ELECTRONIC LETTER

Keratin 8 Y54H and G62C mutations are not associated with liver disease

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The cytoskeleton comprises three filamentous systems: microfilaments, intermediate filaments, and microtubules. In epithelial cells, type I keratins such as keratin 18 (KRT18) and type II keratins such as keratin 8 (KRT8) polymerise to form the intermediate filaments. KRT18 and KRT8 represent the major keratins expressed in single-layered epithelia of the gastrointestinal tract including liver and pancreas.[1]

Animal studies suggest KRT8 and KRT18 have a hepatoprotective role against mechanical and toxic injury.[2] Transgenic mice overexpressing mutant KRT18 display fragile hepatocytes with disrupted cytoskeleton filaments.[3] These mice developed chronic hepatitis and were more susceptible to liver injury in comparison to mice overexpressing wild type KRT18.[4] The viability of *KRT8* null mice depends on the genetic background of the different mouse strains suggesting further genetic factors contribute to the resultant phenotype. For instance, in one mouse strain *KRT8*-deficient mice died during embryonic development due to extensive liver haemorrhage.[5] However, in another strain 55% of the *KRT8*-deficient mice had a normal life expectancy but developed signs of inflammatory bowel disease and in some cases a mild inflammation of the liver.[6] A recent report emphasises the importance of Keratin 8 for the formation of an intact placental barrier function for the viability of *KRT8*-deficient embryos. These findings argue in favour of an extraembryonic defect responsible for lethality of these embryos.[7] Furthermore, *KRT8* null mice showed an abnormal histological liver architecture and were more vulnerable to liver damage after exposure to hepatotoxic substances compared to wild type mice.[8–10]

The above mentioned results support the hypothesis that keratin mutations might predispose humans to liver disease. Indeed, Ku *et al* described an association between two *KRT8* mutations and cryptogenic cirrhosis. A heterozygous single base substitution involving a Gly to Cys at codon 62 (G62C) was found in three out of 55 patients and a heterozygous Tyr to His exchange at position 54 (Y54H) was found in two out of 55 patients with cryptogenic cirrhosis. Neither mutation was detected either in 98 patients with other liver diseases or in 86 control subjects.[11] The role of *KRT8* as a genetic risk factor for developing chronic liver diseases of different aetiologies was further supported by a recently published study.[12]

Following the reports of Ku *et al* we investigated *KRT8* Y54H and G62C mutations in patients with cryptogenic cirrhosis as well as in patients with various other chronic liver diseases such as viral hepatitis, alcoholic cirrhosis, autoimmune hepatitis, primary biliary cirrhosis, and primary sclerosing cholangitis in a large population comprising more than 2000 patients and healthy controls.

Key points

- Keratin 8 is one of the major intermediate filament proteins expressed in single-layered epithelia of the gastrointestinal tract. Animal models provided evidence that keratin 8 (KRT8) has an important protective role against mechanical and toxic stress in hepatocytes. Recently, *KRT8* mutations were identified and linked to cryptogenic and non cryptogenic liver disease.
- In order to investigate the relevance of two hitherto described *KRT8* mutations, Y54H and G62C, we genotyped 1668 patients with liver disorders of different aetiologies (viral, autoimmune, cryptogenic, alcoholic, and others) as well as 679 healthy controls for Y54H and G62C. Samples were analysed by PCR amplification and subsequent melting curve analysis with fluorescence resonance energy transfer (FRET) probes.
- The genotype distributions of both mutations were similar in patients and controls. The G62C variation was detected in 27 out of 1668 patients (1.6%) compared to 12 out of 679 controls (1.8%). The Y54H alteration was found in two patients (0.1%) and one control (0.1%). Patients carrying a mutation suffered from distinct liver diseases and no association with cryptogenic cirrhosis or with any of the other investigated liver diseases was found. Genotype/phenotype analysis revealed no particular findings in patients carrying one mutant allele.
- In contrast to previous reports, *KRT8* mutations neither predispose to cryptogenic cirrhosis nor to chronic liver disease of other aetiologies.

METHODS

Study subjects

We recruited 1668 patients with various liver disorders in our hospital between 1998 and 2003. According to the study protocol, which was approved by the Charité Ethics Committee, blood samples were obtained at the time of routine clinic attendance from patients who had either undergone liver transplantation or who suffer from chronic liver disease. The respective aetiologies of liver disease in our population are shown in table 1. The majority of our patients (1617 out of 1668) were Caucasians with 1400 patients originating from Germany, 131 from Turkey, and 86 from other European countries. Furthermore, 47 Asians and four patients of African origin were included in the study.

We investigated 679 healthy volunteers. Among these control subjects were medical students and medical staff

Abbreviations: BASE, Berlin Aging Study; FRET, fluorescence resonance energy transfer

(n = 168) and parents of healthy newborns (n = 347) as well as healthy volunteers of the Berlin Aging Study (BASE) (n = 164). Information about the ethnic background of the control subjects was available in 547/679 subjects (81%). All parents of healthy newborns and all individuals of the Berlin Aging Study were of German ancestry. Among the medical students and medical staff ethnicity was known in 36 individuals (34 German, one Asian, and one African-German).

Genetic analysis

DNA was extracted from peripheral blood leukocytes using spin columns (Qiagen, Hilden, Germany). We amplified exon 1 of *KRT8* using 5′-CGCTCCTTCTAGGATCTCCG-3′ as forward primer and 5′-GGCACAGTCAGCCACGCAG-3′ as reverse primer. We designed primers and FRET probes according to the published *KRT8* sequence (GenBank #M34482). We performed PCR using 0.75 U AmpliTaq Gold (Applied Biosystems, Weitersstadt, Germany), 400 µM dNTPs, 1.5 mM $MgCl_2$, and 0.1 µM of each primer in a final volume of 25 µl. The reaction mix was denatured at 95℃ for 12 min followed by 48 cycles of denaturation at 95℃ for 20 s, annealing at 64℃ for 40 s, elongation at 72℃ for 90 s, and a final extension step for 2 min at 72℃ in an automated thermocycler (Biometra, Göttingen, Germany).

The detection of the mutant alleles was carried out by melting curve analysis with fluorescence resonance energy transfer (FRET) probes in a LightCycler (Roche Diagnostics, Mannheim, Germany). The probes were designed complementary to the mutant allele of both codons. For detection of Y54H variation the sequence of the sensor fluoresceine labelled probe was 5′-CCCCACCATGGCCGCC-FL and that of the anchor LC Red 705 labelled probe was 5′-LC 705-CCCAGGCCACCGCGAAAGTTGC. For identification of the G62C mutation the sensor probe 5′-LC 640-GTGATGCATCCC ATGCCGCT and the anchor probe 5′-TCAGCAGGCTCTGGTT GACCGTAACTGC-FL were used. All FRET probes were designed and synthesised by TIB MOLBIOL, Berlin, Germany.

We numbered the mutations according to the recommendations of the Nomenclature Working Group for human gene mutations.[13] Thus, we apply the correct numbering Y54H and G62C for the *KRT8* alterations as previously described by Lee *et al*.[14]

Table 1 Frequency of *KRT8* mutations (G62C and Y54H) in patients with chronic liver diseases of different aetiologies and healthy controls

Groups	Number of individuals studied	G62C	Y54H
All patients	1668	27 (1.6%)	2 (0.1%)
Chronic HCV infection	672	13 (1.9%)	1 (0.1%)
Alcoholic liver disease	215	4 (1.9%)	–
Chronic HBV infection	200	1 (0.5%)	1 (0.5%)
Primary biliary cirrhosis	162	1 (0.6%)	–
Autoimmune hepatitis	104	4 (3.8%)	–
Cryptogenic cirrhosis	61	1 (1.6%)	–
Primary sclerosing cholangitis	54	2 (3.7%)	–
Non alcoholic fatty liver disease	52	1 (1.9%)	–
Miscellaneous*	148	–	–
Controls	679	12 (1.8%)	1 (0.1%)

*The group of patients with miscellaneous liver disorders included 21 patients with hepatocellular carcinoma, six patients with cholangiocellular carcinoma, two patients with liver metastases of neuroendocrine tumours, 32 patients with acute liver failure, 14 patients with Budd Chiari's syndrome, one patient with pregnancy induced cholestasis, 54 patients with metabolic/genetic liver disorders, nine patients with drug-induced liver disease, six patients with biliary cirrhosis, two patients with extrahepatic biliary atresia, and one patient with sarcoidosis. HBV, hepatitis B virus; HCV, hepatitis C virus.

Statistical analysis

Statistical analysis was carried out using chi-square test and Fisher's exact test. p Values less than 0.05 were considered to be statistically significant. SPSS software version 11.0 for Windows (Chicago, IL, USA) was used to perform statistical analysis.

RESULTS

The frequency of *KRT8* Y54H and G62C variations in a large cohort of 1668 patients suffering from various liver diseases is shown in table 1. The G62C mutation was detected in 27 patients (1.6%) and 12 healthy controls (1.8%), p = 0.8. The frequency of G62C among the three control populations did not differ significantly. Two patients (0.1%) and one control (0.1%) were heterozygous carriers of the Y54H variation (p = 0.9). We did not detect any homozygous or compound heterozygous carriers of these two mutations. Overall, there were no significant differences in genotype distribution of both mutations between the various groups of liver diseases investigated. Genotype/phenotype analysis of patients carrying *KRT8* mutations revealed no specific characteristics. In particular, the mean age of patients with wild type and mutant *KRT8* was similar (51 years; range 7–87 *v* 51 years, range 20–67). Moreover, we observed no differences concerning disease severity or request for liver transplantation between these two groups of patients. In 794 patients liver transplantation was required for advanced liver disease with 14 (1.8%) of these patients being carriers of a mutant *KRT8* allele compared to 15 (1.7%) out of 874 patients with no need for a liver graft (p = 0.9).

Among those patients carrying a G62C mutation were 26 patients of German ancestry and one Turkish patient. Both patients with an Y54H variation originated from Africa. Eleven control subjects with G62C were of German origin. Information about ethnicity was unavailable in one control with G62C and in the control carrying Y54H.

DISCUSSION

Animal models clearly showed that *KRT8/18* transgenic and knock out mice were predisposed to liver damage. However, these animal studies suggested a complex inheritance pattern for *KRT8/18* mutations. The importance of keratins in the pathogenesis of liver damage was further supported by their modulation of Fas-mediated apoptosis.[15 16]

The relevance of the *KRT8* mutations Y54H and G62C was studied in a large population of 1668 patients with liver disease and 679 healthy controls. In contrast to the results of Ku *et al*,[11 12] the hitherto described human *KRT8* mutations were associated neither with cryptogenic nor with non cryptogenic chronic liver disease in our population. An allele frequency of 0.8% and 0.9% was observed for the G62C alteration in patients and healthy controls, respectively. For the Y54H mutation the respective allele frequencies were 0.06% and 0.08%. The discrepancies between our study and the previous reports are readily explained by the differences in the number of patients and controls investigated in both groups. To investigate whether keratin mutations have any impact on liver disease severity, we studied patients with advanced liver disease requiring liver transplantation as well as patients who suffered from chronic liver disease including all stages of fibrosis. No differences in *KRT8* mutations were found in relation to fibrosis stage indicating that patients carrying *KRT8* mutations are not more prone to develop severe liver disease and cirrhosis. Among our patients were 1617 (97%) Caucasians. Therefore, another possible explanation for the difference between our results and those

of Ku *et al* might be the greater ethnic homogeneity of our patient and control population. It is important to note that both patients with Y54H were of African origin. In the population studied by Ku *et al* three out of five patients and the control subject carrying the Y54H alteration were also African-Americans. These observations suggest that the Y54H variation occurs more frequently among individuals of African origin.

Evidence is increasing that cryptogenic cirrhosis is a heterogeneous disorder.[17 18] Patients presenting no evidence of any known aetiologies for liver disease are usually defined as suffering from cryptogenic cirrhosis though this diagnosis stills depends on how carefully other causes are excluded. We thoroughly re-evaluated all patients with an initial diagnosis of cryptogenic cirrhosis as described previously.[18] Differentiation of cryptogenic liver disease from an underlying autoimmune process is particularly challenging. Ku *et al* reported that two of their five patients with *KRT8* mutations and cryptogenic cirrhosis had some autoimmune features which were not particularly defined. Interestingly, we found the highest frequency of *KRT8* mutations in patients suffering from autoimmune hepatitis and primary sclerosing cholangitis. Therefore, the association of *KRT8* mutations and cryptogenic cirrhosis documented in previous reports might be in part due to an underlying autoimmune liver disease in these patients. Keeping these facts in mind the exact role of *KRT8* in hepatic autoimmune processes remains to be elucidated.

In conclusion, our study clearly demonstrates that carriers of the two *KRT8* mutations, Y54H and G62C, are not at increased risk for developing cryptogenic or non cryptogenic chronic liver diseases. Furthermore, there is conclusive evidence that Y54H occurs mainly among people of African origin. These rare sequence variations may therefore rather represent non pathogenic genetic alterations than disease-causing mutations.

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