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Association of serum IgG *N*-glycome and transforming growth factor-β1 with hepatitis B virus e antigen seroconversion during entecavir therapy

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ABSTRACT

Aberrant serum IgG *N*-glycome has been demonstrated in various autoimmune diseases and viral infections. However, the correlation between serum IgG *N*-glycome and cytokine is unclear. In addition, the clinical relevance of IgG glycosylation and cytokine changes in the treatment outcome of chronic hepatitis B (CHB) has never been assessed. One hundred and three treatment-naive patients with CHB and 101 healthy controls were enrolled in this retrospective cohort study. Serum samples in patients before and after 48 weeks of entecavir treatment were collected. In-gel trypsinized serum IgG heavy chain was analyzed using liquid chromatography–tandem mass spectrometry. Selected ion chromatograms corresponding to 10 *N*-glycoforms on asparagine 297 were individually extracted to calculate the percentage of each glycoforms. Serum cytokine profiles were examined using enzyme-linked immunosorbent assay. Forty-eight weeks of entecavir treatment resulted in decreases in galactose-deficient (total G0) IgG and transforming growth factor (TGF)-β1 levels (both $P < 0.001$) in patients with CHB. The changes in TGF-β1 (ΔTGF-β1) and IgG total G0 (Δtotal G0) levels during treatment were significantly correlated ($r = 0.403$, $P < 0.001$). Furthermore, higher levels of Δtotal G0 ($P < 0.01$) and ΔTGF-β1 ($P < 0.001$) were found in hepatitis B virus e antigen (HBeAg)-positive patients than in HBeAg-negative patients and were also found in patients with HBeAg seroconversion at week 48. The area under the receiver operating characteristic (ROC) curves for Δtotal G0 and ΔTGF-β1 to discriminate a week-48 HBeAg seroconversion were 0.835 and 0.830, respectively. These results suggested a correlation between serum cytokine and IgG *N*-glycome and its effect on the outcome of entecavir treatment in patients with CHB.

1. Introduction

N-linked glycosylation is one of the most important post-translational modifications of proteins; it initiates in endothelium reticulum with modification in Golgi apparatus (Dwek et al., 2002; Helenius and Aebi, 2004). The diversity of *N*-linked glycosylation is derived from the addition of galactose, fucose, GlcNAc, or terminal sialic acid, on the heptasaccharide core [4 *N*-acetylglucosamine (GlcNAc) and 3 mannose residues] to asparagine (Schwarz and Aebi, 2011). Immunoglobulin G (IgG) is the major glycoprotein responsible for the modifications of total serum *N*-glycome in diseases because of its high abundance in serum (Klein et al., 2010a). *N*-glycans are attached to IgG in the CH2 domain of the crystallizable fragment (Fc) at asparagine 297; they are crucial for IgG conformational integrity, secretion, complement activation, and the binding affinity of Fcγ-receptors (FcγRs) (Arnold et al., 2007; Parekh et al., 1985). The alternation of serum IgG *N*-glycome, with

Abbreviations: ALTAMA Manual of Style' has been deleted from the abbreviation list. Please check, and correct if necessary.–>, alanine aminotransferase; AST, aspartate aminotransferase; CHB, chronic hepatitis B; CID, collision-induced dissociation; Fc, crystallizable fragment; FcγR, Fc gamma receptor; G0, galactose-deficient; G1, partially galactosylated; G2, fully galactosylated; GlcNAc, *N*-acetylglucosamine; HBeAg, hepatitis B virus e antigen; HBsAg, hepatitis B virus surface antigen; HBV, hepatitis B virus; IFN, interferon; IgG, immunoglobulin G; IL, interleukin; LC–MS, liquid chromatography–mass spectrometry; ROC, receiver operating characteristic; TGF, transforming growth factor; TNF, tumor necrosis factor.

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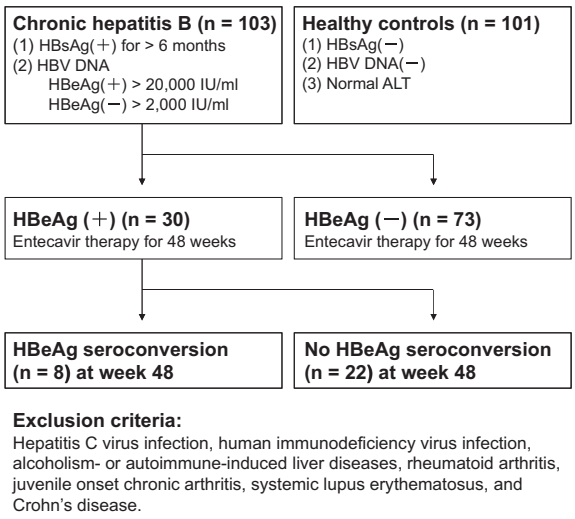

Fig. 1. Flow of study participants.

vague etiology so far, is a potential diagnostic marker for liver diseases. Moreover, it is essential for the modulation of immune responses, particularly against hepatitis viruses or intestinal bacteria (Marth and Grewal, 2008; Mehta and Block, 2008).

Hepatitis B virus (HBV) chronically infects over 350 million people worldwide and it has become a global health threat (Dienstag, 2008). During chronic hepatitis B (CHB), persistent liver injury leads to repetitive healing and scar formation resulting in liver fibrosis, cirrhosis, and eventually hepatocellular carcinoma (Friedman, 2008). HBV e antigen (HBeAg) status is a critical parameter for monitoring or following-up the disease progression and medication of CHB. Seropositivity for HBeAg is marker of active viral replication and it has been shown to be a significant risk factor for the development of liver cirrhosis and hepatocellular carcinoma (Yang et al., 2002). HBeAg seroconversion, the loss of serum HBeAg and the development of anti-HBe antibodies, indicates an increment of immune control against HBV and represents a clinical remission of liver disease (Chu and Liaw, 2007; Hsu et al., 2002). Entecavir, a nucleoside analogue, is an oral antiviral drug for treating HBV infection. It is efficacious in HBV suppression and HBeAg seroconversion in patients with CHB (Chang et al., 2006). We have previously investigated serum IgG *N*-glycosylation pattern in patients with chronic HBV infection (Ho et al., in press; doi: http://dx.doi.org/10.1093/infdis/jiu388). In the present study, we further analyzed serum IgG *N*-glycome in CHB with different HBeAg status and studied its clinical relevance of HBeAg seroconversion. Furthermore, we assessed the correlation between cytokine and serum IgG *N*-glycosylation during entecavir treatment.

2. Materials and methods

2.1. Subject enrollment

This retrospective cohort study was approved by the Institutional Review Board of National Cheng Kung University Hospital (NCKUH) and Keelung Chang Gung Memorial Hospital. Informed consent was obtained from each participant. Fig. 1 shows the inclusion and exclusion criteria for this study. Patients who had HBV surface antigen (HBsAg) for more than 6 months were enrolled from outpatient clinics of both hospitals. HBV DNA was >20,000 IU/ml and >2000 IU/ml in HBV e antigen (HBeAg)-positive and HBeAg-negative patients, respectively. All patients were treatment-naive and received entecavir treatment with regular follow-ups for at least 48 weeks. Serum samples from patients before and after 48 weeks of treatment were collected and stored at −80 °C until use. Healthy controls (n = 101) with normal serum alanine aminotransferase (ALT) level and undetectable HBsAg, were enrolled from the Health Examination Center of NCKUH and were matched for age and gender with CHB patients.

2.2. Biochemical and virological tests

Levels of serum ALT, aspartate aminotransferase (AST), albumin, and total bilirubin were determined using a modular analytics EVO analyzer (Modular DP; Roche Diagnostics). Serum HBV DNA levels were assayed using COBAS Amplicor/COBAS TaqMan HBV Test (Roche Diagnostics, Indianapolis, IN).

2.3. In-gel trypsin digestion of IgG heavy chain

Serum IgG was purified using Protein G 4 Fast Flow Sepharose beads (GE Healthcare, Piscataway, NJ) and resolved using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The protein spot located between 50 and 55 kDa was excised from polyacrylamide gels. Gel slices were destained using destaining buffer (10 mM ammonium bicarbonate, 50% acetonitrile) with a subsequent dehydration using 100% acetonitrile. Peptides in the gel were then digested with 20 ng/μl trypsin (Promega, Madison, WI) in 10 mM ammonium bicarbonate at 37 °C overnight. Tryptic peptides were acidified by 1% trifluoroacetic acid, extracted by vigorous vortexing, and stored at −80 °C.

Table 1
Characteristics of patients with chronic hepatitis B (CHB) before and after 48 weeks of entecavir treatment and healthy controls (HC).

Variable	CHB (*n* = 103)		HC (*n* = 101)	*P*-value[1]	*P*-value[2]
	Baseline	Week 48			
Gender (M:F)	69:34		67:34	0.999[a]	NA
Age (years)	49.3 (11.7)		46.5 (9.2)	0.060	NA
ALT (U/L)	153.6 (172.7)	32.6 (16.5)	21.2 (7.8)	**0.000**	**0.000**
AST (U/L)	88.4 (78.6)	30.5 (11.6)	22.4 (4.3)	**0.000**	**0.000**
Albumin (g/dL)	4.2 (0.5)	4.5 (0.4)	4.7 (0.3)	**0.000**	**0.000**
Total bilirubin (mg/dL)	1.1 (1.0)	0.9 (0.4)	0.8 (0.4)	**0.014**	0.104
Total IgG (mg/mL)	14.5 (5.9)	13.1 (5.7)	9.7 (3.3)	**0.000**	**0.039**
HBV DNA (Log_{10} IU/mL)	6.5 (1.8)	1.0 (1.4)	NA	NA	**0.000**
HBeAg (+:−)	30:73	22:81	NA	NA	0.262[a]

All data are mean (standard deviation); NA, not available.
Bold values indicates the statistical significance of *P*-values
[1] *P*-values for comparisons between CHB baseline and HC are from two-tailed independent *t* tests.
[2] *P*-values for comparisons between CHB baseline and CHB week 48 are from two-tailed paired *t* tests.
[a] *P*-values are from Chi-square tests.