

A Deleterious Mutation in *SAMD9* Causes Normophosphatemic Familial Tumoral Calcinosis

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Familial tumoral calcinosis (FTC) is a rare autosomal recessive disorder characterized by the progressive deposition of calcified masses in cutaneous and subcutaneous tissues, which results in painful ulcerative lesions and severe skin and bone infections. Two major types of FTC have been recognized: hyperphosphatemic FTC (HFTC) and normophosphatemic FTC (NFTC). HFTC was recently shown to result from mutations in two different genes: *GALNT3*, which codes for a glycosyltransferase, and *FGF23*, which codes for a potent phosphaturic protein. To determine the molecular cause of NFTC, we performed homozygosity mapping in five affected families of Jewish Yemenite origin and mapped NFTC to 7q21-7q21.3. Mutation analysis revealed a homozygous mutation in the *SAMD9* gene (K1495E), which was found to segregate with the disease in all families and to interfere with the protein expression. Our data suggest that *SAMD9* is involved in the regulation of extraosseous calcification, a process of considerable importance in a wide range of diseases as common as atherosclerosis and autoimmune disorders.

Extraosseous calcification is considered to play a pivotal role in the pathogenesis of a wide range of common disorders, such as atherosclerosis and renal failure.^{1,2} Recently, the deciphering of the genetic basis of a number of rare monogenic hereditary syndromes has shed light on the molecular mechanisms regulating this process.³

Familial tumoral calcinosis (FTC) is an uncommon life-threatening disorder characterized by massive periarticular—and seldom visceral—deposition of calcified tumors.⁴ When associated with hyperphosphatemia, the disease is known as “hyperphosphatemic FTC” (HFTC [MIM #211900]) and can result from mutations in at least two genes: *GALNT3*, which encodes the glycosyltransferase UDP-N-acetyl- α -D-galactosamine:polypeptide N-acetylgalactosaminyl transferase 3 (ppGalNacT3) that is responsible for initiating O-glycosylation, and *FGF23*, which encodes a potent phosphaturic protein.⁵⁻¹² Recent data suggest that *FGF23* extracellular secretion may depend on ppGalNacT3-mediated O-glycosylation.¹³ A bone disorder termed “hyperostosis-hyperphosphatemia syndrome” (MIM #211900) was also shown to result from mutations in *GALNT3*.¹⁴ To complicate the picture even further, we identified a subset of patients with FTC who display normal circulating levels of phosphate. These individuals were found not to carry mutations in *GALNT3* or *FGF23*, which suggests that HFTC and normophosphatemic FTC (NFTC) are nonallelic.⁵ In the present study, we

identified a deleterious mutation in *SAMD9* that underlies NFTC in five affected families.

All participants or their legal guardians provided written and informed consent, according to a protocol approved by the local Helsinki Committee and by the Committee for Genetic Studies of the Israeli Ministry of Health. Blood samples were drawn from all family members, and DNA was extracted according to standard procedures. Skin biopsy samples were fixed in formaldehyde. Fibroblast cell cultures were established from punch-skin biopsy samples from patients or healthy control individuals, and were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum.

We assessed a total of eight individuals affected with NFTC from five families of Jewish Yemenite origin (fig. 1*a*). All patients reported reddish-to-hyperpigmented skin lesions during the 1st year of life (fig. 2*a*), which preceded the later appearance of calcified nodules, distributed mainly over the extremities (fig. 2*b*). In addition, severe conjunctivitis and gingivitis (fig. 2*c*) were observed in most of the affected individuals. Results of routine laboratory tests—including calcium, phosphate, vitamin D3 metabolites, and parathyroid-hormone levels—were normal. Histopathological examination of lesional skin biopsies disclosed massive calcium deposition in the mid- and lower dermis (fig. 2*d*).

Because all families shared a common ethnic origin and since NFTC is a very rare condition, we postulated the

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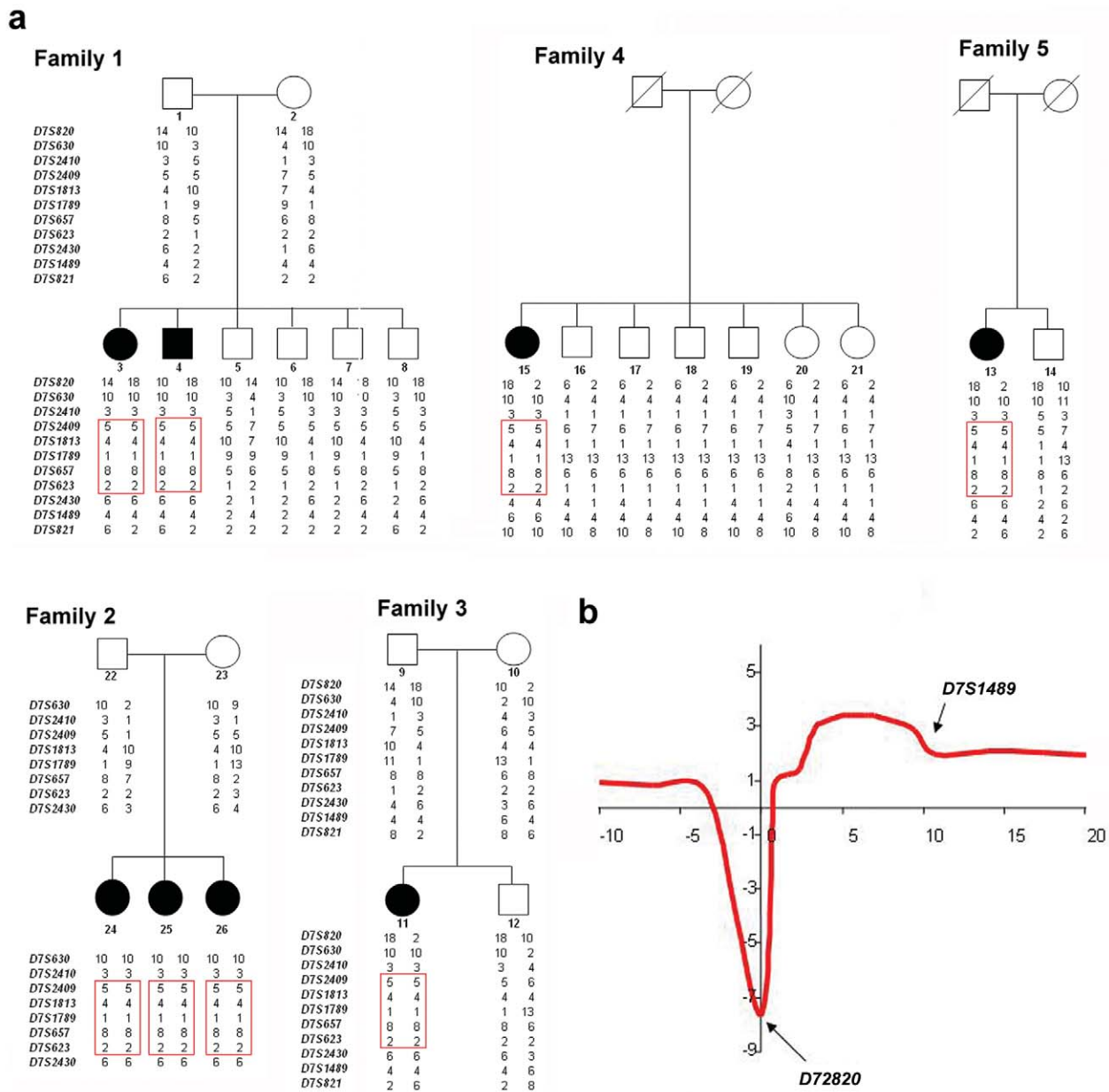


Figure 1. NFTC maps to 7q21-7q21.3. *a*, Haplotype analysis of five NFTC-affected families, through use of polymorphic markers on chromosome 7q21. Blackened symbols represent affected individuals. The disease-associated haplotype found in the five families is boxed in red. *b*, Multipoint linkage analysis, which generated a maximum LOD score of 3.4 between markers *DTS2409* and *D7S623*.

existence of a single homozygous causative mutation and thus used homozygosity mapping to locate the NFTC gene. We performed a genomewide marker screen, using fluorescently labeled primer pairs spanning 731 microsatellite loci (369 markers obtained from PE Biosystems and 362 markers obtained from Research Genetics/Invitrogen) in five affected individuals. We initially identified a 7.6-Mb homozygous interval on 7q21-7q21.3 that was shared by all five patients (not shown). Subsequently, genotypes were obtained for all family members at seven additional microsatellite loci across this region (fig. 1*a*). Multipoint

analysis (with use of Superlink software [Genetic Linkage Analysis Project], as described by Silberstein et al.¹⁵) generated a maximum LOD score of 3.4 between markers *DTS2409* and *D7S623* (fig. 1*b*). Haplotype analysis revealed a critical recombination event in individual 7 between markers *DTS2410* and *D7S2409* and a diverging haplotype in patient 15 at marker *DTS2430*, thereby establishing the centromeric and telomeric boundaries of the disease-associated interval (fig. 1*a*).

Of 23 genes contained within this interval (Map Viewer [build 36]), we excluded *PEX1*, since mutations in this gene



Figure 2. Clinical features of NFTC—including heralding erythematous papular eruption (a), small calcified tumors ulcerating and discharging chalk-like materials on the surface of the skin (b), and severe gingivitis (c)—and calcified material in the upper dermis, as revealed by histopathological examination (d).

have been shown to cause peroxisomal disorders.¹⁶ Twenty-two genes were analyzed for pathogenic mutations by direct sequencing (primer pairs and PCR conditions available on request) of all coding regions, as well as exon-intron boundaries in one or two affected individuals. Only two single-nucleotide substitutions were found to segregate with the disease phenotype in all families. An A→G transition at cDNA position 409 of *MGC40405*, which codes for a protein of unknown function and results in a valine→methionine substitution, was found in 6% of the general Israeli population (4 of 68 healthy controls). The second mutation is an A→G transition at cDNA position 4483 of *SAMD9* (fig. 3a), which is predicted to result in the substitution of a negatively charged glutamic acid residue for a positively charged lysine residue at amino acid position 1495 of the protein sequence. To screen for this mutation, we amplified a 115-bp fragment, using reverse primer 5'-GCAATATAAACATATGCATCGTAC-3' and allele-specific forward primer 5'-CTTCTTAAAGCACTGGTCAATTTTTTC-3'; the latter creates a recognition site for endonuclease *Mbo*II (fig. 3b). Using this assay, we confirmed cosegregation of the mutation with NFTC in all families and excluded the mutation from a panel of 1,042 control chromosomes derived from blood samples obtained from healthy individuals of Jewish origin. We then screened 92 healthy, unrelated Jewish individuals born to couples who immigrated to Israel from Yemen. One individual in this cohort was found to carry both K1495E and the disease

haplotype in a heterozygous state, which corresponds to a carrier rate of ~0.01, fitting the expected prevalence of the disease in the Israeli Jewish Yemenite population.

SAMD9 encodes a 1,589-aa protein of unknown function and is expressed in a wide range of tissues, including skin (fig. 3c). Quantitative real-time PCR revealed that the gene is strongly expressed in endothelial cells and, to a lesser degree, in fibroblasts (not shown). Moreover, *SAMD9* mRNA expression level was found to be sixfold higher in fibroblasts derived from patients with NFTC than in those from healthy controls, which suggests compensatory up-regulation of this gene by a currently unknown mechanism (fig. 3d).

Apart from an N-terminal sterile alpha motif (SAM), a widely distributed putative protein interaction domain,¹⁷ *SAMD9* bears very little homology to other known proteins, except for a paralogous protein termed “*SAMD9L*” (*SAMD9*-like), with which it shares 58% identity at the amino acid-sequence level. K1495 seems to be well conserved across species, suggesting that it is of physiological importance (fig. 3e).

To determine the functional consequences of K1495E mutation in *SAMD9*, a full-length *SAMD9* cDNA was fused to the green fluorescent protein (GFP) sequence in Gateway pDEST53 vector (Invitrogen) and was transfected into HEK293 cells. Confocal microscopy demonstrated clear punctate cytoplasmic expression of the GFP-*SAMD9* fusion protein. We then introduced the K1495E mutation

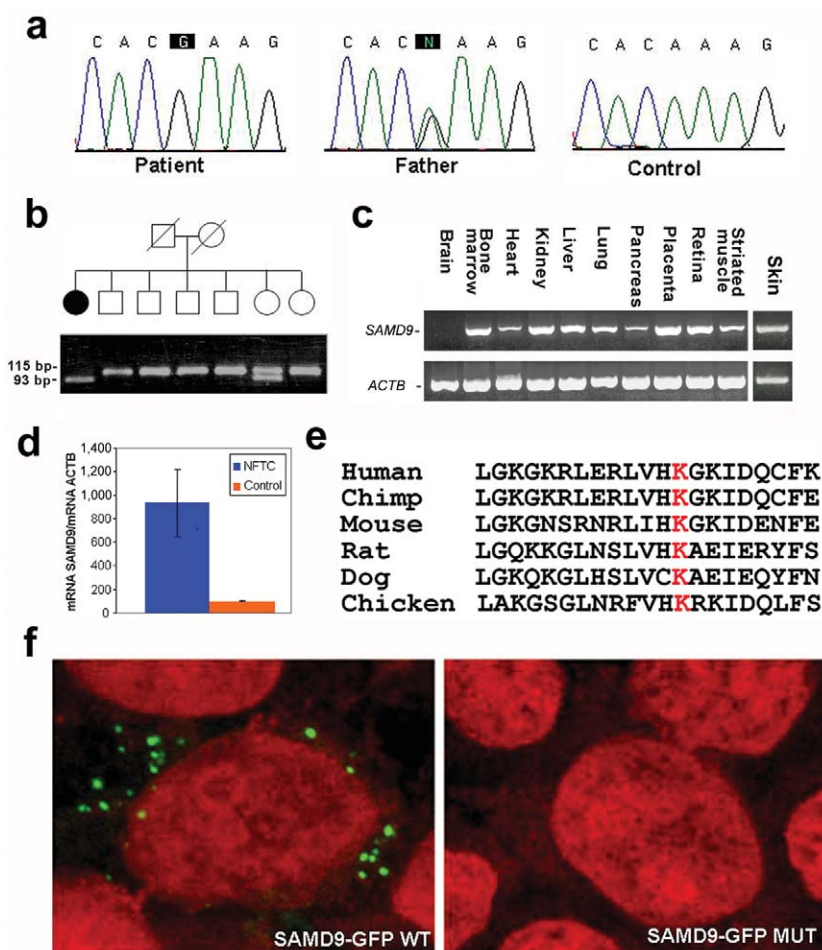


Figure 3. NFTC caused by a mutation in *SAMD9*. *a*, Sequence analysis of *SAMD9* reveals an A→G transition at cDNA position 4483 in patient 3 (*left panel*). The father of the patient carries the mutation in a heterozygous state (*middle panel*). The wild-type sequence is given for comparison (*right panel*). *b*, PCR-RFLP analysis, which confirms segregation of the mutation in family 4. PCR amplification was performed as described in the text. Mutation K1495E creates a novel recognition site for *Mbo*II. Thus, affected patients display a 93-bp fragment (an additional 22 bp not shown), and healthy individuals show a 115-bp fragment, whereas both fragments are found in heterozygous carriers of the mutation. *c*, *SAMD9* gene expression assessed using Clontech tissue blot cDNA array and using total RNA extracted from skin biopsy samples obtained from healthy individuals. Expression of *SAMD9* was compared with that of *ACTB*. *d*, RNA was extracted from fibroblast-cell cultures established from two patients with NFTC and two healthy control individuals. *SAMD9* mRNA expression, normalized to that of *ACTB*, was found to be up-regulated in NFTC fibroblasts (*blue column*) as compared with normal fibroblasts (*orange column*). *e*, ClustalW analysis of the *SAMD9* protein region encompassing the mutation site, which demonstrates that K1495 is conserved across species. *f*, HEK293 cells transfected with wild-type (WT) (*left panel*) and E1495-carrying (*right panel*) GFP-*SAMD9* constructs and examined using confocal microscopy. Note decreased expression in cells transfected with E1495-carrying GFP-*SAMD9*. MUT = mutant.

into the GFP-*SAMD9* cDNA construct, using site-directed mutagenesis (QuikChange JXL Site-Directed Mutagenesis Kit [Stratagene]) according to the manufacturer's instructions. *SAMD9* mRNA levels were shown, by quantitative RT-PCR, to be 4.8-fold higher in HEK293 cells transfected with mutant *SAMD9* than in cells transfected with wild-type *SAMD9*. In seven independent experiments, we observed that K1495E causes loss of punctate expression of the fusion protein after transfection into HEK293 cells, which indicates that this mutation interferes with *SAMD9* expression (fig. 3f).

Collectively, the present data indicate that *SAMD9* may play a crucial role in the pathogenesis of extraosseous calcification; yet, the detailed mechanisms of action of this protein remain to be determined. The conspicuous inflammatory manifestations reported in NFTC,⁴ which are absent in HFTC,⁵ suggest that this protein may be involved in physiological responses to tissue injury.

At this stage, it seems that NFTC represents a clinical entity found specifically in the Jewish Yemenite population. This should not come as a surprise, since it is well known that cultural isolation favors the emergence and

clustering of unique genetic alterations, as shown elsewhere for this community.¹⁸ In spite of the apparent rarity of NFTC, it encompasses clinical features of relevance to the understanding of important and common disorders. Indeed, two major types of acquired skin calcinosis have been described: (1) metastatic calcinosis, which refers to deposition of calcified materials due to abnormal calcium or phosphate metabolism, as seen in chronic renal failure, and (2) dystrophic calcinosis, in which tissue calcification occurs as a consequence of tissue injury/inflammation, as observed in many unrelated conditions, such as vascular disease, cancer, and autoimmune disorders.^{19–21} Whereas HFTC models metastatic calcinosis, NFTC bears striking resemblance to acquired dystrophic calcinosis. A growing body of evidence links tissue inflammation to vascular calcification, a major cause of morbidity and mortality in humans.^{22,23} These observations raise the possibility that alterations in *SAMD9* expression may underlie the varying occurrence of dystrophic calcinosis in a spectrum of human diseases.

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Web Resources

The URLs for data presented herein are as follows:

Genetic Linkage Analysis Project, <http://cbl-fog.cs.technion.ac.il/superlink/> (for Superlink online software)
 Map Viewer, <http://www.ncbi.nlm.nih.gov/mapview/>
 Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for HFTC and hyperostosis-hyperphosphatemia syndrome)

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