



In Vitro Anti-hepatoma Activities of Notoginsenoside R1 Through Downregulation of Tumor Promoter miR-21

Yuan Li¹ · Zhong Li¹ · Yunhao Jia¹ · Bo Ding¹ · Jinsong Yu¹

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Abstract

Background Notoginsenoside R1 (NG-R1) is the predominant active ingredient and a novel triterpene saponin compound extracted from the roots of *Panax notoginseng*. To date, to the best of our knowledge, there are no previous studies concerning the effect of NG-R1 on hepatocellular carcinoma (HCC).

Aims To investigate the effects of NG-R1 on HCC cell growth, apoptosis, and invasion and to explore the underlying mechanisms.

Methods Cell viability and lactate dehydrogenase (LDH) release were evaluated by cell counting kit-8 and LDH assay, respectively. Apoptosis was assessed using flow cytometry analysis and caspase-3/7 activity assay. Cell invasion was detected by Transwell invasion assay and western blot analysis of matrix metalloproteinase (MMP)-2 and MMP-9. The effects of NG-R1 on miR-21 expression and phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathway were examined by qRT-PCR and western blot, respectively.

Results NG-R1 inhibited the viability, increased LDH release and caspase-3/7 activity, induced apoptosis, and suppressed invasion in HCC cells. NG-R1 reduced miR-21 expression in HCC cells. miR-21 overexpression significantly attenuated the effects of NG-R1 on the viability, LDH release, apoptosis, caspase-3/7 activity, and invasion of HCC cells. We further demonstrated that NG-R1 inhibited the activation of the PI3K/Akt pathway in HCC cells, which was abolished by miR-21 overexpression.

Conclusions NG-R1 exerted anti-hepatoma activity through inactivation of the PI3K/Akt pathway by downregulating miR-21, contributing to further understanding of the anti-tumor activities of NG-R1 in HCC.

Keywords Notoginsenoside R1 · Anti-hepatoma · miR-21 · PI3K/Akt pathway · Hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) is one of the most commonly occurring malignancies with high morbidity and mortality, ranking as the third commonest cause of digestive system cancer-associated deaths globally [1]. Despite considerable advances in the early diagnosis and treatments of HCC, the prognosis of advanced HCC patients still remains far from satisfactory due to lack of available treatments, high recurrence and distant metastasis, with a 5-year survival rate for HCC of only about 18% [2, 3]. Many risk factors including alcoholism, consumption of aflatoxins, hepatitis B/C virus infection, liver cirrhosis, and environmental pollution are well documented to contribute to the pathogenesis of HCC [4, 5]. Chemotherapy is one of the major conventional treatments for HCC, but the clinical therapeutic outcomes are still not satisfactory because of strong side effects and

✉ Jinsong Yu
nsyyys@163.com

Yuan Li
2631803326@qq.com

Zhong Li
lizhong116@sohu.com

Yunhao Jia
185725222@qq.com

Bo Ding
1205788013@qq.com

¹ Department of General Surgery, Nanyang First People's Hospital, No. 12 Renmin Road, Nanyang 473012, China

emergence of drug resistance [6]. In this regard, it is of great necessity to develop innovative and effective therapeutic agents with limited side effects for HCC.

Panax notoginseng is a well-known traditional Chinese medicinal herb which has been widely used for over thousands of years in China for the prevention and treatment of cardiovascular and cerebral vascular diseases [7]. Notoginsenoside R1 (NG-R1; Fig. 1) is the predominant active ingredient and a novel triterpene saponin compound extracted from the roots of *Panax notoginseng*. A wide range of documents have shown that NG-R1 possesses multiple pharmacological activities, including neuroprotective, anti-oxidative, anti-inflammatory, anti-proliferative, and pro-apoptotic properties [8, 9]. Moreover, it was previously reported that NG-R1 exerted anticancer effects on colorectal cancer [10, 11]. To date, to the best of our knowledge, there are no previous studies concerning the effect of NG-R1 on HCC.

MicroRNAs (miRNAs) are a group of evolutionary conserved, small, endogenous noncoding RNAs with approximately 18–25 nucleotides in length, which suppress the mRNA translation of related genes or promote their mRNA

degradation at the posttranscriptional level [12]. Modern pharmacological studies have demonstrated that deregulated miRNAs are highly correlated with the carcinogenesis and progression of various types of cancer, exerting cancer-promoting or tumor-suppressive effects, suggesting the potential of miRNAs to be diagnostic and prognostic biomarkers in tumors [13]. Specifically, miR-21, a well-characterized miRNA located at the chromosome of 17q23.2, has been documented to be overexpressed in several human malignancies including HCC [14]. The upregulated miR-21 is therefore considered as a potential prognostic biomarker and therapeutic target in HCC [15]. miR-21 overexpression promotes invasion and migration [16], while miR-21 downregulation induces apoptosis and inhibits proliferation in HCC cells [17]. Interestingly, a previous report demonstrated that NG-R1 exerted anti-atherosclerotic effects on atherosclerosis in part through downregulation of miR-21 [18]. Therefore, we supposed whether NG-R1 could exert its effects via regulating miR-21 expression in HCC.

In the present study, we demonstrated that NG-R1 exerted anti-hepatoma activity by inhibiting cell growth and invasion and promoting apoptosis in HCC cells, which was mediated through inactivation of the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathway via downregulation of miR-21.

Materials and Methods

Cell Culture and Treatments

Human normal liver cell line HL-7702 and human HCC cell lines MHCC97H, BEL7402, Huh7, HepG2, PLC/PRF/5 (PLC), and SMMC7721 were purchased from American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (HyClone, Logan, UT, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C in a humidified atmosphere of 95% and 5% CO₂. miR-21 mimics (miR-21) and its scrambled control (miR-ctrl) were obtained from RiboBio Co., Ltd. (Guangzhou, China). In some experiments, MHCC97H and BEL7402 cells were treated with a series of concentrations of NG-R1 (0, 5, 10, 20, 40, 80, and 160 µM) (purity > 98%, Shanghai Yuanye Biotechnology, Shanghai, China) for 48 h, or transfected with miR-21 or miR-ctrl, followed by exposure to 40 µM NG-R1 for 48 h.

Cell Viability Assay

Cell viability was evaluated by cell counting kit-8 (CCK-8) assay. Briefly, MHCC97H and BEL7402 cells were seeded

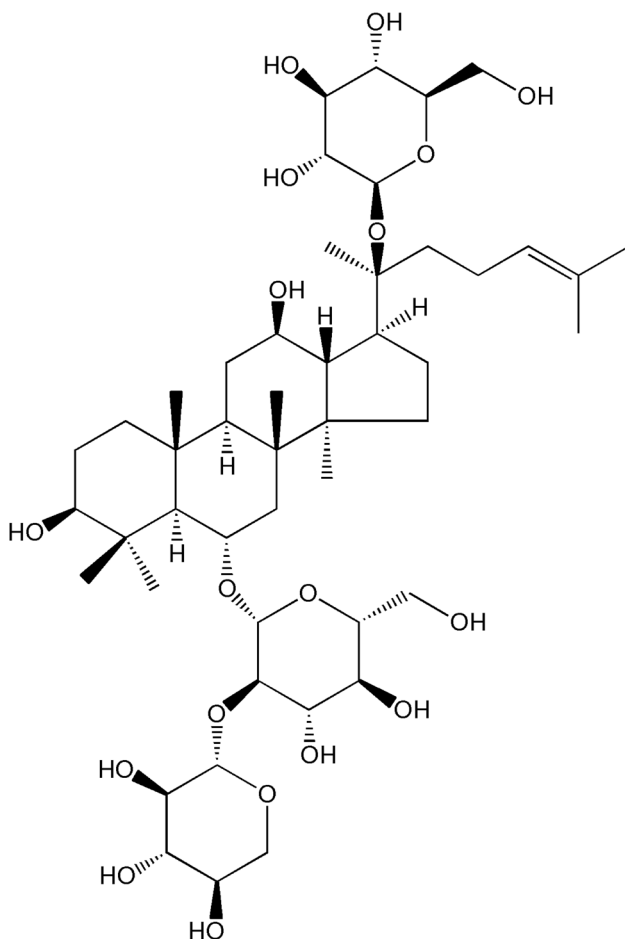


Fig. 1 Chemical structure of NG-R1

into 96-well plates at 2×10^3 cells/well and treated as mentioned above. After 48 h, 10 μ L of CCK-8 reagent (Beyotime, Beijing, China) was added to each well and incubated for 1 h. The optical density at a wavelength of 450 nm was recorded using an Elx800 microplate Reader (Bio-Tek Instruments Inc., Winooski, VT, USA). The 50% growth inhibition concentration (GI50) was calculated using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA).

Lactate Dehydrogenase (LDH) Release Assay

After indicated treatments, the supernatants of MHCC97H and BEL7402 cells were collected for the measurement of LDH release by a commercial LDH Activity Assay Kit (Sigma, St. Louis, MO, USA). The absorbance at 490 nm was read using a microplate reader (Bio-Tek Instruments Inc.).

Flow Cytometry Analysis

Cell apoptosis was detected by an Annexin V-fluorescein isothiocyanate (FITC) Cell Apoptosis Detection kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. In brief, MHCC97H and BEL7402 cells were collected by centrifugation after treatment and washed twice with ice-cold PBS. Subsequently, these cells were resuspended in 100 μ L of $1 \times$ binding buffer containing 10 μ L Annexin V-FITC and 5 μ L propidium iodide (PI) and incubated for 15 min in the dark at room temperature. Finally, the proportion of apoptotic cells was determined by a FACS Calibur flow cytometer (BD Biosciences).

Caspase-3/7 Activity Assay

Caspase-3/7 activity was detected using a commercially available Caspase-Glo 3/7 assay kit (Promega, Madison, WI, USA). The treated MHCC97H and BEL7402 cells were cultured into 96-well plates, and then 100 μ L of Caspase-Glo 3/7 reagent was added into each well. After incubated for 2 h, the luminescence was measured with the FLUOstar OPTIMA plate reader (BMG Labtech, Germany).

Transwell Invasion Assay

Cell invasive ability was evaluated using the 24-well Transwell plate (Corning Incorporated, Corning, NY, USA) pre-coated with Matrigel (BD Biosciences). Following treatments, a total of 2×10^5 MHCC97H and BEL7402 cells resuspended in 200- μ L serum-free culture medium were placed on the upper chamber, while 500 μ L of culture medium containing 20% FBS was inoculated to the lower chamber as a chemoattractant. After culturing for 48 h, the non-invaded cells were wiped off with a cotton swab and

invaded cells in the lower chamber were fixed with 4% paraformaldehyde and then stained with 0.1% crystal violet at room temperature. Finally, the invaded cells were photographed and counted in five randomly fields using an optical microscope (Olympus Corporation, Tokyo, Japan).

Quantitative Real-Time PCR (qRT-PCR)

A miRNeasy kit (Qiagen GmbH, Hilden, Germany) was used to extract miRNA from treated MHCC97H and BEL7402 cells. cDNA was synthesized from total RNA using the RevertAid™ First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). miR-21 expression was detected using a Taqman Universal Master Mix II (Applied Biosystems, Foster City, CA, USA) on a Step one plus system (Roche Molecular Diagnostics, Pleasanton, CA, USA). The relative gene expression was calculated using $2^{-\Delta\Delta CT}$ method and normalized to U6 small nuclear RNA (snRNA).

Western Blot Analysis

Total protein from treated MHCC97H and BEL7402 cells was extracted using a ProteoPrep® Total Extraction Sample kit (Sigma), and protein concentration was measured by a BCA Protein Assay Kit (Thermo Fisher Scientific). Protein samples (20 μ g/lane) were separated on 10% SDS-PAGE, followed by transfer to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After being blocked with 5% non-fat dry milk at room temperature for 1 h, the membranes were incubated overnight at 4 °C with primary antibodies including matrix metalloproteinase (MMP)-2, MMP-9, phosphorylated PI3K (p-PI3K), PI3K, phosphorylated Akt (p-Akt), Akt and β -actin (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA). Then, the membranes were incubated with the horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) for 1 h at room temperature. Protein bands were visualized by an Enhanced Chemiluminescence Detection System (Amersham, Little Chalfont, Buckinghamshire, England) and analyzed using the Image J software (NIH, Bethesda, MD, USA).

Statistical Analysis

Results are shown as mean \pm standard deviation (SD). All statistical analyses were carried out using SPSS 19.0 (SPSS IBM, Armonk, NY, USA). The statistical significance was analyzed by one-way analysis of variance (ANOVA). $P < 0.05$ was considered to be statistically significant. Each experiment was performed at least three times.

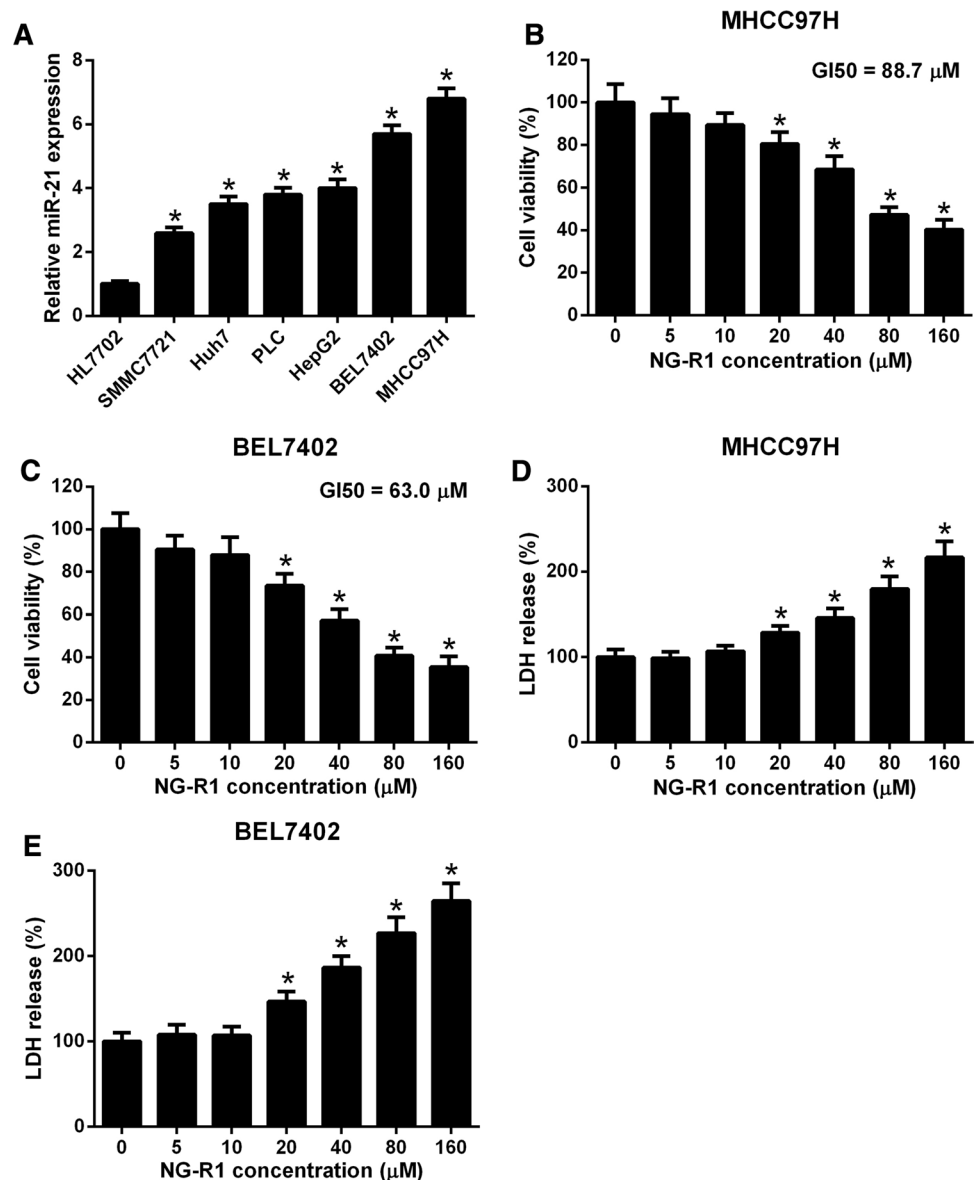
Results

NG-R1 Inhibited the Viability and Increased LDH Release in HCC Cells

Firstly, the expression of miR-21 in human normal liver cell line HL-7702 and human HCC cell lines. As shown in Fig. 2a, miR-21 was highly expressed in human HCC cell lines SMMC7721, Huh7, PLC, HepG2, BEL7402, and MHCC97H compared with that in human normal liver cell line HL-7702. MHCC97H and BEL7402 cell lines were selected for further investigation since the miR-21 expression level was higher in the two cell lines than that in the other HCC cell lines. To determine the cytotoxicity

of NG-R1 on HCC cells, MHCC97H, and BEL7402 cells were suffered from different concentrations of NG-R1 for 48 h, and cell viability and LDH release were evaluated by CCK-8 and LDH assay, respectively. CCK-8 assay showed that cell viability was impeded following exposure to NG-R1 at 20, 40, 80, and 160 μM in a dose-dependent manner in MHCC97H (Fig. 2b) and BEL7402 (Fig. 2c) cells. The GI50 values for NG-R1 were 88.7 and 63.0 μM in MHCC97H and BEL7402 cells, respectively. Moreover, LDH assay revealed that NG-R1 at 20, 40, 80, and 160 μM concentration-dependently enhanced LDH release in MHCC97H (Fig. 2d) and BEL7402 (Fig. 2e) cells. Therefore, we concluded that NG-R1 inhibited the viability and increased LDH release in HCC cells.

Fig. 2 Effects of NG-R1 on cell viability and LDH release in HCC cells. **a** The expression of miR-21 was determined by qRT-PCR in human normal liver cell line HL-7702 and human HCC cell lines SMMC7721, Huh7, PLC, HepG2, BEL7402, and MHCC97H. MHCC97H and BEL7402 cells were exposed to various doses of NG-R1 (0, 5, 10, 20, 40, 80, and 160 μM) for 48 h, followed by assessment of cell viability (**b, c**) and LDH release (**d, e**) by CCK-8 and LDH assays, respectively. * $P < 0.05$ compared with 0 μM NG-R1. Statistical analysis was conducted using one-way ANOVA with Dunnett’s posttest



NG-R1 Promoted Apoptosis of HCC Cells

To investigate the effect of NG-R1 on apoptosis in HCC cells, flow cytometry analysis and caspase-3/7 activity assay were performed. As shown in Fig. 3a and b, NG-R1 treatment induced apoptosis in MHCC97H and BEL7402 cells. Meanwhile, caspase-3/7 activity was increased in MHCC97H (Fig. 3c) and BEL7402 (Fig. 3d) cells in response to NG-R1. These results suggested that NG-R1 promoted apoptosis of HCC cells.

NG-R1 Suppressed Invasion of HCC Cells

We further explored the effect of NG-R1 on cell invasion in HCC cells by Transwell invasion assay. The results implicated that cell invasive ability was impeded after treatment with NG-R1 in MHCC97H (Fig. 4a) and BEL7402 (Fig. 4b) cells. MMP-2 and MMP-9 are well known to play crucial roles in cancer cell metastasis and invasion [19]. The influence of NG-R1 on the expression of MMP-2 and MMP-9 was detected by western blot, and the results presented that 20 and 40 μM NG-R1 both effectively restrained the protein levels of MMP-2 and MMP-9 in MHCC97H (Fig. 4c)

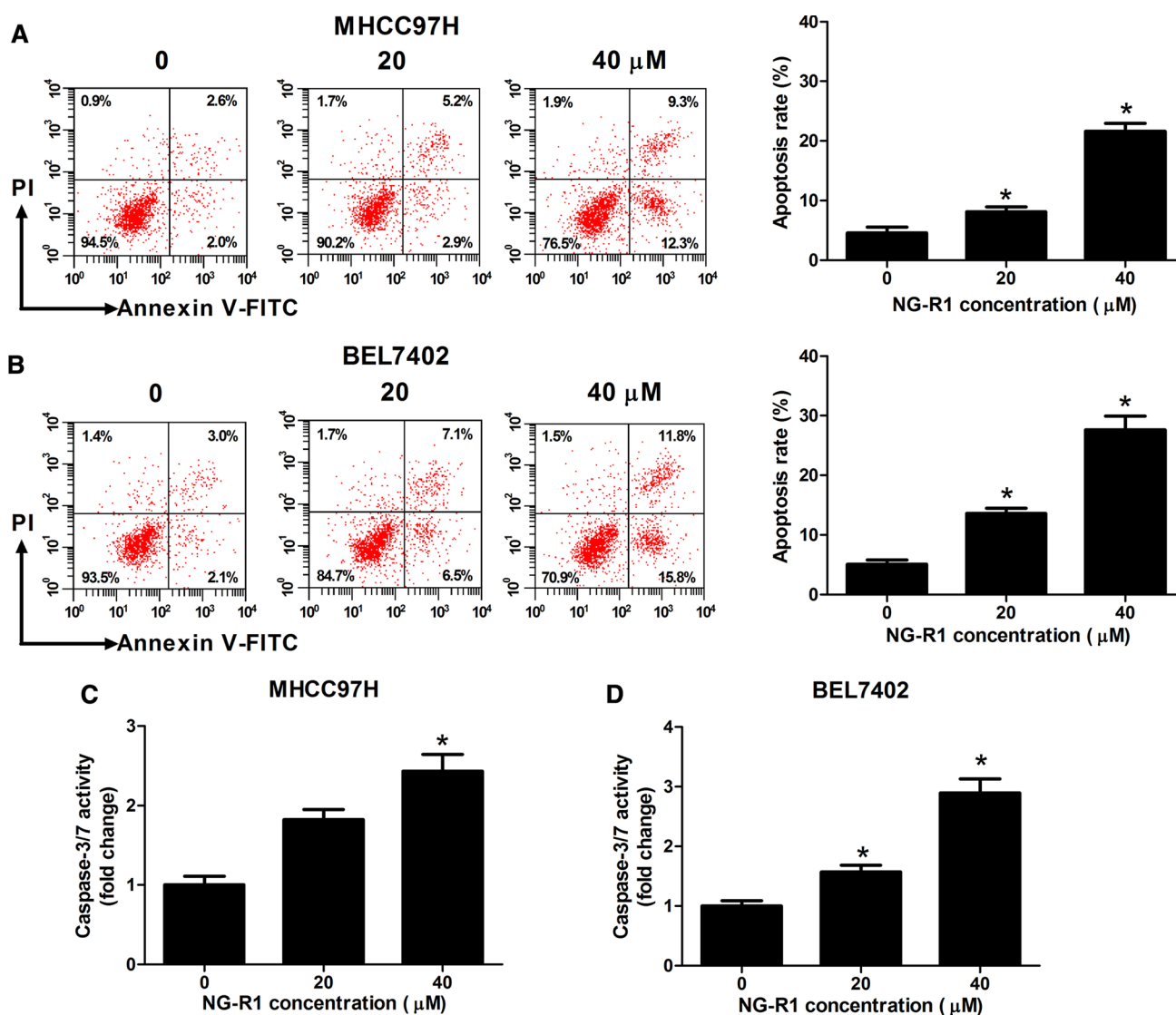


Fig. 3 Effect of NG-R1 on apoptosis of HCC cells. MHCC97H and BEL7402 cells were administrated with 0, 20, and 40 μM NG-R1 for 48 h, and then cell apoptosis (a, b) and caspase-3/7 activity (c, d) were evaluated by flow cytometry analysis and caspase-3/7 activity

assay, respectively. * $P < 0.05$ compared with 0 μM NG-R1. Statistical analysis was conducted using one-way ANOVA with Dunnett's post-test

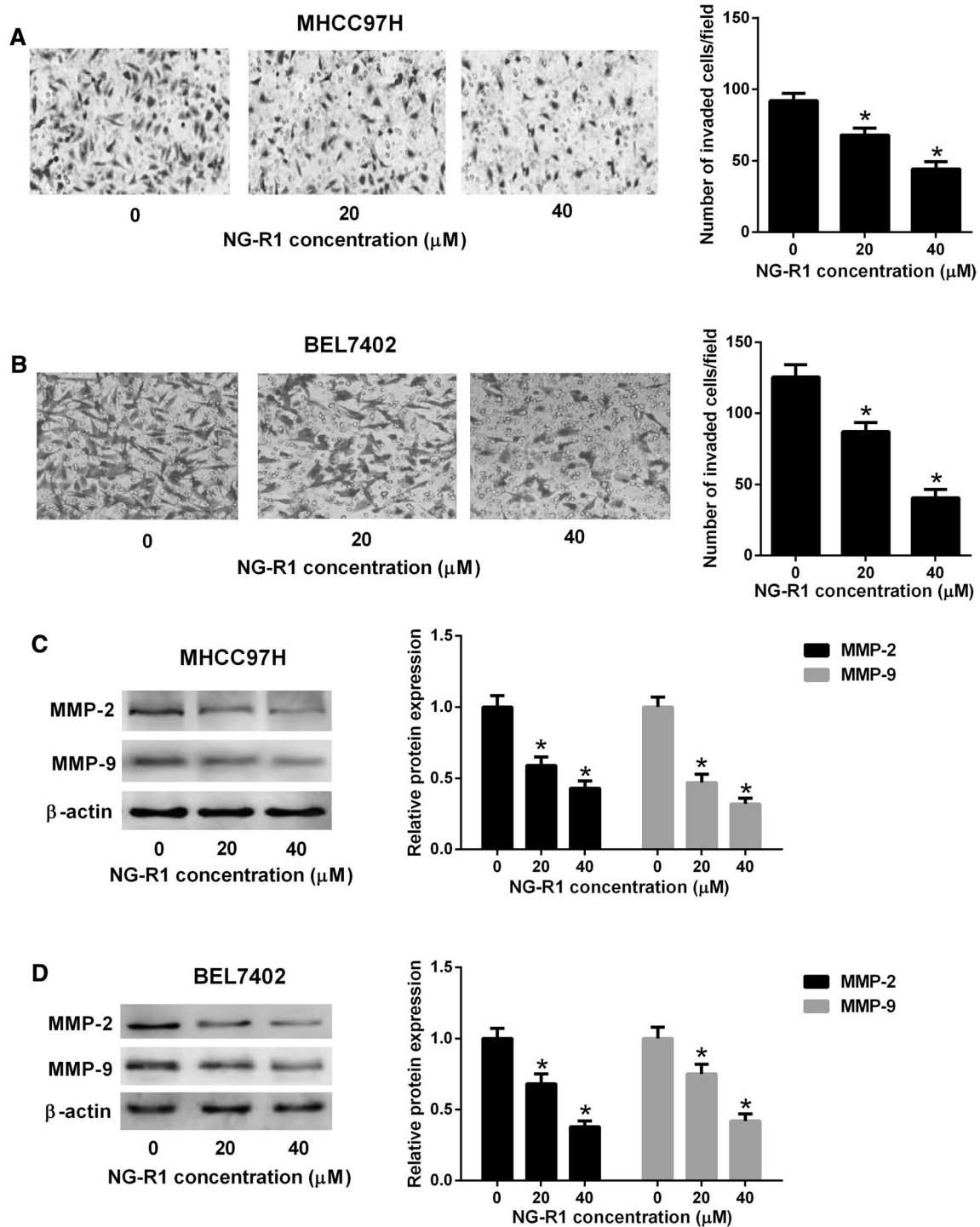


Fig. 4 Effect of NG-R1 on cell invasion in HCC cells. MHCC97H and BEL7402 cells were exposed to 0, 20, and 40 μM NG-R1 for 48 h. Transwell invasion assay was performed to detect cell invasion in the treated MHCC97H (a) and BEL7402 (b) cells. Western blot

was applied to determine the protein levels of MMP-2 and MMP-9 in the treated MHCC97H (c) and BEL7402 (d) cells. * $P < 0.05$ compared with 0 μM NG-R1. Statistical analysis was conducted using one-way ANOVA with Dunnett's posttest

and BEL7402 (Fig. 4d) cells. These results suggested that NG-R1 suppressed invasion of HCC cells.

NG-R1 Downregulated miR-21 Expression in HCC Cells

To explore whether miR-21 was involved in the regulation of NG-R1, we determined the expression of miR-21 in HCC cells following NG-R1 treatment. qRT-PCR analysis revealed that miR-21 expression was dose-dependently inhibited in response to NG-R1 in MHCC97H (Fig. 5a) and BEL7402 (Fig. 5b) cells, suggesting that NG-R1 downregulated miR-21 expression in HCC cells.

miR-21 Overexpression Attenuated the Effects of NG-R1 on the Viability and LDH Release of HCC Cells

To explore whether miR-21 could affect the effects of NG-R1 on HCC cells, MHCC97H and BEL7402 cells were transfected with miR-21 or miR-ctrl, followed by treatment with 40 μ M NG-R1 for 48 h. CCK-8 and LDH assays showed that delivery of miR-21 reversed NG-R1-induced inhibition of cell viability (Fig. 6a and b) and increase in LDH release (Fig. 6c and d) in MHCC97H and BEL7402 cells.

miR-21 Overexpression Attenuated the Effects of NG-R1 on Apoptosis of HCC Cells

Flow cytometry analysis and caspase-3/7 activity assay manifested that NG-R1-induced increase of apoptotic rate (Fig. 7a and b) and caspase-3/7 activity (Fig. 7c and d) in MHCC97H and BEL7402 cells were attenuated by transfection with miR-21, suggesting that miR-21 overexpression attenuated the effects of NG-R1 on apoptosis of HCC cells.

miR-21 Overexpression Attenuated the Effects of NG-R1 on the Invasion of HCC Cells

As displayed in Fig. 8a and b, promotion of miR-21 abolished the suppressive effect of NG-R1 on the invasive ability in MHCC97H and BEL7402 cells. Meanwhile, western

blot analysis proved that cotreatment with NG-R1 and miR-21 overexpression offset the suppressive effect of NG-R1 on MMP-2 and MMP-9 expression in MHCC97H and BEL7402 cells (Fig. 8c and d). Collectively, these results revealed that miR-21 overexpression attenuated the effects of NG-R1 on the invasion of HCC cells.

miR-21 Overexpression Resisted NG-R1-Induced Inactivation of PI3K/Akt Pathway in HCC Cells

To investigate the potential regulatory mechanism of NG-R1 in HCC cells, the PI3K/Akt pathway was examined in this study. As presented in Fig. 9a and b, treatment with NG-R1 significantly inhibited the expression of p-PI3K and p-Akt, but had no impact on PI3K and Akt expression in MHCC97H and BEL7402 cells. However, delivery of miR-21 effectively abrogated the inhibitory effects of NG-R1 on the expression of p-PI3K and p-Akt in MHCC97H and BEL7402 cells. These results indicated that miR-21 overexpression resisted NG-R1-induced inactivation of the PI3K/Akt pathway in HCC cells.

Discussion

HCC is one of the deadliest cancers with increasing incidence and poor prognosis, posing a serious threat to human health [20]. It is indispensable to identify innovative and effective therapeutic agents for the prevention and treatment of HCC. Increasing studies have focused on the anti-tumor activities of various natural or herbal compounds, which provide new avenues for the treatment of cancers. In our study, we studied the anti-tumor effects of NG-R1 on HCC cells. Our study demonstrated that NG-R1 inhibited the viability, increased LDH release, promoted apoptosis, and suppressed cell invasion in HCC cells. Additionally, we found that NG-R1 downregulated miR-21 expression in HCC cells and the anti-tumor effects of NG-R1 on HCC cells were abolished by overexpression of miR-21. Moreover, further

Fig. 5 Effect of NG-R1 on miR-21 expression in HCC cells. **a**, **b** MHCC97H and BEL7402 cells were exposed to 0, 20, 40, and 80 μ M NG-R1 for 48 h, followed by qRT-PCR analysis of miR-21 expression. * $P < 0.05$ compared with 0 μ M NG-R1. Statistical analysis was conducted using one-way ANOVA with Dunnett's posttest

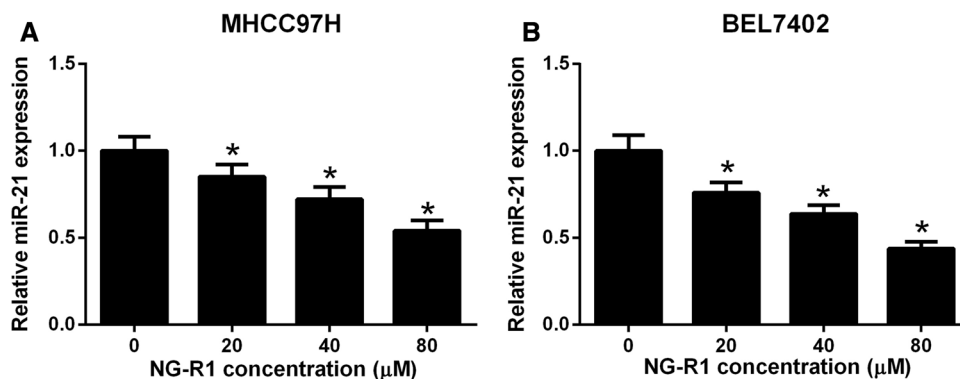
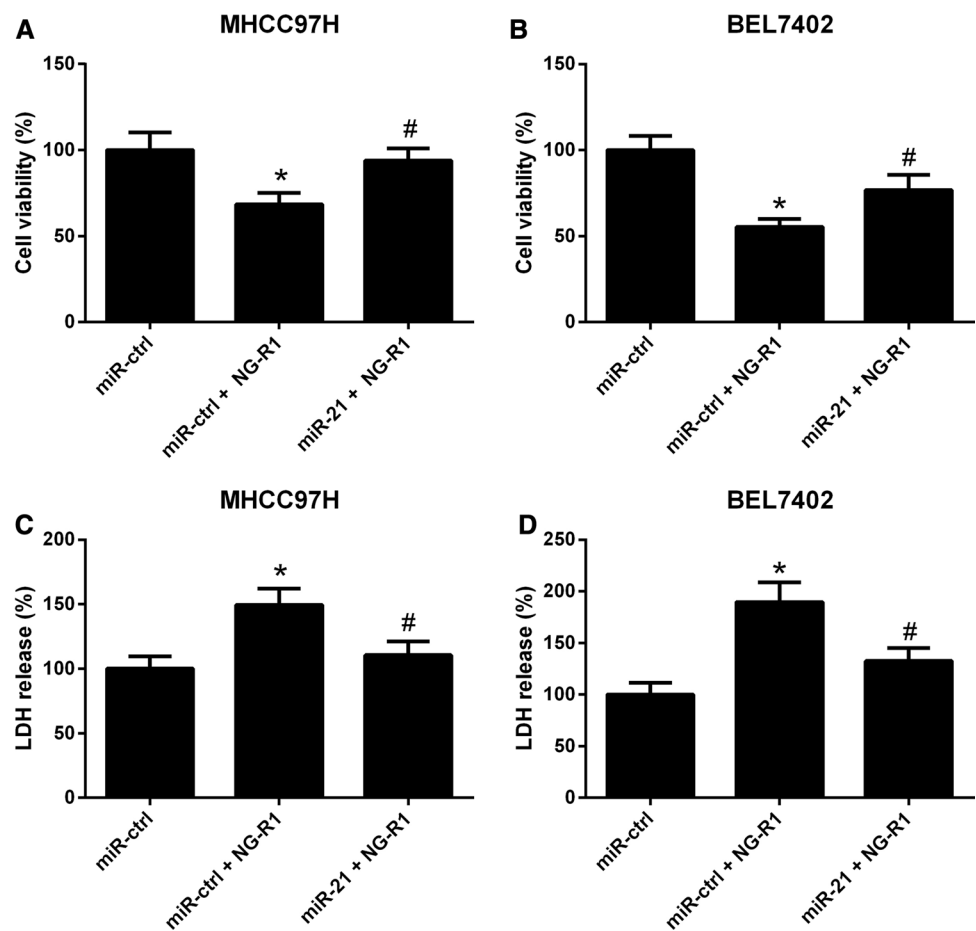


Fig. 6 Effects of NG-R1 or together with miR-21 on the viability and LDH release in HCC cells. MHCC97H and BEL7402 cells were transfected with miR-21 or miR-ctrl, followed by treatment with 40 μM NG-R1 for 48 h. **a, b** Cell viability in the treated MHCC97H and BEL7402 cells was evaluated by CCK-8 assay. **c, d** LDH release in the treated MHCC97H and BEL7402 cells was measured by LDH assay. * $P < 0.05$ compared with miR-ctrl. # $P < 0.05$ compared with miR-ctrl + NG-R1. Statistical analysis was conducted using one-way ANOVA with Tukey's posttest



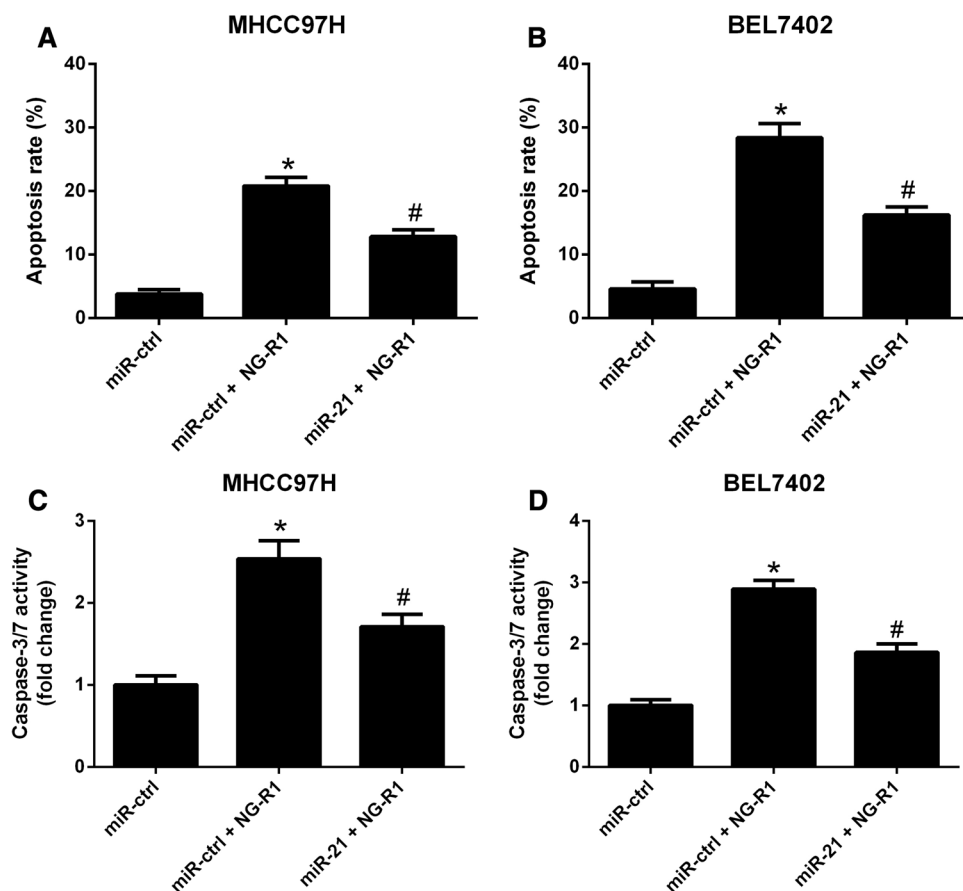
experiments proved that NG-R1 inactivated the PI3K/Akt pathway in HCC cells by downregulating miR-21.

Recently, several lines of evidence have demonstrated that *Panax notoginseng* extract shows anticancer activity [21]. Treatment with *Panax notoginseng* root extract reduced the growth in lung carcinoma cells and a lung carcinoma xenograft mouse model, suggesting that *Panax notoginseng* could serve as a potent anticancer agent [22, 23]. It was demonstrated that *Panax notoginseng* extract exerted anti-metastatic property by inhibiting human colorectal cancer cell migration, invasion, and adhesion [24]. NG-R1, a novel phytoestrogen, is believed to be the characteristic component in the root of *Panax notoginseng* and has multifaceted pharmacologic activity including anticancer activity [25]. For example, NG-R1 showed anti-metastatic properties in colorectal cancer by inhibiting cell migration, invasion, and adhesion and by regulating expression of metastasis-associated signaling molecules [10]. It was also proved that *Panax notoginseng* root extract and its major constituents

including NG-R1 inhibited cell growth, increased apoptosis, arrested cells in the synthesis phase, and enhanced the action of chemotherapeutic agents in colorectal cancer cells [11, 25]. In the present study, we showed that NG-R1 inhibited cell viability, increased LDH release, promoted apoptosis, and suppressed cell invasion in HCC cells, suggesting the anti-tumor effect of NG-R1 in HCC.

In recent years, increasing experimental data have emphasized the importance of miRNA regulation in the pharmacologic activities of drugs with herbal sources [26]. For example, alkannin exerted anti-tumor activity in HCC cells via inactivating phosphatase and tensin homolog (PTEN)/PI3K/Akt signal pathways by downregulation of miR-92a [27]. Schizandrin A inhibited cell proliferation, metastasis, and induced apoptosis in thyroid cancer through inactivation of the Wnt/ β -catenin and mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling pathways by downregulating miR-429 [28]. In addition, it was demonstrated that NG-R1

Fig. 7 Effects of NG-R1 or together with miR-21 on apoptosis in HCC cells. MHCC97H and BEL7402 cells were transfected with miR-21 or miR-ctrl and treated with 40 μ M NG-R1 for 48 h. **a, b** The apoptotic rate was detected by flow cytometry analysis. **c, d** Caspase-3/7 activity was measured by caspase-3/7 activity assay, respectively. * $P < 0.05$ compared with miR-ctrl. # $P < 0.05$ compared with miR-ctrl + NG-R1. Statistical analysis was conducted using one-way ANOVA with Tukey's posttest



prevented oxidized low-density lipoprotein-induced atherogenic response in HUVEC cells via downregulating miR-132 [29]. Interestingly, a previous study proved that NG-R1 reduced miR-21 expression in atherosclerosis [18]. Consistently, we provided the evidence that NG-R1 downregulated miR-21 expression in HCC cells. Moreover, rescue experiments manifested that miR-21 overexpression attenuated the effects of NG-R1 on the viability, LDH release, apoptosis, and invasion of HCC cells, suggesting that NG-R1 exerted anti-tumor activity in HCC cells by downregulating miR-21.

To date, the PI3K/Akt signal pathway, an important intracellular signal transduction pathway, has become a research focus due to its critical role in the regulation of multiple biological processes in cancer cells, including cell proliferation, apoptosis and metastasis [30]. Activation of PI3K/Akt signaling pathway participates in the development and progression of various cancers including HCC [31]. Previously, it was reported that curcumin reduced the proliferation of colorectal cancer cells through inhibiting the PTEN/

PI3K/Akt pathway by targeting miR-21 [32]. Kaempferol inhibited proliferation, migration, and invasion of HCC cells by downregulating miR-21 and inactivating the PI3K/Akt/mammalian target of rapamycin (mTOR) pathway [33]. Accordingly, we supposed whether the anti-tumor activity of NG-R1 in HCC cells was mediated via inactivation of the PI3K/Akt pathway by downregulating miR-21. As expected, our study demonstrated that NG-R1 inhibited the activation of the PI3K/Akt pathway in HCC cells, which was abolished by miR-21 overexpression. Collectively, we concluded that NG-R1 exerted anti-tumor activity in HCC cells through inactivation of the PI3K/Akt pathway by downregulating miR-21.

In summary, to the best of our knowledge, we demonstrated for the first time that NG-R1 exerted anti-hepatoma activity through inactivation of the PI3K/Akt pathway by downregulating miR-21, contributing to further understanding of the anti-tumor activities of NG-R1 in HCC. Our study may provide valuable foundation for further exploring the treatment of HCC using NG-R1.

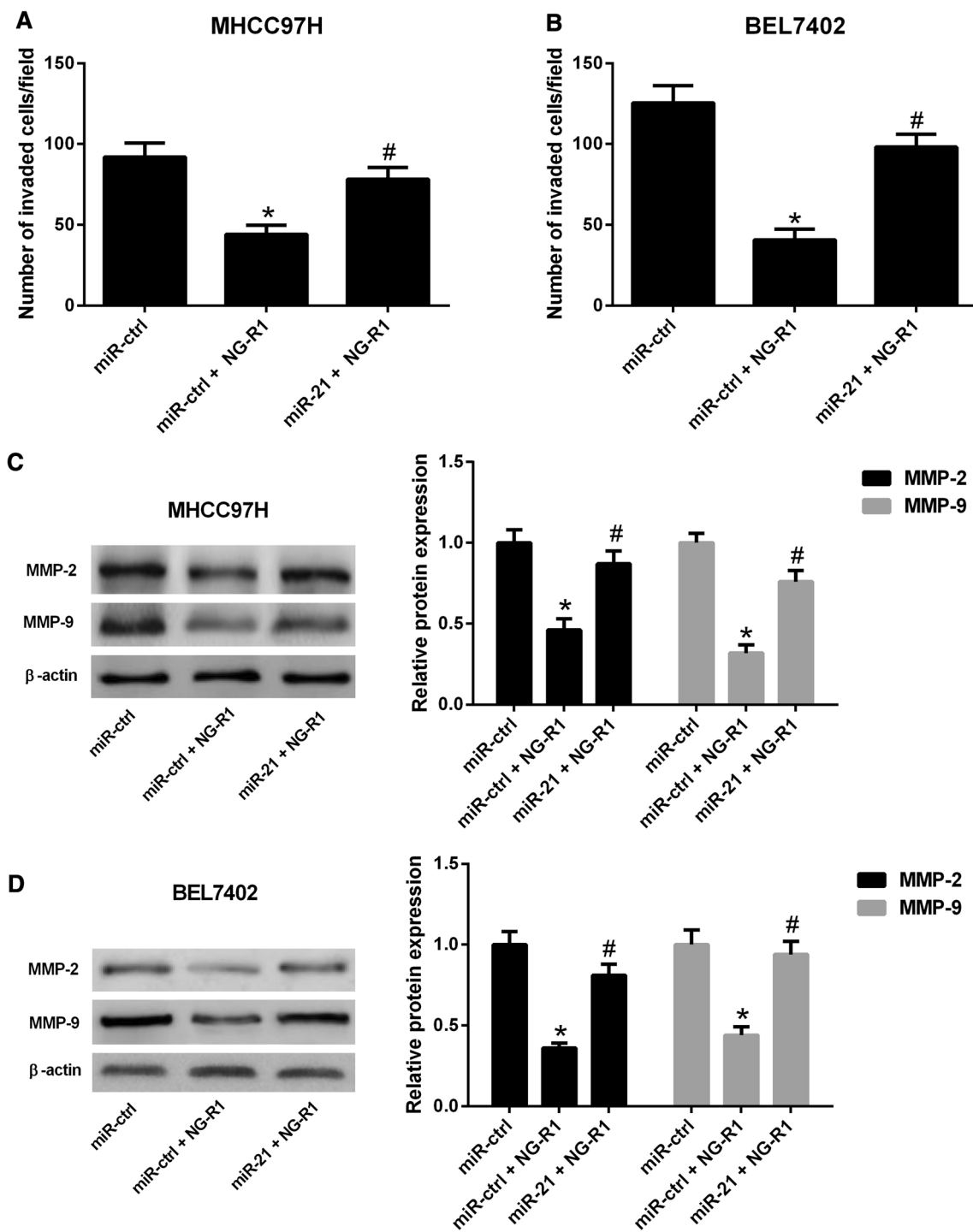


Fig. 8 Effect of NG-R1 or combined with miR-21 on cell invasion in HCC cells. MHCC97H and BEL7402 cells were introduced with miR-21 or miR-ctrl and exposed to 40 μ M NG-R1 for 48 h. **a, b** Transwell invasion assay was conducted to evaluate cell invasive ability in the treated MHCC97H and BEL7402 cells. **c, d** Western blot analysis

was performed to detect the protein levels of MMP-2 and MMP-9 in the treated MHCC97H and BEL7402 cells. * $P < 0.05$ compared with miR-ctrl. # $P < 0.05$ compared with miR-ctrl + NG-R1. Statistical analysis was conducted using one-way ANOVA with Tukey's posttest

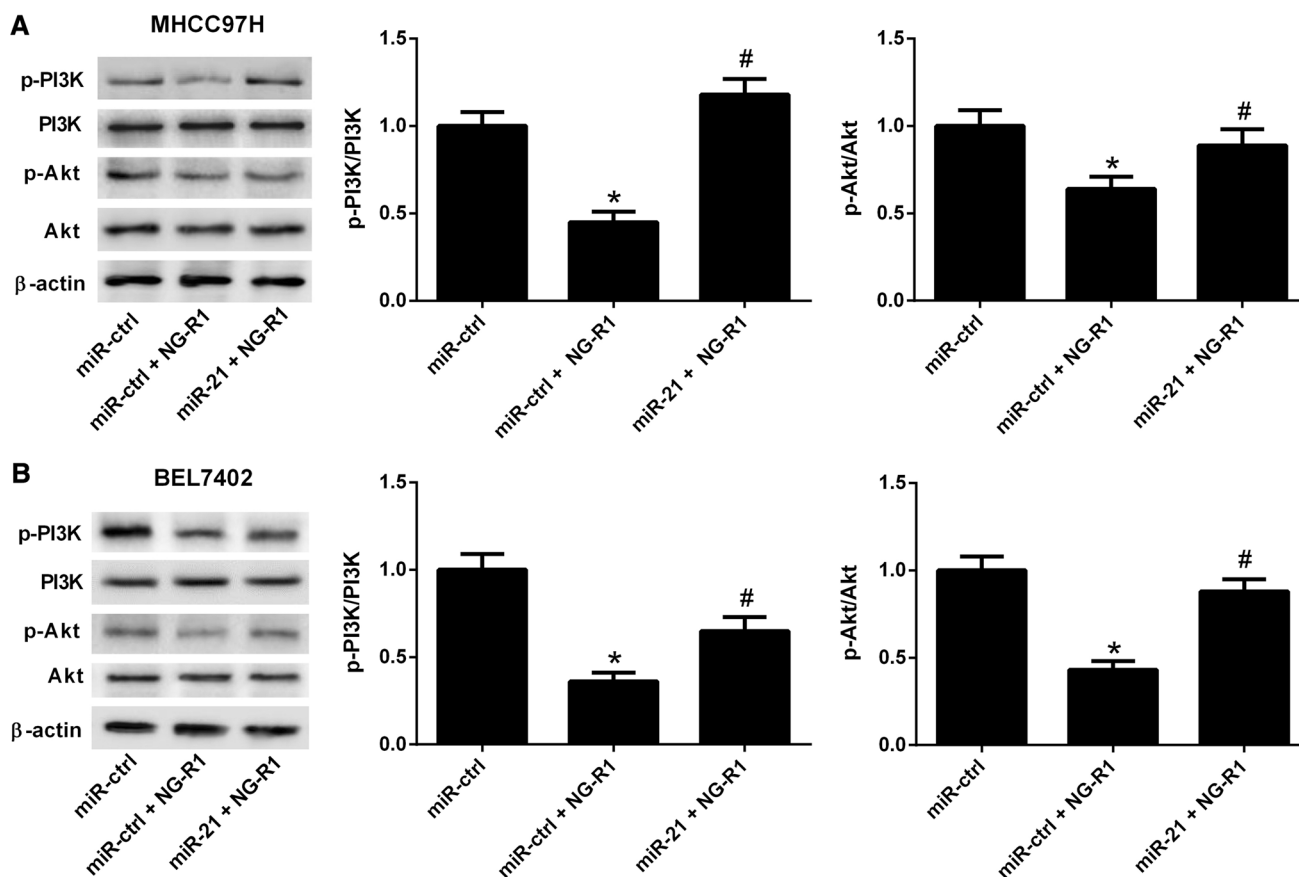


Fig. 9 Effect of NG-R1 or combined with miR-21 on the PI3K/Akt pathway in HCC cells. **a, b** MHCC97H and BEL7402 cells were introduced with miR-21 or miR-ctrl and exposed to 40 μ M NG-R1 for 48 h, followed by western blot analysis of p-PI3K, PI3K, p-Akt,

and Akt expression. * $P < 0.05$ compared with miR-ctrl. # $P < 0.05$ compared with miR-ctrl + NG-R1. Statistical analysis was conducted using one-way ANOVA with Tukey's posttest

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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