Differential altered expression of let-7a and miR-205 tumor-suppressor miRNAs in different subtypes of breast cancer under treatment with Taxol

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Abstract

Background. MicroRNAs (miRNAs) are small non-coding RNAs which have been considered as major players in the process of carcinogenesis and drug responsiveness of breast cancer.

Objectives. In this study, we aimed to investigate the expression pattern of let-7a and miR-205 tumor-suppressor miRNAs in breast cancer cell lines under treatment with paclitaxel.

Material and methods. The half maximal inhibitory concentration (IC_{50}) of paclitaxel was determined for 4 breast cancer cell lines, including MCF-7, MDA-MB-231, SKBR-3, and BT-474 by an MTT assay. The expression level of let-7a and miR-205, and their targets, *K-RAS* and *HER3*, was determined before and after treatment with paclitaxel, using quantitative reverse transcriptase real-time polymerase chain reaction (qRT-PCR).

Results. After treatment, the expression level of both let-7a and miR-205 was significantly increased in *HER2*-overexpressing cell line BT-474 (26.4- and 7.2-fold, respectively). In contrast, the *HER2*-negative cell lines MCF-7 and MDA-MB-231 showed a significantly decreased expression of both let-7a (30.3- and 13.5-fold, respectively) and miR-205 (20- and 18.1-fold, respectively). Controversially, SKBR-3 revealed a significantly decreased expression of both let-7a (1.3-fold) and miR-205 (1.3-fold). The expression level of *K-RAS* as a target of let-7a decreased in all cell lines significantly, but the pattern of alteration in the expression level of *HER3* as a target of miR-205 in all cell lines was the reverse of the pattern of alteration in the expression level of miR-205.

Conclusions. Our results confirmed a better response of *HER2*-overexpressing breast cancer to paclitaxel at the miRNA level. One putative reason could be the upregulation of tumor-suppressor miRNAs after treatment with paclitaxel. On the other hand, *HER2*-negative breast cancer cell lines showed a significantly decreased expression of tumor-suppressor miRNAs, a putative mechanism of resisting the therapy.

Key words: breast cancer, microRNA, tumor-suppressor, Taxol

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Introduction

During the past decades, breast cancer has been the most frequent type of carcinoma and the second most fatal cancer among women worldwide. Importantly, both the prevalence and mortality of breast cancer is increasing[1]; it has been estimated that 249,260 new cases and 40,890 deaths due to breast cancer will occur in the United States in 2016.[2] Breast cancer occurs as a result of a collection of genetic alterations and environmental factors.[3] Among the well-known genetic loci related to breast cancer are: breast cancer susceptibility gene 1 (*BRCA1*) and breast cancer susceptibility gene 2 (*BRCA2*), human epidermal growth factor receptor-2 (*HER-2*) and tumor protein p53 (*TP53*).[4–6] Dietary habits like consuming fat-rich foods, presence of chemical substances in food, long exposure to estrogen, e.g., excessive use of steroids, and use of alcohol are examples of environmental risk factors.[3,7] Breast cancer is a heterogenic disease which includes molecular subtypes, characterized by immunohistochemical expression of estrogen receptor (*ER*), progesterone receptor (*PR*) and *HER-2*. These molecular subtypes differ in response to treatment, progression and preferential organs to metastasis.[8,9] The treatment approaches for breast cancer are surgery, radiation and chemotherapy with different agents, such as taxanes, anthracyclines, hormone-based drugs, and also targeted therapy with monoclonal antibodies.[10]

Taxol (paclitaxel) is a member of the taxane family with a plant origin that is among the effective chemotherapy agents in the treatment of breast cancer. The mechanism of its action is the induction of dimerization and the prevention of depolymerization of tubulins. Cells lose their flexibility and cannot divide, thus, the tumor progression is stopped. Paclitaxel can be used alone or in combination with other chemotherapy or immunological agents as first-line and also salvage therapy in advanced tumors. Particularly, the use of paclitaxel in a regimen including anthracycline significantly improves the chance of survival.[11]

MicroRNAs (miRNAs) are a group of conserved non-coding RNAs which regulate gene expression through the regulation of targeted mRNAs or the prevention of their translation. They play an essential role in cellular processes, and subsequently various physiological and pathological conditions, such as cancer.[12,13] The first study related to the importance of miRNAs in breast cancer was done in 2005, and it demonstrated a significant deregulation of 5 miRNAs in breast cancer.[14] Since then, studies have been improved in this context and shown the importance of miRNAs in the prognosis, metastasis and drug resistance of different subtypes of breast cancer. As a general rule, the miRNAs involved in breast cancer include 2 groups: oncomirs with oncogenic nature (which are upregulated during carcinogenesis) and tumor-suppressor ones with the ability to prevent breast cancer initiation, progression and metastasis (downregulated in malignancy). Several miRNAs have been introduced in breast cancer as tumor-suppressors which target the mRNAs of oncogenes. Among the best-known tumor suppressor miRNAs are the let-7 family and miR-205.[15] Let-7 is the second identified miRNA in *Caenorhabditis elegans* and the first one in humans. The let-7 family contains 13 members encoded from different loci. The role of this family of miRNAs is promoting differentiation, and their expression is low in embryonic and cancerous cells because of poor differentiation.[16] As the let-7 family of miRNAs target mRNAs of some important oncogenes, such as *RAS* and *HMGA2*, several studies have introduced this family of miRNAs as tumor-suppressor miRNAs in breast cancer.[17–21] MiR-205 is located in 1q32.2 and plays a role in the evolution of 3 organs: the breast, prostate and thymus.[22] Studies on the relation of this miRNA to breast cancer have demonstrated the tumor-suppressing nature of miR-205.[19] Among the oncogenes targeted by miR-205 are *HER-3* and vascular endothelial growth factor-A (*VEGF-A*), with roles in promoting cell proliferation, angiogenesis and metastasis.[23]

Because of the role of tumor-suppressor miRNAs in preventing breast cancer progression and the usefulness of them in predicting the outcome and response of cancer to treatment, the aim of this study was to investigate the effect of the anticancer drug paclitaxel on the expression levels of let-7a and miR-205 and their targets in different subtypes of breast cancer cell lines.

Material and methods

Cell culture

The human breast cancer cell lines MCF-7, MDA-MB-231, SKBR3, and BT-474 were purchased from the national cell bank of Iran (Pasteur Institute, Tehran, Iran) and cultured in RPMI1640 (Gibco; Thermo Fisher, Waltham, USA) medium supplemented with 10% fetal bovine serum (FBS) (Gibco), penicillin (100 IU/mL) and streptomycin (100 mg/mL) at 37°C with 5% CO_2 and humidified air.

MTT assay

The inhibitory concentration 50% (IC_{50}) of paclitaxel was determined for each cell line with an MTT assay. Briefly, 15×10^3 cells of each cell line were seeded in 96-well plates. After reaching 70–80% confluency, the cells were treated with different concentrations of paclitaxel. After 24 h of treatment, MTT dye (Sigma-Aldrich, St. Louis, USA), and subsequently its dissolvers, dimethyl sulfoxide (DMSO) and Sorenson's buffer, were added. The optical density (OD) of each concentration was measured at 570 nm and IC_{50} was calculated using Prism v. 6.0 software (Graphpad Software, La Jolla, USA). The MTT assay was performed in 2 steps. First, all cell lines were treated with concentrations of 0.1 nM, 1 nM, 10 nM, 100 nM, 1 µM, 10 µM, and 100 µM. According to the data from step 1 and

determining the narrower range of paclitaxel concentration, the cells were treated with limited concentration ranges (for MCF-7: 125 nM, 250 nM, 500 nM, 1000 nM, 2000 nM, 4000 nM, and 8000 nM; for MDA-MB-231: 153 nM, 306 nM, 612 nM, 1225 nM, 2450 nM, 4900 nM, and 9800 nM; for SKBR3: 100 nM, 1000 nM, 10,000 nM, 25,000 nM, 50,000 nM, 100,000 nM, and 250,000 nM; for BT-474: 0.1 nM, 1 nM, 10 nM, 25 nM, 50 nM, 100 nM, and 250 nM). All assays were done in triplicate.

RNA extraction and complementary DNA synthesis

In the study, 10^5 cells were seeded in a 6-well plate overnight, and paclitaxel was added to the wells in IC_{50} concentrations determined by the MTT assay. After 24 h, total RNA was extracted from both untreated (as a control group) and treated cells by RNX-PLUS reagent (SinaClon; Vivantis, Tehran, Iran) according to the manufacturer's instructions. The quality and quantity of the extracted RNAs were evaluated using spectrophotometry and agarose gel electrophoresis. For miRNA detection, complementary DNA (cDNA) samples were prepared by Universal cDNA Synthesis Kit II (Exiqon A/S, Vedbæk, Denmark) according to kit instructions.

The cDNA synthesis for the quantification of the expression level of target mRNAs was performed as described elsewhere.[24]

Quantitative real-time polymerase chain reaction

The expression levels of all miRNAs and mRNAs in each cell line before and after treatment with paclitaxel were assessed using SYBR green-based quantitative reverse transcriptase real-time polymerase chain reaction (qRT-PCR) (Yekta Tajhiz Azma, Tehran, Iran). We used U6 as an internal control to normalize the miRNAs level expression and β-actin (for cell lines MCF-7, MDA-MB-231 and SKBR3) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (for BT-474) to normalize real-time PCR data for the expression level of the mRNAs of target genes. The sequences of primers used and annealing temperatures are listed in Table 1.

Statistical analysis

Statistical analysis was performed with Prism v. 6.0 software (Graphpad Software). We used a multiple t-test to compare the data of the 2 groups (treated and untreated); p-values <0.05 were considered statistically significant.

Results

Inhibitory concentration 50% of paclitaxel for breast cancer cell lines

To determine IC_{50} concentration of paclitaxel for each cell line, the MTT assay was run in 2 steps, first with a larger range, and then with a limited range. Our results showed specific IC_{50} concentrations of paclitaxel for each cell line: 3.5 μM for MCF-7, 0.3 μM for MDA-MB-231, 4 μM for SKBR3, and 19 nM for BT-474.

The effect of paclitaxel on the expression levels of let-7a and miR-205, and their targets

The results from quantitative real-time PCR and subsequent statistical analysis showed differential effects of paclitaxel on the expression levels of let-7a and miR-205, and their targets, *K-RAS* and *HER-3*. *HER-2* overexpressing BT-474 cell line showed a different pattern of altered expression of both miRNAs, i.e., paclitaxel caused a significant overexpression of let-7a (26.4-fold) and miR-205 (7.2-fold) (Fig. 1A,1C). Inversely, SKBR-3, MCF-7 and MDA-MB-231 showed a significant downregulation of both miRNAs. It is important to note that this effect is much less pronounced in the *HER-2* positive SKBR-3 cell line (1.3-fold

Table 1. Sequences of primers and annealing temperatures used for quantitative real-time PCR

Target gene	Primer sequence	Annealing temperature [°C]
*Let-7a**	5′-UGAGGUAGUAGGUUGUAUAGUU-3′	60
*miR-205**	5′-UCCUUCAUUCCACCGGAGUCUG-3′	60
*U6**	5′-GGG CAG GAA GAG GGC CTA T-3′	60
K-RAS	F: 5′-CTCCCTGTGTCAGACTGCTCTTT-3′ R: 5′-GGCCTTGCAACCTTGGTCTCTTG-3′	60
HER-3	F: 5′-TCTACTCTACCATTGCCCAACC-3′ R: 5′-GGAACCATCGGGAACTGACC-3′	59
β-actin	F: 5′-TCCCTGGAGAAGAGCTACG-3′ R: 5′-GTAGTTTCGTGGATGCCACA-3′	59
GAPDH	F: 5′-CAAGATCATCACCAATGCCT-3′ R: 5′- CCCATCACGCCACAGTTTCC-3′	59

PCR – polymerase chain reaction; * sequence for target gene.

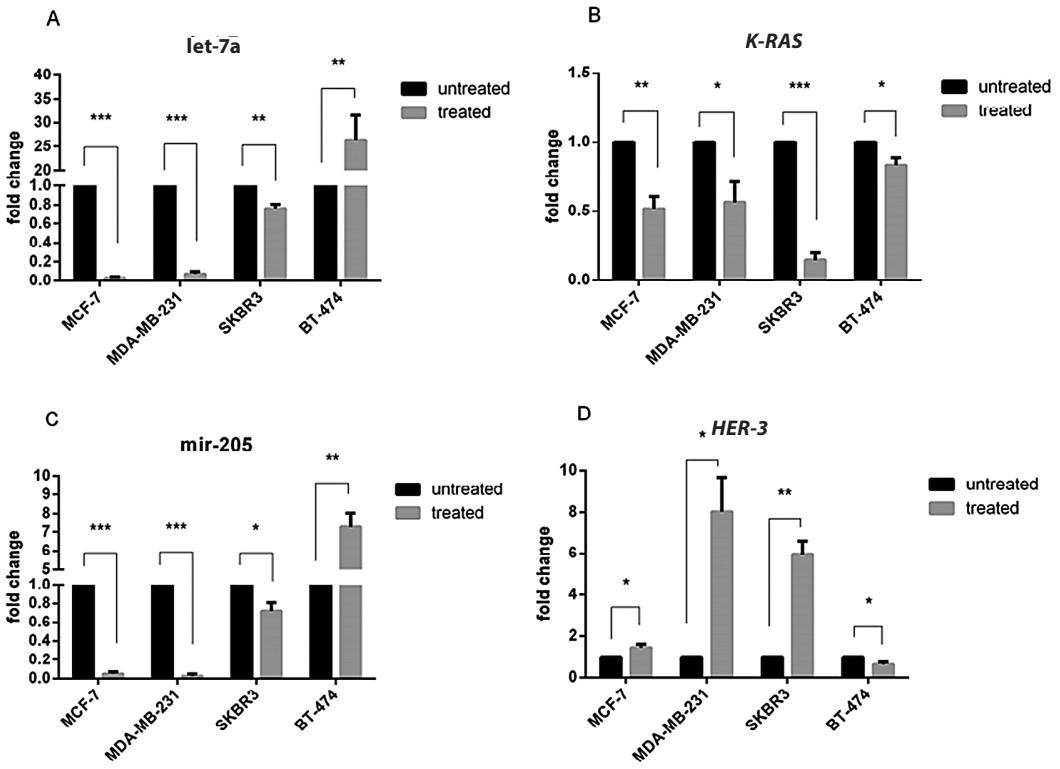

Fig. 1. Alterations in the expression level of miRNAs and targeted mRNAs

The expression level of let-7a (A) and its target, *K-RAS* (B), miR-205 (C) and its target, *HER-3* (D) was quantitatively evaluated before and after treatment of the MCF-7, MDA-MB-231, SKBR3, and BT-474 cell lines with paclitaxel, using quantitative real-time PCR. The expression levels of the 2 miRNAs were significantly decreased in 3 cell lines – MCF-7, MDA-MB-231 and SKBR3 – and significantly increased in BT-474. *K-RAS* showed significantly decreased levels in all cell lines, but *HER-3* was upregulated except for in the BT-474 cell line. * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$.

for both miRNAs, let-7a and miR-205) in comparison to the *HER-2* negative MCF-7 (30.3-fold for let-7a and 20-fold for miR-205) and MDA-MB-231 (13.5-fold for let-7a and 38.4-fold for miR-205) cell lines (Fig. 1A,1C).

Altered expression of *K-RAS* and *HER-3* as targets for let-7a and miR-205, respectively, was found controversial. K-RAS showed a significantly reduced expression in BT-474 (1.2-fold), SKBR-3 (7.4-fold), MCF-7 (1.96-fold), and MDA-MB-231 (1.76-fold) (Fig. 1B). On the other hand, *HER-3* showed an altered expression level related to miR-205. *HER-3* was downregulated in BT-474 (1.58-fold), but upregulated in SKBR-3 (5.9-fold), MCF-7 (1.4-fold) and MDA-MB-231 (8.03-fold) (Fig. 1D).

Discussion

It has been proposed that the altered expression of miRNAs participates in various aspects of cancer, including initiation, progression, metastasis, and drug responsiveness/resistance.[12,25,26] It has been demonstrated that the pattern of miRNA expression can be changed in response to treatment with doxorubicin, 5-fluorouracil and trastuzumab, and this alteration can be useful for predicting the outcome of the therapy.[26–28] However, there is no clear knowledge of the molecular mechanism of paclitaxel with regards to its effect on miRNAs expression. For that reason, we aimed in this study to evaluate the expression level of 2 tumor-suppressor miRNAs, let-7a and miR-205, before and after treatment with paclitaxel. Our results showed that the expression of the miRNAs were significantly altered in response to paclitaxel, but these alterations were different in each subtype of breast cancer.

In our study, the expression level of both tumor-suppressor miRNAs, let-7a and miR-205, showed a significant decrease in 2 *HER-2* negative cell lines, MCF-7 and MDA-MB-231, which was in opposition to the anticancer feature of the drug. Rossi et al. demonstrated that the expression level of miR-21, as an oncomir in various types of cancer, is increased after the administration of 5-fluorouracil in resistant cells of colon cancer. They described this observation as a defense mechanism to resist the chemotherapy.[29] According to the studies done by Konecny et al. and Hayes et al., *HER-2* negative breast tumors show more resistance in response to paclitaxel in comparison to *HER-2* positive ones.[30,31] Our study showed that one of the probable molecular mechanisms of the resistance and a poor response to paclitaxel in *HER-2* negative tumors could be a decreased expression of tumor-suppressor miRNAs let-7a and miR-205.

In contrast, we observed a significant increase in the expression level of both miRNAs in the BT-474 cell line, with the overexpression of the *HER-2* receptor. According to the studies mentioned above, breast cancers with a high expression level of the *HER-2* receptor respond better to paclitaxel therapy, and one molecular mechanism could be the overexpression of tumor-suppressor miRNAs, as we showed.[30,32]

In our study, SKBR-3, which also expresses the *HER-2* receptor, showed a decreased expression of both miR-205 and let-7a after treatment with paclitaxel. According to Ichikawa et al., the genome amplification of the *HER-2* receptor is high, but the expression of this receptor is poor in SKBR-3 in comparison to BT-474.[27] This could justify our results that showed a decreased expression of let-7a and

miR-205 in contrast to BT-474, and much less underexpression than the *HER-2* negative MCF-7 and MDA-MB-231 cell lines. Collectively, our results showed a dependence of the altered expression of let-7a and miR-205 and *HER2*-positivity.

For more evaluation of the molecular aspects of treatment with paclitaxel, we also determined the alterations of expression of *K-RAS* as the target of let-7a and *HER-3* as the target of miR-205.[17,23] Treatment with paclitaxel caused a significant underexpression of *K-RAS* in 4 studied cell lines. It has been previously shown that the downregulation of *K-RAS* is one of the molecular mechanisms of the effect of paclitaxel, and our results confirmed this finding.[33] However, except for in BT-474, the underexpression of let-7a was not in agreement with a decreased expression of *K-RAS* (Fig. 1A,1B). One possible explanation could be multiple regulatory mechanisms for controlling the expression of *K-RAS*, one of the best-known and important oncogenes in most types of cancer.

HER-3, on the other hand, showed a consistent altered expression with the expression of miR-205 in all cell lines (Fig. 1C,1D), albeit with a little inconsistency in the fold change.

Conclusions

Conclusively, treatment of breast cancer cells causes alterations in the expression level of let-7a and miR-205, and also in their targets, *K-RAS* and *HER-3*. The pattern of such alterations is different, depending on the molecular subtype of breast cancer. Such findings could be a molecular reason for the different responsiveness of different subtypes of breast cancer. However, more extensive studies are needed to find the exact and detailed molecular markers for predicting response to therapy.

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