

Association of serum soluble human leukocyte antigen-G levels with chronic hepatitis B virus infection

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Abstract Human leukocyte antigen-G is involved in immunotolerogenic, inflammatory and carcinogenic process. This study investigated serum soluble HLA-G (sHLA-G) levels in patients with chronic hepatitis B virus (HBV) infection according to the infection phases and clinical diagnoses. The study included 223 patients with chronic HBV infection [phases: 38 immune-tolerant (IT), 83 immune clearance (IC), 30 non/low-replicative (LR) and 72 HBeAg negative hepatitis (ENH); diagnoses: 38 asymptomatic

HBV carriers (ASC), 98 chronic hepatitis (CH), 46 cirrhosis (LC) and 41 hepatocellular carcinoma (HCC)], 62 HBV infection resolvers and 66 healthy controls. The sHLA-G levels in patients were elevated compared with resolvers and healthy controls ($P < 0.001$). According to phases, sHLA-G levels were higher in IC and ENH than in IT ($P = 0.017$ and $P = 0.001$, respectively). Serum sHLA-G levels were also higher in ENH than in LR ($P = 0.008$). According to diagnoses, sHLA-G levels in HCC were significantly increased compared with LC, CH and ASC ($P = 0.010$, $P < 0.001$ and $P < 0.001$, respectively). Serum sHLA-G levels were higher in CH than in ASC ($P = 0.039$). The sHLA-G levels in IC, ENH and CH were correlated with alanine aminotransferase levels ($P = 0.011$, $P = 0.010$ and $P < 0.001$, respectively). It is concluded that sHLA-G is involved in the pathogenesis of chronic HBV infection and correlates with infection phases and clinical diseases, suggesting the value in evaluating disease activity and defining clinical diagnosis.

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Introduction

Hepatitis B virus (HBV) infection is a significant cause of a variety of clinical liver diseases such as acute and chronic hepatitis (CH), cirrhosis and hepatocellular carcinoma (HCC). Chronic HBV infection is a dynamic process of complex interactions between the virus and the host's immune response, leading to various disease condition and progression [1, 2]. Typically, the natural course of chronic HBV infection includes several phases: an immune-tolerant (IT) phase, an immune clearance (IC) phase and an

inactive or non/low-replicative (LR) phase [3, 4]. In addition, active hepatitis, referred as HBeAg negative hepatitis (ENH), may sometimes reoccur in individuals already in an inactive phase [3, 5–8]. Intricately, these phases may or may not necessarily and sequentially occur in an infected individual [9]. Furthermore, HBV infection may spontaneously resolve and manifest seroclearance of HBeAg, HBV DNA and HBsAg and seroconversion of anti-HBs in variously low percentages depending on many parameters from the host and the virus [10, 11]. The phases of chronic HBV infection are characterized by different levels of immunoregulatory and inflammatory responses of the hosts to various degrees of the HBV viral replication, in which natural killer (NK) cells [12–14], CD4+ and CD8+ T-lymphocytes [15, 16], dendritic cells [17] and regulatory T cells [18, 19] associated with innate and adaptive immune responses are all involved.

Human leukocyte antigen-G (HLA-G) is a non-classical class I major histocompatibility complex molecule. It was firstly found to be predominantly expressed on the surface of cytotrophoblast cells and is believed to function in establishing the tolerogenic microenvironment at the fetal–maternal interface through inhibiting natural killer cell-mediated lysis and influencing cytokine expression [20, 21]. Subsequent research demonstrated that HLA-G antigens can regulate NK and CD8+ T cell cytotoxicity, modulate the functions of CD4+ T-lymphocytes and dendritic cells and induce regulatory T cells, playing a tolerogenic role in innate and adaptive responses [22–24]. The expression of HLA-G molecules has been associated with a variety of disorders such as septic shock, autoimmune diseases, viral infections, tumors and organ transplantations [25–29], linking HLA-G molecules with the establishment of anti-inflammatory environments.

Alternative splicing of the primary HLA-G transcripts generates membrane-bound and soluble protein isoforms: HLA-G1 to HLA-G4 as membrane-bound and HLA-G5 to HLA-G7 as soluble molecules [30]. Like the cell-surface isoforms, soluble HLA-G (sHLA-G) can also inhibit NK cells and cytotoxic T-lymphocytes [31–33] and suppress CD4+ and CD8+ T cell alloproliferation [34]. Following binding to the CD158d (KIR2DL4) receptor on the surface of human resting NK cells and being then endocytosed, sHLA-G can trigger the expression of a set of chemokines and cytokines, inducing a proinflammatory and proangiogenic response [35].

Because chronic HBV infection is characterized by immunoregulatory and inflammatory responses and associates with the occurrence of HCC, and HLA-G is involved in immunosuppressive, inflammatory and carcinogenic process, the aim of this study was to evaluate serum sHLA-G levels in various phases and clinical diseases of patients with persistent HBV infection. For controls, we also

analyzed sHLA-G levels in individuals resolved from HBV infection and in healthy controls without HBV infection.

Patients and methods

Patients and controls

The study was performed in patients with chronic HBV infection from the First Affiliated Hospital, School of Medicine, Xi'an Jiaotong University, a tertiary hospital in the Northwest China. All patients were negative for markers of hepatitis C virus (HCV) and human immunodeficiency virus (HIV). Coexistence of autoimmune, alcoholic or metabolic liver disease was excluded. Chronic patients who had been never treated with nucleos(t)ide analogs or interferon (IFN)- α were eligible for inclusion.

Resolvers from HBV infection were those who had normal liver biochemistries and were seropositive for anti-HBs and anti-HBc. Healthy controls were those who had normal liver biochemistries, had no history of hepatitis B, and were seropositive for anti-HBs only or seronegative for HBV markers.

Patients were classified according to the phase of chronic HBV infection as IT, immune clearance or immune reactive (IC), LR and ENH and to the clinical disease as asymptomatic HBV carrier status (ASC), CH, liver cirrhosis (LC) and HCC.

The phase of chronic HBV infection in each patient was determined by HBsAg and anti-HBc positivity, HBeAg/anti-HBe serostatus, and serum HBV DNA and alanine aminotransferase (ALT) levels [4]. The IT phase was defined as: HBsAg and anti-HBc positive, HBeAg positive, high viral load, serum ALT $< 2 \times$ upper limit of normal (ULN). The IC phase was defined as: HBsAg and anti-HBc positive, HBeAg positive, moderate to high level of viral load, serum ALT $\geq 2 \times$ ULN. The LR phase was defined as: HBsAg and anti-HBc positive, HBeAg negative, HBV DNA < 2000 IU/ml, normal serum ALT. The ENH was defined as: HBsAg and anti-HBc positive, HBeAg negative, HBV DNA > 2000 IU/ml, serum ALT $\geq 2 \times$ ULN or fluctuation.

The clinical diagnosis of chronic HBV infection in each patient was determined by history of HBV infection, HBsAg/anti-HBs, HBeAg/anti-HBe and anti-HBc serostatus, measurement of HBV DNA, biochemical liver function, α -fetoprotein (AFP) levels, and ultrasonography and/or computerized tomography (CT)/magnetic resonance imaging (MRI) as described previously [36, 37]. The ASC was defined as: HBsAg, HBeAg and anti-HBc positive, high viral load, normal serum ALT level. The CH was defined as: HBsAg and anti-HBc positive, HBeAg and anti-HBe positive or negative, moderate to high level of HBV

DNA, ALT $\geq 2 \times$ ULN or fluctuation, no evidence of LC and HCC. The LC was defined as: HBsAg and anti-HBc positive, HBeAg and anti-HBe positive or negative, detectable HBV DNA, ALT elevation or not, evidence of LC on liver biopsy and/or on ultrasonography and/or CT/MRI and gastroesophageal varices by endoscopy, no evidence of HCC. The HCC was defined as: HBsAg and anti-HBc positive, HBeAg and anti-HBe positive or negative, detectable HBV DNA, ALT elevation or not, evidence of HCC on liver biopsy and/or ultrasonography and/or CT/MRI with or without elevated AFP and gastroesophageal varices by endoscopy. The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Ethics Committee.

Determination of serum sHLA-G levels, HBV markers and liver biochemistry

Serum sHLA-G levels were determined using commercially available Human sHLA-G ELISA kit (Exbio/BioVendor, Praha, Czech Republic) according to the manufacturer's instructions. HBV serologic markers including HBsAg, anti-HBs, HBeAg, anti-HBe and anti-HBc were performed using ELISA from Beijing Wantai Biological Pharmacy (Beijing, China). Biochemical liver function including ALT and aspartate aminotransferase (AST) levels (IU/l) was assayed on the Olympus AU5400 automatic biochemical analyzer (Olympus Corporation, Mishima, Japan). Serum HBV DNA levels (IU/ml) were quantitatively determined using HBV fluorescence polymerase chain reaction diagnostic kit manufactured by Da An Gene Co., Ltd. of Sun Yat-Sen University (Guangzhou, China) according to the instruction. Serum AFP levels (ng/ml) were measured using automated Eleceyes (Roche Diagnostics, Mannheim, Germany).

Statistical analysis

Data were expressed as the mean \pm SD or median (range). Statistical analysis was performed by SPSS software version 16.0 (SPSS, Inc., Chicago, IL). Continuous and categorical variables were compared between groups, using the Mann–Whitney test or Kruskal–Wallis rank-sum test where appropriate. Correlations between variables were assessed using Spearman's correlation coefficients. A P value < 0.05 was considered statistically significant.

Results

Two hundred and twenty-three treatment naïve patients with chronic HBV infection were recruited. Sixty-two HBV infection resolvers and 66 healthy individuals were

recruited as controls. The demographics of the study subjects and the phases and clinical diagnoses of chronic HBV infection in the patients are presented in Table 1. All subjects were of Chinese Han ethnicity. The gender and age between HBV patients, HBV infection resolvers and healthy controls had no statistical difference (Table 1).

Serum sHLA-G levels in HBV patients were significantly elevated compared with HBV infection resolvers ($P < 0.001$) and healthy controls ($P < 0.001$, Table 1). Serum sHLA-G levels between HBV infection resolvers and healthy controls had no significant difference ($P = 0.907$, Table 1).

Serum sHLA-G levels according to the phases of chronic HBV infection

The classifications of patients' phases were as follows: IT ($n = 38$), IC ($n = 83$), LR ($n = 30$) and ENH ($n = 72$) (Table 2). The demographics and serum sHLA-G levels in different phases are shown in Table 2. The distribution of gender among the IT, IC, LR and ENH had no significant difference. The patients in IT were younger than those in IC, LR and ENH ($P < 0.001$). The HBV DNA, ALT, AST, total bilirubin and albumin levels were significantly different between the patients in different phases. Serum HBV DNA levels were higher in IT and IC than in LR and ENH, serum ALT, AST and total bilirubin levels were higher in IC and ENH than in IT and LR, and albumin levels were lower in IC and ENH than in IT and LR (Table 2).

The serum sHLA-G levels in IC and ENH were higher than those in IT ($P = 0.017$ and $P = 0.001$, respectively, Fig. 1). Serum sHLA-G levels in ENH were also higher than those in LR ($P = 0.008$, Fig. 1).

Serum sHLA-G levels according to clinical diseases of chronic HBV infection

The clinical diagnoses of the patients were as follows: ASC ($n = 38$), CH ($n = 98$), LC ($n = 46$) and HCC ($n = 41$) (Table 3). The demographics and serum sHLA-G levels in different diagnoses of chronic HBV infection are shown in Table 3. The distribution of gender among the ASC, CH, LC and HCC had no significant difference. The HBV DNA, ALT, AST, total bilirubin and albumin levels were significantly different between the patients with different diagnoses. HBV DNA levels were higher in ASC patients than in CH patients and lower in HCC patients than in LC patients ($P < 0.001$). The ALT and AST levels in CH patients were higher than those in ASC, LC and HCC patients ($P < 0.001$). The total bilirubin levels in CH, LC and HCC patients were higher than those in ASC patients ($P < 0.001$). Serum albumin levels in LC and HCC

Table 1 Demographics and serum sHLA-G levels in HBV patients, HBV infection resolvers and healthy controls, and infection phases and clinical diagnoses in HBV patients

	HBV patients (<i>n</i> = 223)	HBV infection resolvers (<i>n</i> = 62)	Healthy controls (<i>n</i> = 66)	<i>P</i>
Gender (M/F)	148/75	42/20	43/23	0.953
Age [years, mean ± SD (range)]	38.47 ± 14.00 (17–74)	39.27 ± 13.35 (18–72)	39.30 ± 8.11 (21–54)	0.849
sHLA-G [U/ml, median (range)]	51.56 (6.56–542.78)*	19.80 (2.11–348.26)**	19.43 (2.34–385.26)	<0.0001
Infection phases (<i>n</i>)				
IT	38			
IC	83			
LR	30			
ENH	72			
Clinical diagnoses (<i>n</i>)				
ASC	38			
CH	98			
LC	46			
HCC	41			

IT immune-tolerant phase, IC immune clearance phase, LR non/low-replicative phase, ENH HBeAg negative hepatitis, ASC chronic asymptomatic HBV carrier, CH chronic hepatitis, LC liver cirrhosis, HCC hepatocellular carcinoma

* *P* < 0.001, compared with HBV infection resolvers or healthy controls

** *P* = 0.907, compared with healthy controls

Table 2 Demographics and serum sHLA-G levels in different phases of chronic HBV infection

	IT (<i>n</i> = 38)	IC (<i>n</i> = 83)	LR (<i>n</i> = 30)	ENH (<i>n</i> = 72)	<i>P</i>
Gender (M/F)	23/15	60/23	17/13	48/24	0.373
Age [years, mean ± SD (range)]	26.08 ± 9.06 (17–61)	41.11 ± 13.59 (18–74)	41.37 ± 15.78 (18–73)	40.75 ± 12.49 (18–74)	<0.001
Clinical diagnosis (ASC/CH/LC/HCC)	38/0/0/0	0/41/26/16	0/21/5/4	0/36/15/21	<0.001
HBV DNA (IU/ml, log)	7.44 ± 1.03	6.54 ± 1.19	3.22 ± 0.59	4.45 ± 1.27	<0.001
ALT (IU/l)	26 (12–38)	113 (13–1588)	30.5 (2–60)	156 (14–2729)	<0.001
AST (IU/l)	22.5 (11–92)	99 (17–1612)	32.5 (13–52)	87 (17–2136)	<0.001
Tbil (μmol/l)	13.40 (3–22)	35.38 (2–655)	15.7 (5.5–61)	31.31 (8–506)	<0.001
Albumin (g/l)	41 (36.9–50)	33.6 (14.2–48.7)	40.75 (29.9–50.8)	34.95 (18.7–47.1)	<0.001
sHLA-G [U/ml, median (range)]	29.53 (7.27–388.57)	51.56 (6.56–523.56)	44.88 (7.5–215.61)	71.48 (8.44–542.78)	0.003

IT immune-tolerant phase, IC immune clearance phase, LR non/low-replicative phase, ENH HBeAg negative hepatitis, ASC chronic asymptomatic HBV carrier, CH chronic hepatitis, LC liver cirrhosis, HCC hepatocellular carcinoma, ALT alanine aminotransferase, AST aspartate aminotransferase, HBV hepatitis B virus, Tbil total bilirubin

patients were lower than those in ASC and CH patients (*P* < 0.001).

The serum sHLA-G levels in HCC patients were higher than those in LC (*P* = 0.010), CH (*P* < 0.001) and ASC patients (*P* < 0.001, Fig. 2). Serum sHLA-G levels in CH were also higher than those in ASC (*P* = 0.039, Fig. 2).

Correlations between serum sHLA-G and ALT as well as AFP levels

When all the HBV patients were analyzed, sHLA-G levels were significantly correlated with serum ALT levels (*r* = 0.146, *P* = 0.029).

When the HBV patients were analyzed according to infection phases, sHLA-G levels were significantly correlated with serum ALT levels in IC and ENH patients (*P* = 0.011 and *P* = 0.010, respectively, Fig. 3). The sHLA-G levels in IT and LR patients had no significant correlation with serum ALT levels (Fig. 3).

When the correlation between ALT and serum sHLA-G levels was analyzed according to clinical diagnoses, sHLA-G levels were significantly correlated with serum ALT levels in CH patients (*P* < 0.001, Fig. 4). The sHLA-G levels in ASC, LC and HCC patients had no significant correlation with serum ALT levels (Fig. 4).

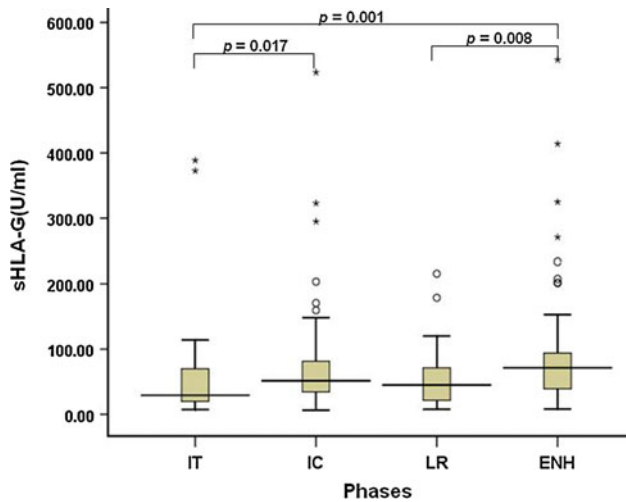


Fig. 1 Serum sHLA-G levels according to phases of chronic HBV infection. *IT* immune-tolerant phase, *IC* immune clearance phase, *LR* non/low-replicative phase, *ENH* HBeAg negative hepatitis

In HCC patients, sHLA-G levels had no significant correlation with AFP levels ($r = 0.264$, $P = 0.096$).

Discussion

This study demonstrated that serum sHLA-G levels were elevated in chronic HBV infection and differed according to infection phases and clinical diseases. HLA-G has anti-inflammatory and immunosuppressive effects by inhibiting CD8+ T cell toxicity [38], CD4+ T cell alloreactivity [39] and NK cell-mediated cytotoxicity [21], and activating regulatory CD4+ T cells [40]. It is suggested that sHLA-G is involved in the immuno-inflammatory process of chronic HBV infection and the elevation of sHLA-G in chronic HBV infection is the consequence of the break of immune-tolerance status and the activation of immune response and inflammatory progress. In accord with these results, studies have shown that sHLA-G was markedly elevated in inflammatory and autoimmune diseases such as septic

shock [25], HIV [28] and HCV infection [27], and systemic lupus erythematosus [26].

Recent studies showed that soluble and membrane-bound HLA-G expression can be observed on hepatocytes and biliary epithelial cells of patients with chronic HBV infection [41] and sHLA-G levels in HBV infection were much higher than those in normal controls [42, 43]. However, these studies did not correlate the sHLA-G levels with the infection phases. We carefully classified our patients according to current definition of the phases of chronic HBV infection. We showed significant correlation between serum sHLA-G and ALT levels in the active phases of chronic HBV infection (IC and ENH) and the diagnosis of CH. This correlation may have relevance to clinical practice. Currently, ALT level is one of the major parameters for evaluating disease activity and predicting treatment response in persistent HBV infection [44, 45]. Taking into account of the close correlation with disease phase and activity, it is plausible to foresee that sHLA-G may be used as another parameter for evaluating disease activity and predicting treatment response in HBV infection.

There has been increasing evidence showing the involvement of HLA-G in carcinogenesis [46]. In terms of HBV-associated HCC, the expression of HLA-G has been found to be a characteristic feature and correlate to disease progression and prognosis [24, 47, 48]. Serum levels of sHLA-G in the HCC were shown to be significantly increased [24, 43, 47]. In support of this, we showed that sHLA-G levels in HCC patients were the highest across all the clinical diagnoses of chronic HBV infection including LC, CH and ASC, suggesting the involvement of sHLA-G in tumor immune escape and the potential usefulness of its determination in diagnosing HCC. However, in accord with previous study [43], serum sHLA-G levels were not found to be associated with AFP levels in our study. The explanations for the lack of correlation between sHLA-G and AFP levels are uncertain. However, previous studies showed that the expressions of HLA-G were differently up-regulated in HCC tissues [49] and the HLA-G

Table 3 Demographics and serum sHLA-G levels in different clinical diagnoses of chronic HBV infection

	ASC (n = 38)	CH (n = 98)	LC (n = 46)	HCC (n = 41)	P
Gender (M/F)	23/15	63/35	29/17	33/8	0.200
Age [years, mean ± SD (range)]	26.08 ± 9.06 (17–61)	34.55 ± 10.84 (18–66)	44.98 ± 12.82 (19–72)	52.00 ± 11.07 (32–74)	<0.001
HBV DNA (IU/ml, log)	7.44 ± 1.03	5.19 ± 1.84	5.48 ± 1.51	4.86 ± 1.66	<0.001
ALT (IU/l)	26 (12–38)	186 (11–2729)	64 (2–836)	53 (10–603)	<0.001
AST (IU/l)	22.5 (11–92)	91 (13–2136)	58.5 (19–667)	71 (17–1348)	<0.001
Tbil (µmol/l)	13.40 (3.1–21.7)	25.4 (2–524.46)	34.59 (6.5–302.6)	30.07 (7.6–655)	<0.001
Albumin (g/l)	41 (36.9–50)	37.1 (23.1–50.8)	30.6 (14.23–50.07)	32.4 (22.6–41.7)	<0.001
sHLA-G [U/ml, median (range)]	29.53 (7.27–388.57)	46.17 (7.27–542.78)	56.6 (6.56–201.55)	75.46 (8.44–413.88)	<0.001

ASC chronic asymptomatic HBV carrier, CH chronic hepatitis, LC liver cirrhosis, HCC hepatocellular carcinoma, ALT alanine aminotransferase, AST aspartate aminotransferase, HBV hepatitis B virus, Tbil total bilirubin

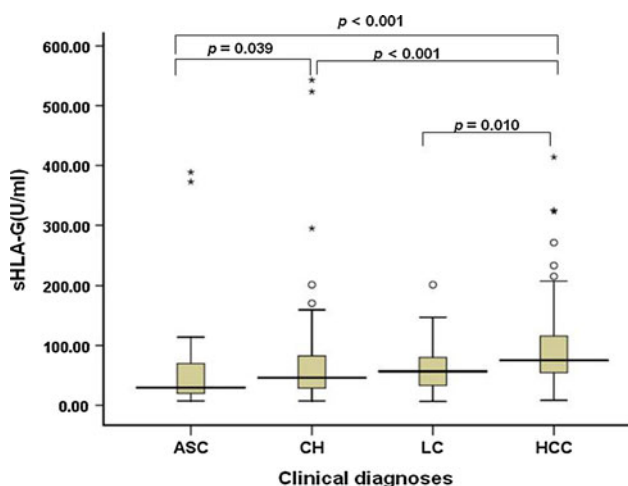


Fig. 2 Serum sHLA-G levels according to clinical diagnoses of chronic HBV infection. *ASC* chronic asymptomatic HBV carrier, *CH* chronic hepatitis, *LC* liver cirrhosis, *HCC* hepatocellular carcinoma

expression in tumors had no significant correlation with AFP level [47]. Therefore, sHLA-G elevation may reflect a major but not all of the characteristics of HCC and may be used as a complementary marker to AFP in presupposing the diagnosis of HCC.

The mechanisms of sHLA-G alteration and the causal relationship between this alteration and the disease phases and activities in chronic HBV infection are largely unclear. However, multiple mechanisms including activation of immune response, reaction of inflammatory process and carcinogenesis may be differently involved in various phases or disease conditions of the infection. In patients in phases of IC and ENH and with diagnosis of CH, the activations of immune response and inflammatory process are probably the major reasons for sHLA-G elevation. In addition to immuno-inflammatory response, the carcinogenesis of HCC may synergistically induce the elevation of sHLA-G, contributing to the striking elevation of sHLA-G in HCC patients. Understanding the changing serum sHLA-G levels across the phases and disease conditions of chronic HBV infection may represent a step forward in not only investigating the influence of the host immune response but also designing immunotherapeutic strategy against chronic HBV infection as well as HCC [46].

Our study has some limitations. One limitation is its cross-sectional design, as it would have been more useful to follow patients longitudinally through different phases of chronic HBV infection. Another limitation is the lack of

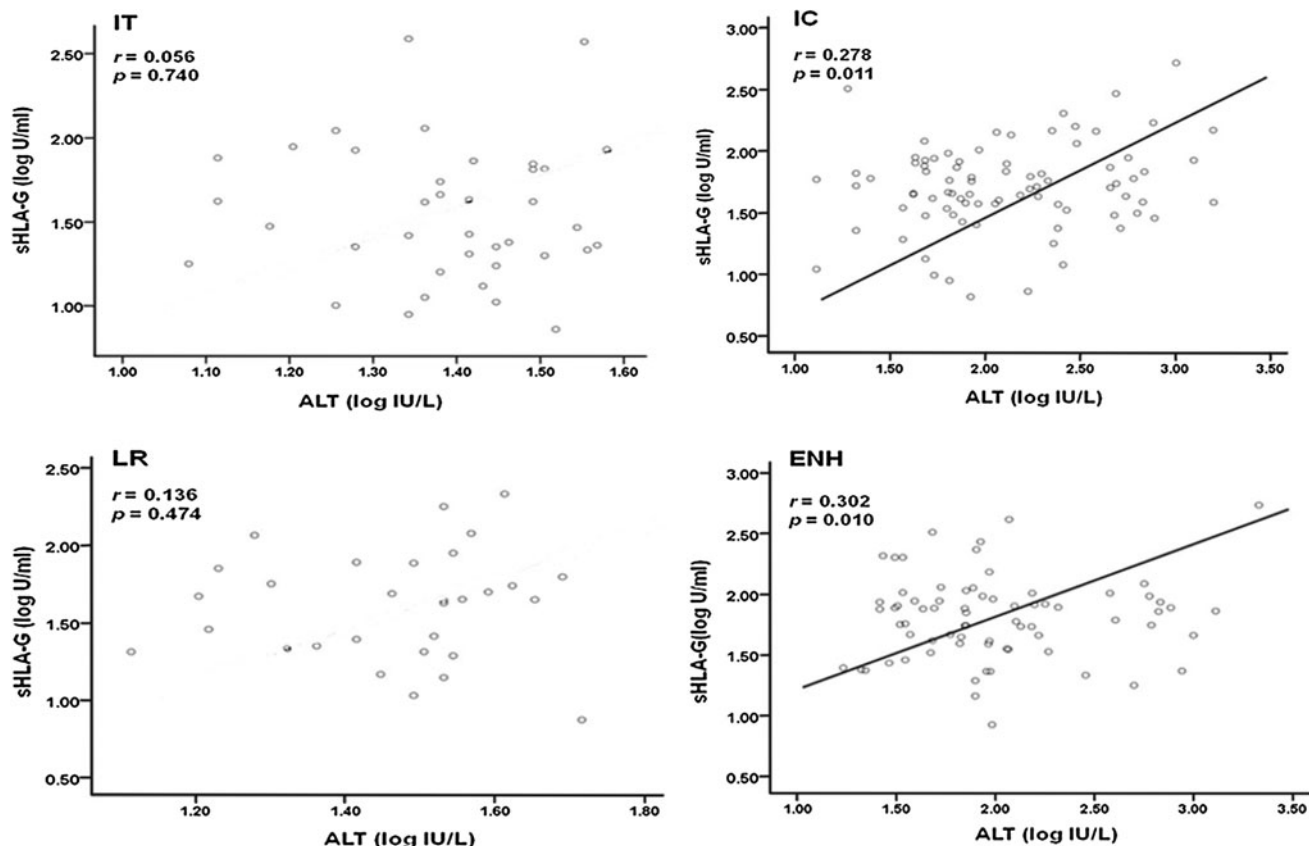


Fig. 3 Correlations between serum sHLA-G and ALT levels according to phases of chronic HBV infection. *IT* immune-tolerant phase, *IC* immune clearance phase, *LR* non/low-replicative phase, *ENH* HBeAg negative hepatitis

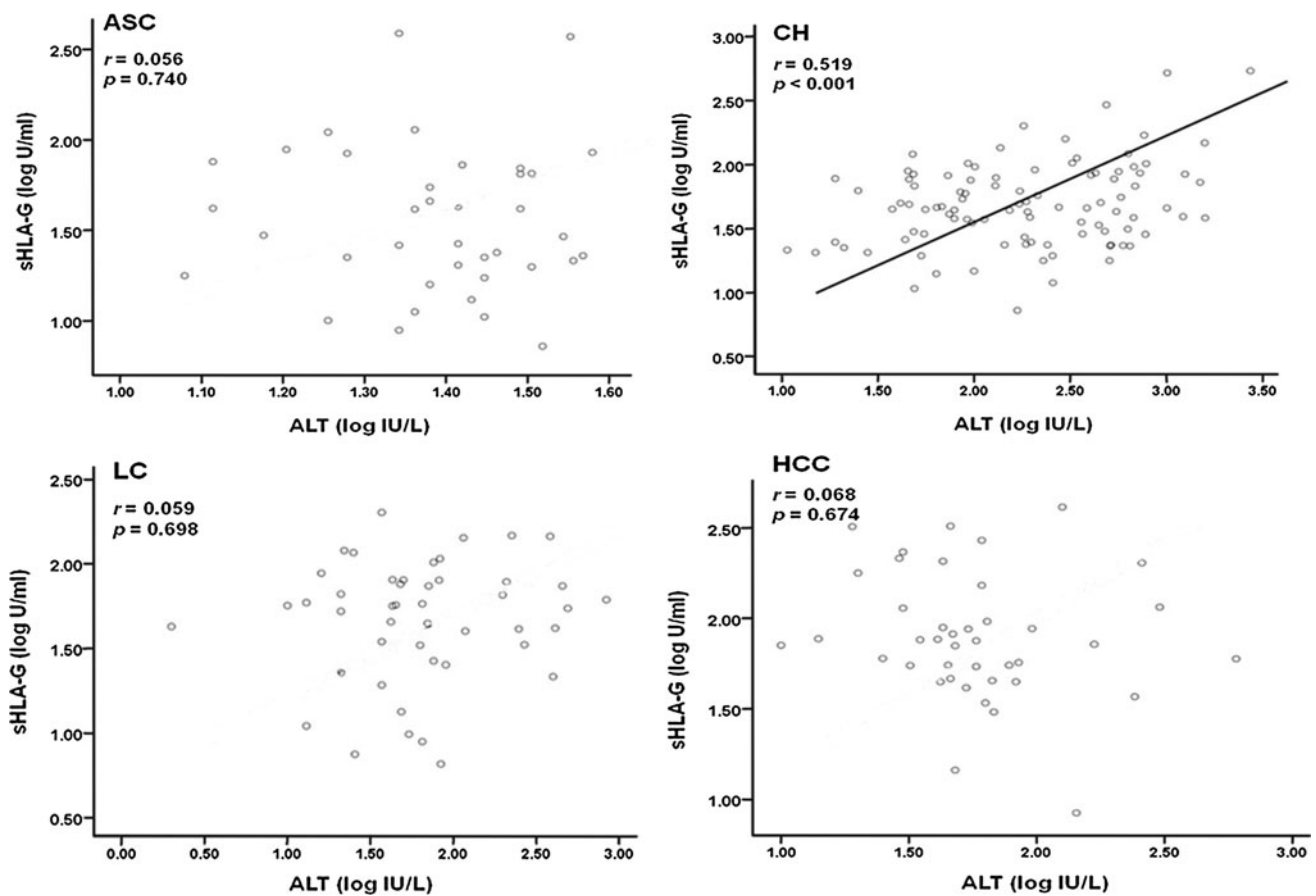


Fig. 4 Correlations between serum sHLA-G and ALT levels according to clinical diagnoses of chronic HBV infection. *ASC* chronic asymptomatic HBV carrier, *CH* chronic hepatitis, *LC* liver cirrhosis, *HCC* hepatocellular carcinoma

dynamic evaluation of the serum sHLA-G levels in relation to treatment response given the implication of this parameter to the immune and inflammatory status of the infected individuals. Larger prospective studies are required to evaluate longitudinal changes in serum sHLA-G levels and the potentials in predicting and monitoring the treatment response of both immune-modulator therapy such as IFN- α and antiviral therapy such as oral nucleos(t)ide analogs.

In conclusion, this study demonstrates significant differences in the serum sHLA-G levels across the different phases and clinical diseases of chronic HBV infection. Since sHLA-G may participate in the immuno-inflammatory process and carcinogenesis of HCC in chronic HBV infection, the levels of sHLA-G are closely correlated with infection phase and disease activity and strikingly elevated in HCC, and quantitation of serum sHLA-G levels is non-invasive, easy to perform and relatively inexpensive, it is suggested that determination of serum sHLA-G levels may be used as an immuno-inflammatory and carcinogenic seromarker to evaluate the disease activity and complementarily diagnose HCC in chronic HBV infection. The role of sHLA-G in predicting disease prognosis, refining

treatment scheme and monitoring treatment response needs to be investigated.

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Conflict of interest No conflict of interest to declare.

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