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Research Article

Evaluating the Role of miR34a in Predicting Early Myocardial Damage in Breast Cancer Patients Undergoing Anthracycline-based Chemotherapy

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Abstract

Objectives: Currently, there are no biomarkers for early detection of anthracycline-induced cardiotoxicity. This study explores whether plasma levels of microRNA34a (miR34a) could serve as predictive markers for cardiotoxicity in breast cancer patients undergoing anthracycline-based chemotherapy.

Methods: Forty-four breast cancer patients receiving anthracycline-based chemotherapy for the first time were enrolled. Plasma samples were collected before and after chemotherapy to assess cardiac troponin-I (cTn-I), miR34a, pre-miR34a levels, and echocardiographic strain.

Results: We observed statistically significant increases in cTn-I, miR34a, and pre-miR34a levels post-treatment, with miR34a and pre-miR34a increasing by 2.5-fold and 2.3-fold, respectively. Echocardiographic analysis showed significant reductions in global longitudinal strain (GLS) from baseline after anthracycline treatment. Increases in plasma miR34a levels post-doxorubicin did not correlate with changes in cTn-I or GLS. Furthermore, while a higher miR34a/pre-miR34a ratio was noted in patients with myocardial deformation compared to those without, this did not reach statistical significance.

Conclusion: Despite increases in miR34a levels following anthracycline-based chemotherapy, there is no clear statistical correlation with early myocardial damage. This suggests that miR34a is not a reliable biomarker for anthracycline-induced cardiotoxicity, underscoring the need for further research to identify more definitive predictive markers for cardiotoxicity in breast cancer patients.

Keywords: Anthracycline, breast cancer, biomarker, cardiotoxicity, microRNA

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Breast cancer accounts for 24.2% of all cancers in women globally.[1] Recent advances in breast cancer treatment modalities have significantly improved patient survival.[2] However, cardiotoxicity-related breast cancer treatment has resulted in increased cardiovascular complications, making cardiovascular disease the leading cause of death among breast cancer survivors.[3] Cardiotoxicity is of particular concern given the broad impact of breast cancer on the female population, with anthracyclines remaining a fundamental component of therapy. The spectrum of cardiovascular complications from anthracycline-based treatments ranges from subclinical ventricular dysfunction to severe heart failure, potentially resulting in mortality. Thus, early recognition of cardiotoxicity is crucial to prevent ventricular complications.[4]

Cardiac imaging, especially echocardiography, is central to assessing cancer therapeutics-related cardiac dysfunction(CTRCD), defined as left ventricular(LV) systolic dysfunction with either greater than 10% reduction in left ventricular ejection fraction(LVEF) or a drop to below 50%.[5, 6] However, significant reductions in LVEF are only noted after considerable myocardial damage has occurred.[7] Global longitudinal strain (GLS) measured by speckle tracking echocardiography(STE), has emerged as a critical parameter for detecting subclinical ventricular dysfunction.[8] GLS is now recommended as a useful tool for detecting early myocardial damage in numerous guidelines.[5, 9] While the effects of anthracyclines on LV dysfunction are well-documented, data on right ventricular(RV) function are limited.[9] Conflicting results have been reported regarding cancer therapy-induced right heart dysfunction, with a growing interest in assessing RV function by STE.[10]

Cardiac troponins (cTn-I/cTn-T) are widely used as circulating markers to identify early myocardial injury caused by anthracycline treatment.[11] Although there is no consensus between cardiology and oncology societies regarding the use of cardiac troponin as a biomarker, the European Society of Cardiology (ESC) considers its use based on patients' cardiovascular risk status.[12] The ongoing search for biomarkers capable of detecting cardiotoxicity at an early and reversible stage has led to several recent studies investigating the role of microRNAs in anthracycline-induced cardiotoxicity.[13]

MicroRNAs, small noncoding RNAs consisting of 22–26 nucleotides, play a crucial role in gene expression regulation.[14] MicroRNA34a (miR34a) influences various cellular processes, including apoptosis, differentiation, senescence, and energy metabolism,[15, 16] and acts as a regulator in cardiac injury and repair.[17-19] Increased levels of miR34a in heart tissue and plasma following doxorubicin exposure in animal models have bolstered its potential as both a biomarker and a therapeutic target.[20-23]

Recent findings suggest various molecules contribute to miRNA biogenesis.[24, 25] The discrepancy between pre-miRNA and miRNA levels arises from the posttranscriptional modification during miRNA biogenesis, potentially due to genetic mutations or signaling pathway defects.[26-28] It is therefore crucial to compare levels of pre-miRNA and mature miRNA to understand the functional effects of the target miRNA.

This study is the first to assess plasma miR34a levels alongside precursor pre-miR34a and GLS as markers of early myocardial damage. Our objective was to identify echocardiographic parameters associated with early subclinical cardiac dysfunction following doxorubicin-based chemotherapy in breast cancer patients.

Materials and Methods

Study Population

The protocol for this prospective cohort study was approved by the local clinical research ethics committee. We included fifty breast cancer patients who sought treatment at our oncology department between May 2017 and December 2017 for their first anthracycline-based chemotherapy. All patients provided consent to participate. Patients with advanced heart failure (New York Heart Association (NYHA) Stage II or higher), a history of myocardial infarction, and renal failure requiring renal replacement were excluded.

A patient flow chart is presented in Figure 1. Out of the initial fifty patients, two were excluded due to poor echo-

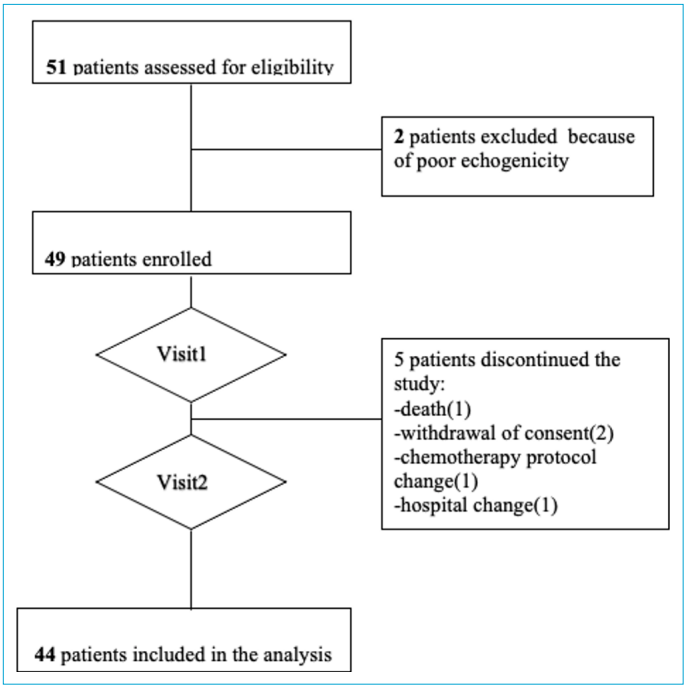

Figure 1. Patient flow chart.

genicity. Post-consent, two patients withdrew, one patient passed away, one had her chemotherapy protocol altered by her oncologist, and another changed hospitals.

All participants received one of the following chemotherapy regimens: doxorubicin-cyclophosphamide (AC), cyclophosphamide-doxorubicin-fluorouracil (CAF), or paclitaxel-doxorubicin-cyclophosphamide (TAC). Treatment regimens were determined by the patients' physicians without intervention from the study team. Each patient was evaluated twice: once before the initial doxorubicin administration (T0) and again within four weeks after the final administration (T1). These evaluations included assessments for symptoms of cardiac dysfunction, echocardiography with strain analysis, serum troponin-I levels, and plasma levels of miR34a and pre-miR34a.

Analysis of Echocardiography Data

A comprehensive two-dimensional echocardiography examination was performed in the left lateral position using an M4S-RS probe (Vivid S6, GE Medical Systems, Horton, Norway) at baseline and after the completion of doxorubicin treatment. Echocardiography images were obtained from the parasternal-short axis, the parasternal-long axis, as well as the apical2- and 4-chamber views. Echocardiographic measurements were made based on the criteria recommended by the European Association of Cardiovascular Imaging (EACVI).[29] Cardiotoxicity was defined as LVEF reduced to below 50% or a greater than 10% reduction from baseline to the lower limit of normal, which is recommended as 54% for women and 52% for men by ESC and EACVI.[5]

LVEF was measured using the biplane Simpson's method. Tricuspid annular plane systolic excursion (TAPSE) was used to assess right ventricular (RV) function. The mitral peak velocity of the early filling(E)and the early diastolic velocity of the lateral and septal mitral annulus (E') were calculated. The E/E' ratio was also recorded as a reliable index of left ventricular filling pressure. Each measurement was obtained after at least three consecutive cardiac cycles.

Measurement of Myocardial Strain

The standard three apical views were used to obtain global longitudinal peak left ventricular strain (LV GLS), and apical four chamber views were used for global longitudinal peak right ventricular strain (RV GLS). A strain analysis was performed using the Echopac PC version BT13 software. According to the position paper from the ESC, a decrease in LV GLS of greater than 15% was considered predictive of cardiotoxicity.[5]

Measurement of Troponin-I Levels

The serum troponin-I level was measured in two hours at our hospital's biochemistry laboratory after blood samples were taken. This study was performed by an immunoassay method using a monoclonal antibody specifically designed for cardiac TnI.

Study of miR-34a and Pre-miR-34a Levels and RNA Isolation

A total of 86 samples were collected from 43 patients in visit 1 and visit 2, and RNA isolation was performed using the miRNeasy Serum/Plasma Kit (Cat. No 217184, QIAGEN, gMBH, D-40724 Hilden, Germany) in accordance with the manufacturer's instructions. The quantities of RNA obtained were measured using 260/230 (an inorganic contaminant) and 260/280 (an organic solvent) values from a 1.5 µl sample in a nano-drop device. A sample of two patients with nano-drop measurement RNA levels below 10 ng/µl was excluded from the study. We proceeded to the complementary DNA (cDNA) synthesis phase with 82 samples from 41 patients with no DNA contamination by measurement. cDNA synthesis was performed according to the protocol for the miScript II RT Kit (Cat No. ID: 218161, QIAGEN, gMBh, D-40724, Hilden, Germany). cDNA reverse transcriptase PCR results were obtained for 20 ng/µl cDNA. The cDNA samples were prepared according to the protocol for the miScript SYBR Green PCR Kit (Cat No. ID: 218075, QIAGEN). Two target gene primers (miR34a-5p and pre-miR34a) and one reference gene primer (RNU6) were used for two samples from each patient. qPCR results were obtained using the Rotor-Gene 1.7.94 program. The value of r2 was greater than 99, and the activity was in the range of 90–110% for all test results (14 runs in total). All samples were triplicated, and the expression of the targeted miR34a-5p and pre-miR34a were analyzed. The Ct (cycle threshold) values determined based on the threshold value were analyzed. The changes in the targeted miR34a and pre-miR34a expressions after anthracycline treatment in the direction of the data obtained by the real-time PCR method were compared. This comparison was determined by normalizing the targeted miRNAs with the expression of the reference gene (RNU6). Real time PCR data were calculated using the Livak model (2-ΔΔCt).[30]

Statistical Analysis

In this analysis, the mean and standard deviation were the descriptive statistics of choice for variables with a normal distribution and for continuous variables, and the median and interquartile range(IQR) were determined for non-normal distributions or ordinal variables. Categorical variables were described using numbers and percentages. A stu-

dent's t-test was used for independent groups if the continuous variables were normally distributed in the binary comparisons, and a Mann–Whitney U test was used if the data were not normally distributed. A chi-square test was used for categorical variables. A Spearman correlation test was used to investigate the correlations between the echocardiographic parameters and the troponin-I and miR34a levels. A Wilcoxon test was used for non-normal distribution dependent variables.

The program SPSS for Windows (version 20 package) was used for statistical analysis, and a $p \leq 0.05$ was considered statistically significant.

Results

The baseline characteristics of the 43 patients who completed the study are provided in Table 1. All patients were administered doxorubicin as a part of their chemotherapy protocol. The mean cumulative doxorubicin dose was calculated as 227±25 mg/m^2. Of these patients, 81% received the AC regimen, 16% received the CAF regimen, and only one patient received the TAC regimen. Additionally, 65% of the patients received adjuvant treatment, 18% received neoadjuvant chemotherapy for local advanced disease, and 16% received treatment for metastatic disease. The pathological characteristics of breast cancer patients and their stages are detailed in Table 1.

Table 1. Patients and Tumor Characteristics

Characteristics	Patients (n=44 (%))
Age (mean±SD)	52±10
BMI (mean±SD)	26.5±4.1
Menopause status [n (%)]	
Premenopause	19 (43)
Postmenopause	25 (57)
Comorbidities [n (%)]	
Diabetes	3 (7)
Hypertension	9 (21)
Vascular disease	0 (0)
Doxorubicin cumulative dose-mg/m^2 (mean±SD)	242.3±26.72
Cardiovascular risk status [n (%)]	
Low	20 (45)
Moderate	19 (44)
High	5 (11)
LVEF ≤ 55% and decrease of LVEF ≥ 10% [n (%)]	2 (5)
Histological subgroups [n (%)]	
IDC	32 (73)
ILC	1 (2)
Mixed (IDC +ILC)	9 (20)
Other	2 (5)
Molecular subtype [n (%)]	
Luminal A	9 (20)
Luminal B	22 (50)
HER2+	7 (16)
Basal	6 (14)
Stage [n (%)]	
1	0
2	18 (41)
3	18 (41)
4	8 (18)

BMI: Body Mass Index; LVEF: Left Ventricular Ejection Fraction; SD: Standard Deviation.

Patients were categorized into three groups based on cardiovascular risk factors according to the latest consensus statement.[5] We found that 42% of patients were in the low-risk group, 39% in the intermediate-risk group, and 19% in the high-risk group. Detailed information on cardiovascular risk factors is presented in Table 2.

Echocardiographic and Laboratory Parameters that Changed After Treatment

All patients were evaluated using echocardiography before and after treatment, and both tissue Doppler imaging and strain analyses were performed. The parameters assessed included left ventricular and right ventricular global longitudinal strain (GLS) for subclinical myocardial damage, tricuspid annular planar systolic excursion (TAPSE) for right ventricular function, and mitral peak early diastolic velocities (E) for diastolic functions. These measurements, along with the changes in cardiac troponin-I levels, are summarized in Table 3. No patients developed symptoms of heart failure and sinus tachycardia was observed in only one patient. In the study population, LVEF showed a mild reduction at T1 compared to baseline, still within normal limit. Only 3 (6%) patients experienced a >10% drop in EF, however their final EF remained >50 %. According to the latest consensus document,[5] no patient developed cardiotoxicity.

The median reduction in LVGLS after doxorubicin was 9% (IQR 3-20). Subclinical myocardial damage,indicated by a reduction

Table 2. Cardiac parameters that changed with treatment.

	Visit 1 (V1)	Visit 2 (V2)	p
Troponin-I (ng/ml)	0.007±0.011	0.061±0.049	<0.001
LVGLS (%)	19.84±3.15	17.92±3.72	0.001
RVGLS (%)	18.22±2.3	17.90±2.8	0.472
TAPSE (mm)	24±3	22±3	0.002
Septal E/e	6±3	7±3	0.005
Lateral E/e	5±2	6±3	0.015
LVEF (%)	64±0.5	62±0.5	<0.001

LVGLS: Left ventricular global longitudinal strain; RVGLS: Right ventricular global longitudinal strain; TAPSE: Tricuspid annular planar systolic excursion; LVEF: Left ventricular ejection fraction.

Table 3. Comparison of patients with and without myocardial deformation.

	ΔGLS <15 n=28	ΔGLS >15 n=13	p
Cumulative doxorubicin dose (mean±SD)	241±29	244±20	0.732
cTn-I-V2 (median (IQR))	0.04 (0.039)	0.062 (0.057)	0.025
TAPSE (mean±SD)	1.14±3.41	3.31±2.90	0.055
Septal E/e (mean±SD)	1.07±1.76	0.69±2.98	0.612
Lateral E/e (mean±SD)	0.79±1.95	1.0±2.31	0.759
LVGLS-V2 (mean±SD)	19.27±3.46	15.43±2.88	0.002
miR34a (median (IQR))	1.82 (3.55)	1.64 (0.82)	0.889
Pre-miR34a (median (IQR))	1.18 (1.81)	1.13 (1.82)	0.427
miR34a/pre-miR34a (median (IQR))	1.61 (1.49)	2.58 (5.23)	0.287

cTn-I: Cardiac troponin-I; TAPSE: Tricuspid annular planar systolic excursion; LVGLS: Left ventricular global longitudinal strain.

of LVGLS >15%, occurred in 35% (n=15) of patients. Additionally, more than a 15% reduction in RVGLS was demonstrated in 7% of patients. A slight but statistically significant decrease in TAPSE was also noted post-treatment, though it remained within normal limits. Only three patients had a decrease in TAPSE below the lower normal limit of <17mm. Significant increases were observed in lateral and septal E/e ratios.

mir34a and Pre-miR34a Expression Changes After Treatment

Expression levels of miR34a before and after treatment were calculated as fold changes using the Livak model, as described in the materials and methods section. As shown in Figure 2, treatment resulted in a statistically significant increase in both miR34a and pre-miR34a expressions. The miR34a/pre-miR34a ratio also indicated that miR34a levels were significantly higher than those of its precursor.

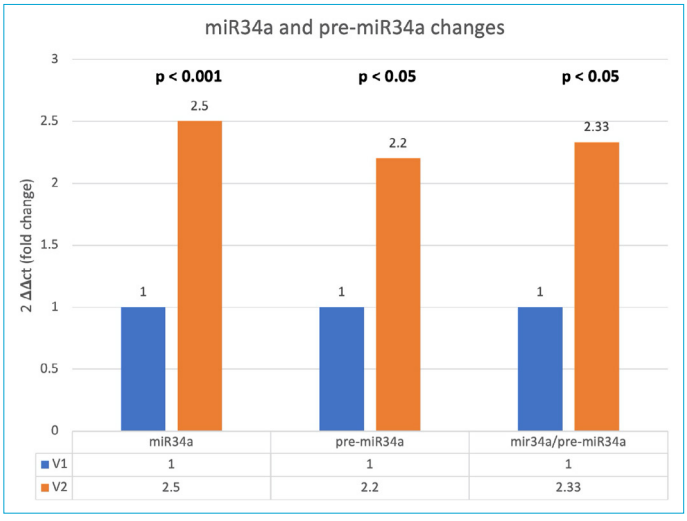

Figure 2. miR34a and pre-miR34a changes after treatment.

Comparison of Patients According to Change in LVGLS

Following the 2020 position statement from the ESC,[6] patients were grouped based on whether their GLS change exceeded 15%. LVGLS analysis for a patient is illustrated in Figure 3. Changes in the echocardiographic parameters, cTn-I levels, and plasma miR34a expressions between the two groups are compared in Table IV. A significant increase in troponin I levels was observed in the group with early subclinical cardiac dysfunction (>15% LVGLS change) compared to the group without dysfunction. No significant differences were found between the groups in terms of miR34a and premiR34a.

Relationship Between Doxorubicin Dose, Troponin-I, miR34a, and Echocardiographic Parameters

In the assessment of cardiac dysfunction based on the GLS changes, no significant differences were found in the cumulative doxorubicin dose between with and without myocardial deformation. However, there was a moderate correlation between cumulative doxorubicin doses and TAPSE (r^2=545 p <0.001) as shown in Figure 4. Increased cu-

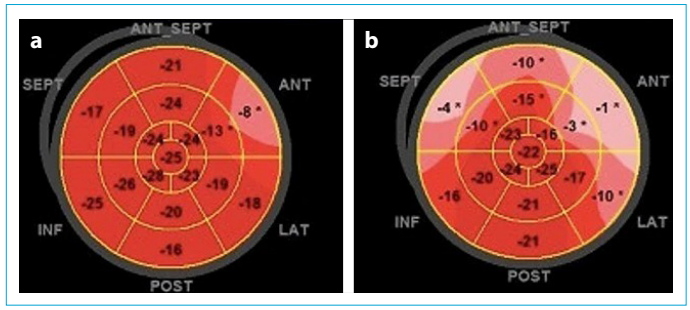

Figure 3. A figure depicting one of patient's LVGLS analysis. **(a)** Baseline LVGLS: 20.6%. **(b)** After treatment LVGLS: 15.3%; change in LVGLS: 25%. (LVGLS: left ventricular global longitudinal strain).

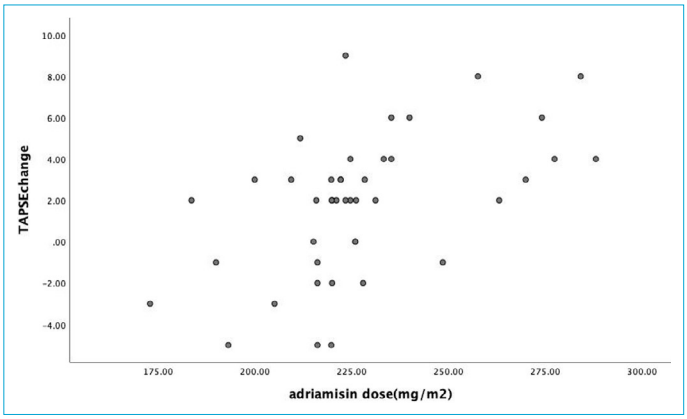

Figure 4. Cumulative Adriamycin Dose (mg/m^2) -TAPSE Correlation Curve.

mulative doses of doxorubicin were associated with greater reductions in TAPSE. A weak moderate correlation was demonstrated between TAPSE-V2 and RVGLS-V2. (r^2=376, p=0.013). No correlations were found between cTn-I, miR34a, and GLS changes.

Discussion

To our knowledge, this is the first study to evaluate miR34a, its precursor pre-miR34a, and cTn-I as markers of early subclinical cardiac dysfunction in breast cancer patients receiving doxorubicin. Our results indicate significant increases in plasma levels of miR34a, pre-miR34a, and cTn-I following doxorubicin administration compared to the baseline measurements.

Previous studies have shown that the miR34 family members, including miR34a, are upregulated in cardiomyocytes under stress conditions such as myocardial infarction, contributing to age-dependent cardiac damage.[17] Desai et al. demonstrated that miR34a is upregulated in doxorubicin-exposed mouse myocardial tissue at very early stages before cardiomyocyte necrosis, unlike troponin.[22] Piegari et al. also reported increased miR34a expression, in cardiac cells, cardiac progenitor cells (CPC), and rat plasma after doxorubicin administration.[20] These findings suggest the potential of miR34a as a biomarker in doxorubicin-related cardiotoxicity. The first human study to explore this was conducted by Fréres et al., which investigated circulating microRNA changes in patients with locally advanced breast cancer following neoadjuvant therapy. Although a secondary outcome, they found a correlation between changes in cTn-T level and miR34a expression after epirubicin treatment.[31] A significant positive correlation was also observed six months post-doxorubicin exposure in a small group of triple-negative breast cancer patients.[32]

While our study was ongoing, Fréres et al. were investigating circulating cardiac biomarkers in patients treated with epirubicin for locally advanced breast cancer. They measured miR34a levels at four time points: before treatment, after two cycles of anthracycline-based chemotherapy, at the end of the chemotherapy and three months post-treatment. Significant increases in miR34a levels were observed at all time points post-epirubisin exposure, with the highest level recorded after two cycles of chemotherapy. However, no correlation was found between the miR34a levels, LVEF, and cardiac troponin T.[33] The higher mean fold change in miR34a expression reported in their studies compared to ours may be attributed to differences in sample collection timing and the proportion of patients receiving adjuvant versus neoadjuvant treatment. In our study, 63% of patients received doxorubicin as adjuvant treatment post-surgery. Plasma miR34a expression and the miR34a/pre-miR34a ratio were higher in patients receiving doxorubicin for neoadjuvant treatment, though this was not statistically significant. Pre-miR34a expression was significantly lower in patients with metastatic disease compared to other patients, aligning with preclinical studies showing lower miR34a expression in metastatic versus non-metastatic breast cancer cells.[34]

mir34a is a well-known tumor suppressor miRNA, transcriptionally regulated by p53 in response to DNA damage.[35, 36] The upregulation of miR-34a following anthracycline treatment may be explained by DNA damage and subsequent p53 activation. Salzman et al. demonstrated that miR34 can also be rapidly activated by phosphorylation in response to DNA damage, explaining the higher plasma miR34a levels compared to its precursor.[37]

In line with the ESC position statement,[5] we compared patients with potential myocardial deformation (LVGLS change >15%) to those without deformation (LVGLS change <15%) in terms of miR34a, pre-miR34a, cTn-I, septal and lateral e / é, TAPSE, and LVEF. Patients with potential myocardial deformation had significantly higher cTn-I levels than the others. Although the change in TAPSE was notable, it was not statistically significant. The miR34a/pre-miR34a ratio was higher in patients with an LVGLS change greater than 15% however, this result was not statistically significant.

We also examined the correlation between cumulative anthracycline doses and myocardial damage parameters. Despite receiving modest doses of doxorubicin(200–300mg/m^2), there was no significant difference in cumulative doses between patients with and without myocardial deformation. However, a positive correlation was observed between cumulative doxorubicin dose and changes in TAPSE. Previous studies have shown that TAPSE decreases after

doxorubicin treatment.[38, 39] Although our study found a significant decrease in TAPSE, the levels remained within the normal range. While the effects of doxorubicin on the left ventricle are well-documented, data on its impacts on the right ventricle are limited.[40] Recent studies highlight the importance of assessing RV function using longitudinal strain analysis.[41] One study reported decreased RVGLS six months post-doxorubicin administration in lymphoma patients.[42] In our study, we assessed RVGLS three months post-treatment and found no significant difference between the V1 and V2 values for RVGLS. However, a correlation was observed between TAPSE-V2 and RVGLS-V2. These findings underscore the importance of evaluating right ventricular function in patients receiving doxorubicin.

The small sample size and short follow-up period are significant limitations of this pioneering study. None of the patients developed heart failure symptoms, and only two patients experienced an asymptomatic decline of LVEF. Long-term follow-up is necessary to determine whether doxorubicin-induced heart failure will manifest. Another limitation is the variability in the sample collection timing for studying miR34a and cTn-I. Despite these limitations, this study is the first to investigate miR34a and pre-miR34a as novel biomarkers for early detection of anthracycline-induced cardiotoxicity in breast cancer patients. The study combines advanced echocardiographic techniques and molecular analyses to provide a thorough assessment of cardiotoxicity.

Conclusion

Our study explored the potential of miR34a as a biomarker for anthracycline-induced cardiotoxicity in breast cancer patients. Although we observed an increase in miR34a and pre-miR34a levels post-chemotherapy, there was no statistically significant correlation with early myocardial damage. These results suggest that miR34a alone may not be a reliable biomarker for predicting anthracycline-induced cardiotoxicity. Further research is needed to identify more definitive biomarkers or a combination of biomarkers to enhance early detection and management of cardiotoxicity in this patient population.

Disclosures

Ethics Committee Approval: The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved. The study was approved by Hacettepe University Non-Interventional Clinical Research Ethics Committee (decision number: GO 17/319-18).

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