Association of MicroRNA Expression in Hepatocellular Carcinomas with Hepatitis Infection, Cirrhosis, and Patient Survival

Jinmai Jiang[1], **Yuriy Gusev**[2], **Ileana Aderca**[3], **Teresa A. Mettler**[3], **David M. Nagorney**[3], **Daniel J. Brackett**[2], **Lewis R. Roberts**[3], and **Thomas D. Schmittgen**[1]

[1]College of Pharmacy, Ohio State University, Columbus, Ohio

[2]Department of Surgery, University of Oklahoma Health Sciences Center and Veterans Affairs Medical Center, Oklahoma City, Oklahoma

[3]Divisions of Gastroenterology and Hepatology and Gastroenterological Surgery, Mayo Clinic College of Medicine, Rochester, Minnesota

Abstract

Purpose—MicroRNA (miRNA) is a new class of small, noncoding RNA. The purpose of this study was to determine if miRNAs are differentially expressed in hepatocellular carcinoma (HCC).

Experimental Design—More than 200 precursor and mature miRNAs were profiled by real-time PCR in 43 and 28 pairs of HCC and adjacent benign liver, respectively, and in normal liver specimens.

Results—Several miRNAs including miR-199a, miR-21, and miR-301 were differentially expressed in the tumor compared with adjacent benign liver. A large number of mature and precursor miRNAs were up-regulated in the adjacent benign liver specimens that were both cirrhotic and hepatitis-positive compared with the uninfected, noncirrhotic specimens ($P < 0.01$). Interestingly, all of the miRNAs in this comparison had increased expression and none were decreased. The expression of 95 randomly selected mRNAs was not significantly altered in the cirrhotic and hepatitis-positive specimens, suggesting a preferential increase in the transcription of miRNA. Comparing the miRNA expression in the HCC tumors with patient's survival time revealed two groups of patients; those with predominantly lower miRNA expression and poor survival and those with predominantly higher miRNA expression and good survival ($P < 0.05$). A set of 19 miRNAs significantly correlated with disease outcome. A number of biological processes including cell division, mitosis, and G_1-S transition were predicted to be targets of the 19 miRNAs in this group.

Conclusion—We show that a global increase in the transcription of miRNA genes occurs in cirrhotic and hepatitis-positive livers and that miRNA expression may prognosticate disease outcome in HCC.

Hepatocellular carcinoma (HCC) is the third most common cause of death from cancer worldwide, with >600,000 cases in 2002 (1). The major etiologies of HCC include chronic liver disease due to chronic hepatitis B or hepatitis C virus infection, metabolic causes such as alcoholic or nonalcoholic steatohepatitis, hereditary hemochromatosis, and α-1-antitrypsin deficiency, or immune-related causes such as primary biliary cirrhosis and autoimmune

Requests for reprints: Thomas D. Schmittgen, College of Pharmacy, Ohio State University, 500 West 12th Avenue, Columbus, OH 43210. Phone: 614-292-3456; Fax: 614-292-7766; E-mail: schmittgen.2@osu.edu.

hepatitis. In parts of Asia and sub-Saharan Africa, dietary fungal aflatoxins have a synergistic effect with chronic hepatitis in the pathogenesis of HCC (2–5).

Chronic liver injury with associated inflammation leads to accelerated cycles of cell death, regeneration, and repair that ultimately lead to premature senescence of the liver. As the regenerative capacity of the liver becomes exhausted, aberrant repair processes in the context of ongoing inflammation result in the development of nodular regeneration, stromal expansion, and fibrosis, the end stage of which is called cirrhosis. Cirrhosis is a major risk factor for the development of HCC; individuals with cirrhosis have a 2% to 6% risk per year of developing HCC (6). Previous studies have identified a number of genetic and epigenetic alterations associated with cirrhosis, including allelic imbalance at multiple genetic loci, p53 mutations, promoter hypermethylation of the p16[INK4a] tumor suppressor gene, and telomere shortening with replicative senescence and associated chromosomal instability. The development of HCC is associated with the development of additional genetic and epigenetic alterations, coupled with telomerase activation and consequent cellular immortalization (7). Important molecules and pathways involved in hepato-carcinogenesis include cell cycle regulatory proteins such as p53, c-Myc, and cyclin D1, the Wnt/β-catenin signaling pathway, and multiple receptor tyrosine kinase growth factor ligands and receptors, including epidermal growth factor, fibroblast growth factor, hepatocyte growth factor, and vascular endothelial growth factor, which activate the mitogen-activated protein kinase and phosphoinositide-3-kinase/AKT kinase pathways (8,9).

MicroRNA (miRNA) are endogenously expressed, small interfering RNAs, discovered during studies of *Caenorhabditis elegans* development (10). miRNAs are transcribed as precursor molecules that are subsequently processed into the active ~21 nucleotide mature miRNA. The mature miRNA binds to the 3′ untranslated region of the target mRNA through imperfect base pairing, producing translational arrest and/or degradation of the mRNA. Conceptually, miRNAs regulate gene expression in a manner similar to transcription factors. Both miRNAs and transcription factors are trans-acting factors that bind to composite cis-regulatory elements that are "hard-wired" into RNA and DNA, respectively (11). Although putative roles for the vast majority of mammalian miRNAs remain unknown, miRNAs have been implicated in a diverse number of mammalian cellular processes including insulin secretion in the pancreas (12), differentiation of adipocytes (13), and regulation of embryonic stem cell development (14).

A growing number of both direct and indirect evidence suggests a relationship between differential miRNA expression and cancer. These include miR-15a and miR-16-1 in chronic lymphocytic leukemia (15,16), miR-143 and miR-145 in colorectal cancer (17), let-7 in lung cancer (18,19), and miR-155 in diffuse large B cell lymphoma (20). Expression profiling has identified other cancers with differential expression of miRNAs including breast cancer (21), papillary thyroid cancer (22), and glioblastoma (23,24). A polycistron encoding miRNAs miR-17, -18, -19a, -19b-1, and -92-1 is amplified in human B-cell lymphomas and forced expression of the polycistron along with c-myc was tumorigenic, suggesting that this group of miRNAs may function as oncogenes (25).

The purpose of this study was to profile the expression of miRNAs in clinical specimens of HCC, adjacent benign tissue, and in liver specimens from nondiseased livers and to compare the miRNA expression profiles among the patients with HCC including those with cirrhosis and hepatitis infection.

Materials and Methods

Tissue procurement and RNA isolation

Fifty-four pairs of HCC tumors and adjacent benign liver were available for the study. For each case, tumor samples with matched adjacent benign tissue were collected during surgical resections at the Mayo Clinic between 1991 and 2001, frozen in liquid nitrogen, and stored at −80°C. The study was approved by the Mayo Clinic Institutional Review Board. Sections from each specimen were examined by a pathologist and graded histologically. Patients were classified for common etiology of HCC including hepatitis B or C, cirrhosis, and alcoholic liver disease. Patients who developed HCC in the context of a normal liver with no known risk factors were negative for cirrhosis, hepatitis B, and hepatitis C. Total RNA was isolated from the tissues using Trizol reagent (Invitrogen). Some of the total RNA specimens were further purified using a Qiagen midi column (Qiagen). Four normal liver samples were purchased from Ambion as part of their First Choice total RNA normal human tissue. These patients died from complications other than liver disease. Eight additional normal liver tissues were received from Dr. Snorri S. Thorgeirsson, National Cancer Institute. Clinical data on the patient specimens are included in Table 1 and Supplemental Table S1. The somewhat higher percentage of patients with no known risk factors in our study is likely due to a combination of factors. First, a number of studies from the United States have documented a relatively high percentage (15–50%) of HCCs that have no known risk factors, this is typical of the population of HCC patients that are seen in the Upper Midwest (26,27). Secondly, patients with cirrhosis are less likely to be candidates for surgical resection. Studies of resected tissues therefore tend to have a higher percentage of samples from patients without cirrhosis and without known risk factors for HCC.

Real-time PCR for miRNA expression

RNA was treated with RNase-free DNase I and cDNA was synthesized from 600 ng of total RNA using gene-specific primers to 182 miRNA precursors and U6 RNA as previously described (28,29). The expression of the miRNA precursors was determined using real-time quantitative PCR assay, as described with the exception that 35 cycles of PCR was used rather than 40 cycles (28,29). TaqMan miRNA Assays (Applied Biosystems) were used to quantify mature miRNA. cDNA was synthesized by priming with 196 different gene-specific looped primers and the reverse primer for 18S rRNA as previously described (30). All real-time PCR data was analyzed using the comparative C_T method, data were multiplied by 10^6 to simplify presentation.

Real-time PCR for mRNA expression

Real-time PCR to measure the mRNA expression of 95 genes was done using SYBR green detection and standard techniques as mentioned above. cDNA was synthesized on total RNA using random primers. PCR primers to the 95 genes were collected from our laboratory archives or those of colleagues or were randomly selected from the PrimerBank database (31). Gene expression was presented relative to 18S rRNA.

Statistics

Differences between the various groups (benign/tumor, cirrhosis/no cirrhosis, virus/nonvirus) were determined using the Student's t test. The Survival Risk Group Prediction algorithm (PAM software package) was used to develop a miRNA expression–based predictor of survival risk groups. The survival risk groups were constructed using the supervised principal component method from ref. (32). This method uses a Cox proportional hazards model to relate survival time to k "supergene" expression levels, where k is selectable by the user (usually 1–3). The supergene expression levels are the first k principal component, i.e., linear combinations of expression levels of the subset of genes that are univariately correlated with survival. In our

analysis, we used the first three principal components. The significance of each gene was measured based on a univariate Cox proportional hazards regression of survival time versus the log expression level for the gene. After selecting the genes, the principal component was computed, and the k variable Cox proportional hazard regression analysis was performed. This provides a regression coefficient (weight) for each principal component. This method provides a prognostic index for a patient with a log expression profile given by a set of miRNA expression data. A high value of the prognostic index corresponds to a high value of hazard of death, and consequently, a relatively poor predicted survival. Unsupervised hierarchical cluster analysis was done for samples and genes using mean centered miRNA expression data, average linkage, and uncentered Pearson correlation as a distance.

Computational prediction of potential miRNA targets

A list of predicted targets was generated for the group of 19 coexpressed miRNAs that were identified by PAM survival analysis as strongly associated with survival of patients with HCC. A combinatorial target prediction algorithm was applied (miRgate 2.2 software suite, Actigenics/Cepheid Europe). Initially, a list of all predicted target genes which are targeted by any miRNA from that group, was generated. Secondly, this list of potential targets was analyzed using gene ontology (GO) enrichment analysis according to the total number of miRNAs that were targeting the same GO categories in order to determine the biological processes and functions that were most likely to be affected by a group of miRNAs. A short list of the three top GO categories, which are targeted by at least 80% of the miRNAs from the group, was selected. A list of 84 target genes from those top three categories was further analyzed using the Ingenuity Pathway Analysis system (IPA 5.0, Ingenuity Systems). This method of miRNA combinatorial target analysis has been described in details elsewhere (33).

Results

miRNA expression in cirrhotic and hepatitis-positive tissues

The expression of 182 miRNA precursors was determined in 43 specimens of HCC, 43 adjacent benign tissues, and in 12 normal liver tissues using real-time PCR. The raw data from the expression profiling is included in Supplemental Table S2. About half of the HCC patients had underlying cirrhosis and one third was infected with hepatitis B and/or hepatitis C (Table 1). To determine the contribution of hepatitis infection or cirrhosis to miRNA precursor expression, the data from the adjacent benign tissues was classified into the following four categories: V^-C^-, V^-C^+, V^+C^-, and V^+C^+, where V represents viral hepatitis infection (hepatitis B or C infection) and C represents cirrhosis. The number of patients in each of the four categories was: V^-C^-, 17 patients; V^-C^+, 10 patients; V^+C^-, 4 patients; and V^+C^+, 12 patients.

Comparing the miRNA precursor expression in the V^-C^- and V^+C^+ groups yielded the largest number of differentially expressed miRNAs, with 50 miRNA precursors differentially expressed in the benign tissue of patients with hepatitis infection and cirrhosis compared with those patients with histologically normal livers (Supplemental Table S3; Fig. 1A). The expression of all 50 of the miRNA precursors is increased in the V^+C^+ group ($\geq$2-fold, $P < 0.01$) and none had reduced expression. When the V^+C^+ benign tissues were compared with normal liver in patients without HCC, 20 miRNA precursors had increased expressions ($\geq$2-fold, $P < 0.01$). Twelve of the 20 miRNAs were increased in the benign tissue comparison (Supplemental Table S3; Fig. 1). These data show that the expression of a large number of miRNA precursors was increased in patients with cirrhosis and concomitant hepatitis infection.

To determine if the increase in miRNA precursor expression was due to cirrhosis or viral hepatitis infection alone, we compared the V^-C^-/V^-C^+ and V^-C^-/V^+C^- groups (benign

tissues). In these comparisons, only one of the miRNA precursors was significantly changed ($\geq$2-fold, $P < 0.01$; Fig. 1A). Comparing the V^-C^+ or V^+C^- to normal liver yielded four and two significantly changed miRNAs, respectively (Fig. 1B). These data suggest that neither hepatitis viral infection nor cirrhosis alone is sufficient to induce major changes in miRNA expression, however the combination of both viral hepatitis infection and cirrhosis significantly enhances the expression of a large number of miRNA precursors.

To determine if the increase in miRNA precursor expression of the virally infected specimens had a preference for hepatitis B or hepatitis C, we compared the differences in the miRNA expression in the hepatitis B ($n = 3$) and hepatitis C ($n = 6$) samples (cirrhosis-positive group only). Comparing the miRNA expression between these groups yielded nine miRNA precursors (miR-145, -9-2, -138-1,2, -320, -33, -10a,b, -21, -146, and -220) that were differentially expressed (>2-fold increased expression; $P < 0.05$). For all nine miRNAs, expression in the hepatitis B–infected patients was greater than those infected with hepatitis C. These data suggest that hepatitis B is a greater contributor to the increase in expression compared with hepatitis C, although it must be noted that the sample size was small.

miRNA versus mRNA expression

Our data describe two rather unusual findings: (*a*) a large percentage of miRNA precursors are differentially expressed in liver tissues that were hepatitis-positive and cirrhotic, and (*b*) in each of these cases, the miRNA expression was increased, not a single statistically significant decrease in miRNA expression was observed. This observation could be explained by biological or technical factors. Possibilities include fundamental differences in the regulation of miRNAs compared with the regulation of mRNAs, or that the sensitive, real-time PCR used to profile miRNA gene expression biased the results in some way. To address the possibility that miRNA genes are expressed differently than mRNA in these tissues, the expression of 95 randomly selected mRNA genes was quantified in cDNA from the identical RNA used in the miRNA expression profiling study. The entire list of genes studied is presented in Supplemental Table S4. Only 3 of the 95 (3%) mRNAs were differentially expressed in the benign tissues, compared with 50 of the 182 (27%) miRNAs ($\geq$2-fold, $P < 0.01$; Fig. 1C). Of the differentially expressed mRNAs, two were up-regulated and one was down-regulated (Supplemental Table S4). These data suggest that transcription of miRNA genes was more predominantly increased compared with mRNA genes in liver tissues that are cirrhotic and infected with hepatitis virus.

Technical validation

The total RNA available in the archives of one of the authors (L.R. Roberts) was isolated with Trizol reagent followed by a clean-up using a Qiagen Midi column (Qiagen). Column purification has the potential of removing smaller RNAs. We determined that the expression of the 106 nucleotide U6 RNA were comparable in filtered and unfiltered RNA (data not shown). The miRNA precursor expression in RNA from normal liver purchased from Ambion (and not filtered) was comparable to that in the filtered, benign tissue (Supplemental Table S2).

The real-time PCR method that was used quantifies the miRNA precursors (28,29) and not the mature miRNA. We and others have shown that in most cases, miRNA precursor expression correlates with the mature miRNA expression (28–30,34–37). However, situations exist in which the precursor expression does not correlate with the mature miRNA expression (29). Although column filtration did not alter the miRNA precursor levels, column filtration did remove a large portion of the ~21-nucleotide mature miRNAs (data not shown). Because the mature miRNA is the active species, we wanted to validate the expression of the miRNA precursors. RNA was isolated by the Trizol procedure from liver tissues that were available in our archives; this included 13 of the 43 specimens listed in Table 1. Total RNA was isolated

from another 13 liver tissues that were not among the 43 profiled for miRNA precursor expression (patient data are presented in Supplemental Table S1). Ten miRNAs were selected from the 50 miRNA precursors that significantly differed among the V^-C^- and V^+C^+ liver tissues (Fig. 1A). The expression of these 10 miRNAs was measured in the 26 benign liver specimens using a real-time PCR assay for mature miRNA (38). Like the miRNA precursors, the mature miRNA expression was increased in the V^+C^+ specimens compared with the V^-C^- specimens, however, only 6 of the 10 comparisons were statistically significant (Fig. 2).

Even though there was good correlation between the expression of precursor and mature miRNA in these samples (Fig. 2), we wanted to profile several hundred mature miRNAs using a commercially available real-time PCR assay (38). RNA was isolated by the Trizol procedure from liver tissues that were available in our archives. This includes another 11 liver tissues that were not among the 43 profiled for miRNA precursor expression. In addition to these 11 patients, we were able to obtain unfiltered RNA from 17 of the original 43 patients. Therefore, mature miRNA was profiled on a total of 28 patients (patient data are presented in Supplemental Table S1). The number of patients in each of the four categories was: V^-C^-, 15 patients; V^-C^+, 4 patients; V^+C^-, 2 patients; and V^+C^+, 7 patients. Fourteen mature miRNAs were increased in the V^+C^+ compared with the V^-C^- group (benign samples, $P < 0.01$; Fig. 1D). Comparing the mature miRNA expression in the V^+C^+ livers to the normal livers showed that 61 mature miRNAs were increased (Fig. 1E). The small number of samples in the V^-C^+ and V^+C^- groups precluded us from analyzing these data. miR-181b and miR-214 were increased in all four comparisons (Fig. 1A, B, D, and E; Supplemental Table S4).

miRNA expression in HCC tissues

The expression of 196 mature miRNAs was compared in 28 specimens of HCC and adjacent benign tissues and in 6 normal liver tissues using real-time PCR. The raw data from the expression profiling is included in Supplemental Table S3. The mature miRNA expression in the HCC was compared with the adjacent benign or the normal liver tissue. Differentially expressed miRNA were defined as those having a 2-fold or greater change in gene expression and $P < 0.05$ (Student's t test). Sixteen miRNAs were differentially expressed when the tumor data was compared with adjacent benign tissue (Table 2). The mature miRNA expression data is presented as a heatmap (Supplemental Fig. S1). Hierarchical clustering yielded four clusters. Clusters 3 and 4 contained only tumor, cluster 1 contained only benign tissue, and cluster 2 contained all benign plus two tumors.

Prognostic benefit of miRNA signature in HCC

To determine if a relationship exists between miRNA expression and the patient's survival, additional analyses were done on the mature miRNA expression data from the tumors of patients with unfiltered RNA and for which the survival time was known (25 patients). A semisupervised analysis of survival risks was conducted using the survival risk prediction algorithm (PAM software package) and Kaplan-Meier estimates of disease-free survival. Unfiltered expression data for all genes and survival times were analyzed using the PAM program and showed two groups of patients with significantly different survival curves; group 1 had better survival rates and group 2 had poor survival rates ($P < 0.05$; Fig. 3A; Table 3). PAM analysis of correlation between miRNA expression and survival determined that a set of 19 genes significantly correlated with the outcome of disease ($P < 0.05$, Supplemental Table S5). These included let-7c, let-7g, miR-26b, -29c, -30e-3p, -31, -99a, -99b, -100, -125b, -139, -148a, -150, -200c, -220, -221, -345, -372, and -377. Hierarchical clustering of the expression data from these 19 genes showed that they correspond (except for two samples) with the patient survival groups; patients in group 1 (good survival rates) had overall higher mature miRNA expression levels compared with those in group 2 (poor survival rates; Fig. 3B).

Prediction of miRNA targets

miRNAs function by binding to conserved sequences within the 3′ untranslated regions of their respective, target mRNAs. Using a combinatorial target prediction algorithm and the gene ontology enrichment analysis, we determined biological processes categories (GO BP) that are targeted by multiple miRNAs from the group of 19 miRNAs which we found to be associated with patient survival. Three of these GO BP categories (cell division, mitosis, and G_1-S transition of mitotic cell cycle) are targeted by at least 80% of miRNAs from this group (Table 4). The top three categories which are most likely to be collectively affected by this group of miRNAs include a total of 84 predicted target genes (Supplemental Table S6), all of which are related to cell division and/or cell cycle regulation. Thirty-nine genes from this group of 84 were reported to be associated with several types of human cancers. Nine of these genes were reported to be associated with liver cancer (*ACVR1B*, *APC11*, *CCND1*, *CDC25A*, *CDKN3*, *HGF*, *PLK1*, *RAN*, and *TPX2*). Because miRNA negatively regulates translation, the expression of these 19 miRNAs could regulate the protein levels of these cell cycle–related targets, for example, by producing reduced protein expression in the good survivors or increased protein levels in the poor survivors. A list of relevant biological functions and diseases that are known to be associated with the 84 genes from Supplemental Table S6 are presented in Supplemental Table S7.

Discussion

We report the results of PCR-based, miRNA expression profiling study in liver tissues. Our data show that a large number of miRNAs have increased expression in the hepatitis-positive and cirrhotic specimens compared with the liver tissues that developed HCC in the context of a histologically normal liver or in normal liver not associated with HCCs. More than 20 miRNA precursors were differentially expressed in the cirrhotic and hepatitis-positive tissues (Fig. 1; Supplemental Table S3).

Surprisingly, the expression of all of these miRNAs was increased in the hepatitis-positive, cirrhotic tissues—and not a single miRNA was reduced to a statistically significant degree. A recent study in human lymphoblastoid cells reports a global increase in miRNA expression in response to stress induced by folate deficiency (39). Many of the miRNAs increased in response to cellular stress (e.g., miR-22, miR-182b, miR-198, miR-221, and miR-222) reported in ref. (39), were increased in cirrhotic and hepatitis-positive livers (Supplemental Table S3), suggesting that increased transcription of certain miRNAs may be a general response to stress induced by a combination of hepatitis and cirrhosis.

Other interesting observations from our study is that the increased miRNA expression in cirrhotic and hepatitis-positive liver specimens is modest in magnitude (2- to 3-fold), consistent across most samples, and highly statistically significant (Supplemental Table S3). The 2- to 3-fold global increase in miRNA expression due to cellular stress from folate deficiency (39) is comparable in magnitude to that reported here. The ability to distinguish modest changes in gene expression was likely facilitated by the fact that we employed sensitive, real-time PCR to profile the miRNA expression. The biological significance of a 2- to 3-fold change in miRNA expression is unknown. Because one miRNA may regulate scores of target genes and one mRNA may be regulated by multiple miRNAs (40), a 2- to 3-fold change in the expression of multiple miRNAs may profoundly alter the regulation of downstream genes.

The only other study to profile miRNA expression in HCC, to our knowledge, is that of Murakami et al. (41). Of the seven miRNAs reported as differentially expressed in HCC by Murakami et al. (41), three were differentially expressed in our study (Mir-199a, Mir-199a*, and Mir-18; Table 2). The number of differentially expressed miRNAs in the entire group of HCC specimens reported in Table 2 is relatively small compared with the large number of

differentially expressed miRNAs identified by expression profiling in other cancers (15,21, 24,37,42–44). However, three of the miRNAs with increased expression here were increased in other solid tumors including Mir-21 in glioblastoma (23), breast cancer (21) and pancreas cancer (30); Mir-221 in pancreas (30,45) and thyroid cancer (22) and Mir-301 in pancreas cancer (30).

Comparing the miRNA expression within the patients with HCC, two different groups of patients emerged: those with reduced miRNA expression and poor survival and those with increased miRNA expression and good survival (Fig. 3; Supplemental Fig. S1). A set of 19 miRNA genes significantly correlated with disease outcome (Fig. 3; Supplemental Table S5). Many of the predicted targets of these miRNAs regulate cell division, mitosis, and G_1-S transition (Table 4). In addition to these theoretical issues, it may be possible to prognosticate patients with HCC based on their miRNA expression, similar to what was done for patients with lung cancer (19,37). Additional testing of the prognostic value of this miRNA expression signature needs to be done on an independent set of samples.

In summary, we have identified a number of miRNAs that are differentially expressed in HCC. Our data also suggest that important changes in miRNA expression occur during the development of chronic viral hepatitis and cirrhosis, and that the combination of viral hepatitis and cirrhosis is significantly more likely to result in changes in miRNA expression. Subsequent changes in miRNA expression in the transition from cirrhosis to HCC are much less marked. It is possible that most of the miRNA changes that occur during carcinogenesis occur early, so that by the stage of replicative senescence, characteristic of cirrhosis, the key miRNA changes that may predispose to carcinogenesis have already occurred. These miRNA changes may reflect and/or mediate a preneoplastic "field effect" in individuals with cirrhosis and hepatitis who are at an increased risk of the development of HCC. Studies of mRNA gene expression analysis during the process of liver carcinogenesis show significant changes in mRNA expression, both in the process of development of cirrhosis and in the transition from cirrhosis to HCC. The results of our miRNA study are in contrast with this and may suggest fundamental differences in the regulation of miRNAs and mRNAs in carcinogenesis that are worthy of further study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References

1. Ferlay, J.; Bray, F.; Pisani, P., et al. IARC Cancer Base no. 5. version 2.0. IARC Press; 2002. GLOBOCAN 2002: cancer incidence, mortality and prevalence world-wide.

2. Bosch FX, Ribes J, Borras J. Epidemiology of primary liver cancer. Semin Liver Dis 1999;19:271–285. [PubMed: 10518307]

3. Jackson PE, Groopman JD. Aflatoxin and liver cancer. Baillieres Best Pract Res Clin Gastroenterol 1999;13:545–555. [PubMed: 10654919]

4. Bruix J, Llovet JM. Hepatitis B virus and hepato-cellular carcinoma. J Hepatol 2003;39:S59–S63. [PubMed: 14708679]

5. Bruix J, Boix L, Sala M, et al. Focus on hepatocellular carcinoma. Cancer Cell 2004;5:215–219. [PubMed: 15050913]

6. Okuda H. Hepatocellular carcinoma development in cirrhosis. Best Pract Res Clin Gastroenterol 2007;21:161–173. [PubMed: 17223503]

7. Herath NI, Leggett BA, MacDonald GA. Review of genetic and epigenetic alterations in hepato-carcinogenesis. J Gastroenterol Hepatol 2006;21:15–21. [PubMed: 16706806]

8. Roberts LR, Gores GJ. Hepatocellular carcinoma: molecular pathways and new therapeutic targets. Semin Liver Dis 2005;25:212–225. [PubMed: 15918149]

9. Lee JS, Thorgeirsson SS. Comparative and integrative functional genomics of HCC. Oncogene 2006;25:3801–3809. [PubMed: 16799621]

10. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004;116:281–297. [PubMed: 14744438]

11. Hobert O. Common logic of transcription factor and microRNA action. Trends Biochem Sci 2004;29:462–468. [PubMed: 15337119]

12. Poy MN, Eliasson L, Krutzfeldt J, et al. A pancreatic islet-specific microRNA regulates insulin secretion. Nature 2004;432:226–230. [PubMed: 15538371]

13. Esau C, Kang X, Peralta E, et al. MicroRNA-143 regulates adipocyte differentiation. J Biol Chem 2004;279:52361–52365. [PubMed: 15504739]

14. Houbaviy HB, Murray MF, Sharp PA. Embryonic stem cell-specific microRNAs. Dev Cell 2003;5:351–358. [PubMed: 12919684]

15. Colin GA, Liu CG, Sevignani C, et al. MicroRNA profiling reveals distinct signatures in B cell chronic lymphocytic leukemias. Proc Natl Acad Sci U S A 2004;101:11755–11760. [PubMed: 15284443]

16. Calin GA, Sevignani C, Dumitru CD, et al. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. Proc Natl Acad Sci US A 2004;101:2999–3004.

17. Michael MZ, SM OC, van Holst Pellekaan NG, et al. Reduced accumulation of specific microRNAs in colorectal neoplasia. Mol Cancer Res 2003;1:882–891. [PubMed: 14573789]

18. Johnson S, Grosshans H, Shingara J, et al. RAS is regulated by the let-7 microRNA family. Cell 2005;120:635–647. [PubMed: 15766527]

19. Takamizawa J, Konishi H, Yanagisawa K, et al. Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoper-ative survival. Cancer Res 2004;64:3753–3756. [PubMed: 15172979]

20. Eis PS, Tam W, Sun L, et al. Accumulation of miR-155 and BIC RNA in human B cell lymphomas. Proc Natl Acad Sci US A 2005;102:3627–3632.

21. Iorio MV, Ferracin M, Liu CG, et al. MicroRNA gene expression deregulation in human breast cancer. Cancer Res 2005;65:7065–7070. [PubMed: 16103053]

22. He H, Jazdzewski K, Li W, et al. The role of micro-RNA genes in papillary thyroid carcinoma. Proc Natl Acad Sci US A 2005;102:19075–19080.

23. Chan JA, Krichevsky AM, Kosik KS. MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells. Cancer Res 2005;65:6029–6033. [PubMed: 16024602]

24. Ciafre SA, Galardi S, Mangiola A, et al. Extensive modulation of a set of microRNAs in primary glioblastoma. Biochem Biophys Res Commun 2005;334:1351–1358. [PubMed: 16039986]

25. He L, Thomson JM, Hemann MT, et al. A microRNA polycistron as a potential human oncogene. Nature 2005;435:828–833. [PubMed: 15944707]

26. El-Serag HB, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. Gastroenterology 2007;132:2557–2576. [PubMed: 17570226]

27. El-Serag HB, Davila JA, Petersen NJ, et al. The continuing increase in the incidence of hepatocellular carcinoma in the United States: an update. Ann Intern Med 2003;139:817–823. [PubMed: 14623619]

28. Schmittgen TD, Jiang J, Liu Q, et al. A high-through-put method to monitor the expression of microRNA precursors. Nucleic Acids Res 2004;32:E43. [PubMed: 14985473]

29. Jiang J, Lee EJ, Gusev Y, et al. Real-time expression profiling of microRNA precursors in human cancer cell lines. Nucleic Acids Res 2005;33:5394–5403. [PubMed: 16192569]

30. Lee EJ, Gusev Y, Jiang J, et al. Expression profiling identifies microRNA signature in pancreatic cancer. Int J Cancer 2007;120:1046–1054. [PubMed: 17149698]

31. Wang X, Seed B. A PCR primer bank for quantitative gene expression analysis. Nucleic Acids Res 2003;31:e154. [PubMed: 14654707]

32. Bair E, Tibshirani R. Semi-supervised methods to predict patient survival from gene expression data. PLoS Biol 2004;2:E108. [PubMed: 15094809]

33. Gusev Y, Schmittgen TD, Lerner MR, et al. Computational analysis of biological functions and pathways collectively targeted by co-expressed microRNAs in cancer. BMC Bioinformatics 2007;8:S16. [PubMed: 18047715]

34. Jiang J, Lee EJ, Schmittgen TD. Increased expression of microRNA-155 in Epstein-Barr virus transformed lymphoblastoid cell lines. Genes Chromosomes Cancer 2006;45:103–106. [PubMed: 16175574]

35. Meng F, Henson R, Lang M, et al. Involvement of human micro-RNA in growth and response to chemo-therapy in human cholangiocarcinoma cell lines. Gastroenterology 2006;130:2113–2129. [PubMed: 16762633]

36. Liu CG, Calin GA, Meloon B, et al. An oligonucleotide microchip for genome-wide microRNA profiling in human and mouse tissues. Proc Natl Acad Sci U S A 2004;101:9740–9744. [PubMed: 15210942]

37. Yanaihara N, Caplen N, Bowman E, et al. Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. Cancer Cell 2006;9:189–198. [PubMed: 16530703]

38. Chen C, Ridzon DA, Broomer AJ, et al. Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res 2005;33:e179. [PubMed: 16314309]

39. Marsit CJ, Eddy K, Kelsey KT. MicroRNA responses to cellular stress. Cancer Res 2006;66:10843–10848. [PubMed: 17108120]

40. Miranda KC, Huynh T, Tay Y, et al. A pattern-based method for the identification of MicroRNA binding sites and their corresponding heteroduplexes. Cell 2006;126:1203–1217. [PubMed: 16990141]

41. Murakami Y, Yasuda T, Saigo K, et al. Comprehensive analysis of microRNA expression patterns in hepato-cellular carcinoma and non-tumorous tissues. Oncogene 2005;25:2537–2545. [PubMed: 16331254]

42. Volinia S, Calin GA, Liu CG, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci US A 2006;103:2257–2261.

43. Lu J, Getz G, Miska EA, et al. MicroRNA expression profiles classify human cancers. Nature 2005;435:834–838. [PubMed: 15944708]

44. Roldo C, Missiaglia E, Hagan JP, et al. MicroRNA expression abnormalities in pancreatic endocrine and acinar tumors are associated with distinctive pathologic features and clinical behavior. J Clin Oncol 2006;24:4677–4684. [PubMed: 16966691]

45. Bloomston M, Frankel WL, Petrocca F, et al. Micro-RNA expression patterns to differentiate pancreatic adenocarcinoma from normal pancreas and chronic pancreatitis. JAMA 2007;297:1901–1908. [PubMed: 17473300]
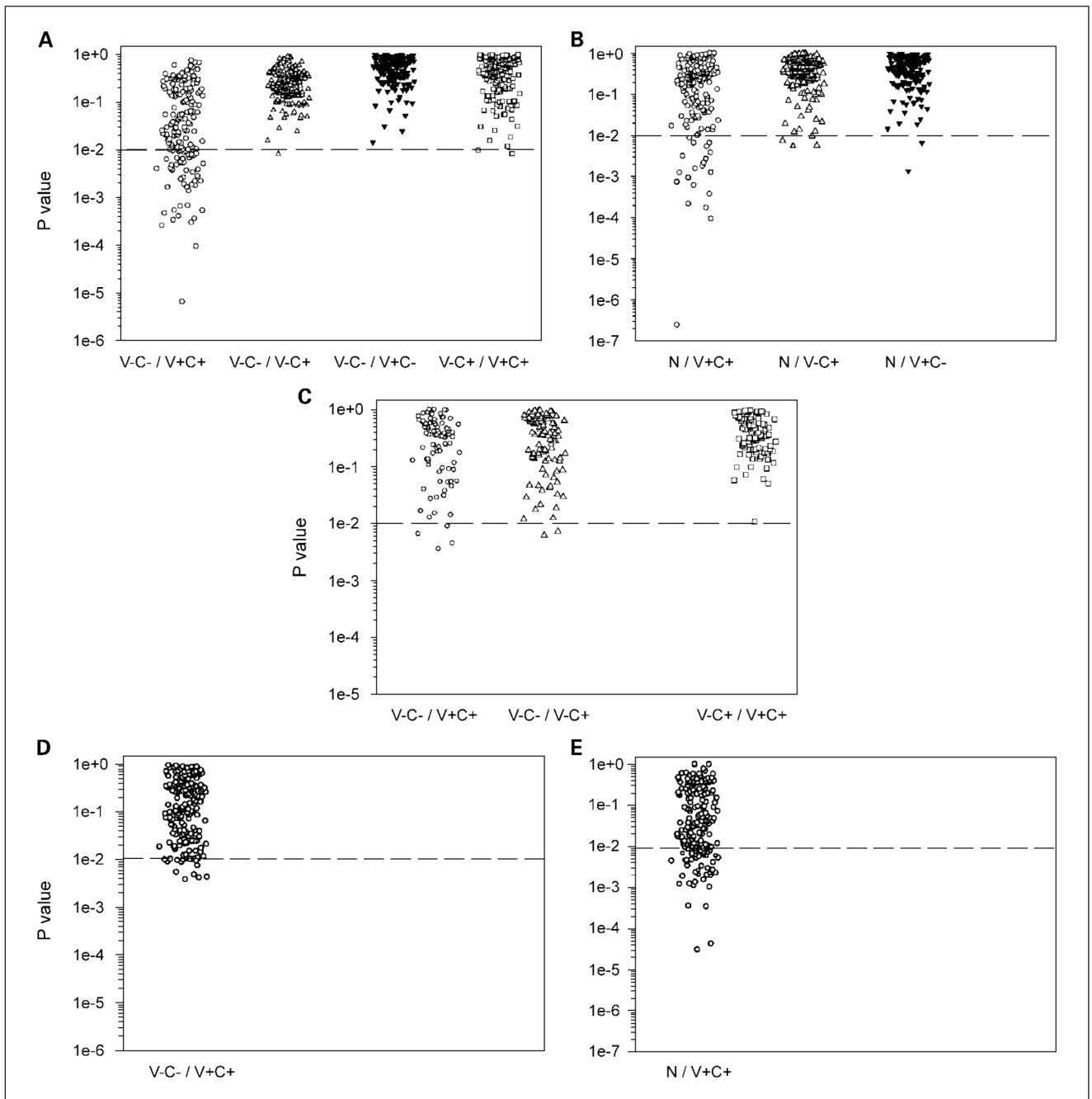
Fig. 1.
miRNA expression in patients with cirrhosis and/or hepatitis infection. The expression of 182 precursors and 196 mature miRNAs was profiled in specimens of adjacent benign tissue or normal liver. Approximately half of the patients had cirrhosis and one third were infected with hepatitis B or C. Comparisons were made between patients infected with hepatitis B or C (V^+) or not (V^-), and those who had cirrhosis (C^+) or not (C^-). *Points*, P values from the Student's *t* test comparing the miRNA expression in the two groups or compared with liver from patients without HCC (*N*). Groups were considered statistically different at $P < 0.01$ (*dashed line*, $P = 0.01$). Precursor miRNAs are presented in corresponding benign (*A*) or normal (*B*) liver tissue. *C*, P values from the expression of 95 randomly selected mRNAs. P values

generated from the mature miRNA expression profiling compared with benign (*D*) or normal (*E*) liver tissue.
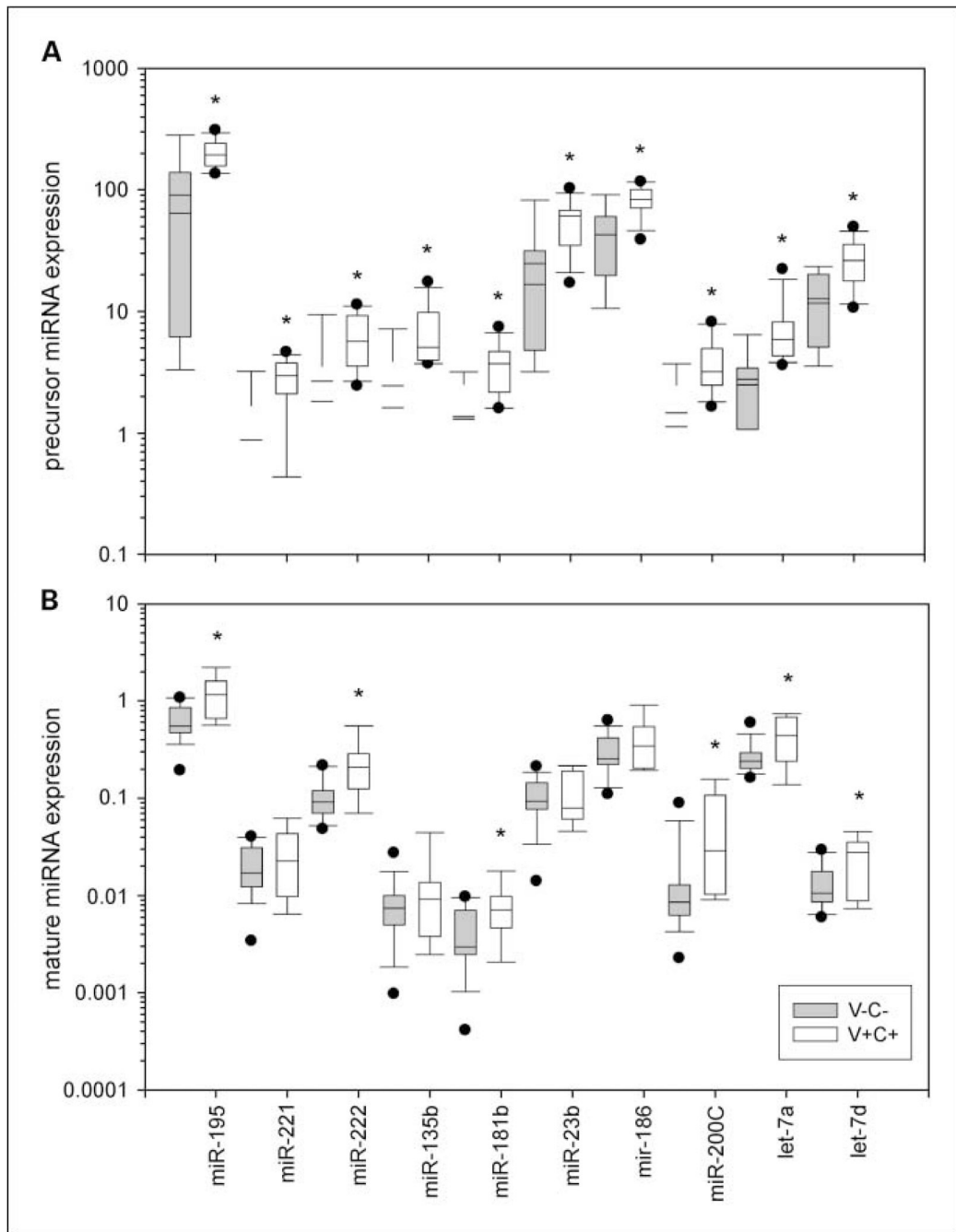
Fig. 2.
Precursor and mature miRNA expression in benign liver tissue. *A*, the expression of miRNA precursors was determined by real-time PCR in specimens of uninfected, noncirrhotic liver (*gray columns*, *n* = 17) and virally infected, cirrhotic livers (*open columns*, *n* = 12). *B*, the expression of mature miRNA was determined by real-time PCR in specimens of uninfected, noncirrhotic liver (*gray columns*, *n* = 17) and virally infected, cirrhotic livers (*open columns*, *n* = 9). *, *P* < 0.05.
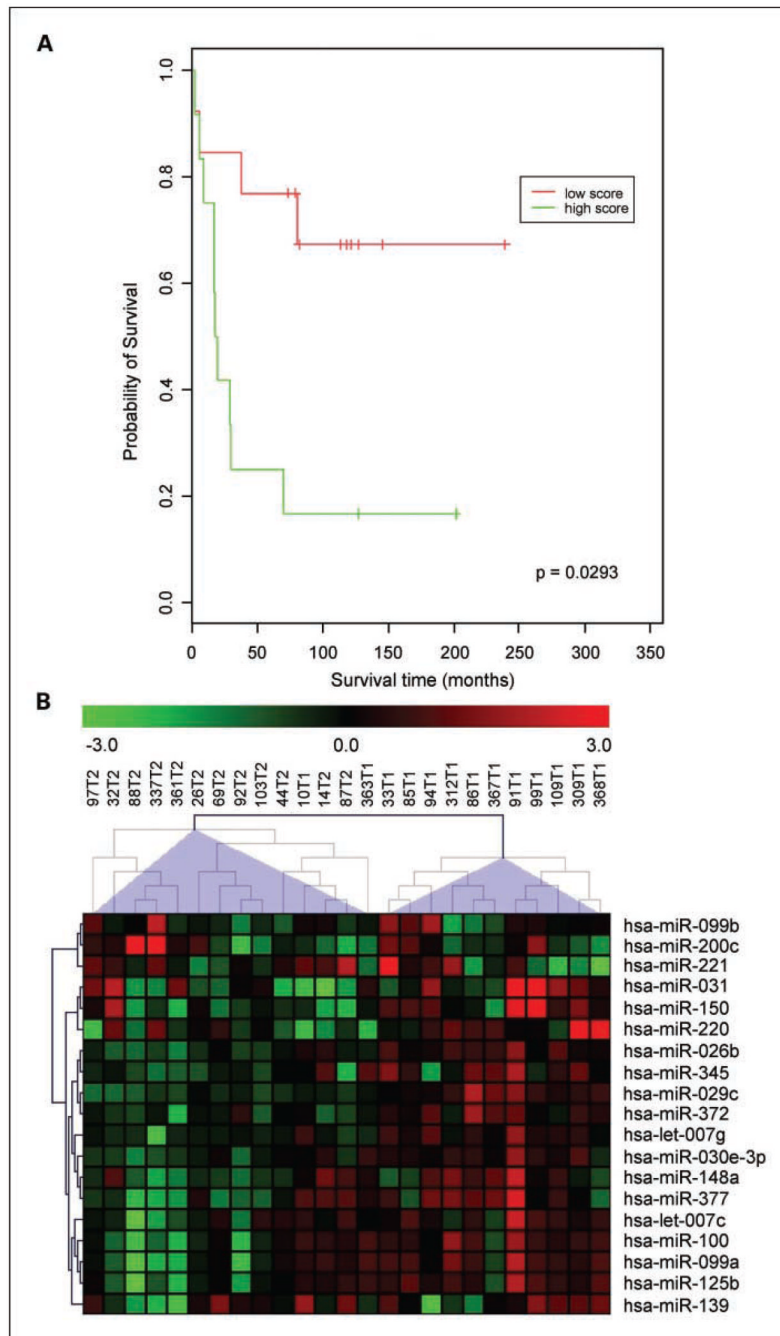
Fig. 3.
Prognostic value of miRNA expression in patients with HCC. *A*, Kaplan-Meyer survival curves for two groups of patients. Prognostic scores for each patient were estimated using semisupervised principal component analysis (PAM algorithm). *Red*, data for patients with low prognostic scores (group 1, higher survival time); *green*, patients with high prognostic scores (group 2, poorer survival; *P* < 0.05). *B*, heatmap of the results of hierarchical clustering of samples and genes. Expression data of 19 miRNAs are presented for 25 tumor samples from patients with known survival times. Nineteen miRNAs were identified by supervised principal component analysis (PAM algorithm) as being significantly associated with survival risk. Two major clusters of samples showed distinct patterns of miRNA expression of these19 genes and

correspond (except for two samples) with two classes of patients predicted by supervised principal component analysis (PAM algorithm) as having good (group 1) and poor survival (group 2). *Red*, expression levels above the pool average; *green*, expression levels below the pool average; black, no difference compared with the pool average; and *gray*, undetectable expression.

Table 1

Clinical and pathologic features of patients with HCC

Age, y (median, range)	74 (36–90)
>60	44 (81.5%)
<60	10 (18.5%)
Gender	
Male	28 (51.9%)
Female	26 (48.1%)
Etiology	
HBV	5 (9.3%)
HCV	9 (16.7%)
HBV + HCV	2 (3.7%)
ALD	6 (11.1%)
ALD + viral infection[*]	1 (1.9%)
Hemochromatosis	3 (5.6%)
Other	10 (18.5%)
No known risk factors	18 (33.3%)
Edmonson grade	
Grade 1	5 (9.3%)
Grade 2	27 (50.0%)
Grade 3	18 (33.3%)
Grade 4	4 (7.4%)
Cirrhosis	
No	24 (44.4%)
Yes	26 (48.1%)
Unknown	4 (9.4%)

[*] Either HBV or HCV infection.

Table 2

Mature miRNA expression in HCC and adjacent benign tissue

miRNA	Relative gene expression		Fold change	*P*
	Mean tumor	**Mean benign**	**Tumor/benign**	**Tumor vs. benign**
miR-18	0.10	0.05	2.03	0.039718
miR-21	15.57	7.38	2.11	0.001165
miR-33	0.03	0.01	3.68	0.000381
miR-101	0.21	0.43	−2.07	0.037011
miR-130b	0.35	0.13	2.67	0.000566
miR-135a	0.40	0.04	10.5	0.029641
miR-139	0.02	0.06	−2.51	0.000002
miR-150	6.09	14.08	−2.31	0.041504
miR-199a	0.01	0.03	−2.59	0.012791
miR-199a*	1.23	3.22	−2.62	0.003364
miR-199b	0.00	0.01	−2.32	0.020543
miR-200b	0.51	1.23	−2.42	0.034570
miR-214	1.99	4.78	−2.40	0.006759
miR-221	0.44	0.13	3.42	0.011714
miR-223	9.59	20.37	−2.12	0.000311
miR-301	0.15	0.07	−2.19	0.021348

Table 3

Patient identifiers along with grouping, survival time, and censored status

Patient ID	Group	Status	Survival (mo)
10T	1	0	240
14T	2	1	29
26T	2	1	17
32T	2	0	201
33T	1	1	3
44T	2	0	127
69T	2	1	70
85T	1	1	81
86T	1	0	146
87T	2	1	9
88T	2	1	18
91T	1	0	79
92T	2	1	18
94T	1	1	38
97T	2	1	20
99T	1	0	73
103T	2	1	30
109T	1	0	127
309T	1	0	118
312T	1	0	82
337T	2	1	3
361T	2	1	6
363T	1	1	6
367T	1	0	114
368T	1	0	122

NOTE: Censoring status: 0, alive; 1, deceased.

Table 4

Enrichment of biologically significant targets of 19 miRNAs from PAM analysis: top three GO biological process categories

Biological process	Significance	No. of target genes	miRNA
Cell division (GO:0051301)	0.0174	61	100% (19/19)
Mitosis (GO:0007067)	0.0126	46	100% (19/19)
G_1-S transition of mitotic cell cycle (GO:0000082)	0.0317	15	84% (16/19)