

## Original Article

# MicroRNA-124 induces apoptosis of MHCC97H cells through downregulation of KIAP

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**Abstract:** Hepatocarcinoma is a great threat to global health. microRNA-124 was proved to influence growth and apoptosis in some cell lines. Our study aimed to investigate the effect of microRNA-124 on growth, proliferation and apoptosis of MHCC97H, a hepatocarcinoma cell line, and explored potential mechanism of such pro-apoptosis effect. microRNA-124 and control microRNA (scramble miRNA, for short as miRNA) were synthesized with routine protocol. Lipofection transfection was performed in hepatocarcinoma cell line MHCC97H. MTT assay, caspase-3 activity detection and flow cytometry were performed to examine growth, proliferation and apoptosis of hepatocarcinoma cell line MHCC97H, respectively. KIAP siRNA was synthesized for inhibition of KIAP. KIAP plasmid was established for activation of KIAP. Western blot was performed to examine protein expression of KIAP and caspase protein family after transfection of KIAP siRNA or KIAP plasmid. Compared with miRNA transfection, microRNA-124 transfection significantly reduced growth of MHCC97H cells, and decreased expression of KIAP. Enhanced translocation of phosphatidylserine and activation of caspase-3 were observed in microRNA-124 transfection cells. Moreover, inhibition of KIAP enhanced pro-apoptosis effect of microRNA-124, while activation of KIAP abrogated pro-apoptosis effect of microRNA-124. microRNA-124 inhibits growth and proliferation of MHCC97H cells, and promotes apoptosis of MHCC97H cells via down-regulating KIAP. KIAP could be a potential therapeutic target for hepatocarcinoma treatment.

**Keywords:** MicroRNA-124, KIAP, MHCC97H, apoptosis

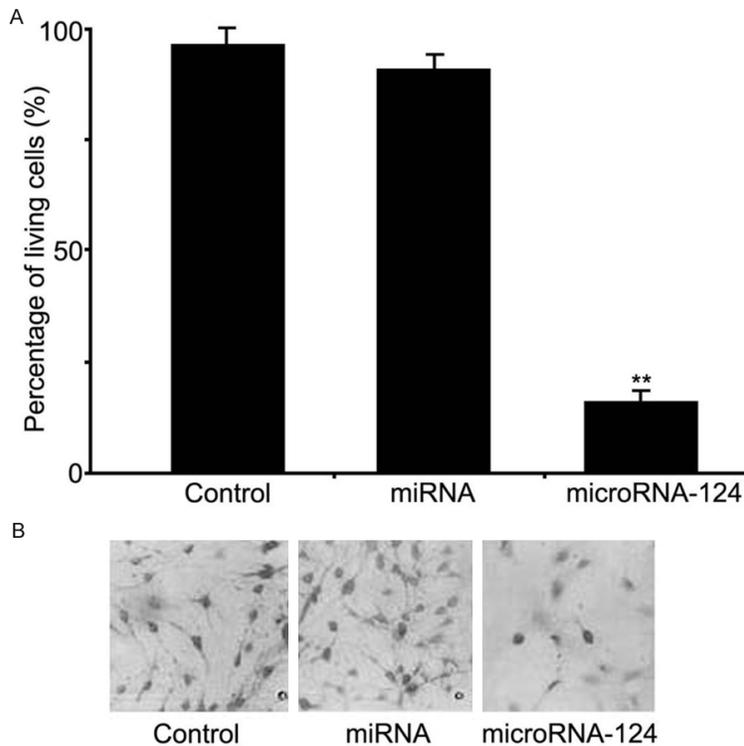
## Introduction

Hepatocarcinoma is one of digestive system diseases with high mortality [1]. Although virus infection has been proved to be the major determinant of liver disease onset, molecular mechanisms of hepatocarcinogenesis are unclear, which need further exploration [2]. Hepatitis virus (HPV) has synergistic effect in the progress of liver cancer [3, 4]. Combined treatment is the most common strategy for liver cancer therapy with promising efficacy, including operative treatment, radioactive therapy and chemotherapy. However, many disadvantages have limited development of combined treatment, such as skin rash, hemorrhage and toxic effect [5-7]. Thus, how to improve efficacy of hepatocarcinoma treatment is the emphasis and difficulty in clinical practice.

As a research hotspot, molecular targeting treatment was rarely applied in liver cancer treatment due to scarce molecular targets [8-10]. Bcl-2 and AIPs are the most reported targets for hepatocarcinoma treatment, but their efficacy was not ideal [11]. Moreover, no microRNA was proved to be an effective target for hepatocarcinoma treatment [12].

microRNA is a kind of non-coding microRNA with multiple biological functions, for example, microRNA-218 was proved to inhibit growth of hepatocarcinoma cells, while microRNA-34a was associated with tumor metastasis, furthermore, expression of microRNA-124 in cancer cells was significantly higher than normal tissues [13-16], suggesting microRNA was involved in occurrence and development of hepatocarcinoma [17]. Our study explored regula-

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**Figure 1.** Analysis of cell viability (A) and morphology (B) in three groups. MHCC97H cells were transferred into 6-well plate for 8 hours followed by addition of MTT working solution for analysis of cell viability (A). Meanwhile, cells morphology was also observed under a microscopy (B). \*\* $P < 0.01$ , versus miRNA (control).

tory effect of microRNA-124 on hepatocarcinoma cell line MHCC97H.

Anti-tumor strategy is aimed to kill cancer cells while not damage normal cells, therefore a potential mechanism is to induce apoptosis via regulating pro-apoptotic protein and inhibitor of apoptosis protein [18, 19]. As an extensively studied inhibitor of apoptosis, KIAP have been used as the target of drugs, but efficacy of such drugs is not promising on decreasing KIAP [20-22].

In summary, our study focused on proliferation, growth and apoptosis of hepatocarcinoma cell line MHCC97H under the influence of microRNA-124, and explored mechanism of pro-apoptosis microRNA-124 via KIAP.

### Materials and methods

#### Reagents and cell model

Fetal bovine serum and DMEM were purchased from HUALAN BIOLOGICAL ENGINEERING, INC

(Beijing, China). Synthesized sequences of RNA were as follows: microRNA-124, 5'-TACT-ATCGCAGGTCATCGCAAT-3' and 5'-AATGTAAGAAAGGGGACG-CA-3'. miRNA, 5'-CCTTACACAT-CTGTGAGGGC-3' and 5'-TACGC-ATTAATGTCCGATT-3'. siRNA KIAP 5'-CTAGCGTGCTACCATGATGC-3' and 5'-TTCTACGATATCACTAGGT-3'.

KIAP plasmid was purchased from Suzhou GenePharma (Suzhou, China). Liposome transfection kit was purchased from Invitrogen (Carlsbad, CA, USA). MTT assay kit was purchased from Beijing Dingguo biotechnology (Beijing, China). FITC-annexin and caspase-3 kits were purchased from Beyotime (Haimen, China). KIAP antibody and actin were purchased from Santa Cruz (Dallas, Texas, USA).

Hepatocarcinoma cell line MHCC97H was purchased from American Type Culture Collection (Manassas, VA, USA). Grouping was as follows: miRNA group, microRNA-124 group, microRNA-124+KIAP siRNA group, microRNA-124+KIAP plasmid group.

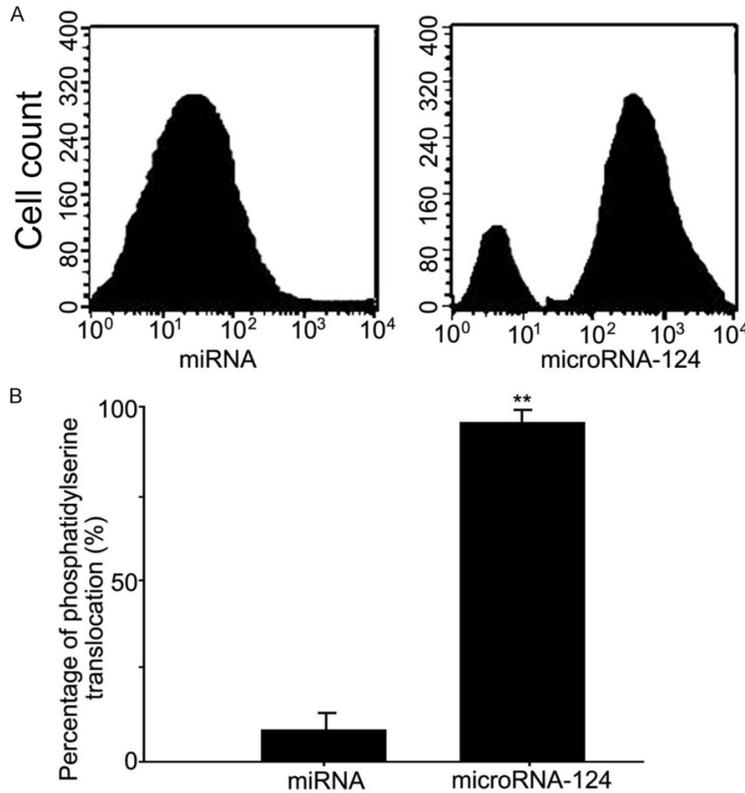
#### Cell culture

Hepatocarcinoma cell line MHCC97H was revived and cultured in DMEM high sugar medium containing 10% FBS and 1% penicillin-streptomycin, in a culture chamber at 37°C with 5% CO<sub>2</sub>. Culture medium was changed every two days. Cells at log-growth phase with good status were used in further experiments.

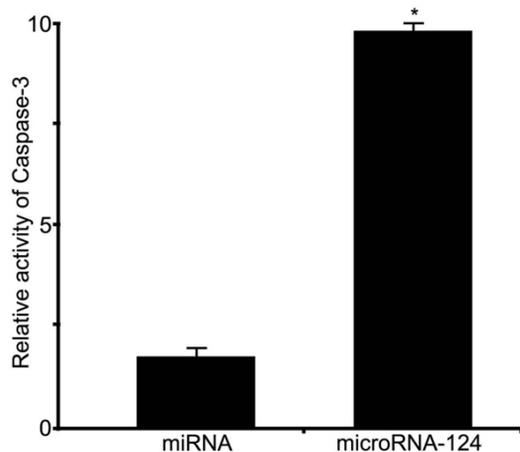
#### Transfection

When they were in logarithmic phase, MHCC97H cells were seeded into 6-well plate at  $4 \times 10^5$  cells/well. 5  $\mu$ l lipo2000 was added into 100  $\mu$ l serum free medium and incubated for 5 min. At the same time, 10  $\mu$ l microRNA-124 or microRNA scramble were mixed with 100  $\mu$ l serum free medium followed by addition of 1800  $\mu$ l serum free medium. After 6 h, medium was replaced by normal culture medium con-

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**Figure 2.** microRNA-124 transfection enhanced translocation of phosphatidyserine.  $**P < 0.01$ , versus miRNA (control). A: Representative histogram graph for expression of phosphatidyserine. B: Quantitative analysis of the expression of phosphatidyserine.



**Figure 3.** microRNA-124 transfection increased caspase-3 activity in MHCC97H cells. Transfected MHCC97H cells were treated with chromophoric substrate followed by transferring into 6-well plate for measurement of caspase-3 activity by a commercial kit according to manufacturer's instructions.  $**P < 0.01$ , versus miRNA (control).

taining serum. After 48 h continuous incubation, cells were collected for further analysis.

### MTT assay

MTT assay was performed with routine protocol [10]. Detailed processes were as follows:

MHCC97H cells were transferred into 6-well plate ( $1 \times 10^6$  cells for each well) for 8 hours. MTT working solution (1 mg/ml for each well) was added into transfected cells. Continue cell culture for 5 hours. DMSO was added to terminate reaction.

Microplate reader was used to examine absorbance value at 420 nm. Growth curve was established with absorbance values.

### Flow cytometry

Flow cytometry was performed to examine apoptosis with Annexin-V-FITC/PI double-staining method as previously described [14]. Briefly, cells after transfection were collected at

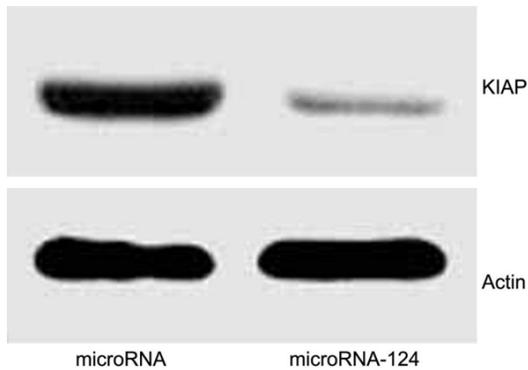
48 h and incubated with FITC-labeled Annexin V at 4°C for 10 min. After that, 5  $\mu$ g/ml PI was added into the cells and incubated for 2 min. At the end time point, cells were re-suspended in buffer and analyzed on flow cytometry.

### Western blot

Proteins were extracted from transfected cells for Western blot with routine protocol [15]. Detailed processes were as follows:

Protein suspension (15  $\mu$ g protein) was prepared for electrophoresis. Block proteins with 5% BSA for 1.5 hours after transferring into a PVDF membrane. After that, the membrane was incubated with primary antibody (1:800 dilutions) at 4°C for 12 hours followed by addition of secondary antibody (1:1500 dilutions) at 37°C for 2 hours. At the end point, the membrane was developed using ECL substance. Gel imaging system was used for analysis of specific protein bands.

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**Figure 4.** microRNA-124 transfection decreased protein expression of KIAP in MHCC97H cells. Protein was isolated from cells after transfection of microRNA-124 for analysis of the KIAP protein expression by western blot. \* $P=0.018$ , versus miRNA (control).

### Examination of caspase-3 activity

Caspase-3 activity was examined with caspase-3 kit [17]. Detailed processes were as follows:

Transfected MHCC97H cells were treated with chromophoric substrate. Transfected MHCC97H cells were transferred into 6-well plate. Absorbance value was assessed with a microplate reader. Relative activity of caspase-3 was analyzed with routine protocol.

### Intervention for expression of KIAP

KIAP siRNA and KIAP plasmid were transfected into normal MHCC97H cells with lipo2000, respectively. microRNA-124 or miRNA was further transfected for MHCC97H cells with KIAP transfection.

### Statistical analysis

SPSS19.0 software was used for data processing. Measurement data are normal distribution to  $X \pm S$ . One-way ANOVA with Newman-Keuls multiple comparison post-hoc analysis was performed for statistical significance.  $P$  value  $< 0.05$  was considered to be statistically significant.

## Results

### microRNA-124 transfection decreased cell viability and reduced cell growth

Compared with miRNA transfection (0.5  $\mu\text{g}$ ), microRNA-124 transfection (0.5  $\mu\text{g}$ ) significantly

decreased cell viability of MHCC97H cells, verified by MTT assays ( $P=0.0047$ ) (Figure 1A) as well by microscopy analysis of cell morphology (Figure 1B). No difference was observed between non-treated MHCC97H cells and miRNA transfection MHCC97H cells (Figure 1A,  $> 0.05$ ). Thus, miRNA transfection was used as control in our study.

### microRNA-124 transfection induced apoptosis of MHCC97H cells

Compared with miRNA transfection, microRNA-124 transfection significantly increased expression of phosphatidylserine, molecular marker of cell apoptosis, verified by Annexin-V-FITC double staining (Figure 2,  $P=0.024$ ). Enhanced translocation of phosphatidylserine suggested apoptosis induced by microRNA-124.

### microRNA-124 transfection activated caspase-3 in MHCC97H cells

Compared with miRNA transfection, microRNA-124 transfection significantly increased caspase-3 activity in MHCC97H cells (Figure 3,  $P=0.025$ ), suggesting microRNA-124 transfection promote apoptosis via activating caspase-3.

### microRNA-124 transfection decreased protein expression of KIAP in MHCC97H cells

Compared with miRNA transfection, microRNA-124 transfection significantly decreased protein expression of KIAP in MHCC97H cells (Figure 4,  $P=0.018$ ), verified by Western blot.

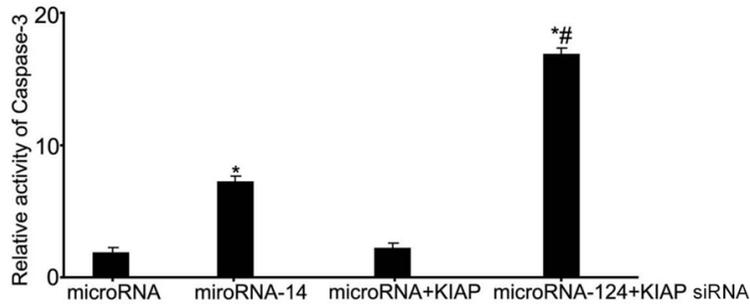
### Inhibition of KIAP enhanced pro-apoptosis effect of microRNA-124

As showed in Figure 5, KIAP siRNA indeed inhibited expression of KIAP, and KIAP siRNA further enhanced decrease of KIAP induced by microRNA-124. Moreover, KIAP siRNA+microRNA-124 group had the highest caspase-3 activity (Figure 5), suggesting inhibition of KIAP enhanced pro-apoptosis effect of microRNA-124.

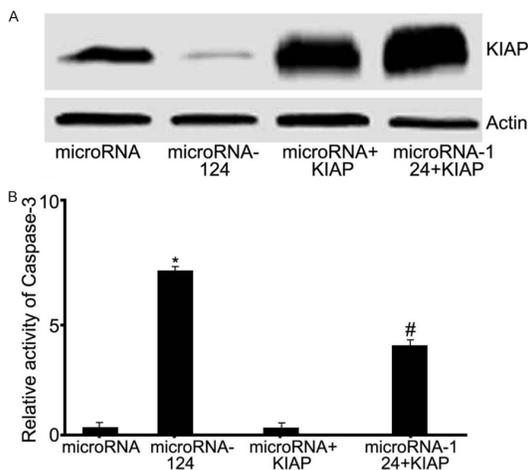
### Activation of KIAP abrogated pro-apoptosis effect of microRNA-124

As showed in Figure 6A, KIAP plasmid indeed enhanced expression of KIAP, and KIAP plas-

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**Figure 5.** Analysis of caspase-3 activity in four groups. Transfected cells was collected for measurement of caspase-3 activity by a commercial kit according to manufacturer's instructions. \* $P < 0.05$  versus miRNA (control). # $P=0.017$ , KIAP siRNA+microRNA-124 versus microRNA-124.



**Figure 6.** A. Western blot results for four groups. B. Analysis of caspase-3 activity in four groups. \* $P < 0.05$  versus miRNA (control). # $P=0.023$ , KIAP+microRNA-124 versus microRNA-124.

mid attenuated decrease of KIAP induced by microRNA-124. Moreover, KIAP plasmid+microRNA-124 group had lower caspase-3 activity than microRNA-124 group (Figure 5), suggesting activation of KIAP abrogated pro-apoptosis effect of microRNA-124.

### Discussion

Our study used hepatocarcinoma cell line MHCC97H to establish cell model, and explored potential mechanisms underlying regulatory effect of microRNA-124 on MHCC97H cells. Our findings indicated that microRNA-124 inhibited the growth and promoted apoptosis of MHCC97H cells, consistent with previous report [3].

Previous studies provided little data on the mechanisms underlying microRNA influenced

hepatocarcinoma [3]. Recent studies showed that microRNA-218 inhibited growth of hepatocarcinoma cells, and microRNA-34a was associated with tumor metastasis [13, 14], suggesting microRNA was involved in progression of hepatocarcinoma.

KIAP was an anti-apoptosis protein [23], while it remains unclear whether KIAP could be regulated by microRNA-124. In addition, specific mechanisms were warranted for better understanding of KIAP [24, 25]. Our study showed that microRNA-124 indeed decreased KIAP. Moreover, MHCC97H apoptosis was enhanced after inhibition of KIAP, which was induced by microRNA-124. Furthermore, overexpression of KIAP abrogated such pro-apoptosis effect of microRNA-124.

Three findings were elucidated in our study, which proved KIAP played a pivotal role in pro-apoptosis effect of microRNA-124 on MHCC97H cells. 1) Protein expression of KIAP was significantly reduced in MHCC97H cells treated with miRNA-124. 2) KIAP siRNA enhanced pro-apoptosis effect of microRNA-124. 3) KIAP plasmid abrogated pro-apoptosis effect of microRNA-124. All these findings suggested that microRNA-124 enhanced apoptosis via down-regulating KIAP. Previous studies showed KIAP was involved in other cancers, while KIAP always inhibited apoptosis of cancer cells and exacerbated tumor lesion [26-28]. However, no report was about relationship between KIAP and hepatocarcinoma.

There are three limitations in our study. 1) We did not collected tumor tissue and para-carcinoma tissue from hepatocarcinoma patients. 2) Our study did not explore long-term prognosis after KIAP intervention. 3) Animal models are needed to explore effect of microRNA-124 on hepatocarcinoma *in vivo*.

### Conclusion

microRNA-124 induced apoptosis of hepatocarcinoma cells via down-regulating KIAP, suggesting KIAP might be a potential target for molecular targeting treatment, and inhibition of KIAP could improve efficacy of clinical treatment.

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## Disclosure of conflict of interest

None.

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