

Methylation-mediated repression of microRNA-129-2 suppresses cell aggressiveness by inhibiting high mobility group box 1 in human hepatocellular carcinoma

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Keywords: miR-129-2, methylation, high mobility group box 1, invasion, hepatocellular carcinoma

Received: November 22, 2015

Accepted: April 16, 2016

Published: May 15, 2016

ABSTRACT

Aberrant expression of microRNAs (miRNAs) and its dysfunction have been revealed as crucial modulators of cancer initiation and progression. MiR-129-2 has been reported to play a tumor suppressive role in different human malignancies. Here, we demonstrated that miR-129-2 was significantly decreased in hepatocellular carcinoma (HCC) tissues and cell lines. Furthermore, miR-129-2 was expressed at significant lower levels in aggressive and recurrent tumor tissues. Clinical analysis indicated that miR-129-2 expression was inversely correlated with venous infiltration, high Edmondson-Steiner grading and advanced tumor-node-metastasis (TNM) stage in HCC. Notably, miR-129-2 was an independent prognostic factor for indicating overall survival (OS) and disease-free survival (DFS) of HCC patients. Ectopic expression of miR-129-2 inhibited cell migration and invasion *in vitro* and *in vivo*. Furthermore, we confirmed that high mobility group box 1 (HMGB1) was a direct target of miR-129-2, and it abrogated the function of miR-129-2 in HCC. Mechanistic investigations showed that miR-129-2 overexpression inhibited AKT phosphorylation at Ser473 and decreased the expression of matrix metalloproteinase2/9 (MMP2/9). Upregulation of p-AKT abolished the decreased cell migration and invasion induced by miR-129-2 in HCC. Whereas inhibition of Akt phosphorylation significantly decreased HMGB1-enhanced HCC cell migration and invasion. Moreover, we found that miR-129-2 was downregulated by DNA methylation, and demethylation of miR-129-2 increased miR-129-2 expression in HCC cells and resulted in significant inhibitory effects on cell migration and invasion. In conclusion, miR-129-2 may serve as a prognostic indicator for HCC patients and exerts tumor suppressive role, at least in part, by inhibiting HMGB1.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common aggressive malignancies and the third-most common cause of cancer-related death worldwide [1]. Despite the advances in diagnosis and treatment, the majority of patients with HCC have an extremely dismal prognosis due to the high frequency of tumor recurrence or distant metastasis after surgical resection [2]. Therefore, a better understanding of the underlying molecular

mechanisms involved in the pathogenesis and progression of HCC is extremely important for the development of more effective therapeutic approaches for the treatment of patients with HCC.

MicroRNAs (miRNAs) are an abundant group of endogenous noncoding RNA molecules of about 22 nucleotides in length [3], which regulate protein-coding gene expression at the post-transcriptional level by base pairing to complementary sites within the 3'-untranslated region (UTR) of target mRNAs and targeting mRNAs

for degradation or translational repression [4]. Thus, aberrant expression of miRNAs are involved in tissue morphogenesis, cell processes such as proliferation, cell cycle, apoptosis, migration, invasion and major signaling pathways [5–11]. Previous studies have identified the critical role of miRNAs in human cancers and suggest that deregulation of miRNAs is involved in tumor development and progression by modulating the expression of oncogenes or tumor suppressors [12]. Recently, accumulating evidence demonstrated that epigenetic aberration may contribute to the down-regulation of tumor suppressing miRNAs [13, 14]. Among which, DNA methylation was frequently observed in the promoter CpG island regions of genes [15]. We therefore speculate whether methylation alteration may occur in miRNAs, resulting in deregulation of its target genes in cancer cells.

MiR-129-2 gene is located in a canonical CpG island on chromosome 11, which was found to be frequently hypermethylated in gastric cancer [16], endometrial cancer [17] and HCC [18]. It plays a critical role in the progression, such as proliferation, migration, invasion of renal cell carcinoma [19], gastric cancer [16], endometrial cancer [17], esophageal carcinoma [20], glioma cells [21] and lung cancer [22]. Furthermore, miR-129-2 suppresses proliferation and migration of esophageal carcinoma cells through downregulation of SOX4 expression [20] and epigenetic repression of miR-129-2 leads to overexpression of SOX4 in gastric cancer [16]. In addition, miR-129-2 function as a tumor suppressor in glioma cells by targeting HMGB1 and is downregulated by DNA methylation [21]. Intriguingly, our studies have demonstrated that HMGB1 was elevated and promoted the migration and invasion of HCC. Moreover, miR-129-2 was methylation-mediated downregulated in HCC [23, 24]. However, the clinical significance and regulatory mechanism between miR-129-2 and HMGB1 are poorly understood.

In this study, we identify miR-129-2 as downregulated in both HCC tissues and cells and a critical suppressor of HCC cells migration and invasion both *in vitro* and *in vivo*. We demonstrate that miR-129-2 is an independent prognostic factor for predicting both the overall and the disease-free 5-year survival of HCC patients. Furthermore, we revealed that miR-129-2 exerted its biological function, at least in part, by inhibiting HMGB1 expression and additional downstream Akt/MMP2/9 pathways. Moreover, demethylation treatment of miR-129-2 inhibited the migration and invasion of HCC. Taken together, our data suggested that miR-129-2 function as a tumor suppressor in HCC cells by directly targeting HMGB1 and is down-regulated by DNA methylation, which may provide a novel therapeutic strategy for treatment of HCC.

RESULTS

miR-129-2 is frequently downregulated in HCC tissues and is negatively associated with metastatic potential

To determine whether miR-129-2 is aberrantly expressed in HCC, we performed qRT-PCR analysis in a 106 pairs of HCC tissues and matched para-tumor tissues. Significantly, there was a remarkable decrease in miR-129-2 expression in approximately 93.4% (99/106) of tumor tissues compared with para-tumor tissues ($P < 0.01$, Figure 1A, 1B). Portal vein tumor thrombus was considered as malignant and aggressive characteristics, as compared with primary tumor tissues, miR-129-2 levels were prominently downregulated in aggressive HCC tissues ($P < 0.05$, Figure 1C). Furthermore, miR-129-2 levels were obviously decreased in tumor tissues arising from patients with tumor recurrence as compared with those without tumor recurrence ($P < 0.05$, Figure 1D). In addition, we found that well-differentiated HCCs showed higher miR-129-2 expression, as compared with those in poorly differentiated HCCs samples ($P < 0.05$, Figure 1E). Similarly, the relative lower expression of miR-129-2 was also observed in a panel of HCC cell lines as compared with a nontransformed hepatic cell line (LO2) ($P < 0.05$, Figure 1F). Together, these data indicated that miR-129-2 may play a protective role in the metastasis or invasion of HCC.

Downregulated expression of miR-129-2 predicts poor prognosis in HCC patients

We determined the mean level of miR-129-2 as a cutoff value to evaluate the significant contribution in the prognosis of HCC patients. As shown in Table 1, the low expression of miR-129-2 was prominently associated with multiple tumor nodes ($P = 0.008$), venous infiltration ($P = 0.001$), high Edmondson–Steiner grading ($P = 0.014$) and advanced tumor-node-metastasis (TNM) tumor stage ($P = 0.001$). Thus, our results indicate that the reduced expression of miR-129-2 is correlated with poor prognostic features of HCC. Furthermore, Kaplan-Meier analysis showed that the higher miR-129-2 expression exhibited better overall survival (OS, median OS time were 53 vs. 20 months, respectively; $P < 0.01$, Figure 2A) and disease-free survival (DFS) median DFS time were 31 vs. 18 months, respectively; $P < 0.01$, Figure 2B). Moreover, miR-129-2 expression level was an independent risk factor for predicting both 5-year OS and DFS of HCC patients ($P = 0.004$ and 0.001 , respectively, Table 2). Taken together, these data indicated that the expression level of miR-129-2 may be used as an independent factor for predicting the prognosis of HCC.

Ectopic expression of miR-129-2 ameliorates HCC migration and invasion, both *in vitro* and *in vivo*

To explore the biologic significance of miR-129-2, we stably overexpressed miR-129-2 in HCC cell lines Hep3B and Huh7 with lentiviruses carrying miR-129-2 and its control. The efficacy of infection was tested by qRT-PCR ($P < 0.01$, Figure 3A). Wound healing assay and Transwell migration assay revealed that exogenous expression of miR-129-2 dramatically inhibited cell migration in comparison with that of control cells ($P < 0.05$, Figure 3B, 3C). As the invasive capacity is a key step during tumor metastasis, we therefore performed Transwell Matrigel invasion assay with miR-129-2-overexpressing Hep3B and Huh7 cells. As shown in Figure 3D, forced expression of miR-129-2 significantly inhibited cell invasion. In addition, cell growth analyses were also carried out by applying MTT assays and no significant differences were observed (Figure 3E).

To verify the consequences of ectopic expression of miR-129-2, we subsequently injected Huh7-miR-129-2 and Huh7-miR-control cells into the lateral veins of the nude mice. We observed that up-regulation of miR-129-2 showed fewer and smaller metastatic growth in the lungs and livers of the nude mice with microscopic evaluation (9 vs. 24 nodules per lung in Huh7-miR-129-2

and miR-control cells, respectively; $P < 0.01$, Figure 3F, Supplementary Figure S1). Collectively, these results indicated that miR-129-2 is capable of manipulating aggressive and metastatic phenotype of HCC both *in vitro* and *in vivo*.

HMGB1 was a direct target of miR-129-2 in HCC

To elucidate the molecular mechanism by which miR-129-2 exerts its inhibitory effect on HCC cells, we predicted potential targets by different miRNA target algorithms and found conserved putative miR-129-2 sites at the 3'-UTR of HMGB1 (Figure 4A). Our previous studies has demonstrated that HMGB1 was highly expressed in HCC cells and tissues and associated with poor prognosis and aggressive phenotype of HCC. To confirm whether the loss of miR-129-2 is an important factor of HCC malignancy by upregulating HMGB1 expression, we performed that qRT-PCR and Western blot analysis and found that ectopic expression of miR-129-2 dramatically decreased the mRNA (Figure 4B) and protein (Figure 4C) expression of HMGB1. In addition, the overexpression of miR-129-2 prominently reduced the luciferase activity of HMGB1 wild-type reporter but not the mutant type (Figure 4D). Moreover, we found the expression of HMGB1 mRNA (Figure 4E) and protein

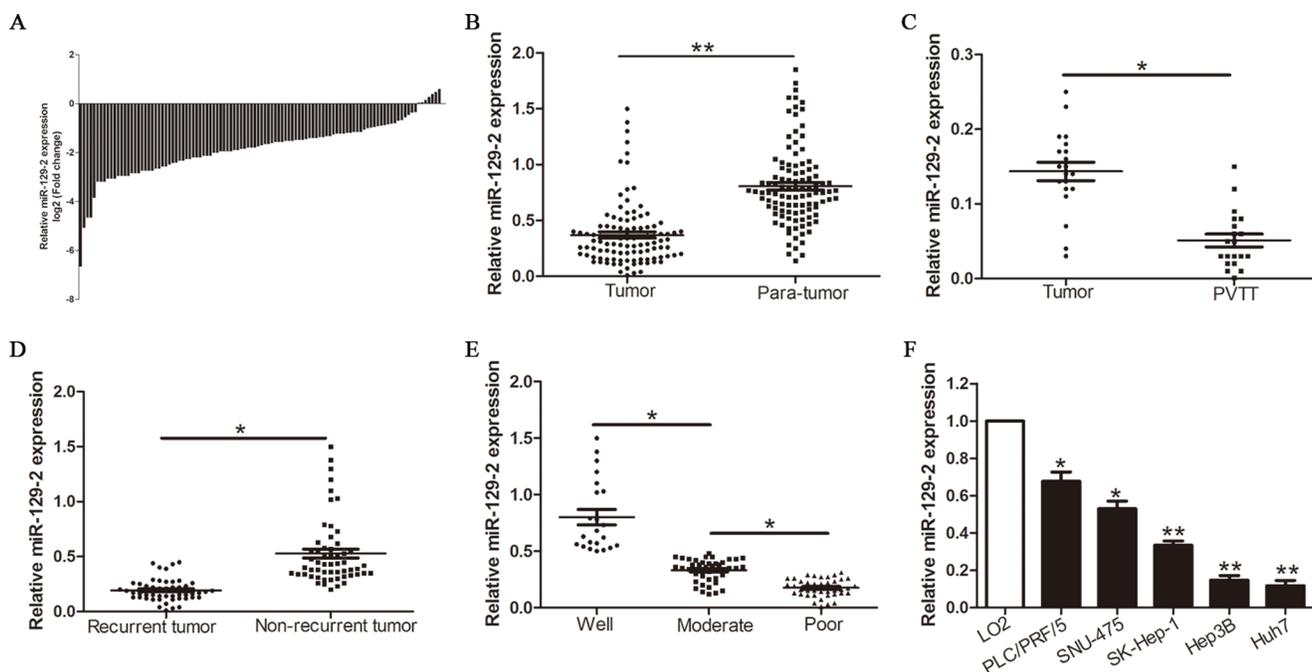


Figure 1: miR-129-2 is frequently downregulated in HCC tissues and is negatively associated with metastatic potential. (A, B) Relative miR-129-2 expression levels in 106 paired HCC tissues and matched adjacent normal tissues were determined by qRT-PCR. (C) Comparison of miR-129-2 expression in 20 paired tumor tissues and the corresponding portal vein tumor thrombus (PVTT) by qRT-PCR. (D) Comparison of miR-129-2 expression in HCC tissues arising from recurrent and non-recurrent groups by qRT-PCR. (E) Comparison of miR-129-2 in differentiated (metastatic potential) HCC tissues was analyzed. (F) The expression of miR-129-2 in five HCC cell lines was significantly decreased compared with that in the LO2 cells. U6 snRNA was used as internal control. * $P < 0.05$, ** $P < 0.01$.

Table 1: Clinical correlation of miR-129-2 expression in HCC

Clinical parameters	Cases (n)	Expression level		P value (* <i>p</i> < 0.05)
		miR-129-2 ^{low} (n = 56)	miR-129-2 ^{high} (n = 50)	
Age (years)				
< 65 years	51	27	24	0.982
≥ 65 years	55	29	26	
Gender				
Male	82	43	39	0.881
Female	24	13	11	
Tumor size (cm)				0.281
< 5 cm	73	36	37	
≥ 5 cm	33	20	13	
Tumor number				0.008*
solitary	92	44	48	
multiple	14	12	2	
Edmondson				
I + II	38	14	24	0.014*
III + IV	68	42	26	
TNM stage				0.001*
I + II	83	37	46	
III + IV	23	19	4	
Venous infiltration				0.001*
Present	20	17	3	
Absent	86	39	47	
AFP				0.643
< 400 ng/ml	32	18	14	
≥ 400 ng/ml	74	38	36	
HBsAg				0.485
positive	100	52	48	
negative	6	4	2	

(Figure 4F, 4G) in the miR-129-2 high-expressing tumors were significantly lower than those in the miR-129-2 low-expressing tumors. Notably, an obvious inverse correlation between miR-129-2 and HMGB1 mRNA was revealed by Spearman's correlation analysis in HCC tissues (Figure 4H). Collectively, these results strongly suggested that HMGB1 is a downstream target of miR-129-2 in HCC.

HMGB1 is a downstream mediator of the biological function of miR-129-2 in HCC

To further validate that miR-129-2 suppressed HCC invasion and metastasis by regulating HMGB1, we performed rescue experiment by transfecting HMGB1 overexpression plasmid or empty vector (EV) in miR-129-2-

overexpressing cells (*P* < 0.05, Figure 5A). We found that HMGB1 overexpression rescued the decreased migration and invasion abilities induced by miR-129-2 overexpressing cells (*P* < 0.05, Figure 5B–5D, respectively). These data confirm that HMGB1 is an essential and functional downstream mediator of miR-129-2 in HCC.

miR-129-2-HMGB1 axis modulates the expression of MMP2 and MMP9 by inhibiting AKT phosphorylation

To investigate the underlying molecular mechanism of the miR-129-2-mediated attenuation of HCC migration and invasion, we extended the studies on the miR-129-2-HMGB1 module to further downstream, based

on reported HMGB1 signaling to AKT and MMPs pathways. Recent studies of MMPs involvement in tumor metastasis such angiogenesis, migration and invasion have received extensive attention that resulted in amounts of experimental data in favor of critical roles of MMPs in these processes [25–29]. Expectedly, overexpression of miR-129-2 significantly decreased the AKT activity and the expression of phosphorylated AKT, MMP2 and MMP9 in HCC cells ($P < 0.05$, Figure 6A). Moreover, the rescue experiment showed that upregulation of HMGB1 expression in miR-129-2-overexpressing cells led to increased phosphorylation of AKT and the expression of MMP2 and MMP9 ($P < 0.05$, Figure 6B). In addition, we demonstrated that overexpression of HMGB1 induced the AKT phosphorylation, in contrast, HMGB1 knockdown inhibited AKT phosphorylation (Supplementary Figure S2). To further study whether the AKT signaling pathway was necessary for miR-129-2-mediated suppression of HCC migration and invasion, we transfected miR-129-2-overexpressing cells with pmyt-AKT (dominant-active AKT) or pcDNA3.1. We noticed that cell migration and invasion were reversed in couple with the increased expression of MMP2 and MMP9 in the pmyt-AKT transfection group ($P < 0.05$, Figure 6C–6F). In addition, the specific AKT inhibitor, MK2206, were applied to further verify whether the rescue experiment are also consistent with previous observations. As shown in Figure 6G–6I, the migration and invasion capability were remarkably attenuated upon AKT inhibitor treatment in miR-129-2-overexpressing and HMGB1 overexpression plasmid cotransfection group, and the attenuation effects were also observed in the expression of MMP2 and MMP9 ($P < 0.05$, Figure 6J). Together, these data suggest that intracellular AKT-dependent proteolytic enzyme MMPs signaling pathway may play the key role in the modulation of miR-129-2-involved HCC migration and invasion.

miR-129-2 selectively inhibits AKT phosphorylation at Ser473

It has been reported that phosphorylation of both Thr308 and Ser473 residues contribute to the full activation of AKT [30]. Therefore, we tested the change of p-AKT (Ser473) and p-AKT (Thr308) in miR-129-2-overexpressing cells. We found the level of p-AKT (Ser473) significantly decreased, whereas p-AKT (Thr308) revealed no obvious difference in miR-129-2-overexpressing cells (Figure 7A). Furthermore, we observed that the upstream regulators of p-AKT (Thr308) [31], including P85, PTEN, and PDK1 had no obvious changes in miR-129-2-overexpressing cells (Figure 7B), which validated that miR-129-2 selectively inhibited AKT phosphorylation at Ser473, not Thr 308. In addition, to confirm the miR-129-2-mediated inhibition effects of p-AKT (Ser473), we examined the correlation between miR-129-2 and p-AKT (Ser473), MMP2 and MMP9 in serial sections of 106 HCC cases by immunohistochemical study. The expression of p-AKT (Ser473), MMP2 and MMP9 in cancer tissues was significantly higher than those in paired noncancerous tissues. Furthermore, IHC scores were used for semiquantitative analysis, we found a strong inverse correlation between miR-129-2 expression and p-AKT (Ser473), MMP2 and MMP9, respectively (Figure 7C). These data revealed that miR-129-2 exerts its biological suppressive function through dephosphorylation of AKT on Ser473 and elevated expression of MMP2 and MMP9.

miR-129-2 expression is epigenetically regulated by DNA methylation in HCC

Our results revealed that miR-129-2 plays an important protective role in HCC migration and invasion by manipulating HMGB1/p-AKT (Ser473)/

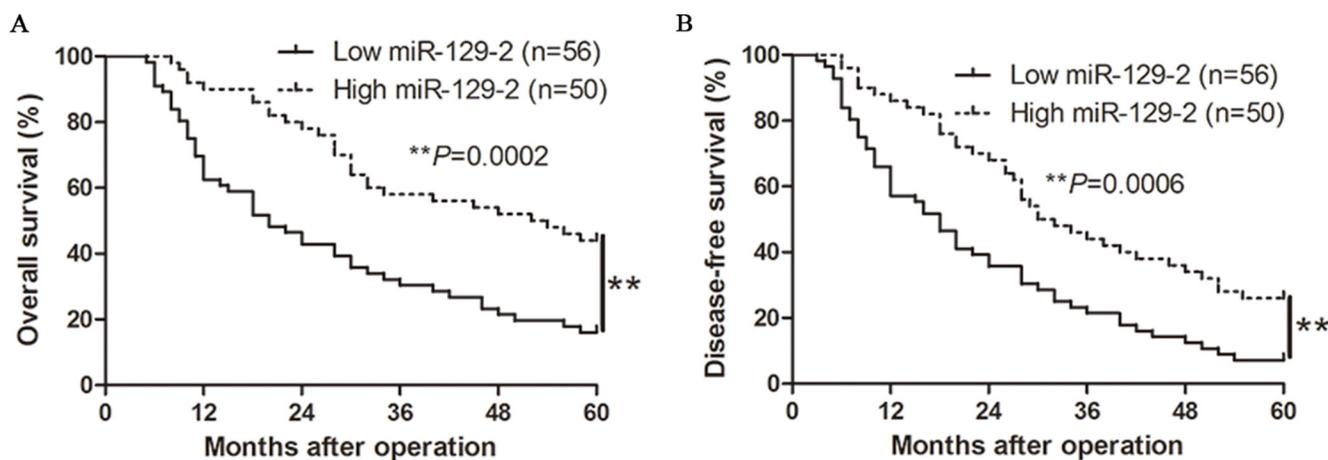


Figure 2: The prognostic value of miR-129-2 for HCC patients. HCC patients with higher expression of miR-129-2 had better (A) overall survival (OS) and (B) disease-free survival (DFS). $**P < 0.01$.

Table 2: Multivariate Cox regression analysis of 5-year overall and disease-free survival of 106 HCC patients

Variables	Overall survival			Disease-free survival		
	HR	95% CI	P	HR	95% CI	p
Tumor number	1.713	0.657–4.134	0.257	1.064	0.218–5.434	0.940
Venous infiltration	1.057	0.986–1.114	0.056	1.058	0.506–2.158	0.865
Edmondson grade	2.230	0.943–5.318	0.070	1.967	0.814–4.576	0.125
TNM stage	1.125	1, 003–1.396	0.028*	1.158	1.039–1.402	0.004*
miR-129-2	2.152	1.237–3.685	0.004*	3.527	1.634–7.753	0.001*

TNM, tumor-node-metastasis; HR, hazard ratio; CI, confidence interval. *Statistically significant.

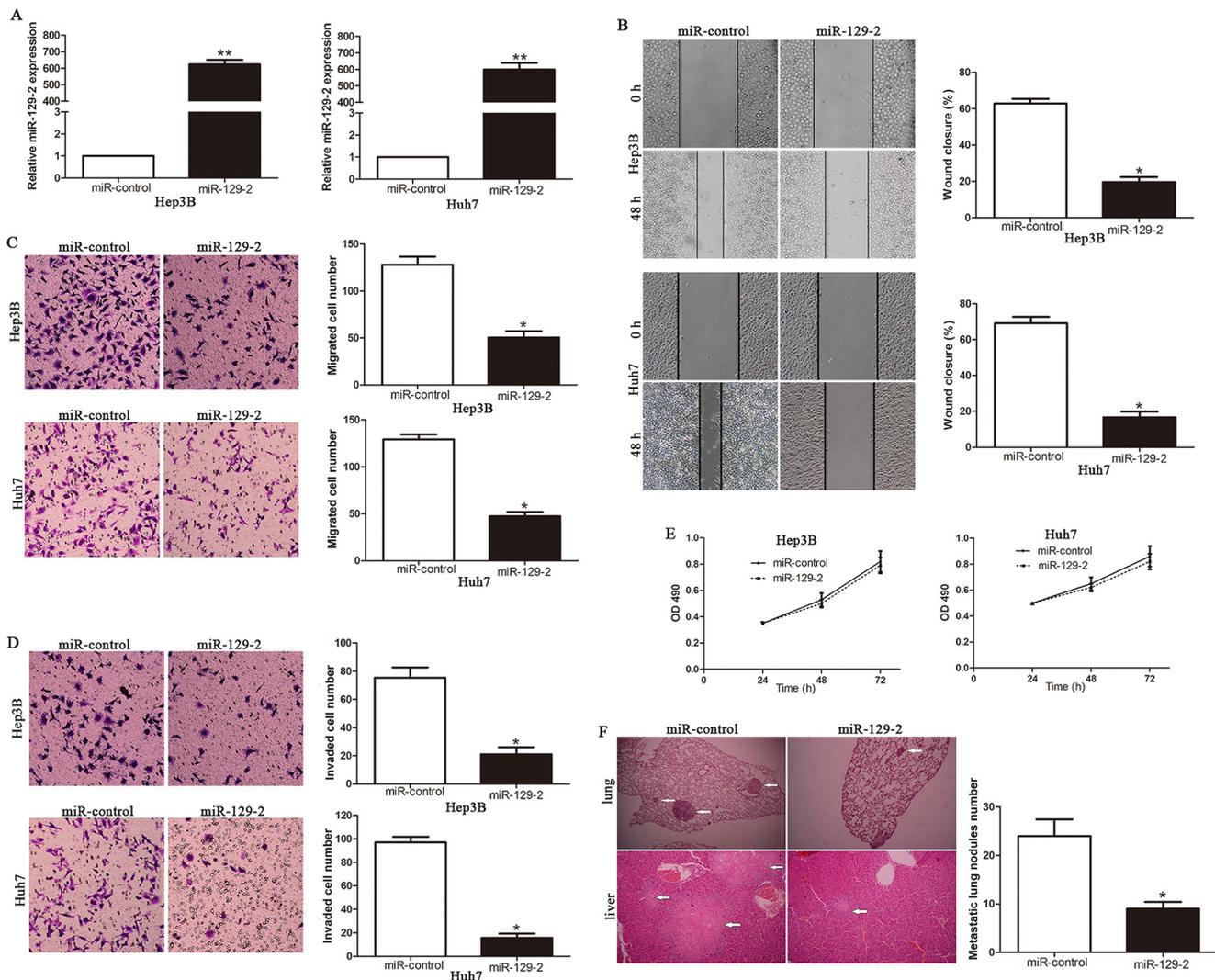


Figure 3: Ectopic expression of miR-129-2 ameliorates HCC migration and invasion, both *in vitro* and *in vivo*. (A) The expression of miR-129-2 was significantly increased in Hep3B and Huh7 cells infected with miR-129-2 expression or control lentiviruses. Cell migration as measured by wound healing assay (B) and Transwell migration assay (C) was inhibited by up-regulation of miR-129-2 in Hep3B and Huh7 cells as compared with control cells. $n = 6$ repeats with similar results. (D) MiR-129-2 over-expressing Hep3B and Huh7 cells conferred a lower number of invaded cells as compared with control cells. $n = 6$ repeats with similar results. (E) Upregulated miR-129-2 had no obvious difference on cell proliferation, as determined by MTT. (F) Representative hematoxylin and eosin (H&E) images of metastatic nodules from the mouse lung and liver tissue sections (left) of the Huh7-miR-control group and the Huh7-miR-129-2 group. The number of metastatic nodules in the lungs of each group was presented as the mean SD (right). Experiments were repeated at least 6 times with similar results, and error bars represent \pm SD. * $P < 0.05$, ** $P < 0.01$.

MMP2/9 signaling pathway, however, the regulatory mechanism of miR-129-2 expression in HCC cells was still unknown. Previous study demonstrated that miR-129-2 downregulation is mediated by epigenetic mechanism, especially DNA methylation. We first detect the promoter methylation level of miR-129-2 in 106 pairs HCC tissues and matched adjacent non-tumor tissues. With threshold set at 10%, we found that the frequency of miR-129-2 gene hypermethylation in tumor tissues was significantly higher than that in matched adjacent non-tumor tissues (50.94% (54/106) in tumor vs. 7.54% (8/106) in para-tumor tissues) ($P < 0.05$, Figure 8A). Furthermore, we investigate whether miR-129-2 was epigenetically regulated in HCC cells. As shown in

Figure 8B, extremely high methylation was observed in Huh7, Hep3B and SK-Hep-1, while PLC/PRF/5 and SNU-475 were unmethylated, which was inversely correlated with the expression of miR-129-2. To confirm that the DNA methylation participated in the regulation of miR-129-2 expression, we treated Hep3B and Huh7 cells *in vitro* with DNA methyltransferase inhibitor 5-Aza-dC. We found miR-129-2 expression was upregulated with 5-Aza-dC compared with the control cells (Figure 8C). Functional analysis also revealed that demethylation-induced expression of miR-129-2 inhibited cell migration and invasion of Hep3B and Huh7 cells (Figure 8D–8F). These results implicated that epigenetic modification may modulate the miR-129-2 expression in HCC.

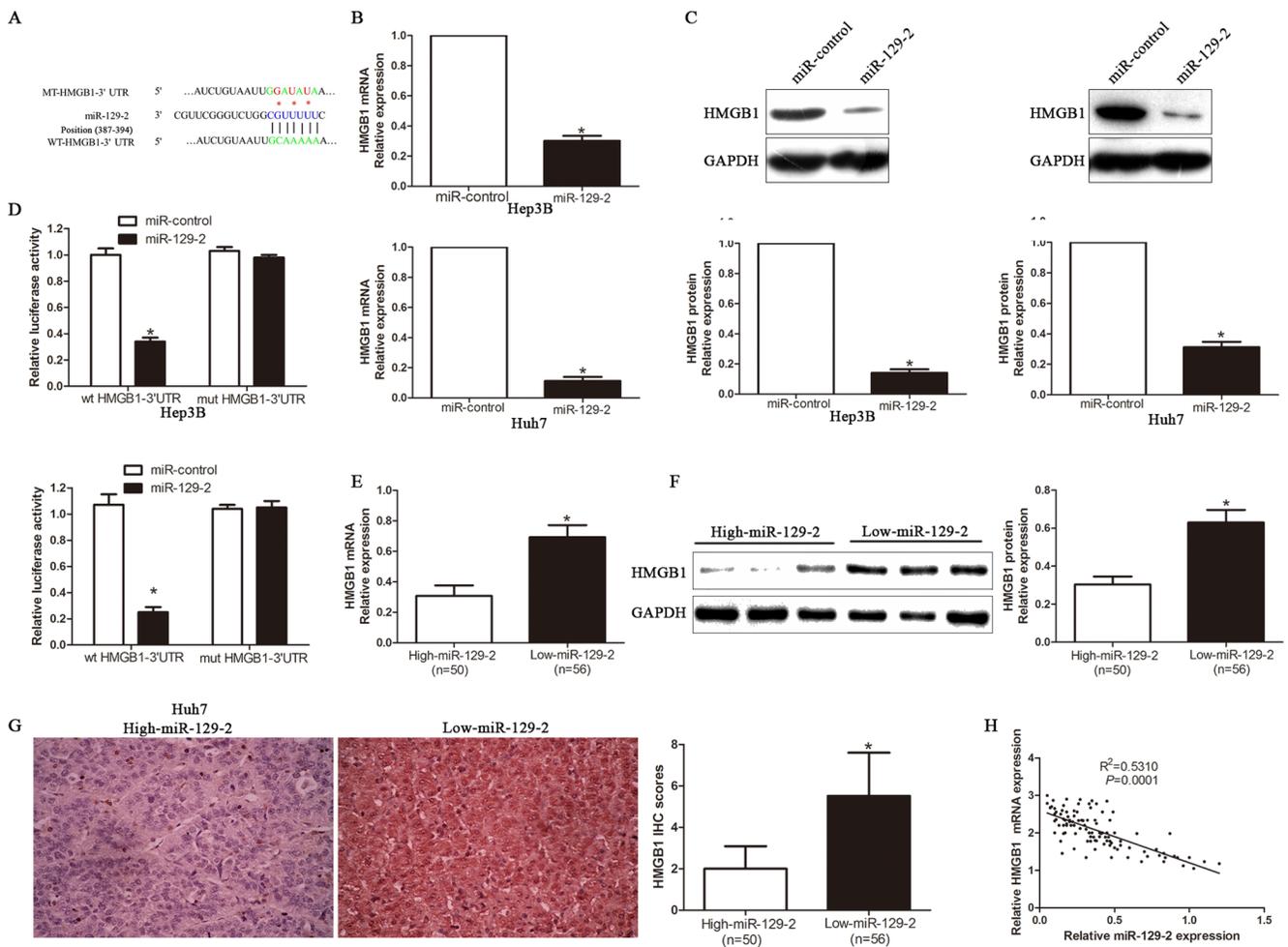


Figure 4: HMGB1 is a direct target of miR-129-2 in HCC cells. (A) The putative binding sequence of miR-129-2 in the 3'-UTR of HMGB1. (B) qRT-PCR analysis of HMGB1 mRNA expression in Hep3B and Huh7 cells after overexpressing miR-129-2. $n = 6$ repeats with similar results. (C) Overexpression of miR-129-2 reduced the expression of HMGB1 protein in Hep3B and Huh7 cells. $n = 6$ repeats with similar results. (D) Luciferase reporter assays showing decreased reporter activity after transfection of wild-type HMGB1 3'-UTR reporter construct in the Hep3B and Huh7 cells overexpressing miR-129-2. The HMGB1 3'-UTR mutant constructs had no effect on reporter activity. $n = 6$ repeats with similar results. (E) The expression of HMGB1 mRNA in miR-129-2 high-expressing tumors was significantly lower than that in miR-129-2 low-expressing tumors. (F) A significantly inverse correlation between miR-129-2 and HMGB1 protein expression was observed in HCC tissues, as determined by Western blot. (G) A significant inverse correlation between miR-129-2 and HMGB1 protein expression was observed in HCC tissues. Representative immunohistochemical staining showed a weak staining of HMGB1 in miR-129-2 high-expressing HCC tissue and strong staining of HMGB1 in the miR-129-2 low-expressing tumor. (H) A significant inverse correlation between miR-129-2 and HMGB1 was observed in HCC tissues. * $P < 0.05$, ** $P < 0.01$.

DISCUSSION

Identification of novel and essential molecular biomarkers is imminent to designate alternative strategies to overcome resistance in HCC therapy [32]. Increasing evidence have demonstrated that miRNAs regulated hepatocarcinogenesis-related gene expression, which indicates a new insight in the initiation and progression of HCC [33, 34]. Recently, epigenetic repression of miR-129-2 was identified as a crucial regulator during the progression of human cancers [18]. Nevertheless, the clinical significance and the underlying molecular mechanisms remain unclear. In our current study, miR-129-2 was found frequently downregulated in aggressive tumors than in nonaggressive tumors. Importantly, our results showed that downregulated miR-129-2 conferred a significant higher recurrence rate for HCC patients. Furthermore, miR-129-2 was reduced in HCC cell lines as compared with a normal hepatic cell line. Clinical analysis revealed that miR-129-2 was expressed at significantly lower levels in HCC patients with multiple tumor nodes,

venous infiltration, high Edmondson-Steiner grading and advanced TNM tumor stage. These results suggest that the reduced expression of miR-129-2 is correlated with poor prognostic features in HCC. In addition, our data demonstrated that the reduced expression of miR-129-2 predicted a significant worse 5-year survival for HCC patients. Multivariate Cox regression analysis indicated that miR-129-2 was an independent prognostic factor for predicting survival of HCC patients. Moreover, ectopic expression of miR-129-2 attenuated cell migration and invasion both *in vitro* and *vivo*. These results suggested that miR-129-2 is critical for prognosis determination in HCC patients and plays an important role in the regulation of tumor migration and invasion in HCC.

It is necessary to identify its downstream functional targets to elucidate its biological effects. HMGB1, the most important member of the high mobility group box protein family [35], plays multiple roles in cancer progression, angiogenesis, invasion, and metastasis development [36–40]. Our previous study identified HMGB1 overexpressed in HCC and associated with poor

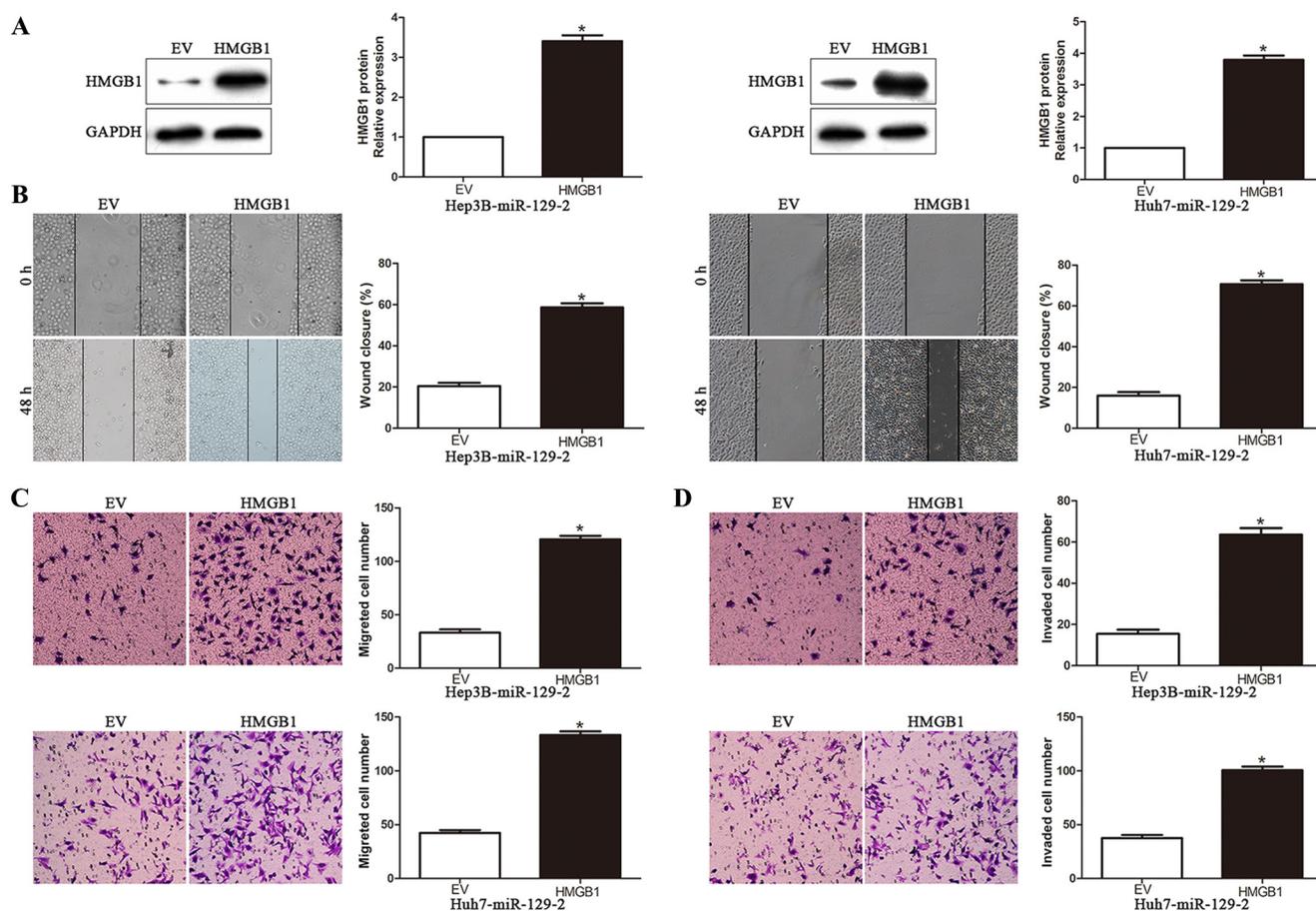


Figure 5: HMGB1 is the functional mediator downstream of miR-129-2 in HCC cells. (A) miR-129-2-overexpressing Hep3B and Huh7 cells that were transfected with empty vector (EV) or HMGB1 expression plasmid were subjected to western blot analysis for HMGB1. $n = 6$ repeats with similar results. Altering HMGB1 expression partly abolished the functional effect of miR-129-2 on cell migration (B) wound healing assay (C) Transwell migration assay and cell invasion (D). $n = 6$ independent experiments. * $P < 0.05$, ** $P < 0.01$.

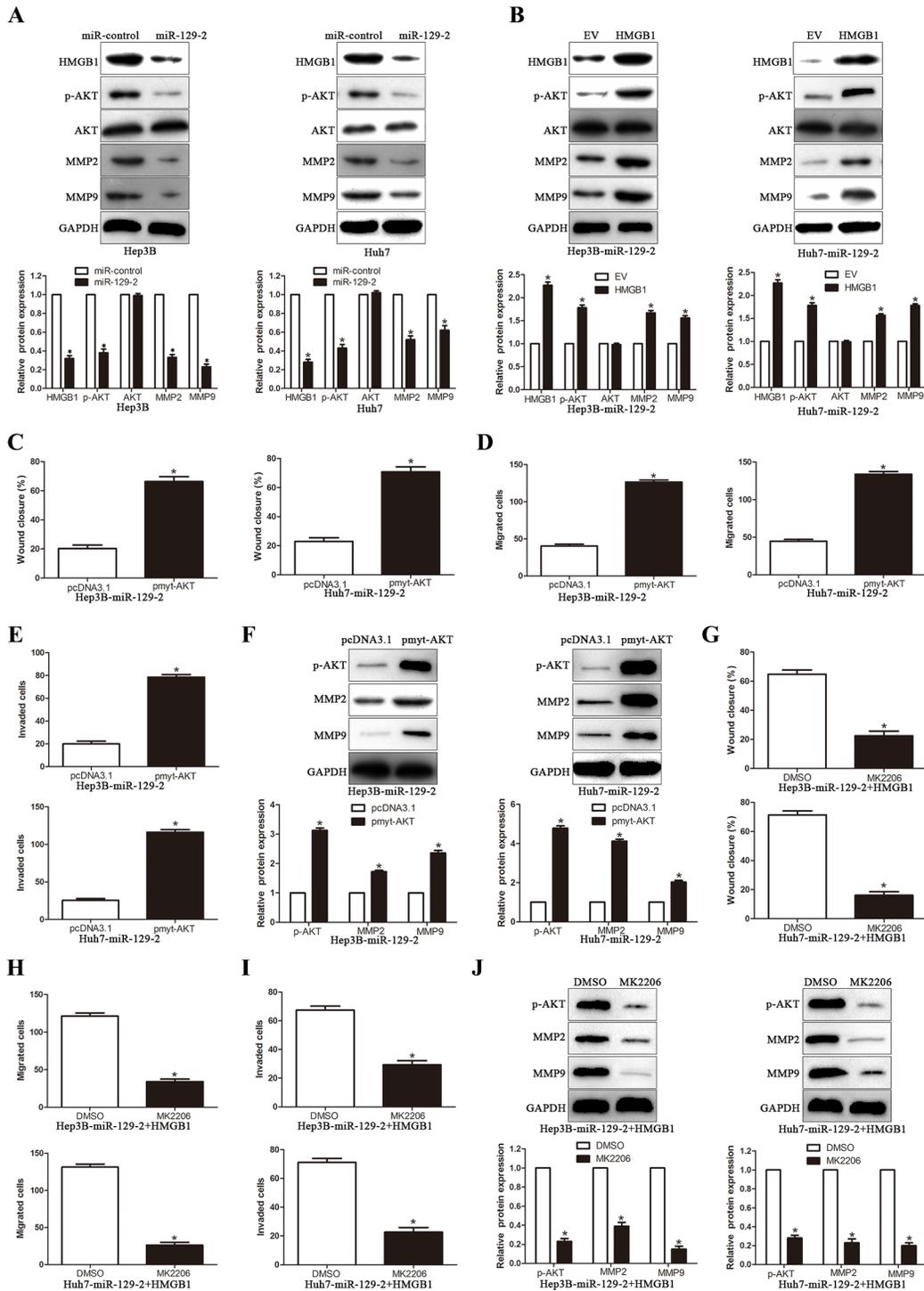


Figure 6: miR-129-2-HMGB1 axis modulates the expression of MMP2 and MMP9 by inhibiting AKT phosphorylation. (A) Western blot analysis showing decreased levels of p-AKT, MMP2/9 in miR-129-2-overexpressed Hep3B and Huh7 cells. (B) Western blot analysis for AKT/p-AKT, MMP2/9 protein expression in miR-129-2-overexpressing Hep3B and Huh7 cells following transfection with HMGB1 plasmid. (C-F) The miR-129-2-overexpressing Hep3B and Huh7 cells were transiently transfected with pmyt-AKT to increase the expression of p-AKT. The cells were subjected to wound healing assay (C) and Transwell migration assay (D) and the Matrigel invasion assay (E) to detect the functional effect and Western blot (F) to investigate the expression of MMP2/9. p-AKT overexpression abrogated the suppressive effects of miR-129-2 overexpressed on HCC cells. (G-J) The HMGB1 rescue experiment that co-transfection of HMGB1 in miR-129-2-overexpressing Hep3B and Huh7 cells, were treated with AKT inhibitors, MK2206 (2 $\mu\text{mol/L}$). Then the capacity of cell migration (G, wound healing assay and H, Transwell migration assay) and cell invasion (I, Matrigel invasion assay), the MMP2/9 expression was determined by Western blot (J). p-AKT inhibitor abolished the HMGB1 rescue experiment on the functional effects and the expression of MMP2/9. $n = 6$ independent experiments. * $P < 0.05$, ** $P < 0.01$.

pathological features. In this study, we confirmed that HMGB1 was a direct downstream target of miR-129-2 and it was implicated in the functional effect of miR-129-2 on HCC. An inverse correlation between the expression of miR-129-2 and HMGB1 mRNA and protein was observed in HCC tissues. Upregulation of miR-129-2 significantly reduced the expression of HMGB1 and decreased the

luciferase reporter activity of HMGB1 wt 3'-UTR but not mt 3'-UTR. Importantly, we confirmed that restoration of HMGB1 expression abrogated the functional effect of miR-129-2 on HCC migration and invasion. Taken together, these data provide competent evidences to support that miR-129-2 exerts its suppressive effect on HCC, at least partly, through inhibiting HMGB1.

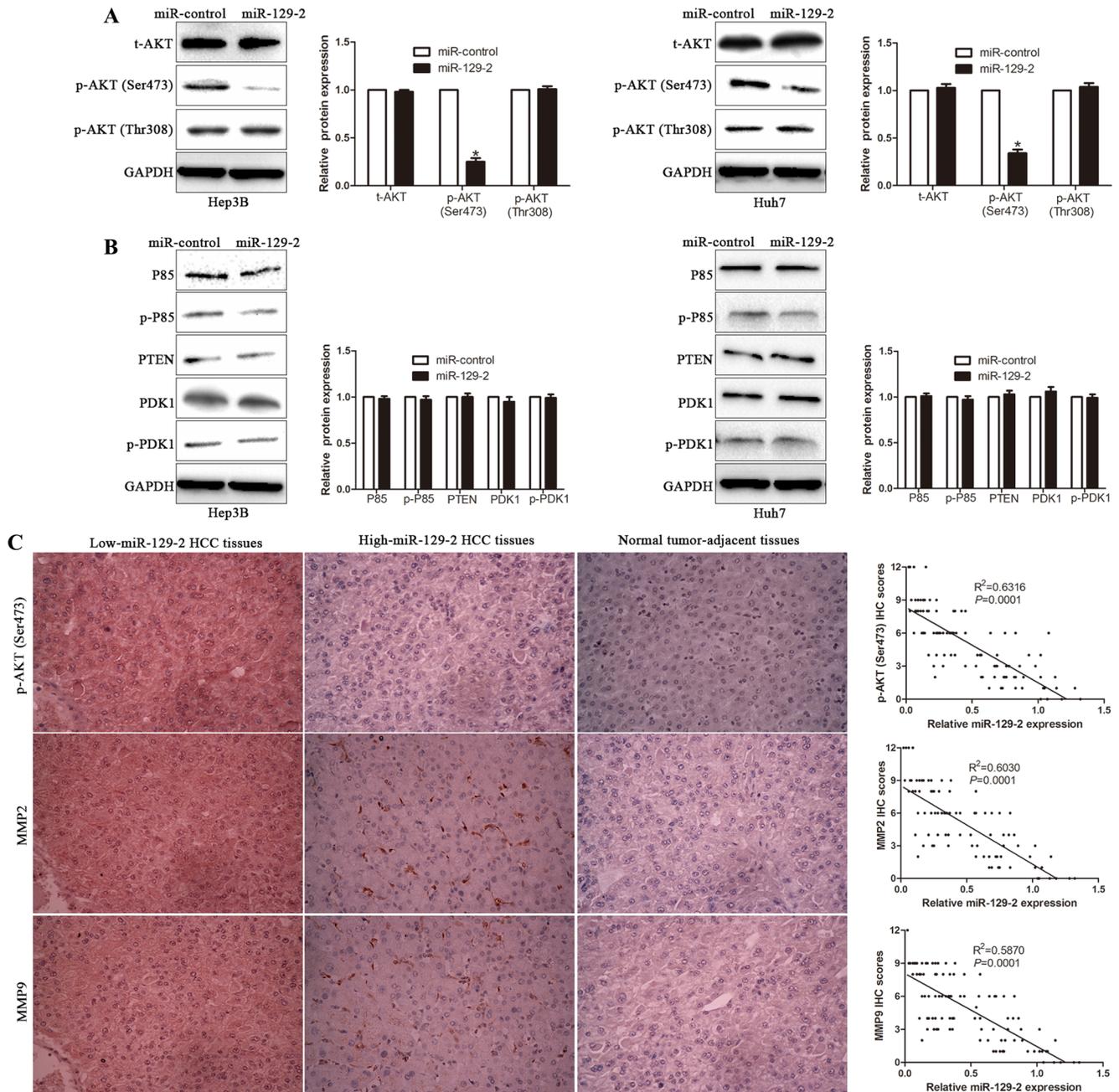


Figure 7: miR-129-2 selectively inhibits AKT phosphorylation at Ser473. (A) The total expression of AKT (t-AKT) and phosphorylation levels of AKT, including p-AKT (Ser473) and p-AKT (Thr308), were analyzed by Western blot. (B) The major upstream regulators of p-AKT (Thr308) were probed by Western blot. (C) Left Panel: the representative picture of immunochemical staining of serial HCC sections for p-AKT (Ser473) and MMP2/9 sections in the different miR-129-2 expression levels tissues. Right Panel: the correlation of miR-129-2 level with those of p-AKT (Ser473) and MMP2/9 in 106 HCC patients. Correlation between two variables was calculated by Spearman rank correlation coefficient. $n = 6$ independent experiments. $*P < 0.05$, $**P < 0.01$.

It has been well documented that MMPs have been implicated in the aggressiveness of HCC [29, 41]. In our study, we indicated that miR-129-2 decreased the expression of MMP2/9 by inhibiting AKT phosphorylation. Upregulation of p-AKT rescued the decreased migration and invasion induced by miR-129-2, whereas Akt phosphorylation inhibition significantly decreased HMGB1-enhanced migration and invasion. AKT is an important kinase mediating survival signaling, which is regulated by phosphorylation on Thr308 and Ser473. In present study, we confirmed p-AKT (Ser473) was obviously inactivated in miR-129-2-overexpressing cells, nevertheless, p-AKT (Thr308) presented no significant difference. Consistently, our data indicated that miR-129-2 expression revealed a significantly inverse correlation with p-AKT (Ser473), MMP2 and MMP9, respectively. Taken together, these data revealed that miR-129-2 exerts its physiologic function

through inhibiting HMGB1, which in turn results in the dephosphorylation of AKT on Ser473 and decreased expression of MMP2/9.

Epigenetic modifications are closely associated with gene inactivation. Several studies revealed that promoter hypermethylation induced downregulation of miRNAs closely correlates with carcinogenesis [42]. Our data demonstrated that hypermethylation of upstream of miR-129-2 led to the downregulation of miR-129-2 in both HCC tissues and cell lines. Moreover, demethylation of miR-129-2 treatment increased miR-129-2 expression in HCC cells and resulted in significant inhibitory effects on cell migration and invasion. These data suggested that miR-129-2 is downregulated by DNA methylation and functions as a tumor suppressor in HCC cells.

In conclusion, we find that miR-129-2 is downregulated in HCC and its decreased expression is associated with poor prognostic features. Moreover, miR-129-2 is an independent prognostic factor for OS

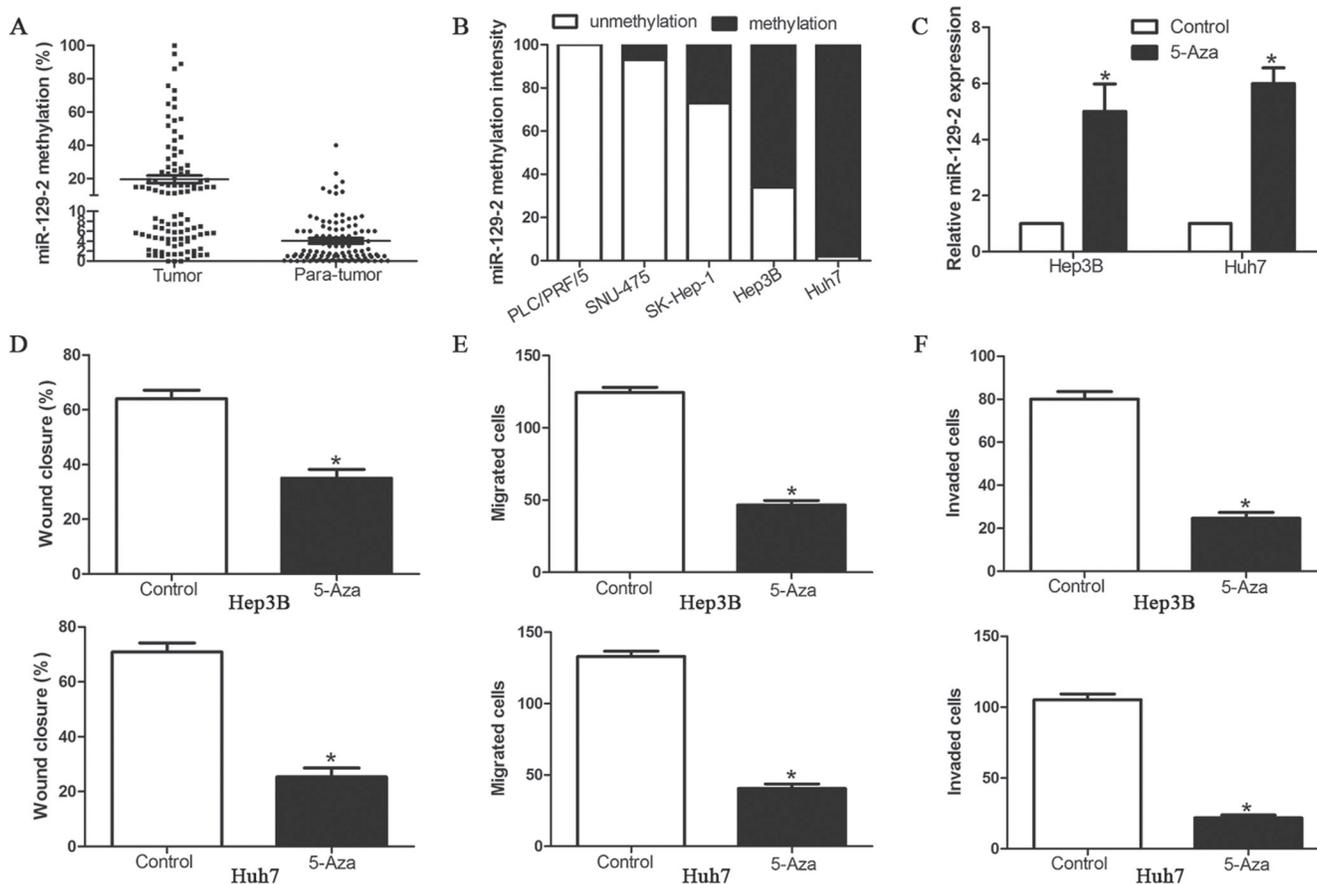


Figure 8: miR-129-2 expression is epigenetically regulated by DNA methylation in HCC cells. (A) The methylation intensity of miR-129-2 promoter in HCC tissues ($n = 106$) and adjacent non-tumor tissues was detected by quantitative methylation specific PCR; (B) Methylation profiles of 5 HCC cell lines created following methylated DNA quantification analysis. (C) Hep3B and Huh7 cells were treated with $4 \mu\text{mol/L}$ 5-Aza-dC. Relative expression levels of miR-129-2 (normalized by U6 RNA expression) in these cells were determined by Taqman qRT-PCR analysis. (D–F) Effects of 5-Aza-dC treatment on cell migration and invasion of Hep3B and Huh7 cells, (D) wound healing assay and (E) Transwell migration assay for cell migration and (F) Matrigel invasion assay for cell invasion. $n = 6$ independent experiments. $*P < 0.05$, $**P < 0.01$.

and DFS of HCC patients. We indicate that miR-129-2 inhibits tumor migration and invasion *in vitro* and *in vivo*. Mechanistically, we suggest that miR-129-2 is downregulated by DNA methylation and functions as a tumor suppressor by targeting HMGB1 in HCC cells, which in turn results in the dephosphorylation of AKT on Ser473 and decreased expression of MMP2/9. Taken together, we consider that miR-129-2 may potentially act as a clinical biomarker and therapeutic target in HCC.

MATERIALS AND METHODS

Clinical samples

Tumor samples and matched tumor-adjacent tissues were obtained from 106 patients undergoing curative resection of their primary HCC in the Department of Hepatobiliary Surgery at the First Affiliated Hospital of Xi'an Jiaotong University during January, 2006 to December, 2009. Written informed consent was obtained from all patients. None of the patients receive any perioperative chemo- or radiotherapy. The demographic and clinicopathologic data of all enrolled patients were obtained through review of hospital records, and were presented in Table 1. And disease recurrence and survival information was updated at each follow-up visit. The protocols of this study were approved by the Xi'an Jiaotong University Ethics Committee according to the Declaration of Helsinki.

Cell culture and treatment

The human immortalized normal hepatic cell line LO2 and HCC cell lines (PLC/PRF/5, SNU-475, SK-Hep-1, Hep3B and Huh7) were obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco, GrandIsland, NY, USA) at 37°C with 5% CO₂. Akt inhibitor MK-2206 (1 μM, Selleck Chemicals, Houston, TX, USA), 5-aza-2'-deoxycytidine (5-Aza-dC) (Sigma-Aldrich, St Louis, MO, USA) was used to treat HCC cells following the manufacturer's instructions.

Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from clinical specimens or HCC cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following manufacturer's instruction. Quantitative real-time PCR was performed using SYBR Premix Ex Taq II (TaKaRa). TaqMan microRNA assays (Applied Biosystems, Foster City, California, USA) were used to

quantify the expression levels of miR-129-2. Forward and reverse primers were used as follows: HMGB1, AGA AGT GCT CAG AGA GGT GGA and CCT TTG GGA GGG ATA TAG GTT; miR-129-2, GCG ACT GAC GTC TTT TTG CGG TCT GG and CAG AAC AGT GTC GTG ACA GTG ACG AT; U6, CGC TTC GGC AGC ACA TAT ACT A and CGC TTC ACG AAT TTG CGT GTC A.

DNA extraction and miR-129-2 methylation assay

DNA was extracted by digestion of frozen samples and cell pellets with 1% protease K, followed by standard phenol/chloroform extraction and ethanol precipitation. The quantificational methylated DNA analysis was performed by combined DNA methylation-sensitive and methylation-dependent restriction endonuclease digestion, followed by subsequent quantitative PCR assay. The chosen target region for quantificational methylation analysis of miR-129-2 gene is a 190 bp long sequence in CpG island. The Primer sequences were as follows: forward: 5'-GGA CGG TCT GGA GAA ATG GAG A-3', reverse: 5'-GAT TCG CGA AGG GCA GAA TAT G-3'.

Western blot analysis

Total protein was extracted from HCC cells and 40 μg of isolated protein was separated by 10% SDS-PAGE and transferred onto a PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were probed with the following primary antibodies: Akt (1:1000, Cell Signaling, Danvers, MA, USA), p-Akt (1:1000, Cell Signaling,), HMGB1 (1:1000, Cell Signaling), MMP2 (1:1000, Cell Signaling), and MMP9(1:1000, Cell Signaling) for overnight. Then the membranes were incubated with the HRP-conjugated goat anti-mouse or anti-rabbit IgG antibody (ZSGB-BIO, China). Protein bands were visualized using an enhanced chemiluminescence kit (Amersham, Little Chalfont, UK).

Immunohistochemical staining

Immunohistochemistry was performed on paraformaldehyde-fixed paraffin sections. HMGB1, MMP2, MMP9 (1:100, Cell Signaling) antibody was used in immunohistochemistry using a streptavidin peroxidase-conjugated (SP-IHC) method. Detailed procedure of immunohistochemistry was performed as previously reported [43].

Plasmids and lentivirus transfection

Lentiviruses containing vector pGCSIL-GFP and pGCSIL-GFP-miR-129-2 were constructed by Genechem (Shanghai, China) and used to infect Hep3B and Huh7 cells at a multiplicity of infection of 10 or 50, respectively, according to the manufacturer's instructions. HMGB1 expressing vector was synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). Cells were transfected with oligonucleotides using Lipofectamine 2000 Reagent (Invitrogen Life Technologies) following the manufacturer's instructions.

Luciferase assay

Cells were seeded in triplicate in 24-well plate and allowed to settle for about 12 h. pGL3-HMGB1 was co-transfected into HCC cells with TK-Renilla plasmid as control signals using Lipofectamine 2000. Luciferase and control signals were measured at 48 h after transfection using the Dual Luciferase Reporter Assay Kit (Promega, Madison, WI, USA), according to a protocol provided by the manufacturer. Three independent experiments were performed and the data were presented as the mean \pm SD.

Wound healing assay

The cells were seeded in 6-well plates at a high density and allowed to form cell monolayers overnight. A 200 μ L sterile plastic tip was used to create a wound line across the surface of plates, and the suspension cells were removed with PBS. Cells were cultured in reduced serum DMEM medium in a humidified 5% CO₂ incubator at 37°C for 48 h, and then images were taken with a phase-contrast microscope. Each assay was replicated three times.

Transwell migration and invasion assay

The migration and invasion assays were performed using Transwell chamber (Millipore, Billerica, USA). For migration assay, the transfected cells were seeded into the upper chamber with serum-free medium (2.5×10^4 cells), and the bottom of the chamber contained the DMEM medium with 10% FBS. While for invasion assay, the chamber was coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA), and the following steps were similar to migration assay. When the cells migrated or invaded for 24 h, the cells were fixed and stained with crystal violet. Migrated and invaded HCC cells were counted under an inverted light microscope.

In vivo metastasis assay

4–6 week-old female BALB/c nude mice (Centre of Laboratory Animals, The Medical College of Xi'an Jiaotong University, Xi'an, China) were randomized

into two groups ($n = 5$), and either Huh7-miR-129-2 or Huh7-miR-control cells (1×10^6) were injected into the tail veins for the establishments of pulmonary metastatic model. Mice were sacrificed 10 weeks post injection and examined microscopically by H&E staining for the development of lung metastatic foci. Animals were housed in cages under standard conditions. All *in vivo* protocols were approved by the Institutional Animal Care and Use Committee of Xi'an Jiaotong University.

Statistical analysis

Data are presented as the mean \pm SD from at least three independent replicates. SPSS software, 16.0 (SPSS, Inc, Chicago, IL, USA) was used to conduct the analysis, and a two-tailed Student *t*-test was employed to analyze the differences between two groups. Pearson's correlation analysis was used to analyze the correlation between two indices. Survival curves were plotted by the Kaplan-Meier method and compared using the log-rank test. Differences were considered statistically significant at $P < 0.05$.

ACKNOWLEDGMENTS AND FUNDING

This work was supported by grants from the National Natural Science Foundation of China (No. 81272645, 81402039, 81502092 and 81572847), the Key Science and Technology Fund of Shaanxi Province (2015SF052), the Zhejiang Provincial Natural Science Foundation of China (LY16H160043) the Natural Science Basic Research Plan in Shaanxi Province of China (No. 2016JQ8047).

CONFLICTS OF INTEREST

All authors declare no conflicts of interest.

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