LOW EXPRESSION OF MIR-34A IN HEPATOCELLULAR CARCINOMA AND ITS EFFECT ON ENHANCING THE PROLIFERATION INHIBITION OF TARGETED C-MET DRUGS

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ABSTRACT

Objective*: The abnormal expression of microRNA-34a (miR-34a) is involved in the tumorigenesis and progression of various malignant tumours. However, its role in hepatocellular carcinoma (HCC) has not been fully clarified in research, to the best of our knowledge. In the present study, we have studied the clinical significance and in vitro effects of miR-34a on the biological function of human HCC.*

Methods: *The tissues of 83 HCC patients were detected by formalin-fixed paraffin embedding (FFPE), using real-time quantitative RT-PCR. The difference between miR-34a in FFPE tissues and surrounding normal tissues was analysed. In the functional test, the effect of miR-34a on cell growth was investigated. Additionally, the effect of miR-34a mimetics on c-met drugs was investigated, the pathway to inhibit tumour miRNA by using miR-34a levels was analysed, and the possibility of targeted therapy was ascertained.*

Results: *In comparison to real-time quantitative RT-PCR, the expression of miR-34a in HCC (FFPE) was significantly lower than that in adjacent liver tissue (P<0.01). The expression of miR-34a in TNM stage I and II had no metastasis and no significant differences in portal vein tumour embolism in comparison to the corresponding group (P<0.05). MiR-34a mimetics could inhibit cell growth migration and invasion, increasing the apoptosis and caspase activity of HCC cells at the same time. MiR-34a mimetics enhanced the inhibitory effect of targeting c-MET (siRNA and small-molecule inhibitor su11274) on cell proliferation and the induction of cystine asparaginase activity.*

Conclusion: *miR-34a can be used as a tumour inhibitor of HCC to inhibit miRNA and increase the miR-34a level, which could be the key to targeted treatment of HCC in the future.*

Keywords: *miR-34a, hepatocellular carcinoma, c-MET, targeting.*

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Introduction

In this study, we used real-time quantitative RT-PCR (RT-qPCR) to study the expression of microRNA-34a (miRNA-34a) in hepatocellular carcinoma (HCC) and its adjacent non-cancerous liver tissue. In addition, we conducted in vitro experiments to study the effects of miR-34a on cell growth, apoptosis, caspase-3/7 activity, migration, and invasion in HCC cell lines. c-MET is a target gene of miR-34a[(1-2)], and c-MET inhibitor exhibits controllable safety and initial anti-tumour activity in the patients with HCC and Child-Pugh A or B liver cirrhosis[(3)]. Therefore, we first studied the combined effects of miR-34a mimetics and c-MET targeting agents (c-MET siRNA or c-MET kinase inhibitor su11274) in HCC cells.

Materials and methods

Subjects

Eighty-three HCC patients were selected for this study, and their average age was 52 years. Hepatectomy was performed within 1-2 years. All cases were treated with initial hepatectomy. The patients' written informed consent was obtained. The study was approved by the local ethics committee. All samples were independently examined and diagnosed by two pathologists.

RT-PCR

The miRNeasy FFPE kit was used to isolate RNA from tumour sections and Nanodrop 2000 (Wilmington, DE 19810 USA) was used to detect RNA concentration. Different internal references were used in the current study, with primers for miR-34a, RNU6B, RNU48, and let-7a included in the TaqManH MicroRNA Assays. Reverse primers were also used in the reverse transcription step of the TaqManH MicroRNA Reverse Transcription Kit, with a total volume of 10 ml. Applied Biosystems PCR7900 was used for real-time qPCR of miRNA. Formula 2-"QUOTEΔcq" was used to calculate the expression of miR-34a in FFPE, and the change ratio in miR-34a in the in vitro experiment was (1-1/2 -" QUOTEΔcq") × 100%.

Re-expression and inhibition of miR-34a in HCC cells

The human HCC-derived cell lines HepB3, HepG2, and SNU449 were purchased from American Type Culture Collection. HCC cells were inoculated on 24-well plates, and subsequently, miR-34a inhibitor, miRNA inhibitor negative control, miR-34a mimetic, and miRNA mimetic negative control were transfected into cells at the final concentration of 200 nmol/L for 96 h. c-MEt specific siRNA and their controls were transfected into HCC cells using the same method described above. The sequences of siRNA are summarized in table 1.

Name of siRNA	Sequence	Location and length (nt)	GC content (%)
c-MET siRNA1188	GAGCCAGCCTGAATGATGA	1188-1206 (19)	53
Scrambled c-MET siRNA1188	GGAGCAACGAGGATTACCT	NA (19)	53
c-MET siRNA2950	GAACAGCGAGCTAAATATA	2950-2968 (19)	37
Scrambled c-MET siRNA2950	GTGACACGAAACAGTATAA	NA (19)	37
c-MET siRNA3191	GTAAGTGCCCGAAGTGTAA	3191-3209 (19)	47
Scrambled c-MET siRNA3191	GTAGCAAGCGACTGATGTA	NA (19)	47
c-MET siRNA4235	GAACTGGTGTCCCGGATAT	4235-4253(19)	53
Scrambled c-MET siRNA4235	GCGATAGCGGTCTGTACTA	NA (19)	53

Table 1: siRNA sequence (NA is not available).

Cell-function detection

To understand cell vitality, cell proliferation, apoptosis, and nuclear morphology, active caspase-3/7 must be researched in relation to miR-34a inhibitor and the role of miR-34a simulacra. Migration and invasion of HepG2 cells were assessed using a hole filter with a diameter of 8 mm (6.5 mm in diameter, 8 mm in diameter) and migration and invasion rates were calculated and compared to the simulated control.

Western-blot analysis

The following primary antibodies were used: c-MET, Caspase-3 (8G10, Cell Signaling) and β-actin (Sigma-Aldrich NV).

Statistical analysis

SPSS19.0 statistical analysis, single-factor analysis of variance (ANOVA) is used to analyse differences between the groups. When ANOVA was statistically significant, multiple comparisons were made using the least significant difference (LSD) method. The Student's paired or unpaired t-test was used to analyse the significance level of differences between paired or unpaired groups. The statistical significance was $P<0.05$.

Results

Expression of miR-34a in FFPE tissues of HCC and its clinical and pathological significance

The expression of miR-34a in HCC tissues was significantly lower than that in adjacent non-cancerous liver tissue ($P<0.01$). MiR-34a was significantly lower in TNM III and IV tissues than in TNM I and II tissues. In addition, miR-34a expression was down-regulated in the metastatic group in comparison to the non-metastatic group ($P<0.05$). When studying the relationship between miR-34a expression and other clinicopathological parameters, it was found that miR-34a level was associated with the status of portal vein tumour emboli. miR-34a levels in patients with portal vein tumours was lower than that in patients without portal vein tumours ($P<0.05$, figure 1).

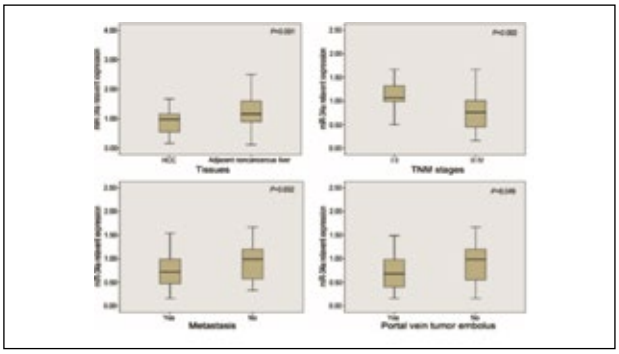

Figure 1: Relationship between miR-34a expression and clinicopathological parameters. miR-34a expression was determined by real-time RT-qPCR. miR-34a is expressed in HCC tissue, and miR-34a is expressed in HCC tissue on the left. TNM stages indicated that the expression of miR-34a in clinical TNM III and IV tissue was lower than that in stage I and II tissues. Metastasis represents the expression of miR-34a in metastatic (left) HCC and unmetastatic HCC (right). The miR-34a level in portal vein tumour patients was lower than that in patients without portal vein tumours.

Effect of miR-34a on the malignant phenotype of HCC cells

Transfection efficiency was monitored using real-time RT-qPCR (figure 2).

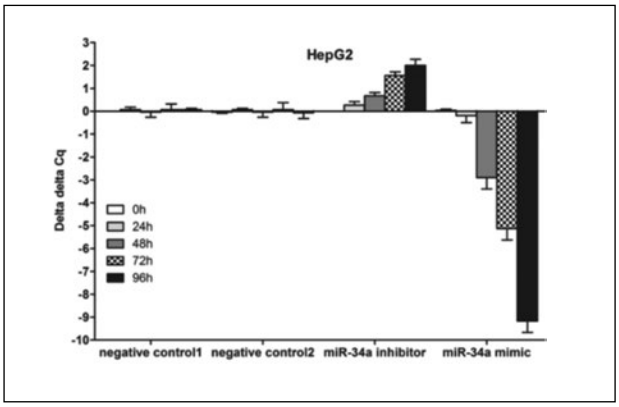

Figure 2: Transfection efficiency of miR-34a inhibitors and miR-34a mimetics in HepG2 cells. HepG2 cells (2.56104 cells per well, using 6-well plates) were transfected with miR-34a inhibitors, miR-34a mimetics, and their negative controls until 96 h. Real-time RT-qPCR detection was used to express miR-34a and calculate the ΔCq.

After transfection with a miR-34a mimetic, a small decrease in viability was observed in all three cell lines at 96 h (figure 3A).

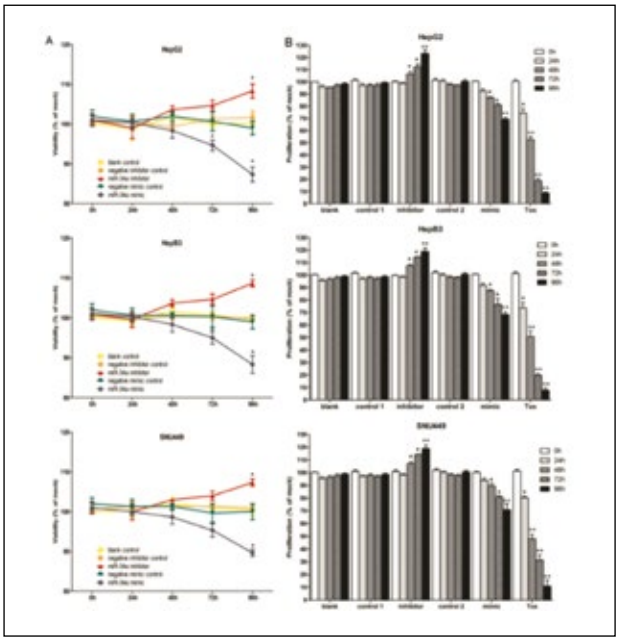

Figure 3: Effect of miR-34a on cell growth in HCC cells. HepG2, HepB3, and SNU449 cells (2.56103 cells per well in a 96-well plate) were cultured for one day and transfected with miR-34a inhibitor, miR-34a mimetic, and a control (200 nM).
*(A) time-dependent effects detected by CellTiter-Blue cell viability assay. (B) cell proliferation was assessed using MTS assay. *P<0.05, **P<0.01, compared with blank and negative control, respectively, at the same time point. TOX: TOX positive transfection control.*

To verify these results, the effect on cell proliferation was assessed using MTS assay (figure 3B), along with a microscopic count of live cells (Hoechst 33342 +/PI negative) (figure 4).

In the miR-34a mimetic, caspase-3/7 activity was significantly enhanced at 72 and 96 h after transfection in all three cell lines (figure 4A).

Its effect on apoptosis was confirmed microscopically by Hoechst 33342 and PI double fluorescence staining (figure 4B), and by the western-blot cutting of caspase-3 (figure 4C).

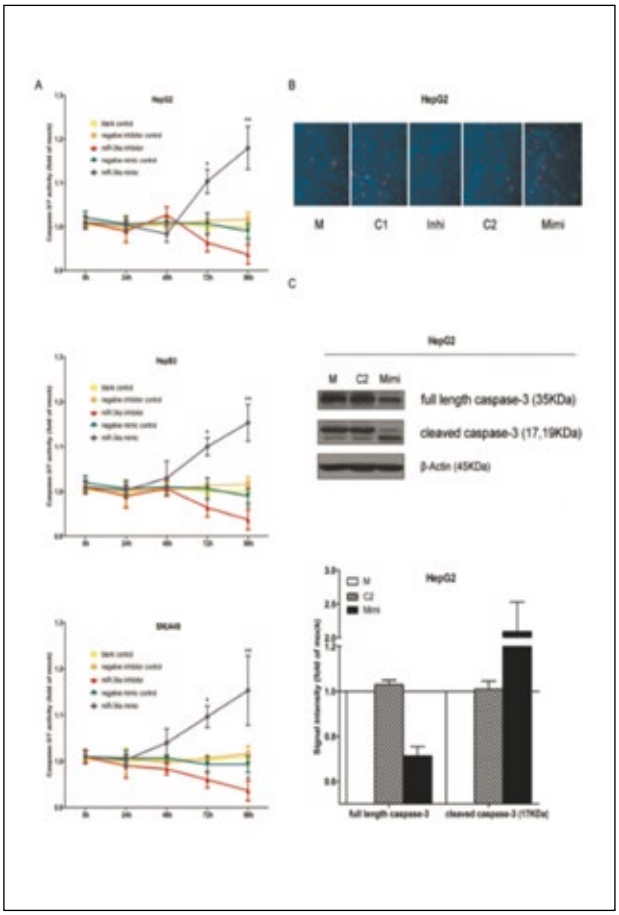

Figure 4: The effect of miR-34a on the apoptosis of HCC cells. HepG2, HepB3, and SNU449 cells were treated as shown in figure 2. (A) caspase-3/7 activity was detected using the Apo-ONE homogeneous Caspase-3/7 assay.
*In contrast to blank and negative controls, at the same time point, *P<0.05, **P<0.01. (B) Hoechst 33342/ PI double-fluorescent chromatin was stained for 96 hours in HepG2 cells. (C) the signal strength of the western blot and the band. The cells were treated for 96 hours. The antibodies include caspase-3, cleaved caspase-3, and caspase-actin. M: analog control; C1: negative control of miRNA inhibitor; Inhi: miR-34a inhibitor; C2: negative control of miRNA mimetic; mimi: miR-34a imitation.*

The effect of miR-34a function on HepG2 cell migration and invasion was subsequently evaluated. miR-34a inhibitors had little effect on migration and

invasion activity. miR-34a mimetics resulted in a modest reduction in the migration and invasion rate in HepG2 (figure 5).

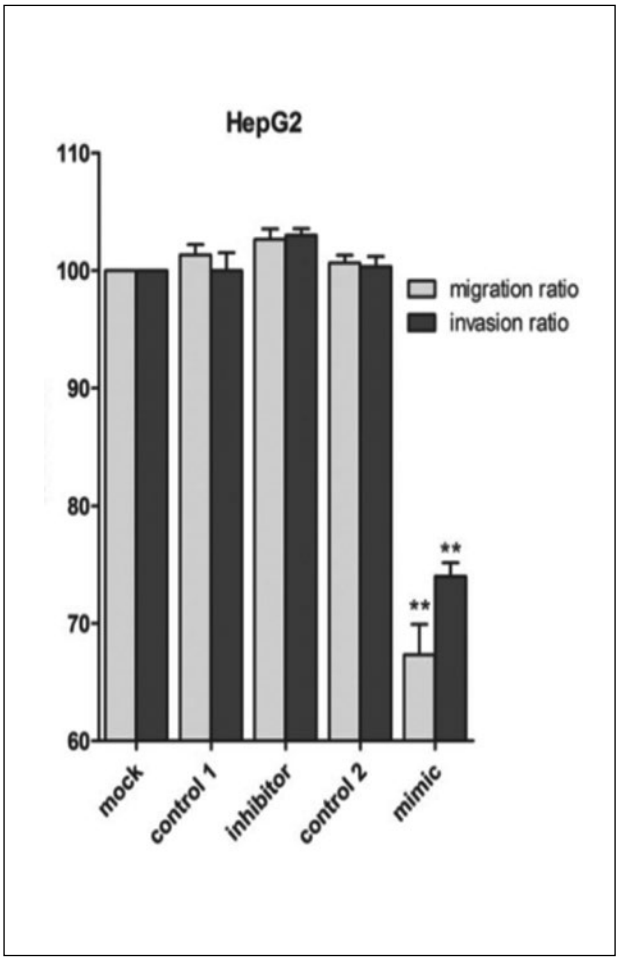

Figure 5: Effects of miR-34a inhibitors and miR-34a mimetics on cell migration and invasion in HepG2 cells. HepG2 cells were cultured to 50% and transfected with miR-34a inhibitors, miR-34a mimetics, and the control. The cells were cultured in a serum-free medium for 96 hours after transfection. The cells were then digested with trypsin and added (56104 cells) for migration and invasion assays. The migration and invasive cells were fixed, stained, and counted, and the ratio of cell migration and cell invasion was calculated.

miR-34a mimetics enhance the inhibition of the proliferation of c-MET siRNA and su11274 cells

It has been reported that c-MET is the target gene of miR-34a (4), and it is important to analyse the combined effect of the miR-34a analog and the targeted c-MET reagent (siRNA or small molecule inhibitor, su11274) through colourimetric MTS proliferation assay. Both the c-MET siRNA and the inhibitor su11274 can down-regulate 70% of the c-MET protein expression (figure 6).

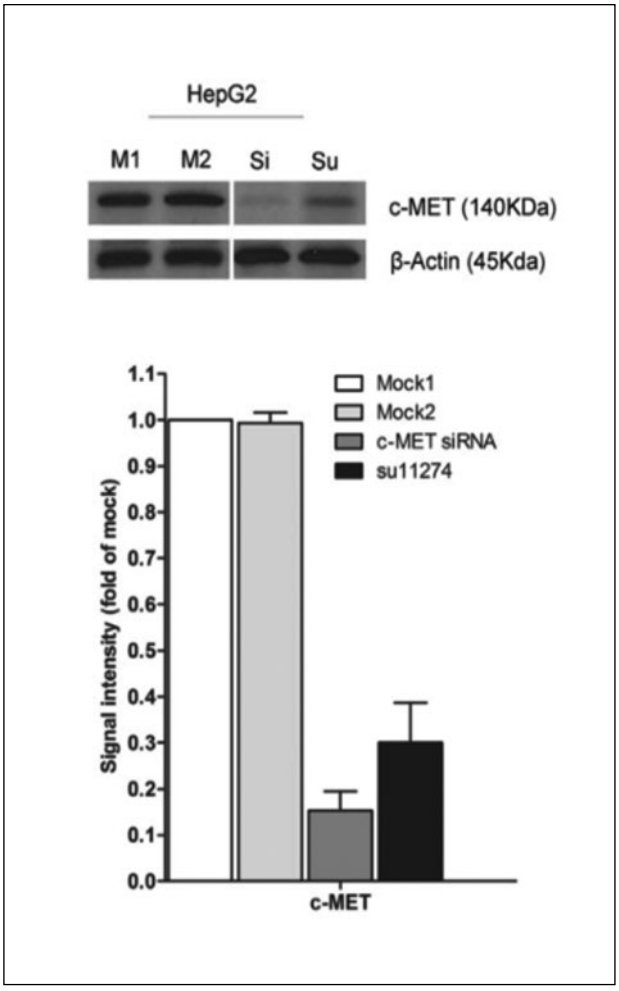

Figure 6: The protein level of c-MET after treatment of siRNA and the small molecule inhibitor su11274. HepG2 cells were transfected with c-MET siRNA (2.56104 cells per well, using 24-well plates) or 96 hours with the c-MET micromolecule inhibitor su11274. The c-MET protein level was determined using a western blot. The c-MET and β-actin protein blot signals were quantified and the c-MET signal strength relative to β-actin was calculated. These values are represented by the bar charts. M1: Mock1, an analog control for siRNA containing only the transfection reagent; M2: an analog control of Mock2, su11274, containing only 0.1% DMSO. Si: c-MET siRNA. Su: su11274.

In comparison to a single drug or single miR-34a mimetic in HepG2 cells, miR-34a mimics the inhibition of cell proliferation and the induction of caspase activity in combination with c-MET siRNA or su11274 (figures 7A-C), ascertained through the western-blot test. The double treatment also enhanced the down-regulation of c-MET protein expression (figure 7D). However, the proliferation profile of the combined treatment was not significantly higher than the Bliss independent curve, indicating an additive effect.

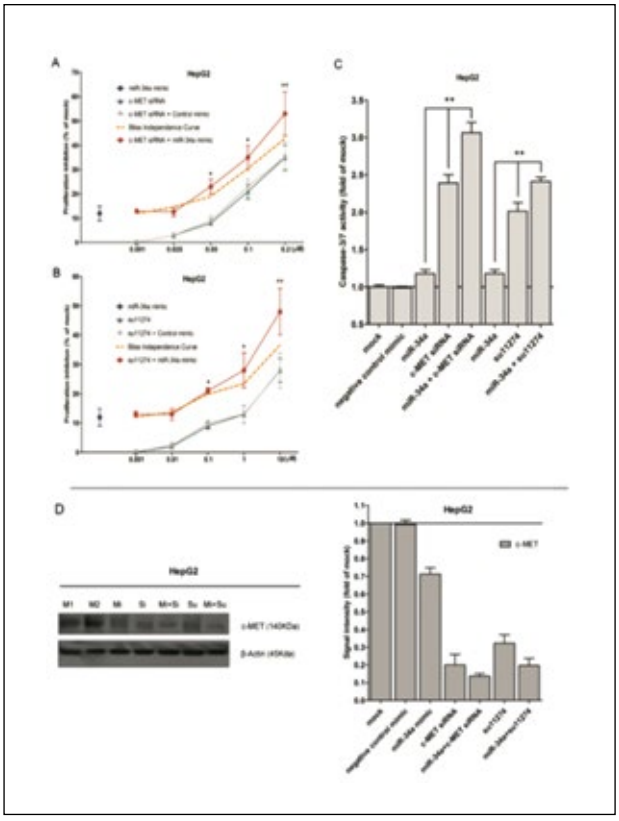

Figure 7: miR-34a enhanced the growth-inhibition effect of c-met targeting agent on HCC HepG2 cells. HepG2 cells were treated with c-met siRNA (A) or su11274 (B) with miR-34a mimetics. MTS is shown in figure 2, and the proliferation-inhibition rate is calculated. The Bliss independence criterion was applied to calculate the theoretical additive effect. (C) caspase-3/7 activity.
Compared with the agent alone, $^{*}P<0.05$, $^{**}P<0.01$, (D) single- or dual-treated c-met protein levels were detected by the western-blot test. The antibodies include c-met and β-actin.

Discussion

It was reported that the down-regulation of miR-34a in a methyl-deficient rat was associated with the occurrence of liver cancer and associated with human viral hepatitis C and hepatitis B infection, alcohol exposure, and metabolic liver disease[5]; in contrast, miR-34a was found to be up-regulated in HCC in comparison to non-neoplastic liver tissue in a chemical-induced HCC F344 rat model[6].

It was reported that the increase in miR-34a in HCC was observed through microarrays and correlated with the progression of the disease from a normal liver, to liver cirrhosis, to the complete occurrence of HCC[7]. In contrast, it was also reported that the down-regulation of miR-34a expression was significant in 19 of 25 (76%) human HCC tissues compared to normal tissue using real-time RT-qPCR[8]. The results of real-time RT-qPCR confirmed that the miR-34a level of HCC was lower than that of the adjacent non-tumour liver tissue in the present study. With respect to the relationship between the expression of miR-34a and clinical pathology, in this study, the expression of miR-34a in the transfer group was down-regulated in comparison to the non-transferred group; in addition, we found that the level of miR-34a was associated with the state of the portal vein tumour embolus. In the case of tumour cell invasion of the portal vein, the expression of miR-34a was reduced. In general, the state of the portal vein tumour embolus reflects the invasion and metastasis of the tumour[9].

Therefore, the results of this study revealed a clear relationship between miR-34a expression and the migration, invasion, and metastasis of HCC. When studying the relationship between miR-34a expression and clinical TNM stages, it was found that the down-regulation of miR-34a is related to the progression of HCC. It is interesting to note that miR-34a expression is of value in the clinical prediction of HCC metastasis and progression. Further, male miR-34a levels are lower than those in women, a fact that has never been reported before, to the best of our knowledge. However, it is necessary to confirm – through a larger cohort study–the relationship between miR-34a levels and sex. For miR-34a mimetics, cell growth inhibition is time-dependent, and cell growth is significantly inhibited 96 hours post-transfection. Hoechst 33342/PI dual-fluorescence staining, caspase-3/7 activity assay, and caspase-3 detection are used to test the effect of miR-34a on the apoptosis of HCC HepG2 cells. The miR-34a mimetic induces apoptosis and caspase activity 72 hours after transfection, and reaches a peak 96 hours after transfection. Therefore, our results show that miR-34a can not only inhibit cell growth, migration, and invasion, but also induce apoptosis.

High expression of c-MET in tumour tissue can lead to angiogenesis, proliferation, cell motion enhancement, cell invasion, and ultimately metastasis[10]. Therefore, c-MET inhibitors are being studied more and more in relation to HCC. For example, oral, selective c-MET inhibitors have shown controlled safety and initial anti-tumour activity in HCC patients in a Phase 1b study[11].

The transfection of miR-34a into HepG2 cells leads to the suppression of the c-MET gene, which is also demonstrated in this study (figure 7D). The addition of miR-34a mimetics to the c-MET siRNA results in a greater effect on cell-growth inhibition and apoptosis induction. A similar combined

effect is observed by the Bliss independent standard and the Biosoft CalcuSyn procedure. Further, a combined effect is also observed when the miR-34a analog is added to the c-MET small-molecule inhibitor su1174. Therefore, we have demonstrated that miR-34a mimetics enhance cell proliferation inhibition through the c-MET siRNA and the c-MET inhibitor su11274.

The present observations demonstrate that miR-34a is a tumour suppressor that plays an important role in the genesis and development of HCC by targeting multiple pathways. On the other hand, the cell growth and invasion inhibition of the miR-34a mimetic and the induction of apoptosis could be associated with its therapeutic effects. The use of miR-34a mimetics with other therapeutic methods, such as a drug targeting of c-MET pathways, could become a new therapy for future HCC.

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