miR-21 Knockdown Reduces Cell Viability and Induces Cell Apoptosis in Breast Cancer Cell Line MDA-MB-231

Accepted 8th August, 2016

ABSTRACT
MicroRNAs (miRNAs) are small non-coding RNAs that negatively modulate gene expression. A key oncomir in breast carcinogenesis is miR-21, which is upregulated in breast tumors. This enhanced expression is associated with advanced clinical stage and metastasis of breast cancer. In this study, we used anti-miR-21 to explore the role of miR-21 in human breast cancer cell line MDA-MB-231. We cultured MDA-MB-231 cells to explore the role of miR-21 in the cell viability using MTT assay and flow cytometry techniques. Using anti-miR-21 transfection, we knockdown miR-21 and then we measured the mRNA expression of its targets (BCL2 and PTEN) using quantitative PCR. Anti-miR-21 inhibited growth of MDA-MB-231 cells. It also changes cell morphology which is a sign of induced apoptosis. Knockdown of miR-21 significantly increased the expression of PTEN and BCL2. Flow cytometry assays showed decrease in cell viability and increased cell portion in late apoptosis. Knockdown of miR-21 MDAMB-231 cells inhibits cell growth and reduces cell viability. Anti-miR-21 transfection also induce cell death which shows its potential as a therapeutic application in breast cancer treatment.

Key words: miR-21, breast cancer, cell death, BCL2, PTEN.

INTRODUCTION
MicroRNAs (miRNAs) are post-transcriptional modulators of gene expression. These small noncoding RNAs (20–24 nucleotides long) negatively regulate the stability or translational efficiency of their target mRNAs (Ambros 2003). miRNAs are involved in various cellular functions like differentiation, proliferation and apoptosis (Chen et al., 2004; Croce et al., 2005). Thus alteration in miRNA expression might change the normal cellular growth, development and function. Previous reports have clearly demonstrated the significant changes in miRNA expression in cancerous tissues as compared to the normal cells (Mattie et al. 2006; Yanaihara et al. 2006). These RNAs may also act as either tumor suppressors or oncogenes (Johnson et al. 2005; He et al. 2005). The previous findings represent the significant potential of miRNAs in cancer research. A specific miRNA, named miR-21, has been found to be upregulated in three most leading and deadly cancers including breast, lung and colorectal cancer (Iorio et al. 2005; Yanaihara et al. 2006; Slaby et al. 2008; Siegel et al. 2015).

According to recent miRNA expression profiling studies, miR-21, which is a oncomiR was found to be significantly upregulated in breast cancer. An overexpressed miR-21 can be associated with advanced clinical stage of cancer and the occurring of metastasis, which shows the importance of this RNA in breast cancer (Iorio et al., 2005; Yan et al., 2016). Regarding its expression pattern in breast cancer, miR-21 is suggested to be a potential prognostic marker for the prompt and effective diagnosis of this disease. (Yan et al., 2016). Moreover, overexpression of miR-21 has been reported to confer chemo-resistance of breast cancer cells against tamoxifen
(Zhao et al., 2008; Miller et al., 2008).

Previous studies have reported that miR-21 plays its role by targeting the key tumor suppressor genes including PTEN and Bcl-2. (Si et al., 2007; Zhang et al., 2012). Regarding the regulatory role of miR-21 on the PTEN, associated with PI3K/AKT signaling pathway in cancer cells, its inhibition would be a good strategy for controlling the tumor growth (Xiong et al., 2013). Although, Bcl-2 protein family has been reported to determine the commitment of cells towards apoptosis and the deregulation of this protein can result in tumor maintenance in different kinds of cancers (Czabotar et al., 2014).

In the present study, we inhibited the miR-21 expression using anti miR-21 transfection in breast cancer cell line MDA-MB-231. Then, we evaluated the effects of miR-21 inhibition on the morphology, growth, viability and apoptotic potential of above-mentioned cells. The PTEN and Bcl-2 expression was also evaluated in these cells following the inhibition of miR-21 level.

MATERIALS AND METHODS

Cells and reagents

Human breast cancer MDA-MB-231 cells were purchased from Avicenna Institute, Tehran, Iran. Cells were grown in Roswell Park Memorial Institute (RPMI)-1640 medium, supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin antibiotics, obtained from Life Technologies GmbH (Darmstadt, Germany). The cells were incubated at 37°C under 5% CO2 atmosphere and 90 to 95% humidity. Lipofectamine 2000, scrambled (non-target) and anti-miR-21 were purchased from Exiqon (Denmark). 3’-(4, 5 dimethylthiazol 2yl)2, 5 diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich (Saint Louis, USA). Ribonuclease A and RT-PCR reagents were provided by iNtRON Biotechnology, Seoul, Korea.

Anti-miR-21 transfection

Cells were seeded under the aforementioned conditions and transfected at 30 to 50% confluency with either of the 50 nM anti-miR-21, 50 nM non-target miRNAs (Scrambled), or the transfection reagent lipofectamine 2000 alone for 8 h without FBS or antibiotics. Then, the culture media were replaced with the medium containing 10% FBS plus antibiotics and incubation was continued for another 48 h.

MTT colorimetric assay

At the end of the incubation period in transfection stage, 10 µl MTT solution (5 mg/ml) was added to each well, followed by incubation at 37°C for 3 h. Then, the formazan crystals were revealed by removing the medium and adding 100 µl of dimethylsulfoxide (DMSO) to each well, followed by gentle shaking for the period of 5 min at room temperature and the absorbance (A) value measured at the wavelength of 570 nm with Elisa reader (Awnernes, USA). Individual samples were analyzed in quintuplet as against a background of blank wells. Cell growth from three independent experiments was expressed as the mean percentage of cell growth relative to the control group.

Flow cytometric analysis of cell cycle

In order to carry out the flow cytometric analysis of the cell cycle arrest, the cells were transfected as follows: The cells floating in the medium and the harvested attached-cells were washed with cold phosphate buffered saline (PBS) and fixed with cold 70% ethanol at 4°C. After 2 h, the fixed cells were pelleted and stained with 10 µl of propidium iodide (2 µg /ml) in the presence of 5 µl Ribonuclease (100 µg /ml) for 30 min at 37°C. Data were analyzed by using Win MDI 2.9 software (Beckman Coulter, CA, USA). Results obtained from three independent experiments (mean ± SD) were expressed as the percentage of alive, apoptotic and necrotic cells.

RNA extraction and real time PCR

Isolation was performed using TRlzlol method according to the manufacturers’ instructions and the RNA concentration was determined using Nanodrop (Thermo scientific, Wilmington, USA). First-strand of cDNA was synthesized from 2 µg total RNA by 1 µl reverse transcriptase with 1 µl oligodTs, 1 µl dNTPs, 0.5 µl ribonuclease inhibitor, 4 µl reaction buffer and the DEPC RNase-free distilled water was used to reach the final volume of 20 µl. The cDNA was amplified by polymerase chain reaction (PCR) using the following primers: Human PTEN, forward: 5- TTTCCAGCTTCTCAGATGGTT-3, and reverse: 5- TTTCCAGCTTCTCAGATGGTT-3 at 58°C and Human BCL2, forward: 5- AACGTCGCTCTAGATGGTT-3, and reverse: 5- TTTCCAGCTTCTCAGATGGTT-3 at 58°C. The human GAPDH expression was assessed as an internal control with the following primers: forward: 5- TGTGAAGTGGCTTGGATTGTT-3 and reverse: 5- TGTGAAGTGGCTTGGATTGTT-3 at 58°C.

Statistical analyses

Data were analyzed using the statistical software package SPSS 19.0 (SPSS, Inc., Chicago, IL, USA). The normality of nominal variables was analyzed by using the Kolmogorov–Smirnov test. One way ANOVA and unpaired 2-tailed
Students' t-test were used for comparing the data among experimental groups. All experiments were conducted in triplicate and the results expressed as mean ± SD. Probability values of ≤0.05 were considered to be statistically significant.

RESULTS

miR-21 inhibition affects the cell growth and morphology

Figure 1 shows that 50 nM anti-miR-21 could inhibit the growth of MDA-MB-231 cells, but also induce some of the MDA-MB-231 cells towards apoptosis or necrosis, which was evaluated by morphological observations. These apoptosis-related features were observed in transfected cells, based on characteristic morphological characters (Figure 1A). To identify the alterations in cell growth, the MTT colorimetric assay was evaluated in MDA-MB-231 cells. The present results showed that the growth of MDA-MB-231 cells significantly decreased 48 h post-transfection of anti-miR-21 transfection (Figure 1B).

Suppression of miR-21 impairs the MDA-MB-231 late apoptosis

To explore the suppression mechanism of miR-21 in MDA-MB-231 cells, the effects of this RNA were evaluated on the
necrosis and early/late apoptosis of experimental cells. The flow cytometric data revealed that cell viability distribution was induced by miR-21 inhibition (Figure 2A). The number of cells in late apoptotic phase increased significantly in this group, but the changes in necrotic and early apoptotic cells was not significant (Figure 2B).

**Bcl-2 and PTEN expression increases after miR-21 inhibition**

Real-time PCR analysis was used to evaluate miRNA level in treatment and control groups. Our data showed 70% increase in Bcl-2 RNA level in anti-miR-21 transfected cells in comparison with scrambled transfected group. PTEN RNA level also increased by near 3 folds in these cells (Figure 3).

**DISCUSSION**

Breast cancer is the 5th leading cause of mortality in Iranian female population (Akbari et al., 2008). Recently, different molecular aspects of breast cancer have been thoroughly investigated by different researchers. One of them is the alteration of miRNA expression pattern, which affects the cells’ potential of proliferation, apoptosis and differentiation, etc (Mattie et al., 2006). Moreover, different studies have observed the over-expression of miRNA in other cancers as well (Kumar et al., 2007; Wang et al., 2010). It has been previously shown that miR-21 is aberrantly overexpressed in human breast cancer as compared with normal breast tissue (Iorio et al., 2005; Si et al., 2007). It has also been reported that anti-miR-21-associated with increased apoptosis and decreased cell proliferation mediates cell growth inhibition. These results
sugges that miR-21 functions as an oncogene and modulates tumorigenesis through the regulation of some important genes, such as Bcl-2 and might serve as a novel therapeutic target (Si et al., 2007).

Given these facts, in this study, we have focused on the impact of miR-21 inhibition on the cell cycle mechanism of MDA-MB-231 cells and the molecular signaling associated with it. As described in manufacturers’ instruction, 50 nM concentration of this RNA was chosen for transfecting the cells. Figure 1A shows that the cell growth significantly decreased after anti-miR-21 transfection, while the cell viability did not change dramatically, but cell morphogenesis changed in transfected cells (Figure 2B).

Anti-miR-21 transfection affected the spherical shape of experimental cells, which is indicative of the morphological sign of apoptosis or necrosis. This suppressive effect of miR-21 on the cell morphology has also been reported on the laryngeal carcinoma cells (Liu et al., 2009). To measure cell viability and identify the cells in early or late apoptosis and necrosis, the flow cytometric assay was performed. The present results showed that anti-miR-21 transfection had a significant role in cell death. Most of the degrading and apoptotic cells were observed to be undergoing late apoptosis, but the ratio of early apoptotic and necrotic cells was not comparatively significant.

For a better understanding of molecular apoptosis-inducing events, quantitative PCR was performed for pro-apoptotic protein, PTEN and anti-apoptotic protein, Bcl-2. PTEN expression increased more than 3 folds in anti-miR-21 transfected cells, which is in line with the previous reports (Zhang et al., 2012; Xiong et al., 2013). The present results showed that PTEN is a target of miR-21 and miR-21 inhibition resulted in the over expression of PTEN and cell arrest as well (Meng et al., 2007; Park et al., 2009). In contrast, the over expression of Bcl-2 was inhibited in the cells treated with miR-21, which does not agree with the previous breast cancer-related studies (Si et al., 2007). Our data have also demonstrated that the anti-miR-21 transfected cells showed almost 70% more RNA expression for Bcl-2. In fact, Si et al. (2007) in their study showed that the inhibition of miR-21 in MCF-7 breast cancer cells decreased the Bcl-2 protein expression, while, in our study, it turned out different for the RNA expression. All the present results suggest a different regulation pattern in MDA-MB-231 cells.

On the other hand, Bax/Bcl-2 ratio is a more important factor in apoptosis related to miR-21 level (Shi et al., 2010). As such, the Bax as a key molecule, affecting the Bcl-2 expression in apoptosis should be considered. There is a possibility of dramatically over expressed Bax after miR-21 inhibition, which neutralizes the over expression of Bcl-2.

**Conclusions**

We have demonstrated that MDA-MB-231 cells, transfected with anti-miR-21 showed growth inhibition and induction of cell death. In addition, PTEN and Bcl-2 are
known to be the target of miR-21 and their overexpression followed by miR-21 inhibition resulted in significant amount of apoptosis. These results suggest that the inhibition of miR-21 by using anti-miR-21 may proved to be the potential agent in therapeutic applications of breast cancer treatment.

REFERENCES


Cite this article as:


Submit your manuscript at http://www.academiaipublishing.org/journals/ajsr