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Original Research

miRNA-223 suppresses FOXO1 and functions as a potential tumor marker in breast cancer

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Abstract: Breast cancer is the most commonly diagnosed cancer in women and a leading cause of cancer mortality. MicroRNAs (miRNAs) have been found to play a key role in proliferation, metastasis and invasion of cancer. In previous study, we found that miRNA-223 was significant expression inexosome derived from peripheral blood serum of breast cancer patients than in samples from control subjects, Therefor,the role ofmiRNA-223willbe researched in MCF-7 breast cancer cells.In this study, to explore the role of miRNA-223in influencing cell proliferation, metastasis and invasion of breast cancer, TargetScan tools (http://www.targetscan.org/vert_71/) was used to scan target genes of miRNA-223, and thenmiRNA expression, real time PCR, Western blotting andluciferase report assay were used to test regulates relationship of miRNA-223and its targets,cell viability and BrdU analysiswere used to test cell proliferation of MCF-7 breast cancer cells after expression miRNA-223inhibitor. Scanning targets of miRNA-223found FOXO1 was listed in targets content, and luciferase reporter assay was used to assess and confirm the binding sequence of 3′untranslated region between FOXO1 and miRNA-223. Results showedthat miRNA-223inhibitorexpression increased protein expression level of FOXO1 in MCF-7 breast cancer cells,meanwhile, cell viability and BrdU analysis showed MCF-7 breast cancer cells were suppressed proliferation after up-regulation of FOXO1.In conclusion, we demonstrated that the miRNA-223can maintain cell proliferation of breast cancer cell through targeting FOXO 1, these results provide a new insight in tumor marker and potential therapeutic targets for breast cancer.

Key words: Breast Cancer; microRNA; FOXO1; Cell proliferation.

Introduction

A novel class of small RNA molecules, miRNAs has been implicated in the post-transcriptional regulation of thousands of mRNA transcripts resulting in decreased protein expression of targetgenes(1-3). MiRNAs are 18~25 nucleotide single stranded RNA molecules that negatively regulate gene expression by binding to the 3'-UTR of a target mRNA molecule resulting in either degradation of the transcript or translational inhibition(2, 4). miRNAs have obtained more attention than the other noncoding RNAs in the past few years, especially for their essential roles in development and tumorigenesis. More than 50% of the known miRNAs have been shown to participate in human tumorigenesis and metastasis by directly targeting oncogenes or tumor suppressor genes(5-8).

In previously research, we detected miRNA expression profile in exosome derived from serums between breast cancer patients and normal control, the miRNA-223was one of significant genes in breast cancer patients, and then we found that FOXO1 was presumptive targets for miRNA-223. Here, we will demonstrate regulates relationship betweenmiRNA-223and FOXO1 in breast cancer cells, and detect role ofmiRNA-223in cell proliferation of breast cancer.

Materials and Methods

This study was approved by the Research Ethics Committees of China Medical University (Licence ID: CMU-CH-2016-2020). Informed and written consent was obtained from each volunteer.

All cell culture media and supplements were from Sigma (Sigma-Aldrich, St, Louis, MO, USA),unless stated otherwise. All cell culture plates were obtained from Nest (Wuxi Nest Biotechnology Co. Ltd.,Wuxi, China).

miRNA isolation and qPCR

Mature miRNAs were obtained from 30 breast cancer patients(details see Table 1) and 10 normal humans serum using the miRcute miRNA Isolation Kit (Tiangen, Beijing, China) respectively, and then miRNAs were reverse-transcribed after being linked poly (A)-tailed using the miRcute miRNA cDNA kit (Tiangen, Beijing, China). qPCR of miRNAs was performed using the miRcute miRNA qPCR Detection Kit (SYBR Green, Tiangen, Beijing, China) and an ABI 7500 real time PCR system according to the manufacturer's instructions under the following conditions: 94°C for 1 min, 40 cycles at 94°C for 20 s, 60°C for 30 s and 70°C for 10 s. U6 small nuclear RNA was used for normalization. Each experiment was performed in duplicate in 96-well

Table 1. Patients details in this research.

Age	Breast Cancer Type			
	Lumina A	Lumina B	Her-2	(TNBC)
~40	3	1	1	1
41-50	3	2	1	0
51-60	2	2	2	0
61-70	1	1	2	0
71~	3	3	1	2
Total number	12	8	7	3
Abnormity of miRNA-223 expression in serum (%)	83.34	87.5	85.71	66.67

plates and repeated three times. Relative amounts of miRNA were calculated by the comparative threshold cycle (CT) method as $2^{-\Delta\Delta CT}$.

Cell culture

MCF-7 breast cancer cells were obtained through American Type Culture Collection (ATCC), and were cultured in Dulbecco's modified Eagle's medium/F-12 media supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. When cultures became 80-90% confluence, cells were passaged using 0.25% trypsin and 0.02% EDTA.

Over-expression of miRNA-223 inhibitor in MCF-7 breast cancer cells

MiRNA-223 inhibitor and a control sequence were synthesized by GenePharma (Shanghai, China) and transfected into MCF-7 breast cancer cells using RNA-Mate reagent. qPCR and western blot analyses were used to detect the expression of miRNA-223 and their target genes, FOXO1.

Western Blotting

FOXO 1, the target gene of miRNA-223, was detected by western blot analysis following overexpression of miRNA-223 inhibitor. Cells were lysed using M-PER Protein Extraction Reagent (Pierce, Colorado,USA) supplemented with a protease inhibitor cocktail (DMSF). Protein concentrations of the extracts were measured using the BCA assay (Pierce, Colorado, USA) and equalized with extraction reagent. Equal amounts of extracts were loaded and subjected to SDS-PAGE, followed by transfer onto nitrocellulose membranes. Primary antibodies and horseradish peroxidase-coupled secondary antibodies were purchased from Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The cells were blocked with 10% normal goat serum for 30 min and then incubated at room temperature for 1 h in PBS containing the following antibodies: mouse anti-FOXO1 (1:400), mouse anti-P53 (1:500), Rabbit anti-P21 (1:500), rabbit anti-Fas (1:400), rat anti-Pro-caspase-3 (1:500), mouse anti-GAPDH (1:2000). Membranes were probed using ultra-enhanced chemiluminescence western blotting detection reagents. GAPDHwas used as an internal control.

Assay of luciferase activity

The 3′UTR fragments for FOXO1 were generated by PCR using the following primers: 5′-CGTGAATTCTGCATTTCGCTACCCGAGTT-3′ (forward) and 5′-TCACTCGAGGTGGCTGACAAGA CTTAACTTAACTCAA-3′ (reverse) and cloned into the psiCHECK-2 vector (Promega) downstream from the Renilla luciferase cassette.The predicted miRNA-223 binding site was mutated using theQuikChange II XL Site-Directed Mutagenesis Kit (Stratagene). HEK293T cells were grown in a 96-well plate and co-transfected with the luciferase reporter vector together with a miRNA precursor or a negative control (20 nM; Ambion) using Attractene (Qiagen) according to the manufacturer's instructions. Activities of firefly and Renilla luciferase were analyzed using the Dual-Luciferase Reporter Assay System (Promega) 24 h after transfection.

Cell viability and BrdU analysis aftermiRNA-223inhibitor transcription

To test cell viability, MCF-7 breast cancer cells were reseeded in 24-well plates, treated with miRNA-223inhibitor or FOXO1 transcription, and analyzed with MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide) (9). Briefly, culture medium in each well was replaced with 500 µl of fresh medium containing 10 µl of 5 mg/ml MTT stock solution (Sigma-Aldrich, St. Louis, MO). Medium was removed after 5 h and replaced with 500 µl dimethyl sulfoxide (DMSO, Sigma-Aldrich) per well and held for 10 min at 37 °C, after which samples were mixed and absorbance was read at 540 nm. Bromodeoxyuridine (BrdU) analyses were used to measure cell proliferation (BrdU incorporation into DNA of proliferating cells). Cells were treated with 10 µM BrdU (Sigma-Aldrich) for 24 h and then fixed for immunofluorescent antibody assays.

Statistical Analysis

Statistical analyses of the data were performed with a one-way ANOVA followed by the Tukey-Kramer honestly significant difference (HSD) test for the three sets of results. A P-value of less than 0.05 was considered significant. Statistical analyses were done with a JMP® Statistical Discovery Software (SAS Institute, Cary, NC).

Results

miRNA-223expression in serum from breast cancer patient

For illustrating expression of miRNA-223in serum from breast cancer patient and normal humans, qPCR were used to test expression level of miRNA-223. As shown in Figure 1A, miRNA-223levels were significantly elevated in serum from

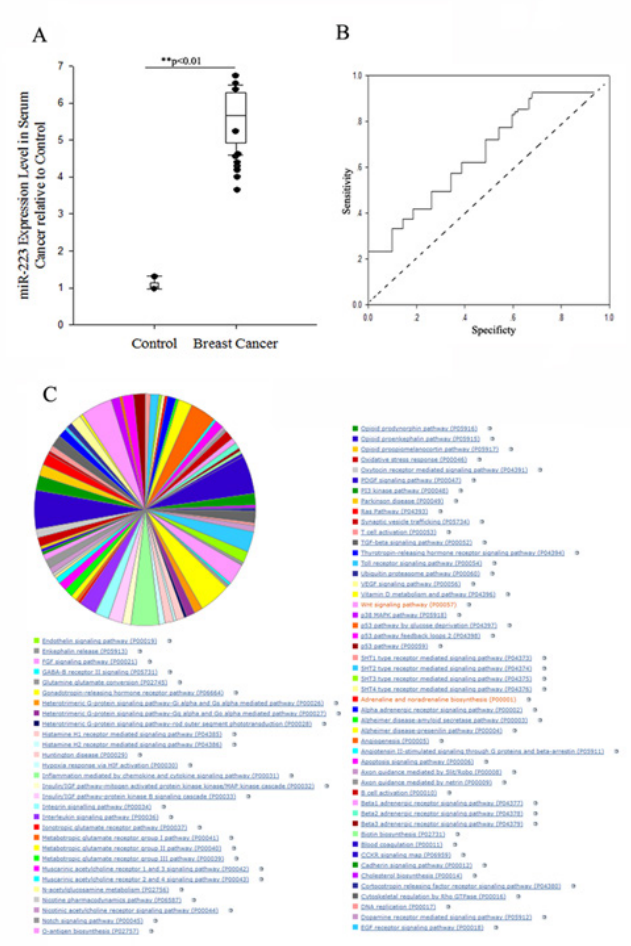

Figure 1. Biological character of miRNA-223in human breast cancer. A. The relative level of miR-223 expression in serum from breast cancer patient and controls, U6 was used for normalization. B. Receiver operating characteristics (ROC) curve analysis using serum miR-223 to differentiate breast cancer. C. Pathway enrichment of miRNA-223 targets using Panther tools.

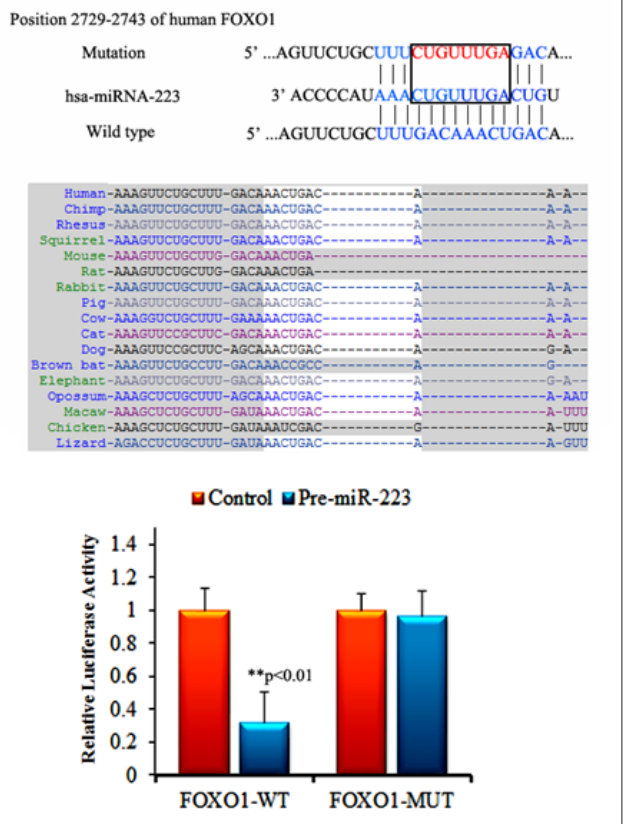

Figure 2. FOXO1 is the direct target of miRNA-223. miR-223 complementary sites with 3′UTR of FOXO1. The mutant sequence (FOXO1-MUT) is identical to FOXO1-WT construct except for five point mutations disrupting base-pairing at the 5′ end of miRNA-223(indicated with red color and a box). Mutating the miRNA-223 target site in the 3′ UTR of FOXO1 abolishes inhibition of luciferase activity by endogenous miRNA-223 in 293 cells.

breast cancer patient ($p<0.01$). To evaluate the diagnostic value of serum miRNA-223 in breast cancer, Receiver operating characteristics (ROC) curve analysis was performed (Figure1 B). The area under the curve (AUC) for serum miRNA-223 was 0.725 (93.63% Cl: 0.603–0.798). At a cutoff value of 41.42, miR-223 had a sensitivity of 68.12% and a specificity of 60.91%.miRNA target genes are likely to have relatively long and conserved 3′UTR (10), so we used the TargetScan algorithm to analyze target gene of miRNA-223, the targets were listed in Table S1. And then pathway of its targets were analyzed using Panther tools, and the results were shown in Figure 1 C and listed in Table S2.

FOXO 1 is a direct target ofmiRNA-223in MCF-7 breast cancer cells

We noticed that FOXO 1 has a long evolutionarily conserved 3′UTR, and the miRNA-223has the same putative target binding sites in FOXO1 in the human genome (Figure2). To directly test whethermiRNA-223targets FOXO1, we cloned the 3′UTRs of FOXO1 downstream of a luciferase reporter, and co-transfected these reporter constructs along with miRNA precursors into the human cell line HEK293T. Co-expression of miRNA-223was found to effectively downregulate luciferase expression in constructs with these 3′UTRs (Figure2). Mutations in the seed sequence of the predicted miRNA-223binding sites within FOXO1 abolished the inhibitory effects ofmiRNA-223on luciferase expression.The miRNA-223inhibitorwas synthesized and expressed in MCF-7 breast cancer cells, expression of miRNA-223was quantified by qPCR and northern blotting at 72 h after transfection. As shown in Figure 3 A and B,miRNA-223levels were significantly decreased after transfection. Protein expression of FOXO1, the putative target gene, was performed on miRNA-223inhibitor-transfected cells using the Image J tools comparative method,the results showed FOXO1 protein expression was up-regulated (Figure 3 C).

miRNA-223and FOXO1in cell proliferation

Cell proliferation was evaluated by MTT and BrdU incorporation assays after miRNA-223inhibitor or FOXO1 over-expressionin MCF-7 cells respectively, results indicated that transfected FOXO1 and miRNA-223inhibitor increased cell viability in MCF-7 breast cancer cells($p<0.01$; Figure 4). Formazan crystal absorbance of formazan in transfected miRNA-223inhibitor or FOXO1 groups were all higher than control group, and BrdU data agreed with PDT assays.

Apoptosis series genes expression after transfected miRNA-223inhibitor and FOXO1

The p53-p21 pathway is crucial for preventing propagation of DNA-damaged cells, and deficiencies in these factors enhances cell viability. To understand a potential mechanism associated with cell proliferation after transfectedmiRNA-223inhibitor, expression of p53,p21 and fas and procaspase-3

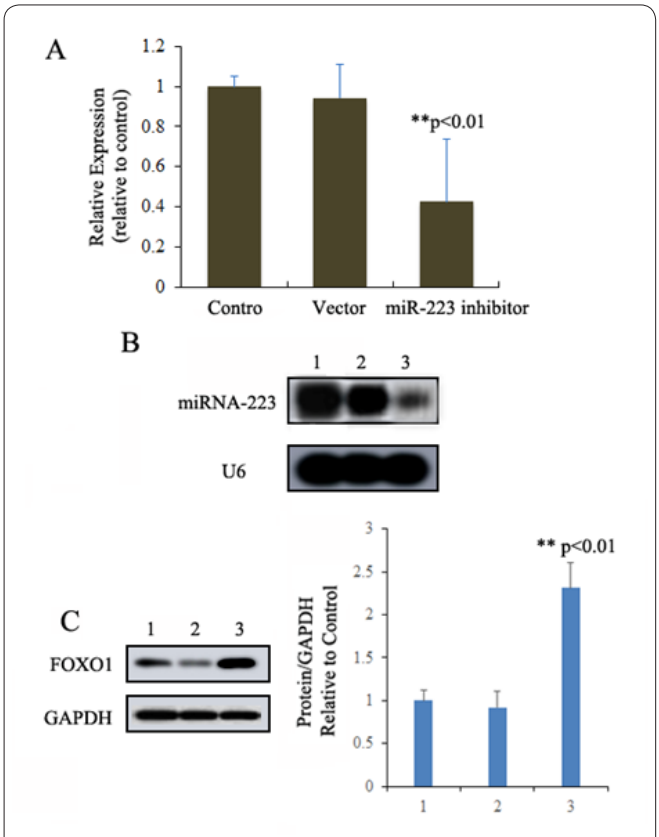

Figure 3. Biological function of miRNA-223in human breast cancer cells. A and B miRNA-223 inhibitor were transfected with adenoviruses as described in the Methods, and the expression of miRNA-223 was quantified by real time PCR (A) and Northern blotting (B). The results demonstrated expression level of miRNA-223 were significantly decreased after anti-miRNA-223 transfection. C. Effect of anti-miRNA-223 on protein level of FOXO1 in human breast cancer cells, quantification of FOXO1 in human breast cancer cells transfected with anti-miRNA-223, vector or control cells for 72 hr. The results demonstrated expression level of FOXO1 was significantly increased after anti-miRNA-223 transfection. 1: Control, 2: Vector, 3: anti-miRNA-223.

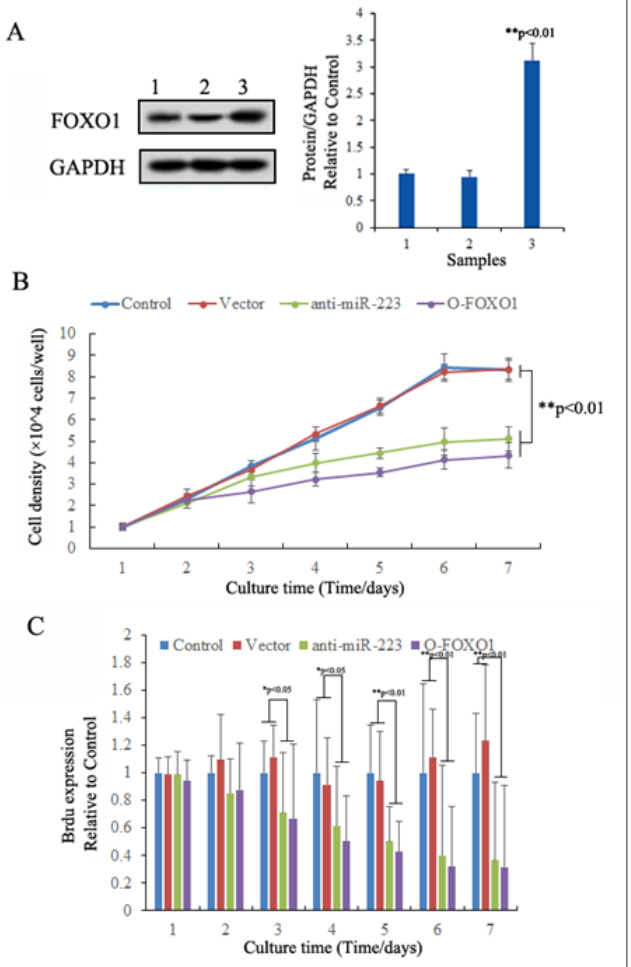

Figure 4. Role of anti-miRNA-223 or FOXO1 in cell proliferation of human breast cancer cells. A. Protein expression of FOXO1 after FOXO1 over-expression in human breast cancer cells, the results showed expression level of FOXO1 was significantly increased after FOXO1 over-expression. B. Cell viability in 1- to 7-day-cultured human breast cancer cells after anti-miRNA-223 or FOXO1 transfection via MTT assay. C. Cell proliferation in 1- to 7-day-cultured human breast cancer cells after anti-miRNA-223 or FOXO1 transfection via BrdU labeling indicating suppressed proliferation with anti-miRNA-223 or FOXO1 transfection.

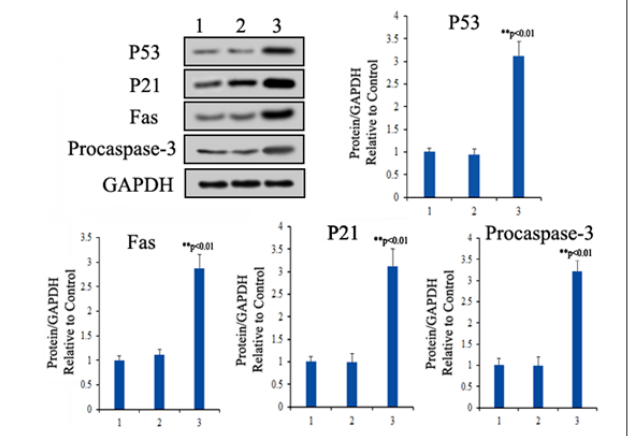

Figure 5. Apoptosis genes expression after miRNA-223 inhibitor transfection in human breast cancer cells. Western blot analysis of P53, P21, Fas and Pro-caspase-3 protein levels after anti-miRNA-223 transfection respectively, the result showed P53, P21, Fas and Pro-caspase-3 were significantly down-regulated.

was assessed using western blotting and found to change significantly after transfected miRNA-223inhibitor and FOXO1 in MCF-7 breast cancer cells ($p<0.01$; Figure 5).

Discussion

MicroRNAs (miRNAs) have been attracting major interest as potential diagnostic biomarkers of cancer,which are a class of small RNAs that regulate mRNA translation and function as oncogenes or tumor suppressor genes, have attracted great research interest. Since the initial description of circulating miRNAs in 2008(11-14), more than 200 articles have reported circulating miRNAs as biomarkers for a range of cancer types and other diseases(15-17). Many miRNAs have been reported as plasma or serum miRNA biomarkers of solid tumors, includingprostate,lung,breast,colon,ovarian,esophageal, melanoma, and gastric cancer.miRNA-223 has been reported to be nearly exclusively expressed in bone marrow(18), its overexpression has been observed in many types of cancer, such as esophageal carcinoma(19), hepatocellular carcinoma(20), and GC(21). In this research, the expression level of miRNA-223 derived from patientserum was significantly higher in breast cancer, and then the targets ofmiRNA-223 was analyzed using bioinformatics tools, we found that FOXO 1 was presumptive targets

formiRNA-223.Analysis of the miRNA binding sites is helpful in understandingregulates relationship between miRNAs and their targets. We mutated binding site within region to demonstrate microRNA target site and regulates relationship in this research. The results showed binding site influenced regulates relationshipafter mutated 8 nucleotides in that binding area, meanwhile, this results also demonstrated this banding site produced effective biological function in post-transcriptional regulation of miRNA-223.

The FOXO gene family encodes tumor-suppressive transcription factors that regulate multiple aspects of cell cycle traverse and survival. The FOXO subfamily of Forkhead transcription factors,including FoxO1 (FKHR), FoxO3a (FKHRL1), FoxO4 (AFX)and FoxO6 contains evolutionarily conserved transcriptionalactivators that are characterized by a highly conserved forkheaddomain with a DNA-binding motif(22). FOXO proteins play apivotal role in biological processes, such as apoptosis, cell cyclecontrol, differentiation, stress response, DNA damage repair andglucose metabolism(23, 24). Activation of each member of the FOXOsubfamily in cells can upregulate cell cycle inhibitors p21 Cip1 andp27 Kip1 and downregulate the cell cycle regulator cyclin D1/2(cell-cycle related genes), consequently leading to G1/S arrest ofcells (25-27). It has been also reported that upregulation of FOXOproteins can induce apoptosis through regulation of multiple pro-apoptotic proteins, including Bim, Puma, Fas ligand and TRAIL(28-30). Meanwhile, FOXO proteins have been associated withDNA damage repair via upregulation of GADD45a or interaction with ATM to promote DNA repair via downstream mediators(31). The FOXO1 transcription factor orchestrates the regulation of genes involved in the apoptotic response, cell cycle checkpoints, and cellular metabolism. One regulatory mechanism of FOXO1 activity is through phosphorylation, primarily downstream of the insulin-stimulated phosphatidylinositol 3-kinase/AKT/protein kinase B signaling pathway, which results in nuclear exclusion (32, 33). FOXO1 activity can also be regulated by acetylation (34) and ubiquitination (35, 36). In addition to insulin, FOXO1 can be down-regulated by other growth factors including gestrogen(37, 38) and epidermal growth factor (39),so, FOXO1 is a putative tumor suppressor, and the expression of this gene is dysregulated in some cancers, including prostate and endometrial cancers. Therefore, FOXO transcription factors are considered key tumor suppressors.

In summary, our study demonstrated that themiRNA-223highly expressed in serum of breast cancer as compared with normal. We also demonstrate that tumor suppressor, FOXO1 expression is regulated by miRNA-223in breast cancer cells.ThemiRNA-223directly target region of the 3'UTR to repress endogenous expression of FOXO1. Blockade of miRNA-223led to restore of FOXO1 expression. The restoration of FOXO1 expression in MCF-7 cells resulted in reduced cell number, decreased cell cycle traverse, andincreasedcellapoptosis. Thesefindingsindicatethat anti-sense targetingofmiRNA-223alongwithmonitoringof miRNA and FOXO1 levels may be of therapeutic andprognostic value in breast cancer.

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