Knockdown of microRNA-29a regulates the expression of apoptosis-related genes in MCF-7 breast carcinoma cells

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Received February 2, 2017; Accepted November 30, 2017

DOI: 10.3892/mco.2017.1528

Abstract. MicroRNA (miR), as non-coding small RNA, are key regulators of cancer-related biological cell processes and contribute to tumor growth through regulation of groups of pro- and anti-apoptotic genes. The present study aimed to investigate the effects of miR-29a on the expression of genes involved in apoptosis, including p21, B-cell lymphoma 2 (BCL-2), p53 and survivin. The MCF-7 breast cancer cell line was transfected with anti-miR-29a and treated with Taxol in subdivided treatment groups including: Scramble; anti-miR-29a; anti-miR-29a + Taxol; Taxol; and control. Expression levels of p21, BCL-2, p53 and survivin were evaluated using reverse transcription-quantitative polymerase chain reaction. miR-29a knockdown resulted in p21 and p53 upregulation and a decrease in survivin expression. These results indicated that miR-29a inhibition regulates apoptosis. The present data suggested that miR-29a inhibition may be a promising strategy for the induction of apoptosis of tumor cells.

Introduction

Breast cancer is the most common cancer and continues to be a major cause of morbidity and mortality among women worldwide (1). Global statistics show that >1.15 million women each year are diagnosed with breast cancer, which results in >502,000 mortalities (2-4). This multifactorial disease occurs as a result of several risk factors, including female gender, increasing age, familial history of breast cancer, diet, obesity and life behavior, such as smoking and alcohol consumption (5,6). Based on the increased incidence of breast cancer (2), early detection will improve treatment success. In this regard, investigating appropriate oncomarkers for early detection, monitoring and treatment appears to be crucial. Due to limited treatment options, resistances to current standard therapies, the short duration of response and rapid relapse (7,8), finding novel therapeutics is considered as extremely important. MicroRNA (miR) may be one of these therapeutic targets.

miR are class of endogenously expressed, single-stranded, non-protein coding small RNA that bind to target mRNA. miR have important roles in diverse biological and pathological processes via both transcriptional and post-transcriptional gene regulation, making them attractive biomarkers in cancer (9,10). Research has demonstrated that miR have important roles in regulating cell processes that are often deregulated in cancer, including proliferation, differentiation and apoptosis (11). The miR-29 family has three main members, including hsa-miR-29a, hsa-miR-29b and hsa-miR-29c (12). Research has indicated that miR-29a negatively regulates the expression of Myb-related protein B, resulting in inhibition of breast cancer growth and arrest of cells in the G0/G1 phase (13). Downregulation of myeloid cell leukemia 1, as an anti-apoptotic protein, by miR-29b in malignant KMCH cholangiocarcinoma cells has been reported (14). Although the mir-29 family is involved in regulating tumorigenesis and development of various types of cancer, the role and the underlying mechanism of miR-29a in breast cancer remains to be fully elucidated (15).

B-cell lymphoma 2 (BCL-2) is a prototypical member of the BCL-2 family, which has been identified as a regulator of apoptosis (16). This molecule prevents the mitochondrial pathway of apoptosis (16,17). It has been reported that the miR-15a and miR-16-1 region is deleted in chronic lymphocytic leukemia, which leads to an aggressive state (18). These miR naturally induce apoptosis in normal cells through regulation of the anti-apoptotic, proto-oncogene BCL-2 (18). BCL-2 expression may be downregulated by miR-34, which is compatible with the role of miR-34 in apoptosis mediated by p53 expression (19). p53 is a pleiotropic regulator of cell fate, which may facilitate

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Key words: microRNA-29a, breast carcinoma, apoptosis, Taxol, MCF-7

apoptosis (20,21). miR have been identified to regulate p53 expression by controlling the upstream regulation of p53 and its pro-apoptotic function, as research has demonstrated that miR-125 negatively regulates p53 (22). Research has indicated that the expression level of p53 protein was decreased and apoptosis was stopped in human neuroblastoma and in lung fibroblasts (22). p21 is induced by p53-dependent and -independent mechanisms implicated in both major functions of the tumor suppressor-cell cycle arrest and apoptosis (23).

Survivin is overexpressed in the majority of human malignancies and functions as a key regulator of mitosis and programmed cell death (24,25). Research has demonstrated that miR-218 is involved in breast cancer evasion of apoptosis by targeting survivin (26). This molecule has been targeted for therapy in cancer based on its selective expression in tumors, but not normal tissues (27,28). The present study aimed to evaluate miR-29a knockdown on the expression of important genes involved in cell apoptosis and proliferation of breast carcinoma. In particular, it was proposed that knockdown of miR-29a may alter the expression profile of p53, p21, BCL-2 and survivin genes.

Materials and methods

Cell line. The MCF-7 human breast cancer line was obtained from the Iranian Stem Cell Technology Research Center (Tehran, Iran) and cultured in RPMI-1640 culture medium supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin (all Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37˚C in a 5% CO_2 humidified incubator. At ~80% confluence, adherent cultured cells were harvested following trypsin treatment at 37˚C for 3 min and used for transfection.

Reverse transfection of MCF-7 cells with anti-miR-29a. Cells were transfected using Lipofectamine reagent (Invitrogen; Thermo Fisher Scientific, Inc.) with anti-miR-29a (MIMAT0000086; 5'-UAGCACCAUCUGAAAUCGGUUA-3'; Qiagen, Inc., Valencia, CA, USA), at a final concentration of 50 nM, and scramble at a final concentration of 5 nM in opti-MEM medium (Invitrogen; Thermo Fisher Scientific, Inc.), as described previously (29). Following transfection, MCF7 cells were treated with Taxol [1, 10 and 50 ng/ml; concentrations determined using MTT assay (data not shown)] to inhibit cell growth and trigger apoptosis. Taxol (Paclitaxel) was purchased from Sobhan Oncology Co., (Tehran, Iran). Cells were harvested after 24 h of incubation with Taxol at 37˚C in a 5% CO_2 humidified incubator.

Viability assessment. Studied cells were categorized into the nine following groups: Non-treated; scramble; anti-miR-29a; anti-miR-29a + Taxol (1 ng/μl); anti-miR-29a + Taxol (10 ng/μl); anti-miR-29a + Taxol (50 ng/μl); Taxol (1 ng/μl); Taxol (10 ng/μl); and Taxol (50 ng/μl). A total of 72 h after anti-miR-29a and Taxol treatment in a 5% humidified CO_2 incubator, 100 μl MTT (5 mg/ml) was added to the cells. Optical absorbance of generated formazan was evaluated at a wavelength of 590 nm in each group 4 h following replacement of supernatant by dimethyl sulfoxide. All experiments were conducted three times.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cultured cells in the different groups using an easy-BLUE TM Total RNA Extraction kit (iNtRON Biotechnology, Inc., Sungnam, Korea), according to the manufacturer's instructions, 72 h after transfection and treatment with Taxol. RNA quality and concentration were determined after extraction using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Inc.) and electrophoresis on 2% agarose gel. cDNA was generated using a cDNA Synthesis H Minus First Strand Revert Aid kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Sequences of specific primers for stem loop RT-PCR cDNA synthesis of miR-29a (target gene) and U6 small nuclear (sn)RNA (reference gene) were 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATCAGACTAACCGAT-3' and 5'-AAAATATGGAACGCTTCACGAATTTG-3', respectively. Using general primers (random and oligo dT primers) and a Maxime RT premix cDNA synthesis (iNtRON Biotechnology, Inc.) the cDNA for quantifying p21, p53, BCL-2 and survivin were synthesized.

qPCR was performed using a 2X Real MOD Green PCR Master Mix kit (Takara Bio, Inc., Otsu, Japan), according to the manufacturer's instructions. Briefly, 2 μl cDNA product was diluted in a final volume of 20 μl, containing 10 pmol of each primer, 10 μl 2x reaction mixture of SYBR Green and 7.4 μl sterile deionized water. The cycling program was as follows: Initial denaturation at 95˚C for 10 min, followed by 40 cycles of denaturation at 95˚C for 10 sec, annealing and extension at 55˚C for 40 sec. U6 snRNA and hypoxanthine-guanine phosphoribosyltransferase (HGPRT) were used as internal housekeeping controls. All reactions were performed in triplicate and relative quantity of gene expression was analyzed using the Pfaffl method (30). Sequences of primers used for quantification of the desired genes involved in apoptosis are presented in Table I.

Statistical analysis. Results were expressed as the mean ± standard deviation and analyzed using SPSS v. 16.0 software (SPSS, Inc., Chicago, IL, USA). Differences between groups was evaluated by Kruskal-Wallis tests, and comparison of studied groups for the expression of the desired genes was assessed by Mann-Whitney U tests. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Knockdown of the miR-29a gene in the MCF-7 cell line. Downregulating the expression of miR-29a was the initial aim of the present experiment. In order to achieve this, cells were treated with anti-miR-29a and scramble. The cycle threshold of miR-29a was normalized with U6 snRNA as a reference gene and compared with the non-treated group as a calibrator. As demonstrated in Fig. 1, a significant reduction of miR-29a expression was observed following anti-miR-29a treatment compared with the scramble treatment ($P<0.05$). In cells transfected with anti-miR-29a, the expression of miR-29a was decreased by up to 75% compared with the scramble-treated group.

Effect of miR-29a suppression on cell viability. The viability of MCF-7 cells following knockdown of miR-29a was evaluated

Table I. Primers used for reverse transcription-quantitative polymerase chain reaction.

Gene	Direction	Primer sequence
MicroRNA-29a	F	5'-TGCGCTAGCACCATCTGAAA-3'
	R	5'-CAGTGCAGGGTCCGAGGAT-3'
U6 small nuclear RNA	F	5'-CTCGCTTCGGCAGCACATATAC-3'
	R	5'-ACGCTTCACGAATTTGCGTGTC-3'
p21	F	5'-GAGGCCGGGATGAGTTGGGAGGAG-3'
	R	5'-CAGCCGGCGTTTGGAGTGGTAGAA-3'
p53	F	5'-TAACAGTTCCTGCATGGGCGGC-3'
	R	5'-AGGACAGGCACAAACACGCACC-3'
B-cell lymphoma 2	F	5'-GGTGGGGTCATGTGTGTGG-3'
	R	5'-CGGTTCAGGTACTCAGTCATCC-3'
Survivin	F	5'-AGAACTGGCCCTTCTTGGAGG-3'
	R	5'-CTTTTTATGTTCCTCTATGGGGT-3'
Hypoxanthine-guanine phosphoribosyltransferase	F	5'-GGACAGGACTGAACGTCTTG-3'
	R	5'-ATAGCCCCCCTTGAGCACAC-3'

F, forward; R, reverse.

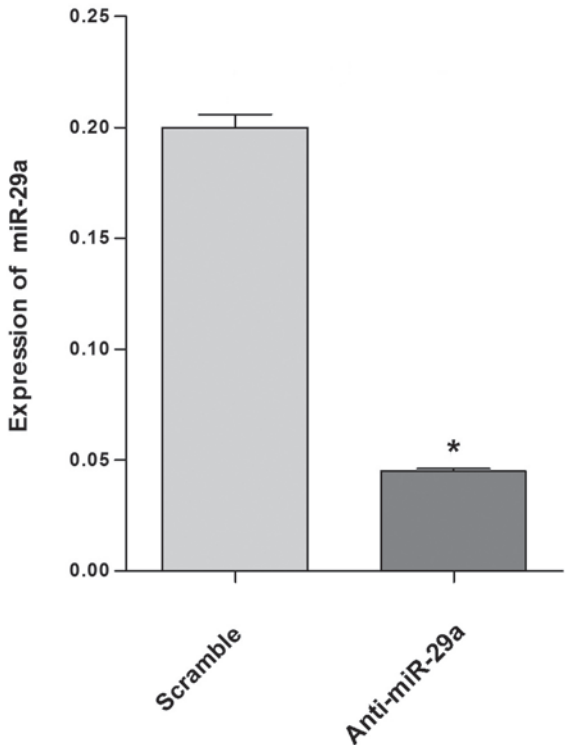

Figure 1. miR-29a gene expression in MCF-7 cells. Cells transfected with anti-miR-29a demonstrated a 75% reduction in miR-29a expression compared with the scramble. Gene expression levels were evaluated relative to the non-treated group and calibrated by the hypoxanthine-guanine phosphoribosyltransferase control gene. Data are presented as the mean ± standard deviation. *P<0.05 vs. scramble. miR, microRNA.

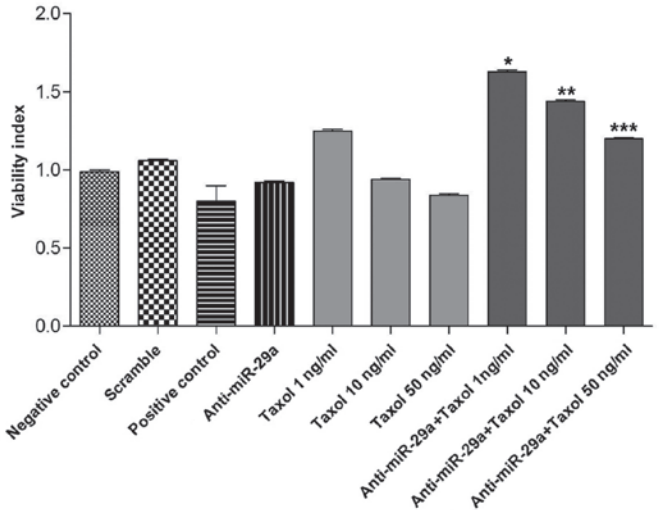

Figure 2. Viability of MCF-7 breast cancer cells following knockdown of miR-29a. Data demonstrate the ratio of obtained absorbance of formed formazan for treated groups and the non-treated group (calibrator). *P<0.05 vs. Taxol 1 ng/ml; **P<0.05 vs. Taxol 10 ng/ml; ***P<0.05 vs. Taxol 50 ng/ml. miR, microRNA.

72 h post-transfection with anti-miR-29a. As demonstrated in Fig. 2, the viability of cells transfected with anti-miR-29a was reduced compared with the scramble and negative control groups; however, this difference was not significant (P>0.05). According to MTT assay, 10 ng/ml Taxol was selected as the optimal concentration. With increasing concentrations of Taxol, cell viability of MCF-7 cells decreased; however, transfection with anti-miR-29a and treatment with Taxol simultaneously resulted in significant increases in the viability of cells compared to treatment with Taxol alone (P<0.05). Therefore, anti-miR-29a induces modification of cell viability following treatment with Taxol.

BCL-2 gene expression decreases following knockdown of miR-29a. Fold changes in the expression levels of genes, including p21, p53, BCL-2 and survivin, are represented in Table II. The cycle threshold of target genes was normalized against HGPRT as a reference gene. Fold changes were obtained by dividing the cycle threshold of each treatment group by the

Table II. Fold changes in p21, p53, BCL-2 and survivin gene expression in the experimental groups.

Group	Gene			
	p21	p53	BCL-2	Survivin
Scramble	0.32±0.070	1.9±0.080	3.27±0.701	0.97±0.021
Anti-miR-29a	1.06±0.030	1.4±0.051	2.1±0.070	0.8±0.020
Anti-miR-29a + Taxol 1 ng/ml	1.46±0.090	1.06±0.011	2.3±0.061	1.07±0.020
Anti-miR-29a + Taxol 10 ng/ml	0.4±0.005	0.5±0.003	0.49±0.030	1.6±0.061
Anti-miR-29a + Taxol 50 ng/ml	0.47±0.011	0.78±0.110	0.62±0.021	0.6±0.040
Taxol 1 ng/ml	0.2±0.006	0.42±0.070	0.16±0.003	1.2±0.090
Taxol 10 ng/ml	0.1±0.005	0.24±0.030	0.12±0.003	1.16±0.051
Taxol 50 ng/ml	0.19±0.020	0.14±0.011	0.43±0.008	1.28±0.010

Data are presented as the mean ± standard deviation. BCL-2, B-cell lymphoma 2; miR, microRNA.

cycle threshold of the non-treated group. Nine groups of MCF-7 cells for each gene were treated: Non-treated (calibrator); treated by scramble; anti-miR-29a; anti-miR-29a + Taxol 1 ng/ml; anti-miR-29a + Taxol 10 ng/ml; anti-miR-29a + Taxol 50 ng/ml; Taxol 1 ng/ml; Taxol 10 ng/ml; and Taxol 50 ng/ml. Kruskal-Wallis and Mann-Whitney U non-parametric tests were used for evaluating the differences in expression levels of the desired genes between the experimental groups.

Results demonstrated that BCL-2 gene expression decreased following knockdown of miR-29a. It has been indicated that BCL-2 has a critical role in the pathogenesis of various types of cancer by regulating apoptosis (31). Thus, the expression of BCL-2 was measured in the different treatment groups. Based on RT-qPCR, it was observed that in cells transfected with anti-miR-29a, the gene expression of BCL-2 was downregulated by ~36% compared with the scramble; however, this difference was not significant (P>0.05). As demonstrated in Fig. 3, transfection with anti-miR-29a + Taxol and Taxol alone caused 50 and 87% reduction, respectively, in expression levels of BCL-2 compared with the non-treated group; however, these differences were not statistically significant. Thus, treatment with anti-miR-29a + Taxol could reduce the expression of BCL-2; however, it could not reduce the level to as low as that observed following treatment with Taxol alone, which was significantly lower than the expression level in the anti-miR-29a group (P<0.05).

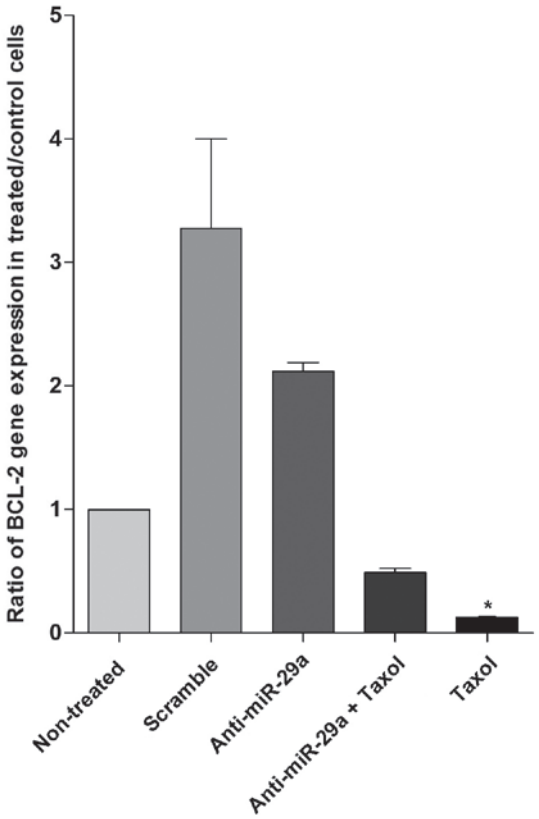

Figure 3. BCL-2 gene expression in MCF-7 cells. Cells were transfected with scramble, anti-miR-29a, anti-miR-29a + Taxol or Taxol alone. Transfection with anti-miR-29a resulted in a 17.5-fold increase in expression of BCL-2 compared with Taxol treatment alone. Data are presented as the mean ± standard deviation. *P<0.05 vs. anti-miR-29a. BCL-2, B-cell lymphoma 2; miR, microRNA.

p53 gene expression increases in anti-miR-29a-treated cells. To further understand the role of miR-29a in the apoptosis of cancer cells, the expression of p53 as a regulatory gene of apoptosis was analyzed by suppressing miR-29a with anti-miR-29a. Based on RT-qPCR, expression levels of p53 increased by ~40% in cells transfected with anti-miR-29a and scramble treatment induced a ~1.9-fold increase in p53 gene expression compared with the non-treated control group. Treatment with anti-miR-29a + Taxol and Taxol alone caused a reduction of 46 and 70%, respectively, in p53 expression compared with the non-treated group (Fig. 4). These results also indicated that p53 gene expression in the anti-miR-29a-treated group was significantly upregulated (5.8-fold higher) compared with the Taxol-treated group (P<0.05).

Inhibition of miR-29a has no effect on the expression level of p21. The role of miR-29a in apoptosis of cancer cells was explored through investigating the gene expression of p21 in MCF-7 cells. There were no significant differences between p21 gene expression levels in cells transfected with anti-miR-29a and the non-treated controls. As demonstrated in Fig. 5**,** transfection with scramble, anti-miR-29a + Taxol and Taxol alone resulted in significant reductions of 80, 60 and

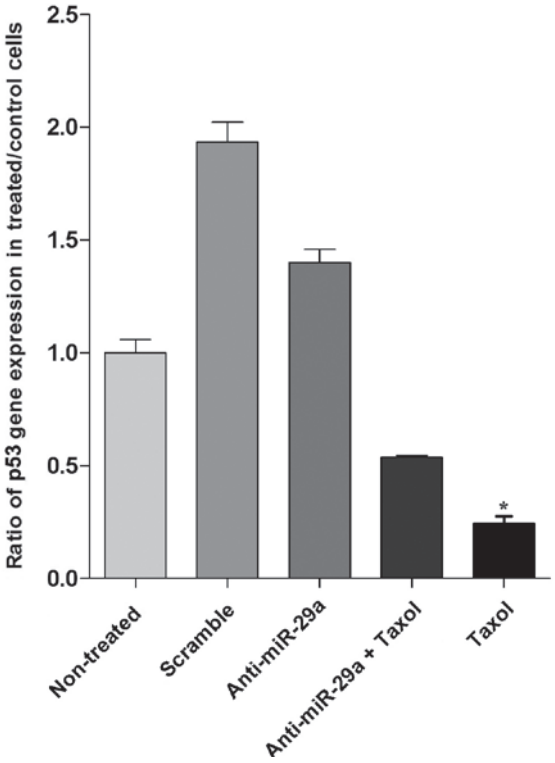

Figure 4. Expression of p53 in MCF-7 cells transfected with scramble, anti-miR-29a, anti-miR-29a + Taxol or Taxol alone. There was an increase in p53 gene expression in anti-miR-29a-transfected MCF-7 cells compared with the non-treated control group; however, this difference was not significant. Data are presented as the mean ± standard deviation. *P<0.05 vs. scramble and anti-miR-29a groups. miR, microRNA.

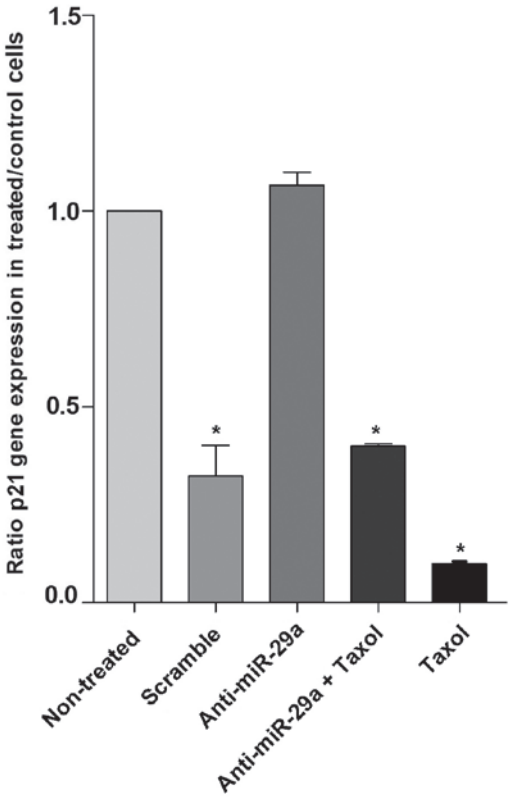

Figure 5. Results of reverse transcription-quantitative polymerase chain reaction for p21 gene expression in treated cells. Cells were transfected with scramble, anti-miR-29a, anti-miR-29a + Taxol and Taxol alone. There were no differences between P21 gene expression in cells transfected cells with anti-miR-29a compared with the non-treated control group. Data are presented as the mean ± standard deviation. *P<0.05 vs. anti-miR-29a. miR, microRNA.

90%, respectively, in gene expression of p21 compared with cells transfected with anti-miR-29a (P<0.05). The expression of p21 in Taxol-treated cells decreased by 10.5-fold compared with cells treated with anti-miR-29a (P<0.05).

Survivin gene expression is reduced in cells transfected with anti-miR-29a. Transfection of MCF-7 cells with anti-miR-29a caused reduction of survivin expression by 17% compared with the non-treated control group. However, there was no significant difference in expression of survivin in cells transfected with scramble and the non-treatment group. The present results demonstrated that transfection with anti-miR-29a significantly reduced the expression of survivin 1.43-fold lower than the Taxol-treated group (P<0.05). As indicated in Fig. 6, survivin gene expression increased significantly, by 1.7- and 1.2-fold, in cells transfected with anti-miR29a + Taxol and Taxol alone compared with the scramble, respectively (P<0.05).

Expression of target genes following transfection with anti-miR-29a. The present experiment demonstrated that inhibition of miR-29a could alter the expression profiles of target genes. As indicated in Fig. 7, following transfection with anti-miR-29a, the expression of BCL-2 increased 2.6-fold higher and 2-fold higher than survivin and p21, respectively. The difference between the expression levels of BCL-2 and survivin were significant (P<0.05). Also, the expression of p21 and survivin decreased 1.3- and 1.7-fold lower than p53 expression, respectively; however, these differences were not significant (P>0.05). Thus, transfection with anti-miR-29a resulted in increases of p53 and BCL-2 gene expression compared to p21 and survivin.

Anti-miR-29a transfection combined with treatment with various concentrations of Taxol results in different expression of target genes. To further analyze the expression of target genes (p21, p53, BCL-2 and survivin), in anti-miR-29a + Taxol-treated cells, it was hypothesized that various concentrations of Taxol would have different effects on gene expression. Therefore, transfection with anti-miR-29a was conducted, along with treatment with 1, 10 or 50 ng/ml Taxol. Results demonstrated that treatment with 1 ng/ml Taxol and anti-miR-29a transfection resulted in increased expression of target genes p21, p53 and BCL-2, compared with the other Taxol concentrations. As demonstrated in Fig. 8, cells treated with anti-miR-29a + Taxol 1 ng/ml showed a significant 3.6-, 1.9- and 4.7-fold increase in p21, p53 and BCL-2 expression, respectively, compared with cells treated with anti-miR-29a + Taxol 10 ng/ml (P<0.05). p21, p53 and BCL-2 levels were also significantly lower in the anti-miR-29a + Taxol 50 ng/ml groups compared with the anti-miR-29a + Taxol 1 ng/ml (P<0.05). Also, in cells treated with anti-miR-29a + Taxol 1 ng/ml, the expression of survivin was reduced by 1.5-fold compared with the expression levels in cells treated with anti-miR-29a + Taxol 10 ng/ml; however, this difference was not significant.

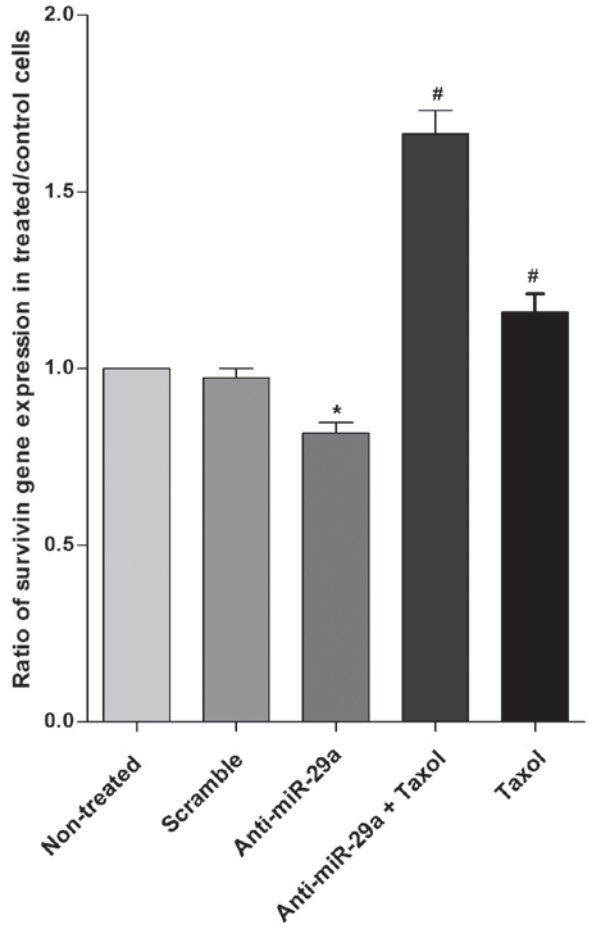

Figure 6. Results of reverse transcription-quantitative polymerase chain reaction for survivin gene transcript in MCF-7 cells transfected with scramble, anti-miR-29a, anti-miR-29a + Taxol or Taxol alone. Survivin gene expression significantly reduced in cells transfected with anti-mir-29a compared with those treated with anti-miR-29a + Taxol and Taxol alone. Data are presented as the mean ± standard deviation. *P<0.05 vs. anti-miR-29a + Taxol and Taxol alone; #P<0.05 vs. scramble. miR, microRNA.

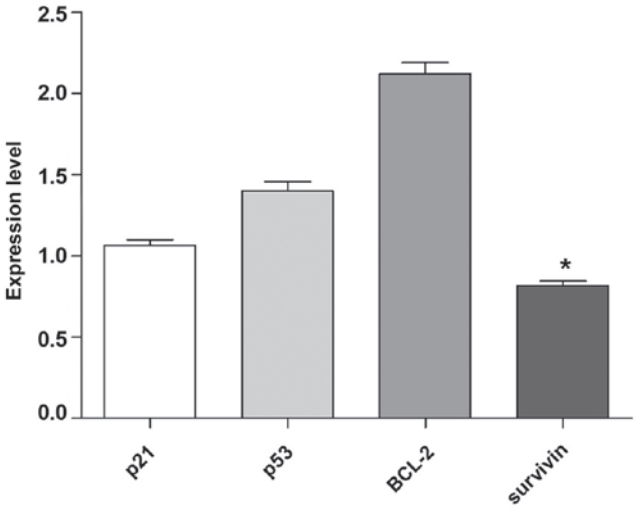

Figure7. Comparison of the expression level of desired genes following transfection with anti-miR-29a. Following transfection with anti-miR-29a, the expression of BCL-2 and p53 increased compared with p21 and survivin in MCF-7 cells. Gene expression levels were evaluated relative to the non-treated group and calibrated by the hypoxanthine-guanine phosphoribosyltransferase control gene. Data are presented as the mean ± standard deviation. *P<0.05 vs. BCL-2. miR, microRNA; BCL-2, B-cell lymphoma 2.

Discussion

Cellular expression of p21, p53, BCL-2 and survivin, which are involved in apoptosis and proliferation, is tightly regulated by miR-29a (32). High expression of miR-29a in the MCF-7 human adenocarcinoma breast cancer cell line and sera of patients with breast cancer disease has been reported (33). As described earlier, miR are key regulators in expression of genes involved in the control of tumor development, proliferation, apoptosis and the stress response (34). Aberrant expression of miR in various types of cancer have been demonstrated; however, the molecular mechanisms by which miR modulate the process of tumor genesis and the behavior of cancer cells remains controversial (35). In present study, it was demonstrated that the inhibition of miR-29a could efficiently alter the expression profile of genes that are regulators of apoptosis in MCF-7 cells. First, miR-29a expression was successfully downregulated using anti-miR-29a. The viability of cells treated with Taxol and anti-miR-29a simultaneously increased compared with treatment of Taxol alone, suggesting the interference role of anti-miR-29a in cancer cell apoptosis. Results indicated that miR-29a expression was reduced by up to 75% in cells transfected with anti-miR-29a compared with the non-treated control group.

miR-29 family members have been identified as regulators of p53, leading to upregulation of p53 and apoptosis induction (32). The effect of miR-29a on breast cancer cell growth is largely dependent on upregulation of p53, and the present study shed new insight on the regulation of breast cancer cell apoptosis by miR-29a.The expression of p53 as a tumor suppressor gene was upregulated by 50% in absence of miR-29a, although Taxol treatment caused a 70% reduction in p53 expression compared with non-treated controls. This undesirable reduction in p53 was also observed following treatment with anti-miR-29a + Taxol, with a 46% reduction compared with that seen in cells transfected with the scramble control. p53 expression in anti-miR-29a-treated cells was 5.8-fold higher compared with the Taxol-treated group. Therefore, inhibition of miR-29a alone could increase p53 expression and result in cancer cell cycle arrest and apoptosis.

Of the four genes examined in the present study, p21 was the only gene for which expression was not altered following anti-miR-29a transfection compared with the non-treated control. p21 is a downstream target of p53 and has a role in apoptosis inhibition, which reflects the unwanted performance of this molecule in the cancer process (36). Research has indicated that p21 activity may be regulated by multiple signal transduction pathways, resulting in a stimulatory or inhibitory role of p21 in tumor genesis depending on cellular status (37). The present study demonstrated that treatment of miR-29a + Taxol reduced the p21 expression levels by up to 60% compared with cells transfected with anti-miR-29a. This reduction was 90% in treatment with Taxol alone. The negative effect of Taxol on the expression of p21 was therefore moderated by the use of anti-miR-29a and Taxol simultaneously. The expression of p21 in the Taxol-treated group was reduced by 10.5-fold compared with the anti-miR-29a group. Regarding the dual role of p21 in tumor genesis, using Taxol to reduce p21 expression and resulting in apoptosis of tumor cells appears to be useful; however, on the other hand, this could help tumor progression.

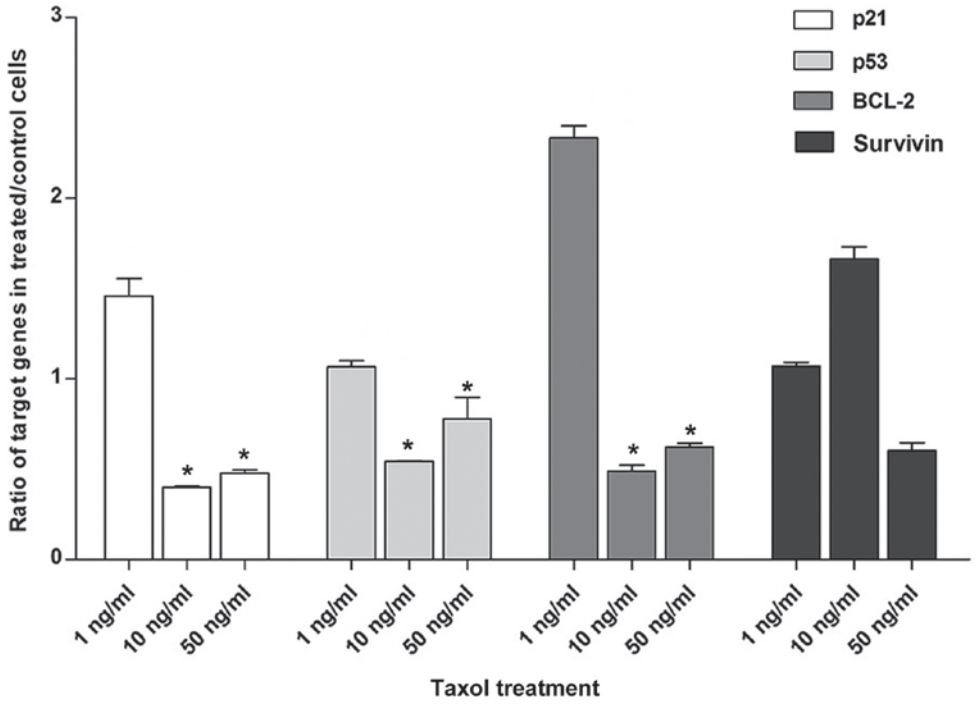

Figure 8. Expression level of p21, p53, BCL-2 and survivin in MCF-7 cells treated with anti-miR-29a and various doses of Taxol. Taxol 1 ng/ml in combination with anti-miR-29a resulted in significantly increased expression of p21, p53 and BCL-2 compared with the two other doses. Data are presented as the mean ± standard deviation. *P<0.05 vs. 1 ng/ml Taxol for the same gene. miR, microRNA; BCL-2, B-cell lymphoma 2.

Furthermore, the present study characterized BCL-2 as a functional target of miR-29a. BCL-2 acts as an apoptosis regulator and it has been reported that BCL-2 expression is associated with a good prognosis in patients with breast cancer (38). It has been reported that in breast cancer cells, silencing miR-15a and miR-16 restore BCL-2 expression (39). Also, due to the activity of miR-15a and miR-16, BCL-2 downregulation results in apoptosis (18). Accordingly, the present results demonstrated that silencing miR-29a increased BCL-2 expression 2-fold higher than the control. Although, in Taxol and anti-miR-29a + Taxol-treated groups, BCL-2 decreased by 87 and 50%, respectively, compared with the non-treated control. It may be concluded that silencing miR-29a could not exert a beneficial effect against tumor genesis through BCL-2. On the other hand, Taxol treatment through reduction of BCL-2 would have a positive effect on apoptosis induction.

Survivin, a multi-functional protein, has an important role in cell cycle regulation, inhibition of apoptosis and stimulation the angiogenesis (40). Survivin expression is upregulated in malignancies, which is associated with upregulation of human epidermal growth factor receptor 2 and vascular endothelial growth factor resulting in angiogenesis (41). It has been demonstrated that survivin leads to resistance to chemotherapy (42). Therefore, inhibition of this molecule could be an important strategy to overcome resistance to chemotherapy and to remove the inhibitory effect of this molecule on apoptosis. It has been reported that survivin expression could be inhibited using deguelin, which results in restoration of the sensitivity of tumor cells to chemotherapy (43). The results of the present study demonstrated that inhibition of miR-29a resulted in the downregulation of survivin by up to 17% compared with the non-treated group. Expression of survivin as a unique inhibitor of apoptosis was upregulated in cells transfected with Taxol alone and in combination with anti-miR-29a compared with cells transfected with anti-miR-29a alone. These findings demonstrated that reduction of survivin expression was achieved through inhibition of miR-29a. This may have a positive impact on apoptosis induction. However, Taxol had negative effects in regards to the levels of survivin.

The anticancer agent, Taxol, is used in chemotherapy regimens against breast cancer and leads to cell cycle arrest and apoptotic cell death (44). However, its application is limited due to adverse effects on normal cells. In the present study, it was demonstrated that lower doses of Taxol in combination with anti-miR-29a treatment induced stronger effects on the expression of target genes. In cells transfected with anti-miR-29a + Taxol 1 ng/ml, the expression profile of p21, p53 and BCL-2 was upregulated significantly compared with anti-miR-29a treatment combined with 10 or 50 ng/ml Taxol. Therefore, anti-miR-29a transfection attracts much attention as an anticancer treatment in the manner of boosting the effect of Taxol.

In conclusion, regarding the important role of miR-29a inhibitors in regulating the apoptosis of breast cancer cells that express a high level of miR-29a, they may be candidates for chemotherapeutic agents. Unlike the adverse effects of Taxol on normal cells and its infilltration throughout the body, as it is not a specific agent, anti-miR-29a may be applied locally in the tumor site. Due to the low stability of miR, the time duration of their application should be considered important. Furthermore, improving the stability of these molecules would be an important research area. However, further investingation in cells and animal models is required to verify the results of the present study.

Acknowledgements

We are grateful to the Research Deputy at Bushehr University of Medical Sciences (Bushehr, Iran) for financially supporting the present study (grant no. 2307-2013.07.16). We wish to thank Bonyakhteh Research Center (Tehran, Iran).

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