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Liver Tumorigenicity Promoted by MicroRNA-221 in a Mouse Transgenic Model

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MicroRNA-221 (miR-221) is one of the most frequently and consistently up-regulated microRNAs (miRNAs) in human cancer. It has been hypothesized that miR-221 may act as a tumor promoter. To demonstrate this, we developed a transgenic (TG) mouse model that exhibits an inappropriate overexpression of miR-221 in the liver. Immunoblotting and immunostaining confirmed a concomitant down-regulation of miR-221 target proteins. This TG model is characterized by the emergence of spontaneous nodular liver lesions in approximately 50% of male mice and by a strong acceleration of tumor development in 100% of mice treated with diethylnitrosamine. Similarly to human hepatocellular carcinoma, tumors are characterized by a further increase in miR-221 expression and a concomitant inhibition of its target protein-coding genes (i.e., cyclin-dependent kinase inhibitor [Cdkn]1b/p27, Cdkn1c/p57, and B-cell lymphoma 2–modifying factor). To validate the tumor-promoting effect of miR-221, we showed that *in vivo* delivery of anti-miR-221 oligonucleotides leads to a significant reduction of the number and size of tumor nodules. *Conclusions*: This study not only establishes that miR-221 can promote liver tumorigenicity, but it also establishes a valuable animal model to perform preclinical investigations for the use of anti-miRNA approaches aimed at liver cancer therapy. (HEPATOLOGY 2012;56:1025-1033)

Several studies revealed that the expression of microRNAs (miRNAs) is deregulated in human hepatocellular carcinoma (HCC), in comparison with non-neoplastic liver tissues, as reviewed recently.[1] Among these, microRNA-221 (miR-221) emerged as consistently up-regulated. In HCC, miR-221 is up-regulated in approximately 70%-80% of cases.[2] Its up-regulation in glioblastoma, pancreatic, kidney, bladder, colon, stomach, prostate, and thyroid cancer strengthened its importance in tumorigenesis.[2-11] The hypothesized tumor-promoting activity was supported by functional and molecular evidence. Forced expression of miR-221 in HCC cells could induce an increase in growth, proliferation, migration, and invasion capabilities *in vitro*.[2,10,12] Conversely, anti-miR-221 oligonucleotides could inhibit *in vitro* growth of liver cancer cells.[13] Importantly, the promotion of tumor progression *in vivo* and the shortening of animal survival was observed when miR-221 was introduced into c-myc-immortalized $P53^{-/-}$ liver progenitor cells, which were implanted into irradiated nude mice.[13] Surprisingly, the almost identical miR-222 miRNA, which shares the same seed sequence of miR-221, did not accelerate tumorigenesis in this model system.

At the molecular level, miR-221 was shown to affect several cancer pathways by modulating multiple gene targets, which included the cyclin-dependent kinase inhibitors CDKN1B/p27[7,11] and CDKN1C/p57,[2,10]

Abbreviations: α1-AT, alpha1 antitrypsin; AMOs, anti-miR oligonucleotides; Bcl2, B-cell lymphoma 2; BMF, Bcl2-modifying factor; CDKN, cyclin-dependent kinase inhibitor; PTEN, phosphatase and tensin homolog; DDIT4, DNA damage-inducible transcript 4; TIMP3, tissue inhibitor of metalloprotease 3; mTOR, mammalian target of rapamycin; DENA, diethylnitrosamine; EII, enhancer II; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; IFN-γ, interferon-gamma; IP, intraperitoneal; IV, intravenous; miR-221, microRNA-221; miRNA, microRNA; PCR, polymerase chain reaction; TG, transgenic; WT, wild type.

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the pro-apoptotic protein B-cell lymphoma 2-modifying factor (BMF),[14] the inhibitor of the phosphoinositide 3-kinase pathway phosphatase and tensin homolog (PTEN),[12] the DNA damage-inducible transcript 4 (DDIT4), a tumor suppressor that modulates kinase activity of mammalian target of rapamycin (mTOR),[13] the tissue inhibitor of metalloproteinase 3 (TIMP3).[12]

From a clinical point of view, it was shown that higher levels of miR-221 in HCC correlated with higher tumor stage and metastasis[15] and were associated with multifocal tumors and a shorter time to recurrence after surgical treatment.[14]

These pieces of evidence strongly suggested an important role of miR-221 up-regulation in hepatocarcinogenesis. To prove the hypothesis and develop a more convenient animal model, we produced a transgenic (TG) mouse model that exhibits an inappropriate overexpression of miR-221 in the liver. This TG model is characterized by the appearance of spontaneous liver tumors in a fraction of male mice and a strong acceleration of tumor development in 100% of mice treated with diethylnitrosamine (DENA). This model represents a valuable tool to perform preclinical investigations on the use of miRNA or anti-miRNA approaches for liver cancer therapy.

Materials and Methods

In Vivo *Studies.* Animal experimentation was approved by the institutional ethical committee. Mice were maintained in a vented cabinet at 25°C with a 12-hour light-dark cycle and were provided food and water *ad libitum*. Ten-day newborn mice received one intraperitoneal (IP) injection of DENA (Sigma-Aldrich, St. Louis, MO) (7.5 mg/kg body weight)[16-19] and then were sacrificed at various ages. All mice were subjected to autopsy, and tissues were partly fixed in 10% formalin and partly frozen in liquid nitrogen. Mice and livers were weighed. The anti-miRNA oligonucleotide (AMO) against miR-221 was: 5'-mG*mA*mA mAmCmC mCmAmG mCmAmG mAmCmA mAmUmG mU*mA*mG* mC*mU-3' (where "m" represents 2'-O-methyl RNA bases and asterisk [*] represents phosphothioate bond) and was obtained from Integrated DNA Technologies (Leuven, Belgium). For *in vivo* evaluation of miR-221 targeting, mice received a single intravenous (IV) dose of 300 μg (10 mg/kg) of anti-miR-221 diluted in saline solution. All animals were sacrificed after 48 hours. Blood and livers were analyzed as described above. For assessing antitumor activity of *in vivo* anti-miR treatments, 10-day newborn mice received one IP injection of DENA (7.5 mg/kg body weight), and after 2 months, each mouse received a single IV dose of anti-miR-221 (10 mg/kg) diluted in saline solution every 15 days for a total of three injections (approximately 1 mg total for each mouse). Mice were sacrificed at 4 and 5 months of age.

Other reagents and methods are described in the Supporting Materials.

Results

Development of a TG Mouse Model Carrying a Liver-Deregulated miR-221. A miR-221 expression vector, based on the pWhere as vector backbone (Invitrogen, Carlsbad, CA), was developed. The pWhere vector is characterized by the presence of two murine H19 insulators, which protect the integrated transcriptional unit from negative, as well as positive, influences from adjacent sequences.

To specifically drive a liver-specific expression, the pWhere vector was modified by inserting a regulatory element that consisted of the liver-specific alpha1-antitrypsin (α1-AT) promoter, coupled with the enhancer II (EII) sequence of human hepatitis B virus (HBV). This chimeric DNA element was previously shown to act as a potent, steady promoter and was able to ensure a constant, high level of gene expression in the liver.[20] The tissue specificity of this EII/α1-AT chimeric promoter, cloned upstream of a luciferase reporter gene into a pGL3 plasmid, was tested in different types of hepatic and nonhepatic cell lines, which confirmed that the highest level of luciferase expression was detectable in hepatocytes, thereby confirming the liver specificity of the promoter (data not shown).

A DNA segment, which included the mmu-mir-221 locus, was amplified from mouse genomic DNA and was cloned into the pWhere/EII/α1-AT vector downstream of the EII/α1-AT promoter (Fig. 1A). Expression of miR-221 from this vector was proven to be functional in a liver-cancer–derived cell line (Supporting Fig. 1).

To generate a line of TG mice, the pWhere/EII/α1-AT/miR221 plasmid was linearized using the *Pac*I

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Additional Supporting Information may be found in the online version of this article.

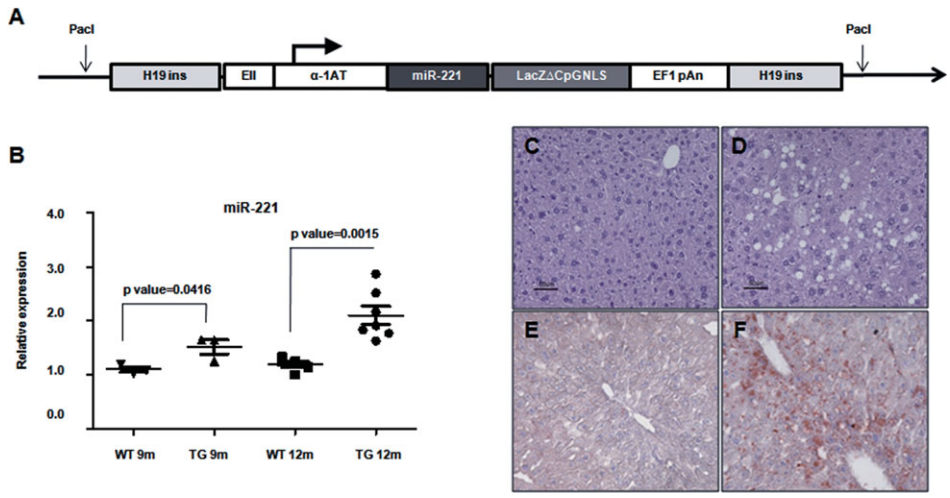

Fig. 1. *In vivo* expression of the vector, pWhere/EII/α1-AT/miR221, and liver histology of transgenic miR-221. (A) Schematic map of the vector that shows the two H19 insulators (H19 ins); the hybrid α1-AT promoter coupled with the EII sequence of human HBV (EII/α1-AT); the mmu-mir-221 miRNA locus (miR-221); gene reporter β-galactosidase without CpG dinucleotides (LacZΔCpG NLS); and the polyadenylation site (EF1 pAn). (B) Expression of miR-221 in the livers of TG and WT mice at the age of 9 or 12 months. Significance of the differences is shown as *P* values. Liver histology of WT (C) and TG mice (D) is shown by hematoxylin and eosin staining. TG livers were characterized by variable extents of steatohepatitic changes, with hepatocyte degeneration characterized by lipidic vacuoli. Oil Red staining for lipid and fat was performed on frozen sections of WT (E) and TG livers (F). Red dots are lipids, whereas nuclei appears in pale blue.

restriction enzyme. The purified 9-kilobase fragment containing the transgene was used to microinject fertilized oocytes of a B6D2F2 mouse strain to complete their development. After several crosses, a homozygous line of TG mice overexpressing the miR-221 in the liver was produced and used in all subsequent experiments.

Characterization of the Livers of miR-221 TG Mice. To assess miR-221 expression levels in the TG model, livers taken from homozygous mice at different ages were analyzed by real-time polymerase chain reaction (PCR). In comparison with wild-type (WT) mice, the analysis revealed a stable, increased expression of miR-221 in the livers of TG animals, thereby confirming the development of homozygous TG mice overexpressing miR-221 in hepatic cells (Fig. 1B).

Macroscopically, livers of TG mice exhibited an increase in volume and weigth in comparison with controls (Supporting Fig. 2). Histologically, though both groups displayed a conserved liver architecture, TG livers were characterized by variable extents of steatohepatitic changes, with hepatocyte degeneration characterized by enlarged cells with large dysplastic nuclei, lipidic vacuole, and focal coagulative necrosis (Fig. 1C-F). These changes were more evident in older TG animals and were absent among WT controls.

To assess whether miR-221 up-regulation could affect the expression of its targets, we performed an immunoblotting analysis to verify the expression of the miR-221 target proteins, Cdkn1b/p27, Cdkn1c/p57, and Bmf.[2,14] In non-neoplastic liver tissue, we confirmed that Bmf and Cdkn1b/p27 were both significantly down-regulated in TG mice. Cdkn1c/p57 was also generally down-regulated, although it did not reach statistical significance (Fig. 2). Immunostaining for Cdkn1b/p27 confirmed the strong reduction of the protein in TG animals (Supporting Fig. 3).

In addition to the above characteristics, gene-expression profiling proved that the livers of TGs differed from WT also at a deeper molecular level (Supporting Fig. 4; Supporting Table 1). Interaction analysis revealed that many of the identified protein-coding genes were connected to the modulation of the interferon-gamma (IFN-γ) pathway (Supporting Fig. 5).

miR-221 Promotes Liver Tumorigenesis. Because it is well established that miR-221 is up-regulated in human cancer, we analyzed whether the miR-221 TG mouse model was predisposed to the development of liver cancer. By monitoring mice at different ages (3, 6, 9, and 12 months), it emerged that a fraction of males developed spontaneous tumors that became visible not earlier than 9 months of age. Four of eight observed male mice (50%), at least 9 months old (range, 9-12) showed evidence of small, but visible, liver tumors. These tumors were characterized by a further up-regulation of miR-221 (Supporting Fig. 6). Females did not develop spontaneous tumors.

TG mice also exhibited an increased susceptibility to treatment with the carcinogen, DENA. TG as well as WT mice were injected IP with 7.5 mg/kg of DENA at 10 days of age. Animals were daily monitored and periodically sacrificed at various ages. An increasing

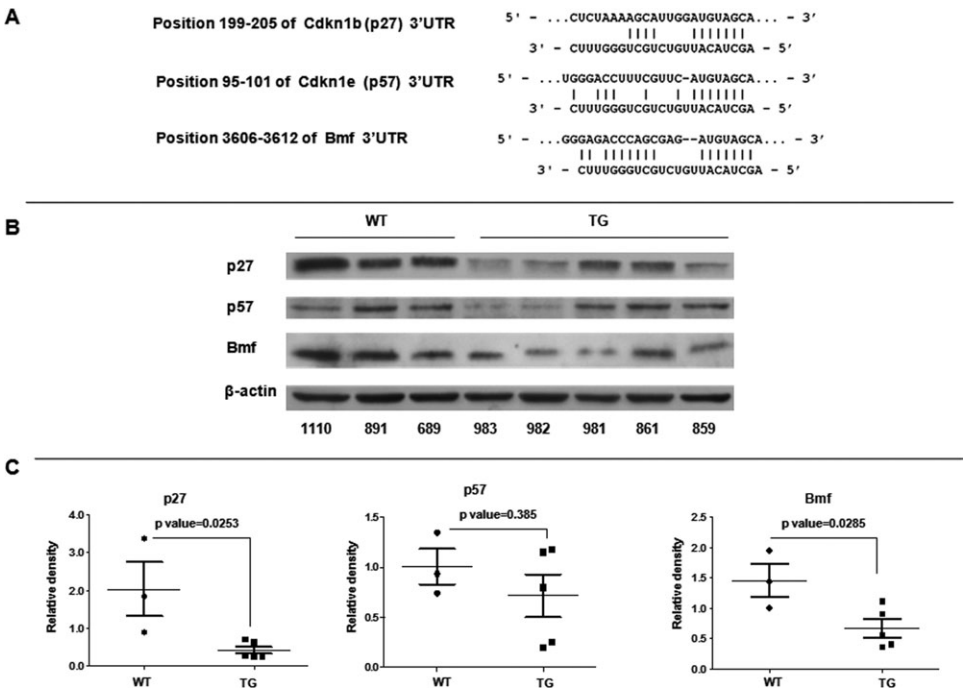

Fig. 2. miR-221 gene targets exhibit a reduced expression in livers of TG mice. (A) miR-221 "seed" matches with 3'-UTR sequences present in mouse Cdkn1b/p27, Cdkn1c/p57, and Bmf genes. (B) Western blotting analyses of p27, p57, and Bmf target proteins and β-actin in healthy livers of TG (TG NL) and WT (WT NL) mice. (C) Protein expression was quantified and normalized versus levels of β-actin. *P* values were calculated using a two-tailed Student's *t* test. UTR, untranslated region.

development of tumors was observed at the different time points in all mice, which was stronger in TG animals than in WT controls (Supporting Fig. 7). At 6 months, all male animals treated with DENA showed evidence of multiple large tumors. TGs exhibited a larger number of foci, which were also larger in size than in WT control mice. Tumor burden caused a significant increase in liver weight. Possibly because of the presence of destructive liver tumors, TG mice exhibited a more significant decrease in body weight than controls (Fig. 3; Supporting Table 2). In females treated with DENA, liver tumors were not visible at 6 months. However, starting at 9 months of age, tumors began to become visible in TG, but not in WT, control females (Supporting Figs. 8 and 9).

In both miR-221 TG mice and controls, multifocal liver nodules were detectable. Their size varied in diameter from 1 mm to 1 cm. Small nodules displayed the histopathological features of liver adenomas or HCCs, whereas large nodules were HCC with either a pseudoglandular or, more often, a trabecular pattern of growth, with some clearly anaplastic HCCs (Supporting Fig. 10A). At 6 months of age, in DENA-treated TG males, tumors almost completely substituted the entire liver by confluent neoplastic nodules, which were characterized by an infiltrative invasive front with no demarcation from the surrounding liver parenchyma, presence of necrotic areas, marked angiogenesis with slit-like sinusoids lined by endothelium, and intravasation of tumor cells (Supporting Fig. 10). Conversely, DENA-treated control mice displayed tumor nodules smaller in size and lower in number, characterized by a better defined tumor margin, even though a fibrous capsule was absent, together with less-evident angiogenesis (Supporting Fig. 10). All tumors were composed almost entirely of basophilic cells that were more evident in zones of trabeculation of large tumors. They were irregularly branched and were composed of cells with a basophilic cytoplasm and central oval nucleus with small nucleoli. Mitoses were rare in adenomas, whereas they were more evident in HCCs.

At the molecular level, tumors were characterized by a further increase in miR-221 expression (Fig. 4). Other miRNAs typically deregulated in human HCC were analyzed: miR-21 was up-regulated, whereas miR-122 and miR-199 were down-regulated, which are results that mimic the human HCC condition. The further increase in miR-221 expression was likely responsible for the strong inhibition detected on its targets, Cdkn1b/p27, Cdkn1c/p57, and Bmf (Fig. 5 and Supporting Fig. 11).

Anti-miR-221 Can Control* In Vivo *Tumorigenicity. Previous studies in mice and primates had shown that AMOs were able to silence miRNAs *in vivo*.[21,22] To support the idea that the up-regulation of miR-221 was important for promoting and maintaining liver tumors as well as investigating the potential antitumor activity of anti-miR-221, we sought to inhibit the endogenous miR-221 through *in vivo* delivery of anti-miR-221 AMOs.

To assess the effects on miR-221 levels, first, a group of 3 TG mice were IV injected through the tail vein with a single dose of an antisense 2'-*O*-methyl

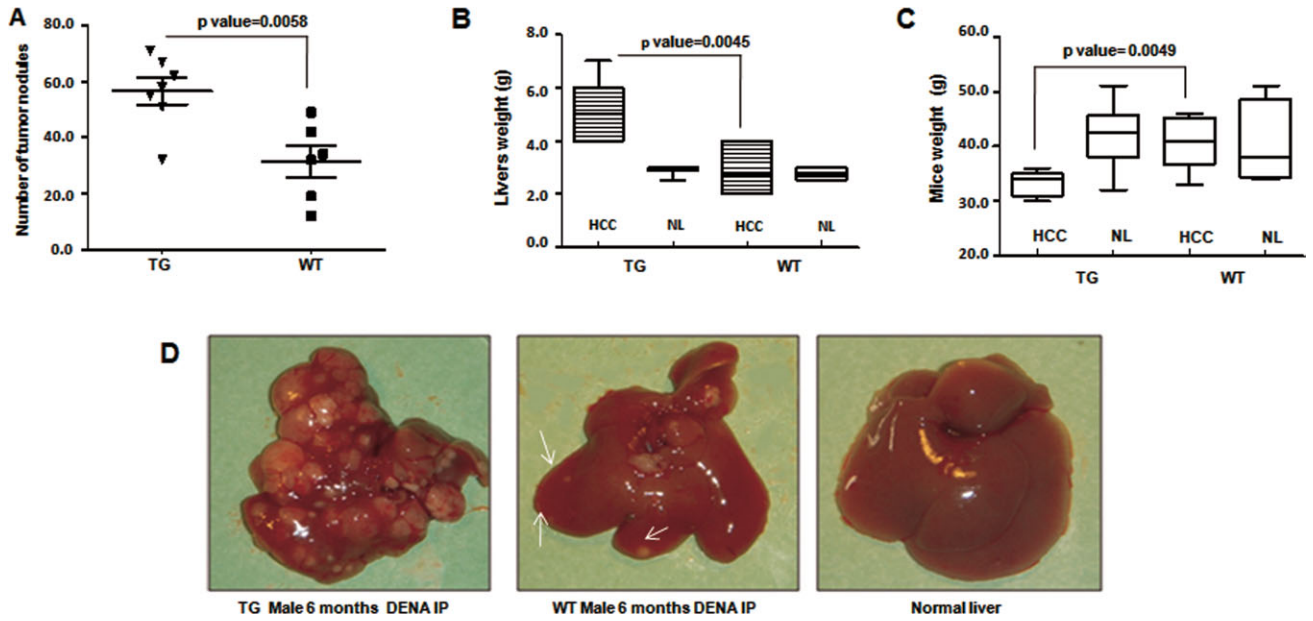

Fig. 3. miR-221 overexpression in mouse liver is correlated with increased cancer development. At 6 months of age, after DENA treatment, TG male mice exhibited an increased number and size of tumors than control WT mice. (A) Distribution of the number of nodules is shown in the table on the left. (B) Tumor burden, deduced by the weigth of livers, is significantly increased in TGs. (C) Conversely, body weight is significantly reduced in TG mice bearing tumors. (D) Examples of liver tumors in TG or WT males at 6 months of age.

oligoribonucleotide targeting miR-221 (10 mg/kg). Forty-eight hours after injection, molecular analysis revealed a significant down-regulation of miR-221 levels, both in liver and plasma of anti-miR-treated mice, in comparison to untreated controls, thus revealing a functional antisense inhibition of miR-221 *in vivo* (Fig. 6A; Supporting Table 3). These effects were also accompanied and supported by a concurrent increase in Cdkn1b/p27 protein expression in the liver (Fig. 6B,C).

Then, to assess the effect of anti-miR-221 oligonucleotides on liver tumorigenicity in this TG mouse model and establish whether miR-221 could represent an antitumor therapeutic target, a group of 5 mice were treated with anti-miR-221 AMOs (10 mg/kg at 60, 75, and 90 days) after IP injection with DENA (at 10 days). Three mice were sacrificed at 120 days and 2 at 150 days of age. Significantly, a reduction in number and size of tumors was observed in anti-miR-221-treated mice, in comparison with same-age (4 or 5 months) mice treated with DENA only (Fig. 7 and Supporting Fig. 12). These antitumor effects were accompanied by a persistent, significant decrease of miR-221 expression in tumors arising in the group of AMO-treated mice.

Discussion

miR-221 is one of the most commonly up-regulated miRNAs in human cancer, including HCC, and is considered an "oncogenic" miRNA, as reviewed recently.[1] To date, the only model aimed at proving its oncogenic role *in vivo* was based on the use of c-myc-immortalized $P53^{-/-}$ liver progenitor cells implanted into irradiated nude mice. The introduction of miR-221 into this model promoted tumor progression *in vivo* and shortened animal survival.[13] Because the reproduction of this model is technically challenging and difficult to compare with human HCC, we addressed the issue of proving the *in vivo* tumor-promoting activity of miR-221 by the generation of a TG mouse model that presents a stable increase of miR-221 in the liver. By using this model, we were able to provide a formal demonstration of miR-221 *in vivo* tumor-promoting capability.

miR-221 TG animals exhibited a strong predisposition to the development of liver tumors. They spontaneously developed visible neoplastic lesions starting at 9 months of age, which were undetectable in WT mice. If treated with DENA, TGs developed a significantly higher number and larger tumor lesions that became evident much earlier than in WT animals treated with the same carcinogen.

Histologically, tumors of TG mice ranged from liver adenomas to typical HCCs characterized by an invasive trabecular growth and a high level of angiogenesis. In comparison, nodules in WT DENA-treated control mice displayed a less-pronounced angiogenesis and a

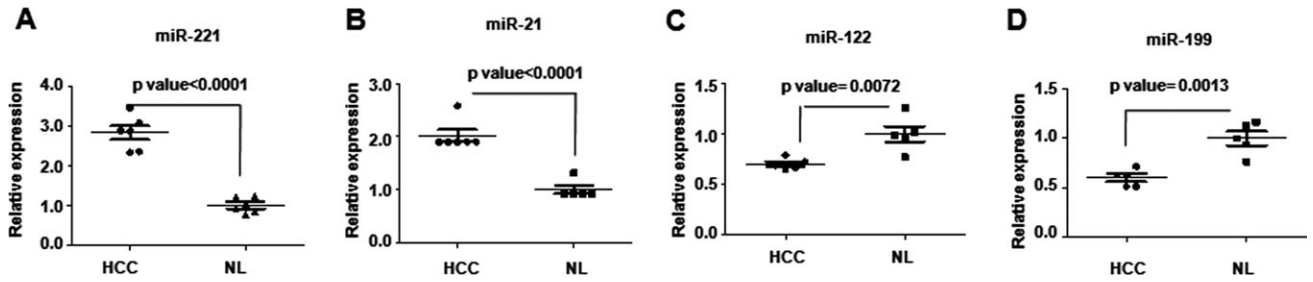

Fig. 4. miRNA expression in liver nodules versus healthy liver of TG mice. (A-D) MicroRNA expression analysis in liver cancer versus healthy livers revealed a statistically significant difference of expression levels: Similarly to human HCC, miR-221 and miR-21 exhibited an increased expression, whereas miR-122 and miR-199 exhibited a down-regulation.

better defined tumor margin, even if no capsule was identifiable.

These tumors did not arise on a cirrhotic background, which is typical of most human HCCs. However, the livers of TG mice exhibited high levels of steatosis, a condition that in humans is frequently observed in the context of metabolic dysfunctions that predispose to HCC.[23,24] Interestingly, gene-expression profiling of non-neoplastic livers of TG versus WT mice provided evidence that a different molecular background driven by the aberrantly expressed miR-221 existed and was likely responsible for the differences in liver phenotypes, including the predisposition to liver cancer. Many of the identified protein-coding genes were connected to the modulation of IFN-γ, which was itself expressed at lower levels in the livers of TG mice. Interestingly, a role of defective IFN-γ response was previously shown to be connected to HCC. Indeed, IFN-γ, through its action on hepatocytes or immune cells, could elicit tumor-suppressive effects by both inhibiting cell-cycle progression and by initiating apoptosis in models of HCC.[25-27]

Similar to human or other mouse models, the predisposition was stronger in males, a result that indicates a protective effect of estrogens and a stimulating effect of androgen hormones in the development of HCC, as previously shown.[28]

At the molecular level, these tumors revealed a further increase of miR-221, which was accompanied by a strong repression of the cell-cycle inhibitors, Cdkn1b/p27 and Cdkn1c/p57, and the proapoptotic Bmf proteins. In addition to miR-221, other miRNAs known to play a key role in human HCC were found to be dysregulated in the tumors arising in this model. Among them, the down-regulated miR-122 and miR-199 or the up-regulated miR-21 were dysregulated in the same direction observed in human HCC. Overall, these findings suggest that this TG mouse overexpressing miR-221 represents a useful *in vivo* liver cancer model for better understanding HCC and testing new anticancer approaches.

To confirm the role of the tumor-driving force of miR-221, we sought to inhibit its activity using an AMO. It was previously established that silencing miRNA activity *in vivo* using synthetic oligoribonucleotides is feasible. Indeed, miR-122 inhibition by AMO administration in mice and primates was shown as a promising approach to reduce miRNA activity in the adult liver.[21,22] In addition, evidences for anti-miR-221 as a potential anticancer molecule were provided through the use of intratumor injections of AMOs targeting miR-221 in PC-3-derived tumors and in melanoma cell xenotransplants.[29,30] Here, we proved that the use of AMO anti-miR-221 could be effectively delivered to the liver, block miR-221, and induce a

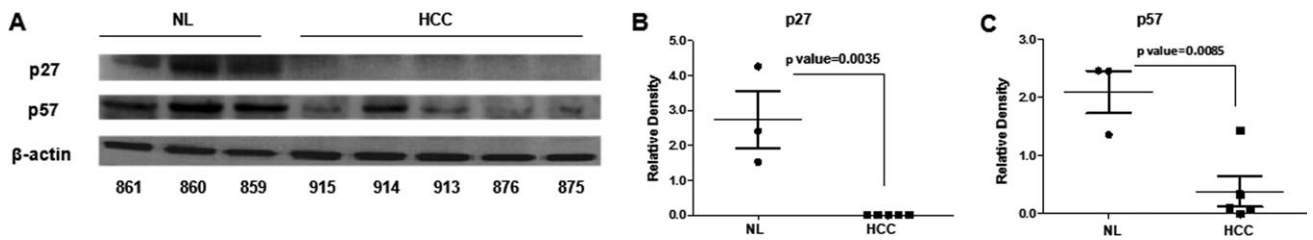

Fig. 5. Cdkn1b/p27 and Cdkn1c/p57 proteins are repressed in liver nodules versus healthy liver of TG mice. (A) Protein levels in tumor and healthy tissues of TG mice were evaluated by western blotting analysis, revealing a strong reduction of miR-221 target genes in tumor tissues. (B and C) Protein expression data were normalized versus levels of β-actin.

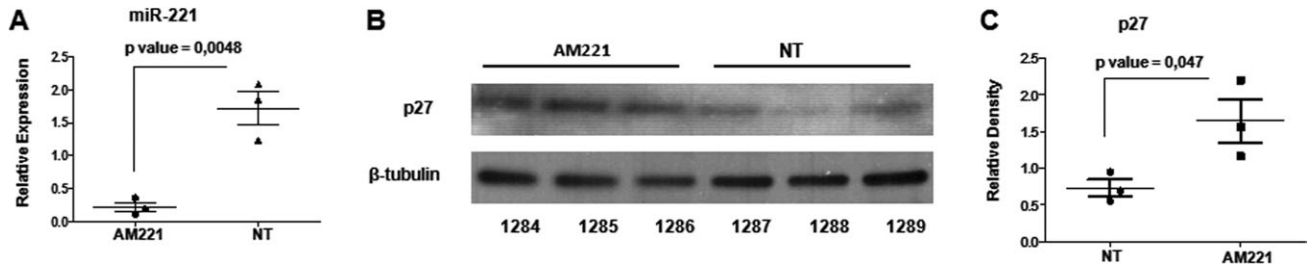

Fig. 6. Expression of miR-221 and Cdkn1b/p27 in livers after *in vivo* delivery of anti-miR-221 oligonucleotides. A group of TG mice received one IV injection of an anti-miR-221 synthetic oligonucleotide into their tail vein. (A) Forty-eight hours after injection, liver and blood of mice were harvested to measure miR-221 expression levels. Quantitative PCR analysis revealed a significant decrease in miR-221 amounts in livers of treated mice, in comparison to untreated ones. Supporting Table 3 indicates a similar reduction in serum. (B and C) Reduced levels of miR-221 correlated with an increase in Cdkn1b/p27 target protein levels. Protein expression data were normalized versus levels of β-tubulin.

significant inhibition of tumor growth. Indeed, the IV injection of synthetic 2'-*O*-methyl modified oligonucleotides targeting miR-221 in TG mice proved the ability of these molecules to specifically silence miRNA expression in the liver, as well as in the circulatory system. Furthermore, in DENA-treated TG mice, systemic administration of AMOs led to a significant containment of liver tumor growth, in comparison to

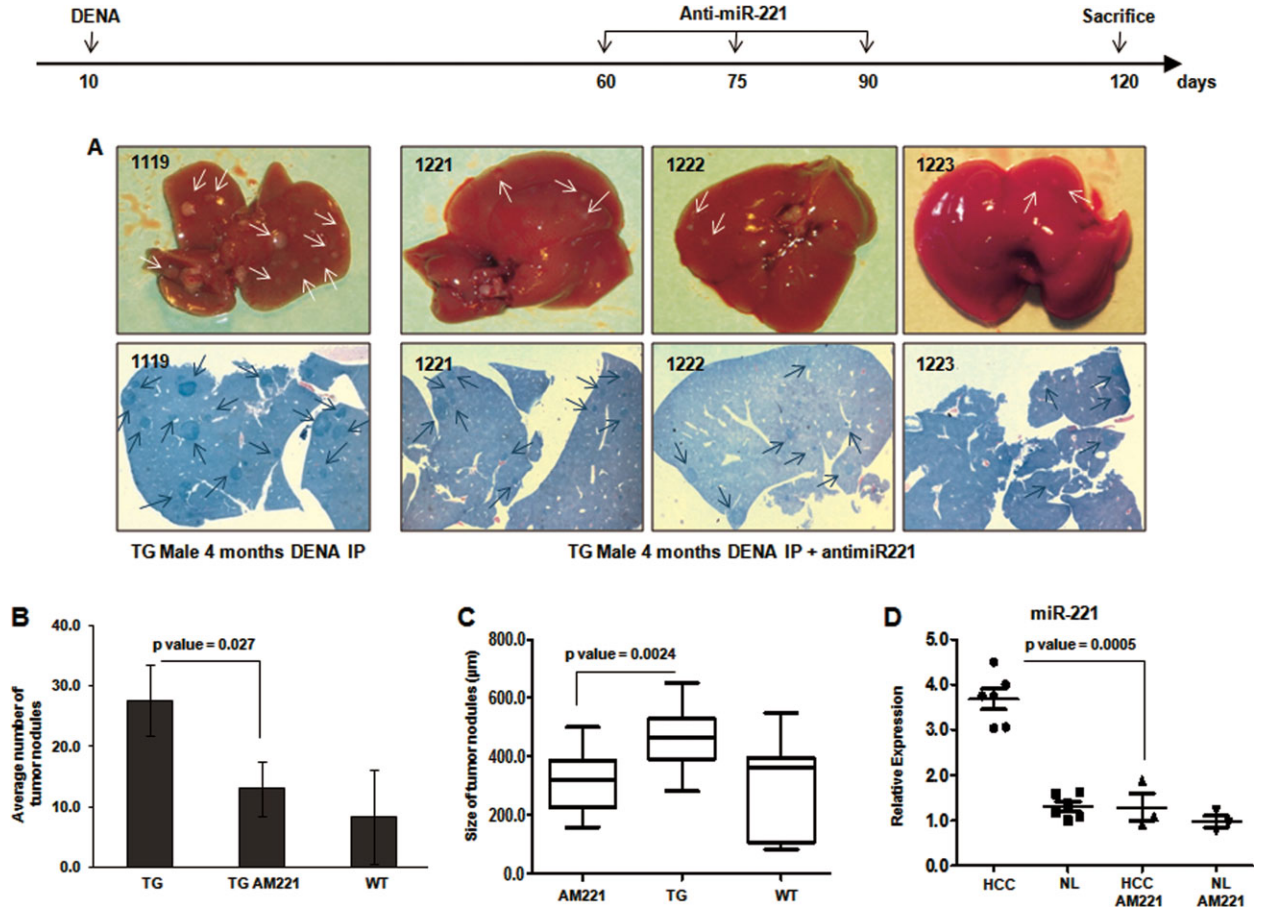

Fig. 7. *In vivo* delivery of AMOs limits tumor growth. A group of TG mice were IP injected with DENA at 10 days of age, and after 2 months, they received three consecutive IV injections every 15 days of an anti-miR-221 synthetic oligonucleotide into their tail vein. (A) At 4 months (120 days of age), anti-miR-221 AMOs-treated and untreated mice were sacrificed. Even macroscopically, the number and size of tumors appeared to be smaller in treated mice. Hematoxylin and eosin–stained histological sections confirmed the results (magnification, x20; visible nodules are indicated by arrows). (B) Liver lesions of AMO-treated mice were significantly fewer than in control animals, and (C) tumor size of nodules was generally smaller. At 5 months, the same pattern was observed (see Supporting Fig. 12). (D) A significant long-term reduction of miR-221 expression was detected in tumors treated with AMOs, in comparison to untreated tumors.

control animals. This finding has two important corollaries: First, it confirms that miR-221 is indeed a tumor driver for liver cancer, and, second, it demonstrates that miR-221 can be effectively targeted to reduce tumor growth. Significantly, this effect was achieved without appreciable toxicity. For HCC, this quality appears to be particularly important. In fact, HCC conveys a very poor prognosis not only because a small fraction of tumors can be curatively treated, but also because systemic chemotherapy in advanced HCC proved to be only marginally effective or too toxic. In addition to AMOs, the use of miRNA-replacement approaches was also reported to be effective as an anticancer approach in animal models: miR-26a transduced by an adeno-associated virus induced a significant reduction of tumors in a *myc* mouse model of HCC[31]; miR-101 was shown to inhibit tumor cells growth in a nude mouse xenograft model[32]; and miR-31 action could alter the invasivity of disseminated tumor cells in an orthotopical cancer metastatic model.[33] Hence, these studies indicate that the use of miRNAs or anti-miRNAs are promising approaches in cancer therapy and, possibly, other noncancer diseases.

The present miR-221 TG animal model represents an important tool not only for investigating liver cancer pathogenesis, but also for testing new miRNA or anti-miRNA therapeutic approaches.

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