Abstract. – OBJECTIVE: microRNA can regulate cell growth, proliferation, and death; also, it can promote or inhibit cell growth and proliferation through regulation of apoptotic proteins. XIAP is a newly discovered pro-apoptotic protein, but how is XIAP regulated remains unclear. This study investigated the role of microRNA-15b in liver cancer cells’ proliferation and apoptosis.

MATERIALS AND METHODS: microRNA15b and control microRNA (miRNA) were purchased and transfected with hepatoma SMCC7721 cells using liposomal transfection techniques. MTT assay, caspase-3 activity assay, and flow cytometry were used to investigate the effects of microRNA-15b on growth, proliferation, and apoptosis of SMCC7721 cells. SiRNA of XIAP and control siRNA were purchased and transfected into SMCC7721 cells. microRNA15b and control microRNA were then transfected into siRNA of XIAP or control siRNA transfected SMCC7721 cells. Western blot analysis was used to detect the expression levels of XIAP and apoptotic proteins. XIAP plasmid and control vector were purchased and transfected into SMCC7721 cells followed by transfection of microRNA15b and control microRNA. Expression levels of XIAP and apoptosis of SMCC7721 cells were analyzed.

RESULTS: Transfection of microRNA15b reduced the growth of SMCC7721 cells, increased phosphatidylserine eversion, activated caspase-3, and decreased the expression levels of XIAP. Silencing of XIAP enhanced microRNA15b-induced apoptosis of SMCC7721 cells. Overexpression of XIAP inhibited microRNA15b-induced apoptosis of SMCC7721 cells. microRNA15b inhibited the growth of SMCC7721 cells.

CONCLUSIONS: microRNA15b induced apoptosis of SMCC7721 cells via down-regulation of XIAP.

Key Words: microRNA15b, XIAP, SMCC7721 cells, Apoptosis.

Introduction

Liver cancer is a cancer of the digestive system with the incidence rate of 0.01%1. Various reasons can cause liver cancer. A recent study suggests that virus infection is a major determining factor2. It also has been shown that gene mutations, drinking, smoking, and staying up late also contribute to the occurrence and development of liver cancer3,4. Therefore, the study of the pathogenesis of liver cancer is of great significance.

With the advancement of science and technology, the detection, prevention, treatment, and prognosis of liver cancer have been greatly improved. Various treatments have been sued in the treatment of liver cancer, including chemotherapy and surgery. However, there are shortcomings and deficiencies of these treatments such as complications, bleeding, and other side effects5-7. How to improve the accuracy and success rate of liver cancer therapy is an important and difficult point for both medical and scientific community.

Currently, targeted molecular therapy has become a hot topic in the treatment of liver cancer8-10. However, only a few targets can be used for the treatment of liver cancer8-10. Targeting anti-apoptotic proteins such as Bcl-2 and AIPs showed less effective in the treatment of liver cancer7,8. microRNA has not been used as a target for liver cancer therapy6. Therefore, there is an urgent need for effective molecular targets for the treatment of liver cancer9.

microRNAs (miRNAs) are a class of small RNAs with 20-24 nucleotides. microRNAs play an important regulatory role in the cells. Studies suggested that microRNA plays a very important role in the diagnosis and treatment of cancer. For example, microRNA-143 can inhibit the growth of esophageal squamous cell carcinoma, and microRNA-34a is associated with tumor metastasis. microRNA-218 can inhibit the growth of liver cancer cells, while microRNA-34a is associated with tumor metastasis3,14, suggesting that microRNA may also be involved in the occurrence and development of liver cancer13-15.
Based on this hypothesis, we designed microRNA specific primers to detect the expression levels of microRNA using RT-PCR in both tumor tissues and adjacent tissues and we found that levels of microRNA-421 (microRNA15b) in tumor tissues were significantly higher than that of the corresponding adjacent tissues, indicating that microRNA15b is linked with liver cancer. This study is designed to investigate the role of microRNA-15b in hepatoma SMCC7721 cells.

One of the anti-tumor strategies is to kill tumor cells without affecting normal cells. Apoptosis is regulated by anti-apoptotic and pro-apoptotic proteins. Ideal anticancer drugs should decrease the levels of anti-apoptotic protein but increase the expression of pro-apoptotic proteins. XIAP is a widely studied anti-apoptotic molecule. There are lots of drugs targeting but the effect of reducing XIAP protein is not ideal. This study will investigate the possibility of using XIAP protein as a potential target.

Therefore, using SMCC7721 cell as a working model, this study was designed to investigate the regulation of microRNA15b on hepatoma SMCC7721 cells and its possible mechanisms, which will provide a theoretical basis for targeted molecular therapy of liver cancer.

Materials and Methods

Reagents and Cell Models

Fetal bovine serum (FBS) and cell culture medium were purchased from Beijing Hualan Biotech (Beijing, China). Methylsulfonyltetrazolium bromide (MTT) assay reagents were purchased from Beijing Dingguo Biotech (Beijing, China). Liposome transfection kit was purchased from Invitrogen (Carlsbad, CA, USA). Apoptosis Detection Reagents FITC-annexin, caspase-3 assay kit, and Western blot reagents were purchased from Beyotime (Shanghai, China).

Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

microRNA-15b (5'-TCAGGTCATCATATCGGCAAT-3' and 5'-AAAGAAAGGGTGTAAAACGCA-3'), control microRNA (5'-CCCATCTATGGGTTACGC-3' and 5'-TTTAATGTCACGCACGATT-3'), siRNA XIAP (5'-CTATGAGGC-TACCATCGTG-3' and 5'-TTTACACTACGATTAGCGT-3'), XIAP plasmid, and control vector were purchased from Suzhou Jima Biotech (Suzhou, China). SMCC7721 cells used in this study were purchased from ATCC (Manassas, VA, USA).

Cell Culture

SMCC7721 cells were cultured in Dulbecco’s Modified Eagle’s medium (DMEM) according to a previous method.

Transfection

microRNA15b and control microRNA were transfected into SMCC7721 cells according to the manufacturer’s instruction. Transfection was performed when cells reached 68% confluence. 1 μl (1 μg/μl) of microRNA15b or control miRNA was mixed with lipo2000 and transfected into cells.

MTT Assay of Cell Viability

MTT test was performed according to conventional methods. MTT solution (2 mg/ml) was added to each well and incubated for 4 h. dimethyl sulfoxide (DMSO) (100 μl) was added to each well to stop reaction. Optical density at 560 nm was recorded using a microplate reader to draw a growth curve of SMCC7721 cells.

Flow Cytometry Measurement of Apoptosis

Flow cytometry was performed to detect the apoptosis of SMCC7721 cells according to conventional methods. Briefly, SMCC7721 cells were transfected with either microRNA15b or control miRNA and cultured for 48 h. Cells were then collected and resuspended. 100 μl of reaction buffer and 2 μl of Annexin-V-FITC (fluorescein isothiocyanate) were added to 500 μl of cell suspension, mixed, and incubated for 17 min in the dark for flow cytometry analysis with 465 nm and 650 nm.

Western Blot

SMCC7721 cells were collected and proteins were extracted and quantified by bicinchonic acid assay (BCA) kit according to manufacturer’s instruction. Equal amounts of proteins (15 μg) were electrophoresed, transferred to membranes, and blocked. After washing with TBST, membranes were incubated with the first antibody (1:1000) over night at 4°C. After washing with Tris Buffered Saline with Tween 20 (TBST), membranes were incubated with secondary antibodies (1:2500) for 2 h at 37°C, and developed with ECL. Expression levels of XIAP were analyzed.

Caspase-3 Activity Assay

Caspase-3 activity was detected according to kit instructions. Briefly, SMCC7721 cells were transfected with either microRNA15b or control miRNA and cultured for 48 h. Cells were then...
collected, lysed, mixed with chromogenic substrate, and incubated at room temperature. The absorbance of each sample was recorded by a microplate reader. Caspase-3 relative activity was calculated as follows: absorption value of cells transfected with microRNA15b - absorption value of cells transfected with control miRNA.

**Effect of Overexpression of XIAP on microRNA15b-transfected SMCC7721 Cells**

SMCC7721 cells were transfected with either XIAP plasmids or siRNA of XIAP. microRNA15b or control miRNA was further transfected into SMCC7721 cells transfected with either XIAP plasmids or siRNA of XIAP using lipofectamine 2000. 2 μl (1.6 μg/μl) of siRNA of XIAP or XIAP plasmid was suspended in lipofectamine 2000. 2 μl (0.6 μg/μl) of control microRNA or microRNA15b was suspended with lipofectamine. DNA and liposome mixtures were than drop-wisely added to cell culture medium.

**Statistical Analysis**

SPSS 11.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. All data are expressed as mean ± standard error. Differences between groups were analyzed by t-test. *p* < 0.05 was considered statistically significant.

**Results**

**Transfection of microRNA15b Reduced Viability of SMCC7721 Cells and Inhibited their Growth**

MTT assay results showed that transfection of microRNA15b (0.5 μg) significantly reduced the viability of SMCC7721 cells, compared with that of SMCC7721 cells transfected with control miRNA (*p* = 0.011) (Figure 1). No significant difference was found in SMCC7721 cells transfected with control miRNA and non-transfected SMCC7721 cells, so SMCC7721 cells transfected with control miRNA were used as controls in this study.

MTT assay was used to analyze the viability of SMCC7721 cells transfected with control miRNA or microRNA15b or non-transfected SMCC7721 cells, ***p* < 0.01, compared with miRNA group.

**Transfection of microRNA15b Induced Apoptosis of SMCC7721 Cells**

Annexin-V-FITC was used to measure the apoptosis of SMCC7721 cells. Flow cytometry analysis showed (Figure 2) that transfection of microRNA15b significantly increased the expression of phosphatidyserine in SMCC7721 cells, compared with that of SMCC7721 cells transfected control miRNA (*p* = 0.0086).

Flow cytometry was used to analyze the apoptosis of SMCC7721 cells transfected with control miRNA or microRNA15b or non-transfected SMCC7721 cells, ***p* < 0.01, compared with miRNA group.

**Transfection of microRNA15b activated caspase-3 in SMCC7721 cells**

Caspase-3 activity assay results showed (Figure 3) that transfection of microRNA15b (0.5 μg) significantly activated caspase-3 in SMCC7721 cells, compared with that of SMCC7721 cells transfected control miRNA (*p* = 0.0062).

Caspase-3 activity assay was used to analyze caspase-3 activity of SMCC7721 cells transfected with control miRNA or microRNA15b or non-transfected SMCC7721 cells, *p* < 0.05, compared with miRNA group.

**Transfection microRNA15b Decreased XIAP Protein Expression in SMCC7721 Cells**

Western blot results showed that transfection of microRNA15b significantly reduced XIAP expression at protein levels in SMCC7721 cells, compared with that of SMCC7721 cells transfected control miRNA (*p* = 0.011) (Figure 4).

**Silence of XIAP Enhanced microRNA15b-induced Apoptosis of SMCC7721 cells**

XIAP was silenced to investigate its role in microRNA15B-induced apoptosis of SMCC7721 cells.
microRNA15b induced SMCC7721 apoptosis via down-regulation of XIAP

cells. Western blot results (Figure 5) showed that expression of XIAP was significantly decreased by siRNA, the expression level of XIAP in SMCC7721-microRNA15b-si-XIAP group was significantly lower than that of SMCC7721-microRNA15b-siControl group ($p = 0.014$). Flow cytometry results showed that decreased XIAP expression by siRNA significantly increased microRNA15b-induced apoptosis of SMCC7721 cells, compared to that of SMCC7721 cells transfected with control siRNA and microRNA15b ($p = 0.013$) (Figure 5).

**Figure 2.** Transfection of microRNA15b induced apoptosis of SMCC7721 cells.

**Figure 3.** Transfection of microRNA15b induced apoptosis of SMCC7721 cells.

**Figure 4.** Transfection of microRNA15b reduced XIAP expression in SMCC7721 cells.
Flow cytometry was used to analyze apoptosis of SMCC7721 cells transfected with control miRNA, microRNA15b, microRNA15b + siXIAP, or control miRNA + siXIAP. * \( p < 0.05 \), compared with control miRNA group, # \( p < 0.05 \), compared with control miRNA + siXIAP group.

**Overexpression of XIAP Inhibited microRNA15b-Induced Apoptosis of SMCC7721 Cells**

To examine the effect of XIAP on microRNA15b-induced apoptosis of SMCC7721 cells, XIAP was over-expressed in SMCC7721 cells. As shown in Figure 6, Western blot showed that XIAP was significantly over-expressed in microRNA15b + XIAP group, compared to that of microRNA15b group (\( p = 0.027 \)). Caspase-3 activity assay results showed that overexpression of XIAP significantly inhibited microRNA15b-induced apoptosis of SMCC7721 cells (\( p = 0.022 \)) (Figure 6). Caspase-3 assay was used to analyze apoptosis of SMCC7721 cells transfected with control miRNA, microRNA15b, or microRNA15b + XIAP group. (A) Western blot assay of XIAP expression. (B) Caspase-3 activity. * \( p < 0.05 \), compared with control miRNA group, # \( p < 0.05 \), compared with microRNA15b + XIAP group.

**Discussion**

In this work, using SMCC7721 cells as cell models, we investigated the roles of microRNA15b in the regulation of MCC7721 cells at the molecular and protein levels.
might be used as a new target for the treatment of liver cancer\textsuperscript{22}. It has been shown that XIAP inhibited apoptosis of other types of cancer\textsuperscript{23,24,25,26,27,28}. However, this is the first time to report that microRNA15b induces apoptosis of SMCC7721 cells via XIAP.

Conclusions

MicroRNA15b induced apoptosis of SMCC7721 cells by downregulating XIAP.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References


