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ORIGINAL ARTICLE

MicroRNA-29-3p Regulates Hepatocellular Carcinoma Progression Through NF-κB Pathway

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SUMMARY

Background: **Hepatocellular carcinoma (HCC) is the most common primary liver cancer and accounts for over 90% of all primary liver cancers. Increasing evidence suggests that microRNAs (miRNAs) mediate signaling pathways by gene expression regulation.**
Methods: **In this study, we evaluated the role of miR-29a-3p in HCC progression. MiR-29a-3p was found significantly down-regulated in HCC tissues compared to adjacent non-tumor tissues. Meantime, PTEN expression was up-regulated in HCC tissues. Moreover, NF-κB activity was decreased following PTEN up-regulation.**
Results: In vitro **assays in the HCC cell line BEL7402 demonstrated that miR-29a-3p suppresses cell proliferation.**
Conclusions: **miR-29a-3p participates in the HCC progression by regulation of NF-κB pathway via targeting PTEN.**
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KEY WORDS

hepatocellular carcinoma, miR-29a-3p, PTEN, NF-κB, cell proliferation

INTRODUCTION

Chronic alcohol abuse is a major risk factor for hepatocellular carcinoma (HCC), the fifth most common cancer worldwide and the third leading cause of cancer deaths worldwide [1]. Eastern Asia is one of the world areas with incidence and mortality rates for HCC. In particular, 55% of all HCC cases worldwide are reported from China [2]. The overall survival of patients with HCC is still unsatisfactory although significant research efforts have been made to improve its prognosis [3,4]. The poor prognosis of HCC promotes tumor metastasis and recurrence [5]. In addition, the proliferative activity of tumor cells is one of the important indictors assessing aggressiveness and could be used to predict prognostic significance.

Like other cancers, dysregulated gene expression features significantly in HCC. MicroRNAs (miRNAs) are small non-coding (~21 - 23 nt) that regulate gene expression at the post-transcriptional/translational levels

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in various biological processes and contribute to many cancers including HCC [6]. Recently, miRNAs have also been reported to regulate the NF-κB pathway [7,8]. miR-224 has been revealed to be up-regulated in HCC and increases cell proliferation [6]. miR-129-2 suppression increases HCC tumorigenesis by enhancing oncogenic SOX4 expression [9].

Although much is known about the profiles of miRNAs in various tumors and tissues, the function of miRNAs in the progression of HCC has still not been fully elucidated. In this study, we identified miR-29a-3p as a down-regulated miRNA in HCC tumors. Additional investigation showed that NF-κB activity was suppressed after miR-29a-3p down-regulation through PTEN. The role of miR-29a-3p in cell proliferation of HCC was also investigated using *in vitro* assays. Our studies provide another layer to the miRNA biological function in HCC and may provide a new therapeutic target for HCC.

MATERIALS AND METHODS

Ethical approval

The study was approved by the Institutional Ethics Committee of our hospitals, and written informed consent was obtained from all participants.

Cell Culture

Human BEL-7402 hepatocellular carcinoma (HCC) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (American Type Culture Collection, Manassas, VA, USA) supplemented with heat-inactivated 10% fetal bovine serum (HyClone Laboratories Inc., Logan, UT, USA), 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C in 5% carbon dioxide in a humidified incubator.

RNA isolation and quantitative real-time PCR

Total RNA extraction was performed as previously described [8]. Residual DNA was removed using DNA-free DNase (Ambion, Austin, TX, USA). RNAs were reverse transcribed into cDNA with 300 units of M-MLV reverse transcriptase (BRL, Gaithersburg, MD, USA). qRT-PCR (TaqMan) assays were used for the detection of gene expression in triplicate on an ABI 7500 system (Applied Biosystems, Foster City, CA, USA). For miRNAs quantification, TaqMan microRNA assays were used to determine levels of mature miRNAs in accordance with instruction (Applied Biosystems). All the gene/miRNA-specific primers and probes were bought from Applied Biosystems.

Western blotting

Cells were lysed in lysis buffer (Thermo Scientific, Waltham, MA, USA) and protein concentrations were determined with the DC Protein Assay (Bio Rad Laboratories, Hercules, CA, USA). Western blot analysis was done according to a previous description with 20 μg of proteins per well [8].

For specific primary antibodies (Ab): anti-PTEN (1: 500; Cell Signaling Technologies, Beverly, MA, USA), anti-p-p65 (1:500 dilution; Abcam) and anti-β-actin (1: 500; Sigma-Aldrich, St Louis, MO, USA) following the addition of related HRP-conjugated secondary Abs (Bio-Rad). All of the results are from separate blots in order to avoid incomplete stripping.

Dual-luciferase reporter assay

For NF-κB pathway activity, signaling pathway luciferase reporter assay was performed as previously described [8]. Briefly, BEL-7402 cells were seeded in 96-well plate (2×10^4 cells/well) and cultured for 24 hours. Fifty nanograms miR-29a-3p and 100 ng NF-κB pathway-luciferase-reporter construct (SA Biosciences, Frederick, MD, USA). miR-con was used as a control. miRNA plasmids were constructed as previously described [8]. NF-κB transcriptional activity was determined by the Dual Luciferase Reporter Assay System. Results are represented by dividing firefly luciferase activity by *Renilla* luciferase activity.

MTT assay

After transfection with miR-29a-3p inhibitor, cell proliferation of BEL-7402 was measured with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) assay (Sigma-Aldrich, St Louis, MO, USA) following the manufacturer's instructions. Briefly, cells were treated with 20 μL of MTT at days 0 - 8 of culture after miR-29a-3p inhibitor addition. Then, cells were incubated at 37°C for 4 hours. The absorbance of each well was determined by absorbance spectrometry at 490 nm using a microplate reader. For PTEN overexpression assay, cell proliferation of BEL-7402 cells overexpressed with PTEN or lentiviral vector control (MOI = 50) was measured by MTT assay as the same as miR-29a-3p inhibitor assay. Both experiments were repeated three times independently.

Statistics

Statistical analyses were carried out with SPSS version 13.0 (SPSS Inc., Chicago, IL, USA) for Windows. Data are presented as mean ± SE (n = 3). Student's *t*-test was performed to compare the means between two groups. $p < 0.05$ was considered as a significant difference.

RESULTS

miR-29a-3p is down-regulated in HCC from chronic alcohol abuse

Based on our preliminary microRNA microarray studies of tissues from 21 patients with HCC from chronic alcohol abuse and 18 normal liver tissue samples, we found that miR-29a-3p was 2.5-fold significantly down-regulated in HCC groups (data not shown). qRT-PCR analysis revealed that 16 cases (76%) exhibited decreased expression of miR-29a-3p in HCC tissues compared

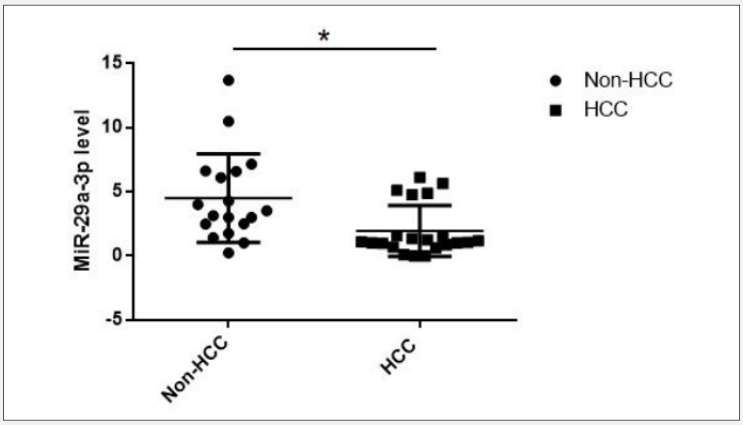

Figure 1. miR-29a-3p expression level in non-HCC and HCC tissues. miR-29a-3p expression level was determined by qRT-PCR. U6 snRNA was used as an internal control. Results are mean ± SE. * p < 0.05 vs. non-HCC.

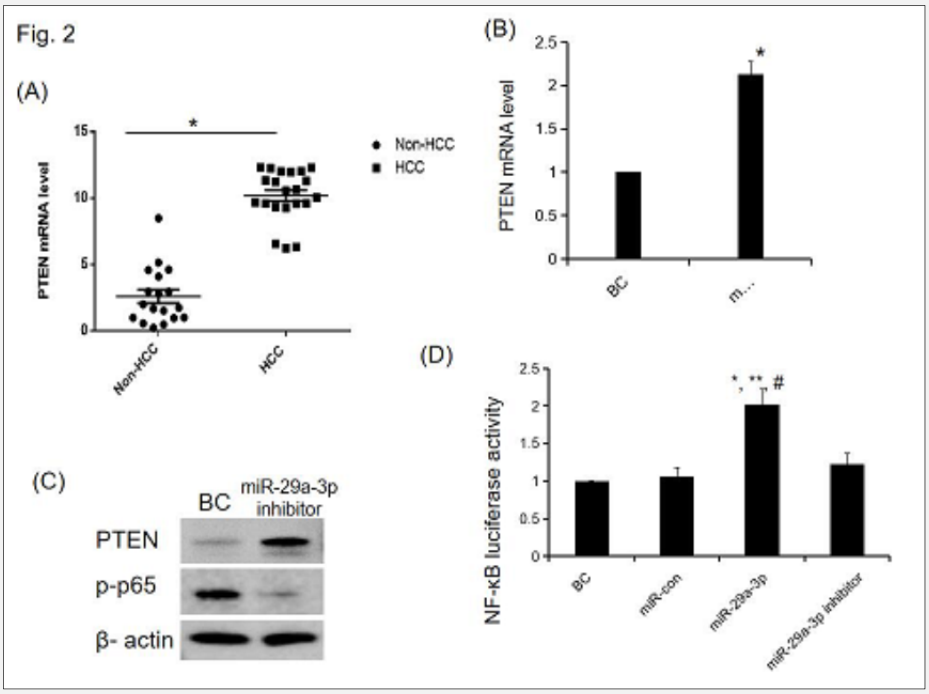

Figure 2. PTEN is the target of miR-29a-3p. PTEN mRNA expression level was determined by qRT-PCR.

(A) PTEN mRNA level in non-HCC and HCC tissues; (B) PTEN mRNA level in BEL-7402 HCC cells after adding miR-29a-3p inhibitor. BC: without miR-29a-3p inhibitor; (C) PTEN and p-p65 protein expression was determined by WB. β-actin was used as an internal control. (D) BEL-7402 HCC cells were co-transferred with NF-κB luciferase-reporter construct and miR-29a-39 or miR-con. NF-κB activity was normalized to Renilla luciferase activity. Results are mean ± SE (n = 3). * p < 0.05 vs. non-HCC or BC; ** p < 0.05 vs. miR-con; # p < 0.05 vs. miR-29a-3p inhibitor.

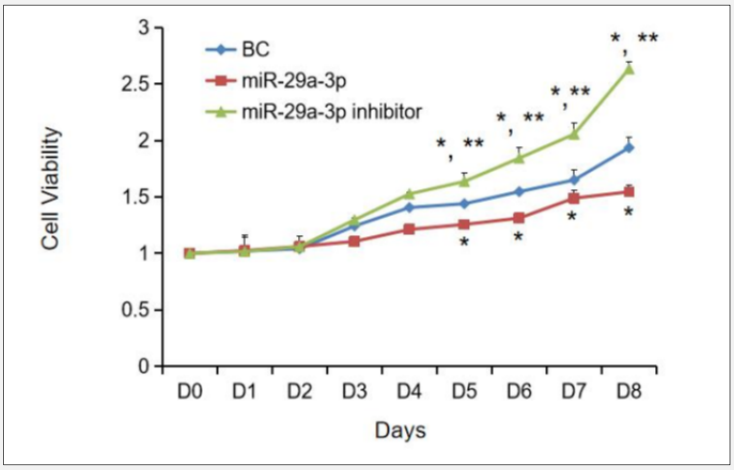

Figure 3. Effect of miR-29a-3p inhibitor on HCC cell growth.

The cell growth curves showed the BEL7402 cells' growth was remarkably increased after day 5 by miR-29a-3p inhibition. Data are mean 6 SE (n = 3 cell preparations). * p < 0.05 vs. cells without miR-29a-3p inhibitor; ** p < 0.05 vs. miR-29a-3p inhibitor.

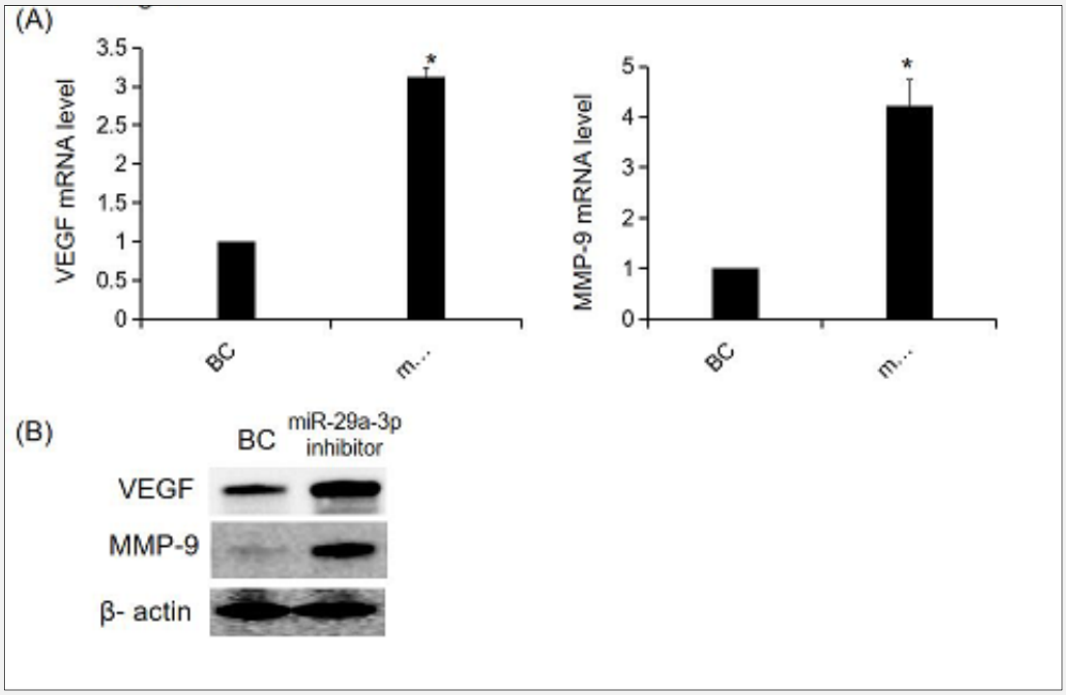

Figure 4. VEGF and MMP-9 expression were increased after adding miR-29a-3p inhibitor to BEL7402 cells.

(A) VEGF and MMP-9 mRNA expression levels were determined by qRT-PCR; (B) VEGF and MMP-9 protein expression were determined by WB. BC: without miR-29a-3p inhibitor. β-actin was used as an internal control. Results are mean ± SE. * p < 0.05 vs. BC.

with non-HCC tissues (Figure 1).

miR-29a-3p down-regulation increases PTEN expression

To investigate the function role of miR-29a-3p in HCC, a bioinformatics tool (TargetScan) was used to predict target genes of miR-29a-3p. Among these potential candidate genes, PTEN was reported to inhibit Akt activation, which consequently activates Ikk, leading to the degradation of IκB and nuclear translocation of the transcription factor NF-κB [8,10].

To verify whether PTEN is a potential target of miR-29a-3p, we determined PTEN mRNA level in HCC tissues (16 cases) and normal tissues. PTEN mRNA expression was up-regulated in HCC tissues (Figure 2A). Furthermore, we also inhibited miR-29a-3p in human BEL-7402 HCC cells. The blank control (BC) group was BEL-7402 HCC cells without treatment. Western blotting and qRT-PCR were used to determine the endogenous PTEN protein and mRNA levels in BEL-7402 HCC cells after adding miR-29-3p inhibitor. The result showed that miR-29a-3p inhibition increased PTEN protein and mRNA levels (Figure 2B, C).

miR-29a-3p inhibition suppresses NF-κB signaling pathway in HCC cells

PTEN is reported to inhibit Akt phosphorylation and, thus, the NF-κB pathway [8,11,12]. Therefore, we next determined whether blocking miR-29a-3p can suppress NF-κB signaling. We found that miR-29a-3p inhibition decreased the phosphorylation of p65 by western blot analysis (Figure 2C).

To double confirm the activity of NF-κB signaling affected by miR-29a-3p, a dual-luciferase reporter assay was used to monitor the activity. We found that miR-29a-3p increased the activity of NF-κB and miR-29a-3p inhibitor blocked the increased NF-κB signaling activity (Figure 2D). This result suggested that miR-29a-3p inhibition down-regulates the NF-κB signaling pathway in HCC cells.

miR-29a-3p inhibition promotes HCC cells proliferation *in vitro*

To investigate the role of miR-29a-3p expression in the growth and metastasis of HCC, the inhibition impact of miR-29a-3p on the proliferation of the HCC cell line BEL-7402 was evaluated.

After transfection with miR-29a-3p, cells effectively decreased the growth rate compared with BC (Figure 3). However, after adding miR-29a-3p inhibitor, BEL-7402 cells growth rate was remarkably promoted compared with BC and miR-29a-3p (Figure 3). Additionally, the protein and mRNA levels of VEGF and MMP-9 protein were significantly increased in BEL-7402 cells with miR-29a-3p inhibitor compared to blank control BEL-7402 cells (Figure 4).

DISCUSSION

The molecular pathogenesis of HCC has not been investigated in detail, and the role of miRNAs in the progression of HCC is not fully understood. In this study, miR-29a-3p was found to be up-regulated in HCC from chronic alcohol abuse. miR-29a-3p also activates the NF-κB pathway through targeting PTEN. Additionally, miR-29a-3p promotes the proliferation of HCC cells. Taken together, these data suggested that miR-29-3p suppresses PTEN and suppresses alcohol-induced HCC progression by the NF-κB pathway. It raises the possibility of the development of novel therapeutic strategies for effectively decreasing HCC progression by regulating miR-29a-3p expression.

In the present study, we found miR-29a-3p down-regulated in HCC tissues and verified the down-regulation by qRT- PCR. miR-29a-3p is involved in many biological processes in cancers. Transcriptional profiling studies of miRNA expression across tumor tissues or cancer cell lines have already revealed that miR-29 is downregulated in the majority of cancers and upregulated in the minority [13]. It was reported that miR-29a-3p suppressed tumor metastasis in gastric cancer from *in vitro* functional assays of wound healing and transwell assays [14]. miR-29a-3p also inhibits cancer cell invasion and long-distance migration through targeting HSP47 in cervical squamous cell carcinoma [15]. The miR-29 family was also found to suppress metastasis-promoting LOXL2 in head and neck squamous cell carcinoma [16]. Despite all of these studies, there are very few *in vitro* functional studies of miR-29a-3p in HCC progression. We demonstrated that miR-29a-3p suppressed HCC progression through the NF-κB pathway via PTEN.

PTEN (also known as MMAC1 or TEP1) is one of the most common tumor-suppressor genes and is frequently mutated in a wide variety of human cancers, including HCC [17]. PTEN has been reported to dephosphorylate PIP3 and phosphatidylinositol and affects Akt which is an upstream component of the NF-κB pathway [18]. Additionally, PTEN silencing has been shown to upregulate the NF-κB signaling pathway [8]. However, it is unknown whether miRNAs acting through PTEN play any role in HCC and whether NF-κB signaling is involved. In this study, PTEN was confirmed as a target of miR-29a-3p. Furthermore, we also demonstrated that NF-κB signaling increased activity caused by miR-29-3p was blocked by miR-29a-3p inhibitor. Moreover, p-p65 protein expression, a NF-κB down-stream component, was down-regulated after PTEN up-regulation. This result provides new insight into the regulatory role that miR-29a-3p plays in the NF-κB signaling pathway in HCC.

CONCLUSION

Taken together, miR-29a-3p suppresses alcohol-induced HCC progression through the NF-κB pathway via targeting PTEN. It may contribute to the clinical value of miR-29a-3p as a target for cancer therapy. In addition, it may possess diagnostic and therapeutic potential for HCC in the future.

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Declaration of Interest:
The authors declare that there is no conflict of interest.

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