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Expression Analysis of MicroRNAs Targeting PIK3CA and AKT1 Genes of PI3K Signaling Pathway in Breast Cancer Cells

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Abstract

Background: MicroRNAs (miRNAs) are key gene regulators that are involved in many biological and also pathological processes, including breast cancer. Breast cancer is the most common form of malignancies in women and requires new therapies and biomarkers. Different signaling pathways, such as Phosphoinositide 3-kinase (PI3K) signaling pathway are involved in breast cancer and can be new candidates for targeted therapies based on miRNAs. The aim of this study was to predict miRNAs targeting Phosphatidylinositol-4, 5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) and serine/threonine kinase 1 (AKT1) genes of PI3K cascade bioinformatically and to analyze their expression in breast cancer. **Materials and Methods:** Bioinformatic software and tools were used to predict miRNAs targeting PIK3CA and AKT1 genes. MCF-7 and MCF-10A cell lines were cultured as breast cancer and control cells respectively. RNA extraction, cDNA synthesis, and quantitative real-time PCR were performed. REST 2009® was utilized to analyze the expression of miRNAs and their target genes. **Results:** The results of our bioinformatic predictions indicated that miR-576-5p, miR-501-3p, and miR-3143 can be the first candidate miRNAs targeting PI3K signaling pathway. Data analyses demonstrated that PIK3CA and AKT1 genes were up-regulated while all bioinformatically predicted miRNAs were down-regulated in MCF-7 cell line compared to the normal cells. **Conclusion:** The results of our study demonstrated that PIK3CA and AKT1 can be targeted by miR-576-5p and miR-501-3p respectively. Furthermore, miR-3143 can target both mRNAs. Since these miRNAs target oncogenes, they can be proposed for new complementary targeted therapies in breast cancer patients. **[GMJ.2017;6(4):338-45] DOI: 10.22086/gmj.v6i4.925**

Keywords: PIK3CA; AKT1; miR-576-5p; miR-501-3p; miR-3143

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Introduction

MicroRNAs (miRNAs) are a class of non-coding RNAs with a length of ~22 nucleotides. These endogenous molecules bind to the 3′-untranslated region (3′-UTR) of their target mRNAs and cause gene silencing at post-transcriptional level [1-3]. Dysregulation of miRNAs is involved in various cancers, like breast cancer and many studies have shown that these molecules can be used as biomarkers for diagnosis, prognosis, or treatment of breast cancer [4, 5]. Breast cancer is a prevalent malignancy adding up to 23% of all cancers in the world. In the USA, after skin cancer, breast cancer is the most common type of cancer among women and includes nearly one-third of all cancers [6-8]. Decline or increase in miRNA expressions in breast cancer is related to the stage of tumor, its resistance to targeted therapies and other pathological features of disease. As for therapeutic application of miRNAs, it is possible to either suppress oncogenic miRNAs by antagomirs or substitute tumor suppressor miRNAs by mimics. miRNAs target different signaling pathways within the cells and affect the expression of different signaling-related genes. PI3K/AKT/mTOR (mechanistic target of rapamycin) is one such signaling cascade, also known as PI3K signaling pathway [6, 9-11]. The binding of growth factors to upstream tyrosine kinase receptor (RTKs) leads to the activation of the PI3K followed by the synthesis of Phosphatidylinositol (3,4,5)-trisphosphate (PIP3) which in turn activates AKT. AKT is a serine/threonine kinase whose over-expression leads the activation of mTORC1 complex to directly pass to translation and protein synthesis. PI3K pathway is the main signaling pathway involved in cell proliferation, survival, metabolism and movement, and its aberrant activation is responsible for cancer. Several studies have shown that the activation of this pathway increases in breast cancer. In fact, changes in the expression and function of different combinations of these signaling pathways, such as PI3K, AKT and many other molecules may lead to its activation. Therefore, reducing the activity of this pathway by miRNAs targeting key genes of this cascade can be considered an interesting clinical strategy for breast cancer treatment [12-17]. In this study, miRNAs which target PIK3CA and AKT1 genes of PI3K pathway were predicted by means of bioinformatics software and the expression level of these novel predicted miRNAs were evaluated.

Materials and Methods

Bioinformatics Prediction

A variety of bioinformatic programs and tools were utilized to predict miRNAs targeting PIK3CA and AKT1 genes of PI3K pathway. These software applications were as follows: TargetScan, DIANA-Tools, miRwalk, microPIR, miRanda, miRDB, microcosm and so on.

Designing the Primers for Genes and miRNAs Expression Analysis

The sequences of target genes mRNA were obtained from GenBank, NCBI (www.ncbi.nih.nlm.gov). Then, forward and reverse primers were designed using Allele ID 7.0 and Oligo7 software application. The stem-loop structures were utilized for cDNA synthesis of miRNAs [18]. The target genes and the specific primers are shown in Table-1.

Cell Culture

MCF-7 is a non-metastatic breast cell line with a high expression of estrogen and progesterone receptors. MCF-10A is a normal epithelial cell line used as control. These cell lines were received from the National cell bank of Iran (Pasteur Institute of Iran, Tehran, Iran). MCF-7 was cultured in DMEM medium with 10% FBS and 1% penicillin-streptomycin and MCF-10A was cultured in DMEM medium containing 10% horse serum, 0.5 µg/ml hydrocortisone, 20 µg/ml EGF and 1% penicillin-streptomycin, at 37°C with 5% CO2 and 98% humidity. The growth and morphology of the cells were daily investigated by light microscopy until they reached desirable confluency.

Extraction of Total RNA and Synthesis of cDNA

Hybrid-RTM RNA purification kit (GeneAll, Korea) was used to extract total RNA and

Table 1. Designed Primers and Probes for Target Genes and mRNAs

miRNAs	**Forward sequence (5′ → 3′)**	**RT Stem-loop sequence (5′ → 3′)**
miR-576-5p	GACCGCATTCTAATTTCTCCAC	GGTGCATTGCAGAGATTGGTCGGAGGTATCCATCGCACGCAACGCTCTGCATTGCGACC TAAGAC
miR-501-3p	CATAGCACGCGGGCTAG	GGTCCTATGGATTTCAGGGTCCGAGGTATCGATCGCACGCTACGCTGTGCATACGACCAGAATC
miR-3143	GACCGGATTACATTCTATAGGC	GGCATCGTATGCAGATGTTTGGTCGCGAGGTATCCATGGCACGCATCGCTCTAGCATAACGACGGCGAAAGAA
SNORD47	ATGACTGTACGACATGTCCA	GTCGTGCAGTAGTTTAGGTCGAGGTATTCCGCACTTGCATACGGACAACTC
Reverse Universal		GACGTGGGACGGAGGT
Probe		**Sequences**
Probe SNORD47		FAM-TGAAAGTGACCTTGTCCGATGCA-BHQ-1
Probe Universal		FAM- 5’ TCG AAC GCA GGC ATC CCA CT- BHQ-1
Genes	**Forward sequence (5′ → 3′)**	**Reverse sequence (5′ → 3′)**
PIK3CA	GTC CAC TAT ACC CTG CTG ATC	AGC ATT TCA TCG CGT ATA TCA TCG
Akt	TGT GCA GCC GCA GCA GTG	GTG GAT CAT CAT CGC GTA GAT GC
HPRT1	CGT GCC GTC GAG ATA ACT G	TGA GTG CTG TGC ATA AAT AGT CC

cDNA synthesis was done applying RevertAid Reverse Transcriptase enzymes (Thermo scientificTM, the USA).

Quantitative Real- time Polymerase Chain Reaction (PCR)

All Real-time PCR reactions were prepared in the final volume of 13µl in triplicates. For expression analysis of miRNAs, reactions were contained 0.4 µl of each primer (10 µM), 0.2 µl TaqMan Probe (Macrogen, Korea), 6.5 µl RealQ Plus 2X Master Mix for Probe, High ROXTM (Ampliqon, Denmark), 4.5 µl dH2O and 1 µl of 1/3 diluted cDNA. Cycling profiles for Real-Time PCR reactions were as follows: 15 minutes enzyme activation step at 95°C followed by 40 cycles of Denaturation at 95°C for 15 seconds, and Annealing and Extension at 60°C for 1 minute. Reactions of Real-time PCR for genes expression analysis contained 0.5 µl of each forward and reverse primer (10 µM), 6.5 µl RealQ Plus 2X Master Mix Green, High ROXTM (Amplicon, Denmark), 4.5 µl dH2O and 1 µl of 1/3 diluted cDNA. Cycling profile of Real-time PCR was as follows: 15 minutes enzyme activation step at 95°C, followed by 40 cycles of Denaturation at 95°C for 15 sec, and annealing and Extension at 60°C for 1 minute. Melting temperature analysis was carried out after the Amplification cycles, with a slow increase in temperature from 60°C to 95°C with 0.2°C/sec increment. Expression of genes was normalized to HPRT1 gene and small nuclear RNA SNORD 47 (U 47) was utilized to normalize the expression of miRNAs.

Results

The results of bioinformatic predictions showed that miR-576-5p, miR-501-3p, and miR-3143 are candidates that target PIK3CA, AKT1 and both of these genes respectively. After performing Real-time PCR and applying ΔΔCT method in REST® 2009 software for data analysis, the final results demonstrated that the expression level of all three miRNAs significantly decreased in MCF-7 cell line compared to the normal breast cell line. Actually, the expression levels of miR-576-5p, miR-501-3p, and miR-3143 in MCF-7 cell line decreased by 10,000, 1818.1 and 10,000 folds respectively (Figure-1). The results also showed that the expression level of both PIK-3CA and AKT1 genes significantly increased by 8.2 and 2 folds respectively in MCF-7 cell line compared to the normal cells (Figure-2).

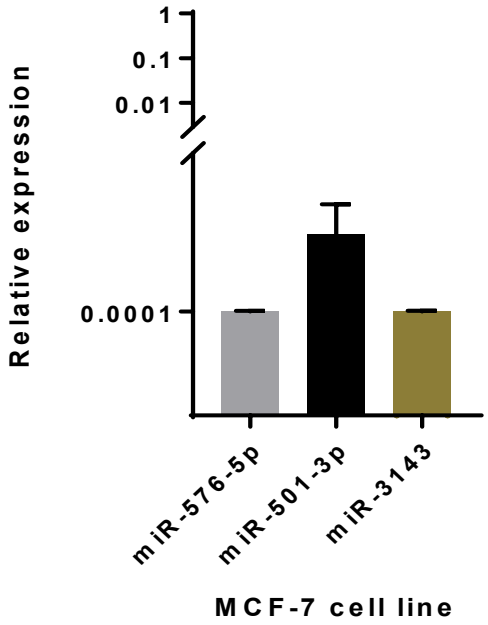

Fig 1: Relative expression of miR-576-5p, miR-501-3p and miR-3143 in MCF-7 cell line compared to normal cell line (MCF-10A).

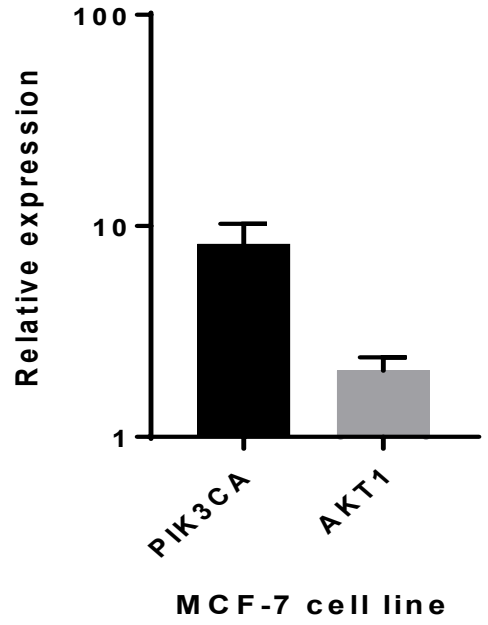

Fig 2: Relative expression of PIK3CA, AKT1 in MCF-7cell line compared to normal cell line (MCF-10A

Discussion

Breast cancer is the most common cause of cancer-related death among women and about 1.4 million women die of it each year [19]. PI3K signaling pathway is an oncogenic cascade that plays crucial roles in breast cancer initiation and progression and it is activated in approximately 70% of breast cancer cases. This pathway is involved in differentiation, cell growth, translation, transcription and many other anabolic and metabolic processes of the cell [20-22]. PI3K and AKT are two important oncogenes in this pathway. PI3K has three classes among which Class IA of PI3K has a catalytic and a regulatory domain named p110 and p85 respectively. p110 catalytic domain has three isoforms. Both PIK3CA encoding p110 α isoform as well as AKT1, which is one of three isoforms of AKT were selected in this study for further investigation. The PIK-3CA gene is located on chromosome 3q and activation of this gene causes cell growth and proliferation [19, 20, 23]. A study performed by Aleskandarany *et al*. to estimate the expression of PIK3CA protein in breast cancer patients. In their study, a long term follow-up was done and immunohistochemistry on a tissue microarrays (TMA) platform was utilized to evaluate PIK3CA protein expression. The result of this study showed a significant association between up-regulation of PIK3CA and poor prognosis of the disease, including higher tumor grade, larger size of tumor, metastasis to axillary nods, and higher proliferation fraction [24]. AKT1 is a serine/threonine kinase which is often activated in human cancers and causes cell proliferation and invasion. AKT1 gene is located on the long (q) arm of chromosome 14. The study of Riggio *et al*. indicated that AKT1 promotes cell proliferation and plays an important role in the growth of breast tumor but inhibits cell migration and invasion [25]. In the study of Li *et al*., high copy numbers of EGFR and AKT1 genes were observed in 205 mammary tumor tissue samples and results of this study showed high copy numbers of AKT1 gene as an approximately frequent event in breast cancer [26]. One of the aims of our study was to apply easily accessible and free bioinformatic software to predict miRNAs targeting these genes. The results of our bioinformatics predictions demonstrated that miR-576-5p targets PIK-3CA, miR-501-3p targets AKT1, and miR-3143 targets both of these oncogenes. At the beginning of this study, there was no report about the effect of these predicted miRNAs on AKT1 and PIK3CA genes in breast cancer, which approved the novelty of the study. Therefore, we examined the expression of these miRNAs and their target genes in MCF-7 which is referred to as non-invasive ductal breast carcinoma cell line [27]. The study of Li *et al*. was conducted on 3 primary colorectal carcinomas and 3 metastatic brain cancers and the expression profile of miRNAs was examined applying Agilent Human miRNA Microarrays V2.0. The results demonstrated that among all detected miRNAs, 2 miRNAs were down-regulated and 17 miRNAs including miR-576-5p were up-regulated in colorectal cancer samples with brain metastasis [28]. The study of Martinez-Ramos *et al*. indicated a different expression pattern of miR-576-5p in asymptomatic patients with systemic lupus erythematosus compared with controls [29]. Diaz-Prado *et al*. analyzed 723 miRNAs in osteoarthritic (OA) and normal human chondrocytes using Agilent Human miRNA Microarray. Among 7 differently expressed miRNAs, 6 miRNAs, including miR-576-5p were down-regulated in OA chondrocytes compared to the normal chondrocytes [30]. Gan *et al*. found that overexpression of miR-576-5p results in down-regulation of its target gene, Integrin beta-like 1 (ITGBL1), and promotes metastasis and invasion in non-small cell lung cancer (NSCLC) [31]. In the study of Ge *et al*., 664 miRNAs were analyzed by miRNA array in pertussis patients in comparison with the control group. 50 miRNAs showed up-regulation and 81 miRNAs were down-regulated. The expression of seven candidate miRNAs was also evaluated by quantitative real-time PCR (qPCR). The results indicated that some miRNAs including miR-576-5p, miR-206, miR-202, miR-487b, and miR-342-5p had an overexpression in pertussis patients [32]. The study performed by Larsen on Conjunctival malignant melanoma showed a significant up-regulation of miR-

501-3p in Conjunctival malignant melanoma, in both T1 and T2 phases of tumor. They also found an association between over-expression of miR-501-3p and the increase in tumor thickness [33]. Our results in breast cancer cells were in accordance with previous studies in other cancers, in which researchers had used expensive techniques, such as microarray The study of Yilmaz *et al*. was the only study about miR-3143 and in this study the expression of many miRNAs that target Leucine-Rich Repeat Kinase 2 (LRRK2) gene, including miR-3143 was examined in peripheral blood of 102 Parkinson patients compared to controls. Data analysis achieved the median expression value of 0.4466 for miR-3143 [34]. Our results demonstrated that this newly reported miRNA, miR-3143, which can target both PI3CA and AKT1 oncogenes, is an effective factor to inhibit breast cancer progression and metastasis.

Conclusion

The results of our study demonstrated that the expression of PIK3CA and AKT1 increased in MCF-7 cell line compared to the normal breast cells and miRNAs targeting them, including miR-576-5p, miR-501-3p, and miR-3143 were down-regulated in cancer cell line. These results indicate that the expression of these miRNAs has a probable inverse correlation with their target genes suggesting the therapeutic potential of these miRNAs in miRNA-based targeted therapies. Since the target genes of these miRNAs are oncogenes, these miRNAs can be considered tumor suppressors with the ability to inhibit growth and the invasion of tumor cells. However, further studies including luciferase assay are required to confirm the exact interaction between predicted miRNAs and their target genes.

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Conflict of Interest
The authors have declared that they have no potential conflicts of interest.

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