• Basic Research •

Effect on proliferation and apoptosis of retinoblastoma cell by RNA inhibiting high mobility group protein box-1 expression

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Abstract

• AIM: To investigate the effect of high mobility group protein box-1 (HMGB1) siRNA on proliferation and apoptosis of retinoblastoma (Rb) cells.

• METHODS: The expression of HMGB1 in Rb cells were detected by real-time polymerase chain reaction (RT-PCR) and Western blot. Chemically synthesized HMGB1 siRNA was transfected into Y79 cells. The inhibitory rate was also examined by RT-PCR and Western blot. After HMGB1 siRNA transfection, the cell proliferation was analyzed by MTT, and cell apoptosis was detected by Caspase-3 active detection kit. Cell cycle distribution and apoptosis were detected by flow cytometry.

• RESULTS: The expression of HMGB1 significantly elevated in Rb cells (P<0.01). After transfected by siRNA, the HMGB1 protein level of Y79 cells was significantly reduced (P<0.01). After siRNA interference HMGB1, the proportion of proliferating cells reduced, and the proportion of quiescent cells increased (P<0.05). In addition, apoptosis rate of Y79 cells increased from 2.03% to 9.10% after interfering with HMGB1 siRNA (P<0.05).

• CONCLUSION: Specific HMGB1 siRNA can inhibit the expression of HMGB1. The effect may be attributed to inhibit the proliferation and promote cell apoptosis.

• KEYWORDS: retinoblastoma; high mobility group protein box-1; proliferation; apoptosis

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INTRODUCTION

Retinoblastoma (Rb) is a most common intraocular malignant tumor of infant and young children[1]. It ranks first in the incidence of malignant tumors of the eye, and there is a gradual upward trend in recent years in China[2]. The existing treatments for Rb include chemotherapy, laser photocoagulation, frozen, radiotherapy and eyeball enucleation *etc*. The preferred treatment option is chemotherapy combined with local consolidation therapy, such as photocoagulation and frozen. However, this scheme has poor efficacy for patients with advanced Rb, while chemotherapy accompanied by severe side effects, and faced with the problem of resistance to chemotherapy[3]. In recent years, the development of tumor molecular biology and genetic engineering techniques makes gene therapy become a research hotspot of cancer therapy[4]. High mobility group protein box-1 (HMGB1) is a kind of non-histone chromatin proteins abundant in eucaryotic cell nucleus, which play an important role in the process of inflammation regulation, migration, survival and other life activities[5-8]. The high expression of HMGB1 in tumor tissue suggests that HMGB1 plays an important role in the regulation of tumor cells occurrence and development. This study investigated the expression of HMGB1 in human Rb cell lines, and observed the inhibiting effect of HMGB1 siRNA and its effect on cell proliferation and apoptosis by use of RNA interference technique to inhibit the gene expression of HMGB1 in human Rb cell lines.

MATERIALS AND METHODS

Cell Culture RMPI-1640 containing 10% fetal bovine serum and MCDB-131 medium were used to culture Y79 and HXO-Rb44 human Rb cell line and ACBRI-181 human retinal vascular endothelial cells (ATCC, Manassas, VA, USA), culture solution was cultured in the incubator with 37℃ , 5% CO_2.

Real-time Polymerase Chain Reaction Collected all the cells take advangtage of Trizol reagent (Invitrogen, Carlsbad, CA, USA), and extract RNA according to reagent kit instructions. Take 1 μg total RNA, reverse transcription using cDNA first strand synthesis kit (fermentas, Invitrogen, USA), and finally take advangtage of SYBR Green master primer kit (Applied Biosystems, USA) to detect HMGB1 mRNA expression. Referred to HMGB1 and GAPDH cDNA sequences

from Genebank, in accordance with the principles of primer design and use Oligo6.22 and Primer Express 5.0 soft-ware designed HMGB1 primers, upstream: TCAAAGGAGAA CATCCTGGCCTGT, downstream: CTGCTTGTCATC TGCAGCAGTGTT; GAPDH primer, upstream: GGTGA AGGTCGGAGTCAACGG; reverse: GGTCAT GAGTCC TTCCACGATACC. Amplification conditions were: 94℃ 3min, 94 ℃ 5s, 55 ℃ 30s, 72 ℃ 30s, a total of 30 cycles, repeated three times. Quantitative genetic level detection by $2^{-\Delta\Delta Ct}$.

Western Blot Take advangtage of RIPA (strong) kit to extract total protein within cell lines, BCA protein quantification kit (BCA kit) test protein concentration of the sample. Each set of samples were loaded 40 μg, after separation by SDS-PAGE, transferred to a PVDF membrane. Of 50 g/L skim milk for 1.5h, plus 50 g/L BSA diluted HMGB1 polyclonal antibody (1:500) and β-actin monoclonal antibody (mAb, 1:1000) after 4 ℃ overnight, TBST (TBS, 1 mL/L Tween-20) washing the membrane three times, 6min/times; each with HRP-labeled secondary antibody (1:5000), room temperature for 2h, ECL chemiluminescence agent darkroom developing.

RNA Interference According to lipofectamine™ 2000 operating instructions for transfection, siRNA screening optimal concentration of siRNA transfected cells was 80 nmol/L, four pairs chemically synthesized HMGB1-specific siRNA were added to the logarithmic growth phase cells, continue to cultrue for 72h, then cells were collected to extract total RNA and protein (Table 1).

Cell Proliferation Inhibition Test MTT assay was used to test. Of 10^4 logarithmic growth phase cells per well/100 μL were seeded in 96-well plates, and transfected cells accordding to the lipofectamine™ 2000 transfected instructions. After 24, 48, 72h test cell viability by MTT. Four hours before the end of the experiment, each hole was added by 20 μL MTT, incubated for 4h. At the end of the experiment, the culture medium by centrifugation was discarded, each well add DMSO 150 μL, at room temperature to dissolve the crystalline slight 15min, each time point set five wells. Microplate absorbance was detected in each well at a wavelength of 490 nm.

Capases-3 Activity Assay Cells were seeded in 6-well cell culture plate, the density of 1×10^6 /well, interference using the above method, continue to culture in incubator with 5% CO_2, 37℃ . Obtain the cells after cultured 24h. The cells were resuspended in 50 μL of cold cell lysis buffer and placed on ice for 10min, 4℃ , 12 000 r/min centrifugation for 10min to remove cellular debris. The supernatant was transferred to another micro centrifuge tube and placed on ice. Each group was added to 50 μL of 2× reaction buffer/dithiothreitol (DTT) mixture. Add 5 μL of 1 mmol/L Capases-3 substrate to each tube. A water bath at 37℃ for 1h. Read OD value at 405 nm wavelength. Calculate Capases-3 activity units according to the description resulting slope value. Capases-3 activity units= (experimental group OD-control group OD)/slope.

Table 1 The sequences of HMGB1-specific siRNAs

Name	Sequences
HMGB1-siRNA-1	
Sense (5'-3')	CCCGUUAUGAAAGAGAAAUTT
Antisense (5'-3')	AUUUCUCUUUCAUAACGGGTT
HMGB1-siRNA-2	
Sense (5'-3')	GGGAGGAGCAUAAGAAGAATT
Antisense (5'-3')	UUCUUCUUAUGCUCCUCCCTT
HMGB1-siRNA-3	
Sense (5'-3')	CUGGGAGAGAUGUGGAAUATT
Antisense (5'-3')	UAUUCCACAUCUCUCCCAGTT
Scramble	
Sense (5'-3')	UUCUCCGAACGUGUCACGUTT
Antisense (5'-3')	ACGUGACACGUUCGGAGAATT

Cell Apoptosis and Cell Cycle Detected by Flow Cytometry According to Annexin V/PI double-labeled kit instructions, buffer solution diluted with distilled water, sample cells were washed with PBS, resuspended cells with dilute binding buffer, and adjust cell concentration of 2×10^5-5×10^5/mL. Take 195 μL of cell suspension, add 5 μL Annexin V mixing, then react 10min at room temperature, PBS washed cells once, then 190 μL of diluted resuspended in binding buffer, plus 10 μL 20 μg/mL PI, analyzed by flow cytometry. Of 1×10^4 cell fluorescence signals were collected from each sample, CellQuest software was used to results analysis.

Statistical Analysis All test experiments were repeated three times, measurement data by Mean±SD, data analysis using SPSS 11.5 statistical software, to deal with treatment group and control groups by *t*-test. $P<0.05$ was considered statistically significant.

RESULTS

Transcription Levels of HMGB1 mRNA in Human Retinoblastoma Cells Detected expression of HMGB1 mRNA in Y79, HXO-Rb44 human Rb cell line and ACBRI-181 normal human retinal vascular endothelial cells by real-time polymerase chain reaction (RT-PCR), and deal with the data by *t*-test, found that the contrast difference of HMGB1 mRNA transcription level between each cell line was statistically significance (Figure 1), the transcription level in human Rb cell lines was significantly higher than in normal retinal vascular endothelial cells ($P<0.01$).

High Mobility Group Protein Box-1 Expressions in Every Human Retinoblastoma Cells by Western Blot Detected HMGB1 protein expression levels in Y79, HXO-Rb44 and ACBRI-181 by Western blot, and get gray value/β-actin gray for each Rb cell line, the *t*-test showed the difference of HXO-Rb44, Y79 expression was statistically significant ($P<0.01$; Figure 2).

siRNA Inhibit High Mobility Group Protein Box-1 mRNA Expression in Y79 Cell Detected by Real-time Polymerase Chain Reaction After siRNA transfected cells with high

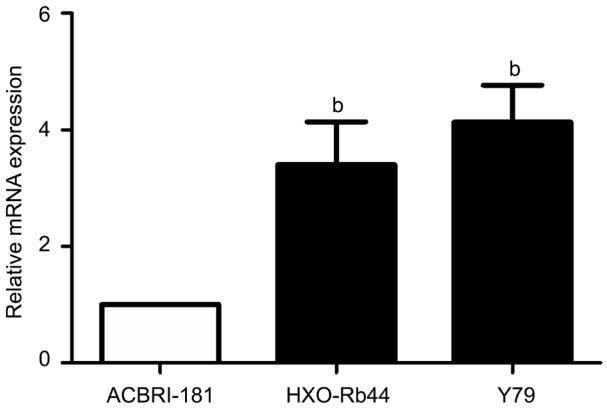

Figure 1 The expression of HMGB1 mRNA in ACBRI-181, HXO-Rb44, Y79 $^{b}P<0.01$ *vs* ACBRI-181.

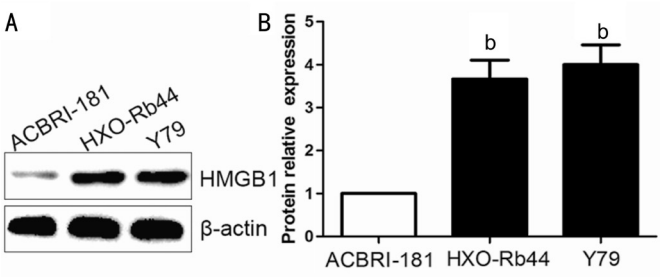

Figure 2 The expression of HMGB1 protein in ACBRI-181, HXO-Rb44, Y79 $^{b}P<0.01$ *vs* ACBRI-181.

expression of HMGB1 Y79, the expression levels of HMGB1 mRNA were detected by RT-PCR, compared siRNA transfected group with control group showed: siRNA had inhibitory effect on the expression of HMGB1, and the inhibitory effect of siRNA1 was better ($P<0.01$; Figure 3).

siRNA Inhibit High Mobility Group Protein Box-1 Protein Expression in Y79 Cell Detected by Western Blot After transfected by siRNA, the HMGB1 protein level of Y79 cells significantly reduced, and siRNA1 could obviously inhibit the expression of HMGB1 protein, and there was statistically significance between the two groups on HMGB1 protein expressions ($P<0.01$; Figure 4).

Effection of High Mobility Group Protein Box-1 siRNA in Y79 Cell Proliferation Detected by MTT Assay After being transfected by the most efficient suppression siRNA1 for 48h, Y79 cells growth was inhibited, compared to the negative control group, the difference was statistically significant ($P<0.01$; Figure 5).

Silence High Mobility Group Protein Box-1 to Induce Caspase-3 Activation in Y79 Cells The results showed that siRNA silencing HMGB1 had significantly enhanced role on Caspase-3 activation in Y79 cells, compared with the control group, the difference was statistically significant ($P<0.01$; Figure 6).

Effect of High Mobility Group Protein Box-1 siRNA on Y79 Cell Cycle After siRNA1 *in vitro* transfection Y79, the ratio of cells in G0-G1 cell cycle phase increased to 66.73%, higher than the negative control group (55.40%), while in

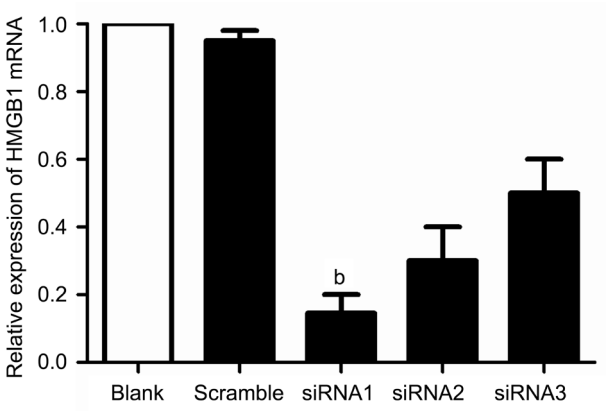

Figure 3 Effect of HMGB1 siRNA on the mRNA expression in Y79 $^{b}P<0.01$ *vs* scramble.

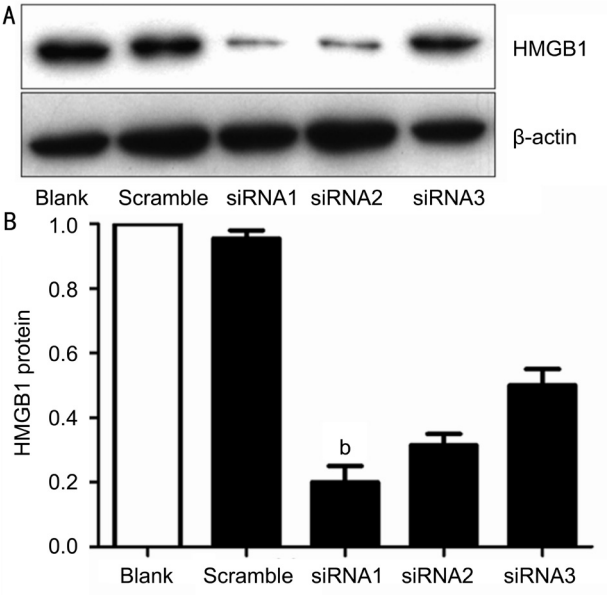

Figure 4 Effect of siRNA on HMGB1 protein in Y79 $^{b}P<0.01$ *vs* scramble.

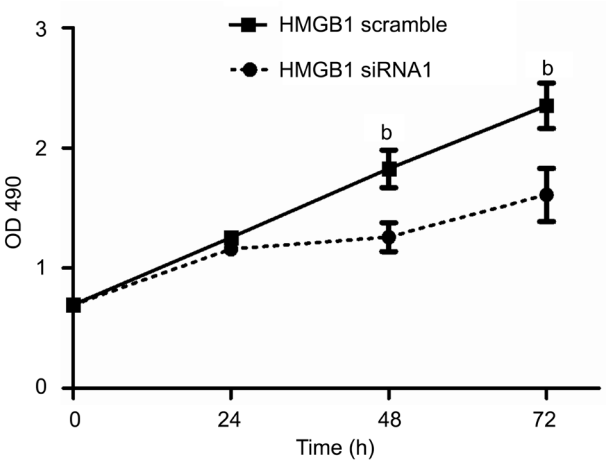

Figure 5 Effect of HMGB1 siRNA on Y79 Cell Proliferation $^{b}P<0.01$ *vs* scramble.

interference group the ratio of cells in G2-M phase fell to 4.42%, lower than the negative control group (13.14%). After siRNA interference HMGB1, the proportion of proliferating cells reduced, and the proportion of quiescent cells increased ($P<0.05$; Figure 7).

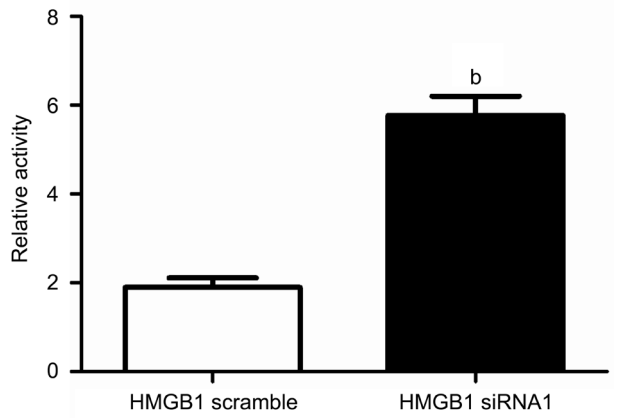

Figure 6 The activity of Caspase-3 after treated with HMGB1 siRNA on Y79 $^{b}P<0.01$ *vs* scramble.

Effect of High Mobility Group Protein Box-1 siRNA on Apoptosis in Y79 Flow cytometry analysis found, after interfering with HMGB1 siRNA1, apoptosis rate of Y79 cells increased from 2.03% to 9.10%, cell apoptosis rate was significantly increased after RNA interference ($P<0.05$; Figure 8).

DISCUSSION

Because of low early attendance rate, the treatment for children with Rb was much more beyond the scope of local adaptation, so oculists often used the traditional model of enucleation, external radiation therapy which have mass greater impact on the psychology and life quality of Rb patients. With the development of molecular biology and genetic engineering, molecular targeted therapy for the treatment of Rb provide a new treatment ideas. But the mechanism of Rb is not very clear. Excessive proliferation and apoptosis inhibition of tumor tissue was considered to be one of mechanisms of Rb occurence[9-10]. Therefore, study of the pathogenesis of Rb may discover new diagnostic markers and find a potential new target for Rb therapy.

As widely present in the nucleus of eukaryotic chromatin non-histone nuclear protein, HMGB1 was named because of fast migration rate in the polyacrylamide gel electrophoresis. HMGB1, an important inflammatory mediator and proinflammatory cytokines, were researched in inflammatory and autoimmune diseases initially[11]. In recent years, because of its regulating transcription activity, people found HMGB1 with high expression levels in a variety of tumors, such as breast cancer, colon cancer, stomach cancer, liver cancer[6,12-15], promoting T98G glioma cell proliferation by RAGE/MEK/ERK signaling pathway, suggested that HMGB1 involving in the regulation of tumor cells occurrence and development[16]. HMGB1 is closely related to several clinicopathological parameters such as clinical staging, recurrence, metastasis and prognosis of tumors. HMGB1 also possibly accelerate tumor metastasis by a number of angiogenic factors such as VEGF, tumor necrosis factor, or secreted interferon itself as an angiogenic factor promotes angiogenesis. HMGB1

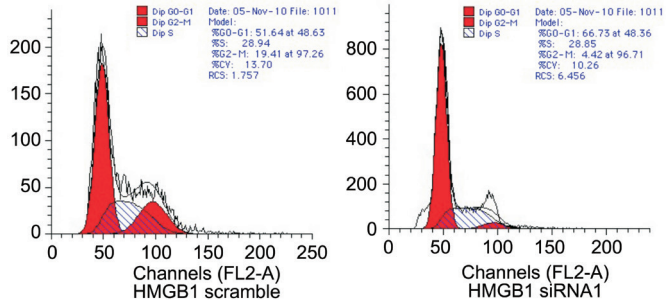

Figure 7 Cell cycle distribution of Y79 cells after interfered by HMGB1-siRNA was detected by flow cytometry.

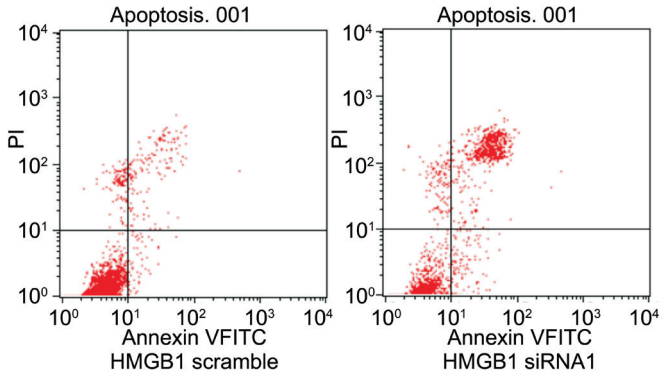

Figure 8 HMGB1 siRNA significantly increased the apoptotic rate of Y79 cell.

can also induce vascular endothelial cell apoptosis. Due to vascular endothelial cells are not belong to quickly update cell population, vascular endothelial cell apoptosis can reduce endothelial cells reserves and then lead to vascular endothelial fissure permeability increased, so that accelerate tumor metastasis and invasion[17]. However, the relationship between HMGB1 and human Rb is still worthy of further study.

Compared with human Rb cell line and normal human retinal vascular endothelial cells lines, we found that HMGB1 expression was significantly increased in the Rb tumor cell lines in our study. It illustrated that the abnormally high expression of HMGB1 was closely related to Rb occurrence and development. In our experiment, highly efficient and specific siRNA were used to transfect human Rb Y79 cells with high expression level of HMGB1 and then made HMGB1 expression blocked. We found that cell proliferation was inhibited after 48h. We also examined the Caspase-3 activity which is closely related to cell apoptosis. Caspase-3 is part of a variety of common downstream effect or apoptosis pathway. It occupies a central position in the process of apoptosis and was called "death execution protease"[18]. Our study found that silencing activity of HMGB1 can obviously promote Caspase-3 activation and inhibit the cell cycle, and induce apoptosis of tumor cells. In our experiment, the silence of HMGB1 gene could increase apoptosis and inhibit cell proliferation in Rb tumor cells, which further proved the important role of HMGB1 in Rb occurrence and development. Some questions should still be further studied including

nature of the tumor, its biological characteristics, as well as the role of multiple genes involved in different stages of tumor and other issues. This study suggests that HMGB1 plays an important role in Rb tumor cells occurrence and development, and provides a new idea for Rb treatment and research. However, there are still some deficiencies in this study. Although the reduced expression of HMGB1 by siRNA interference can inhibit proliferation and induce apoptosis, whether downregulation of HMGB1 can affect invasion and metastasis of Rb, or the specific molecular mechanisms of HMGB1 acts in occurrence and development of Rb, deserve further study.

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Conflicts of Interest: Wang LL, None; **Feng YQ,** None; **Cheng YH,** None.

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