Expression Patterns of miR181a and miR30d in Patients with Breast Cancer

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Abstract

Background: One of the important molecular pathways in breast cancer is the PTEN-PI3K-AKT pathway. Any change in the activity of the PTEN gene can alter the PI3K-AKT pathway. Moreover, there are subsets of genes and pathways their expression changes by post-transcriptional regulations. For instance, gene regulation alters by non-coding RNAs such as micro-RNAs as post-transcriptional regulators that prevent the expression of the target transcript. Therefore, it is essential to assess the related alterations in micro-RNA expression patterns to find out the possible causes of conversions in related transcripts and pathways such as the PTEN-PI3K-AKT pathway in breast cancer.

Methods: To determine the expression level of miR-181a and miR-30d in 30 breast tumor samples and 30 adjacent normal samples, the RNA extraction, and cDNA synthesis was performed by RiboEx (GeneAll, Korea). Finally, the Real-Time PCR method was used for quantitative analysis of the expression levels of these miRNAs. all the experimental part of the project in done at Islamic Azad University in 2017.

Results: After analyzing comparisons in the expression level of miR-181a and miR-30d in tumor and normal tissues, there was a significant increase in the expression level of miR-181a in tumor samples compared with normal samples. Moreover, the expression level of miR-30d in tumor samples reported a significant decrease in comparison with normal samples ($P<0.05$).

Conclusion: Upregulation of miR-181a may affect the transcription of the PTEN gene resulting in the cell progress to cancer. The Downregulation of miR-30d may also lead to cancer cell growth, due to a reduction in the affecting on the CREB gene transcript.

Keywords: Breast Cancer; Micro RNAs; Real-time PCR; Post-transcriptional regulation

Introduction

Breast cancer is the most common cancer and the second leading cause of death for cancer among women in the world (1). Despite several recent advances in breast cancer diagnosis and treatment such as targeted chemotherapies and their combinations, surgery, hormonal therapy, and radiotherapy (2,3), the treatments for Breast cancer still have many limitations, like resistance and a lack of reliable biomarkers (4). Breast cancer still is a major cause of death in women worldwide (3). Realizing the biology and signaling pathways of malignant maladies like breast cancer is a crucial prerequisite to cope with this problem (3).

PTEN pathway is one of the main pathways that play a key role in breast cancer. Since the PTEN network contains cell-surface growth factor receptors for activating transcription factors that are in the nucleus and complemented by interconnecting with other signaling pathways. In breast cancer, the pathway associated with the PTEN gene is the PI3K-AKT signaling pathway that in which PTEN acts as a tumor suppressor (5). Any alteration in this pathway results in irreparable consequences such as the progression of the cell towards cancerous. This pathway could be influenced by various types of genetic factors especially, post-transcriptional regulations. One of the most important elements, which affect this pathway, is microRNAs (6,7).

MicroRNAs (miRNAs) are a group of non-coding RNAs that have a length of about 18-25 nucleotides and after transcription, have direct impact on the transcription of their target transcripts. They trigger this procedure by connecting to complementary sequences, which are often located in the 3'UTR region of the targeted mRNA (8). Of course, these complementary sequences are present in coding regions, 5'UTRs, and even promoters (8,9). Hundreds of genes in the human genome code for these types of RNAs. miRNAs control about 30% of the protein-coding genome (10). Recent studies have shown important roles in many biological functions for these types of RNAs such as their role in differentiating, evolving, metabolism, and cancer-related processes and even diseases such as diabetes, Alzheimer's, as well as heart disease, autism, and fragile X syndrome (11).

According to the undeniable role of miRNAs in biological pathways, we have investigated the miRNAs that have a straight impact on the PTEN pathway. In this paper by considering previous studies on miRNAs and miRNA databases such as mirDB.org and mirbase.org (to identify the score of each miRNA that targeting the PTEN pathways genes); we have investigated the expression level of miRNA181a and miRNA30d as regulators of the PTEN signaling pathway. miR-181a targets very important genes involved in most significant molecular pathways so that the lack of proper alignment of these genes can lead to a variety of cancers, including breast cancer.

Previous studies on this micro-RNA indicated the effect of miR-181a on the development of breast cancer. For instance, in a study, the association between increased expression of miR-181a was detected by regulating the misleading expression of the ATM gene and the development of breast cancer (12). miR-181a acts as a negative regulator of the progesterone receptor (PR) in breast cancer by increasing its expression (13). Moreover, this microRNA is free in body fluids and can be also used as a biomarker for the prognosis of HER2+ breast cancer type (14) (15). On the other hand, since, miR-30d is known as a tumor suppressor, not only acts as a regulator in normal and malignant tissues, including cell development, apoptosis, proliferation, migration, invasion, and angiogenesis but also is significantly associated with several tumor-related pathways, such as Rho family, GTPases and PI3K/AKT signaling pathways (16,17).

Therefore, we aimed to investigate the miR-181a and miR-30d expression patterns related to the

PTEN-PI3K pathway in breast cancer to turn on a peep light in discovering the causes of conversions in this pathway.

Materials and Methods

Patient's characteristics
Human specimens were used to determine the expression of miR-181a and miR-30d. Overall, 30 tumor tissues, and adjacent tumor tissues (as a normal sample) were taken from 30 patients with breast cancer in 2017 (Table 1). In all of the sample tissues, the type of breast cancer was infiltrating ductal, NOS. Besides, this research was performed at Islamic Azad University of Tehran Medical Sciences and human samples were collected from "Biological materials were provided by the Iran National Tumor Bank founded by Cancer Institute of Tehran University of Medical Sciences, for Cancer Research" with the ethical committee with IR.IAU.TMU.REC.1396.213 identification number.

Table 1: Pathological features of Breast Cancer Tissues

Age(yr)		Gender		Tumor size		Tumor Size		
Age groups	Frequency (%)	Male	Female	1cm-3cm	3cm-6cm	I	II	III
20-30	1 (3.%)	0 (0)	30 (100)	18 (60)	12 (40)	4(13)	16(53)	9(30)
30-40	6 (20)							
40-50	9 (30)							
50-60	8 (26.7)							
60-70	6 (20)							

This research was performed at Islamic Azad University of Tehran Medical Sciences branch with the ethical committee identification number: "IR.IAU.TMU.REC.1396.213". Human samples and Biological materials are provided by the Iran National Tumor Bank, founded by the Cancer Institute of Tehran University of Medical Sciences, for Cancer Research.
Concerning Table 2, all of the patients were women and were in stages 1 to 4 of breast cancer. People aged 29-60 yr old.

RNA extraction
Total RNA was isolated from each tumor tissue and its adjacent non-tumor tissue by using Ribo-Ex (GeneAll, Korea) according to the manufacturer's specifications, and the concentration of total extracted RNA in the final volume was determined by spectrophotometry.

cDNA synthesis for miRNA
Concerning the small size of the microRNAs, their cDNA synthesis is accomplished with a stem-loop primer. In which mentioned miRNA primer design, a unique Stem-loop sequence is considered for the synthesis of cDNAs. The length of this sequence is 44 nucleotides. To create the stem-ring primers, 6 nucleotide complements of the end 3', and the studied microRNAs and the desired reference RNA, are added to the end of the sequence (18). In this study, specific and separate stem-loop primers for miR-181a-5p and miR-30d-5p were used to synthesize cDNA (Table 2).

Table 2: Specification and sequences of stem-loop primers used to test the expression of mir-181a-5p and mir30d-5p.

miRNA	Length	Tm	GC %	Sequences
mir-181a-5p	50	79.4	50	5´GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACTCAC3´
Mir-30d-5p	50	85.5	56	5´GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACTCAC3´
RNU44	52	89	57.7	5´GTCGTATCCAGTGCAGGGTCCGACCGGTATTCGACTGGATACGACAGTCAG3´

Real-time quantitative PCR

Real-time PCR was accomplished by using Step One Plus™ Real-Time PCR Systems (ABI Applied Bio-systems, Thermo Fisher Scientific, USA). The reaction was in the volume of 15-µl containing 7.5-µl of RealQ Plus 2x Master Mix Syber Green High ROX™ (Ampliqon, Denmark), 1-µl of cDNA, 5.5-µl of H2O and 1-µl of forward primer (3Pmol/µl concentration) and reverse primer (3Pmol/µl concentration). Real-time PCR amplifications were done as follows:

for two selected microRNAs, PCR amplification was set to an initial 95 °C for 15 min and then for mir181a and mir30d, 40 cycles, 95 °C for 15 sec and 60 °C for 1 min (step and hold). All samples were analyzed by duplicate. RNU44 was used as an internal control for miRNAs. Gene expression was calculated using the comparative threshold cycle (2-$\triangle\triangle$CT) method. Primer sequences of mir181a and mir30d and stem-loop sequences for mir181a and mir30d have been shown in Table 3.

Table 3: Primer Sequences for Amplification of mir181a-5p and mir30d-5p

Target miRNA		Sequences (5´ 3´)
Mir181a-5p	Forward	5´CACGCCAACATTCAACGCTG3´
	Reverse	5´CCAGTGCAGGGTCCGAGGTA3´
Mir30d-5p	Forward	5´CACGCAAGTAAACATCCCCGAC3´
	Reverse	5´CCAGTGCAGGGTCCGAGGTA3
RNU44	Forward	5´CCTGGATGATGATAGCAAATG3
	Reverse	5´TCGTATCCAGTGCAGGG3´

Statistical Analysis

Statistical analysis was performed using the GraphPad Prism v7.03 (GraphPad Software Inc. USA) and *t*-test. For all tests, *P*<0.05 was considered statistically Significant.

Results

Related results to the expression level of miRNA181a

In terms of miR-181a expression, there is a significant difference between the expression levels of mir181a in tumor samples in comparison with normal samples. Twenty-one cases (70%) of the tumor samples showed an increase in expression compared with normal samples, while 9 (30%) of tumor samples showed a decline in expression in comparison with normal samples (*P*=0.040) (Fig. 1).

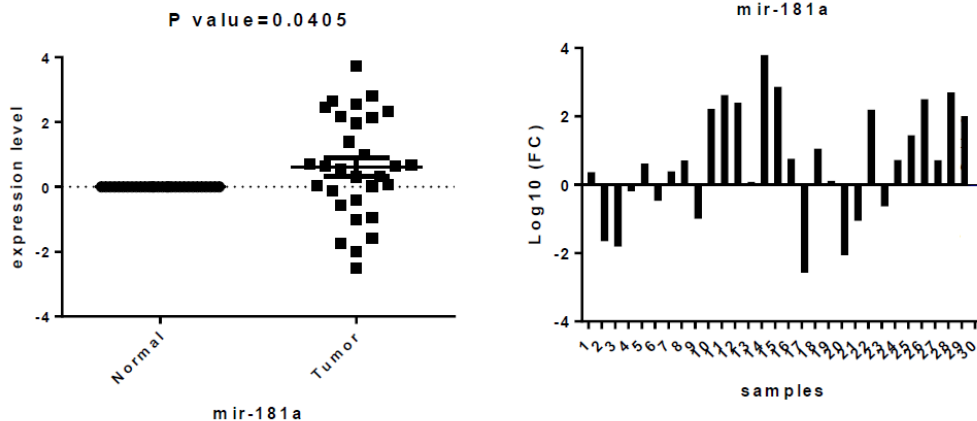**Fig. 1:** Graphs A and B are related to the expression level difference of mir181a in breast tumor samples compared to non-tumor adjacent samples

Outcomes related to the expression level of miRNA30d

The expression level of miR-30d has shown a significant difference between tumor samples in comparison with normal samples. 9 (30%) of the tumor samples showed an increase in expression compared with normal samples, while 21 cases (70%) of tumor samples showed a decline in expression in comparison with normal samples (*P*=0.042) (Fig. 2).
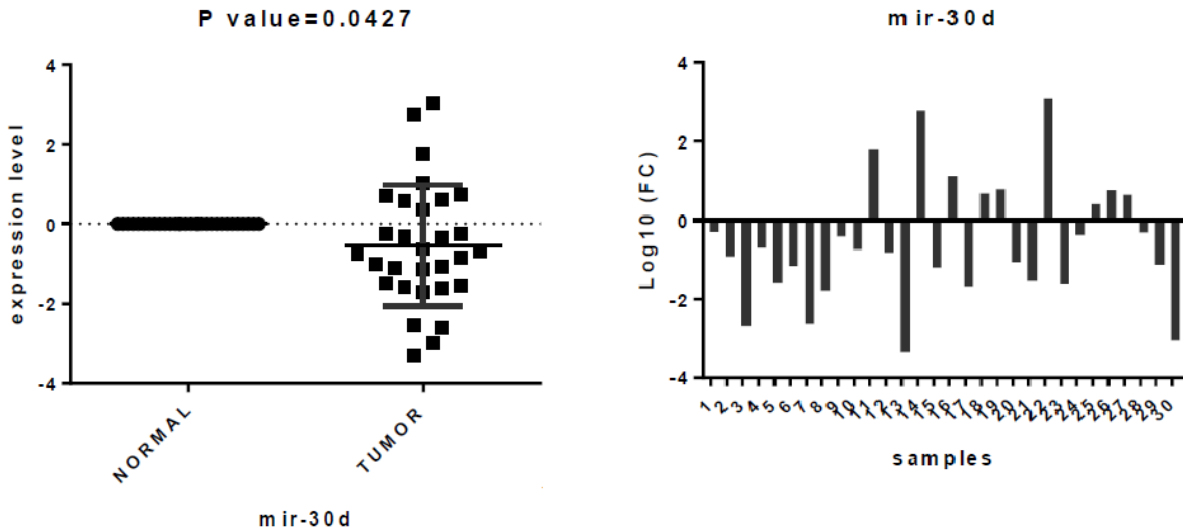

Fig. 2: Graphs A and B are related to the expression level difference of mir30d in breast tumor samples compared to non-tumor adjacent samples

Discussion

About one in nine women, one person develops breast cancer over a lifetime. In Iran, breast cancer with a 32% outbreak is the most common cancer among women (19). About 6.6% of all breast cancers in women under 40 yr of age, 2.4% in women under 35, and 65.6% in women below 30 yr of age have been identified (20). Therefore, the identification of molecular pathways and agents involved in these ways is important for better identification and treatment (21).

PTEN gene is one of the important key roles in Breast cancer that any changes in the mRNA expression of this gene can promote Breast cancer. Previous studies on the PTEN gene have agreed to the reduced expression level of the PTEN gene in most cancers. The most important signaling pathway for the PTEN gene is the PI3K-AKT pathway. This pathway plays a key role in cell cycle regulation, cell proliferation, cell survival, and cancer. Since the PTEN gene plays a key role in the progression of this pathway, any irregularity in the pattern of expression of this gene can disrupt this pathway and thus lead the cell to become cancerous.

MicroRNAs are one of the post-transcriptional regulators for gene expression and by considering the mentioned studies on PTEN expression, now we know that this gene as a tumor suppressor shows decreased expression pattern in breast cancer. Therefore, it would be interesting to investigate the major causes of this decline in expression because by this, we can have more information to find breast cancer related biomarkers.

Following microRNAs related to the PTEN gene, we assessed the expression status of miR-181a and miR-30d. Multitudes of research have been carried out on micro-RNA 181a. The ex-

pression level of miR-181a was examined in patients with gastric cancer by Real-Time PCR. In this study, the miR-181a expression level in tumor samples was found to be higher than expression in normal samples (22). The expression level of miR-181a was investigated in tissue samples of patients with liver cancer using Real-Time PCR. In this study, the expression level of mir181a in tumor samples showed an enhancement in expression level in comparison with normal samples (23). The miR-181a activity in cooperation with the PI3K-AKT pathway was also assessed in lung cancer (24). According to studies, performed on miR-181a and the results obtained from these studies, miR-181a demonstrated upregulation and downregulation in various cancers. In the PI3K-AKT signaling pathway, PTEN acts as a negative regulator and, by its phosphatase action, converts the PIP3 generated by the PI3K activity to PIP2. PTEN, by its phosphatase action, prevents the pathway of PI3K-AKT that confirms PTEN activity as a tumor suppressor gene. In such a case, a change in the activity or pattern of PTEN gene expression can have deleterious effects on important cellular processes. One of the most important factors in PTEN's natural activity is the uncontrolled activity of micro-RNAs as factors regulating gene expression in the post-transcription phase. According to our results, miR-181a has a significant increase in expression. This enhancement in expression may affect the PTEN gene expression by targeting its mRNA that leads to cancer cell growth. In other words, by increasing the activity of miR-181a, this microRNA targets the PTEN gene transcript and leads to downregulation of the PTEN gene. Since this gene is known as a tumor suppressor gene, therefore, this uncontrolled progression may cause impairment in the pathway of PI3K-AKT and, as a result, the progression of the cell to cancerous cell. The significant increase in miR-181a expression with $P=0.04$ can partly prove the role of miR-181a in the disruption of the cell signaling pathway by targeting PTEN gene transcription. Since most specimens were in the early stages of cancer (13% in stage 1 and 53.3% in stage 2 cancers), the alteration in expression of

miR-181a occurred in the early stages of cancer, which shows the role of this microRNA as a factor affecting the PI3K-AKT pathway. Since the increase in the expression level of miR-181a is meaningful in the prototypes that are in the early stages of cancer, if further investigations and other conditions are taken into account, this microRNA can be considered as an early diagnostic biomarker to detect breast cancer in the early stages of cancer.

Besides, the expression level of miR-30d was examined in this study. Wide range of studies have investigated the miR-30d expression level in various cancers. The expression of miR-30d was examined in a sample of intestinal cancer tissue using the Real Time-PCR technique, in which miR-30 d was downregulated in the tumor samples in comparison with normal samples (25). The expression level of miR-30d was investigated in 12 tumor samples and 12 adjacent normal tumor tissues in patients with prostate cancer using Real Time-PCR, in which miR-30d showed a significant increase in tumor samples compared with normal ones (16). The expression pattern of miR-30d was investigated in tissue samples of patients with intestinal cancer using Real Time-PCR, in which miR-30d demonstrated a significant reduction in the expression level of tumor samples compared to normal specimens (26). MiR-30d had reduced and increased expression patterns in various types of cancers. One of the signaling pathways associated with breast cancer is the PI3K-AKT signaling pathway.

In this molecular pathway, after binding the ligand to a receptor and following the receptor activation, the PI3K is activated and connected to the receptor membrane protein and by its kinase activity, can result in the conversion of PIP2 to PIP3. In which case, PIP3 causes the PDK to activate AKT by phosphorylating the threonine 308 region, thereby continuing the signaling pathway in which the AKT is activated and affecting a wide range of proteins and other regulatory factors and the molecular pathways in the direction of the cell targets going ahead. Meanwhile, one of the pathways resulting from AKT activity is the activation of the CREB transcrip-

tion factor. In fact, after AKT phosphorylation by PDK, AKT induces phosphorylation and activation of the CREB transcription factor, and CREB causes the transcription of different genes. One of the goals of CREB activation is the transcription of genes that contribute to cell proliferation and differentiation. In this case, any change in the CREB activity causes uncontrolled expression of genes that contribute to cell proliferation and differentiation, and one of the related results of this uncontrolled expression is cancer.

In the present study, miR-30d showed a significant reduction in expression in tumor samples compared to normal ones. Since the miR-30d expression declines, the regulatory process for the expression of the CREB gene can be impaired and it could be expressed more than usual. Since this reduction is significant in the specimens that are in the early stages of cancer, so it can be considered that if more research is done and other conditions are taken into account, this micro-RNA can be detected as an early diagnostic biomarker to detect breast cancer in the early stages of cancer. Finally, this study was designed as a pilot study, and further investigations are required to confirm our findings.

Conclusion

After evaluating the expression level of mir181a and mir30d, significant upregulation in mir181a, and significant downregulation in mir30d were seen. miR-181a might affect the transcription of the PTEN gene that results in the cell progress to cancer. In addition, the downregulation of miR-30d may also lead to cancer cell growth, due to a reduction in the affecting on the CREB gene transcript

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

The authors declare that there is no conflict of interest.

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