

Design of Lentiviral Vector of Apoptin and Investigating its Cytotoxic Effect on Reh Acute Lymphoblastic Leukemia Cells

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ABSTRACT

BACKGROUND AND OBJECTIVE: Resistance to chemotherapy drugs is one of the most important treatment problems in patients with acute lymphoblastic leukemia. Apoptin due to its ability to induce apoptosis in tumor cells, has an undeniable potential in cancer treatment, especially those that respond lessly to chemotherapy. Therefore, in this study, the effect of induction of apoptin expression on induction of cell death in reh malignant lymphoblasts was investigated.

METHODS: In this experimental study, after culturing of Reh cells (prepared by the Pasteur Institute), the entry of lentiviral vector, metabolic activity and cell proliferation were measured using flow cytometry, MTT and trypan blue at 24, 48, 72 and 96 hours. Real time PCR was also used to determine the apoptin gene expression.

FINDINGS: The results of this study indicate the design and construction of a suitable lentiviral vector that can infect more than 65% of the cells. Also, after infecting the cells with the vector, the apoptin gene expression rate was increased about 10 times to control, and subsequently, the cell viability decreased by 53% time-dependently ($p < 0.01$). The results also showed that the inhibitory effect of apoptin gene expression on the metabolic activity of Reh cells was about 35% ($p < 0.01$).

CONCLUSION: The results of the study showed that apoptin gene expression has anti-tumor activity in Reh cells and can be used as a promising therapy in ALL.

KEY WORDS: *Apoptin, Lentiviral Vector, Acute Lymphoblastic Leukemia, Reh Cell Li.*

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Introduction

Despite significant advances in treatment over the past decades, acute lymphoblastic leukemia is still recognized as one of the deadliest hematologic malignancies worldwide (1, 2). Apoptin due to its ability to induce apoptosis in cancer cells has an undeniable potential for cancer treatment, especially those cases that respond very low to chemotherapy (3). One of the significant attributes of the apoptin gene is its specific function in inducing cell death in a variety of cancer cells including hepatoma, osteosarcoma, melanoma, colon cancer, lung, breast, prostate, cervix and stomach, while no effect on inducing apoptosis in the normal cells of the body has been reported (3-6). The mechanism of apoptin has not yet been completely determined, but specific induction of apoptosis has been shown in some cancer cells (7, 8).

The apoptin gene is isolated from the Chicken Anemia Virus. This small virus, through the induction of apoptosis, has the potential to cause cytopathogenic effects in bird thymocytes. In the genome of this virus, three proteins, VP1 (51.6kDa), VP2 (24.0kDa), and VP3 (13.6 kDa) are coded, which the VP1 encodes the viral capsid protein; VP2 encodes the DPS-Dual-Specificity protein phosphatase, which generates an important signal for virus replication, VP3, which is called apoptin, and can induce cell death in many cells alone (3). Gene therapy is a new method that has been widely considered today due to its role in the treatment of genetic diseases and cancers. The main purpose of this method is to transfer a specific gene to the target cell and consequently to express that gene in the cell in order to produce therapeutic effects (9).

Today, lentiviral are used as a way of transferring genes. Lentiviruses are one of the retrovirus subtypes and are RNA viruses that have the ability to convert their own RNA to a two-stranded DNA that can enter in the host cell genome. This feature is used to construct vectors with therapeutic applications. In addition, lentiviral vectors can infect a wide variety of cell types, while they are much less toxic compared to other viral vectors (9, 10). Hence, in this study, apoptin was introduced by lentiviral vector to acute lymphoblastic leukemia cells; the apoptin effect in induction of cell death in Reh malignant lymphoblasts was studied.

Methods

Cell Lines: In this experimental study, Reh cells, which are acute lymphoblastic leukemia, were cultured in

RPMI-1640 medium and HEK293T cells were cultured in DMEM medium containing 10% bovine serum, 100 U / ml penicillin and 100 µg / ml Streptomycin at 37 ° C and 5% Carbon dioxide pressure.

Construction and titration of lentiviral vector expressing the apoptin gene: In order to construct a lentiviral vector from three plasmids of pCDH (gene transfer plasmid), pMD2G (plasmid for the production of lentivirus vector coating), psPAX2 (containing capsid builder genes of lentiviral vector) is used. The lentiviral vector of the apoptin gene (LV-GFP-Apoptin) from plasmid pCDNA3.1-GFP-Apoptin was prepared by Cloning PCR method. For this purpose, primer was designed for apoptin in plasmid pCDNA containing two cutting positions for ECORI and BamHI enzymes on both sides. After performing PCR, electrophoresis band of apoptin was extracted from gel and then transferred to the pCDH plasmid. Subsequently, all three plasmids (pCDH, psAX2 and PMD2G) were transferred to the HEK293T cell line by PolyFect-Transfection-Reagent and the virus was then harvested at 48, 72 and 96 hours. The virus needs to be ultrasounded for 3 hours to concentrate. In order to titrate the concentrated virus, the amount of protein P24 is measured by a quantitative ELISA kit. Calculating the number of viruses is also done by considering every 2,000 p24 molecules for each virus. This method was used for both lentiviral vectors with apoptin and without apoptin and in this study; MOI 5 was used for all tests.

Cell counting by trypan blue: Trypan blue staining and hemocytometer laminae (neobar laminae) were used to evaluate the cell proliferation. The basis of this test is that living cells are impassable to the color entry while dead cells absorb color. For counting the number of live cells, after adjacent cells with LV-GFP-Apoptin, at 24, 48, 72, and 96 hours, the number of non-colored cells in each of the four houses of sixteen houses (home of WBC count) was counted and averaged.

Metabolic cellular activity measurement by MTT assay: MTT assay was used to evaluate the cytotoxic effect of apoptin gene expression in cells and determination of IC50 values. For this purpose, 10000 cells were added to each well of 96 home-containing plates with and without LV-GFP-Apoptin and incubated for 24, 48, 72 and 96 hours in a CO2-incubator. After the desired time, the MTT (5mg / ml) solution was added to each well and incubated in 37°C incubator for 3 hours. The plate was then centrifuged at about 1000 g for 10 minutes, and after removing the supernatant, 100 µl DMSO solution was added to each well. The optical

absorption of each well was read by the plate Eliza reader at a wavelength of 570 nm.

RNA Extraction and Synthesis of cDNA and Real Time PCR: After treatment of Reh cells with lentiviral vector for 48 hours, RNA was extracted according to the instructions of the high-pure RNA isolation kit (Roche) and it was used to produce cDNA. Revertaid first strand cDNA synthesis kit was used to synthesize cDNA. The volume was 20 μ l for this reaction and the contents of the above solution were incubated for 5 minutes at 65 °, 5 min at 25 ° C and one hour at 42 ° C. Finally, the cDNA synthesis reaction with 5 minutes incubation at 70 ° ends. The prepared cDNA is stored at -20 °C. Real-Time PCR was performed on a light-cycler (Roche) machine in a volume of 20 μ l. For each reaction, 10 μ l

of Syber premix Ex Taq (Takara), 2 μ l of cDNA, 0.5 μ l of each primer (Table 1) and 7 μ l of Nuclease-free water were used. The used temperature conditions include an initial activation step at 95 ° C for 30 seconds, followed by 40 cycles for denaturation (5 seconds at 95 ° C) and annealing / extension (20 seconds at 60 ° C). To investigate the product specificity, the melting curve was investigated. At the end, for the calculation of the relative number of mRNA copies the $2^{-\Delta\Delta Ct}$ formula was used.

Statistical analysis: All experiments were performed in the form of three independent tests and reported values were as Mean \pm SD. T-test and SPSS17 and GraphPad Prism7 software were used for statistical analysis and $p < 0.05$ was considered as significant.

Table1. The primer sequence used in the Real Time Quantitative RT-PCR Assay

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Size (bp)
Apoptin	ACTCTATCGCTGTGTGGC	TCGCTTACCCTGTACTCG	366

Results

Evaluation of the titration and vector entry rate of lentiviral vector into Reh cells: LV-GFP-Apoptin entry into Reh cells was studied by fluorescence microscopy and flow cytometry. The amount of GFP-positive cells in Reh cells exceeded 65% in comparison with control cells, which had less than 1% GFP-positive cells. These results correlate with photos taken from cells after 48 hours of exposure to the lentiviral vector expressing the apoptin gene and high levels of GFP positive cells which seen green indicate high intake lentiviral vector (Fig 1).

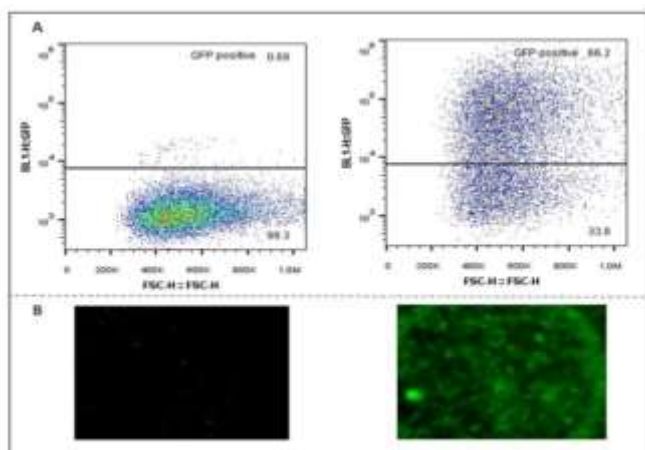


Figure1. Quantitative and qualitative study of lentiviral vector input into Reh cells A) for quantitative study of entry rate of vector, flow cytometry and fluorescence of GFP was used. B) An invert-fluorescent microscope was used to determine the quality of GFP positive cells.

Apoptin gene expression: In order to evaluate the expression of apoptin after entry of lentiviral vector containing apoptin gene into Reh cells, cells were collected at 48 and 72 hours, and for apoptin, RT-PCR was performed. The results showed that expression of apoptin in LV-GFP-Apoptin-infected cells increased about 5 times ($p \leq 0.01$) in 48 hours and increased to about 9 times ($p < 0.001$) in 72 hours (Fig. 2).

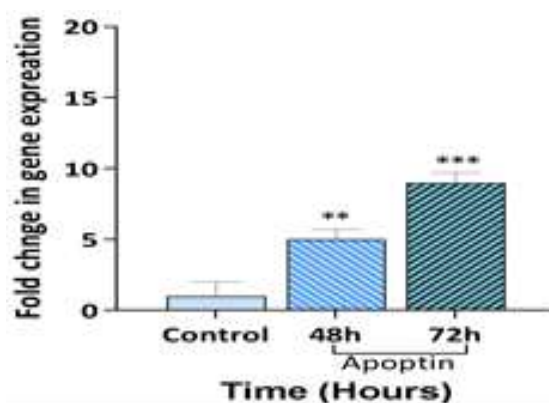


Figure 2. Comparison of apoptin gene expression after entry of lentiviral vector expressing the apoptin gene. To evaluate the expression of apoptin gene after vector entry into cells, the Real Time test was carried out at 48 and 72 hours and increased expression was observed. The mean and standard deviation of the results from the processes (mean \pm SD) and the obtained p (, representing $p \leq 0.01$, $p < 0.001$) indicate that the results are statistically significant compared to control sample.**

Time-dependent apoptin gene expression reduces the count of Reh cells. Trypan blue test was used to determine the effect of LV-GFP-Apoptin on Reh cell proliferation. The results indicate that increasing apoptin expression in Reh cells after 48 hours dependent on time at 72, 96 hours significantly reduced cell proliferation compared to control (Fig. 3). Viability analysis of cells showed that at 48, 72 and 96 hours, 70%, 62% ($p \leq 0.01$) and 53% ($p \leq 0.01$) of the cells were alive. In order to investigate the effect of lentivirus insertion into cells, evaluation of the effect of apoptin gene expression in cells, lentiviral vector without apoptin gene in adjacent to cells, has a negligible effect on cell proliferation, and the vitality percentage of the cells did not change.

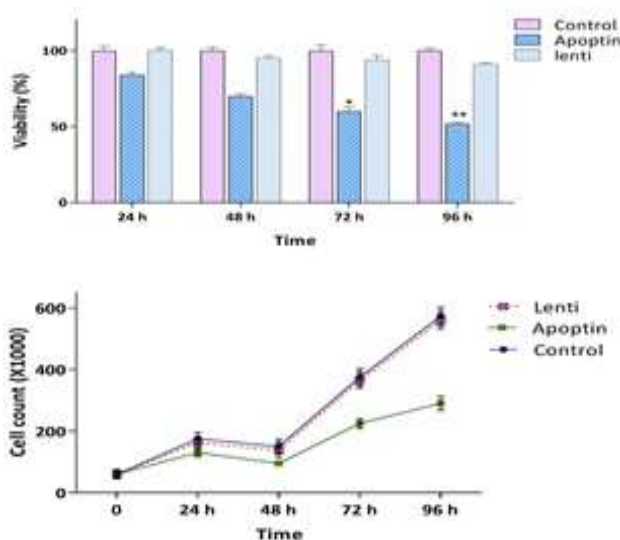


Figure3. The effect of apoptin gene expression on proliferation and viability percentage of Reh cells. The mean and standard deviation of the results from the processes (mean \pm SD) were calculated and the obtained p value (*, indicates $p < 0.05$, **, indicates $p < 0.01$) showed statistically significant in comparison with control sample.

Apoptin gene expression in time-dependent Reh cells decreases metabolic activity of the cell. To evaluate the metabolic activity of Reh cells after adjacent cells to LV-GFP-Apoptin, metabolic activity of the cells was evaluated by MTT. The results showed that the expression of the apoptin gene can reduce the metabolic activity of Reh cells by time, thus applying its cytotoxic effect to this cell line. As shown in Fig. 4, the metabolic activity at 48 and 72 hours was reduced by 20% and 28%, while the metabolic activity decreased by 35% compared to control at 96 hours (Fig. 4). To evaluate the effect of vector entry and GFP expression

on the metabolic activity of apoptin-free lentiviral vector was also adjacent to the cell that the comparison of its effect with control indicated a negligible vector effect and expression of GFP gene in reducing the metabolic activity at 48, 72 and 96 hours.

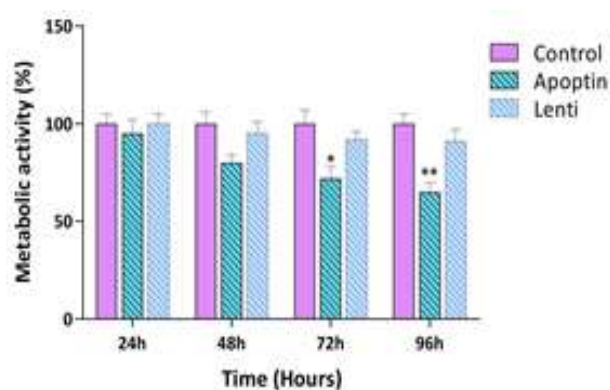


Figure 4. Investigation of LV-GFP-apoptin adjacent effect on metabolic activity of cells. The results show that apoptin gene expression in Reh cells reduces metabolic activity at 48, 72, and 96 hours. The mean and standard deviation of the results of the three different processes (mean \pm SD) were calculated and the p-value obtained (, indicates $p < 0.01$) indicates that the results were statistically significant compared to the control sample.**

Discussion

The results of this study showed that LV-GFP-Apoptin has an antitumor activity in Reh cells, and the expression of this gene in leukemia cells inhibits cell proliferation and induces cell death. There are several methods for expressing the protein inside the cell, which is one of the best methods for transferring the gene into lymphocytic cells due to the high resistance of these types of cells to other vectors.

The results of the study of high levels of GFP fluorescence show the proper function of LV-GFP-Apoptin in entering Reh cells. These results coincide with the findings of research by Jie Jiang and his colleagues, which have been able to infect a high level of malignant cells using the lentiviral vector of the apoptin gene (11).

In addition, apoptin gene expression was measured by Real Time PCR and the results indicated that apoptin gene expression in Reh cells was increased. In order to investigate the cytotoxic effect of apoptin gene expression in Reh cells trypan blue test carried out, and the results show that time-dependent apoptin can not only reduce the proliferation of Reh cells but also can reduce viability percentages, so that the decrease in cell

survival begins at 48 hours and peaked at 96 hours. Previous studies have pointed to the cytotoxic effect of this gene on breast and liver cancers. In addition, studies done by MTT assay showed that the apoptin gene transduction in Reh cells resulted in a decrease in cell metabolic activity. In Guelen et al., study the effect of apoptin expression on apoptosis induction in the Saos-2 and HSC-3 cell lines were determined (12). The inhibitory effect of apoptin gene expression was not due to the viral vector itself and the expression of the GFP gene, and the viral vector without apoptin had a negligible effect on the viability of cells and the metabolic activity (6).

In general, the results of previous studies have shown that lenti viral vectores are one of the most effective methods for gene transfer, and the apoptin gene is also an effective gene for inducing apoptosis in cancer cells (13-16). Clinical and genetic studies of ALL pathogenesis mechanisms indicate that PI3K / Akt pathway is active in over 80% of acute lymphoblastic leukemia cases. The research shows that the activity of this signaling pathway not only enhances the proliferation potential of leukemic cells, but also has a significant effect on the resistance to chemotherapy drugs. The results from previous studies indicate that apoptin, dependent on PI3K/Akt pathway and with accumulation of Akt in the nucleus, induces apoptosis in cancer cells (17,18). Our results in this study showed

that in cells derived from acute lymphoblastic leukemia, Reh, which PI3K/Akt pathway is active, reduces the cell viability. The same effect of transferring apoptin to lymphoblastic cancer cells can cause cellular death in these cells due to the permanent activity of the PI3K pathway. In addition, apoptin, as a viral gene, has no known inhibitors in human cells, and its expression in cancer cells does not increase the compensatory inhibition of its inhibitors.

The results of this study indicate that one of the best methods for transferring the gene into leukemia cells with high resistance to the vector is using lentiviral vector. In this research, after transferring of apoptin gene by lentiviral vector, the time-dependent expression of apoptin gene increased and the expression of this gene resulted in decreased cell proliferation and viability percentage. In addition, the effect of apoptin expression on metabolic activity of the cell shows its role in reducing metabolic activity. For this reason, it may be possible in the future to express this gene to induce cancer cell susceptibility to chemotherapy drugs.

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