A STAT4 variant increases liver fibrosis risk in Caucasian patients with chronic hepatitis B

STAT4 variants increase liver fibrosis risk in chronic hepatitis B through impaired STAT4-dependent NK cell IFN-γ production

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Abstract (N=223 words)

Host genetic modifiers of the natural history of chronic hepatitis B (CHB) infection remains poorly understood. Recently, a GWAS identified polymorphism in the *STAT4* gene that contributes to the risk for hepatocellular carcinoma (HCC) was shown to be associated with the full spectrum of HBV outcomes in Asian patients. However, the functional mechanisms for this effect are unknown and the role of the variant in modulating HBV disease in Caucasians has not been investigated. We determined whether *STAT4* genetic variation is associated with liver injury and investigated for potential mechanisms mediating this effect in Caucasians with CHB. In 1085 subjects (830 with CHB and 255 healthy controls), *STAT4* rs7574865 genotype was independently associated with hepatic inflammation (OR: 1.42, 95% CI: 1.07-2.06, p=0.02) and advanced fibrosis (OR: 1.83, 95% CI: 1.19-2.83, p=0.006). The minor allele frequency (MAF) of rs7574865 was significantly lower than that in healthy controls, suggestive of an association between *STAT4* variants and HBV persistence. rs7574865 GG risk carriers expressed lower levels of STAT4 in liver and in PBMCs, while NK cells from patients with the rs7574865 GG genotype had impaired STAT4 phosphorylation following stimulation with IL-12/IL-18 and a reduction in secretion of the anti-fibrotic cytokine interferon-gamma (IFN-γ). **Conclusion**: Genetic susceptibility to HBV persistence, hepatic inflammation and fibrosis in Caucasians associates with variation in STAT4 at the rs7574865 locus. Downstream effects on NK cell function through STAT4 phosphorylation-dependent interferon gamma production likely contributes to these effects.

Introduction

Two billion people worldwide have been exposed to the hepatitis B virus (HBV) of whom ~350 million are chronically infected, with 800,000 deaths annually from complications [1]. HBV is not directly cytopathic to hepatocytes, with liver damage a consequence of the host immune response directed towards eliminating the virus [2]. A complex interplay between the immune response, viral and host genetic factors, shapes the final outcome of infection [3].

One of the host mechanisms mediating HBV activity appears to be JAK-STAT-signaling. This pathway plays a critical role in viral clearance on the one hand, and hepatic inflammation and fibrosis on the other [4, 5]. This is not unexpected since STAT4, a member of the STAT (signal transducers and activators of transcription) protein family is activated by diverse cytokines including interleukin-12 and interferon alpha (IFN-α) in response to viral infections [6] to regulate tissue inflammation, fibrosis, and antiviral activities [7]. The precise role of STAT4 in the pathogenesis of liver injury is largely unknown, but data from STAT4 knockout mice suggest context-dependent effects with mice being more susceptible to acute T-cell hepatitis [8], while having equal liver injury after ischemia/reperfusion [9].

The host genetic milieu is an important contributor to the risk of HBV persistence and progression as reviewed elsewhere [10]. Recently, a single nucleotide polymorphism (SNP), rs7574865 in the third intron of the *STAT4* gene was reported to be associated with the entire spectrum of HBV infection including spontaneous clearance, response to IFN-α based therapy and the risk of HBV-related cirrhosis and hepatocellular carcinoma (HCC), with risk conferred by the GG genotype [11-14] in Asian patients. The role of this variant in other HBV-infected populations and the effect size, if any, is still unknown. Furthermore, how this variant functionally contributes to HBV pathogenesis and outcomes is not understood.

We recently reported that rs7574865 does not play any role in either the response to interferon-based treatment, spontaneous clearance or fibrosis progression in Caucasian patients with chronic

hepatitis C [15]. Whether this lack of association is due to ethnic differences or more likely to differences in the hepatic immune response to HBV and HCV is unknown, but intriguing. Understanding this dichotomy is important because unravelling differential immune pathways related to viral clearance and liver injury will enhance our understanding of disease pathogenesis [10]. The aim of this study was to examine the effect of *STAT4* rs7574865 variation to liver injury in Caucasian patients with CHB and to gain insights on the functional mechanisms for this effect.

Methods

Patient cohort

The study comprised 1085 subjects (830 histologically characterized Caucasian CHB patients and 255 healthy Caucasian controls). All consecutive patients who had detectable hepatitis B surface antigen (HBsAg), persistently or intermittently abnormal alanine aminotransferase (ALT) values and serum HBV DNA >2,000 IU/mL for >6 months with at least one liver biopsy prior to any therapy were included. Patients were excluded if they had evidence of co-infection with either hepatitis C virus (HCV), hepatitis delta virus (HDV) or human immunodeficiency (HIV) virus, were diagnosed with or suspected to have HCC or alpha-fetoprotein >100 ng/mL, or had evidence of other liver diseases by standard tests. Patients with a current or previous episode of hepatic decompensation defined as (i) ascites (overt or by ultrasound), (ii) hepatic encephalopathy (HE), (iii) gastroesophageal variceal bleeding (GEVB), (iv) jaundice or (v) hepatorenal syndrome (HRS) were excluded. The healthy Caucasian control group was enrolled at Westmead hospital and has been described previously [16]. These patients reported no history of chronic liver disease and did not abuse alcohol (< 20 gm of alcohol daily). Ethics approval was obtained from the Human Research Ethics Committees of the Sydney West Local Health District and the University of Sydney. All other sites had ethics approval from their respective ethics committees. Written informed consent for genetic testing was obtained from all participants.

Clinical and laboratory assessment

The following data were collected at time of liver biopsy from all patients: gender, age, ethnicity, recruitment center, body mass index (BMI), HBV-DNA level, HBe-Ag status and routine laboratory tests. BMI was calculated as weight divided by the square of the height (kg/m^2).

Liver Histopathology

Liver histopathology was scored by expert pathologists according to METAVIR[17]. Fibrosis was staged from F0 (no fibrosis) to F4 (cirrhosis). Necroinflammation (A) was graded as A0 (absent), A1 (mild), A2 (moderate), or A3 (severe). The inter-observer agreement between pathologists was studied previously and was good ($\kappa = 77.5$) for METAVIR staging using κ statistics [18].

Genotyping

Genotyping for *STAT4* rs7574865 was undertaken using the TaqMan SNP genotyping allelic discrimination method (Applied Biosystems, Foster City, CA, USA). Genotyping was blinded to clinical variables.

Cell culture and HBV Transfection

Huh7 cells lines were incubated in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO_2. To examine the effect of HBV on STAT4 expression, 6-well plates were seeded with 5 × 105 Huh7 cells. On the next day, cells were transiently transfected with an HBV expressing plasmid (wild type HBV, genotype D) using the Fugene transfection reagent (Promega) and harvested 72 hours later. Transfection efficiency was evaluated using the Great EscAPe secreted alkaline phosphatase reporter system 3 (Clontech) in which 10 ng/ml of a reporter plasmid expressing secreted alkaline phosphatase (SEAP) is co-transfected. Experiments were performed at least in triplicate.

Quantitative real time reverse transcription PCR

To determine the functional effects of *STAT4* rs7574865, we examined for the association of hepatic and peripheral blood mononuclear cell (PBMC) STAT4 expression according to *STAT4* rs7574865 genotype in a cohort of 45 liver biopsies from CHB patients having stored liver tissue available for analysis as described previously [19], and in PBMCs from healthy controls (n=30). EDTA

tubes of blood were collected from healthy participants and separated on Ficoll-Paque to obtain PBMCs.

STAT4 mRNA expression was also assessed in human primary hepatocytes, human primary hepatic stellate cells, human primary hepatic sinusoidal endothelial cells (ScienceCell) and human primary Kupffer cells (Thermofisher). mRNA expression levels were determined using TaqMan probes and master mix (Life Technologies) according to the manufacturer's protocol. RNA was extracted using the RNeasy kit (Qiagen) according to the manufacturer's instructions. RNA quality and concentration was assessed using the Agilent 2100 Bioanalyser (Agilent, Waldbronn, Germany). cDNA was prepared using qscript (Quanta Biosciences, Gaithersburg, MD, USA) in a Mastercycler gradient 5331 (Eppendorf AG, Hamburg, Germany). GAPDH was used as the house keeping gene. Expression was measured using CT values, normalized to that of GAPDH (ΔCT= CT (GAPDH) - CT (target) and then expressed as $2^{\Delta CT}$.

Intracellular pSTAT4 assay

Frozen PBMCs were thawed and incubated with or without IL-12 (10 ng/mL; R&D Systems) plus IL-18 (100 ng/mL R&D Systems) for 10 minutes at 37ºC. pSTAT4 was measured using the Phosflow kit (BD Biosciences) according to the manufacturer's protocol. Briefly, cells were fixed in fixation buffer and then stained with anti-CD56-BU737 (BD Biosciences) and anti-human CD19 APC/CY7(biolegend) to identify NK cells and B cells, respectively, and with anti-CD3-pacific blue (BD Biosciences) to exclude T cells. Cells were then permeabilized with Perm III buffer (BD Biosciences), blocked with 10 % goat block buffer and additionally stained with anti-STAT4 (Abcam), or anti-pSTAT4-Alexa, Fluor647 (BD Biosciences). Stained cells were analyzed with FACScan (Becton Dickinson, Mountain View, CA, USA) LSRII and the data processed using the Flow-Jo program (Tree Star Inc., Ashland, OR, USA).

IFN-γ Production

Frozen PBMCs were thawed and incubated with or without IL-12 (1 ng/mL; R&D Systems) plus IL-18 (100 ng/mL R&D Systems) for 14 hours, followed by the addition of brefeldin A for 4 hours and stained for intracellular IFN-γ. For confirmation, levels of IFN-γ were measured using Ready-Set-Go ELISA kits (R&D) according to the manufacturer's instructions. All measurements were done in duplicate.

Statistical Analysis

Data are shown as mean and standard deviation (SD), median and range or number and proportion as appropriate. The distribution of *STAT4* rs7574865 genotypes between the different groups was compared using Fisher's exact test. The Cochran–Armitage test was used for assessment of trend. The Student's *t*-test or non-parametric, i.e. Wilcoxon-Mann-Whitney U-test or Kruskal-Wallis tests were used to compare quantitative data, as appropriate. All tests were two-tailed and p values <0.05 were considered significant. For rs7574865, a recessive model (GG versus GT/TT) was adopted as reported previously in the literature [11, 12]. Hardy-Weinberg equilibrium tests of *STAT4* rs7574865 in patients were performed using the chi squared test.

Binary logistic regression was used to evaluate the effect of *STAT4* rs7574865 on significant fibrosis (METAVIR score F2-4) and included other risk factors (age, gender, BMI, HBV-DNA and recruitment centre). The odds ratio estimates the relative change in the rate of the outcome per unit increase in the explanatory variable. HBV-DNA levels were log-transformed before entry into the model. Results are expressed as odds ratios and 95% confidence intervals (CI). Statistical analyses were performed using the statistical software package SPSS for Windows, version 21 (SPSS, Chicago, IL).

Results

Patient characteristics

The demographic, biochemical, and virologic characteristics of the studied CHB patients are presented in **Supplementary Table 1**. The median age was 45 years and 72% were male. 384 (46%) patients had moderate to severe inflammation (A2-A3) and 281 (34%) had advanced fibrosis (METAVIR score F3-4).

STAT4 rs7574865 minor allele frequency in the HBV cohort

The genotype distribution of *STAT4* rs7574865 in CHB patients and in the healthy Caucasian population is shown in **Supplementary Table 2**. Genotype distribution was in Hardy-Weinberg equilibrium in both groups (p=0.6 and 0.9, respectively). The minor allele (T) frequency (MAF) of rs7574865 was 0.19, significantly lower than that observed in a reference Caucasian population sample (MAF 0.23, http://browser.1000genomes.org) [20] and in our healthy cohort (MAF 0.24) (χ^2 for trend=p=0.04). This data is supportive for an association between STAT4 variation and persistent HBV infection.

Association of STAT4 rs7574865 genotype with clinical variables

Next, we explored if baseline clinical variables differed between CHB patients according to *STAT4* rs7574865 genotype; the results are presented in **Supplementary Table 3**. There was no evidence of significant association between rs7574865 genotype (GG versus GT/TT) and any of the clinical variables (i.e. age, BMI, baseline levels of ALT, AST, GGT, bilirubin, albumin or HBV DNA, gender frequency, HBe-Ag status and diabetes status).

STAT4 rs7574865 genotype and severity of hepatic inflammation

We assessed the association of rs7574865 with hepatic inflammation. The distribution of rs7574865 genotypes according to hepatic inflammation is depicted in **Figure 1A.** In multivariate ordinal regression analysis adjusted for covariates including age, gender, BMI, recruitment centre and viral load, rs7574865 was associated with the severity of necroinflammation (β=0.097±0.041, p=0.01) (**Supplementary Table 4**). In further analysis subdividing the cohort into those with mild (A0–1) versus severe hepatic inflammation (A2–A3), again the rs7574865 GG genotype was associated with severe hepatic inflammation by multiple logistic regression analysis adjusting for the same variables (OR: 1.42, 95% CI: 1.07-2.06, p=0.02) (**Table 1**).

STAT4 rs7574865 genotype and stage of liver fibrosis

We assessed the association of rs7574865 with fibrosis stage. The distribution of rs7574865 genotypes according to hepatic fibrosis is depicted in **Figure 1B.** In multivariate ordinal regression analysis adjusted for the covariates mentioned above, rs7574865 was associated with the severity of fibrosis (β=0.114±0.043, p=0.005) (**Supplementary Table 4**). In further multiple logistic regression analysis adjusting for the same variables after subdividing the cohort into those without advanced fibrosis (F0–F2) versus those with advanced fibrosis (F3–F4), rs7574865 GG genotype associated with F3-F4 fibrosis (OR: 1.83, 95% CI: 1.19-2.83, p=0.006) (**Table 1**).

STAT4 expression is decreased in HBV infected patients and in HBV-expressing cells

To explore the role of STAT4 in HBV pathogenesis, we first determined hepatic expression of STAT4 in patients with chronic HBV and in healthy controls using RT-PCR. HBV-infected patients exhibited significantly lower hepatic levels of STAT4 than healthy subjects (p<0.0001) (**Figure 2A**). To further investigate the association, we examined the expression of STAT4 in Huh-7 cells transiently

transfected with an HBV plasmid (wild type HBV, genotype D); RT-PCR was used to quantify expression (**Figure 2B**). Consistently, STAT4 mRNA levels after HBV-transfection was decreased compared to control uninfected cells transfected with SEAP expressing plasmid (**Figure 2C**). Thus, STAT4 expression is decreased during HBV infection and in cells transfected with an HBV plasmid.

Hepatic STAT4 is negatively correlates with markers of liver injury and inflammation

Based on the finding that hepatic expression of STAT4 is reduced in patients with chronic HBV, we explored the impact of disease progression on STAT4 expression and correlated the latter with clinical parameters. According to the Metavir inflammation/activity grade, patients with significant hepatic inflammation (A2-A3) had lower STAT4 mRNA levels than those with none or mild inflammation (A0–A1) (**Figure 2D**). Negative correlations were also found between hepatic STAT4 and several biochemical parameters of liver injury, including AST (r=-0.43, p=0.004) and ALT (r=-0.32, p=0.03) by Spearman's rank correlation. No significant correlation was observed with age, gender, BMI, Platelets, albumin, HB-DNA levels, HBe-Ag status or fibrosis stage.

To further tease out the STAT4 data from liver, we investigated STAT4 expression in primary human hepatocytes, Kupffer cells, hepatic sinusoidal endothelial cells and hepatic stellate cells by ultrasensitive droplet digital PCR (ddPCR). The highest expression of STAT4 was observed in Kupffer cells followed by hepatocytes, while stellate cells and sinusoidal cells had low expression (**Figure 2E**). Expression of STAT4 was also low in the human hepatic stellate cell line (LX2 cells), in both freshly plated and 1 day culture activated cells (threshold cycle=32) and was not significantly altered in response to the archetypal profibrotic cytokine (TGF-β) (**data not shown**). In total, these data suggest that the effect of STAT4 on liver inflammation is likely mediated by immune cells rather than direct effects on stellate cells.

STAT4 expression in liver is rs7574865 genotype dependent

To investigate potential mechanisms of rs7574865 function and given the intronic location of rs7574865, we considered that STAT4 expression might be modulated in an rs7574865 genotype–specific manner. To examine for this, we measured mRNA levels of STAT4 in liver biopsies from individuals with CHB (n=45) using quantitative RT-PCR stratified according to rs7574865 genotype. In this analysis, patients with the risk genotype GG at rs7574865 had lower mRNA levels of STAT4, compared to patients with the GT/TT genotype ($p< 0.05$) (**Figure 3A**).

rs7574865 regulates induction of pSTAT4 in NK Cells

To avoid the confounding effects of a chronic inflammatory state and/or disease fluctuation in HBV patients, we studied the impact of the risk genotype on STAT4 expression in a healthy population. To do this, we isolated PBMCs from healthy subjects and STAT4 expression was quantified by real-time PCR (n=30). As expected, STAT4 mRNA expression was reduced in subjects with the rs7574865 risk GG genotype compared to those with GT/TT genotypes ($p< 0.05$) (**Figure 3B**).

Next, we measured intracellular STAT4 protein by flow cytometry. Because NK cells are major contributors to innate IFN-γ production, and activation of STAT4 is important for peak responses [21], we focussed on these cells. Under basal (unstimulated) conditions, no difference in total STAT4 protein levels was observed between subjects with rs7574865 GG versus GT/TT genotypes (**Figure 3C**). We then reassessed STAT4 phosphorylation following stimulation of PBMCs with IL-12 plus IL-18; the CD56 NK cell population was studied by flow cytometry. After stimulation, there was significantly lower STAT4 phosphorylation in subjects with rs7574865 GG, compared to subjects with the GT/TT genotypes ($p< 0.05$) (**Figure 3D**). These data suggest reduced STAT4 activity in the risk rs7574865 GG genotype that is associated with increased hepatic inflammation and fibrosis.

IFN-γ Production by NK Cells is STAT4 rs7574865 genotype dependent

IFN-γ has well known anti-fibrotic effects mediated via inhibition of HSC activation and enhancement of NK cell cytotoxic activity towards HSCs [22]. IFN-γ also contributes to HBV clearance [23]. The ability of NK cells to produce IFN-γ in response to IL-12 is dependent on STAT4 phosphorylation [24]. Therefore, to evaluate whether IFN-γ secretion by NK cells is STAT4 rs7574865 genotype dependent and thus may ultimately lead, in the appropriate context, to genotype-dependent effects on fibrosis. To this end, PBMCs were stimulated with IL-12 and IL-18 and the CD56 NK cell population was studied by flow cytometry. As shown in **Figure 4A**, consistent with the decrease in *ex vivo* pSTAT4 in subjects with the rs7574865 GG risk genotype, there was a decrease in the percentage of IFN-γ production by NK cells. The latter was confirmed by ELISA for IFN-γ secretion in the medium (**Figure 4B**).

Discussion

We have demonstrated that the GG genotype at rs7574865 in the *STAT4* gene confers susceptibility to more severe liver injury and fibrosis in a large cohort of Caucasian patients with chronic hepatitis B. Individuals with this genotype also appear more likely to develop chronic HBV infection. Finally, we show that those with the risk GG genotype have lower STAT4 mRNA expression in liver and PBMCs, while their NK cells display decreased pSTAT4 upon *in vitro* stimulation with IL-12 and IL-18, with consequent functional deficiency in STAT4 dependent IFN-γ production. This model is summarized in a schematic **Figure 5**.

The rs7574865 polymorphism was originally identified by GWAS as a risk variant for HBV associated HCC in Chinese patients when compared to those with HBV and no HCC [11]. Subsequently, another study by the same authors suggested that rs7574865 is associated with the risk of cirrhosis based on computed tomography (CT) or ultrasonography in 712 Chinese patients with CHB [13]. However in that study, liver histology data were not available and hence, associations to earlier disease stages or to inflammation could not be discerned. Apart from demonstrating for the first time significant associations with hepatic inflammation grade and fibrosis stage, we observed a higher frequency of the risk (G) allele in Caucasians with CHB suggesting a role for this genetic variation in HBV persistence. In Asian populations, it has been reported that the GG genotype is more likely to have a lower rate of HBeAg seroconversion as well as response to IFN-α therapy than those with the (GT/TT) genotype (again, liver histology was not available) [12]. Hence, several levels of evidence in addition to the current data reinforce the notion that STAT4 is pivotal in determining the outcome of HBV infection.

In patients with CHB, NK cells exhibit a dichotomy in effector functions characterized by conserved or enhanced cytolytic activity but at the same time, reduced IFN-γ production [23]. The latter is

a major determinant of the anti-viral and anti-fibrotic capabilities of NK cells [23] and a determinant of immunomodulatory regulatory T cell (Treg) recruitment to the liver [25]. Thus, NK cell-derived IFN-γ plays a key role in promoting HBV viral clearance, [26] while it inhibits stellate cell activation and enhances NK cell cytotoxic activity towards these cells [22, 27, 28]. NKT cell-derived IFN-γ also triggers secretion of chemokine (C-X-C motif) ligand 10 (CXCL10) that in turn attracts Tregs to limit hepatic inflammation [25]. These functional data are supported by a recent study suggesting that IFN-γ production by intrahepatic NK cells is reduced in patients with advanced HBV-related liver fibrosis [29]. Notably, the ability of NK cells to produce IFN-γ in response to IL-12 is dependent on STAT4 phosphorylation [30].

Aside from genetic association studies, detailed functional mechanisms by which the *STAT4* rs7574865 variant contributes to CHB pathogenesis is unknown. In this work we extend knowledge to include functional genomics, demonstrating that the (G) risk allele is associated with lower STAT4 mRNA expression in liver and PBMCs, reduced STAT4 phosphorylation and reduced production of IFNγ by NK cells following *ex-vivo* stimulation. These effects likely contribute to impaired NK cell antiviral immune responses leading to virus persistence, and to the promotion of liver fibrosis and inflammation. The latter likely explains the negative correlations to indicators of liver cell injury and histological inflammation score that we observed. In support, knockout of STAT4 renders mice susceptible to concanavalin A-induced hepatitis [8], lethal endotoxemia [31], and pancreatitis-associated lung injury[32].

Another highlight of the present study is the differential role of rs7574865 in HBV and HCV infected Caucasian patients. In a recent study, we did not observe any association between rs7574865 genotype and spontaneous HCV viral clearance, response to IFN-α therapy, fibrosis severity or fibrosis progression (n=1211)[15]. At present it is not clear why STAT4 rs7574865 predicts liver injury only in

patients with CHB. Interestingly, in work utilizing laser capture microdissection from liver of patients with CHB and CHC, it was shown that STAT3, which has high amino acid sequence homology to STAT4 [33], was strongly upregulated in CHB, when compared to CHC [34]. STAT3 is a major mediator of HCC risk and suggests that the role of the JAK/STAT pathway in the tumorigenic process in CHB and CHC may differ.

In conclusion, we show that *STAT4* genetic variation at rs7574865 is associated with greater hepatic inflammation and fibrosis in Caucasian patients with chronic HBV infection; this effect is at least in part regulated by STAT4 expression and phosphorylation that differentially modulates IFNγ production by NK cells.

FIGURE LEGENDS

Figure 1: Association of *STAT4* rs7574865 genotypes with (A) inflammation degree and B) fibrosis stage (n=830). The rate of moderate/severe hepatic inflammation (METAVIR A2-A3) and advanced fibrosis (METAVIR F3-F4) is shown according to rs7574865 genotype. Numbers per each genotype is in parentheses under the genotype. P-values are univariate and provided for the recessive model of inheritance.

Figure 2: STAT4 expression is decreased in patients with HBV infection and in HBV-expressing cells. A) Comparison of hepatic STAT4 mRNA levels in hepatitis B patients (n=45) and controls (n=28). B) qRT-PCR was used to determine the HBV copy numbers in Huh-7 cells transiently transfected with plasmid HBV/Dw or control vector. C) mRNA levels of STAT4 in Huh-7 cells transiently transfected with plasmid HBV/Dw relative to GAPDH by qRT-PCR. D) Hepatic STAT4 mRNA levels according to hepatic inflammation score. The x axis represents hepatic inflammation dichotomized as absent/mild (METAVIR score A0-A1) (n=15) or moderate/severe (METAVIR score A2-A3) (n=30), and the y axis represents hepatic STAT4 expression relative to GAPDH by qRT-PCR. The number of independent samples tested in each group is shown in parentheses. Data are shown as median and interquartile range or mean and sem and the P-value was calculated using the two-tailed Mann–Whitney test or t-test. E) STAT4 mRNA expression in human primary hepatic cells types. Gene expression level was measured as number of copies/50 ng of total RNA by ultrasensitive droplet digital PCR (ddPCR) ddPCR. KC, kupffer cells; HH, human hepatocytes; HHSEC, human hepatic sinusoidal endothelial cell; HSC, hepatic stellate cells.

Figure 3: STAT4 expression according to rs7574865 genotype in liver and peripheral blood mononuclear cells (PBMCs). A) Association between *STAT4* rs7574865 genotype and hepatic STAT4 mRNA levels (n=45). The x axis shows the genotype at rs7574865 using a recessive model (GG versus GT/TT) (GG=28, GT/TT=17) and the y axis shows STAT4 expression level relative to GAPDH by qRT-PCR. B) Association between *STAT4* rs7574865 genotype and PBMC STAT4 mRNA levels (n=30). The x axis shows the genotype at rs7574865 using a recessive model (GG versus GT/TT) (GG=16, GT/TT=12) and the y axis shows STAT4 expression level relative to GAPDH by qRT-PCR. C) Summary of basal STAT4 levels in natural killer (NK) cells from subjects with the GG genotype (n=10) and GT/TT (n=10) as determined by flow cytometry. The mean fluorescence intensity of

STAT4 is plotted. D) Representative flow cytometry histograms showing interleukin (IL) 12 and 18 stimulated pSTAT4 staining in subjects with GG and GT/TT genotype. E) Summary of pSTAT4 inducibility reflects the pSTAT4 MFI of NK cells after *in vitro* stimulation of PBMCs with IL1-2/IL-18 normalized to the respective MFI of unstimulated cells in subjects with GG (n=7) and GT/TT genotypes (n=7). The number of independent samples tested in each group is shown in parentheses. Data are shown as median and interquartile range or mean; P-value was calculated using the two-tailed Mann–Whitney test.

Figure 4: The rs7574865 GG risk genotype has decreased pSTAT4- dependent IFN-γ production by NK cells. A) Frequency of NK cells that produced IFN-γ in response to *in vitro* stimulation with IL-12 and IL-18 was measured by flow cytometry and B) the increase in production of IFN-γ by stimulated NK cells over unstimulated cells was quantified in the supernatants by ELISA. Charts show median and interquartile range; the P-value was calculated using the two-tailed Mann–Whitney test.

Figure 5: Postulated mechanism based on the present study for the role of STAT4 rs7574865 genetic variation in regulating hepatitis B virus clearance, hepatic fibrosis and HCC.

Table 1. **Independent predictors of severe inflammation (≥A2) and advanced fibrosis (≥F3) by logistic regression analysis in the HBV patient cohort (n=830).**

	Severe inflammation (≥A2)			Advanced fibrosis (≥F3)		
	OR	**95% CI**	***P*- value**	**OR**	**95% CI**	***P*- value**
Age, years	1.01	1.001-1.03	0.03	1.06	1.04-1.08	0.0001
Gender, male	1.41	0.94-2.11	0.08	1.63	1.03-2.6	0.03
BMI, Kg/m^2	1.07	1.02-1.13	0.02	1.05	1.004-1.11	0.03
HBV-DNA (Log_{10} IU/mL)	1.08	0.99-1.17	0.057	1.13	1.03-1.24	0.01
STAT4 rs7574865	1.42	1.07-2.06	0.02	1.83	1.19-2.83	0.006

Genetic analyses were undertaken using a recessive model as previously reported. OR: odds ratios, 95% CI: 95% confidence interval. Multiple Logistic regression models were used to test the association of STAT4 rs7574865 with liver histology outcomes (inflammation and fibrosis). In addition to predictors shown here, models were adjusted for recruitment centre. For rs7574865 analysis, a recessive model (GG versus GT/TT) was adopted as reported previously in the literature because the GG genotype was found to be the only risk genotype in the recent HBV-related HCC GWAS. The reference group was defined by absent/mild (METAVIR score A0-A1) inflammation, and absence of advanced fibrosis (METAVIR F0-F2).

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Figure 1:

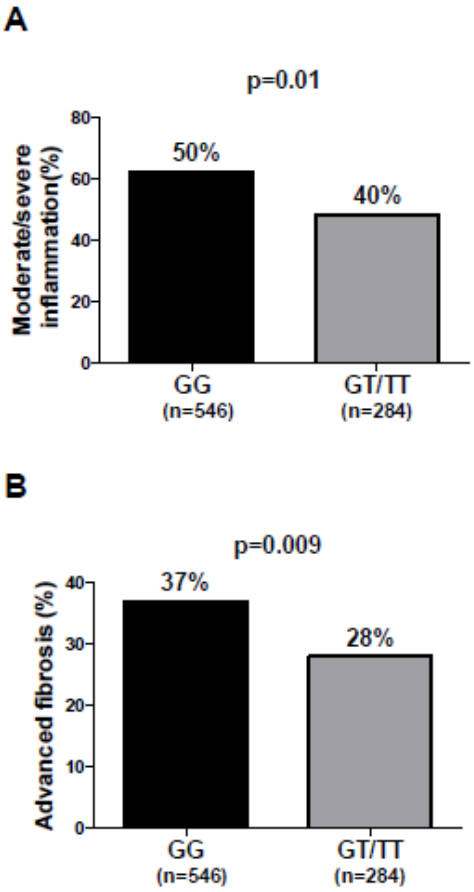

A
p=0.01
Moderate/severe inflammation(%)
80
60
40
20
0
50%
40%
GG
(n=546)
GT/TT
(n=284)
B
p=0.009
Advanced fibrosis (%)
40
30
20
10
0
37%
28%
GG
(n=546)
GT/TT
(n=284)

Figure 2:

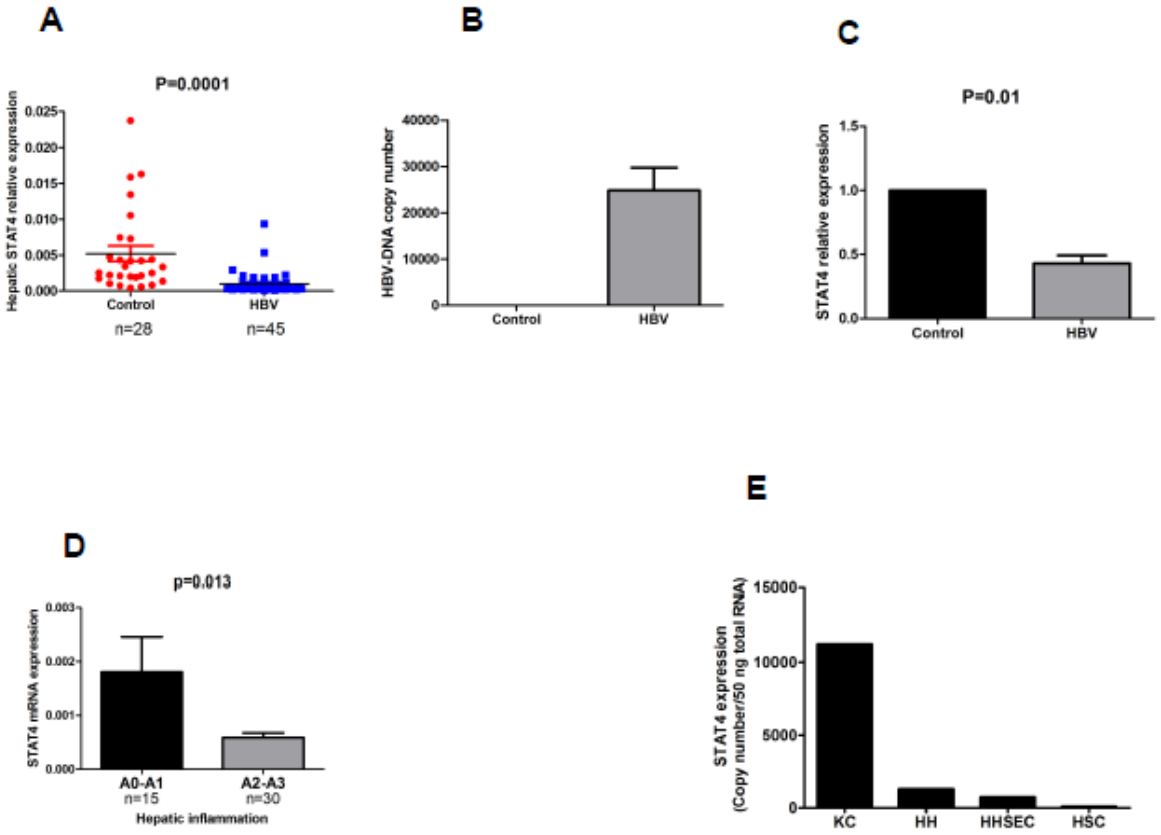

A
P=0.0001
Hepatic STAT4 relative expression
0.025
0.020
0.015
0.010
0.005
0.000
Control
HBV
n=28
n=45
B
HBV-DNA copy number
40000
30000
20000
10000
0
Control
HBV
C
P=0.01
STAT4 relative expression
1.5
1.0
0.5
0.0
Control
HBV
D
p=0.013
STAT4 mRNA expression
0.003
0.002
0.001
0.000
A0-A1
n=15
A2-A3
n=30
Hepatic inflammation
E
STAT4 expression
(Copy number/50 ng total RNA)
15000
10000
5000
0
KC
HH
HHSEC
HSC

Figure 3:

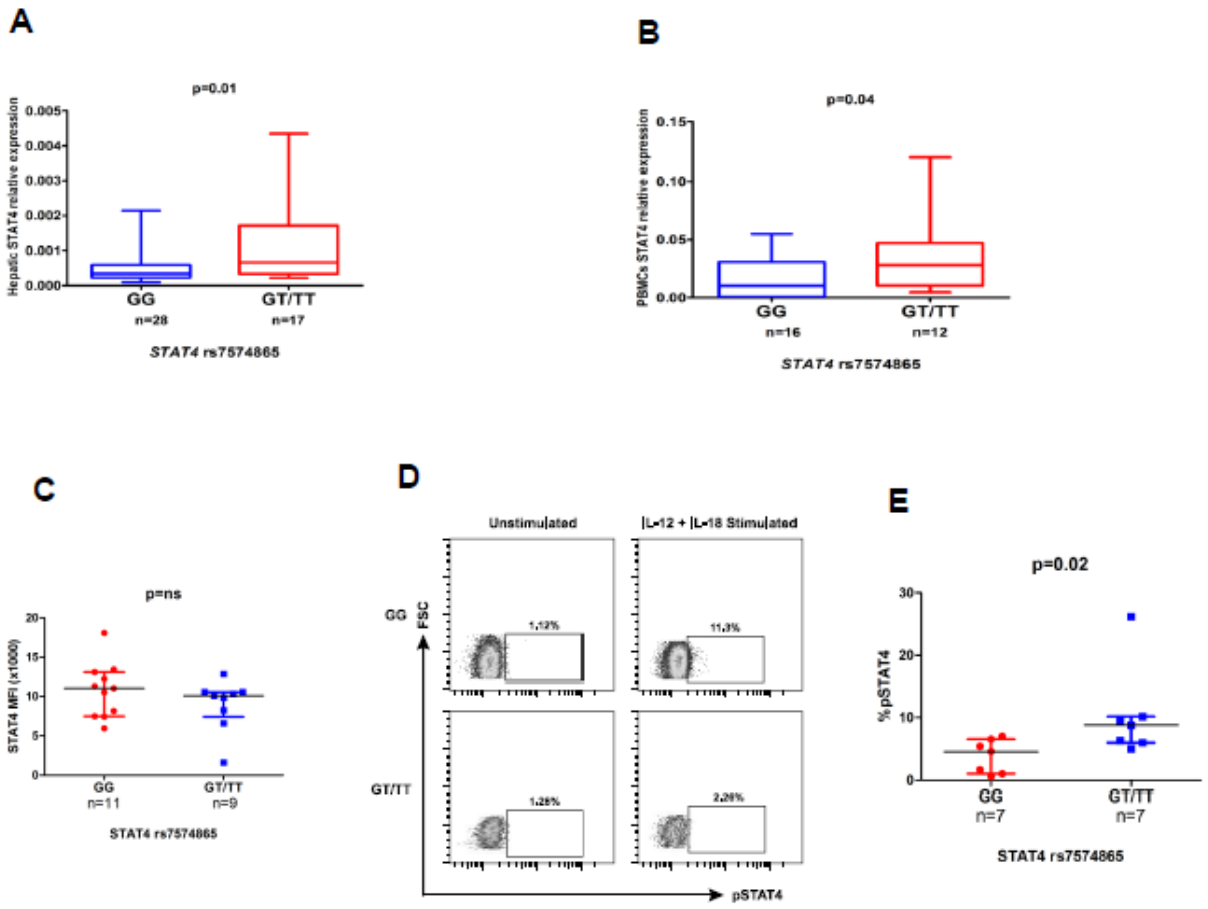
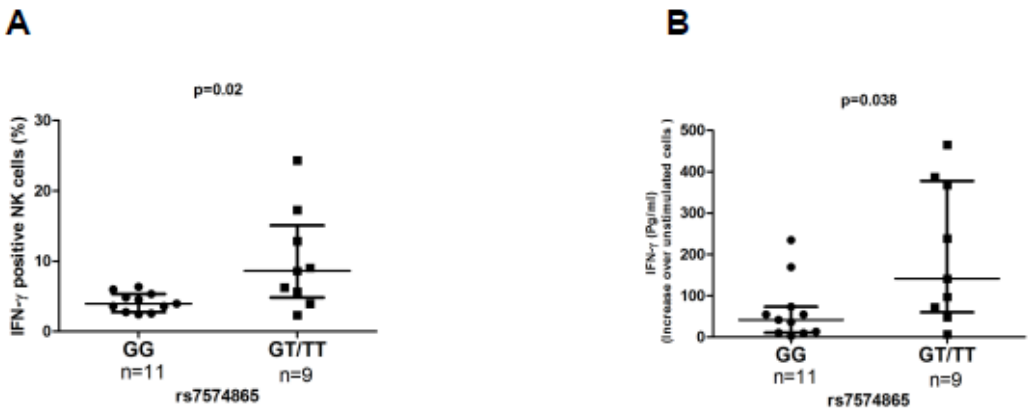


Figure 4:



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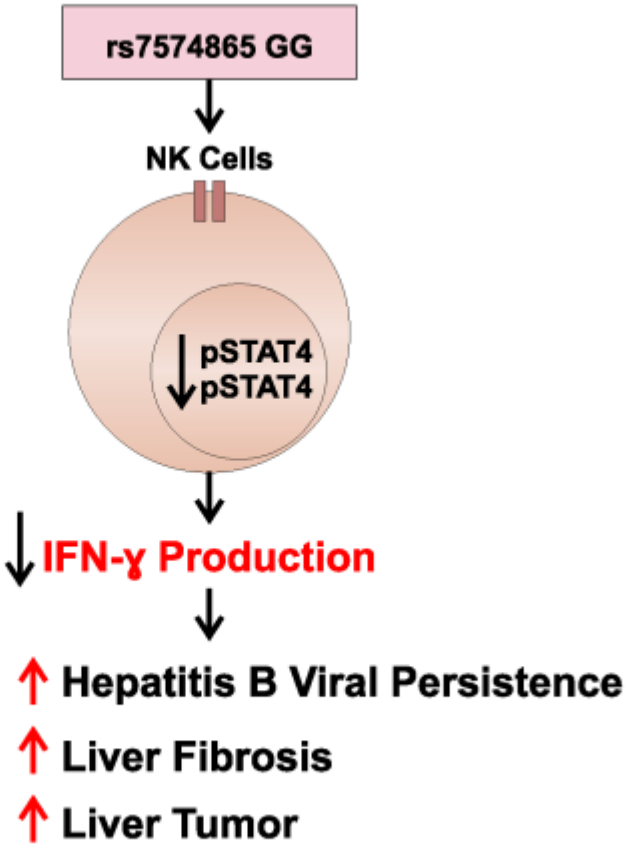
rs7574865 GG
NK Cells
pSTAT4
pSTAT4
IFN-γ Production
Hepatitis B Viral Persistence
Liver Fibrosis
Liver Tumor