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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (9): Hepatitis B virus

Regulation of microRNA by hepatitis B virus infection and their possible association with control of innate immunity

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Abstract

Hepatitis B virus (HBV) chronically infects more than 350 million people worldwide. HBV causes acute and chronic hepatitis, and is one of the major causes of cirrhosis and hepatocellular carcinoma. There exist complex interactions between HBV and the immune system including adaptive and innate immunity. Toll-like receptors (TLRs) and TLR-signaling pathways are important parts of the innate immune response in HBV infections. It is well known that TLR-ligands could suppress HBV replication and that TLRs play important roles in anti-viral defense. Previous immunological studies demonstrated that HBV e antigen (HBeAg) is more efficient at eliciting T-cell tolerance, including production of specific cytokines IL-2 and interferon gamma, than HBV core antigen. HBeAg downregulates cytokine production in hepatocytes by the inhibition of MAPK or NF-κB activation through the interaction with receptor-interacting serine/threonine protein kinase. MicroRNAs (miRNAs) are also able to regulate various biological processes such as the innate immune response. When the expressions of approximately 1000 miRNAs were compared between human hepatoma cells HepG2 and HepG2.2.15, which could produce HBV virion that infects chimpanzees, using real-time RT-PCR, we observed several different expression levels in miRNAs related to TLRs. Although we and others have shown that HBV modulates the host immune response, several of the miRNAs seem to be involved in the TLR signaling pathways. The possibility that alteration of these miRNAs during HBV infection might play a critical role in innate immunity against HBV infection should be considered. This article is intended to comprehensively review the association between HBV and innate immunity, and to discuss the role of miRNAs in the innate immune response to HBV infection.

Key words: Hepatitis B virus; HepG2.2.15; Innate immunity; MicroRNA; Persistent infection; Toll-like receptor

Core tip: Hepatitis B virus (HBV) is the leading cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma in the world. HBV could interact with the host's innate and adaptive immune responses to establish chronic infection. HBV also interacts with Toll-like receptors (TLRs) and TLR signaling pathways, and regulates host immune responses through the regulation of microRNAs (miRNAs) to some extent. This article fo-

cuses on the involvement of miRNA in the association between HBV and TLR signaling pathways and reviews the miRNAs involved in HBV infection.

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INTRODUCTION

Hepatitis B virus (HBV), a member of hepadona viridae, has partially circular double-stranded DNA genome, 3.2 kb in length[1]. It contains four overlapping open reading frames that encode seven proteins: the precore protein, also known serologically as HBe antigen (HBeAg), the core protein (HBcAg), viral polymerase, three forms of the envelope protein known as S antigen (HBsAg) and X (HBx) protein[1,2]. HBV as well as hepatitis C virus (HCV) causes acute and chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC)[3]. Hepatic cirrhosis and HCC are the most common causes of death in patients with chronic liver disease[4].

The outcome of HBV infection is the result of complex interactions between HBV and the immune system including adaptive and innate immunity[5,6]. Toll-like receptors (TLRs) are important parts of the innate immune response in hepatitis virus infections[7]. There are several reports about the important role of TLRs and TLR-mediated signaling in the pathogenesis and outcome of HBV infection[2,5-11].

MicroRNA (miRNA) is one of the endogenous noncoding small RNAs, approximately 18-22 nucleotides in size, a post-transcriptional regulator that binds to the 3'-untranslated region (UTR) of the target gene messenger RNA, usually resulting in cleavage or inhibiting translation of the target gene mRNA[12,13]. It is estimated that the human genome may encode over 2000 miRNAs, which may control about 60% of the human genome[14,15]. Physiologically, miRNAs are able to regulate various biological processes such as cell proliferation, differentiation and apoptosis, neuroprocesses, carcinogenesis and immune response[16-18]. This article is intended to comprehensively review the association between HBV and innate immunity, and to discuss the role of miRNAs in the innate immune response to HBV infection.

INNATE IMMUNITY IS IMPORTANT FOR THE ERADICATION OF HBV

Interferons (IFNs) play an important role in the innate immune response to virus infection. IFN-α and IFN-β (type I IFNs) are secreted by almost all virus-infected cells including hepatocytes and by specialized blood lymphocytes. In contrast, the production of IFN-γ (type II IFN) is restricted to cells of the immune system, such as natural killer (NK) cells, macrophages, and T cells. On the other hand, tumor necrosis factor alpha (TNF-α) primarily initiates innate immune response and triggers acquired immune responses[19]. TNF-α-induced apoptosis is important for clearance of hepatocytes infected with HBV and HCV, and IFN-γ accelerates the killing of theses hepatocytes[19,20]. The previous studies demonstrated that TNF-α and IFN-γ downregulate HBV gene expression in the liver of HBV transgenic mice by post-transcriptionally destabilizing the viral mRNA[21-23]. It has been widely believed that the cytotoxic T lymphocyte response clears viral infections by killing infected cells. However, Chisari's group[21-24] reported that noncytopathic clearance of HBV from hepatocytes by cytokines, which abolish viral replication and HBV gene expression, is another important mechanism. Isogawa *et al*[24] reported that TLR3, TLR4, TLR5, TLR7 and TLR9 ligands could induce antiviral cytokines and inhibit HBV replication in HBV transgenic mice, thereby indicating TLR activation as a powerful strategy for the treatment of chronic HBV infection. HBV replication can be controlled by innate immune response, involving TLRs, if it is activated in hepatocytes[24]. Together, these facts indicate that innate immunity including TLR signaling plays an important role in the pathogenesis of HBV infection.

TOLL-LIKE RECEPTORS AND ANTI-VIRAL DEFENSES

TLRs, germline-encoded pattern recognition receptors (PRRs), can play a central role in host cell recognition and response to various pathogens such as viruses[25]. TLR1, TLR2, TLR4, TLR5, and TLR6 are expressed on the cell surface while TLR3, TLR7, TLR8 and TLR9 are expressed within intracellular vesicles. TLR3, TLR7/8 and TLR9 are involved in the recognition of viral nucleotides such as double-stranded RNA, single-stranded RNA and DNA, respectively[26]. Other than TLRs, membrane-bound C-type lectin receptors (CLRs), cytosolic proteins such as NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs), which include retinoic acid-inducible gene I (RIG- I), melanoma differentiation antigen 5 (MDA5), and lipophosphoglycan biosynthetic protein 2 (LPG2), and unidentified proteins that mediate sensing of cytosolic DNA or retrovirus infection, are also involved in the recognition of pathogen-associated molecular patterns (PAMPs)[25].

TLRs play a crucial role in defending against pathogenic infection through the induction of inflammatory cytokines and type I IFNs by myeloid differentiation primary response 88 (MYD88)-dependent and MYD88-independent pathway. In the MYD88-dependent pathway, MYD88 recruits a set of signal cascades such as MAPK and NF-κB through receptor-interacting serine/

threonine protein kinase (RIPK/RIP). In the MYD88-independent pathway, TLR3 activates NF-κB and MAPKs through RIPK. TLR3 also activates IFN regulatory factor 3 (IRF3) and IRF7 *via* TRIF/TICAM-1, inducing the production of type I IFN. The activated NF-κB and IRFs are translocated to the nucleus. NF-κB and MAPKs initiate the transcription of inflammatory cytokine genes, whereas IRFs initiate the transcription of type I IFN[2]. RIG- I and MDA5 pathways can also activate IRF3 to produce type I IFNs. RNA helicases RIG- I and MDA5, specific receptors for double-stranded RNA, and the downstream mitochondrial effector known as CARDIF/MAVS/VISA/IPS-1, are also major pathways for type I IFN induction.

ASSOCIATION BETWEEN HBV AND TOLL-LIKE RECEPTORS

TLRs have been recognized as playing an important role in the pathogenesis of chronic hepatitis B[8]. NF-κB is activated by three TLR adaptors, MYD88, Toll/interleukin (IL)-1 receptor (TIR)-domain-containing adaptor-inducing IFNβ (TRIF), and IFN promoter stimulator 1 (IPS-1), to elicit anti-HBV response in both HepG2 and Huh7 cells[27]. Down-regulations of TLR7 and TLR9 mRNA were observed in peripheral blood mononuclear cells (PBMC) of HBV-infected patients[28]. Chen *et al*[29] reported that TLR1, TLR2, TLR4 and TLR6 transcripts were also downregulated in PBMC of chronic hepatitis B patients. After being challenged by TLR2 and TLR4 ligands, cytokine production was impaired in PBMC of chronic hepatitis B patients on the basis of the levels of plasma HBsAg[29]. Xie *et al*[30] reported that HBV infection results in reduced frequency of circulating plasmacytoid dendritic cells (pDCs) and their functional impairment *via* inhibiting TLR9 expression. HBV replication suppresses the TLR-stimulated expression of pro-inflammatory cytokines (TNF, IL6) and the activation of IRF3[31]. It has also been reported that HBV could target RIG- I signaling by HBx-mediated IPS-1 down-regulation, thereby attenuating the antiviral response of the innate immune system[32].

HBV E ANTIGEN DOWNREGULATES CYTOKINE PRODUCTION

The HBV precore/core region of HBV genome also encodes HBeAg as well as the HBV core. The precore stop codon prevents the formation of precore protein and HBeAg[2,33]. The existence of HBeAg in serum is known to be a marker of a high degree of viral infectivity. In Japan, the major HBV genotypes are B and C, but our previous study[34] revealed that the precore mutation A1896 and the core promoter mutations at nt1762 and 1764 were found more frequently in acute liver failure than in acute hepatitis, and HBV genotype B was predominant in acute liver failure. It has also been shown that acute liver failure occasionally occurs in persons who are negative for HBeAg[35,36]. It is well known that perinatal transmission of HBV occurs in about 10%-20% of HBeAg-negative mothers without prevention of perinatal HBV transmission by combined passive and active immunoprophylaxis, and the babies are at risk of developing fulminant hepatitis[37]. Chronic hepatitis B with high HBV DNA and ant-HBe is associated with a severe and evolutive liver disease[38]. These clinical findings could be assumed to have immune tolerance for HBeAg, although the function of HBV precore or HBeAg is unknown. Previous immunological studies[39-41] demonstrated that HBeAg is more efficient at eliciting T-cell tolerance, including production of its specific cytokines IL-2 and IFN-γ, than HBV core antigen. We also demonstrated that HBeAg expression inhibits IFN and cytokine production[2] and that HBeAg physically associates with RIPK2 and regulates IL-6 gene expression[6]. Visvanathan *et al*[42] reported that the expression of TLR2 on hepatocytes, Kupffer cells, and peripheral monocytes was significantly reduced in HBeAg-positive chronic hepatitis B patients. Thus, HBV seems to have evolved strategies that block the effector mechanisms induced through IFN and/or cytokine signaling pathways, similar to other viruses[19].

MIRNAS WERE DIFFERENTIALLY EXPRESSED IN HEPG2.2.15 AND HEPG2

HepG2.2.15 cells assemble and secrete HBV virion that infects chimpanzees[43,44]. We examined the expression of approximately 1000 miRNAs in the human hepatoma cells HepG2.2.15 and HepG2 using real-time RT-PCR, the most sensitive technique for mRNA detection and quantification[45,46].

First, 1008 miRNAs were examined in the hepatoma cells HepG2.2.15 and HepG2, using quantitative real-time RT-PCR with specific primers (Qiagen, Hilden, Germany). SNORD61, SNORD68, SNORD72, SNORD95, SNORD96A and RNU6-2 were used as endogenous controls to normalize expression to determine the fold-change in miRNA expression between the test sample (HepG2.2.15) and control sample (HepG2) by 2-ddCT (comparative cycle threshold) method[21]. MiRNAs were annotated by Entrez Gene (NCBI, Bethesda, MD, United States), accessed on 2/27/2013. Data were analyzed with miRNA PCR array data analysis software (http://www.sabiosciences.com/mirnaArrayDataAnalysis.php). Scatter plot analysis is shown in Figure 1A. There were differences in expression between HepG2 and HepG2.2.15 (Figure 1B).

We then excluded 599 miRNAs according to the following criteria: (1) average threshold cycle was relatively high (> 30) in either HepG2 or HepG2.2.15, and was reasonably low in the other samples (< 30); (2) average threshold cycle was relatively high (> 30), meaning that its relative expression level was low, in both HepG2 and

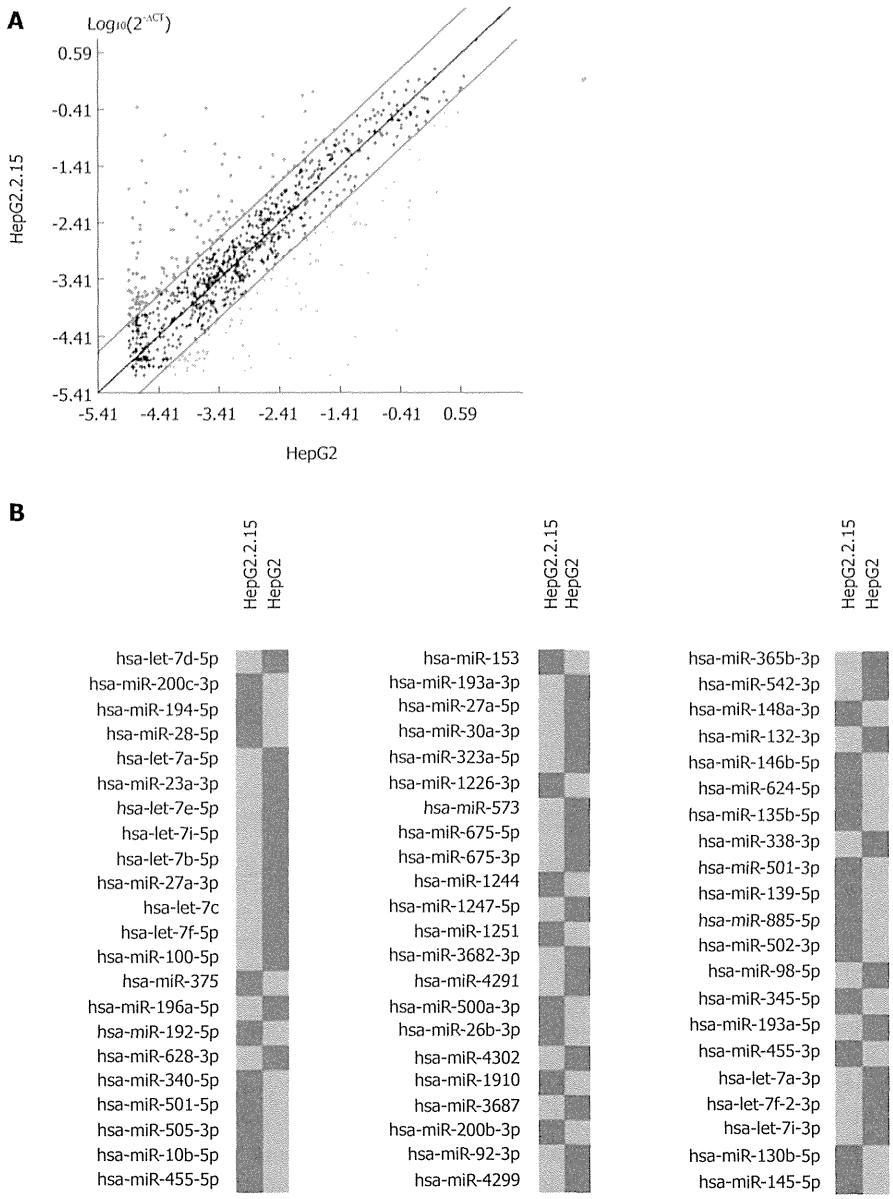

Figure 1 MicroRNAs expression in hepatoma cells HepG2.2.15 and HepG2. A: Scatter plots of 1008 miRNAs indicate 2^{-dCT} numerical values in HepG2 cells (x-axis) and HepG2.2.15 cells (y-axis). The black line indicates fold changes of 1. The pink lines indicate 5-fold change in miRNA expression threshold, comparing HepG2.2.15 with HepG2. Red + indicates miRNA expressed at least 5-fold higher in HepG2.2.15 than in HepG2 cells. Green + indicates miRNA expressed at least 5-fold lower in HepG2.2.15 than in HepG2 cells. Black + indicates that the difference of miRNA between the two cells was within 5-fold; B: Comparison of miRNAs expression between HepG2 and HepG2.2.15 cells. Red color indicates miRNA expressed at least 5-fold higher in HepG2.2.15 than in HepG2 cells. Green color indicates miRNA expressed at least 5-fold lower in HepG2.2.15 than in HepG2 cells. miRNAs: MicroRNAs.

HepG2.2.15; and (3) average threshold cycle was either not determined or was greater than the defined cut-off value (default 35) in both samples, meaning that its expression was undetected, making this fold-change result erroneous and uninterpretable.

Out of 409 miRNAs examined, 30 (7.3%) were upregulated by 5-fold or greater in HepG2.2.15 compared to HepG2. Twelve miRNAs (miR-200b-3p, miR-505-3p, miR-148a-3p, miR-145-5p, miR-194-5p, miR-885-5p, miR-192-5p, miR-146b-5p, miR-340-5p, miR-375, miR-139-5p and miR-200c-3p) were upregulated 10-fold or more in HepG2.2.15 cells. MiRNAs upregulated 5-fold or more are shown in Figures 1B and 2A. On the other hand, out of 409 miRNAs, 35 (8.6%) were downregulated 5-fold or more in HepG2.2.15 compared to HepG2. Twenty-two miRNAs (let-7c, miR-573, let-7b-5p, miR-338-3p, miR-100-5p, miR-92b-3p, miR-542-3p, miR-4302, miR-4291, miR-193a-5p, miR-98-5p, miR-4299, miR-132-3p, let-7f-2-3p, let-7f-5p, let-7i-5p, let-7d-5p, miR-193a-3p, let-7a-5p, let-7i-3p, miR-196a-p and let-7a-3p) were downregulated 10-fold or more in HepG2.2.15 cells. MiRNAs downregulated 5-fold or

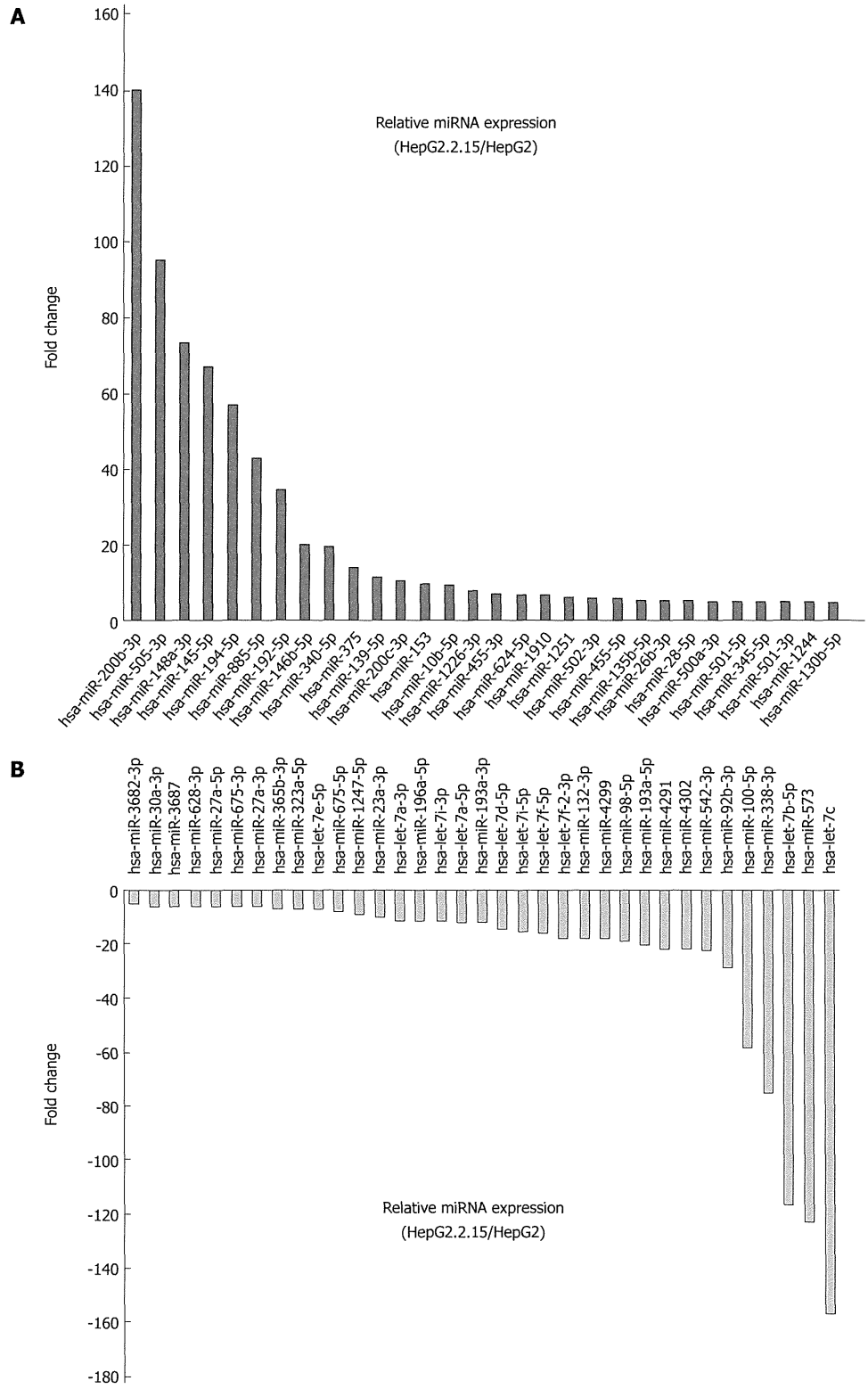

Figure 2 MicroRNAs expressed at more than 5-fold difference between hepatoma cells. HepG2.2.15 and HepG2 cells. A: MiRNA expressed at least 5-fold higher in HepG2.2.15 than in HepG2 cells; B: MiRNA expressed at least 5-fold lower in HepG2.2.15 than in HepG2 cells. miRNAs: MicroRNAs.

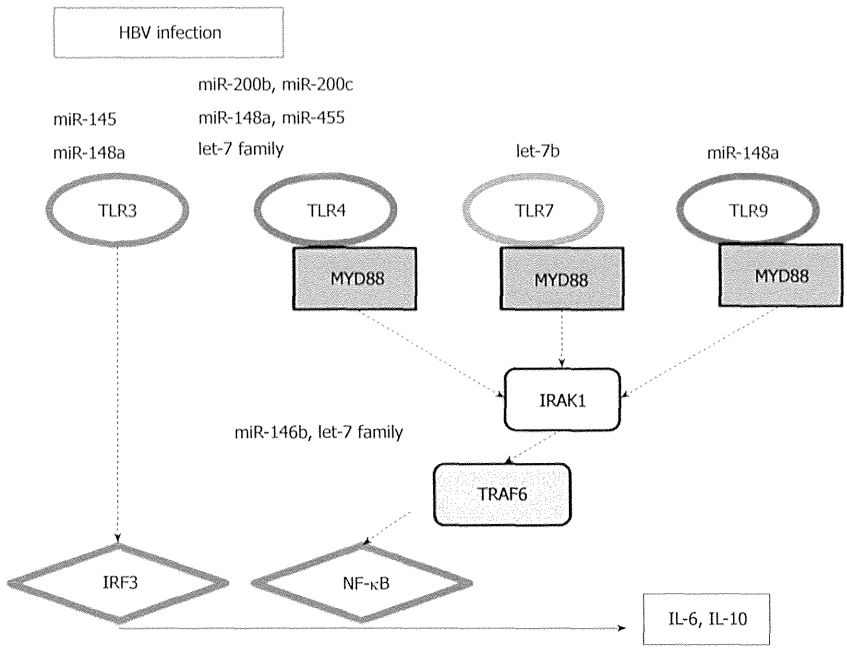

Figure 3 MicroRNAs and Toll-like receptor signaling pathway in hepatitis B virus infection. IRAK: Interleukin (IL)-1 receptor-associated kinase 1; IRF: Interferon regulator factor; miRNA: MicroRNA; MYD88: Myeloid differentiation factor 88; NF-κB: Nuclear factor-κB; TLR: Toll-like receptor; TRAF: Tumor necrosis factor receptor-associated factor.

Table 1 MicroRNAs associated with Toll-like receptor signaling pathways, upregulated by 5-fold or greater in HepG2.2.15 than in HepG2

MicroRNAs	Genomic location	Fold changes	Description of target molecules/pathways	Ref.
miR-200b-3p	1p36.33	140.15	TLR4 signaling through MyD88-dependent pathway	[47]
miR-148a-3p	7p15.2	73.36	TLR3, TLR4 and TLR9 agonists upregulated miR-148/152 expression	[48]
miR-145-5p	5q32	66.97	miR-145 promoted interferon-β induction by SOCS7	[49]
miR-146b-5p	10q24.32	20.05	TNF receptor-associated factor 6 and IL-1 receptor-associated kinase 1	[50]
miR-200c-3p	12p13.31	10.75	TLR4 signaling through MyD88-dependent pathway	[47]
miR-455-3p	9q32	7.36	miR-455 was involved in TLR4 signaling pathway through E2F1 transcription factor	[51]
miR-455-5p	9q32	5.76	miR-455 was involved in TLR4 signaling pathway through E2F1 transcription factor	[51]

Genomic location was analyzed using GeneCards (http://gene4.weizmann.ac.il/). TLR: Toll-like receptor.

more are shown in Figures 1B and 2B.

MIRNAS RELATED TO TLR PATHWAY UPREGULATED IN HEPG2.2.15 CELL LINES

Innate immunity represents the first line of defense against HBV, and we and others have reported its importance in the persistence of HBV infection[2,5-11]. So, we focused on miRNAs related to the TLR pathway. Among miRNAs upregulated 5-fold or more in HepG2.2.15 cells, 7 miRNAs (miR-200b-3p, miR-148a-3p, miR-145-5p, miR-146b-5p, miR-200c-3p, miR-455-3p and miR-455-5p) were reported to be related to TLR pathways (Table 1). MiRNAs miR-200b and miR-200c are the factors that modify the efficiency of TLR4 signaling through MYD88 in HEK293 cells[47]. TLR3, TLR4 and TLR9 agonists upregulated miR-148/152 expression and downregulated calcium/calmodulin-dependent protein kinase II (CaMK II) in dendritic cells (DCs) on maturation[48]. Thus miR-148/152 can act as fine-tuners in regulating the innate response and antigen-presenting capacity of DCs[48]. Exogenous miR-145 promoted IFN-β induction by targeting the suppressor of cytokine signaling 7 (SOCS7), through the nuclear translocation of signal transducer and activator of transcription 3 (STAT3) and SOCS7-silencing enhanced IFN-γ induction by stimulation with TLR3 ligand, poly(I-C)[49]. MiR-146 plays a role in the control of TLR and cytokine signaling through a negative feedback regulation loop involving down-regulation of interleukin (IL)-1 receptor-associated kinase 1 (IRAK1) and tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) protein levels[50]. MiR-455 was involved in the TLR4 signaling pathway through E2F1 transcription factor[51].

Table 2 MicroRNAs associated with Toll-like receptor signaling pathways, downregulated by 5-fold or greater in HepG2.2.15 than in HepG2

MicroRNAs	Genomic location	Fold changes	Description of target molecules/pathways	Ref.
let-7e-5p	19q13.33	-7.29	Akt1 activated by TLR4-ligand LPS, positively regulated let-7e	[52]
let-7a-3p	9q22.32	-11.44	Repression of let-7 family relieves IL-6 and IL-10 mRNAs from negative post-transcriptional control in TLR4 signaling pathway	[53]
	11q24.1			
	22q13.31			
let-7i-3p	12q14.1	-11.57	let-7i regulates Toll-like receptor 4 expression	[54]
let-7a-5p	9q22.32	-11.96	Repression of let-7 family relieves IL-6 and IL-10 mRNAs from negative post-transcriptional control in TLR4 signaling pathway	[53]
	11q24.1			
	22q13.31			
let-7d-5p	9q22.32	-14.03	Repression of let-7 family relieves IL-6 and IL-10 mRNAs from negative post-transcriptional control in TLR4 signaling pathway	[53]
let-7i-5p	12q14.1	-15.10	let-7i regulates Toll-like receptor 4 expression	[54,55]
miR-132-3p	17p13.3	-18.18	TNF receptor-associated factor 6 and IL-1 receptor-associated kinase 1	[56]
let-7b-5p	22q13.31	-116.31	let-7b activates TLR 7	[56]

Genomic location was analyzed using GeneCards (http://gene4.weizmann.ac.il/). TLR: Toll-like receptor.

MIRNAS RELATED TO TLR PATHWAY DOWNREGULATED IN HEPG2.2.15 CELL LINES

Among miRNAs downregulated 5-fold or more in HepG2.2.15 cells, 8 miRNAs (let-7e-5p, let-7a-3p, let-7i-3p, let-7a-5p, let-7d-5p, let-7i-5p, miR-132-3p and let-7b-5p) were reported to be related to TLR pathways (Table 2). Protein kinase Akt1, which is activated by the TLR4-ligand lipopolysaccharide (LPS), positively regulated let-7e and miR-181c but negatively regulated miR-155 and miR-125b[52]. Repression of the let-7 family relieves IL-6 and IL-10 mRNAs from negative post-transcriptional control in the TLR4 signaling pathway[53], and the miRNAs let-7i and let-7b activate TLR4 and TLR7, respectively[54-56].

ROLE OF MIRNAS IN REGULATION OF INNATE IMMUNE RESPONSE IN HBV INFECTION

In the present study, 30 and 35 miRNAs were upregulated and downregulated, respectively, by 5-fold or greater in HepG2.2.15 compared to its parental cell line HepG2. These results indicate that miRNAs could play an important role in chronic persistent HBV infection. Su *et al*[57] reported that miR-155 enhances innate antiviral immunity through promoting the JAK/STAT signaling pathway by targeting SOCS1, inhibiting HBV replication. The possibility cannot be ruled out that HBV persistently infects hepatocytes through the regulation of miRNAs.

We also speculated that several of the miRNAs involved in the TLR signaling pathway play a critical role in innate immunity against HBV infection[5,24] (Figure 3). It has been reported that miR-21[58], miR-22[59,60], miR-122[58], miR-194[61] and miR-219-1[62] are associated with chronic persistent HBV infection as well as its clearance. In the present study, miR-194 was upregulated 10-fold or more in HepG2.2.15 cells.

CONCLUSION

MicroRNAs miR-122 and miR-130a play an important role in chronic hepatitis C[63,64]. Regulation of miRNAs also plays an important role in HIV infection[65]. In HCV infection, a set of miRNAs that regulate host immune response are modulated[66]. We and others have demonstrated that HBV modulates the host immune response. It might be possible that HBV as well as HCV regulates host immune response through the regulation of miRNAs in some steps toward chronic infection. MiRNAs and their regulation play a critical role in HBV infection, and HBV may regulate the TLR signaling pathway through the regulation of miRNAs.

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Original Article

Risk of hepatocellular carcinoma in cirrhotic hepatitis B virus patients during nucleoside/nucleotide analog therapy

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Aim: Some patients develop hepatocellular carcinoma (HCC) during nucleoside/nucleotide analog (NA) therapy even if alanine aminotransferase (ALT) or hepatitis B virus (HBV) DNA levels are sufficiently reduced. The aim of this study is to identify the risk factors of development of HCC during NA therapy.

Methods: Six hundred and two patients were analyzed who were continuously receiving NA for chronic HBV infection. The patients who developed HCC previously or within 1 year of therapy were excluded. In the patients studied, the median duration of therapy was 90 months. A total of 492 patients had chronic hepatitis (CH) and 110 had liver cirrhosis (LC).

Results: In 602 patients, the rate of normalization of ALT, loss of serum HBV DNA and development of HCC were 90.4%, 55.4%, and 6.1%, respectively. The significant risk factors of development of HCC were LC status and duration of therapy. The annual incidence of HCC in LC patients was 2.53%/year, compared with 0.34%/year in CH patients. When the relation between the incidence of HCC and the response to therapy was evaluated, in patients with normalization of ALT level, loss of HBV DNA by real-time polymerase chain reaction or hepatitis B e-antigen seroconversion, the incidences of HCC was reduced to some extent. However, none of the patients who achieved hepatitis B surface antigen (HBsAg) seroclearance during NA therapy developed HCC.

Conclusion: LC status was the significant risk factor of development of HCC during NA therapy. However, none of the patients who showed HBsAg seroclearance developed HCC. The ultimate goal of therapy for reduced risk of HCC may be HBsAg seroclearance.

Key words: hepatitis B surface antigen seroclearance, hepatitis B virus DNA, hepatocellular carcinoma, liver cirrhosis, nucleoside/nucleotide analog therapy, risk factors

INTRODUCTION

HEPATITIS B VIRUS (HBV) can cause chronic hepatitis (CH), liver cirrhosis (LC) and hepatocellular carcinoma (HCC).[1–3] To prevent progression of liver diseases, nucleoside/nucleotide analogs (NA), such as lamivudine, adefovir or entecavir, are used widely for antiviral therapy of chronic HBV infection.[4–6] During NA therapy, alanine aminotransferase (ALT) and HBV DNA levels are often reduced within normal ranges or under the detection limit. However, it is true that some patients develop HCC during NA therapy.[7]

The conventional goals of antiviral therapy for patients with chronic HBV infection should be hepatitis B e antigen (HBeAg) seroconversion, normalization of ALT level and loss of serum HBV DNA. Ultimately, loss of hepatitis B surface antigen (HBsAg), namely HBsAg seroclearance, is desirable. However, HBsAg seroclearance during NA therapy is very rare, especially in Asian countries.[8] It was reported that low serum HBsAg levels were associated with a low risk of HCC in patients with low HBV DNA levels.[9] Hence, the surrogate goals of NA therapy could be normalization

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of ALT levels, loss of HBV DNA and HBeAg seroconversion.

Recent studies have reported that the incidence of development of HCC in patients receiving NA was significantly reduced, compared with non-therapy patients.[10,11] In addition, it was shown that a high proportion of patients with LC during NA therapy carried a higher risk of HCC, compared with those with CH.[11–13] However, the relationship between the risk of HCC during NA therapy and the responses of NA therapy is not clear.

The aim of the present study was to identify the risk factors for the development of HCC during NA therapy and the relation with the responses to the therapy.

METHODS

Patients

SEVEN HUNDRED AND seventy-two patients receiving NA therapy were recruited retrospectively from the 15 hospitals in the Japanese Red Cross Liver Network. All patients were HBsAg positive for more than 1 year, serum HBV DNA positive before NA therapy, and negative for anti-hepatitis C virus or anti-HIV. Of 772 patients, 25 were excluded because they developed HCC before commencement of NA therapy or developed HCC within 1 year of NA therapy. In addition, 145 patients were excluded because of lack of data of quantitative HBsAg levels. Therefore, 602 patients were analyzed in this study.

Of 602 patients, 492 had CH and 110 had LC. Two hundred and ten patients were diagnosed by liver biopsy with their written informed consent, and the other patients were diagnosed by clinical findings, laboratory data and computed tomography or magnetic resonance imaging. Of 602 patients, 405 patients were receiving entecavir, 56 were receiving lamivudine, 67 switched from lamivudine to entecavir and 74 switched from lamivudine alone to lamivudine and adefovir. All patients were receiving NA continuously for more than 1 year until the end of follow up.

Methods

To detect development of HCC during NA therapy, all patients underwent ultrasound, computed tomography or magnetic resonance imaging for screening HCC at least every 6 months.

The duration of NA therapy was defined as the months from the start of therapy to the final month of the continuous therapy. If HCC was detected during therapy, the duration was stopped at the month of detection of HCC.

The normal ALT level was defined as less than 40 IU/L in this study. The serum HBV DNA level was determined by real-time polymerase chain reaction (PCR) or transcription-mediated amplification (TMA).

Loss of HBV DNA was defined as negative state by real-time PCR. In only 53 patients, HBV DNA were determined by TMA. Thus, analysis of the relationship between the incidence of HCC and loss of HBV DNA was evaluated in the patients determined only by real-time PCR.

Hepatitis B surface antigen levels were quantitatively determined by chemiluminescent immunoassay (CLIA) or chemiluminescence enzyme immunoassay (CLEIA). The upper limits of detection of CLIA or CLEIA were 2000 or 250 IU/mL, respectively. The lower limits of detection of CLIA or CLEIA were 0.05 or 0.03 IU/mL, respectively. Thus, in this study, HBsAg seroclearance was defined as HBsAg levels of less than 0.05 IU/mL by CLIA or less than 0.03 IU/mL by CLEIA.

Because only approximately 35% of all the patients were diagnosed by liver biopsy, we used two other methods to confirm the risk of HCC in cirrhotic patients with advanced liver fibrosis. The first method was a stratification by platelet count less than or $10 \times 10^4/\mu L$ or more.[14,15] In addition, the other method was a stratification by FIB-4 index, less than or 3.25 or more. These platelet count or FIB-4 index were considered to be good indicators of cirrhosis or advanced liver fibrosis.[16,17]

This study was designed and performed in accordance with the provision of the Declaration of Helsinki and Good Clinical Practice Guidelines, and was approved by the institutional review board in all attending hospitals.

Statistical analyses were carried out using the Wilcoxon rank sum test, the χ^2-test or Fisher's exact test in the univariate analyses, and by the Cox proportional hazard model in the multivariate analysis. Statistical significance level was set at $P < 0.05$.

RESULTS

THE BASELINE CHARACTERISTICS of the patients studied are shown in Table 1. The median age was 52 years (range, 21–79), and the male : female ratio was 381:221. The median duration of NA therapy was 90 months (range, 12–204). The ratio of CH : LC disease status was 492:110, and the family history of yes : no : unknown HCC was 64:375:163. The laboratory findings were: median ALT, 69 IU/L (range, 9–2821); median platelet count, $16.1 \times 10^4/\mu L$ (range,

Table 1 Baseline characteristic of the patients studied

Characteristics	$n = 602$
Age (years)	52 (21–79)
Sex (M:F)	381:221
Nucleotide analogs (Ent : Lam→Ent : Lam : Lam + Ade)	405:67:56:74
Duration of nucleotide analog therapy (months)	90 (12–204)
Disease (CH : LC)	492:110
Family history of HCC (yes : no : unknown)	64:375:163
AST (IU/L)	58 (14–1752)
ALT (IU/L)	69 (9–2821)
Albumin (mg/dL)	4.2 (1.7–5.5)
Platelet count ($\times 10^4/\mu L$)	16.1 (3.1–41.7)
HBV genotype (A : B : C : D : NT)	4:23:231:2:342
HBeAg status (positive : negative)	295:305
HBV DNA level (log copies/mL)	6.8 (2.3–9.1)

Median (range).
Ade, adefovir; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CH, chronic hepatitis; Ent, entecavir; HBV, hepatitis B virus; HBeAg, hepatitis B e-antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; Lam, lamivudine; LC, liver cirrhosis; N, no; NT, not tested; Y, yes.

3.1–41.7); HBV genotype A : B : C : others : unknown, 4:23:231:2:342; HBeAg positive : negative status, 295:305; and median HBV DNA level, 6.8 log copies/mL (range, 2.3–9.1).

Of 602 patients at the final follow up during NA therapy, 90.4% showed a normal ALT level, 55.4% lost serum HBV DNA, 16.3% showed HBeAg seroconversion, 2.2% revealed HBsAg seroclearance and 6.1% developed HCC during therapy.

Risk factors of development of HCC during NA therapy

The risk factors associated with the development of HCC during therapy were identified to be pretreatment disease status (LC) ($P < 0.001$), duration of NA therapy ($P < 0.001$), ALT levels ($P < 0.001$) and platelet counts ($P < 0.001$) by univariate analyses (Table 2). By multivariate analyses, LC status and duration of therapy were demonstrated to be the most significant risk factors ($P < 0.001$ and $P < 0.001$, respectively).

Cumulative incidence of development of HCC

The cumulative incidence of the development of HCC was analyzed by the Kaplan–Meier method (Fig. 1a).

Table 2 Significant risk factors related with development of HCC during NA therapy

Characteristics	Development of HCC during therapy		Analysis	
	Yes $n = 37$ (6.1%)	No $n = 565$ (93.9%)	Univariate P	Multivariate P
Age (years)	57.2 (41–76)	51.5 (21–79)	0.036	0.446
Sex (M : F)	25:12	356:209	0.725	
Disease (CH : LC)	13:24	479:86	<0.001	<0.001
Duration of NA therapy (months)	37 (12–98)	94.6 (12–204)	<0.001	<0.001
Family history of HCC (yes : no)	6:21	58:354	0.509	
Pretreatment data				
ALT (IU/L)	55 (17–274)	72 (9–2821)	<0.001	0.401
Platelet count ($\times 10^4/\mu L$)	10.9 (4.0–30.3)	16.4 (3.1–41.7)	<0.001	0.146
HBeAg status (positive : negative)	17:20	280:285	0.798	
HBV DNA level (log copies/mL)	6.6 (2.5–8.9)	6.8 (2.3–9.1)	0.090	
HBsAg level >250 IU/mL (Y : N : NT)	24:6:7	410:61:94	0.519	
Final data during therapy				
ALT (<40 : ≥40 IU/L)	28:9	501:64	0.098	
HBeAg seroconversion in HBeAg positive patients (Y : N)	2:6	46:106	0.749	
Loss of HBV DNA by real-time PCR (Y : N)	14:17	247:193	0.330	
HBsAg seroclearance (Y : N)	0:37	13:552	0.351	

ALT, alanine aminotransferase; AST, aspartate aminotransferase; CH, chronic hepatitis; HBeAg, hepatitis B e-antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; LC, liver cirrhosis; N, no; NA, nucleoside/nucleotide therapy; NT, not tested; PCR, polymerase chain reaction; Y, yes.

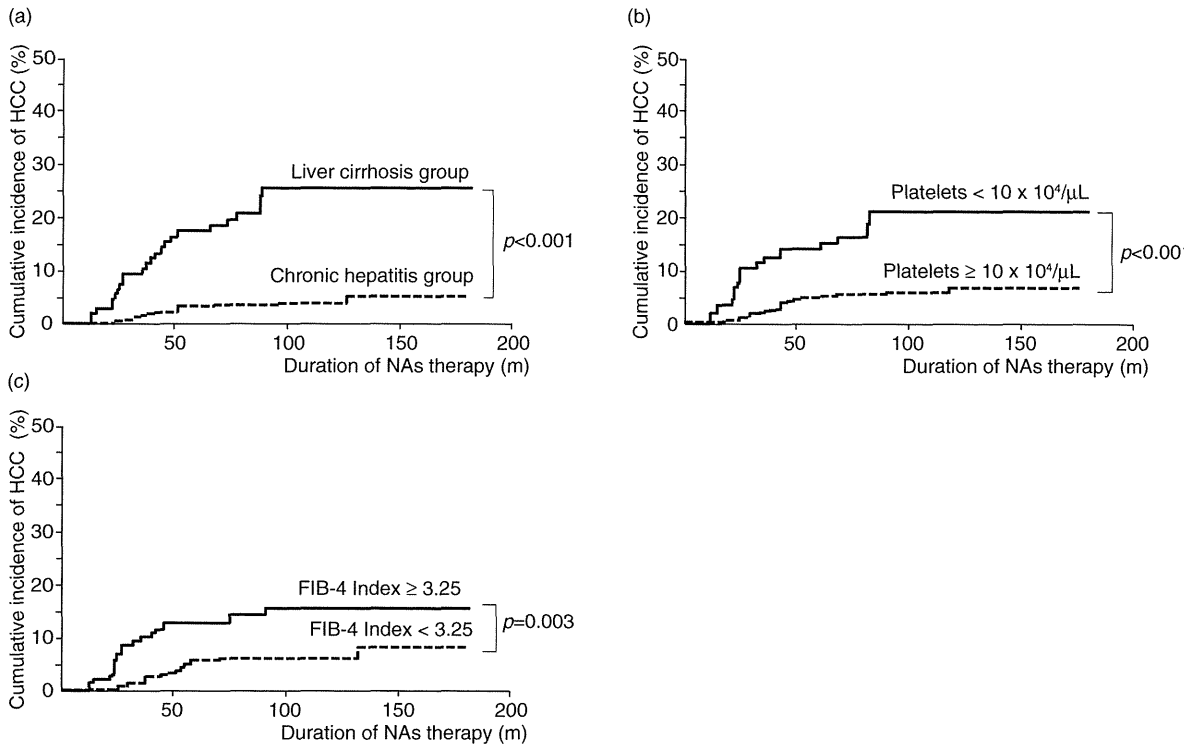

Figure 1 Cumulative incidence of development of hepatocellular carcinoma (HCC) during nucleoside/nucleotide analog (NA) therapy by Kaplan–Meier analysis. (a) Stratification by clinical or histological diagnosis, liver cirrhosis (LC) or chronic hepatitis (CH). (b) Stratification by platelet count. (c) Stratification by the FIB-4 Index. All these analyses indicated that cirrhotic patients carried a higher risk of HCC than non-cirrhotic patients.

The incidence of HCC was significantly higher in the LC group than in the CH group ($P < 0.001$). The annual incidence of development of HCC in the LC group and the CH group was 2.53%/year and 0.34%/year, respectively.

Only approximately 35% of the patients were diagnosed by liver biopsy. Hence, we employed two other methods to confirm the higher risk of HCC in cirrhotic patients with advanced liver fibrosis. By the first method of a stratification according to platelet count ($\geq 10 \times 10^4/\mu L$ or $<10 \times 10^4/\mu L$), the cumulative incidence of HCC was significantly high in the patients with a platelet count of less than $10 \times 10^4/\mu L$, compared with those with a platelet count of $10 \times 10^4/\mu L$ or more ($P < 0.001$) by the Kaplan–Meier analysis (Fig. 1b). Second, when the incidence of HCC was stratified by the FIB-4 index, the patients with a FIB-4 index of 3.25 or more had a significantly high risk, compared with those with a FIB-4 index of less than 3.25 ($P = 0.003$) (Fig. 1c).

Relationship between response to NA and incidence of HCC

To identify the goal of NA therapy for suppression of development of HCC, the relationship between response to NA therapy and incidence of HCC is important. First, when the relationship between normalization of ALT (<40 IU/L) and incidence of HCC was compared, there was no significant difference in the incidence of HCC between the abnormal ALT group and the normalized ALT group in patients with CH and those with LC (Fig. 2a). Second, when the relationship between loss of serum HBV DNA by real-time PCR and incidence of HCC was compared, there was no significant difference between positive and negative HBV DNA groups in CH patients and LC patients (Fig. 2b).

Third, when the relationship between HBeAg seroconversion and incidence of HCC was compared in the patients with positive HBeAg at commencement of the

therapy, there was no significant difference between the group with HBeAg seroconversion and the group without HBeAg seroconversion (Fig. 2c).

When the patients achieved these three goals of NA therapy, namely, normalization of ALT, loss of HBV DNA and HBeAg seroconversion, the incidence of HCC reduced only to some extent.

Finally, the relationship between HBsAg seroclearance and incidence of HCC was compared, none of the patients who achieved HBsAg seroclearance developed HCC in this study (Fig. 2d).

Relationship between duration of NA therapy and incidence of HCC

Duration of NA therapy was a significant risk factor associated with the development of HCC by multivariate analysis (Table 2). Thus, the relationship between duration of NA therapy and incidence of HCC was studied by receiver–operator curve (ROC) analysis. The area under the ROC was 0.802 (95% confidence interval [CI], 0.749–0.856; $P < 0.001$). Duration of therapy of less than 57 months was demonstrated to be the nearest cut-off value with a sensitivity of 76.1% and specificity of 76.3%. When compared with the groups with therapy duration of 57 months or more, and less than 57 months, a significantly higher incidence of development of HCC was observed in the group with therapy duration of less than 57 months than the group with therapy duration of 57 months or more in CH patients and LC patients ($P < 0.001$).

DISCUSSION

IN THE PRESENT study, we indicated that LC patients have a significantly higher risk of development of HCC than CH patients during NA therapy; and that the risk of HCC still existed even if the conventional goals of therapy like normalization of ALT, loss of serum HBV DNA or HBeAg seroconversion were achieved during therapy. However, it was demonstrated that none of the patients who achieved HBsAg seroclearance during therapy developed HCC in this study. It was confirmed that the ultimate goal of antiviral therapy for patients with chronic HBV infection should be HBsAg seroclearance.

Generally, patients with chronic HBV infection are at a high risk of development of HCC.[18] Even during antiviral therapy, a proportion of patients develop HCC.[7,10,11] Furthermore, some patients whose serum HBV DNA levels are under the detection limit level and ALT levels are within normal range develop HCC.

It has been widely stated previously that the conventional goals of therapy for patients with chronic HBV infection are normalization of ALT level, loss of serum HBV DNA and HBeAg seroconversion.[19–21] Of course, the ultimate goal of the therapy should be HBsAg seroclearance. However, HBsAg seroclearance is not common during antiviral therapy. Therefore, achievable conventional goals like normalization of ALT, serum HBV DNA and HBeAg seroconversion are usually pursued.

To achieve these goals, knowing the risk factors of development of HCC during NA therapy is important. We identified LC status and short duration of therapy to be the most significant factors associated with HCC during therapy. That is, the incidence of HCC during therapy in CH patients with a favorable virological response was very low, compared with cirrhotic patients with a favorable response.

Recently, it was reported that the risk of HCC in the patients undergoing NA therapy was reduced, compared with untreated patients, and that LC status was a significant factor of HCC during NA therapy.[10,11] Therefore, NA therapy is thought to be useful for reducing risk of development of HCC in CH and LC patients. However, some risk of HCC during NA therapy was noted in only LC patients.

In our study, the number of patients whose histological diagnosis was confirmed by liver biopsy was limited. The remainder of patients were diagnosed by clinical findings and CT or MRI. Thus, to confirm that LC status was a risk factor for HCC, all patients were analyzed by stratification of platelet count and FIB-4 index.[14–17] It was indicated that lower platelet count or higher FIB-4 index were strongly associated with cirrhotic condition. Using these two additional methods, cirrhotic patients with advanced liver fibrosis were confirmed to have a higher risk of HCC than non-cirrhotic patients.

So far, the relationship between response to NA therapy and risk of HCC is still unclear. Some previous papers have reported that low HBV DNA and normal ALT were associated with lower risk of development of HCC.[9,22–24] In this study, it was demonstrated that in CH patients who achieved normalization of ALT, loss of serum HBV DNA and HBeAg seroconversion, the risk of HCC was reduced remarkably, whereas the risk of HCC was not related to virological response to the therapy in LC patients. Hence, we should observe LC patients carefully for development of HCC during NA therapy, irrespective of a good virological response.

Because this was a multicenter study, HBsAg levels were determined by CLIA or CLEIA. These two methods

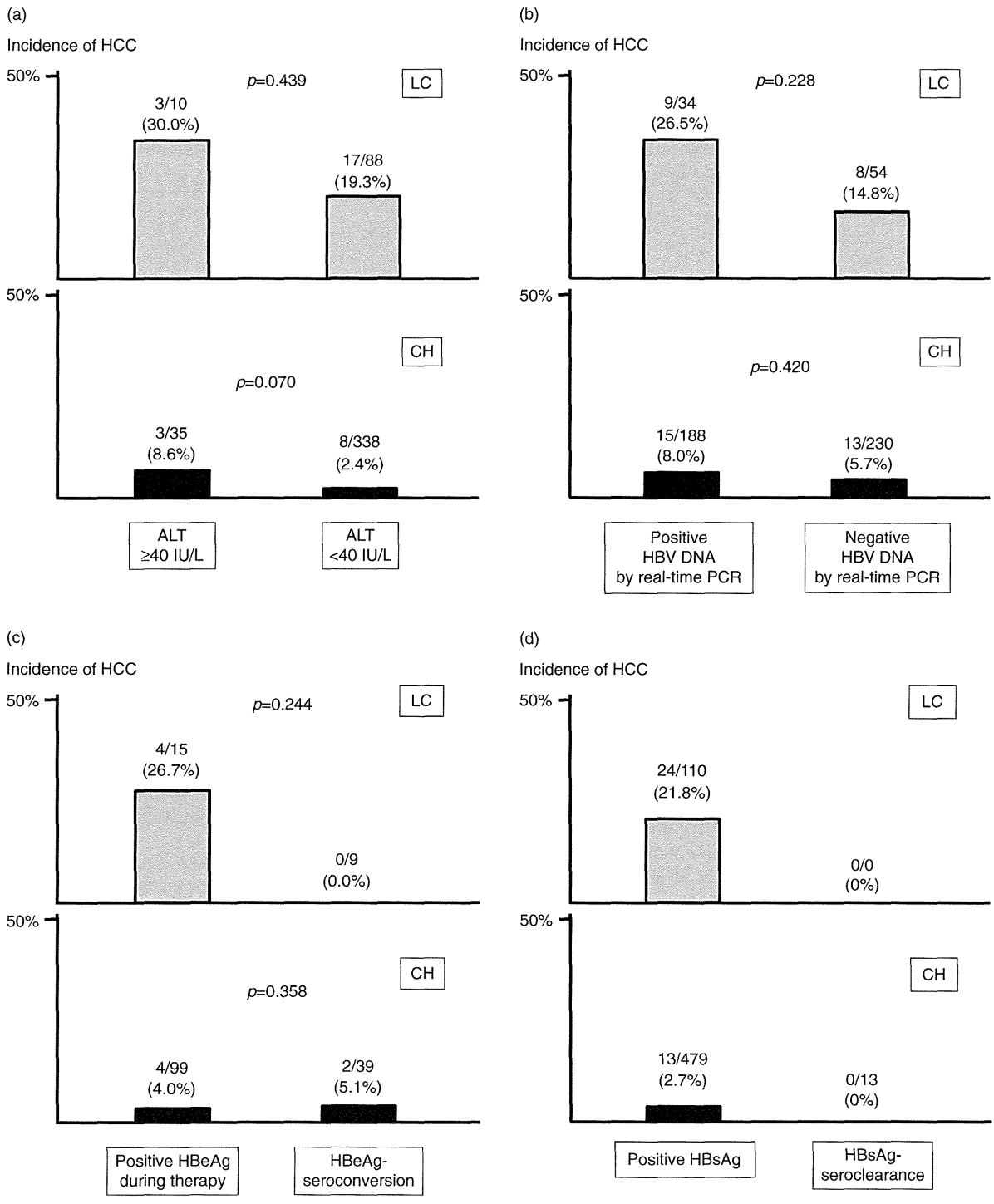

Figure 2 Relationship between incidence of hepatocellular carcinoma (HCC) and various virological responses. (a) Comparison between those with alanine aminotransferase (ALT) of ≥40 IU/L and <40 IU/L. (b) Comparison between positive and negative hepatitis B virus (HBV) DNA groups by real-time polymerase chain reaction (PCR). (c) Comparison between the positive hepatitis B e-antigen (HBeAg) group and the HBeAg seroconversion group. (d) Comparison between the positive hepatitis B surface antigen (HBsAg) group and HBsAg seroclearance group (HBsAg <0.05 IU/mL by chemiluminescent immunoassay or <0.03 IU/mL by chemiluminescence enzyme immunoassay). In these comparisons, in chronic hepatitis (CH) patients, a very low risk of HCC was observed, compared with liver cirrhosis (LC) patients, irrespective of conventional virological responses. However, none of the patients who achieved HBsAg seroclearance developed HCC during the therapy.

were demonstrated to be in good correlation with each other in previous studies.[25] In this study, HBsAg seroclearance was defined as less than 0.05 IU/mL by CLIA or less than 0.03 IU/mL by CLEIA. Only 13 out of 602 patients achieved HBsAg seroclearance in this study. While HBsAg seroclearance was not common, none of the 13 patients developed HCC during the therapy. Thus, HBsAg seroclearance was indicated to be the ultimate goal of the therapy.

In this study, a short duration of NA therapy, especially if less than 57 months, was revealed to carry a high risk of HCC in both CH and LC patients. In the early duration of therapy, inflammation in liver still may be active. It was supposed that a long enough duration of suppression of HBV and ALT by NA therapy was needed for suppression of development of HCC. However, because this study was retrospective, some selection bias of the patient data might not have been excluded. It was a concern that rather more patients who did not develop HCC during long-term therapy were collected in this study. However, careful observation for risk of development of HCC is necessary in the early stage of therapy.

In summary, we demonstrated that during NA therapy for chronic HBV infection, cirrhotic status was a significant risk factor of development of HCC. In such a scenario, careful observation is necessary irrespective of various virological responses. Finally, the ultimate goal of NA therapy, as well as other antiviral therapy, should be HBsAg seroclearance.

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