The Possible Impact of Sortilin in Reducing HBsAg Expression in Chronic Hepatitis B

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Hepatitis B virus (HBV) infection is a major global health problem. Chronically infected people are at risk for progressive hepatic fibrosis and consequent cirrhosis. Hepatitis B surface antigen (HBsAg) level in serum is a complementary marker for intrahepatic HBV DNA and covalently closed circular DNA (cccDNA). Sortilin-1 (SORT1) has been reported to be involved in the post-Golgi vesicle trafficking of Apo lipoproteins degradation pathways. This study was designed to evaluate the hepatic and serum expression of HBsAg and its association with hepatic SORT1 gene expression in patients with chronic HBV. Thirty chronic hepatitis B patients with histological examination results were enrolled in this study. Liver biopsies were analyzed for hepatic HBsAg and SORT1 gene expression by immunohistochemistry and quantitative real time PCR (qRT-PCR), respectively. Twenty seven out of 30 (90%) liver biopsies had positive staining for HBsAg and showed a significant inverse association with hepatic SORT1 fold change gene expression ($\beta = -0.5$, $P = 0.042$). There was significant association between HBV DNA levels and HBsAg expression in hepatocyte or serum titer of HBsAg ($r = 0.39$, $P = 0.029$; $r = 0.39$, $P = 0.032$ respectively). Serum ALT was also correlated with hepatic activity index (HAI) score ($\beta = 0.6$, $P = 0.001$). Inverse association between hepatic SORT1 gene expression and hepatic HBsAg expression indicates the possible role of sortilin in HBsAg particle formation. ***J. Med. Virol.***

KEY WORDS: sortilin (SORT1); HBsAg; chronic hepatitis B; HBV DNA

INTRODUCTION

Chronic active hepatitis B patients characterized by high or fluctuating liver transaminases and/or HBV DNA levels are at great risk for subsequent development of chronic liver disease, including cirrhosis and hepatocellular carcinoma (HCC) [Wang et al., 2008; Mohamadkhani et al., 2010]. Hepatitis B surface antigen (HBsAg) positivity in serum is the hallmark for the diagnosis of chronic hepatitis B virus infection. Studying the membrane structure of HBsAg particles by fluorescence correlation spectroscopy (FCS) demonstrated that the HBsAg particles, like low density lipoproteins (LDLs), exhibit a lipoprotein-like structure in which a phospholipid monolayer surrounds a more hydrophobic inner core, likely composed of 40% lipids including sterol esters, triglycerides, and fatty acids [Diminsky et al., 1997; Greiner et al., 2010]. Therefore, it is probable that lipids metabolism affect the structure and the exposure of specific epitopes and immunogenicity of HBV [Satoh et al., 2000; Mohamadkhani et al., 2012].

The protein sortilin is a multi-ligand type-1 receptor and a member of vacuolar protein sorting 10 protein (VPS10P) domain receptor family that has been identified in association with LDL-C levels in humans [Willnow et al., 2011; Strong et al., 2012]. The gene encoding human sortilin, SORT1, consists of 22 exons that maps on the short arm of

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chromosome 1 (1p21.3-p13.1) [Musunuru et al., 2010]. The subcellular localisation of SORT1 limits mainly to the Golgi apparatus and attaches to a variety of ligands on its extracellular VPS10 domain to transport them to the lysosome then back to the Golgi by the retromer complex [Strong and Rader, 2012]. Recent studies have suggested SORT1 in lipoproteins metabolism in the liver and adipose tissue [Dube et al., 2011]. Indeed, genome wide association studies (GWAS) in human have shown a strong association of a specific SNP in the SORT1 gene with increase in SORT1 hepatic expression and considerable decreased serum levels of LDL and coronary heart disease [Musunuru et al., 2010; Strong et al., 2012]. On the other hand higher activity of mTORC1 in obese mice enhance secretion of Apo B, a main principal protein in lipoproteins and their metabolism, by reducing hepatic Sort1 [Ai et al., 2012]. Moreover Strong et al., presented that hepatic sortilin both decreases hepatic APOB secretion and reduce the catabolism of LDL [Willnow et al., 2011; Strong et al., 2012].

As sortilin binds a number of ligands and participates in a wide range of cellular processes and regarding to the lipoprotein-like structure of HBsAg particles, we hypothesized for a probable role of sortilin in intracellular trafficking of HBsAg and consequently its effect on concentration of hepatic and serum HBsAg. The assumed association of *SORT-1* gene expression and intracellular sortilin levels has been previously studied [Strong et al., 2012; Ghaemimanesh et al., 2014]. Therefore, the main objective of this study was to evaluate hepatic and serum expression of HBsAg and its association with hepatic SORT1 gene expression in patients with chronic HBV.

PATIENTS AND METHODS

Study Population

Thirty patients with e-antigen negative chronic hepatitis B (73% (n = 22) male) referred to Shariati Hospital, Tehran University of Medical Sciences from Jan 2009 to Jan 2011, were enrolled in this study. Patients had no evidence of other liver diseases such as hepatitis C and autoimmune hepatitis. Demographic data and blood samples were collected at the initial assessment (first visit). Serum samples were stored at −70°C prior to analysis. Formalin-fixed paraffin-embedded liver biopsies from these HBeAg negative patients were used in this study. Biopsies were either performed for therapeutic purposes or as a part of a protocol with the objective of evaluating response to therapy after 2 years of lamivudine therapy. Patients with a HBV viral load of more than 2000 IU/ml and significant (moderate-severe) necroinflammation and/or at least moderate fibrosis, on biopsy (n = 14) were considered for antiviral treatment after liver biopsy. None of the patients received (antiviral) treatment prior to liver biopsy. Formalin-fixed paraffin-embedded of three specimens with no histological liver abnormalities were used as control samples. All patients or their legal representatives signed an informed consent according to the ethical guidelines of the 1975 Declaration of Helsinki and its subsequent 1983 revision prior to blood sample collection. The study protocol was approved by the Ethics Committee of the Digestive Disease Research Institute, Tehran University of Medical Sciences.

Quantification of HBsAg and HBV DNA in Serum

HBV DNA levels in serum samples were quantified by LightCycler v 3.5 (Roche Applied Science, Mannheim, Germany) using the RealART^{TM} HBV LC PCR (QIAGEN, Hilden, Germany) with a detection limit of 10 IU/ml according to the manufacturer's instructions.. HBsAg titers were determined with the Architect HBs-Antigen QT assay (Abbott Laboratories, Wiesbaden, Germany) based on an automated chemiluminescent microparticle immunoassay, following the manufacturer's recommendation. The Architect HBsAntigen QT assay measures a range of HBsAg from 0.05 to 250 IU/ml. Therefore, all samples were primarily tested at dilutions of 1 in 1000 within Architect HBsAg Manual Diluent.

Liver Biopsies Examination and Immunohistochemical Staining

Liver biopsies from all patients were evaluated for histological activity index (HAI); rating necroinflammation with a score between 0–18, and fibrosis stage using Ishak's scoring system; rating fibrosis with a score between 0–6. The presence of steatosis was considered as negative or positive. Deparaffinized sections were stained with mouse anti human monoclonal antibody against HBsAg (dilution 1/20, clone 3E7, Dako, Carpinteria, CA) to evaluate the level and pattern of HBsAg expression in the hepatocytes. The staining was developed with standard avidin-biotin peroxidase method (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA). The immunohistochemical staining for HBsAg was quantified visually. The number of $HBsAg^{+}$ cells in the parenchymal area was counted in 10 low power fields and the results were reported by IHC score as a percentage of the overall parenchymal area; 0 = absent, $+1 < 25\%$, $+2 = 25$–50%, $+3 = 50$–75%, and $+4 > 75\%$. The staining results were separately evaluated by two gastrointestinal pathologists who were blinded to the clinical information.

RNA Extraction and Real-Time PCR

Total liver RNA was extracted from four 20 μm sections from formalin fixed paraffin embedded tissue using RNeasy FFPE Kit (Qiagen, country) according to the manufacturer's instructions. The concentration of purified RNA sample was measured by NanoDrop ND-1000^{TM} (Nanodrop Technologies) spectrophotometer.

To synthesis cDNA, 5 μg of total RNA was used in 20 μL reaction mixture of Sensiscript® Reverse Transcription Kit (QIAGEN, Hilden, Germany), incubated at 42°C for 45 min and stored at −20°C until use. The housekeeping gene β-actin was used for the normalization of qPCR SORT1 gene. The specific primer pairs used were precise primers of "5′-CAGTCCAAGCTATATCGAAGTGAGG-3′" as forwards and "5′-AAGATGGTGTTGTCTGATCCC-CATTT-3′" as reverse for SORT1 and "5′-CTGGAACGGTGAAGGTGACA-3′" as forward and "5′-AAGGGACTTCCTGTAACAATGCA-3′" as reverse primers for β-actin (Operon, Köln, Germany).

A light Cycler® FastStart DNA Master^{PLUS} SYBR Green I (QIAGENHilden, Germany) reaction was performed on Roche light cycler version 3.5 according to the manufacturer's protocol. PCR amplification for both SORT1 and β-actin was conducted for 40 cycles with the following conditions: denaturation for 30 sec at 95°C, annealing for 30 sec at 60°C, and extension for 1 min at 70°C. The expression level of SORT1 was normalized to the β-actin. Relative Quantification (RQ), which defines the fold change or how much the gene is differentially expressed, was calculated using the comparative CT method ($2^{-\Delta\Delta CT}$) [Pfaffl, 2001; Spinsanti et al., 2008].

Statistical Analysis

Variables were expressed as mean (± standard deviation), and number (percentage) where appropriate. The HBsAg and HBV-DNA levels were logarithmically transformed for statistical analyses. Multivariate logistic regression analysis was used to identify the predictor for hepatic sortilin expression and all variables with *P*-value of less than 0.5 in univariate analysis were entered into multivariate analysis. The statistical analysis was performed using the SPSS software (version 19; SPSS, Chicago, IL). *P* value of less than 0.05 was considered significant.

RESULTS

Characterization of the Study Population

There were a total of 30 HBsAg positive patients with mean (± SD) age of 34 (± 9) years. The mean (± SD) of $\log_{10}$ of serum HBV DNA was 3.39 (± 1.33) IU/ml. Male to female ratio was 2.8 (22 males and 8 females). Demographic characteristics of these patients along with the biochemical profiles are summarized in Table I.

TABLE I. Summary of Clinical Characterization of Chronic Patients With HBV Infection

Variables	Mean (± SD)
Age (years)	34 (± 9.6)
Log_{10} HBV DNA (IU/ml)	3.39 (± 1.33)
ALT (IU/L)	46 (± 27)
HAI score	4.6 (± 2.0)
Histological fibrosis score	1.8 (± 1.3)
Quantitative HBsAg titer (IU/ml)	5062 (± 8854)
Cholestrol (mg/dL)	203 (± 45)
LDL(mg/dL)	122 (± 31)
Triglycerides(mg/dL)	124 (± 57)
SORT-1 fold changes	2.7 (± 2.1)
Liver HBS Ag expression score (IHC)	1.7 (± 1.1)

Histological Findings and Their Correlation With Clinical Variables

HBsAg immunohistochemical staining was positive in 27 (90%) of the patients with focal or diffuse cytoplasmic pattern (Fig. 1). Histological examination showed a mean (± SD) of 1.8 ± 1.3 and 4.6 ± 2.0 for fibrosis stage and total HAI score respectively. To study the factors associated with liver fibrosis, patients were divided into two groups according to the stage of fibrosis. Patients with a stage of 0–2 were defined as low fibrosis while those with a score above 2 were defined as high fibrosis. No significant differences were seen with respect to the mean of age, $\log_{10}$ HBV DNA, HBsAg titre, serum cholesterol, triglyceride, LDL, and sortilin fold change between high and low fibrosis groups (Table II). There was a significant association between serum ALT levels and liver HAI score ($\beta = 0.6$, $P = 0.001$) and patients with greater fibrosis stage had higher levels of serum ALT compared to lower fibrosis stage (63 ± 41 vs. 38 ± 15 IU/ml, $P = 0.02$). The association between serum ALT levels and HAI score remained significant after adjustment for Log_{10} HBV DNA, cholesterol, SORT1 fold change and HBsAg expression score in a multivariate analysis.

Univariate analysis revealed no significant association between the presence of steatosis with gender, age, SORT1 fold change, HBsAg level, cholesterol and triglyceride levels. Serum HBV DNA level was significantly correlated with serum HBsAg levels ($r = 0.39$, $P = 0.032$) and hepatic expression of HBsAg ($r = 0.39$, $P = 0.029$). Furthermore, we found a correlation between serum HBV DNA and cholesterol levels ($r = 0.36$, $P = 0.045$

Factors Associated With Sortilin Expression

Univariate analysis of all variables (age, gender, HBV viral load, serum ALT, HAI score, cholestrol, triglycerides, LDL, HBsAg expression score (in hepatocytes) and serum HBsAg titer), showed a significant inverse association between SORT1 fold change and hepatic HBsAg expression ($\beta = -0.42$, *P*-value = 0.022) (Fig. 2). In multivariate analysis, we adjusted for all variables with a $P < 0.5$, except serum HBsAg titer due to the correlation with HBsAg expression IHC score. This inverse association was seen again between hepatic HBsAg expression and SORT1 fold changes ($\beta = -0.5$, $P = 0.042$), (Table III).

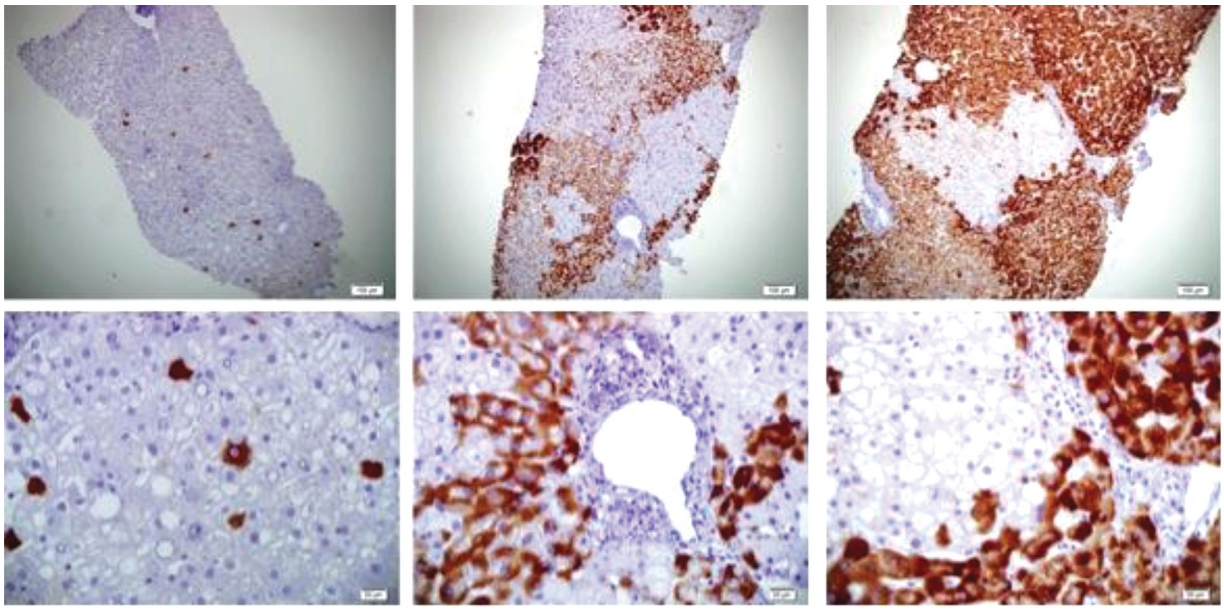

Fig. 1. Hepatic HBsAg expression shows both cytoplasmic and membranous pattern (magnification x100) in first row, (magnification x400) in second row.

DISCUSSION

We showed that higher expression of hepatic SORT1 in patients with chronic hepatitis B is associated with decreased levels of HBsAg in hepatocytes. In addition, we found a correlation between hepatic expression of HBsAg and levels of serum HBV DNA in accordance with previous studies [Brunetto et al., 2010; Su et al., 2010].

Hepatic HBsAg with a typical ground-glass form in hepatocytes is a marker of HBV infection as well as source of sub-viral particles in circulation. The expression pattern of these particles is influenced by the natural course of chronic hepatitis in patients with HBeAg-positive [Hsu et al., 1988; Wang et al., 2003]. Several studies have shown a positive correlation between serum HBsAg and HBV DNA, therefore serum HBsAg titer could be a surrogate marker of intrahepatic HBV DNA and cccDNA [Su et al., 2010; Tseng et al., 2011].

Persistent HBsAg production independent of viral load indicates that viral DNA could integrate into the host chromosomes in the course of infection and provide a template for HBsAg transcription [Wang et al., 2003]. The synthesis of HBsAg occurs at the endoplasmic reticulum more than necessary. Intermolecular disulphide bonds within extra particles produce and secret non-infectious filamentous or spherical particles into the peripheral circulation. The lipoprotein structure of HBsAg particles is composed of host-derived lipids and virus encoded glycoproteins. These particles exhibit a lipoprotein-like structure in which a compact phospholipid monolayer surrounds a more hydrophobic and fluid inner core, likely composed of triglycerides, fatty acids and sterol esters [Satoh et al., 2000; Greiner et al., 2010].

Quantification of HBsAg is important to refine treatment algorithms. In this way many viable assays can detect as little as a few nanograms of HBsAg per milliliter of serum [Satoh et al., 2000; Su et al., 2010]. HBsAg quantification detects all three forms of systemic HBsAg (part of HBV virion, spherical, filamentous), differentiation between the relative proportions is currently not routine. Most of chronic patients has HBsAg titers greater than 1 mg per ml of blood and could play a role to paralyses the host immune response [Satoh et al., 2000; Greiner et al., 2010]. Therefore, reducing production of HBsAg represents a step forward to future successful immune response and HBsAb seroconversion. It has

TABLE II. Comparison of Demographic, Biochemical and Virologic Characteristics Between Patients With Low and High Fibrosis

Fibrosis	# patient	Age	$\log_{10}$ HBV DNA	ALT	Quantitative HBsAg (IU/ml)	Chol	LDL	TG	SORT-1 fold change	HBsAg score
Low	21	34 ± 10	3.14 ± 1.29	38 ± 15	4289 ± 9110	200 ± 46	121 ± 31	118 ± 40	2.90 ± 2.36	1.6 ± 1.2
High	9	34 ± 9	3.98 ± 1.29	63 ± 41	6866 ± 8451	209 ± 43	124 ± 33	137 ± 87	2.22 ± 1.56	2.0 ± 1.0
P value		0.8	0.9	0.02	0.4	0.6	0.7	0.4	0.4	0.4

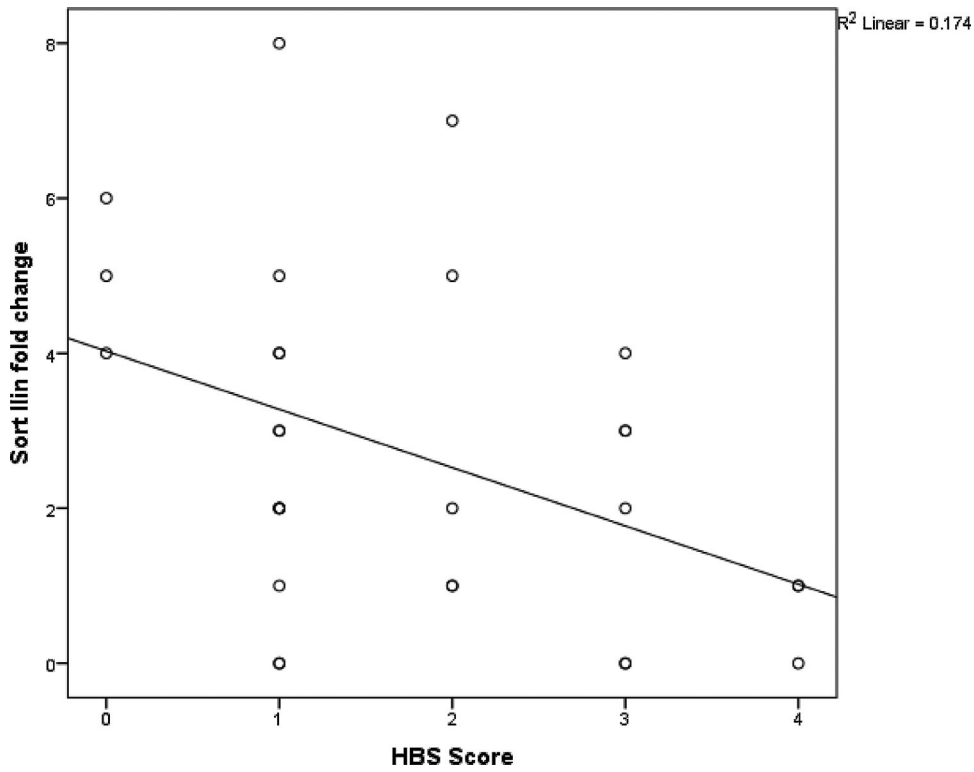

Fig. 2. Scatter plot of correlation between hepatic SORT-1 fold change and HBsAg expression score.

been demonstrated that decreased levels of serum HBsAg in adult chronic hepatitis B patients indicate for favorable outcomes [Tseng et al., 2011].

The comparable structure of HBsAg and LDL suggest that factors involving lipid metabolism would be valuable in researching the hepatitis B virus life cycle and mechanisms for HBsAg degradation. It is well known that posttranslational presecretory degradation of hepatic lipoproteins is regulated via both proteasomal-dependent and lysosomal-dependent pathways. Sortilin-1 promotes trafficking of ApoB-containing lipoproteins into intracellular, possibly lysosomal, degradation pathways [Strong et al., 2012]. These findings are suggestive of the clinical status/importance of hepatic sortilin in the management of CHB patients. Therefore, we hypothesized that sortilin can act as a sorting receptor to bind intracellular HBsAg and trafficking them to degradation pathways. Although we could not find any association between SORT1 gene expression and

TABLE III. Linear Regression Model of Factors Related to the SORT-1 Folds Changes in Cases of Chronic Hepatitis B

	Univariate		Multivariate	
Variables*	Unstandardized β	*P*	Standardized β	*P*
Age(year)*	0.2	0.3	0.2	0.3
Gender	−0.16	0.4	−0.002	1
Male (reference)				
Female				
Viral load (IU/ml)*	−0.23	0.2	0.02	1
ALT (IU/ml)*	0.06	0.8	–	–
HAI score*	−0.2	0.4	−0.1	0.6
Cholesterol(mg/dL)*	−0.2	0.3	0.3	0.6
LDL(mg/dL)*	−0.2	0.3	−0.4	0.3
TG(mg/dL)*	−0.04	0.9	–	–
HBsAg titer*	−0.33	0.07	–	–
HBsAg expression score*	−0.42	0.02	−0.5	0.04

*Mean ± SD.

serum LDL levels probably because of low sample size, however, we further demonstrated that hepatic sortilin is inversely associated with HBsAg expression in hepatocytes. To investigate the potential underlying mechanisms of sortilin in reducing HBsAg levels metabolic features of host cells infected with HBV need to be characterized. Future studies with sufficient numbers of both HBeAg negative and HbeAgpositive patients should be performed for validation of our findings.

In conclusion, we found, for the first time, an inverse association between hepatic SORT1gene expression and hepatic HBsAg expression suggestive of the possible role of sortilin in HBsAg metabolism. Hepatic sortilin could be proposed as potential factor in reducing HBsAg levels in chronic hepatitis.

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