Do the heterozygous carriers of a *CYP24A1* mutation display a different biochemical phenotype than wild types?

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Abstract

Context. Human cytochrome P450 24 subfamily A member 1 (*CYP24A1*) loss-of-function mutations result in impaired activity of the 24-hydroxylase involved in vitamin D catabolism, thus inducing a vitamin D-dependent hypercalcemia. Homozygotes often present an overt clinical phenotype named Idiopathic Infantile hypercalcemia (IIH), whereas it is debated whether heterozygotes display an abnormal phenotype.

Objectives. To compare the clinical and biochemical features of heterozygous carriers of *CYP24A1* variant and healthy wild-type controls, sharing the same genetic and environmental exposure.

Methods. A large family harboring the nonsense c.667A>T, p.Arg223* pathogenic variant in the *CYP24A1* gene was evaluated. All subjects underwent clinical and biochemical evaluation and complete analysis of vitamin D metabolites using mass-spectroscopy including $1,24,25(OH)_3D_3$. Subjects were divided according to their genotype in two groups: heterozygotes and wild-type for the *CYP24A1* variant.

Results. The proband, a 40-year-old man, homozygous for p.Arg223* pathogenic variant, had a history of mild hypercalcemia with a seasonal trend, recurrent nephrolithiasis, and no episodes of acute hypercalcemia. He showed the highest serum levels of FGF23, the highest $25(OH)D_3/24,25(OH)_2D_3$ ratio and undetectable levels of $1,24,25(OH)_3D_3$ which represent indicators of a loss-of function CYP24A1. Compared to the wild-types, heterozygotes had higher serum calcium and $25(OH)D_3$ concentrations (P=0.017 and P=0.025, respectively), without any difference in the other biochemical parameters and in the rate of nephrolithiasis.

Conclusions. Heterozygotes exhibit a biochemical phenotype different from that of wild-type subjects. In clinical practice, these individuals might require surveillance because of the potential risk of developing hypercalcemia and related clinical manifestations if exposed to triggering factors.

Key words: Vitamin D, Hypercalcemia, Idiopathic Infantile Hypercalcemia, FGF23, $25(OH)D_3$

Introduction

Pathogenic variants in the human cytochrome P450 24 subfamily A member 1 (*CYP24A1*) gene were recently associated with Idiopathic Infantile Hypercalcemia (IIH, OMIM 143880) (1). This gene encodes for the 25-hydroxyvitamin D-24-hydroxylase, which plays a key role in the catabolism of vitamin D by catalyzing the inactivation of the active metabolite 1α,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] and its precursor $25(OH)D_3$ into inactive, 24-hydroxylated products (2,3). Loss-of-function mutations in the *CYP24A1* gene can lead to elevated serum $1,25(OH)_2D_3$ concentration which may be associated with various degrees of hypercalcemia and hypercalciuria and low to undetectable plasma parathyroid hormone (PTH) levels (4,5).

The clinical manifestations of IIH embrace different scenarios, from severe forms diagnosed early in the infancy characterized by severe hypercalcemia, dehydration, vomiting and very rarely death, to milder forms, often diagnosed in the adulthood during workout for recurrent nephrolithiasis (6–9). After the identification in 2011 that homozygous mutations of *CYP24A1* are responsible for IIH, many case reports and few studies have investigated clinical, biochemical and genetic features of this disease. Currently, many questions remain unanswered, namely the specific prevalence of the disease, the existence of a genotype-phenotype correlation and the best treatment of hypercalcemia (5,9). In addition, the type of inheritance of the disease is still debated because of the conflicting data on heterozygote individuals (10–12). Indeed, in some kindreds heterozygotes exhibit normal or slightly elevated serum calcium levels whereas in others an overtly hypercalcemic clinical and biochemical phenotype. Conventionally, the inheritance of a genetic disorder is established by comparing the phenotype of heterozygotes to that of the wild-type subjects (13). To date, however, no study has compared the phenotype of subjects carrying a heterozygous *CYP24A1* mutation to that of wild-types.

The present study was designed to shed light on the phenotype of heterozygous carriers of *CYP24A1* variant. To this end, we analyzed their clinical and biochemical features in comparison with wild type subjects belonging to the same family. Of note, we extended the number of vitamin D metabolites analyzed, by including measurements of serum $1,24,25(OH)_3D_3$, an initial degradation product of $1,25(OH)_2D_3$ and evaluated serum FGF23 levels .

Materials and methods

Study design and study population

We investigated a large family harboring a nonsense *CYP24A1* gene mutation (Fig.1). The study included the proband and 18 family members: 12 relatives, 6 spouses. All family members gave written informed consent for i) creation of clinical chart; ii) genetic analysis; and iii) use of the data for scientific purposes and publication.

Clinical and biochemical evaluation

Each member was tested for total calcium, albumin, phosphate, magnesium, creatinine, PTH, $25(OH)D_3$, $1,25(OH)_2D_3$, $24,25(OH)_2D_3$, $1,24,25(OH)_3D_3$, and FGF23 and genetic analysis of *CYP24A1* gene. To reduce differences in sunlight exposure, especially between young subjects and elderly, all serum samples were collected at the same time in winter (February 2019). Moreover, sunlight exposure was evaluated in all subjects by using a questionnaire previously used in people living at the same latitude (14). Vitamin D supplementation and past medical history about symptomatic nephrolithiasis were investigated in all subjects. Kidney ultrasound was available in 5 individuals (II.2, III.2, III.8, III.10 and IV.1). In addition, the proband (Figure 1, III.7) and five first degree relatives (Fig.1, II.3; II.4; III.6; IV.5; IV.6) were evaluated at Endocrine Unit of University Hospital of Pisa and underwent abdominal ultrasound to exclude nephrolithiasis.

Biochemical assays

Fasting serum or plasma samples were collected at the same time in all subjects. Serum calcium, phosphate, creatinine and albumin were measured using standard methods. Plasma PTH was measured by the third-generation assay (DiaSorin LIAISON® 1-84 PTH assay (Saluggia, Italy) [chemiluminescent immunoassay (CLIA)]. Plasma FGF23 was tested by DiaSorin LIAISON® assay . All these measurement were performed at the same laboratory.

The complete biochemical profile of vitamin D metabolites was studied using liquid chromatography tandem mass spectrometry (LC-MS/MS) at Department of Biochemical and Molecular Sciences, Queen's University, Kingston Canada. Serum samples were prepared by immunoextraction and derivatized with 4-[2-(6,7-dimethoxy-4-methyl-3,4-dihydroquinoxalinyl)ehtyl]-1,2,4-triazoline-3,5-dione (DMEQ-TAD), as previously reported

(15). A more rigorous chromatographic method was also used to test the serum extracts of the proband with inappropriately low levels of $24,25(OH)_2D_3$ (16).

The reference ranges are reported in Table 1.

Genetic analysis

Genomic DNA was extracted from peripheral blood leukocytes by standard procedures. DNA sample of the proband was analyzed by Next Generation Sequencing (NGS) using a customized SureSelectXT (Agilent) panel targeting selected vitamin D associated genes, according to the protocol supplied. Sequencing was carried out in a MiSeq sequencer (Illumina) using the pair end format. The sequencing results were analyzed by using the application Variant Interpreter (Illumina), sequence revision against human genome was carried out by using the Integrative Genomes Viewer (IGV). Direct Sanger sequencing (BigDye Terminator v3.1 Cycle sequencing Kit, Life Technologies, Italy) was performed to confirm genetic variants individuated by NGS and to screen the other members of the family. DNA from the non-blood relatives was analysed by NGS, using the same customized SureSelectXT (Agilent) panel targeting selected vitamin D associated genes.

Statistical analysis

Data were expressed as median and interquartile range for continuous variables, and as frequency and percentage for categorical variables. Patients were grouped according to the results of the genetic testing (heterozygotes and wild types), in order to study differences in terms of biochemical and clinical phenotype. Groups were compared using Mann-Whitney U test for continuous variables and by chi-squared test for categorical variables. A P value <0.05 was considered statistically significant. All computations were performed using the SPSS v.25 statistical package (SPSS, Chicago, IL, USA).

Results

Proband history

The proband (Fig. 1, III.7) was a 40-year-old man born from non-consanguineous parents living in a village situated in the North of Tuscany, with a population of 452. He was referred in June 2017 to our outpatient clinic for long lasting PTH-independent hypercalcemia and recurrent symptomatic nephrolithiasis. The past medical history of the patient was unremarkable with an uneventful neonatal period and infancy. At the age of 16 years, he presented with abdominal pain due

to bilateral nephrolithiasis. Given the recurrence of symptomatic nephrolithiasis (at least 5 episodes), at age of 37 years, the patient underwent biochemical evaluation that showed high serum calcium (11.1 mg/dL) and undetectable PTH levels. The patient was extensively evaluated elsewhere in the suspicion of malignancy, including serum electrophoresis and tumor markers, total body computed tomography (CT) scan and 18-FDG PET TC, gastroscopy and colonoscopy that did not reveal any pathology. Family history was unremarkable for hypercalcemia and nephrolithiasis.

Physical examination was negative. Laboratory tests revealed mild hypercalcemia, low-undetectable PTH levels and normal level of phosphate. Total 25(OH)D [(measurement of both $25(OH)D_2$ and $25(OH)D_3$)] was initially tested with chemiluminescent assay at our laboratory (IDS ISYS, UK) and resulted slightly increased (Table 1). Twenty-four hour urinary calcium was elevated, tubular reabsorption of phosphate (TRP) was slightly reduced, as well as tubular maximum reabsorption of phosphate per liter of glomerular filtration rate (TmP/GFR) (Table 1). Given the mild hypercalcemia and the general good health, no specific treatment was advised. In January 2018, the patient was further evaluated, and at this time total serum calcium and PTH levels, as well as 24-h urinary calcium, were within the normal range (Table 1). A careful review of all past medical charts showed a seasonal trend of serum calcium concentration and PTH levels (Figure 2, Panels A and B). The finding of longstanding hypercalcemia and low PTH levels with episodes of normocalcemia and normal PTH levels suggested the possibility that the patient might have an impairment of vitamin D catabolism. Therefore, a complete analysis of vitamin D_3 metabolites was carried out and showed a high $25(OH)D_3/24,25(OH)_2D_3$ ratio, and undetectable $1,24,25(OH)_3D_3$, suggestive of an impairment of CYP24A1 enzyme activity (Table 1)

Given the mild biochemical profile and the general good health, we suggested implementing a low calcium diet, avoiding dehydration and unprotected sunlight exposure. Subsequently, the patient was evaluated every six months. Serum calcium concentration remained in the high normal range without any clinical manifestation of hypercalcemia.

Genetic analysis

NGS analysis of the proband revealed the rare mutation c.667A>T (NM_000782.4) in exon 5 of the *CYP24A1* gene (NG_008334.1) in homozygous condition, that causes the formation of a stop codon at position 223 of the protein (p.Arg223*) (Fig.3). The same mutation was found in heterozygous proband's parents (Fig. 1, II.3; II.4), in the offspring (Fig. 1, IV.5; IV.6), and in the proband's sister (Fig. 1, III.6).

The extension of genetic analysis to the other family members allowed the identification of an additional (Fig. 1, II.7) heterozygote, whereas the other six relatives and spouses were not carriers of any pathogenic variant in the coding regions and exon/intron borders of the *CYP24A1* gene.

Clinical and biochemical features of family members

Biochemical data are summarized in Table 2. Nephrolithiasis was present in one heterozygote subject (Fig. 1, II.2) and in two wild-type (Fig. 1, III.8 and III.6). No subject was taking vitamin D supplements in the six months before the biochemical analyses. None was hypercalcemic, even though two individuals had serum calcium concentration at the upper limit of the normal range (Fig. 1, II.7; IV.6)

No subject showed undetectable PTH concentration nor high $25(OH)D_3$ concentration. $25(OH)D_3/24,25(OH)_2D_3$ ratio was normal in all relatives except for subject IV.4 who is vitamin D deficient by biochemical criteria [$25(OH)D_3$=10.05 ng/mL; $25(OH)D_3/24,25(OH)_2D_3$ ratio= 59.7; PTH=40 pg/mL].

Comparison between groups

Subjects were clustered in two different groups according to the results of genetic analysis: heterozygous (n=6), and wild-type (n=12). Mean age of the two groups did not differ (54 years vs. 57 years, respectively). Moreover, the two groups did not differ in sunlight exposure estimated using the questionnaire (median score 19/42 vs. 21/42, P=0.57). Serum total calcium concentration was significantly higher in heterozygotes than in the wild-type subjects (median 9.8 mg/dL vs. 9.5 mg/dL, P=0.017) (Fig. 4). By excluding from the analysis the two children (Fig 1, IV.1 and IV.5) with different age-specific reference range for total calcium, the statistical analysis confirmed the difference in serum calcium levels between heterozygotes and wild-type subjects (median 9.75 mg/dL vs. 9.44 mg/dL, P=0.037). Serum $25(OH)D_3$ concentration was higher in heterozygotes compared to wild-type subjects (median 36.9 ng/mL vs. 18.7 ng/mL, P=0.025). No other biochemical difference was found between heterozygotes and wild-type individuals (Fig.4). In addition, no difference was observed in the rate of nephrolithiasis between the two groups (P=0.689).

Discussion

Hypercalcemia due to loss-of-function mutations of the *CYP24A1* gene is a genetic disorder recently described in patients with IIH (1). IIH is now considered a misnomer since the genetic cause can be detected and the diagnosis is not restricted to the infant and may be made also in the adult (4) (12).

Loss-of-function mutations of *CYP24A1* gene impairs the catabolism of metabolites of vitamin D [$25(OH)D_3$ and $1,25(OH)_2D_3$] which may lead to PTH-independent hypercalcemia, hypercalciuria associated with normal levels of serum phosphate. To date, about 20 pathological variants of *CYP24A1* have been reported in literature (10) and more than forty pathogenic variants are annotated in the Human Gene Mutation database (HGMD public and professional, September 2020 update) (http://www.hgmd.cf.ac.uk/ac/index.php).

Biallelic mutations carriers (homozygotes) usually display clinical manifestations of IIH that range from severe to mild forms. The severity of disease seems to be age-related. Most cases diagnosed in early infancy present the classic phenotype of IIH, namely severe hypercalcemia, dehydration, polyuria, vomiting, nephrocalcinosis, and lethargy leading to death (1,6,17,18). Conversely, in the adulthood, patients display a mild phenotype characterized by mild to moderate hypercalcemia and recurrent nephrolithiasis (5,19,20).

Loss-of-function mutations of *CYP24A1* should be considered in patients with a clinical scenario similar to that of the proband, namely good health, nephrolithiasis and, as reported in other cases, hypercalcemia and hypercalciuria, with concomitant low/undetectable serum PTH, thus avoiding the extensive evaluation, as occurred in our patient, aimed to exclude hypercalcemia of malignancy (5,19). The seasonal trend of serum calcium with a mild hypercalcemia associated with low PTH levels in the summer and normal serum calcium and PTH in the winter was indicative of a vitamin D_3 catabolism disorder. It is reasonable to believe that sunlight exposure in the summer could lead to an increase of serum levels of activated vitamin D_3 metabolites and serum calcium concentrations in patients with loss-of-function mutations of *CYP24A1* (21,22). Moreover, the proband showed a lower level of $24,25(OH)_2D_3$ and, as a consequence, an increased $25(OH)D_3/24,25(OH)_2D_3$ ratio, as already reported in other homozygous patients harboring mutations of *CYP24A1* (10,16). In addition, the proband showed undetectable level of $1,24,25(OH)_3D_3$. This result is in agreement with a recent finding in an animal model in which knockout mice for a region downstream of cyp24a1 showed lower level of $1,24,25(OH)_3D_3$ compared to wild-type mice (23). Although further studies in humans are necessary, we believe that the measurement of this metabolite

may represent an additional useful tool in the diagnosis and characterization of patients with loss-of-function mutations of *CYP24A1*.

Furthermore, it is worth noting that the proband presented high serum levels of FGF23 and slightly low levels of both TRP and TmP/GFR. These data are in agreement with those reported by Meusburger et al. who found increased levels of FGF23 in a homozygous patient harboring the c.628T>C *CYP24A1* variant (24). A very recent elegant study in mice showed the key role of FGF23 in the regulation of the expression of both *cyp24a1* and cytochrome P450 family 27 subfamily B member 1 (cyp27b1), the enzyme that mediates 1α-hydroxylation of $25(OH)D_3$ in $1,25(OH)_2D_3$ (25). In this model, FGF23 reduced the expression of *cyp27b1* and stimulated the expression of *cyp24a1*, in a finely balanced homeostatic mechanism to prevent from $1,25(OH)_2D_3$ toxicity. Thus, the increased levels of FGF23 in patients with loss-of-function mutations of *CYP24A1* could represent an adaptative reaction to chronic high levels of $1,25(OH)_2D_3$ (25). Accordingly, the TRP and TmP/GFR were decreased, as in other clinical settings characterized by an increase in the rate of phosphate flow into the extracellular space from gut, cells or bone (i.e. vitamin D intoxication, and sarcoidosis) (26). Thus, it is possible that chronic hyperphosphaturia might represent a cofactor in the pathogenesis of nephrolithiasis and nephrocalcinosis in subjects carrying *CYP24A1* mutations and further studies are needed to investigate phosphate metabolism in these patients (24).

Data about clinical and biochemical phenotype of monoallelic mutations carriers (heterozygotes) are lacking and conflicting. In some studies, heterozygotes usually present lower levels of serum calcium and lower frequency of nephrolithiasis compared to homozygotes. Indeed, the majority of authors, considering such subjects as unaffected, postulated a recessive trait of inheritance (10). Conversely, Tebben et al. reported an overtly hypercalcemic clinical and biochemical phenotype in two children with a heterozygous *CYP24A1* intron-exon splice junction variant, supposing a dominant transmission (11). The main limitation of these studies was the lack of comparison between heterozygotes and a control population of wild-type subjects, a not negligible aspect in the study of inheritance of a genetic disorder. In this regard, we compared the clinical and biochemical features of heterozygotes with wild-type subjects living in a small village in the north of Tuscany who shared the same genetic (relatives) and environmental (both relatives and spouses) background. Compared to wild-type subjects, heterozygotes had significantly higher levels of serum total calcium and $25(OH)D_3$ concentrations, values closer to those observed in the proband. The calculation of $25(OH)D_3/24,25(OH)_2D_3$ ratio, a useful diagnostic clue in the identification of homozygous patients with *CYP24A1* variants (15,16), failed to discriminate heterozygotes from wild-types. These results are in line with those already reported (10). Furthermore, though as expected the proband showed undetectable serum $1,24,25(OH)_3D_3$, heterozygotes and wild-type subjects all exhibited detectable levels of this CYP24A1 product. However, no difference was found in the serum levels of

1,24,25$(OH)_3D_3$ between heterozygotes and wild-type subjects, although this result needs further validation in humans. Though it is not clear whether the biochemical differences in serum calcium and 25$(OH)D_3$ levels result in clinical manifestations, we can speculate that heterozygotes could be more prone to develop hypercalcemia and clinical manifestations if exposed to triggering factors such as vitamin D supplementation, sunlight exposure, dehydration or pregnancy (11,18). In this regard, different studies have reported a high incidence of symptomatic nephrolithiasis among heterozygotes ranging from 25 to 75% (5,11,27). Moreover, even in the study of Molin et al. who postulated an autosomal recessive transmission of IIH, a high percentage (25%) of heterozygotes displayed hypercalcemia and hypercalciuria (10). The mutation herein identified (p.Arg223*) is rare (gnomAD Exomes frequency 0.0000159) (2/125568), and has been previously reported in two other families (20,28). Although no functional study of this specific mutation has been performed, it is interesting to note that p.Arg223* is a stop mutation that occurs early in the gene, producing a protein so short without a heme group compared to wild type protein that accounts for 514 amino acids (NP_000773.2). As heme group is fundamental for the hydroxylation process and the likelihood that the protein would not have folded correctly, it is reasonable to believe that the enzyme is completely non-functional. Nevertheless, the proband exhibited a mild phenotype with an uneventful neonatal period and infancy. In the other families, probands were compound heterozygotes p.Arg223*/p.Glu143del and p.Arg223*/p.L148P, respectively, showing a phenotype superimposable of that of our proband. More interestingly, p.Arg223* was also found in heterozygote state in four relatives who showed hypercalciuria, asymptomatic nephrolithiasis (three of four) and mild hypercalcemia (one of four) (20,28). Moreover, by reviewing the literature, we found only eight other subjects harboring a truncating mutation of *CYP24A1* (1,10,22,29–32). Of these, four patients carried a biallelic truncating mutation, with variable clinical presentation at different age (1,10,22,32). The first case, a 6 month-year old child (homozygous p.Ala475fx*490) showed the classical features of severe IIH in infancy (1). The second, a 7 year-old child (homozygous p.Val403Phefs*15) showed a severe hypercalcemia in childhood (10). The third, a 45 year-old woman (compound heterozygous p.Pro21Argfs*8 and p.Glu469Alafs*22) showed a severe hypercalcemia in adulthood while taking vitamin D supplementation (22). Finally, the fourth, a 20 year-old woman (homozygous p.Ser334Valfs*9) experienced a moderate hypercalcemia complicated by pancreatitis during pregnancy (32). However, the small number of patients reported thus far does not allow for drawing a significant genotype-phenotype correlation and it is possible that other catabolic pathways of vitamin D involving cytochrome P450 family 3 subfamily A member 4 (CYP3A4) or the formation of inactive C3-epimers of vitamin D_3, or environmental influences might contribute to the phenotype (33–36).

Our study has several strengths: i) a large family of subjects with different genotype for *CYP24A1*, sharing a similar genetic background and the same environmental exposure was studied; ii)

all biochemical measurements were collected at same time and analyses performed at the same hospital laboratory; iii) a complete analysis of vitamin D_3 metabolites including $1,24,25(OH)_3D_3$ using mass-spectroscopy and FGF23 measurement were carried out. The main limitation is the relatively small numbers of subjects included in the study, which may impact the statistical power of some comparisons. Our data need further validation in larger cohorts of heterozygotes harboring different pathogenic variants compared to larger control population.

In conclusion, the results of our study provide insight on the biochemical profile of patients harboring *CYP24A1* pathogenic variants. We found that heterozygotes exhibit a borderline biochemical phenotype, different from that of wild-type subjects. In clinical practice, we suggest that these individuals might require surveillance because of the potential risk of developing hypercalcemia and related clinical manifestations if exposed to triggering factors.

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Compliance with ethical standards

Conflict of interest

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Informed consent

Written informed consent was obtained from the subjects for publication of this study

Contributorship statement

A.B, D.C and F.C planned the study. G.J and M.K performed the measurements of vitamin D metabolites. S.B. collected biochemical samples. F.B and M.A.C performed the genetic analyses. P.P. performed the statistical analysis. All authors discussed the results and revised the manuscript.

Data availability statement

Some or all data generated or analyzed during this study are included in this published article or in the data repositories listed in References.

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Legends of figures

Figure 1: Pedigree diagram of the family. Circles, women; boxes, males; crossed symbols, deceased; arrow, proband. Black fill, biallelic mutation carrier; black dot, monoallelic mutation carrier; white fill, wild-type carrier; gray fill, subjects not tested. Family members are indicated by generation (Roman number) and individual (Arabic number).

Figure 2: Seasonal trend of calcium (Panel A) and PTH (Panel B) in the proband. To note the mild to moderate hypercalcemia associated with low levels of PTH in summer and the normal values of serum calcium and PTH in winter. Data were obtained by the review of medical records. Regarding the PTH values, we considered only the measurements performed using a third-generation assay. Gray background identifies the normal reference range (Total calcium 8.5-10.2 mg/dL; PTH 8.0-40.0 pg/mL).

Figure 3: Sequencing results (NGS and sanger) in the proband and his relatives. IGV screenshot image in the proband (a); partial sanger sequencing diagram of the exon 5 of the proband (b) a carrier relative (c) and normal subject (d) of the family. The arrows indicate the nucleotide variation in homozygous (a and b) and heterozygous (c) condition or the wild type nucleotide (d).

Figure 4: Box-and-whiskers plots for the comparisons according to the three groups identified by the genetic analysis. For each panel the central box represents the values from the lower to the upper quartile (25^{th} to 75^{th} percentile) and the middle line represents the median; the vertical lines extend from the 10^{th} to 90^{th} percentile. The results of the comparisons performed using the Mann-Whitney test for continuous variables are reported above: the *P* on the top refers to the comparison between the two identified groups. The proband's values are also shown.

Table 1. Biochemical features of the proband at the admission and six months later.

Analytes	Normal range	June 2017	January 2018
Total serum calcium	8.6-10.2 mg/dL	10.8	10.0
Ionized calcium	1.13-1.32 mmol/L	1.36	1.24
Albumin	3.5-5 g/dL	4.6	4.5
Phosphate	2.5-4.5 mg/dL	3.2	2.8
PTH	8-40 pg/mL	4.0	18
Creatinine	0.6-1.2 mg/dL	1.0	1.1
*25(OH)D		35.0	
24 h urinary calcium	< 300 mg/24h	420	220
TRP	80-90%	78	
TmP/GFR	0.8-1.4	0.7	
#25(OH)D_3			47.3
#24,25$(OH)_2D_3$			0.14
#25(OH)D_3/24,25$(OH)_2D_3$			325.6
FGF23	23.2-95.4 pg/mL		150.1

* Using immuchemiluminescent assay; #Using mass-spettroscopy.

PTH=parathyroid hormone; TRP=tubular reabsorption of phosphate; TmP=tubular maximum phosphate reabsorption; GFR=glomerular filtration rate; 25(OH)D_3=25-hydroxyvitamin D_3; 24,25 $(OH)_2D_3$=24,25-dihydroxyvitamin D_3; FGF23=Fibroblast Growth Factor 23

Table 2. Clinical and biochemical features of the study population

Patient ID (Age, yrs)	Genotype	Nephrolithiasis (YES/NO)	Creatinine (N.R. 0.6-1.2 mg/dL)	Albumin (N.R. 3.5-5 g/dL)	Total Calcium (N.R. 8.5-10.2 mg/dL)	Phosphate (N.R. 2.5-4.5 mg/dL)	PTH (N.R. 8-40 pg/mL)	$25(OH)D_3$ (N.R. 14-57 ng/mL)	$1,25(OH)_2D_3$ (N.R. 16-84 pg/mL)	$24,25(OH)_2D_3$ (N.R.0.5-4.2 ng/mL)	$1,24,25(OH)_3D_3$ (N.R. 4.7-49.2 pg/mL)	$25(OH)D_3/24,25(OH)_2D_3$ (N.R. 8-38)	FGF23 (N.R. 23.2-95.4 pg/mL)
III.7 (45)	p.Arg223* (Hom)	YES	1.03	4.6	10	2.4	10.1	46.56	59.9	0.14	< 4	325.6	150.1
II.3 (82)	p.Arg223* (Het)	NO	0.55	4.2	9.7	3.5	11.0	48.06	42.1	2.10	20.1	22.9	40.9
II.4 (89)	p.Arg223* (Het)	NO	1.15	4.1	9.6	3.0	38.2	33.56	34.6	0.83	7.6	40.6	84.1
II.7 (84)	p.Arg223* (Het)	NO	0.77	4.8	10.1	3.0	25.0	49.23	46.1	3.85	27.0	12.8	80.7
III.6 (56)	p.Arg223* (Het)	YES	0.62	4.2	9.4	3.9	17.0	23.43	37.8	1.13	10.5	20.8	71.9
IV.5 (14)	p.Arg223* (Het)	NO	0.53	4.9	9.9	4.4	15.2	31.08	80.7	0.88	11.7	35.3	54.4
IV.6 (10)	p.Arg223* (Het)	NO	0.59	4.7	10.1[†]	4.4	20.0	40.22	34.9	1.53	20.2	26.3	N.A.
II.1 (78)	WT	NO	0.71	4.3	9.6	3.1	21.8	51.71	50.4	2.57	18.0	20.2	45.9
II.2 (85)	WT	YES	0.94	4.6	9.6	3.3	34.5	17.21	52.7	0.51	10.5	33.7	57.4
II.8 (83)	WT	NO	0.95	4.5	9.3	2.4	18.7	23.35	42.4	1.19	13.7	19.7	63.5

III.1 (49)	WT	NO	0.88	4.7	9.8	3.6	24.2	16.35	48.6	0.71	13.3	23.2	72.6
III.2 (44)	WT	NO	0.65	4.1	8.9	2.6	23.6	15.70	39.4	0.5	6.8	31.3	54.6
III.4 (55)	WT	NO	0.59	4.2	8.6	2.6	28.6	20.22	49.5	0.92	13.6	21.9	28.4
III.5 (56)	WT	NO	1.05	4.5	9.6	2.6	38.2	13.03	37.2	0.53	7.8	24.8	65.5
III.8 (46)	WT	YES	0.69	4.6	9.2	3.4	38.7	46.28	93.7	2.24	12.6	20.7	42.5
III.10 (68)	WT	NO	0.94	4.5	9.4	3.4	12.4	24.11	38.7	1.43	13.2	16.9	48.0
IV.1 (11)	WT	NO	0.47	4.6	9.6†	4.4	15.7	30.47	59.6	0.85	8.9	36.1	52.3
IV.4 (20)	WT	NO	0.95	4.9	9.5	3.7	40.0	9.25	54.1	0.16	7.7	59.7	34.7
IV.8 (44)	WT	NO	0.77	4.9	9.5	4.2	10.9	15.61	33.9	0.92	8.3	17.1	39.5

†Reference range adjusted for age: 9.1-11.0 mg/dL

Hom=homozygous; Het=heterozygous; WT=wild type; N.R.=normal range; N.A.=not available; PTH=parathyroid hormone; $25(OH)D_3$= 25-hydroxyvitamin D_3; $1,25(OH)_2D_3$=1,25-dihydroxyvitamin D_3; $24,25(OH)_2D_3$= 24,25-dihydroxyvitamin D_3; $1,24,25(OH)_3D_3$=1,24,25-trihydroxyvitamin D_3

FGF23= Fibroblast Growth Factor 23

Patient ID refers to pedigree diagram. All serum samples were collected in February.

Figure 1

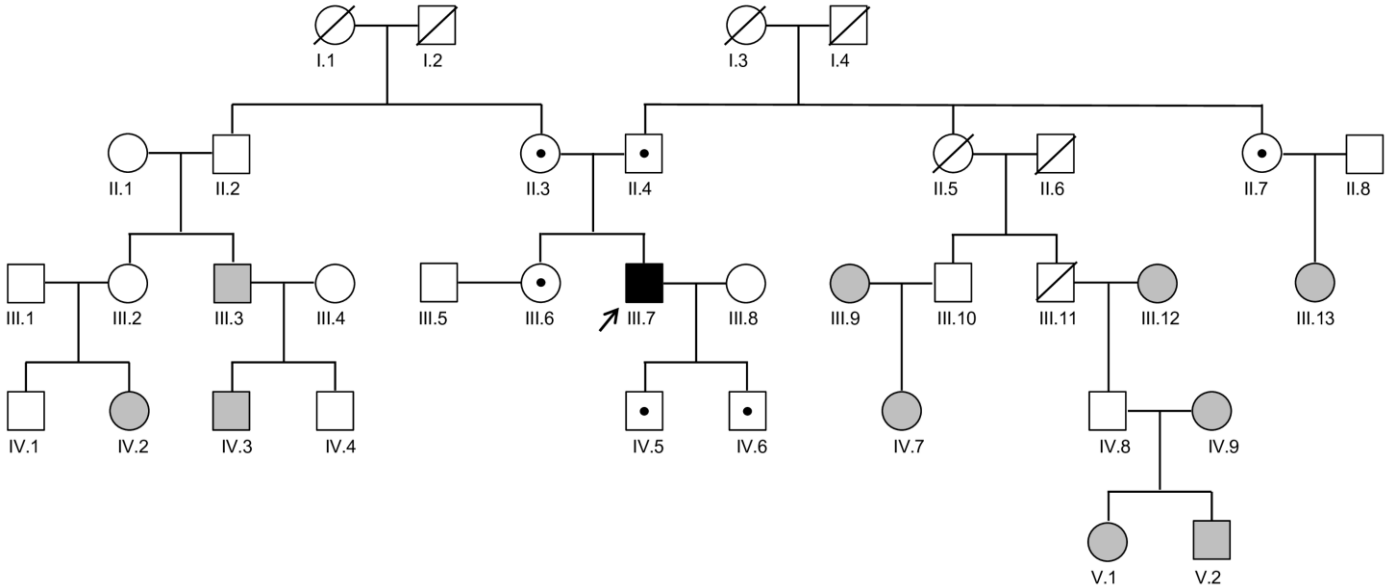

Figure 2

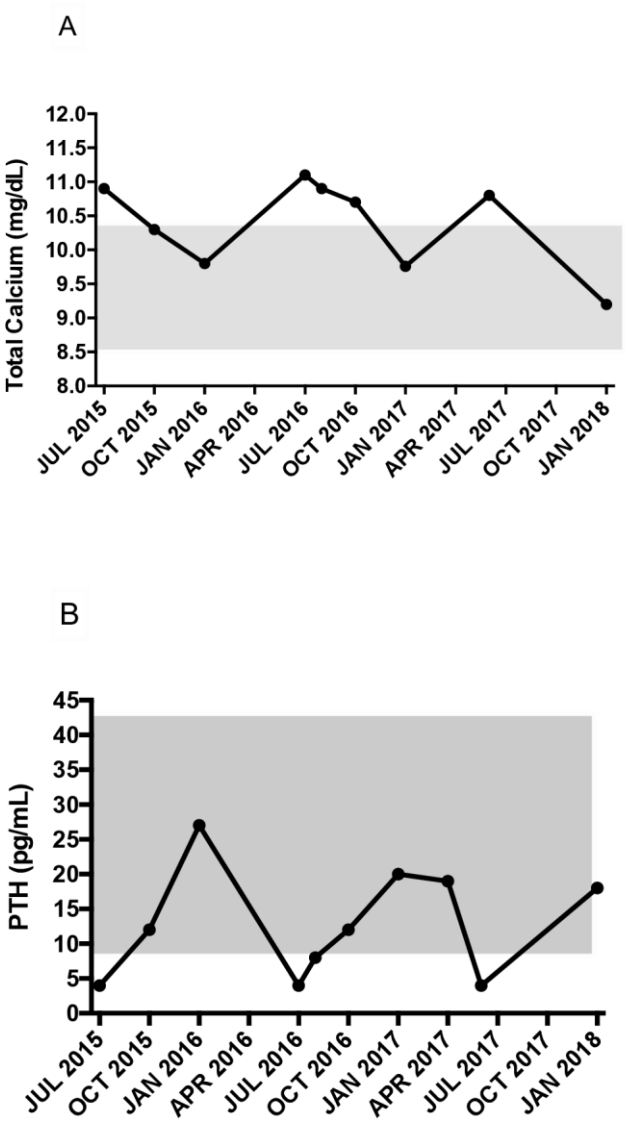

Fig 3

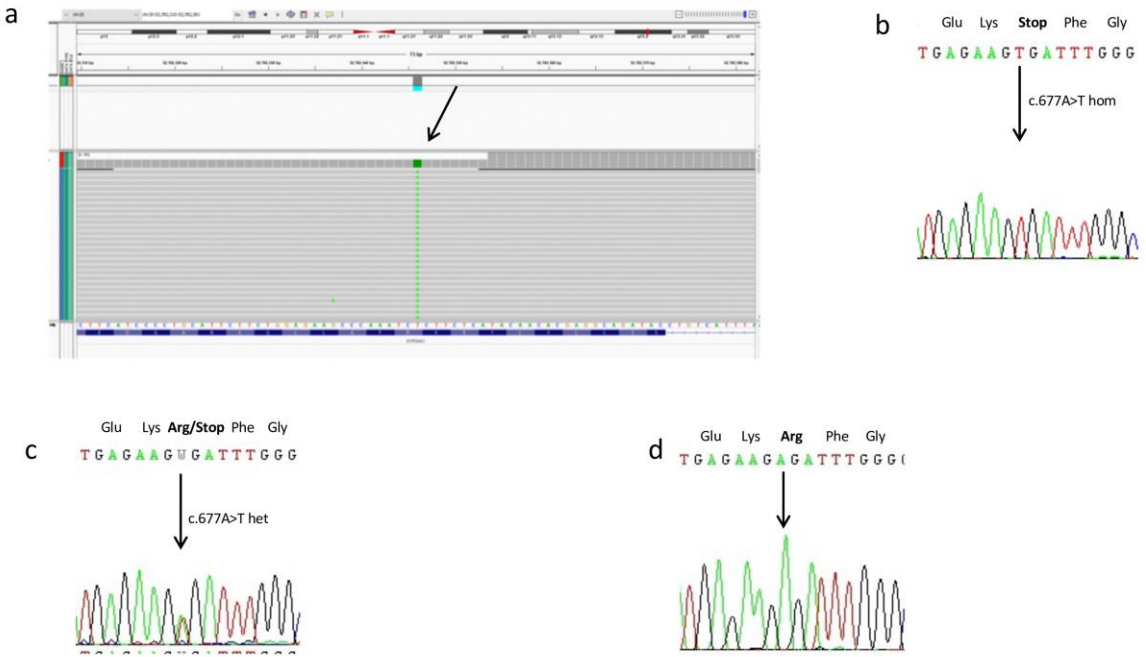

Figure 4