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CASE REPORT

Mutation analysis and serum FGF23 level in a patient with pulmonary alveolar microlithiasis

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Abstract Pulmonary alveolar microlithiasis (PAM) is a rare, hereditary disorder characterized by ectopic formation of calcium-phosphate microliths in the alveolar space. PAM has been reported to arise from inactivating mutations in *SLC34A2*, encoding a sodium-dependent phosphate co-transporter essential for phosphate transport in the lungs and small intestine. Serum levels of the phosphaturic hormone fibroblast growth factor-23 (FGF23) in PAM have not been determined. Our objectives were to investigate the genetic etiology and circulating level of FGF23 in a 50-year-old male with clinical characteristics of PAM and extra-pulmonary calcifications. The *SLC34A2* and *FGF23* genes were sequenced for mutations and serum FGF23 analyzed by ELISA. We found no disease-causing mutations or single nucleotide polymorphisms in the genes investigated. Importantly, repeated measurements revealed undetectable or markedly low serum FGF23 (<3–11 RU/ml). Surprisingly, in the face of low serum FGF23, 1,25-dihydroxy vitamin D_3 level was low-normal and parathyroid hormone mildly elevated. Total 24-h urinary excretion of phosphate and calcium were low, as was fractional urinary excretion of calcium. In contrast, fractional excretion of phosphate was above normal, likely due to elevated PTH. Collectively, PAM may be a polygenic disorder that arises from mutations other than in *SLC34A2*. The low FGF23 level in our PAM patient supports an intestinal-bone axis, leading to decreased FGF23 expression when intestinal phosphate absorption is compromised.

Keywords PAM · SLC34A2 · Npt2b · NaPi-2b · Fibroblast growth factor-23

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Introduction

Pulmonary alveolar microlithiasis (PAM; OMIM #265100) is a rare, autosomal recessive disorder characterized by deposition of calcium-phosphate microliths throughout the lungs [1–3]. In sporadic cases it is associated with calcifications of extrapulmonary organs, such as gonads [4] and pericardium [5]. Even with extensive radiologic findings ("sandstorm lung") the clinical symptoms are often mild. Progression of the disease is usually slow, eventually leading to respiratory failure and pulmonary heart disease in middle age. Diagnosis is achieved through radiographic examination, and confirmed by bronchial lavage or transbronchial biopsy. There is currently no effective treatment other than lung transplantation.

Inactivating mutations in the gene *solute carrier family 34 (sodium-phosphate), member 2* (*SLC34A2*) were recently identified as causative of PAM [6, 7]. *SLC34A2* encodes a sodium-dependent phosphate co-transporter (SLC34A2, Npt2b, NaPi-2b) that is highly expressed in the lungs [8], primarily by alveolar type II cells [9]. SLC34A2 is responsible for uptake of phosphate released from phospholipids in outdated surfactant [9, 10] and the formation of calcium-phosphate microliths in PAM could be explained by the inability to clear phosphate from the alveolar space.

SLC34A2 is also expressed in the small intestine, mediating dietary phosphate absorption [8, 11]. The expression of SLC4A2 is hormonally regulated and the stimulation of intestinal phosphate uptake by 1,25-dihydroxy vitamin D_3 ($1,25(OH)_2D_3$) is attributed to an increase in SLC34A2 expression [12].

Despite abrogated function of SLC34A2 and impaired intestinal phosphate uptake, most PAM patients have normal serum phosphate levels [13]. This may be achieved by, yet unknown, compensatory mechanisms. One possible adaptive response could be altered expression of fibroblast growth factor-23 (FGF23), a circulating hormone that inhibits phosphate reabsorption in the kidney proximal tubule [14, 15]. Serum FGF23 levels in PAM patients have not been determined.

Herein, we aimed to elucidate the genetic etiology and assess the FGF23 level in a 50-year-old male Moroccan patient diagnosed with PAM and concomitant medullary nephrocalcinosis.

Results

Clinical and radiological findings

A 29-year-old Moroccan presented for the first time in 1988 with atypical chest pain upon exertion. Physical examination and laboratory values were unremarkable. Chest X-ray revealed extensive bilateral pulmonary infiltrates with "sandstorm"-like opacifications. The diagnosis of PAM was confirmed by transbronchial biopsy. Subsequent ultrasound examination showed medullary nephrocalcinosis. Family history was unremarkable and the patient has no siblings or children. Over the next 20 years the patient was closely followed at the University Hospital Aachen. There was a moderate expansion of the pulmonary infiltrates (Fig. 1) and pulmonary function testing revealed the beginning of a restrictive pattern, whereas the patient was almost symptom free. Clinical findings of this patient have been reported previously [16]. Dietary intake has not been monitored, however, throughout the disease history the patient has reportedly been on a standard Western diet with normal dietary intake of calcium and phosphate.

Mutation analysis

In order to determine the genetic cause of PAM, we performed sequence analysis of the *SLC34A2* gene, including the intron–exon boundaries. Importantly, no disease-causing mutations or single nucleotide polymorphism (SNPs) were found. In order to exclude a primary defect in the *FGF23* gene, we also sequenced the coding regions and intron–exon boundaries of *FGF23*, but found no mutations or SNPs (data not shown).

FGF23 and other biochemistries

In repeated measurements, serum FGF23 level was undetectable or markedly low (<3–11 RU/ml; reference interval 20–80 RU/ml). Throughout the disease history of more than 20 years, the patient had serum calcium and phosphate levels within or close to the normal ranges. For unknown reasons, intact PTH has been mildly elevated, while 25-hydroxy vitamin D_3 ($25(OH)D_3$) and $1,25(OH_2)D_3$ have ranged from normal to low. Total 24-h urinary excretion of phosphate and calcium were low, as was fractional urinary excretion of calcium. In contrast, fractional excretion of phosphate was above normal and percent maximum tubular reabsorption of phosphate per glomerular filtration rate below the reference range (Table 1).

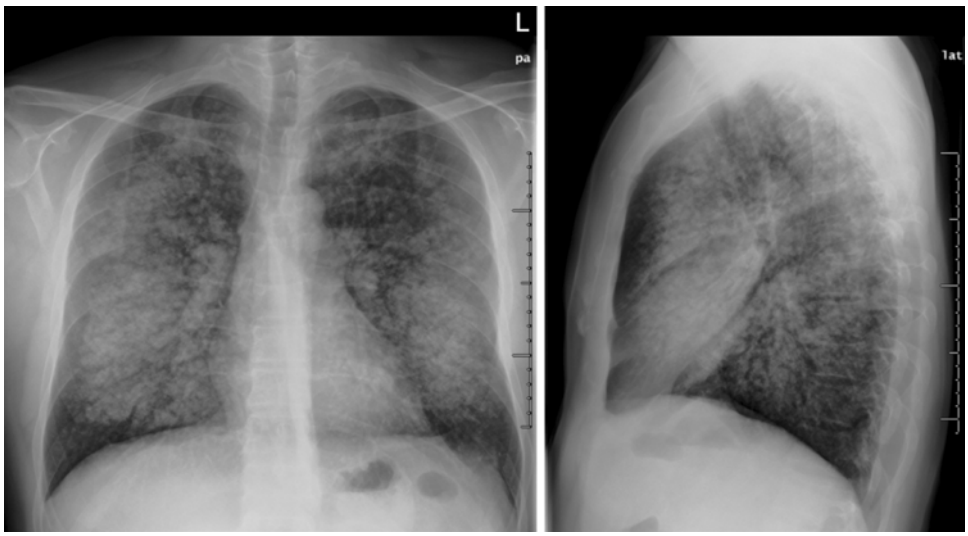

Fig. 1 Chest X-ray examination, posterior–anterior, and lateral view. Predominantly the middle zones of the lungs reveal bilateral "sand-storm" like calcifications as characteristic radiological features of PAM

Table 1 Laboratory analysis of the PAM patient

Variable	Value	Reference interval
Serum calcium (mmol/l)	2.44	2.10–2.44
Serum phosphate (mmol/l)	**0.79**	0.84–1.48
Serum C-terminal FGF23 (RU/ml)	**11**	20–80
Serum total protein (g/l)	76	66–83
Serum C-reactive protein (mg/l)	4	<5
Serum bone-alkaline phosphatase (U/l)	32	15–41
Serum intact PTH (ng/l)	**98**	10–65
Serum $1,25(OH_2)D_3$ (ng/l)	16	16–57
Serum $25(OH)D_3$ (μg/l)	11	9–38
Urinary phosphate (mmol/24 h)	**23**	24–48
Urinary calcium (mmol/24 h)	**1.9**	2.5–7.5
Fractional excretion of phosphate (%)	**31**	5–25
Fractional excretion of calcium (%)	**0.8**	1.5–3
Tubular maximum of phosphate reabsorption/ glomerular filtration rate (mmol/l)	**0.5**	0.8–1.35

Values outside the normal reference ranges are shown in bold

Discussion

In the present study, we sought to determine the genetic etiology in a sporadic case with clinical and radiological signs of PAM and idiopathic medullary nephrocalcinosis. We found no mutations or disease-causing SNPs in *SLC34A2*, implying that PAM may be a heterogenous disorder, or caused by mutations at sites other than in the coding regions of *SLC34A2*. Similar negative findings have been reported by at least one other group [17]. We also excluded the possibility of a primary genetic defect in *FGF23*, as observed in other patients with low intact FGF23 levels, such as familial hyperphosphatemic tumoral calcinosis (FHTC; OMIM # 211900) [18, 19]. Extrapulmonary calcifications present in our patient is not obligate in PAM but have been described previously [20]. It remains to be determined whether this is an unusual PAM manifestation or represents a novel disorder genetically distinct from PAM.

Although screening for mutations in the *SLC34A2* gene was negative, we speculate that the SLC34A2 protein function is compromised in our patient. This is supported by the severe phenotypic changes characteristic for PAM and the fact that SLC34A2 is the only phosphate-transporter present in the lungs. However, we cannot rule out the possibility of an alternative, SLC34A2-independent mechanism as the cause of PAM in our patient. Continuing this speculation, also intestinal phosphate absorption is presumably diminished, explaining the low-normal serum phosphate level in our patient.

Importantly, we for the first time report serum level of the phosphaturic hormone FGF23 in PAM, which was low or undetectable. This is consistent with *SLC34A2* knockout mice that recently were reported to have lower serum FGF23 levels than wild-type controls [21]. Low FGF23 expression in PAM is presumably a compensatory mechanism to maintain a normal phosphate balance through increasing the renal reabsorption of phosphate. Our patient may provide evidence for a, yet unidentified, intestinal-bone axis in which the intestine can sense the impaired phosphate uptake and signal to the osteocyte where FGF23 expression is suppressed. Indeed, intestinal phosphate transport may be a more important regulator of FGF23 expression than serum phosphate level [22, 23]. Alternatively, there may be a local phosphate-sensing mechanism in bone cells that regulate FGF23 expression, however, this appears unlikely given the complete suppression of FGF23 in the face of normal or only mildly decreased phosphate level over time. In the setting of low-undetectable FGF23, it is surprising that $1,25(OH)_2D_3$ was in the lower normal range and PTH was elevated given that FGF23 is a negative regulator of 1α-hydroxylase [14, 24] and PTH may be a stimulator of FGF23 expression [25]. The reasons for the low $1,25(OH)_2D_3$ and elevated PTH levels remains unclear, but subnormal serum $25(OH)D_3$ levels may contribute. Alternatively, the high PTH may be due to reduced intestinal calcium absorption in our patient although there is no data supporting such a hypothesis.

Most PAM patients have normal serum calcium and phosphate levels despite impaired intestinal uptake of phosphate [13]. This suggests a renal-mediated compensatory mechanism for maintaining serum phosphate through an increase in renal reuptake, as evidenced by a reduction in the FGF23 level. In support, we found that total 24-h urinary excretion of phosphate and calcium were low, as was fractional urinary excretion of calcium. In contrast, percent maximum tubular reabsorption of phosphate per glomerular filtration rate was below normal, but

this may be, at least partially, attributed to a dominant phosphaturic effect of elevated PTH level.

In summary, we herein describe an unusual case of PAM with extra-pulmonary calcifications and without mutations in *SLC34A2*. Serum FGF23 level in this PAM patient is low-undetectable, supporting the existence of an intestinal–skeletal feedback loop that needs to be further addressed in experimental studies.

Patients and methods

Study subject

Written, informed consent was obtained from the patient before participation in the study.

SLC34A2 and FGF23 mutational analysis

Genomic DNA was extracted from blood samples using the Wizard Genomic DNA Purification Kit (STS Promega, Madison, USA). All exons and intron–exon boundaries in the *SLC34A2* gene, including the non-translating exon 1, were PCR amplified using the protocol stated by Corut et al. [6]. Exons and intron–exon boundaries of *FGF23* were PCR amplified with AmpliTaq Gold DNA (Applied Biosystems, Foster City, USA) polymerase using 200 ng genomic DNA. PCR conditions were as follows: 10 min at 95°C, followed by 35 cycles of 45 s at 95°C, 1 min at 60°C, 1 min at 72°C, and a final extension of 7 min at 72°C. All PCR products were electrophorized on 1% agarose gels, visualized under UV-light, and subsequently gel purified using the QIAquickGel ExtractionKit (Qiagen). Purified products were sequenced from forward and reverse primers with BigDye v3.1 and the 3130Xl GeneticAnalyser (AppliedBiosystems). Sequencing primers for *SLC34A2* and *FGF23* are available upon request.

Biochemistries

Routine biochemistries were measured at the department of clinical chemistry, Aachen University Hospital, Germany. PTH determination was performed using a second-generation electrochemiluminescence PTH assay on the Elecsys 2010 platform (Roche Diagnostics, Mannheim, Germany). 1,25-Dihydroxy-vitamin D3 was determined using a human 1,25-Dihydroxy vitamin D EIA Kit from IDS Ltd (St. Joseph, MI, USA). Urine was collected for 24 h and fractional excretion of calcium (FECa, %) and phosphate (FEP, %) was determined as follows: $\frac{u-\text{calcium}\times s-\text{creatinine}}{s-\text{calcium}\times u-\text{creatinine}}$; and $\frac{u-\text{phosphate}\times s-\text{creatinine}}{s-\text{phosphate}\times u-\text{creatinine}}$, respectively, and tubular maximum of phosphate reabsorption per glomerular filtration rate (TmP/GFR, mmol/l) as $s-\text{phosphate}-\frac{u-\text{phosphate}\times s-\text{creatinine}}{u-\text{creatinine}}$.

FGF23 serum assays

Circulating FGF23 was analysed using a sandwich ELISA detecting both intact FGF23 and C-terminal FGF23 fragments (Immutopics Inc, San Clemente, USA) according to the manufacturer's protocol. The intraassay coefficient of variation is 5.0%, the interassay coefficient of variation of 5.0–7.3%, and the lower detection limit 3.0 relative units (RU)/ml.

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