RESEARCH COMMUNICATION

# Expression Analysis of MiR-21, MiR-205, and MiR-342 in Breast Cancer in Iran

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## Abstract

**MicroRNAs (miRNAs) are short non-coding RNA molecules characterized by their regulatory roles in cancer and gene expression. We analyzed the expression of miR-21, miR-205, and miR-342 in 59 patients with breast cancer. Samples were divided into three different groups according to their immunohistochemistry (IHC) classification: ER- positive and/or PR-positive group ($ER^+$ and/or $PR^+$; group I); HER2-positive group ($HER2^+$; group II); and ER/ PR/ HER2- negative ($ER^-$/ $PR^-$/ $HER2^-$; group III) as the triple negative group. The expression levels of the 3 miRNAs were analyzed in the tumor samples and the compared with the normal neighboring dissected tumor (NNDT) samples in all three groups. The expression of miR-21 was similar in all three groups. In patients positive for P53 by IHC, positive for axillary lymph node metastasis and higher tumor stages, it appeared to have significantly elevated. However, significant increase was not found among the 18 fibroadenoma samples. Both miR-205 and miR-342 expressions were significantly down regulated in group III. We conclude that miR-21 does not discriminate between different breast cancer groups. In contrast, miR-205 and miR-342 may be used as potential biomarkers for diagnosis of triple negative breast cancer.**

**Keywords:** MiR-21 - MiR-205 - MiR-342 - P53 - breast cancer - sybr green I real time RT-P.C.R.

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## Introduction

Cancer is a genetic disease but only a small percentage of cancers are inherited. Breast cancer is the most common cancer in women after dermatologic carcinomas (McCaskill et al., 2011). About 5-10% of breast cancers are inherited, one-third of which are caused by dominant BRCA-gene mutations (Xu et al., 2011). Research has previously focused on the protein-coding genes such as oncogenes, tumor suppressor genes, DNA repair genes, and anti-metastatic genes in cancer genetics (Jacob & Praz , 2002; Rabien et al., 2011).

*P53* is the most common gene which is mutated in malignant cancers (Gasco et al., 2002). *P53*, which encodes a tumor suppressor protein, has various effects on cell cycle arrest and apoptosis, and it also increases the expression of the proteins involved in DNA repair (Gasco et al., 2002). Mutations in receptor coding genes, HER2 (Human Epidermal growth factor Receptor 2), ER (Estrogen Receptor) and PR (Progesterone Receptor) are very important because they may change the treatment strategy of cancer. Patients who have an ER and/or PR over expression are candidates for hormonal therapy, but the HER2/*neu* -positives can be treated with Herceptin (Gelbfish et al., 1988; Arteaga, 2003).

The discovery of non-coding genes and their regulation was an important accomplishment in the field of oncology. Research has shown that miRNA profiles are different in certain cancer types (Mattie et al., 2006). MicroRNAs (miRNAs) are short (20-24 nucleotides) non-coding RNAs. These small molecules enforce their regulatory roles by adhering to the target mRNAs with Watson-and-Crick base pairing. The mRNA target is then cleaved when there is no mismatches between the miRNA and its target (Ambros 2003; Lewis et al., 2005). If there is a mismatch, the mRNA will not be translated. Approximately, one-third of the human gene expression is regulated with such mechanisms at the level of post-transcription (Obernosterer et al., 2006).

MiR-21 is one of the most important miRNAs that deregulated and over-expressed in many malignant tumors, such as glioblastomas and breast cancers (Chan et al., 2005). Some studies have reported that miR-205 and miR-342 are also deregulated in different types of breast cancer (Lowery et al., 2009; Adachi et al., 2011).

Fibroadenoma is the most common benign mass in the female breast. Abnormal growth and hyperplasia of the breast lobular tissue may lead to the formation of fibroadenoma that are normally diagnosed in young women and are typically seen as multiple masses (Cotran et al., 1989; Noguchi et al., 1993).

In this study, we tried to identify whether the expression

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ratios of miR-21, miR-205, and miR-342 is different in the following groups, 1) ER and/or PR- positive ($ER^+$ and/or $PR^+$; group I), 2) HER2/*neu* -positive ($HER2^+$; group II), and 3) ER/ PR/ $HER2^-$ negative ($ER^-$/ $PR^-$/ $HER2^-$; group III). Moreover, we analyzed the expression of miR-21 with other tumor factors; also expression of miR-21 was analyzed in fibroadenomas.

## Materials and Methods

*Patients and samples*

A total of 18 breast fibroadenoma samples, and 59 primary invasive ductal carcinoma samples, plus the normal neighboring dissected tumor (NNDT) were obtained during 2009-2010 period from the Cancer Institute, Tehran, Iran. Patients who had received medical therapies, such as radiation and/or chemotherapy before the surgical procedure were excluded. An informed consent was obtained from each of the patients, and the study was approved by the Ethics Board of the Cancer Institute.

*Immunohistochemical analysis*

Breast cancer samples were evaluated for receptor status with the use of specific antibodies. Estrogen receptors (ER) and progesterone receptors (PR) were evaluated with the 1D5 antibody and PGR-1A6 antibody, respectively (Dako). HER2/*neu* was detected with CB11 (Dako). The HER2/*neu* expression was scored according to the degree of membrane staining (Rhodes et al., 2002). Samples with HER2/*neu* scores of 2 or more were considered as positive for grouping in this study. Samples with distinct nuclear staining for ER and PR in more than 10% of the tumor cells were reported as ER and/or PR-positive (Iorio et al., 2005). A commercial antibody was purchased for the detection of *P53* N-terminal (Dako).

*MiRNA extraction and quality analysis*

We applied MirVana PARIS Kit (Ambion, AM1556) on 10 milligram of breast tissue for extraction of small RNA. Using this method, small RNAs (< 200 nucleotides) which contained miRNAs were separated from the total RNA. The RNA concentration was measured with a NanoDrop ND-1000 Spectrophotometer. The RNAs were stored at -80 ˚C until cDNA synthesis. The integrity of the miRNAs was checked using agarose gel electrophoresis stained with an intercalating dye.

*cDNA synthesis from small RNA*

cDNAs were synthesized using the MiScript Reverse Transcription Kit protocol (Qiagen) with some minor modifications. According to the protocol, poly A tails were added to the 3' end of all small RNAs. Reverse transcription was carried out using oligo dT VN primers (which have a unique 5'-overhang sequence) to produce the cDNAs.

*Real time RT-PCR*

Real time RT-PCR was carried out using all three miRNA forward (Qiagen) and a reverse primer that is complementary to the unique sequence of the oligo dT VN primer which obtained from the MiScript SYBR Green PCR Kit (Qiagen). Ten ng of the cDNA for each sample in triplicate were used for real time RT-PCR reaction according to the above mentioned protocol.

Expression data was acquired in 36-well Rottor gene Q (Qiagen). For normalization of the real time RT-PCR results, *RNU6B* expression was used as an internal control.

*Statistical analysis*

Corrected baseline optical data was exported from the Rottor gene Q into Excel (Microsoft) sheets and analyzed with the LinRegPCR.11.0 software (Ramakers et al., 2003; Ruijter et al., 2009). The program determines the fluorescence intensity curve as it reaches the threshold cycle, crossing point (Cp), for each sample and a mean PCR efficiency corresponding to a primer pair in the exponential phase of each reaction.

Based on the formula shown below, analyses of the gene expression ratios for the test miRNAs and *RNU6B* in tumor samples were performed with the REST-2009 software (Pfaffl et al., 2002). LinRegPCR data was used for analysis and pair wise fixed reallocation randomization tests were performed with 2,000 iterations to assess the significance by the REST software.

$$\text{MiRNA Expression Ratio} = \frac{(E^{*}_{miR})^{\Delta cp\ miR\ (mean\ NNDT - mean\ Tumor)}}{(E_{U6})^{\Delta cp\ U6\ (mean\ NNDT - mean\ Tumor)}}$$

*E: Efficiency

## Results

Expression analysis of miR-21, miR-205, and miR-342, was performed by Real-time RT-PCR. The melting-curves of all three miRNAs and *RNU6B* showed a narrow peak that corresponded to the RT-PCR products (Figure 1). The presence of single bands for the miRNAs and housekeeping gene, *RNU6B*, on gel electrophoresis confirmed the specificity of the PCR. As shown in the amplification curve of miR-21 in stage III tumor samples, the crossing point value (Cp) was lower in the tumor sample compared with the normal neighboring dissected tumor (NNDT). Therefore, it appears that there were more copies of miR-21 in stage III tumor sample than in NNDT (Figure 2), as indicated by the equal Cp of internal control in both sample and NNDT. The expression ratio of miR-21 showed no significant differences between the 3 different groups. In addition, the fibroadenoma samples did not show any significant over expression of miR-21 compared with the normal control samples.

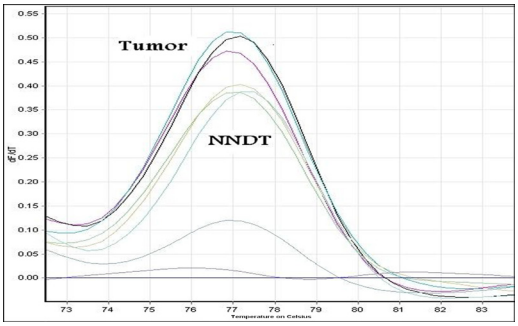


**Figure 1. Melting Curve of miR-21 in a Sample (Tumor) and NNDT, Each in Triplicate**

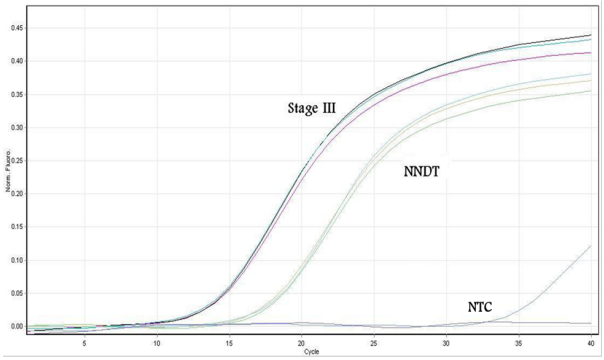


**Figure 2. The Melting Curve of miR-21 in Stage III Sample, Normal Neighboring Dissected Tumor (NNDT) and Non Template Control (NTC).**

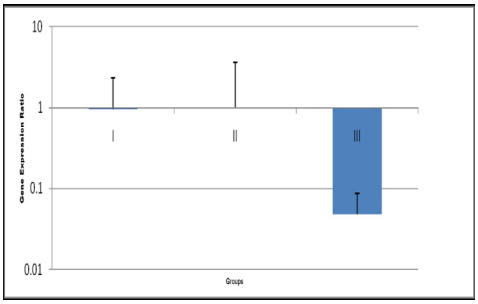


**Figure 3. Expression Ratios of miR-205 in Groups I, II, and III.** Only group III had a significant value. (Vertical axis shows Gene Expression Ratio, for example this ratio is about 0.05 in group III means miR-205 expression of sample to NNDT is about one-twentieth).

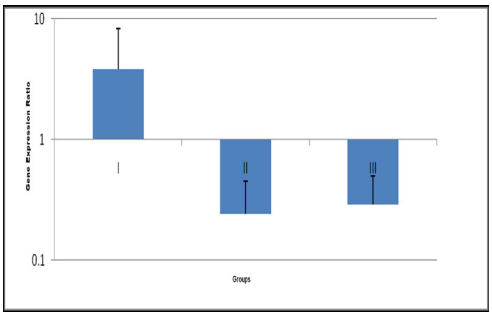


**Figure 4. Expression Ratios of miR-342 in Groups I, II and III.** Only group III had a significant value. (Vertical axis shows Gene Expression Ratio, for example 0.1 in vertical axis means miR-342 expression of sample to NNDT is one-tenth).

We also examined the miR-21 expression ratio in relation to the tumor size. Overall, there were no significant correlation between the miR-21 expression and the size of the tumors ($p>0.05$). However, tumors greater than 2 cm showed an over expression of miR-21. In addition, the expression ratio of miR-21 in tumor samples that had lymph node metastasis was 2.856. The average expression ratio of miR-21 in tumor samples obtained from stage III patients was 3.451 with significant P-value. The expression ratio of miR-21 in *P53* positive tumor samples to NNDT was 2.490 ($p$=0.038) (Table 1).

MiR-205 was under-expressed in all three groups, but its down regulation was significant only in group III (Table 2). A depletion of expression for miR-342 was seen in both groups II and III but over-expression was observed in group I (Figure 3 and 4).

**Table 1. Expression Ratio of miR-21 and its Correlation with different clinicopathologic features and P53 expression.**

| Clinicopathologic Feature | | No. (%) | Mir-21 expression ratio | P-Value |
|---|---|---|---|---|
| Group: | I | 22 (37.2%) | 2.449 | 0.35 |
| | II | 20 (33.9%) | 2.015 | 0.244 |
| | III | 17 (28.9%) | 1.557 | 0.272 |
| Tumor size (cm): | <2 | 25 (42.4%) | 0.843 | 0.822 |
| | 2≤ | 34 (57.6%) | 2.985 | 0.294 |
| Clinical stage : | I , II | 46 (80%) | 1.284 | 0.127 |
| | III | 13 (20%) | 3.451* | 0.041 |
| Lymph node Metastasis 3≤ | | | | |
| | Ve+ | 21 (35.6%) | 2.856* | 0.023 |
| | Ve- | 38 (64.4%) | 1.061 | 0.739 |
| I.H.C. of *P53* : | Ve+ | 27 (45.8%) | 2.490* | 0.038 |
| | Ve- | 32 (54.2%) | 1.2 | 0.527 |

* 'significant

**Table 2. Expression Ratios of MiR-21, MiR-205, and MiR-342 and Correlations with Breast Cancer Groups**

| GROUP No. (%) | GROUP I 22 (37.2%) | GROUP II 20 (33/9%) | GROUP III 17 (28/9%) |
|---|---|---|---|
| Mir-21 | 2.449 (0.350) | 2.015 (0.244) | 1.557 (0.272) |
| Mir-205 | 0.947(0.960) | 0.992(0.455) | 0.048 (0.001)* |
| Mir-342 | 3.861(0.125) | 0.239(0.054) | 0.287 (0.013)* |

Data are Expression Ratio (P Value)

## Discussion

The molecular characteristics of tumor cells and miRNA profiling have come to interest in recent years (Lowery et al., 2009). Examination of the 3 important miRNAs (miR-21, miR-205, and miR-342) involved in the breast tumor's invasive behavior was the main aim of this study. The long-term goal is to use these miRNAs as a practical tool for a better diagnosis of breast cancer and distinguishing between different stages of the tumor. MiR-21 is the most important miRNAs that is over-expressed in cancer tissues (Huang et al., 2009). MiR-21 over-expression has been assayed with different types of methods (Tang et al., 2006; Blenkiron et al., 2007). Here, we used the real-time RT-PCR using SYBR Green I technique for analysis. Although the Taqman probe is more specific, the method used in this study is cost effective and sensitive enough for assessing the expression of miRNA.

Based on the relationship between the clinicopathological status and the miR-21 expression, examined by the real time RT-PCR, Some studies reported that over expression of miR-21 correlates with advanced tumor stages (Iorio et al., 2005). Our results show that invasive tumors have a high miR-21 expression level. However, the size of the tumor does not appear to correlate significantly with miR-21 over expression. Furthermore, in the three cancer groups and fibroadenoma samples examined here, miR-21 over expression was not significant. In addition, based on our results, miR-21 does not appear to discriminate between the different receptor statuses in breast tumors. Nonetheless, miR-21 might still be a potential prognostic marker for predicting lymph node involvement and advanced stages of breast cancer, and

possibly a potential target for antisense therapy.

Different targets for miR-21, PTEN, Tropomyosin 1 (TPM 1), Programmed Cell Death 4 (PDCD 4), (Qi et al., 2009) and some component of P53 such as HNRPK have been reported previously (Papagiannakopoulos et al., 2008). P53 protein was stabilized by HNRPK that interferes with *MDM2* (Papagiannakopoulos et al., 2008). Previous studies showed no significant correlation between P53 and miR-21(Rask et al., 2011). It is known that certain missense mutations of P53 lead to a longer half life for the protein, which its accumulation in the cells can be detected with specific antibody. By contrast, wild type P53 does not have sufficient half life to be detected with an antibody and shows as P53 negative in IHC analysis. In our study, the expression ratio of miR-21 in P53 positive tumors to NNDTs was found to be 2.490 (p=0.038). This over expression may be due to the missense mutations of P53. It has been reported that MDM2 cannot degrade the mutant P53 (Li et al., 2011). In addition, miR-21 could be over-expressed in P53-positive samples because of advanced tumor stages or lymph node involvement.

MiR-205 has been reported as a tumor suppressor in the breast, and its down regulation has been previously reported in the triple-negative group of patients (Radojicic et al., 2011). This microRNA is negatively regulated by HER2/*neu* over expression (Adachi et al., 2011). We found that miR-205 was down regulated in all three groups. However, statistically significant down regulation of miR-205 was only seen in group III (i.e., the triple negative group). We found that miR-342 was also down regulated in groups II and III and up regulated in group I. However, in line with a previous study (Adachi et al., 2011) only down regulation in group III was significant. In conclusion, miR-205 and miR-342 both individually and together can discriminate between the triple-negative breast cancers and other groups.

Identification of the full set of miRNAs involved in breast cancer requires further investigation and it could unveil miRNAs candidates for differentiating the receptor status in breast cancer.

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