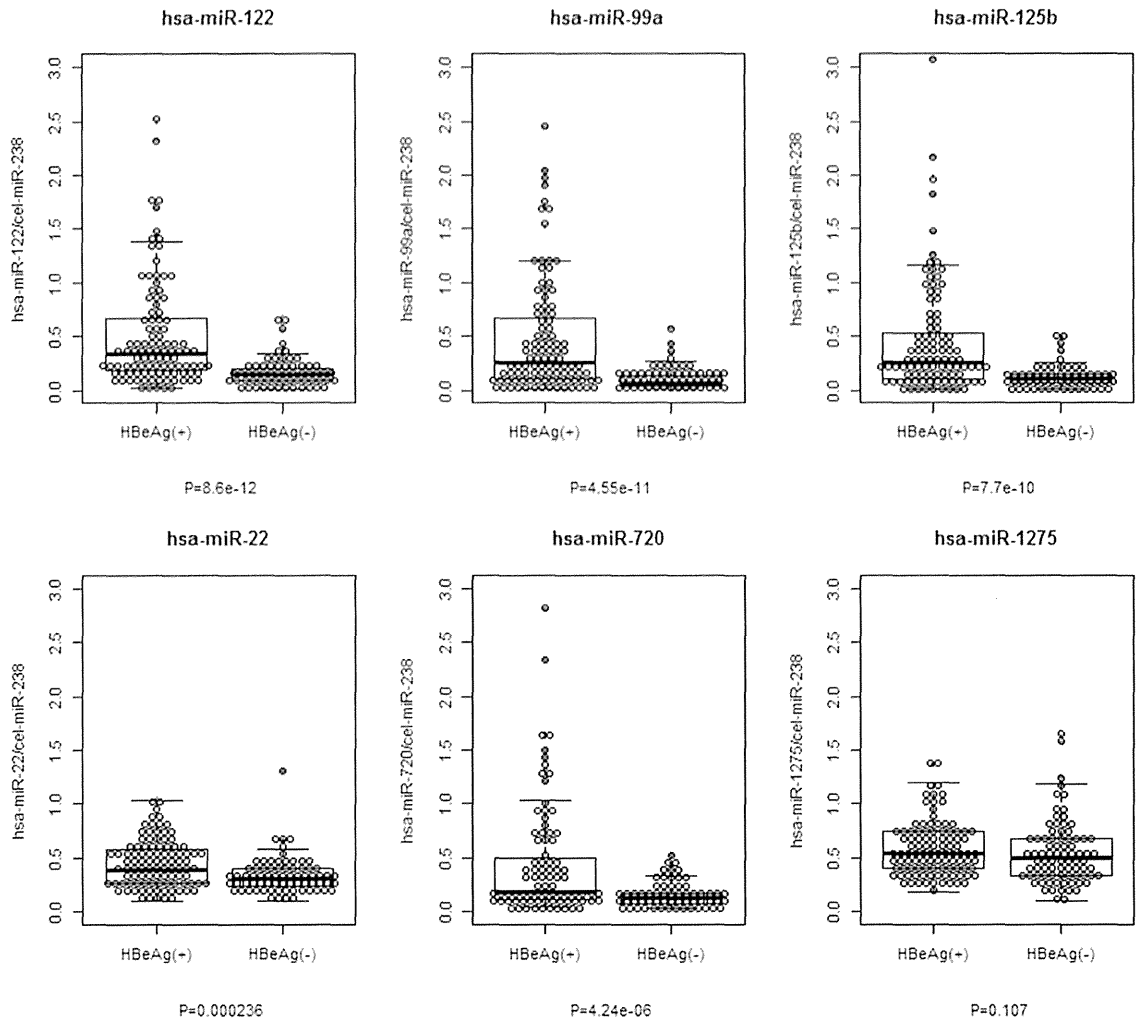

Figure 2 Serum microRNA expression in HBe antigen positive and negative individuals. qRT-PCR microRNA expression levels normalized by cel-miR-238 are shown. *P*-values represent the difference in median values using the non-parametric Kruskal–Wallis rank sum test.

qRT-PCR data (Table 5). MiR-122 was independently associated only with HBV DNA level, whereas miR-125b was independently associated with HBV DNA, HBsAg, HBeAg, and HBeAb levels. MiR-99a was also independently associated with HBeAb levels, and miR-720 was independently associated with HBsAg. While these microRNAs were associated with viral components, miR-22 and miR-1275 were independently associated with γGTP levels. rs8099917 SNP genotype TT in the IFNL3 locus was independently associated with necroinflammatory activity. MiR-125b was the strongest independent factor associated with HBeAg levels, and miR-125b and miR-99a and HBV DNA were each independently associated with HBeAg level. Pairwise expression levels of serum microRNAs were highly correlated, e.g., miR-22 and miR-99a ($R^2 = 0.97$), miR-99a and miR-125b ($R^2 = 0.96$), and miR-122 and miR-125b ($R^2 = 0.96$).

Pathway analysis

To determine which pathways HBV or HCV-associated microRNAs affected, gene targets were predicted using the miRWalk database, and predicted gene targets were compared against pathways in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Predicted targets were found to be significantly overrepresented in the "Pathways in Cancer" gene set. Several of the genes in this set (*AKT1*, *AKT3*, *PTEN*, *BCL2*, *CDKN1B*, *CCND1*, and *TP53*) were also targeted by multiple microRNAs as part of a complex regulatory network. To further examine differences between HBV and HCV infection, predicted gene targets were analyzed using Ingenuity Pathway Analysis software. Significant associations were found between predicted targets and "Cancer," "Cell Cycle," and "Cell Death and Survival" networks in HCV patients and between

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Table 5 Univariate and multivariate linear/logistic regression analysis of associations between clinical data and quantitative RT-PCR serum microRNA levels (relative to cel-miR-238) in patients with chronic HBV infection. Independent factors (bold) were determined using forward-backward stepwise selection based on the Akaike information criterion (AIC) using factors with a univariate *P*-value less than 0.05.

Variable	Factor	N	Coef.	P_{uni}	Coef.	P_{multi}	
HBV DNA (IU/ml)	**hsa-miR-122**	185	2.6	6.1E-17	3.8	7.43E-05	***
	hsa-miR-22	185	3.1	4.3E-06			
	hsa-miR-99a	185	2.3	3.7E-15			
	hsa-miR-720	185	1.5	4.0E-08	−0.5	1.08E-01	
	hsa-miR-125b	185	2.3	2.1E-13	−1.8	2.57E-02	*
	hsa-miR-1275	184	0.4	4.1E-01			
	HBsAg (IU/l)	185	0.0	6.7E-11			
	HBeAg (IU/l)	185	0.0	2.5E-13			
	HBeAb (+/−)	185	−2.2	1.8E-18	−1.5	9.76E-10	***
	rs8099917 TT	167	0.8	5.0E-03			
	AST	185	0.0	4.2E-04	0.0	6.60E-02	.
	ALT	185	0.0	7.4E-04			
	γ-GTP(IU/l)	179	0.0	2.3E-01			
	Liver fibrosis	171	0.2	3.8E-01			
	Activity	171	0.9	4.0E-06	0.6	2.00E-05	***
	Genotype C	145	−0.3	5.4E-01			
HBsAg (IU/l)	hsa-miR-122	185	62950.0	7.6E-60			
	hsa-miR-22	185	59425.0	1.1E-08			
	hsa-miR-99a	185	60936.0	6.9E-66			
	hsa-miR-720	185	41920.0	5.1E-31	14228.0	4.47E-08	***
	hsa-miR-125b	185	62707.0	9.0E-62	51193.0	7.20E-39	***
	hsa-miR-1275	184	2856.0	7.2E-01			
	HBeAg (IU/l)	185	34.0	3.6E-18			
	HBeAb (+/−)	185	−25347.0	1.7E-09			
	rs8099917 TT	167	12077.0	1.2E-02			
	HBV DNA (IU/ml)	185	7119.0	6.7E-11			
	AST	185	−10.3	6.6E-01			
	ALT	185	1.2	9.1E-01			
	γ-GTP	179	−12.6	7.3E-01			
	Liver fibrosis	171	−5283.0	8.4E-02			
	Activity	171	3301.0	3.1E-01			
	Genotype C	145	−16648.0	4.3E-02			
HBeAg (IU/l)	hsa-miR-122	185	751.0	2.8E-20			
	hsa-miR-22	185	872.0	1.3E-06			
	hsa-miR-99a	185	700.0	1.7E-19			
	hsa-miR-720	185	464.0	2.1E-11			
	hsa-miR-125b	185	741.0	3.4E-20	544.0	4.90E-13	***
	hsa-miR-1275	184	101.0	4.6E-01			
	HBsAg (IU/l)	185	0.0	3.6E-18			
	HBeAb (+/−)	185	−609.0	3.8E-19	−395.0	3.14E-10	***
	rs8099917 TT	167	121.0	1.4E-01			
	HBV DNA (IU/ml)	185	135.0	2.5E-13			
	AST	185	0.9	3.3E-02	0.6	3.50E-02	*
	ALT	185	0.4	2.2E-02			
	γ-GTP	179	0.4	5.3E-01			
	Liver fibrosis	171	−22.3	6.7E-01			
	Activity	171	94.1	9.2E-02			
	Genotype C	145	−1.5	9.9E-01			
HBeAb (+/−)	hsa-miR-122	184	−52.1	1.0E-12			
	hsa-miR-22	184	−65.8	2.4E-05			
	hsa-miR-99a	184	−49.8	7.4E-13	−55.3	3.90E-03	**
	hsa-miR-720	184	−32.2	1.3E-07			
	hsa-miR-125b	184	−46.4	2.6E-10	51.3	9.53E-03	**

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Table 5 (*continued*)

Variable	Factor	N	Coef.	P_{uni}	Coef.	P_{multi}	
	hsa-miR-1275	183	−19.4	9.6E-02			
	HBsAg (IU/l)	184	0.0	1.3E-10			
	HBeAg (IU/l)	184	−0.1	7.4E-18	0.0	8.67E-07	***
	rs8099917 TT	166	−10.6	1.2E-01			
	HBV DNA (IU/ml)	184	−13.9	5.4E-20	−8.7	2.84E-08	***
	AST	184	−0.1	8.4E-02			
	ALT	184	0.0	2.9E-02			
	γ-GTP	178	0.0	6.9E-01			
	Liver fibrosis	170	−1.2	7.8E-01			
	Activity	170	−3.9	4.1E-01			
	Genotype C	144	−11.4	3.3E-01			
ALT (IU/l)	hsa-miR-122	185	17.0	6.3E-01			
	hsa-miR-22	185	337.0	1.0E-06	48.2	1.29E-01	
	hsa-miR-99a	185	−18.8	5.7E-01			
	hsa-miR-720	185	15.5	5.8E-01			
	hsa-miR-125b	185	−1.6	9.6E-01			
	hsa-miR-1275	184	9.0	8.6E-01			
	HBsAg (IU/l)	185	0.0	9.1E-01			
	HBeAg (IU/l)	185	0.1	2.2E-02			
	rs8099917 TT	167	18.2	5.5E-01			
	HBV DNA (IU/ml)	185	25.1	7.4E-04			
	AST	185	1.9	2.6E-66	1.8	2.20E-47	***
	γ-GTP	179	2.0	2.1E-20	0.4	6.05E-04	***
	Liver fibrosis	171	35.8	7.6E-02			
	Activity	171	74.8	4.3E-04	−19.0	4.58E-02	*
	Genotype C	145	30.0	5.6E-01			
AST (IU/l)	hsa-miR-122	185	0.2	9.9E-01			
	hsa-miR-22	185	148.0	8.2E-06			
	hsa-miR-99a	185	−15.1	3.4E-01			
	hsa-miR-720	185	4.1	7.6E-01			
	hsa-miR-125b	185	−7.3	6.6E-01			
	hsa-miR-1275	184	10.3	6.8E-01			
	HBsAg (IU/l)	185	0.0	6.6E-01			
	HBeAg (IU/l)	185	0.0	3.3E-02			
	rs8099917 TT	167	18.3	2.0E-01			
	HBV DNA (IU/ml)	185	12.6	4.2E-04			
	ALT	185	0.4	2.6E-66	0.4	1.05E-59	***
	γ-GTP	179	0.9	8.1E-18			
	Liver fibrosis	171	27.2	4.8E-03			
	Activity	171	48.6	1.5E-06	17.4	1.98E-04	***
	Genotype C	145	4.0	8.7E-01			
γ-GTP (IU/l)	hsa-miR-122	179	−5.3	6.4E-01			
	hsa-miR-22	179	46.4	4.2E-02	−48.0	1.95E-02	*
	hsa-miR-99a	179	−10.1	3.4E-01			
	hsa-miR-720	179	3.9	6.7E-01			
	hsa-miR-125b	179	−9.7	3.8E-01			
	hsa-miR-1275	178	33.9	4.3E-02	43.2	2.70E-03	**
	HBsAg (IU/l)	179	0.0	7.3E-01			
	HBeAg (IU/l)	179	0.0	5.3E-01			
	rs8099917 TT	161	10.9	2.7E-01			
	HBV DNA (IU/ml)	179	3.0	2.3E-01			
	AST	179	0.4	8.1E-18			
	ALT	179	0.2	2.1E-20	0.2	5.35E-19	***
	Liver fibrosis	166	24.1	1.7E-04	15.9	1.59E-03	**
	Activity	166	23.5	7.4E-04			
	Genotype C	140	15.7	3.3E-01			

(*continued on next page*)

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Table 5 (*continued*)

Variable	Factor	N	Coef.	P_{uni}	Coef.	P_{multi}	
Liver fibrosis	hsa-miR-122	171	−0.3	6.4E-02			
	hsa-miR-22	171	0.0	9.3E-01			
	hsa-miR-99a	171	−0.3	5.3E-02			
	hsa-miR-720	171	−0.1	4.6E-01			
	hsa-miR-125b	171	−0.2	7.7E-02			
	hsa-miR-1275	170	0.2	2.6E-01			
	HBsAg (IU/l)	171	0.0	8.4E-02			
	HBeAg (IU/l)	171	0.0	6.7E-01			
	rs8099917 TT	160	0.4	1.8E-04			
	HBV DNA (IU/ml)	171	0.0	3.8E-01			
	AST	171	0.0	4.8E-03			
	ALT	171	0.0	7.6E-02			
	γ-GTP	166	0.0	1.7E-04	0.0	3.79E-02	*
	Activity	171	0.6	4.8E-15	0.5	1.35E-09	***
	Genotype C	139	0.4	3.0E-02	0.4	2.63E-02	*
Activity	hsa-miR-122	171	0.2	1.6E-01			
	hsa-miR-22	171	0.4	1.3E-01			
	hsa-miR-99a	171	0.2	1.7E-01			
	hsa-miR-720	171	0.2	1.4E-01			
	hsa-miR-125b	171	0.2	1.1E-01			
	hsa-miR-1275	170	0.1	7.4E-01			
	HBsAg (IU/l)	171	0.0	3.1E-01			
	HBeAg (IU/l)	171	0.0	9.2E-02			
	rs8099917 TT	160	0.9	1.9E-17	0.6	3.80E-13	***
	HBV DNA	171	0.1	4.0E-06	0.1	1.51E-03	**
	AST	171	0.0	1.5E-06	0.0	5.66E-04	***
	ALT	171	0.0	4.3E-04			
	γ-GTP	166	0.0	7.4E-04			
	Liver fibrosis	171	0.5	4.8E-15	0.4	7.00E-11	***
	Genotype C	139	0.0	8.1E-01			

predicted targets and "Cancer," "Hematological Disease," and "Gastrointestinal Disease" networks in HBV patients. To determine if the HBV-associated serum microRNAs shared common transcriptional regulators, upstream transcription factors for each up-regulated microRNA were retrieved from ChIPBase (http://deepbase.sysu.edu.cn/chipbase/ accessed on 14 September 2014).[23] NRSF, JunD, c-Jun transcription have been reported to regulate expression of miR-125b, miR-22, and miR-99a. ZNF11 regulates both miR-125b and miR-99a, and NANOG, E2F4, and HNF4A have been reported to regulate miR-122 and miR-22.

Discussion

This study reports a set of microRNAs that were up- or down-regulated in serum of patients with chronic HBV or HCV compared to healthy subjects. MiR-122 was significantly up-regulated in serum of patients with HBV or HCV, whereas elevated miR-22, miR-99, and miR-125b levels were more characteristic of chronic HBV infection. A number of microRNAs were up-regulated in HBeAg-positive patients compared to HBeAg-negative patients. The HBeAg-associated microRNAs are regulated by a small set of shared transcription factors, including c-Jun, ZNF11, and HNF4A.[23] Expression levels of most HBeAg-associated microRNAs were highly correlated, but individual microRNAs were independently associated with different aspects of HBV infection. MiR-122 was independently associated with HBV DNA, whereas miR-125b was associated with multiple aspects of viral replication, including HBV DNA, HBsAg, and HBeAg, and miR-22 and miR-1275 were independently associated with serum levels of γGTP, a liver enzyme normally associated with alcoholic liver disease or biliary obstruction but which may be elevated in the event of severe viral hepatitis.[24] These results suggest that serum microRNA profiles might serve a diagnostic role in monitoring different aspects of viral infection, although their specific roles in pathogenesis of viral hepatitis remain to be worked out.

The presence of specific serum microRNA profiles associated with chronic HCV or HBV infection suggests involvement of these microRNAs in host-mediated antiviral defense or pathogenesis. Hepatic microRNAs enter the serum via apoptosis or necrosis, or they may be actively secreted within exosomes or viral particles.[14] MiR-122 is abundantly expressed in hepatocytes, and its presence in the serum has been shown to correlate with ALT levels and liver damage.[25,26] MiR-122 strongly suppresses HBV replication both through direct binding to HBV RNA as well as indirectly through cyclin G1-modulated p53 activity.[27–31] MiR-125a-5p, miR-199a-3p and miR-210 also

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inhibit viral replication by directly binding to and suppressing HBV RNA.[30,32,33] MiR-99a is abundantly expressed in the liver and in exosomes and acts as a tumor suppressor by targeting IGF-1R and inducing cell cycle arrest.[16,34] In addition, miR-99 suppresses activity of NF-κB, a transcription factor associated with inflammation and tumorigenesis.[35] In HCC, miR-99a may be severely down-regulated in liver tissue, which is associated with poor prognosis and shorter survival time.[34] As with miR-99a, miR-22 is also abundantly expressed in hepatocytes and exosomes and acts as a tumor suppressor.[16] MiR-22 induces cellular senescence by directly targeting CDKN1A, CDK6, SIRT1, and Sp1 HCC[36,37] and is down-regulated in HBV-related HCC.[37]

Two serum microRNAs investigated in this study (miR-1246 and miR-1275) are part of a set of 13 mitomiRs that have been reported to be significantly enriched in the mitochondrial RNA fraction.[38] Mitochondria play a central role in oxidative stress and apoptosis and are targeted by the HBV X (HBx) protein and the HCV p7 protein.[39] Most mitomiRs, including miR-1246 and miR-1275, are predicted to target COX1, ND5, or other components of the respiratory chain.[38] In this study miR-1275 was significantly up-regulated in patients with HBV and was independently associated with γGTP level, whereas miR-1246 was marginally up-regulated in patients with HCV. MiR-720 has been reported to target the oncogene TWIST1 involved in tumor metastasis in breast cancer,[40] but its status as a microRNA has been challenged due to a possible mis-annotation of what may be a tRNA fragment instead.[41]

An unexpected result of this study is that serum levels of a number of microRNAs were elevated in HBeAg-positive patients compared to HBeAg-negative patients, even though expression levels of both HBeAg-positive and negative patients were both higher than in healthy subjects. The role of the HBe antigen in HBV infection remains unclear, as it is not required for infection but may serve an immunomodulatory role and contribute to chronic infection through vertical transmission by crossing the placenta. However, the HBV precore region that codes for the HBe antigen is highly conserved among hepadnaviruses, which also infect avian hosts lacking a placenta, suggesting that the protein has a more fundamental function. The precore protein contains a signal peptide, causing it to be secreted.[42] However, up to 30% of the protein is retained in the cytoplasm.[43] While secreted HBeAg may have an immunosuppressive role, intracellular HBeAg instead promotes inflammation.[44] However, HBeAg has been shown to inhibit Toll-like receptor signaling and suppress NF-kB and interferon-beta promoter activity.[45] HBeAg also inhibits IL-6 production by blocking activation of RIPK2-mediated activation of NF-kB.[46] Therefore HBeAg may have a complex roles in both intracellular and extracellular immune modulation.

Seroconversion of HBeAg-positive patients to HBe antibody (HBeAb)-positive patients is usually accompanied by a stop codon mutation within the precore open reading frame.[47] This region has been identified as a mutation hotspot for APOBEC3G, an interferon-stimulated deaminase that inhibits HBV replication by hyper-editing of single-stranded HBV DNA[22] as well as by directly blocking reverse transcription.[48] While hypermutation is deleterious to the virus, a small fraction may acquire mutations conferring a selective advantage.[22] Warner et al. proposed a frequency-dependent selection model positing that while HBeAg suppresses the immune response, HBeAg-negative strains may have an initial competitive advantage by benefitting from HBeAg-mediated immune suppression conferred by HBeAg-positive strains while expending fewer of its resources.[49] However, as the frequency of the HBeAg-positive strain falls, the immune system begins to mount a defense against HBeAg-negative viruses, leading to seroconversion.

It is not clear why serum microRNA levels of several microRNAs, including miR-122, miR-22, miR-125, and miR-99a, tended to be higher in HBeAg-positive individuals compared to HBeAg-negative individuals and are higher in HBV-infected individuals compared to healthy subjects. However, Winther et al. reported similar results in children with chronic hepatitis B and found that plasma levels of a subset of microRNAs decreased significantly in one child before and after HBe seroconversion.[50] We have previously shown that both HBc and HBs proteins co-localize and physically interact with AGO2 in hepatocytes and that siRNA ablation of AGO2 suppressed HBV DNA and HBsAg production,[10] suggesting that components of the RNA silencing machinery are recruited during HBV replication. HSP90 has been reported to act as a chaperone during RNA loading of Argonaute proteins[51] and is also essential in catalyzing HBV reverse transcription and capsid formation by interacting with the pregenomic RNA encapsidation signal, reverse transcriptase, and the core protein.[52] Interestingly, APOBEC3G has been shown to interfere with microRNA regulation by disrupting assembly of the miRNA-inducing silencing complex (miRISC).[53] APOBEC3G itself is also incorporated into nucleocapsids by directly binding to the core protein.[54] While microRNA-mediated gene silencing is associated with accumulation in P-bodies, microRNAs may also be sorted into multivesicular bodies by ESCRT proteins and secreted as exosomes.[55] MiR-122, miR-125b, miR-199a, miR-210, and possibly other microRNAs bind directly to targets within the HBV genome. MiR-199a and miR-210 have been shown to suppress HBsAg production in cell culture. However, HBV has been shown to enhance autophagy without a corresponding increase in protein degradation by HBsAg-mediated activation of the unfolded protein response, and disruption of autophagy inhibits HBV production.[56] Although it is not clear how or if HBeAg is involved in this process, it is possible that the loss of non-secreted intracellular HBeAg or a conformational change in precore RNA resulting from precore mutations interferes with viral control of autophagy or suppression of innate immune signaling. This loss of control over the intracellular environment might result in suppressed viral replication and decreased secretion of exosome-associated microRNAs.

The millions of people chronically infected with HBV or HCV pose a serious public health challenge. While cirrhosis and HCC may develop over a span of decades, HCC is often not detected until late in development, resulting in poor prognosis and leaving few treatment options. Sensitive, non-invasive methods able to detect subtle changes in disease state are needed for early identification of individuals at increased risk. Serum microRNAs may improve

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early detection by providing an indirect means to monitor changes in gene and microRNA expression in the liver.

Conflicts of interest

None.

Acknowledgments

This work was supported by Grants-in-Aid for scientific research and development from the Ministry of Health, Labor and Welfare and Ministry of Education Culture Sports Science and Technology, Government of Japan. No writing assistance was provided for this manuscript.

Appendix A. Supplementary data

Supplementary data related to this article can be found at doi:10.1016/j.jinf.2014.10.017.

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Please cite this article in press as: Akamatsu S, et al., Differences in serum microRNA profiles in hepatitis B and C virus infection, J Infect (2014), http://dx.doi.org/10.1016/j.jinf.2014.10.017

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Please cite this article in press as: Akamatsu S, et al., Differences in serum microRNA profiles in hepatitis B and C virus infection, J Infect (2014), http://dx.doi.org/10.1016/j.jinf.2014.10.017

JOURNAL OF VIRAL HEPATITIS
JVH

Journal of Viral Hepatitis, 2014, **21**, e89–e97 doi:10.1111/jvh.12240

Human microRNA hsa-miR-1231 suppresses hepatitis B virus replication by targeting core mRNA

T. Kohno,[1,2*] M. Tsuge,[1,2,3*] E. Murakami,[1,2] N. Hiraga,[1,2] H. Abe,[1,2] D. Miki,[1,2,4] M. Imamura,[1,2] H. Ochi,[1,2,4] C. N. Hayes[1,2] and K. Chayama[1,2,4] [1]*Department of Gastroenterology and Metabolism, Applied life sciences, Institute of Biomedical and Health sciences, Hiroshima University, Hiroshima, Japan;* [2]*Liver Research Project Center, Hiroshima University, Hiroshima, Japan;* [3]*Natural Science Center for Basic Research and Development, Hiroshima University, Hiroshima, Japan; and* [4]*Laboratory for Liver Diseases, SNP Research Center, The Institute of Physical and Chemical Research (RIKEN), Hiroshima, Japan*

Received November 2013; accepted for publication February 2014

SUMMARY. Pathogen-specific miRNA profiles might reveal potential new avenues for therapy. To identify miRNAs directly associated with hepatitis B virus (HBV) in hepatocytes, we performed a miRNA array analysis using urokinase-type plasminogen activator (uPA)–severe combined immunodeficiency (SCID) mice where the livers were highly repopulated with human hepatocytes and human immune cells are absent. Mice were inoculated with HBV-infected patient serum samples. Eight weeks after HBV infection, human hepatocytes were collected from liver tissues, and miRNAs were analysed using the Toray 3D array system. The effect of miRNAs on HBV replication was analysed using HBV-transfected HepG2 cells. Four miRNAs, hsa-miR-486-3p, hsa-miR-1908, hsa-miR-675 and hsa-miR-1231 were upregulated in mouse and human livers with HBV infection. These miRNAs were associated with immune response pathways such as inflammation mediated by chemokine and cytokine signalling. Of these miRNAs, hsa-miR-1231, which showed high homology with HBV core and HBx sequences, was most highly upregulated. In HBV-transfected HepG2 cells, overexpression of hsa-miR-1231 resulted in suppression of HBV replication with HBV core reduction. In conclusion, a novel interaction between hsa-miR-1231 and HBV replication was identified. This interaction might be useful in developing new therapeutic strategies against HBV.

Keywords: HB core, hepatitis B virus, hsa-miR-1231, human hepatocyte chimeric mouse, microRNA.

INTRODUCTION

Hepatitis B virus (HBV) is a member of the *Hepadnaviridae* family, which contains a group of hepatotropic small DNA viruses that infect their respective animal hosts [1–3]. Once HBV infects human hepatocytes, the HBV genome translocates into the nucleus. Some genome copies are converted into a covalently closed circular DNA (cccDNA) form and organized into a minichromosome with histone and nonhistone proteins [4–8]. HBV cccDNA utilizes the cellular transcriptional machinery to produce all viral RNAs including the pregenomic RNA [9], and these gene products regulate viral replication and pathogenesis by regulating host gene expression [10,11].

MicroRNAs (miRNAs) are small noncoding RNAs of 21-25 nucleotides in length, processed from hairpin-shaped transcripts [12]. MiRNAs can bind the 3′-untranslated regions (UTRs) of messenger RNAs and downregulate gene expression by cleaving messenger RNA or inhibiting translation. Several miRNAs associated with HBV infection, HBV replication and hepatocarcinogenesis have recently been identified [13–19]. However, the direct influence of HBV infection on miRNA expression is still unclear.

MicroRNAs are currently being investigated for their therapeutic potential in antiviral therapy. As several studies have demonstrated that hsa-miR-122, which is specifically and abundantly expressed in hepatocytes, supported hepatitis C virus (HCV) replication by improving RNA

Abbreviations: HBc, hepatitis B core; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; miRNA, microRNA; RI, replication intermediates.

Correspondence: Kazuaki Chayama, Department of Gastroenterology and Metabolism, Applied life sciences, Institute of Biomedical and Health sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima-shi, Hiroshima, 734-8551, Japan.
E-mail: chayama@hiroshima-u.ac.jp
*These authors contributed equally to this work.
This study was supported in part by a Grant-in-Aid for Scientific Research from the Japanese Ministry of Labor and Health and Welfare.

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stability [20–24], small molecules or siRNAs which are able to knock down miR-122 expression have been explored as a new therapeutic agent for HCV eradication.

A similar microRNA-based antiviral approach is also sought for the treatment of chronic hepatitis B, as it is difficult to eradicate HBV genomes converted into cccDNA or minichromosomes under present antiviral therapies. To develop new strategies for complete eradication of the viral genome from hepatocytes, it is important to clarify the direct associations between hepatic miRNAs and HBV infection.

In this study, miRNA microarray analysis was performed using human hepatocyte chimeric mouse livers to assess the direct impact of HBV infection on miRNA expression. We successfully demonstrated that HBV infection attenuated the expression of miRNAs under immunodeficient conditions to protect early viral propagation. A novel interaction between hsa-miR-1231 and HBV replication was identified.

MATERIALS AND METHODS

Human serum inoculum

Serum samples were obtained from a carrier infected with HBV genotype C after obtaining written informed consent for the donation and evaluation of blood samples. Inoculum was positive for HBs and HBe antigens with high-level viremia (HBV DNA: 7.1 log copies/mL). The experimental protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the Hiroshima University Hospital ethical committee (Approval ID: D08-9).

Human hepatocyte chimeric mice experiments

Human hepatocyte chimeric mice (PXB mice), in which human hepatocytes were transplanted into $uPA^{+/+}/SCID^{+/+}$ mice, were purchased from Phoenix Bio (Hiroshima, Japan). Mouse experiments were performed in accordance with the guidelines of the local committee for animal experiments at Hiroshima University.

Six chimeric mice, in which more than 90% of the liver tissue was replaced with human hepatocytes, were divided into two experimental groups. Group A contained three uninfected mice. Group B consisted of three mice that were inoculated via the mouse tail vein with human serum containing 6×10^6 copies of HBV. Serum HBV DNA titres were quantified every 2 weeks by real-time PCR, and human albumin levels were measured using the Human Albumin ELISA Quantitation kit (Bethyl Laboratories Inc., Montgomery, TX, USA) as described previously [25]. Eight weeks after inoculation, all three infected mice were sacrificed. Infection, extraction of serum samples and sacrifice were performed under ether anaesthesia as described previously [26].

miRNA microarray analysis

Human hepatocytes were finely dissected from the mouse livers and stored in liquid nitrogen after submerging in RNA*later*® solution (Applied Biosystems, Foster City, CA, USA). Experimental sample RNAs were isolated using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and analysed using TORAY 3-D Gene Chip human miRNA ver. 12.1 (TORAY, Chiba, Japan).

Data analysis

Gene expression profiles were analysed using GeneSpring GX 10.0.2 software (Tomy Digital Biology, Tokyo, Japan). Expression ratios were normalized per chip to the 50th percentile. To determine whether there were miRNAs differentially expressed among samples, we performed two Welch's *t*-tests ($P < 0.01$) on this prescreened list of miRNAs with Benjamini and Hochberg's correction. Complete linkage hierarchical clustering analysis was applied using Euclidean distance.

Pathway analysis

The miRNA target genes were predicted by the online database miRWalk (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/index.html). Target prediction was performed using 3′-UTR sequences of mRNAs, and the probability distributions were calculated using the Poisson distribution [27]. The mRNAs with *P* values <0.01 were considered significant. To improve the accuracy of target gene selection, the predicted genes were screened using other prediction programs, including miRanda (August 2010 release), miRDB (April 2009 release) and TargetScan version 5.1 (Whitehead Institute for Biomedical Research, Cambridge, MA, USA). Genes that were predicted by at least two alternate programs were selected. Pathway analysis was performed by PANTHER version 8.1 (http://www.pantherdb.org/) to determine the effects of the predicted target genes on pathways.

Quantification of miRNAs

Small RNAs were extracted from liver tissues or HepG2 cells with mirVana™ miRNA Isolation Kit (Applied Biosystems) and reverse-transcribed according to the manufacturer's instructions. The selected miRNAs were quantified with TaqMan® MicroRNA Assays (Applied Biosystems) using the 7300 Real-Time PCR System (Applied Biosystems), and the expression of RNU6B served as a control.

Quantification of mRNAs

Total RNA was extracted from HepG2 cells transfected with control miRNA or miR-1231 expression plasmid using

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RNeasy Mini Kit and reverse-transcribed (RT) using ReverTra Ace (TOYOBO, Osaka, Japan) with random primer according to the manufacturer's instructions. The selected cDNAs were quantified by real-time PCR. Differences between groups were examined for statistical significance using Student's *t*-test. The primer sequences were as follows: GAPDH forward 5′-ACAACAGCCTCAAGATCATCAG-3′ and reverse 5′-GGTCCACCACTGACACGTTG-3′; Mx1 forward 5′- TTCGGCTGTTTACCAGACTCC-3′ and reverse 5′- CAAAGCCTGGCAGCTCTCTAC-3′; 2′–5′ oligoadenylate synthetase 1 (OAS1) forward 5′- ACCTGGTTGTCTTCCTCA GTCC-3′ and reverse 5′- GAGCCTGGACCTCAAACTTCAC-3′; double stranded RNA dependent protein kinase (PKR) forward 5′- TGGCCGCTAAACTTGCATATC-3′ and reverse 5′- AGTTGCTTTGGGACTCACACG-3′; and SOCS1 forward 5′-ACGAGCATCCGCGTGCACTT-3′ and reverse 5′-AAGAGG CAGTCGAAGCTCTC-3′.

Plasmid construction

The construction of wild-type HBV 1.4 genome length, pTRE-HB-wt, was described previously [25]. The nucleotide sequence of the cloned HBV genome was deposited into GenBank AB206817. The HBc and HBx genes, amplified from pTRE-HB-wt, were cloned into pcDNA3 and p3xFLAG-CMV10 vectors and designated pcDNA-HBc and p3FLAG-HBx, respectively. The human miR-1231 precursor expression plasmid (HmiR0554-MR04) and the control miRNA plasmid (CmiR0001-MR01), which was a miRNA-scrambled control clone, were commercially produced (GeneCopoeia™, Rockville, MD, USA).

Transfection of HepG2 cell lines with the plasmids

The HBV expression plasmid was transfected into HepG2 cells with control miRNA or miR-1231 expression plasmid using *Trans*IT-LT1 (Mirus, Madison, WI, USA) reagent according to the manufacturer's instructions. 24–48 h after transfection, core-associated HBV DNA and HBV RNA were extracted and quantified by real-time PCR or RT real-time PCR, respectively [28]. For identifying targets within the HBV genome, HBc or HBx expression plasmids were transiently transfected with miR-1231 expression plasmid into HepG2 cells. Twenty-four hours after transfection, the cells were harvested to perform Western blot analysis.

Analysis of HBV replication intermediates

Quantitative analysis of HBV replication intermediates was performed as described previously [29]. The HBV-specific primers used for amplification were 5′-TTTGGGCATGGAC-ATTGAC-3′ and 5′-GGTGAACAATGTTCCGGAGAC-3′. The lower detection limit of this assay was 300 copies.

Western blot analysis

Cell lysates, prepared with RIPA like buffer [50 mM Tris-HCl (pH 8.0), 0.1% SDS, 1% NP-40, 150 mM sodium chloride, and 0.5% sodium deoxycholate] containing protease inhibitor cocktail (Sigma-Aldrich, Tokyo, Japan), were separated on 5–20% (wt/v) SDS-polyacrylamide gels (Bio-Rad Laboratories, Inc., Tokyo, Japan). Immunoblotting was performed with anti-FLAG M2 monoclonal antibody (Sigma-Aldrich) or anti-HBV core monoclonal antibody HB91 (Advanced Life Science Institute Inc., Saitama, Japan) or anti β-actin monoclonal antibody (Sigma-Aldrich) followed by incubation with horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin (GE Healthcare, Buckinghamshire, UK). Expression of HBc protein was quantified based on the densities of the immunoblot signals by Quantity One® software (Bio-Rad Laboratories, Inc.).

RESULTS

miRNA expression alterations associated with HBV infection

To analyse the influence of HBV infection on human hepatocytes, miRNA microarray expression profiles were compared between groups A (mice without HBV infection) and B (mice with HBV infection). Among the 900 miRNAs on the microarray, 10 miRNAs showed a more than 2.0-fold change with HBV infection. Five of the 10 miRNAs were upregulated, and the remaining five were downregulated (Fig. S1). Because immunity was severely suppressed in the chimeric mice, changes in miRNA expression are thought to be closely associated with HBV infection, and the upregulated miRNAs might play a protective role against HBV infection. Thus, we focused on these 5 upregulated miRNAs.

Comparison of expression of the 5 upregulated miRNAs in human liver tissues

To verify the microarray results, quantitative analysis of miRNAs was performed using liver tissues from the chimeric mice. Three of the 5 miRNAs were significantly upregulated by HBV infection (Fig. 1). Expression changes in the other 2 miRNAs (hsa-miR-675 and hsa-miR-1908) showed a similar trend but were not significant due to individual variation. Therefore, further quantitative analysis was performed using human liver tissues. Nine liver tissue samples were obtained from patients with chronic hepatitis B ($N = 3$), chronic hepatitis C ($N = 2$) or alcoholic liver dysfunction ($N = 4$), and miRNA expression levels were compared. Expressions of all miRNAs except for miR-886-5p were significantly higher in liver tissues with chronic hepatitis B than in those with other liver diseases (Fig. 2).

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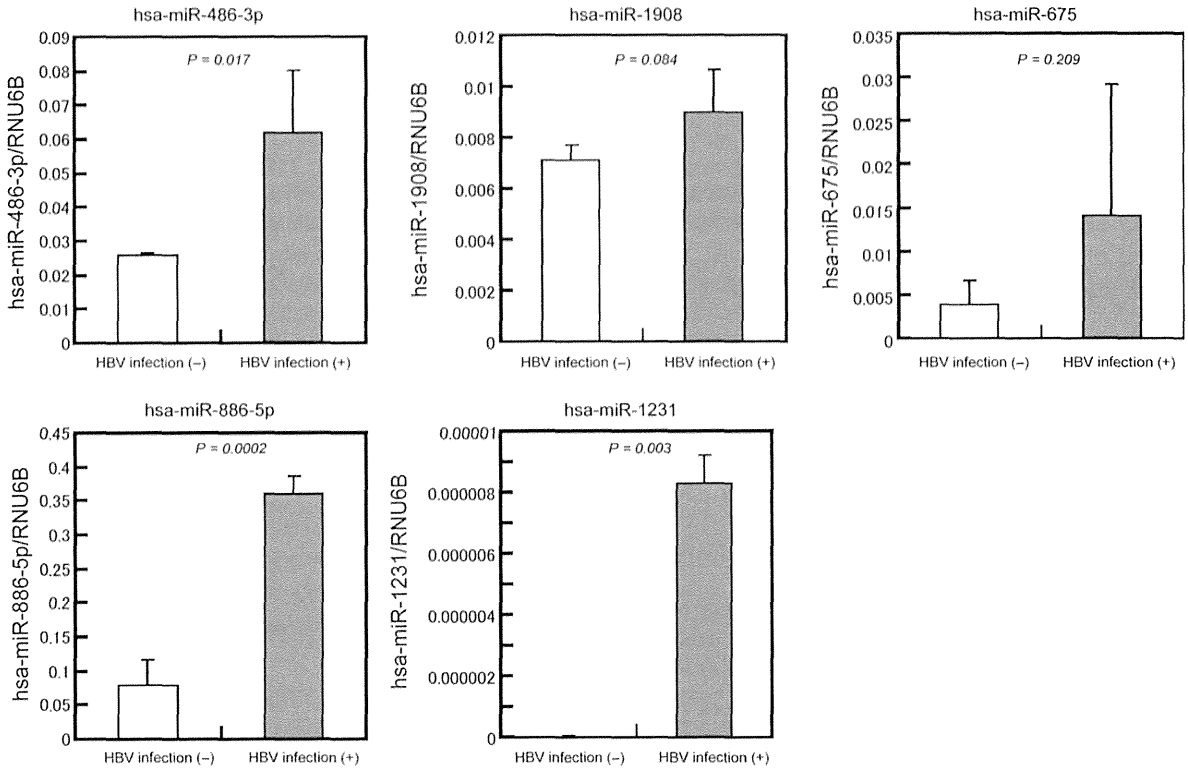

Fig. 1 Upregulation of microRNA by HBV infection. Signal intensities of five upregulated miRNAs were compared between HBV-infected and noninfected mouse livers. All 5 miRNAs were significantly upregulated by HBV infection. *P* values were calculated by the Mann–Whitney *U*-test.

Associations between signalling pathways and the upregulated miRNAs

To analyse the influence of miRNA upregulation on signalling pathways, pathway analysis was performed. However, there are several obstacles in analysing the association between miRNAs and pathways, such as the lack of reliable miRNA target prediction algorithms, differences in the results among target prediction systems, and the small number of validated target genes. To improve the reliability of the targets, we performed the pathway analysis in combination with four prediction tools (miRWalk, TargetScan, miRanda and miRDB). After this operation, 482 targets were predicted (hsa-miR-1231: 203 targets, hsa-miR-1908: 3 targets, hsa-miR-486-3p: 251 targets, hsa-miR-675: 25 targets), and these 482 targets were submitted to the PANTHER classification system for pathway analysis. As shown in Table 1, several immunological pathways such as inflammation mediated by chemokine and cytokine signalling pathway, and the interleukin signalling pathway were identified, but it was difficult to identify characteristic pathways.

Suppression of HBV replication with miR-1231 overexpression

Because hsa-miR-1231 was most the highly upregulated among these four miRNAs and had a high homology with the HBV genome, we focused on hsa-miR-1231. Using GENETYX ver. 8.2.1 (GENETYX, Tokyo, Japan), the hsa-miR-1231 sequence was predicted to hybridize at the HB core and X regions of the HBV genome (Fig. 3). To analyse the influence of hsa-miR-1231 on HBV replication, changes in HBV replication intermediates were evaluated using an *in vitro* HBV replication model. As shown in Fig. 4a, HBV replication intermediates were significantly reduced by hsa-miR-1231 overexpression, and the suppression of HBV RNA and HBc proteins were also observed by hsa-miR-1231 overexpression (Figs 4b,c). Thus, HBV replication was concluded to be inhibited by hsa-miR-1231 at the post-transcriptional level.

Specific regulation of HBV-related protein levels with hsa-miR-1231 overexpression

As the preceding results indicated an association between the production of HBV-related protein or HBV particles and

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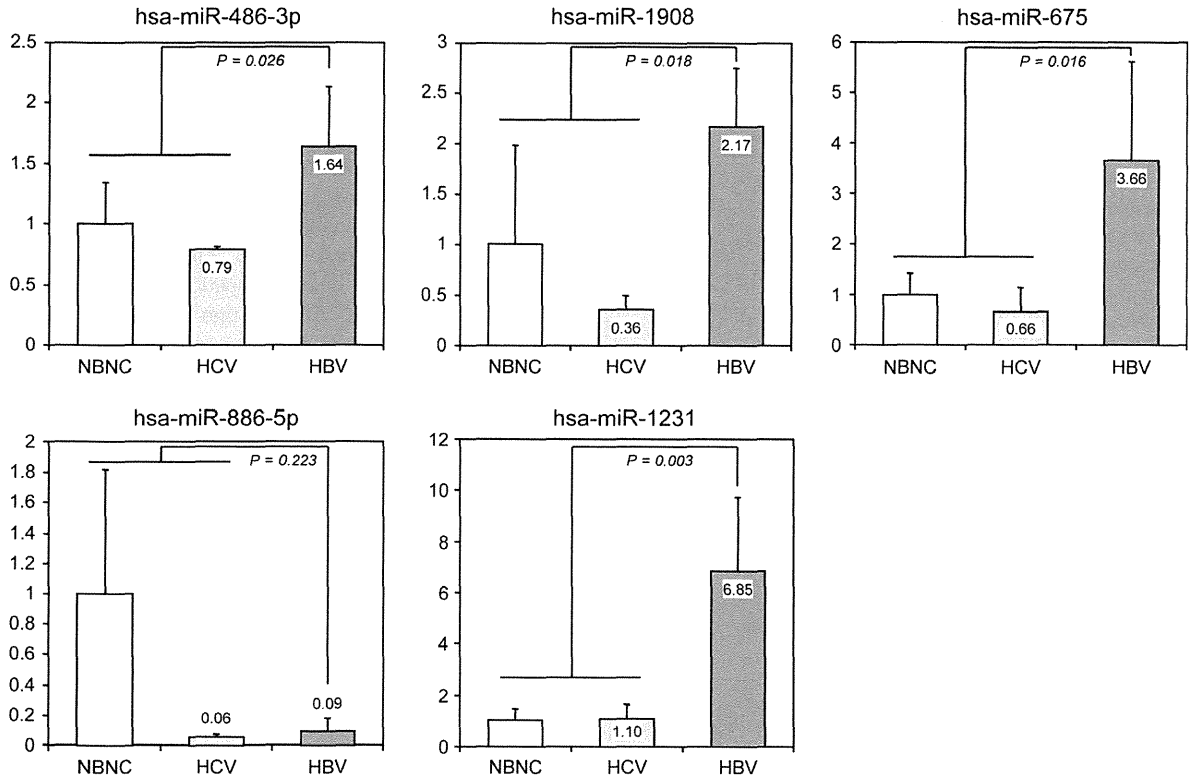

Fig. 2 Comparison of microRNA expression in clinical liver tissues. Quantification of miRNAs was performed by real-time PCR using nine human liver tissues obtained from the patients who had chronic hepatitis B (N = 3), C (N = 2) or alcoholic liver dysfunction (N = 4). Expression levels of four miRNA were significantly higher in the chronic hepatitis B patients than in those of other liver diseases. The results of miR-886-5p levels were not statistically significant. P values were assessed by Mann–Whitney U-test.

hsa-miR-1231 expression, further analysis was performed to identify the region hybridized by hsa-miR-1231. As shown in Fig. 5, HBc protein expression was remarkably reduced by hsa-miR-1231 expression, but no reduction in HBx protein was observed. These results indicate that hsa-miR-1231 might interact with HBV core mRNA and suppress HBV replication by inhibiting HBV core protein production.

The effects of hsa-miR-1231 on the expression of interferon-stimulated genes

Alternatively, hsa-miR-1231 might suppress HBV replication through activation of the interferon signalling pathway. We thus evaluated mRNA expression of interferon-stimulated genes (ISGs) with or without hsa-miR-1231 overexpression. None of the examined ISGs (MxA, PKR, OAS-1 and SOCS1) were regulated by hsa-miR-1231 expression (Fig. S3). These results suggest that hsa-miR-1231 suppresses HBV replication at the post-transcriptional level but not through the activation of interferon signalling.

DISCUSSION

Previously, we have demonstrated that human hepatocyte chimeric mice can be chronically infected with hepatitis B and C viruses [25,30,31]. This mouse model facilitates analysis of the effect of viral infection under immunodeficient conditions. In the present study, we performed miRNA array analysis using this mouse model and obtained miRNA expression profiles reflecting the direct influence of HBV infection on human hepatocytes. Furthermore, we found a novel mechanism for HBV replication mediated by hsa-miR-1231.

To avoid contamination with mouse tissue, human hepatocyte chimeric mice were used in which liver tissue was largely (>90%) replaced by human hepatocytes. Although it is feasible to use microarray analysis in this chimeric mouse model [32], signals from miRNA array analysis may be influenced by cross-hybridization with mouse miRNA from a small amount of contaminated mouse-derived cells because of the high homology between the human and mouse genomes. To compensate

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Table 1 Pathways associated with the 4 miRNAs upregulated by HBV infection

Pathway	Number of gene hits	Ratio of genes %
Inflammation mediated by chemokine and cytokine signalling pathway (P00031)	11	2.60
Angiogenesis (P00005)	10	2.30
Integrin signalling pathway (P00034)	9	2.10
Gonadotropin releasing hormone receptor pathway (P06664)	7	1.60
Wnt signalling pathway (P00057)	7	1.60
Parkinson disease (P00049)	7	1.60
EGF receptor signalling pathway (P00018)	7	1.60
Alzheimer's disease–presenilin pathway (P00004)	6	1.40
PDGF signalling pathway (P00047)	6	1.40
B-cell activation (P00010)	6	1.40
Interleukin signalling pathway (P00036)	5	1.20
Huntington disease (P00029)	5	1.20
FGF signalling pathway (P00021)	5	1.20
Cadherin signalling pathway (P00012)	5	1.20
VEGF signalling pathway (P00056)	4	0.90
Toll receptor signalling pathway (P00054)	4	0.90
T-cell activation (P00053)	4	0.90
Ras pathway (P04393)	4	0.90
Heterotrimeric G-protein signalling pathway-Gi alpha and Gs alpha-mediated pathway (P00026)	4	0.90
Endothelin signalling pathway (P00019)	4	0.90

for contamination, mice that were negative for HBV infection were set up as negative controls.

Only 5 miRNAs showed more than 2.0-fold upregulation with HBV infection under miRNA array analysis using chimeric mouse livers (Fig. S1). Comparing these results with our previous study using patient sera, only hsa-miR-486-3p showed a similar change in sera from chronic hepatitis B patients, but no upregulation of the other 4 miRNAs was observed [15]. These results suggest that miRNA expression in sera from chronic hepatitis B patients might be regulated not only by HBV infection but also by human immune responses. In addition, it might be difficult to analyse changes in expression of miRNAs that are expressed at low levels in human hepatocytes, including hsa-miR-1231, using human serum.

To identify targets of miR-1231, we searched using four prediction systems. Although 632 target genes were identified (data not shown), and involvement of a number of pathways was indicated (Table S1), critical targets associated with human immunity or HBV replication could not be identified. Interferon signalling was also a potential mechanism of HBV suppression, but several ISG mRNAs were not induced by hsa-miR-1231 overexpression *in vitro* (Fig. S2). Therefore, we concluded that hsa-miR-1231 does not suppress HBV replication via interferon signalling.

To examine the possibility that miR-1231 directly regulates HBV replication by interacting with HBV-related mRNAs, we searched for hsa-miR-1231-binding motifs and found two candidate sequences in the HBV core and X genes (Fig. 3). As shown in Fig. 5, one target in the HBV core region could hybridize with hsa-miR-1231, and HBc expression was found to be suppressed by hsa-miR-1231 overexpression. The hsa-miR-1231-binding motif in the HBV core region was conserved in more than 90% of the HBV sequences in GenBank, regardless of HBV genotype (data not shown). Thus, we speculate that hsa-miR-1231 binds to the HBc target region and suppresses HBc production to inhibit HBV replication.

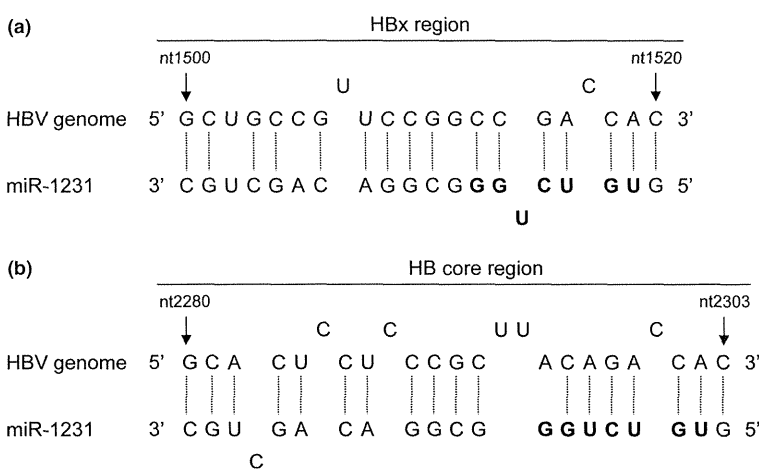

Fig. 3 Alignment of hsa-miR-1231 to HBV genome. Alignment of hsa-miR-1231 to the HBV genome was performed. MiR-1231 sequence was predicted to hybridize at the HBV core **(a)** and HBV X region **(b)**.

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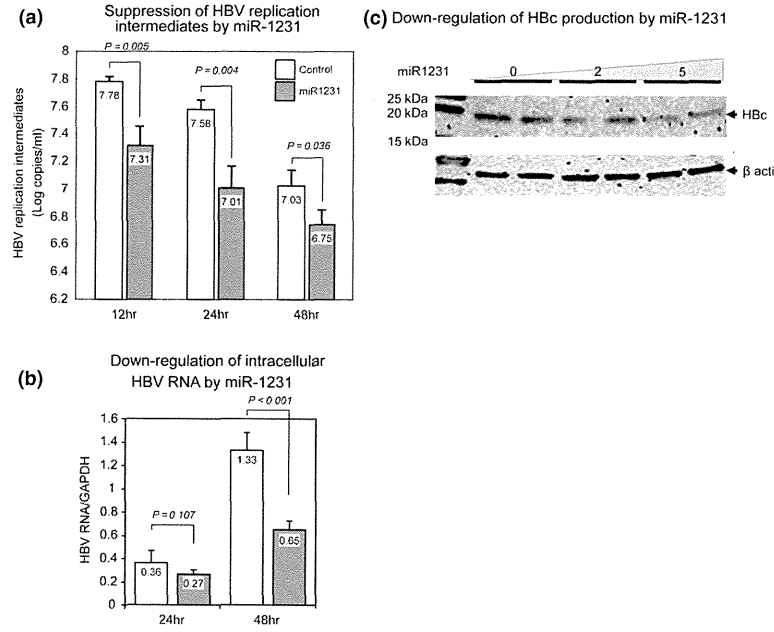

Fig. 4 Suppression of HBV replication by miR-1231. HBV replication intermediates were measured using an *in vitro* HBV replication model. **(a)** Production of HBV replication intermediates was significantly suppressed in cells transfected with both HBV and miR-1231 expression plasmids. **(b, c)** The levels of HBV RNA and HBc protein were also reduced by miR-1231 expression at 24 and 48 h after transfection.

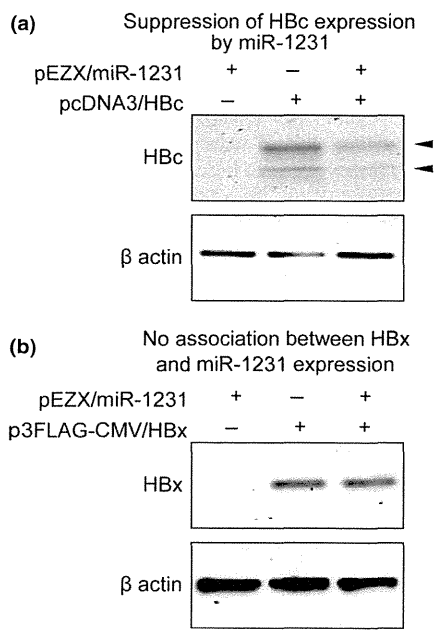

Fig. 5 Identification of miR-1231 target region in HBV genome. To determine the target for miR-1231, HBc or HBx expression plasmid was transfected into HepG2 cells with miR-1231 expression plasmid, and changes in protein levels were analysed by Western blot. HBc protein levels were reduced by miR-1231 expression **(a)**, but HBx protein levels were not reduced **(b)**.

To confirm the association between hsa-miR-1231 and HBV replication, we also tried to suppress hsa-miR-1231 expression using a miRNA inhibitor *in vitro*. However, no significant effects of miR-1231 inhibition on HBV replication were observed *in vitro*. As mentioned previously, expression levels of hsa-miR-1231 are quite low in HepG2 cells and human hepatocytes, and therefore, significant effects of hsa-miR-1231 inhibition could not be observed. The level of hsa-miR-1231 activity was also a factor. As shown in Fig. 4, HBV replication intermediates and HBc expression were significantly suppressed by hsa-miR-1231 overexpression, but the reduction rate was quite small even when 5-fold volume of hsa-miR-1231 plasmid and a volume of HBV expression plasmid were transfected into HepG2 cells. Therefore, it was difficult to observe changes in HBV replication by miRNA inhibition when HBV was replicating vigorously.

In conclusion, we performed miRNA array analysis using human hepatocyte chimeric mice and were able to analyse the direct effects of HBV infection without the confounding effects of the lymphocyte immunological response. We obtained evidence that hsa-miR-1231 was upregulated in response to HBV infection in human hepatocytes, whereupon hsa-miR-1231 suppressed replication of HBV.

ACKNOWLEDGEMENTS

This work was carried out at the Research Center for Molecular Medicine, Faculty of Medicine, Hiroshima University and the Analysis Center of Life Science,

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Hiroshima University. The authors thank Rie Akiyama for her excellent technical assistance and Akemi Sada and Emi Nishio for clerical assistance. This study was supported in part by a Grant-in-Aid for Scientific Research from the Japanese Ministry of Labor and Health and Welfare.

FINANCIAL DISCLOSURE

Kohno T, Tsuge M, Murakami E, Hiraga N, Abe H, Miki D, Imamura M, Takahashi S, Ochi H, Hayes CN, Chayama K: None to declare.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1: HBV infection regulated expression of several microRNAs. Complete linkage hierarchical clustering analysis was performed using Euclidean distance. Among the 900 miRNAs, 10 miRNAs showed more than 2.0-fold change between groups. Five of the 10 miRNAs were upregulated by HBV, and the other five were downregulated.

Figure S2: No effect of miR-1231 expression on IFN signalling. To analyse the influence of miR-1231 expression on interferon signalling, four interferon-stimulated genes (ISGs) were quantified by real-time PCR. None of the four ISGs (MxA, PKR, OAS-1 and SOCS1) were suppressed by miR-1231 expression.

Table S1: Pathway analysis of miR-1231 target genes.

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Antiviral Therapy: in press (doi: 10.3851/IMP2777)

Original article

On-treatment low serum HBV RNA level predicts initial virological response in chronic hepatitis B patients receiving nucleoside analogue therapy

Yi-Wen Huang[1,2,3], Shoichi Takahashi[4,5], Masataka Tsuge[4,5], Chi-Ling Chen[6], Ting-Chuan Wang[7], Hiromi Abe[4,5], Jui-Ting Hu[1,8], Ding-Shinn Chen[3,6,9,10], Sien-Sing Yang[1,8], Kazuaki Chayama[4,5], Jia-Horng Kao[3,6,9,11]**

[1]Liver Center, Cathay General Hospital Medical Center, Taipei, Taiwan
[2]School of Medicine, Taipei Medical University, Taipei, Taiwan
[3]Division of Gastroenterology, Department of Internal Medicine, National Taiwan University College of Medicine and Hospital, Taipei, Taiwan
[4]Department of Gastroenterology and Metabolism, Applied Life Sciences, Institute of Biomedical & Health Sciences, Hiroshima University, Hiroshima, Japan
[5]Liver Research Project Center, Hiroshima University, Hiroshima, Japan
[6]Graduate Institute of Clinical Medicine, National Taiwan University College of Medicine, Taipei, Taiwan
[7]Department of Medical Research, Cathay General Hospital Medical Center, Taipei, Taiwan
[8]School of Medicine, Fu-Jen Catholic University College of Medicine, Taipei, Taiwan
[9]Hepatitis Research Center, National Taiwan University Hospital, Taipei, Taiwan
[10]Genomics Research Center, Academia Sinica, Nankang, Taiwan
[11]Department of Medical Research, National Taiwan University Hospital, Taipei, Taiwan

*Corresponding author e-mails: chayama@mba.ocn.ne.jp; kaojh@ntu.edu.tw

Background: Serum HBV RNA is detectable during nucleoside/nucleotide analogue therapy as a result of unaffected RNA replicative intermediates or interrupted reverse transcription. We studied the predictive value of serum HBV RNA for initial virological response during nucleoside analogue therapy.

Methods: Serum HBV RNA was quantified before and at 12 and 24 weeks of lamivudine or entecavir therapy. Serum HBV DNA was measured every 4–12 weeks during treatment to define initial virological response.

Results: Serum HBV RNA was detectable in 21 of 52 (40%) consecutive patients with a mean of 5.2 log copies/ml (male/female 35/17, mean age of 60 years with a range of 31–82, 44% HBeAg-positive, and 26 with lamivudine and 26 with entecavir) before treatment. Serum HBV RNA level at week 12 in patients with an interval from detectable to undetectable serum HBV DNA level <16 weeks was significantly lower than those with an interval ≥16 weeks (3.8 ±3.8 versus 6.6 ±3.5 log copies/ml, P=0.013). After adjustment for serum HBV DNA level at week 12, serum quantatitive HBsAg level at week 12 and pretreatment ALT level, low serum HBV RNA level at week 12 predicted a shorter interval to undetectable serum HBV DNA level (adjusted hazard ratio =0.908, 95% CI 0.829, 0.993, P=0.035).

Conclusions: Low serum HBV RNA level at week 12 of nucleoside analogue therapy independently predicts initial virological response in treated chronic hepatitis B patients. Serum HBV RNA levels may thus be useful for optimizing treatment of chronic hepatitis B.

Introduction

Although effective vaccines against HBV infection have been available for more than three decades, HBV infection remains a global health problem. It is estimated that more than 350 million people are chronic carriers of HBV worldwide [1,2]. In the United States, 1.2 million individuals have chronic HBV infection [3]. HBV infection causes a wide spectrum of clinical manifestations, ranging from acute or fulminant hepatitis to various forms of chronic liver disease, including inactive carrier state, chronic hepatitis, cirrhosis and even hepatocellular carcinoma [2,4,5].

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Nucleoside/nucleotide analogues (NAs) are widely approved for the treatment of chronic hepatitis B (CHB). HBV is a unique DNA virus that replicates via pregenomic RNA. Lamivudine, as well as other NAs, do not affect the HBV cccDNA and its transcripts – the RNA replicative intermediates [6]. Thus, long-term NA therapy is needed for continued viral suppression in CHB patients. Other studies and ours have shown that serum HBV RNA can be detectable during NA therapy as a result of unaffected RNA replicative intermediates or interrupted reverse transcription [7–10].

For CHB patients with NA therapy, the most important determinant of therapeutic outcomes is the degree of on-treatment viral suppression [11]. Although the correlation of baseline parameters and therapeutic outcomes of NA-treated patients has been reported, little is known about the predictive value of on-treatment predictors [12–14]. For example, a roadmap approach by using on-treatment monitoring of serum HBV DNA levels has been proposed [15]; however, the role of on-treatment serum quantitative HBsAg (qHBsAg) levels in predicting outcomes of NA-treated patients is not satisfactory [16]. To seek better on-treatment predictors, we thus evaluated the predictive value of serum HBV RNA for initial virological response in CHB patients receiving NA therapy.

Methods

Subjects

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We consecutively enrolled 52 CHB patients treated with either lamivudine or entecavir at Hiroshima University Hospital or other hospitals of the Hiroshima Liver Study Group [17]. Serum samples from enrolled patients were obtained just before the initiation of therapy and every 4–12 weeks during therapy. These samples were stored at -80°C until use. Serum HBV RNA was quantified at pretreatment and at treatment weeks 12 and 24. Serum HBV DNA was measured every 4–12 weeks during treatment to record the time of initial undetectable HBV DNA (that is, initial virological response). The lower detection limit of this assay was 2.2 log copies/ml. Informed consent was obtained from each patient.

Extraction of HBV nucleic acid and reverse transcription

Extraction of HBV nucleic acid and reverse transcription with subsequent quantification were performed as previously described [8]. Nucleic acid was extracted from 100 μl serum using SMITEST EX-R&D (Genome Science Laboratories, Tokyo, Japan) and dissolved in 18 μl of ribonuclease-free H_2O. The extract was then divided into two parts with equal amounts. Solution I was mixed with equal amounts of H_2O for DNA quantification. Solution II underwent reverse transcription using random primers (Takara Bio Inc., Shiga, Japan) and M-MLV reverse transcriptase (ReverTra Ace; TOYOBO Co., Osaka, Japan), with subsequent DNA plus cDNA quantification. Low-level pretreatment serum HBV RNA might be masked by serum HBV DNA with this quantification method. This limitation was overcome by treating nucleic acid extracts with deoxyribonuclease digestion before reverse transcription.

The steps in reverse transcription are follows: 25 pM random primer was added and heated at 65°C for 5 min, the mixture was then put on ice for 5 min, 4 μl of 5X reverse transcription buffer, 2 μl of 10 mM dNTPs, 2 μl of 0.1 M dithiothreitol (DTT), 8 units of ribonuclease inhibitor and 100 units of M-MLV reverse transcriptase was then added to each sample and, lastly, the mixture was incubated at 30°C for 10 min, 42°C for 60 min and inactivation was carried out at 99°C for 5 min.

Quantification of HBV DNA and cDNA by real-time PCR

HBV DNA and cDNA quantification were performed as previously described [8]. 1 μl of each solution I and solution II was amplified by real-time PCR with an ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) according to the instructions provided by the manufacturer. Amplification was performed in a 25 μl reaction mixture containing SYBR Green PCR Master Mix (Applied Biosystems), 200 nM of forward primer (5′-TTTGGGGCATGGACATTGAC-3′, nucleotides 1893–1912), 200 nM of reverse primer (5′-GGTGAACAATGGTCCGGAGAC-3′, nucleotides 2029–2049) and 1 μl of solution I or solution II. The steps in real-time PCR are as follows: the mixture was incubated at 50°C for 2 min, denaturation was carried out at 95°C for 10 min, and the PCR cycling programme comprised 40 two-step cycles of 15 s at 95°C and 60 s at 60°C. The HBV RNA quantity was obtained by subtracting the quantification result of solution I from solution II, that is, HBV nucleic acid determined by real-time PCR after reverse transcription reaction minus HBV DNA determined by real-time PCR.

Serological assays

Serum HBeAg and anti-HBe were tested using chemiluminescent immunoassays (Architect HBeAg and Architect HBeAb; Abbott Japan, Tokyo, Japan). Serum HBsAg levels were quantified by Architect HBsAg (Abbott Japan). The dynamic range of the assay was 0.05–250 IU/ml. High HBsAg titre was measured with 1,000-fold diluted serum.

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Statistical analyses

Continuous variables were expressed as mean ±SD and evaluated by Student's *t*-test. Categorical variables were expressed as frequencies with proportions and compared using Pearson's χ^2 test, and Fisher's exact test was applied when at least one cell of the table had an expected frequency <5. All of the tests were two-tailed and a *P*-value <0.05 was considered statistically significant. The correlation between serum HBV RNA and serum HBV DNA as well as with serum qHBsAg was analysed by Pearson's correlation using SPSS programme for Windows 10.0 (SPSS Inc., Chicago, IL, USA). Cox regression analysis was applied for predictors of duration to undetectable serum HBV DNA using SAS version 9.2 (SAS Institute, Inc, Cary, NC. USA).

Results

Demographic profiles of patients

Baseline characteristics of CHB patients treated with lamivudine or entecavir are shown in Table 1. There was no significant difference in terms of age, gender ratio, HBeAg status, serum ALT level, serum HBV DNA level and serum qHBsAg level between the two groups.

Table 1. Baseline characteristics of chronic hepatitis B patients treated with lamivudine or entecavir

Variable	Lamivudine	Entecavir	*P*-value
Patients, *n*	26	26	-
Mean age, years (±SD)	61 ±10	59 ±13	0.609
Male, *n*/total *n* (%)	15/26 (57.7)	20/26 (76.9)	0.139
HBeAg positivity, *n*/total *n* (%)	12/26 (46.2)	11/26 (42.3)	0.780
Mean ALT, U/l (±SD)	641 ±1,837	122 ±209	0.158
Mean log HBV DNA, copies/ml (±SD)	9.9 ±2.1	9.7 ±1.8	0.739
Mean quantitative HBsAg, IU/ml (±SD)	4,537.5 ±6,091.3	6,363.7 ±7,064.9	0.323

Serum HBV RNA and qHBsAg levels before and during lamivudine versus entecavir therapy

The detectability and quantification of serum HBV RNA level at baseline, week 12 and 24 of lamivudine versus entecavir therapy are shown in Table 2. The detectability and quantity of serum HBV RNA level was comparable before the initiation of NA therapy. At week 12 and 24 of therapy, entecavir-treated patients had a higher proportion of detectable serum HBV RNA (50% versus 84.6% [*P*=0.008] and 38.5% versus 76.9% [*P*=0.005], respectively) and a higher quantity (3.8 ±4.1 versus 6.5 ±3.1 log copies/ml, [*P*=0.011] and 2.9 ±3.9 versus 6.2 ±3.8 log copies/ml, [*P*=0.003], respectively) when compared with lamivudine-treated patients. In addition, most of them had detectable HBV RNA at 12 weeks of therapy (lamivudine in 13 and entecavir in 22). Serum qHBsAg at week 12 and 24 of therapy as well as the interval to undetectable serum HBV DNA were not different between the two groups (Table 2).

At week 12 of NA therapy, the correlation of serum HBV RNA levels with serum qHBsAg levels and serum HBV DNA levels is shown in Figure 1. Serum HBV RNA levels tended to correlate better with serum qHBsAg levels (R square 0.407) than with serum HBV DNA levels (R square 0.321).

On-treatment predictors of initial virological response

CHB patients with interval from detectable to undetectable serum HBV DNA level <16 weeks (*n*=23) had a significantly lower serum HBV RNA level at week 12 of NA therapy than those with interval ≥16 weeks (*n*=21; 3.8 ±3.8 versus 6.6 ±3.5 log copies/ml [*P*=0.013]; Figure 2A). The time interval based on entecavir and lamivudine therapy is shown in Figure 2B.

Low serum HBV RNA level at week 12 of therapy predicted a shorter interval to undetectable serum HBV DNA (adjusted hazard ratio =0.908, 95% CI 0.829, 0.993, *P*=0.035), after adjustment for pretreatment serum ALT level as well as serum HBV DNA level and

Table 2. Serum HBV RNA and quantitative HBsAg during lamivudine versus entecavir therapy

Variable	Lamivudine	Entecavir	*P*-value
HBV RNA detectability			
Pre-treatment, *n*/total *n* (%)	9/26 (34.6)	12/26 (46.1)	0.396
At 12 weeks, *n*/total *n* (%)	13/26 (50)	22/26 (84.6)	0.008
At 24 weeks, *n*/total *n* (%)	10/26 (38.5)	20/26 (76.9)	0.005
Log HBV RNA			
Mean pre-treatment, copies/ml (±SD)	5.2 ±1.1	5.2 ±1.4	0.892
Mean at 12 weeks, copies/ml (±SD)	3.8 ±4.1	6.5 ±3.1	0.011
Mean at 24 weeks, copies/ml (±SD)	2.9 ±3.9	6.2 ±3.8	0.003
Mean quantitative HBsAg at 12 weeks	2,633.8 ±3,423	4,170.9 ±4,599	0.178
Mean quantitative HBsAg at 24 weeks, IU/ml (±SD)	2,566.5 ±3,814.3	3,763.1 ±4,707.6	0.319
Mean duration to undetectable HBV DNA, months (range)	4 (1–28)	5.9 (1–15)	0.232

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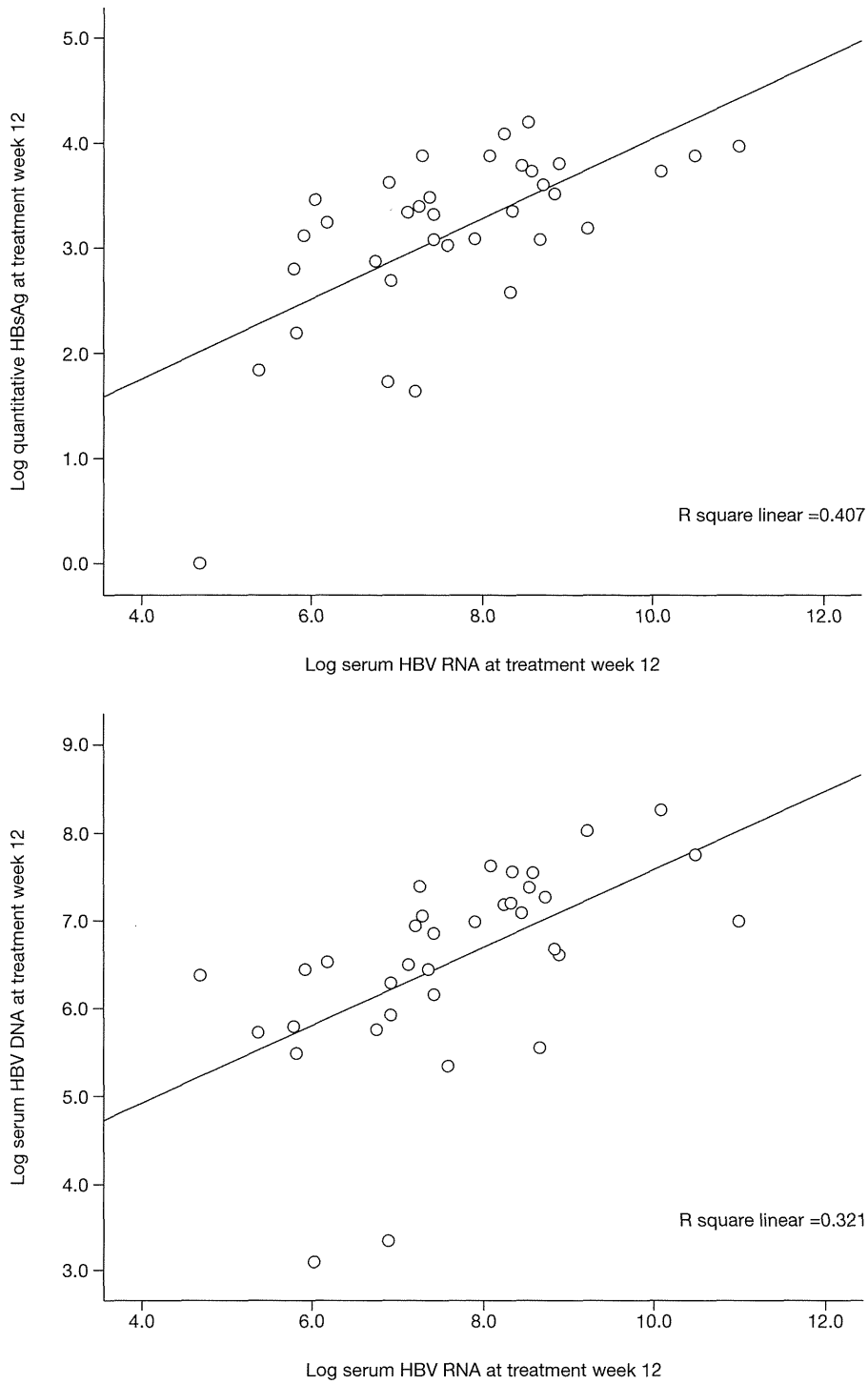
Figure 1. Correlation of serum HBV RNA with quantitative HBsAg and serum HBV DNA at treatment week 12 of NAs



Serum HBV RNA at treatment week 12 correlates better with **(A)** serum quantitative HBsAg at treatment week 12 (R square 0.407) than **(B)** serum HBV DNA at treatment week 12 (R square 0.321).

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