Original Research Article

Targeted silencing of CDX2 gene with siRNA enhanced vincristine-induced inhibition of proliferation and induction of apoptosis of leukemia K562 cells

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Abstract

Purpose: To determine the effect of targeted silencing of CDX2 with siRNA on vincristine-induced inhibition of proliferation and apoptosis induction in leukemia K562 cells.

Methods: K562 cells were divided into untreated group, vincristine group, blank group, and CDX2-siRNA group. The expression of CDX2 gene after CDX2-siRNA transfection was determined by reverse-transcription-polymerase chain reaction (RT-PCR) and immunoblotting, while MTT and flow cytometric procedures were used to evaluate the effect of vincristine on proliferation and apoptotic changes in K562 cells.

Results: Protein expression of CDX2 did not change significantly in non-treated cells, vincristine group and blank group, while the expression of CDX2 protein in cells of CDX2-siRNA group was decreased significantly ($p < 0.05$) while MTT assay results showed that the absorbance of CDX2-siRNA cells was significantly lower than those of the other three groups at 12, 24 and 48 h after CDX2 gene silencing. Flow cytometry showed markedly higher percentage apoptosis of CDX2-siRNA group than in other groups at 12, 24 and 48 h after CDX2 gene silencing. There was no difference in apoptosis level between vincristine group and blank group, but apoptosis was higher in these groups than in untreated group ($p < 0.05$).

Conclusion: Silencing CDX2 gene via targeting with siRNA enhances vincristine-induced suppression of growth and apoptotic changes in leukemia K562 cells, thereby enhancing the anti-tumor effect of vincristine.

Keywords: CDX2 gene, Vincristine, Leukemia, Proliferation, Apoptosis

INTRODUCTION

Hematological malignancy is a cancer originating from bone marrow hematopoietic stem cells. The disease is gradually becoming prevalent in humans, especially in children. The pathogenesis of hematological malignancy is a complicated process caused by continuous accumulation of multiple factors and stages, and it involves a variety of different genes [1,2].

There are abnormal growth and apoptotic changes in hematopoietic stem cells in the blood stream of leukemia patients, and the abnormal

pathological cells increase in large numbers. In the course of continuous clinical chemotherapy, patients experience a relapse which makes it more difficult to treat the disease. The relapse is due to resistance of leukemia malignant tumor cells to chemotherapeutic drugs. Therefore, it is important to develop new ways of increasing the sensitivity of leukemia cells to chemotherapeutic drugs, preventing abnormal proliferation of the cells, and enhancing their apoptosis, as strategies for improving the effect of clinical chemotherapy on leukemia.

EXPERIMENTAL

Reagents and equipment

Human erythroid leukemia cell line K562 was purchased from the Cell bank of Chinese Academy of Sciences. Vincristine was product of Shandong Dezhou Pharmaceutical Co., while CDX2 and CDX2-SiRNA primers were obtained from Shanghai Bioengineering Co. Ltd. The RT-PCR assay kit, MTT assay reagents, and Annexin V-FITC/PI kit were bought from Beijing Solebo Company. In addition, CDX2 polyclonal antibody and other antibodies were purchased from Santa Cruz Company, while American Thermo Company was the maker of ultra-low temperature refrigerators, inverted microscopes and ultrasonic machines.

K562 cell line culture

The K562 cells purchased from Chinese Academy of Sciences were transferred to petri dishes for further culture. Under the microscope, the cell fusion was allowed to reach about 90 %, prior to passage. After digestion, the cells were separated from the bottom of the petri dish, and the digestion was terminated. Then, the cells were prepared into cell suspension and evenly transferred to new petri dishes. Cells in good growth condition and no pollution were selected for experimental treatment. The cultured cells were divided into untreated group, vincristine group, blank group and CDX2-siRNA group. Each group of cells in a petri dish was labeled.

Cell transfection

The culture medium was replaced with pure medium in blank group and CDX2-SiRNA group. Then, pure RPMI1640 culture medium was used to prepare CDX2-SiRNA blank, CDX2-SiRNA and cationic Lipofectamine 2000TM, each in a separate petri dish. The blank had culture medium containing CDX2-SiRNA blank and cationic Lipofectamine 2000TM. Culture medium

containing CDX2-SiRNA blank and cationic Lipofectamine 2000TM was added to CDX2-SiRNA group. Thereafter, the cells were cultured for 6 h, after which the culture medium containing serum and antibiotics was again replaced. The untreated group was cultured in normal medium, while the cells in vincristine, blank and CDX2-SiRNA groups were cultured with vincristine at a concentration of 25 μg/mL.

Determination of mRNA expression of CDX2

Total RNA extraction from cells in each group was done using TRIzol one-step method. Complementary DNA was obtained by reverse-transcribing the extracted RNA using reverse transcription kit in accordance with the kit instructions, and then, polymerase chain reaction (PCR) was performed. The upstream and downstream primers and cDNA (20 μL) in double-distilled water (ddH$_2$O) were denatured, annealed, extended, cycled and extended for agarose-gel identification. Image J software was used for gray scale analysis. The sequences of primers used are shown in Table 1.

Table 1: Sequences of primer used

Gene	Upstream primers	Downstream primers
β-Actin	TTAGTTGCGTTAC ACCCTTTC	ACCTTCACCGTTCC AGTT
CDX 2	GAACCTGTGCGAG TGGATG	GGATGGTGATGTAG CGACTG

Western blot analysis of CDX2 protein

Logarithmic growth-stage cells in each group were subjected to RIPA lysis, boiled, ultrasonicated and centrifuged. Total protein concentration was determined with BCA method. Then, the proteins were separated on SDS-polyacrylamide gel electrophoresis, followed by transfer to PVDF membranes and incubation with CDX2 polyclonal antibody overnight at 4 ℃. This was followed by incubation of the membranes with horse radish peroxidase-linked 2° immunoglobulin for 1 h. Then, the membranes were subjected to enhanced chemiluminescence, development and photography. The Western blot images were analyzed using Image J software.

Determination of K562 cell proliferation

The cells in each group were made into cell suspension, inoculated separately into 96-well plates, and cultured for 12, 24 and 48 h. Then, MTT solution was added to each well and cultured for 12, 24 and 48 h, after which the medium in each well was replaced with DMSO to solubilize the resultant formazan crystals. The

absorbance of each well was read at 470 nm, and mean absorbance was obtained from triplicate assays.

Evaluation of apoptosis of K562 cells

Cells in the untreated group, vincristine group, blank group and CDX2-SiRNA group were cultured for 12 h, 24 h and 48 h. Cell suspension was prepared in binding buffer, and the cells were incubated sequentially with Annexin V-FITC (5 μL) and PI for 15 min in the dark. Then, the cells in each group were subjected to flow cytometric analysis (Attune NxT, ThermoFisher, USA) to determine apoptosis.

Statistical analysis

The SPSS 22.0 software was used for statistical treatment of data on CDX2 mRNA expression, gray level of CDX2 protein expression, MTT absorbance values, apoptosis, and other normal-distribution measurement data which are expressed as mean ± standard deviation (SD. Comparison between two groups was done with F-test, and statistical significance was assumed at $p < 0.05$.

RESULTS

CDX2 mRNA expression in K562 cells after CDX2-siRNA transfection

After CDX2-siRNA transfection, the expression levels of CDX2 mRNA in the untreated group, vincristine group and blank group did not change significantly, while the expression level of CDX2 mRNA in the CDX2-SiRNA group was decreased significantly ($p < 0.05$; Figure 1).
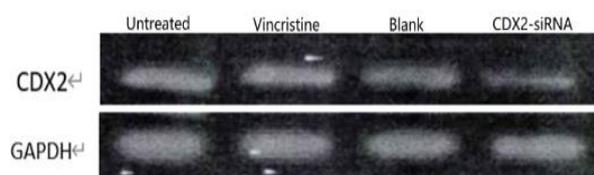

Figure 1: Expression of CDX2 mRNA after transfection

CDX2 protein expression in K562 cells after CDX2-siRNA transfection

After CDX2-siRNA transfection, the expressions of CDX2 protein in the untreated group, vincristine group and blank group did not change significantly, while the expression of CDX2 protein in the CDX2-siRNA group was decreased significantly ($p < 0.05$; Table 2).

Table 2: Protein and mRNA expressions after transfection (mean ± SD)

Group	CDX2 mRNA grayscale (bit)	CDX2 grayscale (bit)
Blank	133.67±11.85	119.82±11.36
Low dose	132.89±15.97	120.03±9.12
Medium dose	131.58±18.26	119.77±11.97
Medium dose	92.56±9.05	89.08±10.85
F	397.65	293.74
P-value	0.000	0.000

Effect of CDX2-siRNA transfection on vincristine-induced inhibition of proliferation and induction of apoptosis in K562 cells

Results from MTT assay showed markedly lower absorbance values in CDX2-SiRNA group than in each of the other three groups at 12, 24 and 48 h after CDX2 gene silencing. The absorbance of the vincristine and blank groups were similar, but absorbance values of the three groups were lower than those of the untreated group ($p < 0.05$).

Table 3: Proliferation of K562 cells after transfection (mean ± SD)

Group	12h (A)	24h (A)	48h (A)
No treatment	0.79±0.02	0.72±0.03	0.69±0.02
Vincristine	0.63±0.01	0.60±0.01	0.58±0.02
Blank	0.62±0.01	0.59±0.01	0.57±0.01
CDX2-siRNA	0.45±0.02	0.41±0.02	0.38±0.01
F	185.20	259.88	376.77
P-value	0.000	0.000	0.000

(A = absorbance value)

Effect of CDX2-siRNA transfection on the apoptosis of vincristine-treated K562 cells

Flow cytometry results showed that the percentage apoptosis of CDX2-siRNA group was significantly higher than that of each of the other three groups at 12, 24 and 48 h after CDX2 gene silencing. Percentage apoptosis was similar in vincristine and blank groups, but there were greater percentage apoptosis values in the three groups than in untreated cells.

Table 4: Apoptosis of K562 cells after transfection

Group	12h (%)	24h (%)	48h (%)
No treatment	0.29±0.01	0.30±0.01	0.32±0.03
Vincristine	3.49±1.18	3.95±1.58	4.17±2.06
Blank	3.45±2.02	3.28±2.11	3.07±1.94
CDX2-siRNA	11.36±6.26	14.94±5.88	16.82±6.75
F	207.49	327.86	383.52
P-value	0.000	0.000	0.000

DISCUSSION

Leukemia comprises two categories: acute leukemia and chronic leukemia. These categories are further divided into acute lymphoblastic leukemia and acute myeloid leukemia, as well as chronic lymphocytic leukemia, and a variety of types e.g., chronic myelogenous leukemia. These forms of leukemia at different onset ages and clinical manifestations, affect patients physically and psychologically, with devastating effects. Leukemia cells stagnate at different stages of cell differentiation, a process which is closely associated with abnormal regulation of multiple genes.

Tail homolobox gene (CDX2) is a nuclear transcription factor involved in embryo development, and it is an extremely important regulatory gene in subsequent growth and development [3]. In normal human body, only intestinal epithelial cells express CDX2, while abnormal overexpression of CDX2 has been found in most gene studies related to leukemia [4,5]. This indicates that unusual elaboration of CDX2 may be associated with the occurrence of leukemia, and it may affect the therapeutic effect of drugs. Thus, interference with the expression of CDX2 gene may have a great impact on incidence of leukemia. This speculation formed the basis of investigating the expression of CDX2 gene interference in this study. In essence, CDX2-siRNA was used to transfect K562 cells. Double verification at RNA and protein levels showed that CDX2 gene was silenced and down-regulated after CDX2-siRNA transfection, and the decreased expression was evident at both RNA level and protein level. These results indicate that CDX2-SiRNA was successfully transfected, thereby indicating the reliability and credibility of subsequent experiments.

The chemotherapeutic drug vincristine blocks spindle formation, arrests cell mitosis, selectively accumulates in tumor tissue, synchronizes cell proliferation, and enhances anti-tumor effect. However, vincristine is still not very beneficial against leukemia due to disease remission in patients. Therefore, it is important to effectively improve the anti-tumor effect of vincristine.

The results of studies in China using other leukemia cell lines suggest that correcting CDX2 overexpression may reduce the proliferation of leukemia cells and increase apoptosis [6,7]. Therefore, the researchers in the present study boldly speculated that down-regulation of CDX2 expression in leukemia cells could enhance the vincristine-induced suppression of growth and initiation of apoptosis of the cells. Based on the successful silencing of CDX2, this study further monitored the proliferation and apoptosis of leukemia cells after CDX2 gene silencing. The cells in the untreated group were ordinary K562 cells without any treatment.

Compared with vincristine group, the cells had higher proliferation potential and fewer apoptotic cells. These results indicate that vincristine reduced the population of K562 cells, decreased cell multiplication and increased cell death. In CDX2-siRNA transfected cell group, the absorbance value in MTT assay for K562 cells decreased significantly. The results showed that cell proliferation decreased significantly, while flow cytometric analysis showed increased cell apoptosis. After CDX2 gene silencing, the anti-cancer efficacy of vincristine was significantly improved.

Researchers have reported that when CDX2 gene is expressed in hematopoietic stem cells, the blood cell differentiation process of HOX dionon is impaired [8,9]. Thus, the cell differentiation process is disrupted, resulting in leukemia. Analysis of the potential mechanism of this effect indicated that exogenous apoptosis pathway was activated after CDX2 gene silencing, and the increases in Bax and Caspase contents promoted cell apoptosis [10-13]. The results of this study are consistent with those of other related investigations which reported that CDX2 gene silencing may promote the therapeutic effects of chemotherapy drugs on leukemia [14]. However, hitherto, the specific mechanism associated with this effect was unclear. This formed the focus of the present study.

CONCLUSION

The findings of this study show that the use of siRNA for targeted silencing of CDX2 gene enhances vincristine-induced suppression of multiplication of K562 cells, and increases their apoptosis, thereby enhancing the anti-tumor effect of vincristine.

DECLARATIONS

Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article, and all liabilities pertaining to claims relating to the contents of this article will be borne by the authors. Yun Li designed the study, supervised the data collection, and analyzed the data. Hong Li interpreted the data and prepared the manuscript for publication. Hong Li, Zhongjing Han, Xue Jiang and Lanzhi Ma supervised the data collection, analyzed the data and reviewed the draft of the manuscript. Hong Li and Zhongjing Han contributed equally to this work as co-first authors.

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