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Molecular epidemiology of hepatitis delta virus and host genetic factors in the progression of HBV related liver diseases in patients with HBV and HDV coinfections

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1. INTRODUCTION

1.1. Hepatitis B virus

1.1.1. Epidemiology

Despite 40 years of the implementation of hepatitis B vaccine (McAleer et al., 1984, Komatsu, 2014), hepatitis B virus (HBV) infection continues to be a global health problem. It accounts for approximately 257 million cases and causes more than 800,000 deaths annually (WHO, 2018a). In addition, poor vaccination coverage (Ott et al., 2012, Zoulim and Durantel, 2015) and population migration (Kowdley et al., 2012, MacLachlan et al., 2013) have led to an increased incidence of HBV in many geographical regions (Figure 1). Large parts of South-East Asia, Sub-Saharan Africa, and distinct regions in Amazon basin report high rates of HBV infection, with HBV prevalence greater than 8%, and are classified as HBV endemic regions (Liaw and Chu, 2009, Ott et al., 2012, Schweitzer et al., 2015). In contrast, among economically developed countries such as the United States, Canada and Australia, low prevalence (<2%) is documented. The prevalence of HBV infections in few East European countries and among Americas range between 2% and 7% (Liaw and Chu, 2009, Lok and McMahon, 2007) with moderate endemicity. Vietnam, a country located in South-East Asia reports on high HBV prevalence, ranging between 10-20% in the population (Nguyen, 2012, WHO, 2018a).

Currently, 10 HBV genotypes have been identified and are distinctly distributed geographically (Tran et al., 2008, Tatematsu et al., 2009, Velkov et al., 2018). They include HBV genotypes A to J. Classification of HBV genotypes is based on nucleotide divergence > 7.5% among HBV isolates. In addition, analogues of a given HBV genotype can further be classified based on sub-genotypes. The classification is

based on HBV nucleotide sequence diversity which varies between 4% and 7.5% (Tatematsu et al., 2009, Norder et al., 2004). The geographical distribution of HBV genotypes differ over regions (Velkov et al., 2018) with HBV genotype A observed commonly in most European countries, North America, Caribbean, and in Madagascar. The HBV genotypes B and C are observed in Asia, the United Kingdom, Denmark, and Australia (Velkov et al., 2018, Liu C-J, 2005, Schaefer, 2007a), genotype D predominant in Africa, parts of Europe, and Northern America (Velkov et al., 2018, Schaefer, 2007a, Sunbul, 2014), genotype E mostly in Sub-Saharan Africa and HBV genotype F in Latin American countries (Arauz-Ruiz et al., 1997, Velkov et al., 2018). HBV genotype G and H were identified in Mexico (Velkov et al., 2018). HBV genotypes I and J were only identified in Japan (Tatematsu et al., 2009).

Figure 1: Global rate of HBV chronicity. Adopted with permission from (MacLachlan and Cowie, 2015)

1.1.2. Transmission and prevention

HBV is transmitted by exposure of the infected blood or blood particles and by body fluids (Lok and McMahon, 2007, WHO, 2018a). Transmission mostly occurs from mother-to-child (vertical transmission) and also by unprotected sex (WHO, 2018a, Lok and McMahon, 2007, Prevention, 2018). Other modes of HBV transmission include transfusion of blood and blood products, unsafe medical practices, tattooing, and also by acupuncture therapy (Lok and McMahon, 2007). Although HBV can be transmitted through breast milk, tears, and urine, there is limited evidence supporting this mode of transmission (Zheng et al., 2011, Prevention, 2018).

HBV transmission control relies on deployment of efficient universal vaccination program and safe blood transfusion practices (Chang, 2006, WHO, 2018a). Vaccination programs contribute significantly towards reduction of HBV transmission. The mammalian cell-derived recombinant vaccine, second-generation HBV vaccine (Meireles et al., 2015), was adopted into immunization programs globally since 1986 (Perez, 2007, Komatsu, 2014). The vaccination regimen entails three to four shots of hepatitis B immunization with an efficacy achieved between 70-80% in at least 20 years (Beasley, 2009, Komatsu, 2014, WHO, 2018a). Combining the hepatitis B vaccine with hepatitis B immunoglobulin booster, the efficacy of hepatitis B vaccine is up to 95%, particularly against perinatal transmission (Komatsu, 2014, Beasley, 2009). WHO recommends this vaccine formulation for neonates within 24 hours after birth (WHO, 2018a).

1.1.3. HBV genome

Human HBV is a member of the *Hepadnaviridae* family (Schaefer, 2007b) and has a genome that is approximately 3200 nucleotides long. The genome consists of a relaxed circular partially double-stranded DNA and surfaced proteins. To date, four open reading frames (ORF), have been identified in the HBV genome, namely the preS/S, Precore/core, X and Pol (Pollicino et al., 2014, Chen, 2018) (Figure 2).

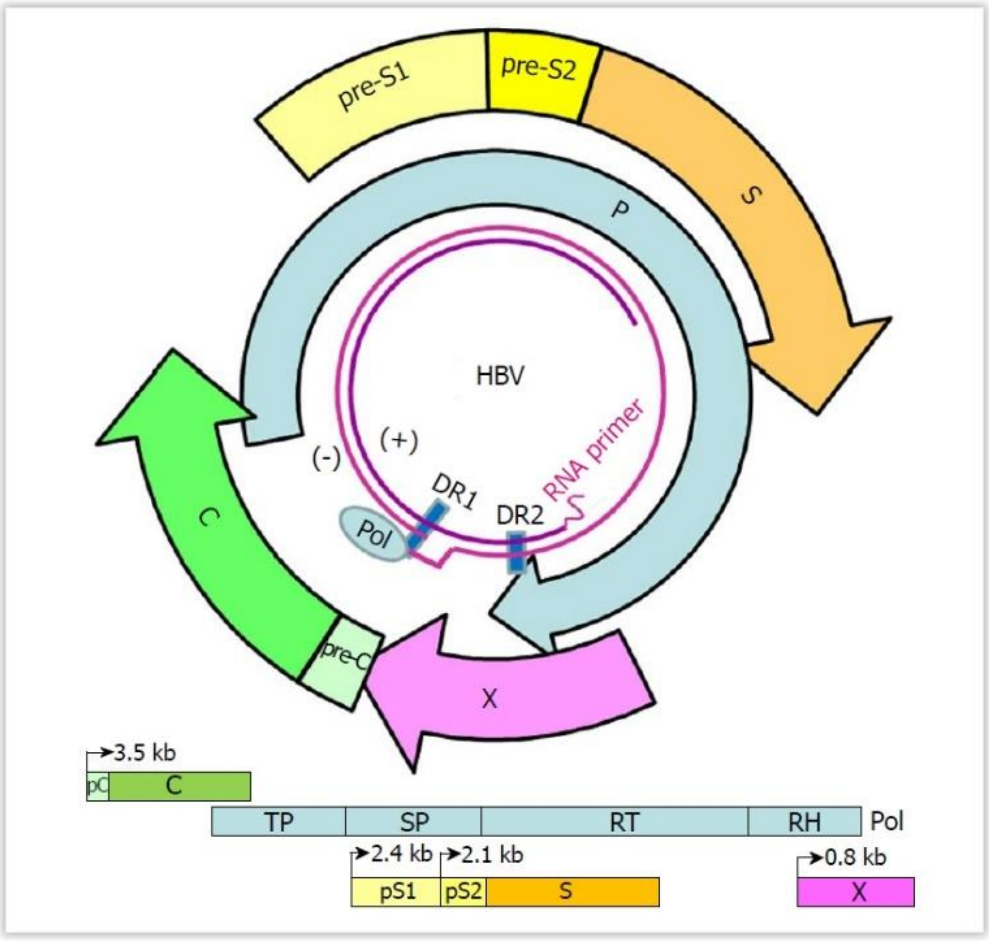

Figure 2: Schematic illustration of the HBV genome. The four main open reading frames (OFRs): preS/S, Precore/core (preC/C), X and Pol are annotated in the structure of the genome. The relaxed-circular DNA genome includes a complete minus strand and a typical incomplete plus strand. The minus (-) and plus (+) DNA strands are marked. Adopted from (Chen, 2018).

The *PreS/S* gene codes for HBV envelope proteins, as called HBV surface proteins (HbsAg). Based on three alternative location, the ORF is translated to three distinct protein domains. These include preS1 (can be either 108 or 119 amino acids long based on the genotype), preS2 (55 amino acids) and S (226 amino acids) (Schadler and Hildt, 2009, Chen, 2018). Depending on the ORF or the combinations ORFs being expressed, three different HBV envelope proteins are synthesized; the independent S domain also known as the small-HBV surface antigen (S-HBsAg) that contain only the S-protein domain, the middle-HBV envelope (M-HBsAg; comprises preS2 and S envelope protein domains), and the large- HBV envelope (L-HBsAg) which comprises the three HBV envelope protein domains (Chen, 2018, Schadler and Hildt, 2009, Pollicino et al., 2014). Of these, current diagnostic tools and HBV vaccines target the surface antigen HBsAg (Meireles et al., 2015, Song and Kim, 2016). The core protein (HBcAg) is encoded by preC/C gene and consist of HBV nucleocapsid with either 183 or 185 amino acids. This protein has two parts: N-terminal containing 149 or 151 amino acid, depending on the genotype, followed by C-terminal containing 34 amino acid. The former promotes the initial self-assembly of capsids while the latter is essential for the packaging of the pregenome/HBVPol complex (Gallina et al., 1989, Zlotnick et al., 1997). The *X* gene is around 0.7 kb that code for the X protein (Hbx). The HBx is vital for the initiation and maintenance of HBV replication. It is also involved in the suppression of the host immune responses, thus preventing HBV clearance. Therefore, HBx is crucial in establishing chronic HBV infections (Zoulim et al., 1994, Keasler et al., 2007). HBx also interacts with various cellular pathways, such as the transforming growth factor beta 1 pathway and Ca^{2+}-dependent signaling pathway (Feitelson et al., 2014), which are associated with hepatocellular carcinoma development. The polymerase (*P*) gene accounts for approximately 80% of the HBV

genome. The *P* gene encodes for a multifunctional polymerases required during HBV replication (Bartenschlager et al., 1990, Fouillot et al., 1993). Due to its crucial role of this gene in the HBV life cycle, these polymerases serve as potential targets in nucleos(t)ide analogue drugs used in HBV treatment (Zhang et al., 2016, Locarnini and Yuen, 2010).

1.1.4. Hepatitis B virus life cycle

The life cycle of HBV (Figure 3) is initiated by the binding of HBV, via the envelope proteins, to the Sodium taurocholate cotransporting polypeptide (NTCP) situated on the surface of hepatocytes (Yan et al., 2012). Sequentially, HBV invades the hepatocytes by endocytosis and subsequently by the fusion of the viral envelope with the hepatic cell membrane. HBV is uncoated in the cytoplasm followed by the translocation of viral genome to the nucleus. Here, the HBV relaxed circular DNA is repaired and subsequently converted into a covalently closed circular DNA (cccDNA). The cccDNA is key for the reactivation of viral replication and the persistence of viral infection (Tang et al., 2018, Zoulim, 2005). As minichromosome (Bock et al., 1994, Bock et al., 2001), the cccDNA transcribes as different viral mRNAs. These viral mRNAs are subsequently exported to the cytoplasm for the synthesis of seven HBV proteins. The largest mRNA, pgRNA, is translated into viral polymerase. In the cytoplasm, the pgRNA, HBV polymerase, and core protein are packaged, forming progeny virus. Then, double-strand HBV DNA is created through reverse transcription catalyzed by RNaseH of the HBV polymerase. The resultant premature capsids are either recycled to the nucleus for further replication or they are assembled with the HBV envelope proteins to form mature viruses. The mature HBV is then released and

subsequently infects other hepatocytes (Tang et al., 2018, Wu et al., 1990, Rehermann and Nascimbeni, 2005).

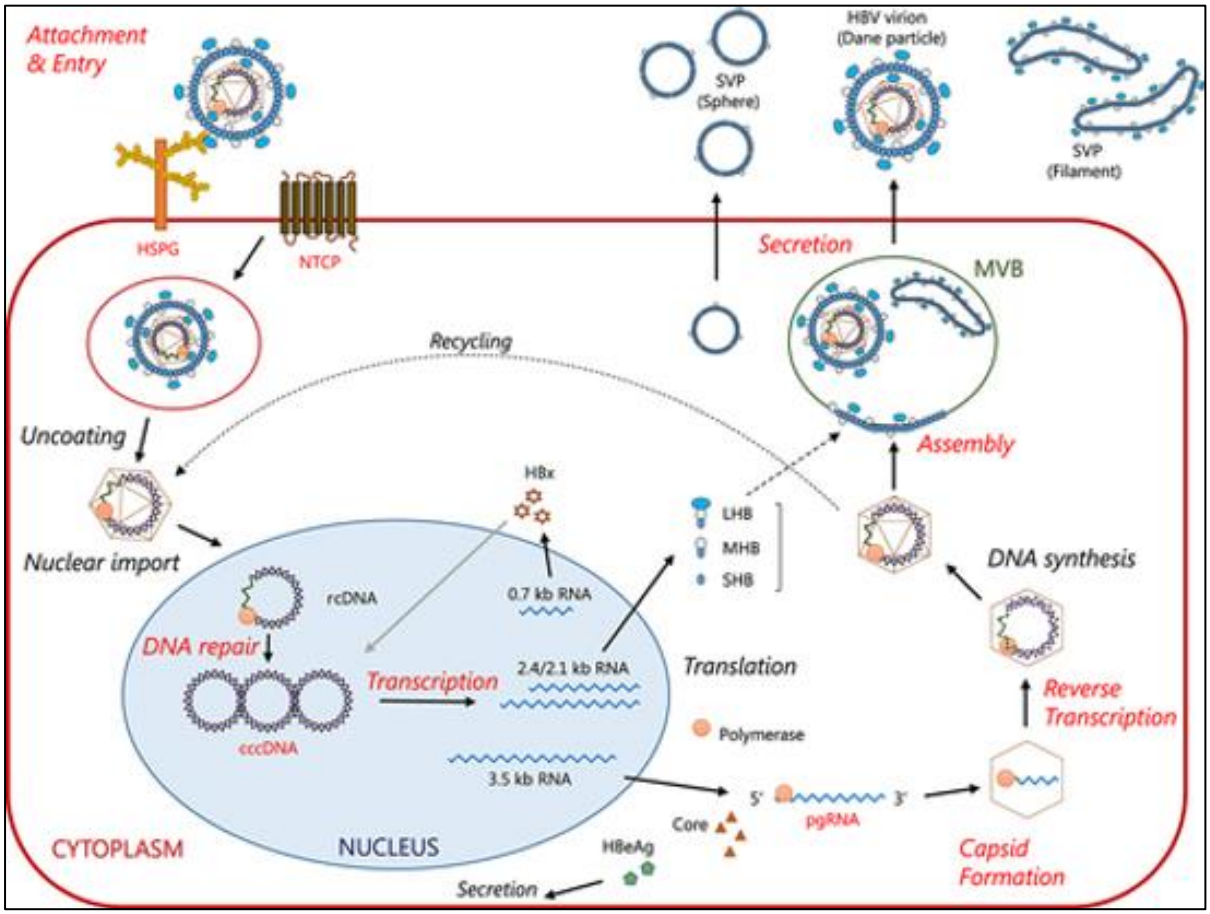

Figure 3: The HBV life cycle. Adopted with permission from (Morikawa et al., 2016)

1.1.5 HBV Clinical progression

The clinical progression of HBV infections has two phase, namely acute and chronic phase (Liang, 2009). The acute phase always occurs when a person experience HBV infection for the first time. After exposure, HBV-infected individuals may develop hepatitis syndrome after the incubation period which takes one to four months. The hepatitis syndrome is characterized by symptoms such as fatigue, anorexia, nausea, jaundice, and right upper quadrant discomfort. In addition, irregular laboratory findings may be observed such as elevated liver enzyme levels, high bilirubin levels and high

viral titers. The acute phase symptoms typically resolve after one to three months. Remarkably, the severity of acute phase varies with age. About 50% of children in this phase develop icteric hepatitis. Less than a third of adult acute infections are symptomatic and <1% of adults develop fulminant hepatitis (Liaw and Chu, 2009, Rehermann and Nascimbeni, 2005, Pan and Zhang, 2005).

Majority of HBV acute phase cases recover completely. The unresolved cases progress to the HBV chronic phase (Rehermann and Nascimbeni, 2005) The quota of HBV chronicity decreases with age (Liaw and Chu, 2009, Tassopoulos et al., 1987). The clinical manifestation of chronic HBV infections can be categorized into three groups. These include chronic hepatitis B (CHB), HBV-related liver cirrhosis (LC), and hepatocellular carcinoma (HCC) (Rehermann and Nascimbeni, 2005). Generally, CHB stage is infectious, mostly occurring early in life and is characterized by four phases: immune tolerance, immune clearance, immune control, and immune escape phases (Tan et al., 2015, MacLachlan and Cowie, 2015). The immune-tolerant phase is defined by high HBV-DNA levels, presence of HBeAg with minimal liver damage with normal alanine aminotransferase (ALT) levels (Wu and Chang, 2015). In the immune clearance phase, individuals are able to control HBV replication showed by HBeAg negativity, but also experience hepatic damage as a result of host immune responses (Wu and Chang, 2015). The HBeAg seroconversion occurs frequently in adults and rarely occurs in infants (Terrault et al., 2016). For the CHB immune control phase, as known as inactive immune phase, low viremia, HBeAg negativity and low inflammation of the liver are observed. In the last phase, immune escape phase, the immune system is reactivated causing variable infectivity and recurrent liver inflammation. The triggering host and viral factors play an important role in the modulation and course of

CHB infections. Consequently, this translates to life-long risk of liver injuries including LC and HCC (Wu and Chang, 2015, Tan et al., 2015, MacLachlan and Cowie, 2015).

1.2. Hepatitis delta virus

1.2.1. Epidemiology

Hepatitis delta virus (HDV) was first identified in 1977 (Rizzetto et al., 1977) and is the leading cause of dual-infections among HBV patients globally (WHO, 2018c, Noureddin and Gish, 2014), as HDV largely depends on HBsAg for their replication. The epidemiology of this virus varies considerably, even within a given geographical area (Figure 4). In Europe, HDV infections are relatively limited because of effective HBV control strategies (Jefferies et al., 2018). Low HDV prevalence is observed in some Asian countries such as Indonesia with <0.5% (Lusida et al., 2003), 5% in Malaysia (Tan et al., 1989), and 4% in S.Korea (S et al., 2017). However, HDV is largely prevalent in HBV-endemic settings. For example, the prevalence of HDV in Pakistan is 35% (Mumtaz et al., 2011) and 45% in Mongolia (Baatarkhuu et al., 2017). HDV positivity is 25% in Gabon (Groc et al., 2019), whereas it ranges from 1.3% to 50% in other sub-Saharan African countries (Andernach et al., 2014).

So far, eight distinct HDV genotypes have been identified and classified as HDV1 to HDV8 (Le Gal et al., 2006, Barros et al., 2011). The distribution of these genotype varies greatly (Le Gal et al., 2006, Wedemeyer and Manns, 2010, Alvarado-Mora et al., 2013). HDV-1 appears worldwide and is commonly found in Europe (Hughes et al., 2011, Wedemeyer and Manns, 2010). Furthermore, HDV-1 is associated with severe and mild cases of liver diseases. HDV-2 is mainly found in East Asia where it causes mild liver failure (Pascarella and Negro, 2011). For HDV-3, it mostly occurs in

South America and has been linked to severe hepatitis epidemics (Alvarado-Mora et al., 2011). HDV-4 occurs mainly in Japan and Taiwan where it is associated with mild liver disease (Lin et al., 2015). The epidemiology of HDV genotypes 5 to 8 is still unclear due to lack of data. Nevertheless, data presently available show that these genotypes occur in originally among distinct ethnicities among African populations (Hughes et al., 2011, Le Gal et al., 2006, Pascarella and Negro, 2011).

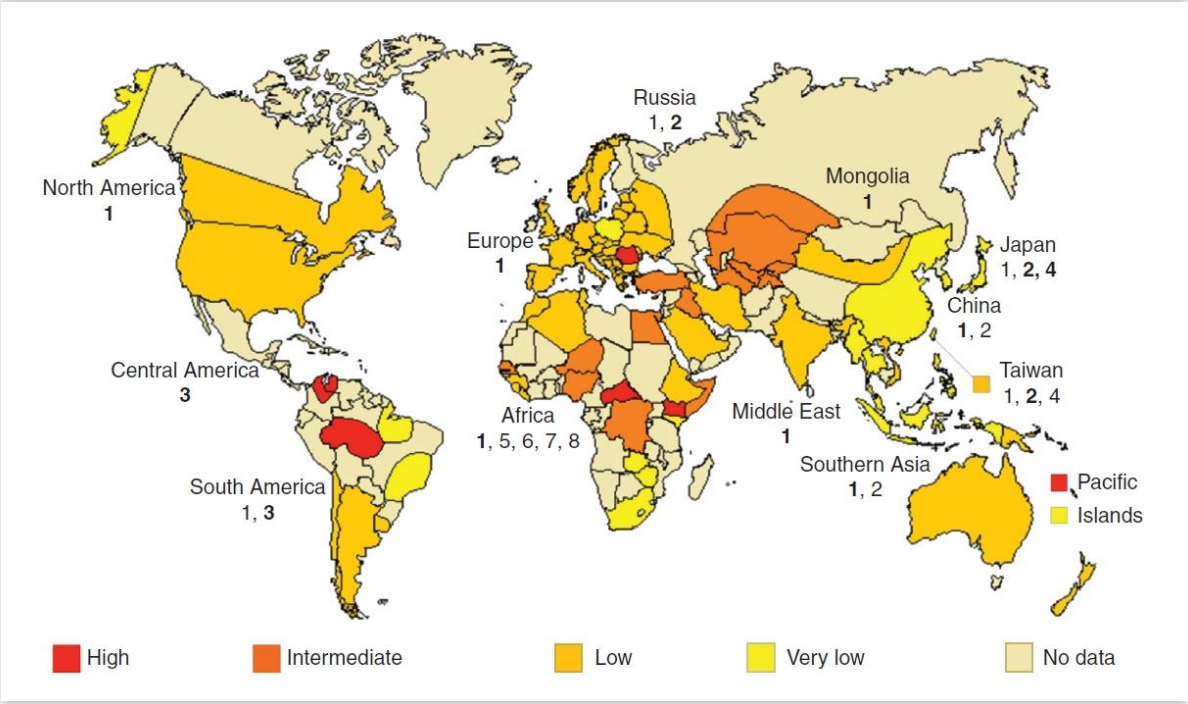

Figure 4: Geographic data of HDV genotypes. Adopted from (Rizzetto, 2015).

There are conflicting reports on the epidemiology HDV infection in Vietnam. This is because that high HBV burden may also relate to high HDV incidence (WHO, 2018b, Nguyen, 2012), low rate of HDV was reported in several regions of Vietnam (Hall et al., 2015). Over 15% HDV-RNA positivity was reported in 2013 among HBV patients from northern Vietnam that recruited in a period spanning over ten years ago (Sy et al., 2013). Another study conducted in central Vietnam in 2015 reported a 10% HDV-RNA positivity among HBV patients (Nguyen et al., 2017). Interestingly, cases of HDV infections in different regions is attributed by distinct HDV genotype distribution. HDV1

is predominant in northern Vietnam (90%) whereas it is infrequently detected (20%) in the central region (Nguyen et al., 2017, Sy et al., 2013).

1.2.2. HDV Transmission and prevention

HDV is transmitted through the exposure to infected human body fluids (WHO, 2018c). A successful transmission can be achieved even with a very low amount of HDV (Ponzetto et al., 1987). As a result, percutaneous contacts can easily transmit HDV. Unprotected sexual intercourse is another mode of HDV transmission (Wu et al., 1995, Osiowy et al., 2017, Rosenblum et al., 1992). It is worth noting that vertical HDV transmission is rare unlike HBV (WHO, 2018c). The control of HDV transmission is tightly tied to HBV vaccination programs since prevention of HBV transmission decreases the risk of HDV infection (WHO, 2018c). This is because of the HDV dependency on HBV for transmission. The HBV vaccine has been shown to highly effective in averting HDV transmission (Alfaiate et al., 2015). Currently, an effective HDV vaccine is not available yet. However, the prevention of HBV transmission contributes to decreasing risk of HDV infection (WHO, 2018c).

1.2.3. The structure of HDV

HDV is a small, circular RNA virus of ~36 nm in diameter (Figure 5). The outer region of HDV contains three HBV proteins referred to as small, medium and large hepatitis B surface antigen (HBsAg) (Hughes et al., 2011). These proteins are essential for HDV's invasion of hepatocytes (Sureau and Negro, 2016). In addition, the outer coat of HDV contains host lipids (Hughes et al., 2011). The HBV surface proteins share a common C-terminus in the S domain (Urban et al., 2014). In contrast with HBV which

relies on L-HBsAg for assembly and infectivity, HDV assembly is facilitated by the S-HBsAg alone. The absence of L-HBsAg in the outer coat renders HDV to be non-infectious (Sureau et al., 1993, Gudima et al., 2007).

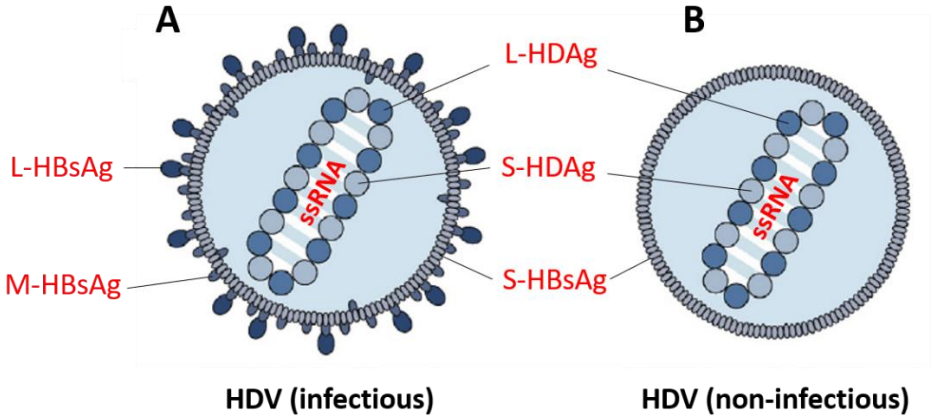

Figure 5: HDV organization. (A) An infectious HDV particle that coats with the large, medium, and small HBV envelop proteins. (B) A non-infectious HDV particle that contains only the small HBV surface protein in its envelope. L-HBsAg, the large HBV envelop protein; M-HBsAg, the medium HBV envelop protein, S-HBsAg, the small HBV envelop protein; L-HDAg, the large HDV antigen; S-HDAg, the small HDV antigen. Adopted with permission from (Urban et al., 2014).

The inner part of HDV is comprised of an inner nucleocapsid that is composed of small and large hepatitis delta antigens (HDAg) and a 1679 nucleotide long single-stranded circular RNA (ssRNA). The HDV antigen proteins are synthesized from a linear HDV mRNA. Two HDAg isoforms are translated from the same ORF, namely small HDV antigen (S-HDAg, 24 kDa) and large HDV antigen (L-HDAg, 27 kDa). S-HDAg containing 195 amino acids can be translated directly from the first round of HDV

genome transcription whereas L-HDAg is produced as a result of RNA modification which leads to the production of a protein with an additional 19-20 amino acids (Zheng et al., 1992, Casey, 2012). They play distinct roles among the HDV life cycle. S-HDAg is necessary for HDV replication (Yamaguchi et al., 2001, Taylor, 2006). The extra amino acids of L-HDAg signal viral assembly and nuclear export, and serves as a prenylation site (Lee et al., 2001). Therefore, L-HDAg inhibits HDV-RNA synthesis, and promote the HDV assembly (Yamaguchi et al., 2001, Taylor, 2006, Hughes et al., 2011).

1.2.4. Hepatitis D virus life cycle

The life cycle of HDV (Figure 6) begins when the L-HBsAg of the virus binds to the human NTCP receptor. The human NTCP is a hepatocyte-specific membrane receptor for HDV as well as HBV (Yan et al., 2012). After HDV enters the hepatocyte, it is uncoated in the cytoplasm. Next, following shipment of nucleocapsid to the nucleus, the replication of HDV genome and S-HDAg-mediated transcription occurs (Chou et al., 1998). Replication of the HDV genome depends entirely on host-cell transcriptional machinery, particularly RNA polymerase I and II. This because HDV lacks RNA-dependent RNA polymerase and does not exploit the polymerase of its helper virus (Lai, 2005, Huang et al., 2008). RNA polymerase I is involved in the transcription, while polymerase II catalyzes the HDV genome replication from the antigenome and transcription of mRNA in the nucleoplasm (Huang et al., 2008).

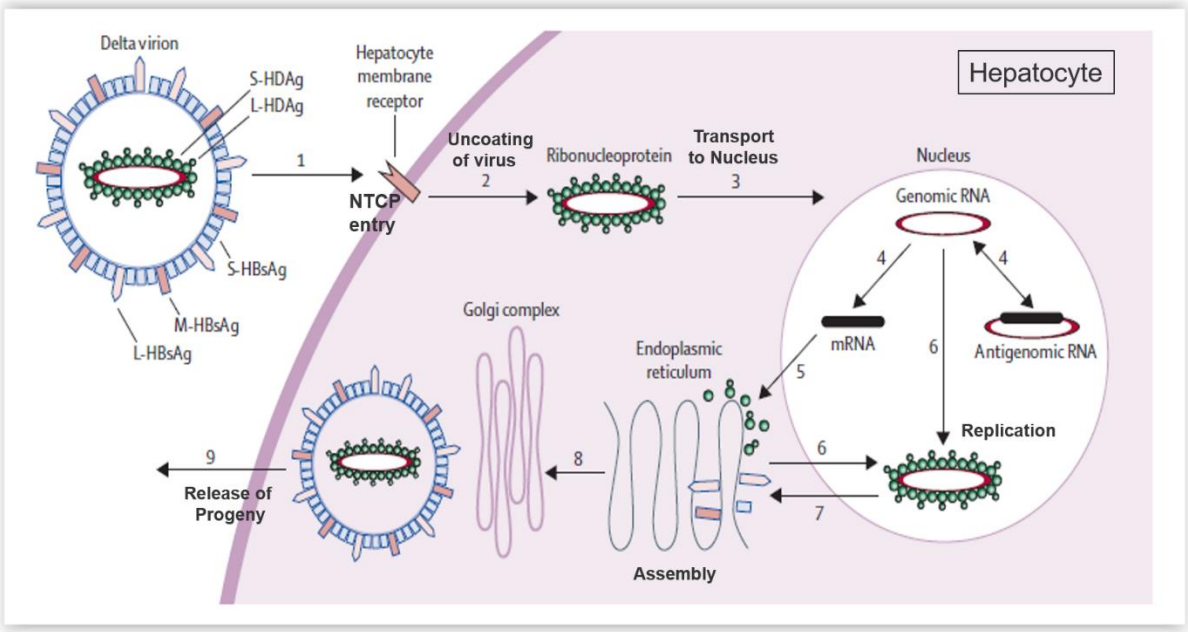

Figure 6: HDV life cycle. Adopted with permission from (Hughes et al., 2011)

Replication of HDV starts when the genomic RNA is transcribed into unmodified mRNA and antigenomic RNA. The mRNA is then translocated to the endoplasmic reticulum (ER). Here, it is translated into the S-HDAg. At the same time, in the nucleosome, an antigenomic RNA is synthesized from the genomic RNA via a transcriptional process of complementary RNA template. Consequently, it self-cleaves into monomers by intrinsic ribozymes. The individual monomers ligate to form circular genomic and antigenomic molecules. Ligation of the monomers is mediated by the host-cell ligases (Abbas and Afzal, 2013). The production of new genomic RNA from the circular antigenome marks the end of HDV replication cycle. The new genomic RNA under editing of ADAR1 translates to a modified mRNA coding for L-HDAg (Alfaiate et al., 2015). The newly formed genomic RNA molecules can stay in the nucleus to enroll new rounds of replication; or be exported to the cytoplasm to participate the viral assembly (Macnaughton and Lai, 2002). The two viral proteins are

subsequently imported into the nucleus to activate viral replication and combine with the HDV genomic RNA to form the HDV nucleocapsid (Abbas and Afzal, 2013)

The final steps of HDV life cycle, viral assembly and release, are dependent on HBsAg availability. The HDV nucleocapsid is enveloped with HBV surface envelope proteins in the Golgi. HDV assembly involves the interaction between the C-terminal sequence of L-HDAg mediates and the S region of HBsAg (Hwang and Lai, 1993). At this point, HDV progenies are released from the host cells and initiate another life cycle.

1.2.5. HDV clinical course

The incubation period for HDV takes one to two months, followed by an acute phase. This phase of HDV infection is characterized by fatigue, lethargy, digestive symptoms, and abnormal elevation of liver enzymes. The icteric form of HDV is rarely observed. It is worth noting that both HDV and HBV symptoms are clinically indistinguishable. HDAg is produced shortly after infection and it is short-lived. Anti-HDV IgMs are generated early in the acute phase whereas low levels of anti -HDV IgG are produced late in the acute phase. Currently, HDV viremia is estimated by RT-PCR detection of HDV RNA in serum (Negro, 2014, Grabowski and Wedemeyer, 2010, Romeo and Perbellini, 2015).

Chronic HDV infections are associated with asymptomatic cases, rapidly progressive hepatitis and prolonged persistence of abnormal liver enzymes levels. Since it is difficult to detect HDAg during chronic HDV infections, RT-PCR detection of HDV RNA in serum has been developed with enough sensitivity. The major limitation of this methodology is that some patients have low viral titers (Negro, 2014, Grabowski and Wedemeyer, 2010, Romeo and Perbellini, 2015).

The course of HDV disease varies tremendously depending on the complexity and infection period (Wedemeyer and Manns, 2010, Negro, 2014, Romeo and Perbellini, 2015). There are two forms of HDV infections, namely the coinfections and superinfections. HDV coinfection occurs when an individual acquires concurrent HBV and HDV infections, whereas the HDV superinfection refers to HDV infection that occurs among persons with an already established HBV chronic infection (Negro, 2014). The former induces fulminant hepatitis and 95% of these cases clear the infection. In contrast, ninety percent of the latter cases become chronic. The superinfections have a more severe course of liver disease than HBV mono-infections. (Negro, 2014, Grabowski and Wedemeyer, 2010, Romeo and Perbellini, 2015). Chronic HDV infection usually exacerbates preexisting HBV-related liver diseases (Smedile et al., 1981).

1.2.6. HDV treatment

Although the establishment HDV infection is dependent on the presence of HBV infections, the existence of HDV in hepatocytes is not completely dependent on HBV transcription machinery. HDV also lacks genes encoding enzymes involved in its replication (Rizzetto, 2018, Botelho-Souza et al., 2017). As a result, the anti-HBV drugs - the nucleoside/ nucleotide analogues, as called HBV polymerase inhibitors, including lamivudine, adefovir, and entecavir have a short-term antiviral effect on HDV infections and cannot halt HDV replication (Wedemeyer et al., 1999, Yurdaydin et al., 2002, Niro et al., 2005a, Kabacam et al., 2012). Interferon-alpha is the only recommended treatment for HDV infections currently (Heidrich et al., 2014), despite having an average viral response rate of 25% (Niro et al., 2005b). Current efforts in

HDV drug development aim at disrupting critical steps of the life cycle as illustrated in Figure 7

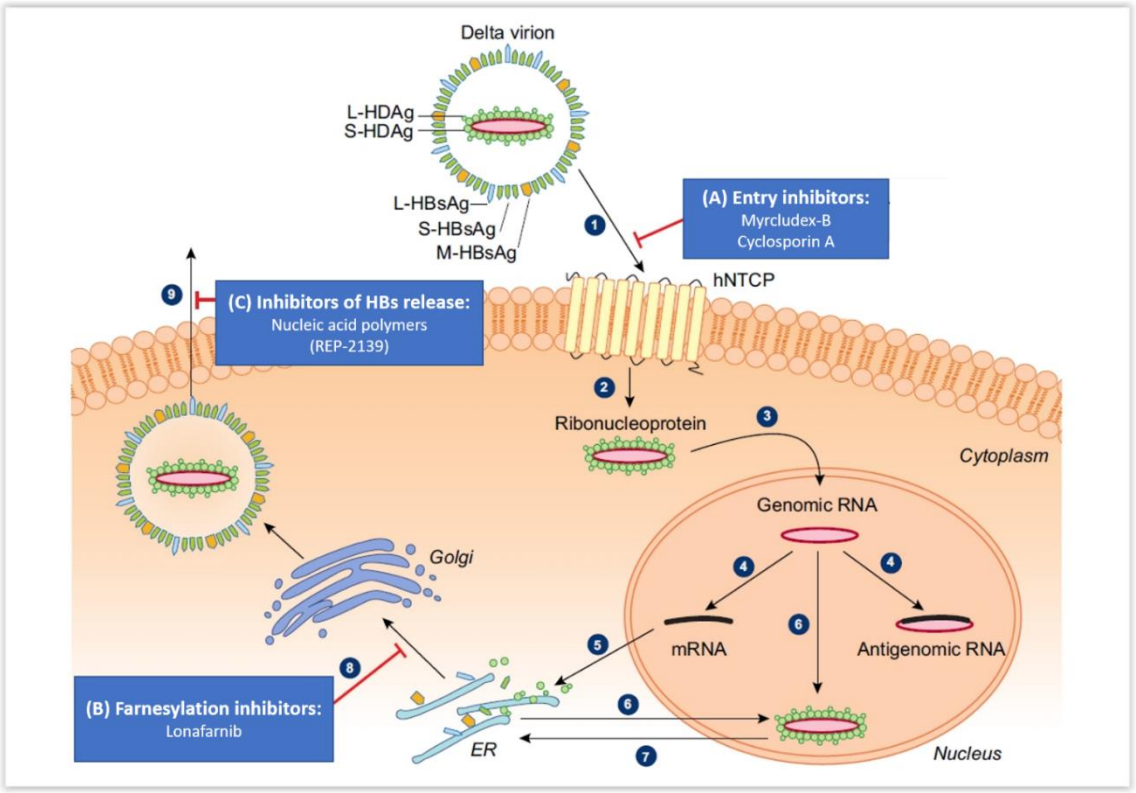

Figure 7: New therapeutic strategies against HDV. Adopted with permission from (Petersen et al., 2016).

Blocking HDV entry into hepatocytes (**A**); The strategy is to inhibit the binding of HBV envelope proteins to NTCP entry. As a result, HBV and HDV may not infect the hepatocytes. Representative of this group are Myrcludex-B and Cyclosporin A that were demonstrated to prevent importation of HDV into cells (Urban et al., 2014). Disruption of HDV assembly (**B**); The initial HDV assembly is the prenylation (covalent addition of prenyl lipids to proteins) of L-HDAg by a host cellular farnesyl transferase. Consequently, the prenylated L-HDAg interacts with the HBsAg for virion morphogenesis (Glenn et al., 1992). Lonafarnib, a prototype inhibitor of

farnesyltransferase, may disrupt the assembly of HDV (Glenn, 2006, Rizzetto, 2018). Inhibition of HBsAg release(**C**); Nucleic acid polymers (NAPs; phosphorothioate oligonucleotides) bind to amphipathic proteins on the viral surface, leading to the destruction of viral adsorption on the cell surface. NAPs also successfully block entry of a few RNA viruses e.g. HIV, and hepatitis C virus (Noordeen et al., 2013). Moreover, NAPs have recently been demonstrated to have an effect against HBV (Guillot et al., 2017, Noordeen et al., 2013). Thus, NAPs could be a potential anti-HDV treatment. Combination of NAP-REP-2139 and Peg IFN has been reported to be auspicious treatment for HDV (Bazinet et al., 2017).

1.3. Viral and host genetic factors and HBV/HDV related liver diseases

In general, clinical manifestations of chronic liver diseases include CHB, LC, and liver cancer (Fattovich, 2003, McMahon, 2009, Liaw and Chu, 2009). Liver cancer has been on top of general cancer incidence with about 800,000 new cases annually. It additionally has caused near 800,000 deaths every year, and ranks fourth in terms of fatalities in 2018 (Bray et al., 2018). Approximately 80% of primary neoplasm of the liver cases are identified as HCC. The leading causes of HCC are chronic HBV and HCV infections and alcohol intake (Bray et al., 2018). With approximately 8.6 million infected people (10-20% of the general population), Vietnam is still one of countries with high rates of HBV infection (Nguyen, 2012, WHO, 2018b), incidences and mortality. HCC accounted for >25,000 cases reported in 2018 in Vietnam (Bray et al., 2018, GLOBOCAN, 2018).

Development of HBV chronic liver diseases usually take a very long time, even more than 20 years. During this progression, liver diseases are under the influence of not only viral genetic factors but also the host genetic factors (Figure 8).

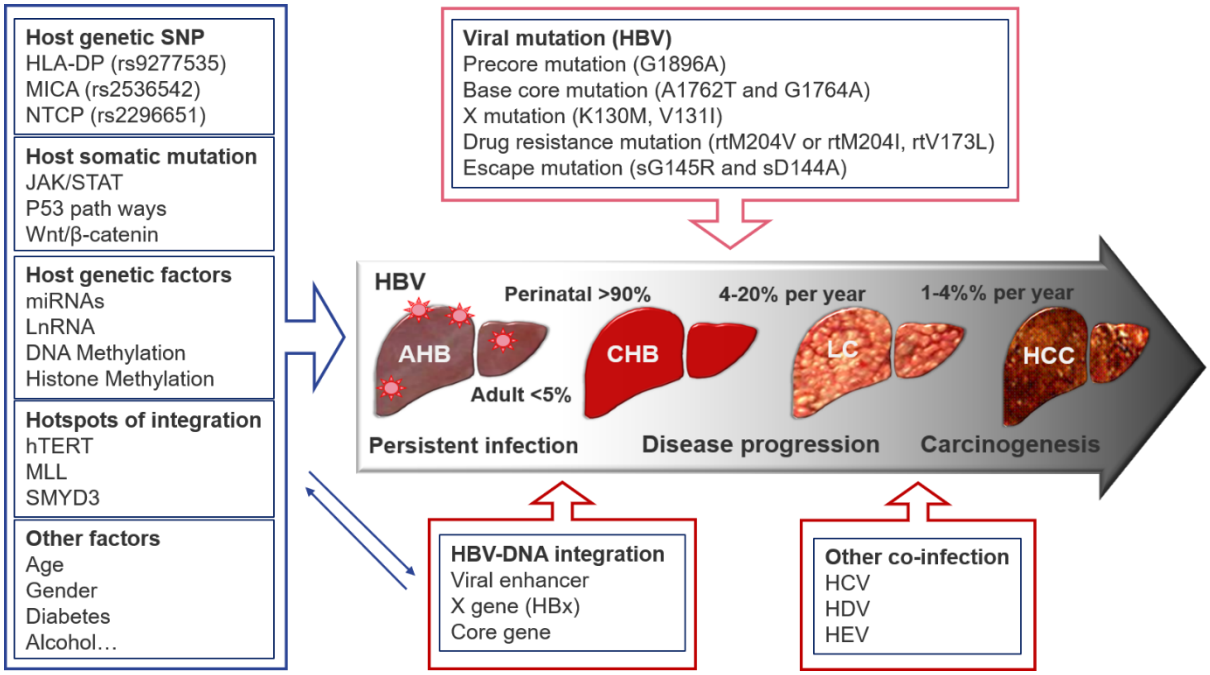

Figure 8: Interaction of viral and host genetic factors impacts the progression of liver diseases related to HBV infection. Adopted and modified with permission from (Tong et al., 2014)

1.3.1. Viral factors

HBV infection as such lead to the development of liver diseases. Most acute HBV infections are self-limited. In contrast, chronic infections usually lead to persistent hepatic damage which induce CHB, LC, and HCC (Fattovich, 2003, McMahon, 2009, Liaw and Chu, 2009). After a five-year window, approx. 10 to 20% of CHB patients have an increased risk of LC development (Liu and Fan, 2007). Remarkably, HBV is one of the main pathogen causing HCC (Nguyen, 2012). A HBV patient has 40 fold increased chance of acquiring HCC compared to a non-HBV individual (Lee et al.,

2013). The integration of HBV-related circular DNA into the host genome is the basis of indefinite persistence of HBV infection (Tang et al., 2018, Zoulim, 2005). Integration of HBV into the host genome occurs in the early phase of HBV infection. Up to 90% of HCC patients have HBV genome integrated into their genome (Bonilla Guerrero and Roberts, 2005, Neuveut et al., 2010, Pollicino et al., 2011, Sung et al., 2012). The cellular topoisomerase-1, has DNA endonuclease and strand transferase activities and is vital for HBV genome integration and viral double-stranded liner DNA importing into the damaged host genome of breakpoints or instabilities (Tokino and Matsubara, 1991). Therefore, increased double-strand breaks increases DNA replication and damage, thus increasing the chance for HBV DNA being integrated to the host genome (Bonilla Guerrero and Roberts, 2005, Tong et al., 2014). Recently, HBV integration breakpoints were reported more frequently in HCC tumor tissues compared to adjacent healthy liver tissues (Sung et al., 2012). The complex integration of host-viral genetic factors is believed to induce the progression of liver diseases. The persistence inflammation of the liver from HBV infection and the integration of viral-host genome contribute to the progression, in particular to LC and HCC. Studies have shown that many somatic mutations induce hepatocarcinogenesis and were observed in high frequencies among HCC tissues than in non-tumors (Huang et al., 2012, Chen et al., 2013a, Chen et al., 2013b, Kan et al., 2013) using whole-genome sequencing and next-generation sequencing methodologies. Mutagenesis caused by HBV integration leads to structural and functional alterations of gene expression and hepatocyte transformation, thus promoting hepatocarcinogenesis.

Chronic HDV infection has been linked to the worsening of HBV-related liver diseases; frequent LC and increased risk of HCC development (Figure 9). HDV/HBV coinfections have been shown to promote rapid LC development in up to 90% of cases due to

increased liver damage (Smedile et al., 1982). Cirrhotic patients account for 15% of chronic HDV after 1-2 years and 70-80% within 5-10 years of infection (Negro, 2014). The European Concerted Action on Viral Hepatitis reported that HDV/HBV cirrhotic patients have up to 18% of hepatic decompensation in their observation with median 6.6 years. They further indicated that the coinfected LC patients experience a triple risk of HCC and double mortality compared to the HBV monoinfected (Fattovich et al., 2000). Romeo et al. reported that newly diagnosed HCC among co-infected individuals is 2.8% per year, whereas nearly 60% of deaths are caused by liver failure (Romeo et al., 2009). The mechanism of how HDV drives HCC remains to be elucidated. Nevertheless, HCC development during HDV infection has been suggested to be due to severe inflammation and necrosis, that induce oxidative stress and/or activate a few oncogenic pathways (Wedemeyer and Manns, 2010).

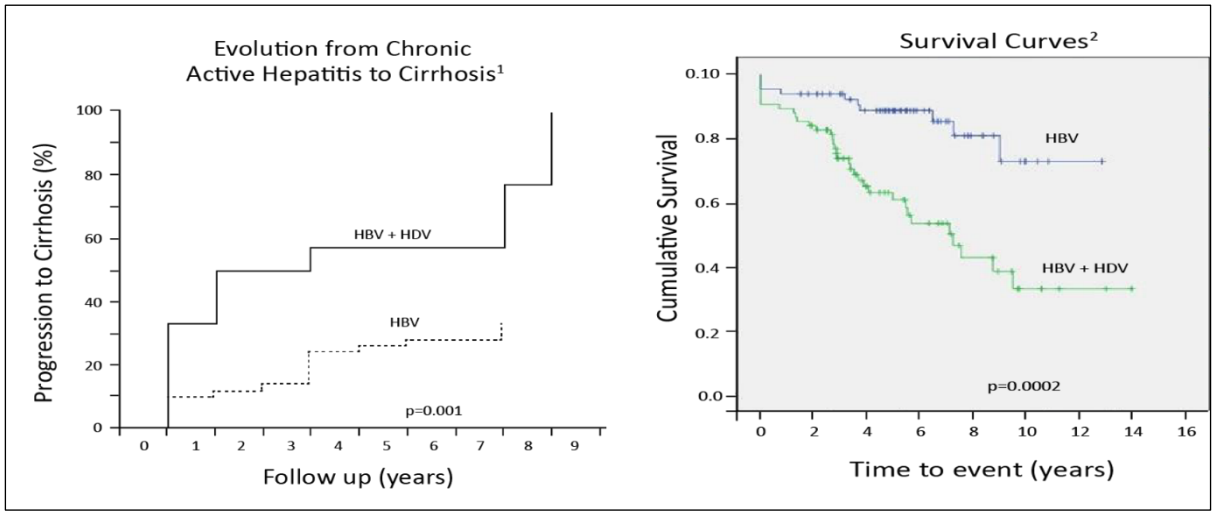

Figure 9: Cirrhotic incidence and mortality among HBV/HDV coinfection patients. Adopted with permission from (Fattovich et al., 1987, Calle Serrano et al., 2011)

1.3.2 Host factors

Host genetic factors also play an important role in the course of HBV infection. They are believed to effect on both viral infections and the hepatic diseases. This exemplifies the high and low occurrence of HBV chronic infection among infants infected at the birth and adults with HBV acute infection (Liaw and Chu, 2009, Tassopoulos et al., 1987). In addition, twin studies demonstrated the important contribution of host genetic factor to the HBV infections. They reported the significant differences in rate of HBV infections, patterns of HBV clinical outcome and response to HBV vaccine among monozygotic, dizygotic twins and controls (Lin et al., 1989, Hohler et al., 2002, Xu et al., 2004).Therefore, numerous genetic risks could thus modulate the progression of liver diseases related to HBV.

1.3.2.1 Sodium taurocholate co-transporting polypeptide

The solute carrier family 10, member 1 gene (*SLC10A1*) on chromosome 14 encodes the human sodium taurocholate co-transporting polypeptide (NTCP). *SLC10A1* expression results to a 349 amino acid glycosylated phosphoprotein (Doring et al., 2012). NTCP act as the main co-transporter for sodium and taurocholate across the membrane of hepatocytes. The non-synonymous R252H of NTCP gene (*NTCP*) are associated with reduced hepatocyte uptake of bile salts in NTCP (Vaz et al., 2015).

NTCP is an important and specific receptor for HBV/HDV hepatocyte invasion (Yan et al., 2012, Ni et al., 2014). This receptor interacts with HBsAg on the surface of the viruses. In particular, the preS1 domain of HBsAg is responsible for the recognition of the NTCP receptor (Le Seyec et al., 1999). Amino acids 2-48 of preS1 interact explicitly with NTCP, whereas amino acids 49-75 are required for hepatocyte invasion (Urban et al., 2014). On the other hand, amino acid residues 157-165 and amino acids

84-87 of NTCP are important for the interaction and infection of HBV and HDV (Yan et al., 2013).Since both HBV and HDV share HBV envelope proteins, it is therefore believed that HBV and HDV share a common utilize the same mechanism to invade hepatocytes via NTCP (Hughes et al., 2011, Yan et al., 2012, Yan et al., 2013, Ni et al., 2014, Yan et al., 2014).

Genetic variations of the *NTCP* gene may alter the structure of the protein as well as the, function of the protein. The prevalence of *NTCP* single nucleotide polymorphisms (SNPs) generally depends on ethnicity (Ho et al., 2004, Pan et al., 2011). For example, the SNP I223T (c.668 T>C) occurs in 5.5% of African Americans whereas S267F (c.800 C>T) and I279T (c.836 T>C) are Asian-specific SNPs with a frequency of 7.5% and 0.5%, respectively. Recently, some of these SNPs were reported to reduce NTCP efficiency in the uptake of bile acids efficiency (Ho et al., 2004, Choi et al., 2011). For example, S267F (rs2296651) has been shown to significantly reduce uptake of bile acids is caused by; phenylalanine replaces serine at position 267 of NTCP (Ho et al., 2004). Based on these findings of alternative proteins, *NTCP* S267F has been hypothesized as a potential genetic factor that could possibly influence HBV and HDV infection. Previous studies indicate that the *NTCP S267F* variant is associated with the restraint of both infected HBV and the progression of HBV hepatic diseases (Peng et al., 2015, Hu et al., 2016, Lee et al., 2017, Wu et al., 2018, Wang et al., 2017). Importantly, data on whether *NTCP S267F* correlates with susceptibility or resistance to HDV infection is still limited.

1. 3.2. 2 The SET and MYND domain-containing protein3

Methylation of histone proteins is an established mechanism for development multiple types of cancers, including HCC (Ma et al., 2014, McCabe et al., 2017). Methylation of lysine residues in histone proteins can lead to the regulation of various cellular processes such as chromatin remodeling, transcription, and signal transduction (Spellmon et al., 2015). Among them, the SET and MYND domain-containing protein3 (SMYD3) are potential carcinogenic factors.

SMYD3 acts mainly as a histone lysine methyltransferase (Taylor, 2006). It has been extensively associated with various kinds of cancers (Huang and Xu, 2017). In particular, SMYD3 methylases H3K4 and H4K5 histone in a process catalyzed by heat shock protein 90α (HSP90), that activates transcriptional tumorgenesis, (Kunizaki et al., 2007, Zou et al., 2009) SMYD3 upregulation also induces abnormal cell proliferation, an indication of SMYD3 involvement in carcinogenesis (Hamamoto et al., 2004, Luo et al., 2009, Liu et al., 2015). Several studies have reported SMYD3 upregulation in HCC, human colorectal cancer, breast cancers, and lung cancer (Hamamoto et al., 2004, Hamamoto et al., 2006, Mazur et al., 2014).

The upregulation of SMYD3 may also arise from other cellular processes. *In-vitro* experiments using HepG2 cell line have shown that overexpression of SMYD3 may be driven by SMYD3 interaction with HBx protein (Yang et al., 2009) and in turn induce overexpression of activator protein 1 (AP1). Also, high levels of AP1 increases the risk of HCC development (Guo et al., 2005, Hayashi et al., 2016). Changes in the variable number of tandem repeats (VNTR; CCGCC) of *SMYD3* promoter have also been associated with some human cancers (Tsuge et al., 2005). An identified putative E2F-1-binding element (5'-CCGCC/G-3') was shown to induce SMYD3 expression by transcriptional activation. A three-repeat of CCGCC motif promotes SMYD3 binding affinity to E2F-1, thus leads to enhance the transactivation of SMYD3 in cancer cells

compared to the two repeats (Tsuge et al., 2005). As a result, cancer progression may be promoted by activating the transcription of multiple oncogenes such as Myc, STAT3, and β-cat (Tsuge et al., 2005, Liu et al., 2015, Sarris et al., 2016). In colorectal cancer, HCC or breast cancer patients, *SMYD3* genotype 3/3 was found to be frequent than others *SMYD3* genotypes (Tsuge et al., 2005, Wang et al., 2008, Liu et al., 2016). Particularly in liver diseases, approximately three fold high risk of HCC was associated with the *SMYD3* VNTR genotype 3/3 in Asian populations (Tsuge et al., 2005, Li et al., 2018).

2. SCOPE AND SPECIFIC OBJECTIVE

This thesis is structured as three chapters. The chapter one aims to assess the prevalence of HDV infection and molecular genotypes distributions in Vietnam. The chapter two aimed to perform a genetic association study on the role of the *NTCP* S267F variant in HBV and HBV/HDV infections. The third chapter aimed to investigate the association of VNTR polymorphisms in the promoter region of *SMYD3* and SMYD3 expression with HBV infection. In particular, this doctoral dissertation investigated three specific objectives:

1. To assess the prevalence of HDV infection and possible changes in genotype distributions over the last decade in the northern Vietnam. In addition, I assessed effects of HDV/HBV coinfections on the clinical staging of HBV-related liver diseases.

2. To determine potential associations of the NTCP S267F variant (rs2296651, c.800C>T) with HBV and HBV plus concomitant HDV infection as well as with the progression of related liver diseases.

3. To investigate the association of VNTR polymorphisms in the promoter region of *SMYD3* and SMYD3 expression with HBV infection and clinical progression of HBV-related liver diseases, in particular progression to HCC.

3. RESULTS

3.1 Chapter 1: HDV infection rates in northern Vietnam

Scientific paper No.1

HDV infection rates in northern Vietnam

Binh MT, Hoan NX, Tong HV, Giang DP, Sy BT, Toan NL, Song LH,
Bang MH, Wedemeyer H, Meyer CG, Kremsner PG, Bock CT, Velavan TP

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HDV infection rates in northern Vietnam

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Hepatitis D caused by the hepatitis delta virus (HDV) is a serious health problem in many regions of the world. A total of 546 HBV-infected patients were enrolled from 2013 to 2015 and classified clinically into the subgroups of chronic hepatitis B (CHB, n = 191), liver cirrhosis (LC, n = 147) and hepatocellular carcinoma (HCC, n = 208). The patients were screened for HDV-RNA by nested PCR assays. HDV genotypes were assessed by direct sequencing, followed by phylogenetic analysis. HDV-RNA was identified in 13% (71/546) of HBV-infected patients. The highest HDV prevalence was found in the LC group (19.7%), followed by the HCC (12%) and CHB (8.9%) groups ($P = 0.017$). HDV/HBV coinfections were significantly associated with a rather unfavourable clinical outcome, in particular with LC development compared to HBV monoinfection. Phylogenetic analyses indicated that the genotype HDV1 was, with a prevalence of 91%, by far the most common genotype in Vietnam, followed by HDV2 with 9%. Other HDV genotypes were not observed. In accordance with previous data obtained a decade ago, our results confirm a continuing high prevalence of HDV infection in hepatitis B patients in northern Vietnam with the HDV1 genotype still being the predominant genotype. HDV nucleic acid testing to minimize the associated risk should be considered.

Hepatitis delta virus (HDV), firstly identified in 1977[1], is a defective virus which uses hepatitis B virus (HBV) envelope proteins for successful infection of hepatocytes[2]. The HDV virion is composed of an outer coat containing HBV envelope proteins and host lipids surrounding an inner nucleocapsid that consists of small and large hepatitis delta antigens (HDAg) and a single-stranded circular RNA of 1679 nucleotides[3]. Both HBV and HDV use the Na+-taurocholate cotransporting polypeptide (NTCP) bile transporter to gain entry to the hepatocytes. HBV infections produce envelope proteins to assemble new HBV particles. HDV utilizes these envelope proteins for its own assembly. Given that HBV and HDV share the same envelope proteins, they enter hepatocytes by a similar mechanism. The viruses are transmitted to healthy individuals by concurrent infection either with HBV (coinfection) or by superinfection (superimposed on chronic hepatitis B). The HDV superinfection progress to an acute infection, leading to severe liver damage than HBV monoinfection.

HDV/HBV coinfections are a global health problem affecting 15–20 million people worldwide[4,5]. Although HDV infection usually is associated with an increased risk of liver cirrhosis (LC) and hepatocellular carcinoma (HCC)[3], it is frequently underdiagnosed due to a lack of awareness and the unavailability of appropriate diagnostic tools in hospitals of developing countries. Moreover, HDV infection is not routinely tested in clinical practice in many of these countries and treatment of hepatitis D infections is challenging and largely ineffective[6,7].

Currently, eight HDV genotypes (HDV1–8) had been recognized with distinct geographic distributions and associated clinical features[8–10]. The HDV1 genotype occurs worldwide and is associated with both severe and mild clinical forms of viral hepatitis[3]. The HDV2 genotype is mainly found in East Asia and causes mild disease[11]. HDV3 is predominantly found in South America[12], and HDV4 preferentially occurs in Japan and Taiwan[13]. Genotypes HDV5-8 were identified in indigenous African ethnic groups, however, these genotypes, their prevalence and clinical relevance are less well characterized[3,9,11].

The HDV prevalence in HBV infected patients is commonly described as percentage of anti-HDAg positive individuals in HBsAg-positive patients. Prevalences vary considerably across geographical regions. While HDV

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Characteristics	Total (n = 546)	CHB (n = 191)	LC (n = 147)	HCC (n = 208)	*P* value
Age (years)	53 [12–86]	37 [12–73]	57 [15–86]	60 [15–81]	<0.0001[#]
Male (%)	85.1	77	85	92.8	<0.0001[#]
Child-Pugh classification					
Child A		NA	60/132	153/208	
Child B		NA	61/132	47/208	
Child C		NA	11/132	8/208	
Missing		NA	15	0	
Clinical parameters					
AST (IU/L)	62.5 [14–6206]	47 [14–6206]	78 [18–1221]	58 [17–670]	0.0045[‡]
ALT (IU/L)	54 [8–3390]	60 [8–3390]	55 [8–1426]	48 [11–934]	0.0048[‡]
Total bilirubin (μmol/L)	18 [4.1–571]	16.3 [5.5–551]	31.65 [4.1–593]	17 [6–282]	<0.0001[‡]
Direct bilirubin (μmol/L)	6 [0.4–349]	5.9 [1–349]	11.5 [0.4–350.22]	5.5 [0.4–189.39]	<0.0001[‡]
albumin (g/L)	39 [9.8–49]	42 [9.8–48]	31 [15–47]	38 [21–49]	<0.0001[‡]
Prothrombin (% of standard)	85 [13–269]	92.5 [17–267]	60 [13–120]	83.5 [19.6–269]	<0.0001[‡]
PLT ($\times 10^3$/ml)	174 [3.7–426]	210 [65–416]	89 [3.7–325]	166 [42–426]	<0.0001[‡]
HBV DNA ($\log_{10}$ copies/ml)	7.0 [2–10.3]	5.4 [2–10.3]	4.5 [2–9]	4.6×10^3 [2–9.2]	0.019[‡]
AFP (IU/L)	8.4 [1.06–400]	3 [1.06–400]	7.9 [1.18–400]	133 [1.38–400]	<0.0001[‡]

Table 1. Clinical characteristics of 546 patients with chronic hepatitis B. CHB, chronic hepatitis B; LC, liver cirrhosis; HCC, hepatocellular carcinoma; PLT, platelets. AST and ALT, aspartate and alanine amino transferase; AFP, alpha-fetoprotein; IU, international unit. Values given are medians and ranges or percentile where appropriate. [(‡)] Kruskal-Wallis test. [(#)] Chi-square test.

infections are rather rare in Europe due to largely effective HBV vaccination programs and screening of blood products, hepatitis D remains of concern with highest rates in low-income HBV-endemic countries with insufficient HBV vaccination coverage[14]. For example, high HDV seroprevalences were reported in Pakistan where vaccination campaigns are difficult to conduct (35.2%)[15], Mongolia (67%)[16], Gabon (15.6% to 70.6%)[17]. Recently, the HDV prevalence in sub-Saharan African countries was estimated to be 1.3% to 50%[18].

In Vietnam, HBV infection rates range from 10% to 15% in the general population[19], suggesting a high prevalence of HDV infection in HBsAg positive patients. Few studies only have assessed the epidemiological and clinical importance of HDV infection between 2000 and 2015 in Vietnam, indicating different results in terms of HDV infection prevalences across regions. In particular, the distribution of HDV genotypes differed between northern and southern Vietnam[20–23]. A high predominance of 90% of the HDV1 genotype in northern Vietnam was shown in HBV-infected patients recruited between 2000 and 2003[23]. In contrast, a recent study has reported that HDV2 was the predominant genotype (80%) in southern Vietnam[22]. However, both studies were conducted with small sample sizes of HBV patients only (n < 300). In the present study, a larger group of HBV-infected patients was involved in order to assess the prevalence of HDV infection and possible changes in genotype distributions over the last decade in the northern Vietnam. In addition, we assessed effects of HDV/HBV coinfections on the clinical staging of HBV-related liver diseases.

Results

Baseline characteristics of chronic hepatitis B patients. The demographic characteristics of the 546 HBV-infected study participants are summarized in Table 1. Of the 546 patients, 465 (85%) were male and 81 (15%) were female. In our study cohort, the mean age of patients was 53 (12–86 years). Eight individuals were at early adolescence (12–18 years old). Among those eight infected individuals, six were experiencing CHB (≤18 years) and one each were with LC and HCC (≤15 years). The HBV transmission was documented from mother to child in all those six patients among the infected eight. All eight patients were not vaccinated against HBV. As expected, patients in the CHB group were younger than those in LC and HCC groups (37 vs. 57 and 60 years, respectively). Albumin and prothrombin levels as well as platelet counts were significantly lower in the LC group compared to the other groups ($P < 0.001$) and direct and indirect bilirubin levels were higher in the LC compared to the CHB and HCC groups ($P < 0.0001$). Liver enzyme levels (AST, ALT) were significantly higher in the CHB group compared to the other groups ($P = 0.0045$ and 0.0048, respectively). AFP levels were significantly higher among HCC patients compared to the subgroups of CHB and LC patients ($P < 0.0001$).

Prevalence of HDV infection in patients with HBV-related liver diseases. HDV-RNA was detected in 71 (13%) among the 546 HBV-infected. Of the 71 HDV-positive patients, 66/71 (93%) were male and 5/71 (7%) were female ($P < 0.05$). The prevalence of HDV infection among clinical groups is presented in Table 2. HDV-RNA was observed more frequently among LC (20%; 29/147) and HCC patients (12%; 25/208) compared to CHB patients (9% or 17/191) ($P = 0.017$). When LC patients were stratified according to child-pugh scores, HDV infections were significantly higher in patients with Child B or C compared to Child A score ($P = 0.047$), however such a significant trend could not be observed when stratified among HCC patients. These results suggest HDV infection as a significant risk factor for the progression of liver disease, esp. for liver cirrhosis.

Groups	HDV/HBV coinfection		HBV monoinfection		p value
Clinical groups	**n/total**	**(%)**	**n/total**	**(%)**	
CHB	17/191	8.9	174/191	91.1	0.017
LC	29/147	19.7	118/147	80.3	
HCC	25/208	12	183/208	88	
Child-Pugh score					
Child A	25/213	11.7	188/213	88.3	0.047
Child B	24/108	22.2	84/108	77.8	
Child C	3/19	15.8	16/19	84.2	
BCLC staging					
BCLC A	5/66	7.6	61/66	92.4	NS
BCLC B	13/76	17	63/76	83	
BCLC C	3/37	8	34/37	92	
BCLC D	1/10	10	9/10	90	

Table 2. Association of HDV infection with liver disease progression. CHB, chronic hepatitis B; LC, liver cirrhosis; HCC, hepatocellular carcinoma; BCLC: Barcelona Clinic Liver Cancer; *P* values calculated by Chi-square test.

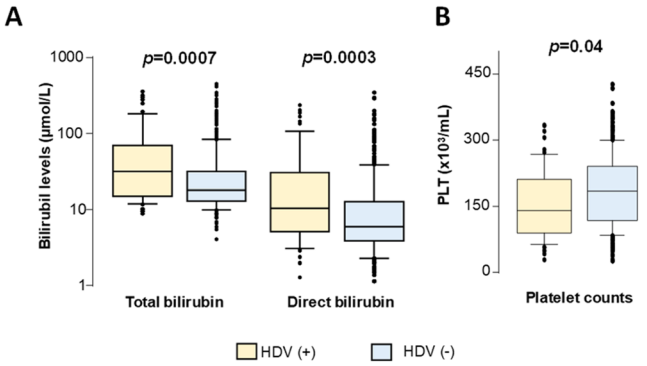

Figure 1. Association of HDV infection with subclinical parameters. Bilirubin levels (**A**) and Platelet counts (**B**) in HBV infected patients with HDV and without HDV coinfection. Other parameters (liver enzymes: AST and ALT, HBV DNA loads, albumin, prothrombin, AFP) that did not reach the statistical significance were not presented in the figure. Box-plots illustrate medians with 25 and 75 percentiles with whiskers to 10 and 90 percentiles. *P* values were calculated by Mann-Whitney-Wilcoxon test.

HDV/HBV coinfection and biochemical parameters. In order to analyze the influence of HDV infection on the clinical outcome in HBV-infected patients, we compared HBV-DNA loads, levels of liver enzymes as well as those of bilirubin, albumin, prothrombin and platelet counts between HBV patients with and without patent HDV infection. HBV-DNA levels were lower in HDV/HBV coinfected patients; however, the difference was not significant. Platelet counts were lower in coinfected patients compared to HBV monoinfected patients ($P = 0.04$). Both total and direct bilirubin levels were higher in HDV-infected patients than in those without HDV infection ($P = 0.0007$ and 0.0003, respectively) (Fig. 1). Comparisons of other laboratory parameters between HDV-positive and HDV-negative patients were not statistically significant (data not shown).

We then analyzed associations between HDV infection and liver function parameters in the HBV subgroups of CHB, LC and HCC. The results are given in Fig. 2 and Table 3. In the CHB group, there were no differences of all biochemical parameters between patients with and without HDV infection. In the LC group, AST and ALT levels were significantly higher in HDV/HBV coinfected patients than in HBV monoinfected patients ($P < 0.05$). In the HCC group, a similar trend applied to the total and direct bilirubin levels in comparison between HCC patients with and without HDV infection (median: 22 *vs*. 17 μmol/L, $P = 0.017$ and 9.8 *vs*. 5 μmol/L, $P = 0.005$, respectively). This was also seen when comparing patients with advanced liver disease (LC and/or HCC patients) with CHB patients (median: 35.5 vs. 19 μmol/L, $P = 0.0014$ and 12 vs. 6.7 μmol/L, $P = 0.002$, respectively).

HDV genotype distribution and its relation to clinical outcomes. Among the 71 HDV-RNA positive samples, 57 samples were successfully sequenced and genotyped. Phylogenetic analyses of the amplified fragment (235 bp) from HDV genomes of 57 sequences showed that HDV1 was the most frequent genotype (52/57, 91%); HDV2 was found in 5/57 (9%) only of HDV/HBV-coinfected patients (Fig. 2A,B). All sequences were submitted to the GenBank database (MG722912-MG722968).

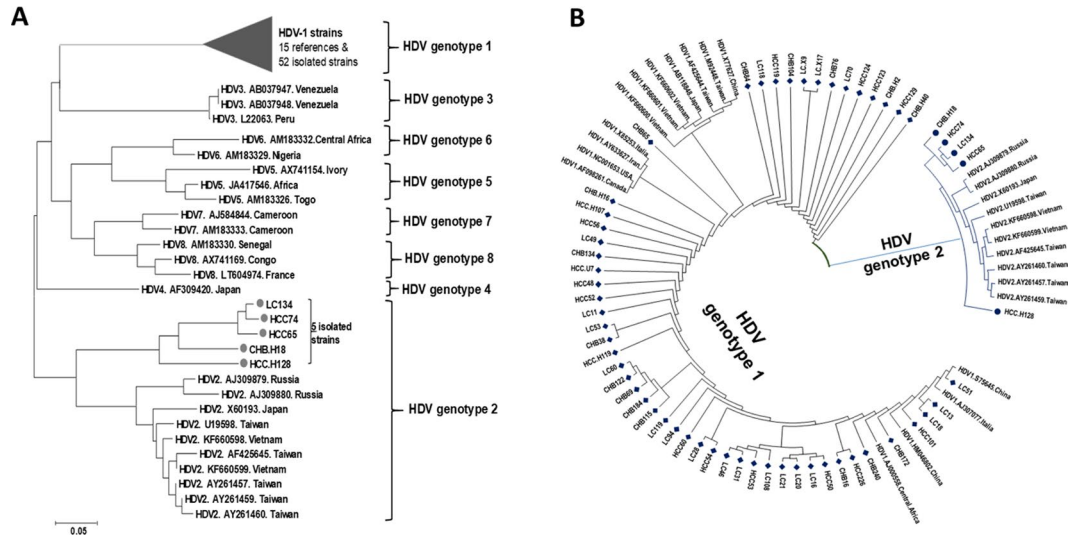

Figure 2. Phylogenetic analysis of isolated HDV genotypes. (**A**) A phylogenetic tree was constructed based on the alignment of 235 bp of 57 nucleotide sequences isolated from HDV/HBV co-infected patients. 39 full-length HDV genomes through HDV1-8 retrieved from NCBI database along with GenBank accession numbers were included for the analysis. A neighbor-joining tree was constructed with a bootstrap of 1000 replicates. The bar at the base of the tree indicates the scale for nucleotide substitutions per position. (**B**) The phylogenetic tree was constructed only for HDV genotype 1 and 2 sequences and involves 82 nucleotide sequences (25 references of full-length HDV genome retrieved from NCBI database, 52 strains of HDV genotype 1 (denoted as ♦) and 5 HDV genotype 2 (denoted as ●) from HBV/HDV coinfected patients in our study group.

Groups	HDV status	HBV DNA (log_{10} copies/mL)	PLT ($\times 10^3$/L)	AST (IU/L)	ALT (IU/L)	Total bilirubin (μmol/L)	Direct bilirubin (μmol/L)	Albumin (g/L)	Prothrombin (%)
CHB (n = 191)	HDV (−)	5.4 [2–10.3]	210 [65–416]	46 [14–6206]	60 [8–3390]	16.3 [5.5–551]	5.7 [1–349]	42 [9.8–51]	92 [35–267]
	HDV (+)	5.7 [2–8.9]	203 [95–262]	67 [26–883]	59 [26–1630]	17 [9.6–321]	7.35 [2.9–168]	42 [25–67]	98 [17–127]
LC (n = 147)	HDV (−)	5.4 [2–9]	89 [17.1–325]	74 [18–1221]	51 [8–1426]	30 [4.1–593]	10 [0.4–350]	31 [15–46]	57 [13–120]
	HDV (+)	3.9 [2–7.2]	91 [28–306]	**98 [19–712]***	**66 [14–1354]***	38 [9–358]	12 [2–238]	32 [26–47]	71.5 [29–100]
HCC (n = 208)	HDV (−)	4.6 [2–9.1]	166 [50–426]	58 [17–670]	49 [11–934]	17 [6–214]	5 [0.4–133]	38 [21–49]	85 [19–269]
	HDV (+)	4.5 [2–9.2]	166.5 [42–334]	56 [23–356]	38 [12–565]	**21.7 [9.7–282]***	**9.8 [1.3–189]****	36 [21–45]	78 [40–103]
HCC + LC (n = 355)	HDV (−)	4.67 [2–9.1]	128 [17.1–426]	63 [17–1221]	49 [8–1426]	19 [4.1–593]	6.7 [0.4–350]	36 [15–49]	77 [13–269]
	HDV (+)	4.3 [2–9.2]	104 [28–334]	78.5 [19–712]	55.5 [12–1354]	**35.5 [9–358]****	**12 [1.3–238]****	34 [21–47]	76 [29–103]

Table 3. Association of HDV infection and clinical parameters in each HBV subgroup. CHB: Chronic Hepatitis B; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma; PLT: Platelet count; AST: Alanine aminotransferase; ALT: Aspartate aminotransferase. Values given are median and range; IU: international unit; *denotes $P < 0.05$; **denotes $P < 0.005$.

We evaluated the impact of the HDV1 and HDV2 genotypes on the clinical outcome in HBV-infected patients by comparing the levels of biomedical parameters, including HBV-DNA loads, liver enzymes, bilirubin, albumin, prothrombin, and platelet counts. HBV-DNA levels were lower in HDV1-infected patients compared to HDV2-infected patients (not significant) (Fig. 1A). Comparisons of biochemical parameters were also not significant (data not shown).

Discussion

In spite of effective HBV vaccines available, HDV/HBV coinfections are still of concern and underreported in many HBV-endemic regions. In Vietnam, although a universal hepatitis B vaccination program has been implemented since 2003 with a vaccine coverage was 97% in 2012 and a birth dose coverage increased to 75% in 2012 from 65% in 2006. The main route of transmission of hepatitis B in Vietnam is from mother to child. HBV infection still is one of the most serious public health problems with 10–15% of infected individuals in the general population and 20–40% among high risk groups such as drug users and HIV-positive individuals[19,24]. This suggests a high prevalence of HDV infection among Vietnamese HBsAg positive individuals. The minimum age of HBsAg positive patients was 12 years in our 2013 cohort, signifying that these individuals were not vaccinated by 2003. Furthermore, our study indicates that individuals at early adolescence are still at high risk, in a Vietnamese population that has a median age of 30 years. Therefore, in next decades, HBV and HBV/HDV coinfection may pose a significant health burden in Vietnam. Few data on the molecular epidemiology of HDV infection are available and the results are inconsistent regarding both the prevalence of infection and the distribution of HDV genotypes

in Vietnam[20–23]. Here we describe a high HDV prevalence and confirm the predominance of the HDV1 genotype in northern Vietnam.

HDV infection rates vary considerably between countries in Africa, South America and parts of Asia[4,10,20,21,25], while prevalences are low in northern Europe and North America, where HDV infections occur mostly among intravenous drug users[25]. A distinct geographic distribution of the HDV prevalence has been described for Vietnam. One study has shown that HDV infection rates varied in five regions across Vietnam, including Hanoi, Hai Phong, Da Nang, Khanh Hoa and Can Tho[21]. HDV seroprevalences were high in northern Vietnam (30.2% and 29.4% in Hanoi and Hai Phong, respectively), but lower in southern Vietnam (8.1% in Khanh Hoa and 12.5% in Can Tho) and in Central Vietnam (5.3% in Da Nang). A previous study conducted in a cohort of HBV-infected patients collected over a decade ago had already shown a high HDV-RNA prevalence in northern Vietnam (15.4%)[23]. The prevalence of HDV-RNA was 10% in chronic hepatitis B carriers collected in 2015 in Central Vietnam[22]. The difference in HDV-RNA prevalences between these two studies reflects distinct geographic distributions of the infection. Our findings of a high HDV infection rate in northern Vietnam (13%) corroborate the earlier results[21,23] and indicate that HDV infection rates did not decline during the last decade in northern Vietnam, although the immunization coverage against HBV is well established. However, it is early enough to see a potential influence of HBV vaccination on the burden of HDV infection after only 10–12 years of vaccination campaign by which the target population of the vaccination program represents for/by newborns and infants.

Varying distributions of HDV genotypes in North and South Vietnam have recently been reported[21–23], with the HDV1 genotype being predominant in all parts of Vietnam[21]. In contrast, HDV1 has been shown to prevail in northern Vietnam with 90%[23], but accounted for only 20% in central Vietnam[22]. We here confirm the predominance of HDV1 (HDV1, 91% *vs*. HDV2, 9%) in northern Vietnam, consistent with the finding that HDV1 is worldwide the most common genotype, while the other genotypes are rather restricted to distinct geographical regions[8,11,26,27].

Concomitant HDV and HBV infections significantly increase the risk of HCC development and liver decompensation at an early stage of coinfection[8,10,28,29]. In our study, HDV positivity rates were particularly high in the LC group, followed by the HCC and CHB groups. Patients with Child-Pugh scores B and C had higher infection rates compared to patients scored as Child-pugh A, indicating that HDV infection is in fact associated with progression to liver cirrhosis. The levels of liver enzymes and bilirubin in patients with advanced liver diseases (LC, HCC) in HDV/HBV-coinfected patients were elevated compared to HDV-negative patients. Like HBV/HCV coinfection, HBV/HDV coinfection is associated with diverse patterns of reciprocal inhibition of viral replication[30]. Although the difference was not significant, our data show that HBV-DNA loads were lower in HDV/HBV coinfection compared to HBV monoinfection, in part supporting the previous finding that HDV-infection suppresses HBV replication[31,32]. While other studies have demonstrated an impact of HDV genotypes on the clinical presentation of HBV-infected patients[3,10,22], our study did not show any significant association of HDV genotypes with liver function test, HBV-DNA loads, and AFP levels. This lack of association may result from the small number of patients infected with HDV2.

In conclusion and in comparison with the situation a decade ago, HDV infection is still a serious medical problem in Vietnam. HDV-RNA positivity in HBV infected patients is high and HDV1 is the predominant genotype circulating in northern Vietnam. A continuing high prevalence of HDV infection in hepatitis B patients increases the disease burden. The National authorities in Vietnam should reconsider the operational clinical protocols to include HDV nucleic acid testing to minimize the associated risk of liver cirrhosis and hepatocellular carcinoma in patients infected with chronic HBV.

Subjects and Methods

Clinical patients. Five hundred forty-six Vietnamese HBV-infected patients were consecutively enrolled at the 108 Military Central Hospital, Hanoi, Vietnam, between 2013 and 2015. All participants were negative for anti-HCV and anti-HIV antibodies as assessed by routine ELISA assays. None of the study participants had a history of alcohol or drug abuse. All LC and HCC patients and most CHB patients were admitted to hospital for specific treatment. HBV-DNA loads and biochemical liver function parameters, including the levels of alanine transaminase (ALT), aspartate transaminase (AST), total bilirubin and direct bilirubin as well as albumin and prothrombin were assessed. Patients were classified into the clinical subgroups of chronic hepatitis B (CHB, n = 191), liver cirrhosis (LC, n = 147), and hepatocellular carcinoma (HCC, n = 208). The diagnostic criteria applying to each subgroup have previously been described[33]. The liver function of LC and HCC patients were categorized according to Child-Pugh scores (Child-A, B, and C)[34]. Staging of HBV-related HCC was assessed according to the Barcelona Clinic Liver Cancer (BCLC) strategy[35]. Blood sampling of all patients were performed on admission. Whole blood and serum samples were stored at −80 °C until use.

Ethics statement. Informed written consent was obtained after detailed explanation of the study at the time of blood and serum sampling from all participants or from their parents if subjects were <18 years old. The study was approved by the institutional Review Board of the 108 Military Central Hospital, Hanoi, Vietnam. All experiments were performed in accordance with relevant guidelines and regulations.

Nucleic acid extraction and cDNA synthesis. Viral RNA was isolated from serum obtained from the study participants (QIAamp Viral RNA Mini Kit; Qiagen GmbH, Hilden, Germany). HDV-RNA was reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Foster City, CA, USA) following the manufacturer's instructions.

HDV-RNA detection. Nested PCR assays specific for the identification of HDV were performed using four highly conserved primer pairs (Table 4), representing all eight currently recognized HDV genotypes as previously described[22]. PCR amplification was carried out in 25 µl reaction volumes (5 ng cDNA, 10x buffer [20 nM

Primers	Sequence (5′-3′)	Position	PCR round
HDV04_F	GGATGCCCAGGTCGGACCG	856–874	1st round PCR
HDV05_R	AAGAAGAGRAGCCGGCCCGY	1159–1179	1st round PCR
HDV06_F	ATGCCATGCCGACCCGAAGA	888–907	2nd round PCR
HDV07_R	GGGGAGCGCCCGGDGGCGG	1104–1122	2nd round PCR
HDV57_F	GAGAAMYCACCTCCAGAGGA	299–318	1st round PCR
HDV60_R	TCCCATTCGCCATTACCGA	752–770	1st round PCR
HDV48_F	AGAGGACCCCTTCAGCGAAC	313–332	2nd round PCR
HDV54_R	CCGGGATAAGCCTCACTCG	467–485	2nd round PCR

Table 4. Primers used for HDV detection.

Tris-HCl, 50 nM KCl, 2 nM $MgCl_2$], 0.2 nM dNTPs, 0.4 nM $MgCl_2$, 0.6 μM specific primer pairs, and 1 unit Taq polymerase). The first PCR round was performed with primer pairs HDV57_F and HDV60_R (nucleotides 299 to 770, according to NC001653). PCR conditions included an initial denaturation step (94 °C, 4 min), 32 cycles of 30 sec at 94 °C denaturation, 30 sec at 54 °C annealing, 30 sec at 72 °C extension, followed by a final extension at 72 °C for 10 min. The second PCR round was performed using the primer pairs HDV48_F and HDV54_R to amplify the inner fragment (nucleotides 313 to 485, according to NC001653). PCR conditions included initial denaturation for 4 min at 94 °C, followed by 35 cycles (denaturing at 94 °C for 30 sec, annealing at 58 °C for 30 sec and extension at 72 °C for 30 sec), with a final 10 min extension step at 72 °C. Amplicons obtained in the second PCR round were visualized on 1.5% agarose gels. Samples were considered positive when HDV-RNA was detected at least twice in three consecutive PCR runs.

HDV genotyping and phylogenetic analysis. In order to define HDV genotypes, nested PCRs were performed using primer pairs HDV04_F and HDV05_R for the first round for amplification of the outer fragment (nucleotides 856 to 1179, according to NC001653; Table 4). For the second round we used primer pairs HDV06_F and HDV07_R to amplify the inner fragment (nucleotides 888 to 1122, according to NC001653). Cycling parameters were identical with those applied for HDV detection as described above. The inner PCR amplicons obtained in the second round were purified using either the GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Europe GmbH, Freiburg, Germany) or the Exo-SAP-IT kit (USB, Affymetrix, MA, USA) following the manufacturer's instructions. Purified PCR products were subsequently used as sequencing templates using the ABI 3130XL system and the BigDye terminator v.1.1 sequencing kit (Applied Biosystems, Foster City, CA, USA).

Phylogenetic trees were constructed using the MEGA7 software (www.megasoftware.net). References of the full genomes of eight HDV genotypes (HDV1-8) were retrieved from available NCBI GenBank data, including the sequences of genotypes HDV1 (AB118848, AF098261, AF425644, AJ000558, AJ307077, AY633627, HM046802, KF660600, KF660601, KF660602, M92448, NC001653, S75645, X77627, X85253), HDV2 (KF660598, AF425645, AJ309879, AJ309880, AY261457, AY261459, AY261460, KF660599, U19598, X60193), HDV3 (AB037947, AB037948, L22063), HDV4 (AF309420, HM309420), HDV5 (AM183326, AX741154, JA417546), HDV-6 (AM183329, AM183332), HDV7 (AJ584844, AM183333) and HDV8 (AM183330, AX741169, LT604974). All sequences were aligned using BioEdit software version 7 (http://www.mbio.ncsu.edu/BioEdit/page2.html) and the CLUSTAL Muscle algorithm[36]. Phylogenetic trees were constructed using the neighbour joining method[37] and the Kimura-2 model[38]. Statistical robustness and reliability of the branching order was confirmed by bootstrap analysis using 1000 iterations.

Statistical analysis. For statistical analyses the R software (https://www.r-project.org) and GraphPad Prism 7 (http://www.graphpad.com) were applied. Chi-square or Fisher's exact test were performed to test for differences of categorical variables between two or more than two groups. Wilcoxon-Mann-Whitney and Kruskal-Wallis tests were used to compare nonparametric data of quantitative variables between two or more than two groups. The level of significance was $P < 0.05$.

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Author Contributions

T.P.V. designed, supervised the study, contributed materials and reagents, interpreted data and wrote the manuscript. M.T.B. recruited patients, performed the experiments, carried out the statistical analyses and wrote the manuscript. N.X.H. recruited patients and collected samples, performed the experiments, carried out the statistical analyses and wrote the manuscript. H.V.T. contributed to the analysis. D.P.G. performed the experiments. B.T.S. contributed to the experimental design. N.L.T. recruited patients and collected samples. L.H.S. recruited patients and collected samples. M.H.B. recruited patients. H.W. contributed to the study design. C.G.M. revised the draft. P.G.K. contributed materials and reagents. C.T.B. contributed to standard experimental procedures. All authors agreed with the results and conclusions.

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3.2 Chapter 2: NTCP S267F variant associates with decreased susceptibility to HBV and HDV infection and decelerated progression of related liver diseases

Scientific paper No.2

NTCP S267F variant associates with decreased susceptibility to HBV and HDV infection and decelerated progression of related liver diseases

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NTCP S267F variant associates with decreased susceptibility to HBV and HDV infection and decelerated progression of related liver diseases

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ABSTRACT

Objectives: To determine potential associations of the rs2296651 variant (c.800C > T, S267F) of NTCP with HBV and HBV plus concomitant HDV infection as well as with the progression of related liver diseases.
Methods: The S267F variant was genotyped by DNA sequencing in 620 HBV-infected patients and 214 healthy controls (HCs). Among the patients, 450 individuals were tested for HDV by a nested PCR assay. Logistic regression was applied to examine the association.
Results: The S267F variant was found more frequently among HCs (16%) compared to HBV-infected (6%) and HBV-HDV co-infected patients (3%) (HBV patients vs HC: OR = 0.32, $P = 0.00002$ and HDV patients vs. HC: OR = 0.17, $P = 0.018$). The frequency of S267F variant was inversely correlated with CHB, LC or HCC patients compared with HCs (OR = 0.31, $P = 0.001$; OR = 0.32, $P = 0.013$; OR = 0.34, $P = 0.002$, respectively). S267F variant was also associated with decreased risk of the development of advanced liver cirrhosis (LC) and hepatocellular carcinoma (HCC) (Child B and C vs. Child A, OR = 0.26, adjusted $P = 0.016$; BCLC B,C,D vs. BCLC A, OR = 0.038, $P = 0.045$, respectively). In addition, patients with the genotype CT had lower levels of AST, ALT, total and direct bilirubin as well as higher platelet counts, indicating an association with a more favorable clinical outcome.
Conclusion: The NTCP S267F variant of the *SLC10A1* gene exhibits protective effects against HBV and HDV infection and is associated with a reduced risk of developing to advanced stages of LC and HCC.

Introduction

Although effective hepatitis B virus (HBV) vaccines are in use worldwide, HBV-related liver diseases are still a major public health concern, causing considerable morbidity and mortality. Approximately 257 million people are currently suffering from chronic hepatitis B and 887,000 deaths have been recorded in 2015 due to HBV infection (WHO, 2017a). HBV causes various clinical conditions, including acute hepatitis B, chronic hepatitis B (CHB), liver cirrhosis (LC) and hepatocellular carcinoma (HCC) (Liaw and Chu, 2009; WHO, 2017a). The risk of HCC development in chronic

Abbreviations: NTCP, sodium taurocholate co-transporting polypeptide; S267F, substitution of serine at position 267 of NTCP with phenylalanine; SNP, single nucleotide polymorphism; HC, healthy control; HBV, hepatitis B virus; HDV, hepatitis delta virus; CHB, chronic hepatitis B; LC, liver cirrhosis; HCC, hepatocellular carcinoma; HBsAg, Hepatitis B surface antigen; HDAg, Hepatitis D antigen; BCLC, Barcelona Clinic Liver Cancer staging; AFP, alpha-fetoprotein; PLT, platelets; AST and ALT, aspartate and alanine amino transferase.

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HBV carriers is approximately 40 times higher than that in non-carriers (Lee et al., 2013).

Hepatitis D virus (HDV), an RNA virus first identified in 1977 (Rizzetto et al., 1977), can cause disease only in the presence of preexisting HBV infection, as it requires HBV envelope proteins for effective infection of hepatocytes (Sureau and Negro, 2016). HDV particles contain a circular single-stranded RNA of 1679 nucleotides and two viral proteins, the small and large hepatitis D antigens, which are surrounded by an outer coat containing HBV-derived envelope proteins and host phospholipids (Hughes et al., 2011). HDV coinfection affects 15–20 million HBV carriers worldwide (Noureddin and Gish, 2014; WHO, 2017b). HDV infection is associated with an increased risk of LC and HCC development (Hughes et al., 2011).

As both HDV and HBV utilize identical proteins, they may enter the hepatocytes through similar mechanisms. Recently, the sodium taurocholate co-transporting polypeptide (NTCP) receptor has been identified as a cellular receptor for both HBV and HDV entry (Ni et al., 2014; Yan et al., 2012). A homozygous non-synonymous Arg252His substitution in the *NTCP* was associated with the impaired uptake of bile salts into hepatocytes, confirming the important role of this hepatic bile acid transporter (Vaz et al., 2015). NTCP is encoded by the *SLC10A1* gene (Solute Carrier family 10, member 1) located on chromosome 14. It is a transmembrane protein and involved in transport of sodium and bile acids across cellular membranes. The N-terminus of the pre-S1 domain of the large HBV envelope protein binds to NTCP, which is predominantly expressed at the basolateral membrane of hepatocytes, supporting HBV and HDV entry into hepatocytes (Ni et al., 2014; Yan et al., 2012).

The missense rs2296651 variant (*c.800C > T*, S267F; substitution of serine by phenylalanine at position 267) of the *SLC10A1* gene may influence HBV infection by modifying the structure of the membrane receptor, resulting in decreased susceptibility of hepatocytes to HBV/HDV infection. Several studies have shown that the S267F variant influences susceptibility to HBV infection, but not in HDV infection and that it is associated with a decreased risk of liver disease progression (Hu et al., 2016; Lee et al., 2017; Peng et al., 2015).

Vietnam is highly endemic for both HBV and HDV infections (Mai et al., 2018; Nguyen et al., 2017; Sy et al., 2013). The functional role of the missense S267F variant in HBV infection has not yet been investigated, and data on whether S267F correlates with susceptibility or resistance to HDV infection is still limited. We conducted a genetic association study on the role of the NTCP S267P variant in HBV and HBV/HDV infection as well as its association with clinical progression of related liver diseases.

Materials and methods

Patients

We randomly recruited 620 HBV-positive patients and 214 healthy controls (HCs) at 108 Military Central Hospital, Hanoi, Vietnam, between 2013 and 2015. Patients and healthy controls represent individuals from the Hanoi metropolitan area and were of Kinh ethnicity. The patients were clinically characterized and HBsAg-positive for at least 6 months. Patients were classified into clinical subgroups, including CHB patients without LC or HCC (n = 176), HBV-related LC patients (n = 144) and HBV-related HCC patients (n = 300). The clinical and diagnostic characteristics applying to each subgroup have been described previously (Hoan et al., 2017). LC and HCC patients were further grouped according to the Child-Pugh scores A, B, and C (Cholongitas et al., 2005). HCC patients were categorized according to the Barcelona Clinic Liver Cancer (BCLC) staging (Diaz-Gonzalez et al., 2016). HCs were civilian individuals and were blood donors tested to be HBsAg seronegative. Both patients and HCs were negative for anti-HCV and anti-HIV antibodies as assessed by routine ELISA assays. None of the study participants had a history of alcohol or drug abuse. Of the 620 HBV-infected patients, 450 individuals were tested for concomitant HDV-infection. Blood sampling of all patients was performed on hospital admission. Whole blood and serum samples were stored at −80 °C until further use.

Ethics statement

Informed written consent was obtained from all study participants after detailed explanation of the study at the time of blood and serum sampling. The study protocol was approved by the Institutional Review Board of the 108 Military Central Hospital, Hanoi, Vietnam. All experiments were performed in accordance with applying guidelines and regulations.

NTCP genotyping

Genomic DNA was isolated from whole blood using a DNA purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Exon 4 of the *SLC10A1* gene was amplified using primers NTCP_4F (5′-CCA TCG CTG CGA AAC TC-3′) and NTCP_4R (5′-GGG CTA CCT GGT TCT TAG TGA-3′). PCR amplification was carried out in 20 μl reaction volumes containing 1 X PCR buffer, 1 X Q solution, 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. Thermal cycles consisted of initial denaturation (95 °C, 5 min), 36 cycles of denaturation (30 s, 95 °C), annealing (20 s, 55 °C), extension (30 s, 72 °C), followed by a final extension step (10 min, 72 °C). PCR products were visualized on 1.2% agarose gels. Amplicons were purified by Exo-SAP-IT (USB, Affymetrix, CA, 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, CA, USA). Genotypes of SNP rs2296651 were wild-type (*CC*), heterozygous (*CT*) and homozygous (*TT*).

HDV detection

Viral RNA was isolated from serum (QIAamp Viral RNA Mini Kit; Qiagen GmbH, Hilden, Germany) and RNA was subsequently reversely transcribed into cDNA using the High-Capacity cDNA Reversers Transcription Kit (Thermo Fisher Scientific, Foster City, CA, USA) following the manufacturer's instructions. HDV-specific nested PCR was employed for HDV detection as described previously (Mai et al., 2018; Nguyen et al., 2017; Sy et al., 2013).

Statistical analysis

Clinical and demographic data are given in medians with ranges for quantitative variables and categorical data, provided as numbers and percentages. Hardy-Weinberg equilibrium was assessed. Binary logistic regression models adjusted for age and gender were applied to determine NTCP S267F associations with HBV and HBV/HDV-related liver diseases. Adjusted odds ratios (OR) with 95% confidence intervals (CI) were calculated. Chi-square and Fisher's exact tests were used to test for differences in categorical variables. Kruskal–Wallis and Mann–Whitney–Wilcoxon tests were applied to compare quantitative variables. Statistical analyses were performed using SPSS version 22 (SPSS Statistics, IBM, Armonk, NY, USA) and GraphPad Prism 7 (http://www.graphpad.com). Significance was set at a value of $P < 0.05$.

Table 1
Demographic and clinical characteristics of healthy controls and HBV patients.

Characteristics	HC (n = 214)	HBV (n = 620)	CHB (n = 176)	LC (n = 144)	HCC (n = 300)	*P* value
Age (years)	46 [18–69]	55 [18–90]	39 [18–85]	57 [20–86]	60.5 [18–90]	<0.0001‡
Male (%)	66.8	85.8	77.3	83.3	92	<0.0001 β
Child-Pugh	NA					
Child A			NA	50/144	217/300	
Child B			NA	55/144	67/300	
Child C			NA	38/144	16/300	
Clinical parameters						
AST (U/L)	NR	63 [14–6206]	44 [14–6206]	60 [15–1221]	60 [17–983]	0.0096‡
ALT (U/L)	NR	52 [8–3390]	64 [9–3390]	56 [8–1426]	47 [10–934]	<0.0001
Total bilirubin (μmol/L)	NR	19 [4.1–571]	17 [5.5–551]	31.3 [4.1–571]	17 [4.3–392]	<0.0001‡
Direct bilirubin (μmol/L)	NR	6.5 [0.4–349]	6.2 [0.7–349]	12 [0.4–291]	5.4 [0.4–247.3]	<0.0001‡
Albumin (g/L)	NR	39 [12–51]	42 [12–51]	31 [15–47]	38 [18–49]	<0.0001‡
Prothrombin (%)	NR	85 [13–269]	92 [17–267]	58.5 [13–101]	86 [20–269]	<0.0001‡
WBC ($x10^3$/mL)	NR	6.13 [1.7–20.5]	6.2 [4.1–13.44]	5.6 [1.7–20.5]	6.15 [2.7–17.8]	0.0011‡
RBC($x10^6$/mL)	NR	4.51 [1.7–6.8]	4.9 [3.1–6.8]	4.04 [1.9–6.7]	4.5 [1.7–6.3]	<0.0001‡
PLT ($x10^3$/ml)	NR	170 [17–441]	211 [89–360]	90 [17–441]	159 [35–432]	<0.0001‡
HBV DNA (log_{10} copies/ml)	NR	5 [2–10]	6 [2–10]	5 [2–10]	5 [2–9]	0.018‡
AFP (IU/L)	NR	9.8 [0.84–300]	3.5 [1–300]	6.8 [1.18–300]	111.4 [0.84–300]	<0.0001‡

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; NR, normal range, NA, not applicable. Values given are medians and ranges. (‡) Kruskal–Wallis test. (β): chi-square test.

Results

Baseline characteristics of study subjects

The demographic, laboratory and clinical parameters of the 834 participants are summarized in Table 1. In the HC group, the mean age was 46 years (range: 18–69), and the majority of HCs were male (67%). Of the 620 patients, 532 (86%) were male. The mean age of patients was 55 years (18–90), and the median age of patients increased according to the degree of progression of liver diseases ($P < 0.0001$). The albumin and prothrombin levels and platelet counts were higher among CHB patients compared to HCC and LC patients ($P < 0.0001$). We also observed high HBV DNA levels in CHB compared to LC and HCC patients ($P = 0.018$). Higher total bilirubin and direct bilirubin were observed in LC patients compared to the subgroups of CHB and HCC patients ($P < 0.0001$). AFP levels were significantly higher among HCC patients compared to the subgroups of CHB and LC patients ($P < 0.0001$).

Association of the S267F variant with HBV infection and clinical outcome

The genotype and allele frequencies of the S267F variant (rs2296651) in the 620 HBV patients and 214 HCs are shown in Table 2. Genotype frequencies of both HCs and cases were in Hardy Weinberg equilibrium ($P = 0.6$ and 0.09, respectively). The genotype frequency of S267F (*CT/TT*) was significantly lower in HBV patients (6%) compared to HCs (16.4%) (OR = 0.32, 95% CI = 0.19-0.54, adjusted $P = 0.00002$; Table 2); indicating that genotypes *CT* and *TT* are associated with relative resistance to HBV infection. Similarly, the frequency of the *T* allele was also significantly lower among HBV patients (3%) compared to HCs (8.4%), suggesting that the *T* allele exerts a protective role in HBV infection (OR = 0.34, 95% CI = 0.2–0.57, adjusted $P = 0.00004$) (Table 2). In order to confirm the protective role of S267F in HBV infection, we compared the frequency of the *CT* genotype in HBV monoinfection with that found in the HCs. The analysis showed that this genotype contributes to a significantly decreased risk of HBV monoinfection (OR = 0.39, 95% CI = 0.22-0.69, adjusted $P = 0.0012$) (Table 4). Frequencies of genotypes and alleles did not differ between the patient subgroups of CHB, LC and HCC (data not shown).

Next, we analyzed the association of S267F with clinical outcomes of the HBV patients. Individuals carrying the genotype *CT* had significantly higher platelet counts ($P = 0.002$), but lower serum AST as well as total and direct bilirubin levels ($P = 0.012$; 0.031; 0.038, respectively) compared to patients with the genotype *CC* (Figure 1). Although not statistically significant, individuals with genotype *CT* had a similar trend of ALT, prothrombin, total protein and albumin levels compared to those carrying the genotype *CC* (data not shown).

Association of S267F with LC and HCC

We applied a logistic regression model adjusted for age and gender to analyze the association of S267F with LC and HCC. Compared to the HCs, individuals with the *CT* genotype had a 3-fold decreased risk of both LC and HCC (LC vs. HC: OR = 0.32, 95% CI = 0.13–0.79, adjusted $P = 0.013$; HCC vs. HC: OR = 0.34, 95% CI = 0.17–0.68, $P = 0.002$) (Table 2).

Patients with LC were classified into the Child-Pugh subgroups A, B and C. Genotype *CT* was significantly more frequent in patients with more advanced LC compared to patients with less advanced LC (Child-Pugh scores B and C *vs.* Child-Pugh score A: OR = 0.26, 95% CI = 0.09–0.78, $P = 0.016$). Patients with HCC were classified into the BCLC subgroups stage A, B, C and D. The frequency of the genotype *CT* was significantly higher among patients with intermediate and advanced stages of HCC compared to those with an early stage of HCC (BCLC staging B, C and D *vs.* BCLC staging A: OR = 0.38, 95% CI = 0.15-0.97, $P = 0.045$) (Table 3).

S267F in HDV infection

To explore the protective role of genotype *CT* in HDV infection, we compared the frequency of genotype *CT* in HBV-HDV co-infected patients with HCs. The analyses indicated that S267F variant contributes significantly to a decreased risk of HBV-HDV coinfection (OR = 0.17, 95% CI = 0.04–0.74, adjusted $P = 0.018$). Although not statistically significant, chronic HBV patients with genotype CT had a reduced risk of concomitant HDV infection compared to those carrying genotype *CC* (Table 4).

Table 2
Association of NTCP S267F variant with HBV-related liver diseases.

NTCP rs2296651 (S267F)	CHB n = 176 (%)	LC n = 144 (%)	HCC n = 300 (%)	HBV n = 620 (%)	HC n = 214 (%)	HBV patients vs. HCs		CHB vs. HC		LC vs. HC		HCC vs. HC	
						OR (95%CI)	*P* value	OR (95%CI)	*P*	OR (95%CI)	*P*	OR (95%CI)	*P*
Genotype													
CC	165 (93.8)	137 (95.1)	281 (93.7)	583 (94)	179 (83.6)	Reference		Reference		Reference		Reference	
*CT+TT**	11 (6.2%)	7 (4.9)	19 (6.3)	37 (6)	35 (16.4)	**0.32 (0.19–0.54)**	**0.00002**	**0.31 (0.15–0.64)**	**0.001**	**0.32 (0.13–0.79)**	**0.013**	**0.34 (0.17–0.68)**	**0.002**
Allele													
C	341 (96.9)	281 (97.6)	581 (96.8)	1203 (97)	392 (91.6)	Reference		Reference		Reference			
T	11 (3.1%)	7 (2.4)	19 (3.2)	37 (3)	36 (8.4)	**0.34 (0.2–0.57)**	**0.00004**	**0.32 (0.16–0.63)**	**0.001**	**0.32 (0.13–0.78)**	**0.012**	**0.33 (0.17–0.63)**	**0.001**

CHB, chronic hepatitis B; LC, liver cirrhosis; HCC, hepatocellular carcinoma; HC, healthy controls; n, numbers individuals; OR, Odd ratio. *P* values were calculated using binary logistic regression model adjusted for age and gender. (*), one healthy carried TT genotype.
Bold values reflect statistical significance.

Discussion

NTCP is a member of the solute carrier family of transporters. Its major physiological function is the transport of bile acids from portal blood into hepatocytes (Claro da Silva et al., 2013; Hagenbuch and Meier, 1994). NTCP is the hepatocytic receptor for HBV and HDV (Ni et al., 2014; Yan et al., 2012); thus, genetic variation of the gene encoding NTCP might be associated with HBV and HDV susceptibility. Previous studies have indicated the clinical significance of the NTCP variant S267F only in HBV infection (Hu et al., 2016; Lee et al., 2017; Peng et al., 2015; Wang et al., 2017). Our data confirm the protective role of S267F in HBV infection, including the stages of HBV-related liver disease progression and especially also in HBV-HDV coinfection.

Several studies have reported that the genotype and allele frequencies of S267F vary considerably between different study groups and geographical regions (Ezzikouri et al., 2017; Hu et al., 2016; Lee et al., 2017; Li et al., 2014; Pan et al., 2011; Peng et al., 2015; Yang et al., 2016; Zhang et al., 2017). Here, we report that the frequencies of the genotypes CC, CT and TT are 83.6%, 15.9% and 0,5%, respectively, among 214 Vietnamese healthy individuals. The frequency of the S267F genotypes *CT* and *TT* in our study was lower than in other Asian populations, e.g. in the Chinese Han (20.4%) and Taiwanese (18.5%) (Hu et al., 2016; Peng et al., 2015), but more common than in the Korean population (5.7%) (Lee et al., 2017). Although the occurrence of S267F was significantly lower in HBV patients than in HCs in most studies, including our present study, its frequency is regionally different, ranging from 0.9% to 18% (Ezzikouri et al., 2017; Hu et al., 2016; Lee et al., 2017; Li et al., 2014; Pan et al., 2011; Peng et al., 2015; Yang et al., 2016; Zhang et al., 2017).

A protective effect of S267F in HBV infection has been reported in previous studies, indicating that individuals with the S267F variant were 2–5-fold less susceptible to chronic HBV infection (Hu et al., 2016; Lee et al., 2017; Peng et al., 2015). Our study yields corresponding results (OR = 0.3, 95% CI = 0.19–0.54). The protective effect of S267F on HBV infection has also been demonstrated in *in vitro* experiments, showing that in mixed cells of wild-type NTCP and S267F at a 1:1 ratio the efficiency of HBV infection was higher than 70% (Yan et al., 2014), suggesting that the *T* allele of S267F contributes to a certain degree to resist HBV infection. Similarly, we observed that the *T* allele of S267F contributes to reducing the risk of CHB (OR = 0.34).

The earlier studies have also shown that S267F is independently associated with a decreased risk of progression to LC and/or HCC (Hu et al., 2016; Lee et al., 2017; Wang et al., 2017). In our study, when comparing HBV patients with advanced stages of LC and HCC with HCs, those carrying S267F had a significantly decreased risk of developing LC or HCC, indicating a lower probability of unfavorable clinical outcome compared to individuals carrying the wild-type genotype. The substitution of serine, a hydrophilic residue, by phenylalanine, a large hydrophobic residue, alters the structure of NTCP and causes a modification of the HBV/HDV receptor function (Yan et al., 2012), but also results in a reduced function of bile acid transport (Ho et al., 2004). Bile acids are cytotoxic compounds, and as their concentrations increase in the liver, they trigger hepatocyte apoptosis by activating the death receptor interactive signaling pathway, thereby promoting persistent inflammatory injury (Faubion et al., 1999; Ho et al., 2004; Miyoshi et al., 1999). The NTCP S267F variant decreases uptake of bile acids into hepatocytes, thus reducing accumulation of intrahepatic cytotoxic bile salts (Ho et al., 2004). Decreased uptake of bile acids into hepatocytes is associated with mild hypotonia, growth retardation, and delayed motor milestones (Vaz et al., 2015). However, the relationship between reduced bile salt uptake into hepatocytes

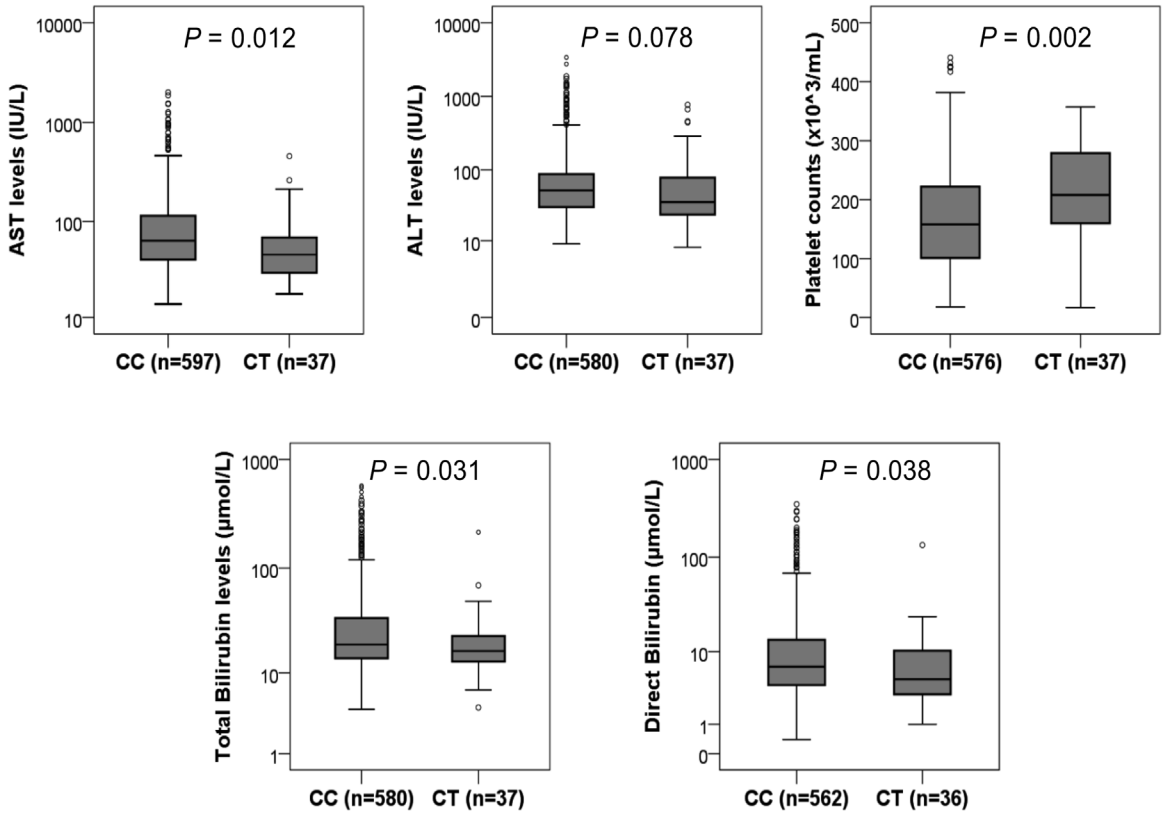

Figure 1. Association of NTCP S267F variant with clinical outcome of HBV infection.
Laboratory parameters were compared between patients with genotype *CC* and those with genotype *CT*. Box plots illustrate medians with interquartile range. *P* values were calculated by using Mann–Whitney–Wilcoxon test.

Table 3
Association of NTCP S267F variant with LC and HCC progression.

Child-Pugh score									
NTCP rs2296651 (S267F)	Child A n = 266 (%)	Child B n = 123 (%)	Child C n = 54 (%)	Child B vs. Child A		Child C vs. Child A		Child B/C vs. Child A	
				OR (95%CI)	*P*	OR (95%CI)	*P*	OR (95%CI)	*P*
CC	245 (92.1)	120 (97.6)	52 (96.3)	Reference		Reference		Reference	
CT	21 (7.9)	3 (2.4)	2 (3.7)	**0.19 (0.04–0.81)**	**0.025**	0.44 (0.1–1.9)	0.28	**0.26 (0.09–0.78)**	**0.016**
BCLC classification									
NTCP rs2296651 (S267F)	Stage A n = 94	Stage B n = 132	Stage C/D n = 55	Stage B vs. Stage A		Stages C/D vs. Stage A		Stages B/C/D vs. Stage A	
				OR (95%CI)	*P*	OR (95%CI)	*P*	OR (95%CI)	*P*
CC	83 (88.3)	127 (96.2)	52 (94.6)	Reference		Reference		Reference	
CT	11 (11.7)	5 (3.8)	3 (5.4)	**0.33 (0.11–0.99)**	**0.049**	0.54 (0.06–4.5)	0.5	**0.38 (0.15–0.97)**	**0.045**

Child A, B, C: Child-Pugh score A, B, C; Stage A, B, C, D: Barcelona Clinic Liver Cancer stage A, B, C, D; *P* values were calculated using binary logistic regression model adjusted for age and gender.
Bold values reflect statistical significance.

Table 4
Association of NTCP S267F variant with HBV and HDV infections.

NTCP rs2296651 (S267F)	HC n = 214 (%)	HBV monoinfection n = 386 (%)	HDV/HBV coinfection n = 64 (%)	HBV monoinfection vs. HC		HDV/HBV coinfection vs. HC		HDV/HBV coinfection vs. HBV monoinfection	
				OR (95%CI)	*P* value	OR (95%CI)	*P* value	OR (95%CI)	*P* value
Genotype									
CC	179 (83.6)	361 (93.1)	62 (96.8)	Reference		Reference		Reference	
*CT + TT**	35 (16.4)	25 (6.9)	2 (3.2)	**0.39 (0.22–0.69)**	**0.0012**	**0.17 (0.04–0.74)**	**0.018**	0.44 (0.1–1.9)	0.27
Allele									
C	392 (91.6)	747 (96.8)	126 (98.4)	Reference		Reference		Reference	
T	36 (8.4)	25 (3.2)	2 (1.6)	**0.38 (0.22–0.66)**	**0.0006**	**0.17 (0.04–0.73)**	**0.017**	0.45 (0.11–1.93)	0.28

HC, healthy controls; HBV mono, hepatitis B monoinfection; HDV/HBV, HDV and HBV coinfection, n = numbers of individuals; OR, Odd Ratio. *P* values were calculated using binary logistic regression model adjusted for age and gender. (*), one healthy carried TT genotype.
Bold values reflect statistical significance.

and lower risk of LC and HCC development warrants further investigation.

Regarding the association of the S267F variant with HDV infection, individuals carrying S267F had a lower risk of concomitant HDV infection. However, we did not observe any difference of S267F genotype and allele frequencies when the comparison between HBV monoinfection and HDV/HBV coinfection was considered. This may be explained by the fact that HBV and HDV share NTCP as hepatocytic receptor and the minor allele of S267F contributes to impair entry of both HBV and HDV.

A larger number of HDV-HBV coinfected patients is desirable to confirm any association of S267F with HDV infection as well as to correlate the genetic findings with the clinical outcome of HBV-HDV coinfection. In conclusion, the NTCP S267F variant was frequent in the Vietnamese population. It is associated with decreased susceptibility to HBV and HDV infection, as well as with a decreased occurrence of LC and HCC and advanced stages of HBV-related liver diseases.

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Conflict of interests

All authors have no conflicts of interest to declare.

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Author's contributions

TPV designed, supervised the study, contributed materials and reagents, and wrote the manuscript. PGK contributed materials and reagents. MTB recruited patients and collected samples, performed the experiments, carried out the statistical analyses, interpreted data and wrote the manuscript. NXH recruited patients and collected samples, carried out the statistical analyses and edited the manuscript. HVT contributed to the analysis, interpreted data and edited the manuscript. BTS and NTT contributed to the experimental design. LHS recruited patients. MHB recruited patients. CGM revised the draft and edited the final version of the manuscript. All authors agreed with the results and conclusions.

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3.3 Chapter 3 3: Association of *SMYD3* VNTR, SMYD3 serum levels and SMYD3 expression in the clinical courses of HBV infection

Scientific paper No.3

Upregulation of *SMYD3* and *SMYD3* VNTR 3/3 polymorphism increase the risk of hepatocellular carcinoma

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Under review

Upregulation of *SMYD3* and *SMYD3* VNTR 3/3 polymorphism increase the risk of hepatocellular carcinoma

Abbreviations: SMYD3, SET and MYND domain-containing protein 3; VNTR, variable number of tandem repeats; SNP, single nucleotide polymorphism, HC, healthy control; HBV, hepatitis B virus; CHB, chronic hepatitis B; LC, liver cirrhosis; HCC, hepatocellular carcinoma; HBsAg, Hepatitis B surface antigen; BCLC, Barcelona Clinic Liver Cancer staging; AFP, alpha-fetoprotein; PLT, platelets; AST and ALT, aspartate and alanine amino transferase.

ABSTRACT

Background: SMYD3 (SET and MYND domain-containing protein 3) is involved in histone modification, which initiates oncogenesis by activating transcription of multiple downstream genes.

Objective: To investigate associations of variable numbers of tandem repeats (VNTR) variants in the *SMYD3* gene promoter, SMYD3 serum levels and *SMYD3* mRNA expression in hepatitis B virus (HBV) infection and clinical progression of related liver disease.

Methods: *SMYD3* VNTRs were genotyped in 756 HBV patients and 297 healthy controls. SMYD3 serum levels were measured in 293 patients and *SMYD3* mRNA expression was quantified in 48 pairs of hepatocellular tumor and adjacent non-tumor liver tissues.

Results: Genotype *SYMD3* VNTR 3/3 was more frequent among HCC patients than in controls ($P_{adjusted}$=0.037). SMYD3 serum levels increased according to clinical progression of liver diseases (P=0.01); HCC patients had higher levels than non-HCC patients (P=0.04). Among patients with *SMYD3* VNTR 3/3, HCC patients had higher SMYD3 levels than others (P<0.05). *SMYD3* mRNA expression was up-regulated in HCC tumor tissues compared to other tissues (P=0.008).

Conclusion: Upregulation of *SMYD3* correlates with the occurrence of HCC and *SMYD3* VNTR 3/3 appears to increase the risk of HCC through increasing SMYD3 levels. SMYD3 may be an indicator for HCC development in HBV patients.

Keywords: HBV infection; hepatocellular carcinoma; SMYD3; SMYD3 VNTRs;SMYD3 serum levels; *SMYD3* mRNA expression.

Key summary

- SMYD3 is involved in oncogenesis and is upregulated in various cancer types.
- *SMYD3* VNTR 3/3 appears to increase the risk of HCC through increasing SMYD3 levels
- SMYD3 serum levels were increased according to the clinical progression.
- Upregulation of *SMYD3* correlates with the occurrence of HCC.
- SMYD3, a potential indicator for HCC development in patients with HBV infection.

Introduction

Although effective hepatitis B virus (HBV) vaccines are in use worldwide, HBV-related liver diseases are still a major health concern with approximately 257 million chronic infections and 887,000 deaths in 2015.[1] HBV is the main cause of primary hepatocellular carcinoma (HCC)[2]. The risk of HCC development is approximately 40 times higher in chronic HBV carriers (CHB) than in non-carriers.[3] Vietnam has a high prevalence of HBV infections, ranging from 10-20% in the general population and 20-40% among high-risk groups.[2,4] As a result, Vietnam is one of the countries with a high incidence of HCC with >25,000 new cases reported in 2018.[5,6]

Further to viral factors contributing to cancer development, methylation of histone proteins is an important mechanism involved in multiple types of cancers including HCC.[7,8] Methylation of histone proteins at lysine residues can lead to chromatin remodelling, transcription, and signal transduction.[9] The SET and MYND domain containing proteins (SMYDs) belong to a family of the multi-domain SET-containing histone lysine methyltransferases and play a crucial role in histone methylation. To date, five SMYD family members have been recognized (SMYD1-SMYD5). SMYD3 is the most important member, as several findings have demonstrated its role in tumor cell growth and its increased expression in various cancers [reviewed in Ref. 10].

SYMD3 promotes dimethylation and trimethylation of histone H3K4, which initiates oncogenesis by activating transcription of multiple downstream target genes.[11,12] SYMD3 overexpression causes cell proliferation, migration and adhesion, whereas suppression by RNAi inhibits cell proliferation and migration, indicating that SMYD3 plays an important role in carcinogenesis.[13-15] SYMD3 was found upregulated in HCC, colorectal and in breast and lung cancers.[13,16,17] *In vitro* interaction of SMYD3 with HBV has been demonstrated, showing that SMYD3 expression was upregulated by HBx in HepG2 cells, promoting HCC development and clinical progression.[18] Moreover, SMYD3 is a HBx-interacting protein, and this interaction induces activation of the activator protein 1 (AP-1), which increases the risk of HCC formation.[19,20]

The common variable number of tandem repeat (VNTR) CCGCC sequence in the *SMYD3* promoter region is the binding site for the transcription factor E2F-1 and shown to be a susceptibility factor for human malignancies.[21] It has been suggested that, compared to the genotype containing two copies, the genotype involving three copies of the CCGCC motif might enhance the binding affinity to E2F-1 and, as a result, promote cancer progression by activating transcription of multiple oncogenes such as myc, STAT3 and β-cat.[15,21,22] Moreover, the VNTR genotype 3/3 was associated with a higher risk of colorectal cancer, hepatocellular carcinoma, breast cancer, ovarian cancer and esophageal squamous cell carcinoma.[21,23,24] Particularly in liver diseases, VNTR genotype 3/3 contributed an over 3-fold increased risk of HCC in a Japanese and a Chinese population.[21,25] The present study aimed to investigate the association of VNTR polymorphisms in the *SMYD3* promoter and SMYD expression with HBV infection and clinical progression of HBV-related liver diseases, in particular progression to HCC.

Patients and methods

Patients

Seven hundred and fifty-six Vietnamese chronic HBV-infected patients enrolled between 2013 and 2015 at the 108 Military Central Hospital, Hanoi, Vietnam, were recruited. All patients were negative for anti-HCV and anti-HIV antibodies nor had a history of alcohol or drug abuse. Patients were categorized into the subgroups of chronic hepatitis B (CHB) without liver cirrhosis (LC) or HCC (n=246), HBV-related LC patients (n=174) and HBV-related HCC patients (n=336). The clinical and diagnostic characteristics of the study group have been described previously.[26] LC and HCC patients were further classified according to Child-Pugh scores A, B, and C[27] and HCC patients were categorized according to the Barcelona Clinic Liver Cancer (BCLC) staging system.[28] The control group (healthy controls; HC) consisted of 297 healthy blood donors. All HCs were HBsAg, anti-HCV and anti-HIV negative. Five mls of venous blood were collected from each participant. Serum was stored at -80 °C until further use.

In order to determine *SMYD3* mRNA expression, pairs of HCC tumor and adjacent non-tumor tissue specimens were obtained from 48 HCC patients who underwent surgery at the 108 Military Central Hospital. The tumor stage was scored following the BCLC system.[29]

Ethics statement

Informed written consent was obtained from all participants after detailed explanation of the study at the time of blood sampling. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee. The Institutional Review Board of the 108 Military Central Hospital, Hanoi, Vietnam (108MCH/Res/Epi-HBV-HDV-HEV-D2-14-03-2014) and the Ethics committee of University of Tübingen (206/2012B02) approved the study.

SMYD3 VNTR genotyping

Genomic DNA of all participants was isolated from whole blood (DNA purification kit; Qiagen, Hilden, Germany). The promoter region of the *SMYD3* gene was amplified using primers SMYD3_F (5'-CGC CTG TCT TCT GCG CAG TCG-3') and SMYD3_R (5'-CCC GAG AAG GCA GCG GTC G-3'). Amplicons underwent DNA sequencing.

Quantification of SMYD3 serum levels by ELISA

Of the 756 patients, 293 individuals were tested for SMYD3 serum levels, measured by a commercially available human SMYD3 sandwich ELISA kit (Wuhan Fine Biological Technology Co. Ltd, Wuhan City, China). In order to determine SMYD3 concentrations, a standard curve was plotted (https://www.curveexpert.net/) based on mean of OD values and the known concentration of the standards. Finally, SMYD3 concentrations were interpolated based on the standard curve. The detectable range of the kit was 31.25-2000 pg/ml.

Quantification of SMYD3 mRNA by RT-PCR

Total RNA of the liver tissues was extracted with Trizol reagent (Life Technologies, Carlsbad, CA, USA), followed by reverse transcription into cDNA (QuantiTect Reverse Transcription Kit; Qiagen, Hilden, Germany). *SMYD3* mRNA levels were assessed through quantification of *SMYD3* cDNA by qRT-PCR using SYBR Green PCR master mix (Bioline, Luckenwalde, Germany). The *GAPDH* gene (glyceraldehyde-3-phosphate dehydrogenase) was used as reference. Primer sequences were SMYD3_F: 5'-GTT GGC CTA TAT CCC AGT ATC TCT TTG CTC -3', and SMYD3_R: 5'-ACC AGT TAG CAT ATC AGC ATC CTT GTC CTG -3', GAPDH_F: 5'-CCA CCC ATG GCA AAT TCC ATG GCA-3' and GAPDH_R: 5'-TCT AGA CGG CAG GTC AGG TCC ACC-3'. All qRT-PCR reactions were performed in duplicate and repeated twice (LightCycler® 480 real-time PCR system; Roche, Basel, Switzerland). The fold change of *SMYD3* mRNA was normalized based upon the ΔΔCt method against expression of *GAPDH*.

Statistical analysis

Chi-square and Fisher´s exact tests were used to test for differences of categorical variables. Kruskal-Wallis and Mann-Whitney-Wilcoxon tests were applied to compare quantitative variables. Hardy-Weinberg equilibrium was assessed. Binary logistic regression models adjusted for age and gender were applied to determine *SMYD3* VNTR associations with HBV-related liver diseases. Adjusted odds ratios (OR) with 95% confidence intervals (CI) were calculated. Paired-samples t-test was used to compare *SMYD3* mRNA levels in tumor and adjacent non-tumor tissues. A linear regression model was applied to analyze the relationship of clinical parameters of patients and SMYD3 levels. Pearson's correlation coefficient test was used to analyze correlations between SMYD3 levels and clinical parameters. Statistical analyses were performed using SPSS version 22 (SPSS Statistics, IBM, Armonk, NY, USA) and GraphPad Prism 7 (http://www.graphpad.com). Significance was set at $P<0.05$.

Results

Patient characteristics

Demographic, laboratory and clinical parameters of the 1053 study participants are summarized in Table 1. In the HC group, the mean age was 43 years (range: 18-69), and majority of HCs were male (66.7%). Of the 756 patients, 630 (83.3%) were male; the mean age was 52 years (12-91). The median age of patients and the proportion of males increased according to the degree of progression of liver diseases (P<0.0001). As expected, albumin, prothrombin levels and platelet counts were higher among CHB patients compared to the other patient subgroups (P<0.0001). HBV DNA levels did not differ among patient subgroups (P=0.4). Higher total bilirubin and direct bilirubin were observed in LC patients compared to the other groups (P<0.0001). AFP levels were significantly higher in HCC patients compared to CHB and LC patients (P<0.0001) (Table 1).

SMYD3 VNTRs and HBV-related liver diseases

The allele frequencies and genotype distributions of *SMYD3* VNTR polymorphisms are given in Table 2. Allele frequencies of VNTR 2 and 3 were 21.7% and 78.3% among controls and 12% and 88% in HBV patients, respectively. The three tandem repeat allele (VNTR 3) was more prevalent among HBV patients than HCs (OR=1.4, 95% CI=1.07-1.86, adjusted P=0.017,). Genotype VNTR 3/3 was observed more frequently in patients compared to HCs (OR=1.4, 95% CI=1.02-1.97, adjusted P=0.036). Although the frequencies of VNTRs 2/2 and 2/3 were higher among HCs than in the patient group, the differences were not significant.

We compared the distribution of allele and genotype frequencies of the *SMYD3* VNTRs in the patient subgroups (CHB, LC, HCC) with those in HCs. Genotype VNTR 3/3 occurred more frequently in HCC patients than in HCs (OR=2.9, 95%CI=1.09-8, adjusted P=0.037,), while genotype VNTR 2/2 was nearly three times more frequent in HCs compared to HCC patients (HCC vs HC, OR=0.34, 95% CI=0.13-0.93, adjusted P=0.036). However, no differences were observed in pairwise comparisons between the CHB, LC and HC groups.

We also analyzed associations of *SMYD3* VNTR polymorphisms with laboratory parameters of HBV infection (AFP, AST, ALT, total and direct bilirubin, prothrombin, albumin, viral loads). The levels of these parameters were not different in all patients with different *SMYD3* VNTR genotypes as well as in patient subgroups (CHB, LC and HCC) (P>0.05).

SMYD3 serum levels in HBV-related liver diseases

Levels of SMYD3 increased significantly according to the progression of liver diseases (Figure 1A, 1B). The SMYD3 median was 227.1 pg/mL in CHB patients, 287.3 pg/mL in LC patients and 311.8 in HCC patients and 292.6 pg/mL in patients with both HCC plus LC (P<0.05). The post-hoc analysis showed a significant difference between the HCC and CHB groups (P=0.012). A corresponding result was observed when comparing the median of SMYD3 levels in non-HCC and in HCC patients (251.1 pg/mL and 311.8 pg/mL, respectively, P=0.04; Figure 1D), whereas the median of SMYD3 levels did not differ between non-LC and LC individuals (Figure 1C). These results indicate that SMYD3 plays a role in HCC development. When analyzing the correlation of SMYD3 serum levels with the laboratory parameters in all HBV patients and in patient subgroups, SMYD3 serum levels did not correlate with these parameters in all HBV patients and in the subgroups.

SMYD3 VNTRs and SMYD3 serum levels and progression of HBV-related liver diseases

We analyzed the relationship between *SMYD3* VNTR genotypes and SMYD3 serum levels in HBV patients (Figure 2). SMYD3 levels in patients with *SMYD3* genotype 3/3 were similar to that in patients with genotypes 2/2 or 2/3 (P>0.05, Figure 2A). In the HCC group, patients with genotype VNTR 3/3 had higher SMYD3 levels than those with genotypes VNTR 2/2 and 2/3 (P=0.03, Figure 2D). This was not observed in CHB and LC patients (Figure 2B, 2C). In addition, in patients with genotypes 2/2 and 2/3 the levels of SYMD3 did not differ between the CHB, LC and HCC groups. However, patients with genotype VNTR 3/3 had increased SMYD3 serum levels according to the

progression of liver diseases (HCC vs. CHB: *P*=0.0005, LC vs. CHB: *P*=0.036, Figure 2F). These results indicate that genotype VNTR 3/3 may increase SMYD3 levels and, thus, increase the risk of HCC development.

SMYD3 mRNA expression in HCC

In order to examine whether *SMYD3* mRNA is upregulated in cancer stages as seen in other cancers[15,16], we analyzed the expression of *SMYD3* mRNA in HCC tissue specimens and in adjacent non-tumor liver tissues obtained from 48 HBV-related HCC patients. Expression of *SMYD3* mRNA in tumor tissues was significantly higher than that in adjacent non-tumor tissues (*P*=0.008, Figure 3A). We then examined whether *SMYD3* mRNA expression was associated with development of HCC by correlating *SMYD3* mRNA expression with BCLC stages. *SMYD3* mRNA expression was higher in stage-B tumor tissues compared to that in stage-A tissues (Figure 3B). A similar trend was seen when *SMYD3* mRNA expression was compared between non-tumor tissues obtained from stage-A and stage-B HCC patients; however, the difference did not reach significance (Figure 3C). These results indicate that SMYD3 expression is associated with HBV-related HCC.

Discussion

HBV infection is a major cause of HCC. In Vietnam, the prevalence of HBV infection is over 10% in the general population[1,5]. Further to HBV, other factors contributing to the development of HCC include epigenetic changes such as methylation and histone modifications of regulatory genes.[7,8,26] SMYD3, a histone H3-K4 specific methyltransferase, is an example of histone modification which is considered a crucial epigenetic factor contributing to the development of various human cancers, including liver cancer.[13,16] So far, the role of SYMD3 and its encoding gene *SYMD3* in HBV-related liver diseases is not clear. We studied the association of genetic variation and expression of variant *SYMD3* VNTRs with susceptibility to HBV infection and with

liver disease progression. We show that both *SMYD3* VNTRs in the promoter region and *SYMD3* overexpression are associated with HBV-related HCC.

SMYD3 VNTR variability has been reported to be a significant factor in HCC.[21,25] An earlier study has reported that VNTR 3/3 homozygosity conferred an over 3-fold increased risk of HCC in a Japanese population.[21] Accordingly, a study in a Chinese population found that the frequency of the *SMYD3* VNTR 3/3 genotype was higher in HCC patients than in controls.[25] Consistent with these studies, we observed that the frequencies of the *SMYD3* VNTR three repeat allele and the 3/3 genotype were higher in HBV patients than in controls (Table 2). Further analysis showed that in the HCC group the *SMYD3* repeat 3/3 genotype was more frequent than in HCs (OR=2.9, $P<0.05$, Table 2). Notably, Wang et al.[30] did not find an association between the risk of HCC and the three tandem repeat allele.

The *SMYD3* VNTRs have been shown to be a susceptibility factor for human cancers, especially for colorectal cancer, breast cancer and HCC.[21] We observed that the *SMYD3* VNTR 3/3 genotype increases the risk of HBV-related HCC. A possible explanation is that *SMYD3* VNTR 3/3 can effectively promote its affinity with E2F-1, which is considered an important transcription factor stimulating cellular proliferation and cell cycle progression.[21,31,32] In HCC development, overexpression of SMYD3 was previously found in disttinct cell lines, but an association between SMYD3 serum levels and HCC has not been shown so far.[18,33] In our study, SMYD3 serum levels were significantly increased according to the various HBV-related liver disease stages. SMYD3 levels were higher in advanced liver disease (LC, HCC) compared to CHB, and HCC patients had higher SMYD3 levels than non-HCC patients. These findings indicate that increased SMYD3 levels may be associated with the occurrence of HCC, and SMYD3 serum levels may be considered a potential marker for HCC. Moreover, SMYD3 serum levels in HCC patients with the *SMYD3* VNTR genotype 3/3 were higher than in those with genotypes 2/2 and 2/3; this difference was not observed in CHB and LC patients.

Finally, we assessed *SMYD3* mRNA expression in liver tissues. *In vitro* evidence indicates that SMYD3 expression is up-regulated in HCC cell lines.[13,33] SMYD3 expression was analyzed in HCC tissues, and upregulation was significantly associated with an unfavourable prognosis of HCC.[34] *SMYD3* mRNA expression was upregulated in HCC tumor compared to adjacent non-tumor tissues, providing further evidence of a role of SMYD3 in HCC development. Several studies have shown that SMYD3 can interact with the HBV-HBx protein, which can induce upregulation of SMYD3 in HCC; these interactions promote development of HCC.[18-20] Although our data underline the significance of the *SMYD3* VNTR 3/3 genotype and SMYD3 overexpression in HBV-related HCC development, the interaction between HBV and SYMD3 needs to be investigated further.

In conclusion, our study shows the association of the *SMYD3* VNTR 3/3 as well as upregulation of SMYD3 serum levels and mRNA expression with HCC development, suggesting that SYMD3 has some potential for the prediction of HCC in HBV patients.

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Conflict of interests

All authors have no conflicts of interest to declare.

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Author's contributions

TPV designed, supervised the study, contributed materials and reagents, and wrote the manuscript. PGK contributed to materials and reagents. MTB recruited patients and collected samples, performed the experiments, carried out the statistical analyses, interpreted data and wrote the manuscript. NXH recruited patients and collected samples, carried out the statistical analyses. HVT, CTB and HW contributed to the analysis, interpreted data and the manuscript. DPG contributed to the experimental design. LHS, NLT and MHB recruited patients. CGM revised the draft and edited the final version of the manuscript. All authors agreed with the results and conclusions.

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Table 1: Demographic and clinical characteristics of healthy controls and HBV patients

Characteristics	HC (n=297)	HBV patients (n=756)	CHB (n=246)	LC (n=174)	HCC (n=336)	*P* value
Age (years)	43 [16-69]	52 [12-91]	41 [12-85]	56 [20-86]	57 [15-91]	<0.0001[#]
Male (%)	66.7	83.3	75.2	82.8	93.7	<0.0001[β]
Child-Pugh	NA					
Child A			NA	53/169	249/335	
Child B			NA	75/169	65/300	
Child C			NA	41/169	21/335	
Missing			NA	5	1	
Clinical parameters						
AST (IU/L)	NR	132 [14-6206]	187 [14-6206]	119 [15-1221]	101 [17-983]	<0.0001[#]
ALT (IU/L)	NR	132 [8-3390]	222 [9-3390]	82 [8-1426]	72 [11-1095]	0.04[#]
Total bilirubin (µmol/L)	NR	39.1 [4.1-571]	34 [5.5-551]	65.2 [4.1-571]	29.4 [4.3-392]	<0.0001[#]
Direct bilirubin (µmol/L)	NR	17.2 [0.4-349]	16.1 [0.7-349]	29.5 [0.4-291]	11.6 [0.4-247.3]	<0.0001[#]
Albumin (g/L)	NR	37 [15-48]	42 [25-48]	31.8 [15-47]	37 [15-48]	<0.0001[#]
Prothrombin (% of standard)	NR	82 [13-269]	94 [17-267]	60 [13-101]	84 [20-269]	<0.0001[#] <0.0001[#]
WBC ($x10^3$/mL)	NR	6.6 [1.7-20.5]	6.7 [4.1-13.44]	6.2 [1.7-20.5]	6.8 [2.7-17.9]	<0.0001[#]
RBC($x10^6$/mL)	NR	4.5 [1.7-6.8]	4.9 [3.1-6.8]	3.9 [1.9-6.7]	4.5 [1.7-6.8]	<0.0001[#]
PLT ($x10^3$/ml)	NR	174 [17-441]	218 [66-379]	106 [17-441]	177 [34-432]	<0.0001[#]
HBV DNA (log_{10} copies/ml)	NR	5.1 [1-10]	5.2 [2-10]	5 [1-10]	5.1 [1-9]	0.4[#]
AFP (IU/L)	NR	142 [1-4029]	7.4 [1-250]	40 [1.18-707]	280 [1-4029]	<0.0001[#]

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 aminotransferase; AFP, alpha-fetoprotein; NR, normal range, NA, not applicable. Values given are medians and ranges. (#) Kruskal-Wallis test (β): chi-square test.

Table 2: Association of the variable number of tandem repeats of SMYD3 with HBV-related liver diseases

SMYD3 tandem repeat	HC n=297 (%)	CHB n=246 (%)	LC n=174 (%)	HCC n=336 (%)	CHB + LC n=420 (%)	HBV total n=756 (%)	CHB vs HC		LC vs HC		HCC vs HC		HBV vs HC	
							OR(95%CI)	*P* value	OR(95%CI)	*P* value	OR(95%CI)	*P* value	OR(95%CI)	*P* value
Genotypes														
VNTR 2/2	21 (7.1)	14 (5.7)	6 (3.4)	7 (2.1)	20 (4.8)	27 (3.6)	Reference		Reference		Reference		Reference	
VNTR 2/3	64 (21.5)	37 (15)	29 (16.7)	61 (18.2)	66 (15.7)	127 (16.8)	0.75 (0.32-1.76)	0.5	1.8 (0.59-5.6)	0.3	2.7 (0.89-7.92)	0.079	1.2 (0.58-2.44)	0.6
VNTR 3/3	212 (71.4)	195 (79.3)	139 (79.9)	268 (79.8)	334 (79.5)	602 (79.6)	1.2 (0.6-2.6)	0.5	2.2 (0.81-6.12)	0.1	**2.9(1.07-8)**	**0.037**	1.7 (0.88-3.15)	0.1
Allele														
VNTR 2	106 (21.7)	65 (13.2)	41 (11.8)	75 (11.2)	106 (12.6)	181 (12)	Reference		Reference		Reference		Reference	
VNTR 3	488 (78.3)	427 (86.8)	307 (88.2)	597 (88.8)	734 (87.4)	1331 (88)	1.4 (0.97-1.92)	0.078	1.5 (1-2.38)	0.05	**1.5 (1-2.14)**	**0.05**	1.4 (1.07-1.86)	**0.017**
Dominant														
VNTR 2/2&2/3	85 (28.6)	51 (20.7)	35 (20.1)	68 (20.2)	86 (20.5)	154 (20.4)	Reference		Reference		Reference		Reference	
VNTR 3/3	212 (71.4)	195 (79.3	139 (79.9)	268 979.8)	334 (79.5)	602 (79.6)	1.5 (0.99-2.23)	0.057	1.4 (0.88-2.4)	0.1	1.3 (0.86-2.05)	0.2	1.4 (1.02-1.97)	**0.036**
Recessive														
VNTR 2/2	21 (7.1)	14 (5.7)	6 (3.4)	7 (2.1)	20 (4.8)	27 (3.6)	Reference		Reference		Reference		Reference	
VNTR 2/3&3/3	276 (92.9)	232 (94.3)	168 (96.6)	329 (97.9)	400 (95.2)	729 (96.4	1.1 (0.55-2.35)	0.7	2.2 (0.8-6.02)	0.1	2.9 (1.07-8.03)	**0.036**	1.6 (0.82-2.94)	0.2

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

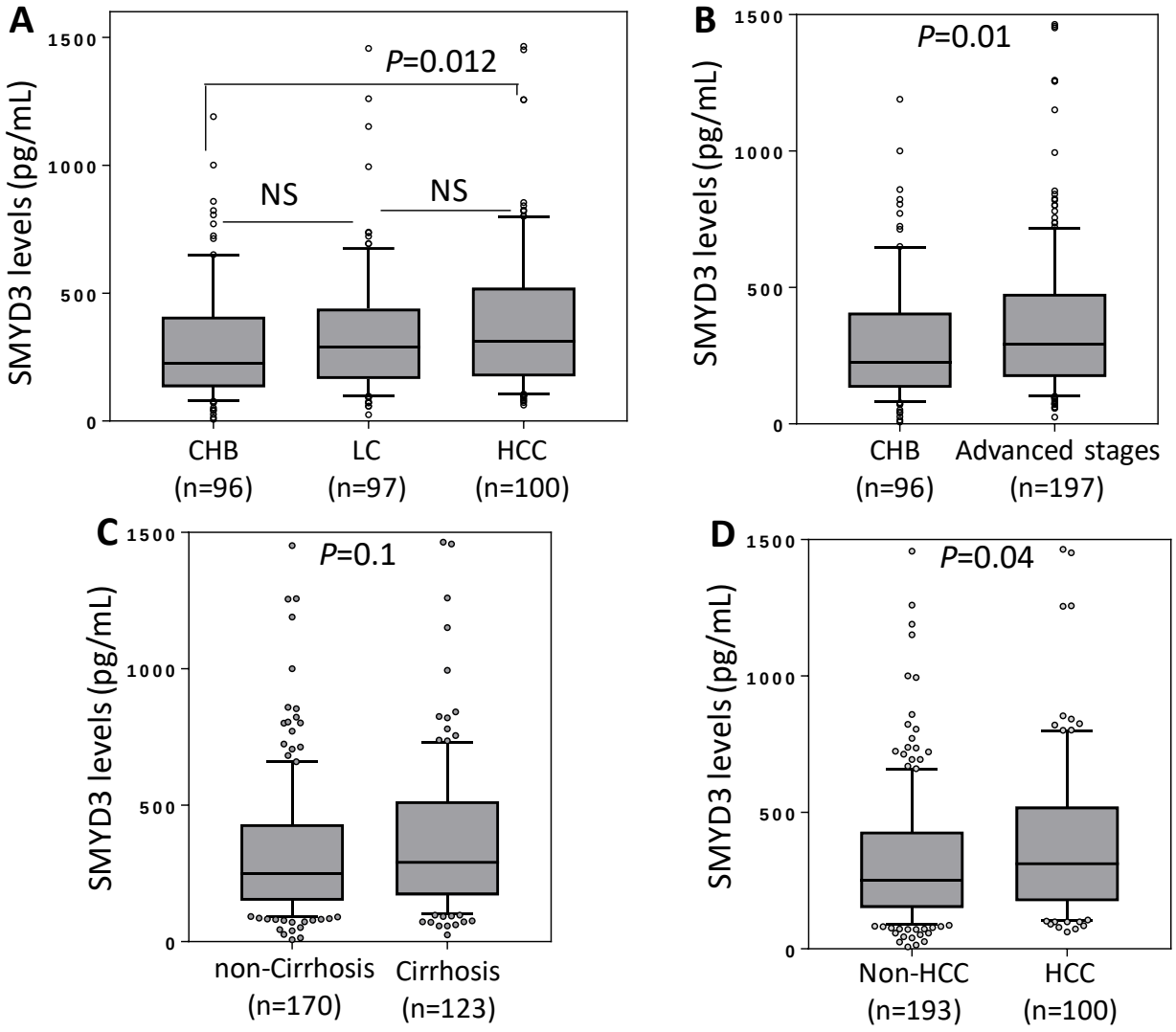

Figure 1: Association of the SMYD3 serum levels with HBV related liver diseases. The comparison of SMYD3 levels (A) among groups of patients according to HBV-related diseases, CHB, chronic hepatitis B; LC, liver cirrhosis without HCC; HCC, hepatocellular carcinoma; (B) between CHB patients with advanced stages patients (including LC and HCC), (C) between patients with and without cirrhosis, (D) between patients with and without HCC. Box-plots illustrate medians with 25 and 75 percentiles with whiskers to 10 and 90 percentiles; *P* value were calculated by using Kruskal-Wallis test or Mann-Whitney-Wilcoxon test.

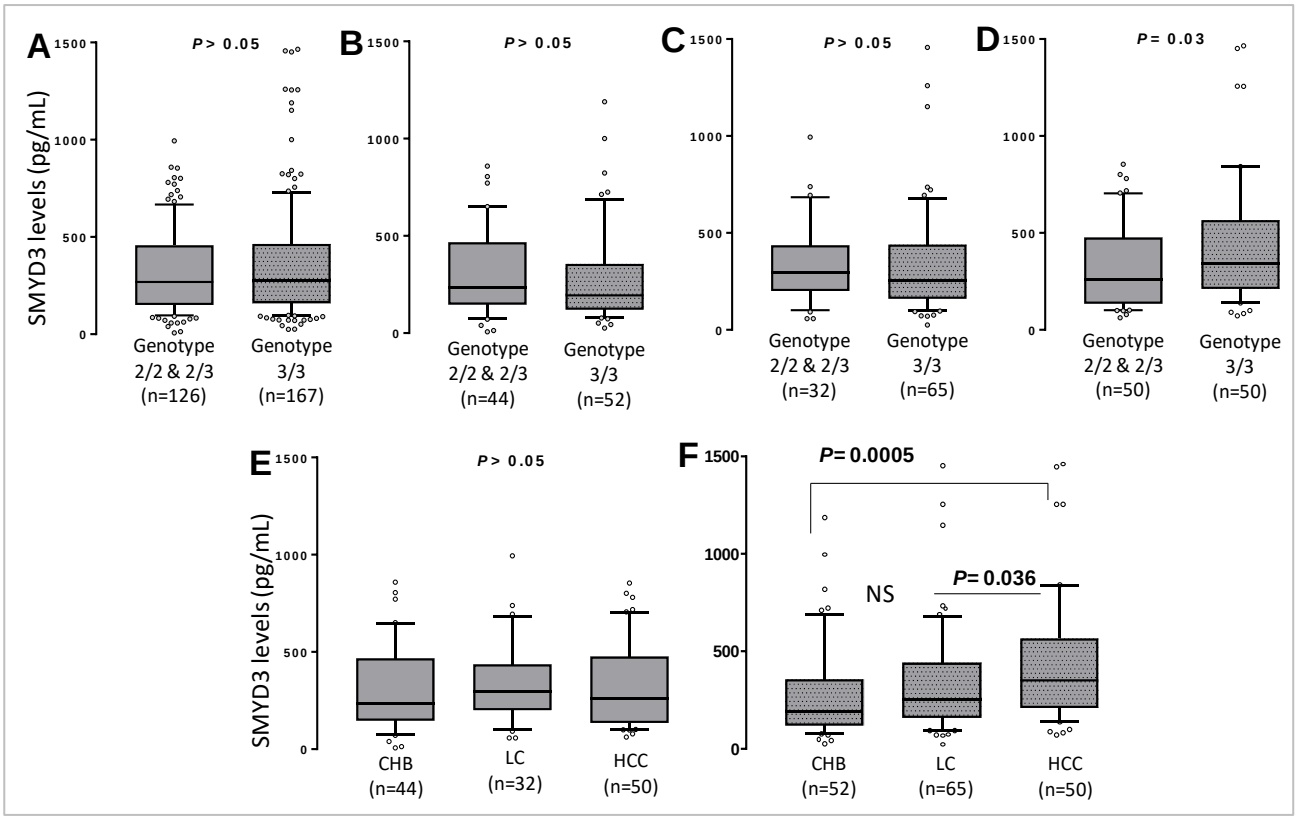

Figure 2: Association of the VNTRs of SMYD3 with SMYD3 serum levels in HBV-related liver diseases. Comparison of the SMYD3 serum levels among patients with HBV-related liver diseases who carry different *SMYD3* VNTR genotypes. CHB, chronic hepatitis B, LC, liver cirrhosis without HCC, HCC, hepatocellular carcinoma. (A) among total HBV patients, (B) among CHB patients, (C) among LC patients, (D) among HCC patients, (E) among patients with the VNTR genotypes 2/2 and 2/3, (F) among patients with genotype 3/3. Box-plots illustrate medians with 25 and 75 percentiles with whiskers to 10 and 90 percentiles; *P* values were calculated by using Mann-Whitney-Wilcoxon test or Kruskal-Wallis test.

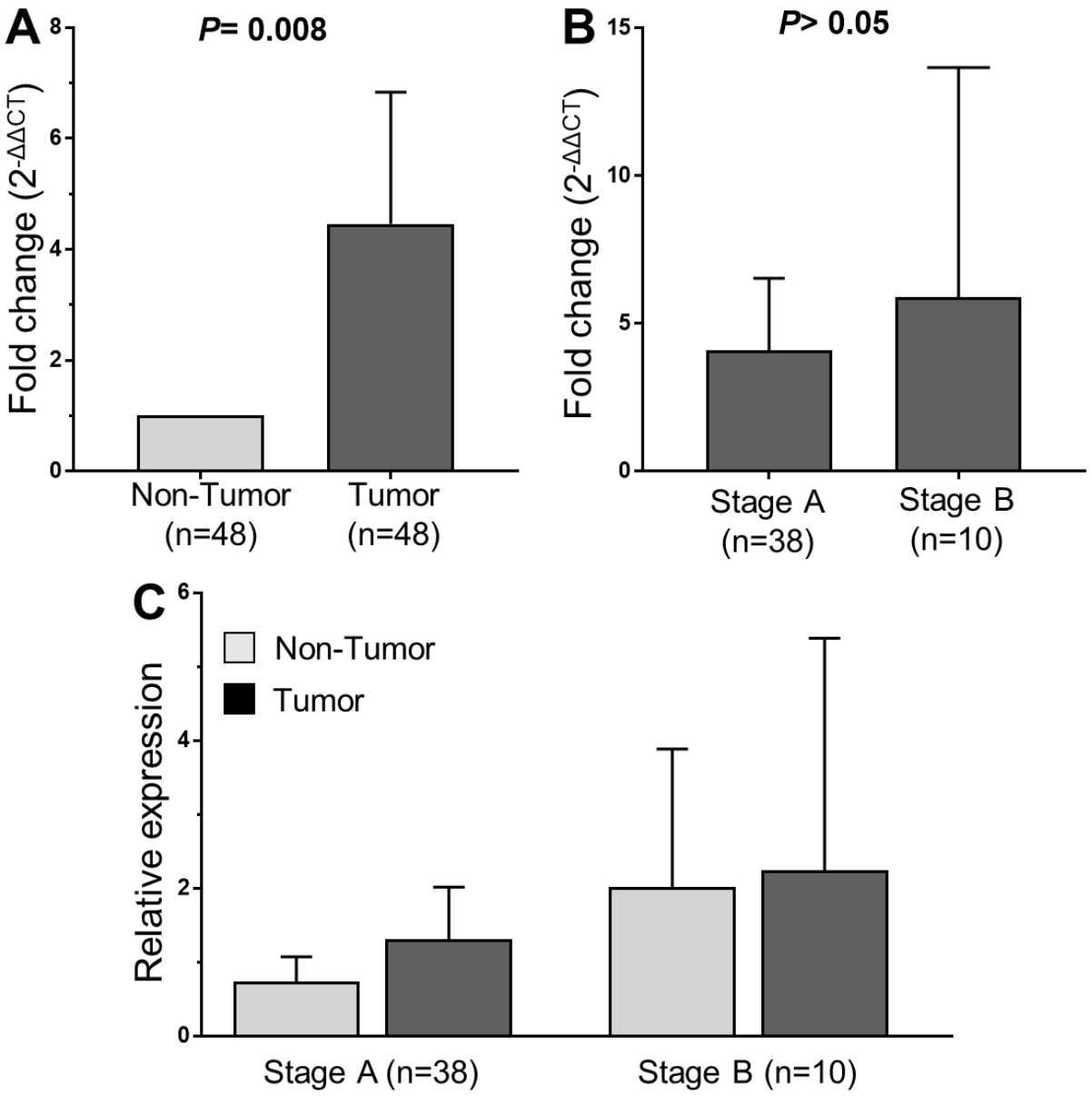

Figure 3: *SMYD3* mRNA expression in liver specimens from 48 HCC patients. Relative quantitative real-time PCR analysis of *SMYD3* mRNA levels. (A) Relative *SMYD3* mRNA expression in tumor tissues and in adjacent non-tumor tissues, (B) The fold changed *SMYD3* mRNA levels between the group of stage A-HCC patients and the group of stage B-HCC patients, (C) SMYD3 mRNA expression in stage-A and stage-B tumor tissues and in adjacent non-tumor tissues. *P* values were calculated by using Mann-Whitney-Wilcoxon test. Data are shown as mean values with 95% confidence intervals.

4. DISCUSSION

Despite the availability of effective HBV vaccine, hepatitis B continues to be a public health problem and accounts for of a large number of chronic cases with high incidence and mortality. The most severe consequence of HBV is advanced liver diseases, in particular HCC. HBV-infected individuals have 40 fold-chance of developing HCC (Lee et al., 2013). Besides HBV, other hepatitis viruses such as HCV, HDV, and HEV may also cause liver damage and promote hepatic failure. Host genetic factors also influence and play an essential role in the development of hepatic illness. This thesis aimed to understand how HDV infection may aggravate HBV-related liver diseases and subsequently investigated on the potential association of host genetic factors such as *NTCP S267F* variant with HBV, HDV, and their clinical courses. Furthermore, this thesis also investigated on the SMYD3 expression and SMYD VNTR genotype 3/3 on HCC development using a large cohort of Vietnamese patients with HBV.

4.1. Hepatitis delta virus infections

4.1.1. HDV infection in Vietnamese population

Commercial HBV vaccines have been deployed in Vietnam since 2003. However, the prevalence of HBV infections is believed to be greater than 10% (Nguyen, 2012, Nguyen et al., 2008), and this indicates a large health burden in the country. Existing data show extensive HDV variation across Vietnamese provinces (Dunford et al., 2012, Sy et al., 2013, Hall et al., 2015, Nguyen et al., 2017). Overall, HDV seropositivity was 15% (Hall et al., 2015). The highest HDV seroprevalences is observed in the northern Vietnam whereas both the south and the central Vietnam

exhibited low seroprevalences (Hall et al., 2015). HDV infections in these settings are mainly observed in persons who inject drugs in northern Vietnam (Hall et al., 2015). Analyses of HBV samples collected over a ten-year period revealed that HDV prevalence in northern Vietnam was 15% (Sy et al., 2013). Moreover, HDV RNA positivity was counted of 10% in central Vietnam (Nguyen et al., 2017). Our study estimated HDV positivity of 13%, thus corroborating earlier findings in northern Vietnam (Hall et al., 2015, Sy et al., 2013) Our findings suggest that the number of HDV infections in northern Vietnam have not decreased in the last decade. However, the effective HBV vaccination campaign show promising HDV control measures, in particular for Northern Vietnam.

HDV genotyping in the present study revealed that only HDV-1 and HDV-2 occurs in Vietnam. Although HDV-1 seems to be predominant in Vietnam, recent studies show that ratio of HDV-1 and HDV-2 in northern and southern regions of Vietnam is quite distinct (Sy et al., 2013, Hall et al., 2015, Nguyen et al., 2017). In particular, HDV-1 was preferentially identified (90%) in northern Vietnam (Sy et al., 2013) whereas in southern region it has a prevalence of 20% (Nguyen et al., 2017). Consistent with previous study, our study observed a 91% HDV-1 prevalence in northern Vietnam. Our findings cumulate an evidence for the globally dominant genotype of HDV-1 (Alvarado-Mora et al., 2013, Pascarella and Negro, 2011, Wedemeyer and Manns, 2010, Hughes et al., 2011, Barros et al., 2011).

4.1.2. HDV/HBV coinfections and HBV related liver diseases

Concomitant HDV and HBV infections have been demonstrated to significantly increase the advancement of liver diseases, including LC and HCC (Smedile et al.,

1982, Fattovich et al., 2000, Negro, 2014). The Hepatitis Delta International Network registry shows that 48.7% HDV positive patients have LC (Wranke et al., 2018). Our study observed that LC patients had higher HDV positive rate (20%) than HCC (12%) and CHB (9%). Moreover, advanced LC patients (identified by Child-Pugh scores B and C) accounted for a higher rate of HDV cases than early LC patients with Child-Pugh score of A. Therefore, our findings support that infected HDV may enhance the development LC among HBV patients.

4.2. Association of *NTCP S267F* variant with HBV and HDV

NCTP is the main mode for the transportation of bile acids into the hepatocytes (Vaz et al., 2015, Claro da Silva et al., 2013). It also play a vital role in the life cycle of both HBV and HDV, as a receptor during HBV-HDV invasion into the hepatocytes (Yan et al., 2012, Ni et al., 2014). Genetic variation in the gene encoding NTCP might therefore modulate HBV and HDV susceptibility. Yan et al. published the first report illustrating that *NTCP S267F* allele (rs2296651, c.800C>T) dampens the NCTP-preS1 lipopeptide interaction, thus indicating this allele could avert the establishment of HBV and HDV infections (Yan et al., 2014). In addition, clinical evidence of the protective nature of *NTCP S267F* allele has only been demonstrated in HBV infections (Peng et al., 2015, Hu et al., 2016, Lee et al., 2017, Wang et al., 2017, Wu et al., 2018). Our findings were in congruence with these reports and reaffirmed that NTCP S267F is protective against HBV infection, the progression of HBV-related liver diseases and HBV-HDV coinfections.

4.2.1. *NTCP S267F* frequency distribution

The distribution of SNPs in the *NTCP* varies on the ethnic background of a population (Choi et al., 2011, Pan et al., 2011). *NTCP S267F* variant (rs2296651, c.800C>T) is mostly found in Asia (Ho et al., 2004, Pan et al., 2011, Casillas et al., 2018); In other regions, the frequency of this allele varies considerably in different cohorts representing varied geographical areas (Pan et al., 2011, Peng et al., 2015, Hu et al., 2016, Ezzikouri et al., 2017, Lee et al., 2017, Li et al., 2014, Zhang et al., 2017). In our study, we observed that 83.6% of healthy Vietnamese people (*n*=214) carry the wildtype genotype, *NTCP S267F* genotype CC, whereas the mutant (T) genotypes (CT and TT) (*S267F*) were observed in 15.9% and 0.5% of the healthy participants, respectively. The occurrence of *NTCP S267F* genotypes CT and TT (*S267F*) in the present study was slightly lower than reported in other Asian populations such as Chinese Han (20.4%) and Taiwanese (18.5%)(Peng et al., 2015, Hu et al., 2016), but higher than observed in Korea (5.7%) (Lee et al., 2017). As expected, we found that S267F were less frequent among HBV patients than healthy controls as previously reported (Pan et al., 2011, Li et al., 2014, Peng et al., 2015, Hu et al., 2016, Ezzikouri et al., 2017, Lee et al., 2017, Zhang et al., 2017).

4.2.2. *NTCP S267F* and HBV

Previous studies have shown that HBV-infected individuals carrying *S267F* have reduced susceptibility, 2-5-fold, to HBV chronicity (Peng et al., 2015, Hu et al., 2016, Lee et al., 2017). Our study corroborates these results (OR = 0.3, 95% CI = 0.19–0.54). The protective effect of *S267F* against HBV infection has also been demonstrated in *in vitro* experiments, showing that in mixed cells at a 1:1 ratio of wild-type *NTCP* and *S267F* at a 1:1 the efficiency of HBV infection was around 70%,

whereas the efficiency just reached below 10% in HepG2-S267F mutant cells (Yan et al., 2014). This clearly shows that the T allele of *NTCP S267F* could contribute towards HBV resistance. Similarly, we observed that the *T* allele of *NTCP S267F* reduced the risk of CHB (OR = 0.34). Even though it is evident that the *NTCP S267F* mutant may confer protection, the mechanism through which NTCP mutation blocks or reduces the interaction is not well understood (Yan et al., 2014).

Consistent with previous findings, our data demonstrated that HBV-infected persons carrying *S267F* had a lower risk of developing LC or HCC. (Hu et al., 2016, Lee et al., 2017, Wang et al., 2017). This indicate that *S267F* could be protecting HBV patients recruited in our study against advanced progression of LC and HCC (Child B and C vs. Child A, OR=0.26; and BCLC B, C, D vs. BCLC A, OR=0.38) and poor clinical outcome. The replacement of hydrophobic residue (phenylalanine) by a hydrophilic residue (serine), following *NTCP S267F* mutation, alters the structure of NTCP (Yan et al., 2012). The plausible outcome for this amino acid change is the reduction of NTCP function as a receptor for HBV and transportation of bile acid into hepatocytes (Ho et al., 2004). The *NTCP S267F* reduce the attachment of HBV to hepatocytes. Thus, explaining the reduced severity of HBV infections among *S267F* carriers. Impartment of bile acid uptake due to *NTCP S267F* mutation increases bile acid (taurocholate) levels in blood and trigger hepatic apoptosis. Accumulation of cytotoxic bile acids in the liver induce and sustain lifelong liver inflammation (Faubion et al., 1999, Miyoshi et al., 1999, Ho et al., 2004). Whether reduced transportation of bile salt contributes towards protection against LC and HCC development is still unclear.

4.2.3. *NTCP S267F* and HDV

The role of *NTCP S267F* mutation in HDV infection has not extensively investigated. So far, an *in-vitro* study revealed that HDV has impaired infectivity of liver cell lines carrying the *S267F* (Yan et al., 2014). To the best of our knowledge, we present the first clinical evidence of the impact of *S267F* on HDV infection. Our study showed that *S267F* carriers have lower risk of acquiring HDV infection relative to healthy controls (OR=0.17). This finding support the findings of *in vitro* experiment (Yan et al., 2014) and suggest that impaired binding of preS1 peptide to *NTCP S267F* variant could explain low HDV rate in individuals carrying *S267F*. The similarity in *NTCP S267F* allele frequency among HBV monoinfected and HBV/HDV coinfected patients in this study is not surprising since both HBV and HDV use the same mechanism to infect hepatic cells (Yan et al., 2014).

4.3. SMYD3 levels and SMYD3 VNTR in HBV related liver diseases

Alongside HBV infections, epigenetic modification in the host have associated with the occurrence of HCC (Ma et al., 2014, McCabe et al., 2017, Hoan et al., 2017). SMYD3 is the main histone methyltransferase known to indirectly trigger oncogenesis. It does so by catalyzing methylation of histone H3-K4, in the presence HSP90α, which then activate transcription of oncogenes. SMYD3 is a potential contributing factor for many human cancers (Hamamoto et al., 2004, Hamamoto et al., 2006, Tsuge et al., 2005). However, there are no consensus on the association of SMYD3 gene with clinical HCC development. This is probably due to the existence of different liver disease etiologies (Tsuge et al., 2005, Wang et al., 2007, Fei et al., 2017, Li et al., 2018). In this study, we showed that *VNTRs* in the *SMYD3* promoter, high SMYD3 serum levels and SMYD3 overexpression associates with HCC in HBV-infected individuals.

*SMYD3 VNTR*s have been described as a prominent factor in the development of HCC (Tsuge et al., 2005, Li et al., 2018). Apart from a study conducted by Wang et al, studies published so far demonstrate that the higher proportion of *SMYD3* VNTR 3/3 genotype in HCC patients than in controls (Tsuge et al., 2005, Wang et al., 2007, Li et al., 2018). Similarly, we found that *SMYD3* VNTR three repeats allele and 3/3 genotype were more frequent among HBV patients than in the healthy people. We also observed that *SMYD3* homozygosity of three repeats was significantly more frequent among HCC patients relative to the healthy controls. Our findings therefore indicate that HBV-infected individuals who carry *SMYD3* VNTR 3/3 genotype have nearly three-times higher risk of developing HCC (OR=2.9). Even though the mechanism underlying this observation is not well understood, the tandem repeat CCGCC has been reported to be a binding site for the transcription factor E2F-1 which triggers cell proliferation and may be a potential oncogenesis (Harbour and Dean, 2000, Stevaux and Dyson, 2002, Tsuge et al., 2005, Muller and Helin, 2000). In view of this, increase in *SMYD3* homozygosity of three tandem repeat CCGCC may strengthen the affinity of E2F-1 to SMYD3 promoter and enhance hepatic oncogenesis.

Although several *in vitro* studies have demonstrated SMYD3 overexpression in HCC cell lines, (Chen et al., 2007, Yang et al., 2009, Hamamoto et al., 2004), it remains to be investigated whether SMYD3 serum levels influence the progression of HCC. Our study shows that SMYD3 serum levels increased the progression of HBV-related liver disease. HCC patients had higher SMYD3 levels than non-HCC patients whereas no significant difference of SMYD3 levels was observed among cirrhosis and non-cirrhosis patients. The association of SMYD3 levels with HCC implies that SMYD3 serum levels may be used as an additional marker for HCC diagnosis. Interestingly,

we only observed that the high SMYD3 serum levels among HCC patients were associated with *SMYD3* genotype 3/3. These results therefore show that HCC cases are characterized by high SMYD3 levels and *SMYD3 VNTR3/3* genotype. Based on these findings, we hypothesize that *SMYD3 VNTR* genotype *3/3* may increase the risk of developing HCC which is marked by high SMYD3 levels. Upregulation of SMYD3 expression in HCC tissues is associated with poor HCC prognosis (Fei et al., 2017). Consistent with previous reports, our study showed that SMYD3 mRNA levels in HCC tumors were five times higher than that of adjacent non-tumor tissues. Our data provides additional evidence on the oncogenic activity of SMYD3 in HCC. In HBV infection, overexpression of SMYD3 may have resulted from the interaction of SMYD3 with HBx proteins. Thus, supporting the occurrence of HCC (Guo et al., 2005, Yang et al., 2009, Hayashi et al., 2016). Although the association of either *SMYD3* VNTR 3/3 genotype or SMYD3 upregulation with HCC development is significant, the relationship between HBV and *SMYD3* on hepatic diseases requires further investigations.

4. SUMMARY

Despite the existence of an effective hepatitis B (HBV) vaccination program, HBV is highly endemic in many countries, including Vietnam. The burden of HBV is further aggravated by hepatitis delta virus, a satellite virus that co-occurs with hepatitis B. HDV/HBV coinfections constitute a global health burden and are associated with a higher risk of cirrhosis and liver cancer. In Vietnam, the prevalence of HBV infection is greater than 10%, suggesting a high prevalence of HDV infection in HBsAg positive patients. In addition, the host and viral factors were shown to associate with HDV/HBV clinical outcome and progression of HBV liver diseases. In this context, this dissertation investigated in a large Vietnamese cohort of HBV patients (i) the current epidemiological situation and clinical importance of circulating HDV genotypes among the Vietnamese population; (ii) the HBV entry receptor, sodium taurocholate co-transporting polypeptide (NTCP) and their genetic association with HBV/HDV clinical outcome; (iii) the association of the SET and MYND domain-containing protein 3 (*SMYD3*) gene with HBV-related liver diseases, particularly with hepatocellular carcinoma. This dissertation documents 13% HDV-RNA positivity among HBV-infected patients and that HDV-RNA positivity was associated with liver cirrhosis in HDV/HBV co-infected patients, with HDV-1 being the predominant genotype in Vietnam. The S267F variant of NTCP exhibits protective effects against HBV and HDV infection and was associated with decreased susceptibility to HBV and HDV infection, as well as with a decreased occurrence of liver cirrhosis and liver cancer. Lastly, the investigations on SMYD3 suggest that the *SMYD3* genotype VNTR 3/3 appears to increase the risk of hepatocellular carcinoma through increasing SMYD3 levels. The findings additionally support that upregulation of SMYD3 mRNA expression in tumor tissues correlates with the occurrence of hepatocellular carcinoma. Taken together,

these findings confirm a continuing high prevalence of HDV infection in hepatitis B patients in Vietnam and provide insights on an increased understanding of the host genetic factors influencing the HBV/HDV clinical outcome.

5. ZUSAMMENFASSUNG

Trotz des bereits bestehenden, wirksamen Hepatitis-B-Impfprogramms, ist HBV in vielen Ländern, einschließlich Vietnam, weit verbreitet. Die HBV Belastung wird weiter verschärft durch das Hepatitis-Delta-Virus (HDV), ein Satellitenvirus, das zusammen mit Hepatitis B auftritt. Diese Koinfektionen mit HDV/HBV stellen eine globale Gesundheitsbelastung dar und gehen mit einem höheren Risiko für Leberzirrhose und Leberkrebs einher. In Vietnam liegt die Prävalenz der HBV-Infektion bei über 10%, was auf eine hohe Prävalenz der HDV-Infektion bei HBsAg-positiven Patienten hinweist. Darüber hinaus wurde gezeigt, dass die Wirts- und Virusfaktoren mit dem klinischen Ausgang von HDV/HBV und dem Verlauf von HBV-Lebererkrankungen zusammenhängen. In diesem Kontext untersuchte diese Dissertation in einer umfangreichen vietnamesischen HBV-Patienten-Kohorte: (i) die aktuelle epidemiologische Situation und die klinische Bedeutung der Verbreitung von HDV-Genotypen in der vietnamesischen Bevölkerung; (ii) den HBV-Eintrittsrezeptor, das Natriumtaurocholat-co-transportierende Polypeptid (NTCP), und deren genetische Assoziation mit dem klinischen Ergebnis von HBV / HDV; (iii) die Assoziation des *SET and MYND domain-containing protein 3* (SMYD3)-Gens mit HBV-verwandten Lebererkrankungen, insbesondere dem Hepatozellulären Karzinom. Diese Dissertation dokumentiert 13% HDV-RNA-Positivität in HBV-infizierten Patienten und dass HDV-RNA-Positivität in Zusammenhang mit Leberzirrhose bei HDV/HBV-koinfizierten Patienten steht, wobei in Vietnam HDV-1 der vorherrschende Genotyp ist. Die S267F Variante von NTCP, weist Schutzwirkungen gegen HBV- und HDV-

Infektionen auf und assoziierte mit einer geringeren Anfälligkeit für HBV- und HDV-Infektionen, sowie einem verringerten Auftreten von Leberzirrhose und Leberkrebs verbunden. Schließlich legen die Untersuchungen zu SMYD3 nahe, dass der *SMYD3*-Genotyp VNTR 3/3 das Risiko für ein hepatozelluläres Karzinom, durch gesteigerte der SMYD3-Spiegel, zu steigern scheint. Die Ergebnisse legen weiterhin nahe, dass die Hochregulierung der SMYD3-mRNA-Expression in Tumorgeweben, mit dem Auftreten eines hepatozellulären Karzinoms korreliert. Zusammengefasst bestätigen diese Ergebnisse eine anhaltend hohe Prävalenz der HDV-Infektion bei Hepatitis B-Patienten in Vietnam und liefern Erkenntnisse über ein besseres Verständnis der genetischen Faktoren des Wirts, die das klinische Ergebnis von HBV / HDV beeinflussen.

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7. DECLARATION OF CONTRIBUTIONS

We hereby declare that the doctoral dissertation entitled "**Molecular epidemiology of hepatitis delta virus and host genetic factors in the progression of HBV related liver diseases in patients with HBV and HDV coinfections**" submitted to the members of the PhD Board at the Faculty of Medicine, University of Tübingen is a record of an original work done by Dr. Mai Thanh Binh and co-authors at the Institute of Tropical Medicine, University of Tübingen.

Three publications (Publication 1: Scientific Reports. 2018;8(1):8047. PMID: 29795302; Publication 2: International Journal of Infectious Diseases. 2019;80:147-52. PMID: 30685591; Publication 3: Under review) accomplished by Dr. Mai Thanh Binh as the first author lay as the backbone of his doctoral dissertation. We declare that Dr. Mai Thanh Binh have substantially contributed to all three manuscripts with respect to study design, sampling procedures, patient recruitment, experiment design, data analyses and writing the manuscript. We also state individually the contribution of Ph.D. candidate and all other co-authors in each study as following.

Scientific paper 1: Thirumalaisamy P. Velavan designed, supervised the study, contributed materials and reagents, interpreted data and wrote the manuscript. Mai Thanh Binh recruited patients, performed the experiments, carried out the statistical analyses and wrote the manuscript. Nghiem Xuan Hoan recruited patients and collected samples, performed the experiments, carried out the statistical analyses and wrote the manuscript. Hoang Van Tong contributed to the analysis. Dao Phuong Giang performed the experiments. Bui Tien Sy contributed to the experimental design. Nguyen Linh Toan recruited patients and collected samples. Le Huu Song recruited patients and collected samples. Mai Hong Bang recruited patients. Heiner Wedemeyer

contributed to the study design. Christian G. Meyer revised the draft. Peter G. Kremsner contributed materials and reagents. C.-Thomas Bock contributed to standard experimental procedures.

Scientific paper 2: Thirumalaisamy P. Velavan designed, supervised the study, contributed materials and reagents, and wrote the manuscript. Peter G. Kremsner contributed materials and reagents. Mai Thanh Binh recruited patients and collected samples, performed the experiments, carried out the statistical analyses, interpreted data and wrote the manuscript. Nghiem Xuan Hoan recruited patients and collected samples, carried out the statistical analyses and edited the manuscript. Hoang Van Tong contributed to the analysis, interpreted data and edited the manuscript. Bui Tien Sy and Ngo Tat Trung contributed to the experimental design. Le Huu Song recruited patients. Mai Hong Bang recruited patients. Christian G. Meyer revised the draft and edited the final version of the manuscript.

Scientific paper 3: Thirumalaisamy P. Velavan designed, supervised the study, contributed materials and reagents, and wrote the manuscript. Peter G. Kremsner contributed to materials and reagents. Mai Thanh Binh recruited patients and collected samples, performed the experiments, carried out the statistical analyses, interpreted data and wrote the manuscript. Nghiem Xuan Hoan recruited patients and collected samples, carried out the statistical analyses. Hoang Van Tong, C-Thomas Bock and Heiner Wedemeyer contributed to the analysis, interpreted data and the manuscript. Dao Phuong Giang contributed to the experimental design. Le Huu Song, Nguyen Linh Toan and Mai Hong Bang recruited patients. Christian G. Meyer edited the final version of the manuscript.

Sincerely,

Tübingen……..

Ph.D. candidate. Mai Thanh Binh **Prof. Dr. Thirumalaisamy P Velavan**

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9. CURRICULUM VITAE

Presentation in conference proceedings

1. **Mai Thanh Binh**, Nghiem Xuan Hoan, Hoang Van Tong, Bui Tien Sy, Ngo Tat Trung, C.-Thomas Bock, Nguyen Linh Toan, Le Huu Song, Mai Hong Bang, Christian G. Meyer, Peter G. Kremsner, Thirumalaisamy P. Velavan. NTCP S267F variant associate with decreased susceptibility to HBV infection and decelerated progression of related liver diseases. The International Liver Congress 54th April 2019 in Vienna, Austria. (Peer-reviewed, poster presentation. Journal of Hepatology).

2. Nghiem Xuan Hoan, Hoang Van Tong, Bui Tien Sy, **Mai Thanh Binh**, C-Thomas Bock, Nguyen Linh Toan, Le Huu Song, Thirumalaisamy P. Velavan. Interferon regulatory factor 5 and soluble fibrinogen-like protein 2 in hepatitis B virus related liver diseases. The International Liver Congress 54th April 2019 in Vienna, Austria. (Peer-reviewed, poster presentation. Journal of Hepatology).

3. **Mai Thanh Binh**, Nghiem Xuan Hoan, Bui Tien Sy, C-Thomas Bock, Heiner Wedemeyer, Thirumalaisamy P Velavan. HDV infection rates in northern Vietnam. Grad School Day at the Interfaculty Graduate School of Infection Biology and Microbiology (IGIM). 24th September 2018 in Tuebingen, Germany (Oral Presentation).

4. **Mai Thanh Binh**, Nghiem Xuan Hoan, Bui Tien Sy, Nguyen Minh Hung, Dao Phuong Giang, Nguyen Linh Toan, Le Huu Song, C-Thomas Bock, Heiner Wedemeyer, Thirumalaisamy P Velavan. Virological heterogeneity of hepatitis delta among Vietnamese populations. The International Liver Congress 53th April 2018 in Paris, France. (Peer-reviewed, poster presentation. Journal of Hepatology).

5. Nghiem Xuan Hoan, Trinh Van Son, Dao Phuong Giang, **Mai Thanh Binh**, Dam Tu Anh, Le Huu Song, C-Thomas Bock, Hoang Van Tong, Nguyen Linh Toan, Thirumalaisamy P Velavan. HEV positivity in domesticated pigs and a relative risk of HEV zoonosis among occupationally exposed individuals in Vietnam. The International Liver Congress 53th April 2018 in Paris, France. (Peer-reviewed, poster presentation. Journal of Hepatology).

Publications

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1. **Binh MT**, Hoan NX, Van Tong H, Sy BT, Trung NT, Bock CT, Toan NL, Song LH, Bang MH, Meyer CG, Kremsner PG, Velavan TP. NTCP S267F variant associates with decreased susceptibility to HBV and HDV infection and decelerated progression of related liver diseases. Int J Infect Dis. 2019 Mar;80:147-152.

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