Anti-miR-21 Suppresses Hepatocellular Carcinoma Growth via Broad Transcriptional Network Deregulation

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Abstract

Hepatocellular carcinoma (HCC) remains a significant clinical challenge with few therapeutic options available to cancer patients. MicroRNA 21-5p (miR-21) has been shown to be upregulated in HCC, but the contribution of this oncomiR to the maintenance of tumorigenic phenotype in liver cancer remains poorly understood. We have developed potent and specific single-stranded oligonucleotide inhibitors of miR-21 (anti-miRNAs) and used them to interrogate dependency on miR-21 in a panel of liver cancer cell lines. Treatment with anti–miR-21, but not with a mismatch control anti-miRNA, resulted in significant derepression of direct targets of miR-21 and led to loss of viability in the majority of HCC cell lines tested. Robust induction of caspase activity, apoptosis, and necrosis was noted in anti-miR-21-treated HCC cells. Furthermore, ablation of miR-21 activity resulted in inhibition of HCC cell migration and suppression of clonogenic growth. To better understand the consequences of miR-21 suppression, global gene expression profiling was performed on anti-miR-21-treated liver cancer cells, which revealed striking enrichment in miR-21 target genes and deregulation of multiple growth-promoting pathways. Finally, in vivo dependency on miR-21 was observed in two separate HCC tumor xenograft models. In summary, these data establish a clear role for miR-21 in the maintenance of tumorigenic phenotype in HCC in vitro and in vivo.

Implications: miR-21 is important for the maintenance of the tumorigenic phenotype of HCC and represents a target for pharmacologic intervention. Mol Cancer Res; 13(6); 1009–21. ©2015 AACR.

Introduction

MicroRNAs (miRNAs) belong to the evolutionary conserved class of small noncoding RNAs whose dysfunction and dysregulation have been implicated in a variety of common human diseases ranging from fibrosis, diabetes, and heart disease to cancer (1). miRNAs function to posttranscriptionally modulate gene expression through sequence-specific base pairing with the 3′ untranslated region (UTR) of target mRNA, which leads to destabilization of the mRNA transcript, translational repression, and under certain conditions, upregulation of translation (2, 3). Rather than controlling expression of a single target gene, most miRNAs regulate entire gene networks and thus can influence a range of biologic processes, including cell cycle regulation, metabolism, development, and aging (4).

MicroRNA-21-5p (miR-21) is one of the small RNAs most frequently overexpressed in cancer, showing broad upregulation in a range of solid and hematologic malignancies (5, 6). Several studies have correlated elevated miR-21 expression with poor clinical outcomes (7). miR-21 is thought to act as an oncogene in part by repressing expression of a range of tumor suppressor genes related to metastasis, proliferation, and apoptosis (8, 9). The oncogenic role of miR-21 is supported by several studies in genetically engineered mouse models which demonstrated that overexpression of miR-21 results in the development of a pre–B-cell lymphoma, while loss of miR-21 suppresses tumor development in murine models of skin carcinogenesis and K-ras Non-Small Cell Lung Cancer (10–12). Mice homozygous for miR-21 deletion are fertile and appear phenotypically normal, indicating that miR-21 is dispensable under normal physiologic conditions, yet plays a pivotal prosurvival function during cellular oncogenic transformation (11, 12). In addition, a number of studies have demonstrated antiproliferative and antigrowth effects of miR-21 inhibitors in cancer cell lines in vitro (13–16).

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related death worldwide in men and sixth in women (17). When detected early, HCC is treatable by surgical resection or transplantation, yet most patients present with advanced disease and have limited treatment options due to lack of effective...
therapies. Genomic profiling has identified a number of micro-RNAs that are dysregulated in HCC, including miR-21 (18, 19). However, the precise contribution of miR-21 to the maintenance of oncogenic phenotype in HCC has not been fully established.

In this report, we evaluate the effects of miR-21 inhibition on growth and proliferation of a broad panel of HCC cell lines. Utilizing potent and specific single-strand oligonucleotide inhibitors of miR-21, we demonstrate that this oncomiR is required for survival, migration, and clonogenic outgrowth of the majority of HCC cells examined. Furthermore, inhibition of miR-21 leads to the onset of active cell death and re-expression of miR-21 targets, which may account for the antiproliferative phenotype elicited by anti–miR-21 treatment. Most importantly, we demonstrate for the first time that suppression of miR-21 activity in vivo results in significant inhibition of growth of HCC tumor xenografts. Taken together, our findings suggest that miR-21 plays an important role in promoting HCC tumorigenesis and that inhibition of this oncomiR could be beneficial for the treatment of liver cancer.

Materials and Methods

Cell culture

All cancer cell lines were obtained from the ATCC or JCRB. Normal human hepatocytes were obtained from Life Technologies. Short Tandem Repeat DNA (STR) profiling was performed to verify cell identity. Cells were grown in DMEM (Invitrogen), supplemented with 10% FBS (Invitrogen), and cultured at 37°C in a humidified incubator with 5% CO₂.

Anti-miRNA and miRNA mimic transfection

The miR-21 mimic and negative control mimic were synthesized by Regulus Therapeutics and were composed of a phosphorothioate backbone and a mixture of DNA, constrained ethyl phosphorothioate. The anti-miRNA compounds were synthesized by Regulus Therapeutics and were composed of a phosphorothioate backbone and a mixture of DNA, constrained ethyl phosphorothioate (cEt), 2′-O-methoxymethyl (2′-MOE), 2′-O-methoxyethyl (2′-MOE), or 2′-ribo-F modified nucleotides. The anti–miR-21 is complementary to the mature hsa-miR-21 (5′-UGACGGUAUACUGUAGUUGA-3′), while the mismatch (MM) control contains no complementarity to the miR-21 seed sequence. All transfections were performed using RNAiMAX (Life Technologies) according to the manufacturer’s recommendations, using between 0.15 μL and 0.3 μL of RNAiMAX per well of 96-well plate depending upon the cell line. The anti-miRNAs were diluted in OptiMEM (Life Technologies) to 10X their final concentrations and mixed with equal volumes of RNAiMAX diluted in OptiMEM. Twenty microliters of the anti-miRNA/RNAiMAX mixture was added per well of the 96-well plate, and an 80 μL aliquot of cells (5 × 10⁴–1 × 10⁵ cells/well) was plated. Anti-miRNA compounds were typically tested using a 6-point dose–response format using 2-fold dilutions from 50 nmol/L. Cell viability was assessed at 96 hours after transfection by adding 80 μL CellTiter-Glo (Promega) per well and incubating the plate for 10 minutes. Luciferase measurements were acquired on an Envision plate reader (PerkinElmer). IC₅₀ values were calculated using GraphPad Prism Software version 6.00 for Windows.

Migration assay

A total of 2 × 10⁴ SKHep1, Hep3B, or HepG2 cells were reverse transfected with anti–miR-21 or MM control and plated on collagen-coated 96-well plates in OptiMEM supplemented with 40 ng/mL hepatocyte growth factor (HGF; R&D Systems). At 24 hours after transfection, a pin scratch wound was generated with the Woundmaker (Essen Bioscience) according to the manufacturer’s recommendations. Wound closure was monitored and quantitated using the IncuCyte ZOOM system (Essen Bioscience).

miR-21 analysis in HCC and normal liver samples

Fresh-frozen tissues or optimal cutting temperature–embedded tissues of 207 HCCs and 46 adjacent matched normal liver were obtained from the Bio-Resource Center, Korea Biobank Network (2012–8 (51)) at the Asan Medical Center, Seoul, Republic of Korea, after approval from the Institutional Review Board (2012–3089). Representative frozen sections of HCCs and matched nonneoplastic liver tissues were reviewed in previous study (20). Total RNA containing microRNA was extracted using the mirVana miRNA Isolation kit (Life Technologies). Total RNA integrity was checked using an Agilent Technologies 2100 Bioanalyzer with a RNA integrity number value greater than 8. Small RNA sequencing libraries were prepared according to the manufacturer’s instructions (Illumina Small RNA Prep kit). Briefly, total RNA (1 μg per sample) was ligated with RNA3’ and RNA5’ RNA adapters. Reverse transcription followed by PCR was used to create DNA constructs based on the small RNA ligated 3’ and 5’ adapters. This process selectively enriches those fragments that have adapter molecules on both ends. PCR was performed with two primers that anneal to the ends of the adapters. cDNA was purified with Sage Science’s the Pippin prep electrophoresis platform. The quality of the libraries was verified by capillary electrophoresis (Bioanalyzer; Agilent). Following qPCR using SYBR Green PCR Master Mix (Applied Biosystems), index-tagged libraries were combined in equimolar amounts. Cluster generation occurred in the flow cell on the cBot–automated cluster generation system (Illumina). Sequencing was performed using HISEQ 2500 sequencing system (Illumina) using 1 × 50 bp read length.

Global gene expression profiling and bioinformatic analysis

SKHep1 cells were transfected with 20 nmol/L of anti–miR-21 or treated with PBS in triplicate. Total RNA was isolated from the cells at 16 hours after transfection and processed for microarray analysis (Expression Analysis). The gene expression dataset has been deposited into Gene Expression Omnibus (GEO) under study number GSE65892. The Student t test was performed for each gene between anti–miR-21-treated cells and PBS-treated cells using Array Studio software (OmicSoft). Only “expressed” genes, as defined by log2-transformed expression value greater than or equal to 6, were considered for analysis. To assess miR-21 target derepression after anti–miR-21 treatment, a sylamer plot analysis was performed using SylArray tool (https://www.ebi.ac.uk/ enright-srv/sylarray/search.html). Pathway analysis on significantly regulated genes in anti–miR-21-treated cells was performed using Ingenuity Pathway Analysis tool (Qiagen). miR-21 expression data in The Cancer Genome Atlas (TCGA) HCC dataset were downloaded from the TCGA Research Network (http://cancer-genome.nih.gov/). Sample composition and main characteristics of the Asan HCC dataset have been described previously (20). The expression of miR-21 was examined in TCGA and Asan HCC patient cohorts, which contain both tumor and normal samples. Waterfall plots were generated, and miR-21 expression was compared between tumor and normal samples using the Student t test (P value ≤ 0.05 was considered as significant).
Generation of sorafenib-resistant cell lines

SKHep1 and JHH4 cell lines were grown in the presence of 10 μmol/L sorafenib. Cells were subcultured and media were refreshed every 7 to 10 days. A polyclonal population of SKHep1 and JHH4 cells that proliferated in the presence of 10 μmol/L sorafenib emerged after 8 weeks of continuous sorafenib selection and was used for subsequent experiments.

Caspase activation and Annexin V staining

Cells were transfected using RNAiMAX with 25 to 50 nmol/L of anti-miRNA and incubated between 24 and 72 hours. Caspase 3/7 activity was quantitated using Caspase-Glo 3/7 Assay (Promega) according to the manufacturer's recommendation. Caspase luciferase values were normalized to relative cell number which was determined using CellTiter-Glo. For Annexin V staining, the cells were either treated with 2 μmol/L Camptothecin or transfected with anti–miR-21 or MM control in a 6-well plate. At 48 or 72 hours after transfection, the media and cells were collected and, following washes with PBS, the cells were stained with Annexin V (BD biosciences) according to the manufacturer's recommended protocol.

RNA isolation and quantitative real-time PCR analysis...

Micro and total RNAs were isolated with RNeasy or RNeasy 96 kit (Qiagen). cDNA synthesis was performed with High Capacity RNA to cDNA kit (Applied Biosystems). MicroRNA cDNA synthesis was performed with TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). Primer and probe sets are listed in Supplemental Table S2. TaqMan assays were performed on a Viia7 (Applied Biosystems) or a QuantStudio 12K Flex Real Time PCR System (Applied Biosystems) using the following conditions: 50°C for 2 minutes; 95°C for 10 minutes; 40 cycles of 95°C for 15 seconds; and 60°C for 1 minute. The expression of GAPDH or RPL37A was used for normalization of miRNA samples, whereas RNU48 was used for normalization of small RNA expression.

LDH and HMGB1 quantification...

Cells were transfected with 25 or 50 nmol/L of anti–miR-21 or MM control in 6-well format for the HMGB1 quantification and 96-well format for the LDH assay. Extracellular HMGB1 was quantified at 24, 48, and 72 hours after transfection from the supernatant using HMGB1 ELISA Kit (IBL International GMBH). HMGB1 ELISA was performed according to the manufacturer's recommendation using the high sensitivity range protocol. LDH activity was monitored using Cytoxicity Detection Kit Plus (LDH; Roche Applied Sciences). The LDH assay was performed according to the manufacturer's recommendations.

miR-21 in situ hybridization

Normal liver and HCC tissue blocks were obtained from Sanofi BioBank, an internal repository of commercially available tissue samples. 3'-5' DIG-labeled LNA-modified miR-21 probe and hybridization buffer were purchased from Exiqon. Tris-EDTA antigen retrieval solution (CC1), protease, mouse anti-DIG-HRP, 2x Saline Sodium Citrate (SSC), AmpBenzofozuran (AmpBF) kit, and ChromoMap DAB kit for detection were purchased from Ventana Medical Systems. Sections (5-μm-thick) were generated from formalin-fixed, parafin-embedded human liver tissue blocks. ISH was performed using a Discovery Ultra instrument from Ventana Medical Systems. Briefly, tissue sections were deparaaffinized, followed by antigen retrieval with CC1 buffer and protease treatment for 8 minutes at 37°C. DIG-labeled miR-21 probe was added at a concentration of 2.5 nmol/L and hybridized in Exiqon buffer at 58°C for 1 hour, followed by 3 washes at 60°C with 2× SSC, 1× SSC, and 0.5× SSC for 8 minutes each. After the washes, sections were incubated with mouse anti-DIG horseradish peroxidase (HRP) antibody for 32 minutes at 37°C. Antibody signal was amplified using tyramide-conjugated BF in the presence of H2O2 for 20 minutes followed by incubation with anti-BF HRP antibody for 16 minutes. HRP was visualized with the chromogenic substrate DAB, with the product appearing as a brown precipitate. Slides were counterstained with hematoxylin and mounted using permount. The samples were evaluated by board-certified pathologist (R. Baffa), and miR-21 expression was scored as "strong (+ + +)", "strong diffuse expression, "intermediate (+ +)", "moderate diffuse or multifocal expression, and "low (+)" for weak multifocal expression.

Anchorage-independent colony formation assay

Cells were transfected with 25 to 50 nmol/L of anti–miR-21 or MM control in 6-well plates and incubated for 24 hours. SeaPrep Agarose (Lonza) was dissolved in PBS to 6% (w/v) and allowed to cool to 37°C. The agar, transfected cells, and media were then mixed to achieve a final agar concentration of 1% and a final cell concentration of 50,000 to 100,000 cells/mL. Hundred microliter of cell/agar suspension was added per well of 96-well ultra-low attachment microplate (Corning). Plates were incubated for 7 to 10 days to allow for colony formation. Cell growth was quantitated by addition of 10 μL of AlamarBlue (Invitrogen) per well of plate and fluorescence was read on an Envision plate reader.

miR-21 tough decoy design

The sequences for the Null and miR-21 tough decoys are 5'-CGGCGCTAGGATCATCAATTG AACAGTGTATACGTGATCATC-3' and 5'-CCGCCTAGGATCATCAATTG AACAGTGTATACGTGATCATC-3'. miR-21 is upregulated in HCC tumors and hepatobiliary tumor cell lines. miR-21 is one of the most frequently upregulated miRNAs in human cancers, which acts to create a prosurvival environment through repression of apoptotic and growth-inhibiting pathways. To better understand the extent of miR-21 upregulation in liver
cancer, we examined miR-21 levels in 147 HCC and 50 normal liver samples catalogued in the publicly available database, TCGA. On average, miR-21 was found to be 2.64-fold higher in HCC samples when compared with the normal liver ($P$ value $= 4.06 \times 10^{-22}$). In a fraction of HCC samples (~10%), miR-21 levels were greater than 5-fold above normal liver tissue expression (Fig. 1A). To confirm these findings, we evaluated miR-21 expression in a second cohort of 207 HCC and 46 normal liver samples from Asan Medical Center (20). In agreement with the TCGA data, we observed a 2.5-fold increase in miR-21 expression in the HCC samples when compared with the normal liver ($P$ value $= 7.53 \times 10^{-18}$; Fig. 1A).

Next, we investigated miR-21 expression in a panel of human HCC and cholangiocarcinoma cell lines. Quantitative real-time PCR revealed that expression of mature miR-21 was strongly elevated in malignant cells when compared with normal human hepatocytes (Fig. 1B), thus confirming that high miR-21 levels are maintained in cultured HCC cells.

![Figure 1](image)

Figure 1.
miR-21 is upregulated in HCC tumors and cell lines. A, a plot of miR-21 expression was generated from 147 HCC and 50 normal liver samples catalogued in the publicly available database, TCGA and from 207 HCC and 46 normal liver samples from the Asan Medical Center. The $y$-axis indicates reads per million (RPM) of mapped miRNA reads in the TCGA data and read counts in the Asan data. In both datasets, the horizontal axis represents patients. Differences in miR-21 levels between HCC and adjacent normal liver were highly significant in both datasets (TCGA: $P$ value $= 4.06 \times 10^{-22}$; Asan: $P$ value $= 7.53 \times 10^{-18}$). B, small RNAs were isolated from HCC cell lines or normal hepatocytes, and mature miR-21 levels were measured by qPCR (mean ± SD, $n = 3$). C, normal liver and HCC samples were stained with miR-21 (brown) or scrambled probe, followed by a hematoxylin nuclei counterstain (blue). Intensity of miR-21 staining is indicated as strong (++) or intermediate (+). All samples are 20×.
To better understand the localization and potential intratumoral heterogeneity of miR-21 expression, we performed ISH analysis of miR-21 in 9 HCC and 3 normal liver samples. ISH using specific miR-21 probe demonstrated intense cytoplasmic staining in HCC samples, but not in normal liver parenchyma (Fig. 1C). Within the limited set of HCC samples examined, two were scored as "high," five were "intermediate," and two exhibited "low" miR-21 expression. Notably, HCC samples with "low" miR-21 expression still exhibited markedly elevated miR-21 staining when compared with the normal liver samples. Taken together, our results confirm and extend previous observations that miR-21 levels are elevated in HCC.

Inhibition of miR-21 leads to derepression of endogenous targets and antiproliferative effects in a panel of HCC cell lines

To understand the role of miR-21 in the maintenance of tumorigenic phenotype in HCC, we explored the effect of miR-21 inhibition on the growth and survival across a diverse panel of HCC and cholangiocarcinoma cell lines. To functionally inhibit miR-21, we employed a chemically modified single-stranded oligonucleotide fully complementary to the mature miR-21 sequence (anti–miR-21). Mechanistically, the anti-miRNA inhibits miR-21 by sequestering the miRNA, thus preventing it from associating with its cellular mRNA targets.

We first evaluated the effect of miR-21 inhibition on the expression of three putative miR-21 target genes, ANKR4D6, DDAH1, and RECK (13, 21, 22), using quantitative real-time PCR (qRT-PCR). Each of these genes possesses an octamer sequence motif within their 3′UTR complementary to the mature miR-21 seed. Transfection of anti–miR-21, but not mismatch (MM) control oligonucleotide, into HCC cell lines resulted in upregulation of ANKR4D6, DDAH1, and RECK (Fig. 2A and Supplementary Table S1). Consistent with context-dependent regulation of gene expression by miRNAs, not all cell lines responded equally to anti–miR-21 treatment; however, derepression of at least one target gene was observed in all HCC cell lines examined. Modest changes observed in individual miR-21 target genes upon treatment with anti–miR-21 (1.5–4 fold) are in line with the notion that miRNAs act on gene networks rather than eliciting changes of large magnitude in a single target. To further confirm that ANKR4D6, DDAH1, and RECK are regulated by miR-21 in HCC cell lines, we introduced miR-21 mimic into SKHeP1 cells and examined target modulation by qRT-PCR. As expected, miR-21 overexpression led to a reduction in ANKR4D6, DDAH1, and RECK mRNA levels, thus confirming that these targets are controlled by miR-21 (Supplementary Fig. S1).

Next, we explored the effects of miR-21 inhibition on HCC cell viability and proliferation. Cells were transfected with anti–miR-21 or MM control, and cell viability was monitored at defined time points. Anti–miR-21 treatment potently suppressed HCC cell growth with IC50 that ranged between 10 and 40 nmol/L (Fig. 2B). The majority of the cell lines tested (14/18) exhibited >60% reduction in cell viability at the highest dose of anti–miR-21 tested (50 nmol/L) and thus could be classified as "responders" (Fig. 2C). Importantly, these effects were specific to inhibition of miR-21 as cell viability was not reduced by a MM control anti-miRNA used under the same experimental conditions. Notably, a fraction of HCC cell lines were inherently resistant to anti–miR-21 treatment ("nonresponders"), suggesting that cellular context influences sensitivity to miR-21 ablation and ruling out indiscriminate cytotoxic activity of anti–miR-21 compound. Similar results were obtained using a different anti–miR-21 and MM control, thus eliminating the possibility of compound-specific effects (data not shown).

Collectively, our data demonstrate that anti–miR-21 elicits broad antiproliferative effects in HCC cell lines, which is accompanied by endogenous miR-21 target derepression.

Anti–miR-21 suppresses viability of both naïve and sorafenib-resistant HCC cell lines

The multikinase inhibitor sorafenib is currently the only approved therapy for HCC (23). However, the survival benefit of this drug is rather limited, and acquired resistance arises frequently through a variety of mechanisms. We sought to determine if anti–miR-21 would be efficacious in the treatment of sorafenib-resistant HCC cells. To this end, two HCC cell lines, SKHeP1 and JHH4, were subjected to long-term treatment with 10 μmol/L sorafenib to induce acquired resistance. Surviving cells were pooled and continually cultured in the presence of sorafenib to maintain selection pressure. Characterization of sorafenib-resistant SKHeP1 and JHH4 cell lines revealed an approximately 2-fold shift in sensitivity of these cells to sorafenib in a short-term proliferation assay as compared with their parental counterparts (Fig. 3A). This is comparable with the phenotype of sorafenib-resistant HCC cell lines reported by others (24). Differential response to sorafenib between parental and resistant cells was more evident in long-term clonogenic survival assays. Although the growth of parental cell lines was completely inhibited by high sorafenib concentrations, sorafenib-resistant cells continued to proliferate under the same conditions (Fig. 3B). Next, we tested the activity of anti–miR-21 in sorafenib-resistant SKHeP1 and JHH4 cells using their parental counterparts as controls. Interestingly, anti–miR-21 was equally efficacious in both sorafenib-resistant and parental cell lines (Fig. 3C), suggesting that suppression of miR-21 activity could be beneficial in both naïve HCC patients and those who have failed sorafenib therapy.

Inhibition of miR-21 leads to induction of cell death in HCC cell lines

miR-21 is known to target a network of proapoptotic and antisu survival genes. We hypothesized that antiproliferative effects observed upon miR-21 inhibition may be linked to the induction of apoptosis and other forms of cell death in HCC cell lines. To investigate this, we analyzed caspase 3/7 induction after treatment with anti–miR-21 in representative "responder" and "nonresponder" cell lines. Treatment with anti–miR-21, but not with the MM control, led to significant, time-dependent induction in caspase 3/7 activity in "responder" HepG2, Hep3B, and SKHeP1 cells, while having minimal effect in "nonresponder" JHH2 cells and in diploid human lung fibroblast cell lines IMR-90 and WI-38 (Fig. 4A and Supplementary Fig. S2). Caspase activation in "responder" cell lines was accompanied by concomitant reduction in cell viability (Fig. 4B). In contrast, limited impaired cell viability was observed in "nonresponder" JHH2 cells. To further investigate potential mechanisms contributing to cell death in "responder" cell lines, we analyzed biochemical markers of necrosis, LDH and HMGB1, in the conditioned media of anti–miR-21-treated cells. Both markers were substantially elevated in anti–miR-21-treated, but not in MM control–treated, cells (Supplementary Fig. S3).

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Finally, to confirm that anti–miR-21-treated cells proceed to active apoptosis and/or necrosis, we performed Annexin V and 7-AAD staining of the three responder cell lines. Consistent with caspase activation data, we observed a significant proportion of Annexin V–positive cells indicative of ongoing apoptosis. In addition, a fraction of anti–miR-21-treated cells stained positive for 7-AAD, indicating loss of cellular membrane integrity typical of late apoptosis and/or necrosis (Fig. 4C). Notably, the MM control oligonucleotide had no effect in any of the cell lines tested. Taken together, these results suggest that suppression of miR-21 activity leads to active cell death in HCC cell lines through a variety of mechanisms, including apoptosis and necrosis.

miR-21 is important for anchorage-independent growth and migration of HCC cells

Thus far our data implicated miR-21 in regulating HCC cell survival and proliferation in two-dimensional cell culture on plastic, which does not fully recapitulate the complexity of cancer cell growth in vivo. To assess if miR-21 contributes to HCC cell growth in a three-dimensional environment, we seeded anti–miR-21-treated cells in semi-solid soft agar medium and monitored colony formation over 7- to 10-day period. Treatment with anti–miR-21 resulted in significant inhibition of colony formation in both Hep3B and HepG2 cells, with more modest effects observed in SKHep1 (Fig. 5A).

Figure 2.
Inhibition of miR-21 results in derepression of endogenous target genes and reduction in cell viability. A, HCC and cholangiocarcinoma (CC) cell lines were treated with anti–miR-21, MM control, or PBS, and expression of ANKRD46, DDAH1, and RECK was examined by qPCR. Expression in PBS-treated samples is indicated by the dotted line. $P$ value $\leq 0.05$ except for SNU423, SNU475 (for ANKRD46); Hep3B (for DDAH1); HLE, SNU475 (for RECK), which were not significant (mean $\pm$ SD, $n = 3$). ND, not detected. B, a dose–response measurement of anti–miR-21 or MM control effect on cell viability was performed in each of the HCC and cholangiocarcinoma cell lines, and the data were used to calculate the $IC_{50}$ of anti–miR-21 and MM control for each cell line. C, the percent viability relative to PBS for each cell line after 4 days treatment with 50 nmol/L of anti–miR-21 or MM control (mean $\pm$ SEM, $n = 3$).
Another aspect of tumor growth in vivo is the ability of cancer cells to migrate and invade the surrounding tissues leading to local and distant metastases. Because miR-21 downstream targets include a number of modulators of cell migration, we aimed to investigate the impact of anti–miR-21 on HCC cell motility. To this end, representative responder lines were transfected with anti–miR-21 or MM control and replated at high density on collagen-coated plates. Confluent monolayers were disrupted using a scratch technique, and cell migration in response to HGF was monitored over time in serum-free media. Anti–miR-21 strongly inhibited HCC cell motility compared with cells treated with an MM control (Fig. 5B). Combined with the anti–miR-21 effects observed in the soft agar assay, these results suggest a role for miR-21 in regulating complex cellular behaviors, such as anchorage-independent growth and cell migration, both of which are expected to contribute to the overall tumorigenic phenotype in HCC.

Figure 3.
Sorafenib-resistant HCC cell lines retain sensitivity to miR-21 inhibition in vitro. A, a dose response of sorafenib in parental and sorafenib-resistant SKHep1 and JHH4 cell lines (mean ± SEM, n = 3). B, effect of sorafenib treatment on colony formation of parental and sorafenib-resistant SKHep1 and JHH4 cell lines. C, dose response of anti–miR-21 and MM control in parental and sorafenib-resistant SKHep1 and JHH4 cells (mean ± SEM, n = 3).
Figure 4. Treatment with anti-miR-21 leads to caspase activation and induction of apoptosis. A, caspase 3/7 activation after treatment of SKHep1, HepG2, Hep3B, or JHH2 cells with MM control or anti-miR-21 for 24 hours and 72 hours. Note difference in scale between 24- and 72-hour time points (mean ± SEM, n = 3). B, cell viability of select responder and nonresponder cell lines after treatment with anti-miR-21 (72 hours; mean ± SEM, n = 3). C, SKHep1, Hep3B, and HepG2 cells were treated with anti-miR-21, MM control, or camptothecin, stained with Annexin V and 7-AAD, and analyzed by flow cytometry.
Anti–miR-21 treatment leads to global target derepression and dysregulation of multiple pathways in HCC cells

Given significant phenotypic effects observed in HCC cell lines upon anti–miR-21 treatment, we sought to obtain additional mechanistic insights into the consequences of miR-21 inhibition. To accomplish this, SKHep1 cells were treated with anti–miR-21 and then subjected to global gene expression profiling. Notably, changes in gene expression in anti–miR-21-treated cells most likely represent a combination of direct changes related to miR-21 inhibition along with secondary indirect responses. Sylamer plot analysis revealed significant enrichment in miR-21 target sequences among upregulated transcripts, confirming the specificity and global nature of anti–miR-21 effects (ref. 25; Fig. 6A).

Greater than 10% of significantly upregulated transcripts (fold induction ≥ 1.5, P value ≤ 0.05) contained miR-21 recognition site in their 3’ UTR (Fig. 6B and Supplementary Data S1). Next, we analyzed the biologic pathways and processes that were affected by the anti–miR-21 treatment. Gene Ontology (GO) pathway mapping identified perturbations in cellular metabolism as one of the major consequences of miR-21 inhibition in HCC cells. A host of other pathways were affected as well, including apoptosis, DNA methylation, and cytokine signaling, among others (Supplementary Fig. S4).

To confirm changes identified by global gene expression profiling, we analyzed a subset of significantly modulated transcripts using qRT-PCR in three HCC cell lines treated with anti–miR-21 in an independent experiment. ANKR D46, RECK, and DD AH1 were once again confirmed as some of the most upregulated miR-21 targets in all three cell lines. In addition, derepression of a number of other known miR-21 target genes with established roles in the inhibition of cell growth and induction of apoptosis was confirmed, including PDCD4, TGFBR3, BTG2, and PELI1 (Fig. 6C). These results indicate that anti–miR-21 treatment induces robust global target engagement in HCC cells leading to changes in multiple prosurvival pathways which ultimately results in the inhibition of cell growth and induction of cell death.

Inhibition of miR-21 suppresses HCC tumor growth in vivo

In vivo dependency on miR-21 in HCC has not been established thus far. We utilized a tough decoy (TuD) approach to inhibit miR-21 in HCC tumor xenografts. Tough decoys are lentiviral vector-based miRNA inhibitors that can be stably introduced into virtually any cell type, resulting in long-term suppression of microRNA function (26). Large numbers of Hep3B or HepG2 cells were infected with lentiviruses encoding either miR-21 TuD or control (Null) TuD, and stable cell pools were obtained by rapid selection with puromycin. Immediately before implantation in vivo, on-target inhibition of miR-21 was confirmed by analyzing levels of endogenous miR-21 targets ANKR D46, DDAH1, and RECK (Fig. 7A). Similar to anti–miR-21 treatment, cells expressing miR-21 TuD displayed upregulation of the miR-21 targets ANKR D46, DDAH1, and RECK. Next, parental cells (Hep3B or HepG2), as well as Null- and miR-21 TuD-expressing cells, were implanted subcutaneously into immunocompromised
Figure 6. Anti–miR-21 causes global deregulation of essential cellular functions in HCC cells. A, Syamer enrichment plot analysis for 6-, 7-, and 8-mer miR-21 seed sequences in SKHep1 cells treated with anti–miR-21. The x-axis displays a sorted gene list from most upregulated (left) to most downregulated (right). The y-axis indicates enrichment (log10 P-value). Gray lines represent unrelated seed sequences. Colored lines represent miR-21 seed regions. B, Venn diagram showing overlap of miR-21 target genes and genes upregulated by anti–miR-21 treatment in SKHep1. C, SKHep1, HepG2, and Hep3B cell lines were treated with PBS, anti–miR-21, or MM control. Total RNA was isolated and analyzed by qRT-PCR for the indicated miR-21 target genes. Expression in PBS-treated samples is indicated by the dotted line (mean ±SD, n = 2; *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001).
mice, and tumor growth was monitored over 4 to 5 weeks. Animals implanted with parental and Null TuD cells formed large tumors that grew robustly over the course of the experiment. In contrast, animals implanted with miR-21 TuD-expressing cells initially formed tumors of approximately 200 mm³, but their growth was significantly inhibited over the course of the experiment (Fig. 7B). miR-21 TuD-mediated tumor growth inhibition was more pronounced in Hep3B (T/C = 0.2, P value = 0.002) than in HepG2 (T/C = 0.61, P value = 0.005), although the effect was statistically significant in both models. Taken together, these data reveal a critical role for miR-21 in the maintenance of tumorigenic phenotype in HCC tumor xenografts in vivo.

Discussion

miR-21 is one of the best-known oncomiRs discovered to date. Its overexpression is well-documented across a diverse array of solid and hematologic human malignancies, including HCC. In addition, multiple studies have demonstrated strong association between increased miR-21 expression and poor clinical outcomes in various cancers (7). Nonetheless, oncogenic dependency on miR-21 in vitro and in vivo is only now starting to be established. In this study, we investigated miR-21 contribution to the oncogenic phenotype in liver cancer cell lines and tumor xenografts. Utilizing a large panel of HCC and cholangiocarcinoma cell lines, we show that blocking miR-21 function using highly potent and specific anti-miRNA compounds leads to endogenous target derepression and the onset of cell death through a variety of mechanisms, including apoptosis and necrosis.

Consistent with the multitargeted nature of miRNA regulation, we detected broad changes in gene expression upon anti-miR-21 treatment, including upregulation of multiple direct targets of miR-21 that are involved in cancer cell proliferation, survival, and migration (BTG2, PELI1, TGFBR3, and RECK). Notably, expression of BTG2 has been shown to correlate with HCC tumor grade and is reported to be lost in around 30% of HCC tumor samples (27), suggesting a tumor-suppressive role for this protein in liver cancer. Overexpression of pheochromocytoma cell-3 (PC3), the rat homologue of BTG2, is also known to lead to cell cycle arrest at the G1-S transition (28). PELI1, an E3 ubiquitin ligase, is repressed by

Figure 7. Inhibition of miR-21 suppresses HCC tumor growth in vivo. A, expression of the miR-21 target genes RECK, ANKRD46, and DDALH1 in Hep3B and HepG2 cell lines expressing either miR-21 TuD or NULL TuD before implantation in vivo. B, immunocompromised mice were implanted subcutaneously with the parental, miR-21, and NULL TuD-expressing cells, and tumor growth was monitored over 5 weeks. Hep3B (mean ± SEM, n = 5–8); HepG2 (mean ± SEM, n = 6–15). *, P ≤ 0.05; **, P ≤ 0.01.
miR-21 during liver regeneration and is proposed to serve as a feedback mechanism for modulating NF-kappaB signaling (29). Furthermore, both TGFBR3 and RECK function as important regulators of cellular migration and invasion (22, 30). Global gene expression analysis following anti–miR-21 treatment revealed nearly 30 different miR-21 target genes to be upregulated, suggesting the strong phenotypic effect observed in HCC cells upon miR-21 inhibition is the result of cumulative changes in the expression of multiple genes rather than a single direct target of miR-21.

In addition to changes in miR-21 target genes associated with cell survival and proliferation, global gene expression analysis demonstrated that anti–miR-21 treatment results in perturbations of multiple cellular metabolic pathways in HCC cells. MiR-21 has previously been implicated in the regulation of cellular metabolism. Specifically, overexpression of miR-21 is known to contribute to the onset of kidney fibrosis through deregulation of lipid metabolism (31). In addition, miR-21 is known to control the levels of PTEN, which in turn regulates the PI3K/AKT pathway linked to metabolic alterations. Curiously, despite strong upregulation of multiple miR-21 target genes, we did not observe changes in PTEN mRNA or protein expression upon anti–miR-21 treatment in HCC cells (data not shown). This may reflect a cellular, context-dependent nature of target gene regulation by miR-21. Our data suggest that additional miR-21 targets are responsible for metabolic changes in anti–miR-21-treated HCC cells. One potential candidate could be the miR-21 target gene DDAH1, as we observed at least a 1.5-fold increase in DDAH1 expression upon anti–miR-21 treatment in 16 of 18 HCC and cholangiocarcinoma cell lines tested herein. DDAH1 is a metabolic enzyme responsible for control of nitric oxide generation by modulating cellular concentrations of methylarginine (32). Ultimately, however, metabolic changes observed by anti–miR-21 treatment may not be due to a single miR-21 target gene, but a result of changes in expression of both direct and indirect miR-21 target genes. Further studies will be required to test this hypothesis and to dissect the metabolic changes upon miR-21 inhibition in HCC.

Significant changes in gene expression after treatment with anti–miR-21 were correlated with the reduction in cell viability in the majority of HCC cell lines tested in this study. However, a small subset of HCC cells was found to be inherently resistant to miR-21 ablation. Both ‘responder’ and ‘nonresponder’ HCC cell lines showed derepression of at least one miR-21 target gene, indicating that intracellular delivery of anti-miRNA was not the cause of resistance. Levels of miR-21 alone may not entirely explain the differential response to miR-21 inhibitors, as the relationship between miRNA abundance and its activity is known to be complex and nonlinear (33, 34). A thorough understanding of the network of interactions between miR-21 and its target genes would likely lead to a better understanding of cellular factors that define sensitivity to miR-21 inhibition. Factors likely to influence the response to anti–miR-21 include the ratio of miR-21 to its target mRNA, expression level of miR-21 target genes, stage of the cell cycle, and the potential impact of competitive endogenous mRNAs. While the precise mechanisms underlying such resistance to anti–miR-21 remain to be elucidated, our data show that it can at least be partially explained by the failure of cancer cells to fully engage apoptotic machinery, including caspase 3/7 activation.

Most importantly, our results provide the first evidence of oncogenic dependency on miR-21 in HCC tumor xenografts in vivo. While we utilized genetic tools to ablate miR-21 functional activity, future experiments will focus on systemic delivery of anti–miR-21 compounds in HCC tumor models. Our data establish an important role for miR-21 in the maintenance of tumorigenic phenotype in HCC and suggest that this miRNA might represent an attractive target for pharmacologic intervention.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
Targeting the microRNA-21 (miR-21) pathway in hepatocellular carcinoma (HCC) is of great importance due to its high incidence and poor prognosis. MiR-21 is overexpressed in various types of cancer, including HCC, and its overexpression is correlated with poor prognosis.

**Pathway Model**

1. **MicroRNA-21 Overexpression**
   - MiR-21 is a well-known oncomiR that is significantly upregulated in HCC.
   - It promotes tumorigenesis and tumor growth by targeting key genes involved in apoptosis, proliferation, and invasion.

2. **Target Genes of MiR-21**
   - MiR-21 targets several genes involved in cell growth, apoptosis, and invasion.
   - Examples include PTEN, BCL2, and NF-kB.

3. **Mechanisms of Action**
   - MiR-21 regulates the expression of its target genes through base-pairing with their 3’UTR.
   - It promotes tumor growth and invasion by inhibiting the translation of its target genes.

4. **Clinical Relevance**
   - MiR-21 overexpression is associated with advanced tumor stage, poor differentiation, and vascular invasion.
   - It can be a potential therapeutic target for the treatment of HCC.

**Future Directions**

- Development of specific inhibitors or delivery systems for miR-21 targeting.
- Integration of miR-21 modulation with other therapeutic strategies.
- Exploration of miR-21 as a diagnostic and prognostic biomarker.

In conclusion, targeting miR-21 in HCC represents a promising therapeutic strategy that could improve patient outcomes.
Molecular Cancer Research

Anti–miR-21 Suppresses Hepatocellular Carcinoma Growth via Broad Transcriptional Network Deregulation

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