Investigating the Expression Level of Mir-361 in Patients with Breast Cancer

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| Article Type	ABSTRACT
Research Paper	**Background and Objective:** MicroRNAs are a new group of small regulatory RNAs, some of which are expressed differently in normal and tumor tissue. The aim of this study was to investigate the expression of miR-361 in breast tumor tissue of patients with breast cancer. **Methods:** In this cross-sectional study conducted from January 2012 to February 2021 in Ahvaz Jundishapur University of Medical Sciences, 30 breast cancer samples were grouped based on clinicopathological characteristics including tumor size, cancer stage, lymphovascular invasion, molecular subtypes, etc. Then, expression analysis was performed using Real-Time PCR, and the expression level of miR-361 gene in tumor and healthy samples and its relationship with clinicopathological characteristics were investigated. **Findings:** The expression of miR-361 was significantly reduced by 1.5-fold in cancerous tissue compared to adjacent normal tissue, and this reduction in expression was only significantly associated with triple-negative breast cancer (TNBC) (p=0.02). The expression level was also significantly reduced in tumors larger than or equal to 3 cm (0.93±0.79) compared to smaller tumors (0.17±0.18) (p=0.031). The ratio of reduction in miR-361 expression in stages (I & II) compared to (III & IV) was 0.29±0.44 and 0.91±1.31, respectively (p=0.308). On the other hand, the ratio of decreased expression in the two groups with and without lymphovascular invasion was determined to be 0.39±0.33 (p=0.682) and 0.15±0.16 (p=0.547), respectively, but this difference was not statistically significant. The AUC for miR-361 for breast cancer diagnosis was 0.71 (p=0.031). **Conclusion:** Based on the results of this study, and considering the role of miR-361 in breast cancer progression and its relationship with clinicopathological factors, it may be possible to introduce it as a new diagnostic marker in breast cancer. **Keywords:** *Breast Cancer, miR-361, Real-Time PCR.*

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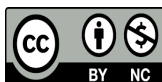
Introduction

Breast cancer is the most common cancer, accounting for 29% of all diagnosed cancers, and is the second leading cause of cancer death in women. Epidemiological studies estimate that 2.3 million new cases of breast cancer were diagnosed in 2020 (1). Clinically, breast cancer is a heterogeneous disease with histopathological subgroups that include estrogen receptor, progesterone receptor positive/negative, and human epidermal growth factor receptor positive/negative based on gene expression profiling (2).

MicroRNAs are small single-stranded non-coding RNAs (18–24 nucleotides) that bind to the 3' untranslated region of target mRNAs and affect their expression levels. The role and function of microRNAs in some cancers, especially breast cancer, has been investigated (3). In breast cancer, microRNAs can act as oncogenes or tumor suppressors. The extensive association of microRNAs and their target mRNAs has revealed important cellular processes related to breast cancer biology and survival. Breast cancer is one of the first cancers for which the expression profile of microRNAs has been described. The expression profile of microRNAs can be used to distinguish between cancerous and normal tissue cells in breast cancer (4). Increased expression of miR-21 is associated with cell survival and proliferation in breast cancer by targeting tumor suppressor genes TPM1, TIMP3, and RECK. Increased expression of miR-21 is also associated with clinical stage progression, poor prognosis, and lymph node metastasis (5). On the other hand, downregulated microRNAs miR-296 and miR-31 regulate the oncogenes HER2 and HER3 receptor tyrosine kinases (6, 7). Some microRNAs play dual roles as oncogenes and tumor suppressors due to the complexity of the target gene networks of the microRNAs and the expression levels of the gene transcripts. This role could serve as key biological hubs contributing to disease outcome (8).

miR-361 is a dual-function miRNA located in the Xq21.2 gene region of the human X chromosome (9). miR-361 is downregulated in breast cancer and has been reported to have tumor suppressive properties in papillary thyroid carcinoma, and lung, and colorectal cancers (10-12). A study on miR-361 expression in malignant prostate adenocarcinoma showed that miR-361 expression was significantly downregulated in tumor tissue compared to healthy tissue (13). miR-361 inhibits the growth, cell proliferation, and metastasis of hepatocellular carcinoma and inhibits Twist1 expression (10). Studies have shown a link between increased miR-361 expression and inhibition of prostate cancer. Induction of miR-361 expression, by downregulating the signal transducer and activator of transcription 6 (STAT6)/B-cell lymphoma 2 (BCL-2) pathway, reduces cell proliferation, induces apoptosis, and inhibits cell migration in malignant prostate cancer cells (13). However, conflicting results have been recently observed regarding the role of miR-361 as an oncogene in cervical cancer development, cell proliferation, and invasion (14).

Apparently, miR-361 expression changes in breast cancer have not yet been fully investigated, and some human studies should focus on the epidemiological characteristics of this disease in Asian populations. In addition, to the best of our knowledge, no study has been conducted in the Iranian population to assess the association of miR-361 expression changes in all molecular subtypes and stages of breast cancer progression. Therefore, the functional and diagnostic significance of miR-361 in breast cancer patients in Ahvaz was evaluated in this study.

Methods

After approval by the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences with the code IR.AJUMS.REC.1398.354, this cross-sectional study was conducted on 30 breast cancer tissue samples and adjacent tissues. A total of 60 samples were collected from patients referred to the surgical center of Apadana Hospital between 2012 and 2021. The obtained samples were immediately stored in

liquid nitrogen. All patients had pathology-confirmed breast cancer of Invasive Ductal Carcinoma (IDC) and Ductal carcinoma in situ (DCIS) types. Patients with any other breast cancer histopathology, patients with incomplete information in their files, and patients who had undergone radiotherapy and chemotherapy were excluded from the study. Written informed consent was obtained from all patients before starting the study.

MicroRNA extraction and synthesis of small cDNAs: The miRNeasy kit (QIAGEN GmbH, Hilden, Germany) was used to extract small RNAs (less than 100 nucleotides). Then, the concentration of small RNAs was measured using NanoDrop Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA), and stored at -80°C. The QuantiTect Kit (QIAGEN GmbH, Hilden, Germany) was used to make cDNA. According to the protocol, poly(A) tail was first added to the 3' end of all microRNAs using Poly(A) Polymerase. Then, the Oligo dT-VN primer was used. When the desired primer fragment was hybridized to the RNA, the reverse transcriptase enzyme made the complementary strand from the end of the primer. With the help of this, cDNA was made from all small RNAs.

Real-time polymerase chain reaction (RT-PCR): RT-PCR was performed using two primers. The forward primer was similar to the miR-361 sequence and the reverse primer was complementary to the unique 5' region of the Oligo dT VN primer. In each reaction, 10 ng/μl of cDNA was used for each sample in duplicate for RT-PCR on Applied Biosystems StepOne (Qiagen, USA). In addition, the housekeeping gene RNU6B was used to normalize expression. Finally, the relative expression level of target genes in tumor samples compared to normal adjacent tissue was calculated based on the formula $2^{-\Delta\Delta CT}$. The sequences of the primers are shown in Table 1.

Immunohistochemical technique: Immunohistochemical technique was used to evaluate the status of estrogen and progesterone receptors and the rate of cell proliferation in breast tissue tumors. In this method, breast cancer tumors were preserved in 10% formalin after surgery. After 3 hours, formalin was replaced, and after 24 hours, the samples were placed in a Tissue Processor for tissue processing. Then, blocking was performed and tissue blocks were cut to a thickness of 4 μm and placed on poly-l-lysine slides. After deparaffinization, dehydration and clearing were performed. Antigen retrieval was performed with citrate buffer solution and a temperature of 98 ° C to inhibit the PBS reaction. After washing the slides in Hydrogen Peroxide 3% Solution, they were placed in methanol. After washing, secondary antibodies conjugated with peroxidase enzyme were used. After washing and exposure to diaminobenzidine peroxidase substrate solution, the slides were dehydrated, cleared, and mounted, and the slides were evaluated under a microscope at 100× magnification.

The data of this study were analyzed after entering into the computer with SPSS 24. The statistical difference of miR-361 levels in tumor samples compared to normal tissue in comparison with various clinicopathological factors was analyzed through two-sided Mann-Whitney and Kruskal-Wallis tests. Receiver-operating characteristic curve (ROC) was also used to determine the diagnostic value of miR-361 levels in molecular differentiation of tumor and non-tumor samples, and $p < 0.05$ was considered significant.

Table 1. Sequences of miR-361 and U6 primers for Real-Time PCR

Primers	Sequence
miR-361 stem-loop	3-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC……-5
Forward	3-CGCTAGTCCCGAAAGGTA-5
Reverse	3-CCAGTGCAGGTCCGAGGTA-5
U6 stem-loop	3′-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GA TAC GAC aaaata-5′
Forward	3′-CTC GCT TCG GCA GCACA-5′
Reverse	3-CCAGTGCAGGTCCGAGGTA-5

Results

In examining the relationship between clinicopathological parameters and miR-361 expression levels, the results showed that there was a significant difference in terms of tumor size and TNBC ($p < 0.05$) (Table 2).

Table 2. Relationship between clinicopathological parameters and miR-361 expression levels in breast cancer patients

Clinicopathological parameters	Number	miR-361 expression level Mean±SD	p-value
Tumor size			
≤3 cm	13	0.79±0.93	0.032[*]
>3 cm	17	0.17±0.18	
Age group			
≤50	16	0.59±1.04	0.638
>50	14	0.47±0.80	
Histological status			
DCIS	10	0.59±0.24	0.718
IDC	14	1.05±0.28	
Histological grade			
I	8	0.30±0.19	
II	6	0.26±0.31	0.677
III	12	0.72±1.15	
Clinical stage			
Primary (I & II)	9	0.29±0.44	0.308
Advanced (III & IV)	19	0.91±1.31	
Lymphatic invasion			
Positive	13	0.39±0.33	0.682
Negative	16	0.58±1.02	
Lymphovascular invasion			
Positive	14	0.15±0.16	0.547
Negative	15	0.71±1.06	
HER2			
Low	7	0.058±0.011	
Moderate	6	1.75±2.41	0.133
High	9	0.89±1.18	
Negative	8	0.26±0.51	
ER			
Low	6	1.60±1.06	
Moderate	12	0.96±1.28	0.192
High	4	0.35±0.32	
Negative	8	0.24±0.55	
PR			
Low	5	0.93±1.44	
Moderate	11	0.69±1.05	0.204
High	7	1.27±1.54	
Negative	7	0.24±0.53	
TNBC			
Positive	7	0.24±0.25	0.02[*]
Negative	23	0.87±0.93	

$p < 0.05$[*]

The melting curve of miR-361 and RNU6B was obtained as a single peak, which indicates only one specific product in PCR (Figure 1). miR-361 has a higher Ct in tumor samples than in the adjacent healthy sample (compared to the Ct of the internal control in the tumor sample compared to the adjacent healthy sample). Real-Time PCR analysis showed a significant decrease in miR-361 expression in breast tumor tissues compared to non-tumor tissues ($p<0.05$) (Figure 2). The association between miR-361-5p gene expression and clinicopathological characteristics, including tumor size, age, histological status, grades, stages, vascular or lymphatic invasion, and molecular subgroups, was evaluated. The data showed that miR-361 gene expression was significantly lower in TNBC breast cancer tissues than in non-TNBC tissues ($p<0.05$).
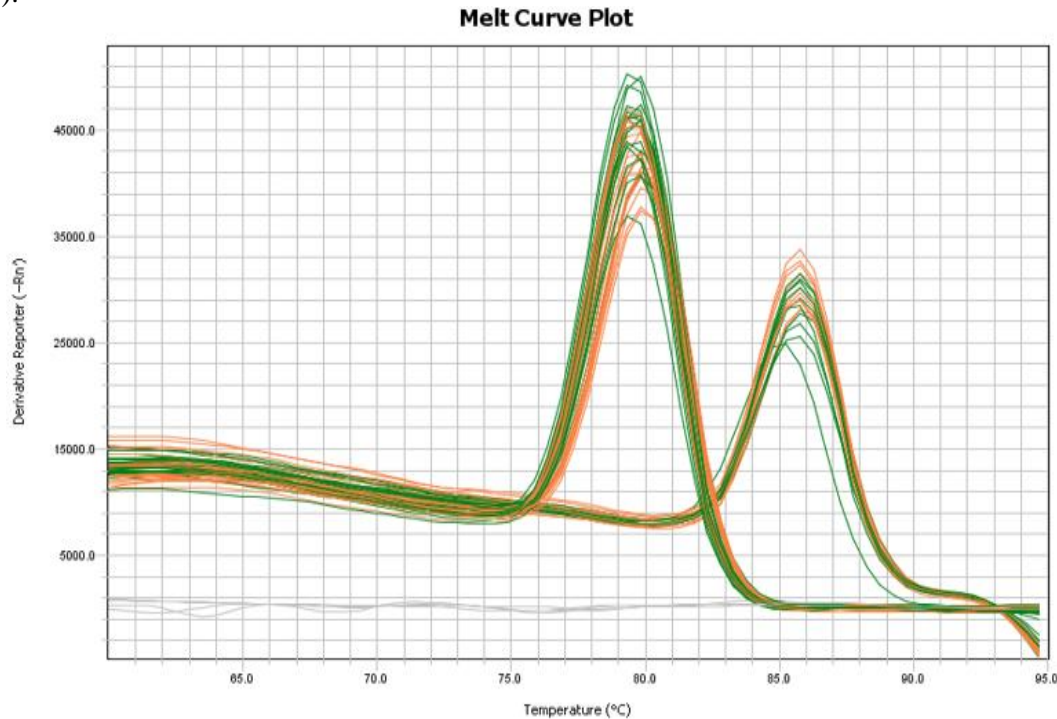
Melt Curve Plot

Figure 1. Melting curve of miR-361 compared to the reference gene (RNU6B) in Real-Time PCR
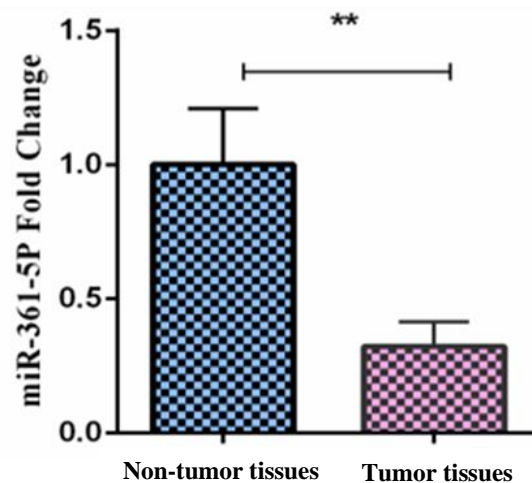

Figure 2. Expression of miR-361 in tumor tissues compared to non-tumor tissues of breast cancer patients

When the samples were divided into two groups based on size: the group of tumors smaller than 3 cm and the group of tumors 3 cm and larger. Both groups showed a decrease in expression; this ratio was 0.79 in the group of tumors smaller than 3 cm and 0.17 in the group of tumors larger than 3 cm. The difference in expression reduction between these two groups was statistically significant ($p < 0.05$). In addition to tumor size, all samples were divided into two groups in terms of lymphovascular invasion, with the expression ratio compared to the adjacent normal tissue being 0.54 and 1.29, respectively. Despite the decrease in expression, this decrease was not significant. Considering the clinical stage of the tumor, samples that were in clinical stages I and II (with an expression ratio of 0.29) showed a greater decrease in expression compared to tumors that were in clinical stages three and four (0.91). However, the difference in expression reduction between these two groups was not statistically significant ($p = 0.308$). No significant association was observed between miR-361 expression and other clinicopathological features.

In this study, the status of HER2, ER and PR receptors was investigated using immunohistochemistry (Figure 3). About 30% and 20% of tumor tissues showed high and moderate HER2 expression, respectively. ER expression was moderate in 40% of cases and high in 13.3%. In addition, about 11 cases (36.6%) and 7 cases (23.4%) of the total 30 tumor samples showed moderate and high PR expression, respectively. The rate of TNBC positive samples was also approximately 25.4%. The results of the Kruskal–Wallis test showed that there was an inverse relationship between the decrease in miR-361 expression and the intensity of low to high HER2, ER and PR expression.
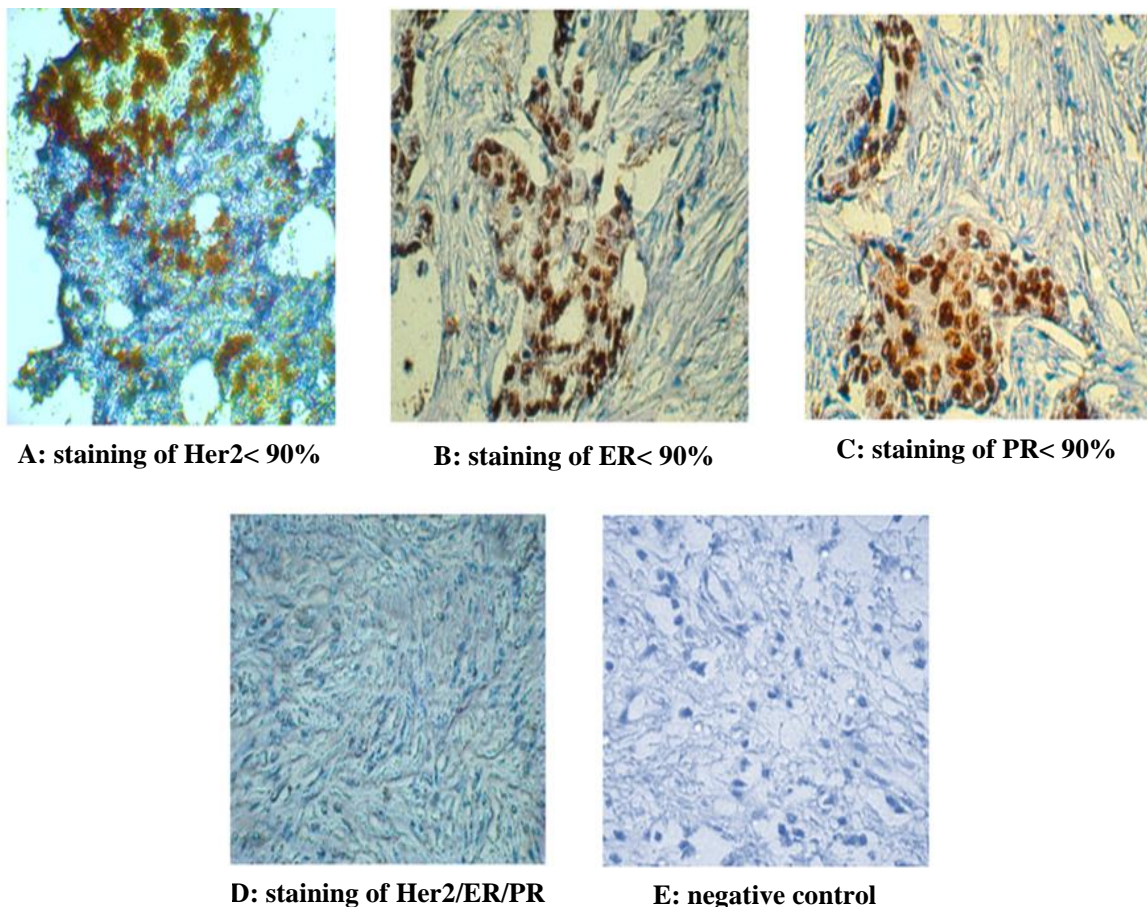

A: staining of Her2< 90%   **B: staining of ER< 90%**   **C: staining of PR< 90%**

D: staining of Her2/ER/PR   **E: negative control**

Figure 3. Staining of tumors

ROC curve analysis showed that tissue miR-361 levels could differentiate well between tumor and non-tumor breast cancer groups. By analyzing the curve and providing a cut-off value (0.6) for this biomarker, it was determined that tissue miR-361 levels above 0.3 times that of healthy individuals (calibrator) could distinguish patients from healthy individuals with a specificity of 65% and a sensitivity of 73% (Figure 4 and Table 3).
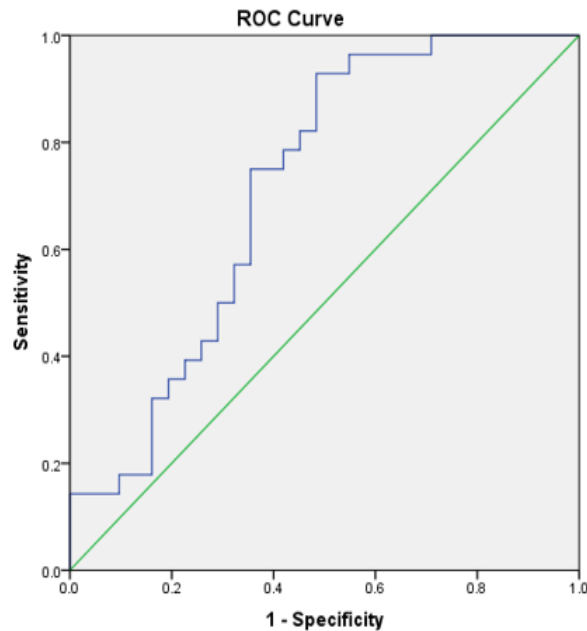

Figure 4. ROC curve of tissue miR-361 levels for molecular differentiation of tumor from non-tumor samples. The area under the ROC curve for this analysis was 0.71 (95% CI: 0.58-0.84)

Table 3. Results of ROC curve analysis related to miR-361

Variable	Area under the curve	Sensitivity	Specificity	95% CI
miR-361	0.71	73%	65%	0.58-0.84

Discussion

The results of this study clearly showed that miR-361 has the potential to be considered as a possible biomarker for breast cancer diagnosis and also as a potential therapeutic target for breast cancer. In various studies, a relationship was found between decreased miR-361 expression and tumor size and lymphovascular invasion (15-17). In the present study, a significant relationship was not observed between decreased miR-361 expression and tumor status and lymphovascular invasion, which was most likely due to the limited sample size, but in samples that were in clinical stages I and II, the decrease in miR-361 expression was greater compared to clinical stages III and IV. Since the diagnosis of cancer at low clinical stages is of great clinical importance, miR-361 seems to have important clinical value as a diagnostic biomarker in breast cancer. Regarding this finding, in a study conducted by Xu et al., it was found that decreased miR-361 expression was inversely and significantly associated with disease stage in breast cancer (18).

Based on the difference in miR-361 expression, the present study showed that miR-361 expression was significantly and significantly reduced in cancer samples compared to normal samples. In this regard, a study conducted by Cao et al. showed that this miRNA is less expressed in tumor tissue compared to normal tissue and is associated with lymph node metastasis, local invasion, and good prognosis of the disease (19). This finding indicates that this biomarker can be used as a factor of disease progression and diagnosis (19). In the study by Ma et al., it was concluded that the reduction in miR-361 expression is associated with the reduction of glycolysis, inhibition of matrix metallopeptidase 1 (MMP-1) and fibroblast growth factor receptor 1 (FGFR1) in breast cancer cells; the induction of increased miR-361 expression causes a decrease in the proliferation, migration, invasion of cancer cells, and an increase in apoptosis in breast cancer cell lines (20).

Breast cancer prognosis is associated with numerous clinical and pathological factors, and various studies have shown that the hormone receptors HER2, ER, and PR are the most important acceptable factors for predicting response or resistance to breast cancer treatment (21). Based on the results of this study, no statistically significant association was observed between HER2, ER, and PR receptors, tumor size, and decreased miR-361 expression. In other similar studies conducted in this field, the association between these parameters and decreased miR-361 expression was also insignificant. However, in the study by Cao et al., the prevalence of tumors with HER2, ER, and PR receptors increased with decreased miR-361 expression, and the prevalence of HER2, ER, and PR receptors decreased with increased tumor grade and tumor size (19). Furthermore, the results of decreased miR-361 expression in TNBC tumor tissues larger than 3 cm in this study were consistent with the results of the study of Han et al., which showed lower miR-361 expression in TNBC tumor tissues larger than 3 cm, aggressive tissues and high-grade ones (22).

In this study, there was no correlation between the decrease in miR-361 expression and tumor grade, but in their study, Yerukala Sathipati et al. observed that the expression level of miR-361 decreased with increase in tumor grade (23). However, Hua et al. did not observe a statistically significant correlation between the expression level of miR-361 and tumor grade in their study (24). Also, in the study of Ma et al., the decrease in miR-361 expression was 0.63 in grade I and II tumors and 0.36 in grade III tumors, and the correlation between miR-361 and histological grade of the tumor was significant (20). Wu et al. also proved that miR-361 and tumors with histological grade III are strongly related to the survival of patients (25).

In the present study, the decrease in miR-361 expression in tissue and serum helps to molecularly differentiate tumor and non-tumor samples, which is consistent with the results obtained by Chang et al., who indicated the association of this miRNA with cancer progression and its decrease in serum expression (26). However, the difference between the present study and this method was that we measured miR-361 in tissue and this researcher examined the decrease in miR-361 expression in serum. miR-361 is directly related to tumor size, tumor stage, and cancer progression, but it is not able to predict the prognosis of the disease. Given this evidence, it can be concluded that miR-361 is produced by breast cancer tumor tissue, and its tissue level is significantly reduced in tumor tissue compared to healthy tissue, thus helping to differentiate these two groups. Moreover, the lower the level of miR-361 expression in tissue, the greater the relationship with tumor size, stage, and disease invasiveness.

It seems that measuring the level of miR-361 in tissue can be used as a suitable method for early diagnosis of breast cancer patients. In fact, the expression of this miRNA in tissue was considered as a diagnostic marker.

Acknowledgment

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