Retinitis Pigmentosa, Cutis Laxa, and Pseudoxanthoma Elasticum–Like Skin Manifestations Associated with GGCX Mutations

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Gamma-glutamyl carboxylase (GGCX) mutations have been reported in patients with a pseudoxanthoma elasticum (PXE)–like phenotype, loose redundant skin, and multiple vitamin K–dependent coagulation factor deficiencies. We report on the clinical findings and molecular results in 13 affected members of two families who had a uniform phenotype consisting of (PXE)-like skin manifestations in the neck and trunk, loose sagging skin of the trunk and upper limbs, and retinitis pigmentosa confirmed by electroretinographies in 10 affected individuals. There were no coagulation abnormalities. Molecular investigations of the ATP-binding cassette subfamily C member 6 did not yield causative mutations. All 13 affected family members were found to be homozygous for the splice-site mutation c.373 + 3G > T in the GGCX gene. All tested parents were heterozygous for the mutation, and healthy siblings were either heterozygous or had the wild type. We suggest that the present patients represent a hitherto unreported phenotype associated with GGCX mutations. Digenic inheritance has been suggested to explain the variability in phenotype in GGCX mutation carriers. Consequently, the present phenotype may not be explained only by the GGCX mutations only but may be influenced by variants in other genes or epigenetic and environmental factors.

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INTRODUCTION

Pseudoxanthoma elasticum (PXE; MIM 264800) is an autosomal recessive condition with manifestations in the skin (yellowish papules in the neck and flexural areas; inelastic and leathery skin), the eyes (angioid streaks; retinal neovascularization, exudations, and hemorrhages), and the cardiovascular system (occlusive arterial disease). The clinical features are secondary to ectopic mineralization of the extracellular matrix, particularly the elastic fibers, but the exact pathophysiological mechanisms still remain unclear (Uitto *et al.*, 2010).

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PXE is caused by mutations in the ABCC6 gene (ATP-binding cassette subfamily C member 6) (Le Saux and Urban, 1999; Bergen *et al.*, 2000; Ringfeil *et al.*, 2001; Miksch *et al.*, 2005; Pfendner *et al.*, 2007), encoding an efflux pump that is mainly expressed in the liver and at lower levels in the proximal tubules of the kidney but hardly, if at all, in the clinically affected tissues (Belinsky and Kruh, 1999; Scheffer *et al.*, 2002; Jiang *et al.*, 2006). In view of the association of PXE with ABCC6 efflux transport, it has been hypothesized that PXE is a systemic metabolic disease caused by lack or accumulation of molecules interacting with the synthesis, turnover, and/or maintenance of the extracellular matrix in the bloodstream (Li *et al.*, 2009b).

PXE(-like) skin manifestations have been reported to co-occur with loose, redundant skin and a deficiency of vitamin K-dependent clotting factors (MacMillan and Vickers 1971; Rongioletti *et al.*, 1989; Le Corvaisier-Pieto *et al.*, 1996). Subsequently, Vanakker *et al.*, 2007 reported six unrelated patients with severe PXE-like skin manifestations, cutis laxa, and relatively mild angioid streaks. Ultrastructurally, fragmentation and calcification of elastic fibers in the reticular dermis were observed, which are commonly seen in PXE, although subtle differences with respect to the localization of the mineral precipitates and the structure of the elastic fiber network could be observed. All six patients had decreased vitamin K-dependent coagulation factor activity. Molecular

studies showed homozygous or compound heterozygous mutations in gamma-glutamyl carboxylase (GGCX) in three of the six patients and in one other patient, previously reported by Le Corvaisier-Pieto *et al.* (1996). In two other patients only a single GGCX mutation could be detected.

GGCX encodes a gamma-carboxylase, which catalyzes gamma-glutamyl carboxylation of coagulation factors as well as of matrix gla protein (MGP). The carboxylated form of MGP serves as a systemic inhibitor of pathologic mineralization. Previously, hereditary deficiency of vitamin K-dependent procoagulants were reported in individuals with GGCX mutations (Brenner *et al.*, 1990, 1998; Rost *et al.*, 2004).

GCCX missense mutations, together with a recurrent ABCC6 nonsense mutation, (p.R1141X) were identified in a mother and maternal aunt of two sibs who had a PXE-like phenotype and vitamin K-dependent coagulation factor deficiency, whereas the mother and aunt had only a PXE-like phenotype (Li *et al.*, 2009a), suggesting digenic inheritance. The sibs themselves had compound heterozygous GGCX mutations (p.R83W; p.Q374X). The authors suggested that the loss-of-function ABCC6 mutations caused reduced vitamin K-dependent gamma-glutamyl carboxylation of MGP, which in turn resulted in connective tissue mineralization (Li *et al.*, 2009a).

Vanakker *et al.* (2010) compared various forms of vitamin K-dependent proteins in the serum, plasma, and dermal tissues in patients with GGCX mutations and in patients with ABCC6 mutations using conformation-specific antibodies and vitamin K levels in serum. GGCX patients showed accumulation of uncarboxylated Gla proteins, MGP, and osteocalcin in the plasma, serum, and dermis, and normal vitamin K levels. ABCC6 patients had similarly elevated Gla protein levels but a significant decrease in serum vitamin K levels. The authors concluded that the ectopic mineralization in PXE and PXE-like patients is caused by deficient carboxylation of vitamin K-dependent inhibitors of calcification. Mutations in GGCX are the cause of this deficiency in PXE-like patients, whereas in ABBC6 patients deficiency of the carboxylation co-factor vitamin K is the cause of the decreased activity of calcification inhibitors. Evidence from studies in abcc6 $-/-$ mice suggests that neither Vitamin K nor $Ca2+$ dietary intake has a role in the progression of PXE. Instead, intake of high $Mg2+$ apparently slows down the progression of PXE (Gorgels *et al.*, 2010; Li and Uitto, 2010).

Here we present two families with a total of 14 affected members, 13 of whom were examined, showing skin manifestations resembling PXE in the neck and flexural areas, loose, sagging skin of the trunk, and retinitis pigmentosa (RP), who were found to harbor homozygous GGCX mutations.

RESULTS

Clinical reports

Family 1. The proband (Figure 1) V12 is the first-born son of first-cousin parents. Pregnancy, delivery, and early physical and psychomotor development were uneventful. The parents noticed progressive reduced vision, especially at night, from the age of 3.5 years onward; otherwise he had no physical complaints. He followed a normal cognitive development for a visually handicapped individual. At 12 years, yellowish papules were noted at the back and sides of the neck and in the chest and flexural areas, and unusually loose skin on the trunk. The loose skin gradually became more marked and also evident on the upper limbs. He was referred because of his disturbed vision and similar problems within the family. Physical examination did not show significant morphological abnormalities. Loose sagging skin was evident on the trunk and upper limbs (Figure 2a and b), and characteristics resembling PXE in the neck and flexural regions (Figure 2c and d). Fundoscopy showed intra-retinal clumps of black pigment, markedly attenuated retinal vessels, loss of retinal pigment epithelium, and pallor of the optic nerve, all of which are findings commonly seen in RP (Figure 2e). Ocular findings associated with PXE, such as angioid streaks, loss of central vision, exudation, and hemorrhage, were absent. An electroretinography confirmed the presence of RP (Supplementary Figure S1 online). Echocardiography yielded normal results. Additional signs and symptoms that can occur in PXE, such as gastrointestinal bleeding, intermittent claudication, loss of peripheral pulses, renovascular hypertension, angina pectoris, and myocardial infarctions, were searched for in the proband and found to be absent. Detailed clotting studies failed to show abnormalities (Supplementary Table S1 online).

Family 2. The proband of family 1 originated from a small village from the north of Iran with 4,000 inhabitants. He informed us that there were nine other affected members in his family and four others from another family living in the same village (Figure 1). All other 10 affected members from family 1 and three out of four affected members from family 2 were available for evaluation. The phenotype in all other affected individuals was very similar, only varying in terms of time of onset of ocular and skin involvement (Supplementary Table S1 online). Electroretinography examination could be performed in 10 patients and showed nondetectable rod responses or rod responses with reduced amplitude and prolonged implicit time confirming the diagnosis of RP (Supplementary Table S1 online). Fundoscopy was performed on 16 members, nine affected and seven unaffected members, which showed no manifestations of PXE but did show the same RP findings as in the proband in all nine investigated affected individuals. Unaffected individuals did not show any ocular findings associated with RP (Supplementary Table S2 online). Clotting studies performed on 10 affected individuals yielded normal values as well. None of the patients reported any abnormal bleeding tendency. Parents and healthy siblings were examined for dermatological and ocular problems, specifically a PXE phenotype, loose sagging skin, and reduced night and peripheral vision, which were normal in all 17 patients.

Histological studies

Light microscopic and electron microscopic evaluation of a full-thickness skin biopsy taken from lesional skin in the upper anterior thigh showed important fragmentation and fragility of the specimens in both families (Figure 3). The presence of thin and fragmented elastic fibers was observed in the superficial, mid-, and reticular dermis, with limited mineralization in these areas. In the very deep dermis, elastic fibers were found to be heavily mineralized, with fine mineral precipitates within the elastic fibers. Collagen fibers were small in some

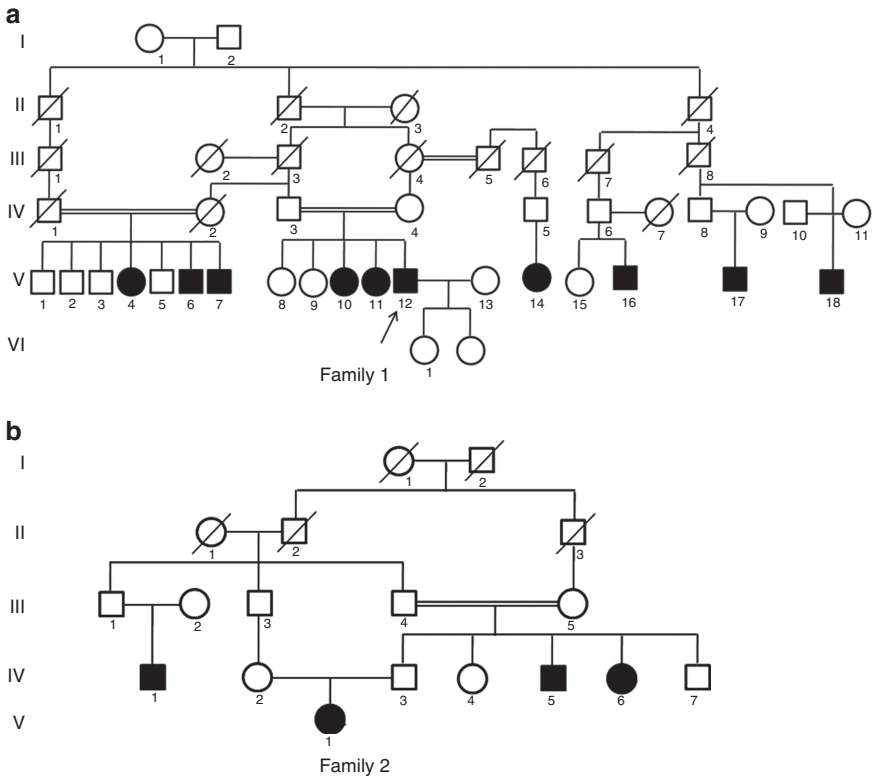

Figure 1. Pedigrees and clinical characteristics of the two families. (**a**, **b**) Affected individuals are indicated with black symbols.

areas and fusion of collagen fibrils was rarely seen; other ultrastructural abnormalities were not observed. Fibroblasts only rarely showed features such as dilatation of the endoplasmic reticulum or large cells described in classical PXE. (Figure 3). Immunohistochemistry with confirmation-specific antibodies against uncarboxylated MGP demonstrates positive labeling compared with control tissue (Figure 4). The staining was clumped, resembling what is observed in the PXE-like phenotype with coagulation factor deficiency, albeit less abundant in our families.

Molecular analyses

Sequences of ABCC6 in the proband and member IV1 of family 2 (Figure 1) were of wild type. Exon 23–29 deletion and other homozygous deletions in ABCC6 were excluded by exon-specific amplifications and sequencing. A pathogenic sequence change was found in GGCX in both individuals: a novel splice-site change c.373 + 3G > T was present homozygously (Figure 5a). We subsequently screened all 30 available family members and found that the GGCX sequence change fully co-segregated with the PXE-like phenotype as a recessive trait.

Alamut software analysis of this variant showed the sequence change to be rare. Three out of five splice-site prediction programs predicted significant aberrant splice patterns for this mutation (Table 1). The c.373 + 3G > T sequence change causes a skip of exon 3 in the GGCX mRNA, and at a protein level results in a deletion of 53 amino acids (p.Phe73_Gly125del) (Figure5b).

The c.373 + 3G > T sequence change in GGCX was not present in human healthy control WWW genome sequence databases (the 1000 genome project (1000 genomes.org) and Exome variant server (http://evs.gs.washington.edu/EVS)).

DISCUSSION

Here we describe two large families with 14 affected individuals from a small village from the north of Iran with skin manifestations on the back and side of the neck resembling PXE, cutis laxa involving the trunk and upper limbs, and RP. The phenotype in the patients showed hardly any variation. The findings in 13 members of the present families are summarized in Supplementary Table S1 online and compared with similar literature reports in Table 2. Table 2 shows that none of the previously published families show the same combination of signs and symptoms. The skin findings in the present patients are very similar with previously reported cases of PXE-like phenotype with GGCX mutations. The degree of sagging, loose skin is more severe in the present patients and in other patients with GGCX mutations compared with that in patients with classical PXE caused by ABCC6 mutations. If PXE patients have sagging and loose skin it is typically located in the flexural regions, but in GGCX-mutated patients this is more widespread and includes the trunk and upper limbs, leaving the face and distal limbs uninvolved. The main differences between the present patients and previously reported cases with GGCX mutations are the histological characteristics of the skin lesions, the presence of RP, and absence of coagulopathy.

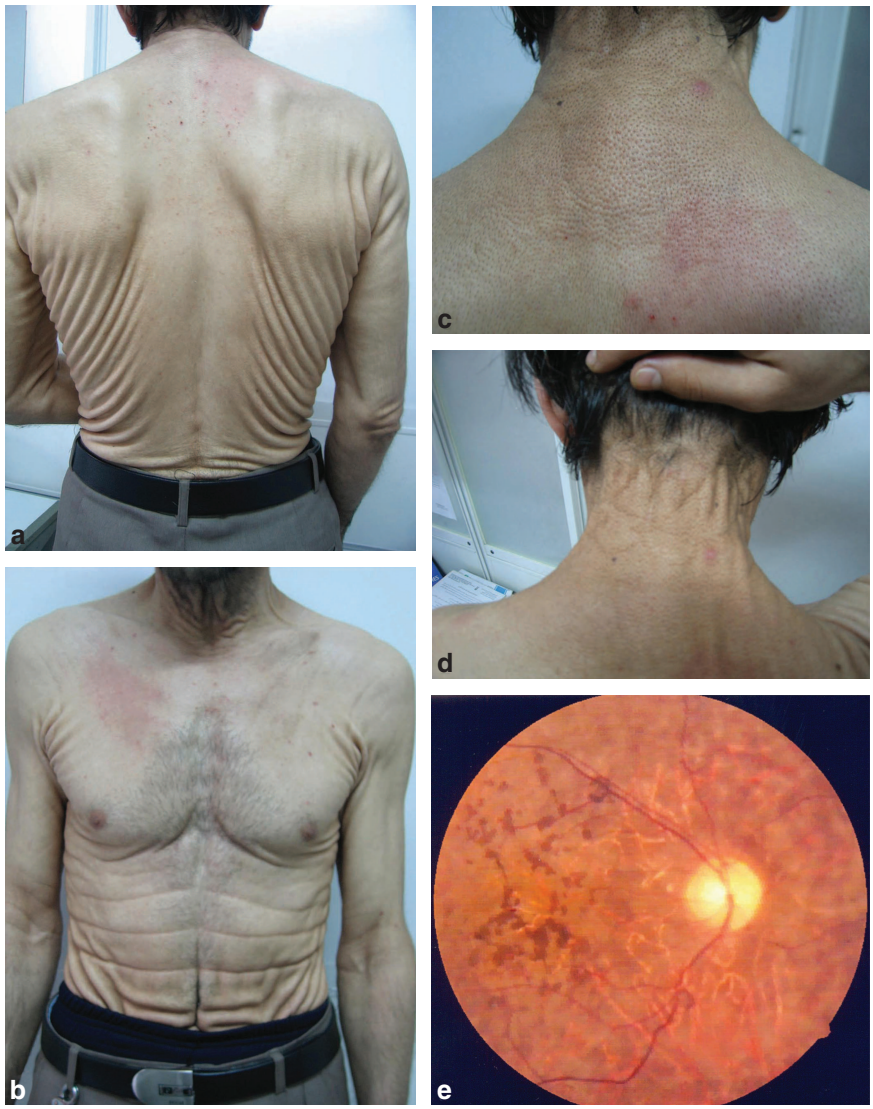

Figure 2. Clinical findings and fundus image of proband. (**a**, **b**) Excessive skin folds and leathery texture mostly prominent in the trunk. (**c**, **d**) Note yellowish papules and depressed dots in the neck. (**e**) Fundus of affected individual V12 of family 1 (Figure 1) showing characteristics of retinitis pigmentosa (RP).

Although fragmentation of the elastic fibers could be observed throughout the dermis, mineralization of the fibers was most prominently present in the deepest layers of the dermis. This localization is very unusual for classical PXE or for the PXE-like phenotype with associated coagulopathy. Unlike in the latter disorders, the morphology of the fibroblasts was not significantly changed in the present families. The remarkable histological features underline the importance of sufficient depth of skin biopsy specimens when evaluating patients with a PXE-related phenotype.

Clinically, all patients reported in this study had poor night vision and reduced peripheral vision. The RP was confirmed by electroretinography in all 10 tested patients (Supplementary Table S1 online). None had eye findings resembling PXE. Four out of 13 previously reported patients with GGCX mutations had ocular abnormalities, including angioid streaks, but none had RP.

None of the present patients had abnormal bleeding tendencies, and coagulopathy tests performed on all 10 tested patients yielded normal results (Supplementary Table SI online). Such bleeding tendencies (meningeal hemorrhages, postpartum hemorrhage, hematemesis, spontaneous gingival bleeding, and severe vaginal hemorrhages) have been reported in other GGCX-mutated patients who were found to have a deficiency of vitamin K-dependent coagulation factors (Vanakker *et al.*, 2007). The reason for the absence of bleeding diathesis in our families is currently unclear, similar to the as-yet-unknown explanation why some patients with GGCX mutations develop a skin and eye phenotype and others do not. A splice-site mutation leading to the deletion of exon 3 of GGCX has been previously described in a patient with isolated hereditary vitamin K combined clotting factors deficiency, in whom it was compound heterozygous with a missense mutation (p.R485P) (Rost *et al.*,

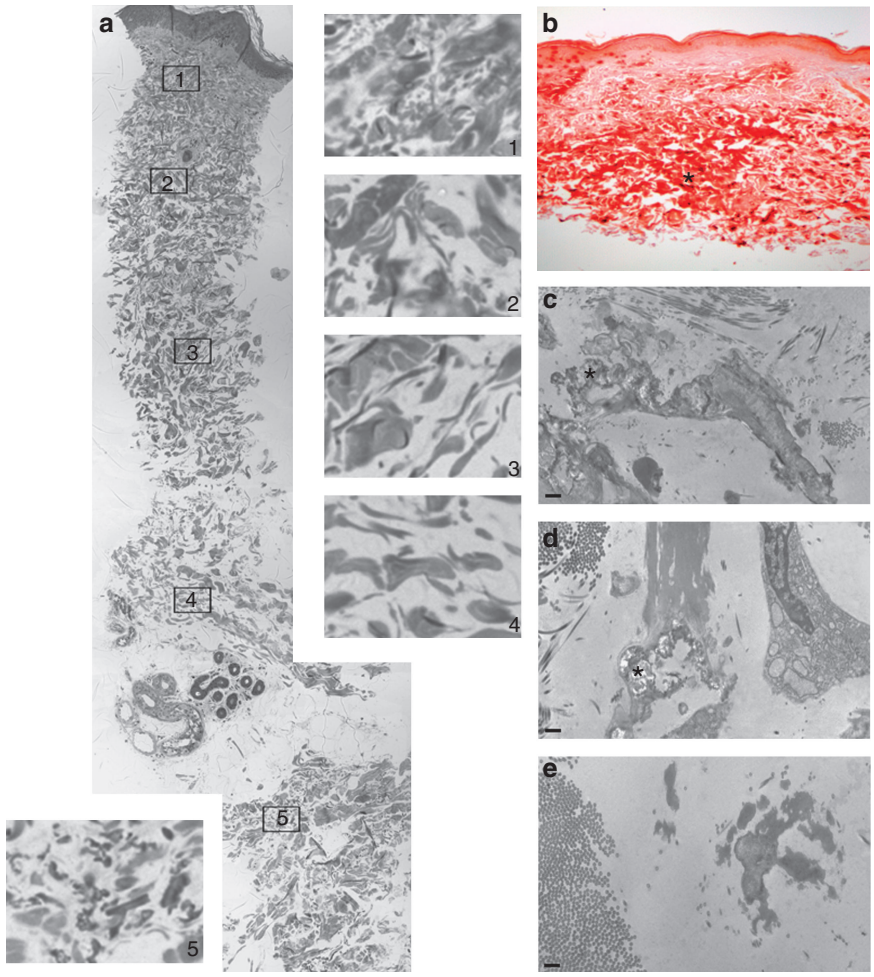

Figure 3. Histology of skin lesions using light and electron microscopy. (**a**) Full-thickness overview of the affected skin shows thin and fragmented elastic fibers in areas 1 through 4. Only in area 5 can mineralization of the fragmented elastic fibers be assumed. This is further documented using Alizarin red staining (**b**; original magnification ×20), showing extensive labeling in the deeper dermis, whereas only limited mineralization is observed in the reticular and mid-dermis. High-resolution electron microscopy of the deeper dermis shows mineralization in the core and periphery of the affected elastic fibers (**c**–**e**; asterisks). Collagen fibers show some variation, some being small, but no other abnormalities. Scale bar = 1 μm.

2004). Although we cannot exclude that the phenotypic effect may differ based on the position of each mutation or that the cumulative effect of two splice-site mutations is different compared with a compound heterozygous splice site and missense variant, no evidence is currently available to substantiate these hypotheses. In addition, the influence of modifier genes on the phenotype cannot be excluded. Besides the two reported phenotypes associated with mutations in GGCX—isolated hereditary vitamin K combined clotting factors deficiency and PXE-like phenotype with multiple coagulation factor deficiency—we propose that *GGCX* mutations can also be associated with a PXE-like phenotype and with RP, without coagulopathy. We cannot exclude with certainty the theory that in the present two families the RP and PXE-like phenotype are two separate entities segregating by coincidence together. Co-segregation of two conditions in at least 13 individuals and absence of isolated occurrence of either of the two conditions in any other family member make this hypothesis extremely unlikely; only in case of linkage disequilibrium may this happen by coincidence. There is, however, no gene known to cause RP located within 10 Mb of *GGCX*, which makes this also highly unlikely. In addition, the absence of vitamin K-dependent clotting factor deficiency and the unique histological features suggest this to be a different GGCX-related phenotype. Although we have no definite explanation on how GGCX malfunctioning can lead to RP, it is interesting that one of the Gla proteins, Gas6, is known to be involved in a recessive form of RP due to mutations in *MERTK* (Hafizi and Dahlbäck 2006; Ksantini *et al.*, 2012). MERTK encodes a specific receptor tyrosine kinase, which on the apical surface of the retinal pigment epithelium interacts with the shed tips of the photoreceptor outer segments via a signaling molecule, Gas6. One could hypothesize that, if *MERTK* mutations cause RP, than a decreased function of its signaling molecule Gas6 due to defective *GGCX* function may result in a similar phenotype. As Gas6 function is cell-type

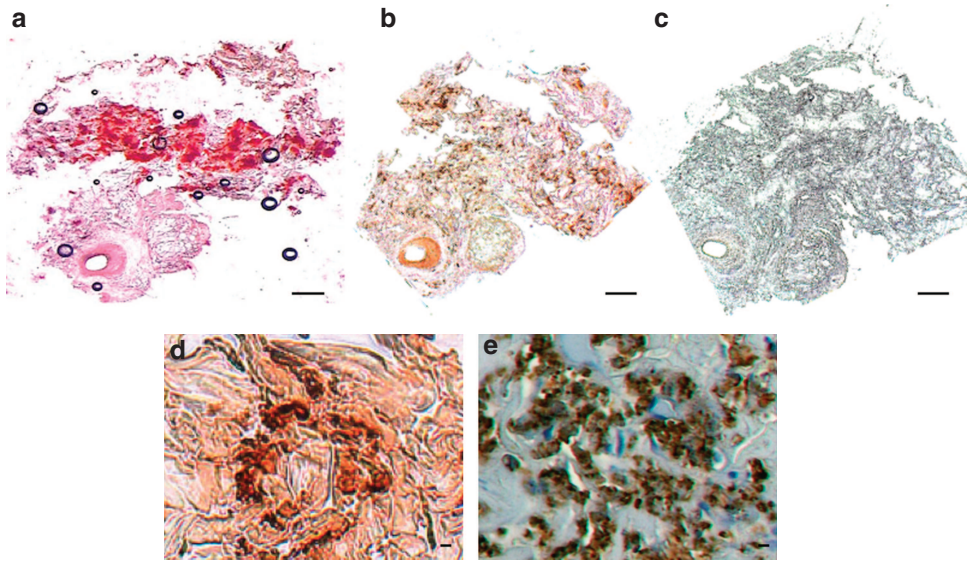

Figure 4. Immunohistochemistry for uncarboxylated (uc) matrix gla protein (MGP). (**a**) Alizarin red staining of the examined skin fragments. All tissues were fragile and heavily fragmented, probably because of the disease itself. (**b**) Staining for ucMGP shows positive labeling compared with a negative control (**c**). The staining, which is clumped (**d**), resembles staining of ucMGP in the pseudoxanthoma elasticum (PXE)-like syndrome with coagulation factor deficiency (**e**), although in the latter ucMGP is more abundant. Scale bar = 200 μm.

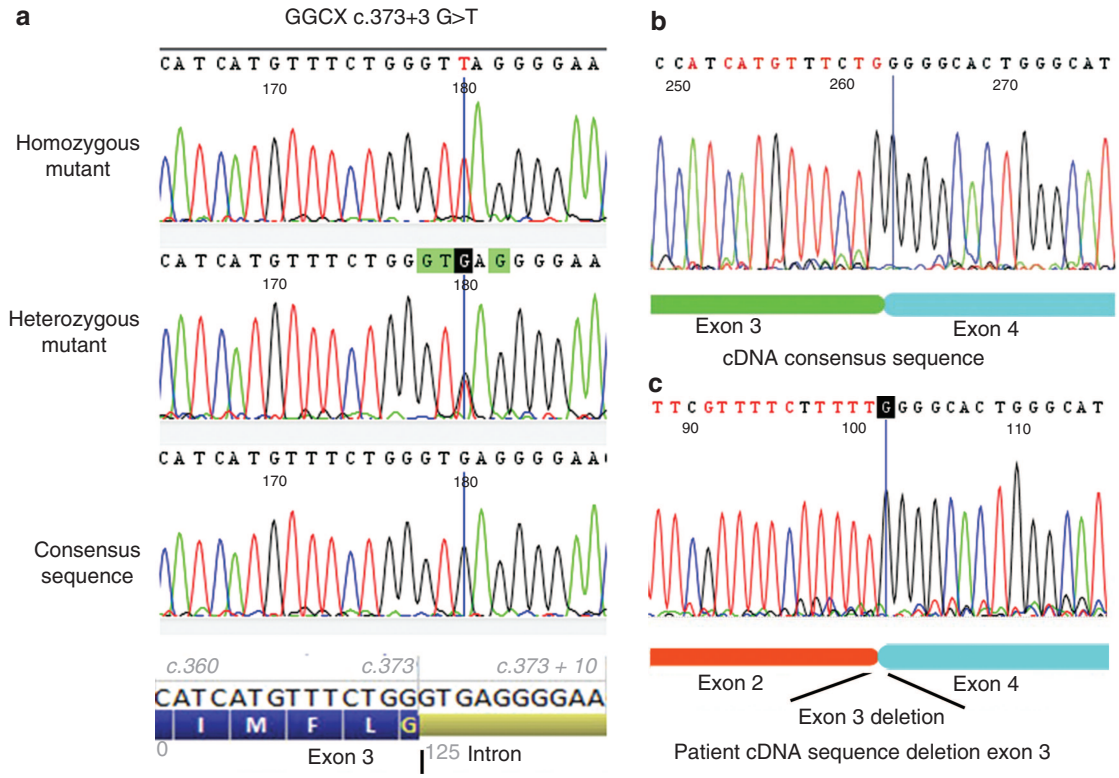

Figure 5. Gamma-glutamyl carboxylase (GGCX) (accession number NM_000821.4) exons were sequenced in DNA samples of this family. (**a**) Chromatograms of the identified sequence change c.373 + 3 G>T are shown for a homozygous, heterozygous, and consensus sequence. The lower bar schematically shows the exon/intron structure of GGCX exon 3. (**b**) The c.373 + 3G>T sequence change causing a skip of exon 3 in the GGCX mRNA. (**b**) Wild-type cDNA sequence; (**c**) patient cDNA sequence.

specific, this implies that such a hypothesis can only be investigated further on retinal tissue, which is for obvious reasons not available from these patients (Healy *et al.*, 2001).

Recently, digenic inheritance of a PXE-like phenotype including loose sagging skin but without coagulopathy was suggested by Li *et al.*, 2009a, caused by mutations in both

GGCX and ABCC6. Similar compound heterozygous ABCC6 mutations and a gain-of-function GGCX variant have been reported by Vanakker *et al.* (2011). The identified mutations could not fully explain the clinical and biochemical findings in their patient and they suggested that variants in other genes involved in calcium homeostasis may have a role in the atypical presentation of this patient. Similarly, the present phenotype may not be explained by the GGCX mutations alone but may also be influenced by variants in other genes. We have excluded an influence by ABCC6 mutations. Epigenetic and also environmental influences cannot be excluded with certainty either.

MATERIALS AND METHODS

Approval

Approval was obtained from the Medical Ethics Committee (Pathology and Genetics Center, Tehran). Written informed consent was obtained from all study participants. The study was conducted in compliance with the guidelines described in the Declaration of Helsinki.

Table 1. Donor-splice prediction scores for the gamma-glutamyl carboxylase c.373 + 3 G > T sequence change calculated using the Alamut software analysis program

Donor-splice prediction program (score range)	Threshold value	Consensus sequence prediction score	Mutation sequence prediction score
SSF (0–100)	⩾70	77.53	72.02 (−7.1%)
MaxEnt (0–12)	⩾0	6.74	—
NNSPLICE (0–1)	⩾0.4	0.92	—
Genesplicer (0–15)	⩾0	0.51	—
HSF (0–100)	⩾60	85.20	78.11 (−8.3%)

The threshold values are according to the default settings of that program.

Histological studies

One patient of each family had a full-thickness skin biopsy taken of a skin lesion. Biopsies were evaluated with light microscopy using hematoxylin and eosin, van Giesson (elastin), Alizarin Red (calcium), and a mAb against ucMGP (EnzoLife Sciences, Antwerp, Belgium). For immunohistochemical analysis, sections were heated in 0.2% citric acid at pH 6.0, washed with phosphate-buffered saline, and incubated with the primary antibody. The antibody was diluted in blocking reagent (Roche Diagnostics, Mannheim, Germany). Negative controls were obtained by omitting the primary antibody. Biotinylated sheep anti-mouse IgG (Amersham Biosciences, Little Chalfont, UK) was used as a secondary antibody (1 hour at room temperature), followed by incubation with the avidin-linked alkaline phosphatase complex (Dako, Golstrup, Denmark); staining was performed with the AEC revelation kit (brown stain; Dako). Sections were counterstained with hematoxylin and mounted with coverslips.

For electron microscopy, skin biopsy fragments were immediately fixed in 3% glutaraldehyde in Tyrode's saline (pH 7.2) for 2–4 hours at room temperature, washed in saline, postfixed in 1% osmium tetroxide in the same buffer for 1 hour, dehydrated in ethanol and propylene oxide, and embedded in spurr resin. Semithin sections were stained with 1% toluidine blue and observed by light microscopy. Thin sections were stained with 1% uranyl acetate in 50% ethanol and lead citrate and observed with a Jeol EM1200 electron microscope (magnification × 10,000).

Molecular analyses

Genomic DNA was isolated from peripheral blood samples according to a standard procedure (Miller *et al.* 1988). The entire coding region and adjacent splice-site sequences were systematically screened in ABCC6 (NM_001171.5) and GGCX_(NM_000821.4) by Sanger

Table 2. Clinical findings in patient previously reported with pseudoxanthoma elasticum-phenotype and cutis laxa

	MacMillan and Vickers, 1971		Rongioletti *et al.*, 1989	Le Corvaisier-Pieto *et al.*, 1996	Vanakker *et al.*, 2007						Li *et al.*, 2009a				Summary present cases
Author	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	***N* = 13**
Gender	F	F	M	M	F	F	F	F	F	M	F	F	F	F	8M/5F
Age	24	24	33	40	46	47	67	32	46	44	16	19	40	40	31.8 (16–52)
Age at onset of skin signs (years)	P	P	P	16	18	13	3	18	NA	NA	10	P	NA	NA	13.5 (10–25)
Cutis laxa	+	+	+	+	+	+	+	+	+	+	+	+	+	+	13/13
Pseudoxanthoma elasticum-like signs	+	+	+	+	−	+	+	+	+	+	−	−	+	+	13/13
Retinitis pigmentosa	−	−	−	−	−	−	−	−	−	−	−	−	−	−	10/10
Other eye findings	−	−	−	−	AS	AS	AS	AS	−	−	−	−	−	−	11/13 Strabismus
Abnormal bleeding tendency	+	+	−	+	−	−	+	−	+	−	+	+	−	−	0/13
Clotting deficiency	+	+	+	+	+	+	+	+	+	+	+	+	−	−	0/10
ATP-binding cassette subfamily C member 6 mutation(s)	NA	NA	NA	−	−	−		−	NA	NA	−	−	+/−	+/−	0/13
Gamma-glutamyl carboxylase mutation(s)	NA	NA	NA	−	+/+	−	+/−	+/−	+/+	+/+	+/+	+/+	+/−	+/−	13/13

Abbreviations: AS, angioid streaks; F, female; M, male; NA, not available; P, puberty.

sequencing (primer sequences are available upon request). Exon 23–29 deletion and other homozygous deletions were excluded by exon-specific amplifications and sequencing. Sequences were analyzed using Codoncode aligner (Codon Code Corporation). The potential pathogenicity of unreported changes was evaluated by segregation analysis of the variant in the families, using the Alamut version 2.1 software mutation assignment package (Interactive Biosoftware, Rouen, France) and by screening of the known variant WWW databases. Blood was collected in PAX gene tubes (QIAGEN, Venlo, The Netherlands) and total RNA was isolated according to the manufacturer's protocol. cDNA was then synthesized using polyT primer and superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) according to the standard procedure. cDNA fragments were amplified using M13-tailed primers specific for exon 2 (5′-tgtaaaacgacggccagtAGGCAGGACAGCCGAATAG-3′) and exon 7 (5′-caggaaacagctatgaccAAAGCTGGGAATTCATGCAG-3′) and amplified fragments were sequenced.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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