Research Article

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Visualization of hepatitis E virus RNA and proteins in the human liver

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Background & Aims: Although hepatitis E constitutes a substantial disease burden worldwide, surprisingly little is known about the localization of hepatitis E virus (HEV) in the human liver. We therefore aimed to visualize HEV RNA and proteins *in situ*.

Methods: A panel of 12 different antibodies against HEV open reading frame (ORF) 1–3 proteins was evaluated for immunohistochemistry (IHC) and two probes for *in situ* hybridization (ISH) in formalin-fixed, paraffin-embedded (FFPE) HuH7 cells transfected with HEV ORF1-3 expression vectors. IHC (and partly ISH) were then applied to Hep293TT cells replicating infectious HEV and liver specimens from patients with hepatitis E (n = 20) and controls (n = 134).

Results: Whereas ORF1-3 proteins were all detectable in transfected, HEV protein-expressing cells, only ORF2 and 3 proteins were traceable in cells replicating infectious HEV. Only the ORF2-encoded capsid protein was also unequivocally detectable in liver specimens from patients with hepatitis E. IHC for ORF2 protein revealed a patchy expression in individual or grouped hepatocytes, generally stronger in chronic compared to acute hepatitis. Besides cytoplasmic and canalicular, ORF2 protein also displayed a hitherto unknown nuclear localization. Positivity for ORF2 protein in defined areas correlated with HEV RNA detection by ISH. IHC was specific and comparably sensitive as PCR for HEV RNA.

Conclusions: ORF2 protein can be reliably visualized in the liver of patients with hepatitis E, allowing for sensitive and specific detection of HEV in FFPE samples. Its variable subcellular distribution in individual hepatocytes of the same liver suggests a redistribution of ORF2 protein during infection and interaction with nuclear components.

Lay summary: The open reading frame (ORF) 2 protein can be used to visualize the hepatitis E virus (HEV) in the human liver. This enabled us to discover a hitherto unknown localization of the HEV ORF2 protein in the nucleus of hepatocytes and to develop a test for rapid histopathologic diagnosis of hepatitis E, the most common cause of acute hepatitis worldwide.

Keywords: Hepatitis E virus (HEV); Histopathology; Immunohistochemistry; *In situ* hybridization; Hepatitis; Human liver.

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Introduction

Hepatitis E virus (HEV) is a non-enveloped, positive-strand RNA virus of the family *Hepeviridae* and genus Orthohepevirus.[1] It is a water- or food-borne, enterically transmitted infectious agent, causing the most common form of acute viral hepatitis worldwide, thus constituting a globally significant health problem.[2,3] The 7.2 kb HEV genome comprises three opening reading frames (ORF) coding for the viral replicase (ORF1), the capsid protein (ORF2), and a small phosphoprotein required for the secretion of viral particles (ORF3), respectively.[4] Currently, four main HEV genotypes (GT1-4) infecting humans are distinguished, with variable geographic distribution.[2,3] GT1 and GT2, mostly occurring in Asia, Africa and Central America, seem to be confined to humans. GT3, occurring worldwide, and GT4, occurring in China and

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Southeast Asia, represent zoonotic infections.[2,3] The incidence of hepatitis E is highest in resource-limited regions in which it also occurs epidemically. However, HEV is more common than initially considered in industrialized countries, as documented by a high seroprevalence in the range of 20% in the United States,[5] and up to 86% in the southwest of France.[6] Although HEV infection generally causes a self-limited acute disease, it can present as fulminant hepatitis and may take a chronic course in immunocompromised patients.[7–9] Diagnosis of HEV infection in the non-epidemic setting is easily missed since the clinical presentation is not specific. Whereas antibody-based serological tests have varying sensitivities and specificities, HEV infection can be reliably diagnosed by testing for viral RNA in blood during viremia lasting from about 2 to 10 weeks post infection in cases of self-limited acute hepatitis E.[3] Even though hepatitis E diagnosis is readily made by serological testing, pathologists are routinely confronted with cases in which hepatitis E is among the differential diagnoses, but serological testing either has not been performed, or results are not yet available. Timely and accurate histopathologic diagnosis of HEV infection is challenging since histological findings are overlapping with other causes of hepatitis and also highly variable, depending, among other factors, on the patients' immune status.[9–12] PCR testing for HEV RNA can be applied to formalin-fixed, paraffin-embedded (FFPE) histopathologic specimens. However, this is rather laborious and available only in a few specialized laboratories. Thus, there is a need for more practicable ancillary tools for the histopathologic diagnosis of hepatitis E.

Precise knowledge of the temporal and spatial viral distribution in the liver during infection would not only be of advantage for developing tissue-based diagnostics, but is also a prerequisite for a better understanding of HEV pathogenesis.[13] Several studies aimed to localize HEV in tissues: Swine HEV has been visualized in livers and extrahepatic tissues of HEV infected pigs by *in situ* hybridization (ISH)[14] and immunohistochemistry (IHC).[15] In human liver tissues, subcellular localization studies of the ORF1 protein[16] and IHC for ORF2 and ORF3 proteins have been reported.[17] More recently, immunostaining of HEV ORF2 and ORF3 proteins was performed in humanized mice.[18,19] However, the field is still hampered by the lack of systematic, comprehensive and rigorously controlled data on HEV proteins and RNA in the human liver. Here, we systematically and comprehensively evaluated IHC and ISH for visualizing HEV proteins and RNA in cell lines and human liver tissues, and determined the value of these tools in a diagnostic setting.

Methods and materials

HuH7 cells expressing HEV proteins

HuH7 human hepatocellular carcinoma cells stably expressing the T7 RNA polymerase were transfected with T7 promoter-driven expression vectors harboring ORF1, ORF2 or all three ORFs (ORF1, ORF2, ORF3) from the GT3 Kernow-C1 strain. All transfected cells were processed as cytospins in paraffin blocks. A mini tissue microarrays (mTMA) was designed comprising four GFP-transfected spots, and two spots each of ORF1-transfected, ORF2-transfected and ORF1/2/3-transfected cells.

HEV-replicating Hep293TT cells

Hep293TT hepatoblastoma cells replicating and producing full-length GT3 virus were electroporated with full-length *in vitro* transcribed p6 viral RNA, and harvested 7 days later. HEV-replicating cells were processed as cytospins in paraffin blocks.

Patients and liver tissue samples

Included were liver biopsies analyzed at the Department of Pathology, University Hospital Zurich (USZ) between 2012 and 2016. The panel comprised cases primarily submitted to the USZ, and cases referred from other centers. In case patients had a positive HEV RNA PCR blood test (n = 20), clinical parameters were retrospectively retrieved from the patients' files. For controls, biopsy material from patients with serologically proven hepatitis B and hepatitis C as well as tissue from non-viral hepatitis (*e.g.* drug, autoimmune) and biopsies without any pathologic changes (taken for living liver donor evaluation) were analyzed. This study was reviewed and approved by the internal review board of the University Hospital Zurich and the Cantonal Ethics Committee of Zurich, Switzerland approval (KEK-ZH-Nr. 2013-0504).

Immunohistochemistry

IHC with antibodies against all three open reading frames was performed on the mTMA as well as on patients' liver biopsies. Three commercially available mouse monoclonal antibodies raised against the HEV ORF2 protein (clones 1E6, 2E2 and 4B2;[20] Millipore Corporation; catalog no. MAB8002, MAB8003 and MAB8006), one commercially available rabbit polyclonal antibody raised against the HEV ORF2 protein (LifeSpan BioScience, Inc.; catalog no. LS-C40560) and two commercially available rabbit polyclonal antibodies raised against the HEV ORF3 protein (LifeSpan BioScience, LS-C414259; and LuBio Science, BS-0212R)[19] were tested. A rabbit polyclonal antibody against the ORF1 methyltransferase domain (anti-MT) was kindly provided by Tero Ahola (University of Helsinki, Finland).[16] All other antibodies were developed at the Laboratory of the Division of Gastroenterology and Hepatology, Centre Hospitalier Universitaire Vaudois, University of Lausanne, Lausanne, Switzerland.

Immunohistochemical staining using the ORF2 antibody clone 1E6 comprized 32 min pretreatment at 100°C with buffer CC1 from Ventana, dilution 1:500 and direct detection with OptiView Kit from Ventana.

In situ hybridization

ISH for HEV RNA was performed according to the manufacturer's protocol on FFPE liver tissues using two commercially available HEV-specific probes designed to detect +strand RNA: 1) A probe designated V-HEV (by Advanced Cell Diagnostics (ACD); ACD catalog number #468111), and 2) A probe designated HEV-GT3-ORF3 (by Advanced Cell Diagnostics (ACD); ACD catalog number #442761).

Molecular testing (HEV RNA detection by semi-nested RT-PCR and sequence analysis)

For HEV RNA detection, two different semi-nested RT-PCR protocols were applied to FFPE liver tissue specimens as described before.[9,12]

Please find further information on the materials and methods used in Supplementary data, including the Supplementary CTAT table.

Results

Implementation of IHC with antibodies against HEV ORF1, ORF2 and ORF3 proteins on mini tissue microarrays generated from HEV protein-expressing HuH7 cells

To implement a reliable IHC protocol, we first evaluated a panel of antibodies raised against all three ORF proteins. To this end, HuH7 cells were transfected with expression vectors for either the green fluorescent protein (GFP) (as positive control for transfection efficiency and negative control for HEV antibodies), or for HEV ORF1, ORF2, as well as a combination of all three viral ORFs, respectively, and processed to FFPE cytoblocks. Cytoblock material was punched to create mTMA as versatile tools for simultaneous antibody incubation on both, expected positive and negative samples (Fig. 1). After optimizing immunostaining protocols with various antibodies (Table 1), a reliable staining pattern was obtained with the expected distribution of negative and positive