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ABSTRACT

Objective: Hepatocellular carcinoma (HCC) is the most prevalent primary tumor of the liver. About a third of these tumors presents activating mutations of the β-catenin gene. The molecular pathogenesis of HCC has been elucidated, but mortality remains high and new therapeutic approaches, including treatments based on microRNAs, are required. We aimed to identify candidate microRNAs, regulated by β-catenin, potentially involved in liver tumorigenesis. **Design:** We used a mouse model, in which β-catenin signaling was overactivated exclusively in the liver, by the tamoxifen-inducible and Cre-Lox-mediated inactivation of the *Apc* gene. This model develops tumors with properties similar to human HCC. **Results:** We found that miR-34a was regulated by β-catenin, and significantly induced by the overactivation of β-catenin signaling in mouse tumors and in HCC patients. An inhibitor of miR-34a (LNA-34a) exerted anti-proliferative activity in primary cultures of hepatocyte. This inhibition of proliferation was associated with a decrease in cyclin D1 levels, orchestrated principally by HNF-4α, a target of miR-34a considered to act as a tumor suppressor in the liver. *In vivo*, LNA-34a approximately halved progression rates for tumors displaying β-catenin activation together with an activation of caspases 2 and 3.**Conclusion:** This work demonstratesthe key oncogenic role of miR-34a in liver tumors with β-catenin gene mutations. We suggest that patients diagnosed with HCC with β-catenin mutations could be treated with an inhibitor of miR-34a. The potential value of this strategy lies in the modulation of the tumor suppressor HNF-4α, which targets cyclin D1, and the induction of a pro-apoptotic program.

SUMMARY BOX

What is already known on this subject ?

Hepatocellular carcinoma (HCC) is the most common form of primary liver cancer and the third leading cause of death by cancer worldwide, due to the lack of effective treatments. We, and others, have shown that mutations of the *CTNNB1* gene, encoding β-catenin, are observed in 15 to 40% of HCCs. HCCs with β-catenin mutation constitute one of the two subclasses of HCC, and are thought to follow a particular pattern of pathogenesis.

What are the new findings?

Using a transgenic mouse model developing tumors with β-catenin activation, we provide the first evidence of an oncogenic role of miR-34a in liver tumors with β-catenin mutations. An inhibitor against miR-34a slows tumor progression, and inhibits the development of new tumors in this model.

How might it impact on clinical practice in the foreseeable future?

These findings call into question the preclinical trial designed by miRNAtherapeutics, using miR-34a mimics to combat liver tumor growth. Instead, we provide here proof-of-concept that a locked nucleic acid-based inhibitor of miR-34a may be useful for treatment, exclusively in HCCs with β-catenin mutation.

INTRODUCTION

Liver cancer is the third leading cause of cancer-related death worldwide. Hepatocellular carcinoma (HCC) is the most frequent form of primary liver cancer, which mostly affects men with cirrhosis. HCC frequently appears in a context of chronic liver disease caused by infection with the hepatitis B and C viruses (HBV and HCV, respectively), alcohol consumption, obesity or genotoxic exposure ([1] for a review). We, and others, have shown that mutations of the *CTNNB1* gene, encoding β-catenin, are observed in 15 to 40% of HCCs [2, 3]. HCCs with β-catenin mutation constitute one of the two subclasses of HCC [4, 5], and are thought to follow a particular pattern of pathogenesis, as most are well differentiated, cholestatic and with a better prognosis [6]. Several years ago, we engineered a mouse model in which β-catenin signaling was overactivated exclusively in the liver, by the tamoxifen-inducible and Cre-Lox-mediated inactivation of the *Apc* (Apc^{KO}) gene, encoding a tumor suppressor, which is an inhibitor of the Wnt/β-catenin pathway, andthe liver "zonation-keeper"[7, 8]. In this model, the absence of *Apc* in single hepatocytes results in the development of β-catenin-activated liver tumors with properties similar to those observed in human patients [8]. If*Apc*is deleted in all hepatocytes, the β-catenin signaling in hepatocytes leads to the induction of a transcriptional program very similar to that of human HCC with β-catenin mutation[8, 9].

The molecular pathogenesis of HCC has been elucidated [10], but the incidence and mortality of HCC remain high, despite the use of the tyrosine kinase inhibitor sorafenib. Different therapeutic strategies could therefore be used to target tumors in accordance with theirpathogenic pathways. MicroRNAs (miRNAs) have recently emerged as potent diagnostic and prognostic biomarkers, and numbers of

reportsdevoted to miRNAs have shown that these molecules act as oncogenes or tumor suppressors[11]. The global analysis of the miRNAs expressed in liver tumors highlighted a miRNA signature common to all types of HCC [12-14]. A loss of miR-122 [13], miR-29 [15], and miR-124 [16] has been described. Inversely, some oncogenic miRNAs have been identified: miR-21 [17], miR-221/miR-222 [18], and miR-224 [19]. Despite the number of global analyses, the role of miR-34a in liver tumorigenesis remains unclear. Even if this miRNA is currently described as a tumor suppressor in various cancers [20], two studies suggested that miR-34a levels decrease in HCCs, mainly HBV+[21, 22], while other studies reported an induction of miR-34a, particularly in HCC with β-cateninmutations [17, 18].

We aimed to identify candidate microRNAs, regulated by β-catenin, potentially involved in liver tumorigenesis. We observed that miR-34a was overexpressed in response to β-catenin overactivation; the levels of miR-34a being inversely correlated with those of HNF-4α (hepatocyte nuclear receptor α), another key factor for hepatocyte differentiation, which we have shown to antagonize β-catenin function[9]. MiR-34a induction was observed specifically in mouse and human liver tumors with β-catenin mutations, and miR-34a was found to play an oncogenic role in the liver. These findings call into question the systematic use of miR-34a mimics to combat liver tumor growth (http://www.mirnarx.com/pipeline/mirna-MRX34.html). Instead, we provide here proof-of-concept that a locked nucleic acid (LNA)-based inhibitor of miR-34a may be useful for treatment, exclusively in HCCs with β-catenin mutation.

METHODS

Animals and reagents

Transgenic mice, liver sampling and immunostaining have been described elsewhere[7, 9, 23], and detailed in the supporting materials.

Hepatocyte isolation

To directly lyse hepatocytes after collagenase perfusion, i.e. for RNA or protein extraction, the transgenic livers were perfused six days after tamoxifen injection (1.5 mg)as previously described[9, 23]. Non-parenchymal cells were excluded in the supernatant, and hepatocytes preparations were used for subsequent experiments. Periportal and pericentral subpopulations of wt hepatocytes were isolated by perfusion with digitonin and collagenase, as previously reported[24].

Cell culture

For primary culture of hepatocytes, livers were perfused three days after tamoxifen injection to $Apc^{lox/lox}$ mice, the cells were dispersed in William's medium (supplemented with 10% fetal bovine serum, penicillin-streptomycin, fungizone, 25nM dexamethasone, insulin 4 µg/mL and 1% BSA), and plated in wells coated with rat-tail collagen I (GIBCO).

Transfection assays

The cells were transfected 4h after plating, as described in the supporting materials.

xCELLigence assay

Hepatocyte adhesion and cell cycle progression were measured with the xCELLigence system (Roche). Cells were transfected with 100 nMLNA or mimic 4 h after plating, as described above (see supporting Materials). The impedance was then measured for 48 h.

Western-Blotting

The membrane with 50µg proteins was probed overnight with a primary antibody against the protein of interest (see Supporting Materials), and then incubated with a HRP-conjugated secondary antibody (Cell Signaling) for detection with the ECL system (Biorad).

***In situ* hybridization**

Sections were deparaffinized and treated with 0.1 mg/ml proteinase K, then hybridized for one hour at 55°C with 100 nmol digoxigenin-labeled-LNA-scramble or LNA-34a (Exiqon). Signals were detected with anti-digoxigenin (Roche) and NBT/BCIP (Roche).

Samples processing for miRNA-seq:

Total RNA was extracted from purified hepatocytes six days after tamoxifen injection with Trizol® reagent (Life Technologies). For each set of conditions, we analyzed four distinct hepatocyte preparations by deep sequencing on an Illumina platform (Fasteris, Switzerland). The reads generated by the HiSeq were analyzed with miRDeep2 and miRAnalyzer software (See Supporting Materials).

Chromatin sonication and immunoprecipitation

The ChIP protocol was adapted from that of Nelson [25], as previously described [9](See Supporting Materials).

Human samples

We included 44 patients treated for liver cancer at Cochin hospital in this study (Table S3). All tumor samples were frozen after surgery.

RNA extraction and RT-qPCR

Total RNA was extracted from purified hepatocytes with Trizol® reagent (Life Technologies). Levels of miRNAwere determinedon 2 ng total mRNA, with a specific Taqman miRNA assay (Applied Biosystems). Levelof cyclin D1 and HNF-4α was determined on 100 ng total mRNA relative to 18S RNA (see Supporting Materials).

Statistical analysis

We assessed the significance of differences between two groups of samples in Wilcoxon tests if n<30 and Student tests if n>30. ANOVA was used to compare three groups of samples. $P<0.05$ was considered statistically significant.

RESULTS

MiR-34a is induced following β-catenin activation

We searched for candidate microRNAs regulated by β-catenin in hepatocytes, by deep-sequencing the miRNAs expressed in the pre-tumoral *Apc* deletion (Apc^{KO}) model, in which there is pan-lobular β-catenin overactivation, and comparing the results with those for wild-type (wt) hepatocytes. A statistical analysis showed that

123 miRNAs discriminated between Apc^{KO} and wt hepatocytes (Tables S1 and S2), and that these miRNAs mostly targeted the Wnt/β-catenin pathway (Figure S1). We confirmed that miR-375 levels were significantly decreased by β-catenin activation (Table S2), as reported in HCCs with β-catenin mutation [13]. We also identified 54 miRNAs generated from the imprinted Dlk1/Gtl2 locus as positive targets of β-catenin (Table S1). We discarded this locus for further studies, as this is not a relevant target of β-catenin in human HCCs (see the supplemental materials, Tables S1, S3 and Figure S2). We thus focused on miR-34a, which was induced by a factor of about three after β-catenin activation, with respect to wild-type hepatocytes, in the miRNA-seq analysis (Table S1), and in qPCR (Figure 1A). This miRNA displayed the most significant induction following β-catenin activation after the miRNAs from the Dlk1/Gtl2 locus, and its overexpression in HCC with β-catenin mutation has already been reported [18]. We collected 24 human tumors with a mutation of *CTNNB1* leading to β-catenin activation, and 20 tumors without *CTNNB1* mutation. This cohort covered the major etiologies observed for HCC (HBV, HCV, alcohol abuse) (Table S3). In our cohort of patients, miR-34a levels were higher in tumors than in normal tissue (by a factor of 2 in the absence of *CTNNB1* mutation, and 5 in presence of *CTNNB1* mutation, Figure 1B); this induction was significantly stronger in tumors with β-catenin mutations ($p<0.005$). The overexpression of miR-34a in tumors with mutations of β-catenin was correlated with a context of HCV infection or no particular risk factors (Figure 1C). The sample size was too small to obtain a significant difference in miR-34a overexpression as a function of alcohol abuse status (Figure 1C). The overexpression of miR-34a was confirmed by *in situ* hybridization in another panel of tumors with β-catenin mutation (Figure 1D). Thus, miR-34a is overexpressed

in human HCCs with *CTNNB1* mutations, and could constitute a major mediator of the oncogenic signal initiated by β-catenin activation in hepatocytes.

β-catenin directly regulates miR-34a expression

For confirmation of miR-34a regulation by β-catenin, we transfected isolated wt and Apc^{KO} hepatocytes with a siRNA against β-catenin. β-catenin silencing decreased miR-34a levels by about 47% for wt hepatocytes and 33% for Apc^{KO} hepatocytes (Figure 2A). We thus investigated whether β-catenin could directly control miR-34a expression, by ChIP experiments with an antibody against TCF-4 (T-cell factor 4), the main LEF/TCF factor associated with β-catenin in liver[9], and an antibody against β-catenin. We measured the binding of TCF-4 and β-catenin tosites upstream from miR-34a identified as a TCF-4 binding site in our ChIP-seq data[9]. β-catenin activation led to a significant increase in the binding of TCF-4 (Figure 2B) and β-catenin (Figure 2C) to a site 21kb upstream from miR-34a. β-catenin binding was also significantly increased following *Apc* depletion on a more proximal TCF-4 site (Figure S3). Overall, our data suggest that an excess of β-catenin could significantly increase miR-34a transcription in hepatocytes.

MiR-34a zonal redistribution after β-catenin overactivation

We then assessed the hepatic distribution of miR-34a by *in situ* hybridization. Exceptions, such as cyclin D1 [23], are known, but the direct targets of β-catenin are generally expressed in pericentral (PC) hepatocytes, in which Wnt/β-catenin signaling is activated [7]. Counterintuitively, in normal livers, miR-34a was found to be preferentially expressed in hepatocytes around the portal vein and, more particularly, in bile duct cells (Figure 3A). For confirmation of this prevalence of miR-

34a in the periportal (PP)subpopulation, we isolated PP hepatocytes and PC hepatocytes separately, by injecting digitonin into the central vein and the portal vein, respectively. This differential perfusion also showed that PP samples were enriched in miR-34a (Figure 3B). The results were non-significant, since using this technique, biliary cells are mainly lost during washing steps. In addition, β-catenin overactivation led to a loss of miR-34a zonation, and to miR-34a expression of in all hepatocytes (Figure 3A). In conclusion, miR-34a appears to be expressed in hepatocytes throughout the liver only when β-catenin is overactivated.

We then searched the DIANA database (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=microT_CDS/index), a database with experimentally validated targets for miRNAs, for potential targets of miR-34a. Number of potent targets have been identified (Table S4), but we focused on three interesting proteins: LEF-1, a transcription factor associated with β-catenin, caspase 2, recently identified as a direct target of miR-34a in the liver [26], and HNF-4α, which we recently showed to be an antagonist of β-catenin in the liver[9]. These opposing activities result in a reciprocal physiological distribution in the liver, with miR-34a found in biliary cells (Figure 3A) and HNF-4α in hepatocytes, consistent with the hypothesis that miR-34a could repress HNF-4α expression in biliary cells [27].

MiR-34a inhibition improves HNF-4α transactivation capacity in hepatocytes displaying β-catenin-activation

We confirmed by western blotting that the levels of HNF-4α, caspase 2, and LEF-1 proteins (Figure 3C) were inversely correlated with miR-34a levels in Apc^{KO} mice. Similarly, the overexpression of miR-34a in human HCCs with *CTNNB1* mutations was also associated with weaker immunostaining for HNF-4α in these tumors (Figure

S4). In a previous study, we showed that β-catenin impaired the transcriptional activity of HNF-4α on a luciferase reporter gene under the control of an HNF-4α-response element (FOP)[9]. Having shown that β-catenin induces miR-34a, which could in turn regulate HNF-4α levels, we investigated the involvement of miR-34a in the antagonism between HNF-4α and β-catenin in Apc^{KO} hepatocytes. An inhibitor of miR-34a based on locked nucleic acid technology (LNA-34a), which consists of the complementary sequence for miR-34a with a methylene group in the ribose, significantly activated the FOP reporter (Figure 3D). A mimic of miR-34a had no effect, probably due to the maximal miR-34a overexpression in Apc^{KO} hepatocytes (Figure 3C). In conclusion, miR-34a represses HNF-4α transcriptional activity, and appears to be involved in the β-catenin/HNF-4α antagonism in our model of β-catenin overactivation.

MiR-34a is induced in tumors displayingβ-catenin activation

We then assessed the level of miR-34a in liver tumours mutated for β-catenin in mice. We compared 35 tumors with seven normal tissues obtained from wt mice that had received tamoxifen injections. MiR-34a levels in these tumors were twice those in normal tissues (Figure 4A). HNF-4α mRNA levels in these tumors were also half those in normal tissues (Figure 4B), confirming its tumor suppressor role in the liver[28, 29]. Interestingly, the tumors with the highest miR-34a levels also had the lowest HNF-4α levels (Figures 4C and 4D). The deregulation of miR-34a in these tumors was independent of p53 mRNA levels (Figure S5), despite miR-34a being a target of p53[30]. Finally, we confirmed that overexpression of miR-34a in tumors with β-catenin overactivation, by *in situ* hybridization, was associated to a loss of HNF-4α (Figure 4E).

MiR-34ainhibitionby a locked nucleic acid

These data highlight the unique oncogenic role of miR-34a in a context of β-catenin overactivation. This led us to try to block miR-34a activity with a LNA, which has proved promising in human liver disease [31]. We assessed the efficacy of this approach in cultures of hepatocytes isolated from wt and Apc^{KO} mice. No change in the morphology of hepatocytes transfected with LNAs was observed, on microscopy, 72 hours after transfection (data not shown). The efficacy of LNA-34a was assessed by cotransfection with a luciferase reporter construct with a miR-34a binding site in its 3'UTR. As expected, following cotransfection with LNA-34a, miR-34a activity was impaired, resulting in an increase in luciferase activity (about three times higher than that obtained after cotransfection with LNA-scramble, Figure 5A). The LNA efficacy was also confirmed by western blotting for HNF-4α: HNF-4α levels were higher in the presence of LNA-34a (Figure 5B).

LNA-34a impairshepatocyte proliferationafterβ-catenin overactivation

We then investigated the effect of LNA-34a on the proliferation of hepatocytes. We used the xCELLigence system, which measures cell impedance in real-time, through the use of gold microelectrodes placed under each well. WT hepatocytes underwent one round of cell division, due to the proproliferative effect of tamoxifen and collagenase perfusion (Figure S6A). WT hepatocytes had a lower cell proliferation index than Apc^{KO} hepatocytes (Figure S6A), confirming that an absence of *Apc* leads to hepatocyte hyperproliferation[8]. Transfection with LNA-34a significantly impaired Apc^{KO} hepatocyte proliferation (35% inhibition), but had no effect on wt hepatocytes (Figure 5C). Similar results were obtained with the Cell Titer assay, measuring the

reduction of tetrazolium in formazan, a process dependent on NADPH (data not shown). The activity of lactate deshydrogenase in cell supernatant was not altered following LNA-34a transfection, suggesting an absence of LNA-34a toxicity (Figure S6B). This suggests that LNA-34a has a specific antiproliferative effect on hepatocytes displaying β-catenin overactivation.In addition, the LNA-34a transfected three days successively increased the amount of caspase-2 propeptide, as well as caspase-2L, its proapototic isoform, and also decreasedthe level offull-length PARP-1, suggesting its increased cleavage (Figure 5D). This was not observed for wt hepatocytes (Figure S6C). In vivo, the LNA-34a injected to mice only increased caspase-2L level in Apc^{KO} mice (Figure S6D).In conclusion, LNA-34a promotes a pro-apoptotic signal in the Apc^{KO} hepatocytes.

Cyclin D1 has been identified as a target of miR-34a [32]. We therefore assessed cyclin D1 levels after transfection with LNA-34a. Contrary to expectations, cyclin D1 protein and mRNA levels, at a lower extent,were decreased in the presence of LNA-34a (Figure 5E). However, this is consistent with the antiproliferative effects of LNA-34a on Apc^{KO} hepatocytes (Figure 5C). The loss of cyclin D1 may be a consequence of the increase in HNF-4α amount in the presence of LNA-34a (Figure 5B), as HNF-4α has been shown to repress cyclin D1 [33] (Figure 6E). In agreement with this hypothesis, no change in cyclin D1 protein and mRNA levels was observed when Apc^{KO} hepatocytes also lacking *hnf-4α*, were transfected with LNA-34a (Figure 6A). LNA-34a had also a weaker antiproliferative effect on these hepatocytes isolated from a transgenic mouse model, in which both *Apc* and *hnf4a* had been deleted by Cre-Lox strategy(Figure 6B). The higher proliferation rate observed for hnf4/Apc^{KO} hepatocytes in the presence of LNA-34a was associated to a minor induction of caspase-2L level, even if caspase-2 propeptide was efficiently increased (Figure 6C);

PARP amount was also not altered in this case (Figure 6C). Finally, we realized ChIP experiments with an antibody against HNF-4α in Apc^{KO} hepatocytes,and analyzed the binding of HNF-4α in the presence of LNA-34a on two binding sites identified in published ChIP-seq experiments done in HepG2 cells and in murine livers (Figure S7). We showed that the binding of HNF-4α to cyclin D1 promoter tended to increasefollowing exposure to LNA-34a, in particular for peak 1 (Figure 6D). This finding corroborates that HNF-4α mediates the decrease in cyclin D1 levels in response to LNA-34a. In conclusion, following its overactivation, β-catenin binds to the miR-34a promoter, thereby increasing its expression. MiR-34a in turn decreases the amount of HNF-4α, leading to increases in cyclin D1 transcription, and hepatocyte proliferation (Figure 6E).

MiR-34a inhibition reduces tumor progression in Apc^{KO} mice

We thus developed a tumor suppressive strategy based on miR-34a inhibition, which could constitute the first targeted therapy for liver tumors displaying β-catenin activation. We assessed the impact of LNA-34a on liver tumor progression in an Apc^{KO} context. Once the tumors became detectable on ultrasound scans, we injected LNA-34a (10 mg/kg) once weekly, and followed tumor development twice monthly, by 2D-ultrasound (Figure S8). We found that LNA-34a slowed the progression of tumors displaying β-catenin activation, to rates about half those for untreated tumors (Figure 7A and S8). This treatment also inhibited the development of new tumors, with only one new tumor on average occurring on LNA-34a treatment, versus four on LNA-scramble treatment (Figure 7B). As expected, HNF-4α levels increased and cyclin D1 levels decreased in tumors treated with LNA-34a (Figure S9A). Even if cyclin D1 level was impaired, Ki-67 labeling was similar in tumors treated with LNA-34a, and in

those treated with the scramble LNA (Figure S9B). Similar results were obtained with western-blots for phospho-Histone H3 and cyclin A labeling (data not shown).However, LNA-34a promoted caspase 2 and 3 activation in tumors, in both western-blot and immunohistochemistry experiments, confirming its pro-apoptotic effect (Figures 7C and 7E), with minor effects on non-tumoral liver (Figure 7D).Thus, treatment based on the use of LNA-34a could reduce tumor development through an increase in HNF-4α levels, leading to a loss of cyclin D1, and through a pro-apoptotic program associated with the induction of caspase 2 and caspase 3 cleavage.

DISCUSSION

The microRNA miR-34a has been widely described as a tumor suppressor. Indeed, its expression is frequently impairedin various cancers following an hypermethylation of its promoter[20], including breast and prostate cancers[34, 35]. A preclinical trial is currently underway with a mimic of miR-34a encapsulated into liposomes in various murine models of solid tumors (miRNATherapeutics, http://www.mirnarx.com/pipeline/mirna-MRX34.html). However, the role of miR-34a in liver tumorigenesis remains unclear. One study suggested that miR-34a level decrease in HCC [22], andis associated to migration and invasion [21], but another two studies reported the induction of miR-34a in HCC samples [17, 18], in HCV+ sera[36], and in HCC cells in response to alcohol [26]. Our findings suggest that miR-34a is an oncogenic mediator in tumors displaying β-catenin activation, both in mice and humans, confirming the data reported by Pineau *et al.* [18]. Our results could reconcile the data of Lou and Li, which suggested that miR-34a was lost in two thirds of the HCCs studied [21, 22]. These samples corresponded mostly to HBV^+ tumors, a

subgroup with a lower frequency of *CTNNB1* mutations [10]. The other third of patients in their cohort, with HCV infection, displayed an increase in miR-34a expression, and this group is likely to be enriched in *CTNNB1* mutations, according to transcriptomic classifications[4, 5]. In our cohort, tumors without β-catenin mutations displayed heterogeneity in terms of miR-34a amount. A greater understanding of this variability would be very useful,

In our study, we used a LNA approach to block cell cycle progression (Figure 5C), to decrease cyclin D1 protein levels (Figure 5E), and to induce caspase-2 activation (Figure 5D), in isolated Apc^{KO}hepatocytes. The decrease in cyclin D1 protein levels was actually due to HNF-4α, because the invalidation of *hnf4a* in the Apc^{KO} model resulted in an absence of change in cyclin D1 levels (Figure 6A), and a smaller decrease in cell proliferation (Figure 6B), in the presence of LNA-34a. In addition, LNA-34a increases HNF-4α protein level (Figure 5B), but also its transcriptional activity on a luciferase construct under the control of a HNF-4 responsive element (Figure 3D), and on cyclin D1 promoter (Figure 6D).Thus, HNF-4α, a tumor suppressor in the liver [28], appears to be a key mediator of the effects of miR-34a in the Apc^{KO} model (Figure 6E). More importantly, miR-34a emerges as a crucial component of the oncogenic signal induced by β-catenin in hepatocytes.

In normal livers, miR-34a was particularly expressed in bile duct cells (Figure 3A and 3B). This specific pattern of expression may hinder the accumulation of HNF-4α protein in the biliary cells, which is required for bile cell lineage patterning[27],probably independently of β-catenin, which is not activated in normal bile duct cells[37]. Thepattern of miR-34a expression is not consistent with the usual model, in which β-catenin targets are located near the central vein. Cyclin D1 is one exception to this model;its pattern of expression is not restricted to the PC zone [23].

Interestingly, the region bound to TCF-4/β-catenin located in miR-34a promoter contains no classical Wnt-responsive element, suggesting that the control on miR-34a is complex (Figure 2B). This complexityis lost when β-catenin is overactivated, as in HCCs.

The key finding of this study was the decrease in liver tumor growth afforded by the delivery of LNA-34a (Figures 7A and S8). Only a few studies to date have demonstrated the potency of miRNA-based approaches to cancer treatment. However, the great advantage of this approach for HCC is that these molecules are preferentially delivered to the liver [38]. The first authorization for clinical trials with miRNA-based therapies, concernsthe LNA against miR-122 (miravirsen) for HCVtreatment[31]. The use of these modulators poses a number of challenges, particularly for their specificity. Our therapeutic approach, involving the inhibition of miR-34a, displays no toxicity (Figure S6B), and has the great advantage of restoring the tumor suppressor HNF-4α, targeting cyclin D1 and the metabolic pathways regulated by HNF-4α (Figure S9). In addition, this approach may also alter the anti-inflammatory loop orchestrated by HNF-4α, controlling interleukin-6 production by hepatocytes [39, 40]. More importantly, the LNA-34a promotes a pro-apoptotic signal orchestrated by caspase 2 and 3 (Figure 7C-E). In conclusion, our data reveal that miR-34a inhibition mainly induces a pro-apototic program centered onto caspase-2, and could affect cell proliferation through cyclin D1 inhibition. However, the fact that Ki-67 and cyclin A labeling were not affected by LNA-34a treatment, suggeststhat miR-34a effects on cell proliferation are more complex.

In conclusion, despite the current preclinical trials being run by miRNAtherapeutic, our findings suggest that the precise differential diagnosis of HCC, including an assessment of the mutational status of β-catenin, for which miR-34a inhibitors would

be more appropriate than miR-34a mimics, is vital. Using this approach, we hope to affect not only the tumor itself, but also its environment, through the first effective targeted therapy for one third of HCCs worldwide.

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STUDY APPROVAL

All procedures were carried out according to the French guidelines for the care and use of experimental animals. All animal studies were approved by the regional veterinary services of the Paris police headquarters (agreement no. 75-1306) and by the Mouse Facility Core laboratory (Institut Cochin, Inserm U1016, Université Paris Descartes, Paris, France). For HCC patients, samples were obtained with written informed consent, and the study protocol was approved by the ethics committee of the Cochin Hospital.

AUTHOR CONTRIBUTIONS

AG: study concept and design and drafting of the manuscript; CS, LB and CG: technical support; CM and GR: acquisition of ultrasound data; FT and PN: analysis of miRNA-seq data; BT and CC: gift and genotyping of patient tumors; CP and SC: funding, study supervision and critical revision of the manuscript.

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ABBREVIATIONS:

Apc : Adenomatous polyposis coli ; Apc^{ko} : specific inactivation of *Apc* in the liver with a liver-specific and tamoxifen-inducible Cre-loxP ; GS : glutamine synthetase ; HBV : hepatitis B virus ; HCC : hepatocellular carcinoma ; HCV : hepatitis C virus ; HNF-4 : hepatocyte nuclear factor 4 ; LNA : Locked nucleic acid ; mut : mutated ; ns : non-significant ; PC : pericentral ; PP : periportal ; RQ : relative quantity ; Tam : tamoxifen ; TCF-4 : T-cell factor 4.

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FIGURE LEGENDS

Figure 1: miR-34a is induced following β-catenin activation in mouse

hepatocytes and in HCC samples from patients.

A: miR-34a induction in Apc^{KO} hepatocytes isolated at day 6 after tamoxifen injection in miRNA-seq was confirmed in 15 samples by RT-qPCR relative to snoRNA135.**B-D:** miR-34a levels, relative to RNU24 and RNU49 levels, in 24samples of HCC with β-catenin mutation (mut-β-cat) and 20 samples of non-mutated (non-mut-β-cat) HCC, as compared with normal tissues. HCV: hepatitis C virus; HBV: hepatitis B virus; OH: alcohol exposure; others: no specific etiology, ns: non-significant. **C:** Detection of miR-34a in HCC samples with β-catenin mutation by*in situ hybridization*.

Figure 2: miR-34a is induced following β-catenin activation, through the direct binding of β-catenin.

A: wt (CTRL) and Apc^{KO} hepatocytes were transfected 3 days after tamoxifen injection with 100 nM of a siRNA against β-catenin for 24 h. Results are shown as the mean RQ (relative quantity) values for miR-34a for five independent experiments ± SEM, as compared with scramble siRNA. **B-C:** TCF-4 (**B**) and β-catenin (**C**) binding to a site 21 kb upstream from the miR-34a promoter in wt (CTRL) and Apc^{KO}hepatocytes at day 6 after tamoxifen injection, as compared with the isotype control (IgG). Results are expressed as the binding value ± SEM normalized with respect to three control sites in the *myc* and *Alb* genes. The means of at least three independent experiments are shown.

Figure 3A-B: miR-34a induction following β-catenin activation is associated with lower HNF-4α level and activity. A: miR-34a localization by *in situ* hybridization with LNA-34a in comparison with a scramble LNA. PP: periportal zone; PC: pericentral zone. **B:** miR-34a level assessed by RT-qPCR on wt (CTRL)

hepatocytes isolated by digitonin/collagenase perfusion, and expressed as previously. ns: non-significant.# represents the number of samples tested.**C:** HNF4α protein expression in comparison with miR-34a levels in Apc^{KO} hepatocytes, by comparison to wt hepatocytes (CTRL). # represents the sample number. **D:** Apc^{KO} hepatocytes transfected with the FOP construct (HNF-4α response element), with LNA-scramble or LNA-34a or a mimic of miR-34a. Results are expressed as the mean luciferase induction of three independent experiments carried out in duplicate.

Figure 4: miR-34a is induced in tumors from Apc^{KO} mice.

A: miR-34a levels in 42 tumors with β-catenin mutations are compared with those for normal tissues, and are expressed as previously described. **B-D**: HNF-4α levels, relative to 18S, are determined by qPCR, and represented as previously, as compared with non-tumor tissues. **D**: Correlation between miR-34a and HNF-4α RNA levels. **E:** Correlation between miR-34a by*in situ hybridization* and HNF-4α by immunohistochemistry in Apc^{KO} tumors.NT: non-tumoral tissue, Tum: tumor.

Figure 5: LNA-34a inhibits Apc^{KO} hepatocyte proliferation, decreases cyclin D1 levels, and promotes apoptosis. A: Isolated Apc^{KO} hepatocytes were transfected with a luciferase construct with a miR-34a binding site in 3'UTR, and with LNA-scramble or LNA-34a. Results are expressed as the mean luciferase activity in three experiments performed in duplicate. **B:** Confirmation of LNA efficacy on HNF-4α protein levels. Isolated Apc^{KO} hepatocytes were transfected with LNA-scramble or LNA-34a for 48 h. **C:** Cell proliferation was assessed, for 48 h on wt or Apc^{KO} cells, transfected with LNA-scramble or LNA-34a. Results are expressed as the mean % proliferation normalized against cells treated with LNA-scramble at time *t* in three

independent experiments carried out in quadriplicate ± SEM. Caspase 2 and PARP protein level (**D),** andCyclin D1 protein and mRNA levels (**E**) in isolated Apc^{KO} hepatocytes transfected with LNA-scramble or LNA-34a for 48 h.

Figure 6: LNA-34a impact depends on HNF4α expression

A: Cyclin D1 mRNA and protein level in isolated hnf-$4\alpha^{KO}$/Apc^{KO} hepatocytes transfected with LNA-scramble or LNA-34a for 48 h. **B:** Cell proliferation of Apc^{KO} or Apc/hnf-$4\alpha^{KO}$ cells, transfected with LNA-scramble or LNA-34a for 48 h. Results are expressed as in figure 5. **C:** Caspase 2 and PARP protein level protein levels in isolated hnf4/Apc^{KO} hepatocytes transfected with LNA-scramble or LNA-34a for 48 h **D:**HNF4α binding to the cyclin D1 promoter in Apc^{KO}hepatocytes, as compared with the isotype control. Results are expressed as in figure 1. They represent the means of at least three independent experiments. **E:** The cascade orchestrated by miR-34a.

Figure 7: LNA-34a slows the development of tumors from APC^{KO} mice, by activating caspases 2 and 3. A: Tumor development was followed by 2D ultrasound. The results are expressed as the fold-change in volume over 15 days ± SEM normalized with respect to *t0*, for mice with and without LNA-34a treatment (10 mg/kg). **B**: Numbers of new tumors detected during the treatment of mice with LNA-scramble or LNA-34a. **C-E:** caspase 2 and 3 expression. Immunohistochemistry experiments have been performed on parallel sections.