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Fully galactosyl-fucosyl-bisected IgG_1 reduces anti-HBV efficacy and liver histological improvement

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ABSTRACT

N-glycosylation on the crystallizable fragment (Fc) governs antibody-mediated immune responses. This study addressed the relevance of *N*-acetylglucosamine (GlcNAc)-bisected IgG_1 on the disease progression and treatment efficacy in the immune active phase of chronic hepatitis B virus (HBV) infection. Serum IgG_1 *N*-glycan patterns from 166 HBV e antigen (HBeAg)-positive patients were analyzed using liquid chromatography-tandem mass spectrometry. The proportion of GlcNAc-bisected IgG_1 on the disease severity and efficacy of nucleos(t)ide analogue treatment were investigated. Cytokine-dependent regulations of IgG_1 GlcNAc bisection were also addressed using mouse IgG_1-producing hybridoma cells. We found that IgG_1 bearing a fully galactosyl-fucosyl-*N*-acetylglucosamine-bisected (G2FN) glycoform in HBeAg-positive patients was associated with high levels of HBV DNA or HBV surface antigen, alanine aminotransferase < 2 upper limits of normal, and a mild liver injury. Moreover, baseline IgG_1-G2FN ≧ 1.5% was linked to lower probabilities of virological response (HBV DNA undetectable in serum), HBeAg seroconversion, HBV core antigen loss, and liver histological improvement after treatment. Cox and logistic regression analyses revealed that IgG_1-G2FN was an unfavorable factor for the virological response (hazard ratio = 0.620, 95% confidence interval = 0.466–0.825, $P = 0.001$) or liver histological improvement (odds ratio = 0.513, 95% confidence interval = 0.279–0.943, $P = 0.032$), respectively. Results from in vitro studies showed that transforming growth factor (TGF)-β1 treatment downregulated mannosyl β-1,4-*N*-acetylglucosaminyltransferase 3 and β-1,4-galactosyltransferase 1 activities and thereby IgG_1-G2FN production, and this phenomenon reflected an inverse correlation between IgG_1-G2FN and TGF-β1 in sera of patients ($r = -0.431$, $P < 0.001$). In conclusion, IgG_1-G2FN was related to an attenuated liver inflammation and unfavorable treatment responses in patients with HBeAg-positive chronic hepatitis B.

1. Introduction

Chronic hepatitis B (CHB) is a worldwide threat and a high risk of the development of liver cirrhosis or hepatocellular carcinoma. Oral nucleos(t)ide analogue (NA)-related therapies that suppress viral genome replication with few adverse effects have revolutionized the management of CHB in the past decade. However, this regimen is unable to eliminate intranuclear hepatitis B virus (HBV) covalently closed circular DNA in infected hepatocytes. Therefore a long-term, even lifelong, administration of NA is indispensable for patients with CHB. Early virological and serological responses lead to a satisfactory medication outcome, a high probability of treatment discontinuation, and a low incidence of end-stage liver failure (Chen et al., 2014; Hsu et al., 2002; Kim et al., 2013; Lee et al., 2011; Shin et al., 2012; Terrault et al., 2016). In addition to antiviral agents, a solid immunity in hosts substantially improves the treatment response by inhibiting virus propagation and preventing the deterioration of liver injury. Nevertheless, the efficiency of humoral immunity on HBV clearance upon chronic infection remains controversial. Until recently B cell and antibody hyper-activities have been demonstrated in HBeAg-positive immune-active phase of CHB (Vanwolleghem et al., 2015), which attracts our great interest to further examine whether circulating antibodies with

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different post-translational modification influence CHB progression and prognosis.

Immunoglobulin G (IgG) is the major component of the antibody pool. It contains a conserved *N*-linked glycosylation on the crystallizable fragment (Fc). The composition of *N*-glycans determines the steric structure of Fc portion and modulates binding affinities of IgG to different Fc gamma receptors (FcγRs), thereby regulating effector functions and properties of immune cells (Nimmerjahn and Ravetch, 2008). The heterogeneity of IgG *N*-glycans comes from the editing of sugar nucleotides, such as galactose, fucose, sialic acid, and bisecting *N*-acetylglucosamine (GlcNAc), on the pentasaccharide (two GlcNAcs and three mannoses) core. The proinflammatory potency of glycan-aberrant IgG has been implicated in the progression of various autoimmune and infectious diseases. However, most reports mentioning this issue are cross-sectional assays and focus mainly on galactosylation, core fucosylation, or sialylation rather than GlcNAc bisection. Reported herein is a clinical glycoproteomic study showing the relevance of GlcNAc-bisected IgG in not only the disease severity but also treatment efficacy of HBeAg-positive CHB. Furthermore, the regulation of IgG GlcNAc bisection was assessed to better understand the mechanical underpinnings of aberrant IgG *N*-glycosylation upon CHB.

2. Materials and methods

2.1. Study design and patients

This study was approved by the Institutional Review Board of National Cheng Kung University Hospital (No B-ER-102-445) and conducted in accord with the guidelines of the Declaration of Helsinki. Informed consent was obtained from each participant. One hundred and sixty-six patients with HBeAg-positive CHB, who had HBV surface antigen (HBsAg) for more than 6 months and baseline serum HBV DNA greater than 20,000 IU/mL, were retrospectively enrolled if there were no concomitant hepatitis C virus infection, alcoholism- or autoimmune-induced liver diseases, gastrointestinal cancers, and any disorders that have been reported to affect serum IgG glycosylation (Huhn et al., 2009). All patients had received nucleos(t)ide analogue monotherapy and had regular follow-ups for at least 48 weeks. Sixty-three patients have been described previously (Ho et al., 2014, 2015).

2.2. Virological, biochemical, and serological tests

Serum HBV DNA and HBsAg levels were quantified using COBAS Amplicor/COBAS TaqMan HBV Test (Roche Diagnostics, Indianapolis, IN) and the ARCHITECT HBsAg assay (Abbott Diagnostics, Sligo, Ireland), respectively. Virological response is defined as an undetectable HBV DNA (< 20 IU/ml) in serum. HBV genotype was determined in 150 patients using a real-time polymerase chain reaction-based analysis or next generation sequencing as previously described (Liu et al., 2006, 2016). Levels of human transforming growth factor (TGF)-β1 in sera of the patients were measured using Ready-Set-Go ELISA kits (eBioscience, San Diego, CA).

2.3. Liver histology

Liver biopsy was performed in 107 patients at baseline and 95 of them after 48 weeks of treatment. Liver necroinflammation and fibrosis stages were evaluated according to Knodell histology activity index and Ishak fibrosis score, respectively, by a single hepatopathologist who was delinked to clinical data of the patients. A liver histological improvement was characterized as a decrease in the fibrosis score or a decline in total necroinflammation score ≧2 without an exacerbation of liver fibrosis.

2.4. Immunohistochemistry

Immunohistochemistry for detecting HBV core antigen (HBcAg) at baseline and week 48 was executed in 68 patients. Formalin-fixed, paraffin-embedded tissue sections were de-paraffinized, rehydrated, and heated in 10 mM Sodium (pH 6.0) for antigen retrieval. Hydrogen peroxide (3% in methanol) and blocking reagent were used to minimize endogenous peroxidase activity and non-specific signal. Tissue sections were blocked using 5% bovine serum albumin and then incubated with mouse anti-HBcAg monoclonal antibody (Leica Biosystems, Nussloch, Germany) at 4 °C overnight and horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody at room temperature for 1 h. All slides were stained with 3-amino-9-ethylcarbazole substrate and counterstained with hematoxylin.

2.5. Glyco-profiling of serum IgG-Fc

Purification and analysis of IgG glycosylation pattern using liquid chromatography–tandem mass spectrometry have been previously reported (Ho et al., 2014, 2015). The liquid chromatography system was equipped with a C18 pre-column and a C18 nano-column (CVC Technologies, Fontana, CA) and was coupled online to a mass spectrometer (LTQ-Orbitrap XL; Thermo Fisher Scientific). A survey MS spectrum (m/z 300–2000) with a mass resolution of 60,000 at m/z 400 (with an ion target value of 5×10^5 ions) was acquired, followed by 5 sequential collision-induced dissociation-MS^2 scans using the mass spectrometer in a data-dependent mode. The percentage of each glycoform that was attached to the peptide EEQYNSTYR of IgG_1 was taken from the average of three runs. MS^2 spectra of each extracted glycopeptide were manually inspected to match all highly abundant product ions with a precursor ion mass accuracy less than 5 ppm to confirm their assignments.

2.6. Cell culture and cytokine treatment

A generation of mouse hybridoma cells that produce mouse IgG_1 has been previously described (Chuang et al., 2016). Cells were cultured in Hybridoma serum-free medium (Thermo Fisher Scientific) at 37 °C in 5% CO_2. Cells in fresh media at the density of 5×10^5/mL were treated with different recombinant mouse cytokines (Cell Guidance Systems, Carlsbad, CA). Cells and secreted IgG_1 in culture media were harvested after 48 h of treatment.

2.7. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA purification and reverse transcription were performed using REzol C&T (Protech Technology, Taipei, Taiwan) and Superscript III First-Strand Synthesis System (Thermo Fisher Scientific), respectively. PCR was performed using Power SYBR Green PCR Master Mix and StepOne Real-Time PCR System (Thermo Fisher Scientific). The PCR program was set in an initial step at 95 °C for 10 min with subsequently 40 cycles at 95 °C for 15 s and 56 °C for 1 min. Sequences of primers for detecting mouse glycosyltransferases and β-actin are shown in Supplementary Table 1. Ct value was analyzed using StepOne Software version 2.3.

2.8. Statistical analysis

SPSS 17.0 for Windows was used for all statistical analyses. The Pearson Chi-square test was used for nominal variables. Non-parametric and parametric continuous variables between two groups were compared using Mann-Whitney *U* test and Student's *t*-test, respectively. Kruskal-Wallis test was used for comparing non-parametric continuous variables among 3 groups. Stepwise Cox and logistic regression analyses were used to identify factors that were related to the virological

Table 1
Characteristics of patients with HBeAg-positive chronic hepatitis B (n = 166).

Variable	
Demography	
Sex (Male: Female)	112: 54
Age (years)	48.0 (30.0–78.0)
Biochemistry and virology	
ALT (ULN)	2.3 (0.4–16.63)
AST (ULN)	1.2 (0.5–7.7)
Albumin (g/dL)	4.5 (3.3–5.5)
Total bilirubin (mg/dL)	0.8 (0.2–3.7)
HBV DNA (Log_{10} IU/mL)	8.0 (14.2–5.2)
HBV genotype (B: C; n = 150)	94: 56
HBsAg (Log_{10} IU/mL)	4.3 (0.5–5.3)
Liver histology	
Periportal/bridging necrosis	1.0 (0.0–6.0)
Intralobular necrosis	1.0 (0.0–4.0)
Portal inflammation	3.0 (0.0–4.0)
Fibrosis	1.0 (0.0–5.0)

Continuous variables are shown as median (range).
Liver biopsies are obtained from 107 patients before treatment.
Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBeAg, hepatitis B virus e antigen; HBsAg, hepatitis B virus surface antigen; HBV, hepatitis B virus; ULN, upper limits of normal.

response and liver histological improvement during NA treatment, respectively. Kaplan-Meier analyses and log-rank tests were used to assess the significance of IgG_1-G2FN on the virological response, HBeAg seroconversion, or HBsAg decline. Pearson's correlation coefficient (r) was used to evaluate the relationship between parameters. Significance was defined as $P < 0.05$, and all P-values were two-tailed.

3. Results

3.1. Characteristics and serum IgG_1-Fc glycoprofiles of the patients

Clinical data of 166 HBeAg-positive patients are shown in Table 1. Patients were at the median age of 48. There was a 2:1 ratio of men to women. Median levels of HBV DNA and HBsAg from sera of the patients were 8.0 and 4.3 logs IU/ml, respectively, and the median level of alanine aminotransferase (ALT) of them was > 2 upper limits of normal. A virus genotyping analysis from 150 patients revealed that 62.7% had HBV genotype B and 37.3% had HBV genotype C. Most of the patients had a mild-to-moderate liver necroinflammation or fibrosis.

Serum IgG_1-Fc glyco-profiling showed that proportions of G0F (fucosylated and agalactosylated), G1F (fucosylated and partially galactosylated), G2F (fucosylated and fully galactosylated), and G2FS (sialylated G2F) glycoforms in HBeAg-positive patients were all greater than 10% (Fig. 1). Four IgG_1 glycoforms with bisecting GlcNAc (N), including G0FN, G1FN, G2FN, and G2FNS, in these patients were all below 7%.

3.2. An inverse correlation between IgG_1 GlcNAc bisection and liver inflammation

At baseline, HBV DNA level in HBeAg-positive patients was positively correlated with the proportions of IgG_1-G2FN and IgG_1-G2FNS (Fig. 2A). Moreover, the proportion of IgG_1-G2FN was correlated with HBsAg titer. The patients with genotype C HBV infection had a lower proportion of IgG_1-G2FN (Fig. 2B), a higher grade of liver fibrosis, and a higher level of transforming growth factor (TGF)-β1 (Supplementary Fig. 1) than the patients with genotype B HBV infection. Furthermore, a low percentage of IgG_1-G2FN was found in patients with ≧2 upper limits of normal of ALT or with severe liver tissue damage (Fig. 2C and D). Of note, IgG_1-G2FN owns a stronger relation to weak liver inflammation and injury than sialylated IgG_1 glycoforms (G1FS and IgG_1-G2FS), which have been proved to be anti-inflammatory (Supplementary Fig. 2).

3.3. Regulation of IgG_1 GlcNAc bisection

A mouse hybridoma cell line with abundant, priming stimuli-free IgG_1 production was leveraged to investigate the regulation of IgG_1-G2FN. We found that TGF-β1 and interferon (IFN)-γ treatments downregulated messenger RNA level of mannosyl β-1,4-*N*-acetylglucosaminyltransferase 3 (*mgat3*; for bisecting GlcNAc editing) but had opposite effects on β-1,4-galactosyltransferase 1 (*b4galt1*; the major

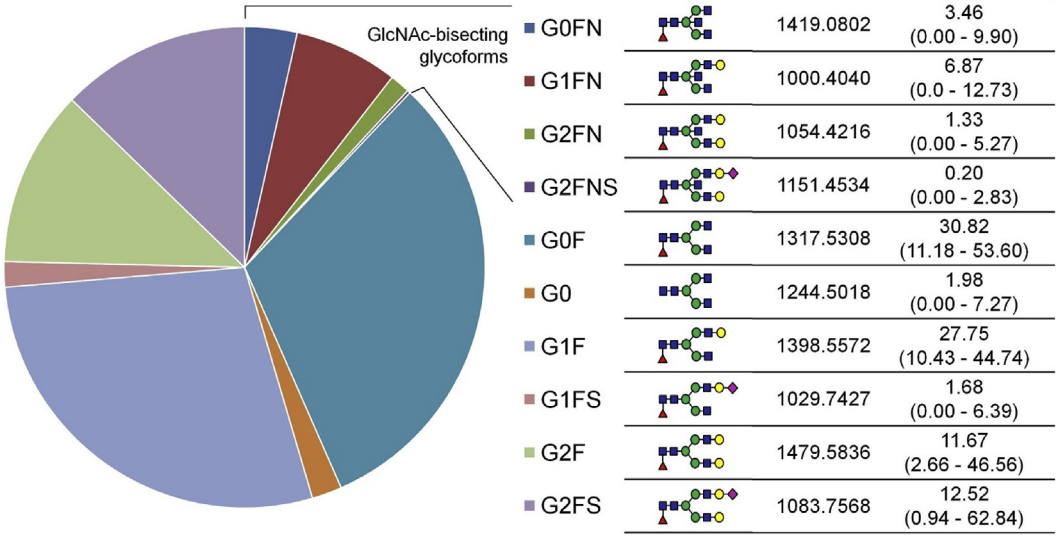

Fig. 1. Serum IgG_1-Fc *N*-glycan profiles. A result from a liquid chromatography–tandem mass spectrometry-based analysis of serum IgG_1-Fc *N*-glycosylation pattern in patients with HBeAg-positive chronic hepatitis B (n = 166) is shown. Red triangle, fucose; blue square, *N*-acetylglucosamine; green circle, mannose; yellow circle, galactose; purple diamond, sialic acid. F, fucosylated; G0, galactose-deficient; G1, partially galactosylated; G2, fully galactosylated; m/z, mass-to-charge number; N, bisected *N*-acetylglucosamine; S, sialylated.

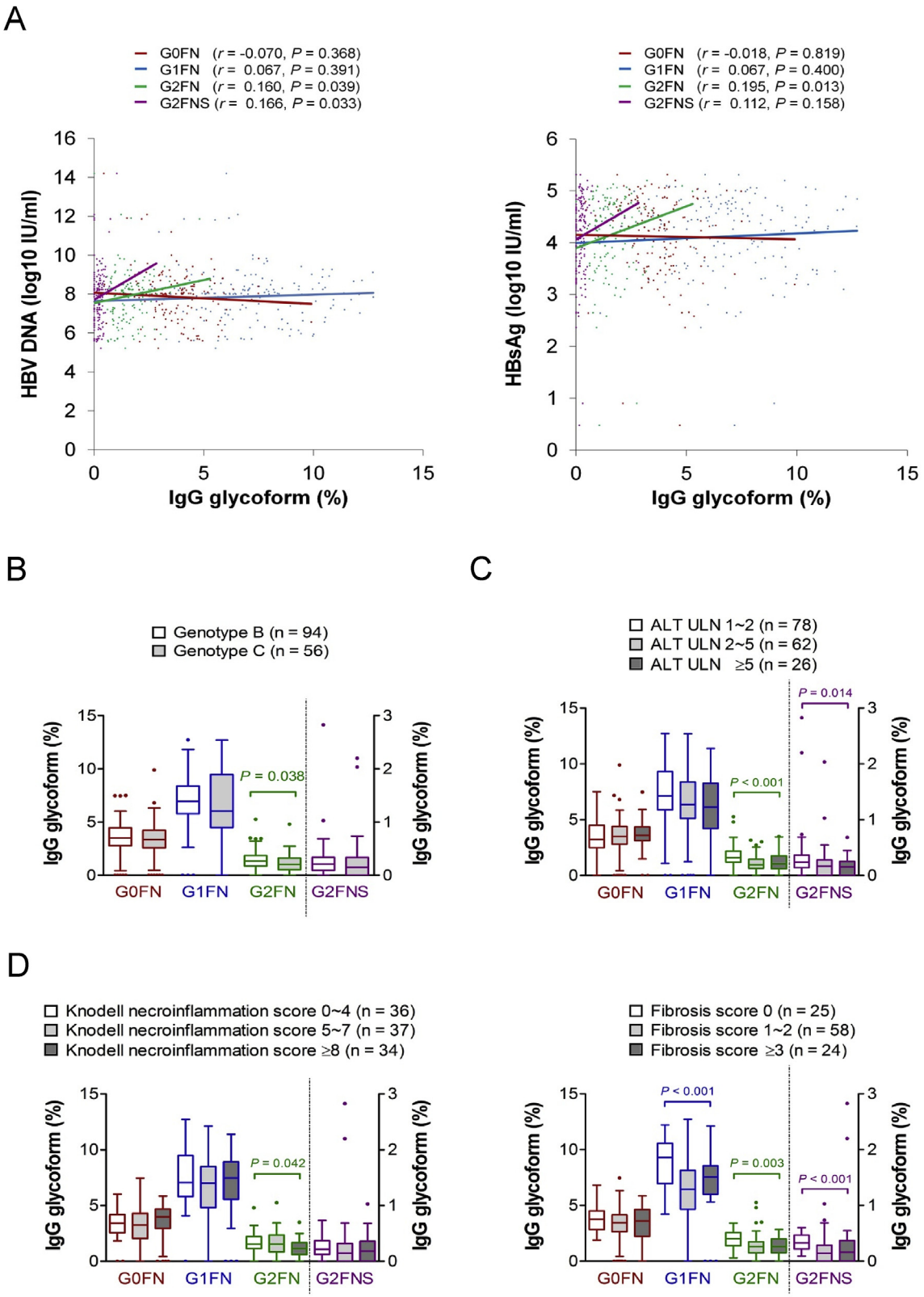

Fig. 2. Association between IgG_1 GlcNAc bisection and the disease severity of HBeAg-positive chronic hepatitis B. (A) Correlations of IgG_1 containing bisecting GlcNAc glycoforms with HBV DNA level and HBsAg titer at baseline are shown. The coefficient *r* and *P* value are obtained from the Pearson correlation tests. Proportions of serum IgG_1 bisecting GlcNAc glycoforms in different (B) HBV genotypes, (C) ALT level, (D) severity of liver tissue damage, including necroinflammation and fibrosis stage, are shown as Tukey box-and-whisker plots. *P* values in (B) are obtained from the Mann–Whitney *U* tests. *P* values in (C) to (D) are obtained from Kruskal-Wallis tests. ALT, alanine aminotransferase; F, fucosylated; G0, galactose-deficient; G1, partially galactosylated; G2, fully galactosylated; HBsAg, hepatitis B virus surface antigen; HBV, hepatitis B virus; N, bisected *N*-acetylglucosamine; S, sialylated; ULN, upper limit of normal.

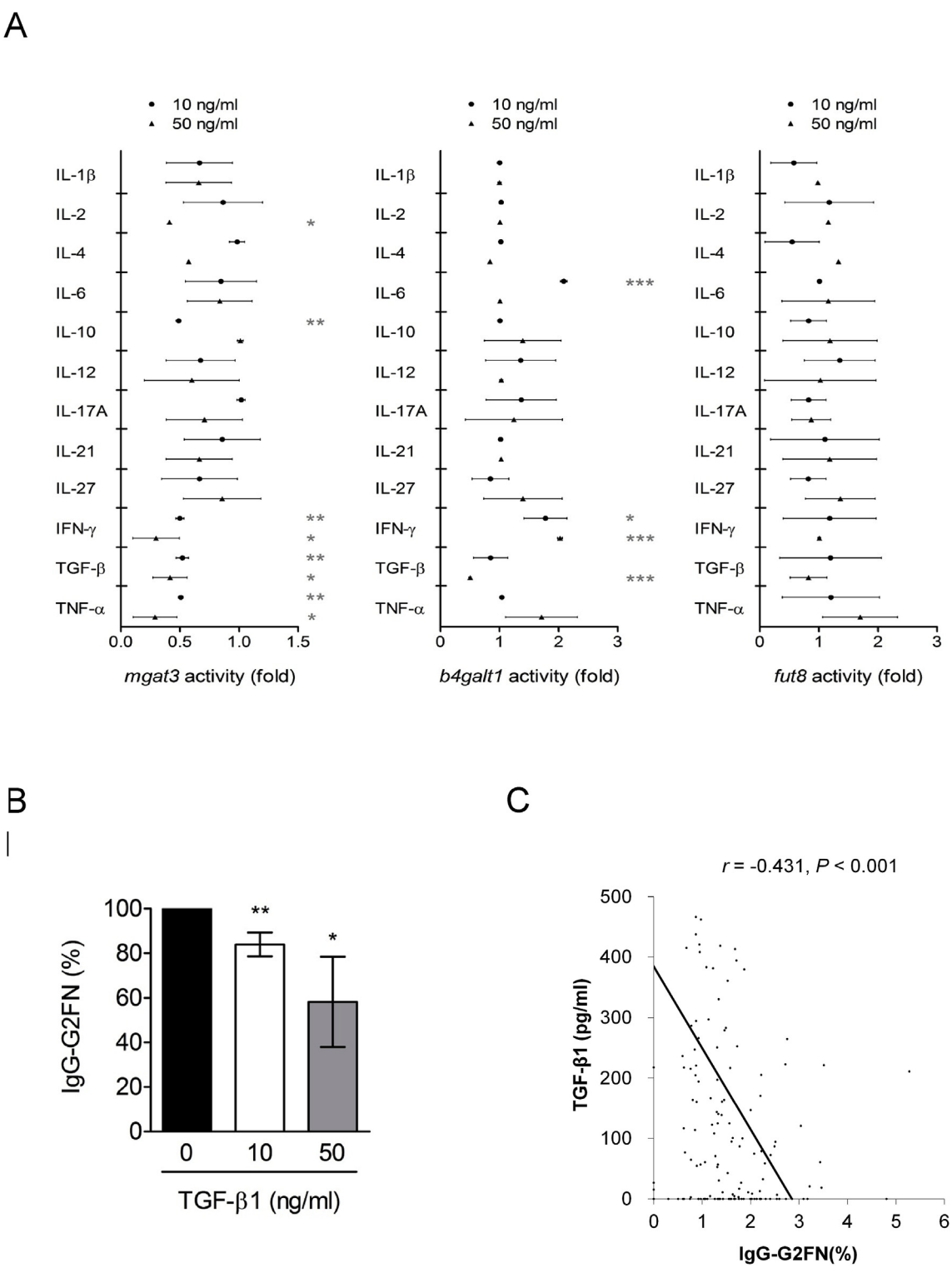

Fig. 3. Effects of cytokines on IgG_1 GlcNAc bisection. (A) Messenger RNA levels of β-1,4-*N*-acetylglucosaminyltransferase-3 (*mgat*3), β-1,4- galactosyltransferase 1 (*b4galt*1), and α-1,6-fucosyltransferase (*fut*8) in mouse hybridoma cells after 48 h of cytokine treatment, and (B) the proportion of mouse IgG_1-G2FN secreted from hybridoma cells after 48 h of TGF-β1 treatment, are shown in graphs as means with standard deviations. Results are obtained from three independent experiments. Student's *t* tests (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) are used for comparisons between the control and treatment groups. (C) The correlation between IgG_1-G2FN and TGF-β1 in sera from 166 HBeAg-positive patients is shown. The coefficient *r* and *P* value are from the Pearson correlation test. IFN, interferon; IgG_1-G2FN, fully galactosyl-fucosyl-*N*-acetylglucosamine-bisected IgG_1; IgG_1-G2FN, fully galactosyl-fucosyl-*N*-acetylglucosamine-bisected IgG_1; IL, interleukin; TGF, transforming growth factor; TNF, tumor necrosis factor.

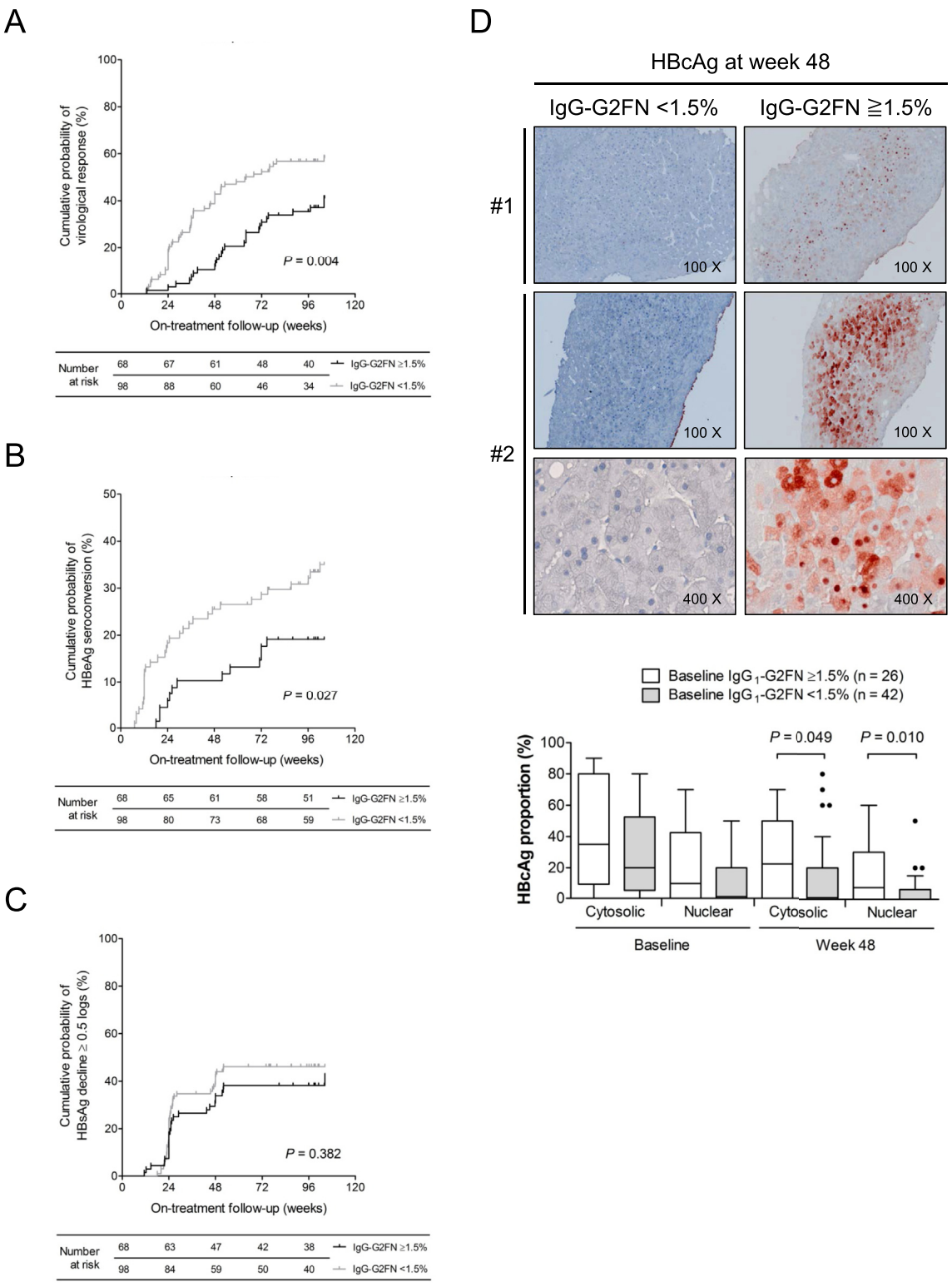

Fig. 4. The effect of IgG_1-G2FN at baseline on the clearance of hepatitis B virus (HBV) during treatment. Kaplan-Meier analyses to identify associations of baseline level of IgG_1-G2FN with (A) virological response, (B) HBV e antigen (HBeAg) seroconversion, and (C) HBV surface antigen (HBsAg) decline by > 0.5 logs, during the course of anti-HBV nucleos(t)ide analogue treatment in HBeAg-positive patients, are shown. *P*-values are obtained from log-rank tests. (D) Representative sections for HBV core antigen (HBcAg) immunohistochemistry in patients after 48 weeks of treatment are shown. Proportions of cytosolic or nuclear HBcAg expression in the liver tissue in different levels of IgG_1-G2FN are shown as Tukey box-and-whisker plots. *P* values are from Mann–Whitney *U* tests.

b4galt isoform for galactose editing) (Fig. 3A). No implicated cytokines regulated α-1,6-fucosyltransferase (*fut8*; for core fucose editing) activity in hybridoma cells. Looking at IgG_1 glycan phenotype, TGF-β1 treatment drastically reduced the production of IgG_1-G2FN from hybridoma cells (Fig. 3B). IFN-γ treatment did not change the proportion of IgG_1-G2FN (data not shown). A result from ELISA showed that TGF-β1 expression in sera from 166 HBeAg-positive patients was inversely correlated with IgG_1-G2FN ($r = -0.431$; $P < 0.001$) (Fig. 3C).

Table 2
Regression analyses for identifying factors that are associated with virological response (n = 166) and liver histological improvement (n = 95) in patients with HBeAg-positive chronic hepatitis B.

Variable	Cox regression for virological response (n = 166)				Logistic regression for 1-year liver histological improvement			
	Univariate		Multivariate		Univariate		Multivariate	
	Hazard ratio (95% CI)	*P*-value	Hazard ratio (95% CI)	*P*-value	Odds ratio (95% CI)	*P*-value	Odds ratio (95% CI)	*P*-value
Sex (Male = 1, Female = 0)	0.869 (0.555–1.360)	0.539			1.111 (0.455–2.715)	0.817		
Age (years)	1.010 (0.988–1.031)	0.372			0.982 (0.942–1.024)	0.396		
ALT (ULN)	1.174 (1.109–1.243)	< 0.001	1.155 (1.083–1.232)	< 0.001	1.583 (1.168–2.146)	0.003	1.001 (0.693–1.446)	0.995
Albumin (g/dL)	0.568 (0.301–1.074)	0.082			0.854 (0.243–2.997)	0.805		
Total bilirubin (mg/dL)	1.340 (0.929–1.932)	0.117			2.202 (0.810–5.985)	0.122		
HBV DNA (Log_{10} IU/mL)	0.715 (0.597–0.857)	< 0.001	0.892 (0.733–1.086)	0.255	0.842 (0.632–1.121)	0.239		
HBV genotype (C = 1, B = 0)	0.789 (0.471–1.323)	0.370			0.809 (0.344–1.904)	0.628		
HBsAg (Log_{10} IU/mL)	0.548 (0.442–0.680)	< 0.001	0.563 (0.421–0.751)	< 0.001	0.514 (0.270–0.978)	0.042	1.492 (0.540–4.121)	0.440
Drug								
Entecavir: Lamivudine	0.491 (0.301–0.802)	0.004	0.655 (0.397–1.079)	0.097	0.522 (0.142–1.919)	0.328		
Entecavir: Adefovir dipivoxil	0.543 (0.292–1.009)	0.054	0.520 (0.274–0.985)	0.045	0.741 (0.187–2.939)	0.670		
IgG_1-G2FN (%)	0.559 (0.425–0.737)	< 0.001	0.620 (0.466–0.825)	0.001	0.528 (0.331–0.840)	0.007	0.513 (0.279–0.943)	0.032
IgG_1-G1FS (%)	0.946 (0.760–1.177)	0.617			1.063 (0.742–1.521)	0.740		
IgG_1-G2FS (%)	0.943 (0.901–0.987)	0.012	0.991 (0.951–1.033)	0.664	0.985 (0.935–1.038)	0.573		
Liver necroinflammation score					1.812 (1.430–2.296)	< 0.001	1.889 (1.356–2.631)	< 0.001
Liver fibrosis score					2.503 (1.551–4.038)	< 0.001	0.990 (0.497–1.974)	0.978

Virological response is defined as an undetectable hepatitis B virus DNA in serum. Liver histological improvement is characterized as a decrease in the fibrosis score or a decline in total necroinflammation score ≧2 without exacerbation of fibrosis.
Abbreviations: ALT, alanine aminotransferase; CI, confidence interval; F, core fucosylation; G, galactosylation; HBsAg, hepatitis B virus surface antigen; HBV, hepatitis B virus; N, bisected *N*-acetylglucosamine; S, sialylation; ULN, upper limits of normal.

These findings, in combination with previous results, depict intricate loops among TGF-β1, IgG_1-G2FN, HBV genotype, and liver fibrosis.

3.4. Associated of IgG_1-G2FN with an attenuated HBV clearance

We assessed whether IgG_1-G2FN linked to the treatment efficacy of antiviral therapy in HBeAg-positive patients. Entecavir had a higher potency on HBV suppression than lamivudine and adefovir dipivoxil, and 3 NAs had similar efficacies on HBeAg seroconversion and liver histological improvement (Supplementary Figs. 3A–C). A Kaplan-Meier analysis revealed that HBeAg-positive patients with baseline IgG_1-G2FN % ≧1.5% had a remarkably lower probability to achieve virological response when compared to their counterparts (Fig. 4A), and this phenomenon could be seen in each NA group (Supplementary Fig. 3D). A stepwise Cox regression analysis revealed that the proportion of IgG_1-G2FN at baseline was an independent factor that was inversely associated with the incidence of virological response (Table 2). Other anti-inflammatory IgG glycoforms G1FS and G2FS were not significant factors on this issue. Because of the inverse correlation between IgG_1-G2FN and liver inflammation, the proportion of IgG_1-G2FN in both virological responders and non-responders increased with the emergence of a decline in ALT level after 48 weeks of treatment (Supplementary Fig. 4).

A low percentage of IgG_1-G2FN was associated with HBeAg seroconversion (Fig. 4B) but not HBsAg decline (Fig. 4C). In addition, immunohistochemistry analyses revealed that patients whose IgG_1-G2FN < 1.5% had a lower HBcAg expression after treatment when compared to the patients with IgG_1-G2FN ≧ 1.5% (Fig. 4D). These results indicated that a low proportion of serum IgG_1-G2FN in HBeAg-positive patients at baseline was prone to an efficacious on-treatment HBV clearance.

3.5. A relation between IgG_1-G2FN and liver histological improvement

Representative liver biopsies showed that a patient with baseline IgG_1-G2FN < 1.5% had a more severe lymphocyte infiltration in the hepatic tissue at baseline but a greater improvement in liver inflammation after 48 weeks of treatment in comparison to a patient with baseline IgG_1-G2FN ≧ 1.5%, (Fig. 5A). A low proportion of serum IgG_1-G2FN at baseline to a liver histological improvement at week 48 could be seen (Fig. 5B). A stepwise logistic regression analysis demonstrated that IgG_1-G2FN was an independent factor that disadvantaged the amelioration of liver injury (Table 2). These results, together with those shown in Fig. 2D, indicated that a high percentage of serum IgG_1-G2FN was associated with a mild liver necroinflammation at baseline but an unsatisfactory post-treatment amelioration of liver tissues.

4. Discussion

Although being a routine test for liver function, serum ALT may fail to reflect the degree of liver necroinflammatory activity and fibrosis (Kumar et al., 2008; Pratt and Kaplan, 2000; Tsang et al., 2008). IgG_1-Fc *N*-glycome is an extrahepatic manifestation to get an overall picture about a cohort on the liver physiology over a period of time owing to a higher abundance and a longer half-life of IgG_1 (approximately 3 weeks) than traditional parameters, e.g. ALT (Callewaert et al., 2004; Ho et al., 2014; Ho et al., 2015; Klein et al., 2010). The present study demonstrates a relation of IgG_1-G2FN with mild liver necroinflammation, thus leading to a poor efficacy of antiviral treatment in patients with HBeAg-positive CHB.

The glycan structure of IgG determines the binding affinity of Fc to different FcγRs on immune cells or mannan-binding lectin (Malhotra et al., 1995; Nimmerjahn and Ravetch, 2008), and it, therefore, influences various immune activities including complement activation, antibody-dependent cellular cytotoxicity (ADCC), opsonophagocytosis, and interferon secretion (Guilliams et al., 2014; Nimmerjahn and Ravetch, 2008; Salmon and Pricop, 2001; Takai, 2002). Recombinant IgG deficient in core fucose, galactose, or terminal sialic acid favors activating FcγRI or FcγRIII (Niwa et al., 2005; Shields et al., 2002; Yamane-Ohnuki et al., 2004). These IgGs can induce an even stronger inflammation, which can be proven by a mouse arthritis models (Nimmerjahn et al., 2007; Rademacher et al., 1994). At the other extreme, fully galactosylated and sialylated IgGs are anti-inflammatory because of their tropism to the inhibitory FcγRIIB, resulting in a high threshold for immune-complexes-mediated cell activation (Bohm et al., 2012; Kaneko et al., 2006). Moreover, an interaction between GlcNAc-bisected IgG_1 and FcγRIII is shown to be able to augment ADCC response against tumor cells (Davies et al., 2001; Umana et al., 1999).

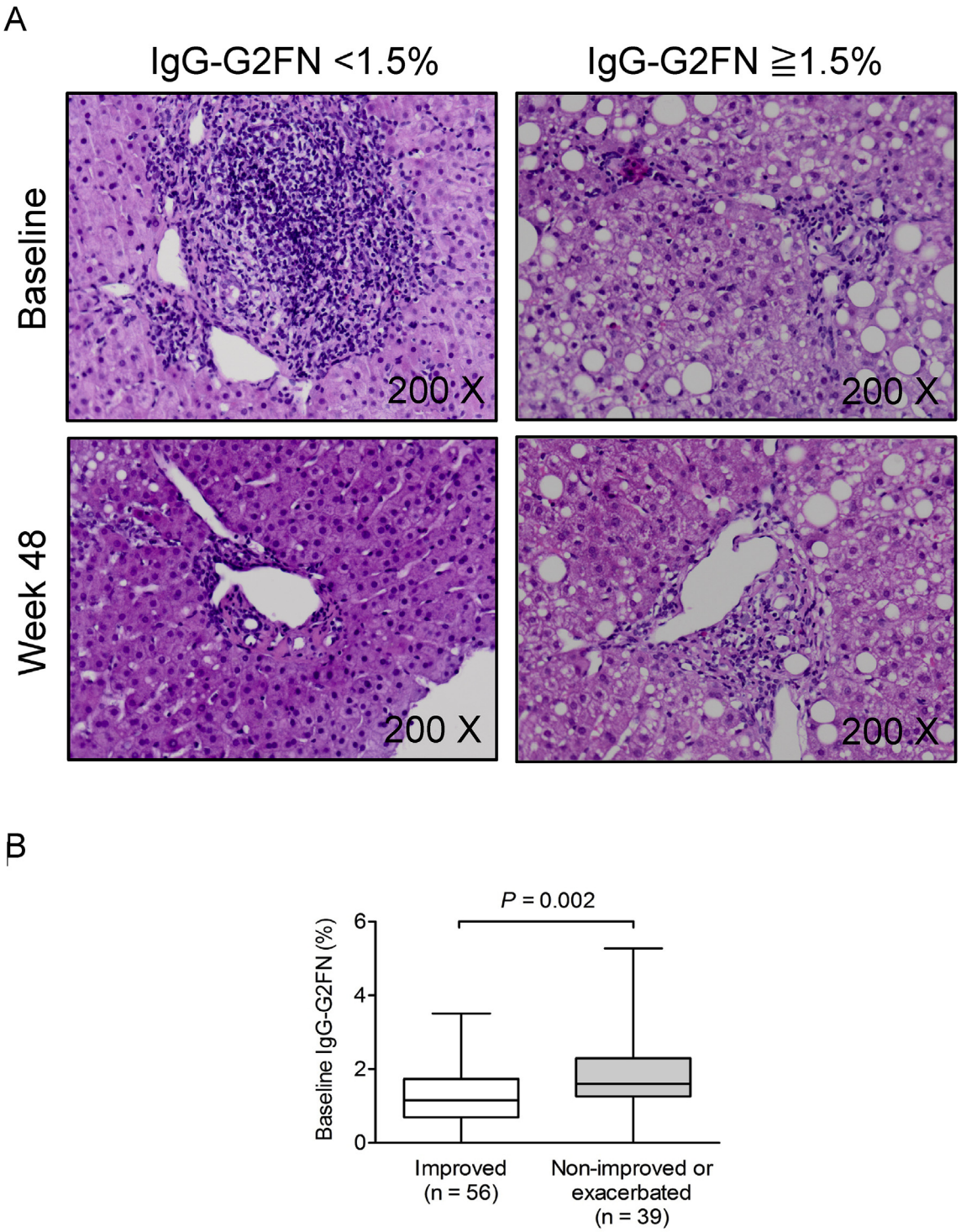

Fig. 5. Association between IgG_1-G2FN and liver histological improvement. (A) Representative liver sections before and after 48 weeks of treatment in 2 patients with different baseline levels of serum IgG_1-G2FN are shown. (B) Baseline levels of IgG_1-G2FN in patients with and without liver histological improvement after 48 weeks of treatment are shown as Tukey box-and-whisker plots. The *P* value is obtained from Mann–Whitney *U* test.

Nevertheless, this theory was refuted by a later paper, in which ADCC driven by GlcNAc-bisected IgG antibody was caused by Fc core afucosylation (Shinkawa et al., 2003). Our results showing the linkage between IgG_1-G2FN instead of IgG_1-G0FN and IgG_1-G1FN to the disease status lead to a new hypothesis stating that Fc-bisected GlcNAc-related immunomodulation might rely on the content of galactose on the IgG.

Data from other groups and from us indicated that CHB patients with a mild liver inflammation respond worse to oral NA therapy than those with ALT levels ≧2 ULN (Chien et al., 1999; Perrillo et al., 2002; Wu et al., 2010). From a clinical point of view, the correlation between high IgG_1-G2FN and ALT < 2 ULN makes it plausible to assume that IgG_1-G2FN possesses an anti-inflammation activity and this property disadvantages HBV clearance and liver histological improvement. When taking the amount into consideration, the effect of IgG_1-G2FN (only 1.3% of total IgG_1) might be huge, even surpasses those IgG_1 with sialylated glycoforms (more than 14% of total IgG_1). Unfortunately,

synthesis or purification of abundant IgG_1-G2FN currently remains an obstacle and thus limits our further quests for the binding preference of IgG_1-G2FN to FcγRs, especially FcγRIIB, and detailed mechanism about IgG_1-G2FN-dependent anti-inflammation.

One may empirically speculate that cytokines are mediators between liver inflammation and serum IgG glycosyl change. Interleukin-21 has been reported to decrease the yield of GlcNAc-bisected IgG from human peripheral $CD19^+$ B cells (Wang et al., 2011). However, priming stimuli for activating peripheral B cells make it difficult to dissect essential factors that modulate of IgG glycan biosynthesis. Therefore, we used mouse hybridoma cells as an in vitro model because they generate antibodies in a stimulation-free mode. Our results showed that a high dosage of mouse interleukin-21 had a borderline effect on the inhibition of *mgat3* activity ($P = 0.106$, data not shown) and TGF-β1 was a pivotal cytokine repressing IgG_1-G2FN secretion. A similar result by Xu et al. reported that TGF-β1 decreased MGAT3 expression in a mouse integrin-β1 knock-out embryonic stem cell line (Xu et al., 2012). Functionwise, it is well identified that TGF-β1 is a pleiotropic cytokine on inflammation but also a notorious inducer of tissue fibrosis (Bataller and Brenner, 2005; Bi et al., 2012; Gressner and Weiskirchen, 2006; Gressner et al., 2002; Kisseleva and Brenner, 2007). Beyond the paradox shown here is a reciprocal action between TGF-β1 and IgG_1-G2FN in both anti-inflammation and fibrogenesis aspects. Viewing it from the macroside, patients with a feeble TGF-β1 expression may tend to increase IgG_1-G2FN to avoid excessive immune responses and liver damages. In contrast, patients with a vigorous TGF-β1 secretion are inclined to constrict IgG_1-G2FN to maintain sufficient immune activities. Interestingly, HBV genotype was also implicated in the interplay between TGF-β1 and IgG_1-G2FN. B and C are the two most common HBV genotypes in East Asia and Taiwan (Sunbul, 2014). Several lines of evidence demonstrate that HBV genotype C is a high-risk factor for the development of liver cancer (Chan et al., 2004; Chen et al., 2004; Yang et al., 2008). A lower level of IgG_1-G2FN and a higher level of TGF-β1 in genotype C HBV correspond to previous cohorts, of which patients with genotype C HBV infection suffered more advanced liver fibrosis and a higher probability of hepatocarcinogenesis than patients with genotype B HBV infection (Chan et al., 2009; Chu and Liaw, 2005; Sumi et al., 2003). Our findings not only form a specific bridge among TGF-β1, IgG_1-G2FN, HBV genotypes, and liver fibrosis but also provide new insights into the value of antibody glycome for balancing host immunity and liver pathophysiology during chronic hepatitis.

Competing financial interests

All authors declare no conflicts of interest.

Submission declaration and verification

This manuscript has not been submitted or accepted elsewhere. All authors have read and approved the manuscript for submission and have contributed significantly to the work. There is no writing assistance other than copy editing was provided in the preparation of the manuscript.

Author contributions

Cheng-Hsun Ho was responsible for study design, experiment performance, and manuscript writing; Shu-Hui Chen supervised the LC-MS/MS instrumentation and IgG-Fc glycan analyses; Hung-Wen Tsai was responsible for liver histology and immunohistochemistry; I-Chin Wu assisted in analyzing clinical data and manuscript writing; Ting-Tsung Chang coordinated the study, supervised the medication of patients, and composed the manuscript.

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Abbreviations used in this paper

ADCC	antibody-dependent cell-mediated cytotoxicity
ADV	adefovir dipivoxil
ALT	alanine aminotransferase
AST	aspartate aminotransferase
B4GALT1	β-1,4-galactosyltransferase 1
CHB	chronic hepatitis B
ELISA	enzyme-linked immunosorbent assay
ETV	entecavir
F	for glycoform, fucosylation
Fc	crystallizable fragment
FcγR	Fc gamma receptor
FUT8	α-1,6-fucosyltransferase
G	for glycoform, galactosylation
G0	galactose-deficient
G1	partially galactosylated
G2	fully galactosylated
GlcNAc	*N*-acetylglucosamine;
HBcAg	hepatitis B virus core antigen
HBeAg	hepatitis B virus e antigen
HBsAg	hepatitis B virus surface antigen
HBV	hepatitis B virus
IgG	immunoglobulin G
IHC	immunohistochemistry
LAM	lamivudine;
LC-MS/MS	liquid chromatography–tandem mass spectrometry
MGAT3	β-1,4-*N*-acetylglucosaminyltransferase 3
N	for glycoform, bisected *N*-acetylglucosamine;
NA	nucleos(t)ide analogue
S	for glycoform, sialylation
TGF	transforming growth factor
ULN	upper limit of normal

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2018.12.021.

References

Bataller, R., Brenner, D.A., 2005. Liver fibrosis. J. Clin. Investig. 115, 209–218.
Bi, W.R., Yang, C.Q., Shi, Q., 2012. Transforming growth factor-beta1 induced epithelial-mesenchymal transition in hepatic fibrosis. Hepatogastroenterology 59, 1960–1963.
Bohm, S., Schwab, I., Lux, A., Nimmerjahn, F., 2012. The role of sialic acid as a modulator of the anti-inflammatory activity of IgG. Semin. Immunopathol. 34, 443–453.
Callewaert, N., Van Vlierberghe, H., Van Hecke, A., Laroy, W., Delanghe, J., Contreras, R., 2004. Noninvasive diagnosis of liver cirrhosis using DNA sequencer-based total serum protein glycomics. Nat. Med. 10, 429–434.
Chan, H.L., Hui, A.Y., Wong, M.L., Tse, A.M., Hung, L.C., Wong, V.W., Sung, J.J., 2004. Genotype C hepatitis B virus infection is associated with an increased risk of hepatocellular carcinoma. Gut 53, 1494–1498.
Chan, H.L., Wong, G.L., Tse, C.H., Chim, A.M., Yiu, K.K., Chan, H.Y., Sung, J.J., Wong, V.W., 2009. Hepatitis B virus genotype C is associated with more severe liver fibrosis than genotype B. Clin. Gastroenterol. Hepatol. 7, 1361–1366.
Chen, C.H., Chiu, Y.C., Lu, S.N., Lee, C.M., Wang, J.H., Hu, T.H., Hung, C.H., 2014. Serum

hepatitis B surface antigen levels predict treatment response to nucleos(t)ide analogues. World J. Gastroenterol. 20, 7686–7695.
Chen, J.D., Liu, C.J., Lee, P.H., Chen, P.J., Lai, M.Y., Kao, J.H., Chen, D.S., 2004. Hepatitis B genotypes correlate with tumor recurrence after curative resection of hepatocellular carcinoma. Clin. Gastroenterol. Hepatol. 2, 64–71.
Chien, R.N., Liaw, Y.F., Atkins, M., 1999. Pretherapy alanine transaminase level as a determinant for hepatitis B e antigen seroconversion during lamivudine therapy in patients with chronic hepatitis B. Asian hepatitis lamivudine trial group. Hepatology 30, 770–774.
Chu, C.M., Liaw, Y.F., 2005. Genotype C hepatitis B virus infection is associated with a higher risk of reactivation of hepatitis B and progression to cirrhosis than genotype B: a longitudinal study of hepatitis B e antigen-positive patients with normal aminotransferase levels at baseline. J. Hepatol. 43, 411–417.
Chuang, Y.C., Lin, J., Lin, Y.S., Wang, S., Yeh, T.M., 2016. Dengue virus nonstructural protein 1-induced antibodies cross-react with human plasminogen and enhance its activation. J. Immunol. 196, 1218–1226.
Davies, J., Jiang, L., Pan, L.Z., LaBarre, M.J., Anderson, D., Reff, M., 2001. Expression of GnTIII in a recombinant anti-CD20 CHO production cell line: expression of antibodies with altered glycoforms leads to an increase in ADCC through higher affinity for FC gamma RIII. Biotechnol. Bioeng. 74, 288–294.
Gressner, A.M., Weiskirchen, R., 2006. Modern pathogenetic concepts of liver fibrosis suggest stellate cells and TGF-beta as major players and therapeutic targets. J. Cell Mol. Med. 10, 76–99.
Gressner, A.M., Weiskirchen, R., Breitkopf, K., Dooley, S., 2002. Roles of TGF-beta in hepatic fibrosis. Front. Biosci. 7, d793–807.
Guilliams, M., Bruhns, P., Saeys, Y., Hammad, H., Lambrecht, B.N., 2014. The function of Fcgamma receptors in dendritic cells and macrophages. Nat. Rev. Immunol. 14, 94–108.
Ho, C.H., Chien, R.N., Cheng, P.N., Liu, C.K., Su, C.S., Wu, I.C., Liu, W.C., Chen, S.H., Chang, T.T., 2014. Association of serum IgG N-glycome and transforming growth factor-beta1 with hepatitis B virus e antigen seroconversion during entecavir therapy. Antivir. Res. 111, 121–128.
Ho, C.H., Chien, R.N., Cheng, P.N., Liu, J.H., Liu, C.K., Su, C.S., Wu, I.C., Li, I.C., Tsai, H.W., Wu, S.L., Liu, W.C., Chen, S.H., Chang, T.T., 2015. Aberrant serum immunoglobulin G glycosylation in chronic hepatitis B is associated with histological liver damage and reversible by antiviral therapy. J. Infect. Dis. 211, 115–124.
Hsu, Y.S., Chien, R.N., Yeh, C.T., Sheen, I.S., Chiou, H.Y., Chu, C.M., Liaw, Y.F., 2002. Long-term outcome after spontaneous HBeAg seroconversion in patients with chronic hepatitis B. Hepatology 35, 1522–1527.
Huhn, C., Selman, M.H., Ruhaak, L.R., Deelder, A.M., Wuhrer, M., 2009. IgG glycosylation analysis. Proteomics 9, 882–913.
Kaneko, Y., Nimmerjahn, F., Ravetch, J.V., 2006. Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation. Science 313, 670–673.
Kim, S.S., Lee, D., Lee, M.H., Cheong, J.Y., Cho, S.W., 2013. Association of on-treatment serum hepatitis B surface antigen level with sustained virological response to nucleos(t)ide analog in patients with hepatitis B e-antigen positive chronic hepatitis B. Hepatol. Res. 43, 219–227.
Kisseleva, T., Brenner, D.A., 2007. Role of hepatic stellate cells in fibrogenesis and the reversal of fibrosis. J. Gastroenterol. Hepatol. 22 (Suppl. 1), S73–S78.
Klein, A., Carre, Y., Louvet, A., Michalski, J.C., Morelle, W., 2010. Immunoglobulins are the major glycoproteins involved in the modifications of total serum N-glycome in cirrhotic patients. Proteom. Clin. Appl. 4, 379–393.
Kumar, M., Sarin, S.K., Hissar, S., Pande, C., Sakhuja, P., Sharma, B.C., Chauhan, R., Bose, S., 2008. Virologic and histologic features of chronic hepatitis B virus-infected asymptomatic patients with persistently normal ALT. Gastroenterology 134, 1376–1384.
Lee, J.M., Ahn, S.H., Kim, H.S., Park, H., Chang, H.Y., Kim do, Y., Hwang, S.G., Rim, K.S., Chon, C.Y., Han, K.H., Park, J.Y., 2011. Quantitative hepatitis B surface antigen and hepatitis B e antigen titers in prediction of treatment response to entecavir. Hepatology 53, 1486–1493.
Liu, W.C., Lin, C.P., Cheng, C.P., Ho, C.H., Lan, K.L., Cheng, J.H., Yen, C.J., Cheng, P.N., Wu, I.C., Li, I.C., Chang, B.C., Tseng, V.S., Chiu, Y.C., Chang, T.T., 2016. Aligning to the sample-specific reference sequence to optimize the accuracy of next-generation sequencing analysis for hepatitis B virus. Hepatol. Int. 10, 147–157.
Liu, W.C., Mizokami, M., Buti, M., Lindh, M., Young, K.C., Sun, K.T., Chi, Y.C., Li, H.H., Chang, T.T., 2006. Simultaneous quantification and genotyping of hepatitis B virus for genotypes A to G by real-time PCR and two-step melting curve analysis. J. Clin. Microbiol. 44, 4491–4497.
Malhotra, R., Wormald, M.R., Rudd, P.M., Fischer, P.B., Dwek, R.A., Sim, R.B., 1995. Glycosylation changes of IgG associated with rheumatoid arthritis can activate complement via the mannose-binding protein. Nat. Med. 1, 237–243.
Nimmerjahn, F., Anthony, R.M., Ravetch, J.V., 2007. Agalactosylated IgG antibodies depend on cellular Fc receptors for in vivo activity. Proc. Natl. Acad. Sci. U. S. A. 104, 8433–8437.
Nimmerjahn, F., Ravetch, J.V., 2008. Fcgamma receptors as regulators of immune responses. Nat. Rev. Immunol. 8, 34–47.
Niwa, R., Sakurada, M., Kobayashi, Y., Uehara, A., Matsushima, K., Ueda, R., Nakamura, K., Shitara, K., 2005. Enhanced natural killer cell binding and activation by low-fucose IgG1 antibody results in potent antibody-dependent cellular cytotoxicity induction at lower antigen density. Clin. Cancer Res. 11, 2327–2336.
Perrillo, R.P., Lai, C.L., Liaw, Y.F., Dienstag, J.L., Schiff, E.R., Schalm, S.W., Heathcote, E.J., Brown, N.A., Atkins, M., Woessner, M., Gardner, S.D., 2002. Predictors of HBeAg loss after lamivudine treatment for chronic hepatitis B. Hepatology 36, 186–194.
Pratt, D.S., Kaplan, M.M., 2000. Evaluation of abnormal liver-enzyme results in asymptomatic patients. N. Engl. J. Med. 342, 1266–1271.
Rademacher, T.W., Williams, P., Dwek, R.A., 1994. Agalactosyl glycoforms of IgG autoantibodies are pathogenic. Proc. Natl. Acad. Sci. U. S. A. 91, 6123–6127.
Salmon, J.E., Pricop, L., 2001. Human receptors for immunoglobulin G: key elements in the pathogenesis of rheumatic disease. Arthritis Rheum. 44, 739–750.
Shields, R.L., Lai, J., Keck, R., O'Connell, L.Y., Hong, K., Meng, Y.G., Weikert, S.H., Presta, L.G., 2002. Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human Fcgamma RIII and antibody-dependent cellular toxicity. J. Biol. Chem. 277, 26733–26740.
Shin, J.W., Jung, S.W., Park, B.R., Kim, C.J., Eum, J.B., Kim, B.G., Jeong, I.D., Bang, S.J., Lee, S.H., Kim, S.R., Park, N.H., 2012. Prediction of response to entecavir therapy in patients with HBeAg-positive chronic hepatitis B based on on-treatment HBsAg, HBeAg and HBV DNA levels. J. Vir. Hepat. 19, 724–731.
Shinkawa, T., Nakamura, K., Yamane, N., Shoji-Hosaka, E., Kanda, Y., Sakurada, M., Uchida, K., Anazawa, H., Satoh, M., Yamasaki, M., Hanai, N., Shitara, K., 2003. The absence of fucose but not the presence of galactose or bisecting N-acetylglucosamine of human IgG1 complex-type oligosaccharides shows the critical role of enhancing antibody-dependent cellular cytotoxicity. J. Biol. Chem. 278, 3466–3473.
Sumi, H., Yokosuka, O., Seki, N., Arai, M., Imazeki, F., Kurihara, T., Kanda, T., Fukai, K., Kato, M., Saisho, H., 2003. Influence of hepatitis B virus genotypes on the progression of chronic type B liver disease. Hepatology 37, 19–26.
Sunbul, M., 2014. Hepatitis B virus genotypes: global distribution and clinical importance. World J. Gastroenterol. 20, 5427–5434.
Takai, T., 2002. Roles of Fc receptors in autoimmunity. Nat. Rev. Immunol. 2, 580–592.
Terrault, N.A., Bzowej, N.H., Chang, K.M., Hwang, J.P., Jonas, M.M., Murad, M.H., 2016. AASLD guidelines for treatment of chronic hepatitis B. Hepatology 63, 261–283.
Tsang, P.S., Trinh, H., Garcia, R.T., Phan, J.T., Ha, N.B., Nguyen, H., Nguyen, K., Keeffe, E.B., Nguyen, M.H., 2008. Significant prevalence of histologic disease in patients with chronic hepatitis B and mildly elevated serum alanine aminotransferase levels. Clin. Gastroenterol. Hepatol. 6, 569–574.
Umana, P., Jean-Mairet, J., Moudry, R., Amstutz, H., Bailey, J.E., 1999. Engineered glycoforms of an antineuroblastoma IgG1 with optimized antibody-dependent cellular cytotoxic activity. Nat. Biotechnol. 17, 176–180.
Vanwolleghem, T., Hou, J., van Oord, G., Andeweg, A.C., Osterhaus, A.D., Pas, S.D., Janssen, H.L., Boonstra, A., 2015. Re-evaluation of hepatitis B virus clinical phases by systems biology identifies unappreciated roles for the innate immune response and B cells. Hepatology 62, 87–100.
Wang, J., Balog, C.I., Stavenhagen, K., Koeleman, C.A., Scherer, H.U., Selman, M.H., Deelder, A.M., Huizinga, T.W., Toes, R.E., Wuhrer, M., 2011. Fc-glycosylation of IgG1 is modulated by B-cell stimuli. Mol. Cell. Proteomics 10 M110 004655.
Wu, I.C., Lai, C.L., Han, S.H., Han, K.H., Gordon, S.C., Chao, Y.C., Tan, C.K., Sievert, W., Tanwandee, T., Xu, D., Neo, B.L., Chang, T.T., 2010. Efficacy of entecavir in chronic hepatitis B patients with mildly elevated alanine aminotransferase and biopsy-proven histological damage. Hepatology 51, 1185–1189.
Xu, Q., Isaji, T., Lu, Y., Gu, W., Kondo, M., Fukuda, T., Du, Y., Gu, J., 2012. Roles of N-acetylglucosaminyltransferase III in epithelial-to-mesenchymal transition induced by transforming growth factor beta1 (TGF-beta1) in epithelial cell lines. J. Biol. Chem. 287, 16563–16574.
Yamane-Ohnuki, N., Kinoshita, S., Inoue-Urakubo, M., Kusunoki, M., Iida, S., Nakano, R., Wakitani, M., Niwa, R., Sakurada, M., Uchida, K., Shitara, K., Satoh, M., 2004. Establishment of FUT8 knockout Chinese hamster ovary cells: an ideal host cell line for producing completely defucosylated antibodies with enhanced antibody-dependent cellular cytotoxicity. Biotechnol. Bioeng. 87, 614–622.
Yang, H.I., Yeh, S.H., Chen, P.J., Iloeje, U.H., Jen, C.L., Su, J., Wang, L.Y., Lu, S.N., You, S.L., Chen, D.S., Liaw, Y.F., Chen, C.J., Group, R.-H.S., 2008. Associations between hepatitis B virus genotype and mutants and the risk of hepatocellular carcinoma. J. Natl. Cancer Inst. 100, 1134–1143.