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Original Article

An inbred family with pulmonary alveolar microlithiasis in China: a genome-wide SNP study

Duchao Zhang[1*], Kun Xiao[1*], Wei Guan[1*], Xiaohong Hu[2], Peng Yan[1], Lixin Xie[1]

[1]*Department of Pulmonary & Critical Care Medicine Chinese PLA General Hospital, Beijing, China;* [2]*Department of Pediatrics, Hospital Affiliated to Chinese PLA General Hospital (304 Hospital), Beijing, China.* [*]*Co-first authors.*

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Abstract: Pulmonary alveolar microlithiasis (PAM) is a rare genetic disease that is characterized by the accumulation of calcium phosphate deposits in the alveolar spaces of the lung. The clinical characteristics of the patients with PAM in Mainland China were analyzed, and a high-density single nucleotide polymorphism (SNP) was used to analysis genome-wide of the patients' genomic DNA. The two patients were sisters of an inbred family whose parents were cousins and presented typical manifestation of recurrent cough, progressive dyspnea. High resolution computed tomography (HRCT) demonstrated the pulmonary was full of high density reflection of intraalveolar microliths especially in double lower lobe, and calcification was found in the pericardial, aorta and pleural. We found homozygous mutation of the SLC34A2 gene, c.910A>T (p.K304X) in exon 8 in two patients, and heterozygous mutation in consanguineous marriage of parents and the other family members. We concluded that a patient with an inbred family history and typical radiological features of high density intra-alveolar microlith, PAM should be highly suspected. The homozygous mutation in SLC34A2 gene, leading to a premature stop codon and a truncated protein, was responsible for PAM in the inbred family.

Keywords: Pedigree research, pulmonary alveolar microlithiasis, whole exon sequencing, single nucleotide polymorphism

Introduction

Pulmonary alveolar microlithiasis (PAM) is a rare chronic lung disease with many microliths of calcium phosphate accumulate in intra-alveolar [1]. Many patients with asymptomatic or only minor recurrent cough have normal pulmonary function or a mild restrictive pattern. Typical chest radiograph reveals sand-like micronodulation of calcified densities bilaterally, mainly in the middle and lower zones [2-4]. Recently, mutation of SLC34A2 gene, which encodes the sodium phosphate co-transporter (NaPi-IIb), is considered to be responsible for PAM [5-7]. However, its mutation symbols in different cases are not investigated yet for its limited data.

In this study we conducted human whole exon sequencing for an inbred family with pulmonary alveolar microlithiasis, screened the related gene mutation, in order to discover disease associated mutations of SLC34A2 gene and provide meaningful references for the study of etiology and diagnosis of rare disease PAM.

Materials and methods

Subjects

Two PAM patients and other members of an inbred family (**Figure 1**) were recruited for this study. Patients were diagnosed based on characteristic computer tomography (CT) and pathology findings. Written informed consent was obtained from either the patient or from an authorized family member. This study was approved by the Ethics Committee of Chinese People's Liberation Army General Hospital (approval number, S2015-067-01).

Case one

The proband (V4) was a 52-year-old female. In 2003, the patient was admitted to the hospital complaining of recurrent cough and dyspnea.

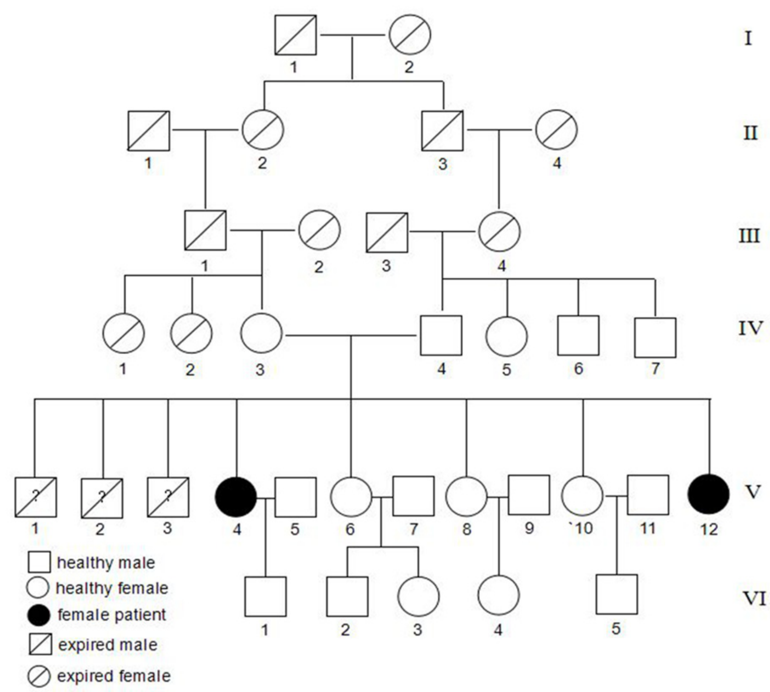

Figure 1. Pedigree Chart of Family History.

She was healthy in the past and denied history of smoking or medication. Her parents are consanguineous, and she has a son and four sisters. One sister (V12) of the patient had the same symptom, neither of the other family members complained of discomfort and their chest CT scans were normal. Physical examination revealed crackle rales in both lung fields, without cyanosis of lips, venous varicose or bulb fingers. Laboratory tests revealed a decreased level of PaO2 (84.3 mmHg). The tumor index and concentration of serum calcium were within the normal range. Spirometry examination showed slight restrictive ventilatory disturbances and moderate decreased diffusing function (vital capacity, 77.2% of predicted, forced expiratory volume in 1 s, 82.5% of predicted, carbon monoxide transfer factor-single breath, 47.0% of predicted). Chest computed tomography (CT) scan showed high density reflection of intraalveolar sand-stones especially in double lower lobe, and calcification of the mediastinal and interlobular pleura as well as the pericardium. Pathology of lung biopsy showed irregular microlith with lamination and massive calcification by hematoxylin-eosin (HE) stain. The final diagnosis was PAM based on characteristic CT and pathology findings.

Case two

The proband (V12) was a 39-year-old female. In 2015, the patient was admitted to the hospital with the same complaining of recurrent cough and exertional dyspnea. She was healthy in the past and denied history of smoking or medication. Physical examination revealed normal breath sound. Laboratory tests revealed a decreased level of PaO2 (86.6 mmHg). Classification of bronchoalveolar lavage fluid (BALF) revealed increased lymphocyte (19%, normal <13%) and decreased macrophage (78%, normal >84%). The tumor index, rheumatoid factor, immunoglobulin, C-reactive protein, and concentration of serum calcium and phosphorus were within the normal range. Pulmonary function test demonstrated moderate impairment of ventilation function (vital capacity, 95.2% of predicted, forced expiratory volume in 1 s, 94.8% of predicted, carbon monoxide transfer factor-single breath, 56.1% of predicted). CT scan showed high density reflection of intraalveolar sand-stones especially in double lower lobe, pericardium and subpleural calcification shadow, and multiple calcified plaque of aortic and coronary artery (**Figure 2A**). Pathology of lung biopsy showed irregular microlith with lamination and massive calcification by HE stain (**Figure 2B**). The final diagnosis was PAM based on characteristic CT and pathology findings.

Genome whole exon sequencing

Blood samples were collected from the patients, their parents, sisters, and the children. Genome DNA was extracted using the human blood DNA extraction kit (QIAGEN). The exon regions were enriched by SeqCap EZ human whole exon capture system of NimbleGen (Roche). After database setup, pair-end (double ends) sequencing was conducted following the instruction brochure using Illumina HiSeq2500 sequencing system. Preliminary data analysis and quality control were conducted for sequencing results.

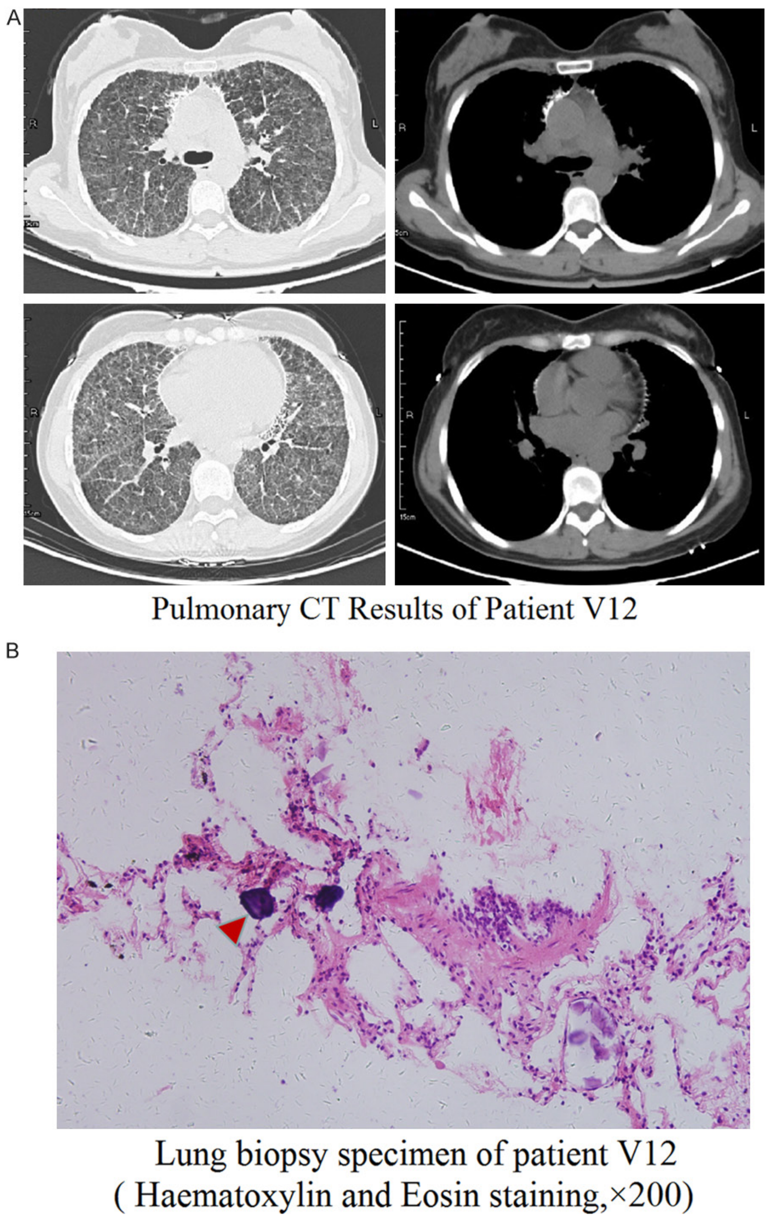

Figure 2. A. Pulmonary CT Results of Patient V12. Lung window image of CT scan of the chest showing intraalveolar sand-stones especially in double lower lobe, pericardium and subpleural calcification shadow, and multiple calcified plaque of aortic. Mediastinal window image showing calcification of the mediastinal pleura as well as the pericardium. B. Lung biopsy specimen of patient V12. Lung biopsy specimen of patient V12 stained with hematoxylin and eosin (×200) showing calcium in alveolar spaces and in the lung parenchyma (red triangle).

Bioinformatic analysis of SNPs of SLA34A2

Based on the above analyzed results, low quality SNPs were eliminated. The variants published in normal control individuals were also eliminated, including the common variant carried by normal individuals in the public genetic mutation database 1000 gemomes, Hapmap and dbSNP. Analysis of related functional pathway regulation and the history of PAM revealed that SLC34A2 gene mutation was probably related to encode the sodium phosphate cotransporter (NaPi-IIb), which is considered to be responsible for PAM.

Results

Data analysis of whole exon sequencing

In order to decrease the impact of error rate from Solexa data on the results, paired reads containing adaptors and low quality segments were eliminated by sliding window approach from raw data obtained from whole exon sequencing. Quality control was conducted using fastQC (**Figure 3A**). Reads alignment on genome hg19, sequencing depth and exon coverage were analyzed (**Figure 3B**). After Indel region realignment and base quality score recalibration (BQSR), variants were detected and eliminated by GATK in order to obtain the mutations or candidate genes which might affect protein functions (**Figure 4A**, **4B**).

New SNPs in SLC34A2 gene

After pre-processing and quality control, low quality SNPs were eliminated from the results of whole exon sequencing. The published variants in normal control individuals were also eliminated, including the common variant carried by normal individuals in the public genetic mutation database 1000 gemomes, Hapmap and dbSNP (**Table 1**). Analysis of related functional pathway regulation and the history of PAM revealed that SLC34A2 gene mutation was probably associated the sodium

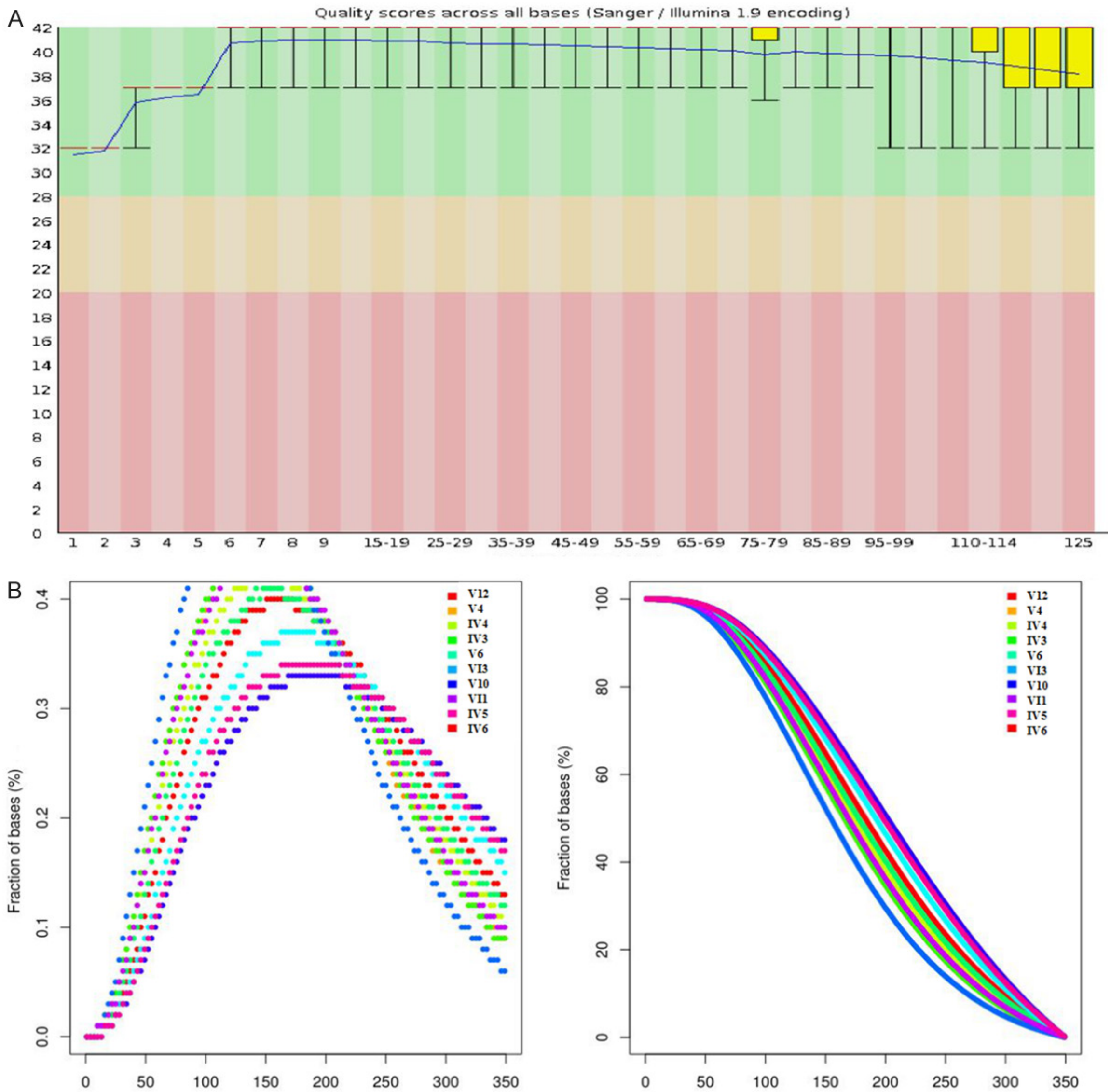

Figure 3. A. Data Quality Score before and after Processing (per base quality). B. Sequencing Depth Distribution of the Target Region.

phosphate co-transporter (NaPi-IIb), which is considered to be responsible for PAM.

Compared with ClinVar database and Uniprot variants database, there were 3 variants found in SLC34A2 gene, including 1 common variants (recorded in public genetic variant database) and 2 unknown variants newly discovered (**Table 1**). The influence on gene expression by the variant located at the untranslated region variant 3 prime (UTR3) named rs3733545 was unclear. From the annotation of the function of variants, the mutation from A to T located on 25672438bp of 4 chromosome was identified in exon 8 (c.910A>T) and determined to be nonsense mutation. This nonsense mutation made codon AAA transcribing lysine change into stop codon TAA (p.K304X), which resulted in dysfunction of SLC34A2. Another missense mutation from A to G on its coding amino acid, Aspartic acid to Glycine (Chr4: 25678199, c.1901A>G (p.D252G)), was not recorded in ClinVar database.

Distribution of SLC34A2 SNPs in the family members

The target region of mutation was sequenced in order to demonstrate the presence of this mutation in family members and whether

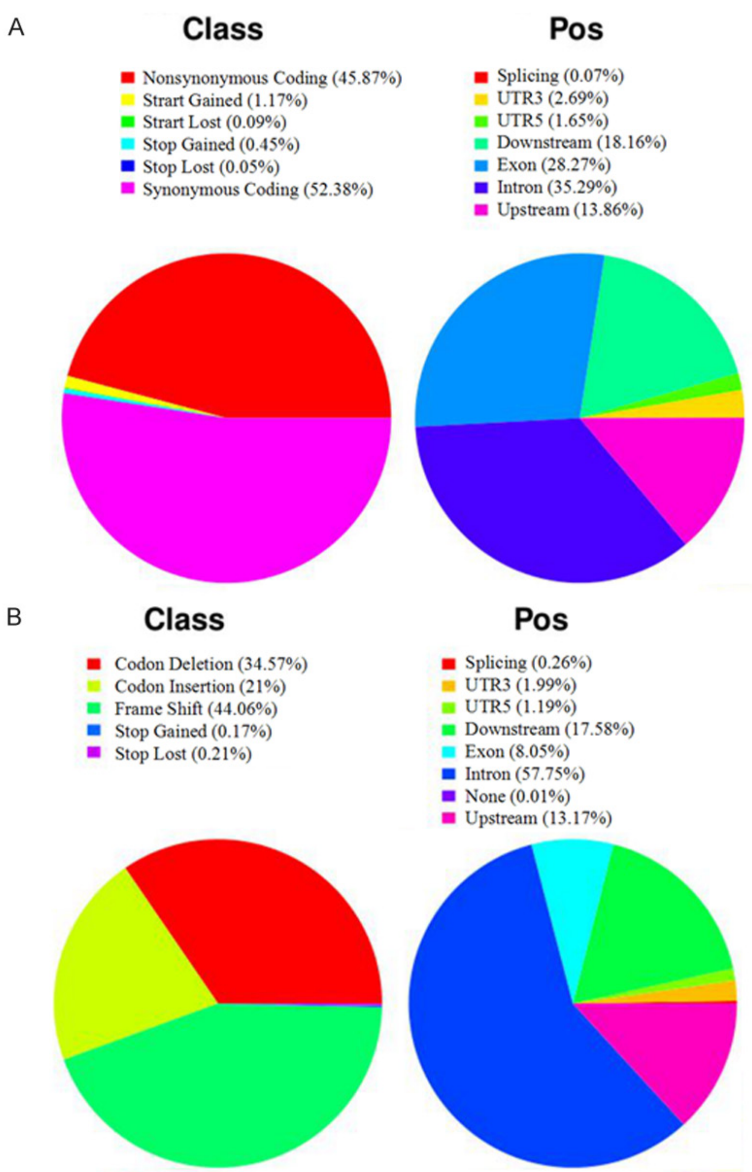

Figure 4. A. Distribution of different types of SNPs in coding region and genomic region. B. Distribution of different types of Indels in coding region and genomic region. Note: UTR3, untranslated region variant 3 prime; UTR5, untranslated region variant 5 prime.

this mutation affected expression of NaPi-IIb protein. Sequencing of exon 8 on SLC34A2 gene revealed that the wild type allele was A, while the was T found in patient V4 and V12, mother IV3, father IV4, the fourth sister V10, the son of patient V4 (VI1), aunt IV5 and uncle IV6, located on Chr4: 25672438, c.910A>T (p.K304X). Among these mutants, patient V4 and V12 were homozygous mutation, and the other members were heterozygous carriers. While the second sister V6 and the daughter VI3, were not carriers. Another missense mutation from A to G, located on Chr4: 25678199, c.1901A>G (p.D252G) was fund in all members with homozygous mutation. The homozygous mutation located at UTR3 was also fund in all members, except the uncle IV6 (**Table 2**).

Discussion

PAM is a rare idiopathic disease characterized by microliths of calcium phosphate accumulate in alveoli. The etiology and pathogenesis of this disease is still unknown and many researches has supposed that PAM is an autosomal recessive hereditary disease as a result of the presence of familial cases [8, 9].

The disease affects both sexes and the cases of PAM are described at all ages, from newborns to an 84-year-old female [4, 11, 12]. More than half of the patients are asymptomatic, and complained symptoms such as dyspnoea, cough or chest pain in chronic progressive course. Lung function showed impairment of ventilation function and diffusing function [4, 13]. Microscopic (biopsy or bronchoalveolar lavage) examination revealed characteristic microliths into the alveoli. Characteristic sand-like appearance of Chest x-rays and computed tomography are sufficient to diagnose especially in cases with other affected family members [14, 15]. Although develops slowly, the disease will progress into pulmonary fibrosis, respiratory failure and cor pulmonale [4]. To date, there is no effective therapy except for lung transplantation [16].

In this study, the two patients were sisters of an inbred family whose parents were cousins and presented typical manifestation of recurrent

Table 1. 3 SNPs on SLC34A2 gene

Chr	Start	End	Gene region	Ref	Alt	AA change	Effect
Chr4	25672438	25672438	Exon 8	A	T	c.910A>T (p.K304X)	Stopgain
Chr4	25678396	25678396	UTR3	G	T	--	Unknown
Chr4	25678199	25678199	Exon 13	A	G	c.1901A>G (p.D634G)	Nonsynonymous

Note: UTR3, untranslated region variant 3 prime.

Table 2. Distribution and Gene type of SLC34A2 SNPs in the Family Members

Location	IV3	IV4	IV5	IV6	V4	V6	V10	V12	VI1	VI3
Chr4: 25672438	het	het	het	het	hom	-	het	hom	het	-
Chr4: 25678396	hom	hom	hom	het	hom	hom	hom	hom	hom	hom
Chr4: 25678199	hom	hom	hom	hom	hom	hom	hom	hom	hom	hom

cough, progressive dyspnea. HRCT demonstrated the pulmonary was full of high density reflection of intraalveolar microliths especially in double lower lobe. Pulmonary function showed impairment of small airway and dispersion function. Pathology of lung biopsy showed irregular microlith with lamination and massive calcification by HE stain. The diagnosis of PAM was therefore established based on the symptoms, thorax imaging and the biopsy.

It has been confirmed that more than one third of cases are familial, which suggested genetic basis might be the etiology of PAM [4]. The mutation of SLC34A2 gene, which encodes the NaPi-IIb, is considered to be responsible for PAM. SLC34A2 gene has 13 exons, and exon 1, 2, 3, 4, 6, 7, 8, 11, 12 and 13 were involved mainly in this disease [4, 7, 17-21]. Chinese patients usually have mutation in exon8 [6-8]. In this study, we found 3 SNPs of ALC34A2 in an inbred PAM patient's family, using whole exon sequencing technique combined with bioinformatic analysis. One of the three variants located at UTR3, which impact on gene expression could not be decided. Through the annotation of function of the mutation, the mutation from A to T located on 25672438bp of 4 chromosome was identified in exon 8 (c.910A>T) and determined to be nonsense mutation. This nonsense mutation made codon AAA transcribing lysine change into stop codon TAA (p. K304X), which resulted in dysfunction of SLC34A2. This nonsense variant was found in patient V4 and V12, mother IV3, father IV4, the fourth sister V10, and the son of patient V4 (VI1), aunt IV5 and uncle IV6. Among these mutants, patient V4 and V12 were homozygous mutation, and the other members were heterozygous carriers. Based on this result, we speculated that nonsense mutation resulted in dysfunction of SLC34A2 and was responsible for PAM in the inbred family. Another missense mutation from A to G on its coding amino acid, Aspartic acid to Glycine (Chr4: 25678199, c.1901A>G (p.D252G)), was fund in all members with homozygous mutation. The homozygous mutation located at UTR3 was also fund in all members, except the uncle IV6. These two variants, which were not recorded in ClinVar database, displayed the same mutation type among the patients and their relatives and indicated polymorphism of SLC34A2.

Taken together, for a patient with an inbred family history and typical radiological features of high density intraalveolar microliths, the diagnosis should be highly suspected and pathology of lung biopsy with irregular microlith is the gold standard. The novel homozygous mutation in exon 13 (Chr4: 25678199, c.1901A>G (p.D252G)) and UTR3 (Chr4: 25678396) indicate polymorphism of SLC34A2. Homozygous mutation (Chr4: 25672438, c.910A>T (p. K252X)) of SLC34A2 gene can change codon AAA transcribing lysine change into stop codon TAA (p.K304X), which resulted in defects of SLC34A2 function and led to PAM in the inbred family. Our result was consistent with the cases reported in Chinese PAM family, and exon8 might be the screen target for Chinese PAM patients.

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Disclosure of conflict of interest

None.

Address correspondence to: Lixin Xie, Department of Pulmonary & Critical Care Medicine, Chinese PLA General Hospital, Beijing 100853, China. Tel: 86-10-55499128; Fax: 86-10-55499128; E-mail: xielx-301@126.com

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