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RESEARCH ARTICLE

The level of circulating miRNA-10b and miRNA-373 in detecting lymph node metastasis of breast cancer: potential biomarkers

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Abstract MicroRNAs (miRNAs) are a class of small non-coding RNAs whose expression changes are associated with cancer development and invasion. We hypothesized that miR-10b and miR-373, which are increased in lymphatic metastatic tissues, could be directly assayed in the plasma and used to detect the lymph node status of breast cancer patients. Between November 2009 and January 2012, 35 breast ductal carcinoma patients with lymph node metastasis (N patients), 25 ductal carcinoma patients without lymph node metastasis (N_0 patients), and ten healthy female donors were enrolled in the study. Circulating miR-10b and miR-373 were determined in preoperative plasma samples by reverse transcription quantitative real-time PCR assay. In preliminary tests, the plasma levels of circulating miR-10b and miR-373 were found to be significantly higher in ten breast cancer patients with lymph node metastasis compared to ten N_0 patients and ten normal donors ($P<0.01$). On validation analysis, the median value level of miR-10b in the 35 N patients was 4.44-fold ($P<0.01$) increased, and miR-373 was 4.38-fold ($P<0.01$) increased in comparison to the 25 N_0 patients. MiR-10b was used for differentiation of N patients from N_0 patients; the odds ratio was 2.19, and the value of the area under the receiver-operating curve (AUC) was 0.80, with sensitivity of 71 % and specificity of 72 %. For miR-373, the odds ratio was 2.62, and the AUC was 0.84, with sensitivity of 68 % and specificity of 89 %. A combination of the two circulating miRNAs further enhanced the sensitivity to 72 % and the specificity to 94.3 %. Our data suggest that circulating miRNA-10b and miRNA-373 are potential biomarkers for detecting the lymph node status of breast cancer.

Keywords Breast cancer · MicroRNA · Plasma · Biomarker

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Introduction

Breast cancer cases are steadily increasing, and breast cancer is one of the leading causes of cancer death among women. Each year, more than 1.3 million women will be diagnosed with breast cancer, and approximately 4.6 million will die from the disease [1]. For significantly improving prognosis of cancer patients, surgery is still the first and most important treatment for breast cancer. Therefore, many studies focused on the improvement of prognosis by radical removal of the tumor with the lowest damage. However, another promising approach is to detect lymph node metastasis at an early stage [2] because lymph node status is one of the most important prognostic factors after breast cancer diagnosis. It dominates the clinical decision making, the choice of tailor-made surgery, and the treatment outcomes [3].

Unfortunately, the existing diagnostic tools and biomarkers have many inherent deficiencies and are not sensitive enough to detect every early presence of lymph node metastasis in breast cancer patients. Mammography and ultrasound are currently the standard diagnostic tools in clinics for detection of breast cancer, but the sensitivity and specificity of mammography can be compromised by dense breast tissue in younger women [4], and it is difficult to find every tumor site or metastatic lymph node [5]. Early metastasis in lymph nodes or other organs is often missed, especially progressive occult breast cancer with micrometastasis. Although lymphatic mapping and sentinel lymph node biopsy are widely accepted and considered as diagnostic criteria for early breast cancer

patients, they are also controversial or contraindicated in certain patient populations [3]. For pregnant patients, blue dye or radiocolloid is considered to be unsafe and is not an option [3]. In addition, patients who want lymph node biopsy should have lymph node dissection as their initial procedure. On the other hand, numerous plasma biomarkers such as estrogen receptor, HER2/*neu*, carcinoembryonic antigen, and carbohydrate antigen are widely used in the detection and evaluation of breast cancer; however, few of them are effective in the detection of lymph node metastasis. Therefore, novel minimally invasive diagnostic tools and biomarkers for detecting lymph node metastasis are urgently needed.

miRNAs are a novel class of endogenous, noncoding, single-stranded RNAs, approximately 22 nucleotides long. They posttranscriptionally inhibit gene expression by either degrading or blocking translation of messenger RNA target [6]. miRNAs are involved in various regulations of cellular processes such as differentiation, proliferation, metabolism, and apoptosis and have been implicated in several diseases including cancer [7]. All these physical or pathological conditions could influence plasma levels of miRNAs. Heneghan et al. reported that miR-195 levels were found to be breast cancer specific and could differentiate breast cancer patients with a sensitivity of 88 % and a specificity of 91 % [8]. Moreover, the discriminative power could be up to 94 % when combined with levels of miRNA let-7a and miR-155. This level of predictive accuracy was not previously achieved by well-known serum immunoassays for circulating antigens such as carcinoembryonic antigen, carbohydrate antigen 153, maspin, and mammaglobin, and thus, circulating miRNAs could be potential biomarkers for breast cancer.

miR-10b and miR-373 could be potential biomarkers for breast cancer patients due to their differentially expressed and close association with tumor invasion and metastasis in tumor cells and tissue studies [9–13]. Ma et al. found that miR-10b was highly expressed in metastatic breast cancer cells and positively regulated cell migration and invasion [13], but Iorio et al. reported that miR-10b was downregulated in breast cancer tissues in comparison with the normal control [14]. Expression of miR-373 is not as controversial. It was found that significant over-expression of miR-373 existed in clinical breast cancer metastatic samples and stimulated cancer cell migration and invasion in vitro and in vivo [12]. Previous studies were performed in metastatic cells and tissues of breast cancer; however, the relationship between circulating miRNAs and lymph node metastasis is unclear yet. Here, we hypothesized that circulating miR-10b and miR-373 might be biomarkers for lymph node metastasis in breast cancer patients and tried to assess specificity and sensitivity of circulating miR-10b and miR-373 as biomarkers in the early detection of lymph node metastasis in breast cancer.

Patients and method

Patients and samples

Between November 2009 and December 2010, for test scale analysis, 20 consecutive patients who were pathological diagnosed as breast ductal carcinoma patients, as well as ten healthy volunteers, were enrolled in the study. In these 20 ductal carcinoma patients, ten had lymph node metastasis (N patients), while the other matched ten had no lymph node metastasis (N_0 patients). Patients who underwent chemotherapy or radiotherapy prior to the surgery and had coexisting malignant diseases, debilitating disease, unresolved psychiatric illness, substance abuse, or recurrence of breast cancer were excluded. Plasma samples were collected before the operation from patients and healthy volunteers. After the initial quantitative analysis of plasma miRNAs, to validate whether plasma miRNAs could detect the metastasis of breast cancer, preoperative plasma samples were collected from another 40 patients with breast ductal carcinoma before January 2012. Thus, a total 60 patients with breast ductal carcinoma and ten healthy female volunteers were enrolled in this study. Each individual signed the written informed consent before blood was taken, and the whole study was approved by the local institutional review board (Ethic commission beider Basel).

Protocols for the detection of miRNAs

Total RNA, including miRNA from plasma, was isolated from 200 μL plasma using the miRNeasy Mini kit (Qiagen GmbH, Hilden, Germany) and eluted into 50 μL elution solution. Then, RNA was purified using the RNeasy MinElute Cleanup kit (Qiagen) to remove impurities and finally eluted into 21 μL of elution solution according to the manufacturer's protocol.

The amount of miRNAs (miR-10b and miR-373) was quantified in duplicate by reverse transcription quantitative real-time PCR (RT-qPCR) using human TaqMan MicroRNA Assay kits (Applied Biosystems, Foster City, CA, USA). MiR-16 was used as a reference for normalization of expression levels of the miRNA panel according to the manufacturer's recommendation (Applied Biosystems) and previous report [15]. Five microliters eluted RNA was reverse transcribed with specific looped RT primers for each miRNA evaluated using the Taqman MicroRNA Reverse Transcription kit (Applied Biosystems). Subsequently, 1.33 μL reverse transcription products were used as templates in RT-qPCR using TaqMan MicroRNA Assays (Applied Biosystems). All reactions were run on the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The quantification cycle (C_q) values were calculated with SDS 1.2 software (Applied Biosystems). Expression of miRNAs

from plasma was normalized using the 2^{-dCq} method relative to miR-16. The dC_q was then calculated by subtracting the C_q values of miR-16 from the C_q values of the miRNAs of interest. The ddC_q was then calculated by subtracting the dC_q of the non-metastatic patients' plasma from the dC_q of the metastatic patients. Fold change in the gene was calculated by the equation 2^{-ddCq} [16].

Statistical analysis

Statistical analyses were performed using the SPSS software (SPSS Inc., Chicago, IL, USA). The chi-square test and Fisher's exact probability test (categorical data analysis) were used to analyze the characteristics between the two groups. The Wilcoxon signed-rank test, Wilcoxon analysis, and Kruskal–Wallis test (nonparametric statistics) were used to compare dC_q values of miRNAs. Logistic regression analysis was used to measure the influence of different variables. Receiver-operating characteristic (ROC) curves were generated to assess the diagnostic accuracy of each parameter, and the sensitivity and specificity of the optimum cutoff point were defined as those values that maximized the area under the ROC curve (AUC). The Youden index (J) was used for the identification of the optimal cutoff point, which allowed the selection of an optimal cutoff point under the assumption that sensitivity and specificity were equally weighted ($J = \text{Maximum}(\text{Sensitivity} + \text{Specificity} - 1)$) [17]. All P values were two tailed, and $P<0.05$ was considered statistically significant.

Results

Study design and subjects

Peripheral blood specimens from 60 breast ductal carcinoma patients with or without lymph node metastasis and ten normal female donors were collected between November 2009 and January 2012, in Basel University Women's Hospital. There were 25 N_0 patients, aged 34 to 72 years with a median of 47 years and 35 N patients, aged 33 to 69 years with a median of 49 years. There were no statistical differences in age, estrogen receptor, progesterone receptor, and Her2/*neu* between the two groups (Table 1).

Table 1 Patients' and tumor characteristics at time of plasma collection

Characteristic	N_0 patients (n)	N patients (n)
Total	25	35
Age (years)	47 (range 34–72)	49 (range 33–69)
Tumor		
pT1	15	7
pT2	9	12
pT3–4	1	16
Lymph node involvement		
pN0	25	0
pN1	0	22
pN2	0	9
pN3	0	4
Grading		
G1	15	0
G2	10	15
G3	7	20
Estrogen receptor status		
Positive	16	20
Negative	9	15
Progesterone receptor status		
Positive	11	17
Negative	14	18
Her2/*neu* status		
Positive	17	23
Negative	8	12

N_0 patients breast cancer patients without lymph node metastasis, *N patients* breast cancer patients with lymph node metastasis

The levels of plasma miR-10b and miR-373 in different groups

We investigated the stability of miRNAs in three plasma samples randomly selected from the study patient groups. Using RT-qPCR assay, circulating miRNAs such as miR-10, miR-373, and miR-16 were detectable in all samples (data not shown). After four cycles of freezing at −80 °C and thawing at 23 °C, there was no significant difference in C_q values ($P>0.05$). Expression of circulating miR-16 in the plasma of patients and control was also analyzed, and it was found that the levels remained stable across the plasma samples. There was no statistical difference in the level of miR-16 between groups ($P=0.35$).

Subsequently, we performed a pilot study to compare miRNAs levels between healthy donors, N_0 patients, and N patients (matched ten patients for each group). The result of RT-qPCR showed that median dC_q values (range) of miR-10b were 8.06 (5.1–10.89) in healthy donors, 7.47 (4.74–9.12) in N_0 patients, and 4.61 (2.15–5.52) in N patients (Fig. 1a). The median level of miR-10b in N patients was significantly higher than that in N_0 patients and healthy female donors ($P<0.01$), but there were no differences between N_0 patients and healthy donors. Median dC_q values (range) of miR-373 were 8.73 (7.12–10.01), 7.57 (5.6–8.93), and 5.80 (3.02–6.95) in healthy donors, N_0 patients, and N patients, respectively (Fig. 1b). The median level of miR-373 in N patients was also significantly higher than that of N_0 patients. Also, the median level in N_0 patients was higher than that of healthy donors ($P<0.01$).

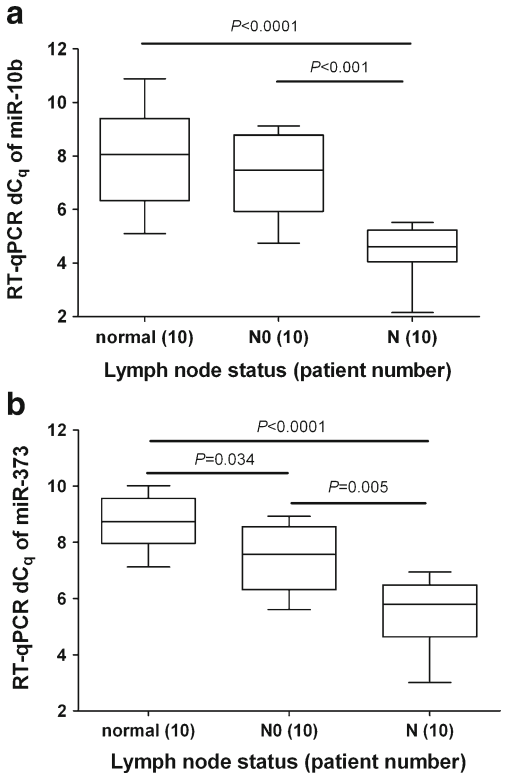

Fig. 1 Pilot study comparing dC_q values of circulating miR-10b and miR-373 in healthy female donors and breast cancer patients

Validation study of clinical application as a diagnostic plasma biomarker

Based on the result of the pilot study with 30 participants, we further validated the clinical utility of circulating miR-10b and miR-373 levels in all patients. The median (range) dC_q values of miR-10b were 6.56 (3.27–10.89) and 4.41 (1.18–7.29), and median (range) dC_q values of miR-373 were 7.63 (4.21–9.64) and 5.5 (1.90–7.79) in N_0 patients and N patients, respectively (Fig. 2). The dC_q values of miR-10b and miR-373 were significantly lower in N patients compared to those in N_0 patients ($P<0.01$). The median level of miR-10b in N patients was 4.44-fold increased, and miR-373 was 4.38-fold increased in comparison to N_0 patients.

Logistic regression and ROC analyses were subsequently used to assess the odds ratio and sensitivity and specificity of the miRNAs. Using miR-10b to distinguish N patients from N_0 patients, the odds ratio was 2.19 (95 % confidence interval (CI) 1.43–3.61), and the AUC was 0.80; when the cutoff value was set to the optimal point of 4.98, sensitivity was 71 %, and specificity was 72 % (Fig. 2c). For miR-373, the odds ratio was 2.62 (95 % CI 1.57–4.37), and the AUC was 0.84; when the cutoff value was set to the optimal point of 6.97, sensitivity was 68 %, but specificity was 89 % (Fig. 2d).

To investigate more sensitivity or specificity of diagnostic biomarkers in plasma, we analyzed the product of dC_q value of miR-10b and miR-373 as a combined biomarker. As a result, the product of miR-10b's and miR-373's dC_q values was significantly higher in the N_0 group (Fig. 3a). The combined biomarker had larger AUC (0.88), more sensitivity (72 %), and more specificity (94.3 %, Fig. 3b).

Correlation between plasma miRNAs and other clinicopathological factors

The correlation between circulating miR-10b and miR-373 and other clinicopathological factors was also assessed (Table 2). The level of miR-10b and miR-373 in grade 3 patients was higher than that in grade 1 or 2 ($P<0.05$), but no statistical significance of miRNA's level between different tumor sizes could be found. Moreover, there was no statistical significance in the level of miR-10b and miR-373 between negative receptor status of the estrogen receptor, progesterone receptor, and Her2/*neu*, and positive receptor.

Discussion

The treatment of breast cancer is systemic, including surgery, chemotherapy, radiation therapy, endocrinotherapy, and immunotherapy, and individual. The surgery, for example, can include radical mastectomy, extensive radical mastectomy, modified radical mastectomy, total mastectomy, or breast-conserving surgery (partial mastectomy, lumpectomy, tylectomy, wide local excision, or quadrantectomy) [18]. Overtreatment of breast cancer might extend the duration of the surgery, decrease immunity, increase postoperative pain, the risk of lymphedema onset, and hospital confinement [19]. But undertreatment could influence the prognosis and recurrence of breast cancer. The detection of lymph node invasion has gained an increasingly important role in the treatment of the breast carcinoma because it dominates the choice of surgery and the following systemic treatment. Over the past 10 years, a 2.3 % annual decline in breast cancer death rates resulted from early detection and appropriate treatment [20]. However, there is still a high missed diagnosis of the incidence of lymph node micro-metastasis in ductal carcinoma, especially high-risk ductal carcinoma, due to invasive characteristics [21, 22].

In this study, it was found that miR-10b and miR-373 are significantly over-expressed in breast cancer patients with lymph node metastasis and could discriminate breast cancer patients with metastasis from those without metastasis. We performed a pilot study on plasma specimens from 30 breast cancer patients and normal donors using miR-16 as the reference. Two candidate miRNAs (miR-10b and miR-373), which were reported in breast cancer cell studies but

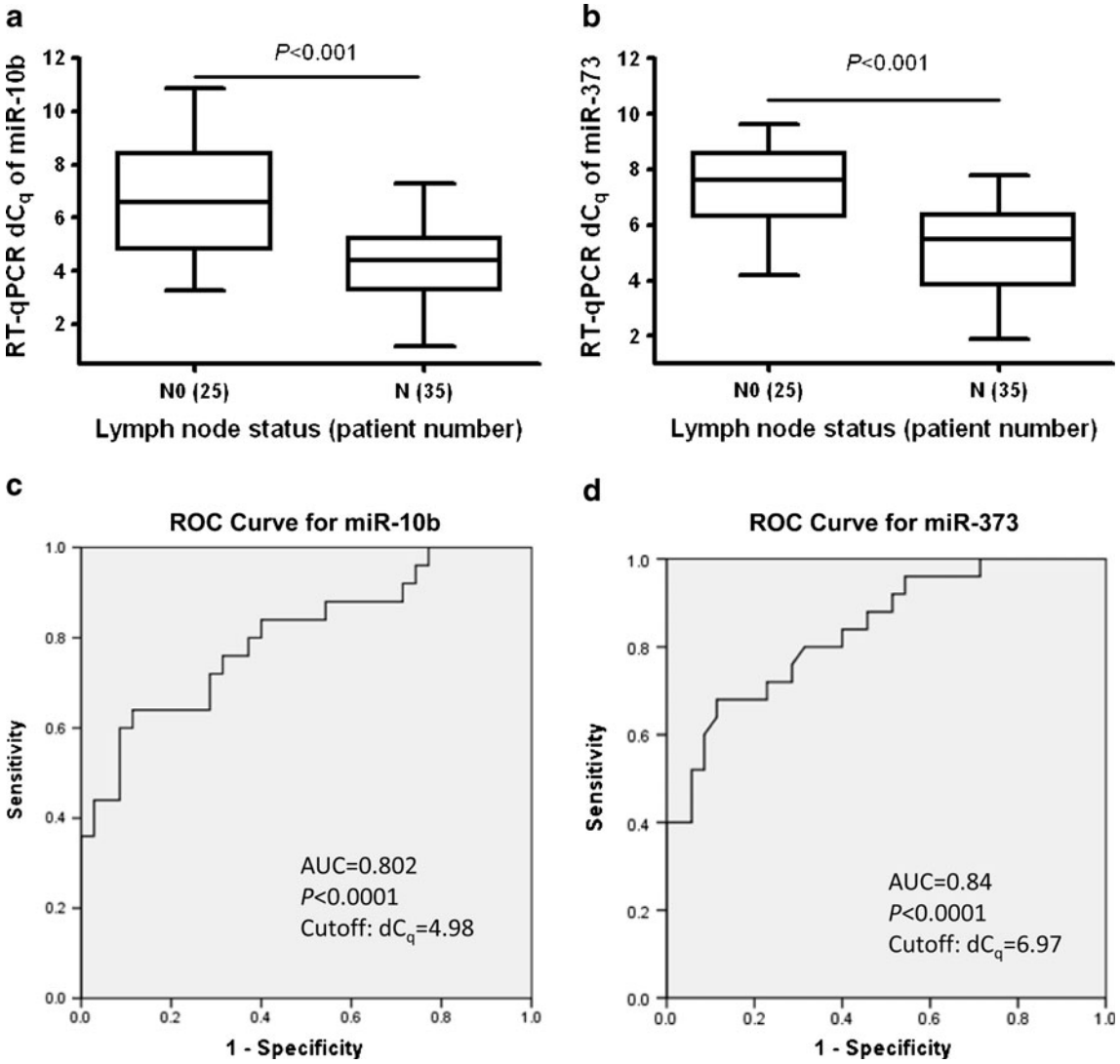

Fig. 2 Identification of the presence and extent of breast cancer by RT-qPCR of circulating miR-10b and miR-373. **a**, **b** The dC_q values of circulating miR-10b and miR-373 in non-metastatic breast cancer patients and lymph node metastatic patients. **c**, **d** Receiver-operating characteristic analysis for patients with vs without lymph node metastasis

scarcely in plasma, were examined to determine whether their expression profile in plasma was associated with lymph node metastasis of breast cancer. Results showed that significant differences of expression were found between N_0 and N patients, but there were no differences of expression between different tumor sizes. This corresponds to several

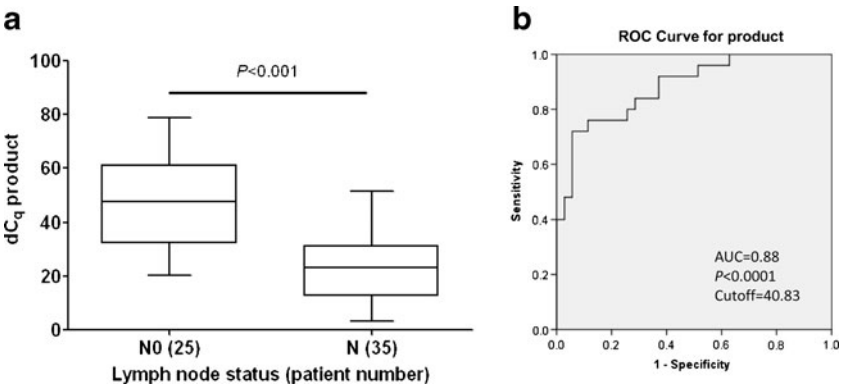

Fig. 3 Comparison and receiver-operating characteristic analysis of dC_q product of circulating miR-10b and miR-373. **a** The product of miR-10b's and miR-373's dC_q values in non-metastatic breast cancer patients and lymph node metastatic patients. **b** ROC analysis of dC_q product for patients with vs without lymph node metastasis

Table 2 Clinicopathologic characteristics of breast ductal carcinoma patients

Characteristic	Patients, *n*	dC_q value of miR-10b, mean (SD)	dC_q value of miR-373, mean (SD)
Patient age, years			
≥50	25	4.21 (1.56)	5.09 (1.80)
≤49	35	5.95 (2.04)	6.87 (1.61)
Tumor			
pT1	22	5.65 (2.16)	6.87 (1.65)
pT2	21	5.59 (2.02)	6.18 (1.85)
pT3–4	17	4.21 (1.58)	5.09 (1.88)
Lymph node involvement			
pN0	25	6.55 (2.06)	7.41 (1.42)
pN1	22	4.34 (1.26)[a]	5.46 (1.69)[a]
pN2–3	13	4.18 (1.68)[a]	4.78 (1.56)[a]
Grading			
G1	15	6.27 (2.23)	7.53 (1.40)
G2	25	5.49 (1.92)	6.42 (1.54)
G3	20	4.12 (1.51)[a]	4.71 (1.73)[a]
Estrogen receptor			
Positive	36	5.10 (2.13)	6.15 (1.66)
Negative	24	5.41 (1.91)	6.09 (2.25)
Progesterone receptor			
Positive	28	4.88 (1.65)	6.14 (1.54)
Negative	32	5.52 (2.30)	6.11 (2.19)
Her2			
Positive	40	5.14 (2.11)	6.18 (1.68)
Negative	20	5.38 (1.91)	6.01 (2.32)

dC_q normalized quantification cycle, *SD* standard deviation

[a] Statistically significant

cell and serum studies. Ma et al. reported that miR-10b was highly expressed in metastatic breast cancer cells and positively regulated cell migration and invasion [13]. MiR-10b inhibits translation of the mRNA encoding homeobox D10, leading to increased expression of a well-characterized pro-metastatic gene, RHOC. Therefore, increased expression of miR-10b might correlate with progression and metastasis of breast cancer. Zhao et al. found that circulating miR-10b could be used for the identification of bone metastatic breast cancer [23]. Roch et al. confirmed that expression of miR-10b in blood serum correlated with the presence of overt metastasis after surgery [24]. Iorio et al., however, reported that miR-10b was downregulated in breast cancer tissue in comparison with the normal control [14]. An explanation of these controversial results is that these miRNAs may confer specific invasive or other properties only in metastatic cells since they are ubiquitously downregulated in primary breast cancer cells [25]. MiR-373 is not as controversial. It was found that over-expression in clinical breast cancer metastasis samples could stimulate cancer cell migration and invasion in vitro and in vivo and inversely correlate with CD44 expression [12]. Much other literature also described invasion characteristics of miR-10b and miR-373 in other tumors, and the aberrant expressions could be used to detect the metastasis of colorectal cancers [26], hepatocellular carcinoma [27], renal cell carcinoma [28], and prostate cancer [29].

Subsequently, our validation study found that in using miR-10b to distinguish N patients from N_0 patients, the sensitivity was 71 %, and the specificity was 72 %, and while using miR-373, the sensitivity was 68 %, but the specificity was 89 %. MiR-10b has higher sensitivity, while miR-373's specificity is higher. When using the product of miR-10b and miR-373 as a combined biomarker, the sensitivity was 72 %, and the specificity was enhanced to 94.3 %. They showed great promise regarding sensitivity and specificity. Therefore, both circulating miR-10b and miR-373 might be potential biomarkers for detecting the lymph node metastasis of breast cancer patients.

Although the kinetics and metabolism of circulating miRNA are not clear, differentially expressed circulating miRNAs open up a new field for investing biomarkers in the screening and monitoring of cancer patients. Circulating RNAs have been identified in the plasma or serum of cancer patients for more than a decade [30]. Many miRNAs were detected in exosomes and then hypothesized to be involved in intercellular communication [31]. But a large part of extracellular miRNA in blood plasma is independent of exosomes and might be a by-product of dead or dying cell [32]. In breast cancer, miRNAs involved in tumor genesis, and the breakdown of cells often happen in tumor tissues. This might be the reason that many aberrant expressions can be found in the plasma in breast cancer patients and can be influenced by cancer-dependent variables such as tumor stage, treatment, and other risks, and prognosis-related factors. The presence of circulating miRNAs and their potential use as novel biomarkers were also demonstrated in various other cancers, such as prostate cancer, leukemia, oral cancer, lung cancer, pancreatic cancer, colorectal cancer, and breast cancer [16].

Moreover, miR-10b and miR-373 have the potential to serve as ideal biomarkers because of their stability. As RNase is present in the blood and other extracellular environment, one would expect circulating RNA (including miRNAs) in the blood to be rapidly destroyed. However, intracellular miRNAs could be well preserved in the extracellular environment. It was reported that intracellular miRNAs could be well preserved in the extracellular environment for at least 2 months after cell lysis [32]. Beside plasma, significant amounts of miRNA have been found in extracellular human body fluids including urine, saliva, and semen [33–35]. The stability might be due to bonding to the Ago protein, a part of the RNA-induced silencing complex,which provides protection

of miRNA [36]. This unusual stability of miRNA was also vertificated by repeated freezing and thawing in our study. After four cycles of freezing at −80 °C and thawing at 23 °C, there was no significant difference in C_q values of miRNAs.

In conclusion, we observed that circulating miR-10b and miR-373 levels could distinguish breast cancer patients with lymph node metastasis from non-metastatic patients. Considering the importance of lymph nodes in the choice of treatment, circulating miR-10b and miR-373 might be potential biomarkers for screening lymph node metastasis in breast cancer patients. Our findings warrant further studies with a large cohort of patients to validate and develop the plasma biomarker as a critical tool for breast cancer care.

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Conflicts of interest None.

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