor but this overstimulation of AKT does not correlate with the decreased phosphorylation at Tyr751 observed in this mutant. The reason for the increased AKT phosphorylation for this mutation remains therefore unclear but could be unrelated to the PDGF-B/PDGFR β pathway. Alternatively, it is possible that mutations at this position disrupt the regulation of the activation of downstream pathways, more specifically in the Ras/Gap pathway. Together with the recent description of activating mutations in positions 660 and 666 of the receptor hypothetically inducing PDGFR β activation and tumor growth in infantile myofibromatosis (Martignetti, et al., 2013) our data related to the p.L658P and p.R695C mutations suggest that neighboring mutations in PDGFR β kinase domain may differently affect receptor activation and the activation of its downstream effectors.

Despite the recent advances in the knowledge of factors involved in the brain calcification, it remains unclear how mutations in *PDGFRB*, *PDGFB* and *SLC20A2* specifically contribute to the formation of calcifications in IBGC patients. Neuropathological studies of brain tissue of IBGC patients strongly suggest that the brain vasculature is the main target for the calcification process (Kobayashi, et al., 1987; Miklossy, et al., 2005; Wszolek, et al., 2006). More specifically, *in vitro* and *in vivo* studies point to the vascular smooth muscle cells (vSMCs) and pericytes as the main cell types involved in ectopic calcification (Giachelli, 2009); therefore, mutations in *PDGFRB*, *PDGFB* and *SLC20A2* most likely have an important effect on these vascular cells and Pi homeostasis in the brain.

To explain how *PDGFRB* mutations may be involved in the calcification observed in IBGC, Nicolas et al. proposed two alternative physiopathologic hypotheses (Nicolas, et al., 2013). First, loss-of-function of PDGFR β may impact blood-brain barrier integrity and secondarily induce vascular and perivascular calcium deposition. According to this hypothesis, the impaired integrity of the blood-brain barrier observed in IBGC (Miklossy, et al., 2005) results from abnormal function of blood vessel cells, most likely pericytes. Supporting this idea, deficient signaling through PDGF-B/PDGFR β has been shown to be highly detrimental to vSMCs and pericytes resulting in complete lack of pericytes or pericyte hypoplasia, endothelial hyperplasia, increased vessel diameter, increased vascular permeability and vessel instability (Hellström, et al., 2001; Lindahl, et al., 1997; Tallquist, et al., 2003), and impacting the normal maturation of the blood-brain barrier (Armulik, et al., 2010; Keller, et al., 2013). Brain calcification and impaired brain-blood barrier have been repeatedly found to co-occur in IBGC, but a causal link between these two processes is still missing. Importantly, our data of a loss-of-function mechanism associated with the reported *PDGFRB* mutations identified in BGC patients would provide support for this hypothesis. An alternative hypothesis has been proposed that suggests that mutations in *PDGFRB* would overactivate the receptor which would impact the PDGF-Pit-1 pathway and induce Pit-1 overactivity leading to calcifications. Different lines of evidence have provided clues for a link between Pit-1 and the PDGF-B/PDGFR β pathway in vascular calcification and support this second hypothesis. Pit-1, encoded by *SLC20A1*, is one of the two members of the type III sodium-phosphate (Na/Pi) co-transporter family. The second member of this family of transporters is Pit-2, encoded by *SLC20A2*. Pit-1 and Pit-2 share 62% homology and are both important for Pi homeostasis. Although Pit-1 and Pit-2 are expressed in a broad range of cells throughout the body, including the brain, the expression distribution is different for these two transporters. Pit-2 is predominantly expressed in neurons (Inden, et al., 2013) whereas Pit-1 is the predominant Pi transporter in vSMCs (Jono, et al., 2000). This selective expression pattern of Pit-1 together with the finding that Pi transport through Pit-1 is required for calcification of vSMCs (Jono, et al., 2000) has led to the idea that overstimulation of Pi transport through Pit-1 may underlie abnormal brain calcification. Interestingly, calcification of vSMCs in a study by Jono et al. was induced by homodimeric PDGF-BB stimulation in the same way as it is induced by increased Pi levels, directly linking PDGFR β stimulation with calcification. In addition, PDGF-B stimulation has also been shown to upregulate Pit-1 expression (Kakita, et al., 2004). Although these findings support that PDGF-B overstimulation of Pit-1 may be involved in the calcification process, our current work on *PDGFRB* and the published work on *PDGFB* mutations suggest that the identified mutations induce a loss-of-function, and are not activating mutations. However, our results cannot exclude that *PDGFRB* mutations may also exert an activating effect on *SLC20A1* by different, as of yet unknown, mechanisms.

Finally, recent studies provide support for an alternative way to explain Pit-1 overactivity as the cause of vascular calcification that may not involve an overactive PDGF-B/PDGFR β pathway. The authors found that calcification-prone mice genetically modified to carry a deletion of Pit-1 in vSMCs did not differ in the arterial medial calcification from calcification-prone mice with no deletion demonstrating that Pit-1 and Pit-2 have a redundant role in vascular cells (Crouthamel, et al., 2013). Since complementary functional

studies now suggest that mutations in *SLC20A2* result in deficient transport of Pi through Pit-2 in the plasma membrane (Wang, et al., 2012), it is possible that Pit-2 deficient activity is compensated by Pit-1 overactivity in order to maintain Pi homeostasis. Additionally, different from Pit-1, the function of Pit-2 can be regulated by the filament rearrangement induced by activation of PKC ϵ via PDGFR β stimulation (Jobbagy, et al., 1999) suggesting that deficient signaling via the PDGF-B/PDGFR β pathway may also lead to Pit-2 dysfunction and secondarily induce Pit-1 overactivity. It therefore remains possible that a combination of overactive Pit-1 transport and a deficient PDGF-B/PDGFR β /Pit-2 pathway may be responsible for brain calcification in IBGC but the currently available genetic and functional evidence is limited to fully prove this hypothesis.

In conclusion, based on the existing data, it is likely that a more elaborate pathogenic mechanism needs to be reconstructed that involves the function of Pit-2 and the PDGF-B/PDGFR β pathway in the brain vasculature and the specific role of these components in the calcification of affected brain regions in IBGC. Moreover, the study of familial and sporadic BGC cases that remain unexplained may provide important information on potential alternative pathways leading to brain calcification in IBGC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Grant Sponsor: Morris K. Udall Parkinson's Disease Research Center of Excellence (National Institute of Neurological Disorders and Stroke NINDS P50NS072187). NINDS is part of the NIH.

We would like to thank all those who have contributed to our research, particularly the patients and families who donated DNA samples for this work. This work is supported by a Morris K. Udall Parkinson's Disease Research Center of Excellence (National Institute of Neurological Disorders and Stroke P50NS072187).

References

- Andrae J, Gallini R, Betsholtz C. Role of platelet-derived growth factors in physiology and medicine. *Genes & Development*. 2008; 22(10):1276–1312. [PubMed: 18483217]
- Armulik A, Genove G, Mae M, Nisancioglu MH, Wallgard E, Niaudet C, He L, Norlin J, Lindblom P, Strittmatter K, et al. Pericytes regulate the blood-brain barrier. *Nature*. 2010; 468(7323):557–561. [PubMed: 20944627]
- Baker M, Strongosky A, Sanchez-Contreras M, Yang S, Ferguson W, Calne D, Calne S, Stoessl AJ, Allanson J, Broderick D, et al. *SLC20A2* and *THAP1* deletion in familial basal ganglia calcification with dystonia. *Neurogenetics*. 2013;1–8. [PubMed: 23142975]
- Collins J, Bai L, Ghishan F. The SLC20 family of proteins: dual functions as sodium-phosphate cotransporters and viral receptors. *Pflügers Archiv*. 2004; 447(5):647–652. [PubMed: 12759754]
- Crouthamel MH, Lau WL, Leaf EM, Chavkin NW, Wallingford MC, Peterson DF, Li X, Liu Y, Chin MT, Levi M, et al. Sodium-Dependent Phosphate Cotransporters and Phosphate-Induced Calcification of Vascular Smooth Muscle Cells: Redundant Roles for PiT-1 and PiT-2. *Arteriosclerosis, Thrombosis, and Vascular Biology*. 2013; 33(11):2625–2632.
- Fantl WJ, Escobedo JA, Martin GA, Turck CW, del Rosario M, McCormick F, Williams LT. Distinct phosphotyrosines on a growth factor receptor bind to specific molecules that mediate different signaling pathways. *Cell*. 1992; 69(3):413–423. [PubMed: 1374684]

- Franke TF, Yang S-I, Chan TO, Datta K, Kazlauskas A, Morrison DK, Kaplan DR, Tsichlis PN. The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell*. 1995; 81(5):727–736. [PubMed: 7774014]
- Giachelli CM. The emerging role of phosphate in vascular calcification. *Kidney Int*. 2009; 75(9):890–897. [PubMed: 19145240]
- Heldin C-H, Östman A, Rönstrand L. Signal transduction via platelet-derived growth factor receptors. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer*. 1998; 1378(1):F79–F113.
- Hellström M, Gerhardt H, Kalén M, Li X, Eriksson U, Wolburg H, Betsholtz C. Lack of Pericytes Leads to Endothelial Hyperplasia and Abnormal Vascular Morphogenesis. *The Journal of Cell Biology*. 2001; 153(3):543–554. [PubMed: 11331305]
- Hsu S, Sears R, Lemos R, Quintáns B, Huang A, Spiteri E, Nevarez L, Mamah C, Zatz M, Pierce K, et al. Mutations in SLC20A2 are a major cause of familial idiopathic basal ganglia calcification. *neurogenetics*. 2013; 14(1):11–22. [PubMed: 23334463]
- Inden M, Iriyama M, Takagi M, Kaneko M, Hozumi I. Localization of type-III sodium-dependent phosphate transporter 2 in the mouse brain. *Brain Research*. 2013; 1531(0):75–83. [PubMed: 23911649]
- Inui H, Kitami Y, Kondo T, Inagami T. Transduction of mitogenic activity of platelet-derived growth factor (PDGF) AB by PDGF-beta receptor without participation of PDGF-alpha receptor in vascular smooth muscle cells. *Journal of Biological Chemistry*. 1993; 268(23):17045–50. [PubMed: 8349593]
- Jobbagy Z, Olah Z, Petrovics G, Eiden MV, Leverett BD, Dean NM, Anderson WB. Up-regulation of the Pit-2 Phosphate Transporter/Retrovirus Receptor by Protein Kinase C ϵ . *Journal of Biological Chemistry*. 1999; 274(11):7067–7071. [PubMed: 10066763]
- Jono S, McKee MD, Murry CE, Shioi A, Nishizawa Y, Mori K, Morii H, Giachelli CM. Phosphate Regulation of Vascular Smooth Muscle Cell Calcification. *Circulation Research*. 2000; 87(7):e10–e17. [PubMed: 11009570]
- Kakita A, Suzuki A, Nishiwaki K, Ono Y, Kotake M, Ariyoshi Y, Miura Y, Itoh M, Oiso Y. Stimulation of Na-dependent phosphate transport by platelet-derived growth factor in rat aortic smooth muscle cells. *Atherosclerosis*. 2004; 174(1):17–24. [PubMed: 15135246]
- Kasuga K, Konno T, Saito K, Ishihara A, Nishizawa M, Ikeuchi T. A Japanese family with idiopathic basal ganglia calcification with novel SLC20A2 mutation presenting with late-onset hallucination and delusion. *Journal of Neurology*. 2014; 261(1):242–244. [PubMed: 24323245]
- Keating MT, Williams LT. Processing of the platelet-derived growth factor receptor. Biosynthetic and degradation studies using anti-receptor antibodies. *Journal of Biological Chemistry*. 1987; 262(16):7932–7937. [PubMed: 3034910]
- Keller A, Westenberger A, Sobrido MJ, Garcia-Murias M, Domingo A, Sears RL, Lemos RR, Ordonez-Ugalde A, Nicolas G, da Cunha JEG, et al. Mutations in the gene encoding PDGF-B cause brain calcifications in humans and mice. *Nature genetics*. 2013; 45(9):1077–1082. [PubMed: 23913003]
- Kobayashi S, Yamadori I, Miki H, Ohmori M. Idiopathic nonarteriosclerotic cerebral calcification (Fahr's disease): an electron microscopic study. *Acta Neuropathologica*. 1987; 73(1):62–66. [PubMed: 3604574]
- Lemos RR, Oliveira MF, Oliveira JRM. Reporting a new mutation at the SLC20A2 gene in familial idiopathic basal ganglia calcification. *European Journal of Neurology*. 2013; 20(3):e43–e44. [PubMed: 23406454]
- Lennartsson J, Burovic F, Witek B, Jurek A, Heldin C-H. Erk 5 is necessary for sustained PDGF-induced Akt phosphorylation and inhibition of apoptosis. *Cellular Signalling*. 2010; 22(6):955–960. [PubMed: 20138986]
- Lindahl P, Johansson BR, Levéen P, Betsholtz C. Pericyte Loss and Microaneurysm Formation in PDGF-B-Deficient Mice. *Science*. 1997; 277(5323):242–245. [PubMed: 9211853]
- Martignetti John A, Tian L, Li D, Ramirez MCM, Camacho-Vanegas O, Camacho SC, Guo Y, Zand Dina J, Bernstein Audrey M, Masur Sandra K, et al. Mutations in PDGFRB Cause Autosomal-Dominant Infantile Myofibromatosis. *The American Journal of Human Genetics*. 2013; 92(6):1001–1007.

- Miklossy J, Mackenzie I, Dorovini-Zis K, Calne D, Wszolek Z, Klegeris A, McGeer P. Severe vascular disturbance in a case of familial brain calcinosis. *Acta Neuropathologica*. 2005; 109(6): 643–653. [PubMed: 15937691]
- Nicolas G, Pottier C, Maltête D, Coutant S, Rovelet-Lecrux A, Legallic S, Rousseau S, Vaschalde Y, Guyant-Maréchal L, Augustin J, et al. Mutation of the PDGFRB gene as a cause of idiopathic basal ganglia calcification. *Neurology*. 2013; 80(2):181–187. [PubMed: 23255827]
- Nicolas G, Rovelet-Lecrux A, Pottier C, Martinaud O, Wallon D, Vernier L, Landemore G, Chapon F, Prieto-Morin C, Tournier-Lasserre E, et al. PDGFB Partial Deletion: a New, Rare Mechanism Causing Brain Calcification with Leukoencephalopathy. *Journal of Molecular Neuroscience*. 2014:1–5.
- Tallquist MD, French WJ, Soriano P. Additive Effects of PDGF Receptor β Signaling Pathways in Vascular Smooth Muscle Cell Development. *PLoS Biol*. 2003; 1(2):e52. [PubMed: 14624252]
- Wang C, Li Y, Shi L, Ren J, Patti M, Wang T, de Oliveira JR, Sobrido MJ, Quintans B, Baquero M, et al. Mutations in SLC20A2 link familial idiopathic basal ganglia calcification with phosphate homeostasis. *Nature genetics*. 2012; 44(3):254–6. [PubMed: 22327515]
- Williams DR, de Silva R, Paviour DC, Pittman A, Watt HC, Kilford L, Holton JL, Revesz T, Lees AJ. Characteristics of two distinct clinical phenotypes in pathologically proven progressive supranuclear palsy: Richardson's syndrome and PSP-parkinsonism. *Brain*. 2005; 128(Pt 6):1247–58. [PubMed: 15788542]
- Wszolek ZK, Baba Y, Mackenzie IR, Uitti RJ, Strongosky AJ, Broderick DF, Baker MC, Melquist S, Hutton ML, Tsuboi Y, et al. Autosomal dominant dystonia-plus with cerebral calcifications. *Neurology*. 2006; 67(4):620–625. [PubMed: 16924015]
- Yamada M, Tanaka M, Takagi M, Kobayashi S, Taguchi Y, Takashima S, Tanaka K, Touge T, Hatsuta H, Murayama S, et al. Evaluation of SLC20A2 mutations that cause idiopathic basal ganglia calcification in Japan. *Neurology*. 2014; 82(8):705–712. [PubMed: 24463626]
- Zhang Y, Guo X, Wu A. Association between a Novel Mutation in *SLC20A2* and Familial Idiopathic Basal Ganglia Calcification. *PLoS ONE*. 2013; 8(2):e57060. [PubMed: 23437308]

Figure 1. Expression of total PDGFRβ receptor in HeLa cells transfected with WT and BGC-associated mutant forms

A. Representative western blot showing total levels of PDGFRβ of WT and the p.L658P, p.R695C, p.R987W and p.A1096V mutations (top panel). Each genotype is shown in duplicates. Arrows indicate the electrophoretic mobility of the three maturation forms of PDGFRβ 180, 160 and 130 kDa. Alpha-tubulin is shown in the bottom panel as a protein loading control. **B.** Graphic representation of the total levels of PDGFRβ observed by western blot. Data represents the percentage of WT PDGFRβ. **C.** Graphic representation of the three maturation forms of total PDGFRβ observed by western blot. The percentage of the each PDGFRβ band was calculated from the total PDGFRβ for each mutation. **D.** Quantitative analysis of the levels of total PDGFRβ and PDGFRβ forms represented in B and C. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 2. Altered PDGFR β processing associated with the p.R987W mutation

A. Representative western blot showing total levels of PDGFR β of WT and the p.R987W mutant expressed from two different expression vectors (pCMV and pAAV). **B.** Quantification of mRNA levels of HeLa cells transfected with WT and p.R987W PDGFR β in two different expression vectors (pCMV and pAAV). **C.** Representative western blot showing total levels of WT and p.R987W PDGFR β transfected HeLa cells treated with 10 μ M MG-132 for 8 hours (+MG132) and non-treated (–MG132). **D.** Quantification of the levels of total PDGFR β in WT (WT –MG132) and p.R987W PDGFR β transfected HeLa cells treated with 10 μ M MG-132 for 8 hours (R987W +MG132) and non-treated transfected HeLa cells (R987W –MG132). Values represent the percentage as compared to WT PDGFR β . **p<0.01, ***p<0.001, ****p<0.0001.

Figure 3. Time-course for the phosphorylation of PDGFR β receptor and downstream signaling molecules in BGC-associated mutant forms

A. Representative western blots of transfected HeLa cells treated with PDGF-BB at 5, 15, 30 and 60 minutes blotted to detect total PDGFR β , phosphorylated PDGFR β at tyrosine 751 (p-Tyr751), 1009 (p-Tyr1009) and 1021 (p-Tyr1021), phosphorylated AKT (pAKT), total AKT, phosphorylated ERK (pERK) and total ERK. Alpha-tubulin is shown in the bottom panel as a protein loading control. **B.** Schematic representation of the quantitative data obtained by western blot of the time-dependent change in the PDGF-BB induced phosphorylation of PDGFR β and downstream pathways. Data is expressed as the fold change (average $\pm$ S.E.M.) in p-Tyr751, p-Tyr1009 and p-Tyr1021 immunoreactivity compared to total PDGFR β 180 kDa band, pAKT fold change compared to total AKT and pERK fold change compared to total ERK. Significant changes from WT are indicated by * for p.L658P and \$ for p.R695C. */\$ $p<0.05$, **/\$\$ $p<0.01$, ***/\$\$\$ $p<0.001$, ****/\$\$\$\$ $p<0.0001$.