

Alma Mater Studiorum Università di Bologna  
Archivio istituzionale della ricerca

MiR-30e-3p influences tumor phenotype through MDM2/TP53 axis and predicts sorafenib resistance in hepatocellular carcinoma

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

*Published Version:*

MiR-30e-3p influences tumor phenotype through MDM2/TP53 axis and predicts sorafenib resistance in hepatocellular carcinoma / Gramantieri L.; Pollutri D.; Gagliardi M.; Giovannini C.; Quarta S.; Ferracin M.; Casadei-Gardini A.; Callegari E.; De Carolis S.; Marinelli S.; Benevento F.; Vasuri F.; Ravaioli M.; Cescon M.; Piscaglia F.; Negrini M.; Bolondi L.; Fornari F.. - In: CANCER RESEARCH. - ISSN 0008-5472. - STAMPA. - 80:8(2020), pp. 1720-1734. [10.1158/0008-5472.CAN-19-0472]

*Availability:*

This version is available at: <https://hdl.handle.net/11585/796808> since: 2021-02-09

*Published:*

DOI: <http://doi.org/10.1158/0008-5472.CAN-19-0472>

*Terms of use:*

Some rights reserved. The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>).  
When citing, please refer to the published version.

(Article begins on next page)

This is the accepted manuscript of:

S, Benevento F, Vasuri F, Ravaioli M, Cescon M, Piscaglia F, Negrini M, Bolondi L, Fornari F. MiR-30e-3p Influences Tumor Phenotype through MDM2/TP53 Axis and Predicts Sorafenib Resistance in Hepatocellular Carcinoma. Cancer Res. 2020 Apr 15;80(8):1720-1734

Final peer reviewed version available at: <https://doi.org/10.1158/0008-5472.can-19-0472>

Rights / License:

The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

*This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>)*

***When citing, please refer to the published version.***

1 **MiR-30e-3p influences tumor phenotype through *MDM2/TP53* axis and predicts sorafenib**  
2 **resistance in hepatocellular carcinoma**

3 Laura Gramantieri<sup>1\$</sup>, Daniela Pollutri<sup>1,2</sup>, Martina Gagliardi<sup>1,3</sup>, Catia Giovannini<sup>1,3</sup>, Santina  
4 Quarta<sup>4</sup>, Manuela Ferracin<sup>2</sup>, Andrea Casadei-Gardini<sup>5</sup>, Elisa Callegari<sup>6</sup>, Sabrina De Carolis<sup>1,2</sup>,  
5 Sara Marinelli<sup>1,3</sup>, Francesca Benevento<sup>3</sup>, Francesco Vasuri<sup>7</sup>, Matteo Ravaoli<sup>8</sup>, Matteo Cescon<sup>3,8</sup>,  
6 Fabio Piscaglia<sup>3</sup>, Massimo Negrini<sup>6</sup>, Luigi Bolondi<sup>1,3</sup>, Francesca Fornari<sup>1,3,\$</sup>

7 <sup>1</sup> Center for Applied Biomedical Research, St.Orsola-Malpighi University Hospital, Bologna,  
8 Italy

9 <sup>2</sup> Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna, Italy

10 <sup>3</sup> Department of Medical and Surgical Sciences, University of Bologna, Bologna, Italy

11 <sup>4</sup> Department of Medicine, University of Padua, Padua, Italy

12 <sup>5</sup> Department of Oncology and Hematology, Division of Oncology, University of Modena and  
13 Reggio Emilia, Italy

14 <sup>6</sup> Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Italy

15 <sup>7</sup> Pathology Unit, St.Orsola-Malpighi University Hospital, Bologna, Italy

16 <sup>8</sup> General Surgery and Transplant Unit, St.Orsola-Malpighi University Hospital, Bologna, Italy

17 <sup>\$</sup>Corresponding Authors

18

19 **Running title:** MiR-30e predicts sorafenib escape in HCC

20 **Key words:** HCC, microRNA, miR-30e, Sorafenib

21

22 **Corresponding Authors:**

23 Francesca Fornari, Laura Gramantieri

24 Address: Via Massarenti, 9, 40138, Bologna, Italy.

25 Tel/Fax: +390512143902

26 E-mail: francesca.fornari2@unibo.it, laura.gramantieri@aosp.bo.it

27

28 **Conflict of interest:** The Authors have no conflict of interest to declare

29

30    **Abstract**

31    The molecular background of hepatocellular carcinoma (HCC) is highly heterogeneous and  
32    biomarkers predicting response to treatments are an unmet clinical need. We investigated miR-  
33    30e-3p contribution to HCC phenotype and response to sorafenib, as well as the mutual  
34    modulation of *TP53/MDM2* pathway, in HCC tissues and preclinical models. MiR-30e-3p was  
35    downregulated in human and rat HCCs and its downregulation associated with *TP53* mutations.  
36    *TP53* contributed to miR-30e-3p biogenesis and *MDM2* was identified among its target genes,  
37    establishing a miR-30e-3p/*TP53/MDM2* feedforward loop and accounting for miR-30e-3p dual  
38    role based on *TP53* status.

39    EpCAM, PTEN and p27 were demonstrated as miR-30e-3p additional targets mediating its  
40    contribution to stemness and malignant features. In a preliminary cohort of HCC patients treated  
41    with sorafenib, increased miR-30e-3p circulating levels predicted the development of resistance.

42    In conclusion, molecular background dictates miR-30e-3p dual behavior in HCC. Mdm2  
43    targeting/p53 axis plays a predominant tumor-suppressor function in wild type *TP53* contexts,  
44    whereas other targets such as PTEN, p27 and EpCAM gain relevance and mediate miR-30e-3p  
45    oncogenic role in non-functional *TP53* backgrounds. Increased circulating levels of miR-30e-3p  
46    predict the development of sorafenib resistance in a preliminary series of HCC patients and  
47    deserve future investigations.

48

49    **Statement of significance**

50    The dual role of miR-30e-3p in hepatocellular carcinoma clarifies how the molecular context  
51    dictates the tumor suppressor or oncogenic function played by microRNAs.

52

53     **Introduction**

54     Hepatocellular carcinoma (HCC) accounts for 90% of primary liver cancers representing the  
55     second leading cause of cancer mortality with increasing incidence in western countries [1].  
56     HCC is characterized by a high inter- and intra-individual tumor heterogeneity: the former  
57     ascribed to different risk factors and genomic contexts [2] and the latter to clonal evolution of  
58     cancer cells [3], both contributing to limited targeted therapy efficacy. Primary resistance and  
59     escape from antitumor strategies remain poorly understood and no biomarker predicting response  
60     to sorafenib or other targeted treatments has been identified so far, highlighting the need for  
61     novel tools to allocate patients to the best treatment.

62     The deregulation of tumor-specific microRNAs (miRNAs) with tumor suppressor or oncogenic  
63     properties has been described [4, 5]. Our group and others demonstrated the key role of  
64     deregulated miRNAs in tumor progression and metastasis [6, 7], as well as their involvement in  
65     drug resistance phenotype [8-10]. Aberrant expression of miR-30 family members is a frequent  
66     event in solid tumors [11, 12] displaying a prominent connection with other deregulated  
67     miRNAs in neoplastic tissues [13]. The miR-30 family contains five members encoded by six  
68     genes located on three distinct loci of the human genome (chromosomes 1, 6 and 8) giving rise  
69     to six mature miRNAs (miR-30a, -30b, -30c-1, -30c-2, -30d, -30e) with the same seed sequence.  
70     The downregulation of these miRNAs was associated with proliferation and invasion, as well as  
71     with induction of epithelial-to-mesenchymal transition (EMT), exerted by direct targeting of  
72     EMT-associated factors [14, 15] and cell adhesion molecules [16]. Remarkably, besides studies  
73     supporting the tumor suppressor role of miR-30 family, other studies point to its opposite  
74     behavior [17, 18].

75     *TP53* is one of the most frequently mutated gene in human cancers with about 30% of affected  
76     HCC cases [19]. *TP53* regulates miRNA expression by acting at two different levels: on one  
77     hand, it activates their transcriptional regulation [20-22] and, on the other hand, it interferes with  
78     their maturation process by interacting with key molecules, such as Drosha and p68 RNA

79 helicase [23]. Notably, *TP53* regulates the expression of miR-30 family members contributing to  
80 cell invasion and distal spreading as reported in colorectal and breast cancers [24, 25].  
81 Despite the prominent role of miR-30 family as tumor suppressor miRNAs, there are conflicting  
82 data regarding its aberrant expression in HCC. Previous studies reported the downregulation of  
83 miR-30a and miR-30e and the upregulation of miR-30d [26-28]. Although miR-30 was reported  
84 as participating in hepatobiliary development [29], its functional roles in HCC remain poorly  
85 understood. Here, we investigated the expression of miR-30 family members in two HCC patient  
86 cohorts, in a HCC animal model and in HCC cells, and focused on miR-30e-3p, characterizing  
87 its biological activity as well as its involvement in drug resistance and modulation of  
88 *MDM2/TP53* and *PTEN/AKT* axes. Moreover, we investigated circulating miR-30e-3p levels in a  
89 preliminary series of HCC patients and in preclinical models in order to evaluate its contribution  
90 as a non-invasive biomarker of treatment response.

91 **Materials and Methods**

92 **HCC study cohorts**

93 HCC and cirrhotic tissues were obtained from two independent cohorts of patients undergoing  
94 liver surgery for HCC at the Department of Surgery and Transplant Unit of St. Orsola-Malpighi  
95 University Hospital of Bologna. The discovery cohort consists of 22 patients, whereas the  
96 validation cohort consists of 48 patients, all enrolled at the Department of Surgery of St. Orsola-  
97 Malpighi University Hospital. We collected tissue samples at surgery and stored as previously  
98 described [30]. Local ethics committee of St. Orsola-Malpighi University Hospital approved this  
99 study (138/2015/O/Tess). Normal liver tissues were from patients undergoing liver surgery for  
100 traumatic lesions or haemangioma resection. The clinical characteristics of patients are detailed  
101 in Supplementary Table S1. *TP53* mutations were identified as previously described [20].

102 A further cohort of sorafenib-treated advanced HCC patients (Table S2) was tested for serum  
103 miR-30e-3p levels before treatment and at two-month follow-up assessment. Sera samples,  
104 obtained after the local committee approval (271/2012/O/Oss), were processed as previously  
105 described [31]. Informed written consent has been obtained from patients enrolled in this study.

106

107 **HCC animal models**

108 The diethyl-nitrosamine (DEN)-induced HCC rat model and the xenograft model were  
109 established and treated as previously described [9]. Total RNA was extracted from frozen tissues  
110 by using TRIzol Reagent (Invitrogen) and was analyzed by microarray and QPCR. Local ethics  
111 committee approved the study protocols (14/70/12 and 23/79/14).

112

113 **Microarray analysis**

114 RNAs from 22 rat samples (12 HCCs, 8 surrounding livers and 2 normal livers) were hybridized  
115 on Agilent rat whole-genome miRNAs microarray (#G4471A\_046066 Release 19.0, Agilent



Technologies). One-color gene expression was performed according to the manufacturer's procedure. Raw data are available in ArrayExpress repository (accession number E-MTAB-7624).

Technical details are described in Supplementary section.

## **Cell culture and treatments**

HCC cell lines were cultured as previously described [9]. Starvation was obtained culturing cells in media without FBS for 48-60 hours. Cells were treated with 5.0-7.5  $\mu$ M sorafenib-tosylate (Bayer) or Nutlin-3 (Sigma-Aldrich) or with 1.0-2.0  $\mu$ g/ml of doxorubicin (Pfizer) for 48 hours. Cell transfection and proliferation, clonogenic and sphere formation assays are detailed in Supplementary Material. Genetics Unit at S.Orsola-Malpighi Hospital performed HCC authentication and cell identification was obtained by online STR analysis (<https://www.dsmz.de/>).

## **Reporter assays**

3' untranslated regions (3'UTR) of *MDM2*, *CDKN1B/p27*, *PTEN* and *EpCAM* mRNAs were amplified by PCR as reported in Supplementary Table S2. Mutagenesis of miR-30e-3p seed sequence was performed by using QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies) following manufacturer's instruction (Supplementary Table S3). Sanger sequencing verified mutated sequences. Dual-luciferase reporter and p53 activity assays were performed as previously described [32].

## **Real-time PCR**

TaqMan MicroRNA Assays (Applied Biosystems) were used to evaluate miRNA expression, as previously described [30]. RNU6B was used as housekeeping gene. Quantitative PCR (qPCR)

141 was used for gene expression analysis.  $\beta$ -actin and GAPDH housekeeping genes were considered  
142 for gene normalization. QPCR experiments were run in triplicate. Primers and conditions are  
143 detailed in Supplementary Table S4.

144

#### 145 **Western blot**

146 Western blot (WB) was used to analyze protein extracts (30  $\mu$ g) from cell and tissues with  
147 antibodies reported in Supplementary Table S5. ChemiDoc<sup>TM</sup> XRS+ (Image Lab<sup>TM</sup> Software,  
148 Bio-Rad) was employed to quantify digital images of X-ray films. WB analysis was performed  
149 in duplicate.

150

#### 151 **Chromatin immunoprecipitation and electrophoretic mobility shift assay**

152 HepG2 cells were subjected to chromatin immunoprecipitation (ChIP) with a polyclonal p53  
153 antibody (Novocastra) as previously described [21]. Primers and conditions are reported in  
154 Supplementary Table S4. Amplicon design has been detailed in Supplementary Material.  
155 Electrophoretic mobility shift assay (EMSA) was performed with nuclear extract (NE) from  
156 Nutlin-3 treated HepG2 cells by using LightShift Chemiluminescent EMSA Kit (Thermo  
157 Scientific) as previously described [21]. Probe sequences are reported in Supplementary Table  
158 S6.

159

#### 160 **Flow cytometry**

161 Annexin-V assay was performed in duplicate by flow cytometry (FACSaria I, BD) as previously  
162 reported [33]. Annexin-V assay in miR-30e-3p overexpressing HepG2 cells was assessed on  
163 Cytoflex S (Beckam Coulter). Immunophenotype analysis of EpCAM was performed by using  
164 CD326 monoclonal antibody (MH99)-Alexa Fluor 488 (eBioscience).

165

166 **Cell invasion and wound healing assay**

167 Real-time cell invasion, performed on xCELLigence DP instrument (ACEA), and wound healing  
168 assays were executed as previously described [34].

169

170 **Caspase activity assay**

171 Caspase pathway activation was evaluated by Caspase-Glo 3/7 assay (Promega) according to  
172 manufacturer's instructions. Each sample was performed in quadruplicate in two independent  
173 experiments.

174

175 **Serum and exosome miRNA extraction**

176 Isolation of circulating miRNAs from exosomes, cell culture supernatant, and serum was  
177 executed as previously reported [9, 31].

178

179 **Statistical analysis**

180 Differences between two or more groups were analyzed using unpaired Student's t-test or  
181 ANOVA. Tukey's post hoc test was used for comparisons among groups after ANOVA analysis.  
182 Pearson's correlation coefficient was used to investigate relationships between two variables.  
183 Time to recurrence (TTR) curve based on miR-30e-3p levels was computed by Kaplan-Meier  
184 product-limit method and compared using a log-rank test. Paired t-test was used to evaluate the  
185 relationship between circulating miR-30e-3p and response to sorafenib in HCC patients analyzed  
186 both before and on treatment. Reported p-values were two-sided and considered significant when  
187 lower than 0.05. Statistical calculations were executed using SPSS version 20.0 (SPSS inc). \*  
188  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ .

## 189    **Results**

### 190    **MiR-30e-3p is downregulated in HCC and associates with *TP53* status**

191    We previously reported a deregulation of miR-30 family members in histologically aggressive  
192    HCCs [35]. Here, we confirmed in a first patient cohort, a decrease of miR-30e-3p in 73% of  
193    HCCs in comparison to matched cirrhotic livers (t-test;  $p=0.0002$ ), showing a mean  
194    downregulation of 1.7-fold (**Figure 1A**). Similarly, a decrease of miR-30e-5p and miR-30a-3p  
195    was detected in 64% of tumors with an average 1.6-fold change (t-test;  $p=0.003$  and  $p=0.008$ ,  
196    respectively) (**Figure 1B, C**). A positive correlation between these miR-30 family members was  
197    detected in HCC and cirrhotic human samples (**Figure S1A-F**), suggesting common regulation  
198    mechanisms for these miR-30 family members.

199    We next tested the DEN-induced HCC rat model [9] by microarray analysis on 12 HCCs and 10  
200    non-cancerous livers. The downregulation of five miRNAs belonging to miR-30 family was  
201    observed in tumors compared to surrounding livers (**Figure S2 and Table S7**). In rat HCC, miR-  
202    30e-3p decreased in 64% of tumors, showing a 1.5-fold change (t-test;  $p=0.034$ ), mirroring  
203    findings from human HCCs (**Figure 1D**). MiR-30e-5p and miR-30a-3p were downregulated in  
204    59% and 32% of rat HCCs, showing no difference between tumor and non-tumor samples  
205    (**Figure 1E, F**). The discrepancy between human and rat HCCs might be ascribed to the lack of  
206    cirrhotic microenvironment in the rat model.

207    In the validation cohort, we confirmed miR-30e-3p, miR-30e-5p and miR-30a-3p  
208    downregulation in HCCs with respect to both matched cirrhosis (Tukey's post hoc test;  $p=0.012$ ,  
209     $p=0.013$  and  $p=0.025$ , respectively) and normal livers (Tukey's post hoc test;  $p=0.046$ ,  $p=0.001$   
210    and  $p=0.044$ , respectively) (**Figure 1G-I**). Here, we focused on miR-30e-3p, which is the most  
211    downregulated miRNA in human and rat HCCs.

212    Since *TP53* is mutated in about 30% of HCCs and it establishes complex regulatory networks  
213    with miRNAs [1, 36], we investigated the relationship between *TP53* status and miR-30e-3p in

HCC. Lower miR-30e-3p levels were observed in *TP53* mutated HCCs while no difference was found for primary miR-30e (pri-miR-30e) levels (**Figure 1J, K**). Remarkably, a negative correlation between primary and mature miR-30e levels was found in HCCs (Pearson's correlation;  $R=-0.32$ ) (**Figure S3A**), suggesting a role for post-transcriptional mechanisms in miR-30e biogenesis regulation. Interestingly, after splitting HCCs according to *TP53* status, a negative correlation was confirmed between primary and mature miRNA levels in *TP53* mutated cases, while a trend towards a positive correlation was found in *TP53* WT cases (**Figure S3B, C**). These findings suggest an impairment of miR-30e-3p maturation by mutant p53 isoforms which were previously reported to interfere with the functional assembly of Drosha/p68 processing complex [23].

#### ***TP53* influences miR-30e-3p transcription and biogenesis in HCC cells**

To start dissecting p53 involvement in miR-30e biogenesis in HCC, we modulated its expression in *TP53* WT HepG2 cells by using different strategies. First, we investigated the influence of p53 silencing on miR-30e primary transcript and mature isoforms. As showed in Figure 2A, a decrease of primary and mature miR-30e-3p and miR-30e-5p levels was detected in p53-silenced cells. MiR-34a was used as a positive control since it represents the first identified p53-target miRNA [37], whereas p53 silencing was verified by WB and qPCR analyses of target genes (**Figure 2A**). Secondly, we investigated the effect of p53 overexpression in HepG2 cells following transfection of a p53-overexpressing vector or treatment with Nutlin-3, a *MDM2* inhibitor. In line, an increase of miR-30e primary transcript and mature miRNA isoforms was detected in both experimental settings (**Figure 2B, C**). Notably, Nutlin-3 administration led to a stronger p53 transcriptional activation with respect to p53-overexpressing vector, as confirmed by the extent of variation of *CDKN1A/p21* and *MDM2* mRNAs. In turn, this resulted in higher pri-miR-30e levels, but not mature isoforms (**Figure 2B, C**), letting us to speculate that

mdm2/p53 axis might have an additional role in miR-30e biogenesis. To investigate p53 involvement in miRNA maturation, we quantified primary and mature miR-30e levels in p53-deleted (CRISPR/Cas9 technology) HepG2 cells and p53-null Hep3B cells following Nutlin-3 treatment. In line with p53 absence, no increase of miR-30e primary transcript was observed, whereas increased mature miRNA isoforms were detected in both treated cell lines (**Figure 2D, E**), confirming p53 role in miRNA processing impairment during Nutlin-3 administration. Since Dicer1 promoter contains several p53/p53 responsive elements [38], we investigated Dicer1 expression in HepG2 cells following p53 overexpression or Nutlin-3 treatment in both p53 WT and p53-deleted cells. A downregulation of Dicer1 was observed in Nutlin-3 treated HepG2 cells, but not in p53-overexpressing cells, which is in line with miRNA maturation impairment detected after Nutlin-3 administration in p53 WT cells (**Figure S3D, E**). On the contrary, higher Dicer1 levels were detected in p53-deleted HepG2 and p53 null Hep3B cells (**Figure S3F, G**), suggesting that mdm2 inhibition by Nutlin-3 influences miR-30e processing by regulating Dicer1 transcription in a p53-dependent and independent manner.

To go deeper into p53-mediated mechanisms regulating miR-30e expression, we overexpressed WT and mutant (truncated, dominant negative) *TP53* isoforms in p53-deleted Hep3B and p53 WT HepG2 cells, respectively. As displayed in Figure 2F, an increase of primary and mature miR-30e isoforms was observed in *TP53*-overexpressing Hep3B cells confirming its role in miRNA transcription. Interestingly, mutant p53 expression in HepG2 cells decreased miR-30e transcription and, consequently, mature isoforms (**Figure 2G**), suggesting a negative role for p53 mutations in miR-30e regulation. These findings demonstrate that *TP53* WT isoform is necessary to induce miR-30e transcription in HCC cells and that p53/mdm2 axis is involved in a multi-step regulation of miR-30e biogenesis.

To prove p53 binding to hypothetical consensus elements in proximity of miR-30e precursor region, a ChIP experiment was conducted in control and Nutlin-3 treated HepG2 cells. Six amplicons containing *TP53* hypothetical binding sites were designed in a DNA region spanning

265 from -4000 to +1000 nucleotides, considering +1 the first nucleotide of miR-30e precursor  
266 (**Figure S3H**). This region was chosen based on described promoter distance for intronic  
267 miRNA [39] and due to the presence of histone modifications (**Figure S3I**). The ChIP analysis  
268 showed an enrichment of three out of six p53 hypothetical binding sites, namely BS1, BS2 and  
269 BS6, in Nutlin-3 treated cells only (**Figure S3J**), demonstrating that p53 activation strengthen its  
270 binding to miR-30e-3p genomic region favoring pri-miR-30e regulation. EMSA assay further  
271 confirmed p53 binding to hypothetical consensus sites identified in the ChIP experiment,  
272 showing a specific shift for BS1, BS2 and BS6 probes in presence of nuclear extracts from  
273 Nutlin-3 treated HepG2 cells (**Figure S3K**).

274 Notably, a positive correlation (Pearson's correlation;  $R=0.71$ ) exists between pri-miR-30e and  
275 its host gene, nuclear transcription factor Y subunit gamma (*NFYC*, NM\_014223) in HCCs,  
276 whereas a negative correlation (Pearson's correlation;  $R=-0.76$ ) was found between mature miR-  
277 30e-3p and *NFYC*, outlining the relevance of maturation process in the regulation of miR-30e  
278 levels (**Figure S3L, M**).

279

#### 280 **MiR-30e-3p establishes a *TP53* positive feedback loop through *MDM2* targeting**

281 To investigate the interplay between miR-30e-3p and *TP53*, we interrogated bioinformatics tools  
282 and identified the principal p53 negative regulator, *MDM2* (NM\_002392), as a hypothetic miR-  
283 30e-3p target gene containing three complementary binding sites (**Figure 3A**). We quantified  
284 miR-30e-3p levels in seven HCC cell lines and compared its expression with mean values  
285 observed in HCCs, cirrhosis and normal livers (**Figure 3B**). In order to avoid a confounding  
286 effect due *TP53/MDM2* auto-regulatory loop, we performed a functional analysis in HCC cell  
287 lines with mutated or null p53 isoform. Huh-7, Hep3B and SNU475 cells were chosen based on  
288 their low/intermediate miR-30e-3p basal levels, whereas SNU449 cells were selected because of  
289 their high basal levels (**Figure 3B**). MiR-30e-3p overexpression in Huh-7, Hep3B and SNU475

290 cells decreased mdm2 mRNA and protein levels, whereas miRNA silencing in SNU449 cells  
291 increased its mRNA and protein expression (**Figure 3C-F**). Subsequently, we explored *MDM2*  
292 targeting by miR-30e-3p in HepG2 cells. We observed increased mRNA and protein levels  
293 following transient miRNA silencing in p53 WT and silenced (1.3 and 1.5-fold, respectively)  
294 HepG2 cells (**Figure S4A, B**). To assess if miR-30e-3p regulation of *MDM2* is long lasting, we  
295 evaluated its expression in miR-30e-3p stably silenced (MZIP-30e-3p) HepG2 cells and detected  
296 increased protein levels (**Figure S4C**), highlighting a prolonged post-transcriptional regulation.  
297 Finally, we verified miRNA/mRNA interaction by performing a reporter assay in HepG2 cells.  
298 MiR-30e-3p co-transfection decreased luciferase activity of WT *MDM2*-3'UTR vectors, but not  
299 that of mutant ones, proving miRNA direct interaction with its complementary sequence in  
300 *MDM2* mRNA (**Figure 3G**). To verify the existence of a possible miR-30e/*MDM2*/*TP53*  
301 feedback loop, we measured p53 transcriptional activity and expression in WT and mutant *TP53*-  
302 bearing cells following miR-30e-3p overexpression. An increase of luciferase signal and p53  
303 protein expression was registered in miR-30e-3p transfected HepG2 cells; whereas no change  
304 was observed in Huh-7 cells harboring an inactive p53 isoform (**Figure 3H, S4D**). These data  
305 demonstrated that, in p53 WT contexts, miR-30e-3p increases p53 expression and activity by  
306 targeting mdm2, leading to a feedforward loop sustained by p53-dependent transcription of miR-  
307 30e-3p itself. Conversely, in *TP53* mutated backgrounds this positive loop is hampered,  
308 suggesting that other targets might mediate miR-30e-3p functions in these contexts.

309

### 310 **MiR-30e-3p influences cell proliferation and invasion of HCC cells by targeting *MDM2*,** 311 ***PTEN* and *CDKN1B/p27***

312 The regulation of cell proliferation and invasion by miR-30 family members has been reported in  
313 tumors [40, 41]; nevertheless, miR-30e-3p, the most downregulated miR-30 member in our  
314 patient cohorts and preclinical model, was not previously studied. Besides *MDM2*, a



315 bioinformatics analysis highlighted *p27* and *PTEN* among miR-30e-3p hypothetic targets  
 316 (**Figure S5A**). Due to their key role in hepatocarcinogenesis, it is conceivable that miR-30e-3p  
 317 establishes multi-target networks driving HCC phenotype. QPCR and WB analyses in  
 318 transfected HCC cells proved *p27* and *PTEN* inhibition by miR-30e-3p at mRNA and protein  
 319 levels (**Figure S5B**). A reporter assay showed decreased luciferase activity of p27 and PTEN  
 320 3'UTR WT vectors upon miR-30e-3p overexpression, demonstrating that miR-30e-3p directly  
 321 regulates these targets through mRNA degradation (**Figure S5C**).

322 We subsequently explored the effect of miR-30e-3p on HCC cell proliferation by dissecting the  
 323 contribution of mdm2/p53 axis and PTEN/AKT pathway. In order to investigate the former, we  
 324 performed the proliferation assay in *TP53* WT and p53-silenced HepG2 cells. Cell growth  
 325 inhibition (1.2-fold) occurred upon miR-30e-3p enforced expression in *TP53* WT cells, whereas  
 326 an increase of cell proliferation (1.4-fold) was detected in *TP53*-silenced HepG2 cells (**Figure**  
 327 **4A, B**), suggesting p53 as a pivotal factor modulating miR-30e-3p effect. The relevance of *PTEN*  
 328 targeting on cell proliferation was next assessed in p53-silenced cells by evaluating *AKT*  
 329 phosphorylation upon miR-30e-3p enforced expression. An increase of phospho-AKT occurred  
 330 in both *TP53* WT and p53-silenced HepG2 cells highlighting AKT, beside p53, as pivotal factors  
 331 mediating miR-30e-3p effects. We next assayed p53-mutated Huh-7 and p53-deleted Hep3B  
 332 cells displaying high constitutive pten levels [20]. MiR-30e-3p overexpression increased  
 333 proliferation of Huh-7 and Hep3B cells (1.9 and 2.1-fold, respectively). Enhanced AKT  
 334 phosphorylation confirmed PTEN/AKT targeting as a trigger for proliferation in these non-  
 335 functional p53 backgrounds (**Figure 4C, D**). To check whether p27 contributes to cell  
 336 proliferation, we chose p53-mutated SNU475 cells displaying null pten levels [20]. MiR-30e-3p  
 337 increased proliferation of SNU475 cells without changing phospho-AKT levels, but strongly  
 338 decreasing p27 (**Figure 4E**). These findings support the crucial role of mdm2/p53, p27 and AKT  
 339 in mediating miR-30e-3p regulation of cell proliferation. We hence analyzed cell cycle  
 340 regulators p21 and p27 in miR-30e-3p overexpressing HCC cells. P21 modulation reflected p53

status, showing an increase in *TP53* WT HepG2 cells only, while it did not change in *TP53*-mutated cell lines (**Figure 4A-E**). Conversely, p27 downregulation was observed in all cell lines, confirming its targeting by miR-30e-3p (**Figure 4A-E**). Opposite proliferative changes induced by miR-30e-3p in HCC cell lines can be explained by its multi-target activity. Indeed, the inhibition of mdm2 prevents cell cycle acceleration through p53/p21 signaling in *TP53* WT contexts only. On the contrary, p27 and PTEN inhibition prevails in *TP53*-mutated/deleted backgrounds, leading to increased proliferation.

We next explored miR-30e-3p ability to modulate invasive properties of HepG2 and SNU449 cells. These cell lines were chosen due to high miR-30e-3p constitutive levels that allowed the investigation of miR-30e-3p stable silencing (**Figure S5D**) in a p53 WT (HepG2) and p53-mutated context (SNU449). No change was observed in miR-30e-3p silenced HepG2 cells, whereas decreased invasive and migratory potentials were detected in miRNA-silenced SNU449 cells (Student's t-test;  $p < 0.0001$ ) (**Figure 4F, S5E**). Despite decreased phospho-AKT levels in both cell lines, their different invasion capability highlights cell context as pivotal in driving miR-30e-3p influence on cell aggressiveness.

356

#### 357 **MiR-30e-3p influences stem cell properties of HCC cells through *EpCAM* targeting**

We investigated miR-30e-3p driven changes on stemness in HCC and observed a negative correlation between miR-30e-3p and both AFP and EpCAM mRNAs in HCC patients (Pearson's correlation;  $R = -0.45$  and  $-0.36$ , respectively) (**Figure 5A**) and cell lines (Pearson's correlation;  $R = -0.62$ ) (**Figure S6A**). Analyzing HCCs according to *TP53* status, an even stronger correlation was observed in *TP53* mutated patients (Pearson's correlation;  $R = -0.78$ ) (**Figure S6B**), suggesting a stronger activity of miR-30e-3p in the absence of miR-30e-3p/mdm2/p53 regulatory loop. An opposite behavior was detected for pri-miR-30e that positively correlated with EpCAM mRNA (Pearson's correlation;  $R = 0.39$ ) (**Figure S6C**). Accordingly, a change of EpCAM mRNA

366 and immunophenotype was observed following miR-30e-3p modulation in HCC cells (**Figure**  
367 **5B and S6D**). To investigate how miR-30e-3p influences stem cell properties, we interrogated  
368 bioinformatics algorithms and identified two hypothetical miRNA binding sites in EpCAM  
369 3'UTR (**Figure S6E**). A decreased luciferase activity was observed for WT EpCAM-3'UTR  
370 vector in presence of miR-30e-3p overexpression (Student's t-test;  $p=0.00045$ ), demonstrating a  
371 direct regulation of this target in HCC (**Figure 5C**). We next performed clonogenic and sphere  
372 formation assays in miR-30e-3p stably silenced (MZIP-30e-3p) p53 WT HepG2 and p53-  
373 mutated SNU449 cells (**Figure S5E, S6F**), chosen on the basis of their different stemness  
374 features (**Figure S6G**). The clonogenic assay displayed an increase of colony number (t-test;  
375  $p=0.0005$ ) and OD 595 (t-test;  $p=0.001$ ) in MZIP-30e-3p HepG2 cells, whereas an opposite  
376 behavior was observed in SNU449 cells displaying a decreased colony number (t-test;  $p<0.0001$ )  
377 and OD 595 (t-test;  $p=0.025$ ) in miR-30e-3p silenced cells (**Figure 5D, E**). Finally, we  
378 performed colony-forming unit and sphere formation assays in miR-30e-3p stably  
379 overexpressing Huh-7 cells exhibiting high stem cell characteristics and a mutated p53 isoform  
380 (**Figure S6F, G**). A decrease of colony and sphere number (t-test;  $p=0.0033$  and  $p=0.010$ ,  
381 respectively) was observed in miR-30e-3p-Huh-7 cells (**Figure 5F, G**). Similarly, miR-30e-3p  
382 silencing determined an increased sphere formation (Student's t-test;  $p<0.0001$ ) in HepG2 cells  
383 (**Figure 5G, S6H**). Due to low expression of stem cell characteristics, SNU449 cells displayed  
384 no capability to sphere formation (**Figure S6I**). An increase of AFP and EpCAM levels was  
385 observed in MZIP-30e-3p HepG2-derived spheres, whereas their decrease was detected in  
386 pMXs-miR-30e-3p Huh-7-derived spheres (**Figure 5H**), confirming findings observed in HCC  
387 specimens and cell lines.

388 In primary HCCs, low miR-30e-3p expression associated with microvascular invasion (Student's  
389 t-test;  $p=0.012$ ) and increased recurrence rate (log-rank test;  $p=0.027$ ) (**Figure 5I, J**). These  
390 findings demonstrated that miR-30e-3p downregulation participates to boost stemness properties  
391 in HCC cells characterized by a stem-like phenotype.

392

393

394 **MiR-30e-3p regulates treatment response in HCC preclinical models through *MDM2* and**  
395 ***PTEN* targeting**

396 Due to the relevance of miR-30e-3p targets in stressful conditions and drug response, we  
397 evaluated apoptotic cell death in HepG2 and Huh-7 cells following starvation and doxorubicin or  
398 sorafenib treatment. Firstly, we considered *TP53* WT HepG2 cells and observed that miR-30e-3p  
399 overexpression had no effect in basal conditions (**Figure 6A**). On the contrary, it increased early  
400 apoptosis (2.3-fold) following serum deprivation and both early and late apoptosis (1.3 and 7.8-  
401 fold, respectively) in doxorubicin treated cells (**Figure 6B, C**), which is consistent with p53  
402 activation in these conditions (**Figure S7A**). WB and caspase assay confirmed increased p53  
403 expression, apoptotic markers and caspase-3/7 activity upon miR-30e-3p enforced expression in  
404 serum-deprived and doxorubicin treated HepG2 cells (**Figure 6C-E**).

405 Similarly, sorafenib increased early apoptosis (3.1-fold increase) in miR-30e-3p transfected  
406 HepG2 cells (**Figure 6F-H**). Since both sorafenib (**Figure S7A**) and miR-30e-3p induces p53  
407 expression (**Figure 6G**), we wondered if p53 might account for increased apoptotic cell death in  
408 miR-30e-3p overexpressing cells. To prove a p53-dependent response, we performed the same  
409 assays in p53-silenced HepG2 cells. In this setting, miR-30e-3p overexpression decreased (4.2-  
410 fold) early apoptotic cells (**Figure 6I-K**), demonstrating the central role of p53 in triggering  
411 miR-30e-3p-mediated apoptosis. In agreement, miR-30e-3p overexpression in *TP53* deleted  
412 (CRISPR/Cas9 technology) HepG2 cells determined a resistance to both starvation and sorafenib  
413 (**Figure S7B-E**), confirming p53 as a determinant of miR-30e-3p duality in HCC. To  
414 demonstrate the relevance of *PTEN/AKT* axis in the absence of p53, we coupled miR-30e-3p  
415 inhibition with *PTEN* silencing in p53-deleted HepG2 cells. Annexin-V assay after sorafenib  
416 administration showed that *PTEN* inhibition decreases early apoptosis (1.5-fold) in miR-30e-3p

417 silenced/p53-knockout HepG2 cells confirming PTEN/AKT pathway as a driver of sorafenib-  
418 resistance in the absence of p53 (**Figure S7F, G**). Finally, since sorafenib triggers p27 in HepG2  
419 cells (**Figure S7H**), we wondered whether p27 targeting by miR-30e-3p might influence  
420 sorafenib response. Strikingly, when both *TP53* and *PTEN* were inhibited, miR-30e-3p  
421 overexpression decreased early apoptotic cell population (1.4-fold) in sorafenib treated HepG2  
422 cells, suggesting p27 participation to miR-30e-3p-mediated sorafenib resistance in p53-depleted  
423 cells (**Figure S7I**). These findings confirm the importance of PTEN and p27 in driving miR-30e-  
424 3p-associated phenotype in *TP53* mutated backgrounds. In line, miR-30e-3p overexpression in  
425 *TP53* mutated Huh-7 cells determined a resistance to starvation, doxorubicin and sorafenib  
426 administration (**Figure S8A-H**) and high miR-30e-3p levels associated with increased tumor cell  
427 proliferation in sorafenib-treated Huh-7-derived xenograft mice (**Figure S8I, J**), confirming its  
428 oncogenic role in p53-mutated cells.

429 In sorafenib-treated DEN-HCC rats, a downregulation of miR-30e-3p expression was found in  
430 73% of nodules (Student's t-test;  $p=0.02$ ) (**Figure 7A**). Sorafenib resistant tumors showed lower  
431 miR-30e-3p tissue levels (Student's t-test;  $p=0.004$ ) (**Figure 7B**), suggesting miR-30e-3p  
432 downregulation as an adverse event with respect to sorafenib efficacy in this model. In  
433 agreement, an inverse correlation between miR-30e-3p and tumor size (Pearson's correlation;  
434  $R=-0.69$ ) (**Figure 7C**) and a positive correlation between miR-30e-3p and apoptotic markers  
435 (Pearson's correlation;  $R=0.70, 0.71$  and  $0.49$ ) were identified in treated rat HCCs (**Figure S9A-**  
436 **C**). Finally, the positive correlation between miR-30e-3p and p21 levels in tumor tissues  
437 (Pearson's correlation;  $R=0.52$ ) (**Figure S9D**) from *TP53* WT rat DEN-HCCs [42], led us to  
438 speculate that miR-30e-3p/mdm2/p53 axis might take part to sorafenib sensitization also in rat  
439 tumors.

440

441 **Circulating miR-30e-3p levels predict treatment response in HCC**

442 Circulating miRNAs were proposed as biomarkers of treatment response [43] and TKIs, such as  
443 gefitinib, promote exosomal secretion [44]. Thus, we assayed if sorafenib could modulate miR-  
444 30e-3p extracellular levels. An exosome-mediated miR-30e-3p extrusion was confirmed in all  
445 HCC cell lines treated by sorafenib, except for SNU449 cells. Sorafenib decreased intracellular  
446 miR-30e-3p levels in sensitive cell lines (HepG2, Hep3B and Huh-7), while it increased miRNA  
447 levels in the resistant (SNU475) one (**Figure 7D-F**). Aiming at identifying molecular  
448 mechanisms underneath miR-30e-3p extrusion triggered by sorafenib, we focused on p53, a  
449 known promoter of exosomal secretion [45]. We assayed p53 activity following sorafenib  
450 treatment in WT *TP53* HepG2 cells, in SNU475 cells that harbor a double *TP53* heterozygous  
451 mutation but maintain a residual *TP53* transcriptional activity and in SNU449 cells with a  
452 mutated and transcriptionally inactive *TP53* isoform (**Figure 3B**). Sorafenib increased *TP53*  
453 activity in both HepG2 and SNU475 cells, but not in SNU449 cells (**Figure 7G**). We next  
454 performed *TP53* silencing and deletion in HepG2 cells and observed that p53 absence prevented  
455 miR-30e-3p extracellular and exosomal rising (**Figure 7H and S9E**), confirming that p53  
456 contributes to sorafenib-mediated miR-30e-3p exosomal secretion by HCC cells.

457 Remarkably, an inverse correlation between tissue and serum miR-30e-3p levels was detected in  
458 both DEN-HCC rats and xenograft mice subjected to sorafenib treatment (Pearson's correlation;  
459  $R=-0.58$  and  $R=-0.88$ , respectively) (**Figure S9F, G**). In addition, a negative correlation between  
460 miR-30e-3p circulating levels and apoptotic molecules was found in sorafenib-treated HCC rats  
461 (Pearson's correlation;  $R=-0.82$ ,  $-0.65$  and  $-0.51$ , respectively) (**Figure S9H-J**) and higher  
462 circulating miR-30e-3p levels were detected in sorafenib-resistant group (Student's t-test;  
463  $p=0.05$ ) (**Figure 7I**). Taken together, these correlative findings suggested miR-30e-3p as a  
464 potential biomarker of treatment response.

465 We thus tested circulating miR-30e-3p levels in patients with advanced HCC (**Table S2**),  
466 assaying sera samples collected before (46 patients, 25 responders and 21 non-responders at two  
467 months) and on sorafenib treatment (49 patients, 23 responders and 26 non-responders at the 4

months follow-up assessment). Circulating miR-30e-3p levels did not differ between responders and non-responders (mean circulating miR-30e-3p: 2.24 in R vs 1.96 in NR; unpaired t-test,  $p=0.3$ , **Figure 7J**) ruling out any predictor role of primary response. Conversely, higher circulating miR-30e-3p was found at the two months follow-up in patients experiencing escape to treatment at the four months TC (circulating miR-30e-3p at two months: 2.13 in responders vs 3.18 in non-responders, unpaired T-test,  $p<0.0001$ ; **Figure 7K**), suggesting its early elevation in acquired drug resistance. In a subgroup of 28 patients (13 responders and 15 non-responders at the four months follow-up), variations of circulating miR-30e-3p were tested over time (basal versus two months follow-up). Even though the cohort is small, preventing any conclusion, an increase in circulating miR-30e-3p levels at the two months assessment was observed in patients developing escape at the subsequent CT-scan (**Figure S9K, L**). Notably, circulating miR-30e-5p levels did not predict sorafenib response neither before nor on treatment (**Figure S9M, N**) further highlighting the specificity of miR-30e-3p variations in acquired resistance to sorafenib. In conclusion, even though these data are very preliminary and circumstantial, the early rising of circulating miR-30e-3p in patients developing sorafenib resistance makes this miRNA appealing for further exploitation, in a field where non-invasive biomarkers are needed.

## 484    **Discussion**

485    MiRNAs act as tumor suppressors (TS) or oncogenes depending on targets' core, driver gene  
486    mutations and tumor microenvironment. Here we report the downregulation of miR-30e-3p in  
487    human and rat HCCs and its association with poor prognosis, proliferation, stemness phenotype  
488    and drug resistance. The modulation of different biological functions has to be ascribed to the  
489    simultaneous targeting of both oncogenes and tumor suppressor genes.

490    Previous studies reported miR-30 as a 'dual' miRNA, with opposite functions in NSCLC and  
491    pancreatic cancer [15, 18]. Here, we describe the 'dual' role of miR-30e-3p within the same  
492    tumor type, highlighting opposite effects based on different molecular backgrounds and in  
493    particular to *TP53* status. On one hand, miR-30e-3p decreased cell proliferation and induced  
494    sorafenib sensitization in *TP53* WT HepG2 cells, behaving as a TS miRNA. On the other hand, it  
495    induced proliferation, and drug resistance in *TP53*-mutated/deleted HCC cells, behaving as an  
496    oncomiR. Indeed, both tumor suppressors (*CDKN1B/p27* and *PTEN*) and oncogenes (*MDM2*,  
497    *EpCAM*) were demonstrated to be miR-30e-3p targets. Specifically, miR-30e-3p exerts TS  
498    functions in *TP53* WT contexts where *mdm2* targeting is prevalent, leading to p53 pathway  
499    activation. On the contrary, tumor-promoting effects take place in non-functional p53 contexts,  
500    where p27 and PTEN targeting becomes prevalent. In particular, p27 drives the proliferating  
501    phenotype; EpCAM modulates staminal properties, whereas PTEN contributes to drug resistance  
502    by triggering AKT activation [46]. Similarly, we previously reported a dual behavior for miR-  
503    221 with respect to p53 status, highlighting the importance of molecular characterization when  
504    antagomiR strategies are considered [21]. Indeed, both miR-221 and miR-30e-3p modulate the  
505    *TP53* axis through *mdm2* direct regulation explaining, at least in part, their opposite activity in  
506    different *TP53* contexts.

507    As established for miR-30a and miR-30e-5p [24, 25] here we proved that p53 activates miR-30e  
508    transcription. Nevertheless, we observed a negative correlation between primary and mature



509 miR-30e-3p levels, envisaging regulatory events on miRNA biogenesis. Strikingly, mature but  
510 not primary miRNA profiles clustered normal and tumor samples into separate nodes, suggesting  
511 maturation processes as more relevant than altered transcription for aberrant miRNA expression  
512 [47]. Gain-of-function of mutated p53 isoforms hampers miRNA processing machinery leading  
513 to miRNA downregulation due to interference with both p68 and p72/82 RNA helicases,  
514 impairing Drosha microprocessor activity [23, 48]. Moreover, R273H mutated p53 inhibits miR-  
515 30a transcription contributing to its aberrant expression in breast cancer [49]. We observed an  
516 association between p53 mutations and miR-30e-3p downregulation, as well as the inhibition of  
517 miRNA maturation following Nutlin-3 treatment, suggesting the enrollment of other mdm2  
518 targets, such as p53 family members (p63 and p73), as possible regulators of miRNA biogenesis.  
519 In this regard, an elegant study by Su and coworkers demonstrated that Dicer1 is a  
520 transcriptional target of TAp63 but not of p53 [50]. Our descriptive findings on p53 involvement  
521 in miRNA biogenesis led us to speculate that p53 accumulation, as in the case of *MDM2*  
522 permanent inhibition by Nutlin-3, might be responsible for the transcriptional inhibition of  
523 Dicer1 through saturation of p63/p53-REs, preventing p63-mediated transcription. Dicer1  
524 disruption in knockout (KO) mice promoted hepatocarcinogenesis and led to the deregulation of  
525 several miRNAs, including all miR-30 family members, suggesting a strict regulation of miR-30  
526 maturation by Dicer1 in HCC [51].

527 Rat HCCs, characterized by a *TP53* WT background [42], showed an inverse correlation  
528 between miR-30e-3p and tumor size, as well as reduced miR-30e-3p tissue levels in sorafenib  
529 resistant tumors, suggesting miR-30e-3p downregulation as a contributor to sorafenib resistance.  
530 New molecules, beside sorafenib, were recently registered for advanced HCC [52], however  
531 biomarkers predicting treatment outcome are lacking. In the last two decades, miRNAs have  
532 emerged as attractive diagnostic, prognostic and therapeutic tools [53, 54] and reliable markers  
533 [43]. Decreased miR-30e and miR-223 serum levels were reported in HCC patients irrespective

534 of tumor etiology [55], whereas miR-30e-enriched vesicles decreased mesenchymal properties in  
535 cholangiocarcinoma cells [56].

536 Our and other groups reported sorafenib as a trigger for *TP53* activation and demonstrated *TP53*  
537 as a key player for exosomal secretion in stressful conditions [45] even though mechanisms  
538 governing exosomal secretion remain poorly understood. Here we confirmed the contribution of  
539 the p53 pathway to miR-30e-3p exosomal secretion in sorafenib-treated HCC cell lines. In  
540 addition, as reported for the TKI gefitinib, sorafenib triggers exosome secretion in HCC cells  
541 [44]. Increased miR-30e-3p circulating levels were found in sorafenib resistant rat and human  
542 HCCs. In particular, higher miR-30e-3p circulating levels, deriving from exosomal secretion,  
543 were associated with subsequent escape to sorafenib. This does not prove a causative effect and  
544 may simple represent a bystander event; however, miR-30e-3p role as a non-invasive predictor  
545 of treatment escape can be envisaged and should be further investigated in larger patient cohorts.  
546

547    **Acknowledgements and Financial Support Statement**

548    We thank Dr. Vilma Mantovani and Dr. Marinella Cenci of Genitics Unit at S.Orsola-Malpighi  
549    University Hospital for cell line authentication. We thank Dr. Clarissa Patrizi of Modena and  
550    Reggio Emilia University for her precious help with CRISPR technology.

551    Financial support: (1) Programma di Ricerca Regione-Università, Regione Emilia-Romagna,  
552    Bando "Alessandro Liberati", to F.F. "Identification of innovative microRNAs-based biomarkers  
553    and anti-cancer strategies for the treatment of Hepatocellular carcinoma". (2) Programma di  
554    Ricerca Regione-Università, Regione Emilia-Romagna, Bando "Ricerca Innovativa", to L.B. and  
555    L.G. "Innovative approaches to the diagnosis and pharmacogenetic-based therapies of primary  
556    hepatic tumors, peripheral B and T-cell lymphomas and lymphoblastic leukaemias".

557

558 **References**

- 559 1. Llovet JM, Zucman-Rossi J, Pikarsky E, Sangro B, Schwartz M, Sherman M, et al.  
560 Hepatocellular carcinoma. *Nat Rev Dis Primers* 2016;**2**:16018.
- 561 2. Llovet JM, Villanueva A, Lachenmayer A, Finn RS. Advances in targeted therapies for  
562 hepatocellular carcinoma in the genomic era. *Nat Rev Clin Oncol* 2015;**12**:408-24.
- 563 3. Colombo F, Balcan F, Mazzucchelli S, Martin-Padura I, Marighetti P, Cattaneo A, et al.  
564 Evidence of distinct tumour-propagating cell populations with different properties in primary  
565 human hepatocellular carcinoma. *Plos One* 2011;**6**:e21369.
- 566 4. Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F, et al. A microRNA  
567 expression signature of human solid tumors defines cancer gene targets. *PNAS* 2006;**103**:2257-  
568 61.
- 569 5. Zhang B, Pan X, Cobb GP, Anderson TA. microRNAs as oncogenes and tumor  
570 suppressors. *Dev Biol* 2007;**302**:1-12.
- 571 6. Budhu A, Jia HL, Forgues M, Liu CG, Goldstein D, Lam A, et al. Identification of  
572 metastasis-related microRNAs in hepatocellular carcinoma. *Hepatology* 2008;**47**:897-907.
- 573 7. Gramantieri L, Fornari F, Ferracin M, Veronese A, Sabbioni S, Calin GA, et al.  
574 MicroRNA-221 targets Bmf in hepatocellular carcinoma and correlates with tumor multifocality.  
575 *Clin Cancer Res* 2009;**15**:5073-81.
- 576 8. Bai S, Nasser MW, Wang B, Hsu SH, Datta J, Kutay H, et al. MicroRNA-122 inhibits  
577 tumorigenic properties of hepatocellular carcinoma cells and sensitizes these cells to sorafenib. *J*  
578 *Biol Chem* 2009;**284**:32015-27.
- 579 9. Fornari F, Pollutri D, Patrizi C, La Bella T, Marinelli S, Casadei Gardini A, et al. In  
580 Hepatocellular Carcinoma miR-221 Modulates Sorafenib Resistance through Inhibition of  
581 Caspase-3-Mediated Apoptosis. *Clin Cancer Res* 2017;**23**:3953-65.

- 582 10. Garofalo M, Di Leva G, Romano G, Nuovo G, Suh SS, Ngankekou A, et al. miR-221&222  
583 regulate TRAIL resistance and enhance tumorigenicity through PTEN and TIMP3  
584 downregulation. *Cancer Cell* 2009;**16**:498-509.
- 585 11. Ichimi T, Enokida H, Okuno Y, Kunimoto R, Chiyomaru T, Kawamoto K, et al.  
586 Identification of novel microRNA targets based on microRNA signatures in bladder cancer. *Int J*  
587 *Cancer* 2009;**125**:345-52.
- 588 12. Zhao JJ, Lin J, Zhu D, Wang X, Brooks D, Chen M, et al. miR-30-5p functions as a  
589 tumor suppressor and novel therapeutic tool by targeting the oncogenic Wnt/beta-catenin/BCL9  
590 pathway. *Cancer Res* 2014;**74**:1801-13.
- 591 13. Volinia S, Galasso M, Costinean S, Tagliavini L, Gamberoni G, Drusco A, et al.  
592 Reprogramming of miRNA networks in cancer and leukemia. *Genome Res* 2010;**20**:589-99.
- 593 14. Chen Z, Zhang J, Zhang Z, Feng Z, Wei J, Lu J, et al. The putative tumor suppressor  
594 microRNA-30a-5p modulates clear cell renal cell carcinoma aggressiveness through repression  
595 of ZEB2. *Cell Death Dis* 2017;**8**:e2859.
- 596 15. Kumarswamy R, Mudduluru G, Ceppi P, Muppala S, Kozlowski M, Niklinski J, et al.  
597 MicroRNA-30a inhibits epithelial-to-mesenchymal transition by targeting Snail and is  
598 downregulated in non-small cell lung cancer. *Int J Cancer* 2012;**130**:2044-53.
- 599 16. Zhang Q, Yu L, Qin D, Huang R, Jiang X, Zou C, et al. Role of microRNA-30c targeting  
600 ADAM19 in colorectal cancer. *PloS One* 2015;**10**:e0120698.
- 601 17. Su W, Hong L, Xu X, Huang S, Herpai D, Li L, et al. miR-30 disrupts senescence and  
602 promotes cancer by targeting both p16(INK4A) and DNA damage pathways. *Oncogene*  
603 2018;**37**:5618-32.
- 604 18. Tsukasa K, Ding Q, Miyazaki Y, Matsubara S, Natsugoe S, Takao S. miR-30 family  
605 promotes migratory and invasive abilities in CD133(+) pancreatic cancer stem-like cells. *Hum*  
606 *cell* 2016;**29**:130-7.

- 607 19. Shimada S, Mogushi K, Akiyama Y, Furuyama T, Watanabe S, Ogura T, et al.  
608 Comprehensive and integrative genomic characterization of HCC. *EBiomedicine* 2019;**40**:457-  
609 70.
- 610 20. Fornari F, Milazzo M, Chieco P, Negrini M, Marasco E, Capranico G, et al. In  
611 hepatocellular carcinoma miR-519d is up-regulated by p53 and DNA hypomethylation and  
612 targets CDKN1A/p21, PTEN, AKT3 and TIMP2. *J Pathol* 2012;**227**:275-85.
- 613 21. Fornari F, Milazzo M, Galassi M, Callegari E, Veronese A, Miyaaki H, et al. p53/mdm2  
614 feedback loop sustains miR-221 expression and dictates the response to anticancer treatments in  
615 hepatocellular carcinoma. *Mol Cancer Res* 2014;**12**:203-16.
- 616 22. Raver-Shapira N, Marciano E, Meiri E, Spector Y, Rosenfeld N, Moskovits N, et al.  
617 Transcriptional activation of miR-34a contributes to p53-mediated apoptosis. *Mol Cell*  
618 2007;**26**:731-43.
- 619 23. Suzuki HI, Yamagata K, Sugimoto K, Iwamoto T, Kato S, Miyazono K. Modulation of  
620 microRNA processing by p53. *Nature* 2009;**460**:529-33.
- 621 24. di Gennaro A, Damiano V, Brisotto G, Armellin M, Perin T, Zucchetto A, et al. A  
622 p53/miR-30a/ZEB2 axis controls triple negative breast cancer aggressiveness. *Cell Death Differ*  
623 2018;**25**:2165-80.
- 624 25. Laudato S, Patil N, Abba ML, Leupold JH, Benner A, Gaiser T, et al. P53-induced miR-  
625 30e-5p inhibits colorectal cancer invasion and metastasis by targeting ITGA6 and ITGB1. *Int J*  
626 *Cancer* 2017;**141**:1879-90.
- 627 26. Deng L, Tang J, Yang H, Cheng C, Lu S, Jiang R, et al. MTA1 modulated by miR-30e  
628 contributes to epithelial-to-mesenchymal transition in hepatocellular carcinoma through an  
629 ErbB2-dependent pathway. *Oncogene* 2017;**36**:3976-85.
- 630 27. Liu Z, Tu K, Liu Q. Effects of microRNA-30a on migration, invasion and prognosis of  
631 hepatocellular carcinoma. *FEBS Lett* 2014;**588**:3089-97.

- 632 28. Yao J, Liang L, Huang S, Ding J, Tan N, Zhao Y, et al. MicroRNA-30d promotes tumor  
633 invasion and metastasis by targeting Galphai2 in hepatocellular carcinoma. *Hepatology*  
634 2010;**51**:846-56.
- 635 29. Hand NJ, Master ZR, Eauclore SF, Weinblatt DE, Matthews RP, Friedman JR. The  
636 microRNA-30 family is required for vertebrate hepatobiliary development. *Gastroenterology*  
637 2009;**136**:1081-90.
- 638 30. Gramantieri L, Ferracin M, Fornari F, Veronese A, Sabbioni S, Liu CG, et al. Cyclin G1  
639 is a target of miR-122a, a microRNA frequently down-regulated in human hepatocellular  
640 carcinoma. *Cancer Res* 2007;**67**:6092-9.
- 641 31. Fornari F, Ferracin M, Trere D, Milazzo M, Marinelli S, Galassi M, et al. Circulating  
642 microRNAs, miR-939, miR-595, miR-519d and miR-494, Identify Cirrhotic Patients with HCC.  
643 *PloS One*. 2015;**10**:e0141448.
- 644 32. Fornari F, Gramantieri L, Giovannini C, Veronese A, Ferracin M, Sabbioni S, et al. MiR-  
645 122/cyclin G1 interaction modulates p53 activity and affects doxorubicin sensitivity of human  
646 hepatocarcinoma cells. *Cancer Res* 2009;**69**:5761-7.
- 647 33. Fornari F, Milazzo M, Chieco P, Negrini M, Calin GA, Grazi GL, et al. MiR-199a-3p  
648 regulates mTOR and c-Met to influence the doxorubicin sensitivity of human hepatocarcinoma  
649 cells. *Cancer Res* 2010;**70**:5184-93.
- 650 34. Pollutri D, Patrizi C, Marinelli S, Giovannini C, Trombetta E, Giannone FA, et al. The  
651 epigenetically regulated miR-494 associates with stem-cell phenotype and induces sorafenib  
652 resistance in hepatocellular carcinoma. *Cell Death Dis* 2018;**9**:4.
- 653 35. Fittipaldi S, Vasuri F, Bonora S, Degiovanni A, Santandrea G, Cucchetti A, et al. miRNA  
654 Signature of Hepatocellular Carcinoma Vascularization: How the Controls Can Influence the  
655 Signature. *Dig Dis Sci* 2017;**62**:2397-407.
- 656 36. Pollutri D, Gramantieri L, Bolondi L, Fornari F. TP53/MicroRNA Interplay in  
657 Hepatocellular Carcinoma. *Int J Mol Sci* 2016;**17**(12).

37. He L, He X, Lim LP, de Stanchina E, Xuan Z, Liang Y, et al. A microRNA component of the p53 tumour suppressor network. *Nature* 2007;**447**:1130-4.
38. Boominathan L. The tumor suppressors p53, p63, and p73 are regulators of microRNA processing complex. *PloS One* 2010;**5**(5):e10615.
39. Ozsolak F, Poling LL, Wang Z, Liu H, Liu XS, Roeder RG, et al. Chromatin structure analyses identify miRNA promoters. *Genes Dev* 2008;**22**:3172-83.
40. Ning ZQ, Lu HL, Chen C, Wang L, Cai W, Li Y, et al. MicroRNA-30e reduces cell growth and enhances drug sensitivity to gefitinib in lung carcinoma. *Oncotarget* 2017;**8**:4572-81.
41. Schepeler T, Holm A, Halvey P, Nordentoft I, Lamy P, Riising EM, et al. Attenuation of the beta-catenin/TCF4 complex in colorectal cancer cells induces several growth-suppressive microRNAs that target cancer promoting genes. *Oncogene* 2012;**31**:2750-60.
42. Sasaki Y, Tsujiuchi T, Murata N, Kubozoe T, Tsutsumi M, Konishi Y. Absence of p16, p21 and p53 gene alterations in hepatocellular carcinomas induced by N-nitrosodiethylamine or a choline-deficient L-amino acid-defined diet in rats. *Cancer Lett* 2000;**152**:71-7.
43. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, et al. Circulating microRNAs as stable blood-based markers for cancer detection. *PNAS* 2008;**105**:10513-8.
44. Li XQ, Liu JT, Fan LL, Liu Y, Cheng L, Wang F, et al. Exosomes derived from gefitinib-treated EGFR-mutant lung cancer cells alter cisplatin sensitivity via up-regulating autophagy. *Oncotarget* 2016;**7**:24585-95.
45. Yu X, Harris SL, Levine AJ. The regulation of exosome secretion: a novel function of the p53 protein. *Cancer Res* 2006;**66**:4795-801.
46. Chen KF, Chen HL, Tai WT, Feng WC, Hsu CH, Chen PJ, et al. Activation of phosphatidylinositol 3-kinase/Akt signaling pathway mediates acquired resistance to sorafenib in hepatocellular carcinoma cells. *J Pharmacol Exp Ther* 2011;**337**:155-61.



683 47. Thomson JM, Newman M, Parker JS, Morin-Kensicki EM, Wright T, Hammond SM.  
684 Extensive post-transcriptional regulation of microRNAs and its implications for cancer. *Genes*  
685 *Dev* 2006;**20**:2202-7.

686 48. Garibaldi F, Falcone E, Trisciuglio D, Colombo T, Lisek K, Walerych D, et al. Mutant  
687 p53 inhibits miRNA biogenesis by interfering with the microprocessor complex. *Oncogene*  
688 2016;**35**:3760-70.

689 49. Guo F, Chen H, Chang J, Zhang L. Mutation R273H confers p53 a stimulating effect on  
690 the IGF-1R-AKT pathway via miR-30a suppression in breast cancer. *Biomed Pharmacother*  
691 2016;**78**:335-41.

692 50. Su X, Chakravarti D, Cho MS, Liu L, Gi YJ, Lin YL, et al. TAp63 suppresses metastasis  
693 through coordinate regulation of Dicer and miRNAs. *Nature* 2010;**467**:986-90.

694 51. Sekine S, Ogawa R, Ito R, Hiraoka N, McManus MT, Kanai Y, et al. Disruption of  
695 Dicer1 induces dysregulated fetal gene expression and promotes hepatocarcinogenesis.  
696 *Gastroenterology* 2009;**136**:2304-15 e1-4.

697 52. Kudo M. A New Era of Systemic Therapy for Hepatocellular Carcinoma with  
698 Regorafenib and Lenvatinib. *Liver Cancer* 2017;**6**:177-84.

699 53. Janssen HL, Reesink HW, Lawitz EJ, Zeuzem S, Rodriguez-Torres M, Patel K, et al.  
700 Treatment of HCV infection by targeting microRNA. *New Engl J Med* 2013;**368**:1685-94.

701 54. Rupaimoole R, Slack FJ. MicroRNA therapeutics: towards a new era for the management  
702 of cancer and other diseases. *Nat Rev Drug Dis* 2017;**16**:203-22.

703 55. Bhattacharya S, Steele R, Shrivastava S, Chakraborty S, Di Bisceglie AM, Ray RB.  
704 Serum miR-30e and miR-223 as Novel Noninvasive Biomarkers for Hepatocellular Carcinoma.  
705 *Am J Pathol* 2016;**186**:242-7.

706 56. Ota Y, Takahashi K, Otake S, Tamaki Y, Okada M, Aso K, et al. Extracellular vesicle-  
707 encapsulated miR-30e suppresses cholangiocarcinoma cell invasion and migration via inhibiting  
708 epithelial-mesenchymal transition. *Oncotarget* 2018;**9**:16400-17.

709 **Figure Legends**

710 **Figure 1. Deregulated expression of miR-30 family in human and rat HCCs.** (A-C) Box plot  
711 graphs of miR-30e-3p, miR-30e-5p and miR-30a-3p in human and (D-F) rat tumor and non-  
712 tumor (NT) samples or liver cirrhosis (LC). (G-I) Box plot graph of miR-30e-3p, miR-30e-5p  
713 and miR-30a-3p in the validation cohort and in normal livers (NL). On the top of each graph is  
714 reported the p-value relative to ANOVA, whereas stars represent comparison between groups  
715 (Tukey's post hoc test). (J, K) Box plot graphs of mature and primary (pri-miR) miR-30e-3p in  
716 wild type (WT) and mutated (MUT) *TP53* HCC specimens. Y-axes report  $2^{-\Delta\Delta Ct}$  values  
717 corresponding to miRNA levels.

718

719 **Figure 2. *TP53* regulates miR-30e expression in HCC cells.** (A) QPCR analysis of primary  
720 miR-30e (pri-miR-30e) and mature isoforms (miR-30e-3p, miR-30e-5p, miR-34a) in *TP53*  
721 silenced, (B) *TP53* overexpressing (pCMV-p53) and (C) Nutlin-3 treated HepG2 cells or (D-E)  
722 Nutlin-3 treated p53-deleted HepG2 (CRISPR) and p53-null Hep3B cells. (F) QPCR analysis of  
723 primary miR-30e and mature isoforms in *TP53* overexpressing Hep3B cells and (G) mutated  
724 *TP53* (pBabe-p53-mut) overexpressing HepG2 cells. (A-G) WB and qPCR analyses of *TP53*,  
725 *MDM2*, *CDKN1A/p21* in the same settings. U6RNA and  $\beta$ -actin were used as housekeeping  
726 genes. QPCR experiments were performed twice in triplicate; p-values from unpaired t-test are  
727 shown in each graph. Scr: scramble oligonucleotide; pCMV and pBabe: control vectors; DMSO:  
728 vehicle.

729

730 **Figure 3. MiR-30e-3p targets *MDM2* in HCC cells.** (A) MiR-30e-3p hypothetical binding sites  
731 in *MDM2* 3'UTR, as identified by Targetscan. Stars represent mutated bases. (B) QPCR analysis  
732 of miR-30e-3p in HCC, liver cirrhosis (LC) and normal liver (NL) as well as in HCC cell lines.

733 *TP53* status and relative transcriptional activity are reported in the table below the graph, *TP53*  
734 activity has been normalized on that detected in HepG2 cells. (C-E) QPCR and WB analyses of  
735 miRNA and *MDM2* expression in miR-30e-3p overexpressing Huh-7, Hep3B and SNU475 cells  
736 and (F) miR-30e-3p silenced SNU449 cells. Time after transfection: 24 h. Y-axes represent  
737 relative expression levels. U6RNA and  $\beta$ -actin were used as housekeeping genes. (G) Luciferase  
738 reporter assay in HepG2 cells co-transfected with wild type (WT) or mutated (MUT) pGL3-  
739 MDM2-3UTR vectors and miR-30e-3p or negative control (NC). This experiment was repeated  
740 three times in triplicate. (H) P53 reporter assay and WB analysis in miR-30e-3p overexpressing  
741 HepG2 and Huh-7 cells. (G, H) Y-axes report relative values with respect to negative controls.  
742 NC: pre-miR negative control; NCi: anti-miR negative control; AM-30e-3p: anti-miR-30e-3p.

743

744 **Figure 4. MiR-30e-3p influences cell growth and invasion of HCC cells.** (A, B) Growth  
745 curves of miR-30e-3p and negative control (NC) transfected HepG2 cells in basal conditions and  
746 following p53 silencing. (C-E) Growth curves of miR-30e-3p transfected Huh-7, Hep3B and  
747 SNU475 cells with respect to negative control (NC) cells. (A-E) WB analysis of p53, PTEN,  
748 pAKT, p27 and p21 levels normalized on  $\beta$ -actin housekeeping gene. (F) Real time invasion  
749 assay of miR-30e-3p stably silenced (MZIP-30e-3p) HepG2 and SNU449 cells with respect to  
750 controls (MZIP-shRNA).

751

752 **Figure 5. MiR-30e-3p influences stem cell properties of HCC cells.** (A) Correlation graphs  
753 between miR-30e-3p and AFP or EpCAM mRNAs in HCC patients (N=30). Axes report  $2^{-\Delta\Delta Ct}$   
754 values transformed in a log2 form. (B) QPCR analysis of EpCAM expression in miR-30e-3p  
755 overexpressing Huh-7 cells (transient transfection) and miR-30e-3p silenced HepG2 cells (stable  
756 infection). Y-axis reports relative gene expression. (C) Luciferase reporter assay in HepG2 cells

757 co-transfected with WT or mutated (MUT) pGL3-EpCAM-3UTR vectors and miR-30e-3p or  
 758 negative control (NC). **(D-F)** Six-well plate images of clonogenic assay in MIZ-30e-3p and  
 759 control (MZIP-shRNA) HepG2 and SNU449 cells, as well as in miR-30e-3p overexpressing  
 760 (pMXs-miR-30e-3p) and control (pMXs-shRNA) Huh-7 cells. Column graphs represent colony  
 761 count and OD 595. Columns and bars represent average  $\pm$  SD values. **(G)** Sphere formation  
 762 assay in MZIP-30e-3p and control HepG2 cells as well as in pMXs-miR-30e-3p and control  
 763 (pMXs-shRNA) Huh-7 cells. Average  $\pm$  SD values from two independent experiments are  
 764 shown. **(H)** WB analysis of stemness markers in MZIP-30e-3p HepG2 and pMXs-miR-30e-3p  
 765 Huh-7 spheres. **(I)** Box plot graph displaying miR-30e-3p expression in patients with or without  
 766 microvascular invasion (MVI). **(J)** Kaplan-Meier survival curve in HCC patients with high or  
 767 low miR-30e-3p expression. High or low miRNA values were considered with respect to median  
 768 value.

769

770 **Figures 6. MiR-30e-3p induces drug sensitization in HepG2 cells.** **(A)** Annexin-V assay in  
 771 untreated or **(B)** serum deprived (60h) miR-30e-3p overexpressing and control cells. **(C-E)**  
 772 Annexin-V images, WB analysis and caspase activity assay in miR-30e-3p overexpressing cells  
 773 following doxorubicin (2.0  $\mu$ g/ml, 48h) or **(F-H)** sorafenib treatment (7.5  $\mu$ M, 48h). **(I-K)**  
 774 Annexin-V images, WB analysis and caspase activity assay in p53-silenced miR-30e-3p  
 775 overexpressing HepG2 cells following sorafenib treatment.

776

777 **Figure 7. Increased miR-30e-3p serum levels associate with sorafenib escape in HCC.** **(A)**  
 778 Box plot graph displaying miR-30e-3p levels in sorafenib-treated rat HCCs with respect to  
 779 surrounding livers or **(B)** in responder (R) and non-responder (NR) groups. **(C)** Correlation  
 780 graph between tissue miR-30e-3p levels and tumor size in sorafenib-treated rat HCCs. **(D)**

781 QPCR analysis of intracellular, (E) extracellular and (F) exosomal miR-30e-3p levels in  
782 untreated and sorafenib treated HCC cells. Y-axes report relative values with respect to not-  
783 treated cells. DMSO was used as vehicle in untreated cells. Numbers below histograms represent  
784 relative cell viability values (%) of sorafenib treated versus untreated cells (48 h) identifying  
785 sensitive and resistant cell lines. (G) *TP53* activity assay in sorafenib treated HepG2, SNU449  
786 and SNU475 cell lines (5  $\mu$ M for 48 h). Y-axis reports relative p53 activity with respect to  
787 untreated cells (vehicle: DMSO). (H) QPCR analysis of intracellular, extracellular and exosomal  
788 miR-30e-3p levels in scramble and p53-silenced HepG2 cells after sorafenib treatment (5  $\mu$ M for  
789 48 h). U6RNA and cel-miR-39 were used as housekeeping genes for intracellular and  
790 extracellular/exosomal miRNA levels, respectively. Y-axis reports relative values with respect to  
791 not-treated cells. (I) Box plot graph displaying circulating miR-30e-3p levels in responder (R)  
792 and non-responder (NR) animals. (J) Box plot graph displaying circulating miR-30e-3p levels in  
793 serum samples from responder and non-responder HCC patients collected before treatment start  
794 and (K) at two-month follow-up. Response/escape to sorafenib was assessed at the subsequent  
795 (2-months or 4 months respectively) TC scan. Axes report  $2^{-\Delta\Delta C_t}$  values transformed in a log2  
796 form.

# Figure 1

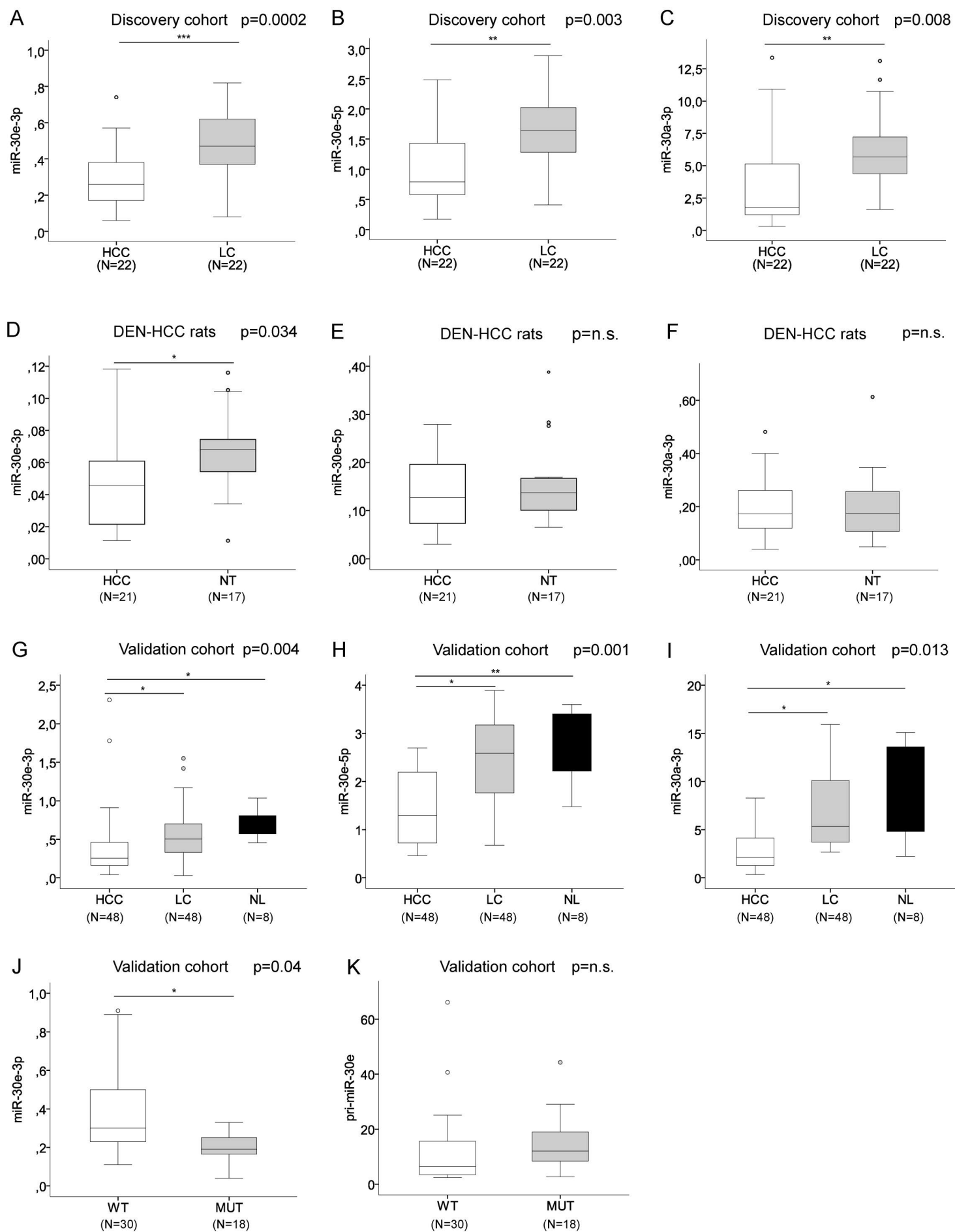


Figure 2

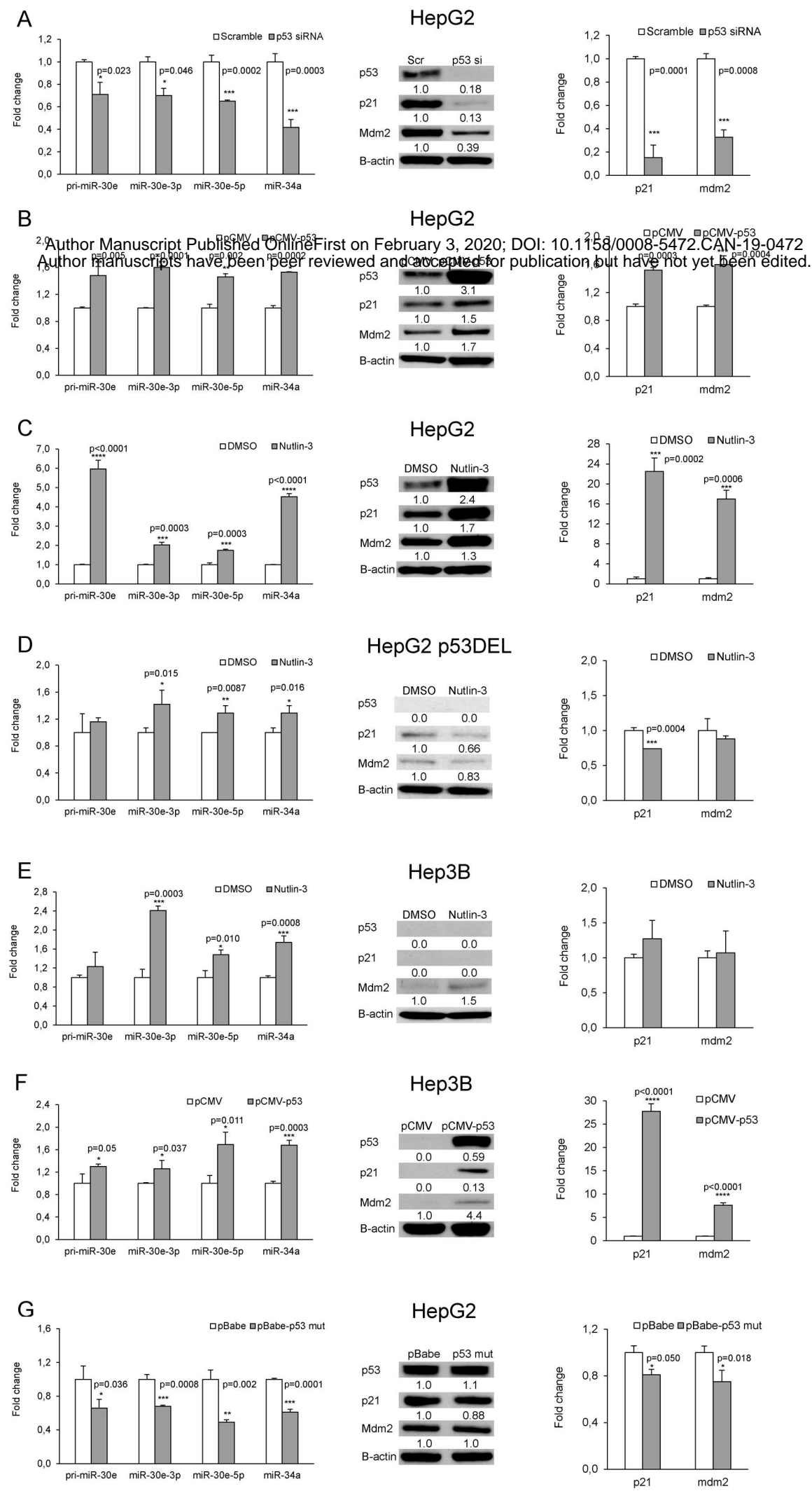
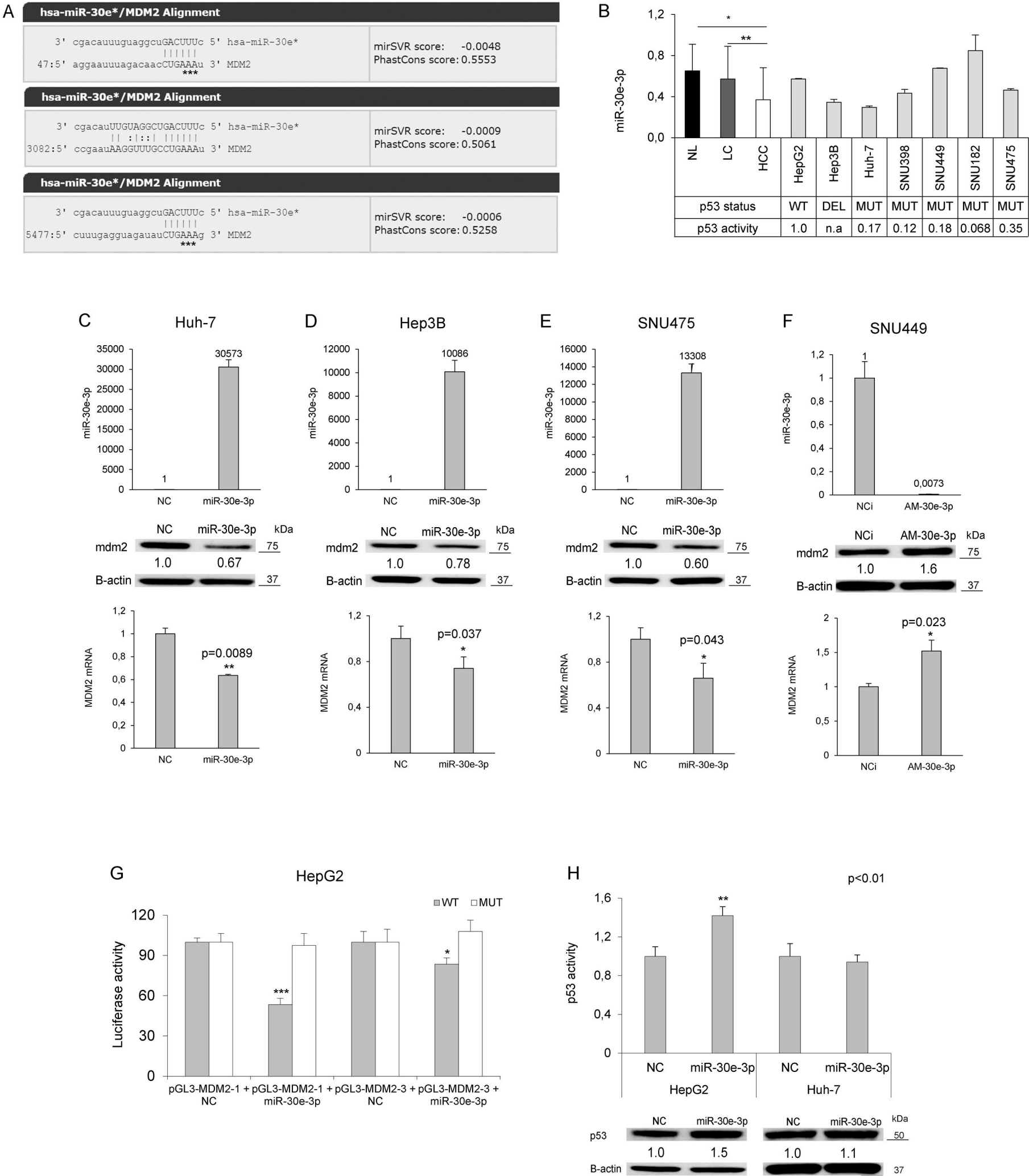
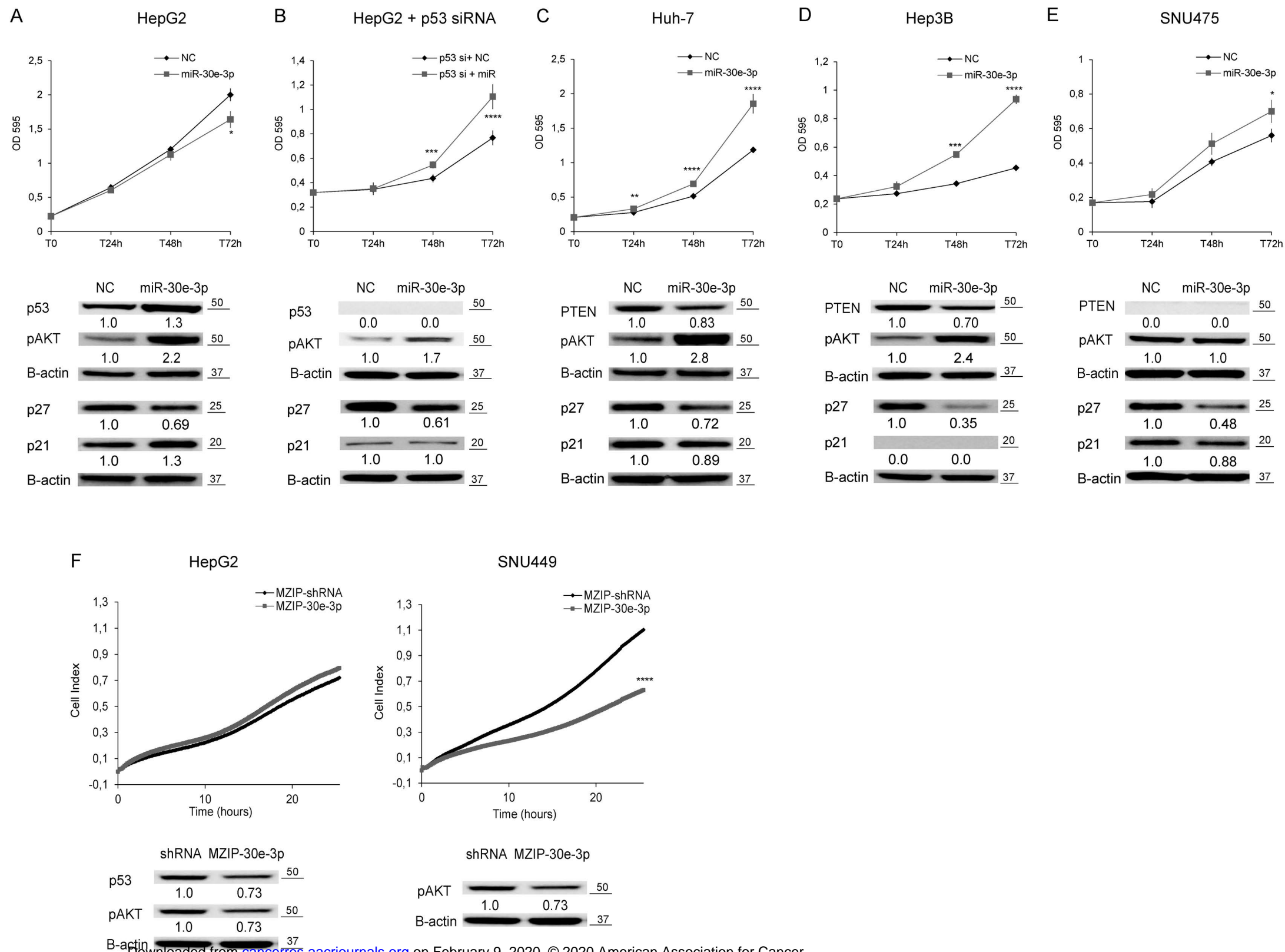


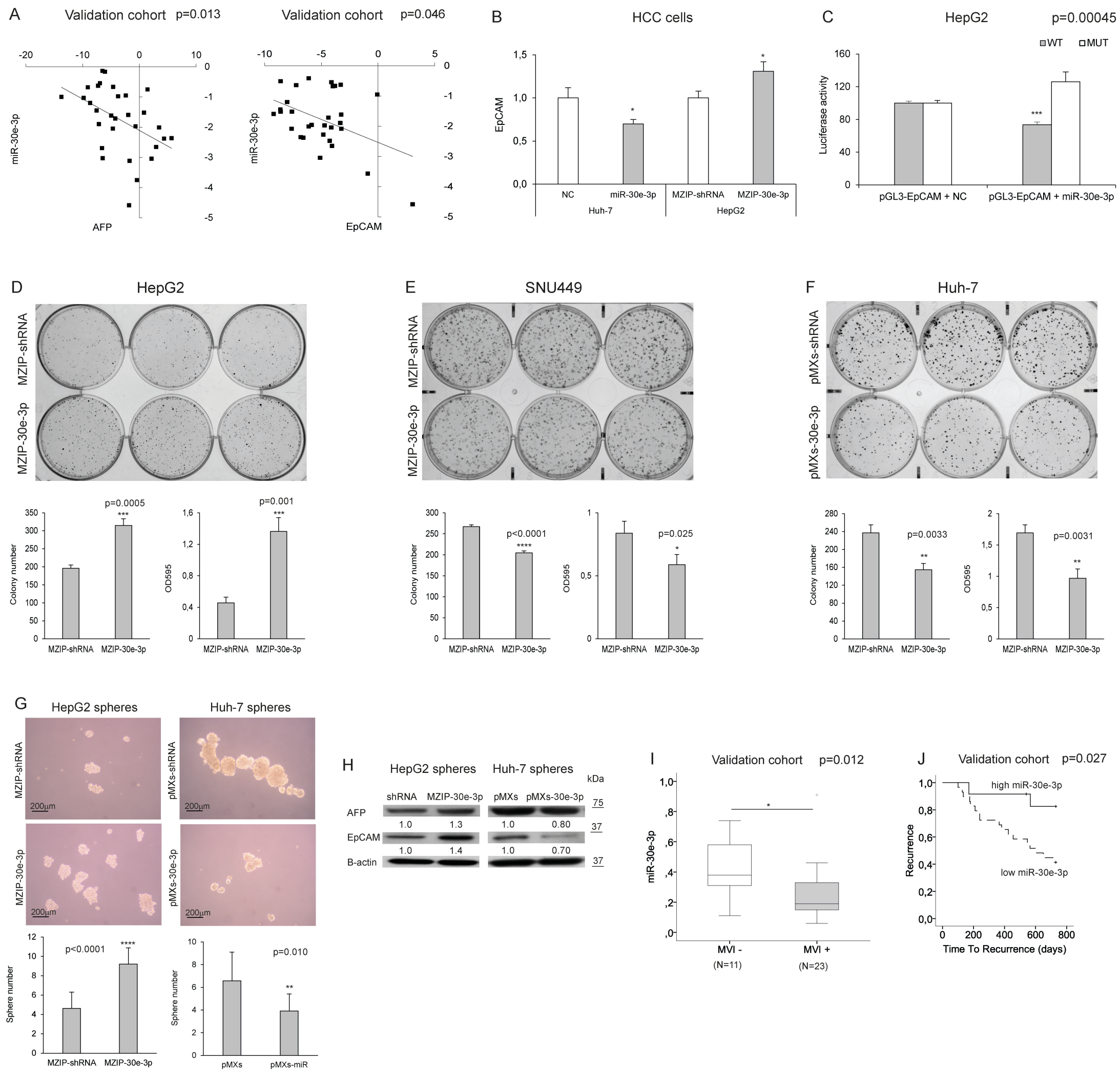
Figure 3



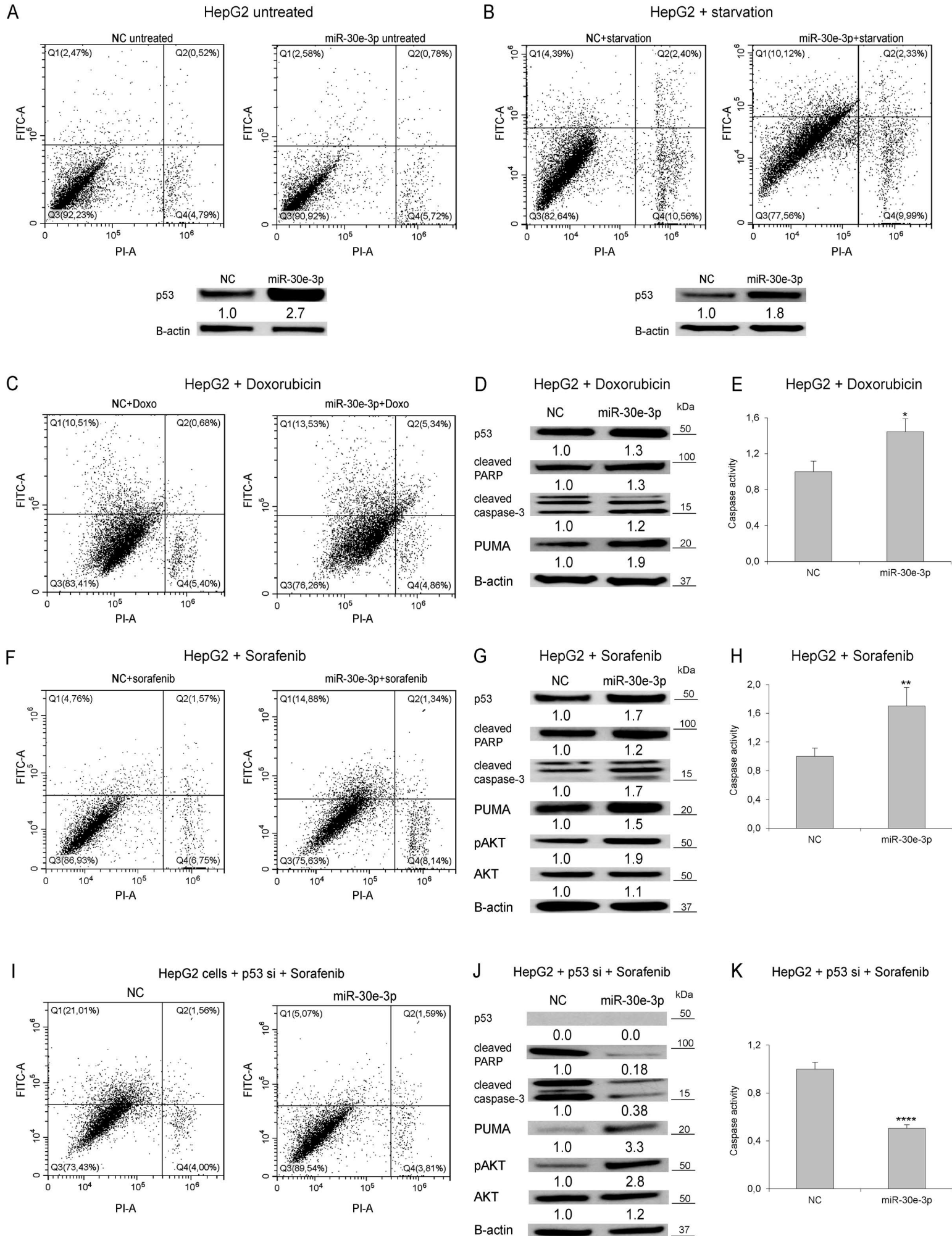


# Figure 4





# Figure 6



# Figure 7

