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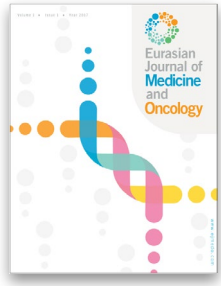

Research Article

PIWI Interacting RNA-823: Epigenetic Regulator of The Triple Negative Breast Cancer Cells Proliferation

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Abstract

Objectives: Triple negative breast cancer cells are estrogen, progesterone, and HER receptor negative, malign breast cancer cells. These cells have high telomerase activity, and this activity gives these cells high proliferation and evading apoptosis abilities. In this study, we aimed to determine the affect piR-823 on genetic parameters of proliferation and ERα status in MDA-MB-231 cells.

Methods: After anti-piR-823 transfection, proliferation of cells was tested by XTT. Gene expressions were determined by RT-PCR. Protein expressions were determined by ELISA.

Results: The proliferation decreased after inhibition ($p<0.001$). Gene and protein expressions of ERα were upregulated while hTERT was downregulated after inhibition ($p<0.001$). Furthermore, piR-823 inhibition cause to decrease PI3K/AKT/mTOR gene expressions and miR-126 expression ($p<0.001$).

Conclusion: Obtained data indicates that piR-823 inhibition lead MDA-MB-231 cells to increase ERα expression. Decreased expressions of hTERT and PI3K/AKT/mTOR pathway affect cell proliferation. Moreover, miR-126 decrease indicates that piRNAs and miRNAs can share the same target molecules and piRNA expression changes might have an impact on miRNA expressions. All obtained data is important on the perspective of piRNAs and their effect on cellular characteristics of triple negative breast cancer cells.

Keywords: Phosphatidylinositol-3-Kinase, piR-823, Triple Negative Breast Cancer

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P-Element induced wimpy testis (PIWI) interacting RNA-823 (piR-823) is a member of the longest and novel small non-coding RNAs. piRNAs are thought to regulate the expression of molecules epigenetically. Their expression profiles can change according to the cancer type.[1] piR-823 can exhibit different characteristics in various cancer types. piR-823 was first identified as a tumor suppressor in gastric cancer.[2] In renal cell carcinoma patients, piR-823 expression was determined in high levels and was thought to be associated with shorter disease-free survival.[3, 4] The expression pattern of piR-823 in colorectal cancer acts as an oncogene, and its expression provides cancer cells to avoid apoptosis and upregulate proliferation.[5] Overexpression of piR-823 has been reported to force the expressions of vascular endothelial growth factor (VEGF), interleukin-6 (IL-6), and intercellular adhesion molecule-1 (ICAM-1) to reduce apoptosis and to increase the proliferation of cells significantly.[6] Furthermore, piRNA-823 induces apoptosis-associated protein expression by regulating de novo DNA methylation and angiogenesis.[7]

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Triple-negative breast cancer (TNBC) represents 15%–20% of all breast cancers, which is more common in women under the age of 50 and those who have the BRCA-1 mutation.[8] TNBC cells are heterogeneous tumor cells with a histology and genetic makeup with low expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2.[9] Treatments targeting PR, ER, and HER2 receptors in benign breast cancer cells might not be effective for TNBC treatments.[10] Radiotherapy, chemotherapy, and surgical therapies have been determined to be inadequate to treat TNBC cells effectively. One-third of patients with TNBC tend to metastasize to distant sites.[11] The prognosis for TNBC patients is poor because of its propensity for recurrence and metastasis and a lack of clinically established targeted therapies.[12] Tumor ERα expression plays an important role in the clinical care of breast cancer patients as a prognostic factor and therapeutic target. ERα absence is correlated with malignant disease and poor prognosis. Up to one-third of breast cancers lack ERα at the time of diagnosis, and a fraction of breast cancers that are initially Erα-positive lose ER expression during tumor progression.[13] This deprives of an important possibility of tumor patient care by endocrine therapy and increases poor clinical outcome. For patients with ER-positive breast cancer, approximately 5 years of adjuvant endocrine therapy reduces the annual breast cancer death rate by approximately 30%.[14]

The human telomerase reverse transcriptase (hTERT) promoter has been shown to selectively promote hTERT gene expression in tumor cells, but not in normal cells.[15] hTERT expression is upregulated in tumors through multiple genetic and epigenetic mechanisms, including hTERT amplifications, hTERT structural variants, hTERT promoter mutations, and epigenetic modifications via hTERT promoter methylation.[16] Genetic (hTERT promoter mutations) and epigenetic (hTERT promoter methylation and miRNAs) events have been shown to have clinical implications in cancers due to hTERT activation.[17, 18]

Phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) is a classical signaling pathway identified to be important in different vital cellular processes.[19] Furthermore, PI3K/AKT/mTOR signaling pathway is well known as the crucial pathway in the regulation of genes used to enhance growth, viability, proliferation, and survival of cancer cells.[19, 20]

In this study, we aimed to determine the impact of piR-823 inhibition on the proliferation and main characteristics of TNBC cells as low ERα and high hTERT expressions. Furthermore, we also aimed to determine the possible relationship between miR-126 and piR-823 via observing gene expression changes of PI3K/AKT signaling pathway, which is one of the targets of miR-126.

Methods

Cell Culture and Transfection

MDA-MB-231 cells were maintained in a humidified atmosphere with 5% CO2 at 37°C. Phenol red-free Dulbecco's Modified Eagle's Medium (Gibco, USA) with 10% FBS (Gibco, USA) and 1% Penicillin/Streptomycin (Capricorn, Germany) were used to culture cells. Non-target and anti-piR-823 sequences were transfected to cells according to the manufacturer protocol of transfection reagent (TaKaRa, Japan).

Proliferation

MDA-MB-231 cells were seeded to a 96-well cell culture plate and transfected with non-target and anti-piR-823 as described previously. The proliferation of MDA-MB-231 cells was observed by using the 2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide (XTT) method (Biological Industries, Israel) at the 24th, 48th, and 72nd hour. The absorbance of cells at 450 nm was determined using a microplate reader (BioTek, Japan).

Real-Time Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated in accordance to the manufacturer protocol (Macherey-Nagel, Germany). The total RNAs were converted to cDNA through a reverse transcription. After obtaining cDNA, miR-126, ERα, hTERT, and PI3K, AKT and mTOR gene expressions were analyzed by Roche LightCycler 96 (Vedbaek, Denmark). ΔΔCT formula was used to determine the gene expressions.

Enzyme Linked Immunosorbent Assay (ELISA)

Protein expressions of ERα and hTERT were determined by ELISA based on the kit manufacture (Elabscience, USA). Protocol started with the incubation of samples for 90 min at 37 °C. Then, biotinylated detection antigens were added and incubated for 60 min at 37 °C. All wells were washed with phosphate buffered saline (PBS) at the next step. After washing, the samples were incubated with horseradish peroxidase (HRP) for 30 min at 37 °C. Stop solution was added and then was read at 450 nm using a microplate reader (BioTek, Japan).

Statistical Analysis

A normal distribution of the continuous variables was performed using the Kolmogorov-Smirnov test. Comparisons between groups of normally distributed variable were evaluated by one-way variance analysis (ANOVA). The Tukey Honestly Significant Difference (HSD) test was used

for multiple comparisons. All analyses were performed using IBM SPSS Statistics 21.0 software package.

Results

Anti-piR-823 Impact on the Proliferation of MDA-MB-231 Cells

Proliferation was noted to decrease at 24th, 48, and 72nd hours after transfecting anti-piR-823 compared to the control group ($p<0.001$, Fig. 1). No statistical significant changes were determined between control and non-target groups at the 24th, 48th, and 72nd hours ($p>0.05$, Fig. 1).

piR-823 Inhibition Effect on PI3K/Akt/mTOR Gene Expressions

The PI3K gene expression decreased in piR-823 inhibited cells (0.467±0.00424) as compared to the control group (0.595±0.00342; $p<0.001$). AKT gene expressions were downregulated in inhibited group (1.516±0.0408) as compared to the control group (6.916±0.00394; $p<0.01$). mTOR gene expressions in inhibited group (1.866±0.00216) was noted to reduce as compared to the control group (7.7270±0.00548; $p<0.001$; Fig. 2).

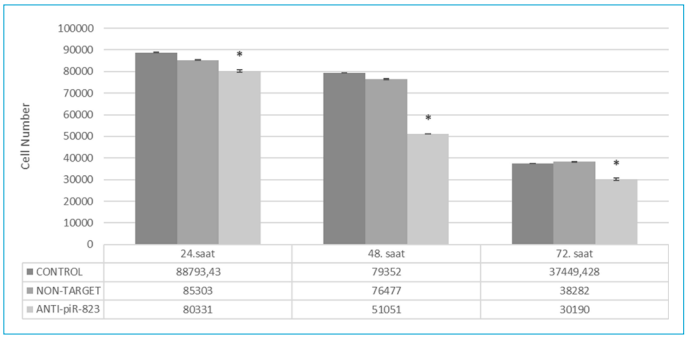

Figure 1. The Proliferation of MDA-MB-231 cells at 24th, 48th and 72nd hours after transfections ($p<0.001$).

miR-126 and piR-23 Expressions

The aberrant reduction in miR-126 expression was determined in anti-piR-823 transfected group (5.242±0.00455) compared to the control group (210.839±0.0051; $p<0.001$; Fig. 2).

Estrogen Receptor Alpha (ERα) Gene and Protein Expressions

ERα gene and protein expressions were increased in anti-piR-823 transfected cells as compared to the control group ($p<0.001$). The ERα gene expression of anti-piR-823 transfected cells (18.507±0.00529) increased as compared to the control group (11.158±0.00294; $p<0.001$; Fig. 2). After piR-823 inhibition, the ERα protein expression also induced in transfected group (412.388±0.23973) compared to the control group (245.1±3.00832; $p<0.001$; Fig. 3).

Human Telomerase Reverse Transcriptase (hTERT) Gene and Protein Expressions

A decrease in hTERT gene was observed in anti-piR-823 transfected cells (0.49±0.0387) compared to the control group (0.66±0.0265; $p<0.001$; Fig. 2). hTERT protein expression in anti-piR-823 transfected cells (0.466±0.0057) was

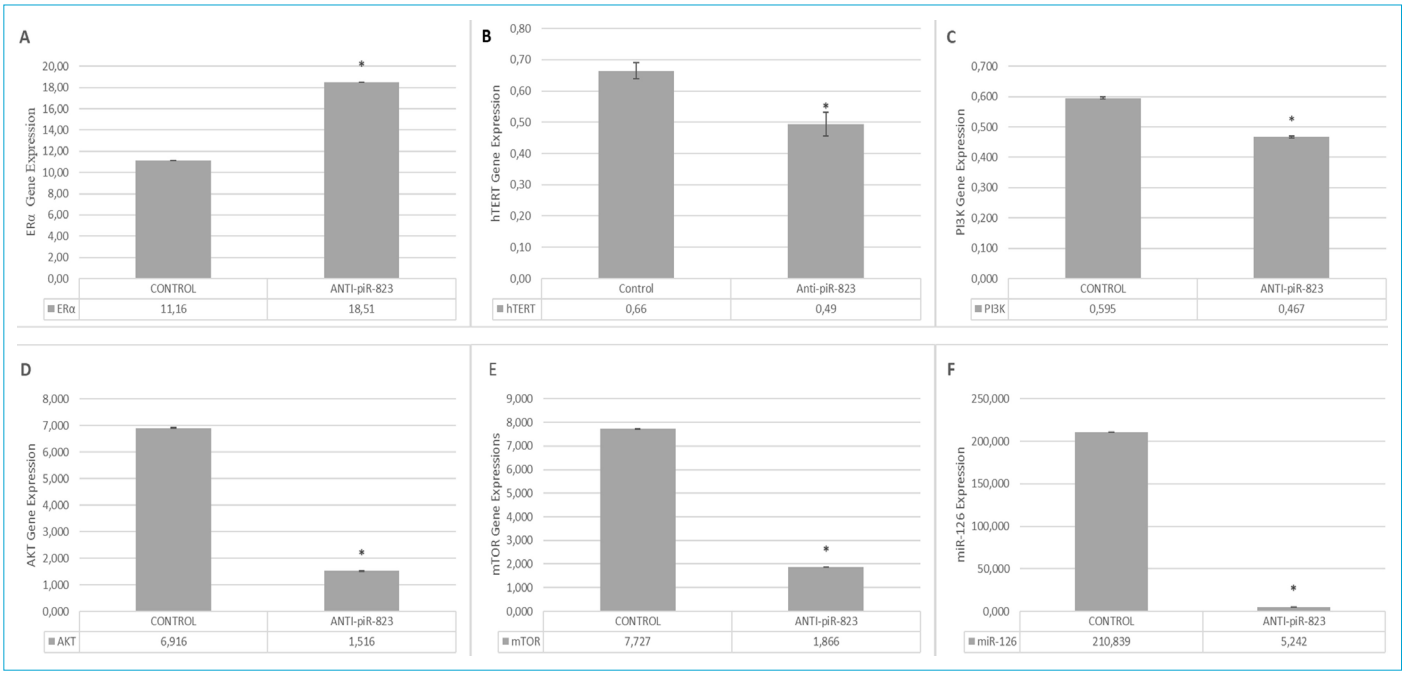

Figure 2. The gene expression changes of cellular targets (ERα, hTERT, PI3K, AKT, mTOR and miR-126) of piR-823 ($p<0.001$).

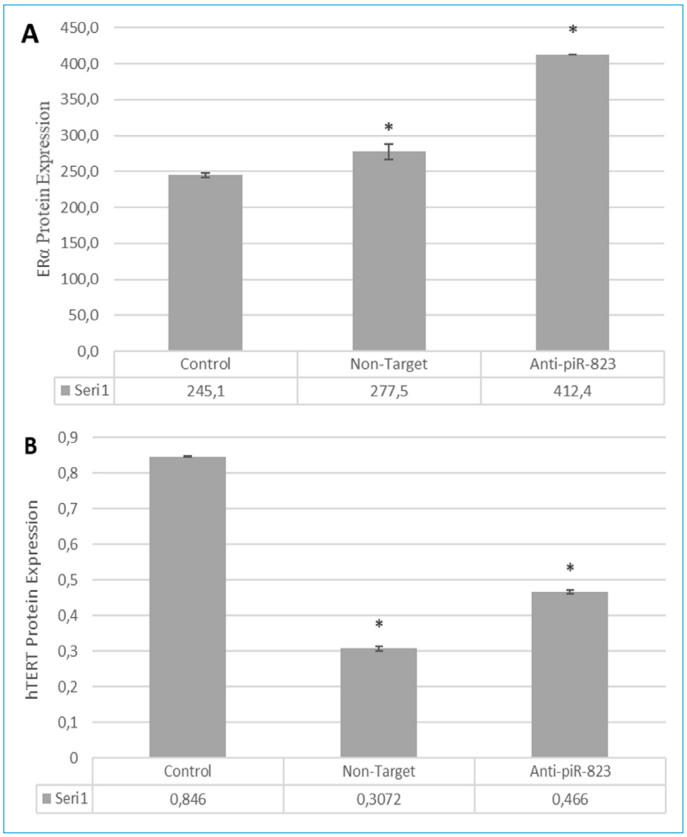

Figure 3. ERα and hTERT protein expressions after transfections in MDA-MB-231 cells (The unit of hTERT is ng/ml; ERα is pg/ml; p<0.001).

determined to decrease as compared to the control group (0.846±0.00224; p<0.001; Fig. 3).

Conclusion

Silencing piR-823 can effectively inhibit tumor growth in therapeutic xenograft models, as evidenced in the data by Yan H. et al. (2015) in multiple myeloma (MM).[21] piR-823 effects on TNBC cells were thought to be oncogenic as in MM and can result in a decrease in the proliferation of cells. Our observed results supported the expected results, and the reduced proliferation was determined in TNBC cells after anti-piR-823 transfection. Cancer cells, especially the invasive ones, are known to have high telomerase (hTERT) activity. High telomerase activity supplies cancer cells to proliferate without aging and avoid apoptosis. Epithelial-to-mesenchymal transition confirms the relation between hTERT and differentiation mechanisms.[16] Lord et al. (2000) have found that hTERT expression levels rise with the progression from metaplasia to dysplasia and to malignancy. They reported that hTERT expression is significantly higher in malignancy such that it may be a clinically useful marker.[22] In a study examining 134 breast cancer specimens, Biéche et al. (2000) detected hTERT mRNA in 101 of 134 unilateral invasive breast cancers (sensitivity = 75.4%). They found a significant shortening in the length of disease-free survival for the patients with hTERT-positive tumors (p=0.017) and a trend toward hormone receptor negativity and increasing tumor grade.[23] TNBC cells do not need any ER activity to survive or proliferate. As opposite to TNBC cells, the induced enhancer of zeste homolog 2 (EZH2; histone methyltransferase of polycomb repressive complex 2) inhibitor and histone deacetylation molecule (HDAC) have decreased ERα gene expression in MCF-7 cells, which had estrogen receptor signaling disruption.[24] ERα signaling cascade can regulate hTERT activity and increase hTERT expression in ER-dependent breast cancer cases.[25] Leu et al. (2004) determined the strong relationship between ERα and DNA methyltransferases (DNMT1), which can regulate hTERT expressions[26] in breast cancer cells.[24] Obtained results indicate that piR-823 affect ERα and hTERT both transcriptionally and translationally. The decrease of proliferation is suggested because of converting low ERα expression to high and high hTERT expression to low.

PI3K/AKT/mTOR pathway plays an important role in the survival and proliferation of cancer cells. It is known that small non-coding RNAs, like miRNAs and piRNAs, can induce or reduce the activity of PI3K/AKT/mTOR pathway. piR-55490 is a kind of tumor-suppressive piRNA that especially inhibits the lung cancer cell growth via degrading the 3'UTR region of mTOR. Furthermore, high expressions of piR-55490 can suppress the activation of AKT/mTOR pathway in lung cancer.[19, 27] Ma et al. (2020) suggested that sphingosine-1-phosphate receptor regulates PI3K/AKT/mTOR signaling pathway via piR-004800 by promoting MM.[28] In hepatocellular carcinoma, piR-020498, piR-013306, piR-LLi-30552, and piR-00823 expressions increased; as per functional analysis, p53, PI3K/AKT signaling pathway molecules could be the target of these piRNAs.[29, 30] PI3K/AKT/mTOR signaling pathway is activated in cancer cells on the perspective of cell proliferation especially. We determined that piR-823 inhibition might reduce proliferation of MDA-MB-231 cells and downregulate the main molecules of PI3K/AKT/mTOR signaling pathway.

Methylation and histone modifications are the general inhibition mechanisms of piRNAs. Peng et al. suggested that piRNAs might have similar functions and targets with miRNAs in cancer cells.[27] By recognizing 3'OH region of target molecule and inhibiting its expression, the target molecules of piRNAs might be reduced in cancerous cells. Peng et al. (2016) have suggested mTOR as the main target molecule of both piRNA and miRNAs in lung cancer,[27] although Law et al. (2013) suggested AKT is the main target of both piRNA and miRNA in gastric cancer.[31] We suggest PI3K/AKT/mTOR molecules might be the main target molecules of both piR-823 and miR-126 in TNBC cells. piR-823 inhibition was noted to decrease gene expressions of PI3K,

AKT, mTOR, and miR-126 in TNBC cells.

Taking control of cancer cell proliferation known to have invasive characteristics and restoring the properties they lost in the tumorigenesis are important parameters for cancer researchers. These results suggest that piR-823 inhibition led to change some special characteristics of MDA-MB-231 cells which are TNBC cells. The conversion of low ERα expression to high and high hTERT expression to low and decreased proliferation via PI3K/AKT/mTOR signaling pathway are the main signatures of piR-823 usage as an epigenetic target for gene treatment in the future. The usage of piR-823 for future treatments is argued that it may be useful in controlling proliferation. Moreover, oncogenic function of piR-823 is indicated in TNBC cells and can be used as a diagnostic marker considering its expression.

Disclosures

Ethics Committee Approval: This is a cell culture study so there is no need to approve by the Local Ethics Committee.

Peer-review: Externally peer-reviewed.

Conflict of Interest: None declared.

Authorship Contributions: Concept – Ç.Ö.; Design – Ç.Ö.; Supervision – Ç.Ö.; Materials – Ç.Ö.; Data collection &/or processing – Ç.Ö., E.Ç.; Analysis and/or interpretation – Ç.Ö., E.Ç.; Literature search – Ç.Ö.; Writing – Ç.Ö.; Critical review – Ç.Ö.

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