RESEARCH ARTICLE

# Micro RNA 34a and Let-7a Expression in Human Breast Cancers is Associated with Apoptotic Expression Genes

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## Abstract

**Breast cancer is the most common cause of cancer-related death among women in the whole world. MiR-34a and let-7a are well known tumor suppressors that participate in the regulation of apoptosis, invasion and other cellular functions. In this study, expression of miR-34a, let-7a and apoptosis pathway genes such as Bcl-2, Caspase-3 and P53 were evaluated using quantitative real-time PCR in 45 paired samples of normal margin and tumor tissue collected from breast cancer patient at advanced stage (3-4). MiR-34a, let-7a, caspase-3 and P53 expression are reduced and Bcl-2 expression is increased within tumoral tissues in comparison with normal margin tissues. P53 expression directly or indirectly was correlated with miR-34a, let-7a, Bcl-2 and caspase-3 expression. In This study we found that MiR-34a and let-7a expression are reduced in the tumoral tissues. Down-regulation of these two molecules correlated with expression of genes associated with apoptosis. These results suggest that due to the correlation of miR-34a and let-7a with apoptotic and anti-apoptotic pathways these molecules could participate as regulators in advanced clinical stages of breast cancer and should be considered as markers for diagnosis, prognostic assessment and targeted therapy.**

**Keywords:** Breast cancer - miR-34a - let-7a - Bcl-2 - caspase-3 - P53 - apoptosis

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## Introduction

Breast cancer is the most common cause of cancer-related death among women worldwide, and is the molecular and clinically heterogeneous (Ong et al., 2015). The mechanisms leading to the progression of this cancer has not been determined and the molecular and genetic changes in this disease has not been fully realized (Thun et al., 2010).

In recent years, small RNA regulator in the field of cancer research was noted. MicroRNAs are small non-coding RNAs which have 19-22 nucleotides (Mansoori et al., 2014) and participitated in the stability or translation of mRNA in post-transcriptional regulation of gene expression in multicellular organisms (Davis et al., 2006; Costa and Pedroso de Lima, 2013). Current evidence have shown that miRNAs aberrantly are expressed in various types of tumors (Shi et al., 2008; Reis et al., 2010) and by the direct targeting of oncogenes or tumor suppressor participate in human Tumorgenesis and / or metastatic process (Chou et al., 2013; Fang and Gao, 2014). The altering expression of MiRNAs profile have been identified as modulators of proliferation, apoptosis and resistance treatment in breast cancer (Iorio et al., 2008). Indeed, The dysregulation of specific miRNAs was correlated with breast cancer (Hannafon et al., 2011). The concept of specific miRNA expression pattern and the expression of related gene with this, insight into choose a best targeted therapy for these patients (Yao et al., 2015).

MiR-34a and let-7are known as a tumor suppressors. These are expressed in healthy cells, however, their expression are reduced in cancers such as breast cancers. The aim of this study was to investigate the expression of let-7 and miR-34a and apoptotic-related genes in breast cancer with stage 3 and 4. Then comparing the expression of them in normal margine and tumoral tissues will be carried out.

## Materials and Methods

*Ethics statement*

For the analyzed tissue specimens, all patients gave informed consent to use excess pathological specimens for research purposes. The protocols employed in this study and the use of human tissues was approved by the Ethics Committee of Immunology research center affiliated of Tabriz University of medical sciences. The manuscript was accompanied by a statement that the experiments were

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undertaken with the understanding and written consent of each subject and according to the above mentioned principles.

*Human tissue specimens*

Forty five pathologically diagnosed of stage 3–4 breast cancer samples and marginal normal tissues were collected from patients with breast cancer. Breast cancer patient tissues were immediately frozen in liquid nitrogen and were stored at -80 °C until further use. The cancer tissue samples were all obtained from the Imam Reza Hospital affiliated to Tabriz University of Medical Sciences.

*Quantitative real-time PCR*

Total RNA was extracted from tissues and cells with Trizol reagent (Takara), and the reverse transcription reactions were performed with randomhexamer primer and a Reverse Transcriptase M-MLV (Takara) following the manufacturer's protocol.

Quantitative Real-time PCR was performed by using a standard SYBR Green PCR master mix (Takara) protocol on a Roche LightCycler 96 system (Roche) according to the instructions. GAPDH was used as references for Caspase 3, Caspase 8, Caspase 9, P53 and Bcl2, and miR-103 was used as references for (Peltier and Latham, 2008) miR-34a and Let-7a. The primer sequences were as follows in table 1. Each sample was analyzed in triplicate. The $2^{-\Delta\Delta Ct}$ method was used to determine the relative quantitation of gene expression levels (Livak and Schmittgen, 2001).

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7 µl of Trizol extracted RNA, in 20 µl of total volume, were subjected to reverse transcription with miRCURY LNA™ Universal cDNA synthesis kit (Exiqon, Vedbæk Denmark), incubated for 60 min at 42 °C followed by enzyme heat-inactivation for 5 min at 95 °C. Quantitative RT-PCR was carried out for miR-34a and Let-7a in total volume of 10 µl reaction mixture using miRCURY LNA™ Universal RT microRNA PCR, SYBR Green master mix (Exiqon, Vedbæk Denmark) according to the manufacturer's protocol.

Amplification was performed as follows: 95 °C for 10 min, 40 cycles of 95 °C for 10 s and 60 °C for 10 s, ramp rate 100% under standard condition. miR-103 was used as references.

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**Table 1. Primer sequences**

| | | |
|---|---|---|
| GAPDH | F | 5'- CAAGATCATCACCAATGCCT -3' |
| | R | 5'- CCCATCACGCCACAGTTTCC-3' |
| Caspase 3 | F | 5´ TGTCATCTCGCTCTGGTACG 3´ |
| | R | 5´ AAATGACCCCTTCATCACCA 3´ |
| P53 | F | 5´ ACTTGTCATGGCGACTGTCC 3´ |
| | R | 5´ CACCCCTCAGACACACAGGT 3´ |
| Bcl2 | F | 5´ CCTGTGGATGACTGAGTACC 3 |
| | R | 5´ GAGACAGCCAGGAGAAATCA 3´ |
| miR-103-3p | Target sequence | 5´ AGCAGCAUUGUACAGGGCUAUGA 3´ |
| Let-7a-5p | Target sequence | 5´ UGAGGUAGUAGGUUGUAUAGUU 3´ |
| miR-34a-5p | Target sequence | 5´ UGGCAGUGUCUUAGCUGGUUGU 3´ |

**Table 2. Apoptosis Related gene Differentially Expressed Between Marginal and Tumoral Breast Tissue**

| | Marginal tissues | | | Tumoral tissues | | |
|---|---|---|---|---|---|---|
| | Median | Range | | Median | Range | |
| | Normalized | Min | Max | Normalized | Min | Max |
| Caspase 3 | 1.4 | 0.85 | 1.89 | 0.78 | 0.51 | 1.13 |
| P53 | 0.64 | 0.37 | 1.42 | 1.64 | 0.79 | 4.32 |
| Bcl2 | 1.63 | 0.72 | 2.64 | 0.92 | 0.43 | 2.57 |

**Table 3. miR-34a and Let7a Differentially Expressed Between Marginal and Tumoral Breast Tissue**

| | Marginal tissues | | | Tumoral tissues | | |
|---|---|---|---|---|---|---|
| | Median | Range | | Median | Range | |
| | Normalized | Min | Max | Normalized | Min | Max |
| miR-34a | 1.54 | 1.16 | 2.58 | 0.66 | 0.43 | 0.95 |
| Let-7a | 1.72 | 0.45 | 6.41 | 0.76 | 0.37 | 1.66 |

## Results

*Expression pattern and clinicopathological signification of Apoptosis related genes in breast cancer tissues*

Importantly, bcl2 expression was mainly observed in the advanced front of the tumoral tissues, interestingly caspase3, p53 expression was decreased in tumoral tissues compared to normal marginal tumoral tissue, suggesting its effect on tumor progression. To determine differentially expressed caspase 3, bcl2 and p53 the ANOVA analysis of normalized data generated (P<0.05) between normal marginal tissues and tumoral breast cancers shown in Table 2 and Figure 1.

*Down expression of miR-34a and let-7a in stage 3-4 of breast cancer tissues*

We used a QRT-PCR to evaluate miR-34a and let-7a expression profiles of 24 normal marginal and 45 tumoral breast cancer tissues. To identify miRNA whose expression was significantly different between normal and tumor samples and could identify the different nature of these breast tissues, we made use of ANOVA and class prediction statistical tools. To identify differentially expressed miR-34a and let-7a, the ANOVA analysis of normalized data generated (P<0.05) between normal marginal tissues and tumoral breast cancers (Table 3 and Figure 2).

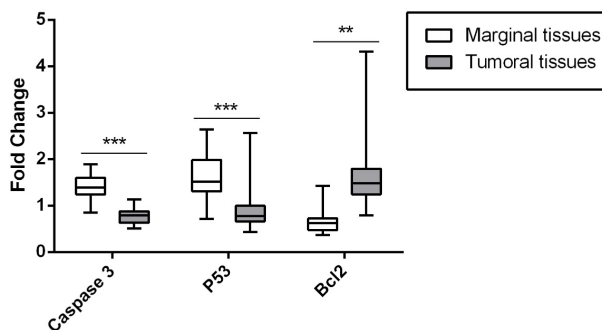


**Figure 1. Levels of Caspase 3, Bcl2 and p53 Expression Tumoral and Margin Tissues of Breast Cancer.** Gene expression was measured by qRT-PCR. The results are expressed as mean±SD (n=3); **p<0.001, *** p= 0.0001

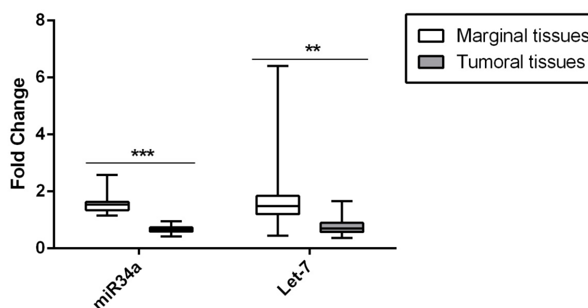


**Figure 2. Levels of miR-34a and Let-7 Expression in Tumoral and Margin Tissues of Breast Cancer.** miRNA expression was measured by qRT-PCR. The results are expressed as mean±SD (n=3);**p<0.001, *** p= 0.0001

## Discussion

Deregulation of the p53 tumor suppressor protein is a causal event in the different of malignancies. p53 introduced as a transcription factor that involves in different cellular responses to stresses such as oncogene activation (Fridman and Lowe, 2003). P53 deregulation caused alters in expression of a large number of target genes leading to cellular hemostasis including, apoptosis, cell-cycle arrest and inhibition of angiogenesis (Giono and Manfredi, 2006). P53 has regulated genes including: bcl2, caspase3, miR34a and let7a (Zhang et al., 2014). The p53/miR-34 pathway regulates apoptosis and is altered in cancer. In this pathway p53 activates through DNA damage by activation of ATM. p53 induces apoptosis through different targets including Puma and cell cycle arrest through p21. miR-34a is a direct target of p53 and in case of a functional pathway, miR-34a will be up-regulated. miR-34a induces cell death through apoptosis by silencing of its potential targets BCL2 and caspases specially caspase 3 (Zenz et al., 2009). let-7a miRNAs are down regulated in several malignancies (Garzon et al., 2009). highly characterized example is non-small cell lung cancer in which down regulation of let-7a miRNA is well correlated with poor prognosis in patients (Kumar et al., 2008). In breast cancer, some reports showed down regulation of let-7, while others did not (Yu et al., 2007). In the present study, let-7a miRNA was under-expressed at less than 45% of the normal level in approximately half of the tumoral compare to normal marginal tissues.

Further study is needed to determine the clinical significance of p53/miR34, let-7 miRNA in breast cancer.

let-7 miRNA is supposed to attend as a tumour suppressor (Büssing et al., 2008) In this path way P53 activated miR-34a expression, down regulation of bcl2 and up regulation of caspase-3.

In the present study, we have demonstrated that miR34a is a direct target for p53 miRNA, implying that this well-known tumour suppressor miRNA directly regulates apoptosis, another important process in malignancy. As we expected, p53, miR34a, caspase 3 and let-7a significantly down regulate in tumoral tissues compare to normal marginal tissues, but, bcl2 expression notably increase in tumoral tissues. miR-34a and let7a replacement can introduced as new therapeutic strategies in stage 3-4 breast cancer, also this miRNA can be a good diagnostic and prognostic and therapeutic marker in stage 3 breast cancer. In summary, we illustrated that bcl2 expression notably increase in tumoral tissues but p53, miR34a, caspase 3 and let-7a significantly down regulate in tumoral tissues compare to normal marginal tissues. This study helps researcher to approach new diagnostic and prognostic and therapeutic marker in their further experiments.

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