Original Article
Middle East Journal of Cancer; October 2022; 13(4): 565-572

The Efficacy of siRNA Specific MDM2 in the Induction of Apoptosis in MCF-7 Breast Cancer Cell Line

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Please cite this article as: Kalantari T, Mohseni-Aghdam B, Nasri F, Tamaddon G, Kalantari M. The efficacy of siRNA specific MDM2 in the induction of apoptosis in MCF-7 breast cancer cell line. Middle East J Cancer. 2022;13(4):565-72. doi: 10.30476/mejc.2021.89554. 1532.

Abstract

Background: A high number of human breast cancers overexpress the murine double minute (MDM2) gene which blocks the p53 protein which plays an important role in arresting the cell growth. The present study aimed to investigate the efficacy of siRNA specific MDM2 in knocking down MDM2 and its subsequent effects on p53 to exert antiproliferative effects on Michigan Cancer Foundation-7 (MCF-7) breast cancer cells.

Method: In this in vitro study, we used the specific siRNA of the MDM2 gene to knock down the expression of the MDM2 protein in the MCF-7 cell line. The expression of MDM2, BCL2-associated X (BAX), BH3 interacting-domain death agonist (BID), and B cell lymphoma 2 (BCL2) genes was evaluated using the real-time polymerase chain reaction (PCR) technique. The apoptosis level was also assessed using the flow cytometry technique by the Annexin V test.

Results: The results showed that the entry of MDM2 siRNA into MCF-7 cells significantly reduced the mRNA expression of MDM2 gene (P-value < 0.05). Besides, the expression of the antiapoptotic gene of BCL2 significantly decreased (P-value < 0.05) in transfected MCF-7 cells, while that of BAX and BID genes increased (P-value < 0.05).

Conclusion: Based on the results, MDM2 inhibition is conducive to prevent cancer metastasis by the induction of cancer cell apoptosis. Moreover, it can be considered in cancer therapy along with chemotherapy.

Keywords: Transfected MCF-7 cells, Breast neoplasms, Bcl-2-associated X protein, BH3 interacting domain, BCL-2, p53

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Introduction

Breast cancer is a heterogeneous disease with five subtypes based on the genes expressed in cancer, and it is considered as the second cause of cancer-related mortality among

Received: December 31, 2020; Accepted: March 28, 2021

women.[1] Determining the molecular pathways and the imbalanced expression of the genes would pave the way for interventional therapy. In this regard, the murine double minute (MDM) family members are major regulators of protective responses to genotoxic condition.[2] The members of the MDM family include MDM4 and MDM2, as an E3 ubiquitin ligase.[3] MDM2 was cloned from a transformed mouse BALB/c cell line [3T3-DM] whose expression is more than 50-fold in 3T3-DM. MDM2 contains four functional domains, among which the RING finger domain is necessary for its E3 ubiquitin ligase activity, and the N-terminal domain recognizes the N-terminal Box-I domain of p53.[4, 5] MDM2 is known to negatively regulate p53 tumor suppressor gene through binding to the N-terminal domain of p53 and lead to its proteasomal degradation.[2] In the absence of stress, p53 is ubiquitinated by MDM2, as E3 ligase, resulting in the degradation of ubiquitinated p53 by the proteasome; in this way, p53 molecules are maintained at low levels through the MDM2 activity.[6] In response to DNA damage provoked by exposure to carcinogens or radiation, MDM2, as a negative regulator of p53, is ubiquitinated and destroyed in the proteasome.[6, 7] Consequently, p53 molecules are released to function in a remedial pathway such that the activated p53 potentially triggers DNA repairing where damage happens and prevents the uncontrolled proliferation of the cells with oncogene potential.[8] When p53 is activated, MDM family levels increase and enhance the positive feedback loop function of p53 and MDM2.[3, 9] Therefore, a good interaction between MDM2 and p53 is of great significance in healthy and genotoxic conditions and, apparently, the deregulation of these molecules poses a risk for cancer.[10] For more illustration, in the context of cancer, Wild-type p53 exists in approximately 50% of human cancers, in which p53 pathway is inactivated due to the overexpression of negative regulator molecules, particularly MDM2. In fact, 17% of the tumors, which show MDM2 gene amplification, have poor prognosis and unsuccessful response to chemotherapy.[11, 12] The MDM-2 and MDM-4 molecules along with tumor suppressor p53 maintain genomic integrity in mature breast epithelial cells.[13, 14] The MDM family is only expressed on the epithelial cells surrounding the lumen of the milk ducts of the adult breast.[3] Considering the modulatory role of MDM2 in maintaining the genomic stability of the breast cells, a study showed the oncogenic capacity of MDM2 as a contributory factor in the formation of mammary tumors and the development of ductal hyperplasia in transgenic MDM2-overexpressing mice. In line with this animal study, the increased level of MDM2 protein is also associated with the development of breast cancer in humans.[16] In fact, high levels of MDM2 protein have been detected in approximately 38% of human breast cancer.[17] The elevated MDM2 protein levels reported in breast cancer result from several contributors such as gene amplification, cellular location, genetic modification, HBV virus, and (TGF) β1 signaling.[1,18-21] Following the MDM2 overexpression, p53 molecules are degraded, reducing DNA repair and accelerating the tumorigenesis of cancers. There is probably a positive relationship between increased MDM2 expression and chemotherapy resistance in malignancies.[22, 23] Some studies showed that MDM2 inhibited the apoptotic effect of cisplatin drug, possibly causing drug resistance in human malignancies.[24, 25] Accordingly, defining the expression status of vital genes in the repairing pathway can help find out an interventional therapy for a better response to chemotherapy regimen in cancer.

In this study, we aimed to investigate the efficiency of MDM2 knockdown using a specific siRNA method and evaluating the expression level of BAX, BH3 interacting-domain death agonist (BID), and BCL2 genes in Michigan Cancer Foundation-7 (MCF-7) cell lines using real-time polymerase chain reaction (PCR) technique. The apoptosis level was further assessed using a flow cytometry technique by the Annexin V test.

Methods and Materials

Cell culture

In this in vitro study, MCF-7 cell lines

purchased from Pasture institute were cultured in RPMI 1640 medium (Gibco, USA) supplemented with 10% FBS (Gibco, USA) and 1% penicillin-streptomycin (Gibco, USA). CO2 incubator was used for the optimal cell growth of the MCF-7 cell line.

Small interfering RNA transfection

The cells were harvested with Trypsin-EDTA and diluted with media to a cell density of 2.5×10^6 of cells/ml. Typically, 5µl of siRNA against MDM2 and 5µl Fluorescein-5-isothiocyanate (FITC)-labeled unspecific siRNA (Santa Cruz, USA) were added to the cell suspension. The siRNA-cells mixtures were transferred into 2 mm Biorad Gene Pulser cuvettes and electroporated at 250 volts and 600µF capacity condition by Gene Pulser X cell Electroporation system (BioRad, USA). Following electroporation, an appropriate amount of the complete medium was rapidly added to each aliquot of the cells and re-plated onto the T25 flask to prepare for other tests.

Small interfering RNA was directed against MDM2 (cat.no.sc-29394) and an unspecific siRNA (sc-36869) was obtained from Santa Cruz Biotechnology, Dallas, Texas, USA.

Efficiency of transfection

We used FITC-labeled unspecific siRNA (Santacruz, USA) to evaluate the efficiency of transfection. Unspecific siRNA-treated MCF7 cells were incubated for 48 h. The percentage of FITC-labeled cells was estimated with a fluorescence microscope.

Flow cytometric apoptosis assay

The percentage of apoptotic cells was determined on a FACSCalibur (BD, Heidelberg, Germany). Cells were washed with PBS and resuspended in 100 ml annexin V/7-AAD incubation buffer and incubated for 15 min at room temperature in the dark. 400 ml of binding buffer (1X) was added to each sample and analyzed with flow cytometry.

RNA isolation and cDNA synthesis

Cells were harvested, centrifuged, and washed with PBS. The total RNA was isolated with RNA-isolation RNAX-plus kit according to the manufacturer's protocol (USA). The cDNA was synthesized from 1000 ng of total RNA based on the manufacturer's protocol (Cinnagene, Iran).

Real-time PCR

MDM2-up:5'CAGGGGAGAGTGATACA-GATT3'down:5'GCTTGTGTTGAGTTTTCCAG TT-3'- BAX-up: 5'-AAGAAGCTGAGCGAGT-GTCT-3' down: 5'GTTCTGATCAGTTCCGGCA C-3'- BID-up: 5'-CATCCGGAATATTGCCAGG C-3' down: 5'CCATGTCTCTAGGGTAGGCC-3'- B cell lymphoma 2 (BCL-2)- up: 5'-GGGATGCGGGAGATGTGG-3'down: 5'-GTAGCGG CGGGAGAAGTC-3'

Thermal cycling consisted of 95ºC for 30s,

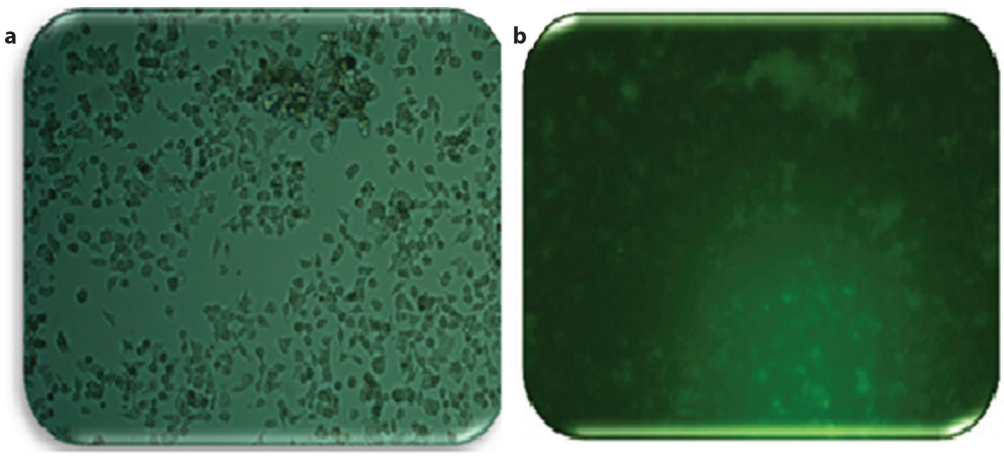

Figure 1. The efficiency of the transfection of MCF7 cells at 250 volts and 600µF capacity conditions using the electroporation system. The cells were incubated with FITC-labeled unspecific siRNA for 48 h; a) transfected MCF-7 cells under the ordinary microscope; and b) under the fluorescent microscope.

MCF-7: Michigan Cancer Foundation-7: FITC: Fluorescein-5-isothiocyanate

followed by 50 cycles at 95ºC for 5 s, 60ºC for 30s, and 72ºC for 30s. All tests were carried out in triplicate.

The study was approved by the ethics committee of Shiraz University of Medical Sciences, Shiraz, Iran with the ethics code of IR.sums.REC.1395.S643.

Statistical analysis

Statistical analysis was performed using SPSS, version 18.0 (SPSS Inc., Chicago, Ill., USA). Student t-test was used to determine the differences between siRNA treated MCF-7 cell lines and scramble-treated ones in terms of MDM-2, BID, BAX, and BCL-2 expressions.

Results

Transfection efficiency

The number of transfected cells in a population is of utmost importance in gene-silencing experiments. In our experiments, FITC-labeled unspecific siRNA or scramble-FITC conjugate was used to assess the transfection efficacy. Transfected cells were observed under a fluorescent microscope 48 hours after transfection. The results showed that 90% of the transfected MCF-7 cells were FITC positive. The FITC fluorescent light emission is shown in figure 1.

Real-time PCR quantification of MDM-2 transcripts after MDM-2 specific siRNA transfection

The down-regulation of the MDM-2 transcript was estimated with real-time PCR. Immediately after electroporation, MDM-2 siRNA-treated MCF-7 cells showed a significant reduction in MDM-2 transcripts compared with scramble-MCF-7 control cells. The expression of MDM-2 decreased 0.4234-fold change compared with scramble control cells (Figure 2).

BID, BAX, and BCL2 mRNA expression in transfected MCF-7 cells

Messenger RNAs encoding BID, BAX, and BCL2 were detectable by real-time qPCR in siRNA specific-MDM-2 transfected MCF-7 cells and scramble transfected MCF-7 cells. As shown in figure 2, real-time PCR data showed the increased mRNA expression levels of BID ($P < 0.05$/ fold change: 2.73) and BAX ($P < 0.05$/fold change:1.29), as apoptotic genes, and the reduced

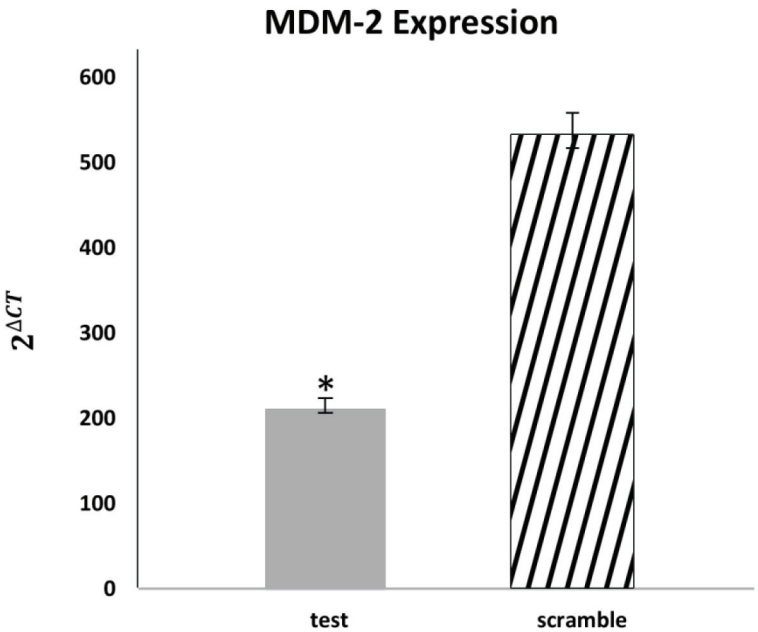

Figure 2. The effect of MDM-2 siRNA transfection on MDM-2 mRNA levels in MCF-7 cells using real-time PCR. The figure represents the expression levels of mRNA normalized with β-actin as a housekeeping gene. *P*-values <0.05 were considered as statistically significant.

MDM-2: Murine double minute-2: MCF-7: Michigan Cancer Foundation-7; PCR: Polymerase chain reaction

mRNA expression level of BCL-2 ($P < 0.05$/fold change:0.41), as an antiapoptotic gene, in siRNA transfected MCF-7 cells compared with scramble transfected MCF-7 cells (Figure 3).

Flow cytometric apoptosis assay

To determine whether apoptosis reduced the cell viability after siRNA treatment, we analyzed the percentage of annexin V/7AAD cells 48 h after treatment with siRNA. Compared with the control cells, the treatment of MCF-7 cells with siRNA showed a significant increase in apoptosis from 32.3% in the controls up to 56.6% in the MCF-7 siRNA-transfected cells (Figure 4).

Discussion

The results showed that the entry of MDM2 siRNA into MCF-7 cells significantly reduced the mRNA expression of MDM2 gene. In addition, the expression of the antiapoptotic gene of BCL-2 was significantly reduced in the transfected MCF-7 cells, whereas the expression of BAX and BID genes increased. MDM2 is expressed in various types of breast cancer and its overexpression is associated with poor prognosis in breast cancer.[26] In fact, MDM2 overexpression promotes tumorigenic potential through blocking p53,[27] which is one of the most important tumor-suppressor proteins with inhibitory effects on cell proliferation and cell growth.[28, 29] p53 promotes the apoptosis of cancerous cells.[29] The tumor suppressor gene, p53, is often blocked in breast cancer cells due to the mutation or upregulation of its inhibitors, including MDM2 and MDM4, which could entail the uncontrolled growth of tumor cells.[4, 27] Therefore, MDM2 inhibition can be helpful in the restoration of p53 activity. Such interventional therapy could be a promising strategy for future interventional therapy of breast cancer. In the present study, we observed the overexpression of MDM2 in the MCF-7 cell lines. Thus, we aimed to investigate the feasibility of siRNA-specific MDM2 in the inhibition of MDM-2 expression and its effect on the induction of apoptosis pathway via the restoration of blocked p53. Apoptosis of cancerous cells is highly important in cancer studies. The importance of p53 in the induction of apoptosis was first confirmed in the murine myeloid leukemia cell lines lacking p53 gene.[30] p53 induces apoptosis through the transcriptional activation of pro-apoptotic target gene of intrinsic or extrinsic apoptosis pathways.[30] Given the involvement of p53 in the induction of the pro-apoptotic BCL-2 family members BAX and the BH3-only proteins BID, PUMA, and Noxa,[31-33] we were prompted to investigate the effect of p53 restoration on the induction of proapoptotic genes of intrinsic or mitochondrial apoptosis pathway.[34] Thus, the antiapoptotic effects of MDM2 on the p53 activity were studied in MDM-2 knockdown MCF-7 cell

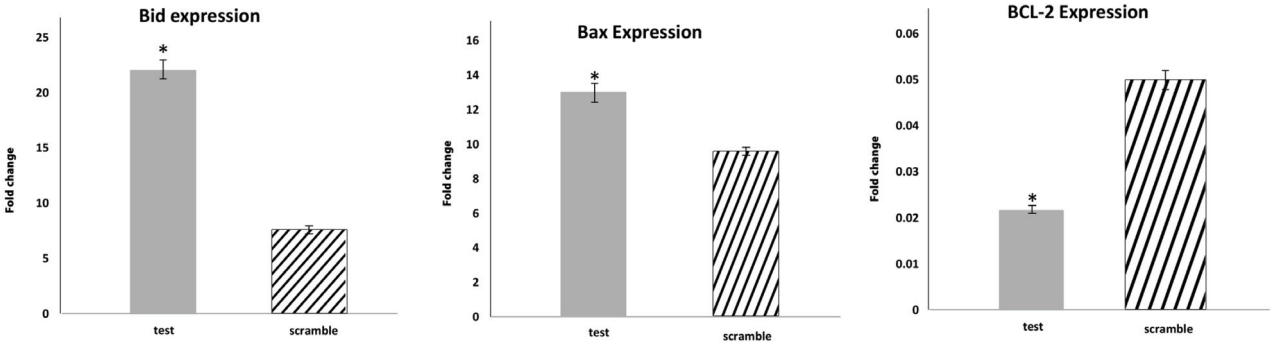

Figure 3. mRNA expression levels of a) BID, b) BAX, and c) BCL2 in MCF-7 cells, siRNA MDM-2 transfected cells, and scramble transfected MCF-7 cells. The figure represents the significant increase in the mRNA expression levels of BID (fold change: 2.73) and BAX (fold change: 1.29) and the significant reduction in the mRNA expression levels of BCL2 (fold change: 0.41). *P*-values < 0.05 were considered as statistically significant.

BID: BH3 interacting-domain death agonist; BAX: BCL-2 -associated X protein; BCL-2: B cell lymphoma 2

lines through studying the apoptosis pathway. For this purpose, we examined the mRNA expression of BID and BAX as apoptotic genes and BCL-2 as an antiapoptotic gene. Based on the results, the entry of MDM2-specific siRNA into MCF-7 cells significantly decreased the MDM2 gene. As expected, following the knockdown of MDM2, the mRNA expression of BID and BAX increased and the mRNA expression of BCL-2 decreased. In line with our study, p53 protein can activate the apoptosis pathway through inhibiting the BCL-2 molecule.[34] As a result, MDM2 inhibition activates p53, so the low BCL-2 expression by the treated MCF-7 is due to the inhibitory activity of p53 on the BCL-2 molecule. A previous study showed that p53 was able to directly activate the PUMA protein, promoting apoptosis pathway through inhibiting BCL-2 molecule, which is in line with our results.[35] In the present study, BCL-2 expression decreased significantly, while BAX and BID genes showed valuable increased expression (P-value < 0.05) leading to the activation of the apoptosis pathway and death of cancerous cells. Our flow cytometry analysis of MCF-7 cell line apoptosis by Annexin/7-AAD showed a significant increase in apoptosis from 32.3% in the control cells to 56.6% in the MCF-7 siRNA-transfected cells. In accordance with the current research, the recombinant proteins inhibiting the expression of MDM2 and MDM4 proteins eventually increased the expression of p21, BAX, and PUMA proteins that regulated the cell cycle progression and cancer cell apoptosis.[35]

Conclusion

In summary, it was shown that the function of apoptotic pathways is directly related to the expression and function of MDM2 protein. Accordingly, inhibiting MDM2 protein by increasing the p53 activity and inducing apoptotic pathways resulted in the expression of pro-apoptotic genes and the death of cancer cells. Finally, it is suggested that after inhibiting the MDM2 protein, the level of p53 expression should be investigated for a more accurate examination of apoptotic death through a p53-dependent pathway. Also, it is recommended that the efficacy of drug therapy should be examined in combination with blocking MDM2 in the tumorigenic animal model.

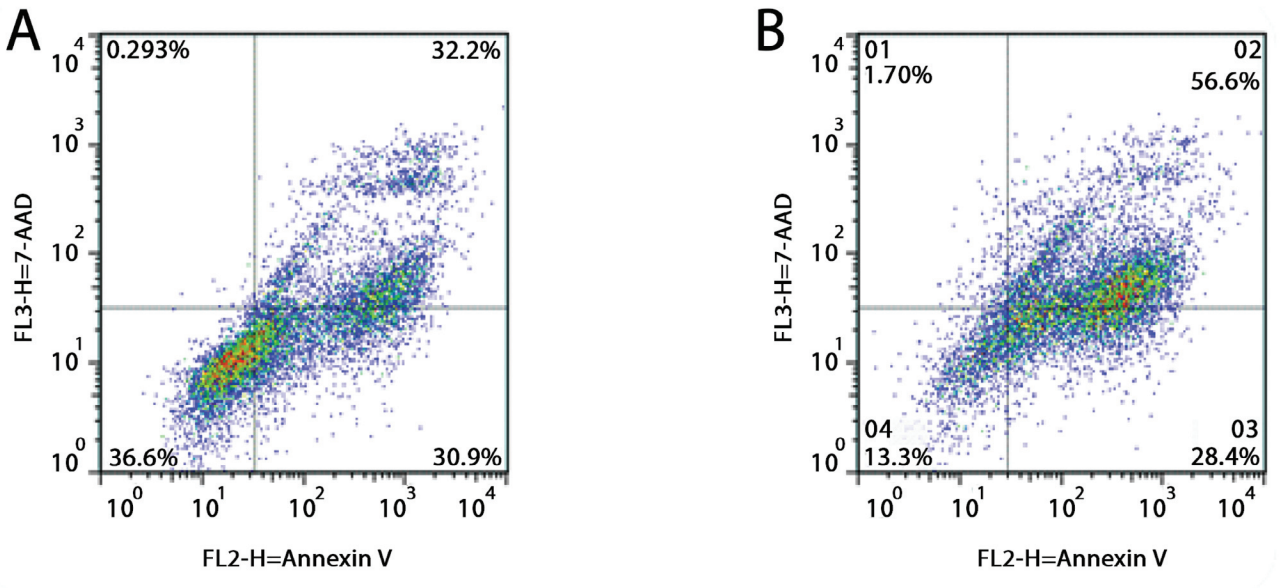

Figure 4. Flow cytometry analysis of apoptosis in MCF-7 siRNA-transfected cells. Compared with the control cells (scramble treated-cells), the treatment of MCF-7 cells with siRNA showed a significant increase in apoptosis from 32.3% in the controls up to 56.6% in the MCF-7 siRNA-transfected cells.
MCF-7: Michigan Cancer Foundation-7

Acknowledgment

This study was extracted from the MSc. thesis of Mr. Bahram Mohseni-aghdam with the ethics code of IR.sums.REC.1395.S643 to complete MSc. degree. Authors would like to thank Dr. Nasrin Shokrpour at the Research Consultation Center of Shiraz University of Medical Sciences for the English editing of the manuscript.

Funding

This study was financially supported by Shiraz University of Medical Sciences, Shiraz, Iran (Grant no:95-01-10-12383).

Conflict of Interest

None declared.

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