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Lipoid proteinosis maps to 1q21 and is caused by mutations in the extracellular matrix protein 1 gene (*ECM1*)

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Lipoid proteinosis (LP), also known as hyalinosis cutis et mucosae or Urbach–Wiethe disease (OMIM 247100) is a rare, autosomal recessive disorder typified by generalized thickening of skin, mucosae and certain viscera. Classical features include beaded eyelid papules and laryngeal infiltration leading to hoarseness. Histologically, there is widespread deposition of hyaline (glycoprotein) material and disruption/reduplication of basement membrane. The aetiology of LP is currently unknown. Using DNA from three affected siblings in a consanguineous Saudi Arabian family we performed genome-wide linkage and mapped the disorder to 1q21 (marker D1S498) with a two-point LOD score of 3.45 at $\theta = 0$. A further 28 affected individuals from five other unrelated consanguineous family groups from different geographical regions also showed complete linkage and resulted in a maximum two-point LOD score of 21.85 at $\theta = 0$. Using available markers in the interval between D1S442 and D1S305, the observed recombinants placed the gene in a 2.3 cM critical interval between D1S2344 and D1S2343 (Marshfield genetic map) corresponding to an ~6.5 Mb region on the UCSC physical map. Using a candidate gene approach (comparison of control versus LP gene expression in cultured fibroblasts) and subsequent direct sequencing of genomic DNA, we identified six different homozygous loss-of-function mutations in the extracellular matrix protein 1 gene (*ECM1*). Although the precise function of ECM1 is not known, our findings provide the first clinical indication of its relevance to skin adhesion, epidermal differentiation, wound healing, scarring, angiogenesis/angiopathy and basement membrane physiology, as well as defining the molecular basis of this inherited disorder.

INTRODUCTION

Lipoid proteinosis (LP) was first described by a Viennese dermatologist and otorhinolaryngologist, Urbach and Wiethe, in 1929 (1), originally using the term 'lipoidosis cutis et mucosae'. The disorder was recognized as an autosomal recessive condition that usually presented in early infancy with a hoarse voice, followed by chicken-pox-like scars, along with infiltration and thickening of the skin and mucous membranes. A decade later,

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Urbach changed the name to 'lipoid proteinosis cutis et mucosae', believing that the condition was associated with abnormal lipid and protein deposition in various tissues. Indeed, the disorder has also been referred to as 'lipid proteinosis', 'lipoglycoproteinosis' or, due to the glass-like (hyaline) appearance of tissue sections under microscopy, 'hyalinosis cutis et mucosae' (2,3). Moreover, some authors have debated whether the condition might be a form of mucopolysaccharidosis or amyloidosis or, given the clinical features, whether it might be a variant of porphyria (3). Attempts to characterize the biochemical abnormalities in LP have not yielded any pathognomonic findings (4), although ultrastructural evidence of abnormal vacuolar changes in fibroblasts has been documented (5,6), as have alterations in basement membrane and interstitial collagen gene expression (7). Protein analysis of the hyaline material has shown an overproduction of normally expressed non-collagenous proteins (8). From a clinical perspective, the features may be protean. Aside from the laryngeal infiltration leading to hoarseness, a characteristic finding is beaded papules along the eyelid margins (9). Other mucocutaneous changes may include thickening of the tongue and frenulum, blisters, warty skin papules, scarring, alopecia, nail dystrophy and dental anomalies (2). Extracutaneous features may include epilepsy and neuropsychiatric abnormalities, sometimes in association with calcification in the temporal lobes or hippocampi (2,10,11).

Lipoid proteinosis is a rare disorder, although the literature contains more than 250 published cases (2). It occurs worldwide, but is more common in certain areas such as the Northern Cape province of South Africa, including Namaqualand. Here, it occurs predominantly in a population of mixed Khoisan and European origin in which a founder effect has long been suspected (12–14). In this study we sought to determine the molecular basis of LP, including that present in South Africa. Our approach was to use genome-wide linkage analysis using genomic DNA from consanguineous pedigrees followed by candidate gene analysis and then protein analysis in skin. Using these techniques, we have mapped LP to 1q21 and identified the extracellular matrix protein 1 gene (*ECM1*) as the LP gene.

RESULTS

The clinicopathological features of LP in the patients were determined by clinical examination and skin biopsy, subdivided for light microscopy, immunohistochemistry, immunofluorescence microscopy and transmission electron microscopy. Six different unrelated consanguineous families (from Saudi Arabia, Kuwait, Pakistan, The Netherlands, UK, and a group of South African families with a probable common ancestor) were available for assessment and investigation. Representative features are shown in Figure 1. In brief, the clinical manifestations included beaded eyelid papules (Fig. 1A), warty papules on the hands and limbs (Fig. 1B and C), acne-like pitted scars of the face and thickening of vocal cords (Fig. 1D). Some inter- and intra-familial variability in skin phenotype was seen, but a hoarse voice was a universal finding. Light microscopy showed prominent periodic acid-Schiff's (PAS) reagent staining (glycoprotein) at the dermal epidermal junction and around dermal blood vessels (Fig. 1E), as well as broad band-like anti-type IV collagen immunostaining at these sites (Fig. 1F). Ultrastructually, dermal blood vessels were surrounded by multilaminated concentric rings of basement membrane (Fig. 1G).

After obtaining informed consent, we performed a genome-wide linkage analysis using DNA from three affected siblings and one unaffected individual from a consanguineous Saudi Arabian family (15). False linkage was detected with marker D13S175 that was excluded by analysis of additional LP families. However, linkage was obtained with the marker D1S498, giving a two-point LOD score of 3.45 at $\theta = 0$. An additional 28 affected individuals from five other unrelated families (22 of whom represented a probable South African founder effect) were analysed for D1S498 and showed complete linkage. A maximum two-point LOD score of 21.85 at $\theta = 0$ was obtained, assuming a founder effect in the South African cohort, since these affected individuals shared the same haplotype across the critical region. Further analysis was performed using available markers in the interval between D1S442 and D1S305. Observed recombinants and the region of shared haplotype observed in the South African families placed the gene in a 2.3 cM interval on 1q21 between D1S2344 and D1S2343 (Marshfield genetic map). This corresponded to an ~6.5 Mb region on the UCSC physical map (http://genome.uscs.edu/) containing 68 known genes or *in silico* predicted genes with supporting expressed sequence tag data.

A positional candidate gene approach was then used to identify the LP gene. Specifically, given previous observations of abnormal vacuoles in skin fibroblasts from LP patients (5,8), we performed RT–PCR on RNA extracted from cultured normal and LP fibroblasts using pairs of 3′-untranslated region (3′-UTR) primers for all 68 possible genes. We presupposed that the LP gene would be expressed in fibroblasts and that its expression in LP would probably be reduced compared to normal controls (akin to many other autosomal recessive skin disorders in which inherent nonsense mutations result in nonsense-mediated mRNA decay) (16–19). Following this rationale, several candidate genes, including *TUFT1*, *PSMB4*, *RFX5*, *PIK4CB*, *SELENBP1*, *PIP5K1A*, *PSMD4*, *SEMAY*, *ANXA9*, *SPEC1*, *SETDB1*, *CTSS*, *CTSK*, *VPS45B* and *CEZANNE*, showed no relative differences in RNA expression. However, RT–PCR for *ECM1* showed a reduction in the intensity of the RT–PCR product in the one cultured LP fibroblast sample available for analysis (Kuwaiti pedigree, family 2). Subsequent direct sequencing of *ECM1* using genomic DNA from this patient identified a homozygous single nucleotide deletion, 1019delA (GenBank accession no. XM_002030, with initiation codon ATG as nucleotide 1-2-3) that causes a frameshift and a premature stop codon 108 bp downstream. This frameshift mutation is in exon 7 of *ECM1* and was also present as a homozygous deletion in genomic DNA from the other two affected subjects in this pedigree: both parents were heterozygous carriers of 1019delA. Presence of this mutation was not detected by direct sequencing of 80 ethnically matched control chromosomes.

Sequencing of genomic DNA for all exons and flanking intronic sequences of *ECM1* in the other pedigrees disclosed five further loss-of-function mutations comprising nonsense (Q276X, family 4; Q346X, family 3; Q359X, family 5), frameshift (501insC, family 6) or deletion (1163del, family 1) mutations (Figs 2 and 3A, sequence analysis; and Fig. 3B, new restriction digest site for Q276X mutation). Every individual tested was homozygous for a particular mutation. None of

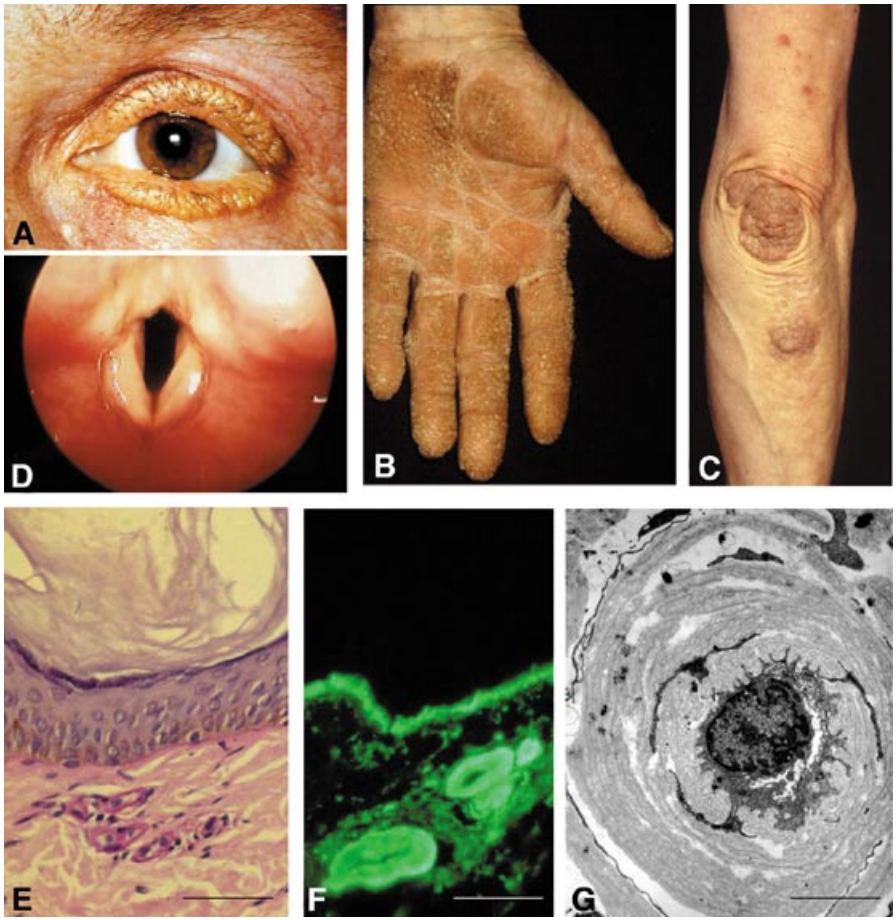

Figure 1. Clinicopathological features of LP. (**A**) Beaded papules along the eyelids (moniliform blepharosis) (family 3); (**B**) warty papules and skin hyperkeratosis on the palm (family 6); (**C**) waxy nodules and scarring on the elbow (family 6); (**D**) thickening of vocal cords (family 3); (**E**) deposition of PAS-positive material in skin at the dermal–epidermal junction and surrounding blood vessels in the dermis. Hyperkeratosis is also present (family 3). Bar = 50 µm. (**F**) Immunofluorescent labelling with an anti-type IV collagen antibody shows bright, thick bands of staining at the dermal–epidermal junction and around blood vessels, consistent with basement membrane thickening (family 3). Bar = 50 µm. (**G**) transmission electron microscopy of a small dermal blood vessel reveals marked concentric reduplication of basement membrane (family 3). Bar = 2 µm.

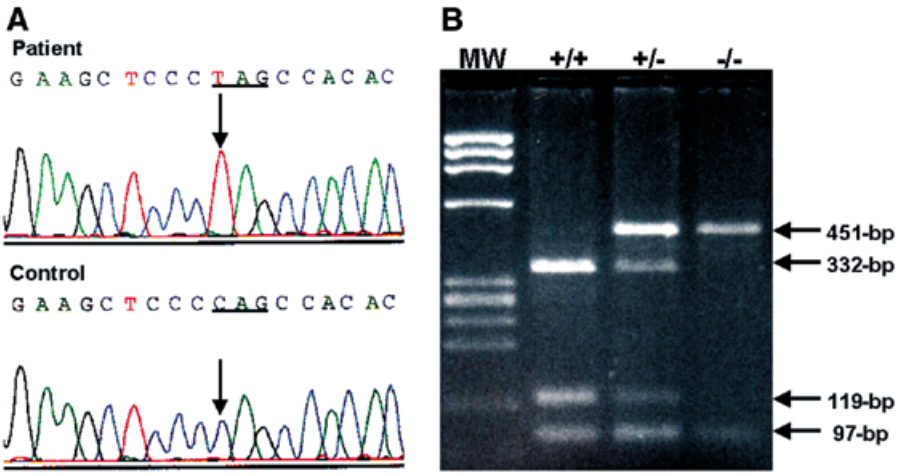

Figure 2. Molecular basis of LP. (**A**) Direct sequencing of exon 7 of *ECM1* in an affected South African patient reveals a C→T transition at nucleotide 826 that converts a glutamine residue (CAG) to a stop codon (TAG); the mutation is designated Q276X. (**B**) Verification of the mutation Q276X by restriction endonuclease digestion with *Bfa*I (New England BioLabs, Hitchen, UK). Wild-type PCR product (–/–) spanning exon 7 and flanking introns (548 bp) is digested into products of 451 and 97 bp, consistent with a solitary cut site for *Bfa*I. In patients homozygous for the mutation Q276X (+/+), the PCR product is digested into fragments of 332, 119 and 97 bp in size, as the mutation introduces a new recognition site for this enzyme. In individuals heterozygous for Q276X (+/–), four digested fragments (451, 332, 119 and 97 bp) are present.

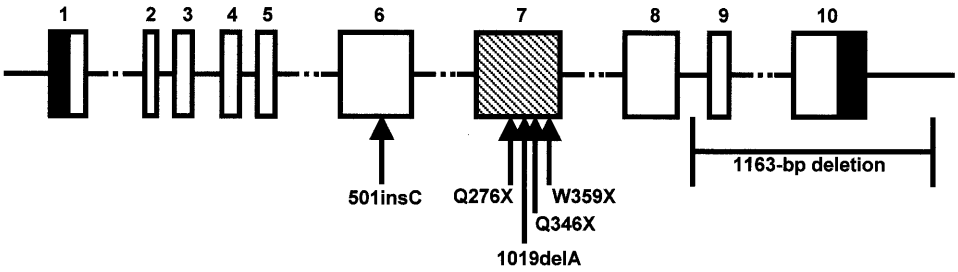

Figure 3. Illustration of the positions of the six different mutations identified in the *ECM1* gene in patients with LP. All patients are homozygous for a single mutation. Four mutations are located within the differentially spliced exon 7 (Q276X, Q346X, W359X and 1019delA). The other mutations comprise a frameshift mutation in exon 6 (501insC) and a 1163 bp deletion starting 34 bp into intron 8 and encompassing all of exon 9, intron 9, exon 10 (including the termination codon) and part of the 3′-UTR.

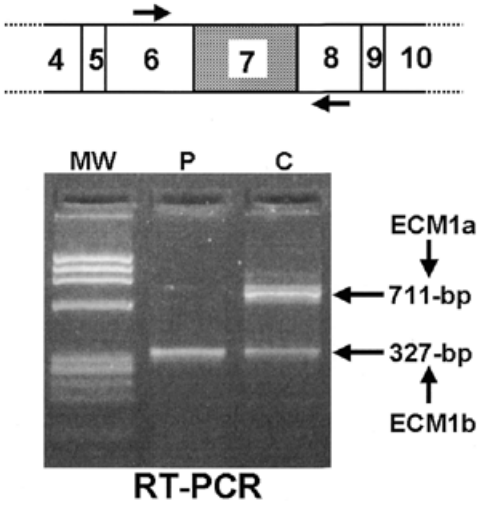

Figure 4. RT–PCR products resolved by 2.5% agarose gel electrophoresis. RT–PCR was performed on RNA extracted from cultured skin fibroblasts with primers spanning the differentially spliced exon 7 show two bands in the control sample (C) corresponding to transcripts for ECM1a (711 bp) and ECM1b (327 bp). In contrast, in a sample from a Kuwaiti patient homozygous for the mutation 1019delA (family 2), only the ECM1b transcript is detected.

these additional *ECM1* sequence variants was detected (by direct sequencing) in 160 ethnically diverse control chromosomes.

Four of the mutations occurred in exon 7 of *ECM1*. This exon is known to be alternatively spliced in some tissues, including skin (19). Specifically, in skin two isoforms of ECM1 are usually present, a longer ECM1a transcript and a shorter ECM1b form that lacks exon 7. Thus, the mutations in exon 7 would be expected to reduce expression of ECM1a but not ECM1b. To assess this we performed RT–PCR using RNA extracted from cultured fibroblasts (family 2) with a forward primer sited in exon 6 and a reverse primer in exon 8. Agarose gel electrophoresis of the RT–PCR products (Fig. 4) showed a near absence of ECM1a transcript in the LP patient sample but no change in ECM1b amplification compared to control fibroblast cDNA. These differences in mRNA expression were confirmed at the protein level by immunohistochemical staining of skin sections using antibodies to ECM1a and b (Fig. 5). In skin from two patients (families 2 and 3) with different mutations in exon 7, labelling revealed a marked reduction in ECM1a immunostaining but no reduction or alteration in the ECM1b immunostaining pattern compared to control skin.

DISCUSSION

We have mapped LP to 1q21 and identified the *ECM1* gene as the mutated gene in this disorder. Human *ECM1* encodes a glycoprotein of unknown function, the counterpart to an 85 kDa secreted protein first identified in a murine osteogenic stromal cell line (19–21). It was so named because it was identified amidst various extracellular matrix proteins (including collagens, osteonectin and sialo bone protein) (22), but thus far there has been no evidence of any relevance or relationship to the family of known extracellular matrix proteins (23). ECM1 has been thought to have a role as a negative regulator of endochondral bone formation, inhibiting alkaline phosphatase activity and mineralization (23). In addition, it has been implicated in aspects of keratinocyte differentiation (24), tumour biology and angiogenesis, selectively stimulating endothelial cell proliferation and promoting blood vessel formation (25). It is expressed as alternatively spliced transcripts 1.8 kb ECM1a and 1.4 kb ECM1b. ECM1a is predominantly present in the placenta and heart (but also in the liver, small intestine, lung, ovary, prostate, testis, skeletal muscle, pancreas, kidney and skin) and ECM1b is found in skin and tonsils (20). The shorter transcript lacks 125 amino acids corresponding to exon 7 of the gene.

The *ECM1* gene is located close, but centromeric, to the epidermal differentiation complex on 1q21 and a role for ECM1 in aspects of keratinocyte differentiation has been investigated (24). Specifically, an association between keratinocyte differentiation and expression of the ECM1b transcript was demonstrated. However, identification of *ECM1* as the causative gene in LP now provides the first insight into the biological significance of ECM1, particularly as the mutations identified are predicted to ablate either ECM1a (four mutations) or both isoforms (two mutations). From a clinical perspective, the two families harbouring mutations outside exon 7 have a similar, but slightly more severe phenotype compared to those with mutations in exon 7. Specifically, erosive, papulovesicular lesions are seen in family 1 and warty palmar hyperkeratosis is present in the affected individual in family 6; neither of these features is prominent in the exon 7 mutation families. Nevertheless, more extensive *ECM1* mutation analysis in further LP patients will be necessary to define

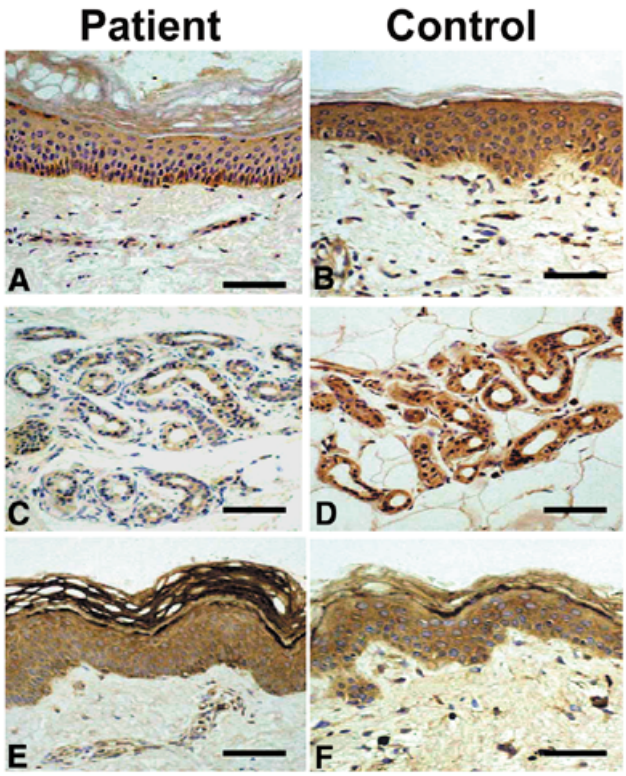

Figure 5. Immunoperoxidase labelling of formalin-fixed, paraffin-embedded skin from a patient homozygous for Q346X in exon 7 (family 3) with antibody 12573 (predominantly to ECM1a) (20,24) is almost completely absent in patient skin (**A**) compared to the pan-epidermal labelling in control skin (**B**). In (A) there is some brown staining within basal keratinocytes but this is endogenous melanin (patient has type V skin) rather than peroxidase reactivity. In the control skin focal labelling of dermal fibroblasts and blood vessel walls is also seen but this is not present in the patient skin. In (**C**) (patient) and (**D**) (control) the labelling intensity with antibody 12573 is very much reduced within deep dermal blood vessel walls, sweat glands and adipocytes in the patient compared to the control. Background melanin is not present in this part of the patient's skin. In (**E**) (patient) and (**F**) (control) there is similar pan-epidermal and upper dermal labelling with antibody 12906 (predominantly to ECM1b) (21). Scale bar = 50 µm. These findings indicate a marked reduction in ECM1a protein in skin from a patient with a homozygous nonsense mutation in exon 7, but no reduction in ECM1b, i.e. from the transcript that normally splices out this exon.

genotype–phenotype correlation more precisely and to determine how particular mutations in *ECM1* preferentially perturb the epidermis or dermis. Overall, our preliminary molecular data demonstrate that the longer isoform, ECM1a, is of more fundamental biological importance, with a minor additional contribution, in terms of the LP phenotype, from loss of the ECM1b isoform. Histologically, the epidermis in LP skin was unremarkable, apart from minor changes (basket-weave hyperkeratosis). In contrast, the major changes in LP skin are in the dermis and sub-cutis, with basement membrane thickening at the dermal–epidermal junction, around blood vessels and adnexal epithelia, and deposition of hyaline material in the dermis. Although the pathophysiology underlying these basement membrane alterations is not clear, one explanation might be that lack of ECM1a leads to defective protein binding. It is known that ECM1 has a pattern of cysteine repeats similar to the ligand binding loops present in serum albumins (19) and, therefore, if ECM1 normally binds type IV collagen, the lack of this potentially regulatory or stabilizing protein–protein interaction in LP could then result in increased type IV collagen expression and the typical histopathological changes. Failure to bind other non-collagenous proteins would also account for the hyaline material deposition in skin and other tissues. Further studies to determine the specific protein interactions of ECM1 will be necessary to provide a more comprehensive understanding of the skin changes in LP as well as defining the precise role of ECM1 in connective tissue homeostasis. Exon 7 of ECM1 also contains a calcium-binding domain (19,24) and an absence of this motif in LP might help explain the intracranial calcification seen in some LP patients, although calcification is not a recognized feature at other sites.

The molecular basis of LP results from loss of ECM1 expression, particularly the ECM1a isoform. This discovery now provides an opportunity to further assess the protean clinical manifestations in this disorder as well to determine whether other inherited disorders such as infantile systemic hyalinosis (which may have clinical overlap with LP) also involve ECM1 pathology (26). Likewise, delineation of ECM1 as the LP gene may provide new insight into the pathogenesis of some acquired or autoimmune disorders such as lichen sclerosus, systemic sclerosis or graft-versus-host-disease that may be associated with hyperkeratosis of the epidermis and hyaline changes in the dermis. In addition, the ultrastructural changes affecting basement membrane material seen around blood vessels in LP are reminiscent of those occurring in diabetes mellitus, systemic sclerosis and porphyria (2), observations that might also indicate a role for ECM1 in other aspects of angiopathy or angiogenesis (2,25). The discoveries also suggest that ECM1 contributes to aspects of skin adhesion, epidermal differentiation, wound healing and scarring and collectively highlight that ECM1 is an important secreted protein with diverse biological functions.

MATERIALS AND METHODS

Subjects

Lipoid proteinosis patients and their families were ascertained through clinical dermatology and genetics services. Family 1 is a consanguineous Saudi Arabian family with four affected siblings. Affected individuals (homozygous for the 1163 bp deletion involving exons 8–10), aged 2–10 years, have hoarseness (starting at ~1 year of age), papulovesicular skin lesions, scarring and progressive skin and mucosal thickening. Comprehensive clinicopathological features of this family have been reported recently (15). Family 2 is a consanguineous Kuwaiti family containing three affected siblings (aged 3–7 years); parents are first cousins. Affected individuals (homozygous for the mutation 1019delA in exon 7) have similar but milder features to those seen in family 1. In family 3, there is a single 14-year-old affected Pakistani boy (homozygous for the nonsense mutation Q346X; 1036 C→T nucleotide substitution in exon 7); the parents are first cousins. He has had progressive laryngeal hoarseness since early infancy. Skin signs are relatively mild: there is diffuse thickening (which also affects the tongue) and pitted scarring on the face and upper trunk. Family 4 comprises several South African families with 22 affected individuals (homozygous for the nonsense mutation Q276X; 826 C→T nucleotide substitution in exon 7) from Namaqualand

in the Northern Cape province. The exact inter-relatedness of these individuals is not known, but the population is of mixed Khoisan and European origin and a founder effect has been suspected. Some clinicopathological and epidemiological details have been published previously in that the origins of LP in South Africa are purported to go back to an affected brother and sister from Germany who settled in the Cape in the 1650s (13). The age range of the affected individuals is 7–69 years and inter-individual variability in phenotype is present. Some individuals have mild skin induration, scarring and vocal hoarseness, while others have very marked hoarseness, laryngeal thickening (sometimes necessitating tracheostomy) and skin thickening. Epilepsy and personality disorders have been reported in some of the affected South African individuals although this has not yet been formally reviewed (2,13). Family 5 comprises two affected white Anglo-Saxon brothers (homozygous for the nonsense mutation W359X; 1077 G→A nucleotide substitution in exon 7), aged 38 and 35 years. Details of these individuals have been reported previously (27). In brief, the clinical features have been relatively mild with hoarseness only starting around the age of 5 years and patchy skin thickening developing as teenagers. Family 6 consists of an affected white Dutch male (homozygous for the frameshift mutation 501insC in exon 6), aged 30 years. The clinical features are similar to the other affected individuals but slightly more severe with early onset of laryngeal hoarseness, extensive skin involvement and epilepsy in association with focal intracranial calcification.

Linkage analysis

Initially, genomic DNA from three affected siblings and one unaffected individual from a consanguineous Saudi Arabian kindred (family 1) was used for linkage analysis. A genome-wide screen was performed using the ABI LD5 panel of 811 fluorescent microsatellite markers, analysed on an ABI 310 DNA sequencer using Genescan 2.1 and Genotyper 2.0 software (PE Applied Biosystems, Foster City, CA). Two-point LOD scores were calculated with MLINK algorithm of LINKAGE version 5.1. The mutant allele frequency was assumed to be 0.00001 with 100% penetrance and CEPH/Genethon allele frequencies were used. Markers showing potential linkage were further assessed in an additional 28 affected individuals from five consanguineous pedigrees (22 of the affected cases were from the Namaqualand region of South Africa). Due to the shared haplotype observed in the South African families with markers in the critical region, which confirmed the suspected founder effect, 'fuzzy' inheritance was simulated in the calculation of LOD scores by assuming the affected were the offspring of second cousin marriages. This resulted in a combined two-point LOD score of 21.85 for all families studied. Recalculation of the LOD scores, assuming the South Africans were the offspring of more distantly related parents, did not significantly affect the overall LOD score. Recalculation ignoring the relatedness of the South Africans gave an overall combined LOD score of 8.61 at $\theta = 0$. Loci of interest were investigated further using available markers in the intervals and visible recombinants and a region of shared haplotype in the South African families was used to determine the LP gene locus. Known genes or *in silico* predicted genes were then assessed as possible candidate genes using data from the August 2001 browser of the UCSC physical map (http://genome.ucsc.edu/).

Cell culture

Following informed consent, biopsies from non-lesional, upper arm skin were taken under local anaesthetic from two affected individuals (families 2 and 3) and two control subjects of similar age. The epidermis was separated from the dermis by Dispase (Sigma-Aldrich, Poole, UK) digestion in Dulbecco's phosphate-buffered saline (Gibco BRL, Paisley, UK) for 2 h at 37°C, as described previously (28). Fibroblasts from the dermal extract were cultured in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 10% fetal bovine serum (ICN Biomedicals, Basingstoke, UK) and 1% penicillin/streptomycin (Sigma-Aldrich).

RNA studies

For RNA extraction from cultured fibroblasts, culture medium was removed from a confluent T75 flask and 4 ml of RNAzol B (Biogenesis, Poole, UK) was added. The lysate was collected with a cell scraper and aliqoted into 1 ml samples. Tubes were then placed on ice for 1 min. Aliquots of 100 µl of 10% chloroform were added and the tubes were vortexed for 20 s. The samples were then placed on ice for 10 min before centrifuging at 13 000 r.p.m. for 4 min (rotor: Mistral 3000i, Sanyo UK, Loughborough, UK). The aqueous phase was transferred to a fresh tube and an equal volume of isopropanol was added. Samples were precipitated at –20°C and stored for further analysis. RNA samples were pelleted at 13 000 r.p.m. for 10 min (rotor: Mistral 3000i, Sanyo UK). The pellet was then washed in 70% ethanol, air-dried for 5 min, and resuspended in water (total volume 300 µl). Fifty microlitres of RNA was cleaned using the Qiagen Rneasy mini-cleanup protocol (Qiagen, Crawley, UK) and 5 µl was used for RNA quantification. RNA was quantified in a Model 6200 fluorimeter (Jenway, Dunmow, UK) using SYBR Green I dye (Molecular Probes Europe, Leiden, The Netherlands) and a standard curve created using Excel™ (Microsoft, Reading, UK) for a range of known control concentrations.

cDNA synthesis

For reverse transcription, two identical reactions for each RNA sample were established comprising 5 µg DNase I-treated RNA, 7 µl of 5× M-MLV RT-buffer (Promega, Southampton, UK), 4 µl of 10 mM dNTPs, 1 µl Rnasin (Promega) and 1 µl random primers (ICN). Reactions were incubated at 65°C for 10 min and then placed in ice. After cooling, 2 µl M-MLV reverse transcriptase (400 U) was added to one of the duplicate reactions, whereas 2 µl water was added to the other (negative control). Samples were then incubated at 42°C for 90 min and then stored at –20°C. PCR for house keeping genes L32 and GAPDH were used to compare the quantity of the cDNA template and to exclude genomic contamination. Further RT–PCR was undertaken using a 1:10 dilution of the cDNA template. To assess candidate genes for LP, primers were designed to amplify a 200–400 bp part of the 3′-UTR for known genes or predicted genes. PCR conditions for these primers were optimized using different annealing temperatures on control genomic DNA.

For PCR amplification, 250 ng of genomic DNA was used as template in an amplification buffer containing 6.25 pmol of the primers, 37.5 nmol $MgCl_2$, 5 mmol of each nucleotide and 1.25 U *Taq* polymerase (PE Biosystems, Warrington, UK) in a 25 µl total volume reaction in an Omni-Gene thermal cycler (Hybaid, Teddington, UK). Amplification conditions were 95°C for 5 min; then 95°C for 45 s, annealing temperature (55–60°C) for 45 s, 72°C for 45 s, for 38 cycles. Aliquots of 5 µl of each PCR product were then examined by electrophoresis on 2% agarose. Using fresh primers and PCR reagents, equal quantities of cDNA from the cultured fibroblast samples were then amplified (in duplicate).

Mutation analysis

To amplify genomic DNA for the *ECM1* gene, primers were designed for individual exons (apart from exons 8 and 9 which were amplified together) with upstream and downstream primers sited at least 50 bp into flanking introns. To amplify exon 7, the primers were X7F, 5′-ttatctgcctgcccagtgtc-3′, and X7R, 5′-acatggatggatggactggc-3′ (expected PCR product size 548 bp). To amplify exon 6, the primers were X6F, 5′-agccttgagaagcaggagga-3′, and X6R, 5′-agtgaacgggacctgaggtt-3′ (expected PCR product size 671 bp). The PCR conditions were the same as those described above with an annealing temperature of 55°C for both reactions. All PCR products were sequenced with Big Dye labelling in an ABI 310 genetic analyser (PE Biosystems). Where possible, mutations were verified by restriction endonuclease digestion and confirmed/excluded in other family members. To determine the mutation in family 1, no PCR product could be detected in affected individuals for exons 8–10 (cf detectable PCR products from parents or unaffected sibling). Therefore, serial PCR reactions from a fixed forward primer in intron 7 and variably sited reverse primers in the 3′-UTR were used to establish the nature of the deletion. PCR demonstrated evidence for a deletion of ~1.1 kb, the exact nature of which was determined by direct sequencing.

Microscopy of skin biopsies

Parts of the skin biopsies were either snap-frozen in liquid nitrogen, or fixed in 10% formalin and embedded in paraffin, or fixed in half-strength Karnovsky's fixative for transmission electron microscopy (29). For type IV collagen labelling, 5 µm cryosections of skin were labelled with antibody COL94 (Sigma-Aldrich) diluted 1:100 in phosphate-buffered saline/bovine serum albumin. Labelling was as described previously (30). Fluorescein isothiocyanate-labelled anti-rabbit secondary antibody (Dako, Glostrup, Denmark) was used diluted 1:200 in phosphate-buffered saline/bovine serum albumin. For light microscopy, sections from the paraffin-embedded tissue were stained with either haematoxylin and eosin or PAS. For ECM1 labelling, the following antibodies were used: 12573 (predominantly to ECM1a) and 12906 (predominantly to ECM1b). Further details of the use of these antibodies to label skin sections have been published elsewhere (24), although we used formalin-fixed paraffin-embedded tissue rather than frozen sections as used by Smits *et al.* (24), and employed different methods of antigen retrieval (see below). This alternative approach may explain slight differences in control section staining in our study compared to that in (24). Antibodies were diluted 1:400 in phosphate-buffered saline/bovine serum albumin. Sections were dewaxed in xylene and endogenous peroxidase was blocked by incubation in 3% hydrogen peroxide in methanol for 10 min. The sections were then pre-treated, either with microwaves (placed in 500 ml of 0.01 sodium citrate pH 6.0 and microwaved at 750 W for 10 min before re-immersing in tap water) or trypsin-digested using 0.1 g of trypsin (ICN) and 0.1 M $CaCl_2$ in 100 ml distilled water (pH 7.0) at 37°C for 15 min before re-immersing in tap water. Control sections with no pre-treatment were also included. Diluted primary antibodies were then applied and sections were incubated overnight in a moist incubating chamber at 4°C. After serial washing in Tris-buffered saline, subsequent labelling was performed using the Dako ChemMate streptavidin biotin technique (Dako), which uses a universal biotinylated second layer and a streptavidin peroxidase tertiary layer (both applied for 30 min). Final reaction products were visualized using 3-3 diaminobenzedene for 10 min. All sections were then counterstained in Harris' haematoxylin for 1 min, dehydrated, cleared and mounted in DPX. For transmission electron microscopy, samples were processed largely as described previously (29). Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a JEOL 100CX transmission electron microscope.

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