

ORIGINAL ARTICLE

MiRNA-99a directly regulates AGO2 through translational repression in hepatocellular carcinoma

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The regulation network consisting of microRNAs (miRNAs) and their target genes remains largely elusive in hepatocellular carcinoma (HCC), especially the reciprocal loop between specific miRNAs and the miRNA processing machinery. In this study, we found that miR-99a was remarkably decreased in 111 of 152 (73.03%) primary HCC tissues and low-level expression of miR-99a was correlated with low tumor differentiation ($P = 0.001$), liver cirrhosis ($P = 0.015$), poor tumor-free survival ($P = 0.004$) and overall survival ($P = 0.006$) for HCC patients. By restoration of miR-99a, the HCC growth could be considerably inhibited both *in vitro* and *in vivo*. Subsequently, *Argonaute-2* (*Ago2*), a central component of RNA-induced silencing complex, was found to be directly regulated by miR-99a via translational repression. Overexpression of *Ago2* could partly impair the inhibitory effect of miR-99a on HCC cells *in vitro*. Then, we demonstrated that *Ago2* was upregulated in HCC tissues at both RNA and protein levels and the expression of AGO2 protein and miR-99a was negatively correlated within detected HCC tissues ($r = -0.727$, $P = 0.004$). Interestingly, the tumorigenicity of *Ago2*-knockdown HCC cells was severely impaired (4/10 vs 10/10, $P < 0.05$), and this was in contrast to the miR-99a-overexpressing HCC cells. Functionally, the increased AGO2 protein could specifically facilitate oncogenic miR-21 to repress its targeted gene *phosphatase and tensin homolog* (*Pten*) in HCC, whereas leave the regulatory capacity of let-7a on its targeted oncogenes almost unaltered. In summary, our study has revealed a novel pathway for the tumor suppressor miR-99a to control tumor growth in HCC, via its downstream signaling of AGO2/miR-21/PEN. In addition, this study provides potential strategies for HCC therapy by reintroduction of miRNA suppressors.

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INTRODUCTION

Hepatocellular carcinoma (HCC), the primary cancer of liver, ranks as the fifth most prevalent malignancy worldwide and accounts for ~700 000 deaths per year.¹ In the past decades, great advances have been made in the treatment of this disease; however, relapse and metastasis occurred frequently and the 5-year survival rate remains quite low among patients with HCC.² Although it has been identified that infection of hepatitis B virus or hepatitis C virus, intake of aflatoxin B1, alcohol abuse or obesity are the major causes of HCC, the precise mechanisms of HCC oncogenesis remain elusive.^{3,4}

MicroRNAs (miRNAs) are small endogenous noncoding RNA molecules that have been identified as posttranscriptional regulators of gene expression.⁵ The mature miRNAs can be loaded onto RNA-induced silencing complex and guided to their mRNA targets through interactions with Argonaute proteins (including Ago subfamily and Piwi subfamily in mammals), resulting in mRNA degradation or blockade of mRNA translation.⁶ In recent years, the aberrant expression pattern of specific miRNAs has been repeatedly reported and this phenomenon is linked to carcinogenesis and progression in a wide range of cancers, including HCC.^{7–9} Recently, the miRNomes of human normal liver and HCC tissues have been successfully identified

by using full-scale analysis based on deep sequencing, through which the downregulation of let-7, miR-99a, miR-122, miR-101 and miR-199-3p, as well as the upregulation of miR-21 in HCC are largely verified.¹⁰

In this study, we report that miR-99a, a miRNA abundantly expressed in normal liver,¹⁰ is downregulated in HCC tissues compared with the matched noncancerous tissues. Deregulation of miR-99a can serve as an independent risk factor for HCC patients' poor survival. Furthermore, miR-99a suppresses the growth of HCCs by inhibiting *Ago2*. In addition, we found that *Argonaute-2* (*Ago2*) is increased in HCC tissues and can promote oncogenic miR-21 to negatively regulate its target—tumor suppressor phosphatase and tensin homolog (PTEN). Therefore, our study has revealed a novel pathway for HCC suppressor miR-99a (that is, miR-99a/AGO2/miR-21/PTEN) that may facilitate both diagnosis and therapy of this malignancy.

RESULTS

MiR-99a is deregulated in HCC and correlates with the prognosis of HCC patients

We previously revealed that miR-99a was downregulated in a small amount of HCC tissue samples.¹⁰ To further validate this

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finding, the expression profile of miR-99a in 152 pairs of primary HCC tissues and their matched noncancerous tissues was detected using quantitative reverse transcriptase-PCR (RT-PCR). As a result, we found that miR-99a was significantly decreased (≥ 2 -fold) in 111 of 152 (73.03%) primary HCC tissues, whose average relative quantity of tumor/normal was 0.19 ± 0.14 .

We next sought to explore the association between miR-99a expression and pathological characteristics of HCC patients (Table 1). Results showed that the lower expression level of miR-99a was more frequently observed in tumors from patients with lower tumor differentiation or liver cirrhosis ($P=0.001$ and $P=0.015$, respectively, Figure 1a). Furthermore, Kaplan–Meier analysis revealed that low miR-99a level in HCC tissues significantly correlated with the markedly reduced tumor-free survival ($P=0.004$) and overall survival of HCC patients ($P=0.006$), suggesting the important roles of miR-99a in the prognosis of HCC patients (Figure 1b). In addition, the result of Cox proportional hazards regression analysis identified that the deregulation of miR-99a was an independent risk factor for reduced tumor-free survival (hazard ratio 0.443, 95% confidence interval 0.233–0.842; $P=0.013$; Supplementary Table S1) and overall survival of HCC patients (hazard ratio 0.433, 95% confidence interval 0.209–0.897; $P=0.024$; Supplementary Table S2).

MiR-99a suppresses HCC cell growth *in vitro* and *in vivo*

To further investigate the role of miR-99a in HCC, we restored miR-99a expression in Huh7 and Hep3B cells in which miR-99a was greatly reduced (Supplementary Figure S1) via nonreplicative adenoviral vector Ad5-miR-99a that expresses miR-99a (Supplementary Figure S2a). As a result, the growth of transduced cells was significantly decreased and the capacity to form cell colony was remarkably attenuated (Figure 2a) when compared with the cells transduced with the control adenovirus (that is, Ad-Blank). Furthermore, a significant impairment on cell growth was observed in Huh7 and Hep3B cells with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Figure 2b). These results demonstrated that miR-99a inhibited tumor growth *in vitro*.

In order to investigate the antitumor effect of miR-99a *in vivo*, we established a human HCC-bearing mouse model by

subcutaneously implanting Huh7 cells to BALB/c nude mice (nu/nu). By intratumoral injection of Ad5-miR-99a, miR-99a expression in tumor tissues was greatly increased (Supplementary Figure S2b), whereas tumor growth was significantly inhibited as compared with the control ($P<0.001$; Figures 2c and d), and more tumor necrosis was observed (Supplementary Figure S3). Thus, our results demonstrated that the restoration of miR-99a expression could dramatically repress HCC growth, suggesting that Ad5-miR-99a might have considerable potential for HCC gene therapy.

MiR-99a directly targets *Ago2* by translational repression

The target genes of miR-99a that mediated its suppressive functions in HCC pathogenesis were further analyzed. By searching predicted target genes of miR-99a overlapped in TargetScan (<http://www.targetscan.org>) and miRNA database (<http://www.microrna.org/microrna/getMirnaForm.do>), and subsequently confirmed in RegRNA (<http://regrna.mbc.nctu.edu.tw/index1.php>), we found that *Ago2* (also known as *Eif2c2*) might be a novel candidate gene targeted by miR-99a (Figure 3a). Interestingly, the putative recognized sites of miR-99 family within the 3'-untranslated region (UTR) of *Ago2* were broadly conserved among vertebrates (Figure 3a). When the miR-99a expression was restored by transduction with Ad5-miR-99a in Huh7 and Hep3B cells, the expression level of AGO2 protein was greatly decreased (Figure 3b), whereas the *Ago2* mRNA was not significantly reduced (Figure 3c), indicating that miR-99a may inhibit *Ago2* via translation repression rather than mRNA degradation.

To verify whether *Ago2* is a direct target of miR-99a, a dual-luciferase reporter containing the 3'-UTR of *Ago2* was constructed and used to transfect Huh7 cells. As shown in Figure 3d, the luciferase activity (Firefly Luciferase) was significantly repressed by co-transfection with a vector expressing miR-99a, whereas it was recovered by reducing the expression of miR-99a with the miR-99a inhibitor. In contrast, when co-transfection was performed with the miR-99a expression vector and the dual-luciferase reporter containing an artificial 3'-UTR of *Ago2*, in which the putative miR-99 target sites were mutated, the luciferase activity was not distinctly changed. Together, these results proved that

Table 1. Correlation analysis between miR-99a expression and clinical pathological characteristics

Variable	miR-99a subgroup ^a		χ^2	P-value
	Low	High		
All cases	111	41		
Age (year), > 50:≤50	53:58	18:23	0.178	0.673
Gender, male:female	90:21	33:8	0.007	0.934
AFP (μg/l), > 20:≤20	75:36	22:18	2.022	0.155
Serum bilirubin (μmol/l), > 17:≤17	31:80	7:34	1.881	0.17
Serum albumin (g/l), > 40:≤40	84:27	30:11	0.100	0.752
ALT (U/l), > 40: ≤40	47:64	16:25	0.136	0.712
HBs antigen, positive:negative	84:27	34:7	0.907	0.341
HBe antigen, positive:negative	33:78	13:28	0.055	0.814
Tumor size (cm), > 5:≤5	53:58	22:19	0.418	0.518
Tumor number, single:multiple	75:36	32:9	1.578	0.209
Encapsulation, complete:no	60:51	18:23	1.235	0.266
Liver cirrhosis, with:without	89:22	25:16	5.889	0.015*
Differentiation, I + II:III + IV	29:82	23:18	11.950	0.001*
Microvascular invasion, yes:no	26:85	8:33	0.264	0.608
TNM stage, I:II:III	39:31:41	21:11:9	4.019	0.134
BCLC stage, A:B:C	33:59:19	19:19:3	4.673	0.097

Abbreviations: AFP, α -fetoprotein; ALT, alanine aminotransferase; BCLC, Barcelona Clinic Liver Cancer; HBe, hepatitis Be; HBs, hepatitis B surface; TNM, tumor node metastasis. Data are expressed as ratios. * $P<0.05$ by χ^2 test. ^aThe relative quantitative expression level was used. Low expression of miR-99a in 111 patients was classified as hepatocellular carcinoma (HCC)/nontumor ratio of ≤ 0.5 . High miR-99a expression in 41 patients was classified as HCC/nontumor ratio of > 0.5 .

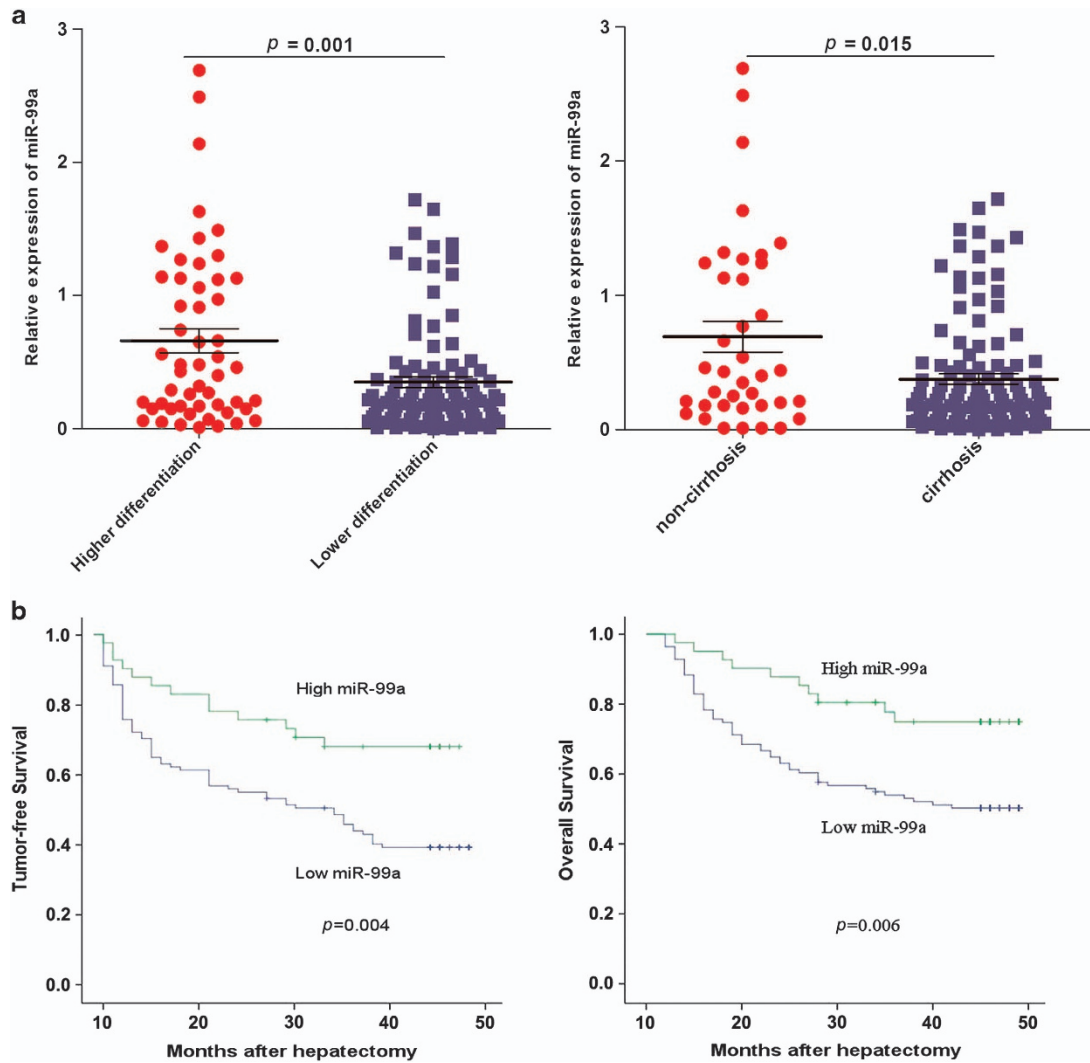


Figure 1. miR-99a is correlated with tumor differentiation, liver cirrhosis and patients' outcome in HCC. **(a)** Relative lower expression of miR-99a preferably observed in HCC tumors from patients with lower tumor differentiation or liver cirrhosis. **(b)** Kaplan–Meier survival curves of tumor-free survival and overall survival according to relative expression level of miR-99a in primary HCC tissues. The median value of this ratio was chosen as the cutoff point.

endogenous *Ago2* was targeted and regulated by miR-99a, mainly through translation repression.

Furthermore, we overexpressed AGO2 with Ad5-Ago2, a recombinant adenoviral vector carrying an expression cassette of 3'-UTR-negative *Ago2* (Figure 3e). When Ad5-Ago2 was co-transduced at defined multiplicity of infection (MOI) with Ad5-miR-99a at a gradient of MOI into Huh7 and Hep3B cells respectively, the inhibitory effect of Ad-miR-99a could be impaired lower on these HCC cells (Figure 3f). However, the ability of *Ago2* overexpression was insufficient to rescue the viability of miR-99a effects when the used MOI of Ad-miR-99a was increased, indicating that miR-99a could also inhibit HCC growth via regulating its other target genes.

Ago2 is upregulated in HCC and negatively correlated with miR-99a. *Ago2* plays an important role in miRNA-mediated posttranscriptional regulation, but its expression profile in HCC remains largely unknown. Thus, the expression profile of *Ago2* in HCC cell lines and primary HCC tissues was investigated by RT-PCR analysis. As a result, overexpression of *Ago2* was found in 5 of 6 detected HCC cells (Supplementary Figure S4) and 105 of 152 cancerous tissues

as compared with their paired noncancerous tissues. However, no distinct correlation was revealed between the expression of miR-99a and *Ago2* at the RNA level ($r = -0.077$, $P = 0.117$, Figure 4a).

Then, protein expression of *Ago2* was examined by immunohistochemistry in 20 pairs of primary HCC tissues with different levels of miR-99a. Among the 10 HCC tissues with lower miR-99a, AGO2 protein was found to be overexpressed (Figure 4b). For the other 10 HCC samples with higher miR-99a, no significant difference of AGO2 expression was detected between the cancerous tissues and the matched noncancerous tissues in 7 samples (Figure 4b). This result was further confirmed by western blotting analysis in which 12 pairs of HCC samples were examined ($r = -0.727$, $P = 0.004$, Figures 4c and d). Taken together, these data showed that AGO2 was overexpressed in HCC tissues and the protein expression was negatively correlated with the expression level of miR-99a that validated that *Ago2* was a novel target gene directly regulated by miR-99a via translation repression.

Knockdown of *Ago2* distinctly inhibits HCC growth *in vivo*

To identify the role of *Ago2* in HCC pathogenesis, the expression of *Ago2* was decreased by lentivirus-mediated RNA interference

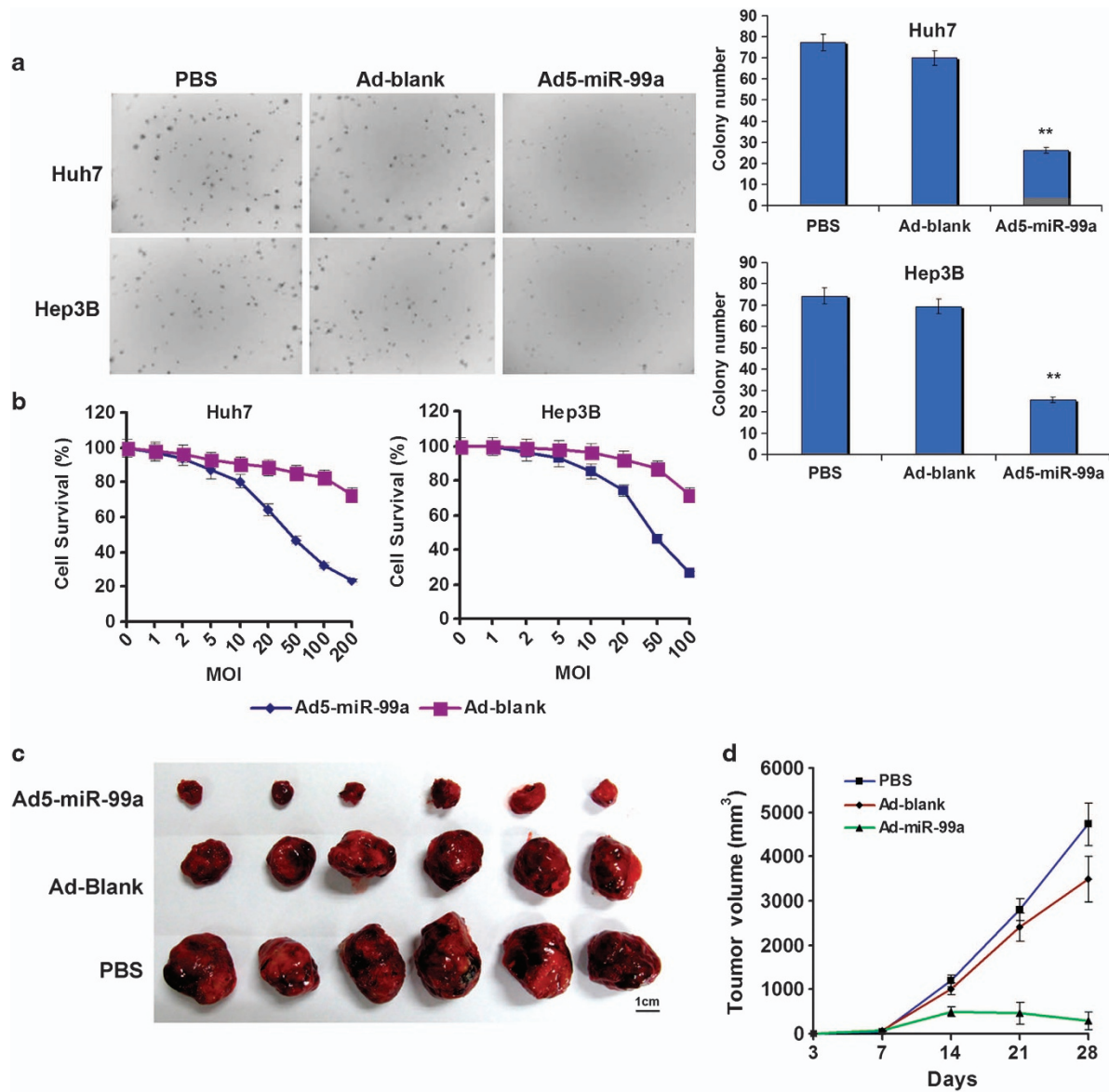


Figure 2. Overexpression of miR-99a inhibits HCC growth *in vitro* and *in vivo*. (a) The influence of miR-99a restoration on colony formation of HCC cells. Shown was the image 15 days post plating (left) and the quantitative results (right). (b) The killing effect on HCC cells by reintroduction of miR-99a *in vitro* as examined by MTT assay. (c, d) The tumor morphology and growth curve of subcutaneous Huh7 xenograft after treatment, respectively. Scale bar = 1 cm.

(Supplementary Figure S5). As shown in Figures 5a and b, the abundance of *Ago2* was successfully downregulated at both RNA and protein levels in HCC Huh7 cells stably expressing *Ago2*-specific small interfering RNA (siRNA). However, not distinct alteration on cell growth was observed when *Ago2* was downregulated *in vitro* (Figure 5c).

Then, *Ago2*-knockdown Huh7 cells were subcutaneously injected into BALB/c nude mice to further analyze the influence of *Ago2* on HCC growth *in vivo*. Surprisingly, nearly half of the mice (4 of 10) in Huh7 *Ago2*-knockdown group failed to generate xenografts, whereas all the mice in control group developed detectable tumors 2 weeks post transplantation (10 of 10), indicating the tumorigenic ability was greatly impaired by *Ago2* knockdown in Huh7 cells ($P < 0.05$). Furthermore, the tumors derived from *Ago2*-knockdown Huh7 cells were much smaller compared with those in the control group transduced with nonsilencing siRNA ($P < 0.001$; Figures 5d and e). The decreased expression of AGO2 and Ki67 in tumors derived from

Ago2-knockdown Huh7 cells was revealed by immunohistochemistry analyses (Figure 5f). These results indicated that the suppression of *Ago2* might greatly inhibit HCC growth *in vivo*.

Ago2 facilitates miR-21 to repress its targeted gene *Pten*

The capacity of *Ago2* knockdown to inhibit HCC growth *in vivo* promoted us to further investigate the mechanism of *Ago2* in HCC carcinogenesis. Considering *Ago2* functions in miRNA-mediated gene silencing, we examined the alteration of miRNA-target gene pairs upon *Ago2* knockdown and miR-99a overexpression, including oncomiRs (for example, miR-21), miRNA suppressors (for example, let-7) and their targets. As detected by RT-PCR, miR-21a was upregulated in HCC cells (Huh7 and Hep3B) but decreased in both *Ago2*-knockdown cells and miR-99a-overexpressing cells, whereas let-7a was almost silenced and this silencing was maintained in both *Ago2*-knockdown and miR-99a-overexpressing HCC cells (Supplementary Figure S6). The results of

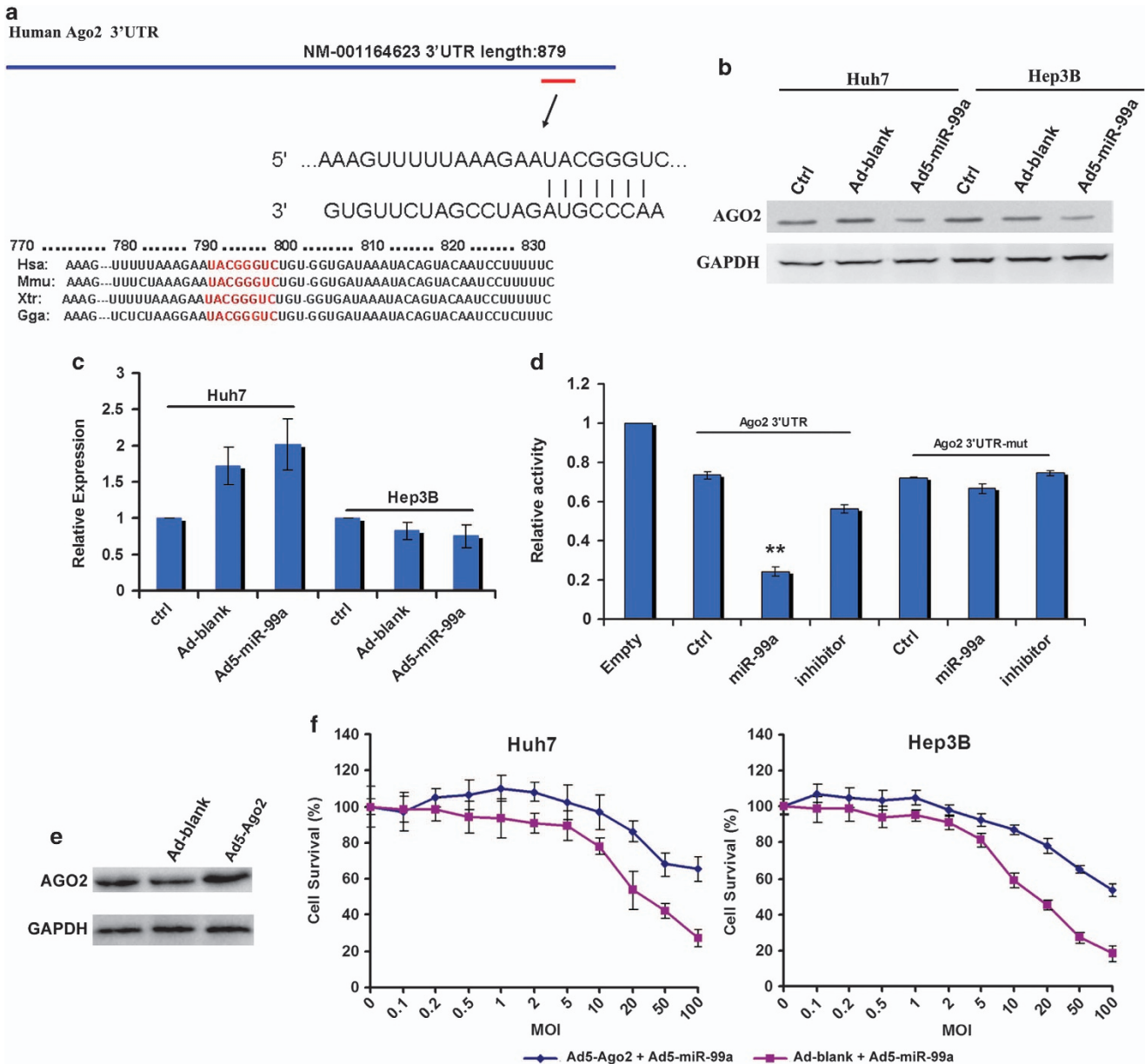


Figure 3. *Ago2* is directly targeted by miR-99a. **(a)** Schematic representation of the 3'-UTR of *Ago2*. Red bars show putative miR-99a target sequences. Sequence alignment of the target site among vertebrate, including amphibians (*Xenopus* (Xtr)), birds (*Gallus* (Gga)) and mammals (human (Hsa) and mouse (Mmu)), is shown below. **(b, c)** The capacity of miR-99a on regulating *Ago2* as detected by western blot and qRT-PCR. Ad-blank, the control adenovirus without transgene; Ad5-miR-99a, the recombinant adenovirus expressing miR-99a; Ctrl, untreated HCC cells. **(d)** Luciferase assay for the regulation capacity of miR-99a on *Ago2*. Both wild and mutated (mut) *Ago2* 3'-UTR (the putative seed binding sequence was mutated from TACGGGTC to ATGCCAG) was used. Empty, Huh7 cells transfected with the empty dual-Luc assay vector, psiCHECK-2; Ctrl, Huh7 cells transfected with psiCHECK-2 containing *Ago2* 3'-UTR (psiCHECK2-*Ago2* UTR) or mutated *Ago2* 3'-UTR (psiCHECK2-*Ago2* mutUTR), respectively. miR-99a, Huh7 cells co-transfected with miR-99a mimic and psiCHECK2-*Ago2* UTR/psiCHECK2-*Ago2* mutUTR; inhibitor, Huh7 cells co-transfected with miR-99a inhibitor, miR-99a mimic and psiCHECK2-*Ago2* UTR/psiCHECK2-*Ago2* mutUTR. * $P < 0.05$; ** $P < 0.01$. **(e)** Overexpression of AGO2 protein by transduction with Ad5-Ago2 (MOI = 5), a recombinant adenoviral vector expressing *Ago2*. **(f)** The counteraction of AGO2 overexpression on miR-99a killing effect on HCC cells as examined by MTT assay.

western blotting showed that the expression of PTEN (targeted by miR-21) was increased, whereas those of NRAS, HMG2 and C-MYC (targeted by let-7) were not distinctly altered in either *Ago2*-knockdown or miR-99a-overexpressing HCC cells (Figure 6a and Supplementary Figure S7), suggesting an indirect regulation of miR-99a on PTEN.

Interestingly, when the let-7 expression was restored by the recombinant adenovirus expressing let-7, NRAS protein could be substantially deregulated, whereas the deregulation was

attenuated if *Ago2* was knocked down (Figure 6b). In contrast, in *Ago2*-knockdown Huh7 cells, PTEN protein was upregulated, whereas the upregulation was considerably disrupted when *Ago2* was restored via Ad5-Ago2 (Figure 6c). In consistency with previously reported result,¹⁰ we found that the tumor-suppressor miRNA let-7a was remarkably downregulated, whereas the oncomiR miR-21 was distinctly upregulated in a list of HCC cell lines (Supplementary Figure S8). Thus, these results had revealed a novel regulation mode of *Ago2* in HCC in which upregulation of

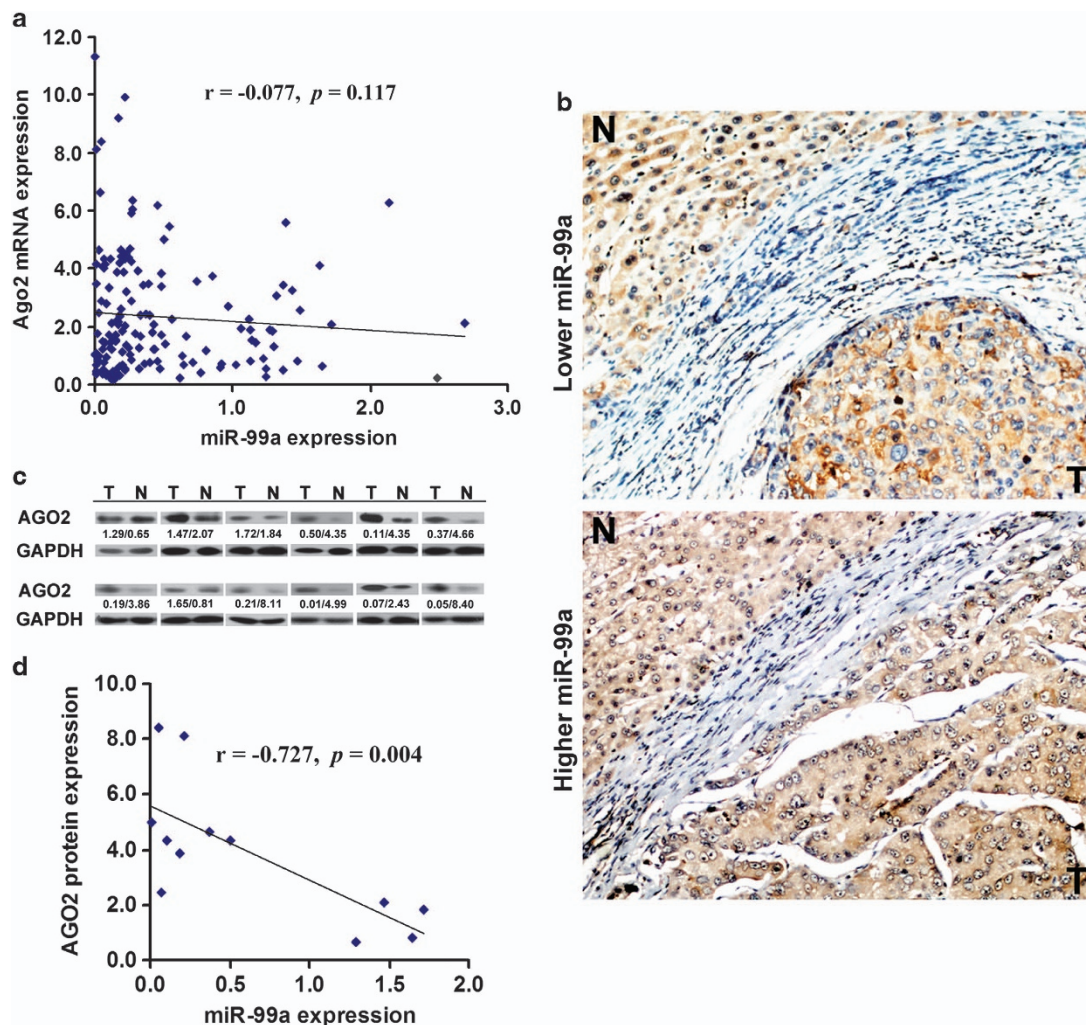


Figure 4. Ago2 is upregulated in HCC and negatively correlated with miR-99a. **(a)** The correlation between relative expression of miR-99a and Ago2 mRNA in HCC tissues as determined by qRT-PCR. **(b)** Increased AGO2 protein as analyzed by immunohistochemistry in cancerous tissues. The patient serial number was 30 and 6, respectively. **(c)** Increased AGO2 protein as detected by western blotting. Expression ratio was calculated by normalizing AGO2 to total protein levels in each case. The value of relative expression of miR-99a/AGO2 protein is shown below. The patient serial number was 6, 8, 9, 11, 30, 41, 53, 68, 73, 90, 121, and 132, respectively. N, matched noncancerous control; T, HCC tissue. **(d)** The correlation between expression of miR-99a and AGO2 protein in 12 pairs of HCC samples mentioned above.

Ago2 specifically facilitates the increased oncomiRs miR-21 to repress its targeted tumor-suppressors PTEN, whereas it leaves the regulatory capacity of the decreased tumor-suppressor miRNAs let-7 on their oncogenic targets at lower level, thereby promoting HCC progression and carcinogenesis (Figure 6d).

DISCUSSION

We have previously reported that miR-99a is the sixth most abundantly expressed miRNA in human normal liver, but it is deregulated in a high proportion of primary HCC tissues relative to their matched noncancerous tissues via deep sequencing.¹⁰ The downregulation of miR-99ab/100 family was also observed in HCC by other groups^{11–14} and in other types of cancers,^{15–19} including prostate cancer, oral cancer, head and neck squamous cell carcinoma, lung adenocarcinoma and renal cell carcinoma, suggesting an important role of miR-99 in malignant tumors. In this study, using an independent cohort of HCC patients, miR-99a was found to be distinctly decreased in primary HCC tissues and significantly correlated with both tumor-free survival ($P = 0.004$) and overall survival in HCC patients ($P = 0.006$). Furthermore, our

result indicated that miR-99a downregulation was more frequently observed in tumors from patients with liver cirrhosis ($P = 0.015$) or lower tumor differentiation ($P = 0.001$), in accord with the finding that miR-99ab/100 dysregulation is an early event maintained along with HCC progression.¹³ Furthermore, the correlation between miR-99a expression and tumor differentiation may be responsible for the poor survival of HCC patients with lower level of miR-99a.

To date, a number of targets for miRNA-99 have been experimentally validated and most of them are oncogenes. Notable targets include *Igf1r* (insulin-like growth factor 1 receptor), *mTOR* (mechanistic target of rapamycin), *Mtmt3* (Myotubularin-related protein 3) and two SWI/SNF chromatin remodeling factor *Smarca5* and *Smarca4*.^{14–21} Here, a novel target gene, *Ago2*, was found to be directly regulated by miR-99a via translational repression and the expression level of AGO2 protein in primary HCC tissue was negatively correlated with miR-99a expression. Thus, a new way that miR-99a functions as a tumor suppressor in HCC has been revealed.

Ago2, also known as *Eif2c2*, is the central component of RNA-induced silencing complex, the protein complex responsible

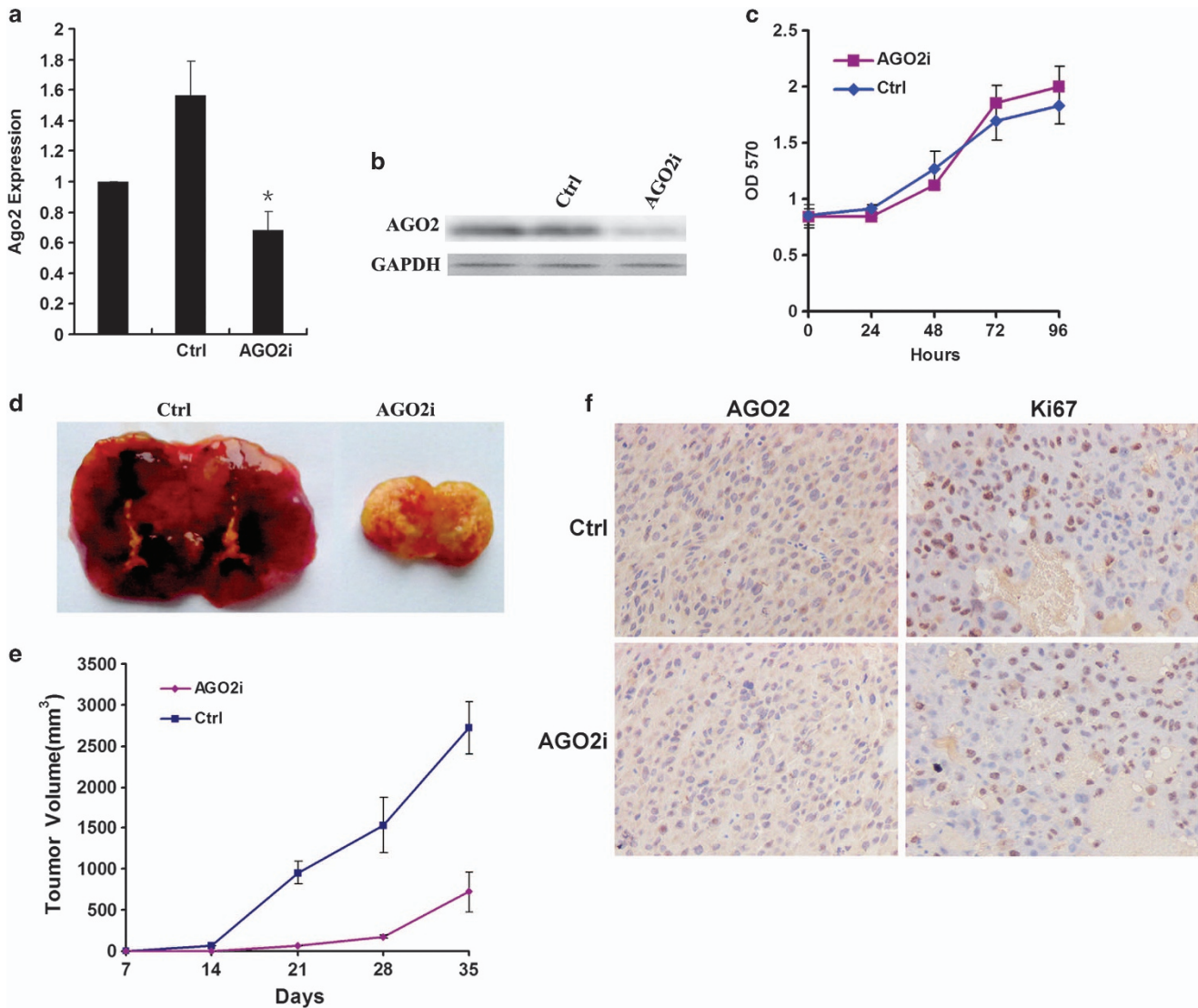


Figure 5. *Ago2* knockdown distinctly inhibits HCC tumor growth *in vivo*. (**a, b**) Downregulation of *Ago2* through lentivirus-mediated RNA interference (RNAi) as detected by RT-PCR and western blot. Ctrl and AGO2i indicated Huh7 cells stably expressing control siRNA and *Ago2*-specific siRNA, respectively. (**c**) The effect on cell proliferation of *Ago2*-knockdown as measured by MTT assay. Data were obtained from three independent experiments and shown as mean \pm s.d. (**d, e**) The influence on tumor growth by *Ago2* knockdown *in vivo*. The growth curve and tumor morphology of *Ago2*-knockdown Huh7 xenograft. (**f**) AGO2 and Ki67 expression examined by immunohistochemistry ($\times 200$). Ctrl and AGO2i indicated Huh7 cells stably expressing control siRNA and *Ago2*-specific siRNA, respectively.

for gene silencing.^{22,23} In addition, *Ago2* has been found in Dicer-independent miRNA biogenesis through its catalytic activity.^{24–25} The *Ago2* gene belongs to an evolutionarily conserved family that is shared among not only eukaryotes, but also archaea and certain bacteria such as *Aquifex aeolicus*.²³ In mice, disruption of *Ago2* leads to embryonic lethality early in development after the implantation.²⁶ Despite the fact that *Ago2* expression is regulated by epidermal growth factor receptor and mitogen-activated protein kinase signaling at the transcriptional level,²⁷ little is known about the posttranslational regulatory networks of *Ago2*. Previously, Dicer, another key component of the miRNA processing machinery, has been found to be regulated by miR-103/107 and miRNA-130a,^{28,29} forming a regulatory feedback loop that mediated tumor metastasis and survival. In this study, *Ago2* is demonstrated to be directly targeted by miR-99a and involved in tumor growth control, and this is the first report revealing the miRNA regulator of *Ago2*.

Compared with individual miRNA, the deregulation of miRNA processing machinery itself can exert more profound effects on miRNA profile.³⁰ Therefore, to accurately maintain the expression levels of miRNA machinery (for example, Dicer, Drosha and AGO2) is crucial for normal cell activities. In recent years, the dysregulation of Dicer and Drosha has been intensively discussed and linked to the carcinogenesis of several cancers, including HCC.^{31–38} Merely, the opposite situations have been revealed between patient's outcome and the expression levels of Dicer and Drosha among different cancer types. As for *Ago2*, overexpression of AGO2 protein was found within 31 pairs of primary HCC samples during the period that this paper was prepared, and it was found that increased AGO2 can promote tumor growth, migration and metastasis.³⁹ In this study, the upregulation of *Ago2* is validated in a large sample of primary HCC tissues (that is, 152) relative to their matched noncancerous tissues at both RNA and protein levels, and this was partly associated with reduced expression of miR-99a. It is noteworthy that the effect of *Ago2* knockdown on

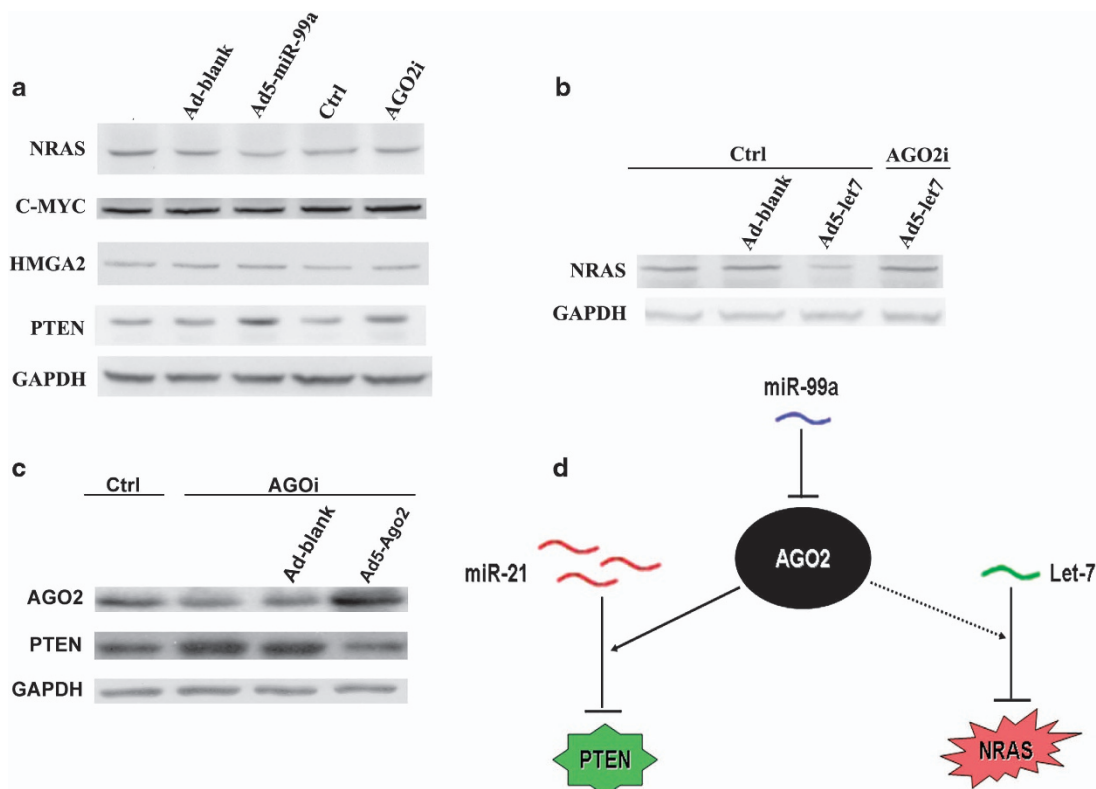


Figure 6. *Ago2* facilitates miR-21 to repress its targeted gene *Pten*. (a) The expression of a list of cancer-associated proteins as analyzed by western blot. GAPDH served as loading control. Ctrl and AGO2i indicated Huh7 cells stably expressing control siRNA and *Ago2*-specific siRNA, respectively. Ad-blank, the control adenovirus without transgene; Ad5-miR-99a, the recombinant adenovirus expressing miR-99a. Data are shown as representative of three independent experiments. (b) The expression of NRAS protein under different AGO2 conditions as determined by western blot. Ctrl and AGO2i indicated Huh7 cells stably expressing control siRNA and *Ago2*-specific siRNA, respectively. Ad5-let7a, the recombinant adenovirus expressing let-7a. (c) The expression of PTEN protein under different AGO2 conditions as determined by western blot. Ctrl and AGO2i indicated Huh7 cells stably expressing control siRNA and *Ago2*-specific siRNA, respectively. Ad5-Ago2, the recombinant adenovirus expressing *Ago2*; Ad-blank, the control adenovirus without transgene. (d) A mode of the regulatory network of miR-99a and *Ago2*. *Ago2* is targeted by miR-99a. On the condition that the oncomiRs (for example, miR-21) are upregulated but the tumor suppressors (for example, let-7a) are downregulated, the increased *Ago2* can promote miR-21 to repress PTEN, whereas leave the regulation capacity of let-7 on NRAS at low level.

cell proliferation *in vitro* and *in vivo* was varied. A plausible reason for this phenomenon is that *Ago2* knockdown can inhibit angiogenesis *in vivo* that is hardly displayed *in vitro* (Jin H, *et al*, unpublished data).

Although several miRNAs are upregulated in specific tumors, a global reduction of miRNA abundance appears a common trait of human cancers.^{7,28,40} Previously, a list of miRNAs suppressors have been found downregulated in HCC, along with the upregulation of some oncoMiRs (for example, miR-21 and miR-221).^{8–10} Among them, miR-122, miR-199a-3p, miR-101, let-7 and miR-99a are the first, third, fourth, fifth and sixth most abundant miRNAs in normal human liver, respectively, accounting for ~71.4% of the miRNome.¹⁰ Thus, the global downregulation of miRNA suppressor also applies to HCC. On this basis, the upregulation of *Ago2* has aggravated the homeostasis of regulatory networks mediated by miRNA, wherein the capacities of oncoMiRs (for example, miR-21) to regulate tumor suppressors (for example, PTEN) are specifically enhanced, whereas those of miRNA suppressors (for example, let-7) on oncogenes (for example, NRAS) is unaltered. The superposition of these two factors promotes the progression of HCC. In turn, the oncogenic effect exerted by the upregulation of *Ago2* in HCC may have also benefitted gene therapy by reintroduction of miR-99a present and miR-199-3p previous.¹⁰ Therefore, our study has implied the promising prospect of miRNA therapy for HCC by restoring the expression of miRNA suppressors.

MATERIALS AND METHODS

Patients and cell lines

A total of 152 pairs of primary HCC tissues and the matched adjacent noncancerous tissues were randomly selected from patients undergone surgical resection at the Eastern Hepatobiliary Surgery Hospital. All samples were collected with the written informed consent of HCC patients and the experiments were approved by the Institutional Review Board of Eastern Hepatobiliary Surgery Hospital Ethics Committee. For the enrolled HCC patients, no local or systemic treatment was conducted before operation. The tumor differentiation was defined according to the Edmondson grading system, in which a higher grade indicated a lower differentiation state.⁴¹ The tumor staging was defined as described previously.⁴² The detailed clinical characteristics of all patients are listed in Table 1. Human HCC cell lines (HepG2, HepG2.15, Hep3B, PLC/PRF/5, Huh7 and SMMC-7721), human embryonic liver cell line (WRL-68) and human embryo kidney cell line (HEK293) were obtained and cultured as described previously.^{42,43}

Quantitative RT-PCR

Total RNA of all test tissues or cells samples was isolated using a miRNeasy mini kit (Qiagen, Valencia, CA, USA). Then, 1 μ g RNA of each sample was reverse transcribed to complementary DNA using a miScript Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. For quantitative PCR analysis, aliquots of complementary DNA were amplified using miScript SYBR Green PCR Kit (Qiagen) and performed on 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The miR-99a expression of all test samples was determined by normalizing to that of

U6. The *Ago2* expression was measured via similar method except for using *Gapdh* as an endogenous control. All reactions were performed in triplicate. The sequences of used primers were listed in Supplementary Table S3.

Luciferase assays

Mutagenesis of the putative miR-99-binding sites within the 3'-UTR of human *Ago2* was performed using overlapped PCR. Then, the natural and modified 3'-UTRs of *Ago2* were cloned into psiCHECK-2 (Promega, Madison, WI, USA), respectively. Cell culture, transfection and luciferase assay were performed as previously described.^{10,43} The miR-99a-specific mimic and inhibitor were used to upregulate or suppress the expression of exogenous miR-99a according to the manufacturer's instructions (Life Technologies, Carlsbad, CA, USA).

Virus construction

The replication-defective recombinant adenovirus expressing miR-99a and *Ago2* was constructed according to the procedure described previously.⁴³ For lentivirus-based shRNA knockdown systems, five RNA interference candidate target sequences to human *Ago2* (Supplementary Table S4) were designed and the oligonucleotides encoding the candidate *Ago2*-siRNA sequence and a loop sequence separating the complementary domains were synthesized and inserted into the lentiviral transfer vector pGC-LV (Genechem, Shanghai, China), respectively. Among them, *Ago2*-Si3 had the best interference efficiency in HEK-293T cells cotransfected with *Ago2*-overexpressing vector and siRNA expression constructs revealed by western blot and was selected to knock down the endogenous *Ago2* in HCC cells. The nonsilencing siRNA was used as a control. Then, the obtained lentiviral transfer vector expressing *Ago2*-specific shRNA was cotransfected with the packaging plasmids pHelper 1.0 and pHelper 2.0 into HEK-293T cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). At 40 h post transfection, the lentiviral particle was harvested from the medium, concentrated by ultracentrifugation and titrated on HEK-293T cells as previously described.⁴⁴

MTT assay

To examine the therapeutic effect of miR-99a restoration, Huh7 and Hep3B cells were planted at a density of 5×10^3 in 96-well plates and infected with Ad5-miR-99a or Ad-blank at a gradient of MOI. In the rescue experiments, Ad5-Ago2 or Ad-blank at 5 MOI was co-transduced with Ad5-miR-99a at a gradient of MOI into Huh7 and Hep3B cells. On the day of harvest, 100 μ l of spent medium was replaced with an equal volume of fresh medium containing MTT 0.5 mg/ml. Plates were incubated at 37 °C for 4 h, and then the medium was replaced by 100 μ l of dimethyl sulfoxide (Sigma, St Louis, MO, USA) and plates shaken at room temperature for 10 min. The absorbance was measured at 570 nm.

Proliferation and colony formation assays

Cells (5×10^3) suspended in 1 ml 0.3% top agarose were plated onto 2 ml 0.6% base agarose in six-well plate and maintained for 15 days. Then, complete medium (500 μ l) was added every 5 days. On day 15, colonies were counted and photographed by light microscopy. For cell proliferation assay, 1×10^4 Huh7 or *Ago2*-knockdown Huh7 cells were planted in 96-well plates, and then cells were harvested at a series of time point and detected with MTT assay. Data were obtained from three independent experiments.

Western blot

Protein lysates from cell lines or tissues were prepared in lysis buffer and centrifuged at 12000 *g* at 4 °C. The procedure of western blotting was according to the procedure described previously.⁴³ Primary antibodies used were rabbit anti-AGO2 (Abcam, Cambridge, UK), rabbit anti-PTEN (SAB, Pearland, TX, USA), rabbit NRAS (Abgent, San Diego, CA, USA), rabbit HMGA2 (Abgent), rabbit C-MYC (Santa Cruz, CA, USA) and mouse anti-GAPDH (Santa Cruz Ltd.).

In vivo assay

All animal experiments were undertaken in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of Second Military Medical University, Shanghai. Human HCC-bearing male nude mice with Huh7 cells were used for evaluating the antitumor effect of miR-99a *in vivo*.

To prepare the subcutaneous model, mice were injected subcutaneously in the right flank with 1×10^7 Huh7 cells in matrigel (injection volume of 100 μ l). At 2 weeks post injection, mice with the xenografts tumor were randomly distributed into three groups ($n = 6$ per group) and injected intratumorally with Ad-blank (at a dose of 5×10^8 plaque-forming units (PFUs)), Ad5-miR-99a (at a dose of 5×10^8 PFUs) or placebo five times (one time every other day), respectively. Tumor volumes (volume = $(W^2 \times L)/2$; W, width; L, length, in cubic mm) were measured once weekly after the first adenovirus injection on day 1. To evaluate the effect of *Ago2* knockdown *in vivo*, Huh7 cells stably expressing *Ago2*-specific siRNA or nonsilencing-siRNA were injected subcutaneously (1×10^7 /mouse, $n = 10$ per group). At 2 weeks post injection, tumor volumes were measured once weekly if the xenografts tumors were formed.

Immunohistology

Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded tissue sections using ABC (avidin biotin-peroxi-dase complex) method. The used primary antibodies were AGO2 (1:100, Abcam) and Ki67 (1:200, Sigma). The quantification of immunohistology for primary tissues was performed according to the procedure previously described with minor modification.⁴⁵ In brief, positive reaction was determined based on the presence and intensity of cytoplasm immunoreaction deposits. Tissues were graded using the following criteria: 0, low <25% of total cells; 1, 25–50% of total cells; 2, 50–75% of total cells; 3, >75% of total cells. Two pathologists without knowledge regarding patients' clinical characteristics were independently responsible for the scoring. The concordance between scores from different sections of the same tissue was >90%. All discrepancies in scoring were reviewed, and a consensus was reached.

Statistical analysis

All data are presented as mean \pm s.d. unless otherwise stated. The independent Student's *t*-test was used to analyze the variation of two selected groups and Pearson's χ^2 test was used to analyze the correlation between two clinical pathologic parameters. $P < 0.05$ was considered significant statistically and is marked with an asterisk. $P < 0.01$ was considered highly significant statistically and is marked with a double asterisk. All statistical analyses were performed with SPSS version 18.0 software (IBM, Chicago, IL, USA).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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