

## SCIENTIFIC REPORT

# A novel arginine substitution mutation in 1A domain and a novel 27 bp insertion mutation in 2B domain of keratin 12 gene associated with Meesmann's corneal dystrophy

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**Aim:** To determine the disease causing gene defects in two patients with Meesmann's corneal dystrophy.

**Methods:** Mutational analysis of domains 1A and 2B of the keratin 3 (K3) and keratin 12 (K12) genes from two patients with Meesmann's corneal dystrophy was performed by polymerase chain reaction amplification and direct sequencing.

**Results:** Novel mutations of the K12 gene were identified in both patients. In one patient a heterozygous point mutation (429A→C=Arg135Ser) was found in the 1A domain of the K12 gene. This mutation was confirmed by restriction digestion. In the second patient a heterozygous 27 bp duplication was found inserted in the 2B domain at nucleotide position 1222 (1222ins27) of the K12 gene. This mutation was confirmed by gel electrophoresis. The mutations were not present in unaffected controls.

**Conclusion:** Novel K12 mutations were linked to Meesmann's corneal dystrophy in two different patients. A missense mutation replacing a highly conserved arginine residue in the beginning of the helix initiation motif was found in one patient, and an insertion mutation, consisting of a duplication of 27 nucleotides, was found before the helix termination motif in the other.

Meesmann's corneal dystrophy is a disease of impaired keratin function<sup>1</sup> inherited in an autosomal dominant pattern. This disease presents early in life with numerous minute intraepithelial cysts seen best on retroillumination.<sup>2–3</sup> Histologically, the epithelium appears thickened and irregular with cysts that react with periodic acid Schiff stain.<sup>3</sup>

Keratin, like vimentin and desmin, is an intermediate filament (10 nm in diameter).<sup>4</sup> However, unlike vimentin and desmin, keratins are heteropolymers, containing one member from the type I subfamily of keratins (K9–K20) and one member from the type II subfamily (K1–K8).<sup>5–6</sup> Keratin intermediate filaments are expressed solely in epithelial cells and different types of epithelia will express unique keratins each consisting of a specific type I keratin paired with a specific type II keratin.<sup>7</sup>

Structural analysis of intermediate filaments reveals widespread alpha helical content.<sup>8</sup> More specifically, the structure consists of a conserved central alpha helical domain with variable amino terminal and carboxy terminal extensions.<sup>9–10</sup> The central domain contains several short non-helical linker regions which interrupt the alpha helix. The helix is a "coiled coil" structure with every third and seventh residue exhibiting hydrophobic interactions.<sup>11</sup>

A mutation causing an interruption in the alpha helix structure of intermediate filaments may compromise protein

function. In particular, the ends of the keratin alpha helix, termed the helix initiation motif and the helix termination motif, are crucial in establishing proper filament formation.<sup>12–13</sup> The helix initiation motif lies in the beginning of the first alpha helix region termed 1A. The helix termination motif is located at the end of the fourth alpha helix region termed 2B. These domains are separated from the other helix domains (1B and 2A) by non-helical linker regions termed L1, L12, and L2.<sup>14</sup> A pathogenic mutation in the highly conserved 1A and 2B helix boundary motif regions may exert dominant negative effects, specific to the epithelia in which the keratin is expressed.

A wide range of keratin based diseases stem from genetic mutations of the keratin gene including epidermolytic hyperkeratosis, epidermolysis bullosa simplex, ichthyosis bullosa of Siemens, epidermolytic palmoplantar keratoderma, pachyonychia congenita, and white sponge naevus.<sup>4</sup> Most of these diseases are caused by mutations located within the helix boundary motif regions. In Meesmann's corneal dystrophy, the stratified non-cornified epithelium of the anterior cornea express keratin intermediate filaments consisting predominantly of the type II keratin 3 (K3) and the type I keratin 12 (K12).<sup>7–15</sup> Genetic analysis of families with Meesmann's corneal dystrophy has revealed mutations in the helix boundary motif regions of K3 and K12 (table 1).<sup>1–16–21</sup>

In this study we present clinical and genetic data from two patients with novel mutations to the K12 gene associated with Meesmann's corneal dystrophy. One K12 mutation represents a novel missense mutation within the helix initiation motif of the gene, while the other mutation represents a 27 bp duplication insertion lying just outside of the helix termination motif of the K12 gene, a type of mutation not previously described in Meesmann's corneal dystrophy.

## SUBJECTS AND METHODS

### Subjects

This study was approved by the institutional review board at the University of California, San Francisco. After obtaining informed consent, members from two consecutive generations of two families with Meesmann's corneal dystrophy were enrolled. Family A was an American family of Japanese descent. Family B was an American family of European descent. Fifty unrelated volunteers were recruited to serve as controls. All study subjects were interviewed at UCSF, where slit lamp examination and collection of buccal mucosal swabs was performed. Genetic analysis of all samples was performed at the Francis I Proctor Foundation at UCSF. Medical records were reviewed for all affected family members.

**Table 1** Review of known mutations in Meesmann's corneal dystrophy

<b>Keratin 3</b>			
Exon 7	1525G→A	E509K	Irvine <i>et al</i> <sup>f</sup>
<b>Keratin 12</b>			
Exon 1	410T→C	M129T	Corden <i>et al</i> <sup>9</sup>
Exon 1	413A→C	Q130P	Corden <i>et al</i> <sup>8</sup>
Exon 1	423T→G	N133K	Irvine <i>et al</i> <sup>20</sup>
Exon 1	427A→G	R135G	Nishida <i>et al</i> <sup>6</sup>
Exon 1	428G→T	R135I	Nishida <i>et al</i> <sup>6</sup>
Exon 1	428G→C	R135T	Irvine <i>et al</i> , <sup>1</sup> Corden <i>et al</i> <sup>9</sup>
Exon 1	429A→C	R135S	Current report
Exon 1	433G→C	A137P	Takahashi <i>et al</i> <sup>21</sup>
Exon 1	443T→G	L140R	Nishida <i>et al</i> <sup>6</sup>
Exon 1	451G→C	V143L	Irvine <i>et al</i> <sup>f</sup>
Exon 6	1222ins27	400ins9	Current report
Exon 6	1300A→G	I426V	Coleman <i>et al</i> <sup>7</sup>
Exon 6	4064T→G	Y429D	Nishida <i>et al</i> <sup>6</sup>

Author numbered from K12 DNA (Genbank accession number AF137286). Numbering from K12 mRNA (Genbank accession number D78367) would be 1309T→G.

### DNA preparation

DNA was obtained by scraping buccal mucosal epithelium of affected patients and controls using a CytoSoft brush CP-5B (Medical Packaging Corporation, Camarillo, CA, USA), and purified using the QIAamp DNA Mini Kit spin protocol (Qiagen, Valencia, CA, USA).

### Mutation detection

Domains 1A and 2B of both the K3 and K12 genes were amplified by the polymerase chain reaction (PCR). The 1A domain in exon 1 of K3 was amplified using the following primers: FK3.1 (5' CTA TGG AGG TGG CTT TGG TGG T 3'), and RK3.1 (5' ACA GGA TGT GTG GTG CTC AGG T 3'). The 2B domain in exon 7 of K3 was amplified using the following primers: FK3.7 (5' GGA GGA GGA ATG TTC CCT GAC T 3'), and RK3.7 (5' TAA AAG TGC CCT GAA CCC AGA A 3'). The 1A domain in exon 1 of K12 was amplified using the following primers: FK12.1 (5' TTG TGA GCT GGC CAA AAA CC 3'), and RK12.1 (5' CAA AGC GCC TCC AAA AGA GA 3'). The 2B domain in exon 6 of K12 was amplified using the following primers: FK12.6 (5' CAA ACA GAC GTA GCA TCC TTT GG 3'), and RK12.6 (5' CCC CAT TCC TTC TAT TTC TGC TG 3').

PCR was carried out in a volume of 50 µl mixture containing 1 µM of each primer, 0.5 units of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA), 1 µl dNTP mixture (Applied Biosystems), 5 µl of 10×PCR Buffer with MgCl (Applied Biosystems), and approximately 100 ng of human genomic DNA. Thermal cycling was performed using a GeneAmp PCR System 9700 (Applied Biosystems) with the following programme: (95°C

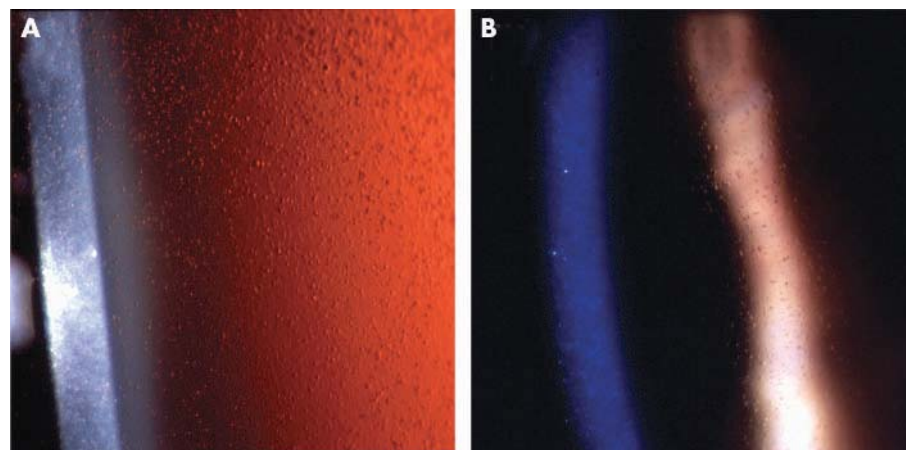
5 minutes for 1 cycle); (95°C 1 minute, 60°C 1 minute, ×40 cycles); (72°C 10 minutes for 1 cycle). The annealing temperature for the FK3.1/RK3.1 primer reaction was 68°C.

Amplified DNA was purified using the QIAquick PCR purification kit (Qiagen). The DNA was sequenced using the BigDye Terminator protocol (2 µl DNA, 8 µl BigDye Ready Reaction Mix, 0.33 µM of primer, in a total volume of 20 µl). An ABI Prism 310 Genetic Analyser (Applied Biosystems) was used to collect and analyse the sequence data. DNA was sequenced in both the forward and reverse directions. Nucleotide sequences were compared with the published human K3 and K12 DNA sequences (GenBank accession nos X54021 and AF137286).

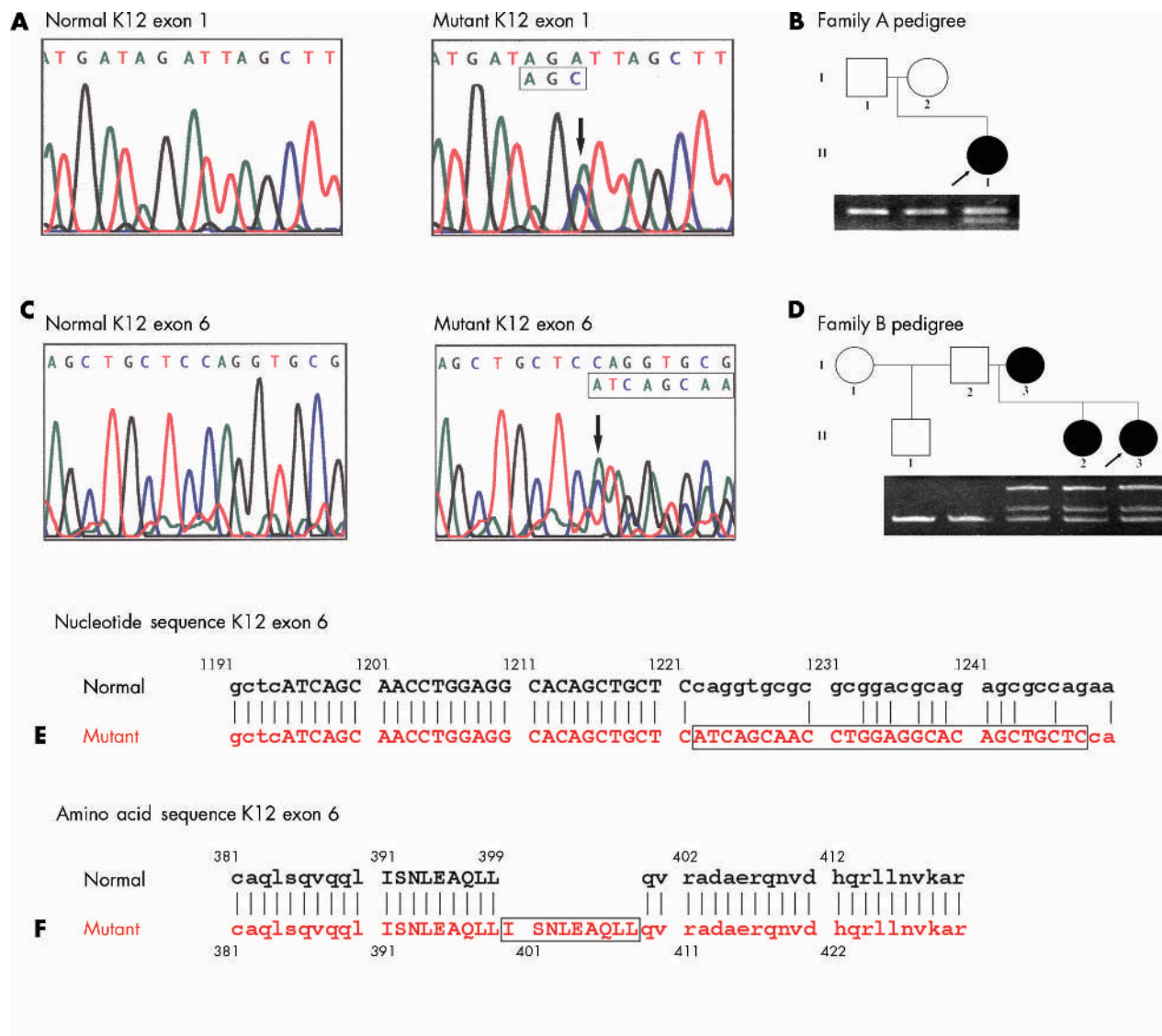
### Mutation confirmation

Mutation R135S generated a novel *Blp* I restriction enzyme site in exon 1 of K12. PCR products from members of the two affected families and 50 control individuals were amplified as above and subjected to restriction digestion to assay for the R135S mutation. To accomplish this, *Blp* I (10 U) (New England BioLabs, Beverly, MA, USA), 2.5 µl 10X NEBuffer 4 (100 mM NaCl, 50 mM TRIS-HCl, 10 mM MgCl<sub>2</sub>, 1 mM DTT, pH 7.9 at 25°C), and 15 µl of the PCR reaction product were combined in a total volume of 25 µl and incubated at 37°C for 2 hours. Bands of digested DNA were resolved on a 1% agarose gel and visualised by ethidium bromide staining.

The 27 bp insertion mutation (1222ins27) did not alter any recognition site for known restriction enzymes. For this reason, confirmation of the mutation was based on gel migration differences between the normal product and the mutant product which was 27 bp longer. A new primer pair



**Figure 1** Clinical photographs of affected family members. (A) Slit lamp photograph of proband of family A. Red reflex retroillumination demonstrates the numerous translucent corneal epithelial microcysts characteristic of Meesmann's dystrophy. (B) Slit lamp photograph of proband of family B. Iris retroillumination reveals the diffuse pattern of corneal epithelial involvement characteristic of family B.



**Figure 2** Mutational analysis of families A and B. (A) Left side of panel shows normal sequence of K12 exon 1 from unaffected member of family A. Right side of panel shows mutant sequence of K12 exon 1 from affected member of family A, representing a heterozygous A→C transversion (arrow) at the third position of codon 135 (boxed sequence), resulting in an arginine to serine replacement (R135S). (B) Pedigree of family A, which had no previous evidence of family history of disease before the proband (II-1, marked by arrow). The mutation R135S created a novel restriction enzyme site (*Bsp*I). Upon digestion, the PCR product was visualised as two distinct bands. Unaffected family members and 50 control individuals produced a single band, and were screened using this digest. (C) Left side of panel shows normal sequence of K12 exon 6 from unaffected member of family B. Right side of panel shows mutant sequence of K12 exon 6 from affected member of family B. Sequence begins to diverge at nucleotide 1222 (arrow), which represents an insertion of duplicated nucleotides (boxed sequence) from 27 base pairs earlier (1222ins27). (D) Pedigree of family B, which shows proband (II-3, marked by arrow), affected family members, and unaffected family members. The mutation 1222ins27 created a PCR product that consisted of a normal product and a slightly longer mutant product. These products were visualised on a polyacrylamide gel at 109 bp (normal product, bottom band), at 136 bp (mutant product, middle band), and a heteroduplex of the two products (top band). Affected family members produced the same gel pattern. Unaffected family members and 50 control individuals produced a single band, and were screened in this manner. (E) Nucleotide sequence data of family B mutant K12 exon 6, with duplication of sequence first seen at position 1222. Normal sequences indicated in black, mutant sequences in red. Regions of duplication are in capital letters. Insertions of duplications into mutant sequences are boxed. (F) Amino acid sequences. Duplication of sequence first seen at codon position 400.

(F2K12.6: 5'AGC CGA GGG CGA TTA CTG C 3' and R2K12.6: 5' GTC CAC GTT CTG GCG CTC T 3') producing a much smaller product was designed in order to resolve the 27 bp difference and assay for this insertion mutation. PCR amplification with this new set of primers (as above except for annealing temperature of 63°C for 15 seconds per cycle) was carried out on genomic DNA from members of the two affected families and 50 control individuals. Amplified DNA was then electrophoresed on 5% TBE polyacrylamide gel (Bio-Rad, Hercules, CA, USA) and visualised with ethidium bromide staining.

## RESULTS

The proband of family A had worn glasses since age 5, but was not diagnosed with Meesmann's corneal dystrophy until routine slit lamp examination at age 10 revealed numerous corneal epithelial microcysts in both eyes (fig 1A). The translucent microcysts involved all layers of the corneal epithelium, with both positive and negative fluorescein staining evident. A 3–4 mm trapezoidal island of sparing was seen centrally in both eyes. Spectacle corrected vision was 20/20 in both eyes. She remained asymptomatic until age 15 when she suffered a traumatic corneal



**Table 2** Review of known in-frame insertion/deletion mutations in keratin disorders

Disorder	Mutation	Keratin	Domain	Reference
WSN	3 bp insertion	K4	1A	Terrinoni <i>et al</i> <sup>67</sup>
WSN	3 bp deletion	K4	1A	Rugg <i>et al</i> <sup>68</sup>
PC	3 bp deletion	K6a	1A	Bowden <i>et al</i> <sup>69</sup>
EPPK	3 bp insertion	K9	2B	Coleman <i>et al</i> <sup>60</sup>
MCD	27 bp insertion	K12	2B	Current report
EBS	3 bp deletion	K14	2B	Chen <i>et al</i> <sup>60</sup>

WSN = white sponge naevus; PC = pachyonychia congenita; EPPK = epidermolytic palmoplantar keratoderma; MCD = Meesmann's corneal dystrophy; EBS = epidermolysis bullosa simplex.

abrasion in the left eye, followed by a recurrent erosion 4 months later.

The proband of family B was also diagnosed at age 10, during an evaluation for contact lenses. She had a history of amblyopia in the left eye and infantile esotropia, and had undergone strabismus surgery at age 4. She had also required glasses since early childhood. Slit lamp examination revealed diffuse involvement of the corneal epithelium with translucent microcysts in both eyes (fig 1B). She did well until the insidious onset and gradual progression of photophobia, foreign body sensation, and fluctuating vision as a teenager. At age 19 she began to suffer from recurrent erosions in both eyes, and by age 21 she was forced to discontinue contact lens wear. Vision remained correctable in spectacles to 20/20 right eye, and 20/25 left eye. The proband's mother and sister were subsequently examined and found to be similarly affected. They both wear spectacles with vision correctable to approximately 20/25 in both eyes. Each reports a similar history of light sensitivity, foreign body sensation, and fluctuating vision.

Analysis of the sequence data from the affected patient from family A revealed a heterozygous point mutation in the region of the K12 gene encoding the helix initiation motif (fig 2A). The mutation was 429A→C substitution, resulting in a predicted arginine to serine amino acid change at codon 135 (R135S). This mutation was confirmed by analysis of the reverse sequence data and was absent in an unaffected family member. Since this mutation created a novel restriction enzyme site, restriction fragment analysis enabled rapid detection of the substitution in family members and control subjects (fig 2B). As assayed in this manner this mutation was not detected in two unaffected family members and 50 unaffected unrelated individuals.

Analysis of the sequence data from the affected patient from family B revealed overlapping sequences starting at nucleotide 1222 of the K12 gene (numbering per Genbank accession number D78367). As can be seen in figures 2C and 2E, these overlapping sequences correspond to a heterozygous insertion mutation in which the genetic code from 27 bases is repeated. This mutation was confirmed by sequencing in the reverse direction. This repetition of the genetic code represents a heterozygous 27 base pair in-frame duplication insertion mutation (1222ins27) resulting in a duplication of nine codons (fig 2F). The mutation was absent in an unaffected family member.

Since the original PCR product of the K12 gene was 424 bp long, the 27 extra nucleotides in the mutant product could not be resolved as a distinct band on a 1% agarose gel. To confirm the mutation, PCR amplification of a smaller 109 bp product of the K12 gene was performed. The duplication insertion mutation could then be resolved as a separate band of 136 bp by polyacrylamide gel electrophoresis. A slowly migrating third band representing a mutant and wild type heteroduplex product was also resolved. Gel electrophoresis analysis enabled rapid detection of the insertion in family members and control subjects (fig 2D). As assayed in this

manner the same mutation was detected in two affected family members, not detected in two unaffected family members, and not detected in 50 unaffected unrelated individuals.

## DISCUSSION

Meesmann's corneal dystrophy is an autosomal dominant genetic disease resulting in an irregular corneal epithelium. Here, we report two novel mutations of the K12 gene in patients with Meesmann's corneal dystrophy. The proband of family A carries a novel heterozygous missense mutation resulting in the substitution of an arginine residue (R135S) in the helix initiation motif of the K12 gene. This is the fifth different novel mutation of arginine at codon 135 that has been reported. This arginine, which may represent a hot spot mutation site,<sup>16,19</sup> is particularly important for keratin assembly and functionality.<sup>4,22-25</sup> In other keratin disorders, hot spot mutation sites containing arginine in the rod 1A region have been reported.<sup>26-29</sup>

The proband of family B carries a novel duplication insertion of 27 nucleotides (1222ins27). This type of mutation is unique for a number of different reasons. Firstly, in-frame insertion or deletion mutations are extremely rare in keratin diseases (table 2), and this is the first reported case of such a mutation in Meesmann's corneal dystrophy. Secondly, whereas previous insertion/deletion mutations in other keratin diseases have all been one to three nucleotides long, the insertion in our study patient is 27 nucleotides long. It has been postulated that these duplications result from slipped mispairing during DNA replication owing to the somewhat repetitive DNA sequence.<sup>30</sup> Thirdly, the site of the mutation is unique. Up to now, all reported mutations of Meesmann's corneal dystrophy have been restricted to the helix boundary motifs. The duplication mutation in our study patient is located before the start of the helix termination motif at the end of the 2B domain of the K12 gene.

The predicted nine amino acid insertion in this novel duplication mutation could be disruptive in two ways. Firstly, changing the highly conserved heptad periodicity of the amino acid sequence (a-g)<sub>n</sub> could disrupt proper alpha helix formation and filament assembly of the keratin 12 protein. Secondly, keratin heterodimer alignment is extremely sensitive to change,<sup>12</sup> and altering the length of the keratin 12 protein could potentially disrupt proper alignment with the keratin 3 protein.

The majority of keratin disease is linked to mutations in the highly conserved helix boundary motifs in regions 1A and 2B of the keratin genes,<sup>12,13</sup> but a number of recent studies have found disease causing mutations outside of conserved regions.<sup>25,31-36</sup> We have observed two novel mutations in both conserved and non-conserved regions of the genome. In the proband of family A, we observed a mutation of a highly conserved arginine residue of domain 1A, analogous to mutations in other keratin diseases. In the proband of family

B, we observed a large 27 bp insertion mutation in domain 2B just before the start of the helix termination motif.

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