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MicroRNA-185 inhibits cell proliferation and epithelial-mesenchymal transition in hepatocellular carcinoma by targeting Six2

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Abstract. – OBJECTIVE: Epithelial-mesenchymal transition (EMT) plays an imp[illegible]t role in the malignant transformation of [illegible]. MicroRNAs are a group of small non-c[illegible]g RNA molecules that down-regulate the ex[illegible]- sion of genes involved in tumorigenesis. [illegible]though microRNA-185 (miR-185) participat[illegible] in the pathogenesis of sev[illegible]s of ca[illegible]cer, its relationship with E[illegible]n h[illegible]n hepatocellular carcinoma (H[illegible] has n[illegible]een investigated. The prese[illegible]udy ai[illegible]- date the regulatory [illegible]fec[illegible]mi[illegible]s [illegible] in HCC cells.

MATERIALS A[illegible]METHOD[illegible]T and an in vitro wound-he[illegible]say were pe[illegible]ed to determine cell [illegible]th [illegible]metastasis p[illegible]ential, respectively. R[illegible]l-time [illegible]was used to measure the mRN[illegible]pression o[illegible]185 and Six2. In addition, [illegible]ein expression l[illegible]f Six2 and EMT-relat[illegible]arkers [illegible]ere determin[illegible]d by western blot.

[illegible]ULTS: [illegible] study showed that miR-185 w[illegible]nifi[illegible]y dow[illegible]regulated in HCC cells. Also[illegible]erase [illegible]rter gene assay con-[illegible]med [illegible]s a d[illegible] target of miR-185. Func-[illegible]l an[illegible]ated that miR-185 up-regula[illegible] rema[illegible] suppressed cell growth and th[illegible]etastatic [illegible]otential of HCC cells. We also [illegible]ectopic expression of miR-185 re-[illegible]rse[illegible]via the up-regulation of E-cadherin and down-regulation of vimentin in epithelial [illegible]d mesenchymal HCC cells.

[illegible]ONCLUSIONS: miR-185 suppresses cell g[illegible]wth and EMT progression by targeting Six2, prov[illegible] target for the molecular treatment of [illegible]alignancies.

[illegible]rds:
[illegible]cellular carcinoma, microRNA-185, Six2, Epithelial-mesenchymal transition.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most frequent type of malignant tumor and the third leading cause of cancer-related death in the world[1]. Despite the fact that much is known about the genetic and epigenetic regulation contributing to HCC, the molecular mechanisms underlying the pathogenesis have not been fully elucidated[2].

MicroRNAs are a class of small, non-coding RNAs, which are capable of modulating gene expression at the post-transcriptional level[3]. A large body of evidence suggests that aberrant microRNA expression features significantly in various types of cancer[4,5]. Functional studies have demonstrated that microRNAs play diverse roles in tumorigenesis, where they may act as tumor suppressors, oncogenes, and modulators of tumor cell survival, apoptosis, and metastasis[6,7]. mi-

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croRNA-185 (miR-185) is one of the most commonly down-regulated miRNAs in several cancers including HCC, prostate carcinoma, breast cancer, and gastric cancer, suggesting that miR-185 may play an important role in carcinogenesis[8-11]. In addition, the potential anti-cancer properties of miR-185 have been demonstrated through the regulation of critical cellular behaviors such as cell proliferation, apoptosis, cell migration, and invasion[12-14].

Epithelial-mesenchymal transition (EMT) is a complex, reversible process that induces epithelial cells to transform to a mesenchymal phenotype[15]. Advances in research have highlighted the role of EMT in regulating the cellular process of liver carcinoma, but the molecular mechanisms regulating EMT remain poorly understood[16].

In this current study, we focused on miR-185 in HCC and its relationship with EMT, and our results would complement the current knowledge of miR-185 in liver cancer.

Materials and Methods

Cell Culture

A normal liver cell line (LO2) and four human HCC cell lines (HepG2, HuH7, SNU-387, and SNU-449) were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO, USA) and 1% penicillin-streptomycin (Sigma, St. Louis, MO, USA). The cells were maintained at 37 °C in a humidified air atmosphere containing 5% carbon dioxide.

Cell Proliferation Assay

Cell proliferation was measured using an MTT (3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay. Briefly, cells at a density of 5×10^4 (cells/well) were seeded in 96-well plates. Then 15 μl of MTT (5 mg/ml) was added to the wells and incubated in dark at 37□ for 4 h. Absorbance was measured with a microplate reader at a wavelength of 490 nm (Bio-Rad, CA, USA).

Cell Migration Assay

Cell migration was measured using an *in vitro* wound-healing assay. Cells were cultured in 6-well plates overnight. Experimental wounds were made by dragging a rubber policeman (Fisher Scientific, Hampton, VA, USA) across the cell culture. The cultures were rinsed with PBS and placed in fresh quiescence medium. Three wounds were created for each specimen. The relative distance traveled by the cells was determined.

RNA Extraction and Real-Time PCR

Total miRNA was extracted from the harvested cells using an RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. cDNA was synthesized from total RNA using a TaqMan miRNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). miR-185 expression was detected using a miRNA-specific TaqMan MiRNA Assay Kit (Applied Biosystems, Carlsbad, CA, USA). PCR reactions were performed on an ABI 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) with the following conditions: 95°C, 10 min for 1 cycle, then 95°C, 15 sec, 60°C, 1 min for 40 cycles. The U6 small nuclear RNA was used as a loading control. The mRNA expression of Six2 was measured using real-time PCR in an ABI 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA), with U6 as a control.

Plasmid Construction and Luciferase Activity Assay

For the fluorescent reporter assay, the following primers were used to amplify the 3'UTR of the Six2 gene: forward primer 5'- CTTGGTACCGAGCTCTCCTAGAGCTCTGTTCGCCT-3'; reverse primer 5'- -TGCTGGATATCTGCACGAACATTCACATGAGGGCG-3'.

A plasmid containing the Six2 3'UTR and a fluorescent reporter was constructed. HepG2 cells were seeded into 48-well plates and co-transfected with a negative control, a miR-185 mimic or a miR-185 inhibitor. Luciferase activity was measured using a dual-luciferase reporter assay system (Promega, Madison, WI, USA). Firefly luciferase activity was normalized to renilla activity.

Western Blot

Cells were lysed using RIPA buffer and total cell extracts were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). For immunoblotting, the membranes were blocked with 5% non-fat milk in Tris-buffered saline then incubated with antibodies against Six2, E-cadherin, vimentin, and GAPDH (Santa Cruz, CA, USA). The membranes were washed thrice

and incubated with horseradish peroxidase-conjugated secondary antibody. Protein bands were detected using an enhanced chemiluminescence (ECL) kit (Thermo Scientific, Waltham, MA, USA) and a ChemiGenius bioimaging system (Syngene, Frederick, MD, USA).

Statistical Analysis

All data are presented as mean ± SD. For comparisons between two groups, statistical significance was determined using the Student's *t*-test. Comparisons between three or more groups were performed using Least-significant difference (LSD) following analysis of variance (ANOVA). A value of $p < 0.05$ was considered significant.

Results

Down-Regulation of miR-185 in HCC Cells

miR-185 is known to play a central role in various types of cancers including HCC. However, the biological function of miR-185 has not been fully elucidated. In the current study, we used web-based target analysis tools like TargetScan, miRanda, and pictar to predict potential target genes that may be regulated by miR-185. In this analysis, Six2 was identified as a potential target of miR-185. We then went on to examine the expression of miR-185 and *Six2* in four human HCC cell lines (HepG2, HuH7, SNU-387 and SNU-449) and a normal human liver cell line (LO2) by real-time PCR. Our results revealed that miR-185 expression was significantly reduced in HCC cells compared with normal liver cells (Figure 1A). Conversely, we found the mRNA and protein expression of *Six2* was increased in HCC cells (Figure 1B and 1C). These results imply that miR-185 may be one of the upstream molecules that mediate *Six2* expression in liver cancer.

miR-185 Modulates Cell Growth and Migration in HCC Cells

To investigate the biological role of miR-185 in HCC cell growth and migration, four HCC cell lines were transiently transfected with either miR-185 mimics or a negative control. MTT assays were then performed to determine the effect of miR-185 on cell proliferation. Our results showed that overexpression of miR-185 significantly suppressed the growth of HCC cells (Figure 2A). Moreover, we found that ectopic expression of miR-185 reduced the metastatic potential of HCC cells (Figure 2B). These results indicate

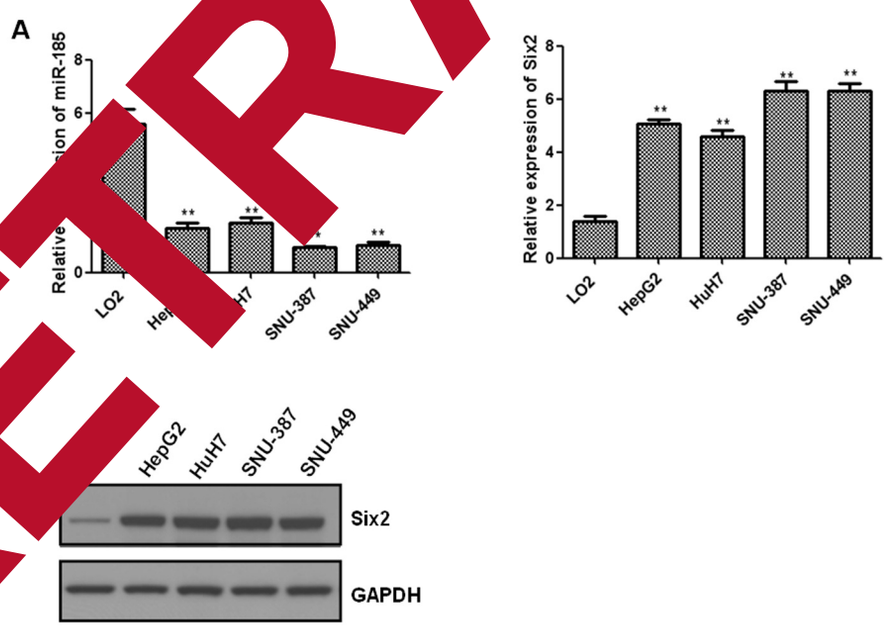

Figure 1. Negative correlation between miR-185 and Six2 expression in HCC cells. (A) The expression of miR-185 was determined in four HCC cell lines (HepG2, HuH7, SNU-387 and SNU-449) and a normal human liver cell line (LO2) by real-time PCR. (B and C) mRNA and protein levels of Six2 were assessed by real-time PCR and Western blot, respectively. $^{*}p < 0.05$, $^{**}p < 0.01$.

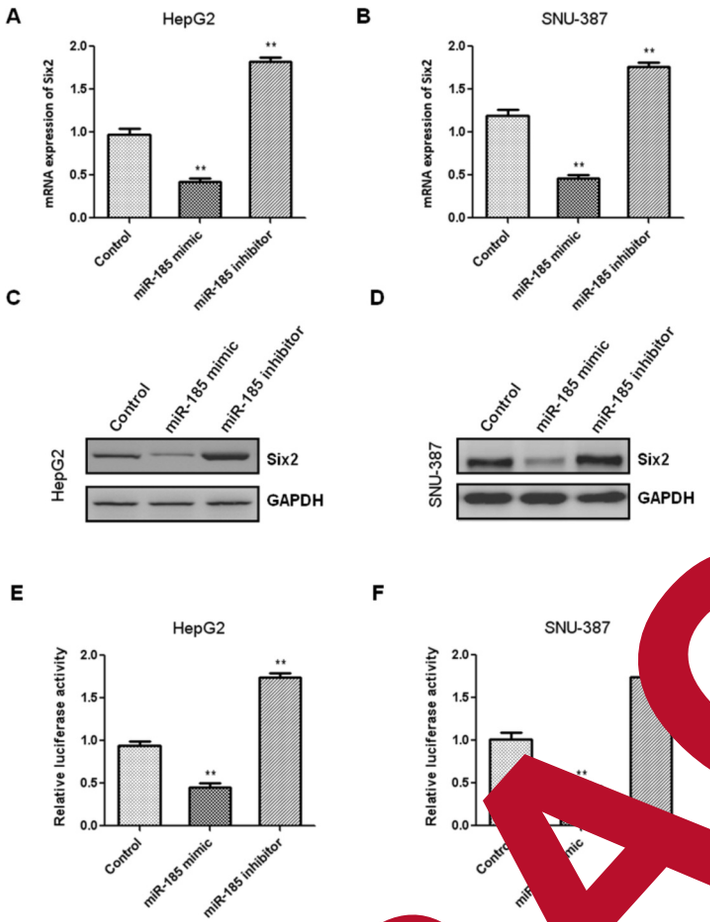

Figure 2. Ectopic expression of miR-185 suppresses HCC cell growth and migration. Four HCC cell lines were transfected with a miR-185 mimic or a negative cont... proliferation ***(A)*** and migrat... HepG2, HuH7, SNU-387 ... NU-449 cells were determined by ... assay and a wound-healing assay, res... ly.

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miR-185 Direc... argets Si... in HCC Cell...

To determ... whe... ix2 is directly regulated by mi...85, we trans... HepG2 and SNU-387 cel... ith a miR-185 m... or inhibitor, then mea... *Six2* mRNA expres...on using real-time PC... Our re... show that miR-185 up-regulation ... repres... *Six2* mRNA expression comp... control... hereas inhibition of miR-... resu... ased *Six2* levels in HepG2 a... NU-3... (Figure 3A and B). Western b... nalysis al... showed that over-expression of ...ted in a markedly decrease in Six2 ...otein, w...reas a reduction in miR-185 remarkably increased Six2 protein levels in HepG2 and ...-387 cells (Figure 3C and D). We then per-f...ed fluorescent reporter assays to determine whether Six2 is a direct target of miR-185. As predicted, miR-185 over-expression significantly suppressed luciferase activity in HepG2 and SNU-387 cells. Furthermore, firefly luciferase activity was significantly increased in HepG2 and SNU-387 cells after transfection with miR-185 inhibitor (Figure 3E and F). These results indicate that Six2 is a direct functional target of miR-185.

Determination of the Phenotypes of Four HCC Cell Lines

EMT progression is characterized by the loss of expression of epithelial cell junction proteins, such as E-cadherin, and a gain of mesenchymal marker expression, such as vimentin. In this study, we found that E-cadherin was primarily expressed in HepG2 and HuH7 cells, but was absent in SNU-387 and SNU-449 cells. In contrast, vimentin expression was higher in SNU-387 and SNU-449 cells than in HepG2 and HuH7 cells (Figure 4). These findings indicate that HepG2 and HuH7 cells exhibit an epithelial character while SNU-387 and SNU-449 cells have a mesenchymal phenotype.

Up-regulation of miR-185 Reverses EMT in HCC Cell lines

We used epithelial (HepG2 and HuH7) and mesenchymal (SNU-387 and SNU-449) cell lines to investigate the effect of miR-185 on EMT. We transfected a miR-185 mimic into HepG2 (epithelial) and SNU-387 (mesenchymal) cells and showed that ectopic miR-185 expression led to increased E-cadherin expression in the HepG2 epithelial cells (Figure 5A and B) and suppressed vimentin expression in SNU-387 mesenchymal cells (Figure 5C and D). Thus, these results demonstrate that up-regulation of miR-185 caused a reversal of EMT in HCC cell lines.

Discussion

To date, a large body of data has shown that miRNAs exhibit altered expression levels in multiple types of cancer, where they play key roles in tumor cell behavior through the regulation of target gene expression[17,18]. Thus, the key to understanding miRNA function is to identify their functional targets. In this study, we demonstrated that miR-185 suppresses cell growth a progression in HCC cells by regulati x2 expression.

miR-185 is one of the most wel d miRNAs in cancer biology. Several iga-tions[13,19-21] have demonstrat at miR- ts as a tumor suppressor i tric cancer, b cancer, prostate carcin and col tal canc However, although th r s essive effects of miR-185 have ts pre ole in tumorigenesi not bee y elu ed.

Homeobox s encode tra n factors that act as f embryoni velopment, where they e invo n cell growth and differentiation[22]. It has also hown that homeobox gen n essential ro mor initiation and p ession[23]. Six2, a member of the SIX family omeobox ge has been given much atten- due to its di e roles in cancer. Early stud-

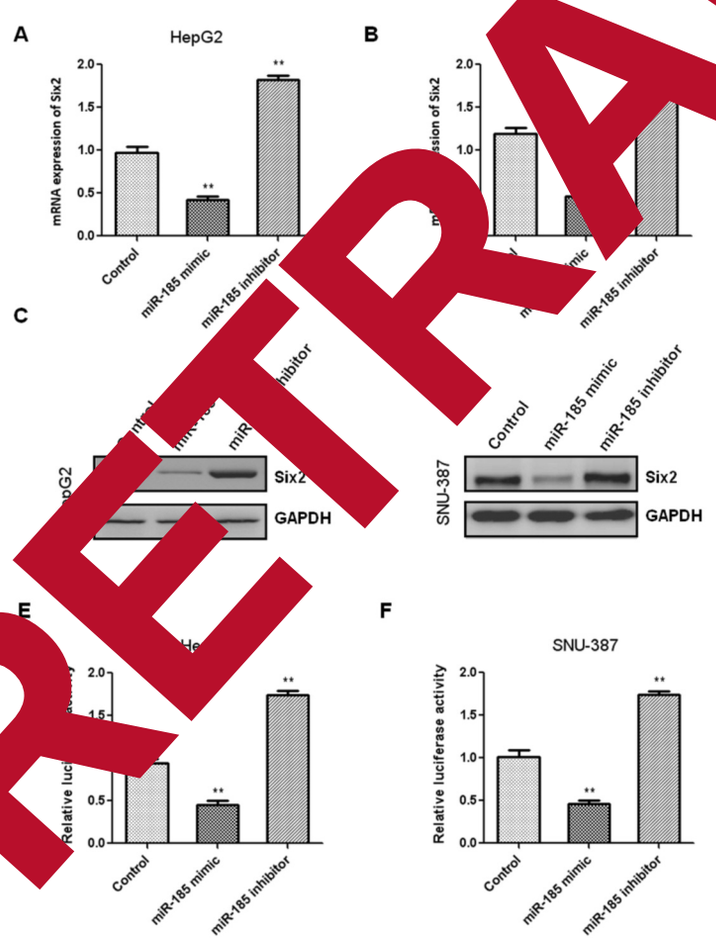

Figure 3. Six2 is a target of miR-185 in HCC cells. HepG2 and SNU-387 cells were transfected with a negative control, a miR-185 mimic or a miR-185 inhibitor. mRNA ***(A*** and ***B)*** and protein ***(C*** and ***D)*** expression of Six2 were determined by real-time PCR and Western blot, respectively. ***(E*** and ***F)*** Luciferase activity was assessed 48 h after transfection using a dual luciferase reporter assay, normalized to a renilla control. $^{*}p < 0.05$, $^{**}p < 0.01$.

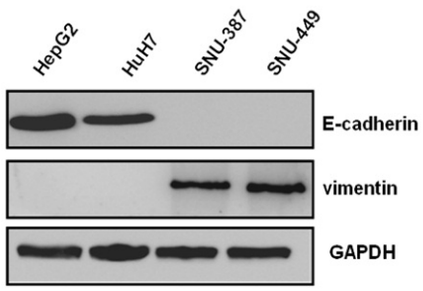

Figure 4. Expression of EMT-related markers in four HCC cell lines. Western blot analysis was performed using antibodies against E-cadherin (epithelial marker) and vimentin (mesenchymal marker) in HepG2, HuH7, SNU-387 and SNU-449 cell lines. GAPDH was used as an internal control.

ies[24,25] suggested that Six2 regulates kidney development through the suppression of premature nephrogenesis and maintenance of mesenchymal progenitor populations. The pro-proliferative and pro-migratory effects of Six2 have been reported in the pathogenesis of several types of cancers, including breast cancer, nephroblastomas, and renal clear cell carcinomas[26,27]. In the current repo observed the concomitant down-regula miR-185 and up-regulation of *Six2* in HCC ls, suggesting that Six2 could be a potential t gene of miR-185. As expected, transfection v miR-185 significantly down-regulated the expression of *Six2*. Also, luciferase activity assays further confirmed the direct regulation of Six2 by miR-185 in HCC cells.

Functional researches have sug d that miR-185 could induce cell cycle t and repress cell proliferation in gastric r-19, colorectal cancer[20], non-small-cell lung c 8 and glioma[29]. Moreover, miR-1 as also e-ported to be involved in stasis in colo cancer and glioma[29,30]. istent these p vious works, our resu non that the ectopic expression ifican up-pressed cell gro nd mig in H cells.

EMT is a hogenetic p n which cells lose e atures such ll polarity, and gain senchy properties such as increased motility[31]. Acq on of EMT enhances the properties cancer cells. During E , the loss of cell-cell adhesion is a critical in tumor inv on and metastases, and is often accompanied the down-regulation of E-ca in[32]. The helial molecule E-cadherin play role in epithelial polarization and acts mor suppressor in many types of cancers[33]. It has been suggested that decreased E- expression is a major hallmark of EMT, closely associated with the malignant progression of HCC[34]. A recent study in breast

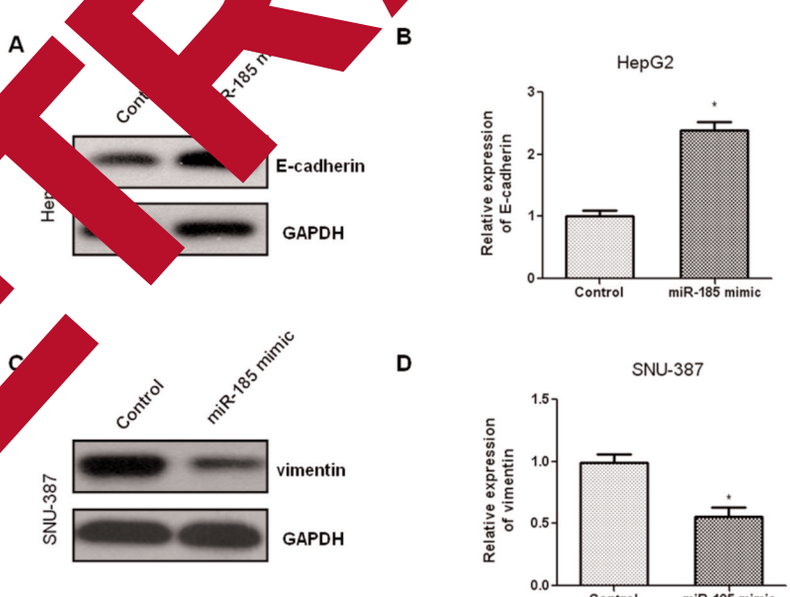

Figure 5. Up-regulation of miR-185 reversed EMT in HCC cell lines. HepG2 and SNU-387 cells were transfected with a miR-185 mimic or a negative control. 72 h after transfection, western blot was performed to determine the expression of E-cadherin ***(A*** and ***B)*** and vimentin ***(C*** and ***D)*** in HepG2 and SNU-387 cells, respectively. $^{*}p < 0.05$, $^{**}p < 0.01$.

cancer suggested that Six2 functions as a regulator of metastasis through the epigenetic regulation of E-cadherin, implicating a regulatory role for Six2 in EMT progression[26]. In our paper, we found that up-regulation of miR-185 significantly increased the expression of E-cadherin in epithelial (HepG2) cells and reduced the expression of vimentin in mesenchymal (SNU-387) cells. Taken together, these results indicate that up-regulation of miR-185 could reverse EMT in liver cancer cells via the regulation of Six2.

Conclusions

We identified miR-185 as a potential regulator of EMT in HCC cells. In addition, the up-regulation of miR-185 or the inhibition of Six2 could be potential therapeutic strategies for the treatment of HCC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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