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MicroRNA-23a induces apoptosis of hepatocarcinoma cell line MHCC97H via down-regulating KIAP: a mechanism study

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Abstract. – OBJECTIVE: Hepatocarcinoma is a great threat to global health. MicroRNA-23a was suggested to regulate growth and apoptosis in certain cell lines. Our study was focused on growth, proliferation, and apoptosis of hepatocarcinoma cell line MHCC97H under the influence of microRNA-23a, and explored the mechanism of pro-apoptosis microRNA-23a.

MATERIALS AND METHODS: MicroRNA-23a and control microRNA (scramble miRNA, for short as miRNA) were synthesized with the routine protocol. Lipofection transfection was performed in hepatocarcinoma cell line MHCC97H. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay, caspase-3 activity detection, and flow cytometry were performed to examine growth, proliferation, and apoptosis of hepatocarcinoma cell line MHCC97H, respectively. Kidney inhibitor of apoptosis protein (KIAP) and small interfere RNA (siRNA) was synthesized for inhibition of KIAP. KIAP plasmid was established for activation of KIAP. Western blot was performed to examine the protein expression of KIAP and caspase protein family after transfection of KIAP siRNA or KIAP plasmid.

RESULTS: Compared with miRNA transfection, microRNA-23a transfection significantly reduced the growth of MHCC97H cells, and decreased the expression of KIAP ($p < 0.05$). Enhanced translocation of phosphatidylserine and activation of caspase-3 were observed in microRNA-23a transfection cells. Moreover, inhibition of KIAP enhanced the pro-apoptosis effect of microRNA-23a, while activation of KIAP abrogated pro-apoptosis effect of microRNA-23a.

CONCLUSIONS: MicroRNA-23a inhibits growth and proliferation of MHCC97H cells, and induces apoptosis of MHCC97H cells via down-regulating KIAP. KIAP could be a potential therapeutic target for hepatocarcinoma treatment.

Key Words:
MicroRNA-23a, KIAP, MHCC97H, Apoptosis.

Introduction

Hepatocarcinoma is one of the digestive system diseases with high mortality[1]. Molecular mechanisms of hepatocarcinogenesis are unclear and need further exploration[2]. Combined treatment is the common strategy for hepatocarcinoma treatment with promising efficacy, including operative treatment, radioactive therapy, and chemotherapy[3,4]. However, multiple disadvantages have limited development of combined treatment, such hemorrhage and toxic effect[5-7]. Therefore, how to improve the efficacy of hepatocarcinoma treatment is the emphasis and difficulty in a clinical scenario.

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As a research hotspot, molecular targeting treatment was less applied in hepatocarcinoma treatment due to scarce molecular targets[8-10]. B-cell lymphoma 2 (Bcl-2) and apoptosis-inducing proteins (AIPs) are the most reported targets for hepatocarcinoma treatment, but their efficacy was not ideal[11]. Moreover, no microRNA was proven 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, the expression of microRNA-23a 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 the regulatory effect of microRNA-23a 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, kidney inhibitor of apoptosis protein (KIAP) have been used as the target of drugs, but the efficacy of such drugs is not promising on decreasing KIAP[20-22]. Our study aimed to establish as effective was to decrease KIAP.

We focused our investigation on growth, proliferation, and apoptosis of hepatocarcinoma cell line MHCC97H under the influence of microRNA-23a, and explored the mechanism of pro-apoptosis microRNA-23a via KIAP.

Materials and Methods

Reagents and Cell Model

Fetal bovine serum (FBS) and Dulbecco's Modified Eagle Medium (DMEM) were purchased from Hualan Biological Engineering, Inc. (Beijing, China). Synthesized sequences of RNA were as follows: microRNA-23a, 5'-CATCACTGCAATCGTCAGGTAT-3' and 5'-AAGACAAAGGTAGGTAAA GCA-3'. miRNA (Scrambled miRNA), 5'-CCTGAGGGTTCAACTCTAGC-3' and 5'-TTACGATTGTCACGTACAT-3', siRNA KIAP, 5'-CTAGCGTGCTAC CATGATGC-3' and 5'-TTCTACGATATCACT AGGT-3'. siRNA NC (Scrambled siRNA) 5'-TTCTCCGAACGTGTCACGTTT-3' and 5'-ACGTGACA CGTTCGGAGAATT-3'.

KIAP plasmid was purchased from Suzhou GenePharma (Suzhou, China). Liposome transfection kit was purchased from Invitrogen/Life Technologies (Carlsbad, CA, USA). 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay kit was purchased from Beijing Dingguo Biotechnology (Beijing, China). FITC-Annexin and caspase-3 kits were purchased from Beyotime Biotechnology (Shanghai, China). KIAP antibody and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Hepatocarcinoma cell line MHCC97H was purchased from American Type Culture Collection (ATCC Cell Bank, Manassas, VA, USA). Grouping was as follows: miRNA group, microRNA-23a group, microRNA-23a+KIAP siRNA group, microRNA-23a+KIAP plasmid group.

The present study was approved by the Ethical Committee of China-Japan Union Hospital of Jilin University, Changchun, China.

Cell Culture

Hepatocarcinoma cell line MHCC97H was revived and cultured in DMEM high sugar medium with routine protocol[9].

Transfection

MicroRNA-23a, scrambled siRNA (negative control), KIAP-specific siRNA, KIAP over-expression vector, and negative control vector were transfected into hepatocarcinoma cell line MHCC97H with routine protocol[9], respectively. Liposome lipo2000 was used for transfection.

MTT Assay

MTT assay was performed with routine protocol[10]. Detailed processes were as follows: MHCC97H cells were transferred into 6-well plate (1×10^6 cells for each well) for 8 h. MTT working solution (1 mg/ml for each well) was added into transfected cells. Continue cell culture for 5 h. Dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) was added to terminate the reaction. Microplate reader was used to examine the absorbance value at 420 nm. The growth curve was established with absorbance values.

Flow Cytometry

Flow cytometry was performed to examine apoptosis with Annexin-V-FITC double-staining method[14].

Western Blot

Proteins were extracted from transfected cells for Western blot with routine protocol[15]. Detailed processes were as follows: protein suspension (15 μg protein) was prepared for electrophoresis. Block proteins for 1.5 h after transferring. Incubate with primary antibody (1:800) at 4°C for 12 h. React with second antibody (1:1500) at 37°C for 2 h. Gel imaging system was used for the analysis of specific protein bands.

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 the 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 the routine protocol.

Intervention for Expression of KIAP

KIAP siRNA and KIAP plasmid were transfected into normal MHCC97H cells with lipo2000, respectively. MicroRNA-23a or miRNA was further transfected for MHCC97H cells with KIAP transfection.

Statistical Analysis

All data analyses were performed on SPSS 14.0 software (SPSS, Inc., Chicago, IL, USA). The measurement data were presented as mean ± standard deviation ($\bar{x} \pm$ SD). The Student's *t*-test was used to compare the differences between the two groups. The Tukey's post hoc test was used to validate the ANOVA for comparing measurement data between groups. $p < 0.05$ was considered statistically significant.

Results

MicroRNA-23a Transfection Decreased Cell Viability and Reduced Cell Growth

MicroRNA-23a transfection efficacy was almost 100% as demonstrated by RT-PCR (Figure 1). Compared with miRNA transfection (0.5 μg), microRNA-23a transfection (0.5 μg) significantly decreased the cell viability of MHCC97H cells, verified by MTT assays ($p = 0.0047$). No difference was observed between normal MHCC97H cells and miRNA transfection MHCC97H cells (Figure 2, $p > 0.05$). Therefore, miRNA transfection was used as control in our study.

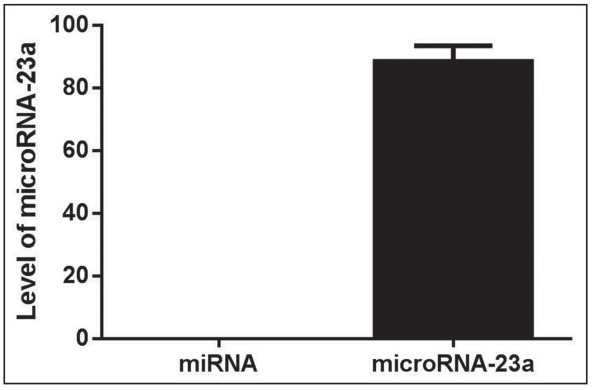

Figure 1. RT-PCR analysis of microRNA-23a expression after transfection.

MicroRNA-23a Transfection Induced Apoptosis of MHCC97H Cells

Compared with miRNA transfection, microRNA-23a transfection significantly increased expression of phosphatidylserine, verified by Annexin-V-FITC double staining (Figure 3, $p = 0.024$). Enhanced translocation of phosphatidylserine suggested apoptosis induced by microRNA-23a.

MicroRNA-23a Transfection Activated Caspase-3 in MHCC97H Cells

Compared with miRNA transfection, microRNA-23a transfection significantly increased caspase-3 activity in MHCC97H cells (Figure 4, $p = 0.025$), suggesting microRNA-23a transfection promote apoptosis via activating caspase-3.

MicroRNA-23a Transfection Decreased Protein Expression of KIAP in MHCC97H Cells

Compared with miRNA transfection, microRNA-23a transfection significantly decreased protein expression of KIAP in MHCC97H cells (Figure 5, $p = 0.018$), verified by Western blot.

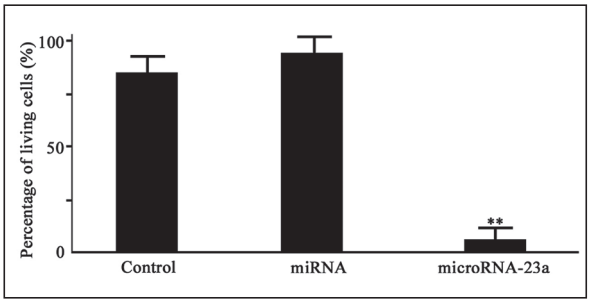

Figure 2. Analysis of cell viability in three groups. $^{**}p = 0.0047$, *versus* miRNA (control).

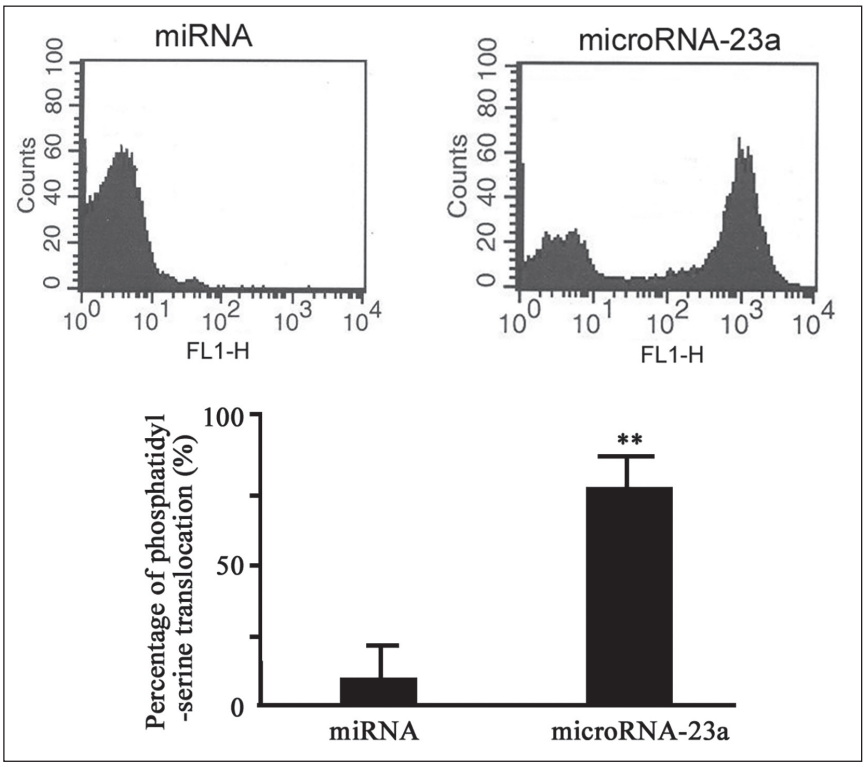

Figure 3. microRNA-23a transfection enhanced translocation of phosphatidylserine. $^{**}p = 0.024$, *versus* miRNA (control).

Inhibition of KIAP Enhanced Pro-Apoptosis Effect of MicroRNA-23a

To evaluate the transfection efficacy of KIAP transfection, we measured KIAP mRNA expression by Real Time-PCR (RT-PCR) and showed that KIAP expression was reduced by the transfection of either siKIAP or microRNA-23a (Figure 6A). Of note, the inhibitory effect on the KIAP mRNA level was more evident after the co-transfection of siKIAP and microRNA-23a. Interestingly, as showed in Figure 6B, though no induction of caspase-3 activity was observed by the transfection of KIAP siRNA, the down-regulation of KIAP expression combined with microRNA-23a significantly enhanced the promoting function of microRNA-23a on the increase

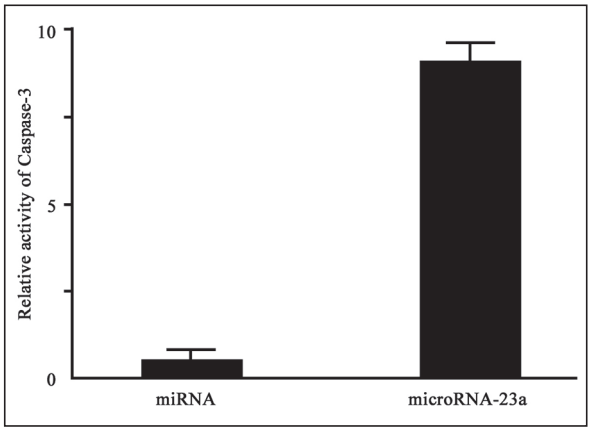

Figure 4. microRNA-23a transfection increased caspase-3 activity in MHCC97H cells. $^{**}p = 0.025$, *versus* miRNA (control).

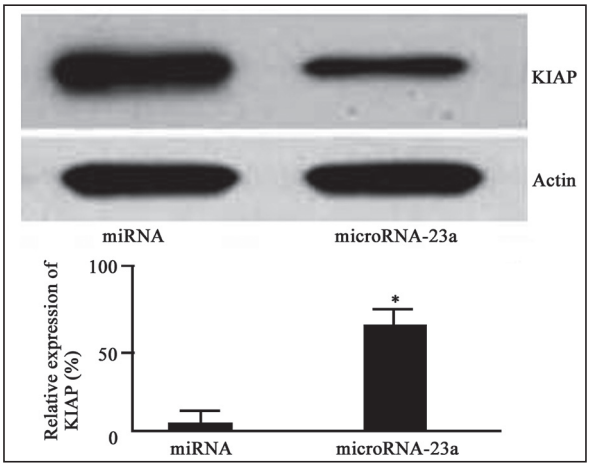

Figure 5. microRNA-23a transfection decreased protein expression of KIAP in MHCC97H cells. $p = 0.018$, *versus* miRNA (control).

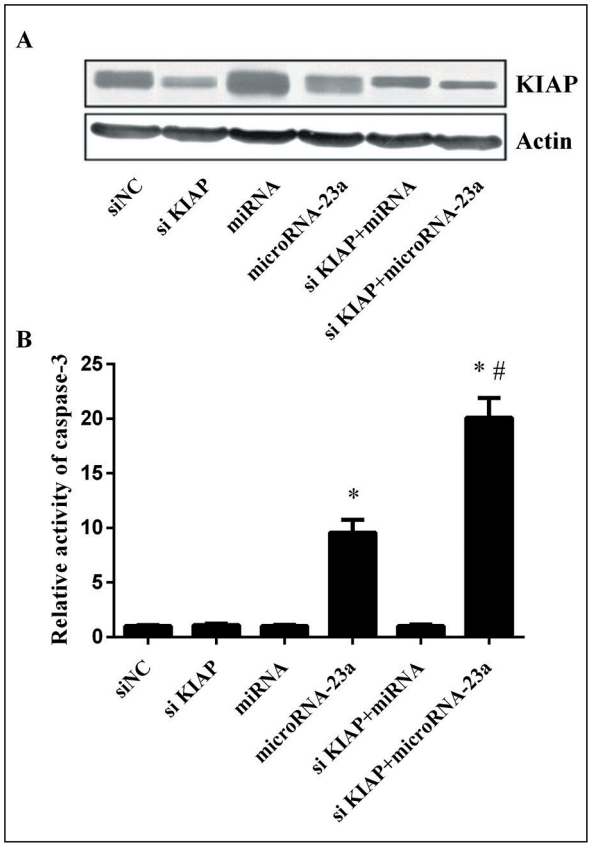

Figure 6. KIAP mRNA expression after siRNA ***(A)*** or overexpression vector transfection ***(B)***. ***(C)*** Western blot results for four groups. ***(D)*** Analysis of caspase-3 activity in four groups. *$p < 0.05$ *versus* miRNA (control). $^{\#}p = 0.017$, KIAP siRNA+microRNA-23a *versus* microRNA-23a.

of caspase-3 activity, compared with siNC or microRNA control group ($p < 0.05$), suggesting inhibition of KIAP enhanced pro-apoptosis effect of microRNA-23a.

Activation of KIAP Abrogated Pro-Apoptosis Effect of MicroRNA-23a

We also determined the effect of KIAP on the apoptosis by overexpression of KIAP protein. As showed in Figure 7A, the plasmid vectored KIAP up-regulated the expression of KIAP, compared to blank vector control and remarkably attenuated the suppression of microRNA-23a on KIAP level in comparison with the expression of KIAP in microRNA-23a group. Intriguingly, the overexpression of KIAP statistically neutralized the inducing effect of microRNA-23a on caspase-3 activity ($p < 0.05$) (Figure 7B), indicating that KIAP abrogated pro-apoptosis function of microRNA-23a.

Discussion

Our study used hepatocarcinoma cell line MHCC97H to establish cell model, and explored potential mechanisms underlying the regulatory effect of microRNA-23a on MHCC97H cells. Our findings demonstrated that microRNA-23a inhibited the growth of MHCC97H cells and induced apoptosis, consistent with previous report[3].

Previous studies offered scarce data on how microRNA influenced hepatocarcinoma[3]. Some studies[13,14] showed that microRNA-218 inhibited growth of hepatocarcinoma cells, and microRNA-34a was associated with tumor metastasis, suggesting microRNA was involved in the progression of hepatocarcinoma.

KIAP was an anti-apoptosis protein[23], while it remained unclear whether KIAP could be regulated by microRNA-23a. In addition, specific mechanisms were warranted for better understanding of KIAP[24-26]. Our research showed that microRNA-23a indeed decreased KIAP. Moreover, MHCC97H apoptosis was enhanced after inhibition of KIAP, which was induced by

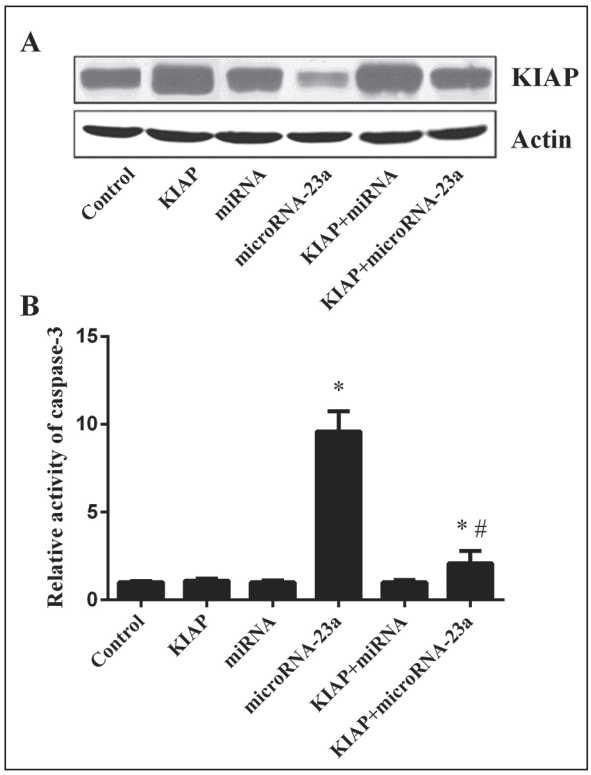

Figure 7. ***(A)*** Western blot results for four groups. ***(B)*** Analysis of caspase-3 activity in four groups. *$p < 0.05$ *versus* miRNA (control). $^{\#}p = 0.023$, KIAP siRNA+microRNA-23a *versus* microRNA-23a.

microRNA-23a. Furthermore, overexpression of KIAP abrogated such pro-apoptosis effect of microRNA-23a.

Three findings were elucidated in our study, which proved KIAP played a pivotal role in the pro-apoptosis effect of microRNA-23a on MHCC97H cells. (1) Protein expression of KIAP was significantly reduced in MHCC97H cells. (2) KIAP siRNA enhanced the pro-apoptosis effect of microRNA-23a. (3) KIAP plasmid abrogated the pro-apoptosis effect of microRNA-23a. All these findings suggested that microRNA-23a enhanced the apoptosis via down-regulating KIAP. Previous studies[26-28] showed that KIAP was involved in other cancers, while KIAP always inhibited apoptosis of cancer cells and exacerbated tumor lesion. However, no report was about the relationship between KIAP and hepatocarcinoma. Our study firstly demonstrated KIAP also inhibited apoptosis of hepatocarcinoma, suggesting that it was a potential target for molecular targeting treatment.

There are three limitations in our study. (1) We did not collect 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 the effect of microRNA-23a on hepatocarcinoma *in vivo*.

Conclusions

We found that microRNA-23a induced apoptosis of hepatocarcinoma cells via down-regulating KIAP. KIAP was a potential target for molecular targeting treatment, and inhibition of KIAP could improve the efficacy of clinical treatment.

Disclosure of Conflict of Interest

The authors declare no competing financial or commercial interests in this manuscript.

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