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Abstract

Background: Abnormal expressions of microRNAs are related to various cancers such as breast cancer for which paclitaxel is widely used as a chemotherapeutic agent. We aimed to investigate the effect of paclitaxel treatment on the expression level of miR-199a-5p and miR-10b, involved in epithelial-mesenchymal transition (EMT) process in breast cancer cell lines.

Methods: Human breast cancer cell lines BT-474, SKBR-3, MDA-MB-231, and MCF-7 were cultured and MTT assay was used to determine IC50 of paclitaxel. RNA was extracted, cDNA was synthesized, and the expression level of miRNAs and genes was quantitatively determined using real-time PCR.

Results: After treatment with paclitaxel, the expression level of miR-199a-5p significantly decreased in MCF-7 and SKBR-3 cell lines, while it increased in MDA-MB-231 and BT-474. The expression level of miR-10b was also significantly reduced in MCF-7, MDA-MB-231, and SKBR-3 and increased in BT-474 cell lines following treatment with paclitaxel. Our results further indicated that paclitaxel reduced the expression level of vimentin and MMP-9 in MDA-MB-231 cell line.

Conclusion: Our findings revealed the increased expression of EMT-inhibitor miR-199a-5p and the decreased expression of metastamir miR-10b after treatment of MDA-MB-231 metastatic breast cancer cell line. Reduced expressions of vimentin and MMP-9 were also observed, corroborating the inhibition of metastasis markers in this type of breast cancer. The therapeutic effect of paclitaxel may in part be due to the change in the balance of EMT-promoting and EMT-inhibiting miRNAs.

Keywords: Breast cancer, MiR-199a-5p, MiR-10b, Paclitaxel, Vimentin, MMP-9
Introduction

Breast cancer is among the three common cancers and the most common malignancy among women worldwide. Breast cancer is still the most and second most common fatal cancer in less and more developed countries, respectively.1 This cancer is a heterogeneous disease which cannot be explained only through clinical parameters or biomarkers such as epidermal growth factor receptor-2 (HER-2), estrogen receptor (ER) and progesterone receptor (PR). However, based on gene expression profiling, breast tumors are grouped into six subtypes, namely HER-2-enriched, luminal A, luminal B, basal-like, normal breast, and claudin-low. Basal-like subtype, which lacks hormone receptors and HER-2 expression, is also called triple-negative breast cancer (TNBC).2, 3 As treatment methods for breast cancer, surgery, radiation therapy, chemotherapy, and targeted therapy are selected based on the type and stage of cancer.4 For certain biological tumor subtypes such as TNBC, Anthracycline and taxane-containing chemotherapy could be recommended.1 Paclitaxel, a member of taxane antitumor agents, is a chemotherapeutic agent utilized in the treatment of several cancers including breast cancer. Paclitaxel inhibits the depolymerization of the microtubules and blocks cell mitosis cycle in G2/M phase, ultimately entailing apoptotic cell death.5, 6

MiRNAs are among the important classes of regulatory mechanisms that target about 5300 human genes7 and play critical roles in various biological processes; the abnormal expression of these regulatory mechanisms are related to various human diseases and cancers.8, 9 The expression profile of miRNAs in breast cancer was for the first time elucidated in 2005 by Iorio et al.10 Two groups of miRNAs including tumor-suppressor and oncogenic miRNAs (oncomiRs) are involved in breast cancer.11 Moreover, it has been found that certain miRNAs are involved in a metastasis called “metastamirs”. Metastasis causes approximately 90% of cancer-related mortalities.12 The role of miRNAs in drug resistance is also under vigorous investigations.13

Initially introduced by Greenburg and Hay in 1982,14 EMT is identified as an initial step in the metastatic cascade.15 EMT is a process where cells lose their epithelial properties and acquire mesenchymal characteristics through decreasing in E-cadherin expression and increasing in the expression of mesenchymal markers such as vimentin, Fibronectin, and N-cadherin, which eventually leads to cell migration and invasiveness.16 Physiologically, EMT process plays a critical role in embryonic development, tissue repair, and wound healing. Pathologically, EMT contributes to chemo-resistance, metastasis and fibrosis.17 Involved in EMT are various signaling pathways such as Transforming Growth Factor-Beta (TGF-β), Sonic Hedgehog (SHH) and Wingless-type MMTV integration site family member (WNT) pathways, and effector molecules such as vimentin and MMP-9, and MMP-2.18 Several miRNAs have been identified to target the EMT regulatory factors in order to inhibit or induce EMT process. The first evidence linking miRNAs and metastasis was provided by Ma et al. who demonstrated the initiation of tumor invasion and metastasis by miR-10b in breast cancer.19 MiR-10b is the most well-known metastamir and EMT-promoting miRNA in breast cancer. MiR-199a-5p is down-regulated in breast cancer and has a tumor-suppressor role.20

In this study, we investigated the effect of anticancer agent paclitaxel on the expression level of miR-199a-5p, miR-10b, vimentin and MMP-9 in breast cancer cell lines.

Materials and Methods

Cell Culture

In this experimental in vitro study, human breast cancer cell lines including BT-474, SKBR-3, MDA-MB-231, and MCF-7 were purchased form National Cell Bank of Iran (Pasteur Institute, Tehran, Iran) and cultured in RPMI 1640 medium (GIBCO, USA) supplemented with 10% fetal bovine serum (FBS) (GIBCO, USA), 100 units/ml penicillin and 100 µg/mL streptomycin. Cells were grown at 37°C in a humidified atmosphere with 5% CO₂.
MTT assay

In order to determine the IC50 (half maximal inhibitory concentration) of paclitaxel in the studied cell lines, MTT assay was performed. First, 15×10^3 cells of each cell line were seeded with 200 μl culture medium in 96-well plates. In 70-80% confluency, the medium was removed, various concentrations of paclitaxel (0.1 nM, 1 nM, 10 nM, 100 nM, 1 μM, 10 μM and 100 μM) with 200 μl of fresh media were added, and cells were incubated for 24 hours at 37°C with 5% CO₂. After 24 hours, the medium was removed, and after washing the cells by phosphate buffered saline (PBS), 50 μl of 2 mg/mL MTT solution (Sigma, USA) and 150 μl medium were added to each well and incubated for 4 hours at 37°C with 5% CO₂. Supernatants were removed and dimethyl sulfoxide (DMSO) and Sorenson’s buffer were added. After 30 minutes, the optical density of each concentration was read at 570 nm, and the IC50 of paclitaxel for each cell line was further calculated. To obtain more accurate results for the IC50 of paclitaxel in cell lines, we limited the range of paclitaxel concentrations (Table 1) in the second step. All assays were run as triplicate.

RNA extraction and cDNA synthesis

Total RNA of both untreated and treated cells were extracted using RNX-PLUS reagent (CinnaGen, Iran) according to the manufacturer’s instructions. RNA concentration was determined using Nano Drop Spectrophotometer (Thermo Scientific Nanodrop 2000c, USA) and the quality of the extracted RNAs was evaluated by agarose gel electrophoresis.

Because primers were used based on locked nucleic acid (LNA) technology (Exiqon, Denmark), cDNA for microRNA detection was synthesized by Universal cDNA Synthesis Kit II (Exiqon, Denmark) according to the manufacturer’s protocol. As described before, cDNA for mRNAs was synthesized from the extracted RNA.21

Quantitative Real-time PCR

The expression levels of miRNAs before and after treatment with paclitaxel in each cell line were evaluated by quantitative real-time PCR using SYBR Green master mix (Yekta Tajhiz Azma, Iran) on a Corbett Rotor-gene 6000 system (Corbett Life Science, Australia). MiRNA primers with LNA technology were purchased from Exiqon, and real-time PCR was performed according to the manufacturer’s protocol. U6 snRNA was used as an internal control to normalize miRNA expression levels. Further evaluated were the expressions of vimentin and MMP-9 via real-time PCR, and β-actin was used as an endogenous control. The sequences of the primers are shown in table 2.

Statistical analysis

Data were analyzed using Prism Software version 6.01 (Irvine, CA). Multiple t-test was used to compare the data of two groups (treated and untreated) and P-values less than 0.05 were considered as statistically significant.

Results

MTT assay results

To determine the IC50 concentration of paclitaxel after 24 hours, MTT assay was dose dependently performed for each cell line. The

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Concentration</th>
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<tbody>
<tr>
<td>BT-474</td>
<td>0.1 nM, 1 nM, 10 nM, 25 nM, 50 nM, 100 nM and 250 nM.</td>
</tr>
<tr>
<td>SKBR3</td>
<td>100 nM, 1000 nM, 25000 nM, 50000 nM, 100000 nM and 250000 nM</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>153 nM, 306 nM, 612 nM, 1225 nM, 2450 nM, 4900 nM and 9800 nM</td>
</tr>
<tr>
<td>MCF-7</td>
<td>125 nM, 250 nM, 500 nM, 1000 nM, 2000 nM, 4000 nM and 8000 nM</td>
</tr>
</tbody>
</table>
IC50 concentrations of paclitaxel for breast cancer cell lines including BT-474, SKBR3, MDA-MB-231 and MCF-7 were 19 nM, 4 µM, 0.3 µM and 3.5 µM, respectively (Figure 1).

![MTT results for breast cancer cell lines](image)

**Figure 1.** MTT results to determine the IC50 concentration of paclitaxel for breast cancer cell lines. To determine the IC50 concentration of paclitaxel in the studied cell lines, MTT assay was performed in different serial concentrations of paclitaxel, for four different breast cancer cell lines.

**Table 2.** The sequences of primers used for the detection of the expression of miR-199a-5p, miR-10b, and vimentin and MMP-9 in this study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>miR-199a-5p*</td>
<td>5′-CCCAGUGUUCAGACUACCUGUUC-3′</td>
</tr>
<tr>
<td>miR-10b*</td>
<td>5′-UACCCUGUAGAACCAGAUAUGUG-3′</td>
</tr>
<tr>
<td>U6*</td>
<td>5′-GGG CAG GAA GAG GCC TTA T-3′</td>
</tr>
<tr>
<td>Vimentin</td>
<td>F: 5′-GCGCAAAATCCCTTCTACC-3′</td>
</tr>
<tr>
<td></td>
<td>R: 5′-ATCCGTTGAGCAATTCCTTGCC-3′</td>
</tr>
<tr>
<td>MMP-9</td>
<td>F: 5′-GGTTCTCTTCTCGCTACTGCTG-3′</td>
</tr>
<tr>
<td></td>
<td>R: 5′-GTCGTAGGGCTGCGAAGG-3′</td>
</tr>
<tr>
<td>β-actin</td>
<td>F: 5′-TCCCTGGAAAGAGCTACG-3′</td>
</tr>
<tr>
<td></td>
<td>R: 5′-GTAGTTTCGATGCAGCCACA-3′</td>
</tr>
</tbody>
</table>

*The sequence for target sequence is shown.
Paclitaxel alters the expression level of miR-199a-5p and miR-10b in breast cancer cell lines

The results showed that following treatment with IC50 concentration of paclitaxel, the expression level of miR-199a-5p decreased in MCF-7 and SKBR-3 cell lines, while increased in MDA-MB-231, and BT-474 (Figure 2 A). Moreover, the expression level of miR-10b was reduced in MCF-7, MDA-MB-231, and SKBR-3, while increased in BT-474 cell lines (Figure 2 B). *P*-values and fold changes of the studied miRNAs are shown in table 3.

Down-regulation of EMT effector molecules in triple-negative metastatic breast cancer cell line, MDA-MB-231 in response to treatment with paclitaxel

The expression level of vimentin and MMP-9 mRNAs, two EMT effector molecules, in MDA-MB-231 breast cancer cell line was studied before and after treatment with paclitaxel. Our findings indicated that both vimentin (41.6 fold) and MMP-9 (83.3 fold) were reduced in response to treatment with paclitaxel (Figure 3).

Discussion

MiR-199a-5p (also called miR-199a) plays different oncogenic or tumor suppressor roles in various types of cancer. It has been shown that miR-199a is down-regulated in ovarian cancer, breast cancer and advanced small cell carcinoma of the cervix. Other studies have shown that miR-199a suppresses cell growth in renal cancer and acts as a tumor suppressor in HCC. In contrast, the oncogenic role of miR-199a has been shown in gastric cancer. Regarding breast cancer, Chen et al. have shown the inhibitory role of miR-199a in proliferation via inducing G0/G1 phase arrest and apoptosis in MDA-MB-231 cells. Our results indicated that following treatment with paclitaxel, the expression level of miR-199a-5p was increased in triple-negative breast cancer cell line, MDA-MB-231. Our findings are justifiable since paclitaxel and miR-199a-5p cause cell cycle arrest and paclitaxel exerts synergic effects with miR-199a-5p in MDA-MB-231 cells. Considering the findings of the two previous studies, it could be suggested that miR-199a-5p may sensitize the TNBC to paclitaxel chemotherapy. Because of the unre sponsiveness of targeted therapies such as Tamoxifen (an antagonist to the ER) or Tratuzumab (monoclonal antibody against HER2 receptor) in TNBC, and due to the lack of ER,
PR and HER2, systemic chemotherapy with paclitaxel and its sensitization by miR-199a-5p mimics could be considered as an approach to treating TNBC.

Chen et al. also found that miR-199a-5p significantly inhibited cell migration and invasion ability in MDA-MB-231 cells through EMT process. Another study has demonstrated that miR-199a-5p suppresses invasion in breast cancer by regulating β1 integrin through Ets-1. It has also been shown that miR-199a-5p inhibits the invasion in human hepatocellular carcinoma by targeting DDR1. In our study, the up-regulation of miR-199a-5p in breast cancer metastatic cells, MDA-MB-231, was observed after treatment with paclitaxel. These findings are consistent with the usage of paclitaxel in combination with other chemotherapeutic agents as the first line chemotherapy in metastatic breast cancer.

Since miR-10b was identified as the starter of tumor invasion and metastasis in 2007, other studies have reported the involvement of miR-10b in the metastasis of cancers including breast cancer. Liu et al. observed that miR-10b targeted E-cadherin and modulated breast cancer metastasis. It was shown that miR-10b targeted syndecan-1 and promoted breast cancer cell motility and invasiveness by mechanisms dependent on Rho-GTPase and E-cadherin. Furthermore, miR-10b plays a critical role in TGF-β1-induced EMT in breast cancer, and the up-regulation of miR-10b is related to the brain metastasis of breast cancer. Considering all the foregoing reports, miR-10b, as the most well-known metastamir, plays a major role in EMT process and metastasis in breast cancer. In line with previous studies, our results showed that after treatment with paclitaxel, the expression level of miR-10b was reduced in MDA-MB-231. Therefore, it the down-regulation of miR-10b could be considered as a probable mechanism for paclitaxel effects.

We further investigated the effect of paclitaxel on two EMT effectors, namely vimentin and MMP-9 in MDA-MB-231 cell line, and observed their significant down-regulation after treatment. Yang et al. demonstrated that paclitaxel-resistant breast cancer cells displayed EMT phenotype with the up-regulation of vimentin. Also, it has been shown that vimentin is up-regulated in Paclitaxel-resistant ovarian cancer cells. In line with these studies, paclitaxel reduced the expression level of vimentin in MDA-MB-231 breast cancer cell line in the present research. Furthermore, the expression level of MMP-9 was reduced in MDA-MB-231 cell line after treatment with paclitaxel, a finding in agreement with Ruan et al. who showed that paclitaxel down-regulated MMP-9 expression in glioblastoma cells. According to these results, it could be proposed that paclitaxel inhibits EMT process in part via the reduction of vimentin and MMP-9 expression levels.

**Conclusion**

Defining the exact molecular action mechanism
of chemotherapeutic agents such as paclitaxel improves cancer therapy regimens. Given the increased expression level of miR-199a-5p and the decreased expression level of miR-10b, and vimentin and MMP-9 following the treatment of triple-negative metastatic MDA-MB-231 breast cancer cell line, it is concluded that changing the expression of EMT-prompting and EMT-inhibiting miRNAs and genes can be a role of paclitaxel chemotherapeutic agents.

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Conflict of Interest
None declared.

References


