**Cell Cycle and Senescence**

**p53/mdm2 Feedback Loop Sustains miR-221 Expression and Dictates the Response to Anticancer Treatments in Hepatocellular Carcinoma**

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**Abstract**

The overexpression of microRNA-221 (miR-221) is reported in several human cancers including hepatocellular carcinoma, and its targeting by tailored treatments has been proposed. The evidence supporting the role of miR-221 in cancer is growing and has been mainly focused on the discovery of miR-221 targets as well as on its possible therapeutic exploitations. However, the mechanism sustaining miR-221 aberrant expression remains to be elucidated. In this study, MDM2 (E3 ubiquitin-protein ligase homolog), a known p53 (TP53) modulator, is identified as a direct target of miR-221, and a feed-forward loop is described that sustains miR-221 aberrant expression. Interestingly, miR-221 can activate the p53/mdm2 axis by inhibiting MDM2 and, in turn, p53 activation contributes to miR-221 enhanced expression. Moreover, by modulating the p53 axis, miR-221 impacts cell-cycle progression and apoptotic response to doxorubicin in hepatocellular carcinoma–derived cell lines. Finally, CpG island methylation status was assessed as a causative event associated with miR-221 upregulation in hepatocellular carcinoma cells and primary tumor specimens. In hepatocellular carcinoma–derived cell lines, pharmacologically induced DNA hypomethylation potentiated a significant increase in miR-221 expression. These data were confirmed in clinical specimens of hepatocellular carcinoma in which elevated miR-221 expression was associated with the simultaneous presence of wild-type p53 and DNA hypomethylation.

**Implications:** These findings reveal a novel miR-221–sustained regulatory loop that determines a p53-context-specific response to doxorubicin treatment in hepatocellular carcinoma. *Mol Cancer Res; 12(2); 203–16.*

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**Introduction**

microRNAs (miRNA) play a pivotal role in cancer cell biology. Because of their ability to simultaneously control several cancer-related pathways, miRNAs represent attractive therapeutic targets. Each miRNA triggers different molecular events depending both on the cell type and on the molecular context. Indeed, the same miRNA can exert diverse functions in different settings (1). Thus, it is not surprising that both oncogenes and tumor suppressor (TS) genes coexist among the targets predicted for each tumor-related miRNA. Most studies have been focused on the confirmation of tumor suppressor genes as targets of cancer-promoting miRNAs as well as on the validation of oncogenes as targets of miRNAs downregulated in tumor tissues. Nevertheless, for an exhaustive miRNA-based cancer classification, it is important to explore the action of deregulated miRNAs on molecules playing relevant roles in each cancer type, even though the relationship cancer-promoting miRNA/TS gene or TS-miRNA/oncogene is not satisfied. Moreover, in the perspective of molecularly driven treatments, it is mandatory to characterize miRNAs’ functions not only in specific cancer types, but also in definite molecular subgroups of each tumor.

miRNA-221 (miR-221) is a tumor-related miRNA commonly upregulated in different cancers, (2–7) as well as in hepatocellular carcinoma (8). We have shown its tumor promoting functions in the liver of a miR-221 transgenic mouse, confirming its role as a therapeutic candidate (9). In addition, several direct targets of miR-221 have been assessed so far (10–13). Despite miR-221 upregulation being a common event in solid malignancies, factors contributing
to its overexpression are still poorly understood and they deserve attention to select the optimal cellular contexts in which miR-221 targeting is expected to be more effective, as well as to identify possible therapeutic combinations.

The TP53/MDM2 axis represents a key mechanism controlling stress responses, cell-cycle progression, and DNA repair (13). MDM2 (E3 ubiquitin-protein ligase homolog) encodes for the oncogenic mdm2 phosphoprotein (14), which inhibits DNA repair and apoptosis by complexing and ubiquitinating p53 for proteasome degradation (15). In hepatocellular carcinoma, p53 accumulation and mdm2 overexpression are negatively associated with survival (16). MDM2 is a predicted target of miR-221 as proved in normal chondrocytes (17). This interaction might seem conflicting in cancer, due to the oncogenic role of both miR221 and mdm2. Nevertheless, the assessment of MDM2 targeting by miR-221 might contribute to elucidate events sustaining the neoplastic phenotype.

Epigenetic changes promote cancer development and progression by regulating multiple cancer-associated factors (18). Concerning miRNAs, several evidences support the role of aberrant methylation in the modulation of cancer-associated miRNAs (19, 20). We have identified the hypomethylation of the chromosome 19 miRNA cluster (C19MC) region as a relevant factor determining the increased expression of some members of the C19MC (20). Similarly, the aberrant methylation of miR-29a and miR-148a regions was described as a contributor to the neoplastic phenotype (21, 22).

Here, we define the modulation of MDM2 expression by miR-221 in hepatocellular carcinoma, identifying for the first time, an oncogene as a target of a tumor-promoting miRNA in cancer cells. This molecular mechanism is involved in cell-cycle regulation and response to treatment in a TP53-dependent manner in hepatocellular carcinoma cells. Remarkably, TP53/MDM2 axis and aberrant DNA methylation of the miR-221 upstream region were assessed as contributors to miR-221 overexpression in hepatocellular carcinoma.

Materials and Methods

Patients

Hepatocellular carcinoma and cirrhotic tissues were obtained from 47 consecutive patients (38 males and 9 females; median age ± SD, 67.9 ± 8.3; range, 49–82 years) undergoing liver resection for hepatocellular carcinoma. Tissues were collected at surgery after obtaining an informed consent, and stored as previously described (23). Histopathological grading was scored according to Edmondson and Steiner criteria (24). No patient received anticancer treatment before surgery. The characteristics of patients are detailed in Supplementary Table S1.

Cell culture and treatments

HepG2 and Hep3B cell lines were purchased from American Type Culture Collection. Huh-7 cells are from Prof. Alberti’s laboratory, University of Padova, Padua, Italy. Cells were treated with 2.5 and 10 μg/mL of doxorubicin (Pfizer) for 6 and 24 hours, or with 2.0–5.0 μmol/L of 5-Aza-2’-deoxycytidine or Nurtilin-3 (Sigma-Aldrich) for 24 and 48 hours. All experiments were performed in triplicate. Trypan blue (Sigma-Aldrich) was added with a ratio of 1:1 to cells immediately before cell counting with Burker chamber.

Cell transfection

Hepatocellular carcinoma–derived cell lines were transfected with 100 nmol/L of pre-miR-221, anti-miR-221, or Negative Control#1 precursor and inhibitor miRNAs (Ambion). Gene silencing was carried out by using MDM2 (Santa Cruz Biotechnology) and TP53 siRNAs pools and Stealth RNA Negative Control (Life Technologies). Oligonucleotide transfection was performed by using Lipofectamine 2000 (Life Technologies) according to the manufacturer’s instructions.

Stable transfection

DNA sequence of mature miR-221 was inserted into the BamHI–HindIII cloning sites of pGFP-V-RS short hairpin RNA (shRNA) vector according to manufacturer’s instructions (OriGene Technologies). Retroviral particle production was obtained by calcium phosphate precipitation of plasmidic vector into Phoenix A packaging cells (kindly provided by Dr. Gary Nolan, Department of Microbiology and Immunology, Stanford University, Stanford, California, USA). Viral infection was performed in Huh-7 cells by spinoculation (centrifugation at 2,200 rpm for 45 minutes at 32°C) of 2,5 ml of undiluted virus in the presence of 8 μg/mL of Polybrene, followed by incubation of cells at 32°C for 5 hours. Subsequently, viral supernatants were replaced with fresh medium and cells were incubated at 37°C. Selection with RPMI containing puromycin was started 48 hours after infection.

MTT assay

HepG2 cells were seeded in a 96-well plate at a concentration of 8,000 cells per well. At the end of the specific treatment, 10 μL of MTT (Sigma-Aldrich) was added to each well and cells were incubated at 37°C for 4 hours. The formazan salts were then dissolved by adding 100 μL of dimethyl sulfoxide (DMSO) to each well. The colorimetric measurement was performed by using Multiskan Ascent microplate reader (Thermo Labsystems) with a 550-nm filter. The experiment was performed in sextuplicate.

Luciferase activity assay

The 3’ untranslated (3’UTR) region of human MDM2 gene was amplified by PCR using primers and conditions reported in Supplementary Table S2. Luciferase reporter assay of wild-type (WT) and mutagenized 3’UTR containing vectors and TP53 reporter (pp53-TA-luc, Clontech) assay were performed as previously described (25).

Caspase-3/7 activity assay

The enzymatic activation of effector caspases 3 and 7 was evaluated in transfected hepatocellular carcinoma cells following doxorubicin treatment (10 μg/mL for 6 hours) by
using Caspase-Glo 3/7 assay (Promega) accordingly to manufacturers’ instructions. These experiments were performed in triplicate.

**Real-time PCR**

TaqMan MicroRNA Assays (Applied Biosystems) were used for quantifying miRNA expression, as previously described (23). Real-time qPCR (RT-qPCR) quantification (IQ SYBR Green Supermix; Bio-Rad Laboratories) of MDM2, CDKN1A/p21, and pri-miR-221 genes was performed by using primer and conditions reported in Supplementary Table S3. A TaqMan probe (primeTime qPCR assay; IDT) was used for β-actin. Real-time experiments were performed in triplicate.

**Western blot analysis**

Hepatocellular carcinoma cells and tissues were assayed with anti-mdm2 (N-20), anti-p21 (F-5), anti-p57 (H-91), anti-β-actin (C4; Santa Cruz Biotechnology), anti-p53 (clone DO-7, Dakocytomation and clone FL-393, Santa Cruz Biotechnology), anti-p27 (57; BD Biosciences), anti-cleaved-caspase-3 (Asp175) and caspase-7 (Asp198), antibax, and anti-puma (Cell Signaling Technology) antibodies. Digital images were acquired and quantified with ChemiDoc XRS+ (Image Lab Software; Bio-Rad). Western blot analysis was performed in triplicate.

**Semiquantitative RT-PCR**

Semiquantitative RT-PCR was performed using primers and amplification conditions detailed in Supplementary Table S3.

**Flow cytometry analysis**

Cell-cycle analysis was performed as previously described (12). Detection of apoptotic cells was performed by flow cytometry with Annexin V/Propidium Iodide (PI) detection kit (Bender MedSystems). Bromodeoxyuridine (BrdUrd) incorporation, performed for 1 hour at the end of the specific transfection period, was evaluated by using anti-BrdU-FITC antibody (clone B44; Becton Dickinson) according to the manufacturer’s instructions. All these analysis were performed in triplicate.

**Methylation analysis**

A 194-bp CpG island located 7,769 base pairs upstream of miR-222 precursor was predicted by using MethPrimer algorithm (http://www.urogene.org/methprimer/). DNA methylation status was analyzed by methylation-specific PCR as previously described (20). Primers and conditions are reported in Supplementary Table S3.

**Chromatin immunoprecipitation**

Untreated and treated HepG2 cells were subjected to chromatin immunoprecipitation (ChIP) with a polyclonal p53 antibody (clone CM1; Novocastra) as previously described (20). Primers and conditions are reported in Supplementary Table S3. Putative binding sites (BS) for TP53 were identified by using the PROMO algorithm (http://alggen.lsi.upc.es/; ref. 26).

**Electrophoresis mobility shift assay**

Nuclear extracts were obtained from untreated and Nutlin-3 (5 μmol/L) treated HepG2 cells by using NE-PER Nuclear and Cytoplasmatic Extraction Reagents (Thermo Scientific) according to the manufacturer’s instructions. Forward and reverse oligonucleotide sequences for miR-221_p53_BS2, BS3, and BS4 are reported in Supplementary Table S4. Forward strands were 5'-biotinylated for labeled probes. Complementary strands were annealed in a thermocycler by using the following program: 95°C for 5 minutes, 95°C (−1°C/cycle) for 70 repeats. Electrophoresis mobility shift assay (EMSA) was performed by using Light Shift Chemiluminescent EMSA Kit (Thermo Scientific) according to the manufacturer’s instructions. The following cofactors were added to the binding reaction: glycerol (5%), MgCl2 (2 mmol/L), poly (dIdC; 50 ng), DT5 (5 mmol/L), and bovine serum albumin (BSA; 20 mg) and the reaction mix together with nuclear extracts was incubated for 5 minutes at room temperature. After the addition of biotin-labeled DNA (40 fmol), the binding reaction was incubated for other 30 minutes at room temperature. A 200-fold molar excess of unlabelled probe was used in the competition assay. For supershift assay, the same antibody of ChIP was used. The binding reactions were transferred to Hybond-N+ nylon membrane (GE Healthcare). Digital images were acquired with ChemiDoc XRS+ (Image Lab Software; Bio-Rad).

**TP53 sequencing**

TP53 mutations were identified as previously reported (20).

**Animal model**

A miR-221 transgenic mouse was obtained by microinjection of fertilized oocytes of a B6D2F2 mouse strain with a DNA fragment containing the mmu-miR-221 locus cloned into the pWhere vector downstream the EII/al-AT enhancer II of hepatitis B virus coupled with the liver-specific α-1-antitrypsin promoter), which guarantees a liverspecific expression. After several crosses, a homozygous line of transgenic mice overexpressing miR-221 in the liver about 2-folds (1.7 folds in the cases assayed in this study: 8.66±1.25 and 4.98±1.12) with respect to controls was produced (9). Hepatocellular carcinoma development was elicited by diethylnitosamine (DENA) treatment both in transgenic and WT mice. Hepatocellular carcinomas arisen in transgenic mice were analyzed for TP53 and mdm2 protein expression and were compared with hepatocellular carcinomas arisen in WT mice.

**Statistical analysis**

Differences between groups were analyzed using the conventional Student t test, both for in vitro assays and for the analysis of hepatocellular carcinoma–related variables, including miR-221 expression in primary tumors, which was analyzed against TP53 and DNA methylation status. The
Results

**TP53 participates to miR-221 modulation in hepatocellular carcinoma cells**

To investigate whether TP53 might exert any effect in the control of miR-221 expression, we performed p53 overexpression and silencing in HepG2 cells, harboring a WT TP53. TP53 silencing was carried out by a siRNAs pool, whereas TP53 overexpression was carried out by inhibiting MDM2 with Nutlin-3, as well as by doxorubicin treatment, which is known to trigger TP53 expression (27). TP53 silencing determined a reduction of both mature miR-221 and primary miR-221 transcript (pri-miR-221) in HepG2 cells (Fig. 1A). A time-dependent increase of both miR-221 and pri-miR-221 expression was observed in HepG2 cells treated with Nutlin-3, at a concentration of 2 μmol/L (Fig. 1B). Similarly, an increase of both miR-221 and pri-miR-221 levels was detected in HepG2 cells, following doxorubicin treatment (Supplementary Fig. S1A). Remarkably, a higher Nutlin-3 concentration (5 μmol/L), determining a stronger TP53 expression, resulted in an increased transcriptional activation of pri-miR-221 but a diminished mature miR-221 expression, letting us to hypothesize a dose-dependent regulation of miR-221 maturation process by TP53 (Fig. 1B). The treatment of HepG2 cells with the highest Nutlin-3 dose for 48 hours determined a 9% decrease of cell viability, as determined by the MTT assay (Supplementary Fig. S1B) and a 22% increase of cell death, as assayed by Trypan blue exclusion assay (Supplementary Fig. S1C). No variation in cell viability and cell death rate was observed with the lowest Nutlin-3 dose (Supplementary Fig. S1B and S1C).

To investigate whether TP53 might directly regulate miR-221 expression, a bioinformatic analysis was performed to identify hypothetical TP53-binding sites in a DNA region spanning from −1,000 to +1,000 nucleotides, considering +1 the first nucleotide of miR-222 precursor. Six TP53-binding sites were identified (Fig. 1C). To analyse p53 binding to these consensus sequences, a ChIP assay was performed in HepG2 cells. ChIP analysis enriched the p53-binding site B54 in both untreated and (2 and 5 μmol/L) Nutlin-3–treated cells (Fig. 1D and Supplementary Fig. S1D and S1E). To explore if methylation status could influence DNA binding by TP53, ChIP analysis was also performed in HepG2 cells following 5-Aza-2′-deoxycytidine treatment, which resulted to be a slight inducer of TP53 expression itself (Fig. 1E). P53 protein levels of Nutlin-3 and Aza-treated HepG2 cells are displayed in Supplementary Fig. S1D. An enrichment of p53-binding sites B52 and B53, but not B54, was observed upon Aza treatment alone or in combination with 0.5 μmol/L Nutlin-3 (Fig. 1D and Supplementary Fig. S1D and S1E), suggesting the influence of epigenetic mechanisms in p53 binding at specific sites of the miR-221 upstream region. The p53 interaction with B52, B53, and B54 was also verified by EMSA assay, which confirmed the data from the ChIP analysis (Supplementary Fig. S1F). This experiment further showed that, in the absence of DNA demethylation, p53 binding to B54 is stronger than that to B52 and B53.

In line with previous data, an increase of pri-miR-221 and mature miR-221 expression was shown in 5-Aza-2′-deoxycytidine–treated HepG2 cells, whereas no synergetic effect was observed following 5-Aza-2′-deoxycytidine and low-dose Nutlin-3 combined treatment. On the contrary, the combination of 5-Aza-2′-deoxycytidine with high-dose Nutlin-3 treatment inhibited miR-221 expression, whereas it greatly increased pri-miR-221 levels (Fig. 1F). These findings suggest that, despite increased expression of p53, which triggers pri-miR-221 transcription, a stronger p53 accumulation might inhibit miR-221 maturation, resulting in mature miR-221 reduction.

**MiR-221 upregulation by p53 exerts a positive feedback loop by targeting MDM2**

To elucidate the mechanisms sustaining miR-221 overexpression in hepatocellular carcinoma and to identify
miR-221 targets involved in positive-feedback loops, a bioinformatic analysis was performed. This analysis highlighted the presence of p53-transcriptionally activated genes, including MDM2, the principal inhibitor of p53 itself.

Two bioinformatic algorithms (miRanda and TargetScan) revealed two complementary binding sites for miR-221 in human MDM2 (NM_002392) 3′ UTR (Fig. 2A). miR-221 and MDM2 basal levels were analyzed in hepatocellular carcinoma–derived cell lines (Fig. 2B). A positive correlation was found between miR-221 and p53 (Pearson correlation, P = 0.025), whereas an inverse correlation was found between miR-221 levels and mdm2 protein (Pearson correlation, P = 0.021), as well as between p53 and mdm2 protein levels (Pearson correlation, P = 0.01) and miR-221 expression and MDM2 mRNA levels (Pearson correlation, P = 0.03; Supplementary Fig. S2A and S2B). A direct correlation linked MDM2 mRNA and protein levels (Pearson correlation, P = 0.02; Supplementary Fig. S2C). These findings are in line with a possible role of miR-221 as a regulator of MDM2 mRNA stability.

To investigate if in vitro data are representative of hepatocellular carcinomas, we performed an ex vivo analysis assessing miR-221 expression and mdm2 protein levels in 17 hepatocellular carcinoma tissues. An inverse correlation between miR-221 and mdm2 levels (Pearson correlation, P = 0.005; Fig. 2C and D) was shown, suggesting that aberrant miR-221 expression might modulate mdm2 expression in hepatocellular carcinoma.

The 17 hepatocellular carcinoma tissues previously assessed for miR-221 and mdm2 expression (Fig. 2D) were also tested for p53 expression. No significant correlation was found between p53 and mdm2, even though a trend toward an inverse correlation was observed (Pearson correlation, P = 0.078). Notably, this analysis was performed on both WT and mutated TP53 cases, thus making not fully reliable any correlation between mdm2 and p53, because an accumulation of p53 protein was observed at Western blot analysis in most of the cases presenting TP53 mutations in the coding sequence. A representative case selection is showed in Fig. 2C.

To demonstrate that miR-221 was involved in mdm2 regulation, a functional analysis was performed in hepatocellular carcinoma cells. Transfection of miR-221 in Huh-7 cells resulted in a decrease of mdm2 protein levels when...
compared with negative-control cells (Fig. 3A), whereas transfection of anti-miR-221 in SNU449 and SNU182 cells determined an increase of mdm2 protein (Fig. 3B and Supplementary Fig. S2D).

To verify whether miR-221 regulates MDM2 mRNA, p53-deicient Hep3B cells were chosen to avoid the confounding effect due to p53 transcriptional regulation of MDM2. MiR-221 overexpression and silencing in Hep3B cells determined a reduction and an increase of MDM2 mRNA and protein levels, respectively (Fig. 3C and Supplementary Fig. S2E), suggesting that miR-221 regulates MDM2 mRNA stability.

To assay whether miR-221 variations within the physiologic range are still effective in the modulation of mdm2 levels, we also established by retroviral infection a Huh-7 cell clone stably overexpressing miR-221. RT-qPCR analysis determined a 6.2-fold increase of miR-221 in stably transfected Huh-7 cells with respect to controls. Western blot analysis of miR-221 known targets (p27, puma, and mdm2) showed a significant decrease of these targets (Fig. 3D).

The direct interaction between miR-221 and its binding sites in MDM2 3’UTR was proved by a luciferase reporter assay. A decrease of the luciferase activity was observed in HepG2 cells for both 3’-UTR–containing vectors in presence of miR-221 with respect to negative-control cotransfected cells (Fig. 3E). Cotransfection of pGL3-MDM2 3’-UTR–containing vectors and anti-miR-221 in HepG2 and SNU449 cells displayed an increase of the luciferase activity for both hypothetical binding sites when compared with controls (Supplementary Fig. S2F and S2G). Taken together, these data demonstrated that miR-221 directly binds MDM2 mRNA, determining its degradation in hepatocellular carcinoma cells.

Because mdm2 is the principal regulator of p53 stability and functionality, we performed a reporter assay to investigate p53 transcriptional activity following miR-221 modulation in HepG2 cells. MiR-221 overexpression determined an increase of p53-mediated reporter gene activity, whereas miR-221 silencing caused a decrease of the luciferase signal (Fig. 3F). Western blot analysis showed a modulation of mdm2 and p53 protein levels following miR-221 or anti-miR-221 transfection, suggesting that the regulation of p53 protein expression is a relevant event triggered by miR-221 and it is associated with a variation in p53 activity. The confirmation of p53 transcriptional activity modulation was done by investigating CDKN1A/p21 mRNA levels in the same experimental settings (Fig. 3F and Supplementary Fig. S2H).

MDM2 silencing in HepG2 cells (Fig. 3G and Supplementary Fig. S2I) determined an increase of p53 activity similar to that observed following miR-221 overexpression. Finally, the cotransfection of anti-miR-221 and MDM2 siRNAs in HepG2 cells confirmed that miR-221 regulates p53 activity and expression through MDM2 targeting (Fig. 3F and Supplementary Fig. S2H).

To assay whether miR-221–mediated modulation of p53 expression also occurs in primary tumors, we analyzed hepatocellular carcinomas from miR-221 transgenic mice and WT controls. In line with our in vitro findings, transgenic mice displayed lower mdm2 and higher p53 protein expression levels (t test, P = 0.048 and P = 0.044 respectively, Fig. 3H) when compared with controls. Notably, hepatocellular...
Figure 3. miR-221 targets MDM2 and regulates TP53 expression. A, quantitative PCR (qPCR) of miR-221 in transfected Huh-7 cells. Western blot analysis revealed a decrease of mdm2 levels in miR-221–transfected cells (miR). NC, negative control. B, qPCR of anti-miR-221–transfected SNU449 cells. Western blot analysis displayed an increase of mdm2 expression in anti-miR-221 (AM)–transfected cells. NC, miRNA negative-control inhibitor. C, qPCR of miR-221 expression in miR-221 or anti-miR-221–transfected Hep3B cells. Western blot and RT-PCR analyses showed MDM2 modulation by miR-221. Control cells are represented by Huh-7 cells stably transfected with pGFP-V-RS shRNA vector. D, qPCR of miR-221 expression in miR-221 stably transfected Huh-7 cells. Western blot analysis showed a decrease of miR-221 target genes. E, a decrease of luciferase activity for both MDM2 3′-UTR–containing vectors was observed in miR-221 cotransfected HepG2 cells. Any change of the reporter gene activity was observed following mutagenesis of miR-221 seed sequence in 3′-UTR vectors. F, determination of TP53 activity by using a TP53-responsive vector. An increase of 2.4-fold and 1.5-fold of the luciferase activity was observed in miR-221 and MDM2 siRNA-transfected cells, respectively. A decrease of 2.4-fold was shown following anti-miR-221 transfection. Western blot and RT-PCR analyses showed the expression of mdm2, p53, and CDKN1A/p21 in transfected cells. E and F, columns, average values of two independent experiments; bars, SD. Each experiment was performed in triplicate.
carcinomas arisen in miR-221 transgenic mice display a miR-221 overexpression of about 1.7-folds with respect to hepatocellular carcinomas arisen in WT mice (9). These data confirm that even a 2-fold increase of miR-221 in hepatocellular carcinoma tissue is associated with a significant modulation of mdm2 and TP53 expression.

MiR-221–mediated cell-cycle modulation is cell-context specific

MiR-221 overexpression was previously shown to speed up proliferation and cell-cycle progression through p27 and p57 inhibition in Hep3B and SNU398 cells (12), even though no change in proliferation was observed in other hepatocellular carcinoma–derived cell lines such as PLC/PRF5 and HuH6 cells (28). Here, we investigated the effects of miR-221 enforced expression on cell cycle in two hepatocellular carcinoma–derived cell lines: HepG2 cells harboring WT TP53, high basal levels of p21 and p27 proteins, and low levels of p57 protein and Hep3B cells that are TP53−/− and display high p27 and p57 levels in the presence of low p21 levels. miR-221 overexpression in HepG2 cells caused a cell-cycle arrest in the G1 phase (Fig. 4A). In line with these data, miR-221 overexpression determined a 13% decrease of BrdUrd incorporation with respect to negative control cells (Student t test, P = 0.04; Fig. 4A). To demonstrate that not only p27 and p57 inhibition, but also miR-221/mdm2/p53 control loop might influence cell-cycle regulation, fluorescence-activated cell sorting (FACS) analysis was performed in HepG2 cells following TP53 silencing and miR-221 overexpression. A decrease of the G1 phase and an increase of the S phase cell populations were observed in miR-221/TP53 siRNAs cotransfected cells with respect to TP53-silenced cells (Fig. 4B). Remarkably, miR-221/TP53 siRNAs cotransfected HepG2 cells displayed a 32% increase of BrdUrd incorporation with respect to TP53-silenced cells (Student t test, P = 0.002, Fig. 4B). The increase of the principal p53 transcription target, p21, in p53 WT HepG2 cells following miR-221 overexpression might be responsible for the G1-phase arrest, functionally overcoming miR-221–mediated p27 inhibition (Fig. 4C). On the contrary, miR-221 silencing in HepG2 cells led to an increase of the S-phase population and a decrease of G1-phase cells together with a reduction of p21 levels (Fig. 4D and E). A further confirmation of p53’s role in miR-221 cell-cycle regulation was derived from miR-221–transfected Hep3B cells, which displayed an increase of the G1 to S phase transition and a cell-cycle acceleration, together with a very slight decrease of p21 protein levels (Fig. 4F and G).

miR-221 modulates the response to genotoxic damage

To evaluate the contribution of miR-221/mdm2/p53 control loop to genotoxic damage response, doxorubicin challenge was chosen because it is one of the most commonly used chemotherapies for intermediate hepatocellular carcinoma. The extent of apoptotic cell death was investigated in two hepatocellular carcinoma–derived cell lines, HepG2 (TP53 WT; Fig. 5) and SNU449 (TP53−/−) following miR-221 modulation and doxorubicin challenge. In HepG2 cells, miR-221–enforced expression caused an increase of caspase activity both in untreated and doxorubicin-treated cells. This unexpected behaviour was associated with an upregulation of proapoptotic genes in the treated cells, whereas in untreated cells, the increased caspase activity is mainly due to caspase-7 cleavage. The extent of caspase activation was stronger in miR-221–transfected cells than in MDM2-inhibited cells, confirming that MDM2 is not the only target through which miR-221 modulates apoptosis.

In untreated cells, the inhibition of miR-221 determined a decrease of the caspase activity in HepG2 cells in the absence of any phenotypic effect on cell death. On the other side, neither increased apoptotic activity, nor molecular changes in apoptotic gene expression or phenotypic effect on apoptotic cell death were observed in SNU449 cells following miR-221 inhibition.

Conversely, after doxorubicin treatment, the inhibition of miR-221 turned out to be relevant in both settings, in which a different behaviour was, however, observed. miR-221-silenced HepG2 cells displayed a lower caspase activity, confirmed by a reduced caspase-3/7 cleavage and down-regulation of apoptotic genes. These events resulted in a reduced apoptotic cell death evaluated by FACS analysis for Annexin V/PI staining. On the contrary, the inhibition of miR-221 in SNU449 cells, which display a very low residual functionality of TP53 as assessed by p21 mRNA expression in TP53-silenced cells, was associated with an increased caspase activity, higher caspase-3/7 cleavage and apoptotic genes’ overexpression, and increased apoptotic cell death.

To confirm these data, the same experiment was conducted in Hep3B cells that are TP53−/−. In these cell lines, miR-221 silencing determined an increased sensitization to doxorubicin challenge in terms of caspase-3/7 activity assay and Western blot analysis of proapoptotic genes (Supplementary Fig. S2L).

These data let us to hypothesize that miR-221 inhibition might determine a different response to drug-induced cell death, depending on specific cell context and on TP53 functionality. These data suggest that miR-221 silencing, in association with traditional treatments such as doxorubicin, depends on cell contexts and might be more effective, in terms of apoptotic cell death, in TP53-mutated hepatocellular carcinomas.

MiR-221 upregulation is associated with WT TP53 and DNA hypomethylation in primary hepatocellular carcinomas

Because DNA hypomethylating agents determined an overexpression of miR-221 in HepG2 cells (Fig. 1F), we investigated whether CpG island hypomethylation might be responsible for the modulation of miR-221 expression. A bioinformatic analysis revealed the presence of a 194-bp long CpG island, approximately 7.8 kb upstream of miR-222 precursor. MSP analysis was performed in HepG2 cells treated with 5-Aza-2′-deoxycytidine and in 43 randomly selected matched hepatocellular carcinoma and liver cirrhosis tissues. A hypomethylation profile of this CpG island was
identified in 5-Aza-2'deoxyctydine–treated HepG2 cells and in 27 of 43 examined hepatocellular carcinomas (62%) with respect to matched nonneoplastic tissues (Supplementary Fig. S3A).

The TP53 status and the DNA methylation profile at miR-221–222 locus were evaluated against miR-221 levels in hepatocellular carcinoma tissues. Missense, nonsense, splicing, and stop-codon mutations were identified in 11 of 42 cases.
Figure 5. miR-221 regulates apoptosis following doxorubicin treatment in HepG2 cells. A, caspase-3/7 activity assay in transfected HepG2 cells. A 1.4-fold increase of caspase activity was observed in miR-221 (miR)-transfected cells, whereas a 3.0-fold decrease of caspase activity was shown in anti-miR-221 (AM)-transfected cells. RT-PCR analysis of CDKN1A/p21 and GADD45A in the same experimental setting. B, caspase-3/7 activity assay in transfected HepG2 cells following doxorubicin treatment. A 1.5- and 1.3-fold increase of caspase activity was observed in miR-221 and MDM2 siRNA transfected cells. A 1.5-fold decrease of caspase activity was shown in anti-miR-221 -transfected cells. RT-PCR analysis of CDKN1A/p21 and GADD45A in the same experimental setting. C and D, Western blot analysis of apoptotic genes in miR-221 overexpressing and silenced HepG2 cells with no treatment or following doxorubicin treatment. E, Annexin V/PI FACS analysis of transfected HepG2 cells with no treatment. No significant change in apoptotic cell populations was observed. F, Annexin V/PI FACS analysis of transfected HepG2 cells following doxorubicin challenge. An 88% increase of Q2 cell population was observed in miR-221 overexpressing cells with respect to negative controls (Student t test, P < 0.05). A 45% and 54% decrease of Q1 and Q2 cell populations was detected in anti-miR-221 -transfected cells (Student t test, P < 0.05).
and in none of the matched cirrhotic tissues, as previously detailed (20). A correlation was found between higher miR-221 expression and DNA hypomethylation at miR-221–222 locus (Student t test, $P = 0.02$, Supplementary Fig. S3B), whereas no correlation was found with $TP53$ status (Supplementary Fig. S3C). Remarkably, when both $TP53$ and DNA methylation status were considered, the simultaneous presence of WT $TP53$ and DNA hypomethylation associated with higher miR-221 levels ($t$ test, $P = 0.009$, Supplementary Fig. S3D), suggesting that these mechanisms might play an effective role in primary hepatocellular carcinomas.

Therefore, notwithstanding the high molecular heterogeneity of hepatocellular carcinoma, the analysis of primary tumors is in line with the in vitro findings.

Figure 6. miR-221 regulates apoptosis following doxorubicin treatment in SNU449 cells. A, caspase-3/7 activity assay in transfected SNU449 cells with no treatment. Any change of the caspase activity was displayed in anti-miR-221–transfected SNU449 cells. Western blot analysis of cleaved caspase-3 and caspase-7, puma, and bax expression levels in the same experimental setting. B, caspase-3/7 activity assay in transfected SNU449 cells following doxorubicin treatment. A 1.6-fold increase of the caspase activity was observed in anti-miR-221–transfected SNU449 cells. Western blot analysis of cleaved caspase-3 and caspase-7, puma, and bax expression levels in the same experimental setting. C, Annexin V/PI FACS analysis of untreated transfected SNU449 cells. No significant change in apoptotic cell populations was observed. D, Annexin V/PI FACS analysis of transfected SNU449 cells following doxorubicin challenge. A 71% increase of Q1 cell population was observed in anti-miR-221–transfected cells with respect to negative control cells (Student t test, $P < 0.01$). E, Western blot and RT-PCR analyses of $TP53$ and CDKN1A/p21 expression in $TP53$ siRNA transfected SNU449 cells at 24 hours. Numbers represent the ratio between genes and $\beta$-actin expression. $P$ values were obtained by the Student t test. "$P < 0.05.$
Discussion

The upregulation of miR-221 has been reported in several cancer types, including hepatocellular carcinoma (2–6, 23). In this study, we identified two mechanisms contributing to miR-221 overexpression in hepatocellular carcinoma, specifically DNA hypomethylation and TP53 activation. This last process may be sustained by miR-221 itself and occurs through direct targeting of MDM2.

The presence of oncogenes among the predicted targets of tumor-associated miRNAs has not been reported in the literature so far and might seem conflicting. Here, we show that oncogenes such as MDM2 can be directly targeted by oncomiRNAs such as miR-221 (Supplementary Table S5). Mdm2 targeting by miR-221 was confirmed in primary tumors and in hepatocellular carcinomas arisen in miR-221 transgenic mice. Remarkably, this context displays a 2 to 3-fold upregulation of miR-221 (9), reflecting the situation observed in about 70% of human hepatocellular carcinomas (25). Because this model of hepatocellular carcinoma is obtained by DENA administration, it is conceivable to hypothesize TP53 activation as in vitro setting.

miRNAs play essential roles in the modulation of cancerous phenotype, still the mechanisms sustaining their aberrant expression need to be explored. We identify TP53 as a trigger of miR-221 expression in the presence of DNA hypomethylation at miR-221/222 locus. MiR-221 overexpression partakes to a self-maintaining loop: on the one hand, miR-221 is able to increase p53 levels through mdm2 downregulation and, on the other hand, p53 triggers miR-221 transcription by binding its upstream region. Although a moderate TP53 activation is associated with increased mature miR-221 expression, higher extents of TP53 expression, such as those obtained upon high doses of Nutlin-3, determine a reduction of miR-221 levels, likely resulting from its impaired maturation.

We can thus, hypothesize a possible role of different extents of TP53 activation in the modulation of mature miR-221 levels; however, experimental explorations in contexts like stress response or apoptosis induction are needed to confirm this hypothesis. In line with these speculations, a TP53-dependent regulation of miRNA maturation was recently reported by Suzuki and colleagues (27).

Because hepatocellular carcinomas display mutated isoforms of TP53 in approximately 30% of cases and an impaired TP53 function in an even wider proportion of cases, we assayed the miR-221/MDM2/TP53 regulatory loop in different cell contexts. Our findings suggest that modulation of miR-221 differently impacts the cell cycle and the response to genotoxic damage, depending on TP53 status. Similarly, here, we report that in different cellular contexts characterized by a different TP53 functionality, the regulation of the known target PUMA (29, 30) by miR-221 determines completely different outcomes. When the miR-221-mdm2-TP53 loop is functioning, as in HepG2 cells, overexpression of miR-221 surprisingly increases puma expression, instead of inhibiting it. This is likely due to the prevalence of the effect of the tested loop over the direct effects of miR-221. Conversely, in TP53-mutated HuH-7 cells, in which the miR-221-mdm2-TP53 loop cannot work, an even mild overexpression of miR-221 causes puma inhibition.

Our data might contribute to elucidate the reason why in some contexts, an increased miR-221 level does not stimulate cell growth (28). In addition, because miR-221 inhibition has been proposed as a possible treatment for cancer, our findings might deserve attention when evaluating molecular characteristics, predicting who will take more advantage from this kind of treatment. The identification of epigenetic changes sustaining aberrant miRNA expression is not novel in the field of cancer, even though they were not described so far, concerning miR-221. Although DNA hypermethylation is reported as a cause of tumor-suppressor miRNA silencing (20), DNA hypomethylation was described as responsible for C19MC miRNAs’ upregulation in hepatocellular carcinoma and in gastric cancer cells (19, 20). Here, we report DNA hypomethylation at miR-221/222 locus as a relevant event contributing to miR-221 overexpression in hepatocellular carcinoma. Higher miR-221 levels were found in hepatocellular carcinomas displaying miR-221 locus hypomethylation associated with wt TP53, suggesting that in vitro findings might reflect primary hepatocellular carcinomas.

Several mechanisms were shown to sustain miR-221 expression in cancer cells. C-met activation induces miR-221 expression through c-jun DNA binding (10), underlying that our findings are not the only ones responsible for miR-221 overexpression in hepatocellular carcinoma. The mechanisms identified here deserve attention because they are part of the feed-forward loop which inhibition might be relevant in a therapeutic perspective. Indeed, both MDM2/TP53 regulators and DNA demethylating agents are under investigation in clinical trials. Therefore, a molecular characterization of hepatocellular carcinomas might add relevant information for optimization of treatment, especially when planning an anti-miR-221–based therapeutic strategy (31).

Disclosure of Potential Conflicts of Interest
L. Bolondi received commercial research grants from Bayer, Bristol-Myers Squibb, Daiichi-Sankyo, and Polaris; has honoraria from Bayer, Bristol-Myers Squibb, Bracco, and Esaote; and is consultant/advisory board member of Bayer and Bristol-Myers Squibb. No potential conflicts of interest were disclosed by the other authors.

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References


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