**Original Article**

**MicroRNA-219-5p targets NEK6 to inhibit hepatocellular carcinoma progression**

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**Abstract:** MicroRNA-219-5p (miR-219-5p) is a key post-transcriptional regulator of gene expression that is known to regulate cancer progression, but its role in the context of hepatocellular carcinoma (HCC) remains to be fully elucidated. Herein, it was found that this miRNA functions as a tumor suppressor. Specifically, significant decreases in miR-219-5p expression were detected in HCC cells and patient serum samples relative to that found in the serum of 15 healthy people, and it was concluded that miR-219-5p overexpression was sufficient to impair HCC cell proliferation in vitro and vivo and migration in vitro. At the mechanistic level, it was found that miR-219-5p was able to suppress the expression of NEK6 (never in mitosis gene a-related kinase 6), thereby resulting in dysregulated β-catenin/c-Myc-regulated gene expression. When NEK6 was overexpressed in HCC cells, this was sufficient to reverse the inhibitory impact of miR-219-5p on HCC cell proliferation both in vitro and vivo and metastasis in vitro. Bioinformatics analyses were also conducted, and both miR-219-5p and Nek6 were linked to disease progression in HCC patients with advanced disease. More importantly, the serum specimen data showed that reduced perioperative plasma miR-219-5p correlated significantly with increased risk of early recurrence after curative hepatectomy, whereas it was opposed to NEK6. Together, these findings highlight miR-219-5p as a potentially valuable diagnostic biomarker that can potentially be leveraged to improve clinical outcomes in HCC patients.

**Keywords:** Hepatocellular carcinoma, miR-219-5p, Nek6, β-catenin/c-Myc, proliferation

**Introduction**

Hepatocellular carcinoma (HCC) is among the most prevalent forms of cancer, causing over 700,000 deaths globally each year [1]. The majority of HCC cases are associated with hepatitis B and C virus infections [2]. While patients with early-stage HCC can often undergo curative surgery to resect tumors, up to 70% of these patients ultimately go on to suffer from disease recurrence within a five-year period [3]. It is therefore vital that the mechanisms governing HCC development and recurrence be better understood so as to facilitate the more reliable development of diagnostic and therapeutic approaches for treating this disease.

The fungal protein, NIMA (never in mitosis, gene A), is a protein kinase that is essential for both mitotic initiation and exit [4], with NIMA abundance and activation being well-documented in fungal cells during the G2/M cell cycle transition in fungi [5]. NIMA-related kinases (NEKs) are proteins that share significant NIMA homology [6]. In human cells, NEK6 undergoes phosphorylation and activation during the M stage of the cell cycle and is essential for mitotic progression in these cells [7]. The disruption of NEK6 functionality by either knocking down this protein or overexpressing an inactive isoform thereof is sufficient to trigger M phase arrest and apoptotic cell death [8, 9]. NEK6 upregulation has also been detected in a range of human tumor types, including lung, breast, colorectal, and laryngeal cancers [10-12]. NEK6 has also been found to be independently predictive of poor prognosis in HCC patients [13], and there is additional evidence that it may function to
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promote HCC development and progression in humans [14]. As such, NEK6 is a promising target for the development of novel therapeutics designed to treat HCC and other forms of cancer.

MicroRNAs (miRNAs) are short RNAs (~22 nucleotides long) that lack coding potential but are able to suppress target mRNA expression at the post-transcriptional level [15]. This suppression is mediated by complementary base pairing of these miRNAs with specific 3’ untranslated regions (UTRs) in target mRNAs, resulting in mRNA degradation or translational arrest [16]. There is substantial recent evidence that miR-219-5p can serve to modulate proliferative, metastatic, and apoptotic activity in a range of human cancer types, including ovarian [17], colorectal [18], gastric [19], and thyroid cancers [20]. HCC patients typically exhibit miR-219-5p downregulation, with this downregulation correlating with larger tumor size, poorer histological differentiation, and decreased patient overall survival [21]. The mechanisms with miR-219-5p influence HCC progression and its targets within these tumor cells. However, remain to be fully clarified. Herein, it is determined that miR-219-5p can suppress HCC progression, at least in part, by inhibiting the expression of NEK6, which is a miR-219-5p target mRNA.

Materials and methods

Cell culture

HCC-LM3 and Huh7 cells were obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China), and were cultured in high-glucose DMEM (Life Technologies, NY, USA) containing 10% FBS (Life Technologies), 1% MEM essential amino acids (Corning), and 1% penicillin/streptomycin (Gibco, NY, USA) in a 37°C 5% CO₂ humidified incubator.

Lentiviral transduction

Lentiviral particles encoding the hsa-miR-219-5p precursor or a corresponding control (Scramble) sequence were obtained from GeneChem (Shanghai, China). Lentivirus supernatants were used to infect HCC-LM3 and Huh7 cells, after which FACS sorting (FACS Aria III, BD Bioscience, UT, USA) was used to isolate successfully transduced cells based on the presence of green fluorescence. The overexpression of miR-219-5p in these cells was then confirmed via RT-qPCR.

RT-qPCR

Gene expression was quantified via RT-qPCR. Briefly, 1 μg of total RNA per sample was used to prepare the cDNA, after which, the RT-qPCR reactions were performed using SYBR-Green (Takara Biotech) and appropriate primers. The primers included miR-219-5p: Forward ACACCCAGCTGGGTGATTGTCCAAACGCAAT, Reverse: CTCAACTGTTGTCGTGGAGTCGGC; Nek6: Forward: AGAGTGGAGCAGACCTTCCC, Reverse: CCCCGTGCTGTCTCTA (Table S2).

Western blotting

RIPA lysis buffer containing protease inhibitors was used to isolate protein from the cells, and then these samples were separated via SDS-PAGE and transferred onto PVDF membranes (Millipore). The blots were then blocked using 5% non-fat milk for 1 h followed by incubation overnight with either anti-NEK6 (1:1000, Abcam, Cambridge, UK) or anti-GAPDH (1:1000, CST, MA, USA). Proteins were then detected using a chemiluminescent HRP detection system (Millipore) (Table S1).

MTT assay

An MTT assay was employed to evaluate the HCC cell viability. Briefly, 1,000 cells were added to each well of a 96-well plate in a 100 μL volume, and the cells were cultured for four days. At appropriate time points, 20 μL of MTT solution (Sigma, MO, USA) was added per well for 4 h. Next, 150 μL DMSO (Sigma) was added per well, and the absorbance at 490 nm was evaluated using a microplate reader.

Colony formation assay

A total of 100 HCC cells were added per well to a six-well plate, and the cells were incubated for two weeks. Next, the cells were washed two times using PBS followed by staining with 0.1% crystal violet. All of the colonies that were made up of at least 50 cells were then counted via microscope, with colony formation efficiency being determined based upon the following equation: (number of colonies/number of inoculated cells) × 100%.
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**Transwell assays**

HCC cell migration and invasion were assessed in vitro using Transwell inserts (8 μm pore size, Corning, USA) that were either uncoated or coated with 20 μL of Matrigel (BD Bioscience), respectively [25]. Briefly, the inserts were affixed to 24-well plates, and $1.5 \times 10^5$ cells in 100 μL of serum-free media were added to the upper chamber of these wells, with 500 μL of DMEM supplemented with 10% FBS being added to the lower chamber. After 24 h incubation, the inserts were washed twice with PBS, and the non-migratory/invasive cells were gently removed using a cotton swab. The remaining cells were then fixed for 15 min with 1% formaldehyde, washed two times using PBS, stained with 0.1% crystal violet, and assessed via microscopy (Leica, Wetzlar, Germany).

**The HCC xenograft model system**

The impact of miR-219-5p on HCC tumor growth in vivo was assessed by subcutaneously implanting Huh7 cells that had been transfected with either mock constructs or with hsa-miR-219-5p ($5.0 \times 10^6$ cells/animal) into female BALB/c nude mice (4-5 weeks old). Tumor growth was monitored every three days for an 18-day period using electronic calipers (Fowler Sylvac UltraCal Mark III, Sylvac, Lausanne, Switzerland). The tumor volume ($V$, in mm$^3$) was calculated as follows: $V = \pi W^2 \times L/6$, where $L$ and $W$ corresponded to the largest and smallest diameters of the tumor, respectively. After this 18-day period, the animals were euthanized, and tumor tissue samples were collected for histological analyses. Briefly, the paraffin-embedded sections of these tumors were prepared for immunohistochemistry (IHC) to assess NEK6 expression levels via an indirect streptavidin peroxidase approach as per recommended protocols. All of the animal experiments were approved by the Ethics Committee of ZSSOM on Laboratory Animal Care (Shenzhen Hospital of the Southern Medical University, No. 2020-008).

**Survival analyses**

The association between NEK6 and miR-219-5p expression and HCC patient survival was evaluated using a Kaplan-Meier plotter (https://kmplot.com/analysis/index.php). Briefly, the patients were separated into groups of miR-219-5p-low and miR-219-5p-high patients (51 and 104, respectively) based on expression levels of this miRNA, and they were also separated into NEK6-low and NEK6-high groups (54 and 33, respectively, among stage 3+4 HCC patients) in the same manner. The NEK6 survival curve correlations were only evaluated for HCC patients with advanced (Stage 3+4) disease.

**Serum sample information**

A total of 15 pairs of serum samples, including 15 healthy people and 15 patients with HCC, were collected from patients undergoing surgery at Shenzhen Hospital of the Southern Medical University in China (obtained by collecting venous blood at the time of the primary diagnosis before operation and one week after surgical resection). These were used in this study for measuring the serological level of miR-219-5p by qPCR and NEK6 by ELISA. The serum samples were frozen and stored at -80°C until measurement. All of the samples of this study received approval from the Ethics Committee of ZSSOM (Shenzhen Hospital of the Southern Medical University, SZYYEC20-20R001).

**Statistical analysis**

SPSS 19.0 (IBM, NY, USA) was used for all statistical testing, and all data were given as means ± SEM from three or more independent experiments. The results of the RT-qPCR, western blotting, migration, invasion, MTT assay, and hsa-miR-219-5p expression were analyzed using Student’s t tests. The data integration and statistical analysis of the microRNA datasets were conducted using GraphPad Prism 6.0 (GraphPad Inc., San Diego, CA, USA). Patient sample data were compared using Student’s t tests, one-way ANOVAs, and parametric generalized linear models with random effects for two-way and multiple-groups, respectively. A value of $P < 0.05$ was statistically significant, and single, double, and triple asterisks indicated statistical significance of *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

**Results**

**HCC samples exhibited miR-219-5p downregulation**

In order to assess the role of miR-219-5p in HCC, the expression of this miRNA in several
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HCC cell lines (Huh7, HCC-LM3, Hep-3B, BEL-7402, SMMC-7721, HepG2, and HT29) and in the control LO2 cell line were examined. A significant miR-219-5p downregulation was observed in HCC cells relative to these control cells (Figure 1A). These analyses were then extended to 15 pairs of serum samples, including 15 healthy people serums and 15 patient serums with primary HCC. In agreement with the cell line results, a significantly reduced miR-219-5p expression was observed in patient serum samples relative to those from the 15 healthy people (Figure 1B). Together, these findings revealed that miR-219-5p downregulation occurs in HCC, potentially suggesting that this miRNA plays a role in HCC onset and/or progression.

NEK6 is a miR-219-5p target gene

In order to detect potential miR-219-5p target genes within HCC cells, the TargetScan and RNAhybrid algorithms were employed to identify a potential miR-219-5p binding site in the NEK6 3'-UTR (Figure 2A). A luciferase reporter assay approach was then used to directly evaluate the ability of miR-219-5p to bind to this putative 3'-UTR binding sequence. Decreased luciferase activity was detected when the cells that were transfected with a wild-type (WT) NEK6 3'-UTR reporter vector were co-transfected with miR-219-5p mimics (Figure 2B, lane 1), while miR-219-5p inhibitor transfection resulted in the opposite phenotype (Figure 2B, lane 3). In contrast, when a mutated version of this NEK6 3'-UTR reporter was transfected into cells, miR-219-5p levels did not impact luciferase activity (Figure 2B, lanes 5 and 6). These results thus validated NEK6 as a miR-219-5p target mRNA. Consistent with these results, a decreased NEK6 expression was observed at the mRNA and protein levels in the Huh7 and HCC-LM3 cells overexpressing miR-219-5p relative to the control cells (Figure 2C-F). These findings thus supported a model wherein miR-219-5p can bind to the NEK6 3'-UTR and thereby suppress NEK6 expression in HCC cells.

NEK6 is a key kinase that is essential for mitotic progression in human cells, undergoing phosphorylation and consequent activation during the M phase of the cell cycle [7]. Wei et al. previously demonstrated that miR-219-5p is capable of disrupting ovarian cancer cell proliferation, invasion, and migration, at least in part via disrupting the Wnt/β-catenin signaling pathway [22]. As such, the protein level expression of β-catenin and its downstream target c-Myc was evaluated in HC cells expressing different levels of miR-219-5p. These analyses revealed that miR-219-5p overexpression was linked to β-catenin and c-Myc downregulation, whereas
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Figure 2. NEK6 is a miR-219-5p target mRNA. (A) Complementarity between miR-219-5p and the NEK6 3’-UTR was detected using bioinformatics analyses. (B) A luciferase reporter assay using reporters bearing a WT or mutated version of the miR-219-5p binding site confirmed the ability of miR-219-5p to directly target this NEK6 3’-UTR region. Data are means ± SEM from triplicate experiments and were compared using one-way ANOVAs with Dunnett’s multiple comparison test. (C-F) Expression of miR-219-5p and NEK6 was assessed via RT-qPCR (C, D) and western blotting (E, F) in Huh7 and HCC-LM3 cells that had been transduced with a control (Mock) or miR-219-5p constructs. GAPDH served as a normalization control in these analyses. (G-I) NEK6, β-catenin, and c-Myc protein levels were assessed via Western blotting in cells overexpressing miR-219-5p and in control cells. *P < 0.05, **P < 0.01, ***P < 0.001.
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NEK6 overexpression was sufficient to reverse this phenotype (Figure 2G-I), indicating that miR-219-5p is capable of disrupting the β-catenin/c-Myc pathway activation in HCC cells via targeting Nek6.

**NEK6 overexpression partially reverses the ability of miR-219-5p to inhibit HCC cell growth in vitro**

As the above results identified NEK6 as a miR-219-5p target gene in HCC cells, the ability of NEK6 overexpression to reverse the ability of miR-219-5p to inhibit HCC cell proliferation was then examined. To that end, the proliferation of Huh7 and HCC-LM3 cells that had been transduced with either Vector + Vector, miR-219-5p overexpression (OE) + Vector, or miR-219-5p OE + NEK6 OE was compared. When RNA was isolated from these cells and assessed via RT-qPCR, it was confirmed that NEK6 overexpression did not alter miR-219-5p expression levels via any form of feedback mechanism (Figure 3A, 3B). An MTT assay was then used to assess the proliferation of these differentially-transduced cell lines, revealing that miR-219-5p OE + Vector Huh7 and HCC-LM3 cells exhibited proliferation that was just 45.3% and 40.5% of that observed for the control (Vector + Vector) cells, respectively. Notably, when NEK6

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**Figure 3.** NEK6 overexpression partially reversed the ability of miR-219-5p to inhibit HCC cell proliferation in vitro. Huh7 and HCC-LM3 cells were transduced with either control vectors (Vector + Vector), miR-219-5p overexpression (OE) + Vector, or miR-219-5p OE + NEK6 OE. The expression of miR-219-5p and NEK6 in these cells was then assessed via RT-qPCR (A, B). An MTT assay was employed to evaluate the proliferation of the transduced cells (C, D), and their colony-forming capabilities was also assessed following crystal violet staining (E, F). Data are means ± SEM from triplicate experiments. *P < 0.05, **P < 0.01, ***P < 0.001.
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was overexpressed in these Huh7 and HCC-LM3 cells (miR-219-5p OE + Nek6 OE), their proliferations were 81.7% and 85.6% of those observed in control (Vector + Vector) cells, respectively (Figure 3C, 3D). Consistent results were also observed in a colony formation assay (Figure 3E, 3F). Together, these findings indicated that the ability of miR-219-5p to impair HCC cell proliferation was partially reversed when NEK6 was ectopically overexpressed in these same cells.

**NEK6 overexpression partially reverses the ability of miR-219-5p to inhibit HCC cell metastasis in vitro**

Using the same experimental approach as above, NEK6 overexpression was assessed to see if it was sufficient to reverse the miR-219-5p-dependent changes in HCC cell migration and invasion in vitro using a Transwell assay system. It was found that Huh7 and HCC-LM3 cells overexpressing miR-219-5p (miR-219-5p OE + Vector) exhibited migration activity that was 26.4% and 30.9% that observed in control (Vector + Vector) cells, respectively. Importantly, NEK6 overexpression in these same Huh7 and HCC-LM3 cells (miR-219-5p OE + Nek6 OE) resulted in their exhibiting migratory activity that was 90.8% and 93.2% of that observed in control cells (Figure 4A, 4B). Comparable results were also obtained in an invasion assay using Matrigel-coated Transwell inserts (Figure 4C, 4D). These findings thus suggested that ectopic NEK6 overexpression was
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sufficient to partially reverse the observed miR-219-5p-mediated inhibition of HCC cell migratory and invasive activity.

miR-219-5p overexpression suppresses HCC tumor growth in vivo

Given that that above results indicated that miR-219-5p overexpression can suppress in vitro HCC tumor cell growth, an assessment was further conducted to assess whether it similarly impacted tumor growth in vivo in a murine xenograft model system. For this experiment, Huh7 cells that had been transduced with the control and miR-219-5p-encoding vector or miR-219-5p-encoding + NEK6 overexpression vectors were subcutaneously implanted into the flanks of BALB/c nude mice (female; 4-5 weeks old) and monitored for tumor growth every three days for a total of 18 days. It was found that tumors overexpressing miR-219-5p grew significantly more slowly than did tumors transduced with the control vectors, whereas tumors overexpressing miR-219-5p-encoding + NEK6 rescue grew (922.5 ± 32.4 mm³ vs. 436.7 ± 25.3 mm³ vs. 819.6 ± 30.7 mm³ on day 18; Figure 5A, 5B). Consistent with this, the reduced tumors weighed in the miR-219-5p-overexpressing group were partially rescued by the simultaneous overexpression of NEK6 (1416.7 ± 51.6 mg vs. 815.9 ± 48.2 mg vs. 1341.7 ± 50.4 mg on day 18; Figure 5C). As expected, significantly elevated miR-219-5p expressions in the miR-219-5p-overexpressing tumors were detected relative to the control tumors, confirming that stable knockdown of this miRNA was maintained in vivo (Figure 5D). Then the NEK6 and c-Myc protein levels in the tumor samples from these mice were assessed using IHC. Importantly, the reduced NEK6 and c-Myc protein levels in the miR-219-5p-overexpressing tumors were partially rescued by the co-overexpression of miR-219-5p-encoding and NEK6 (Figure 5E). Together, these results thus showed that miR-219-5p is capable of suppressing HCC tumor growth in vitro and in vivo.

miR-219-5p and NEK6 expression correlated with HCC patient prognosis

As it was discovered that miR-219-5p was able to suppress HCC tumor growth in vitro and in a murine xenograft model, next, the prognostic relevance of miR-219-5p expression in HCC patients was assessed. To that end, extant patient datasets (https://kmplot.com/analysis/index.php) were queried, with patients being separated into NEK6 and miR-219-5p low and high expression groups based upon the median expression of these two respective targets. The relapse-free survival of all patients as a function of their intratumoral NEK6 and miR-219-5p expression (low vs. high) was then assessed. This analysis revealed that decreased miR-219-5p expression was correlated with a poor prognosis (Figure 6A; low, n = 104, high, n = 54; P = 0.0031), suggesting a negative correlation between HCC patient outcomes and miR-219-5p expression levels. However, it was found that elevated NEK6 expression was associated with a poorer prognosis among patients with advanced stage HCC (stage 3+4) (Figure 6B; low, n = 54, high, n = 33; P = 0.011). Significantly reduced miR-219-5p expression in patient serum samples relative to those in 15 healthy people serum was observed (Figure 1B). The expression of NEK6 was further tested in these serum samples. Compared with healthy control individuals and patients, it was found that NEK6 was significantly higher in HCC patient serum (Figure 6C). In addition, in the nonearly recurrence subgroup, plasma miR-219-5p levels elevated more dramatically after operation, and some were even increased to the normal range over a certain time, whereas this was opposed to NEK6 (Figure 6D, 6E). To analyze the value of the serum miR-219-5p and NEK6 in predicting the prognosis of HCC, the receiver operating characteristic (ROC) curve for serum miR-219-5p and NEK6 expression was prepared according to early recurrence. Interestingly, a low serum miR-219-5p level correlated significantly with the cumulative recurrence after curative hepatectomy, and at the same time the low serum NEK6 level correlated significantly with the cumulative recurrence after curative hepatectomy (Figure 6F, 6G). Next, the predictive performance was compared between serum preoperative and postoperative miR-219-5p and NEK6. This data showed that the ROC curve using serum biomarkers indicated that the postoperative serum miR-219-5p and NEK6 had significant predictive abilities for HCC early recurrence (Figure 6H-K). Together, these findings thus indicated that both NEK6 and miR-219-5p expression are associated with HCC patient outcomes, with opposing trends being observed for these two targets.
miR-219-5p inhibits HCC by targeting NEK6

Discussion

HCC development can be profoundly impacted by dysregulated miRNA expression [23], and miR-219-5p has been reported as a tumor suppressor in gastric cancer, glioblastoma, and esophageal adenocarcinoma [19, 24-26]. It also works as a negative regulator of cell proliferation in CRC by targeting PGFR [27]. However, the mechanisms with miR-219-5p influencing HCC onset and progression have not been previously well characterized. A previous study used a microarray analysis for the investigation of gene expression signatures associated with HCC and found that miR-219-5p was downregulated [33], which implicated miR-219-5p in the development of HCC. Furthermore, low miR-219-5p expression was found to be correlated

Figure 5. miR-219-5p overexpression suppressed HCC tumor growth in vivo. Female BALB/c mice (4-5 weeks old) were subcutaneously implanted with Huh7 cells that had been transduced with control or miR-219-5p-encoding vectors, after which tumor growth over time was monitored. A. Representative tumor images. B. Tumor volumes were assessed every three days for 18 days (n = 5). Data are means ± SEM. C. Tumors were excised and weighed after the mice were euthanized. D. The expression of miR-219-5p in tumors was assessed via RT-qPCR. Data are means ± SEM (n = 5). E. NEK6 and c-Myc protein levels in xenograft tumors were assessed via IHC. Scale bar: 30 mm. *P < 0.05, **P < 0.01, ***P < 0.001.

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A. hsa-miR-219-5p

HR = 0.42 (0.24 - 0.76)  
logrank P = 0.0031

B. NEK6 (10783)

HR = 2.12 (1.18 - 3.82)  
logrank P = 0.011

C. Serum NEK6 level (pg/ml)

Healthy controls (n=15)  HCC (n=15)

D. miR-219-5p level (Relative expression)

Preoperative  Postoperative

E. Serum NEK6 level (pg/ml)

Preoperative  Postoperative

C. p < 0.001  D. p < 0.05  E. p < 0.01
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Figure 6. Reduced perioperative plasma miR-219-5p correlated significantly with increased risk of early recurrence after curative hepatectomy. (A, B) HCC patients in an extant public dataset (https://kmplot.com/analysis/index.php) were separated into miR-219-5p and NEK6 high and low expression groups based upon the median expression of these two respective targets. Relapse-free survival was then compared among these patient groups using Kaplan-Meier curves, with the association between miR-219-5p and prognosis being assessed in all the HCC patients (low, \( n = 104 \), high, \( n = 54 \); \( P = 0.0031 \)), and the relationship between NEK6 and prognosis was specifically assessed in those with advanced-stage HCC (low, \( n = 54 \), high, \( n = 33 \); \( P = 0.011 \)). (C) Compared with the healthy control individuals and patients, miR-219-5p was significantly lower in patients with HCC serum (\( P < 0.001 \)), whereas NEK6 was significantly higher in patients with HCC serum (\( P < 0.001 \)). (D, E) In the non-early recurrence subgroup, plasma miR-219-5p levels decreased dramatically after operation, and some were even reduced to the normal range in a certain time, whereas this was the opposite for NEK6. (F, G) A low serum miR-219-5p level correlated significantly with cumulative recurrence after curative hepatectomy (\( P < 0.05 \)), and at the same time the low serum NEK6 level correlated significantly with the cumulative recurrence after curative hepatectomy (\( P < 0.05 \)). (H-K) The ROC curve constructed using serum biomarkers indicated that postoperative serum miR-219-5p and NEK6 had a significant predictive ability of HCC early recurrence. *\( P < 0.05 \), **\( P < 0.01 \).
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with larger tumors and poorer differentiation [28]. These studies suggested that miR-219-5p could act as a candidate tumor suppressor in HCC [21, 29]. Herein, the miR-219-5p level in HCC cell lines and patient samples was first analyzed, and it was found that this miRNA functions as a tumor suppressor. Specifically, significant decreases in miR-219-5p expression in HCC cells and patient serum samples were detected relative to those in 15 healthy people serum samples. It was further determined that miR-219-5p overexpression was sufficient to impair HCC cell growth in vivo and in vitro. Thus, further evidence was provided that miR-219-5p had the potential to be a novel prognostic biomarker for HCC patients.

By binding with complementary 3'-UTR sequences in target mRNAs, miRNAs can promote the degradation and/or translational suppression of these target genes [30]. Individual miRNAs can target many different proteins, with miR-219-5p having previously been shown to suppress colorectal cancer cell metastasis and the proliferation via targeting calcyphosin [31], while also targeting Twist in epithelial ovarian cancer cells [22] and EGFR in glioblastoma cells [24]. Li et al. demonstrated that the overexpression of miR-219-5p can decrease the proliferation and invasion of gastric cancer cells by targeting the liver receptor homolog-1, meanwhile repressing activation of the Wnt/β-catenin signaling pathway [19]. This result was partially consistent with the findings from the HCC model. In this study, the TargetScan and RNAhybrid algorithms were used to identify NEK6 as an additional miR-219-5p target gene, and this prediction was confirmed experimentally using a luciferase reporter assay.

NEK6 has previously been shown to facilitate thyroid cancer cell proliferation at least in part by activating Wnt/β-catenin signaling [32], and it has additionally been identified as a putative oncogene in a range of tumor types [7, 33], including HCC [14]. Zhang et al. showed that NEK6 is upregulated in 79.1% of HCC patients, and it presents significantly increased expression in the Huh7, HepG2, Hep3B, and PLC/PRF/5 cell lines. Furthermore, NEK6 overexpression has been shown to increase the proliferation and viability of HCC cells, while its silencing had the opposite effect in vitro. The same study revealed that NEK6 silencing induced G2/M phase arrest and delayed the G0/G1 cell cycle phase in Hep3B cells, since NEK6 is involved in mitotic cell cycle progression. Additionally, NEK6 promotes cell proliferation and hepatic tumorigenesis by modulating the Cyclin B protein levels. This process is mediated by increased CDC2 expression [13].

Herein, it was found that the negative impact of miR-219-5p overexpression on HCC tumor cell proliferation and invasion was partially reversed upon NEK6 overexpression, thus confirming that NEK6 is a functionally relevant target of miR-219-5p in these HCC cells. Importantly, it was determined that miR-219-5p overexpression was sufficient to suppress HCC tumor growth in vivo, with NEK6 expression being decreased in these miR-219-5p-overexpressing tumors. Interestingly, when NEK6 was overexpressed, it was sufficient to reverse the inhibitory impact of miR-219-5p on HCC tumor growth in vivo. Bioinformatics analyses also established that both miR-219-5p and Nek6 were linked to disease progression in HCC patients with advanced disease. Since liquid biopsy has been widely applied in diagnostic situations and the detection of many cancers [34, 35], serum biomarkers are also recommended to be used in surveillance for HCC early recurrence. Compared to the serum of healthy people, it was found that NEK6 was also significantly higher in HCC patient serum, and this predicted a poor prognosis and high recurrence risk. Data from online sources confirmed this result. In addition, in the no nearly recurrence subgroup, plasma miR-219-5p levels elevated more dramatically after operation, and some even increased to the normal range over a certain time, whereas this was the opposite for NEK6. In addition, a low serum miR-219-5p level correlated significantly with cumulative recurrence after curative hepatectomy, and at the same time the high serum NEK6 level correlated significantly with cumulative recurrence after curative hepatectomy. HCC tissues are not always readily obtained during routine follow-up visits, so miR-219-5p and NEK6 have meaningful potential to be translated and applied for serum detection, although further verification based on large sample tests is required. These findings confirm that NEK6 represents a direct miR-219-5p target gene, and thus indicates that both NEK6 and miR-219-5p expression are associated with HCC patient outcomes.
Regarding the mechanism, it was found that miR-219-5p was able to suppress the expression of NEK6, thereby resulting in dysregulated β-catenin/c-Myc-regulated gene expression. It has been shown that β-catenin is a key transcription factor in the Wnt signaling pathway, which enhanced tumor stemness and invasion [19, 22, 32]. Thus, it was proposed that the change in cell proliferation and metastasis by miR-219-5p might be mediated indirectly by the NEK6-induced change in the transcription factors, such as β-catenin and c-Myc. To prove this possibility, which is beyond the scope of this current study, several strategies, such as CHIP-seq, immunoprecipitation, RNA-seq, and other techniques, may be required. In addition, the function of miR-219-5p was focused upon, but the mechanism of its up and downstream processes in HCC progression remains unclear. Further studies are required to fully understand the complex mechanism behind miR-219-5p expression and maturation. Meanwhile, more patient samples for further analysis of the association between miR-219-5p expression level and recurrent or overall survival are needed to clarify whether miR-219-5p can be used as a stable and reliable prognostic biomarker. Nevertheless, these findings position miR-219-5p as an interesting signaling mediator in the β-catenin/c-Myc axis, which may contribute to the progression of HCC.

In summary, these findings highlight miR-219-5p as a potentially valuable diagnostic biomarker and suggest that the miR-219-5p-mediated targeting of NEK6 may be a viable therapeutic approach to treating HCC.

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Disclosure of conflict of interest

None.

References

miR-219-5p inhibits HCC by targeting NEK6


Table S1. Antibodies used in the present study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Cat. No</th>
<th>Company</th>
<th>Mol weight</th>
<th>Dilution (WB/IHC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-Myc</td>
<td>ab32072</td>
<td>Abcam</td>
<td>57 kDa</td>
<td>1:1000 (WB)</td>
</tr>
<tr>
<td>β-catenin</td>
<td>9582</td>
<td>CST</td>
<td>92 kDa</td>
<td>1:1000 (WB)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1:100 (IHC)</td>
</tr>
<tr>
<td>Nek6</td>
<td>ab133494</td>
<td>Abcam</td>
<td>36 kDa</td>
<td>1:1000 (WB)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1:500 (IHC)</td>
</tr>
<tr>
<td>GAPDH</td>
<td>2547</td>
<td>CST</td>
<td>37 kDa</td>
<td>1:1000 (WB)</td>
</tr>
</tbody>
</table>

Table S2. Primers and oligonucleotides used in the present study

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5’-3’)</th>
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<tr>
<td>Nek6 Forward</td>
<td>AGAGTGGAGCAGACCTTCCC</td>
</tr>
<tr>
<td>Nek6 Reverse</td>
<td>CCCCCGTCGCTCTCTAA</td>
</tr>
<tr>
<td>β-catenin Forward</td>
<td>GATGGTTGCCCCCAAATCTGC</td>
</tr>
<tr>
<td>β-catenin Reverse</td>
<td>CACCAGTGAAAGGTGCTGGA</td>
</tr>
<tr>
<td>c-Myc Forward</td>
<td>GCTGGACCAGATGTATGTCCC</td>
</tr>
<tr>
<td>c-Myc Reverse</td>
<td>ATCATTTCCATGACGGCCCTG</td>
</tr>
<tr>
<td>miR-219-5p mimics Forward</td>
<td>AACTCCAGCTGGGATTTGCTCCAAAACGCAAT</td>
</tr>
<tr>
<td>miR-219-5p mimics Reverse</td>
<td>CTCAACTGGTGCTGGAGTCG</td>
</tr>
<tr>
<td>miR-219-5p</td>
<td>ACCTCCAGCTGGTGATTGCTCCCAAACGCAAT</td>
</tr>
<tr>
<td>miR-219-5p inhibitor</td>
<td>CTCAACTGGTGCTGGAGTCG</td>
</tr>
<tr>
<td>snRNA U6</td>
<td>AACGCTTACAGAATTTCG</td>
</tr>
</tbody>
</table>

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