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Original Article

MicroRNA-181a is a predictor of poor survival and a prognostic biomarker of chemoresistance in triple negative breast cancer

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Abstract: Acquired chemoresistance represents a major obstacle in TNBC treatment, the underlying mechanism of which is complex and not well understood. Recently, miR-181a has been reported to be implicated in tumorigenesis and chemoresistance in a few cancer types. The aim of this study is to evaluate the expression of miR-181a and its clinical significance in TNBC patients. To this end, quantitative real-time PCR was performed to measure miR-181a expression level in 47 pairs of TNBC tissues and the non-tumor adjacent tissues (NATs) as well as tissue samples from 46 TNBC patients who respond to neo-adjuvant chemotherapy (NAC) and 42 patients who do not. Correlation between miR-181a expression and overall survival (OS) was evaluated using Kaplan-Meier analysis. Prognostic value of miR-181a was assessed using Cox proportional hazards regression analysis. We found that miR-181a expression was significantly increased in TNBC tissue samples compared with the neighboring non-tumor tissues. High miR-181a expression was associated with higher tumor grade, positive lymph node metastasis and chemoresistance. Furthermore, Kaplan-Meier analysis showed that miR-181a expression was significantly correlated with OS in TNBC patients with or without receiving chemotherapy. In addition, multivariate analyses revealed that miR-181a was an independent prognostic factor of OS in TNBC. Thus, we concluded that miR-181a is up-regulated in TNBC patients who are chemoresistant, and miR-181a may serve as a predictive marker of poor outcome for TNBC patients undergoing chemotherapy.

Keywords: TNBC, miR-181a, chemoresistance, biomarker

Introduction

Breast cancer is the most common type of tumor among women in both developing and developed countries as it was estimated to affect about 1.7 million women in 2012 worldwide (American Cancer Society) [1]. Primary breast cancer is usually classified into 5 main intrinsic molecular subtypes based on the gene expression profile: luminal A, luminal B, human epidermal growth factor receptor 2 (HER2) enriched, basal like, and claudin low [2]. Triple-negative breast cancer (TNBC) is a kind of invasive carcinoma of primary breast cancer that lack the expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 or HER2 gene amplification [3]. TNBC accounts for approximately 20% of all breast cancer cases in the United States and is regarded as a very heterogeneous disease with various molecular, genetic, and clinical subgroups [4]. Due to the absence of hormone receptors and HER2 expression, endocrine therapy and HER2 targeted therapy are not available for TNBC. Despite initial good response to chemotherapy, TNBC often recurs with chemotherapy resistance, visceral and brain metastasis [5]. The risk of relapse for TNBC patients in the first 3-5 years is significantly higher than for non-TNBC breast cancer women [6, 7]. In addition, patients with residual TNBC disease post neo-adjuvant chemotherapy (NAC) have been shown to have a worse prognosis than those presenting with hormone positive breast cancer [8]. Therefore, there is an urgent need to identify predictive biomarkers for chemosensitivity in TNBC.

Studies have shown that many factors and genes are involved in chemoresistance of breast cancer, such as gene mutation, gene amplification, epigenetic changes, and microR-

NA expression etc [9]. MicroRNA (miRNA) is an abundant class of conservative and small non-coding RNAs which post-transcriptionally regulate genes expression by inducing the degradation or translational repression of target mRNAs [10]. Aberrant expression of miRNAs contributes to tumorigenesis process through their functions in cell cycle control, DNA repair, apoptosis, invasion/metastasis and angiogenesis, etc. in various cancer types [11]. Previous studies have also shown that miRNAs are involved in regulating the sensitivity of cancer cells to chemotherapeutic agents and dysregulation of miRNA function might contribute to the acquisition of chemoresistance [12, 13].

In our study, we evaluated the expression of miR-181a in TNBC patient samples and its association with clinicopathological features and prognosis. Our results suggest that miR-181a is significantly higher in TNBC tissues compared with matched non-cancerous tissues. Cox regression analysis reveals that miR-181a expression level may serve as a prognostic biomarker for survival and predictor of chemoresistance in TNBC patients.

Method

Patients and tissue samples

The study was approved by the Ethical Committee of Xinjiang Medical University, and informed consent was obtained from all patients. TNBC surgical specimens from 47 patients with locally advanced primary BC (LAP-TNBC) (stage IIIA-C) for whom matching biopsies were available for pathological and immunohistochemical analysis were included between March 2009 and July 2014. All tissues were immediately snap-frozen in liquid nitrogen and stored at -80°C until use. In addition, the patients with any other tumor were excluded from the study. None of the subjects had received any therapeutic procedures prior to this study, in-cluding surgery, chemotherapy, and radiotherapy. In addition, a total of 88 patients who re-ceived six cycles of an anthracycline based-therapy (FEC: 5-fluorouracil (5-FU) 500 mg m-2, epirubicin 75-100 mg m-2, cyclophosphamide 500 mg m-2, on day 1 of a 21-day cycle) were also included. All patients underwent surgery (mastectomy and axillary node clearance) 4 weeks after the last cycle of chemotherapy, followed by radiotherapy to the chest wall. The pathological complete response (pCR) was de-fined as the absence of any residual invasive carcinoma at both the primary site and in axillary lymph nodes. Patients with pCR were defined as responders whereas others were defined as non-responders. The prognosis was evaluated in all TNBC patients in January 2015. Overall survival was defined as the time from cancer onset until death or by censoring at the last follow-up date.

Total RNA extraction and RT-qPCR analysis

Total RNAs of tissues were extracted with Qiagen miRNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, and then miRNAs was reverse transcribed to cDNA. TaqMan miRNA assays (Applied Biosystems, Foster City, USA) with specific RT primers and probes were used to quantify the expression of mature miR-181a. cDNA was generated from 500 ng of total RNA using PrimeScript™ RT Master Mix Perfect Real Time (TaKaRa, Dalian, China). U6 was used for miRNA template normalization. All samples were performed in triplicate and independently repeated three times.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics Version 16 (SPSS Inc, Chicago, IL, USA) and GraphPad Prism v5.0 (Graphpad Software Inc.). The Wilcoxon test was used to compare miR-181a expression in paired tumor tissue samples and NATs. The Mann-Whitney U test was used to perform statistical analysis of miR-181a level between unpaired groups. The Pearson's chi-squared test and Fisher's exact test were used to evaluate the association between tissue miRNA levels and clinicopathological parameters. In addition, survival curves were constructed with the Kaplan-Meier method and compared using log-rank test. Cox proportional hazards regression analysis was used for univariate and multivariate analyses of prognostic values. *P* value of two-sided less than 0.05 was considered statistically significant.

Results

miR-181a expression was up-regulated in TNBC tissues

Quantitative real-time PCR was performed to evaluate the expression levels of miR-181a in

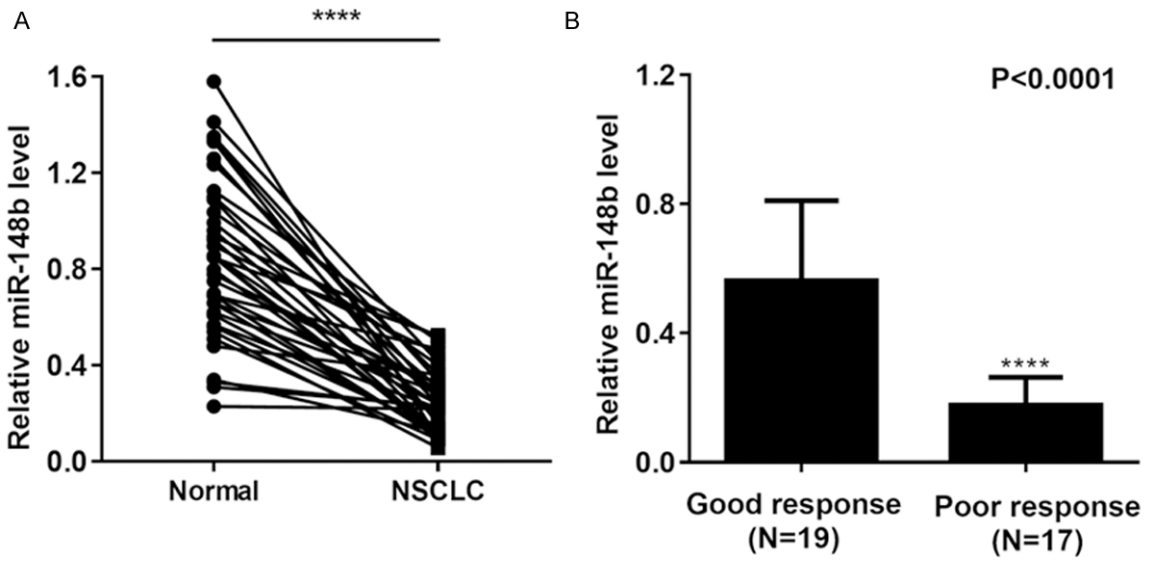

Figure 1. miR-181a expression levels in TNBC tissue samples and normal controls. A. Quantitative PCR analysis of relative miR-181a expression in tissue 47 pairs of TNBC patients and matched NATs. B. Quantitative PCR analysis of relative miR-181a expression in tissue samples from TNBC patients who are responders (n=46) or non-responders (n=42) to NAC. Data represent mean ± SD. ****, P<0.0001.

Table 1. Correlation between tissue miR-181a expression level and clinicopathological characteristics or chemosensitivity

Characteristics	Number of patients	miR-181a low expression	miR-181a high expression	*P* value
Age (years)				
≤40	25	5	20	0.1155
>40	22	10	12	
Tumor size (cm)				
≤5	18	5	13	0.7526
>5	29	10	19	
Tumor stage				
T1-2	30	12	18	1.0000
T3-4	17	6	11	
Tumor grade				
G1	32	20	12	0.0144*
G2/G3	15	3	12	
Lymph node metastasis				
Negative	20	14	6	0.0392*
Positive	27	10	17	
Clinical response to NAC				
Responder	46	29	17	0.0014*
Non-responder	42	12	30	

*, Statistical significance (P<0.05).

47 pairs of TNBC tissues and the NATs. We observed significantly higher levels of miR-181a in tumor tissues compared with NATs (P<0.0001) (**Figure 1A**). We also measured the miR-181a expression levels in tissue samples from 46 TNBC patients who respond to NAC and 42 patients who do not. We found that the expression levels of miR-181a were significantly higher in the non-responder group than the responder group (P<0.0001) (**Figure 1B**).

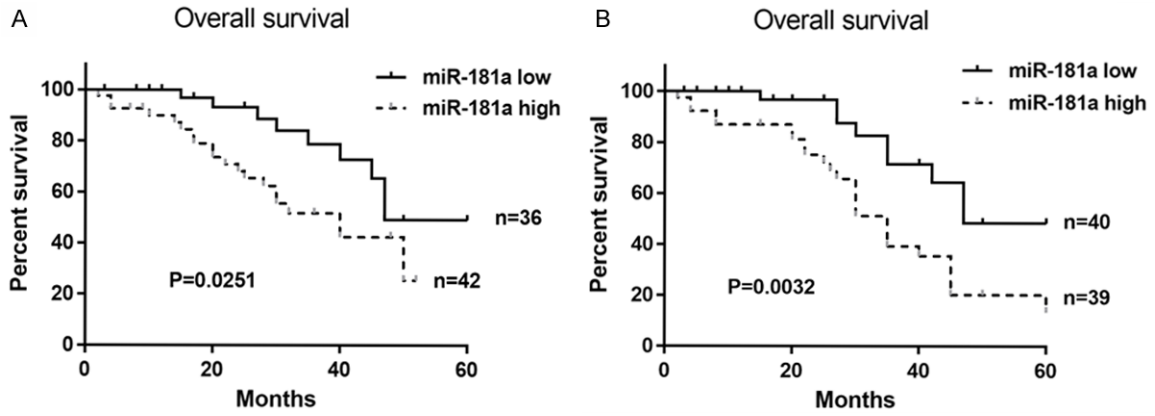

Figure 2. Correlation between miR-181a expression and OS in TNBC patients with or without receiving NAC. A. Kaplan Meier OS of TNBC patients without receiving NAC. Patients with high miR-181a expression had a shorter survival (P=0.0251). B. Kaplan Meier OS of TNBC patients receiving NAC. Patients with high miR-181a expression had a shorter survival (P=0.0032).

Correlation between tissue miR-181a expression level and clinicopathological characteristics or chemotherapy response

The correlation between miR-181a expression level and clinicopathological factors or chemotherapy response was assessed using X^2 test. As shown in **Table 1**, high levels of miR-181a were significantly correlated with tumor grade (P=0.0144), lymph node metastasis (P=0.0392) and response to NAC (P=0.0014). However, there were no significant association between miR-181a expression levels and other factors including age, tumor size or tumor stage (P=0.1155, 0.7526, 0.2268, and 1.0000 respectively).

High expression of miR-181a predicted the poor prognosis in TNBC patients with or without receiving NAC

To determine whether miR-181a expression level can predict prognosis, we performed a survival analysis of OS in patients with or without NAC treatment. The estimated Kaplan-Meier OS curves showed that high expression of miR-181a was significantly correlated with poor OS in patients without receiving NAC (P=0.0251) (**Figure 2A**). In patients receiving NAC treatment, the OS was significantly worse in patients with high levels of miR-181a (P=0.0032) with a median survival of 35 months (**Figure 2B**). Univariate and multivariate survival analyses were also performed to examine the effects of clinicopathological factors and miR-181a expression on prognosis in patients receiving NAC. As shown in **Table 2**, tumor grade (P=0.036), lymph node metastasis (P=0.029), miR-181a expression (P=0.009) and response to NAC (P=0.004) were significantly associated with overall survival (OS). Multivariate analysis indicated that lymph node metastasis (P=0.037, HR=2.36, 95% confidence interval: 1.17-3.16), miR-181a expression (P=0.018, HR=2.75, 95% confidence interval: 1.84-3.98) and response to NAC (P=0.008, HR=4.56, 95% confidence interval: 3.31-6.24) were independent predictors of OS in these patients (**Table 2**). These results suggest that high miR-181a expression was associated with poor OS in patients with or without NAC treatment and was an independent prognostic factor.

Discussion

Due to the lack of ER, PR and HER2/neu expression, which conventionally determine the therapeutic response and general disease prognosis of primary breast cancer, chemotherapy has been the primary methods for the treatment of TNBC patients. However, chemoresistance has severely limits its application and efficacy and this has emerged as a major obstacle in the treatment success of TNBC. In the past two decades, although significant progress has been made in understanding drug resistance in breast cancer, more amenable therapeutic targets and novel biomarkers are still urgently needed to improve the overall survival and refine the therapeutic strategy for TNBC patients. Our study demonstrated the association

Table 2. Univariate and multivariate analysis of factors associated with overall survival for patients with LAP-TNBC (stage IIIA-C) who received NAC prior to surgery

	Univariate		Multivariate	
Variables	P	HR	95% CI	P
Age	0.336			NA
Tumor size	0.724			NA
Tumor stage	0.477			NA
Tumor grade	0.036*			0.069
Lymph node metastasis	0.029*	2.36	1.17-3.16	0.037*
miR-181a expression (positive vs. negative)	0.009*	2.75	1.84-3.98	0.018*
Response to NAC (non-responder vs. responder)	0.004*	4.56	3.31-6.24	0.008*

*, Statistical significance (P<0.05). HR: hazard ratio; CI: confidence interval.

between miR-181a expression and locally advanced TNBC. We observed significantly higher levels of miR-181a in tumor tissues compared with NATs (**Figure 1A**). In addition, miR-181a expression levels were significantly correlated with tumor grade, lymph node metastasis, and response to NAC (**Table 1**). Moreover, the OS was significantly lower in patients with elevated levels of miR-181a (**Figure 2A**). These results suggest that miR-181a may function as an oncomir in TNBC tumorigenesis.

Accumulating evidence has suggested that miRNAs may be involved in chemotherapy resistance in many cancer types, including breast cancer [14]. For example, miRNA-34a-5p has been shown to enhance sensitivity to chemotherapy by targeting AXL in hepatocellular carcinoma cells [15]. The plasma miR-1914 and miR-1915 has been shown to suppresses chemoresistant in colorectal cancer patients by down-regulating NFIX [16]. Numerous studies have demonstrated the implication of miR-181a in various aspects of tumorigenesis. It was initially found to function as a tumor suppressor in human glioma cells [17]. Later, miR-181a was found to have tumor suppressive effect by down-regulating K-ras in oral squamous cell carcinoma cells [18]. TRIM3, a close family member to one of the target genes for miR-181a, has been reported to function as a tumor suppressor in transformed human colonic epithelial cells [19]. In addition, miR-181a has been shown to regulate cell migration, invasion, epithelial-mesenchymal transition in pancreatic cancer, prostate cancer, ovarian cancer, respectively [20-22]. Aberrant expression of miR-181a was observed in various cancer types and may serve as a potential biomarker for hepatocellular carcinoma, colorectal cancer, esophageal cancer, etc [23-25]. More recently, miR-181a has been implicated in regulation of radio- or chemo-sensitivity. For example, miR-181a was reported to sensitize human malignant glioma U87MG cells to radiation by targeting Bcl-2 [26]; It has been shown to modulate sensitivity to cisplatin via suppressing autophagy in gastric cancer cells [27]. More recently, Jiao et al. showed that miR-181a could enhance drug sensitivity in mitoxantone-resistant breast cancer cells by targeting breast cancer resistance protein (BCRP/ABCG2) [28]. However, the expression of miR-181a and its association with chemo-sensitivity has not been investigated in TNBC patients. In our study, we found that the expression of miR-181a was elevated in tissue samples from patients who do not respond to NAC compared to those who respond (**Figure 1B**). miR-181a expression levels were significantly correlated with chemosensitivity in those patients (**Table 1**). In addition, high miR-181a expression levels predicted poor survival in patients receiving NAC treatment (**Figure 2B**). Moreover, multivariate analysis demonstrated that miR-181a was an independent prognostic factor for OS in TNBC patients receiving NAC (**Table 2**).

In summary, our study showed that miR-181a was up-regulated in TNBC tissues and was significantly correlated with chemoresistance in patients receiving NAC. miR-181a may serve as prognostic biomarker for poor survival and a predictive factor for NAC response in TNBC patients. Further studies regarding the detailed molecular mechanism may lend support to the development of new miRNA based therapeutic strategies to overcome chemoresistance in TNBC.

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Disclosure of conflict of interest

None.

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