

Investigation into the Relation between miR-31 and *RhoA* Expressions in Breast Cancer Clinical Samples and Cell Lines: A Controversial Matter

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HIGHLIGHTS

- miR-31 is an important miRNA implicated in different cellular processes as well as cancer.
- The protein product of *RhoA* gene plays a role in actin-myosin contraction and cell motility in cancer metastasis.
- We approved bioinformatically and experimentally that *RhoA* is one of the genes regulated by miR-31.

ABSTRACT

Keywords:

Breast cancer

miR-31

Metastasis

RhoA

Breast cancer is the most prevalent diagnosed cancer and the second cause of cancer death among women worldwide. There are different mechanisms that play crucial roles in the onset and progression of breast cancer including microRNAs. MicroRNAs are small noncoding RNAs that regulate gene expression by repressing translation post-transcriptionally. MiR-31 is an integrin modulator implicated in different cellular processes such as apoptosis, cell cycle control, and DNA repair. According to the literature, *RhoA* is one of the genes regulated by miR-31. It has an important role in actin-myosin contraction and subsequently in cell motility and migration in metastasis cascade. Breast cancer cell lines, MCF-7 and MDA-MB-231, as well as normal breast cells, MCF-10A, were cultured. RNA extraction, cDNA synthesis, and SYBR Green I quantitative real-time PCR were used to investigate the expression of miR-31 and *RhoA*. In addition, 10 metastatic breast cancer clinical samples were analyzed to assess miR-31 and *RhoA* expression, and normal cells from the same patients were used as controls. Pearson's correlation co-efficient was applied to find out any probable relation between miR-31 and *RhoA* expression. Gene expression analyses in MCF-7 cell line showed downregulation of miR-31 while *RhoA* was upregulated in the cell line (inverse correlation). MiR-31 and *RhoA* were both upregulated in metastatic MDA-MB-231 cell line and downregulated in 90% of clinical samples. Pearson's correlation co-efficient showed complete positive correlation between miR-31 and *RhoA* expression. The expression of miR-31 and *RhoA* is positively correlated, and it is declined in metastatic breast cancer clinical samples save MDA-MB-231 cells. Unlike previous reports, we found that miR-31 is not the main silencer of *RhoA* expression. Therefore, more investigation on genes and miRNAs affecting metastasis process can elucidate new biomarkers and therapeutic targets for metastatic breast cancer.

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Introduction

According to published statistics, breast carcinoma is the second most common cancer after skin cancer among women (Takebe et al., 2011), and it is still the most prevalent cause of cancer-related death in women. It is reported that just in 2012, breast cancer caused 550,000 deaths (Yi et al., 2014). Invasion and metastasis are responsible for 90% of deaths in breast cancer patients (Marino et al., 2013).

There are different signaling pathways involved in progression and metastasis of breast cancer including Wnt pathway (Nwabo Kamdje et al., 2014). Different proteins in Wnt pathway participate in cancerous cells migration and metastasis, one of which is *RhoA* protein (Roarty and Rosen, 2010). *RhoA* is a monomeric G-protein encoded by *RhoA* gene. It is a noticeable regulatory factor usually found in the cytoplasm of cells and regulates cell division, cell motility, transcription, cell cycle progression, and cell shape (Howe and Brown, 2004). *RhoA* regulates the expression of sodium channel nav1.5 in breast cancer cells. Voltage-gated sodium channels are highly expressed in breast cancer that intensifies migration and invasion of cancer cells (O'Connor and Chen, 2013). In addition, dysregulation of *RhoA* expression has been detected in a number of cancers including breast cancer (Chen et al., 2013).

MicroRNAs (miRNAs) are group of biomolecules involved in the development and metastasis of the cancers. These are small RNAs, approximately 18-25 nucleotide in length, originated from a stem-loop precursor. These short sequences regulate gene expression by inhibiting translation (Takahashi et al., 2015). The biological roles of miRNAs include the development of vertebrates and plants, differentiation, metabolism, and other cellular processes like those involved in cancerous cells. miRNAs, which can act as tumor suppressors or oncogenes by inhibiting a target gene expression, can be used as biomarkers in cancers (Augoff et al., 2011; Reddy, 2015; Takahashi et al., 2015).

Dysregulation of miR-31 expression has been reported to be connected with different cancer events (Schmittgen, 2010; Valastyan and Weinberg, 2010; Rasheed et al., 2015). Researches show that re-expression of miR-31 in breast cancer cells suppresses metastasis, and repression of miR-31 in cells results in metastatic phenotype of breast cancer cells (Lu et al., 2012; Laurila and Kallioniemi, 2013). Nevertheless, all together, these studies are not informative enough since there are contradictory results showing that miR-31 can have both tumor suppressive and oncogenic role in different tumors.

Because of the lack of enough supporting evidence on miR-31 and *RhoA* expression and their correlation in metastatic breast cancers, we investigated the level of their expression in breast cancer cell lines and metastatic

clinical samples to explore and suggest new biomarkers for diagnosis of metastatic breast cancer.

Materials and Methods

Materials

All primer sets were synthesized by Macrogen Inc. (Korea). Breast cancer cell lines MCF-7, MDA-MB-231, and MCF-10A were purchased from NCBI (National Cell Bank of Iran, Tehran). SYBR Premix Ex Taq II (Tli RNase H Plus) was supplied from Takara Bio Inc. (Japan). Hexamer primer and dNTP mix was purchased from CinnaGen Company (Tehran, Iran). RT buffer, and reverse transcriptase enzyme was supplied from Fermentas Inc. (USA).

Designing primers to analyze miR-31 and *RhoA* expression

The sequence of *RhoA* was retrieved from GenBank, NCBI (www.ncbi.nlm.nih.gov). Using Primer3 and Oligo7 software applications, the best primer sets were designed and selected. To assess the expression of miRNA, we utilized a homebrew stem-loop system, developed and validated previously, for reverse transcription of miRNA (Mohammadi-Yeganeh et al., 2013; Mohammadi-Yeganeh et al., 2015). Specific forward and reverse primers were also designed for real-time PCR gene expression analysis.

Cell culture

MCF-7 and MDA-MB-231 cells were cultured in high glucose DMEM medium containing 10% FBS, and 1% penicillin-streptomycin. Normal breast cell line, MCF-10A, was cultured in high glucose DMEM medium containing 5% horse serum, 10 µg/ml insulin, 20 µg/ml EGF, 0.5 µg/ml hydrocortisone, and 1% penicillin-streptomycin. The cells were incubated in humidified atmosphere with 5% CO₂ at 37°C to reach more than 80% confluency.

Total RNA extraction from cell lines and cDNA synthesis

In order to analyze *RhoA* gene expression, total RNA was extracted from cell lines using RNX™-Plus (Cinnaclon, Tehran, Iran) according to the manufacturer's instruction. The quantity and quality of extracted RNA were determined using spectrophotometer (Eppendorf, Germany) at 260 and 280 nm and gel electrophoresis, respectively.

One microgram of extracted RNA was mixed with random hexamer primer in deionized water and incubated for 5 minutes at 65°C. Then, dNTP mix (10 mM), RT buffer, and reverse transcriptase enzyme were added to the mixture and cDNA synthesis was performed in a thermal cycler (Eppendorf, Germany).

Quantitative real-time PCR

In order to analyze the expression of *RhoA* and β -actin housekeeping gene, triplicate real-time PCR reactions were performed in a final volume of 13 μ L containing 6.25 μ L SYBR Premix Ex Taq II, 0.2 μ L of each primers (Forward and Reverse primers, 0.4 μ M), and 6.35 μ L double-distilled water. Amplification condition was as following: enzyme activation step at 95 °C for 30 s, followed by 35 cycles of 95 °C for 5 s and 60 °C for 30 s. Melting curve analysis was performed from 60 °C to 95 °C (1 °C increments). Amplification, data acquisition, and analysis were performed using Rotor-Gene Q instrument (Qiagen, Germany). Fold change in gene expression was determined using the Relative Expression Software Tool (REST®) (Pfaffl et al., 2002). Non-template control and RT-minus were used in all of the experiments.

miRNA expression analysis

Because of short length of miRNAs, primer design was carried out by stem-loop primer method as previously explained (Mohammadi-Yeganeh et al., 2013). One microgram of total extracted RNA was mixed with stem-loop primer and deionized water and incubated for 5 minutes at 65 °C. dNTP mix (10 mM), RT buffer, and reverse transcriptase were then added to the mixture for cDNA synthesis. To ensure that cDNA synthesis was performed properly, PCR was applied using specific primers for SNORD 47 (U47) housekeeping gene, and the products were then electrophoresed. Afterwards, Real-time PCR was performed, according to the previously mentioned protocol (Mohammadi-Yeganeh et al., 2016), to assess miR-31 and U47 expression in cell lines. The results were analyzed using REST® 2009.

Clinical sample collection and storage

Ten metastatic breast cancer samples and 10 adjacent normal samples from the same patients were obtained from different hospitals in Tehran, Iran. Parallel sections were paraffin embedded and prepared for Hematoxylin-Eosin (H&E) staining and histological examination. The fresh specimens were immediately placed in an RNA-preserving solution, RNA Later (Qiagen, Germany), stored at 4 °C for 24 hours, and then transferred to -80 °C until use. None of the patients were undergone previous surgery, chemotherapy, or radiotherapy. Written informed consent for biologic studies was obtained from all patients and analyzed in accordance with the Declaration of Helsinki (Ethics committee No IR.SBMU.SM.REC.1394.37, Shahid Beheshti University of Medical Sciences).

Total RNA extraction, cDNA synthesis, and RT-qPCR on clinical samples

After homogenizing clinical samples with TissueLyser LT instrument (Qiagen, Germany), RNA was extracted from cancer and normal samples using RNX™-Plus (Cinnaclon, Tehran, Iran) according to the manufacturer's instructions. CDNA synthesis was performed using random hexamer primers to analyze *RhoA* and β -actin genes. Stem-loop primers were applied for miR-31 and U47 reverse transcription as mentioned earlier. Finally, triplicate Real-time PCR was performed using StepOne® instrument (ABI, USA). Melting curve analysis was then performed to ensure specific amplification of the product, and the final Ct results were analyzed using REST® 2009 software.

Statistical analysis

Correlation co-efficient calculation for miR-31 and *RhoA* expression was performed using SPSS software (version 16.0; SPSS Inc, Chicago, IL). Graphs were created in Microsoft Excel 2010. Real-time PCR results were analyzed using REST® 2009 software, which compares target and control samples using 2000 iterations.

Results

miR-31 and *RhoA* are two contributing factors in breast cancer progression and metastasis. Since previous studies had shown that miR-31 targeted 3'-UTR of *RhoA* mRNA, we hypothesized that the expression of *RhoA* and miR-31 may be inversely correlated. To test the hypothesis, we recruited a sensitive quantitative real-time PCR based on SYBR Green I to evaluate the expression of *RhoA* and miR-31 in breast cancer cell lines, clinical cancer samples, and adjacent normal breast tissue samples. The sequence of primers designed for miR-31, U47, *RhoA*, and β -actin are presented in Table 1. All the primer and reaction setups were manually performed. As shown in Fig. 1, *RhoA* expression increased nearly 2.5 folds in MCF-7 cells while miR-31 expression showed a 250-fold decline in this cell line. In MDA-MB-231 cells, the expression of miR-31 and *RhoA* were increased by a factor of 151 and 1351, respectively.

Breast cancer and normal adjacent tissue samples were retrieved from women undergoing breast cancer surgery. Confirmed by pathologist, 10 tumor samples were identified as invasive carcinoma and 10 normal adjacent tissues were selected for further analyses. The mean age of recruited patients in this study was 48.5 years old. Histopathology results revealed that eight of the samples were ER⁺PR⁺ and the two remaining ones were ER⁻PR⁻. Her-2/neu overexpression was positive in 3 samples, negative in 6 samples, and equivocal in one sample.

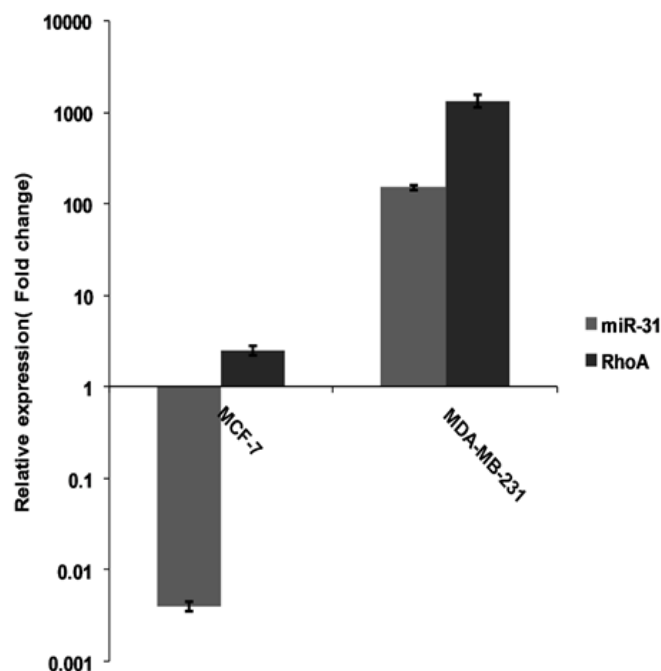


Figure 1. Relative expression of miR-31 and *RhoA* in MDA-MB-231 and MCF-7 cell lines.

Real-time PCR analysis was performed to assess miR-31 and *RhoA* expression in these samples. Results, analyzed by REST[®]2009, are presented in Fig. 2. In 9 of the tumor samples, the expression of both miR-31 and *RhoA* were significantly decreased compared to the normal tissue. MiR-31 and *RhoA* expression were increased in only one sample.

Pearson's correlation co-efficient determines the correlation of two variables and ranges from -1 to +1. Positive values indicate direct relationship of variables, and negative values mean inverse relationship between them. Zero means that there is no relation between the variables. The correlation co-efficient calculated for miR-31 and *RhoA* was 0.962 (p value=0.01), which means a positive correlation between *RhoA* and miR-31 expressions.

Discussion

Breast cancer is still the most prevalent cause of death in women worldwide. Metastasis of cancer cells is one of the major problems and leading causes of death in these patients. Therefore, gaining a detailed knowledge about the molecular mechanism controlling cancer cell metastasis can be useful in developing new diagnostic and therapeutic tools (Pourteimoor et al., 2016). miRNAs are among the factors affecting signaling pathways in

cancers. It is noteworthy that the expression of miR-31 in cancers is debatable. For example, its declined expression in breast cancer is accompanied with high probability of metastasis while its increased expression correlates with acute phase of colorectal cancer (Valastyan et al., 2009).

Because of the inconsistency among research papers on the role of miR-31 in breast cancer and its relation with *RhoA* expression, we performed a bioinformatic prediction to find miRNAs targeting 3'-UTR of *RhoA* mRNA. Interestingly, miR-31 was one of the high-ranking predicted miRNAs targeting *RhoA* mRNA. To confirm the result of bioinformatic predictions and to shed light on probable correlation between miR-31 and *RhoA* expression, we conducted a research to evaluate their expression in widely used breast cancer cell lines, i.e MCF-7 and MDA-MB-231, as well as clinical samples. These cell lines are the models of breast cancer research. Therefore, we aimed to determine whether these cell lines could be used with certainty in research concerning breast cancer metastasis. In addition, since previous reports had indicated the inhibitory role of miR-31 on *RhoA* expression (Valastyan et al., 2009; Valastyan et al., 2009; Valastyan and Weinberg, 2010), we decided to find any probable correlation between miR-31 and *RhoA* in breast cancer cell lines and clinical samples.

MCF-7 is a human breast adenocarcinoma cell line isolated from pleural effusion. We observed an inverse

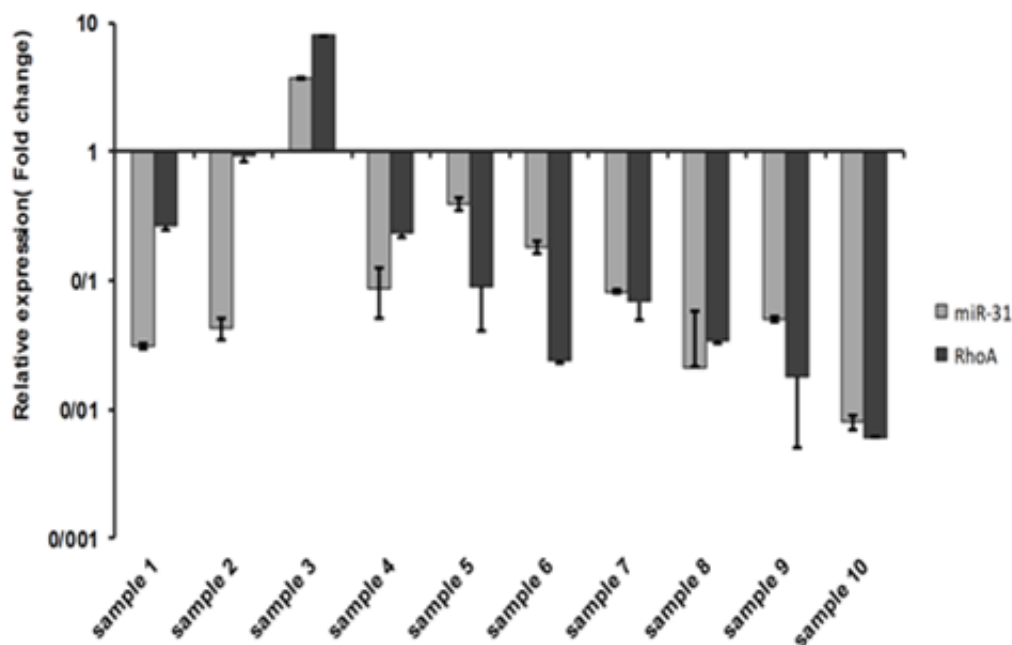
Table 1. The sequences of specific primers for *RhoA* gene and miRNA expression analysis.

Primer	Sequence	Length	T _m
<i>RhoA</i> -F	CAACACAAATGGCTGGTACG	20 bp	60.03
<i>RhoA</i> -R	GTGTGGTTGAAATGGTGCTG	20 bp	60.01
B-ACT-F	CTTCCTTCCTGGGCATG	17 bp	54.1
B-ACT-R	GTCTTTGCGGATGTCCAC	18 bp	56.1
miR-31-F	AGAGGCAAGATGCTGGC	17 bp	60.3
miR-31-R	GAGCAGGGTCCGAGGT	16 bp	59
SNORD-F	AAG GAT GAC ACG CAA ATT C	19 bp	60.1
SNORD-R	GAGCAGGGTCCGAGGT	16 bp	59

correlation between miR-31 and *RhoA* expression in this cell line. MDA-MB-231 is a more invasive human breast adenocarcinoma cell line, and the expression of *RhoA* was increased in line with miR-31 in this cell line. We propose that molecular mechanism of metastasis in this cell line differs from that of MCF-7 cells due to the fact that MDA-MB-231 is a drug resistant triple negative cell line. As indicated by our results, the molecular characteristics of MCF-7 and MDA-MB-231 cell lines are different. In addition, we demonstrated that the expression of miR-31 and *RhoA* differs in the mentioned cell lines and clinical samples. Therefore, it seems these cell lines are not reliable models for *in vitro* experiments concerning all aspects of breast cancer.

In 9 clinical samples, declined expression of both miR-31 and *RhoA* were observed, and only in one sample increased expression of both genes was detected. After more comprehensive pathological examination of the samples, we noticed that the latter sample was still in the first stage of metastasis. Pearson's correlation co-efficient (0.962) showed a positive correlation between miR-31 and *RhoA* expression.

In 2009, Valestyan S. and colleagues for the first time showed that miR-31 acts as a repressor of 6 different key genes, including *RhoA*, radixin, and integrin- $\alpha 5$ in MDA-MB-231 cell lines (Valastyan et al., 2009; Valastyan et al., 2009). In our study, we found increased expression of both miR-31 and *RhoA* in MDA-MB-231 cells while their

**Figure 2.** Relative expression of miR-31 and *RhoA* gene in clinical samples.

expressions declined in clinical samples. Although this seems contradictory, two research papers published by Valestyan's research group were retracted in 2015 because of inconsistency of their results with other groups.

In 2012, Lu Z and colleagues examined 67 plasma and tissue samples from breast cancer patients and found out that miR-31 increased in the patients' plasma, but there was no significant change in its expression in tissue samples (Lu et al., 2012).

Another study in 2013 confirmed that declined expression of miR-31 was related to high risk of metastasis (Laurila and Kallioniemi, 2013). Mulrane L. et al (2014) also found that miR-31 is an inhibitor of metastasis and was decreased in breast cancer. The result of these two studies are in line with what we observed in clinical samples. In 2015, Rasheed SA. and colleagues found that re-expression of miR-31 in metastatic cell lines resulted in declined invasiveness properties of cancer cells (Rasheed et al., 2015). In line with our results, Augoff et al. also discovered that miR-31 expression in cancer cells leads to post-transcriptional repression of integrin subunits, and thus inhibits metastasis (Augoff et al., 2011). Altogether, along with 2 retracted researches of Valestyan, these researches probably indicate that cell lines are not as trustworthy as considered to be, and the results of experiments on cell lines are not extendable to clinical samples.

Conclusion

In this study, we observed declined expression of miR-31 and RhoA in most cases of invasive and metastatic breast cancer clinical samples. Moreover, we found a completely positive correlation between miR-31 and RhoA expression in metastatic breast cancer, which is in opposition to Valestyan's findings. Design and development of new therapeutic agents to increase the expression of miR-31 and RhoA can be suggested to hamper breast cancer metastasis. Finally, we also suggest expression analysis of new genes and miRNAs such as RhoA and miR-31 in clinical samples as a potential biomarker for diagnosis of invasive breast cancer.

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Competing Interests

The authors declared that they have no conflict of interests.

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