

Figure 4: Downstream proteins of CASP8 within the distance 2 in apoptosis pathway maps. CASP8 is highlighted with the double circles. We excluded CASP8 from this subnetwork not to select it as a reporter.

edges were selected as the optimal reporter nodes in IP2.

5.2 Artificial Scale-free Networks

It is known that many real biological networks have the scale-free property [24]. In particular, it is observed that gene regulatory networks have the power-law out-degree distribution and the Poisson indegree distribution [25]. Thus, we generated scale-free networks with a power-law outdegree distribution ($\propto k^{-2.5}$) and Poisson indegree distribution as follows. We first choose the outdegree for each node from a power-law distribution. That is, the outdegree d_i of node v_i is drawn from a power-law distribution. Then, we choose d_i output nodes randomly with uniform probability from n nodes. Thus, the indegree distribution should follow a Poisson distribution.

Table 4 shows the average CPU time over 100 networks for each case. For large n ($= 1000, 5000, 10000$), the elapsed time was sufficiently short (even in the case of $L = 3$ and $K = 5$). This result suggests that the proposed methods are scalable to realistic size instances. The elapsed time of IP2 was shorter than that of IP1 for almost all cases. It is reasonable because IP1 has twice as many integer variables as IP2, and the number of constraints in IP1 is larger than that in IP2.

6 Concluding Remarks

We defined two problems P1 and P2 to allocate reporter genes that are effective for observing behaviors of various biological networks. We showed hardness results

Table 3: Selected proteins as reporters for each L and K in the downstream proteins of CASP8.

| L | K | IP1 | Reporters |
|-----|----------|-----|---|
| 1 | 1 | 6 | BCL2 |
| 1 | 2 | 10 | BID, CASP7 |
| 1 | 3 | 13 | BCL2, BID, BIRC4 |
| 1 | 10 (IP2) | 23 | CASP9, RAD9B, BCL2, BAK1, DIABLO, CASP3, DFFA, NUMA1, PAK2, PARP1 |
| 2 | 1 | 13 | BCL2 |
| 2 | 2 | 18 | BCL2, BIRC4 |
| 2 | 3 | 19 | BCL2, DIABLO, NUMA1 |
| 2 | 7 (IP2) | 23 | BCL2, BAK1, DIABLO, DFFA, NUMA1, PAK2, PARP1 |
| 3 | 1 | 16 | BAD |
| 3 | 6 (IP2) | 23 | CASP9, BAK1, DFFA, NUMA1, PAK2, PARP1 |
| 4 | 5 (IP2) | 23 | BAK1, DFFA, NUMA1, PAK2, PARP1 |
| 23 | 1 | 19 | BAK1 |
| 23 | 5 (IP2) | 23 | BAK1, DFFA, NUMA1, PAK2, PARP1 |

on approximation of these problems. On the other hand, by means of reduction to the set cover problem, we showed that P1 and P2 can be approximated within a factor of $e/(e-1)$ and $O(\log n)$, respectively.

We proposed integer programming-based methods IP1 and IP2 for solving practical instances of P1 and P2, respectively. We applied them to apoptosis pathway maps, and found that such proteins as TP53, BCL2 and BAX selected by our methods often correspond to hubs in the network. These proteins are also considered to play important biological roles. Furthermore, we applied our methods to artificial scale-free networks with up to 10,000 nodes, and we showed that our methods can compute optimal solutions for these networks in practical time.

Table 4: Elapsed time (sec.) of solving IP1 and IP2 for each n , L and K .

| n | L | K | IP1 | IP2 |
|-------|-----|-----|-----------|------------|
| 1000 | 1 | 1 | 0.0147972 | 0.00932519 |
| 1000 | 3 | 5 | 0.904964 | 0.0526494 |
| 5000 | 1 | 1 | 0.102972 | 0.0485728 |
| 5000 | 3 | 5 | 2.90922 | 0.841976 |
| 10000 | 1 | 1 | 0.276991 | 0.101553 |
| 10000 | 3 | 5 | 5.62986 | 4.01971 |

Though we considered directed and unweighted networks in this paper, IP1 and IP2 can be modified for undirected and/or weighted networks. Furthermore, we can add various kinds of constraints to IP1 and IP2 because these are based on integer programming. Such a flexibility would be useful for modifying the proposed methods according to requirements from experimental biologists.

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

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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.)

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.

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MicroRNA (miRNA) is an endogenous, small, single-strand, noncoding 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

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

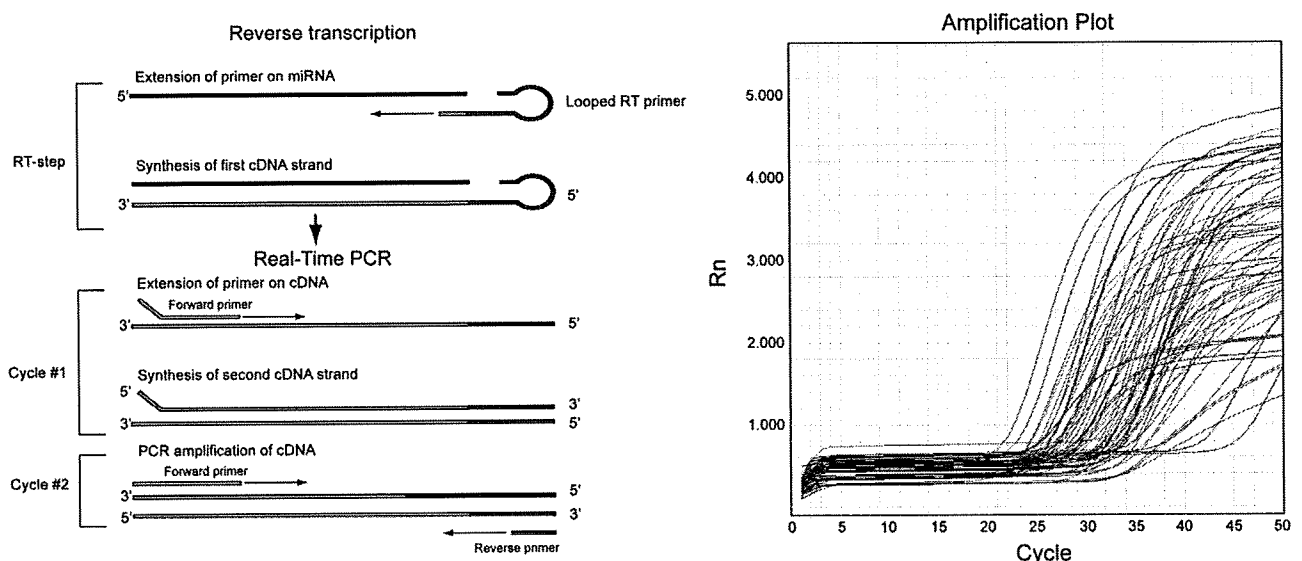


Fig. 1. (A) miRNA-specific RTD-PCR using sheet hairpin primers. (B) miRNA amplification curves by RTD-PCR.

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 \times 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 $-\Delta\text{Ct}$ 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

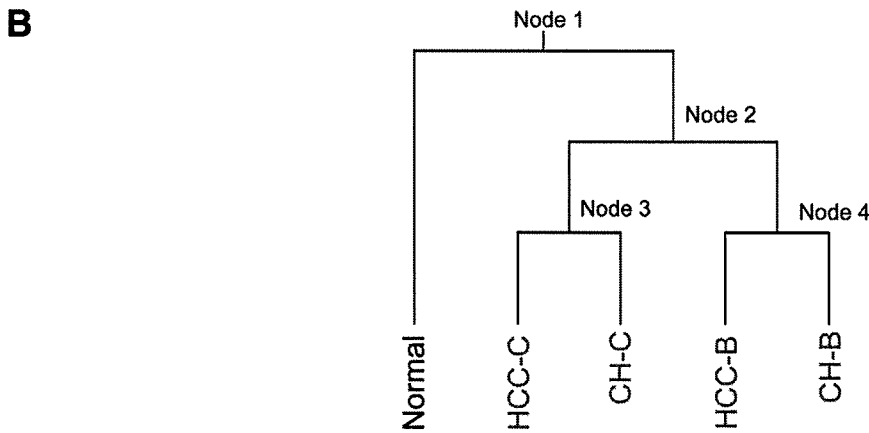
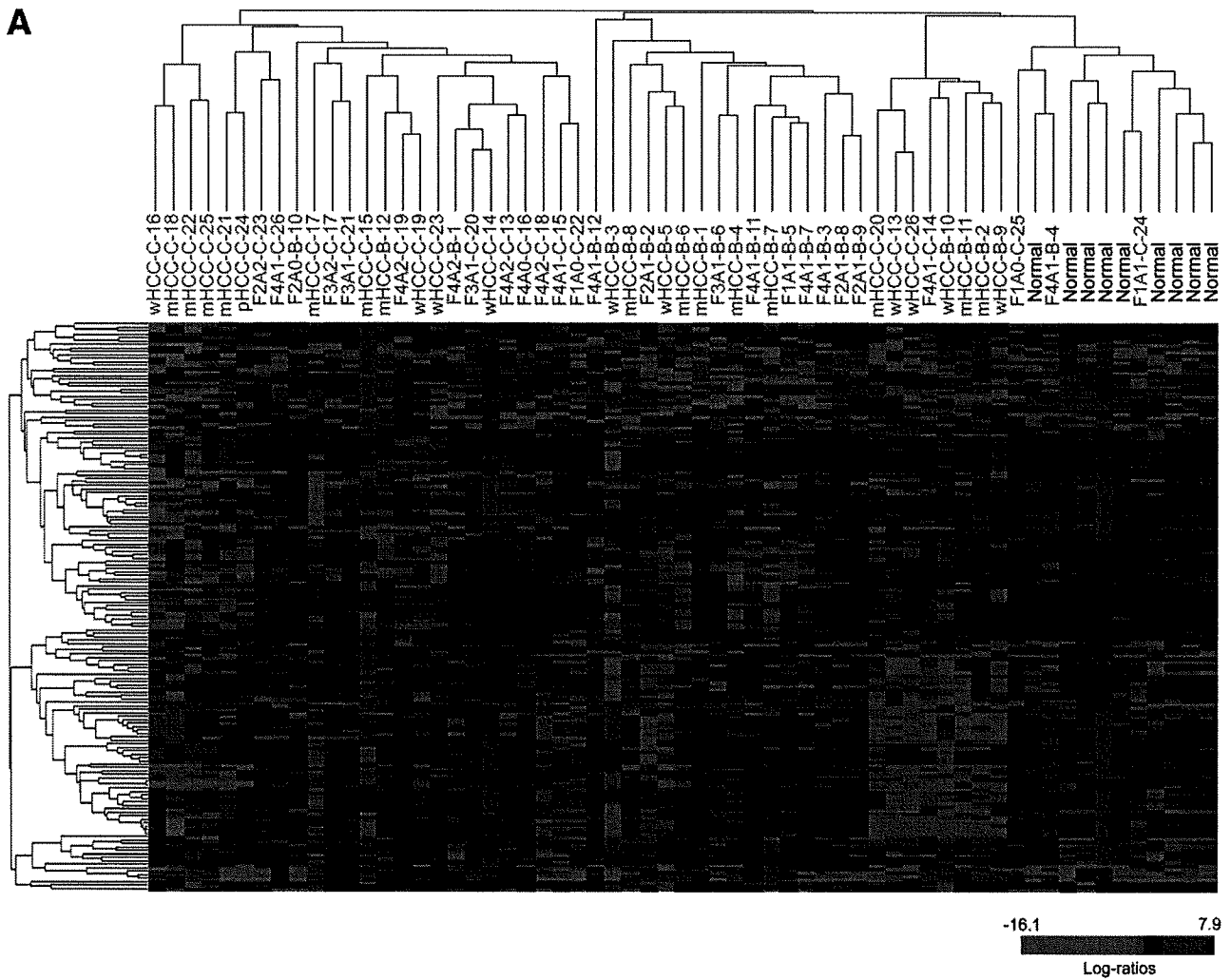
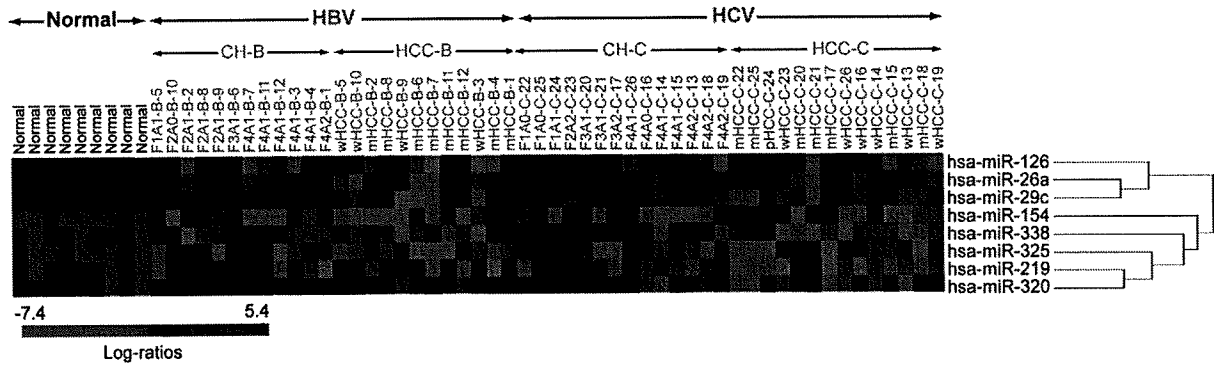
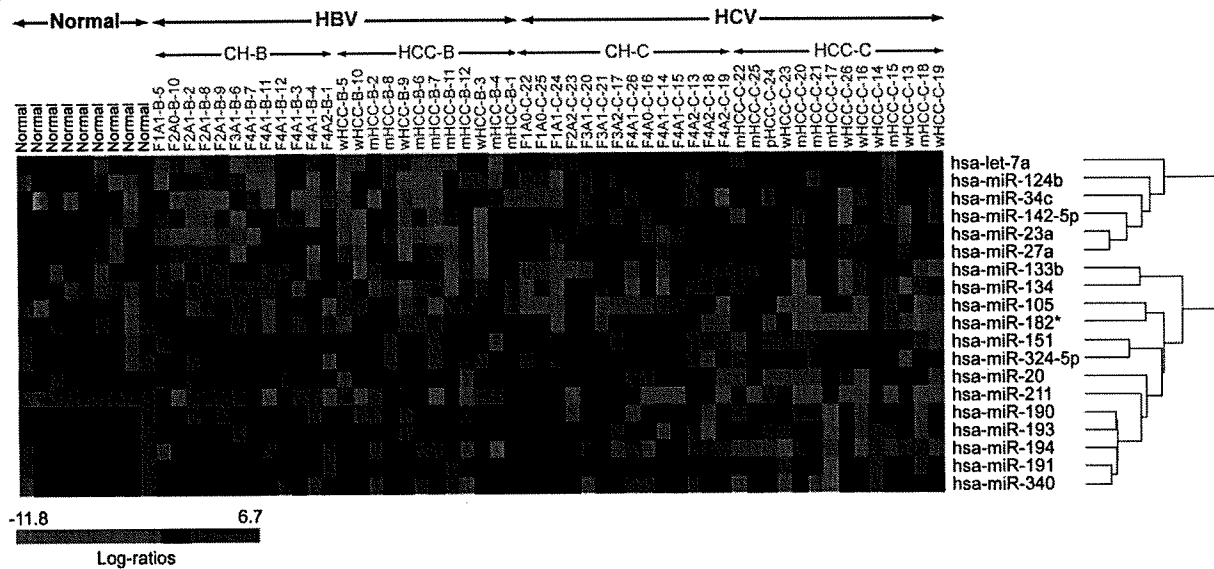


Fig. 2. (A) Hierarchical cluster analysis using total miRNA. Chronic hepatitis is indicated by histological stage and grade (F, fibrosis; A, activity) and type of infecting virus (B or C). HCC is indicated by histological grade (w, well differentiated; m, moderately differentiated; p, poorly differentiated) 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).

Cluster 1



Cluster 2



Cluster 3

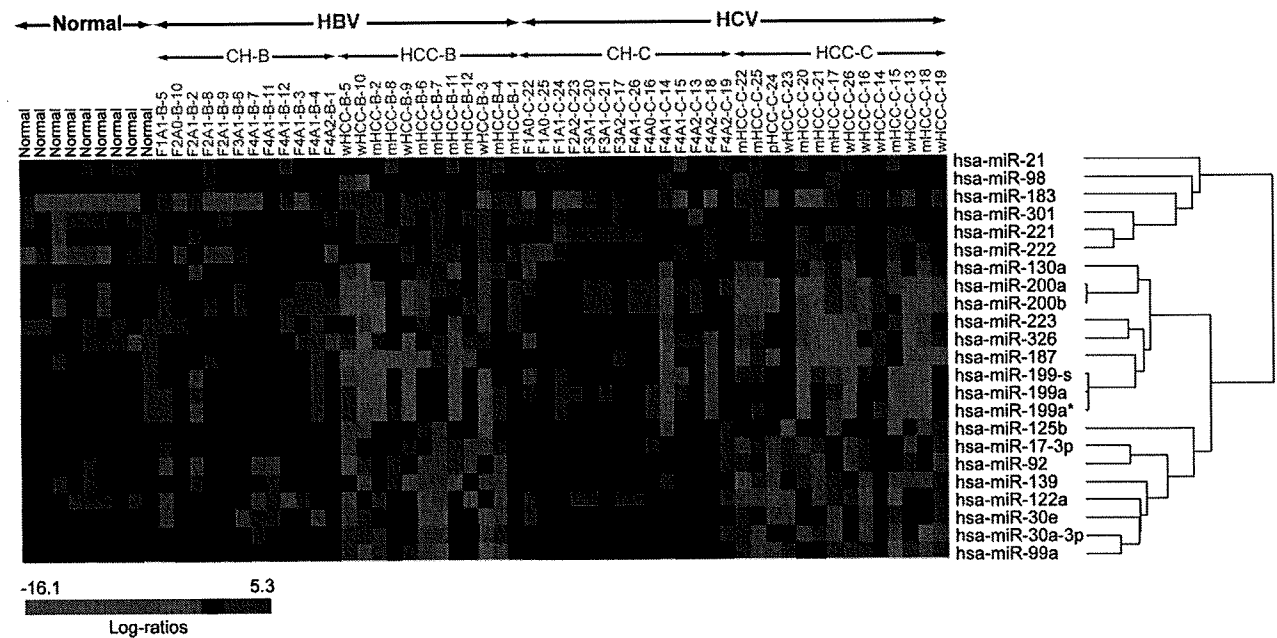


Fig. 3. Cluster 1: Eight miRNAs specifically differentiated node 1 classification. Cluster 2: Nineteen miRNAs specifically differentiated node 2 classification. Cluster 3: Twenty-three miRNAs differentiated CH-B and HCC-B as well as CH-C and HCC-C.

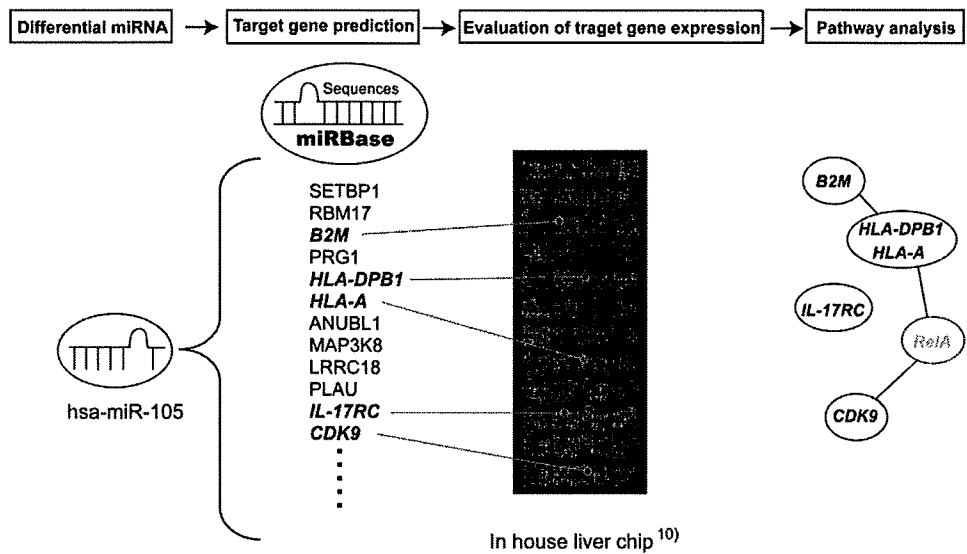


Fig. 4. Analysis of miRNA expression data. Target genes of miRNAs were predicted using MIRANDA Pro3.0; candidate target genes spotted on microarray were identified; number of genes that actually exhibit significant ($P < 0.05$) changes in expression among the genes was determined; and signal pathways 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 test¹² (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 classification 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 ACOX1 | Regulatory T cell differentiation Fatty acid beta-oxidation |
| hsa-miR-320 | 9.8E-05 | 0.50 | 26/88 | 3.50E-06 | NF-X1 Vimentin, ALP (N-acetyltransferase-like), SEC61 beta, G-protein alpha-i2, Filamin A | MHC class II biosynthetic process Protein kinase cascade |
| hsa-miR-154 | 2.7E-04 | 0.15 | 22/70 | 5.40E-06 | Rac1, RhoG Vinexin beta, Profilin 1, Ca-ATPase3 OTR, NET1(TSPAN1), NAP1, Vimentin, PDIA3, cytochrome P-450 reductase | Organelle organization and biogenesis Actin cytoskeleton organization and biogenesis Regulation of apoptosis |
| hsa-miR-29c | 1.8E-03 | 0.55 | 53/133 | 1.00E-06 | DLX2 GUAC, ACAT1 | Morphogenesis Branched chain family amino acid catabolic process |
| hsa-miR-338 | 5.2E-03 | 0.46 | 30/101 | 3.60E-06 | FBX07, ASPP1, HSPA4, Cathepsin O, PDF, COL4A1, HSPA4, TIP30, CXADR NS1-BP, ALP (N-acetyltransferase-like), ACTR10, Beclin 1 SMAD6, LTBR(TNFRSF3), ENPP7 | Cell-substrate adhesion Transcription, DNA-dependent Apoptosis |
| hsa-miR-26a | 6.3E-03 | 0.70 | 37/119 | 2.64E-05 | ID3, GATA-4, NFIA, FR-beta, CREST, HYOU1 G3ST1, CAD, FKBP12, LZIP, PDIA3, Schwannomin (NF2), CREST | Developmental process Immune effector process Immune system process |
| hsa-miR-126 | 8.1E-03 | 0.65 | 27/101 | 4.04E-03 | LIG4, c-FLIP, GADD45 beta, DAPK1, PRDX4, LRP130 Cyclin E, ZDHHC6, Tx1, ATG8 (GATE-16), WASP, C1s COPG1 | Response to stimulus DNA replication initiation Ion transport |
| hsa-miR-325 | 8.7E-03 | 0.20 | 18/63 | 2.03E-04 | ANP32B (april), HSPA4, RLI, LIV-1 (SLC39A6), PTP-MEG2, CD97, DHPR NFKBIA, NMI, MDH1, PDCC2 SMAD6, ATP6AP2, ANP32B (april), NMI, HSPA4 | Regulation of cellular protein metabolic process Response to stress Apoptosis |
| | | | | | TRADD, CREST, NEDD8, annexin IV, GPX2, PDF, TNFAIP1 Glypican-3, ID1, PC-TP, SNRNP (Sm-B) | Developmental process Multicellular organismal development 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 \pm 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 Expressed 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 |
| hsa-miR-134 | 2.3E-04 | 5.74 | 11/58 | 3.40E-06 | AP1S2, RNASE4 PPP2R1B, ARHGAP15, UBPY VKDGC, SH2B, MALS-1, DDB2 BCRP1 DDB2 | Mitotic cell cycle Negative regulation of apoptosis Multicellular organismal process Regulation of viral reproduction Lipid biosynthetic process |
| hsa-miR-151 | 2.8E-04 | 1.82 | 12/62 | 6.41E-01 | RGS2, UFO, AK2, USP7 eIF4G2, USP7 SLC22A7 | G-protein signaling Regulation of translation Organic anion transport |
| hsa-miR-193 | 5.0E-04 | 1.67 | 23/95 | 9.30E-01 | G-protein alpha-11, p130CAS, VAV-1, PDCD11 Colipase, ACSA DCOR | Cell motility Energy coupled proton transport Intracellular signaling cascade |
| hsa-miR-133b | 1.7E-03 | 2.42 | 20/97 | 3.69E-02 | DDB2, Bcl-3, Cystatin B Rab-3, RAG1AP1, KCNH2, DCOR AL1B1 | Proteasomal protein catabolic process Regulation of biological quality Carbohydrate metabolic process |
| hsa-miR-324-5p | 2.9E-03 | 1.51 | 27/121 | 1.90E-06 | SKAP55, VAV-1, DDB2, E2A, NIP1 MEMO (CGI-27), Rab-3 COPG1, GPX3, OAZ2 | Cellular developmental process Cellular structure morphogenesis Glutathione metabolic process |
| hsa-miR-182* | 3.1E-03 | 2.23 | 28/123 | < 1e-07 | Alpha-endosulfine, HCCR-2, Thioredoxin-like 2, TPT1, USP7 DDB2, TPT1 JIP-1 | Translation initiation in response to stress Cellular developmental process JNK cascade |
| hsa-miR-105 | 4.6E-03 | 4.38 | 18/68 | 4.74E-05 | Beta-2-microglobulin, HLA-B27 PIMT, IL-17RC MHC class I, CDK9, ERG1, Desmocollin 3 PSMD5, SLC26A6 | Antigen processing and presentation Immune response Proteasomal protein catabolic process |
| hsa-miR-211 | 5.3E-03 | 25.61 | 10/56 | 2.00E-04 | Noelin, SC4MOL, Thioredoxin-like 2, CCL5, NALP3 Hic-5/ARA55, USP16, MAP4, Ferroportin 1 | Regulation of apoptosis Positive regulation of cellular process |
| hsa-miR-20 | 5.7E-03 | 1.52 | 27/113 | 5.28E-03 | TOP3A, PLRP1 CDK9, GPS2, CLTA, LXR-alpha ACSA UGCGL1, SGPP1 | Oxygen transport Nucleic acid metabolic process Acetyl-CoA biosynthetic process Metal ion transport |
| hsa-miR-191 | 6.7E-03 | 1.39 | 25/79 | 7.55E-04 | FKBP12, DCOR, Gelsolin, VAV-1, ARF6 | Calcium ion transport Actin cytoskeleton organization and biogenesis |
| hsa-miR-340 | 8.5E-03 | 1.48 | 17/81 | 3.73E-03 | HXK3 Cyclin B1, Serglycin PTE2 SLC7A6 | Glucose catabolic process M phase of mitotic cell cycle Acyl-CoA metabolic process Carbohydrate utilization |
| hsa-miR-194 | 8.7E-03 | 1.67 | 13/74 | 5.90E-01 | RGL2, MANR, MEK1 (MAP2K1), Caspase-3, AZGP1 FRK, Pyk2(FAK2), CSE1L AZGP1 | Protein kinase cascade Cellular developmental process Defense response |
| hsa-miR-23a | 1.9E-04 | 0.46 | 14/97 | < 1e-07 | Sirtuin4, PAI2, PSAT, RIL, CDC34, SPRY1 E4BP4, DNAJC12, WWP1, PAIP1, PASK, rBAT VCAM1, CaMK I, WWP1, FHL3 | Metabotropic glutamate receptor Regulation of gene expression Cell-matrix adhesion |
| hsa-miR-142-5p | 4.9E-04 | 0.40 | 25/89 | 9.10E-06 | Diacylglycerol kinase, zeta, PLC-delta 1, ATP2C1, PAI2 MLK3(MAP3K11), MEK1(MAP2K1), CDC25C, MRF-1, XPC GNT-IV | Manganese ion transport Protein kinase cascade Inflammatory cell apoptosis |
| hsa-miR-34c | 5.1E-04 | 0.20 | 31/129 | 7.30E-06 | | |

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 MLK3(MAP3K11), RGS1 COL16A1 | Muscle development Intracellular signaling cascade MAPKKK cascade |
| hsa-let-7a | 1.0E-03 | 0.45 | 28/136 | 9.35E-04 | RAD51C, CoAA, hASH1, Cockayne syndrome B, Caspase-1, PP5 PLC-delta 1, MANR, ACADVL HGF, NGF | Response to DNA damage stimulus Fibroblast proliferation Cellular developmental process |
| hsa-miR-27a | 3.9E-03 | 0.59 | 18/108 | 1.19E-02 | COL16A1, RIL, RhoGDI gamma, ANP32B (april) VE-cadherin, NTH1, GATA-2, E4BP4 RAD51C | Cytoskeleton organization and biogenesis Response to external stimulus 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 Expressed miRNA Between CH and HCC and Their Representative Target Genes (Cluster 3)

| miRNA | Parametric p-value | Ratio* | No. of Significant Genes/Predicted Target Genes† | Hottelling 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 RBCK1, SYHH | Mitotic cell cycle Protein catabolic process |
| hsa-miR-30a-3p | 2.50E-05 | 0.49 | 26/144 | 1.73E-02 | ILK, IGFBP7, SAFB, CTR9 GGH, Pirin, ZNF207, Annexin VII ILK, LTA4H, ABC50, GNPAT DLC1 | Response to external stimulus Regulation of oxidoreductase activity Cell-matrix adhesion Morphogenesis |
| hsa-miR-130a | 7.00E-05 | 0.50 | 22/108 | 1.07E-02 | SPHM, PPP2R5D, RHEB2, SPHM MLK3(MAP3K11), Otubain1, TIMP4 NRBP | Mitotic cell cycle Protein modification process Cell differentiation |
| hsa-miR-223 | 3.40E-04 | 0.39 | 14/90 | 6.52E-03 | Ephrin-A1, Midkine, FDPS K(+) channel, subfamily J | Cell morphogenesis Notch signaling pathway |
| hsa-miR-187 | 3.55E-04 | 0.12 | 16/66 | 6.76E-04 | HFE2, Otubain1 | Negative regulation of programmed cell death |
| hsa-miR-200a | 6.86E-04 | 0.18 | 20/141 | 2.15E-02 | PRSS11, SUPT5H, RAG1AP1 PLOC3 CDC25B, KAP3, CDK2AP2, CHKA POLD CPSF4 | Developmental process Mitochondrial ornithine transport Cell communication DNA replication RNA splicing |
| hsa-miR-17-3p | 8.42E-04 | 0.58 | 28/108 | 8.98E-04 | MLK3(MAP3K11), Tip60, ACBD6, DOC-1R, DAX1, RBCK1 WNT5A, 14-3-3 gamma, DHX15 HFE2, MCM5 | Protein kinase cascade BMP signaling pathway DNA recombination |
| hsa-miR-99a | 1.17E-03 | 0.53 | 33/163 | 9.52E-03 | Calpain small subunit, Thoredoxin-like 2, Survivin IBP2, DNA-PK, KAP3, NFE2L1, PARP-1, HDAC11 | Cytokinesis Intracellular signaling cascade Regulatory T cell differentiation |
| hsa-miR-200b | 1.57E-03 | 0.18 | 24/147 | 2.72E-02 | HSP47, HMG2, NRBP SNX17 Ephrin-A1 | Regulation of cell cycle Cell motility Receptor protein signaling pathway |
| hsa-miR-125b | 1.82E-03 | 0.55 | 26/114 | 1.03E-01 | COL4A2, TIP30, HSP47, MSP58 MLK3(MAP3K11), ERK2 (MAPK1), ERK1 (MAPK3), PLOC3 Otubain1, SCN4A(SKM1) | Cell adhesion Nuclear translocation of MAPK Ubiquitin-dependent protein catabolic process Mitotic cell cycle |
| hsa-miR-30e | 2.10E-03 | 0.65 | 24/151 | 4.30E-02 | Cyclin B1, XTP3B, GAK, Annexin VII, MIC2, NRBP MSS4 S100A10 | Protein localization Calcium ion transport Mitotic cell cycle |
| hsa-miR-199a* | 4.26E-03 | 0.35 | 11/71 | 7.16E-02 | BUB3, Cyclin B1, LMNBR PRAME | Cardiac muscle cell differentiation Base-excision repair Translational initiation |
| hsa-miR-122a | 6.31E-03 | 0.51 | 11/80 | 1.01E-03 | JAB1, APEX, Clathrin heavy chain PARN DDAH2 | Regulation of cellular respiration Protein amino acid phosphorylation |
| hsa-miR-199a | 8.77E-03 | 0.35 | 18/94 | 3.56E-02 | IL-13, MLK3(MAP3K11), CLK2, ACP33 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 Thy-1, MCM6, Tip60, VILIP3 COMP, Cathepsin A | Regulation of programmed cell death Cell-matrix adhesion Blood vessel development |
| hsa-miR-92 | 9.60E-03 | 0.81 | 28/140 | 2.47E-02 | TUBGCP2, Fibrillin 1, PIPKI gamma, KAP3 SNX15, BCAT2 IGFBP7, FZD6, COPS6 | Rho protein signal transduction LDL receptor and BCAA metabolism 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 KYNLU, PMCA3 | Immune response-activating signal transduction 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 |
| hsa-miR-301 | 5.22E-05 | 1.96 | 14/71 | 1.16E-01 | PGAR KYNU Beta-2-microglobulin, PPCKM, PRC, Fra-1, PPCKM, ACAT2 | Triacylglycerol metabolic process DNA replication Antigen processing and presentation |
| hsa-miR-21 | 7.67E-03 | 1.57 | 19/81 | 1.86E-04 | BMPR1B, ARMER, EHM2, RBBP8 Neuromodulin, LDLR | Meiotic recombination Cell motility |
| hsa-miR-183 | 2.46E-02 | 3.51 | 13/86 | 3.36E-01 | Btk, Fra-1, MSH2, Collectrin, Adipophilin RNASE4, AGXT2L1 SARDH | Regulation of T cell proliferation Peptidyl-tyrosine phosphorylation Natural killer cell activation during immune response |
| hsa-miR-98 | 5.22E-02 | 1.32 | 24/130 | 2.95E-04 | Hdj-2, PEMT, Lck, MKP-5, Chondromodulin-I, ABCA8 IL-16, MTRR, SerRS ACAA2, LTB4DH, ACADVL, DECR, S14 protein, Rapsyn, Kallistatin, ENPEP, Beta crystallin B1 CYP4F8 | Cell differentiation Methionine biosynthetic process Fatty acid metabolic process Multicellular organismal process 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 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.

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, hierar-

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 |

chical 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 test¹² (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.

| | 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 |

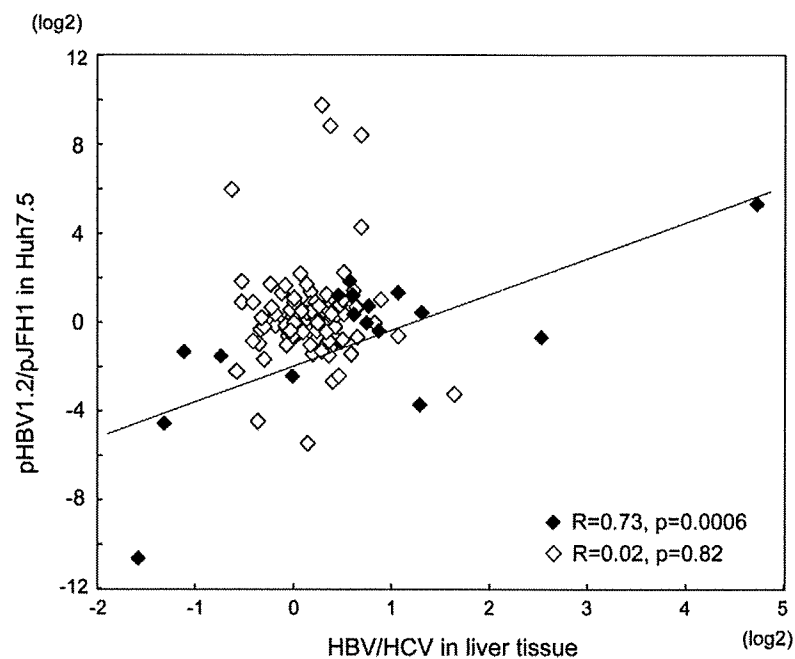


Fig. 5. Correlation between differences in miRNA expression between liver tissues infected with HBV and HCV and those in miRNA expression between cultured cell models of HBV and HCV infections. A total of 140 of 188 miRNAs were confirmed to be expressed in Huh7.5 cells. There was a significant correlation 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-

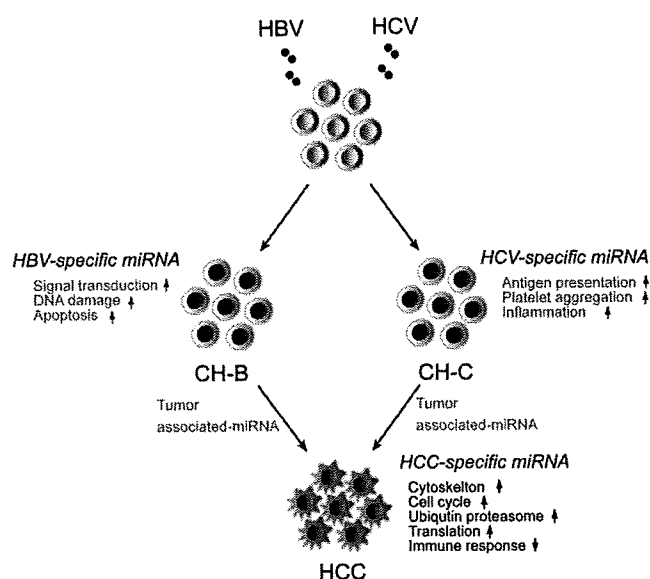


Fig. 6. Infection-associated and HCC-specific miRNAs and liver disease progression.

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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EpCAM-Positive Hepatocellular Carcinoma Cells Are Tumor-Initiating Cells With Stem/Progenitor Cell Features

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Background & Aims: Cancer progression/metastases and embryonic development share many properties including cellular plasticity, dynamic cell motility, and integral interaction with the microenvironment. We hypothesized that the heterogeneous nature of hepatocellular carcinoma (HCC), in part, may be owing to the presence of hepatic cancer cells with stem/progenitor features. **Methods:** Gene expression profiling and immunohistochemistry analyses were used to analyze 235 tumor specimens derived from 2 recently identified HCC subtypes (EpCAM⁺ α -fetoprotein [AFP⁺] HCC and EpCAM⁻ AFP⁻ HCC). These subtypes differed in their expression of AFP, a molecule produced in the developing embryo, and EpCAM, a cell surface hepatic stem cell marker. Fluorescence-activated cell sorting was used to isolate EpCAM⁺ HCC cells, which were tested for hepatic stem/progenitor cell properties. **Results:** Gene expression and pathway analyses revealed that the EpCAM⁺ AFP⁺ HCC subtype had features of hepatic stem/progenitor cells. Indeed, the fluorescence-activated cell sorting-isolated EpCAM⁺ HCC cells displayed hepatic cancer stem cell-like traits including the abilities to self-renew and differentiate. Moreover, these cells were capable of initiating highly invasive HCC in nonobese diabetic, severe combined immunodeficient mice. Activation of Wnt/ β -catenin signaling enriched the EpCAM⁺ cell population, whereas RNA interference-based blockage of EpCAM, a Wnt/ β -catenin signaling target, attenuated the activities of these cells. **Conclusions:** Taken together, our results suggest that HCC growth and invasiveness is dictated by a subset of EpCAM⁺ cells, opening a new avenue for HCC cancer cell eradication by targeting Wnt/ β -catenin signaling components such as EpCAM.

Tumors originate from normal cells as a result of accumulated genetic/epigenetic changes. Although considered monoclonal in origin, tumor cells are heterogeneous in their morphology, clinical behavior, and mo-

lecular profiles.^{1,2} Tumor cell heterogeneity has been explained previously by the clonal evolution model³; however, recent evidence has suggested that heterogeneity may be owing to derivation from endogenous stem/progenitor cells⁴ or de-differentiation of a transformed cell.⁵ This hypothesis supports an early proposal that cancers represent “blocked ontogeny”⁶ and a derivative that cancers are transformed stem cells.⁷ This renaissance of stem cells as targets of malignant transformation has led to realizations about the similarities between cancer cells and normal stem cells in their capacity to self-renew, produce heterogeneous progenies, and limitlessly divide.⁸ The cancer stem cell (CSC) (or tumor-initiating cell) concept is that a subset of cancer cells bear stem cell features that are indispensable for a tumor. Accumulating evidence suggests the involvement of CSCs in the perpetuation of various cancers including leukemia, breast cancer, brain cancer, prostate cancer, and colon cancer.⁹⁻¹³ Experimentally, putative CSCs have been isolated using cell surface markers specific for normal stem cells. Stem cell-like features of CSC have been confirmed by functional in vitro clonogenicity and in vivo tumorigenicity assays. For example, leukemia-initiating cells in nonobese diabetic, severe combined immunodeficient (NOD/SCID) mice are CD34⁺CD38⁻.¹¹ Breast cancer CSCs are CD44⁺CD24^{-/low} cells, whereas tumor-initiating cells of the brain, colon, and prostate are CD133⁺.^{10,12,13} CSCs are considered more metastatic and drug-/radiation-resistant than non-CSCs in the tumor, and are responsible for cancer relapse. These findings warrant the development of treatment strategies that can specifically eradicate CSCs.^{14,15}

Abbreviations used in this paper: AFP, α -fetoprotein; BIO, 6-bromoindirubin-3'-oxime; CSC, cancer stem cell; FACS, fluorescence-activated cell sorting; 5-FU, 5-fluorouracil; HpSC, hepatic stem cell; IF, immunofluorescence; IHC, immunohistochemistry; MACS, magnetic-activated cell sorting; MeBIO, 1-methyl-BIO; MH, mature hepatocyte; PCNA, proliferating cell nuclear antigen; siRNA, small interfering RNA.

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