MicroRNA-127-5p targets the biliverdin reductase B/nuclear factor-κB pathway to suppress cell growth in hepatocellular carcinoma cells

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It has been well established that chronic inflammation promotes the initiation and progression of cancers. The first evidence linking inflammation to cancer was presented in 1863 by Virchow, who observed leukocytes in tumor tissues.1 Later, that observation was confirmed in several clinical cases.2 Chronic inflammation occurs when inflammatory signaling cannot be stopped, resulting in recruitment of inflammatory cells, increased production of reactive oxygen species and reduction of DNA repair.3 Moreover, chronic inflammation is regarded as an important cause of tumorigenesis in humans, accounting for ~20% of human cancers worldwide.4 The development of hepatocellular carcinoma (HCC) is one of the most extensively illustrated inflammation-induced carcinogenic processes because more than 90% of HCCs are attributed to chronic liver damage and inflammation.4 Hepatocellular carcinoma represents the fifth most common cancer in men and the seventh most common cancer in women and is the third leading cause of cancer-related death worldwide.5

In recent years, research has been dedicated to exploring the underlying mechanisms of inflammation-induced cancers. Two major regulating signaling pathways have been shown to participate in the crosstalk between inflammation and cancers. One is the signal transducer and activator of transcription (STAT) pathway, in particular, the STAT pathway member STAT3, which can be activated by a series of inflammation factors.6 Another is the nuclear factor-kappa B (NF-κB) signaling pathway, which is recognized as a critical player in incipient neoplasia and progression of inflammation-induced cancers.7 Under normal circumstances, NF-κB is a heterodimer of p50 and p65, and it binds to inhibitory molecules of the IκB family. Nuclear factor-κB is activated by a number of signals from the cell surface. The IκB kinase (IKK) complex, composed of the IKKa and IKKb catalytic subunits and the regulatory subunit, is activated, result-
Fig. 1. MicroRNA (miR)-127-5p inhibits nuclear factor (NF)-kB transcriptional activity in hepatocellular carcinoma cells. (a) SMMC-7721 and SNU-449 cells were cotransfected with NF-kB luciferase reporter and miRNA mimic. A luciferase assay was carried out after 48 h. (b) The mRNA levels of NF-kB downstream effectors were determined by quantitative real-time PCR analysis after transfection with miR-127-5p mimic or negative control (NC) in SMMC-7721 and SNU-449 cells. β-actin served as an internal control. (c) Separation and preparation of cytoplasmic and nuclear extracts were carried out in SMMC-7721 and SNU-449 stable cells. The protein level of p65 was determined by Western blot analysis. (d) Confocal microscopic images show that ectopic expression of miR-127 inhibited the nuclear translocation of p65, which suggests that NF-kB signaling is suppressed. (e) The protein levels of NF-kB signaling were analyzed by Western blot after transfection with miR-127-5p. (f) The protein levels of NF-kB signaling were determined by Western blot in lenti-miR-127 stable cell lines. **P < 0.01.
ing in I-κB phosphorylation and degradation. Once released from I-κB family members, the phosphorylation of RelA results in NF-κB translocation into the nucleus and binds to the κB motif of its target genes.\(^8\) Nuclear factor-κB signaling can be regulated by many positive and negative regulatory factors. Recently, regulation by non-coding RNAs, especially micro-RNAs (miRNAs), has been discovered as a new mechanism of NF-κB signaling regulation.

MicroRNAs constitute a class of short endogenous non-coding RNAs. Increasing evidence has shown that miRNAs play a conserved role in various biological processes by regulating the expression of sets of genes.\(^9\) In previous work, we used high-throughput luciferase reporter assays to screen for miRNAs that regulate the NF-κB signaling pathway. For example, miR-195 targets the NF-κB signaling pathway by directly downregulating I-κBα and TAB 3.\(^10\) In addition to miRNAs that direct target molecules in NF-κB signaling pathway, we also identified miRNAs that regulate NF-κB activities without targeting molecules that directly participate in NF-κB signaling pathway. Among these miRNAs, miR-127-5p significantly decreased NF-κB activities and inhibited cell growth by targeting biliverdin reductase B (BLVRB) in HCC.

Materials and Methods

Cell culture. HEK-293T, SMMC-7721, and SNU-449 cells were cultured in DMEM supplemented with 10% FBS and antibiotics at 37°C in an atmosphere of 5% CO₂.

Oligonucleotide transfection. MicroRNA-127-5p mimic and BLVRB siRNA were synthesized by GenePharma (Shanghai, China). The sequences are shown in Table S1. In each well of

Table 1. Correlation of the clinicopathological features with tumor miR-127-5p expression in HCC

| Clinical variables | High miR-127-5p | Low miR-127-5p | Total no. | Unknown no. |
|-------------------|-----------------|----------------|-----------|-------------|
| Case no.                                     | Case no.                        |
| %                          | %                          |           |           |       |
| Age                                     |                              |           |           |       |
| >50                             | 47.36                       | 63.64     | 110       | 1     |
| ≤50                             | 23.64                       | 16.36     | 120       | 1     |
| Gender                                  |                              |           |           |       |
| Male                              | 60.00                       | 52.63     | 110       | 1     |
| Female                            | 40.00                       | 47.37     | 110       | 1     |
| Risk factor                          |                              |           |           |       |
| Hepatitis                         | 36.96                       | 52.63     | 110       | 1     |
| Other                             | 63.04                       | 47.37     | 110       | 1     |
| AFP                                |                              |           |           |       |
| Positive                          | 74.19                       | 52.63     | 110       | 1     |
| Negative                          | 25.81                       | 47.37     | 110       | 1     |
| Inflammation extent                |                              |           |           |       |
| Mild/Severe                       | 46.43                       | 41.86     | 71        | 40    |
| None                              | 53.57                       | 58.14     | 71        | 40    |
| Liver fibrosis score               |                              |           |           |       |
| ≥1                                | 34.38                       | 42.11     | 70        | 41    |
| 0                                 | 65.62                       | 57.89     | 70        | 41    |
| G2/G3/G4                          |                              |           |           |       |
| G1                                |                              |           |           |       |
| Pathologic stage                  |                              |           |           |       |
| II/III/IV                         | 64.71                       | 57.14     | 100       | 11    |
| I                                 | 35.29                       | 42.86     | 96        | 15    |
| Vascular invasion                 |                              |           |           |       |
| Micro/Macro                       | 21.28                       | 42.86     | 96        | 15    |
| None                              | 78.72                       | 57.14     | 96        | 15    |
Fig. 3. MicroRNA (miR)-127-5p suppresses the proliferation of hepatocellular carcinoma cells. (a) Proliferation of SMMC-7721 and SNU-449 cells transfected with miR-217-5p mimic or negative control (NC) was measured by CCK8 assay. (b) Proliferation of SMMC-7721 and SNU-449 stable cells overexpressing miR-127 were measured by CCK8 assay. (c) Colony formation assay for SMMC-7721 and SNU-449 cells infected with lentivirus expressing miR-127 or mock control. A total of 1000 cells per well were seeded and cultivated for 2 weeks. The colonies were fixed and stained in a dye solution containing 0.1% crystal violet and 20% methanol. *P < 0.05, **P < 0.01, ***P < 0.001.

Fig. 4. Biliverdin reductase B (BLVRB) is a direct target of microRNA (miR)-127-5p. (a) Candidate genes identified by prediction algorithms based on gene expression profiles. (b) Putative miR-127-5p binding sites in the BLVRB 3'-UTR. Mutant binding sequences are highlighted in gray. (c) Luciferase activity assays of luciferase reporters with wild-type or mutant BLVRB 3'-UTRs were carried out after cotransfection with miR-127-5p mimic or negative control (NC) in HEK-293T cells. The mean ± SEM of a representative experiment in triplicate is shown. (d) mRNA levels of BLVRB in SMMC-7721 and SNU-449 stable cells were determined by quantitative real-time PCR analysis. (e) Protein levels of BLVRB in SMMC-7721 and SNU-449 stable cells were determined by Western blot analysis. *P < 0.05, ***P < 0.001, ns, not significant.
MicroRNA-127-5p inhibits NF-κB activation in HCC cells. We first determined the effect of miR-127-5p on NF-κB activation. A luciferase reporter harboring four inducible NF-κB binding sites upstream of the luciferase gene was co-transfected with miR-127-5p mimic or negative control (NC) into two HCC cell lines, SMMC-7721 and SNU-449. The results showed that luciferase activity was notably decreased by miR-127-5p in both cell lines (Fig. 1a), which indicates that miR-127-5p influenced NF-κB activation. A set of NF-κB downstream effectors were detected after miR-127-5p overexpression, and the results showed miR-127-5p also decreased the expression of these targets (Fig. 1b). Furthermore, a negative correlation between miR-127-5p expression and NF-κB activity was noted in six HCC cell lines (Fig. S1). While exploring the mechanism through which miR-127-5p regulated NF-κB activation, we found that ectopic expression of miR-127-5p decreased the nuclear fraction of the RelA/p65 subunit of NF-κB in SMMC-7721 and SNU-449 cells (Fig. 1c). Moreover, immunofluorescence assays also indicated that the nuclear location of RelA/p65 was reduced after miR-127-5p overexpression (Figs. 1d, S2). We next examined the influence of miR-127-5p on the major components of the NF-κB signaling pathway and found that ectopic expression of miR-127-5p in HCC cells reduced the phosphorylated form of RelA/p65 (Fig. 1e,f). Taken together, overexpression of miR-127-5p inhibited NF-κB activation by reducing phosphorylation and nuclear translocation of RelA/p65 proteins in HCC cells.

MicroRNA-127-5p frequently downregulated and suppresses cell proliferation in HCC. As miR-127-5p inhibited the activation of NF-κB in HCC cells, we wondered whether miR-127-5p could act as a tumor suppressor in HCC. Therefore, we investigated the expression of miR-127-5p in HCC samples reported in the Cancer Genome Atlas (http://cancergenome.nih.gov/) database. Compared to normal liver, miR-127-5p expression is lower in HCC samples.
(a) SMMC-7721 and SNU-449

Relative NF-κB activity

NC | siBLVRB
---|---
NC | siBLVRB

(b) SMMC-7721 and SNU-449

p-P65
BLVRB
β-actin
NC | siBLVRB
---|---
NC | siBLVRB

(c) SMMC-7721 and SNU-449

Relative expression level

IL6 | XAP | c-TUP | Survivin | c-FLIP | CD44 | CD34 | MYC | Cyclin D1
NC | siBLVRB
---|---
NC | siBLVRB

(d) SMMC-7721 and SNU-449

P65
DAPI
Merged
NC | siBLVRB
---|---
NC | siBLVRB

(e) SMMC-7721 and SNU-449

OD450

Time (days)

NC | siBLVRB
---|---
NC | siBLVRB

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in HCC tissues (Fig. 2a,b, Table 1). Downregulation of miR-127-5p (greater than twofold change) was observed in 58% (27/47) of HCC tissues compared to matched adjacent normal tissues (Fig. 2c). In addition, HCC tissues with poor histologic grade showed significantly lower expression of miR-127-5p than tissues with higher histologic grade (Fig. 2d, Table S1). These results, together with the known function of miR-127-5p, indicate that miR-127-5p is a potential tumor suppressor in HCC. Furthermore, we found that miR-127-5p mimic suppressed the growth of SMMC-7721 and SNU-449 cells (Fig. 3a). Moreover, stable expression of miR-127-5p through a lentiviral system also decreased the growth of both cell types (Fig. 3b). We also examined the effects of miR-127-5p on the colony formation ability of HCC cells and found that stable expression of miR-127-5p significantly reduced the colony formation ability of SMMC-7721 and SNU-449 cells (Fig. 3c).

Biliverdin reductase B is a direct downstream target of miR-127-5p in HCC cells. To determine the mechanism of the inhibition of proliferation and NF-κB signaling in HCC by miR-127-5p, we attempted to identify the target genes of miR-127-5p. We carried out a microarray analysis to determine differentially expressed genes after miR-127-5p transfection and combined the results with the targets predicted by three bioinformatics tools (TargetScan, miRanda, and DIANA) (Fig. 4a). Four candidate genes were identified, and BLVRB was confirmed by qPCR to be the most significantly downregulated by miR-127-5p (Fig. S3). Therefore, we selected BLVRB for further exploration. The 3′-UTR of BLVRB contains one binding site for miR-127-5p. We constructed luciferase reporter plasmids containing the 3′-UTR of BLVRB with a wild or mutant miR-127-5p binding site and cotransfected those plasmids with NC or miR-127-5p into HEK-293T cells (Fig. 4b). The luciferase assays showed that miR-127-5p significantly reduced luciferase activity in the wild-type group, but not in the mutant group (Fig. 4c). In addition, ectopic expression of miR-127 suppressed the expression of BLVRB at both the mRNA and protein levels (Fig. 4d,e). A negative correlation between miR-127-5p and BLVRB was noted among 346 patients from the Cancer Genome Atlas database (Fig. S4). These findings indicate that BLVRB is a direct target of miR-127-5p in HCC cells.

MicroRNA-127-5p inhibits NF-κB signaling and cell proliferation in HCC cells. To determine whether BLVRB is a functional target of miR-127-5p, the expression of BLVRB was restored in cells overexpressing miR-127-5p. We cotransfected plasmids expressing BLVRB with miR-127-5p mimic or NC into SMMC-7721 and SNU-449 cells (Fig. S5). The CCK-8 assays showed that restoration of BLVRB expression abrogated the inhibition of proliferation and NF-κB signaling by miR-127-5p (Fig. 6a,b). To determine whether BLVRB is a functional target of miR-127-5p, the expression of BLVRB was restored in cells overexpressing miR-127-5p. The CCK-8 assays showed that miR-127-5p significantly inhibited proliferation of HCC cells and suppressed the phosphorylation of p65, and restoration of BLVRB expression abrogated the inhibition of proliferation and NF-κB signaling by miR-127-5p (Fig. 6a,b).
NF-κB activation and proliferation of HCC by miR-127-5p (Fig. 6b,c).

Discussion

This research has been dedicated to determining the mechanisms underlying NF-κB signaling dysregulation in the development of cancer. Nuclear factor-κB acts as the central coordinator of inflammatory signaling and has a critical role in cancer initiation and progression. Activation of NF-κB enhances the anti-apoptotic ability of cells. (11) Deficiency in the NF-κB component RelA in mouse embryos facilitates apoptosis in the liver. (12)

In the past decade, an increasing number of studies have shown that miRNAs have important roles in the initiation and progression of cancers. Our group and others have found that miRNAs also regulate the NF-κB signaling pathway by directly or indirectly targeting genes that participate in this signaling pathway. (10,13,14) In this study, we first observed that miR-127-5p, which is frequently downregulated in HCC, regulates NF-κB signaling activity in HCC cells. Previous studies have reported that miR-127 is downregulated in various cancers, including glioblastoma, (15) gastric cancer, (16) and HCC, (17) which indicated that downregulation of miR-127-5p is also a common event in cancers. In addition, expression of miR-127-5p is negatively associated with the histological grade of HCC. Li Wang’s group reported that miR-433 and its twin miR-127 were regulated by DNA methylation in HCC cells. (18)

Treatment with the DNA methylation inhibitor 5′-aza-2′-deoxycytidine resulted in a dose-dependent induction of miR-127-5p. Consistently, a reduction of BLVRB was noted in cells treated with DNA methylation inhibitor (Fig. S6). Further functional studies showed that miR-127-5p significantly suppresses HCC cell proliferation. Together, these results indicate that suppression of this regulatory miR-127-5p facilitated the proliferation of HCC cells.

In the current study, we found that BLVRB is a novel downstream target of miR-127-5p. Biliverdin reductase B has two activities in the cell: riboflavin reductase activity and biliverdin reductase activity. (19–21) It was previously identified as a component of antioxidant stress that shows a cell protective function. (22) Several studies have reported that BLVRB is upregulated at the protein level in esophageal squamous cell carcinoma, (23) acute lymphoblastic leukemia, (24) and HCC. (25) Here, we found that the knockdown of BLVRB inhibited the proliferation of HCC cells and inhibited NF-κB activity. Accordingly, the ectopic expression of BLVRB promoted the proliferation of HCC cells. It has been reported that an isozyme of BLVRB, biliverdin reductase A, enhanced the activation of NF-κB in HCC cells. Biliverdin inhibited NF-κB activity in a concentration- and time-dependent manner. (26)

In addition, analysis of the structure of BLVRB showed that it accommodates a wide range of tetrapyrrole substrates, including biliverdin IX beta, met-biliverdin IV alpha, and biliverdin IX alpha (27). We infer that BLVRB may regulate NF-κB activity by the same mechanism. In summary, our findings show that miR-127-5p, which is frequently downregulated in HCC, indirectly affects the NF-κB signaling pathway by targeting BLVRB (Fig. 6d). Moreover, miR-127-5p was found to suppress the proliferation of HCC. This newly found miR-127-5p/BLVRB axis provides another link between microRNA, inflammation, and cancer.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Table S1. Sequences of oligonucleotide used in this study.
Table S2. Probes used in this study.
Table S3. Primer sequences used in this study.
Fig. S1. Negative correlation between microRNA-127-5p and nuclear factor-kB activity.
Fig. S2. Establishment of stable cell lines expressing microRNA-127.
Fig. S3. Screening of potential target of microRNA-127-5p in the cell.
Fig. S4. Correlation between microRNA-127-5p and BLVRB.
Fig. S5. Establishment of stable cell lines expressing BLVRB.
Fig. S6. MicroRNA-127-5p was silenced by DNA methylation.