ORIGINAL ARTICLE

Loss-of-function of miR-142 by hypermethylation promotes TGF- β -mediated tumour growth and metastasis in hepatocellular carcinoma

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Abstract

Objectives: Hypermethylation-induced epigenetic silencing of tumour suppressor genes (TSGs) are frequent events during carcinogenesis. MicroRNA-142 (miR-142) is found to be dysregulated in cancer patients to participate into tumour growth, metastasis and angiogenesis. However, the tumour suppressive role of miR-142 and the status of methylation are not fully understood in hepatocellular carcinoma (HCC).

Methods: Hepatocellular carcinoma tissues and corresponding non-neoplastic tissues were collected. The expression and function of miR-142 and TGF- β in two HCC cell lines were determined. The miRNA-mRNA network of miR-142 was analysed in HCC cell lines.

Results: We found that the miR-142 expression was reduced in tumour tissues and two HCC cell lines HepG2 and SMMC7721, which correlated to higher TNM stage, metastasis and differentiation. Moreover, miR-142 was identified to directly target and inhibit transforming growth factor β (TGF- β), leading to decreased cell vitality, proliferation, EMT and the ability of pro-angiogenesis in TGF- β -dependent manner. Interestingly, the status of methylation of miR-142 was analysed and the results found the hypermethylated miR-142 in tumour patients and cell lines. The treatment of methylation inhibitor 5-Aza could restore the expression of miR-142 to suppress the TGF- β expression, which impaired TGF- β -induced tumour growth.

Conclusion: These findings implicated that miR-142 was a tumour suppressor gene in HCC and often hypermethylated to increase TGF- β -induced development of hepatocellular carcinoma.

1 | INTRODUCTION

Primary hepatic malignancy is the second leading causes of cancer-related death worldwide, the majority of which are

hepatocellular carcinoma (HCC) with over 5 00 000 new cases diagnosed every year and about 10% of all cancer deaths. Asia and Africa have the highest incidence rates of liver cancer all over the world.¹ Because of the characteristics of HCC that typically late-stage

TABLE 1 qPCR primers

Gene	Primer
hsa-miR-142b-R	GTCGTATCCAGTGCAGGGTCCG AGGTGCACTGGATACGACTCCATAA
hsa-miR-142b-F	TGCGGTGTAGTGTTTCCTACTT
U6 F	CTCGCTTCGGCAGCAC
U6 R	AACGCTTCACGAATTTGCGT
GAPDH-F	ACACCACTCCTCCACCTTT
GAPDH-R	TTACTCCTTGGAGGCCATGT
TGFB1-F	AGCAACAATTCCTGGCGATAC
TGFB1-R	CAACCACTGCCGCACAACT

presentation and aggressive nature, there are limited treatment options which lead to the very poor 5-year survival of patients with HCC (less than 40%). Importantly, patients with advanced disease are not eligible for curative hepatectomy or hepatic transplantation, and even for those patients who undergo surgical resection, the high recurrence rate is the main causes for low survival time.² The molecular pathogenesis involved into its aggressive nature and high recurrence rate are complicated, thus understanding the potential mechanisms of HCC pathogenesis, including genetic and epigenetic alterations, is crucial to uncover efficient new treatment.

MicroRNAs (miRNAs) are non-coding RNAs of 20-22 bases in length that regulate gene expression post-transcriptionally *via* binding to the 3' untranslated regions (UTRs) of mRNAs.³ Numerous evidences indicated that miRNAs influence numerous cancer-relevant processes such as proliferation, cell cycle, apoptosis, differentiation, metastasis and metabolism.⁴ Many miRNAs could function as oncogenes or tumour suppressors during carcinogenesis and their dysregulations in tumours were frequently occurred, including in HCC. miR-21 was reported to be highly overexpressed in HCC tumours and contributed to HCC growth and spread by modulating the tumour suppressor PTEN expression.⁵ miR-29 down-regulation was significantly associated with worse disease-free survival of HCC patients and two anti-apoptotic molecules, Bcl-2 and Mcl-1, as direct targets of miR-29 for the regulation of apoptosis.⁶ However, the function of miR-142 had not reached inconsistency. miR-142 was identified to dysregulated in different types of tumours. miR-142 was up-regulated in human breast cancer stem cells (BCSCs) as compared to the non-tumorigenic breast cancer cells⁷ but obviously down-regulated in human cervical cancer tissues⁸ and HCC tissues.⁹ But, to date, the courses of this dysregulation in HCC and related function have not fully understood.

Epigenetic disorders have been discovered to be associated with many kinds of significant human diseases including cancer.¹⁰ As the first type of epigenetic mark, DNA methylation is the most characterized epigenetic phenomenon, hypermethylation and epigenetic silencing of tumour suppressor genes are frequent events during the development of cancer. In bladder cancer, the tumour suppressor miR-127 was down-regulated and silenced by promoter DNA hypermethylation in tumour tissues.¹¹ The methylation of promoter CpG islands of miR-34a was found in the majority of primary prostate carcinoma samples,

which led to the loss expression of miR-34a.¹² However, the correlation between methylation and the loss of miR-142 in HCC is unclear.

In this study, we investigated the function of miR-142 in HCC and its relationship with methylation. We found that miR-142 was down-regulated in HCC tissues and cell lines which contributed to the up-regulation of its direct target transforming growth factor β (TGF- β) to promote tumour growth, metastasis and angiogenesis. The decreased expression of miR-142 attributed to the hypermethylation and the methylation inhibitor 5-Aza could restore the expression of miR-142 to suppress the TGF- β expression and related pro-tumour functions in hepatocellular carcinoma.

2 | MATERIAL AND METHODS

2.1 | Tumour samples

Hepatocellular carcinoma tissues (n = 50) and corresponding relative-normal tissues (n = 50) were collected from Nanfang Hospital Affiliated to Southern Medical University to determine the expression of miR-142 and TGF- β . This study was approved by the Research Ethics Committee of Nanfang Hospital Affiliated to Southern Medical University. All these retrospective specimens were handled and made anonymous according to the ethical and legal standards. Written informed consent was obtained from all of the patients. According to WHO classifications, all of the patients diagnosed with primary hepatocellular carcinoma were confirmed by haematoxylin and eosin staining by experienced pathologists.

2.2 | Cell lines and reagents

The hepatocellular carcinoma cell line HepG-2, SMMC-7721 and one normal human liver cell line HL-7702 were purchased from the cell bank of the Chinese academy of sciences (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (Life Technologies, USA), ampicillin and streptomycin at 37°C, 5% CO₂ conditions. Oligonucleotide sequences of miR-142 mimics, inhibitors or negative control was purchased from RiboBio (Guangzhou, China). Anti-GAPDH, TGF- β , E-cadherin, N-cadherin, and Vimentin antibodies were obtained from Cell Signaling Tech (Denver, MA) and Abcam (USA). 5-Aza was purchased from Sigma (St. Louis, MO). For expression of TGF- β , the overexpression vector pcDNA3.0-TGFB1 was conducted by GenePharma (Shanghai, China). Reporter plasmid of full-length 3'-UTR (wild-type or mutant) of TGFB1 was conducted by GenePharma (Shanghai, China).

2.3 | RNA isolation and qRT-PCR

After the cells were harvested, PBS was used to wash for three times, total RNA from tissues or HepG-2 and SMMC-7721 cells was extracted using Trizol reagent (Invitrogen) according to the standard RNA isolation protocol. Quantitative real-time RT-PCR (qRT-PCR) was performed, and the expression levels of miR-142 and TGFB1 were normalized to GAPDH and U6 for gene expression. The primers were listed as Table 1.

2.4 | Western blots

According to the manufacturer's instructions, the whole cell protein extracts were prepared and all proteins were resolved on a 10% SDS denatured polyacrylamide gel and were then transferred onto a nitrocellulose membrane, which were blocked in 5% non-fat milk in TBST buffer (Tris buffer saline containing 0.1% Tween-20) for 1 hour at room temperature, and subsequently incubated with primary antibodies overnight at 4°C. After washing with TBST buffer, the blots were then incubated with HRP-conjugated secondary antibody for 1 hour at room temperature. After washing with TBST buffer, the blots were visualized using the ECL-Plus reagent (Millipore, Billerica, MA). GAPDH was used as the loading control in the Western blotting.

2.5 | Cell transfection

The HepG-2, SMMC-7721 cells were seeded into 12 plates and transfected with miR-142 mimics, inhibitors, pcDNA3.0-TGFB1 or negative control for indicated time at a concentration of 1 ng/mL by Lipofectamine 2000 (Invitrogen, CA) according to the manufacturer's instructions.

2.6 | Dual-luciferase assay

To investigate the direct target of miR-142 on the transcription of TGFB1, the HepG-2, SMMC-7721 cells were co-transfected containing 200 ng firefly Luciferase vector, 40 ng Renilla luciferase pRL-TK vector (Promega, WI) and psiCHECK2-UTR (wild-type or mutant) of TGFB1 (wild-type or mutant). Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, WI). Firefly luciferase acted as a reporter gene and Renilla luciferase as a normalized control.

2.7 | Flow cytometry assay

After the indicated treatment, the cells were harvested and suspended with PBS, then cells were stained by for BrdU incorporation 8 hours after all experimental conditions with the BrdU Labeling and Detection Kit I (Roche). Cells pre-labelled with BrdU were fixed with ethanol and then incubated with monoclonal antibodies against BrdU mixed with nucleases, followed by fluorescein-conjugated secondary antibodies according to the manufacturer's instructions and quantified by flow cytometry on a FACS Calibur instrument.

2.8 | Transwell assay

For the migration assay, after the indicated treatment, 2×10^4 HepG-2, SMMC-7721 cells were cultured in the upper chamber of a non-coated transwell insert. The lower chamber contained 600 μ L medium with 10% bovine serum which was used as a chemo-attractant to encourage cell migration. For the invasion assay, the upper chamber of the transwell inserts were coated with 50 μ L of 2.0 mg/mL Matrigel, the cells were plated in the upper chamber and incubated for 24 hour and cells that did not migrate or invade were removed using

a cotton swab. Using crystal violet staining, the cells were stained and counted under an inverted microscope. Five random views were selected to count the cells and the independent experiments were repeated three times.

2.9 | HUVEC tube formation assay

The conditional supernatants of tumour cells were collected. The HUVEC capillary-like tube formation assay was performed on Matrigel (Solarbio). After the Matrigel coagulated, the plate was added with 5×10^4 cells/well and equal volume of supernatants. Cells on Matrigel were incubated for 8 hour and five random fields were choose to calculate the tubes length of tube-like capillary by image pro plus software.

2.10 | Methylation analysis

To detect the status of methylation of miR-142 in HCC tissues and cells, genomic DNA was extracted and modified with sodium bisulfite (2 μ g) using an EpiTect Bisulfite Kit (Qiagen, Hilden, Germany). Methylation was determined by methylation-specific PCR, and bisulfite sequencing, and details of methods are shown as previously.¹³

2.11 | Statistical analysis

All statistical analyses were performed by Statistical Program for Social Sciences (SPSS) 16.0 software (SPSS, Chicago, IL) and GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA). Unpaired *t* tests or Mann-Whitney U tests were used to compare the two groups, and multiple group comparisons were analysed with one-way ANOVA. *P* < .05 was considered to be statistically significant. Data were presented as the mean $\pm$ standard deviation (SD).

3 | RESULTS

3.1 | Decreased expression of miR-142 correlates to poor clinical outcomes in HCC

To investigate the clinical role of miR-142 in hepatocellular carcinoma, we collected HCC tissues (*n* = 50) and corresponding non-neoplastic tissues (*n* = 50), and the expression of ETS2 was analysed by Q-PCR. The results showed that the expression of miR-142 was significantly reduced in HCC tissues than that in the corresponding non-neoplastic tissue (Figure 1A). The human HCC tumour cell lines HepG2 and SMMC7721 and human normal liver cell line HL-7702 were used to confirm the miR-142 level in vitro (Figure 1B). We found that the miR-142 level was lower in HepG2 and SMMC7721 than that in HL-7702, which was consistent with the clinical finding. Moreover, the clinicopathological characteristics were collected and its relationships to miR-142 were determined. Patients with lower expression of miR-142 had high stage of TNM and differentiation and had more metastasis (Figure 1C-E). These data suggested that miR-142 was down-regulated to be involved into the progression of HCC.

FIGURE 1 miR-142 is down-regulated in HCC patients. A, The expression pattern of miR-142 in hepatocellular carcinoma (HCC) and relatively normal tissues were determined by Q-PCR. B, The expression of miR-142 in HCC cell lines HepG2, SMMC-7721 and one normal human liver cell line HL-7702 were analysed. C-E, The clinicopathological characteristics of HCC patients were analysed according to the expression of miR-142. * $P < .05$, ** $P < .01$, data represent the mean $\pm$ SD

3.2 | TGF β 1 is a direct target of miR-142 in HCC

Although some genes (eg *TGF- β 2*, *HSP70*, *HMGB1* and *smad3*) were demonstrated to be the targets of miR-142 in different cancers, the targets of miR-142 in HCC are unclear. According to miRWalk (<http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/>), we next determined the potential targets of miR-142, the 3'-UTR of *TGF- β* , which encoded TGF- β , harboured a binding site of miR-142 which indicated that TGF- β could be targeted by miR-142 (Figure 2A). Therefore, we assessed the expression of TGF- β in HCC tissues and cell lines and found that the TGF- β level was highly increased in tumour samples and cell lines, and negatively correlated to the miR-142 level in clinical samples (Figure 2B-E). Besides, overexpression of miR-142 mimics in HepG2 and SMMC7721 cells inhibited the expression of TGF- β but the miR-142 inhibitors could restore this process in mRNA and protein level (Figure 2F,G).

To conform the directly target relationship, a luciferase reporter vector with full-length 3'-UTR (wild-type or mutant) of TGF- β was transfected into HepG2 and SMMC7721 cells to perform the luciferase reporter assays and the results showed that miR-142 mimics could significantly impair the luciferase activity of wild-type TGF- β -3'UTR, but not the mutant type (Figure 2H).

3.3 | miR-142 inhibit the progression of HCC via TGF- β

We next investigated the role of miR-142/TGF- β signals in HCC cell lines, the cell vitality, proliferation, EMT and angiogenesis were estimated. After transfection of miR-142 mimics, the cell vitality of two cells were significantly reduced but could be rescued by the

overexpression of TGF- β (Figure 3A). Considering that miR-142 could impair the cell vitality of tumour cells, the proliferation was also determined by BrdU proliferation assay (Figure 3B) and found that miR-142 inhibited the proliferation of HepG2 and SMMC7721 cells and TGF- β could repress miR-142-induced inhibition of proliferation.

Moreover, the effects of miR-142 on the ability of migration and invasion were analysed (Figure 3C). Similarly, the results of transwell assay indicated that miR-142 mimics inhibited the migration and invasion of HepG2 and SMMC7721 cells *via* TGF- β . We further hypothesized that the impacts on metastasis was due to EMT. Thus, the expression of epithelial-specific marker E-cadherin, and mesenchymal markers N-cadherin, and Vimentin were estimated (Figure 3D). We found that miR-142 was able to elevate the expression of E-cadherin but reduce the expression of N-cadherin and Vimentin, which were restored partly by the introduction of TGF- β . The role of miR-142 in angiogenesis was determined by HUVECs tube formation assay (Figure 3E). The conditional culture supernatant of tumour cells was treated to the HUVECs and the cells overexpressed of miR-142 in HCC cells inhibited the tube formation but TGF- β could restore miR-142-induced inhibition of tube formation. These data suggested that miR-142 inhibit the tumour characteristics of HCC *via* TGF- β .

3.4 | The loss expression of miR-142 is attributed to hypermethylation in HCC

To find out the causes of the loss expression of miR-142 in HCC, the status of methylation of miR-142 was analysed in HCC tissues, and HepG2 and SMMC7721 cells. Bisulfite sequencing analysis

FIGURE 2 TGF β 1 is a direct target of miR-142. A, Sequence alignment of putative miR-142-5p binding sites in the TGF β 1 3'UTRs. B-D, The expression of TGF β 1 in HCC tissues was determined by Q-PCR and its relationship with miR-142 was also analysed. E, The expression of TGF β 1 in HepG-2, SMMC-7721 and HL-7702 were analysed. F and G, After the transfection of miR-142 mimics, inhibitors or negative control, the expression of TGF- β was determined by Q-PCR and WB. H, HepG-2, SMMC-7721 cells were transfected with full-length 3'-UTR (wild type or mutant) of TGF β 1, and the luciferase reporter was performed to confirm the direct target sites. * $P < .05$, ** $P < .01$, data represent the mean $\pm$ SD

FIGURE 3 miR-142 inhibits the tumour characteristics of HCC cells. A and B, after the indicated treatments in HepG-2, SMMC-7721 cells, the proliferation and cell vitality was estimated by CCK-8 and BrdU assay. C, The migrations and invasions of two tumour cells were determined by transwell assay. D, The expressions of EMT markers were estimated by WB. E, Different conditional supernatants were collected and the HUVEC capillary-like tube formation assay was performed on Matrigel. * $P < .05$, ** $P < .01$, data represent the mean $\pm$ SD

FIGURE 4 The status of methylation of miR-142 in HCC samples and cells were analysed by methylation specific PCR (A), and bisulfite sequencing (B). The HepG-2, SMMC-7721 cells were transfected with miR-142 inhibitors and negative control, and then the cells were treated with methylation inhibitor 5-Aza ($5 \mu\text{mol L}^{-1}$), the expression of (C) miR-142 and (D) TGFβ1 were determined by Q-PCR. * $P < .05$, ** $P < .01$, data represent the mean $\pm$ SD

showed that the HCC tissues had more hypermethylated miR-142 than normal tissues (Figure 4A). The similar result was confirmed in HL-7702, HepG2 and SMMC7721 cells, which emphasized the frequently hypermethylated miR-142 in HCC. Moreover, the treatment of methylation inhibitor 5-Aza could inhibit the methylation of miR-142 (Figure 4B) to restore the expression of miR-142 in HepG2 and SMMC7721 cells which was accompanied by elevated expression of TGF-β (Figure 4C,D).

3.5 | The methylation inhibitor 5-Aza could rescue the miR-142 level for anti-tumour effects

Given that hypermethylated miR-142 in HCC induced the loss-of-function of miR-142, the methylation inhibitor 5-Aza might restore the anti-tumour effects of miR-142. Thus, two cell lines were

transfected with miR-142 inhibitors with or without the treatment of 5-Aza, the cell vitality, proliferation, EMT and angiogenesis were estimated. The results showed that 5-Aza impaired the cell vitality (Figure 5A) and proliferation (Figure 5B) of tumour cells which could be abrogated by the miR-142 inhibitors. In addition, the ability of migration and invasion were also inhibited by 5-Aza *via* miR-142-regulated EMT (Figure 5C,D). Similarly, 5-Aza-treated tumour cells had impaired ability of pro-angiogenesis and miR-142 inhibitors could repress 5-Aza-induced inhibition of angiogenesis (Figure 5E).

4 | DISCUSSION

Cancers are far more complex than realized due to the genetically heterogeneity, which involve with the protean miRNA and long

FIGURE 5 The HepG-2, SMMC-7721 cells were transfected with miR-142 inhibitors and negative control, and then the cells were treated with methylation inhibitor 5-Aza ($5 \mu\text{mol L}^{-1}$), the proliferation and cell vitality was estimated by (A) CCK-8 and (B) BrdU assay. C, The migrations and invasions of two tumour cells were determined by transwell assay. D, The expressions of EMT markers were estimated by WB. E, Different conditional supernatants were collected and the HUVEC capillary-like tube formation assay was performed on Matrigel. * $P < .05$, ** $P < .01$. Data represent the mean $\pm$ SD

non-coding RNAs profiling, copy number aberrations, hemizygous and homozygous deletions, and promoter methylation. Patients with cirrhosis are predisposed to hepatocellular carcinoma and the aberrant TGF- β expression is one of culprits of cirrhosis and tumorigenesis.¹⁴ In this study, miR-142 functioned as tumour suppressor was dysregulated in HCC and was hypermethylated to be down-regulated. TGF- β was found to be the direct target of miR-142 and highly up-regulated for the progression of HCC, including proliferation, EMT and the ability of pro-angiogenesis. Interestingly, the methylation inhibitor 5-Aza could restore the expression of miR-142 for tumour elimination.

miRNA mutations or aberrant expressions are demonstrated to be associated with various human cancer, including lung, breast, liver, colon, oral cancer and leukaemia.^{3,15,16} Depending on the distinct cellular function of their targets, miRNAs may function as oncogenes or tumour suppressors in tumour development. Indeed, miR-142 and TGF- β functioned inconsistently in different types of cancers contexts. In cervical cancer, miR-142 was obviously down-regulated in human cervical cancer tissues which correlated to advanced FIGO stage and lymphatic metastasis. HMGB1 was a direct target of miR-142 and regulated cervical cancer cell proliferation and invasiveness via miR-142.⁸ Increased levels of circulating miR-142 were associated with aggressive non-small cell lung cancer (NSCLC) disease and a high risk of relapse in patients with early-stage NSCLC. miR-142 could directly bind to 3'-UTR of TGF β R1 and inhibit the expression of TGF β R1 and impair TGF- β -induced

growth inhibition in NSCLC cells.¹⁷ Dickman et al. reported that miR-142 in small extracellular vesicles of oral cancer was associated with increased vascular density in vivo.¹⁸ We here found that miR-142 was significantly reduced during the development of HCC and TGF- β could be targeted to inhibit the cell vitality, proliferation, EMT and angiogenesis of HCC cells. Other molecular in TGF- β pathway, eg SMAD3, was also reported to be targeted and inhibited by miR-142.¹⁹ Ma et al. found that miR-142-5p promoted tumour cell proliferation and inhibited apoptosis and play as a negative regulator in TGF- β pathway by targeting SMAD3.²⁰ These inconsistent researches emphasized the multifunction of miR-142 in distinct cancer environments.

Currently, the mechanisms of regulation of differentially expressed miRNAs in cancer mainly consisted of transcriptional regulation, genetic alterations in miRNA biogenesis pathways and epigenetic mechanisms.⁴ Representative examples such as miR-9-1 in breast cancer²¹ and miR-124a in colorectal tumours²² was suppressed by CpG island hypermethylation in tumour tissues when compared to normal controls. Watanabe et al. reported that miR-142 was highly methylated in fibroblasts²³ and other found that miR-142 was epigenetically repressed by DNA methylation in mesenchymal cells,²⁴ whereas miR-142 was found to be hypomethylated in cutaneous T-cell lymphoma for its up-regulation.²⁵ But the status of methylation of miR-142 in cancer was unclear. We here demonstrated that the down-regulation of miR-142 was attributed to its hypermethylation in HCC. The methylation inhibitor 5-Aza

could restore the expression of miR-142 in HCC cells. Similarly, in osteosarcoma (OS), the hypermethylation of miR-142 was found in OS samples and led to its loss-of-function, which promoted the cell invasion and growth.²⁶

5 | CONCLUSIONS

In summary, we uncovered the hypermethylated status of miR-142 in HCC, which explained the decreased expression of miR-142. The methylation inhibitor 5-Aza could restore the expression of miR-142 for targeting and inhibiting the TGF- β -dependent tumour proliferation, EMT and the ability of pro-angiogenesis.

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CONFLICT OF INTEREST

No competing financial interests exist.

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