

Original Article

Circulating MiR-663 as a novel biomarker for chemo-resistance in breast cancer of neoadjuvant chemotherapy

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Abstract: Neoadjuvant chemotherapy has become prevalent in breast cancer therapies. Our previous research has reported that overexpression of miR-663 was associated with chemoresistance in breast cancer cells by targeting Heparin Sulfate Proteoglycan 2 (HSPG2). This study focused on exploring the potential role of miR-663 as a novel biomarker for chemotherapy in breast cancer patients who previously received neoadjuvant chemotherapy. A total of 35 healthy individuals and 116 breast cancer patients who received neoadjuvant chemotherapy were included in this study. The expression of circulating miR-663 in the plasma of all individuals was measured and the correlation between miR-663 expression and clinicopathological features was analyzed. The expression level of miR-663 was significantly associated with estrogen (ER) and partial clinical remission (PR) status. Patients with diverse miR-663 expression levels varied in the overall response rate but not in the pathologic complete response rate. Our results imply that plasma miR-663 may be a potential predictive biomarker for chemosensitivity of neoadjuvant chemotherapy in breast cancer patients.

Keywords: MiR-663, Neoadjuvant chemotherapy, breast cancer biomarker

Introduction

Since the novel concept of intrinsic molecular subtypes sorted by gene expression profiles was established and prevalently accepted, the treatment strategies for cancer have changed dramatically in clinical therapy. As an available and reasonable alternative to adjuvant chemotherapy for operable breast cancer, neoadjuvant chemotherapy can increase the rates of breast-conserving surgery [1-5]. Although neoadjuvant chemotherapy that can increase the rates of pathological complete response (pCR) and may be likely to improve the outcomes in breast cancer patients, neoadjuvant chemotherapy could not necessarily improve breast cancer patient survival. Furthermore, the efficacy of neoadjuvant chemotherapy, which can be measured by the pathological complete response rate, varied significantly according to the breast cancer subtype [6-9]. There are a series of factors which can be a potential predictive marker to the response of breast cancer patients to neoadjuvant chemotherapy, such as

hormone receptor (HR) status, Her2 expression level, tumor stage, clinical stage, lymph node status [10-12]. However, there is still unclear which breast cancer patients of phenotype can benefit from neoadjuvant chemotherapy.

MicroRNAs (miRNAs) are a class of conserved, short (approximately 22 nucleotides), single-stranded noncoding RNAs that control diverse biological functions by regulating gene expression at the post transcriptional level through inducing of target mRNA degradation [13-15]. Increasing evidences have demonstrated that miRNAs regulated a variety of major cellular functions, such as proliferation, migration, and apoptosis; thus, they have been also implicated in the development and progression of various types of cancers [16-18]. Abundant researches also revealed that some miRNAs expression may associate with particular human tumor phenotypes and biological characters, such as response to treatment and prognosis [19, 20]. More and more studies have illustrated that plasma miRNAs expression could be a novel

Table 1. Clinicopathological features of all 116 patients

Characteristic	n (%)
Age	
<45	38 (32.76)
≥45	78 (67.24)
Tumour size	
T1	6 (5.17)
T2	80 (68.97)
T3+T4	30 (25.86)
Lymph node	
Positive	81 (69.83)
Negative	35 (30.17)
Histological grade	
I+II	77 (66.38)
III	39 (33.62)
ER	
Positive	80 (68.97)
Negative	36 (31.03)
PR	
Positive	55 (47.41)
Negative	61 (52.59)
Her2	
Positive	33 (28.45)
Negative	83 (71.55)
ER/PgR/Her2	
Triple negative	20 (17.24)
Not triple negative	96 (82.76)
Histology	
Ductal	106 (91.38)
Nonductal	10 (8.62)
Ki-67	
High	78 (67.24)
Low	38 (32.76)
Pathological response	
CR	25 (19.83)
PR	79 (68.1)
Others	12 (10.34)

potential biomarker for the diagnosis of cancer.

In previous studies, miR-663 has been reported as distinct roles during tumor progression. In one hand, as tumor suppressor gene, miR-663 attenuates tumor growth and invasiveness by targeting eEF1A2, PIK3CD and H-Ras in pancreatic cancer, human glioblastoma and chronic myelogenous leukaemia [21-23]. In the other hand, miR-663 promotes tumor proliferation

and tumorigenesis by targeting TGFB1, p21WAF1/CIP1, and HSPG2 in lung cancer, nasopharyngeal carcinoma and breast cancer [24-26]. Particularly, miR-663 was significantly up-regulated in adriamycin-resistant MDA-MB-231/ADM cells comparing with the expression in the parental MDA-MB-231 cells and overexpression of miR-663 was closely associated with chemoresistance. To further explore whether miR-663 can be a potential predictive biomarker for breast cancer to the response of neoadjuvant chemotherapy, we compared the circulating miR-663 expression in 116 breast cancer patients who had previously received neoadjuvant chemotherapy in our hospital to those in 35 healthy individuals by quantitative real-time polymerase chain reaction (qPCR) from plasma samples. We found that the expression of miR-663 was significantly associated with HR status. The group with high plasma miR-663 expression had a tendency to be HR-negative. When comparing patients in the high and low miR-663 expression groups, a significant difference was observed regarding the overall response rate (ORR) but not the pCR rate.

Materials and methods

Patients and treatment

A total of 116 patients' blood samples with locally breast cancer, who received neoadjuvant chemotherapy from October 2012 to June 2015 at Department of Surgery, the First People's Hospital of Lianyungang, were collected and studied retrospectively. Meanwhile, 35 healthy individuals' plasmas were included as normal controls. All individuals were informed consent for the use of the blood samples and this project was approved by the Clinical Research Ethics Committee of Lianyungang People's Hospital. The clinicopathological features details were shown in **Table 1**.

All patients received core needle biopsy before neoadjuvant chemotherapy was implemented and all patients received cyclophosphamide at a dosage of (500 mg/m²), epirubicin (90 mg/m²) or pirarubicin (50 mg/m²) and docetaxel (75 mg/m²) for four cycles every 3 weeks. None of the patients received targeted drug treatment during neoadjuvant chemotherapy. The assessment of tumor response to neoadjuvant chemotherapy was defined as pCR (pathologi-

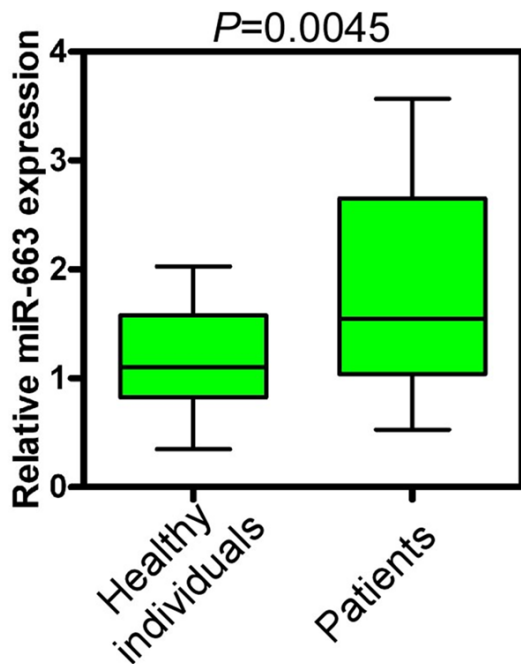


Figure 1. The expression of miR-663 in the plasma of breast cancer patients was significantly higher than that in the plasma of healthy individuals.

cal complete remission), partial response (PR; partial clinical remission), and others (including stable disease and progressive disease). pCR and PR contributed to the ORR of neoadjuvant chemotherapy and others indicated resistance to neoadjuvant chemotherapy. pCR was defined as complete disappearance of the invasive component of the primary tumor after neoadjuvant chemotherapy treatment.

All the patients' ER, PgR and Her2 expression status were measured by immunohistochemistry (IHC) in the Pathology Department of our hospital. All patients who finished neoadjuvant chemotherapy received surgery and standard adjuvant chemotherapy, endocrine therapy or radiotherapy. All of the tests were conducted at the time of primary diagnosis before treatment.

RNA extraction and quantitative RT-PCR

The miRNeasy Mini Kit (Qiagen, USA) was used to isolate the total RNA from human blood serum according to the manufacturer's instructions. For quantitative real-time PCR, the specific miRNA TaqMan MicroRNA Assay (Applied Biosystems) for miR-663 was used as described

by the manufacturer's protocol. Briefly, 100 ng of total RNA was reverse transcribed using specific primers to miR-663 and followed by real-time PCR on a 7900 HT Fast Real-Time PCR System using TaqMan miRNA primers and probes (Applied Biosystems). All the assays were measured and validated by endogenous controls in triplicate throughout the experiment.

Statistical analysis

Statistical analysis was performed using a SPSS software package (SPSS Standard version 18.0, SPSS Inc). (SPSS, Chicago, IL, USA) Differences between variables were assessed by the χ^2 test according to Fisher's exact test. Multivariate analysis was performed on all parameters that were found to be significant in univariate analysis using the Cox regression model. Two-tailed Student's t-tests were used to determine statistical significance for all results. $P < 0.05$ was considered to be statistically significant in all cases.

Results

Characteristics of study population

In total, 116 blood serum samples of breast cancer patients and 35 healthy control individuals were analyzed in this study. The clinicopathological features of the 116 patients and tumor characteristics are shown in **Table 1**. At the time of diagnosis, the median age of these patients was 43 years (range, 25-66 years). Most of the cases were histologically confirmed as early stage invasive ductal carcinoma and more than half of the patients had T2 tumors and positive nodal status. All breast cancer cases hormone receptor status (estrogen/progesterone receptors (ER/PgR), and Her2 status were available for all patients (**Table 1**).

MiR-663 was up-regulated in breast cancer patient plasma

The miR-663 expression level in blood serum of all 116 patients and 35 healthy control individuals were measured. Comparing with the expression levels of miR-663 in healthy individuals, the expression of miR-663 in the plasma of breast cancer patients was significantly higher than that in the plasma of healthy individuals ($P = 0.0045$, **Figure 1**).

Circulating MiR-663

Table 2. Correlation between plasma miR-663 expression level and clinicopathological characteristics in breast cancer patients

Characteristics	miR-663 Expression level		P
	Low (%)	High (%)	
Age			
<45	26 (46.43)	19 (31.67)	0.103
≥45	30 (53.57)	41 (68.33)	
Tumour size (cm)			
>5	18 (32.14)	15 (25)	0.393
≤5	38 (67.86)	45 (75)	
Lymph node			
Positive	33 (58.93)	43 (71.67)	0.149
Negative	23 (41.07)	17 (28.33)	
Histological grade			
I+II	37 (66.07)	38 (63.33)	0.752
III	19 (33.93)	22 (36.67)	
ER and PR status			
Positive	43 (76.79)	31 (51.67)	0.005
Negative	13 (23.21)	29 (48.33)	
HER2 status			
Positive	15 (26.79)	21 (35)	0.34
Negative	41 (73.21)	39 (65)	
Histology			
Ductal carcinoma	48 (85.71)	53 (88.33)	0.671
Nonductal	8 (14.29)	7 (11.67)	
Ki-67			
Low	26 (46.43)	22 (36.67)	0.286
High	30 (53.57)	38 (63.33)	

Table 3. Relationship between miR-663 expression and response to chemotherapy

Variables	miR-663 Expression level		P
	Low (%)	High (%)	
pCR			
Yes	17 (28.6)	10 (22)	0.081
No	39 (71.4)	50 (78)	
ORR			
Yes	41 (77.4)	33 (55)	0.041
No	15 (22.6)	27 (45)	

Correlation analysis between blood serum miR-663 expression level and breast cancer patient clinicopathological characteristics

To further investigate the correlation between miR-663 expression and the prognosis, the relationships between plasma miR-663 expression levels and clinicopathological characteristics

were analyzed in all 116 cases of breast cancer patients. The results indicated that miR-663 expression was significantly associated with HR status. The high plasma miR-663 expression group exhibited a tendency of HR negativity (**Table 2**). However, the miR-663 expression level was not related to other characteristics such as patient age, tumor size, lymph node status, Her2 status, Ki-67 level, histological grade and histology.

Plasma miRNA-663 expression level correlates significantly with that in breast cancer and can be a potential biomarker for resistance to chemotherapy

Next, correlation analysis was performed in all the samples which were be divided into high and low miR-663 expression groups, a significant difference was obtained regarding the ORR but not the pCR rate (**Table 3**). We also performed univariate statistical analysis with the Cox proportional hazards model to evaluate the factors predicting patient outcomes. And as the results shown that the histological grade, HR status, Her2 status, Ki-67 and miR-663 expression were associated with chemoresistance (**Table 4**). When these factors, which were significantly associated with the ORR in the univariate, were further analyzed with multivariate Cox regression model, we found that HR status, Her2 status and plasma miR-663 expression were still identified as independent prognostic factors for chemotherapeutic response (**Table 5**).

Discussion

Despite recent controversy regarding the attainment of pCR in luminal tumors, there could be a sub-population who would benefit from neoadjuvant chemotherapy. At previously, abundant studies have reported and demonstrated the efficacy of neoadjuvant chemotherapy, which is now widely accepted and used in clinical surgery of early stage breast cancer patients for shrinking the breast tumor to facilitate surgical resection [27, 28]. Neoadjuvant chemotherapy might also increase possibilities for breast conserving surgery, another advantage for patients. However, it has been found in clinical therapy that different patients respond variable strikingly to treatment. Some patients may achieve pCR after neoadjuvant chemotherapy, whereas others may not. Consequently,

Table 4. Univariate analysis of clinical factors of total response in breast cancer patients (complete response and partial response)

Characteristics	ORR		P
	HR	95% CI	
Age	1.036	0.393-2.81	0.656
Lymph node status	1.036	0.393-2.81	0.656
Tumour size	1.337	0.51-3.709	0.392
Histological grade	0.375	0.13-1.068	0.058
Hormone receptor status	3.894	1.301-13.552	0.013
Her2 status	0.189	0.039-0.693	0.015
Histology	0.511	0.114-2.857	0.507
Ki67	0.268	0.123-0.695	0.006
Plasma miR-663 expression	9.813	3.379-39.827	0.003

HR: Hazard Ratio; CI: Confidence Interval.

Table 5. Multivariate logistic regression analysis of clinical factors of total response in breast cancer patients (complete response and partial response)

Characteristics	ORR		P
	HR	95% CI	
Histological grade	0.872	0.229-3.352	0.85
Hormone receptor status	5.767	1.623-30.929	0.018
HER2 status	0.188	0.031-0.772	0.03
Ki-67	0.503	0.155-1.864	0.269
Plasma miR-663 expression	8.116	2.208-23.695	0.003

HR: Hazard Ratio; CI: Confidence Interval.

it is very important to seek a stable and reliable biomarker to predict the response to neoadjuvant chemotherapy for different patient is clinical treatment. Numerous researches have focused on exploring novel tumor markers for predicting the response to neoadjuvant chemotherapy in multiple tumors with conflicting conclusion [29, 30]. Therefore, more studies should be done to deeply elucidate reliable markers for chemosensitivity in tumor patients with neoadjuvant chemotherapy treatment.

In present study, our results demonstrated that high expression of miR-663 was related to a poor response to chemotherapy and bad pCR. In previous studies, miR-663 has been reported to play distinct roles involving in multiple cancer progression both in attenuating and promoting cancer proliferation and progression. By comparing the expression of miR-663 between patients and healthy individuals, our results implied that blood serum miR-663 levels were higher in breast cancer patients than

that in healthy individuals ($P = 0.0045$). In our study, the patients who had previously received neoadjuvant chemotherapy ($n = 116$) and a small group ($n = 35$) of healthy individuals were recruited; therefore, the sample is small and our findings might not reflect the whole breast cancer patients and healthy individuals. Through analyzing the correlation between plasma miR-663 expression and the clinicopathological characteristics of breast cancer patients, we found that the miR-663 level was negatively associated with HR status ($P = 0.005$). In other studies, miR-663 has been demonstrated to promote tumor growth and proliferation by regulating target genes of p21WAF1/CIP1. However, we failed to observe the association between miR-663 expression and Ki-67 expression, tumor size, and lymph node status [31]. Moreover, the miR-663 expression level was closely related to ORR but not to pCR, suggested that the miR-663 expression can be a potential predictive marker for chemoresistance in breast cancer patients who previously received neoadjuvant chemotherapy. Although miR-663 expression was negatively correlated with HR expression, which is a predictive factor for chemoresistance, the univariate and multivariate analysis finding suggested that miR-663 expression was an independent factor for chemoresistance.

We had previously demonstrated that miR-663 was significantly up-regulated in adriamycin-resistant MDA-MB-231/ADM cells comparing with the expression in the parental MDA-MB-231 cells and overexpression of miR-663 was closely associated with chemoresistance. These data suggest that miR-663 represents a promising therapeutic target for treating hormone-independent breast cancer.

In our multivariate Cox regression analysis, some other factors such as HR status and Her2 status were also identified as independent predictive factors for the response to chemotherapy. However, we failed to identify Ki-67 as an independent predictive marker for neoadjuvant chemotherapy. This discrepancy may be due to small sample size in this study. There are some limitations to our study. Firstly, the sample size of patients and healthy individuals were small. Second, this was a retrospective study in a single centre. In conclusion, our study demonstrated that the expression of miR-663 was up-reg-

ulated in the blood serum of breast cancer patients. The miR-663 expression was negatively associated with HR expression and patients with high miR-663 expression were more likely to be chemoresistant. Based on these results, it is believed that plasma miR-663 expression might be a predictive biomarker for response in breast cancer patients before neoadjuvant chemotherapy.

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Disclosure of conflict of interest

None.

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