

# MiR-199a-3p Regulates mTOR and c-Met to Influence the Doxorubicin Sensitivity of Human Hepatocarcinoma Cells

Francesca Fornari<sup>1</sup>, Maddalena Milazzo<sup>1</sup>, Pasquale Chieco<sup>1</sup>, Massimo Negrini<sup>3</sup>, George Adrian Calin<sup>4</sup>, Gian Luca Grazi<sup>2</sup>, Daniela Pollutri<sup>1</sup>, Carlo Maria Croce<sup>5</sup>, Luigi Bolondi<sup>1</sup>, and Laura Gramantieri<sup>1</sup>

## Abstract

MicroRNAs (miRNA) have rapidly emerged as modulators of gene expression in cancer in which they may have great diagnostic and therapeutic import. MicroRNA-199a-3p (miR-199a-3p) is downregulated in several human malignancies including hepatocellular carcinoma (HCC). Here, we show that miR-199a-3p targets mammalian target of rapamycin (mTOR) and c-Met in HCC cells. Restoring attenuated levels of miR-199a-3p in HCC cells led to G<sub>1</sub>-phase cell cycle arrest, reduced invasive capability, enhanced susceptibility to hypoxia, and increased sensitivity to doxorubicin-induced apoptosis. These *in vitro* findings were confirmed by an analysis of human HCC tissues, which revealed an inverse correlation linking miR-199a-3p and mTOR as well as a shorter time to recurrence after HCC resection in patients with lower miR-199a-3p expression. These results suggest that tactics to regulate mTOR and c-Met by elevating levels of miR-199a-3p may have therapeutic benefits in highly lethal cancers such as HCC. *Cancer Res*; 70(12); 5184–93. ©2010 AACR.

## Introduction

In the recent years, great advances in the molecular characterization of human hepatocellular carcinoma (HCC) have been reported (1, 2) and molecular pathways involved in neoplastic transformation and progression have been addressed as targets for nonconventional treatments. Nevertheless, the molecular classification of HCC has not been completely assessed and HCC still confers a poor prognosis when diagnosed at advanced stages. This is partially due to the intrinsic resistance of HCC to traditional chemotherapies, linked to the multidrug-resistant phenotype, and to the coexistence of the underlying chronic liver disease, which hinders the adoption of efficacious therapeutic regimens. Furthermore, despite apparent survival benefits (3), the efficacy of molec-

ularly targeted therapy is impaired by the activation of redundant pathways driving cell proliferation and inhibition of apoptosis (4). Moreover, recent findings show an increased drive toward metastatization following treatment with anti-angiogenic drugs, confirming the need to hit multiple targets at one time (5). Thus, the identification of molecular targets for effective and nontoxic therapeutic approaches against angiogenesis, tumor growth, and invasion is urgently needed.

Evidence collected to date shows the involvement of microRNA (miRNA) in cancer initiation, development, and progression, and identify this class of regulatory RNAs as diagnostic and prognostic cancer biomarkers, as well as additional therapeutic tools (6). Indeed, growing evidence links miRNA deregulation to carcinogenesis in several human neoplasms, including HCC. Molecular signatures of deregulated miRNAs have been associated with HCC clinical features, histotype, etiology, and metastatization (7–10). MiRNAs are small (~22 nucleotides) RNA sequences that bind to target RNA acting as posttranscriptional gene expression regulators. By pairing to complementary binding sites within the 3' untranslated region (3'UTR) of hundreds of target mRNAs, miRNAs impair their translation or promote their degradation, thus participating in the control of crucial processes, such as development, cell proliferation, apoptosis, and differentiation. Compelling evidence underscores the ability of a single miRNA to target multiple molecules belonging to the same, as well as to redundant, pathways in neoplastic and normal cells. Thus, in tumor cells, the restoration of deregulated miRNAs to physiologic levels could provide valuable information that will facilitate our understanding of miRNA involvement in the regulation of molecular networks sustaining cancerous phenotypes.

**Authors' Affiliations:** <sup>1</sup>Centro di Ricerca Biomedica Applicata e Dipartimento di Medicina Interna, Policlinico S.Orsola-Malpighi e Università di Bologna and <sup>2</sup>Dipartimento di Chirurgia e dei Trapianti, Università di Bologna, Policlinico S. Orsola-Malpighi, Bologna, Italy; <sup>3</sup>Dipartimento di Medicina Sperimentale e Diagnostica e Centro Interdipartimentale per la Ricerca sul Cancro, Università di Ferrara, Ferrara, Italy; <sup>4</sup>Department of Experimental Therapeutics, University of Texas, MD Anderson Cancer Center, Houston, Texas; and <sup>5</sup>Comprehensive Cancer Center, Ohio State University, Columbus, Ohio

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

**Corresponding Authors:** Laura Gramantieri, CRBA, Via Massarenti, 9, 40138, Bologna, Italy. Phone: 39-051-6364903; Fax: 39-051-6363902; E-mail: [laura.gramantieri@aosp.bo.it](mailto:laura.gramantieri@aosp.bo.it) or Luigi Bolondi, Dipartimento di Medicina Clinica e CRBA, Via Massarenti, 9, 40138, Bologna, Italy. Phone: 39-051-6362260; Fax: 39-051-6362725; E-mail: [luigi.bolondi@unibo.it](mailto:luigi.bolondi@unibo.it)

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Among deregulated miRNAs, the miR-199-miR-214 cluster is of particular interest because it is downregulated in the majority of HCCs (11), in addition to other human malignancies (12, 13), in cancer-derived cell lines (14) and in experimental neoplastic and preneoplastic conditions (15). miR-199a-3p was linked to the modulation of different target genes, such as c-Met (16), Smad1 (17), and leukemia inhibitory factor (LIF; ref. 18), and its exogenous expression was reported to reduce the motility and viability of melanoma, gastric, and lung cancer cells (19).

Notably, with the help of a bioinformatic analysis, we found that a key promoter of cell growth, the mammalian target of rapamycin (mTOR), is a hypothetical target of miR-199a-3p. The mTOR pathway is activated by multiple extracellular signals, such as growth factors, nutrients, amino acids, hormones, and mitogens leading to the phosphorylation of the translational regulator, phospho-p70S6 kinase, which, in turn, regulates cell proliferation, regulates protein synthesis, and allows progression from the G<sub>1</sub> to the S phase of the cell cycle. The relevance of the mTOR pathway in the carcinogenic process is a common feature of several human cancers, including HCC (20), in which its overexpression is associated with a poor prognosis, invasion, and metastasis (21). The downregulation of miR-199a-3p in the majority of HCC samples, together with the upregulation of mTOR, led us to hypothesize a causal link between these two molecules. In addition, a very low rate of genetic alterations affecting mTOR pathway in HCC was recently reported (20).

The present study was undertaken to define the role of miR-199a-3p in HCC through the identification of new target genes, and to analyze its biological functions in HCC-derived cell lines. Here, we identified mTOR as a target of miR-199a-3p and we found that miR-199a-3p restoration blocks the G<sub>1</sub>-S transition of the cell cycle, impairs invasion capability, and sensitizes HCC cells to doxorubicin challenge. Considering that miR-199a-3p and miR-199a-5p are downregulated in the majority of HCCs, our results suggest that miR-199a-3p restoration could be a possible therapeutic approach worthy of evaluation in HCC.

## Materials and Methods

### Patients

HCC and cirrhotic tissues were obtained from 39 consecutive patients (33 males and 6 females; median age, 70 y, range, 51–82 y) who underwent liver resection for HCC at the Department of Surgery of the University of Bologna. Normal liver tissues were obtained from 10 patients undergoing liver surgery for traumatic lesions (8 cases) or hemangioma resection (2 cases). Tissue samples were collected at surgery and stored as previously described (11). Informed consent was obtained from each patient. Histopathologic grading was scored according to Edmondson and Steiner's criteria (22). Exclusion criteria were a previous history of local or systemic treatments for HCC and the presence of necrotic tissue surrounding the HCC nodule/s. The characteristics of HCC patients included in this study are described in Supplementary Table S1.

### Cell culture and treatments

HepG2 [American Type Culture Collection (ATCC) no. HB-8065] cell line was cultured with MEM (Eagle), whereas Huh-7 and SNU475 (ATCC no. CRL-2236) cell lines were cultured with RPMI 1640; both media were supplemented with 10% fetal bovine serum (FBS) and antibiotics.

Cells were treated with 10 ng/mL of rapamycin (Sigma-Aldrich) and collected 24, 48, and 72 hours after treatment for Western blotting (WB) and flow cytometry (FC) analyses. Doxorubicin (2.5 µg/mL) was administered for 24 hours and cells were collected for WB and FC analysis. Hypoxic conditions were created by using 250 µmol/L of Deferoxamine (Sigma) for 24 hours before cell collection for FC Annexin V assay.

### Cell transfection with RNA oligonucleotides

HCC-derived cell lines were transfected with 100 nmol/L of pre-miR-199a-3p, anti-miR-199a-3p, or Negative Control#1 precursor and inhibitor miRNAs (Ambion). Gene silencing was obtained by using 20 nmol/L of mTOR small interfering RNA (siRNA; DsiRNA Duplexes, HSC.RNAI.N004958.10.10, IDT), c-Met siRNA (Sigma-Aldrich), and Stealth RNA Negative Control. siRNAs sequences are reported in Supplementary Table S2. Oligonucleotide transfection was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Samples were collected at 24, 48, and 72 hours after the transfection and were stored at -80°C for subsequent analysis.

### Luciferase activity assay

The portion of the 3'UTR region (908 bp) of human mTOR gene containing the hsa-miR-199a-3p binding site was amplified by PCR using the following primers: mTOR-3'UTR-F: 5'-CTT TCT AGA AAC TGG AGG CCC AG-3' and mTOR-3'UTR-R: 5'-TGG TGT CTA GAC ATG GCT ACA CTT TAT AC-3'. This portion was cloned using the XbaI site in a pGL3 Control vector (Promega) downstream of the reporter gene. This construct, named pGL3-mTOR, was used for transfection experiments in Huh-7 and SNU475 cells. Both cell lines were cultured in 24-well plates and transfected with 500 ng of either pGL3-mTOR or pGL3 Control vector in association with 50 ng of pRL-TK vector (Promega) and 100 nmol/L of miR-199a-3p, anti-miR-199a-3p, or negative controls. Mutagenesis of miR-199a-3p seed sequence in the mTOR 3'UTR region was executed by Genscript Corp. (<http://www.genscript.com>). Twenty-four hours after the transfection, luciferase activity was measured by Dual-Luciferase Reporter Assay (Promega). Each transfection was repeated twice in triplicate.

### Real-time reverse transcription-PCR analysis of miR-199a-3p and miR-199a-5p

Accurate quantitation of mature miR-199a-3p and miR-199a-5p was obtained using the Taqman MicroRNA Assays (hsa-miR-199a-3p, N/P:4378068 and has-miR-199a-5p, N/P:4373272, Applied Biosystems) as previously described (11). Taqman endogenous control (RNU6B, N/P:4373381, Applied Biosystems) was used to normalize miRNAs expression levels. Analysis of miR-199a-3p and miR-199a-5p expression

was carried out using the cycle threshold method. A pool of five liver cirrhosis samples not included in this study was used as a reference. To show that miR-199a-3p levels fell in the linear range of the amplification assay, a standard curve for Taqman MicroRNA Assays was generated by using serial dilutions of the mirVana miRNA Reference Panel v9.1 (Applied Biosystems), with concentrations ranging from  $1.0 \times 10^{-6}$  to 1.0 fmol.

### WB analysis

Transfected HCC-derived cell lines and primary HCC tissues were assayed with anti-mTOR, anti-phospho-S6 Ribosomal Protein (Ser-240/244), anti-c-Met, anti-cleaved caspase-3 (Cell Signaling), anti-p27 (clone 57, BD Biosciences), anti-p21 (clone F-5), and anti- $\beta$ -actin (Santa Cruz Biotechnology) antibodies. Digital images of X-ray films were acquired and quantified with Fluor-S MultiImager (Quantity-one, Bio-Rad) as previously reported (11).

### Semiquantitative reverse transcription-PCR

Semiquantitative reverse transcription-PCR (RT-PCR) was performed using the primers and the amplification conditions detailed in Supplementary Table S3.

### Cell cycle and Annexin V assays

Transfected HCC cells were collected and fixed in 70% ethanol at  $-20^{\circ}\text{C}$  for 16 hours. FC analysis of cell cycle was performed as previously described (23), using the FACSaria cell sorter (BD Biosciences). Detection of apoptotic cells after doxorubicin treatment was performed by using the Annexin V/Propidium Iodide detection kit (Bender MedSystems) according to the manufacturer's instructions and analyzed by FC.

### Cell invasion assay

Cell invasion was assessed by Boyden blind-well chambers (New Technologies Group) containing poly-vinyl-pyrrolidone-free polycarbonate filters, 8- $\mu\text{m}$  pore size coated with Matrigel (Sigma). Twenty-four hours after the transfection,  $5.0 \times 10^4$  HepG2 and  $3.0 \times 10^4$  SNU475 cells were resuspended in serum-free medium and added to the upper chamber. A medium supplemented with 30% FBS was used as chemoattractant to the lower chamber. HepG2 and SNU475 cells were incubated at  $37^{\circ}\text{C}$  for 48 and 24 hours, respectively, and non-

invading cells were removed with cotton swabs. Invasive cells were fixed, stained with Giemsa (Sigma), and counted.

### Statistical analysis

Differences between groups were tested using paired or unpaired Student's *t* test or ANOVA as appropriate. Pearson's correlation coefficient, or paired or unpaired Student's *t* test were used to explore any relationship between miR-199a-3p expression and tumor characteristics including  $\alpha$ -fetoprotein (AFP) serum levels, tumor size, grade, etiology, and focality. Patients survival and time to recurrence (TTR) curves based on miR-199a-3p levels were computed by Kaplan-Meier product-limit method and compared using a log-rank test. Reported *P* values were two sided and were considered significant when lower than 0.05. Statistical calculations were performed using SPSS version 8.0 (SPSS, Inc.).

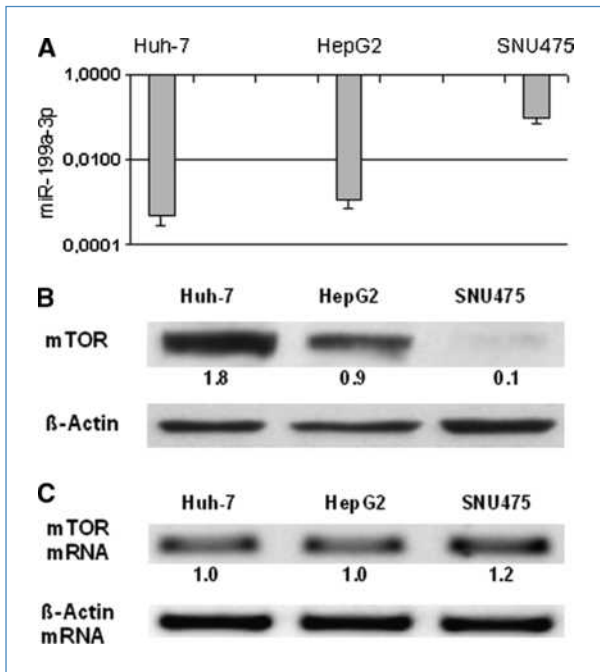
## Results

### mTOR is a target of miR-199a-3p

A bioinformatic analysis identified mTOR as a hypothetical target gene of miR-199a-3p, as identified by MICRO-COSM, Pictar, and TargetScan (Table 1) algorithms. A further hint for the potential role of miR-199a-3p in the regulation of mTOR expression came from the analysis of three different HCC-derived cell lines (Huh-7, HepG2, and SNU475) that showed an inverse correlation between miR-199a-3p levels and mTOR protein (Fig. 1A and B). Conversely, no correlation was observed between miR-199a-3p and mTOR mRNA levels (Fig. 1C). To show that miR-199a-3p participates to the regulation of mTOR expression, we performed an *in vitro* functional analysis by either restoring or silencing miR-199a-3p. HepG2 and Huh-7 cell lines were chosen for the enforced expression of miR-199a-3p because they display the lowest constitutive levels of this miRNA and a high mTOR protein expression (Fig. 1A and B). The enforced expression of miR-199a-3p in HCC-derived cell lines at levels similar to those observed in normal liver tissue (Supplementary Fig. S1) led to a decrease of mTOR protein, without any variation in mTOR mRNA levels (Fig. 2A and B). To investigate the potential effect of the restored expression of miR-199a-3p to the mTOR downstream pathway, we analyzed the phosphorylation status of the ribosomal protein S6, a

**Table 1.** TargetScan prediction of miR-199a-3p pairing site in mTOR 3'UTR

	Predicted consequential pairing of target region (top) and miRNA (bottom)	Seed match
Position 128–134 of FRAP1 3' UTR	5'...AUGUAAAUGAAAAGAACUACUGU... 	7mer-m8
hsa-miR-199b-3p	3' AUUGGUUACACGUC-UGAUGACA	
Position 128–134 of FRAP1 3' UTR	5'...AUGUAAAUGAAAAGAACUACUGU... 	7mer-m8
hsa-miR-199a-3p	3' AUUGGUUACACGUC-UGAUGACA	



**Figure 1.** Inverse correlation between miR-199a-3p and mTOR protein levels in HCC-derived cell lines. A, real-time RT-PCR analysis of miR-199a-3p expression in Huh-7, HepG2, and SNU475 cells.  $2^{(-\Delta\Delta Ct)}$  value is reported in the Y-axis with a logarithmic scale. Each sample was run in triplicate. B, WB analysis of mTOR protein expression in HCC cells. C, RT-PCR analysis of mTOR mRNA expression in HCC cells. Numbers, the ratio between mTOR and  $\beta$ -actin expression.

target of the p70-S6K protein kinase. miR-199a-3p transfection determined a downregulation of phosphorylated-S6 protein both in HepG2 and Huh-7 cells (Fig. 2A and B).

SNU475 cells were chosen for miR-199a-3p silencing because they express the highest miR-199a-3p basal levels associated with low mTOR protein levels (Fig. 1A and B). The inhibition of miR-199a-3p in SNU475 cells determined an increase of both mTOR and P-S6 proteins, with no variations in the mRNA level (Fig. 2C). These data suggested a posttranscriptional regulation of mTOR by miR-199a-3p in HCC-derived cell lines.

To prove a direct interaction between miR-199a-3p and its binding site within mTOR mRNA, pGL3-mTOR vector was cotransfected in Huh-7 and SNU475 cells in association with miR-199a-3p, anti-miR-199a-3p, or negative controls and then assayed by a luciferase reporter. The choice of these cell lines was intended to obtain the greatest variations upon miR-199a-3p modulation. Huh-7 cells transfected with pGL3-mTOR vector showed a 1.3-fold decrease of the luciferase activity when cotransfected with miR-199a-3p (*t* test,  $P = 0.03$ ). Similarly, SNU475 cells transfected with pGL3-mTOR vector displayed a 3.0-fold increase of the luciferase activity when cotransfected with anti-miR-199a-3p (*t* test,  $P = 0.006$ ; Fig. 2D). miR-199a-3p seed sequence was mutagenized in the cloned mTOR 3'UTR region to generate pGL3-mTOR-m1 vector. No significant change of the luciferase activity

was observed following the cotransfection of this mutagenized vector with miR-199a-3p or anti-miR-199a-3p (Fig. 2D). Taken together, these findings showed a direct interaction between miR-199a-3p and mTOR mRNA in HCC-derived cell lines.

#### miR-199a-3p affects cell cycle of HCC-derived cell lines

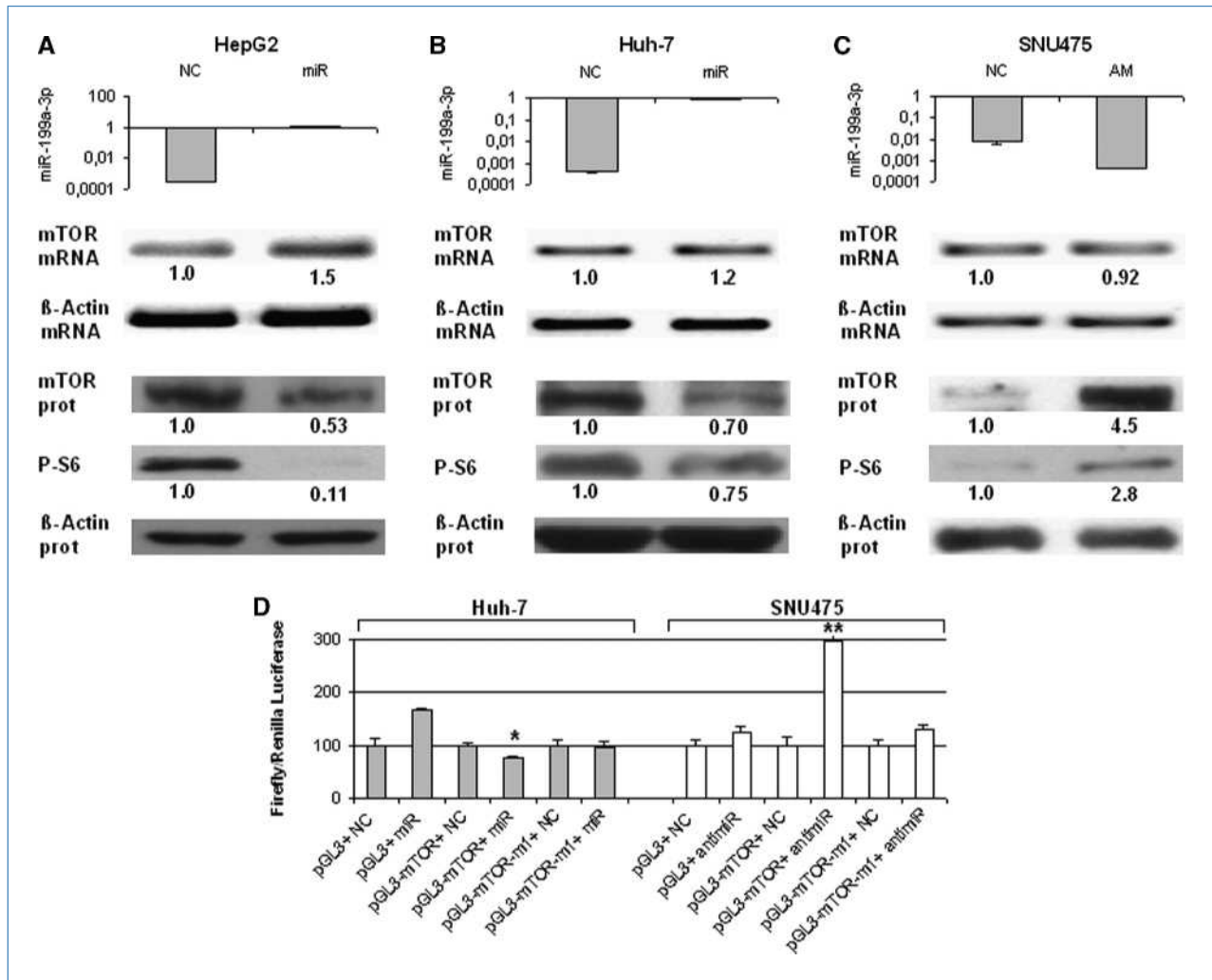
To analyze the role of miR-199a-3p in cell cycle regulation, we restored miR-199a-3p expression in HepG2 cells and evaluated the cell distribution through the cell cycle. An increase in the  $G_1$ -phase population and a decrease of the S phase followed the restoration in miR-199a-3p expression. Accordingly, miR-199a-3p restoration determined an upregulation of two crucial cell cycle inhibitors, CDKN1A/p21 and CDKN1B/p27, at both protein and mRNA levels (Fig. 3A).

It has been recently reported that c-Met proto-oncogene is a direct target of miR-199a-3p in melanoma cells and gastric and lung cancer cells (16). This downregulation affects cell proliferation, motility, and invasive capability. To discriminate which of the miR-199a-3p targets, mTOR or c-Met, mostly contributes to the regulation of cell cycle, we silenced both genes by transfecting HepG2 cells with miR-199a-3p or specific siRNAs (Fig. 3A–C). The transfection of HepG2 cells with either mTOR or c-Met siRNAs determined an arrest in the  $G_1$  phase. Moreover, and similarly to miR-199a-3p restoration, mTOR and c-Met silencing determined an increase of CDKN1B/p27 expression at both mRNA and protein levels. miR-199a-3p restoration and c-Met silencing also determined an increase in CDKN1A/p21 expression, whereas mTOR silencing determined a decrease of CDKN1A/p21 levels (Fig. 3B and C).

To compare our findings with those reported in the literature (24), we blocked mTOR pathway by treating HepG2 cells with the mTOR inhibitor rapamycin. This treatment determined an arrest of cell cycle in the  $G_1$  phase, similar to that observed after miR-199a-3p restoration, and prevented the phosphorylation of the ribosomal subunit S6 (Fig. 4A). Again, following rapamycin treatment, a decrease of CDKN1A/p21 expression was observed in HepG2 cells. To further investigate miR-199a-3p target contribution to cell cycle regulation, miR-199a-3p silencing was performed in SNU475 cells followed by mTOR or c-Met siRNAs-mediated inhibition. In line with our previous findings, fluorescence-activated cell sorting analysis revealed that a reduction of  $G_1$  phase occurred in SNU475 cells after miR-199a-3p silencing (Fig. 4B). Notably, both mTOR and c-Met silencing in anti-miR-199a-3p-transfected cells partially rescued the phenotype induced by anti-miR-199a-3p transfection, with an increase of the  $G_1$  phase (Fig. 4C). Taken together, these data showed that miR-199a-3p contributes to the cell cycle modulation of HCC cells by silencing both mTOR and c-Met.

#### miR-199a-3p and miR-199a-5p increase doxorubicin sensitivity and apoptosis in hypoxic conditions

Animal models of HCC showed that mTOR inhibition with rapamycin or its analogues has a synergic antitumoral



**Figure 2.** mTOR is a target of miR-199a-3p. A, transfection of miR-199a-3p (miR) in HepG2 cells increased its expression to levels comparable with normal liver with a corresponding 1.9- and 9.1-fold decrease of mTOR and P-S6 protein levels, respectively, when compared with negative control (NC)-transfected cells. No decrease of mTOR mRNA was observed in transfected cells. B, transfection of miR-199a-3p in Huh-7 cells increased its expression to levels comparable with normal liver with a corresponding 1.4- and 1.3-fold decrease of mTOR and P-S6 protein levels, respectively, without any modification in the mRNA level. C, transfection of anti-miR-199a-3p (AM) in SNU475 determined an 18-fold decrease of miR-199a-3p expression ( $t$  test,  $P = 0.01$ ) and a 4.5- and 2.8-fold increase of mTOR and P-S6 protein expression, respectively. No modifications were observed in the mRNA level. D, luciferase assay in Huh-7 and SNU475 cell lines. Wild-type (pGL3-mTOR 3'UTR) and mutagenized (pGL3-mTOR-m1) reporter vectors were cotransfected with miR-199a-3p, anti-miR-199a-3p, or negative controls. Top, B and C, representative of miR-199a-3p levels in miR- and anti-miR-199a-3p-transfected cell lines used for Western blot and Luciferase assays.

activity when coadministered with doxorubicin (25). Therefore, we investigated whether miR-199a-3p restoration and/or mTOR and c-Met silencing might modulate doxorubicin-induced apoptotic cell death. Because rapamycin analogues trigger apoptosis in a p53-dependent manner (26), we assayed HepG2 cells that express wild-type p53. Following doxorubicin treatment, flow cytometric analysis for Annexin V-positive cells revealed an increase of apoptosis upon miR-199a-3p restoration. Similarly, an increase of apoptotic cells was observed in either mTOR- or c-Met-silenced cells, with the highest increase found in mTOR-silenced cells (Fig. 5A). As a further proof, an increase of the cleaved fragments of caspase-3 was observed in miR-199a-3p-transfected and

mTOR-knocked down cells. Moreover, an upregulation of Puma mRNA, assessed as a p53 transcriptional target, was observed following miR-199a-3p restoration and mTOR/c-Met silencing (Fig. 5B). These data showed that miR-199a-3p increases doxorubicin sensitivity of HCC cells to a similar extent of mTOR siRNAs. This increased is, at least in part, mediated by the activation of the caspase cascade.

Because it is known that the mTOR pathway enhances HIF-1 $\alpha$  stability under hypoxic conditions (27) and that miR-199a-5p directly regulates HIF-1 $\alpha$  expression (28), we tested whether the restoration of miR-199a-3p or miR-199a-5p in HepG2 cells would modify their susceptibility to hypoxia. FC analysis showed an increase of apoptotic cell death for

both miR-199a-3p and miR-199a-5p-transfected cells maintained in hypoxia-mimetic conditions (Fig. 5C).

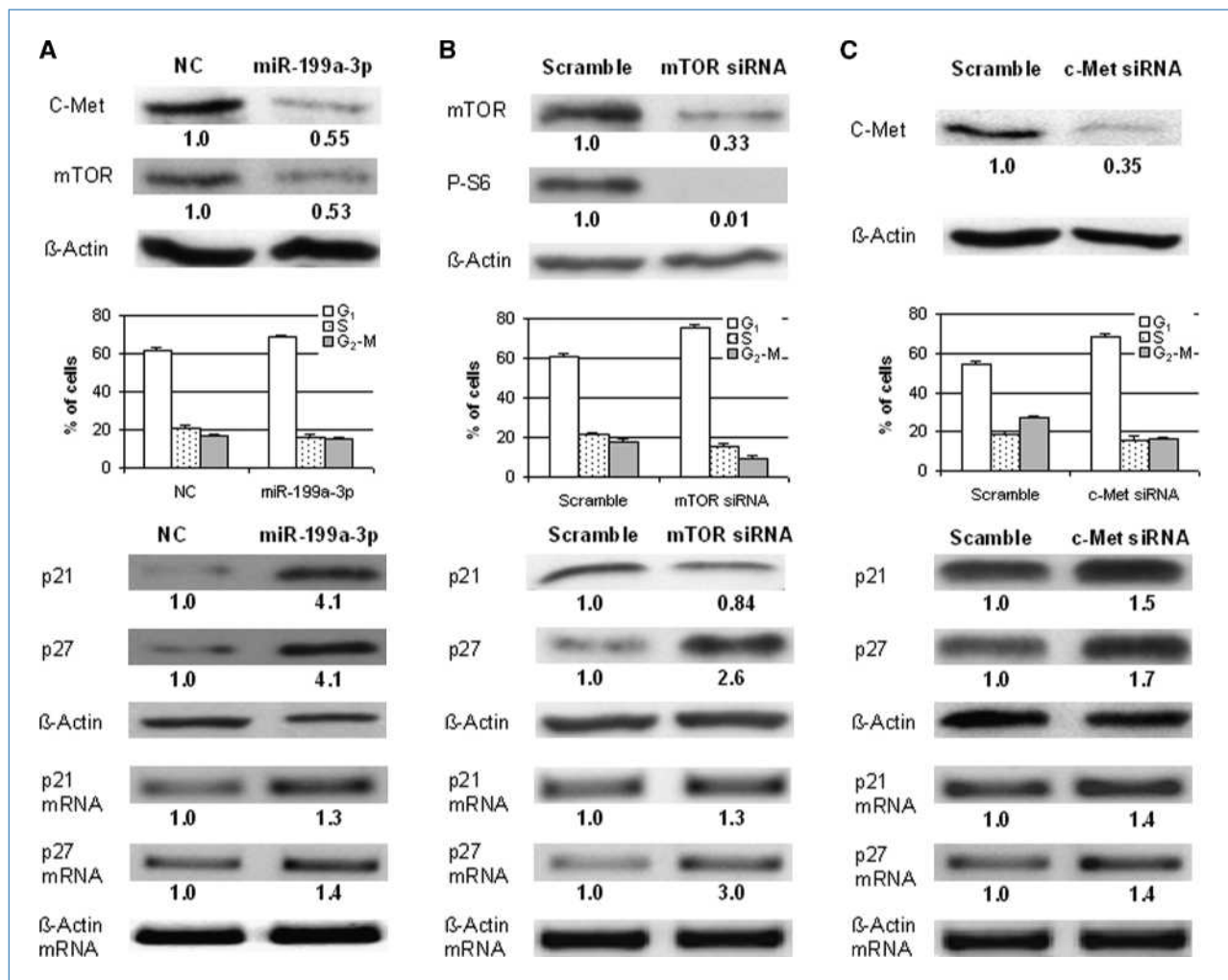
### miR-199a-3p affects the invasion capability of HCC-derived cell lines

The role of miR-199a-3p and of its target genes in the modulation of the invasion capability was investigated by Matrigel invasion assay. The restoration of miR-199a-3p in HepG2 cells caused a 60% decrease of the invasive potential, overcoming the effects of mTOR and c-Met silencing (20% and 40% decrease, respectively). Accordingly, a 2.2-fold increase of invading cells was observed following miR-199a-3p silenc-

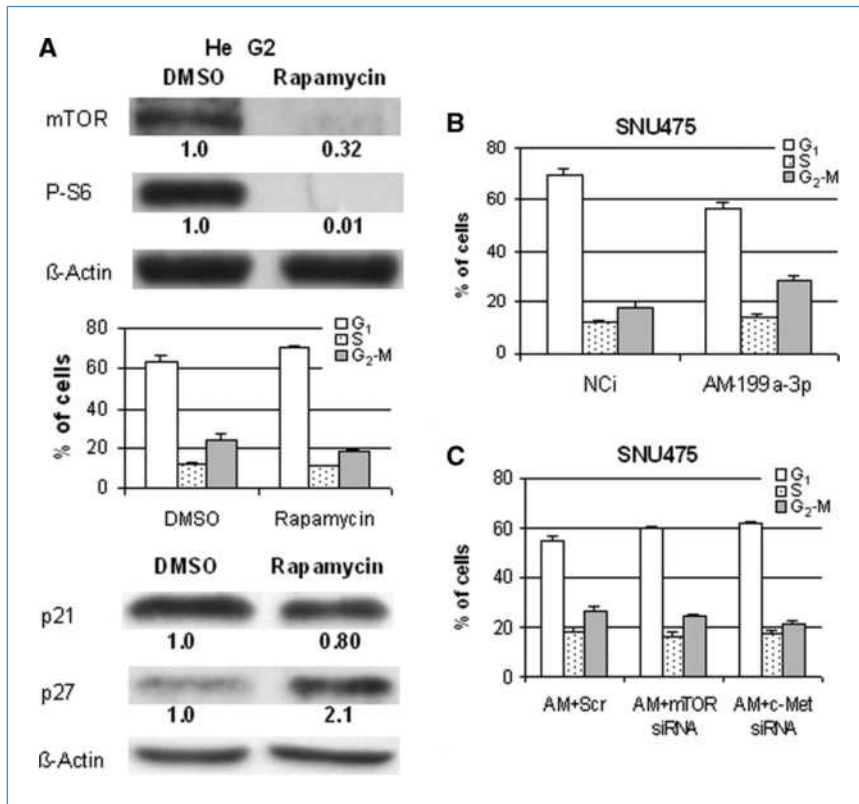
ing in SNU475 cells (Fig. 5D). These findings showed that miR-199a-3p counteract the invasive potential of HCC cells, even if this event is not likely related to mTOR inhibition.

### Expression of miR-199a-3p and mTOR in HCC tissues and correlation with clinicopathologic variables

Based on our previous findings obtained with microarray analysis (11), real-time RT-PCR was used to assess miR-199a expression in 10 normal livers and 39 matched HCC and cirrhotic tissues. A significant downregulation of both miR-199a-3p and miR-199a-5p expression was observed in HCC tissues when compared with cirrhotic ones (Fig. 6A and B) and



**Figure 3.** miR-199a-3p affects the cell cycle of HepG2 cells. A, WB analysis of miR-199a-3p-transfected HepG2 cells showed a 1.8-fold decrease of both mTOR and c-Met protein expression. FC analysis of miR-199a-3p-transfected cells displayed an arrest in the G<sub>1</sub> phase (11% increase; paired *t* test, *P* < 0.01) and a decrease of the S phase (22%, paired *t* test, *P* < 0.01) when compared with negative control (NC)-transfected cells. WB and RT-PCR analysis revealed an increase of cyclin-dependent kinase inhibitors (CDKI) in miR-199a-3p-transfected cells. B, WB analysis of mTOR-silenced HepG2 cells displayed a 3.0-fold decrease of mTOR expression and a corresponding decrease of P-S6 expression. FC analysis of mTOR-silenced cells showed an arrest of cell cycle in the G<sub>1</sub> phase (24% increase; paired *t* test, *P* < 0.01) and a decrease (28%, paired *t* test, *P* < 0.01) of the S phase, when compared with scramble-transfected cells. RT-PCR analysis of CDKIs in mTOR-silenced cells showed an increase of both p27 and p21 levels, whereas WB analysis revealed a downregulation of p21 levels. C, WB analysis of c-Met-silenced HepG2 cells displayed a 2.8-fold decrease of c-Met protein expression when compared with scramble-transfected cells. FC analysis of c-Met-silenced cells displayed an arrest of the cell cycle in the G<sub>1</sub> phase (25% increase; paired *t* test, *P* < 0.01) and a decrease (15%, paired *t* test, *P* < 0.01) of cell population in the S phase. WB and RT-PCR analysis of CDKIs in c-Met siRNA-transfected cells showed a concordant increase of both p21 and p27 levels. In the FC analysis, columns represent the average values of three independent experiments with corresponding SDs.



**Figure 4.** Rapamycin treatment affects cell cycle of HepG2 cells. A, WB analysis of rapamycin-treated HepG2 cells showed a 3.1-fold decrease of mTOR protein expression and a corresponding decrease of P-S6 expression. Rapamycin treatment determined an arrest of cell cycle in the G<sub>1</sub> phase (11%, *t* test, *P* = 0.04) and a decrease of cell population in the S phase (9%). B, FC analysis of anti-miR-199a-3p-transfected SNU475 cells revealed a 20% decrease of the G<sub>1</sub>-phase cell population and an increase of S-phase cells (*t* test, *P* < 0.01). C, FC analysis of mTOR- and c-Met-silenced SNU475 following anti-miR-199a-3p transfection. An increase of the G<sub>1</sub>-phase cells was observed following both mTOR and c-Met silencing (8% and 12%, respectively; *t* test, *P* = 0.028 and *P* = 0.014).

normal liver (ANOVA: *P* = 0.0009), whereas mTOR upregulation was observed in 23 of 39 HCCs. Subsequent analyses were carried out with miR-199a-3p because it modulates mTOR and c-Met. An inverse correlation between miR-199a-3p and mTOR protein expression in HCC samples (Pearson's correlation: *P* < 0.0001) is shown in Fig. 6C. No association was found between miR-199a-3p expression and serum AFP, tumor size, focality, grading, and etiology of the liver disease. Because HCC recurrences occurring within 2 years after surgery are considered as true recurrences due to a "seeded field" (29), TTR within this time frame was compared in patients with different miR-199a-3p expression. A shorter TTR was associated with lower miR-199a-3p values (log-rank test, *P* = 0.04; Fig. 6D), whereas no association was identified with patients survival.

## Discussion

Human miR-199a-3p and miR-199a-5p are processed from the same precursor, hsa-miR-199a, and are downregulated in several human cancers, including HCC (7, 12, 13). It was recently reported that miR-199a-3p regulates c-Met proto-oncogene and inhibits the "invasive growth" program when transfected in cancer cells other than HCC (16). In this study, we showed that an important function of miR-199a-3p is to repress the translation of mTOR and c-Met in HCC cells. In addition, we showed that miR-199a-3p contributes to the modulation of cell cycle, invasiveness, and sensitizes HCC cells to doxorubicin treatment and to hypoxia-induced apoptosis.

The serine/threonine kinase mTOR plays a key role in cell growth, protein translation, metabolism, cell invasion, and apoptosis (30). The mTOR pathway turned out to be implicated in the initiation and progression of several cancers, including HCC, and is therefore a promising therapeutic target, the potential of which is under clinical investigation (20, 31).

Recent reports identified c-Met, Smad1, and LIF (16–18) as miR-199a-3p targets. In addition, among genes with oncogenic functions, SEMA3A, ErbB4, ZEB1, ZEB2, and ITGB8 are predicted as putative, but not yet ascertained, miR-199a-3p targets. Conversely, c-Met plays a well-defined role in hepatocarcinogenesis (32–34) and was previously confirmed as a miR-199a-3p target. Therefore, here, we investigated to what extent miR-199a-3p contributes to the HCC phenotype by inhibiting mTOR or c-Met.

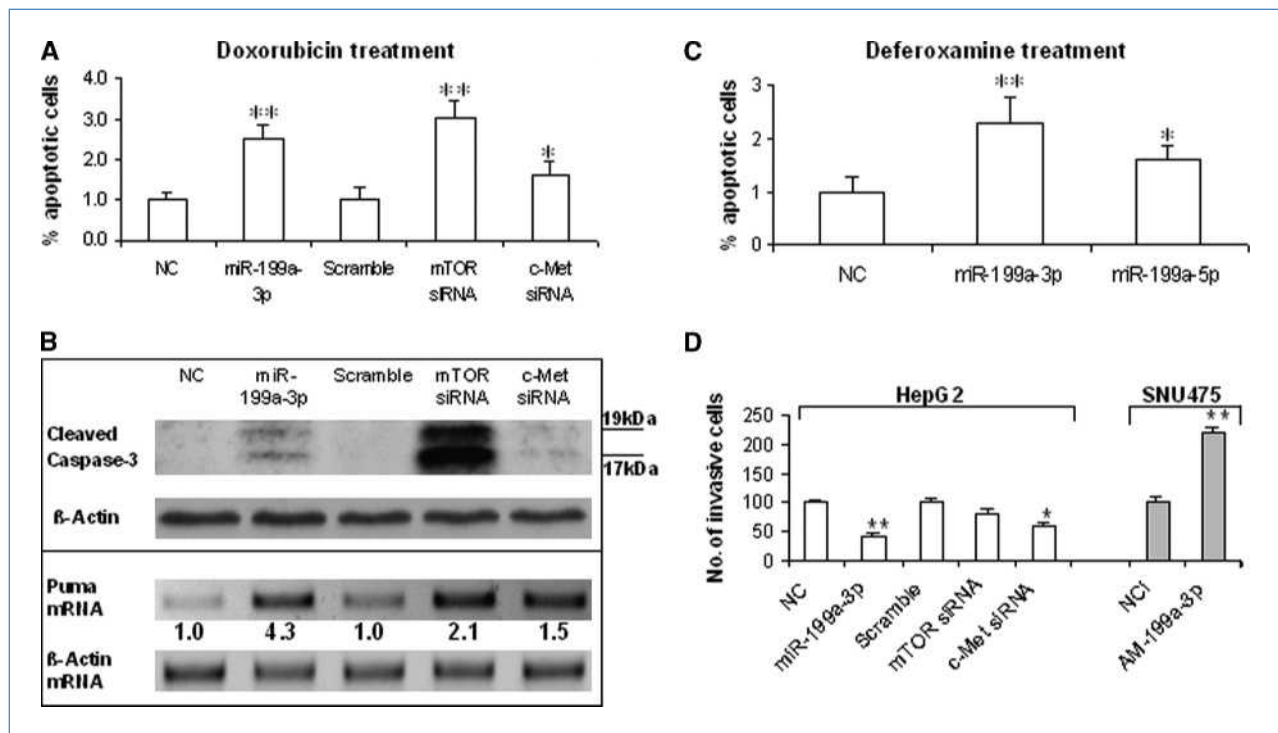
Cell cycle modulation by miR-199a-3p, resulted in increased G<sub>1</sub> and decreased S phases, though at less impressive levels than mTOR or c-Met silencing by siRNAs. These findings might be explained by considering the lower inhibition of mTOR and c-Met obtained by miR-199a-3p with respect to the more specific siRNAs. Indeed, because of their physiologic role and intrinsic nature, miRNAs regulate multiple targets and pathways, partitioning their inhibitory effect within the cell, which makes their effects difficult to be compared with the nearly total silencing of a single gene. Notably, in our experiments, we transfected cell lines with miR-199a-3p concentrations, mimicking those observed in normal liver tissue (Supplementary Fig. S1).

To gain a further insight into the mechanisms involved in the miR-199a-3p cell cycle modulation, we found an upregulation of CDKN1A/p21 and CDKN1B/p27 upon its restored expression. The same effect was determined by mTOR and c-Met silencing by siRNAs, except for CDKN1A/p21 downregulation upon mTOR-specific silencing. Indeed, only CDKN1A/p21 mRNA was upregulated, whereas the protein appeared downregulated. In line with our data, the inhibition of CDKN1A/p21 translation by mTOR has been reported in tumors other than HCC (35, 36). About cell cycle regulation, Li and colleagues (34) reported that c-Met silencing had no effect on the cell cycle of HepG2 cells. We can speculate that the discrepancy with our findings might be due to different experimental settings, such as cell confluence that indeed seems to have a fundamental role in the regulation of CDKN1A/p21 and CDKN1B/p27 through the hepatocyte growth factor/c-MET axis (37).

It was previously reported that mTOR inhibition by temsirolimus increases the sensitivity to chemotherapeutic agents through the downregulation and the accelerated deg-

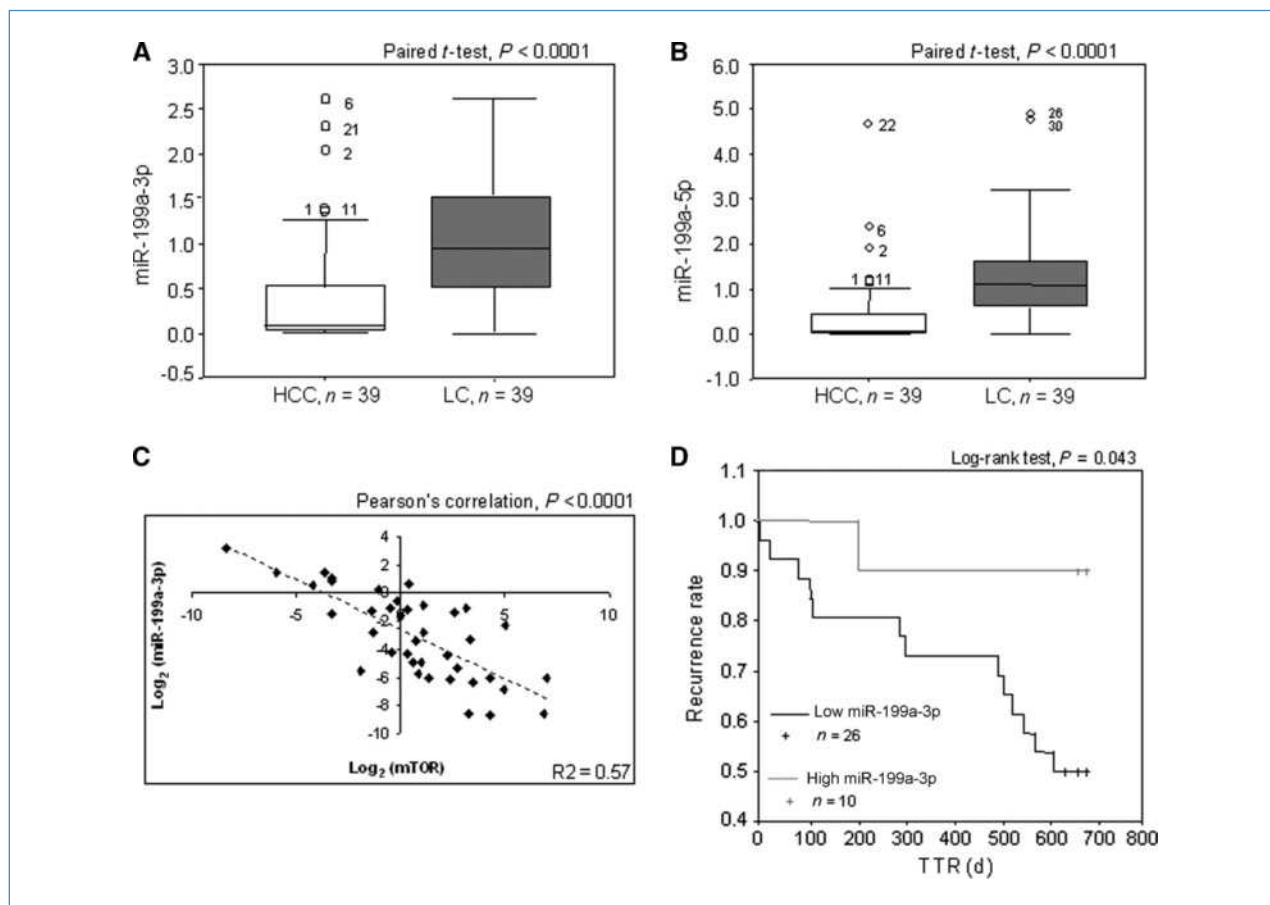
radation of CDKN1A/p21, which is thought to lower the apoptotic threshold to DNA damage (25, 38). Accordingly, we observed an increased sensitivity to doxorubicin in terms of apoptotic cell death upon both miR-199a-3p restoration and mTOR silencing, whereas the contribution of c-Met turned out to be marginal.

c-Met proto-oncogene is involved in a complex biological program named "invasive growth" that results from stimulation of cell motility, invasion, and protection from apoptosis (39). It has been previously reported that c-Met is downregulated by miR-199a-3p in several cancer cells other than HCC (16). Here, we report the role of miR-199a-3p in the modulation of the invasion capability of HCC cells through c-Met inhibition. HepG2 cells transfected with miR-199a-3p, as well as c-Met siRNAs, display a decreased invasion capability and in this experimental setting, miR-199a-3p seems to be more effective than inhibition of c-Met, whereas mTOR silencing does not affect the invasion capability of HepG2 cells. Accordingly, SNU475 cells, constitutively expressing high miR-199a-3p levels, displayed a higher invasion capability upon



**Figure 5.** miR-199a-3p affects doxorubicin sensitivity, growth in hypoxic conditions, and cell invasion. **A**, FC Annexin V assay in transfected HepG2 cells following doxorubicin treatment. A 2.5- and 3.0-fold increase in Annexin V-positive cells was observed in miR-199a-3p-transfected and mTOR-silenced cells, respectively (*t* test,  $P = 0.005$ ) when compared with negative controls. A 1.6-fold increase in apoptotic cells was observed in c-Met-silenced cells (*t* test,  $P = 0.03$ ). **B**, WB analysis of the cleaved caspase-3 in HepG2 cells transfected with miR-199a-3p, mTOR, and c-Met siRNAs following doxorubicin challenge. Cleaved caspase-3 fragments were upregulated in miR-199a-3p-transfected and mTOR-silenced cells. RT-PCR analysis of HepG2 cells displayed an increase of Puma expression following miR-199a-3p transfection or mTOR/c-Met silencing. **C**, FC Annexin V assay in transfected HepG2 cells following deferoxamine treatment. A 2.3- and 1.6-fold increase in Annexin V-positive cells was observed in miR-199a-3p- and miR-199a-5p-transfected cells, respectively (*t* test,  $P = 0.006$  and  $0.03$ ). **D**, HepG2 cells transfected with miR-199a-3p, mTOR, c-Met siRNAs, or negative controls (NC and Scramble) were tested by the Matrigel invasion assay. A significant reduction of invading cells was observed in miR-199a-3p- and c-Met siRNA-transfected cells (*t* test,  $P = 0.009$  and  $0.017$ ), but not in mTOR-silenced cells (*t* test,  $P = 0.15$ ). SNU475 cells were transfected with anti-miR-199a-3p or miRNA inhibitor negative controls (NCi) and then assayed by the Matrigel invasion assay. A significant increase of invading cell number was observed following miR-199a-3p silencing (*t* test,  $P = 0.0005$ ). Columns, mean of three independent experiments; bars, SD; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$  at paired *t* test.





**Figure 6.** miR-199a-3p and miR-199a-5p expression in HCC samples and correlation with mTOR protein and TTR. A and B, box plot graphic displays of 39 HCC and matched cirrhotic (LC) tissues grouped according to miR-199a-3p and miR-199a-5p expression. The three horizontal bars from top to bottom are the 75th, 50th (median), and 25th percentiles, respectively. Circles, outliers. C, inverse correlation (two-tailed Pearson's test,  $P < 0.0001$ ) between miR-199a-3p and mTOR protein in human HCC. Each data from HCC samples was referred to matched cirrhosis and expressed with a  $\log_2$  scale. D, TTR analysis by Kaplan-Meier survival curves exhibits a higher recurrence rate in HCC patients with lower miR-199a-3p tissue levels (categorized on the basis of the median value).

miR-199a-3p inhibition. The involvement of matrix metalloproteinases (MMP) in the invasion process was analyzed by zymography assay in transfected cells (data not shown). MMP-2 and MMP-9 decreased in c-Met-silenced HepG2 cells, but not in miR-199a-3p-transfected cells, suggesting c-Met as a major activator of MMPs. The higher invasion capability observed upon miR-199a-3p inhibition let us hypothesize the regulation of multiple targets, beside c-Met, as well as the involvement of other mechanisms different from MMPs modulation.

To confirm the relevance of our *in vitro* findings, we identified an inverse correlation between miR-199a-3p expression and mTOR protein levels in human HCC samples. Notably, a shorter time to recurrence was observed in patients with lower miR-199a-3p levels. These findings further suggest that miR-199a-3p may influence the invasion capability *in vitro*, as well as *in vivo*.

In conclusion, our findings emphasize the role of miR-199a-3p as a modulator of cell cycle, invasion capability, and response to doxorubicin treatment. The identification

of mTOR and c-Met among miR-199a-3p targets further emphasizes the roles of this miRNA in HCC.

The regulation of multiple, key, cancer-associated pathways by miR-199a-3p in HCC cells emphasizes out the importance of its replacement and offers the theoretical possibility to counteract the malignant phenotype. Moreover, endogenous and nontoxic molecules such as miRNAs analogues might represent a promising tool for cancer gene therapy (40), particularly when combined with doxorubicin.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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## References

- Laurent-Puig P, Legoix P, Bluteau O, et al. Genetic alterations associated with hepatocellular carcinomas define distinct pathways of hepatocarcinogenesis. *Gastroenterology* 2001;120:1763–73.
- Chiang DY, Villanueva A, Hoshida Y, et al. Focal gains of VEGFA and molecular classification of hepatocellular carcinoma. *Cancer Res* 2008;68:6779–88.
- Llovet JM, Ricci S, Mazzaferro V, et al. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008;359:378–90.
- Ajani J, Allgood V. Molecular mechanisms in cancer: What should clinicians know? *Semin Oncol* 2005;32:2–4.
- Ebos JM, Lee CR, Cruz-Munoz W, Bjarnason GA, Christensen JG, Kerbel RS. Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis. *Cancer Cell* 2009;15:232–9.
- Iorio MV, Croce CM. MicroRNAs in cancer: small molecules with a huge impact. *J Clin Oncol* 2009;27:5848–56.
- Jiang J, Gusev Y, Aderca I, et al. Association of MicroRNA expression in hepatocellular carcinomas with hepatitis infection, cirrhosis, and patient survival. *Clin Cancer Res* 2008;14:419–27.
- Budhu A, Jia HL, Forgues M, et al. Identification of metastasis-related microRNAs in hepatocellular carcinoma. *Hepatology* 2008;47:897–907.
- Gramantieri L, Fornari F, Callegari E, et al. MicroRNA involvement in hepatocellular carcinoma. *J Cell Mol Med* 2008;12:2189–204.
- Ladeiro Y, Couchy G, Balabaud C, et al. MicroRNA profiling in hepatocellular tumors is associated with clinical features and oncogene/tumor suppressor gene mutations. *Hepatology* 2008;47:1955–63.
- Gramantieri L, Ferracin M, Fornari F, et al. Cyclin G1 is a target of miR-122a, a microRNA frequently down-regulated in human hepatocellular carcinoma. *Cancer Res* 2007;67:6092–9.
- Ichimi T, Enokida H, Okuno Y, et al. Identification of novel microRNA targets based on microRNA signatures in bladder cancer. *Int J Cancer* 2009;125:345–52.
- Iorio MV, Visone R, Di Leva G, et al. MicroRNA signatures in human ovarian cancer. *Cancer Res* 2007;67:8699–707.
- Landgraf P, Rusu M, Sheridan R, et al. A mammalian microRNA expression atlas based on small RNA library sequencing. *Cell* 2007;129:1401–14.
- Dolganiuc A, Petrasek J, Kodys K, et al. MicroRNA expression profile in Lieber-DeCarli diet-induced alcoholic and methionine choline deficient diet-induced nonalcoholic steatohepatitis models in mice. *Alcohol Clin Exp Res* 2009;33:1704–10.
- Kim S, Lee UJ, Kim MN, et al. MicroRNA miR-199a\* regulates the MET proto-oncogene and the downstream extracellular signal-regulated kinase 2 (ERK2). *J Biol Chem* 2008;283:18158–66.
- Lin EA, Kong L, Bai XH, Luan Y, Liu CJ. MiR-199a, a bone morphogenic protein 2-responsive MicroRNA, regulates chondrogenesis via direct targeting to Smad1. *J Biol Chem* 2009;284:11326–35.
- Oskowitz AZ, Lu J, Penforis P, et al. Human multipotent stromal cells from bone marrow and microRNA: regulation of differentiation and leukemia inhibitory factor expression. *Proc Natl Acad Sci U S A* 2008;105:18372–7.
- Migliore C, Petrelli A, Ghiso E, et al. MicroRNAs impair MET-mediated invasive growth. *Cancer Res* 2008;68:10128–36.
- Villanueva A, Chiang DY, Newell P, et al. Pivotal role of mTOR signaling in hepatocellular carcinoma. *Gastroenterology* 2008;135:1972–83.
- Zhou L, Huang Y, Li J, Wang Z. The mTOR pathway is associated with the poor prognosis of human hepatocellular carcinoma. *Med Oncol* 2010;27:255–61.
- Edmondson HA, Steiner PE. Primary carcinoma of the liver: a study of 100 cases among 48,900 necropsies. *Cancer* 1954;7:462–503.
- Fornari F, Gramantieri L, Ferracin M, et al. MIR-221 controls CDKN1C/p57 and CDKN1B/p27 expression in human hepatocellular carcinoma. *Oncogene* 2008;27:5651–61.
- Huynh H, Chow KH, Soo KC, et al. RAD001 (everolimus) inhibits tumor growth in xenograft models of human hepatocellular carcinoma. *J Cell Mol Med* 2009;13:1371–80.
- Piguet AC, Semela D, Keogh A, et al. Inhibition of mTOR in combination with doxorubicin in an experimental model of hepatocellular carcinoma. *J Hepatol* 2008;49:78–87.
- Buitrago-Molina LE, Pothiraju D, Lamle J, et al. Rapamycin delays tumor development in murine livers by inhibiting proliferation of hepatocytes with DNA damage. *Hepatology* 2009;50:500–9.
- Land SC, Tee AR. Hypoxia-inducible factor 1 $\alpha$  is regulated by the mammalian target of rapamycin (mTOR) via an mTOR signaling motif. *J Biol Chem* 2007;282:20534–43.
- Rane S, He M, Sayed D, et al. Downregulation of miR-199a derepresses hypoxia-inducible factor-1 $\alpha$  and Sirtuin 1 and recapitulates hypoxia preconditioning in cardiac myocytes. *Circ Res* 2009;104:879–86.
- Imamura H, Matsuyama Y, Tanaka E, et al. Risk factors contributing to early and late phase intrahepatic recurrence of hepatocellular carcinoma after hepatectomy. *J Hepatol* 2003;38:200–7.
- Vignot S, Faivre S, Aguirre D, Raymond E. MTOR-targeted therapy of cancer with rapamycin derivatives. *Ann Oncol* 2005;16:525–37.
- Meric-Bernstam F, Gonzalez-Angulo AM. Targeting the mTOR signaling network for cancer therapy. *J Clin Oncol* 2009;27:2278–87.
- Salvi A, Arici B, Portolani N, Giuliani SM, De Petro G, Barlati S. *In vitro* c-met inhibition by antisense RNA and plasmid-based RNAi downmodulates migration and invasion of hepatocellular carcinoma cells. *Int J Oncol* 2007;31:451–60.
- Salvi A, Sabelli C, Moncini S, et al. MicroRNA-23b mediates urokinase and c-met downmodulation and a decreased migration of human hepatocellular carcinoma cells. *FEBS J* 2009;276:2966–82.
- Li N, Fu H, Tie Y, et al. miR-34a inhibits migration and invasion by down-regulation of c-Met expression in human hepatocellular carcinoma cells. *Cancer Lett* 2009;275:44–53.
- Lin PY, Fosmire SP, Park SH, et al. Attenuation of PTEN increases p21 stability and cytosolic localization in kidney cancer cells: a potential mechanism of apoptosis resistance. *Mol Cancer* 2007;6:16–30.
- Beuvink I, Boulay A, Fumagalli S, et al. The mTOR inhibitor RAD001 sensitizes tumor cells to DNA-damaged induced apoptosis through inhibition of p21 translation. *Cell* 2005;120:747–59.
- Yang H, Magilnick N, Noureddin M, Mato JM, Lu SC. Effect of hepatocyte growth factor on methionine adenosyltransferase genes and growth is cell density-dependent in HepG2 cells. *J Cell Physiol* 2007;210:766–73.
- Tam KH, Yang ZF, Lau CK, Lam CT, Pang RW, Poon RT. Inhibition of mTOR enhances chemosensitivity in hepatocellular carcinoma. *Cancer Lett* 2009;273:201–9.
- Comoglio PM, Trusolino L. Invasive growth: from development to metastasis. *J Clin Invest* 2002;109:857–62.
- Tong AW, Nemunaitis J. Modulation of miRNA activity in human cancer: a new paradigm for cancer gene therapy? *Cancer Gene Ther* 2008;15:341–55.