ORIGINAL ARTICLE

Molecular Basis of Kindler Syndrome in Italy: Novel and Recurrent Alu/Alu Recombination, Splice Site, Nonsense, and Frameshift Mutations in the *KIND1* Gene

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Kindler syndrome (KS) is a rare autosomal recessive disorder characterized by skin blistering in childhood followed by photosensitivity and progressive poikiloderma. Most cases of KS result from mutations in the *KIND1* gene encoding kindlin-1, a component of focal adhesions in keratinocytes. Here, we report novel and recurrent *KIND1* gene mutations in nine unrelated Italian KS individuals. A novel genomic deletion of approximately 3.9 kb was identified in four patients originating from the same Italian region. This mutation deletes exons 10 and 11 from the *KIND1* mRNA leading to a truncated kindlin-1. The deletion breakpoint was embedded in AluSx repeats, specifically in identical 30-bp sequences, suggesting Alu-mediated homologous recombination as the pathogenic mechanism. *KIND1* haplotype analysis demonstrated that patients with this large deletion were ancestrally related. Five additional mutations were disclosed, two of which were novel. To date, four recurrent mutations have been identified in Italian patients accounting for approximately ∼75% of KS alleles in this population. The abundance of repetitive elements in intronic regions of *KIND1*, together with the identification of a large deletion, suggests that genomic rearrangements could be responsible for a significant proportion of KS cases. This finding has implications for optimal *KIND1* mutational screening in KS individuals.

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INTRODUCTION

Although Kindler syndrome (KS, OMIM 173650) was first described over 50 years ago (Kindler, 1954), the underlying molecular pathology was only established in 2003 (Jobard *et al*., 2003; Siegel *et al*., 2003). The disorder results from loss-of-function mutations on both alleles of the *KIND1* gene that encodes the protein kindlin-1 (also known as kindlerin). The clinical characteristics of KS include congenital skin blistering and mild photosensitivity, which improve with age, and generalized progressive poikiloderma with extensive atrophy. Other features include webbing of the fingers, palmoplantar keratoderma, nail abnormalities, involvement of the oral cavity, and of esophageal and urethral mucosae. Squamous cell carcinoma of the skin and mucous membranes has been reported as a complication of KS (Ashton, 2004).

The *KIND1* gene, mapped to chromosome 20p12.3, spans 48.5 kb of genomic DNA and consists of 15 exons. However, the physiological functions of the gene product, kindlin-1, are not yet fully understood. Kindlin-1 exhibits N-terminal homology to filopodin, a *Dictyostelium* talin homologue (Siegel *et al*., 2003; White and McLean, 2005) and C-terminal homology to talin. It also possesses two regions of homology with the four-point one ezrin radixin moesin domain, which is shared by several other proteins involved in cytoskeleton attachment to the plasma membrane. The bipartite four-point one ezrin radixin moesin domain is interrupted by a pleckstrin homology domain. Pleckstrin homology domains mediate associations with phosphatidylinositol phospholipids in the plasma membrane and are a feature of cytoskeleton associated and/or cell-signalling molecules. *In vitro* studies demonstrated that in cultured epithelial cells kindlin-1 associates with vinculin and actin at focal adhesions (Siegel *et al*., 2003). Furthermore, kindlin-1 forms complexes with

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Abbreviations: KS, Kindler syndrome; RT, reverse transcriptase

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the cytoplasmic domains of integrin β subunits, and its expression is transforming growth factor-β dependent (Kloeker *et al.*, 2004). These observations implicate kindlin-1 in cell adhesion and other integrin-mediated cellular processes (Lanschuetzer *et al.*, 2003), but more studies on normal and mutated kindlin-1 are required to elucidate all functions and binding partners of this protein.

Thus far, approximately 20 different *KIND1* mutations have been reported in KS (Ashton, 2004; Fassihi *et al.*, 2005; Sethuraman *et al.*, 2005). These include nonsense, frameshift and splice site mutations, and evidence for some recurrent and founder mutant alleles has been published. Specifically, the mutations p.R271X and p.R288X have been described in individuals with KS from different ethnic backgrounds and with different *KIND1* haplotypes, whereas propagation of the mutations c.676_677insC, IVS7-1G>A, p.E304X, and p.W616X have been documented in the Pakistani, Italian, Caucasian European, and Omani populations, respectively (Ashton *et al.*, 2004; Has and Bruckner-Tuderman, 2004). In this study, we have investigated the gene pathology of KS in nine further unrelated Italian individuals and disclosed new and recurrent mutations in this population, including, for the first time, a large internal deletion resulting from Alu-mediated homologous recombination. These findings have implications for understanding the molecular basis of KS and the design of mutation detection strategies in the *KIND1* gene.

RESULTS

Clinical and skin biopsy features of Italian subjects with KS

Nine unrelated patients with KS from different regions of Italy were studied (Table 1). Consanguinity was reported in three families. Acral poikiloderma with skin atrophy and dyschromia (Figure 1a) were the main clinical features in adult patients, while trauma- or sun-induced bullous skin lesions were observed in the younger patients. The 6-year-old patient 4 had acral blistering present since birth and was initially diagnosed as having epidermolysis bullosa. Recently, however, pronounced skin atrophy on the dorsal aspects of her hands and feet and webbing of the fingers (Figure 1b) gave the clue to the actual diagnosis of KS. Except for patient 4, all showed some degree of oral involvement. Ectropion was present in five patients and digit webbing in six. In patient 3, esophageal dilatation was performed at the age of 23 years to release strictures. The 52-year-old patient 6 was treated for a squamous cell carcinoma of the lower lip. Ultrastructurally, reduplication of the lamina densa was detected in all patients examined (Figure 1c), while hemidesmosomes and anchoring fibrils appeared morphologically normal. Microdetachments within the lamina lucida were also observed in two patients (Figure 1d). Skin biopsies from patients 3–5, 7, and 8 were studied by immunofluorescence microscopy with antibodies to proteins involved in dermal–epidermal adhesion. In all cases, there was thickened and interrupted basement membrane labelling at the dermal–epidermal junction (Figure 1f and h). The abnormal staining pattern was prominent for collagens IV, VII (Figure 1f), and XVII (Figure 1h), as well as laminin 5, while linear staining for $\alpha6\beta4$ integrin was similar to control.

Characterization of a 3.9 kb deletion mutation in four unrelated families

Using the mutation detection strategy of sequencing PCR products amplified from genomic DNA (for details see Siegel *et al.*, 2003), no *KIND1* mutations were detected in patients 1–3 and their parents. However, DNA samples from these patients repeatedly failed to yield PCR amplification products for exons 10 and 11. Therefore, we assumed the presence of a genomic deletion spanning exons 10–11. In patient 4 and her mother, a heterozygous mutation c.910G>T (p.E304X), was disclosed, but the second, paternal mutation, could not be identified. No heterozygous single nucleotide polymorphisms were present in exons 10 and 11 and their flanking intronic regions. As a result of the homozygosity in this region, an identical single nucleotide polymorphism haplotype with patients 1–3 (see below) and the fact that families 1–3 came from Calabria, we also tested family 4 for the same putative deletion of exons 10 and 11.

To confirm the suspected deletion, quantitative real-time PCR was performed using two primer pairs for each of exon 10 or exon 11 (Table S1). Calculation of the relative copy number of *KIND1* gene exons 10 and 11 yielded values consistent with (i) the absence of these exons in both alleles of patient 3 (ratio = 0), (ii) the absence of the target exons in one allele of parents of patient 3, patient 4 and her father (ratio ≅ 0.5), and (iii) the presence of the target exons in both alleles of the mother of patient 4 (ratio ≅ 1) (Figure 2a). Results similar to those of family 3 were obtained for families 1 and 2. Additional quantitative PCR assays were performed with primer pairs positioned in the middle of intron 9 (qi9F and R) and in the middle of intron 11 (qi11F and R). The resulting amplification plots were consistent with homozygosity for the two *KIND1* target amplicons in all families and allowed us to circumscribe the deleted interval (not shown).

In order to identify the deletion breakpoints, primers qi9F and qi11R were then used to amplify across the deletion (Figure 2b, upper panel). A fragment of ~1.5 kb, instead of the ~5.5 kb wild-type PCR product, was obtained with template DNA from affected patients and healthy carrier family members, while it could not be amplified using the DNA of the mother of patient 4 and a control subject (Figure 2b, middle panel). Sequencing of the ~1.5 kb fragments resulted in the identification of the 5′ and 3′ breakpoints within introns 9 and 11 (g.70250_74168del with respect to GenBank AL118505, reverse complemented strand; Figure 2b, lower panel), and demonstrated that the four families carried the same deletion of 3,919 bp.

To establish an easy and rapid test for detecting this homozygous or heterozygous mutation, we developed a PCR reaction that makes use of three primers, two of them being positioned outside the deletion (primers a and c), and the third one placed within the deletion (primer b) (Figure 3a, upper panel). In the presence of a normal allele, an 835-bp fragment is generated by using primers b and c, whereas in the presence of the deleted allele a 362-bp product is amplified with primers a and c. As shown in Figure 3a (lower panel), this diagnostic PCR generated a single band of 362 bp in patients 1–3, whereas two bands of 362 and 835 bp were

Table 1. Mutations and major clinical features of Italian individuals with Kindler syndrome

Patient	Age/sex	Consan-guinity	Geographic origin	Mutations[1]	Atrophy of the dorsum of hand and feet/webbing	Poikilo-derma	Photo-sensitivity	Trauma- and sun-induced blistering	Tumor development
1	41/F	No	Southern Italy (Calabria)	g.70250_74168del/g.70250_74168del	+/–	+	+	–	–
2	16/F	Yes	Southern Italy (Calabria)	g.70250_74168del/g.70250_74168del	+/–	+	+	+	–
3	25/F	Yes	Southern Italy (Calabria)	g.70250_74168del/g.70250_74168del	+/+	+	+/–	–	–
4	6/F	No	Father from Southern Italy (Calabria)	g.70250_74168del/c.910G > T	+/+	–	+	+	–
5	18/F	Yes	Northern Italy (Emilia Romagna)	IVS13-1G > A/IVS13-1G > A	+/–	+	+	+	–
6	52/F	No	Sardinia	c.95_96delGA/c.95_96delGA	+/+	+	+	–	+
7	13/M	No	Central Italy (Tuscany)	c.1161delA/c.1161delA	+/+	+	+	+	–
8	23/M	No	Central Italy (Marche)	c.1161delA/c.1161delA	+/+	+	+	–	–
9	42/M	No	Central Italy (Lazio)	c.373delT/c.373delT	+/+	+	+	–	–
Previously reported Italian KS individuals									
10	NA	NA	NA	c.373delT/c.373delT[2]	NA	NA	NA	NA	NA
11	48/M	Yes	Central Italy (Lazio)	IVS7-1G > A/IVS7-1G > A[3]	+/+	+	+	–	+
12[4]	36/M	No	Central Italy (Lazio)	IVS7-1G > A/IVS7-1G > A[3]	+/–	+	+	–	–
13[4]	38/M	No	Central Italy (Lazio)	IVS7-1G > A/IVS7-1G > A[3]	+/+	+	+	–	–
14[5]	21/M	Yes	North Africa	c.1714_1715insA/c.1714_1715insA[3,5]	+/+	+	+/–	+	–

F, females; M, males; NA, data not available.
[1]Mutations are indicated on the basis of the nucleotide change in the cDNA (GenBank AY137240) or genomic (GenBank AL118505, reverse complemented strand) sequence of *KIND1*.
[2]Siegel *et al*. (2003).
[3]Ashton *et al*. (2004).
[4]These patients are siblings.
[5]Mutation observed in this patient was also detected in a North African Arab reported in Jobard *et al*., 2003.

observed in patient 4 and several healthy carriers belonging to the patients' families. Finally, a solitary band of 835 bp was observed in healthy controls, indicating absence of the deletion. Using this assay, the deletion mutation was not detected in any allele of 102 control Italian individuals.

The consequences of the genomic deletion on *KIND1* expression were also investigated by reverse transcriptase (RT)-PCR analysis of the mRNA purified from cultured keratinocytes of patient 1 (homozygous for the g.70250_74168del mutation), using primers spanning exons 5–13. With the patient's sample, a single band of 796 bp was generated, whereas a wild-type fragment of 1,028 bp was evident in a control sample (Figure 3b). Sequencing of the 796-bp cDNA fragment showed the deletion of exons 10 and 11 which introduces a frameshift starting with codon 381 and terminating with a premature termination of translation 36 codons downstream. This predicts elimination of the entire C-terminal four-point one ezrin radixin moesin domain of kindlin-1 (Figure 3b).

Haplotype analysis of intragenic single nucleotide polymorphisms (for details see Ashton *et al*., 2004) showed that the 3.9 kb deletion occurred on the same genetic background in all patients. We extended the analysis of the haplotype found to be associated with the recurrent mutation p.E304X (Ashton *et al*., 2004) by analyzing patient 4 and her family and an additional European patient with the homozygous mutation p.E304X (Has and Bruckner-Tuderman, 2004); they also had the same ancestral *KIND1* allele (Table S2).

Novel and recurrent point mutations in *KIND1*

Sequencing of all *KIND1* exons led to the identification of a homozygous mutation in five other individuals (Table 1). Two of these mutations (IVS13-1G > A and c.95_96delGA (p.R32fsX63)) were novel and not found in 102 control Italian individuals. Mutations IVS13-1G > A led to aberrant splicing of exon 14, as demonstrated by RT-PCR analysis of total RNA purified from cultured keratinocytes of patient 5 (not shown). In patients 7 and 8, the same homozygous single nucleotide deletion, c.1161delA (p.A388fsX14), was identified within exon 10. This mutation has been previously documented in a Caucasian patient from the UK (Ashton *et al*., 2004). Haplotype analysis of patients 7 and 8 showed that the mutation c.1161delA is associated with a common genetic background (not shown). In patient 9, a homozygous deletion mutation, c.373delT (p.C125fsX4), was disclosed. This mutation has been previously described in an Italian patient (Siegel *et al*., 2003).

DISCUSSION

This report has identified the molecular basis of KS in nine Italian individuals with KS and, taken together with other published reports, *KIND1* gene mutations have now been identified in 14 subjects from 13 Italian families (Siegel *et al*., 2003; Ashton *et al*., 2004). Interestingly, in a number of families with KS reported in the literature, mutations in *KIND1* could not be disclosed, in spite of homozygosity by descent at the *KIND1* locus (Jobard *et al*., 2003; Siegel *et al*.,

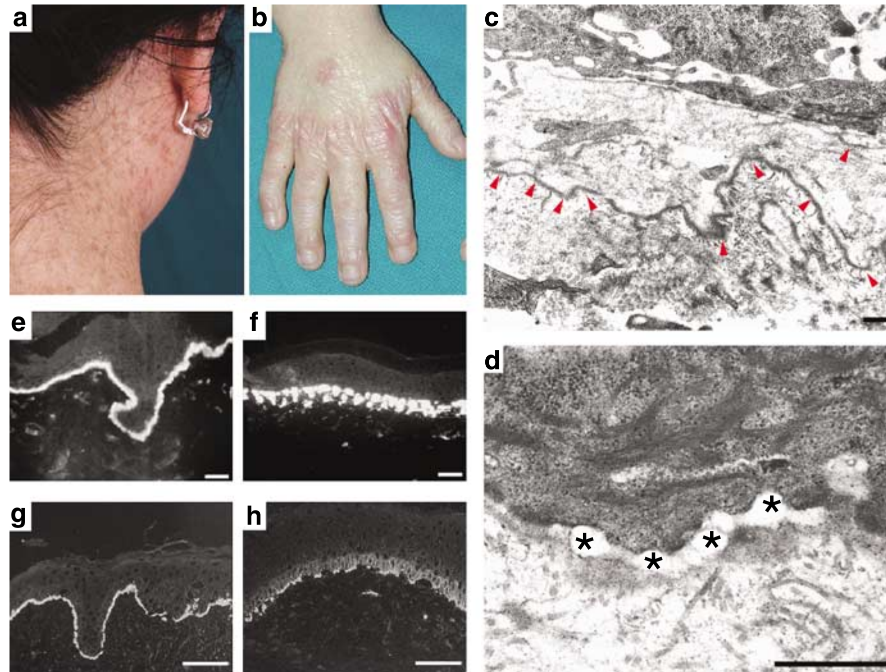

Figure 1. Clinical and skin biopsy findings in KS. (**a**) In patient 3, poikiloderma is evident on the neck and non-sun exposed skin behind the ear. (**b**) Cigarette paper-like atrophy is present on the dorsal aspect of the right hand of patient 4. (**c**) Ultrastructural analysis of KS skin showing reduplication of the lamina densa (red arrows) in patient 7, and (**d**) microdetachments (asterisks) within the lamina lucida in patient 8. (**e**) Linear labelling at the dermal epidermal junction for collagen VII in normal skin. (**f**) Markedly thickened, reticular, and patchy pattern of collagen VII labelling in patient 7. (**g**) Indirect immunofluorescence with antibodies to collagen XVII produced a linear staining in normal skin, but (**h**) an interrupted pattern in the skin of patient 4. Bars = (**c** and **d**) 0.85 μm and (**e–h**) 20 μm.

2003). This suggested that the commonly used mutation-detection strategies based on screening or sequencing of exons and intron–exon boundaries in genomic DNA fail to detect a certain number and type of mutations. Indeed, using established protocols we found mutations in only six of nine patients in this study.

Quantitative PCR and long-range PCR techniques were employed to search for larger rearrangements in genomic DNA of four patients. These techniques led to the identification of the first large deletion mutation in the *KIND1* gene. Quantitative PCR was shown to be a useful method to detect the deletion in the heterozygous state and to narrow down the deleted interval. In addition, a straightforward and accurate genomic DNA-based PCR test for routine diagnostic detection of the deletion was developed. RT-PCR and sequence analysis of the resulting cDNA fragment confirmed that the mutant g.70250_74168del mRNA variant contains an aberrant splicing junction between exons 9 and 12 and terminates translation 116 nucleotides upstream of splicing-generated exons 12–13 junction. Notably, nonsense-mediated mRNA decay in mammals downregulates spliced mRNA that harbors premature termination codon followed by an exon–exon junction located more than 50–55 nucleotides downstream (Inoue *et al*., 2004; Maquat, 2005). Therefore, it is expected that kindlin-1 nonsense-mediated mRNA decay occurs in cells bearing the g.70250_74168del mutation. The visualization of a detectable amount of aberrant mRNA by RT-PCR may be explained by the ability of this technique to amplify unstable mRNA, even if strongly reduced and almost undetectable by Northern blot analysis (Aberdam *et al*., 1994; Baudoin *et al*., 1994). Although the synthesis of a residual amount of polypeptide from mutant mRNA cannot be formally excluded, this abnormal protein is predicted to be nonfunctional. Indeed, the deletion of 232 nucleotides corresponding to exons 10 and 11 results in the elimination of 77 amino acids within the pleckstrin homology domain of kindlin-1 and generates a frameshift leading to truncation of the entire C-terminal four-point one ezrin radixin moesin domain. Both these domains are believed to be critical for the function of kindlin-1.

Computational analysis of the *KIND1* gene was performed to investigate the mechanism underlying this large deletion. NCBI Map Viewer inspection revealed that introns 9 and 11 contain 15 and five repetitive sequence elements, respectively, accounting for 48 and 59% of the intronic length. These sequences comprise several members of distinct subfamilies of Alu repeats as well as other interspersed repetitive elements (Figure 4a) (Batzer and Deininger, 2002). Mutation g.70250_74168del in *KIND1* is likely to be the result of homologous unequal recombination as the sequences spanning the junction region show high homology (83%) with Alu-repetitive elements. In particular, a recombination event in this deletion appears to have occurred between an almost uninterrupted 5′ AluSx repeat located within intron 9 and another AluSx repeat within intron 11 near the 3′ breakpoint (almost completely deleted as a

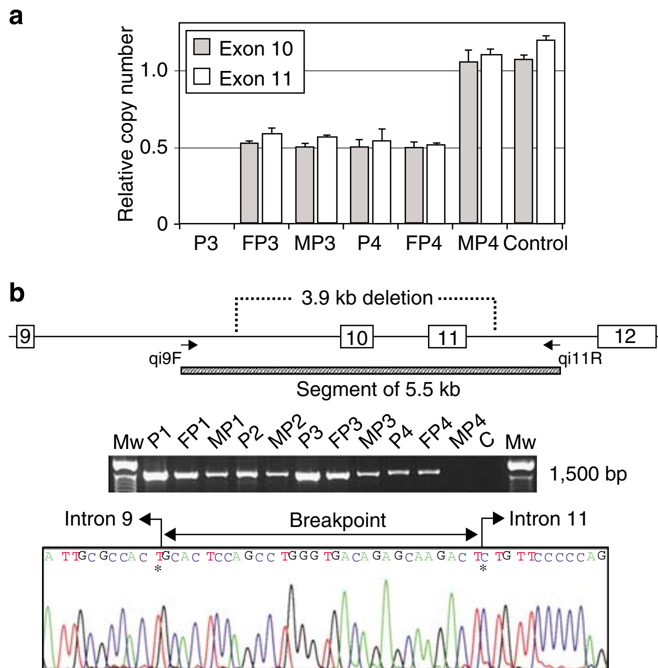

Figure 2. Characterization of the 3.9 kb deletion (g.70250_74168del) in *KIND1*. (**a**) Relative copy number of *KIND1* gene exons 10 and 11 in the genomic DNA of patients 3 and 4 (P3, P4) and their parents (FP3, MP3, FP4, and MP4) measured by quantitative real-time PCR. (**b**, upper panel) Schematic representation of the *KIND1* genomic region spanning exons 9–12. The 3.9 kb deletion mutation is depicted by the dashed line. Primers qi9F and qi11R were used to amplify a 5.5 kb region (hashed bar). (**b**, middle panel) A PCR product of ~1.5 kb instead of the expected ~5.5 kb amplicon was obtained with genomic DNA from patients 1 to 4 (P1–P4) and patients' parents (MP1, MP2, MP3, FP1, FP3, and FP4). Mw, 100 bp DNA ladder. (**b**, lower panel) Sequencing of the 1.5 kb-PCR products showed identical deletion breakpoints within introns 9 and 11. The precise breakpoint lies somewhere in the 30 bp between the 5′ variant base in intron 9 and the 3′ variant base in intron 11 (asterisks).

result of the recombination). The precise site of the recombination event has been narrowed to a 30-bp sequence, (gcactccagcctgggtgacagagcaagact), which shows 100% homology in the two AluSx-repeat regions involved (Figure 4b) (Deininger and Batzer, 1999; Ringpfeil *et al.*, 2001).

We have calculated that 52% of all noncoding regions of *KIND1* are represented by repetitive sequences, and almost half of these repeats are Alu elements (24% of all intronic sequences). Of note, the two AluSx elements involved in the deletion presented here harbor two common 26-bp core sequences, one of which is present near the crossover site. This 26-bp core sequence has been proposed to be highly recombinogenic because it lies frequently at, or close to, the sites of recombination and also in view of its structural similarity with recombinational hotspot sequence present in *E. coli* (Figure 4b) (Rüdiger *et al.*, 1995; Le Saux *et al.*, 2001). Eleven out of 16 AluSx repeats dispersed along the intronic sequences of *KIND1* contain such 26-bp core sequences that are more than 95% conserved. Our findings support the hypothesis that DNA rearrangements might account for a significant number of the KS cases in whom pathogenic mutations could not be detected using previously published genomic PCR amplification of *KIND1* exons (Jobard *et al.*, 2003; Siegel *et al.*, 2003) and suggest that screening for *KIND1* rearrangements should be performed in these patients.

To date, four recurrent *KIND1* mutations, comprising g.70250_74168del (23.8% of the mutated alleles) c.1161delA (19.0%) IVS7-1G>A (14.3%), and c.373delT (19.0%) have been identified in Italian patients with KS (Table 1). Each of these has been detected in families from similar parts of Italy, and haplotype analysis of highly polymorphic single nucleotide polymorphisms within the coding region of *KIND1* in the patients carrying these mutations indicates a common ancestral origin for the corresponding mutant allele. In the case of extremely rare autosomal recessive genetic diseases, such as KS, most alleles from patients originating from a restricted geographical area will be identical-by-descent due to genetic drift and hidden inbreeding. Indeed, Italian patients with KS are all, except one, homozygous for the causative mutation. Homozygosity for more than one recurrent mutation is the result of distinct founder effects following the occurrence or the introduction of different *KIND1* mutations in various regions of Italy. Altogether, the frequent mutations account for ~75% of the KS alleles in the Italian population and therefore, for optimal mutation detection, new KS patients should first be screened for the presence of these mutations. Interestingly, the mutation c.1161delA have been also detected in a British patient and the p.E304X mutation has been identified in a Swiss, an Austrian, and three British patients (Lanschuetzer *et al.*, 2003; Ashton *et al.*, 2004; Has and Bruckner-Tuderman, 2004), suggesting propagation of ancestral mutant alleles in the Caucasian European population due to both genetic drift and geographic migration of ancient populations across the Alps.

Assessment of skin biopsies by immunofluorescence microscopy and transmission electron microscopy of the dermal–epidermal junction can give good clues to the diagnosis of KS (Fassihi *et al.*, 2005), but optimization of mutation detection strategies requires knowledge of the molecular basis of KS within particular countries or ethnic groups. As such, the findings in our study help refine mutation detection strategies for patients with this genodermatosis.

MATERIALS AND METHODS

Patients and biological materials

Nine Italian unrelated KS patients were studied (Table 1). The diagnosis was based on physical examination and clinical history. Skin biopsies were obtained from six patients (subjects 1, 3–5, 7, 8) and processed for ultrastructural examination and/or immunofluorescence analysis with antibodies to basement membrane zone (Hammami-Hauasli *et al.*, 1998; Terracina *et al.*, 1998; Castiglia *et al.*, 2001). In three patients, a skin biopsy specimen was also processed for keratinocyte culture. Keratinocytes were cultivated on a feeder layer of lethally irradiated 3T3-J2 murine fibroblasts (a gift from H. Green, Harvard Medical School,

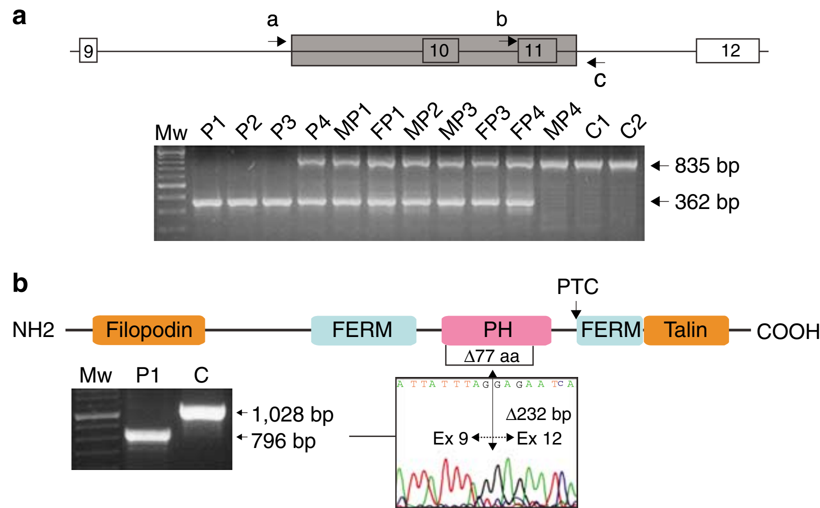

Figure 3. Verification of the g.70250_74168del mutation in the affected families and mutation consequences at mRNA level. (**a**, upper panel) PCR-based diagnostic test for rapid screening of the deletion g.70250_74168del using primers a–c. Shaded region represents the 3.9 kb deletion. (**a**, lower panel) A single band of 362 bp was visualized in patients 1–3 (P1–P3), whereas two bands of 362 and 835 bp were observed in patient 4 (P4) and in healthy carriers from these families (MP1, FP1, MP2, MP3, FP3, and FP4). The mother of patient 4 (MP4) and two unaffected controls (C1 and C2) generated a 835 bp band (lanes 11–13). Mw, 100 bp DNA ladder. (**b**) RT-PCR analysis of total RNA from patient 1 (P1) resulted in a unique PCR product of 796 bp instead of the normal product of 1,028 bp amplified from control cells (**C**). The 796-bp fragment corresponded to an aberrant out-of-frame transcript carrying the deletion of exon 10 and 11 (232 bp). Mw, 100 bp DNA ladder. A schematic representation of kindlin-1 structure with mutation consequences at protein level is also shown. PTC, premature termination codon. FERM, four-point one ezrin radixin moesin domain; PH, pleckstrin homology domain.

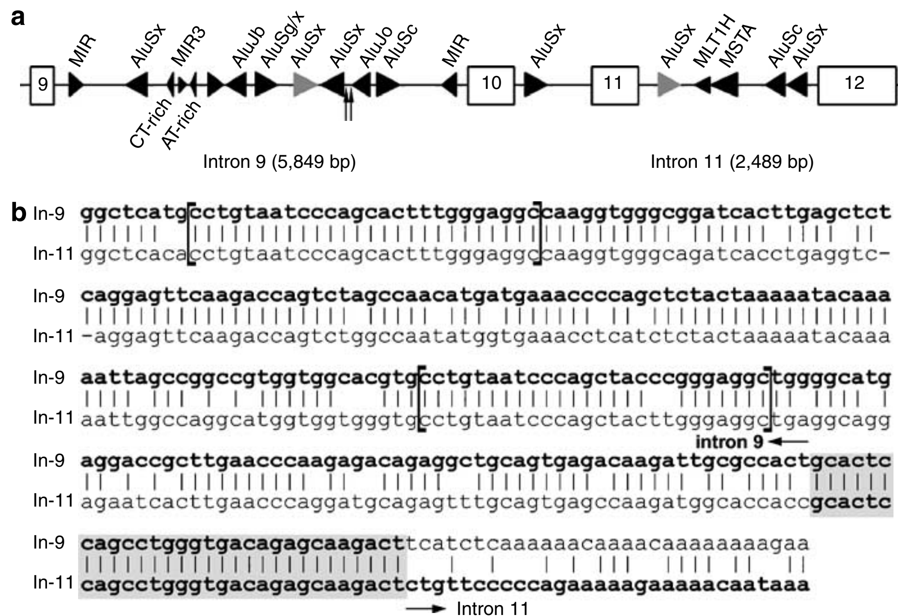

Figure 4. Abundance of repetitive elements within introns 9 and 11 of *KIND1*. (**a**) Schematic representation of the genomic region spanning exons 9–12. Orientation and type of the repetitive sequence elements comprised in this region are indicated by arrowheads, the size of which is proportional to the length of the corresponding repeat. The two AluSx sequences involved in the 3.9 kb deletion are indicated by gray arrowheads. (**b**) Sequence alignment between the AluSx repeats containing the deletion breakpoints. The sequences show 83% identity. The 30-bp sequence which identifies the precise site of the crossover event is highlighted by the gray box and the two 26-bp core sequences are depicted between the square brackets. The boldface type represents the sequence of the *KIND1* mutated allele that results from the recombination event.

Boston, MA), as described (Zambruno *et al.*, 1995). This study was conducted in accordance to the Declaration of Helsinki Principles; skin biopsies and blood samples were obtained from all patients and their parents after written informed consent and approval by the ethical committees of IDI-IRCCS, St Thomas' Hospital London, and of the University of Freiburg. Genomic DNA was extracted from blood using a standard kit (Qiagen, Hilden, Germany).

Mutation detection strategy

PCR amplification of the *KIND1* gene was performed on genomic DNA (100 ng) as template and using intronic primers specific for each *KIND1* exon, as detailed elsewhere (Jobard *et al.*, 2003; Ashton *et al.*, 2004). PCR products were directly sequenced in both orientations in an ABI 3100 genetic analyzer (ABI, Darmstadt, Germany). Each mutation was confirmed by re-sequencing the PCR product obtained from a second amplification reaction.

Quantitative real-time PCR

Detection of the large genomic deletion was carried out with quantitative PCR and SYBR-Green I detection method, as described (Boehm *et al.*, 2004; Borozdin *et al.*, 2004). Primers for generation of small amplicons corresponding to exons 10 and 11 were designed with the software http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi. Amplicons corresponding to exon 4 (hsall 4F/R) of the *SALL4* gene were used as an internal reference. For real-time detection, the ABI Prism 7900 system and 384-well plates (both from PE Applied Biosystems, Norwalk, CT) were used. Reactions contained 0.25 mM of each primer and 5 μl of QuantiTect SYBR® Green PCR Master Mix (Qiagen, Hilden, Germany) in a final volume of 10 μl. Each run included samples of serially diluted control DNA (40, 20, 10, and 5 ng) for the generation of standard curves for each primer pair, and genomic DNA samples from patients, parents and controls (25 ng). The PCR program consisted of 50°C for 2 minutes, 95°C for 15 minutes, and 40 cycles of 94°C for 15 seconds, 54°C/60°C for 15 seconds, and 72°C for 1 minute. To verify the specificity of the primers, melting curve analysis of the amplicons was performed and the results were evaluated as previously described (Borozdin *et al.*, 2004). Sequence of primers and amplification conditions used for quantitative real-time PCR are given in Table S1.

Cloning and sequencing of the junction fragments

The forward primer qi9F and the reverse primer qi11R (Table S1) were used for amplification of the genomic region spanning exons 10 and 11. The PCR reaction contained 0.9 μg of genomic DNA, $1 \times$ PCR buffer, 2.5 mM $MgCl_2$, 250 μM of each deoxynucleoside triphosphate, 2.5 μM of each primer, and 2.5 U of TaKaRa LA *Taq* enzyme (TAKARA Bio Inc., Otsu, Japan) in a final volume of 50 μl. An initial denaturation step at 94°C for 3 minutes was followed by 30 cycles of 94°C for 15 seconds, 60°C for 30 seconds, and 72°C for 7 minutes, and a final elongation step at 72°C for 10 minutes. The PCR product was subcloned with the TOPO cloning kit for sequencing (Invitrogen, San Diego, CA). Plasmid DNA was isolated from bacterial colonies by routine methods and sequenced using T7, T3 primers and the primer seqKSIntron9: 5′-cagtctagccaacatgatga-3′. The *KIND1* sequence (GenBank accession number NT_011387) surrounding the breakpoint was masked for repeats with the online software BCM Search Launcher (http://searchlauncher.bcm.tmc.edu/seq-search/alignment.html) and AluSx elements involved in the deletion were aligned.

PCR-based detection of the 3.9-kb deletion in genomic DNA

After the junction fragment had been sequenced and the deletion breakpoints precisely identified, primers a–c (Table S1) were used for detection of the deletion mutation in KS families. These three primers were used in a single PCR reaction at the following concentration: primer a, 0.25 μM, primer b, 1.5 μM, and primer c, 2 μM (annealing temperature 60°C), in a volume of 50 μl. Standard cycling conditions were used for amplification.

RT-PCR analysis

Total RNA was purified from cultured cells using the NucleoSpin® RNA II kit (Mackerey-Nagel, Düren, Germany) and converted to cDNA using the SuperScript™ Rnase H free reverse transcriptase (Invitrogen) and an oligo dT as primer. PCR amplifications of the *KIND1* cDNA was carried out with primers RT12S and RT15AS (nucleotides 1,494–2,153) spanning exons 12–15 in patient 5, and using primers RT5S and RT13AS (nucleotides 637–1,664) spanning exons 5–13 in patient 1 (GenBank accession number NM_017671) (Table S1). PCR products were analyzed on 1.5% agarose gel electrophoresis and specific bands were excised from the gel, purified, and directly sequenced.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Table S1. Primers used in this study.

Table S2. *KIND1* alleles associated with the deletion g.70250_74168del and with p.E304X.

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