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Doxorubicin Induces Apoptosis through down Regulation of miR-21 Expression and Increases miR-21 Target Gene Expression in MCF-7 Breast Cancer Cells

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Abstract

miRNAs play an important regulatory role in variety of cellular functions and several diseases, including cancer. MicroRNA-21 (miR-21) is overexpressed in almost all types of human cancers. Studies revealed that the knockdown of miR-21 results in reduced tumor cell growth, cell cycle arrest and cell apoptosis. In this study, we evaluated the effect of doxorubicin on miR-21 expression in mcf-7 breast cancer cells. miRNA was extracted from mcf-7 cells treated with doxorubicin and untreated cells using miRNeasy Kit (Qiagen) according to the manufacturer's instructions. cDNA synthesis was performed using miScript II RT Kit (Qiagen) and Real Time-PCR was performed using Real Q Plus 2x Master Mix Green-(Ampliqon, Denmark). The relative expression of miR-16 and miR-21 was calculated using comparative Ct method. All tests were run in triplicate to minimize the experimental errors. Samples with a Ct > 37 were excluded from the analysis. Statistically, a significant decrease in cell proliferation of mcf-7 cells was found in doxorubicin group compared with control groups 24 hours after transfection, dose dependently (p value< 0.001). After 24 hours, Doxorubicin (100 μm) significantly decreased miR-21 expression in mcf-7 cells (p = 0.0001). Also, the expression of caspase 9 sig-

nificantly increased after Doxorubicin (100 μm) treatment (p = 0.0003). Together, these findings indicate that miR-21 plays a key role in regulating cell apoptosis in mcf-7 cells and may serve as a target for effective therapies.

Keywords

miR-21, mcf-7 Cells, Caspase 9, Cancer

1. Introduction

Cancers are a group of disease with abnormal cell growth and potential to spread to different tissues of body [1]-[6] Despite much progress in the management of cancer, cancer is still a major public health problem and one of the deadliest diseases worldwide, with approximately 14 million new cases and 8.2 million death each year [7] [8]. Breast cancer is a very common malignant tumor among female patients and it is estimated that 1 in 10 women worldwide is affected by breast cancer during their lifetime [9]. Many studies were interested to identify specific molecules involved in breast cancer and understand their characteristics [10]. The rapidly increasing technology development leads to the identification of many biomarkers which are easily detectable, measurable, dependable, and inexpensive with a high sensitivity and specificity which play a critical role in breast cancer [11] [12] [13]. MicroRNAs (miRNAs) are a class of small, non-coding, single-stranded RNAs with a 19 - 25 nucleotide length which are found in both animals and plants [14]. MiRNAs are a novel group of gene regulators, binding to complementary sequences in the 3' untranslated region (UTR) of their target mRNAs [15]. MiRNAs are negative regulators of gene expression which induce mRNA degradation or translational repression [16]. Over the past years, it was shown that many miRNAs are influential in the development of many human cancers [17]. miRNA dysregulation is shown to contribute to cancer development through a range of mechanisms [18]. Identified in many types of tumors, miRNAs can act as oncogenic or tumor suppressors [19] Dysregulation of miRNA expression has been implicated in estrogen-related diseases including breast cancer and endometrial cancer [20]. MiR-21 is one of the most extensively investigated miRNAs which is tightly regulated by a variety of extracellular and intracellular signaling molecules. The important target genes of miR-21 are involved in cell proliferation, activation, and apoptosis [21]. MicroRNA-21 (miR-21) is overexpressed in almost all types of human cancers [22] [23]. Several studies using cell lines revealed that miR-21 knockdown results in reduced tumor cell growth, cell cycle arrest and cell apoptosis [24] [25]. In this study, we evaluated the effect of doxorubicin on miR-21expression in mcf-7 breast cancer cells.

2. Methods & Materials

Doxorubicin was purchased from Sigma. All primers were produced by Pishgam

company (Iran). The media, FBS, trypsin and antibiotics were purchased from Gibco.

2.1. Cell Culture

Mcf-7 cells were purchased from the Pasteur institute of Iran and were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin (100 µg/ml), incubated at 37˚C in a humidified atmosphere of 5% CO_2 95% air. All experiments were performed in a similar medium contain.

2.2. MTT Assay

Cell survival was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as previously described [20]. Cells were cultivated at sub confluence before being washed twice with phosphate-buffered saline (PBS). Cells were then resuspended in culture medium with FBS, counted, and plated in 100 µL media at 15×10^3 cells/well in 96-well microliter plates. After 24 hours, the cells were washed and treated with doxorubicin. The best concentrations of doxorubicin were calculated. MTT absorbance was measured at 492 nm.

2.3. miRNA and Total RNA Extraction and First Strand cDNA Synthesis

miRNA was extracted from treated and control cells using the miRNeasy Kit (Qiagen) according to the manufacturer's instructions. First strand cDNA was synthesized from miRNA using high Specificity miRNA 1st-Strand cDNA Synthesis Kit (Agilent Technologies, USA). cDNA synthesis was performed in 2 steps. First, a polyadenylation reaction was performed at 37˚C for 30 minutes and then the reverse transcription step was conducted.

2.4. Quantitative RT-PCR for miRNA Expression

Quantitative Real-Time PCR reactions were performed in Rotogene Q (Qiagen, Hilden, Germany) in 20 µl of PCR master mix containing 10 µl of SYBR-Green QPCR Master Mix, 1 µl of primer of miR-21, 1 µl universal primer, 1 µl cDNA products and 8 µl of RNase free water. Quantitative Real Time-PCR was performed using SYBR® Premix EX Taq II (Takara, biotechnology, LTD, Dalian, Japan). miR-16 and miR-21 forward primers were CTCGCTTCGGCAGCACA and TAGCTTATCAGACTGATGTTGA, respectively. Forward and reverse primers of caspase-9 were CTCAGACCAGAGATTCGCAAAC and GCATTTCCCCTCAAACTCTCAA respectively and forward and reverse primers of b-actin were CATGTACGTTGCTATCCAGGC and CTCCTTAATGTCACGCACGAT respectively. The relative expression of caspase-9, miR-16 and miR-21 was calculated using comparative Ct method. All tests were run in triplicate to minimize the experimental error. All assays were inspected for distinct melting curves and the Tm was checked to be within known specifications for each particular

assay. Furthermore, the samples must be detected with a Ct < 37 to be included in the analysis.

3. Statistical Analyses

All statistical analyses were carried out using the statistical program SPSS (version 22, SPSS, Chicago, IL, USA); p-values are two-sided throughout, and $p < 0.05$ was considered significant. Baseline quantitative results are expressed as mean ± SD; Comparison between groups was performed using unpaired student's t test.

4. Discussion and Conclusions

Doxorubicin induced cell proliferation in mcf-7 cells. After 24 h of incubation with different doxorubicin concentrations (50 nm to 500 μM), a decline of about 30% in cell numbers was observed (**Figure 1**). Among different concentrations, 100 μM concentration was chosen for next experiments. A significant decline was observed in miRNA-21 expression in MCF cells which were treated with 100 μM doxorubicin (**Figure 2**). Considering the role of mir-21 in breast cancer, the results show that doxorubicin can be effective in breast cancer. Next, we investigated the effect of doxorubicin in caspase-9 expression, because caspase-9 is an important upstream factor leading to apoptosis. Consistent with the result, we found that 100 μM doxorubicin caused a significant increase in caspase-9 expression in mcf-7 cells (**Figure 3**).

In this study, we demonstrate that doxorubicin decreases mir-21 expression and increases caspase-9 expression in mcf-7 cells. Breast cancer is one of the most commonly diagnosed types of cancer among women [26] [27] [28]. Chemotherapy is an important component in the treatment of breast cancers [29] [30]. Recent studies have focused on new approaches to treat breast cancer [31]. Thus, understanding the molecular mechanisms involved in the progression of

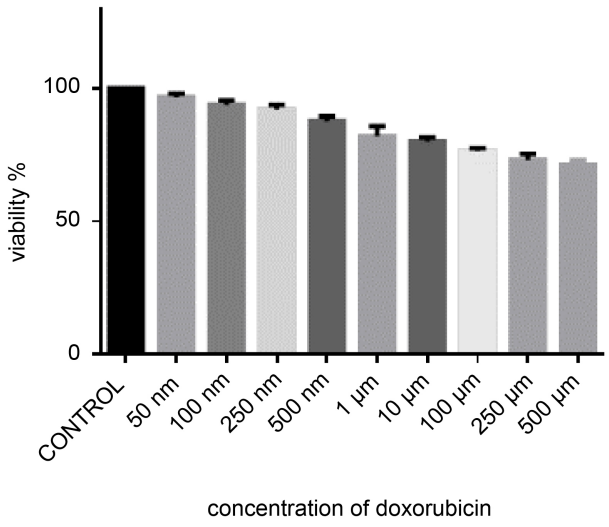

Figure 1. MTT assay for mcf-7 cells treated with different concentration of doxorubicin.

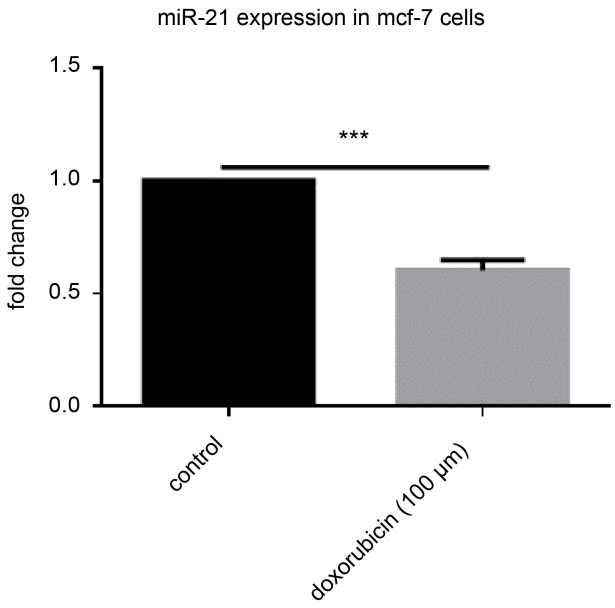

Figure 2. The expression of miR-21 in mcf-7 cells treated with doxorubicin 100 µm and control cells. miR-21 expression was significantly reduced in mcf-7 cells treated with doxorubicin 100 µm. *** p value < 0.001.

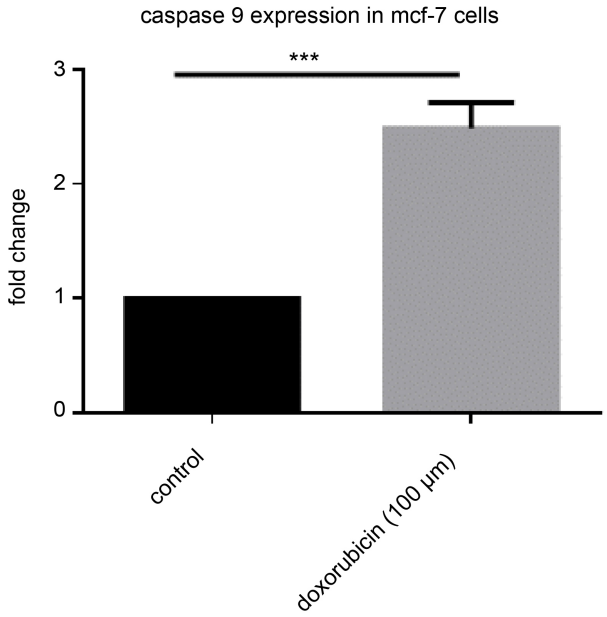

Figure 3. The expression of caspase9 in mcf-7 cells treated with doxorubicin 100 µm and control cells. Caspase 9 expression was significantly increased in mcf-7 cells treated with doxorubicin 100 µm. **** p value < 0.001.

breast cancer is crucial. In the recent years, micro-RNAs have attracted the attention of many researchers [32]. As a result, it has become clear that the dysregulation in the expression of microRNA (miRNA) genes contributes to the pathogenesis of most human cancers and these dysregulations can be caused by

different mechanisms [33]. According to the relationship between dysregulated expression of miRNA genes and the development of cancers, these miRNAs provide important opportunities for the development of future miRNA-based therapies [34] [35]. miR-21 has been found to be overexpressed in many cancers, including breast cancer [36] [37] [38]. In a study conducted by Yan L. X *et al*. in 2008, it was shown that miR-21 is highly up-regulated in breast cancer cell lines, which suggests that miR-21 overexpression is correlated with specific breast cancer bio pathologic features, such as advanced tumor stage, lymph node metastasis, and poor survival of the patients, indicating that miR-21 may serve as an oncogene [39]. Another study also confirmed that the down-regulation of miR-21 can lead to apoptosis caused by increased amounts of caspases-9 [40]. To our knowledge, this is the first report that doxorubicin down-regulates miR-21 and thus, it upregulates the protein expression of miR-21 target gene caspase-9 in MCF-7 human breast cancer cells. The results of our study demonstrated that suppressing miR-21 by doxorubicin increases caspas-9 expression and increases apoptosis. Li Xu Yan *et al*. showed that the knockdown of miR-21 in MCF-7 cells inhibits in vitro and in vivo growth as well as in vitro migration. Also, they suggest that inhibitory strategies against miR-21 using antimiR-21 may provide potential therapeutic applications in breast cancer treatment [40]. Taken together, miR-21 is shown to affect several targets which are very effective in apoptosis process including caspase-9. The results of this study suggest that miR-21 is an oncogenic miRNA and plays a role in apoptotic pathways.

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