University of Sistan and Baluchestan

Journal of Epigenetics

Online ISSN: 2676 - 4350

Homepage: https://jep.usb.ac.ir

Iranian Genetics Society

The Potential Role of hsa_circ_0004214 as a Biomarker for Breast Cancer Prognosis

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ARTICLE INFO

Article type:
Research Article

Article history:
Received:9 April 2024
Revised:1 June 2024
Accepted:22 September 2024

Keywords:
Neoplasia,
CircRNA,
Biomarker,
Breast Cancer.

A B S T R A C T

Breast cancer is the most important cause of cancer-related death among women. In this respect, finding cancer management biomarkers may improve cancer management strategies, patient outcomes, and quality of life. Recently, circular RNA (circRNA) expression has attracted huge attention in cancer management. CircRNAs have a covalently closed loop structure that makes them resistant to exonuclease degradation and more stable than their linear counterparts. The present study investigates the potential of hsa_circ_0004214 as a circRNA derived from the angiomotin gene on chromosome 11 as a candidate possible prognostic biomarker.

SYBR Green real-time reverse transcription–polymerase chain reaction (RT-PCR) was used to assess the expression level of hsa_circ_0004214 in 40 breast ductal carcinoma tumors and whole blood samples from breast cancer patients, compared to the match control samples. Afterward, the association between the expression levels of this circRNA and the clinical characteristics of breast cancer patients was assessed.

The findings indicate that hsa_circ_0004214 expression elevated in triple-negative cases and at higher stages of the disease ($P< 0.05$).

The hsa_circ_0004214 can be proposed as a potential biomarker for breast cancer prognosis. Overall, this biomarker can be proposed for use in clinical settings, considering its elevated expression in advanced breast cancer situations. However, further research is warranted to establish its prognostic value and potential implications in breast cancer management.

Introduction

Breast cancer (BC) is the leading cause of cancer-related deaths among women (Arnold et al., 2022). In Iran, BC ranks first among malignancies diagnosed in females, comprising 24.4% of all cancers with an age-standardized rate (ASR) of 23.1 per 100000 (48, 49). Also, it is the fifth most common cause of death related to cancers. According to the new statistics in Iran, 6160 breast malignancies are diagnosed annually in the country, of which 1063 cases cause death (Farhood et al., 2018). The most well-defined environmental risk factors causing BC are radiation exposure and alcohol ingestion. Diet is related to the increased incidence of breast cancer in developed countries, although its precise role is not yet established (Rodgers et al., 2018). The progression of breast cancer involves a complex interaction of genetic interactions that cause the activation of oncogenes in primary tumors. These genomic changes have the potential to produce cancer, promote tumor progression, and initiate metastasis. Early detection of breast cancer and the prognosis of

DOI: https://doi.org/ 10.22111/JEP.2024.48402.1072

Publisher: University of Sistan and Baluchestan

How to Cite: Beikzadeh, M., Saliml, M. (2024). The Potential Role of hsa_circ_0004214 as a Biomarker for Breast Cancer Prognosis. *Journal of Epigenetics*, 5(2), 16-23. https://doi.org/ 10.22111/JEP.2024.48402.1072

cancer status are very important in terms of improving the patient's survival rate and treatment outcomes (Wilkinson et al., 2022). Biomarkers have emerged as a key approach in diagnosing and prognosis of various diseases (Zhang et al., 2018).

A biomarker is a measurable indicator of biological states or conditions with clinical applications, aiding diagnosis and treatment decisions. Biomarkers should be measurable, specific, sensitive, reproducible, and valid. Besides, they must cover a dynamic range, non-invasive, and have clinical utility (Califf et al., 2018). Any substances (whether metabolites or molecules) that change in response to the creation and development of cancer can be considered biomarkers. Among these are proteins, DNA, RNA, metabolites in body fluids and tissue samples. Cancer biomarkers are used in various fields, including cancer management, diagnosis, prognosis, treatment selection, and monitoring (Sarhadi et al., 2022). Circular RNAs (circRNAs) are a type of non-coding RNA molecule that form closed-loop structures (Liu et al., 2020). Unlike linear RNAs, which have distinct start and end points, circRNAs form continuous loops by joining the ends of the RNA molecule together. This circular structure provides circRNAs stability and resistance to degradation by cellular enzymes called RNases (Liu et al., 2020; Nisar et al., 2021).

There are different types of receptors in breast cancer, such as the Estrogen Receptor (ER), Progesterone Receptor (PR), Human Epidermal Growth Factor Receptor 2 (HER2), and Androgen Receptor (AR). These receptors significantly impact the growth and progression of the disease. ER and PR respond to estrogen and progesterone hormones, respectively, contributing to the proliferation of cancer cells in hormone receptor-positive breast cancers. HER2 is a protein involved in cell growth regulation. When overexpressed, this protein can drive aggressive growth in HER2-positive breast cancers. The role of AR, found in a subset of breast cancers, is less well-understood but holds the potential to influence cancer growth. Understanding the presence and status of these receptors is crucial in guiding treatment decisions and predicting prognosis in breast cancer patients. Hormone receptor-positive cancers can be effectively targeted with hormone therapies. Meanwhile, HER2-positive cancers may benefit from targeted treatments against HER2, highlighting the importance of receptor status in personalized breast cancer management (Wei et al., 2023).

In addition, circRNAs have important biological functions, including regulatory roles in cellular processes, which accomplish this work in different ways. Among them, miRNAs can be inhibited by sponging in special binding sites by circRNAs. Moreover, circRNAs can interact with other proteins that bind to mRNA and affect the expression regulation at two levels of transcription and post-transcription. Besides, they can affect processes such as alternative splicing, mRNA stability, and translation (Gopikrishnan et al., 2023).

Various evidences indicate that circRNAs are involved in various biological processes, including development, cell differentiation, and disease (Gopikrishnan et al., 2023). In this respect, circRNAs have been reported in the pathogenesis of several diseases, including cancer (Kristensen et al., 2022), cardiovascular diseases (Singh et al., 2023), and neurological disorders (Wang et al., 2018). Shreds of evidence of their role in tumor growth, metastasis, and drug resistance have also been reported, making them potential targets for diagnosis and treatment (Guo et al., 2022).

In the context of breast cancer, circRNAs affect various fields, including the genesis of cancer, its progression, the occurrence of special features, and the characteristics of the disease. Research has shown that circRNAs regulate the proliferation, invasion, and metastasis of cancer cells in their progression. Accordingly, identifying cancer-related circRNAs in body fluids, especially blood, holds promise for non-invasive breast cancer diagnosis. Using these circRNAs as new biomarkers can help in the early detection of breast cancer and cancer management strategies (Li et al., 2019).

Considering the importance of identifying biomarkers associated with breast cancer, finding new biomarkers with different clinical values in cancer management is crucial. In this study, we investigated the expression level of hsa circ 0004214 (circAmotl1) derived from the angiomotin gene and located on chromosome 11 in the tissues and blood of both healthy individuals and breast cancer patients. This study aims to assess and track this circular RNA in the free nucleic acids in the whole blood to find possible candidate biomarkers associated with breast cancer.

The rationale for selecting hsa_circ_0004214 as the focus of this research stems from two main factors. First, our literature review revealed an upregulation of its expression in cervical cancer (Sun et al., 2020) and glioma (Zhou et al., 2023), suggesting a potential role in cancer development. Second, the novelty of studying hsa_circ_0004214 in breast cancer arises from the absence of prior reports on its involvement in this specific cancer type. Hence, considering the shared hormonal interactions in females between cervical cancer and breast cancer further justifies exploring the potential impact of hsa_circ_0004214 in breast cancer pathogenesis.

Materials and Methods

Sampling

During the years 2019 to 2021, 40 breast tumor tissues and 40 normal adjacent breast tissues, along with 40 whole blood samples from breast cancer patients and 40

whole peripheral blood samples from normal individuals, were collected at Imam Khomeini Hospital. Blood samples were transferred to the laboratory in tubes containing EDTA, and tissue samples were transferred in nitrogen liquid. All patients signed a consent form, and the sampling was carried out following the Helsinki Declaration (52d/4922, 6.10.2016).

All samples were obtained from patients diagnosed with ductal carcinoma of the breast. Inclusion criteria were having access to the patient's medical records and pathological reports and having tumor tissue and whole peripheral blood samples.

Table 1 presents the clinical and demographic characteristics of both the patients and the control group.

Table 1- Table 1. Clinical characteristics of the specimens

	Patient N (%)	Control N (%)
number	40	40
Age (years)		
Mean	43.6±10.7	46.8± 11.4
Range	32-82	24-80
Stage at diagnosis		
Stage I	4 (8%)	
Stage II	18 (46%)	
Stage III	16 (41%)	
Stage IV	2 (5%)	
Lymph node status		
N0	19 (47.5%)	
N+	21(52.5%)	
Distance metastasis		
yes	2 lung (5%)	
No	38 (95%)	
Hormone receptor status (IHC)		
ER positive	23 (57.5%)	
ER negative	17 (42.5%)	
PR positive	25 (62.5%)	
PR negative	15 (37.5%)	
HER-2 status (IHC)		
+++	12 (30%)	
Negative	28 (70%)	
Triple-negative breast cancer	4 (10%)	

IHC: Immunohistochemistry, ER: Estrogen receptor, PR: Progesterone receptor, HER2: Human epidermal growth factor receptor 2

RNA extraction, cDNA synthesis

Following the manufacturer's instructions, total RNA from the whole blood and breast tissues was extracted using RNX-Plus reagent (Cat. No. : EX6101). Both blood and tissue samples were processed using the RNX-Plus method with minor adjustments following the manufacturer's protocol. During RNA isolation, the Guanidine salt facilitated the simultaneous precipitation of DNA and proteins in the phenol phase from the homogenized sample. The resulting aqueous phase contained high-quality genomic RNA of all types. The extracted RNAs were quantified using the NanoDrop ND-ONE instrument (Thermo-USA). Complementary DNA (cDNA) was generated using the YT4500 cDNA Synthesis Kit (Yekta Tajhiz Azma, Cat No: YT4500). The kit offers high-quality reagents designed to facilitate effective first-strand cDNA synthesis from mRNA or total RNA templates. In this process, M-MuLV is used as a reverse transcriptase enzyme that exhibits optimal activity at 37℃ and lacks RNase H activity, starting from random hexamer primers.

Real-Time Reverse Transcription PCR (Q-RT-PCR)

Hsa_circ_0004214 expression was assessed by Comparative Real-Time RT-PCR using the SYBR Green qPCR Master mix 2x (Yekta Tajhiz Azma, Iran) on the Corbett Rotor-Gene 6000 instrument (QIAGEN, USA). In this research, the *Beta-actin* gene was used to ensure normalization and internal control. The specificities and sequence details of the distinct primers used in this study are outlined in Table 2. Thermal cycling conditions were applied to analyze the expression of circRNA and Beta-actin. This process involved an initial denaturation step at 95℃ for 5 minutes, followed by 40 cycles at 95℃ for 30 seconds, 57℃ for 30 seconds, and 72℃ for 30 seconds. Each data point was tested in triplicate, with negative test controls included in each reaction. The efficiency of each primer set for both the experimental target and reference gene was determined using LinRegPCR software (approximately 100%). Subsequently, the $2^{-\Delta\Delta CT}$ method was used for comparative relative quantification. In this method, the data are expressed as the fold change in gene expression normalized to an endogenous reference gene (Beta-actin) and relative to the control samples. RNA expression showing a fold change of two or more was classified as upregulated. Accordingly, an expression between 0.5 and 2-fold was considered normal, and 0.5-fold or less was deemed downregulated.

Table 2. Primer sequences for circRNA and *Beta-actin*

Circular RNA name	Primer sequence	Annealing temperature (℃)	Amplicon length
hsa_circ_0004214	F: 5′- GAG ATG GTC AAG CCC TAC -3′ R:5'- TCG GAA CCT CTC ATT TCC -3′	57	165
Beta-actin	F:5'GAGACCTTCAACAC CCCAGC-3′ R:5′- AGACGCAGGATGGCAT GG-3′	57	161

Characterization of hsa_circ_0004214 Expression in Patient Subgroups based on Clinical and Demographic Features

Patients were divided into different groups based on clinical and demographic characteristics shown in Table 1. The expression level of hsa_circ_0004214 was measured in each group and compared with the corresponding group. The investigated characteristics are age, stage of the disease, involvement or non-involvement of lymph nodes, metastasis or non-metastasis to distant sites, and the status of estrogen and progesterone hormone receptors and HER2 on the tumor surface. This information was obtained by studying the clinical files of the patients and using the pathology reports in the file.

Statistical analysis

Subsequently, the results were evaluated using GraphPad Prism 8.0.2 (California Corporation, USA). The Kolmogorov-Smirnov test was applied to determine the normal distribution of the data in each group. In statistics, a normal distribution refers to a symmetric bell-shaped curve where the data are evenly distributed around the mean, following a certain pattern. This distribution is also known as a Gaussian distribution. On the other hand, a non-normal distribution refers to a distribution where the data does not follow the typical bell-shaped curve of a normal distribution. Such a pattern could include distributions that are skewed to one side, have multiple peaks, or do not follow a specific pattern. When data do not follow a normal distribution, different statistical techniques (e.g., non-parametric tests) may need to be used for analysis. Since our data did not pass the normality test, non-parametric analysis methods were employed for statistical analysis. The non-parametric Mann-Whitney and Kruskal-Wallis tests were used to assess circRNA expression in various clinical, pathological, and molecular characteristics in cancer samples. The correlation between the studied circRNA expression in tumor and whole blood was measured using the Pearson correlation test. Data were expressed as mean ± standard deviation. Differences were deemed significant if $P < 0.05$.

Results

Expression Analysis of hsa_circ_0004214 in Breast Cancer Patients

Figure 1 indicates that the average expression of has_circ_0004214 in breast cancer patients' tumor tissue and blood samples, compared to the control group, is 1.6±0.67 and 1.1±0.52, respectively. However, for an expression level higher than twofold (i.e., as an indication of increased expression), hsa_circ_0004214 did not show a significant increase in breast cancer patients. It is worth noting that the observed difference in expression is statistically significant in breast tumors compared to the control ($p<0.0001$).

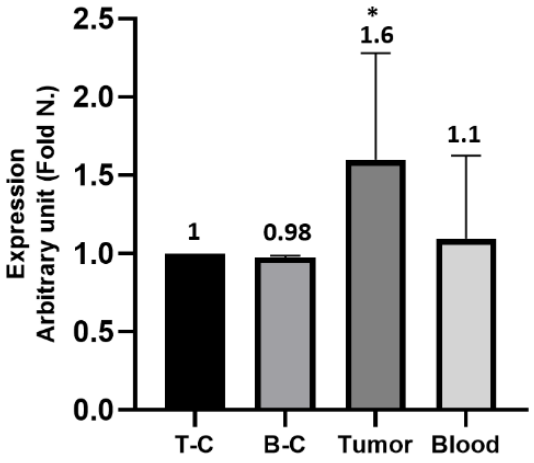

Figure 1. Comparison of the expression level of circ0004214 in tissue and blood samples of breast cancer patients compared to the control group; the results are reported as fold change relative to the control expression.
T-C: tumor samples control
B-C: Blood samples control
***: p<0.0001**

Furthermore, as depicted in Figure 2, 85% of the breast cancer patient samples exhibited normal expression of hsa_circ_0004214, while only 15% displayed more than twofold increased expression. The correlation between the expression of hsa_circ_0004214 in tissue and whole blood samples is illustrated in Figure 3.

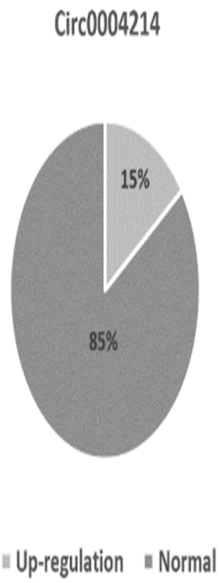

Figure 2. The expression status of Circ0004214 in tissue and blood samples of breast cancer patients
Up-regulation: Fold change expression ≥ 2
Normal: 0.5< Fold change expression<2
Figure 3. The correlation between the expression level of Circ0004214 in tissue and blood samples of breast cancer patients using the Pearson correlation test.

Differential Expression Analysis of hsa_circ_0004214 in Breast Cancer Patients Based on Clinical Characteristics

The expression levels of hsa_circ_0004214 were assessed in distinct patient groups, taking into account clinical attributes such as lymph node involvement, tumor receptor status, and disease stage. The findings (Table 3) demonstrate a noteworthy upregulation in hsa_circ_0004214 expression within both tumor tissue and blood samples derived from breast cancer patients. The results show the presence of lymph node involvement, advanced disease stages, and triple-negative receptor situation there. However, there is no other significant association between hsa_circ_0004214 expression and other mentioned characteristics such as age.

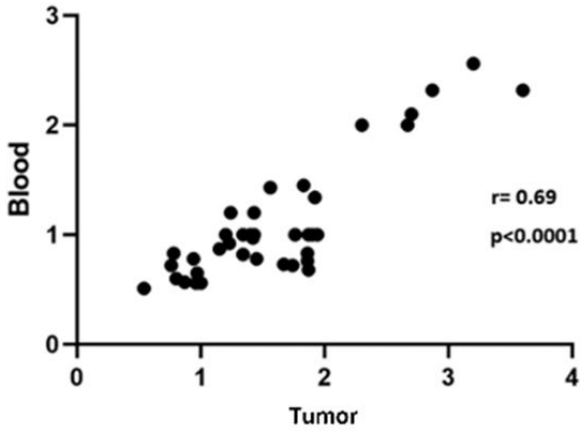

Figure 3. The correlation between the expression level of Circ0004214 in tissue and blood samples of breast cancer patients using the Pearson correlation test.

Differential Expression Analysis of hsa_circ_0004214 in Breast Cancer Patients Based on Clinical Characteristics

The expression levels of hsa_circ_0004214 were assessed in distinct patient groups, taking into account clinical attributes such as lymph node involvement, tumor receptor status, and disease stage. The findings (Table 3) indicate a substantial upregulation in hsa_circ_0004214 expression within both tumor tissue and blood samples derived from breast cancer patients presenting lymph node involvement and advanced disease stages and triple-negative receptor situation.

Table 3 . Comparative analysis of Circ0004214 expression in breast cancer samples

Variable factors	Circ0004214 exp. Mean ± SD Breast Tumor	P value	Circ0004214 exp. Mean ± SD Blood	P value
ER⁺	1.55 ±0.79	0.134	1.03 ±0.53	0.23
ER⁻	1.65±0.38		1.18±0.49	
PR⁺	1.59±0.56	0.7	1.02±0.48	0.23
PR⁻	1.60±0.81		1.20±0.65	
HER2⁺	1.46± 0.50	0.86	0.95± 0.42	0.65
HER2⁻	1.42±0.46		0.97±0.33	
Triple-negative	**3.04±0.38**	**0.032 ***	**2.24±0.21**	**0.013 ***
Non-triple negative	**1.43±0.47**		**0.96±0.37**	
Lymph node +	**2.32±0.85**	**0.04 ***	**2.12±0.51**	**0.02 ***
Lymph node -	**1.12±0.34**		**0.98±0.31**	
Stage 1	**1.61 ±0.23**	**<0.0008 *****	**1.25 ±0.18**	**<0.001 *****
Stage 2	**1.53±0.34**		**0.93±0.21**	
Stage 3	**1.12±0.34**		**0.77±0.17**	
Stage 4	**2.89±0.41**		**2.21±0.20**	

The study compares the expression levels of Circ0004214 in tissue and blood samples of breast cancer patients versus the control group across various clinicopathological conditions, presenting the results as fold changes relative to the control expression.

* $P<0.05$

** $P< 0.01$

*** $P<0.001$

Discussion

In the present study, we investigated the expression of hsa_circ_0004214 in tumor tissue and whole blood samples obtained from breast cancer patients, comparing them to normal control groups. Based on the obtained results, only 15% of patients exhibited a significant increase of more than two-fold in hsa_circ_0004214 expression. Notably, the upregulation of hsa_circ_0004214 was significantly observed in patients categorized as triple negative, lacking the hormone receptors ER, PR, and HER2, and in higher stages of the disease. The presence and activity of hormone receptors in breast tissue are fundamental in both normal and cancerous states. Estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) are key biomarkers used in breast cancer diagnosis and treatment decision-making. In normal breast tissue, these receptors play essential roles in regulating cell growth, differentiation, and function. However, in breast cancer, aberrant expression or signaling of these receptors can drive tumorigenesis and disease progression. Understanding the intricate interplay between hormone receptors and breast tissue biology is critical for developing targeted therapies and personalized treatment strategies for breast cancer patients (Mohanty et al., 2020).

Triple-negative breast cancer (TNBC) represents a subtype of breast cancer characterized by the absence of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) expression. TNBC is known for its aggressive nature, high rates of recurrence, and limited treatment options compared to other breast cancer subtypes. The lack of specific targeted therapies for TNBC poses a significant challenge in clinical management. Thus, exploring alternative treatment strategies such as chemotherapy and immunotherapy is necessary. Understanding the molecular and genetic features that drive TNBC development and progression is crucial for identifying novel therapeutic targets and improving patient outcomes in this challenging breast cancer subtype (Aysola et al., 2013).

Concerning diagnosis, treatment, and prognosis, triple-negative breast cancer presents several challenges. As mentioned earlier, lack of targeted therapies is among the major difficulties as this type of cancer does not have any hormone receptors or HER2 protein usually addressed by other sorts of cancer treatments. Such features prevent triple-negative breast cancer patients from having sufficient treatment options and contribute to its aggression, limited biomarkers, chemoresistance, and increased chances of metastasis (Luo et al., 2022; Agostinetto et al., 2022; Chakrabarty et al., 2022).

Improving our understanding of the molecular mechanisms underlying this subtype is crucial for developing targeted therapies and personalized treatment strategies. Previous studies have reported the diagnostic potential of hsa_circ_0004214 in glioma (Zhou et al., 2023). These authors found that hsa_circ_0004214 (i.e., a novel circular RNA) is important for the migration of tumors. By expanding the number of human glioma and control samples used in this study, they were able to analyze the expression of circRNAs that were independently detected in tissue samples from glioma patients. In addition, they analyzed the diagnostic value of hsa_circ_0004214 in different grades of glioma and evaluated the correlation between various pathological factors and hsa_circ_0004214. Besides, they preliminarily verified the value of hsa_circ_0004214 in judging the migration capacity of glioma cells in vitro and constructed the molecular network that hsa_circ_0004214 regulates the occurrence and development of tumors. Finally, for the first time, they identified that hsa_circ_0004214 is significantly upregulated in glioma and proposed it as a potential and stable biomarker to diagnose this cancer type. Altogether, these findings support that circRNAs play important roles in cancer and may serve as new strategies for diagnosis and treatment.

Some significant research findings reveal that circRNAs regulate miRNA activity as competing endogenous RNAs (ceRNAs) through base complementary pairing, influencing miRNA interactions with target genes

(Huang et al., 2020). Studies indicate that the upregulation of hsa_circ_0004214 is a common occurrence in Olp-associated Oral Squamous Cell Carcinoma (OSCC) (Bagley et al., 2022) and Cervical Cancer (CC) (Ou et al., 2020), suggesting a potential role as a molecular sponge for miRNAs in modulating gene expression within tumors through the miR-526b/SIK2 axis (Sun et al., 2020). Additionally, research has shown that circ Amotl1 (hsa_circ_0004214) enhances the invasive capacity of MDA-MB-231 breast cancer cells and promotes proliferation and resistance to serum-free conditions (Sadlak et al., 2023). These findings suggest that circ Amotl1 (hsa_circ_0004214) contributes to cancer progression through various mechanisms, including proliferation, survival, and apoptosis. Its altered expression has been observed in uterine cervical cancer (Sun et al., 2020; Ou et al., 2020) and Oral Squamous Cell Carcinoma (Liu et al., 2020). Therefore, it is inferred that they are potentially involved in tumor progression, migration, invasion, and cell proliferation. Further investigation into the role of circ Amotl1 (hsa_circ_0004214) in cancer development is warranted to better understand its molecular mechanisms and potential therapeutic applications. All these studies reported upregulation of hsa_circ_0004214 in cancerous tissues.

The present study, for the first time, investigated the expression of hsa_circ_0004214 in breast cancer patients using tumor tissue and whole blood samples. The findings showed that 15% of patients experienced a significant up-regulation of hsa_circ_0004214 expression, specifically in triple-negative cases and advanced stages. The correlation was observed between tumor and whole blood levels of hsa_circ_0004214. Blood biomarkers present several advantages over breast tumor biomarkers in clinical practice. They offer a minimally invasive sampling method, making them more patient-friendly compared to invasive procedures for obtaining tissue samples from breast tumors. Besides, blood biomarkers provide insights into systemic changes, offering a comprehensive view of disease progression and treatment response beyond localized markers.

Furthermore, they enable longitudinal monitoring, allowing for real-time assessment of disease status and treatment efficacy. The ease of monitoring and versatility of blood biomarkers make them valuable tools for early detection, prognosis, and personalized treatment approaches in breast cancer care (Oloomi et al., 2020). Triple-negative breast cancer presents unique difficulties due to the absence of targeted therapies, limited markers, and higher chances for metastasis. Therefore, comprehension of the molecular pathogenesis of TNBC is important in developing targeted therapies and individualized treatment approaches. To this end, it is necessary to research how hsa_circ_0004214 influences malignant transformation, tumor growth, and its migratory capacities and invasiveness during carcinogenesis. Such knowledge could improve our understanding of the tumorigenesis process at molecular levels, thus helping us develop newer methods of treatment for these neoplasms. Although the current understanding of circRNAs expression is still in its infancy, this research highlights its potential and guides the development of future methods for clinical evaluation.

Acknowledgements

We sincerely thank all the individuals who participated in this study. This project was supported by the National Institute of Genetic Engineering and Biotechnology (NIGEB).

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