Profiling MicroRNA Expression in Hepatocellular Carcinoma Reveals MicroRNA-224 Up-regulation and Apoptosis Inhibitor-5 as a MicroRNA-224-specific Target*

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Like other cancers, aberrant gene regulation features significantly in hepatocellular carcinoma (HCC). MicroRNAs (miRNAs) were recently found to regulate gene expression at the post-transcriptional/translational levels. The expression profiles of 157 miRNAs were examined in 19 HCC patients, and 19 up-regulated and 3 down-regulated miRNAs were found to be associated with HCC. Putative gene targets of these 22 miRNAs were predicted in silico and were significantly enriched in 34 biological pathways, most of which are frequently dysregulated during carcinogenesis. Further characterization of microRNA-224 (miR-224), the most significantly up-regulated miRNA in HCC patients, revealed that miR-224 increases apoptotic cell death as well as proliferation and targets apoptosis inhibitor-5 (API-5) to inhibit API-5 transcript expression. Significantly, miR-224 expression was found to be inversely correlated with API-5 expression in HCC patients (p < 0.05). Hence, our findings define a true in vivo target of miR-224 and reaffirm the important role of miRNAs in the dysregulation of cellular processes that may ultimately lead to tumorigenesis.

Hepatocellular carcinoma (HCC) is among the top 10 most prevalent cancers worldwide (1), accounting for ~600,000 deaths annually (2). The overall 5-year survival rate for HCC patients is less than 5% (3). Chemotherapeutic interventions are ineffective, and surgical resection/liver transplantation is the only treatment modality to confer survival benefit in HCC patients. Late clinical presentations have also led to poor prognosis for HCC patients. It is thus necessary to elucidate the molecular mechanisms underlying HCC and identify novel therapeutic targets and biomarkers for the early detection of HCC.

Like other cancers, aberrant gene regulation features significantly in HCC. Several reports on gene expression profiling of HCC patients have identified numerous pathways (e.g. proliferation, cell cycle regulation, apoptosis, angiogenesis, etc.) that may be dysregulated during hepatocarcinogenesis (see review in Ref. 4). Recently, an increasing number of reports have described a new class of small noncoding RNAs that are implicated in the regulation of gene expression at the post-transcriptional and translational level. These regulators are termed microRNAs (miRNAs), and their dysregulation may have implications in carcinogenesis.

miRNAs represent a class of noncoding RNAs whose processed products are ~22 nucleotides in length and regulate gene expression in plants and animals (5). To date, more than 500 miRNAs are predicted to be expressed in humans (6, 7). These miRNAs are estimated to regulate the expression of >5000 human genes or ~30% of all human proteins (8). It is likely that the interaction between miRNAs and their numerous mRNA targets may represent an important level of gene regulatory control in the cell (5).

The importance of miRNAs in cancer is highlighted by the observation that ~50% of miRNA genes are located in cancer-associated genomic regions or fragile sites (9–11). Importantly, miRNA expression is frequently dysregulated in several cancers including B-cell chronic lymphocytic leukemia (9, 12), Burkitt lymphoma (13), colorectal cancer (14), lung cancer (15, 16), and hepatocellular carcinoma (17). Additionally, differential expression of miRNAs have been found to be associated with post-operative survival in lung cancer patients (15) and are diagnostic and prognostic markers of lung cancer (16), miRNAs have been implicated to play both tumor suppressor and oncogenic roles (18).

Although much is known about the profiles of miRNAs in the various tissues/developmental stages, embryonic stem cell dif-
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...ferentiation, brain development, hematopoietic lineage differentiation, as well as their deregulation in various cancers, less is known about the function of each of these miRNAs or their true cellular targets. Numerous algorithms have been developed to predict the putative cellular targets of these miRNAs including PicTar (19), miRBase Targets (7), and TargetScan (8). Some of the cellular targets that have been experimentally validated for the various miRNAs are HoxB8 (miR-196) (20), Hand2 (miR-1) (21), E2F1 (miR-17–5p and miR-20a) (22), Hox-A11 (miR-181) (23), LATS2 (miR-372/3) (24), Rb1 (miR-106a), TGFBR2 (miR-20a) (25), as well as IRAK1 and TRAF6 (miR-146) (26). Here, we characterized the expression profiles of 157 miRNAs in 19 HCC patients and identified a set of significantly differentially expressed miRNAs associated with HCC. We also functionally characterized one of the differentially expressed miRNAs and identified its gene target.

**EXPERIMENTAL PROCEDURES**

**Samples**—Paired tumorous and adjacent nontumorous liver tissues from 19 hepatocellular carcinoma patients were obtained from the National Cancer Centre of the Singapore Tissue Repository with prior approval from the National Cancer Centre Institutional Review Board.

**miRNA Extraction and Expression Profiling**—The MirVana™ miRNA isolation kit (Ambion, Austin, TX) was used to isolate total RNA including low molecular weight RNA from patient samples and cell lines according to the manufacturer’s protocol. Expression of 157 verified human microRNAs was analyzed using the TaqMan MicroRNA assay human panel early access kit (Applied Biosystems), according to the manufacturer’s instructions as previously described (27). Briefly, patient RNA samples were used as template for reverse transcription. Together with the high capacity cDNA archive kit, RNase inhibitors, and miRNA-specific reverse transcription primers (Applied Biosystems), the reverse transcription reactions were carried out in a 96-well plate format. Real time PCR was then performed with the reverse transcription products, TaqMan 2× Universal PCR Master Mix without UNG Amplerase (Applied Biosystems), miRNA-specific TaqMan probes, and primers (Applied Biosystems) on an Applied Biosystems 7500 Fast Real Time PCR system with an initial denaturation at 95 °C, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The threshold cycle (Ct) was then defined and determined as the fractional cycle number at which the fluorescence detected passes a fixed threshold. The Applied Biosystems 7500 Fast software was used to analyze the Ct values of different miRNAs normalized to an endogenous control (let-7a or U6). The normalized values (ΔΔCt) from tumorous tissue were then compared with its paired nontumorous tissue, yielding miRNA differential expression profiles.

**In Silico Analyses of miRNA Expression Data, Identification of Putative miRNA Targets, and Categorization of Biological Processes of These Putative miRNA Targets**—Relative quantitation of the expression of miRNA was determined using the 2−ΔΔCt method (28), and the results were expressed as log2 of the relative quantity (RQ) of the target miRNA normalized against hsa-let-7a (log2RQ). Differentially expressed miRNAs were identified by significant analysis of microarrays (29), with the false discovery rate threshold set at <5%. Clustering and visualization of the normalized data were performed with Cluster and TreeView (30), using average linkage and Pearson’s correlation as a measurement for similarity. Computational identification of the putative miRNA targets was performed using PicTar (19), miRBase Targets Version 3.0 (7), and TargetScan Release 3.0 (8). A gene was considered to be a putative target of a given miRNA only if it was predicted by at least two of the three methods. Categorization of the biological processes of the putative miRNA gene target was performed with gene ontology (GO) using the Database for Annotation, Visualization, and Integrated Discovery GoCharts module (31), at level 5 annotations.

**Validation of the Expression of miR-224 in Tumors of HCC Patients**—Northern blot analysis was performed to validate miR-224 overexpression in the tumors of HCC patients. Briefly, 1 μg of total RNA (HCT116 cells transfected with Pre-miRTM miR-224 precursor) or 2 μg of low molecular weight RNA from both the tumor and adjacent nontumorous tissues of a few of the same HCC patients that were previously profiled was separated on a 15% denaturing polyacrylamide gel and electroblotted onto a nylon membrane (Schleicher & Schuell GmbH; Dassel, Germany) at 300 mA for 30 min. The miRNA-224 probe (5’-TAAACGGAACCACTAGTGACTTG-3’ (17)) and U6-probe (5’-ATGTGCTGCGAAGCGACAC-3’) were end-labeled with Redivue [γ-32P]ATP (Amersham Biosciences) using T4 Polynucleotide Kinase (New England Biolabs, Ipswich, MA) and purified using the nucleotide removal kit according to the manufacturer’s instructions (Qiagen). Hybridization was performed using Express Hybridization solution (Clontech, Mountain View, CA) at 42 °C for 16 h, and the blots were exposed to Hyperfilm MP (Amersham Biosciences).

**Growth and Viability of HCT116 Cells Transfected with miR-224**—The human colon cancer cell line, HCT116, was grown in McCoy’s medium supplemented with 10% fetal calf serum at 37 °C in a humidified atmosphere of 5% CO2. To determine the effect of miR-224 on growth and viability properties of HCT116 cells, 4 × 105 cells were transfected with either 30 nm of Pre-miRTM miR-224 precursor molecule (miR-224 precursor) or 30 nm of Pre-miRTM miRNA Precursor Molecules-Negative Control 1 (Control) (AM17110) (Ambion) using siPORT™ amine transfection agent (Ambion) following the manufacturer’s protocol. The time when transfection commenced was considered as time 0. Sixteen hours after incubation in medium containing siPORT™ amine transfection agent, these transfected cells were transferred into normal growth medium. Viable cells were determined through trypan blue dye exclusion. The growth properties of these cells were expressed as percentages of viable cells at the respective time points relative to time 0. Viability of these cells was expressed as the percentages of viable cells relative to the total number of cells (both dead and alive) at each individual time point. The results were obtained by counting cells from the same experiment twice in three independent experiments.

**Apoptotic and Cell Proliferation Properties of HCT116 Cells Transfected with miR-224**—HCT116 cells were transfected using siPORT™ amine transfection agent with either 60 nm control or 30 nm miR-224 precursor and 30 nm control or 30 nm
miR-224 precursor and 30 nm anti-miRTM miR-224 inhibitor (miR-224 inhibitor; AM17000, Ambion). Apoptosis assay was performed 48 h post-transfection using the Annexin V-PE apoptosis detection kit I (BD Biosciences) according to the manufacturer’s protocols and analyzed using a FACSCalibur flow cytometer (BD Biosciences). Apoptotic cells were represented by high PE-Annexin V and low 7-aminactinomycin fluorescence signals. Cell proliferation assays were performed 24 h post-transfection using the BrdUrd cell proliferation assay kit (Calbiochem, San Diego, CA) following the manufacturer’s protocol. BrdUrd incorporation was measured as absorbance at A450 in a SpectroMAX PLUS microplate reader (Molecular Devices, Sunnyvale, CA) 48 h after it was added to the cells.

**Apoptotic Properties of Primary Liver Cell Line Immortalized with SV40 T-antigen, THLE-3, Transfected with miR-224 Inhibitor**—To evaluate whether miR-224 influence the apoptotic potential of a primary liver cell line transformed with SV40 T-antigen, THLE-3 cells were transfected in collagen-coated plates with either 60 nm control or 60 nm miR-224 inhibitor using siPORT™ amine transfection agent. The transfected cells were then treated with ~20 J/m² UV 24 h post-transfection, and apoptosis assay was performed 48 h after transfection.

**Generation of the miR-224 Target, API-5 3'-UTR-Reporter Construct**—To experimentally validate whether the API-5 gene is an in vivo target of miR-224, the 3'-UTR of the API-5 gene was amplified from nontumorous human liver tissue using primers API5primer1 and API5primer2 as shown in supplemental Fig. S1. The 3'-UTR was then cloned downstream to a β-galactosidase (β-gal) reporter gene driven by the human multidrug resistance-associated protein 1 (MRP1) promoter in a construct that also contained the enhanced green fluorescence protein (EGFP) gene for normalization of transfection efficiency (32) (see Fig. 3B). The human MRP1 promoter was chosen over the constitutive human cytomegalovirus promoter, because the MRP1 promoter is ~30 times weaker than the cytomegalovirus promoter (data not shown), which will facilitate the measurement of subtle changes in reporter gene activity. A mutant pAPI5-3UTR-MUT was also generated by PCR mutagenesis using primers as shown in supplemental Fig. S1. Three point mutations were generated on each of the three miR-224 target recognition sites/seed as shown in Fig. 3C. These mutant recognition sites were verified in silico not to bind to any of the known human miRNAs using miRBase (Release 8.1, May 2006). The mutant construct generated was confirmed by sequencing.

**Characterization of the Effect of miR224 on API-5 3'-UTR-Reporter Construct**—HCT116 cells were transfected in 6-well plates by using siPORT™ amine transfection agent (Ambion, Austin, TX) according to the manufacturer’s instructions with either 1.0 μg of the β-gal reporter construct containing the wild type 2035-bp 3'-UTR sequence of human API-5 (termed pAPI5-3UTR-WT) or β-gal reporter construct containing the mutant 3'-UTR sequence of human API-5 (termed pAPI5-3UTR-MUT) and co-transfected with either 30 nm of miR-224 precursor (Ambion) or 30 nm of control (Ambion). β-Gal reporter gene activity was also assayed kinetically using chlorophenol red-β-D-galactopyranoside as substrate and measured at 1-min intervals over 60 min at 570 nm in a SpectraMax PLUS microplate reader (Molecular Devices) with crude lysate from the transfected cells harvested 24 h post-transfection. To normalize for differences in transfection efficiencies, Western blot analyses were performed using 0.02 μg/ml mouse anti-EGFP (Roche Applied Science) and 1:100,000 horseradish peroxidase-conjugated goat anti-mouse (Pierce) secondary antibodies. β-Gal activity was then normalized against EGFP expression levels. The data was also normalized against differences in basal β-gal activity when either the pAPI5-3UTR-WT or pAPI5-3UTR-MT construct, but not miR-224 precursor or Control, was transfected.

**Quantitation of the API-5 mRNA Levels in Patient Samples and HCT116/THLE-3 Cells Transfected with miR-224**—Reverse transcription real time PCR was performed to quantitate the API-5 transcript levels in patient samples and transfected HCT116/THLE-3 cells. cDNA was synthesized from total RNA using a high capacity cDNA archive kit (Applied Biosystems) according to the manufacturer’s instructions. Real time PCR was performed in an Applied Biosystems 7500 real time PCR system using the Quantitect™ SYBR Green PCR kit (Qiagen). Amplification reactions included cDNA template (25 ng), API-5 primers (forward, 5'-TAGTGGGTTTGGAAAAGTTC-3'; reverse, 5'-TCATTTGATAGGCATCTTTATG-3') (0.25 pmol/μl), and 2× PCR Master Mix (5 μl; Qiagen) in a total volume of 10 μl. Amplification conditions include an initial denaturation at 95 °C for 15 min, followed by 40 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. SYBR Green fluorescence was measured after each extension step.

**Statistical Analysis of Experimental Data**—Student’s t test was performed to analyze the significance of differences between sample means obtained from at least three experiments.

**RESULTS**

**miRNA Expression Profiling Identifies Dysregulation of miRNAs That Are Associated with HCC**—In this study, the expression profiles of 157 mature miRNAs were determined in 19 HCC tumors and adjacent nontumorous liver tissues using the TaqMan MicroRNA assays human panel early access kit (Applied Biosystems) and normalized against hsa-let-7a as recommended by the manufacturer. Similar results were obtained when the same data set was also normalized against U6 RNA levels (data not shown) (27). Of the 157 miRNAs, 133 exhibited differential expression in at least 50% of the HCC patients, and these are presented in Fig. 1A as a TreeView Heat Map. From the TreeView Heat Map, there seem to be clusters of 20 miRNAs (red box) and 14 miRNAs (green box) that were up-regulated and down-regulated, respectively, in the tumors of HCC patients. The trends of differential expression of some of these miRNAs (miR-199a, miR-200a, miR-125a, and miR-224) were consistent with those observed in the only other previous report on miRNA expression in HCC (17). Significance analysis of microarrays (21) was then utilized to identify miRNAs that displayed significant differential expression between the tumor and adjacent nontumorous liver tissues of HCC patients (supplemental Fig. S2). When the false discovery rate was set to <5%, only 19 miRNAs were found to be significantly up-regulated, whereas three were determined to be significantly down-regulated, with the mean fold change of the most highly up-reg-
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TABLE 1. miRNAs Differentially Regulated between Tumor and Adjacent Nontumorous Liver Tissue from HCC Patients

| miRNA   | Mean fold change (log2RQ) | No. of predicted targets |
|---------|--------------------------|--------------------------|
| hsa-miR-224 | 3.50                   | 114                      |
| hsa-miR-182 | 3.46                   | 447                      |
| hsa-miR-183 | 3.36                   | 201                      |
| hsa-miR-96  | 2.64                   | 498                      |
| hsa-miR-9*   | 2.61                   | 46                       |
| hsa-miR-9    | 2.56                   | 538                      |
| hsa-miR-222 | 2.32                   | 107                      |
| hsa-miR-216 | 2.10                   | 77                       |
| hsa-miR-21  | 2.07                   | 125                      |
| hsa-miR-186 | 2.01                   | 136                      |
| hsa-miR-301 | 1.59                   | 315                      |
| hsa-miR-221 | 1.57                   | 115                      |
| hsa-miR-155 | 1.44                   | 151                      |
| hsa-miR-182* | 1.44                 | 12                       |
| hsa-miR-137 | 1.34                   | 307                      |
| hsa-miR-25  | 1.32                   | 229                      |
| hsa-miR-324-5p | 1.28             | 32                       |
| hsa-miR-151 | 1.26                   | 22                       |
| hsa-miR-374 | 1.23                   | 133                      |

**Significantly Up-regulated in Tumors (19)**

**Significantly Down-regulated in tumors (3)**

FIGURE 1. Profiles of miRNA expression in HCC patients. miRNA expression was examined between the HCC tumor and adjacent nontumorous liver tissues from 19 HCC patients as described under “Experimental Procedures.” A, hierarchical clustering of 133 miRNAs that are differentially expressed in the tumors of at least 50% of the 19 HCC patients. The different HCC patients are represented on the x axis, and the results are presented as the mean fold change in miRNA expression of tumor versus adjacent nontumorous tissue in each patient. Red represents miRNAs that are overexpressed in the tumors, whereas green represents miRNAs whose expression is down-regulated in tumors. The green box shows a cluster of miRNAs that are down-regulated in HCC tumors, whereas the red box display the cluster of miRNAs whose expression of are up-regulated in the tumor. B, top panel, hierarchical clustering of the 22 significantly up- and down-regulated miRNAs obtained using significance analysis of microarrays with false discovery rate of <.05. Bottom left panel, table of the 19 miRNAs that are significantly up-regulated in the tumors of HCC patients represented as mean fold change of tumor versus nontumorous tissue and expressed as log2RQ. Bottom right panel, table of the three miRNAs that are significantly down-regulated in the tumors of HCC patients represented as mean fold change of tumor versus nontumorous tissue and expressed as log2RQ.
transcription, regulation of progression through cell cycle and Wnt receptor signaling pathway (supplemental Table S1). 80% of these 35 biological pathways have been reported to be implicated in the carcinogenesis process (supplemental Table S1), strongly suggesting that the miRNAs we observed to be differentially expressed in HCC patients target genes whose dysregulation may play significant roles in carcinogenesis.

**miR-224 Overexpression Decreases Cell Viability and Sensitizes Cells to Apoptotic Cell Death**—To gain further insights into how dysregulation of these miRNAs may play a role in carcinogenesis, we further characterized miR-224, the most up-regulated miRNA in our study and the only miRNA that was also reported in another study to be up-regulated in HCC patients. Northern blot analysis confirmed that miR-224 was up-regulated in the tumors of HCC patients (Fig. 2A). The functional significance of increased miR-224 expression in the cells was evaluated by transfecting miR-224 precursor into the human colorectal HCT116 cells, which exhibit low endogenous miR-224 expression (data not shown). miR-224 expression peaked 24 h post-

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**FIGURE 2. Functional characterization of miR-224.** A, Northern blot analyses to validate the increased miR-224 expression in tumor tissues of representative HCC patients. *P* (number) represent the identity of the patients corresponding to the same patients in Fig. 1. 7 denotes tumor tissue from indicated HCC patients, whereas *N* denotes the paired nontumorous liver tissues. Control represents total RNA isolated from HCT116 cells 72 h after transfection with miR-224 precursor and hybridized with miR-224 probe. B, miR-224 expression in HCT116 cells. The left panel shows results from TaqMan MicroRNA individual assay for hsa-miR-224 (Applied Biosystems) in HCT-116 cells after transfection with miR-224 and normalization against hsa-Let-7a for the various time points. The results are expressed as the fold difference between miR-224 expression in miR-224 precursor transfected cells versus control transfected cells. The right panel shows Northern blot Analyses of untransfected HCT116 cells or HCT116 cells carrying control or miR-224 precursor, 72 h after transfection. C, left panel, cell growth expressed as percentage of viable cells at the respective time points relative to the transfection start time, time 0. miR-224 precursor transfected cells are represented by squares, and control transfected cells are represented by triangles. Right panel, viability of cells expressed as the percentage of viable cells relative to the total number of cells at a particular time point. The results were obtained by counting cells from the same experiment twice in three independent experiments. D, panel i, miRNA expression of cells transfected with control, miR-224 precursors, or miR-224 precursors and miR-224 inhibitors. Panel ii, relative apoptosis of similarly treated cells, as assayed using PE-conjugated annexin V staining. Apoptotic cells were detected as high in PE-annexin V staining and low in 7-amino-actinomycin (7-AAD) staining. The profiles shown are representative from three independent experiments. Panel iii, relative cell proliferation of similarly treated cells assayed using the BrdUrd proliferation assay kit. In B–D, data are from at least three independent experiments and shown as the means ± S.E. *, *p < 0.05; **, *p < 0.01; ***, denotes *p < 0.001.
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transfection, but its expression remained high (>100-fold higher than that of Control transfected cells) even at 168 h post-transfection (Fig. 2B). Cell growth, viability, and apoptotic profiles, some of the primary hallmarks of cancer (37), were examined on miR-224 expressing cells. Cell growth remained generally unaffected in cells transfected with either miR-224 precursor or Control (Fig. 2C, left panel), but cell viability was significantly reduced (up to 20% reduced cell viability; p < 0.05) in cells transfected with miR-224 precursor compared with cells transfected with Control (Fig. 2C, right panel). These data suggest that miR-224 seems to affect the viability rather than the growth of these cells.

Annexin V labeling was performed to delineate whether the reduced viability of cells overexpressing miR-224 is due to increased cell death through apoptosis. miR-224 precursor- or control-transfected HCT116 cells were harvested 48 h post-transfection, stained with PE-conjugated annexin V and 7-amino-actinomycin, and analyzed on the flow cytometer. Apoptotic cells are represented as cells with high PE-conjugated annexin V fluorescence signals and low 7-amino-actinomycin fluorescence signals. As shown in Fig. 2D (panel ii), ~2-fold greater apoptosis was observed in cells expressing miR-224 than in cells expressing the control precursors (p < 0.001). Significantly, when miR-224 inhibitor was introduced into cells overexpressing miR-224, the miR-224 expression was significantly reduced (p < 0.0001) (Fig. 2D, panel i), and significantly less apoptosis (p < 0.001) (Fig. 2D, panel ii) was observed, consistent with cells not overexpressing miR-224. Hence, the reduced cell viability observed in miR-224-expressing cells is likely due to increased apoptosis of these cells in the presence of miR-224 as evident from the increased apoptosis observed in miR-224-overexpressing cells and the restoration of its normal phenotype when miR-224 inhibitor was also introduced.

miR-224 Overexpression Increases Cell Proliferation—Curiously, although miR-224 overexpression reduced cell viability (Fig. 2C, right panel) and increased apoptosis (Fig. 2D, panel ii), it does not seem to affect cell growth (Fig. 2C, left panel) as measured by the percentage of viable cells at each time point relative to time 0. We thus hypothesize that this observation could be due to miR-224, also increasing cell proliferation in addition to increasing cell apoptosis, thus resulting in a similar number of viable cells in miR-224-expressing cells compared with control cells at any time point. The proliferation potential of these cells was then examined using the BrdUrd incorporation assay. As shown in Fig. 2D (panel iii), transfection of miR-224 precursor into these cells significantly increased the proliferation potential of these cells (p < 0.001), whereas the co-transfection of the miR-224 inhibitor with the miR-224 precursors restored the cell proliferation potential to normal.

miR-224 Targets API-5—To elucidate the gene target that miR-224 acts through to sensitize cells to apoptosis, we examined the putative targets of miR-224 more closely and shortlisted seven (H3F3B, API-5, ARM2X2, NUP153, FOSB, TRIM9, and HOXD10) putative targets that ranked among the top five in at least one in silico target prediction program and is also identified by at least one other prediction program (Fig. 3A). These seven putative targets were found to be involved in several biological processes that are significantly over-represented. FOSB and HOXD10 function as transcriptional regulators. HOXD10 has also been implicated as a tumor suppressor (38) and inhibitor of angiogenesis (39), whereas API-5 has been shown to be an apoptosis regulator (40–42). Other significant biological processes that involve these putative genes include chromosome organization for H3F3B (43), microtubule binding for TRIM9 (33), and intracellular/nuclear transport for NUP153 (44). Although the gene targets of miR-224 reside in varied biological pathways that are true for any miRNA (35), the pathways of nearly all of the top seven miR-224 gene targets are primarily those most associated with cancer, namely transcription, cell cycle, or apoptosis regulation and nucleus/chromosomal organization. Of these seven top miR-224 gene targets, API-5 seems to be the most appropriate candidate through which miR-224 may act to increase apoptosis in cells because API-5 has previously been reported to be an anti-apoptotic gene (40–42).

We proceeded to validate that API-5 was in fact a true gene target of miR-224. Three miR-224-binding sites were identified along the 2035-bp-long 3' UTR of API-5 (miRBase release 8.1, May 2006) (Fig. 3C). The entire wild type 3' UTR of API-5 as well as a mutant form in which all three putative miR-224-binding sites were mutated was then cloned downstream the β-gal reporter gene (Fig. 3B). The specific interaction between miR-224 and API-5 3' UTR to inhibit reporter gene activity was evident in Fig. 3D. When miR-224 precursor was co-transfected with wild type API-5 3' UTR reporter construct, significantly reduced β-gal activity was observed compared with cells co-transfected with miR-224 precursor and mutant API-5 3' UTR reporter construct (p < 0.01). No significant difference in β-gal activity was observed between HCT116 cells carrying either the wild type or mutant API-5 3' UTR reporter construct when co-transfected with control precursor molecules (Fig. 3D).

To evaluate whether miR-224 can affect the endogenous expression of API-5, HCT116 cells were initially transfected with either miR-224 precursor or control. miR-224 inhibitor or control were then introduced into these transfected cells 24 h later, and reverse transcription real time PCR was performed at various time points after this second transfection. As shown in Fig. 3E, API-5 expression was significantly lower in miR-224 precursor transfected HCT116 cells compared with the control cells (p < 0.001) across time points examined, consistent with previous reports that miRNAs can also down-regulate gene expression at the transcript level (45). When miR-224 inhibitor was transfected into miR-224-overexpressing cells, the API-5 transcript levels significant increased (p < 0.001) at the two time points examined, suggesting that the miR-224 inhibitor was able to rescue the inhibition of API-5 expression by miR-224. This observation that miR-224 decreased API-5 expression corroborates with our earlier observation that miR-224 increases apoptotic cell death and strongly suggests that miR-224 exerts this effect through regulating the expression of API-5.

Inhibition of the Endogenous Expression of miR-224 Up-regulates API-5 Expression and Reduced Apoptotic Cell Death in the Immortalized Primary Liver Cell Line, THLE-3—Thus far, we observed that miR-224 is overexpressed in the majority of
HCC patients examined and demonstrated experimentally that
the overexpression of miR-224 in HCT116 cells resulted
increased apoptosis and reduced expression of the apoptosis
inhibitor, API-5 gene. We proceeded to evaluate the functional
consequences of inhibiting the endogenous expression of miR-
224 in an immortalized primary liver cell line, THLE-3. The
introduction of miR-224 inhibitor into THLE-3 cells was found
to significantly inhibit the endogenous expression of miR-224
in these cells (p < 0.001) (Fig. 4A, top panel) and significantly
increased the expression of the API-5 gene (Fig. 4A, bottom
panel). Inhibiting endogenous miR-224 expression in the
THLE-3 primary liver cells was also found to protect these cells
from UV-induced apoptotic cell death (p < 0.05) (Fig. 4B).

The Level of API-5 mRNA Expression Was Inversely Correlated
with miR-224 Expression in 18 HCC Patients—We pro-
cceeded to examine whether there is any correlation between
miR-224 and API-5 expression in HCC patients to evaluate the
clinical significance of our observations. As evident in Fig. 6, a
statistically significant inverse correlation was observed
between miR-224 and API-5 expression in HCC patients (Pear-
son Coefficient r = −0.471, R^2 = 0.221 at p < 0.05).

DISCUSSION

MicroRNAs have recently been implicated to play important
roles in cancers because >50% of miRNA genes reside in can-
cer-associated genomic regions, and their expression has been
found to be dysregulated in various cancers. Thus far, only one
study examined miRNA profiles in HCC patients, and they
identified five miRNAs that were significantly down-regulated
(miR-199a, miR-199a*, miR-200a, miR-125a, and miR195) and
three that were significantly up-regulated (miR-224, miR-18,
and miR-p18) (17). miR-18 and miR-p18 were not examined in
this study, whereas miR-199a, miR-199a*, miR-200a, miR-125a,
and miR-195 were also generally down-regulated in our study,
although they were not statistically significant. Only miR-224 was also found to be significantly up-regulated in our study.

Our study identified 19 up-regulated and three down-regulated miRNAs that may be associated with HCC. Interestingly, four of the 19 significantly up-regulated miRNAs (miR-182, miR-182*, miR-183, and miR-96) reside together on chromosome 7q32.2 in the intergenic region between two protein coding genes, namely the transcription factor α palindrome-binding protein, α-pal (also known as nuclear respiratory factor 1) and the ubiquitin-conjugating enzyme, E2H. The coordinate up-regulation of these miRNAs that resides within the same cluster suggests that this region of chromosome 7 may be amplified. A search through the Comparative Genome Hybridization data base revealed that gain of chromosome 7 is commonly observed in HCC and other cancers including breast, prostate, colorectal, gastro-esophageal, lung and bronchial, head and neck, as well as aggressive lymphomas. Such coordinate expression of several miRNA and/or genes was also observed at the miR-17–92 cluster whose expression is up-regulated in lung cancer and plays a role in cell proliferation (46). However, further examination of nuclear respiratory factor 1 and E2H transcript expression in these HCC patients revealed that these genes were not significantly overexpressed in the tumors of these patients (data not shown).

Although our study found that more miRNAs are up-regulated in HCC patients, another study that examined 334 tissues from patients with different cancers found that there is a general trend of down-regulation of miRNA expression (47). Nonetheless, a recent study that examined 540 samples from patients with different cancers identified a solid tumor miRNA signature that primarily comprises up-regulated miRNAs (25).

Some of the miRNAs that we observed to be dysregulated in HCC patients exhibited similar trends of dysregulation in other cancers. miR-96 and miR-183 were also found to be up-regulated in colorectal cancer (27), whereas miR-21 and miR-155 were similarly overexpressed in breast, lung, and colon cancers (25). miR-224 was also up-regulated in HCC patients in another report (17), and miR-221 was also up-regulated in colon, pancreas, and stomach tumors (25). These observations suggest a common miRNA regulation pathway shared by different solid tumors.

FIGURE 4. Inhibiting endogenous miR-224 in THLE-3, an immortalized primary liver cell line, increased API-5 expression and decreased number of apoptotic cells. A, miR-224 (top panel) and API-5 (bottom panel) expression in THLE-3 cells transfected with either control or miR-224 inhibitor. B, relative apoptosis of THLE-3 cells transfected with either control or miR-224 inhibitor. The results show data from at least three independent experiments, expressed as the means ± S.E.*, p < 0.05; **, p < 0.01; ***, p < 0.001.
The biological pathways that are affected through the dysregulation of these 22 miRNAs were further examined. Gene targets for all 22 miRNAs were predicted in silico and GO/DaBase for Annotation, Visualization, and Integrated Discovery was employed to functionally annotate these gene targets. We found that nearly 50% of the nonoverlapping gene targets reside within 34 biological processes. 80% of these 34 biological processes has been implicated in carcinogenesis (supplemental Table S1) including proliferation (GO terms: regulation of nucleobase, nucleoside, nucleotide, and nucleic acid metabolism; regulation of progression through cell cycle; cell growth; positive regulation of cellular metabolism; cell death (GO term: apoptosis); metastasis (GO term: cell migration); and Wnt (48) and Notch (49) signaling pathways.

To gain a better understanding of how dysregulation of these miRNAs may play a role in carcinogenesis, we further characterized miR-224, the most up-regulated miRNA in our study. We observed that HCT116 cells overexpressing miR-224 exhibited similar rate of cell growth but significantly lower cell viability than control cells (Fig. 2C). This decrease in cell viability was found to be due to a significant increase of apoptosis in miR-224-expressing cells that can be reversed by the addition of miR-224 inhibitors (Fig. 2D, panel ii). Interestingly, miR-224-overexpressing cells also significantly increase the proliferation potential of the cells, which can be reversed by the addition of miR-224 inhibitors (Fig. 2D, panel iii). The ability of a single gene to be involved in both proliferation and apoptosis has been previously demonstrated (e.g. myc) (50).

To elucidate the gene target of miR-224 that may play a role in regulating apoptosis and hence account for the phenotype we observed, we shortlisted seven putative miR-224 gene targets that are ranked highly by at least one prediction program (Fig. 3A). Of these seven putative targets, apoptosis inhibitor 5 (API-5), also known as AAC-11, seems to be the most likely candidate because API-5 was reported as an anti-apoptotic gene (40–42). Three potential miR-224-binding sites were identified at the 3’-UTR of API-5 (Fig. 3C). Our results showed that miR-224 specifically interacted with the 3’-UTR of API-5 to inhibit reporter activity because no inhibition of reporter activity was observed when the miR-224-binding sites in API-5 3’-UTR was mutated (Fig. 3D). We further demonstrated that miR-224 can inhibit endogenous API-5 expression (Fig. 3E), which is consistent with our earlier observation that increased miR-224 expression leads to increased cell death (Fig. 2D) because API-5 is an anti-apoptotic gene.

Earlier studies suggest that mRNA acts at the translational level because only significant differences in the protein but not the mRNA levels were observed (51). Our observations that increased miR-224 reduced the endogenous API-5 mRNA levels in cells thus suggest that it is likely that miR-224 inhibits API-5 expression at the post-transcriptional level by degrading API-5 transcript, resulting in reduced steady-state API-5 transcript levels (Fig. 3E). Reduced API-5 levels will then sensitize these cells to apoptosis (Fig. 2D).

To evaluate the functional consequences of inhibiting the endogenous expression of miR-224 in primary liver cells, miR-224 inhibitor was transfected into THLE-3 cells, an immortalized primary liver cell line. Inhibiting miR-224 expression in these primary liver cells significantly inhibited ($p < 0.001$) the endogenous expression of miR-224 in these cells (Fig. 4A, top panel) and significantly increased the physiological expression of the API-5 gene (Fig. 4A, bottom panel). Notably, reduced physiological levels of miR-224 expression in the THLE-3 primary liver cells was also found to protect these cells from UV-induced apoptotic cell death ($p < 0.05$) (Fig. 4B).

Taken together, our data suggest that miR-224 influences both the proliferation and apoptotic potential of cells. It has been proposed that a single miRNA may regulate different unrelated target genes to control opposing activities like cell proliferation and apoptosis (52). miR-224 may thus represent such a miRNA. The regulation of the cell proliferation potential is likely to be mediated by a target gene of miR-224, which is currently unknown, whereas the regulation of the apoptotic potential is likely to be mediated via API-5, a known gene that regulates apoptosis (Fig. 5). API-5 was previously reported to act downstream E2F and inhibit E2F-dependent apoptosis without affecting E2F-dependent transcription (42). We thus hypothesize that miR-224 may influence E2F-dependent apoptosis via API-5.

Notably, there is a significant inverse correlation between miR-224 expression and API-5 expression in HCC patients ($p < 0.05$) (Fig. 6). The significant correlation between miR-224 and API-5 in HCC patients lends credence to the experimental observation that miR-224 negatively regulates API-5 expression and highlights the clinical relevance of this observation.

The observation that miR-224 is overexpressed in the tumors of HCC patients and that it plays a role in sensitizing cells to apoptosis via the inhibition of API-5 expression seems to contradict conventional wisdom that apoptosis is reduced during carcinogenesis. Nonetheless, similar to miR-224, oncoproteins such as c-Myc and E1A were found to sensitize cells to apoptosis upon minor insults that normal cells can usually resist, for example, serum depletion, DNA-damaging agents, hypoxia, etc. (53, 54). Oncogenic changes that promote apoptosis are thought to provide the selective pressure for cells to override apoptosis during the multitarget process of carcinogenesis (53), resulting in a resistant population of cells that accumulate heritable genetic mutations (55) during its increased lifespan, thus facilitating oncogenic transformation. The dual role of miR-224 to influence both cell proliferation and cell death simultaneously may thus potentially hasten this selection process favoring cells that accumulate sufficient heritable genetic mutations to override apoptosis during carcinogenesis.

In summary, we have identified 19 up-regulated and three down-regulated miRNAs that are associated with HCC. Pre-
miR-224 Is Up-regulated in HCC and Targets the API-5 Gene

dicted putative gene targets of these differentially expressed miRNAs in HCC were found to belong to 34 significant biological processes, most of which have been implicated in carcinogenesis. Further characterization of one of these dysregulated miRNAs revealed that miR-224, which is up-regulated in the tumors of HCC patients, sensitizes cells to apoptosis through the inhibition of API-5 at the mRNA levels and increases cell proliferation. Significantly, miR-224 expression was found to be inversely correlated with API-5 expression in HCC patients. These findings reaffirm the important role of miRNAs in regulating gene expression and suggest that dysregulation of the expression of miRNAs may lead to dysregulated target gene expression resulting in dysregulated cellular processes that may ultimately lead to tumorigenesis.

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