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Case Report

Alterations in *SAMD9*, *AHSG*, *FRG2C*, and *FGFR4* Genes in a Case of Late-Onset Massive Tumoral Calcinosis

Melvin Khee Shing Leow, MD, PhD, MMed (Int Med) [1, 2, 3, 4, 5], Joshur Ang, MRes [1], Xinyan Bi, PhD [6], Ee Tzun Koh, MD, MMed (Int Med), MRCP (UK) [2, 3, 7], Craig McFarlane, PhD [8, *]

[1] *Singapore Institute for Clinical Sciences (A*STAR), Brenner Centre for Molecular Medicine, Singapore*
[2] *Yong Loo Lin School of Medicine, National University of Singapore, Singapore*
[3] *Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore*
[4] *Cardiovascular and Metabolic Disorders Program, Duke-NUS Medical School, Singapore*
[5] *Department of Endocrinology, Division of Medicine, Tan Tock Seng Hospital, Singapore*
[6] *Singapore Institute of Food and Biotechnology Innovation (A*STAR), Singapore*
[7] *Department of Rheumatology, Allergy and Immunology, Tan Tock Seng Hospital, Singapore*
[8] *Department of Molecular & Cell Biology, College of Public Health, Medical and Veterinary Sciences, James Cook University, Townsville, QLD, Australia*

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ABSTRACT

Background/Objective: Tumoral calcinosis (TC) is a rare, arcane, and debilitating disorder of phosphate metabolism manifesting as hard masses in soft tissues. Primary hyperphosphatemic TC has been shown to be caused by pathogenic variants in the genes encoding FGF23, GALNT3, and KLOTHO. We report a case of massive TC mechanistically associated with phosphatonin resistance associated with heterozygous alterations in the sterile alfa motif domain–containing protein-9 gene (*SAMD9*), alfa 2-Heremans-Schmid glycoprotein gene (*AHSG*), FSHD region gene 2-family member-C gene (*FRG2C*), and fibroblast growth factor receptor-4 gene (*FGFR4*).
Case Report: A middle-aged Malay woman with systemic sclerosis presented with painful hard lumps of her axillae, lower limbs, and external genitalia. She was eucalcemic with mild hyperphosphatemia associated with reduced urinary phosphate excretion. Magnetic resonance imaging revealed calcified soft tissue masses. Paradoxically, the serum intact FGF23 level increased to 89.6 pg/mL, corroborated by Western blots, which also showed overexpression of sFRP4 and MEPE, consistent with phosphatonin resistance.
Discussion: Whole genome sequencing identified 2 heterozygous alterations (p.A454T and p.T479M) in *SAMD9*, 2 heterozygous alterations (p.M248T and p.S256T) in *AHSG*, a frameshift alteration (p.Arg156fs) in *FRG2C*, and a heterozygous alteration (p.G388R) in *FGFR4*, all of which are associated with calcinosis. Nonsynonymous alterations of *FRP4* and *MEPE* were also detected.
Conclusion: This highlights that the simultaneous occurrence of alterations in several genes critical in phosphate homeostasis may trigger massive TC despite their heterozygosity. These findings should prompt functional studies in cell and animal models to reveal mechanistic insights in the pathogenesis of such crippling mineralization disorders.

Abbreviations: AHSG, alpha 2-Heremans-Schmid glycoprotein gene; Amino acids, Ala (alanine), Arg (arginine), Ser (serine), Thr (threonine), Gly (glycine), Met (methionine); ClinVar, public archive of interpretations of clinically relevant variants; CREST, calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly and telangiectasia; CRISPR-Cas9, clustered regularly interspaced short palindromic repeats-CRISPR associated protein 9; CT, computed tomography; DNA bases, A (adenine), T (thymine), C (cytosine), G (guanine); ELISA, enzyme-linked immunosorbent assay; FEPi, fractional excretion of phosphate index; FGF23, fibroblast growth factor subtype 23; FGFR1-3, fibroblast growth factor receptor subtypes 1 to 3; FGFR4, fibroblast growth factor receptor-4 gene; FRG2C, FSHD region gene 2-family member C gene; GALNT3, Udp-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3; HFTC, hyperphosphatemic familial tumoral calcinosis; kDa, kilodalton; KLOTHO, name originated from Klotho, one of the 3 Fates of Greek mythology who spins the thread of human life; MEPE, matrix extracellular phosphoglycoprotein; MRI, magnetic resonance imaging; NFTC, normophosphatemic familial tumoral calcinosis; PO_4, inorganic phosphate; Ras GTPase, rat sarcoma guanine triphosphatase; sFRP4, secreted frizzled related protein 4; SAMD9, sterile alpha motif domain-containing protein-9 gene; SNP, single nucleotide polymorphism; TC, tumoral calcinosis.

* Address correspondence to Dr Craig McFarlane, Department of Molecular and Cell Biology, College of Public Health, Medical, and Veterinary Sciences, James Cook University, 1 James Cook Dr., Townsville, QLD 4811, Australia.
E-mail address: craig.mcfarlane@jcu.edu.au (C. McFarlane).

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Introduction

Described over a century ago, tumoral calcinosis (TC) is a rare, arcane, and debilitating disorder of phosphate metabolism manifesting as hard masses in soft tissue.[1,2] Primary normophosphatemic familial tumoral calcinosis (NFTC) occurs largely among Middle Eastern people, whereas primary hyperphosphatemic familial tumoral calcinosis (HFTC) has a predilection for those of African descent.[3] Both forms exhibit autosomal recessive Mendelian genetics. The sex ratio is equal, and disease starts in childhood or adolescence.[3] Secondary TC can occur in hyperparathyroidism, hypervitaminosis D and severe hyperphosphatemia from uremia; resolution after parathyroidectomy with daily hemodialysis has been documented.[4,5] The differential diagnosis includes tophaceous gout, myositis ossificans, calcific myonecrosis, synovial osteochondromatosis, calcinosis universalis, and calcinosis circumscripta. Serum biochemistry typically shows normal or mildly elevated phosphate and alkaline phosphatase levels with normocalcemia and abnormally increased 1,25-dihydroxyvitamin D levels and tubular reabsorption of phosphate.[3] Radiologically, amorphous calcifications on plain roentgenography are observed, whereas computed tomography shows "sedimentation sign" and magnetic resonance imaging reveals bright, alternating high signal intensity and signal voids with fluid levels. Scintigraphy shows intense radionuclide uptake in these masses.[6] Histologically, encapsulated masses with septations comprising calcium phosphate, carbonate/hydroxyapatite with granulomatous multinucleated giant cells, and epithelioid elements are observed microscopically.[7] HFTC is associated with pathogenic variants in genes encoding fibroblast growth factor-subtype 23 (FGF23), GALNT3 (responsible for FGF23 glycosylation), and KLOTHO (coreceptor for FGF23 signaling).[8] An acquired autoimmune form has been reported.[9] More recently, drug-induced TC due to pemigatinib, an FGFR1-3 inhibitor for cholangiocarcinoma with FGFR gene fusion/rearrangement, has been described.[10]

Case Report

A 53-year-old Malay woman with systemic sclerosis presented with progressively enlarging masses over her body (Fig. *A*). She noticed these lesions 3 years prior, which began as small painless lumps 1 to 2 cm over her left axilla. These "tumorous lumps" grew, some ulcerating painfully through the skin. Surgical extirpation was attempted at her left axilla; however, new lumps surfaced elsewhere, including her left upper arm, right thigh, buttocks, and vulva. Wound healing was impaired by a persistently sterile calcareous discharge through a small sinus. Apart from her Middle Eastern ancestry and nonconsanguineous parents, her family history was unremarkable. Physical examination revealed massive lumps up to approximately 15 × 20 cm over her posterolateral right thigh, axillae, posterior trunk, and vulval and gluteal regions with a "rocky" consistency. Sclerodermatous facies with CREST syndrome were noted. Clinical biochemistries (Table) included serum and urine calcium, serum and urine inorganic phosphate, serum intact parathyroid hormone, and serum 25-hydroxycholecalciferol.

Her fractional excretion of phosphate index, computed as follows:

Fractional excretion of phosphate index = (PO4 [urine]/PO4 [serum]) × (creatinine [serum]/creatinine [urine]) × 100, equals (13.9/1.6) × (40/5.9) × 100 = 5.89% (normal, 10%-20%), suggestive of phosphatonin activity deficiency. Magnetic resonance imaging showed calcified masses in her right medial upper to mid-thigh with dimensions up to 10 × 15 cm across (Fig. *B*).

Highlights

- *SAMD9*, *AHSG*, *FRG2C* and *FGFR4* are genes linked to massive tumoral calcinosis
- High circulating phosphatonins with hypophosphatemia implies phosphatonin resistance
- Heterozygotes of several calcinosis-associated genes can develop tumoral calcinosis

Clinical Relevance

Massive tumoral calcinosis is an extremely rare, arcane, and debilitating disorder that appear bizarre to both affected patients and their clinicians alike. Ectopic metastatic calcification, extraosseous ossification, and heterotopic mineralization are relevant initial clinical diagnostic considerations. However, in the diagnostic workup, it is reasonable to evaluate for phosphatonins, including fibroblast growth factor-subtype 23, frizzled-related protein-4, and matrix extracellular phosphoglycoprotein, and examine for alterations of GALNT3 and KLOTHO. This case illustrates that when none of these are involved, other less commonly involved genes, such as SAMD9, AHSG, FRG2C, and FGFR4 alterations, may have to be considered. Multiple heterozygous alterations of calcinosis-related genes may cause hyperphosphatemia via phosphatonin resistance. Its orphan disease status qualifies for more funding to support research that generates insights on its etiology and pathogenesis to help elucidate the function of these proteins with the hope that effective novel therapeutic biologics and drugs may emerge in the near future.

The circulating intact FGF23 level by enzyme-linked immunosorbent assay increased to 89.6 pg/mL, corroborated by FGF23 on Western blots, which also showed overexpression of 2 other phosphatonins, secreted frizzled-related protein-4 (FRP4) and matrix extracellular phosphoglycoprotein (MEPE), relative to a healthy age-/sex-matched control (Fig. *C* and *E*). Such hyperphosphatoninemia should have expectedly induced phosphaturia and intractable hypophosphatemia. Instead, a paradoxical phenotype of hyperphosphatemia with diminished urinary phosphate excretion was observed, suggesting phosphatonin resistance. Plausible mechanisms include FGF23 resistance due to nonfunctional FGFR or inactivating alterations of KLOTHO. Alternatively, mutant FGF23, FRP4, and/or MEPE lacking phosphatonin action could be responsible.

Whole genome next-generation sequencing revealed 2 heterozygous alterations in the sterile alfa motif domain–containing protein-9 gene (*SAMD9*) (Fig. *F*). The first alteration (single-nucleotide variation [SNV] [formerly SNP] rs117649834) was c.1360G>A (p.Ala454Thr), likely benign with respect to NFTC. The second alteration (SNV rs78564070) was c.1436C>T, (p.Thr479Met), reported in ClinVar as benign. Although the threonine residue at position Thr479 is only conserved in humans and chimpanzees, the alanine residue at position Ala454 is highly conserved across species (Fig. *G*). Additional alterations in alfa 2-Heremans-Schmid glycoprotein gene (*AHSG*), FSHD region gene-2-family member-C gene (*FRG2C*), and fibroblast growth factor receptor-4 gene (*FGFR4*) were also found. For *AHSG*, there were 2 heterozygous alterations. The first alteration (SNV rs4917) was c.743T>C (p.Met248Thr), which was reported previously in ClinVar as benign with respect to the "alopecia-intellectual disability syndrome 1." The second alteration (SNV rs4918) was c.767G>C (p.Ser256Thr), which was

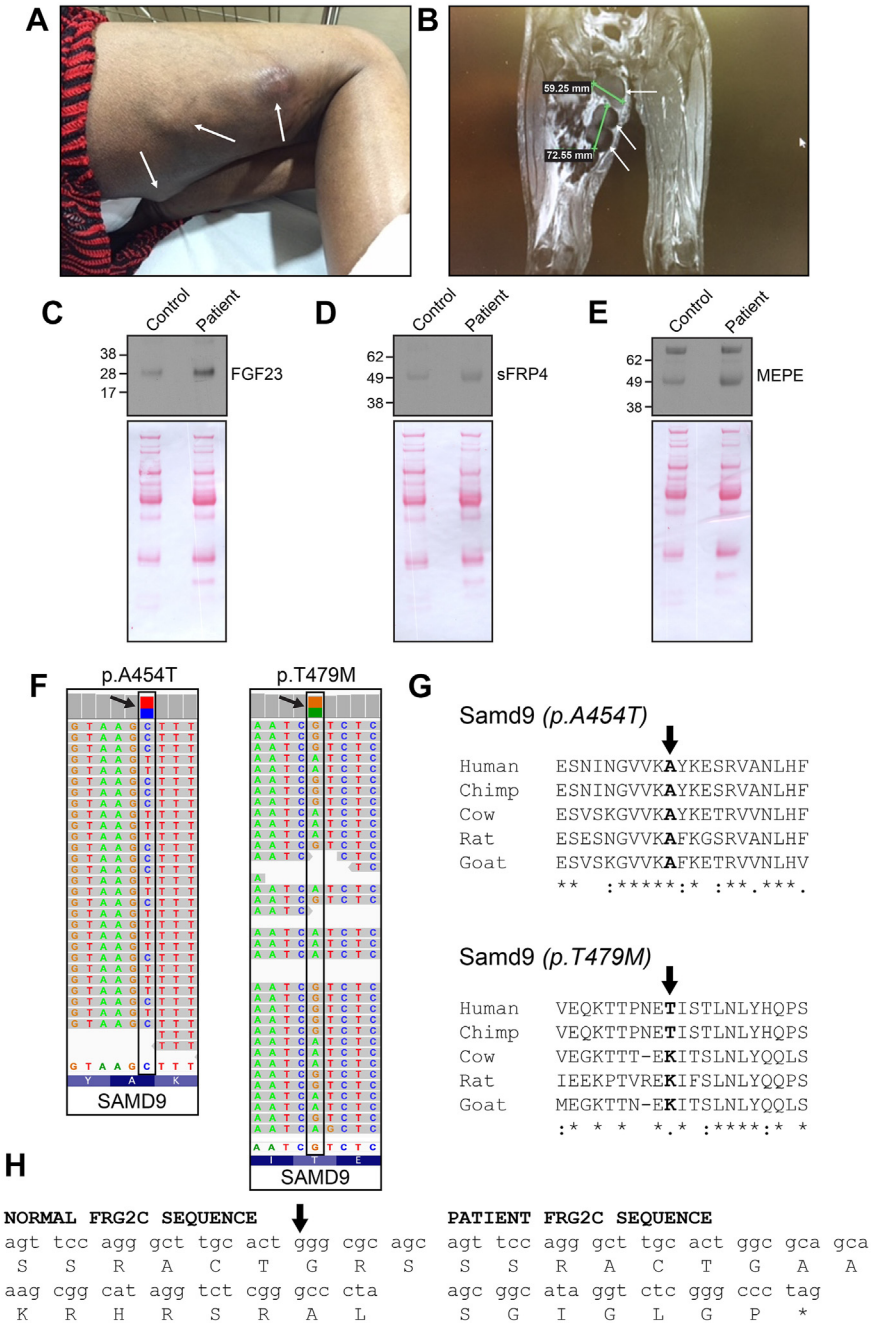

Fig. *A*, Posterolateral aspect of the right thigh showing multiple bulky subcutaneous masses. *B*, Magnetic resonance imaging revealed extraosseous calcified masses in the location of the right thigh in this coronal view. Western blot analysis of the circulating levels of the phosphatonins fibroblast growth factor-subtype 23 (FGF23) (*C*), secreted frizzled-related protein-4 (sFRP4) (*D*), and matrix extracellular phosphoglycoprotein (MEPE) (*E*) in the control and patient plasma samples. *F*, Illumina HiSeq whole genome sequencing analysis of the heterozygous p.A454T and p.T479M alterations in the *SAMD9* gene. The reverse-direction sequence was given for the HiSeq sequencing reads. *G*, Conservation of Samd9 protein sequences in different species, as determined by Clustal Omega sequence alignment. The location of the heterozygous p.A545T (left) and p.T479M (right) alterations are indicated by arrows. *H*, Images showing the region of the *FRG2C* gene that contained the c.465delG (p.Arg156Alafs*10) alteration. The normal *FRG2C* sequence (left) and patient *FRG2C* sequence (right) are provided. The arrow indicates the g base pair that was deleted, which resulted in a frameshift and an early stop codon (*).

also previously reported in ClinVar as benign with respect to the "alopecia-intellectual disability syndrome 1." For *FRG2C*, the alteration (SNV rs144577984), yet to be reported in ClinVar, was c.465delG (p.Arg156Alafs*10), a frameshift alteration that creates a new reading frame ending at a premature stop codon at position 10 (Fig. *H*), likely impacting its protein function. The *FGFR4* alteration (SNV rs351855) was c.1162G>A (p.Gly388Arg), reported in ClinVar as "uncertain significance, benign (conflicting evidence)." Apart from 1 neutral nonsynonymous alteration each in *FRP4* and *MEPE*, both encoding phosphatonins, no deleterious alterations of other phosphatonin genes or their receptors were identified, including *FGF23*, *KLOTHO*, and *GALNT3*.

Discussion

SAMD9, a gene located on chromosome 7q21.2, encodes a 1589-amino-acid-long cytoplasmic protein (molecular weight, approximately 184.3 kd) expressed in a broad range of tissues with

Table
Clinical Biochemistries of Relevance to This Patient

Analyte	Patient's values	Reference intervals
Serum calcium	2.38 (adjusted for serum albumin)	2.10-2.60 mmol/L
Urinary calcium	<0.3	0.05-7.25 mmol/L
Serum phosphate	1.6	0.80-1.50 mmol/L
Urinary phosphate	13.9	14.5-43.9 mmol/L
Serum magnesium	0.7	0.7-1.0 mmol/L
Serum urea	2.8	2.8-9.3 mmol/L
Serum creatinine	40	40-75 μmol/L
Urine creatinine	5.9	1.1-20.3 mmol/L
Creatinine clearance	96 mL/min (BSA, 1.37 m^2)	62-139 mL/min/BSA (1.73 m^2) (for the ages of 50-59 y)
Calcium-phosphate product ($Ca \times PO_4$)	3.8	<4.4 $mmol^2/L^2$
Fractional excretion of phosphate index	5.89%	10%-20%
Serum intact PTH	4.4	0.8-6.8 pmol/L
Serum intact FGF23	89.6	30-46 pg/mL
Serum 1,25-dihydroxyvitamin D	24	20-50 μg/L

Abbreviations: BSA = body surface area; FGF23 = fibroblast growth factor-subtype 23; PTH = parathyroid hormone.

functions yet to be fully defined although its alterations are associated with NFTC seemingly confined to the Jewish Yemenite population.[11] Remarkably, Yemen is located in the Middle East, and our patient coincidentally was of Arabian extraction based on her ancestry. Unlike NFTC, the effects of 2 heterozygous alterations in our patient manifested much later. A late disease onset implies the requirement for gene-environmental interactions and effects of aging for a phenotype to manifest. This includes background concomitant disease, diet, and the exposome. These 2 *SAMD9* variants we report are novel and have never been published. Both *SAMD9* variants have been reported as "likely benign" for NFTC on ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), although no functional evidence is provided to support this. *SAMD9* is known to be involved in tissue response to injury and plays a role in dystrophic calcification. To date, *SAMD9* has only been implicated in NFTC but not in HFTC.

Her serum calcium-phosphate solubility product of 3.8 $mmol^2/L^2$ was close to the saturation threshold of 4.4 $mmol^2/L^2$ established to heighten the risk of mineral crystallization and systemic calciphylaxis.[12,13] Her *SAMD9* alterations may have synergized with systemic sclerosis, the latter being known to cause calcinosis (CREST syndrome).[14] Her whole genome sequencing data also revealed heterozygous alterations in 3 more genes newly associated with TC, namely, *AHSG*, *FRG2C*, and *FGFR4*. Interestingly, it is increasingly being recognized that differences in specific alterations site(s) within diseases-causing genes(s) can dictate disease inheritance patterns (dominant vs recessive).[15] Thus, a heterozygous alteration may occasionally manifest a disease phenotype due to an autosomal dominant behavior. Additionally, double alterations in a gene resulting in more than 1 amino acid residue substitution carry a greater chance of structural alteration that impacts function, which implies that the sum of 2 otherwise "relatively benign" alterations may occasionally yield a pathogenic phenotype.[16,17] *AHSG*, localized to chromosome 3q27.3, encodes fetuin-A, a major inhibitor of systemic calcification.[18] The level of fetuin-A is inversely correlated with the serum phosphate in a dose-dependent relationship with the number of copies of the *AHSG2* allele (ie, homozygous vs heterozygous; double missense alterations Thr248 and Thr256 are denoted by *AHSG1*, whereas alterations Met248 and Ser256 are denoted by *AHSG2*).[19] Our patient can, thus, be similarly genotyped as *AHSG1/2*, which was correlated well with her mildly elevated phosphate level, based on the serum phosphate-genotype regression plot by Osawa et al.[19] Although no functional evidence is provided on ClinVar, both *AHSG* variants have been reported as "benign" with respect to the alopecia-intellectual disability syndrome 1. However, no interpretation for this variant in the context of TC has ever been reported, thus making our case possibly the first to associate these 2 alterations with HFTC. As for *FRG2C*, localized to chromosome 3p12.3, this gene encodes a 30.8-kd protein with histone demethylase function that negatively regulate Ras GTPase activity recently associated with HFTC, thereby lending credence to a relevant functional consequence in our patient for the frameshift alteration with subsequent premature stop codon we found. *FGFR4*, on chromosome locus 5q35.2, encodes the FGF receptor-type 4, which exhibits multiple functions including phosphate regulation. FGFR4 alterations can lead to aberrant FGF23 signaling, which may precipitate HFTC.[20] Finally, the nonsynonymous alterations in *FRP4* and *MEPE* probably diminished phosphatonin activity, hence resulting in loss of negative feedback and compensatory reciprocal overexpression on Western blots. Our case illustrates how multiple alterations in 4 genes associated with calcinosis plus defective FRP4 and MEPE phosphatonins in scleroderma can trigger massive TC. The predilection for calcinosis in scleroderma itself should not be underestimated, given that up to 40% of patients with scleroderma are affected by calcinosis.[14]

Conclusion

Functional elucidation of these alterations could be aided by precise CRISPR-Cas9 gene-editing experiments, siRNA targeted against calcinosis-linked genes in animal models or in vitro tissue calcification assays. As genome sequencing of the patient's parents was not performed, we cannot deduce if the alterations were inherited from 1 or both parents. Its orphan disease status qualifies massive TC for more funding to support research with the hope that effective novel therapeutic biologics and drugs may emerge in the near future.

Disclosure

The authors have no multiplicity of interest to disclose.

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Author Contributions

M.K.S.L. and E.T.K. comanaged the patient and were in charge of her clinical care both during her in-patient hospitalization and during her outpatient office visits; M.K.S.L. helped with the collection of blood samples and made the correct preliminary clinical diagnosis, which steered the subsequent confirmatory molecular testing, and drafted and extensively edited the manuscript to reach the finalized form accepted for publication; X.B. performed the FGF23 enzyme-linked immunosorbent assays; J.A. and C.M. performed the Western blots and whole genome sequencing; M.K.S.L. interpreted the clinical laboratory results and guided on the necessary molecular experiments based on these results; and C.M. contributed to the interpretation of the molecular tests and whole genome sequencing data. All authors helped to review, edit, and amend the manuscript draft, and all approved the final version of the revised manuscript.

References

1. Giard A. Sur la calcification hibernale. *C R Soc Biol*. 1898;10:1013–1015.
2. Duret MH. Tumeurs multiples et singulieres des bourses sereuses. *Bull Mem Soc Ant Paris*. 1899;74:725–731.
3. Smack D, Norton SA, Fitzpatrick JE. Proposal for a pathogenesis-based classification of tumoral calcinosis. *Int J Dermatol*. 1996;35(4):265–271.
4. Li SY, Chuang CL. Rapid resolution of uremic tumoral calcinosis after parathyroidectomy. *Mayo Clin Proc*. 2012;87(11):e97–e98.
5. Kim SJ, Goldstein M, Szabo T, Pierratos A. Resolution of massive uremic tumoral calcinosis with daily nocturnal home hemodialysis. *Am J Kidney Dis*. 2003;41(3):E12.
6. Farzan N, Farhoud AR. Tumoral calcinosis: what is the treatment? Report of two cases of different types and review of the literature. *Am J Orthop (Belle Mead NJ)*. 2011;40(9):E170–E176.
7. Pakasa NM, Kalengayi RM. Tumoral calcinosis: a clinicopathologic study of 111 cases with emphasis on the earliest changes. *Histopathology*. 1997;31(1):18–24.
8. Boyce AM, Lee AE, Roszko KL, Gafni RI. Hyperphosphatemic tumoral calcinosis: pathogenesis, clinical presentation, and challenges in management. *Front Endocrinol (Lausanne)*. 2020;11:293.
9. Roberts MS, Burbelo PD, Egli-Spichtig D, et al. Autoimmune hyperphosphatemic tumoral calcinosis in a patient with FGF23 autoantibodies. *J Clin Invest*. 2018;128(12):5368–5373.
10. Puar A, Donegan D, Helft P, et al. Hyperphosphatemic tumoral calcinosis with pemigatinib use. *AACE Clin Case Rep*. 2022;8(5):217–220.
11. Topaz O, Indelman M, Chefetz I, et al. A deleterious mutation in SAMD9 causes normophosphatemic familial tumoral calcinosis. *Am J Hum Genet*. 2006;79(4):759–764.
12. Alfrey AC, Ibels LS. Role of phosphate and pyrophosphate in soft tissue calcification. *Adv Exp Med Biol*. 1978;103:187–193.
13. Hebert LA, Lemann Jr J, Petersen JR, Lennon EJ. Studies of the mechanism by which phosphate infusion lowers serum calcium concentration. *J Clin Invest*. 1966;45(12):1886–1894.
14. Burgess KA, Herrick AL, Watson REB. Systemic sclerosis skin is a primed microenvironment for soft tissue calcification-a hypothesis. *Rheumatology (Oxford)*. 2021;60(6):2517–2527.
15. You W, Yang J, Wang L, et al. Case report: a Chinese family of type A insulin resistance syndrome with diabetes mellitus, with a novel heterozygous missense mutation of the insulin receptor gene. *Front Endocrinol (Lausanne)*. 2022;13, 895424.
16. Gress A, Ramensky V, Kalinina OV. Spatial distribution of disease-associated variants in three-dimensional structures of protein complexes. *Oncogenesis*. 2017;6(9), e380.
17. Tenaillon O, Matic I. The impact of neutral mutations on genome evolvability. *Curr Biol*. 2020;30(10):R527–R534.
18. Brylka L, Jahnen-Dechent W. The role of fetuin-A in physiological and pathological mineralization. *Calcif Tissue Int*. 2013;93(4):355–364.
19. Osawa M, Tian W, Horiuchi H, Kaneko M, Umetsu K. Association of alpha2-HS glycoprotein (AHSG, fetuin-A) polymorphism with AHSG and phosphate serum levels. *Hum Genet*. 2005;116(3):146–151.
20. Gattineni J, Twombley K, Goetz R, Mohammadi M, Baum M. Regulation of serum 1,25(OH)2 vitamin D3 levels by fibroblast growth factor 23 is mediated by FGF receptors 3 and 4. *Am J Physiol Renal Physiol*. 2011;301(2):F371–F377.