ELECTRONIC LETTER

A frequent keratin 8 p.L227L polymorphism, but no point mutations in keratin 8 and 18 genes, in patients with various liver disorders

M Hesse, T Berg, B Wiedenmann, U Spengler, R P Woitas, T M Magin

J Med Genet 2004;41:e42 (<http://www.jmedgenet.com/cgi/content/full/41/4/e42>). doi: 10.1136/jmg.2003.012393

Keratins (K) 8 and 18 are the first intermediate filament proteins present in the early embryo, and form the backbone of the intermediate filament cytoskeleton in all simple internal epithelia. Mice with a combined deficiency in K8/K19 and K18/K19 suffer from midgestational lethality with full penetrance, which strongly supports an essential keratin function in extraembryonic epithelia. Moreover, mutations in K8 and K18 have been reported to present risk factors in liver disease of multiple aetiologies. This mutation analysis of the complete K8 and K18 genes, using DHPLC technology, involved screening blood samples from 256 patients diagnosed positive for various liver disorders, and from 100 individuals serving as controls. None of the previously reported mutations in K8 and K18 was found in any of the samples, nor was a positive correlation observed between K8 and K18 mutations to cryptogenic cirrhosis or to chronic liver disease of other origin. However, a novel polymorphism was detected in exon 4 of the K8 gene, leaving L227 unaltered, in both patient and control samples.

Type I and type II keratins belong to the large gene family coding for intermediate filament proteins. Among the 27 type I and 27 type II keratins known to exist in the human genome, type I K18 and type II K8 form copolymers and are coexpressed in all embryonic and internal epithelia.¹ DNA linkage analysis and transgenic animal experiments have established that dominant acting point mutations in epidermal keratin genes lead to a large number of skin fragility syndromes, including epidermolysis bullosa simplex. The majority of these mutations are highly conserved with regard to their position.² The molecular mechanism leading to cell fragility is not well understood, but it is clear that an intact cytoskeleton is required for epithelial homeostasis. A more detailed biochemical analysis has demonstrated a strong genotype-phenotype correlation, in the sense that mutations located in conserved protein coding motifs produce more severe pathology than mutations elsewhere.^{3–5}

With respect to internal and embryonic epithelia, studies of keratin gene knockout mice so far have underlined an essential function of these proteins in extraembryonic tissues,^{6–8} but have failed to support a vital role in adult internal epithelia.⁴ Expression of a mutated human K18 gene in transgenic mice, on the other hand, has revealed fragile hepatocytes and supports a hepatoprotective role for this keratin.¹¹ There is evidence that such a function includes modulation of apoptotic pathways by interaction of keratins with effectors of TNF and Fas pathways.^{9 21–24} Mutant K8 has so far not been tested in transgenic mice. Collectively, these and additional data⁴ support the hypothesis that K8 and K18 have a non-structural function in digestive epithelia and that corresponding mutations might predispose individuals to liver or gastrointestinal disease. In fact, several reports have now identified mutations in either K8 or K18 genes as risk factors for liver disease of multiple aetiologies.¹⁰ Of these

Key points

- Mutations in K8 and K18 genes may constitute risk factors for liver disease of multiple aetiologies.
- Other data support a hepatoprotective role for human K18 in transgenic mice, but K8 has not so far been tested similarly.
- The present mutation analysis showed none of these mutations in 256 patients with liver disorder, but did show a novel polymorphism in the K8 gene.
- Further work with animal models and European liver patients is needed.

mutations, six of each were found in K8 and K18 genes, respectively, in non-conserved locations with regard to those observed in epidermal keratin genes. All of these represent heterozygous single base exchanges. The total frequency of K8 and K18 mutations amounted to 0.036, compared with 0.0057 in control groups.

In order to assess the role of K8 and K18 mutations as potential risk factors for patients with various chronic liver disorders in the European Caucasian population, samples from more than 250 subjects suffering from a range of liver disorders, and from a control group of 100 unaffected individuals, were analysed and compared.

MATERIALS AND METHODS

Patients

Genomic DNA from peripheral leucocytes or liver biopsies was prepared using standard procedures. Of the 256 patients recruited for this study, 21 were infected with hepatitis B virus (HBV); 126 with hepatitis C virus; 11 had non-alcoholic steatohepatitis; 32 had primary biliary cirrhosis; 18 had primary sclerosing cholangitis; 22 had autoimmune hepatitis; five were in fulminant hepatic failure; 10 were suffering from cryptogenic cirrhosis; and 11 had liver disorder of unknown aetiology. These patients were chosen because alterations in the keratin cytoskeleton, such as reorganization and formation of aggregates, have been described in these disorders,²⁰ and because certain viral proteases cleave keratins 8 and 18.^{18 19} As a reference group, 100 white blood donors from the Bonn University transfusion centre were selected randomly.

PCR amplification

PCR was performed using 1U Platinum Taq (Invitrogen, Karlsruhe, Germany), 200 µM dNTPs, 1.5–2.0 mM MgCl₂ (see table), 5% DMSO, and 0.5 µM of the corresponding primer (table 1), in a total volume of 25 µl. Conditions were

as follows: five minutes at 94°C, (25 seconds at 94°C, 25 seconds at 61.3–65.6°C (see table), and one minute at 72°C) × 35 cycles. Of the reaction, 10 µl was checked for the expected product on an agarose gel. PCR products from genomic DNA of one blood donor were fully sequenced and used as controls. Equal amounts of control product were mixed with the patient samples, denatured for 10 minutes at 94°C, and allowed to cool slowly to room temperature for heteroduplex formation.

DHPLC analysis and sequencing

Mixed amplicons were analysed by DHPLC using the Transgenomic WAVE system (Transgenomic, Omaha, Neb., USA). For validation, positive control samples with the p.Y54H and p.G62C variations in K8 and the p.R90C variation in K18 were used (gift from B Omary). These variations could be detected, using exactly the same conditions as for the screening protocol: 5 µl of the mixed amplicons were loaded on a DNasep column and eluted on a linear acetonitril gradient in a 0.1 M triethylamine acetate buffer (pH 7) with a constant flow rate of 1.5 ml/minute. All samples were injected at different temperatures, to minimise the risk of missing mutations (table 1). The optimal melt temperature for detection of the A-G polymorphism in K8 exon 4 was 60°C; for the C>G polymorphism in K18, Intron 1, it was 60.7°C. Sequencing was performed by automated fluorescent dideoxy sequencing (AGOWA, Berlin, Germany).

RT-PCR

Total RNA from a biopsy of the transplanted liver of a patient carrying the g.418-4C>G variation, and from a control, was isolated using TRIzol Reagent (Gibco-BRL, Gaithersburg, MD, USA). For the RT-reaction, the Expand Reverse Transcriptase (Roche; Mannheim, Germany) was used. PCR reaction was performed with the primer 5'-GCAAAGCCTGAGTCCTGTCC-3', specific for the 5'-end, and 5'-TTTATTGGCCTCCTGCTC CCC-3', specific for the 3'-end of the K18 cDNA. Conditions

were as follows: 94°C for two minutes, (94°C for 25 seconds, 65°C for 25 seconds, and 72°C for one minute) × 35 cycles, and 72°C for 10 minutes, with Taq polymerase (MBI Fermentas, Walldorf, DC, USA).

RESULTS

Genomic DNA from peripheral blood samples was used for PCR amplification with primers specific for all exons of K8 and K18. After analysis of fragments on a DHPLC high throughput system, all detected mutations were identified by sequencing. In none of 256 patients suffering from a variety of liver diseases could mutations altering amino acids be detected. However, a g.740A>G sequence variation p.L227L was found very frequently in all patients (fig 1A, B). Of the 256 patients, 54% were heterozygous, 26% homozygous at this CTG, and 20% homozygous for CTA. This polymorphism was observed in the analysed controls as well: 46% were heterozygous, 24% homozygous at this CTG, and 30% homozygous for CTA. Another polymorphism in the K18 gene was detectable in one patient suffering from HBV at the end of intron 1 (fig 1C, D). This g.418-4C>G variation could possibly affect the nearby splicing site and lead to an alternative spliced product. To test this, RT-PCR with primers specific for the 5'- and 3'-end of the human K18 cDNA was performed on total liver RNA. Only one product with the expected size of 1.3 kb was obtained from the RNA of the patient carrying the g.418-4C>G variation as well as from the control RNA (fig 2). This clearly demonstrates that the g.418-4C>G variation does not affect the splicing of the K18 cDNA.

DISCUSSION

Data from transgenic mice have suggested a correlation between mutations in keratin 8 and 18 and liver diseases in humans.¹¹ These mice carried a point mutation in keratin 18, in analogy to a hot spot mutation in epidermolysis bullosa simplex, and as a consequence suffered from chronic hepatitis. The mutations found in patients with cryptogenic

Table 1 Primer sequences, PCR conditions, temperatures, and temperature gradients used for DHPLC mutation screening of genes K8 and K18					
Exon	Sequence	MgCl ₂	Anealing temp	Product size	Temp gradient
K8 Ex1 5'	5'-AGAAGCAGCTTCTCCGCTC-3'	2.0 mM	64.1°C	452	62.5–65.5°C
K8 Ex1 3'	5'-GGACTACCAGGAGAAAGGGG-3'				
K8 Ex2 5'	5'-AAAAGACTGCTTGCTTTCCTC-3'				
K8 Ex2 3'	5'-AGGTGACTTCAGTTGGGTGG-3'	1.5 mM	64.1°C	322	60.0–62.0°C
K8 Ex3 5'	5'-AGGGCAACCTCCCTTTTG-3'				
K8 Ex3 3'	5'-CAGTGTCCAGATAGGAGAAGGG-3'	2.0 mM	64.1°C	148	59.5+60.5°C
K8 Ex4 5'	5'-GGCTCCCCCTCTCTCATC-3'				
K8 Ex4 3'	5'-TCTCTGCGACCAGGAAC-3'	2.0 mM	62.7°C	142	60.0+61.0°C
K8 Ex5 5'	5'-ACCACTGCCCCTCTTCCC-3'				
K8 Ex5 3'	5'-AGCTGAAGTGTGGCTGCC-3'	1.5 mM	65.6°C	458	60.0–62.0°C
K8 Ex6 5'	5'-GAAGAGAAGGCATACCCAACC-3'				
K8 Ex6 3'	5'-ACAGGACCCCAAGTCCAAG-3'	1.5 mM	65.6°C	373	63.5–65.5°C
K8 Ex7 5'	5'-GAAGGGCTAGGAGTTGGTCC-3'				
K8 Ex7 3'	5'-TGAGCGACTGAGCACAGC-3'	1.5 mM	65.6°C	188	61.0–66.0°C
K8 Ex8 5'	5'-TGTGGCTACCTCTGTCCCTC-3'				
K8 Ex8 3'	5'-GAGGGGTAGGCTGGGAGG-3'	1.5 mM	62.7°C	246	64.0–66.0°C
K18 Ex1 5'	5'-CCTGCTCTTCTCTCTCCCC-3'				
K18 Ex1 3'	5'-TCCCTCTACCCCTTACCTG-3'	1.5 mM	61.3°C	461	62.0–67.0°C
K18 Ex2 5'	5'-TGGGACCAGGAAGTTTTCAC-3'				
K18 Ex2 3'	5'-GTCCTTCTTAGCTCCACCCC-3'	1.5 mM	62.7°C	241	60.0+61.0°C
K18 Ex3 5'	5'-TGGATCAGAGATAGGGGGCC-3'				
K18 Ex3 3'	5'-TTCTTTGGCCCCCTCAATC-3'	1.5 mM	62.7°C	248	62.0+63.0°C
K18 Ex4 5'	5'-AGAGGCAATCACAGAAGAAAGG-3'				
K18 Ex4 3'	5'-CACACCTTGGCAGCCATC-3'	1.5 mM	62.7°C	276	60.5–63.5°C
K18 Ex5 5'	5'-TCTCAGCAGGTGCGTGAG-3'				
K18 Ex5 3'	5'-GGGCAGGTGATGTGAAGG-3'	1.5 mM	62.7°C	245	61.5–63.5°C
K18 Ex6 5'	5'-GATGAGCAGTCTGGGACTC-3'				
K18 Ex6 3'	5'-CAGTGAACAGACACATGGGG-3'	1.5 mM	62.7°C	377	62.0–64.0°C
K18 Ex7 5'	5'-GCTGTTTAACTTGGGCTTGG-3'				
K18 Ex7 3'	5'-TCCTGCTCCCAAAGGGTAC-3'	1.5 mM	62.7°C	190	61.0+62.0°C

Temp, temperature.

Figure 1 (A) Detection of a heterozygous g.740A>G p.L227L polymorphism by DHPLC (blue wild type, red polymorphism). (B) Automated fluorescent dideoxy sequencing. (C) Detection of a variation of g.418-4C>G in the sequence of intron 1 (I1) adjacent to exon 2 (E2) in a patient suffering from HBV by DHPLC (blue wild type, green mutation). (D) Automated fluorescent dideoxy sequencing. The elution time is on the x axis; the ultraviolet absorbance at 260 nm (mV) is on the y axis.

cirrhosis were predominantly and most frequently p.Y54H and p.G62C in the head domain of K8.^{10–12–13} Cells transiently transfected with keratin 8 carrying those mutations showed a normal keratin cytoskeleton under standard culture conditions, but displayed alterations in the filaments after applying pharmacological stress, including okadaic acid, hydrogen peroxide, or heat.¹³

However, there is no evidence yet from mouse models that the p.Y54H and p.G62C-mutations in K18 predispose to liver disease or cause an increased susceptibility to stress factors. Mutations used so far for liver studies in transgenic mice were p.R90C,¹¹ p.S34A,¹⁴ p.S53A¹⁵ in K18, K8^{null},¹⁶ and K18^{null}.¹⁷ Such mutations have not been detected in humans so far. It would be useful to develop mouse models carrying p.Y54H- and p.G62C-mutations in K8 in order to investigate conditions under which these mutations affect disease progression.

The contrary result of our study to the mutations reported so far suggests that allele frequencies might possibly differ between European and North American populations. It might be also possible that additional risk factors coincide with K8 and K18 mutations in Northern American but not European liver patients. The question arises as to whether patients suffering from liver diseases are the most likely disease group

to carry mutations in K8 and K18. Evidence from animal models strongly suggests an essential role for K8, 18, and 19 during embryonic development. Mice deficient for K8 in the C57BL/6 strain and with a compound deficiency for K18/19 and K8/19 display embryonic lethality during mid gestation.^{6–8} It seems likely that mutations in analogy to hot spot mutations in human skin fragility syndromes at the ends of the rod domain are able to cause early embryonic lethality in humans.

Authors' affiliations

M Hesse, T M Magin, Institut für Physiologische Chemie, Abteilung fuer Zellbiochemie, Universitätsklinikum Bonn, Germany

R P Woitas, U Spengler, Klinik für Innere Medizin I, Rheinische Friedrich Wilhelms Universität Bonn, Germany

T Berg, B Wiedenmann, Medizinische Klinik mit Schwerpunkt Hepatologie und Gastroenterologie, Medizinische Fakultät der Humboldt-Universität zu Berlin, Germany

Correspondence to: Prof. T M Magin, Institut für Physiologische Chemie, Abteilung fuer Zellbiochemie, Bonner Forum Biomedizin und LIMES, Universitätsklinikum Bonn, Nussallee 11, 53115 Bonn, Germany; t.magin@uni-bonn.de

REFERENCES

- Hesse M, Magin TM, Weber K. Genes for intermediate filament proteins and the draft sequence of the human genome: novel keratin genes and a surprisingly high number of pseudogenes related to keratin genes 8 and 18. *J Cell Sci* 2001;**114**:2569–75.
- Irvine AD, McLean WH. Human keratin diseases: the increasing spectrum of disease and subtlety of the phenotype-genotype correlation. *Br J Dermatol* 1999;**140**(5):815–28.
- Fuchs E, Cleveland DW. A structural scaffolding of intermediate filaments in health and disease. *Science* 1998;**279**(5350):514–19.
- Coulombe PA, Omary MB. 'Hard' and 'soft' principles defining the structure, function and regulation of keratin intermediate filaments. *Curr Opin Cell Biol* 2002;**14**(1):110–22.
- Herrmann H, Hesse M, Reichenzeller M, Aebi U, Magin TM. Functional complexity of intermediate filament cytoskeletons: from structure to assembly to gene ablation. *Int Rev Cytol* 2003;**223**:83–175.

Figure 2 RT-PCR of RNA from a patient carrying the g.418-4C>G variation and a control. In both reactions a band with a size of 1.3 kb was obtained, indicating no splicing variants.

- 6 Hesse M, Franz T, Tamai Y, Taketo MM, Magin TM. Targeted deletion of keratins 18 and 19 leads to trophoblast fragility and early embryonic lethality. *EMBO J* 2000;**19**(19):5060–70.
- 7 Tamai Y, Ishikawa T, Bosl MR, Mori M, Nozaki M, Baribault H, Oshima RG, Taketo MM. Cytokeratins 8 and 19 in the mouse placental development. *J Cell Biol* 2000;**151**(3):563–72.
- 8 Jaquemar D, Kupriyanov S, Wankell M, Avis J, Benirschke K, Baribault H, Oshima RG. Keratin 8 protection of placental barrier function. *J Cell Biol* 2003;**161**(4):749–56.
- 9 Oshima RG. Apoptosis and keratin intermediate filaments. *Cell Death Differ* 2002;**9**(5):486–92.
- 10 Ku NO, Darling JM, Krams SM, Esquivel CO, Keffe EB, Sibley RK, Lee YM, Wright TL, Omary MB. Keratin 8 and 18 mutations are risk factors for developing liver disease of multiple etiologies. *Proc Natl Acad Sci U S A* 2003;**100**(10):6063–8.
- 11 Ku NO, Michie S, Oshima RG, Omary MB. Chronic hepatitis, hepatocyte fragility, and increased soluble phosphoglycokeratins in transgenic mice expressing a keratin 18 conserved arginine mutant. *J Cell Biol* 1995;**131**(5):1303–14.
- 12 Ku NO, Wright TL, Terrault NA, Gish R, Omary MB. Mutation of human keratin 18 in association with cryptogenic cirrhosis. *J Clin Invest* 1997;**99**(1):19–23.
- 13 Ku NO, Gish R, Wright TL, Omary MB. Keratin 8 mutations in patients with cryptogenic liver disease. *N Engl J Med* 2001;**344**(21):1580–7.
- 14 Ku NO, Michie S, Resurreccion EZ, Broome RL, Omary MB. Keratin binding to 14-3-3 proteins modulates keratin filaments and hepatocyte mitotic progression. *Proc Natl Acad Sci U S A* 2002;**99**(7):4373–8.
- 15 Ku NO, Michie SA, Soetikno RM, Resurreccion EZ, Broome RL, Omary MB. Mutation of a major keratin phosphorylation site predisposes to hepatotoxic injury in transgenic mice. *J Cell Biol* 1998;**143**(7):2023–32.
- 16 Zatloukal K, Stumptner C, Lehner M, Denk H, Baribault H, Eshkind LG, Franke WW. Cytokeratin 8 protects from hepatotoxicity, and its ratio to cytokeratin 18 determines the ability of hepatocytes to form Mallory bodies. *Am J Pathol* 2000;**156**(4):1263–74.
- 17 Magin TM, Schroder R, Leitgeb S, Wanninger F, Zatloukal K, Grund C, Melton DW. Lessons from keratin 18 knockout mice: formation of novel keratin filaments, secondary loss of keratin 7 and accumulation of liver-specific keratin 8-positive aggregates. *J Cell Biol* 1998;**140**(6):1441–51.
- 18 Seipelt J, Liebig HD, Sommergruber W, Gerner C, Kuechler E. 2A proteinase of human rhinovirus cleaves cytokeratin 8 in infected HeLa cells. *J Biol Chem* 2000;**275**(26):20084–9.
- 19 Chen PH, Ornelles DA, Shenk T. The adenovirus L3 23-kilodalton proteinase cleaves the amino-terminal head domain from cytokeratin 18 and disrupts the cytokeratin network of HeLa cells. *J Virol* 1993;**67**(6):3507–14.
- 20 Denk H, Stumptner C, Zatloukal K. Mallory bodies revisited. *J Hepatol* 2000;**32**(4):689–702.
- 21 Ku NO, Soetikno RM, Omary MB. Keratin mutation in transgenic mice predisposes to Fas but not TNF-induced apoptosis and massive liver injury. *Hepatology* 2003;**37**(5):1006–14.
- 22 Inada H, Izawa I, Nishizawa M, Fujita E, Kiyono T, Takahashi T, Momoi T, Inagaki M. Keratin attenuates tumor necrosis factor-induced cytotoxicity through association with TRADD. *J Cell Biol* 2001;**155**(3):415–26.
- 23 Gilbert S, Loranger A, Daigle N, Marceau N. Simple epithelium keratins 8 and 18 provide resistance to Fas-mediated apoptosis. The protection occurs through a receptor-targeting modulation. *J Cell Biol* 2001;**154**(4):763–73.
- 24 Caulin C, Ware CF, Magin TM, Oshima RG. Keratin-dependent, epithelial resistance to tumor necrosis factor-induced apoptosis. *J Cell Biol* 2000;**149**(1):17–22.