

Downregulation of Expression of CHFR in RAJI Cells after Transfection with shRNA Promotes Growth and Inhibits Apoptosis

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Abstract: To investigate the proliferation and cell-cycle changes in the human RAJI cells after CHFR gene silencing. Cells were transfected with CHFR silencing RNA (shRNA) lentiviral vectors. CHFR silencing was verified in these cells 72h post-transfection using real-time polymerase chain reaction and western blot. Cell proliferation was analyzed by a standard Cell Counting Kit-8 (CCK-8) assay, and the cell cycle changes were detected by flow cytometry. The mRNA expression of CHFR in the ShRNA group was significantly down-regulated compared with that in the control and normal groups (both $P < 0.01$). The results of western blot showed that CHFR protein expression decreased significantly compared with their corresponding levels in blank control group. The CCK results suggesting that the decrease in CHFR expression promoted cell proliferation ($P < 0.05$). And Flow cytometry results demonstrated that the cell apoptosis rate was decreased after CHFR silencing ($P < 0.05$). The decrease in CHFR expression led to promoted cell proliferation and the cell apoptosis rate was decreased. And CHFR gene may play a negative role during the growth and proliferation.

Keywords: CHFR gene; Raji cell; Lentivirus; Proliferation; Apoptosis

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1. Introduction

Lymphoma is one of the most common malignant tumors that originates in the lymphatic system and in recent years, and the incidence of lymphoma has been increasing [1,2]. Patients with B-cell non-Hodgkin's lymphoma (NHL) usually respond to initial conventional chemotherapy, they often relapse and mortality has continued to increase over the last three decades in spite of salvage therapy or high dose therapy and stem cell transplantation. Outcomes vary by subtype, but there continues to be a need for novel options that can help overcome chemotherapy resistance, offer new options as consolidation or maintenance therapy post-induction, and offer potentially less toxic combinations [3]. So it is very important to find a more effective treatment for improving the rate of complete remission with disease free survival.

CHFR is an antephasis checkpoint gene that functions to delay cell cycle entry into metaphase in response to mitotic stress [4,5]. Cells that are deficient in this checkpoint undergo mitotic catastrophe and apoptosis, explaining the increased sensitivity of CHFR negative tumors towards micro tubular targeted therapies. Mice deficient in CHFR develop spontaneous malignancies and are more susceptible to chemical carcinogenesis [6]. CHFR prevents errors in chromosome segregation which can lead to neoplasia. CHFR is ubiquitously expressed in normal human tissues, while the loss of CHFR expression has been observed in human tumors, in which it fails to prevent

proliferation of abnormal cells from G2 to M phase, then abnormal differentiation and proliferation of cells occurs [7]. This study find CHFR gene can promoting growth and inhibit apoptosis in Raji cells, which may contribute to the occurrence of B-NHL, and it aimed to investigate the relationship between CHFR gene expression and lymphoma, and to find a new idea of molecule marks and therapy.

2. Materials and methods

2.1. Materials

2.1.1. Chemical and reagents

RPMI-1640 medium, HEPES and fetal bovine serum (FBS) were purchased from Hyclone. QrT-PCR kit was purchased from Takara (Dalian, China). The primary anti-CHFR and β -actin antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Anti-mouse IgG HRP-linked and anti-rabbit IgG HRP-linked secondary antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

2.1.2. Cell lines

Raji cell line was purchased from the Chinese Academy of Sciences Committee Type Culture Collection (Shanghai, China). The cell was cultured in RPMI 1640 medium (HyClone), the culture media contain 10% fetal bovine serum (FBS) (HyClone) and penicillin 100 Unit/ml. All cell cultures were at 37 °C with 5% CO₂.

2.2. Methods

2.2.1. Lentiviral shRNA vector construction and transfection

The ShRNA were purchased from jikai (Shanghai, China). The shRNA-CHFR lentiviral vectors were constructed according to manufacturers suggestion. The target sequences of CHFR for production of shRNA were 5'-GTCAGACATCCTGAAGAAT-3', and an unrelated 5-Aza-dC negative control group (NC) sequence was 5'-TTCTCCGAACGTGTACAGT-3'. The Raji cells were cultured in RPMI-1640 medium containing 10% FBS and when they reached the exponential growth phase, 5.0×10^4 cells per well were plated in 96 plates. Next, 100 μ l complete culture medium, containing recombinant lentiviruses, control lentiviruses or RPMI-1640 medium (all containing 10 μ g/ml polybrene; jikai) was added into the plates when the cells reached 50–60% confluence. Two days later, the virus-containing medium was added with fresh complete medium. The expression level of green fluorescent protein was observed under a microscope after 3 days. There are three groups in the study: normal RAJI group, ShRNA negative control group and ShRNA group.

2.2.2. Determination of the optimal multiplicity of infection (MOI)

To assess the efficiency of lentiviral transduction in the human RAJI cells, the cells were infected with pLenti-GFP at various MOIs for 24h. The supernatant was then changed to fresh complete medium every other day. After 72h, green fluorescent protein-expressing cells were detected by fluorescence inverted microscope (Olympus; Tokyo, Japan) (MOI=15).

2.2.3. Quantitative real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, Tokyo, Japan), and 5mg of extracted RNA samples was reverse transcribed into cDNA by a One-step Primescript cDNA Synthesis Kit (TaKaRa Dalian, China) according to the manufacturer's protocol. The resulting cDNAs were subjected to qRT-PCR analysis to evaluate the relative expression levels of CHFR, PARP-1 and β -actin (an internal control). The cDNAs were amplified by qPCR using SYBR Premix DimerEraser (Takara, Dalian, China) on the LightCycler 96 system (Roche) according to the normalized PCR conditions. β -actin was used for normalization. Primers for CHFR, PARP-1 and β -actin were synthesized by Sangon (Shanghai, China). All

reactions were run in triplicate.

PCR with primer of β -actin; forward, 5'-AGCTACGAGCTGCCTGAC-3'; reverse, 5'-AAGGTAGTTTCGTGGATGC-3'. CHFR forward 5'-CCTCAACAACCTCGTGGGAAGCATAAC-3'; reverse- 5'TCCTGGCATCCATACTTTGCACAT-3'.

2.2.4. Western blot assay

After being transfected with ShRNA cells were collected. Total protein was extracted after cell disruption, and protein concentrations were estimated with the bicinchoninic acid (BCA) method. Then, the proteins were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), in which gel separation was performed at 80V, and gel concentration was performed at 120V, and subsequently transferred to polyvinylidene fluoride (PVDF) membranes using 280Ma for 100 min. Primary antibodies against CHFR and actin were added to the membranes, which were incubated at 4 °C overnight. After the membranes were rinsed with Tris-buffered saline with Tween-20 (TBST), the appropriate secondary antibodies were added. After development, protein expression was detected.

2.2.5. Cell counting kit-8 (CCK-8) assay

The cells were collected on 96-well plates (concentration >5000 cells/mL) and cultured for 2-3 days. The cells were again cultured with 100mL volume of the culture medium, and then 10mL CCK-8 solution (BOSTER, Wuhan, China) was added; the cells were incubated at 37 °C in 5% CO₂ for 1 h according to the manufacturer's protocol. The absorbance was measured at 450nm using Thermo-plate microplate reader (Rayto Life and Analytical Science Co. Ltd, Germany) to observe the speed of proliferation.

2.2.6. Detection of cell cycle by flow cytometry

Cells (1×10^6 cells/mL) were collected, centrifuged, and the culture medium was discarded. The cells were washed with PBS, fixed in 800mL/L ethanol at low temperature for 5min and centrifuged. The fixing solution was discarded. The cells were then resuspended in PBS, 5 μ L of Annexin V-APC, and finally, 5 μ L of 7-AAD (BD Biosciences) staining solution for 15min, and detected by NovoCyte Flow Cytometer (ACEA Biosciences, China).

2.2.7. Statistical analysis

Data were analyzed by the SPSS 17.0 software. Correlation of every group was assessed by one way Anov. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Fluorescence detection in RAJI cells at 72h after lentiviral transfection

After RAJI cells were transfected with the ShRNA vector for 72h, GFP fluorescence intensity was observed using a fluorescence microscope to determine transfection efficiency. As seen in Figure 1, the fluorescence demonstrated high transfection efficiency of over 90 % at 72h.

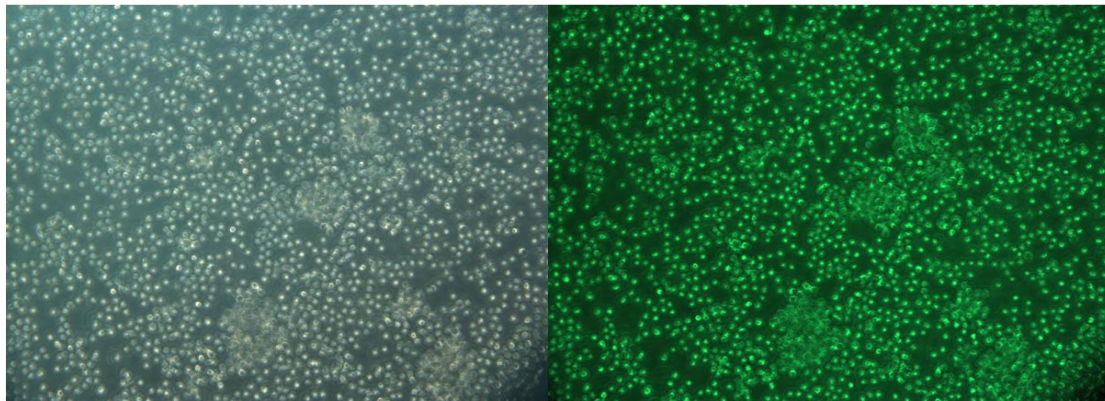


Figure 1. Fluorescence detection in RAJI cells at 72 h after lentivirus transfection:1:10.

3.2. Quantitative real-time PCR and Western blot

In order to assess the ability and specificity of the shRNA inhibit CHFR gene, qRT-PCR analysis revealed that when lentiviral was transfected, expression of the corresponding target mRNA was decreased by at least 90%; The results showed that the mRNA of CHFR expression was significantly down-regulated in the shRNA group compared with

the negative control and blank groups (both $P < 0.01$) (Figure 2A). The level of CHFR expression in the shRNA negative control (NC) group was not significantly different from that of the blank group ($P > 0.05$). The results of western blot showed that CHFR protein expression decreased significantly compared with their corresponding levels in blank control group ($P < 0.05$).

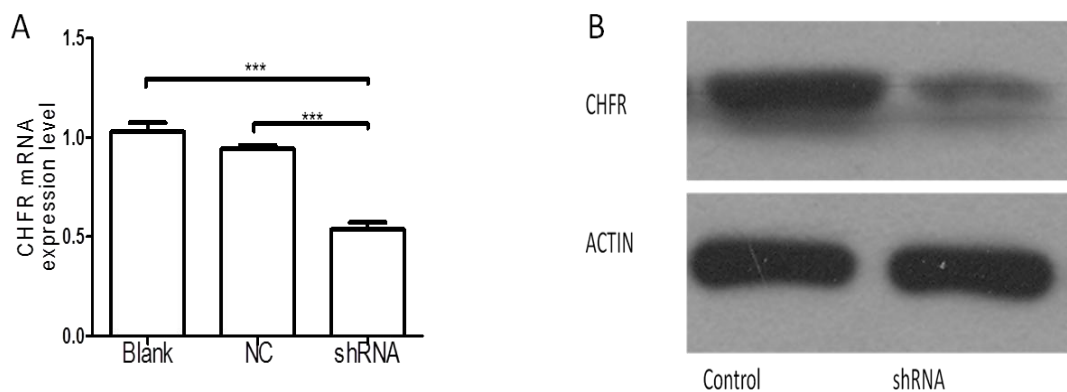


Figure 2. mRNA of CHFR expression down-regulated in the shRNA group with the negative control and blank groups.

3.3. Analysis of Raji cells rate after treatment with lentivirus by CCK

The proliferation of CHFR-silenced cells was analyzed by detecting the optical density at 450nm (OD450) 24 and 48h after the CCK-8 test. The experiment was performed in 5 wells. One-way

analysis of variance indicated that the cell proliferation was significantly promoted ($P < 0.05$). The Dunnett test revealed that the control and experimental groups had significantly different outcomes ($P < 0.05$), suggesting that the decrease in CHFR expression promoted cell proliferation (Figure 3).

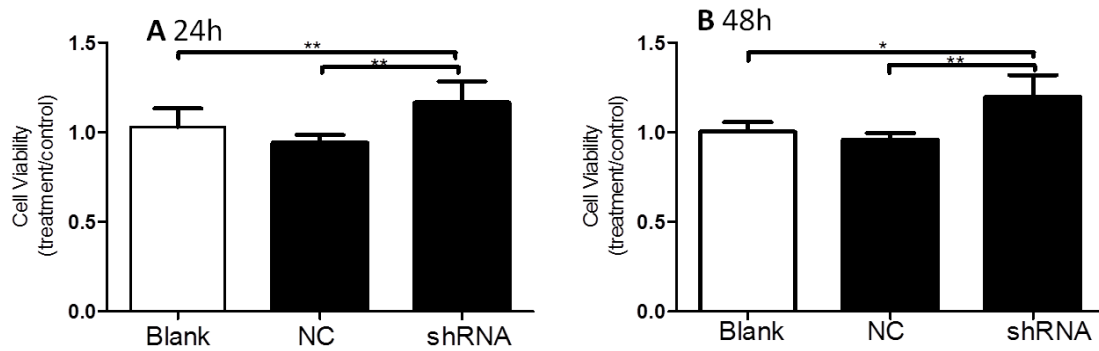


Figure 3. RAJI cell proliferation after CHFR silencing in different time was detected by CCK-8 assay.

3.4. Flow cytometry results

The Annexin-APC/7-AAD staining method to detect cell apoptosis demonstrated that the cell apoptosis rate was decreased after CHFR silencing compared with

the negative control and blank groups ($P < 0.05$) (Figure 4). The apoptosis rates of RAJI cells and transfected RAJI cells compared with NC were $15.61 \pm 0.24\%$ and $9.21 \pm 0.53\%$, respectively.

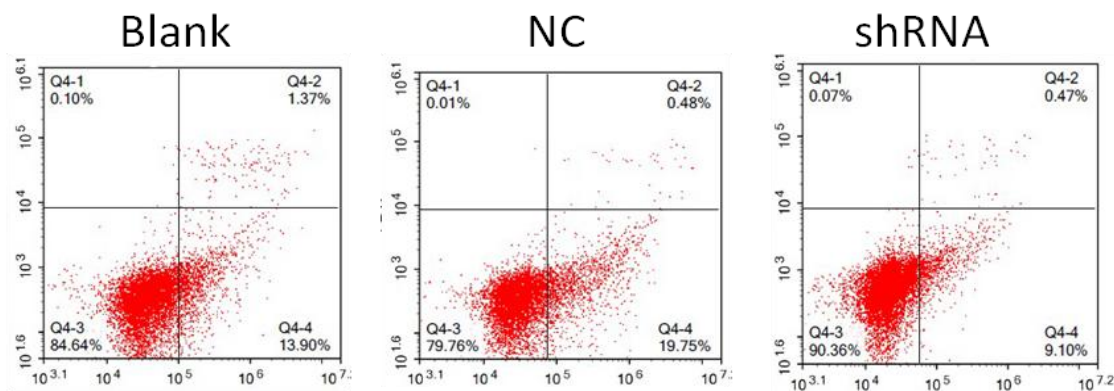


Figure 4. Flow cytometry results of RAJI cells after CHFR silencing. A. Blank group. B. NC group. C. RNAi group.

4. Discussion

CHFR is a nuclear protein and plays an important role in the early mitotic checkpoint by actively delaying passage into mitosis in response to mitotic stress caused by microtubule inhibitors. CHFR contains an N-terminal FHA domain, a central RING finger domain, and a C-terminal cysteine-rich (CR) region. The FHA domain is required for the mitotic checkpoint [4]. Some existing data have suggested that CHFR maintain genomic stability to inhibit tumorigenesis by regulating multiple mechanisms and decrease or loss expression of CHFR has been detected in many tumors [8-10]. Chfr knockout mice develop invasive lymphomas and solid tumors (lung, liver, gastrointestinal) after 40 weeks and have an increased susceptibility to chemical carcinogenesis [11]. Our previous evidence suggested CHFR may be associated with the pathogenesis, progression for B-NHL, which might be a novel molecular marker as prognosis and treatment for B-NHL [12].

The evidence shows that the green fluorescence observed 72 h after transfection indicated the successful transfection of RAJI cells by this lentivirus.

Both real-time PCR and western blot analyses showed that the shRNA interference lentiviruses were capable of disrupting CHFR expression in RAJI cells. With the reduction of CHFR protein levels resulted in down-regulation promote the growth and inhibit apoptosis in Raji cells. Our data suggested that RAJI cell apoptosis arrest by decreasing CHFR levels. The decreased or lost CHFR expression may lead to genomic instability by impairing the spindle assembly checkpoint, which can lead to cells a high mitotic, suggesting that CHFR gene function as a tumor suppressor gene [13,14]. Mitotic progression is regulated by co-ordinated action of several proteins and is crucial for the maintenance of genomic stability. Currently CHFR is known to regulate the stability of very limited substrates such as Aurora A, Plk1, Kif22 and PARP1, which are critical for proper mitotic transitions [15]. CHFR has been found to be either lost or down regulated or its promoter being hypermethylated in several human cancers including breast, prostate, lung and esophageal cancers. The crucial role of CHFR in mitotic checkpoint control and

a clear prognostic and predictive power highlights the clinical potential of CHFR as biomarker [16].

5. Conclusion

This study found CHFR gene expression had down-regulation promote the growth and inhibit apoptosis in Raji cells. In conclusion, CHFR gene plays a negative role during the growth and proliferation of Raji cells. CHFR may be used as a molecular target to cure the lymphoma in the future.

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