Original Article
Diagnostic value of circulating miR-155, miR-21, and miR-10b as promising biomarkers in human breast cancer

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Abstract: Background: Breast cancer (BC) remains one of the top threats to the health of women and so far, there are no powerful and convenient methods to diagnose BC. In this study, we selected 3 microRNAs, miR-155, miR-21, and miR-10b, to assess their diagnostic value in BC screening. Methods: From March, 2014 to March, 2015, 106 BC patients and 106 age-match healthy participants were recruited in our study. Blood samples were collected from the total 206 participants. MicroRNAs were extracted from plasma and quantified by RT-qPCR, which their relative expressions were normalized by external control, cel-miR-39. An new miRNA Score including miR-155, miR-21 and miR-10b, was constructed using multivariate logistic regression. Statistical analyses were conducted to compare microRNAs level as well as other clinical characteristics between two groups. Results: The levels of circulating miR-155, miR-21, and miR-10b were significantly up-regulated in BC patients compared with healthy participants. ROC curve analyses revealed that the AUC (95% CI, sensitivity, specificity) value for miR-10b, miR-29c, and miR-205 were 0.692 (95% CI: 0.625-0.754; sensitivity=66.0%, specificity=68.9%), 0.748 (95% CI: 0.684-0.805; sensitivity=77.4%, specificity=67.9%), 0.794 (95% CI: 0.733-0.846; sensitivity=68.9%, specificity=75.3%), respectively. The new miR-score had the best performance with AUC (95% CI: 0.806-0.903; sensitivity=83.0%, specificity=77.3%). Conclusions: The 3 selected microRNAs, miR-155, miR-21, and miR-10b, were significantly up-regulated in BC patients and may be an ideal, noninvasive screening tool for BC detection.

Keywords: microRNA, breast cancer, diagnose

Introduction

Breast cancer (BC) is the second most common cancer in the world, and by far, it remains one of the top threats to the health of women, contributing to an estimated 25% of all new cancers or cases diagnosed in 2012 [1]. Early detection of breast cancer is vital to reduce the mortality of this disease [2, 3]. Several biological features are routinely used for the diagnosis and prognosis of patients with BC and for determining the therapy, such as histological grade, lymph node status, hormone receptor status, and human epidermal growth factor receptor type 2 status [4]. However, some patients, with a similar combination of BC features, may have different clinical outcomes. And, even with the most acceptable methods, such as mammography, ultrasonography and magnetic resonance imaging, for breast cancer detection, concerns remain for rates of misdiagnosis, missed diagnosis and the overdiagnosis [2, 3].

New affordable methods are therefore needed to help diagnosis and to suggest the most appropriate treatment for patients with BC on an individual basis. As a solution, microRNAs (miRNAs) have been proposed as promising biomarkers of BC because they can be readily detected in tumor biopsies and are also stably found in body fluids, particularly in blood, plasma, serum, and saliva [5-7]. miRNAs are endogenous, noncoding, single-stranded RNAs of approximately 22 nucleotides, that regulate gene expression across a wide spectrum of biologic and pathologic processes [8]. Recently, several reported studies have investigated...
serum/plasma miRNAs levels in breast cancer. Lodes et al. [9] used a panhuman microarray platform to evaluate serum miRNAs expression patterns of five types of cancer, including breast cancer, and found that miRNAs expression patterns could discriminate normal and breast cancer patients. Zhao et al. [10] performed a pilot study to compare the levels of plasma miRNAs from early-stage breast cancer patients and healthy controls and also confirm a difference expression of plasma miRNAs between two groups. Thus it may be now clear that miRNAs have the potential to provide new diagnostic, prognostic, and predictive biomarkers for BC, with a great impact on the clinical management of patients with BC.

In present study, we selected 3 candidate circulating miRNAs (miR-155, miR-21, and miR-10b) to assess their diagnostic values in BC screening by comparing their expression level in serum between BC patients and healthy controls.

Materials and methods

Subjects and sample collection

From March, 2014 to March, 2015, a total of 106 breast cancer (BC) participants from the Ruijin Hospital, Shanghai Jiaotong University School of Medicine, were enrolled in our study. 106 age-matched normal subjects from Medical Examination Center in the same period were included. Each enrolled patient has to meet the following criteria: 1) diagnosis of primary BC was clinically confirmed by histopathology or biopsy; 2) patients have no severe infection, active clinical comorbidities, or a history of any other malignancy; 3) patients have not received chemotherapy, radiotherapy, or operation. The stage of tumor was determined according to the tumor-node-metastasis (TNM) staging system. The research was conducted in strict accordance with the protocol approved by the Ethics Committee of Ruijin Hospital, Shanghai Jiaotong University School of Medicine, and a written informed consent was obtained from each subject before their participation in the study.

Up to 8 mL of whole blood were collected from each participant in an ethylenediamine tetraacetic acid tube. Blood samples were centrifuged at 12000 rpm for 15 min at 4°C to completely remove cellular contaminants. Then plasma were aliquoted into microcentrifuge tubes, marked and stored at -80°C until use. Blood samples were processed and plasma was frozen within 2 hours of collection.

RNA extraction

Total RNA was extracted from 0.2 ml plasma samples using the Norgen RNA Purification Kit (Norgen Biotek Corporation, Thorold, Ontario, Canada) following the manufacturer’s protocol. Briefly, lysis solution was added to 200 μL of plasma, and then ethanol was added. The lysates were then loaded onto the provided column, and most of the contaminating cellular proteins were removed as they flowed through it. The column was then washed 3 times with 400 μL of wash solution. The purified total RNA was eluted into as much as 50 μL of elution buffer. The concentration and purity of the RNA solution was measured by detecting its absorbance at 260/280 and 260/230 nm with NanoDrop 1000A spectrophotometer (NanoDrop Technologies, Wilmington, DE). All the purified RNA samples were stored at -80°C for further processing.

Reverse transcription and real-time quantitative PCR (RT-qPCR)

Reverse transcription for total RNA was performed by the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Darmstadt, Germany). The 30 μl reverse transcription reaction contained: 1 μL Oligo(dt)$_{18}$, 1 μL Random Hexamer primer, 1 μL stem loop primer, 1 μL RNase Inhibitor, 1 μL M-MLV Rtase, 4 μL Reaction Buffer, 2 μl 10 mM dNTP Mix, 10 μl total RNA, 9 μL ddH$_2$O (DNase-free). The reaction was performed on a MJ Research PTC-200 Peltier Thermal Cycler (Global Medical Instrumentation) at 16°C for 30 min, 42°C for 40 min, and 85°C for 5 min.

Then RT-qPCR was performed to quantify the expression level of miR-155, miR-21 and miR-10b with SYBR Green PCR Master Mix (Thermo) following the manufacturer’s instructions. Cel-mir-39 was used as an extrinsic control. The 25 μl-amplifications reaction contained: 10 μL SYBR Green Mix, 2 μL miR-specific forward-primer, 2 μL miR-specific revere-primer, 2 μl total cDNA and 14 μl ddH$_2$O (DNase-free). The RT-PCR reaction was performed at 95°C for 10
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Table 1. Characteristics of normals and breast cancer patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Normals (n=106)</th>
<th>BC patients (n=106)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>57.7 ± 7.6</td>
<td>56.9 ± 6.7</td>
<td>0.416</td>
</tr>
<tr>
<td>Alcohol use</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>4 (3.8%)</td>
<td>7 (6.6%)</td>
<td>0.353</td>
</tr>
<tr>
<td>No</td>
<td>102 (96.2%)</td>
<td>99 (93.4%)</td>
<td></td>
</tr>
<tr>
<td>Tobacco use</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>3 (2.8%)</td>
<td>2 (1.9%)</td>
<td>1.000</td>
</tr>
<tr>
<td>No</td>
<td>103 (97.2%)</td>
<td>104 (98.1%)</td>
<td></td>
</tr>
<tr>
<td>Family history of BC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>4 (3.8%)</td>
<td>9 (8.5%)</td>
<td>0.152</td>
</tr>
<tr>
<td>No</td>
<td>102 (96.2%)</td>
<td>97 (91.5%)</td>
<td></td>
</tr>
<tr>
<td>TNM stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>11 (10.4%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>43 (40.5%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>32 (30.2%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>20 (18.9%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Statistical analysis

Continuous variables of normal distributions are expressed as mean ± standard deviation (SD) and skewed distributions are expressed as median (interquartile range). Categorical values were expressed by absolute and relative frequencies. Differences in variables were analyzed using Student t tests (for normally distributed data), Wilcoxon signed rank test (for skewed distributed data) or the chi-square test (for categorical data). Receiver operating characteristic (ROC) curves were constructed based on the miRNAs levels between groups. Area under the ROC curve (AUC) was generated to assess the diagnostic values of the candidate microRNAs. The cutoff values for microRNA levels were determined by Youden index. In addition, we performed multivariate logistic regression to construct a new miRNA Score including miR-155, miR-21, and miR-10b, which may have a more powerful diagnosability. A P value less than 0.05 was considered as statistically significant. All statistical analysis were performed by STATA version 12.0 software (Stata Corp, College Station, TX), and the graphs were obtained from GraphPad Prism 5.0 (GraphPad Software Inc., CA).

Results

Clinical characteristics of study population

Serum samples were acquired from 216 subjects (106 BC patients, 106 healthy participants). As presented in Table 1, no significant difference in age (57.7 ± 7.6 vs. 56.9 ± 6.7, P=0.416), alcohol use (3.8% vs. 6.6%, P=0.353) as well as tobacco use (2.8% vs. 1.9%, P=1.00) were observed between healthy participants and BC patients group. A higher family history of BC was observed in BC patients, however the difference was not statistically significant (3.8% vs. 8.5%, P=0.152). Among 106 BC patients, 11 were diagnosed as Stage I, 43 as stage II, 32 as stage III, and 20 as stage IV.

MicroRNAs level and BC susceptibility

Three microRNAs (miR-155, miR-21, and miR-10b) were examined in serum samples from 106 BC patients and 106 healthy participants. Serum relative levels of miR-155, miR-21, and miR-10b were plotted in the form of scatter dots in Figures 1A-3A. As shown, relative expression of miR-155, miR-21, and miR-10b in BC patients were 2.87 (1.43, 4.97), 6.74 (4.36, 9.71), 0.78 (0.34, 1.42) respectively. And the relative expression of 3 miRNAs were 1.44 (0.69, 3.05), 3.19 (1.86, 5.94), 0.25 (0.11, 0.47) in healthy participants. Statistically significant difference can be observed between BC patients and healthy participants (all P<0.001). Hence, these 3 miRNAs were able to discriminate BC patients from healthy controls based on their aberrant expression patterns. Then we performed multivariate logistic regression to construct a new miRNA score: Score=0.25*miR-155+0.2*miR-21+1.59*miR-10b. As shown in Figure 4A, the score of BC patients was also higher than that of healthy
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Figure 1. Diagnostic performance of serum miR-155 in differentiating breast cancer (BC) patients from participants. A. Relative expression levels of serum miR-155 in BC patients and participants. B. ROC curve analysis of serum miR-155 in differentiating BC patients from participants.

Figure 2. Diagnostic performance of serum miR-21 in differentiating breast cancer (BC) patients from participants. A. Relative expression levels of serum miR-21 in BC patients and participants. B. ROC curve analysis of serum miR-21 in differentiating BC patients from participants.

participants [3.86 (2.67, 5.60) vs. 1.76 (1.27, 2.46), all P<0.001].

Diagnostic performance of MicroRNAs in BC detection

We further evaluated the diagnostic value of 3 selected miRNAs and the new miRNA score by ROC curves and AUC values. Figures 1B-4B shows the ability of the 3 miRNAs and miR-score to distinguish between BC patients and healthy participants. The AUC of miR-155, miR-21, and miR-10b were 0.692 (0.625-0.754), 0.748 (0.684-0.805), 0.794 (0.733-0.846), respectively. miR-Score had a highest AUC of 0.860 (0.806-0.903). More information about the diagnosability were shown in Table 2. Among 3 selected miRNAs, miR-21 had a higher sensitivity of 77.4% and miR-10b had a higher specificity of 75.5%. We also can observe that miR-10b had a higher+LR of 2.81 and miR-21 had a lower-LR of 0.33. However, compared with these 3 miRNAs, miR-Score showed the best diagnosability, with the highest sensitivity of 83.0%, specificity of 77.3%, +LR of 3.67 and lowest-LR of 0.22.

Discussion

Currently, the identification of cancer-specific miRNAs profiles in the circulation is an emerging field of particular interest. The current
Diagnostic value of miRNAs to BC

Figure 3. Diagnostic performance of serum miR-10b in differentiating breast cancer (BC) patients from participants. A. Relative expression levels of serum miR-10b in BC patients and participants. B. ROC curve analysis of serum miR-10b in differentiating BC patients from participants.

Figure 4. Diagnostic performance of miR-score in differentiating breast cancer (BC) patients from participants. A. Relative expression levels of miR-score in BC patients and participants. B. ROC curve analysis of miR-score in differentiating BC patients from participants. miR-score=0.25 miR-155+0.2 miR-21+1.59 miR-10b.

Table 2. The diagnosis value of microRNAs normal and breast cancer patients

<table>
<thead>
<tr>
<th>miRNAs</th>
<th>AUC</th>
<th>95% CI</th>
<th>P value</th>
<th>Youden</th>
<th>Cut-off</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>+LR</th>
<th>-LR</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-155</td>
<td>0.692</td>
<td>0.625-0.754</td>
<td>&lt;0.001</td>
<td>2.2</td>
<td>0.321</td>
<td>66.0%</td>
<td>68.9%</td>
<td>2.03</td>
<td>0.53</td>
</tr>
<tr>
<td>miR-21</td>
<td>0.748</td>
<td>0.684-0.805</td>
<td>&lt;0.001</td>
<td>4.32</td>
<td>0.453</td>
<td>77.4%</td>
<td>67.9%</td>
<td>2.41</td>
<td>0.33</td>
</tr>
<tr>
<td>miR-10b</td>
<td>0.794</td>
<td>0.733-0.846</td>
<td>&lt;0.001</td>
<td>0.46</td>
<td>0.443</td>
<td>68.9%</td>
<td>75.5%</td>
<td>2.81</td>
<td>0.41</td>
</tr>
<tr>
<td>miR-Score</td>
<td>0.860</td>
<td>0.806-0.903</td>
<td>&lt;0.001</td>
<td>2.5</td>
<td>0.604</td>
<td>83.0%</td>
<td>77.3%</td>
<td>3.67</td>
<td>0.22</td>
</tr>
</tbody>
</table>

Note. AUC, area under the receiver operating characteristic curve; CI, confidence interval; +LR, positive likelihood ratio; -LR, negative likelihood ratio; P value, compared with AUC of 0.5. miR-Score=0.25 miR-155+0.2 miR-21+1.59 miR-10b.

results suggested that plasma levels of miR-155, miR-21 and miR-10b in BC patients were higher than those in healthy participants. Receiver operating characteristic (ROC) curves were used to assess the diagnostic values of the candidate microRNAs. We can observe ideal diagnosability of 3 selected miRNAs, with AUCs of 0.692, 0.748 and 0.794, respectively. Furthermore, we constructed a new score, named miR-score, which show greater diagnosability than 3 selected miRNAs. Collectively, our study provides evidences that serum level of
miR-155, miR-21 and miR-10b have great clinical value as promising biomarkers in BC preliminary screening.

Advanced technologies, such as microarray expression data, have shown that aberrant miRNAs expression is the rule rather than the exception in BC [11, 12]. BC-relative miRNAs, which have an important role in the pathophysiology of the disease, facilitating invasion, epithelial to mesenchymal transition (EMT), and maintenance of BC stem cells, have become an interesting topic in BC management [13, 14]. Recently, some attempts have been made to identify affordable BC signatures for diagnosis, and prediction of the therapeutic response. With respect to diagnosis, Lorio et al. [15] found that miRNAs aberrantly expressed in human breast cancer could clearly separate normal versus cancer tissues with 100% accuracy and the conclusion were confirmed by microarray and Northern blot analyses. Blenkiron et al. [16] identified 133 miRNAs that displayed aberrant expression levels in breast tumor tissues compared with normal breast tissues and bead-based flow cytometric miRNAs expression profiling might be a suitable platform to classify breast cancer into prognostic molecular subtypes. With respect to prediction, Rothé F et al. [17] study showed that expression of miR-210 is linked to tumor proliferation and appears to be a strong potential biomarker of clinical outcome in BC.

Although direct measurements of tissue gene biomarkers have greatly improved BC diagnosis, the invasive and unpleasant nature of the diagnostic procedures limits their application. Isolation and subsequent characterization of circulating miRNAs provide the opportunity to bypass the problems associated with tissue biopsy. Gloria Bertoli et al. [18] have depicted an overview of circulating miRNAs that can already be considered as BC biomarkers, such as miR-195, miR-16, miR-25, miR-222, miR-324-3p and so on. In the present study, we screened the level of circulating miR-155, miR-21 and miR-10b in BC patients and healthy participants by using RT-qPCR. Mir-155 is a robust oncogenic miRNA. A number of studies have built a critical role of miR-155 in breast carcinogenesis and suggested overexpressed circulating miR-155 can be released from tumor mass, as demonstrated in a xenograft mouse model [19-21]. It was reported that miR-155 down regulates SOCS1 in breast cancer, in turn leading to persistent activation of STAT3 signaling, which significantly promoted the proliferation of cancer cells [21]. MiR-21 has been reported to promote oncogenesis and progression of various carcinomas via direct targeting of tumor suppressing phosphatase and tensin homolog (PTEN) [22]. Then Liu et al. [23] found miR-21 is up-regulated in breast cancer bell lines, clinical specimens, and serum samples and demonstrated miR-21 could promote oncogenesis by miR-34b/c through affecting PTEN/PI3K/AKT/FOXO3a signaling. A correlation between elevated miR-10b expression and poor prognosis was recent reported in gastric cancer, renal cancer, colorectal tumors, and bladder tumors [24-26]. miR-10b is a particularly interesting candidate given its close correlation with metastatic behaviors [27]. Moreover, higher miR-10b expression level was recently detected in serum from metastatic breast cancer patients [28], consisting with our result.

Obviously, the studies on circulating miRNA profiles offer an exciting expectation. A reliable miRNA biomarker in circulation will dramatically facilitate the management of BC. However, the development of miRNA biomarker is cumbersome. A major concern is the different normalization strategies. Internal controls, like miR-16, may not be ideal for they are not always consistent across BC patients and controls [29, 30]. In present study, we took advantage of spiked-in cel-miR-39 for normalization. However, these synthetic miRNAs are less stable than endogenous miRNAs. Thus, finding an ideal miRNA for normalization should be imperative. Also, other certain limitations in our study should be mentioned. The population of enrolled patients and healthy controls was relatively small. Further study on a larger sample is needed to confirm our results. Secondly, the plasma has been stored at -80°C for average 7 months before use. Immediate analysis should be taken after blood samples are collected for a better reflection of real condition.

Taken together, this study extended the findings of previous studies about the serum levels of miR-155, miR-21 and miR-10b in breast cancer patients. Our data provided complementary information on its diagnostic value, indicating new insights into the diagnosability of serum level of the 3 selected miRNAs. However, whether this correlation is exactly proportional requires carefully scrutiny.
Acknowledgements

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Disclosure of conflict of interest

None.

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References


