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Underestimated disease prevalence and severe phenotypes in patients with biallelic variants: A cohort study of primary familial brain calcification from China

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ABSTRACT

Background: Primary familial brain calcification (PFBC) is a rare calcifying disorder of the brain with extensive clinical and genetic heterogeneity. Its prevalence is underestimated due to clinical selection bias (compared with symptomatic PFBC patients, asymptomatic ones are less likely to undergo genetic testing).
Methods: A total of 273 PFBC probands were enrolled in a multicenter retrospective cohort study by two different approaches. In Group I (nonsystematic approach), 37 probands diagnosed at our clinic were enrolled. In Group II (systematic approach), 236 probands were enrolled by searching the medical imaging databases of 50 other hospitals using specific keywords. Genetic testing of four genes known to be causative of autosomal dominant PFBC was performed in all probands using cDNA. All identified variants were further confirmed using genomic DNA and classified according to ACMG-AMP recommendations.
Results: Thirty-two variants including 22 novel variants were detected in 37 probands. Among these probands, 83.8% (31/37) were asymptomatic. Two probands with homozygous pathogenic *SLC20A2* variants presented more severe brain calcification and symptoms. Based on the variant detection rate of probands in Group II, we extrapolated an overall minimal prevalence of PFBC of 6.6 per 1,000, much higher than previously reported (2.1 per 1000).
Conclusions: We identified a higher proportion of genetically confirmed PFBC probands who were asymptomatic. These patients would be overlooked due to clinical selection bias, leading to underestimation of the disease prevalence. Considering that PFBC patients with biallelic variants had more severe phenotypes, this specific condition should be focused on in genetic counseling.

1. Introduction

Primary familial brain calcification (PFBC), also known as Fahr's disease or idiopathic basal ganglia calcification, is a rare neurodegenerative disease with bilateral brain calcification mainly affecting the basal ganglia. The clinical manifestations include movement disorders, neuropsychiatric symptoms, and other associated symptoms with diverse severity, while up to 36%–42% of patients remain asymptomatic [1–3]. Typically, PFBC is inherited as an autosomal dominant trait with four causative genes (*SLC20A2*, *PDGFRB*, *PDGFB*, and *XPR1*) identified so far [4–7]. Recently, biallelic mutations in the *MYORG* gene have also been identified as a novel genetic cause of autosomal recessive PFBC (AR-PFBC) [8,9]. Nicolas et al. estimated that the minimal prevalence of PFBC caused by a variant in one of the four causative genes for autosomal dominant PFBC (AD-PFBC) was 4.5 per 10,000 and further extrapolated an overall minimal prevalence of PFBC of 2.1 per 1,000, which is much higher than previously thought [10]. However, the high proportion (36%–42%) of asymptomatic PFBC patients [2,3] and clinical selection bias (compared with symptomatic PFBC patients, asymptomatic ones are less likely to undergo a brain CT scan and be diagnosed genetically) might still undermine our ability to accurately estimate the disease prevalence.

In this study, we attempted to minimize clinical selection bias and collected 273 unrelated Chinese PFBC probands (including 24 probands with a positive family history) by two different approaches (see Study design). We performed genetic screening of four genes causative of AD-PFBC to help elucidate the clinical features of genetically confirmed patients and estimate the minimal disease prevalence of PFBC.

2. Methods

2.1. Study design

In this multicenter retrospective cohort study, patients were

collected by two different approaches (from March 1, 2016, through October 1, 2018). In Group I (nonsystematic approach), 37 probands diagnosed at the Neurology Clinic of the Second Affiliated Hospital of Zhejiang University School of Medical were enrolled. In Group II (systematic approach), 236 probands were enrolled by searching the medical imaging databases of 50 other hospitals using specific keywords (see online Supplementary Methods). All patients met the following criteria for the diagnosis of PFBC: a) bilateral and symmetrical calcifications present in basal ganglia and/or dentate nucleus on brain CT scans; b) the total calcification score (TCS) rated above the age-specific thresholds; c) normal assessments of calcium, phosphate, and parathyroid hormone (PTH) in blood; and d) absence of other known etiology [2,11,12]. Demographic, clinical, and imaging (brain CT scan) data were evaluated by at least two different investigators.

This study was approved by the Ethics Committee of the Second Affiliated Hospital of Zhejiang University School of Medicine. Written informed consent was obtained from all participants.

2.2. Clinical data

Detailed clinical data were collected, including age at clinical onset (only for symptomatic patients), age at examination (brain CT scan), sex, PFBC-associated symptoms, and the reason why brain CT scans were performed (see online Supplementary Tables S1–S4). Patients were considered "symptomatic" only when they presented with at least one PFBC-associated symptom as listed by Manyam et al. [1]. For the brain CT scans, TCS was evaluated independently by two different investigators and discussed to reach a consensus if there was any disagreement [11].

2.3. Genetic screening

Blood samples of PFBC probands and their available family members were collected. Total RNA was extracted and cDNA was synthesized using PrimeScript™ 1st Strand cDNA Synthesis Kit (TaKaRa), in accordance with the kit's manual. Genomic DNA of the participants was extracted from peripheral blood leukocytes using phenol and chloroform extraction. We used the following transcript and genomic references (hg19) for variant nomenclature and exon numbering: NM_006749.4 for *SLC20A2*, NM_002608.2 for *PDGFB*, NM_002609.3 for *PDGFRB*, and NM_004736.3 for *XPR1*. A two-step genetic screening strategy was designed: 1) PCR amplification of four genes was performed using cDNA samples with two to five pairs of primers (see online Supplementary Methods). PCR products were purified and then directly sequenced using a standard method. 2) Candidate variants identified in the first step were verified by Sanger sequencing or quantitative PCR using the genomic DNA. Co-segregation analysis of verified variants was performed on all available family members.

2.4. Variant assessment

All variants were classified following ACMG-AMP recommendations

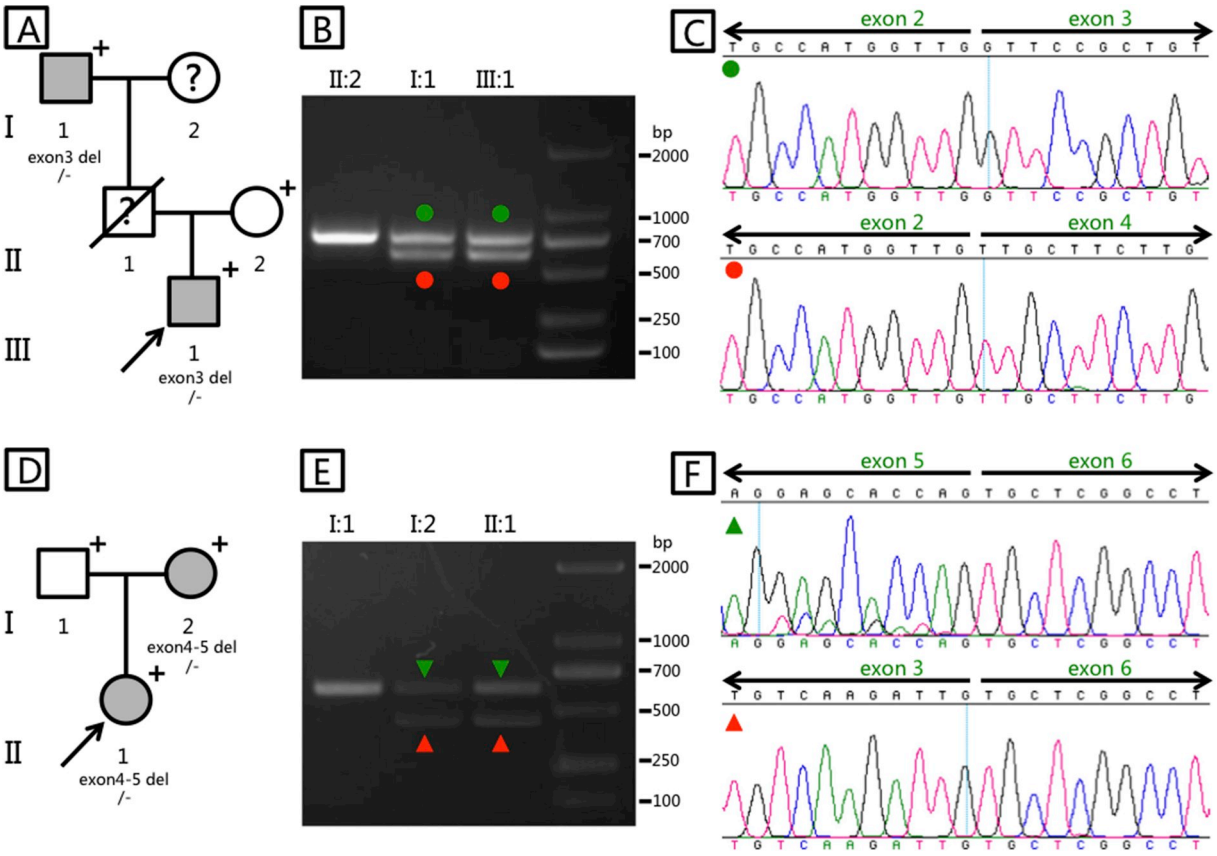

Fig. 1. Sanger sequencing of cDNA found two exonic deletions in the *SLC20A2* gene. A and D, Pedigrees of families GI-F12 and GI-F13. The arrow indicates the proband. Gray symbols represent asymptomatic PFBC patients, while unfilled symbols represent individuals without brain calcification on brain CT scans. "+" indicates individuals whose DNA and RNA samples were available. "?" indicates that brain CT scans of those individuals were unavailable. B and E, PCR amplification of the *SLC20A2* gene (exons 2–4 and exons 3–7) using cDNA samples from probands and available members of families GI-F12 and GI-F13 showed mutant alleles (red spots and triangles) and normal alleles (green spots and triangles) in the gel diagram. C and E, Sanger sequencing of mutant alleles and normal alleles showed exon 3 deletion [c.(289 + 1_290–1)_(429 + 1_430–1)del] (red spot) in family GI-F12 and exon 4–5 deletion [c.(430 + 1_431–1)_(613 + 1_614–1)del] (red triangle) in family GI-F13, which co-segregated with the disease in these families. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

[13], which included prior identification as a variant causative of PFBC (reported in the literature, HGMD, Clinvar, and/or the PFBC variant database https://coppolalab.ucla.edu/lovd/genes), allele frequency in population databases (gnomAD) [14], computational and predictive data (Polyphen2, SIFT, MutationTaster), functional studies (cDNA sequencing data evaluating whether variants affected splicing in this study and other functional studies reported in the literature), and segregation data. Each variant was classified into one of the five ACMG-AMP classes by two investigators independently and discussed to reach a consensus if there was any disagreement.

2.5. Statistical analysis

Minimal disease prevalence with 95% exact confidence interval (CI) was estimated based on the variant stringency criteria (A, B, or C) [10]. Variant detection rates in Group I, Group II, and Groups I + II were

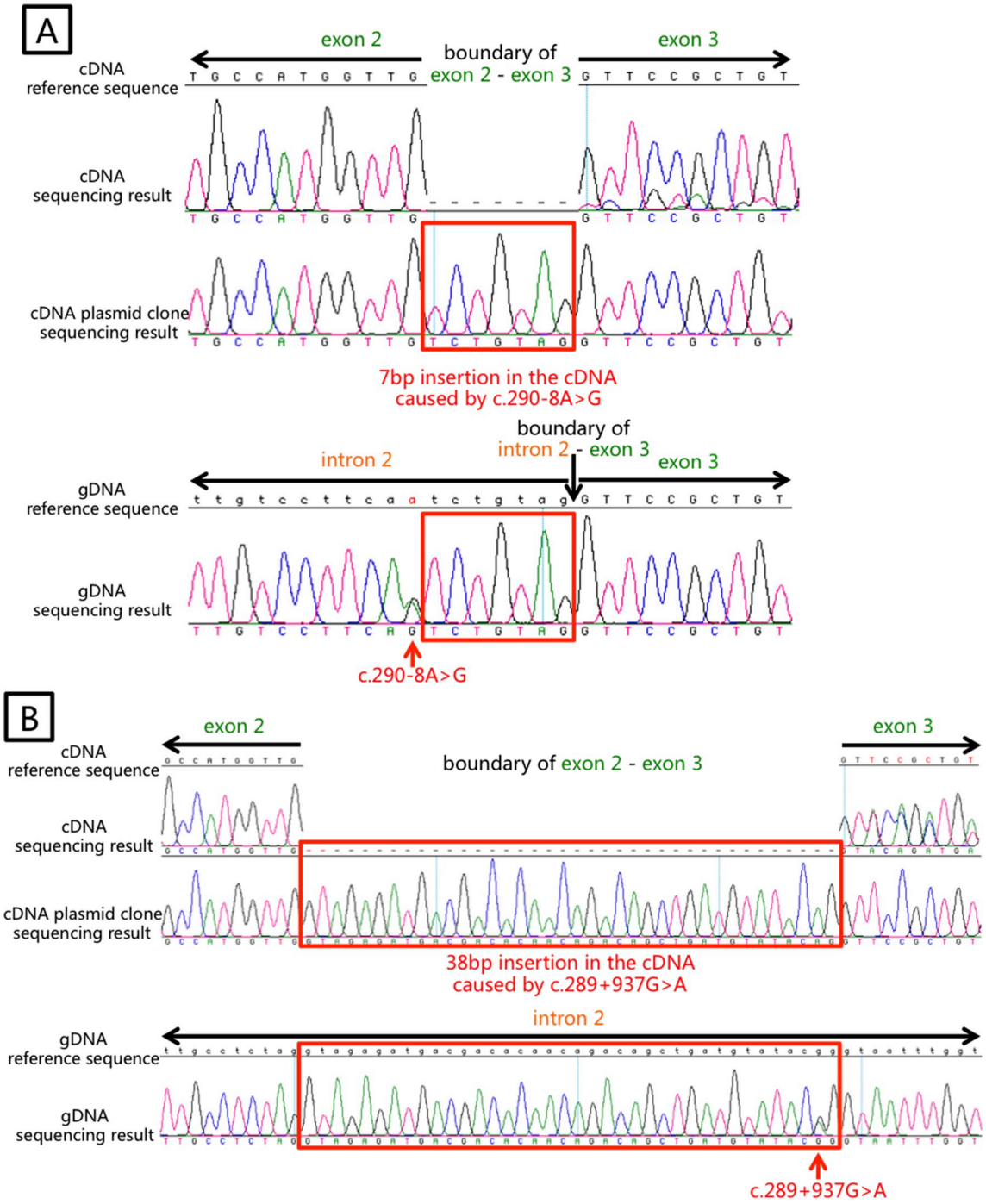

Fig. 2. Sanger sequencing of cDNA and genomic DNA revealed two intronic splicing site variants in the *SLC20A2* gene. A, Sanger sequencing of cDNA and genomic DNA (GI-F3-II:1) identified a cryptic intronic splicing site variant (c.290-8A > G) (red arrow), which produced a 7-bp intronic insertion (red box) between exon 2 and exon 3, and resulted in a truncated protein (p.G97Afs*163). B, Sanger sequencing of cDNA and genomic DNA (GII-F24-P) identified a cryptic deep intronic splicing site variant (c.289 + 937G > A) (red arrow), which led to a 38-bp intronic insertion (red box) between exon 2 and exon 3, and produced a truncated protein (p.S98Rfs*9). Nonsense-mediated mRNA decay might occur with reduced peaks corresponding to the mutant allele in the sequence reads of cDNA sequencing. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

used to estimate the minimal overall prevalence of PFBC.

Covariance analysis (SPSS software, version 21; SPSS, Inc., Chicago, IL) involving TCS, age at examination, and sex was performed to investigate the phenotypic difference between the genetically confirmed PFBC probands from Group I and Group II. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Mutational spectrum of AD-PFBC genes

By genetic screening of the four genes causative of AD-PFBC in 273 unrelated probands, we identified 37 probands (37/273, 13.6%) carrying a variant classified as pathogenic (class 5) (16/32, 50.0%), likely pathogenic (class 4) (8/32, 25.0%), or of uncertain significance (VUS) (class 3) (8/32, 25.0%) (see online Supplementary Tables S1–S4). Among these variants, those in *SLC20A2* made the highest contribution (30/32, 93.8%), followed by *PDGFB* (1/32, 3.1%) and *XPR1* (1/32, 3.1%). No variant in *PDGFRB* was detected. Four variants, c.290-8A > G (n = 2) c.660_660delT (n = 2), c.1157_1158insA (n = 3), and c.1876T > A (n = 2) in *SLC20A2*, were detected repeatedly in unrelated probands.

SLC20A2 was the main genetic cause of PFBC in our study, with 30 variants identified in 35 probands. Two exonic deletions [c.(289 + 1_290–1)_(429 + 1_430–1)del and c.(430 + 1_431–1)_(613 + 1_614–1)del] were detected using cDNA (Fig. 1) and further verified by quantitative PCR in genomic DNA (data not shown). In three probands' cDNA, a 7-bp intronic insertion (GI-F3-II:1 and GII-F4-P) and a 38-bp intronic insertion (GII-F24-P) were detected between exon 2 and exon 3 (Fig. 2). A reported splicing site variant (c.290-8A > G, p.G97Afs*163) and a novel deep intronic splicing site variant (c.289 + 937G > A, p.S98Rfs*9) were detected in the genomic DNA and considered as the causes of the intronic insertions identified in the cDNA (Fig. 2). Ten of the *SLC20A2* variants detected in our study were previously reported in PFBC patients, including five variants (three missense variants, one splicing site variant, and one exonic deletion) whose pathogenicity could be upgraded from likely pathogenic to pathogenic: c.290-8A > G and c.(430 + 1_431–1)_(613 + 1_614–1)del, or from VUS to likely pathogenic: c.248C > T, c.1711G > A, and c.1790G > A [4,11,12,15–21]. Twenty novel variants were identified in our study, including ten pathogenic ones, three likely pathogenic ones, and seven VUSs. The genetic screening of three other genes causative of AD-PFBC revealed only one previously reported likely pathogenic variant in *PDGFB* and one novel VUS in *XPR1*.

Notably, two different novel homozygous pathogenic variants (c.1168A > G and c.1765G > A) in *SLC20A2* were detected in two unrelated probands, both from consanguineous families (Fig. 3). Detailed clinical and neuroradiological data of the probands and family members are described here.

3.2. Phenotypes of patients with biallelic mutations in SLC20A2

Proband ZY (family GI-F6, II:5): The proband ZY (GI-F6-II:5) of family GI-F6 was a 59-year-old woman born to a consanguineous family (Fig. 3A). She presented bradykinesia and gait unsteadiness for 5 years, and gradually developed festinating gait, which caused several falls. She also exhibited mild cognitive decline as measured by Mini-Mental State Examination (MMSE score: 17). Neurological examination revealed wide-based gait and tandem gait difficulty. There were no pathological reflexes. Her brain CT scan revealed severe calcification at the bilateral basal ganglia, thalamus, dentate nuclei, subcortical white matter, and cortex (TCS: 56) (Fig. 3B). The serum calcium, phosphate, and PTH levels were all within the normal range. Her elder brother (GI-F6-II:1) was reported to have presented dysarthria, dysphagia, and gait unsteadiness, while her younger brother (GI-F6-II:7) was reported to have exhibited bradykinesia and gait unsteadiness (Fig. 3A). However, both of these symptomatic brothers were deceased, and no brain CT scans or blood samples for them were available. A homozygous variant (c.1168A > G, p.T390A) in *SLC20A2* was detected in the proband ZY. Two elder sisters (GI-F6-II:2 and GI-F6-II:3), and this proband's son (GI-F6-III:1) and daughter (GI-F6-III:2) all carried the heterozygous variant. They were all diagnosed as having asymptomatic PFBC with mild brain calcification on brain CT scans (Fig. 3B).

Proband WG (family GI-F9, II:2): The proband WG (GI-F9-II:2) of family GI-F9 was a 64-year-old woman born to a consanguineous family (Fig. 3C). She presented moderate parkinsonism (bradykinesia and rigidity) predominantly affecting her right side for 3 years. She developed hand tremor 1 year before consultation. She also had slurred

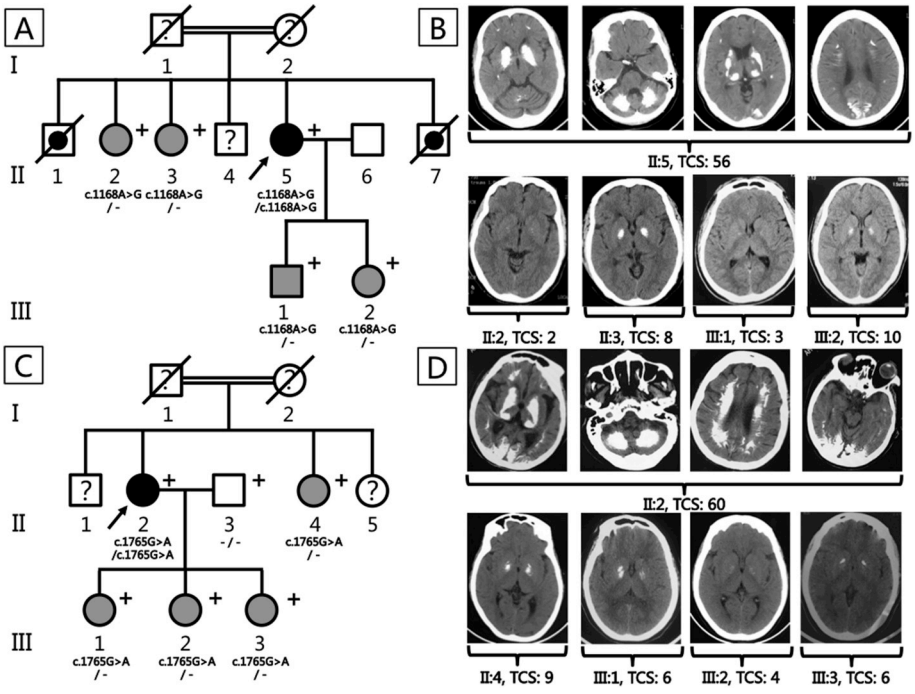

Fig. 3. Two consanguineous families with PFBC probands carrying homozygous pathogenic variants in the *SLC20A2* gene. A and C, Pedigrees of families GI-F6 and GI-F9. The arrow indicates the proband. Black symbols represent symptomatic PFBC patients. Gray symbols represent asymptomatic PFBC patients, while unfilled symbols represent individuals without brain calcification on brain CT scans. "+" indicates individuals whose DNA and RNA samples were available. "?" indicates that brain CT scans of those individuals were unavailable. A black spot indicates deceased individuals who were reported to present PFBC-associated symptoms but had no brain CT scans available. B and D, Brain CT scans and calculated TCS of all probands and their family members whose brain CT scans were available.

speech, a gradual decline in memory, and changes in mood. Neurological examination revealed resting, postural, and action tremors in both hands, bradykinesia, and shuffling gait. Her brain CT scan demonstrated severe calcification at bilateral basal ganglia, dentate nuclei, cortex, thalamus, and subcortical white matter (TCS: 60) (Fig. 3D). The serum calcium, phosphate, and PTH levels were all within the normal range. A homozygous variant (c.1765G > A, p.G589R) in *SLC20A2* was detected in GI-F9-II:2. One of this proband's younger sisters (GI-F9-II:4) and her three daughters (GI-F9-III:1, GI-F9-III:2, and GI-F9-III:3) all carried the heterozygous variant (Fig. 3C). They were all diagnosed as having asymptomatic PFBC with mild brain calcification on brain CT scans (Fig. 3D).

Both novel variants could be classified as pathogenic by adding the evidence that patients with homozygous variants had more severe symptoms and higher TCS than their family members who were heterozygous.

3.3. Estimation of minimal disease prevalence of PFBC

As we used the same criteria for inclusion in this study as applied by Nicolas et al. [11,13], we attempted to extrapolate the overall minimal prevalence of PFBC using the same method as Nicolas et al. did [10]. For the analysis of variant detection rates in Group I, two probands with exon deletions were regarded as undetected, as genomic deletions were not available for gnomAD. For the analysis of variant detection rates in Group II, GII-F4-P with a deep intronic variant was regarded as undetected because no deep intronic variants were evaluated in the study by Nicolas et al. [10]. The estimated minimal overall prevalence (95% CI) in Group I was 1.7 per 1000 (1.5–1.9 per 1000) (Table 1), which was similar to the previously reported rate of 2.1 per 1000 (1.9–2.4 per 1000)[10]. In Group II, thought to suffer less from clinical selection bias, the estimated minimal overall prevalence (95% CI) was 6.6 per 1000 (6.2–7.0 per 1000) (Table 1).

3.4. Clinical features of genetically confirmed PFBC patients

Co-segregation analysis was performed on all available family members. In total, these variants were detected in 54 individuals (37 probands and 17 family members from 11 families). All family members carrying the variants were asymptomatic and had TCSs above their age-specific threshold, except one carrier of c.1168A > G (GI-F6-II:2, 66 years old), who had very mild brain calcification (TCS: 2). PFBC-associated symptoms were also uncommon in our probands (16.2%, 6/37), being found only in four probands from Group I (30.8%, 4/13) and two probands from Group II (8.3%, 2/24). In total, only 11.1% (6/54) of the genetically confirmed PFBC patients exhibited PFBC-associated symptoms. Excluding the two probands with homozygous pathogenic variants described above, the other probands' PFBC-associated symptoms were parkinsonism (n = 2), epilepsy (n = 1), and dysarthria (n = 1). Headache (13/37, 35.1%) was the most common cause of probands attending hospital and undergoing a brain CT scan, followed by trauma (7/37, 18.9%), dizziness (3/37, 8.1%), syncope (2/37, 5.4%), stroke (2/37, 5.4%), and other factors (diabetes, nasosinusitis, and health checkup).

As PFBC-associated symptoms were relatively uncommon, which could not perform reliable statistical analysis on them, so we chose TCS as a reliable index for further analysis. A covariance analysis was used to analyze the factors potentially influencing TCS. Previous studies suggested that TCS might be affected by age and sex. Age was thus included as a covariate and sex was considered as one of the factors in the model. The results revealed that sex was not related to TCS ($p = 0.78$), while there was a statistically significant correlation between TCS and age at examination ($p = 0.002$) (Fig. 4A). Regardless of the influence of age at examination, the TCS of probands (n = 13) from Group I was significantly higher than in those from Group II (n = 23, GII-F22-P's TCS was not available) (32.08 ± 17.33 vs. 21.43 ± 14.59,

Table 1
Estimation of the minimal prevalence of PFBC in Group I, Group II, and Groups I + II.

	Variant detection rates (%)	Variant stringency category A	Variant stringency category B	Variant stringency category C
Minimal prevalence of PFBC due to a variant in four known AD-PFBC genes [95%CI][7]		4.5 p. 10,000 [3.4–5.5 p. 10,000]	6.9 p. 10,000 [5.6–8.4 p. 10,000]	8.9 p. 10,000 [7.4–1.1 p. 10,000]
Minimal overall prevalence of PFBC [95%CI] in Group I (n = 37)	27.0% (10/37) for A and B, 29.7% (11/37) for C	1.7 p. 1000 [1.5–1.9 p. 1000]	2.6p. 1000 [2.3–2.8 p. 1000]	3.0 p. 1000 [2.7–3.3 p. 1000]
Minimal overall prevalence of PFBC [95%CI] in Group II (n = 236)	6.8% (16/236) for A and B, 9.7% (23/236) for C	6.6 p. 1000 [6.2–7.0 p. 1000]	10.2 p. 1000 [9.7–10.8 p. 1000]	9.2 p. 1000 [8.7–9.7 p. 1000]
Minimal overall prevalence of PFBC [95%CI] in Groups I + II (n = 273)	9.5% (26/273) for A and B, 12.5% (34/273) for C	4.7 p. 1000 [4.3–5.1 p. 1000]	7.3 p. 1000 [6.8–7.7 p. 1000]	7.2 p. 1000 [6.7–7.6 p. 1000]
Minimal overall prevalence of PFBC [95%CI] by Nicolas et al. [7]. (n = 90)	21.1% for (19/90) A and B, 27.8% (25/90) for C	2.1 p. 1000 [1.9–2.4 p. 1000]	3.3 p. 1000 [3.0–3.6 p. 1000]	3.2 p. 1000 [2.9–3.5 p. 1000]

Minimal overall prevalence of PFBC = Minimal prevalence of PFBC due to a variant in four known AD-PFBC genes/Variant detection rate.

$p = 0.004$) (Fig. 4B).

4. Discussion

To date, four genes causative of AD-PFBC and one of AR-PFBC have been identified [4–8]. A large number of genetically confirmed PFBC patients have been reported, which greatly improves our understanding of the disease. PFBC is a genetically and clinically heterogeneous disorder, the clinical picture of which is further confounded by the following factors: 1) diverse associated symptoms, some of which are difficult to evaluate, and 2) a large proportion of asymptomatic patients who remain unidentified. Compared with symptomatic PFBC patients, the asymptomatic carriers are less likely to be genetically identified, which might lead to overestimation of the clinical penetrance (57.9%–81.5%) and underestimation of the prevalence [2,3,12]. These problems limit our understanding of the clinical features and prevalence of PFBC.

4.1. High proportion of asymptomatic genetically confirmed PFBC patients

Compared with the full penetrance (100%) of the imaging phenotype, the penetrance of the clinical phenotype (symptomatic) in PFBC varied from 57.9% to 81.5% [1–3,11,12]. The proportion of symptomatic genetically confirmed patients was mainly affected by how many family members were included in studies [2,12]. Different proportions (81.5% vs. 57.9%) of symptomatic genetically confirmed patients were reported with different numbers of enrolled family members carrying pathogenic variants (11/56 vs. 35/57)[2,12]. The inevitable problem is that clinical selection bias makes asymptomatic patients less likely to be genetically identified. In China, brain CT scanning is a widely used technique for diagnosis in a clinical context, especially in primary hospitals lacking magnetic resonance imaging (MRI) machines. Patients consulting for a headache or dizziness undergo brain CT scans as a routine examination in many hospitals. This particular medical environment allows us to enroll PFBC patients with relatively low clinical selection bias.

As such, in this study, we considered that the PFBC probands in Group II were enrolled with less clinical selection bias and could be a better representative cohort of the overall population. We supposed that there was lower penetrance of the clinical phenotype in probands from Group II, but were still surprised to find that the proportion of symptomatic genetically confirmed probands was only 8.3% (2/24). The proportion of symptomatic genetically confirmed probands in Group I was 30.8% (4/13), higher than that in Group II, but still lower than previously reported [2,3,11,12]. Taking all probands and their family members as a whole, up to 88.9% (48/54) of genetically confirmed PFBC patients were asymptomatic. Such high proportions (91.7% among probands from Group II and 88.9% among all patients) indicate that numerous asymptomatic PFBC patients remain unidentified in the general population, being undiagnosed or overlooked in hospitals even after undergoing a brain CT scan.

4.2. Objectively estimated disease prevalence with less clinical selection bias

In our clinical practice in China, brain calcification can be coincidentally identified in many patients with other unrelated neurological (headache, dizziness) or non-neurological symptoms (trauma) by a brain CT scan. This indicates that PFBC was an underestimated disease because of the incomplete penetrance of its clinical phenotype (57.9%–81.5% in previous reports and extremely low in this study, at only 8.3%) [2,3,11,12]. Nicolas et al. reported the minimal prevalence of 2.1 per 1,000, which improved our understanding of the prevalence of PFBC [10]. However, owing to the clinical selection bias, 92.0% (23/25) of probands in their cohort exhibited PFBC-associated symptoms [10,12]. This bias might have led to underestimation of the true prevalence. In this study, PFBC probands in Group II constituted a large PFBC cohort (n = 236) with less clinical selection bias. Using the detection rates of PFBC probands in Group II, we estimated a relatively objective minimal disease prevalence rate of 6.6 per 1,000, which was three times that estimated by Nicolas et al. [10]. Using the detection rates of PFBC probands in Group I, we extrapolated a minimal disease prevalence of 1.7 per 1000. Two factors might explain the lower estimated minimal disease prevalence of Group I: 1) the sample size was relatively small as only 37 probands were enrolled; and 2) probands from Group I had more severe symptoms and brain calcification, leading to a higher detection rate. This second factor was supported by a higher proportion of genetically confirmed probands from Group I (30.8%, 4/13) exhibiting PFBC-associated symptoms than from Group II (8.3%, 2/24). The covariance analysis involving TCS, different approaches (Group I and Group II), age at examination, and sex also revealed a significantly higher TCS in genetically confirmed probands from Group I than in those from Group II. These results suggested that 6.6 per 1000 is a more objective minimal disease prevalence with minimized clinical selection bias compared with 1.7 per 1000 estimated for Group I and 2.1 per 1000 estimated by Nicolas et al. [10].

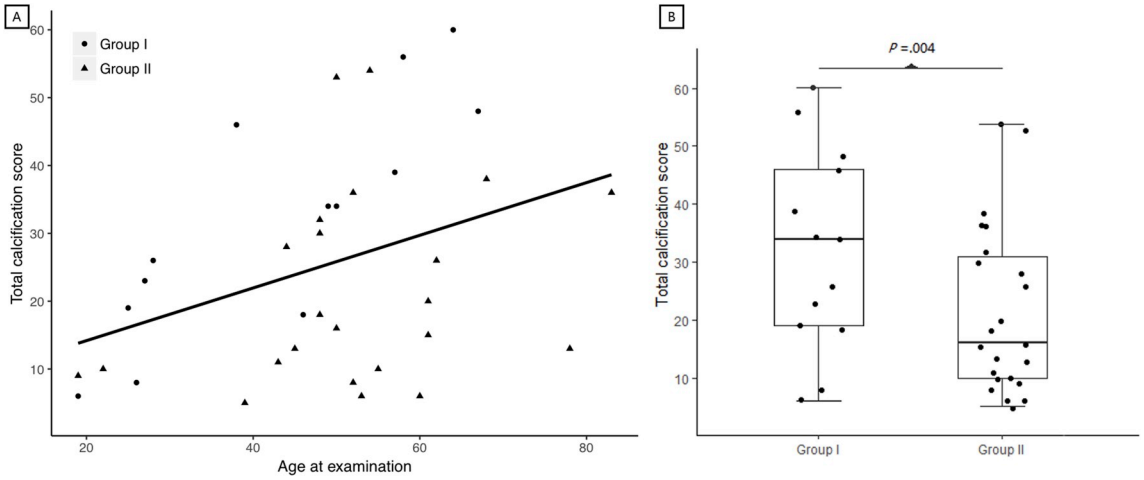

Fig. 4. Statistical analysis of TCS and its potential influencing factors. A, Scatterplot of TCS (y-axis) and age at examination (x-axis) of probands from Group I (spots) and Group II (triangles). A statistically significant correlation between TCS and age at examination ($p = 0.002$) was revealed. B, Covariance analysis including the age at examination as a covariate revealed significantly higher TCS in probands from Group I (32.08 ± 17.33, n = 13) than in those from Group II (21.43 ± 14.59, n = 23) ($p = 0.004$).

4.3. PFBC patients with biallelic pathogenic variants in SLC20A2

Two probands (GI-F6-II:5 and GI-F9-II-2) in our cohort were detected with homozygous pathogenic variants in *SLC20A2*. They both presented parkinsonism and had severe calcification on brain CT scans (TCS: 56 and 60). Both probands came from consanguineous families. Their family members with heterozygous pathogenic variants were asymptomatic with mild calcification (TCS: 2–10). Very rarely, PFBC patients with biallelic mutations have been reported. In our previous report, two siblings with compound heterozygous *SLC20A2* pathogenic variants (c.1802C > G and c.362C > G) were described as presenting recurrent seizures and mental retardation since infancy, with extremely severe brain calcification [4,22]. PFBC probands with biallelic *SLC20A2* pathogenic variants in the current study had relatively moderate symptoms and later ages of onset compared with their siblings with compound heterozygous *SLC20A2* mutations. The different phenotypes might have been caused by the different effects of these missense variants on protein function, as family members with heterozygous pathogenic variants in this study showed less brain calcification than the previously reported siblings. All known PFBC patients with biallelic *SLC20A2* pathogenic variants revealed extremely severe brain calcification compared with their family members with a heterozygous variant. Most family members (13/14, one family member of the siblings presented parkinsonism at 73 years of age) were asymptomatic and had mild brain calcification, which indicated that the second mutation could drastically increase the severity of the disease.

Considering the much more severe phenotypes in patients with biallelic pathogenic variants and our newly estimated disease prevalence of 6.6 per 1,000, it is worth performing genetic screening in more PFBC patients (symptomatic and asymptomatic) and even in the general population, to offer more appropriate genetic counseling for better decision-making.

4.4. Sanger sequencing in PFBC patients' cDNA

Exonic deletion was previously reported in *SLC20A2* and *PDGFB*. To detect all different variant types by an efficient and economic procedure, we designed primers covering all cDNA sequences of four AD-PFBC-related genes. In addition to two exonic deletions, two splicing site variants were unexpectedly detected. The variant c.290-8A > G was previously reported and predicted to result in the loss of the wild-type 3′ splicing acceptor site. Our Sanger sequencing of cDNA in GI-F7-II:1 and GII-F4-P found a 7-bp intronic insertion between exon 2 and exon 3, which was caused by the splicing variant c.290-8A > G. This proved that c.290-8A > G did not cause the loss of the wild-type 3′ splicing acceptor site as predicted by computational tools. In fact, this variant offered a new 3′ splicing acceptor site, which caused a frameshift (p.G97Afs*163). Interestingly, in GII-F24-P, a 38-bp intronic insertion was found between exon 2 and exon 3. A deep intronic variant c.289 + 937G > A was found in the 3′ boundary of this 38-bp intronic insertion. This variant was absent from dbSNPs and gnomAD and the 38-bp intronic nucleotide insertion would cause a frameshift (p.S98Rfs*9). To our knowledge, such a deep intronic variant causing a 38-bp intronic insertion in the cDNA and resulting in a frameshift is reported here for the first time in PFBC patients. Our findings show that Sanger sequencing of PFBC patients' cDNA is efficient and convenient for variant detection.

This study had several limitations. First, regarding the diagnostic criteria of PFBC, we used the same TCS age-specific thresholds as established by Nicolas et al. [11]. However, the age-specific thresholds of TCS are based on a non-Chinese population, which might cause inclusion bias. Second, as a multicenter retrospective cohort study, quality control of the evaluation of clinical symptoms was not feasible, especially in primary hospitals. To reduce the influence of incomplete clinical information, at least one investigator conducted face-to-face medical interviews and physical examinations of the patients in the clinic. Third, Sanger sequencing of cDNA might miss some large deletions spanning multiple exons that could not be covered by our cDNA primers. So far, only a few studies have reported exonic deletion in *SLC20A2* and *PDGFB*. Compared with DNA sequencing, RNA sequencing would miss fewer genomic rearrangements. However, this would not cause bias in the estimation of minimal disease prevalence as we did not include genomic deletions for estimation. Moreover, nonsense-mediated mRNA decay (NMD) might lead to truncated mutations being missed in cDNA sequencing. However, the impact of NMD on the mRNA level is variable and, in most cases, does not necessarily result in 100% decay [23–25]. Actually, variants that might undergo NMD could still be detected by cDNA sequencing in the current study (Fig. 2A) and previous ones [26,27]. Further studies comparing the results of gDNA and cDNA sequencing would help to reveal the influence of NMD in this sequencing strategy.

In conclusion, in this multicenter retrospective cohort study with low clinical selection bias (Group II), we identified a higher proportion of genetically confirmed PFBC probands that exhibited no PFBC-associated symptoms. Overall minimal prevalence of PFBC of 6.6 per 1000 was extrapolated, which is much higher than the previously reported rate of 2.1 per 1,000[10]. Considering that PFBC patients with biallelic variants had more severe phenotypes, it would be worth being aware of this specific condition in genetic counseling.

Conflicts of interest

None declared.

Ethical statement

The study was approved by the Ethics Committee of the Second Affiliated Hospital of Zhejiang University School of Medicine. Written informed consent was obtained from all participants.

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Authors' contributors

SC, ZDC, YC, and WL contributed to the study concept and design; SC, ZDC, YC, XHC, FF, DHY, HTW, HWW, XSZ, FX, WGT, SHZ, LLY, YQZ, PYM, XZZ, HWZ, FFJ, KYZ, JPH, DHZ, HQM, DQS, ZPZ, YL, QG, YKS, XXW, HYT, CLW, and WQC contributed to the acquisition and analysis of data; SC, ZDC, YC, and XHC contributed to drafting of the manuscript; ZDC, YC, WL, and JYL contributed to critical revision of the manuscript for important intellectual content; SC, XHC, ZDC, and DHY contributed to statistical analysis; WL, ZDC, FF, and JYL contributed to obtaining funding; WL, ZDC, JYL, BRZ, YC, and ZYOY contributed to administrative, technical, or material support; and WL, ZDC, and JYL contributed to study supervision.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.parkreldis.2019.04.009.

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