

Prognostic Significance of Circular *HER2* and Its Association with EMT Markers in Advanced Breast Cancer

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Abstract

Introduction: Globally, breast cancer (BC) represents the most prevalent malignancy among females. In the field of epigenetic regulation, non-coding circular RNAs have recently been identified as key contributors to BC pathogenesis. Among this class, circular *HER2* (*Circ-HER2*) has been documented to be overexpressed in various cancers, including BC. Particularly, in triple-negative breast cancer (TNBC), elevated *Circ-HER2* expression correlates with increased metastatic potential, invasiveness, and cell proliferative capacity. Nevertheless, the precise molecular mechanisms through which *Circ-HER2* is implicated in TNBC remains inadequately understood and further investigations are still needed. Consequently, this study aimed to investigate the *Circ-HER2* gene expression profile in a sample of BC patients and to elucidate its association with other prognostic biomarkers and disease aggressiveness phenotypes. **Patients and Methods:** This study was conducted on 40 breast cancer patients, two tissue specimens were collected from each patient: one from the malignant tumor (MT) and the other sample from the corresponding adjacent, microscopically normal breast tissue (TAN). q-RT-PCR was employed for gene expression profiling of *Circ-HER2*, *E-Cadherin*, *ILE2*, and *Twist1* in malignant as well as normal tissues. **Results:** Our results revealed a significant upregulation of *Circ-HER2*, *ILE2*, and *Twist1* in malignant tissues compared to the matched adjacent normal tissues. Furthermore, *Circ-HER2* expression demonstrated a statistically significant correlation with *E-Cadherin* expression, *ILE2*, and *Twist1*. However, when the TNBC patients analyzed separately, *Circ-HER2* was significantly higher than *E-Cadherin*, while its expression was not significantly different than the other metastatic markers. **Conclusion:** In conclusion, the significant *Circ-HER2* overexpression in breast cancer tissues, along with significant correlation of *Circ-HER2* with increased levels of key EMT biomarkers, especially in TNBC patients, suggest a potential role for *Circ-HER2* in promoting an aggressive, pro-metastatic phenotype in breast cancer. These findings provide preliminary clinical evidence supporting the oncogenic function of *Circ-HER2* and underscore the need for further mechanistic investigations to delineate its precise molecular pathway and validate its potential as a prognostic biomarker or therapeutic target in breast cancer, particularly in the aggressive subtypes such as TNBC, where therapeutic options are limited.

Keywords: TNBC, *Circ-HER2*, EMT markers, *Twist1*, *ILE2*

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Introduction

Breast cancer (BC) is a female malignancy with high prevalence worldwide. According to estimates, BC incidence and mortality would increase by 38% and 67%, respectively by 2050, with a greater impact on countries with low human development index [1].

BC cases continue to rise amongst Egyptian females making it the most prevalent female malignancy in Egypt. A recent metanalysis reported diagnosis at young age and advanced stage BC are common presentation of BC amongst Egyptian women [2].

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Breast cancer has four main classes according to the status of estrogen and progesterone hormone receptors (HoR) and human epidermal growth factor receptor 2 (*HER2*); these classes are HoR+/HER2+, HoR+/HER2-, HoR-/HER2+ and triple-negative breast cancer (TNBC). With respect to HoR expression status, two BC molecular classes have been described, luminal A (estrogen receptor +, progesterone receptor +, and *HER2*-) and luminal B (estrogen receptor +, progesterone receptor -, and *HER2*+) [3]. Two other molecular subclasses have been also described based on gene expression profile; basal-like BC and normal-like BC. Basal-like BC expresses genes of basal or myoepithelial cells and is TNBC with high aggressiveness and poor prognosis. Normal-like BC has a gene expression profile similar to normal cells and HoR and *HER2* status varies; likewise, aggressiveness and prognosis vary [3]. *HER2* exerts protooncogenic influence in nearly 20% of BC, which is related with more aggressiveness and poor prognosis, particularly in metastatic TNBC [4].

In this regard, overexpression of non-coding circular RNAs play a role in progression of several types of cancer including BC; and among those circular RNAs, circular *HER2* (*Circ-HER2*) overexpression and oncogenic potential in BC has been postulated [5, 4]. In this context, overexpression of *Circ-HER2* was reported in TNBC, and its silencing, demonstrated antitumor capacities in TNBC in in vitro and in vivo models [6]. Moreover, it was reported that overexpression of *Circ-HER2* in TNBC is associated with enhanced metastatic behavior, invasiveness and cell proliferation (reported in [7]). The tumorigenic role of *Circ-HER2* in BC, particularly TNBC, was also reported by Zonglin and colleagues [8] confirming the importance of *Circ-HER2* as a biomarker and therapeutic target in BC. Therefore, the aim of the current study was to investigate the gene expression profile of *Circ-HER2* in a group of BC patients and to correlate its expression pattern with other prognostic biomarkers and diseases aggressiveness phenotypes.

Materials and Methods

Ethical approval

All sampling procedures and research methodologies employed in the present study were reviewed and approved by Research Ethics Committee of Faculty of Medicine at Alexandria University (#0307252). Written informed consent was obtained from every participant before their enrollment in the study.

Sampling

This study was conducted on 40 breast cancer patients recruited from the Cancer Management and Research Department, Medical Research Institute, Alexandria University, Egypt. During surgical resection, two tissue specimens were collected from each patient: one from the malignant tumor (MT) and a matched sample from the corresponding adjacent, microscopically normal breast tissue (TAN). Relevant clinicopathological data, including patient age, pathological tumor type, histological

grade, lymph node status, and estrogen receptor (ER), progesterone receptor (PR), and *HER2* status, were also collected. All collected specimens were subsequently stored at -80°C until time of investigations.

Quantitative reverse transcriptase polymerase chain reaction (q-RT-PCR)

Total RNA extraction

Total RNA was extracted from malignant and normal tissues using RNeasy Mini Kit total RNA extraction kit (Qiagen) as per manufacturer instructions. A nano-drop spectrophotometer was employed to monitor RNA purity and concentration, through the measurement of absorbance at 230, 260 and 280 nm.

cDNA synthesis & amplification

Complementary DNA (cDNA) was synthesized from the extracted RNA using the Omniscript RT Kit (Qiagen) in accordance with the manufacturer's instructions. Quantitative real-time PCR (qPCR) reactions were carried-out in 20 µL final volume, comprising 1 µL of cDNA, 1 µL each of forward / reverse primers (10 pM/µL; primer sequences are provided in Supplementary File 1, Table S1), 10 µL of SYBR Green Master Mix, and 7 µL of RNase-free water. Following gentle mixing, reaction plates were sealed and loaded into a Bio-Rad thermal cycler. The amplification protocol consisted of an initial denaturation at 95°C for 10 minutes, followed by 40 cycles as follows: denaturation at 95°C for 15 seconds, annealing at 55°C for 10 seconds, and extension at 72°C for 15 seconds. The relative expression of target genes in malignant tissues was determined using the $2^{-\Delta\Delta CT}$ method, with gene expression normalized to the reference gene GAPDH as a housekeeping gene within each sample.

Results

Patients' characteristics

This study was conducted on forty breast cancer patients, including patients with luminal (10 patients) and TNBC (30 patients). Patients age ranged from 37 -73 years with a mean age of 57 years. Cancer stage and grade are summarized in Figure 1 showing that patients were in stage II or III and the grade of cancer was II or III. All patients had undergone modified radical mastectomy or other types of surgeries with/without axillary clearance.

Gene expression profiles in normal and malignant tissues

Gene expression levels of *Circ-HER2*, *E-Cadherin*, *ILE2*, and *Twist1* are presented in Figure 2. Results showed that the malignant tissues express markedly higher levels of *Circ-HER2*, *ILE2* and *Twist1* than their corresponding normal tissues, while the mean values of *E-Cadherin* in malignant and normal tissues were almost similar. There was an apparent difference between the maximum values of expression levels in malignant tissues and normal tissues, except for *E-Cadherin*, where a small difference was observed as compared to the other target genes. To compare between expression levels of target genes between malignant and normal tissues, non-parametric

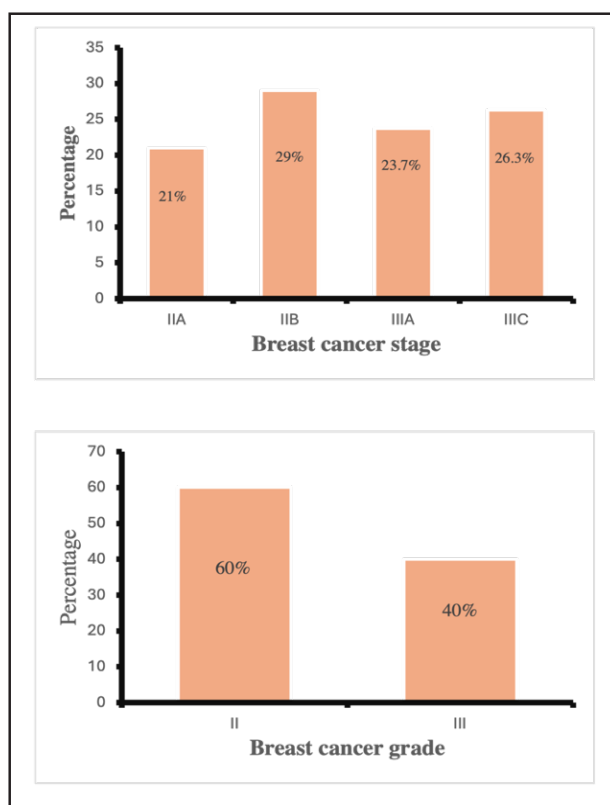


Figure 1. Patients Distribution Regarding Stage and grade of Breast Cancer in the whole Study Cohort. All patients in the study sample were diagnosed with breast cancer, stage and grade II or III.

statistics was used following ROC curve analysis that was used to determine the cutoff point for a better classification of expression levels as high or low.

ROC curve analysis and comparison of gene expression levels in normal and malignant tissues

To determine the best cut off point of *Circ-HER2* expression level and the other advanced breast cancer markers, ROC curve (Figure 3) analysis was performed. The cut off values that were used to classify gene expression levels in malignant and normal tissues are presented in Table 1. The results of Mann Whitney test showed that there is a significant difference between the ranks of gene expression levels (being classified as high or low based on the cut off values) of *Circ-HER2*, *E-Cadherin*, *ILE2*, and *Twist1* in normal and malignant tissues (p values = 0.003, 0.02, 0.014, and 0.005, respectively) (Figure 4).

Table 1. Cut off Points of *Circ-HER2*, *E-Cadherin*, *ILE2*, and *Twist1*

Gene	Cutoff
<i>Circ-HER2</i>	2.7373
<i>E-Cadherin</i>	2.1696
<i>ILE2</i>	2.3224
<i>Twist1</i>	2.5871

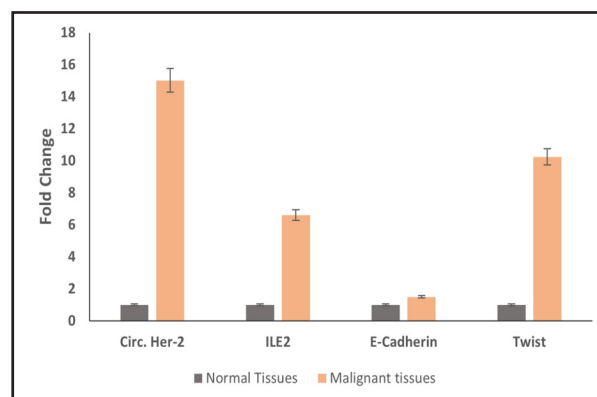


Figure 2. Gene Expression of Target Genes in Malignant and Normal Tissues. The Figure shows that *Circ. HER-2*, *ILE2* and *Twist* levels were markedly higher in malignant tissues of all BC cohort than their corresponding normal, while the mean values of *E-Cadherin* in malignant and normal tissues were similar.

Correlation between *Circ-HER2* expression and the other target genes

Spearman's correlation (Table 2) was performed to assess the possible association between gene expression levels of *Circ-HER2* and the other studied genes in malignant tissues. The results revealed a moderately positive association between *Circ-HER2* and *E-Cadherin* and *ILE2*, which was statistically significant ($p < 0.001$ for both correlations); whereas, a weak positive association, which was statistically significant, was observed with *Twist1* ($p = 0.022$).

Correlation between gene expression levels and breast cancer stage and grade and number of lymph nodes

Expression level of genes was classified into high or low to assess the potential association with disease severity represented by disease's stage and grade. The association was evaluated using Chi-square and ordinal by ordinal Spearman's correlation. The results presented in Tables 3 and 4, respectively summarize Chi-square and Spearman's correlation coefficients values for disease's stage and grade with target gene expression. There was only a significant association between *ILE* expression levels and disease's stage ($p = 0.02$), which was confirmed by both Chi-square and Spearman's correlation tests and was weak positive association. On the other hand, neither disease's stage nor grade showed a statistically significant association with all other target genes.

Regarding correlation with lymph nodes number, there was a weak positive, but nonsignificant correlation

Table 2. Correlation between *Circ-HER2* Expression in Malignant Tissues and Other Target Genes

	Malignant <i>E-Cadherin</i>	Malignant <i>ILE2</i>	Malignant <i>Twist1</i>
Malignant <i>Circ-HER2</i>			
Correlation coefficient	0.548	0.633	0.362
p value	< 0.001	< 0.001	0.022

Table 3. Correlation between Gene Expression Levels in Malignant Tissues and Breast Cancer's Stage

		Stage		Total	Chi-Square value	P value	Ordinal by Ordinal Spearman's correlation	P value
		II	III					
Malignant <i>Circ-HER2</i>	low	14	9	23	2.754	0.097	0.269	0.102
	high	5	10	15				
Total		19	19	38				
Malignant <i>E-Cadherin</i>	low	15	11	26	1.949	0.163	0.226	0.172
	high	4	8	12				
Total		19	19	38				
Malignant <i>ILE2</i>	low	15	8	23	5.397	0.02*	0.377	0.02*
	high	4	11	15				
Total		19	19	38				
Malignant <i>Twist1</i>	low	13	10	23	0.991	0.319	0.62	0.333
	high	6	9	15				
Total		19	19	38				

between gene expression level of all candidate genes and number of lymph nodes, where ≤ 5 lymph nodes was considered less aggressive disease, while > 5 lymph nodes was considered more aggressive disease (Table 5).

Expression profile of *Circ-HER2* and other metastatic markers in TNBC patients

To have a better understanding on the status of *Circ-HER2* expression amongst the studied metastatic markers, a separate analysis was performed on the TNBC patients' data to compare the gene expression profile in their malignant tissues with the normal corresponding

tissues as well as with the expression in the malignant tissues form the luminal BC patients. Results showed that *Circ-HER2* and *Twist1* expression in TNBC patients was significantly higher the corresponding normal control ($p = 0.04$ and 0.03 , respectively). On the other hand, there was no significant difference between *E-Cadherin* and *ILE2* expression level in TNBC malignant tissues and the corresponding normal tissues ($p = 0.3$ and 0.06 , respectively). Additionally, *Circ-HER2* expression in the TNBC malignant tissues was significantly higher than that in the malignant tissues from the luminal BC patients ($p = 0.04$). Expression levels of *E-Cadherin*, *ILE2*,

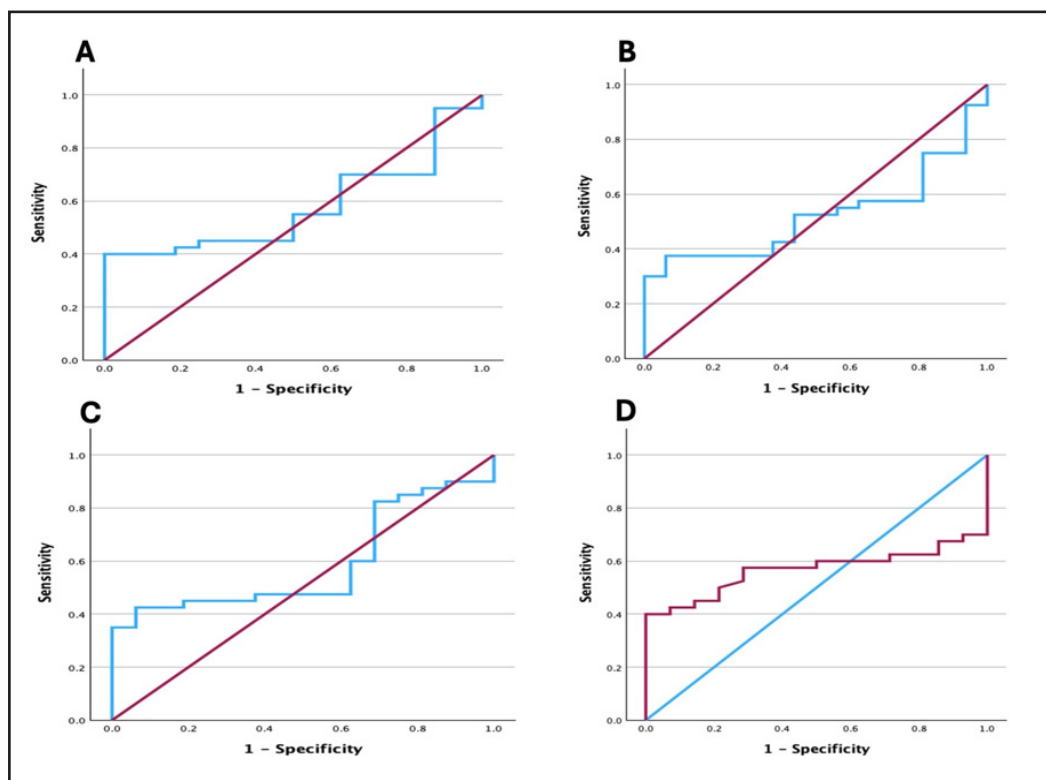


Figure 3. ROC Curves Used for Classification of Gene Expression in Normal and Malignant Tissues from the whole Study Cohort. The Figure shows ROC curves used to determine the cutoff values to discriminate between expression levels in malignant and normal tissues of *Circ-HER2* (A), *E-Cadherin* (B), *ILE* (C) and *Twist* (D).

Table 4. Correlation between Gene Expression Levels in Malignant Tissues and Breast Cancer's Grade

		Grade		Total	Chi-Square value	P value	Ordinal by Ordinal Spearman's correlation	P value
		II	III					
Malignant <i>Circ-HER2</i>	low	12	12	24	2.5	0.114	-2.5	0.12
	high	12	4	16				
Total		24	16	40				
Malignant <i>E-Cadherin</i>	low	16	11	27	0.019	0.89	-0.022	0.894
	high	8	5	13				
Total		24	16	40				
Malignant <i>ILE2</i>	low	15	8	23	0.614	0.433	0.124	0.446
	high	9	8	17				
Total		24	16	40				
Malignant <i>Twist1</i>	low	16	8	24	1.11	0.292	0.167	0.304
	high	8	8	16				
Total		24	16	40				

Twist1 in malignant tissues from TNBC and luminal BC patients were not statistically different ($p = 0.9$, 0.7 , and 0.7 , respectively). This suggests that *Circ-HER2* could be a better prognostic factor in the more aggressive BC, TNBC in this study settings. Moreover, we have compared the expression level of *Circ-HER2* with the expression level of the other studied metastatic markers in TNBC patients. Results revealed that the difference between *Circ-HER2* expression and *ILE2* and *Twist1* was not statistically significant, while *Circ-HER2* expression level was significantly higher than *E-Cadherin*. This suggests that *Circ-HER2* expression profile is in concordance

with that profile of known positive metastatic markers whose upregulation supports the metastatic potential. Finally, Fisher's exact analysis showed that there was no association between expression level of *Circ-HER2* and the other metastatic markers with disease stage, which might be due to unavailability of stage IV patients in the study sample.

Discussion

Breast cancer is currently the most commonly diagnosed malignancy worldwide, surpassing lung cancer

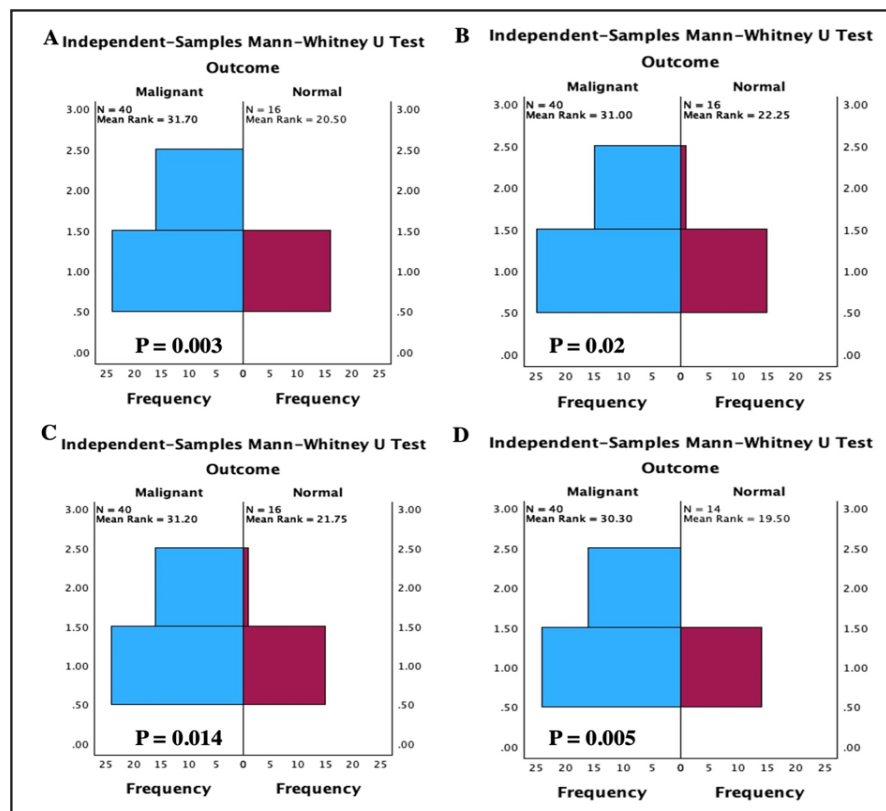


Figure 4. Mean ranks of *Circ-HER2*, *E-Cadherin*, *ILE2*, and *Twist1* in Normal and Malignant Tissues from the whole Study Cohort. Mann-Whitney U test results revealed statistically significant difference between the mean ranks of all tested genes in normal and malignant tissues. A = *Circ-HER2*, B = *E-Cadherin*, C = *ILE2*, and D = *Twist1*

Table 5. Correlation between Gene Expression Levels in Malignant Tissues and Number of Lymph Nodes

		Disease aggressiveness based on number of lymph nodes		Total	Chi-Square value	P value	Ordinal by Ordinal Spearman's correlation	P value
		Less severe	More severe					
Malignant <i>Circ-HER2</i>	low	17	7	24	0.02	0.8	0.02	0.8
	high	11	5	16				
	Total	28	12	40				
Malignant <i>E-Cadherin</i>	low	21	6	27	2.4	0.12	0.245	0.12
	high	7	6	13				
	Total	28	12	40				
Malignant <i>ILE2</i>	low	17	6	23	0.4	0.5	0.1	0.5
	high	11	6	17				
	Total	28	12	40				
Malignant <i>Twist1</i>	low	17	7	24	0.02	0.88	0.02	0.8
	high	11	5	16				
	Total	28	12	40				

in incidence. The elevation in incidence and mortality rates of breast cancer leads to a great burden on public health [9]. With metastasis being one of the most difficult challenges in the management of BC [10], extensive research is directed toward understanding the mechanisms implicated in BC development and metastasis.

Recently, a newly emerging category of non-coding RNAs has attracted more attention, namely circRNAs, because of their pivotal functions in various cancers including BC, with some circRNAs have been elucidated as BC biomarkers [11, 12]. On the other hand, there are few reports regarding the role of many circRNAs in the progression and metastasis of breast cancer. Among this category, *Circ-HER2* still needs extensive research to deepen the understanding of its role in aggressiveness and metastasis of BC.

Therefore, the present study was conducted to highlight the role of *Circ-HER2* in BC, through determining the level of gene expression of *Circ-HER2* in malignant tissues, and to correlate this expression with markers of epithelial mesenchymal transition (EMT) as key regulators of the metastatic potential. In addition, we correlated the gene expression of *Circ-HER2* with clinicopathological features including tumor stage, grade, and lymph node infiltration. Moreover, a separate analysis of the status of *Circ-HER2* expression and association with the profile of other metastatic markers was conducted in patients with the more aggressive form of BC, the TNBC.

Our data revealed a significant upregulation of *Circ-HER2* in the malignant BC tissues as compared to TAN tissues, this finding is consistent with the previous results that reported an elevated expression of *Circ-HER2* in malignant tissues rather than normal tissues [4]. In support to this, *Circ-HER2*, has attracted great attention due to its function in regulation of various tumors, it enhances tumor growth and reduces overall survival in gastric cancer patients [13]. Additionally, it is abnormally expressed and exerts carcinogenic effects in different cancer cells including those of BC [13, 14].

One of the proposed mechanisms to explain the role

of *Circ-HER2* in BC, is through its ability to encode a novel protein identified as *HER2-103*, which promotes downstream malignant phenotypes through interaction and activation of EGFR/HER3 and promoting AKT phosphorylation [6]. A considerable part of triple negative breast cancer, which has the highest metastatic potential and worst prognosis, shows an increased expression of *Circ-HER2*, contributing to the tumorigenicity through promotion of EGFR/HER3 pathway. Furthermore, Depriving TNBC cells from *HER2-103* inhibits their proliferation, invasion and tumorigenicity in vitro and in vivo [6]. This is in concordance with the significant overall increase in *Circ-HER2* in all BC tissues in comparison to TAN tissues. In support to the important role of *Circ-HER2* in TNBC progression, our results have also shown that *Circ-HER2* expression was significantly higher in TNBC than in luminal BC patients. Interestingly, *HER2-103* Shares same antigen-recognition domain of *HER2* which can be targeted with Pertuzumab and Trastuzumab. This could represent a new avenue for treatment of triple negative breast cancer, whose only treatment option is chemotherapy [6].

Regarding EMT marker, our results indicated a significant upregulation of *E-Cadherin*, *ILE2*, and *Twist1* genes in the malignant tissues as compared to TAN tissues. These results are in agreement with previous studies that reported abnormal elevation of these genes in malignant tissues [15]. In this context, it was reported that *Twist1* activation promotes tumor invasiveness through EMT in *HER2+* breast cancer [16]. A recent report has also reported *Twist1* amongst others as a mediator of EMT in metastatic breast cancer, a process that was induced by activation of STAT3 signaling [17]. In the same context, it was reported that *Twist1* promotes EMT by silencing epithelial gene markers such as *FOXAI* and *E-Cadherin* through its interaction with nucleosome remodeling and deacetylase resulting in its recruitment and repression of these genes [18]. Another study, has also supported that *Twist1* suppression of *FOXAI* sustains the metastatic potential and invasiveness of BC [15]. The important

contribution of *Twist1* to the EMT in BC was further confirmed in another study [19]. Moreover, we have shown a significant correlation between these gene and *Circ-HER2* gene expression patterns. However, its noteworthy that overexpression of *E-Cadherin* suppresses EMT [20], the correlation between *Circ-HER2* and *E-Cadherin* levels in BC tissues could be interpreted as some aggressive cancers could oppose overexpression of *E-Cadherin* or other tumor suppressors by increasing the expression of counteracting genes, in this case *Circ-HER2*, *ILE2* and *Twist1* counteract *E-Cadherin* influence reducing its anti-EMT action. This explanation is supported by the clinicopathological features of patients; however, these results were observed when all patients were analyzed as a single cohort, and further research is needed to investigate this assumption. Additionally, *Circ-HER2* expression was significantly higher than *E-Cadherin* in TNBC patients, while its expression was not different than the other metastatic markers. Moreover, the significant higher expression of *Circ-HER2*, rather than the other studied metastatic markers, in TNBC than the luminal BC cases support the superior possible diagnostic/prognostic utility of *Circ-HER2* in TNBC. In concordance with these results, a recent article reported the importance of circular RNAs, including *Circ-HER2* in TNBC [21].

In line with the finding of this study, it was reported that expression of *Circ-HER2* and its encoded protein *HER2-103* is associated with worse prognosis in TNBC patients [6]. Additional evidence was reported on the link between *Circ-HER2* expression and tumor aggressiveness represented by enhanced invasiveness, migration and cell proliferation in HER-2+ breast cancer [4]. This supports our findings that *Circ-HER2* was overexpressed in malignant breast tissues and its association with EMT markers in the study's cohort. In this context, it was recently reported that circular RNAs are involved in breast carcinogenesis, progression and resistance to therapy [22], which is in harmony with our study results. Notably, circular RNAs functions in breast cancer progression could be achieved through their ability to act as protein and miRNA sponges and regulation of gene expression [8]. In agreement with this, it was recently reported that circular RNA UBAP2 contributes to cisplatin resistance in TNBC suggesting involvement of other circular RNA in carcinogenesis of the most aggressive BC, TNBC and emphasizing the importance of circular RNAs in breast tumorigenesis and progression [23]. However, it is noteworthy that circular RNAs is a big family comprising many members, and some of these members might exert a protective function in breast cancer as recently discussed in a literature review [24], though *Circ-HER2* was not discussed in this article.

In conclusion, the emerging role of circular RNAs in human malignancies including breast cancer presented this family of RNAs as potential markers and therapeutic targets. Of these RNAs, *Circ-HER2* gained a lot of attention due to the growing evidence on its contribution to breast cancer aggressiveness. Herein, we are adding to the scientific literature another evidence on *Circ-HER2* oncogenic potential in TNBC by showing: 1) its

overexpression in malignant tissues as compared to the TAN and its overexpression in TNBC in comparison to luminal BC, and 2) significant association between *Circ-HER2* and positive EMT genes such as *Twist1* and *ILE2*, particularly in TNBC. Having said that, further research is needed to confirm these findings in a larger study sample and investigate the functional aspects of *Circ-HER2* in in vitro and in vivo studies to understand the exact mechanisms of these RNAs in promoting TNBC progression and, perhaps resistance to therapies.

However, the results of the present study should be interpreted with careful consideration of the small sample size of the study cohort and the heterogeneity of the patients' population.

Acknowledgements

Authors contribution

Fawziya A.R Ibrahim suggested the research topic and experimental design, performed biochemical analysis, participated in data analysis and interpretation wrote and revised the paper, Nesma A. Ghazal, Hanan Mohamed Nomeir, and Shaymaa Ali Elhamshary, participated in the experimental design, biochemical analysis, and data interpretation, Ola A. Sakr, participated in the experimental design, and in analysis and interpretation of clinicopathological data Omar Al-Masry participated in formulating research topic, carried out the statistical analysis, participated in data analysis and interpretation, and wrote and revised the paper. All authors have read and approved the final manuscript.

Ethical approval

The study was reviewed and approved by the Research Ethics Committee of the Faculty of Medicine at Alexandria University (#0307252).

Data availability

All data generated or analyzed during this study are included in the article.

Competing interests

The authors declare no competing interests.

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