Mutation of Human Keratin 18 in Association with Cryptogenic Cirrhosis

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Abstract

Mutations in 11 of the more than 20 keratin intermediate filaments cause several epidermal and oral associated diseases. No disease-associated mutations have been described in keratin 8 or 18 (K8/18) which are the major keratin pair in simple-type epithelia, as found in the liver, pancreas, and intestine. However, transgenic mice that express mutant keratin 18 develop chronic hepatitis, and have an increased susceptibility to drug-induced hepatotoxicity. Also, ectopic expression of epidermal K14 in mouse liver results in chronic hepatitis, and disruption of mouse K8 leads to embryo lethality with extensive liver hemorrhage. We tested if patients with liver disease of unknown cause may harbor mutations in K18. We describe a his127→leu (H127L) K18 mutation in a patient with cryptogenic cirrhosis that is germline transmitted. The K18 H127L isolated from the liver explant, or after expression in bacteria, showed an altered migration on two-dimensional gel analysis as compared with normal human liver or bacterially expressed K18. Electron microscopy of in vitro assembled K18 H127L and wild type K8 showed an assembly defect as compared with normal K8/18 assembly. Our results suggest that mutations in K18 may be predispose to, or result in cryptogenic cirrhosis in humans. (*J. Clin. Invest.* 1997. 99:19–23.) Key words: cirrhosis • keratin 18 mutation • intermediate filaments • liver • cytokeratin

Introduction

Intermediate filament (IF)[1] proteins make up a large family of cytoskeletal proteins that are found in higher eukaryotes, with tissue/cell specific expression of its members (1, 2). For example, keratin (K) IF are expressed in epithelial cells, neurofilaments in neuronal cells, desmin in muscle, and vimentin in mesenchymal cells. Two important features of keratins are their obligate noncovalent heteropolymeric association and their cell-type preferential expression in specific keratin heteropolymeric combinations (3). As such, epithelial cells express at least one type I keratin (K9-K20) and one type II keratin (K1-K8) which oligomerize to form soluble tetramers or cytoskeletal filaments. For example, simple-type glandular epithelia such as the intestine, liver, and pancreas preferentially express K8/18, whereas basal keratinocytes express K5/14.

Although the function of IF proteins, including keratins, is poorly understood, mutations in 11 of the more than 20 keratins have been described in several oral and epidermal diseases (4–11). In addition, mutations in neurofilament heavy chain have been described in several patients with sporadic amyotrophic lateral sclerosis (12). One important clue that helped lead to the subsequent search for epidermal keratin mutations in patients was based on a transgenic animal model. This model expressed a mutant form of K14, with a resultant disease phenotype that mimicked epidermolysis bullosa simplex (13), and subsequent identification of a K14 mutation in a patient with the equivalent human disease (14). In the case of K8 and K18, no mutations have been described in any human disease. However, transgenic mice that expressed an arg89→cys K18 developed chronic hepatitis with increased hepatocyte fragility (15), and an increased susceptibility to drug-induced liver disease (16). In this animal model, arg89 was chosen as a K18 mutation site because it is a highly conserved residue that is associated with mutations in several epidermal diseases (4–7). Two other independent lines of evidence, based on animal studies, suggested that the liver is a likely target organ if mutations in K8/18 are to be involved in human disease. First, ectopic transgene expression of epidermal K14 in mouse liver resulted in chronic hepatitis in association with keratin filaments reorganization (17). Second, 94% of K8-null mice die at a late embryonic stage in association with extensive spontaneous liver hemorrhage (18). Given the K8/18 transgenic animal results, and the previous findings of human epidermal keratin diseases that were similar to animal models, we asked if patients with liver disease of unknown etiology harbor mutations in K18. As described below, we identified one of 28 cryptogenic cirrhosis patients who had a his127→leu (H127L) K18 mutation. This mutation causes an in vitro defect in filament assembly, and leads us to propose that K18 mutations may predispose to, or may directly cause liver disease.

Methods

Patient samples. Liver explants were obtained during liver transplantation or as part of liver resection done for clinically indicated

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1. *Abbreviations used in this paper:* H127L, his127→leu mutation; IF, intermediate filaments; K, keratin; SSCP, single-strand conformation polymorphism; WT, wild type.

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reasons. The use of remaining surgically resected tissues was approved by Human Subject Committees at California Pacific Medical Center and the University of California San Francisco. Liver specimens were either snap frozen then stored in liquid nitrogen or were stored at −80°C after resection. Patient 459, the only patient who was found to have a K18 mutation within the region that we examined, was diagnosed with cryptogenic cirrhosis at the age of 61 when he presented for the first time with ascites and esophageal variceal bleeding. He had no previous history of liver disease, and other potential causes of known liver diseases were excluded with the appropriate tests (see below). It is not known if patient 459 had any family history of liver disease.

Criteria used for the diagnosis of cryptogenic cirrhosis. The criteria included absence of serologic markers for known liver diseases, absence of a history of chronic ingestion of alcohol and any potentially hepatotoxic drug, and a supporting liver biopsy. Serologic testing included absent hepatitis B surface antigen, lack of antibodies to hepatitis C and to nuclear/smooth muscle/mitchondrial antigens, transferrin saturation < 60%, ferritin < 500, and normal ceruloplasmin and α1-antitrypsin. In the case of patient 459 liver biopsy, no Mallory bodies were noted.

Single strand conformation polymorphism (SSCP) and DNA sequencing. Genomic DNA was isolated from the liver specimen using a QIAamp Tissue Kit (QIAGEN Inc., Chatsworth, CA). For SSCP analysis, a 240-bp fragment of exon 1 that corresponds to amino acid residues 59–138 was amplified in duplicates/liver specimen using the γ-^{32}P-end–labeled primers: (+) 5′-TCCGGGGGCCTGGCCAC-CGGGATA-3′ and (−) 5′-CTGAGCCCTCAGGTCCTCGAT-3′. This includes amino acids 59–66 and 132–138 which correspond to primer sequences. Amplifications were done at 94°C (4 min) then 35 cycles of 94°C for 1 min, 55°C for 1 min, then 72°C for 1 min. The PCR products were analyzed using nondenaturing polyacrylamide (6%) gels containing 10% glycerol.

For sequencing, a 650-bp fragment which includes exon 1 and part of intron 1 was amplified from patient 459 genomic DNA and from DNA isolated from whole blood obtained from his daughter, and from a normal liver specimen using the primer pairs: (+) 5′-CGCCCAGCTACGGCGCCCGGCCGGTCAGCA-3′ and (−) 5′-GTCTCCTCCTCTTTACAGGCCTTTCCTTAC-3′. Sequencing was done using an automated sequencer. The 650-bp product was also subcloned into pBluescript SK+ and 13 individual clones were then sequenced.

Expression of wild type and mutant K18. Site-directed mutagenesis, using a Transformer kit (Clontech, Palo Alto, CA), was done using wild type K18 cDNA as described (19). Bacterial expression of K8 and K18 was done using the pET system (Novagen Inc., Madison, WI) whereby human K8 and K18 cDNA (19) were individually subcloned into the EcoRI site of pET-24a(+) and pET-23a(+), respectively. In both cases, vector sequences between the NdeI and EcoRI sites of the multiple cloning region were deleted to allow expression of intact K8 or K18 without any fusion component arising from vector sequences (not shown). K8 and K18 constructs were also subcloned into a pMRB101 mammalian expression vector as described (19). Transfection into NIH-3T3 cells was done using LipofectAMINE liposomes (Life Technologies Inc., Gaithersburg, MD) as recommended by the supplier. Cells were used three days after transfection for immunoprecipitation or immunofluorescence analysis.

Characterization of the K8/18 protein. Bacterially expressed K8 and K18 were purified using Fast Protein Liquid Chromatography as described (20). Immunoprecipitation of K8/18 from cultured human colonic HT29 cells or from transfected NIH-3T3 cells was done using monoclonal antibody L2A1 which specifically recognizes human K18 (19). Immunofluorescence staining of transfected NIH-3T3 cells (grown on cover slips) was done using monoclonal antibody L2A1 with visualization using Texas-red conjugated goat anti–mouse IgG (19). In vitro filament assembly was done as described (20), except that dialysis of the keratins (150 μg/ml of bacterially expressed type I and II keratins at a 1:1 ratio [wt/wt]) was done in 10 mM Tris–HCl

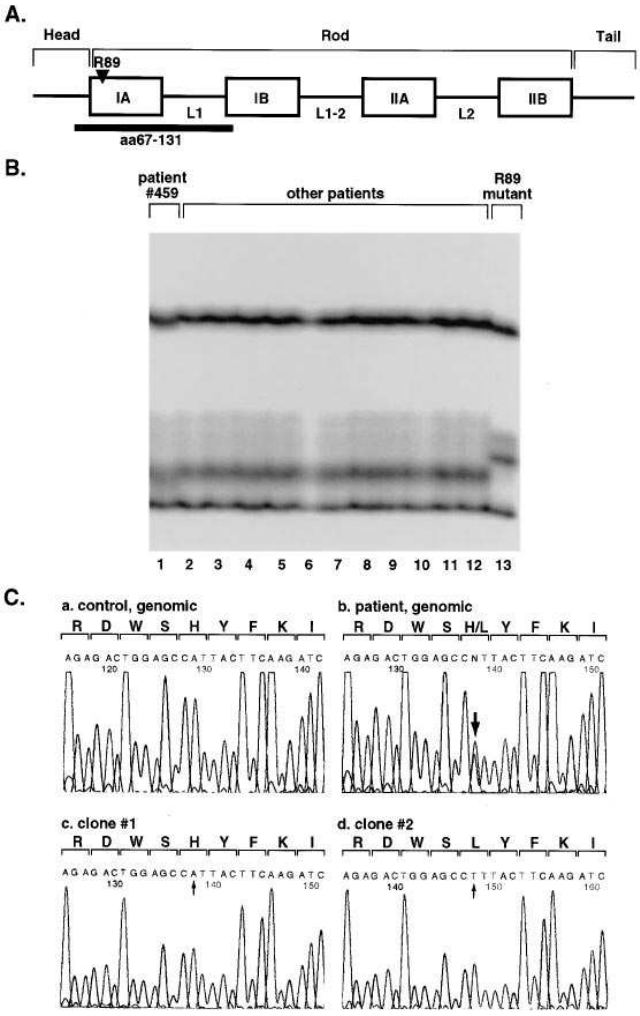

Figure 1. Analysis of K18 mutations in genomic DNA from patients with liver disease. (*A*) IF proteins (including K18) consist of two globular domains (head and tail) and a central coil-coil α-helix (rod). The rod domain consists of relatively conserved subdomains IA, IB, IIA, and IIB which are divided by less conserved linker (L) subdomains L1, L1-2, and L2. A hot mutation spot in epidermal keratin diseases involves the proximal portion of region IA in the rod domain, which includes a highly conserved arg in K18 (R89), which was mutated in the transgenic mouse model that develops chronic hepatitis (15). (*B*) SSCP was used to amplify genomic DNA from liver explants as described in Methods. The screened region corresponds to amino acids (aa) 67–131. Lane *1* shows a slight shift in mobility of amplified DNA isolated from a patient with cryptogenic cirrhosis (patient 459), as compared with other amplified DNA specimen (lanes *2–12*) isolated from liver explants of other patients. The SSCP profiles for the remaining patient DNA specimen were identical in migration to that in lanes *2–12* (not shown). Lane *13* shows SSCP analysis of amplified genomic K18 that contains an in vitro introduced point mutation that results in an arg89 → cys K18 (used as an SSCP positive control). (*C*) DNA sequencing of amplified genomic DNA from a normal liver explant (*a*), patient 459 (*b*), and two clones isolated from subcloned patient 459 genomic DNA (*c*, *d*). Downward arrow in *b* shows overlapping nucleotides A/T (read as N) which are resolved on sequencing the individual clones in *c* and *d* (*upward arrows*). The A→T missense mutation results in a his 127→leu change.

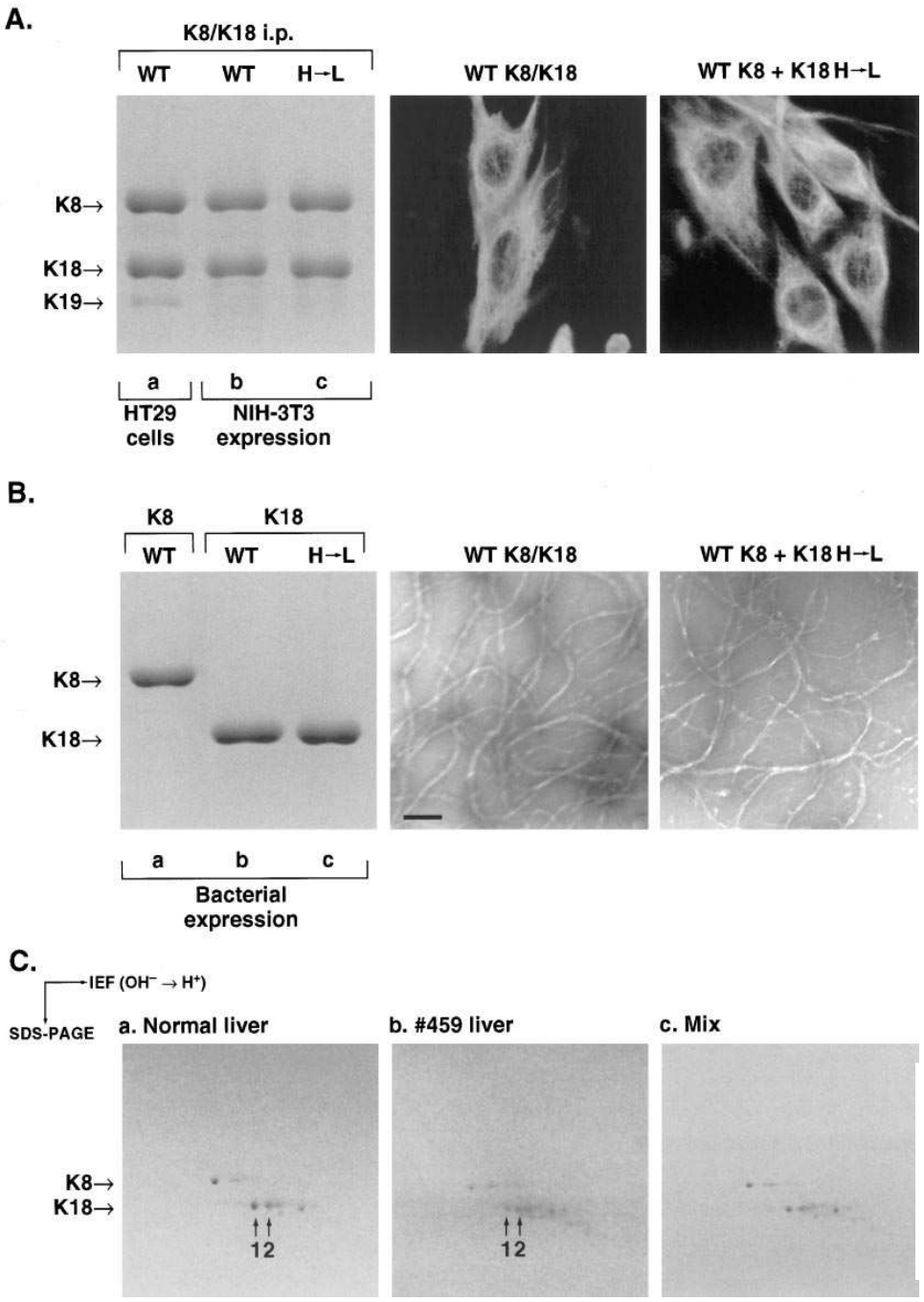

Figure 2. Comparison of WT and his 127→leu K18 expressed in bacteria and transfected NIH-3T3 cells. (*A*) A his127→leu K18 (H→L) was generated by introducing a point mutation into K18 cDNA. WT K8/K18 were immunoprecipitated (i.p.) from cultured human colonic HT29 cells and compared with WT K18 or K18 H→L that were cotransfected with WT K8 into NIH-3T3 cells. K19, which coimmunoprecipitates with K8/18 in HT29 cells is indicated by the arrow (lane *a*). K8/K18 filament formation in transfected NIH-3T3 cells was also examined by immunofluorescence staining. (*B*) WT K8, WT K18, or K18 H→L were expressed in bacteria followed by purification then SDS-PAGE analysis as described in Methods. WT K8/K18 or WT K8/K18 H→L, in 6 M urea/10 mM Tris-HCl, pH 8 (150 μg/ml), were dialyzed followed by negative staining then analysis by electron microscopy. Bar = 111 nm, and both filament assembly pictures have the same magnification. (*C*) K8/18 were immunoprecipitated from a normal human liver specimen or from a liver explant specimen from patient No. 459. The immunoprecipitated keratins were analyzed by isoelectric focusing (horizontal) followed by SDS-PAGE in the second dimension (vertical). A mix of the immunoprecipitates was also analyzed and followed by Coomassie staining of the gels. Isoforms 1 and 2 (arrows) represent two major K18 isoforms.

(pH 7.3) to decrease filament clumping. The density of the in vitro assembled filament arrays were estimated by assigning a density of low, medium, or heavy to individual windows within negatively stained grids. The assigner did not know the sample source (wild type versus mutant) on the grids. Statistical analysis was done using the χ^2 test in JMP version 3.1 software program (SAS Institute Inc., Cary, NC). Two-dimensional gel analysis was done as described (20).

Results

We analyzed genomic DNA isolated from liver explant specimen from 120 individuals, most of whom had chronic or acute liver disease of unknown (cryptogenic) or known causes that resulted in liver transplantation (28 cryptogenic cirrhosis, 20 alcoholic liver disease, 30 autoimmune hepatitis, 12 acute fulminant hepatitis, 11 viral hepatitis B or C, 7 drug induced hepatitis, 6 other liver diseases, 3 neonatal hepatitis, and 3 normal), using SSCP analysis. Cryptogenic cirrhosis accounts for ~ 10% of patients who undergo liver transplantation (21, 22). As shown in Fig. 1 *A*, the region that was examined corresponds to amino acids 67–131 that is part of the head, IA, and L1 domains and includes the first four amino acids of domain IB. This region was chosen for screening since it contains the highly conserved helix initiation subdomain that harbors ~ 70% of the mutations in the epidermal skin diseases (4–11). In the case of K18, this subdomain includes arg89 which was mutated

and expressed in transgenic mice with resultant keratin filament disruption and development of chronic hepatitis (15). Analysis of DNA specimen from the 120 patients showed one cryptogenic cirrhosis patient (459) who had a shift on SSCP that suggested a mutation (Fig. 1 *B*, lane *1*). The remaining 119 DNA samples (which include the normal livers) had an identical SSCP pattern (exemplified in Fig. 1 *B*, lanes *2–12*) that was clearly distinct from patient 459 (Fig. 1 *B*, lane *1*) or from a positive control DNA that had an in vitro introduced C→T mutation to convert arg89→cys (Fig. 1 *B*, lane *13*). Sequencing of the PCR amplified fragment, obtained from patient 459 liver DNA, showed a heterozygous mutation with one normal allele and one allele having an A→T transversion that converts K18 his127→leu (Fig. 1 *C*). This mutation is not related to a PCR artifact since it was reproducible ($n = 6$, not shown; see protein characterization in Fig. 2). In addition, subcloning of the generated PCR product and sequencing 13 individual subclones resulted in four clones having the wild type (WT) sequence (Fig. 1 *C*, *c*) and nine clones with the mutant sequence (Fig. 1 *C*, *d*). The mutation is germline since sequencing of amplified genomic DNA, isolated from a blood sample of the daughter of patient 459, showed that the H127L mutation is inherited in a heterozygous fashion (not shown).

We then analyzed the effect of the K18 H127L mutation on protein migration in one and two-dimensional gels, and its effect on filament assembly as visualized by immunofluorescence staining and electron microscopy. First, the his127→leu mutation was introduced into WT K18 followed by cotransfection of WT K18 (or K18 H127L) and WT K8 into NIH-3T3 cells then immunoprecipitation of K8/18 (Fig. 2 *A*). Cotransfection of K8 with K18 is needed in order to mimic their in vivo coexpression and allow for filament formation and partner keratin protein stabilization which does not occur if one partner of the keratin pair is missing (23, 24). K18 H127L comigrates with WT K18 that is transfected into NIH-3T3 cells or endogenously expressed in human cultured colonic HT29 cells (Fig. 2 *A*). In addition, the immunofluorescence filament staining pattern of WT K18 or K18 H127L, after cotransfection with K8, is indistinguishable upon transfection into NIH-3T3 cells (Fig. 2 *A*). However, a prominent effect of the his→leu mutation in K18 can be appreciated by electron microscopy after in vitro assembly of bacterially expressed then purified K8/18 (Fig. 2 *B*). To that end, the K18 H127L/K8 filaments form significantly less extended arrays and are heterogeneous as compared with WT K8/18 ($P < 10^{-4}$, for the difference in filament density between WT and mutant K18, see Methods). Although the physiologic ramifications of the K18 his→leu mutation are not known, it is clear that it does have a phenotype in vitro in terms of interfering with the ability to form normal filaments.

In order to confirm the presence of the H127L K18 mutant protein in patient No. 459 liver, we asked if we can distinguish WT K18 from K18 H127L using two-dimensional gels, because a histidine (pI 7.59) to leucine (pI 5.98) may be expected to shift K18 to a slightly more acidic form upon isoelectric focusing. An acidic shift of K18 H127L as compared with WT K18 was confirmed by comparing the two-dimensional profile of the bacterially expressed proteins (not shown). Furthermore, immunoprecipitation of K8/18 from normal liver and from the liver explant of patient 459 showed a reciprocal ratio of isoforms 1 and 2 (Fig. 2 *C*, compare *a* and *b*) such that normal liver had a $1 > 2$, whereas patient 459 had a $2 > 1$, Coomassie staining intensity of the isoforms. This is consistent with the presence of both alleles (WT K18 and K18 H127L) in patient 459.

Discussion

The his127→leu mutation in K18 involves the last amino acid of the L1 region, which represents the first described mutation in L1 and the first described mutation in human K18. This histidine is conserved in mouse and human K18 and K19, and mouse K15, but not in other type I keratins (i.e., K9 to K20; Fig. 3). The major location of other known keratin mutations in the epidermal/oral keratin diseases involve the helix termination region at the end of domain IIB and within the L1-2 region (4–11) which had normal sequences in patient 459 (not shown).

Our results suggest that the K18 mutation that we identified in patient 459 and the expression of the mutant protein resulted in, or predisposed to the development of cryptogenic cirrhosis. Distinction between these two possibilities is not possible given the information that we are able to obtain. For example, the patient's daughter, who also harbors the K18 mutation, is still too young to draw any conclusions regarding the penetrance of this mutation. The late onset of cryptogenic cirrhosis and the lack of well described autosomal dominant

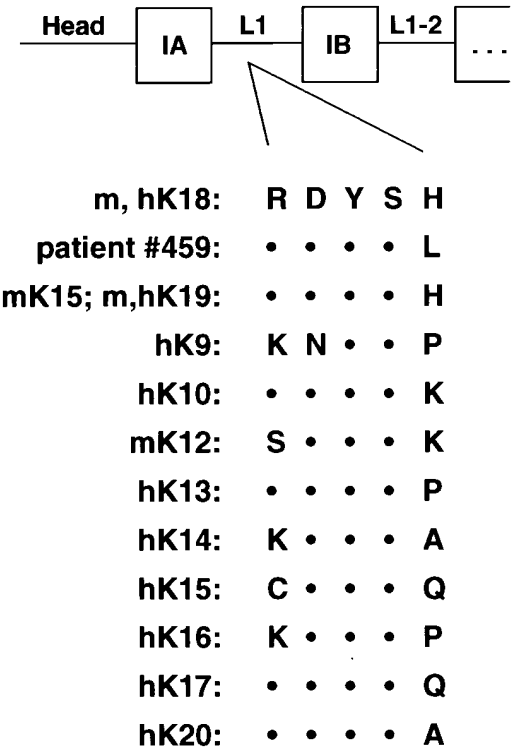

Figure 3. Comparison of linker 1 (L1) sequence of type I keratins with patient 459 K18 sequence. Single letter abbreviations are used to show all known sequences of the L1 region (last five amino acids) of type I keratins. Human (h) and mouse (m) his127 of hK18 is the last residue of the L1 sequence which is then followed by domain IB sequence (not shown). Amino acids that are identical to the h,m K18 sequence are represented by bold dots. The indicated sequences are for m,hK18 (25); mK15 (26); m,hK19 (27); hK9 (28); hK10 (29); mK12 (30); hK13 (31); hK14 (32); hK15 (33); hK16 (34); hK17 (35); and hK20 (36).

cases (in contrast to the epidermal keratin inherited diseases) make the potential for K18 mutations to be a predisposition rather than a direct cause of liver disease more likely. More patients with idiopathic acute or chronic liver disease need to be examined for K18 (and possibly K8) mutations to have an appreciation for the extent and consequences of K8/18 mutations in liver disease of unknown cause, and the role of the location of the mutation and any genetic/environmental modifiers. Strong support for the association of liver disease with K18 mutations is also based on the transgenic animal models (15–17). Although it is possible that the identified mutation may represent a polymorphism, this is unlikely because the mutation was found in only one of 240 alleles. More importantly, the K18 his127→leu mutation manifests a filament assembly defect. The biologic consequences of this defect remain to be investigated but possibilities include an increase in hepatocyte fragility (15) and/or an effect on the ability to handle oxidative (16) or other stresses (37, 38).

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