Hepatitis E virus superinfection and human *SOCS3* and *ISG15* in hepatitis B virus-related liver diseases

Thesis submitted as requirement to fulfill the degree "Doctor of Philosophy" (Ph.D.)

at the
Faculty of Medicine
Eberhard Karls University
Tübingen

by

Xuan Hoan Nghiem

from

Hanoi, Vietnam

2017

Dean:	Professor Dr. B. Autenrieth
1. Reviewer:	Privatdozent Dr. Thirumalaisamy P. Velavan
2. Reviewer:	Professor Dr. M. Schindler

CONTENTS

1. INTRODUCTION 1

1.1. Hepatitis B virus infection 1

1.1.1. Epidemiology, transmission and prevention 1

1.1.2. Hepatitis B virus: structure, genome organization and viral proteins 3

1.1.3. HBV life cycle 6

1.1.4. Natural course of HBV infection 7

1.1.5. Host immune responses to HBV infection 11

1.1.6. Host genetic/epigenetics on HBV-related liver diseases 16

1.2. Hepatitis E virus infection 18

1.2.1. Epidemiology and transmission routes 18

1.2.2. Hepatitis E virus: structure and genome organization 19

1.2.3. Clinical pathology 20

1.2.4. Treatment and prevention 22

1.3. Scope and specific objectives 22

2. RESULTS AND DISCUSSION 24

2.1. Chapter 1: Hepatitis E virus superinfection in patients infected with hepatitis B virus 24

Hepatitis E virus superinfection and clinical progression in hepatitis B 24-35

patients (publication No.1)

2.2. Chapter 2: The role of human *SOCS3* and *ISG15* in HBV infection 36

SOCS3 genetic variants and promoter hypermethylation in patients with chronic hepatitis B (publication No.2) 36-55

Interferon-Stimulated gene 15 in hepatitis B-related liver diseases (publication No.3) 56-70

3. DISCUSSION 71

3.1. Hepatitis E virus superinfection in HBV patients 71

3.2. *SOCS3* and *ISG15* in HBV-related liver diseases 73

Conclusions 79

4. SUMMARY 81

5. ZUSAMMENFASSUNG 83

6. BIBLIOGRAPHY 85

7. DECLARATION OF CONTRIBUTIONS 108

8. ACKNOWLEDGEMENTS 110

9. CURRICULUM VITAE 111

1. INTRODUCTION

1.1. Hepatitis B virus infection

1.1.1. Epidemiology, transmission and prevention

Epidemiology

Hepatitis B virus (HBV) infection is one of the major public health problems affecting approximately two billion people worldwide. HBV infection causes a range of clinical manifestations that include asymptomatic carriers, acute hepatitis (AHB), chronic hepatitis (CHB), liver cirrhosis (LC), and hepatocellular carcinoma (HCC) (Lozano et al. 2012; Schweitzer et al. 2015; Trepo et al. 2014; WHO. 2017). An estimated 257 million people were reported to be chronically infected and approximately 887 000 deaths were reported in 2015 due to the complications of HBV infection (WHO. 2017). The infection gathers pace from CHB to LC and HCC over years and the current five-year cumulative risk for the development of HBV-related LC ranges between 10% and 20% (Liu et al. 2007). Furthermore, chronic HBV infection largely contributes to 50% of the HCC cases caused by a multitude of factors including hepatitis B and among those HCC cases, 70%-80% are reported in patients with HBV-related LC (El-Serag. 2011).

Taking into consideration global HBV prevalence, distinct geographical distribution can be observed that ranges between 0.1 and > 20% (Lok. 2002; Ott et al. 2012). The global endemicity of hepatitis B surface antigen (HBsAg) is classified in four specific groups, namely the low prevalence (<2%), low-intermediate (2-5%), high–intermediate (5-8%) and the high endemic (>8%) (Ott et al. 2012). A high burden of HBV infection is documented particularly in Asia, sub-Saharan Africa, and in countries of the Western Pacific (Ott et al. 2012; Schweitzer et al. 2015). Few countries in the Middle East, Eastern and Southern Europe, Southern America are with high-intermediate HBV prevalence, whereas low endemic zones include countries from Western and Northern Europe, North America, Central America, and

the Caribbean (Lok et al. 2001a; Lok et al. 2007). Vietnam is one of the South-East Asian countries, where HBV is highly endemic. The prevalence of chronic HBV infections ranges between 10% and 15% (Nguyen. 2012). HBV-related liver diseases are foreseen to become a significant public health burden in the next decades.

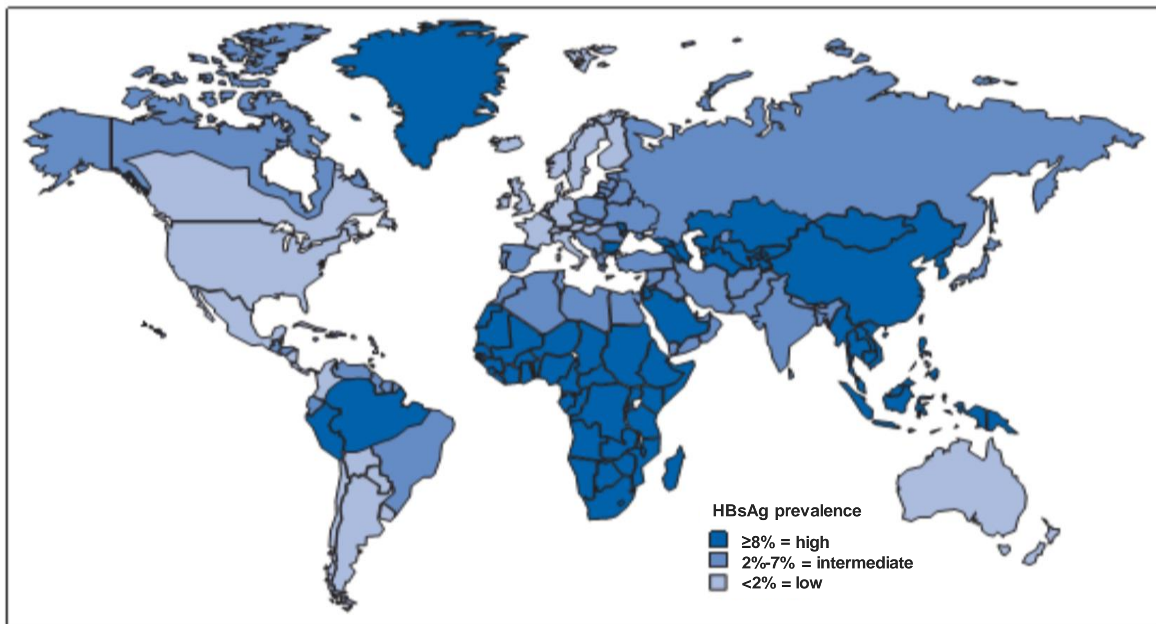


Figure 1: Geographic distribution of chronic HBV infection.

Source: *https://www.cdc.gov/hepatitis/hbv/pdfs/hbv_figure3map_08-27-08.pdf*

Transmission and prevention

HBV is a contagious virus that remains viable in the environment and can survive on inanimate surfaces for at least a week. Thus, transmission may occur even in the absence of visible blood or residue (Lok and McMahon. 2007; Stroffolini et al. 2007). HBV is spread predominantly by exposure to infected blood and other body fluids from infected persons. The frequency of infection and transmission vary in different regions of the world. For instance, in a HBV endemic area, the most common route of vertical transmission is perinatal (from mother to infant) and horizontal transmission occurs by person-to-person contact (CDC. 2015). Other modes of transmission reported in the spread of HBV infections are through drug use, sexual

and occupational exposure, blood transfusions, tattooing, and by acupuncture therapy. So far, there is no direct evidence that HBV can be spread through breastfeeding (WHO. 2001).

Although HBV infection remains highly endemic in certain parts of the world, global expansion of universal hepatitis B vaccination programs has effectively reduced the rates of acute and chronic HBV infections as well as related complications (Chang. 2006). The development of HBV vaccine using purified HBsAg protein as an immunogen to induce the protective antibody (anti-HBs), has paved the way for eliminating HBV infection. Active immunization with three to four regimens of HBV vaccine is 95% effective in preventing infection (WHO. 2015) and the incidence of HCC has been reduced to approximately 25%, since the launch of the vaccination program (Chang. 2006). Active HBV vaccine plus passive immunization with HBIG (hepatitis B immune globulin) showhigh effectiveness in infants and in HBeAg positive mothers (94% and 71% respectively) (Lo et al. 1985). Other measures to prevent horizontal transmission include screening among the donated blood, proper sterilization of injection needles and syringes, and any potential risk avoidance (tattoo, skin piercing).

1.1.2. Hepatitis B virus: structure, genome organization and viral proteins

HBV structure

HBV belongs to the genus *Orthohepadnavirus*, of the *Hepadnaviridae* family (Schaefer. 2007). HBV was first described in an Australian Aborigine in 1967 (Blumberg et al. 1967). Prior to the discovery of HBV, the hepatitis B surface antigen (HBsAg) known as the Australian antigen was isolated in patients who received large number of transfusions for the treatment of anemia (Blumberg et al. 1965). Subsequently, this antigen was linked to the particulate form of the so-called Dane particle (Cossart et al. 1970; Dane et al. 1970), which is the complete form of the HBV virion (H.C.Thomas et al. 2013; Sherlock. 1970). In addition to Dane particles,

sera of HBV-infected persons contain 1000-fold excessive amounts of sub-viral particles that are either 22 nm spheres or 22 nm-diameter filaments with variable lengths. These sub-viral particles do not contain genomic material and are therefore non-infectious.

Dane particles, spheres, and filaments consist of hepatitis B surface antigen (HBsAg). The hepatitis B surface protein comprises of three different protein domains, namely the S domain, the preS1, and the PreS2. The S domain encodes the small HBs protein (HBSs), whereas the preS1 and preS2 encode the large and middle HBs protein (LHBs and MHBs), respectively. Dane particle has an inner capsid, which includes the 3.2kb of the viral dsDNA. Each virion also contains kinase and heat shock proteins hsp70 and hsp90.

Genome organization and viral proteins

The HBV genome is composed of partial double-stranded DNA, also known as relaxed-circular DNA. Neither of the two strands of this relaxed circular DNA is covalently closed. The minus strand which is complementary to pre-genomic RNA, has a short terminal redundancy and carries the whole genome (3.2kb). The incomplete noncoding positive strand is heterogeneous in length with 3' end terminating hundreds of nucleotides (H.C.Thomas et al. 2013). The 5' end of the minus strand is located in the DR1 (direct repeats), while the positive strand starts at the DR2. The repeats are involved in priming the synthesis of the respective DNA strands. On the genome, six start codons, four promoters, and two transcription elements were identified. The four distinct open reading frames (ORF) are PreS/S, Precore/core, P and X. The ORF preS/S encodes the three viral surface proteins (LHBs, MHBs and SHBs or HBsAg), depending on the usage of one of the three translational start codons. HBsAg is the main target of host immune response during HBV infection. The ORF precore/core encodes core protein known as HBcAg and non-structural precore protein known as HBeAg. The ORF P is the longest HBV ORF and overlaps, at least partially, with all other HBV ORFs. It encodes a multifunctional

viral DNA polymerase (Pol, 90 kd) with RNAase H activities and terminal protein (TP). The ORF X codes for the small regulatory X protein, which has been shown to be essential in vivo for viral replication and is capable of transactivating numerous cellular and viral genes (Lucifora et al. 2011). The two enhancer elements (Enh I and Enh II) control the four defined overlapping open reading frames (pre-S/S, core/e, X, and P gene), which promote transcription and expression of the seven different hepatitis B virus proteins.

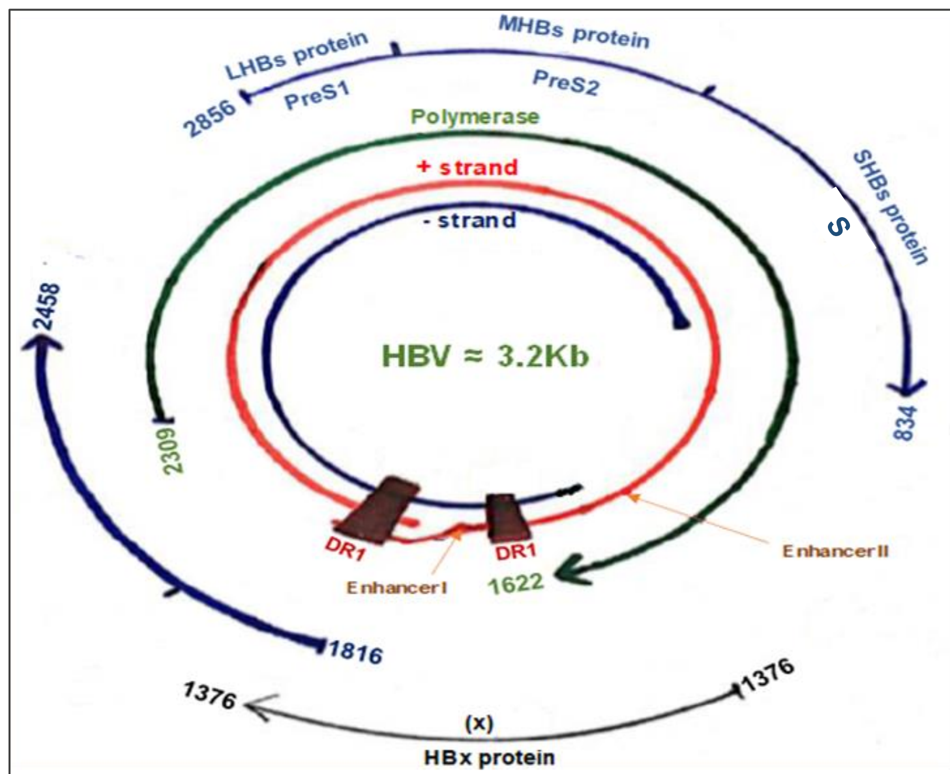


Figure 2. HBV-DNA structure and genome organization. The HBV genome is ~3.2 kb in size with a circular partially double-stranded DNA composed of a complete coding minus strand (–) and an incomplete noncoding positive strand (+) with variable 3 ′end size. Two direct repeats (DRs) of 11 nucleotides (DR1 and DR2) are located at the 5 ′ends of both strands. The viral genome contains four promoters and two transcription enhancers (Enh1 and Eh2), which control the transcription of four genes (preC/C, P, preS/S, and X). This figure is redrawn and adapted from the original picture (Kao. 2011).

1.1.3. HBV life cycle

During the last 30 years, the HBV replication in hepatocytes has been extensively studied to understand the mechanism of infection (Allweiss et al. 2016; Schultz et al. 2004). The viral replication takes place entirely in the hepatocytes and is illustrated in Figure 3. The virus entry is initiated by the interaction of surface lipoproteins with the host specific receptors on the liver cells. HBV uses the carbohydrate side chains of hepatocyte-associated heparan sulfate proteoglycans as receptors (Leistner et al. 2008; Schulze et al. 2007). This interaction requires the integrity of LHBs protein (Engelke et al. 2006; Gripon et al. 2005; Meier et al. 2013). Studies have demonstrated that HBV and HDV (hepatitis D virus) can be blocked by the competition of a small protein (Myrcludex-B; described as HBVpreS/2-48^{myr}), which contains the same amino acid sequences as LHBs (Glebe et al. 2007; Lutgehetmann et al. 2012; Petersen et al. 2008). Recently, the sodium taurocholate co-transporting polypeptide (NTCP) has been identified as a cellular entry receptor for HBV (Yan et al. 2012, 2014). This entry mechanism is inhibited by myrcludex-B, cyclosporin A and NTCP inhibitors (Watashi et al. 2014)

Following entry into hepatocytes, HBV undergoes uncoating of its capsid and releases its relaxed circular-DNA. The relaxed circular-DNA is then translocated to the nucleus, where it is converted to covalently close circular DNA (cccDNA). This intra-nuclear and episomal form of the viral genome serves as a transcriptional template for viral RNAs and subsequent generation as virions. Thus, the cccDNA is crucial for HBV replication and persistence of HBV infection (Levrero et al. 2009a). In short, cccDNA functions as the template for the transcription of four viral RNAs (of 0.7 kb, 2.1 kb, 2.4 kb and 3.5 kb), which are exported to the cytoplasm and used as mRNAs for the translation of the seven HBV proteins. The longest pre-genomic RNA (3.5 kb) functions as the template for replication, which occurs within nucleocapsids in the cytoplasm. Nucleocapsids are enveloped to become the complete viral particles. The mature HBV virions will be released from the cells through the endoplasmic reticulum and/or golgi complex (Rehermann et al. 2005).

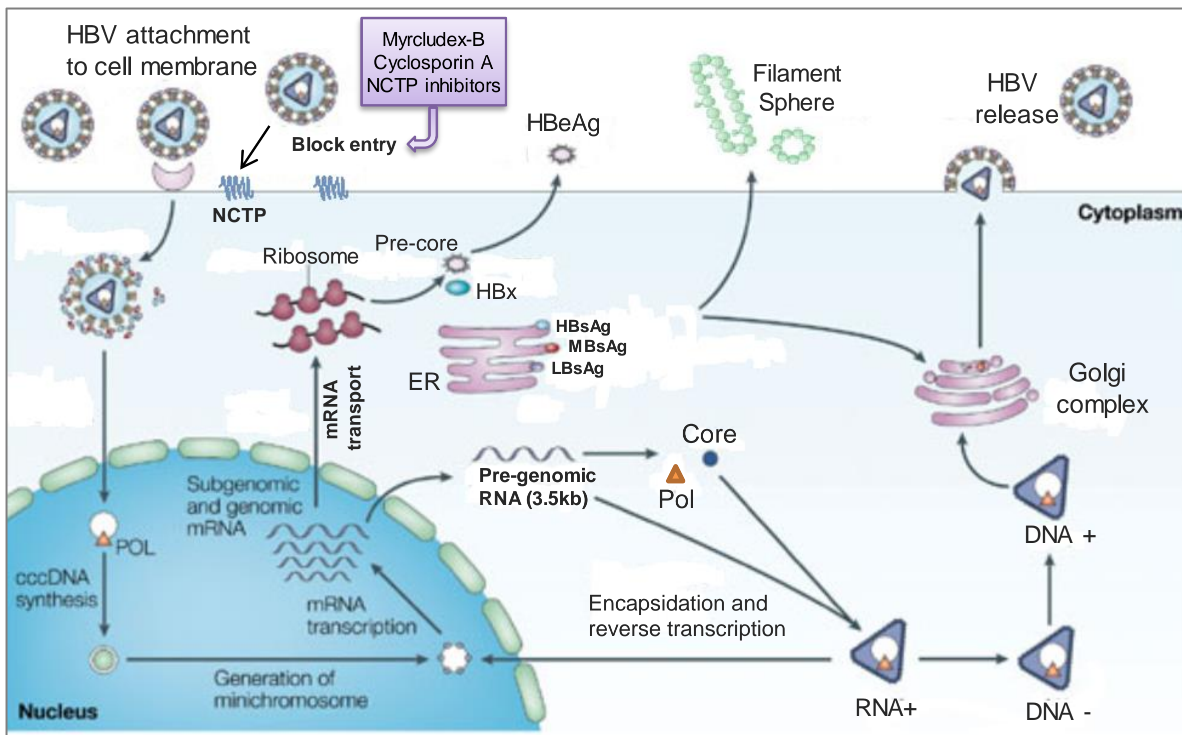


Figure 3. The replication cycle of hepatitis B virus in the hepatocytes and the inhibitors of HBV attachment to the cell membrane. This picture is adapted and used with the permission from (Rehermann et al. 2005).

1.1.4. Natural course of HBV infection

Children are at greater risk of being infected with HBV. Over 90% of infants were infected from mothers and 30–50% of children were infected before the age of 6 years and develop chronic HBV infections. In contrast, ~5% of immunocompetent adults will progress to chronic infection. (Dandri et al. 2012; Fattovich. 2003; Ganem et al. 2004; WHO. 2015). HBV infection can lead to inflammation of the liver with variable severity, in both acute and chronic phases. During the acute phase, clinical manifestations vary from a self-limiting, transient liver disease to an anicteric and icteric hepatitis, and fulminant hepatitis in some cases. During the chronic phase, clinical manifestations range from an asymptomatic carrier state to chronic hepatitis, cirrhosis and HCC (Fattovich. 2003; Fattovich et al. 2008; Hadziyannis. 2011; Lok et al. 2001b; Villeneuve. 2005).

Acute hepatitis: After exposure, the incubation of HBV lasts from 1 to 4 months. During this period, flu-like syndrome develop. Fever, skin rash, fatigue, typically arthralgia occur and usually cease with the onset of hepatitis. At least 70% of patients have subclinical or anicteric hepatitis and less than 30% will develop an icteric hepatitis (S.Mauss et al. 2015). In typical cases of acute hepatitis, abnormal elevation of liver enzymes (alanine and aspartate aminotransferases: ALT and AST) and bilirubin were detected in the serum. In addition, HBV-DNA levels are usually high and several HBV-related markers were also present in the serum including HBsAg, HBeAg, followed by anti-HBe and anti-HBc antibodies (Figure 4A). In acute self-limiting hepatitis, sero-conversion of HBeAg and HBsAg to anti-HBe and anti-HBs as well as absence of HBV DNA from the serum occur within about six months after exposure. Fulminant hepatic failure is an unusual event that occurs in 0.1-0.5% of patients with acute hepatitis. The pathology of fulminant hepatitis is not well understood. However, it is believed to be immune-mediated that might be a result of interaction between the virus and host immune response (S.Mauss et al. 2015).

Chronic hepatitis: The natural course of chronic hepatitis B is age dependent and varies between individuals, depending on the interaction between the virus and host responses. In general, chronic hepatitis B has been well characterized into four different phases in parentally acquired infections namely; the immune-tolerant, immune clearance, inactive carrier state, and an immune-reactive phase (Figure 4B).

The immune-tolerant phase lasts between 10 and 30 years with high levels of HBV replication (HBeAg positive and HBV-DNA >10^7-10^8 copies/ml), low levels of liver necroinflammation (biopsies are without signs of significant inflammation or fibrosis), and with no evidence of active liver disease (normal ALT concentration) (Fattovich. 2003; Fattovich et al. 2008; Ganem and Prince. 2004; Sarin et al. 2016; Terrault et al. 2016). During this phase, the spontaneous clearance rate of HBeAg is expected to occur between 15-20% (S.Mauss et al. 2015).

The HBeAg-positive immune-active phase or immune clearance is characterized by irregular HBV-DNA levels and ALT levels in the serum (Figure 4B) and hepatic necroinflammation (Fattovich. 2003; Fattovich et al. 2008; Ganem and Prince. 2004; S.Mauss et al. 2015; Sarin et al. 2016; Terrault et al. 2016). During this phase, HBeAg sero-conversion may occur. The rate of seroconversion is less than 2% per year in children < 3 years of age and among adults this may range between 8% to 12% per year (Terrault et al. 2016). Some patients will develop acute hepatitis or fulminant hepatic failure as a result of the high levels of immune-mediated lysis of infected hepatocytes (Elgouhari et al. 2008). Also in this phase, periodic loss of serum HBV-DNA and HBeAg also occur (S.Mauss et al. 2015).

The inactive carrier state is an outcome of the immune-active phase, marked by sero-conversion from HBe antigenemia to anti-HBe antibody positivity. During this phase, one may observe low and/or undetectable HBV-DNA with normal ALT levels, and minimal necro-inflammation with variable fibrosis, reflecting previous liver injury during the immune-active phase (Fattovich. 2003; Ganem and Prince. 2004; S.Mauss et al. 2015; Sarin et al. 2016). Approximately between 4% and 20% of inactive carriers have one or more reversions back to HBeAg positivity (Terrault et al. 2016). However, a few clinical-based cross-sectional studies have demonstrated that relatively few patients in the inactive HBV phase have experiencedmoderate to severe liver fibrosis (Kumar et al. 2008a; McMahon. 2009).

The HBeAg-negative immune-reactive phase is characterized by elevated ALT levels from moderate to severe necroinflammation with HBeAg negativity, anti-HBe positivity and detectable HBV-DNA (Sarin et al. 2016). As HBV cccDNA persists in hepatocytes, reactivation of HBV can be spontaneously triggered during or after inactive carrier state (Fattovich et al. 2008). Reactivation of viral replication may occur due to reactivation with the wild type virus or much more frequently with HBV variants that prevent HBeAg expression. The precore mutation produces a stop codon in a region of the HBV genome that prevents the formation of HBeAg, whereas the basal core promoter mutation affects HBeAg transcription (Chen et al. 2011;

Jammeh et al. 2008; Tong et al. 2016). These mutations allow HBV replication in the absence of HBeAg.

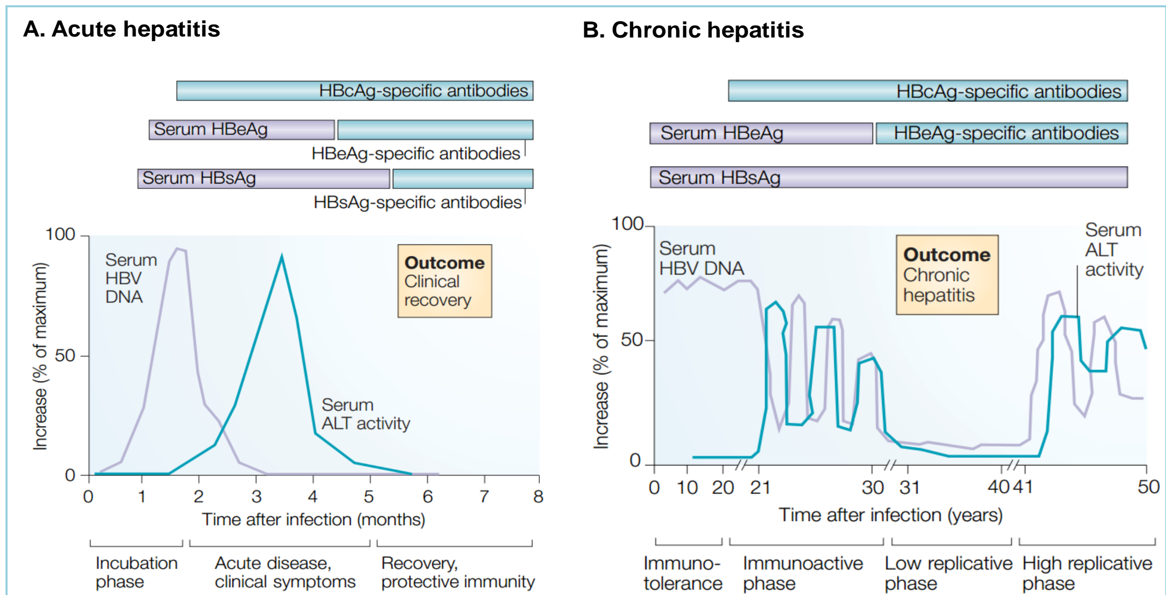


Figure 4. Clinical, serological and virological course of HBV infection. This picture is adapted and used with the permission from (Rehermann et al. 2005).

New nomenclature redefined for the chronic hepatitis B infection

A recent guideline for the nomenclature for the chronic states (European Association for the Study of the Liver. Electronic address et al. 2017) has been released. The natural course of chronic HBV infection is now classified into five distinct phases, considering the presence of HBeAg, HBV DNA levels, ALT values and eventually the presence or absence of liver inflammation. The new nomenclature is based on the description of the two main characteristics: infection versus hepatitis. The phases of chronic HBV infection are not necessarily sequential but includes: ***Phase 1***: HBeAg-positive chronic HBV infection, previously termed ‘‘immune tolerant’’; ***Phase 2***: HBeAg-positive chronic hepatitis B; ***Phase 3***: HBeAg-negative chronic HBV infection, previously termed ‘inactive carrier’; ***Phase 4:*** HBeAg-negative chronic hepatitis B; ***Phase 5:*** HBsAg-negative phase is characterized by the absence of HBsAg and presence of antibodies to HBcAg (anti-HBc) in the serum, with or without

detectable antibodies to HBsAg (anti-HBs). This phase is also known as ''*occult HBV infection*".

1.1.5. Host immune responses to HBV infection

Innate immune responses to HBV infection

HBV is a noncytopathic virus as seen in a number of asymptomatic HBV carriers who have minimal hepatocellular injury as well as liver necroinflammation, despite high levels of HBV replication. Most of the clinical syndromes are as a result of interaction between the virus and the host defense (Chang et al. 2007; Guidotti et al. 2006). Following HBV infection, pathogens are recognized by a subset of immune receptors so-called pattern recognition receptors (PRRs) e.g. toll-like receptors (TLRs) (Akira et al. 2006; Boehme et al. 2004) initiating the process of antiviral responses of innate immune system, which functions as the first line of defense. Innate immune system consists of several components including epithelial barriers, innate immune host cells [phagocytes: granulocytes, monocytes, macrophages; natural killer (NK) cells, natural killer T (NKT) cells, dendritic cells (DC), kupffer cells, the complement system, and cytokines released as cellular responses.

Cytokines play a key role in cell to cell communication and are required for the defense against hepatitis viruses and determines liver disease progression (Dunn et al. 2007; Koziel. 1999a; Larrubia et al. 2009). It is widely believed that interferons (IFNs) are the most important cytokines, which are produced by interferon-producing cells in response to viral infections (Colonna et al. 2002). These cells were primarily termed "plasmacytoid T cells" because of their location and abundance in the lymph nodes and in their expression of CD4 (Kadowaki et al. 2002; Vollenweider et al. 1983). Today, many host cells (innate and adaptive immune cells) were shown to induce IFNs. Interferons are divided into type I (IFN-α/β), type II (IFN-γ), and type III (IFN-λ). Type I IFNs are produced by most innate cells and affect most phases of cycle including entry/uncoating, transcription, RNA stability,

translation, maturation, assembly, and release. Type II interferon (IFN-γ) is exclusively produced by T cells and NK cells and is often considered in the context of adaptive immune response as a key antiviral T-cell cytokine (Koyama et al. 2008). Type III IFNs bind to receptors distinct from type I IFNs and activate intracellular signals, which overlap with IFN-α/β in downstream JAK/STAT signaling pathway (Donnelly et al. 2010).

During HBV infection, the secretion of IFNs and other cytokines (such as IL-2, IL-6, IL-10 and IL-12) is controlled by suppressors of cytokine signaling (SOCS) family proteins (CISH and SOCS1 to SOCS7). These SOCS proteins belong to a classical negative feedback system that regulates cytokine transduction via JAK/STAT signaling pathway (Carow et al. 2014; Yoshimura et al. 2007a). Of these, SOCS family proteins, suppressor of cytokine SOCS3 was shown as a key regulator in regulating various intracellular activities. Subsequently, SOCS3 can inhibit the process of cell proliferation and cell survival through inhibition of STAT3, which is an oncogene by itself (Levy et al. 2006; Wake et al. 2015; Yasukawa et al. 2003; Yoshimura et al. 2007a; Yu et al. 2007; Yu et al. 2009). In chronic HBV infection, the persistent activation of STAT3 mediated by JAK/STAT signaling pathway was shown to induce inflammation and subsequent carcinogenesis (Jiang et al. 2011; Yang et al. 2016; Yu et al. 2007).

IFNs mediate their signal transduction by binding to their cell surface receptors (IFNAR) activating the downstream IFN-signaling pathway. This process leads to the induction of numerous IFN-stimulated genes (ISGs) that activate a wide biologic responses (e.g. antiviral, immune modulatory, antiproliferative, antiangiogenic, and antitumor effects) (Platanias. 2005). Among these ISGs, the ubiquitin-like molecule, ISG15, is one of the most strongly induced proteins linked to a variety of cellular proteins, suggesting regulation of different cellular processes.

ISG15 is a major effector molecule of the innate immune responses (Estrabaud et al. 2010). Although ISG15 was discovered early, the anti-viral effects of ISG15 were

only extensively studied in recent years. ISG15 induces type I interferon responses and exert responses against many viruses including Influenza, HIV-1, Ebola and HPV (Campbell et al. 2013b). ISG15 also plays an important role in several immunomodulatory activities, including induction of natural killer (NK) cell proliferation, triggering the T-cell reaction, augmentation of lymphokine-activated killer activity, stimulating IFN-γ production, inducing dendritic cell maturation and also functions as a chemotactic factor for neutrophils (Lenschow. 2010). Although ISG15 is a 15 kDa protein with a sequence homology and immune-cross reactivity to ubiquitin (Ub), limited knowledge still exists on the role of ISG15 and its conjugates towards antiviral activities and on carcinogenesis (Campbell and Lenschow. 2013b; Desai. 2015a; Jeon et al. 2010a; Skaug et al. 2010).

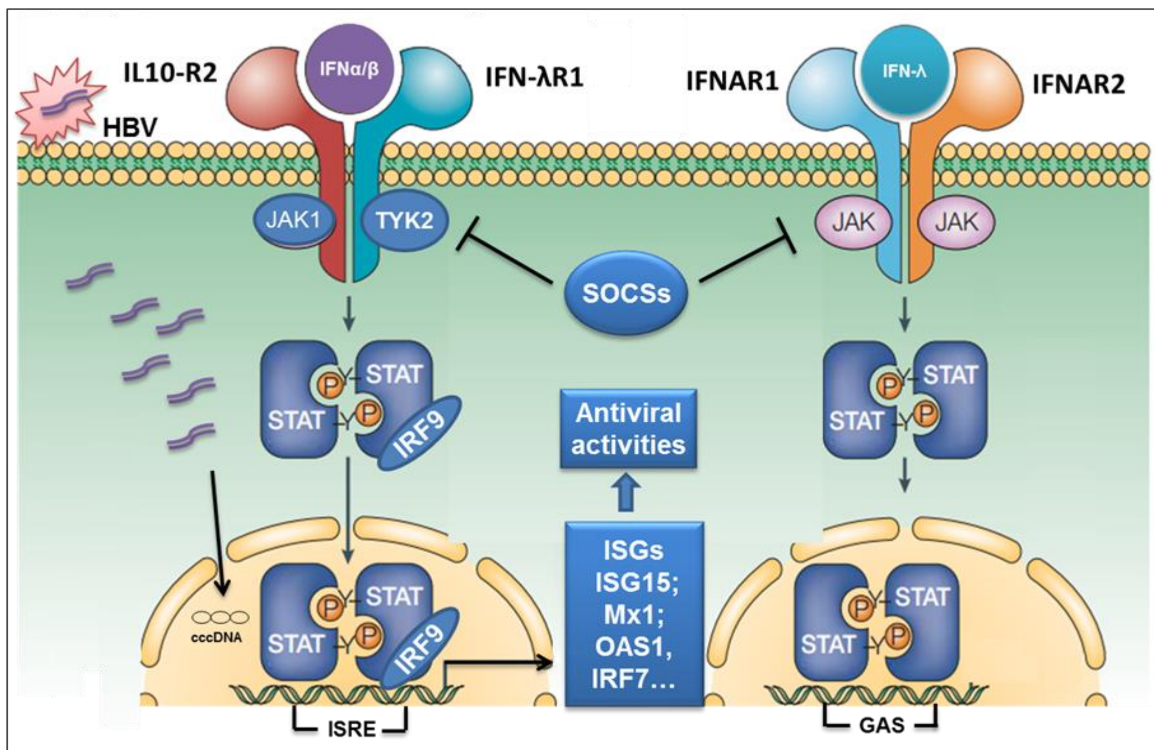


Figure 5. Innate immune response to HBV infection through JAK/STAT signaling pathway. During HBV infection, type I interferons (IFN α/β/γ) bind to specific surface receptors and induce antiviral effects by activating the JAK/STAT signaling pathway. In brief: the binding of IFNs to receptors activate both janus kinase 1 (JAK1) and tyrosine kinase 2

(TYK2). The activated JAK1 and TYK2 phosphorylate tyrosine residues remain as the binding site for STAT proteins. Two activated STATs form hetero-or homodimer complexes, which then translocates into the nucleus and acts as an active transcription factor. Generally, IFN regulatory factor 9 binds to STAT1/STAT2 heterodimers to form the transcription complex. This complex translocates into nucleus and binds to IFN stimulatory response elements (ISREs) within the promoters of ISGs to produce hundreds of ISG proteins. In addition, the homo and heterodimers of STAT1 and STAT3 bind to gamma activated response elements. These ISG proteins play key roles in the induction of innate and adaptive immune responses. This figure was redrawn and adapted from (Gao et al. 2012).

Adaptive immune response to HBV infection

Adaptive immunity is mediated by humoral B cell and cellular T cell responses that are activated by antigen-presenting cells including Kupffer cells and, in particular, the dendritic cells.

T-cell response

The cellular immune response is believed to play a role in disease pathogenesis during HBV infection which is mediated by $CD8^+$ and $CD4^+$ T cell activity. (Chang and Lewin. 2007; Chisari et al. 1995; Guidotti et al. 1999; Rehermann and Nascimbeni. 2005; Shuai et al. 2016). The naive T cells are usually activated by antigen-presenting cells in the lymph nodes and other lymphoid organs however, these T cells can readily be detected in peripheral blood (Maini et al. 2000; Maini et al. 1999; Shuai et al. 2016) and can also be activated locally in the liver during acute HBV infection (Sprengers et al. 2006). HBV clearance is mediated by the destruction of infected hepatocytes by the antiviral effects (cytopathic and perhaps more importantly by noncytolytic) of HBV-specific cytotoxic T lymphocytes (CTLs) (Guidotti and Chisari. 2006; Guidotti et al. 1999; Schuch et al. 2014).

HBV-specific CTLs are associated with HBV resolution by producing several anti-viral cytokines during acute infection (Guidotti et al. 1996; Kakimi et al. 2000; Phillips

et al. 2010; Webster et al. 2000). These cytokines can suppress HBV gene expression and replication. In contrast, the HBV-specific CTL responses are weak during chronic HBV infection (Bertoletti et al. 2003; Chang and Lewin. 2007; Phillips et al. 2010; Webster et al. 2000; Yang et al. 2010). The liver injuries are associated with the activity of CTLs (Guidotti et al. 1999; Thimme et al. 2003). The dysfunction of HBV-specific CTL responses can aggravate the liver damage (Kakimi et al. 2001) and subsequent liver disease progression in patients with chronic hepatitis B (Thimme et al. 2003).

Increasing knowledge on immune regulation contributes to HBV chronic infection and disease progression. One such immune regulation is the involvement of the regulatory T cells ($CD4^+$, $CD25^+$ T_{reg} cells) which suppresses the HBV-specific T cell responses. In chronic HBV patients, these regulatory T cells are observed to be in increased numbers in peripheral blood. The presence of HBV-specific Tregs could lead to an inadequate immune response against the virus, and thus establishment of a chronic infection (Peng et al. 2008; Stoop et al. 2005; Xu et al. 2006).

B-cell response

B cells mediate the humoral adaptive immune responses by producing antibodies that are antigen-specific. Therefore, the production of anti-HBs antibodies is key in in the clearance of HBV and protection against reinfection. The anti-HBs antibodies can limit the viral load, spread, andresolve infections either by direct binding to the virions or by blocking viral entry. Protection by neutralizing anti-HBs antibodies remains a basis for successful vaccine implementation and passive immunization by HBIG (hepatitis B immune globulins) for newborns from HBV infected mothers. It has been demonstrated that lack of HBsAg-specific B cells and anti-HBsAg antibodies are associated with HBV persistence (Barnaba et al. 1985; Bocher et al. 1996) and that the restoration of B-cell hyperactivation and HBsAg-specific B-cell impairment was associated with HBsAg seroconversion in chronic HBV infection (Oliviero et al. 2011; Xu et al. 2015).

1.1.6. Host genetic/epigenetics on HBV-related liver diseases

The clinical outcome HBV infection is markedly heterogeneous and is a consequence of the complex interaction between viral and host factors in patients with persistent HBV infection. The factors influencing the progression are age of the patient, the duration of the HBV infection, HBV genotypes, and various host and viral factors. It has been widely accepted that host genetic factors influence the outcome of a given disease. Substantial evidences from the twin study (Lin et al. 1989) and genome wide association studies (GWASs) (Al-Qahtani et al. 2013; Chang et al. 2014; Hu et al. 2013; Jiang et al. 2013; Kim et al. 2013; Li et al. 2012; Mbarek et al. 2011; Nishida et al. 2014; Nishida et al. 2012) were documented. The GWASs examined the association of large number of genetic variants across the entire human genome with a specific disease phenotype (Thursz et al. 2011). Recently, many candidate genes were identified, which are significantly associated with the susceptibility to HBV infection, persistence of the viruses, liver diseases, and HCC development.

HCC is believed to develop from chronic liver injury, inflammation, and cirrhosis and is considered to be multifactorial. Transformation of normal hepatocytes to malignancy is associated with altered gene expression and accumulated genetic alterations. Among genetic alterations, DNA methylation is of particular interest and can aid in early detection of carcinogenesis (Mikeska et al. 2014). DNA methylation is a multistep event, which occurs at the early stage of HBV infection (Zhao et al. 2014). As a normal process, methylation of DNA cytosine residues at the carbon 5 position (5 mC) in cytosine-guanine dinucleotides (CpG sites) is believed to be essential for cell differentiation and embryonic development, playing important roles in regulation of gene expression. In carcinogenesis, tumor suppressor genes (TSGs) are often silenced following hypermethylation of CpG islands located in the promoter regions (Herman et al. 2003). There is increasing evidence that support the notion that hypermethylation in the CpG islands of TSG promoter region can be a prognostic indicator in many cancers (Mikeska and Craig. 2014; Ozen et al. 2013;

Rakyan et al. 2011; Tischoff et al. 2007). Studies on DNA hypermethylation in HCC tumor and adjacent tissues have identified several candidate genes involvement, including GSTP1, RASSF1A, APC, SOCS1, E-cadherin, and EDKN2A (Gao et al. 2008; Hernandez-Vargas et al. 2010; Li et al. 2010; Lou et al. 2009; Moribe et al. 2009; Shen et al. 2012; Um et al. 2011; Yong et al. 2016; Zhong et al. 2002).

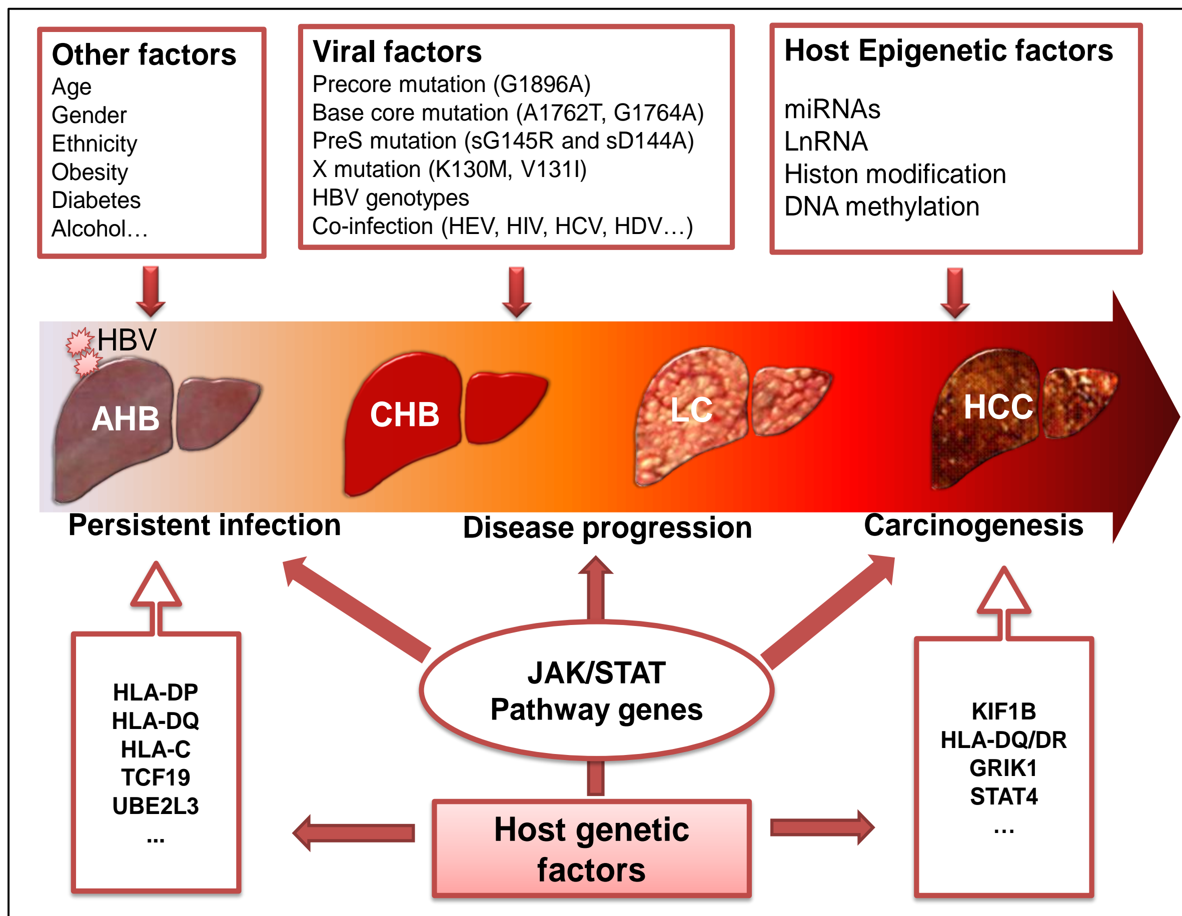


Figure 6. The contribution of host genetic/epigenetic factors to the clinical course in patients with HBV infection. The human genes presented in the boxes have been identified as associated with susceptibility to persistent HBV infection, disease progression, and hepatocarcinogenesis by GWAS (genome-wide association studies). In addition, mutations in HBV genome, HBV genotypes, coinfection with other viruses such as HIV, HCV, HDV, and HEV are the crucial factors that significantly contribute to the HBV replication, immune response escape and subsequently the progression of liver diseases. This figure was redrawn and adapted from (Matsuura et al. 2016; Tong et al. 2014b).

1.2. Hepatitis E Virus infection

1.2.1. Epidemiology and transmission routes

Hepatitis E virus infection is a significant public health problem worldwide. It is estimated that one third of the world population lives in areas endemic for HEV and are at risk. Each year approximately 20 million HEV infections were documented, with an estimated 3.3 million hepatitis E symptomatic cases, and 56,600 hepatitis E-related deaths (WHO. 2016). HEV is considered to be the most common etiology of acute viral hepatitis and several outbreaks were reported, especially in resource-limited countries. Limited access to clean water, poor sanitation, hygiene, and inadequate health care services are the major contributing factors in resource-limited countries. HEV occurs as sporadic outbreaks (Kamar et al. 2012; Kamar et al. 2014) and more than 60% of infections and 65% of deaths occur in Asia, where seroprevalence exceed 25% in certain age groups (WHO. 2016).

Although HEV infection is sporadic in industrialized countries, it is described as an emerging disease and the transmission also occurs by zoonoses. In particular, intake of raw or undercooked meat from wild boar, pigs, and deer increases the risk of being infected (Arends et al. 2014). Studies have reported on a high HEV seroprevalence (5 to 20%) among general populations in developed countries (Emerson et al. 2003; Mansuy et al. 2011; Mansuy et al. 2008). Seroprevalence might be even higher, if high sensitive tests are utilized for anti-HEV antibody detection. This is substantiated by a seroprevalence study conducted in Toulose, France where the HEV seroprevalence rose from 16 to 52% (Dalton et al. 2008; Dalton et al. 2013; Kamar et al. 2012).

In the new nomenclature, HEV belongs to the family of Hepeviridae, divided into the two genera: members of Piscihepevirus and Orthohepevirus. The genus of Orthohepevirus can infect several mammalian and avian species and comprises four species (Orthohepevirus A-D). In which, Orthohepevirus A is the most important

species and consists of eight HEV genotypes (HEV-1 to HEV-8) that are identified based on the phylogeny of entire viral genomes (Smith et al. 2016; van Tong et al. 2016a; Woo et al. 2016). In high endemic areas, HEV-1 and -2 are responsible for most epidemics and is usually transmitted by the fecal-oral route and outbreaks occur particularly during monsoons and floods (Bile et al. 1994; Teshale et al. 2010). HEV-3 and -4 can cause sporadic infections in both humans and animals and are associated with zoonotic transmission in developed countries (Kamar et al., 2012; Kamar et al., 2014; WHO, 2016). HEV isolated from wild boars in Japan are designated as HEV-5 and HEV-6 (Smith et al. 2014; Takahashi et al. 2011). Very recently, two novel HEV strains isolated from camel were classified as HEV-7 and HEV-8 (Lee et al. 2016; Sridhar et al. 2017; Woo et al. 2016). Of which, HEV-7 was shown to cause chronic hepatitis in a liver transplanted patient (Lee et al. 2016; Sridhar et al. 2017).

Waterborne transmission is the key mode in many outbreaks, which are related to poor sanitation and hygiene, whereas the zoonotic transmission is associated with sporadic cases in developed countries. Phylogenetic evidence reveals homology between the human and swine strains isolated in different geographic areas (Banks et al. 2004; Lu et al. 2006; Meng. 2010; Wenzel et al. 2011). Other transmission routes through blood transfusion and vertical transmission from mother to child have been documented, however, there is no direct evidence by sexual transmission (Bose et al. 2011; Kamar et al. 2012; Scobie et al. 2013).

1.2.2. Hepatitis E virus: structure and genome organization

HEV is a small, non-envelope Hepevirus of 27-34 nm in size. The virus consists of a 7.2 kb single-stranded, positive-sense RNA genome harboring three open reading frames (*ORF1*, *2*, *3*), 5´- and 3´-untranslated regions (UTRs), and a polyA-tail at the 3´-end (Kamar et al. 2012). *ORF1* encodes enzymes for RNA replication; *ORF2* encodes the viral capsid and O*RF3* encodes a multifunctional protein that can modulate cellular signaling and is related to particle secretion (Parvez et al. 2015).

Recently, a novel ORF4 of 158 amino acids within ORF1 has been described for HEV-1. ORF4 is involved in HEV replication by interacting with multiple viral proteins and host factors such as eEF1α1 (eukaryotic elongation factor 1 isoform-1) and β-tubulin (Nair et al. 2016; van Tong et al. 2016b).

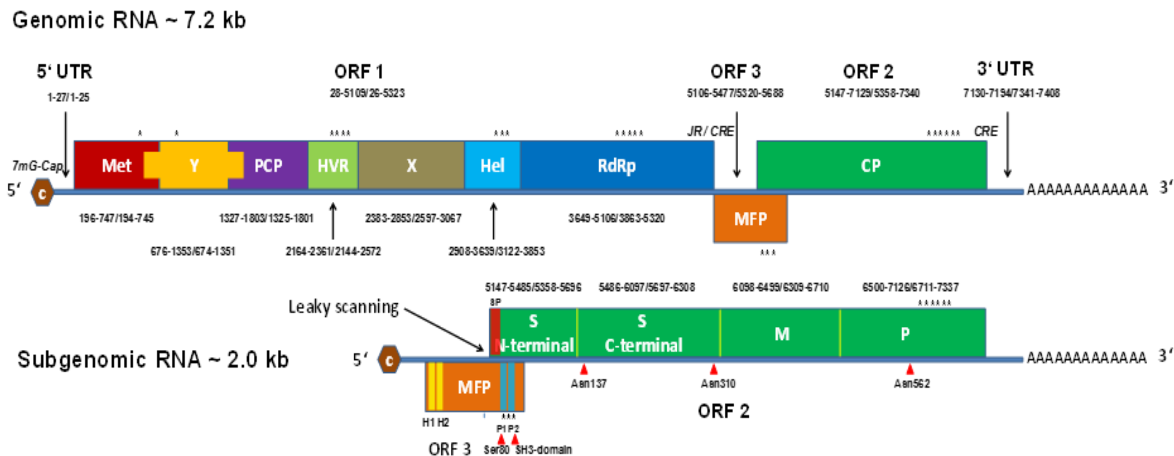


Figure 7. HEV genome structure and viral proteins. HEV is a single-stranded RNA virus. Its genome is about 7.2kb and is composed of 3 open reading frames. ORF1 encodes the nonstructural polyprotein, including methyltransferase (Met), Y-domain (Y), papain-like cysteine protease (PCP), hypervariable region (HVR), macro-domain (X), RNA helicase (Hel) and RNA-dependent RNA polymerase (RdRp). ORF2 encodes the capsid protein (CP), containing S domain (S), M domain (M) and P domain (P). ORF3 encodes a small multifunctional protein (MFP) including hydrophobic regions (D1, D2) and proline-rich regions (P1, P2). JR is ORF2 and ORF3 overlapping/intergenic-junction region; CRE is cis-reactive element; SP is signal peptide. Nucleotide positions are relative to the HEV-1 Burmese strain (Acc. No. M73218)/HEV-3 47832 strain (Acc. No. KC618402). Asterisk (*) indicates the hot spot region for clinical mutations (this figure was used with the permission from Dr. Hoang van Tong (van Tong et al. 2016a).

1.2.3. Clinical pathology

HEV can cause both acute and chronic hepatitis. Among acute cases, HEV infections are mainly asymptomatic or self-limited. In rare cases, HEV infection can

be symptomatic and sometime results in severe acute and fulminant hepatitis (acute liver failure), which may lead to life-threatening conditions (Dalton et al. 2013; Kamar et al. 2012; WHO. 2016). Acute Hepatitis E is usually a self-limiting disease and last less than 6-10 weeks showing symptoms typically presented also for other viral hepatitis such as HAV and HBV. Notably, HEV3 (and HEV4) infections are more frequent in males with a median age of 65 years while HEV1 and HEV2 infections in endemic countries affect young adults from 10-40 years (Aggarwal. 2013). These differences may be due to different routes of transmission of the HEV genotypes (zoonotic or waterborne) or other yet unknown environmental, host, and viral factors. Alcohol consumption, pre-existing liver diseases, pregnancy, and co-infection with other hepatitis viruses (e.g. HBV) are considered risk factors. However, knowledge of interference mechanisms in co-infection with other hepatitis viruses is poor and needs further investigation.

Hepatitis E infection during the third trimester of pregnancy is associated with increased risk of stillbirth and mortality. The case fatality rate is as high as 20–23% among pregnant women in their third trimester (Bose et al. 2011; Lhomme et al. 2016; Navaneethan et al. 2008; WHO. 2016). In addition, abortion and stillbirth are common. This can be explained by the evidence of HEV replication in the human placenta and thus an increased neonatal risk (Bose et al. 2014; Bose et al. 2011; Khuroo et al. 2009).

Chronic hepatitis E is defined by HEV genome persistence (RNA) and/or anti-HEV IgM for more than 6 months. To date, HEV genotype 3 is reported to cause chronic hepatitis in patients who were immune compromised, such as those with organ transplant, stem cell recipients, hematological patients receiving chemotherapy and immunotherapy (Donnelly et al. 2017; Kamar et al. 2008; Pischke et al. 2012). Very recently, HEV genotype 4 was also reported to cause chronic HEV infection (Wu et al. 2017). The chronicity of HEV infection largely depends on host immune responses. However, also few cases with chronic or persistent HEV infection are also reported in healthy and in immune competent individuals (Gonzalez Tallon et

al. 2011; Grewal et al. 2014). Until to-date, there were no studies carried out to understand, whether viral factors esp. the genetic variability is associated with HEV chronic infection.

1.2.4. Treatment and prevention

Prevention of HEV infection is largely associated with appropriate hygiene and sanitary measures, thereby, avoidance of fecal-oral transmission. In regions where HEV infection is sporadic, the consumption of raw meat products shall be avoided. HEV infection can be prevented with an effective immunization program. Although HEV has become a significant health problem worldwide, to date, vaccine against HEV infection is not available globally. The first vaccine for HEV was licensed in 2011 for use only in China and recently a recombinant HEV vaccine (Hecolin) sustained protection against HEV for up to 4.5 years in a Chinese population (Zhang et al. 2015a).

Both these HEV vaccines (recombinant ORF2 and HEV 293) based on the viral capsid protein have shown protective efficacy (Zhang et al. 2015a). However, there is no direct evidence of these vaccine usees among pregnant women, patients with pre-existing liver damage, and protective efficacy across HEV genotypes (HEV1-HEV4). There are no specific drugs that are approved for HEV treatment. In 2010, an initial case study demonstrated the effectiveness of PEG-interferon-α in combination with ribavirin for HEV infection. This finding was further confirmed by a larger multi-center study (Pischke et al. 2013; Wedemeyer et al. 2012). The data from these studies revealed that antiviral resistance mutations can occur in HEV genome (RdRp-domain) under treatment with PEG-IFN/ribavirin (Debing et al. 2014).

1.3. Scope and specific objectives

The present thesis describes the contribution of host candidate genes in the JAK/STAT signaling pathway to HBV susceptibility and its clinical course. The

association of SOCS3 hypermethylation, ISG15 genetic variants with HBV disease susceptibility and its clinical outcome was studied. Also, this thesis describes how HEV superinfection in HBV patients may influence HBV disease and its course in liver disease progression. This study especially on HEV epidemiology is first of its kind in the Vietnamese population, where HBV is epidemic. Based on the objectives, the thesis is structured as two independent chapters, which are published as three full length original articles in peer reviewed journals. In particular, I investigated the

1. Clinical significance of hepatitis E virus superinfection in hepatitis B virus infected patients.

2. Role of SOCS3 genetic variants and promoter hypermethylation in patients with chronic hepatitis B.

3. Association of ISG15 variants, ISG15 expression with outcomes of HBV infection and progression of HBV-related diseases.

2. RESULTS AND DISCUSSION

Chapter 1

Hepatitis E virus superinfection in patients infected with hepatitis B virus

Publication No.1

Hepatitis E Virus superinfection and clinical progression in hepatitis B patients

Hoan NX, Tong HV, Hecht N, Sy BT, Marcinek P, Meyer CG, Song le H, Toan NL, Kurreck J, Kremsner PG, Bock CT, Velavan TP.

EBioMedicine. 2015 Nov 11;2(12):2080-6. PMID: 26844288.

EBioMedicine 2 (2015) 2080–2086

Contents lists available at ScienceDirect

EBioMedicine

journal homepage: www.ebiomedicine.com

Research Article

Hepatitis E Virus Superinfection and Clinical Progression in Hepatitis B Patients

Nghiem Xuan Hoan [a,1], Hoang Van Tong [a,1], Nicole Hecht [b], Bui Tien Sy [b,c], Patrick Marcinek [a], Christian G. Meyer [a], Le Huu Song [d], Nguyen Linh Toan [e], Jens Kurreck [f], Peter G. Kremsner [a], C-Thomas Bock [b,2], Thirumalaisamy P. Velavan [a,⁎,2]

[a] *Institute of Tropical Medicine, University of Tübingen, Tübingen, Germany*
[b] *Department of Infectious Diseases, Robert Koch Institute, Berlin, Germany*
[c] *Department of Molecular Biology, Tran Hung Dao Hospital, Hanoi, Viet Nam*
[d] *Institute of Clinical Infectious Diseases, Tran Hung Dao Hospital, Hanoi, Viet Nam*
[e] *Department of Pathophysiology, Vietnam Military Medical University, Ha Dong, Hanoi, Viet Nam*
[f] *Department of Biotechnology, Technical University of Berlin, Berlin, Germany*

A R T I C L E   I N F O

Article history:
Received 25 August 2015
Received in revised form 21 October 2015
Accepted 10 November 2015
Available online 11 November 2015

Keywords:
Hepatitis E virus
HEV seroprevalence
HEV superinfection
HBV infection
HBV-related liver diseases

A B S T R A C T

Hepatitis E virus (HEV) infection may cause acute hepatitis and lead to hepatic failure in developing and developed countries. We studied HEV seroprevalences in patients with hepatitis B virus (HBV) infection to understand the consequences of HEV superinfection in a Vietnamese population. This cross-sectional study was conducted from 2012 to 2013 and included 1318 Vietnamese patients with HBV-related liver diseases and 340 healthy controls. The case group included patients with acute (n = 26) and chronic hepatitis B (n = 744), liver cirrhosis (n = 160), hepatocellular carcinoma (n = 166) and patients with both liver cirrhosis and hepatocellular carcinoma (n = 222). Anti-HEV IgG and IgM antibodies were assessed in patients and controls by ELISA. HEV-RNA was identified by PCR assays and sequencing. Seroprevalences of anti-HEV IgG among hepatitis B patients and controls were 45% and 31%, respectively (adjusted $P = 0.034$). Anti-HEV IgM seroprevalences were 11.6% and 4.7% in patients and controls, respectively (adjusted $P = 0.005$). Seroprevalences were higher among the elder individuals. When stratifying for patient groups, those with liver cirrhosis had the highest anti-HEV IgG (52%) and anti-HEV IgM (19%) seroprevalences. Hepatitis B patients with current HEV infection had abnormal liver function tests compared to patients with past or without HEV infection. One HEV isolate was retrieved from a patient with both liver cirrhosis and hepatocellular carcinoma and identified as HEV genotype 3. This study indicates high prevalences of HEV infection in Vietnamese HBV patients and among healthy individuals and shows that HEV superinfection may influence the outcome and progression of HBV-related liver disease.

© 2015 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Hepatitis E virus (HEV) may cause acute hepatitis. Pregnant women are vulnerable to fulminant courses (Boccia et al., 2006; Hamid et al., 2002). Chronic HEV infection may occur mainly in immunocompromised patients with HIV, organ transplants and during cancer chemotherapy (Dalton et al., 2009; Kamar et al., 2008). Large hepatitis E outbreaks with 120,000 cases occurred in China in 1986–1988 (Dalton et al., 2013), followed by >30,000 cases in India in 1956 (Shukla et al., 2011). Global annual incidences are 20 million asymptomatic infections and 3.4 million acute cases with 70,000 deaths (Rein et al., 2012). Seroprevalences of 16 to 52% and incidences between 0.2 to 1.7% are reported from regions with poor sanitation (Dalton et al., 2013). More than 60% of infections and 65% of deaths occur in Asia, where seroprevalences exceed 25% in certain age groups (WHO, 2014). The first vaccine for HEV was licensed in China in 2011 and very recently a recombinant HEV vaccine (Hecolin) sustained protection against HEV for up to 4.5 years after first vaccination tested in a Chinese population (Zhang et al., 2015).

HEV is a hepevirus of 27–34 nm in size (Kamar et al., 2012). Its genome consists of ssRNA of 7.2 kb, containing three open reading frames

Abbreviations: HEV, hepatitis E virus; HBV, hepatitis B virus; AHB, acute hepatitis B; CHB, chronic hepatitis B; LC, liver cirrhosis; HCC, hepatocellular carcinoma; IgG, immunoglobulin G; IgM, immunoglobulin M; *ORF*, open reading frame; WBC, white blood cells; RBC, red blood cells; PLT, platelets; AST, aspartate amino transferase; ALT, alanine amino transferase; AFP, alpha-feto protein.

⁎ Corresponding author at: Institute of Tropical Medicine, Wilhelmstraße 27, 72074 Tübingen, Germany.
E-mail address: velavan@medizin.uni-tuebingen.de (T.P. Velavan).

[1] These authors contributed equally to this work.
[2] These authors share the last authorship.

http://dx.doi.org/10.1016/j.ebiom.2015.11.020
2352-3964/© 2015 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

(*ORF1*, *2*, *3*) (Kamar et al., 2012). *ORF1* encodes enzymes for RNA replication while *ORF2* and *ORF3* encode the capsid and a multifunctional protein, respectively. However, viral replication in hepatocytes is not fully understood (Cao and Meng, 2012). Four HEV genotypes have been identified. Genotypes 1 and 2 (HEV1, HEV2) only infect humans, and HEV genotypes 3 and 4 (HEV3, HEV4) can cause human and animal disease (Kamar et al., 2012, 2014). HEV1 is widely distributed in Asia and HEV2 predominates in Africa and Mexico. HEV3 and HEV4 are distributed globally. HEV1, HEV2 and HEV4 are transmitted fecal–orally, while HEV3 infections occur also by consumption of undercooked meat (Kamar et al., 2012).

Hepatitis B virus (HBV) infections are frequent in sub-Saharan Africa and Asia with infection rates between 8% and 20% (WHO, 2013). In Vietnam, approximately 10 million (10%) individuals live with chronic hepatitis B (Dunford et al., 2012; Nguyen, 2012). HEV superinfection in patients with chronic HBV or HCV infections and autoimmune hepatitis has been found associated with clinical outcomes in several geographical settings (Atiq et al., 2009; Bayram et al., 2007; Cheng et al., 2013; Marion-Audibert et al., 2010; Monga et al., 2004; Pischke et al., 2014). This study aims to assess prevalences and consequences of HEV infection in patients with HBV-related liver diseases and to compare it to uninfected Vietnamese controls.

2. Materials and Methods

2.1. Study Design and Sample Collection

A cross-sectional study was implemented between June 2012 and December 2013. A total of 1318 HBV patients and 340 healthy controls were recruited (108 Military Central Hospital and 103 Hospital of the Vietnam Military Medical University, Hanoi). Based on clinical manifestations and laboratory parameters, patients were assigned to the different clinical subgroups as previously described (Song et al., 2003). Briefly, the acute hepatitis B (AHB, n = 26) are patients who presented with the prodromal symptoms preceded the onset of jaundice (e.g. fever, anorexia, nausea, vomiting, and fatigue), an onset of clinical jaundice, the constitutional prodromal symptoms were diminished, hepatomegaly, jaundice, hyperbilirubinemia, serum ALT, and AST at least 5-fold higher than normal range, HBsAg positive, anti-HBc IgM positive, anti-HBc IgG negative. The chronic hepatitis B (CHB, n = 744) were characterized based upon clinical syndromes such as fatigue, anorexia, jaundice, hepatomegaly, hard density of the liver, splenomegaly, hyperbilirubinemia, elevated levels of AST and ALT, HBsAg positive for longer than 6 months. The HBV-related liver cirrhosis (LC, n = 160) were characterized as patients infected with HBV (HBsAg positive) showing the clinical manifestations such as anorexia, nausea, vomiting, malaise, weight loss, abdominal distress, jaundice, edema, cutaneous arterial "Spider" angiomas, palma erythema, ascites, shrunken liver, splenomegaly, hyperbilirubinemia, elevated levels of AST and ALT, prolonged serum prothrombin time, and decreased serum albumin. The HBV-related hepatocellular carcinoma (HCC, n = 166) were characterized as patients infected with chronically HBV (HBsAg positive), abdominal pain, an abdominal mass in the right upper quadrant, blood-tinged ascites, weight loss, anorexia, fatigue, jaundice, prolonged serum prothrombin time, hyperbilirubinemia, elevated levels of AST, ALT and serum a-fetoprotein (AFP), ultrasound showed tumor, liver biopsy and histopathology showing tumor cells. The patients with liver cirrhosis and hepatocellular carcinoma (LC + HCC, n = 222) were characterized if the patients showed clinical manifestations and laboratory tests of both LC and HCC. In addition, the patients with LC were also categorized as Child-A, Child-B and Child-C based on Child–Pugh scores (Cholongitas et al., 2005). We also included 340 healthy individuals without any history of alcohol or drug use as healthy controls (HC). Biochemical and serological tests were performed for all participants. HBV viral loads were measured by quantitative real-time PCR as described previously (Song et al., 2003). Five milliliters of venous blood was collected from all participants. Serum or plasma was used for biochemical and laboratory assays.

2.2. Ethics Statement

Informed consent was obtained at sampling from all participants or from parents if subjects were <18 years. The study was approved by the institutional review board of the 108 Military Central Hospital and the 103 Military Hospital, Hanoi, Vietnam.

2.3. Serology Testing for HEV Infection

Anti-HEV IgG and IgM levels were determined in serum from patients and healthy controls through ELISA kits (MP Biomedicals, Santa Ana, California, USA). The MP HEV IgG and IgM EIA test system was chosen for our analysis on the basis of own round robin test and due to recently reported comparative analysis of commercial available HEV IgG/IgM EIA assays showing no inconsistent performances of sensitivity and specificity among different assays including the MP EIA (Avellon et al., 2015; Wu et al., 2013).

2.4. HEV-RNA Detection

Viral RNA was isolated from serum obtained from patients and controls (QIAamp Viral RNA Mini Kit; Qiagen GmbH, Hilden, Germany). HEV-RNA was reverse transcribed into cDNA (QuantiTect Reverse Transcription Kit; Qiagen GmbH, Hilden, Germany). Presence of HEV-RNA was examined in all the patients and controls using a nested PCR assay. Primers were designed based on conserved regions of the overlapping HEV *ORF1* region. Outer primer pairs were HEV-38 (sense) 5′-GAGGCYATGGTSGAGAARG-3′ and HEV-39 (antisense) 5′-GCCATGTTCCAGACRGTRTTCC-3′; inner primers were HEV-37 (sense) 5′-GGTTCCGYGCTATTGARAARG-3′ and HEV-27 (antisense) 5′-TCRCCAGAGTGYTTCTTCC-3′.

PCR amplification was carried out in 25 μl volumes [5 ng viral cDNA, 1× PCR buffer (20 mM Tris–HCl, 50 mM KCl, 2 mM $MgCl_2$), 0.2 mM dNTPs, 0.4 mM $MgCl_2$, 0.6 μM specific primer pairs, 1 unit Taq polymerase (Qiagen GmbH, Hilden, Germany)]. Cycling parameters were denaturation (94 °C, 5 min), 35 cycles of 30 s at 94 °C denaturation, 30 s at 54 °C annealing, 30 s at 72 °C extension, followed by final extension of 10 min (72 °C). Parameters for nested PCR were: denaturation (94 °C 5 min), followed by 40 cycles of 30 s at 94 °C denaturation, 30 s at 56 °C annealing temperature, 30 s at 72 °C extension, followed by 10 min extension (72 °C). A plasmid containing HEV cDNA served as positive control. Amplicons (306 bp) were visualized on 1.5% agarose gels stained with SYBR green.

In addition, the HBV patients were confirmed for the presence of HEV-RNA by applying the same PCR conditions by, however, using other set of primers which amplified a 497 bp *ORF2* fragment. Outer primers were HEV-34 (sense) 5′-CCGACGTCYGTYGAYATGAA-3′ and HEV-36 (antisense) 5′-TTRTCCTGCTGAGCRTTCTC-3′; inner primers were HEV-35 (sense) 5′- AAGTGAGCGCCTACAYTAYCG-3′ and HEV-29 (antisense) 5′-CTCGCCATTGGCTGAGAC-3′.

2.5. HEV Genotyping

PCR products were purified (Exo-SAP-IT kit; USB, Affymetrix, USA) and used as sequencing templates (BigDye terminator v.1.1 sequencing kit; Applied Biosystems, USA, ABI 3130XL sequencer). HEV genotyping was performed by phylogenetic analyses based on the amplified *ORF2* sequences using the MEGA 5 software (www.megasoftware.net). HEV reference sequences were obtained from the NCBI GenBank database.

2.6. Statistical Analysis

Analyses were performed using the SPSS software (SPSS Statistics, IBM, Armonk, NY) and Intercooled Stata (Stata Corporation, College Station, TX, USA). Prevalences and quantitative variables are given as percentages and medians with ranges. Categorical variables were compared applying Chi square or Fisher's exact tests. Kruskal–Wallis or Mann–Whitney U tests were used to compare quantitative variables. A logistic regression model was used to compare HEV seroprevalences between groups and to analyze associations of HEV seroprevalences with disease progression. Odds ratios (OR) and 95% confidence intervals (CI) were calculated. The level of significance was $P < 0.05$.

3. Results

3.1. Demographic and Clinical Characteristics of Hepatitis B Patients and Controls

The baseline characteristics of the 1318 Vietnamese hepatitis B patients and 340 healthy controls are provided in Table 1. The progression of liver disease increased according to median age of patients. Most patients were male (82%). Differences of means were observed for several clinical parameters, including white and red blood cell counts, platelets, AST/ALT, total and direct bilirubin, albumin, prothrombin, AFP and HBV viral loads. Platelet counts were lower in patients with LC compared to non-LC patients ($P < 0.001$). Levels of AST, ALT and total and direct bilirubin were higher in AHB patients compared to other subgroups ($P < 0.001$). Albumin and prothrombin levels were lower in patients with LC and/or with HCC ($P < 0.001$). As expected, AFP levels were higher in HCC compared to non-HCC patients ($P < 0.001$). HBV viral loads were higher in chronic hepatitis B patients without LC and/or HCC than in chronic hepatitis B patients with LC and/or HCC (Table 1).

3.2. Seroprevalence of HEV Infection in Patients With HBV Infection and in Healthy Population

Seroprevalence rates of anti-HEV IgG were higher in patients (45%) compared to controls (31%) ($P = 0.034$). When stratified for the clinical subgroups of HBV infection, the anti-HEV IgG seroprevalence was 42% (11/26) in AHB, 41% (305/744) in CHB, 52% (83/160) in LC, 48% (79/166) in HCC and 49% (108/222) in patients with LC and HCC (Fig. 1A, Supplementary Table 1). Anti-HEV IgM was observed in 19% (30/160) of LC patients, followed by 15% (4/26) of AHB patients, 15% (33/222) of patients with both LC and HCC, 12% (20/166) of HCC patients, 9% (65/744) of CHB patients and 5% (16/340) of controls. The seroprevalence rate of anti-HEV IgM was also higher among patients with HBV infection (11.6%) compared to the controls (4.7%; adjusted $P = 0.005$) (Fig. 1A, Supplementary Table 1). In the HBV group positive for anti-HEV IgG, we observed a higher positivity of anti-HEV IgM in the AHB (36%) and LC (36%) subgroups, followed by patients with both LC and HCC (29%), HCC (20%) and CHB (20%) (Fig. 1B).

3.3. Association of Higher HEV Seroprevalence With Increasing Age

Seroprevalences of anti-HEV IgG and IgM were higher in elder compared to younger subgroups of HBV patients and controls. In the HBV group, the mean age of patients positive for anti-HEV antibodies was higher than that in HBV patients negative for anti-HEV antibodies ($P < 0.001$ and $P = 0.002$ for anti-HEV IgG and IgM). A similar trend applied to the controls ($P < 0.001$ and $P = 0.022$ for anti-HEV IgG and IgM). A prevalence of 51% and 46% of anti-HEV IgG was observed in HBV patients and controls >40 years of age, respectively. The anti-HEV IgG prevalence in HBV patients between 30 to 40 years was higher compared to that in controls of the same age group ($P = 0.03$). No difference was seen between HBV patients and controls <30 years of age (Fig. 1C and Supplementary Table 2). The prevalence of anti-HEV IgM in HBV patients and in controls >40 years of age was 13.7% and 9.2%, respectively. When stratified for age groups and sex, no differences of anti-HEV IgM prevalence was observed between HBV patients and controls (Fig. 1D and Supplementary Table 2).

3.4. HEV Seroprevalence and Progression of HBV-Related Liver Diseases

We found higher anti-HEV IgM prevalences in LC patients (16.8%) compared to patients without LC (9.5%) (OR = 1.64; 95% CI = 1.1–2.4; adjusted $P = 0.01$), indicating that anti-HEV IgM was associated with LC in chronic hepatitis B patients (Table 2). Significance did not apply to anti-HEV IgG seroprevalences. A similar trend was found when comparing anti-HEV IgG and IgM prevalences between HCC and non-HCC patients (Supplementary Table 3).

To analyze associations of HEV seroprevalences with underlying LC and its prognosis, we categorized LC patients into three subgroups based on Child–Pugh scores. Anti-HEV IgG prevalences in Child-B and

Table 1
Demographic and clinical characteristics of the studied HBV patients and controls.

Characteristics	AHB (n = 26)	CHB (n = 744)	LC (n = 160)	HCC (n = 166)	LC and HCC (n = 222)	Healthy controls (n = 340)
Age (years)	34 [20–49]	41 [9–84]	57 [15–84]	55 [15–81]	59 [26–81]	33 [15–69]
Gender (M/F)	21/5	557/187	135/25	159/7	210/12	223/117
WBC* (10^3/ml)	NA	6.5 [4–17]	5.6 [1.7–20.5]	6.3 [3–16]	6 [2.5–17]	Normal
RBC* (10^6/ml)	NA	4.9 [3–6.8]	4.2 [1.9–9.2]	4.7 [2.1–6.8]	4.3 [2.2–6.2]	Normal
PLT* (10^3/ml)	NA	223 [19–401]	90 [3.7–441]	208 [68.6–389]	122 [34–361]	Normal
AST* (IU/l)	1064.5 [316–4425]	44 [14–1600]	76.5 [15–1221]	48.5 [17–2158]	72 [14–670]	<30
ALT* (IU/l)	1125.5 [309–3328]	48 [8–2924]	58.5 [8–1426]	40.5 [10–832]	54.5 [10–805]	<30
Total bilirubin* (μmol/l)	184.8 [21.8–558]	15 [4.9–452]	31 [4.1–690]	14 [5–160]	22 [7–419]	<17
Direct bilirubin* (μmol/l)	137.9 [15–353]	5 [1–298]	12 [0.4–440]	4.9 [1–80]	8.2 [0.4–214]	<5
Albumin* (g/l)	36 [27–42]	41.8 [26–48]	34 [20–47]	39 [27–49]	37 [24–47]	>35
Prothrombin* (% of standard)	70 [58–127]	93 [45–215]	56.5 [13–101]	88 [43–172]	75 [19.6–158]	>70
HBV viral load* (copies/ml)	8.8×10^3 [1062–3.7×10^4]	1.1×10^6 [189–4.3×10^{12}]	6.8×10^4 [180–4.7×10^9]	8.56×10^5 [450–1.44×10^9]	5.85×10^4 [190–3.04×10^{10}]	NA
Alpha-feto protein (AFP)* (mg/l)	NA	4.3 [1–200]	7.3 [1.18–300]	196 [1.1–305]	170 [1.6–880]	NA

AHB: acute hepatitis B; CHB: chronic hepatitis B; LC: liver cirrhosis; HCC: hepatocellular carcinoma; WBC: white blood cells; RBC: red blood cells; PLT: platelets; AST and ALT: aspartate and alanine amino transferase; IU: international unit; NA: not available. Values given are medians and range.

* $P < 0.001$ for comparison with all other groups.

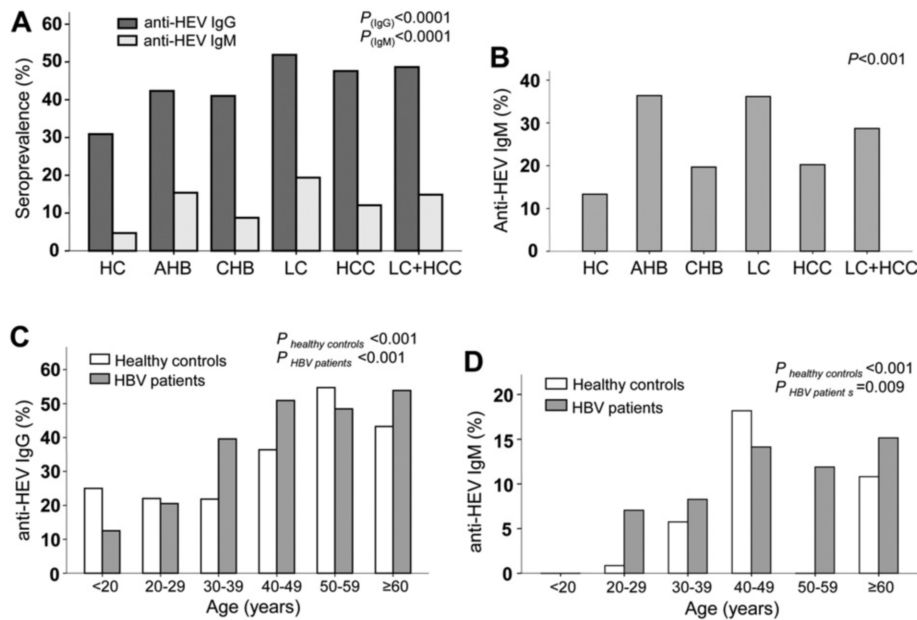


Fig. 1. Seroprevalence of HEV infection in patients with HBV infection and in healthy population. (A): Anti-HEV IgG and anti-HEV IgM in healthy controls (HC) and in HBV patients including acute hepatitis B (AHB), chronic hepatitis B (CHB), patients with only liver cirrhosis (LC), patients with only hepatocellular carcinoma (HCC) and patients with both LC and HCC; (B): Anti-HEV IgM positivity in individuals positive for anti-HEV IgG; (C): Anti-HEV IgG seroprevalence increasing with age; (D): Anti-HEV IgM prevalence in different age groups. *P* values were calculated by Chi square or Fisher's exact tests for comparisons of the seroprevalence among different groups.

Child-C LC patients were higher compared to those in Child-A patients (adjusted $P < 0.001$ and $P = 0.002$, respectively). A similar result was observed for anti-HEV IgM (adjusted $P = 0.007$ and $P < 0.001$, respectively) (Table 2), indicating that higher seroprevalences of anti-HEV IgG and IgM are associated with an increase of LC severity.

3.5. Significance of HEV Coinfection in the Outcome of HBV Patients

To analyze the significance of HEV superinfection in HBV patients, we categorized HBV patients into three subgroups based on HEV serology results. Patients negative for anti-HEV IgG and IgM were categorized as “no HEV infection”. Patients who were positive for anti-HEV IgG and negative for IgM were categorized as “past HEV infection”. “Current HEV infection” was defined as HBV patients positive for anti-HEV IgM (anti-HEV IgG positive or negative). Levels of AST, total and direct bilirubin and HBV-DNA viral loads were elevated in the “current HEV infection” group compared to the “no HEV infection” and “past HEV infection” groups ($P = 0.023$ for AST, $P = 0.01$ for total bilirubin, $P = 0.005$ for direct bilirubin, $P = 0.026$ for HBV-DNA viral loads) (Fig. 2). In contrast, levels of albumin, prothrombin and platelet counts in the “current HEV infection” group were lower compared to the “no HEV infection” and “past HEV infection” groups ($P < 0.001$ for albumin and prothrombin, $P = 0.018$ for platelets) (Fig. 2).

We formed three subgroups of patients with “no HEV infection”, “past HEV infection” and “current HEV infection” for each patient group including AHB, CHB, LC, HCC and patients with both LC and HCC. We observed that AST levels were increased, while prothrombin levels were decreased in the “current HEV infection” patients in CHB group ($P = 0.01$) and for other clinical parameters no differences were observed (Fig. 3).

3.6. Prevalence of HEV-RNA and Sequencing Analysis of HEV Isolates

HEV-RNA was detected in only one serum sample from a patient with both LC and HCC. The phylogenetic analysis revealed that the HEV isolate was close to a HEV genotype 3 (NCBI #ssID 1825526) (Supplementary Fig. 1). The patient was negative for both anti-HEV IgG and IgM, indicating that he was in the early phase of HEV infection.

4. Discussion

HEV superinfection in HIV-infected patients, but also in patients with chronic HBV and HCV infections may aggravate the course of underlying conditions (Dalton, 2012; Pischke et al., 2014). We describe

Table 2
Association of HEV seroprevalence with liver cirrhosis (LC) and prognosis of LC in HBV patients.

Patient group	Anti-HEV IgG				Anti-HEV IgM			
	Total n	Positive n (%)	OR (95% CI)	*P* value	Total n	Positive n (%)	OR (95% CI)	*P* value
Cirrhosis status								
Non-cirrhosis	936	395 (42.2)	1	Reference	936	98 (9.5)	1	Reference
Cirrhosis	382	191 (50)	0.94 (0.7–1.22)	0.65	382	64 (16.8)	1.64 (1.1–2.4)	0.01
Child–Pugh								
Child A	235	91 (38.7)	1	Reference	235	25 (10.6)	1	Reference
Child B	117	79 (67.5)	3.3 (2.05–5.2)	<0.0001	117	31 (21.4)	2.3 (1.3–4.3)	0.007
Child C	30	9 (70)	1.9 (1.3–2.9)	0.002	30	14 (46.4)	2.7 (1.8–4.2)	<0.0001

Non-cirrhosis: HBV patients without liver cirrhosis; Cirrhosis: hepatitis B patients with liver cirrhosis (with or without hepatocellular carcinoma); odds ratio (OR) and *P* values were calculated by using logistic regression model and adjusted for age and gender.

Fig. 2. Clinical outcomes of HEV superinfection in HBV patients. Based on the positivity of anti-HEV IgG and anti-HEV IgM, HBV patients were categorized into three different groups as "no HEV infection", "past HEV infection" and "current HEV infection". Different clinical and biochemical parameters including AST (A), ALT (B), albumin (C), total bilirubin (D), direct bilirubin (E), prothrombin (F), platelets (G) and HBV-DNA viral loads (H) were compared. Box-plots illustrate medians with 25 and 75 percentiles and *P* values were calculated by using Kruskal–Wallis test. The number in parenthesis indicates number of samples analyzed and those numbers in the respective groups vary for the clinical parameters because some patients were not tested for all the clinical parameters.

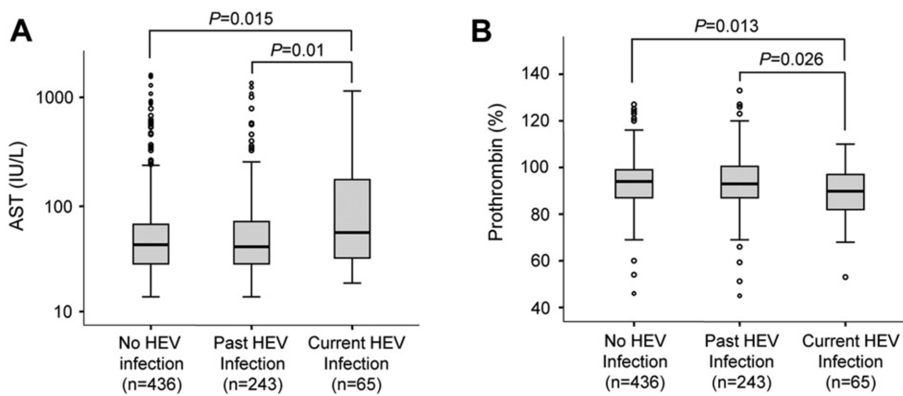


Fig. 3. Clinical outcomes of HEV superinfection in patients with chronic HBV. HBV patients were categorized into three different groups as "no HEV infection", "past HEV infection" and "current HEV infection" based on the positivity of anti-HEV IgG and anti-HEV IgM. The levels of aspartate aminotransferase (AST) (A) and prothrombin (B) were compared. Box-plots illustrated medians with 25 and 75 percentiles and *P* values were calculated by using Kruskal–Wallis test. The number in parenthesis indicates number of analyzed subject samples.

higher HEV seroprevalences in patients with HBV-related liver disease compared to healthy individuals. The frequency of HEV superinfection in HBV patients was differentially distributed among progressive stages of HBV infection. HEV infection was independently associated with the prognosis of HBV-related LC and the clinical outcome of HBV infection.

HEV seroprevalences have previously been determined in general populations and blood donors from various geographical settings (Boutrouille et al., 2007; Cleland et al., 2013; Gallian et al., 2014; Guo et al., 2010; Hau et al., 1999; Pittaras et al., 2014; Ren et al., 2014). Our study shows prevalences of 31% and 5% of anti-HEV IgG and IgM, respectively, in healthy Vietnamese individuals. Anti-HEV IgG seropositivity was considerably higher than previously reported in rural areas of Vietnam (Hau et al., 1999). This difference might result from the sensitivity and specificity of ELISA tests applied (Abravanel et al., 2014; Mansuy et al., 2011). Comparative analysis of the sensitivity and specificity of commercial test systems have been described in recent reports showing comparable performance in terms of negative predictive value and slightly variable performance in relation to sensitivity and specificity (Avellon et al., 2015; Pas et al., 2013; Wenzel et al., 2013; Wu et al., 2014). In our study, the used MP HEV EIA showed minor limitation with regard to sensitivity (IgM and IgG, approx. 80% and approx. 75%, respectively) in agreement to recent reports. However, the MP HEV EIA assay seemed to be robust and suitable for our analysis generating a feasible and true number of positive HEV IgM and IgG samples. Social and demographic characteristics may also cause differences of seroprevalences (Hau et al., 1999; Mansuy et al., 2011). Our results support studies in Chinese blood donors, where anti-HEV IgG and IgM prevalences were approximately 30% and 1%, respectively (Guo et al., 2010; Ren et al., 2014). Somewhat lower anti-HEV IgG prevalences of 3% to 17% occur among most European populations (Beale et al., 2011; Boutrouille et al., 2007; Cleland et al., 2013; Gallian et al., 2014; Juhl et al., 2014; Pittaras et al., 2014; Ren et al., 2014). HEV seroprevalences were, however, exceptionally high in southern France (53%) and Denmark (21%) (Christensen et al., 2008; Mansuy et al., 2011).

This is the first study on HEV superinfection in Vietnamese HBV patients. Prevalences of anti-HEV IgG and IgM in patients were 45% and 12%, respectively. The HEV seroprevalence in our patient group differed from other studies on HBV, HCV and HIV infections (Atiq et al., 2009; Bayram et al., 2007; Feldt et al., 2013; Hamid et al., 2002). Anti-HEV IgG prevalences in chronic HBV Turkish and American patients were 14% and 8% (Atiq et al., 2009; Bayram et al., 2007). In agreement with other findings (Atiq et al., 2009), our results indicate that the HEV seroprevalence was higher in patients with HBV-related liver diseases compared to healthy individuals, suggesting patients with HBV-related liver diseases might have a higher risk for HEV infection (Hamid et al., 2002). Nevertheless, similar findings were not observed in other studies (Bayram et al., 2007; Hamid et al., 2002). A probable explanation could be the sample size utilized in these studies and the study area. These both factors may largely contribute for such dissimilarities in seroprevalence rates. In Europe, HEV infection was observed to be higher in autoimmune hepatitis patients and not in patients with either chronic hepatitis B or C (Pischke et al., 2014), whereas in endemic areas like Vietnam, individuals usually acquire the HBV early in their life and HEV infection subsequently through the fecal–oral transmission later. A plausible explanation is that the patients primarily infected with HBV may show an altered immune response and thus likely to be more susceptible for HEV as a secondary infection. In addition, HEV superinfection in HIV patients and development of chronic hepatitis E has also been described (Dalton et al., 2009). Recently, a study has found a seroprevalence of anti-HEV IgG in Ghana (45%) and Cameroon (14%) (Feldt et al., 2013). However, no contribution of HEV infection to liver pathology was observed. The HEV seroprevalence in our study group was significantly associated with age, both in HBV patients and controls, indicating cumulative exposure to HEV. Aging of the immune system might also favor acquisition of HEV infection.

Although most HEV-infected individuals are asymptomatic, the outcome of HEV superinfection in HBV patients appears more severe (Cheng et al., 2013; Marion-Audibert et al., 2010; Monga et al., 2004). Our results show increased levels of liver enzymes and total and direct bilirubin and decreased levels of albumin, prothrombin and platelet counts in HBV patients with concomitant HEV infection. In contrast to an earlier study suggesting that chronic HBV infections may be inactive during HEV–HBV coinfections (Cheng et al., 2013), we found higher HBV-DNA loads in coinfections. Biochemical and serological tests suggest that HEV superinfection contributes to inflammation and liver failure. HBV-DNA loads were lower in HBV patients with past HEV infection compared to those with no or patent HEV infection, suggesting that host immune responses contribute to control HBV replication. HBsAg positive individuals had a poorer prognosis after HEV superinfection (Chow et al., 2014; Wu et al., 2013).

Although associations of HEV infection with development and progression of LC were reported (Gerolami et al., 2008; Kumar et al., 2007; Marion-Audibert et al., 2010), the mechanisms of LC induction are unclear. According to previous studies, we show that HEV infections were independently associated with underlying LC and progression in chronic hepatitis B. Hepatocyte damage and immune responses during HEV superinfection that leads to increased liver inflammation can progress to LC. The severity of LC is classified based on the Child–Pugh score, determined by a number of clinical and laboratory parameters such as bilirubin, albumin, and prothrombin levels. The association of HEV superinfection with increased abnormalities of bilirubin, albumin, and prothrombin levels supports that HEV superinfection contributes to severity of HBV infection. Although the difference was not significant, the prevalence of anti-HEV antibodies was increased among patients with HBV-related HCC. Therefore, tumorigenesis of HEV cannot be excluded.

In this study, we detected the presence of HEV-RNA in only one serum sample from patient with both LC and HCC (negative for both anti-HEV IgG and IgM) showing that this patient was in the early stage of HEV infection. However, we could not follow up longitudinally the patient confirmed positive for HEV-RNA to verify the chronicity of HEV infection. HEV-RNA can persist longer in the stool than in the blood (Kamar et al., 2012), therefore another limitation of the study is that the nested PCR for detection of HEV-RNA from stool of the HBV patients and controls had not been performed due to the unavailability of stool samples.

In conclusion, this study indicates high HEV seroprevalences in Vietnamese patients with HBV-related liver diseases and in healthy individuals. HEV infection may aggravate the clinical outcome of HBV infection, especially in liver cirrhosis.

Author Contributions

NXH, HVT, NH, BTS, and PM contributed to performing of the experiments. LHS, NLT, PGK, CTB and TPV contributed materials and reagents. CTB, PGK and TPV designed the study. NXH, LHS and NLT recruited patients and collected samples. NXH and HVT performed statistical analyses. HVT, NXH, CTB and TPV wrote the manuscript. CGM and JK corrected the manuscript and contributed to the study design. NXH and HVT contributed equally to this work. All authors agreed with the results and conclusions. All authors agreed with the results and conclusions.

Acknowledgments and Disclosures

We would like thank the staffs in Tran Hung Dao and 103 Military hospitals for their support during sample collection and all the study subjects and blood donors for their participation. We also would like to extend our thanks to Andrés Lamsfus Calle for excellent technical help. We acknowledge support from DAAD-PAGEL (57140033) for student fellowship. The funders have no role in the study design,

data collection and analysis, decision to publish or preparation of the manuscript.

Conflict of Interest

All authors have no conflicts of interest to declare.

Appendix A. Supplementary Data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2015.11.020.

References

Abravanel, F., Lhomme, S., Chapuy-Regaud, S., Mansuy, J.M., Muscari, F., Sallusto, F., Rostaing, L., Kamar, N., Izopet, J., 2014. Hepatitis E virus reinfections in solid-organ-transplant recipients can evolve into chronic infections. J. Infect. Dis. 209, 1900–1906.

Atiq, M., Shire, N.J., Barrett, A., Rouster, S.D., Sherman, K.E., Shata, M.T., 2009. Hepatitis E virus antibodies in patients with chronic liver disease. Emerg. Infect. Dis. 15, 479–481.

Avellon, A., Morago, L., Garcia-Galera Del, C.M., Munoz, M., Echevarria, J.M., 2015. Comparative sensitivity of commercial tests for hepatitis E genotype 3 virus antibody detection. J. Med. Virol. 87, 1934–1939.

Bayram, A., Eksi, F., Mehli, M., Sozen, E., 2007. Prevalence of hepatitis E virus antibodies in patients with chronic hepatitis B and chronic hepatitis C. Intervirology 50, 281–286.

Beale, M.A., Tettmar, K., Szypulska, R., Tedder, R.S., Ijaz, S., 2011. Is there evidence of recent hepatitis E virus infection in English and North Welsh blood donors? Vox Sang. 100, 340–342.

Boccia, D., Guthmann, J.P., Klovstad, H., Hamid, N., Tatay, M., Ciglenecki, I., Nizou, J.Y., Nicand, E., Guerin, P.J., 2006. High mortality associated with an outbreak of hepatitis E among displaced persons in Darfur, Sudan. Clin. Infect. Dis. 42, 1679–1684.

Boutrouille, A., Bakkali-Kassimi, L., Cruciere, C., Pavio, N., 2007. Prevalence of anti-hepatitis E virus antibodies in French blood donors. J. Clin. Microbiol. 45, 2009–2010.

Cao, D., Meng, X.J., 2012. Molecular biology and replication of hepatitis E virus. Emerg. Microbes Infect. 1, e17.

Cheng, S.H., Mai, L., Zhu, F.Q., Pan, X.F., Sun, H.X., Cao, H., Shu, X., Ke, W.M., Li, G., Xu, Q.H., 2013. Influence of chronic HBV infection on superimposed acute hepatitis E. World J. Gastroenterol. 19, 5904–5909.

Cholongitas, E., Papatheodoridis, G.V., Vangeli, M., Terreni, N., Patch, D., Burroughs, A.K., 2005. Systematic review: the model for end-stage liver disease—should it replace Child–Pugh's classification for assessing prognosis in cirrhosis? Aliment. Pharmacol. Ther. 22, 1079–1089.

Chow, C.W., Tsang, S.W., Tsang, O.T., Leung, V.K., Fung, K.S., Luk, W.K., Chau, T.N., 2014. Comparison of acute hepatitis E infection outcome in patients with and without chronic hepatitis B infection: A 10 year retrospective study in three regional hospitals in Hong Kong. J. Clin. Virol. 60, 4–10.

Christensen, P.B., Engle, R.E., Hjort, C., Homburg, K.M., Vach, W., Georgsen, J., Purcell, R.H., 2008. Time trend of the prevalence of hepatitis E antibodies among farmers and blood donors: a potential zoonosis in Denmark. Clin. Infect. Dis. 47, 1026–1031.

Cleland, A., Smith, L., Crossan, C., Blatchford, O., Dalton, H.R., Scobie, L., Petrik, J., 2013. Hepatitis E virus in Scottish blood donors. Vox Sang. 105, 283–289.

Dalton, H.R., 2012. Hepatitis: hepatitis E and decompensated chronic liver disease. Nat. Rev. Gastroenterol. Hepatol. 9, 430–432.

Dalton, H.R., Bendall, R.P., Keane, F.E., Tedder, R.S., Ijaz, S., 2009. Persistent carriage of hepatitis E virus in patients with HIV infection. N. Engl. J. Med. 361, 1025–1027.

Dalton, H.R., Hunter, J.G., Bendall, R.P., 2013. Hepatitis E. Curr. Opin. Infect. Dis. 26, 471–478.

Dunford, L., Carr, M.J., Dean, J., Nguyen, L.T., Ta Thi, T.H., Nguyen, B.T., Connell, J., Coughlan, S., Nguyen, H.T., Hall, W.W., Thi, L.A., 2012. A multicentre molecular analysis of hepatitis B and blood-borne virus coinfections in Viet Nam. PLoS One 7, e39027.

Feldt, T., Sarfo, F.S., Zoufaly, A., Phillips, R.O., Burchard, G., van, L.J., Jochum, J., Chadwick, D., Awasom, C., Claussen, L., Drosten, C., Drexler, J.F., Eis-Hubinger, A.M., 2013. Hepatitis E virus infections in HIV-infected patients in Ghana and Cameroon. J. Clin. Virol. 58, 18–23.

Gallian, P., Lhomme, S., Piquet, Y., Saune, K., Abravanel, F., Assal, A., Tiberghien, P., Izopet, J., 2014. Hepatitis E virus infections in blood donors, France. Emerg. Infect. Dis. 20, 1914–1917.

Gerolami, R., Moal, V., Colson, P., 2008. Chronic hepatitis E with cirrhosis in a kidney-transplant recipient. N. Engl. J. Med. 358, 859–860.

Guo, Q.S., Yan, Q., Xiong, J.H., Ge, S.X., Shih, J.W., Ng, M.H., Zhang, J., Xia, N.S., 2010. Prevalence of hepatitis E virus in Chinese blood donors. J. Clin. Microbiol. 48, 317–318.

Hamid, S.S., Atiq, M., Shehzad, F., Yasmeen, A., Nissa, T., Salam, A., Siddiqui, A., Jafri, W., 2002. Hepatitis E virus superinfection in patients with chronic liver disease. Hepatology 36, 474–478.

Hau, C.H., Hien, T.T., Tien, N.T., Khiem, H.B., Sac, P.K., Nhung, V.T., Larasati, R.P., Laras, K., Putri, M.P., Doss, R., Hyams, K.C., Corwin, A.L., 1999. Prevalence of enteric hepatitis A and E viruses in the Mekong River Delta region of Vietnam. Am. J.Trop. Med. Hyg. 60, 277–280.

Juhl, D., Baylis, S.A., Blumel, J., Gorg, S., Hennig, H., 2014. Seroprevalence and incidence of hepatitis E virus infection in German blood donors. Transfusion 54, 49–56.

Kamar, N., Selves, J., Mansuy, J.M., Ouezzani, L., Peron, J.M., Guitard, J., Cointault, O., Esposito, L., Abravanel, F., Danjoux, M., Durand, D., Vinel, J.P., Izopet, J., Rostaing, L., 2008. Hepatitis E virus and chronic hepatitis in organ-transplant recipients. N. Engl. J. Med. 358, 811–817.

Kamar, N., Bendall, R., Legrand-Abravanel, F., Xia, N.S., Ijaz, S., Izopet, J., Dalton, H.R., 2012. Hepatitis E. Lancet 379, 2477–2488.

Kamar, N., Dalton, H.R., Abravanel, F., Izopet, J., 2014. Hepatitis E virus infection. Clin. Microbiol. Rev. 27, 116–138.

Kumar, A.S., Kumar, S.P., Singh, R., Kumar, M.S., Madan, K., Kumar, J.J., Kumar, P.S., 2007. Hepatitis E virus (HEV) infection in patients with cirrhosis is associated with rapid decompensation and death. J. Hepatol. 46, 387–394.

Mansuy, J.M., Bendall, R., Legrand-Abravanel, F., Saune, K., Miedouge, M., Ellis, V., Rech, H., Destruel, F., Kamar, N., Dalton, H.R., Izopet, J., 2011. Hepatitis E virus antibodies in blood donors, France. Emerg. Infect. Dis. 17, 2309–2312.

Marion-Audibert, A.M., Tesse, S., Graillot, E., Phelip, G., Radenne, S., Duperret, S., Durieux, M., Rode, A., Mabrut, J.Y., Souquet, J.C., Nicand, E., 2010. Lethal acute HEV superinfection on hepatitis B cirrhosis. Gastroenterol. Clin. Biol. 34, 334–336.

Monga, R., Garg, S., Tyagi, P., Kumar, N., 2004. Superimposed acute hepatitis E infection in patients with chronic liver disease. Indian J. Gastroenterol. 23, 50–52.

Nguyen, V.T., 2012. Hepatitis B infection in Vietnam: current issues and future challenges. Asia Pac. J. Public Health 24, 361–373.

Pas, S.D., Streefkerk, R.H., Pronk, M., de Man, R.A., Beersma, M.F., Osterhaus, A.D., van der Eijk, A.A., 2013. Diagnostic performance of selected commercial HEV IgM and IgG ELISAs for immunocompromised and immunocompetent patients. J. Clin. Virol. 58, 629–634.

Pischke, S., Gisa, A., Suneetha, P.V., Wiegand, S.B., Taubert, R., Schlue, J., Wursthorn, K., Bantel, H., Raupach, R., Bremer, B., Zacher, B.J., Schmidt, R.E., Manns, M.P., Rifai, K., Witte, T., Wedemeyer, H., 2014. Increased HEV seroprevalence in patients with autoimmune hepatitis. PLoS One 9, e85330.

Pittaras, T., Valsami, S., Mavrouli, M., Kapsimali, V., Tsakris, A., Politou, M., 2014. Seroprevalence of hepatitis E virus in blood donors in Greece. Vox Sang. 106, 387.

Rein, D.B., Stevens, G.A., Theaker, J., Wittenborn, J.S., Wiersma, S.T., 2012. The global burden of hepatitis E virus genotypes 1 and 2 in 2005. Hepatology 55, 988–997.

Ren, F., Zhao, C., Wang, L., Wang, Z., Gong, X., Song, M., Zhuang, H., Huang, Y., Shan, H., Wang, J., Liu, Q., Ness, P., Nelson, K.E., Wang, Y., 2014. Hepatitis E virus seroprevalence and molecular study among blood donors in China. Transfusion 54, 910–917.

Shukla, P., Nguyen, H.T., Torian, U., Engle, R.E., Faulk, K., Dalton, H.R., Bendall, R.P., Keane, F.E., Purcell, R.H., Emerson, S.U., 2011. Cross-species infections of cultured cells by hepatitis E virus and discovery of an infectious virus–host recombinant. Proc. Natl. Acad. Sci. U. S. A. 108, 2438–2443.

Song, L.H., Binh, V.Q., Duy, D.N., Kun, J.F., Bock, T.C., Kremsner, P.G., Luty, A.J., 2003. Serum cytokine profiles associated with clinical presentation in Vietnamese infected with hepatitis B virus. J. Clin. Virol. 28, 93–103.

Wenzel, J.J., Preiss, J., Schemmerer, M., Huber, B., Jilg, W., 2013. Test performance characteristics of anti-HEV IgG assays strongly influence hepatitis E seroprevalence estimates. J. Infect. Dis. 207, 497–500.

WHO, 2013. Hepatitis B. Fact sheet N° 204 (Ref Type: Report).

WHO, 2014. Hepatitis E. Fact sheet N° 280 (Ref Type: Report).

Wu, T., Huang, S.J., Zhu, F.C., Zhang, X.F., Ai, X., Yan, Q., Wang, Z.Z., Yang, C.L., Jiang, H.M., Liu, X.H., Guo, M., Du, H.L., Ng, M.H., Zhang, J., Xia, N.S., 2013. Immunogenicity and safety of hepatitis E vaccine in healthy hepatitis B surface antigen positive adults. Hum. Vaccin. Immunother. 9, 2474–2479.

Wu, W.C., Su, C.W., Yang, J.Y., Lin, S.F., Chen, J.Y., Wu, J.C., 2014. Application of serologic assays for diagnosing acute hepatitis E in national surveillance of a nonendemic area. J. Med. Virol. 86, 720–728.

Zhang, J., Zhang, X.F., Huang, S.J., Wu, T., Hu, Y.M., Wang, Z.Z., Wang, H., Jiang, H.M., Wang, Y.J., Yan, Q., Guo, M., Liu, X.H., Li, J.X., Yang, C.L., Tang, Q., Jiang, R.J., Pan, H.R., Li, Y.M., Shih, J.W., Ng, M.H., Zhu, F.C., Xia, N.S., 2015. Long-term efficacy of a hepatitis E vaccine. N. Engl. J. Med. 372, 914–922.

Supplementary data

Supplementary table 1: HEV seroprevalence in patients with HBV-related liver diseases

Groups	Total n	Positive n (%)	OR (95% CI)	*P* value
anti-HEV IgG				
HC	340	105 (30.9)	1	Reference
AHB	26	11 (42.3)	1.97 (0.9-4.5)	0.11
CHB	744	305 (41.0)	**1.2 (1-1.34)**	**0.043**
LC	160	83 (51.9)	1.14 (0.98-1.3)	0.091
HCC	166	79 (47.6)	1.05 (0.9-1.2)	0.4
LC+HCC	222	108 (48.6)	1.05 (0.96-1.15)	0.25
HBV patients	1318	586 (44.5)	**1.34 (1.02-1.75)**	**0.034**
anti-HEV IgM				
HC	340	16 (4.7)	1	Reference
AHB	26	4 (15.4)	4.7 (1.37-16)	0.014
CHB	744	65 (8.7)	**1.3 (1-1.76)**	**0.056**
LC	160	30 (18.8)	**1.7 (1.3-2.1)**	**<0.0001**
HCC	166	20 (12)	1.2 (0.97-1.5)	0.089
LC+HCC	222	33 (14.9)	1.15 (0.99-1.35)	0.074
HBV patients	1318	153 (11.6)	**2.2 (1.3-3.75)**	**0.005**

HC: healthy controls; AHB: acute hepatitis B; CHB: chronic hepatitis B; LC: patients with only liver cirrhosis; HCC: patients with only hepatocellular carcinoma; LC+HCC: patients with both liver cirrhosis and hepatocellular carcinoma; Odds ratio (OR) and *P* values were calculated by using logistic regression model and adjusted for age and gender.

Supplementary table 2: HEV seroprevalence in patients with HBV-related liver diseases and in healthy controls according to age groups

Age Group	Healthy controls		AHB		CHB		LC		HCC		LC+HCC		HBV patients	
	Number (pos./total)	% positive	Number (pos./total)	% positive	Number (pos./total)	% positive	Number (pos./total)	% positive	Number (pos./total)	% positive	Number (pos./total)	% positive	Number (pos./total)	% positive
Anti-HEV IgG														
<20	3/12	25.0	0/0	0.0	2/28	7.1	1/2	50.0	1/2	50.0	0/0	0.0	4/32	12.5
20-29	26/118	22.0	2/8	25.0	29/137	21.2	1/4	25.0	0/6	0.0	0/1	0.0	32/156	20.5
30-39	19/87	21.8	7/12	58.3	67/176	38.1	5/15	33.3	5/15	33.3	7/12	58.3	91/230	39.6
40-49	12/33	36.4	2/6	33.3	82/173	47.4	19/29	65.5	16/34	47.1	25/41	61.0	144/283	50.9
50-59	29/53	54.7	0/0	0.0	69/144	47.9	24/52	46.2	32/54	59.3	30/70	42.9	155/320	48.4
≥60	16/37	43.2	0/0	0.0	56/86	65.1	33/58	56.9	25/55	45.5	40/98	40.8	160/297	53.9
Total	105/340	30.9	11/26	42.3	305/744	41.0	83/160	51.9	79/166	47.6	108/222	48.6	586/1318	44.5
Anti-HEV IgM														
<20	0/12	0.0	0/0	0.0	0/28	0.0	0/2	0.0	0/2	0.0	0/0	0.0	0/32	0.0
20-29	1/118	0.0	0/8	0.0	11/137	8.0	0/4	0.0	0/6	0.0	0/1	0.0	11/156	7.1
30-39	5/87	5.7	3/12	25.0	6/176	3.4	5/15	33.3	3/15	20.0	2/12	16.7	19/230	8.3
40-49	6/18	33.3	1/6	16.7	20/173	11.6	10/29	34.9	3/34	8.8	6/41	14.6	40/283	14.1
50-59	0/53	0.0	0/0	0.0	16/144	11.1	8/52	15.4	7/54	13.0	7/70	10.0	38/320	11.9
≥60	4/37	10.8	0/0	0.0	12/86	14.0	8/58	13.8	7/55	12.7	18/98	18.4	45/297	15.2
Total	16/340	4.7	4/26	15.4	65/744	8.7	31/160	19.4	20/166	12.0	33/222	14.9	153/1318	11.6

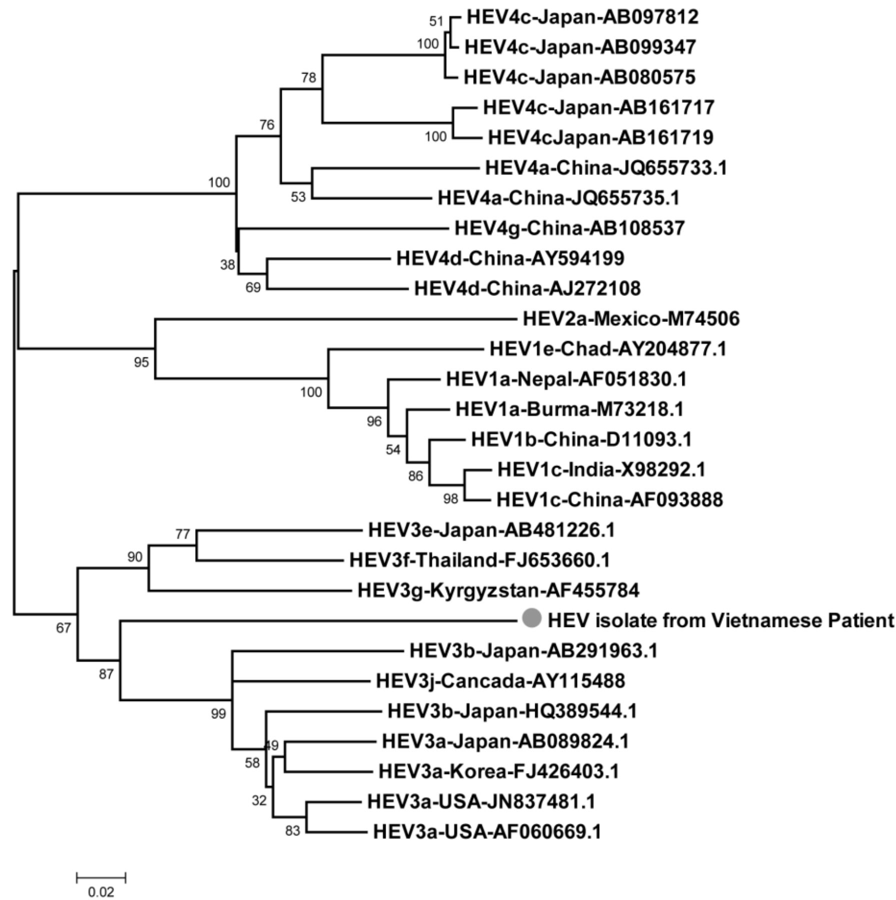
HC: healthy controls; AHB: acute hepatitis B; CHB: chronic hepatitis B; LC: patients with only liver cirrhosis; HCC: patients with only hepatocellular carcinoma; LC+HCC: patients with both liver cirrhosis and hepatocellular carcinoma.

Supplementary table 3: Association of HEV seroprevalence with HCC

Patient Group	Total n	Positive n(%)	OR (95% CI)	P value
anti-HEV IgG				
Non-HCC	930	399 (42.9)	1	Reference
HCC	388	187 (48.2)	0.8 (0.6-1.1)	0.17
anti-HEV IgM				
Non-HCC	930	100 (10.8)	1	Reference
HCC	388	53 (13.7)	1.06 (0.7-1.56)	0.78

Non-HCC: Hepatitis B patients without hepatocellular carcinoma; HCC: patients with hepatocellular carcinoma (with or without LC); Odds ratio (OR) and P values were calculated by using logistic regression model and adjusted for age and gender.

Supplementary figures



Supplementary figure 1: Phylogenetic analysis of HEV RNA sequence

Phylogenetic tree was reconstructed from amplicons representing the overlapping *ORF2/3* region of the identified HEV genome. The reference sequences were obtained from NCBI database along with GenBank accession numbers. A neighbor-joining tree was reconstructed with a bootstrap of 1,000 replicates. The scale indicates the number of nucleotide substitutions per position.

Chapter 2

The role of human *SOCS3* and *ISG15* in HBV infection

Publication No.2

SOCS3 genetic variants and promoter hypermethylation in patients with chronic hepatitis B

Hoan NX, Van Tong H, Giang DP, Cuong BK, Toan NL, Wedemeyer H, Bock CT, Kremsner PG, Song LH, Velavan TP.

Oncotarget. 2017 Mar 7;8(10):17127-17139. PMID: 28179578.

---

www.impactjournals.com/oncotarget/ Oncotarget, 2017, Vol. 8, (No. 10), pp: 17127-17139

Research Paper

SOCS3 genetic variants and promoter hypermethylation in patients with chronic hepatitis B

Nghiem Xuan Hoan[1,2,3,*], Hoang Van Tong[1,3,4,*], Dao Phuong Giang[1,2,3,*], Bui Khac Cuong[3,4], Nguyen Linh Toan[3,4], Heiner Wedemeyer[5], C. Thomas Bock[6], Peter G. Kremsner[1,3], Le Huu Song[2,3,], Thirumalaisamy P. Velavan[1,3,4,7,**]**

[1]Institute of Tropical Medicine, University of Tübingen, Tübingen, Germany

[2]Institute of Clinical Infectious Diseases, 108 Military Central Hospital, Hanoi, Vietnam

[3]Vietnamese-German Center for Medical Research (VG-CARE), Hanoi, Vietnam

[4]Department of Pathophysiology, Vietnam Military Medical University, Hanoi, Vietnam

[5]German Center for Infection Research, Department for Gastroenterology, Hepatology, and Endocrinology, Medical School Hannover, Germany

[6]Department of Infectious Diseases, Robert Koch Institute, Berlin, Germany

[7]Faculty of Medicine, Duy Tan University, Da Nang, Vietnam

[*]Equal contributions

[**]Shared senior authorship

Correspondence to: *Thirumalaisamy P. Velavan,* ***email:*** *velavan@medizin.uni-tuebingen.de*

Keywords: *HBV infection, liver diseases, SOCS3 variants, SOCS3 methylation*

Received: *November 10, 2016* ***Accepted:*** *January 11, 2017* ***Published:*** *February 04, 2017*

ABSTRACT

The clinical manifestations of hepatitis B viral infection (HBV) include chronic hepatitis B (CHB), liver cirrhosis (LC) and hepatocellular carcinoma (HCC). The contribution of negative regulator suppressor of cytokine signaling-3 (SOCS3) promoter variants in HBV disease and *SOCS3* hypermethylation in tumor tissues were investigated. The *SOCS3* promoter region was screened for polymorphisms in 878 HBV patients and in 272 healthy individuals. *SOCS3* promoter methylation was examined by bisulfite sequencing. *SOCS3* mRNA expression was quantified in 37 tumor and adjacent non-tumor liver tissue specimens. The minor allele *rs12953258A* was associated with increased susceptibility to HBV infection (OR=1.3, 95%CI=1.1-1.6, adjusted *P*=0.03). The minor allele *rs111033850C* and *rs12953258A* were observed in increased frequencies in HCC and LC patients compared to CHB patients (HCC: OR=1.7, 95%CI=1.1-2.9, adjusted *P*=0.046; LC: OR=1.4, 95%CI=1.1-1.9, adjusted *P*=0.017, respectively). HBV patients with *rs111033850CC* major genotype had decreased viral load (*P*=0.034), whereas the *rs12953258AA* major genotype contributed towards increased viral load (*P*=0.029). Tumor tissues revealed increased hypermethylation compared to adjacent non-tumor tissues (OR=5.4; 95%CI= 1.9-17.1; *P*=0.001). Increased *SOCS3* expression was observed in HBV infested tumor tissues than non-HBV related tumor tissues (*P*=0.0048). *SOCS3* promoter hypermethylation was associated with relatively low mRNA expression in tumor tissues (*P*=0.0023). In conclusion, *SOCS3* promoter variants are associated with HBV susceptibility and SOCS3 hypermethylation stimulates HCC development.

INTRODUCTION

Hepatitis B virus (HBV) infection is a major health problem affecting approximately two billion people worldwide. Approximately 240 million individuals are chronically infected with 780,000 annually reported deaths due to HBV infection [1]. HBV infection causes a wide spectrum of clinical manifestations of liver diseases. Besides asymptomatic carriers, HBV causes chronic hepatitis B (CHB), liver cirrhosis (LC) and hepatocellular

carcinoma (HCC). The five-year cumulative risk for the development of HBV-related LC ranges between 10% and 20% [2]. In addition, chronic HBV infection accounts for 50% of all HCC cases and most HCC cases (70%-80%) occur in patients with HBV-related LC [3].

During the course of HBV infection, the mechanism of liver injury is dependent on the host immune responses [4]. The innate immune responses play a major role in suppression of viral replication and in inflammatory activity during the early stage of HBV infection. These responses include the secretion of interferons (IFNs) and cytokines, which are regulated by Janus kinase/ Signal Transducer and Activator of Transcription (JAK/ STAT) signaling [5, 6] and involvement of JAK/STAT pathway in HBV infection had been well documented [7, 8]. Suppressors of cytokine signaling (SOCS) family proteins (CISH and SOCS1 to SOCS7) belong to a classical negative feedback system that regulates cytokine transduction via JAK/STAT signaling pathway [9]. Of these SOCS family, SOCS3 is a key regulator of interleukin (IL)-6 and IL-10, which are activated by Toll-like receptor stimulation. SOCS3 can inhibit the process of cell proliferation and cell survival through inhibition of STAT3 activation [6, 10]. *STAT3,* an oncogene, is largely correlated with NF-kβ activation [11, 12]. The activation of STAT3 by cytokines (e.g. IL-6 and IL-22) mediated by JAK/STAT signaling pathway was shown earlier to induce inflammation and subsequent carcinogenesis [10–12]. SOCS3 functionally suppresses STAT3 activation and negatively regulate tumor development. Therefore, SOCS3 is a vital regulator of several diseases including atopic, autoimmune and infectious diseases, inflammation, and cancer development [13, 14].

SOCS3 overexpression has been shown in the liver tissue of CHB patients and is associated with the severity of inflammation suggesting that JAK/STAT signaling pathway is dysregulated in HBV-infected hepatocytes [15]. The hypermethylation in the CpG (5'-Cytosine-phosphate-Guanine-3') islands of the *SOCS3* promoter can be a prognostic indicator in cancer development [16]. In addition, *SOCS3* expression is further influenced by *SOCS3* polymorphisms, especially in the *SOCS3* promoter. To date, *SOCS3* polymorphisms were documented in several diseases including HCV infection and colorectal cancer [14, 17–20]. However, there are so far no available data on association of *SOCS3* promoter variants with susceptibility to HBV infection and the clinical course of HBV-related liver diseases. In addition, the involvement of epigenetics during the clinical course of HBV infection needs to be studied. Therefore, this study aims to investigate whether *SOCS3* promoter variants are associated with HBV infection and HBV-related liver diseases and to investigate the hypermethylation in the *SOCS3* promoter region and corresponding *SOCS3* mRNA expression in HBV-related HCC.

RESULTS

Baseline characteristics of study participants

The baseline characteristics of the 878 HBV-infected patients and 272 healthy controls (HC) are shown in Table 1. Most HBV patients and HCs were male (86% and 66%, respectively). The median age of patients increased according to the clinical progression of the liver disease. HCs were younger than patient groups (*P*<0.05). The levels of liver enzymes ALT, AST and HBV loads were higher in CHB patients compared to other subgroups (*P*<0.01). As expected, albumin and prothrombin levels and platelet counts were lower in LC patients compared to the other patient groups (*P*<0.001). AFP levels were higher in HCC patients compared to CHB and LC patients (*P*<0.001). The clinical profile of the 37 HCC patients who underwent surgery and corresponding data of their liver specimens are described in Table 2. Most patients were male (89%) and were between 40-60 years of age (73%). The HCC patients were in early and/or at intermediate stage of liver cancer according to the Barcelona clinic liver cancer (BCLC) staging criteria (stage A: 70% and stage B: 30%). All the patients were Child-Pugh class A group according to Child-Pugh classification. HBV was the common etiology of the liver cancer in this study (46%), while 8% suffered from HCV infection, and 46% showed non-HBV/non-HCV related HCC.

SOCS3 promoter variants and HBV-related liver diseases

The genotype and allele frequencies of two *SOCS3* promoter SNPs (rs111033850T/C, rs12953258C/A) in clinically classified 878 HBV patients and 272 HCs are described in Table 3 and Supplementary Table 2. The analyzed SNPs in healthy controls were in Hardy-Weinberg equilibrium (*P*>0.05). We compared the genotype and allele frequencies between HBV patients and HCs. We observed that heterozygous genotype *rs111033850TC* and minor allele *rs111033850C* were less frequent in HBV patients compared to HCs (OR=0.4, 95%CI=0.3-0.6, adjusted *P*<0.0001 and OR=0.6, 95%CI=0.4-0.8, adjusted *P*<0.0001; respectively). In contrast, the homozygous genotype *rs12953258AA* and the minor allele *rs12953258A* were more frequent in patients compared to HCs (OR=2.0, 95%CI=1.3-3.2, adjusted *P*<0.0001 and OR=1.3, 95%CI=1.1-1.6, adjusted *P*=0.03; respectively). A similar trend was observed for the SNP rs111033850T/C in the dominant genetic model and for the SNP rs12953258C/A in the recessive genetic model. These results indicated that the *rs111033850TC* contributes to a decreased risk of HBV infection while the genotype *rs12953258AA* contributes to increased susceptibility to HBV infection.

Table 1: Clinical profiles of 878 HBV-infected patients and 272 healthy controls

Characteristics	CHB (n=212)	LC (n=243)	HCC (n=220)	LC + HCC (n=203)	HC (n= 272)
Age (years)	43 [18-82]	55 [18-84]	55 [18-81]	50 [19-81]	36 [18-69] ‡α
Male (%)	74	84.4	92.7	94.1	66 †α
Child-Pugh classification (n)					
Child A	NA	117/236	101/173	93/164	NA
Child B	NA	81/236	56/173	52/164	NA
Child C	NA	38/236	16/173	19/164	NA
Missing	NA	7	47	39	NA
Clinical parameters					
AST (IU/L)	72 [15-3253] ‡β	52 [15-1221]	50 [17-2158]	49 [21-737]	NR
ALT (IU/L)	69 [9-3382] ‡β	46 [8-1426]	46 [10-832]	44 [10-1095]	NR
Total bilirubin (µmol/l)	17 [8-788]	29 [3-752]	14 [5-235]	22 [7-419]	NR
Direct bilirubin (µmol/l)	7 [1-472]	12 [1-450]	5 [1.2-167]	8 [1-214]	NR
Albumin (g/L)	42 [23-48]	30 [20-47]‡β	39 [27-49]	38 [23-47]	NR
Prothrombin (% of standard)	87 [30-180]	53.5 [15-101]§β	80 [31-115]	74 [19.6-118]	NR
PLT (10^3/ml)	208 [19-360]	90 [18-441]§β	203 [20-389]	122 [34-361]	NR
HBV-DNA (copies/ml)	$1.6x10^7$ [$2x10^2$- $8.4x10^{10}$]§β	$6.8x10^4$ [$1.8x10^2$- $4.7x10^9$]	$7.4x10^5$ [$2.9x10^2$-$1.4x10^9$]	$1.6x10^5$ [$1.9x10^2$- $3.1x10^{10}$]	NA
Alfa Feto Protein (IU/L)	4.3 [1.5-300]	7.4 [1.2-400]	196 [1.1- 438] §β	168 [1.6-489] §β	NR

Abbreviations: CHB: chronic hepatitis B; LC: liver cirrhosis; HCC: hepatocellular carcinoma; HC: healthy control; PLT: platelets. AST and ALT: aspartate and alanine amino transferase; IU: international unit; NR: Normal range, NA: not applicable. Values given are medians and range. *P* values were calculated by student's t-test, Fisher exact test, and Mann-Whitney- Wilcoxon test where appropriate. (†) $P<0.05$, (‡) $P<0.01$ and (§) $P<0.001$. (α) for comparison with LC, HCC and LC+HCC group; (β) for comparison with all other groups.

Subsequently, we compared the genotype and allele frequencies between different subgroups of HBV patients. The genotype *rs12953258AA* was significantly more frequent in LC patients compared to CHB patients (OR=1.7, 95%CI=1.02-2.8; adjusted *P*=0.036). The genotype *rs111033850TC* was also significantly more frequent in LC, HCC and HCC+LC groups compared to CHB patients (LC vs. CHB: OR=2.6, 95%CI=1.4-5.0, adjusted *P*=0.002; HCC vs. CHB: OR=2.6, 95%CI=1.3-5.0, adjusted *P*=0.005; LC+HCC vs. CHB: OR=2.0, 95%CI=1-4.4, adjusted *P*=0.048). The alleles *rs111033850C* and *rs12953258A* were more frequent in HCC and in LC patients compared to CHB patients, respectively (OR=1.7, 95%CI=1.1-2.9, adjusted *P*=0.046 and OR=1.4, 95%CI=1.1-1.9, adjusted *P*=0.017).

We also observed the gene dose effect of the allele *rs111033850C* when compared CHB with HCC and HCC+LC groups (*P* for trend =0.046 and 0.026, respectively). This indicate that the allele *rs111033850C* was associated with an increased risk of HCC and that the allele *rs12953258A* was associated an increased risk of LC in CHB patients. There were no significant differences when comparing the genotype and allele frequencies of the two *SOCS3* SNPs in LC and HCC groups with HCC+LC group.

Table 2: Characteristics of 37 HCC patients

Characteristics	n (%)
Age (years)	
< 40	4/37 (10.8)
40 - 60	27/37 (73)
> 60	6/37 (16.2)
Gender	
Male	33/37 (89.2)
Female	4/37 (10.8)
Etiology	
HBV	17/37 (46)
HCV	3/37 (8)
Non-HBV/HCV	17/37 (46)
Child-Pugh classification	
Child A	37/37 (100)
BCLC staging Classification	
Stage A	26/37 (70.3)
Stage B	11/37 (29.7)
Stage C and D	0/ 37 (0)
Clinical parameters	**Median (Range)**
AFP (IU/ml)	240 [4.6 - 300]
HBV-DNA	NA
PLT (10^3/ml)	211 [153 - 461]
AST (IU/ml)	52 [21 - 415]
ALT (IU/ml)	66.5 [17 - 242]
Total Bilirubin (µmol/l)	27.8 [8.9 - 315]
Direct Bilirubin (µmol/l)	6.7 [1 - 178]
Prothrombin (% of standard)	93 [75 - 125]
Protein (g/l)	73 [62 - 78]
Allbumin (g/l)	40 [32 - 48]

Abbreviations: BCLC: Barcelona Clinic Liver Cancer; HCC: hepatocellular carcinoma; AFP: Alpha feto protein; PLT: platelets; AST and ALT: aspartate and alanine amino transferase; IU: international unit; NA: not applicable.

SOCS3 promoter haplotypes and HBV-related liver diseases

The haplotypes were reconstructed based on the two SNPs (rs111033850T/C, rs12953258C/A) and the frequencies were presented in Table 4. Haplotype *CC* was found more frequently in HCs compared to HBV patients (OR=0.5, 95%CI=0.4-0.75, adjusted *P*=0.001) indicating that this haplotype contributes to a decreased risk of HBV infection. We further compared haplotype frequencies between different subgroups of HBV patients and observed that the frequencies of haplotypes *TA* and *CC* were significantly higher in LC, HCC and patients with both LC and HCC compared to CHB patients (*P*<0.01) (Table 4). This result indicates that the haplotypes *TA* and *CC* contribute to an increased risk of progression to

Table 3: Association of *SOCS3* variants with HBV-related liver diseases

SOCS3 variants	CHB n (%)	LC n (%)	HCC n (%)	HCC+LC n (%)	HC n (%)	Cases vs. HC		LC vs. CHB		HCC vs. CHB		HCC+LC vs. CHB	
	n=212	n=243	n=220	n=203	n=272	OR (95%CI)	*P* value	OR (95%CI)	*P* value	OR (95%CI)	*P* value	OR (95%CI)	*P* value
rs111033850													
TT	190 (89.6)	198 (81.5)	178 (80.9)	162 (79.8)	191 (70.3)	Reference		Reference		Reference		Reference	
TC	16 (7.5)	43 (17.7)	36 (16.4)	35 (17.2)	76 (27.9)	**0.4 (0.3-0.6)**	**<0.0001**	**2.6 (1.4-5.0)**	**0.002**	**2.6 (1.3-5.0)**	**0.005**	**2.0 (1- 4.4)**	**0.048**
CC	6 (2.9)	2 (0.8)	6 (2.7)	6 (3.0)	5 (1.8)	1.1 (0.4 -3.0)	0.89	0.4 (0.1-2.0)	0.2	0.9 (0.2-3.1)	0.8	1.2 (0.3 - 5.3)	0.8
P for trend							1.99		0.12		**0.046**		**0.026**
Allele													
T	396 (93.4)	439 (90.3)	392 (89)	359 (88.4)	458 (84.2)	Reference		Reference		Reference		Reference	
C	28 (6.6)	47 (9.7)	48 (11)	47 (11.6)	86 (15.8)	**0.6 (0.4-0.8)**	**<0.0001**	1.6 (0.9-2.6)	0.07	**1.7 (1.1-2.9)**	**0.046**	1.7 (0.9-3.0)	0.09
Dominant													
TT	190 (89.6)	198 (81.5)	178 (80.9)	162 (79.8)	191 (70.3)	Reference		Reference		Reference		Reference	
TC & CC	22 (10.4)	45 (18.5)	42 (19.1)	41 (20.2)	81 (29.7)	**0.5 (0.3-0.7)**	**<0.0001**	**2.0 (1.1-3.7)**	**0.014**	**2.0 (1.1-3.8)**	**0.017**	**1.9 (1.1-3.7)**	**0.046**
Recessive													
TT & TC	206 (97.2)	241 (99.2)	214 (97.3)	197 (79.8)	267 (98.2)	Reference		Reference		Reference		Reference	
CC	6 (2.9)	2 (0.8)	6 (2.7)	6 (3.0)	5 (1.8)	1.3 (0.5-3.7)	0.64	0.3 (0.1-1.8)	0.2	0.8 (0.2-2.8)	0.7	1.1 (0.3-5.3)	0.8
P for trend							0.058		0.059		0.55		0.33
rs12953258													
CC	94 (44.3)	88 (36.2)	86 (39.1)	75 (36.9)	101 (37.1)	Reference		Reference		Reference		Reference	
AC	72 (34)	88 (36.2)	88 (40)	84 (41.4)	140 (51.5)	**0.7 (0.5-0.98)**	**<0.0001**	1.3 (0.8-2.0)	0.27	1.34 (0.8-2.2)	0.21	1.4 (0.8-2.4)	0.22
AA	46 (21.7)	67 (27.6)	46 (20.9)	44 (21.7)	31 (11.4)	**2.0 (1.3-3.2)**	**<0.0001**	**1.7 (1.02-2.8)**	**0.036**	1.0 (0.6-1.7)	0.96	1.4 (0.7-2.7)	0.26
Allele													
C	260 (61.3)	264 (54.3)	260 (59.1)	172 (42.4)	342 (62.9)	Reference		Reference		Reference		Reference	
A	164 (38.7)	222 (45.7)	180 (40.9)	234 (57.6)	202 (37.1)	**1.3 (1.1-1.6)**	**0.03**	**1.4 (1.1-1.9)**	**0.017**	0.9 (0.7 - 1.2)	0.87	1.3 (0.9-1.8)	0.15
Dominant													
CC	94 (44.3)	88 (36.2)	86 (39.1)	75 (36.9)	101 (37.1)	Reference		Reference		Reference		Reference	
AC & AA	118 (55.7)	155 (63.8)	134 (60.9)	128 (63.1)	171 (62.9)	1.0 (0.7-1.3)	0.72	1.45 (0.9-2.2)	0.07	1.2 (0.8-1.8)	0.38	1.4 (0.8-2.3)	0.16
Recessive													
CC & AC	166 (78.3)	176 (72.4)	174 (79.1)	159 (78.3)	241 (88.6)	Reference		Reference		Reference		Reference	
AA	46 (21.7)	67 (27.6)	46 (20.9)	44 (21.7)	31 (11.4)	**2.4 (1.6-3.7)**	**<0.0001**	1.5 (0.9-2.4)	0.08	0.9 (0.5-1.5)	0.61	1.2 (0.7-2.1)	0.47

Abbreviations: CHB: Chronic hepatitis B; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma; HC: Healthy control; Cases = all HBV infected patients; n= Number of chromosomes; OR: adjusted Odd Ratio; ORs and *P* values were calculated by using binary logistic regression model adjusted for age and gender. *P* for trend was calculated by Cochran-Armitage test. Bold values present the statistical significance.

LC and HCC in HBV patients. However, no significant difference was observed when haplotype frequencies of LC and HCC patients were compared.

SOCS3 promoter variants and clinical parameters

The HBV loads were lower in HBV patients with *rs111033850CC* compared to those with *rs111033850TT* and *rs111033850TC* (*P*=0.034). In contrast, viral loads were higher in HBV patients with *rs12953258AA* compared to those with *rs12953258CC* and *rs12953258AC* (*P*=0.029) (Figure 1A and 1C). To further examine this possible association, we compared HBV loads according to different genotypes for each SNP in the subgroups of HBV patients. A similar trend of HBV loads was observed in HCC+LC group for rs111033850 (*P*=0.045) and in LC group for rs12953258 (*P*=0.039) (Figure 1B and 1D).

Table 4: Association of *SOCS3* haplotypes with HBV-related liver diseases

SOCS3 Haplotype	HC	CHB	LC	HCC	HCC+LC	Cases vs. HC		LC vs. CHB		HCC vs. CHB		HCC+LC vs. CHB	
	n=544	n= 424	n= 486	n= 440	n= 406	OR (95%CI)	*P*	OR (95%CI)	*P*	OR (95%CI)	*P*	OR (95%CI)	*P*
TC	*256 (47.0)*	*244 (57.5)*	*216 (44.4)*	*204 (46.4)*	196 (48.3)	Reference		Reference		Reference		Reference	
TA	*194 (35.7)*	*154 (36.3)*	*228 (46.9)*	*186 (42.3)*	162 (39.9)	*1.2 (0.9-1.5)*	0.17	***1.89 (1.4-2.54)***	**0.0001**	***1.46 (1.1 - 2.0)***	**0.017**	***1.49 (1.1-2.13)***	**0.026**
CC	92 (16.9)	24 (5.7)	*42 (8.7)*	46 (10.5)	46 (11.3)	***0.5 (0.4 - 0.8)***	**0.001**	***2.1 (1.2-3.9)***	**0.008**	***2.76 (1.5 -5)***	**0.001**	***2.49 (1.3-4.8)***	**0.007**
CA	2 (0.4)	2 (0.5)	0 (0)	4 (0.9)	2 (0.5)	*0.9 (0.2 - 4.8)*	0.96	NA	NA	*1.89 (0.4-11.6)*	0.49	*1.47 (0.1 - 17)*	0.75

Abbreviations: CHB: Chronic hepatitis B; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma; HC: Healthy control; Cases = all HBV infected patients; n= Number of chromosomes; NA: not applicable; OR: Adjusted Odd Ratio; ORs and *P* values were calculated by using binary logistic regression model adjusted for age and gender. Bold values present the statistical significance.

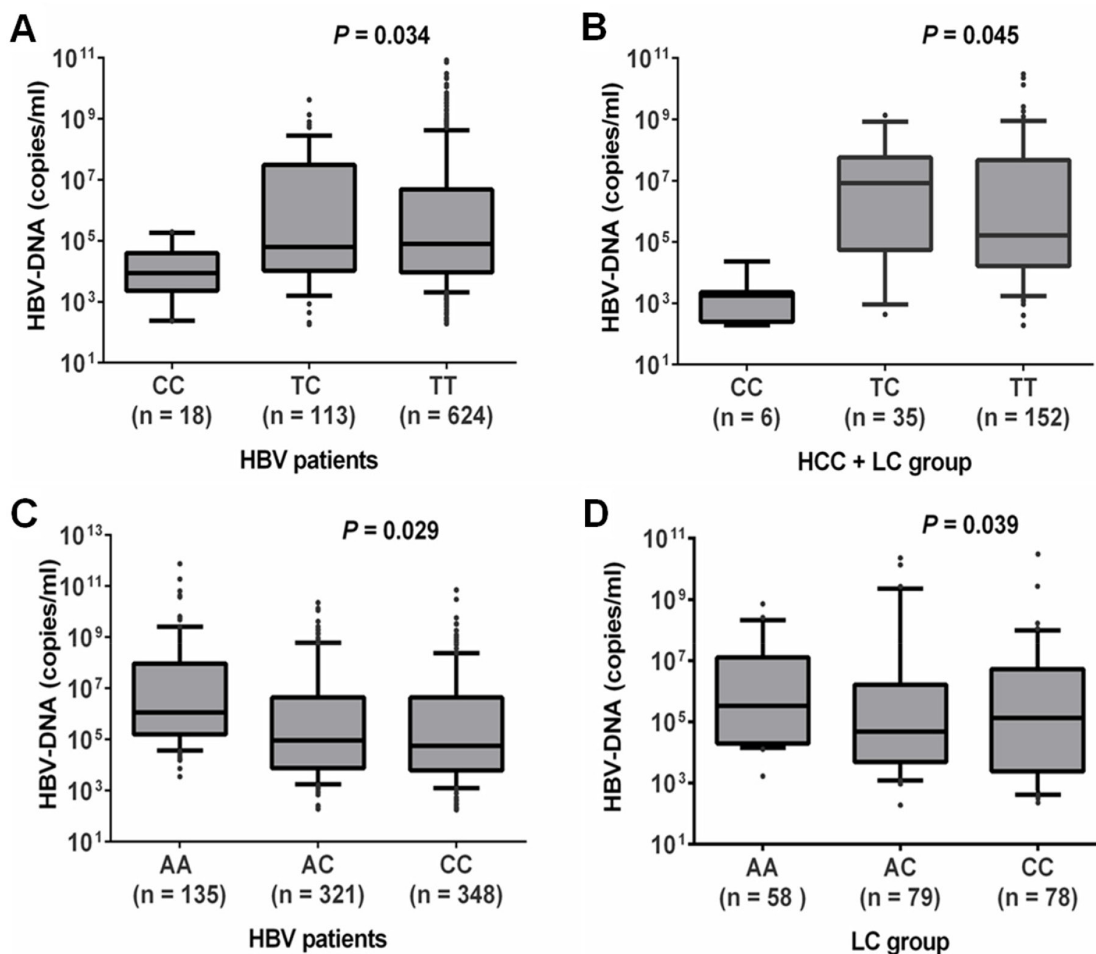


Figure 1: Association of HBV loads with *SOCS3* SNPs. A. and **B.** HBV viral loads according to different genotypes of SNP rs111033850T/C in all HBV patients and in patients with both liver cirrhosis and hepatocellular carcinoma, respectively. **C.** and **D.** HBV loads according to different genotypes of SNP rs12953258C/A in all HBV patients and in patients with liver cirrhosis, respectively. Box-plots illustrate medians with 25 and 75 percentiles with whiskers to 10 and 90 percentiles; *P* values were calculated by Krusskal-Wallis test.

However, there was no significant association of these SNPs with clinical parameters: ALT, AST, total and direct bilirubin, albumin, prothrombin, platelet counts and AFP ($P>0.05$) (Supplementary Figure 1 and 2). In addition, we also compared clinical parameters according to different haplotypes however we did not observe any significant association of *SOCS3* promoter haplotypes with laboratory parameters.

Methylation status of *SOCS3* promoter region in primary HCCs

In total, 127 CpG islands in the fragment of 1150 bp (-1091 through +60) in the *SOCS3* promoter region were investigated. We found that CpG islands were unmethylated in the fragment 1 (-1091 through -679) while were hypermethylated in the fragment 2 (-425 through -217) and in the fragment 3 (-140 through -28) (Figure 2 and 3A). Subsequently, we analyzed methylation status in the *SOCS3* promoter in 37 pairs of tissue samples (tumor and adjacent non-tumor tissues). We observed that the *SOCS3* promoter region was methylated in 26/37 (70.3%) liver tumor tissues while only in 11/37 (29.7%) adjacent non-tumor tissues showed methylation patterns (OR=5.4, 95%CI=1.9-17.1, $P=0.0011$) (Figure 3A). This result indicates that *SOCS3* promoter methylation occurs more frequently in tumor tissues compared to adjacent non-tumor tissues. In addition, we analyzed the intensity of *SOCS3* promoter methylation in 11 pairs of tissue samples, in which *SOCS3* promoter methylation was detected in both tumor and adjacent non-tumor tissues. We observed that the methylation intensity was higher in tumor tissues compared to adjacent non-tumor tissues ($P=0.012$) (Figure 3B). In addition, we compared the status and intensity of *SOCS3* promoter methylation between tissue samples (tumor and non-tumor) with and without HBV infection. However, no statistical significance was observed.

SOCS3 mRNA expression in primary HCCs

The mean levels of *SOCS3* mRNA expression did not differ significantly between tumor and non-tumor tissues (Figure 4A and Supplementary Figure 3). The hypermethylated tumor tissues had significantly decreased *SOCS3* mRNA expression than non hypermethylated tumor tissues ($P=0.0023$). However, there were no significant differences in *SOCS3* mRNA expression between hypermethylated tumor and non-tumor liver tissues. (Figure 4B and Supplementary Figure 3). Our data demonstrate that the hypermethylation status in the *SOCS3* promoter is associated with downregulation of the *SOCS3* mRNA expression in tumor tissues.

SOCS3 mRNA expression discriminating between HCC and non-HCC tissue samples showed that *SOCS3* mRNA expression was significantly higher in HBV-related HCC tissues compared to non-HBV-related HCC tissues ($P=0.0048$) (Figure 4C and Supplementary Figure 3). In order to examine whether *SOCS3* mRNA expression was associated with the development of liver cancer, we analyzed *SOCS3* mRNA expression according to the BCLC staging classification. However, *SOCS3* mRNA expression was not different between stage A and B HCC tissues (Figure 4D and Supplementary Figure 3).

DISCUSSION

The negative regulator SOCS3 is a key player in the modulation of the JAK/STAT signaling that control a number of inflammatory cytokines such as IL6 and IL16 [6] and is involved in infectious diseases and cancers [13, 14]. In addition, gene silencing mediated by aberrant methylation of CpG islands in the *SOCS3* promoter frequently occurs in malignancies [16, 21]. In this study, we investigated the possible association of *SOCS3* promoter variants with the progression of HBV-related liver diseases and *SOCS3* methylation with HBV-induced HCC**.** We showed that the *SOCS3*

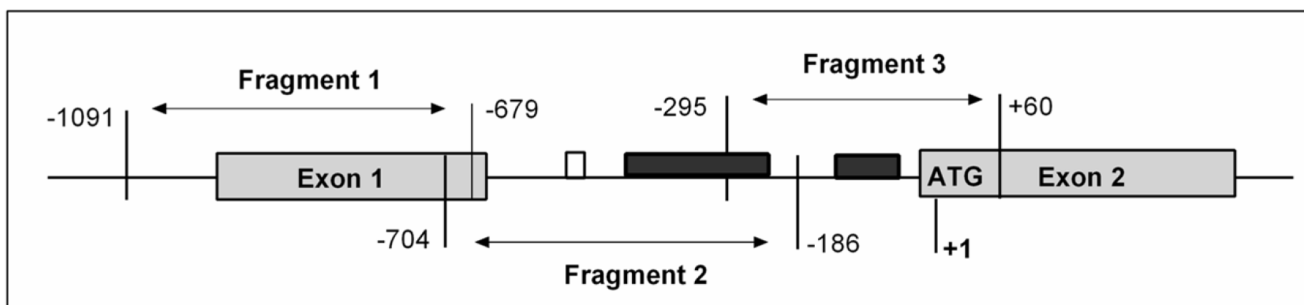


Figure 2: Schematic structure of *SOCS3* gene presenting the location of CpG island. Methylation status of *SOCS3* promoter region was analyzed by bisulfite sequencing (BS). The start codon site for *SOCS3* gene is defined as +1. The shaded boxes depict the exons of the *SOCS3* gene and open box represents the reported STAT3-binding site and the black boxes represent the region with aberrant methylation in the fragments 2 and 3.

promoter variants are associated with HBV infection and HBV-related liver diseases. *SOCS3* mRNA expression was higher in tumor tissues infected with HBV than non-infected tumor tissues. The aberrant methylation of the CpG islands in the *SOCS3* promoter is associated with relatively low mRNA expression in tumor tissues.

This first study reports on the association of *SOCS3* variants with HBV susceptibility and progression of HBV-related liver diseases. We have shown that the variants rs111033850T/C and rs12953258C/A are associated with HBV infection and the progression of HBV-related liver diseases. Particularly, the variant rs111033850T/C shows a heterozygous advantage in HBV susceptibility but might be a risk factor for the disease progression. The contribution of the minor allele *rs111033850C* to the increased risk of HCC in CHB patients is through the gene dose manner. In studies on hepatitis C, the *rs4969170AA* genotype was associated with antiviral IFN-α resistance with increased *SOCS3* expression in HCV patients [14, 18]. The rs4969170A/G polymorphism was associated with HCV treatment-induced neutropenia

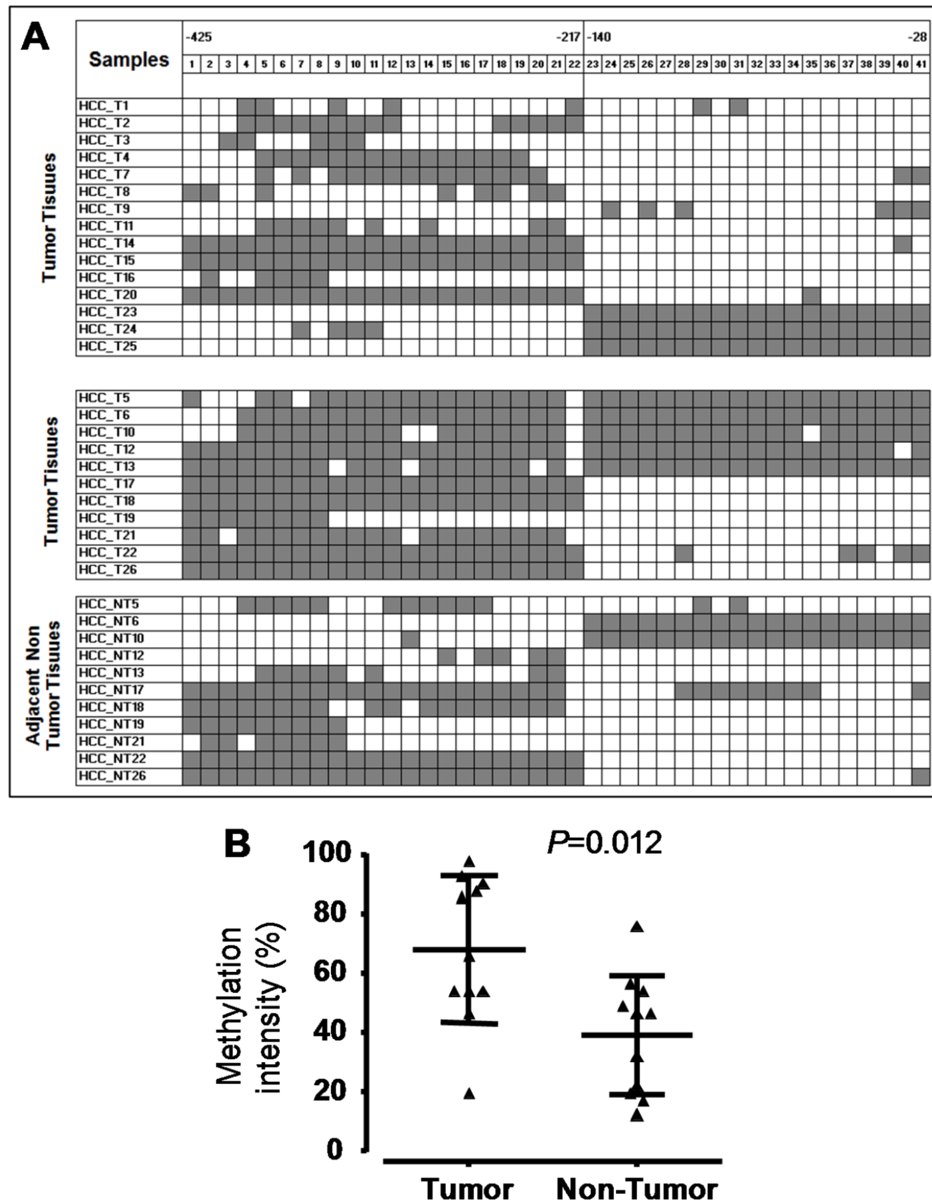


Figure 3: Methylation status of the *SOCS3* CpG islands in the promoter region. A. Methylation status of HCC tumor and non-tumor tissues. We randomly sequenced five to eight clones of PCR products amplified from Bisulfite treated genomic DNA for each liver tissue sample. The highly methylated CpG islands were found in two regions (from positions -425 to -217 and from positions -140 to -28). Each square shape presents one CpG island, black color shows CpG islands with methylation and white color shows CpG islands without methylation. **B.** Methylation intensity of the 11 HCC tumor and 11 adjacent non-tumor tissue samples. *P* values were calculated by Mann-Whitney test.

and thrombocytopenia in antiviral therapy with pegylated interferon alpha [17]. The rs4969170A/G polymorphism was associated with clinical features and prognosis of HCC after surgical treatment [22]. Our results support earlier findings that *SOCS3* polymorphisms influence liver disease progression by modulating the SOCS3 protein expression and thus down-regulate the JAK/STAT signaling.

In this study, we showed that HBV-DNA loads were associated with the *SOCS3* promoter polymorphisms rs111033850T/C and rs12953258C/A. The HBV-DNA loads are an important and independent risk factor for liver disease progression in CHB patients [23, 24]. The effects of HBV replication during HBV persistence were regulated by many host factors [25]. Previous studies have shown that the control of HBV replication was regulated by JAK/STAT signaling, which is activated by IFNs [15, 26, 27]. IFNs play a central role in control of viral replication including HBV [28] and IFNs are controlled by JAK/STAT signaling, which is regulated by SOCS3 protein [9]. Therefore, *SOCS3* promoter variants might have contributed to control of HBV replication through IFN signaling, which is also regulated by JAK/STAT signaling. However, the effects of *SOCS3* and its variants on the cytokine signaling that subsequently influence the HBV replication are required further studies.

Cytokines are involved in cell communication and are required for the defense against hepatitis viruses [28]. Previous study has shown that the HCV core protein impairs IFN-α-induced signal transduction via induction of *SOCS3* expression [29] and therefore influences the outcome of antiviral therapy [30]. SOCS3 was

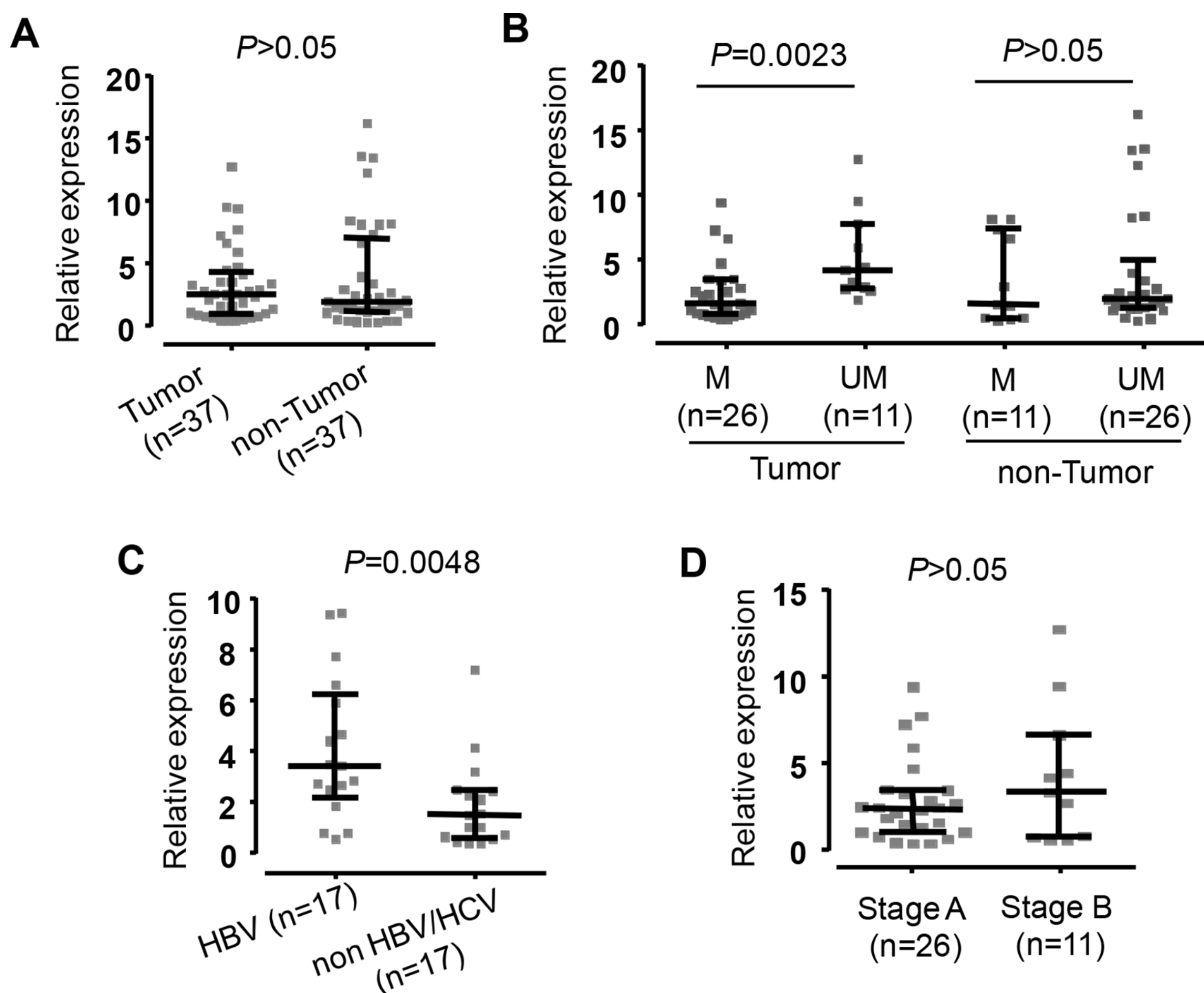


Figure 4: Expression of *SOCS3* mRNA in liver specimens from HCC patients. Quantitative real-time PCR (qRT-PCR) analysis presents comparison of *SOSC3* mRNA level. **A.** The *SOSC3* mRNA level in the tumor tissues and adjacent non-tumor tissues. **B.** The *SOSC3* mRNA level in the tumor and in non-tumor tissues with methylation (M) and in the tumor and in non-tumore tissues with un-methylation (UM). **C.** The *SOSC3* mRNA level in patients positive for HBV and in patients negative for both HBV and HCV. **D.** The *SOSC3* mRNA level in patients at early HCC stage (Stage A) and in patients at intermediate HCC stage (Stage B). The *GAPDH* gene was used as a reference gene. The data are shown as the medians with inter-quartile range. *P* values were calculated by Mann-Whitney test.

overexpressed in liver tissues and was strongly associated with severity of hepatic inflammation in CHB patients [15, 27]. In accordance, SOCS3 overexpression was observed in liver tissues from HBV-infected patients rather than non-HBV patients. These results may indicate that HBV can induce *SOCS3* expression, which in turn inhibits IFN signaling transduction resulting in the progression of liver diseases and failure of IFN treatment of HBV infection [27].

HCC development is often due to chronic liver injury, inflammation, and cirrhosis caused by the persistence of HBV infection. However, the interaction between HBV and SOCS3 in infected hepatocytes has not been clearly understood. DNA hypermethylation in the promoter region can lead to the silence of *SOCS3* in HCC [16, 31]. *SOCS3* silencing by promoter methylation is possibly involved in the progression of HBV-related liver cancer. In line with previous studies, our results showed that aberrant methylation in the *SOCS3* promoter region was observed more frequently in tumor tissues compared to adjacent non-tumor tissues. Hypermethylation status in the *SOCS3* promoter region may be a crucial factor for HCC development. However, *SOCS3* expression in tumor and adjacent non-tumor tissues was not significantly different, suggesting that other factors such as phosphorylation, acetylation and microRNAs may also involve in the regulation of *SOCS3* expression during progression of liver diseases [32]. In addition, both *SOCS3* methylation status and intensity were not significantly different between tissue samples (tumor and non-tumor) from patients with and without HBV infection. This indicates that HBV may not promote DNA methylation of host genes in infected hepatocytes. However, further studies are needed to verify this preliminary observation since the number of samples used for this analysis was rather small.

Although our data indicate that SOCS3 expression is associated with HBV infection and may involve in the progression of HBV-related liver diseases, the study has several limitations. A limited number of HCC tumor and non-tumor tissues were utilized. Due to the study design as a case-control study, *SOCS3* expression over the course of HBV infection were not assessed longitudinally and therefore the causative effect of *SOCS3* expression on progression of HBV-related liver diseases could not conclusively be determined. The insufficiency of some clinical and laboratory parameters such as HBV serology tests and HBV genotypes may weaken the findings indicating the crucial role of SOCS3 in the immune response to HBV infection and the disease outcomes.

In conclusion, the *SOCS3* promoter variants rs111033850 and rs12953258 are associated with HBV infection and HBV-related liver diseases. DNA methylation in the *SOCS3* promoter region is related to the regulation of *SOCS3* expression and occurs frequently in HCC tumors of HBV-infected patients. Our study suggests that *SOCS3* polymorphisms and methylation play an important role in regulation of *SOCS3* expression and thus influences the progression of HBV-related liver diseases.

MATERIALS AND METHODS

Patients and liver specimens

878 unrelated Vietnamese HBV-infected patients were randomly recruited in a case-control design at 108 Military Central Hospital and 103 Military Hospital of the Vietnam Military Medical University, Hanoi, Vietnam, between 2012 and 2013. With this sample size, we can detect the significance of common studied *SOCS3* variants according to the sample size estimation based on the 95% confidence interval, the lowest detection rate of minor allele set at the lowest value of 5%, a significance level of 5% and a power >90%. Patients were assigned to subgroups of disease based on clinical manifestations and liver function tests. Subgroups included chronic hepatitis (CHB, n=212), liver cirrhosis (LC, n=243), hepatocellular carcinoma (HCC, n=220) and patients with LC and HCC (LC+HCC, n=203). The diagnostic criteria for the CHB patients and the HBV-related LC were previously described [33]. The HBV-related HCC group was characterized as patients infected with HBV and was diagnosed based on the American Association for the Study of Liver Diseases (AASLD) practice guideline for HCC [34]. The patients with LC and HCC were characterized if the patients showed clinical manifestations and laboratory tests of both LC and HCC. The patients with LC were also categorized as Child-A, Child-B and Child-C based on Child-Pugh scores [35]. None of these HBV-infected patients had a history of alcohol or drug abuse. All participants were confirmed negative for anti-HCV and anti-HIV by ELISA assays. HBV-DNA loads and liver function tests including alanine transaminase (ALT), aspartate transaminase (AST), total bilirubin and direct bilirubin, albumin, prothrombin were quantified. 272 blood samples from healthy individuals were collected from blood bank as the control group. In addition, we analyzed 37 dyads of liver specimens (tumor and adjacent non-tumor) collected from HCC patients who underwent surgery at the 108 Military Central Hospital between 2013 and 2014. The HCC patients who underwent surgery were independent from 878 HBV patients. HCC was confirmed by histology and classified based on the BCLC classification [36]. All specimens were frozen at -80°C until use.

Ethics statement

Informed written consent was obtained after explanation of the study at the time of sampling from all participants. The study was approved by the institutional review board of the 108 Military Central Hospital and the

103 Military Hospital of the Vietnam Military Medical University, Hanoi, Vietnam.

Genotyping of *SOCS3* promoter variants

Genomic DNA was isolated from blood using DNA purification kits (Qiagen, Hilden, Germany). The *SOCS3* promoter region (nucleotides -1109 to -772) including two pre-described SNPs (rs111033850 and rs12953258) were amplified by PCR using primer pair *SOCS3_PrF* and *SOCS3_PrR* (Supplementary Table 1). PCR components, thermal conditions and sequencing procedures are presented in the Supplementary Materials.

SOCS3 promoter methylation analysis

Liver tissues were grounded using liquid nitrogen. Genomic DNA was extracted from liver powder using DNA purification kit (Qiagen, Hilden, Germany). Extracted DNA (2μg) was subjected to bisulfite conversion using EZ DNA Methylation-Direct™ Kit (Zymo Research Corp, the USA), according to the manufacturer's protocol. Three different fragments were amplified from bisulfite-treated genomic DNA using three specific primer sets (Figure 2 and Supplementary Table 1). The PCR products were subsequently cloned into TOPO-TA pCR2.1 vector (Life Technologies, the USA). Eight clones were randomly picked from each transformation (each tissue sample) and were analyzed for methylation by direct sequencing.

SOCS3 mRNA expression

Total RNA was extracted from 37 dyads of liver biopsy tissues with Trizol reagent (Life Technologies, the USA). RNA was reverse transcribed into cDNA using QuantiTect Reverse Transcription Kit (Qiagen GmbH, Hilden, Germany). cDNA quantification was performed by qRT-PCR and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) gene was used as a reference gene. The specific primers used for evaluating *SOCS3* mRNA expression as well as the PCR components and thermal conditions of qRT-PCR are presented in the Supplementary Materials. Calculation of normalized gene expression was based upon the ΔΔCT method.

Statistical and genetic analysis

All statistical analysis was performed using R version 3.1.2 (http://www.r-project.org) and GraphPad Prism 6 (http://www.graphpad.com). Genotype and allelic frequencies were determined by simple gene counting and the haplotype frequency was estimated using the expectation-maximum algorithm method implemented in the Arlequin v.3.5.2.2. The deviations from Hardy-Weinberg equilibrium were calculated for each group. We used a binary logistic regression adjusted for age and gender to analyze association of *SOCS3* variants with HBV-related liver diseases applying for different genetic models. In the binary logistic regression model, the disease subgroups and control group are considered as dependent variables. The genetic data are considered as independent variables (predictors) while age (treated as a continuous variable) and gender (treated as a binary variable) are independent confounding factors. In addition, the Cochran-Armitage test for trend was used to examine the gene dose effect of risk allele. Fisher's exact test was used to test the difference of categorical variables between two groups. Student's t-test and Mann Whitney Wilcoxon test were used to compare the parametric and non-parametric data of quantitative variables between two groups, respectively. Kruskal-Wallis test was used to compare non-parametric data of quantitative variables among more than two groups. The level of significance was set at a value of $P < 0.05$.

Abbreviations

CHB, chronic hepatitis B; HBV, hepatitis B virus; HC, healthy control; HCC, hepatocellular carcinoma; LC, liver cirrhosis; SNP, single nucleotide polymorphism; SOCS3, suppressor of cytokine signaling-3.

ACKNOWLEDGEMENTS

We thank all study subjects for their participation. We acknowledge Marisa Diehl, Nina Eiermann, and Kristin Patzwaldt for experimental helps and Le Duc Anh, Nguyen Viet Ha during sampling.

CONFLICTS OF INTEREST

All authors have no conflicts of interest to declare.

FINANCIAL SUPPORT

This study was supported from DAAD-PAGEL (57140033) for student fellowship. The authors greatly acknowledges the support by the Deutsche Forschungsgemeinschaft (DFG) and Open Access Publishing Fund of Tuebingen University. The funder has no role in the study design, data collection and analysis, decision to publish or preparation of the manuscript.

Author's contributions

NXH, HVT, CTB, HW and TPV designed the study and wrote the manuscript. NXH, HVT, and DPG performed the experiments and contributed equally to this work. NXH and HVT performed the statistical analyses and interpreted data. NXH, LHS, NLT and BKC recruited patients and collected samples. PGK and TPV contributed

to materials and reagents. All authors agreed with the results and conclusions.

REFERENCES

1. WHO. Hepatitis B. 2015. Report No.: Fact sheet N°204.
2. Liu J, Fan D. Hepatitis B in China. Lancet. 2007; 369:1582-3.
3. El-Serag HB. Hepatocellular carcinoma. N Engl J Med. 2011; 365:1118-27.
4. Rehermann B, Bertoletti A. Immunological aspects of antiviral therapy of chronic hepatitis B virus and hepatitis C virus infections. Hepatology. 2015; 61:712-21.
5. Carow B, Rottenberg ME. SOCS3, a Major Regulator of Infection and Inflammation. Front Immunol. 2014; 5:58.
6. Yoshimura A, Naka T, Kubo M. SOCS proteins, cytokine signalling and immune regulation. Nat Rev Immunol. 2007; 7:454-65.
7. Chang J, Block TM, Guo JT. The innate immune response to hepatitis B virus infection: implications for pathogenesis and therapy. Antiviral Res. 2012; 96:405-13.
8. Chang JJ, Lewin SR. Immunopathogenesis of hepatitis B virus infection. Immunol Cell Biol. 2007; 85:16-23.
9. Kubo M, Hanada T, Yoshimura A. Suppressors of cytokine signaling and immunity. Nat Immunol. 2003; 4:1169-76.
10. Yu H, Kortylewski M, Pardoll D. Crosstalk between cancer and immune cells: role of STAT3 in the tumour microenvironment. Nat Rev Immunol. 2007; 7:41-51.
11. Jiang R, Tan Z, Deng L, Chen Y, Xia Y, Gao Y, Wang X, Sun B. Interleukin-22 promotes human hepatocellular carcinoma by activation of STAT3. Hepatology. 2011; 54:900-9.
12. Yu H, Pardoll D, Jove R. STATs in cancer inflammation and immunity: a leading role for STAT3. Nat Rev Cancer. 2009; 9:798-809.
13. Yoshimura A, Suzuki M, Sakaguchi R, Hanada T, Yasukawa H. SOCS, Inflammation, and Autoimmunity. Front Immunol. 2012; 3:20.
14. Persico M, Capasso M, Russo R, Persico E, Croce L, Tiribelli C, Iolascon A. Elevated expression and polymorphisms of SOCS3 influence patient response to antiviral therapy in chronic hepatitis C. Gut. 2008; 57:507-15.
15. Koeberlein B, zur HA, Bektas N, Zentgraf H, Chin R, Nguyen LT, Kandolf R, Torresi J, Bock CT. Hepatitis B virus overexpresses suppressor of cytokine signaling-3 (SOCS3) thereby contributing to severity of inflammation in the liver. Virus Res. 2010; 148:51-9.
16. Niwa Y, Kanda H, Shikauchi Y, Saiura A, Matsubara K, Kitagawa T, Yamamoto J, Kubo T, Yoshikawa H. Methylation silencing of SOCS-3 promotes cell growth and migration by enhancing JAK/STAT and FAK signalings in human hepatocellular carcinoma. Oncogene. 2005; 24:6406-17.
17. Vidal F, Lopez-Dupla M, Laguno M, Veloso S, Mallolas J, Murillas J, Cifuentes C, Gallart L, Auguet T, Samperiz G, Payeras A, Hernandez P, Arnedo M, et al. Pharmacogenetics of efficacy and safety of HCV treatment in HCV-HIV coinfected patients: significant associations with IL28B and SOCS3 gene variants. PLoS One. 2012; 7:e47725.
18. Zheng YY, Wang LF, Fan XH, Wu CH, Huo N, Lu HY, Xu XY, Wei L. Association of suppressor of cytokine signalling 3 polymorphisms with insulin resistance in patients with chronic hepatitis C. J Viral Hepat. 2013; 20:273-80.
19. Fischer-Rosinsky A, Fisher E, Kovacs P, Bluher M, Mohlig M, Pfeiffer AF, Boeing H, Spranger J. Lack of association between the tagging SNP A+930–>G of SOCS3 and type 2 diabetes mellitus: meta-analysis of four independent study populations. PLoS One. 2008; 3:e3852.
20. Igci M, Cakmak EA, Oztuzcu S, Bayram A, Arslan A, Gogebakan B, Igci YZ, Cengiz B, Ozkara E, Camci C, Demiryurek AT. Mutational screening of the SOCS3 gene promoter in metastatic colorectal cancer patients. Genet Test Mol Biomarkers. 2012; 16:1395-400.
21. He B, You L, Uematsu K, Zang K, Xu Z, Lee AY, Costello JF, McCormick F, Jablons DM. SOCS-3 is frequently silenced by hypermethylation and suppresses cell growth in human lung cancer. Proc Natl Acad Sci U S A. 2003; 100:14133-8.
22. Jiang BG, Yang Y, Liu H, Gu FM, Yang Y, Zhao LH, Yuan SX, Wang RY, Zhang J, Zhou WP. SOCS3 Genetic Polymorphism Is Associated With Clinical Features and Prognosis of Hepatocellular Carcinoma Patients Receiving Hepatectomy. Medicine (Baltimore). 2015; 94:e1344.
23. Chen CJ, Yang HI, Iloeje UH. Hepatitis B virus DNA levels and outcomes in chronic hepatitis B. Hepatology. 2009; 49:S72-S84.
24. Zacharakis G, Koskinas J, Kotsiou S, Tzara F, Vafeiadis N, Papoutselis M, Maltezos E, Sivridis E, Papoutselis K. The role of serial measurement of serum HBV DNA levels in patients with chronic HBeAg(-) hepatitis B infection: association with liver disease progression. A prospective cohort study. J Hepatol. 2008; 49:884-91.
25. Levrero M, Pollicino T, Petersen J, Belloni L, Raimondo G, Dandri M. Control of cccDNA function in hepatitis B virus infection. J Hepatol. 2009; 51:581-92.
26. Robek MD, Boyd BS, Wieland SF, Chisari FV. Signal transduction pathways that inhibit hepatitis B virus replication. Proc Natl Acad Sci U S A. 2004; 101:1743-7.
27. Du LY, Cui YL, Chen EQ, Cheng X, Liu L, Tang H. Correlation between the suppressor of cytokine signaling-1 and 3 and hepatitis B virus: possible roles in the resistance to interferon treatment. Virol J. 2014; 11:51.

28. Koziel MJ. Cytokines in viral hepatitis. Semin Liver Dis. 1999; 19:157-69.

29. Bode JG, Ludwig S, Ehrhardt C, Albrecht U, Erhardt A, Schaper F, Heinrich PC, Haussinger D. IFN-alpha antagonistic activity of HCV core protein involves induction of suppressor of cytokine signaling-3. FASEB J. 2003; 17:488-90.

30. Persico M, Capasso M, Persico E, Svelto M, Russo R, Spano D, Croce L, La M, V, Moschella F, Masutti F, Torella R, Tiribelli C, Iolascon A. Suppressor of cytokine signaling 3 (SOCS3) expression and hepatitis C virus-related chronic hepatitis: Insulin resistance and response to antiviral therapy. Hepatology. 2007; 46:1009-15.

31. Zhang X, You Q, Zhang X, Chen X. SOCS3 Methylation Predicts a Poor Prognosis in HBV Infection-Related Hepatocellular Carcinoma. Int J Mol Sci. 2015; 16:22662-75.

32. Boosani CS, Agrawal DK. Methylation and microRNA-mediated epigenetic regulation of SOCS3. Mol Biol Rep. 2015; 42:853-72.

33. Song lH, Binh VQ, Duy DN, Juliger S, Bock TC, Luty AJ, Kremsner PG, Kun JF. Mannose-binding lectin gene polymorphisms and hepatitis B virus infection in Vietnamese patients. Mutat Res. 2003; 522:119-25.

34. Bruix J, Sherman M. Management of hepatocellular carcinoma: an update. Hepatology. 2011; 53:1020-2.

35. Cholongitas E, Papatheodoridis GV, Vangeli M, Terreni N, Patch D, Burroughs AK. Systematic review: The model for end-stage liver disease--should it replace Child-Pugh's classification for assessing prognosis in cirrhosis? Aliment Pharmacol Ther. 2005; 22:1079-89.

36. Llovet JM, Bru C, Bruix J. Prognosis of hepatocellular carcinoma: the BCLC staging classification. Semin Liver Dis. 1999; 19:329-38.

SOCS3 genetic variants and promoter hypermethylation in patients with chronic hepatitis B

SUPPLEMENTARY DATA

SUPPLEMENTARY MATERIALS AND METHODS

Genotyping of *SOCS3* promoter variants

Genomic DNA was isolated from whole blood using a DNA purification kit (Qiagen, Hilden, Germany). The *SOCS3* promoter region (nucleotides -1109 to -772) including two pre-described SNPs (rs111033850 and rs12953258) were amplified by PCR using primer pair *SOCS3_PrF* and *SOCS3_PrR* (Supplementary Table 1). PCR amplifications were carried out in a 25µl reaction volume containing (Qiagen): 1X PCR buffer, 0.2mM dNTPs, 1mM MgCl2, 0.15mM of each primer, 1unit of Taq polymerase and 50ng of genomic DNA. The cycling conditions were as follows: denaturation at 95°C for 5min, followed by 40 cycles of three-step cycling with denaturation at 94°C for 30s, annealing at 63°C for 35s, and extension at 72°C for 45s and a final extension at 72°C for 7min. PCR products were purified using Exo-SAP-IT (USB, Affymetrix, USA). 5µl of purified PCR products were used as templates. The sequencing was performed using the BigDye terminator v.1.1 cycle sequencing kit (Applied Biosystems, Foster city, USA) on an ABI 3130XL DNA sequencer according to the manufacturer's instructions. Sequencing results were finally analysed using vector NTI v.10 software.

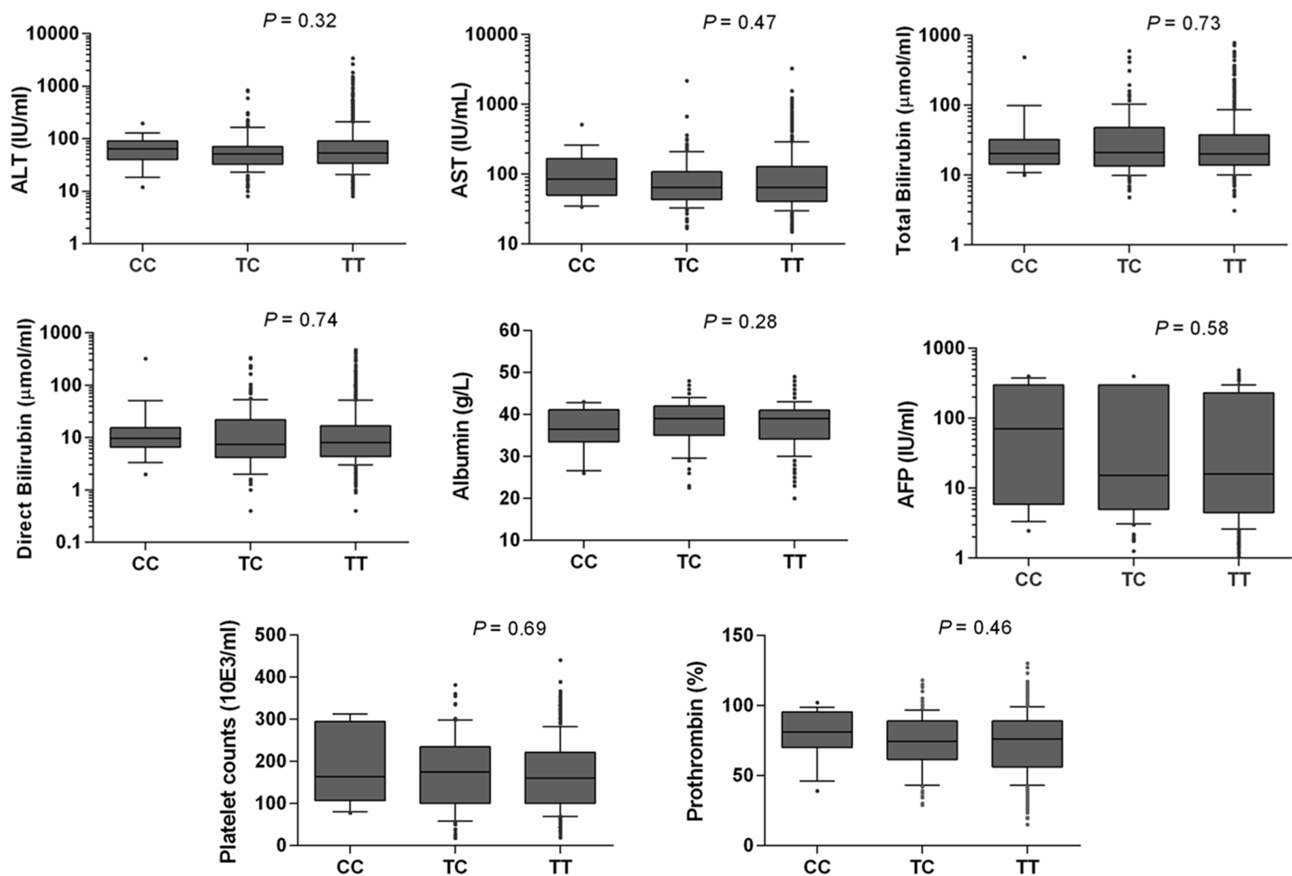
SOCS3 mRNA expression

Total RNA was extracted from 37 dyads of liver biopsy tissues with Trizol reagent (Life Technologies). RNA was reverse transcribed into cDNA using QuantiTect Reverse Transcription Kit (Qiagen GmbH, Hilden, Germany). Quantification of cDNA was performed by qRT-PCR using SYBR Green PCR mix (Bioline, Germany). All reactions were performed in triplicate using the LightCycler®480 real-time PCR system (Roche, Switzerland). The *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) gene was used as a reference gene. In addition, the *TGFBRAP-1* gene (transforming growth factor, beta receptor associated protein 1) was selected as an additional reference gene using Genevestigator (https://genevestigator.com/gv/). The specific primers used for evaluating the *SOCS3* mRNA expression were presented in the Supplementary Table 1. The thermal cycling conditions were as follows: 2min at 95°C followed by 45 cycles of denaturation at 95°C for 5s and annealing at 58°C for 10s and extension at 72°C for 20s. The specificity of each reaction was confirmed by melting curve analysis. Calculation of normalized gene expression was based upon the ΔΔCT method. The fold change in *SOCS3* expression was normalized to the expressed reference genes and then compared to the mean level expression in non-tumor tissues as the calibrator sample as follows: $2^{-\Delta\Delta CT}$, where $\Delta\Delta Ct=(Ct_{socs3}-Ct_{ref})_{cancer\text{-}sample}-(Ct_{socs3}-Ct_{ref})_{calibrator\text{-}sample}$ [1].

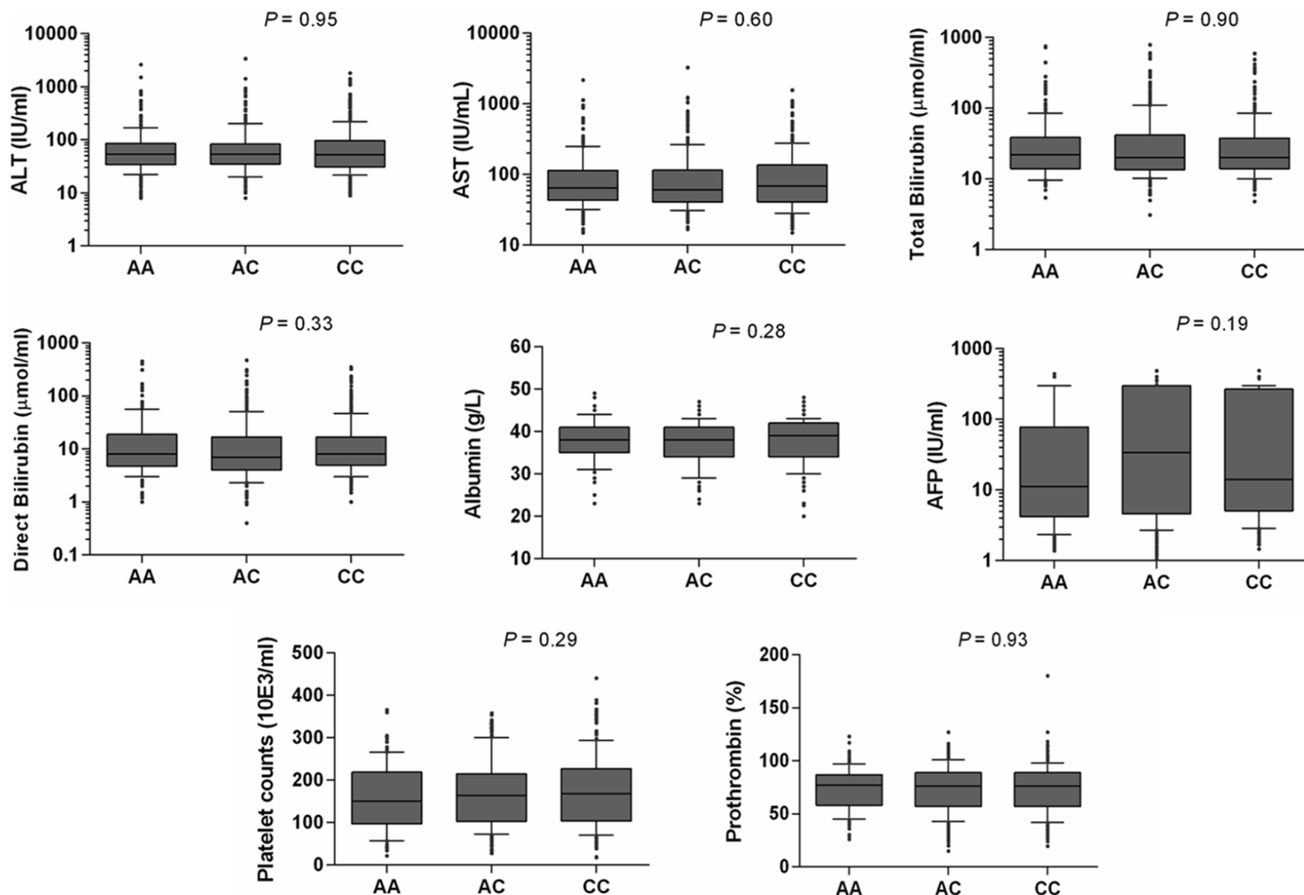
REFERENCES

1. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001; 25:402-8.
2. He B, You L, Uematsu K, Zang K, Xu Z, Lee AY, Costello JF, McCormick F, Jablons DM. SOCS-3 is frequently silenced by hypermethylation and suppresses cell growth in human lung cancer. Proc Natl Acad Sci U S A. 2003; 100:14133-8.
3. Niwa Y, Kanda H, Shikauchi Y, Saiura A, Matsubara K, Kitagawa T, Yamamoto J, Kubo T, Yoshikawa H. Methylation silencing of SOCS-3 promotes cell growth and migration by enhancing JAK/STAT and FAK signalings in human hepatocellular carcinoma. Oncogene. 2005; 24:6406-17.
4. Walsh MJ, Jonsson JR, Richardson MM, Lipka GM, Purdie DM, Clouston AD, Powell EE. Non-response to antiviral therapy is associated with obesity and increased hepatic expression of suppressor of cytokine signalling 3 (SOCS-3) in patients with chronic hepatitis C, viral genotype 1. Gut. 2006; 55:529-35.
5. Imai K, Kamio N, Cueno ME, Saito Y, Inoue H, Saito I, Ochiai K. Role of the histone H3 lysine 9 methyltransferase Suv39 h1 in maintaining Epsteinn-Barr virus latency in B95-8 cells. FEBS J. 2014; 281:2148-58.

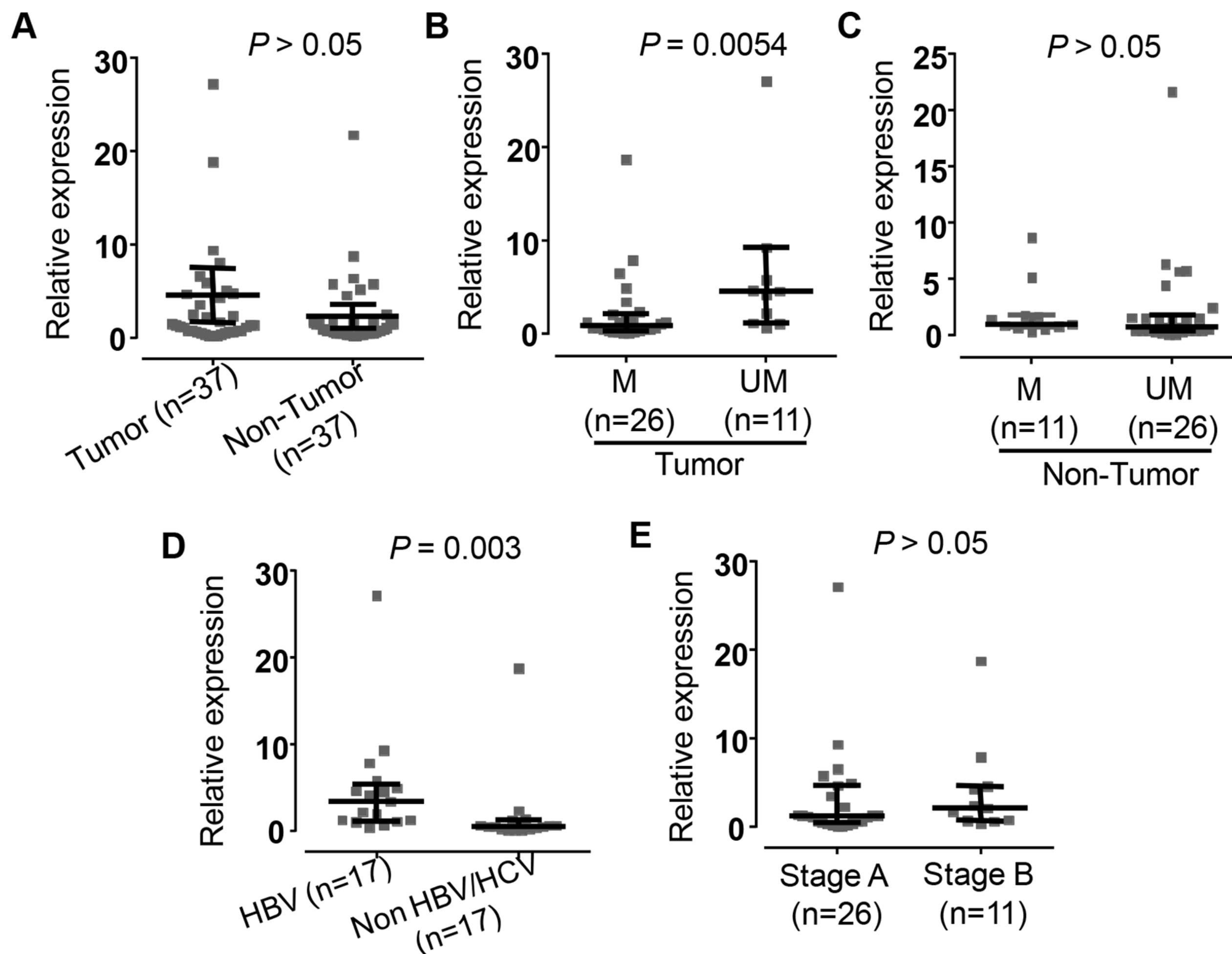
SUPPLEMENTARY FIGURES AND TABLES



Supplementary Figure 1: Association of clinical parameters with *SOCS3* rs111033850T/C variants. Box-plots illustrate medians with 25 and 75 percentiles with whiskers to 10 and 90 percentiles; P values were calculated by Krusskal - Wallis test.



Supplementary Figure 2: Association of clinical parameters with *SOCS3* rs12953258C/A variants. Box-plots illustrate medians with 25 and 75 percentiles with whiskers to 10 and 90 percentiles; P values were calculated by Krusskal - Wallis test.



Supplementary Figure 3: Expression of *SOCS3* mRNA in liver specimens from HCC patients. Quantitative real-time PCR (qRT-PCR) analysis presents comparison of *SOSC3* mRNA level. **A.** The *SOSC3* mRNA level in the tumor tissues and adjacent non-tumor tissues. **B, C.** The *SOSC3* mRNA level in the tumor and in non-tumor tissues with methylation (M) and in the tumor and in non-tumore tissues with un-methylation (UM). **D.** The *SOSC3* mRNA level in patients positive for HBV and in patients negative for both HBV and HCV. **E.** The *SOSC3* mRNA level in patients at early HCC stage (Stage A) and in patients at intermediate HCC stage (Stage B). The *TGFBRAP-1* gene was used as a reference gene. The data are shown as the medians with inter-quartile range. P values were calculated by Mann-Whitney test.

Supplementary Table 1: Primers used in this study

Primer	Sequence	Application
SOCS3_PrF	5'- CCG CGC TCA GCC TTT CTC TGC TGC GA-3'	*SOCS3* genotyping
SOCS3_PrR	5'-AGT CCA CAA AGG AGC CTT CGC GCG CG-3'	*SOCS3* genotyping
SOCS3_Fr1_F	5'-GTG TAG AGT AGT GAT TAA ATA-3'	*SOCS3* promoter methylation
SOCS3_Fr1_R	5'-TCC TTA AAA CTA AAC CCC CTC-3'	*SOCS3* promoter methylation
SOCS3_Fr2_F	5'-GAT TYG AGG GGG TTT AGT TTT AAG GA-3'	*SOCS3* promoter methylation
SOCS3_Fr2_R	5'-CCA CTA CCC CAA AAA CCC TCT CCT AA-3'	*SOCS3* promoter methylation
SOCS3_Fr3_F	5'- GGG AAG GGG TTG TTY GGG GTT ATT TTG -3'	*SOCS3* promoter methylation
SOCS3_Fr3_R	5'- CAA ACT AAT ATC CAA AAA ACA ACT CAT CC -3'	*SOCS3* promoter methylation
SOCS3_Exp_F	5'- CCC TCG CCA CCT ACT GAA -3'	SOCS3 mRNA expression
SOCS3_Exp_R	5'- TCC GAC AGA GAT GCT GAA GA -3'	SOCS3 mRNA expression
GAPDH_F	5'-TGCACCACCAACTGCTTAGC-3'	SOCS3 mRNA expression
GAPDH_R	5'-GGCATGGACTGTGGTCATGAG-3'	SOCS3 mRNA expression
TGFBRAP-1_F	5'-GCG GCT GTG TCC TTT CCA TA-3'	SOCS3 mRNA expression
TGFBRAP-1_R	5'-GCG TCT GCT TCT GTT GCT GAT-3'	SOCS3 mRNA expression

The specific primer pair *SOCS3_PrF* and *SOCS3_PrR* were designed to amplify nucleotides -1109 to -772 from start codon and were used for amplification and sequencing of the SOCS3 promoter region. Primers for fragment 1 were *SOCS3_Fr1_F* and *SOCS3_Fr1_R* [2], primers for fragment 2 were *SOCS3_Fr2_F* and *SOCS3_Fr2_R* [3], and primers for fragment 3 were *SOCS3_Fr3_F* and *SOCS3_Fr3_R*. These primers were designed to amplify nucleotides -1091 to -679; - 704 to -186, and -295 to +60, respectively. The PCR amplification of the three fragments were performed as described previously (2;3). The specific primers used for evaluating the expression of *SOCS3* mRNA were *SOCS3_Exp_F* and *SOCS3_Exp_R* [4]. Primers used for the reference genes *were GAPDH_F* and *GAPDH_R* (*GAPDH)* [5] and were *TGFBRAP-1_F* and *TGFBRAP-1_R* (*TGFBRAP-1*).

Supplementary Table 2: Allelic and genotypic frequencies of *SOCS3* variants in sub-HBV patient groups and healthy controls

SOCS3 variants	CHB (%)	LC (%)	HCC (%)	HCC+LC (%)	HC (%)	CHB vs. HC		LC vs. HC		HCC vs. HC		HCC+LC vs. HC	
	n=212	n=243	n=220	n=203	n=272	OR (95%CI	*P* value	OR (95%CI	*P* value	OR (95%CI	*P* value	OR (95%CI	*P* value
rs111033850T/C													
Genotype													
TT	190(89.6)	198(81.5)	178(80.9)	162(79.8)	191(70.3)	Reference		Reference		Reference		Reference	
TC	16(7.5)	43(17.7)	36(16.4)	35(17.2)	76(27.9)	**0.2(0.1-0.4)**	< .0001	**0.6 (0.4-0.9)**	**0.02**	**0.6(0.4-1.0)**	**0.045**	**0.6(0.3-0.9)**	**0.03**
CC	6(2.9)	2(0.8)	6(2.7)	6(3.0)	5(1.8)	1.2(0.4 - 4.1)	0.73	1.7(0.3 - 9.9)	0.5	1.26 (0.3-5.1)	0.7	1.9(0.4 -9.1)	0.43
Allele													
T	396(93.4)	439(90.3)	392(89)	359(88.4)	458(84.2)	Reference		Reference		Reference		Reference	
C	28(6.6)	47(9.7)	48(11)	47(11.6)	86(15.8)	**0.4(0.2-0.6)**	< .0001	**0.6(0.4-0.9)**	**0.03**	**0.49(0.3-0.8)**	**0.001**	**0.7(0.5-1.2)**	0.21
Dominant													
TT	190(89.6)	198(81.5)	178(80.9)	162(79.8)	191(70.3)	Reference		Reference		Reference		Reference	
TC & CC	22(10.4)	45(18.5)	42(19.1)	41(20.2)	81(29.7)	**0.3(0.2-0.5)**	< .0001	**0.6(0.4-0.9)**	**0.02**	0.6(0.4-1.1)	0.07	0.6(0.4-1.1)	0.08
Recessive													
TT & TC	206(97.2)	241(99.2)	214(97.3)	197(79.8)	267(98.2)	Reference		Reference		Reference		Reference	
CC	6(2.9)	2(0.8)	6(2.7)	6(3.0)	5(1.8)	1.6 (0.5-5.3)	0.45	0.7(0.1 - 4.0)	0.7	1.4(0.4-5.6)	0.6	2.1(0.4-10.2)	0.34
rs111033850C/A													
Genotype													
CC	94(44.3)	88(36.2)	86(39.1)	75(36.9)	101(37.1)	Reference		Reference		Reference		Reference	
AC	72(34)	88(36.2)	88(40)	84(41.4)	140(51.5)	**0.6(0.4 - 0.8)**	**0.00014**	0.8(0.5 - 1.2)	0.21	0.8(0.5-1.2)	0.27	0.9(0.5-1.5)	0.61
AA	46(21.7)	67(27.6)	46(20.9)	44(21.7)	31(11.4)	1.63(1 - 2.8)	0.08	**3.0(1.69-5.32)**	**< 0.0001**	**1.8(1.0-3.3)**	**0.047**	**2.8 (1.3-5.6)**	**0.004**
Allele													
C	260(61.3)	264 (54.3)	260 (59.1)	172(42.4)	342(62.9)	Reference		Reference		Reference		Reference	
A	164(38.7)	222 (45.7)	180 (40.9)	234(57.6)	202 (37.1)	1.1(0.8-1.4)	0.58	**1.6 (1.2-2.1)**	**0.001**	1.2(0. 9 - 1.6)	0.25	**1.5(1.1-2.1)**	**0.022**
Dominant													
CC	94(44.3)	88 (36.2)	86(39.1)	75(36.9)	101(37.1)	Reference		Reference		Reference		Reference	
AC & AA	118(55.7)	155 (63.8)	134(60.9)	128(63.1)	171(62.9)	0.7(0.5-1.1)	0.12	1.2(0.8-1.7)	0.48	1(0.6 - 1.5)	0.86	1.2(0.7-1.9)	0.48
Recessive													
CC & AC	166(78.3)	176(72.4)	174(79.1)	159(78.3)	241(88.6)	Reference		Reference		Reference		Reference	
AA	46(21.7)	67(27.6)	46(20.9)	44(21.7)	31(11.4)	**2.2(1.3-3.6)**	**0.0019**	**3.5(2.1-5.7)**	**< 0.0001**	**2.0(1.1-3.6)**	**0.015**	**3(1.6-5.7)**	**0.0007**

CHB: Chronic hepatitis B; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma; HC: Healthy control; Cases = all HBV infected patients; n= Number of chromosomes; OR: adjusted Odd Ratio; ORs and *P* values were calculated by using binary logistic regression model adjusted for age and gender. Bold values present the statistical significance.

Interferon-stimulated gene 15 in hepatitis B-related liver disease

Hoan NX, Van Tong H, Giang DP, Toan NL, Meyer CG, Bock CT, Kremsner PG, Song LH, Velavan TP.

Oncotarget. 2016 Oct 18;7(42):67777-67787. PMID: 27626177

Research Paper: Pathology

Interferon-stimulated gene 15 in hepatitis B-related liver diseases

Nghiem Xuan Hoan[1,2,3,*], Hoang Van Tong[1,3,*], Dao Phuong Giang[1,2,3,*], Nguyen Linh Toan[3,4], Christian G. Meyer[1,3], C.-Thomas Bock[5], Peter G. Kremsner[1,3], Le Huu Song[2,3,] and Thirumalaisamy P. Velavan[1,3,4,**]**

[1] Institute of Tropical Medicine, University of Tübingen, Tübingen, Germany

[2] 108 Military Central Hospital, Hanoi, Vietnam

[3] Vietnamese-German Center for Medical Research, Hanoi, Vietnam

[4] Department of Pathophysiology, Vietnam Military Medical University, Hanoi, Vietnam

[5] Department of Infectious Diseases, Robert Koch Institute, Berlin, Germany

[*] These authors have contributed equally to this work

[**] Shared senior authors

Correspondence to: *Thirumalaisamy P. Velavan,* ***email:*** *velavan@medizin.uni-tuebingen.de*

Keywords: HBV infection, liver diseases, ISG15, *ISG15* polymorphism, ISGlation, Pathology Section

Received: *June 11, 2016* ***Accepted***: *September 05, 2016* ***Published***: *September 10, 2016*

ABSTRACT

This study investigates the association of Interferon-stimulated gene 15 (*ISG15*) polymorphisms, ISG15 serum levels and expression with HBV-related liver diseases. The *ISG15* promoter and the two exons of the gene were screened for polymorphisms in 766 HBV-infected patients and in 223 controls. Soluble ISG15 levels were measured by ELISA. *ISG15* mRNA expression was quantified by qRT-PCR in 36 tumor and adjacent non-tumor tissues. The exon 2 allele *rs1921A* was found associated with decreased progression of HBV-related liver diseases (LC *vs.* CHB: OR = 0.6, 95%CI = 0.4-0.8, adjusted *P* = 0.003; HCC *vs.* CHB: OR = 0.6, 95%CI = 0.4-0.9, adjusted *P* = 0.005). The *rs1921AA* genotype was associated with low levels of AST, ALT and total bilirubin, but with high prothrombin levels (*P* < 0.05). ISG15 serum levels were higher among HBV patients compared to controls (*P* < 0.0001) and positively associated with HBV-related liver diseases, with highest levels among LC patients. ISG15 levels were correlated with HBV-DNA loads (*P* = 0.001). In non-tumor tissues from HCC patients, *ISG15* mRNA expression was increased in HBV compared to non-HBV infection (*P* = 0.016). The *ISG15* rs1921 variant and *ISG15* expression are associated with HBV-related liver diseases. Taken together, ISG15 appears to be a proviral factor involved in HBV replication and triggering progression of HBV-related liver diseases.

INTRODUCTION

Although effective vaccines for hepatitis B virus (HBV) infections are available, HBV-related liver diseases remain a health problem of considerable concern with 250 million chronic carriers globally [1]. In Vietnam, the prevalence of HBV infection currently ranges from 10% to 20% in general population and HBV-related liver diseases are foreseen and predicted to be a notable public health burden in the next decades [2]. HBV causes various forms of infection, ranging from an asymptomatic carrier status to liver cirrhosis (LC) and life-threatening hepatocellular carcinoma (HCC) [1]. Important factors influencing disease progression include the patient's age, duration of HBV infection, and host-virus interactions. HBV has evolved various mechanisms to evade both innate and adaptive immune responses in order to establish persistent infections [3].

Currently, nucleos(t)ide analogues and Peg-Interferon alpha are the main classes of antivirals to treat chronic hepatitis B (CHB) [4,5]. IFN-α mediates signal transduction by binding to its receptors. Tyrosine phosphorylation of signal transducer and activator of transcription 1 (STAT1) and STAT2 leads to the formation

Table 1: Characteristics HBV patients segregated according to clinical presentation and healthy controls

Characteristics	CHB (n = 262)	LC (n = 241)	HCC (n = 263)	HC (n = 223)
Age (years)	42 [18-82]	54 [18-84]	58 [18-81]	37 [18-69]
Male (%)	74.5	83.7	92.2	66.4
AST (IU/L)	49 [14-7700] ‡ β	75 [15-1221]	60 [17-2158]	<40
ALT (IU/L)	58 [9-4908] ‡ β	56 [8-1426]	46 [11-832]	<40
Total bilirubin (mg/dL)	16 [6-357] ‡ β	30 [3-752]	18 [6-419]	<17
Direct bilirubin (mg/dL)	5 [1-226] ‡ β	12 [1-450]	6.5 [1.2-214]	<5
Albumin (g/L)	41 [23-50]	33 [20-47] ‡β	38 [22-49]	>35
Prothrombin (% of standard)	87 [30-180]	54 [15-101] ‡β	80 [20-149]	>70
WBC ($x10^3$/mL)	6 [3.6-13.9]	5.6 [1.7-20.5)	6 [6.6-16]	4 - 10
RBC($x10^6$/mL)	4.8 [3.2-6.8)	3.9 [1.9-6.7)	4.5 [2.1-6.8)	4 - 9
PLT ($x10^3$/mL)	195 [19-472]	89 [18-441] ‡ β	166 [34-389]	150 -300
HBV-DNA (copies/mL)	$5.8x10^5$ [$2x10^2$- $8.4x10^{10}$] ‡ β	$1.8x10^4$ [$1.8x10^2$- $4.7x10^9$]	$8.2x10^5$[10^2- $3x10^{10}$]	NA
AFP (IU/L)	2.9 [1.5-320]	36 [1.2-400]	196 [1.1-480] ‡ β	<5

CHB, chronic hepatitis B; LC, liver cirrhosis; HCC, hepatocellular carcinoma; HC, healthy control; RBC, red blood cells; WBC, white blood cells; PLT, platelets. AST and ALT, aspartate and alanine amino transferase; AFP, alpha-fetoprotein; IU, international unit; NR, normal range, NA, not applicable. Values given are medians and ranges. (β) Kruskal-Wallis test was used to test differences of nonparametric data. (‡): $P < 0.0001$ for comparisons with other groups.

of transcriptional complexes, which translocate to the nucleus and activate expression of certain genes [6]. IFN-stimulated gene factor 3 (ISGF3) is composed of phosphorylated forms of STAT1, STAT2 and interferon regulatory factor 9 (IRF9). This complex binds to interferon (IFN)-stimulated response elements (ISREs) located in the promoters of interferon-stimulated genes (ISGs) [6]. Among ISGs, the human ISG15 is a 15 kDa protein encoded by *ISG15* located on chromosome 1p36.33 (OMIM# 147571) [7]. ISG15 is a strongly induced protein in various cellular processes [8,9]. It exists in free and/or conjugated forms (ISGlation), covalently conjugated to protein targets via consecutive action of conjugating enzymes such as ubiquitin activating E1 (UbE1L), E2-conjugating enzyme (UbcH8) and E3 ligases. The ubiquitin-specific protease 18 (USP18), which has deconjugating protease functions, cleaves ISG15's substrates and removes ISG15 (deISGylation) from ISG15 conjugates [8].

Several studies have indicated that aberration of cell signaling in the ISG15 pathway perturbates ISG15 regulation and causes malignant transformation of various human cancers [10-14]. ISG15 overexpression in liver tumor tissue is associated with pathology and poor outcome of HCC patients [12,13]. ISG15 plays also a role in the response to many viral infections [9,15-18]. A recent study has shown that knockdown of *ISG15* results in suppression of hepatitis C virus (HCV) replication *in vitro* by promoting the IFN response, suggesting involvement of ISG15 in regulating HCV replication [19,20]. Furthermore, patients who respond favorably to IFN treatment of hepatitis C have low expression levels of ISG15 and its conjugates in the liver compared to non-responders [21,22]. So far, two studies using mouse models have documented the role of ISG15 in HBV pathogenesis. They concluded that mice injected with murine IFN-α expression plasmid along with HBV had higher expression levels of ISG15 [23], and an ISG15-deconjugating enzyme (USP18) reduced expression is associated with rapid HBV clearance [24]. However, the functional role of ISG15 in the HBV replication cycle, immune response and clinical progression of HBV-related liver diseases remains poorly understood. We investigated possible *ISG15* associations, of ISG15 serum levels and *ISG15* expression with outcomes of HBV infection and progression of HBV-related diseases.

RESULTS

Study participants

Baseline characteristics of the clinically well-characterized 766 HBV patients and of the 223 healthy controls (HCs) are given in Table 1. The median age of patients increased according to the progression of disease ($P < 0.001$). ALT, AST, total bilirubin and direct bilirubin levels as well as HBV loads were higher among CHB patients compared to other subgroups ($P < 0.0001$). As expected, albumin and prothrombin levels and platelet counts were lower in LC patients compared to the other patient groups ($P < 0.0001$). AFP levels were higher in HCC patients compared to the CHB and LC subgroups ($P < 0.0001$). Of the 36 HCC patients who underwent surgery, 32 were males and most patients (27/36, 75%) were aged between 40-60 years. According to Barcelona Clinic Liver Cancer (BCLC) staging [25], 25/36 (69%) HCC patients were in stage A and 11/36 (31%) HCC patients in stage B.

Among all HCC patients, 17/36 (47%) had HBV infection, 2/36 (6%) had HCV infection, and 17/36 (47%) showed non-HBV/HCV-related HCC (Suppl. Table 1).

Association of *ISG15* rs1921 variant with HBV-related liver diseases

The genotype frequencies of the *ISG15* rs1921 variant in HCs were in Hardy-Weinberg equilibrium (P = 0.166), whereas other promoter and exonic variants were not. Therefore, only *ISG15* rs1921 was considered for further analyses. The genotype and allele frequencies in different subgroups and the association analyses are presented in Table 2. Genotype and allele frequencies of *ISG15* rs1921 did not differ between HBV patients and controls, indicating that *ISG15* rs1921 is not associated with HBV infection *per se*. However, the *rs1921GA* genotype occurred more frequently among CHB patients compared to the LC and HCC subgroups (LC vs. CHB, OR = 0.5, 95%CI = 0.3-0.8, adjusted P = 0.036; HCC *vs.* CHB, OR = 0.5, 95%CI = 0.3-0.8, adjusted P = 0.014). The minor allele *rs1921A* was more frequent in CHB than in HCC and LC patients (LC *vs.* CHB, OR = 0.6, 95%CI = 0.4-0.8, adjusted P = 0.003; HCC *vs.* CHB, OR = 0.6, 95%CI = 0.4-0.9, adjusted P = 0.005). In a dominant genetic model, we also observed that minor allele *rs1921A* was associated with an increased protection against LC and HCC (LC *vs.* CHB, OR = 0.5, 95%CI = 0.3-0.7, adjusted P = 0.0016; HCC *vs.* CHB, OR = 0.5, 95%CI = 0.4-0.8, adjusted P = 0.003) (Table 2). These results indicate that *rs1921A* may contribute to a decreased risk of progression to LC and HCC in HBV infection.

In order to explore the influence of *ISG15* rs1921 on the clinical outcome of HBV-related liver disease, we compared clinical parameters among patients with different *ISG15* rs1921 genotypes (*GG*, *GA* and *AA*). Pathological liver function tests as indicated by high levels of AST, ALT, total bilirubin, direct bilirubin and by low prothrombin levels were observed rather in HBV patients with the *rs1921GG* genotype (P < 0.05) (Figure 1) than in the other subgroups. AFP levels and viral loads were higher in patients with *rs1921GG* compared to those with either *rs1921AA* or *rs1921GA*. However, the difference was not significant (P>0.05) (Figure 1 and Suppl. Figure 1A).

ISG15 serum levels and HBV-related liver diseases

We quantified ISG15 levels in serum samples of 470 HBV patients and 175 healthy controls. ISG15 serum levels were significantly lower in the control

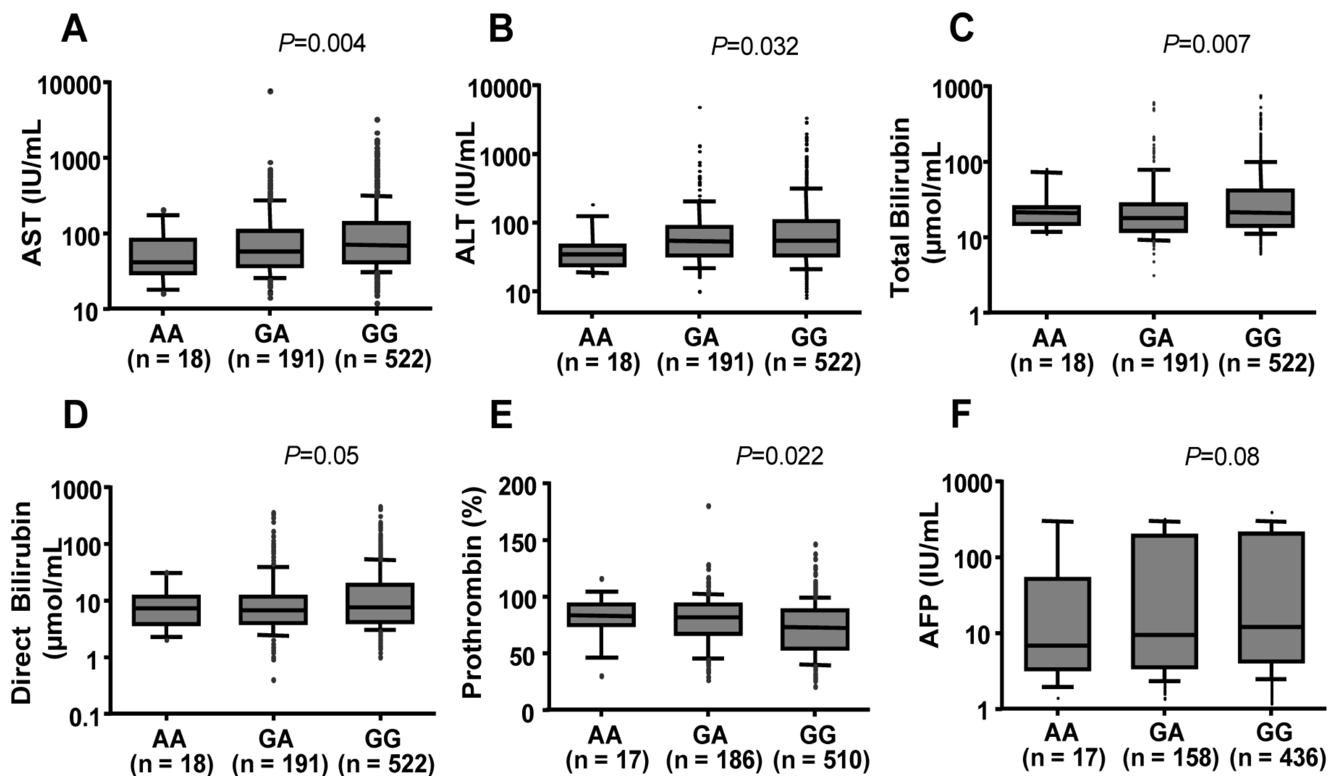


Figure 1: Association between *ISG15* variant with clinical parameters in HBV patients. Box-plots illustrate median values with 25 and 75 percentiles with whiskers to 10 and 90 percentiles; P values were calculated by Kruskal-Wallis tests. AST and ALT, aspartate and alanine amino transferase; AFP, alpha-fetoprotein.

Table 2: Association of *ISG15* variant (rs1921) with HBV-related liver diseases

ISG15 variant	CHB *n*(%)	LC n(%)	HCC *n*(%)	HC *n*(%)	HBV patients vs. HCs		LC vs. CHB		HCC vs. CHB		HCC vs. LC	
(rs1921)	*n* = 262	*n* = 241	*n* = 263	*n* = 223	OR (95%CI)	*P*	OR (95%CI)	*P*	OR (95%CI)	P	OR (95%CI)	*P*
Codominant												
GG	161(61.5)	180(74.7)	198(75.3)	158(70.7)	Reference		Reference		Reference		Reference	
GA	92(35.1)	57(23.7)	58(22.1)	56(25.2)	1.2(0.8-1.6)	0.43	**0.5 (0.3-0.8)**	**0.006**	**0.5 (0.3-0.8)**	**0.014**	0.9(0.6-1.4)	0.53
AA	9(3.4)	4(1.7)	7(2.6)	9(4.1)	0.7 (0.3-1.6)	0.89	0.4 (0.1-1.5)	0.17	0.3 (0.1-1.2)	0.12	1.9(0.5-6.7)	0.47
Allele												
G	414(79)	417(86.5)	454(86.3)	416(84.9)	Reference		Reference		Reference		Reference	
A	110(21)	65(13.5)	72(13.7)	74(15.1)	1.1(0.8-1.4)	0.90	**0.6 (0.4-0.8)**	**0.003**	**0.6 (0.4-0.9)**	**0.005**	1.0(0.7-1.5)	0.95
Dominant												
GG	161(61.5)	180(74.7)	198(75.3)	157(70.7)	Reference		Reference		Reference		Reference	
GA&AA	101(38.5)	61 (25.3)	65 (24.7)	65(29.3)	1.1(0.8-1.5)	0.62	**0.5 (0.3-0.7)**	**0.0016**	**0.5 (0.3-0.8)**	**0.003**	0.9(0.6-1.4)	0.83
Recessive												
GG&GA	253(96.6)	237 (98.3)	256(97.4)	213(95.9)	Reference		Reference		Reference		Reference	
AA	9(3.4)	4(1.7)	7(2.6)	9(4.1)	0.7(0.3-1.5)	0.34	0.5 (0.1-1.8)	0.25	0.6 (0.2-1.9)	0.32	1.9(0.5-68)	0.31

CHB, chronic hepatitis B; LC, liver cirrhosis; HCC, hepatocellular carcinoma; HC, healthy controls; n = numbers genotyped; OR, Odd Ratio. *P* values were calculated using binary logistic regression model adjusted for age and gender. Bold values reflect statistical significance.

group (median: 3.4 ng/ml) compared to all HBV patients (median: 8.1 ng/ml), and compared to the median values of the different subgroups (CHB, 6.5 ng/ml; LC, 12 ng/ml; HCC 8.0 ng/ml; $P < 0.0001$) (Figure 2A). Among the HBV patients, ISG15 levels were lower in the CHB than in the combined LC and HCC subgroups ($P = 0.008$ and 0.04; respectively) (Figure 2A, 2B). In addition, all LC, including HCC patients with concomitant LC, had significantly higher ISG15 levels than non-LC patients ($P = 0.00083$) (Figure 2C). However, ISG15 levels did not differ significantly between patients with and without HCC (HCC vs. CHB+LC: $P>0.05$) (Figure 2A and Suppl. Figure 2). These results show that ISG15 induced by HBV infection may play a role in progression of HBV-related liver diseases.

ISG15 rs1921 variant and ISG15 serum levels

We analyzed the association of the *ISG15* rs1921 variant with ISG15 serum levels in HBV patients and controls. ISG15 serum levels in HBV patients with the genotype *rs1921GG* were marginally higher than those in HBV patients with either *rs1921AA* or *rs1921GA* genotypes ($P = 0.089$) (Suppl. Figure 1B). Among controls, ISG15 levels did not differ among individuals with the various genotypes ($P>0.05$).

ISG15 serum levels and viral loads

Of 766 HBV patients, 222 were available for analyses of the correlation between ISG15 serum levels and HBV-DNA viral loads. ISG15 serum levels were significantly higher in patients with high viral loads (viral loads $\geq 10^5$ copies/ml) compared to patients with lower levels (viral loads $< 10^5$ copies/ml) (Figure 3A). In a simple linear regression analysis, the ISG15 serum levels were positively correlated with HBV-DNA loads ($r = 0.28$, $P < 0.0001$) (Figure 3B). High levels of HBV replication and LC were, in a multivariate linear regression model, independently associated with increased ISG15 serum levels ($P = 0.0011$ and 0.008, respectively) (Table 3).

ISG15 mRNA expression in primary HCC

We also analyzed expression of *ISG15* mRNA in HCC tissue specimens and in adjacent non-tumor liver tissues retrieved from 36 primary HCC patients. The mean of *ISG15* mRNA expression in liver tumor tissues was higher than in adjacent non-tumor tissues (not significant; Figure 4A). We examined whether *ISG15* mRNA expression can be up-regulated during HCC development, however, *ISG15* mRNA expression did not differ between stage A and stage B HCC tissues (Figure 4B). We further examined whether *ISG15* mRNA expression is associated with HBV infection. *ISG15* mRNA expression was significantly higher in HBV-related non-tumor tissues compared to non-HBV-related non-tumor tissues ($P = 0.016$). A similar trend was observed when comparing HBV-related and non-HBV-related tumor tissues, but the difference was not significant (Figure 4C). These results indicate that increased expression of *ISG15* in liver tissues is regulated by HBV infection.

DISCUSSION

The interferon signaling pathway constitutes the first-line defense against viral infections. ISGs can regulate the host immune response, which in turn may inhibit viral replication [8,9,26,27]. The role of ISG15 in host

defense against invading viral pathogens has previously been documented [9,19,26,27]. ISG15 and its conjugates exhibit both antitumor and oncogenic properties [8,28]. We have investigated the role of *ISG15* variants and ISG15 expression in the progression of HBV-related liver diseases and could show that the non-synonymous *ISG15* rs1921 variant, ISG15 levels and *ISG15* expression are associated with HBV-related liver diseases.

Host genetic factors contribute to the progression of HBV-related liver diseases [29]. To the best of our knowledge, this is the first study showing an association of the *ISG15* rs1921 variant with the clinical outcome of HBV-related liver diseases. The frequency of the *rs1921G* allele in different Vietnamese HBV patient subgroups and controls fits in the range estimated for East Asian of the 1000 Genomes project. The *rs1921GG* genotype and *rs1921G* allele are associated with progression of liver disease and contributes to poor laboratory parameters. The *ISG15* rs1921 exon 2 variant harbors a missense mutation (S355N) and, thus, may influence gene expression and protein modification. ISG15 targets many cellular proteins, including Janus kinase 1 (JAK1), STAT1 and ISGs via ISGlation [30], a process that can regulate HBV pathogenesis. However, our study failed to detect a significant association of *ISG15* rs1921 with ISG15 expression. Our result is similar to a recent observation showing that rs1921 variant had no influence on ISG15 expression in European human immunodeficiency virus (HIV)-1 patients and healthy individuals [31]. It may be more likely that this missense variant may alter the binding affinity to its conjugate. Polyphen and Sift algorithms used for predicting functional consequences of nucleotide

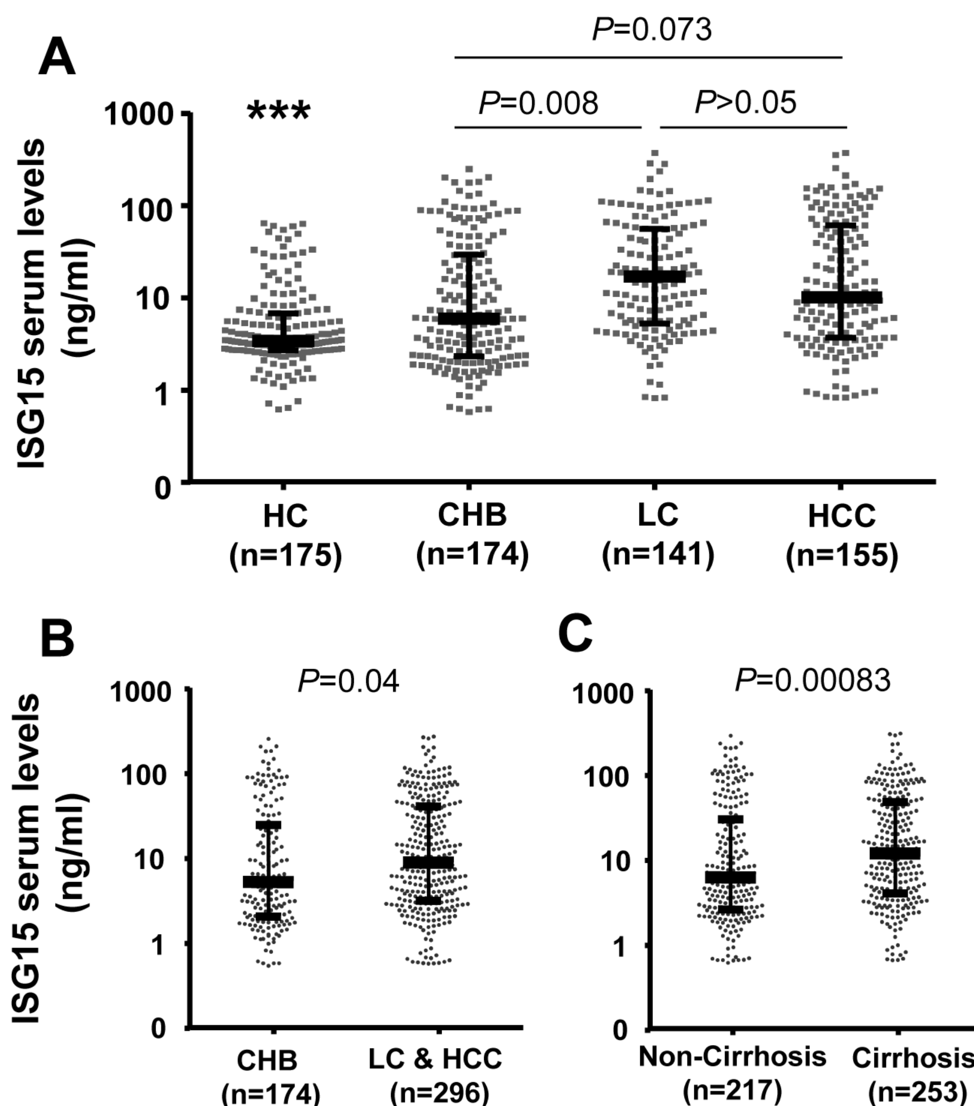


Figure 2: ISG15 serum levels in healthy individuals and in HBV patient sub-groups. HC, healthy controls; CHB, chronic hepatitis B; LC, liver cirrhosis; HCC, hepatocellular carcinoma; Cirrhosis: combination of patients with LC and patients with both HCC and LC; non-cirrhosis: combination of CHB patients and HCC patients without liver cirrhosis. (***): $P < 0.0001$ for comparison with other groups. Dot plots illustrate medians with inter-quartile range. *P* values were calculated by Mann-Whitney-Wilcoxon test.

Table 3: Factors associated with ISG15 serum levels in HBV patients

Variables	Univariate analysis	Multivariate analysis	
	***P* value**	***P* value**	**Coefficient β**
Age	0.31	0.57	-0.0096
Gender (male vs. female)	0.4	0.3	0.13
HBV-DNA (log10 copies/mL)	**<0.0001**	**<0.0001**	0.14
ALT (IU/L)	0.42	0.57	-0.00013
AST (IU/L)	0.46	0.88	-0.00003
Albumin (g/L)	0.30	0.12	0.02
Total Bilirubin (µmol/L)	0.13	0.8	-0.00017
Prothrombin (% of standard)	**0.0015**	0.16	-0.0004
Platelets (x10^3/mL)	0.47	0.11	-0.0011
HCC vs. non-HCC	0.9	0.12	0.2
Cirrhosis vs. non-Cirrhosis	**0.00032**	**0.009**	0.3
ISG15 genotype (GA vs. AA)	0.55	0.77	-0.66
ISG15 genotype (GG vs. AA)	0.51	0.62	-1.08

Univariate analysis and multivariate linear regression model for independent factors to correlate ISG15 serum levels with clinical parameters.

substitutions classify this variant as "benign" and "tolerated", respectively. The association may be due to linkage disequilibrium with another variant (a hitch-hiking effect). However, the effect of rs1921 on ISG15 function and the ISGlation process requires further clarification.

ISG15 and its ISGlation form mediate innate immune responses through IFNs, lipopolysaccharide and double-stranded RNA (dsRNA) stimulation [30]. Higher ISG15 serum levels in HBV patients than in healthy individuals and an increased *ISG15* mRNA expression

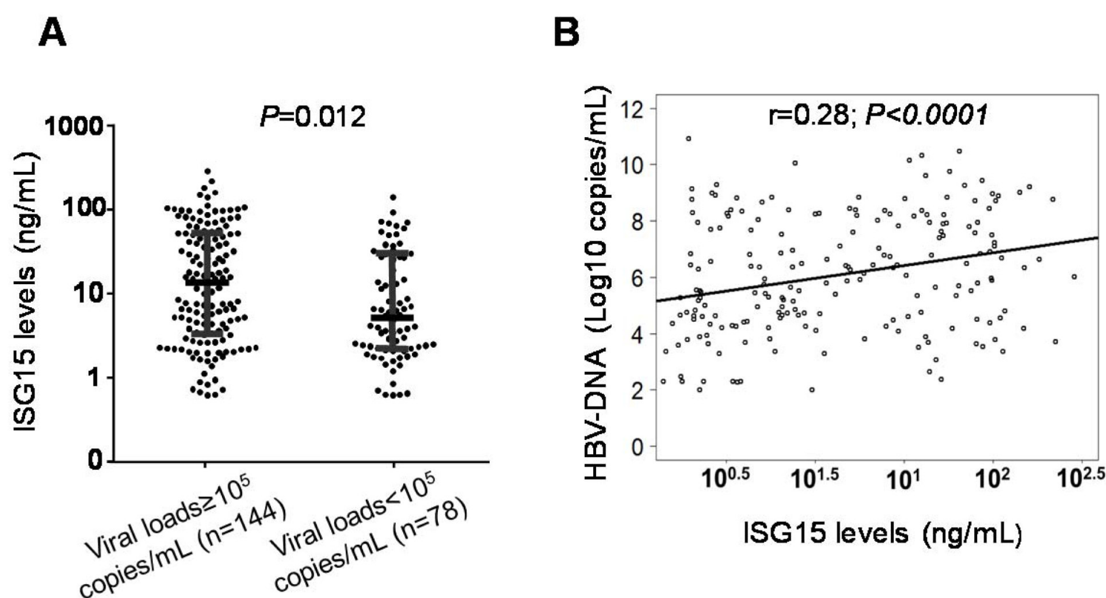


Figure 3: Association of ISG15 serum levels with viral loads in HBV patients. A. Dot plots illustrate median values with 25 and 75 percentiles with whiskers to 10 and 90 percentiles. *P* values were calculated by Mann-Whitney-Wilcoxon test. **B.** Linear regression analysis indicates a positive correlation between ISG15 serum levels and HBV-DNA in CHB patients. Non parametric Spearman's rank correlation coefficient (r) and *P* values are presented.

observed in HBV-related liver tissues compared to non-HBV/HCV-related liver tissues demonstrate that HBV infection induces *ISG15* expression. This is in agreement with the fact that the major host response to viral infection is the production of IFNs, which in turn stimulate *ISG15* expression. The notion of antiviral activities of both ISG15 and ISGlation came from studies using an *ISG15* knockout mice model [15,32]. Mice lacking ISG15 expression were more susceptible to influenza, sindbis and herpes simplex viruses [15]. Recent studies have explored the biological functions of ISG15 and related conjugates that can impair viral replication *in vivo* [18,32-34]. In contrast, our study showed that ISG15 levels were positively correlated with viral loads, implying a contradictory effect of ISG15 on antiviral activities. This is consistent with studies showing that ISG15 can promote HCV replication [19,35,36].

Host immune factors are essential in the immune-pathogenesis of HBV infection through genetic and epigenetic modifications [37,38] and via the effects of cytokines [39]. An ineffective immune response against HBV may result in persistent virus replication and liver inflammation, leading to CHB, LC and HCC [39]. ISG15 appears to act as an immune-modulator regulating the expression of cytokines, in particular of the IFN signalling pathway. Previous studies have indicated that *ISG15* upregulation leads to a blunted immune response to IFN signalling and contributes to a poor outcome of IFN-based therapy in HCV patients [19,40]. In addition, higher levels of ISG15 were observed in treatment failure compared to responders to IFN-based treatment [36,40]. Therefore, high levels of ISG15, combined with clinical, biochemical and histological analyses may be useful to predict the outcome of HBV-related liver disease and may help to identify HBV-infected individuals positively responding to IFN treatment.

The biological function of ISG15 promoting or suppressing tumor growth remains controversial [28] and antagonistic roles of ISG15 in tumorigenesis have been

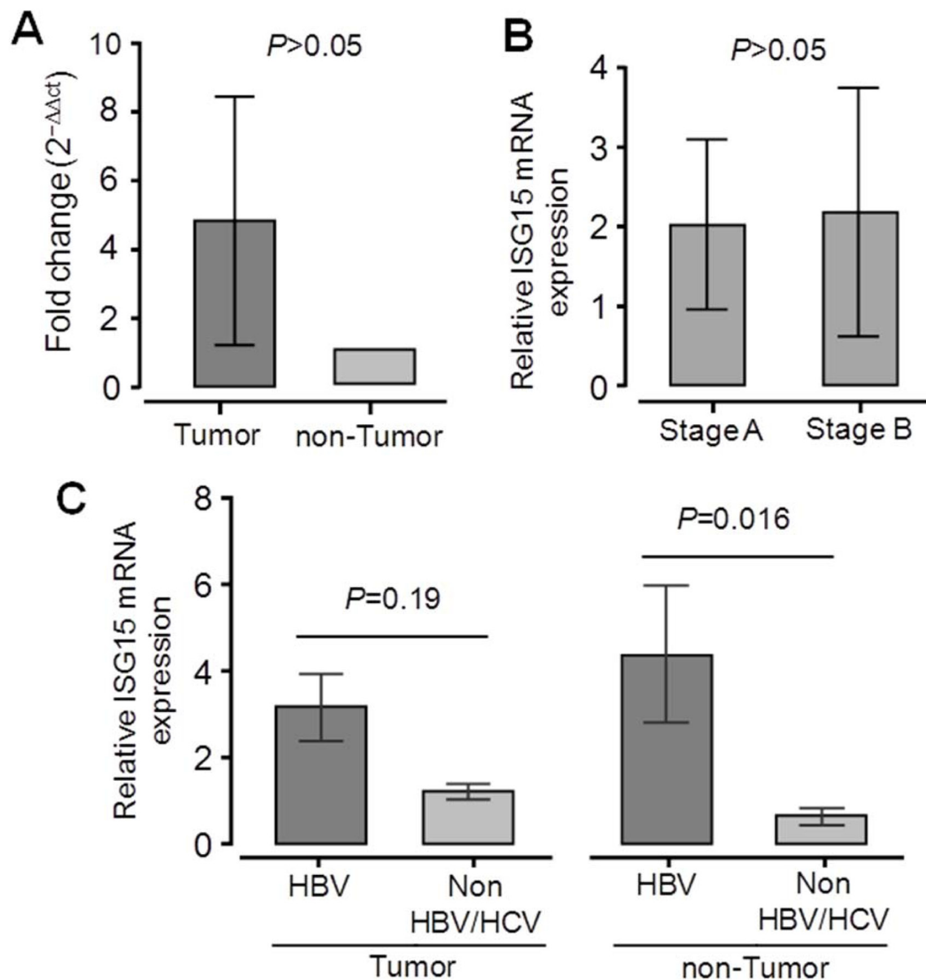


Figure 4: Expression of *ISG15* mRNA in liver specimens from 36 primary HCC patients. Relative quantitative real-time PCR (qRT-PCR) analysis of *ISG15* mRNA levels **A.** Relative *ISG15* mRNA expression in tumor and adjacent non-tumor tissues. **B.** Relative *ISG15* mRNA expression in tumor tissues from HCC stage A patients and HCC stage B patients according to the BCLC staging classification. **C.** Relative *ISG15* mRNA expression in liver tissues from patients positive for HBV and in patients negative for both HBV and HCV. Data are shown as mean values with 95% confident intervals. *P* values are calculated by Mann-Whitney-Wilcoxon tests.

reported [28,41,42]. Several studies have demonstrated that ISG15 is an oncoprotein, as *ISG15* gene expression and its protein conjugates were found elevated in tumor cell lines and in various human malignancies [10,12-14,41,43-46]. High levels of ISG15 serum protein in HCC patients and mRNA expression in liver tumor tissues also suggest that ISG15 may serve as a protumor factor. In contrast, other studies reported that free ISG15 has the potential to induce antitumor responses [47,48]. These discrepancies could be due to the functional differences of free ISG15 and ISG15 conjugates [28,42]. So far it is clear that ISG15 overexpression is crucial in modulating cell growth and in the progression of breast cancer [10,41]. The functional role of ISG15 in HCC, however, is still unclear. ISG15 overexpression is associated with poor clinical outcomes [12,13]. Moreover, knocking down ISG15 by shRNA ISG15 can lead to a remarkable reduction of HCC cell proliferation and migration [13].

Although our data indicate that *ISG15* over-expression is regulated by HBV infection and may trigger liver disease progression, the study has limitations. Due to the study design as a case-control study, ISG15 levels over the course of HBV infection were not assessed longitudinally and the causative effect of ISG15 levels on progression of HBV-related liver diseases could not conclusively be determined. HCC patients who donated liver tissues were in early and intermediate stages of liver cancer, which also might influence our results. Therefore, further studies in HCC patients with advanced stages of liver cancer are required to correlate *ISG15* mRNA expression with cancer progression.

In conclusion, our study shows that both the *ISG15* rs1921 variant and ISG15 overexpression are associated with HBV-related liver diseases and indicate that ISG15 may be a proviral factor and trigger progression of HBV-related liver diseases.

MATERIALS AND METHODS

Study design and sample collection

We randomly recruited 766 unrelated Vietnamese HBV-infected patients in a case-control design at 108 Military Central Hospital and 103 Military Hospital of the Vietnam Military Medical University, Hanoi, Vietnam, between 2012 and 2015. Patients were assigned to subgroups of disease based on clinical manifestations and liver function tests. Subgroups included chronic hepatitis B (CHB, n = 262), HBV-related liver cirrhosis (LC, n = 241) and HBV-related hepatocellular carcinoma (HCC, n = 263). Criteria for the patient classification have been described previously [49]. HBV load is quantified by qPCR and the presence of HBsAg by ELISA. Among the 263 HCC patients, 180 (68.4%) had concomitant LC and the remaining 83 patients (21.6%) did not have LC. As the healthy control (HC) group, we collected 223 blood samples from blood donors from blood bank and these healthy individuals were devoid of HBsAg, HCV and HIV infections. Neither the HCs nor HBV patients had a history of alcohol or drug use. Five ml of venous blood were collected from all participants. Serum/plasma was separated and stored at -80°C until further use. In addition, 36 dyads of liver tissue specimens (tumor and adjacent non-tumor specimens) from HCC patients undergoing surgery were collected between 2013 and 2014. HCC was confirmed histologically and classified based on the Barcelona Clinic Liver Cancer (BCLC) classification [25]. The clinical profiles of the HCC patients and data of the liver tissue specimens were shown in the Suppl. Table 1. All specimens were frozen at -80^{0}C until use.

Ethics statement

Informed written consent was obtained after explanation of the study at the time of sampling from all participants or from their parents if subjects were less than 18 years. The study was approved by the institutional review board of the 108 Military Central hospital and the 103 Military Hospital of the Vietnam Military Medical University, Hanoi, Vietnam.

ISG15 genotyping

Genomic DNA was isolated from whole blood using a DNA purification kit (Qiagen, Hilden, Germany). The *ISG15* promoter and the two exons of the gene were PCR amplified using specific sets of primers (Suppl. Table 2). PCR amplification was carried out in a 25 µl volume containing 1X PCR buffer, 0.2 mM dNTPs, 1 mM $MgCl_2$, 0.15 mM of each primer, 1 unit of Taq polymerase and 50 ng of genomic DNA. Cycling conditions were denaturation at 95°C for 5 min, followed by 40 cycles of three-step cycling with denaturation (94°C, 30 sec), annealing (63°C, 35 sec), and extension (72°C, 45 sec) and final extension (72°C, 7 min). PCR products were purified by Exo-SAP-IT (USB, Affymetrix, USA) and 5 µl of products were used as sequencing templates (BigDye terminator v.1.1 cycle sequencing kit, ABI 3130XL DNA sequencer; Applied Biosystems, Foster City, USA).

Quantification of ISG15 levels by ELISA

Soluble ISG15 serum levels were assessed in 174, 141 and 155 samples from CHB, LC and HCC patients, respectively and in 175 samples from HCs by the sandwich ISG15 ELISA kit (LifeSpan BioSciences, Eching, Germany, catalog number: MBS9302876-96). The lower detection limit was 0.5 ng/ml.

ISG15 mRNA expression

Total RNA was extracted from 36 dyads of liver biopsy tissues with Trizol reagent (Life Technologies, California, USA). RNA was transcribed into cDNA (QuantiTect Reverse Transcription Kit; Qiagen, Hilden, Germany). Quantification of cDNA was performed by qRT-PCR using SYBR Green PCR mix (Bioline, Luckenwalde, Germany). All reactions were performed in duplicate (LightCycler®480 real-time PCR system; Roche, Basel, Switzerland). The glyceraldehyde 3-phosphate dehydrogenase (*GAPDH)* gene was used as the reference gene. The specific primers used for evaluating the relative expression of *ISG15* mRNA were presented in Suppl. Table 2. Thermal cycling conditions were 2 min (95°C) followed by 45 cycles of (95°C, 5 sec), annealing (58°C, 10 sec) and extension (72°C, 20 sec). Reaction specificity was confirmed by melting curve and electrophoresis analyses. Calculation of normalized gene expression was based on the ΔΔCT method. The fold change in *ISG15* mRNA was normalized against the expressed *GAPDH* reference gene and adjusted to the calibration sample [50].

Statistical and genetic analyses

Statistical analyses were performed using R version 3.1.2 (http://www.r-project.org) and GraphPad Prism 6 (http://www.graphpad.com). Hardy-Weinberg equilibrium deviations were calculated according to the Guo & Thompson approach by using R software. We applied binary logistic regression models adjusted for age and gender to determine *ISG15* associations with HBV-related liver diseases in co-dominant, dominant and recessive models. Univariate analysis and a multivariate linear regression model for independent factors were used to correlate ISG15 serum levels with clinical parameters. Adjusted odds ratios (OR) with 95% confidence intervals (CI) were calculated. Chi-square tests were used to test for differences of categorical variables and Mann-Whitney-Wilcoxon and Kruskal-Wallis tests were applied to compare quantitative variables. Significance was set at a value of $P < 0.05$.

ACKNOWLEDGMENTS

We thank all study subjects and blood donors for their participation. We appreciate experimental support of Maximilian Schulz and Amanda Link. We also extend our deep thanks to Le Duc Anh, Nguyen Viet Ha for sample collection.

CONFLICTS OF INTEREST

All authors have no conflicts of interest to declare.

FINANCIAL SUPPORT

This study acknowledges DAAD-PAGEL (57140033) for the support towards student fellowships. The funder has no role in the study design, data collection and analysis, decision to publish or preparation of the manuscript. The authors wish to acknowledge the financial publication support for this study from NAFOSTED-Vietnam.

Author's contributions

NXH, HVT, CTB and TPV designed the study. NXH, HVT and DPG performed the experiments. NXH, HVT, CGM and TPV performed the statistical analyses and interpreted the data. NXH, DPG, LHS, and NLT are involved in patient recruitment. CTB, PGK and TPV contributed to study materials and consumables. NXH, HVT, CGM and TPV wrote the manuscript. NXH, HVT and DPG contributed equally to this work. All authors agreed with the results and conclusions.

REFERENCES

1. WHO. Hepatitis B. 2016. Report No.: Fact sheet N°204. http://www.who.int/mediacentre/factsheets/fs204/en/.
2. Nguyen VT, Law MG, Dore GJ. An enormous hepatitis B virus-related liver disease burden projected in Vietnam by 2025. Liver Int. 2008; 28:525-31.
3. Rehermann B, Bertoletti A. Immunological aspects of antiviral therapy of chronic hepatitis B virus and hepatitis C virus infections. Hepatology. 2015; 61:712-21.
4. Baltayiannis G, Karayiannis P. Treatment options beyond IFNalpha and NUCs for chronic HBV infection: expectations for tomorrow. J Viral Hepat. 2014; 21:753-61.
5. Durantel D, Zoulim F. New antiviral targets for innovative treatment concepts for hepatitis B virus and hepatitis delta virus. J Hepatol. 2016; 64:S117-S131.
6. Platanias LC. Mechanisms of type-I- and type-II-interferon-mediated signalling. Nat Rev Immunol. 2005; 5:375-86.
7. Blomstrom DC, Fahey D, Kutny R, Korant BD, Knight E Jr. Molecular characterization of the interferon-induced 15-kDa protein. Molecular cloning and nucleotide and amino acid sequence. J Biol Chem. 1986; 261:8811-6.
8. Jeon YJ, Yoo HM, Chung CH. ISG15 and immune diseases. Biochim Biophys Acta. 2010; 1802:485-96.
9. Campbell JA, Lenschow DJ. Emerging roles for immunomodulatory functions of free ISG15. J Interferon Cytokine Res. 2013; 33:728-38.
10. Bektas N, Noetzel E, Veeck J, Press MF, Kristiansen G, Naami A, Hartmann A, Dimmler A, Beckmann MW, Knuchel R, Fasching PA, Dahl E. The ubiquitin-like

molecule interferon-stimulated gene 15 (ISG15) is a potential prognostic marker in human breast cancer. Breast Cancer Res. 2008; 10:R58.

11. Wan XX, Chen HC, Khan MA, Xu AH, Yang FL, Zhang YY, Zhang DZ. ISG15 inhibits IFN-alpha-resistant liver cancer cell growth. Biomed Res Int. 2013; 2013:570909.
12. Qiu X, Hong Y, Yang D, Xia M, Zhu H, Li Q, Xie H, Wu Q, Liu C, Zuo C. ISG15 as a novel prognostic biomarker for hepatitis B virus-related hepatocellular carcinoma. Int J Clin Exp Med. 2015; 8:17140-50.
13. Li C, Wang J, Zhang H, Zhu M, Chen F, Hu Y, Liu H, Zhu H. Interferon-stimulated gene 15 (ISG15) is a trigger for tumorigenesis and metastasis of hepatocellular carcinoma. Oncotarget. 2014; 5:8429-41. doi: 10.18632/oncotarget.2316.
14. Satake H, Tamura K, Furihata M, Anchi T, Sakoda H, Kawada C, Iiyama T, Ashida S, Shuin T. The ubiquitin-like molecule interferon-stimulated gene 15 is overexpressed in human prostate cancer. Oncol Rep. 2010; 23:11-6.
15. Lenschow DJ, Lai C, Frias-Staheli N, Giannakopoulos NV, Lutz A, Wolff T, Osiak A, Levine B, Schmidt RE, Garcia-Sastre A, Leib DA, Pekosz A, Knobeloch KP, et al. IFN-stimulated gene 15 functions as a critical antiviral molecule against influenza, herpes, and Sindbis viruses. Proc Natl Acad Sci U S A. 2007; 104:1371-6.
16. Hsiang TY, Zhao C, Krug RM. Interferon-induced ISG15 conjugation inhibits influenza A virus gene expression and replication in human cells. J Virol. 2009; 83:5971-7.
17. Zhao C, Hsiang TY, Kuo RL, Krug RM. ISG15 conjugation system targets the viral NS1 protein in influenza A virus-infected cells. Proc Natl Acad Sci U S A. 2010; 107:2253-8.
18. Okumura A, Lu G, Pitha-Rowe I, Pitha PM. Innate antiviral response targets HIV-1 release by the induction of ubiquitin-like protein ISG15. Proc Natl Acad Sci U S A. 2006; 103:1440-5.
19. Broering R, Zhang X, Kottilil S, Trippler M, Jiang M, Lu M, Gerken G, Schlaak JF. The interferon stimulated gene 15 functions as a proviral factor for the hepatitis C virus and as a regulator of the IFN response. Gut. 2010; 59:1111-9.
20. Broering R, Trippler M, Werner M, Real CI, Megger DA, Bracht T, Schweinsberg V, Sitek B, Eisenacher M, Meyer HE, Baba HA, Weber F, Hoffmann AC, et al. Hepatic expression of proteasome subunit alpha type-6 is upregulated during viral hepatitis and putatively regulates the expression of ISG15 ubiquitin-like modifier, a proviral host gene in hepatitis C virus infection. J Viral Hepat. 2016; 23:375-86.
21. Chen L, Sun J, Meng L, Heathcote J, Edwards AM, McGilvray ID. ISG15, a ubiquitin-like interferon-stimulated gene, promotes hepatitis C virus production in vitro: implications for chronic infection and response to treatment. J Gen Virol. 2010; 91:382-8.
22. Chen L, Li S, McGilvray I. The ISG15/USP18 ubiquitin-like pathway (ISGylation system) in hepatitis C virus infection and resistance to interferon therapy. Int J Biochem Cell Biol. 2011; 43:1427-31.
23. Song J, Zhou Y, Li S, Wang B, Zheng X, Wu J, Gibbert K, Dittmer U, Lu M, Yang D. Susceptibility of different hepatitis B virus isolates to interferon-alpha in a mouse model based on hydrodynamic injection. PLoS One. 2014; 9:e90977.
24. Kim JH, Luo JK, Zhang DE. The level of hepatitis B virus replication is not affected by protein ISG15 modification but is reduced by inhibition of UBP43 (USP18) expression. J Immunol. 2008; 181:6467-72.
25. Llovet JM, Bru C, Bruix J. Prognosis of hepatocellular carcinoma: the BCLC staging classification. Semin Liver Dis. 1999; 19:329-38.
26. Skaug B, Chen ZJ. Emerging role of ISG15 in antiviral immunity. Cell. 2010; 143:187-90.
27. Harty RN, Pitha PM, Okumura A. Antiviral activity of innate immune protein ISG15. J Innate Immun. 2009; 1:397-404.
28. Andersen JB, Hassel BA. The interferon regulated ubiquitin-like protein, ISG15, in tumorigenesis: friend or foe? Cytokine Growth Factor Rev. 2006; 17:411-21.
29. Tong S, Revill P. Overview of hepatitis B viral replication and genetic variability. J Hepatol. 2016; 64:S4-S16.
30. Sadler AJ, Williams BR. Interferon-inducible antiviral effectors. Nat Rev Immunol. 2008; 8:559-68.
31. Scagnolari C, Monteleone K, Selvaggi C, Pierangeli A, D‘Ettorre G, Mezzaroma I, Turriziani O, Gentile M, Vullo V, Antonelli G. ISG15 expression correlates with HIV-1 viral load and with factors regulating T cell response. Immunobiology. 2016; 221:282-90.
32. Werneke SW, Schilte C, Rohatgi A, Monte KJ, Michault A, Arenzana-Seisdedos F, Vanlandingham DL, Higgs S, Fontanet A, Albert ML, Lenschow DJ. ISG15 is critical in the control of Chikungunya virus infection independent of UbE1L mediated conjugation. PLoS Pathog. 2011; 7:e1002322.
33. Okumura A, Pitha PM, Harty RN. ISG15 inhibits Ebola VP40 VLP budding in an L-domain-dependent manner by blocking Nedd4 ligase activity. Proc Natl Acad Sci U S A. 2008; 105:3974-9.
34. Malakhova OA, Zhang DE. ISG15 inhibits Nedd4 ubiquitin E3 activity and enhances the innate antiviral response. J Biol Chem. 2008; 283:8783-7.
35. Broering R, Trippler M, Werner M, Real CI, Megger DA, Bracht T, Schweinsberg V, Sitek B, Eisenacher M, Meyer HE, Baba HA, Weber F, Hoffmann AC, et al. Hepatic expression of proteasome subunit alpha type-6 is upregulated during viral hepatitis and putatively regulates the expression of ISG15 ubiquitin-like modifier, a proviral host gene in hepatitis C virus infection. J Viral Hepat. 2016.
36. Chen L, Sun J, Meng L, Heathcote J, Edwards AM, McGilvray ID. ISG15, a ubiquitin-like interferon-stimulated gene, promotes hepatitis C virus production in vitro:

implications for chronic infection and response to treatment. J Gen Virol. 2010; 91:382-8.

37. Tong H, Bock CT, Velavan TP. Genetic insights on host and hepatitis B virus in liver diseases. Mutat Res Rev Mutat Res. 2014; 762:65-75.

38. Koumbi L, Karayiannis P. The Epigenetic Control of Hepatitis B Virus Modulates the Outcome of Infection. Front Microbiol. 2015; 6:1491.

39. Li X, Liu X, Tian L, Chen Y. Cytokine-Mediated Immunopathogenesis of Hepatitis B Virus Infections. Clin Rev Allergy Immunol. 2016; 50:41-54.

40. Chen L, Borozan I, Feld J, Sun J, Tannis LL, Coltescu C, Heathcote J, Edwards AM, McGilvray ID. Hepatic gene expression discriminates responders and nonresponders in treatment of chronic hepatitis C viral infection. Gastroenterology. 2005; 128:1437-44.

41. Burks J, Reed RE, Desai SD. ISGylation governs the oncogenic function of Ki-Ras in breast cancer. Oncogene. 2014; 33:794-803.

42. Desai SD. ISG15: A double edged sword in cancer. Oncoimmunology. 2015; 4:e1052935.

43. Wood LM, Pan ZK, Seavey MM, Muthukumaran G, Paterson Y. The ubiquitin-like protein, ISG15, is a novel tumor-associated antigen for cancer immunotherapy. Cancer Immunol Immunother. 2012; 61:689-700.

44. Andersen JB, Aaboe M, Borden EC, Goloubeva OG, Hassel BA, Orntoft TF. Stage-associated overexpression of the ubiquitin-like protein, ISG15, in bladder cancer. Br J Cancer. 2006; 94:1465-71.

45. Desai SD, Haas AL, Wood LM, Tsai YC, Pestka S, Rubin EH, Saleem A, Nur-E-Kamal, Liu LF. Elevated expression of ISG15 in tumor cells interferes with the ubiquitin/26S proteasome pathway. Cancer Res. 2006; 66:921-8.

46. Kiessling A, Hogrefe C, Erb S, Bobach C, Fuessel S, Wessjohann L, Seliger B. Expression, regulation and function of the ISGylation system in prostate cancer. Oncogene. 2009; 28:2606-20.

47. Burks J, Reed RE, Desai SD. Free ISG15 triggers an antitumor immune response against breast cancer: a new perspective. Oncotarget. 2015; 6:7221-31. doi: 10.18632/oncotarget.3372.

48. D‘Cunha J, Knight E Jr, Haas AL, Truitt RL, Borden EC. Immunoregulatory properties of ISG15, an interferon-induced cytokine. Proc Natl Acad Sci U S A. 1996; 93:211-5.

49. Hoan NX, Tong HV, Hecht N, Sy BT, Marcinek P, Meyer CG, Song lH, Toan NL, Kurreck J, Kremsner PG, Bock CT, Velavan TP. Hepatitis E Virus Superinfection and Clinical Progression in Hepatitis B Patients. EBioMedicine. 2015; 2:2080-6.

50. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001; 25:402-8.

Interferon-stimulated gene 15 in hepatitis B-related liver diseases

Supplementary Materials

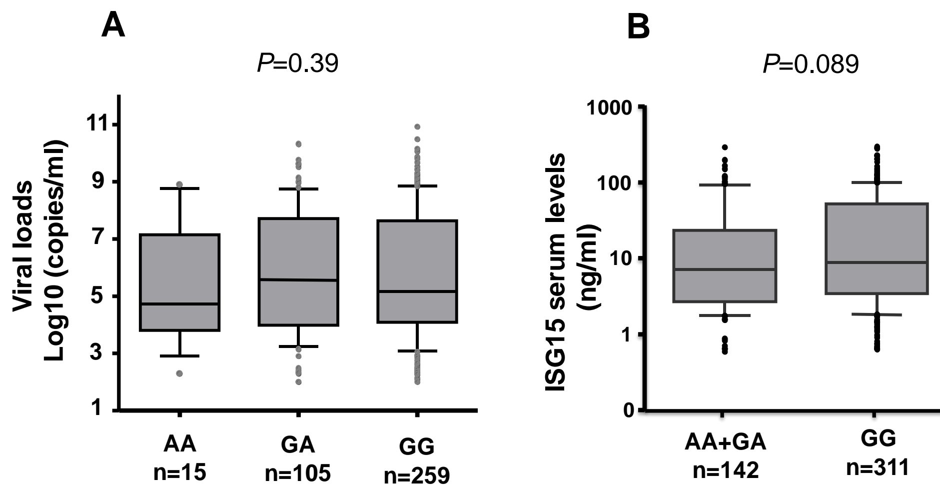
Suppl. Table 1: Characteristics of 36 HCC patients

Characteristics	n (%)
Age (years)	
< 40	3/36 (8.3)
40 - 60	27/36 (75)
> 60	6/36 (16.7)
Gender	
Male	32/36 (89)
Female	4/36 (11)
Etiology	
HBV	17/36 (47)
HCV	2/36 (6)
Non-HBV/HCV	17/36 (47)
BCLC staging Classification	
Stage A	25/36 (69)
Stage B	11/36 (31)
Stage C and D	0/ 36 (0)
Clinical parameters	**Median (Range)**
AFP (IU/ml)	240 [4.6 - 300]
HBV-DNA	NA
PLT (10^3/ml)	211 [153 - 461]
AST (IU/ml)	52 [21 - 415]
ALT (IU/ml)	66.5 [17 - 242]
Total Bilirubin (µmol/l)	27.8 [8.9 - 315]
Direct Bilirubin (µmol/l)	6.7 [1 - 178]
Prothrombin (% of standard)	93 [75 - 125]
Protein (g/l)	73 [62 - 78]
Allbumin (g/l)	40 [32 - 48]

Abbreviation: BCLC: Barcelona Clinic Liver Cancer; HCC: hepatocellular carcinoma; AFP: Alpha feto protein; PLT: platelets; AST and ALT: aspartate and alanine amino transferase; IU: international unit; NA: not applicable.

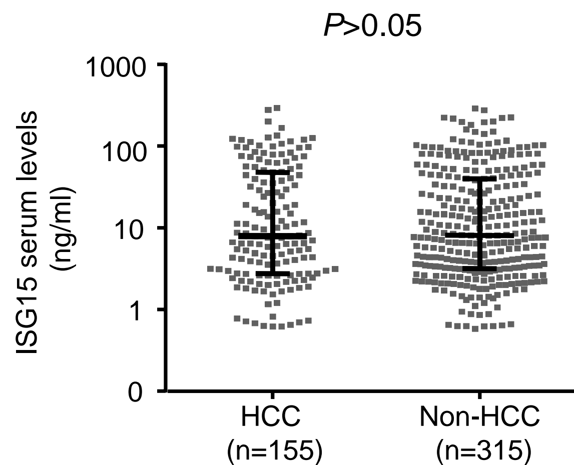
Suppl. Table 2: Primers used for this study

Primer	Sequence	Application	Fragment length
ISG15_Pr_F	5'- GAG GCT GAG GTG AGA GGA TC -3'	ISG15 promoter genotyping	715 bp
ISG15_Pr_R	5'- GAG GGA GAC GAA AAT TGG CTG -3'		
ISG15_E1_F	5'- CAG TGC CTT GTG TGT GGT GG -3'	ISG15 exon1 genotyping	578 bp
ISG15_E1_R	5'- GAT GCT GGT GGA GGC CCT TAG -3'		
ISG15_Exp_F	5'- GAG AGG CAG CGA ACT CAT CT -3'	ISG15 mRNA expression	157 bp
ISG15_Exp_R	5'- CTT CAG CTC TGA CAC CGA CA -3'		
GADPH_F	5'-TGC ACC ACC AAC TGC TTA GC-3'	ISG15 mRNA expression	87 bp
GADPH_R	5'-GGC ATG GAC TGT GGT CAT GAG-3'		



Suppl. Figure 1: Association of viral loads and ISG15 serum levels with *ISG15* rs1921G/A variant.

Box-plots illustrate medians with 25 and 75 percentiles with whiskers to 10 and 90 percentiles; *P* values were calculated by Krusskal-Wallis-Wilcoxon test.



Suppl. Figure 2: Distribution of ISG15 serum levels in HCC and non-HCC patients.

Scatter dot plots illustrate medians with 25 and 75 percentiles with whiskers to 10 and 90 percentiles; *P* values were calculated by Krusskal-Wallis-Wilcoxon test.

3. DISCUSSION

Hepatitis B can lead to several liver disease phenotypes, ranging from asymptomatic carrier status and chronic infections to life-threatening hepatocellular carcinoma. The factors influencing such liver disease progression are modulated by various host and viral factors. This thesis describes how HEV superinfection in HBV patients may influence HBV disease and its course in liver disease progression. Also, investigations were carried out to determine the contribution of host candidate genes in the JAK/STAT signaling pathway to HBV susceptibility and its clinical course.

3.1. Hepatitis E virus superinfection in HBV patients

It is well known that HBV co-infection with HIV, HDV, and HCV contribute to a poor clinical outcome (Kruse et al. 2014; Kumar et al. 2008c; Rajbhandari et al. 2016; Wedemeyer et al. 2010). In Vietnam, HBV prevalence is documented to be greater than 10% in the population (Nguyen. 2012), and thus poses a significant health burden. Similar to other comorbidities, HEV superinfections also contribute to the HBV clinical outcome. However, only limited knowledge exists on the prevalence of HEV infection and its consequence in HBV related liver diseases in Vietnam. My current thesis aims to bridge this gap, thereby providing actual data on the burden of HEV infection.

3.1.1 HEV infection in Vietnamese population

The seroprevalence of HEV in general populations and blood donors vary from one geographical setting to another (Boutrouille et al. 2007; Cleland et al. 2013; Guo et al. 2010; Petrovic et al. 2014; Pittaras et al. 2014; Ren et al. 2013) and this also depends on the performance of the immunoassay used (Abravanel et al. 2014; Mansuy et al. 2011). Several reports have raised concerns about the importance of HEV infection. The results from our study show a prevalence of 31% and 5% for anti-HEV IgG and IgM respectively, in healthy Vietnamese individuals. Anti-HEV IgG positivity was considerably higher than previously reported in rural areas of Vietnam

(Hau et al. 1999). This difference might be due to the applied ELISA assays. Differences in social and demographic features may also affect seroprevalences (Hau et al. 1999; Mansuy et al. 2011). However, our results are in agreement with studies in Chinese blood donors, where anti-HEV IgG and IgM prevalences were about 30% and 1%, respectively (Guo et al. 2010; Ren et al. 2014). Until a decade ago, hepatitis E infection was thought to be restricted to Asian countries and European travelers returning from endemic areas. However, it is now well established that HEV is also endemic in Europe. Indeed, several studies have shown lower anti-HEV IgG prevalences of 3% to 17% occur among most European populations (Adlhoch et al. 2016; Beale et al. 2011; Boutrouille et al. 2007; Gallian et al. 2014; Juhl et al. 2014; Pittaras et al. 2014; Ren et al. 2014). HEV seroprevalences were, however, exceptionally high in southern France and Denmark (Christensen et al. 2008; Mansuy et al. 2011; Mansuy et al. 2016).

Prevalences of anti-HEV IgG and IgM in hepatitis B patients were 45% and 12%, respectively. The HEV seroprevalence in our patient group was different from other reports on HBV, HCV, and HIV infections (Atiq et al. 2009; Bayram et al. 2007; Feldt et al. 2013; Hamid et al. 2002). Anti-HEV IgG prevalences in American and Turkish chronic HBV patients were 8% and 14% respectively (Atiq et al. 2009; Bayram et al. 2007). Our results show that HBV patients might be at risk for an HEV infection (Hamid et al. 2002). HEV superinfection in HIV patients and the development of chronic hepatitis E has also been described (Dalton et al. 2009). A recent study reported a seroprevalence of anti-HEV IgG in Cameroon (14%) and Ghana (45%) (Feldt et al. 2013). However, no contribution of HEV infection to liver pathology was detected. The HEV seroprevalence in our study group was significantly associated with age, both in HBV patients and controls, indicating cumulative exposure to HEV. Ageing of the immune system might also favor acquisition of an HEV infection.

3.1.2 HEV superinfection and HBV clinical outcome.

Although most people infected with HEV are asymptomatic, the outcome of HEV superinfection in HBV patients generally appears more severe (Cheng et al. 2013; Marion-Audibert et al. 2010; Monga et al. 2004). Our results show high levels of liver enzymes and bilirubin and low levels of albumin, prothrombin, and platelet counts in the serum of HBV patients coinfected with HEV. In contrast, an earlier report suggested that chronic HBV infections may remain inactive during HEV-HBV coinfections (Cheng et al. 2013), however, we found higher HBV-DNA loads in coinfected patients. Biochemical and serological tests indicate that HEV superinfection contribute to inflammation and liver failure. HBV-DNA loads were lower in HBV patients with previous HEV infection compared to those currently infected or with no infection, suggesting that host immune responses control HBV replication. HBsAg positive individuals had a poorer prognosis following HEV superinfection (Chow et al. 2014; Wu et al. 2013).

HEV infection had been reported to be associated with the development and progression of LC (Gerolami et al. 2008; Kumar et al. 2008b; Marion-Audibert et al. 2010), but the mechanisms of LC induction are not clear. Previous studies have demonstrated that HEV infections are correlated with underlying LC and progression of chronic hepatitis B. The association of an HEV superinfection with abnormal levels of bilirubin, albumin, and prothrombin supports the fact that HEV superinfections contribute to the severity of HBV infections. Although the difference was not significant, the prevalence of anti-HEV antibodies were higher among patients with HBV-related HCC. A HEV-induced tumorigenesis cannot be excluded.

3.2. *SOCS3* and *ISG15* in HBV-related liver diseases

3.2.1. SOCS3 promoter variants and methylation

The negative regulator SOCS3 (suppressor of cytokine signaling-3) is a key player in the modulation of the JAK/STAT signaling that regulate several inflammatory

cytokines such as IL6 and IL16 (Yoshimura et al. 2007b) and is involved in infectious diseases and cancers (Persico et al. 2008; Yoshimura et al. 2012). Additionally, gene silencing mediated by aberrant methylation of CpG islands in the *SOCS3* promoter frequently occurs in malignancies (He et al. 2003; Niwa et al. 2005). In this current thesis, I investigated the association of *SOCS3* promoter variants with the progression of HBV-related liver diseases and explored the association of *SOCS3* methylation with HBV-induced HCC**.** We demonstrated that the *SOCS3* promoter variants are associated with HBV infection and HBV-related liver diseases. *SOCS3* mRNA expression was higher in infected tumor tissues than non-tumor tissues. The aberrant methylation of the CpG islands in the *SOCS3* promoter is associated with relatively low mRNA expression in tumor tissues.

SOCS3 promoter variants and HBV-related liver diseases

This first study reports on the association of SOCS3 variants with HBV susceptibility and progression of HBV-related liver diseases. We have demonstrated that infection and the progression of HBV-related liver diseases are associated with rs111033850T/C and rs12953258C/A variants. In particular, the variant rs111033850T/C shows a heterozygous advantage in HBV susceptibility, but might be a risk factor for disease progression. The contribution of the derived allele *rs111033850C* to a higher risk of HCC in CHB patients is through the gene dose manner. In studies on hepatitis C, the *rs4969170AA* genotype was associated with antiviral IFN-α resistance with increased *SOCS3* expression in HCV patients (Persico et al. 2008; Zheng et al. 2013). The rs4969170A/G mutation was associated with HCV treatment-induced neutropenia and thrombocytopenia in antiviral therapy with pegylated interferon alpha (Vidal et al. 2012). The rs4969170A/G polymorphism was associated with clinical features and prognosis of HCC after surgical treatment (Jiang et al. 2015). Our results support earlier findings that *SOCS3* polymorphisms affect liver disease progression by regulating the SOCS3 protein expression, and thus down-regulate the JAK/STAT signaling.

In this study, we showed that HBV-DNA loads are associated with the *SOCS3* promoter polymorphisms rs111033850T/C and rs12953258C/A. The HBV-DNA load is an essential and independent risk factor for liver disease progression in CHB patients (Chen et al. 2009; Zacharakis et al. 2008). HBV replication is regulated by many host factors (Levrero et al. 2009b). Previous studies have shown that the control of HBV replication is regulated by JAK/STAT signaling induced by the Interferon responses (Du et al. 2014; Koeberlein et al. 2010; Robek et al. 2004). IFNs play a vital role in controlling HBV viral replication (Koziel. 1999b) and SOCS3 protein involved in the JAK-STAT pathway, is one such host factor that can suppress interferon responses (Kubo et al. 2003). Therefore, *SOCS3* promoter polymorphisms might contribute to HBV multiplication by modulation of IFN signaling.

Cytokines play a central role in cell to cell communication and are required for the defense against hepatitis viruses (Koziel. 1999b). A previous study has demonstrated that the HCV core protein impairs IFN-α-induced signal transduction via induction of *SOCS3* expression (Bode et al. 2003) and thereby influencing the outcome of antiviral therapy (Persico et al. 2007). An intense expression of SOCS3 in liver tissues was strongly associated with severity of hepatic inflammation in CHB patients (Du et al. 2014; Koeberlein et al. 2010). In accordance, a high SOCS3 expression in liver tissues was observed in HBV compared to non-HBV patients. These results indicate that HBV can induce *SOCS3* expression, which in turn inhibits IFN signaling transduction resulting in the progression of liver diseases and failure of IFN treatment of HBV infection (Du et al. 2014).

SOCS3 promoter hypermethylation and HCC development

HCC development commonly results from chronic liver injury, inflammation, and cirrhosis caused by HBV infection. However, the interaction between HBV and SOCS3 in infected hepatocytes is not well understood. DNA hypermethylation in the promoter region can lead to the silence of *SOCS3* in HCC (Niwa et al. 2005; Zhang et al. 2015b). In agreement with previous studies, our results indicated that aberrant

methylation in the *SOCS3* promoter region can be detected more frequently in tumor tissues compared to adjacent non-tumor tissues. Hypermethylation status in the *SOCS3* promoter region is an important factor for HCC development. However, SOCS3 mRNA expression in tumor and adjacent non-tumor tissues was not significantly different, indicating that other factors such as acetylation, phosphorylation, and microRNAs may also be involved in the regulation of SOCS3 expression during progression of liver diseases (Boosani et al. 2015). In addition, both SOCS3 methylation status and intensity were similar in tissue samples (tumor and non-tumor) from patients with and without HBV infection. This suggests that HBV may not enhance DNA methylation of host genes in infected hepatocytes. However, further studies are needed to verify this observation.

Although our data indicate that SOCS3 expression is associated with HBV infection and may be involved in the progression of HBV-related liver diseases, the study has a number of limitations. A limited number of HCC tumor and non-tumor tissues were utilized. Due to the study design as a case-control study, SOCS3 expression over the course of HBV infection were not assessed longitudinally and therefore the causative effect of SOCS3 expression on progression of HBV-related liver diseases could not be conclusively determined. The insufficiency of some clinical and laboratory parameters such as HBV serology tests and HBV genotypes may weaken the findings indicating the crucial role of SOCS3 in the immune response to HBV infection and the disease outcomes.

3.2.2. The role of *ISG15* in the progression of liver diseases.

The interferon signaling pathway forms the first-line defense against viral infections. ISGs can regulate the host immune response, which in turn may restrain viral replication (Campbell et al. 2013a; Harty et al. 2009; Jeon et al. 2010b; Skaug and Chen. 2010). The role of ISG15 in host defense against invading viral pathogens has formerly been documented (Broering et al. 2010; Campbell and Lenschow. 2013a; Harty et al. 2009; Skaug and Chen. 2010). ISG15 and its conjugates display both

antitumor and oncogenic features (Andersen et al. 2006b; Jeon et al. 2010b). We have investigated the role of *ISG15* variants and ISG15 expression in the progression of HBV-related liver diseases and showed that both the *ISG15* rs1921 variant polymorphisms and ISG15 overexpression are associated with HBV-related liver diseases and suggest that ISG15 may be a proviral factor and trigger progression of HBV-related liver diseases.

ISG15 rs1921 variant and HBV-related liver diseases

Host genetic factors influence the progression of HBV-related liver diseases (Tong and Revill. 2016). To the best of our knowledge, this is the first study showing an association of the *ISG15* rs1921 variant with the clinical outcome of HBV-related liver diseases. The *rs1921GG* genotype and *rs1921G* allele are associated with progression of liver disease. The *ISG15* rs1921 exon 2 variant harbors a missense mutation (S355N) and, thus, may affect gene expression and protein modification. ISG15 targets many cellular proteins, including JAK1, STAT1 and ISGs through ISGlation (Sadler et al. 2008), a process that can regulate HBV pathogenesis. However, our study could not detect any association of *ISG15* rs1921 with ISG15 expression. A recent study also drew the same conclusion that rs1921 had no influence on ISG15 expression in HIV-1 patients (Scagnolari et al. 2016). It is most likely that this missense variant alters the binding affinity to its conjugate. However, the effect of rs1921 on ISG15 function and the ISGlation process requires further clarification.

Overexpression of ISG15 and clinical liver disease outcomes

ISG15 and its ISGlation form mediate innate immune responses through IFNs, lipopolysaccharide and dsRNA stimulation (Sadler and Williams. 2008). Higher ISG15 serum levels in HBV patients than in healthy individuals and an increased *ISG15* mRNA expression observed in HBV-related liver tissues compared to non-HBV/HCV-related liver tissues demonstrate that HBV infection triggers *ISG15*

expression. This is in accordance with the fact that the major host response to viral infection is the production of IFNs, which in turn stimulate *ISG15* expression. The notion of antiviral activities of both ISG15 and ISGlation came from studies using an *ISG15* knockout mice model (Lenschow et al. 2007; Werneke et al. 2011). Mice lacking ISG15 expression were more susceptible to influenza, sindbis, and herpes simplex viruses (Lenschow et al. 2007). Recent studies have explored the biological functions of ISG15 and related conjugates that can impair viral replication *in vivo* (Malakhova et al. 2008; Okumura et al. 2006; Okumura et al. 2008; Werneke et al. 2011). In contrast, our study showed that ISG15 levels were positively associated with high viral loads, implying an opposing effect of ISG15 on antiviral activities. This is consistent with studies showing that ISG15 can promote HCV replication (Broering et al. 2016; Broering et al. 2010; Chen et al. 2010).

Host immune factors are important in the immune-pathogenesis of HBV infection through genetic and epigenetic modifications (Koumbi et al. 2015; Tong et al. 2014a) and via the effects of cytokines (Li et al. 2016). An unsuccessful immune response against HBV may result in persistent virus replication and liver inflammation, leading to CHB, LC and HCC (Li et al. 2016). ISG15 appears to act as an immune-modulator, regulating the expression of cytokines, in particular of the IFN signaling pathway. Earlier studies have indicated that *ISG15* upregulation results in a reduced immune response to IFN signaling and contributes to a poor outcome of IFN-based therapy in HCV patients (Broering et al. 2010; Chen et al. 2005). In addition, higher levels of ISG15 were observed in treatment failure compared to responders to IFN-based treatment (Chen et al. 2005; Chen et al. 2010). Consequently, levels of ISG15 combined with clinical, biochemical, and histological analyses may be useful to predict the outcome of HBV-related liver disease and may help to evaluate the response of HBV-infected individuals to IFN treatment.

The biological function of ISG15 in enhancing or suppressing tumor growth remains controversial (Andersen and Hassel. 2006b) although antagonistic roles of ISG15 in tumorigenesis have been documented (Andersen and Hassel. 2006b; Burks et al.

2014; Desai. 2015b). Several studies have shown that ISG15 is an oncoprotein, as *ISG15* gene expression and its protein conjugates were found elevated in tumor cell lines and in various human malignancies (Andersen et al. 2006a; Bektas et al. 2008; Burks et al. 2014; Desai et al. 2006; Kiessling et al. 2009; Li et al. 2014; Qiu et al. 2015; Satake et al. 2010; Wood et al. 2012). High serum ISG15 levels in HCC patients and mRNA expression in liver tumor tissues also suggest that ISG15 may serve as a protumor factor. In contrast, other studies reported that free ISG15 has the potential to induce antitumor responses (Burks et al. 2015; D'Cunha et al. 1996). These differences could be due to the functional differences of free ISG15 and ISG15 conjugates (Andersen and Hassel. 2006b; Desai. 2015b). So far it is clear that an intense ISG15 expression is vital in modulating cell growth and in the progression of breast cancer (Bektas et al. 2008; Burks et al. 2014). The functional role of ISG15 in HCC, however, is still unclear. ISG15 overexpression is associated with poor clinical outcomes (Li et al. 2014; Qiu et al. 2015). Moreover, knocking down ISG15 by shRNA ISG15 can lead to a remarkable reduction of HCC cell proliferation and migration (Li et al. 2014).

Although our data suggest that ISG15 over-expression is modulated by HBV infection and may induce liver disease progression, the study has some limitations. Due to the study design as a case-control study, levels of ISG15 over the course of HBV infection were not measured and the causative effect of ISG15 levels on progression of HBV-related liver diseases was not decisively assessed. HCC patients who donated liver tissues were in early and intermediate stages of liver cancer, which might have also affected our results. Therefore, additional studies in HCC patients with advanced stages of liver cancer are needed to associate ISG15 mRNA expression with cancer progression.

In conclusion, viral hepatitis caused by HBV remains a critical public health concern. Worldwide, more than 2 billion people are affected during their lifetime, and 350-400 million people are chronically infected. Chronic cases can develop into severe forms of liver diseases such as LC and HCC. The clinical course and pathogenesis of HBV-

related liver diseases are multifactorial and essentially influenced by the coinfection with other viruses and host factors. My dissertation has explored the impact of HEV superinfections and the role of human genetic and epigenetic factors in the JAK/STAT pathway-related genes towards susceptibility to HBV infection and the outcome of HBV-associated liver diseases. The findings in the first publication reflect high endemicity of HEV infection in Vietnam, a country where HBV is also heavily endemic. High prevalences of HEV superinfection in all clinically classified HBV patients is also reported. Findings of this study also support that HEV plays an undesirable role in liver disease progression in HBV-infected patients, especially in liver cirrhosis patients. The findings extracted from the second and third publication have demonstrated for the first time that variants of *SOCS3* and *ISG15* genes (*SOCS3* rs111033850, *SOCS3* rs12953258 and *ISG15* rs1921), relating to JAK/STAT signaling pathway, play a critical role in HBV susceptibility and liver disease outcomes. Moreover, the expression of SOCS3 and ISG15 at the mRNA and protein level is also associated with liver disease progression in HBV-infected patients. In addition to the importance of genetics, the role of epigenetics reveals that SOCS3 promoter hypermethylation stimulates HCC development. Taken together, this dissertation provides an increased understanding about how host genetic factors might influence the HBV liver diseases and how another hepatitis superinfection may aggravate the existing HBV liver disease.

4. SUMMARY

Although the introduction of the Hepatitis B virus (HBV) vaccine has significantly reduced mortality and morbidity in many parts of the world, few pockets of high endemicity still remains in South-East Asia and in Sub-Saharan Africa. Despite, Vietnam initiated universal immunization for hepatitis B for infants in 2003, the rates of chronically infected patients with hepatitis B remains high with an estimated prevalence of >10%. The clinical course of HBV infection is influenced by both host and viral factors. In this thesis, I utilized a cohort of patients well characterized for clinical HBV infections, including acute and chronic hepatitis B, liver cirrhosis, and hepatocellular carcinoma. In chapter one of my thesis, I investigated 1318 Vietnamese HBV patients to elucidate if superinfection by other hepatitis viruses exists in this study population. In particular, I studied the HEV seroprevalences in patients with HBV and characterized specific HEV isolates at the molecular level to understand the consequences of HEV superinfections on the clinical course of HBV infections. This study showed that HEV may aggravate the clinical outcome of HBV infection. In chapter two of my thesis, I studied the contribution of negative regulator suppressor of cytokine signaling-3 (SOCS3) promoter variants in HBV disease. I genotyped 878 HBV patients and 272 healthy controls for SOCS3 promoter variants. SOCS3 promoter hyper methylation in HBV tumor tissues was examined by bisulfite sequencing and mRNA expression was quantified in tumor and non-tumor tissues. The results revealed that *SOCS3* promoter variants are associated with HBV susceptibility and *SOCS3* hypermethylation stimulates HCC development. Additionally in chapter 2, investigations were carried out to associate Interferon-stimulated gene 15 (ISG15) polymorphisms, ISG15 serum levels, and relative mRNA expression with HBV-related liver diseases. *ISG15* exon2 variant rs1921 was associated with HBV susceptibility. ISG15 serum levels were higher among HBV patients and were positively correlated with HBV-DNA loads. ISG15 mRNA expression was increased in HBV patients. The results reveal that ISG15 appears to

be a proviral factor involved in HBV replication and triggers the progression of HBV-related liver diseases.

Taken together, this dissertation serves as a basis to understand the association of host and viral factors with HBV susceptibility and subsequent clinical outcomes of HBV related liver diseases.

5. ZUSAMMENFASSUNG

Trotz der Einführung eines effektiven Hepatitis-B-Virus (HBV) Impfstoffs, der weltweit die Mortalität und Morbidität einer HBV-Infektion signifikant reduziert hat, gibt es dennoch einige Regionen, insbesondere Südostasien und Sub-Sahara Afrika, in denen HBV-Infektionen hoch endemisch sind. Obwohl Vietnam im Jahr 2003 eine generelle Immunisierung gegen Hepatitis B für Säuglinge und Jugendliche eingeführt hat, ist in diesem Land die Rate von chronisch HBV-Infizierten mit einer geschätzten HBV-Prävalenz von >10% noch immer hoch. Bekannt ist, dass der klinische Verlauf einer HBV Infektion sowohl von Wirt- als auch viralen Faktoren beeinflusst wird. Um dies weiter zu untersuchen wurde in der vorliegenden Doktorarbeit Proben von einer gut charakterisierten Kohorte von Patienten mit HBV-Infektion eingesetzt, die Patienten mit akuter und chronischer Hepatitis B, Leberzirrhose und hepatozellulärem Karzinom einschloss. In Kapitel 1 der vorliegenden Dissertation wurden Proben von 1318 vietnamesischen HBV-infizierten Patienten untersucht, um zu klären, ob Superinfektionen mit anderen Hepatitisviren in dieser Studienpopulation auftreten. Im Einzelnen wurde die Hepatitis E Virus (HEV) Seroprävalenz in den HBV-positiven Patienten analysiert. Spezifische HEV-Stränge wurden weiter molekular-genetisch charakterisiert um die Auswirkung einer HEV-Superinfektion auf den klinischen Verlauf einer HBV-Infektion besser zu verstehen. Diese Studie zeigte, dass HEV den klinischen Verlauf einer HBV-Infektion verschlimmern kann. In Kapitel 2 dieser Arbeit wurde der Beitrag von negative regulator suppressor of cytokine signaling-3 (SOCS3) Promotorvarianten auf den Verlauf einer HBV-Infektion analysiert. Dazu wurden SOCS3 Promotorvarianten, isoliert aus 878 HBV-positive Patienten und 272 gesunden Kontrollindividuen, näher charakterisiert. SOCS3 Promotor-Hypermethylierung in Tumorgewebe von HBV-positiven Patienten wurde mittels Bi-Sulfit-Sequenzierung analysiert und zudem die SOCS3 mRNA-Expression in Tumor und Nicht-Tumorgewebe dieser Patienten quantifiziert. Die Ergebnisse dieser Untersuchungen zeigten, dass SOCS3 Promotorvarianten mit der HBV-

Suszeptibilität assoziiert waren und SOCS3 Promotor-Hypermethylierung die HCC Entwicklung stimulierte. Zusätzlich dazu wurde in Kapitel 2 weiterführende Experimente durchgeführt, die eine Assoziation von Interferon-stimulated gene 15 (ISG15)-Polymorphismen, ISG15 Serumspiegel und relativer ISG15 mRNA Expression mit HBV-bedingten Leberentzündungen aufdecken sollten. Hierbei zeigte sich, dass die ISG15 exon2 Variante rs1921 mit der HBV-Suszeptibilität assoziiert war und ISG15 Serumspiegel in HBV-positiven Patienten höher und positiv korreliert mit der HBV-DNA Viruslast waren. Zudem war die ISG15 mRNA Expression in HBV-positiven Patienten erhöht. Die Ergebnisse belegten, dass ISG15 ein proviraler Faktor ist, der bei die HBV-Replikationskontrolle involviert ist und die Progression HBV-bedingter Leberentzündungen triggert.

Zusammenfassend bildet die vorliegende Dissertation eine Grundlage für das bessere Verständnis essentieller Assoziationen von Wirt- und viralen Faktoren mit der HBV Suszeptibilität und folgend dem klinischen Verlauf HBV-bedingter Lebererkrankungen.

6. BIBLIOGRAPHY

Abravanel F, Lhomme S, Chapuy-Regaud S, Mansuy J M, Muscari F, Sallusto F, Rostaing L, Kamar N, and Izopet J (2014) Hepatitis E virus reinfections in solid-organ-transplant recipients can evolve into chronic infections. J Infect. Dis 209: 1900-1906.

Adlhoch C, Avellon A, Baylis S A, Ciccaglione A R, Couturier E, de Sousa R, Epstein J, Ethelberg S, Faber M, Feher A, Ijaz S, Lange H, Mandakova Z, Mellou K, Mozalevskis A, Rimhanen-Finne R, Rizzi V, Said B, Sundqvist L, Thornton L, Tosti M E, van Pelt W, Aspinall E, Domanovic D, Severi E, Takkinen J, and Dalton H R (2016) Hepatitis E virus: Assessment of the epidemiological situation in humans in Europe, 2014/15. J Clin Virol 82: 9-16.

Aggarwal R (2013) Hepatitis E: clinical presentation in disease-endemic areas and diagnosis. Semin Liver Dis 33: 30-40.

Akira S, Uematsu S, and Takeuchi O (2006) Pathogen recognition and innate immunity. Cell 124: 783-801.

Al-Qahtani A, Khalak H G, Alkuraya F S, Al-hamoudi W, Alswat K, Al Balwi M A, Al A, I, Sanai F M, and Abdo A A (2013) Genome-wide association study of chronic hepatitis B virus infection reveals a novel candidate risk allele on 11q22.3. J.Med.Genet. 50: 725-732.

Allweiss L, and Dandri M (2016) Experimental in vitro and in vivo models for the study of human hepatitis B virus infection. J.Hepatol. 64: S17-S31.

Andersen J B, Aaboe M, Borden E C, Goloubeva O G, Hassel B A, and Orntoft T F (2006a) Stage-associated overexpression of the ubiquitin-like protein, ISG15, in bladder cancer. Br. J. Cancer 94: 1465-1471.

Andersen J B, and Hassel B A (2006b) The interferon regulated ubiquitin-like protein, ISG15, in tumorigenesis: friend or foe? Cytokine Growth Factor Rev 17: 411-421.

Arends J E, Ghisetti V, Irving W, Dalton H R, Izopet J, Hoepelman A I, and Salmon D (2014) Hepatitis E: An emerging infection in high income countries. J Clin Virol 59: 81-88.

Atiq M, Shire N J, Barrett A, Rouster S D, Sherman K E, and Shata M T (2009) Hepatitis E virus antibodies in patients with chronic liver disease. Emerg. Infect. Dis 15: 479-481.

Banks M, Bendall R, Grierson S, Heath G, Mitchell J, and Dalton H (2004) Human and porcine hepatitis E virus strains, United Kingdom. Emerg Infect Dis 10: 953-955.

Barnaba V, Valesini G, Levrero M, Zaccari C, Van D A, Falco M, Musca A, and Balsano F (1985) Immunoregulation of the in vitro anti-HBs antibody synthesis in chronic HBsAg carriers and in recently boosted anti-hepatitis B vaccine recipients. Clin.Exp.Immunol. 60: 259-266.

Bayram A, Eksi F, Mehli M, and Sozen E (2007) Prevalence of hepatitis E virus antibodies in patients with chronic hepatitis B and chronic hepatitis C. Intervirology 50: 281-286.

Beale M A, Tettmar K, Szypulska R, Tedder R S, and Ijaz S (2011) Is there evidence of recent hepatitis E virus infection in English and North Welsh blood donors? Vox Sang 100: 340-342.

Bektas N, Noetzel E, Veeck J, Press M F, Kristiansen G, Naami A, Hartmann A, Dimmler A, Beckmann M W, Knuchel R, Fasching P A, and Dahl E (2008) The ubiquitin-like molecule interferon-stimulated gene 15 (ISG15) is a potential prognostic marker in human breast cancer. Breast Cancer Res 10: R58.

Bertoletti A, and Naoumov N V (2003) Translation of immunological knowledge into better treatments of chronic hepatitis B. J.Hepatol. 39: 115-124.

Bile K, Isse A, Mohamud O, Allebeck P, Nilsson L, Norder H, Mushahwar I K, and Magnius L O (1994) Contrasting roles of rivers and wells as sources of drinking water on attack and fatality rates in a hepatitis E epidemic in Somalia. Am J Trop Med Hyg 51: 466-474.

Blumberg B S, ALTER H J, and VISNICH S (1965) A "NEW" ANTIGEN IN LEUKEMIA SERA. JAMA 191: 541-546.

Blumberg B S, Gerstley B J, Hungerford D A, London W T, and Sutnick A I (1967) A serum antigen (Australia antigen) in Down's syndrome, leukemia, and hepatitis. Ann.Intern.Med. 66: 924-931.

Bocher W O, Herzog-Hauff S, Herr W, Heermann K, Gerken G, Meyer Zum Buschenfelde K H, and Lohr H F (1996) Regulation of the neutralizing anti-hepatitis B surface (HBs) antibody response in vitro in HBs vaccine recipients and patients with acute or chronic hepatitis B virus (HBV) infection. Clin.Exp.Immunol. 105: 52-58.

Bode J G, Ludwig S, Ehrhardt C, Albrecht U, Erhardt A, Schaper F, Heinrich P C, and Haussinger D (2003) IFN-alpha antagonistic activity of HCV core protein involves induction of suppressor of cytokine signaling-3. FASEB J 17: 488-490.

Boehme K W, and Compton T (2004) Innate sensing of viruses by toll-like receptors. J.Virol. 78: 7867-7873.

Boosani C S, and Agrawal D K (2015) Methylation and microRNA-mediated epigenetic regulation of SOCS3. Mol. Biol. Rep 42: 853-872.

Bose P D, Das B C, Hazam R K, Kumar A, Medhi S, and Kar P (2014) Evidence of extrahepatic replication of hepatitis E virus in human placenta. J Gen Virol 95: 1266-1271.

Bose P D, Das B C, Kumar A, Gondal R, Kumar D, and Kar P (2011) High viral load and deregulation of the progesterone receptor signaling pathway: association with hepatitis E-related poor pregnancy outcome. J Hepatol 54: 1107-1113.

Boutrouille A, Bakkali-Kassimi L, Cruciere C, and Pavio N (2007) Prevalence of anti-hepatitis E virus antibodies in French blood donors. J Clin. Microbiol 45: 2009-2010.

Broering R, Trippler M, Werner M, Real C I, Megger D A, Bracht T, Schweinsberg V, Sitek B, Eisenacher M, Meyer H E, Baba H A, Weber F, Hoffmann A C, Gerken G, and Schlaak J F (2016) Hepatic expression of proteasome subunit alpha type-6 is upregulated during viral hepatitis and putatively regulates the expression of ISG15 ubiquitin-like modifier, a proviral host gene in hepatitis C virus infection. J. Viral Hepat.

Broering R, Zhang X, Kottilil S, Trippler M, Jiang M, Lu M, Gerken G, and Schlaak J F (2010) The interferon stimulated gene 15 functions as a proviral factor for the hepatitis C virus and as a regulator of the IFN response. Gut 59: 1111-1119.

Burks J, Reed R E, and Desai S D (2014) ISGylation governs the oncogenic function of Ki-Ras in breast cancer. Oncogene 33: 794-803.

Burks J, Reed R E, and Desai S D (2015) Free ISG15 triggers an antitumor immune response against breast cancer: a new perspective. Oncotarget 6: 7221-7231.

Campbell J A, and Lenschow D J (2013a) Emerging roles for immunomodulatory functions of free ISG15. J. Interferon Cytokine Res 33: 728-738.

Campbell J A, and Lenschow D J (2013b) Emerging roles for immunomodulatory functions of free ISG15. J.Interferon Cytokine Res. 33: 728-738.

Carow B, and Rottenberg M E (2014) SOCS3, a Major Regulator of Infection and Inflammation. Front Immunol. 5: 58.

CDC (2015). Epidemiology and Prevention of Vaccine-Preventable Diseases (www.cdc.gov/vaccines/pubs/pinkbook/hepb.html).

Chang J J, and Lewin S R (2007) Immunopathogenesis of hepatitis B virus infection. Immunol.Cell Biol. 85: 16-23.

Chang M H (2006) Impact of hepatitis B vaccination on hepatitis B disease and nucleic acid testing in high-prevalence populations. J.Clin.Virol. 36 Suppl 1: S45-S50.

Chang S W, Fann C S, Su W H, Wang Y C, Weng C C, Yu C J, Hsu C L, Hsieh A R, Chien R N, Chu C M, and Tai D I (2014) A genome-wide association study on chronic HBV infection and its clinical progression in male Han-Taiwanese. PLoS.One. 9: e99724.

Chen C J, and Yang H I (2011) Natural history of chronic hepatitis B REVEALed. J.Gastroenterol.Hepatol. 26: 628-638.

Chen C J, Yang H I, and Iloeje U H (2009) Hepatitis B virus DNA levels and outcomes in chronic hepatitis B. Hepatology 49: S72-S84.

Chen L, Borozan I, Feld J, Sun J, Tannis L L, Coltescu C, Heathcote J, Edwards A M, and McGilvray I D (2005) Hepatic gene expression discriminates responders and nonresponders in treatment of chronic hepatitis C viral infection. Gastroenterology 128: 1437-1444.

Chen L, Sun J, Meng L, Heathcote J, Edwards A M, and McGilvray I D (2010) ISG15, a ubiquitin-like interferon-stimulated gene, promotes hepatitis C virus production in vitro: implications for chronic infection and response to treatment. J. Gen. Virol 91: 382-388.

Cheng S H, Mai L, Zhu F Q, Pan X F, Sun H X, Cao H, Shu X, Ke W M, Li G, and Xu Q H (2013) Influence of chronic HBV infection on superimposed acute hepatitis E. World J Gastroenterol 19: 5904-5909.

Chisari F V, and Ferrari C (1995) Hepatitis B virus immunopathogenesis. Annu.Rev.Immunol. 13: 29-60.

Chow C W, Tsang S W, Tsang O T, Leung V K, Fung K S, Luk W K, and Chau T N (2014) Comparison of acute hepatitis E infection outcome in patients with and without chronic hepatitis B infection: a 10 year retrospective study in three regional hospitals in Hong Kong. J Clin. Virol 60: 4-10.

Christensen P B, Engle R E, Hjort C, Homburg K M, Vach W, Georgsen J, and Purcell R H (2008) Time trend of the prevalence of hepatitis E antibodies among farmers and blood donors: a potential zoonosis in Denmark. Clin. Infect. Dis 47: 1026-1031.

Cleland A, Smith L, Crossan C, Blatchford O, Dalton H R, Scobie L, and Petrik J (2013) Hepatitis E virus in Scottish blood donors. Vox Sang 105: 283-289.

Colonna M, Krug A, and Cella M (2002) Interferon-producing cells: on the front line in immune responses against pathogens. Curr Opin Immunol 14: 373-379.

Cossart Y E, and Field A M (1970) Virus-like particles in serum of patients with Australia-antigen-associated hepatitis. Lancet 1: 848.

D'Cunha J, Jr K E, Haas A L, Truitt R L, and Borden E C (1996) Immunoregulatory properties of ISG15, an interferon-induced cytokine. Proc. Natl. Acad. Sci. U. S. A 93: 211-215.

Dalton H R, Bendall R, Ijaz S, and Banks M (2008) Hepatitis E: an emerging infection in developed countries. Lancet Infect Dis 8: 698-709.

Dalton H R, Bendall R P, Keane F E, Tedder R S, and Ijaz S (2009) Persistent carriage of hepatitis E virus in patients with HIV infection. N. Engl. J Med 361: 1025-1027.

Dalton H R, Hunter J G, and Bendall R P (2013) Hepatitis E. Curr Opin Infect Dis 26: 471-478.

Dandri M, and Locarnini S (2012) New insight in the pathobiology of hepatitis B virus infection. Gut 61 Suppl 1: i6-17.

Dane D S, Cameron C H, and Briggs M (1970) Virus-like particles in serum of patients with Australia-antigen-associated hepatitis. Lancet 1: 695-698.

Debing Y, Gisa A, Dallmeier K, Pischke S, Bremer B, Manns M, Wedemeyer H, Suneetha P V, and Neyts J (2014) A mutation in the hepatitis E virus RNA polymerase promotes its replication and associates with ribavirin treatment failure in organ transplant recipients. Gastroenterology 147: 1008-1011 e1007; quiz e1015-1006.

Desai S D (2015a) ISG15: A double edged sword in cancer. Oncoimmunology. 4: e1052935.

Desai S D (2015b) ISG15: A double edged sword in cancer. Oncoimmunology 4: e1052935.

Desai S D, Haas A L, Wood L M, Tsai Y C, Pestka S, Rubin E H, Saleem A, Nur-E-Kamal, and Liu L F (2006) Elevated expression of ISG15 in tumor cells interferes with the ubiquitin/26S proteasome pathway. Cancer Res 66: 921-928.

Donnelly M C, Scobie L, Crossan C L, Dalton H, Hayes P C, and Simpson K J (2017) Review article: hepatitis E-a concise review of virology, epidemiology, clinical presentation and therapy. Aliment Pharmacol Ther 46: 126-141.

Donnelly R P, and Kotenko S V (2010) Interferon-lambda: a new addition to an old family. J.Interferon Cytokine Res. 30: 555-564.

Du L Y, Cui Y L, Chen E Q, Cheng X, Liu L, and Tang H (2014) Correlation between the suppressor of cytokine signaling-1 and 3 and hepatitis B virus: possible roles in the resistance to interferon treatment. Virol. J 11: 51.

Dunn C, Brunetto M, Reynolds G, Christophides T, Kennedy P T, Lampertico P, Das A, Lopes A R, Borrow P, Williams K, Humphreys E, Afford S, Adams D H, Bertoletti A, and Maini M K (2007) Cytokines induced during chronic hepatitis B virus infection promote a pathway for NK cell-mediated liver damage. J.Exp.Med. 204: 667-680.

El-Serag H B (2011) Hepatocellular carcinoma. N.Engl.J.Med. 365: 1118-1127.

Elgouhari H M, Abu-Rajab Tamimi T I, and Carey W D (2008) Hepatitis B virus infection: understanding its epidemiology, course, and diagnosis. Cleve.Clin.J.Med. 75: 881-889.

Emerson S U, and Purcell R H (2003) Hepatitis E virus. Rev Med Virol 13: 145-154.

Engelke M, Mills K, Seitz S, Simon P, Gripon P, Schnolzer M, and Urban S (2006) Characterization of a hepatitis B and hepatitis delta virus receptor binding site. Hepatology 43: 750-760.

Estrabaud E, and Asselah T (2010) Interferon-stimulated gene 15: a dual activity during hepatitis C virus infection. Gut 59: 1017-1019.

European Association for the Study of the Liver. Electronic address e e e, and European Association for the Study of the L (2017) EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection. J Hepatol.

Fattovich G (2003) Natural history of hepatitis B. J.Hepatol. 39 Suppl 1: S50-S58.

Fattovich G, Bortolotti F, and Donato F (2008) Natural history of chronic hepatitis B: special emphasis on disease progression and prognostic factors. J.Hepatol. 48: 335-352.

Feldt T, Sarfo F S, Zoufaly A, Phillips R O, Burchard G, van L J, Jochum J, Chadwick D, Awasom C, Claussen L, Drosten C, Drexler J F, and Eis-Hubinger A M (2013) Hepatitis E virus infections in HIV-infected patients in Ghana and Cameroon. J Clin. Virol 58: 18-23.

Gallian P, Lhomme S, Piquet Y, Saune K, Abravanel F, Assal A, Tiberghien P, and Izopet J (2014) Hepatitis E virus infections in blood donors, France. Emerg. Infect. Dis 20: 1914-1917.

Ganem D, and Prince A M (2004) Hepatitis B virus infection--natural history and clinical consequences. N.Engl.J.Med. 350: 1118-1129.

Gao B, Wang H, Lafdil F, and Feng D (2012) STAT proteins - key regulators of anti-viral responses, inflammation, and tumorigenesis in the liver. J Hepatol 57: 430-441.

Gao W, Kondo Y, Shen L, Shimizu Y, Sano T, Yamao K, Natsume A, Goto Y, Ito M, Murakami H, Osada H, Zhang J, Issa J P, and Sekido Y (2008) Variable DNA methylation patterns associated with progression of disease in hepatocellular carcinomas. Carcinogenesis 29: 1901-1910.

Gerolami R, Moal V, and Colson P (2008) Chronic hepatitis E with cirrhosis in a kidney-transplant recipient. N. Engl. J Med 358: 859-860.

Glebe D, and Urban S (2007) Viral and cellular determinants involved in hepadnaviral entry. World J.Gastroenterol. 13: 22-38.

Gonzalez Tallon A I, Moreira Vicente V, Mateos Lindemann M L, and Achecar Justo L M (2011) [Chronic hepatitis E in an immunocompetent patient]. Gastroenterol Hepatol 34: 398-400.

Grewal P, Kamili S, and Motamed D (2014) Chronic hepatitis E in an immunocompetent patient: a case report. Hepatology 59: 347-348.

Gripon P, Cannie I, and Urban S (2005) Efficient inhibition of hepatitis B virus infection by acylated peptides derived from the large viral surface protein. J.Virol. 79: 1613-1622.

Guidotti L G, and Chisari F V (2006) Immunobiology and pathogenesis of viral hepatitis. Annu.Rev.Pathol. 1: 23-61.

Guidotti L G, Ishikawa T, Hobbs M V, Matzke B, Schreiber R, and Chisari F V (1996) Intracellular inactivation of the hepatitis B virus by cytotoxic T lymphocytes. Immunity. 4: 25-36.

Guidotti L G, Rochford R, Chung J, Shapiro M, Purcell R, and Chisari F V (1999) Viral clearance without destruction of infected cells during acute HBV infection. Science 284: 825-829.

Guo Q S, Yan Q, Xiong J H, Ge S X, Shih J W, Ng M H, Zhang J, and Xia N S (2010) Prevalence of hepatitis E virus in Chinese blood donors. J Clin. Microbiol 48: 317-318.

H.C.Thomas, S.M.Lemon, and A.J.Zuckerman (2013). Structure and molecular virology. In Viral hepatitis (Wiley Blackwell), pp. 65-80.

Hadziyannis S J (2011) Natural history of chronic hepatitis B in Euro-Mediterranean and African countries. J.Hepatol. 55: 183-191.

Hamid S S, Atiq M, Shehzad F, Yasmeen A, Nissa T, Salam A, Siddiqui A, and Jafri W (2002) Hepatitis E virus superinfection in patients with chronic liver disease. Hepatology 36: 474-478.

Harty R N, Pitha P M, and Okumura A (2009) Antiviral activity of innate immune protein ISG15. J. Innate. Immun 1: 397-404.

Hau C H, Hien T T, Tien N T, Khiem H B, Sac P K, Nhung V T, Larasati R P, Laras K, Putri M P, Doss R, Hyams K C, and Corwin A L (1999) Prevalence of enteric hepatitis A and E viruses in the Mekong River delta region of Vietnam. Am. J Trop. Med. Hyg 60: 277-280.

He B, You L, Uematsu K, Zang K, Xu Z, Lee A Y, Costello J F, McCormick F, and Jablons D M (2003) SOCS-3 is frequently silenced by hypermethylation and suppresses cell growth in human lung cancer. Proc. Natl. Acad. Sci. U. S. A 100: 14133-14138.

Herman J G, and Baylin S B (2003) Gene silencing in cancer in association with promoter hypermethylation. N.Engl.J.Med. 349: 2042-2054.

Hernandez-Vargas H, Lambert M P, Le Calvez-Kelm F, Gouysse G, McKay-Chopin S, Tavtigian S V, Scoazec J Y, and Herceg Z (2010) Hepatocellular carcinoma displays distinct DNA methylation signatures with potential as clinical predictors. PLoS.One. 5: e9749.

Hu Z, Liu Y, Zhai X, Dai J, Jin G, Wang L, Zhu L, Yang Y, Liu J, Chu M, Wen J, Xie K, Du G, Wang Q, Zhou Y, Cao M, Liu L, He Y, Wang Y, Zhou G, Jia W, Lu J, Li S, Liu J, Yang H, Shi Y, Zhou W, and Shen H (2013) New loci associated with chronic hepatitis B virus infection in Han Chinese. Nat.Genet. 45: 1499-1503.

Jammeh S, Tavner F, Watson R, Thomas H C, and Karayiannis P (2008) Effect of basal core promoter and pre-core mutations on hepatitis B virus replication. J.Gen.Virol. 89: 901-909.

Jeon Y J, Yoo H M, and Chung C H (2010a) ISG15 and immune diseases. Biochim.Biophys.Acta 1802: 485-496.

Jeon Y J, Yoo H M, and Chung C H (2010b) ISG15 and immune diseases. Biochim. Biophys. Acta 1802: 485-496.

Jiang B G, Yang Y, Liu H, Gu F M, Yang Y, Zhao L H, Yuan S X, Wang R Y, Zhang J, and Zhou W P (2015) SOCS3 Genetic Polymorphism Is Associated With Clinical

Features and Prognosis of Hepatocellular Carcinoma Patients Receiving Hepatectomy. Medicine (Baltimore) 94: e1344.

Jiang D K, Sun J, Cao G, Liu Y, Lin D, Gao Y Z, Ren W H, Long X D, Zhang H, Ma X P, Wang Z, Jiang W, Chen T Y, Gao Y, Sun L D, Long J R, Huang H X, Wang D, Yu H, Zhang P, Tang L S, Peng B, Cai H, Liu T T, Zhou P, Liu F, Lin X, Tao S, Wan B, Sai-Yin H X, Qin L X, Yin J, Liu L, Wu C, Pei Y, Zhou Y F, Zhai Y, Lu P X, Tan A, Zuo X B, Fan J, Chang J, Gu X, Wang N J, Li Y, Liu Y K, Zhai K, Zhang H, Hu Z, Liu J, Yi Q, Xiang Y, Shi R, Ding Q, Zheng W, Shu X O, Mo Z, Shugart Y Y, Zhang X J, Zhou G, Shen H, Zheng S L, Xu J, and Yu L (2013) Genetic variants in STAT4 and HLA-DQ genes confer risk of hepatitis B virus-related hepatocellular carcinoma. Nat.Genet. 45: 72-75.

Jiang R, Tan Z, Deng L, Chen Y, Xia Y, Gao Y, Wang X, and Sun B (2011) Interleukin-22 promotes human hepatocellular carcinoma by activation of STAT3. Hepatology 54: 900-909.

Juhl D, Baylis S A, Blumel J, Gorg S, and Hennig H (2014) Seroprevalence and incidence of hepatitis E virus infection in German blood donors. Transfusion 54: 49-56.

Kadowaki N, and Liu Y J (2002) Natural type I interferon-producing cells as a link between innate and adaptive immunity. Hum Immunol 63: 1126-1132.

Kakimi K, Guidotti L G, Koezuka Y, and Chisari F V (2000) Natural killer T cell activation inhibits hepatitis B virus replication in vivo. J.Exp.Med. 192: 921-930.

Kakimi K, Lane T E, Wieland S, Asensio V C, Campbell I L, Chisari F V, and Guidotti L G (2001) Blocking chemokine responsive to gamma-2/interferon (IFN)-gamma inducible protein and monokine induced by IFN-gamma activity in vivo reduces the pathogenetic but not the antiviral potential of hepatitis B virus-specific cytotoxic T lymphocytes. J.Exp.Med. 194: 1755-1766.

Kamar N, Bendall R, Legrand-Abravanel F, Xia N S, Ijaz S, Izopet J, and Dalton H R (2012) Hepatitis E. Lancet 379: 2477-2488.

Kamar N, Dalton H R, Abravanel F, and Izopet J (2014) Hepatitis E virus infection. Clin Microbiol Rev 27: 116-138.

Kamar N, Mansuy J M, Cointault O, Selves J, Abravanel F, Danjoux M, Otal P, Esposito L, Durand D, Izopet J, and Rostaing L (2008) Hepatitis E virus-related cirrhosis in kidney- and kidney-pancreas-transplant recipients. Am J Transplant 8: 1744-1748.

Kao J H (2011) Molecular epidemiology of hepatitis B virus. Korean J.Intern.Med. 26: 255-261.

Khuroo M S, Kamili S, and Khuroo M S (2009) Clinical course and duration of viremia in vertically transmitted hepatitis E virus (HEV) infection in babies born to HEV-infected mothers. J Viral Hepat 16: 519-523.

Kiessling A, Hogrefe C, Erb S, Bobach C, Fuessel S, Wessjohann L, and Seliger B (2009) Expression, regulation and function of the ISGylation system in prostate cancer. Oncogene 28: 2606-2620.

Kim Y J, Kim H Y, Lee J H, Yu S J, Yoon J H, Lee H S, Kim C Y, Cheong J Y, Cho S W, Park N H, Park B L, Namgoong S, Kim L H, Cheong H S, and Shin H D (2013) A genome-wide association study identified new variants associated with the risk of chronic hepatitis B. Hum.Mol.Genet. 22: 4233-4238.

Koeberlein B, zur H A, Bektas N, Zentgraf H, Chin R, Nguyen L T, Kandolf R, Torresi J, and Bock C T (2010) Hepatitis B virus overexpresses suppressor of cytokine signaling-3 (SOCS3) thereby contributing to severity of inflammation in the liver. Virus Res 148: 51-59.

Koumbi L, and Karayiannis P (2015) The Epigenetic Control of Hepatitis B Virus Modulates the Outcome of Infection. Front Microbiol 6: 1491.

Koyama S, Ishii K J, Coban C, and Akira S (2008) Innate immune response to viral infection. Cytokine 43: 336-341.

Koziel M J (1999a) Cytokines in viral hepatitis. Semin.Liver Dis. 19: 157-169.

Koziel M J (1999b) Cytokines in viral hepatitis. Semin. Liver Dis 19: 157-169.

Kruse R L, Kramer J R, Tyson G L, Duan Z, Chen L, El-Serag H B, and Kanwal F (2014) Clinical outcomes of hepatitis B virus coinfection in a United States cohort of hepatitis C virus-infected patients. Hepatology 60: 1871-1878.

Kubo M, Hanada T, and Yoshimura A (2003) Suppressors of cytokine signaling and immunity. Nat. Immunol 4: 1169-1176.

Kumar M, Sarin S K, Hissar S, Pande C, Sakhuja P, Sharma B C, Chauhan R, and Bose S (2008a) Virologic and histologic features of chronic hepatitis B virus-infected asymptomatic patients with persistently normal ALT. Gastroenterology 134: 1376-1384.

Kumar M, Sharma B C, and Sarin S K (2008b) Hepatitis E virus as an etiology of acute exacerbation of previously unrecognized asymptomatic patients with hepatitis B virus-related chronic liver disease. J Gastroenterol. Hepatol 23: 883-887.

Kumar R, Singla V, and Kacharya S (2008c) Impact and management of hepatitis B and hepatitis C virus co-infection in HIV patients. Trop Gastroenterol 29: 136-147.

Larrubia J R, Benito-Martinez S, Miquel-Plaza J, Sanz-de-Villalobos E, Gonzalez-Mateos F, and Parra T (2009) Cytokines - their pathogenic and therapeutic role in chronic viral hepatitis. Rev.Esp.Enferm.Dig. 101: 343-351.

Lee G H, Tan B H, Teo E C, Lim S G, Dan Y Y, Wee A, Aw P P, Zhu Y, Hibberd M L, Tan C K, Purdy M A, and Teo C G (2016) Chronic Infection With Camelid Hepatitis E Virus in a Liver Transplant Recipient Who Regularly Consumes Camel Meat and Milk. Gastroenterology 150: 355-357 e353.

Leistner C M, Gruen-Bernhard S, and Glebe D (2008) Role of glycosaminoglycans for binding and infection of hepatitis B virus. Cell Microbiol. 10: 122-133.

Lenschow D J (2010) Antiviral Properties of ISG15. Viruses 2: 2154-2168.

Lenschow D J, Lai C, Frias-Staheli N, Giannakopoulos N V, Lutz A, Wolff T, Osiak A, Levine B, Schmidt R E, Garcia-Sastre A, Leib D A, Pekosz A, Knobeloch K P, Horak I, and Virgin H W (2007) IFN-stimulated gene 15 functions as a critical antiviral molecule against influenza, herpes, and Sindbis viruses. Proc. Natl. Acad. Sci. U. S. A 104: 1371-1376.

Levrero M, Pollicino T, Petersen J, Belloni L, Raimondo G, and Dandri M (2009a) Control of cccDNA function in hepatitis B virus infection. J.Hepatol. 51: 581-592.

Levrero M, Pollicino T, Petersen J, Belloni L, Raimondo G, and Dandri M (2009b) Control of cccDNA function in hepatitis B virus infection. J. Hepatol 51: 581-592.

Levy D E, and Inghirami G (2006) STAT3: a multifaceted oncogene. Proc.Natl.Acad.Sci.U.S.A 103: 10151-10152.

Lhomme S, Marion O, Abravanel F, Chapuy-Regaud S, Kamar N, and Izopet J (2016) Hepatitis E Pathogenesis. Viruses 8.

Li B, Liu W, Wang L, Li M, Wang J, Huang L, Huang P, and Yuan Y (2010) CpG island methylator phenotype associated with tumor recurrence in tumor-node-metastasis stage I hepatocellular carcinoma. Ann.Surg.Oncol. 17: 1917-1926.

Li C, Wang J, Zhang H, Zhu M, Chen F, Hu Y, Liu H, and Zhu H (2014) Interferon-stimulated gene 15 (ISG15) is a trigger for tumorigenesis and metastasis of hepatocellular carcinoma. Oncotarget 5: 8429-8441.

Li S, Qian J, Yang Y, Zhao W, Dai J, Bei J X, Foo J N, McLaren P J, Li Z, Yang J, Shen F, Liu L, Yang J, Li S, Pan S, Wang Y, Li W, Zhai X, Zhou B, Shi L, Chen X, Chu M, Yan Y, Wang J, Cheng S, Shen J, Jia W, Liu J, Yang J, Wen Z, Li A, Zhang Y, Zhang G, Luo X, Qin H, Chen M, Wang H, Jin L, Lin D, Shen H, He L, de Bakker P I, Wang H, Zeng Y X, Wu M, Hu Z, Shi Y, Liu J, and Zhou W (2012) GWAS

identifies novel susceptibility loci on 6p21.32 and 21q21.3 for hepatocellular carcinoma in chronic hepatitis B virus carriers. PLoS.Genet. 8: e1002791.

Li X, Liu X, Tian L, and Chen Y (2016) Cytokine-Mediated Immunopathogenesis of Hepatitis B Virus Infections. Clin. Rev. Allergy Immunol 50: 41-54.

Lin T M, Chen C J, Wu M M, Yang C S, Chen J S, Lin C C, Kwang T Y, Hsu S T, Lin S Y, and Hsu L C (1989) Hepatitis B virus markers in Chinese twins. Anticancer Res. 9: 737-741.

Liu J, and Fan D (2007) Hepatitis B in China. Lancet 369: 1582-1583.

Lo K J, Tsai Y T, Lee S D, Yeh C L, Wang J Y, Chiang B N, Wu T C, Yeh P S, Goudeau A, Coursaget P, and . (1985) Combined passive and active immunization for interruption of perinatal transmission of hepatitis B virus in Taiwan. Hepatogastroenterology 32: 65-68.

Lok A S (2002) Chronic hepatitis B. N.Engl.J.Med. 346: 1682-1683.

Lok A S, Heathcote E J, and Hoofnagle J H (2001a) Management of hepatitis B: 2000--summary of a workshop. Gastroenterology 120: 1828-1853.

Lok A S, and McMahon B J (2001b) Chronic hepatitis B. Hepatology 34: 1225-1241.

Lok A S, and McMahon B J (2007) Chronic hepatitis B. Hepatology 45: 507-539.

Lou C, Du Z, Yang B, Gao Y, Wang Y, and Fang S (2009) Aberrant DNA methylation profile of hepatocellular carcinoma and surgically resected margin. Cancer Sci. 100: 996-1004.

Lozano R, Naghavi M, Foreman K, Lim S, Shibuya K, Aboyans V, Abraham J, Adair T, Aggarwal R, Ahn S Y, Alvarado M, Anderson H R, Anderson L M, Andrews K G, Atkinson C, Baddour L M, Barker-Collo S, Bartels D H, Bell M L, Benjamin E J, Bennett D, Bhalla K, Bikbov B, Bin A A, Birbeck G, Blyth F, Bolliger I, Boufous S, Bucello C, Burch M, Burney P, Carapetis J, Chen H, Chou D, Chugh S S, Coffeng L E, Colan S D, Colquhoun S, Colson K E, Condon J, Connor M D, Cooper L T, Corriere M, Cortinovis M, de Vaccaro K C, Couser W, Cowie B C, Criqui M H, Cross M, Dabhadkar K C, Dahodwala N, De L D, Degenhardt L, Delossantos A, Denenberg J, Des Jarlais D C, Dharmaratne S D, Dorsey E R, Driscoll T, Duber H, Ebel B, Erwin P J, Espindola P, Ezzati M, Feigin V, Flaxman A D, Forouzanfar M H, Fowkes F G, Franklin R, Fransen M, Freeman M K, Gabriel S E, Gakidou E, Gaspari F, Gillum R F, Gonzalez-Medina D, Halasa Y A, Haring D, Harrison J E, Havmoeller R, Hay R J, Hoen B, Hotez P J, Hoy D, Jacobsen K H, James S L, Jasrasaria R, Jayaraman S, Johns N, Karthikeyan G, Kassebaum N, Keren A, Khoo J P, Knowlton L M, Kobusingye O, Koranteng A, Krishnamurthi R, Lipnick M, Lipshultz S E, Ohno S L, Mabweijano J, MacIntyre M F, Mallinger L, March L, Marks G B, Marks R, Matsumori

A, Matzopoulos R, Mayosi B M, McAnulty J H, McDermott M M, McGrath J, Mensah G A, Merriman T R, Michaud C, Miller M, Miller T R, Mock C, Mocumbi A O, Mokdad A A, Moran A, Mulholland K, Nair M N, Naldi L, Narayan K M, Nasseri K, Norman P, O'Donnell M, Omer S B, Ortblad K, Osborne R, Ozgediz D, Pahari B, Pandian J D, Rivero A P, Padilla R P, Perez-Ruiz F, Perico N, Phillips D, Pierce K, Pope C A, III, Porrini E, Pourmalek F, Raju M, Ranganathan D, Rehm J T, Rein D B, Remuzzi G, Rivara F P, Roberts T, De Leon F R, Rosenfeld L C, Rushton L, Sacco R L, Salomon J A, Sampson U, Sanman E, Schwebel D C, Segui-Gomez M, Shepard D S, Singh D, Singleton J, Sliwa K, Smith E, Steer A, Taylor J A, Thomas B, Tleyjeh I M, Towbin J A, Truelsen T, Undurraga E A, Venketasubramanian N, Vijayakumar L, Vos T, Wagner G R, Wang M, Wang W, Watt K, Weinstock M A, Weintraub R, Wilkinson J D, Woolf A D, Wulf S, Yeh P H, Yip P, Zabetian A, Zheng Z J, Lopez A D, Murray C J, AlMazroa M A, and Memish Z A (2012) Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet 380: 2095-2128.

Lu L, Li C, and Hagedorn C H (2006) Phylogenetic analysis of global hepatitis E virus sequences: genetic diversity, subtypes and zoonosis. Rev Med Virol 16: 5-36.

Lucifora J, Arzberger S, Durantel D, Belloni L, Strubin M, Levrero M, Zoulim F, Hantz O, and Protzer U (2011) Hepatitis B virus X protein is essential to initiate and maintain virus replication after infection. J.Hepatol. 55: 996-1003.

Lutgehetmann M, Mancke L V, Volz T, Helbig M, Allweiss L, Bornscheuer T, Pollok J M, Lohse A W, Petersen J, Urban S, and Dandri M (2012) Humanized chimeric uPA mouse model for the study of hepatitis B and D virus interactions and preclinical drug evaluation. Hepatology 55: 685-694.

Maini M K, Boni C, Lee C K, Larrubia J R, Reignat S, Ogg G S, King A S, Herberg J, Gilson R, Alisa A, Williams R, Vergani D, Naoumov N V, Ferrari C, and Bertoletti A (2000) The role of virus-specific CD8(+) cells in liver damage and viral control during persistent hepatitis B virus infection. J.Exp.Med. 191: 1269-1280.

Maini M K, Boni C, Ogg G S, King A S, Reignat S, Lee C K, Larrubia J R, Webster G J, McMichael A J, Ferrari C, Williams R, Vergani D, and Bertoletti A (1999) Direct ex vivo analysis of hepatitis B virus-specific CD8(+) T cells associated with the control of infection. Gastroenterology 117: 1386-1396.

Malakhova O A, and Zhang D E (2008) ISG15 inhibits Nedd4 ubiquitin E3 activity and enhances the innate antiviral response. J. Biol. Chem 283: 8783-8787.

Mansuy J M, Bendall R, Legrand-Abravanel F, Saune K, Miedouge M, Ellis V, Rech H, Destruel F, Kamar N, Dalton H R, and Izopet J (2011) Hepatitis E virus antibodies in blood donors, France. Emerg. Infect. Dis 17: 2309-2312.

Mansuy J M, Gallian P, Dimeglio C, Saune K, Arnaud C, Pelletier B, Morel P, Legrand D, Tiberghien P, and Izopet J (2016) A nationwide survey of hepatitis E viral infection in French blood donors. Hepatology 63: 1145-1154.

Mansuy J M, Legrand-Abravanel F, Calot J P, Peron J M, Alric L, Agudo S, Rech H, Destruel F, and Izopet J (2008) High prevalence of anti-hepatitis E virus antibodies in blood donors from South West France. J Med Virol 80: 289-293.

Marion-Audibert A M, Tesse S, Graillot E, Phelip G, Radenne S, Duperret S, Durieux M, Rode A, Mabrut J Y, Souquet J C, and Nicand E (2010) Lethal acute HEV superinfection on hepatitis B cirrhosis. Gastroenterol. Clin. Biol 34: 334-336.

Matsuura K, Isogawa M, and Tanaka Y (2016) Host genetic variants influencing the clinical course of hepatitis B virus infection. J.Med.Virol. 88: 371-379.

Mbarek H, Ochi H, Urabe Y, Kumar V, Kubo M, Hosono N, Takahashi A, Kamatani Y, Miki D, Abe H, Tsunoda T, Kamatani N, Chayama K, Nakamura Y, and Matsuda K (2011) A genome-wide association study of chronic hepatitis B identified novel risk locus in a Japanese population. Hum.Mol.Genet. 20: 3884-3892.

McMahon B J (2009) The natural history of chronic hepatitis B virus infection. Hepatology 49: S45-S55.

Meier A, Mehrle S, Weiss T S, Mier W, and Urban S (2013) Myristoylated PreS1-domain of the hepatitis B virus L-protein mediates specific binding to differentiated hepatocytes. Hepatology 58: 31-42.

Meng X J (2010) Hepatitis E virus: animal reservoirs and zoonotic risk. Vet Microbiol 140: 256-265.

Mikeska T, and Craig J M (2014) DNA methylation biomarkers: cancer and beyond. Genes (Basel) 5: 821-864.

Monga R, Garg S, Tyagi P, and Kumar N (2004) Superimposed acute hepatitis E infection in patients with chronic liver disease. Indian J Gastroenterol 23: 50-52.

Moribe T, Iizuka N, Miura T, Kimura N, Tamatsukuri S, Ishitsuka H, Hamamoto Y, Sakamoto K, Tamesa T, and Oka M (2009) Methylation of multiple genes as molecular markers for diagnosis of a small, well-differentiated hepatocellular carcinoma. Int.J.Cancer 125: 388-397.

Nair V P, Anang S, Subramani C, Madhvi A, Bakshi K, Srivastava A, Shalimar, Nayak B, Ranjith Kumar C T, and Surjit M (2016) Endoplasmic Reticulum Stress Induced Synthesis of a Novel Viral Factor Mediates Efficient Replication of Genotype-1 Hepatitis E Virus. PLoS Pathog 12: e1005521.

Navaneethan U, Al Mohajer M, and Shata M T (2008) Hepatitis E and pregnancy: understanding the pathogenesis. Liver Int 28: 1190-1199.

Nguyen V T (2012) Hepatitis B infection in Vietnam: current issues and future challenges. Asia Pac.J.Public Health 24: 361-373.

Nishida N, Sawai H, Kashiwase K, Minami M, Sugiyama M, Seto W K, Yuen M F, Posuwan N, Poovorawan Y, Ahn S H, Han K H, Matsuura K, Tanaka Y, Kurosaki M, Asahina Y, Izumi N, Kang J H, Hige S, Ide T, Yamamoto K, Sakaida I, Murawaki Y, Itoh Y, Tamori A, Orito E, Hiasa Y, Honda M, Kaneko S, Mita E, Suzuki K, Hino K, Tanaka E, Mochida S, Watanabe M, Eguchi Y, Masaki N, Murata K, Korenaga M, Mawatari Y, Ohashi J, Kawashima M, Tokunaga K, and Mizokami M (2014) New susceptibility and resistance HLA-DP alleles to HBV-related diseases identified by a trans-ethnic association study in Asia. PLoS.One. 9: e86449.

Nishida N, Sawai H, Matsuura K, Sugiyama M, Ahn S H, Park J Y, Hige S, Kang J H, Suzuki K, Kurosaki M, Asahina Y, Mochida S, Watanabe M, Tanaka E, Honda M, Kaneko S, Orito E, Itoh Y, Mita E, Tamori A, Murawaki Y, Hiasa Y, Sakaida I, Korenaga M, Hino K, Ide T, Kawashima M, Mawatari Y, Sageshima M, Ogasawara Y, Koike A, Izumi N, Han K H, Tanaka Y, Tokunaga K, and Mizokami M (2012) Genome-wide association study confirming association of HLA-DP with protection against chronic hepatitis B and viral clearance in Japanese and Korean. PLoS.One. 7: e39175.

Niwa Y, Kanda H, Shikauchi Y, Saiura A, Matsubara K, Kitagawa T, Yamamoto J, Kubo T, and Yoshikawa H (2005) Methylation silencing of SOCS-3 promotes cell growth and migration by enhancing JAK/STAT and FAK signalings in human hepatocellular carcinoma. Oncogene 24: 6406-6417.

Okumura A, Lu G, Pitha-Rowe I, and Pitha P M (2006) Innate antiviral response targets HIV-1 release by the induction of ubiquitin-like protein ISG15. Proc. Natl. Acad. Sci. U. S. A 103: 1440-1445.

Okumura A, Pitha P M, and Harty R N (2008) ISG15 inhibits Ebola VP40 VLP budding in an L-domain-dependent manner by blocking Nedd4 ligase activity. Proc. Natl. Acad. Sci. U. S. A 105: 3974-3979.

Oliviero B, Cerino A, Varchetta S, Paudice E, Pai S, Ludovisi S, Zaramella M, Michelone G, Pugnale P, Negro F, Barnaba V, and Mondelli M U (2011) Enhanced B-cell differentiation and reduced proliferative capacity in chronic hepatitis C and chronic hepatitis B virus infections. J.Hepatol. 55: 53-60.

Ott J J, Stevens G A, Groeger J, and Wiersma S T (2012) Global epidemiology of hepatitis B virus infection: new estimates of age-specific HBsAg seroprevalence and endemicity. Vaccine 30: 2212-2219.

Ozen C, Yildiz G, Dagcan A T, Cevik D, Ors A, Keles U, Topel H, and Ozturk M (2013) Genetics and epigenetics of liver cancer. N.Biotechnol. 30: 381-384.

Parvez M K, and Al-Dosari M S (2015) Evidence of MAPK-JNK1/2 activation by hepatitis E virus ORF3 protein in cultured hepatoma cells. Cytotechnology 67: 545-550.

Peng G, Li S, Wu W, Sun Z, Chen Y, and Chen Z (2008) Circulating CD4+ CD25+ regulatory T cells correlate with chronic hepatitis B infection. Immunology 123: 57-65.

Persico M, Capasso M, Persico E, Svelto M, Russo R, Spano D, Croce L, La M, V, Moschella F, Masutti F, Torella R, Tiribelli C, and Iolascon A (2007) Suppressor of cytokine signaling 3 (SOCS3) expression and hepatitis C virus-related chronic hepatitis: Insulin resistance and response to antiviral therapy. Hepatology 46: 1009-1015.

Persico M, Capasso M, Russo R, Persico E, Croce L, Tiribelli C, and Iolascon A (2008) Elevated expression and polymorphisms of SOCS3 influence patient response to antiviral therapy in chronic hepatitis C. Gut 57: 507-515.

Petersen J, Dandri M, Mier W, Lutgehetmann M, Volz T, von W F, Haberkorn U, Fischer L, Pollok J M, Erbes B, Seitz S, and Urban S (2008) Prevention of hepatitis B virus infection in vivo by entry inhibitors derived from the large envelope protein. Nat.Biotechnol. 26: 335-341.

Petrovic T, Lupulovic D, Jimenez de O N, Vojvodic S, Blazquez A B, Escribano-Romero E, Martin-Acebes M A, Potkonjak A, Milosevic V, Lazic S, and Saiz J C (2014) Prevalence of hepatitis E virus (HEV) antibodies in Serbian blood donors. J Infect. Dev. Ctries 8: 1322-1327.

Phillips S, Chokshi S, Riva A, Evans A, Williams R, and Naoumov N V (2010) CD8(+) T cell control of hepatitis B virus replication: direct comparison between cytolytic and noncytolytic functions. J.Immunol. 184: 287-295.

Pischke S, Hardtke S, Bode U, Birkner S, Chatzikyrkou C, Kauffmann W, Bara C L, Gottlieb J, Wenzel J, Manns M P, and Wedemeyer H (2013) Ribavirin treatment of acute and chronic hepatitis E: a single-centre experience. Liver Int 33: 722-726.

Pischke S, Stiefel P, Franz B, Bremer B, Suneetha P V, Heim A, Ganzenmueller T, Schlue J, Horn-Wichmann R, Raupach R, Darnedde M, Scheibner Y, Taubert R, Haverich A, Manns M P, Wedemeyer H, and Bara C L (2012) Chronic hepatitis e in heart transplant recipients. Am J Transplant 12: 3128-3133.

Pittaras T, Valsami S, Mavrouli M, Kapsimali V, Tsakris A, and Politou M (2014) Seroprevalence of hepatitis E virus in blood donors in Greece. Vox Sang.

Platanias L C (2005) Mechanisms of type-I- and type-II-interferon-mediated signalling. Nat.Rev.Immunol. 5: 375-386.

Qiu X, Hong Y, Yang D, Xia M, Zhu H, Li Q, Xie H, Wu Q, Liu C, and Zuo C (2015) ISG15 as a novel prognostic biomarker for hepatitis B virus-related hepatocellular carcinoma. Int. J. Clin. Exp. Med 8: 17140-17150.

Rajbhandari R, Jun T, Khalili H, Chung R T, and Ananthakrishnan A N (2016) HBV/HIV coinfection is associated with poorer outcomes in hospitalized patients with HBV or HIV. J Viral Hepat 23: 820-829.

Rakyan V K, Down T A, Balding D J, and Beck S (2011) Epigenome-wide association studies for common human diseases. Nat.Rev.Genet. 12: 529-541.

Rehermann B, and Nascimbeni M (2005) Immunology of hepatitis B virus and hepatitis C virus infection. Nat.Rev.Immunol. 5: 215-229.

Ren F, Zhao C, Wang L, Wang Z, Gong X, Song M, Zhuang H, Huang Y, Shan H, Wang J, Liu Q, Ness P, Nelson K E, and Wang Y (2013) Hepatitis E virus seroprevalence and molecular study among blood donors in China. Transfusion.

Ren F, Zhao C, Wang L, Wang Z, Gong X, Song M, Zhuang H, Huang Y, Shan H, Wang J, Liu Q, Ness P, Nelson K E, and Wang Y (2014) Hepatitis E virus seroprevalence and molecular study among blood donors in China. Transfusion 54: 910-917.

Robek M D, Boyd B S, Wieland S F, and Chisari F V (2004) Signal transduction pathways that inhibit hepatitis B virus replication. Proc. Natl. Acad. Sci. U. S. A 101: 1743-1747.

S.Mauss, T.Berg, J.Rockstroh, C.Sarrazin, and H.Wedemeyer (2015). Hepatitis B. In Hepatology - A Clinical Textbook (Flying), pp. 36-49.

Sadler A J, and Williams B R (2008) Interferon-inducible antiviral effectors. Nat. Rev. Immunol 8: 559-568.

Sarin S K, Kumar M, Lau G K, Abbas Z, Chan H L, Chen C J, Chen D S, Chen H L, Chen P J, Chien R N, Dokmeci A K, Gane E, Hou J L, Jafri W, Jia J, Kim J H, Lai C L, Lee H C, Lim S G, Liu C J, Locarnini S, Al M M, Mohamed R, Omata M, Park J, Piratvisuth T, Sharma B C, Sollano J, Wang F S, Wei L, Yuen M F, Zheng S S, and Kao J H (2016) Asian-Pacific clinical practice guidelines on the management of hepatitis B: a 2015 update. Hepatol.Int. 10: 1-98.

Satake H, Tamura K, Furihata M, Anchi T, Sakoda H, Kawada C, Iiyama T, Ashida S, and Shuin T (2010) The ubiquitin-like molecule interferon-stimulated gene 15 is overexpressed in human prostate cancer. Oncol. Rep 23: 11-16.

Scagnolari C, Monteleone K, Selvaggi C, Pierangeli A, D'Ettorre G, Mezzaroma I, Turriziani O, Gentile M, Vullo V, and Antonelli G (2016) ISG15 expression correlates with HIV-1 viral load and with factors regulating T cell response. Immunobiology 221: 282-290.

Schaefer S (2007) Hepatitis B virus taxonomy and hepatitis B virus genotypes. World J.Gastroenterol. 13: 14-21.

Schuch A, Hoh A, and Thimme R (2014) The role of natural killer cells and CD8(+) T cells in hepatitis B virus infection. Front Immunol. 5: 258.

Schultz U, Grgacic E, and Nassal M (2004) Duck hepatitis B virus: an invaluable model system for HBV infection. Adv.Virus Res. 63: 1-70.

Schulze A, Gripon P, and Urban S (2007) Hepatitis B virus infection initiates with a large surface protein-dependent binding to heparan sulfate proteoglycans. Hepatology 46: 1759-1768.

Schweitzer A, Horn J, Mikolajczyk R T, Krause G, and Ott J J (2015) Estimations of worldwide prevalence of chronic hepatitis B virus infection: a systematic review of data published between 1965 and 2013. Lancet 386: 1546-1555.

Scobie L, and Dalton H R (2013) Hepatitis E: source and route of infection, clinical manifestations and new developments. J Viral Hepat 20: 1-11.

Shen J, Wang S, Zhang Y J, Kappil M, Wu H C, Kibriya M G, Wang Q, Jasmine F, Ahsan H, Lee P H, Yu M W, Chen C J, and Santella R M (2012) Genome-wide DNA methylation profiles in hepatocellular carcinoma. Hepatology 55: 1799-1808.

Sherlock S (1970) Progress report: hepatitis-associated (Australia) antigen. Gut 11: 185-188.

Shuai Z, Leung M W, He X, Zhang W, Yang G, Leung P S, and Eric G M (2016) Adaptive immunity in the liver. Cell Mol.Immunol. 13: 354-368.

Skaug B, and Chen Z J (2010) Emerging role of ISG15 in antiviral immunity. Cell 143: 187-190.

Smith D B, Simmonds P, International Committee on Taxonomy of Viruses Hepeviridae Study G, Jameel S, Emerson S U, Harrison T J, Meng X J, Okamoto H, Van der Poel W H, and Purdy M A (2014) Consensus proposals for classification of the family Hepeviridae. J Gen Virol 95: 2223-2232.

Smith D B, Simmonds P, Izopet J, Oliveira-Filho E F, Ulrich R G, Johne R, Koenig M, Jameel S, Harrison T J, Meng X J, Okamoto H, Van der Poel W H, and Purdy M

A (2016) Proposed reference sequences for hepatitis E virus subtypes. J Gen Virol 97: 537-542.

Sprengers D, van der Molen R G, Kusters J G, De Man R A, Niesters H G, Schalm S W, and Janssen H L (2006) Analysis of intrahepatic HBV-specific cytotoxic T-cells during and after acute HBV infection in humans. J.Hepatol. 45: 182-189.

Sridhar S, Teng J L L, Chiu T H, Lau S K P, and Woo P C Y (2017) Hepatitis E Virus Genotypes and Evolution: Emergence of Camel Hepatitis E Variants. Int J Mol Sci 18.

Stoop J N, van der Molen R G, Baan C C, van der Laan L J, Kuipers E J, Kusters J G, and Janssen H L (2005) Regulatory T cells contribute to the impaired immune response in patients with chronic hepatitis B virus infection. Hepatology 41: 771-778.

Stroffolini T, Gaeta G B, and Mele A (2007) AASLD Practice Guidelines on chronic hepatitis B and HBV infection in Italy. Hepatology 46: 608-609.

Takahashi M, Nishizawa T, Sato H, Sato Y, Jirintai, Nagashima S, and Okamoto H (2011) Analysis of the full-length genome of a hepatitis E virus isolate obtained from a wild boar in Japan that is classifiable into a novel genotype. J Gen Virol 92: 902-908.

Terrault N A, Bzowej N H, Chang K M, Hwang J P, Jonas M M, and Murad M H (2016) AASLD guidelines for treatment of chronic hepatitis B. Hepatology 63: 261-283.

Teshale E H, Howard C M, Grytdal S P, Handzel T R, Barry V, Kamili S, Drobeniuc J, Okware S, Downing R, Tappero J W, Bakamutumaho B, Teo C G, Ward J W, Holmberg S D, and Hu D J (2010) Hepatitis E epidemic, Uganda. Emerg Infect Dis 16: 126-129.

Thimme R, Wieland S, Steiger C, Ghrayeb J, Reimann K A, Purcell R H, and Chisari F V (2003) CD8(+) T cells mediate viral clearance and disease pathogenesis during acute hepatitis B virus infection. J.Virol. 77: 68-76.

Thursz M, Yee L, and Khakoo S (2011) Understanding the host genetics of chronic hepatitis B and C. Semin.Liver Dis. 31: 115-127.

Tischoff I, Hengge U R, Vieth M, Ell C, Stolte M, Weber A, Schmidt W E, and Tannapfel A (2007) Methylation of SOCS-3 and SOCS-1 in the carcinogenesis of Barrett's adenocarcinoma. Gut 56: 1047-1053.

Tong H, Bock C T, and Velavan T P (2014a) Genetic insights on host and hepatitis B virus in liver diseases. Mutat. Res. Rev. Mutat. Res 762: 65-75.

Tong H, Bock C T, and Velavan T P (2014b) Genetic insights on host and hepatitis B virus in liver diseases. Mutat.Res.Rev.Mutat.Res. 762: 65-75.

Tong S, and Revill P (2016) Overview of hepatitis B viral replication and genetic variability. J.Hepatol. 64: S4-S16.

Trepo C, Chan H L, and Lok A (2014) Hepatitis B virus infection. Lancet 384: 2053-2063.

Um T H, Kim H, Oh B K, Kim M S, Kim K S, Jung G, and Park Y N (2011) Aberrant CpG island hypermethylation in dysplastic nodules and early HCC of hepatitis B virus-related human multistep hepatocarcinogenesis. J.Hepatol. 54: 939-947.

van Tong H, Hoan N X, Wang B, Wedemeyer H, Bock C T, and Velavan T P (2016a) Hepatitis E Virus Mutations: Functional and Clinical Relevance. EBioMedicine 11: 31-42.

van Tong H, Hoan N X, Wang B, Wedemeyer H, Bock C T, and Velavan T P (2016b) Hepatitis E Virus Mutations: Functional and Clinical Relevance. EBioMedicine.

Vidal F, Lopez-Dupla M, Laguno M, Veloso S, Mallolas J, Murillas J, Cifuentes C, Gallart L, Auguet T, Samperiz G, Payeras A, Hernandez P, Arnedo M, Gatell J M, and Richart C (2012) Pharmacogenetics of efficacy and safety of HCV treatment in HCV-HIV coinfected patients: significant associations with IL28B and SOCS3 gene variants. PLoS. One 7: e47725.

Villeneuve J P (2005) The natural history of chronic hepatitis B virus infection. J.Clin.Virol. 34 Suppl 1: S139-S142.

Vollenweider R, and Lennert K (1983) Plasmacytoid T-cell clusters in non-specific lymphadenitis. Virchows Arch B Cell Pathol Incl Mol Pathol 44: 1-14.

Wake M S, and Watson C J (2015) STAT3 the oncogene - still eluding therapy? FEBS J. 282: 2600-2611.

Watashi K, Urban S, Li W, and Wakita T (2014) NTCP and beyond: opening the door to unveil hepatitis B virus entry. Int.J.Mol.Sci. 15: 2892-2905.

Webster G J, Reignat S, Maini M K, Whalley S A, Ogg G S, King A, Brown D, Amlot P L, Williams R, Vergani D, Dusheiko G M, and Bertoletti A (2000) Incubation phase of acute hepatitis B in man: dynamic of cellular immune mechanisms. Hepatology 32: 1117-1124.

Wedemeyer H, and Manns M P (2010) Epidemiology, pathogenesis and management of hepatitis D: update and challenges ahead. Nat Rev Gastroenterol Hepatol 7: 31-40.

Wedemeyer H, Pischke S, and Manns M P (2012) Pathogenesis and treatment of hepatitis e virus infection. Gastroenterology 142: 1388-1397 e1381.

Wenzel J J, Preiss J, Schemmerer M, Huber B, Plentz A, and Jilg W (2011) Detection of hepatitis E virus (HEV) from porcine livers in Southeastern Germany and high sequence homology to human HEV isolates. J Clin Virol 52: 50-54.

Werneke S W, Schilte C, Rohatgi A, Monte K J, Michault A, Arenzana-Seisdedos F, Vanlandingham D L, Higgs S, Fontanet A, Albert M L, and Lenschow D J (2011) ISG15 is critical in the control of Chikungunya virus infection independent of UbE1L mediated conjugation. PLoS. Pathog 7: e1002322.

WHO (2001). Introduction of hepatitis B vaccine into childhood immunization services: management guidelines, including information for health works and parents. (Geneva).

WHO (2015). Hepatitis B.

WHO (2016). Hepatitis E: Fact sheet.

WHO (2017). Global hepatitis report, 2017.

Woo P C, Lau S K, Teng J L, Cao K Y, Wernery U, Schountz T, Chiu T H, Tsang A K, Wong P C, Wong E Y, and Yuen K Y (2016) New Hepatitis E Virus Genotype in Bactrian Camels, Xinjiang, China, 2013. Emerg Infect Dis 22: 2219-2221.

Wood L M, Pan Z K, Seavey M M, Muthukumaran G, and Paterson Y (2012) The ubiquitin-like protein, ISG15, is a novel tumor-associated antigen for cancer immunotherapy. Cancer Immunol. Immunother 61: 689-700.

Wu C H, Ho C M, Tsai J H, Sun H Y, Hu R H, and Lee P H (2017) First Case Genotype 4 Hepatitis E Infection After a Liver Transplant. Exp Clin Transplant 15: 228-230.

Wu T, Huang S J, Zhu F C, Zhang X F, Ai X, Yan Q, Wang Z Z, Yang C L, Jiang H M, Liu X H, Guo M, Du H L, Ng M H, Zhang J, and Xia N S (2013) Immunogenicity and safety of hepatitis E vaccine in healthy hepatitis B surface antigen positive adults. Hum. Vaccin. Immunother 9: 2474-2479.

Xu D, Fu J, Jin L, Zhang H, Zhou C, Zou Z, Zhao J M, Zhang B, Shi M, Ding X, Tang Z, Fu Y X, and Wang F S (2006) Circulating and liver resident CD4+CD25+ regulatory T cells actively influence the antiviral immune response and disease progression in patients with hepatitis B. J.Immunol. 177: 739-747.

Xu X, Shang Q, Chen X, Nie W, Zou Z, Huang A, Meng M, Jin L, Xu R, Zhang J Y, Fu J, Wang L, Tang Z, Xie Y, Yang X, Zhang Z, and Wang F S (2015) Reversal of

B-cell hyperactivation and functional impairment is associated with HBsAg seroconversion in chronic hepatitis B patients. Cell Mol.Immunol. 12: 309-316.

Yan H, Zhong G, Xu G, He W, Jing Z, Gao Z, Huang Y, Qi Y, Peng B, Wang H, Fu L, Song M, Chen P, Gao W, Ren B, Sun Y, Cai T, Feng X, Sui J, and Li W (2012) Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. Elife. 1: e00049.

Yan H, Zhong G, Xu G, He W, Jing Z, Gao Z, Huang Y, Qi Y, Peng B, Wang H, Fu L, Song M, Chen P, Gao W, Ren B, Sun Y, Cai T, Feng X, Sui J, and Li W (2014) Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. Elife. 3.

Yang P L, Althage A, Chung J, Maier H, Wieland S, Isogawa M, and Chisari F V (2010) Immune effectors required for hepatitis B virus clearance. Proc.Natl.Acad.Sci.U.S.A 107: 798-802.

Yang Y, Zheng B, Han Q, Zhang C, Tian Z, and Zhang J (2016) Targeting blockage of STAT3 inhibits hepatitis B virus-related hepatocellular carcinoma. Cancer Biol.Ther. 17: 449-456.

Yasukawa H, Ohishi M, Mori H, Murakami M, Chinen T, Aki D, Hanada T, Takeda K, Akira S, Hoshijima M, Hirano T, Chien K R, and Yoshimura A (2003) IL-6 induces an anti-inflammatory response in the absence of SOCS3 in macrophages. Nat.Immunol. 4: 551-556.

Yong W S, Hsu F M, and Chen P Y (2016) Profiling genome-wide DNA methylation. Epigenetics.Chromatin. 9: 26.

Yoshimura A, Naka T, and Kubo M (2007a) SOCS proteins, cytokine signalling and immune regulation. Nat.Rev.Immunol. 7: 454-465.

Yoshimura A, Naka T, and Kubo M (2007b) SOCS proteins, cytokine signalling and immune regulation. Nat. Rev. Immunol 7: 454-465.

Yoshimura A, Suzuki M, Sakaguchi R, Hanada T, and Yasukawa H (2012) SOCS, Inflammation, and Autoimmunity. Front Immunol 3: 20.

Yu H, Kortylewski M, and Pardoll D (2007) Crosstalk between cancer and immune cells: role of STAT3 in the tumour microenvironment. Nat.Rev.Immunol. 7: 41-51.

Yu H, Pardoll D, and Jove R (2009) STATs in cancer inflammation and immunity: a leading role for STAT3. Nat.Rev.Cancer 9: 798-809.

Zacharakis G, Koskinas J, Kotsiou S, Tzara F, Vafeiadis N, Papoutselis M, Maltezos E, Sivridis E, and Papoutselis K (2008) The role of serial measurement of serum

HBV DNA levels in patients with chronic HBeAg(-) hepatitis B infection: association with liver disease progression. A prospective cohort study. J. Hepatol 49: 884-891.

Zhang J, Shih J W, and Xia N S (2015a) Long-term efficacy of a hepatitis E vaccine. N Engl J Med 372: 2265-2266.

Zhang X, You Q, Zhang X, and Chen X (2015b) SOCS3 Methylation Predicts a Poor Prognosis in HBV Infection-Related Hepatocellular Carcinoma. Int. J. Mol. Sci 16: 22662-22675.

Zhao Y, Xue F, Sun J, Guo S, Zhang H, Qiu B, Geng J, Gu J, Zhou X, Wang W, Zhang Z, Tang N, He Y, Yu J, and Xia Q (2014) Genome-wide methylation profiling of the different stages of hepatitis B virus-related hepatocellular carcinoma development in plasma cell-free DNA reveals potential biomarkers for early detection and high-risk monitoring of hepatocellular carcinoma. Clin.Epigenetics. 6: 30.

Zheng Y Y, Wang L F, Fan X H, Wu C H, Huo N, Lu H Y, Xu X Y, and Wei L (2013) Association of suppressor of cytokine signalling 3 polymorphisms with insulin resistance in patients with chronic hepatitis C. J. Viral Hepat 20: 273-280.

Zhong S, Tang M W, Yeo W, Liu C, Lo Y M, and Johnson P J (2002) Silencing of GSTP1 gene by CpG island DNA hypermethylation in HBV-associated hepatocellular carcinomas. Clin.Cancer Res. 8: 1087-1092.

7. DECLARATION OF CONTRIBUTIONS

We hereby declare that the doctoral dissertation entitled "**Hepatitis E virus superinfection and human *SOCS3* and *ISG15* in hepatitis B-related liver diseases**" submitted to the members of the PhD Board at the Faculty of Medicine, University of Tuebingen is a record of an original work done by Dr. Xuan Hoan Nghiem and co-authors at the Institute of Tropical Medicine, University of Tuebingen.

Three publications (Publication 1: EBioMedicine. 2015 Nov 11;2(12):2080-6. PMID: 26844288; Publication 2: Oncotarget. 2017 Mar 7;8(10):17127-17139. PMID: 28179578; Publication 3: Oncotarget. 2016 Oct 18;7(42):67777-67787. PMID: 27626177) accomplished by Dr. Xuan Hoan Nghiem as the first author lay as the backbone of his doctoral dissertation. We declare that Dr. Xuan Hoan Nghiem have substantially contributed to all three manuscripts with respect to study design, sampling procedures, patient's recruitment, experimental design, data analyses and writing of the manuscript. We also state individually the contribution of of all the co-authors in each study as following,

Contributions of PhD candidate and other co-authors

Publication 1: Nghiem Xuan Hoan, Hoang Van Tong, Nicole Hecht, Bui Tien Sy, and Patrick Marcinek contributed to performing of the experiments. Le Huu Song, Nguyen Linh Toan, Peter G Kremsner, C- Thomas Bock and Thirumalaisamy P Velavan contributed to materials and reagents. C- Thomas Bock and Thirumalaisamy P Velavan designed the study. Nghiem Xuan Hoan, Le Huu Song and Nguyen Linh Toan recruited patients and collected samples. Nghiem Xuan Hoan and Hoang Van Tong performed statistical analyses. Nghiem Xuan Hoan, Hoang Van Tong, C- Thomas Bock and Thirumalaisamy P Velavan wrote the manuscript. Christian G Meyer and Jens Kurreck contributed to the revision.

Publication 2: Nghiem Xuan Hoan, Hoang Van Tong, C- Thomas Bock, Heiner Wedemeyer and Thirumalaisamy P Velavan designed the study and wrote the

manuscript. Nghiem Xuan Hoan, Hoang Van Tong, and Dao Phuong Giang performed the experiments. Nghiem Xuan Hoan and Hoang Van Tong performed the statistical analyses and interpreted data. Nghiem Xuan Hoan, Le Huu Song and Nguyen Linh Toan, Bui Khac Cuong recruited patients and collected samples. Peter G Kremsner and Thirumalaisamy P Velavan contributed to materials and reagents.

Publication 3: Nghiem Xuan Hoan, Hoang Van Tong, C- Thomas Bock, and Thirumalaisamy P Velavan designed the study. Nghiem Xuan Hoan, Hoang Van Tong and Dao Phuong Giang performed the experiments. Nghiem Xuan Hoan, Hoang Van Tong, Christian G Meyer and Thirumalaisamy P Velavan performed the statistical analyses and interpreted the data. Nghiem Xuan Hoan, Dao Phuong Giang, Le Huu Song and Nguyen Linh Toan are involved in patient recruitment. C-Thomas Bock, Peter G Kremsner and Thirumalaisamy P Velavan contributed to study materials and consumables. Nghiem Xuan Hoan, Hoang Van Tong, Christian G Meyer and Thirumalaisamy P Velavan wrote the manuscript.

Sincerely,

Tuebingen 26.09.2017

PhD candidate. Xuan Hoan Nghiem

Professor, Dr. Peter G. Kremsner
Director of Institute of Tropical Medicine
Universitätsklinikum Tübingen

PD Dr. Thirumalaisamy P Velavan

8. ACKNOWLEDGEMENTS

First and foremost, I would like to thank Prof. Peter G. Kremsner for giving me an opportunity to work at the Institute of Tropical Medicine, University of Tübingen (ITM-UKT). I am heartily thankful to PD. Dr. Thirumalaisamy P Velavan. He encouraged me to improve my scientific skills and helped me to deal with all my research problems. I really appreciate you for your continuous support. Thank you Prof. Dr. Le Huu Song and Prof. Dr. Nguyen Linh Toan for recommending me to start my doctoral thesis in Tübingen and I am grateful for all your support. A very special thanks goes to the doctoral committee members, Prof. Dr. C. Thomas Bock and Prof. Dr. Ruben R. Plentz for your valuable time and guidance. A special thanks goes to Prof. Dr. Christian Meyer for his constructive criticisms on my manuscripts. My sincere thanks go to my colleague Dr. Hoang Van Tong for his valuable suggestions, practical advises, and for contributions related to my work. I would like to thank my colleague Dr. Patrick Marcinek for all your helps and support during my initial PhD life. Furthermore, I thank to all colleagues and staffs, working in the ITM-UKT for their assistance offered every time, when needed. It was memorable to work with you during my PhD years. I am highly indebted to my beloved wife Dao Phuong Giang for her constant forbearance, unconditional support and being there for me when needed. My special thanks and all my love go to my dearest family, including my parents, my parents-in-law, my older and younger sisters for their understanding, fortitude, and taking care my little boys: Nghiem Hoang Minh and Nghiem Quang Vinh. I sincerely thank all staffs of the 108 Military Central hospital, Hanoi for your continued support. I sincerely apologize to those, whom I might have missed to acknowledge here due to space constraint.

Xuan Hoan Nghiem

9. CURRICULUM VITAE

Personal and contact Information

Full name:	Xuan Hoan Nghiem
Date of birth:	May 19th, 1983
Place of birth:	Hanoi, Vietnam
Gender:	Male
Nationality:	Vietnam
Marital status:	Married and two children
Language:	English (working knowledge), Vietnamese (native)
Address:	Eberhard-Karls University, Institute of Tropical Medicine, Wilhelmstr. 27 D- 72074 Tübingen, Germany
Tel:	0049-7071 2982197 (Office)
Email:	nghiemxuanhoan@108-icid.com

Education background

2001-2007:	Hanoi Medical University, Vietnam; Medical Doctor
1999-2001:	Phuong Duc School, Hanoi, Vietnam General Certificate of Secondary Education

Working experience

2007 - 12/2013:	Medical Doctor in Institute of clinical infectious diseases in Tran Hung Dao hospital, Hanoi, Vietnam.
03/2014 - 03/2015:	Molecular biology trainee in Institute of Tropical Medicine Eberhard Karls Universität Tübingen, Germany
04/2015 - to date:	PhD student - Institute of Tropical Medicine - Faculty of Medicine Eberhard Karls Universität Tübingen, Germany

Research interests

- Infectious diseases, Viral hepatitis infection, Clinical course, diagnostics, molecular mechanisms, epigenetics, immune responses, host directed therapy.

Presentations in conference proceedings

1. **Nghiem Xuan Hoan**, Hoang Van Tong, Dao Phuong Giang, Nguyen Linh Toan, Le Huu Song, C.-Thomas Bock, Peter G Kremsner, Thirumalaisamy P Velavan. Functional variants and aberrant Methylation in the SOCS3 promoter region influence the progression of HBV-related liver diseases. The International Liver Congress 51th. 13-17th April 2016 in Barcelona, Spain. (Peer-reviewed, poster presentation. Journal of Hepatology).

2. Hoang Van Tong, **Nghiem Xuan Hoan**, Dao Phuong Giang, Nguyen Linh Toan, Le Huu Song, C.-Thomas Bock, Peter G Kremsner, Thirumalaisamy P Velavan. Interferon-stimulated gene 15 in HBV infection and progression of HBV-related liver diseases. The International Liver Congress 51th. 13-17th April 2016 in Barcelona, Spain. (Peer-reviewed, poster presentation. Journal of Hepatology).

3. **Nghiem X. Hoan,** Hoang Van Tong, Nicole Hecht, Bui Tien Sy, Patrick Marcinek, Christian G. Meyer, Le Huu Song, Nguyen Linh Toan, Jens Kurreck, Peter G. Kremsner, C-Thomas Bock, Thirumalaisamy P. Velavan. Hepatitis E Virus Superinfection and Clinical Progression in Hepatitis B Patients. Grad School Day at the Interfaculty Graduate School of Infection Biology and Microbiology (IGIM). 12th February 2016 in Tuebingen, Germany (Oral Presentation).

4. Hoang V. Tong, **Nghiem Xuan Hoan**, Adam Clark, Franziska Gerlach, Nguyen Linh Toan, Le Huu Song, C.-Thomas Bock, Peter G Kremsner, Thirumalaisamy P Velavan. JAK/STAT signaling pathway and susceptibility to hepato]it is B virus infection and related liver diseases. The International Liver Congress 50th. 13-17th April 2016 in Vienna, Austria. (Peer-reviewed, poster presentation. Journal of Hepatology).

5. Hoang V. Tong, Le Huu Song, **Nghiem Xuan Hoan**, Bui Khac Cuong, Bui Tien Sy, Ho Anh Son, Do Quyet, Vu Quoc Binh, Peter G Kremsner, Claus Thomas Bock, Thirumalaisamy P Velavan and Nguyen Linh Toan. Soluble MICB protein levels and platelet counts during hepatitis B virus infection and response to hepatocellular carcinoma treatment. HCC Summit. 13-16th February 2014 in Geneva, Switzeland. (Poster Presentation).

Publications

https://www.ncbi.nlm.nih.gov/pubmed/?term=Hoan+NX

(*Shared first authors)

1. Sy BT*, **Hoan NX***, Tong HV*, Meyer CG, Toan NL, Song LH, Bock CT, Velavan TP. Genetic variants of interferon regulatory factor 5 associated with chronic hepatitis B infection. World J Gastroenterol 2017; In press
2. Amorima LM, Tong HV, **Hoan NX**, Vargas LB, Enilze Maria de Souza Fonseca Ribeiroa, Petzl-Erler LM, Angelica B.W. Boldt, Toan NL, Song LH, Velavan TP, August DG. KIR-HLA distribution in a Vietnamese population from Hanoi. Human Immunology; Accepted 24.11.2017.
3. Adedoja A, **Hoan NX**, van Tong H, Adukpo S, Tijani DB, Akanbi AA 2nd, Meyer CG, Ojurongbe O, Velavan TP. Differential contribution of interleukin-10 promoter variants in malaria and schistosomiasis mono- and co-infections among Nigerian children. Trop Med Int Health. 2017 Nov 13.
4. Nguyen HM, Sy BT, Trung NT, **Hoan NX**, Wedemeyer H, Velavan TP, Bock CT. Prevalence and genotype distribution of hepatitis delta virus among chronic hepatitis B carriers in Central Vietnam. PLoS One. 2017 Apr 12;12(4):e0175304.
5. **Hoan NX**, Van Tong H, Giang DP, Cuong BK, Toan NL, Wedemeyer H, Bock CT, Kremsner PG, Song LH, Velavan TP. SOCS3 promoter variants and hypermethylation influence the progression of HBV-related liver diseases. Oncotarget. 2017 Mar 7;8(10):17127-17139.
6. **Hoan NX,** Khuyen N, Binh MT, Giang DP, Van Tong H, Hoan PQ, Trung NT, Anh DT, Toan NL, Meyer CG, Kremsner PG, Velavan TP, Song LH. Vitamin D deficiency influences the clinical course of Hepatitis B virus - related liver diseases. BMC Infect Dis. 2016 Sep 23;16(1):507.
7. **Hoan NX,** Van Tong H, Giang DP, Toan NL, Meyer CG, Bock CT, Kremsner PG, Song LH, Velavan TP. Interferon-stimulated gene 15 in hepatitis B-related liver diseases. Oncotarget. 2016 Sep 10. doi: 10.18632/oncotarget.11955.
8. Van Tong H, **Hoan NX**, Wang B, Wedemeyer H, Bock CT, Velavan TP. Hepatitis E Virus Mutations: Functional and Clinical Relevance. EBioMedicine. 2016 Sep;11:31-42.
9. **Hoan NX,** Tong HV, Hecht N, Sy BT, Marcinek P, Meyer CG, Song le H, Toan NL, Kurreck J, Kremsner PG, Bock CT, Velavan TP. Hepatitis E Virus Superinfection and Clinical Progression in Hepatitis B Patients. EBioMedicine. 2015 Nov 11;2(12): 2080-6.

10. Tong HV, Song le H, **Hoan NX**, Cuong BK, Sy BT, Son HA, Quyet D, Binh VQ, Kremsner PG, Bock CT, Velavan TP, Toan NL. Soluble MICB protein levels and platelet counts during hepatitis B virus infection and response to hepatocellular carcinoma treatment. BMC Infect Dis. 2015 Jan 23;15:25.
11. Clark A, Gerlach F, Tong HV, **Hoan NX**, Song le H, Toan NL, Bock CT, Kremsner PG, Velavan TP. A trivial role of STAT4 variant in chronic hepatitis B induced hepatocellular carcinoma. Infect Genet Evol. 2013 Aug;18:257-61.