

## Original Article

# Expression of microRNA-10b is associated with the pathology of early breast invasive ductal cancer and is a prognostic predictor

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**Abstract:** The present study is to investigate the correlation of microRNA (miR)-10b with the clinical-pathological factors of early breast invasive ductal cancer. A total of 193 patients with early breast invasive ductal cancer and tumor size  $\leq 2$  cm were selected. miR-10b expression was determined using in situ hybridization. Pearson Chi-square test and COX regression model were used to analyze the relationship of miR-10b with clinical-pathological factors. Kaplan-Meier method was used to calculate cumulative tumor-free survival time. miR-10b was related with lymph node, tumor-free survival, or recurrence and metastasis of early breast invasive ductal cancer patients. Patients with negative miR-10b expression had longer tumor-free survival time than patients with positive miR-10b expression in the presence of negative lymph node. Endocrine therapy and miR-10b were independent prognostic factors for early breast invasive ductal cancer, with positive miR-10b expression as a prognostic risk factor, and endocrine therapy as a prognostic protection factor. In the presence of negative lymph node, factors of age, ethnic group, endocrine therapy and miR-10b were independent prognostic factors. Positive miR-10b expression and ethnic group were prognostic risk factors, and age  $\leq 50$  years and endocrine therapy were prognostic protection factors. In the presence of positive lymph node, positive miR-10b expression was a prognostic risk factor, but not a prognostic independent factor. The miR-10b expression is closely associated with the recurrence and metastasis of early breast invasive ductal cancer and may be used as a clinical indicator for predicting the prognosis of early breast invasive ductal cancer.

**Keywords:** Early breast invasive ductal cancer, miR-10b, clinical pathology, prognosis

## Introduction

Breast cancer is one of the most common malignant tumors in women at present. Breast cancer seriously threatens the health and life of women because its etiology is not clear. The smaller the primary focus is, the better the prognosis of breast cancer is. The 10-year survival rates of patients with T<sub>1</sub>, T<sub>2</sub>, T<sub>3</sub> and T<sub>4</sub> stages of breast cancer are 87.8%, 62.6%, 46.0% and 19.7%, respectively [1]. One of the biological behaviors of breast cancer is characterized by lesions at early stage, after which cancer cells may have occult metastasis through blood or lymphatic pathway, leading to the formation of recurrence or metastasis foci. Therefore, even if the primary tumor is small, local recurrence and distant metastasis may still occur

after axillary lymph node dissection of negative and early-stage breast tumor [2, 3].

MicroRNA (miRNA or miR) is a class of endogenous non-encoding single-stranded small RNA in eukaryotes that is conservative, temporal and tissue-specific. Recently, it has been found that the expression of about 1/3 genes in human genome is regulated by miRNA molecules [4]. The effect of miRNA in the occurrence and development of breast cancer is closely concerned, and it is shown that some miRNA molecules promote the invasion and metastasis of breast cancer. miR-10b is a member of miRNA family that can activate breast tumor infiltration in vivo and facilitate cancer cell metastasis in vitro. Therefore, overexpression of miR-10b is considered to play an important

role in the invasion and metastasis of non-metastatic breast cancer [5]. miR-10b is highly expressed in breast cancer cells with high metastatic ability, and participates in the balance mechanism of breast cancer metastasis. As a result, miR-10b endows non-metastatic breast cancer cells with strong ability in invasion and metastasis [6-8]. In the present study, we determine the expression of miR-10b in 193 cases of early breast invasive ductal cancer, and investigate how miR-10b expression is related with the clinical pathological index and prognosis of early breast invasive ductal cancer.

### Materials and methods

#### *Patients*

About 2,600 patients with breast cancer admitted at our hospital between January 2000 and December 2013 were followed up every year until 31 December 2015 with postoperative durations of 2 to 15 years, and their clinical, imaging, pathological and therapeutic information was complete. By the end of follow-ups, patients without recurrence or metastasis were considered to have tumor-free survival, and those with metastatic foci and/or pathological evidence of metastasis according to imaging were considered to have recurrence and metastasis. The follow-up lost rate was 6%. A total of 193 patients with early breast invasive ductal cancer and tumor size  $\leq 2$  cm were selected from the 2,600 patients for the present study. The 193 patients were aged between 34 and 78 years (average age, 46.5 years). All procedures were approved by the Ethics Committee of Xinjiang Medical University. Written informed consents were obtained from all patients or their families.

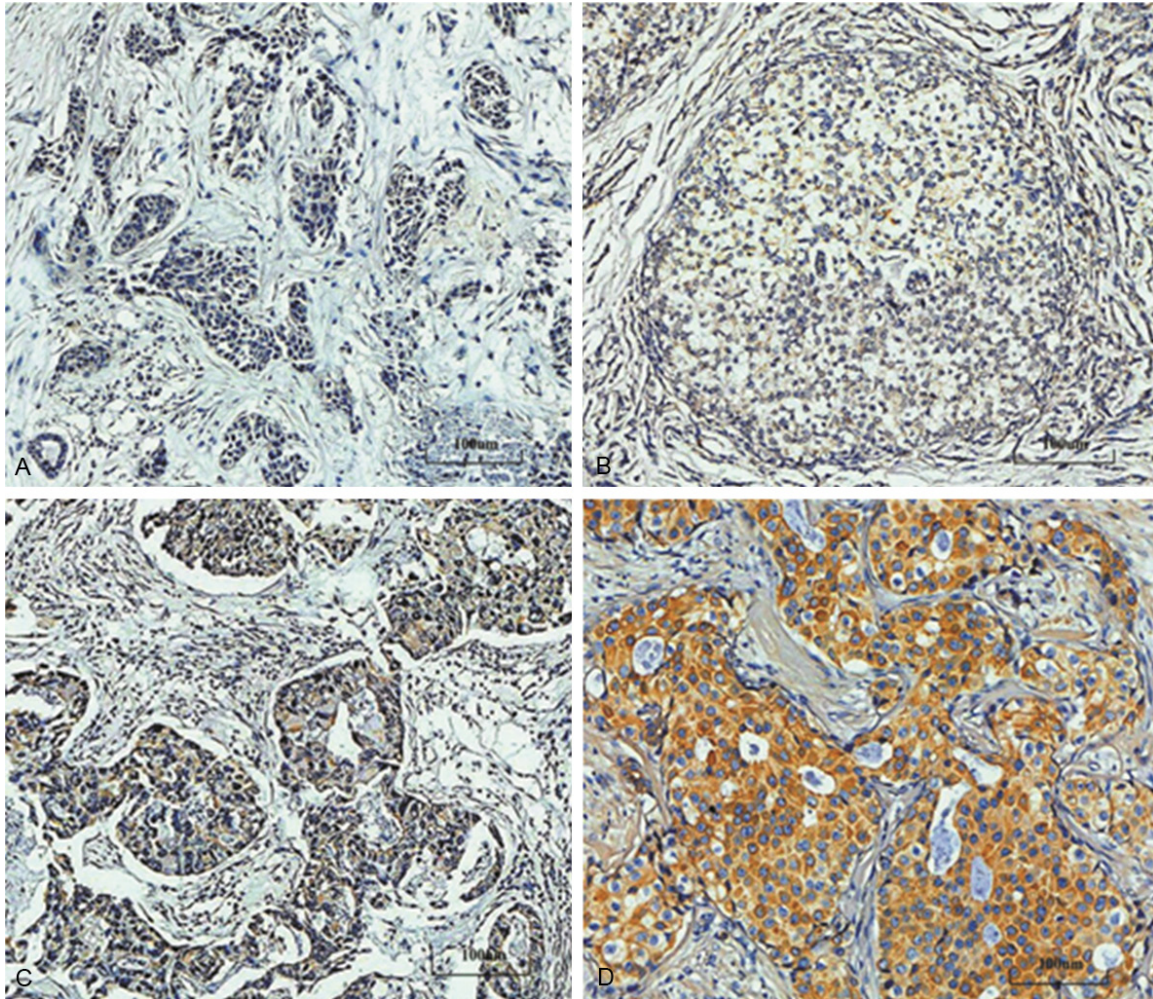
#### *In situ hybridization (ISH)*

Tissue section was paraffin-embedded and attached on APES glass slides for incubation at 70°C overnight before dewaxing and dehydration. Then, the section was treated with 3% H<sub>2</sub>O<sub>2</sub> at room temperature for 10 min, and washed twice with distilled water. After digestion with pepsin at 37°C for 15 min, the section was washed with 0.5 M phosphate-buffered saline for three times of 5 min followed by

washing with distilled water once. After pre-hybridization for 3 h at 37°C in wet box, digoxigenin-labeled oligonucleotide probe (2 µg/ml; hsa-miR-10b) was added. Hybridization solution (20 µl; Boster Biological Technology, Wuhan, China) was added on each section, and covered with coverslip. The section was incubated at 37-40°C overnight for hybridization. Afterwards, the coverslip was removed and the section was washed with 2× SSC at 37-40°C for 5 min twice, 0.5× SSC for 15 min once, and 0.2× SSC for 15 min once. After adding blocking solution, the section was incubated at 37°C for 30 min before addition of biotinylated mouse anti-digoxigenin. After incubation for 120 min, the section was washed with 0.5 M phosphate-buffered saline for 4 times of 5 min. Then, streptavidin-biotin complex was added, and the section was incubated at room temperature for 30 min and washed with 0.5 M phosphate-buffered saline for 3 times of 5 min. After addition of biotinylated peroxidase, the section was incubated at room temperature for 30 min before washing with 0.5 M phosphate-buffered saline for 4 times of 5 min. Then, DAB reagents (Exiqon, Vedbaek, Denmark) were added for color development of 10-30 min, followed by washing with water. The section was stained with hematoxylin and dehydrated using gradient alcohol. Finally, the section underwent xylene transparency and mounting.

#### *Scoring standards for staining*

According to American Society of clinical Oncology/College of American Pathologists Guideline Recommendations for Human Epidermal Growth Factor Receptor 2 Testing In Breast Cancer published in 2007, cytoplasm of positive cells was stained brown yellow. Scores of staining were evaluated by staining intensity and the number of positive cells [9]. According to staining intensity, cells with no staining scored 0 point, those with light yellow staining scored 1 point, those with brown yellow staining scored 2 points, and those with brown staining scored 3 points. According to the number of positive cells (5 high power fields), cells with positive rate of 1-25% scored 1 point, those with positive rate of 26-50% scored 2 points, those with positive rate of 51-75% scored 3 points, and those with positive rate > 75% scored 4 points. The final score of each



**Figure 1.** Analysis of miR-10b expression by in situ hybridization. Representative images were shown. Scores of staining were evaluated by staining intensity and the number of positive cells. (A) miR-10b (-), (B) miR-10b (+), (C) miR-10b (++) and (D) miR-10b (+++). Magnification, 100 X.

sample was obtained by the score of positive cell number multiplied by the score of staining intensity. Samples with 0 point were considered as (-), those with 1-2 points were considered as (+), those with 3-4 points were considered as (++) and those with > 4 points were considered as (+++). The representative images were shown in **Figure 1A-D**.

#### Statistical analysis

The results were analyzed using Pearson Chi-square test by SPSS v17.0 statistical software (IBM, Armonk, NY, USA). Kaplan-Meier method was used to calculate cumulative tumor-free survival time. Log-rank test was used to compare differences of tumor-free survival time between groups. COX regression model was

used for multivariate analysis.  $P \leq 0.05$  was considered statistically significant.

#### Results

*Expression of miR-10b is related with lymph node, tumor-free survival, or recurrence and metastasis of patients with early breast invasive ductal cancer*

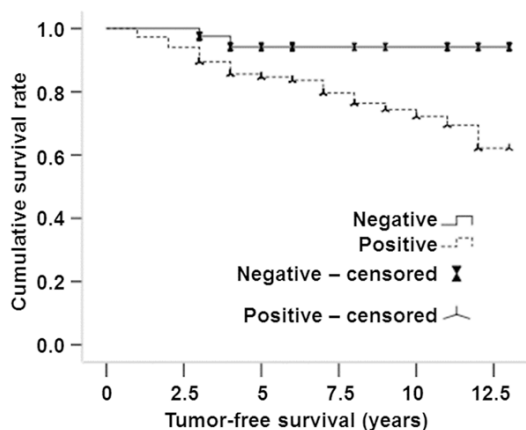
To study the relationship between miR-10b expression and clinical pathological index, the expression of miR-10b in 193 cases of early breast invasive ductal cancer was measured using ISH. The miR-10b positive rate of these patients was 78.8%. The miR-10b positive rate in patients with negative lymph node was significantly higher than that in patients with positive lymph node ( $\chi^2 = 3.847$ ,  $P = 0.005$ ).

## miR-10b and early breast invasive ductal cancer

**Table 1.** Relationship between miR-10b expression in early breast invasive ductal cancer and clinical pathological index

Clinical index	n	miR-10b positive rate (%)	miR-10b negative rate (%)	$\chi^2$	P
<b>Menstruation</b>					
Amenorrhoea	97	76 (78.4)	21 (21.6)	0.019	0.890
No amenorrhoea	96	76 (79.2)	20 (20.8)		
<b>Age</b>					
≤ 50 years	104	79 (76.0)	25 (24.0)	1.053	0.305
≥ 50 years	89	73 (82.0)	16 (18.0)		
<b>Total lymph node</b>					
L (-)	124	103 (83.1)	21 (16.9)	3.847	0.050
L (+)	69	49 (71.0)	20 (29.0)		
<b>ER-<math>\alpha</math></b>					
(+)	127	99 (78.0)	28 (22.0)	0.143	0.705
(-)	66	53 (80.3)	13 (19.7)		
<b>PR</b>					
(+)	129	102 (79.1)	27 (20.9)	0.023	0.880
(-)	64	50 (78.1)	14 (21.9)		
<b>Her-2</b>					
(+)	106	85 (80.2)	21 (19.8)	0.288	0.591
(-)	87	67 (77.0)	20 (23.0)		
<b>Prognosis</b>					
Tumor-free survival	158	119 (75.3)	39 (24.7)	6.162	0.013
Recurrence and metastasis	35	33 (94.3)	2 (5.7)		

Note: ER, estrogen receptor; PR, progesterone receptor; Her-2, human epidermal growth factor receptor-2.



**Figure 2.** Cumulative survival curve for patients with different expression of miR-10b.

Similarly, miR-10b positive rate in patients with recurrence and metastasis was significantly higher than that in patients in tumor-free survival group ( $\chi^2 = 6.162$ ,  $P = 0.013$ ). In addition, miR-10b positive rate was not significantly different in patients with different menstruation

status, ages, estrogen receptor (ER)- $\alpha$  expression, progesterone receptor (PR) expression, or human epidermal growth factor receptor-2 (Her-2) expression ( $P > 0.05$ ) (Table 1). These results suggest that expression of miR-10b is related with lymph node, tumor-free survival, or recurrence and metastasis of patients with early breast invasive ductal cancer.

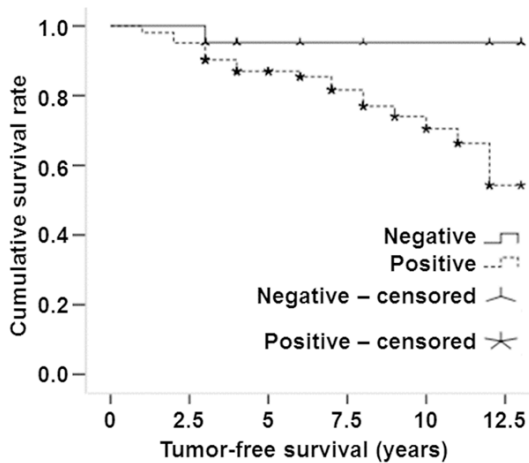
*Patients with negative expression of miR-10b have longer tumor-free survival time than patients with positive expression of miR-10b*

To compare the tumor-free survival time between patients with positive and negative expression of miR-10b, Kaplan-Meier survival analysis was performed. The data showed that the median tumor-free survival time for miR-10b negative expression was 12.453 years, being significantly longer than that for miR-10b positive expression (10.689 years) (Log-rank:  $\chi^2 = 5.230$  and  $P = 0.022$ ) (Figure 2). The result indicates that patients with negative expression of miR-10b have longer tumor-free survival time than patients with positive expression of miR-10b.

*Patients with negative expression of miR-10b have longer tumor-free survival time in the presence of negative lymph node*

To compare the tumor-free survival time between patients with positive and negative expression of miR-10b in the presence of negative lymph node, Kaplan-Meier survival analysis was also used. The data showed that the median tumor-free survival time for miR-10b negative expression (12.524 years) was significantly longer than that for miR-10b positive expression (10.666 years) (Log-rank:  $\chi^2 = 3.847$  and  $P = 0.05$ ) (Figure 3). The result suggests that patients with negative expression of miR-10b have longer tumor-free survival time in the presence of negative lymph node.

## miR-10b and early breast invasive ductal cancer



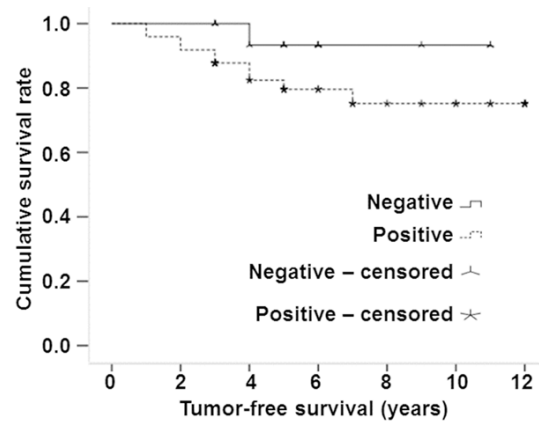
**Figure 3.** Cumulative survival curve for patients with different expression of miR-10b in the presence of negative lymph node.

*Tumor-free survival time of patients with negative expression of miR-10b is not significantly different from that of patients with positive expression of miR-10b in the presence of positive lymph node*

To compare the tumor-free survival time between patients with positive and negative expression of miR-10b in the presence of positive lymph node, Kaplan-Meier survival analysis was also used. The data showed that the median tumor-free survival time for miR-10b negative expression (10.533 years) was not significantly different from that for miR-10b positive expression (9.930 years) (Log-rank:  $\chi^2 = 2.225$  and  $P = 0.136$ ) (Figure 4). The result indicates that the tumor-free survival time of patients with negative expression of miR-10b is not significantly different from that of patients with positive expression of miR-10b in the presence of positive lymph node.

*Positive expression of miR-10b and endocrine therapy are independent prognostic factors for early breast invasive ductal cancer*

To examine the correlation of miR-10b with the prognosis of patients with early breast invasive ductal cancer, COX multivariate analysis was used to analyze prognosis-related factors such as age, ethnic group, staging, miR-10b, ER- $\alpha$ , PR, Her-2, and postoperative chemotherapy, radiotherapy and endocrine therapy. The data showed that endocrine therapy and miR-10b were independent prognostic factors for early



**Figure 4.** Cumulative survival curve for patients with different expression of miR-10b in the presence of positive lymph node.

breast invasive ductal cancer ( $P = 0.013$  and  $P = 0.035$ , respectively). Of note, positive expression of miR-10b was a prognostic risk factor (OR = 4.726), and endocrine therapy was a prognostic protection factor (OR = 2.938) (Table 2). The result suggests that positive expression of miR-10b and endocrine therapy are independent prognostic factors for early breast invasive ductal cancer.

*Age, ethnic group, endocrine therapy and miR-10b are independent prognostic factors for early breast invasive ductal cancer in the presence of negative lymph node*

To examine the correlation of negative lymph node with the prognosis of patients with early breast invasive ductal cancer, COX multivariate analysis was used to analyze prognosis-related factors such as age, ethnic group, staging, miR-10b, ER- $\alpha$ , PR, Her-2, and postoperative chemotherapy, radiotherapy and endocrine therapy. The data showed that age, ethnic group, endocrine therapy and miR-10b were independent prognostic factors for early breast invasive ductal cancer ( $P = 0.015$ ,  $P = 0.019$ ,  $P = 0.043$  and  $P = 0.016$ , respectively). Moreover, positive expression of miR-10b and ethnic group were prognostic risk factors (OR = 8.773 and OR = 6.104, respectively), and age  $\leq 50$  years and endocrine therapy were prognostic protection factors (OR = 0.228 and OR = 4.172, respectively) (Table 3). These results indicate that age, ethnic group, endocrine therapy and miR-10b are independent prognostic factors for early breast invasive ductal cancer in the presence of negative lymph node.

## miR-10b and early breast invasive ductal cancer

**Table 2.** COX multivariate analysis for the correlation between the expression of miR-10b and prognosis

Variables	$\beta$	Standard error	Wald value	P value	OR	95% confidence interval	
Age	-0.765	0.442	2.996	0.083	0.465	0.196	1.106
Ethnic group	0.562	0.461	1.487	0.223	1.754	0.711	4.326
Staging	-0.411	0.440	0.872	0.350	0.663	0.280	1.570
miR-10b	1.533	0.737	4.446	0.035	4.726	1.116	20.021
ER- $\alpha$	0.146	0.446	0.108	0.743	1.158	0.483	2.773
PR	-0.280	0.427	0.429	0.512	0.756	0.327	1.746
Her-2	0.040	0.380	0.011	0.916	1.041	0.494	2.192
Chemotherapy	-0.809	1.054	0.589	0.443	0.445	0.056	3.514
Radiotherapy	0.588	0.440	1.788	0.181	1.800	0.760	4.262
Endocrine therapy	1.078	0.435	6.124	0.013	2.938	1.251	6.897

**Table 3.** COX multivariate analysis for the correlation between negative lymph node and prognosis in early breast invasive ductal cancer

Variables	$\beta$	Standard error	Wald value	P value	OR	95% confidence interval	
Age	-1.480	0.609	5.904	0.015	0.228	0.069	0.751
Ethnic group	1.809	0.768	5.544	0.019	6.104	1.354	27.514
miR-10b	2.172	1.073	4.096	0.043	8.773	1.071	71.872
ER- $\alpha$	-0.254	0.573	0.197	0.657	0.776	0.253	2.383
PR	-0.118	0.533	0.049	0.825	0.889	0.312	2.528
Her-2	0.342	0.483	0.501	0.479	1.408	0.546	3.628
Chemotherapy	-1.149	1.071	1.151	0.283	0.317	0.039	2.585
Radiotherapy	0.853	0.557	2.344	0.126	2.347	0.787	6.992
Endocrine therapy	1.429	0.591	5.851	0.016	4.172	1.311	13.277

**Table 4.** COX multivariate analysis for the correlation between positive lymph node and prognosis in early breast invasive ductal cancer

Variables	$\beta$	Standard error	Wald value	P value	OR	95% confidence interval	
Age	-0.226	0.660	0.118	0.732	0.797	0.219	2.908
Ethnic group	-1.017	0.744	1.866	0.172	0.362	0.084	1.556
miR-10b	1.181	1.097	1.159	0.282	3.257	0.379	27.962
ER- $\alpha$	1.239	0.887	1.950	0.163	3.451	0.607	19.624
PR	-0.735	0.865	0.722	0.396	0.480	0.088	2.613
Her-2	-0.996	0.764	1.698	0.192	0.369	0.083	1.652
Chemotherapy	-10.907	752.267	0.000	0.988	0.000	0.000	.
Radiotherapy	-0.455	0.941	0.234	0.629	0.635	0.100	4.012
Endocrine therapy	0.053	0.764	0.005	0.945	1.054	0.236	4.711

*Positive expression of miR-10b is a prognostic risk factor for early breast invasive ductal cancer in the presence of positive lymph node*

To examine the correlation of positive lymph node with the prognosis of patients with early breast invasive ductal cancer, COX multivariate

analysis was used to analyze prognosis-related factors such as age, ethnic group, staging, miR-10b, ER- $\alpha$ , PR, Her-2, and post-operative chemotherapy, radiotherapy and endocrine therapy. The data showed that positive expression of miR-10b is prognostic risk factor (OR = 3.257), but not a prognostic independent factor (P = 0.282) (Table 4). The result suggests that positive expression of miR-10b is a prognostic risk factor for early breast invasive ductal cancer in the presence of positive lymph node.

### Discussion

Current surgery and post-operative comprehensive treatment for early breast invasive ductal cancer can achieve good effect and prognosis, but recurrence and metastasis may still occur [2, 3]. The effect of miRNA in the occurrence and development of breast cancer has become a hot research topic. For example, miR-10b is a member of miRNA family that is related with tumor recurrence and metastasis [10-12]. However, the expression of miR-10b in early breast invasive ductal cancer is never reported before. Our study has shown that the expression of miR-10b is elevated in patients with negative lymph node, recurrence and metastasis, suggesting that miR-

10b expression may be related with the occurrence and development of early breast invasive ductal cancer.

It is reported that positive expression of miR-10b is related with the metastasis of early breast invasive ductal cancer. Francesca et al.

show that higher positive expression of miR-10b is related with shorter tumor-free survival time, while negative expression of miR-10b corresponds to longer tumor-free survival time [13]. Ma et al. report that positive expression of miR-10b leads to the recurrence and distal metastasis of early breast cancer, suggesting that miR-10b plays important roles in the recurrence and metastasis of early breast cancer [8]. Moreover, elevated expression of miR-10b in early breast cancer leads to enhanced invasion and metastasis of breast cancer cells [14]. Consistently, our results show that the median tumor-free survival time of patients with positive expression of miR-10b is significantly decreased compared with that with negative expression of miR-10b, suggesting that positive expression of miR-10b may lead to the recurrence and metastasis of early breast invasive ductal cancer.

Furthermore, the present study on differences between negative and positive lymph node shows that, in the presence of negative lymph node, the median tumor-free survival time of patients with positive expression of miR-10b was shorter than that with negative expression of miR-10b, suggesting that miR-10b may lead to the recurrence and metastasis of early breast invasive ductal cancer and shorten tumor-free survival time.

There are many factors affecting the prognosis of breast cancer. It is reported that age and endocrine therapy are prognostic factors [15]. In addition, high expression of miR-10b is an indicator for the poor prognosis of breast cancer [14, 16]. Multivariate analysis results in the present study demonstrate that positive expression of miR-10b and endocrine therapy are independent prognostic factors, positive expression of miR-10b is prognostic risk factor and endocrine therapy is prognostic protection factor. Consistent with these results, patients with positive expression of miR-10b and those who receive no endocrine therapy have high rate of recurrence and metastasis.

It is reported that age and endocrine therapy are indicators for the evaluation of the prognosis of breast cancer, and positive expression of miR-10b is a prediction indicator for breast cancer [17]. It is widely accepted that age and endocrine therapy are prognostic factors [18, 19]. Multivariate survival analysis in the pres-

ent study shows that age, ethnic group, endocrine therapy and positive expression of miR-10b are independent prognostic factors for early breast invasive ductal cancer patients with negative lymph node. Consistent with previous report [20], ethnic group and miR-10b are risk factors, and age  $\leq 50$  years and endocrine therapy are protection factors. For early breast invasive ductal cancer patients with positive lymph node, positive expression of miR-10b is a prognostic risk factor. In conclusion, the prognosis of early breast invasive ductal cancer is correlated with the expression of miR-10b. Positive expression of miR-10b plays important roles in invasion and metastasis, and participates in the recurrence and metastasis of early breast invasive ductal cancer. Of note, patients with negative lymph node tend to have recurrence and metastasis. In addition, miR-10b is an independent risk factor for the prognosis of early breast invasive ductal cancer. Therefore, positive expression of miR-10b can be an indicator for the judgment of the prognosis of early breast invasive ductal cancer.

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### Disclosure of conflict of interest

None.

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