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Suppressing the molecular signaling pathways involved in inflammation and cancer in breast cancer cell lines MDA-MB-231 and MCF-7 by miR-590

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Abstract

Breast cancer is the most frequent cancer among women worldwide. Tumor immunology suggests relationships between the immune system, chronic inflammation, and cancer. The immune system may either prevent or promote carcinogenesis. Here, we evaluated molecular signaling pathways common in inflammation and cancer and detected the microRNAs which play pivotal roles in mediating these pathways. Using bioinformatics assays, signaling pathways common in inflammation and cancer, and microRNAs mediating these pathways were identified. MiR-590 was selected and cloned into the pLenti-III-eGFP vector and transfected into the breast cancer cell lines. The expression level of microRNA and the candidate genes was evaluated by real-time quantitative reverse transcription polymerase chain reaction, and the apoptosis level in transfected cells was measured by Annexin V-7AAD assay. The cell migration was tested by real-time quantitative reverse transcription polymerase chain reaction for MMP2/MMP9. The expression levels of miR-590 and the selected genes (i.e. JAK2, PI3K, MAPK1, and CREB) were measured 72h after transfection. While miR-590 showed an over-expression, the genes were significantly down-regulated. A significant increase was observed in apoptosis level in both cell lines and MMP2/MMP9 was significantly decreased in MDA-MB-231 cells. MiR-590 was selected as a microRNA which triggers and down-regulates critical genes of signaling pathways similar in cancer and inflammation. Following the miR-590 treatment, JAK2, PI3K, MAPK1, and CREB were down-regulated and the apoptosis level was increased in breast cancer cell lines. Apparently, some microRNAs can be good candidates for novel treatments of cancer. Although miR-590 showed good results in this study, further studies are required to investigate the role of miR-590 in breast cancer therapy.

Keywords

Breast cancer, inflammation, microRNAs, hsa-miR-590

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Introduction

Breast cancer is the second most frequent cancer among women around the world. The disease shows increasing incidence rates in different countries and is associated with high death rates, as it is the fifth most common cause of cancer-related deaths in the world.[1] It is highly heterogeneous and can present in a variety of forms. The presence or absence of estrogen receptors (ERs), progesterone receptors (PRs), and human epidermal growth factor receptor 2 (HER2) can be partly responsible for such a heterogeneity.

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Triple-negative breast cancer (TNBC) is an important clinical subtype of breast cancer which owes its name to the absence of all the above-mentioned receptors. TNBCs are generally diagnosed in younger patients. They lead to the development of larger tumors and are accompanied by a higher risk of distant metastasis and death within 5 years of diagnosis. Despite their low incidence rate (10%–24% of all breast cancer diagnoses), TNBCs are receiving increasing attention due to their aggressive clinical behavior and unresponsiveness to common targeted anti-hormone therapies.[2,3]

Inflammation is the body's innate response to various stress conditions. Since this process involves the activation of different signaling pathways, it may be both in favor and against cancer development. In some cases, especially in acute inflammatory responses, the immune system up-regulates the activity of cytotoxic immune cells which fight aberrant cells such as cancer cells.[4] However, in some other cases where cancer itself triggers inflammation by activating various signaling pathways, inflammatory responses may promote tumorigenesis. The higher risk of cancer in immunosuppressed patients, such as organ transplant recipients, highlights the significance of the immune system in preventing malignant cell transformation. Nevertheless, since the antitumorigenic activity of the immune system may lack the required potency to eliminate all malignant cells, some cancer cells may survive the body's immune response.[5] Since these challenges rise during chronic inflammatory conditions, such conditions can increase the risk of both immunosuppression and cancer. For instance, the activation of nuclear factor-kappa B (NF-κB) pathway, a well-known pro-inflammatory signaling pathway, generally increases the expression of anti-apoptotic genes and promotes defense against the stressors that stimulated the inflammatory response by enhancing cell survival mechanisms. Apparently, complex mechanisms determine the role of inflammation in the development and progression of cancer.[6,7]

According to previous research, tamoxifen-resistant breast cancers can be predicted or classified according to immune responses and inflammatory gene expression. This confirms the idea that endocrine resistance is associated with a dysregulated immune response and/or excessive inflammation in the tumor microenvironment. A recent research has indicated that the immune response profile and inflammatory signature in breast cancer may provide helpful information on patient prognosis and treatment. Therefore, research about inflammation and the immune system may lead to the development of new therapeutic methods for breast cancers, especially for cases resistant to endocrine therapies.[8,9]

Three major types of inflammation, that is, chronic inflammation occurring before tumor development, tumor-associated inflammation, and therapy-induced inflammation, can be related with tumorigenesis and cancer. Inflammation has also been reported to activate the angiogenic switch.[10] The roles of pro-inflammatory cytokines and chemokines, such as interleukin-1 (IL-1), (IL-6), (IL-8), tumor necrosis factor alpha (TNF-α), monocyte chemoattractant protein-1 (MCP-1), chemokine ligand 5 (CCL5), and C-X-C motif chemokine 12 (CXCL12), in breast cancer have been previously reviewed.[11] Immune cells and their related inflammatory mediators in the tumor microenvironment, that is, the balance between antitumorigenic and tumorigenic inflammatory factors, play a critical role in the initiation and metastasis or control and elimination of breast tumors. Inflammation induced during the natural tumor progression is probably one of the main reasons preventing the immune system from efficiently controlling the development of cancer. Moreover, therapy-induced inflammation has tumorigenic properties, that is, it causes resistance to treatment and might result in treatment-induced metastasis and relapse. Actually, improved outcomes have been observed in patients receiving anti-inflammatory treatment in combination with standard breast cancer treatments.[12,13]

MicroRNAs (miRNAs) are non-coding RNA molecules consisting of approximately 22 nucleotides. These molecules regulate gene expression through their role in the mediation of translational repression and RNA degradation.[14] Over the past decade, miRNAs have become a new paradigm of gene regulation. miRNAs are stable, easily detectable, and capable of serving as endogenous antisense mediators of entire gene sets that regulate cancer development. They can, thus, be regarded as potential targets in the treatment of cancer.[15,16] The significance of miRNAs in breast cancer has been documented by several studies. Iorio et al.[17] were the first to identify the significant relationships between the expression of several miRNAs and both breast cancer subtypes and clinicopathological characteristics of the disease, for example, hormone receptor status, clinical stage, and proliferation index. They, hence, introduced miRNA expression as a valuable biomarker in diagnosis and prognosis of breast cancer.[17] Numerous studies have confirmed the role of miRNAs not only in the development and progression of different cancers but also in various physiological functions such as immune responses, cell proliferation and apoptosis, and inflammation.[18] Therefore, this study investigated the convergence of miRNAs and their target genes with inflammatory signaling cascades and molecular pathways that are critical to tumor development and malignant progression.

Materials and methods

In Silico miRNA selection

Currently, it is believed that many diseases, such as cancers, are triggered by inflammation. Therefore, we searched the Kyoto Encyclopedia of Genes and Genomes (KEGG) to find the molecules, genes, and molecular

signaling pathways similar in cancer and inflammatory diseases such as systemic lupus erythematosus, rheumatoid arthritis, autoimmune thyroid disease, and inflammatory bowel disease (IBD). After selecting the genes, potential miRNAs involved in their regulation were predicted and independently analyzed using a number of relevant algorithms including PicTar (http://pictar.mdc-berlin.de/), TargetScan (http://www.targetscan.org/), miRanda (http://www.microrna.org/), and DIANA-microT (http://diana.imis.athena-innovation.gr/DianaTools/index.php). The score tables were prepared for each gene in Excel, and the tables were merged to obtain a single score table in which the miRNAs were sorted based on their repeat rates (in a descending order). Finally, the most proper miRNA, that is, miR-590, was selected from the score table.

Primer design and plasmid construction

Mesenchymal stem cells were derived from human bone marrow and used to extract human genomic DNA. The extracted DNA was then applied as a template for the polymerase chain reaction (PCR) of the miRNA gene. The primer for selected miRNA was designed in order to produce miRNA clones. The PCR product was extracted from the gel after electrophoresis. The candidate miRNA gene was cloned into the vector pLenti-III-eGFP. Two enzymes, namely EcoR I and BamH I, were used in the digestion of the PCR product and pLenti-III-eGFP vector. Ligation was then performed, and the obtained product was transformed into DH5á competent cells. Then, miRNA-pLenti-III-eGFP was verified by PCR and sequencing.

Cell culture

In this experimental study, two human breast cancer cell lines (MDA-MB-231 and MCF-7 cells) were purchased from the Iranian Biological Resource Center (IBRC). All cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The culture was conducted in a humidified atmosphere (5% CO_2) at 37°C. The cells were plated approximately 24 h before transfection at optimal confluence of about 70%. On the transfection day, the cells were divided into two groups. The cells in the test group were transfected with miRNA vector and those in the control group received no treatment.

Transfection

In order to transfect the vector, plasmid DNA was diluted with serum-free medium in a sterile tube and gently pipetted to mix. X-tremeGENE HP DNA Transfection Reagent (cat# 06366236001; Roche, Germany) was then added to the diluted DNA and incubated at 15°C–25°C for 15 min. The cells were transfected with either miR-590-pLenti-III-eGFP or the control vector. This transfection complex was added to the cells in a drop-wise manner. The wells were gently shaken and swirled to ensure even distribution over the entire plate. After incubation for 72 h, the expression levels of the candidate miRNA and candidate genes were measured by real-time PCR.

Real-time quantitative reverse transcription PCR (qRT-PCR) for miR-590

RNX-Plus (SinaClon, Iran) was used for the isolation of total RNA from the cells. The procedure was performed 72 h after the transfection of miR-590-pLenti-III-eGFP. In order to measure miR-590 expression, the isolated RNA underwent a reverse transcription reaction (to obtain cDNA) using a two-step reverse transcription PCR (RT-PCR) kit (Vivantis, Malaysia). The manufacturer's instructions were followed during the whole process with the exception that RT primer was used instead of oligo d(T), and SNORD47 was used as the internal control for miRNA. RealQ Plus 2x Master Mix Green (Ampliqon, Denmark) was used for real-time RT-PCR. The expression of miRNA was normalized using SNORD47 as internal control, and the $2^{-\Delta\Delta CT}$ method was used to examine the relative expression levels in treated and control cells. Each test was run in triplicate. The primers' sequences are represented in Table 1.

Real-time qRT-PCR for candidate genes

In order to perform the real-time qRT-PCR for the candidate genes, the procedure described above was followed. Real-time PCR was performed 72 h after the transfection of miR-590-pLenti-III-eGFP to measure the expression level of four candidate genes, that is, JAK2, PI3K, MAPK1, and CREB. The expression of target genes was normalized using beta-actin as internal control, and the $2^{-\Delta\Delta CT}$ method was used to examine the relative expression

Table 1. Details of primers' sequences for selected miRNA.

Primer name	Primer sequence
RT 590-5p	GGTCGTATGCAGAGCAGGGTCCGAGGTATCCATCGCACGCATCGCACTGCATACGACCCTGCA
590-5p-F	GCCGAGCTTATTCATAAAAG
590-5p-R	GAGCAGGGTCCGAGGT
RT SNORD47	GTCGTATGCAGAGCAGGGTCCGAGGTATTCGCACTGCATACGACAACCTC
SNORD47-F	ATCACTGTAAAACCGTTCCA

levels in treated and control cells. Each test was run in triplicate. The primers' sequences of candidate genes are represented in Table 2.

Detection of cell apoptosis

Flow cytometry was applied to determine the percentage of Annexin $V^{positive}$/7-$AAD^{negative}$ cells and measure apoptosis. An Annexin V apoptosis detection kit with 7-AAD (A9210/A9400; Sigma–Aldrich, UK) was used to measure the percentage of apoptotic cells 72 h after miRNA transfection.

Real-time qRT-PCR for MMP2 and MMP9

Total RNA was isolated from the cells 72 h after the transfection of miR-590-pLenti-III-eGFP using RNX-Plus (SinaClon) and was reversely transcribed into cDNA using two-step RT-PCR kit (Vivantis). Real-time PCR was performed using RealQ Plus 2x Master Mix Green (Ampliqon). The expression of target genes was normalized using beta-actin as internal control, and the $2^{-\Delta\Delta CT}$ method was used to examine the relative expression levels in treated and control cells. Each test was run in triplicate. The primers' sequences of candidate genes are represented in Table 3.

Results

Since we focused on the molecular signaling pathways similar in cancer and inflammation, KEGG database and literature study led us to some pathways and some molecules and genes which behave similarly in cancer and inflammatory diseases including JAK2, MAPK1, PI3K, and CREB. Potential miRNAs regulating selected genes were analyzed using publicly available algorithm-based databases. A score table was prepared in which the miRNAs were sorted based on repeat rate of more to less, and finally, miR-590 was selected. Then miR-590 was cloned into the vector pLenti-III-eGFP, and miRNA-pLenti-III-eGFP was purchased with the schematic map shown in Figure 1 (MBA Company, Canada).

Two cell lines, MCF-7 and MDA-MB-231, were cultured in 24-well plate, and when reached to almost 70% confluency, they were transfected with the vector in which the presence of green fluorescent protein (GFP) as a fluorescent dye could report the validity and the percentage of the vector entrance (Figure 2).

The entrance of miR-590 into the cell lines was confirmed with real-time qRT-PCR. The result showed that miR-590 was significantly up-regulated in both MCF-7 and MDA-MB-231 cells (Figure 3). In addition, the expression level of four selected genes, PI3K, JAK2, CREB, and MAPK1, which show over-expression in cancer and in chronic inflammation, was evaluated by qRT-PCR 3 days after miR-590 transfection into the cells to measure the probable changes on their expression. The results showed that all the genes were significantly down-regulated ($p \leq 0.05$) (Figure 4).

We examined cell surface expression of phosphatidylserine by Annexin V staining, and fluorescently labeled Annexin V (green) bound to phosphatidylserine in early apoptotic cells. Significant increase in Annexin $V^{positive}$ was seen in both MCF-7 and MDA-MB-231 cells transfected with miR-590 as compared to control cells (Figures 5 and 6). This increase was more in MDA-MB-231 cells (48.55%) compared to MCF-7 cells (29.30%) (Figures 5 and 6).

MMP2 and MMP9 are related to tumor invasion and metastasis by their capacity for tissue remodeling via ECM, as well as their involvement in epithelial mesenchymal transition (EMT). Actually, MMPs are key players in invasion since they promote digestion of the ECM components. Real-time PCR for MMP2 and MMP9 in

Table 2. Details of primers' sequences for selected genes.

Primer name	Primer sequence
JAK2-F	5′-TAGATGAGTCAACCAGGCATAATG-3′
JAK2-R	5′-CCGCCACTGAGCAAAGAG-3′
PI3K-F	5′-CTTGGAGGACGATGATGTTCTG-3′
PI3K-R	5′-TCTGCTGATAGTGTCTGGACTGG-3′
MAPK1-F	5′-CATGGTGTGCTCTGCTTATG-3′
MAPK1-R	5′-GTAGGTCTGGTGCTCAAAGG-3′
CREB-F	5′-AACCAGCAGAGTGGAGATGCA-3′
CREB-R	5′-GGCATAGATACCTGGGCTAATGTG-3′
Beta-actin-F	5′-CTTCCTTCCTGGGCATG-3′
Beta-actin-R	5′-GTCTTTGCGGATGTCCAC-3′

Table 3. Details of primers' sequences for MMP2 and MMP9 and beta-actin.

Primer name	Primer sequence
H-MMP2-F	5′-GCTCGTGCCTTCCAAGTC-3′
H-MMP2-R	5′-AGTCCGTCCTTACCGTCAA-3′
H-MMP9-F	5′-CGGACCAAGGATACAGTTTGT-3′
H-MMP9-R	5′-CTCAGTGAAGCGGTACATAGG-3′
Beta-actin-F	5′-CTTCCTTCCTGGGCATG-3′
Beta-actin-R	5′-GTCTTTGCGGATGTCCAC-3′

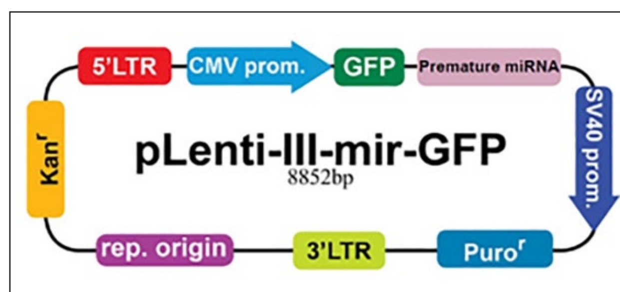

Figure 1. Schematic map of vector miRNA-pLenti-III-eGFP.

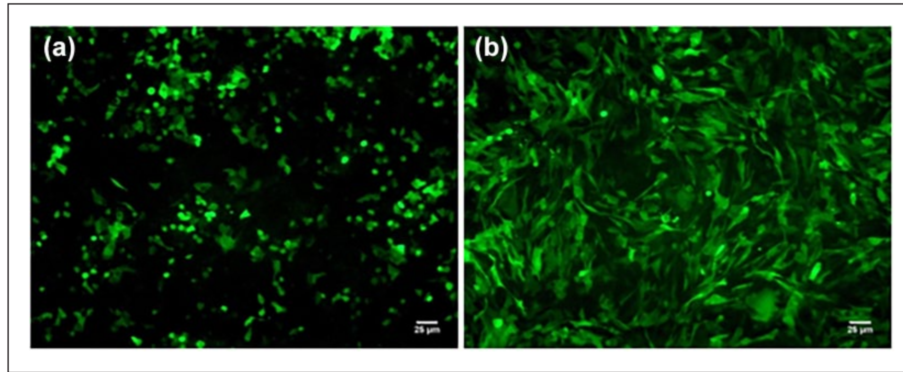

Figure 2. Two breast cancer cell lines transfected with miR-590-5p-pLenti-III-eGFP: (a) MCF-7 cell line and (b) MDA-MB-231 cell line.

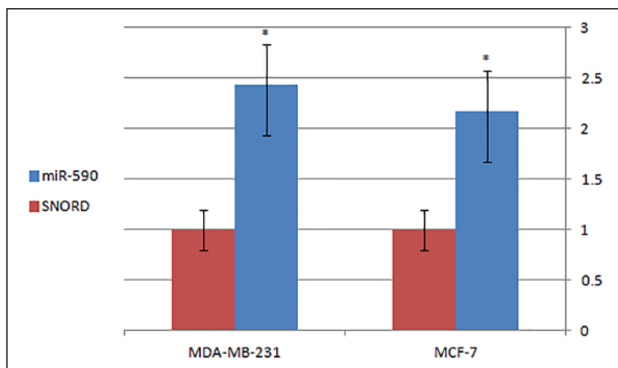

Figure 3. Relative quantification of miR-590 by real-time RT-PCR (fold change based on $2^{-\Delta\Delta CT}$ method). MiR-590 shows over-expression in both cell lines, MDA-MB-231 and MCF-7, after transfection.
*$p \leq 0.05$.

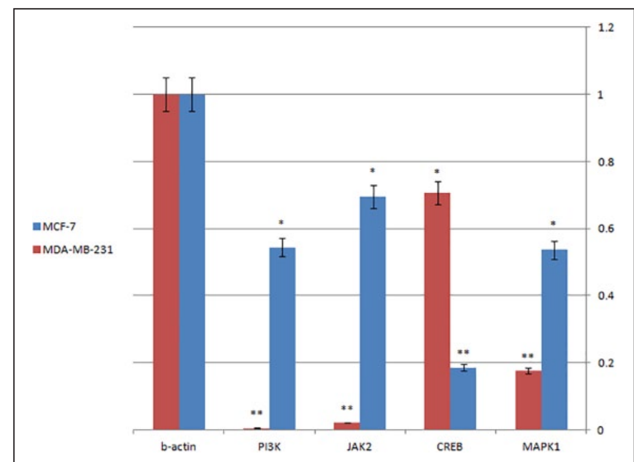

Figure 4. Relative quantification of candidate genes by real-time RT-PCR (fold change based on $2^{-\Delta\Delta CT}$ method). The genes PI3K, JAK2, CREB, and MAPK1 are down-regulated in both cell lines, MDA-MB-231 and MCF-7, after transfection.
*$p \leq 0.05$; **$p \leq 0.01$.

both MCF-7 and MDA-MB-231 cells was performed 72 h after miR-590 transfection. Both MMP2 and MMP9 were strongly down-regulated in MDA-MB-231 cells. No significant change was observed in MCF-7 cells which might be due to their non-aggressive nature, as MCF-7 cells are not migratory and invasive compared to MDA-MB-231 cells, whereas MMPs are molecular indicators of migration and invasion in cancer cells and so, MCF-7 cells do not show down-regulation in MMPs (Figure 7).

Discussion

Chronic inflammation involves proliferation, migration, and recruitment of tissue and inflammatory cells which can be extremely damaging to normal tissue. Researchers have highlighted potential associations between chronic inflammation and not only various types of cancer but also several other conditions such as rheumatoid arthritis, diabetes, and cardiovascular diseases.[19] Rudolph Virchow (1863) was the first to argue that cancer initially developed at sites of chronic inflammation.[20] Recent studies have also increased the existing knowledge about the role of complex inflammatory processes in breast cancer. A real breakthrough in this field occurred in 2010 when inflammation in the breast was confirmed as an indicator of the development and progression of breast cancer. A previous study also reported a two- to three-fold higher risk of breast cancer recurrence and premature death in patients who had high levels of C-reactive protein and serum amyloid A (two major inflammatory markers).[21,22] Among the various inflammatory substances with potential roles in cancer, cyclooxygenase (COX), lipoxygenase (LOX), and NF-κB have received the greatest levels of attention. The significance of the balance between pro- and anti-inflammatory factors in human health is undeniable.[10] According to available literature, pro-inflammatory cytokines may facilitate tumor growth and metastasis through changing tumor cell biology and activating stromal cells in vascular

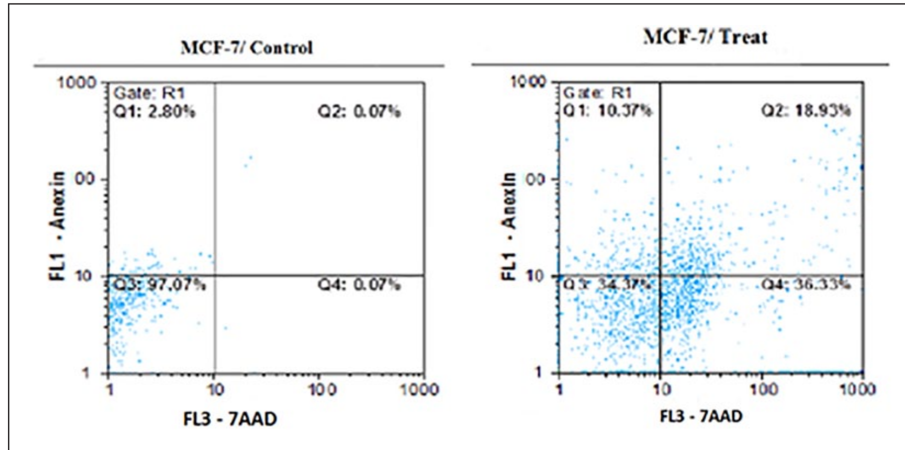

Figure 5. Flow cytometric results of MCF-7 cells for Annexin V. Left panel: control group; 2.87% of cells expressed Annexin V. Right panel: test group; 29.30% of transfected cells expressed Annexin V.

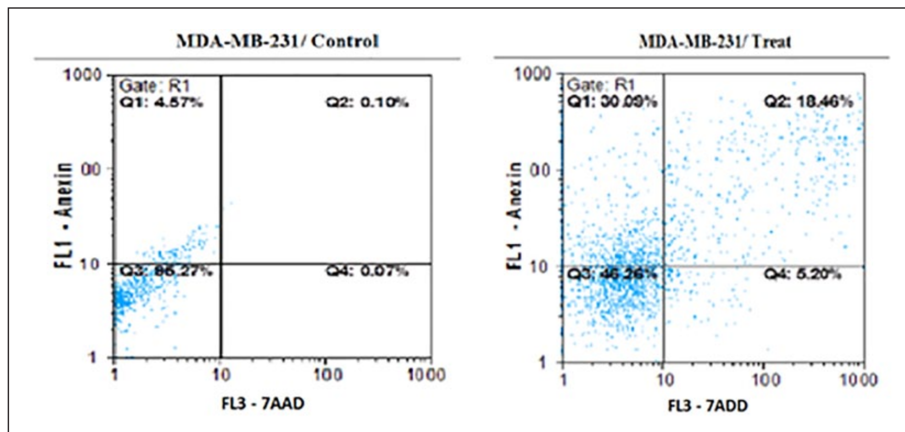

Figure 6. Flow cytometric results of MDA-MB-231 cells for Annexin V. Left panel: control group; 4.67% of cells expressed Annexin V. Right panel: test group; 48.55% of transfected cells expressed Annexin V.

endothelial cells, tumor-associated macrophages, fibroblasts, and other components of the tumor microenvironment. Systemic inflammation can also alter vasculature and promote extravasation, engraftment, growth of micrometastases, and distant metastases.[23,24]

Inflammatory status can also be a prognostic factor for breast cancer. Clinical and experimental data indicate that chronic inflammation promotes mammary tumor development through mechanisms involving chronic activation of humoral immunity and infiltration of Th2 cells and innate inflammatory cells.[25] Higher mutation rates and greater proliferation of mutated cells have been reported in inflammatory microenvironments. Nevertheless, evidence suggests mutual interactions between inflammation and tumor initiation, that is, DNA damage can cause inflammation and thus promote carcinogenesis. A major tumorigenic mechanism involves the production of tumor-promoting cytokines by immune and inflammatory cells. This stimulates transcription factors such as NF-κB, signal transducer

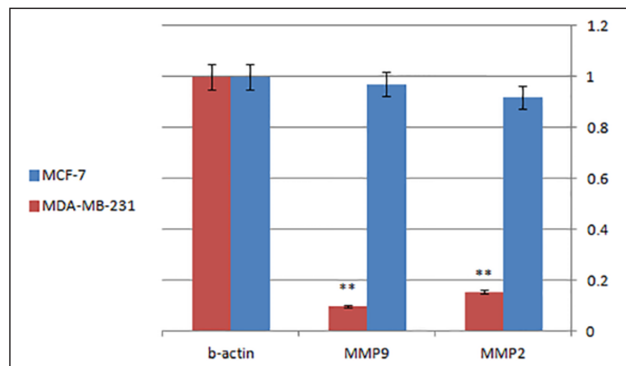

Figure 7. MMP2 and MMP9 are down-regulated in MDA-MB-231 cells after miR-590 transfection, whereas no significant change in MMP2 and MMP9 is detectable in MCF-7 cells. **$p \leq 0.01$.

and activator of transcription 3 (STAT3), and activator protein-1 (AP-1) in pre-malignant cells and thus increases

the expression of genes enhancing cell proliferation and survival.[26,27]

Many questions about the breast cancer origination still remain unanswered. Recently, miRNAs have been identified as effective factors in breast cancer. The expression levels of these molecules vary in different stages of breast cancer, and they play an important role in disease progression and metastasis.[28,29] The levels of miRNAs are regulated by different factors such as hormones. Since the miRNAs are rendered through a secondary post-transcriptional regulation, their regulation further alters the pathways in breast cancer.[30] Up- or down-regulation of miRNAs can mediate the pathway of a gene regulation.[31] Research has indicated miRNAs to be involved in not only cancer development and progression but also a variety of physiological processes including immune responses, cell proliferation, cell death, and inflammation. Since such processes are controlled by NF-κB, researchers have tried to clarify the convergence of miRNAs and their target genes with NF-κB pathways necessary for tumor initiation and progression.[32]

A previous study found down-regulated levels of miR-590-5p and up-regulated levels of S100A10 in six hepatocellular carcinoma cell lines. S100A10, a member of the S100 calcium-binding family of proteins, is critical to the migration of macrophages to the tumor site.[33] The simultaneous over-expression of miR-590-5p and underexpression of S100A10 inhibited cell growth and induced gap 1 (G1) arrest in HepG2 cells. Moreover, the up-regulation of miR-590-5P suppressed the expression of two oncogenes called Wnt5a and c-myc. This, in turn, prevented the proliferative activity of HepG2. Another detected change was the down-regulation of cyclin D1, a major regulator of G1 to S-phase transition, which promoted the G1 arrest of HepG2 cells.[34] Furthermore, since the over-expression of miR-590-5p also increased caspase 3, miR-590-5p might promote apoptosis under the experimental conditions. The mentioned changes can justify the inhibitory effect of miR-590-5p on HepG2 growth and suggest 590-5P as a potential target molecule for the treatment of hepatocellular carcinoma. However, more research is warranted to confirm these mechanisms.[35]

The underexpression of pre-miR-590, caused by changes in its target activating transcription factor 3 (ATF-3), has also been reported in human breast cancer. ATF-3 is a stress response gene product with confirmed roles in breast cancer metastasis and cell invasion and proliferation. It was reported that there is a negative feedback regulation of expression between pre-mir-590 and ATF-3 in human breast cancer cells.[36]

Conclusion

The effects of miR-590 on some major genes in breast cancer cell lines may be notable. No previous studies used bioinformatics to predict a miRNA based on the relationship between inflammation and cancer. In this study, miR-590 was introduced as a miRNA which regulates molecular signaling pathways similar in inflammation and breast cancer. A significant reduction was seen in major genes involved in inflammation and cancer followed by miR-590 transfection in breast cancer cells. This reduction led to considerable changes in the behavior of cells and resulted in increased apoptosis and decreased migration markers. Our proposed method for the identification of miRNAs responsible for mediating genes involved in cancer and inflammation can be beneficial in the clarification of tumor biology and development of novel treatments for cancer. Nevertheless, further research is required to investigate the role of miR-590 in cancer and inflammation.

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Declaration of conflicting interests

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