

RESEARCH ARTICLE

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

Behzad Mansoori^{1,2}, Ali Mohammadi¹, Solmaz Shirjang¹, Elham Baghbani¹, Behzad Baradaran^{1*}

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

Asian Pac J Cancer Prev, 17 (4), 1887-1890

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 participated 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 Tumorigenesis 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-7a are 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 margin 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

¹Immunology Research Center, ²Student Research Committee, Tabriz University of Medical Sciences, Tabriz, Iran *For correspondence: behzad_im@yahoo.com

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).

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. QReal-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.

7 μ l of Trizol extracted RNA, in 20 μ l 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 μ l 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.

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).

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).

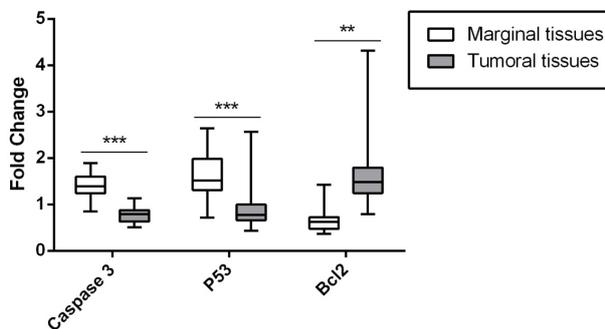


Figure 1. Levels of Caspase 3, Bcl2 and p53 Expression Tumoral and Margin Tissues of Breast Cancer. Gene expression was measured by qRT-PCR. The results are expressed as mean \pm SD (n=3); **p<0.001, *** p= 0.0001

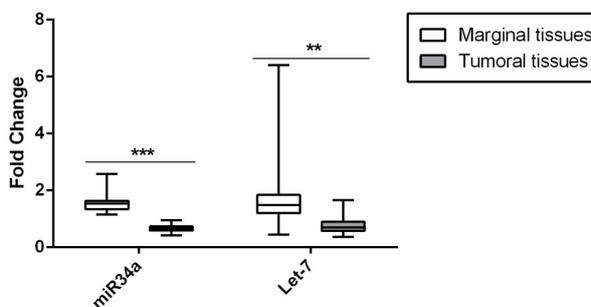


Figure 2. Levels of miR-34a and Let-7 Expression in Tumoral and Margin Tissues of Breast Cancer. miRNA expression was measured by qRT-PCR. The results are expressed as mean \pm SD (n=3);**p<0.001, *** p= 0.0001

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.

References

- Büssing I, Slack FJ, Großhans H (2008). let-7 microRNAs in development, stem cells and cancer. *Trends Molecular Med*, **14**, 400-9.
- Chou J, Shahi P, Werb Z (2013). microRNA-mediated regulation

- of the tumor microenvironment. *Cell Cycle*, **12**, 3262-71.
- Costa PM, Pedroso de Lima MC (2013). MicroRNAs as molecular targets for cancer therapy: on the modulation of microRNA expression. *Pharmaceuticals*, **6**, 1195-220.
- Davis S, Lollo B, Freier S, et al (2006). Improved targeting of miRNA with antisense oligonucleotides. *Nucleic Acids Res*, **34**, 2294-304.
- Fang Y, Gao W (2014). Roles of microRNAs during prostatic tumorigenesis and tumor progression. *Oncogene*, **33**, 135-47.
- Fridman JS, Lowe SW (2003). Control of apoptosis by p53. *Oncogene*, **22**, 9030-40.
- Garzon R, Calin GA, Croce CM (2009). MicroRNAs in cancer. *Annual Review Med*, **60**, 167-79.
- Giono LE, Manfredi JJ (2006). The p53 tumor suppressor participates in multiple cell cycle checkpoints. *J Cellular Physiol*, **209**, 13-20.
- Hannafon BN, Sebastiani P, de las Morenas A, et al (2011). Expression of microRNA and their gene targets are dysregulated in preinvasive breast cancer. *Breast Cancer Res*, **13**, 24.
- Iorio MV, Casalini P, Tagliabue E, et al (2008). MicroRNA profiling as a tool to understand prognosis, therapy response and resistance in breast cancer. *Eur J Cancer*, **44**, 2753-9.
- Kumar MS, Erkland SJ, Pester RE, et al (2008). Suppression of non-small cell lung tumor development by the let-7 microRNA family. *Proc Natl Acad Sci USA*, **105**, 3903-8.
- Mansoori B, Mohammadi A, Shirjang S, et al (2014). MicroRNAs: The new potential biomarkers in cancer diagnosis, prognosis and cancer therapy. *Cellular Molecular Biol*, **61**, 1-10.
- Ong CC, Gierke S, Pitt C, et al (2015). Small molecule inhibition of group I p21-activated kinases in breast cancer induces apoptosis and potentiates the activity of microtubule stabilizing agents. *Breast Cancer Res*, **17**, 59.
- Peltier HJ, Latham GJ (2008). Normalization of microRNA expression levels in quantitative RT-PCR assays: identification of suitable reference RNA targets in normal and cancerous human solid tissues. *RNA*, **14**, 844-52.
- Reis LO, Pereira TC, Lopes-Cendes I, et al (2010). MicroRNAs: a new paradigm on molecular urological oncology. *Urol*, **76**, 521-7.
- Shi X-B, Tepper CG, deVere White RW (2008). Cancerous miRNAs and their regulation. *Cell Cycle*, **7**, 1529-38.
- Thun MJ, DeLancey JO, Center MM, et al (2010). The global burden of cancer: priorities for prevention. *Carcinogenesis*, **31**, 100-10.
- Yao Y, Hu J, Shen Z, et al (2015). MiR 200b expression in breast cancer: a prognostic marker and act on cell proliferation and apoptosis by targeting Sp1. *J Cellular Molecular Med*, **19**, 760-9.
- Yu F, Yao H, Zhu P, et al (2007). let-7 regulates self renewal and tumorigenicity of breast cancer cells. *Cell*, **131**, 1109-23.
- Zenz T, Mohr J, Eldering E, et al (2009). miR-34a as part of the resistance network in chronic lymphocytic leukemia. *Blood*, **113**, 3801-8.
- Zhang D-G, Zheng J-N, Pei D-S (2014). P53/microRNA-34-induced metabolic regulation: new opportunities in anticancer therapy. *Molecular Cancer*, **13**, 1-7.