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ORIGINAL ARTICLE

Serum microRNA-21 predicted treatment outcome and survival in HER2-positive breast cancer patients receiving neoadjuvant chemotherapy combined with trastuzumab

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Abstract

Purpose The purpose of this study was to evaluate the expression of ser-miRNAs at different periods during treatment and analyze their relationship with therapeutic response and prognosis in HER2-positive breast cancer patients receiving neoadjuvant chemotherapy combined with trastuzumab (NCCT).

Methods Venous blood was drawn from patients at different periods during NCCT. The expression of ser-miRNAs was assessed by qRT-PCR and their relation to treatment response and survival was analyzed.

Results The results showed the expression of miR-10b, -21, -34a, -125b, -145, -155, and -373 in patients before the start of treatment was significantly higher, ser-miR-210 was lower, and ser-miR-122 was comparable to the levels in healthy controls. Changes in ser-miR-21 levels during NCCT were significantly correlated to clinical response and survival and, however, were not associated with pathology response. The expression levels of ser-miR-21 were decreased from the start of NCCT to the end of the second cycle and from the start to the end of NCCT in clinical responders; however, there was no significant difference in non-responders. The patients with decreased ser-miR-21 expression from the start to the end of the second cycle and from the start to the end of NCCT had better overall survival (OS) and disease-free survival (DFS) than those with elevated ser-miR-21 expression.

Conclusion These results showed that changes in ser-miR-21 levels were significantly related to NCCT clinical response and prognosis. Ser-miR-21 may serve as a non-invasive biomarker to predict NCCT response in HER2-positive breast cancer.

Keywords Serum miR-21 · HER2-positive breast cancer · Trastuzumab · Survival

Introduction

Breast cancer (BC) is one of the leading causes of cancer-related death in women worldwide [1]. Human epidermal growth factor receptor 2 (HER2) gene amplification is reported in approximately 20–30% of BC patients. HER2 amplification is associated with poor survival and cancer recurrence [2]. Although trastuzumab monotherapy or its use in combination with chemotherapy significantly improved the outcomes of HER2-positive breast cancer (HPBC) patients, the efficacy of trastuzumab is limited by the development of resistance to this targeted drug [3–5]. To date, no reliable and effective method has been identified for predicting the therapeutic effect before the treatment. Therefore, identifying specific biomarkers to assess the effects of chemotherapy combined with trastuzumab at the start or at the early stages of therapy is important and may help the

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design of individual treatment strategies as well as reducing the incidence of adverse effects.

MicroRNAs (miRNAs) are a class of small, evolutionarily conserved non-coding RNAs of 20–23 nucleotides that repress gene expression by specifically targeting mRNA transcripts. MiRNAs are aberrantly expressed in HPBC patients and associated with resistance to chemotherapy combined with trastuzumab [6–8]. Increasing evidence has suggested that miRNAs are stably present in the peripheral blood, serum, and plasma [9]. Serum miRNAs (ser-miRNAs) have been identified as non-invasive biomarkers for the diagnosis of cancer [10]. Recent studies have shown a relationship between several ser-miRNAs, such as miR-10b, miR-21, miR-34a, miR-122, miR-125b, miR-145, miR-155, miR-210, and miR-373, and chemotherapy outcome in BC [11–18]. However, the conclusions from these studies are inconsistent and contradictory. In a previous study, we showed that ser-miR-21, miR-34a, and miR-125b are associated with neoadjuvant chemotherapy response in HER2-negative and ER-positive BC patients receiving preoperative neoadjuvant chemotherapy [19, 20].

The aim of the present study was to determine the predictive value of ser-miRNAs including miR-10b, miR-21, miR-34a, miR-122, miR-125b, miR-145, miR-155, miR-210, and miR-373 regarding treatment response and survival in HPBC patients receiving neoadjuvant chemotherapy combined with trastuzumab (NCCT).

Patients

A total of 83 stage II and III HPBC patients were included in the present study. Patients were treated in the first and second affiliated Hospital of Harbin Medical University, Harbin, China, between January 2010 and December 2013. All the patients received preoperative NCCT with a combination of taxotere (75 mg/m^2), paraplatin (area under the curve = 5), and trastuzumab (4 mg/m^2 on the first day and 2 mg/m^2 thereafter). One cycle of therapy consisted of 21 days, and the patients underwent 4–6 cycles of NCCT (six patients received six cycles of NCCT and the rest received four cycles). Venous blood samples were harvested before the start of treatment (baseline, BL), at the end of the second cycle (first detection during NCCT, FDN), and at the end of NCCT (second detection during NCCT, SDN). Forty age-matched healthy adult women were included as controls. To clarify the relationship between ser-miRNA expression and BC presence, 30 HPBC patients who underwent mastectomy and did not receive neoadjuvant or adjuvant chemotherapy were also included in the present study. Paired pre-operative and post-operative blood samples were collected from these 30 patients. Survival data from all 83 HPBC patients receiving NCCT were collected. The follow-up period ranged from 13 to 36 months (mean period, 23.6 months).

Analyses of ser-miRNAs expression

Total RNA was isolated using the miRNeasy Serum/Plasma Kit (Qiagen, Hilden, Germany) from 200 µL serum in accordance with the manufacturer's instructions. The concentration and quality of eluted RNA were quantified using the NanoDrop-2000 (Thermo Fisher Scientific, Waltham, MA, USA). The reverse transcriptional reactions were run using the PrimeScript RT reagent Kit (Takara Biotechnologies Inc., Tokyo, Japan). The expression of miRNAs was evaluated by quantitative polymerase chain reaction (qRT-PCR) using the SYBR Premix Ex Taq II (Takara, Tokyo, Japan) on the LightCycler 96 Real-Time qPCR System (Roche, Basel, Switzerland). MiR-484 was selected as the endogenous control because several researches reported that its expression was stable between the healthy subjects and cancer patients [11, 21]. The relative expression levels of miRNAs were calculated using the $2^{-\Delta\Delta Ct}$ method. The ΔCt is equal to the Ct value of the interest miRNA minus the Ct value of miR-484, and the ΔΔCt is equal to the Ct value of HPBC patient minus the Ct value of healthy controls.

Evaluation of the treatment efficacy

Clinical and pathological examination was used to evaluate the NCCT response of HPBC patients. Clinical response was evaluated using the Response Evaluation Criteria in Solid Tumors (RECIST 1.1) [22]. The radiologic examination including chest and abdomen computerized tomography (CT) scans and X-rays was used to estimate tumors. According to these criteria, the patients having complete or partial response were regarded as the clinical responders and those having stable or progressive disease were regarded as the clinical non-responders. Pathological response was evaluated according to the Residual Cancer Burden (RCB) score described by Symmans [23]. The patients having RCB score of zero were considered as the pathologic complete response (pCR) and those having RCB score of ≥ 1 were considered as the non-pCR.

Statistical analysis

Statistical analysis was carried out using SPSS 20.0 software. The expressions of selected miRNAs were compared using the independent sample *t* test or paired sample *t* test, and one-way analysis of variance. Receiver operating characteristic (ROC) curve was plotted and the area under the ROC curves (AUC) was calculated to assess the predictive capability of selected miRNAs for the treatment

response. Survival analysis was used to assess the association between ser-miRNA expression and overall survival (OS), and disease-free survival (DFS). $P < 0.05$ was considered statistically significant.

Results

MiRNA expression in the serum of HPBC patients and healthy controls

The expression levels of the nine ser-miRNAs were first quantified by qRT-PCR at BL in HPBC patients and healthy volunteers. The results showed that the mean expression levels for miR-10b, miR-21, miR-34a, miR-125b, miR-145, miR-155, miR-373, miR-210, and miR-122 were 6.45, 3.22, 5.26, 4.43, 3.21, 3.18, 2.65, 0.67, and 0.93 fold change in HPBC patients compared with healthy controls. The results indicated that the levels of seven ser-miRNAs (miR-10b, -21, -34a, -125b, -145, -155, and -373) were significantly higher in patients than in healthy controls ($P < 0.001$ for all seven miRNAs), one ser-miRNA (miR-210) showed higher expression levels in healthy controls than in HPBC patients ($P < 0.001$), and one ser-miRNA (miR-122) was expressed at the same level in patients and in healthy volunteers. Evaluation of the correlation between ser-miRNAs expression and the clinicopathological parameters of HPBC patients showed that ser-miR-10b, -125b, and -373 upregulation was related to advanced clinical stage and lymph node metastasis (LNM). In addition, miR-145 upregulation was significantly associated with ER positive, and miR-10b upregulation was significantly associated with PR negative (Fig. 1, Table 1).

Relationship between ser-miRNAs expression and NCCT response

After the completion of NCCT, patients were divided according to clinical and pathological response. Among 83 HPBC patients, 31 showed complete response, 31 showed partial response, 18 achieved stable disease, and 3 showed progressive disease. Therefore, according to previous definitions, 62 patients were divided as clinical responders and 21 as clinical non-responders. Patients were also classified according to pathological response. The results showed that 18 patients achieved the RCB score of 0, and 32, 23, and 10 patients achieved the score of 1, 2, and 3, respectively. Therefore, 18 patients were classified as the pCR and 65 as the non-pCR.

The expression of the nine ser-miRNAs was compared in clinical responders and non-responders to clarify the relationship between ser-miRNAs expression and treatment response. The results showed no significant differences in ser-miRNAs expression between clinical responders and non-responders at BL, FDN, and SDN. The expression of the nine ser-miRNAs was also compared in pathological responders and non-responders (pCR vs non-PCR). The results showed no significant association between ser-miRNAs expression and pathological response at BL, FDN, and SDN. Next, the changes in the nine ser-miRNAs expression levels at different periods during NCCT were analyzed. The results showed that only changes in ser-miR-21 expression during the treatment were significantly associated with NCCT. The mean levels of ser-miR-21 were significantly decreased following NCCT. The results showed that the expression levels of ser-miR21 were significantly lower at FDN and SDN than at BL in all patients ($P < 0.001$ both). Further analysis revealed that the decrease in ser-miR-21

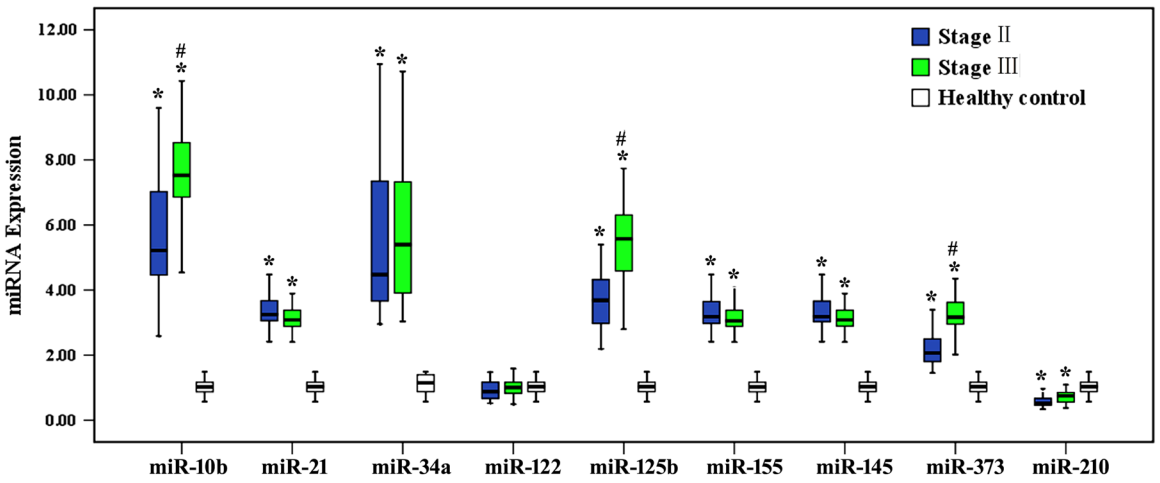

Fig. 1 Expression levels of ser-miRNAs in HPBC patients and healthy controls. *$P < 0.05$ compared with healthy controls; #$P < 0.05$ compared with stage II

Table 1 Clinicopathologic characteristic and the expression of ser-miRNAs in HPBC patients at BL

Characteristic	*n*	Mean expression levels of ser-miRNAs(SD)								
		miR-10b	miR-21	miR-34a	miR-122	miR-125b	miR-155	miR-145	miR-373	miR-210
Age (years) 47.6 (31–75)										
< 47.6	25	6.66 (1.98)	3.27 (0.65)	5.16 (1.98)	0.94 (0.24)	4.64 (1.27)	3.24 (0.65)	3.26 (0.65)	2.86 (0.72)	0.71 (0.21)
≥ 47.6	58	6.63 (2.23)	3.33 (0.59)	5.67 (2.28)	0.95 (0.29)	4.52 (1.61)	3.30 (0.58)	3.33 (0.55)	2.67 (0.85)	0.66 (0.33)
Histological type										
Invasive ductal carcinoma	70	6.61 (2.19)	3.33 (0.62)	5.58 (2.21)	0.95 (0.28)	4.54 (1.48)	3.29 (0.61)	3.33 (0.59)	2.72 (0.81)	0.67 (0.31)
Others	13	6.82 (1.97)	3.22 (0.54)	5.23 (2.19)	0.94 (0.24)	4.62 (1.75)	3.22 (0.54)	3.19 (0.49)	2.77 (0.86)	0.71 (0.24)
Tumor grade										
Well	24	7.07 (2.19)	3.39 (0.72)	5.59 (2.30)	0.90 (0.23)	4.39 (1.29)	3.38 (.073)	3.39 (0.72)	2.72 (0.68)	0.66 (0.19)
Moderate	42	6.63 (2.29)	3.28 (0.55)	5.38 (2.07)	0.97 (0.27)	4.73 (1.64)	3.23 (0.55)	3.30 (0.50)	2.81 (0.92)	0.68 (0.32)
Poor	17	6.07 (1.63)	3.30 (0.59)	5.77 (2.45)	1.02 (0.30)	4.35 (1.49)	3.25 (0.52)	3.24 (0.56)	2.52 (0.73)	0.76 (0.46)
Clinical stage										
Stage II	47	5.88 (2.10)*	3.40 (0.55)	5.35 (2.18)	0.93 (0.28)	3.84 (1.18)*	3.36 (0.56)	3.37 (0.53)	2.29 (0.68)*	0.66 (0.38)
Stage III	36	7.63 (1.79)*	3.21 (0.67)	5.75 (2.13)	1.00 (0.25)	5.49 (1.38)*	3.18 (0.64)	3.24 (0.64)	3.30 (0.59)*	0.72 (0.20)
LNM										
(−)	47	5.89 (2.09)*	3.39 (0.55)	5.47 (2.23)	0.93 (0.28)	3.84 (1.18)*	3.34 (0.56)	3.36 (0.53)	2.29 (0.68)*	0.66 (0.39)
(+)	36	7.64 (1.80)*	3.22 (0.67)	5.59 (2.17)	0.99 (0.23)	5.51 (1.39)*	3.19 (0.64)	3.25 (0.64)	3.31 (0.60)*	0.73 (0.21)
ER status										
(−)	14	6.65 (2.72)	3.05 (0.54)	5.20 (2.38)	0.95 (0.30)	3.89 (1.36)	3.01 (0.49)	3.02 (0.49)*	2.49 (0.88)	0.81 (0.55)
(+)	69	6.64 (2.03)	3.37 (0.61)	5.59 (2.17)	0.95 (0.27)	4.69 (1.51)	3.33 (0.60)	3.37 (0.58)*	2.77 (0.80)	0.65 (0.21)
PR status										
(−)	26	7.58 (2.26)*	3.37 (0.72)	5.41 (2.02)	1.02 (0.26)	4.68 (1.47)	3.33 (0.70)	3.39 (0.65)	2.99 (0.91)	0.74 (0.34)
(+)	57	6.21 (1.96)*	3.29 (0.55)	5.57 (2.29)	0.92 (0.27)	4.50 (1.54)	3.26 (0.55)	3.27 (0.55)	2.61 (0.75)	0.65 (0.28)
Clinical response to NCCT										
Responders	62	6.81 (2.21)	3.34 (0.65)	5.49 (2.08)	0.98 (0.27)	4.54 (1.43)	3.29 (0.64)	3.33 (0.61)	2.76 (0.82)	0.71 (0.29)
Non- responders	21	6.15 (1.90)	3.25 (0.48)	5.62 (2.56)	0.89 (0.25)	4.61 (1.77)	3.25 (0.48)	3.25 (0.48)	2.63 (0.82)	0.62 (0.39)
Pathological response to NCCT										
pCR	18	6.04 (1.84)	3.28 (0.57)	4.93 (1.50)	0.85 (0.22)	4.02 (0.96)	3.25 (0.55)	3.29 (0.45)	2.56 (0.68)	0.64 (0.17)
Non-pCR	65	6.81 (2.20)	3.32 (0.62)	5.47 (2.28)	0.97 (0.28)	4.70 (1.61)	3.30 (0.61)	3.32 (0.61)	2.77 (0.85)	0.69 (0.32)

SD standard deviation; *$P < 0.05$

expression was primarily contributed by clinical responders. In clinical responders, the expression of ser-miR-21 was significantly lower at FDN and SDN than at BL ($P < 0.001$ both); however, there was no significant difference in non-responders. In addition, the proportion of patients showing decreased expression of ser-miR-21 was higher in clinical responders than in non-responders. In 87.10% (54/62) of clinical responders, ser-miR-21 expression decreased from BL to FDN and in 83.87% (52/62) it decreased from BL to SDN, whereas the corresponding numbers were 28.57% (6/21) and 33.33% (7/21) in clinical non-responders, and the difference between the two groups was significant ($P < 0.001$ both). However, there was no significant difference in changes of ser-miR-21 expression between pCR and non-pCR. The results showed that the expression of ser-miR-21 was significantly lower at FDN and SDN than at BL in pCR and non-pCR patients (Fig. 2, Table 2).

To evaluate the ability of changes of ser-miR-21 expression to predict the clinical response to treatment, ROC curve was plotted and AUC was calculated. The AUC values (95% CI) from BL to FDN and from BL to SDN were 0.862 (0.773–0.951) and 0.750 (0.626–0.874), respectively,

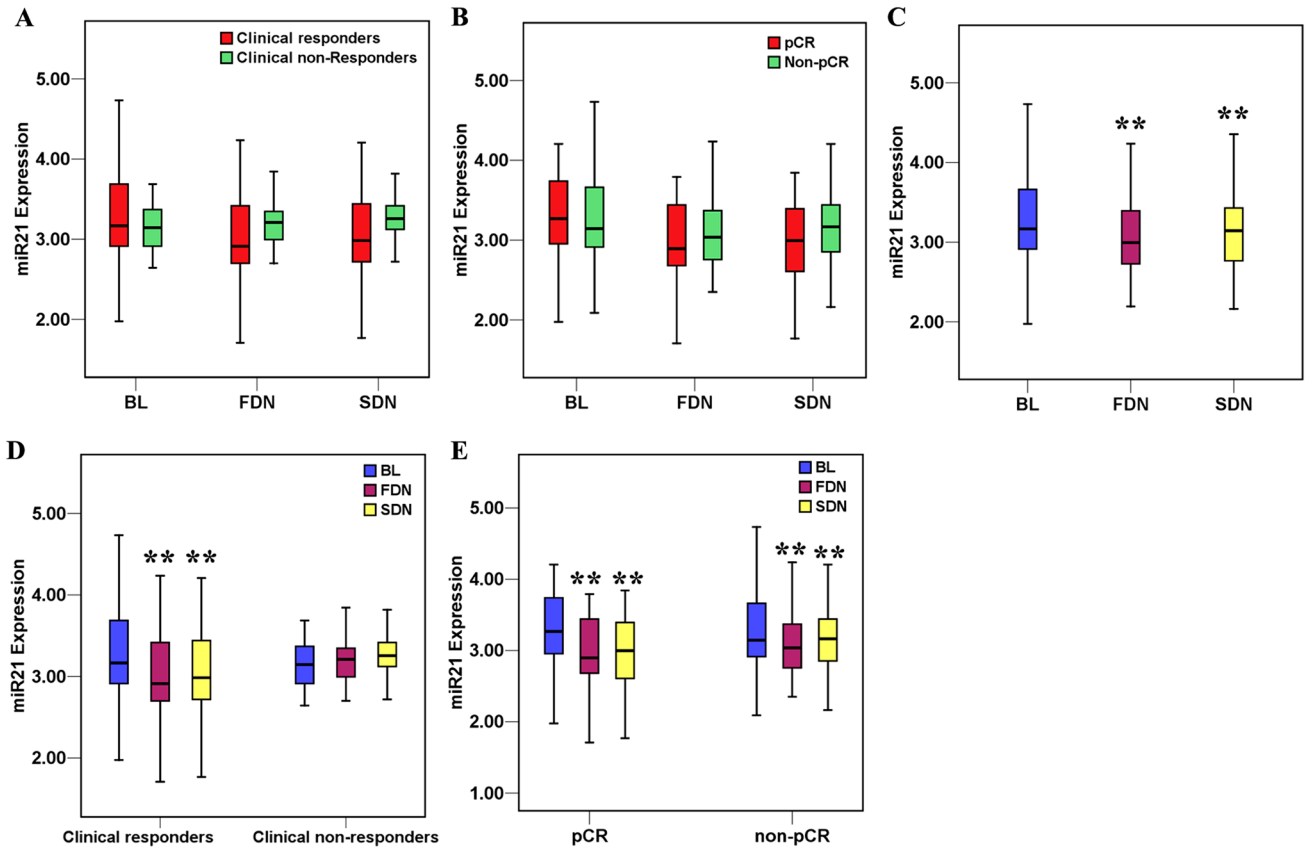

Fig. 2 Expression levels of ser-miR-21 in HPBC patients during NCCT. **a**, **b** Expression of ser-miR-21 in different responding groups at BL, FDN and SDN. **c** Expression of ser-miR-21 in all patients at BL, FDN and SDN. **d**, **e** Changes in ser-miR-21 expression levels from BL to FDN and from BL to SDN in different responding groups. $^{**}P < 0.01$ compared with BL

Table 2 Changes in ser-miR-21 expression levels during NCCT

Groups	*n*	BL (SD)	FDN (SD)	SDN (SD)	*P* value (BL to FDN)	*P* value (BL to SDN)
All patients	83	3.32 (0.61)	3.12 (0.55)	3.14 (0.52)	< 0.001	0.001
Clinical responders	62	3.34 (0.65)	3.06 (0.56)	3.09 (0.56)	< 0.001	< 0.001
Clinical non-responders	21	3.25 (0.48)	3.30 (0.49)	3.29 (0.37)	0.204	0.599
pCR	18	3.28 (0.57)	2.96 (0.55)	2.99 (0.56)	< 0.001	< 0.001
Non-pCR	65	3.32 (0.62)	3.16 (0.54)	3.17 (0.51)	< 0.001	0.003

($P < 0.001$ and $P = 0.001$, respectively; Fig. 3). These results demonstrated that ser-miR-21 could accurately distinguish clinical responders from non-responders but not distinguish pCR from non-pCR.

Relationship between ser-miR-21 expression and prognosis

During follow-up, 24 patients died and 29 patients relapsed. First, the prognostic values of ser-miR-21 expression levels at BL, FDN, and SDN were analyzed. The patients were divided into high expression and low expression groups according to the median level of miR-21 expression at BL, FDN, and SDN, respectively. Kaplan–Meier analysis showed that the expression of ser-miR-21 at BL, FDN, and SDN was not related to OS and DFS. Next, the prognostic values of the changes of ser-miR-21 expression from BL to FDN and from BL to SDN were also analyzed. The results showed that changes in ser-miR-21 expression during NCCT were significantly associated with OS and DFS. Patients in which ser-miR-21 expression decreased from BL to FDN and from BL to SDN had better OS ($P < 0.001$ both) and DFS ($P < 0.001$ both) than patients with elevated expression (Fig. 4).

The Cox regression model was used to confirm the effect of ser-miR-21 and clinicopathological characteristics on prognosis. Univariate analysis showed that five variables, namely clinical stage, LNM, clinical response to NCCT, change of ser-miR-21 from BL to FDN, and change of ser-miR-21 from BL to SDN, were significantly related to OS. Multivariate analysis showed that only the change of ser-miR-21 from BL to FDN was significantly associated with OS (Table 3). Univariate and multivariate analyses for DFS indicated same results with OS (Table 4). However, age, histological type, tumor grade, ER, PR, and pathological response to NCCT were not significantly correlated with OS or DFS. These results identified the change of ser-miR-21 from BL to FDN as an independent prognostic indicator.

Relationship between the expression of ser-miR-21 and HPBC presence

The expression levels of miR-21 in paired pre- and post-operative serum samples from 30 HPBC patients who underwent mastectomy were measured to confirm the relationship between ser-miR-21 expression and HPBC presence. Of 30 HPBC patients, 29 showed lower ser-miR-21 expression levels post-operatively than pre-operatively, whereas 1 patient showed higher levels post- than pre-operatively (Fig. 5). In addition, the mean expression levels of ser-miR-21 were significantly lower in post-operative samples (2.74 ± 0.51) than in pre-operative samples (3.24 ± 0.55; $P < 0.001$). This result indicates that the expression of ser-miR-21 is significantly associated with HPBC burden.

Discussion

Although studies indicate that miRNAs can be used as markers for the diagnosis, prediction of chemotherapy response, and prognosis in BC, most studies are based on the analysis of tumor tissues and cell lines [24, 25]. However, the analysis of tumor tissues is often inconvenient and impractical for clinical application, whereas the analysis of serum is less invasive and shows reproducible results. Therefore, ser-miRNAs show great promise for cancer diagnosis, prognosis, and prediction of treatment response.

HPBC has aggressive features and high metastatic potentials because overexpression of HER2 induces the activation of growth factor signaling pathways [26]. Despite the clinical benefits of trastuzumab, almost 50% of HPBC patients receiving trastuzumab have residual cancer because of resistance to the drug [5, 27]. Therefore, large-scale studies are needed to explore biomarkers for predicting treatment response. Studies show that the relative miRNA expression is associated with chemotherapy response and prognosis in

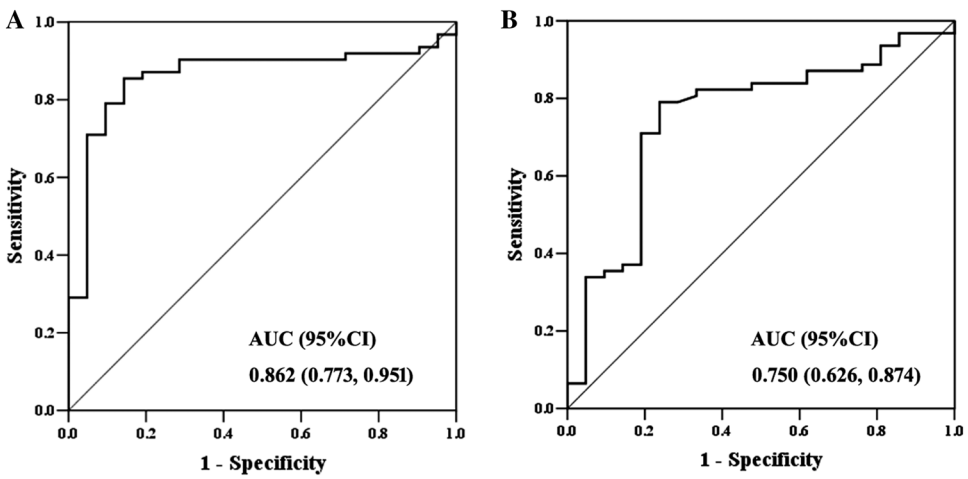

Fig. 3 ROC curves for the identification clinical responding and non-responding HPBC patients based on the changes of ser-miR-21 expression: **a** from BL to FDN and **b** from BL to SDN

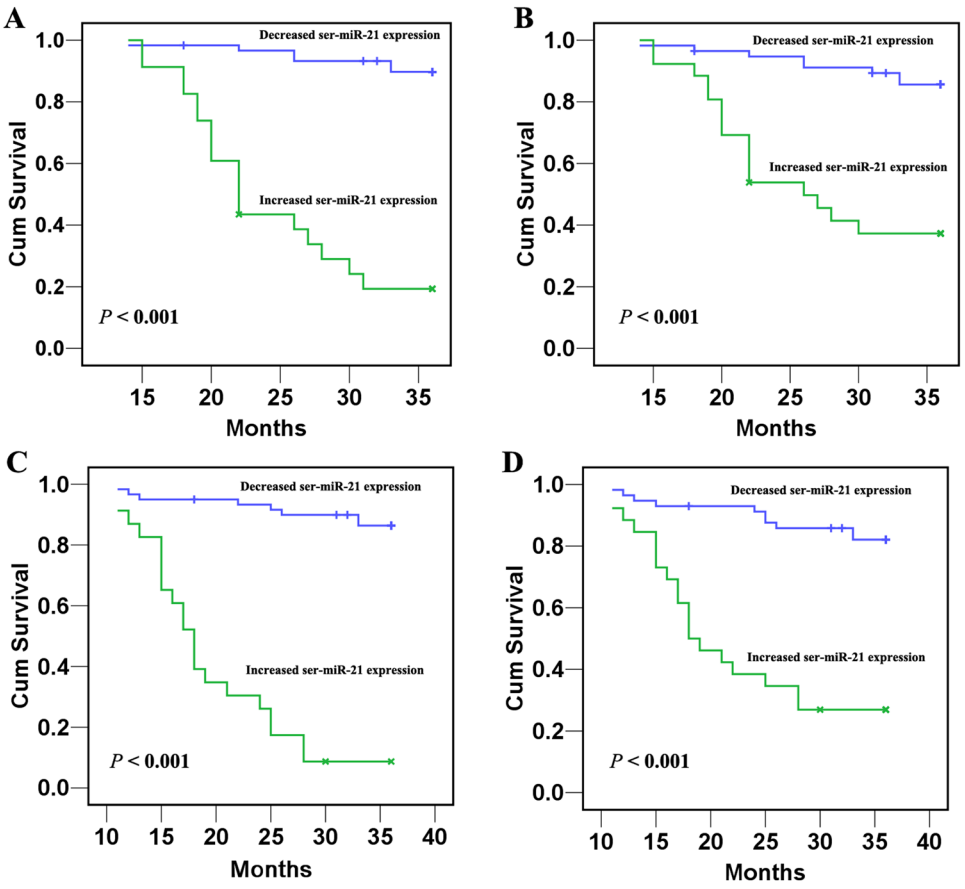

Fig. 4 Survival curves according to the changes of ser-miR-21 expression levels. **a** OS curves according to the changes of ser-miR-21 expression from BL to FDN. **b** OS curves according to the changes of ser-miR-21 expression from BL to SDN. **c** DFS curves according to the changes of ser-miR-21 expression from BL to FDN. **d** DFS curves according to the changes of ser-miR-21 expression from BL to SDN

Table 3 Cox hazards models analysis for OS in HPBC patients

	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P* value	HR	95% CI	*P* value
Age	0.858	0.39 ~ 1.91	0.707			
Tumor grade	1.32	0.79 ~ 2.22	0.288			
Histological type	1.04	0.36 ~ 3.02	0.938			
Clinical stage	2.19	1.02 ~ 4.72	0.046	1.49	0.16 ~ 13.56	0.724
LNM	2.17	1.01 ~ 4.68	0.048	0.91	0.10 ~ 8.27	0.931
ER expression	0.81	0.31 ~ 2.13	0.662			
PR expression	1.13	0.50 ~ 2.59	0.77			
Clinical response to NCCT	5.41	2.50 ~ 11.71	< 0.001	0.79	0.29 ~ 2.11	0.632
Pathological response to NCCT	32.66	0.85 ~ 1260.88	0.061			
Ser-miR-21 expression at BL	0.49	0.21 ~ 1.11	0.089			
Ser-miR-21 expression at FDN	0.88	0.41 ~ 1.87	0.734			
Ser-miR-21 expression at SDN	0.79	0.37 ~ 1.67	0.529			
Change of Ser-miR-21 from BL to FDN	23.78	8.87 ~ 63.73	< 0.001	21.05	5.95 ~ 79.35	< 0.001
Change of Ser-miR-21 from BL to SDN	8.7	3.75 ~ 20.20	< 0.001	1.43	0.45 ~ 4.53	0.546

HPBC. Ohzawa measured the expression of 2024 miRNAs by microarray and established a prediction model including 14 differentially expressed miRNAs for distinguishing pCR from non-pCR in HPBC patients receiving NCCT [28]. Jung detected the expression of plasma miR-210, miR-21, miR-29a, and miR-126 prior to treatment in HPBC patients undergoing NCCT, and found that only plasma miR-210 expression was significantly higher in patients with residual disease than in those achieving pCR [5]. Müller analyzed the relationship between the expression of ser-miR-21, ser-miR-210, and ser-miR-373 and treatment response in HPBC patients receiving chemotherapy combined with

Table 4 Cox hazards models analysis for DFS in HPBC patients

	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P* value	HR	95% CI	*P* value
Age	0.96	0.46 ~ 2.01	0.914			
Tumor grade	1.18	0.74 ~ 190	0.481			
Histological type	0.75	0.31 ~ 1.83	0.531			
Clinical stage	2.04	1.02 ~ 4.08	0.043	1.63	0.24 ~ 11.04	0.619
LNM	2.02	1.01 ~ 4.04	0.046	0.89	0.13 ~ 6.09	0.908
ER expression	1.04	0.40 ~ 2.70	0.937			
PR expression	1.06	0.51 ~ 2.23	0.877			
Clinical response to NCCT	4.01	2.00 ~ 8.05	< 0.001	0.56	0.61 ~ 2.73	0.244
Pathological response to NCCT	33.09	0.99 ~ 1099.77	0.05			
Ser-miR-21 expression at BL	0.51	0.24 ~ 1.08	0.077			
Ser-miR-21 expression at FDN	0.82	0.41 ~ 1.64	0.578			
Ser-miR-21 expression at SDN	0.81	0.41 ~ 1.59	0.533			
Change of Ser-miR-21 from BL to FDN	14.84	6.65 ~ 33.07	< 0.001	19.32	5.94 ~ 62.84	< 0.001
Change of Ser-miR-21 from BL to SDN	5.847	2.86 ~ 11.94	< 0.001	1.22	0.44 ~ 3.41	0.703

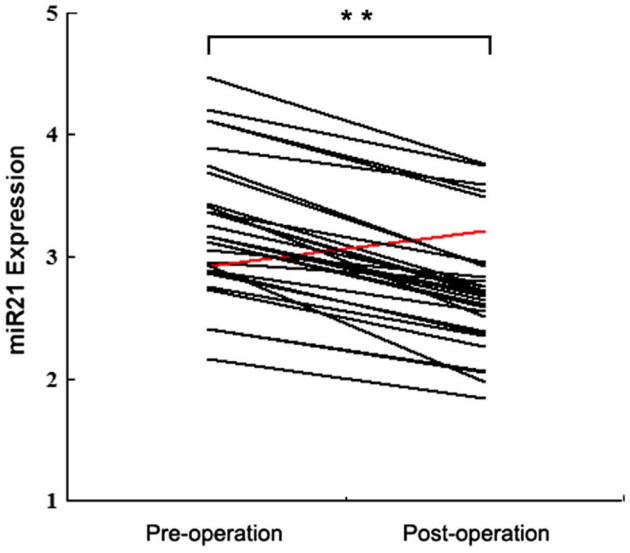

Fig. 5 Changes in miR-21 expression level in paired pre-operative and post-operative serum samples. The black lines represent reduced ser-miR-21 expression and the red line represents elevated ser-miR-21 expression. $^{**}P < 0.01$

either trastuzumab or lapatinib. The results showed that the levels of these three miRNAs were significantly higher in patients before chemotherapy than in healthy controls and increased further after chemotherapy. Although an association between ser-miR-21 expression and OS was found, no significant association between ser-miRNAs and treatment response was detected [13].

Of miRNAs associated with cancer, miR-21 is particularly interesting and compelling. Accumulating evidence shows that the overexpression of miR-21 promotes cell proliferation, migration, self-renewal, and clonogenicity through the induction of epithelial–mesenchymal transition in cancer cell lines and tissues, hence contributing to carcinogenesis, progression, angiogenesis, metastasis, and recurrence [29, 30]. Studies show that the expression of miR-21 is higher in cancer tissues than in normal tissues and significantly associated with worse prognosis [31, 32]. However, contrary to these results, Silva-Santos showed that the expression level of miR-21 was significantly lower in renal cell carcinomas than in normal tissues, although patients with higher expression levels of miR-21 had worse DFS [33]. Cheng showed that the inhibition of miR-21 expression by antisense molecules significantly enhanced growth in HeLa cells [34]. These results suggest that the expression patterns of miR-21 in cancer tissues are complex and may be related to the type of the cancer. In addition, studies show an association between miR-21 expression and drug resistance in BC. Gong demonstrated that miR-21 upregulation contributes to trastuzumab resistance in HER2-positive BT474, SKBR3, and MDA-MB-453 BC cells and in breast xenograft tumors, whereas downregulation of miR-21 by miR-21 antisense oligonucleotides rescues the sensitivity to trastuzumab [7]. In another study, Mattos-Arruda analyzed 14 miRNAs related to epithelial–mesenchymal transition and showed that only miR-21 expression was associated with the response of neoadjuvant trastuzumab and chemotherapy, and the levels of miR-21 increased further after treatment in HPBC [4].

Circulating miR-21 was the first miRNA shown to be present in the biological fluids of cancer patients. In that study, Lawrie showed that ser-miR-21 is upregulated in patients with diffuse large B-cell lymphoma compared with healthy controls, and high ser-miR-21 expression is associated with improved DFS [35]. In recent years, many studies have focused on circulating miR-21 as a non-invasive biomarker

for the diagnosis of certain cancers and for predicting treatment outcome and prognosis. However, research conclusions are controversial, in particular regarding miR-21 as a biomarker for predicting chemotherapy response in BC. Toraih tested ser-miR-21 expression in pre- and post-operative BC patients, and showed that ser-miR-21 is upregulated in BC patients and in asymptomatic high-risk individuals compared with healthy controls. In addition, the author showed that the levels of ser-miR-21 decreased toward normal levels after mastectomy [36]. Yadav showed that ser-miR-21 expression is higher in BC patients than in healthy women and decreases further after chemotherapy. Similar conclusions were drawn by Müller [13] and Yoruker [15]. Importantly, these studies did not demonstrate an association between circulating miR-21 and chemotherapy response.

In the present study, we identified eight ser-miRNAs, including miR-10b, miR-21, miR-34a, miR-125b, miR-145, miR-155, miR-210, and miR-373 that were differentially expressed in HPBC patients and in healthy controls. The results suggested the potential of these ser-miRNAs as candidate diagnostic markers in HPBC. In addition, we found that the expression of nine ser-miRNAs examined in the present study at BL, FDN, and SDN was not associated with clinical and pathological responses. However, changes in ser-miR-21 expression levels from BL to FDN and from BL to SDN during NCCT were significantly associated with clinical response, but not associated with pathological response. The results showed that patients with reduced expression levels of ser-miR-21 during NCCT had a better clinical chemotherapy response than those with increased levels. Survival analysis showed that patients with reduced expression levels of ser-miR-21 had better OS and DFS than those with increased levels. Notably, the ROC curve results indicated that changes in ser-miR-21 expression from BL to FDN had a good discriminatory power between clinical responding and non-responding patients. This result suggested that ser-miR-21 could serve as a biomarker for predicting the chemotherapy response at the early stages of NCCT. Combined with the result that postoperative ser-miR-21 levels decreased compared with preoperative levels, these findings suggest that changes in ser-miR-21 expression could reflect dynamic changes in the disease process in HPBC and hence may serve to monitor disease progression and relapse after treatment. The finding that changes in circulating miRNA expression were related to chemotherapy response during treatment is not surprising. In our previous studies, changes in ser-miR-34a and ser-miR-21 levels were associated with the response to neoadjuvant chemotherapy before surgery in ER-positive and HER2-negative BC [19, 20]. A study by Hansen showed that colorectal cancer patients showing decreased miR-126 expression during neoadjuvant chemotherapy combined with bevacizumab have a better treatment response and prognosis than those with increased expression. MiR-126 plays an important role in the proliferation, differentiation and repair of vascular endothelial cells under disease and health conditions [37, 38]. Vascular endothelial cells are targets of bevacizumab treatment [39]. These studies explain Hansen's results. However, the mechanism underlying the relationship between miR-21 and chemotherapy resistance in BC is complex and further studies are necessary to elucidate the detailed mechanism underlying our present results.

Our present research had some limitations. The subjects of this study were all from China. Therefore, whether the biomarkers identified in the present study are applicable to other ethnic populations remains to be determined. In addition, the conclusion of this study differs from that of our previous study, which recruited ER-positive and HER2-negative BC patients. Our previous study showed that in addition to ser-miR-21, ser-miR-34a and ser-miR-125b were also significantly associated with neoadjuvant chemotherapy response. Further large-scale studies are necessary to determine whether miR-21 is a specific biomarker for HPBC or trastuzumab response.

In summary, our results indicated that changes in ser-miR-21 expression were significantly associated with NCCT clinical response, tumor presence, and prognosis in HPBC patients. Ser-miR-21 has the potential to serve as a non-invasive biomarker to predict therapeutic response, prognosis, and tumor presence in HPBC.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

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