Involvement of Human Micro-RNA in Growth and Response to Chemotherapy in Human Cholangiocarcinoma Cell Lines

FANYIN MENG,* ROGER HENSON,* MOLLY LANG,* HANIA WEHBE,* SHAIL MAHESHWARI,* JOSHUA T. MENDELL,† JINMAI JIANG,§ THOMAS D. SCHMITTGEN,§ and TUSHAR PATEL*

*Department of Internal Medicine, Scott and White Clinic, Texas A&M University System Health Science Center College of Medicine, Temple, Texas; †The Institute of Genetic Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland; and §College of Pharmacy, The Ohio State University, Columbus, Ohio

Background & Aims: **Micro-RNA (miRNA) are endogenous regulatory RNA molecules that modulate gene expression. Alterations in miRNA expression can contribute to tumor growth by modulating the functional expression of critical genes involved in tumor cell proliferation or survival. Our aims were to identify specific miRNA involved in the regulation of cholangiocarcinoma growth and response to chemotherapy.** ***Methods:*** **miRNA expression in malignant and nonmalignant human cholangiocytes was assessed using a microarray. Expression of selected miRNA and their precursors was evaluated by Northern blots and real-time polymerase chain reaction, respectively. The effect of selected miRNA on cell growth and response to chemotherapy was assessed using miRNA-specific antisense oligonucleotides to decrease miRNA expression or with precursor miRNA to increase cellular expression.** ***Results:*** **miRNA expression was markedly different in malignant cholangiocytes, with decreased expression of many miRNA compared with nonmalignant cells. A cluster of miRNA, including miR-320, miR-200b, miR-21, miR-23a, miR-141, miR-27a, and miR-34a, were expressed in all cell lines. MiR-21, miR-141, and miR-200b were highly over-expressed in malignant cholangiocytes. Inhibition of miR-21 and miR-200b increased sensitivity to gemcitabine, whereas inhibition of miR-141 decreased cell growth. Treatment of tumor cell xenografts with systemic gemcitabine altered the expression of a significant number of miRNA. miR-21 modulates gemcitabine-induced apoptosis by phosphatase and tensin homolog deleted on chromosome 10 (PTEN)-dependent activation of PI 3-kinase signaling. Potential target genes that were modulated by selected miRNA were identified.** ***Conclusions:*** **Alterations in miRNA expression contribute to tumor growth and response to chemotherapy. Aberrantly expressed miRNA or their targets will provide mechanistic insight and therapeutic targets for cholangiocarcinoma.**

Micro-RNA (miRNA) are a group of endogenous small, noncoding RNA that can modulate protein expression by regulating translational efficiency or cleavage of target mRNA. Although several hundred miRNA have been cloned or predicted, a functional role for these in many cellular processes has not been fully elucidated.[1] The best characterized role of miRNA has been as a developmental regulator in *Caenorhabditis elegans* and Drosophilia. More recently, several other roles have been described including regulation of apoptosis and differentiation. miRNA are expressed as long precursor RNA that are processed by a single nuclease Drosha prior to transport into the cytoplasm by an exportin-5-dependent mechanism. In the cytoplasm, miRNA are further cleaved by Dicer. The result is a 17–24 nucleotide (nt) small miRNA that can complex with sequence complementary in a manner similar to the RNA-induced silencing complex, which is also formed during the RNA interference process. The miRNA are then targeted to sequences that are at least partially complementary to the miRNA. The translation of the bound mRNA is then inhibited or the mRNA transcript degraded based on the sequence match. Although the targets of miRNA have not been fully characterized, potential targets can be predicted based on sequence complementarity.

A role for miRNA in cancer is emerging with the recent identification that miRNA may be important contributors to cancer development. Studies of miRNA expression profiles in cancer samples have identified a handful of miRNA that are differentially regulated in tumors, suggesting a possible link between miRNA and oncogenesis.[2,3] miRNA targets can include genes involved in differentiation or transformation, such as transcription factors and cell-cycle control. Tumorigenesis might result from the perturbation of these pathways by mutations in the miRNA genes, their 3′-untranslated region (UTR) miRNA-binding sites, or in pathways that regulate their expression. Through modulation of cell

Abbreviations used in this paper: miRNA, micro-RNA.

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differentiation and proliferation, miRNA can control and regulate many cellular processes germane to tumor genesis, growth, and survival. Many miRNA genes are located at chromosomal fragile sites or regions of cytogenetic abnormalities associated with cancer.[4] Moreover, the pattern of expression of miRNA has been shown to define accurately the specific cancer types.[5] However, the biologic consequences of alterations in miRNA expression in cancer cells remain undefined.

Cholangiocarcinomas are enigmatic malignancies of the biliary tract that are highly chemoresistant.[6–8] The need for improved treatments for this cancer is highlighted by the dramatic increases in the incidence of cholangiocarcinoma in several regions of the world.[9–11] The mechanisms regulating cholangiocarcinoma growth and resistance to chemotherapy are poorly understood. Although miRNA are becoming increasingly recognized as regulatory molecules in human cancers, their involvement in functional responses to environmental changes such as exposure to chemotherapy is unknown. We postulated that aberrant expression of miRNA in cancer cells could modulate the functional expression of critical genes involved in tumor cell behavior, such as genes contributing to cell survival in response to chemotherapeutic stress. Our aims were, thus, to identify the miRNA that were differentially expressed in malignant human cholangiocytes and to recognize specific miRNA involved in the regulation of tumor cell growth and response to chemotherapy. We asked the following questions: Are miRNA differentially activated in malignant cholangiocytes? If so, does their expression reflect cytogenetic abnormalities? Can aberrantly expressed miRNA regulate tumor cell growth or survival? Are miRNA involved in chemotherapeutic responses in cholangiocarcinoma?

Materials and Methods

Cell Lines and Cultures

The KMCH-1, Mz-ChA-1, and TFK human cholangiocarcinoma cell lines and the nonmalignant human cholangiocyte H69 cell lines were obtained as previously described.[12–14] Mz-ChA-1 cells are derived from metastatic gallbladder cancer, TFK-1 cells from common bile duct cancer, and KMCH from an intrahepatic mixed cholangiocellular-hepatocellular carcinoma. H69 cells are derived from nonmalignant cholangiocytes and immortalized by SV40 transfection.[15] KMCH and Mz-ChA-1 cells were cultured in CMRL 1066 media with 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% antimycotic antibiotic mix. H69, KMCH, and TFK cells were cultured in Dulbecco's modified Eagle medium (DMEM)/F-12 and minimum essential media (MEM) as previously described.[12] Normal human intrahepatic biliary epithelial cells and epithelial cell medium used for their growth were obtained from ScienCell research laboratories (San Diego, CA). All other cell culture media and supplements were obtained from Invitrogen (Carlsbad, CA).

Transfections

Transfections were performed by electroporation using the Nucleofector system (Amaxa Biosystems, Koln, Germany). Transfection conditions for each cell type were first optimized to result in 20%–30% transfection efficiency with viability >80%. All studies were performed in quadruplicate. Cells ($1–2 \times 10^6$) were spun down at 1000 rpm for 5 minutes, and the medium was removed. Cells were then resuspended in 100 μL Nucleofector solution (Amaxa Biosystems) at room temperature followed by addition of 20 μL of 100 nmol/L microRNA precursor, antisense inhibitor, or controls (all obtained from Ambion Inc., Austin, TX). Electroporation was performed using the Nucleofector (Amaxa Biosystems). Transfected cells were then resuspended in regular culture media containing 10% serum for 48–72 hours prior to study.

Isolation of miRNA

Total RNA from cell lines and tumor tissue samples were isolated using the Totally RNA isolation kit (Ambion). The miRNA fraction was obtained by mixing RNA with 2X sample buffer and flashPAGE purification using flashPAGE precast Gels and the flashPAGE Fractionator System (Ambion). The size of the miRNA fractions were confirmed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Palo Alto, CA).

miRNA Array Hybridization and Analysis

The isolated miRNA from the pooled sample were appended 3′ amine-modified tails using the mirVana miRNA Array Labeling Kit (Ambion) and then fluorescently coupled with Cy3 or Cy5 using the Post Labeling Reactive Dye kit (Amersham Bioscience, Pittsburgh, PA). The Cy3 (control) and Cy5 (treated) labeled samples were washed 3 times using miRNA washing buffer, mixed in the same labeling cartridge, eluted, and stored at −70°C or analyzed by hybridizing to miRNA arrays. miRNA arrays were generated on glass slides using the mirVana miRNA Array Probe Set (Ambion) and an OmniGrid Microarrayer (Gene Machines, San Carlos, CA). Each probe was printed in duplicate. Following hybridization, the miRNA arrays were scanned using a GenePix 4000A array scanner (Axon Instruments, Union City, CA). Raw data were normalized and analyzed using GeneSpring 7.0 Software (Silicon Genetics, Redwood City, CA). Normalization was performed by expressing each miRNA replicate relative to a control miRNA (Ambion) added to each sample, thus allowing comparisons between chips. An average value of the median intensity of each replicate in 4 groups was generated. Heat maps were generated using HeatMap Builder version 1.0 (accessed on-line at http://quertermous.stanford.edu/heatmap.htm) to illustrate the relative differences. The heat maps are row normalized between samples based on the minimum and maximum values for each miRNA and thus provided a visual representation of relative differences between

each miRNA in a nonquantitative manner. miRNA expression levels were clustered using a hierarchical clustering algorithm and self-organizing tree algorithm clustering performed using Multiexperiment Viewer Version 3.1 from The Institute for Genomic Research.[16]

Real-Time Polymerase Chain Reaction Assay for miRNA Precursors

Total RNA was extracted from the cultured cells using Trizol (Invitrogen), and the concentration of total RNA was quantitated by measuring the absorbance at 260 nm. Expression of miRNA precursors was assessed using real-time polymerase chain reaction (PCR) as previously described.[17,18] One microgram of total RNA was briefly exposed to RNase-free DNase I and reverse transcribed to cDNA using gene-specific primers and Thermoscript, thermostable reverse transcriptase (Invitrogen) as described.[17] Triplicate reverse transcriptions were performed from the RNA of each cell line; 0.5 μL of dilute cDNA (1:50) and additional PCR reagents from the SYBR green core reagent kit (Applied Biosystems) were added to the reaction along with 800 nmol/L of the PCR primers to each miRNA precursor.[18] PCR was performed in duplicate in the Applied Biosystems 7900HT real-time PCR instrument equipped with a 384-well reaction block. PCR was performed for 15 seconds at 95°C and 1 minute at 60°C for 40 cycles followed by the thermal denaturation protocol. The expression of each miRNA relative to U6 RNA was determined using the $2^{-\Delta CT}$ method.[17] To simplify the presentation of the data, the relative expression values were multiplied by 10^5.

Northern Blotting

Northern blotting was performed as previously reported.[19] Briefly, total RNA (20 μg) was resolved on 15% polyacrylamide urea gels and transferred to Genescreen Plus membranes (Perkin Elmer, Boston, MA). Oligonucleotides complementary to the mature miRNA were end labeled with γ-^{32}P adenosine triphosphate (ATP) and T4 kinase. The membranes were incubated with labeled probe (1.5×10^6 CPM per mL hybridization buffer) prior to visualization using phosphorimaging. Blots were stripped once and reprobed using a different oligonucleotide.

Cell Proliferation Assay

Cell proliferation was assessed using the CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI), which uses a tetrazolium compound as substrate. Following transfection, cells (10,000/well) were plated in 96-well plates (BD Biosciences, Rockville, MD) and incubated at 37°C, and cell proliferation was assessed after 3 days as previously described.[20]

Cytotoxicity Assay

Transfected cells were seeded into 96-well plates (10,000 viable cells/well) and incubated with gemcitabine or appropriate diluent controls in a final volume of 200 μL medium containing 0.5% FBS. After 48 hours, cell viability was assessed using a commercially available tetrazolium bioreduction assay for viable cells, and cytotoxicity was assessed as previously described.[13]

Apoptosis Assay

Cells were washed with phosphate-buffered saline (PBS) and then resuspended in PBS containing 0.1% Triton X for 10 minutes on ice, prior to resuspension (5000 cells/μL) in 4% PBS-buffered paraformaldehyde solution containing 10 μg/mL 4′6-diamidino-2-phenylindole (DAPI). Ten microliters of this suspension was placed on a glass slide and covered with a coverslip. Nuclear morphology was assessed using a fluorescence microscope (Olympus BH, Tokyo Japan). Apoptotic nuclei were identified by condensed chromatin or nuclear fragmentation. More than 150 cells were counted, and the percentage of apoptotic nuclei was determined.

Western Blotting

For immunoblot analysis of cells in culture, cells grown in 100-mm dishes were washed twice with ice-cold PBS and then lyzed by incubation for 20 minutes in 1 mL of ice-cold cell lysis buffer (1% Nonidet P-40, 50 mmol/L HEPES, pH 7.4, 150 mmol/L NaCl, 2 mmol/L EDTA, 2 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium vanadate, 1 mmol/L sodium fluoride, and 1X protease inhibitor mixture). Modified cell lysis buffers (without phosphatase inhibitors sodium vanadate and sodium fluoride) were used for PTPN12 and Src[P-Tyr418] immunoblots only. For analysis of xenograft tissue, the tissue was homogenized, and lysates were obtained. The protein concentrations of the lysates were measured using a Bradford protein assay kit (Bio-Rad, Hercules, CA). Equivalent amounts of protein were resolved and mixed with 6X SDS-PAGE sample buffer, electrophoresed in a 4%–20% linear gradient Tris-HCl-ready gel (Bio-Rad), and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline, pH 7.4, containing 0.05% Tween 20, and were incubated with primary antibodies and IRDye700 and IRDye800-labelled secondary antibodies (Rockland, Gilbertsville, PA) according to the manufacturer's instructions. The protein of interest was visualized and quantitated using the LI-COR Odyssey Infrared Imaging System (LI-COR Bioscience, Lincoln, NE).

Xenograft Model

Eight-week-old male athymic *nu/nu* mice were obtained from Charles River Laboratories (Wilmington, MA) and fed food and water ad libitum. The mice were maintained in accordance with the Institutional Animal Care and Use Committee (IACUC) procedures and guidelines. They were housed 3 or 4 per cage, and fluorescent light was controlled to provide alternate light and dark cycles of 12 hours each. Mz-ChA-1 cells (5×10^6 cells) were suspended in 0.25 mL of extracellular matrix gel, and the mixture was injected subcutaneously into the right and left flanks. Xenograft growth was estimated by serial measurements obtained twice a week. Once xenograft tumor volume had reached 150 mm^3, mice were randomized to

receive either gemcitabine (120 mg/kg, intraperitoneally [IP]) or control diluent every 3 days for a total of 5 doses. For analysis of chemotherapy-induced miRNA and protein expression in vivo, the xenografts were excised, and tissue was homogenized. An aliquot of the lysates was used for miRNA isolation as described above and the remainder used for protein expression studies.

Luciferase Expression Constructs

The pGL3-PTEN-3′-UTR construct, which contains the putative binding site for mir-21 downstream of the stop codon in the pGL3 Firefly luciferase reporter, was constructed as reported.[21] Mz-ChA-1 cells were plated (2×10^6 cells/well) in 6-well plates. One microgram of pGL3-PTEN-3′-UTR construct was cotransfected with 1 μg of a Renilla luciferase expression construct pRL-TK (Promega), using Trans-It (Mirus, Madison, WI). Luciferase assays were performed 24 hours after transfection using the dual Luciferase Reporter Assay System (Promega).

Reagents

Pre-miR miRNA precursors and Anti-miR miRNA-specific inhibitors of miR-141, miR-21, and miR-200b were purchased from Ambion. Peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibodies against circadian locomotor output cycles kaput protein (CLOCK) and phospho-PI-3 kinase p85α [Tyr^{508}] were from Santa Cruz Biotechnology Inc; PTEN, phospho-Akt [Ser^{473}], and phospho-mTOR [Ser^{248}] from Cell Signaling, Inc. (Beverly, MA); PTPN12 from Orbigen, Inc. (San Diego, CA); and phospho-Src [Tyr^{418}] and α-tubulin from Sigma Chemical Co. (St. Louis, MO). DAPI was obtained from Sigma Chemical Co., and 1L-6-Hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate (Akt inhibitor), LY294002, and rapamycin were obtained from Calbiochem (San Diego, CA).

Statistical Analysis

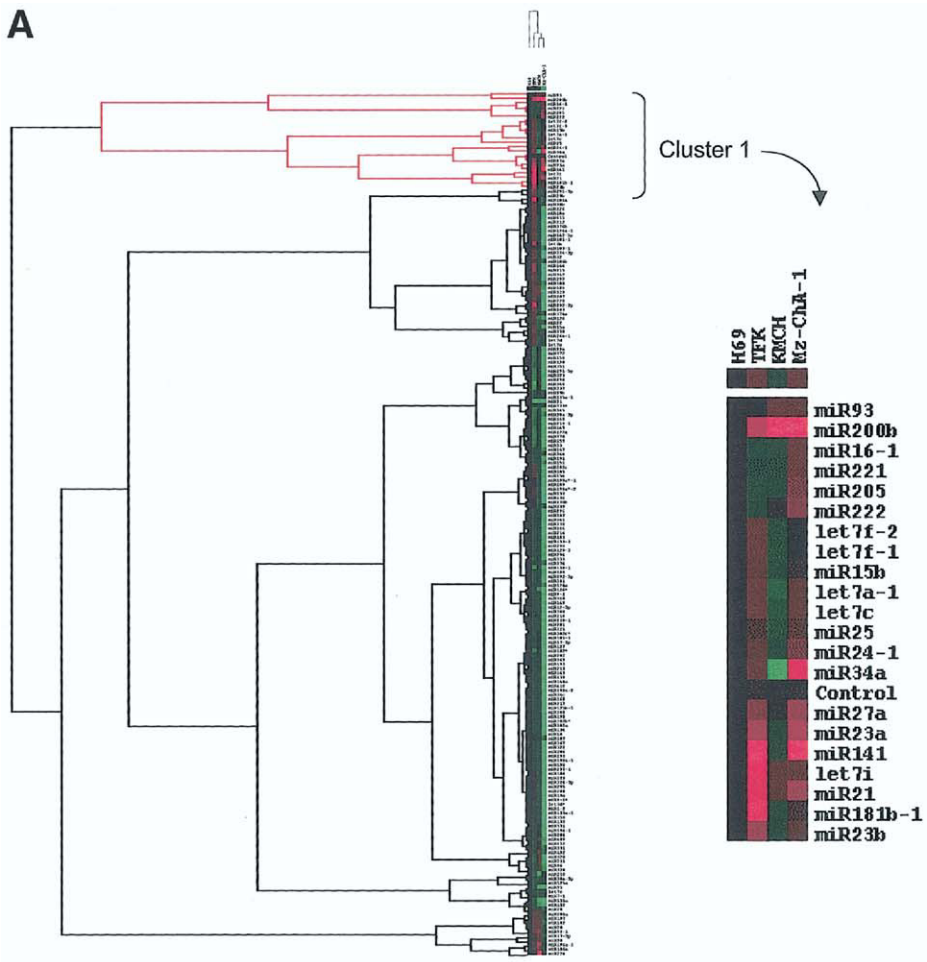
Data are expressed as the mean ± standard error (SE) from at least 3 separate experiments performed in triplicate, unless otherwise noted. The differences between groups were analyzed using a double-sided Student *t* test when only 2 groups were present and the null hypothesis was rejected at the .05 level.

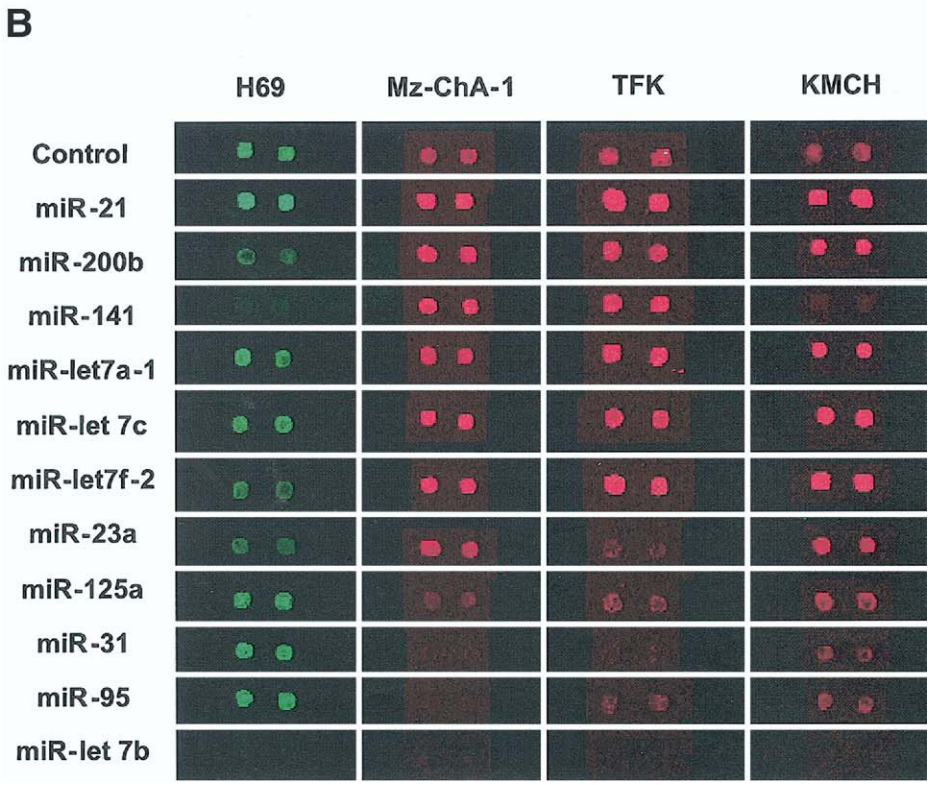
Results

miRNA Are Differentially Expressed in Human Malignant Cholangiocytes

We began by assessing the expression of miRNA in several cholangiocyte cell lines (Figure 1*A*). In each cell type, there was a wide range of relative miRNA expression ranging from undetectable to greater than 12-fold that of an internal standard control miRNA. A list of the miRNA that were expressed at relatively higher levels than the other miRNA on the array in each cell line is provided in Table 1. Expression of miR-141 and miR-21 was verified by Northern blot analysis and paralleled the expression pattern observed on the miRNA array (Figure 1*C*). The level of expression of miRNA with the highest (miR-21) or lowest (let-7b) expression for each cell type using the miRNA array correlated with expression of their respective miRNA precursors using real-time PCR analysis (Figure 1*D*). Although the expression patterns varied with different cell types, increased expression of several miRNA was seen in all cell types. These include miR-21, let7a-1, let7c, let7f-2, miR-320, and miR-16-1. A greater than 5-fold increase in expression was noted for miR-21, and greater than 5-fold decrease in expression was noted for let7b, miR-326, miR-351, miR-373, and miR-150 for all cell types. Because the pattern of miRNA expression can occur in a tissue-specific manner, these specific miRNA may reflect potential markers of a biliary tract lineage. The pattern of expression for TFK-1 and Mz-ChA-1 cells was similar to each other and distinct from KMCH-1 cells, which likely reflects differences in their developmental origins.

Next, we compared miRNA expression between malignant and nonmalignant cholangiocytes. The pattern of miRNA expression in malignant cells was markedly different from that of either normal human intrahepatic biliary tract epithelia or the nonmalignant H69 cells. A heat map showing the relative, nonquantitative differences between cell lines is shown in Figure 2. In general, expression of most miRNA was lower for each of the malignant cells compared with the nonmalignant cells. For nonmalignant H69 cells, the expression of 22.2% of miRNA was increased relative to an internal standard control miRNA (Ambion), greater than for Mz-ChA-1 (6.6%), TFK-1 (14.6%), or KMCH-1 (10.6%) cells. When the relative expression was compared between cell lines, expression of a group of miRNA was differentially increased in malignant cells (Table 2). These include the human miR-200b, miR-21, miR-23a, miR-141, and miR-27a, all of which were expressed >2.0-fold in Mz-ChA-1 and TFK malignant cells compared with nonmalignant H69 cells. These miRNA may play an important role in malignant transformation or tumor cell behavior. miRNA that have been reported to be associated with certain cancers, such as the miR-17-92 polycistron (B-cell lymphoma)[22]; miR-143 and miR-145 (colon cancer)[23]; and miR-15 and miR-16 (chronic lymphocytic leukemia)[24] or miR-155 (Burkitt's lymphoma)[25] or miR-192, miR-194, and miR-215 (various gastrointestinal


A
Cluster 1
H69
TFK
KMCH
Mz-ChA-1
miR93
miR200b
miR16-1
miR221
miR205
miR222
let7f-2
let7f-1
miR15b
let7a-1
let7c
miR25
miR24-1
miR34a
Control
miR27a
miR23a
miR141
let7i
miR21
miR181b-1
miR23b


B
H69
Mz-ChA-1
TFK
KMCH
Control
miR-21
miR-200b
miR-141
miR-let7a-1
miR-let 7c
miR-let7f-2
miR-23a
miR-125a
miR-31
miR-95
miR-let 7b

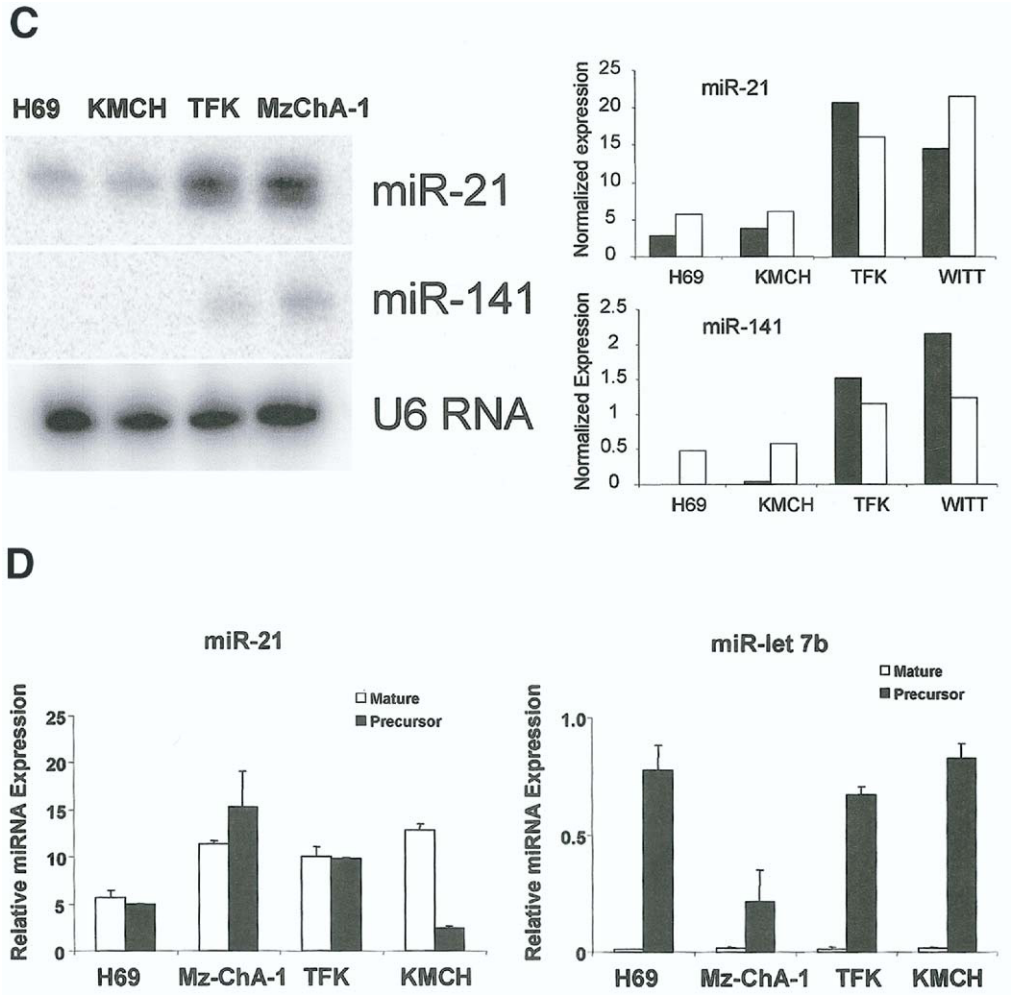


Figure 1. Expression of miRNA in human cholangiocyte cell lines. (*A*) miRNA was isolated and profiling performed as described in the Materials and Methods section by hybridization to miRNA-specific probes on epoxy-coated slides. Samples from H69 nonmalignant cholangiocytes were labeled with Cy3, whereas samples from malignant cholangiocytes were labeled with Cy5. Cluster analysis identifies a group of miRNA that are increased in expression in malignant cholangiocytes (*cluster 1*). An enlarged view of this group is shown. (*B*) Representative spots for selected miRNA for which expression is differentially increased (*miR-21*, *miR-200b*, *miR-141*), decreased (*miR-31*, *miR-95*, *miR-125a*), or remains unchanged in malignant cholangiocytes (*let7a-1*, *let 7c*, *let-7f-2*) are illustrated. (*C*) Northern blotting was performed on the RNA from the cell lines and probed for miR-21, miR-141, and U6 RNA. The normalized data from the Northern blots (*solid bars*) and the microarray (*open bars*) are shown in the Figure. (*D*) The expression of miRNA precursors was assessed using real-time PCR and correlated with expression of mature miRNA using the miRNA array. The relative expression levels for miRNA that were expressed at very high levels on the array (*miR-21*) or at very low levels (*let-7b*) are illustrated by the *open bars* (n = 8), whereas relative expression of their respective miRNA precursors by real-time PCR is shown by the *solid bars* (n = 3). Data represent mean ± standard error.

tract cancers),[5] were not consistently altered in cholangiocarcinoma cells. Furthermore, the expression of several miRNA, such as miR-125a, miR-31, and miR-95, was decreased in malignant cholangiocytes. These observations raise the possibility that cholangiocarcinoma-specific miRNA expression profiles may be useful to develop diagnostic assays. Moreover, the data suggest that specific miRNA may be involved in malignant transformation. These miRNA may contribute to cancer cell behavior through modulating gene expression either by gene silencing or gene activation as a result of termination of miRNA-catalyzed gene silencing.

Correlation Between Genomic Sites of Human miRNA and Sites of Chromosomal Gain or Loss in Human Cholangiocarcinoma

To determine whether the pattern of miRNA expression reflects cytogenetic abnormalities, we correlated the genomic site of miRNA that were differentially expressed in malignant cells with reported regions of chromosomal gain or loss in human cholangiocarcinoma. Of miRNA that were increased in expression, the chromosomal site of miR-141 (12p13.31) was most often associated with a region of chromosomal gain in biliary

Table 1. Highly Expressed miRNA in Human Cholangiocyte Cell Lines

H69		Mz-ChA-1		TFK		KMCH	
miRNA	Mean ± SE	miRNA	Mean ± SE	miRNA	Mean ± SE	miRNA	Mean ± SE
miR21	5.67 ± 0.18	miR21	11.42 ± 0.27	miR21	9.98 ± 1.09	let7d	12.90 ± 0.63
let7a-1	3.66 ± 0.75	let7a-1	3.50 ± 0.80	miR16-1	4.42 ± 0.45	let7a-1	9.57 ± 0.58
let7f-2	3.16 ± 0.34	let7c	3.05 ± 0.61	miR320	4.05 ± 0.26	let7f-2	9.55 ± 0.47
miR290	3.06 ± 2.58	miR16-1	2.41 ± 0.36	let7a-1	3.79 ± 0.64	miR21	7.05 ± 0.28
let7c	2.84 ± 0.46	let7f-2	2.34 ± 0.34	miR202	3.44 ± 0.27	let7c	6.22 ± 0.22
miR215	2.75 ± 0.26			let7f-2	3.25 ± 0.37	miR16-1	5.04 ± 0.21
miR202	2.65 ± 0.24			let7d	3.04 ± 0.31	miR106a	5.02 ± 0.33
miR320	2.54 ± 0.16			let7c	3.00 ± 0.52	miR17-5p	4.78 ± 0.62
miR1-2	2.32 ± 0.20					let7f-1	4.70 ± 0.36
miR16-1	2.03 ± 0.23					miR31	3.53 ± 0.47
						miR24-1	3.46 ± 0.35
						miR320	2.15 ± 0.34

NOTE. For each cell type, miRNA expression is relative to that of a control miRNA, added at a constant amount to the hybridization mix. All miRNA expressed greater than 2-fold relative to expression of the control miRNA, with a *P* value of <.05, are shown. The H69 cells are nonmalignant, whereas the Mz-ChA-1, TFK, and KMCH are malignant cholangiocyte cell lines. A group of miRNAs, namely, miR-21, let7a-1, let7 f-2, let 7c, miR320, and miR16-1, are highly expressed in all cell types and may thus constitute a "cholangiocyte-miRNA cluster."

tract cancers (3/6 gallbladder cancers, and 1/19 intrahepatic cholangiocarcinomas).[26,27] However, for most other differentially expressed miRNA, the miRNA chromosomal sites were not associated with reported regions of chromosomal loss or gain in biliary tract cancers. Amplification of DNA sequences on chromosomes 2p25.3 and 7q11.23 has been reported in cholangiocarcinoma, but there are no known miRNA encoded at these genomic sites.[28] Similarly, chromosomal locations of miRNA that are markedly decreased in expression in malignant cholangiocytes, such as miR-376b and miR-215, were not associated with regions of reported chromosomal loss in biliary tract cancers. Unlike other cancers such as chronic lymphocytic leukemia or Burkitt's lymphoma, altered miRNA expression in cholangiocarcinoma does not appear to occur as a result of major cytogenetic abnormalities.[24,25]

Regulation of Proliferation by miRNA

miR-141, miR-21, and miR-200b were selected for further study because they are over expressed in malignant cholangiocytes, and their genomic locations exist within regions of reported chromosomal gain in human cholangiocarcinoma. In Mz-ChA-1 cells, expression of miR-141 increased by 5.5- ± 0.9-fold, miR-21 increased by 2.7- ± 0.1-fold, and miR-200b increased by 7.6- ± 0.9-fold relative to nonmalignant cells. The effects of these 3 miRNA on cell proliferation were further characterized using specific antisense oligonucleotide miRNA specific inhibitors. Transfection of Mz-ChA-1 cells with inhibitors of miR-141, miR-21, and miR-200b decreased proliferation to 82.3% ± 1.6%, 71.1% ± 12.0%, and 77.2% ± 5.4%, respectively (all $P < .05$), of controls after 72 hours (Figure 3). However, cell proliferation was not significantly altered (93.2% ± 4.6% of controls) by inhibitors to miR-17, whose expression is not altered in malignant cells. Moreover, proliferation was increased to 121.7% ± 5.9%, 149.8% ± 16.8%, and 145.0% ± 19.0% of controls (all $P < .05$) after 72 hours in cells transfected with miR-141, miR-21, and miR-200b precursors, respectively, to over express miRNA expression (Figure 3). We hypothesize that aberrant expression of selected miRNA can enhance tumor cell growth.

miRNA as Regulators of Cell Survival In Vitro

Cholangiocarcinomas are highly chemoresistant tumors. The aberrant activation of survival signaling in tumor cells can enhance chemoresistance. Recently, miR-21 was reported to be an antiapoptotic factor in human glioblastoma cells, suggesting that aberrantly expressed certain miRNA may contribute to the malignant phenotype by blocking expression of critical apoptosis-related genes.[29] To explore a role for miRNA in cell survival and the response to chemotherapy, Mz-ChA-1 cells were transfected with miRNA-specific antisense inhibitors, and their sensitivity to gemcitabine was assessed. Anti-miR-21 and anti-miR-200b both increased gemcitabine-induced cytotoxicity by 12.4% and 14.4%, respectively, whereas anti-miR-141 failed to show a significant effect (Figure 4*A*). Next, we assessed cell viability in response to gemcitabine in H69 nonmalignant cholangiocytes transfected with miRNA precursors. Cell viability was increased by 18.7%, 12.0%, and 14.0% of controls in cells transfected with miR-141, miR-21, and miR-200b precursors, respectively (Figure 4*B*). To determine the specific effect of miRNA on

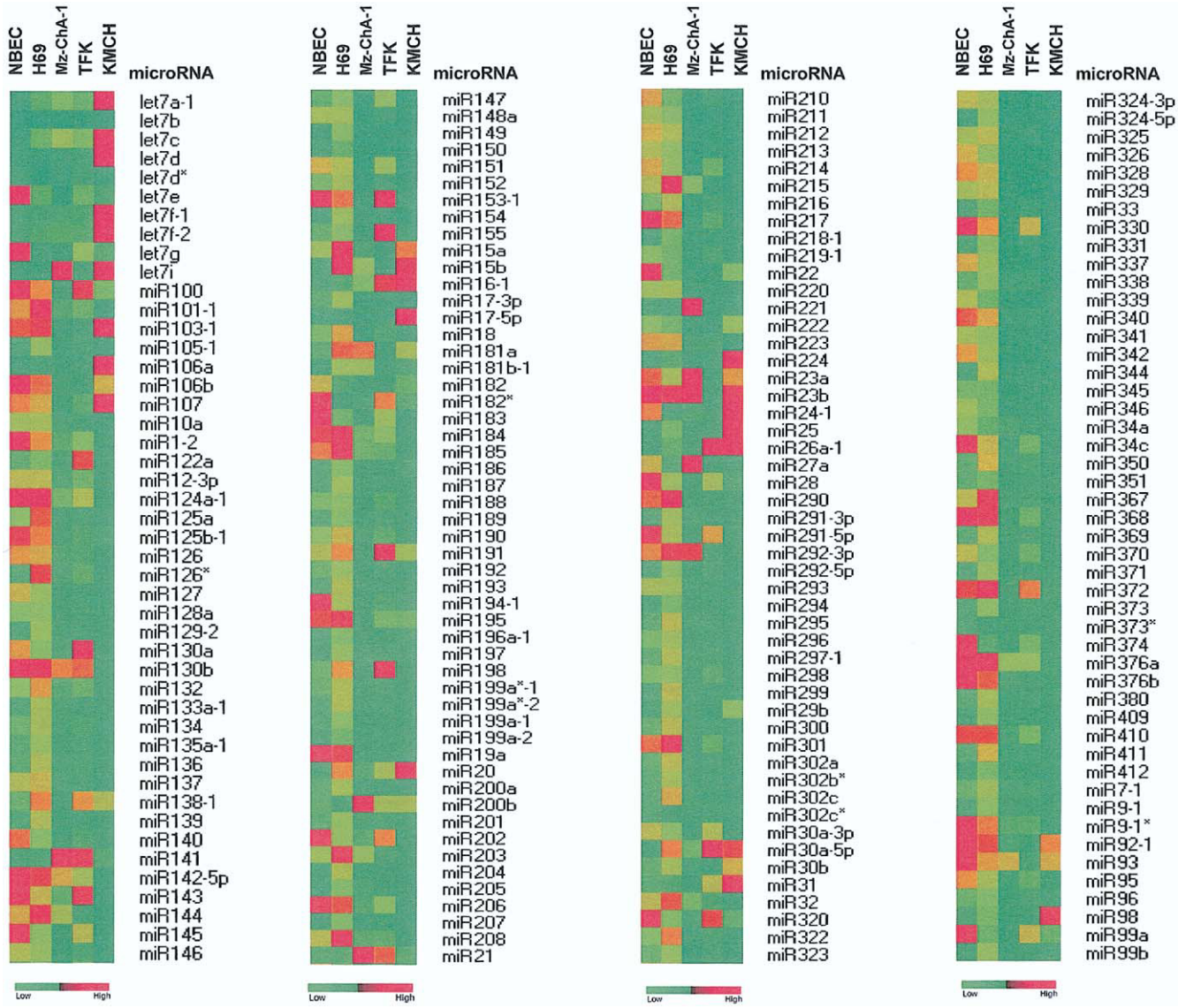


Figure 2. miRNA expression profiles in malignant and nonmalignant cholangiocytes. miRNA was isolated and profiling performed by miRNA microarray as described in the Materials and Methods section. miRNA was obtained from normal intrahepatic biliary tract epithelial cells (NBEC); H69 nonmalignant cholangiocytes; and the human cholangiocarcinoma cell lines Mz-ChA-1, TFK-1, and KMCH-1. A heat map was generated from the average of normalized log-transformed fluorescent intensity for each data set (n = 4 separate arrays, each with 2 probes for each miRNA). The expression relative to the median values is shown with increased relative amounts in *red* and decreased amounts in *green*. miRNA are grouped based on cell type in ascending order of the name list.

apoptosis, the extent of apoptosis was quantitated during incubation of Mz-ChA-1 cells with gemcitabine in the presence or absence of mir-141, miR-200b, or miR-21 inhibitors (Figure 5). Similar to the results of the cell viability studies, inhibition of miR-21 or miR-200b, but not miR-141, decreased gemcitabine-induced apoptosis. These results support a functional role for these miRNA in mediating cell survival in both malignant and nonmalignant cells and suggest a mechanism by which over expression of miR-21 and miR-200b miRNA may contribute to chemoresistance in cholangiocarcinoma by modulating chemotherapy-induced apoptosis.

Chemotherapy Alters miRNA Expression In Vivo

To evaluate the biologic relevance of alterations in miRNA expression to chemotherapeutic responses in vivo, we profiled miRNA expression in Mz-ChA-1 tumor cell xenografts in nude mice randomized to treatment with either gemcitabine or control diluent (Figure 6). Gemcitabine treatment resulted in a greater than 2-fold increase in the expression of a group of miRNA that included miR-202, miR-320, miR-374, miR-1-2, miR-16-1, miR-24-1, let7f-2, let7a-1, and miR-374

Table 2. miRNA Differentially Expressed in Malignant Cholangiocytes

Mz-ChA-1		TFK		KMCH	
miRNA	Mean ± SE	miRNA	Mean ± SE	miRNA	Mean ± SE
miR200b	7.607 ± 0.953	miR141	10.535 ± 1.377	miR224	8.531 ± 0.154
miR141	5.481 ± 0.941	miR181a	5.394 ± 0.446	miR220b	7.314 ± 0.420
miR34a	4.371 ± 0.714	miR181b-1	5.019 ± 0.403	miR196a-1	3.986 ± 0.405
miR23a	3.020 ± 0.121	let7i	4.591 ± 0.329	miR92-1	3.066 ± 0.073
miR21	2.742 ± 0.112	miR21	4.350 ± 0.136	miR106a	2.525 ± 0.080
miR27a	2.602 ± 0.090	miR292-3p	3.951 ± 0.361	miR17-5p	2.367 ± 0.040
miR222	2.314 ± 0.122	miR200b	3.360 ± 0.191	miR182	2.281 ± 0.067
miR205	2.149 ± 0.101	miR23a	2.944 ± 0.139	miR328	2.158 ± 0.226
miR24-1	2.099 ± 0.156	miR23b	2.710 ± 0.098	miR200a	2.020 ± 0.132
		miR215	2.367 ± 0.289		
		miR27a	2.325 ± 0.072		
		miR144	2.287 ± 0.228		

NOTE. For each malignant cell type, the miRNA expression is represented as a ratio of normalized expression in that cell type relative to the normalized expression in nonmalignant H69 cells. All miRNA that are expressed in the malignant cells at greater than 2-fold the level of expression in the nonmalignant cells, and with a P value $< .05$, are represented in the Table.

(Table 3). Among these is a group including let7a-1, let7f-2, and miR-16-1 that are highly expressed in both nonmalignant and malignant cholangiocytes. Although the expression of miR202 and miR320 is considerably decreased in Mz-ChA-1 malignant cholangiocytes when compared with nonmalignant cholangiocytes, their expression was highly increased in response to gemcitabine. Thus, there are dynamic regulatory mechanisms involved in their decreased expression in tumor cells. Consistent with their expression in vitro, miR-21, miR-200b, and miR-141 were all up-regulated in vivo by 2.0- ± 0.1-, 2.3- ± 0.1-, and 1.5- ± 0.1-fold, respectively, when compared with control miRNA (Figure 6*B*). Moreover, expression of miR-21 and miR-200b were further increased by 4.1- ± 0.1- and 1.5- ± 0.1-fold in tumor cell xenografts treated with gemcitabine compared with controls (Table 3). However, miR-141 expression was decreased to 0.6- ± 0.1-fold in tumors treated with gemcitabine. These results suggest differences between these miRNA in their mechanisms of regulation of expression and their functional effects on cell survival in response to chemotherapeutic stress.

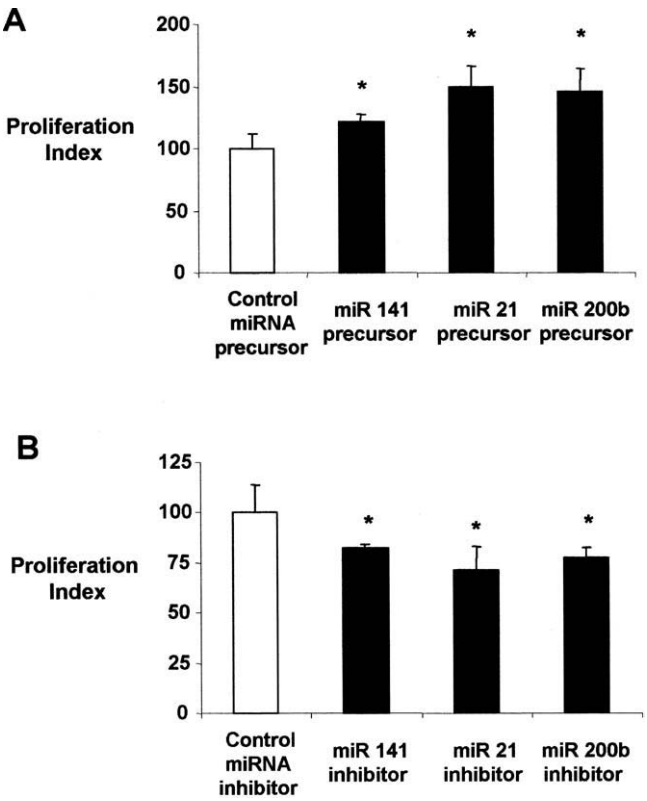


Figure 3. Modulation of cholangiocarcinoma cell proliferation by miR-141, miR-21, and miR-200b. Mz-ChA-1 cells (5×10^4/well) in 96-well plates were transfected with miRNA-specific inhibitors or precursors to miR-141, miR-21, or miR-200b or with control precursors or inhibitors. Cell proliferation was assessed after 72 hours using a viable cell assay and the proliferation index derived. The mean ± standard error from 4 separate experiments are illustrated. *$P < .05$ when compared with controls.

Identification of Potential miRNA Targets for miR-21, miR-200b, and miR-141

There have been several reported efforts to identify biologically relevant targets of miRNA. To identify potential targets that may contribute to miRNA regulation of cholangiocarcinoma growth and survival, we used a bioinformatics approach to identify potential candidates for further study using the rules proposed by Doench and Sharp with slight modifications.[30] The criteria used include >85% complementarity between positions 2 and 9 of the miRNA and the candidate target and the overall T_m of the miRNA/mRNA interaction. This core sequence has been identified as critical to the activities of miRNA. Similarly, overall binding stability between miRNA and mRNA has been shown to be an

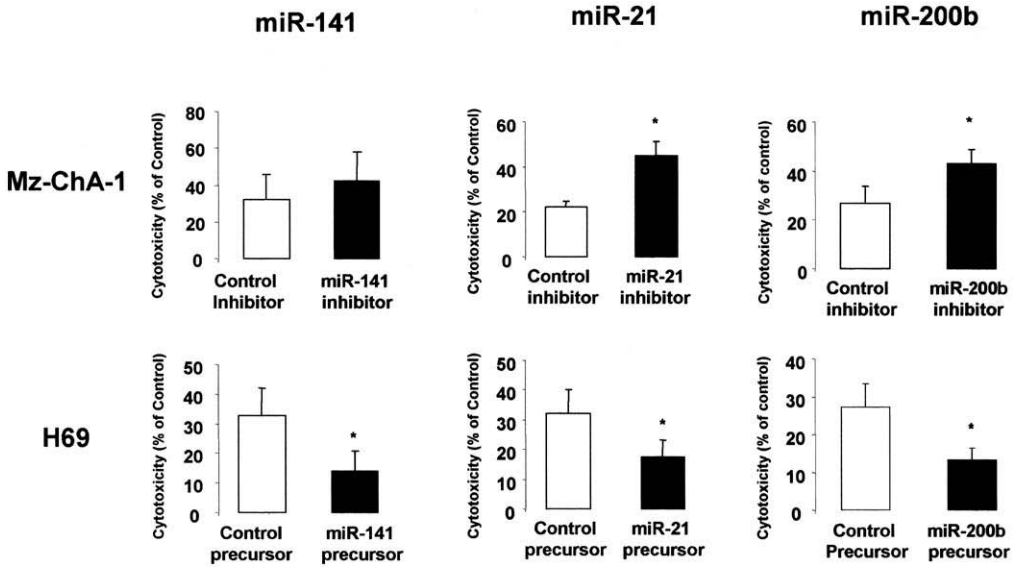


Figure 4. miRNA regulation of gemcitabine-induced cytotoxicity in vitro. *Mz-ChA-1 cells*, shown in the *top panels*, were transfected with control anti-miRNA or miRNA specific inhibitors to miR-141, miR-21, or miR-200b. *H69 nonmalignant cholangiocytes*, shown in the *bottom panels*, were transfected with miRNA-specific precursors to miR-141, miR-21, or miR-200b or miRNA precursor control. After 48 hours, cells were incubated with 30 μmol/L gemcitabine or diluent (treatment control) in medium containing 0.5% FBS. Cell viability was assessed after 48 hours, and the cytotoxicity index was calculated. The cytotoxicity index is the percentage reduction in cell viability in cells incubated with gemcitabine relative to untreated controls. The results shown represent the mean ± SE from 4 independent experiments. $*P < .05$ when compared with control miRNA precursor or inhibitor group.

important indicator of miRNA activity. Using these criteria, we scanned all known mRNA 3′-UTRs as potential targets for miR-21, miR-200b, or miR-141.

Among the predicted mRNA that may be regulated by miR-141 miRNA is the CLOCK gene, which regulates circadian rhythms. The regulation of CLOCK expression by miR-141 has been experimentally studied.[31] The intracellular circadian clockwork can control the cell-division cycle directly and unidirectionally in proliferating cells. CLOCK can act as a tumor-suppressor by regulating the mechanisms by which cells stop dividing or undergo apoptosis following DNA damage, a response that may be dependent on the circadian rhythm. Over expression of mir141 may decrease the expression of CLOCK. This could contribute to aberrant responses to DNA damage in cancer cells. Compared with controls, levels of clock protein were increased in a time-dependent manner after transfection with the specific miR-141 inhibitor (Figure 7*A*).

Potential targets for miR-200b were also identified using our modified criteria. For one of the identified candidates, the protein tyrosine phosphatase, nonreceptor type 12 (PTPN12), the putative target sequence was conserved across species. Protein tyrosine phosphatases

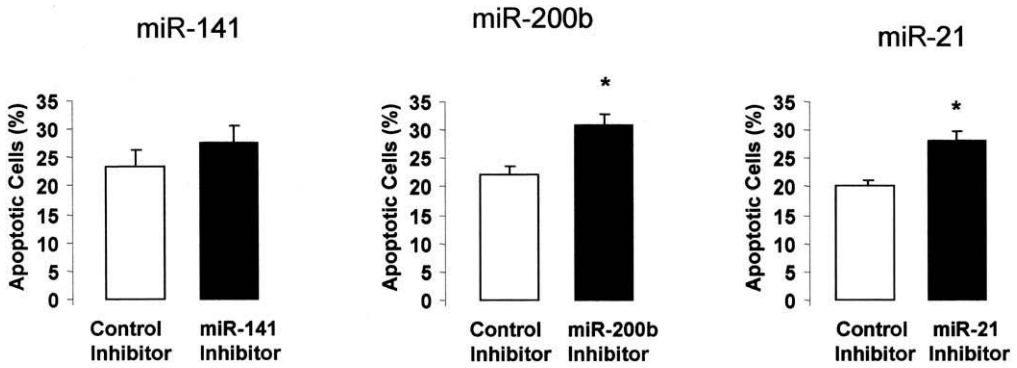


Figure 5. miRNA regulation of gemcitabine-induced apoptosis. Mz-ChA-1 cells were transfected with control anti-miRNA or miRNA-specific inhibitors to miR-141, miR-21, or miR-200b. After 48 hours, cells were incubated with 30 μmol/L gemcitabine or diluent (treatment control) in medium containing 0.5% FBS. Apoptosis was quantitated after 48 hours by counting the number of cells showing nuclear morphologic changes of apoptosis after staining with DAPI. The results shown represent the mean ± SE from 3 separate experiments. $*P < .05$ when compared with control miRNA inhibitor group.

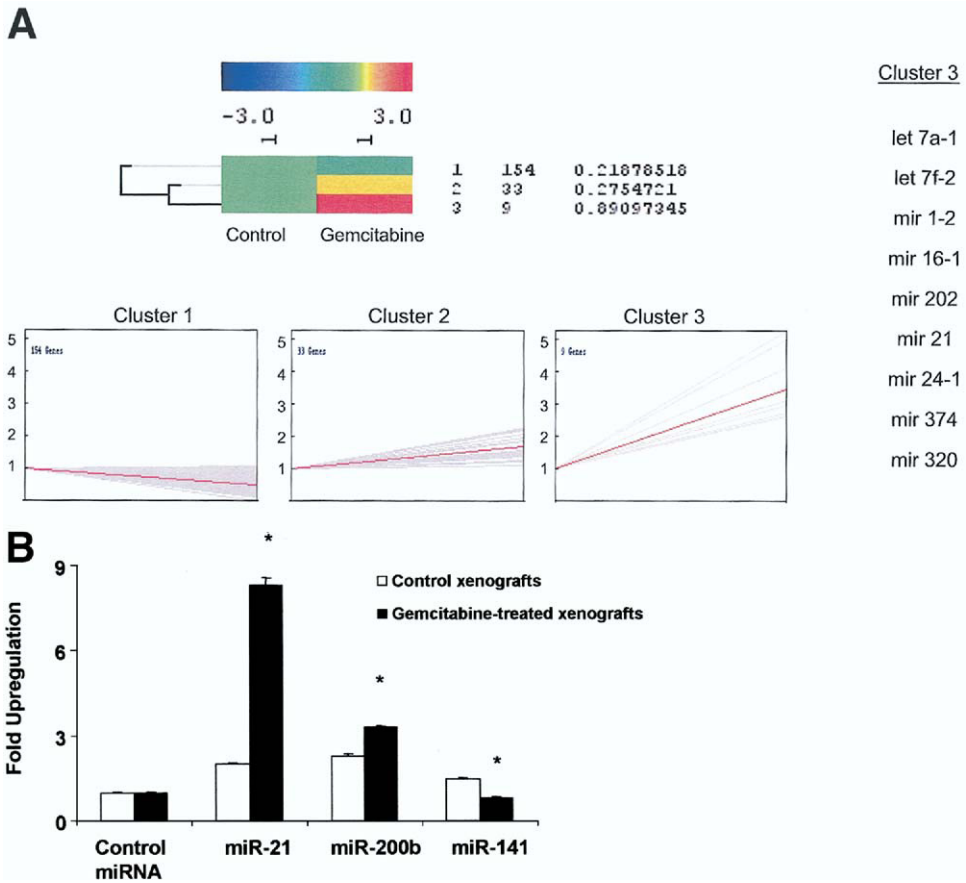


Figure 6. Alterations in miRNA expression in response to gemcitabine in tumor cell xenografts. Mz-ChA-1 cell xenografts were established in nude mice as described. Once xenograft tumors had formed to a predetermined size, 120 mg/kg gemcitabine or an equivalent volume of diluent control were administered IP every 3 days for a total of 5 doses. Tumors were then excised, and miRNA were isolated, labeled, and analyzed by miRNA microarray as described in Materials and Methods section. (*A*) miRNA expression patterns were analyzed using a self-organizing tree algorithm using the Multiexperiment Viewer (version 3.1) from the Institute for Genomic Research. A dendrogram showing 3 clusters was generated. miRNA expression following gemcitabine treatment is shown on the *right axis* relative to controls on the *left axis*. *Cluster 1* defined a group of 154 miRNA, which were unchanged or minimally decreased in expression. *Cluster 2* included 33 miRNA that showed small increases in expression, and *cluster 3* defined a group of 9 miRNA that had a marked increase following gemcitabine treatment and are listed. (*B*) Differential expression of selected miRNA in vivo following treatment with gemcitabine is shown. Data represent fold-change in expression in gemcitabine treated tumors compared with controls. For each miRNA, the difference between groups was statistically significant using Welch 2-sample *t* test with a *P* value $< .05$.

are signaling molecules that have been implicated in cellular regulation of oncogenic transformation, cell growth, cell-cycle progression, and cell differentiation. PTPN12 can bind and dephosphorylate the product of oncogenes such as c-Abl or Src and inactivate the Ras pathway.[32,33] Thus, deregulation of PTPN12 expression may contribute to tumor cell survival and oncogenesis. In cells transfected with anti-miR-200b, PTPN12 expression was increased to 132.2% ± 7.2% of control after 48 hours and 147.3% ± 12.8% of control after 72 hours. Moreover, inhibition of miR-200b significantly reduced the tyrosine phosphorylation of a downstream target Src, a key mediator of tumor cell proliferation and differentiation (Figure 7*B*). The altered expression of miR-200b in malignant cholangiocytes, and in response to gemcitabine treatment in vivo, may contribute to oncogenesis by functional deregulation of expression of key signaling intermediates.

Of the miR-21 targets identified, we selected the phosphatase and tensin homolog deleted on chromosome 10 (*PTEN*) for further study because of the well-characterized role for this gene in tumor biology. *PTEN* is a tumor-suppressor gene that, in mutated form, has been found to be associated with a number of different tumors.[34] It encodes a phosphatase that normally inhibits the survival/growth-promoting activity of the phosphoinositide 3-kinase (PI-3 kinase)-signaling pathway.[35] The PI-3 kinase pathway is constitutively active in many tumors and mediates a cellular-signaling pathway involved in cell survival. Inhibitors of this prosurvival network have been shown to sensitize tumor cells to death stimuli.[36] We assessed cellular expression of the

Table 3. Alterations in miRNA Expression in Tumor Xenografts Following Gemcitabine Treatment In Vivo

Expression increased >2-fold			Expression decreased <3-fold		
miRNA	Mean ± SE	*P* value	miRNA	Mean ± SE	*P* value
miR202	5.274 ± 0.312	2.65E-09	let7b	0.010 ± 0.001	.002
miR320	4.986 ± 0.362	1.68-08	miR328	0.097 ± 0.001	.013
miR21	4.130 ± 0.312	6.06E-08	miR220	0.110 ± 0.005	.015
miR374	3.106 ± 0.343	5.89E-06	miR197	0.123 ± 0.003	.017
miR1-2	2.968 ± 0.348	1.21E-05	miR326	0.139 ± 0.005	.019
miR16-1	2.735 ± 0.311	1.75E-05	miR204	0.142 ± 0.002	.020
miR24-1	2.716 ± 0.335	3.26E-05	miR346	0.156 ± 0.003	.022
let7f-2	2.663 ± 0.352	6.02E-05	miR207	0.157 ± 0.002	.022
let7a-1	2.601 ± 0.362	9.88E-05	miR412	0.157 ± 0.003	.022
miR372	2.274 ± 0.314	2.68E-04	miR324-3p	0.183 ± 0.005	.026
let7c	2.244 ± 0.346	5.91E-04	miR211	0.185 ± 0.005	.027
let7d	2.199 ± 0.337	6.63E-04	miR329	0.186 ± 0.010	.027
miR26a-1	2.192 ± 0.328	6.00E-04	miR341	0.197 ± 0.009	.029
miR217	2.097 ± 0.307	7.57E-04	miR345	0.208 ± 0.006	.031
miR194-1	2.076 ± 0.331	.001	miR337	0.213 ± 0.007	.032
			miR373*	0.220 ± 0.009	.033
			miR294	0.224 ± 0.005	.034
			miR213	0.226 ± 0.006	.034
			miR324-5p	0.229 ± 0.005	.035
			miR212	0.229 ± 0.004	.035
			miR299	0.230 ± 0.005	.035
			miR149	0.234 ± 0.008	.036
			miR205	0.246 ± 0.005	.038
			miR290	0.247 ± 0.005	.038
			miR133a-1	0.254 ± 0.006	.040
			miR302c*	0.259 ± 0.009	.041
			miR199a-2	0.262 ± 0.006	.042
			miR409	0.266 ± 0.006	.043
			miR154	0.267 ± 0.005	.043
			miR342	0.269 ± 0.006	.043
			miR339	0.280 ± 0.006	.046
			miR187	0.281 ± 0.005	.046
			miR186	0.283 ± 0.006	.047
			miR344	0.283 ± 0.005	.047
			miR139	0.289 ± 0.006	.048
			miR128a	0.291 ± 0.007	.049
			miR291-3p	0.293 ± 0.005	.049
			miR129-2	0.294 ± 0.005	.049

PTEN in Mz-ChA-1 cells in response to inhibition of miR-21 using specific anti-miR 21 inhibitors. The expression of the *PTEN* was 2.1- ± 0.3-fold higher in the miR-21 inhibitor transfected cells after 3 days (Figure 7*C*). Meanwhile, there was a concomitant reduction of phosphorylated PI-3 kinase p85 subunit to 0.49- ± 0.02-fold of controls after transfection with the miR-21 inhibitor. Expression of *PTEN* was unchanged in malignant cholangiocytes treated with an inhibitor targeting miR-141 (data not shown), which also modulated cell survival after chemotherapy. Although miR-21 is upregulated in malignant cholangiocytes, there is a marked >4-fold increase in expression of miR-21 following treatment with gemcitabine chemotherapy in vivo. Moreover, the phosphorylation of PI-3 kinase was significantly increased to 160.0% ± 10.4% in xenografts treated with gemcitabine. In addition, there was a decrease in expression of *PTEN* (Figure 7*D*). Taken together, these data are consistent with the hypothesis that the *PTEN* is a biologically relevant miR-21 target gene and indicate that down-regulation of *PTEN* is not a general result of inhibition of cell survival.

miR-21 Directly Alters *PTEN* Expression and Modulates Gemcitabine-Induced Apoptosis by Activating PI 3 Kinase-Signaling Pathways

To assess whether miR-21 can directly alter the expression of *PTEN*, we performed luciferase expression studies using a construct in which a fragment of the 3′-UTR of *PTEN* mRNA, containing the putative miR-21-binding sequence was cloned into a firefly luciferase reporter construct. This construct was cotransfected with

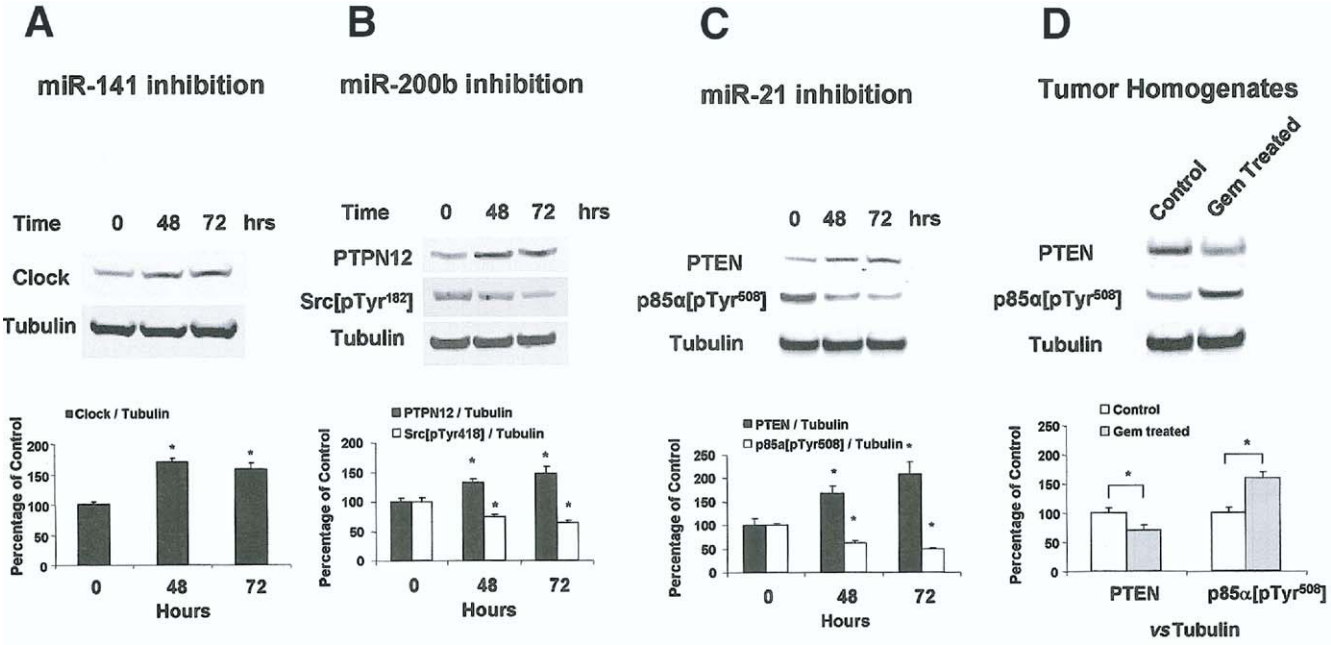


Figure 7. Inhibition of miRNA modulates expression of miRNA targets. Mz-ChA-1 cells were transfected with 30 nmol/L antisense inhibitor targeting miR-21, miR-200b, or miR-141. Cell lysates were obtained at baseline and after 48 and 72 hours for immunoblot analysis of protein expression of predicted target proteins for (*A*) *miR-141* (*CLOCK*); (*B*) *miR-200b* (*PTPN12* and its downstream target *Src*); and (*C*) *miR-21* (*PTEN* and its downstream target *PI-3K p85α* subunit). The blots were stripped and reprobed for α-tubulin as a loading control and for quantitation. Representative immunoblots are shown along with quantitative data that show the mean ± standard error from 4 separate blots. $*P < .05$ relative to baseline value. (*D*) miR-21 expression is increased in vivo following gemcitabine treatment. To assess the effect of increased miR-21 on potential downstream targets, immunoblots for *PTEN*, phosphorylated PI-3 kinase p85 subunit, and α-tubulin were performed in the homogenates from tumor cell xenografts following treatment with gemcitabine or diluent control. A decrease in expression of *PTEN*, the miR-21 predicted target, along with increased phosphorylation of p85 was observed in vivo. Representative immunoblots are shown along with quantitative data from 3 separate experiments. $*P < .05$ relative to control.

a control Renilla luciferase reporter construct in Mz-ChA-1 cells along with control or miR-21-specific inhibitor or precursor miRNA. An increase in relative luciferase activity was noted with anti-miR-21 and a corresponding decrease with the miR-21 precursor, indicating that miR-21 can directly modulate gene expression at the *PTEN* 3′-UTR (Figure 8*A*). We next assessed the potential involvement of PI-3 kinase-dependent mechanisms in the regulation of cell survival by miR-21. *PTEN* is a negative regulator of PI-3 kinase-dependent signaling. Activation of PI-3 kinase results in the activation of Akt and downstream mediators involved in cell survival such as the mammalian target of rapamycin (mTOR). Introduction of the miR-21 precursor into Mz-ChA-1 cells decreased *PTEN* expression (Figure 8*B*). Moreover, an increase in basal expression of phosphorylated AKT and mTOR was noted in cells transfected with the miR-21 precursor, which was decreased by preincubation with 100 μmol/L LY294002, a PI-3 kinase inhibitor, or 20 μmol/L AKT inhibitor. Furthermore, Mz-ChA-1 cells transfected with the miR-21 precursor had decreased sensitivity to gemcitabine-induced apoptosis, but this effect was abrogated in the presence of either the AKT inhibitor or the mTOR inhibitor rapamycin (Figure 8*C*). These observations implicate *PTEN*-dependent activation of PI-3 kinase and activation of AKT/mTOR signaling as a mechanism by which increased miR-21 modulates chemosensitivity in cholangiocarcinoma cells.

Discussion

Aberrant regulation of gene expression by translational or posttranslational mechanisms is emerging as an important contributor to cancer cell growth.[37] miRNA are key regulators of mRNA degradation and, hence, gene expression. Tissue-specific patterns of miRNA expression have been reported and postulated to reflect embryologic development. Emerging evidence implicates alterations in miRNA expression in cancer, based on the demonstration of an important functional role of miRNA such as let-7a in development.[38] Aberrant miRNA expression profiles have been recently reported in cancer cells compared with normal cells and have been postulated to reflect embryologic development.[5] Although these deviant patterns of miRNA ex-

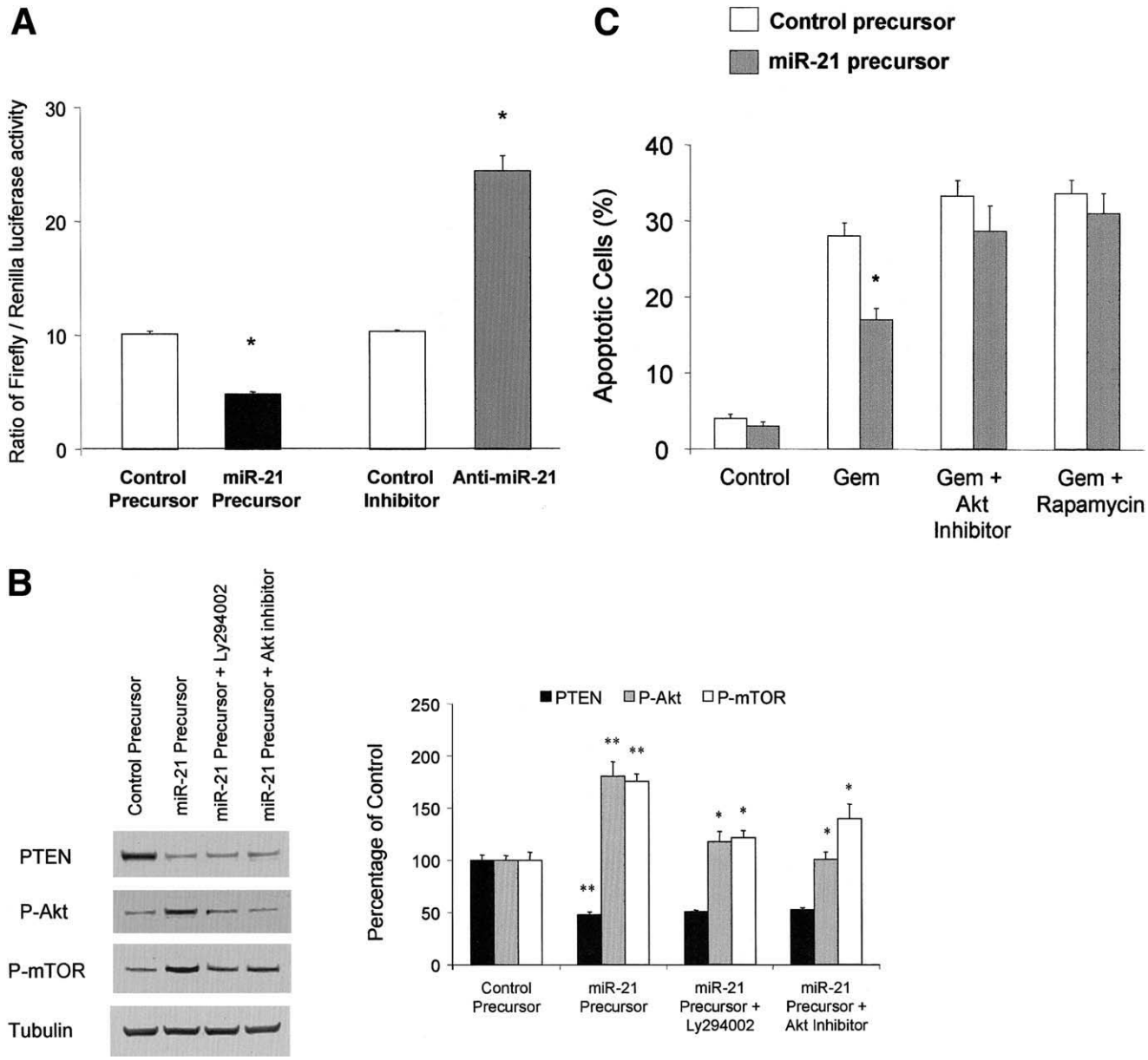


Figure 8. miR-21 regulates *PTEN* expression and PI 3-kinase signaling. (*A*) Mz-ChA-1 cells were plated (2×10^6 cells/well) in 6-well plates. One microgram of the pGL3-PTEN-3′-UTR firefly luciferase expression construct and 1 μg of a Renilla luciferase expression construct pRL-TK (Promega) were cotransfected with the miR-21 or control precursor or inhibitor. Luciferase assays were performed 48 hours after transfection using the dual Luciferase Reporter Assay System (Promega, Madison, WI). Firefly luciferase activity was normalized to Renilla luciferase activity for each sample. The results represent the mean and standard deviation of 8 separate determinations. (*B*) Mz-ChA-1 cells were transfected with 30 nmol/L miR-21 precursor miRNA or control miRNA. After 48 hours, media was replaced with CMRL medium containing 0.5% FBS and either 100 μmol/L LY294002, a PI 3-kinase specific inhibitor, or 20 μmol/L 1L-6-Hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate (Akt inhibitor). Cell lysates were obtained after 24 hours for immunoblot analysis of *PTEN*, phospho-Akt Ser(P)^{473}, and phospho-mTOR Ser(P)^{248}. The blots were stripped and reprobed for α-tubulin as a loading control and for quantitation. Representative immunoblots are shown along with quantitative data that show the mean ± standard error from 4 separate blots. $*P < .05$ relative to expression in the presence of miR-21 precursor. $**P < .05$ relative to expression in the presence of control precursor. (*C*) miR-21-dependent inhibition of gemcitabine-induced apoptosis is decreased by either the Akt inhibitor or the mTOR inhibitor rapamycin. Mz-ChA-1 cells were transfected with miR-21 or control precursor in CMRL medium with 10% FBS. After 48 hours, the medium was replaced with CMRL medium containing 0.5% FBS and 100 μmol/L gemcitabine, with or without 20 μmol/L Akt inhibitor, or 100 nmol/L rapamycin. Apoptosis was quantitated by DAPI staining after 48 hours. The results shown represent the mean ± SE from 3 separate experiments. $*P < .05$ relative to control precursor group.

pression may contribute to malignant transformation, the central role of miRNA in the regulation of gene expression indicates that aberrant miRNA expression may also determine transformed cell behavior. miRNA are likely to have a broad targeting ability because only a small number of nucleotide bases are needed to target mRNA. Thus, changes in expression of even a single miRNA can have a significant impact and result in diverse cellular effects by "re-programming" gene expression. The identification of alterations in miRNA expression in malignant cells in a tissue-specific manner is therefore likely to be essential to understand the mechanisms and role of altered gene expression in cancer. Thus, we sought to identify aberrantly expressed miRNA in malignant cells and to begin to define their potential relevance to tumor cell growth or chemotherapeutic resistance.

Our studies identify several miRNA that are consistently altered in expression in malignant cholangiocytes. It should be noted that these data were derived from cholangiocarcinoma cell lines, which may have a different profile from primary tumors. Thus, future studies to assess variations in miRNA profiles in primary tumors are warranted. Such studies should include analysis of an adequate number of samples, matched as far as possible for prior exposure to chemotherapy. Although we have focused our studies on miRNA that are differentially up-regulated in cancer cells, it is equally likely that down-regulated miRNA may also contribute to tumor cell behavior. However, we have observed a decrease in expression of several miRNA in all malignant cells. These observations are consistent with the identified role of miRNA in normal cellular differentiation and development and may potentially reflect a loss of cellular differentiation. These observations are similar to those made recently in other cancer cell types. Moreover, our findings add to the emerging body of evidence suggesting tissue-specific expression of miRNA. A cluster of miRNA is highly expressed in epithelial tumors originating from colon, pancreas, liver, and stomach (miR-192, miR-194, miR-215) and is thought to reflect a common developmental origin.[5] However, this cluster was not over-expressed in cholangiocarcinoma. Thus, cholangiocarcinoma-associated miRNA that are differentially up-regulated may play functional tissue-specific roles rather than recapitulate developmental origin.

There are important clinical implications of our observations. A group of miRNA, namely, miR-21, let7a-1, let7 f-2, let 7c, miR-320, and miR-16-1, are highly expressed in human cholangiocytes. Expression of these miRNA varies from that reported for hepatocytes. This group of miRNA, therefore, represents a "cholangiocyte-miRNA cluster," which may be helpful for developmental or cell type-specific cellular responses. miR-122 is highly overexpressed in the liver and has been implicated in the replication of hepatitis C virus replication.[39] However, expression of miR-122 in cholangiocytes is much lower. It is tempting to speculate that these differences may account for cell type-specific differences in viral persistence between hepatocytes and cholangiocytes. Other miRNA such as let-7i may contribute to cholangiocyte-specific responses to environmental pathogens such as *Cryptosporidium parvum*.

Techniques to isolate and amplify miRNA and to perform global miRNA expression profiling are rapidly advancing, making it likely that miRNA profiling in clinical tissues will become feasible.[5,40–42] The diagnosis of cholangiocarcinoma is often difficult because of histologic similarities with metastatic tumors from other primary epithelial tumors. Moreover, the diagnosis of malignancy in biliary tract strictures remains a perplexing clinical challenge because of the limitations of cytologic techniques to distinguish cancer cells. Assays based on profiling cancer-associated changes in miRNA expression or selective assays using highly over expressed miRNA may be potentially useful to diagnose cholangiocarcinoma. Thus, further studies to analyze the patterns of miRNA expression in human cholangiocarcinoma are warranted.

The mechanisms by which miRNA expression is altered in cholangiocarcinoma, or in other cancers, is a subject of considerable interest. Other than for miR-141, miRNA chromosomal sites were not associated with regions of chromosomal loss in biliary tract cancers. Because of the small size of miRNA genes, it is nevertheless possible that genetic abnormalities such as loss of heterozygosity or mutations may account for differential expression in tumor cells. Other mechanisms may include dysregulated expression by transcription factors such as c-Myc, which has been shown to modulate the expression of a cluster of miRNA.[21] Because miRNA are developmental regulators, differential expression of miRNA may reflect the effect of biliary tract differentiation factors.

Of the cholangiocarcinoma-associated miRNA, miR-141 appears to be an important regulator of tumor cell proliferation in vitro but does not appear to be involved in tumor cell responses to chemotherapeutic stress. Strategies to decrease miR-141 overexpression may be potentially useful to limit tumor growth, especially for tumors associated with regions of chromosomal gain involving the genomic location of miR-141. In addition, identifi-

cation of specific miR-141 gene targets involved in tumor cell proliferation may provide much needed insights into the mechanisms of growth regulation in cholangiocarcinoma.

Chemotherapeutic stress results in altered expression of a surprising number of diverse miRNA. These include miRNA such as miR-21 and miR-200b that are overexpressed in malignant cells and miRNA such as miR-145, miR-23a, and miR-34c that are not differentially expressed in cancer cells. We speculate that the former group of miRNA may be involved in survival responses that are altered in malignant cells, whereas the latter may reflect the general cellular response to death-inducing stimuli. However, detailed further evaluation will be necessary to assess whether or not an individual miRNA reflects a cause or a consequence of exposure to chemotherapy. miR-21 is overexpressed in many cancers and has been implicated as having a role in inhibiting apoptosis.[29] Increased miR-21 expression following exposure to cytotoxic drugs suggests that miR-21 may mediate a general response to adverse conditions that would promote cell death and supports a central role for this miRNA in malignant transformation. Characterization of miR-21-regulated survival mechanisms may eventually lead to improved therapeutic strategies. Although we have identified some potential targets of miR-21 and miR-200b, several others may exist, and their interactions will need to be studied. Because of the promiscuity of mRNA targets for individual miRNA, the cellular responses to chemotherapeutic stress in this experimental framework may be more complex than previously appreciated.

The modulation of aberrantly expressed miRNA may be a useful strategy to limit tumor cell proliferation or improve responses to cytotoxic therapies. Further work based on selected targets identified by our studies needs to be done to evaluate their efficacy in tumor models in vivo and to develop subsequently the miRNA targeting therapeutic strategies. Furthermore, the identification of specific miRNA gene targets that are involved in tumor cell behavior, such as CLOCK, *PTEN*, and PTPN12; elucidation of the mechanisms by which they are regulated; and their functional effects will provide potential targets to reduce chemoresistance and tumor growth. Knowledge of specific processes that are regulated in a miRNA manner and identification of critical targets for individual miRNA will yield useful information and novel insights into the mechanisms of tumorigenesis and chemoresistance in cholangiocarcinoma and other cancers.

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Address requests for reprints to: Tushar Patel, MBChB, Scott and White Clinic, Texas A&M University Health Science Center, 2401 South 31st St, Temple, Texas 76508. e-mail: tpatel@swmail.sw.org; fax: (254) 724 8276 or (254) 742 7181.

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