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Keratin 8 and 18 variants are associated with ethnic background and adverse outcome from acute liver failure

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Abstract

BACKGROUND & AIMS—Keratins 8 and 18 (K8/K18) provide anti-apoptotic functions upon liver injury. The cytoprotective function of keratins explains the over-representation of K8/K18 variants in patients with cirrhosis. However, K8/K18 variant-associated susceptibility to acute liver injury, which is well-described in animal models, has not been studied in humans.

METHODS—We analyzed the entire coding regions of the *KRT8* and *KRT18* genes (15 total exons and their exon-intron boundaries) to determine the frequency of K8/K18 variants in 344

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acute liver failure (ALF) patients (49% acetaminophen-related) and two control groups [African-Americans (245 subjects) and previously-analyzed Caucasians (727 subjects)].

RESULTS—There were 45 ALF patients with significant amino-acid-altering K8/K18 variants including 23 with K8 R341H and 11 with K8 G434S. K8 variants were significantly more common (total of 42 patients) than K18 variants (3 patients) ($p < 0.001$). We found an increased frequency of variants in Caucasian ALF patients (9.1%) versus controls (3.7%) ($p = 0.01$). K8 R341H was more common in Caucasian ($p = 0.01$) and G434S was more common in African-American ($p = 0.02$) ALF patients versus controls. Furthermore, Caucasians with K8/K18 variants were less likely to survive ALF without transplantation ($p = 0.02$). K8 A333A and G434S variants associated exclusively with African-Americans (23% combined frequency in African-American but none in Caucasian controls; $p < 0.0001$), while overall K18 variants were more common in non-Caucasian liver disease subjects compared to Caucasians (2.8% versus 0.6%, respectively, $p = 0.008$).

CONCLUSIONS—*KRT8* and *KRT18* are important susceptibility genes for ALF development. The presence of K8/K18 variants predisposes to an adverse ALF outcome, and some variants segregate with unique ethnic/race backgrounds.

Keywords

intermediate filaments; keratin mutations; acute hepatitis; acetaminophen toxicity

INTRODUCTION

Keratins (K), the largest subgroup of the intermediate filament cytoskeleton, are found predominantly in epithelial cells and skin appendages and consist of >50 individual proteins.^{1,2} They are subdivided into type-I (K9–28, K31–40) and type-II (K1–8, K71–86) proteins; and members of the two types co-polymerize to produce the typical filamentous cytoplasmic arrays. Keratins are tissue-specific proteins and their variants result in a variety of organ-specific human diseases.^{3,4} In single-layered (also called ‘simple’) epithelia, K8/K18 are the major keratins, but variable amounts of K7/K19 and K20 are also found depending on the epithelial cell type.^{5,6} Adult hepatocytes are unique in that they express K8/K18 only, whereas other digestive-organ epithelial cells, such as ductal or intestinal epithelia, exhibit a more complex keratin-expression pattern.^{5,6} The limited hepatocyte keratin composition is likely responsible for the predominant liver phenotype seen in various K8/K18-deficient mouse models.⁶ For example, mice that express human K18 R90C (a variant not found in humans that disrupts keratin cytoplasmic filaments) have mild chronic hepatitis but a remarkable predisposition to acute hepatotoxicity due to acetaminophen (APAP), microcystin-LR or Fas-induced apoptosis.^{6,7} The findings in K8/K18-deficient/absent mice led to human association studies that established K8/K18/K19 variants as a risk factor for the development of end-stage cirrhosis of multiple etiologies, and liver fibrosis progression in patients with chronic hepatitis C infection (K8) or primary biliary cirrhosis (K8/K19).^{8–11} One major human liver disease-predisposing variation (K8 G62C), when expressed in mice, renders them highly susceptible to liver-related lethality when challenged with Fas ligand.¹² While the animal model data clearly demonstrate the keratin variant-based predisposition to acute liver injury, human studies to date have focused exclusively on chronic liver diseases.⁶

Acute liver failure (ALF) involves ~2000 US cases/year and is characterized by sudden loss of hepatocyte function in previously liver-healthy subjects that may lead to profound morbidity and mortality.^{13,14} Drug-induced liver toxicity is the most common cause of adult ALF mainly because of APAP poisoning (~1/2 of US ALF cases). Other frequent ALF etiologies include hepatitis B (7%), indeterminate (14%), autoimmune (5%) and drugs other

than APAP (11%).¹³ Liver transplantation represents the only effective ALF therapy, unless patients recover spontaneously, although the decision for a liver transplantation is challenging because of the scarcity of donor organs, the need of a lifetime immunosuppressant therapy and the fast pace of disease progression.¹³ Therefore, a better predictability of the disease outcome is of great importance. A number of prognostic scores combining laboratory and host parameters with clinical observations were developed, but there are insufficient data to recommend a particular one.¹⁴ In addition, human genetic variations may be of importance as it was shown for chronic liver diseases.^{15,16} Given the abundant evidence from animal models supporting the importance of K8/K18 variants in predisposing to acute liver injury,⁶ we hypothesized that keratin variants predispose their carriers to ALF and may have prognostic implications once ALF develops.

METHODS

Human Subjects

We utilized clinical data and genomic DNA from 346 adult ALF patients prospectively identified and consecutively enrolled (2003–2007) at 21 tertiary US centers participating in the Acute Liver Failure Study Group (ALFSG).¹⁷ Patients were selected based on standard ALF criteria that include presence of coagulopathy (INR $\geq$ 1.5) and hepatic encephalopathy within 26 weeks of the first symptoms in previously liver-healthy individuals.¹⁸ All subjects provided informed consent and the study was approved by the Human Subjects Committees of all participating centers.

To estimate the frequency of selected K8 variants in African-Americans, 236 non-ALF subjects were analyzed. These donors included 136 healthy blood-bank donors who provided blood at the American Red Cross Northern California Blood Services Region in Oakland during 2007, and 100 donors to the Coriell Institute for Medical Research. Previously-published cohorts of healthy Caucasian subjects were used as controls.^{9,19,20} Nearly 10% of the enrolled ALF patients received N-acetylcysteine as part of an independent drug-efficacy study.²¹ However, there was no association between N-acetylcysteine administration and any of our findings.

ALF etiology, patient management and liver transplant candidacy were determined at the enrollment sites and all diagnoses were reviewed at the ALFSG central site. Indeterminate ALF etiology was assigned when extensive clinical, radiographic, and laboratory workup revealed no known ALF cause. Disease outcomes were evaluated 3 weeks after study admission. Given that the incidence of K8/K18 variants may depend on the individuals descent,⁶ patients race was derived from the patient's chart and his/her ethnicity (Latino/Hispanic: yes or not) was determined from patient's name and/or caring physician impression. For outcome analysis, hepatic encephalopathy/coma was scored with a standard scale of 1–4. For bilirubin, INR and creatinine, the maximum value from the pre-admission and admission data was used. When patients died during the observation period, the maximal coma score was set as 4. Of 346 initially-enrolled patients, two were excluded (one did not meet entry criteria and one because of inability to amplify the DNA).

Genetic Analysis

Genomic DNA was isolated from EDTA-anticoagulated blood with a DNeasy-tissue-kit (Qiagen). All 15 total K8/K18 exons and the exon-intron boundaries were PCR-amplified using established primers and a mixture of T-Taq and Optimase Polymerases (Transgenomic).¹⁰ Amplified samples were screened for the presence of heterozygous variants with a WAVE DNA Fragment Analysis system (Transgenomics).²² Samples yielding a shifted elution pattern were purified then sequenced bi-directionally. Assignment

of K8/K18 variants was based on the mRNA sequences NM 002273.2/NM 000224.2. Our analysis may miss homozygous variants since such polymorphisms/mutations may not reveal a 'shifted elution pattern' but the frequency of such variants is likely to be very small given the already low frequency of individual K8/K18 heterozygous variants that we identified in patients with chronic liver disease.²³

Statistical Methods

We analyzed the association of K8/K18 variants with ALF development and outcome. The frequency of individual K8 variants and total significant K8/K18 variants was first compared between ALF patients and controls as well as ethnic groups using Chi-square test. We use the term 'ethnic' to represent both ethnicity (e.g., Hispanic) or race (e.g., Caucasian or African American). Association of K8/K18 variants with ethnicity/ALF etiology/enrolment site was examined using two-tailed Fisher's exact test. Univariate logistic regression analysis was performed to assess the association of K8/K18 variants with different variables and outcomes (STATA software, version-10; stata.com). Since the presence of selected K8/K18 variants affected spontaneous survival (without liver transplantation), a multivariate analysis was performed to adjust for the simultaneous effects of other variables. We used a multivariate-logistic-regression model with age; African-American race (yes/no); and ALF etiology [acetaminophen-related (yes/no), ischemic-related (yes/no) and "other category" of ALF] as predictors. We determined estimates and 95%-confidence intervals for the adjusted odds ratios. All p-values in the logistic regression analysis were computed with a standard Wald test.

RESULTS

K8/K18 Variants are Overrepresented in ALF and in Subjects with Unique Ethnic Backgrounds

The composition of the analyzed ALF cohort (age, male/female ratio, frequency of ALF etiologies) is comparable with the data previously published on adult US patients with ALF.¹³ To that end, acetaminophen is the most frequent cause of ALF (49%), while 13% of ALF cases are of indeterminate etiology (Table 1). On the other hand, the spontaneous survival (i.e. the survival without a transplantation), was slightly higher in our cohort (57 vs. 45%, $p < 0.001$) (Table 1).^{13,18} Caucasian and African-American patients were the most common races, making up 73% and 12% of the cohort, respectively. As previously shown,¹³ patients with APAP and ischemic ALF are more likely to survive without transplantation when compared to the other ALF etiologies ($p < 0.01$ for each of the subgroups).

Genetic analysis of the entire coding regions of the *KRT8/KRT18* genes in 344 ALF patients identified 15 amino-acid-altering, and 11 non-amino-acid-altering K8/K18 variants which include 6 non-coding (promoter, intronic and 3' untranslated regions) variants (Table 2, Supplemental Table 1). Apart from the common L22/L polymorphism, which was found both in homozygous and heterozygous form, all observed variants were heterozygous. Five patients harbored two compound heterozygous amino-acid altering K8 variants (2 K8 A319S+R341H; 1 K8 T153A+R341H; K8 R341H+I466V; 1 K8 I63V+V380I). There were 10 newly identified variants, five of which led to amino acid substitutions (Table 2, Supplemental Table 1) in residues that are conserved across mammalian species except for K8 T153 (Supplemental Figure 1). Eleven K8/K18 variants involving 45 patients were assigned as significant (Table 2) based on several criteria including previous human association studies, their documented biological effects^{6,23} or protein structure considerations. The other variants (K8 I63V/A319S/V480I) are deemed of unknown significance (Supplemental Table 1) because they are silent or lead to conservative amino-acid substitutions or were detected at similar or higher frequency in the control group. Non-

coding variants are also classified in the category of ‘unknown significance’ given the lack of biological data and that they did not affect the development of liver fibrosis in patients with hepatitis C.¹⁰

Henceforth, the term variants will describe the significant variants only. Of the 11 K8/K18 variants, K8 R341H was the most common (seen in 23 subjects, Table 2), and as shown previously was uniformly associated with the intronic single nucleotide deletion (K8 IVS7+10delC)¹⁰. Combining findings herein with prior published data,^{10,20} patients with K8 R341H are more likely to also carry K8 A319S ($p<0.0001$; Supplemental Table 1). Overall, K8 variants are significantly more common (total of 42 patients) than K18 variants (3 patients) ($p<0.001$). Notably, autoimmune ALF patients had the highest frequency of K8/K18 variants while no K8/K18 variants were seen in subjects with rare ALF etiologies (Supplemental Table 2). When the analyzed races were considered separately, African-American ALF patients had the highest frequency of K8/K18 variants (e.g., 31.7% versus 9.1% in Caucasians, $p=0.0003$) with K8 G434S being the most common (Table 3, Supplemental Table 3). Among the variants with unknown biological significance, K8 A333A and K8 IVS7+8C>T were also frequently observed in the African-American ALF subjects (Table 3, Supplemental Table 3). Interestingly, none of these variants were seen in Caucasian liver disease patients ($p<0.001$ for each variant; Table 4, Supplemental Table 4).

In order to determine the overall frequency of K8 variants in African-Americans, we analyzed K8 exons 1,6 and 8, which include the K8 variant hot-spots, in 236 control African-American subjects (See Methods for how the control group was obtained). We identified 7 amino-acid-altering K8 variants (3 novel), and 7 non-amino-acid-altering variants (5 novel, including 3 non-coding) (Table 5). K8 A333A and K8 G434S were the most frequent variants (>10%) and similar to three additional K8 variants (Y54H/E576E/V480I) appear to be largely restricted to African-Americans in that they have not been described in Caucasian subjects^{9,19,20} (Table 5, Supplemental Table 5). On the other hand, the K8 R341H variant is underrepresented in African-Americans compared to Caucasian subjects (1.3% versus 4.1%, $p=0.02$ when both liver disease patients and control subjects are considered; Tables 3 and 4).

Next, we compared the frequency of K8/K18 variants in ALF patients versus controls (Table 3). In Caucasians, the frequencies of total K8/K18 variants and the K8 R341H variant were significantly higher than in controls (9.1% versus 3.4%, $p=0.01$ for total variants; and 7.1% versus 3.2%, $p=0.01$ for K8 R341H) (Table 3). Similarly, K8 G434S was more frequent in African-American ALF patients versus African-American controls ($p=0.02$).

Keratin Variants Associate with Adverse ALF Outcome

We used univariate analysis to examine the association of K8/K18 variants with clinical prognostic markers related to ALF patients (Table 6). As highlighted above, K8 A333A/A338A, K8 G434S and overall K8/K18 variants were unevenly distributed among races. K8 A333A/A338A concentrated in subjects with viral and indeterminate ALF, and patients harboring K8 A333A/A338A had higher bilirubin levels and were significantly more likely to undergo liver transplantation (Table 6). Importantly, subjects with K8/K18 variants were less likely to survive ALF without liver transplantation ($p=0.034$). We used transplant-free survival as an important, previously employed outcome variable,²¹ because other scoring systems (e.g., Model-for-End-Stage-Liver-Disease; MELD) do not apply for ALF.²⁴ To address whether the presence of K8/K18 variants affected patient survival without liver transplantation, we performed a multivariate analysis adjusted for age, African-American race and the APAP-related and ischemic ALF etiologies. Presence of K8 R341H associated significantly with lower survival without transplantation, while patients with K8/K18 variants exhibited a trend towards lower survival both in the overall cohort and APAP

subgroup (Table 7). In Caucasian patients, there was a significant association between the presence of either K8 R341H or significant K8/K18 variants and lower survival without transplantation (Table 7). Hence, K8/K18 variants appear to predispose to the development of ALF and are also associated with an adverse outcome once ALF develops.

DISCUSSION

The overall frequency of biologically-relevant K8/K18 variants in our cohort (13.1%) was similar to that in patients with chronic end-stage liver disease (13.4%),⁹ but significantly higher than in blood-bank donors (3.7%). This highlights the importance of *KRT8/KRT18* gene variants in both acute and chronic liver disease. However, these percentages need to be taken in the context of K8/K18 variant frequency differences between races, which is an emerging theme.^{6,9,25} For example, K8 R341H is the most frequent amino-acid-altering variant in Caucasians^{9,10} and was found at increased frequency in Caucasian ALF patients versus ethnic-matched controls ($p=0.01$). K8 R341H is even more prevalent in Hispanic acute and chronic liver disease patients (14.3% vs. 4.8%, $p=0.0006$; Table 4), while it is seen at relatively low levels in African-American subjects (1.4%, Table 3). For the ethnic background frequencies shown in Table 4, we combined the patients with acute and chronic liver disease in order to enhance the power of analysis. K8 R341H also associates exclusively with the intronic K8 IVS7+10delC deletion,¹⁰ as confirmed herein, and appears to segregate with K8 R319S. This suggests that nucleotide changes at one site trigger errors at other sites.

An important finding is that K8 G434S appears to be the most common variant in African-Americans and its presence associates preferentially with ALF patients versus ethnic-matched controls ($p<0.02$). Six K8 variants were seen at significantly higher frequencies in African-American versus Caucasian controls (K8 Y54H/A333A/E376E/G434S/V480I) and four variants are noted primarily in liver disease patients (K8 Y54H/A333A/G434S/IVS7+8C>T) including Y54H and G434S which were identified in earlier studies involving chronic liver disease.^{8,9,25} Collectively, these exonic variants were observed in 33% of African-Americans and <1% of Caucasians, which renders them useful tools in ethnic background association analyses. However, one needs to consider, that K8 Y54H, A333A and G434S are also found in Hispanic subjects, albeit at significantly lower levels (Table 4) which also raises the possibility of ancestry confounding variables.

Additional rare variants also display ethnicity-dependent distribution. For example, K8 A338A was identified in 2 African-American control subjects and one African-American ALF patient, but in none of 868 Caucasians analyzed to date (Tables 2,5; Supplemental Table 4). Overall, significant K18 variants were more common in non-Caucasian liver disease patients compared to Caucasians (2.8% versus 0.6%, $p=0.008$; Supplemental Table 4). In additions, several variants (K8 T153A; K18 V48A and T103A) were found only in the Hispanic samples analyzed up to date, but not in the much larger cohort of Caucasian subjects (Supplementary Table 4). Further studies are needed to determine the racial distribution of these variants.

The autoimmune and APAP ALF subgroups had unique K8/K18 variant-related significant associations. Autoimmune ALF patients had the highest percentage of K8/K18 variants while in the ALF-subgroup afflicted with APAP poisoning, presence in Caucasians of any K8/K18 variant or of K8 R341H is significant when compared with Caucasian controls (10.1 versus 3.4%, $p=0.01$; and 7.2 versus 3.2%, $p<0.05$, respectively).

How keratin variants predispose to liver injury appears to be multi-factorial and depends on the site. For example, K8 G62C shunts stress-activated kinase phosphorylation away from

K8 S74 (a major stress-kinase phospho-site) to other phosphorylation-driven proapoptotic substrates, while K18 R90C has a profound effect on hepatocyte fragility.^{6,12} Similarly, K8 G434S limits the physiologic phosphorylation of K8 S432 by a likely proximity effect.⁶ Based on protein structure predictions, K8 R341H may destabilize the K8 protein and its mode of subunit interaction.⁹ Other potential mechanisms include keratin functions in subcellular protein-targeting and organelle function and positioning based on finding in K8-null mice.^{26,27} For example, K8-null hepatocyte mitochondria have altered morphology and positioning and become 'primed' to release cytochrome-c upon oxidative stimulation,²⁷ which may be relevant given the importance of mitochondrial function in APAP-induced cell death.²⁸ In addition, K8/K18 bind the tumor necrosis factor (TNF) receptor-2²⁹ and K8-null hepatocytes appear to have increased cell surface Fas receptor distribution.³⁰

Our findings highlight the importance of genetic variations in ALF, which is supported by few other studies. For example, the development of diclofenac-induced severe liver injury and/or ALF might be affected by the presence of allelic variants in diclofenac's metabolizing enzymes and the biliary excretion transporter MRP2.³¹ Similarly, flucloxacillin liver toxicity strongly associates with the HLA-B*5701 genotype.³² Also, in APAP-induced ALF, an intronic nucleotide polymorphism of the TNF- β gene of undefined biologic significance is more frequent in patients with fulminant hepatitis compared to controls and associated with the development of severe encephalopathy.^{33–35}

In conclusion, we used a large prospectively-identified cohort of ALF patients to identify *KRT8/KRT18* as important susceptibility genes for ALF development. K8/K18 variants predispose to an adverse ALF outcome in humans which raises the possibility for the utility of K8/K18 variants as prognostic biomarkers. The finding that specific keratin variants segregate with unique ethnic backgrounds may also have broad implications. Collectively, these findings provide a unique link of the cytoskeleton to ALF.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard abbreviations used

Ab	antibody
ALF	acute liver failure
ALT	alanine aminotransferase
APAP	acetaminophen
AST	aspartate aminotransferase
H&E	hematoxylin and eosin

h	human
INR	international normalized ratio
K	keratin
m	mouse
MELD	Model for End Stage Liver Disease
p	phospho
WT	wild type

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Table 1

Characteristics of the ALF patient cohort

	Male	Female	Total
Gender	120	224	344
Cancer (yes/no)	2/118	3/221	5/339
Alive (yes/no)	88/32	180/44	268/76
Transplant (yes/no)	28/92	50/174	78/266
Spon Survival (yes/no)	63/57	133/91	196/148
Latino (yes/no)*	9/111	17/206	26/317
Age (average±SD)	38.1±15.2	40.1±13.7	39.4±14.3
Cr max (average±SD) ^	3.5±2.5	2.9±2.3	3.1±2.3
INR max (average±SD) ^	5.7±5.1	5.4±4.8	5.5±4.9
Bili max (average±SD) ^	19.5±14.2	15.5±12.6	16.9±13.3
MELD score (average±SD) *	33.7±9.8	29.4±8.0	30.9±8.9
Coma Score **	I	II	III IV
Patient # (M/F)	19/34	15/39	18/34 68/117
Race	AA	Asian	LA NH OT CA
Patients # (M/F)	12/29	12/8	8/16 2/1 1/1 0/2 85/167
ALF Etiology	AP	AU	DI ISCH RA Viral
Patients # (M/F)	46/121	4/19	10/29 20/24 10/9 9/11 21/11
Spon Survival (yes/no)	126/41\$	5/18	20/19 14/30 14/5 7/13 10/22

AA, African-American; AP, Acetaminophen; AU, autoimmune; Bili, bilirubin; CA, Caucasian; Cr, creatinine; DI, Drug-induced; F, Female; IN, Indeterminate etiology; ISCH, ischemic; LA, Latino/Hispanic; M, Male; NA, Native American, NH, Native Hawaiian, RA, patients with rare ALF etiology; OT, patients, in which the race was not specified; SD, standard deviation; Spon, spontaneous survival without transplantation.

^ 'Max' represents the highest value seen in a given patient both prior to and after admission.

° The values indicate MELD scores obtained immediately after hospital admission. These initial MELD scores were unknown in 7 patients.

* There were 26 patients with Hispanic/Latino ethnicity, in whom 24 the 'race' was either 'Caucasian' (11) or 'Other' (13). In these cases, the race was changed to Hispanic/Latino. One patient with Hispanic/Latino ethnicity was labelled as African-American and one as 'Asian'. In these cases, original race was kept as it was. In one patient, the ethnicity is unknown.

** Represents the highest value in a given patient both prior to and after admission. Deceased patients were assigned coma score 4.

This group includes patients with fatty liver disease during pregnancy (2), Budd-Chiari syndrome (2), Mushroom intoxication (3), Wilson disease (2) and two ALF patients with unknown diagnosis due to insufficient data. The other cause(s) is/are associated with non-alcoholic steatohepatitis and occurred after gastric bypass (1), associated with multi-organ failure and Flu-/Voriconazole administration (1), with partial ornithine transcarbamylase deficiency (1), cirrhotic liver disease with steatosis (1), HELLP syndrome (1), T-cell lymphoma (1), non-compliance with immunosuppressive therapy/allograft rejection (1), hemochromatosis (1) and non-alcoholic fatty liver disease (1).

Table 2

Significant K8 and K18 variants in ALF patients

Variant	Nucleotide	# of patients (%)
K8 Y54H	TAT→CAT	1 (0.3)
K8 G62C	GGC→TGC	4 (1.2)
K8 T153A	ACT→GCT	1 (0.3)*
K8 R341H	CGT→CAT	23 (6.7)**
K8 V380I	GTC→ATC	2 (0.6)
K8 G422V	GGT→GTT	1 (0.3)
K8 G434S	GGC→AGC	11 (3.2)
K8 I466V	ATC→GTC	1 (0.3)*
K18 V48A	GTG→GCG	1 (0.3)
K18 G69A	GGG→GCG	1 (0.3)
K18 Δ65–72	N/A	1 (0.3)
Total		45 (13.1)

All described variants are heterozygous. Keratin variants were assigned as significant based on previous human association studies, documented biological effects or protein structure considerations. Five patients harbored two independent amino-acid altering K8 variants (2 K8A319S+R341H; 1 K8T153A+R341H; K8 R341H+I466V; 1 K8 I63V+V380I).

* Represents compound heterozygous variants.

** Two of the highlighted variants represent compound heterozygous amino-acid-altering variants.
 Bold lettering signifies novel variants which were not previously described.

Table 3

Distribution of selected K8 and K18 variants in the ALF and control groups

	Subgroup	Y54H	A333A	R341H	G434S	Total*
ALF	Caucasian	0/252	0/252 ⁷	18/252 ¹	0/252 ⁸	23/252 ^{3,9}
	African-American	1/41	5/41 ⁷	0/41	10/41 ^{2,8}	13/41 ⁹
	APAP	0/167	0/167	12/167	3/167	21/167
	Total	1/344	6/344	23/344	11/344	45/344
Control [¶]	Caucasian	0/338 ⁴	0/338 ⁵	23/727 ¹	0/338 ⁶	9/268 ³
	African-American	10/245 ⁴	31/245 ⁵	4/245	25/245 ^{2,6}	N.A.

* Includes all variants which are likely biologically significant based on previous human association studies or their biological effects.

[¶]The “control” group consists of a cohort of 236 African-Americans analyzed in this study (see Methods section for details) as well as 9 additional healthy African Americans reported in a prior study.⁹ The number of Caucasian controls was different (i.e., 268, 338 and 727) depending on the variants/exons analyzed in 3 independent studies. The complete exonic regions of K8/K18 were analyzed in 268 Caucasian controls.⁹ For K8 Y54H, A333A and G434S, 338 Caucasian controls were examined.^{9,20} For the K8 R341H there were 727 patients that were analyzed.^{9,19,20}

¹ p=0.01 (7.1% vs. 3.2%; OR 2.35, 95%CI 1.2–4.6)

² p=0.02 (24.4% vs. 10.2%; OR 2.84, 95%CI 1.1–6.8)

³ p=0.01 (9.1% vs. 3.4%; OR 2.89, 95%CI 1.3–7.2)

⁴ p=0.0002 (0% vs. 4.1%)

⁵ p<0.0001 (0% vs. 12.7%)

⁶ p<0.0001 (0% vs. 10.2%)

⁷ p<0.0001 (0% vs. 12.2%)

⁸ p<0.0001 (0% vs. 24.4%)

⁹ p=0.0003 (9.1% vs. 31.7%; OR 4.62, 95%CI 1.9–10.7)

N.A., not applicable since only the K8 exons 1,6 and 8 were analyzed in the majority of African-American control samples, while all the K8 exons were analyzed for the ALF group.

Table 4
Ethnic distribution of select K8 variants in acute and chronic liver disease patients

K8 Variant	Keratin Domain	AA (69)	Asian (92)	Caucasian (868)	Hispanic (91)
Y54H	Head	7.2 ^{a,b}	0 ^a	0.1 ^b	1
G62C	Head	2.9	0	1.5	0
A333A	Rod	11.6 ^c	0	0 ^c	4.2
R341H	Rod	0 ^d	1 ^e	4.8 ^f	14.3 ^{d,e,f}
G434S	Tail	18.8 ^{g,h,i}	0 ^g	0 ^h	1 ⁱ
IVS7+8C>T [†]	Intronic	11.6 ^k	0	0 ^k	0

AA, African-American. Summarized are the percent of K8 variants in the indicated ethnic groups reported collectively in the study herein and in two prior independent chronic liver disease patient groups.^{9,10} For each ethnic category the number in parenthesis represents the total patients examined [for some variants, one or two of the studies did not include the listed ethnic background; see Supplemental Table 4 for details regarding the specific number of patients for each category)

[†] Not all patients were analyzed for the presence of the variant.

^a p=0.03;

^b p<0.001;

^c p<0.001;

^d p=0.003;

^e p=0.002;

^f p=0.0006;

^g p<0.001;

^h p<0.001;

ⁱ p=0.0003;

^k p<0.001

Table 5*KRT8* variants in African-American control subjects**

Variant	Nucleotide	# of variants (%)
F38F	TTC→TTT	1 (0.4)
Y54H	TAT→CAT	9 (3.8)
IVS1+9C>A		2 (0.8)
A333A	GCC→GCT	31(13.1)*
A338A	GCC→GCT	2 (0.8)
R341H	CGT→CAT	4 (1.7)*
R362Q	CGG→CAG	1 (0.4)*
R369W	CGG→TGG	1 (0.4)
E376E	GAG→GAA	5 (2.1)
A433T	GCC→ACC	1 (0.4)
G434S	GGC→AGC	24 (10.2)
V480I	GTC→ATC	8 (3.4)
IVS8+31delC		2 (0.8)
IVS8+38del5nt		1 (0.4)

* One of the highlighted variants is compound heterozygous.

** The total number of tested controls is 236.

Bold lettering signifies novel variants which were not previously described.

Table 6

Univariate analysis of *KRT8/KRT18* exonic variant associations

Factor	Total*	R341H	G434S	Comp.	A333A/ A338A	Test
Race	0.004	0.145	0.000	n.s.	0.000	F.E.
ALF Etiology	0.09	n.s.	0.19	n.s.	0.009	F.E.
Center Number	n.s.	n.s.	n.s.	n.s.	n.s.	F.E.
Age	n.s.	n.s.	n.s.	n.s.	n.s.	L.R.
Coma total**	n.s.	n.s.	n.s.	0.147	n.s.	L.R.
Survival	n.s.	n.s.	n.s.	n.s.	n.s.	L.R.
Transplant	0.150	n.s.	0.079	n.s.	0.045	L.R.
Spon Survival	0.034	0.181	0.172	n.s.	0.148	L.R.
Cr max [^]	n.s.	0.10	n.s.	0.069	n.s.	L.R.
INR max [^]	n.s.	n.s.	0.100	n.s.	n.s.	L.R.
Bili max [^]	n.s.	n.s.	0.083	n.s.	0.041	L.R.

* Total significant variants (see Table 2).

** Represents the highest value in a given patient both prior to and after admission. Deceased patients were assigned coma score 4.

[^] 'Max' represents the highest value seen in a given patient both prior to and after admission.

Comp., Patients harbouring two independent amino-acid altering heterozygous variants; F.E., Fisher Exact test; L.R., Linear regression analysis; Spon Survival, survival without the need of a transplantation

Table 7

Multivariate Analysis of the impact of K8/K18 variants on survival without transplantation

Subgroup	Variants	% Nonsurvivors/Total		p-value	OR	95% CI
		With Variants	Without Variants			
Total	Significant	58	41	<0.1	1.8	0.90–3.78
Total	R341H	57	42	<0.05	2.6	1.01–6.61
Acetaminophen	Significant	43	22	0.05	2.6	0.99–6.77
Acetaminophen	R341H	42	23	0.14	2.5	0.75–8.56
Caucasians	Significant	57	37	0.02	3.1	1.19–7.98
Caucasians	R341H	61	37	<0.02	3.6	1.23–10.51

Analysis was adjusted for age, African-American race (yes/no) and the acetaminophen-related (yes/no) and ischemic (yes/no) ALF etiology. Nonsurvivors were defined as patients who either died or received a liver transplantation. Of note, we used the initial MELD score to determine if the transplanted group differed from those who died without transplant and found no statistically significant difference between both groups ($p=0.09$ in all subjects, $p=0.40$ in Caucasians). In contrast, patients who survived transplant-free displayed significantly lower initial MELD scores than patients who were either transplanted or who died without transplant (27.6 ± 8.0 , 34.2 ± 8.0 and 36.4 ± 8.3 , respectively; $p<0.001$ in both cases overall and in Caucasians). Although transplant criteria differ among transplant centers, and MELD scores are not used for listing patients with ALF,²⁴ these MELD estimations help justify using transplant-free survival as an important endpoint indicative of less severe disease.