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Original Article

MicroRNA-543-3p a potential chemotherapy marker regulates the messenger RNA expression of survivin in patients with advanced breast cancer

Saleh Ali Alghamdi[1], Ayman Alhazmi[1], Ashjan Shami[1], Amal F. Gharib[1,2], Wael Hassan Elsawy[3], Majed Al Mourgi[4]

[1]Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, Taif University, Saudi Arabia, [2]Department of Medical Biochemistry and Molecular Biology, Faculty of Medicine, Zagazig University Egypt, [3]Department of Clinical Oncology, Faculty of Medicine, Zagazig University, Egypt, [4]Department of Surgery, College of Medicine, Taif University, Taif, Saudi Arabia

Address for correspondence:
Wael Hassan Elsawy, Department of Clinical Oncology, Faculty of Medicine, Zagazig University, Egypt. Tel.: +9660566482653.
E-mail: whelsawy@gmail.com

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ABSTRACT

Objectives: Induction chemotherapy (ICT) is the standard of care for patients with locally advanced breast cancer. The major criticism about ICT is the delay of surgery, which may affect the local control. Survivin functions by the regulation of mitosis and inhibition of apoptosis. The miR-542-3p regulates survivin mRNA, upregulation of miR-542-3p leads to the downregulation of survivin and arrest of the cell cycle at G1 and G2/M phases resulting in tumor growth suppression. We studied whether we can use survivin mRNA and miRNA-542-3p as potential biomarkers to predict response to ICT.

Methods: Fifty-one patients with locally advanced breast cancer were treated with ICT. miRNA-542-3p and survivin mRNA were determined in breast cancerous tissues and their nearby healthy breast tissues by a real-time quantitative polymerase chain reaction.

Results: Our results revealed a negative correlation between miRNA and survivin. miRNA-542-3p levels were elevated in normal tissues and in patients with good prognostic features and response to ICT. On the contrary, survivin was upregulated in malignant tissues in patients with adverse prognostic features and patients with no response to neoadjuvant chemotherapy.

Conclusions: ICT is a promising option for the treatment of patients with locally advanced carcinoma of the breast. The studied miRNA-542-3p and its target survivin correlate inversely with each other in both malignant and their nearby normal tissues. miRNA-542-3p and survivin can be used as possible molecular markers for the prediction of response to ICT in locally advanced carcinoma of the breast.

Keywords: LABC, miRNA-542-3p, NAC, real-time quantitative polymerase chain reaction, survivin mRNA

Introduction

Patients with locally advanced carcinoma of the breast usually have a bad outcome when they managed by surgery and or radiotherapy.[1] Therefore, induction chemotherapy (ICT) was used.[2] ICT helps to improve the survival and locoregional of breast carcinoma.[3] ICT provides many benefits: Large infiltrative malignant lesions can efficiently being reduced and removed by lumpectomy. Decreasing the extent of the surgery results in better cosmetic outcomes and reducing the locoregional recurrence.[4] The other advantage of ICT is the testing of tumor sensitivity to chemotherapy.[5] The major drawbacks of ICT are: Pre-operative treatment causes a lack of data on the status of the axillary nodes before chemotherapy and lengthens the time to surgery.[6] Many clinical trials were conducted using ICT with variable numbers of cycles before surgical interventions. The published clinical response to ICT ranges between 30 and 90% with a 10–35% complete clinical response.[1,7-9]

Apoptosis is the process by which the body eliminates the senescent or damaged cells. This process is regulated by proteins that oppose or enhance cell death. Many cytotoxic drugs destroy malignant cells by interference with apoptosis.[10] The malignant transformation in the cell is promoted by abnormal inhibition of apoptosis.[11] Survivin is a protein that prevents the apoptotic pathways dependent or independent on caspase described as apoptosis inhibitors. It is located on chromosome 17q25.[12] Survivin levels are usually low in non-malignant cells, but it is usually increased in several malignancies such as prostatic, colon, pancreas, lung, and lymphoid malignancies.[13] Survivin expression showed a poor prognosis in most malignancies studied.[14]

MiRNAs are short non-coding molecules consists of 20–22 nucleotides which negatively control gene expression at the post-transcriptional level. By sequence-guided recognition, miRNAs bound to the 3'-UTR of the target mRNA to induce mRNA degradation or translation repression.[15-18] Many miRNAs seem to be important regulators in breast cancer cells such as cell division, metastases, and invasion. miR-542-3p is a part in these miRNAs, distinguished by the regulation of essential tumor-related mechanisms inhibiting malignant transformation.[19,20] However, a little is published concerning the role of miR-542-3p in breast carcinoma. miR-542-3p regulates survivin mRNA by binding to one of three putative binding sites on its 3'-UTR. Upregulation of miR-542-3p leads to the downregulation of survivin and arrest of the cell cycle at G1 and G2/M phases resulting in tumor growth suppression.[21]

The current study aims to evaluate the role of survivin and miRNA-542-3p in breast carcinoma and to study their role as molecular biomarkers for the prediction of the response to ICT.

Materials and Methods

Patients

The study conducted at the Clinical Oncology Department, Faculty of Medicine, Zagazig University, during the period between June 2013 and February 2020. The study included 51 female patients with histopathologically proved Stage II and III breast cancer, according to the American Joint Committee on Cancer.[22] The study protocol was approved by the Ethical Committee of the Zagazig University and informed consent was taken from each patient.

All patients were subjected before enrollment in the study to a careful history and physical examination, Complete Blood Picture (CBC), liver and kidney functions, electrocardiogram, X-ray of the chest, abdominal ultrasonography, echocardiogram, mammograms, and magnetic resonance imaging of both breasts, and isotopic bone scintigraphy. All patients had been diagnosed by a core biopsy. Determination of hormone receptors (estrogen and progesterone) and HER2 receptors were carried out by immunohistochemistry.

ICT (FEC) regimen was administered at 3 weeks interval until the achievement of complete response (CR) or maximal partial response. The number of chemotherapy cycles ranged from 3 to 5, with a median of 4 cycles. The response to chemotherapy was evaluated according to the revised Response Evaluation Criteria in Solid Tumors criteria.[23] Evaluation of response was carried out by clinical examination, mammography, and breast ultrasonography. Conservative surgery was done for eligible patients, while the others underwent a modified radical mastectomy.

Complete axillary dissection was done in complete clinical responders with a re-biopsy of previous tumor sites. Radiation therapy was planned in patients with complete pathologic response (pCR) and patients with a partial pathological response after excision of the residual tumor site.

The pathologic response was evaluated on the tissues excised during breast preservation or mastectomy or dissected axillary lymphatics. No evidence of residual malignant cells in the excised specimens was identified as pCR.[24]

Tissue specimens were obtained during surgery from freshly resected tumors and nearby normal breast tissues. All sections were cleaned in saline followed by storage at −80°C for further analysis.

RNA extraction and quantitative real-time-PCR

To isolate the RNA from the tissues, we utilized TRIzol (Invitrogen, Carlsbad, CA, USA) and Taqman miRNA package for reverse transcription was used to synthesize the cDNA (Applied Biosystems, Carlsbad, CA, USA), and evaluation of the levels of survivin-mRNA and miR-542-3p was carried out by TaqMan MicroRNA analysis Kit (Applied Biosystems, Carlsbad, CA, USA). The real-time PCR was conducted by TB Green Premix Ex Taq II (Tli RNase H Plus) by Takara Bio (Takara Bio USA Inc., Mountain View, CA 94043 USA).

B-actin and RNA U6 were used as internal controls for survivin and miR-542-3p, respectively. The following sequences of the primers were used: Survivin sense: 5'-TCCGCAGTTTCCTCAAATTC-3′ and reverse: 5′- TTGCGCTTTCCTTTCTGTC-3′; β-Actin: sense: 5'- CCTTGCACATGCCGGAG-3′ and reverse: 5′- GCACAGAGCCTCGCCTT-3′; miR-542-3p: sense: 5'- TGTGACAGATTGATAACTGAAA-3′ and reverse: 5′- GTGCAGGGTCCGAGGT-3′; and U6: sense: 5'-GCTTCGGCAGCACATATACTAAAAT-3′ and reverse: 5′-CGCTTCACGAATTTGCGTGTCAT -3′.

The PCR reactions were utilized in triplicate and the relative expressions of survivin mRNA and miR-542-3p were standardized to β-actin and U6 levels, respectively. The mean value of the triplicate PCR after standardization with the internal controls was used to compute the relative quantity of survivin mRNA and miR-542-3p according to $2^{-\Delta\Delta Ct}$ model.[25]

Statistical analyses

Statistical analysis was conducted by SPSS® version 23.0 (SPSS Inc., Chicago, IL, USA) for Windows®.

Results

Clinical and pathological features of included patients

Table 1 represents the clinical and pathological features of all patients. Their ages ranged from 25 to 65 years, with a median of 52.

Survivin and miR-542-3p in breast cancer tissues and adjacent normal breast tissues (ANBT)

Survivin-mRNA levels were increased in malignant tissues significantly relative to their ANBT. The levels of expression were expressed as the mean of $2^{-\Delta\Delta ct}$. The mean level of survivin-mRNA was 12.76 ± 5.08 (mean ± SD) in cancer tissues versus 1.35 ± 0.83 (mean ± SD) in ANBT (t = 15.82, P < 0.0001) [Figure 1a]. Meanwhile, miR-542-3p was markedly elevated in ANBT relative to BC. The mean level in ANBT was 17.19 ± 2.12 (mean ± SD) versus 1.52 ± 0.94 in BC (t = 47.97, P <0.0001) [Figure 1b]. The relationship between Survivin-mRNA and miR-542-3p levels in BC and their ANBT were found to be statistically significant (one-way ANOVA f = 408.2, P < 0.0001) [Figure 1c]. The relationship between both Survivin and miR-542-3p in BC and ANBT was found to be statistically significant (Kruskal-Wallis Test= 158.2586, P < 0.00001). Pearson correlation showed a strong correlation between survivin-mRNA and miR-542-3p, (r^2 = 0.8042, P < 0.0001) [Figure 1d].

Table 1: Patient's characteristics

Age		
Range	25–65 years	
Median	52 years	
Menopausal status		
Postmenopausal	28	54.9%
Premenopausal	23	45.1%
Clinical stage		
IIA	15	
IIB	11	
IIIA	15	
IIIB	5	
IIIC	5	
Pathology		
Infiltrative duct carcinoma	42	82.4
Infiltrative lobular carcinoma	9	17.6
Grade		
I	12	
II	16	
III	17	
IV	6	
ER		
Positive	31	60.8%
Negative	20	39.2%
PR		
Positive	26	50.9%
Negative	25	49.1%
Her-2		
Positive	8	15.7%
Negative	43	48.3%

Clinical and pathological response after chemotherapy

The response after ICT was recorded in 88.2% of patients (45/51), 29.4% of patients (15/51) have a CR (χ^2 = 17.294, P < 0.001) (Table 2).

Following ICT and surgery, pathological examination of surgical specimens revealed that 7 patients (13.7%) achieved a pCR and 13 still had a microscopic residual disease (25.5%), the difference was statistically significant between groups as regards the residual disease after surgery (χ^2 = 18.353, P < 0.001), and without a significant relation regarding CR and pCR at the primary site after surgery (χ^2 = 5.776, P = 0.056) [Table 3].

Our results revealed that 66.7% of our patients with locally advanced breast cancer who were not candidates for breast conservative surgery preserved their breast after neoadjuvant chemotherapy versus 37.3% underwent a modified radical mastectomy.

Survivin and miR-542-3p expression and the clinical response to ICT

Analysis of the levels of survivin-mRNA and miR-542-3p in BC and response to treatment revealed a significant correlation between survivin-mRNA levels and response. In patients with CR, survivin-mRNA was decreased with a mean ± SD value of survivin of 6.7 ± 0.11 while in patients with no response, it was elevated 18.97 ± 1.18 (mean ± SD). This variation was significant (P < 0.0001) [Figure 2a]. On the contrary, miR-542-3p was elevated in the complete responder with 2.85 ± 0.14 mean and SD value versus 0.39 ± 0.04 mean and SD value in patients with no response (P < 0.0001) [Figure 2b].

Table 2: Assessment of clinical response to induction chemotherapy

Response	Tumor		Nodes		χ^2	P
	No.	%	No.	%		
CR	15	29.4	15	29.4	17.3	0.001
PR	30	58.8	30	58.8		
SD	6	11.8	6	11.8		
Total	51	100	51	100		

CR: Complete response, PR: Partial response, SD: Stable disease

Table 3: Assessment of pathological and clinical response at the tumor site after surgery

Pathological response**	Clinical response**		Total	
	CR	PR	No.	%
No residual disease*	5	2	7	13.7
Microscopic residual disease*	11	2	13	25.5
Macroscopic residual disease*	3	28	31	60.8
Total	19	32	51	100

*(χ^2 =18.353, P<0.001), **(χ^2 =5.776, P=0.056)

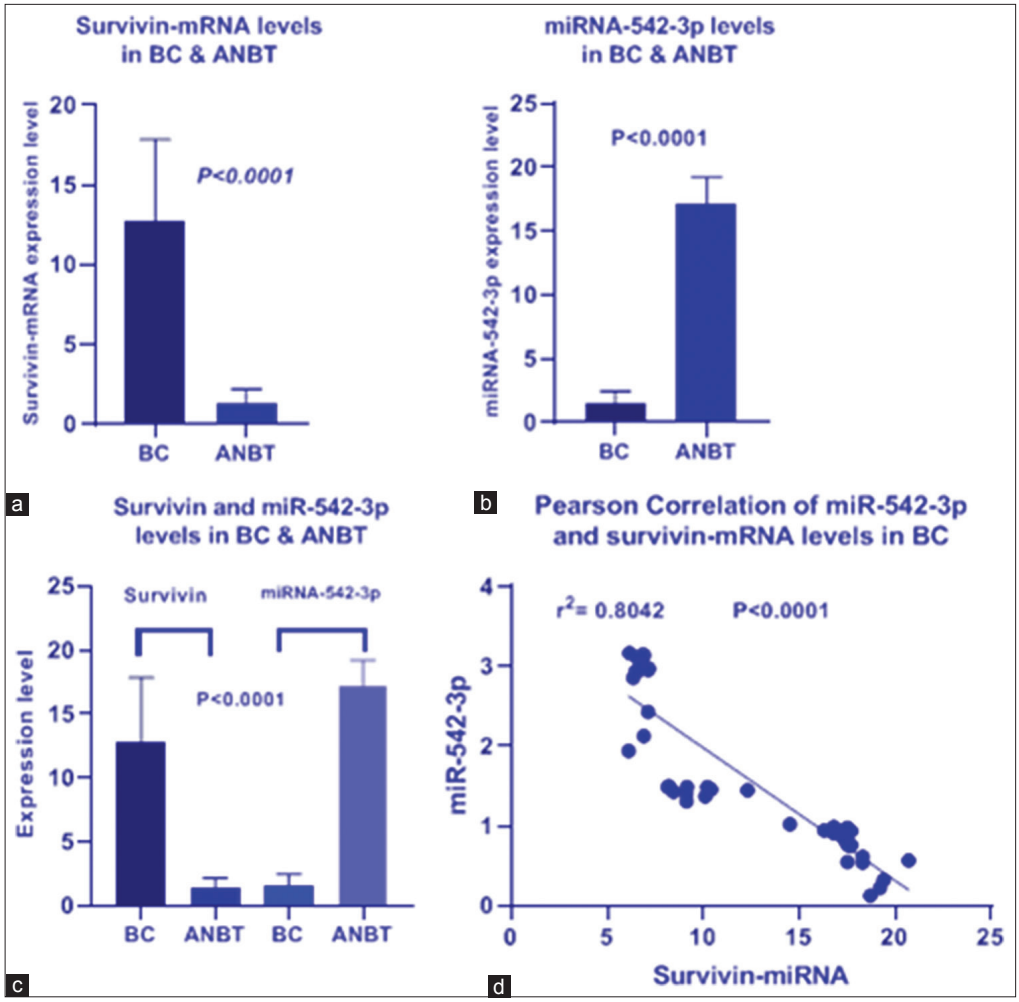

Figure 1: Survivin-mRNA and miR-542-3p levels in BC and NABT. (a) Survivin-mRNA levels in BC and ANBT ($P < 0.0001$), (b) miR-542-3p levels in BC and ANBT ($P < 0.0001$), (c) Comparison of levels of survivin-mRNA and miR-542-3p in BC and ANBT, ($P < 0.0001$), and (d) relationship between levels of survivin-mRNA and miR-542-3p in BC revealed strong correlation (Pearson correlation)

Survivin-mRNA and clinicopathological features

In our series of patients, we noted that increased levels of survivin-mRNA expression were significantly linked to poor clinical and pathological features. Increased expression was correlated with advanced-stage tumors (<0.00001), with premenopausal patients (<0.00001), with infiltrative lobular carcinomas (ILC) (<0.00001), with poorly differentiated tumors (<0.00001), with estrogen and progesterone negative tumors (<0.00001), and with HER2 positive tumors ($P = 0.000132$) [Table 4].

When we analyzed whether survivin-mRNA expression was linked to a worse prognosis, we calculated a univariate regression analysis with the clinicopathological features. No significant correlation between survivin-mRNA levels and other parameters except HER2 positivity ($P = 0.0252$). However, in a multivariate study, progesterone receptors ($P < 0.05$) and undifferentiated tumors ($P = 0.018$) were strongly correlated with survivin-mRNA expression [Table 5].

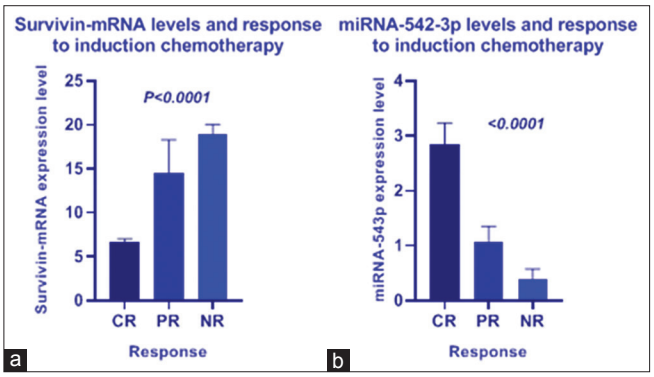

Figure 2: Levels of survivin-mRNA and miR-542-3p and treatment response. (a) Patients with complete response (CR) have low levels of survivin-mRNA ($P < 0.0001$). (b) Patients with CR have high levels of miR-542-3p ($P < 0.0001$). PR: Partial response, NR: No response

miR-542-3p and clinicopathological features

On the contrary to survivin-mRNA, miR-542-3p elevations were strongly related to good clinical and pathological

Table 4: Survivin-mRNA levels and clinicopathological features

Histopathological parameters	Number	Mean	Variance	F ratio	*P*
Stage					
IIA	15	6.7	0.11	323.1	<0.00001*
IIB	11	10.0	3.71		
IIIA	15	17.1	0.14		
IIIB	5	17.7	0.11		
IIIC	5	19.3	0.83		
Menopausal status					
Postmenopausal	28	8.71	9.36	57.7[a]	<0.00001*
Premenopausal	23	17.69	1.05		
Histopathological type					
Infiltrative duct carcinoma	42	1.19	0.45	67.7[a]	<0.00001*
Infiltrative lobular carcinoma	9	3.06	0.01		
Histopathological grade					
I	12	18.27	1.17	167.9	<0.00001*
II	16	16.15	4.41		
III	17	7.82	1.91		
IV	6	6.71	0.12		
Estrogen receptors (ER)					
Positive	31	2.03	0.75	42.4[a]	<0.00001*
Negative	20	0.73	0.07		
Progesterone receptors (PR)					
Positive	26	8.08	4.27	435.3[a]	<0.00001*
Negative	25	17.63	1.00		
Her-2 Receptors					
Positive	8	18.7	18.7	1.1[a]	0.000132*
Negative	43	11.7	11.7	22.6	

*Statistically significant, ANOVA: single factor, [a]Student's *t*-test

Table 5: Multivariate regression analysis of Survivin mRNA and clinical and pathological parameters

	Coefficients	Standard error	*t*-Stat	*P*-value	Lower 95%	Upper 95%
Intercept	3.608	3.195	1.129	0.265	−2.839	10.055
Stage	0.264	0.647	0.408	0.686	−1.042	1.570
miRNA-542-3p	−1.287	0.668	−1.926	0.061	−2.635	0.061
Response	1.045	0.866	1.207	0.234	−0.703	2.793
Pathological type	1.178	0.667	1.768	0.084	−0.167	2.524
Histopathological grade	−0.973	0.394	−2.469	0.018*	−1.769	−0.178
HER2	0.826	0.755	1.094	0.280	−0.697	2.349
PR	5.604	0.819	6.842	0.000*	3.951	7.257
ER	−0.318	0.528	−0.603	0.550	−1.383	0.747

features such as postmenopausal status ($P < 0.00001$), early-stage tumors ($P < 0.00001$), highly differentiated tumors ($P < 0.00001$), infiltrative duct carcinomas ($P < 0.00001$), tumors with positive estrogen ($P < 0.00001$), and progesterone receptors ($P < 0.000001$) and negative HER2 expression ($P = 0.0003$) [Table 6].

We evaluated whether elevated levels of miRNA-542-3p were related to a good prognosis. A strong relationship between elevated levels and HER2 negativity was noted by univariate analysis ($P = 0.0003$) and confirmed also by multivariate analyses ($P = 0.041$) in addition to the histopathological type of the tumor ($P = 0.000$) [Table 7].

Discussion

ICT is a standard of care for women with locally advanced, non-metastatic breast cancer and who needs to preserve their

Table 6: Clinical and pathological parameters and miR-542-3p levels

Histopathological parameters	No	Mean	Variance	F ratio	*P*
Stage					
IIA	15	2.85	0.143	201.2	<0.00001*
IIB	11	1.40	0.019		
IIIA	15	0.94	0.002		
IIIB	5	0.69	0.010		
IIIC	5	0.36	0.038		
Menopausal status					
Postmenopausal	28	2.15	0.69	19.1[a]	<0.00001*
Premenopausal	23	0.76	0.07		
Histopathological type					
Infiltrative duct carcinoma	42	1.19	0.45	67.7[a]	<0.00001*
Infiltrative lobular carcinoma	9	3.06	0.01		
Histopathological grade					
I	12	0.58	0.06	60.16	<0.00001*
II	16	1.01	0.03		
III	17	2.11	0.50		
IV	6	3.09	0.01		
Estrogen receptors					
Positive	31	2.03	0.75	42.47[a]	<0.00001*
Negative	20	0.73	0.07		
Progesterone receptors					
Positive	26	2.24	0.62	38.5[a]	<0.00001*
Negative	25	0.77	0.06		
Her-2 receptors					
Positive	8	0.46	0.05	15.38[a]	0.0003*
Negative	43	1.72	0.80		

*Statistically significant, ANOVA: single factor, [a]Student's *t*-test

Table 7: Multivariate regression analysis of miRNA-542-3p and clinical and pathological parameters

	Coefficients	Standard error	*t*-Stat	*P*-value	Lower 95%	Upper 95%
Intercept	3.398	0.491	6.925	0.000	2.407	4.388
Survivin	−0.063	0.033	−1.926	0.061	−0.129	0.003
Stage	−0.136	0.142	−0.955	0.345	−0.422	0.151
Response	−0.601	0.172	−3.501	0.001*	−0.947	−0.255
Pathological type	0.597	0.122	4.888	0.000*	0.351	0.843
Histopathological Grade	0.054	0.093	0.584	0.562	−0.133	0.242
HER2	−0.340	0.161	−2.108	0.041*	−0.665	−0.014
PR	0.105	0.263	0.399	0.692	−0.426	0.636
ER	0.001	0.117	0.010	0.992	−0.236	0.238

breasts by tumor reduction and at the same time keen about reducing locoregional failure.[26] Besides these considerable advantages, ICT offers a basic advantage for evaluating the effect of chemotherapy on the tumor during surgery.[27]

ICT acts by reducing the size and infiltration of breast primary and eliminating the systemic spread of malignant cells.[28] Unfortunately, eliminating the systemic spread, malignant cells were widely neglected by the most of the studies, although cancer metastases are the primary cause of death in breast cancer and precise determination of micrometastatic cells the circulation is feasible,[29,30] with a clinical validity in different types of cancer.[31,32]

In the present study, we recorded 88.2% of the patients had a response to chemotherapy and CR was noted in 29.4% of them. Asselain *et al*.,[2] in their meta-analysis, reported a 28% complete clinical response and 41% partial response

after neoadjuvant chemotherapy. Furthermore, Klein *et al*.[33] observed a CR in 25.2% of his patients and a partial response in 61.2%. These results support our results.

In the ACOSOG Z1071 clinical trial, Haffty *et al*. reported that 40.4% of their patients preserved their breast after ICT, 72.2% of their patients still have microscopic residue at the primary tumor site and 27.8% had pCR.[34] In the current study, after chemotherapy and surgery, pathological examination of surgical specimens recorded 13.7% of patients with pCR to chemotherapy and 25.5% with microscopic residual disease. Our study revealed that 60.7% of our patients preserved their breasts after ICT.

To improve the outcome of women with advanced breast carcinoma, we sought to identify molecular markers to predict the chemotherapy response and try tailoring the therapy according to the specific features of the individual tumor.

We studied the relationship between survivin-mRNA and miRNA-542-3p in breast cancer as they are not fully investigated in breast cancer. The major criticism about ICT is the delay of surgery which may affect the local control. Hence, we studied whether we can use survivin-mRNA and miRNA-542-3p as potential molecular markers to predict response to chemotherapy.

Survivin acts as a controller for mitosis and an inhibitor of apoptosis. miR-542-3p target survivin at 3′-UTR regions inhibiting cell proliferation by inducing arrest of the cell cycle at G1 and G2/M. This means that miR-542-3p is the key regulator of survivin.[21]

miR-542-3p was confirmed as the main regulator of survivin-mRNA in several malignant tumors,[19,35-37] we evaluated if survivin is affected by miR-542-3p in breast cancer. At first, we estimated the levels of survivin-mRNA and miR-542-3p in breast cancer and then studied their clinical relation to breast cancer. Our finding showed a negative impact of the downregulation of miR-542-3p on survivin-mRNA.

In the current study, we noted a negative relationship between miRNA-542-3p and survivin. Upregulation of miRNA-542-3p results in the down-regulation of survivin mRNA. In the ANBT, the levels of miRNA-542-3p were significantly elevated and survivin-mRNA was significantly reduced. The same observation was also noted in neuroblastoma, bladder cancer, astrocytoma, and colorectal cancer.[19,35-37]

In our included patients, we found a strong relationship between levels of survivin-mRNA, miR-542-3p, and response. In complete responders to treatment, survivin-mRNA levels were low compared with high levels of miR-542-3p. On the other side, patients with no response to chemotherapy, survivin-mRNA levels were highly increased compared to miR-542-3p levels which were significantly reduced.

In our series of patients, increased levels of survivin-mRNA had a significant relationship with adverse clinical and pathological features such as premenopausal status, estrogen and progesterone receptors negative tumors, HER2 receptor-positive tumors, ILC pathology, poorly differentiated tumors, and advanced tumor stage. Meanwhile, miR-542-3p levels were highly elevated in patients with good clinical and pathological parameters. Our observations were also reported by Zhang *et al*. in bladder cancer, they found that increased levels of survivin-mRNA and low miRNA-542-3p levels were linked to an advanced tumor and high rate of local recurrence.[37] Ye *et al*. also noted that the relationship between low levels of miR-542-3p and bad prognostic features such as lymphatic and vascular infiltration, systemic metastases, and advanced disease in colorectal cancer.[38]

On the contrary to our results and the others, Takeyama *et al*. have shown in their study in colorectal cancer that patients with hepatic metastases had an elevated miR-542-3p more than those without metastases.[38] Such variation may be attributed to differences in the patient's pathological features or genetic factors in the studied patients.

Since the miRNA-542-3p-survivin signal axis is not fully studied, and their role in breast cancer requires further studies and additional ideas into its functional mechanisms in breast cancer can be useful.

Conclusions

ICT is a reliable option for the treatment of advanced breast carcinoma. The studied miRNA-542-3p and its target survivin-mRNA correlate negatively with each other in both malignant and their nearby normal tissues. miRNA-542-3p and survivin can be used as possible molecular markers to predict the results of treatment with chemotherapy.

Ethics Approval and Consent to Participate

The study was approved by the Committee of Ethics of research, Zagazig University, Egypt. Informed consent was obtained from all participating patients before enrollment in the study.

Availability of Data and material

The data used in this study are available and will be provided by the corresponding author on a reasonable request.

Competing Interests

The authors declare no conflicts of interest.

Funding Statement

Not applicable.

Authors' Contributions

Initial manuscript preparation: Wael H. Elsawy. Study concepts and design: Amal F. Gharib, Ashjan Shami, and Saleh Ali Alghamdi. Data Acquisition: Amal F. Gharib, Wael H. Elsawy, and Majed Al Mourgi. Statistical analysis: Wael H. Elsawy. Data Analysis and interpretation: Wael H. Elsawy, Amal F. Gharib, Ashjan Shami, Alhazmi Ayman, and Majed Al Mourgi. Manuscript editing: All authors. Manuscript review: All authors.

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