Am. J. Hum. Genet. 56:577–585, 1995

Epidermolysis Bullosa Simplex: A Keratin 5 Mutation Is a Fully Dominant Allele in Epidermal Cytoskeleton Function

Karen Stephens,[1] Abraham Zlotogorski,[7] Lynne Smith,[2,3] Pamela Ehrlich,[1] Ellen Wijsman,[1,4] Robert J. Livingston,[1] and Virginia P. Sybert[1,2,5,6]

Department of Medicine, Divisions of [1]Medical Genetics and [2]Dermatology, and Departments of [3]Biological Structure, [4]Biostatistics, and [5]Pediatrics, University of Washington, and [6]Children's Hospital and Medical Center, Seattle; and [7]Department of Dermatology, Hadassah University Hospital, Jerusalem

Summary

To explore the relationship between abnormal keratin molecules, 10-nm intermediate filament (IF) organization, and epidermal fragility and blistering, we sought to determine the functional consequences of homozygosity for a dominant keratin defect. We describe a family with an autosomal dominant skin-blistering disorder, epidermolysis bullosa simplex, Koebner subtype (EBS-K), that has a novel point mutation, occurring in the keratin 5 gene (KRT5), that predicts the substitution of an evolutionarily conserved lysine by an asparagine residue (K173N). Unlike previous heterozygous mutations located within the initial segment of domain 1A of keratin molecules, K173N heterozygosity did not result in severe disease or clumping of keratin filaments. One family member was found to be homozygous for the K173N allele, having inherited it from each of her affected first-cousin parents. Despite a lack of normal keratin 5 molecules, and an effective doubling of abnormal molecules, available for heterodimerization with keratin 14 during IF formation, there were no significant differences in the clinical severity or the ultrastructural organization of the keratin IF cytoskeleton of the homozygous individual. These data demonstrate that the K173N mutation behaves as a fully dominant allele and indicate that a limited number of abnormal keratin molecules are sufficient to impair cytoskeletal function and elicit epidermal fragility and blistering.

Introduction

Several disorders of blistering and fragility of the epidermis result from defects in the keratin intermediate filament (KIF) cytoskeleton. KIFs of epithelial cells are assembled from cytoplasmic type I (acidic) and type II (neutral basic) keratin polypeptides that individually are unstable and immediately form a coiled-coil heterodimer. The heterodimers are the building blocks for the higher-order interactions necessary for assembly of the 10-nm-diameter KIF (reviewed by Steinert 1993). The tissue- and differentiation-specific expression of each mutated keratin gene appears to be the primary determinant of the clinical phenotype in epidermal blistering disorders. A single mutation in either gene encoding the coexpressed basal cell–specific keratins 5 (K5, type II) and 14 (K14, type I) causes at least three of the autosomal dominant disorders known as "epidermolysis bullosa simplex" (EBS) (Bonifas et al. 1991; Coulombe et al. 1991; Dong et al. 1993; Rugg et al. 1993; Stephens et al. 1993; Chan et al. 1994*a*), which are characterized primarily by nonscarring intraepidermal blisters resulting from basal cell cytolysis (Fine et al. 1991). Mutations in the suprabasal cell–specific keratins underlie several autosomal dominant conditions characterized by epidermolytic hyperkeratosis with cytolysis of the suprabasal cells of the spinous layers. For example, mutation of keratins 1 or 10 (K1, type II; and K10, type I) result in the generalized blistering disorder bullous congenital ichthyosiform erythroderma (BCIE; or epidermolytic hyperkeratosis [EH]; Traupe 1989), while mutation of keratin 9 (K9), expressed in the suprabasal cells of sole and palm, results in the localized blistering disorder epidermolytic palmar plantar keratoderma (EPPK; Reis et al. 1994; Torchard et al. 1994).

Identified keratin gene defects, which include point mutations and in-frame deletions (reviewed by Compton 1994), produce abnormal molecules that disrupt heterodimerization and/or interactions between heterodimers necessary for KIF assembly/function. Several lines of evidence provide support for dominant-negative effects of keratin mutations. Disorganized clumps of KIF in basal and suprabasal cells are hallmarks of Dowling-Meara, the most severe type of EBS (EBS-DM), and of BCIE and EPPK (Traupe 1989; Fine et al. 1991), respectively. Perturbation of KIF networks has also been induced in vitro by synthetic mutant oligopeptides or during transient transfections by bacterially expressed mutant K5 or K14 (Coulombe et al. 1991; Letai et al. 1993; Steinert et al. 1993). In addition, basal cell KIF networks and epidermal tissue architecture in the mouse were altered by a K14 transgene with a C-terminal deletion (Vassar et al. 1991).

Received September 28, 1994; accepted for publication December 19, 1994.

Address for correspondence and reprints: Dr. Karen Stephens, University of Washington, Division of Medical Genetics, RG-25, 1959 N.E. Pacific Street, Seattle, WA 98195.

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0002-9297/95/5603-0004$02.00

The identification of keratin mutations that underlie human skin-blistering disorders may reveal critical residues or domains involved in assembly and/or function of normal KIF and may explain the molecular basis for the clumping of certain abnormal KIFs. In contrast to the histopathology of EBS-DM, basal cell KIFs do not aggregate into clumps in other EBS disorders, specifically the Koebner subtype (EBS-K) with generalized blistering and the Weber-Cockayne subtype (EBS-WC) with blistering primarily localized to the hands and feet (reviewed by Fine et al. 1991). Although all three EBS subtypes result from mutations in K5 or K14, the molecular basis for clumping of certain abnormal KIF molecules is not known. Functional assays suggest that mutations at the ends of the keratin central rod domain are the most effective in perturbing KIF organization (Coulombe et al. 1991; Letai et al. 1993; Steinert et al. 1993). Further investigations are necessary to determine the effects that specific mutated residues or domains and/or altered ratios of defective to normal peptides have on KIF organization and function.

In an attempt to identify an individual homozygous for a keratin mutation, we have studied a large family with a consanguineous mating between two related individuals with dominant EBS. The phenotype of an individual homozygous for a defective keratin would reveal the functional consequences of effectively doubling the number of abnormal keratin molecules available for heterodimer formation, in a background of no normal molecules. Although individuals homozygous for mutations for dominant human disease can provide insight into pathogenesis, they are rarely encountered (Wilkie 1994). Examples of disorders in which homozyogotes were found to have more severe disease than did their heterozygous relatives include thalassemia (Weatherall 1994), familial hypercholesterolemia (Hobbs et al. 1992), Charcot-Marie-Tooth syndrome (Lupski et al. 1991), and achondroplasia (Shiang et al. 1994). Fewer dominant disorders in which homozygotes and heterozygotes are affected to a similar degree have been reported, perhaps because clinical recognition of homozygosity is precluded and homozygosity can only be identified by molecular studies. Mutational and/or linkage analyses have identified individuals with fully dominant mutant alleles for amyloidotic polyneuropathy (Holmgren et al. 1992), Huntington disease (The Huntington's Disease Colaborative Research Group 1993), and multiple endocrine neoplasia type 1 (Brandi et al. 1993).

In the present article, we describe a multigenerational family with autosomal dominant EBS-K with a mutation in the beginning of the evolutionarily conserved central rod domain of the K5 gene (KRT5). One affected individual was found to be homozygous for the KRT5 mutation, having inherited the same mutated allele from each of her affected first-cousin parents. KRT5 homozygosity did not affect significantly either the clinical severity of the dominant EBS disorder or the ultrastructure of the basal cell KIF network.

Material and Methods

Family-Tissue Studies

Family EB13, identified and published initially as EBS-DM (Hacham-Zadeh et al. 1988), was recontacted, interviewed, physically examined, and sampled for blood; the homozygous status of individual 31 was unknown at time of exam. Affected family members had nonscarring generalized blistering with obvious exacerbation during the summer and with fever, few or no milia, and no tooth involvement, as reported elsewhere (Hacham-Zadeh et al. 1988). Immortalized cell lines were established by transformation of peripheral lymphocytes with Epstein-Barr virus (Nietzel 1986). From a total of eight individuals, skin biopsies (2 mm), taken from the upper outer arm after rubbing or, in one case, without rubbing, were analyzed (see Results). Skin biopsies were transported in glutaraldehyde and were processed for electron microscopy as described elsewhere (Smith and Sybert 1990).

Linkage Studies

For genotyping, Southern blots were prepared and hybridized as described elsewhere (Kayes et al. 1992), to probes pEFD33.2 (D12S14) and pCMM86 (D17S74). LOD scores were calculated with the computer program LIPED (Ott 1976) modified to handle large numbers of alleles (Schellenberg et al. 1992). To reduce computation time, sampled but unaffected individuals in consanguineous marriages were assumed to be unrelated to the rest of the pedigree. Analyses were done under the assumption that the disease locus and marker loci were in linkage equilibrium. The EBS disease allele was assumed to have a frequency of .005. Allele frequencies used for the marker loci were estimated from the observed alleles in the pedigree, because frequency information was unavailable for a representative control population. It should be noted that, in the case of G531C, this most likely overestimates the frequency of the mutant allele. Analysis with a low allele frequency of .0001 for the G521C mutation gave essentially the same results, however, as were obtained with the estimated allele frequencies.

Mutation Detection

To identify the KRT5 mutation, each exon, along with adjacent intron boundaries, was amplified using flanking intronic primers (P. Ehrlich and K. Stephens, unpublished data) and was sequenced directly (Kretz et al. 1989). The KRT5 G521C mutation was amplified from genomic DNA by PCR with primers K5-672, 5′GCTATGGCTTTGGAGGTGGT; and K5-I1-49, 5′CCTTCTTTCTCTCTCTTTGGC. The primer names designate their positions at bp 672 of KRT5 cDNA sequence (GenBank accession

number M21389; Eckert and Rorke 1988) and bp 49 of intron 1 (I1; P. Ehrlich and K. Stephens, unpublished data). Two hundred fifty nanograms of genomic DNA was amplified in a 50-μl volume with 0.5 μM each primer, 40 nmol dNTPs, 2 Units *Taq* polymerase, and 1 × PCR buffer with 1.5 μM Mg^{++} (Perkin Elmer), for 35 cycles of 94°C for 45 s, 55°C for 30 s, and 72°C for 45 s. Allele-specific hybridization was performed by making duplicate Southern blots (Kayes et al. 1992) of PCR products with the aforementioned primers, prehybridized for >1 h at 55°C as previously described, except that the formamide concentration was reduced to 20% and hybridized to 0.2 × 10^6 dpm ^{32}P-γ-ATP–labeled oligonucleotide/ml. One each of the blots was hybridized, for >1 h, to either the wild type–specific oligonucleotide 5′GCAGATCAAGACCCTCAACA or the mutant-specific oligonucleotide 5′GCAGATCAACACCCTCAACA. Nonspecific hybridization signal was removed with washes in 6 × SSC, 0.1% SDS for 67°C for 10 min.

Results

Linkage of EBS to the Keratin Type II Gene Cluster

To determine whether the skin-blistering disorder in family EB13 was due to a defect in a keratin gene, we analyzed the segregation of polymorphic loci that mapped near the keratin type I and type II gene clusters on chromosomes 17 and 12, respectively. Figure 1 shows the 39 members of family EB13 who participated in our study; this family was described elsewhere as having EBS-DM (Hacham-Zadeh et al. 1988). As shown in table 1, free recombination was observed between the EBS phenotype and D17S74, a locus linked to the type I keratin gene cluster that includes KRT14, the gene encoding K14. These data provided significant evidence to exclude the EBS gene from a location within 19% recombination of either side of D17S74. The maximum LOD score (Z_{max}) between the EBS phenotype and the D12S14 locus was 7.60 at a recombination fraction (θ) of .0. These data provided significant evidence for linkage, in this family, between the defective EBS gene and D12S14, a locus tightly linked to KRT5 (Bonifas et al. 1991). Together, these data strongly indicated that the defective gene responsible for EBS in this family was located in or near the keratin type II gene cluster.

The segregation pattern of D12S14 alleles in this family demonstrated that all affected individuals coinherited the EBS phenotype and the 9.8-kb D12S14 allele from their affected parent; no recombination events were observed (fig. 1). Affected individual 31 inherited a 9.8-kb allele from each of her affected parents, individuals 10 and 11, which, in the absence of genetic recombination, suggested that individual 31 was homozygous for the mutated EBS allele.

Identification of a KRT5 Homozygote

To determine if the EBS defect resided in KRT5, the gene was amplified from genomic DNA of an affected individual and was sequenced directly. A single-base G→C substitution was identified in individual 11 and was confirmed by

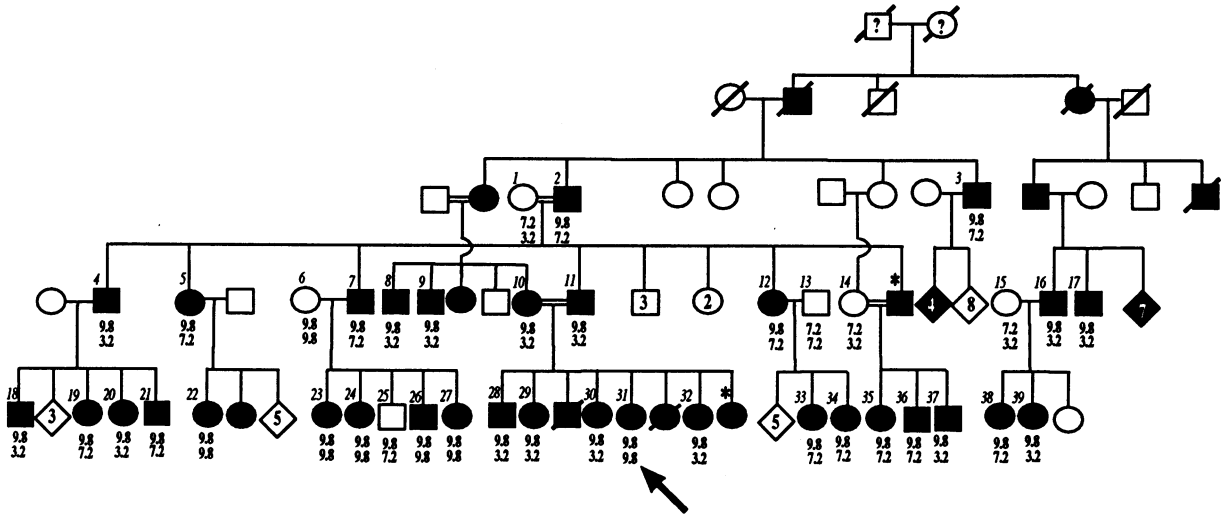

Figure 1 Pedigree of EBS family and segregation of marker D12S14. The four-generational pedigree of family EB13, including four documented consanguineous marriages (*double lines*), is depicted. Numbers to the upper left of symbols are identifiers for each individual who donated blood for this study. The alleles for the polymorphism revealed by D12S14 (9.8, 7.2, and 3.2 kb) are given below each sampled individual. The arrow denotes homozygous individual 31; two of her siblings died in infancy, from causes unrelated to EBS (Hacham-Zadeh et al. 1988). □ = Unaffected male; ○ = unaffected female; ■ = affected male; ● = affected female; ◇ = unaffected, sex not designated; and ◆ = affected, sex not designated. A question mark (?) within a symbol indicates that the affection status was unknown; a diagonal line (/) through a symbol indicates that the individual is deceased; and numbers within the symbols are used to indicate individuals of similar status. Two individuals whose blood was not collected and who were sampled for skin biopsies are indicated by asterisks (*).

Table 1

Genetic Linkage Analysis

Locus	LOD Score at θ = .0	.001	.05	.1	.2	.3	.4	$\hat{Z}_{max}$	$\hat{\theta}$
D17S74	−∞	−26.64	−7.84	−4.65	−1.88	−.66	−.12	.0	.5
D12S14	7.60	7.59	6.87	6.12	4.60	3.04	1.44	7.60	.0
KRT5 (G521C)	7.13	7.12	6.42	5.69	4.20	2.70	1.24	7.13	.0

NOTE.—$\hat{Z}_{max} = Z_{max}$ at θ (maximum estimated recombination fraction).

direct sequencing of this region in affected individuals 2, 10, 31, and 32 and in an unaffected, unrelated individual used as a control. As illustrated in figure 2*B*, for affected individual 2, comigrating G and C fragments of approximate one-half intensity were detected at base number 521 (the first base of the initiating methionine is assumed to be position 1). This transversion, designated "G521C," predicts the replacement of the lysine residue at KRT5 codon 173 by an asparagine (K173N). Direct sequencing demonstrated unambiguously that individual EB13-31 was homozygous for the G521C mutation (fig. 2*C*) and that both of her affected parents were G521C heterozygotes (data not shown).

To assay each family member for the G521C substitution, an allele-specific oligonucleotide (ASO) hybridization assay was developed. With a single exception, all affected family members were heterozygous for the G521C mutation and showed positive hybridization signals to both the wild type– and mutant-specific oligonucleotides (data not shown). The exception was individual 31, the putative homozygote, for whom a positive hybridization signal was detected only with the mutant-specific ASO.

When the KRT5 G521C mutation was used as a genetic marker, Z_{max} was 7.13 (table 1). No recombination events were detected between the mutation and the EBS phenotype. Of the six unaffected family members sampled (fig. 1), none carried the mutation. Although ethnically matched, unrelated, unaffected individuals were not available, we did determine that the mutation was not a common polymorphism in the Caucasian population; no instances of G521C mutation were observed among 92 independent alleles (data not shown).

KIF Histopathology

To verify the previously reported diagnosis of EBS-DM (Hacham-Zadeh et al. 1988) in family EB13 and to analyze the KIF of the homozygous individual, skin biopsies of unaffected and affected family members were examined by electron microscopy. Verification of the EBS-DM diagnosis was critical because the published electron micrographs (Hacham-Zadeh et al. 1988) were not considered by us to demonstrate the characteristic KIF clumping that is diagnostic for EBS-DM. Biopsies were evaluated in a blinded fashion with an initial designation of "EBS-unknown subtype." Examination of unaffected individual 25 revealed KIF of normal organization and abundance, with no evidence of basal cytolysis (fig. 3*a*). Basal cells of affected heterozygous individual 24 (fig. 3*b*) and homozygous individual 31 (fig. 3*c*) were lysed or showed regions of cytoplasmic dissolution, an early indicator of cell lysis. In both

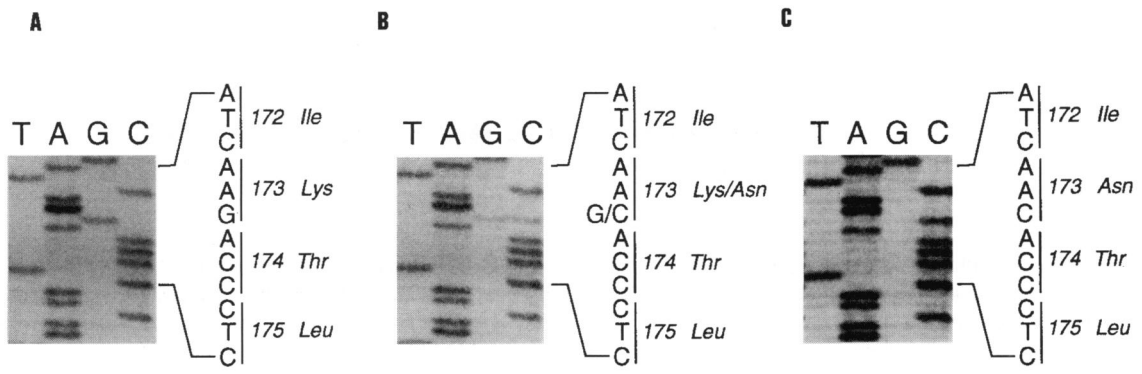

Figure 2 KRT5 sequence of family EB13. The KRT5 gene was amplified from genomic DNA and was sequenced directly as described. The sequence surrounding codon 173 is depicted for an unaffected, unrelated control individual (*A*), affected individual 2 (*B*), and affected individual 31 (*C*).

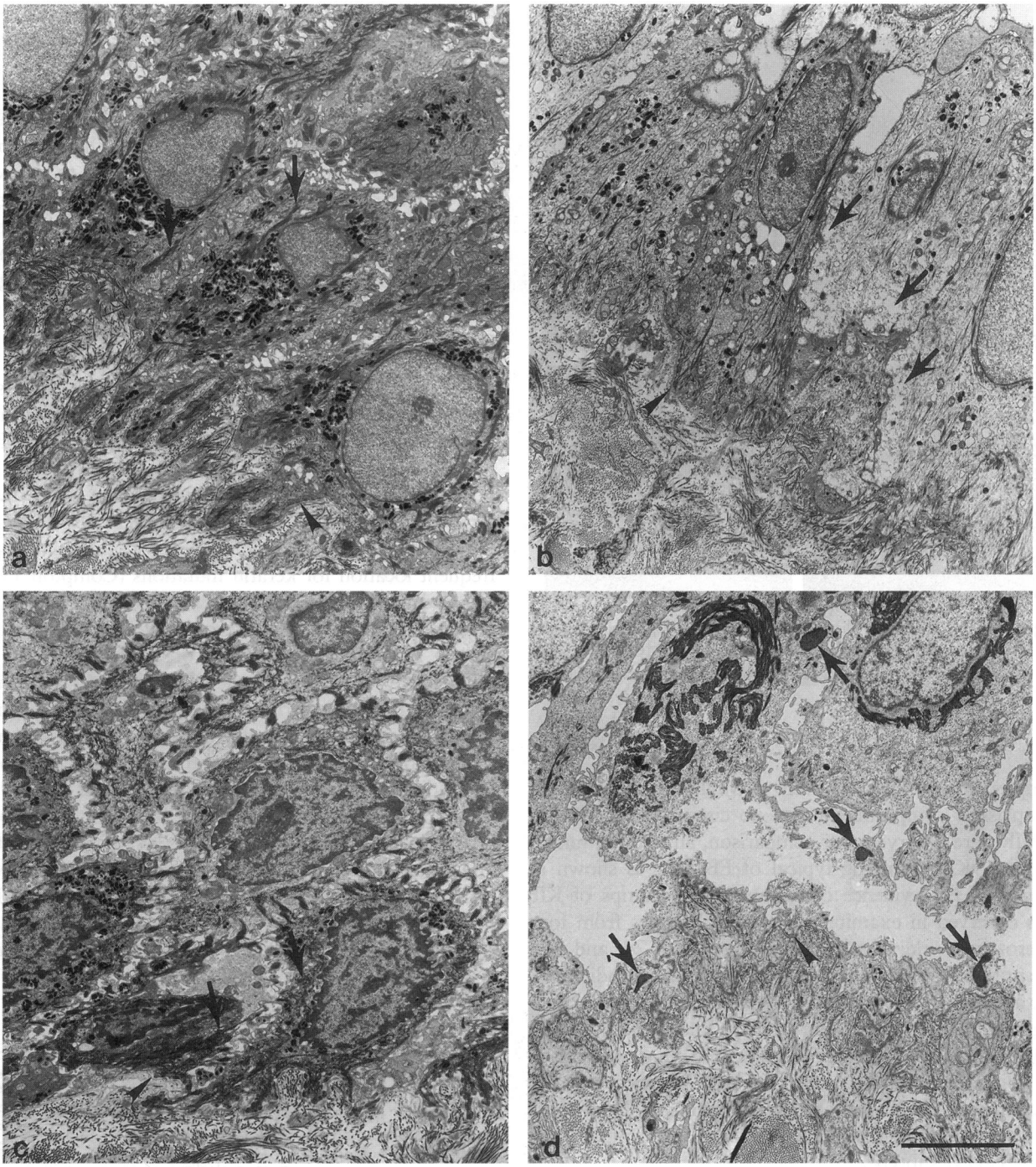

Figure 3 Electron microscopy of skin. *a,* Unaffected individual 25, with basal cell KIF of normal appearance and distribution in the cytoplasm (*arrows*). *b,* Affected heterozygous individual 24, with normal appearing KIF and areas of cytoplasmic dissolution (*arrows*). *c,* Affected homozygous individual EB13-31, with normal appearing KIF (*arrows*). The normal focusing of KIF into desmosomes can be observed at the periphery of some cells. The dermal-epidermal junction is indicated (*arrowhead*), and the scale bar (5 microns) applies to all panels. For comparison, panel *d,* which shows the epidermis of an unrelated patient with EBS-DM (patient EB2-1 in Stephens et al. 1993), reveals deep basal intracellular cleavage of keratinocytes accompanied by circumscribed clumps of keratin filaments (*arrows*) and dense bundles or whorls of keratin filaments that leave the remaining cytoplasm with diminished keratin filament networks.

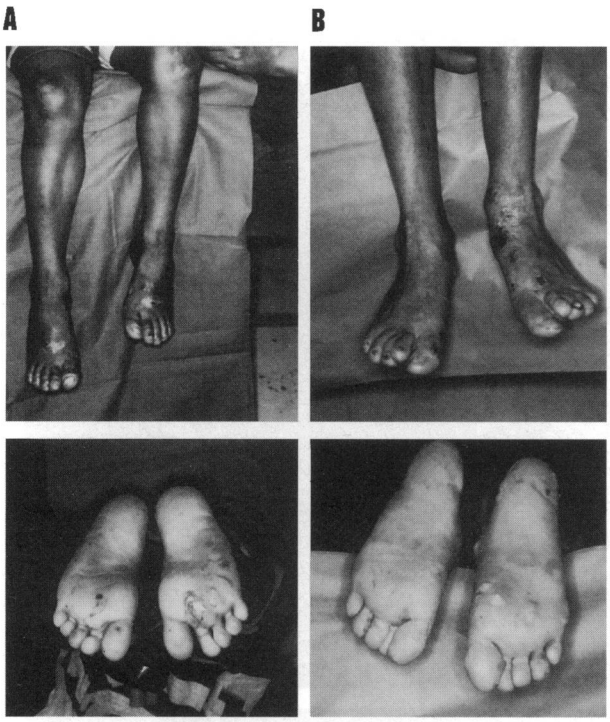

Figure 4 Epidermal blistering: a comparison of blistering severity and extent on legs, feet, and plantar surfaces of G521C heterozygous individual 37 (age 7–8 years; panel *A*) and G521C homozygous individual 31 (age 11 years; panel *B*).

samples, keratin filaments were distributed throughout the cytoplasmic compartment, and no circumscribed clumps of KIF were observed. For comparison, an electron micrograph of KIF clumping typical of EBS-DM is shown in figure 3*d*. No evidence of circumscribed clumps of KIF was observed in examination of skin biopsies from four heterozygous individuals (individuals 7, 23, 24, and 37), one presumably heterozygous (by pedigree data) individual, one affected female who was either heterozygous or homozygous (fig. 1, *asterisks*), and from the known homozygous individual 31. No significant differences were observed in the epidermal ultrastructure of the KRT5 homozygote 31, as compared with her heterozygous family members. These data supported the diagnosis of EBS-Koebner, rather than the EBS-DM subtype.

Clinical Features

Affected individuals had generalized blistering that worsened with heat and improved with age. Both the absence of KIF clumping in basal cells and the worsening with heat are consistent with a diagnosis of EBS-K and do not support the previous diagnosis of EBS-DM (Hacham-Zadeh).

The clinical features of the KRT5 homozygous individual 31 fell within the spectrum of EBS-K disease. Her mother considered her to be more clinically involved than her relatives, because she failed to improve significantly with cold weather, did not show a diminution in her blistering at as early an age as did other affected family members, and required hospitalization at birth. Nevertheless, the clinical findings for homozygous individual 31 were considered not to be outside the spectrum of the disease within her family. Her clinical features did not differ sufficiently to raise concern by her physicians that she had a different or significantly worse disease. Clinical photographs demonstrate the similarity, in blistering severity and extent, observed in the homozygous individual and a heterozygous relative (fig. 4).

Discussion

We have described several lines of evidence demonstrating that a GC base-pair substitution at base-pair position 521 (G521C) of the KRT5 gene is responsible for the EBS-K phenotype observed in a multigenerational family. The G521C mutation predicts a substitution of lysine by an asparagine at codon 173 (designated "K173N"), which is an evolutionarily conserved residue within domain 1A, a frequent location for keratin mutations (Compton 1994). The codon 173 lysine residue was invariant among type II keratin polypeptides, suggesting that it plays an important role in KIF assembly, stability, or function (fig. 5). The mutated K173N residue is the fifth residue of domain 1A located at the amino-terminal end of the central rod domain (fig. 6). Functional studies have shown that the beginning segment of domain 1A is a region critical for formation of heterodimers (Albers and Fuchs 1989; Steinert et al. 1993). The substitution of a charged lysine by an uncharged polar asparagine may disrupt both the secondary structure of K5 and consequent interactions with its keratin mate, K14, or may interfere with higher-order interactions of K5/K14 heterodimers. Further support for a critical function for this initial segment of domain 1A is derived from evidence

Accession Number	Gene	Domain 1A
M21389	human K5	R E Q I K T L N N K F A S F I D
M98776	human K1	R E Q I K S L N N Q F A S F I D
S43646	human K2	R E Q I K T L N N K F A S F I D
X05418	human K3	R E Q I K T L N N K F A S F I D
X07695	human K4	R E Q I K L L N N K F A S F I D
	human K6b	R E Q I K T L N N K F A S F I D
X13320	human K7	S E Q I K T L N N K F A S F I D
M34225	human K8	K E Q I K T L N N K F A S F I D
M10937	mouse K1	R E Q I K S L N D K F A S F I D
X03491	mouse K6	R E Q I K T L N N K F A S F I D
X12789	mouse K8	K E Q I K S L N N K F A S F I D
M63482	rat K8	K E Q I K T L N N K F A S F I D
X14427	frog K5/6	R E Q I K T L N N K F A S F I D
M13811	frog K8	K E Q I K T L N N K F A S F I D

Figure 5 Conservation of K173 residue. The peptide sequences of different type II vertebrate keratins were aligned for comparison of the first 16 residues of domain 1A. The invariant lysine residue is highlighted, and GenBank accession numbers are given; the sequence for human gene K6b is from Tyner et al. (1985).

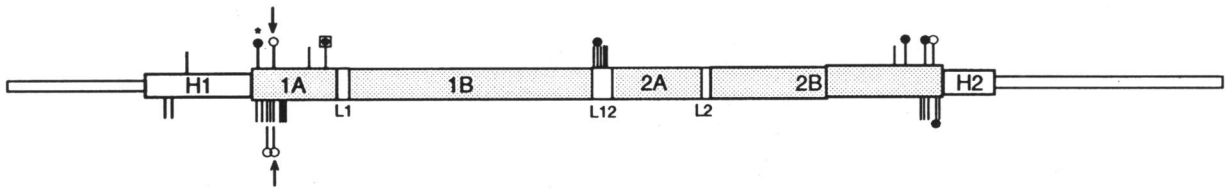

Figure 6 Mutational analysis of keratin peptides. The generic secondary structure of IF proteins, including type I and type II keratins, is depicted with a central rod domain comprising four helical subdomains (1A, 1B, 2A, and 2B), separated by three linker regions (L1, L12, and L2) and flanked by head-and-tail regions of variable sequence and length. Mutated residues in K5 or K14 that are responsible for clinical subtypes of dominant EBS are indicated above the diagram, as EBS-WC (|), EBS-K (♦), and EBS-DM (♀) (Rugg et al. 1993; Chan et al. 1994*a*; Yamanishi et al. 1994; reviewed in Compton 1994). An asterisk (*) denotes the EBS-K mutation (K173N) at residue 5 of domain 1A reported in this article. A K14 mutation reported in an apparent recessive EBS-K family (Hovnanian et al. 1993) is indicated (♕). Below the diagram, keratin mutations underlying other skin-blistering disorders include K1 or K10 mutations in BCIE (|), K9 mutations in EPPK (♁), and K2e mutations in IBS (♦) (Rothnagel et al. 1993, 1994; reviewed in Compton 1994). The arrow denotes an evolutionarily conserved arginine, at residue 10 of domain 1A, that is frequently mutated in K14 for EBS-DM, in K10 for EH, and in K9 for EPPK (Rothnagel et al. 1993; Stephens et al. 1993; Reis et al. 1994).

that mutations in different type I and type II keratins underlie several disorders of keratinization (fig. 6). For example, several type I keratins have a mutational hotspot in arginine, the 10th residue of domain 1A (fig. 6, *arrow*), presumably because of spontaneous deamination of a methylated CpG dinucleotide (Rothnagel et al. 1993; Stephens et al. 1993; Reis et al. 1994). Depending on the tissue- and differentiation-specific expression of the type I keratin, these mutations result in EBS-DM, BCIE, or EPPK (fig. 6).

To date, mutations underlying several disorders characterized by KIF aggregation—specifically, EBS-DM, BCIE, EPPK, and ichthyosis bullosa of Siemens (IBS)—are clustered at either the beginning of domain 1A or the end of domain 2B (fig. 6). In contrast, the mutations identified in blistering disorders without KIF clumping (EBS-K and EBS-WC) are located farther inside the rod domain. The mutation in family EB13 did not result in KIF clumping, despite its location in the initial segment of domain 1A. Three other EBS-K point mutations include one each in the L12 and 2B domains of K14 and one in the 2B domain of K5 (fig. 6). These data, in conjunction with data correlating the position of domain 1A substitutions with the degree of in vitro KIF disruption, support the hypothesis that mutations at the ends of the central rod are more detrimental to KIF structure than are mutations mapping farther within the rod (Letai et al. 1993). However, the K173N mutation in the fifth amino acid residue of domain 1A in this family demonstrates that not all substitutions in the beginning of domain 1A disrupt the KIF structure sufficiently to result in clumping. Perhaps the fifth residue of K5 domain 1A is less critical, or the Asn substitution less detrimental, than amino acid substitutions in the neighboring residues 4, 6, 8, 9, and 10 (the last of which is the mutational hotspot) of K1, K10, and K14 (fig. 6), amino acid substitutions that all resulted in KIF clumping. Another mutation occurring in the initial rod segment and resulting in apparent EBS-K disease was reported recently by Yamanishi et al. (1994). However, the effect on KIF clumping of the Leu→Phe substitution of the seventh residue of domain 1A of K14 cannot be assessed, because electron-microscopic analyses were not reported. Heterozygous mutations in the initial segment of domain 1A that give rise to milder disease without KIF clumping could also result from reduced expression of the mutant protein. In this case, fewer abnormal keratin molecules would be available for dominant-negative interactions with their normal keratin mate during heterodimer formation. Milder disease due to reduced production of abnormal subunits of multimeric or dimeric proteins is well documented for the COL1A1 and c-kit genes responsible for the dominant disorders osteogenesis imperfecta and piebaldism, respectively (Willing et al. 1990, 1992; Spritz et al. 1992). However, this is an improbable explanation for our family, since it would predict increased clinical and histopathological severity for the homozygous individual, which was not the case.

An important result from this study was that K173N homozygosity had no significant effect on either clinical severity or KIF clumping. The similar clinical phenotype of individuals in the heterozygous and homozygous states demonstrated that a level of 50% abnormal K5 molecules, which would result in approximately one-half abnormal K5/K14 heterodimers (under the assumption of equal expression of mutant and normal alleles), was sufficient for epidermal fragility resulting in the EBS-K phenotype. In the epidermis of homozygous individual 31, only mutant K5 molecules were produced, and every K5/K14 heterodimer was abnormal. Yet, there were no ultrastructurally detectable KIF differences observed in the epidermis of the homozygote compared with the epidermis of heterozygous relatives. These data demonstrate that KIF function can be disrupted by a limited number of abnormal K5 molecules and that increasing this number has no further detectable effects on KIF organization or function. The relative number of abnormal keratin molecules necessary for phenotypic expression of epidermal fragility and blistering may be quite small. In vitro, KIFs were disrupted by the presence of

1% abnormal keratin molecules with deletion of amino or carboxy ends of the rod domain (Coulombe et al. 1990).

Current evidence suggests that protein expression of the mutant keratin allele, rather than the specific residue mutated, is a critical determinant in inheritance and phenotype of EBS. As summarized earlier, dominant EBS disease results from heterozygous mutant alleles that produce abnormal keratin molecules, which incorporate into KIF and disrupt their structure and function. As summarized in figure 6, heterozygous missense or in-frame mutations underlying EBS and BCIE have been identified at residues throughout the keratin molecule. The K5 homozygosity documented here has expanded our understanding of dominant EBS disease by demonstrating that a dominant-negative missense mutation of keratin is fully dominant. While the presence of abnormal K5 or K14 molecules underlies dominant EBS disease, the lack of all K5 or K14 molecules, normal or abnormal, appears to underlie recessive EBS disease. Two independent cases of a homozygous K14 null mutation have been reported in an affected child of a consanguineous marriage, resulting in lack of detectable K14 mRNA, K14 protein, and basal cell KIF (Chan et al. 1994*b*; Rugg et al. 1994). In these two families, the heterozygous parents were asymptomatic, presumably because of the lack of disrupting abnormal K14 molecules, while the homozygous offspring was affected because of lack of normal K14 molecules. Interestingly, a precedent for these observations has been established by another multimeric structural protein. Heterozygous mutations producing abnormal procollagen subunits that disrupt collagen fiber assembly/function underlie dominant osteogenesis imperfecta, while homozygous null mutations producing neither normal nor abnormal procollagen result in recessive osteogenesis imperfecta (Schnieke et al. 1983; Pihlajaniemi et al. 1984; Cohn et al. 1990; Willing et al. 1990, 1992). In addition to providing insight into KIF structure and function, the completely dominant behavior of the KRT5 homozygous allele predicts that homozygosity for dominant-negative alleles of subunits of other multimeric structural proteins may behave in a similar fashion.

Note added in proof.—GenBank accession numbers for KRT5 intronic sequences are U05838–U05849.

Acknowledgments

We thank the family members for their willing participation. This research was supported by USPHS grant P01 AR21557. We thank the Welch's Genesis fund for support for sample collection, Drs. Philip Fleckman and Sidney N. Klaus for facilitating access to the family, The Dystrophic Epidermolysis Bullosa Research Association of America, Inc., for support for immortalization of cell lines, and Karen Holbrook for evaluation of previously published micrographs.

References

Albers K, Fuchs E (1989) Expression of mutant keratin cDNAs in epithelial cells reveals possible mechanisms for initiation and assembly of intermediate filaments. J Cell Biol 108: 1477–1493

Bonifas JM, Rothman AL, Epstein EH Jr (1991) Epidermolysis bullosa simplex: evidence in two families for keratin gene abnormalities. Science 254:1202–1205

Brandi ML, Weber G, Svensson A, Falchetti A, Tonelli F, Castello R, Furlani L, et al (1993) Homozygotes for the autosomal dominant neoplasia syndrome (MEN1). Am J Hum Genet 53:1167–1172

Chan Y-M, Anton-Lamprecht I, Yu Q-C, Jackel A, Zabel B, Ernst J-P, Fuchs E (1994*a*) A human keratin 14 "knockout": the absence of K14 leads to severe epidermolysis bullosa simplex and a function for an intermediate filament protein. Genes Dev 8:2574–2587

Chan Y-M, Yu Q-C, LeBlanc-Straceski J, Christiano A, Pulkkinen L, Kucherlapati RS, Uitto J, et al (1994*b*) Mutations in the non-helical linker segment L1-2 of keratin 5 in patients with Weber-Cockayne epidermolysis bullosa simplex. J Cell Sci 107:765–774

Cohn DH, Starman BJ, Blumberg B, Byers PH (1990) Recurrence of lethal osteogenesis imperfecta due to parental mosaicism for a dominant mutation in a human type I collagen gene (COL1A1). Am J Hum Genet 46:591–601

Compton JG 91994) Epidermal disease: faulty keratin filaments take their toll. Nat Genet 6:6–7

Coulombe PA, Chan Y-M, Albers K, Fuchs E (1990) Deletions in epidermal keratin leading to alterations in filament organization in vivo and in intermediate filament assembly in vitro. J Cell Biol 111:3049–3064

Coulombe PA, Hutton ME, Letai A, Hebert A, Paller AS, Fuchs E (1991) Point mutations in human keratin 14 genes of epidermolysis bullosa simplex patients: genetic and functional analyses. Cell 66:1301–1311

Dong W, Ryynanen M, Uitto J (1993) Identification of a leucine-to-proline mutation in the keratin 5 gene in a family with the generalized Kobner type of epidermolysis bullosa simplex. Hum Mutat 2:94–102

Eckert RL, Rorke EA (1988) The sequence of the human epidermal 58-kD (#5) type II keratin reveals an absence of 5′ upstream sequence conservation between coexpressed epidermal keratins. DNA 7:337–345

Fine J-D, Bauer EA, Briggaman MD, Carter DM, Eady RAJ, Esterly MB, Holbrook KA, et al (1991) Revised clinical and laboratory criteria for subtypes of inherited epidermolysis bullosa. J Am Acad Dermatol 24:119–135

Hacham-Zadeh S, Rappersberger K, Livshin R, Konrad K (1988) Epidermolysis bullosa herpetiformis Dowling-Meara in a large family. J Am Acad Dermatol 18:702–706

Hobbs HH, Brown MS, Goldstein JL (1992) Molecular genetics of the LDL receptor gene in familial hypercholesterolemia. Hum Mutat 1:445–466

Holmgren G, Bergstrom S, Drugge U, Lundgren E, Nording-Sikstrom C, Sandgren O, Steen L (1992) Homozygosity for the transthyretin-Met30-gene in seven individuals with familial amyloidosis with polyneuropathy detected by restriction en-

zyme analysis of amplified genomic DNA sequences. Clin Genet 41:39–41

Hovnanian A, Pollack E, Hilal L, Rochat A, Prost C, Barrandon Y, Goossens M (1993) A missense mutation in the rod domain of keratin 14 associated with recessive epidermolysis bullosa simplex. Nat Genet 3:327–331

Huntington's Disease Collaborative Research Group, The (1993) A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. Cell 72:971–983

Kayes LM, Schroeder WT, Marchuk DA, Collins FS, Riccardi VM, Duvic M, Stephens K (1992) The gene for a novel epidermal antigen maps near the neurofibromatosis 1 gene. Genomics 14:369–376

Kretz KA, Carson GS, O'Brien JS (1989) Direct sequencing from low-melt agarose with Sequenase. Nucleic Acids Res 17:5864

Letai A, Coulombe PA, McCormick MB, Yu Q-C, Hutton E, Fuchs E (1993) Disease severity correlates with position of keratin point mutations in patients with epidermolysis bullosa simplex. Proc Natl Acad Sci USA 90:3197–3201

Lupski JR, de Oca-Luna RM, Slaugenhaupt S, Pentae L, Gussetta V, Trask BJ, Saucedo-Cardenas O, et al (1991) DNA duplication associated with Charcot-Marie-Tooth disease type 1A. Cell 66:219–232

Nietzel H (1986) A routine method for the establishment of permanent growing lymphoblastoid cell lines. Hum Genet 73:320–326

Ott J (1976) A computer program for linkage analysis of general human pedigrees. Am J Hum Genet 28:528–529

Pihlajaniemi T, Dickson LA, Pope FM, Korhonen VR, Nicholls A, Prockop DJ, Myers JC (1984) Osteogenesis imperfecta: cloning of a pro-2(I) collagen gene with a frameshift mutation. J Biol Chem 259:12941–12944

Reis A, Henniew H-C, Langbein L, Digweed M, Mischke D, Drechsler M, Schrock E, et al (1994) Keratin 9 gene mutations in epidermolytic palmoplantar keratoderma (EPPK). Nat Genet 6:174–179

Rothnagel JA, Fisher MT, Axtell SM, Pittelkow MR, Anton-Lamprecht I, Huber M, Hohl D, et al (1993) A mutational hot spot in keratin 10 (KRT10) in patients with epidermolytic hyperkeratosis. Hum Mol Genet 2:2147–2150

Rothnagel JA, Traupe H, Wojcik S, Huber M, Hohl D, Pittelkow MR, Saeki H, et al (1994) Mutations in the rod domain of keratin 2e in patients with ichthyosis bullosa of Siemens. Nat Genet 7:485–489

Rugg EL, McLean WHI, Lane EB, Pitera R, McMillan JR, Dopping-Hepenstal PJC, Navsaria HA, et al (1994) A functional "knockout" of human keratin 14. Genes Dev 8:2563–2573

Rugg EL, Morley SM, Smith FJD, Boxer M, Tidman MJ, Navsaria H, Leigh IM, et al (1993) Missing links: Weber-Cockayne keratin mutations implicate the L12 linker domain in effective cytoskeleton function. Nat Genet 5:294–300

Schellenberg GD, Bird TD, Wijsman EM, Orr HT, Anderson L, Nemens E, White JA, et al (1992) Genetic linkage evidence for a familial Alzheimer's disease locus on chromosome 14. Science 258:668–671

Schnieke A, Harbers K, Jaenisch R (1983) Embryonic lethal mutation in mice induced by retrovirus insertion into the 1(I) collagen gene. Nature 304:315–320

Shiang R, Thompson LM, Zhu YZ, Church DM, Fielder TJ, Bocian M, Winokur ST, et al (1994) Mutations in the transmembrane domain of FGGR3 cause the most common genetic form of dwarfism, achondroplasia. Cell 78:335–342

Smith LT, Sybert VP (1990) Intra-epidermal retention of type VII collagen in a patient with recessive dystrophic epidermolysis bullosa. J Invest Dermatol 94:261–264

Spritz RA, Giebel LB, Holmes SA (1992) Dominant negative and loss of function mutations of the c-*kit* (mast/stem cell growth factor receptor) proto-oncogene in human piebaldism. Am J Hum Genet 50:261–269

Steinert PM (1993) Structure, function, and dynamics of keratin intermediate filaments. J Invest Dermatol 100:729–734

Steinert PM, Yang J-M, Bale SJ, Compton JG (1993) Concurrence between the molecular overlap regions in keratin intermediate filaments and the locations of keratin mutations in genodermatoses. Biochem Biophys Res Commun 197:840–848

Stephens K, Sybert VP, Wijsman EM, Ehrlich P, Spencer A (1993) A keratin 14 mutational hot spot for epidermolysis bullosa simplex, Dowling-Meara: implications for diagnosis. J Invest Dermatol 101:240–243

Torchard D, Blanchet-Bardon C, Serova O, Langbein L, Narod S, Janin N, Goguel AF, et al (1994) Epidermolytic palmoplantar keratoderma cosegregates with a keratin 9 mutation in a pedigree with breast and ovarian cancer. Nat Genet 6:106–110

Traupe H (1989) The ichthyoses. Springer, Berlin

Tyner A, Eichman M, Fuchs E (1985) The sequence of a type II keratin gene expressed in human skin: conservation of structure among all intermediate filament genes. Proc Natl Acad Sci USA 76:4350–4354

Vassar R, Coulombe PA, Degenstein L, Albers K, Fuchs E (1991) Mutant keratin expression in transgenic mice causes marked abnormalities resembling a human genetic skin disease. Cell 64:365–380

Weatherall DJ (1994) The thalassemias. In: Stamatoyannopoulos G, Nienhuis AW, Majerus PW, Varmus H (eds) The molecular basis of blood diseases. WB Saunders, Philadelphia, pp 157–205

Wilkie AOM (1994) The molecular basis of genetic dominance. J Med Genet 31:89–98

Willing MC, Cohn DJ, Byers PH (1990) Frameshift mutation near the 3′ end of the COL1A1 gene of type 1 collagen predicts an elongated pro(I) chain and results in osteogenesis imperfecta type 1. J Clin Invest 85:282–290

Willing MC, Pruchno CJ, Atkinson M, Byers PH (1992) Osteogenesis imperfecta type I is commonly due to a COL1A1 null allele of type I collagen. Am J Hum Genet 51:508–515

Yamanishi K, Matsuki M, Konishi K, Yasuno H (1994) A novel mutation of Leu^{122} to Phe at a highly conserved hydrophobic residue in the helix initiation motif of keratin 14 in epidermolysis bullosa simplex. Hum Mol Genet 3:1171–1172