Long-Range Polymerase Chain Reaction for Specific Full-Length Amplification of the Human Keratin 14 Gene and Novel Keratin 14 Mutations in Epidermolysis Bullosa Simplex Patients

To the Editor:

Epidermolysis bullosa simplex (EBS) is an autosomal dominant genetic skin disorder characterized by intraepidermal blistering in response to mild physical trauma (Irvine and McLean, 1999). In EBS, histologic and/or ultrastructural examination shows that blistering occurs in the subnuclear cytoplasm of basal keratinocytes. The disorder can be subdivided into three subgroups depending on the severity of the disease (Fine *et al*, 2000). The EBS Dowling–Meara variant is the most severe variant with widespread herpetiform blistering occurring within the first week following birth. EBS Weber–Cockayne and EBS Köbner are milder forms of the disease where blistering is mainly localized to hands and feet. These forms of EBS are predominantly caused by dominant-negative mutations, either missense or small in-frame insertion–deletion mutations, in keratin 5 (K5) or K14 (Irvine and McLean, 1999). K5 and K14 polymerize to form the intermediate filament cytoskeleton of basal keratinocytes (Coulombe and Omary, 2002). Structural weakening of this cytoskeletal system due to genetic mutations leads to fragility of basal keratinocytes and therefore to hereditary skin blistering.

Mutation detection strategies for the K5 gene (*KRT5*) have been described previously (Stephens *et al*, 1997; Whittock *et al*, 2000) but reliable mutation detection for the K14 gene (*KRT14*) has proved to be more difficult due to the presence of pseudogenes. One intron-containing pseudogene for K14 was reported some time ago (Savtchenko *et al*, 1988); however, a more recent survey of the emerging human genome data reported a second partial pseudogene for K14 (Hesse *et al*, 2001). Mutational analysis for K14 has previously been carried out using cDNA prepared from skin biopsies or, more often, using pairs of polymerase chain reaction (PCR) primers specific for each exon of the functional K14 gene (Chen *et al*, 1995; Müller *et al*, 1998; Shemanko *et al*, 1998). Another strategy based on long-range amplification of *KRT14* and its pseudogene(s) involved restriction digestion of genomic DNA (Hut *et al*, 2000). Here, we present a robust long-range PCR method that specifically amplifies the whole of the K14 gene from genomic DNA. This allows sequencing of the entire K14 gene from one PCR product using internal primers. Using this method, we have identified three novel mutations in K14 in EBS patients.

The reported K14 genomic sequence was used for homology searching of the April 2002 version of the human genome sequence, via the UCSC website (http://genome.ucsc.edu). This revealed two full-length intron-containing pseudogenes, which were 94.9% and 94.8% identical to the functional gene. This was consistent with the recent survey of keratin genes in the emerging genome data (Hesse *et al*, 2001). These pseudogenes appear to be located several megabases of DNA centromeric to the type I keratin gene cluster on chromosome 17, assuming the current genome assembly to be correct in this region (data not shown). Alignment of the flanking regions of the K14 gene and its two pseudogenes identified a number of places where primers might be placed to specifically amplify the functional gene. A number of these were tested for ease of amplification and specificity, of which the following primers and conditions were found to be optimal. Forward primer K14spf2 (5′-ATT TCA GGG ATG GGA CAG AC-3′) and reverse primer K14spr2 (5′-GAG GGG ATC TTC CAG TGG GAT CT-3′) were used in 50 μl reactions containing 100 ng genomic DNA, 2 μg per ml each primer, 250 μM of each dNTP and Expand Long Template PCR system, 0.2 μl Long Template enzyme mix, and Long Template reaction buffer 1 (Roche Diagnostics, Lewes, U.K.). A "touchdown" PCR program was used. The PCRs were initially denatured at 94°C for 2 min followed by 94°C 30 s, 62°C 30 s, 72°C 4 min for two cycles; then 94°C 30 s, 60°C 30 s, 72°C 4 min for two cycles; 94°C 30 s, 59°C 30 s, 72°C 4 min for 25 cycles; followed by 72°C for 5 min. PCR was performed in a computer-controlled ThermoHybaid MBS thermocycler with heated lid, without use of mineral oil. Simulated tube control with a 50 μl volume setting were used for the PCR program within the MBS software.

The resultant 5099 bp PCR product (**Fig 1**) spans all of the *KRT14* gene including 624 bp upstream of the initiation codon ATG of K14 and 69 bp downstream of the TGA codon. Using this method we identified three novel EBS mutations found at either end of the K14 gene, by sequencing with internal primers (**Fig 1**). Two mutations, R148C and S128del, were found in the 1A domain, encoded by exon 1 of *KRT14*. The third mutation, R416P, was found in the helix termination motif at the end of the 2B domain, encoded by exon 6 (**Fig 1**). The mutations were confirmed by tried and tested PCRs specific for the individual exons concerned, exons 1 and 6 (Shemanko *et al*, 1998). The same mutations were found by sequencing of these fragments (data not shown). Mutation R148C creates a novel *Bgl* II site, S128del ablates an *Mnl* I site, and R416P destroys an *Fnu4H* I site. All three novel mutations were excluded from 50 normal ethnically matched controls by the appropriate restriction digests (data not shown). Mutations S128del and R416P are located in the helix initiation and termination motifs of K14, respectively. These are known mutation hotspots for EBS Dowling–Meara (Irvine and McLean, 1999), consistent with the observed phenotypes of these two patients. Mutation R148C is located in the second half of the 1A domain, a known hotspot associated with milder phenotypes (Irvine and McLean, 1999), consistent with the diagnosis of EBS Weber–Cockayne in the patient examined here. Clinical details of these patients will be reported elsewhere.

Avoiding pseudogene amplification is a particular problem in the molecular diagnosis of keratin disorders as many keratins have multiple pseudogenes. A recent survey of the human genome revealed that, of >250 intermediate filament genes, about 75% are keratin-related pseudogenes (Hesse *et al*, 2001). The problem is particularly prominent in diagnosis of the two most common types of pachyonychia congenita (PC), where the PC-1 phenotype is caused by mutations in K6a or K16 and the PC-2 variant

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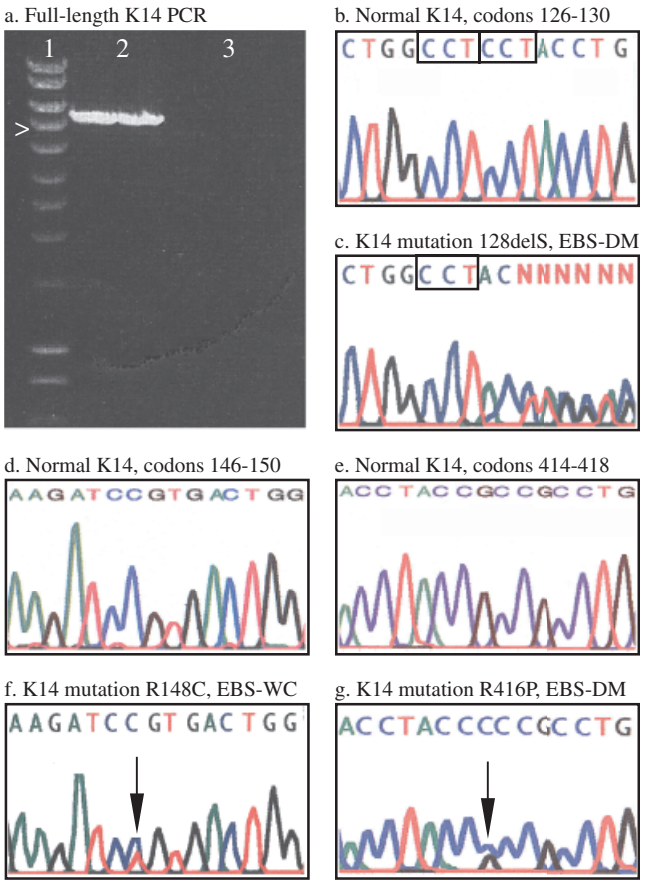

Figure 1. Long-range PCR to detect novel KRT14 mutations in EBS patients. (*a*) PCR amplification of a 5.1 kb genomic DNA fragment containing all exons and flanking regions of the functional *KRT14* gene analyzed on a 1% agarose/0.5 x TBE gel. *Lane 1*, molecular weight markers (Hyperladder I, Bioline, London, U.K.), the *arrow* indicates 5 kb marker; *lane 2*, typical PCR product amplified from a normal control DNA; *lane 3*, negative control showing no amplification. (*b*)–(*g*) Direct sequencing of *KRT14* long-range PCR products with internal primers. Sequences shown in *panels b*, *c*, *d*, and *f* were generated with sequencing primer K14p8 (+ strand, 5′ GGG GGA GCC TAT GGG TTG GGG 3′). Sequences shown in *panels e* and *g* were generated with sequencing primer K14pm20 (+ strand, 5′ CAG AGC GGC AAG AGC GAG AT 3′). (*b*) Normal control sequence from exon 1, showing codons 126–130. A CCT repeat is present in this region of the gene (*boxed*). (*c*) The same region as shown in (*b*) derived from a patient with EBS Dowling–Meara, showing heterozygous deletion mutation 383delCCT, predicting the amino acid change 128delS in the helix initiation motif of the K14 polypeptide. The first CCT repeat is unchanged (*boxed*) but the second CCT is deleted, leading to overlapping sequence traces. This mutation occurs in a known mutation hotspot associated with severe EBS phenotypes. (*d*) Normal control sequence from exon 1, showing codons 146–150. (*e*) Normal control sequence from exon 6, showing codons 414–418. (*f*) The same region as shown in (*d*) derived from a patient with EBS Weber–Cockayne, showing heterozygous transition mutation 442C→T, predicting the amino acid change R148C in the latter half of the 1A domain of the K14 polypeptide. This is a known mutation hotspot associated with milder EBS phenotypes. (*g*) The same region as shown in (*e*) derived from a patient with EBS Dowling–Meara, showing heterozygous transversion mutation 1247G→C, predicting the amino acid change R416P in the helix termination motif of the K14 polypeptide. This is a known mutation hotspot associated with more severe EBS phenotypes.

is caused by mutations in K6b or K17. Humans possess at least six homologous K6-like isogenes and/or pseudogenes (Takahashi *et al*, 1995). K16 and K17 each have two pseudogenes (Troyanovsky *et al*, 1992; Smith *et al*, 1999a). Over the last few years, we have developed robust long-range PCR strategies to enable mutation screening for all coding regions of the K6a, K16, and K17 genes (Smith *et al*, 1999a; 1999b; Terrinoni *et al*, 2001), thus enabling mutation detection and prenatal diagnosis based on genomic DNA. Similarly, the long-range PCR strategy described here is suitable for detection of mutations in all exons of the K14 gene, circumventing the need for enzyme digestion of PCR fragments or calibration of specific PCR for each of the individual exons. A further advantage of this system is that it may detect larger heterozygous genomic deletions, say involving one or two exons, that would be missed by PCR of individual exons. For example, a homozygous 1163 bp intragenic deletion mutation was recently reported in the ECM1 gene in a lipoid proteinosis family (Hamada *et al*, 2002). A similar mutation would be missed in heterozygotes by PCR of individual exons but would be detected by the method described here. Therefore, the long-range PCR method should be considered for other genes where there is no particular pseudogene problem but where a group of patients exists that lack mutations. The only drawback that we have observed in application of this methodology is that DNA samples that are partially degraded can be more difficult to amplify for obvious reasons; however, peripheral blood lymphocyte DNA and mouthwash DNA samples prepared in our laboratory by a standard high-salt extraction method generally work well.

In conclusion, the method described here should ease the molecular diagnosis of EBS in the routine laboratory and perhaps identify mutations missed by other strategies.

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