JOURNAL OF VIRAL HEPATITIS
JVH

Journal of Viral Hepatitis, 2011, **18,** e167–e174 doi:10.1111/j.1365-2893.2010.01410.x

What factors determine the severity of hepatitis A-related acute liver failure?

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Received July 2010; accepted for publication October 2010

SUMMARY. The reason(s) that hepatitis A virus (HAV) infection may progress infrequently to acute liver failure are poorly understood. We examined host and viral factors in 29 consecutive adult patients with HAV-associated acute liver failure enrolled at 10 sites participating in the US ALF Study Group. Eighteen of twenty-four acute liver failure sera were PCR positive while six had no detectable virus. HAV genotype was determined using phylogenetic analysis and the full-length genome sequences of the HAV from a cute liver failure sera were compared to those from self-limited acute HAV cases selected from the CDC database. We found that rates of nucleotide substitution did not vary significantly between the liver failure and non-liver failure cases and there was no significant variation in amino acid sequences between the two groups. Four of 18 HAV isolates were sub-genotype IB, acquired from the same study site over a 3.5-year period. Sub-genotype IB was found more frequently among acute liver failure cases compared to the non-liver failure cases (chi-square test, $P < 0.01$). At another centre, a mother and her son presented with HAV and liver failure within 1 month of each other. Predictors of spontaneous survival included detectable serum HAV RNA, while age, gender, HAV genotype and nucleotide substitutions were not associated with outcome. The more frequent appearance of rapid viral clearance and its association with poor outcomes in acute liver failure as well as the finding of familial cases imply a possible host genetic predisposition that contributes to a fulminant course. Recurrent cases of the rare sub-genotype IB over several years at a single centre imply a community reservoir of infection and possible increased pathogenicity of certain infrequent viral genotypes.

Keywords: acute liver failure, epidemiology, genome sequencing, hepatitis A.

INTRODUCTION

Hepatitis A virus (HAV), a single stranded RNA virus, is endemic throughout most of the world and is also highly genetically conserved [1,2]. In the United States, rates of infection have dropped significantly with increased use of the hepatitis A vaccine, however the estimated incidence is still above 30 000 new infections per year [3]. The infection usually follows an innocuous course, remaining sub-clinical in most children, while appearing as acute self-limited hepatitis in adults. Less than 1% of acute hepatitis A cases result in acute liver failure [4,5]. HAV infection currently accounts for only 3% of adult acute liver failure (ALF) cases in the United States [4,6]. Although hepatitis A-related ALF patients experience a relatively high spontaneous survival rate (69%), the remaining patients either die or require emergency liver transplantation [7].

The relationship between self-limited hepatitis A cases and those HAV infections resulting in ALF (HAV ALF) is poorly understood. Host factors including age and underlying liver disease are thought to increase the likelihood of a fulminant course [8,9]. Viral factors including low viral load and a higher rate of substitution in the 5′ untranslated region (UTR) of the viral RNA have also been correlated with increased frequency of HAV-related ALF [10,11]. Another potential viral factor is genotype. Epidemiologic studies in the United States have demonstrated that sub-genotype IA is most common, comprising 98% of infections, while

Abbreviations: ALF, acute liver failure; HAV, hepatitis A virus; INR, international normalized ratio; UTR, untranslated region.

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*See Appendix for The Acute Liver Failure Study Group 1998–2004

sub-genotype IB is found in only 2%; most IB infections have been associated with international travel [12]. To date, no correlation between ALF and HAV genotype has been evident but this has not been systematically examined. Previous studies have focused on the 5′ UTR and VP1-P2B regions, while only small case series have evaluated the entire HAV genome of HAV ALF cases [13]. This study aimed to further explore both host and viral factors, including genotype and nucleotide sequence variation in the entire HAV genome among consecutive adult cases of HAV ALF from the multicentre adult US acute liver failure registry. To identify factors contributing to variation in outcomes, full genome sequences of 18 HAV ALF cases were compared to geographically matched controls: patients with self-limited acute HAV identified in the CDC database.

PATIENTS AND METHODS

The adult US Acute Liver Failure Study Group (ALFSG) is a cooperative effort of 23 tertiary liver centres to study the aetiology and outcomes of ALF [14]. Twenty-nine patients from the ALFSG registry were diagnosed with HAV ALF between January 1998 and May 2004 and all of these patients were included in this study. ALF was defined as the onset of encephalopathy and coagulopathy within 26 weeks of first symptoms of hepatic illness with no prior history of liver disease [15]. Encephalopathy was defined as any degree of altered mentation while coagulopathy was defined by an international normalized ratio (INR) ≥1.5. ALF was attributed to acute HAV infection when a positive IgM anti-HAV antibody test was obtained in conjunction with consistent clinical findings by the site investigator and the data-coordinating centre. All patients tested negative for other markers of acute viral infection including HBsAg, anti-HBc IgM, anti-HCV with the exception of two patients. One had typical acute HAV infection but was hepatitis C virus (HCV) antibody positive without detectable HCV RNA on repeated testing, unlikely to represent acute hepatitis C. One patient had evidence of both chronic hepatitis B and C with detectable HBsAg, anti-HCV, HBV DNA and HCV RNA detected but was shown to be anti-HAV IgM positive and recovered fully from ALF.

Informed consent, approved by the Institutional Review Board of each of the respective centres, was obtained from the next of kin due to the patients' altered mental status and all cases were coded to maintain anonymity. Detailed data including the patients' demographics, history, laboratory results, and outcome were obtained. Sera were collected daily for up to 7 days, stored at −80 °C and shipped to the central repository for continued storage prior to testing.

A series of randomly selected sera from US and Mexican patients with acute hepatitis A infection in the CDC database was used as controls for the HAV whole-genome analysis. These control sera included 20 sub-genotype IA and 8 sub-genotype IB specimens obtained over a similar time frame from a comparable US geographic distribution as the HAV ALF cases.

HAV RNA amplification and sequencing

Serum samples (collected on day 1 or no later than day 4) were available from 24 of 29 patients. These samples were used for HAV whole-genome amplification. HAV surveillance databases at the Division of Viral Hepatitis, Centers for Disease Control and Prevention, Atlanta, GA, USA were accessed to form a demographically comparable control group of acute hepatitis cases for comparison of the entire viral RNA genome. RNA was extracted using the Total Nucleic Acid Isolation kit in conjunction with a MagNAPure LC system (Roche Applied Science, Indianapolis, IN, USA). Extracted RNA was used as a template in a one step RT-PCR reaction (QIAGEN, Valencia, CA, USA) to amplify 14 different overlapping fragments encompassing the entire length of the HAV genome. Nested PCR was carried out for each amplicon to generate fragments suitable for sequencing. Sequencing of both DNA strands was carried out with the corresponding internal primers using BigDye v3.1 chemistry (Applied Biosystems, Foster City, CA, USA). The resulting products from the sequencing reaction were cleaned using the Millipore Montage SEQ96 Cleanup Kit (Millipore, Billerica, MA, USA) and later run on 3130xl Genetic Analyzer (Applied Biosystems). All reactions were performed on a Biomek 3000 Laboratory Automation Workstation (Beckman Coulter, Fullerton, CA, USA). Sequences were assembled and preliminarily analysed using SeqMan and MegAlign programs from the Lasergene DNA & Protein analysis package v8.0.2 (DNASTAR Inc., Madison, WI, USA) The Accelrys GCG Package v11.0 (Accelrys Inc. San Diego, CA, USA) was used for phylogenetic analysis. Shannon entropy was expressed as mean + standard deviation (SD). Differences in Shannon entropy and viral genetic identity were tested by ANOVA. SAS for Windows version 9.2 (SAS Institute Inc., Cary, NC, USA) was used for statistics analysis.

Measurement of acetaminophen protein adducts

Adducts in sera were measured using a high-performance liquid chromatography method with electrochemical detection as previously described [16,17].

RESULTS

Epidemiological data

The 29 patients were obtained from 10 of the 23 ALFSG sites; demographic and laboratory data are shown in Table 1. Four patients with sub-genotype IB were from the same site (site 23, University of Michigan) and presented over the course of 3.5 years. That same site demonstrated

Table 1 Demographic and clinical data for 29 patients with HAV-related acute liver failure

No.	Gender	Age	APAP use	Adducts	Serum	PCR Status	Genotype	Peak ALT	Peak AST	Outcome
1	Male	23	−	<LLQ	+	+	1A	2946	331	SS
2	Male	52	−	N/A	−	N/A	N/A	364	262	LTx
3	Female	32	+	N/A	+	+	1A	3333	3212	SS
4	Male	72	−	N/A	−	N/A	N/A	1214	2340	LTx
5	Female	21	+	<LLQ	+	−	1A	2664	1039	SS
6	Male	55	+	<LLQ	+	−	1A	170	204	Died
7	Female	39	+	0.238	+	+	1A	607	763	SS
8	Male	47	+	<LLQ	+	−	1A	2404	892	LTx
9	Male	41	−	<LLQ	+	−	1A	1516	4000	LTx
10	Female	60	−	<LLQ	+	+	1A	7560	1353	LTx
11	Male	48	−	<LLQ	+	+	1A	4134	924	SS
12	Female	72	−	N/A	−	N/A	N/A	3266	1235	SS
13	Female	41	−	<LLQ	+	−	1A	947	2241	LTx
14	Female	57	−	<LLQ	+	+	1A	4914	4784	SS
15	Male	57	−	N/A	−	N/A	N/A	3147	896	LTx
16	Male	64	−	N/A	−	N/A	N/A	3052	5470	Died
17	Male	40	−	<LLQ	+	−	1A	1898	3346*	LTx & Died
18	Male	55	−	<LLQ	+	+	1A	1659	2157*	LTx
19	Female	46	+	<LLQ	+	+	1B	6591	5993	SS
20	Male	21	−	<LLQ	+	+	1B	3440	2384	SS
21	Female	66	−	<LLQ	+	+	1B	2102	734	Died
22	Male	32	−	< LLQ	+	+	1B	9817	>9000	SS
23	Female	57	+	0.18	+	+	1A	2622	623	SS
24	Female	45	+	0.338	+	+	1A	1367	330	SS
25	Male	50	+	0.279	+	+	1A	2658	583	Died
26	Female	53	+	<LLQ	+	+	1A	2482	607	SS
27	Female	43†	+	<LLQ	+	+	1A	1989	2486	SS
28	Female	69	+	<LLQ	+	+	1A	4440	2069	SS
29	Male	44	−	<LLQ	+	+	1A	6795	4417	SS

SS, spontaneous survival; <LLQ, below lower limit quantification.
*Anti-HCV positive.
†Concomitant chronic HBV/HCV.

one sub-genotype IA case during the study period as well. Site 35, Albert Einstein Medical College, recorded two related HAV ALF patients admitted within 1 month of each other, a mother and son. The mother's infection preceded the son's. Although they did not live together they had shared meals during the mother's period of infection. There were no other known interactions between patients or common risk factors other than geographic location and none of the patients had migrated from an endemic area outside of the United States.

Patient outcomes

Sixteen of twenty-nine (55%) patients recovered spontaneously (e.g. without transplantation). Nine of twenty-nine (31%) received a liver transplant and eight survived at least 3 weeks post transplant. Four of twenty-nine patients (14%) died from renal failure, brain oedema, respiratory failure or cardiac arrest.

HAV genetic analyses

Eighteen of twenty-four (75%) samples were RT-PCR positive (Table 1). To confirm the PCR negative results, we repeated testing on later (day 3) samples. Five of the six remained negative. The entire genome of 15 of the 18 initially PCR positive cases was sequenced. Within the three cases that could not be completely sequenced; the VP1-P2B (nt 2912–3277) region and 5′ UTR were sequenced successfully. Based on phylogenetic analysis of the VP1-P2B region 14 cases were classified as sub-genotype IA and 4 cases as sub-genotype IB. The complete HAV-genome sequences of the 15 ALF cases were compared with a control group of sequences of sub-genotype IA (n = 20) and sub-genotype IB (n = 8) retrieved from the CDC surveillance databases of acute hepatitis A cases (Fig. 1). The nucleotide and amino acid sequences were compared between ALF cases and controls within the same sub-genotype. There were no apparent sequence differences or specific nucleotide/

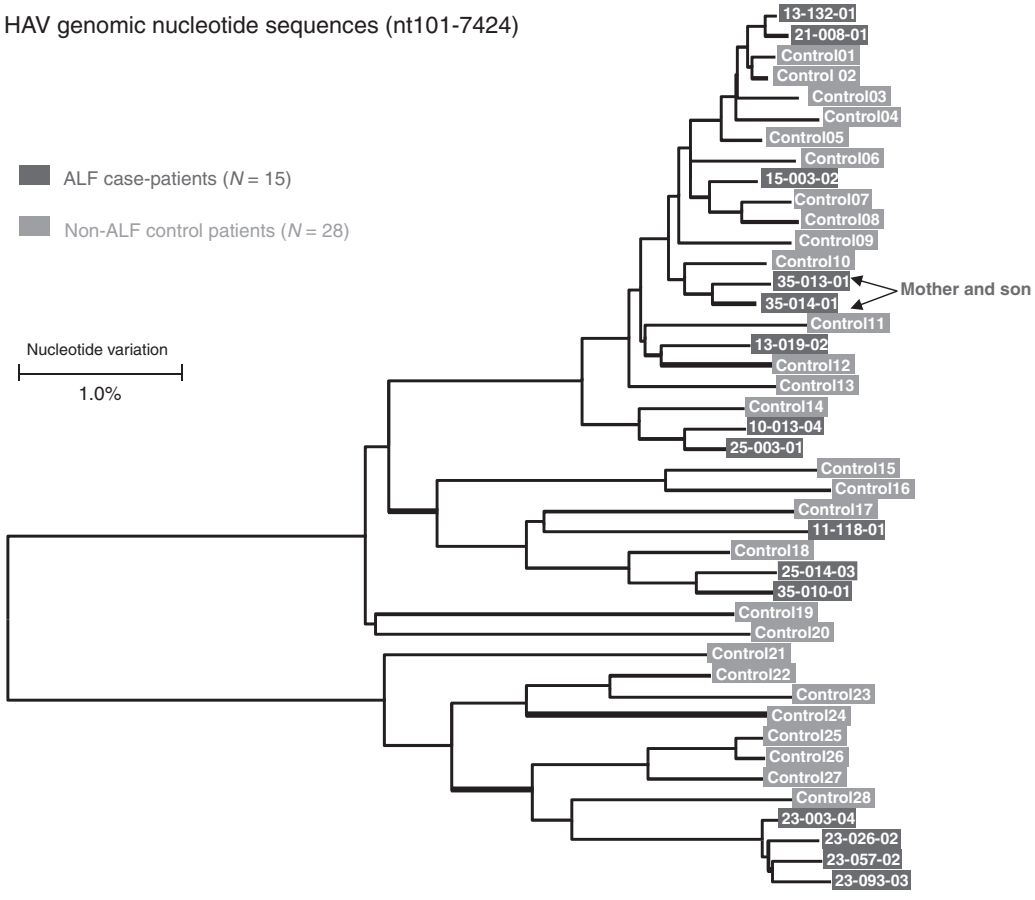

Figure 1 Genomic tree for 15 HAV ALF patients for whom adequate PCR products were available for sequencing, compared with 28 'control' HAV patients without ALF. The mother and son who both developed acute liver failure after hepatitis A had nearly identical viruses. The four patients from one site who were infected with sub-genotype IB were also remarkably similar in sequence. Otherwise, there was no clustering of the HAV ALF cases. Number sequence of ALF patients = site number–patient number–day of study.

amino acid substitutions between ALF cases and controls. Also, no significant differences by ANOVA were observed in the Shannon entropy and genetic similarity between the ALF case and control sequences among sub-genotype IA. The mean nucleotide identity among ALF cases and control sequences were 96.8 per 100 nucleotides (SD = 1.78) and 96.6% (SD = 1.81) among the sub-genotype IA (ANOVA, $P = 0.447$; Table 2). However, similar analyses of the HAV sub-genotype IB sequences showed a higher nucleotide and amino acid identity among HAV variants from ALF cases than controls (ANOVA, $P < 0.01$; Table 2). Rates of spontaneous survival were not significantly different between the two sub-genotypes (chi-square test, Table 3).

PCR negative cases

The six PCR negative samples had viral loads below the lower limit of detection of the assay of 0.1 plaque forming units/100 μL [18]. PCR negative patients experienced worse outcomes than those who were PCR positive (Table 3); transplant free survival amongst PCR positive cases was 77.8% while PCR negative cases experienced a transplant free survival of 16.7% (chi-square test, $P < 0.01$). Being PCR positive was associated with a decreased risk of death or transplant with an odds ratio of 0.057 (95% confidence interval 0.005–0.641, $P = 0.02$). Of interest, the PCR negative cases as a group had lower mean peak ALT levels: 1414 IU/L compared to 3113 IU/L for the PCR positive ALF patients. Prothrombin time/international normalized ratio (INR) values were slightly more prolonged: 3.9 in the PCR negative patients *vs* 2.9 for PCR positive cases.

HAV genotype IB

Four out of eighteen (22%) of the PCR positive ALF patients were infected with sub-genotype IB, which is rare in the United States. Though not identical, the sequences were remarkably similar although the cases occurred over a three and a half year period. None of the patients were born abroad nor had they visited countries where genotype IB is

Table 2 Patients with HAV ALF and control HAV patients were compared for genetic complexity and identity. Sub-genotype IB cases differed somewhat from controls but there were no differences between sub-genotype IA cases and controls

Genotype	ALF patients	Nucleotides Shannon entropy (mean ± SD)	Amino acids Shannon entropy (mean ±SD)	Nucleotides identity (mean ± sd)	Amino acids identity (mean ±SD)
IA	Cases (n = 11)	0.0176 ±0.0593	0.0036 ±0.0261	96.8 ±1.78	99.4 ±0.21
	Controls (n = 20)	0.0170 ±0.0463	0.0037 ±0.0198	96.6 ±1.81	99.4 ±0.27
	Sub-Total	0.0173 ±0.0532	0.0036 ±0.0231	96.6 ±1.80	99.4 ±0.25
IB	Cases (n = 4)	0.0045 ±0.0434**	0.0043 ±0.0419	99.5 ±0.12**	99.5 ±016**
	Controls (n = 8)	0.0228 ±0.0737	0.0057 ±0.0364	96.2 ±1.16	99.2 ±0.23
	Sub-total	0.0137 ±0.0611	0.0049 ±0.0393	96.8 ±1.63	99.2 ±0.24

**$P < 0.01$.

Table 3 Outcomes by sub-genotype and by PCR status on admission to study. Genotype was not associated with a difference in outcome but early clearance, defined as PCR negative on admission, was associated with much poorer survival

Subgroup/ outcome	Spontaneous survivors	Death or LTx	P-value
Genotype 1B	3 (75%)	1 (25%)	0.88
Genotype 1A	11 (78.6%)	3 (21.4%)	
PCR+	14 (77.8%)	4 (22.2%)	0.007
PCR−	1 (16.7%)	5 (83.3%)	

more common. During the same observation period (1998–2004), only 19 (2.2%) sub-genotype IB cases were detected in the United States among 834 hepatitis A cases identified through different surveillance projects and this was also highly significant ($P < 0.01$) by chi-square test.

Patient co-factors

Case report data revealed that 12 of 29 patients (41.4%) reported having taken more than 1g of acetaminophen/day for at least 3 days preceding hospital admission (Table 1), therapeutic dosing that might indicate possible toxicity in the setting of viral hepatitis. Four patients reported regular alcohol consumption (>2 drinks per night), one of whom also took acetaminophen. There was no correlation between a history of acetaminophen use and poorer outcomes. Acetaminophen adducts were measured in all 24 for whom sera were available; 20 were undetectable and four demonstrated levels below 1.0 nmol a-cys adducts/mL (range 0.18–0.34). None were considered compatible with liver toxicity due to acetaminophen [16]. A more detailed clinical description of these cases has been published recently. As in our prior report of the same 29 cases, subject age, gender, and MELD score were not predictive of patient outcomes [19].

DISCUSSION

Since prior studies had suggested a possible difference in viral substitutions between HAV ALF patients and other patients with acute HAV, we were interested in further exploring whether unique viral genome patterns might predispose hepatitis A patients to liver failure. We did not find a clear difference in genomic sequences between patients with HAV ALF and those with self-limited hepatitis A.

The rates of single nucleotide substitutions in the complete HAV genome including both the 5′ UTR and the VP1-P2B regions showed no significant variation between those with HAV ALF and patients with uncomplicated acute hepatitis A infection. This finding suggests that variation in severity of hepatitis A infection is not dependent upon rates of substitution in the internal ribosomal entry site of the 5′ UTR or elsewhere in the genome. Furthermore, the differences in rates of substitution in the transcribed amino acid residues were also not significant in the two groups (Table 2).

Only a small fraction (8%) of acute HAV sera from a previous large CDC cohort study was shown to be PCR negative during the acute illness [12]. Among our HAV ALF study patients, 25% (6/24) were PCR negative and this status correlated with significantly worse outcomes defined by a lower rate of spontaneous survival. The inability to obtain PCR products in our study corresponds with an undetectable or very low viral load. Low or undetectable viral load at presentation has been previously cited as a significant factor differentiating fulminant from non-fulminant cases for both hepatitis A [11] and hepatitis B [20].

In vitro studies suggest that the immunologic response is primarily responsible for the liver disease in HAV infections [21]. In our study, undetectable viral load not only correlated with more severe disease, but also indicated a worse outcome amongst the ALF cases. However, the small number of patients with undetectable HAV RNA may limit the generalizability of this finding. Quantitative PCR was not available for this study.

With regard to use of acetaminophen, 12 of 29 patients gave a history of acetaminophen ingestion of at least 1 g/day for a minimum of 3 days (Table 1). We could not relate a history of acetaminophen ingestion to poorer outcomes or to more severe disease in this small case series, nor could we identify other demographic or clinical factors such as age or gender to be associated with poor outcome (Table 1).

Additional data were obtained on two clusters of HAV ALF cases with highly conserved viral RNA genomes. The first revealed a mother and son, both of whom presented with HAV ALF within 1 month of each other to the same clinical centre and without pre-existing liver disease. Although they did not live together, they shared meals together during the time of the first patient's infection and the close genetic similarity of their viruses confirms that infection appears to have been transmitted from mother to son. HAV ALF cases transmitted between family members have been reported in the literature, specifically the occurrence of HAV ALF among siblings [22,23]. Often the virus was transmitted to the family via a community outbreak of routine HAV infections, suggesting that host factors contributed to these more severe outcomes. Furthermore, all reported inter-familial occurrences of HAV ALF have been among patients with no pre-existing liver condition. In both previously reported studies of siblings, the infections occurred in young patients whose age ranged from 16 to 24 years. Genetic predisposition to a fulminant course could be a factor considering the inter-familial HAV ALF outbreak we observed in our study, emphasizing the possible importance of the immunological response to outcome.

A second cluster of HAV ALF cases came from a single centre. Four of five cases from this site were sub-genotype IB, which occurs very infrequently in the United States. This finding coincides with epidemiological data from typical acute hepatitis A cases that suggest that community reservoirs of infection may be present for long periods of time under certain circumstances [12]. The near identity of all four IB sequences, coupled with the rarity of genotype IB, suggest ongoing transmission of this one strain within the community and that it may be somewhat more virulent than other subtypes but this remains highly speculative.

Our study was limited in that we had serum samples on only 24 of the 29 patients with HAV ALF in the study. In addition, we could not perform phylogenetic analysis on five of the six patients because they were PCR negative repeatedly during the study period. Finally, the case controls used in this analysis of whole genome sequencing were not obtained from the same local hospital as the ALF index case due to the limited number of annual HAV cases that the CDC has access to. Nonetheless, matching based upon medical centre location and year of infection was undertaken. The small number of cases in this study of acute liver failure provides further evidence of the declining incidence of HAV infection in the United States [19].

In conclusion, viral factors including substitution rate in the entire genome do not seem to differ between HAV ALF patients and those with uncomplicated hepatitis A infection in North America. While this study is limited in the number of cases presented, the rapidly declining rate of HAV and HAV associated ALF make it unlikely that larger studies will be possible in the future at least in the United States. However, further analysis of genotype I should be performed, particularly in locations where HAV is still endemic. An undetectable viral load at the onset of liver failure correlated in our study with worse outcomes. HAV RNA testing by PCR, if it were readily obtainable, might help determine prognosis but should not be used to preclude referral and listing for transplantation. The association of undetectable viral load with poor outcome suggests a genetic predisposition to a 'excessive' host immune response contributes to the more severe disease observed. This is further supported by the occurrence of familial ALF case clustering. Whole genome sequence analyses of patients with ALF of diverse aetiologies compared to ethnically matched controls may prove useful in identifying the genetic basis for differences in outcomes with hepatotrophic viruses [24].

ACKNOWLEDGEMENTS

This work was funded by NIH grant DK U-01 58369 for the Acute Liver Failure Study Group. Additional funding provided by the Tips Fund of Northwestern Medical Foundation and the Jeanne Roberts and Rollin and Mary Ella King Funds of the Southwestern Medical Foundation. VA received a summer research fellowship as a first year medical student at UT Southwestern Medical School.

The authors gratefully acknowledge the excellent technical assistance provided by Dr. Patricia Jones, Jerry Ashmore, Jason A Feinberg, Barbara Slocum, Barbara Duke, Yousuf Parupia, Dr. Martin Kroll, Johanna J. Koch, Viraj Mehta, and Rosamond Klick in the completion of the work presented in this manuscript. In addition, the continued support of the coordinators and investigators participating in the ALF Study Group has been essential to this work and is gratefully acknowledged.

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APPENDIX: THE ACUTE LIVER FAILURE STUDY GROUP 1998–2004

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