


# MicroRNA-493 is a prognostic factor in triple-negative breast cancer

Ling Yao<sup>1</sup> | Yirong Liu<sup>1</sup> | Zhigang Cao<sup>1</sup> | Junjing Li<sup>2</sup> | Yanni Huang<sup>1</sup> | Xin Hu<sup>1</sup> | Zhiming Shao<sup>1</sup> 

<sup>1</sup>Key Laboratory of Breast Cancer in Shanghai, Department of Breast Surgery, Fudan University Shanghai Cancer Center, Shanghai, China

<sup>2</sup>Department of Breast Surgery, Affiliated Union Hospital, Fujian Medical University, Fuzhou, China

## Correspondence

Zhiming Shao, Key Laboratory of Breast Cancer in Shanghai, Department of Breast Surgery, Fudan University Shanghai Cancer Center, Shanghai, China.  
Email: shao\_zhi\_ming@126.com

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Breast cancer is one of the most common malignant diseases in women. Triple-negative breast cancer (TNBC) shows higher aggressiveness and recurrence rates than other subtypes, and there are no effective targets or tailored treatments for TNBC patients. Thus, finding effective prognostic markers for TNBC could help clinicians in their ability to care for their patients. We used tissue microarrays (TMAs) to detect microRNA-493 (miR-493) expression in breast cancer samples. A miR-CURY LNA detection probe specific for miR-493 was used in in situ hybridization assays. Staining results were reviewed by two independent pathologists and classified as high or low expression of miR-493. Kaplan–Meier survival plots and multivariate Cox analysis were carried out to clarify the relationship between miR-493 and survival. In the Kaplan–Meier analysis, patients with high miR-493 expression had better disease-free survival than patients with low miR-493 expression. After adjusting for common clinicopathological factors in breast cancer, the expression level of miR-493 was still a significant prognostic factor in breast cancer. Further subtype analysis revealed that miR-493 expression levels were only significantly prognostic in TNBC patients. These results were validated in the Molecular Taxonomy of Breast Cancer International Consortium database<sup>1</sup> for overall survival. We proved the prognostic role of miR-493 in TNBC by using one of the largest breast cancer TMAs available and validated it in a large public RNA sequencing database.

## KEYWORDS

breast cancer, DFS, microRNA 493, prognostic factor, triple-negative

## 1 | INTRODUCTION

Breast cancer is one of the most common malignant diseases in women, and is responsible for the second highest cancer-specific death rates.<sup>1–5</sup> According to the American Cancer Society Surveillance Information Services, Surveillance and Health Services' report, there will be 252 710 new breast cancer cases, which account for 30% of all new cancer cases diagnosed in US women in 2017; and

about 40 610 patients will die of breast cancer, accounting for 14% of all cancer-related deaths.<sup>1</sup> The 5-year survival rate of breast cancer can be as high as 90%,<sup>1</sup> and many patients will experience disease recurrence or metastasis. Once this occurs, oncologists have little to offer in the way of treatment.<sup>6–9</sup> Therefore, finding useful approaches or markers for predicting breast cancer recurrence can allow treatment in the adjuvant setting to be tailored to these patients, which can be effective in decreasing relapse and cancer-related deaths.<sup>10–12</sup> This kind of strategy might be more significant

Yao, Liu and Cao equally contributed to this work.

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in triple-negative breast cancer (TNBC; estrogen receptor-negative, progesterone receptor-negative, and human epidermal growth factor receptor 2 [HER2]-negative), which usually shows higher aggressiveness and recurrence rates and is widely accepted as a heterogeneous disease. For TNBC patients, there are no effective targets or tailored treatments. The standard adjuvant treatment for TNBC is chemotherapy, the doses and regimens of which can be modified according to the patient's risk of recurrence. Thus, finding effective prognostic markers for TNBC might help clinicians in their ability to take better care of their patients.<sup>11,12</sup>

MicroRNA (miRNA) are small non-coding elements, usually 21–25 nt in length, which regulate expression of various genes and some important cellular processes.<sup>13,14</sup> The role of miRNAs in breast cancer has been widely studied.<sup>15–18</sup> There are oncogenic miRNAs, like miR-21,<sup>15,16</sup> miR-155,<sup>17,18</sup> miR-10b,<sup>19,20</sup> and miR27a,<sup>21,22</sup> which are frequently upregulated in breast cancer and influence tumor cell survival, promote proliferation and metastasis, and inhibit cell death. There are also tumor-suppressive miRNAs, like the let-7 family,<sup>23,24</sup> the miR-200 family,<sup>25,26</sup> miR-145,<sup>27</sup> and miR-205,<sup>28,29</sup> which are significantly downregulated in breast cancer and can silence different target genes, and negatively regulate epithelial–mesenchymal transition and tumor development.

MicroRNA-493 has been explored in cancer in previous studies. According to these studies, the roles of miR-493 in cancer are mainly suppressive. In lung cancer, Gu et al<sup>30</sup> found that miR-493 was markedly reduced in pulmonary carcinomas and that it could directly target E2F1, which in turn decreased growth, invasion, and metastasis of lung cancer cells. Another study by Cui et al<sup>31</sup> showed that overexpression of miR-493 suppressed cell proliferation and the cell cycle in human melanoma cells. Furthermore, miR-493 was a new tumor suppressor miRNA in bladder cancer and inhibited cell motility through downregulation of RhoC and FZD4.<sup>32</sup> Moreover, the suppressor role of miR-493 has also been reported in colon cancer metastasis. Sakai et al<sup>33</sup> observed that miR-493 inhibited MAPK kinase 7 expression by targeting the binding site at the 3'-UTR of the *mkk7* gene, which resulted in marked suppression of liver metastasis of colon cancer cells. Also, it was shown in the Okamoto et al<sup>34</sup> study that in a subset of colon cancer, upregulation of miR-493 during carcinogenesis prevented liver metastasis through the induction of cell death of metastasized cells.

Several studies have shown that miRNAs can be prognostic factors in breast cancer.<sup>13,35,36</sup> In the present study, we focused on miR-493, and have used our center's well-developed tissue microarray (TMA) to explore its prognostic value in breast cancer, especially in TNBC.

## 2 | MATERIALS AND METHODS

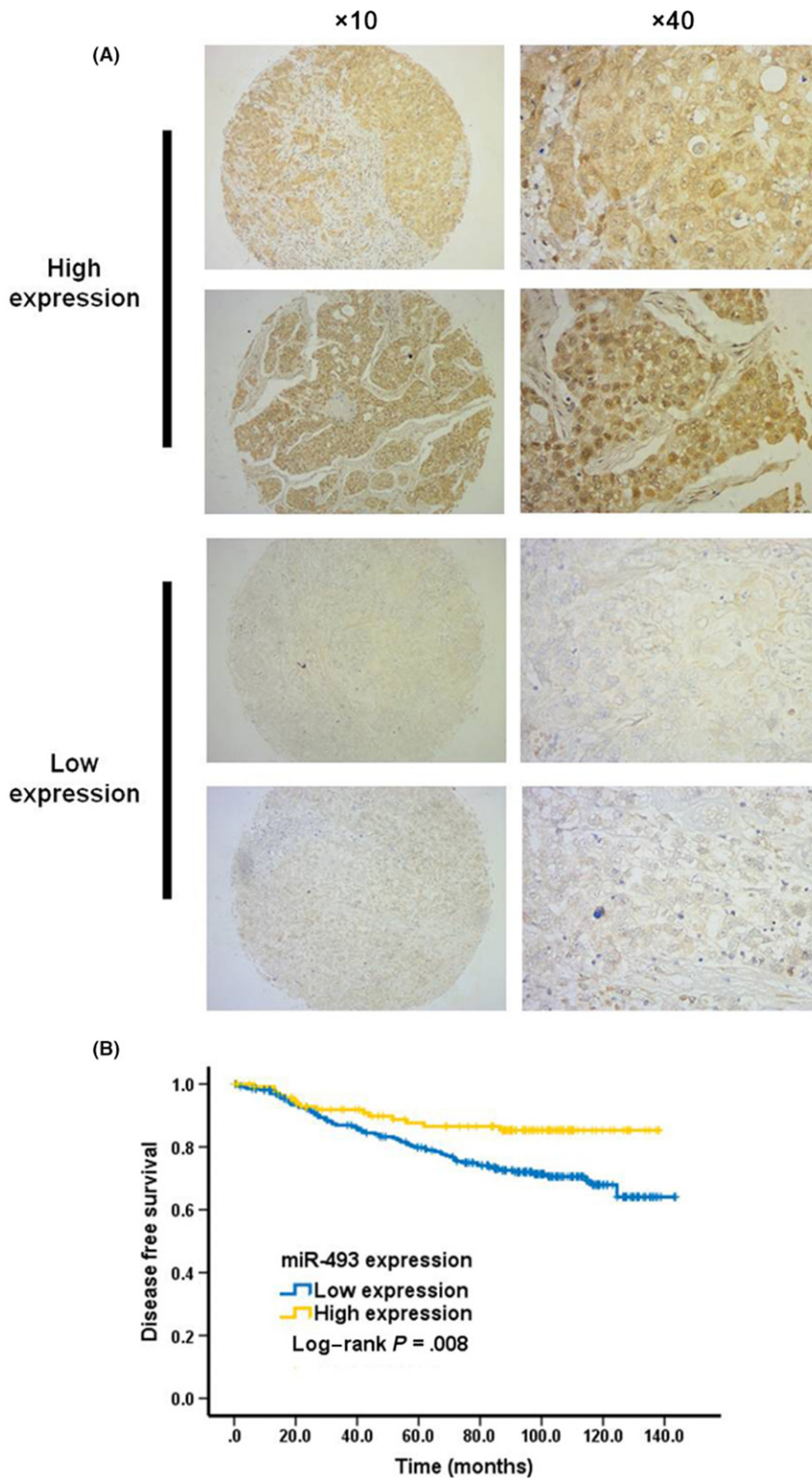
### 2.1 | Study cohort

We used our prepared TMAs, which have been described previously,<sup>35–38</sup> to detect miR-493 expression in breast cancer samples. A total of 382 breast cancer patients were included in the present

study, with their miR-493 expression levels available. All analyzed patients were diagnosed with stage I–III breast cancer from August 2001 to November 2007 at Fudan University Shanghai Cancer Center (FUSCC; Shanghai, China), and confirmed using pathology. Clinical and pathological factors were collected by using the FUSCC inpatient system, and survival data was retrieved through the FUSCC outpatient system or by telephone. Disease-free survival (DFS) events were recorded with any of the following situations: the first recurrence of invasive disease at a local, regional, or distant site, contralateral breast cancer, and death from any cause. Patients without DFS events were censored at the last follow-up. The median DFS and overall survival (OS) times for the cohorts were 90.28 and 94.73 months, respectively. The independent ethics committee/institutional review board of FUSCC (Shanghai Cancer Center Ethics Committee) approved the present study. All patients gave their written informed consent.

### 2.2 | In situ hybridization of miR-493

All staining was undertaken on the well-developed TMAs. The detailed method used in the development of the TMAs has been described previously.<sup>35–38</sup> A miRCURY LNA detection probe specific for miR-493 was purchased from Exiqon (Vedbaek, Denmark). The probe, 5'-CCTGGCACACAGTAGACCTTCA-3', was 5'-digoxigenin- and 3'-digoxigenin-labeled. For in situ hybridization, we used Enhanced Sensitive ISH Detection I (POD) kits from Boster (Wuhan, China). All experiments were undertaken according to the manufacturer's protocol and described previously. Briefly, TMAs were rewarmed at 65°C for 4 hours, deparaffinized in xylene, and sequentially hydrated in gradient ethanol solutions (three times in 100%, once in 95%, once in 85%, and once in 75%). After this procedure, TMAs were washed with PBS three times and incubated with 3% hydrogen peroxide for 10 minutes at room temperature to block endogenous peroxidase activity. Then TMAs were washed with 0.1% DEPC-H<sub>2</sub>O for 5 minutes, and then incubated with pepsin diluted 10-fold by citrate at 37°C for 20 minutes to expose the nucleic acid fraction of RNA. After the digestion procedure, TMAs were washed with PBS three times for 5 minutes each and with 0.1% DEPC-H<sub>2</sub>O once for 5 minutes. After incubation with prehybridization solution for 3 hours at 37°C, the TMAs were incubated with 200 µL miRNA probe (20 nmol/L) that had been preheated for 10 minutes at 80°C and quickly transferred to an ice/water mixture for 5 minutes, in a hybridization box at 60°C overnight. The next day, TMAs were subjected to a stringent washing procedure with 2× saline sodium citrate (SSC; preheated at 37°C, two washes, 5 minutes each), 0.5× SSC (one wash, 15 minutes), and 0.2× SSC (one wash, 15 minutes). After a 30-minute wash in blocking solution, TMAs were sequentially incubated with biotinylated digoxin (60 minutes), streptavidin–biotin complex (20 minutes), and peroxidase (20 minutes) with a 5-minute wash in 0.5 mol/L PBS between each. The results were visualized after staining with 3, 3'-diaminobenzidine and counterstained with Gill hematoxylin. For all experiments, a U6 probe was used as a positive control.



**FIGURE 1** MicroRNA-493 (miR-493) expression is correlated with breast cancer survival. A, Examples of high and low expression of miR-493 staining in samples at ×10 and ×40 magnification. B, Kaplan-Meier plot of miR-493 in relation to disease-free survival

Two experienced pathologists reviewed the in situ hybridization staining independently, using the staining index (SI), which incorporates intensity and percentage of positive cells. The strength of the staining was scored as: 0, no staining; 1, weak; 2, moderate; and 3, strong. The percentage of cells stained was scored as: 0, no staining; 1, <10%; 2,

10%-50%; and 3, >50% tumor cells. If there was a disagreement between the two pathologists, a third pathologist would be consulted. The SI was derived by multiplying the two results mentioned above. Samples with SIs >4 were considered as miR-493 high expression. Examples of high and low expression stains are shown in Figure 1(A).

## 2.3 | MicroRNA-493 expression in METABRIC database

The Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) is an integrated genomic/transcriptomic database of breast cancers.<sup>39</sup> In the following research, Lánczky et al<sup>40</sup> developed a tool, miRpower, which helps to easily access the miRNA expression data in METABRIC, and they provided a website for public use ([http://kmpplot.com/analysis/index.php?p=service&cancer=breast\\_mirna](http://kmpplot.com/analysis/index.php?p=service&cancer=breast_mirna)). All miRNA expression data in the METABRIC database are based on the microarray platform. To validate our finding of the prognostic effect of miR-493 in breast cancer, we downloaded miR-493 expression and responding clinical data from the METABRIC database using miRpower. The cut-off of miR-493 expression value, classifying its expression as high or low, was retrieved by using the

**TABLE 1** Clinicopathological characteristics of microRNA (miR)-493 levels in the Fudan University Shanghai Cancer Center cohort of patients with triple-negative breast cancer

	miR-493 expression level, n (%)		P-value, $\chi^2$ -test
	Low	High	
Age, years			.353
<40	28 (60.9)	18 (39.1)	-
41-60	197 (70.4)	83 (29.6)	-
>61	41 (73.2)	15 (26.8)	-
Menopause			.069
No	129 (65.5)	68 (34.5)	-
Yes	137 (74.1)	48 (25.9)	-
Grade			.328
II	136 (66.3)	69 (33.7)	-
III	74 (71.8)	29 (28.2)	-
Tumor size			.746
T1	125 (69.8)	54 (30.2)	-
T2	123 (69.1)	55 (30.9)	-
T3	14 (77.8)	4 (22.2)	-
Lymph nodes			.236
Negative	155 (72.1)	60 (27.9)	-
Positive	111 (66.5)	56 (33.5)	-
ER			.216
Negative	147 (67.1)	72 (32.9)	-
Positive	119 (73.0)	44 (27.0)	-
PR			.417
Negative	181 (71.0)	74 (29.0)	-
Positive	85 (66.9)	42 (33.1)	-
HER2			.707
Negative	150 (70.4)	63 (29.6)	-
Positive	116 (68.6)	53 (31.4)	-

-, not applicable; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; PR, progesterone receptor.

auto select best cut-off function in miRpower in the whole available METABRIC breast cancer cohort, and applied it to subtype analyses.

## 2.4 | Statistical analysis

The distribution of miR-493 expression in relation with different clinicopathological factors was evaluated using Pearson's chi-square-test. Kaplan–Meier survival plots were used to assess the impact of miR-493 expression on DFS, with log-rank test results reported.

**TABLE 2** Multivariate survival analyses of factors associated with disease-free survival in the the Fudan University Shanghai Cancer Center cohort of patients with triple-negative breast cancer

	HR (95% CI)	P-value
Age, years		
<40	Reference	-
41-60	0.53 (0.25-1.13)	.101
>61	0.27 (0.09-0.80)	.019
Menopause		
No	Reference	-
Yes	1.37 (0.79-2.36)	.260
Grade		
II	Reference	-
III	0.86 (0.49-1.48)	.577
Tumor size		
T1	Reference	-
T2	1.22 (0.72-2.04)	.462
T3	2.16 (0.72-6.45)	.167
Lymph nodes		
Negative	Reference	-
Positive	2.31 (1.40-3.81)	.001
ER		
Negative	Reference	-
Positive	0.92 (0.43-2.00)	.840
PR		
Negative	Reference	-
Positive	0.44 (0.19-1.00)	.051
HER2		
Negative	Reference	-
Positive	0.52 (0.28-0.92)	.018
Chemotherapy		
No	Reference	-
Yes	0.33 (0.13-0.82)	.017
Unknown	0.81 (0.23-2.84)	.744
miR-493 status		
Low expression	Reference	-
High expression	0.51 (0.28-0.92)	.026

-, not applicable; CI, confidence interval; ER, estrogen receptor; FUSCC, Fudan University Shanghai Cancer Center; HER2, human epidermal growth factor receptor 2; HR, hazard ratio; miR, microRNA; PR, progesterone receptor.

Multivariate Cox analysis was carried out to clarify the relationship between miR-493 and survival. All statistical analyses were undertaken using SPSS software (version 22; IBM, Armonk, NY, USA). All *P*-values were two-sided unless stated otherwise and only *P*-values <.05 were considered statistically significant.

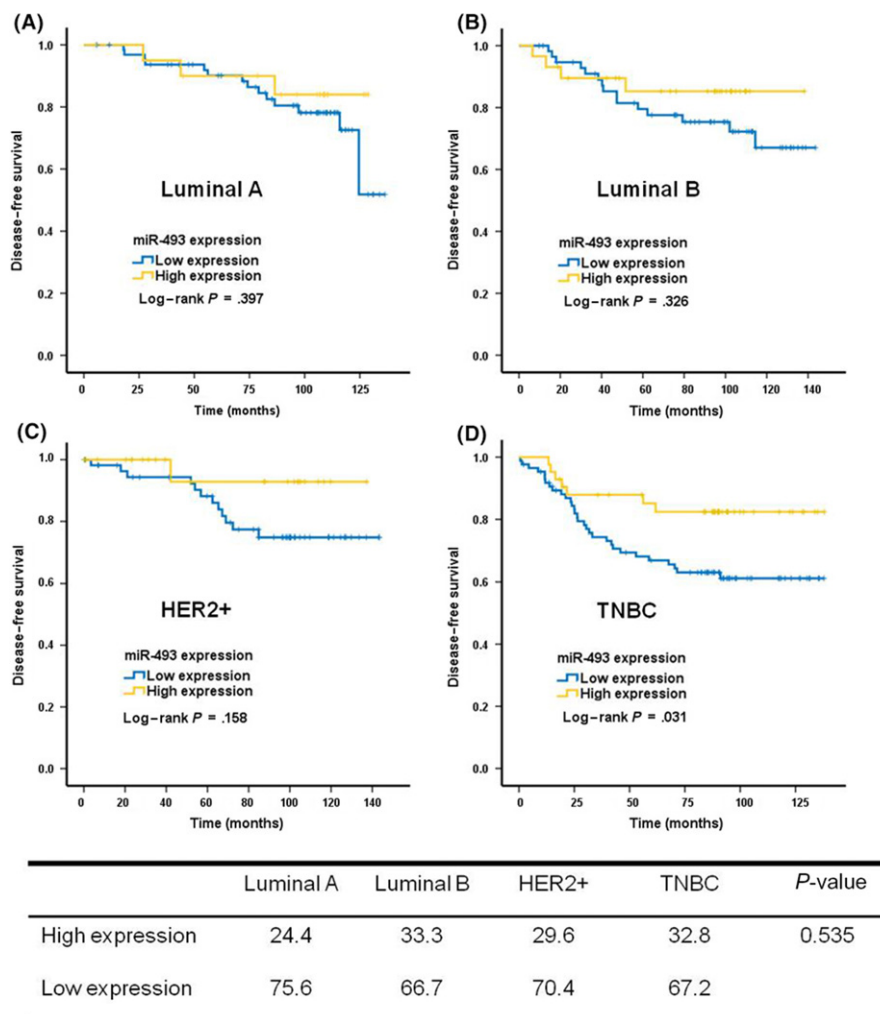
### 3 | RESULTS

Detailed information of miR-493 expression with different clinicopathological factors is listed in Table 1. Among 384 patients, 116 patients (30.1%) were classified as having high miR-493 expression. There was no statistically significant difference in miR-493 expression among any of the clinicopathological factors studied (Table 1).

In the Kaplan–Meier analysis, patients with high miR-493 expression had better DFS than patients with low miR-493 expression (Figure 1B; log-rank, *P* = .008). Although there was no distribution

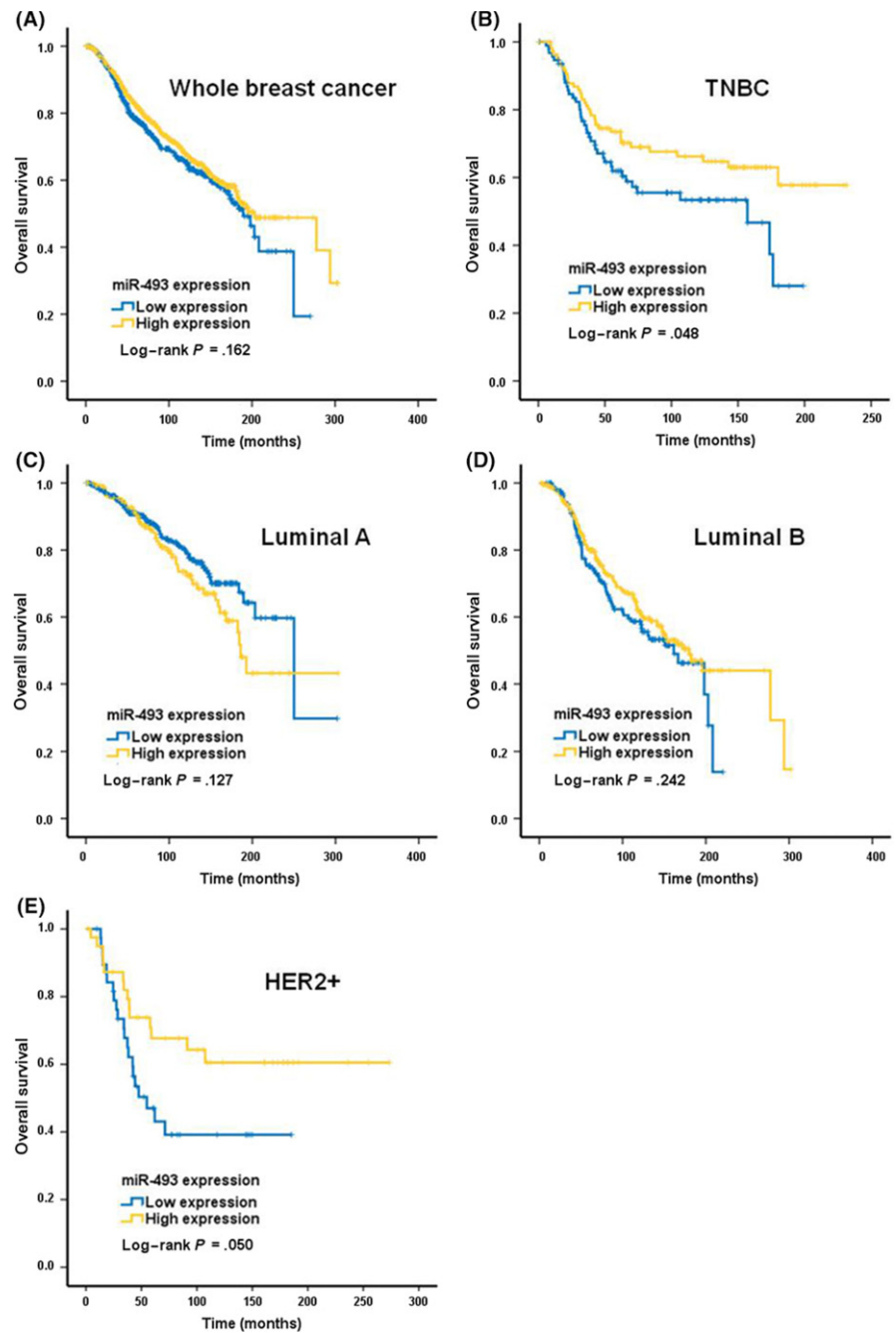
difference of miR-493 expression level with the studied clinicopathological factors, we still undertook multivariate analysis using a Cox proportional hazards regression model to rule out potential confounders. As shown in Table 2, after adjusting for the common clinicopathological factors in breast cancer, the expression level of miR-493 was still a significant prognostic factor in breast cancer. MicroRNA-493 high-expression patients have less risk of disease recurrence compared with low-expression patients (HR = 0.51; 95% CI, 0.28–0.92; *P* = .026). Other significant factors were lymph node status and HER2 status (Table 2).

To further explore miR-493 prognostic value in breast cancer, we carried out Kaplan–Meier analyses in different breast cancer subtypes. Interestingly, miR-493 expression level was only significantly related with DFS in TNBC patients (Figure 2D, *P* = .031), but not in luminal A, luminal B, or HER2-positive groups (Figure 2A–C; *P* = .397, .326, and .159, respectively). This prognostic value difference was not caused by distribution imbalance of miR-493 expression among the different subtypes (Figure 2; *P* = .535).



**FIGURE 2** Prognostic value of microRNA-493 (miR-493) in breast cancer varies among different subtypes. A, Luminal A. B, Luminal B. C, Human epidermal growth factor receptor 2 (HER2)+. D, Triple-negative breast cancer (TNBC). The table shows miR-493 expression in different subtypes





**FIGURE 3** Validation of microRNA-493 (miR-493) prognostic value in the Molecular Taxonomy of Breast Cancer International Consortium database of breast cancers. A, All breast cancers in the database. B, Triple-negative breast cancer (TNBC) subtype. C, Luminal A subtype. D, Luminal B subtype. E, Human epidermal growth factor receptor 2 (HER2)+ subtype

To validate our finding of the prognostic effect of miR-493 in breast cancer, we downloaded miR-493 expression data from the METABRIC database using miRpower. In the whole breast cancer cohort, the difference in expression level of miR-493, the cut-off for which was determined by miRpower automatically, was not significantly related with OS in patients (Figure 3A;  $P = .162$ ). In detailed subtype analyses, we found the same prognostic value of miR-493 in TNBC patients; that is, patients with high miR-493 expression level would have better OS than those with low-level expression (Figure 3B;  $P = .048$ ). In the HER2-positive group, high expression of miR-493 was also correlated with better OS (Figure 3E;  $P = .05$ ); however, only 80 patients were available for this

subtype analysis. As before, miR-493 expression was not prognostic in luminal A or luminal B patients (Figure 3C,D;  $P = .127$  and  $.242$ , respectively).

#### 4 | DISCUSSION

In the present study, we found that miR-493 could be a prognostic factor in breast cancer, especially in the TNBC subtype. Patients with high miR-493 expression had better survival outcomes compared with patients with low expression. Further analysis in the different subtypes showed that the prognostic value of miR-493 was

mainly significant in TNBC patients, but not in luminal A or luminal B patients. More samples are needed to clarify the prognostic role of miR-493 for HER2-positive patients.

In our study, we found that miR-493 could be a prognostic marker for breast cancer, especially in TNBC. Patients with miR-493 high expression had better survival, which was in line with the results shown in studies of other cancer types discussed above. The prognostic value of miR-493 was also validated in the METABRIC database. In breast cancer, few studies have reported investigating the role of miR-493. In a study by Zhao et al,<sup>41</sup> the authors showed that miR493-5p was differentially expressed between breast cancer tissues, matched adjacent tissue samples, and could be a novel suppressor of invasiveness and tumorigenicity of breast cancer cells through targeting of fucosyltransferase IV. Another study reported that miR-493 might be a prognostic factor in breast cancer. Using a miRNA expression profiling technique, Gasparini et al<sup>42</sup> identified a four-miRNA signature that included miR-155, miR-493, miR-30e, and miR-27a, which allowed subdivision of TNBCs into high- and low-risk groups. They proved that this subclassification was a powerful diagnostic and prognostic biomarker in TNBCs. Taken together with our study, these results proved that miR-493 could be an important tumor suppressor and prognostic marker in breast cancer.

Our study had some limitations. First, this was a retrospective analysis in a relatively small patient cohort. More samples will be needed to further validate the prognostic value of miR-493. Second, determining miR-493 expression levels using TMAs in breast cancer might be subjective, even though we used two independent pathologists who were blinded to patients' survival outcomes and consulted a third pathologist to resolve any discord. Use of the METABRIC database in the validation of the results might help in attenuating these limitations.

Taken together, by using one of the largest breast cancer TMAs available in conjunction with a large public RNA sequencing database, we have shown that miR-493 can be used as a prognostic marker in TNBC. Clarifying the mechanism by which miR-493 plays a tumor-suppressor role in breast cancer will require additional functional studies.

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## CONFLICT OF INTEREST

The authors have no conflict of interest.

## ORCID

Zhiming Shao  <http://orcid.org/0000-0001-8896-0652>

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