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Research paper

Dysregulation of fibrosis related genes in HCV induced liver disease

Reham M. Dawood[a,*], Mai Abd el-Meguid[a], Marwa K. Ibrahim[a], Noha G. Bader El Din[a], Ahmed Barakat[b], Khaled El-Wakeel[c], Mohamed Darwish Ahmed Abd Alla[d], George Y. Wu[e], Mostafa K. El Awady[a]

[a] Micrbial Biotechnology Department, National Research Center, Dokki, Cairo, 12622, Egypt
[b] Department of Microbiology, Faculty of Science, Ain Shams University, Cairo, Egypt
[c] Medical Research Division, Biological Anthropology Department, National Research Centre, Dokki, Giza, Egypt
[d] Tropical Medicine Department, Faculty of Medicine, Al-Azhar University, Gouhar Al-KaedStreet, El-Hussein University Hospital, Al-Darasah, Cairo, 11675, Egypt
[e] Department of Medicine, Division of Gastroenterology-Hepatology, University of Connecticut Health Center, Hartford, USA

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ABSTRACT

Background: Liver fibrosis results from a wound healing response to chronic injury, which leads to excessive matrix deposition. Genome wide association studies have showen transcriptional dysregulation in mild and severe liver fibrosis. Recent studies suggested that genetic markers may be able to define the exact stage of liver fibrosis.

Aim: To define genes or genetic pathways that could serve as markers for staging or as therapeutic targets to halt progression of liver fibrosis.

Methods: The study was performed on 105 treatment naïve HCV genotype 4 infected patients [F0–F2, $n = 56$; F3–F4, $n = 49$] and 16 healthy subjects. The study included PCR array on 84 fibrosis related genes followed by customization of a smaller array consisting of 11 genes that were designed on the bases of results obtained from the larger array. Genes that displayed significant dysregulation at mRNA levels were validated at protein levels.

Results and discussion: Two major pathways exhibited high dysregulation in early fibrosis as compared with controls or when compared with late fibrosis, these were the TGFβ - related pathway genes and Matrix - deposition associated genes. Hepatic stellate cell (HSC) activators i.e. TGFβ pathway genes [TGFβ1, 2 and 3, their receptors TGFβR1 and 2, signaling molecules SMAD genes and PDGF growth factors] were considerably over-expressed at transcriptional levels as early as F0, whereas expression of their inhibitor TGIF1 was simultaneously down regulated. Matrix proteins including collagen and MMPs were upregulated in early fibrosis whereas tissue inhibitors TIMPs 1 and 2 began over expression in late fibrosis. Expression at protein levels was concordant with RNA data excluding dysregulation at post transcriptional levels.

Conclusion: Since these 2 gene sets are closely interrelated regarding HSC activation and proliferation, we assume that the current findings suggest that they are favorable targets to further search for stage specific markers.

1. Introduction

Hepatitis C Virus (HCV) infection is a global health problem affecting > 71 millions people worldwide, and the annual infection rate is estimated to be 3 to 4 million new cases (Gower et al., 2014; WHO, 2017). In Egypt, 12% of the population is infected making it the highest prevalence rate worldwide (Waked et al., 2014). Approximately, 80–90% of acute infections progress to the chronic disease. After

Abbreviations: Alb, Albumin; ALK PH, Alkaline phosphatase; ALT, Alanine amino transferase; APO, Apolipoprotein; AST, Aspartate transaminase; AUC, Area Under Curve; BIL.D, Direct bilirubin; BIL T, Total bilirubin; BMI, Body mass index; cDNA, Complementary DNA; CTGF, Connective Tissue Growth Factor; CYP2C8, Cytochrome P4502C8; DAAs, Direct Acting Antivirals; ECM, Extra Cellular Matrix; ELISA, Enzyme Linked Immunosorbent Assay; FAM14B, Family With Sequence Similarity 14, Member B; GAPDH, Glyceraldehyde-3-Phosphate Dehydrogenase; HB, Hemoglobin; HBV, Hepatitis B Virus; HCC, Hepatocellular Carcinoma; HCV, Hepatitis C Virus; HSC, hepatic stellate cells; ICAM-1, Intercellular Adhesion Molecule 1; IFN, Interferon; LAMB1, Laminin Subunit Beta 1; LAMC1, Laminin Subunit Gamma 1; LRP, lipoprotein receptor-related protein; MAP, Mitogen-Activated Protein Kinase; MMPs, Matrix Metalloproteinases; mRNA, Messenger Ribo-Nucleic Acid; NASH, Non-Alcoholic Steatohepatitis; NK, Natural Killer; OAS2, 2′-5′-Oligoadenylate Synthetase 2; PBMCs, Peripheral Blood Mononuclear Cells; PCR, Polymerase Chain Reaction; PDGF, Platelet Derived Growth Factor; PI3, Phosphatidylinositol 3 Kinase; PLT, platelet; ROC, Receiver Operating Characteristic Curve; RNA, Ribonucleic Acid; RT-PCR, Real Time Polymerase chain Reaction; SERPINF2, Serpin Family F Member 2; TGFβ, Transforming Growth Factor β; TGFBR, Transforming Growth Factor β Receptor; TGIF, TG- interacting factor; TIMPs, Tissue Inhibitor of Matrix Metalloproteinases; TNF-α, Tumor Necrosis factor-alpha; WBCS, White blood cells

* Corresponding author.
E-mail address: rmhaemd@hotmail.com (R.M. Dawood).

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15–20 years of chronic infections, 2–3% of cases develop hepatic cirrhosis each year (Thein et al., 2008; Ponziani et al., 2017). Patients with cirrhosis either remain free of major complications for several years known as compensated cirrhosis or may progress to decompensated cirrhosis. The latter is associated with short survival where liver transplantation is often indicated as the only effective management. Hepatic cancer occurs in 1.5–2% of cirrhotic cases per year, making the HCV infection one of the primary causes of hepatocellular carcinoma worldwide (Sarin & Kumar, 2012). Current treatment of HCV infection is based on a variety of combinations from a large panel of direct acting antiviral (DAA) agents which include the following three classes (NS3/4A protease inhibitors, NS5A inhibitors and NS5B polymerase inhibitors) (Thiagarajan & Ryder, 2015; Gogela et al., 2015).

Besides hepatitis viruses, other stimuli such as alcohol, steatohepatitis, toxins and immune disorders can induce liver injury (Bataller & Brenner, 2005; Yanguas et al., 2016). Hepatic fibrosis alters the architecture and disrupts the function of the liver (Suk & Kim, 2015). It is a dynamic process initiated by liver injury that results in increased deposition of extracellular matrix (ECM) proteins (Trautwein et al., 2015). Accumulation of ECM proteins and their decreased removal by matrix metalloproteinases (MMPs) results in a progressive replacement of the liver parenchyma by scar tissue, leading to liver fibrosis and its complications (Singh et al., 2015). Progressive liver fibrosis results in cirrhosis (the end stage of fibrosis) that is complicated by liver failure, portal hypertension and/or hepatocellular carcinoma (HCC) (Hannivoort et al., 2012). Hepatic stellate cells (HSCs) are considered the main cellsproducing ECM proteins (Yanguas et al., 2016; Bachem et al., 1992). Activation and proliferation of HSCs are mediated by two cytokines; transforming growth fact or beta 1 (TGFß1) and platelet-derived growth factor (PDGF). Both are secreted by HSCs, platelets, and other cells (Friedman, 2008; Seki & Schwabe, 2015).The activated HSCs are then converted to myofibroblasts, producing fibrogenic proteins, including collagens and tissue inhibitors of metalloproteinases (TIMPs) that inhibit the ECM degradation through the suppression of MMPs activities (Friedman, 2008; Shimada & Rajagopalan, 2012). The resolution of liver fibrosis with a restoration of near normal architecture has been demonstrated after successful treatment of the underlying disease. This observation has been described in patients with iron and copper overload, alcohol-induced liver injury, chronic hepatitis B, C and D, hemochromatosis, secondary biliary cirrhosis, NASH, and autoimmune hepatitis (Bataller & Brenner, 2005; Milic et al., 2016). Whether end-stage cirrhosis can revert to a normal liver architecture remains controversial (Iredale, 2007). However, significant improvement in hepatic structure and function provides evidence of regression of liver fibrosis (Jung & Yim, 2017). Liver biopsy is referred to as the “gold standard” in assessing both the activity and degree of fibrosis in many chronic liver diseases. Besides the invasive nature of liver biopsy it has several drawbacks, including variable accessibility, high cost, sampling errors and inaccuracy due tointer- and intra-observer variability of pathologic interpretations (Sebastiani & Alberti, 2006; Valva et al., 2016). There is a need to find alternatives less invasive and more accurate diagnostic tools for assesment of fibrosis and follow up after therapeutic intervention. Genome-wide analysis of abnormal gene expression has showen dysregulation differences among normal, mild and severefibrosisas well as during HCC development with identification of novel serum markers for early stages (Valva et al., 2016; Smith et al., 2006). Recent studies suggested that genetic markers may be able to define the exact stage of liver fibrosis. For this purpose, limited but functional studies have proposed quite a few genetic markers using individual genes or group of genes (Ahmad et al., 2012). Genetic markers have an additional advantage of permitting serial measurements over time while; liver biopsy can usually be obtained at only one or at most a few time points (Huang et al., 2006).

Previous studies of liver fibrosis have shown dysregulation of some genes such as CTGF, CXCL10, TGFB1, MMPs, TIMPs, collagens, COL1-A1, Cadherin, CD44, LAMB1, LAMC1, SERPINF2, interleukin, PDGF, ICAM1, TNFα, ITGA, APO, and CYP2C8. This gene portrait can be used to assess liver fibrosis and cirrhosis (Ahmad et al., 2012; Caillot et al., 2009). Other studies on HCVgenotype 3a explored genes that were dysregulated during either early fibrosis process and/or cirrhosis. Real time RT-PCRs have validated the over expression of CASPASE9, OAS2, TGFBR2 and the down regulation of FAM14B gene in early fibrosis (Ahmad et al., 2012). However, there have been few similar profiling studies on HCV genotype 4 infected (G4) patients. Recently we reported on dysregulation of few IFN –related genes in G4 infected subjects (Ibrahim et al., 2016a). Studies on genetic polymorphisms of several fibrosis - related genes in those patients were reported (Dawood et al., 2017; Bader El Din et al., 2015; Bader El Din et al., 2016; Ibrahim et al., 2016b; El Awady et al., 2016).

Profiling of gene expression in PBMC's has been correlated with values obtained from liver tissues regarding several genes including TNF-α (Larrea et al., 1996), genes identifying tolerant and non tolerant reciepients post liver transplantation (Martinez-Llordella et al., 2008) and cytochrome p450 3A4 (Lee et al., 2010), Since PBMC's are easily accessible for investigating gene expression, the aim of the current study was to develop non - invassive tests for fibrosis using; PBMCs RNAs to develop gene expression profiling in G4 infected patients. Two-phase systematic analyses were performed. Phase 1 was explorative, where mRNA expression of 84 fibrosis related genes was examined in 30 treatment- naïve HCV G4 infected patients with various stages of liver fibrosis. Phase 2 included a focused analysis on 75 patients [39 early versus 36 late fibrosis] using 11 genes exhibiting significant dysregulation in phase 1. Selection criteria of genes included dysregulation by 2-fold change [preferably those with p value ≤ 0.05] and interrelated genes within specific cellular pathways.

2. Materials and methods

2.1. Patients

Inclusion criteria: Venous blood was withdrawn from 105 treatment-naïve patients chronically infected with HCV G4. Their ages ranged from 25 to 52 years old. Patients were recruited from El-Hussein University Hospital, Cairo, Egypt. All patients were positive for anti-HCV IgG antibodies [Enzyme linked immunosorbent assays ELISA, Ortho Diagnostics, Neckargmun, Germany] and serum HCV RNA (real time PCR Artus HCV QS RGQ Kit Qiagen, Hilden, Germany). All patients were infected with HCV genotype 4 [InnoLiPA HCV II assay, Innogenetics Inc., Alpharetta, GA, USA]. The severity of liver fibrosis was determined histologically in liver biopsies by Knodell and Metavir scoring system and confirmed by transient elastography (Fibroscan) measurement. Exclusion criteria included, coinfection with HBV or schistosomiases, excessive alcohol intake, NASH or autoimmune disease. Based on the histological assessment of the liver, there are five stages of liver fibrosis: F0 (no fibrosis- $N = 14$), F1 (mild fibrosis without septa- $N = 21$) F2 (moderate fibrosis with few septa- $N = 21$), F3 (severe fibrosis with numerous septa without cirrhosis-$N = 24$) and F4 (cirrhosis- $N = 25$). All experiments were approved by the Institutional Ethical Review Board (medical research ethics committee at the National Research Center, IRB13-106; Cairo, Egypt) according to Helsinki Declaration 1975 revised in 2008 and performed with the understanding and the consent of the human subject. Informed consent was obtained from each subject before collecting blood samples.

2.2. Healthy subjects

Blood samples from 16 healthy individuals were enrolled in this study as a control group. All subjects had normal liver enzymes and were free of liver diseases including viral hepatitis, parasitic or bacterial infections, no autoimmune hepatitis or inborn errors of metabolism, no metabolic disorders and no steatosis.