A Panel of Serum Noncoding RNAs for the Diagnosis and Monitoring of Response to Therapy in Patients with Breast Cancer

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Background:
The aim of this study was to identify a panel of serum noncoding RNAs (ncRNAs) as potential diagnostic and prognostic biomarkers for breast cancer.

Material/Methods:
Patients with breast cancer (n=30), and normal controls (n=30) were included in the ‘training set.’ A ‘validation set’ included cases of breast cancer (n=128) and controls (n=77). All cases provided blood samples for serum analysis. All cases of breast cancer were confirmed histologically and were staged. Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was used to detect the expression of 11 candidate ncRNAs, including long noncoding RNAs (lncRNAs) and microRNAs (miRNAs), in the serum. The expression of the panel of ncRNAs was further analyzed following surgery or chemotherapy.

Results:
The four ncRNAs identified in the serum of patients with breast cancer included let-7a, miR-155, miR-574-5p, and metastasis-associated lung adenocarcinoma transcript 1 (MALAT1). Analysis based on the risk score showed that the panel of these four ncRNAs could effectively distinguish between patients with breast cancer and the control group. For the training set and the validation set, analysis of the receiver-operating characteristic (ROC) curve showed that the areas under the curve (AUCs) were 0.960 and 0.968, respectively. Also, the serum expression levels of the four ncRNAs differed in the pre-treatment and the post-treatment patients with breast cancer, with levels of miR-155 showing a significant decrease following chemotherapy.

Conclusions:
A panel of serum ncRNAs, including let-7a, miR-155, miR-574-5p, and MALAT1, was shown to be present in patients with breast cancer.

MeSH Keywords: Breast Neoplasms • Diagnosis • RNA, Untranslated • Serum

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Background

In 2017 in the USA, breast cancer was the most commonly diagnosed cancer in women, was the second most common cause of death due to cancer in women, and was expected to account for 30% of all new cancer diagnoses in women [1]. The prognosis for patients with breast cancer is strongly associated with the stage of the disease at diagnosis. Therefore, the diagnosis and treatment of breast cancer at an early stage is a clinical priority. Mammography, ultrasonography, and magnetic resonance imaging (MRI) are routinely used for the detection of breast abnormalities. However, the sensitivity of mammography is moderate and can be affected by age and the density of the breast tissue [2]. Breast ultrasonography has a high rate of false-negative results when used in breast cancer screening, particularly in women with dense breast tissue [3]. The high cost of breast MRI makes it inappropriate for use in screening for early breast cancer. Therefore, more sensitive and specific detection methods for early breast cancer are still needed.

There has been recent interest in the detection of expression of noncoding RNAs (ncRNAs) as a biomarker in several types of cancer. The ncRNAs include long noncoding RNAs (lncRNAs), microRNAs (miRNAs), and circular RNAs (circRNAs), which are short noncoding RNAs (ncRNAs). The miRNAs are approximately 19–25 nucleotides in length and are involved in many biological processes, including cell proliferation, cell differentiation, regulation of the cell cycle, and apoptosis. The IncRNAs are more than 200 nucleotides in length and play an important role in gene regulation and carcinogenesis. Recent studies have shown that miRNAs and IncRNAs are abnormally expressed in tumor tissues and have a role as regulators of tumor initiation and development [4,5]. Currently, several miRNAs and IncRNAs have been reported to be potential diagnostic and prognostic tumor biomarkers, and because they are tumor-derived nucleic acids, they can be released into the peripheral circulation and detected in the plasma and serum [6,7]. Therefore, the detection of ncRNAs (miRNAs and IncRNAs) in the circulation, by analysis of plasma or serum, has potential as diagnostic or prognostic biomarkers for specific cancers [6,7].

In this study, 11 candidate ncRNAs (miRNAs and IncRNAs) that have already been reported to show increased expression in breast cancer tissues were selected as candidate serum diagnostic biomarkers. The aim of this study was to identify a panel of ncRNAs, using quantitative reverse transcription polymerase chain reaction (RT-qPCR), that could be detected in the serum from blood samples and that showed potential as either diagnostic or prognostic biomarkers for breast cancer.

Material and Methods

Ethics statement

The study design and the procedures used involved human participants and were performed ethically and approved by the Clinical Research Ethics Committee of the Cancer Institute and Hospital, Chinese Academy of Medical Sciences, as well as in accordance with the 1964 Helsinki declaration and its later amendments. Informed consent was obtained from all of the individual participants included in the study.

Study populations

A total of 158 patients with breast cancer and 107 healthy women participated in this study. Serum samples were collected from a study ‘training set’ of 30 healthy women and 30 patients with breast cancer. The ‘validation set’ consisted of 128 women with breast cancer and 77 healthy women. Serum samples were also collected from 158 women with breast cancer before they had undergone any treatment, such as chemotherapy, surgery, or radiotherapy. Serum samples from 53 patients with breast cancer were collected before and after performing surgery. Serum samples from 12 patients with breast cancer were collected before and after treatment with vinorelbine chemotherapy.

Study design

To identify the optimal biomarker panel of serum noncoding RNAs (ncRNAs) for breast cancer, the study was divided into two phases that included analysis of a ‘training set’ and a ‘validation set’ of study participants. Microsoft Excel was used to divide all of the samples randomly into two groups. There were 60 participants in the ‘training set,’ including 30 women with breast cancer and 30 healthy women. The ‘validation set’ consisted of 128 women with breast cancer and 77 healthy women. All the study participants were registered at the Cancer Institute and Hospital, Chinese Academy of Medical Sciences, between 2013 and 2014. The women with breast cancer all had histologically confirmed diagnoses of primary breast cancer, with tumor grading and staging by surgery or by imaging. For those patients who did not receive surgical treatment, biopsy and imaging technology were used to determine the histopathology and tumor stage. Breast cancer staging was conducted according to the staging system of the American Joint Committee on Cancer (AJCC) Tumour Node Metastasis (TNM) system (6th Edition). The histological typing of the breast tumors were performed according to the current criteria of the World Health Organization (WHO). Healthy people who had...
no evidence of inflammatory conditions or malignancy of the breast were chosen as the control group. Table 1 summarizes the clinical and demographic characteristics of the subjects included in the study. This study was performed according to the current Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) guidelines [8].

**Serum preparation and isolation of RNA**

Venous blood samples (5 mL) were collected from all study participants and maintained for one hour at room temperature. The serum was separated by centrifugation, transferred to new tubes, and were subjected to 10 minutes of high-speed centrifugation at 16,000 g at 4°C to remove the cells. Serum samples were preserved at –80°C until further analysis.

TRizol LS reagent (Invitrogen, Inc.) was used to extract the RNAs from 250 μL of serum according to the manufacturer’s protocol. A synthetic version of a *Caenorhabditis elegans* (*C. elegans*) microRNA (cel-miR-39) (Takara, Japan) was added to each sample at a final concentration of 10⁻⁴ pmol/μL to control for the variability in RNA extraction and purification.

**Quantitative reverse transcription polymerase chain reaction (RT-qPCR) assay**

An equal volume of serum (250 μL) was processed during RNA isolation for each subject. For detection of microRNAs (miRNAs), a TaKaRa SYBR PrimeScript™ miRNA RT-PCR kit (Takara, Japan) was used to reverse transcribe serum RNA into cDNA (Takara, Japan) according to the manufacturer’s protocol.

For the detection of metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), reverse transcriptase M-MLV was used to reverse transcribe serum RNA into cDNA (Takara, Japan) (RNaseH-) according to the manufacturer’s protocol.

An ABI ViIA™ 7 real-time PCR system (Applied Biosystems) and a TaKaRa SYBR PrimeScript™ miRNA RT-PCR kit (Takara, Japan) were used to perform quantitative PCR. The universal primers were provided by the manufacturer. The miRNA-specific forward primers are shown in Supplementary Table 1.

The quantitative PCR reactions were conducted as follows: at 95°C for 30 seconds; at 95°C for 5 seconds; and at 60°C for 31 seconds, for 40 cycles. The expression of miRNA of all of the samples was normalized to cel-miR-39 [9]. GAPDH was used as a reference gene for the gene expression normalization [10]. All the reactions were conducted in triplicate, and the 2⁻ⁿDΔCt method was used to calculate the relative expression levels of the target genes.

**Statistical analysis**

The chi-squared (χ²) test and Student’s t-test were used to compare the clinical features and the demographics of the two groups (women with breast cancer and controls). An independent sample t-test was used to compare the serum concentrations of ncRNA in the serum samples of the patients with breast cancer and the healthy controls. A risk score function method was applied to build a diagnosis model for patients with breast cancer when compared with the controls, as described previously [11–13]. An appropriate cut-off value was identified, and the diagnostic performance of the model was evaluated by using receiver-operating curve (ROC) analysis. The SPSS version 17.0 software (SPSS Inc., Chicago, IL, USA) was used to perform all the statistical analysis, and p<0.05 indicated the statistical significance. Graphpad Prism 5.0 (Graphpad Software Inc., San Diego, CA, USA) was used to generate the graphs. Cluster 3.0 (Berkeley, CA, USA) was used for hierarchical clustering through the complete linkage method.

### Table 1. Clinical character of study samples.

| Variable     | Breast cancer | Control | p-Value | Surgery | Chemotherapy |
|--------------|---------------|---------|---------|---------|--------------|
| Gender       | Female        | 158     | 107     | –       | –            |
| Age          | 51.19±10.39   | 49.93±8.46 | 0.278   | 49.38±10.04 | 50.13±9.89 |
| Smoking      | Ever          | 0       | 0       | –       | 0            |
|              | Forever       | 158     | 107     | –       | 12           |
| Drinking     | Ever          | 0       | 0       | –       | 0            |
|              | Forever       | 158     | 107     | –       | 12           |
| BC History   | Ever          | 3       | 0       | 0.274   | 0            |
|              | Forever       | 154     | 107     | 53      | 12           |
Results

Expression of candidate noncoding RNAs (ncRNAs) in serum samples of patients with breast cancer and healthy controls

A two-phase experiment was designed to identify a panel of serum noncoding RNAs (ncRNAs) as potential breast cancer diagnostic biomarkers. The two groups studied included a ‘training set’ of 30 patients with breast cancer and 30 healthy women controlled for age, and a ‘validation set’ of 128 patients with breast cancer and 77 healthy women. Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was performed to determine the expression of 11 candidate ncRNAs in the training set. The expression of the microRNAs (miRNAs) and the long noncoding RNA (lncRNA), metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), were normalized to the cel-miR-39 and the expression of GAPDH, respectively. A panel of four serum ncRNAs, including let-7a, miR-155, miR-574-5p, and MALAT1, was shown to be present in patients with breast cancer.

The results showed that, compared with the healthy controls, the expression of let-7a of patients with breast cancer was significantly down-regulated, while the miR-574-5p, miR-155, and MALAT1 in the serum from patients in the breast cancer group were upregulated (Table 2, Figure 1). However, the other seven miRNAs in the initial panel (miR-21, miRNA-10b, miRNA-181b, miRNA-1254, miRNA-196a, miRNA-205, and miRNA-195) did not show significant changes (data not shown).

In the validation set, which consisted of 128 patients with breast cancer and 77 healthy women, the four upregulated ncRNAs were then tested further, to verify the reliability of the results. These results were consistent with those of the training set (shown in Table 2, Figure 1). Therefore, it might be concluded that the patients with breast cancer and the healthy controls had significantly different expression levels of let-7a, miR-574-5p, miR-155, and MALAT1 and that these four ncRNAs might represent a serum biomarker panel with potential in the diagnosis of breast cancer.

Diagnostic performance of the four ncRNAs: let-7a, miR-155, miR-574-5p, and MALAT1

The breast cancer diagnosis model of the four ncRNAs was established using the risk score function. To evaluate the diagnostic performance of serum detection of the panel of ncRNAs that included let-7a, miR-155, miR-574-5p, and MALAT1, a ROC curve analysis was carried out to compare the patients with breast cancer with healthy controls. The parameters were determined using the training set and evaluated using the validation test. Youden’s index indicated the difference between specificity and sensitivity, as when this index reached the maximum value, it could identify the optimal cut-off predictive value. The receiver-operating characteristic (ROC) curve analysis indicated that the areas under the ROC curve (AUCs) for miR-155, let-7a, MALAT1, and miR-574-5p were 0.817, 0.783, 0.650, and 0.867, respectively. However, when the risk score function was used to combine the four ncRNAs, the AUC of the ncRNA panel for the training set was 0.960 (95% CI, 0.864–1.000), and the cut-off value was 9.1815. The optimal specificity and sensitivity were 93.3% and 96.7%, respectively, with a Youden’s index of 0.900 (see Table 3). Using this cut-off value, the samples from 128 patients with breast cancer and 77 healthy people were evaluated as ‘the validation set.’ A significantly increased diagnostic performance was shown for the ncRNA panel, with an AUC of 0.968 (95% CI, 0.903–0.985), the Youden’s index was 0.89, and the specificity and sensitivity were 89.6% and 99.2%, respectively (see Table 3, Figure 2A, 2B). Therefore, when compared with a single ncRNA, a combination of multiple ncRNAs was considerably more sensitive for distinguishing between the patients with breast cancer and the healthy controls (Table 3).

Further analysis was performed for the AUC and for the standard error and compared the statistical test results with p<0.05 (reference value, without discriminant ability). The results showed that compared with the AUC for let-7a, miR-155, miR-574-5p, MALAT1, and the combined four ncRNAs at 0.5, the p-values were p=0.000, p=0.000, p=0.000, p=0.046, and p=0.000, respectively, for the training set; and p=0.000, p=0.001, p=0.000, p=0.000, and p=0.000, respectively, for the validation set. The above results indicated that the diagnostic efficiency of the serum ncRNA panel was high, and the clinical validity was reliable.

To determine the value of the serum ncRNA panel as a diagnostic or screening method, the clinical application of the panel required testing. The predicted value (PV), which is the probability that a test will reach an accurate conclusion, is commonly used as an evaluation index. When the cut-off value was 9.1815 for the training set, the positive predictive value (PPV) and the negative predictive value (NPV) of the panel were 0.96 and 0.85, respectively; while for the validation set, the PPV and NPV were 0.97 and 0.85, respectively (see Table 4). The results indicated that the serum breast cancer diagnostic panel, based on the four ncRNAs, let-7a, miR-155, miR-574-5p, and MALAT1, had diagnostic value.

Unsupervised clustering to distinguish between patients with breast cancer from healthy controls using the panel of four ncRNAs

Classification systems that use unlabeled samples are ‘unsupervised.’ In this experiment, all of the samples were mixed,
Table 2. Different expression of four serum non-coding RNAs in control and BC group.

| Group          | RNA     | Control       | BC             | Average fold change | p-Value |
|----------------|---------|---------------|-----------------|---------------------|---------|
| Training set   | let-7a  | 1.21±0.73     | 0.20±0.23       | 0.17                | p=0.000 |
|                | miR-155 | 2.08±2.77     | 17.51±24.04     | 8.42                | p=0.002 |
|                | miR-574-5p | 3.12±4.42    | 56.35±79.36     | 18.06               | p=0.001 |
|                | MALAT1  | 2.62±2.64     | 8.32±12.74      | 3.18                | p=0.022 |
| Validation set | let-7a  | 1.58±1.53     | 0.26±0.33       | 0.16                | p=0.000 |
|                | miR-155 | 2.49±6.66     | 10.52±19.34     | 4.22                | p=0.000 |
|                | miR-574-5p | 2.30±4.92    | 120.74±213.06   | 52.50               | p=0.000 |
|                | MALAT1  | 3.39±7.00     | 17.24±16.59     | 5.09                | p=0.000 |

Figure 1. Serum levels of the four noncoding RNAs (ncRNAs) (let-7a, miR-155, miR-574-5p, and MALAT1) in patients with breast cancer and healthy controls. The quantitative reverse transcription polymerase chain reaction (RT-qPCR) assay-based SYBR was performed to measure the serum expression levels of the four ncRNAs in the training and validation sets. Each assay was performed in triplicate.
Table 3. Diagnostic performance of each non-coding RNA between BC patients and controls.

| Group          | Non-coding RNAs | Cut-off value | AUC    | p-Value | 95% CI      | Sensitivity | Specificity | Youden's index |
|----------------|-----------------|---------------|--------|---------|-------------|-------------|-------------|----------------|
|                 |                 |               | Lower  | Upper   |             |             |             |                |
| Training set    |                 |               |        |         |             |             |             |                |
| let-7a          |                 | 1.8865        | 0.783  | 0.000   | 0.662       | 0.905       | 96.7%       | 60%            | 0.567          |
| miR-155         |                 | 1.4980        | 0.817  | 0.000   | 0.703       | 0.931       | 83.3%       | 80%            | 0.633          |
| miR-574-5p      |                 | 2.2785        | 0.867  | 0.000   | 0.767       | 0.967       | 96.7%       | 76.6%          | 0.734          |
| MALAT1          |                 | 0.6200        | 0.650  | 0.046   | 0.509       | 0.791       | 63.3%       | 66.7%          | 0.300          |
| Combined        |                 | 9.1815        | 0.960  | 0.000   | 0.864       | 1.000       | 96.7%       | 93.3%          | 0.900          |
| Validation set  |                 |               |        |         |             |             |             |                |
| let-7a          |                 | 1.6405        | 0.683  | 0.000   | 0.602       | 0.764       | 97.7%       | 39%            | 0.367          |
| miR-155         |                 | 0.7615        | 0.638  | 0.001   | 0.562       | 0.714       | 40.6%       | 87%            | 0.276          |
| miR-574-5p      |                 | 2.1990        | 0.891  | 0.000   | 0.837       | 0.944       | 93.8%       | 84.4%          | 0.782          |
| MALAT1          |                 | 2.1345        | 0.875  | 0.000   | 0.818       | 0.932       | 94.5%       | 80.5%          | 0.750          |
| Combined        |                 | 9.1815        | 0.968  | 0.000   | 0.903       | 0.985       | 99.2%       | 89.6%          | 0.896          |

Figure 2. Receiver-operating characteristic (ROC) curves of the panel of four noncoding RNAs (ncRNAs) (let-7a, miR-155, miR-574-5p, and MALAT1) to distinguish between the patients with breast cancer and the healthy controls. (A) Training set: area under the curve (AUC), 0.960; sensitivity, 96.7%; specificity, 93.3%; and cutoff value, 9.1815. (B) Validation set: AUC, 0.968; sensitivity, 99.2%; specificity, 89.6%; and cutoff value, 9.1815.

Table 4. Risk scores analysis of BC patients and healthy controls.

| Group          | Score | <9.1815 | >9.1815 | PPV* | NPV* |
|----------------|-------|---------|---------|------|------|
| Training set   |       |         |         |      |      |
| Control        | 29    | 1       | 0.96    | 0.85 |
| BC             | 5     | 25      |         |      |
| Validation set |       |         |         |      |      |
| Control        | 73    | 4       | 0.97    | 0.85 |
| BC             | 13    | 115     |         |      |

* Positive predictive value; * Negative predictive value.
and unsupervised clustering was carried out to distinguish the patients with breast cancer from the healthy controls by the use of the panel of four ncRNAs, let-7a, miR-155, miR-574-5p, and MALAT1. The results indicated that the signatures of the ncRNAs could be used as a basis to separate the samples from patients with breast cancer from the control samples (as shown in Figure 3). In the training set, two patients with breast cancer, but none of the healthy controls were incorrectly classified. In the validation set, the samples collected from 128 patients with breast cancer and 77 healthy controls were divided into two main categories, and only five samples from the control group were misclassified.

**Expression of four ncRNAs in serum samples from patients with breast cancer before and after surgery**

To determine whether the expressions of the four ncRNAs were different in the serum samples of the patients with breast cancer before and after surgery, serum samples were collected from 53 patients with breast cancer before surgery, and one week after surgery. The expression levels of let-7a, miR-574-5p, and miR-155 were slightly increased in the serum one week after surgery when compared with those before surgery, while the expression of MALAT1 was significantly reduced by one week after surgery (Figure 4).

There was a difference in the expression of miR-574-5p and let-7a patients with breast cancer pre-operatively when compared post-operatively, primarily in the patients with stage II breast cancer (p=0.033 and p=0.037, respectively), while a difference in expression of miR-155 was mainly observed in the patients with stage I breast cancer (p=0.031). The difference in the expression of MALAT1, between the patients with breast cancer compared with the controls, increased with the progression of the clinical stage of breast cancer (Supplementary Figure 1).

Similarly, there was a difference between the expression of miR-574-5p and MALAT1 in breast cancer patients pre-operatively and postoperatively, which were most obvious in patients with T1 and T2–3 tumors, indicating that the tumor size was associated with increased serum levels of the ncRNAs. The different expression of let-7a was the most apparent in patients with T2–3 breast cancer, whereas the expression of serum miR-155 did not change significantly with breast tumor size (Supplementary Figure 2).

The different expression of the ncRNAs pre-operatively and postoperatively by lymph node metastasis status was also studied. As shown in Figure S3, the expressions of miR-574-5p and let-7a were increased in the postoperative patients, and the difference was the most significant in patients with breast cancer N1-3. In contrast, reduced expression of MALAT1 in the...
post-operative patients was not associated with the lymph node metastasis status. The expression of miR-155 showed no significant difference between the pre-operative and the post-operative patients (Supplementary Figure 3).

**Changes in expression levels of ncRNAs in patients with breast cancer treated with the chemotherapy agent, vinorelbine**

Serum samples from 12 patients with breast cancer were analyzed for the levels of the four ncRNAs, let-7a, miR-155, miR-574-5p, and MALAT1 before and after the vinorelbine treatment. The results showed that after the vinorelbine treatment, the expression of miR-155 was significantly decreased in 11 cases out of 12 (p=0.0003), while no obvious changes were observed in the other three ncRNAs. These results indicated that the miR-155 expression was sensitive to vinorelbine and might potentially be used to monitor clinical outcome following chemotherapy (Figure 5).

**Discussion**

Tumor cells can to release microRNAs (miRNAs), also known as short noncoding RNAs (sncRNAs), into the blood, which means that miRNAs can be detected in plasma and serum samples. Previously published studies have shown that extracellular secreted miRNA might play a role in intercellular communication by regulating recipient cell gene expression and affecting the target cell function [14,15]. Recently, long noncoding RNAs (lncRNAs), another type of noncoding RNA (ncRNA) that can be detected in plasma and serum, has been shown to have a role in tumor initiation and progression, with studies showing that it can function as a competing endogenous RNA (ceRNA) that regulates other RNA transcripts by regulating specific miRNAs and the corresponding target genes [16]. Therefore, there is a potential clinical application in investigating the ncRNA targets and their relationship.

In this study, the expression of 11 ncRNAs in the serum samples of patients with breast cancer and healthy people were screened, and a ncRNA panel of four was identified, that included let-7a,
miR-155, miR-574-5p, and metastasis-associated lung adenocarcinoma transcript 1 (MALAT1). This panel of four ncRNAs could be measured in serum samples and could distinguish between the patients with breast cancer from the normal controls, with relatively high sensitivity. In particular, this ncRNA panel differed between healthy controls and patients with early-stage breast cancer (stages I–IIa) (Supplementary Figure 4). This finding, of a marker for early-stage breast cancer, is important because patients with breast cancer will have a better prognosis if they are diagnosed at an early stage.

Several previously published studies have shown that the expression of let-7a is reduced in cancer cells and the serum of patients with cancer when compared with normal controls, a finding that has been correlated with the overexpression of RAS and HMGA2 genes [17–19]. Also, let-7a is involved in tumor cell proliferation, invasion, and metastasis and has been shown to have a role in energy metabolism in a variety of tumors, including breast cancer [20,21]. In the present study, the expression of let-7a was reduced in the serum of patients with breast cancer, and one week after surgery, its expression level increased significantly when compared with the control samples. Consistent with previous reports that the reduced expression of let-7a promotes the growth of breast cancer cells, this result indicated that let-7a might have a role in the occurrence and development of human breast cancer. Also, miR-155 has previously been studied in breast cancer [22]. Some studies have shown that miR-155 was more abundant in the serum of patients with breast cancer during diagnosis and treatment [23]. The expression of miR-155 has also previously been shown to be inversely correlated with the expression of the tumor suppressor gene, suppressor of cytokine signaling 1 (SOCS1) in a breast cancer cell line [24]. Also, miR-155 regulates DNA repair and sensitivity to ionizing radiation by inhibiting the expression of RAD51 in breast cancer cells [25]. In the present study, although the expression of miR-155 in serum was almost stable in pre-operative and post-operative patients with breast cancer, the expression levels of miR-155 decreased significantly in patients with breast cancer following chemotherapy with vinorelbine. However, as this data in the present study were obtained from serum samples from only 12 patients with breast cancer following vinorelbine treatment, this preliminary finding requires validation with further studies and larger patient numbers. However, from the findings of the present study, miR-155 might be as a potential target for breast cancer diagnosis and therapy.

The miRNA, miR-574-5p, has previously been reported to be involved in biological processes including the regulation of neurogenesis in the embryonic cerebral cortex [26], and in the development of human cancer [27,28]. The expression of miR-574-5p has been shown to be found in extracellular vesicles

Figure 5. Changes in expression levels in the serum of the panel of four noncoding RNAs (ncRNAs) (let-7a, miR-155, miR-574-5p, and MALAT1) before and after chemotherapy in 12 patients with breast cancer.
in human colon carcinoma cell lines using the technique of miRNA deep sequencing [29], and has a suppressive role in colorectal cancer and liver metastasis by negatively regulating the expression of the MACC-1 gene in the colorectal cancer cells [30]. In the present study, the expression of miR-574-5p was increased in the serum of patients with breast cancer and had a high diagnostic sensitivity and specificity.

MALAT1 is one of the most abundant and highly conserved long ncRNAs, and previous studies have shown that it participates in RNA alternative splicing, protein subnuclear localization, the modulation of protein phosphorylation, and cell differentiation and development [31–33]. Previous studies have shown that MALAT1 plays an important role in tumor cell proliferation and cell migration, including in breast cancer cells [34,35]. In the present study, MALAT1 was over-expressed and found at high levels in the serum of patients with breast cancer. Importantly, in patients with breast cancer, one week after surgery, the serum levels of MALAT1 decreased significantly when compared with pre-operative levels. This result indicated that MALAT1 might be a serum biomarker of potential value in monitoring the development of breast cancer and monitoring response to treatment.

**Conclusions**

The findings of this study identified a panel of serum non-coding RNAs (ncRNAs) as potential diagnostic and prognostic biomarkers for breast cancer that included the ncRNAs let-7a, miR-155, miR-574-5p, and MALAT1. Furthermore, some of these serum ncRNAs might have potential as serum biomarkers of response to therapy. The findings of this initial study require further controlled studies with larger numbers of cases, to support the potential clinical role for the detection of serum ncRNAs in breast cancer diagnosis, prognosis, and response to therapy.

**Conflict of interest**

None.

**Supplementary Files**

**Supplementary Table 1.** PCR primers used in this study.

| Official symbol | Primer (5'-3') |
|-----------------|---------------|
| miR-21          | CGCGCTACCTTATCAGACTGATG |
| miR-10b         | GCTACCTGTAGAACGGAAATTG |
| let-7a          | GCCGGTGAGAGTAGGATTGTTATG |
| miR-155         | CTTAATGCTAATCGTGATAGGGG |
| miR-181b        | AAGACATTGGTGGTGGTGG |
| miR-196a        | cgcgcTGATGTTGATTGTTG |
| miR-1254        | AGCGTGAAAGCCTGAGCCTG |
| miR-574-5p      | GCGTGAGTGTGTGTGTGT |

| Official symbol | Primer (5'-3') |
|-----------------|---------------|
| miR-205         | TCTTCATTCACCCGGAGTCTG |
| miR-195         | CTTTAGCAGCAGGAAATTTG |
| cel-miR-39 F    | TCACCCGGTGGTAAATCAGCTT |
| miR-X R         | Uni-miR qPCR Primer |
| MALAT1 F        | TATGGAAGACCTGAGCCTG |
| MALAT1 R        | GATGCAAATGCGCCTGAGTG |
| GAPDH F         | CTCTGCTCCTCGTTCGAC |
| GAPDH R         | TTTAAAGCAAGCCCTGTTG |

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Supplementary Figure 1. Serum levels of the four noncoding RNAs (ncRNAs) (let-7a, miR-155, miR-574-5p, and MALAT1) of patients with breast cancer of different clinical stages, before and after surgery.

Supplementary Figure 2. Serum levels of the four noncoding RNAs (ncRNAs) (let-7a, miR-155, miR-574-5p, and MALAT1) by tumor size before and after surgery.
**Supplementary Figure 3.** Serum levels of the four noncoding RNAs (ncRNAs) (let-7a, miR-155, miR-574-5p, and MALAT1) in the lymph node metastases of patients with breast cancer, before and after surgery.

**Supplementary Figure 4.** Comparison of risk score values between healthy controls and patients with breast cancer, stages I–IIa. *** p<0.0001, Student’s t-test.

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