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# Identification of miRNA-3652 Signature as a Promising Diagnostic Biomarker in FFPE Tissues of Breast Cancer Patients

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## ABSTRACT

Researchers have recently begun to investigate microRNAs (miRNAs) as a potential new class of biomarkers. In this study, we examine whether miR-3652 may be regarded as a powerful biomarker for predicting the prognosis of breast cancer (BC). In this study, the expression level of miR-3652 was detected in 30 FFPE tissue pairs of tumoral samples and their adjacent non-tumoral tissues using qRT-PCR. The clinicopathological characteristics of patients relative to miR-3652 expression level, along with fold change analysis employing the $2^{-\Delta\Delta CT}$ method, were also investigated. All statistical analyses were conducted using GraphPad Prism version 8.4.3 (686). We found that the miR-3652 level in BC FFPE tissues is notably increased compared to matched non-cancerous specimens. Also, we analyzed the association between the expression level of miR-3652 and the clinicopathological parameters of BC patients. The overexpression of miR-3652 exhibited no significant association with age, tumor stage, ER status, PR status, TNM stage, or calcification. All HER2-negative status patients suggested a potential trend (P = 0.017), indicating that low miR-3652 expression was more common in HER2-negative status subjects. Moreover, the p-value, AUC, Std. Error, sensitivity, and specificity are ($p < 0.018$, 0.6772, 0.06905, 0.53, and 0.80, respectively), which signifies a moderate capacity of the test to differentiate between patients with tumors and controls. The results of fold change analysis employing the $2^{-\Delta\Delta CT}$ method indicated a (6.89)-fold elevation in miR-3652 expression in tumors relative to controls. This suggests that miR-3652 may have an oncogenic role in carcinogenesis, potentially promoting BC development. In conclusion, our results demonstrated that elevated miR-3652 expression associates with the progression of BC, and this indicates that miR-3652 may possess an oncogenic function in tumorigenesis, possibly facilitating BC progression. Additional studies are required.

## 1.Introduction

Breast cancer is the most frequent carcinoma and the second most common cause of cancer-related mortality in women (Afifi et al., 2020, Hussen et al., 2023a). Its heterogeneous character is reflected in the classification into four intrinsic subtypes (luminal A, luminal B, basal-like, and ErbB2+), a normal-like group, and a new subtype, referred to as claudin-low (Russo and Russo, 2021, Dvorkin-Gheva and Hassell, 2014). Histologically, breast cancer can be divided into in situ and invasive carcinoma, both of which can be further subdivided into ductal and lobular (Cserni, 2020). Stratification by integrative clustering is based on genomic and transcriptomic data (Zhang et al., 2016).

MicroRNAs (miRNAs) are diminutive noncoding RNAs that disrupt mRNA translation by complementary base pairing to the 3′ untranslated region (UTR) of the target mRNA, resulting in either mRNA destruction or translational inhibition (Sumaira et al., 2024, Fernandez-Moure et al., 2014). Evidence indicates that miRNAs exert diverse cellular regulatory effects, with certain miRNAs acting as oncogenes or tumor suppressor genes (Svoronos et al., 2016, Otmani and Lewalle, 2021). Numerous studies have underscored the prognostic significance of miRNA-3652 in many malignancies, including ovarian cancer, indicating that elevated or diminished levels of miRNA-3652 are associates with adverse or favorable survival outcomes, respectively (Sathipati and Ho, 2021). These findings indicate that miRNA-3652 may function as a possible prognostic biomarker and underscore the pressing necessity for additional research to elucidate the molecular pathways influenced by this microRNA.

In this study, for the first time, we provided important clinical evidence that miR-3652 expression was upregulated in tumor tissue compared to non-tumoral adjacent tissue in BC and associated with poor prognosis of BC patients. Additionally, we found that there was no substantial association between miR-3652 expression and clinicopathological parameters, except that HER-2 status suggests low miR-3652 expression. This suggests that miR-3652 may have an oncogenic role in carcinogenesis, potentially promoting BC development.

## 2. Materials and methods

### 2.1Cases

Expressions of miR-3652 were quantified in 30 formalin-fixed, paraffin-embedded (FFPE) tissue pairs of tumoral samples and their adjacent non-tumoral tissues. Tissues were obtained from the Par Hospital-Erbil, Kurdistan Region, Iraq. Inclusion criteria encompassed patients with a confirmed breast cancer diagnosis and availability of matched adjacent non-tumoral tissue. Exclusion criteria included patients who had received neoadjuvant therapy prior to tissue collection or had insufficient tissue quality for RNA extraction. All samples were acquired according to the guidelines of Par Hospital's protocol, encompassing patient consent and specimen acquisition. The clinical characteristics of the included patients are listed in (Table 1).

### 2.2. RNA extraction and reverse transcription

Total RNA was isolated from 30 pairs of FFPE of tumoral samples and their adjacent non-tumoral tissues, using the miRNeasy FFPE kit (Qiagen, catalog no 217504) and subsequently reverse transcribed into complementary DNA (cDNA) using the miScript II RT Kit (catalog nos. 218161).

### 2.3. Real-time quantitative PCR

Two-step quantitative Real-time PCR (qRT-PCR) was performed from Immunogen CENTER according to the manufacturer's guidelines utilizing the miScript SYBR Green PCR kit (Qiagen, catalog no. 218073). The forward primers for miR-3652 were (AACAGACGGCTGGAGGTGT), with the reverse primer was (GTGCAGGGTCGGAGGT). Also, the forward primers for endogenous control U6 were (GTGCTGCTTGGGCAGCA) with the U6 reverse primer (GAAATATGGAACGGTTC). The U6 RNA was chosen as an endogenous reference to determine the relative amount of miR-3652 expression in tumor tissues in comparison to control tissues using the $2^{-\Delta\Delta Ct}$ approach, where ΔCT = CT (a target miRNA) – CT (a reference gene).

### 2.4. Statistical analyses

All statistical analyses were conducted utilizing

GraphPad Prism version 8.4.3 (686). The comparison of miRNA expression levels between the FFPE tissue of BC patients and the FFPE tissue of BC controls was performed using the Mann-Whitney test. The Chi-square test ($\chi^2$ test or $X^2$ test) was employed to examine the association between miR-3652 expression and clinicopathological features. The ROC curve analysis was utilized to evaluate the diagnostic importance of miR-3652.

## 3. Results

### 3.1. Relative expression of miR-3652 with clinicopathological features

The results of the examination of miR-3652 expression levels did not show any significant relationships with age (p-value = 0.414), tumor stage (p-value = 0.283), ER status (p-value = 0.125), PR status (p-value = 0.133), TNM stage (p-value = 0.900), or calcification (p-value = 0.189) (Figure 1). All Her2-negative patients demonstrated low miR-3652 expression, although one Her2-positive instance displayed high expression; still, a statistically significant association with Her2 status was noted (p-value = 0.017). This data provides more evidence that Her2 status may affect miR-3652 expression in BC patients.

### 3.2. Relative expression of miR-3652 and HKGs in FFPE tissues of BC patients

In this study, we used the Mann-Whitney test to compare the tumor with the control group, as shown in (Figure 2) The results demonstrated a statistically significant difference, with a p-value = 0.018, suggesting significance at the (p-value < 0.05) level. The median value for the tumor group was 33.99, whereas the control group had a median of 35.18. The true difference between the medians was (1.190). The precise 95% CI for this difference spanned from (0.2400 to 3.030), indicating a continuous and dependable disparity between the two groups.

Moreover, as shown in (Figure 3), the expression level of HKG between the tumor and control groups was also analyzed, revealing no statistically significant difference, yielding an identical (p-value = 0.301). The median value for the tumor group was (35.75), whereas the control group exhibited a median of (34.39). The precise 95 % CI for the difference spanned from (-2.730 to 0.9300), suggesting that the observed difference may result from random variation.

Additionally, as shown in (Figure 4), the examination of the ROC curve analysis revealed an AUC of (0.6772), signifying a reasonable capacity to differentiate between the tumor groups and control groups. The Stan. Error, sensitivity, and specificity were (0.06905, 0.53, and 0.80, respectively), and the 95% CI extended from (0.5419 to 0.8126), indicating that the genuine AUC is likely to be within this range with a high degree of confidence. The result was statistically significant, with a (p-value = 0.018), suggesting that the categorization performance was above random chance.

### 3.3. Folding expression of miR-3652 utilizing 2- ΔΔCT method for qRT-PCR data analysis

The qRT-PCR results demonstrate that miR-3652 expression is significantly increased in BC patients relative to the control group. (Table 2) illustrates that the mean CT value for miR-3652 in tumor tissue was 33.23, whereas in the control it was 35.089. Additionally, the CT values for the reference gene (U6) were 34.554 in patients and 33.62 in control samples, respectively. Furthermore, the ΔCT calculations indicated that the BC tumor group exhibited a ΔCT of (-1.32), while the control group displayed a ΔCT of (1.464). This signifies that the CT value is elevated in the tumor samples relative to the control. The ΔΔCT value, which assesses the expression level of miR-3652 between the tumor group and the control group, was determined as (-1.343), hence further substantiating an increase in malignancies. The fold change analysis employing the 2-ΔΔCT method demonstrated a 6.89-fold elevation in miR-3652 expression levels in tumors relative to controls. This suggests that miR-3652 may have an oncogenic role in carcinogenesis, potentially promoting BC growth.

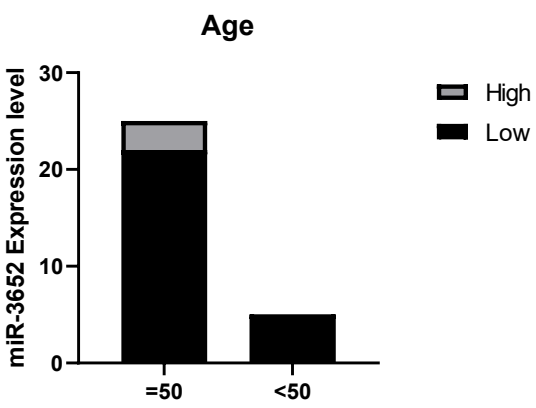
**Table 1.** Clinical and demographic characteristics of the included patients.

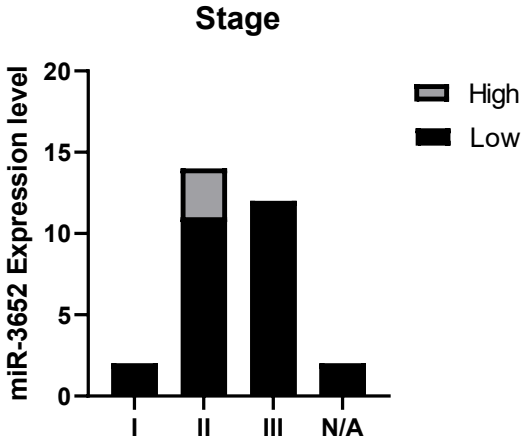
| No | Age | Gender | Tumor Size | Histological Grade | Tumor Stage | TNM Stage | ER | PR | Her-2 | Tumor/ Lymph Node Number | Carcinoma in situ % | Calcification |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 40 | Female | 30 mm | III | IIIA | pT2 N2 | Negative | Negative | Negative | 5/7 | <5 % | Not present |
| 2 | 56 | Female | 26 mm | II | IIB | pT2 N1 | Positive | Negative | Negative | 1/26 | N/A | Not present |
| 3 | 55 | Female | 35 mm | II | II B | pT2 N1 | Positive | Positive | Negative | 3/19 | N/A | N/A |
| 4 | 57 | Female | 24 mm | II | IIIA | pT2 N2 | Positive | Positive | Negative | 6/10 | N/A | N/A |
| 5 | 58 | Female | 28 mm | II | IIB | pT2 N1 | Negative | Negative | Positive | 1/14 | N/A | N/A |
| 6 | 60 | Female | 30 mm | I | IIA | pT2 N0 | Negative | Negative | Negative | 0/12 | N/A | N/A |
| 7 | 61 | Female | 30 mm | I | IIA | pT2 Nx | N/A | N/A | N/A | N/A | 0.1 | Present |
| 8 | 57 | Female | 22 mm | II | IIIA | pT2 N2 | Positive | Positive | Negative | 5/16 | N/A | Not present |
| 9 | 77 | Female | 30 mm | II | IIA | pT2 N0 | Positive | Positive | Negative | 0/33 | N/A | Present |
| 10 | 58 | Female | 6 mm | II | IA | pT1 N0 | Negative | Negative | Negative | 0/15 | N/A | N/A |
| 11 | 61 | Female | 15 & 5 mm | II | IIIA | pT1 N2 Mx | Positive | Positive | Positive | 5/13 | 0.8 | Present |
| 12 | 53 | Female | 40 mm | N/A | N/A | pTis N0 | Negative | Negative | Positive | 0/12 | 1 | Present |
| 13 | 60 | Female | 27 mm | II | IIA | pT2 N0 | Positive | Positive | Negative | 0/22 | N/A | Not present |
| 14 | 62 | Female | 32 mm | II | IIA | pT2 N0 | Positive | Positive | Negative | 0 | <5% | Present |
| 15 | 59 | Female | 25 mm | II | IIIA | pT2 N2 Mx | Positive | Positive | Negative | 4/9 | <5% | Not present |
| 16 | 52 | Female | 40 mm | N/A | N/A | ypTis N1 Mx | Positive | Positive | Negative | 2/6 | 1 | Present |
| 17 | 68 | Female | 25 mm | III | IIIA | pT2 N2 Mx | Negative | Negative | Positive | 6/15 | Rare focal | Not present |
| 18 | 61 | Female | 30 mm | III | IIA | pT2 N0 | N/A | N/A | N/A | 0/5 | 0.05 | Pesent |
| 19 | 66 | Female | 15 mm | II | IA | pT1 N0 | Positive | Positive | Negative | 0/22 | N/A | Not present |
| 20 | 67 | Female | 28 mm | II | IIB | T 2 N1 | Positive | Positive | Negative | 2/21 | N/A | Not present |
| 21 | 49 | Female | 24 mm | II | IIIC | pT2 N3 | Positive | Positive | Negative | 32/42 | 0.1 | Not present |
| 22 | 52 | Female | 70 mm | III | IIIA | pT3 N2 | Positive | Positive | Positive | 9/12 | 0.5 | Present |
| 23 | 49 | Female | 25 mm | II | IIA | pT2 N0 Mx | Positive | Positive | Negative | 0 | Focal, within the tumour | Present |

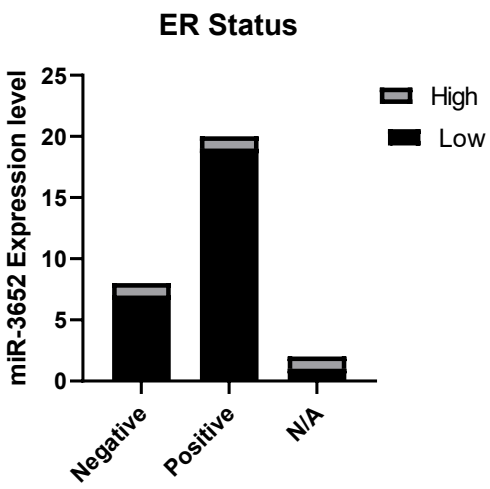
| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 24 | 45 | Female | 21 mm | III | IIA | pT2 N0 | Positive | Positive | Negative | 0 | 0.35 | Not present |
| 25 | 51 | Female | 55 mm | II | IIIA | pT3 N2 | Negative | Negative | Positive | 9/15 | N/A | Not present |
| 26 | 54 | Female | 18 mm | I | IIA | pT1 N1 | Positive | Positive | Negative | 1/11 | 0.1 | Present |
| 27 | 70 | Female | 24mm | II | IIA | T2 N0 Mx | Positive | Positive | Negative | 4 | 5% | Not present |
| 28 | 56 | Female | 45mm | II | IIIC | pT2 N3 | Positive | Positive | Negative | 13/20 | N/A | Not present |
| 29 | 72 | Female | 27 mm | II | IIIA | pT2 N2 | Positive | Positive | Negative | 7/27 | 0.02 | Not present |
| 30 | 69 | Female | 17 mm | III | IIIA | pT1 N2 | Negative | Negative | Positive | 5/16 | N/A | Not present |
| 31 | 59 | Female | 30 mm | II | IIA | pT2 N0 Mx | Positive | Positive | Negative | 11 | Focal, within the tumour | Present |
| 32 | 71 | Female | 30 mm | III | IIB | pT2 N1 Mx | Negative | Negative | Positive | 6-Jan | pleomorphic LCIS | Not present |
| 33 | 59 | Female | 19 mm | II | IIIA | pT1 N2 | Positive | Positive | Positive | 6/21 | 0.02 | Not present |
| 34 | 61 | Female | 30 mm | III | IIA | pT2 N0 | Negative | Negative | Negative | 5 | 0.05 | Present |
| 35 | 74 | Female | 80 mm | II | IIIC | pT3 N3 | Positive | Negative | Negative | 34/34 | N/A | Not present |
| 36 | 61 | Female | 4 mm | II | IIA | pT1 N0 | Positive | Positive | Negative | 0 | N/A | Present |
| 37 | 82 | Female | 36 mm | II | IIIA | pT2 N2 | Positive | Positive | Negative | 4/35 | 0.05 | Not present |
| 38 | 55 | Female | 35 mm | II | IIB | pT2 N Mx | Positive | Positive | Negative | 2/9 | 0.1 | Present |
| 39 | 48 | Female | 18 mm | I | IIA | pT1 N1 | Positive | Positive | Negative | 1/8 | N/A | N/A |

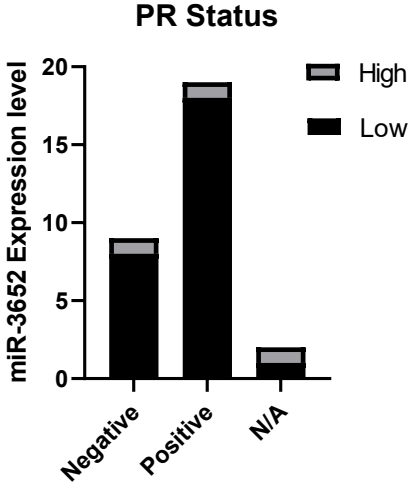
**Table 2.** Shows the results of folding expression of miR-3652 utilizing $2^{-\Delta\Delta CT}$ method for qRT-PCR data analysis.

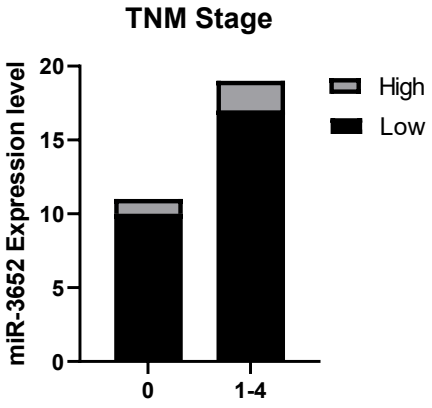
| **Equations** | |
|---|---|
| ΔCT target | ΔCT = CT (target) – CT (a reference gene)<br>ΔCT (target) = 33.23 - 34.554 = -1.32 |
| ΔCT control | ΔCT = CT (Control) – CT (control- reference gene)<br>ΔCT (Control) = 35.089 - 33.62 = 1.464 |
| ΔΔCT | ΔΔCT = ΔCT (a target miR1256) – ΔCT (Control)<br>ΔΔCT = -1.32 – 1.464 =<br>-2.785 |
| Folding Expression | Folding Expression = $2^{-\Delta\Delta CT} = 2^{-(-2.785)} = 6.89$ |

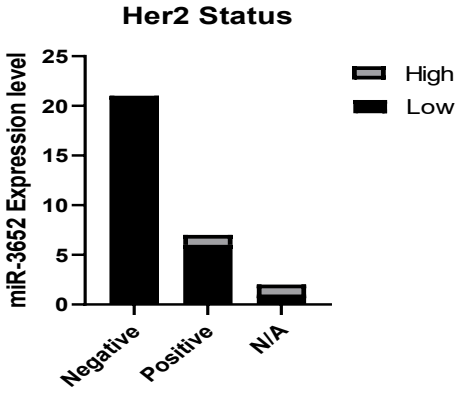

Age
miR-3652 Expression level
30
20
10
0
=50
<50
High
Low


Stage
miR-3652 Expression level
20
15
10
5
0
I
II
III
N/A
High
Low


ER Status
miR-3652 Expression level
25
20
15
10
5
0
Negative
Positive
N/A
High
Low


PR Status
miR-3652 Expression level
20
15
10
5
0
Negative
Positive
N/A
High
Low


TNM Stage
miR-3652 Expression level
20
15
10
5
0
0
1-4
High
Low


Her2 Status
miR-3652 Expression level
25
20
15
10
5
0
Negative
Positive
N/A
High
Low

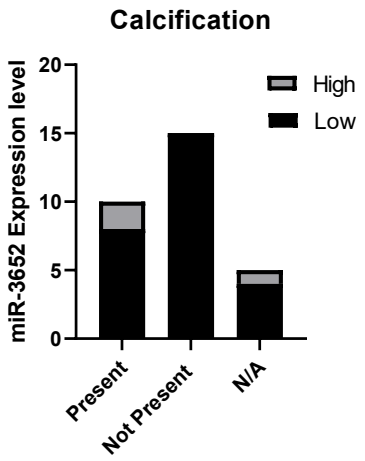



**Figure 1.** Correlation between miR-3652 and clinicopathological parameters.

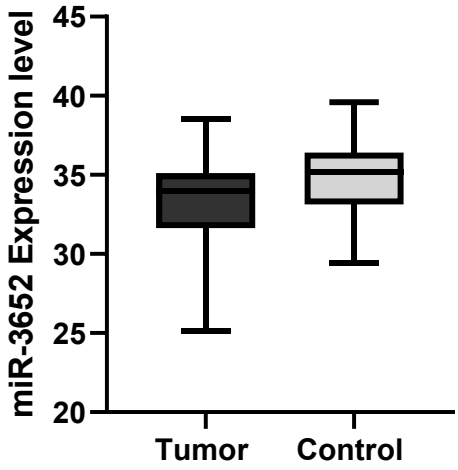



**Figure 2.** Relative expression level of miR-3652 in 30 FFPE tissue pairings of tumor samples and control tissues.

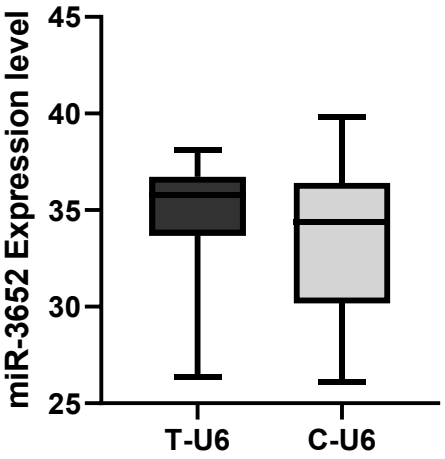



**Figure 3.** Relative expression level of miR-3652 in 30 FFPE tissue pairings of tumor-U6 samples and control-U6 tissues.

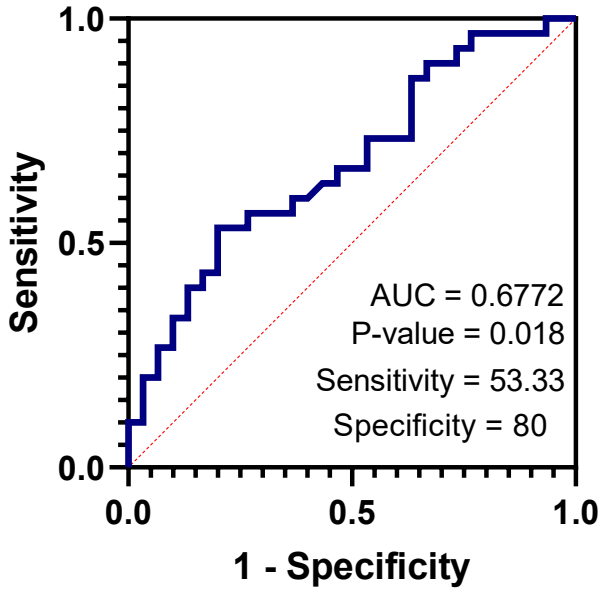



**Figure 4.** The ROC curve of miR-1256 expression for the differentiation of BC tissues from surrounding normal tissues. AUC indicates area under the ROC curve.

## 4. Discussion

Breast cancer impacts women globally and leads to cancer-related deaths (Wilkinson and Gathani, 2022). Breast cancer is commonly categorized into one of four main subtypes (luminal A, luminal B, HER2 positive, and basal), indicating molecular characteristics and informing treatment regimens (Johnson et al., 2021, Cejalvo et al., 2018). The most severe form of breast cancer is metastasis, when the tumour spreads from the breast tissue to other parts of the body (Park et al., 2022).

MicroRNAs are a class of small, non-coding RNA molecules, usually about 21–23 nucleotides in length (Tomasello et al., 2021). Unlike messenger RNAs (mRNAs) that serve as templates for protein synthesis, miRNAs primarily function to regulate gene expression at the post-transcriptional level (Hussen et al., 2023b). The function of an individual miRNA can be context-dependent; a single miRNA can have multiple target mRNAs, and likewise, a single mRNA can be targeted by multiple miRNAs (Hussen et al., 2023c). This creates a complex regulatory network where miRNAs play pivotal roles in fine-tuning gene expression to ensure cellular homeostasis and proper response to environmental cues (Schratt, 2009).

In this study, we investigate the association between the clinical characteristics of patients with the miR-3652 expression level. The results of the examination of miR-3652 expression levels did not show any significant relationships with age, tumor stage, ER status, PR status, TNM stage, or calcification. All Her2-negative patients demonstrated low miR-3652 expression, although one Her2-positive instance displayed high expression; still, a statistically significant association with Her2 status was noted (p-value = 0.017). This data provides more evidence that Her2 status may affect miR-3652 expression in BC patients.

Furthermore, the significant association between HER2 status and miR-3652 expression suggests a potential regulatory interplay. HER2 is a key oncogene that drives breast cancer progression by activating signaling pathways involved in cell proliferation and survival. MiR-3652 may be influenced by HER2 signaling or may itself regulate components of the HER2 pathway, thereby contributing to tumorigenesis. This relationship underscores the biological relevance of miR-3652 in HER2-positive breast cancer and supports its potential role as a biomarker or therapeutic target. Additional functional studies are needed to clarify the molecular mechanisms

underlying this interaction.

Moreover, although our study did not find a statistically significant association between miR-3652 expression and tumor size or lymph node involvement, these clinical parameters remain critical in breast cancer prognosis. Tumor size indicates the extent of primary tumor growth, while the number of metastatic lymph nodes reflects the degree of cancer spread. The elevated expression of miR-3652 in tumor tissues may be related to biological processes promoting tumor growth and metastasis. Therefore, further research with larger sample sizes is necessary to explore possible associations between miR-3652 levels and these important clinical indicators, which could provide deeper insight into the role of miR-3652 in breast cancer aggressiveness and patient outcomes.

In this investigation, we employed the Mann-Whitney test to compare the tumor group with the control group, yielding a statistically significant difference (p-value = 0.018), indicating significance at the p-value < 0.05 level. The median value for the tumor group was 33.99, while the control group had a median of 35.18. The actual disparity between the medians was (1.190). The exact 95% confidence interval for this difference ranged from 0.2400 to 3.030, signifying a consistent and reliable distinction between the two groups.

Furthermore, the expression level of HKG between the tumor and control groups was evaluated, revealing no statistically significant difference, with a p-value of 0.301. The tumor group had a median value of 35.75, while the control group displayed a median of 34.39. The exact 95% confidence interval for the difference ranged from -2.730 to 0.9300, indicating that the observed difference may be attributable to random variation.

Additionally, the ROC curve analysis indicated an AUC of 0.6772, demonstrating a moderate ability to distinguish between tumor groups and control groups. The Stan results indicated an error of 0.06905, sensitivity of 53.33, and specificity of 80, with a 95% confidence interval ranging from 0.5419 to 0.8126, suggesting that the true area under the curve (AUC) is expected to fall within this range with considerable confidence. The outcome was statistically significant, with a p-value of 0.018, indicating that the categorization performance exceeded random chance.

While this study provides valuable preliminary insights into the expression of miR-3652 and its association with HER2 status in breast cancer, several limitations should be acknowledged. The sample size was relatively small, which may limit the statistical power and generalizability of our findings. Additionally, our analysis focused on miR-3652 expression using qRT-PCR, without direct measurement of HER2 protein levels by techniques such as Western blotting. Including protein-level analyses in future studies would strengthen the evidence for a direct regulatory relationship between miR-3652 and HER2 expression. Despite these limitations, our study's use of paired tumoral and adjacent non-tumoral tissues and the identification of a significant association between miR-3652 expression and HER2 status represent important steps toward understanding the potential role of miR-3652 as a biomarker and its biological relevance in breast cancer. Future investigations incorporating protein quantification and larger cohorts are warranted to confirm and expand upon these findings.

Overall, our results suggest that miR-3652 may have an oncogenic role in the development and progression of BC tumors, since increased miR-3652 expression is associated with BC progression.

## 5. Conclusion

This study demonstrates that miR-3652 expression is significantly elevated in breast cancer tissues compared to adjacent non-tumoral tissues, suggesting its potential role as an oncogenic factor in breast cancer development. Although overexpression of miR-3652 showed no significant association with most clinicopathological parameters, a notable association with HER2 status was observed. The moderate diagnostic performance of miR-3652 supports its promise as a biomarker for distinguishing breast cancer patients from controls. Overall, these findings highlight miR-3652 as a potential biomarker involved in breast cancer progression, warranting further investigation in larger cohorts to validate its clinical utility and clarify its biological role.

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