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 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 p16INK4a 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 hepatocarcinogenesis 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 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 Ct method, data were multiplied by 10k 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

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.
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.

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.
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 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 adjacent benign tissue 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.

![Fig. 2. Precursor and mature miRNA expression in noncirrhotic liver. 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.](image-url)
expression in the hepatitis B (or hepatitis C, we compared the differences in the miRNA of the virally infected specimens had a preference for hepatitis B a large number of miRNA precursors. In these comparisons, only one of the miRNA precursors was differentially expressed in liver tissues that were available in our archives; this included 13 of the 43 specimens

| 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    | 0.009759 |
| 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 |

in the V^C^+ group (≥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 (≥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 (≥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, -230, -33, -10a, -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 miRNA 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%) miRNAs were differentially expressed in the benign tissues, compared with 50 of the 182 (27%) miRNAs (≥2-fold, P < 0.01; Fig. 1C). Of the differentially expressed miRNAs, 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: VC+, 15 patients; VC−, 4 patients; V+C, 2 patients; and V+C−, 7 patients. Fourteen mature miRNAs were increased in the V+C− compared with the VC+ 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 VC− 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 G1-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 TXP2). 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 G1-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 miRNA gene expression analysis during the process of liver carcinogenesis show significant changes in miRNA 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.

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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) |
| G1-S transition of mitotic cell cycle (GO:0000082) | 0.0317 | 15                  | 84% (16/19) |
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