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Dominant and Recessive Compound Heterozygous Mutations in Epidermolysis Bullosa Simplex Demonstrate the Role of the Stutter Region in Keratin Intermediate Filament Assembly*

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Keratin intermediate filaments are important cytoskeletal structural proteins involved in maintaining cell shape and function. Mutations in the epidermal keratin genes, keratin 5 or keratin 14 lead to the disruption of keratin filament assembly, resulting in an autosomal dominant inherited blistering skin disease, epidermolysis bullosa simplex (EBS). We investigated a large EBS kindred who exhibited a markedly heterogeneous clinical presentation and detected two distinct keratin 5 mutations in the proband, the most severely affected. One missense mutation (E170K) in the highly conserved helix initiation peptide sequence of the 1A rod domain was found in all the affected family members. In contrast, the other missense mutation (E418K) was found only in the proband. The E418K mutation was located in the stutter region, an interruption in the heptad repeat regularity, whose function as yet remains unclear. We hypothesized that this mutated stutter allele was clinically silent when combined with the wild type allele but aggravates the clinical severity of EBS caused by the E170K mutation on the other allele. To confirm this *in vitro*, we transfected mutant keratin 5 cDNA into cultured cells. Although only 12.7% of the cells transfected with the E170K mutation alone showed disrupted keratin filament aggregations, significantly more cells (30.0%) cotransfected with both E170K and E418K mutations demonstrated keratin aggregation ($p < 0.05$). These transfection assay results corresponded to the heterogeneous clinical findings of the EBS patient in this kindred. We have identified the first case of both compound heterozygous dominant (E170K) and recessive (E418K) mutations in any keratin gene and confirmed the significant involvement of the stutter region in the assembly and organization of the keratin intermediate filament network *in vitro*.

The epidermis plays an important role in the protection from environmental insult by forming an extensive cytoskeletal network within keratinocytes, comprising of keratin intermediate filaments (KIF)[1] belonging to the intermediate filaments (IF) superfamily (1, 2). Keratins can be subdivided into two separate subfamilies, type I, the acidic keratins, and type II, the neutral-basic keratins (3–5). They have a central 310-amino acid α-helical rod domain composed largely of seven residue heptad repeats. This domain exists in four segments (1A, 1B, 2A, and 2B) interrupted by three non-helical linkers (L1, L12, and L2) and a so-called, stutter region (6, 7). The stutter region is an interruption in the regularity of the heptad repeat substructure, which generates a helix phase reversal near the middle of the 2B rod domain segment and is considered to be the result of a deletion of three residues at the junction of two heptads (8). Although the stutter region is highly conserved among all IF (7), its molecular functions in KIF assembly remain unknown.

Mutations in either the *KRT5* or *KRT14* gene, which encode the epidermal keratins K5 and K14, respectively, lead to the epidermolysis bullosa simplex (EBS), the majority of which are inherited in an autosomal dominant manner and are characterized by intraepidermal blister formation (9). According to the clinical severity of blister formation, EBS can be subdivided into three major subtypes (10). The mildest variant is the Weber-Cockayne type EBS (EBS-WC) with blistering restricted to the hands and feet, the more moderate variant is the Koebner type (EBS-K) with generalized blister formation, and the most severe variant (Dowling-Meara type; EBS-DM) is characterized by severe herpetiform blistering (10).

EBS was the first human keratin disease to be identified (11–13) that came from the ultrastructural findings demonstrating the clumping of KIFs in the basal keratinocytes of patients with EBS-DM (14, 15). Transgenic mice engineered to express the mutant human K14 exhibited similar phenotypic and morphological characteristics to EBS-DM, which suggested that keratin mutations disrupt KIF assembly in a dominant negative fashion (16, 17). It showed that if even a small amount of copolymerizing keratin protein is defective, this is sufficient to disrupt the whole KIF network. The severity of EBS has been shown to be dependent upon the structural implications for each mutation, including its location within the protein and its effect upon KIF formation (18, 19). The mutations responsible for EBS-DM lie within the highly conserved ends of the rod domain, which are critical for proper K5 and K14 filament assembly (11, 20, 21). In contrast, EBS-K

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) NM000424, AF237621, AF019084, X05418, X05421, X61028, L42583, BC000163, Y00067, and M13451.

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[1] The abbreviations used are: KIF, keratin intermediate filament; EBS, epidermolysis bullosa simplex; EBS-DM, Dowling-Meara type EBS; EBS-K, Koebner type EBS; EBS-WC, Weber-Cockayne type EBS; HIP, helix initiation peptide; HTP, helix termination peptide; IF, intermediate filament; K5, keratin 5; K14, keratin 14; MDCK, Madin-Darby canine kidney.

mutations are also located within the rod domain but are more centrally located (12, 13) and EBS-WC mutations are mostly found in the non-helical regions (22- 24).

We have studied an EBS family in which the affected individuals showed a variable phenotype and have identified two distinct *KRT5* mutations in the proband, the most severely affected member. He is compound heterozygous for both a dominant mutation in the helix initiation peptide (HIP) and a recessive mutation in the stutter region of K5. To confirm whether the stutter mutation exacerbated the clinical severity of EBS caused by the dominant mutation on the other allele, we examined cultured cells transfected with the mutated *KRT5* cDNA corresponding to the proband's mutations. Here we show evidence that the stutter region plays an important role in the organization of the KIF network.

EXPERIMENTAL PROCEDURES

EBS Family with Heterogeneous Clinical Presentation—The proband (Fig. 1, *III:4*) was a 22-year-old man with blistering over his entire body from birth and was given a diagnosis of EBS-K. Although the blistering on the trunk improved with age, he continued to get severe blisters and erosions on the hands and feet after minor trauma with a worsening of symptoms during the summer months (Fig. 2). Electron microscopy of the proband showed that cytolysis occurred within the basal keratinocytes consistent with EBS, although apparent clumping of keratin filaments was not observed (data not shown). His family had several affected members (Fig. 1). An older brother (III:6) also presented with blistering over the whole body since birth, with similar clinical findings to the proband, but died in a traffic accident in childhood. In contrast, his deceased father (II:6), deceased grandmother (I:2), and his paternal uncle (II:2) developed few blisters that were restricted to the soles only after extensive walking. They seldom exhibited blisters with increasing age; therefore, they were unaware that they had any disease. All of them were given the diagnosis of EBS-WC, not EBS-K. Thus the markedly heterogeneous clinical presentation in this dominantly inherited EBS family could not be explained by a single dominant keratin mutation (Fig. 1).

Mutation Analysis—Genomic DNA extracted from peripheral blood was used as a template for PCR amplification. The *KRT5* and *KRT14* genes were amplified by the methods previously reported (25, 26). Specifically, for amplification of the 407-bp DNA fragment comprising the 339-bp exon 1 of *KRT5* (nucleotides 216–555 in the cDNA; GenBank™ accession number NM000424) containing the paternal mutation E170K, the following primers were used; 5′-GAGGATATCCATCAGCACTA-3′ (forward) and 5′-CCTTCTTTCTCTCTTTGGC-3′ (reverse). For amplification of the 558-bp DNA fragment comprising the 221-bp of exon 7 of *KRT5* (nucleotides 1219–1440 in the cDNA; GenBank™ accession number NM000424) containing the maternal mutation E418K, the following primers were used; 5′-GAGAGCCGAGATTGACAATG-3′ (forward) and 5′-TAGAGCAGCCTCGCTTTATC-3′ (reverse). Fifty-microliter reactions containing 250 ng of DNA, 200 μM of each dNTP, 450 nM of each primer, 2 units of Amplitaq polymerase, and 5 μl of 10× PCR buffer with 1.5 mM $MgCl_2$ (PerkinElmer Life Sciences, San Francisco, CA) were initially denatured at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C. DNA sequencing of all the PCR products was carried out using a Genetic Analyzer 310A automatic sequencer (PerkinElmer Life Sciences-ABI, Foster City, CA).

Verification of the Mutations—Because there was no proper restriction enzyme to verify each mutation, we created restriction enzyme sites recognized by *Bsr*BI. The genomic template was amplified by PCR under the same conditions with the following primers: for amplification of the 333-bp DNA fragment containing the E170K mutation and changing the base from A to G at base 512 in exon 1, forward primer 5′-GTGGTGCCGGTAGTGGATT-3′ and reverse primer 5′-TGTTGAGGGTCTTGATCCGC-3′ were used. For amplification of the 321-bp DNA fragment containing the E418K mutation and changing an A to G at base 1256 in exon 7, forward primer 5′-GAAAGAGGGCAAGGAAGGGGCCTG-3′ and reverse primer 5′-TTGAGGGCCAGCTCCCCACGCCGC-3′ were used. Both mutations (G to A substitutions) resulted in the loss of the restriction enzyme site for *Bsr*BI.

Plasmid Constructions and Cell Transfection—Using human keratinocyte cDNA as a template, full-length human *KRT5* was amplified by PCR with primer pairs to generate artificial *Bam*HI and *Hin*dIII sites and subjected to nucleotide sequencing. After digestion with these

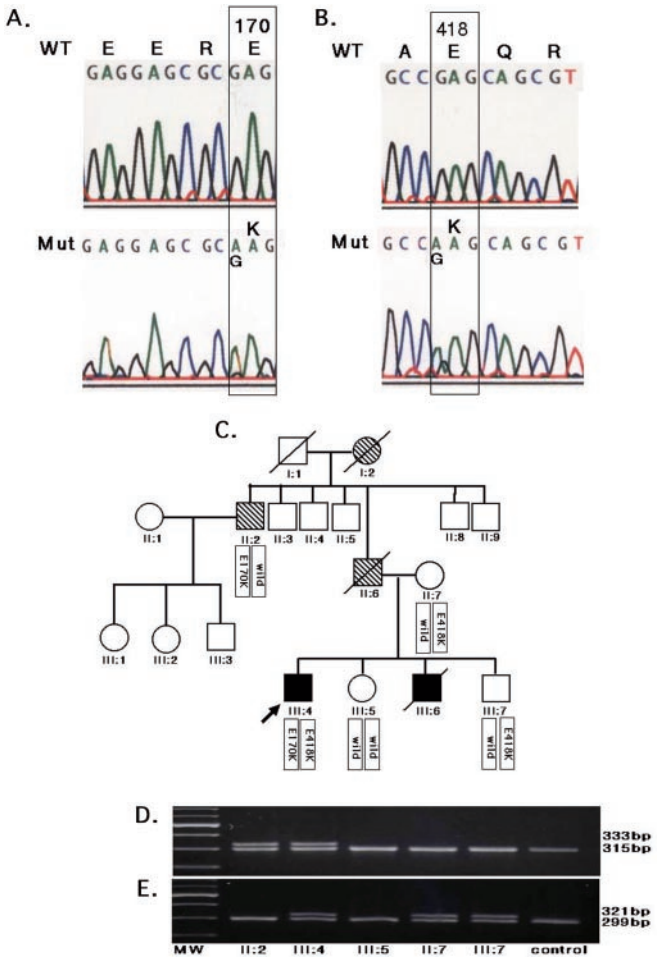

FIG. 1. **The EBS family investigated in this study with results of the genetic mutation in *KRT5*.** *WT*, wild type; *Mut*, mutation. Direct nucleotide sequencing of the proband's genomic DNA revealed two heterogeneous missense mutations. One is a heterozygous G to A transition at nucleotide position 508, which substitutes a glutamic acid to lysine at residue 170 (E170K) (*A*). Another is a heterozygous G to A transition at nucleotide position 1252, which substitutes a glutamic acid to lysine at residue 418 (E418K) (*B*). Males (*squares*), females (*circles*), deceased (*slashed symbols*). The *black square* represents individuals who presented with a moderately severe phenotype (EBS-K). The *striped circle* or *squares* denote individuals with a mild clinical phenotype (EBS-WC). Unaffected individuals are represented by *open symbols*. An *arrow* indicates the proband. The identified mutations E170K and E418K are indicated under the individual's symbol (*C*). To verify mutations, exon 1 and exon 7 were amplified by PCR with primers generating new restriction enzyme sites recognized by *Bsr*BI. E170K and E418K mutations obliterate a *Bsr*BI site. In exon 1, the mutant alleles remain uncut (333 bp) and wild type alleles are digested into 315 and 18 bp in length (*D*), whereas in exon7 the mutant alleles are 321 bp and wild type alleles are digested into 299 and 22 bp in length (*E*). The results confirm heterozygous E170K mutation in the affected family members (*II:2*, *III:4*) and heterozygous E418K mutation in the proband and the unaffected members (*II:7*, *III:4*, *III:7*).

enzymes, the full-length *KRT5* cDNA insert was ligated into pCMV-tag vectors with a FLAG sequence (pK5F) (Stratagene, La Jolla, CA), which allows FLAG epitope tagging of the C terminus of the K5 protein. The point-mutated *KRT5* cDNAs corresponding to the E170K and E418K mutations were generated with the use of the site-directed mutagenesis system Mutan-Super Express kit (TaKaRa, Japan) and subcloned into the same vectors (pK5E170KF and pK5E418KF). Madin-Darby canine kidney (MDCK) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 100 units/ml penicillin and 100 μg/ml streptomycin in the presence of 5% CO_2. The day before transfection, the cells were trypsinized, counted, and plated into a four-welled chamber coverglass at 10^5 cells per well. Four different transfections, including the wild type *KRT5* cDNA alone (pK5F), the combinations with an equimolar ratio of the wild type *KRT5* cDNA and

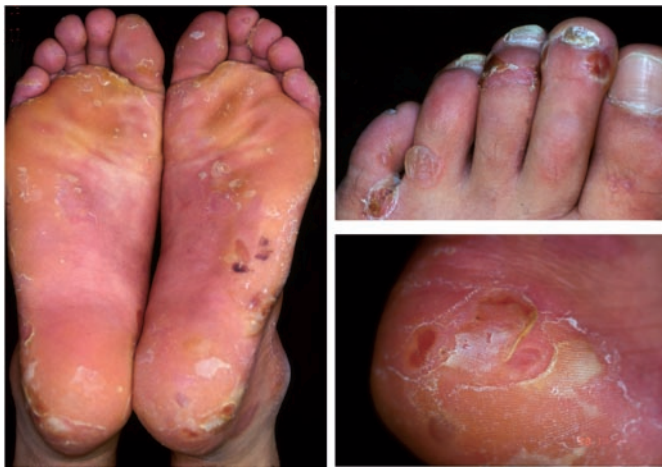

FIG. 2. **Clinical features of the proband.** The proband continues to get occasional blisters and erosion on the soles and palms, particularly in areas of trauma. Old and new blisters and erosion with crusting are seen on the soles, heels, and dorsum of toes. The other affected family members have never presented any similar clinical findings.

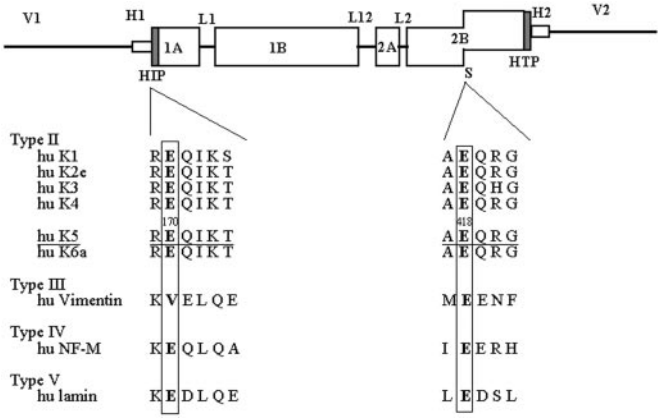

FIG. 3. **Schematic demonstration of the E170 residue and E418 residue in K5 are highly conserved among IF.** The *large boxes* encompass the central α-helical rod domain, separated into four segments (*1A*, *1B*, *2A*, *2B*) by virtue of short non-helical linker segments (*L1*, *L1–2*, and *L2*). Helix initiation peptide (*HIP*) and helix termination peptide (*HTP*) denote the highly conserved end domains of the rod domain. Shown are amino acid sequences of the HIP and the stutter region near the middle of the 2B rod domain segment along with: a selected group of other type II IF keratins, type III IF vimentin, type IV IF neurofilament subunit M (NF-M), type V IF lamin. *V*, variable region; *H*, homology region; *L*, linker; *S*, stutter; *hu*, human. The sequences were obtained from the GenBank™. GenBank™ accession numbers are as follows (*hu K1*, AF237621; *hu K2e*, AF019084; *hu K3*, X05418 and X05421; *hu K4*, X61028; *hu K5*, NM000424; *hu K6a*, L42583; *hu vimentin*, BC000163; *hu NF-M*, Y00067; *hu-lamin*, M13451).

the mutated *KRT5* cDNA (pK5F/pK5E170KF or pK5F/pK5E418KF), and the combinations with an equimolar ratio of the two mutated *KRT5* cDNA (pK5E170KF/pK5E418KF) were performed using LipofectAMINE reagent (Invitrogen-BRI, Bethesda, MD) according to the distributor's recommendation.

Confocal Laser Analysis—At 24 h after transfection, MDCK cells were washed with phosphate-buffered saline and fixed in 2% paraformaldehyde for 10 min and permeabilized by incubation with 0.5% Triton-X for 10 min. To detect transfected cells, an anti-FLAG antibody (Stratagene, La Jolla, CA) was used to recognize the FLAG tag as a primary antibody (1:100 dilution), and fluorescein-conjugated goat IgG against mouse IgG (Jackson ImmunoResearch Laboratories) was used as a secondary antibody. All cells were observed using a confocal laser scanning microscope (Olympus Fluoview FV300). The cells with keratin aggregates were counted in three different areas, two from each experimental replicate, and the results obtained from six counts were expressed as the mean ± S.D.

RESULTS

A Patient with EBS Compound Heterozygous for a Dominant and a Recessive Mutation in KRT5—*KRT5* and *KRT14*, including all the exons and exon-intron borders, were amplified using the proband's genomic DNA and PCR primers and directly sequenced. There was no mutation in *KRT14*, whereas two novel heterozygous point mutations were found in *KRT5*. One mutation was a G to A transversion at base 508, leading to the exchange of a glutamic acid to lysine at residue 170 (E170K) in the 1A domain (Fig. 1*A*). Another mutation was a G to A transversion at base 1252, leading to the same amino acid substitution at residue 418 (E418K) in the 2B domain (Fig. 1*B*). To verify each mutation, *KRT5* was amplified by PCR with primers generating new restriction enzyme sites recognized by *Bsr*BI (Fig. 1, *D* and *E*). Both mutations (G → A substitutions) resulted in the loss of the restriction enzyme site for *Bsr*BI. These mutations were not found among 100 normal alleles, suggesting that they are not polymorphisms within the normal Japanese population. The altered glutamic acid at residue 170 is within the second residue of the HIP, a well conserved motif among all IF types. Notably, another mutated glutamic acid at residue 418 in the stutter region of the 2B domain was also highly conserved among all types of IF (Fig. 3).

Analysis of *KRT5* of the proband's relatives (II:2, II:7, III:5, III:7) revealed that the E170K mutation was on the paternal allele and the E418K mutation on the maternal allele. His affected uncle (II:2), who showed a milder clinical phenotype (EBS-WC) than the proband's (EBS-K), had only the E170K mutation, while his mother (II:7) and his brother (III:7) with the E418K mutation were clinically normal (Fig. 1*C*). We therefore hypothesized that the E170K mutation caused the EBS-WC phenotype in the family, and we suggest that the proband's clinical phenotype was aggravated by the E418K mutation consistent with the proband being a compound heterozygote for dominant and recessive mutations. Although we could not perform mutational analysis with DNA from his deceased older brother (III:6), the similar clinical severity of EBS suggests that he might also be a compound heterozygote for dominant (E170K) and recessive (E418K) mutations.

The Mutated K5 Protein Disrupts the KIF Network in Vitro—We examined whether the clinical heterogeneity of the EBS family could be demonstrated in a cell culture system. Three mammalian expression vectors containing the wild type *KRT5* (pK5F), the mutated *KRT5* correspondent with the E170K mutation (pK5E170KF), and the E418K mutation (pK5E418KF) were constructed. Four distinct gene transfections using the MDCK cells were performed, including the pK5F alone, a combination with equimolar ratio of pK5F and either of pK5E170KF or pK5E418KF, and a combination with equimolar ratio of the two mutated *KRT5* cDNA (pK5E170KF and pK5E418KF). We chose MDCK cells for the transfection study, because they do not express endogenous K5 and K14 (27), and previous studies have demonstrated that MDCK cells transfected with the human *KRT9* gene could readily incorporate K9 into their endogenous KIF network (28).

A monoclonal antibody against the tag sequence, FLAG, was used to detect the KIF network derived from transfected *KRT5*. The cells transfected with pK5F alone showed fine bundles of keratin filaments extended throughout the cytoplasm without disturbing the cells' morphology (Fig. 4, *A–C*), whereas cells cotransfected with the wild type and mutated *KRT5* gene (pK5E170KF/pK5F), (pK5E418KF/pK5F), or both mutated *KRT5* (pK5E170KF/pK5E418KF) exhibited small ball-like filament aggregates indicating a disruption in the keratin network (Fig. 4, *D–I*).

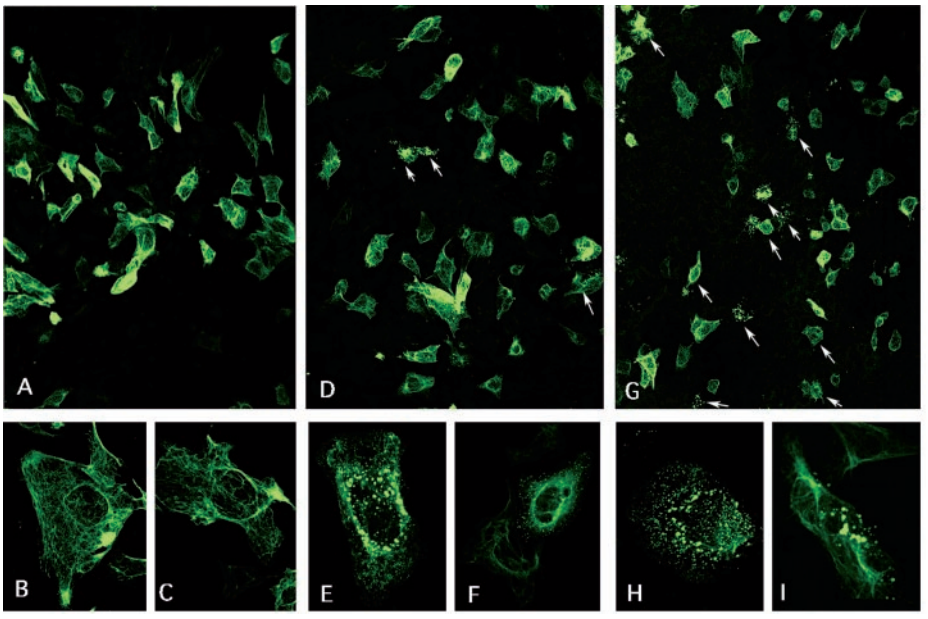

FIG. 4. **The mutated K5 protein disrupts the KIFs network *in vitro*.** MDCK cells were transfected with plasmid pK5F (*A–C*) or cotransfected with pK5E170KF/pK5F (*D–F*) or pK5E170KF/pK5E418KF (*G–I*). To visualize the transfected gene product, cells were stained with a rat monoclonal antibody recognizing the C-terminal sequence of FLAG. Antibody staining was followed by fluorescein isothiocyanate-conjugated goat anti-rat IgG. Cells transfected with pK5F alone had a normal keratin filament network (*A–C*), whereas significantly more cells transfected with pK5E170KF/pK5F (*D–F*), pK5E170KF/pK5E418KF (*G–I*), and a few of the cells transfected with pK5E418KF/pK5F (data not shown) exhibited small ball-like clump formation (*arrows*).

The Stutter Mutation E418K Exacerbated the KIF Network Disturbance—The percentage of the cells with keratin clumping in each transfection assay was as follows, pK5F (1.5 ± 1.8), pK5F/pK5E418KF (4.4 ± 1.7), pK5F/pK5E170KF (12.7 ± 4.3), and pK5E170KF/pK5E418KF (30.0 ± 3.1) (Fig. 5). Statistical analysis showed that significantly more clumped cells were observed in cells cotransfected with pK5E170KF/pK5E418KF than those with pK5F/pK5E170KF ($p < 0.05$). These results suggested that the compound heterozygous mutations (E170K and E418K) within the keratin genes resulted in a more severe keratin network disturbance than the heterozygote for dominant mutation (E170K) alone *in vitro*. This is concordant with the markedly variable severity of EBS phenotype in this family.

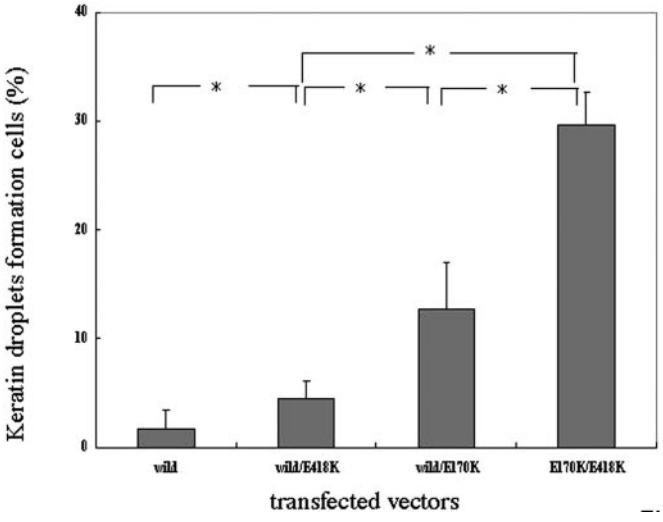

FIG. 5. **The keratin clumps were most frequently observed in cells cotransfected with two mutated *KRT5* (pK5E170KF/pK5E418KF).** The percentage of cells showing keratin aggregate formation among transfected cells was compared. There were significantly more clumps observed in the pK5E170KF/pK5E418KF-transfected cells than in those transfected with pK5E170KF/pK5F. Each value shown represents the mean ± S.D. of six individual samples. Statistical significance of the differences between groups was assessed using the Student's *t* test (*, $p < 0.05$).

DISCUSSION

We have presented the first case of a compound heterozygote with both dominant and recessive mutations, and have demonstrated that the recessive stutter region mutation (E418K) in *KRT5* exacerbated the disruption in the KIF network caused by the dominant mutation (E170K). Although most cases of EBS are autosomal dominant and are caused by missense mutations (9), whether they are fully or partially dominant is still not clear. For example, a partial dominant *KRT14* mutation was found in an EBS family with consanguineous marriage, in which homozygotes had a more severe clinical phenotype than heterozygotes (29).

Usually, compound heterozygous genotypes involve different recessive alleles at the same locus; nevertheless, in some diseases compound heterozygotes with one dominant allele and one recessive allele are known (30). In these cases, although the heterozygous individuals with a recessive allele are phenotypically normal, heterozygotes for the dominant allele develop the disease, and compound heterozygotes usually manifest more severe symptoms than heterozygotes due to the dominant allele (30). For instance, we recently reported such cases of dystrophic epidermolysis bullosa in which type VII collagen was the defective gene (31). Heterozygotes with a dominant glycine substitution mutation in *COL7A1* presented with only toe nail dystrophy without skin fragility, whereas compound heterozygotes for this dominant and recessive mutation led to dystrophic epidermolysis bullosa with marked skin fragility (32–33).

In this study, the mother and brother who were heterozygous for the E418K mutation were clinically normal, however, it was difficult to demonstrate such a subclinical abnormality *in vivo*. We examined how the E418K mutation would influence KIF formation *in vitro* using a transfection assay, for which several studies have been done to examine how EBS mutations perturb the KIF assembly (27, 34, 35). In cultured EBS keratinocytes, keratin aggregates were observed in the majority of EBS-DM keratinocytes but in only a few of the EBS-K cultures (19). Cells transfected with a mutated keratin gene corresponding to each type of EBS showed similar results (19), suggesting that the transfection assay correlated well with the clinical severity of EBS and to the degree to which a particular mutation perturbed filament assembly. In our study, the keratin aggregate formation was observed in significantly more cells when cotransfected with E170K and E418K mutations, which was concordant with the clinical findings. This indicates that the E418K mutation exacerbates KIF disruption, similar to the proband phenotype.

The locations of these EBS mutations in the keratin gene

structure are known to correlate with the disease severity. We know that the ends of the rod domain, HIP and helix termination peptide (HTP), where most EBS-DM mutations are situated, are important areas in which K5 and K14 assemble into filaments (11, 20, 21). Recent research has suggested a role for each region of keratin proteins in heterodimer formation, helix stabilization, and filament assembly (36–38), nevertheless, much about IF structure and function remains unknown.

Particularly, the functions of the IF stutter region have yet to be fully elucidated. The fact that the stutter region is highly conserved among all IFs (7) indicates its importance in IF structure and function. Two computer-based molecular models of the stutter region have been proposed. Steinert and North *et al.* (39, 40) concluded that the stutter was limited to a very short distance and, therefore, did not cause a significant interruption or kink in the coiled-coil α helix structure. Conversely, Brown *et al.* (41) proposed that the unwound coiled-coil model, in which the stutter is located, may contribute to filament flexibility, suggesting that it has a profound effect on the structure and can modify both the assembly and interactions of these protein.

The relatively important contribution of the stutter region in IF formation was shown by Hermann *et al.* (36, 42) using the "stutterless" recombinant type III IF, vimentin. This stutterless vimentin assembled into seemingly normal "uni-length" fibrils, whose formation was the first important step in the assembly, but elongation of these short IFs into longer filaments was slowed down considerably (36, 42). It suggested that the stutter region might play specific roles in the elongation of IFs.

Although glutamic acid at residue 418 in the stutter region has been extremely highly conserved among different IFs (Fig. 3), only a few cells cotransfected with pK5E418K/pK5F exhibited keratin clumping in this study. One reason for this is that, because both glutamic acid and lysine are polar amino acids, this substitution doesn't influence the irregularity of the heptad repeats in the stutter, resulting in a limited influence on KIF formation. Another possibility is that the stutter region may contribute to the elongation of KIFs rather than the coiled-coil heterodimer formation as Hermann suggested (42). Mutations affecting the early stage of KIF assembly would be expected to have the most severe effects on the KIF network for assembly causing EBS-DM, whereas mutations in areas involved in the higher order KIF assembly processes might have more subtle effects similar to EBS-WC. However, the fact the E418K mutation can exacerbate KIF disturbance when combined with a pathogenic mutation on the other allele suggests the stutter region plays some role in the KIF network organization.

In conclusion, we have shown that a compound heterozygote for a dominant and a recessive *KRT5* mutation can result in a more severe disturbance in KIF assembly than a heterozygote for a dominant mutation *in vitro*. We confirmed the fact that the recessive mutation found in the stutter region of the K5 molecule exacerbated this KIF disturbance. These results and transfection assay data emphasize the sensitivity and importance of the stutter region in the structural alterations and organization of the KIF network.

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