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Exploring the Prognostic and chemotherapy Response Potential of CDR1as, circRNA-000284, and circ-ITCH as Circulating Biomarkers in Breast Cancer

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ABSTRACT

Background: Breast cancer is the leading cause of death for women worldwide. Optimal methods for most cancer control require the use of appropriate biomarkers. Recently, circular RNAs (circRNAs), as closed-loop RNA molecules generated through reverse splicing, have emerged as promising biomarkers for cancer development.

Methods: In this observation, the expression of 3 unique circRNAs - cdr1as, circRNA-000284, circ-ITCH - within the peripheral blood of breast cancer patients was studied using Real-Time RT-PCR before and after chemotherapy compared with the control.

Results: The data confirmed that the analyzed circRNAs expression was significantly altered in breast cancer patients compared to the controls ($p< 0.0001$). These alterations have been related to advanced stages, lymph node involvement, and metastasis. CircRNA-000284 expression considerably decreased after chemotherapy ($p< 0.0001$). This may propose its potential as a biomarker for monitoring treatment response.

Conclusion: Data suggests that increased expression of circRNA-cdr1as and circRNA-000284, along with decreased expression of circ-ITCH, are observed in breast cancer patients. These expression changes are associated with poor prognosis and prediction. Additionally, the expression level of circRNA-000284 significantly decreases after chemotherapy, indicating its potential as a candidate marker for studying chemotherapy response. these findings provide valuable insights for the development of novel strategies in the management and treatment of breast cancer. However, further studies are required to validate the clinical utility of these circRNAs as biomarkers and to explore their underlying mechanisms in breast cancer progression.

Keywords: Breast cancer, circRNAs, Biomarkers, Chemotherapy, Prognosis

INTRODUCTION:

Circular RNAs (circRNAs) are a unique magnificence of RNA molecules characterized by the aid of their closed-loop shape, which makes them more stable and resistant to degradation as compared to linear RNAs [1]. CircRNAs were thought to be splicing byproducts with no functional significance, but recent advances in RNA sequencing have discovered that they are abundant in a variety of organisms and tissues. CircRNAs can originate from coding and non-coding genes and play numerous roles in biological strategies [2]. They can regulate gene expression, RNA splicing, translation, and protein interactions. CircRNAs can also act as miRNA sponges or competitive endogenous RNAs and affect the expression of target genes. In addition, circRNAs can interact with RNA-binding proteins and influence their affinity and localization within cells [3]. Some of the circRNAs have been found to undergo translation, challenging the traditional assumption that circular RNAs are exclusively non-coding entities [4].

Dysregulation of circRNAs has been related to various human diseases, which include most cancers, neurological disorders, and cardiovascular sicknesses [5-7]. In most cancers, circRNAs can contribute to tumor initiation, progression, and metastasis, functioning as both oncogenes or tumor suppressors [4]. The specific localization of circRNAs, together with their high abundance, strong expression in unique body fluids and tissues, and precise expression at developmental or tissue levels, make them promising candidates as biomarkers for diagnostic and therapeutic applications [8].

Various circular RNAs (circRNAs) have shown potential as biomarkers in different types of cancer. For example, in laryngeal squamous cell cancer (LSCC) [9], gastric cancer [10], hepatocellular carcinoma [11, 12, 13], colorectal cancer [14-19], and breast cancer[20]. Circular RNAs also have potential as therapeutic goals in most cancers. They act as miRNA sponges, regulating gene expression [21, 22,23]. []. Microarray analysis has discovered forty-one circ RNAs with substantially altered expression in the plasma of breast cancer patients, suggesting their capability as diagnostic markers [24]. Among them, we selected 3 circular RNAs, named CDR1as, Circular RNA-000284, and Cir-ITCH, for additional studies. CDR1as (CiRS-7), a circular RNA derived from CDR1 gene positioned in Xq27.1 indirectly regulates miR-7 target genes concerned in cancer improvement and development [25]. CDR1as, a circular RNA, is overexpressed in various cancers, along with breast, lung, gastric, hepatocellular carcinoma, and colorectal cancers. It promotes cancer proliferation, migration, invasion, metastasis, and resistance to therapy [24]. CDR1as interacts with microRNAs and RNA-binding proteins, revealing its complicated function in cancer [25].

Circular RNA-000284, derived from exon 2 of the HIPK3 gene positioned in chromosome 11p13, is upregulated in cancer acts as a miRNA sponge for miR-506, and suppresses the expression of the Snail-2 gene to improve the invasion in endometrial cancer [26]. Cir-ITCH, (circular RNA itchy E3 ubiquitin protein ligase), derived from the ITCH gene, located at locus 20q11.22 and spans exons 6 to 13, regulates protein stability and plays a role in cancer development [27]. It is downregulated in colorectal [28] and lung [29] cancers but is related to better survival in hepatocellular carcinoma [30]. In bladder cancers, Cir-ITCH interacts with miR-17, miR-224/p21, and PTEN as a tumor suppressor [31]. In thyroid cancers, it creates a signaling pathway concerning circ-ITCH/miR-22-3p/CBL/β-catenin [32]. Additionally, in ovarian cancers, it inhibits cancer using interacting with miR-145 and affecting the RAS1 pathway [33]. CircRNAs, as a type of biomarker, play an important role in breast cancer chemotherapy monitoring. They help measure the cancer's response to treatment, identify patients who are likely to respond, monitor disease progression, and detect recurrence. CircRNAs provide benefits over conventional biomarkers, they are fantastically strong and specific to unique types of breast cancer [34]. Using circRNAs as monitoring biomarkers can result in more customized treatment alternatives and decrease healthcare prices. Overall, the identification of circRNAs as potential biomarkers for breast cancer chemotherapy monitoring is a promising development [35].

This study aims to examine three circular RNAs (Cdr1as, circRNA-000284, and Cir-ITCH) in the peripheral blood of breast cancer patients before and after chemotherapy. It will be the first time these circRNAs are investigated in this context. The objective is to understand the relationship between each candidate circRNA and breast cancer, as well as their association with treatment response following chemotherapy. The study hopes to lay the groundwork for future research on non-invasive biomarkers for investigating and monitoring treatment response.

Methods and Material:

Sampling

Thirty blood samples were collected from breast cancer patients before and after chemotherapy at Imam Khomeini Hospital in Tehran, Iran. Additionally, a control group of thirty blood samples was collected from healthy individuals. The patients, who were diagnosed with ductal carcinoma of the breast, had not initiated any treatment at the time of sampling. The age range of the patients was 32 to 82 years old (Table 1). The inclusion criteria for the normal control group samples were female participants within the age range of the patients, devoid of any history of breast disease or malignancy in both themselves and their first-degree relatives. Ethical considerations and informed consent forms were obtained for this study (IR.MODARES.REC.1398.148).

Chemotherapy

The treatment course for the patients in this study consisted of 4 to 7 sessions of chemotherapy. All patient blood samples were collected three weeks after receiving the first chemotherapy session. The patients in this study received the CMF treatment regimen, consisting of Cyclophosphamide, Methotrexate, and Fluorouracil, with doses of 800, 60, and 800 mg/kg, respectively.

RNA extraction, cDNA synthesis, and Quantitative real-time reverse transcription PCR (QReal-Time RT-PCR)

The extraction of total RNA from plasma samples, both before and after chemotherapy, as well as from healthy individuals, was carried out using RNX-Plus solution (CinnaClone-Iran) following the manufacturer's instructions. The concentration of the extracted RNA was measured using a NanoDrop ND-ONE instrument (Thermo-USA). For plasma preparation, peripheral blood samples (3–4 mL) were collected into EDTA tubes. The tubes were then centrifuged at 1000 g for 15 minutes within 30 minutes of collection. Subsequently, 1 mL of plasma was transferred to a 1.5-mL Eppendorf tube and centrifuged at 11,000 g for 10 minutes to remove any remaining cellular debris.

For cDNA synthesis, the YT4500 cDNA Synthesis Kit (Yekta Tajhiz Azma-Iran) using random hexamer primers was utilized. The Beta-actin gene served as an internal control and normalization factor. The primer specifications and sequences (divergent primers) can be found in Table 2. In this study, the CircInteractome software was utilized for the design of primers targeting circular RNAs. The software, available at https://circinteractome.nia.nih.gov/divergent_primers.html, was employed to facilitate the primer design process for circular RNAs.

To analyze the expression of circRNAs and Beta-actin, thermal cycling conditions were as follows: initial denaturation at 95°C for 5 minutes, followed by 40 cycles at 95°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds. Each data point was tested in duplicate, and negative test controls were included in every reaction.

The relative expression was quantified in the presence of an appropriate internal control, beta-actin. The efficiency of each primer set for the experimental targets and reference gene was calculated using LinRegPCR software (approximately 100%). Comparative relative quantification was performed using the $2^{-\Delta\Delta CT}$ method, and the data were presented as the fold change in gene expression normalized to an endogenous reference gene (beta-actin) relative to the controls. In this study, RNA expression that was two-fold or higher was considered upregulated, expression between 0.5 and 2-fold was considered normal, and expression that was 0.5-fold or lower was considered downregulated.

Statistical Analyses

The obtained results were analyzed using Graph Pad Prism software version 8.2. Since the data did not follow a normal distribution, non-parametric tests were employed for statistical analysis.

To compare circRNA expression between blood samples collected before and after chemotherapy, the non-parametric Mann-Whitney test was used. Additionally, the non-parametric Mann-Whitney test and the independent samples Kruskal-Wallis test were used to compare circRNA expression among cancer samples with different clinical-pathological and molecular indices and to assess the association between tumor molecular and clinical-pathological indices with circRNA expression. The significance level was set at $P<0.05$. Results were reported as mean ± standard deviation.

Results:

Differential Expression of studied circRNAs in Breast Cancer Patients

Based on the results shown in Figure 1, it is evident that the mean expression levels of circRNA-Cdr1as and circRNA-000284 before chemotherapy were significantly higher (9.62 ± 3.43 and 2.01 ± 1.29 times, respectively) compared to the normal control group. This indicates a noteworthy upregulation in their expression. In contrast,

Table 1. Characteristics of breast cancer patients and controls.

	Patient N (%)	Control N (%)
number	30	30
Age (years)		
Mean	43.6±10.7	46.8± 11.4
Range	32-82	24-80
Stage at diagnosis		
Stage I	4 (13.3%)	
Stage II	17 (56.7%)	
Stage III	7 (23.3%)	
Stage IV	2 (6.7%)	
Lymph node status		
N0	14 (46.6%)	
N+	16 (53.4%)	
Distance metastasis		
yes	2 lung (6.7%)	
No	28 (93.3%)	
Hormone receptor status (IHC)		
ER positive	17 (56.6%)	
ER negative	13 (43.4%)	
PR positive	19 (63.3%)	
PR negative	11 (36.7%)	
HER-2 status (IHC)		
+++	9 (30%)	
Negative	21 (70%)	
Triple-negative breast cancer	3 (10%)	
Non- triple negative	27 (90%)	

Table 2. The primer sequences for circRNAs and beta-actin

	Primer sequence	Annealing temperature	Amplicon length
CircRNA-Cdr1as	F: 5′-AGACCTTGAGATTATTGGAAGACT TGA-3′ R:5'- TAC CCA GTC TTC CAT CAA CTG GCT -3′	57	128
CircRNA-000284	F:5'- TATGTT-GGTGGATCCTGTTCGGCA -3′ R:5′- TGG-TGGGTAGACCAAGACTTGTGA -3′	57	134
Circ-ITCH	F:5'- GCAGAGGCCAACACTGGAA-3′ R:5′- TCCTTGAAGCTGACTACGCTGAG-3′	57	150
Beta actin	F:5'- GAGACCTTCAACACCCCAGC-3′ R:5′- AGACGCAGGATGGCATGG-3′	57	161

circ-ITCH exhibited a significant downregulation in expression (0.48 ± 0.31) compared to the control group ($p<0.0001$).

Expression Analysis of circRNAs in Breast Cancer Patients Before and After Chemotherapy

Figure 2 presents the mean expression levels of all three circRNAs before and after chemotherapy. The figure demonstrates a significant reduction in the expression levels of circRNA-Cdr1as and circRNA-000284 following chemotherapy ($p<0.0001$). However, there was no statistically significant difference in the expression level of circ-ITCH before and after chemotherapy. Notably, the decrease in the expression of circRNA-000284 after chemotherapy was substantial. The expression level of circRNA-Cdr1as decreased from 9.59±3.48 before chemotherapy to 6.35±2.30 after chemotherapy. Similarly, the expression levels of circRNA-000284 before and after chemotherapy were 2.14±1.20 and 0.84±0.39, respectively, indicating an approximately 2.54-fold decrease. Conversely, the expression levels of circ-ITCH before and after chemotherapy were 0.48±0.31 and 0.53±0.78, respectively, without a statistically significant difference ($p > 0.05$).

The Association between Different Expression Levels of circRNAs and Clinical Status

As summarized in Table 3, changes in the expression of all three studied circRNAs are associated with lymph node involvement and higher cancer stages. Specifically, an increase in the expression of circRNA-Cdr1as and circRNA-000284 is observed in severe conditions, i.e., higher cancer stages and more lymph node involvement. Conversely, a greater decrease in the expression of circ-ITCH is noticeable in higher cancer stages, greater lymph node involvement, and triple-negative breast cancer. In other words, these changes in expression serve as poor prognostic markers.

Discussion:

Biomarkers play an important role in the diagnosis and treatment of specific types of cancer, including breast cancer. They are useful in determining the prognosis and individuals at risk in susceptible families, choosing the appropriate treatment, monitoring the progress after surgery, and evaluating the effectiveness of treatment in advanced stages. Recently, blood-based biomarkers have been considered a promising approach for cancer diagnosis and treatment [36]. These biomarkers can be evaluated in various biological samples for diagnosis, prognosis, and monitoring of treatment effectiveness. The difference in biomarker expression between healthy and diseased individuals serves as an important indicator to evaluate the importance of the biomarkers. As a result, various DNA, RNA, and protein biomarkers have been introduced to be used as cancer-related biomarkers at different stages of the disease [37]. CircRNAs have

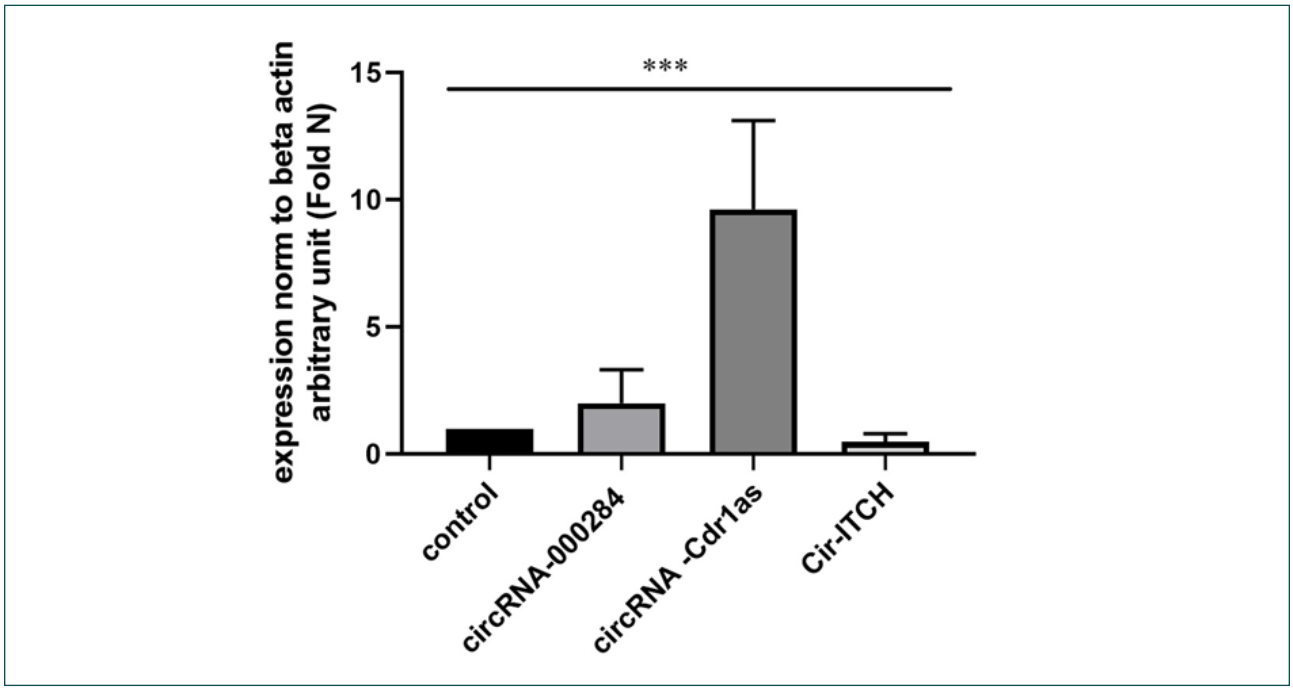

Figure 1. Comparison of the mean expression levels of circRNA-Cdr1as, circ-ITCH, and circRNA-000284 in breast cancer patients in comparison to the normal control group before chemotherapy.
Statistical analysis was performed using the Kruskal-Wallis test. P-value < 0.00

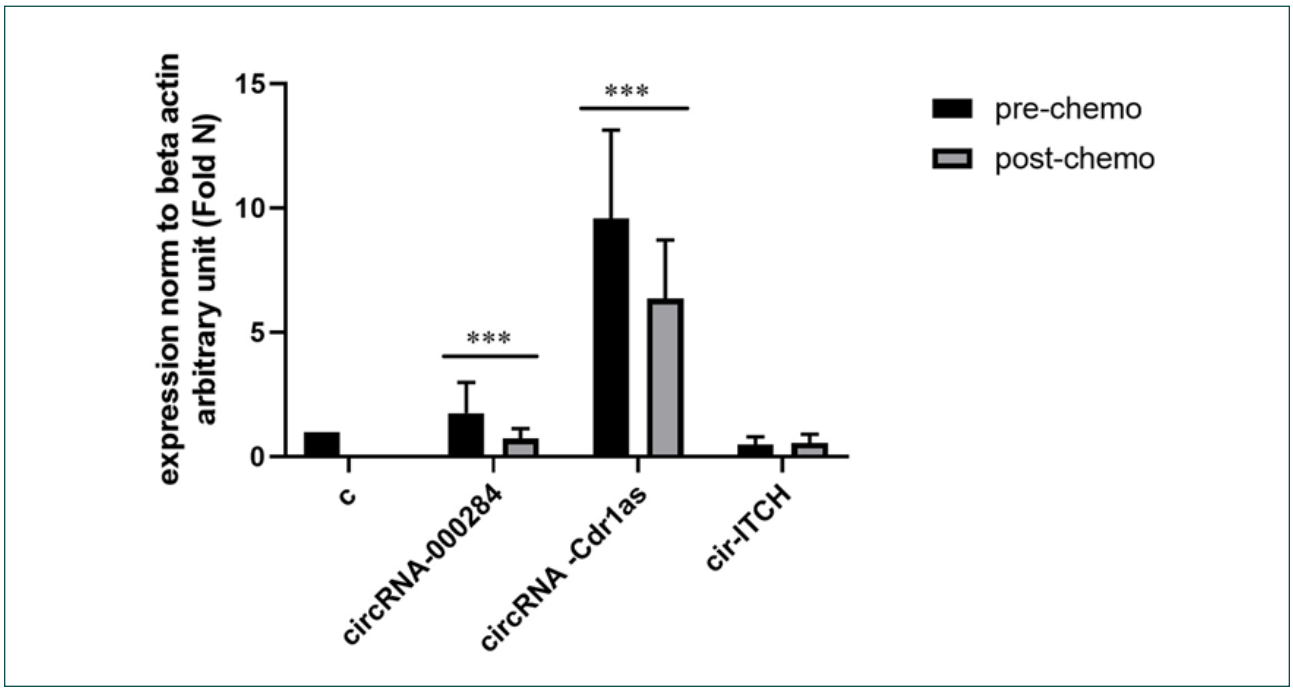

Figure 2. Comparison of the mean expression levels of circRNA-Cdr1as, circ-ITCH, and circRNA-000284 in breast cancer patients before and after chemotherapy.
Statistical analysis was performed using the Mann-Whitney test.
*** represents a highly significant difference ($p<0.0001$) between the groups

Table 3. Correlation of circRNAs Expression with Hormone Receptor Status and Cancer Stages in Breast Cancer Patients

P value	Statistical test	Mean of circ-ITCH exp. ± SD	Mean of circRNA000284 exp. ± SD	Mean of circ-cdr1as exp. ± SD	Various factors
>0.05	Mann-Whitney	0.460.24±	1.851.15±	9.63.67±	ER^+
0.45±0.16		1.96±1.21	9.583±..2	ER^-	
>0.05	Mann-Whitney	0.41±0.19	1.85±1.15	9.35±3.58	PR^+
0.46±0.25		1.96±1.21	9.97±3.28	PR^-	
>0.05	Mann-Whitney	0.47±0.15	1.4±0.62	9.49±3.61	$HER2^+$
0.42±0.24		1.13±1.10	9.64±3.42	$HER2^-$	
>0.05 for cdr1as & circRNA000284	Mann-Whitney	0.11±0.01*	1.84±0.75	9.64±4.66	Triple-negative
0.56±0.28*		1.91±1.22	9.59±3.32	Non-triple negative	
<0.0001 for circ-ITCH					
<0.0001	Mann-Whitney	0.38±0.21*	2.34±1.40*	11.58±2.96*	Lymph node +
0.68±0.30*		1.34±0.31*	6.78±1.84*	Lymph node -	
<0.0001	Kruskal-Wallis	1.15±0.22*	0.97±0.22*	3.8±0.81*	Stage 1
0.62±0.12*		1.39±0.23*	7.88±0.57*	Stage 2	
0.38±0.15*		2.41±1.23*	11.6±2.31*	Stage 3	
0.11±0.01*		4.43±1.34*	16.57±0.57*	Stage 4	

attracted attention as a unique diagnostic agent due to their molecular structure, which makes them resistant to degradation and allows them to persist in body fluids. In this study, we examined the expression of three circRNAs (circRNA-Cdr1as, circRNA-000284, and circ-ITCH) in the peripheral blood of breast cancer patients before and after chemotherapy. We also explored the correlation between circRNA expression and various histopathological features, such as receptor type and cancer stage.

The use of a blood sample in this research method provides a suitable screening option through a simple blood test. Our study showed significant differences in circRNA expression between breast cancer patients before and after chemotherapy as well as compared to normal subjects. These differences in circRNA expression retain potential as indicators of breast cancer pathogenesis. Several studies have investigated circRNAs and their

involvement in cancer. For example, hsa_circ_0025202 has been found to act as a sponge for miR-182-5p and regulate the expression and activity of FOXO3a. Functional studies have shown that hsa_circ_0025202 exerts tumor suppressive and tamoxifen-sensitive effects through the miR-182-5p/FOXO3a axis. Intratumoral experiments have also confirmed its ability to inhibit tumor growth and increase the efficacy of tamoxifen. Interestingly, hsa_circ_0025202 plays an anti-angiogenic role in HR-positive breast cancer and could potentially serve as a novel biomarker for tamoxifen-resistant breast cancer [38]. CircRNA-ABCB10 has been discovered to play a unique role in breast cancer tumorigenesis and act as a regulator through the miR-1271 sponge. This finding shed light on the understanding of breast cancer pathogenesis [39]. High expression of circRNA_100876 is strongly associated with negative prognosis in breast cancer patients. In addition, circRNA_100876 is involved in promoting breast cancer metastasis by affecting the expression of microRNA-361-3p and increasing the proliferation capacity [24]. Another study shows that CircRNA_069718 contributes to the progression of triple-negative breast cancer (TNBC) through the Wnt/β-catenin pathway. This finding suggests that CircRNA_069718 could be a potential therapeutic target for the treatment of TNBC [40]. Our study shows the differential expression of circRNAs in different stages of breast cancer, with an increasing trend as the disease progresses and reaches its peak in stage four. Classification based on hormone receptors showed no significant association between expression of circRNA-Cdr1as, circRNA-000284 and hormone receptors (ER, PR, HER2) or triple-negative breast cancer. However, a significant decrease in circ-ITCH expression was observed in triple-negative patients compared to other patients. Lymph node involvement also affects circRNA expression, with lymph node involvement increasing the expression of circRNA-Cdr1as and circRNA-000284, and decreasing the expression of circ-ITCH compared to non-involvement. In our study, examining the expression of circRNA-cdr1as in breast cancer patients showed a significant increase in its expression in cancer blood samples compared to normal samples (p-value: <0.0001). Recent studies have shown the role of Cdr1as as a miRNA sponge in various diseases. In hepatocellular carcinoma, Cdr1as targets miR-7 and promotes hepatic vascular invasion [41]. In bladder cancer, Cdr1as is downregulated in cancer cells compared to normal tissues. Overexpression of Cdr1as inhibits the proliferation and invasion of bladder cancer cells by regulating p21 expression through the miR-135a sponge [25]. In colorectal cancer (CRC), Cdr1as is upregulated and its upregulation inhibits tumor cell proliferation and invasion. Cdr1as promotes CRC progression by blocking miR-7 and upregulating EGFR and IGF-1R expression [42]. In addition, Cdr1as expression has been reported in various cancers including GBM (glioblastoma multiforme), NRBL (neuroblastoma), SARC (sarcoma), SECR (carcinoma), BRC (breast cancer) and SKCM (melanoma) [43].

CircRNA-000284 promotes cell proliferation and invasion in cervical cancer by sponging miR506 and suppresses Snail-2 [26].

Another circular RNA, ITCH circRNA, was investigated in this study. ITCH circRNA acts as a miRNA sponge and is believed to increase ITCH gene expression while degrading Dvl2 through ubiquitination. This ultimately characterizes the Wnt-directed pathway in particular. In this situation, it is possible to help the initiation and development of cancer. ITCH is involved in the proteasomal degradation of various substrates such as p63, p73, Notch1, and Dvl2. It has been reported to be associated with tumor formation and chemotherapy. In colorectal cancer, Cir-ITCH expression was significantly decreased in cancerous tissue compared to normal tissue. Its role in the regulation of ITCH expression and its implementation in carcinogenesis was highlighted through interference with the β-catenin Wnt guidance pathway [28]. Similar findings were observed in lung cancer [29]. In hepatocellular carcinoma, high expression of Cir-ITCH was associated with improved patient survival rate, confirming its inhibitory role in HCC [30]. In bladder cancer, Cir-ITCH plays a tumor-suppressive role through interaction with miR-17, miR-224/p21, and PTEN [31]. In addition, a novel signaling pathway, circ-ITCH/miR-22-3p/CBL/β-catenin, was identified in cancer development and progression [32]. In ovarian cancer, the inhibitory role of Cir-ITCH in cancer was reported through interaction with miR-145 and its effect on RAS1 signaling pathway [33]. Determining the potential of tumor metastasis is very important in the treatment of patients with breast cancer. Clinical variables such as lymph node involvement, tumor histological grade, and receptor status (estrogen, progesterone, and HER2) influence the probability of metastasis. These prognostic factors are associated with proliferation, mitotic index and clinical behavior of invasive breast cancer [44].

Chemotherapy response monitoring is very important in cancer management. Our study showed that the expression of circRNA-cdr1as was significantly increased in blood samples of patients compared to normal samples, but decreased after chemotherapy. Similarly, the expression of circRNA-000284 was increased in patients' blood samples and decreased after chemotherapy. Monitoring the expression level of circRNA after chemotherapy may allow us to assess the 5-year survival rate of patients.

While the expression of circ-ITCH was decreased in breast cancer patients compared to the control group, no statistically significant difference was observed in its expression level before and after chemotherapy. Regarding lymph node (LN) involvement, the change in the expression of studied circRNAs in patients with lymph node involvement was more than in those without LN involvement. Disease stage is a prognostic indicator in breast cancer, reflecting tumor spread at diagnosis. Comparing the mean expression levels of these circRNAs in patients in stages one to four, as well as in patients with and without distant metastasis, strengthens the possibility that circRNA-cdr1as and circRNA-000284 act as onco-circs. The mean expression of circRNAs was significantly increased in patients with distant metastasis and advanced stages (three and four), indicating their association with invasive and metastatic breast cancer.

Conclusion:

The use of circRNAs in investigating cancer at different stages could be a suitable option for screening tests. These molecules have the potential to become molecular biomarkers in breast cancer management. Data from this study suggests that increased expression of circRNA-cdr1as and circRNA-000284, along with decreased expression of circ-ITCH, are observed in breast cancer patients. These expression changes are associated with poor prognosis and prediction. Additionally, the expression level of circRNA-000284 significantly decreases after chemotherapy, indicating its potential as a candidate marker for studying chemotherapy response.

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Conflict of Interests:

No conflict of interests was declared by all authors.

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