Keratin 9 point mutation in the pedigree of epidermolytic hereditary palmoplantar keratoderma perturbs keratin intermediate filament network formation

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Abstract Keratins form an intracellular keratin filament network in keratinocytes. Point mutations in the epidermal keratins could lead to the disruption of keratin filament formation, developing skin diseases such as epidermolytic hereditary palmoplantar keratoderma (EHPPK). We found a G to A transition in keratin 9 (K9) cDNA, resulting in the substitution of glutamine for arginine at 162, in all patients of a pedigree of EHPPK. Transfection into MDCK cells and DJM-1 cells revealed that the plasmid CMX vector containing normal keratin 9 cDNA showed normal keratin network formation, whereas the vector with a G to A point mutated keratin 9 cDNA showed disrupted keratin filaments with droplet formation in the cells. These results indicate that the point mutation seen in our patients had a dominant-negative effect on keratin network formation.

Key words: Keratin 9; Epidermolytic hereditary palmoplantar keratoderma; Keratin intermediate filament; Point mutation; Transfection

1. Introduction

Keratins are the major structural proteins of the epidermis. The 10-nm keratin filaments belong to the intermediate filament (IF) superfamily including keratins, desmin, vimentin, neurofilament and glial fibrillary acidic protein. All IF proteins have a central α-helical domain, the rod, which is flanked by a non-helical head (amino-end) and tail (carboxy-end) domains. The α-helical sequences have repeats of hydrophobic amino acids, which provide a hydrophobic seal on the helical surface, enabling the coiled-coil structure between two keratin polypeptides. Keratins assemble in vitro as obligatory heteropolymers. The highly conserved rod ends play a crucial role in IF network formation [1]. The point mutation in keratin 5 and keratin 14 in epidermolysis bullosa simplex (EBS), especially the mutation in the α-helical rod domain, perturbs keratin filament structure and was found to be important for 10 nm filament assembly [2]. Transfection of K14 mutant cDNA into PtK2 cells showed a dysfunction in keratin assembly, resulting in a droplet formation of the cytoskeleton [2,3]. EHPPK also shows an abnormality in keratin formation in patients' skin. Thus, we speculated that K9 with a point mutation would lead to a dominant-negative effect on keratin network formation. There are previous reports of point mutations in the K9 gene in EHPPK [4-8] but none showed a function assay with these mutations. Here, we provide the first demonstration that the point mutation found in a pedigree of EHPPK has a dominant-negative effect on the assembly of keratin intermediate filaments in the cells.

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Abbreviations: EHPPK, epidermolytic hereditary palmoplantar keratoderma; IF, intermediate filament; K9, keratin 9; PCR, polymerase chain reaction; HA, hemagglutinin protein of influenza; K14, keratin 14; EBS, epidermolysis bullosa simplex

2. Materials and methods

2.1. PCR and DNA sequence

Genomic DNA was extracted and purified from blood or biopsy specimens from the patients. The primers were designed at nucleotide 263–282 and 664–683 based on the K9 cDNA sequence [9]. PCR products were digested with restriction enzymes *Eco*RI and *Xba*I. The digested 424 bp fragment was ligated to *Eco*RI/*Xba*I digested pBluescript II KS(+) (Stratagene, La Jolla, CA, USA). Double-stranded cDNAs inserted into pBluescript II KS(+) were sequenced using a Sequenase version 2.0 7-deaza-dGTP kit (US Biochemical, OH, USA) according to the manufacturer's recommendation. *Pvu*II polymorphism was tested by digesting the PCR products from the family member of the patients or normal controls. The resultant DNA fragments were applied to 3.5% agarose gels and stained using ethidium bromide.

2.2. Cell culture

MDCK cells were grown in Dulbecco's MEM supplemented with 10% FCS and 100 U/ml penicillin and 100 μg/ml streptomycin. DJM-1 cells were grown in Dulbecco's MEM supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.4 μg/ml hydrocortisone, 10 ng/ml EGF and 84 ng/ml cholera toxin.

2.3. Full cloning of normal and patient K9 cDNA and the construction of expression vectors

PCR cloning was performed via two primer pairs, one pair corresponding to nucleotides 45–66 and 1026–1048 with an artificial *Kpn*I site, the other to nucleotides 963–984 and 1913–1932 with an artificial *Hin*dIII site (Fig. 1B, arrows). The former RT-PCR product was digested with *Kpn*I, the latter with *Hin*dIII and subcloned into pBluescript II KS(+), respectively. Each subclone was digested with *Kpn*I/internal *Bgl*II and internal *Bgl*II/*Hin*dIII, respectively, and these two fragments were ligated into pBluescript II KS(+) to make a full length cDNA (pK9) (Fig. 1B). For the construction of the point mutated K9 cDNA (pK9R162Q), the *Kpn*I/*Xba*I fragment was replaced with the patient-derived *Kpn*I/*Xba*I fragment which has a G to A point mutation (Fig. 1B, bottom). The expression vector plasmid pCMX [10], which has a cytomegalovirus promoter and SV40 poly(A) signal, was digested with *Kpn*I and *Hin*dIII. *Kpn*I/*Hin*dIII fragments from pK9 and pK9R162Q were purified from agarose gels and each fragment was ligated to this vector with the tag sequence derived from hemagglutinin protein of influenza (HA). Thus, each has a normal K9 sequence (K9HA) or point mutated K9 (K9R162QHA) with HA tag (Fig. 1A).

2.4. Transient transfection and immunofluorescent study

Plasmid DNAs were purified by affinity chromatography (Quiagen, Chatsworth, CA, USA) followed by column chromatography with Bio-Gel 150 (Bio-Rad, Boston, MA, USA). MDCK cells or DJM-1 cells were grown on glass coverslips in 6-well plates (Iwaki, Kyoto Japan). The cells were transfected with these DNAs using Lipofectamin reagent (Gibco-BRL, Bethesda, MD, USA) according to the dis-

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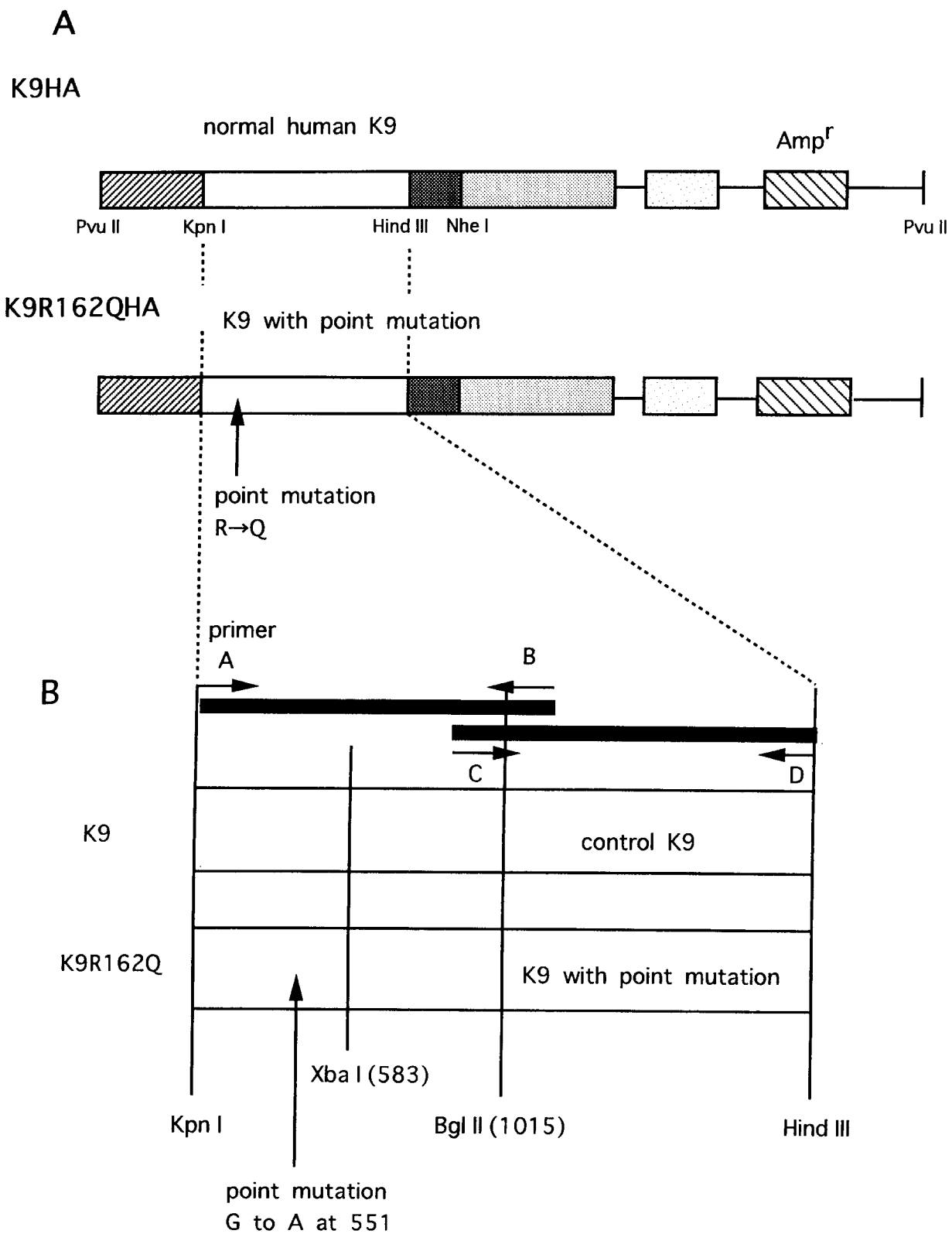


Fig. 1. Construction of K9HA and K9R162QHA: A schematic diagram of the PCR cloning strategy for keratin 9 cDNA and construction of the expression vector. Arrows A and B indicate the RT-PCR primers for the 5′ half of keratin 9 cDNA. Arrows C and D correspond to those of the 3′ half. The restriction site used for full length cDNA construction (*Bgl*II) and point mutation introduction into full length cDNA (*Xba*I) are indicated. (◩) CMV promoter, (▩) Tag (HA), (□) SV40 ori, (◨) SV40 poly(A), (◹) ampicillin resistance.

tributor's recommendation. At 24 h posttransfection, cells were fixed in 3% paraformaldehyde for 10 min and were permeabilized by incubation in 0.5% Triton X-100 for 10 min. Transfected cells were processed for double staining with immunofluorescence. Anti-HA.11

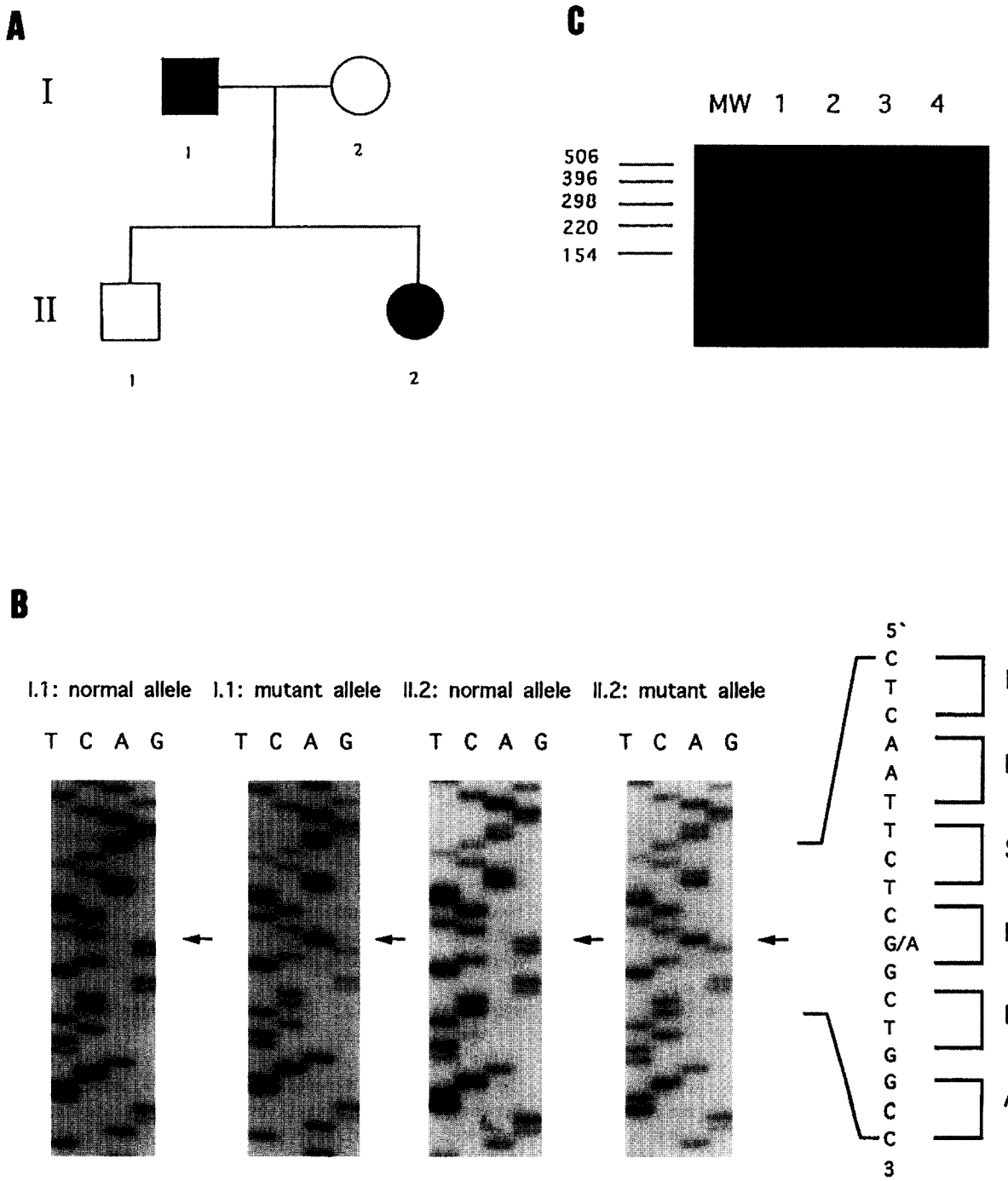


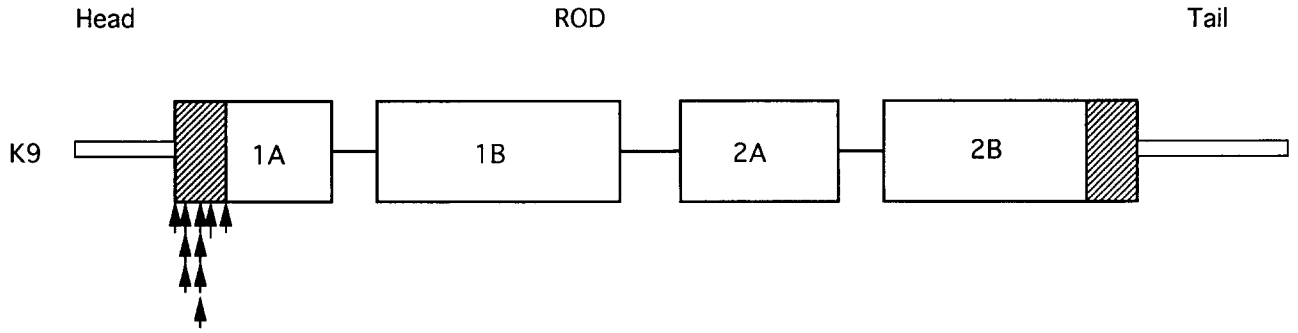
Fig. 2. DNA sequences and *Pvu*II mutation analysis: (A) Pedigree of the family. Solid symbols show affected individuals; open symbols, unaffected; squares, male; circles, female. (B) DNA sequences. Arrows show the G to A transition at nt 551 leading to the substitution of glutamine for arginine at 162. (C) *Pvu*II mutation analysis. This point mutation gave rise to the new restriction site of the *Pvu*II sequence. PCR amplified fragments of 424 bp were digested by *Pvu*II restriction enzyme. Normal alleles do not have a *Pvu*II site. Mutant allele was digested by *Pvu*II (296 and 128 bp fragments). MW: size marker. Lanes 1,2, mutant allele; lanes 3,4, normal allele.

rabbit polyclonal antibody (Berkeley Antibody Co., CA, USA) to recognize the HA tag at a dilution of 1:500 was used as primary antibody and 1:100 diluted rhodamine-conjugated swine anti-rabbit IgG (Dakopatts, Glostrup, Denmark) was used as a second antibody. After HA tag staining, the keratin network was stained with MAB1629 (mouse monoclonal anti-cytokeratin 5, 8; Chemicon, CA, USA) for MDCK cells at a dilution of 1:1000 or mouse monoclonal anti-cytokeratin peptide 14 (Sigma Chemical Co., MO, USA) for DJM-1 cells to recognize the endogenous keratin network at a dilution of 1:100 and finally 1:100 diluted fluorescein-conjugated goat IgG fraction to mouse IgG (Organon Teknika Corp., NC, USA) was used to detect the keratin network. Observations were performed with a Carl Zeiss LSM 410 confocal scanning laser microscope.

3. Results and discussion

3.1. Clinical description of the EHPPK patients

The pedigree of the EHPPK family is described in Fig. 2A.



Human K9	-KSTMQELNSRLASYLDKVQALEEANNDLENKI-	[9]
M156V	-KSTVQELNSRLASYLDKVQALEEANNDLENKI-	[6]
N160Y	-KSTMQELYSRLASYLDKVQALEEANNDLENKI-	[4]
N160S	-KSTMQELSSRLASYLDKVQALEEANNDLENKI-	[7]
N160K	-KSTMQELKSRLASYLDKVQALEEANNDLENKI-	[5]
R162W	-KSTMQELNSWLASYLDKVQALEEANNDLENKI-	[5,7,8]
R162Q	-KSTMQELNSQLASYLDKVQALEEANNDLENKI-	[5]
L167S	-KSTMQELNSRLASYSDKVQALEEANNDLENKI-	[8]
Q171P	-KSTMQELNSRLASYLDKVPALEEANNDLENKI-	[6]

Fig. 3. Location of human EHPPK mutations relative to keratin 9 structure: Positions of mutations are shown by ↑. (Open square) The central rod domain, predicted to be largely α-helical. The rod is subdivided into helices 1A, 1B, 2A and 2B. (Hatched area) Highly conserved end domains of the rod; (open rectangle) non-helical head (N-terminal) and tail (C-terminal) domains.

All affected family members showed typical hyperkeratosis on palms and soles with the characteristic histological finding of granular degradation in the epidermis [11].

3.2. Sequence and mutation analysis of K9

Analysis for the K9 gene mutation was conducted on family members of patients and normal volunteers. By sequencing, we found a G to A transition at nt 551, resulting in the substitution of glutamine for arginine at 162, in patients 1 and 2 (Fig. 2B). A G to A transition at nt 551 gives rise to the new restriction enzyme recognition site corresponding to *Pvu*II. Thus, we analysed PCR products by digestion with this enzyme. Affected patients showed heterogenicity in this site. Fig. 2C shows a non-digested band of 424 bp and *Pvu*II digested bands of 128 and 296 bp in patients, whereas healthy members of this family had no *Pvu*II restriction site within this area. A control study of 20 normal volunteers' genomic DNA revealed that there was no polymorphism in this region (data not shown). In previous reports on the K9 mutation, eight types of point mutations were reported [4-8] as summarized in Fig. 3. (1) M156V: substitution of valine for methionine at amino acid 156 by A to G transition at nt 532 [6]; (2) N160Y: substitution of tyrosine for asparagine at amino acid 160 by the transversion of A to T at nt 544 [4]; (3) N160S: serine for asparagine at 160 by the transversion of A to G transition at nt 545 [7]; (4) N160K: lysine for asparagine at 160 by T to A transversion at nt 546 [5]; (5) R162W: tryptophan for arginine at 162 by C to T transition at nt 550 [5,7,8]; (6) R162Q: glutamine for arginine at 162 by G to A transition at nt 551 [5] (our cases have the same amino acid mutation of glutamine for arginine); (7) L167S: serine for leucine at 167 by transition of T to C at nt 566 [8]; and (8) Q171P: substitution of proline of glutamine at 171 by A to C transversion at nt 578 [6]. Thus, amino acids 156–171, which correspond to the head of the α-helical domain of keratin intermediate filaments, may be a hot spot of mutation and the replacement of these amino acids may be crucial for keratin filament assembly and intermediate filament network formation in the cells.

3.3. Expression of K9 HA and K9R162QHA in MDCK cells and DJM-1 cells

The point mutation of the keratin 14 rod domain seen in the EBS patients has a dominant-negative effect on keratin network formation in epithelial cells [3]. We transfected normal keratin 9 cDNA (K9HA) or point mutated K9 (K9R162QHA) found in our pedigree into MDCK cells or DJM-1 cells, since there is no experimental evidence that the mutations in the rod domain of keratin 9 had a dysfunctioning effect on keratin intermediate filament assembly. Fig. 4 demonstrates the staining results on the Tag sequence (HA) derived from transfected K9HA and K9R162QHA cDNAs and the endogenous keratin network by keratin staining and overlaying. Both the epithelial cell derived cell line, MDCK, and the epidermal cell derived cell line, DJM-1, showed essen-

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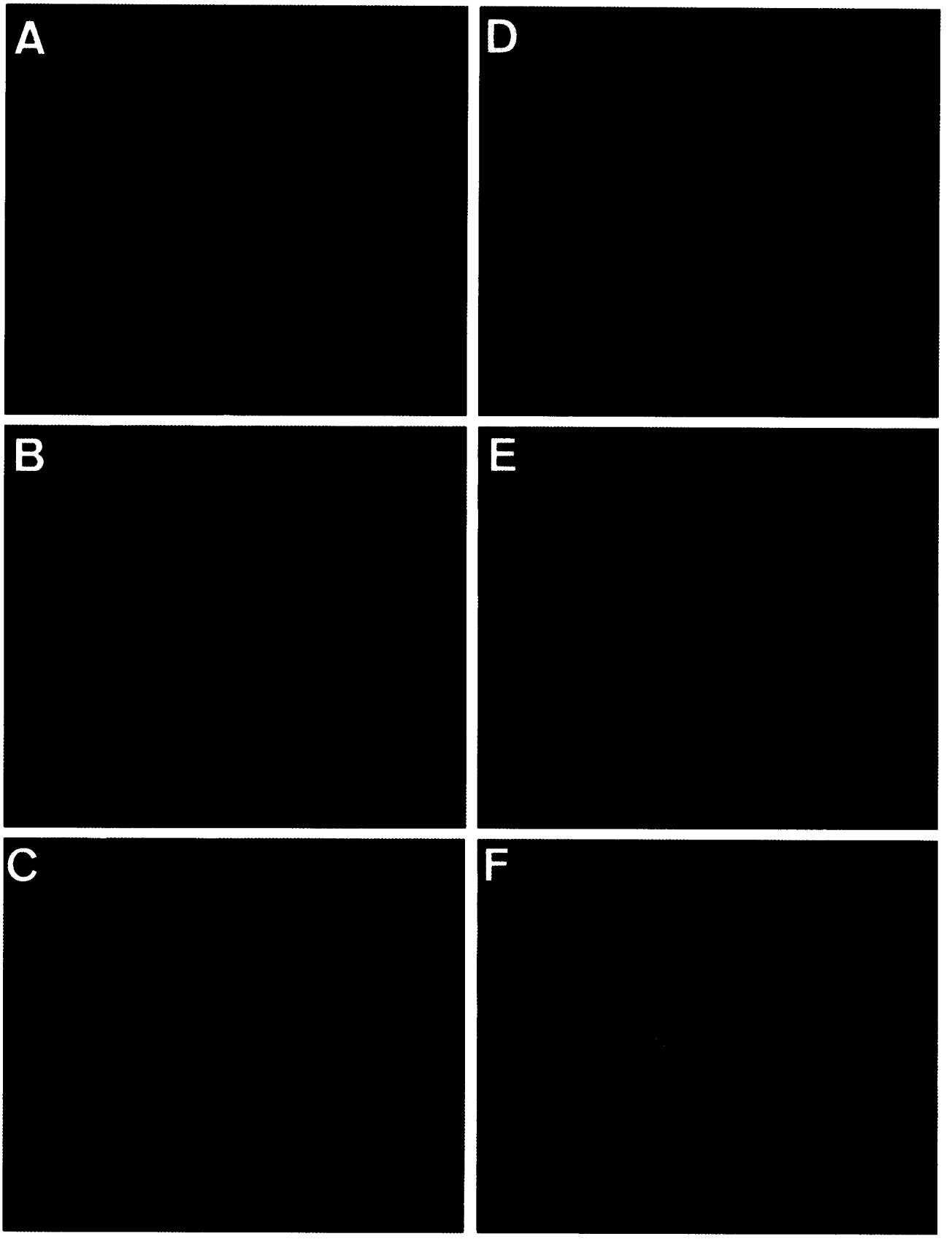
Fig. 4. Immunofluorescence analysis of transfected MDCK cells and DJM-1 cells: MDCK cells (A–F) and DJM-1 cells (G–L) were transfected with pK9HA (A–C, G–I) and pK9R162QHA (D–F, J–L). 24 h posttransfection, cells were fixed and were processed for double immunofluorescence. The pk9HA and pK9R162QHA products were detected by anti-HA.11 rabbit polyclonal antibody through a 488 nm argon laser (A,D,G,J). The endogenous keratins were detected with mouse monoclonal anti-cytokeratin 8 for MDCK cells (B,E) or mouse monoclonal anti-cytokeratin peptide 14 for DJM-1 cells (H,K), through a 543 nm HeNe laser. The yellow color in C, F, I, L demonstrates the superpositioning of the colocalization through overlaying the 488 nm and 543 nm laser.

tially the same result. As shown by the red signal (HeNe 543 nm laser) K9 protein showed filament formation in wild type cDNA transfected cells (Fig. 4A,G), whereas point mutated K9 revealed droplet formation (Fig. 4D,J). Endogenous keratin filament stained by anti-keratin antibody is demonstrated by the green signal (argon 488 nm laser). All the cells revealed



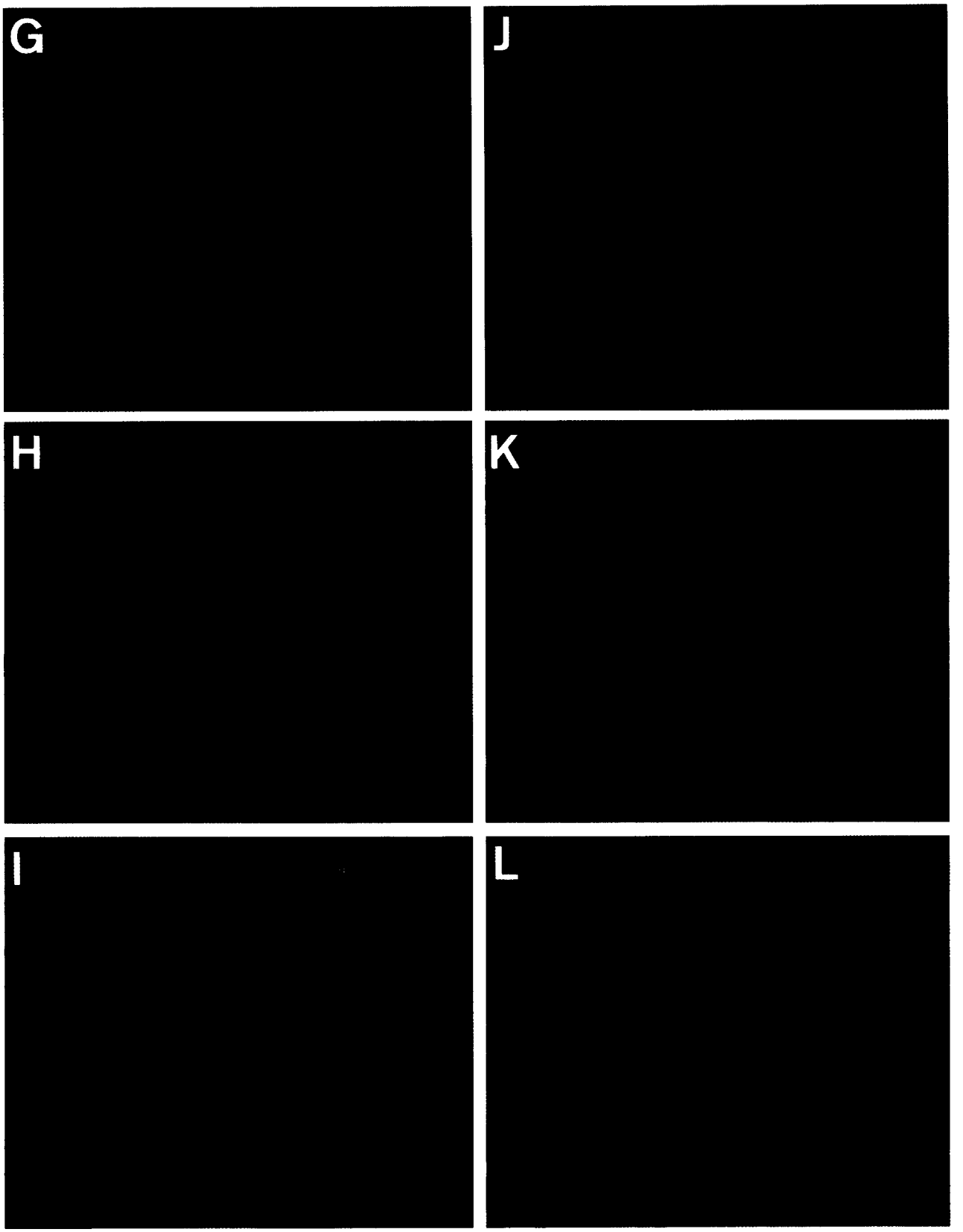


Fig. 4 (*continued*).

keratin fibers. Within these cells, mutated K9 cDNA transfected cells showed droplets within the cell (Fig. 4E,K), whereas wild type K9 cDNA transfected cells showed a fiber structure only (Fig. 4B,H). Thus, the point mutation in K9 disrupts the endogenous keratin network in the cells. These results are in line with the observation of droplet formation with point mutated keratin 14 cDNA transfected cells [3]. To confirm whether the point mutated K9 droplets and endoge-

nous keratin droplets are identical, we performed further analysis by confocal microscopy using 488 nm and 543 nm laser light. The yellow colored droplet in Fig. 4F and L clearly demonstrates the colocalization of the K9R162QHA product and internal keratin in droplet formation within the transfected cells. These results indicate that the keratin intermediate filament structure integrated by the newly expressed K9R162QHA product collapses and shows the formation of droplets in MDCK cells and DJM-1 cells and that the point mutation seen in our pedigree does have a dominant-negative effect on filament formation in the cells.

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