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 posttranscriptionally 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. reported that TLR1, TLR2, TLR4 and TLR6 transcripts were also downregulated in PBMC of chronic hepatitis B patients[29]. 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 proinflammatory 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 (HBeAG) 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 nt1753 and 1754 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 ~1,000 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, USA), 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 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-5p and let-7a-3p) were downregulated 10-fold or more in HepG2.2.15 cells. MiRNAs downregulated 5-fold or 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 (CaMKII) 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-β 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].

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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Figure 1 MicroRNAs (miRNAs) 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.

Figure 2 MicroRNAs (miRNAs) 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.

Figure 3 MicroRNAs and Toll-like receptor (TLR) signaling pathway in HBV infection. IRAK, interleukin(IL)-1 receptor-associated kinase 1; IRF, interferon regulator factor; miR, 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 (TLR) 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/).

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

miRNA ID	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 11q24.1 22q13.31	-11.44	Repression of let-7 family relieves IL-6 and IL-10 mRNAs from negative post-transcriptional control in TLR4 signaling pathway	[53]
let-7i-3p	12q14.1	-11.57	let-7i regulates Toll-like receptor 4 expression	[54]
let-7a-5p	9q22.32 11q24.1 22q13.31	-11.96	Repression of let-7 family relieves IL-6 and IL-10 mRNAs from negative post-transcriptional control in TLR4 signaling pathway	[53]
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/).

Figure 1(A)

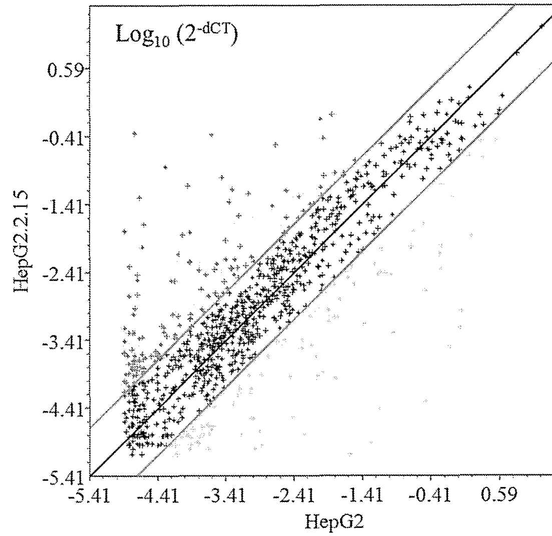


Figure 1(B)

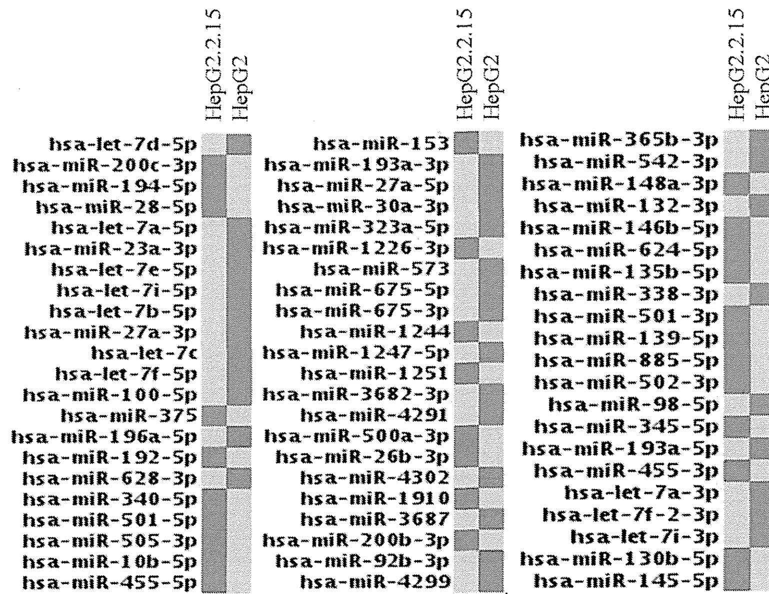
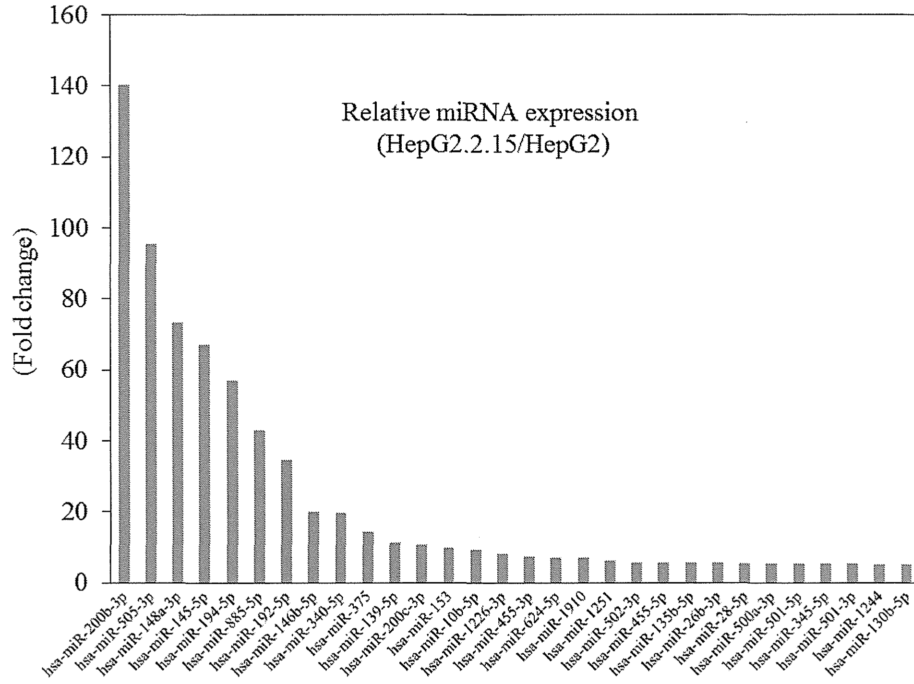


Figure 2(A)



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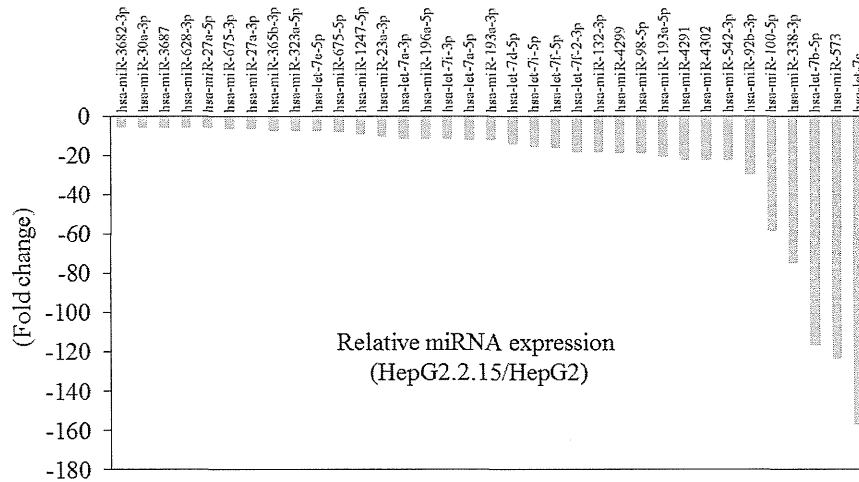

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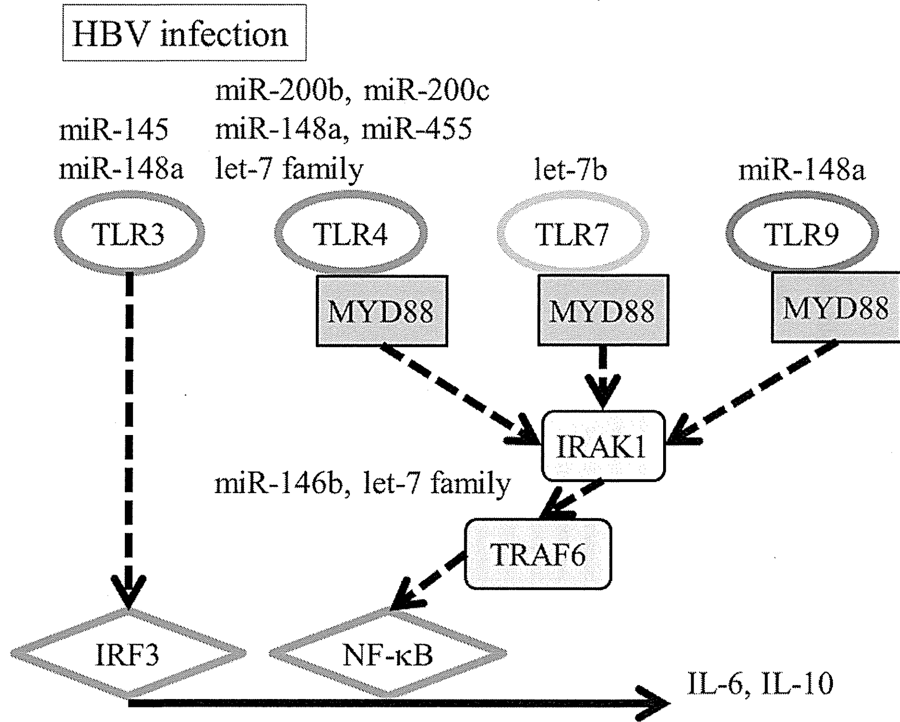
Figure 3



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Increased serum mitochondrial creatine kinase activity as a risk for hepatocarcinogenesis in chronic hepatitis C patients

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Serum mitochondrial creatine kinase (MtCK) activity was reportedly increased in cirrhotic patients although less prominent than that in hepatocellular carcinoma (HCC) patients. To elucidate the clinical significance of serum MtCK activity in chronic liver disease, 171 chronic hepatitis C patients were enrolled. Serum MtCK activity in study subjects was correlated with serum albumin, platelet counts, liver stiffness values and serum aspartate and alanine aminotransferase. In mouse fibrotic liver induced by bile duct ligation, ubiquitous MtCK mRNA and protein expressions were significantly enhanced and its immunoreactivity was increased, predominantly in hepatocytes. During the mean follow-up period of 2.7 years, HCC developed in 21 patients, in whom serum MtCK activity was significantly higher than that in patients without HCC development. Multivariate Cox regression analysis revealed that higher serum MtCK activity was a risk for HCC development. A cutoff value of MtCK for the prediction of HCC development was determined as 9.0 U/L on receiver operating characteristics analysis, where area under receiver operating characteristics curve was 0.754, with a sensitivity of 61.9%, a specificity of 92.8% and a high negative predictive value of 94.2%. Cumulative incidence of HCC was significantly higher in patients with serum MtCK activity of >9.0 U/L compared to those with serum MtCK activity of ≤9.0 U/L even in patients with elevated liver stiffness value, >15 kPa. In conclusion, serum MtCK activity may be increased correlatively with the stage of liver fibrosis and hepatocellular damage. Increased serum MtCK activity is an independent risk for hepatocarcinogenesis in chronic hepatitis C patients.

Early Detection and Diagnosis

Hepatocellular carcinoma (HCC) is one of the common malignancies worldwide,[1] and the number of patients suffering from HCC is currently increasing in many countries.[2,3] As HCC has a specific feature that it usually develops in the setting of chronic liver injury,[2] especially liver cirrhosis,[4] cancer surveillance, when performed intensively in patients with chronic liver injury, could lead to HCC detection in its early stage, where biomarkers for HCC may play an important role. Although novel therapies have been developed to prolong survival in patients with advanced HCC, their effects are rather limited,[5] suggesting that the effective way for early detection of HCC is urgently needed. To this end, many attempts have been made to explore a novel biomarker for HCC,[6,7] among which we have recently found that serum mitochondrial creatine kinase (MtCK) activity was increased in patients with HCC. Among two tissue-specific isozymes of MtCK, that is, ubiquitous MtCK (uMtCK) and sarcomeric MtCK (sMtCK), we have found that the increase in serum MtCK activity in HCC patients was mostly owing to uMtCK, not sMtCK.[8] We have further found high expression of uMtCK mRNA in human HCC cell lines compared to normal human liver tissue.[8] Recently, we have reported that high uMtCK expression in HCC denotes a poor prognosis with highly malignant potential.[9] It is worth noting the increased uMtCK expression occurred not only upon malignant changes in the liver, but also in several other malignant tumors such as gastric cancer, breast cancer and lung cancer.[10–13]

In our previous report, we have observed that serum MtCK activity was also increased in patients with liver cirrhosis compared to healthy control although less prominent than in HCC patients.[8] In fact, an elevated serum MtCK

Key words: ubiquitous mitochondrial creatine kinase, hepatocellular carcinoma, liver fibrosis

Abbreviations: AFP: alpha-fetoprotein; ALT: alanine aminotransferase; AST: aspartate aminotransferase; CT: computed tomography; DCP: des-gamma-carboxy prothrombin; GGT: γ-glutamyltransferase; HCC: hepatocellular carcinoma; HCV: hepatitis C virus; MtCK: mitochondrial isoenzyme of creatine kinase; sMtCK: sarcomeric mitochondrial creatine kinase; uMtCK: ubiquitous mitochondrial creatine kinase

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What's new?
Chronic liver injury such as viral hepatitis increases the risk to develop hepatocellular carcinoma (HCC). Here, the authors show that serum mitochondrial creatine kinase activity, a potential new biomarker for progressive liver damage, was increased in patients with chronic hepatitis C virus infection and correlated with the stage of liver fibrosis and hepatocellular damage. Similar results were reproduced in mice after liver damage via bile duct ligation. Notably, high serum mitochondrial creatine kinase activity was an independent risk factor for hepatocarcinogenesis in viral hepatitis patients, underscoring the promise of this new marker in the prediction and possibly pathogenesis of HCC.

activity was previously reported in patients with liver cirrhosis,[14] where MtCK was described as "Macro CK type 2."[14,15] However, the clinical significance of increased serum MtCK activity in cirrhotic patients has not been clarified yet. In our study, we wondered whether serum MtCK activity might be increased in patients with not only liver cirrhosis but also chronic liver disease, in general, with less fibrosis, and if so, what would be the clinical significance of increased serum MtCK activity in patients with chronic liver disease. To address these questions, we sought to analyze serum MtCK activity in patients with chronic hepatitis C without the presence and the history of HCC.

Material and Methods

Subjects

One-hundred seventy-one patients with chronic hepatitis C, who visited the Department of Gastroenterology, The University of Tokyo Hospital, Tokyo, Japan, between January 2010 and April 2011, were enrolled. Chronic hepatitis C was defined as serum anti-hepatitis C virus antibody positivity and a detectable HCV RNA level, having persistent liver damage for more than 6 months, where other causes of liver disease such as hepatitis B and alcohol abuse had been excluded. Patients with HCC at the time of enrollment or with past history of HCC were excluded from this analysis, where HCC was ruled out by ultrasonography, dynamic computed tomography (CT) and/or magnetic resonance imaging. To assess a potential relationship between serum MtCK activity and liver fibrosis, all the enrolled patients undertook liver stiffness measurement.

Our study was carried out in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Institutional Research Ethics Committees of the authors' institutions. In our study, informed consent was obtained for the use of the samples.

Measurement of MtCK activity

MtCK activity was measured with an immune-inhibition method using two types of anti-MtCK monoclonal antibodies, that is, an anti-uMtCK monoclonal antibody and an anti-sMtCK monoclonal antibody in addition to an anticreatine kinase-M antibody[16] as described previously.[8] JCA-BM8040 (JEOL, Tokyo, Japan) was used as an automatic analyzer. The regression line of this assay was linear up to at least 1,800 U/L. The minimum detection limit was 1.9 U/L. The within-run coefficient variations were 3.1 and 0.8% at the mean MtCK activities of 25.7 and 64.4 U/L, respectively. The between-run coefficient variations were 2.3% for both the mean MtCK activities of 24.0 and 59.5 U/L.

Measurements of other parameters

Ordinary serum chemistry parameters, albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (GGT) and total bilirubin, were analyzed using JCA-BM8040 (JEOL, Tokyo, Japan). Complete blood count examination was performed using XE-5000 (Sysmex, Kobe, Japan). Prothrombin time was measured using ACL TOP (Mitsubishi Chemical Medience, Tokyo, Japan). Alpha-fetoprotein (AFP) and des-gamma-carboxy prothrombin (DCP) were analyzed by a two-site immunoenzymetric assay using ST AIA-PACK AFP (TOSOH, Tokyo, Japan) and Lumipulse Presto PIVKAII (EIDIA, Tokyo, Japan), in automatic analyzers, AIA 2000 (TOSOH) and Lumipulse® PrestoII (FUJIREBIO, Tokyo, Japan), respectively. Liver stiffness was measured using transient elastography (FibroScan 502; EchoSens, Paris, France) as described previously.[17]

Animals and induction of liver fibrosis

Liver fibrosis was induced in C57BL/6N mice (CLEA Japan, Japan) by bile duct ligation at 4 weeks after the operation as described previously.[18]

All animals received humane care and the experimental protocol was approved by Animal Research Committee of the University of Tokyo.

Quantitative real-time polymerase chain reaction

Total RNA of mouse livers was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA). One microgram of purified total RNA was transcribed using a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany). Quantitative real-time polymerase chain reaction (PCR) was performed with a TaqMan Universal Master Mix No AmpErase UNG (Applied Biosystems, Foster City, CA). Mouse uMtCK primers and probe were obtained from Applied Biosystems, TaqMan Gene Expression Assays (Mm00438221_m1). The samples were incubated for 10 min at 95°C, followed by 40 cycles at 95°C for 15 sec and 60°C for 60 sec. The target gene mRNA expression level was relatively quantified to 18S

Early Detection and Diagnosis