Changes in gene expression in liver tissue from patients with fulminant hepatitis E

Anshu Naik, Amit Goel, Vinita Agrawal, Aditya N Sarangi, Nanda Chhavi, Vineeta Singh, Shahid Jameel, Rakesh Aggarwal

Anshu Naik, Amit Goel, Aditya N Sarangi, Nanda Chhavi, Rakesh Aggarwal, Department of Gastroenterology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow 226014, India

Vinita Agrawal, Department of Pathology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow 226014, India

Vineeta Singh, Sandor Proteomics Pvt Ltd, Hyderabad 500034, India

Shahid Jameel, Virology Group, International Centre for Genetic Engineering and Biotechnology, New Delhi 110067, India

Author contributions: Naik A carried out the laboratory work and data analysis, and prepared the first draft of the manuscript; Goel A carried out initial screening and identification of patients and liver biopsies, and helped in drafting and critical revision of the manuscript; Agrawal V supervised the histological and immunohistochemical studies; Sarangi AN did the microarray data analysis and helped in drafting the related segments of the manuscript, and in critical revision of the manuscript; Chhavi N carried out initial screening and identification of patients and liver biopsies; Singh V did the laboratory work related to microarray analysis; Jameel S conceived the idea, participated in design of the study and interpretation of data, and helped revise the manuscript; Aggarwal R conceived the idea, participated in design of the study, clinical identification of patients, supervision of laboratory work, analysis of microarray data and overall supervision of the work, and helped in drafting and revision of the manuscript; all authors read and approved the final manuscript.

Supported by National Institutes of Health, Bethesda, United States, No. 5R01AI076192.

Institutional review board statement: The study was reviewed and approved by Sanjay Gandhi Postgraduate Institute of Medical Sciences institutional review board. Written, informed consent was obtained from and all patient or their families, as appropriate.

Institutional animal care and use committee statement: Exempt, since this study did not include the use of any animals.

Conflict-of-interest statement: None of the authors listed on the above manuscript had any conflict of interest, either financial or non-financial in relation to this paper.

Data sharing statement: Participants gave informed consent for data sharing. The corresponding author would be willing to share the data as required for the above manuscript with the editors.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: http://creativecommons.org/licenses/by-nc/4.0/

Correspondence to: Dr. Rakesh Aggarwal, Department of Gastroenterology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow 226014, India. aggarwal.ra@gmail.com
Telephone: +91-522-2494431
Fax: +91-522-2668017

Received: January 29, 2015
Peer-review started: January 30, 2015
First decision: March 10, 2015
Revised: March 27, 2015
Accepted: May 2, 2015
Article in press: May 4, 2015
Published online: July 14, 2015

Abstract

AIM: To study host gene expression and number...
of immune cells in liver tissues from patients with fulminant hepatitis E (FH-E).

METHODS: Microarray-based expression profiling was done using Illumina Human WG-6_v3_BeadChip arrays on post-mortem liver tissue from 5 patients with FH-E, and compared with similar tissue from 6 patients with fulminant hepatitis B (FH-B; disease controls) and normal liver tissue from 6 persons. Differential expression was defined as ≥ 2.0-fold change with Benjamini-Hochberg false discovery rate below 0.05 using t-test in liver tissue from FH-B and FH-E, than healthy liver tissue. For some genes that showed differential expression in FH-E, microarray data were validated using quantitative reverse transcription PCR. Differentially expressed gene lists were then subjected to “Gene Ontology” analysis for biological processes, and pathway analysis using BioCarta database on the DAVID server. In addition, tissue sections were stained for CD4+, CD8+ and CD56+ cells using indirect immunohistochemistry; cells staining positive for each of these markers were counted and compared between groups.

RESULTS: Compared to normal livers, those from patients with FH-E and FH-B showed differential expression of 3377 entities (up-regulated 1703, downregulated 1674) and 2572 entities (up 1164, down 1408), respectively. This included 2142 (up 896, down 1246) entities that were common between the two sets; most of these belonged to metabolic, hemostatic and complement pathways, which are active in normal livers. Gene expression data from livers of patients with FH-E but not those of FH-B showed activation of several immune response pathways, particularly those involving cytotoxic T cells. The fold-change values of mRNA for selected genes in livers from FH-E than in normal liver tissue determined using quantitative reverse transcription PCR showed excellent concordance with microarray analysis. At immunohistochemistry, CD8+ T cells showed an increase in liver biopsies from both FH-E patients and FH-B with CD4+ and CD56+ T cells showing positive staining for each of these markers were counted and compared between groups.

CONCLUSION: FH-E patients show CD8+ T cell infiltration and increased gene expression of cytotoxic T cell pathways in liver, suggesting a possible pathogenetic role for these cells.

Key words: Cytotoxic T cells; Gene expression; Hepatitis E; Hepatitis E virus; Immune response; Liver biopsy; Microarray; Natural killer cells; Pathogenesis

© The Author(s) 2015. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Data on pathogenesis of hepatitis E virus (HEV) infection, which is a common cause of acute hepatitis in several developing countries, are quite limited. This manuscript reports our data on microarray-based gene expression analysis and immunohistochemistry in liver tissue from patients with HEV infection, as compared to liver tissue from patients with hepatitis B virus infection and normal liver tissue. These data advance the current knowledge about the pathogenesis of HEV infection.
peripheral blood. However, little data are available on the liver tissue from hepatitis E. Therefore, the mechanism of liver injury in this disease remains unclear.

We therefore studied gene expression profile in liver biopsies from patients with FH due to acute hepatitis E (FH-E), in comparison with those from healthy adults and patients with FH due to acute hepatitis B (FH-B; a disease control group).

**MATERIALS AND METHODS**

**Patients and specimens**

Post-mortem needle liver biopsies were obtained from patients dying of FH-E (n = 5) or of FH-B (n = 6). The biopsies were obtained within 30 min after death, and separate pieces were collected in RINa later (Qiagen) and stored at -80 °C for gene expression analysis, and in formalin for histology and immunohistochemistry. In addition, a blood specimen was collected from each subject, for biochemical tests and serological markers of viral hepatitis.

Patients dying more than 2 wk after the onset of disease, and those with major sepsis or clinical evidence of pre-existing liver disease were excluded. Diagnosis of acute hepatitis E was based on detection of IgM anti-HEV (Genelabs, Singapore) in the absence of hepatitis B surface antigen (HBsAg), IgM anti-HBc antibody, anti-hepatitis C virus (HCV) antibody and IgM anti-hepatitis A virus (HAV) antibody (all from BioMerieux, Marcy l'Etoile, France), and that of acute hepatitis B on detection of HBsAg and IgM anti-HBc, in the absence of IgM anti-HEV, anti-HCV and IgM anti-HAV.

In addition, normal liver tissue was obtained from six persons undergoing partial hepatic resection for focal diseases, such as hydatid liver disease, gallbladder cancer without biliary obstruction.

The study was reviewed and approved by Sanjay Gandhi Postgraduate Institute of Medical Sciences institutional review board. Written, informed consent was obtained from all patients or their families, as appropriate.

**RNA isolation**

Total RNA was isolated from tissue biopsies using RNeasy Protect minikit (Qiagen, Carlsbad, CA). RNA concentration was measured using NanoDrop 1000 spectrophotometer (Nanodrop, Wilmington, DE). RNA integrity was assessed using Bioanalyzer 2100 (Agilent, Santa Clara, CA). Specimens with A260/A280 and A260/A230 ratios > 1.9, and RIN > 8.0 were processed further.

**Microarray analysis**

Gene expression profiling was done using Illumina Human WG-6_v3_BeadChip arrays (Illumina, San Diego, CA), each containing more than 46000 entities (gene probes or probesets) derived from NCBI, RefSeq and UniGene databases. Poly(A)-RNA was reverse transcribed to complementary DNA (cDNA), using an oligo(dT)-primer that contained a phage T7 RNA polymerase promoter sequence at its 5'-end, followed by conversion to double-stranded cDNA. The double-stranded cDNA was transcribed in vitro to yield large quantities of biotin-labelled anti-sense RNA, which was then hybridised to the bead arrays at 55 °C for 16-18 h, and scanned using an Illumina iScan reader.

**Bioinformatic analysis**

The array intensity data were initially analysed using Illumina Genome Studio Gene Expression Module (v1.1.1.1) (Illumina, Cambridge, United Kingdom) for visualisation and normalisation. After quantile normalisation and background correction using medians within the BeadStudio software, the data were exported for further analysis in GeneSpring GX software 11.5 (Agilent).

Differential expression between various subject groups was analyzed using Illumina Custom Algorithm. The normalized data were first subjected to quality check using principal component analysis. This was followed by analysis to determine genes showing differential expression (fold-change ≥ 2.0 and Benjamini-Hochberg false discovery rate < 0.05 using t-test) in liver tissue from patients with FH-B and FH-E, as compared to healthy liver tissue. Such gene lists were then subjected to "Gene Ontology" (GO) analysis for biological processes, and pathway analysis using BioCarta database on the DAVID server (http://david.abcc.ncifcrf.gov). Separate analyses were done for genes that were differentially expressed in both FH-E and FH-B, and those differentially expressed in only one of these.

**Validation of microarray results**

For some genes that showed differential expression in FH-E, microarray data were validated using quantitative reverse transcription PCR (qRT-PCR). In brief, cDNA was prepared using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) from 2 µg of RNA. This was followed by real-time PCR in 20-µL reactions comprising of 50 ng cDNA, primers (Appendix 1) and SYBR Green (Applied Biosystems).

The validation assay included liver tissue for all the subjects used for microarray analysis (a biopsy piece other than that used for microarray analysis) and four additional healthy liver tissues. All assays included RNA from peripheral blood mononuclear cells of a healthy person as a calibrator. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and 18S rRNA was used as housekeeping genes. Relative fold-change was determined for each specimen and gene from cycle threshold (Ct) values as follows, and compared to fold-change in expression in microarray data:
Table 1 Characteristics of study subjects in the three groups

| Characteristic          | Healthy controls (n = 6) | Fulminant hepatitis B (n = 6) | Fulminant hepatitis E (n = 5) |
|-------------------------|--------------------------|-------------------------------|------------------------------|
| Age (yr)                | 52 (30-58)               | 24 (16-60)                    | 28 (18-32)                   |
| Gender (Male-Female)    | 4:2                      | 4:2                           | 1:4                          |
| Duration of illness     | -                        | 9 (6-14)                      | 10 (7-13)                    |
| Maximum total serum bilirubin (mg/dL) | 0.7 (0.4-1.4) | 21 (13.1-41.4) | 25 (5.4-32.0) |
| Serum ALT* (IU/L)       | 30 (21-58)               | 1939 (524-5795)               | 770 (240-1302)               |
| Serum AST (IU/L)        | 28 (18-35)               | 859 (250-2345)                | 458 (332-1000)               |
| Alkaline phosphatase    | -                        | 257 (131-427)                 | 306 (164-561)                |
| (IU/L)                  | -                        | 2.8 (2.0-3.3)                 | 2.8 (2.1-3.2)                |

Data are shown as median (range), except for gender distribution; patients with fulminant hepatitis B and fulminant hepatitis E were comparable in all variables except serum ALT levels (P < 0.05, Mann-Whitney U test). ALT: Alanine aminotransferase.

\[
\Delta \Delta C_t = [(\Delta C_t_{\text{gene of interest}} - \Delta C_t_{\text{GAPDH, calibrator sample}})_{\text{patient}} - (\Delta C_t_{\text{gene of interest}} - \Delta C_t_{\text{GAPDH, calibrator sample}})_{\text{control}}] \times 2^{\Delta \Delta C_t/2}
\]

Immunohistochemistry on liver biopsies

Formalin-fixed, paraffin-embedded liver sections were stained with hematoxylin and eosin, and examined to confirm the presence of acute inflammation in patients with FH, and absence of disease in controls.

In addition, immunohistochemistry (IHC) was performed for specific T-cell subsets (CD4+ and CD8+) and NK cells (CD56). In brief, formalin-fixed, paraffin-embedded 3-µm liver sections on silanized glass slides (Dako) were fixed at 60 °C overnight. The slides were deparaffinised in xylene and rehydrated in graded alcohol. Antigen retrieval was done in 10 mmol/L EDTA (pH 9.0) at 98 °C for 30 min. After washing with distilled water and Tris-buffered saline (TBS; pH 7.4), endogenous peroxidase was blocked using 3% hydrogen peroxide in methanol for 30 min in dark. Sections were then incubated with monoclonal primary antibodies [prediluted mouse anti-human CD8 (clone C8/144B), CD4 (clone 4B12) or CD56 (clone 123C3) (Dako, Denmark)] for 1 h at room temperature, followed by peroxidase enzyme-labelled secondary antibody (Dako) for 30 min. Diaminobenzidine was used as chromogen to detect the bound antibodies. Slides were counterstained with Mayer’s hematoxylin for 1 min, cleared and mounted with DPX (Sigma-Aldrich). Stained cells were counted in five fields, averaged and expressed as number in an area measuring 73000 µm².

Statistical analysis

Inter-group comparisons were done using t-test with Benjamini-Hochberg correction in case of gene expression data and Mann-Whitney U test for other quantitative data. Relationship of fold-change in the microarray and real-time PCR data was assessed using Pearson’s correlation coefficient. The statistical methods of this study were reviewed by Dr. Rakesh Aggarwal from Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, India.

RESULTS

Demographic features and biochemical findings in the three subject groups are shown in Table 1. Alanine aminotransferase levels were higher in FH-B than in FH-E.

Principal component analysis

On principal component analysis of gene expression data (Figure 1), all specimens except one (with FH-B) showed clustering with other specimens in the same group.

Microarray gene expression

A total of 3377 entities, each representing a discrete gene, showed differential expression of ≥ 2-fold with P-value of < 0.05 in liver tissue from patients with FH-E than that from healthy persons (Figure 2); this included 1703 entities with over-expression and 1674 showing reduced expression in FH-E. In liver tissue from FH-B, 2572 entities showed differential expression than in healthy livers (up-regulation 1164, down-regulation 1408).

Of the entities differentially expressed in FH-E or FH-B compared to normal liver, 2142 were common (up-regulation in both 896, down-regulation in both 1246) (Figure 2, Appendix 2); none of these entities showed discordance in the direction of differential expression between FH-E and FH-B. In contrast, 1235 entities showed discordance in the direction of differential expression between FH-E and FH-B.
entities (Appendix 3) were differentially expressed in FH-E but not in FH-B, and 430 entities (Appendix 4) were differentially expressed in FH-B but not in FH-E.

**Gene ontology analysis**

**Genes differentially expressed in FH-E and FH-B livers:** Table 2 lists the biological processes that showed differential regulation on GO analysis of the 2142 entities differentially expressed in both FH-E and FH-B. These included several metabolic processes in which the liver plays an active part, such as general body metabolism, or those whose proteins are mainly synthesized in the liver, such as coagulation pathways and complement system. Further, both the disease conditions showed differential regulation of innate immune responses and humoral immune responses mediated by B cells.

**Genes differentially expressed in FH-E but not in FH-B:** On GO analysis, the 1235 entities that showed differential expression in FH-E but not in FH-B were related mainly to the immune system pathways (Table 3); for each of these immune pathways, several genes showed upregulation.

**Genes differentially expressed in FH-B but not in FH-E:** GO analysis of these entities did not reveal significant changes in any specific pathway.

**Pathway analysis**

**Genes differentially expressed in both FH-E and FH-B:** Pathway analysis for the 2142 entities differentially expressed in both FH-E and FH-B using the BioCarta database showed downregulation of several pathways related to metabolic processes, hemostasis, and complement system (Table 4). Appendix 5 shows individual genes involved in each of these pathways.

**Genes differentially expressed in FH-E but not in FH-B:** BioCarta pathways whose genes were significantly over-represented among genes differentially expressed in liver tissue from FH-E compared to normal liver but not in FH-B were mostly related to cellular immune mechanisms (Table 4), e.g., T cytotoxic cell surface molecules, CTL mediated immune response against target cells, T helper cell surface molecules, co-stimulatory signal during T-cell activation, T cell receptor signalling pathway, Lck and Fyn tyrosine kinases in initiation of TCR activation, IL-7 signal transduction, T cell receptor and CD3 complex, IL 17 signalling pathway. Several individual genes involved in these pathways were upregulated in patients with FH-E (Appendix 6).

**Genes differentially expressed in FH-B but not in FH-E:** Pathway analysis of these 430 entities did not reveal significant change in any pathway.

**Validation using qRT-PCR**

The fold-change values of mRNA for selected genes (Appendix 1) in livers from FH-E than in normal liver tissue determined using qRT-PCR showed excellent concordance with those at microarray (Figure 3), using either of the two housekeeping genes.

**Immunohistochemistry**

IHC showed a few CD4+ positive T-cells and occasional NK cells in all the biopsies. In FH-B as well as FH-E, CD8+ T-cells were found in both the liver parenchyma and the portal areas (Figure 4). The median number of CD8+ T-cells was greater in liver tissues from both FH-E [median 53.4 (range 31.2-99.9)]; \( P = 0.005, \text{2-sided Mann-Whitney U test} \) and FH-B [49.3 (19.3-51.0); \( P = 0.005 \)] than in controls 6.9 (3.1-14.9); however, there was no significant difference between FH-E and FH-B.

**DISCUSSION**

Host tissue injury during a viral infection may be caused either by virus-induced cell death, or by immune-mediated killing of infected cells. Understanding the underlying mechanism may not only help understand the pathogenesis of a particular viral disease but also provide a lead to development of strategies for countering the host injury. Infection with hepatitis A, B or C virus, liver injury is mediated primarily by the host immune response[7-11]. However, the mechanisms of liver injury in HEV infection remain unclear.

Initial attempts at delineation of mechanism of liver injury caused by HEV were based on observations during experimental HEV infection in non-human primates. In these studies, liver injury appeared after viremia and fecal viral excretion had started declining.
Table 2  Gene Ontology analysis (biological processes) for gene entities differentially expressed in liver tissue from fulminant hepatitis E as compared to normal liver tissue, as well as in fulminant hepatitis B as compared to normal liver tissue

| Gene ontology accession | Gene Ontology term | P value |
|-------------------------|--------------------|---------|
| GO:00055114             | Oxidation-reduction process | 2.68E-40 |
| GO:0006082              | Organic acid metabolic process | 4.75E-39 |
| GO:0043436              | Oxocarboxylic acid metabolic process | 2.17E-38 |
| GO:0019752              | Carboxylic acid metabolic process | 4.09E-38 |
| GO:0006629              | Lipid metabolic process | 3.94E-28 |
| GO:0044281              | Small molecule metabolic process | 9.06E-27 |
| GO:0032787              | Monocarboxylic acid metabolic process | 3.56E-22 |
| GO:0016054              | Organic acid catabolic process | 4.22E-19 |
| GO:0046395              | Carboxylic acid catabolic process | 4.22E-19 |
| GO:0044255              | Cellular lipid metabolic process | 1.35E-17 |
| GO:0044282              | Small molecule catabolic process | 5.16E-17 |
| GO:0044712              | Single-organism catabolic process | 5.16E-17 |
| GO:0006631              | Fatty acid metabolic process | 2.71E-16 |
| GO:0006520              | Cellular amino acid metabolic process | 4.43E-16 |
| GO:0044771              | Single-organism biosynthetic process | 8.29E-14 |
| GO:1901605              | Alpha-amino acid metabolic process | 1.07E-13 |
| GO:0044283              | Small molecule biosynthetic process | 1.60E-13 |
| GO:1901564              | Organonitrogen compound metabolic process | 2.46E-11 |
| GO:0009063              | Cellular amino acid catabolic process | 4.46E-11 |
| GO:0008610              | Lipid biosynthetic process | 8.46E-11 |
| GO:0072376              | Protein activation cascade | 3.87E-10 |
| GO:0088020              | Steroid metabolic process | 4.10E-10 |
| GO:0016053              | Organic acid biosynthetic process | 6.26E-10 |
| GO:0046394              | Carboxylic acid biosynthetic process | 6.26E-10 |
| GO:1901566              | Organonitrogen compound biosynthetic process | 1.94E-08 |
| GO:0008152              | Metabolic process | 4.34E-08 |
| GO:1901606              | Alpha-amino acid catabolic process | 4.58E-08 |
| GO:0072329              | Monocarboxylic acid catabolic process | 1.40E-07 |
| GO:0007596              | Blood coagulatation | 1.98E-07 |
| GO:2059017              | Coagulation | 1.98E-07 |
| GO:0075999              | Hemostasis | 2.83E-07 |
| GO:1901615              | Organic hydroxy compound metabolic process | 4.14E-07 |
| GO:0042860              | Wound healing | 4.86E-07 |
| GO:006956               | Complement activation | 6.77E-07 |
| GO:0009862              | Fatty acid catabolic process | 1.71E-06 |
| GO:006066               | Alcohol metabolic process | 1.83E-06 |
| GO:008652               | Cellular amino acid biosynthetic process | 2.42E-06 |
| GO:0050878              | Regulation of body fluid levels | 5.46E-06 |
| GO:0051186              | Cofactor metabolic process | 5.50E-06 |
| GO:0006694              | Steroid biosynthetic process | 8.32E-06 |
| GO:0044242              | Cellular lipid metabolic process | 1.09E-05 |
| GO:005996               | Monosaccharide metabolic process | 0.000012 |
| GO:0051346              | Negative regulation of hydrolase activity | 1.28E-05 |
| GO:0050975              | Carbohydrate metabolic process | 1.35E-05 |
| GO:0010876              | Lipid localization | 5.30E-05 |
| GO:0044710              | Single-organism metabolism | 5.82E-05 |
| GO:006732               | Coenzyme metabolic process | 6.57E-05 |
| GO:0010951              | Negative regulation of endopeptidase activity | 7.22E-05 |
| GO:0019318              | Hesoxo metabolic process | 7.95E-05 |
| GO:006669               | Lipid transport | 8.02E-05 |
| GO:0065008              | Regulation of biological quality | 8.96E-05 |
| GO:0010466              | Negative regulation of peptidase activity | 0.0001 |
| GO:006006               | Glucose metabolic process | 0.0001 |

Naik A et al.  Liver changes in fulminant hepatitis E

GO:0042558  Pteridine-containing compound metabolic process  0.0002
GO:0043648  Dicarboxylic acid metabolic process  0.0002
GO:0006958  Complement activation, classical pathway  0.0004
GO:0006991  Generation of precursor metabolites and energy  0.0004
GO:0044262  Cellular carbohydrate metabolic process  0.0004
GO:0006575  Cellular modified amino acid metabolic process  0.0005
GO:0016551  Carbohydrate biosynthetic process  0.0005
GO:0072330  Monocarboxylic acid metabolic process  0.0006
GO:0024555  Humoral immune response mediated by circulating immunoglobulin  0.0010
GO:0044723  Single-organism carbohydrate metabolic process  0.0010
GO:0019320  Hesoxo carbohydrate metabolic process  0.0011
GO:0016052  Carbohydrate catabolic process  0.0014
GO:0044724  Single-organism carbohydrate catabolic process  0.0014
GO:0006820  Anion transport  0.0016
GO:0006007  Glucose catabolic process  0.0017
GO:0046365  Monosaccharide catabolic process  0.0018
GO:0046835  Carbohydrate phosphorylation  0.0020
GO:1901617  Organic hydroxy compound biosynthetic process  0.0020
GO:0002253  Activation of immune response  0.0021
GO:0016125  Sterol metabolic process  0.0022
GO:0006096  Glycolysis  0.0023
GO:0019395  Fatty acid oxidation  0.0037
GO:0034440  Lipid oxidation  0.0037
GO:0046165  Alcohol biosynthetic process  0.0038
GO:0006959  Humoral immune response  0.0042
GO:0009611  Response to wounding  0.0044
GO:0006957  Complement activation, alternative pathway  0.0044
GO:0006760  Folic acid-containing compound metabolic process  0.0044
GO:0045887  Innate immune response  0.0053
GO:0052548  Regulation of endopeptidase activity  0.0077
GO:0046364  Monosaccharide biosynthetic process  0.0077
GO:0006790  Sulfur compound metabolic process  0.0078
GO:0051179  Localization  0.0079
GO:0052547  Regulation of peptidase activity  0.0083
GO:0046942  Carboxylic acid transport  0.0083
GO:0031849  Organic acid transport  0.0095
GO:0016042  Lipid catabolic process  0.0113
GO:0051234  Establishment of localization  0.0113
GO:0006810  Transport  0.0119
GO:0044092  Negative regulation of molecular function  0.0126
GO:0019319  Hesoxo biosynthetic process  0.0131
GO:0072378  Blood coagulation, fibrin clot formation  0.0132
GO:0006558  L-phenylalanine metabolic process  0.0146
GO:0006559  L-phenylalanine catabolic process  0.0146
GO:0000338  Very long-chain fatty acid metabolic process  0.0146
GO:0051289  Protein homotetramerization  0.0146
GO:0002252  Immune effector process  0.0153
GO:0006203  Cholesterol metabolic process  0.0153
GO:009074  Aromatic amino acid family catabolic process  0.0212
GO:0048806  Genitalia development  0.0212
GO:0016064  Immunoglobulin mediated immune response  0.0214
GO:0050819  Negative regulation of coagulation  0.0214
and coincided with the appearance of anti-HEV antibodies\textsuperscript{[17-19]}. These findings suggested that host immune response was responsible for the hepatocyte killing. This was followed by attempts to determine whether HEV was capable of producing a cytopathic effect in in vitro models. However, these efforts were largely unsuccessful due to failure of this virus to grow well in cell culture. Recent successful culture\textsuperscript{[20]}, though in low titers, of some genotypes 3 and 4 HEV isolates has revealed absence of cytotoxicity\textsuperscript{[21,22]}, providing some support to the hypothesis that liver injury in HEV infection is unlikely to be virus-mediated.

Human data on cellular immune responses during HEV infection are limited to studies on peripheral blood mononuclear cells (PBMCs). In these studies, we and others have shown proliferation of HEV-specific CD4\textsuperscript{\textsuperscript{+}} and CD8\textsuperscript{\textsuperscript{+}} cells, and increased production of various cytokines by PBMCs during acute and convalescent phases of hepatitis E\textsuperscript{[14,16]}. In a study that looked at patients with different disease severities, HEV-specific T-cell responses in PBMCs were weaker in patients with FH-E than in those with uncomplicated acute hepatitis E\textsuperscript{[23]}. However, observations made on circulating immune cells may not faithfully represent those in the immune cells infiltrating the liver, because of preferential localization of certain cells, including virus-specific T cell subsets, in the inflamed tissue\textsuperscript{[22,24]}.

Thus, there is a need to study changes in the liver tissue from patients with hepatitis E.

In the current study, we found changes in expression of genes involved in a variety of immune responses, including the innate, humoral and cellular immune pathways in liver tissue from patients with FH-E. Of these, changes in innate and humoral immune responses were also observed in FH-B, indicating that HEV infection may share some of the pathways causing liver cell injury with HBV infection. In contrast, activation of cellular immune pathways was found only in FH-E, and not in FH-B, indicating a specific role for cellular immune response in inducing liver injury during HEV infection.

In particular, we found several T-cell surface molecules such as CD2, CD3 (γ, δ and ε chains) and CD8-alpha to be up-regulated in liver tissue from patients with FH-E. Furthermore, several T-cell signalling molecules, such as Fyn, phospholipase C gamma-1, NF-κB1, protein kinase C and ras-related C3 botulinum

| Gene Ontology term | P value |
|---------------------|---------|
| Immune system process\textsuperscript{1} | 1.67E-11 |
| Immune response\textsuperscript{1} | 4.81E-09 |
| Regulation of immune system process\textsuperscript{1} | 5.06E-08 |
| Positive regulation of immune system process\textsuperscript{1} | 2.72E-06 |
| Defense response | 1.04E-05 |
| Response to wounding | 1.90E-05 |
| Signaling | 7.78E-05 |
| Adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains\textsuperscript{1} | 2.40E-04 |
| Adaptive immune response\textsuperscript{1} | 2.40E-04 |
| Response to stimulus | 3.53E-04 |
| Regulation of cell activation | 4.65E-04 |
| Inflammatory response\textsuperscript{1} | 6.89E-04 |
| Cell adhesion | 0.0010 |
| Biological adhesion | 0.0010 |
| Positive regulation of immune response\textsuperscript{1} | 0.0015 |
| Cell activation | 0.0018 |
| Signal transduction | 0.0019 |
| Regulation of leukocyte activation\textsuperscript{1} | 0.0020 |
| Activation of immune response\textsuperscript{1} | 0.0021 |
| Oxidation reduction | 0.0021 |
| B cell mediated immunity\textsuperscript{1} | 0.0023 |
| Leukocyte mediated immunity\textsuperscript{1} | 0.0026 |
| Positive regulation of response to stimulus | 0.0027 |
| Regulation of locomotion | 0.0028 |
| Regulation of immune response\textsuperscript{1} | 0.0050 |
| Phospholipid efflux | 0.0067 |
| Lymphocyte mediated immunity\textsuperscript{1} | 0.0053 |
| Regulation of cell migration\textsuperscript{1} | 0.0048 |
| Regulation of cell motility\textsuperscript{1} | 0.0048 |
| Response to stress | 0.0050 |
| Regulation of lymphocyte activation\textsuperscript{1} | 0.0056 |
| Regulation of cellular component movement | 0.0064 |
| Signal transmission | 0.0067 |
| Immunoglobulin mediated immune response\textsuperscript{1} | 0.0067 |
| Signalling process | 0.0067 |
| Small molecule metabolic process | 0.0083 |
| Cholesterol efflux | 0.0124 |
| Immune effector process\textsuperscript{1} | 0.0154 |
| Negative regulation of apoptosis | 0.0161 |
| Positive regulation of cell activation | 0.0176 |
| Positive regulation of leukocyte activation\textsuperscript{1} | 0.0176 |
| Regulation of response to stimulus | 0.0177 |
| Negative regulation of cell death | 0.0179 |
| Negative regulation of programmed cell death | 0.0179 |
| Regulation of lipid metabolic process | 0.0180 |
| Signalling pathway | 0.0206 |
| Acute inflammatory response\textsuperscript{1} | 0.0241 |
| Phospholipid transport | 0.0257 |
| Regulation of fatty acid metabolic process | 0.0273 |
| Alcohol metabolic process | 0.0282 |
| Cholesterol transport | 0.0310 |
| Sterol transport | 0.0472 |

\textsuperscript{1}These pathways are related to immune responses.
Some other findings in our study may also point to the role of activated CD8+ T cells in the causation of liver injury in HEV infection. An over-expression of CTLA4, which is expressed only on activated T cells, also supports this hypothesis. An alternative explanation for the overexpression of CD2 in liver tissue could be the presence of this marker on the NK cells. Importantly, the increased expression of CD2 in the liver tissue was also associated with over-expression of perforin, the main cytolytic protein contained in the CD8+ T lymphocytes. The above findings, taken together, indicate that immune-mediated cytotoxicity of CD8+ T cells may play a role in producing liver damage in HEV infection.

Some other findings in our study may also point to alterations in CD8+ cells. In viral infections, the number of effector CD8 T cells contracts over time with the formation of a population of protective memory cells, which is maintained by IL-7 and IL-15. Our finding of activation of IL-7-mediated signalling in patients with hepatitis E possibly reflects this phenomenon. In addition, the genes for signal transducer and activator of transcription 5 (STAT5), a key molecule involved in the survival of effector and memory CD8 cells, and for BCL2, a key survival molecule that is upregulated by STAT5, were overexpressed in FH-E.

The differential expression in livers from patients with FH-E of several genes which were unaffected in FH-B could have been because of changes in any of the several cell types present in the liver, including hepatocytes, Kupffer cells, cholangiocytes, endothelial cells, stellate cells and a variety of immune cells. However, the demonstration of predominant infiltration with CD8+ T cells in the FH-E livers in the current study as well as in two previous reports[25], and the fact that the genes that showed differential expression are not expressed much in other cell types, indicate the CD8+ T cells were the most likely source. It may be pertinent to note that since the immune cells constitute only a minority of all the cells in the liver, the absolute gene expression changes within the intrahepatic immune cells must be more marked than is indicated by the overall gene expression data. It may thus be interesting in future to undertake studies on such immune cells after recovering these using techniques such as laser dissection microscopy.

It may be pertinent to compare our results with those from a recent study of gene expression in serial
liver tissues from chimpanzees with experimental HEV infection. In this study, differential expression was limited to a few genes that belonged predominantly to the innate immune response pathways, and was weaker than that observed in chimpanzees with HCV infection. Further, the number of upregulated genes peaked sooner after the onset of viremia in HEV infection than with HCV infection. We did find altered expression of some genes involved in innate immune responses; however, these genes were different from those showing significant changes during HEV infection in the chimpanzee study. In this context, it is important to note that comparison of our data with those from the experimentally-infected chimpanzees may not be valid. Our patients had severe liver disease, whereas experimental animals had been inoculated by the intravenous route, whereas humans acquire infection through the oral route.

Though we studied patients with FH-B primarily as disease controls, it may be interesting to compare liver in this condition with healthy livers. Livers from patients with FH-B showed infiltration with CD8^+ cells, but no differential expression of genes belonging to cellular immune response pathways. Previous gene expression data in this disease are available from only two Italian patients. These patients too showed prominent CD8^+ T cell infiltration with little change in expression of genes associated with T cell activity and a prominent upregulation of B cell response, similar to

Figure 4 Immunohistochemistry for CD8^+ cells in liver biopsies shows occasional cytotoxic T-cells in portal tracts of controls (A), and moderate infiltration in HBV (C) and HEV (E); immunostaining for CD4 shows absence of helper T-cells in portal areas of control (B), an occasional cell in HBV (D) and some small aggregates of helper T-cells in HEV (F). A and B: Diaminobenzidine × 400; C-F: Diaminobenzidine × 200. HBV: Hepatitis B virus; HEV: Hepatitis E virus.
our observations.

Interestingly, though liver tissues from both FH-E and FH-B in our study showed prominent CD8+ T cell infiltration, only FH-E was associated with a cytotoxic T cell transcriptional signature e.g., increased expression of perforin. This suggests that the infiltrating CD8+ T cells in the two diseases behave quite differently. This may hold the key to pathogenesis of liver injury in HEV infection.

We also found reduced expression of several genes associated with metabolic, hemostatic and complement pathways in liver tissue from FH-E as well as FH-B. Liver is a metabolically active organ which produces several body proteins. Since FH, irrespective of its cause, is characterized by marked destruction of hepatocytes, reduction in the expression of these proteins was thus expected in both FH-E and FH-B, as a consequence of the massive liver injury.

Our data are limited by a small sample size, the use of post-mortem tissue, and of patients with severe liver injury at only one time-point. These limitations are related to the fact that liver biopsy, being invasive, is ethically unacceptable in patients with acute liver disease, forcing us to use tissue collected immediately post-mortem. Though our findings may not be entirely applicable to acute uncomplicated hepatitis E, these should be seen in light of the current absence of any human data on immune events in the liver in acute hepatitis E. Further, we compared our data to those from a control group of post-mortem biopsies from FH-B, which would have been susceptible to similar artefacts as those in FH-E.

In conclusion, liver tissue from our patients with FH-E showed infiltration with CD8+ T cells and overexpression of genes involved in T cell immune responses, especially those related to T cell activation, cytotoxicity and IL-7 signalling. The latter changes were not observed in FH-B, and hence were specific to hepatitis E. These data suggest that the severe liver damage in FH-E is mediated by the host T-cell immune response. Further work on this aspect should help us better understand the pathogenesis of liver injury in hepatitis E.

ACKNOWLEDGMENTS

Microarray data analysis was done at Bio-medical Informatics Centre, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, India; this center is supported by the Indian Council of Medical Research (ICMR) and Department of Biotechnology, Government of India during this work. ANS was supported by ICMR during this work. The authors thank Dr. Vivek Chandra for analysis comparing the liver biopsy data with in vitro data from HEV ORF3-expressing cells.

REFERENCES

1. Aggarwal R, Jameel S. Hepatitis E. Hepatology 2011; 54: 2218-2226 [PMID: 21932388 DOI: 10.1002/hep.24674]
2. Aggarwal R, Naik S. Epidemiology of hepatitis E: current status. J Gastroenterol Hepatol 2009; 24: 1484-1493 [PMID: 19686410 DOI: 10.1111/j.1440-1746.2009.05933.x]
3. Aggarwal R, Shukla R, Jameel S, Agrawal S, Puri P, Gupta VK, Patil AP, Naik S. T-cell epitope mapping of ORF2 and ORF3 proteins of human hepatitis E virus. J Viral Hepat 2007; 14: 283-292 [PMID: 17381721 DOI: 10.1111/j.1365-2893.2006.00796.x]
4. Agrawal V, Goel A, Rawat A, Naik S, Aggarwal R. Histological and immunohistochemical features in fatal acute fulminant hepatitis E. Indian J Pathol Microbiol 2012; 55: 22-27 [PMID: 22499295 DOI: 10.4103/0377-4929.94849]
5. Ahmad I, Holla RP, Jameel S. Molecular virology of hepatitis E virus. Virus Res 2011; 161: 47-58 [PMID: 21345356 DOI: 10.1016/j.virusres.2011.02.011]
6. Exley MA, Koziel MJ. To be or not to be NKT: natural killer T cells in the liver. Hepatology 2004; 40: 1033-1040 [PMID: 15486982 DOI: 10.1002/hep.20433]
7. Farci P, Diaz G, Chen Z, Govindarajan S, Tice A, Aguilo L, Pittaluga S, Boon D, Yu C, Engle RE, Haas M, Simon R, Purcell RH, Zamboni F. B cell gene signature with massive intrahepatic production of antibodies to hepatitis B core antigen in hepatitis B virus-associated acute liver failure. Proc Natl Acad Sci USA 2010; 107: 8766-8771 [PMID: 20421498 DOI: 10.1073/pnas.1003854107]
8. Guidotti LG, Chisari FV. Noncytolytic control of viral infections by the innate and adaptive immune response. Annu Rev Immunol 2001; 19: 65-91 [PMID: 11244031 DOI: 10.1146/annurev.immunol.19.1.65]
9. Guidotti LG, Rochford R, Chung J, Shapiro M, Purcell R, Chisari FV. Viral clearance without destruction of infected cells during acute HBV infection. Science 1999; 284: 825-829 [PMID: 8903145]
Liver changes in fulminant hepatitis E

10221919 DOI: 10.1126/science.284.5415.825

10. Hand TW, Cui W, JungYW, Setk F, Joshi NS, Chandelev A, Liu Y, Kaech SM. Differential effects of STATS and PI3K/AKT signaling on effector and memory CD8 T-cell survival. Proc Natl Acad Sci USA 2010; 107: 16601-16606 [PMID: 20823247 DOI: 10.1073/pnas.1003457107]

11. Husain MM, Aggarwal R, Kumar D, Jameel S, Naik S. Effector T cells immune reactivity among patients with acute hepatitis E. J Viral Hepat 2011; 18: e603-e608 [PMID: 21914082 DOI: 10.1111/j.1365-2893.2011.01489.x]

12. Kamar N, Bendall R, LeGrand-Abravanel F, Xia NS, Ijaz S, Izopet J, Dalton HR. Hepatitis E. Lancet 2012; 379: 2477-2488 [PMID: 22549046 DOI: 10.1016/S0140-6736(11)61849-7]

13. Kamar N, Seljes J, Munsuy JM, Ouezzani L, Pérön JM, Guitard J, Cointault O, Espisitte L, Abravanel F, Danjoux M, Durand D, Vinel JP, Izopet J, Rostaing L. Hepatitis E virus and chronic hepatitis in organ-transplant recipients. N Engl J Med 2008; 358: 811-817 [PMID: 18287603 DOI: 10.1056/NEJMoa0706992]

14. Kleneman P, Thirone T. Cell responses in hepatitis C: the good, the bad and the unconventional. Gut 2012; 61: 1226-1234 [PMID: 21873736 DOI: 10.1136/gut-2011-30620]

15. Krawczynski K, Bradley DW. Enterically transmitted non-A, non-B hepatitis: identification of virus-associated antigen in experimentally infected cynomolgus macaques. J Infect Dis 1989; 159: 1042-1049 [PMID: 2470832 DOI: 10.1093/infdis/159.6.1042]

16. Krawczynski K, Meng XJ, Rybczynska J. Pathogenic elements of hepatitis E and animal models of HEV infection. Virus Res 2011; 161: 78-83 [PMID: 2144365 DOI: 10.1016/j.virusres.2011.03.007]

17. Lu L, Li C, Hagedorn CH. Phylogenetic analysis of global hepatitis E virus sequences: genetic diversity, subtypes and zoonosis. Rev Med Virol 2006; 16: 5-36 [PMID: 16175650 DOI: 10.1002/rmv.482]

18. McCaustland KA, Krawczynski K, Ebert JW, Balalay MS, Andjaparidze AG, Spelbring JE, Cook EH, Humphrey C, Yarbough PO, Favorov MO, Carson D, Bradley DW, Robertson BH. Hepatitis E virus infection in chimpanzees: a retrospective analysis. Arch Virol 2000; 145: 1909-1918 [PMID: 11043950]

19. Nissim O, Melis M, Diaz G, Kleiner DE, Tice A, Fantola G, Zamboni F, Mishra L, Farcy P. Liver regeneration signature in hepatitis B virus (HBV)-associated acute liver failure identified by gene expression profiling. PLoS One 2012; 7: e49611 [PMID: 23185381 DOI: 10.1371/journal.pone.0049611]

20. Okamoto H. Hepatitis E virus cell culture models. Virus Res 2011; 161: 65-77 [PMID: 21316402 DOI: 10.1016/j.virusres.2011.01.015]

21. Pal R, Aggarwal R, Naik SK, Das V, Das S, Naik S. Immunological alterations in pregnant women with acute hepatitis E. J Gastroenterol Hepatol 2005; 20: 1094-1101 [PMID: 15955220 DOI: 10.1111/j.1440-1746.2005.03875.x]

22. Prabhu SB, Gupta P, Durgapal H, Rath S, Gupta SD, Acharya SK, Panda SK. Study of cellular immune response against Hepatitis E virus (HEV). J Viral Hepat 2011; 18: 587-594 [PMID: 20579277 DOI: 10.1111/j.1365-2893.2010.01338.x]

23. Racanelli V, Rehermann B. The liver as an immunological organ. Hepatology 2006; 43: S54-S62 [PMID: 16447271 DOI: 10.1002/hep.21060]

24. Rehermann B. Hepatitis C virus versus innate and adaptive immune responses: a tale of coevolution and coexistence. J Clin Invest 2009; 119: 1745-1754 [PMID: 19587449 DOI: 10.1172/JCI39133]

25. Srivastava R, Aggarwal R, Jameel S, Puri P, Gupta VK, Ramesh VS, Bhatia S, Naik S. Cellular immune responses in acute hepatitis E virus infection to the viral open reading frame 2 protein. Viral Immunol 2007; 20: 56-65 [PMID: 17424521 DOI: 10.1089/vim.2006.0053]

26. Srivastava R, Aggarwal R, Sachdeva S, Alam M, Jameel S, Naik S. Adaptive immune responses during acute uncomplicated and fulminant hepatitis E. J Gastroenterol Hepatol 2011; 26: 306-311 [PMID: 21143520]

27. Takahashi M, Hoshino Y, Tanaka T, Takahashi H, Nishizawa T, Okamoto H. Production of monoclonal antibodies against hepatitis E virus capsid protein and evaluation of their neutralizing activity in a cell culture system. Arch Virol 2008; 153: 657-666 [PMID: 18266052]

28. Tiechhurst J, Rhodes LL, Krawczynski K, Asher LV, Engler WF, Mensing TL, Caudill JD, Sjogren MH, Hoke CH, LeDuc JW. Infection of owl monkeys (Aotus trivirgatus) and cynomolgus monkeys (Macaca fascicularis) with hepatitis E virus from Mexico. J Infect Dis 1992; 165: 835-845 [PMID: 1569334]

29. Yu C, Boon D, McDonald SL, Myers TG, Tomioka K, Nguyen H, Engle RE, Govindarajan S, Emerson SU, Purcell RH. Pathogenesis of hepatitis E virus and hepatitis C virus in chimpanzees: similarities and differences. J Virol 2010; 84: 11264-11278 [PMID: 20739520 DOI: 10.1128/JVI.01205-10]

30. Zhang HY, Chen DS, Wu YQ, He QG, Chen HC, Liu ZF. Both swine and human cells are capable to support the replication of swine hepatitis E virus type 4 in vitro. Virus Res 2011; 158: 289-293 [PMID: 21470567 DOI: 10.1016/j.virusres.2011.03.028]

31. Zhou Y, Callendret B, Xu D, Brasky KM, Feng Z, Hensley LL, Guedj J, Perelson AS, Lemon SM, Lanford RE, Walker CM. Dominance of the CD4(+) T helper cell response during acute resolving hepatitis A virus infection. J Exp Med 2012; 209: 1481-1492 [PMID: 22753925 DOI: 10.1084/jem.20111996]
