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# Identification of mutations in a new gene encoding a FERM family protein with a pleckstrin homology domain in Kindler syndrome

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**Kindler syndrome is a rare autosomal-recessive genodermatosis characterized by bullous poikiloderma with photosensitivity. We report the localization to chromosome 20p12.3 by homozygosity mapping and the identification of a new gene, which we propose to name kindlerin. We found four different homozygous mutations in four consanguineous families from North Africa and Senegal; three are expected to lead to premature stop codons and truncated proteins and the fourth involves a splice site. We were unable to identify a mutation in kindlerin in a fifth consanguineous family from Algeria with a similar phenotype and in which the patient was homozygous for the markers in the 20p12.3 interval. The kindlerin protein contains several domains which are shared by a diverse group of peripheral membrane proteins that function as membrane-cytoskeleton linkers: two regions homologous to band 4.1 domain of which one includes a FERM domain with a NPKY sequence motif, and a third region with a PH or pleckstrin homology domain. Kindlerin might be involved in the bidirectional signaling between integrin molecules in the membrane and the cytoskeleton, and could be involved in cell adhesion processes via integrin signaling.**

## INTRODUCTION

Kindler syndrome (KS) is a rare genodermatosis, which was first described by Theresa Kindler (1). It is characterized by acral blistering and photosensitivity early in life followed by progressive diffuse poikiloderma with cutaneous atrophy. Webbing of the fingers and toes, labial leukokeratosis, gingivitis, esophageal, vaginal and urethral mucosal lesions leading to stenosis, and nail dystrophy have also been described (1–3). Less than 100 patients and only a few families with at least two affected subjects have been reported to date (2–6). Inheritance seems to be autosomal-recessive and a consanguineous loop has been found in half of the families, although OMIM indicates autosomal-dominant transmission (MIM 173650). An overlap between KS and hereditary acrokeratotic poikiloderma, an autosomal-dominant disorder described by Weary, has been suggested, and some have adopted the term Weary–Kindler syndrome (7). Weary and Kindler syndromes have poikiloderma in common, but differ in that bullae and photosensitivity are present in KS but not in Weary syndrome. Histologically, skin lesions of patients affected by KS are characterized by epidermal atrophy, focal vacuolization of the basal layer and pigmentary incontinence in the upper dermis in addition to a mild lymphocytic infiltrate, consistent with poikiloderma. Electron microscopy shows extensive reduplication and disruption of the lamina densa along the dermoepidermal junction beneath the basal cells, and cleft formation which occurs in the lamina lucida (3,5,8,9). Desmosomes, hemidesmosomes, tonofilaments, anchoring filaments and fibrils appear normal. In immunofluorescence studies, the basement membrane components including the integrins ($\alpha3\beta1$ and $\alpha6\beta4$) are normal, with the exception of type VII collagen which is found in abnormal locations deep in the connective tissue stroma (8,9). Laminin 5 and collagen types IV and VII present a broad and reticular labeling pattern (3), corresponding to the reduplication of lamina densa observed by electron

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microscopy. Abnormalities of adhesion molecules linking the lamina densa to the basal cells were thus suggested as the pathological mechanism (3).

A genome-wide scan of a consanguineous Algerian family with three affected children permitted localization of a gene on chromosome 20p12.3 in a 7 cM interval, which we further refined to around 800 kb by analyzing two supplementary consanguineous families. Mutation analysis in a total of five consanguineous kindreds from North Africa and Senegal revealed four homozygous mutations in a new gene, which belongs to the FERM family (band four-point-one/ezrin/radixin/moesin). In one of the Algerian families no mutation was found, which could suggest genetic heterogeneity in this disorder.

## RESULTS

### Clinical features and patient origins

Initially six patients and 11 non-affected subjects from three families were analyzed; two Algerian families (A, B) were known to be consanguineous from first-cousin marriages, and in the Senegalese family (C) consanguinity of the fourth degree was reported. Clinical symptoms of patients are summarized in Table 1 and pedigrees are shown in Figure 1.

The three affected children in family A presented acral blistering at birth and were initially diagnosed with hereditary epidermolysis bullosa. The subsequent observation of photosensitivity, progressive poikiloderma with dyschromatic macules, telangiectases and cutaneous atrophy led to revision of the diagnosis to KS. Further clinical features were sclerodermiform fingers and leucokeratotic plaques on the oral mucosa. In the second Algerian family (B), the 9-year-old daughter (B.4) showed a similar phenotype including congenital blistering, progressive poikiloderma, photosensitivity and xerosis, and she also suffered from aortic insufficiency.

In the family from Senegal, the 18-year-old boy (C.3) presented congenital blistering on the back, buttocks and limbs, and progressive diffuse poikiloderma, xerosis, gingivitis and extensive dental caries at age 4. At age 7, surgical treatment of urethral stenosis and pseudoainhum of three fingers was performed. Physical examination revealed additional features such as diffuse palmar hyperkeratosis and webbing of fingers and toes, microstomia, leucokeratosis of oral mucosa and anal stenosis. The patient complained of dysphagia but barium esophagography was normal. His 17-year-old sister (C.4) presented blistering since birth, and dry skin, erosive gingivitis and caries at age 4. She complained of intermittent dysphagia and bleeding of the gums. The skin on the dorsa of the hands and feet, elbows and knees was pigmented and atrophic with a cigarette-paper-like appearance; diffuse palmoplantar keratoderma, erosive gingivitis, leucokeratosis of oral mucosa and poor dentition were also noted. The 22-year-old sister (C.5) showed a similar phenotype including congenital blisters, progressive poikiloderma, diffuse xerosis, severe gingivitis and anal fissures. DNA from this patient was not available.

After identification of the kindlerin gene, two additional consanguineous families from Tunisia (D) and Algeria (E) including four affected and six non-affected individuals were analyzed (Fig. 1 and Table 1). In the Tunisian family (D), an 11-year-old female (D.3) was referred for poikiloderma, dysphagia and failure to thrive. She was born to healthy first-cousin parents. Spontaneous blisters were noted on the arms and legs since birth. She had marked atrophic, cigarette-paper-like, wrinkled skin on the dorsa of her hands and feet. Dyschromic patches with reticulated erythema and telangiectases and white atrophic macules were noted on the parts of the skin exposed to the sun. Hair, nails and oral mucosa were normal. Dysphagia and failure to thrive were related to esophageal stenosis. A skin biopsy revealed epidermal atrophy and fibrosis of the papillary dermis, in which numerous melanophages and angiogenesis were noted. Collagen and elastic fibres were normal. Her first cousin (D.6), born to related parents, presented similar skin manifestations (Fig. 2A).

In the third Algerian family, a 13-year-old boy (E.2) was referred for poikiloderma with photosensitivity. He was born to healthy parents who were second cousins. Blisters were noted on the arms, legs and oral mucosa and have occurred spontaneously since birth. He presented diffuse ichthyosis and reticular hyperpigmentation on the trunk. Other clinical features were thin, dry hair, nail dystrophy, webbing of fingers and phimosis. He had failure to thrive related to chronic diarrhoea. A skin biopsy showed hyperkeratosis, mild fibrosis of the upper dermis with free melanin and melanophages. His 6-year-old sister (E.3) presented with similar but milder skin manifestations (Fig. 2B).

### Linkage analysis

A genome-wide scan in family A with three affected children was performed using 400 highly polymorphic microsatellite markers from the ABI panel (Linkage Mapping Set 2, LMS2, Applied Biosystems). A co-segregating region of 7 cM at 20p12.3 was found with loss of homozygosity in two affected children limited by the telomeric marker AFM248td1 (D20S116) and the centromeric marker AFM292xb5 (D20S189). Families A, B and C were genotyped with a total of 23 markers (spaced at about 0.3 Mb) from public databases, which permitted us to refine the interval to 834 kb between D20S905 at the telomeric end and D20S192 at the centromeric limit. Only the central five markers of the interval were analysed in the two additional families (D, E); the genotypes are presented in Figure 1. All patients from the five families showed homozygous alleles in the smallest co-segregating interval, with the exception of marker AFM299xd1 (D20S192) in family A. This marker, which is in the middle of a 7 cM-long homozygous haplotype, is heterozygous in the three affected children, most likely as a consequence of a mutation in one of the ancestors (Fig. 1). The frequency of microsatellite mutations has been estimated to be as high as 0.1% (10). There was no common haplotype and no linkage disequilibrium was observed in the five families. The maximum pairwise LOD score at $\Theta = 0.00$ for D20S194 was 8.54 and the multipoint LOD score at this same locus was 9.72.

### Exclusion of candidate genes

In the initial 7 cM interval, we sequenced and excluded the SLC23A1 gene, a sodium-dependent vitamin C transporter.

**Table 1.** Summary of clinical data of the patients

| Patients | A.4 | A.5 | A.9 | B.4 | C.3 | C.4 | C.5 | D.3 | D.6 | E.2 | E.3 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Origin | Algeria | Algeria | Algeria | Algeria | Senegal | Senegal | Senegal | Tunisia | Tunisia | Algeria | Algeria |
| Sex | F | M | F | F | M | F | F | F | M | M | F |
| Age (years) | 20 | 18 | 2 | 9 | 18 | 17 | 22 | 11 | 19 | 13 | 6 |
| Congenital blistering | + | + | + | + | + | + | + | + | + | + | + |
| Progressive poikiloderma | + | + | + | + | + | + | + | + | + | + | + |
| Skin atrophy | + | + | + | + | + | + | + | + | + | + | + |
| Telangiectasia | + | + | + | − | − | + | + | + | + | + | + |
| Dyschromatic patches | + | + | + | − | − | − | − | + | + | − | + |
| Photosensitivity | + | + | + | + | − | − | − | + | + | + | + |
| Ichthyosis or xerosis | + | + | + | + | + | + | + | + | + | + | + |
| Palmoplantar keratoderma | − | − | − | − | Diffuse | Diffuse | − | + | + | − | − |
| Oral mucosa | | | | | | | | | | | |
| Leucokeratotic plaques | + | + | + | + | + | + | − | − | − | − | − |
| Gingivitis | + | + | − | + | + | + | + | − | − | + | + |
| Labial leucokeratosis | − | − | − | − | + | Erosive | − | − | − | − | − |
| Teeth | Caries | N | N | Caries | Caries | Poor dentition | N | N | N | Caries | Caries |
| Hair | N | N | N | N | N | N | N | N | N | Thin, dry | |
| Nails | N | N | N | N | N | N | N | N | N | Dystrophic | |
| Acral abnormalities | Sclerodermiform fingers | | | − | Syndactyly of toes, webbing and pseudo ainhum of fingers | − | − | − | − | Webbing of fingers | |
| Dysphagia | + | − | − | − | + | + | − | + | + | + | − |
| Diarrhoea | − | − | − | − | − | − | + | − | − | + | + |
| Stenosis | Pharynx | − | − | − | Anal and urethral, microstomia | − | − | Anal fissures | Esophagus | Phimosis | − |
| Skin biopsies | + | + | + | + | + | − | − | + | + | + | + |
| Other symptoms | − | − | − | Aortic insufficiency | Anemia | Anemia | − | − | − | − | − |

N = normal.

In the refined interval of 834 kb, eight genes were found in the Ensembl Human Contig view (www.ensembl.org): CHGB, KIAA1153, C20orf154, C20orf155, Q8WUT4, C20orf75, Q8WUS3 and C20orf42. We sequenced and excluded C20orf154, which codes for a novel MCM2/3/5 family member or minichromosome maintenance 8 protein, and C20orf155 (a member of the CDP-alcohol phosphatidyl transferase family), before we identified mutations in the C20orf42 gene which we proposed to name kindlerin.

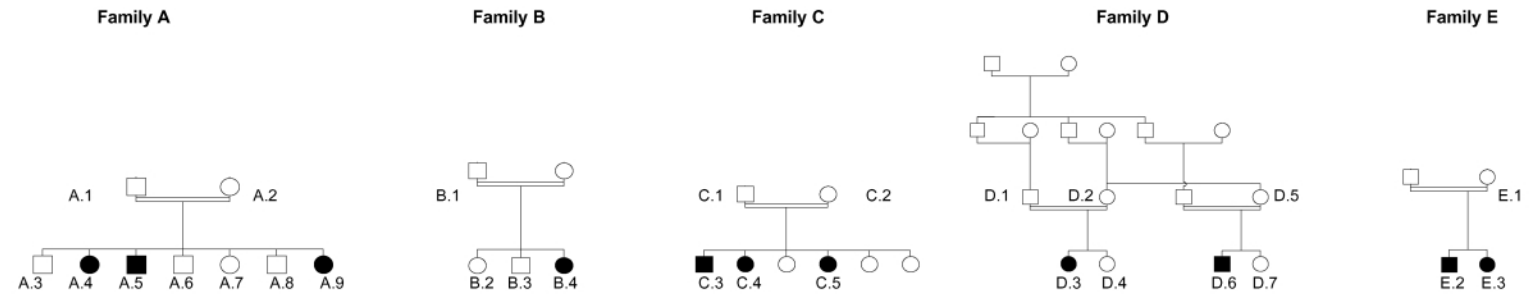
## Structure of the kindlerin gene and cDNA

Various gene annotation programs predicted between 7 and 22 exons for the kindlerin gene, which is transcribed in the reverse direction. The prediction with the Acembly program (www.acedb.org/Cornell/acembly) and curated Sanger reports (www.sanger.ac.uk) indicates 15 exons, of which the first is non-coding, whereas Twinscan (http://genome.cs.wustl.edu/~bio/) predicts 19 and Genscan (http://genes.mit.edu/GENSCAN.html) 22 exons.

To elucidate the gene structure, we performed overlapping RT–PCRs covering the entire sequence of kindlerin. Thus we excluded the additional exons predicted by Twinscan and Genscan; the five exons at the 3′ end proposed by both programs belong to a separate gene transcribed in the same direction encoding a putative leucine-rich protein and not to the kindlerin gene. We determined a gene structure with 15 exons as predicted by Sanger Institute, which was recently confirmed by the submission of a complete cDNA (URP1). The total length of the cDNA is 4720 bp including a complete 5′UTR of 358 bp, a 2034 bp coding region and a long 3′UTR (2328 bp). The coding portion begins with an ATG codon at nucleotide residue 359 in the second exon. This methionine is preceded by an in-frame stop codon 79–81 bp upstream in the 5′UTR, and is also embedded in a consensus sequence described as an 'adequate context' for initiation of translation (RNNatgY, where R is a purine and Y a pyrimidine) (11). Using the CpG_grailexp program (http://compbio.ornl.gov/grailexp/), we identified a 578 bp-long CpG island which includes exon 1 and a 522 bp-long sequence which precedes it (70.21% CG).

We performed 5′-RACE to confirm the 5′UTR mRNA and sequenced the extensions. Two populations of transcripts were found, which differed by 3 bp as a consequence of two contiguous ag acceptor splice sites in the genomic sequence (2 and 5 bp upstream of exon 2). Two transcripts for kindlerin have been described in public databases; one is predicted to code for a protein of 230 aa (BAA90957) and the other to a protein of 677 aa (AAN75822).

Homologs of kindlerin are present in *Mus musculus* (BB440207), *Drosophila melanogaster* (CG14991 and CG7729), *Xenopus laevis* (BJ046068), and *C. elegans* (NP_506628). This latter gene encodes UNC112, which has been recently hypothesized to be a new regulator of cell–extracellular matrix adhesion (12,13). Kindlerin was initially described as a gene coding for a novel protein similar to mitogen-inducible protein MIG-2 (isoform 1), because it is a paralog of MIG-2 (mitogen-inducible gene) on chromosome 14q22.1; both genes have 15 exons. MIG-2 was isolated by




| Marker | A.1 | A.2 | A.3 | A.4 | A.5 | A.6 | A.7 | A.8 | A.9 | B.1 | B.2 | B.3 | B.4 | C.1 | C.2 | C.3 | C.4 | D.1 | D.2 | D.3 | D.4 | D.5 | D.6 | D.7 | E.1 | E.2 | E.3 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| D20S889 | 3 1 | 3 2 | 1 2 | 3 3 | 3 3 | 1 3 | 1 3 | 1 2 | 3 3 | 1 5 | 5 5 | 1 1 | 1 5 | 5 3 | 4 2 | 5 4 | 5 2 | | | | | | | | | | |
| D20S116 | 4 1 | 4 3 | 1 3 | 4 4 | 4 3 | 1 4 | 1 4 | 1 3 | 4 4 | 1 2 | 0 0 | 0 0 | 1 2 | 4 1 | 4 3 | 4 4 | 4 3 | | | | | | | | | | |
| D20S849 | 2 1 | 2 2 | 1 2 | 2 2 | 2 2 | 1 2 | 1 2 | 1 2 | 2 2 | 1 3 | 3 4 | 1 1 | 1 1 | 0 0 | 0 0 | 0 0 | 0 0 | | | | | | | | | | |
| D20S835 | 4 1 | 4 2 | 1 2 | 4 4 | 4 4 | 1 4 | 1 4 | 1 2 | 4 4 | 2 3 | 3 2 | 2 2 | 2 2 | 5 6 | 5 5 | 5 5 | 5 5 | | | | | | | | | | |
| D20S882 | 1 2 | 1 4 | 2 4 | 1 1 | 1 1 | 2 1 | 2 1 | 2 4 | 1 1 | 3 1 | 1 2 | 3 3 | 3 3 | 4 1 | 4 3 | 4 4 | 4 3 | | | | | | | | | | |
| D20S95 | 2 1 | 2 3 | 1 3 | 2 2 | 2 2 | 1 2 | 1 2 | 1 3 | 2 2 | 7 7 | 7 5 | 7 7 | 7 7 | 6 4 | 6 2 | 6 6 | 6 2 | | | | | | | | | | |
| D20S916 | 1 2 | 1 2 | 2 2 | 1 1 | 1 1 | 2 1 | 2 1 | 2 2 | 1 1 | 4 3 | 3 2 | 3 4 | 4 4 | 3 5 | 3 3 | 3 3 | 3 3 | 6 2 | 6 7 | 6 6 | 6 7 | 6 8 | 6 6 | 6 2 | 6 2 | 6 6 | 6 6 |
| D20S905 | 4 3 | 4 3 | 3 3 | 4 4 | 4 4 | 3 4 | 3 4 | 3 3 | 4 4 | 1 1 | 1 2 | 1 1 | 1 1 | 1 5 | 1 3 | 1 1 | 1 3 | 1 7 | 1 4 | 1 1 | 1 4 | 1 7 | 1 1 | 1 7 | 3 6 | 3 3 | 3 3 |
| D20S194 | 2 1 | 2 1 | 1 1 | 2 2 | 2 2 | 1 2 | 1 2 | 1 1 | 2 2 | 3 2 | 2 4 | 2 3 | 3 3 | 5 6 | 5 7 | 5 5 | 5 5 | 6 10 | 6 11 | 6 6 | 6 11 | 6 3 | 6 6 | 6 11 | 8 9 | 8 8 | 8 8 |
| D20S192 | 2 5 | 3 6 | 5 6 | 2 3 | 2 3 | 5 3 | 5 3 | 5 6 | 2 3 | 5 4 | 4 5 | 4 5 | 5 1 | 7 7 | 7 4 | 7 7 | 7 7 | 7 6 | 7 10 | 7 7 | 7 10 | 7 6 | 7 7 | 7 6 | 8 9 | 8 8 | 8 8 |
| D20S892 | 6 3 | 6 1 | 3 1 | 6 6 | 6 6 | 3 6 | 3 6 | 3 1 | 6 6 | 5 4 | 4 2 | 4 5 | 5 2 | 4 7 | 4 5 | 4 4 | 4 4 | 6 6 | 6 3 | 6 6 | 6 3 | 6 6 | 6 6 | 6 7 | 2 6 | 2 2 | 2 2 |
| D20S846 | 4 3 | 4 1 | 3 1 | 4 4 | 4 4 | 3 4 | 3 4 | 3 1 | 4 4 | 2 2 | 2 3 | 2 3 | 2 3 | 3 3 | 3 5 | 3 3 | 3 3 | | | | | | | | | | |
| D20S900 | 1 4 | 1 2 | 4 2 | 1 1 | 1 1 | 4 1 | 4 1 | 4 2 | 1 1 | 2 1 | 1 1 | 1 3 | 2 3 | 4 2 | 4 2 | 4 4 | 4 4 | | | | | | | | | | |
| D20S115 | 4 3 | 4 2 | 3 2 | 4 4 | 4 4 | 3 2 | 3 4 | 3 2 | 4 4 | 2 3 | 3 4 | 3 3 | 2 3 | 2 1 | 2 4 | 2 2 | 2 2 | | | | | | | | | | |
| D20S907 | 1 2 | 1 1 | 2 1 | 1 1 | 1 1 | 2 1 | 2 1 | 2 1 | 1 1 | 1 1 | 1 3 | 1 2 | 1 2 | 4 2 | 4 5 | 4 4 | 4 4 | | | | | | | | | | |
| D20S879 | 1 1 | 1 1 | 1 1 | 1 1 | 1 1 | 1 1 | 1 1 | 1 1 | 1 1 | 1 2 | 2 1 | 2 1 | 1 1 | 1 2 | 1 1 | 1 1 | 1 1 | | | | | | | | | | |
| D20S177 | 3 2 | 3 3 | 2 3 | 3 3 | 3 3 | 2 3 | 2 3 | 2 3 | 3 3 | 2 3 | 3 2 | 3 1 | 2 1 | 2 4 | 2 2 | 2 2 | 2 2 | | | | | | | | | | |
| D20S851 | 7 5 | 7 4 | 5 4 | 7 7 | 7 7 | 5 7 | 5 7 | 5 4 | 7 7 | 1 3 | 3 6 | 3 4 | 1 4 | 2 2 | 2 4 | 2 2 | 2 2 | | | | | | | | | | |
| D20S917 | 5 1 | 5 3 | 1 3 | 5 5 | 5 5 | 1 5 | 1 5 | 1 3 | 5 5 | 2 5 | 5 3 | 5 1 | 2 1 | 5 6 | 5 4 | 5 5 | 5 5 | | | | | | | | | | |
| D20S901 | 2 2 | 2 1 | 2 1 | 2 2 | 2 2 | 2 2 | 2 2 | 2 1 | 2 2 | 4 1 | 1 1 | 1 2 | 4 2 | 5 1 | 5 3 | 5 5 | 5 5 | | | | | | | | | | |
| D20S894 | 4 2 | 4 1 | 2 1 | 4 4 | 4 4 | 2 4 | 2 4 | 2 1 | 4 4 | 4 2 | 2 1 | 2 3 | 4 3 | 5 2 | 5 3 | 5 5 | 5 5 | | | | | | | | | | |
| D20S189 | 3 3 | 3 1 | 3 1 | 3 3 | 3 3 | 3 3 | 3 3 | 3 1 | 3 1 | 3 1 | 1 3 | 1 2 | 3 2 | 3 4 | 3 2 | 3 3 | 3 3 | | | | | | | | | | |
| D20S186 | 2 1 | 2 3 | 1 3 | 2 2 | 2 2 | 1 2 | 1 2 | 1 3 | 2 3 | 0 0 | 0 0 | 0 0 | 0 0 | 0 0 | 0 0 | 0 0 | 0 0 | | | | | | | | | | |

**Figure 1.** Pedigrees of the five families affected by Kindler syndrome, and construction of haplotypes. Genotypes for 23 microsatellite markers from telomere to centromere on chromosome 20p12.3 are shown for families A, B and C, and for five markers in the central part of the interval for families D and E. Affected individuals are indicated by blackened symbols, and the homozygous region that segregates with the disease phenotype is shaded.

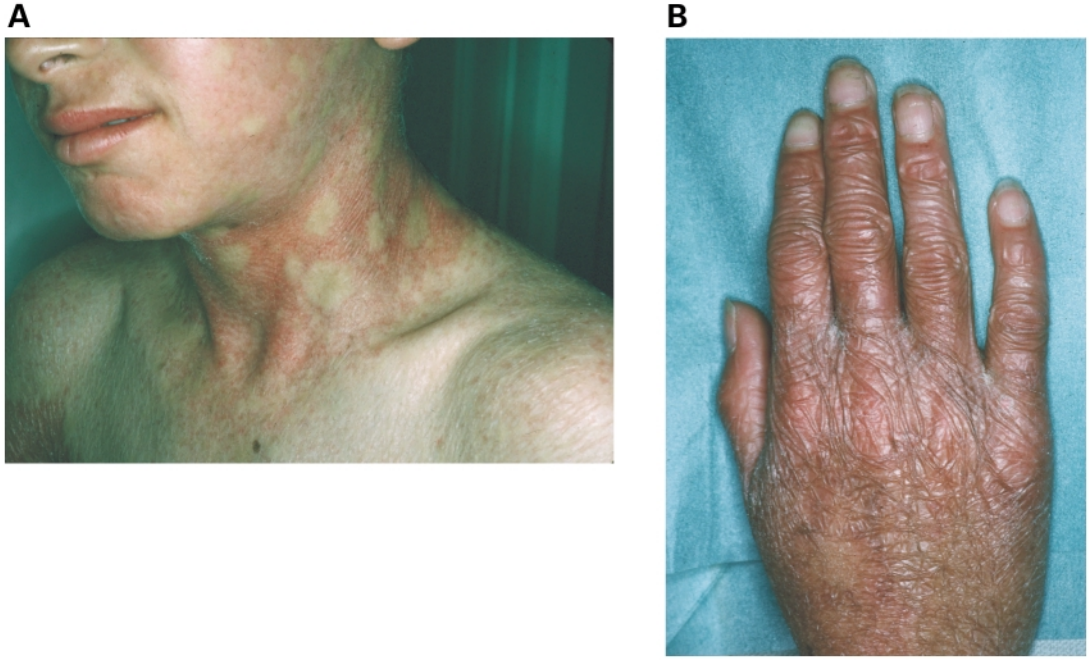



**Figure 2.** (**A**) Typical aspect of poikiloderma on the neck in a 13-year-old boy (E.2) from family E. Note the atrophic skin, telangiectases, reticulated and white atrophic macules. (**B**) Right hand of an 11-year-old girl (D.3) from family D showing atrophic, cigarette-paper-like skin.

differential cDNA library screening of serum-inducible genes in the human fibroblast cell line WI-38 (14) and is predicted to encode a protein with 680 or 720 amino acids. Another paralog (MGC10966, BC015584) on chromosome 11q13 with at least 13 exons codes for a protein with 663 amino acids.

### Kindlerin mutations

Mutation analysis of the 15 exons and of the exon-intron boundaries of kindlerin gene revealed four different homozygous mutations (Fig. 3) in four of the five families we studied. These include a splice-site mutation affecting the invariant T of the donor-splice-site GT dinucleotide in exon 3 ($385+2t>c$) in the Algerian family (A), a point mutation 787C>T (Q263X) in exon 6 in the Senegalese family (C), an insertion of 1 bp (1714insA) in exon 13 in a Tunisian family (D) and a deletion of 1 bp (464delA) in exon 4 in the Algerian family (E). The last three mutations would lead to premature stop codons. A homozygous Alu insertion in intron 14 was also observed in two families from Algeria (A) and Tunisia (D). Because we failed to detect a mutation in kindlerin in family B, we sequenced five exons of a 3′ end neighbouring gene coding for a putative leucine-rich protein, but no sequence variations were found.

### Expression analysis

Using the RAPID-SCAN$^{TM}$ gene expression panel, kindlerin was found to be expressed in 15 of the 24 human tissues analysed (Fig. 4) including skin; the strongest expression was found in colon, lung, placenta, adrenal gland and prostate, but no expression was detectable in adult liver, muscle, salivary gland, leukocytes, bone marrow, fetal brain and fetal liver. Strong expression of kindlerin transcripts was also observed in cultured keratinocytes from normal skin biopsies (Fig. 5).

### Sequence analysis of the kindlerin protein and identification of conserved residues

The longest kindlerin transcript codes for a protein of 677 amino acid residues with a calculated molecular weight of 77.4 kDa. The amino acid sequence lacks a signal peptide and a transmembrane domain, which suggests an intracellular localization of the protein. Using homology searches through protein databases (www.expasy.org/), several functional domains were identified: two regions which are homologous to band 4.1 domain between residues 242–343 and 515–570 (smart00295), of which one contains a FERM domain (residues 250–319, pfam00373), and a third region which contains a pleckstrin homology (PH) domain (residues 391–473, pfam00169; smart00233). An NPKY motif at amino acid positions 281–284 is also seen within the FERM domain, which is a known binding substrate for phosphotyrosine-binding (PTB) domain proteins.

Three of the four mutations we found are predicted to cause loss of the FERM and PH domains of the protein. The fourth mutation is localized in the PH domain and would lead to protein truncation with loss also of the second band 4.1 domain.

## DISCUSSION

The cells of multicellular organisms secrete and shape around them a network of extracellular matrix proteins, including the basement membrane (BM). In the skin, the BM is known as the

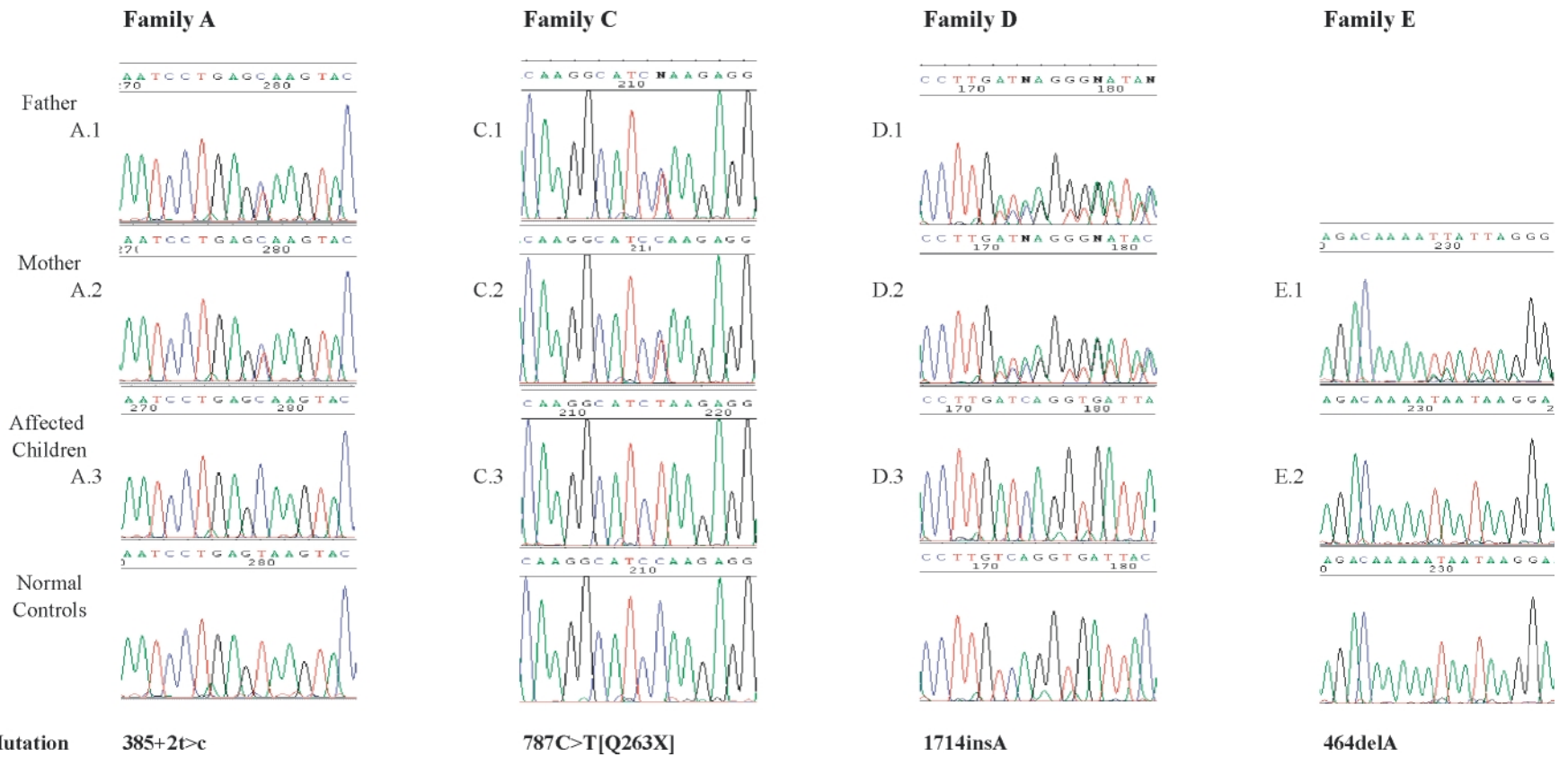



**Figure 3.** Mutations in kindlerin. Sequences are shown in four patients affected by KS, their parents and one normal control.

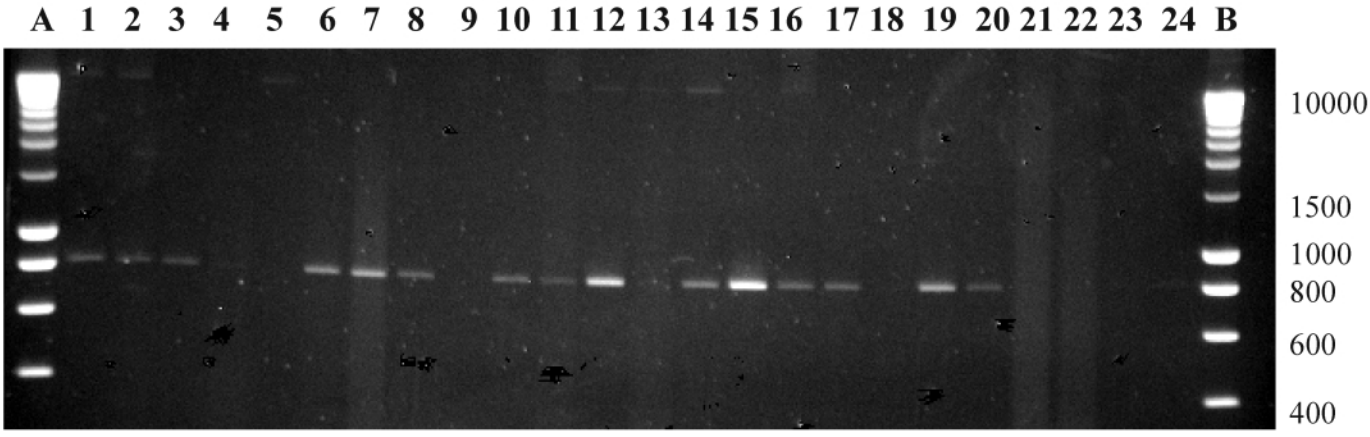



**Figure 4.** Expression of kindlerin transcripts by RT–PCR in 24 different tissues from the Rapid Scan Expression panel (OriGene Technologies). Lanes: 1, brain; 2, heart; 3, kidney; 4, spleen; 5, liver; 6, colon; 7, lung; 8, small intestine; 9, muscle; 10, stomach; 11, testis; 12, placenta; 13, salivary gland; 14, thyroid; 15, adrenal gland; 16, pancreas; 17, ovary; 18, uterus; 19, prostate; 20, skin; 21, peripheral blood lymphocytes; 22, bone marrow; 23, fetal brain; 24, fetal liver. Strong expression was found in colon (lane 6), lung (lane 7), placenta (lane 12), adrenal gland (lane 15) and prostate (lane 19); no expression was detectable in adult liver (lane 5), muscle (lane 9), salivary gland (lane 13), leukocytes, bone marrow, fetal brain and fetal liver (lanes 21–24). The molecular marker for quantification and fragment size determination (lane A and B) is Quanti-Ladder$^{TM}$ DNA.

dermo-epidermal junction (DEJ). It provides mechanical support, a barrier and a dynamic interface governing the overall structural integrity of the skin and the functions of keratinocytes such as cell polarity, proliferation, migration, and differentiation (15). Analyses of several inherited and acquired cutaneous disorders (15) and notably skin blisters (16) have helped in understanding the functions of some of the protein components of the DEJ. The identification of mutations in a new protein in KS adds another piece to the mosaic of the DEJ protein complex. Clinically, this rare disorder may be confused with epidermolysis bullosa in young patients. Partial spontaneous resolution of bullae, photosensitivity and later development of poikiloderma with cutaneous atrophy leads to correction of the initial diagnosis and to suspicion of KS which was the case for the families we studied. The distribution of lesions in KS including epidermis and mucosal membranes of the digestive and urinary tracts, are similar to those described in two subtypes of junctional epidermolysis bullosa associated with pyloric atresia (JEB-PA) which are due to mutations in α6 or β4 integrin genes (17,18). Some types of epidermolysis bullosa implicating structural proteins of the skin have autosomal-dominant inheritance, whereas others are recessive. In all five families that we studied, we found an autosomal-recessive mode of inheritance for KS.

Four different mutations, each of which corresponds to a different haplotype, were found, but we were unable to find a mutation in an Algerian patient from family B, although the phenotype was similar, and a homozygous interval of 2.6 Mb was present on chromosome 20p12.3. Since only one affected subject was found in this family, the analyzed markers could be homozygous in the patient by chance. We felt that the sequence of the kindlerin was complete because we confirmed its structure by 5′- and 3′-RACE and by RT–PCR. No heterozygous mutations or polymorphisms in the kindlerin gene were found in three non-affected members of this family and in the neighbouring gene coding for a putative leucine-rich protein with a fibronectin 3 domain. Complete sequencing of the gene including all introns and the promoter in this family is needed before suspecting genetic heterogeneity in KS.

The kindlerin gene codes for a protein with multiple binding sites (FERM and PH domains) for membrane proteins which may bind in turn other membrane proteins, proteins of the extracellular matrix or proteins of the cytoskeleton. The FERM domain is known to be involved in localizing proteins to the plasma membrane (19–21). This domain is conserved in members of the band 4.1 superfamily which includes cytoskeletal proteins such as erythrocyte band 4.1, talin, proteins of the ezrin–radixin–moesin family, as well as several tyrosine kinases and phosphatases and the tumor suppressor protein merlin (19,22). The PH domain was first identified as a module of ∼100–120 amino acids similar to the N- and C-terminal domains of pleckstrin (23–25). It is found in a wide range of proteins involved in intracellular signaling or as constituents of the cytoskeleton (26,27). PH domains interact directly with the cell membrane by binding to phosphoinositides with a broad range of specificity and affinity (28) and provide ‘a general attraction for the membrane surface that cooperates with a specific regulated interaction mediated by other regions of the host molecule’ (29).

Binding to integrin β subunits via a subdomain named F3 within the FERM domain of talin, an actin-binding protein, has recently been described and results in the activation of integrins (30,31). This F3 subdomain is found in several different structures including PTB and PH domains (31). It has been proposed that other proteins with a FERM domain and NPxY/F sequence motifs might also bind integrins (32,33). Another example of interactions has been described for KRIT1, a microtubule-associated protein (34,35), which also has a FERM domain and a NPKY motif. Mutations of KRIT1 are associated with hereditary cerebral cavernous malformations (CCMs; MIM 604214) (36,37), hyperkeratotic cutaneous capillary–venous malformations associated with cerebral capillary malformation (38) and abnormal basal lamina underlying the endothelial cells (39). KRIT1 binds ICAP-1 (integrin

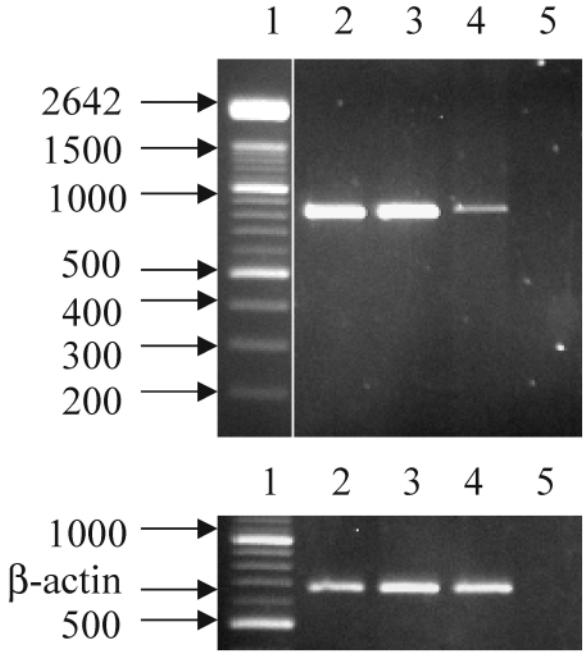



**Figure 5.** Expression analysis of the kindlerin mRNA by RT–PCR. (Top) RT–PCR of kindlerin in cultured keratinocytes and placenta using primers RT-2 amplifying a transcript of 796 bp: lane 1, molecular marker (XIV, Roche Molecular Biochemicals); lanes 2 and 3, mRNA from cultured keratinocytes; lane 4, mRNA from placenta, lane 5, negative control. (Bottom) RT–PCR with β-actin primers as control (661 bp). A stronger expression of kindlerin was observed in keratinocytes than in placenta.

cytoplasmic domain-associated protein) (34,40), which has itself a PTB/PI domain (smart00462) similar to those with pleckstrin homology (PH) and IRS-1-like PTB domains, and which is the binding partner of β1 integrin (40–42). One of the homologs of kindlerin, UNC112, contains three band 4.1 domains as well as a PH domain; it has been shown to colocalize with β-integrin in muscle cell membrane in *C. elegans* (12). Kindlerin could bind integrin β4 and other proteins of the hemidesmosome, which would explain some clinical similarities of KS with JEB-PA. Like the KRIT1 protein which might be involved in the bidirectional signaling between integrin molecules and the cytoskeleton and might affect cell adhesion processes via integrin signaling (34,43), kindlerin could have a similar function in the skin. Finally genetic heterogeneity is also observed in hereditary cerebral cavernous malformations (44). More studies are obviously needed, and in particular analyses of products of co-immunoprecipitation with antibodies against kindlerin or two-hybrid assays to define the binding partners of this protein, and analysis of more families to confirm the suspicion of genetic heterogeneity.

## MATERIALS AND METHODS

### Subjects and samples

Three dermatologists (B.B., F.C. and S.H.) recorded clinical data, pedigree information and performed skin biopsies for histological examination. Blood samples were collected from each participating family member after obtaining written informed consent. DNA extraction from peripheral blood leukocytes was performed using standard procedures.

### Genetic analysis

Genotyping with fluorescent markers was carried out as described previously (45). Three loci, for which the markers were homozygous in patients and heterozygous in the other family members were saturated with three supplementary markers (data not shown). Haplotypes were constructed assuming the most parsimonious linkage phase. Linkage programs were used on the assumption of autosomal recessive inheritance, full penetrance, and a disease frequency of 1/300 000 in the general population. Pairwise LOD scores were calculated with the MLINK program of the LINKAGE 5.1 package (46) incorporating consanguineous loops into the pedigree files. Multipoint analysis was performed with the Allegro 1.1 program (47). For linkage disequilibrium analysis, the excess of the disease-associated alleles was calculated as previously described (48).

### Mutation screening

Mutation analysis was performed in affected patients and in both parents in the five families, and in supplementary non-affected sibs in cases of missing parents. We designed intronic oligonucleotide primers flanking the exons and internal primers from the chromosome 20 genomic contig (NT_011387) for sequencing the kindlerin gene (Table 2) using the Primer3 program (http://intranet.cng.fr/primer3/primer3_www.cgi). Sets of PCR conditions were used as indicated in Table 2. The touch-down PCR reaction was performed in a 50 µl volume containing 50 ng of genomic DNA (in 5 µl) using standard procedures (45). After an initial denaturation step at 96°C for 5 min, *Taq* polymerase was added at 94°C (hot start) and nine cycles of amplification were performed consisting of 40 s at 94°C, 30 s at temperatures between 65–68°C (optimal annealing temperature plus 5°C, see Table 2) and a 30 s elongation step at 72°C, followed by 30 cycles of 40 s at 94°C, 30 s at optimal annealing temperature, 30 s at 72°C and one 10 min terminal elongation step. Purified PCR products, 1–1.5 µl were added to 1 µl of sense or antisense primer (10 µM) and 3 µl of BigDye terminator mix (Applied Biosystems). The linear amplification consisted of an initial 5 min denaturation step at 96°C, 25 cycles of 10 s of denaturation at 96°C and a 4 min annealing/extension step at 60–63°C. The reaction products were purified and sequenced on an Applied Biosystems Sequencer 3700. Both strands from all patients and controls were sequenced for the entire coding region and the exon/intron boundaries.

### Establishment of lymphoblastoid cell lines, keratinocyte cell culture and RNA extraction

Lymphoblastoid cell lines were established for the patients of families A, B and C, and at least one of the parents using standard procedures. Total RNA from lymphocytes was obtained with the RNA-PLUS (Quantum-Appligene) kit

**Table 2.** Primer sequences for the kindlerin gene

| Exon no. | Forward sequences | Reverse sequences | Temperature (°C) | Length (bp) |
|---|---|---|---|---|
| 1 | CTTCGCCAGCGCCCGCTC | GTTTATTTTGCGGCCAGATG | 62 | 293 |
| 2 | GGAACCTTGCAGAGTGTTTTG | GCTCTCCAGGGCATTACAAG | 60 | 417 |
| 3 | CTCCAGGACCCTCCAAGCT | CTTGAAGTAGGCAGAATGCAC | 60 | 382 |
| 4 | GGAGGTCTCTGTTCCCCTTT | GCCTTTCCTCATCACATCAG | 60 | 449 |
| 5 | CACAGTGCCCAGCTTGACT | CACAATCCCTAGGCCTACCA | 60 | 461 |
| 6 | GCCTCAGGTCAGTGCTCAG | GCTAAACAGGCGATCACACA | 62 | 416 |
| 7 | GCTGAAGTTTGCTGCATCTG | GCGTGTGTGGATTATGAGGA | 60 | 432 |
| 8 | ACGGGAAGGAGACCTCTGTT | TTGAAGCTTGTTAGGTGAAGAGC | 60 | 415 |
| 9 | CCTTACTCCCACACCCAGTT | GAACCATGAACCTGCCTCAG | 60 | 354 |
| 10 | CCAGCGCTTGGAAATTAGC | CTCCTGACCTCAGGTGATCC | 63 | 396 |
| 11 | GCAAATCTGAGGGGCTTGTA | TTACAGGTGTGAGCCACTGC | 60 | 408 |
| 12 | GGAAGGTGATGGTTTTGCAC | GCTGGTCTTGAACTCCCAAC | 60 | 493 |
| 13 | CTGGAGCTAACAGGGTGATC | AATGAGAAAACTGGGGCTCCC | 60 | 273 |
| 14 | GTCCATTCCTCTGAAATGATTCAG | GCATAGCAGTTACATCTCCAG | 60 | 622 |
| 15 | CCATCTGTTATCCACCTCAGC | CCAGCGGTGAATGTATGTTG | 60 | 409 |
| | *Internal sequencing* | | | |
| 8 | | GTCAATCAGAAGAAAGCCACAG | 60 | |
| 10 | GACACAAACATCTGATCTGATCTG | | 60 | |
| 11 | CAGATACACACCTGATGTTTATC | | 60 | |
| | *RT–PCR* | | | |
| RT-1 | CTGCGAGGCTGGACGCTA | GCGGTGAATGTATGTTGTCTGT | 60 | 2160 |
| RT-2 | TGAGATGTGACCATGAGAATCA | GCGGTGAATGTATGTTGTCTGT | 60 | 796 |
| RT-3 | CTACGGGCTCCTGGAAAG | CCAGCGGTGAATGTATGTTG | 60 | 2195 |
| RT-4 | CAAGACTGGTCAGACTTTGCTC | GCTTGCCTTGTTGGTGTGAG | 60 | 1914 |
| RT-5 | GAACAAAACTGCAGCATCCTC | CTTGCCTTGTTGGTGTGAGC | 60 | 1436 |
| RT-6 | ACGAGTACATTGGCGGCTAC | AGGCAGAGATGTTCTCAGCAG | 60 | 2031 |
| | *RACE* | | | |
| RACE-5′ | CTGAGAAGCTGACTCGCAACCTCAC | | 70 | |
| RACE-5′nest | CTGGACCCCATATTTGTCCAGGGTC | | 70 | |
| RACE-3′ | CAGCTCCTACCAGCCAGAGGTCC | | 70 | |

following the manufacturer's instructions. Human keratinocytes were obtained from a skin biopsy removed during routine plastic surgery of a normal individual. It was processed for primary keratinocyte culture and cells were grown according to the standard procedure described by Invitrogen Life Technologies using products from the same company in serum-free keratinocyte medium supplemented with bovine pituitary extract (25 µg/ml) and recombinant epidermal growth factor (0.1 ng/ml). Cultures were grown for two passages and harvested when they reached 90% confluence. Total RNA was isolated using the QIAamp RNA Mini Protocol for isolation of total RNA from cultured cells (QIAGEN) following the manufacturer's instructions. The mRNA was isolated following the Oligotex direct mRNA protocol as provided by the manufacturer (QIAGEN).

### 5′- and 3′-RACE (rapid amplification of cDNA ends)

5′- and 3′-RACEs were performed with Marathon-Ready cDNA from placenta (Clontech) using Advantage 2 PCR Enzyme Systems Polymerase (Clontech) following the manufacturer's instructions. A first PCR was run with AP1 primer and the gene-specific antisense or sense primers (GSP1) (Table 2) of the kindlerin cDNA for 5′- or 3′-RACE, respectively, with an initial denaturation step at 94°C for 1 min, five cycles at 94°C for 30 s and 72°C for 2 min, five cycles at 94°C (30 s) and 70°C (2 min), and 25 cycles at 94°C (30 s) and 68°C (2 min). PCR products were loaded on a 2% agarose gel. The purified 5′-RACE PCR products were used for a second nested PCR with AP2 and an internal antisense GSP2. Since the gene was not expressed in lymphoblastoid cell lines, we used cDNAs from placenta and keratinocytes to analyze the structure of the kindlerin gene.

### RT–PCR and Rapid-Scan™ gene expression panel

RT–PCR was performed using the RT–PCR kit (Life Technologies) with hexamer primers to generate the first strand of cDNA. Amplification of cDNA from placenta and keratinocytes was performed with primer pairs (Table 2) covering the entire coding region, the 3′UTR and the 5′UTR region.

The Rapid-Scan gene expression panel including 24 human tissues was tested following the manufacturer's instructions (OriGene Technologies) using the kindlerin mRNA-specific primer pairs (Table 2).

### GenBank accession numbers

Chromosome 20 genomic contig: NT_011387. CHGB: AL035461, BC000375, Y00064. KIAA1153 (LOC51605): AL035461, AB032979, BC001262. C20orf154: AJ439063, AK027644, BC008830. C20orf155 (LOC54675): AF241784, BC015333. Homo sapiens, similar to dJ1056H1.1 putative leucine rich protein: BC027720; Q8WUT4. C20orf75 (partial putative leucine-rich protein) in AL118505. Q8WUS3: BC019703.

Kindlerin: C20orf42-002 (Sanger Center curated known gene): NM_017671(NCBI RefSeq). FLJ20116: AK000123, AK000747. URP1: AF443278.

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