MIR-145's Regulatory Role in Breast Cancer Progression

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ÖZET

Meme kanseri tanısı geç konulduğunda tedavi ve sağkalım oranları azalmaktadır. Her yıl milyonlarca kadın meme kanseri teşhisi almakta ve bu hastalık nedeniyle birçok insan hayatını kaybetmektedir. Ancak, erken teşhisi sağlayacak biyobelirteçlerin keşfi ile tedavi süreçlerinin daha başarılı olacağı ve sağkalım sürelerinin arttırılması hedeflenmektedir. Son yıllarda, mikroRNA'ların biyobelirteç olarak meme kanseri erken teşhisinde kullanımı ile ilgili çalışmalar yapılmaktadır. Araştırmalar, mikroRNA'ların meme kanseri teşhisi ve prognoz tahmini için kullanılabilecek biyobelirteçler olabileceğini göstermektedir. mikroRNA'ların meme kanseri erken tanısında biyobelirteç olarak potansiyel kullanımı üzerine devam eden araştırmalarla birlikte, çalışmamızda da miR-145'in meme kanserinde biyobelirteç olarak kullanılabilme potansiyeli analiz edildi. Çalışmamızda, 200 meme kanseri hastasının ve sağlıklı kontrol grubunda yer alan 100 kişinin kan-serum numunelerinden RNA örnekleri izole edilerek, mir-145 özgün ekspresyon seviyeleri RT-qPCR tekniği ile tespit edildi. Bu analizler sonucunda, miR-145'in meme kanseri hastalarında sağlıklı kontrol grubuna göre daha düşük düzeylerde ekspresyon seviyeleri bulundu. miR-145'in biyobelirteç olabilme niteliğinin daha yüksek sayılı meme kanseri hasta kohortunda araştırıldıktan sonra meme kanseri erken teşhisinde hedef biyobelirteç olarak kullanılabileceği hedeflenmektedir.

Anahtar Kelimeler: Meme kanseri, mikroRNA, miR-145

MIR-145'in Meme Kanserinin İlerlemesinde Düzenleyici Rolü

ABSTRACT

Delayed diagnosis of breast cancer results in lower treatment success rates and lower survival rates. Every year millions of women are diagnosed with breast cancer and many of them die from this disease. However, the discovery of biomarkers that enable early diagnosis should increase the success of treatment processes and extend survival times. In recent years, studies have been carried out to use microRNAs as biomarkers in the early detection of breast cancer. Research shows that microRNAs can serve as potential biomarkers for the diagnosis and prognosis of breast cancer. In addition to ongoing research on the possible use of microRNAs as biomarkers for early breast cancer diagnosis, our study also analyzed the possible use of miR-145 as a biomarker in breast cancer. In our study, RNA samples were isolated from blood serum samples of 200 breast cancer patients and 100 people in the healthy control group, and the specific expression levels of miR-145 were determined using RT-qPCR technique. As a result of these analyses, it was found that miR-145 had lower expression levels in breast cancer patients than in healthy controls. After further investigation of the biomarker potential of miR-145 in a larger group of breast cancer patients, it will be used as a target biomarker for early breast cancer diagnosis.

Keywords: Breast cancer, microRNA, miR-145

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INTRODUCTION

In recent years, molecular biology and biomedical research have focused a lot of attention on small noncoding RNA molecules known as microRNAs, or miRNAs. The regulation of gene expression is greatly aided by these small, non-coding RNA molecules. miRNAs play a significant role in the etiology of many diseases because they organize into a complex network that regulates cellular processes (Macfarlane, L.A. and Murphy, P.R. 2010). MicroRNAs can be observed in cancer cells, blood, plasma, and even patients' saliva. They are released into the bloodstream by apoptotic cells in two forms. These can be ordered acellular or embedded in membrane vesicles, microvesicles or exosomes (Schwarzenbach et al., 2011). By analyzing the levels and types of microRNAs found in a patient's blood, researchers can identify dysregulated microRNAs and categorize breast cancer patients for specific treatments (Teo et al., 2021).

Unregulated microRNAs, including as like miR-145, are associated with tumor growth, development, cell death, invasion, and cell proliferation. Several studies have identified specific miRNAs that can be used as early detection molecules to detect breast cancer (Iorio et al., 2005; Blenkiron et al., 2007; Roth et al., 2010). It has been noted that miRNAs are involved in a number of signaling pathways in a variety of diseases. According to reports, they actively participate in processes like breast cancer metastasis, tumor formation, and disease progression. Targeting genes involved in growth factor signaling pathways and cell cycle control, microRNAs have been demonstrated to control the proliferation of breast cancer cells. Furthermore, they have been discovered to control apoptosis by focusing on genes linked to cell death pathways. Furthermore, they have been discovered to promote the invasion of breast cancer cells by controlling genes related to the epithelial-mesenchymal transition (EMT) and cell migration (Goh and Kumar, 2015; Hu et al., 2012). miR-145 is a tumor suppressor miRNA, and when it is lost, its expression increases in cancer cells. This downregulation is associated with an increase in cancer cells, emphasizing miR-145's critical role as a tumor suppressor (Otmani and Lewalle, 2021). Consequently, it has been suggested that miR-145 may be useful as a biomarker for the detection of cancer. The molecule's potential as a biomarker in the early detection, prognosis, and staging of breast cancer was investigated. miRNA-145 (miR-145) is thought to play an important role in tumorigenesis and progression. This miRNA interacts with cell invasion and migration signaling pathways (Xu et al., 2019). It has been noted that miR-145 directly associates with the p53 protein in a variety of cancers, including breast cancer, and is thought to function as a tumor suppressor (Sachdeva et al., 2009). In a different study, it was found that miR-145 directly targets ROCK1, and that its downregulation increases the process by which ROCK1 causes invasion and cancer (Zheng and Zuo, 2016). Another study demonstrated that TGF-β1 expression was elevated in breast cancer as a result of downregulating miR-145, which led to cell migration and proliferation (Ding et al. 2017).

As a result, miR-145 functions as a tumor suppressor, and increasing its expression stops cancer cells and tissues from invasively growing. Reduced miR-145 levels in cells encourage the growth and

invasion of tumors by integrating related signaling pathways into higher expression. These findings indicate that miR-145 might be a crucial target for cancer treatments.

Aim

Given that breast cancer is one of the most prevalent cancers in women worldwide, early detection is vital. MicroRNAs (miRNAs) play a significant role in the diagnosis and prognosis of breast cancer, as evidenced by the progress made in biotechnology and cancer research. Specifically, miR-145 is regarded as a tumor-suppressive miRNA, and an increased proliferative capacity of cancer cells is linked to its deficiency. Therefore, new methods for early detection, prognosis, and staging of the disease are promised by continuing research into the potential value of miR-145 as a biomarker for breast cancer diagnosis. In comparison to 100 healthy controls, we compare the expression profile of miR-145 in serum samples from 200 breast cancer patients who are matched for age, gender, and ethnicity. The purpose of this study is to shed new light on miR-145's function in the management of disease by examining its potential as a useful biomarker in the detection and treatment of breast cancer.

MATERIAL AND METHODS

Materials

- CFX96 Real-Time PCR (Biorad)
- Vorteks (Finepzr)
- Micro Star 17R Mini Santrifüj (VWR)
- NEB Monarch Quick-DNA/RNA Miniprep kit
- NEB ProtoScript® II Reverse Transcriptase
- Meridian Sensifast No Rox SYBR qPCR kit
- Forward Primers
- Reverse Primers
- Stem Loop Primers

Methods

The study's sample group was chosen using a Power and Sample Size program. The power analysis showed that a minimum of 182 participants were required for the patient analysis, with a Type I Error of 0.05 and Test Power (Confidence Interval) of 80% being taken into account. It was decided to include 200 patients in the study who had been given a breast cancer diagnosis, searched for treatment at the Istanbul University Institute of Oncology, volunteered to participate, and gave informed consent by signing the necessary paperwork. Comparably, 100 healthy people who were matched for age and gender with the patient group and had no family history of cancer as well as no diagnosis of breast-related disorders made up the control group for the study. Blood serum samples from the patient group and the control group were used for miRNA-145 expression studies. First, total RNA was extracted from

serum samples as a method. The obtained total RNAs were then used to create cDNA using reverse transcriptase and stem loop primers. Using a Thermal Cycler, reactions were incubated at 42°C for 30 minutes and then at 95°C for 5 minutes. The resulting cDNAs were subjected to real-time qPCR in order to examine the variations in miRNA expression levels. The Kolmogorov-Smirnov Test was used to determine whether the distribution between the study groups was assumed to be normal. Consequently, a non-parametric test called the Mann-Whitney U Test was employed to analyze the data. Between the patient and control groups, 2-(ΔΔCt) values were used to conduct the Mann-Whitney U Test.

Obtaining Serum from Blood

In a study conducted at Istanbul University Oncology Institute on patients diagnosed with breast cancer and healthy control group members, the collection of samples for serum collection was carried out with the following protocol steps. First, the blood taken from the patient was transferred to a yellow-capped gel tube. This gel created a barrier between the serum and blood cells, accelerating the clotting of the serum. At least 30 minutes were waited for the blood to clot. Then, it was centrifuged at 1500-2000xg for 10 minutes to accelerate clotting. The clot, serum and gel layers formed after the centrifugation process became evident. The resulting serum was then transferred to Eppendorf tubes and stored at -80°C. These careful processing steps were performed to ensure quality control of the study and to safely use the resulting serum samples.

Total RNA Isolation from Serum

To make the serum samples usable, they were removed from -80°C and solved on ice. Then, the following protocol steps were followed for RNA isolation using the NEB Monarch Quick-DNA/RNA Miniprep kit:

First, 200 µl of RNA preservation buffer and 200 µl of serum sample were mixed and this mixture was thoroughly homogenized by vortex. Then, 10 µl of Proteinase K was added, mixed again by vortex, and incubated at room temperature for 30 minutes. Then, 400 µl of isopropanol was added to the mixture and mixed well. The resulting mixture was then transferred to a dark blue RNA purification filter and centrifuged at 16,000 × g for 30 s. The filtered mixture was discarded. Then, 500 µl of RNA washing buffer was added to the filter and centrifuged at 16,000 x g for 30 seconds. In the next step, 5 µl of DNase I and 75 µl of DNase reaction buffer were mixed in an RNase-free tube. This mixture was then transferred directly onto the filter and incubated at room temperature for 15 minutes. 500 µl of RNA priming buffer was added to the filter and centrifuged at 16,000 x g for 30 seconds. Then, 500 µl of RNA washing buffer was added to the filter and centrifuged at 16,000 x g for 30 seconds. The same process was repeated once more, but this time it was centrifuged at 16,000 x g for 2 minutes and placed on the filter. Finally, to obtain RNA, 50 µl of Elution Buffer was added to the middle of the spin column membrane and then centrifuged at 16,000 x g for 1 minute. These steps were followed to ensure successful isolation of RNA from serum samples.

cDNA Synthesis

Isolated total RNA samples were thawed on ice and purity rates were checked with Nanodrop in addition to the number of ng of nucleic acid per µl. While performing this control, samples at 310 nm and with a 260/230 ratio of ~1.8 were used and 1 ng of RNA entered the reaction. cDNA synthesis was performed with stem-loop primers using NEB ProtoScript® II Reverse Transcriptase.

Real – Time PCR

Real-time qPCR was performed to analyze differences in expression levels of miRNAs. Real-time qPCR was performed using the Meridian Sensifast No Rox SYBR qPCR kit and specific forward and reverse primers. In the real-time PCR process, expression levels were read in the extension phase.

RT-qPCR Result Analysis of Data

Expression differences and fold&change fold change rates of miR-145 selected in the study in patient groups and healthy control group samples were found by RT-qPCR study. In the study, cycle number (ct (cycle threshold)) values of miR-145 and *U6*, which was used as a reference gene (endogeneus control preferred to normalize the study), were found in the samples in the studied group. Finding the fold&change ratios from the ct values found is as follows:

1- In all groups, the ct values of *U6*, the reference gene, were subtracted from the ct values of the target miRNA. Thus, Delta CT values (ΔCT) were found for each miRNA in the healthy control group, stage 1, stage 2, stage 3 and stage 4 patient groups.

2- ΔCT values of miRNAs of the healthy control group were averaged.

3- The average ΔCT values of the control group were subtracted from the ΔCT values of the control group and each stage patient group. Thus, ΔΔCT values of target miRNA were found for all groups.

4- The obtained ΔΔCT values were calculated as two over – (ΔΔCT) ($2^{-\Delta\Delta CT}$). This indicates the fold change in the expression level of the target gene. The explicit form of the formula is $2^{-\Delta\Delta CT}$.

Statistical Analysis of Data

Descriptive statistics of the qualitative variables in the study are given as numbers and percentages, and descriptive statistics of the quantitative variables are given as mean, standard deviation, median, 1st and 3rd quarters. The suitability of quantitative variables to normal distribution was examined with the Kolmogorov Smirnov test. Mann Whitney U test was used to compare the means of two independent groups. Kruskall Wallis test was used for comparison of means of more than two independent groups. Dunn's test was used as a post hoc test for detailed comparison of groups with differences. Statistical significance level was taken as <0.001, and IBM SPSS Statistics for Windows,

Version 26 (Armonk, NY, IBM Corp) was used in the calculations. program was used.

RESULTS

In the study, miR-145 expression profile changes of a total of 300 participants were examined. Samples with RT-qPCR Ct values of 37.00 and above for the relevant miRNA were excluded from the statistical analysis of the study. These samples were marked N/A and were excluded from analysis.

According to the expression results, the target miRNAs' expression levels were statistically significant when compared to the patient control group. The groups in which differences in miR-145 expression were investigated showed statistically significant differences when compared to one another ($p<0.001$ for each) (Table 1). When the means of the stage 1, stage 2, stage 3 and stage 4 patient groups were compared to those of the healthy control group, statistically significant differences ($p<0.001$) were discovered for each. The control group's mean expression of miR-145 was found to be statistically significantly higher than that of the patient groups in stages 1, 2, 3, and 4 ($p<0.001$ for each) (Table 2). Fold change in patient groups at stages 1, 2, 3, and 4. when compared to the control group, the fold change 2-ΔΔCT ratios are shown to have decrease.

Table 1: Statistical values of fold change 2^-ΔΔCT of miR-145 in the control group and patient stages

	Groups	**Number of samples**	**Mean**	**Median**	**Standard Derivation**	**Minimum**	**Maximum**	**p**
Fold change miR-145 ($2^{-\Delta\Delta CT}$)	Control Group	39	1,02	1,01	0,21	0,58	1,53	<0,001
	Stage 1	34	0,51	0,4	0,14	0,29	0,85	
	Stage 2	35	0,35	0,33	0,09	0,26	0,75	
	Stage 3	38	0,22	0,23	0,08	0,05	0,42	
	Stage 4	31	0,11	0,11	0,04	0,05	0,22	

Table 2: Comparison of fold change 2-ΔΔCT of miR-145 according to Control Group and Patient Stages

Group comparisons	Test Statistics	Standard Error	Standard Statistics of the Test	p
Control group- Stage 1	31,54	12,39	2,54	,011
Control group- Stage 2	67,57	12,63	5,34	<,001
Control group- Stage 3	97,47	12,72	7,66	<,001
Control group- Stage 4	137,01	12,32	11,11	<,001
Stage 1- Stage 2	36,02	12,00	3,00	,003
Stage 1- Stage 3	65,92	12,09	5,45	<,001
Stage 1- Stage 4	105,46	11,67	9,03	<,001
Stage 2- Stage 3	29,89	12,33	2,42	,015
Stage 2- Stage 4	69,43	11,92	5,82	<,001
Stage 3- Stage 4	39,53	12,02	3,28	,001

DISCUSSION

The findings show that miR-145 inversely declines as breast cancer stages advance. Results that are statistically significant ($p < 0.001$) highlight the significance of variations in miR-145 expression in breast cancer. Based on these findings, it is thought that miR-145 expression analyses offer an alternative viewpoint to the widely recognized clinical staging approach for breast cancer and can also be applied to the diagnosis of cancer. Furthermore, it is thought that the study's incorporation of various miRNA molecules may serve as a more accurate diagnostic instrument when individual miRNA expression data prove inadequate because breast cancer patients' expression levels vary throughout the course of the disease and their combined assessment can offer a thorough analysis.

CONCLUSION AND RECOMMENDATION

The study's findings demonstrate that miR-145 suppresses tumors in breast cancer and that the expression of the protein declines noticeably with the disease's progression. The utilization of miR-145 as a potential biomarker for breast cancer diagnosis and staging is supported by these results. In order to confirm the significance of miR-145 in the diagnosis and prognosis of breast cancer, additional investigation and clinical trials are required. In order to increase the precision of breast cancer diagnosis and staging, miR-145 expression analysis may be incorporated into standard clinical practices if these findings hold true. It is advisable to take into account a variety of different miRNAs when considering diagnostic testing, since patients with breast cancer may have different miRNA expression profiles. Several miRNAs working together can help provide a more thorough and precise evaluation of the diseases. Researching therapeutic approaches targeted at regaining miR-145 expression in breast cancer cells is crucial because of the tumor-suppressive function of miR-145. The development of novel breast cancer treatment options may be facilitated by miR-145-based therapeutics.

Finally, studies that following changes in miR-145 expression over the course of treatment and the progression of the disease should be carried out over an extended period of time. These investigations may offer important new understandings of the dynamic function of miR-145 in breast cancer. To fully comprehend and utilize miR-145's potential in the detection and treatment of breast cancer, more study and clinical applications are therefore required.

Conflict of Interest

The authors declare that there is no conflict of interest regarding this study.

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