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Overexpression of microRNA-16 declines cellular growth, proliferation and induces apoptosis in human breast cancer cells

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Abstract MicroRNAs (miRNA) are a large family of small single-stranded RNA molecules found in all multicellular organisms. Early studies have been shown that miRNA are involved in cancer development and progression, and this role can be done by working as an oncogenes and tumor suppressor genes, so manipulation of this molecules can be a promising approach in cancer therapy, and experimental results represented that the modification in breast cancer phenotype is possible by miRNA expression alteration. miR-16, which is located in 13q14 chromosome, plays critical roles as a tumor suppressor by targeting several oncogenes which regulate cell cycle and apoptosis. Hence, in the present study, we investigated whether miR-16 could decline growth and survival of MCF-7 cell line as model of human breast cancer. MCF-7 cell line was infected with lentiviruses containing miR-16 precursor sequence. The effects of ectopic expression of miR-16 on breast cancer phenotype were examined by cell cycle analysis and apoptosis assays. miR-16 cytotoxicity effect was measured by the MTT assay. We showed that the miR-16 overexpression reduces Cyclin D1 and BCL2 at messenger RNA (mRNA) and protein levels in MCF-7 cell line. In addition, this is found that enforced expression of miR-16 decreases cell growth and proliferation and induces apoptosis in MCF-7 cells. In conclusion, our results revealed that upregulation of miR-16 would be a potential approach for breast cancer therapy.

Keywords MCF-7 · miR-16 · Cell cycle · Tumor suppressor · Oncogene

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Introduction

Breast cancer is the most prevalent cancer in the women that originates from breast tissue and can be a result from mutations on several tumor suppressor genes including BRCA1, BRCA2, p53, ATM, and PTEN (Buchholz and Wazer 2002). Today, with thanks from miRNA biology, cancer researchers have been able to achieve many dark aspects of breast cancer (He and Hannon 2004; Filipowicz *et al.* 2008). MicroRNAs (miRNAs) are a novel class of small noncoding regulatory RNAs which control gene expression at post-transcriptional levels (Kouhkan *et al.* 2013; Kouhkan *et al.* 2014). MiRNAs play critical roles in various cellular processes such as growth, apoptosis, metabolism, and survival of the cells (Bartel 2004; Kloosterman and Plasterk 2006; Bushati and Cohen 2007; Rana 2007). Official miRBase database lists that up to 30% of protein-coding genes in human genome is controlled by miRNAs (Griffiths-Jones *et al.* 2006). With regard to crucial roles of miRNAs, their expressions have been completely controlled in different organs. Recent studies have indicated that miRNAs can function as tumor suppressors and oncogenes and any changes in their expression correlate with various human cancers such as lung cancer, prostate cancer, breast cancer, gastric cancer, and leukemia (Michael *et al.* 2003; Takamizawa *et al.* 2004; Cimmino *et al.* 2005; Hayashita *et al.* 2005; Iorio *et al.* 2005; Deng *et al.* 2008; Schetter *et al.* 2008; Golestaneh *et al.* 2012). Oncogenic miRNAs cause reduction in expression of the tumor suppressor genes and/or genes that are implicated in cellular differentiation, whereas tumor suppressor miRNAs lead to reduction in the expression levels of oncogenic genes involved in proliferation and apoptosis (Calin and Croce 2006; Cho 2007; Barbarotto *et al.* 2008; Shenouda and Alahari 2009). The first experimental evidence indicating miR-16 involvement in mammalian carcinogenesis was reported by Calin et al. in B-cell chronic lymphocytic leukemia (CLL) (Calin *et al.* 2008). Further investigation indicated that miR-16 was frequently deleted and/or downregulated in solid tumors including breast cancer, suggesting that this miRNA might be important for tumorigenesis (Calin *et al.* 2002; Bonci *et al.* 2008; Rivas *et al.* 2012). miR-16, as an important tumor suppressor, can participate in cell-cycle regulation in several tumor cell lines by targeting *cyclin D* (*CCND*) and *E* (CCNE) and in the induction of apoptosis by targeting BCL2 (Linsley *et al.* 2007; Liu *et al.* 2008). Cyclin E1 (CCNE1) and Cyclin D1 (CCND1) are vital proteins that are synthesized during G1 phase of cell cycle driving the G1/S phase transition. Aberrant expression of these oncogenes has been observed in many tumors, which results in chromosome instability, and thus may contribute to tumorogenesis (Ekholm and Reed 2000; Sandhu and Slingerland 2000; Grillo *et al.* 2006; Alao 2007; Carleton *et al.* 2007). On the other hand, anti-apoptotic B-cell lymphoma 2 (BCL2) is a critical regulator in mitochondrial pathway inhibition of apoptosis in eukaryotic cells, consequently, facilitating their survival (Cory and Adams 2002; Guo *et al.* 2009). Therefore, miR-16 could consider as most important tumor suppressor miRNA. Due to the fact that breast cancer is the most common invasive cancer and the second leading cause of the cancer death in women (Tinoco *et al.* 2013).

The aim of this study was to investigate whether miR-16 expression modulation could influence growth and survival of the breast tumor cells. Our data indicated that enforced expression of miR-16 in MCF-7 cells considerably reduced tumor growth and induced apoptosis. So, ectopic expression of miR16 could be utilized as a novel therapeutic approach in breast cancer.

Materials and Methods

Cell culture. Human breast cancer MCF-7 cell line and human embryonic kidney (HEK) 293 cell lines were obtained from Pasteur Institute of Iran and cultured in high-glucose Dulbecco's modified Eagle medium (DMEM, Gibco BRL, Grand Island, NY) supplemented with 10% Fetal Bovine Serum (FBS, Gibco) and 1% nonessential amino acids (Invitrogen, Waltham, MA), penicillin (100 U/mL, Gibco), streptomycin (0.1 mg/mL, Gibco), and L-glutamine (2 mM, Gibco). Cells were cultured under standard condition in 95% humidity and 5% CO_2 at 37°C.

Recombinant lentivirus generation. A 458-bp DNA fragment containing the miR-16 precursor sequence was targeted for PCR amplification from human genomic DNA (Cinnagen, Tehran, Iran) by the following primers: forward: 5′-ACTC TAGAGCAGCACATAATGGTTTG-3′ and reverse: 5′-TGGATCCCTCTAATGCTGCATAAGC-3′; the *XbaI* and *BamHI* recognition sites were included. The amplified product was cloned into a pCDH-CMV-MCS-EF1-copGFP vector (System Biosciences, Mountain View, CA). Inclusion of the green fluorescent protein (GFP) gene in the vector smooths the tracking of any transduction in subsequent experiments (Supplementary Fig. 1). Lentiviral system expressing miR-16 precursor or control vector, as well as a lentiviral packaging system produced based on before, in brief generation of lentivirus performed at ~50–60% confluency HEK which cultured in cell expansion media (DMEM media supplemented with 10% FBS) on 10-cm plates. Transfection was performed with 10 μg of recombinant miR-16 lentivector plasmid, 10 μg of packaging plasmid pPAX2, and 5 μg of envelop plasmid pMD2. The medium was replaced with new culture medium 12 h after transfection; then, viral supernatants were harvested 36, 48, and 72 h after transfection. Collected viruses were centrifuged using ultracentrifuge at 25,000 rpm for 2.5, flash frozen and stored at −80°C. For downregulation of

endogenous miR-16, pLenti-III-miR-Off-16 construct was purchased from ABM Company (Richmond, Canada) and packaged into lentivirus particles (recombinant anti-miR-16) as mentioned above.

Transduction of MCF-7 cells with miR-16 containing lentiviruses. For lentiviral transduction, 2×10^5 cells were seeded in 6 cm^2 culture plates and the following day transduced with MOI 5 (TU/cell) of recombinant miR-16 lentivirus. Forty-eight hours post-transduction, the cells were selected with puromycin at 2 mg/mL. Efficiency of transduction was also confirmed by flow cytometry (Supplementary Fig. 2). Empty lentivirus was utilized as a negative control for the infection experiments. So, study was performed in three different groups. First, blank control group (the group that was untreated); second, test groups (the group infected by recombinant miR-16 viruses or anti-miR-16); and third, negative control groups (the group infected with nonrecombinant miR-16 or anti-miR-16 viruses). For anti-miR studies, MCF-7 cells were seeded in 24-well plate and transduced following day with recombinant anti-miR-16 viruses.

qRT-PCR for miRNA expression. Total RNA were extracted 36 h after infection, from all kind of samples according to the manufacturer's instruction for total RNA extraction kit (Bioneer, Daejeon, Korea). Real-time PCR for miR-16 was performed using Startagen kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. Briefly, total RNA was polyadenylated by poly(A) polymerase. Then, reverse transcription was performed using poly(A)-tailed total RNA, general RT primer, and reverse transcriptase according to the manufacturer's protocol. Finally, relative fold changes of miR-16 expression in the test group against blank control group were calculated using the comparative CT ($2^{-\Delta\Delta CT}$) method with U6 small nuclear RNA (snRNA) as internal control. All of the experiments were carried out in triplicate.

cDNA synthesis and real time PCR for miR-16 targets. Total RNA was extracted and complementary DNA (cDNA) synthesis performed using Takara Reverse Transcriptase System (TaKaRa, Otsu, Japan); then the RT-PCR products (cDNA) were used for real-time PCR according to the manufacturer's instruction. Expression level of cyclin D1 (CCND1) as important cell cycle target of miR-16 was analyzed using real-time PCR (ABI, Grand Island, NY) by SYBER premix ExTaq kit (TaKaRa). Comparative CT ($2^{-\Delta\Delta CT}$) analysis was used to indicate the quantitative expression of target gene related to GAPDH as an endogenous control to standardize amount of RNA samples. The following primers were used for QRT-PCR reactions, CCND1 FW: 5′-CCGTCCATGCGGAAGATC-3′, CCND1 RW: 5′-GAAGACCTCCTCGCACT-3′; GAPDH FW: 5′-GACAAGCTTCCCGTTCTCAG-3′, GAPDH RW: 5′-GAGTCAACGGAT-TTGGTCGT-3′.

Western blot. Whole-cell proteins were extracted with cell lysis buffer (50 Mm Tris, pH=8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM EDTA) and resolved by 10% sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred onto polyvinylidene fluoride membrane (PDVF, pore size 0.45 mm; Millipore, Billerica, MA) with a semi-dry transfer cell (Model 755, Bio-Rad, Hercules, CA) for 1.5 h at 80 A at room temperature. Then, the membrane was immersed in 5% nonfat milk powder over 2 h. When finished, the membrane was incubated with anti-BCL2, anti-CCND1, and anti-GAPDH antibody. The membrane was further probed with horseradish peroxidase (HRP)-conjugated goat anti-(rabbit Ig) antibody (Sigma, St. Louis, MO). The protein bands were visualized using (ECL®) detection kit (Pierce. Rockford, IL). Finally, chemiluminescence was captured on Kodak X-film (Tokyo, Japan).

Growth curve. MCF-7 cells were seeded into six-well plate and infected with recombinant or nonrecombinant miR-16 viruses (control) particles. Cells were collected and counted at 36, 60, and 84 h using a ViCell counter (Beckman Coulter, Pasadena, CA). Apoptotic rate of miR-16 overexpression at 60 and 84 h was estimated by following equation:

$$\text{Apoptotic rate} = 100\% - \left(\frac{\text{cell count in target period}}{\text{ell count in previous period}} \times 100\right)$$

Cell proliferation assay. MCF-7 cells were cultivated at 5×10^3 in 96-well plates and incubated at 37°C for one night. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays was performed 36, 60, and 84 h after infection of MCF-7 cells with 25×10^3 recombinant or nonrecombinant miR-16 viral particles, 0.5 mg/mL of MTT solution was added to each well in 96-well plate, and incubation was continued for 4 h at 37°C. Then, medium was removed and 100 μL dimethyl sulfoxide (DMSO, Sigma) was added to extract the dye. Finally, test and control absorbance measured at 570 nm with a reference filter of 570 nm. All tests were performed in triplicate. Inhibitory rate of miR-16 overexpression on breast cancer cells proliferation was calculated by following equation: inhibitory rate of cells={(OD control wells−OD treated wells)/OD control wells}×100%.

Cell cycle analysis. For assessment of cell population in different phases of cell cycle, flow cytometric analysis of DNA content was utilized for transduced or non-transduced miR-16 virus particles 36 h after transduction as just described (Gharagozloo *et al.* 2012) briefly, cells were enzymatically

detached from tissue cultures, fixed with ethanol for 2 h, permeabilized with 0.1% Triton X-100, and labelled with propidium iodide (PI). Finally, the distribution of cell-cycle phases was evaluated by flow cytometry (FACScan; BD Biosciences, San Jose, CA) and Modfit 3.0 software.

Apoptotic assay. MCF-7 cells were infected by recombinant or nonrecombinant miR-16 virus particles. After incubation for 60 h in 37°C and 5% CO_2, apoptotic cells were detected using an Annexin-V/PI kit (Sigma) according to the manufacturer's instruction. Then, results were analyzed by flow cytometry.

Statistical analysis. All experiments were performed at least three times. Statistical analysis including mean and standard deviation (SD) were carried out using Statistical Package for the Social Sciences software (SPSS, v.16 for windows; SPSS Inc., Chicago, IL). One-way analysis of variance was used to compare results. The P values of ≤ 0.05 were considered statistically significant. Data are presented as mean±SD.

Results

Infection of MCF-7 cells by recombinant miR-16 lentiviruses leads to overexpression of miR-16. To detect the effect of miR-16 overexpression on proliferation and survival of cancer cells, we cloned the sequence of pre-miR-16 in lentiviral vectors and transduced MCF-7 cells. To assess the expression level of miR-16, we performed miR-quantitative RT-PCR assay, indicating that miR-16 expression was significantly increased by sixfold ($P<0.05$) when MCF-7 cells were infected by recombinant miR-16 virus. However, no remarkable differences were observed between miR-16 expression in the blank control group and the group infected by nonrecombinant miR-16 viruses (Fig. 1*A*, $P<0.05$).

Overexpression of miR-16 decreased expression level of CCND1 and BCL2. *CCND1* and *BCL2* mRNAs are specific targets of miR-16 (Liu *et al.* 2008) that contains only one highly conserved target site for this miRNA, at position 3130–3137 and 3741–3749 of their 3′-UTR, respectively. To

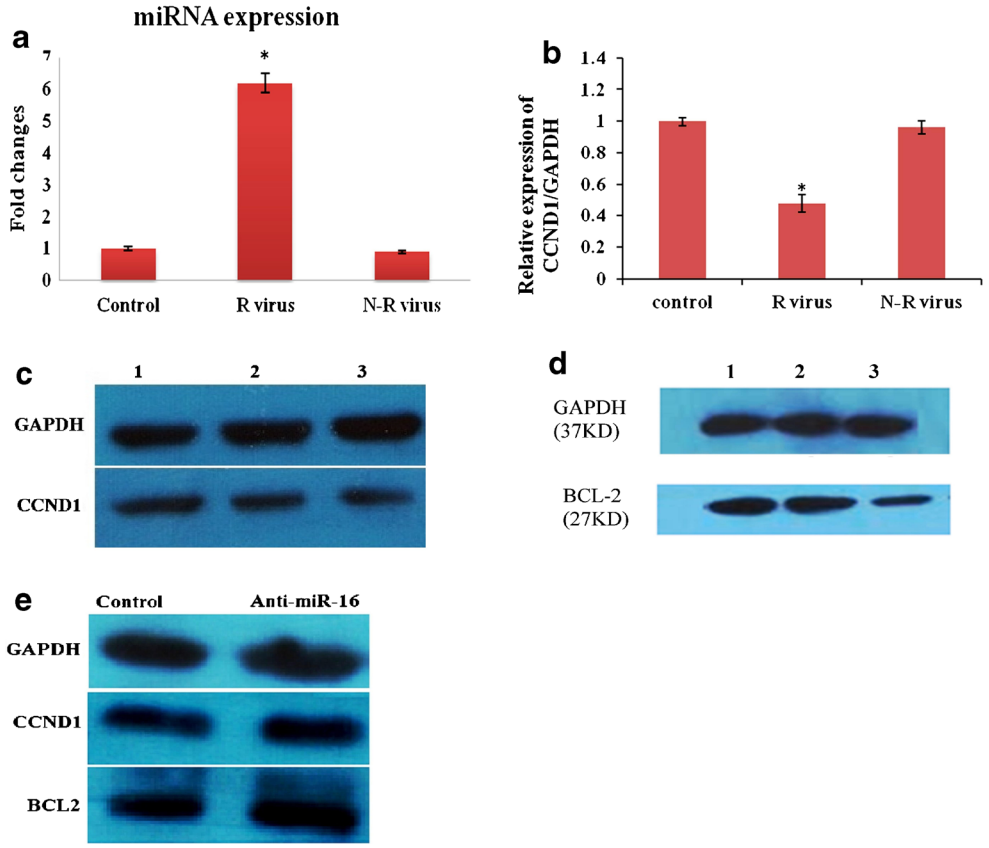

Figure 1. Impact of miR-16 overexpression on mRNA and protein levels of target genes. (*A*) MiRNA expression level was evaluated in different groups using QRT-PCR. miR-16 expression was considerably increased by sixfold ($P<0.05$) when MCF-7 cells were infected by recombinant miR-16 virus. (*B*) qRT-PCR analysis of *CCND1* mRNA level in MCF-7 cells. Overexpression of miR-16 induced a reduction in the *CCND1* mRNA level compared to the blank control (*$P<0.05$). Western blotting analysis of CCND1 (*C*) and BCL2 (*D*) proteins expression in MCF-7 cell line. Ectopic expression of miR-16 induces significant reduction in expression level of these proteins. (*E*) Anti-miR-16 transduction leads to a significant increase in the expression of CCND1 and BCL2 proteins. *R virus* recombinant miR-16 virus, *N-R virus* nonrecombinant miR-16 virus. *1* Blank control group. *2* Nonrecombinant miR-16 infected group. *3* Recombinant miR-16 infected group. All tests were performed in triplicate and data were presented as mean±SD. *$P<0.05$.

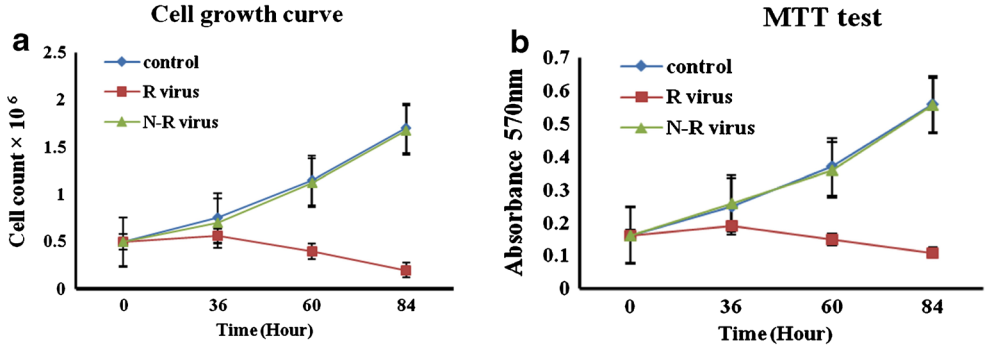

Figure 2. miR-16 reduced cell growth in MCF-7. (*A*) As compared to that of the blank control, miR-16 overexpression reduces cell growth in MCF-7 cell line after 36, 60, and 84 h of infection ($P<0.05$). (*B*) MTT results of MCF-7 cell line after miR-16 induction. miR-16 can considerably reduce the absorbance value of MCF-7 cells after 36, 60, and 84 h upon infection by recombinant miR-16 virus compared with the blank control group ($P<0.05$), while there was no significant difference between the blank control and nonrecombinant miR-16 virus groups ($P<0.05$). *R virus* recombinant miR-16 virus, *N-R virus* nonrecombinant miR-16 virus. Data were shown as mean±SD of at least three separate tests.

assess whether miR-16 could also regulate these targets in breast cancer, QRT-PCR and Western blot were performed for CCND1 and BCL2. qRT-PCR results in Fig. 1*B* indicated that *CCND1* expression in the test group which treated with recombinant miR-16 viruses reduced to 48±1.34% compared with the expression level in the blank control group. However, *CCND1* expression level did not differ between the blank control group and cells infected with nonrecombinant miR-16 viruses ($P<0.05$). We also performed Western blot and found that the protein level of CCND1 decreased into 64% in MCF-7 cell line, with increased miR-16 (Fig. 1*C*). There was no differences in *BCL2* mRNA expression between transduced and control cells. So, BCL2 protein level was analyzed by Western blot and found that miR-16 overexpression dramatically decreased the BCL2 protein level (into 43%, Fig. 1*D*) in MCF-7 cells. Next, we inhibited endogenous miR-16 in MCF-7 cells by using anti-miR-16 lentiviruses. Globally, for both of targets, we observed a trend towards increase in BCL2 and CCND1 proteins, indicating that miR-16 regulates the expression of the two endogenous targets analyzed in MCF-7 cells (Fig. 1*E*).

miR-16 can reduce cell proliferation and induce apoptosis in MCF-7 cells. To study the functional roles of miR-16 overexpression on breast cancer, we examined MCF-7 cell growth curve and performed MTT test, cell cycle analysis, and apoptosis assay. In Fig. 2*A*, we demonstrated that cell growth in MCF-7 cells infected by recombinant miR-16 viruses were significantly decreased in comparison with control groups after 36, 60, and 84 h. According to these results, cell proliferation rate in recombinant miR-16 transduced group was significantly reduced to 74% at 36 h, 38% at 60 h, and 13.5% at 84 h, when compared with the blank control group. On the other hand, apoptotic rate of miR-16 overexpression in cells infected with recombinant miR-16 viruses was approximately 29 and 49%, at 60 and 84 h, respectively, comparing their blank controls. MTT assay results exhibited that there was meaningful statistical difference between the absorbance value of the blank control cells and cells infected with recombinant miR-16 viruses after 36 h (0.250 vs. 0.190), 60 h (0.370 vs. 0.150), and 84 h (0.560 vs. 0.110). Nevertheless, no obvious changes were observed between blank control cells and cells which were infected with nonrecombinant miR-16 viruses in the same intervals: 0.250 vs. 0.248, 0.370 vs. 0.365 and 0.560 vs. 0.556, respectively ($P<0.05$; Fig. 2*B*). Consequently, inhibitory rate of miR-16 on proliferation and survival rate of MCF-7 cells which infected with recombinant miR-16 viruses were 24% in 36 h, 59% in 60 h, and 80% in 84 h. As

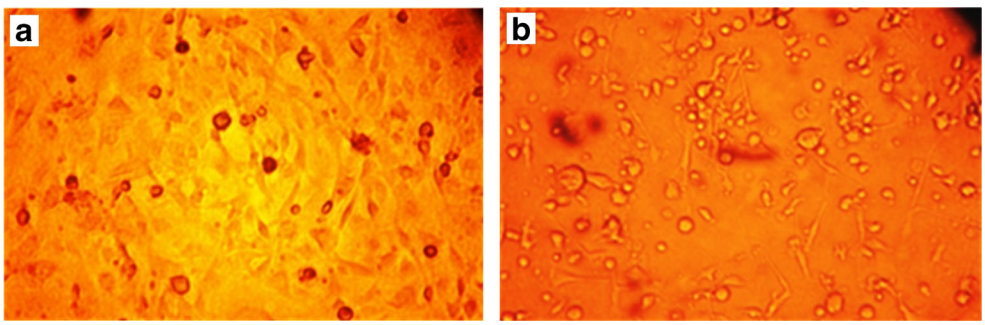

Figure 3. Phase contrast micrograph of MCF-7 cell lines. Impact of miR-16 overexpression on MCF-7 cells morphology and apoptosis by invert microscopy. (*A*) Blank control group that did not receive any treatment (×400); (*B*) MCF-7 cells infected with recombinant miR-16 virus (×400).

Table 1. Cell cycle phase distribution of MCF-7 groups after 36 h

Cell cycle phases	MCF-7 groups		
	Control	N-R virus	R virus
G0/G1%	42.58±1.53	45.90±2.87	62.08±1.21
S%	29.30±0.98	27.17±1.57	20.05±2.98
G2%	28.12±3.02	26.93±2.69	17.87±2.55

Results were reported as mean±SD

N-R nonrecombinant miR-16 virus infected group, *R* recombinant miR-16 virus-infected group

shown in Fig. 3, miR-16 overexpression induced remarkably morphological changes in infected cells with recombinant miR-16 viruses, compared to other groups.

Interestingly, cell cycle analysis by flow cytometry showed that overexpression of miR-16 triggered an increase in the number of cells in G0/G1 phase (62.08%) as compared to that of the blank control group (42.58%) and a reduction in the number of S phase cells (20.05%) as compared with the blank control (29.30%, Table 1). To confirm miR-16 overexpression effect on cell apoptosis, annexin V-PI assay was performed after 60 h upon infection. We found that apoptotic rate in the blank control group, cells treated with nonrecombinant and recombinant miR-16 viruses were 6.5, 8.5, and 35%, respectively, indicating apoptosis rate was elevated by 5.4-fold in cells infected with recombinant miR-16 viruses. As expected, there was no obvious difference between the blank control group and nonrecombinant miR-16 virus-infected cells ($P<0.05$, Fig. 4).

Discussion

Proliferation and apoptosis are two important factors in which regulating by abundant mechanisms ensuring correct cell division and programmed cell death. Defection in these regulated pathways disturbs the controlled proliferation and differentiation of the normal cell homeostasis and consequence in malignant growth. According to many literatures, altered expression of miRNAs has also been revealed in numerous types of human cancer including breast cancer, suggesting the potential of miRNAs as imperative biomarkers for cancer diagnosis, prognosis, and pathogenesis (Guo *et al.* 2012; Harquail *et al.* 2012). On the other hand, miR-16 is the one of the main tumor suppressor miRNAs that reduces cell proliferation, promotes apoptosis of cancer cells, and inhibits tumorigenicity both in vitro and in vivo (Rivas *et al.* 2012). miR-16 functions by targeting multiple oncogenes, including BCL2 (Yang *et al.* 2008), CCND1, CCNE1, and WNT3A. So the aim of the present study was to investigate the miR-16 enforced expression impact on the breast cancer cell behavior. Our results demonstrated that ectopic expression of miR-16 inhibits cell proliferation and induces apoptosis in cell line derived from breast cancer (MCF-7), efficiently through targeting CCND1 and BCL2, respectively. Liu Q et al. has shown that miR-16 regulates Cyclin D1 (CCND1), Cyclin D3 (CCND3), Cyclin E1 (CCNE1), and cyclin-dependent kinase 6 (CDK6) and induces G1 arrest in A549, HepG6, and HeLa cell lines (Linsley *et al.* 2007). In the present study, we have revealed by various methods that miR-16 can also regulate CCND1 and induce G1 arrest in MCF-7 cells. According to qRT-PCR and Western blot results, CCND1 expression in recombinant miR-16 virus-infected cells dramatically decreased into approximately 50 and 36%, respectively, when compared with that in the blank control group. Moreover, cell cycle analysis indicated that enforced expression of miR-16 inhibits the progression of G1 to S phase of MCF-7 cell cycle compared with the blank control group. Also, our experiments designated that miR-16 overexpression in MCF-7 cells after 36, 60, and 84 h upon infection with recombinant miR-16 viruses could reduce cell proliferation dramatically as compared with the blank control group. These results seem to be consistent with previous study by

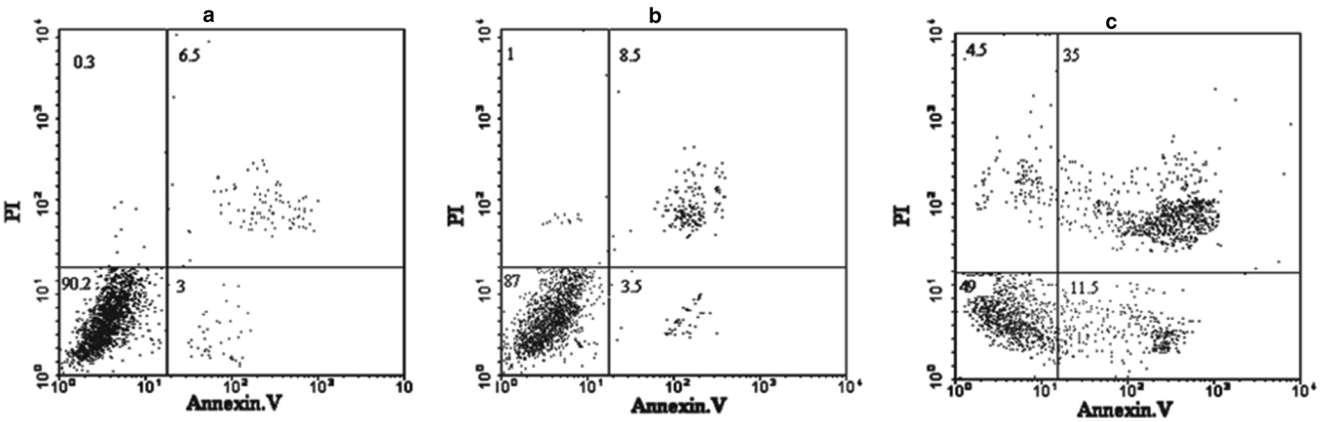

Figure 4 Annexin-V/PI assay results. (*A*) Blank control group; (*B*) MCF-7 cells infected by nonrecombinant miR-16 viruses; (*C*) MCF-7 cells infected by recombinant miR-16 viruses. Results were mean of three independent experiments±SD.

Kaddar T. et al. who found that miR-16 can negatively regulates HMGA1 and caprin-1, which play important roles in the MCF-7 cell proliferation (Kaddar *et al.* 2009). In the present study, MCF-7 cells morphology apparently changed to spherical shape with dense cytoplasm upon approximately 46 h after infection with recombinant miR-16 viruses. Then, detached cells from plate could be observed gradually. These observations demonstrated that overexpression of miR-16 has two functions: not only controls cell cycle and inhibits proliferation but also markedly induces apoptosis in MCF-7 cells. Immortality is a key factor in cancer, as it permits genetically unstable cells to stay alive and accumulate further mutations that ultimately make possible tumorigenesis. Moreover, Cimmino A. et al. indicated that miR-16 induces apoptosis in a leukemic cell line via targeting BCL2 (Kaddar *et al.* 2009). BCL2 is a vital player in the programmed cell death of eukaryotic cells that inhibiting apoptosis and favoring survival. Anti-apoptotic role of BCL2 protein, as an oncogenic protein, was identified for the first time in follicular lymphoma on 1984 (Tsujimoto *et al.* 1984; Bakhshi *et al.* 1985). Further studies demonstrated that overexpression of BCL2 protein is correlated with numerous type of cancers such as lymphoma, leukemia, lung, and breast cancer (Cory and Adams 2002; Youle and Strasser 2008). Until now, various studies are devoting to recognize and target signaling pathway of BCL2 protein, to destroy tumor cells and reverse the phenotype of cancerous cells (Jovanovic and Hengartner 2006; Garofalo *et al.* 2010). For example, Guo C. et al. reported that miR-15 and miR-16 expression are associated inversely with BCL2 expression and that both miRNAs remarkably induce apoptosis by targeting BCL2 in rat hepatic satellite cells (Guo *et al.* 2009). Similar to that, our Western blot results revealed that there was inverse correlation between the expression of miR-16 and BCL2 protein. miR-16 negatively regulates BCL2 expression and promotes apoptosis through this way in MCF-7 cells. Annexin V-PI analysis demonstrated that no effects were observed 36 h upon infection with recombinant miR-16 viruses (data not shown). However, miR-16 overexpression increased apoptosis rate into 35%, upon culturing for 60 h. These findings are disagreement with previous finding as observed with Linsely P. et al. who demonstrated that miR-16 overexpression have no effect on BCL2 level and apoptosis induction in SW1417, HeLa, and DLD-1 cell lines, suggesting that the miR-16 influence on apoptosis is cell type specific. Thus, miR-16 could induce apoptosis in some types of cells, but leads to cell cycle arrest in the others (Linsley *et al.* 2007). There were a little difference between apoptosis rate as measured by cell growth curve and apoptosis assay. These differences might due to accuracy in analysis of apoptosis by flow cytometry and apoptosis kit.

Briefly, achieved data displayed that miR-16 is able to induce apoptosis and cell cycle arrest in MCF-7 cells. It should be noted that anti-proliferation effect of miR-16 overexpression was observed after 36 h of infection with recombinant miR-16 viruses, but anti-apoptotic impact was slowly revealed after 46 h. These observations suggest that anti-proliferating effect of miR-16 is not the consequence of apoptosis. Therefore, it is likely that both of these effects participate in tumor suppressor activity of miR-16 in MCF-7 cells. Taken together, these data suggest that overexpression of miR-16 could be considered as a useful therapeutic agent to treat breast cancer patients in the future. Unfortunately, our study was limited to just MCF-7 cells, and miR-16's capability to induce apoptosis and inhibit cancer cell growth should be assessed in not only other type of breast cancer cell lines and but also in normal cells. However, considering the probability of side effects in adjacent normal cell, cancer cell-specific gene expression can be achieved by using promoters expressed in malignant cells, such as H19 lncRNA promoter (Mizrahi *et al.* 2009). Further in vitro and in vivo investigations in this field could open new insights in use of miRNA-based therapy in breast cancer.

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Conflicts of interest The authors declare that they have no conflict of interest.

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