Large Molecule Therapeutics

Systemic Delivery of a miR34a Mimic as a Potential Therapeutic for Liver Cancer

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Abstract

miR34a is a tumor-suppressor miRNA that functions within the p53 pathway to regulate cell-cycle progression and apoptosis. With apparent roles in metastasis and cancer stem cell development, miR34a provides an interesting opportunity for therapeutic development. A mimic of miR34a was complexed with an amphoteric liposomal formulation and tested in two different orthotopic models of liver cancer. Systemic dosing of the formulated miR34a mimic increased the levels of miR34a in tumors by approximately 1,000-fold and caused statistically significant decreases in the mRNA levels of several miR34a targets. The administration of the formulated miR34a mimic caused significant tumor growth inhibition in both models of liver cancer, and tumor regression was observed in more than one third of the animals. The antitumor activity was observed in the absence of any immunostimulatory effects or dose-limiting toxicities. Accumulation of the formulated miR34a mimic was also noted in the spleen, lung, and kidney, suggesting the potential for therapeutic use in other cancers. Mol Cancer Ther; 13(10); 2352–60. ©2014 AACR.

Introduction

The development of lipid nanoparticle technologies that provide efficient delivery of RNA oligonucleotides to hepatocytes and liver tumors (1) has created an opportunity for RNAi-based therapies. Among the most interesting RNAi therapeutic candidates for cancer are mimics of tumor-suppressor miRNAs. Like multi kinase inhibitors that have increased the survival rates of patients with cancer (2), tumor-suppressor miRNAs inhibit cancer by co-regulating many different oncoproteins and oncogenic pathways. One of the best characterized tumor-suppressor miRNAs, miR34a, functions within the p53 pathway to regulate a diverse set of oncoproteins (reviewed in ref. 3) and reduced levels of the tumor-suppressor miRNA have been observed in a variety of cancer types, including liver cancer (4, 5). When introduced into cancer cells, miR34a induces cell-cycle arrest (6), senescence (7), and apoptosis (7). The miRNA also inhibits cancer stem cell development (8–10).

We have developed a miR34a mimic that has proved to be effective in inhibiting the growth of tumors in mouse models of lymphoma (11), lung cancer (12), and prostate cancer (8). To address whether miR34a can inhibit liver tumor growth, we encapsulated our miR34a mimic with an amphoteric liposomal formulation and evaluated the resulting nanoparticles for therapeutic activity using mice with orthotopic Hep3B and HuH7 liver cancer xenografts. Systemic delivery of the encapsulated miR34a mimic reduced the expression levels of several miR34a target mRNAs and caused significant growth inhibition and even regression of the liver tumors. Toxicity studies revealed no detrimental side effects or immune stimulation at therapeutic doses.

Materials and Methods

Cell culture

Hep3B cancer cells were purchased from ATCC in January 2013. HuH7 cells were purchased from the National Institute of Biomedical Innovation in Japan in January 2013. Hep3B cells were cultured as recommended by the manufacturer (ATCC). HuH7 cells were cultured in DMEM (ATCC) supplemented with 10% FBS (HyClone). All cells were grown at 37°C–95% O2.

miRNAs and amphoteric liposomal formulation

The miR34a mimic was synthesized and purified by Aveca Biotechnology using standard procedures for RNA oligonucleotide synthesis and high-performance liquid chromatography purification. Synthesis of α-(3-O-cholesteryloxycarbonyl)-δ-(N-ethylmorpholine)-succinamide (MoChol) and production of amphoteric liposomes were performed as described (13).

Orthotopic liver cancer models

Female NOD/SCID mice between 7 and 8 weeks of age were purchased from Jackson Laboratory. All animals were housed in disposable Micro-Isolator cages (Innovive) and animal husbandry and procedures were carried out in accordance with the institutional guidelines for proper animal care and maintenance. Animals were maintained...
in aseptic conditions for all aspects of experimentation. Mice were anesthetized under 3% isoflurane/O2 mixture throughout surgery. Once sufficiently anesthetized, the abdominal hair was removed with electric hair clippers and the incision site sterilized with betadine and 70% EtOH. A 1 cm incision was made along the midline of the abdomen with the uppermost portion being just inferior to the sternum. Separate incisions were required to safely expose the peritoneum and the abdominal organs. The left lateral lobe of the liver was exteriorized with cotton swabs and a bolus of 2 × 10^6 Hep3B or 1 × 10^6 HuH7 cells in a 25 μL volume of PBS was injected using a 27-gauge needle (BD Biosciences) attached to a 100 μL glass syringe (Hamilton). Any bleeding was stopped by applying slight pressure to the injection site with cotton swabs. The liver was placed back in the abdomen, the peritoneum was closed with 6.0 silk sutures, and the skin was closed with wound clips. All wound clips were removed 7 days after surgery.

**Animal studies**

**Target gene identification.** NOD/SCID mice with Hep3B or HuH7 orthotopic liver cancer xenografts were subjected to a single tail vein injection with liposome dilution buffer, 1.0 mg/kg of formulated miR34a mimic (MRX34), or liposome formulation alone with lipid concentrations equivalent to 1.0 mg/kg dose of formulated miR34a mimic. Twenty-four hours after injection, tumors were recovered from sacrificed mice and RNA was isolated and subjected to mRNA array analysis using an Illumina Bead (Hep3B) or Affymetrix (HuH7) microArray. Microarray analyses were performed by Asuragen. Array data are available at Gene Expression Omnibus (GEO) using accession numbers GSE58893 (Hep3B) and GSE58800 (HuH7).

**Efficacy studies.** NOD/SCID mice bearing Hep3B or HuH7 orthotopic liver cancer xenografts were subjected to multiple tail vein injections with liposome dilution buffer: 0.5, 1.0, or 3.0 mg/kg MRX34 or liposome formulation alone with lipid concentrations equivalent to 0.3, 1.0, or 3.0 mg/kg MRX34. Tumor growth was assessed by periodic blood collections and subsequent assaying of α-fetoprotein (AFP) from sera. Three doses were given per week (Monday/Wednesday/Friday) for two weeks and a seventh dose was given on Monday. Twenty-four hours after the final injections, animals were subjected to necropsy and tumors were resected and weighed. As both Hep3B and HuH7 tumors typically grow as single bulbous tumor masses, they were easily dissected from surrounding host liver tissue.

**Toxicology assessments.** Seven- to 8-week-old female Balb/C mice (Charles River) were housed appropriately and animal husbandry and procedures were carried out in accordance with institutional guidelines for proper animal care and maintenance. Animals were injected intravenously with MRX34 or dilution buffer. Blood was collected from mice via retro orbital sinus draws. Blood samples were added to serum collection tubes for subsequent serum separation by centrifugation. Serum cytokine levels were determined using a mouse Fluorokine Multianalyte Profiling Kit (R&D Systems) and the LumineX 100 IS instrument. For blood chemistry analyses, serum from animals dosed was sent to the Comparative Pathology Laboratory at University of California, Davis (Davis, CA).

**AFP analysis**

Sera were prepared from mouse blood samples by centrifugation and AFP was measured by ELISA (DRG EIA-4331). A standard curve was produced using purified AFP so that ELISA readings could be converted to ng of AFP/mL of serum.

**Human whole blood immunostimulation assay**

To determine whether the formulated miR34a mimic induces a human cytokine response, formulated miRNAs were incubated for 24 hours in whole blood collected from three healthy male volunteers. Heparin was added to the whole blood to prevent coagulation and the formulated miRNA was present at a final concentration of 600 nmol/L. After the incubation, plasma was separated by centrifugation and IL6, IL8, and TNFα were analyzed by ELISA (R&D Systems).

**Quantitative RT-PCR**

Total RNA was isolated from tissues and blood using miVana PARIS reagents (Ambion). All tissues were homogenized in PARIS lysis buffer and total RNA was isolated according to the manufacturer’s specifications. For mRNA or miRNA analysis, cDNA was generated from 10 ng total RNA, respectively, with MMLV reverse transcriptase (Invitrogen). Quantitative PCR analysis was performed using the 7900 Real-time PCR System. For analysis of miR34 target mRNAs, TaqMan primer/probe sets (Life Technologies) specific for ERC1, RRAS, PHF19, WTAP, CTNNB1, SIPA1, DAB1, MYCN, and PHF19, CTNNB1, SIPA1, DAB1, and MCL1 were used along with ubiquitously expressed GAPDH and CyclinH-A to generate raw mRNA expression data used for relative standard curve PCR analysis (RSC). For RSC, standard curves were generated from diluted negative control samples and raw data from each primer/probe set were expressed in terms of these curves to account for differences in individual target reverse transcription and PCR efficiencies. The normalized data were compared for each test primer/probe set against geometric means of RSC normalized GAPDH and CyclinH-A mRNA levels.

**Statistical analysis**

Results are reported as the mean ± SD. The statistical significance of the differences in tumor weights and AFP levels in the miR34a efficacy experiments was assessed by
one-way ANOVA and the Dunnett posttest. All statistics were generated using GraphPad Prism 5.04 software for Windows, GraphPad Software (www.graphpad.com). The level of significance was set at $P < 0.05$.

Results

Systemic delivery and activity of a miR34a-based therapeutic candidate

A mimic of miR34a was encapsulated using a liposomal formulation featuring amphoteric lipids that are cationic during liposome formation to ensure efficient encapsulation of the miRNA mimic and anionic during storage and delivery to maximize shelf-life, circulation time, and tissue uptake (14). We call the liposome-formulated miR34a mimic MRX34. To characterize the delivery pattern of the amphoteric liposomal formulation, we injected MRX34 into the tail veins of NOD/SCID mice bearing orthotopic Hep3B liver tumor xenografts and measured the levels of miR34a in five tissues of animals 24 hours after injection. A single intravenous injection of MRX34 introduced more than 60 million copies of the miRNA per ng of total RNA from liver and more than 9 million copies per ng of tumor RNA (Fig. 1A). The single injection of MRX34 also increased the levels of miR34a by 100,000 to 4,000,000 copies per ng of total RNA from lung, spleen, and kidney (Fig. 1A). The endogenous levels of miR34a in the five tissues ranged from 10,000 to 100,000 copies per ng of total RNA. The endogenous levels of miR34a and the delivered copies of miR34a were similar for a second orthotopic liver cancer model that featured xenografts of the HuH7 human liver cancer cell line (data not shown).

NOD/SCID mice with orthotopic Hep3B or HuH7 liver tumor xenografts were subjected to a single dose of MRX34, empty liposome, or dilution buffer and sacrificed 24 hours later. RNA samples from the tumors resected from the mice were subjected to microarray analyses and the resulting data were analyzed by ranking genes based upon their level of differential expression in mice dosed with MRX34 compared to mice dosed with dilution buffer or empty liposomes. The levels of 624 mRNAs were

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Systemic delivery and activity of a miR34a mimic in mice with liver tumors. NOD/SCID bearing orthotopic Hep3B or HuH7 liver cancer xenografts were injected with 1 mg/kg MRX34, dilution buffer, or empty liposomes (3 mice/dose group) and sacrificed 24 hours after injection. Tissue samples were recovered and assessed by qRT-PCR analysis. A, copies of miR34a above endogenous levels in liver, Hep3B liver tumor, lung, spleen, and kidney of mice dosed with 1.0 mg/kg MRX34. B, relative Hep3B tumor mRNA expression of nine putative miR34a target genes in liver tumors from mice dosed with dilution buffer, empty liposomes, or MRX34 (3 mice per dose group). C, relative HuH7 tumor mRNA expression of nine putative miR34a target genes in liver tumors from mice dosed with MRX34, dilution buffer, or empty liposomes (4 mice/dose group).
miR34-Based Cancer Therapy Candidate

Table 1. Genes in tumor-suppressor and oncogenic pathways that are commonly altered in liver tumors that were differentially expressed in Hep3B and/or HuH7 xenografts of mice dosed with MRX34

<table>
<thead>
<tr>
<th>p53</th>
<th>Wnt/β-catenin</th>
<th>MapK</th>
<th>Hedgehog</th>
<th>VEGF</th>
<th>c-MET</th>
</tr>
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<tbody>
<tr>
<td>CHEK2 (+)</td>
<td>FRAT1 (−)</td>
<td>PDGF (−)</td>
<td>RAB23 (−)</td>
<td>VEGFB (−)</td>
<td>c-MET (−)</td>
</tr>
<tr>
<td>CDKN1A (+)</td>
<td>CSNK2A1P (−)</td>
<td>RRAS (−)</td>
<td>PRKX (−)</td>
<td>MAPKAPK2 (−)</td>
<td>MAPK3 (−)</td>
</tr>
<tr>
<td>BID (+)</td>
<td>CTNMB1 (−)</td>
<td>PRKX (−)</td>
<td>FBOXW11 (−)</td>
<td>NFATC3 (−)</td>
<td>MAPK1 (−)</td>
</tr>
<tr>
<td>CASP3 (+)</td>
<td>FBOXW11 (−)</td>
<td>NFIXb2 (−)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCNG2 (−)</td>
<td></td>
<td>ELK4 (−)</td>
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<td></td>
<td></td>
<td>THBS1 (−)</td>
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NOTE: The symbols (+/−) in parentheses indicate whether the expression of the gene was elevated (+) or reduced (−) in the tumors of mice dosed with MRX34 compared with tumors from mice dosed with dilution buffer and empty liposomes.

Reduced by at least 30% in the Hep3B liver tumors of mice dosed with MRX34. Thirty-three of the downregulated mRNAs were identified via TargetScan (15) as having potential binding sites for miR34a. The levels of 59 mRNAs were reduced by at least 30% in the HuH7 liver tumors of mice dosed with MRX34. Twenty of the downregulated mRNAs were identified via TargetScan as having potential binding sites for miR34a. Comparing the downregulated mRNAs with miR34a-binding sites from the two cancer models revealed that nine mRNAs are common between the two datasets. Quantitative reverse transcription-PCR (qRT-PCR) analysis confirmed that the mRNA levels of all nine genes were significantly lower in the liver tumors of mice dosed with MRX34 than the mice dosed with dilution buffer or empty liposomes in the Hep3B and HuH7 tumor RNA samples (Fig. 1B and C). The complete list of genes that were differentially expressed (up or down) in the liver tumors of the MRX34-dosed mice by 30% or greater includes 25 genes that are associated with pathways that are commonly altered in primary liver tumors—p53, Wnt/β-catenin, MapK, Hedgehog, VEGF, and c-MET (refs. 16–18; Table 1).

miR34a anticancer effects in Hep3B liver orthotopic mice

We conducted a study to evaluate the therapeutic activity of MRX34 using mice with orthotopic liver cancer xenografts. A total of \(2 \times 10^6\) Hep3B cells were surgically implanted into the left lateral lobes of the livers of female NOD/SCID mice and tumors were allowed to develop for 3 weeks. We used a serum biomarker of liver cancer, AFP, to monitor the growth of the xenografts. Serum AFP levels correspond with tumor mass in orthotopically grown Hep3B xenografts (data not shown) and provide an effective way to assess tumor size without the need for imaging. When AFP reached mean levels of 1,600 ng/mL of serum, mice were separated into test and control groups of 6 animals per group (Fig. 2A). Animals received single intravenous administrations of MRX34 at 0.3 or 3.0 mg of miRNA/kg of body weight (mg/kg), 3.0 mg/kg equivalent (eq) of empty liposomes, or dilution buffer three times per week (Monday/Wednesday/Friday) for 2 weeks with a final injection on the following Monday for a total of 7 doses. To monitor tumor growth in the animals, serum AFP was measured every 3 to 4 days after the initiation of dosing. At every time point following the initiation of treatment, AFP levels in the two MRX34 dose groups were significantly lower than the levels in the two control groups (Fig. 2B). Mean serum AFP levels of animals in the empty liposome and dilution buffer groups were similar in magnitude reaching nearly 1,000,000 ng/mL by the end of 2 weeks of dosing (Fig. 2B). In contrast, the serum AFP levels of the mice on the MRX34 dose groups ranged from less than 2-fold higher than the starting levels to barely above background for the AFP ELIAS (Fig. 2B and C).

Animals were sacrificed from each group 24 hours after the final dose was administered and large tumors were detected in the livers of all mice from the two control groups, while much smaller and paler tumors, in general, were detected in both MRX34 dose groups. In fact, tumors were visibly absent in 1/7 and 3/7 animals dosed at 0.3 and 3.0 mg/kg MRX34, respectively (Fig. 2C and D). These results have been repeated multiple times in the Hep3B liver orthotopic mouse model and these data are representative of those results.

MRX34 inhibits growth of established HuH7 orthotopic liver tumors

We used a second orthotopic liver cancer model featuring HuH7 cells that express mutant p53 (19) to determine whether the therapeutic activity of MRX34 extends beyond Hep3B xenografts. HuH7 and Hep3B cells have distinct genetic backgrounds and cancer models featuring these two cell lines are often used to evaluate how broadly applicable a therapeutic candidate for liver cancer might be. We surgically implanted HuH7 human liver cancer cells into the left lateral lobes of NOD/SCID mice and allowed tumors to develop for 2 weeks. When serum AFP levels in 24 mice averaged 1,200 ng/mL, we separated the mice into three dosing groups (Fig. 3A). The difference in mean serum AFP levels between the HuH7 and Hep3B efficacy studies (1,200 ng/mL vs. 1,600 ng/mL) was due to slightly different growth rates of the tumors in the two
liver cancer models. Tumor-bearing mice received three tail vein injections of dilution buffer, 1.0 mg/kg MRX34, or 1.0 mg/kg equivalent of empty liposomes per week for 2 weeks and all mice received a seventh dose 15 days after the initiation of dosing. Unlike the Hep3B study, where we had enough mice with liver tumors to accommodate two MRX34 dose groups, we only had enough mice with HuH7 tumors to accommodate a single MRX34 dose group. For the HuH7 study, we used an MRX34 dose level (1.0 mg/kg) that was between the 0.3 and 3.0 mg/kg doses that were used for the Hep3B study. Consistent with the study using the Hep3B liver cancer model, tail vein injections of MRX34 significantly reduced the accumulation of human AFP in the sera of mice compared with the dilution buffer and empty liposome dosing groups (Fig. 3B). The serum AFP levels in the mice from both control groups increased on average by more than 115-fold between the first and last day of dosing. In contrast, the average serum AFP levels for the animals in the MRX34 dose group increased by less than 2-fold and 2 of the 8 mice had substantially lower AFP levels at the end of the dosing period than at the beginning (Fig. 3C). We sacrificed animals 24 hours after the final dose and tumors were detected in all of the mice from the two control groups, whereas only 5 of the 8 animals in the MRX34 group had detectable tumors (Fig. 3D). The average liver
tumor masses recovered from the mice in the dilution buffer and empty liposome groups were 155 and 159 mg, respectively. The average tumor mass recovered from the five MRX34-dosed mice that had liver tumors was 8 mg. qRT-PCR analysis of the recovered tumors revealed that animals dosed with MRX34 had approximately 107 more copies of miR34a per ng of total tumor RNA than the animals in the control groups (Fig. 3E). These results have been individually reproduced in a separate efficacy study with MRX34 in the HuH7 liver orthotopic mouse model and these data are representative of those results.

**MRX34 evaluation in immunocompetent systems**

The safety profile of MRX34 was evaluated using immunocompetent BALB/c mice. Three to 4 animals per group were dosed intravenously with 1.5 mg/kg MRX34 every other day (q.o.d.) for 2 weeks. Animal health was monitored daily and liver, spleen, and whole blood were collected 24 hours after the last intravenous administration. Whole organ weights were collected and compared between groups and serum clinical chemistries were analyzed. No body weight changes were observed over the 2-week period between animals in the test (MRX34) and control (dilution buffer) groups (Fig. 4A). Spleen weights were normalized to body weight and no changes in spleen size were observed in mice dosed with MRX34 compared with animals dosed with dilution buffer (Fig. 4B). In a separate study, we measured levels of cytokines indicative of immune stimulation (IL1β, IL6, and TNFα) in BALB/c mouse sera following single administration of...
MRX34 at 1 mg/kg and found no significant elevations compared with normal levels measured in animals dosed with PBS (Fig. 4C). An ex vivo model was used to confirm that MRX34 is not immunostimulatory in human blood (20). MRX34 (600 nmol/L), along with 600 nmol/L formulated NC2 and unformulated PBS, were incubated in whole blood from three male donors for 24 hours and then plasma was prepared and assayed for TNFα, IL6, and IL8. Consistent with the mouse studies, MRX34 failed to stimulate a cytokine response (Fig. 4D).

Discussion

The systemic delivery of a formulated mimic of miR34a caused tumor regression in two different orthotopic models of liver cancer. Repeated dosing of the formulated tumor-suppressor miRNA did not impact normal animal behavior nor did it produce any histologic evidence of toxicity at any dose tested. Furthermore, there was no evidence of immune stimulation in mice or in human whole blood. The combination of antitumor activity and favorable safety profile suggests that the formulated miR34a mimic might be effective as a targeted therapy for cancer.

The profound efficacy exhibited by the systemic delivery of the miR34a mimic likely derives from the ability of the small RNA to regulate multiple genes and pathways that are important for hepatocellular carcinoma development. A single administration of the miRNA inhibited multiple genes within oncopgenic pathways of Wnt/Catenin, MapK, c-Met, Hedgehog, and VEGF and stimulated multiple genes within the p53 pathway. Hyperactivation of any of the five oncopgenic pathways or suppression of the p53 pathway have proved to stimulate the development of hepatocellular carcinoma (HCC) in mice (16–19, 21). p53 is the most commonly mutated gene in patients with HCC (19, 22) and reduced p53 activity appears to be a key early event in the development of liver tumors (23, 24). Not surprisingly, the p53-regulated miR34a has reduced expression levels in liver tumors (4). Consistent with a recent publication that describes the ability of miR34a to stimulate the p53 pathway in cancer cells lacking p53 (25), we observed increased expression levels of the p53-related genes CHEK2, CDKN1A, BID, CASP3, and CCNG2 (21, 26) in the liver tumors of mice dosed a single time with MRX34. This result suggests that the potency of
MRX34 might derive at least partially from its capacity to stimulate the p53 pathway in cancer cells that lack p53 activity as is the case with the Hep3B cell line.

The hyperactivation of the Wnt/b-catenin pathways is an early signature of hepatocellular carcinogenesis (27). Abnormal Wnt/b-catenin signaling impacts cellular proliferation, metabolic function, and stem cell behavior (28) and stimulates HCC formation (29). Following a single administration of MRX34, we observed the inhibition of FRAT1, CSNK2AP, CTNNB1, FBXW1, and NFATC3, genes that are involved in propagating Wnt/b-catenin signaling (30, 31). The ability to suppress Wnt/b-catenin signaling with MRX34 likely contributes to the efficacy observed in the two xenograft models of HCC that we used in our studies.

The MAPK pathway has been shown to be elevated in 91% of human samples of HCC (32). In these tumor types, dysregulation of JNK1 and p38 is facilitated by MAPK signaling and a cascade effect ultimately results in abnormal cellular proliferation, survival, and differentiation (33). With a single injection of MRX34 there were notable reductions in the expression of five genes that are commonly associated with the MAPK pathway, PDGF, RRAS, PRKX, NFkB, and ELK4.

The Hedgehog pathway plays a significant role in HCC proliferation, invasion, and metastasis by upregulating the protein expression of MMP9 via the ERK pathway (34, 35). In fact, formulated siRNA targeting Hedgehog is sufficient to eliminate Hedgehog expression and markedly reduce metastasis in orthotopic mouse models of HCC (36). A single injection of MRX34 reduced the tumor levels of RAB23, PRKX, and FBXW11 mRNAs. All three genes fall within the Hedgehog pathway (37). Activation of the Hedgehog pathway has been detected in 67% of human liver tumors (35).

The VEGF pathway regulates angiogenesis and enhances HCC tumor development when hyperactivated (38). Interestingly, VEGF expression has been reported to be highest in cirrhotic regions of the liver which surround HCC tumors in human patients (39), and agents are currently being designed to target both tumor and surrounding liver tissue as both appear critical in tumor formation and propagation (40, 38). Administering a single injection of MRX34 inhibited mRNA expression of MAPKAPK2, NFATC3, and MAPK1, which all function within the VEGF pathway (41). The ability to diminish angiogenesis via the VEGF pathway is a likely contributor to the efficacy observed in our mouse models of HCC.

c-MET plays a role in early HCC development and cell proliferation (4). In fact, the activation of c-MET is sufficient to initiate hepatocellular carcinogenesis in mice (27), presumably via the activation of the phosphoinositide 3-

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14. Li N, Fu H, Tie Y, Hu Z, Kong W, Wu Y, et al. miR-34a inhibits migration and invasion by down-regulation of c-Met expression in human hepa-


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