Research Article

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A cell culture system for distinguishing hepatitis C viruses with and without liver cancer-related mutations in the viral *core* gene

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Background & Aims: Although patients infected by genotype 1b hepatitis C virus (HCV) with Q^{70} and/or M^{91} *core* gene mutations have an almost five-fold increased risk of developing hepatocellular carcinoma (HCC) and increased insulin resistance, the absence of a suitable experimental system has precluded direct experimentation on the effects of these mutations on cellular gene expression.

Methods: HuH7 cells were treated long-term with human serum to induce differentiation and to produce a model system for testing high-risk and control HCV. For clinical validation, profiles of infected cells were compared to each other and to those of liver biopsies of patients with early-stage HCV-related cirrhosis followed prospectively for up to 23 years (n = 216).

Results: Long-term culture in human serum produced growth-arrested, hepatocyte-like cells whose gene profile overlapped significantly with that of primary human hepatocytes. High-risk (Q^{70}/M^{91}) and control (R^{70}/L^{91}) viruses had dramatically different effects on gene expression of these cells. The high-risk virus enhanced expression of pathways associated with cancer and type II diabetes, while the control virus enhanced pathways associated with oxidative phosphorylation. Of special clinical relevance, the transcriptome of cells replicating the high-risk virus correlated significantly with an HCC high-risk profile in patients (Bonferroni-corrected $p = 0.03$), whereas no such association was observed for non-HCC-related clinical outcomes.

Conclusions: The cell-based system allowed direct head-to-head comparison of HCV variants, and provided experimental support for previous clinical data indicating an oncogenic effect of *core* gene mutations. This simple experimental system distinguished HCV variants and will enable future mechanistic analysis and exploration of interventional approaches.

Keywords: HCC; HCV; Core-mutations; Human serum.

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Abbreviations: HCC, hepatocellular carcinoma; HCV, hepatitis C virus; FBS, fetal bovine serum; HS, human serum; A1AT, alpha-1 antitrypsin; RIG-I, retinoic acid-inducible gene 1; DMSO, dimethyl sulfoxide; RPS11, ribosomal protein subunit 11; TLR3, toll-like receptor 3; MDA5, melanoma differentiation-associated protein 5; IFNAR1, interferon alpha receptor 1; STAT1, signal transducer and activator of transcription 1; IRF9, interferon regulatory factor 9; PKR, protein kinase RNA-activated; OAS1, 2′,5′-oligoadenylate synthetase; ISG15, interferon stimulated gene 15; MX1, myxovirus resistance 1; IFNB, interferon beta; IFNL1, interferon lambda 1; IFITM3, interferon-induced transmembrane protein 3; $TCID_{50}$, tissue culture infectious dose 50; TBP, TATA-binding protein; KEGG, Kyoto Encyclopedia of Genes and Genomes; GSEA, Gene Set Enrichment Analysis; NES, normalized enrichment score; FDR, false discovery rate.

Introduction

Hepatocellular carcinoma (HCC) is the second leading cause of site-specific cancer-related death worldwide [1] and the most rapidly increasing cause of cancer-related death in the United States (US) [2]. Chronic hepatitis C virus (HCV) infection is a leading risk factor for HCC in the US, Japan, and other parts of the world [3]. HCV will remain a major cause of HCC for the foreseeable future despite the introduction of highly effective direct-acting antiviral drugs that increase the percentage of patients who achieve a sustained virological response (SVR) [4]. Many barriers to HCV treatment exist, including the high percentage of patients who are unaware of their infection and the high cost of treatment. Moreover, for poorly understood reasons, successful HCV treatment does not eliminate the HCC risk [5]. Patients with advanced fibrosis/cirrhosis who achieve an SVR are advised to continue life-long screening for HCC [6]. Currently, many patients with HCV-related HCC are diagnosed at a late stage. Only 12% of patients with HCV-related HCC are diagnosed through surveillance in the US [7]. Methods for stratifying HCC risk would allow HCV treatment and HCC surveillance to be focused on the individuals most likely to benefit.

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Data about factors modulating the risk of other types of cancer might provide clues about risk stratification for HCC. Approximately 12% of human cancers are associated with oncogenic viruses [8]. Experimental studies suggest that the HCV *core* gene has oncogenic potential. Expression of the *core* gene in transgenic mice leads to insulin resistance, hepatic steatosis, and liver cancer [9,10]. In cell culture systems, the *core* gene causes cellular transformation and disruption of several growth control pathways [11].

In the case of human papillomavirus, the cancer risk varies by viral strain [12], demonstrating the importance of sequence- and strain-specific differences in viral oncogenesis. In the case of HCV, genotype 1b is reported to increase the HCC risk [13]. Among patients infected by genotype 1b HCV, those with virus harboring mutations in codons 70 and/or 91 in the *core* gene have the highest risk [14]. Patients infected with viruses carrying a mutation in codon 70 (Q^{70}) and/or codon 91 (M^{91}) had a 4.65-fold increased occurrence of HCC ($p = 0.017$) [14]. The close association between codon 70 mutations and HCC risk has been observed in several independent patient cohorts [15–22], primarily in Asian (Japanese) populations. The impact in non-Asian populations is unclear. Significantly, a recent study showed that the elevated HCC risk among patients with Q^{70} persisted even after they achieved an SVR [25]. The Q^{70}/M^{91} mutations are also associated with insulin resistance [23] and failure of interferon (IFN)-based therapies [24].

Despite clinical data showing an association between Q^{70}/M^{91} mutations and HCC, the absence of supporting laboratory data has raised concern that it is an artifact of uncontrolled confounding in the clinical data. To address this concern, we developed a cell culture system for testing the impact of high-risk HCV on the expression of cancer-related molecular pathways. The model utilizes HuH7 cells cultured long-term in media containing human serum (HS), as was done previously for HuH7.5 cells [26]. Under these conditions, HuH7 cells acquire the capacity for contact inhibition and they express many gene pathways that are typical of primary human hepatocytes (PHH). These growth-arrested, hepatocyte-like HuH7 cells support replication of full-length HCV. Matched viruses with and without the high-risk mutations were indistinguishable in their virological properties; however, cells replicating the high-risk variant had enhancement of gene pathways associated with oncogenesis and insulin resistance compared to cells replicating the control HCV, which had enhancement of pathways associated with normal metabolism, such as oxidative phosphorylation. In addition, cells replicating the high-risk virus had a global transcriptome that correlated significantly with a high-HCC risk profile among 216 HCV-infected patients with early-stage cirrhosis who were followed prospectively for up to 23 years to determine clinical outcome. The transcriptome of cells replicating the control virus did not correlate with HCC or any other clinical outcome. Thus, we introduce a simple experimental system that distinguished high-risk and control HCV. When combined with each other, the clinical and experimental data emphasize the importance of HCV mutations as prognostic indicators of liver cancer risk. The simple experimental system will enable detailed biochemical investigation of cellular pathways disrupted by high-risk HCV strains and provide a platform for the discovery of drugs that reverse these disruptions.

Materials and methods

HuH7 cultures

HuH7 cells cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS) are referred to as FBS/HuH7 cells. To induce differentiation, HuH7 cells were maintained in media containing 2% AB HS (Corning-Mediatech), without sub-culturing and with regular media changes as previously described [26] and are referred to as HS/HuH7 cells. Additionally, a range of dimethyl sulfoxide (DMSO) concentrations (1–2.5%) (Sigma-Aldrich) was also tested [27]. PHH were cultured as described in Supplementary methods.

Quantification of hepatocyte differentiation markers and innate antiviral gene expression

RNeasy Plus Mini kits (Qiagen) were used to purify RNA. Reverse transcription (RT) was performed using SuperScript III First-Strand Synthesis (Invitrogen) and random hexamers. RT products were then used for quantitative-PCR (q-PCR) using the LightCycler 480 SYBR Green I Master kit. Expression of hepatocyte-specific genes, *albumin* and *A1AT*, and innate immune antiviral genes was determined and calculated using the $\Delta\Delta C_t$ method. *RPS11* was used for normalization. Primer sets were previously reported [27,28]. Statistical analysis of RNA quantitation was performed using *Student's t test*. A two-tailed p value <0.05 was regarded as statistically significant.

Viral constructs

APP144, a Con1/JFH-1 chimeric construct ($Apath_{LLC}$), has five enhancing mutations (L831F, I857T, I1312V, K2073R and I2266L) and core, E1, E2, P7 and part of NS2 from Con1 (genotype 1b; accession number: AJ238799) and other regions from JFH-1 (genotype 2a; accession number: AB047639). APP144 has Q^{70} (glutamine) and L^{91} (leucine). Mutagenesis was performed as previously described [29], creating constructs with R^{70}/L^{91} and Q^{70}/M^{91}, which were verified by sequencing.

Infectivity assays

Infectious titers were determined by a tissue culture infectious dose 50 assay ($TCID_{50}$) previously described [30]. Standard methods were used for preparation of viral stocks, intracellular HCV RNA quantitation, Western blotting and flow cytometry to assess percentage of infected cells; detailed in Supplementary methods.

Gene expression profiling and bioinformatics analysis

Genome-wide gene expression profiling was performed using the Human-HT-12 Expression Beadchip (Illumina); 200 ng/sample. Hybridized chips were scanned using HiScan Array Scanner, raw data were extracted using Genome Studio software ver.3 (Illumina), and normalized by cubic spline algorithm implemented in Illumina Normalizer module of GenePattern genomic analysis toolkit (www.broadinstitute.org/genepattern). The dataset is available at NCBI Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo, accession number GSE64605). The transcriptome profile of PHH was previously described [31] (www.ncbi.nlm.nih.gov/geo, accession number GSE29907, samples GSM740485 and GSM740486).

The global transcriptome profile of HCV-infected cells was compared to that of liver biopsy specimens from 216 patients with early-stage HCV-associated cirrhosis and no prior history of HCC or complications of cirrhosis. These patients were followed longitudinally for a median of 10 years to identify patients who developed HCC, died from any cause, progressed in Child-Pugh class, and/or experienced hepatic decompensation. The cohort and the transcriptome profile of the liver specimens have been described previously (NCBI Gene Expression Omnibus accession number: GSE15654) [32]. For each gene in the dataset, the association with each clinical outcome was calculated using the Cox score, as previously described [33]. Vectors of Cox scores were merged to create a matrix, and the association between HCV *core* gene variants and clinical outcomes were assessed by using a Subclass Mapping algorithm, a bi-directional gene signature enrichment-based subclass association determination method, as previously described [34]. A mutation-outcome association with a Bonferroni-corrected p value <0.05 was regarded as statistically significant.