Differential MicroRNA Expression Between Hepatitis B and Hepatitis C Leading Disease Progression to Hepatocellular Carcinoma

Shunsuke Ura,[1] Masao Honda,[1,2] Taro Yamashita,[1] Teruyuki Ueda,[1] Hajime Takatori,[1] Ryuhei Nishino,[1] Hajime Sunakozaka,[1] Yoshio Sakai,[1] Katsuhisa Horimoto,[3] and Shuichi Kaneko[1]

MicroRNA (miRNA) plays an important role in the pathology of various diseases, including infection and cancer. Using real-time polymerase chain reaction, we measured the expression of 188 miRNAs in liver tissues obtained from 12 patients with hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC) and 14 patients with hepatitis C virus (HCV)-related HCC, including background liver tissues and normal liver tissues obtained from nine patients. Global gene expression in the same tissues was analyzed via complementary DNA microarray to examine whether the differentially expressed miRNAs could regulate their target genes. Detailed analysis of the differentially expressed miRNA revealed two types of miRNA, one associated with HBV and HCV infections (n = 19), the other with the stage of liver disease (n = 31). Pathway analysis of targeted genes using infection-associated miRNAs revealed that the pathways related to cell death, DNA damage, recombination, and signal transduction were activated in HBV-infected liver, and those related to immune response, antigen presentation, cell cycle, proteasome, and lipid metabolism were activated in HCV-infected liver. The differences in the expression of infection-associated miRNAs in the liver correlated significantly with those observed in Huh7.5 cells in which infectious HBV or HCV clones replicated. Out of the 31 miRNAs associated with disease state, 17 were down-regulated in HCC, which up-regulated cancer-associated pathways such as cell cycle, adhesion, proteolysis, transcription, and translation; 6 miRNAs were up-regulated in HCC, which down-regulated anti-tumor immune response. *Conclusion:* miRNAs are important mediators of HBV and HCV infection as well as liver disease progression, and therefore could be potential therapeutic target molecules. (HEPATOLOGY 2009;49:1098-1112.)

MicroRNA (miRNA) is an endogenous, small, single-strand, noncording RNA consisting of 20 to 25 bases and regulates gene expression of various cell types. It plays an important role in various biological processes, including organ development and differentiation as well as cellular death and proliferation, and is also involved in various diseases such as infection and cancer.[1-3]

miRNAs are produced as follows. A primary miRNA with a hairpin loop structure is cleaved into a precursor miRNA and transported out of the nuclei with a carrier protein (Exportin-5). The precursor miRNA is then processed by Dicer and converted into an active single-strand RNA in the cytoplasm. The miRNA binds to a target messenger RNA in a sequence-dependent manner and induces degradation of the target messenger RNA and translational inhibition. One miRNA regulates the expression of multiple target genes; bioinformatics analyses have suggested that the expression of more than 30% of human genes is regulated by miRNAs.[4-7]

Abbreviations: cDNA, complementary DNA; CH, chronic hepatitis; CH-B, chronic hepatitis B; CH-C, chronic hepatitis C; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCC-B, hepatitis B-related hepatocellular carcinoma; HCC-C, hepatitis C-related hepatocellular carcinoma; HCV, hepatitis C virus; miRNA, microRNA; RTD-PCR, real-time detection polymerase chain reaction; SVM, support vector machine.

From the Departments of [1]Gastroenterology and [2]Advanced Medical Technology, Kanazawa University Graduate School of Medicine, Kanazawa, Japan; and the [3]Biological Network Team, Computational Biology Research Center, National Institute of Advanced Industrial Science and Technology, 2-42 Aomi, koto-ku, Tokyo 135-0064, Japan.

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Address reprint requests to: Masao Honda, M.D., Ph.D., Department of Gastroenterology, Graduate School of Medicine, Kanazawa University, Takara-Machi 13-1, Kanazawa 920-8641, Japan. E-mail: mhonda@m-kanazawa.jp; fax: (81)-76-234-4250.

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Table 1. Characteristics of Patients Used for Analysis of miRNA and Microarray Samples

Patient No.	Virus	Age	Sex	ALT	Histology of Activity	Background Liver Fibrosis	Histological Grade of HCC	Tumor Size (mm)	TNM Staging	HCV-RNA (KIU/mL)	HBV-DNA (LEG/mL)
1	HBV	57	M	16	2	4	Moderate	20	II	–	3.4
2	HBV	51	M	57	1	2	Moderate	48	II	–	< 2.6
3	HBV	61	M	17	1	4	Well	16	II	–	< 3.7
4	HBV	47	M	19	1	4	Moderate	15	I	–	< 3.7
5	HBV	72	M	19	1	1	Well	25	II	–	NA
6	HBV	73	M	62	1	3	Moderate	45	III	–	5.7
7	HBV	42	M	36	1	4	Moderate	18	I	–	< 3.7
8	HBV	63	M	13	1	2	Moderate	15	I	–	2.8
9	HBV	68	F	54	1	2	Well	56	II	–	4.1
10	HBV	70	M	13	0	2	Well	40	II	–	< 3.7
11	HBV	58	M	29	1	4	Moderate	35	IVA*	–	3.3
12	HBV	72	M	22	1	4	Moderate	18	I	–	6
13	HCV	66	F	33	2	4	Well	25	II	423	–
14	HCV	67	M	89	1	4	Well	30	II	> 850	–
15	HCV	64	M	31	1	4	Moderate	75	III	< 5 (+)	–
16	HCV	68	M	30	0	4	Well	23	II	> 850	–
17	HCV	46	M	98	2	3	Moderate	20	I	> 850	–
18	HCV	68	F	32	2	4	Moderate	25	III	< 5 (+)	–
19	HCV	66	F	46	2	4	Well	25	II	> 850	–
20	HCV	47	M	246	1	3	Moderate	20	I	262	–
21	HCV	75	M	27	1	3	Moderate	19	II	85.1	–
22	HCV	77	M	21	0	1	Moderate	20	II	< 5 (−)	–
23	HCV	66	M	46	2	2	Well	60	II	50.3	–
24	HCV	65	M	89	1	1	Poorly	25	III	850	–
25	HCV	53	M	54	0	1	Moderate	28	II	< 5 (−)	–
26	HCV	75	F	212	1	4	Well	19	I	580	–
27	–	51	F	18	0	0	–	–	–	–	–
28	–	78	F	13	0	0	–	–	–	–	–
29	–	75	M	20	0	0	–	–	–	–	–
30	–	34	M	12	0	0	–	–	–	–	–
31	–	64	M	30	0	0	–	–	–	–	–
32	–	78	M	9	0	0	–	–	–	–	–
33	–	53	M	19	0	0	–	–	–	–	–
34	–	64	F	12	0	0	–	–	–	–	–
35	–	60	F	20	0	0	–	–	–	–	–

HCV RNA was assayed via Amplicor Monitor Test (KIU/mL); HBV DNA was assayed via transcription-mediated amplification (LEG/mL).

Abbreviations: ALT, alanine aminotransferase; F, female; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; M, male; TNM, tumor-node–metastasis.

*Vascular invasion (+).

Infection of the human liver with hepatitis B virus (HBV) and hepatitis C virus (HCV) induces the development of chronic hepatitis (CH), cirrhosis, and in some instances hepatocellular carcinoma (HCC).[8] The virological features of these two distinct viruses are completely different; however, the viruses infect the liver and cause CH, which is not distinguished by histological examination or clinical manifestations. We previously reported that gene expression profiles in chronic hepatitis B (CH-B) and chronic hepatitis C (CH-C) are different. Proapoptotic and DNA repair responses were predominant in CH-B, and inflammatory and antiapoptotic phenotypes were predominant in CH-C. However, factors inducing these differences in gene expression remain to be elucidated.[9,10]

We examined miRNA expression in liver tissue with HBV-related liver disease (CH-B and HCC-B) and HCV-related liver disease (CH-C and HCC-C) and in normal liver tissue via real-time detection polymerase chain reaction (RTD-PCR). We also performed global analysis of messenger RNA expression in these tissues using complementary DNA (cDNA) microarray. These analyses allowed us to find characteristic miRNAs associated with HBV or HCV infection as well as the progression of liver disease.

Patients and Methods

Patients. The study subjects included 12 patients with CH-B complicated by HCC and 14 patients with

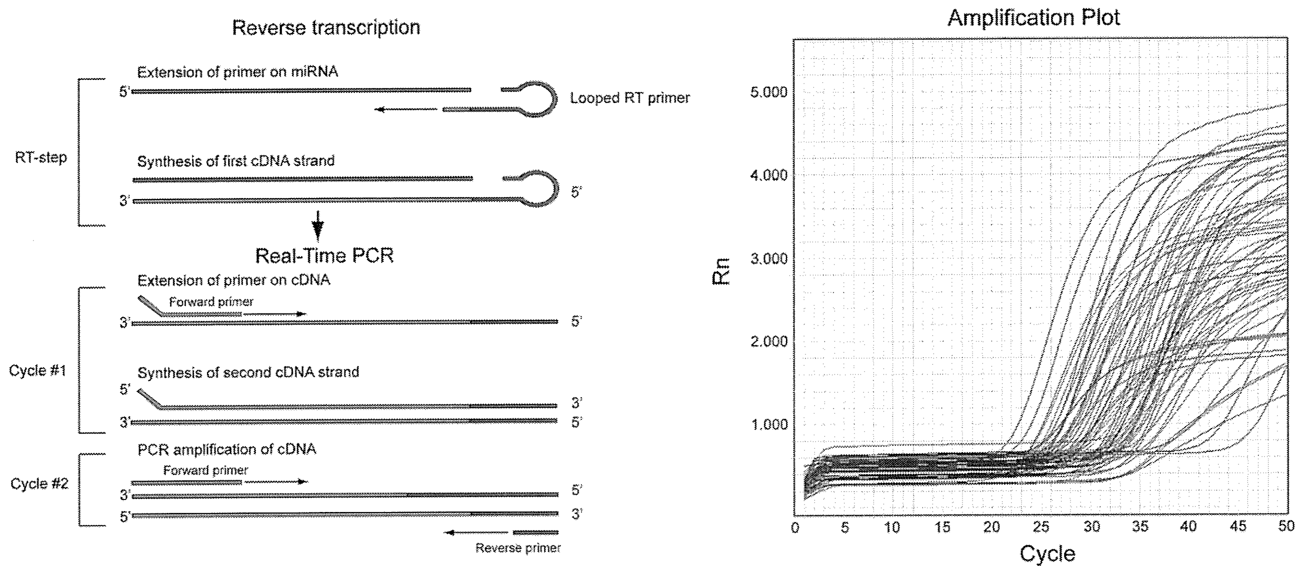

Fig. 1. (A) miRNA-specific RTD-PCR using sheet hairpin primers. (B) miRNA amplification curves by RTD-PCR.

CH-C complicated by HCC. Gene expression analysis was approved by the ethics committee of the Graduate School of Medicine, Kanazawa University Hospital, Japan, between 1999 and 2004. In addition, nine normal liver tissue samples obtained during surgery for metastatic liver cancer were used as control samples. Surgically removed liver tissues were stored in liquid nitrogen until analysis. Histological classification of HCC and histological evaluation of hepatitis in noncancerous regions for each patient are shown in Table 1. HCV viremia in two patients with CH-C was persistently cleared by interferon therapy before HCC development. There were no significant differences in the histological findings of HCC and noncancerous regions, as well as in sex, age, and hepatic function between the HBV and HCV infection groups.

Quantitative RTD-PCR. Approximately 1 mg of each liver tissue sample stored in liquid nitrogen was ground with a homogenizer while still frozen, and total RNA containing miRNA was isolated according to the protocol of the mirVana miRNA Isolation kit (Ambion, Austin, TX) and stored at −80°C until analysis. miRNA expression levels were quantitated using the TaqMan MicroRNA Assays Human Panel Early Access kit (Applied Biosystems, Foster City, CA). cDNA was prepared via reverse transcription using 10 ng each of the isolated total RNA and 3 μL each of the reverse transcription primers with specific loop structures. Reverse transcription was performed using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer's protocol. Then, a mixture of 6.67 μL of nuclease-free water, 10 μL of TaqMan 2 × Universal PCR Master Mix (No AmpErase UNG; Applied Biosystems), and 2 μL of TaqMan MicroRNA Assay Mix, which was included in the kit, was prepared for each sample on a 384-well plate; 1.33 μL of the reverse transcription product was added to the mixture, and amplification reaction was performed on an ABI PRISM 7900HT (Applied Biosystems). Expression levels of 188 miRNAs in each sample were quantitated.

Analysis of RTD-PCR Data. The measured 188 miRNAs included RNU6B, which is commonly used as a control for miRNA. β-Actin and glyceraldehyde 3-phosphate dehydrogenase were also measured simultaneously for correcting RNA amount. The mean Ct values and standard deviations of each miRNA were calculated from expression data of all patients obtained by RTD-PCR. miRNA with the lowest expression variation was used as the internal control. Ct values of each miRNA were then corrected by the Ct value of the internal control to yield −ΔCt values defined as relative miRNA expression levels and used for analyses. Statistical analyses and hierarchical cluster analyses of expression data were performed using BRB ArrayTools (http://linus.nci.nih.gov/BRB-ArrayTools.html). Relative miRNA expression levels were further normalized using the median over the all patients so that the normalized expression levels of each patient have a median log ratio of 0. A class prediction method was used for classifying two patient groups based on the supervised learning method, and a binary tree classification method was used for classifying three or more patient groups with a statistical algorithm of the support vector machine (SVM). Class prediction was performed using SVM incorporating genes differentially expressed at a univariate parametric significance level of $P = 0.01$. The prediction rate was estimated via cross-validation and the bootstrap method for small sample data.[11] (It is worth

A

-16.1 7.9
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B

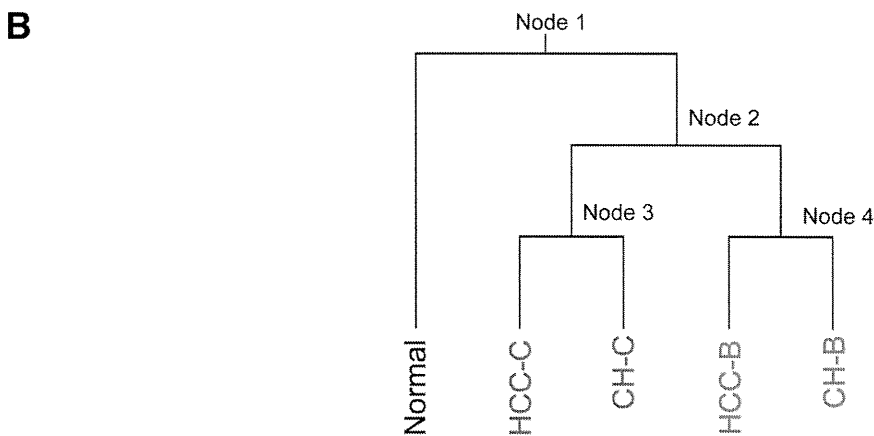
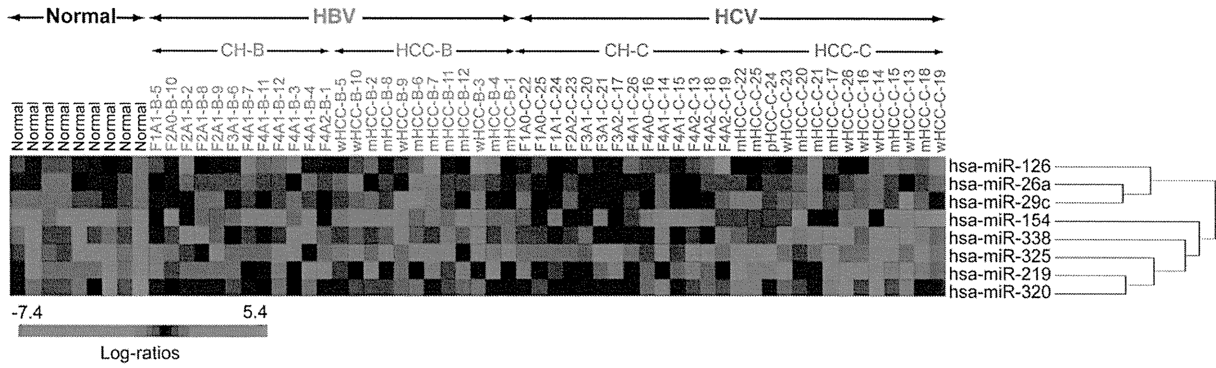
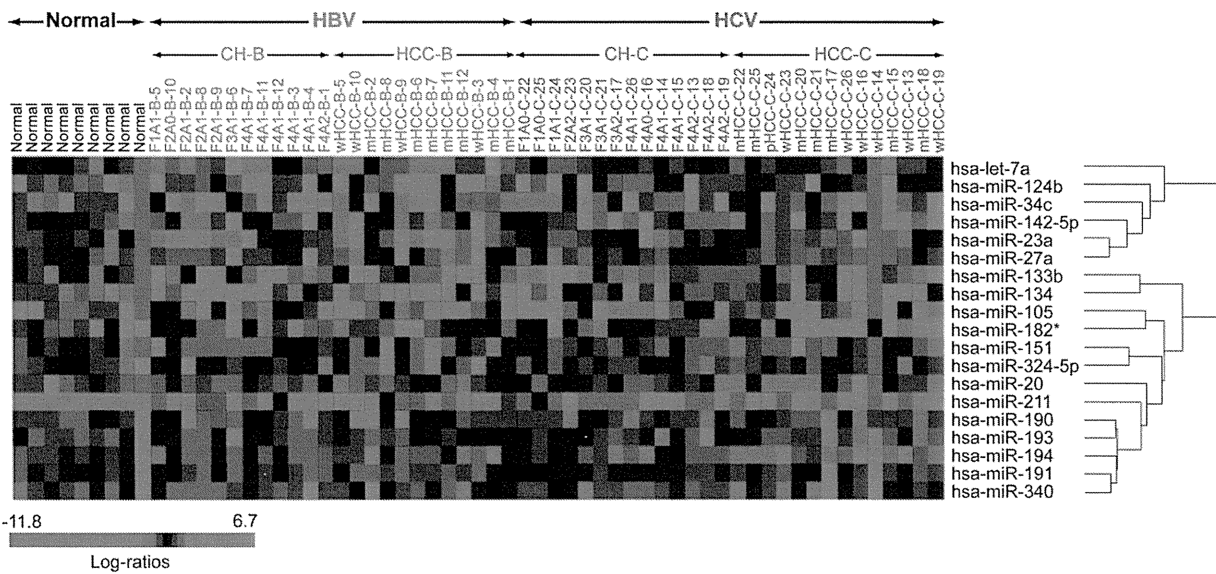


Fig. 2. (A) Hierarchical cluster analysis using total miRNA. Chronic hepatitis is indicated by histological stage and grade (F, fibrosis; A, activity) and type of infecting virus (B or C). HCC is indicated by histological grade (w, well differentiated; m, moderately differentiated; p, poorly differentiated) and type of infecting virus (B or C); with the patient number added at the end. (B) Relationship between five classes divided by binary tree classification. Expression profiles were first classified into normal liver and non-normal liver groups (node 1), then into HBV and HCV groups (node 2). The HBV group was further divided into HCC-B and CH-B (node 3), and the HCV group into HCC-C and CH-C (node 4).





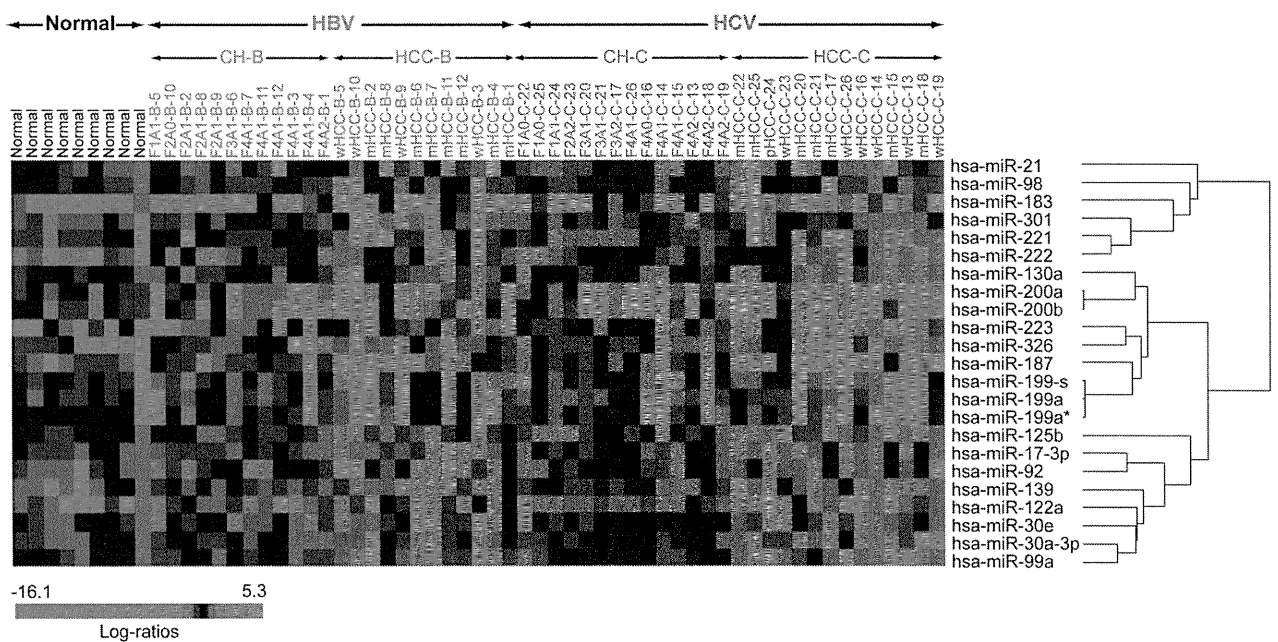


Fig. 3. Cluster 1: Eight miRNAs specifically differentiated node 1 classification. Cluster 2: Nineteen miRNAs specifically differentiated node 2 classification. Cluster 3: Twenty-three miRNAs differentiated CH-B and HCC-B as well as CH-C and HCC-C.

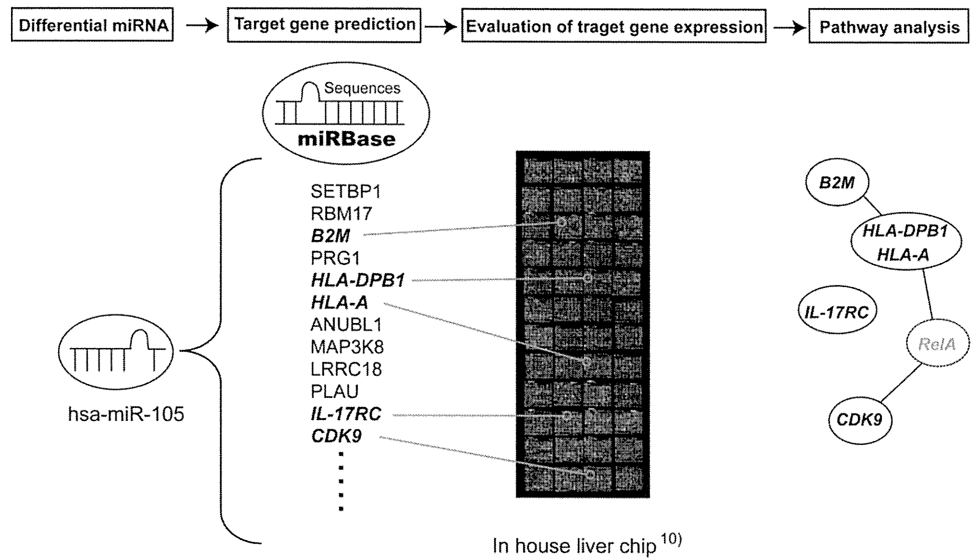


Fig. 4. Analysis of miRNA expression data. Target genes of miRNAs were predicted using MIRANDA Pro3.0; candidate target genes spotted on microarray were identified; number of genes that actually exhibit significant ($P < 0.05$) changes in expression among the genes was determined; and signal pathways involving genes regulated by the miRNAs that had exhibited differential expression between each group were analyzed using MetaCore (Table 4).

noting that the prediction rate may be likely an overestimate of the true rate, given the weaknesses of cross-validation and bootstrapping methods in a strict sense.)

Microarray Analysis. cDNA microarray slides (Liver chip 10k) were used as described.[10] RNA isolation, amplification of antisense RNA, labeling, and hybridization were performed according to the protocols described.[9,10] Quantitative assessment of the signals on the slides was performed by scanning on the ScanArray 5000 (General Scanning, Watertown, MA) followed by image analysis using GenePix Pro 4.1 (Axon Instruments, Union City, CA) as described.[10]

Preliminary Survey of Independency of Paired Samples from the Same Patient. CH and HCC expression data were derived from the same patient. Before further analysis, we examined whether the miRNA expression of paired samples was similar or independent. We compared differences in the expressions of paired and nonpaired CH and HCC samples using the Dunnett test[12] (Supplementary Data). All possible tests performed for data pairs represented no dependency due to the paired data from the same patients. For data analysis, we used the standard pairwise class comparison and prediction tool in BRB ArrayTools.

Identification of Candidate miRNA Target Genes. Candidate target genes predicted to be regulated by miRNAs based on sequence comparison were selected using MIRANDA Pro3.0 (Sanger Institute). Of the selected genes, those represented on a microarray chip were then examined for expression (Fig. 4). The number of genes showing a significant (P <0.05) expression difference among the candidate target genes represented on the chip was statistically analyzed to evaluate the significance of expression regulation by miRNAs. Analysis of significance was performed using Hotelling T2 test (BRB ArrayTools).

Pathway Analysis. Of the candidate miRNA target genes, those showing a significant ($P < 0.01$) expression difference between N, CH-B, HCC-B, CH-C, and HCC-C samples were analyzed for pathways involving these genes using MetaCore software suite (GeneGo, St. Joseph, MI). Significance probability was calculated using

Table 2-1. Class Prediction

No.	Class	Prediction (%)	No. of Predictors	*P* Value
1	HBV versus HCV	87	32	<0.001
2	N versus CH (B+C)	91	26	0.007
3	CH (B+C) versus HCC (B+C)	92	34	0.003

Class prediction algorithm was used for the classification of two groups of patients. Feature selection was based on the univariate significance level (alpha = 0.01). The support vector machine classifier was used for class prediction.

Abbreviations: CH, nontumor lesion of HCC; HCC, hepatocellular carcinoma; N, normal.

Table 2-2 Binary Tree Classification

Node	Group 1 Class	Group 2 Class	No. of Predictors	Misclassification Rate (%)
1	HCC-B, HCC-C, CH-B, CH-C	N	20	4.9
2	HCC-B, CH-B	HCC-C, CH-C	19	13.5
3	HCC-B	CH-B	15	29.2
4	HCC-C	CH-C	14	17.9

Binary tree classification algorithm was used for the classification of each category of patients. Feature selection was based on the univariate significance level (alpha = 0.01). The support vector machine classifier was used for class prediction. There were four nodes in the classfication tree.

Abbreviations: CH-B, non-tumor lesion of HCC-B; CH-C, nontumor lesion of HCC-C; HCC-B, hepatitis B virus-related hepatocellular carcinoma; HCC-C, hepatitis C virus-related hepatocellular carcinoma; N, normal

Table 3-1. Representative miRNAs That Were Commonly Repressed in CH-B, CH-C, HCC-B, and HCC-C Compared with Normal Liver (Cluster)

miRNA	Parametric *P* Value	Ratio*	No. of Significant Genes/Predicted Target Genes†	Hotelling Test *P* Value‡	Differentially Expressed Target Genes§	Pathway of Regulated Genes¶
hsa-miR-219	7.3E-05	0.28	25/109	2.59E-04	Glypican-3, ERP5, PLK2, HIRA, HMG2	Regulatory T cell differentiation
					ACOX1	Fatty acid beta-oxidation
					NF-X1	MHC class II biosynthetic process
hsa-miR-320	9.8E-05	0.50	26/88	3.50E-06	Vimentin, ALP (*N*-acetyltransferase-like), SEC61 beta, G-protein alpha-i2, Filamin A	Protein kinase cascade
					Rac1, RhoG	Organelle organization and biogenesis
					Vinexin beta, Profilin I, Ca-ATPase3	Actin cytoskeleton organization and biogenesis
hsa-miR-154	2.7E-04	0.15	22/70	5.40E-06	OTR, NET1(TSPAN1), NAP1, Vimentin, PDIA3, cytochrome P-450 reductase	Regulation of apoptosis
					DLX2	Morphogenesis
					GUAC, ACAT1	Branched chain family amino acid catabolic process
hsa-miR-29c	1.8E-03	0.55	53/133	1.00E-06	FBXO7, ASPP1, HSPA4, Cathepsin O, PDF, COL4A1, HSPA4, TIP30, CXADR	Cell-substrate adhesion
					NS1-BP, ALP (*N*-acetyltransferase-like), ACTR10, Beclin 1	Transcription, DNA-dependent
					SMAD6, LTBR(TNFRSF3), ENPP7	Apoptosis
hsa-miR-338	5.2E-03	0.46	30/101	3.60E-06	ID3, GATA-4, NFIA, FR-beta, CREST, HYOU1	Developmental process
					G3ST1, CAD,	Immune effector process
					FKBP12, LZIP, PDIA3, Schwannomin (NF2), CREST	Immune system process
hsa-miR-26a	6.3E-03	0.70	37/119	2.64E-05	*LIG4*, c-FLIP, GADD45 beta, DAPK1, PRDX4, LRP130	Response to stimulus
					Cyclin E, ZDHHC6, Tx1, ATG8 (GATE-16), WASP, C1s	DNA replication initiation
					COPG1	Ion transport
hsa-miR-126	8.1E-03	0.65	27/101	4.04E-03	ANP32B (april), HSPA4, RLI, LIV-1 (SLC39A6), PTP-MEG2, CD97, DHPR	Regulation of cellular protein metabolic process
					NFKBIA, NMI, MDH1, PDCD2	Response to stress
					SMAD6, ATP6AP2, ANP32B (april), NMI, HSPA4	Apoptosis
hsa-miR-325	8.7E-03	0.20	18/63	2.03E-04	TRADD, CREST, NEDD8, annexin IV, GPX2, PDF, TNFAIP1	Developmental process
					Glypican-3, ID1, PC-TP,	Multicellular organismal development
					SNRPB (Sm-B)	RNA splicing

*Ratio of HCC-B, HCC-C, CH-B, and CH-C to normal.
†The number of significant genes ($P < 0.05$) out of predicted target genes in which expression was evaluated in microarray.
‡Statistical assessment of presence of differentially expressed genes out of predicted target genes of miRNAs.
§Representative differentially expressed genes out of predicted target genes of miRNAs.
¶Representative pathway of differentially expressed genes out of predicted target genes of miRNAs.

the hypergeometrical distribution based on gene ontology terms. Because one gene is frequently involved in multiple pathways, all pathways corresponding to the genes with significance probability were listed.

Verification of Regulation of Candidate Target Genes by miRNAs. Anti-miRNA (Ambion) specific to 13 miRNAs (has-miR-17*, has-miR-20a, has-miR-23a, has-miR-26a, has-miR-27a, has-miR-29c, has-miR-30a, has-miR-92, has-miR-126, has-miR-139, has-miR-187, has-miR-200a, and has-miR-223) showing significant differences in expression were transfected into Huh7 cells using TransMessenger transfection reagent (QIAGEN, Valencia, CA), and loss of function of each miRNA was evaluated. Similarly, precursor miRNAs of five miRNAs (has-miR-23a, has-miR-26a, has-miR-27a, has-miR-92, and has-miR-200a) were also transfected into Huh7 cells, and gain of function of each miRNA was evaluated. The loss- and gain-of-function of miRNAs were evaluated via RTD-PCR. In addition, different gene expressions regulated by miRNAs were also evaluated via RTD-PCR.

HBV/HCV Infection Model Using Cultured Cells. The plasmid pHBV 1.2 coding the 1.2-fold length of the HBV genome was transfected into Huh7.5 cells using Fugene6 transfection reagent (Roche Applied Science, Indianapolis, IN). HBeAg production in culture medium was measured using Immunis HBeAg/Ab EIA (Institute of Immunology Co., Ltd., Tokyo, Japan).[13] The amount of HBV-DNA was measured via RTD-PCR (Supplementary Fig. 1A,B). JFH1-RNA was transfected into Huh7.5 cells using TransMessenger transfection reagent (QIAGEN) and the expression of the core protein was examined via immunofluorescence staining using anti-HCV core antibody (Affinity BioReagent, CO).[14,15] HCV-RNA amount was also measured via RTD-PCR (Supplementary Fig. 1A,B). JFH1/GND was used as a negative control. miRNA expression was quantitated by RTD-PCR 48 hours after transfection.

Results

Expression of miRNA in Liver Tissue. A panel of miRNA was successfully amplified from liver tissues via RTD-PCR. The representative amplification profile of miRNA as determined with RTD-PCR is shown in Fig. 1. To assess the reliability and reproducibility of this assay system, we first measured RNU6B in duplicate from all samples in different plates. The mean difference in Ct values of RNU6B expression within the same samples was 0.08 ± 0.05 (mean ± standard deviation), indicating the high reproducibility of this assay. All Ct values from each reaction were collected, and Ct variation obtained by each probe from all patients was calculated. Although RNU6B was frequently used as the internal control, the standard Ct variation was relatively high (Ct, 27 ± 1.94), suggesting that the variances in its value depend on the state of liver disease (N, CH and HCC). Therefore, we selected has-miR-328 as the internal control with the smallest standard deviation (Ct, 30 ± 0.60). The relative expression ratio of individual miRNA to has-miR-328 was calculated and applied to the following analysis using a BRB-array tool.

Hierarchical cluster analysis revealed that the expression profiles of the 188 miRNAs from each patient were roughly classified into normal liver, HBV-infected liver (CH-B+HCC-B; HBV group), and HCV-infected liver (CH-C+HCC-C; HCV group) (Fig. 2A). HCV viremia in two patients with CH-C was persistently cleared by interferon therapy before HCC development. The background liver of one of these patients was clustered in the normal group and those of others in the HCV group. Although these two patients were not clearly differentiated from others, some miRNAs such as miR-194, miR-211, and miR-340 that were down-regulated in the HCV group were significantly up-regulated in two patients (Fig. 3, cluster 2).

The present CH and HCC expression data were obtained from the same patient; however, each sample clustered irrespective of pairs in all but two patients. miRNA expression profiling was therefore more dependent on the disease condition than on the paired condition, as also confirmed by the Dunnett test.[12] We then attempted to classify the expression profiles into HBV and HCV groups using supervised learning methods (Table 2-1). HBV and HCV groups were significantly differentiated at an 87% accuracy ($P < 0.001$). The normal liver and CH (CH-B + CH-C) and CH and HCC (HCC-B + HCC-C) were also significantly differentiated at a 90% rate of accuracy. These results suggest that different stages of liver disease (normal, CH, and HCC) can be differentiated from each other based on the miRNA expression profile, as well as HBV and HCV infection.

To examine the relationship among five categories of groups, namely, N, CH-B, CH-C, HCC-B and HCC-C, we attempted to differentiate the five groups using a supervised learning algorithm (binary tree classification) used for classifying three or more groups. SVM was used as a prediction method. Expression profiles were first classified into groups N (normal) and non-N (non-normal) (CH-C, CH-B, HCC-C, and HCC-B) (node 1) ($P < 0.01$). The non-N group was then classified into HBV and HCV (node 2) ($P < 0.01$). The HBV group was further classified into CH-B and HCC-B (node 3) ($P < 0.01$), and the HCV group was further classified into CH-C and HCC-C (node 4) ($P < 0.01$) (Fig. 2B, Table 2-2). Thus, the findings support the notion that differences in miRNA expression between HBV and HCV are as distinct as those between CH and HCC.

Out of 20 miRNAs that differentiated node 1 classification (Table 2-2), 12 also differentiated node 3 or node 4 classification. The remaining eight miRNAs specifically differentiated node 1 classification. They were down-regulated in the HBV and HCV groups compared with the normal group (Fig. 3, cluster 1). Nineteen miRNAs differentiated node 2 classification (Table 2-2) and the hierarchical clustering using these miRNAs clearly differentiated the HBV and HCV groups (Fig. 3, cluster 2). There were 15 and 14 miRNAs that differentiated node 3 and 4 classifications, respectively (Table 2-2). Hierarchical clustering using these miRNAs revealed that these miRNAs differentiated CH-B and HCC-B as well as CH-C and HCC-C, respectively; 17 miRNAs were down-regulated in HCC, and six were up-regulated in HCC (Fig. 3, cluster 3).

Table 3-2. Differentially Expessed miRNA Between HCC-B, CH-B, and HCC-C, CH-C, and Their Representative Target Genes (Cluster 2)

miRNA	Parametric *P* Value	Ratio*	No. of Significant Genes/Predicted Target Genes†	Hotelling Test *P* Value‡	Differentially Expressed Target Genes§	Pathway of Regulated Genes¶
hsa-miR-190	1.2E-05	2.06	21/68	4.47E-02	Chk1, C2orf25, VRK2, USP16, STAF65(gamma)	Regulation of cell cycle
					AP1S2, RNASE4	Mitotic cell cycle
					PPP2R1B, ARHGAP15, UBPY	Negative regulation of apoptosis
hsa-miR-134	2.3E-04	5.74	11/58	3.40E-06	VKDGC, SH2B, MALS-1, DDB2	Multicellular organismal process
					BCRP1	Regulation of viral reproduction
					DDB2	Lipid biosynthetic process
hsa-miR-151	2.8E-04	1.82	12/62	6.41E-01	RGS2, UFO, AK2, USP7	G-protein signaling
					eIF4G2, USP7	Regulation of translation
					SLC22A7	Organic anion transport
hsa-miR-193	5.0E-04	1.67	23/95	9.30E-01	G-protein alpha-11, p130CAS, VAV-1, PDCD11	Cell motility
					Colipase, ACSA	Energy coupled proton transport
					DCOR	Intracellular signaling cascade
hsa-miR-133b	1.7E-03	2.42	20/97	3.69E-02	DDB2, Bcl-3, Cystatin B	Proteasomal protein catabolic process
					Rab-3, RAG1AP1, KCNH2, DCOR	Regulation of biological quality
					AL1B1	Carbohydrate metabolic process
hsa-miR-324-5p	2.9E-03	1.51	27/121	1.90E-06	SKAP55, VAV-1, DDB2, E2A, NIP1	Cellular developmental process
					MEMO (CGI-27), Rab-3	Cellular structure morphogenesis
					COPG1, GPX3, OAZ2	Glutathione metabolic process
hsa-miR-182*	3.1E-03	2.23	28/123	< 1e-07	Alpha-endosulfine, HCCR-2, Thioredoxin-like 2, TPT1, USP7	Translation initiation in response to stress
					DDB2, TPT1	Cellular developmental process
					JIP-1	JNK cascade
hsa-miR-105	4.6E-03	4.38	18/68	4.74E-05	Beta-2-microglobulin, HLA-B27	Antigen processing and presentation
					PIMT, IL-17RC	Immune response
					MHC class I, CDK9, ERG1, Desmocollin 3	
hsa-miR-211	5.3E-03	25.61	10/56	2.00E-04	PSMD5, SLC26A6	Proteasomal protein catabolic process
hsa-miR-20	5.7E-03	1.52	27/113	5.28E-03	Noelin, SC4MOL, Thioredoxin-like 2, CCL5, NALP3	Regulation of apoptosis
					Hic-5/ARA55, USP16, MAP4, Ferroportin 1	Positive regulation of cellular process
					TOP3A, PLRP1	Oxygen transport
hsa-miR-191	6.7E-03	1.39	25/79	7.55E-04	CDK9, GPS2, CLTA, LXR-alpha	Nucleic acid metabolic process
					ACSA	Acetyl-CoA biosynthetic process
					UGCGL1, SGPP1	Metal ion transport
hsa-miR-340	8.5E-03	1.48	17/81	3.73E-03	FKBP12, DCOR,	Calcium ion transport
					Gelsolin, VAV-1, ARF6	Actin cytoskeleton organization and biogenesis
					HXK3	Glucose catabolic process
hsa-miR-194	8.7E-03	1.67	13/74	5.90E-01	Cyclin B1, Serglycin	M phase of mitotic cell cycle
					PTE2	Acyl-CoA metabolic process
					SLC7A6	Carbohydrate utilization
hsa-miR-23a	1.9E-04	0.46	14/97	< 1e-07	*RGL2*, MANR, MEK1 (MAP2K1), Caspase-3, AZGP1	Protein kinase cascade
					FRK, Pyk2(FAK2), CSE1L	Cellular developmental process
					AZGP1	Defense response
hsa-miR-142-5p	4.9E-04	0.40	25/89	9.10E-06	Sirtuin4, PAI2, PSAT, RIL, CDC34, SPRY1	Metabotropic glutamate receptor
					E4BP4, DNAJC12, WWP1, PAIP1, PASK, rBAT	Regulation of gene expression
					VCAM1, CaMK I, WWP1, FHL3	Cell-matrix adhesion
hsa-miR-34c	5.1E-04	0.20	31/129	7.30E-06	Diacylglycerol kinase, zeta, PLC-delta 1, ATP2C1, PAI2	Manganese ion transport
					MLK3(MAP3K11), MEK1(MAP2K1), CDC25C, MRF-1, XPC	Protein kinase cascade
					GNT-IV	Inflammatory cell apoptosis

Table 3-2. Continued

miRNA	Parametric P Value	Ratio*	No. of Significant Genes/Predicted Target Genes†	Hotelling Test P Value‡	Differentially Expressed Target Genes§	Pathway of Regulated Genes¶
hsa-miR-124b	8.6E-04	0.32	25/120	7.10E-05	E2F5, Rad51, Jagged1	Muscle development
					MLK3(MAP3K11), RGS1	Intracellular signaling cascade
					COL16A1	MAPKKK cascade
hsa-let-7a	1.0E-03	0.45	28/136	9.35E-04	RAD51C, CoAA, hASH1, Cockayne syndrome B, Caspase-1, PP5	Response to DNA damage stimulus
					PLC-delta 1, MANR, ACADVL	Fibroblast proliferation
					HGF, NGF	Cellular developmental process
hsa-miR-27a	3.9E-03	0.59	18/108	1.19E-02	COL16A1, RIL, RhoGDI gamma, ANP32B (april)	Cytoskeleton organization and biogenesis
					VE-cadherin, NTH1, GATA-2, E4BP4	Response to external stimulus
					RAD51C	DNA recombination

*Ratio of HCC-B, CH-B, to HCC-C,CH-C.
†The number of significant genes ($p < 0.05$) out of predicted target genes in which expression was evaluated in microarray.
‡Statistical assesment of presence of differentially expressed genes out of predicted target genes of miRNAs.
§Representative differentially expressed genes out of predicted target genes of miRNAs.
¶Representative pathway of differentially expressed genes out of predicted target genes of miRNAs.

These results indicate that there were two types of miRNAs—one associated with HBV and HCV infection (cluster 2), the other associated with the stages of liver disease (clusters 1 and 2) that were irrelevant to the differences in HBV and HCV infection.

Differential miRNAs and Their Candidate Target Genes and Signaling Pathways. Differentially expressed miRNAs are shown in Table 3. In addition to the expression ratios of miRNAs in each group, the number of genes analyzed on the microarray predicted to be the target genes of miRNAs and that which actually showed significant ($P < 0.05$) differences in expression are also shown. Based on the frequencies and levels of expression of differential genes, the significance of regulation of these gene groups by miRNAs was evaluated using Hotelling T2 test (BRB ArrayTools) (Table 3). The representative candidate target genes and their signaling pathways by each miRNA were shown one by one (Table 3). The signaling pathways regulated by all differential miRNAs in each category of groups are shown in Table 4.

Eight miRNAs were down-regulated in the HBV and HCV groups compared with the normal group (Table 3-1; Fig. 3, cluster 1). These miRNAs were associated with an increased expression of genes related to cell adhesion, cell cycle, protein folding, and apoptosis (Tables 3-1, 4-1), and possibly with the common feature of CH irrespective of the differences in HBV and HCV infection.

Nineteen miRNAs clearly differentiated the HBV and HCV groups (Fig. 3, cluster 2, Table 3-2). Thirteen miRNAs exhibited a decreased expression in the HCV group, and six showed a decreased expression in the HBV group. miRNAs exhibiting a decreased expression in the HCV group regulate genes related to immune response, antigen presentation, cell cycle, proteasome, and lipid metabolism. On the other hand, those exhibiting a decreased expression in the HBV group regulate genes related to cell death, DNA damage and recombination, and transcription signals. These findings reflected the differences in the gene expression profile between CH-B and CH-C described (Tables 3-2, 4-2).[10] Interestingly, although these miRNAs were HBV and HCV infection–specific, some of them were reported to be tumor-associated miRNAs, suggesting the possible involvement of infection-associated miRNAs in HCC development.

Twenty-three miRNAs clearly differentiated CH and HCC that were irrelevant to the differences in HBV and HCV infection. Seventeen miRNAs were down-regulated in HCC that up-regulated cancer-associated pathways such as cell cycle, adhesion, proteolysis, transcription, translation, and the Wnt signaling pathway (Tables 3-3, 4-3). Six miRNAs were up-regulated in HCC that down-regulated all inflammation-mediated signaling pathways, potentially reflecting impaired antitumor immune response.

Relationship Between Expressions of Infection-Associated miRNA in Liver and Cultured Cells Infected with HBV and HCV. To clarify whether the expression of infection-associated miRNA is regulated by HBV and HCV infection, we investigated the relationship between changes in miRNA in liver tissues and those in miRNA in Huh7.5 cells in which infectious HBV or HCV clones replicated. To evaluate the replication of each clones in Huh7.5 cells, we measured time-course changes in the amounts of HBV-DNA and HCV-RNA in Huh7.5 cells transfected with pHBV1.2 and JFH1-RNA, respectively, by RTD-PCR (Supplementary Fig. 1A). The expression of HBV proteins was examined by measuring the amount

Table 3-3. Differentially Expessed miRNA Between CH and HCC and Their Representative Target Genes (Cluster 3)

miRNA	Parametric p-value	Ratio*	No. of Significant Genes/Predicted Target Genes†	Hotelling Test *P* Value‡	Differentially Expressed Target Genes§	Pathway of Regulated Genes¶
hsa-miR-139	4.50E-06	0.42	19/106	2.70E-03	Cyclin B1, DHX15, MCM5, Histone H2A	Mitotic cell cycle
					RBCK1, SYHH	Protein catabolic process
					ILK, IGFBP7, SAFB, CTR9	Response to external stimulus
hsa-miR-30a-3p	2.50E-05	0.49	26/144	1.73E-02	GGH, Pirin, ZNF207, Annexin VII	Regulation of oxidoreductase activity
					ILK, LTA4H, ABC50, GNPAT	Cell-matrix adhesion
					DLC1	Morphogenesis
hsa-miR-130a	7.00E-05	0.50	22/108	1.07E-02	SPHM, PPP2R5D, RHEB2, SPHM	Mitotic cell cycle
					MLK3(MAP3K11), Otubain1, TIMP4	Protein modification process
					NRBP	Cell differentiation
hsa-miR-223	3.40E-04	0.39	14/90	6.52E-03	Ephrin-A1, Midkine, FDPS	Cell morphogenesis
					K(+) channel, subfamily J	Notch signaling pathway
hsa-miR-187	3.55E-04	0.12	16/66	6.76E-04	HFE2, Otubain1	Negative regulation of programmed cell death
					PRSS11, SUPT5H, RAG1AP1	Developmental process
					PLOD3	Mitochondrial ornithine transport
hsa-miR-200a	6.86E-04	0.18	20/141	2.15E-02	*CDC25B, KAP3, CDK2AP2,* CHKA	Cell communication
					POLD	DNA replication
					CPSF4	RNA splicing
hsa-miR-17-3p	8.42E-04	0.58	28/108	8.98E-04	MLK3(MAP3K11), Tip60, ACBD6, DOC-1R, DAX1, RBCK1	Protein kinase cascade
					WNT5A, 14-3-3 gamma, DHX15	BMP signaling pathway
					HFE2, MCM5	DNA recombination
hsa-miR-99a	1.17E-03	0.53	33/163	9.52E-03	Calpain small subunit, Thoredoxin-like 2, Survivin	Cytokinesis
					IBP2, DNA-PK, KAP3,	Intracellular signaling cascade
					NFE2L1, PARP-1, HDAC11	Regulatory T cell differentiation
hsa-miR-200b	1.57E-03	0.18	24/147	2.72E-02	HSP47, HMG2, NRBP	Regulation of cell cycle
					SNX17	Cell motility
					Ephrin-A1	Receptor protein signaling pathway
hsa-miR-125b	1.82E-03	0.55	26/114	1.03E-01	COL4A2, TIP30, HSP47, MSP58	Cell adhesion
					MLK3(MAP3K11), ERK2 (MAPK1), ERK1 (MAPK3), PLOD3	Nuclear translocation of MAPK
					Otubain1, SCN4A(SkM1)	Ubiquitin-dependent protein catabolic process
hsa-miR-30e	2.10E-03	0.65	24/151	4.30E-02	Cyclin B1, XTP3B, GAK, Annexin VII, MIC2, NRBP	Mitotic cell cycle
					MSS4	Protein localization
					S100A10	Calcium ion transport
hsa-miR-199a*	4.26E-03	0.35	11/71	7.16E-02	BUB3, Cyclin B1, LMNBR	Mitotic cell cycle
					PRAME	Cardiac muscle cell differentiation
hsa-miR-122a	6.31E-03	0.51	11/80	1.01E-03	JAB1, APEX, Clathrin heavy chain	Base-excision repair
					PARN	Translational initiation
					DDAH2	Regulation of cellular respiration
hsa-miR-199a	8.77E-03	0.35	18/94	3.56E-02	IL-13, MLK3(MAP3K11), CLK2, ACP33	Protein amino acid phosphorylation
					PAFAH beta, SPA1, CLCN4	Small GTPase mediated signal transduction
hsa-miR-326	9.00E-03	0.57	29/147	2.25E-01	Midkine, ENT1, IP3KA, PSMC5, ANCO-1	Regulation of programmed cell death
					Thy-1, MCM6, Tip60, VILIP3	Cell-matrix adhesion
					COMP, Cathepsin A	Blood vessel development
hsa-miR-92	9.60E-03	0.81	28/140	2.47E-02	*TUBGCP2,* Fibrillin 1, PIPKI gamma, KAP3	Rho protein signal transduction
					SNX15, BCAT2	LDL receptor and BCAA metabolism
					IGFBP7, FZD6, COPS6	Adenosine receptor signaling pathway
hsa-miR-221	3.40E-06	3.34	16/67	3.59E-01	Lck, Kallistatin, Neuromodulin, LFA-3, PA24A, AZGP1, MSH2	Immune response-activating signal transduction
					KYNU, PMCA3	DNA repair

Table 3-3. *Continued*

miRNA	Parametric p-value	Ratio*	No. of Significant Genes/Predicted Target Genes†	Hotelling Test *P* Value‡	Differentially Expressed Target Genes§	Pathway of Regulated Genes¶
hsa-miR-222	6.50E-06	2.23	18/85	1.59E-02	Thrombospondin 1, Lck, MSH2, ATF-2, CITED2, Kallistatin	Cell motility
					PGAR	Triacylglycerol metabolic process
					KYNU	DNA replication
hsa-miR-301	5.22E-05	1.96	14/71	1.16E-01	Beta-2-microglobulin, PPCKM, PRC, Fra-1, PPCKM, ACAT2	Antigen processing and presentation
					BMPR1B, ARMER, EHM2, RBBP8	Meiotic recombination
					Neuromodulin, LDLR	Cell motility
hsa-miR-21	7.67E-03	1.57	19/81	1.86E-04	Btk, Fra-1, MSH2, Collectrin, Adipophilin	Regulation of T cell proliferation
					RNASE4, AGXT2L1	Peptidyl-tyrosine phosphorylation
					SARDH	Natural killer cell activation during immune response
hsa-miR-183	2.46E-02	3.51	13/86	3.36E-01	Hdj-2, PEMT, Lck, MKP-5, Chondromodulin-I, ABCA8	Cell differentiation
					IL-16, MTRR, SerRS	Methionine biosynthetic process
hsa-miR-98	5.22E-02	1.32	24/130	2.95E-04	ACAA2, LTB4DH, ACADVL, DECR, S14 protein,	Fatty acid metabolic process
					Rapsyn, Kallistatin, ENPEP, Beta crystallin B1	Multicellular organismal process
					CYP4F8	Prostaglandin metabolic process

*Ratio of HCC to CH.
†The number of significant genes ($P<0.05$) out of predicted target genes in which expression was evaluated in microarray.
‡Statistical assesment of presence of differentially expressed genes out of predicted target genes of miRNAs.
§Representative differentially expressed genes out of predicted target genes of miRNAs.
¶Representative pathway of differentially expressed genes out of predicted target genes of miRNAs.

of HBeAg released in culture medium (Supplementary Fig. 1B). HCV protein expression was examined by evaluating the core protein expression after 48 hours by fluorescence immunostaining (Supplementary Fig. 1C). RNA was extracted from the Huh7.5 cells 48 hours after gene transfection, and miRNA expression pattern in the cells was compared with those in liver tissues. We found a strong correlation between differences in miRNA expression between liver tissues of the HBV and HCV groups, and those in miRNA expression between Huh7.5 cells transfected with HBV and HCV clones ($r = 0.73$, $P = 0.0006$) (Fig. 5). These results revealed that differences in the expression of infection-associated miRNA in the liver between the HBV and HCV groups are explained by changes in miRNA expression caused by HBV and HCV infections.

Verification of Regulation of Candidate Target Genes by miRNA. Anti-miRNAs (Ambion) specific to 13 miRNAs (has-miR-17*, has-miR-20a, has-miR-23a, has-miR-26a, has-miR-27a, has-miR-29c, has-miR-30a, has-miR-92, has-miR-126, has-miR-139, has-miR-187, has-miR-200a, and has-miR-223) showing significant differences in expression were transfected into Huh7 cells to examine loss of function of the miRNAs. Five miRNAs (has-miR-23a, has-miR-26a, has-miR-27a, has-miR-92, and has-miR-200a) showed a decreased expression by more than 50%. Precursor miRNAs of these miRNAs were also transfected into the cells to examine the gain of function of the miRNAs (Supplementary Fig. 2). It was confirmed that the expressions of target genes of the five miRNAs (LIG4 [by has-miR-26a]; RGL2 [by has-miR-23a]; Rad51C [by has-miR-27a]; KAP3, CDC25B, KAP3, CDK2AP2, POLD, and CPSF4 [by has-miR-200a]; and TUBGCP2, SNX15 and BCAT2 [by has-miR-92]) were increased by the suppression of the miRNAs induced by anti-miRNAs and were decreased by the overexpression of precursor miRNAs (Supplementary Fig. 3).

Discussion

miRNA plays an important role in various diseases such as infection and cancer.[1-3] In this study, we examined miRNA expression profiles in normal liver and HCC, including nontumor lesions infected with HBV or HCV. Although the expression profiles of miRNAs in HCC have been reported,[16-18] most of the studies were performed using a microarray system. Because we thought that miRNAs could not produce enough detection signals owing to their short length, we applied a highly sensitive and quantitative RTD-PCR method for miRNAs. Moreover, global gene expression in the same tissues was ana-

Table 4-1. Pathway Analysis of Targeted Genes by miRNAs that Were Commonly Repressed in CH-B, CH-C, HCC-B, and HCC-C Compared with Normal Liver (Cluster 1)

No.	Pathway Name	*P* Value
Down-regulated miRNA in CH-B,HCC-B,CH-C and HCC-C (possibly up-regulating target genes)		
1	Cell adhesion_Platelet-endothelium-leukocyte interactions	1.11E-02
2	Cell cycle_S phase	2.18E-02
3	Protein folding_Protein folding nucleus	2.43E-02
4	Cell cycle_G1-S	3.07E-02
5	Development_Cartilage development	3.89E-02
6	Protein folding_Folding in normal condition	3.89E-02
7	Proteolysis_Connective tissue degradation	3.99E-02
8	Proteolysis_Proteolysis in cell cycle and apoptosis	4.31E-02
9	Signal Transduction_BMP and GDF signaling	5.81E-02
10	Immune_Antigen presentation	6.05E-02

lyzed via cDNA microarray to examine whether the differentially expressed miRNAs could regulate their target genes. Because the absolute standard of miRNA is not available at present, and miRNA expression was compared within the samples and genes analyzed in this study, there might be possible errors when a larger number of samples and genes were analyzed.

Using these systems, we found that the expression profile in miRNAs was clearly different according to HBV and HCV infection for the first time. The differences were confirmed by the nonsupervised learning method, hierarchical clustering (Fig. 2A), and supervised learning methods based on SVM at an 87% accuracy ($P < 0.001$) (Table 2-1). As similarly described, the expression profile in miRNAs was significantly different according to the progression of liver disease (normal, CH, and HCC) in this study. The present CH and HCC expression data were derived from the same patient, and some microarray analyses suggested that the noncancerous liver tissue can predict the prognosis of HCC.[19,20] We examined whether the miRNA expression of paired samples was similar or independent using the Dunnett test[12] (Supplementary Data). Our data indicated that miRNA expression profiling was more dependent on the disease condition than on the paired condition, although the issue of paired samples should be taken into account carefully.

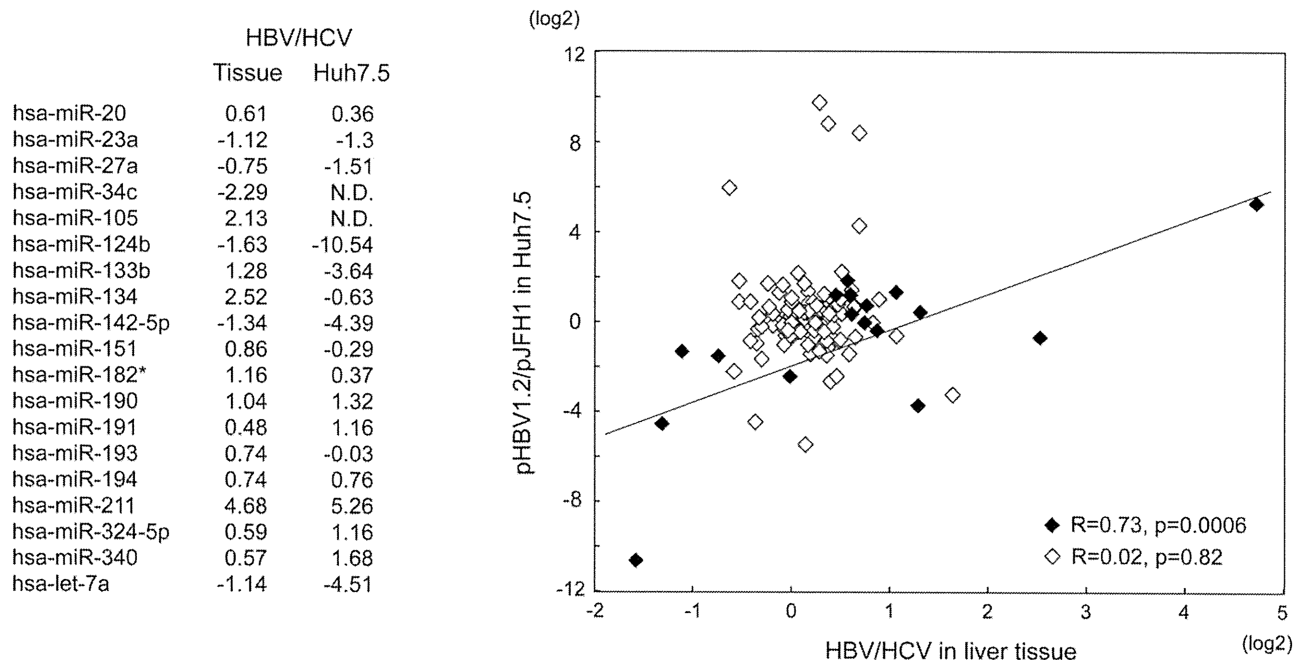
Binary tree prediction analysis and detailed assessment of hierarchical clustering revealed two types of differential miRNAs, one associated with HBV and HCV infection, the other associated with the stages of liver disease that were irrelevant to the differences in HBV and HCV infection. We found that differences in miRNA expression between liver tissues with HBV and HCV (HBV/HCV) were strongly correlated with those in miRNA between cultured cell models of HBV and HCV infection (HBV/HCV) ($r = 0.73$ $P = 0.0006$) (Fig. 5). Thus, there exist HBV- and HCV-infection–specific miRNAs that potentially regulate viral replication and host gene signaling pathways in hepatocytes.

Table 4-2. Pathway Analysis of Targeted Genes by Differentially Expressed miRNAs Between HBV-Related Liver Disease (CH-B,HCC-B) and HCV Related Liver Disease (CH-C,HCC-C Cluster 2)

No.	Pathway Name	*P* Value
Down-regulated miRNA in CH-C,HCC-C (possibly up-regulating target genes)		
1	Immune_Phagosome in antigen presentation	5.80E-04
2	Muscle contraction	1.05E-03
3	Immune_Antigen presentation	5.75E-03
4	Cell cycle_Meiosis	1.49E-02
5	Reproduction_Male sex differentiation	2.06E-02
6	Cell adhesion_Platelet aggregation	2.77E-02
7	Transport_Synaptic vesicle exocytosis	3.56E-02
8	Inflammation_Kallikrein-kinin system	3.73E-02
9	Inflammation_IgE signaling	4.10E-02
10	Development_Skeletal muscle development	5.02E-02
Down-regulated miRNA in CH-B,HCC-B (possibly up-regulating target genes)		
1	Signal Transduction_Cholecystokinin signaling	1.15E-04
2	Inflammation_NK cell cytotoxicity	5.29E-03
3	Signal transduction_CREM pathway	5.31E-03
4	Reproduction_GnRH signaling pathway	7.80E-03
5	DNA damage_DBS repair	1.02E-02
6	Cell cycle_G2-M	1.63E-02
7	Development_Neuromuscular junction	2.07E-02
8	Apoptosis_Apoptosis mediated by external signals	2.42E-02
9	Reproduction_FSH-beta signaling pathway	2.92E-02
10	Cell adhesion_Amyloid proteins	3.81E-02

Table 4-3. The Pathway Analysis of Targeted Genes by Differentially Expressed miRNAs Between CH and HCC (Cluster 3)

No.	Pathway Name	*P* Value
	Down-regulated miRNA in HCC (possibly up-regulating target genes)	
1	Cytoskeleton_Spindle microtubules	2.15E-03
2	Transcription_Chromatin modification	5.27E-03
3	Proteolysis_Ubiquitin-proteasomal proteolysis	6.43E-03
4	Cell adhesion_Cell-matrix interactions	7.30E-03
5	Cell cycle_Meiosis	7.83E-03
6	DNA damage_Checkpoint	1.69E-02
7	Reproduction_Progesterone signaling	1.94E-02
8	Apoptosis_Apoptotic mitochondria	3.14E-02
9	Translation_Regulation of initiation	4.22E-02
10	Signal transduction_WNT signaling	4.26E-02
	Up-regulated miRNA in HCC (possibly down-regulating target genes)	
1	Inflammation_IgE signaling	1.05E-02
2	Inflammation_Kallikrein-kinin system	2.46E-02
3	Inflammation_Innate inflammatory response	2.51E-02
4	Inflammation_Histamine signaling	4.25E-02
5	Inflammation_Neutrophil activation	4.55E-02
6	Chemotaxis	4.68E-02
7	Inflammation_IL-12,15,18 signaling	5.16E-02
8	Inflammation_NK cell cytotoxicity	7.25E-02
9	Cell cycle_G0-G1	7.53E-02
10	Inflammation_Complement system	7.72E-02



	HBV/HCV	
	Tissue	Huh7.5
hsa-miR-20	0.61	0.36
hsa-miR-23a	-1.12	-1.3
hsa-miR-27a	-0.75	-1.51
hsa-miR-34c	-2.29	N.D.
hsa-miR-105	2.13	N.D.
hsa-miR-124b	-1.63	-10.54
hsa-miR-133b	1.28	-3.64
hsa-miR-134	2.52	-0.63
hsa-miR-142-5p	-1.34	-4.39
hsa-miR-151	0.86	-0.29
hsa-miR-182*	1.16	0.37
hsa-miR-190	1.04	1.32
hsa-miR-191	0.48	1.16
hsa-miR-193	0.74	-0.03
hsa-miR-194	0.74	0.76
hsa-miR-211	4.68	5.26
hsa-miR-324-5p	0.59	1.16
hsa-miR-340	0.57	1.68
hsa-let-7a	-1.14	-4.51

Fig. 5. Correlation between differences in miRNA expression between liver tissues infected with HBV and HCV and those in miRNA expression between cultured cell models of HBV and HCV infections. A total of 140 of 188 miRNAs were confirmed to be expressed in Huh7.5 cells. There was a significant correlation of infection-associated miRNA (closed lozenge) in vitro and in vivo (r = 0.73, P = 0.0006), but none for the other 121 miRNAs (open lozenge) (r = 0.02, P = 0.82).

The pathway analysis of targeted genes by miRNAs revealed that 13 miRNAs exhibiting a decreased expression in the HCV group regulate genes related to immune response, antigen presentation, cell cycle, proteasome, and lipid metabolism. Six miRNAs showing a decreased expression in the HBV group regulate genes related to cell death, DNA damage and recombination, and transcription signals. These findings reflected differences in the gene expression profile between CH-B and CH-C as described.[10] Many of the miRNAs were down-regulated in the HCV group rather than in the HBV group. It has been reported that human endogenous miRNAs may be involved in defense mechanisms, mainly against RNA viruses.[21] On the other hand, it is suggested that endogenous miRNAs may be consumed and reduced by defense mechanisms, especially those against RNA viruses.

Although the expressions of these HBV- and HCV-infection–specific miRNAs were irrelevant to the differences in CH and HCC (Fig. 3, cluster 2), some of them have been reported to play pivotal roles in the occurrence of cancer. For example, has-let-7a regulates ras and c-myc genes,[22] and has-miR-34 is involved in the p53 tumor suppressor pathway.[23] These miRNAs were down-regulated in the HBV group, possibly participating in a more aggressive and malignant phenotype in HCC-B rather than in HCC-C. High expression of has-miR-191 was shown to be significantly associated with the worse survival in acute myeloid leukemia,[24] and has-miR-191 was overexpressed in the HBV group compared with the HCV group. On the other hand, has-miR-133b, which was reported to be down-regulated in squamous cell carcinoma,[25] was repressed in the HCV group compared with the HBV group. Some hematopoietic-specific miRNAs such as has-miR-142-5p were up-regulated in the HCV group. Therefore, these miRNAs were not only HBV and HCV infection–associated but also tumor-associated. These findings indicate different mechanisms of development of HCC infected with HBV and HCV (Fig. 6).

Following HCC development, common changes in miRNA expression between HCC-B and HCC-C appeared (Fig. 3, cluster 3). The 23 miRNAs mentioned above clearly differentiated CH and HCC that were irrelevant to the differences in HBV and HCV infections. Seventeen miRNAs were down-regulated in HCC, which up-regulated cancer-associated pathways. Six miRNAs were up-regulated in HCC that down-regulated all inflammation-mediated signaling pathways, potentially reflecting impaired antitumor immune response in HCC. These results suggest that common signaling pathways are involved in HCC development from CH, and that HBV- and HCV-specific miRNAs participate in generating HCC-specific miRNA expressions (Fig. 6). Therefore, these miRNAs might be good candidates for molecular targeting to prevent HCC occurrence, because they reg-

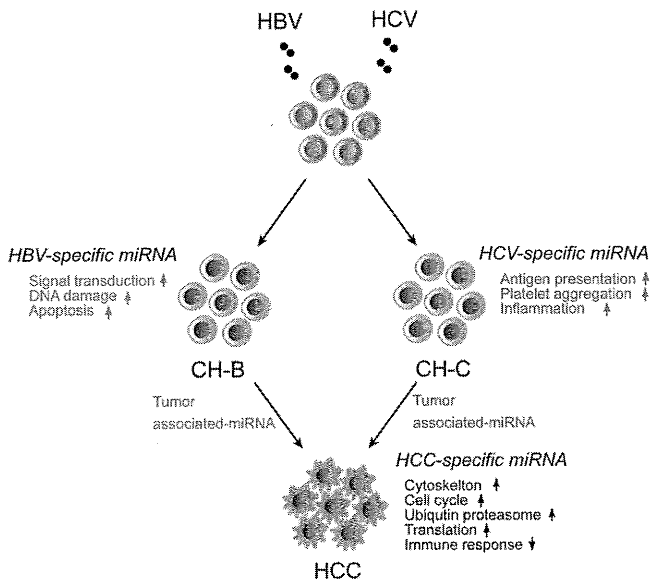


Fig. 6. Infection-associated and HCC-specific miRNAs and liver disease progression.

ulate a common signaling pathway underlying HCC-B and HCC-C development.

In conclusion, we showed that miRNAs are important mediators of HBV and HCV infections as well as liver disease progression. Further studies are needed to enable more detailed mechanistic analysis of the miRNAs identified here and to evaluate the usefulness of miRNAs as diagnostic/prognostic markers and potential therapeutic target molecules.

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MECHANISMS OF GASTROINTESTINAL, PANCREATIC AND LIVER DISEASES

Molecular mechanisms of hepatocarcinogenesis in chronic hepatitis C virus infection

Taro Yamashita, Masao Honda and Shuichi Kaneko

Department of Gastroenterology, Kanazawa University Graduate School of Medical Science, Kanazawa, Ishikawa, Japan

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Correspondence
Professor Shuichi Kaneko, Department of Gastroenterology, Kanazawa University Graduate School of Medical Science, 13-1 Takara-Machi, Kanazawa, Ishikawa 920-8641, Japan. Email: skaneko@m-kanazawa.jp

Abstract

Hepatitis C virus (HCV) infection is a major cause of hepatocellular carcinoma (HCC) and chronic liver disease worldwide. Recent developments and advances in HCV replication systems *in vitro* and *in vivo*, transgenic animal models, and gene expression profiling approaches have provided novel insights into the mechanisms of HCV replication. They have also helped elucidate host cellular responses, including activated/inactivated signaling pathways, and the relationship between innate immune responses by HCV infection and host genetic traits. However, the mechanisms of hepatocyte malignant transformation induced by HCV infection are still largely unclear, most likely due to the heterogeneity of molecular paths leading to HCC development in each individual. In this review, we summarize recent advances in knowledge about the mechanisms of hepatocarcinogenesis induced by HCV infection.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy and the third leading cause of cancer death worldwide.[1] The majority of HCCs arise from a background of chronic liver diseases caused by infection with hepatitis B virus (HBV) or hepatitis C virus (HCV).[2] Although both viruses are hepatotropic and regarded as causative agents of HCC, the underlying mechanisms of hepatocarcinogenesis are considered to be largely different, partly due to differences in the nature of DNA viruses (with an integration capacity for the host genome) and RNA viruses (with no genome integration capacity).

Hepatitis C virus is an RNA virus that is unable to integrate into the host genome but, instead, its proteins interact with various host proteins and induce host responses that potentially contribute to the malignant transformation of cells. In addition, HCC usually develops in the setting of liver cirrhosis after long-term continuous inflammation/regeneration processes; these accelerate the turnover of hepatocytes with increased risk of replication errors and DNA damage. Furthermore, recent genome-wide association studies have suggested that the natural course of HCV infection might be modified by the genetic background of the host.[3,4] Thus, both host and virus factors are considered to affect the process of hepatocarcinogenesis in a complex manner.

In this review, we summarize the current knowledge of the mechanisms of hepatocarcinogenesis induced by HCV infection. We also focus on recent findings of transcriptomic characteristics of HCV-related HCC and summarize the potential signaling pathways that are altered in this condition.

Epidemiology

Chronic HCV infection is a major risk factor for the development of HCC worldwide. According to the World Health Organization (WHO), approximately 170 million people are chronically infected with HCV. Although epidemiological evidence has suggested a clear, close relationship between HCV infection and HCC,[5,6] the prevalence of HCV infection in HCC patients differs noticeably between geographical regions. Thus, HCV infection is found in 70–80% of HCC patients in Japan, 70% in Egypt, 40–50% in Italy and Spain, about 20% in the United States (US), and less than 10% in China.[7–9] In industrialized countries including the US, a recent increase in HCC incidence and mortality has been observed, potentially due to the rising incidence of HCV infection transmitted through contaminated blood.[10]

Hepatitis C virus increases the risk of HCC by promoting inflammation and fibrosis of the infected liver that eventually results in liver cirrhosis. Once HCV-related cirrhosis is established, HCC develops at an annual rate of about 4–7%.[11] Other factors including alcohol intake, diabetes, and obesity have also been reported to increase the risk of HCC development by about two- to fourfold, indicating a strong life-style effect on the process of hepatocarcinogenesis.[12,13] Age and male gender are also contributing risk factors for HCV-related HCC, although the detailed mechanisms are still debatable.

Virus proteins and host responses

Hepatitis C virus belongs to the Flaviviridae family. It has a positive-stranded linear RNA genome of about 9.6-kb containing a

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single large open reading frame encoding three structural (core, E1, and E2) and seven non-structural (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins.[14] The structural proteins form the HCV virions, whereas non-structural proteins are involved in the processes of viral replication, assembly, and maturation. HCV proteins are known to be processed by host and viral proteases. Both structural and non-structural proteins can interact with various host cellular proteins to potentially promote the malignant transformation of hepatocytes (see recent reviews[7,15,16]). In this review, because of space limitations, we focus on the findings of core and NS5A proteins in terms of host responses potentially evoked during the process of HCV-related hepatocarcinogenesis.

Core protein

Hepatitis C virus core is a 21-kDa nucleocapsid protein with an RNA-binding capacity. In addition to its function in regulating HCV-RNA translation and HCV particle assembly, core protein is known to be involved in mediating the alteration of various host cell signaling pathways, transcriptional activation, modulation of immune responses, apoptosis, oxidative stress, and lipid metabolism.[7] Several recent studies have indicated the statistically significant high frequency of mutations in the *core* gene in HCV-infected patients who developed HCC.[17,18] However, the functional relevance of mutant core proteins on the malignant transformation of hepatocytes or the HCV life cycle has yet to be clarified.

Evidence of core protein as a causative agent of HCC was initially obtained from the transgenic mice model in which *core* gene overexpression, under the regulation of the HBV regulatory element used as a promoter, resulted in steatosis of mouse livers in early life, with subsequent development of adenoma and HCC.[19] However, another mouse model using a different promoter and of a different strain background resulted only in steatosis or different phenotypes without HCC development.[20,21] Similar controversial findings were reported in transgenic mice expressing HCV polyprotein or structural protein with regards to the development of HCC.[22,23] Thus, the role of core protein alone in the development of HCC remains unclear in transgenic mouse models.

Although the direct role of core protein in the malignant transformation of hepatocytes is still under investigation, it seems to be related to the development of hepatic steatosis.[19,24] Indeed, steatosis is one of the risk factors for the development of HCV-related HCC,[25,26] and activation of the lipogenic pathway has been reported in a subset of HCC cases.[27] Core protein is associated with the surface of lipid droplets in infected cells and might be directly related to steatosis through several factors responsible for lipid biogenesis and degradation, including peroxisome proliferator-activated receptor alpha and sterol-regulatory element binding protein-1.[21,28–30] Furthermore, core protein is reported to interact with endoplasmic reticulum (ER) or mitochondrial outer membranes and induce ER stress by perturbation of protein folding or by the accumulation of reactive oxygen species (ROS) through mitochondrial dysfunction.[31,32] ROS produced in this way might result in DNA damage to the host genome and accelerate the process of hepatocarcinogenesis. Increased hepatic iron deposition may also induce oxidative stress and lipid peroxidation, thus increasing the risk of HCC development in HCV polyprotein transgenic mice.[33]

Since the discovery of HCV, various studies have investigated the role of core on host cells. Its effects have been demonstrated on signaling pathways responsible for the cell cycle, and apoptosis through interaction with several tumor suppressors including p53, p73, and p21[34–39] as well as apoptosis regulators such as tumor necrosis factor-α (TNF-α) signaling or Bcl-2 members.[40–42] However, the data obtained from these studies are relatively inconsistent with each other and have varied across experimental models. Core protein may influence the growth and proliferation of host cells through activation of signaling pathways such as Raf/mitogen activated protein kinase (MAPK),[43] Wnt/beta catenin,[16] and transforming growth factor-β (TGF-β).[15,44] These pathways are known to be activated in HCC.[45] The findings therefore indicate a potential role for core in cell proliferation or suppression of apoptosis during malignant transformation of hepatocytes in the liver of chronic hepatitis C, where chronic inflammation and regeneration of hepatocytes continuously occurs.

NS5A protein

NS5A is a 56–58-kDa protein phosphorylated at serine residues by serine-threonine kinase[46] and is essential for replication of the HCV genome. NS5A protein forms part of the viral replicase complex and is localized mainly in the cytoplasm of infected cells in association with the ER. NS5A can become a lower molecular weight protein through post-translational modification, after which it can undergo translocation to the nucleus where it acts as a transcriptional activator. High frequencies of wild-type *NS5A* genes were reported to be dominant in liver cirrhosis patients who finally developed HCC compared with those who did not,[47] but the mechanistic significance of the *NS5A* wild/mutant genotypes in the process of HCV-related hepatocarcinogenesis remains uncertain.

NS5A protein has been suggested to interact with various signaling pathways including cell cycle/apoptosis[48] and lipid metabolism[28,49,50] in host cells and shares some signaling targets with core protein. NS5A is recognized as a transcriptional activator for many target genes[51] including p53 and its binding protein, TATA binding protein (TBP). Transcription factor IID activities were reported to be modified by NS5A in the suppression of p53-dependent transcriptional transactivation and apoptosis.[52,53] NS5A may also interact with pathways such as Bcl2,[54] phosphatidylinositol 3-kinase (PI3-K),[55] Wnt/beta catenin signaling,[56] and mTOR[57] to activate cell proliferation signaling and inhibit apoptosis.

Taken together, intriguing data concerning the function of core and NS5A proteins on host cell signaling pathways, transcriptional activation, apoptosis, oxidative stress, and lipid metabolism described above suggest a diverse role for HCV proteins in the pathophysiology of chronic hepatitis C that leads to malignant transformation in infected hepatocytes. Key findings and present concepts are summarized in Figure 1.

Transcriptomic characteristics of HCV-related HCC

As described above, HCV proteins can evoke various host responses in infected cells at transcriptional/translational/post-translational levels. Furthermore, enhanced cell death/regeneration processes are considered to induce DNA damage and accelerate replication errors that cause frequent mutations and genomic alter-

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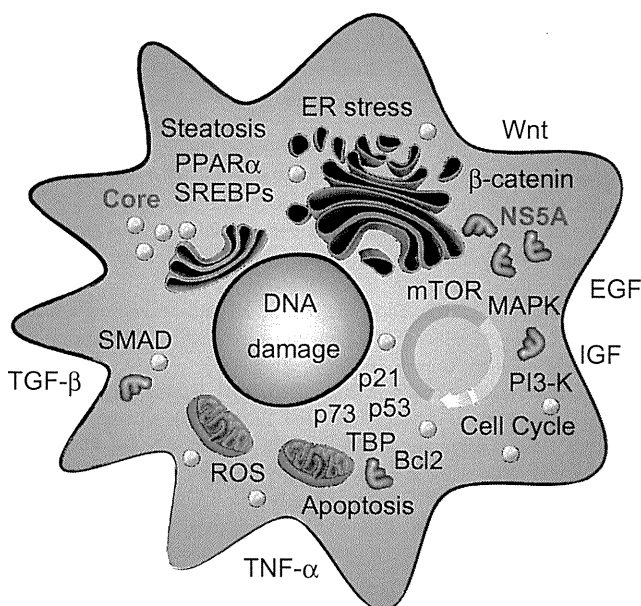

Figure 1 Signaling pathways potentially affected by hepatitis C virus (HCV) proteins. EGF, epidermal growth factor; ER, endoplasmic reticulum; IGF, insulin-like growth factor; MAPK, mitogen activated protein kinase; mTOR, mammalian target of rapamycin; PI3-K, phosphatidylinositol 3-kinase; PPAR, peroxisome proliferator-activated receptor; ROS, reactive oxygen species; SREBP, sterol-regulatory element binding protein; TBP, TATA binding protein.

ation in the host genome. The central dogma is defined as the flow of genetic information from DNA to mRNA and then to protein, so genetic/genomic alterations and transcriptional/translational modifications are ultimately considered to affect the cellular signaling pathway at the transcriptional level.

Over the past decade, several methods (including differential display, serial analysis of gene expression [SAGE], and microarray) have been developed to allow comparative studies of gene expression between normal and cancer cells on a genome-wide scale,[58] and the analysis of a set of all RNA molecules (mainly indicating mRNAs) is termed as whole transcriptome analysis. Extensive transcriptome analysis of HCC and corresponding non-cancerous livers has been performed, and the results have greatly increased our knowledge about the transcriptome characteristics of HCV-related HCC.

Early microarray and SAGE studies investigating the gene expression patterns of chronic hepatitis B and C tissues indicated that these two chronic hepatitis tissues had distinct gene expression profiles; the genes activated in chronic hepatitis C were correlated with signaling pathways associated with apoptosis, oxidative stress responses, and Th1 cytokine signaling.[59,60] An early study comparing genes activated in HCV-related and HBV-related HCCs showed that the genes associated with xenobiotic metabolism were more abundantly expressed in HCV-related HCC,[61] suggesting a detoxification role, which is potentially induced by chronic inflammation and generation of ROS resulting from HCV infection. In contrast, HBV-related HCC might closely correlate with the activation of imprint genes, including insulin-like growth factor-II (IGF-II) as investigated by oligo-DNA microarray,[62] suggesting a role of de-differentiation or epigenetic alteration of the host genome in HBV-related HCC. Activation of genes associated with interferon, oxidative stress, apoptosis, and lipid metabolism signaling was detected in HCV-related HCC and chronic hepatitis C specimens,[27,60,63] consistent with numerous functional studies that have investigated the host response evoked by HCV structural and non-structural proteins.[48]

Transcriptome analysis has also recently shed new light on the transcriptional alteration events occurring in early stages of HCV-related hepatocarcinogenesis. *GPC3* (encoding Glypican 3) was identified as one of the most activated transcripts in the early stage of hepatocarcinogenesis,[60,64] while several recent studies showed that gene signatures including *GPC3* can successfully discriminate HCCs from pre-malignant dysplastic nodules and cirrhosis nodules.[65,66] Close examination of genes differentially expressed among cirrhotic nodules, dysplastic nodules, and early and advanced HCV-related HCC tissues has also suggested roles for Toll-like receptor signaling, Wnt signaling, bone morphogenetic protein (BMP)/TGF-β signaling, JAK-STAT signaling, and DNA repair/cell cycle responses in each step of the malignant transformation processes.[67] These processes might therefore provide candidate molecular targets for the chemoprevention of HCV-related HCC.

Recent advances in transcriptome analysis have also provided detailed information on the status of small noncoding RNAs, microRNAs, that can regulate the expression of target genes and viral replication in normal and cancer tissues. Expression of microRNAs including miR-122 and −199a has been reported to modulate HCV replication,[68–70] and miR-122 expression can be regulated by host interferon signaling and responses.[71] HCV protein expression in turn could induce miRNAs and might affect the tumor suppressor DLC1 and the chemosensitivity of malignantly transformed cells.[72,73] Several microRNAs were also differentially expressed between HCV-related and HBV-related HCCs as well as their corresponding non-cancerous liver tissues. The candidate signaling pathways potentially altered by microRNAs in HCV-related tissues were those associated with antigen presentation, cell cycle, and lipid metabolism,[74] consistent with the mRNA microarray data described above. MicroRNAs have also recently been reported to successfully discriminate between HCC and cirrhotic liver tissues,[75] implicating their role in the early stages of malignant transformation. These data suggest that microRNAs may be good targets for the eradication of HCC as well as hepatocytes infected with HCV.

Conclusion

The heterogeneity of genetic/transcriptomic/proteomic events observed in hepatocytes or cell lines expressing HCV proteins and HCV-related HCCs reported thus far has suggested that complex mechanisms underlie malignant transformation induced by HCV infection. These potentially act through convoluted virus-host interactions including HCV replication with host cell cycles, apoptosis, proliferation, quality control of protein synthesis, lipid metabolism, and DNA damage responses. Indeed, HCC is a heterogeneous disease in terms of drug sensitivity, metastatic capacity, and clinical outcome. The heterogeneity of HCV-related HCC may closely correlate with the origin of malignantly transformed cells where multifaceted cellular reactions including apoptosis and

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cell proliferation are induced by HCV infection. An in-depth understanding of these molecular complexities associated with HCV-related HCC may provide the opportunity for effective chemoprevention of HCC among those with HCV-cirrhosis, and to design tailor-made treatment options for HCV-related HCC patients in the future.

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