

Dephosphorylated cofilin expression is associated with poor prognosis in cases of human breast cancer: a tissue microarray analysis

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Background: Proteins in the cofilin pathway regulate actin dynamics and may be involved in cancer cell migration and invasion. However, there are no direct data that suggest that dephosphorylated cofilin can affect breast cancer prognosis.

Methods: We assessed the expressions of cofilin and phosphorylated cofilin (P-cofilin) in breast cancer tissue microarrays (290 patients, mean follow-up: 95.7±2.49 months) to evaluate dephosphorylated cofilin and its relationship with breast cancer prognosis. The associations of pathological characteristics with cumulative survival were evaluated using Kaplan–Meier analysis.

Results: Univariate analyses revealed that overall survival was associated with cofilin levels, N category, TNM stage, estrogen receptor status, progesterone receptor status, and molecular subtypes. Cofilin status and TNM stage independently affected overall survival, although P-cofilin expression was not associated with patient survival. In the P-cofilin-negative subgroup, cofilin expression was significantly associated with patient survival, although cofilin expression was not significantly associated with patient survival in the P-cofilin-positive subgroup. We further analyzed the P-cofilin-negative cases and found that Ki-67 expression was significantly elevated in the subgroup that was strongly positive for cofilin ($P=0.002$).

Conclusion: Among P-cofilin-negative patients with breast cancer, cofilin expression defines a population of patients with lower overall survival, which suggests that dephosphorylated cofilin expression might predict the prognosis in cases of P-cofilin-negative breast cancer. Furthermore, our results suggest that inhibitors of dephosphorylated cofilin expression may provide therapeutic benefits in patients with breast cancer.

Keywords: cofilin, P-cofilin, poor prognosis, breast cancer

Introduction

Breast cancer is the most common carcinoma among women and the second most common cause of cancer-related death among women. Unfortunately, the incidence of breast cancer has been increasing during recent decades.^{1–3} Nevertheless, considerable progress in breast cancer treatments (eg, surgery, chemotherapy, radiation, and hormone and targeted therapies) has recently led to decrease in breast cancer-related mortality rates.⁴ However, not all cases benefit from these treatments. Therefore, it would be useful to develop additional biomarkers to predict the prognosis of patients with breast cancer.

Cancer cell migration and invasion lead to cancer metastasis, which accounts for a majority of cancer-related deaths. The increased motility of cancer cells helps drive migration and invasion and is an essential step in breast cancer metastasis.^{5,6}

Therefore, targeting tumor cell motility is a potential antitumor strategy.⁷ As proteins in the cofilin pathway are key regulators of actin dynamics at the leading edge of motile cells, these proteins are likely to be involved in cancer cell migration and invasion. Recent data also indicate that components of the cofilin pathway are frequently misregulated in cancer cells, although there are conflicting reports regarding how cofilin and the upstream regulators contribute to the malignant phenotype (via overexpression or suppression). These data suggest that the balance between the cofilin pathway regulators and the “output” of the cofilin pathway (cofilin activity, most contributed by dephosphorylated cofilin) determines the invasiveness of tumor cells.⁸ However, there is currently no direct evidence that suggests that dephosphorylated cofilin expression can affect breast cancer prognosis. Our previous data revealed that cofilin expression was associated with poor outcomes, although cofilin expression itself is not a direct indicator of dephosphorylated cofilin expression. Therefore, this study used immunohistochemistry (IHC) to analyze the expressions of cofilin and phosphorylated cofilin (P-cofilin) in tissue microarrays of tumors from 290 patients with breast cancer (mean follow-up: 95.7±2.49 months). Our data provide insight regarding the role of cofilin levels in invasive breast cancer and highlight the correlations between cofilin levels and various clinical and pathological parameters.

Materials and methods

Tissue microarrays and immunostaining

We obtained breast cancer tissues from 300 patients who were diagnosed with primary breast cancer at the Institute of the National Engineering Center for BioChips in Shanghai, People’s Republic of China. This study and the study design were approved by the Human Research Ethics Committee of Taizhou Hospital (Zhejiang Province), and written informed consent to be included in the study was obtained from each patient before their original examination. All tissues had originally been fixed using formalin and embedded in paraffin. Due to lack of tumor tissues in some certain individual punches, we finally evaluated 290 cases of breast cancer solid tumors successfully. Clinicopathological data were obtained from the records and pathology reports of the Breast Cancer Surgery Department.

The expressions of cofilin, P-cofilin, estrogen receptor (ER), progesterone receptor (PR), Ki-67, and human epidermal growth factor receptor 2 (HER-2) were determined in the arrays and normal samples using IHC. To evaluate cofilin expression, we used a cofilin-specific antibody from Abcam (ab42824; Cambridge, UK) at a dilution of 1:1,500, based on

the manufacturer’s protocol. To evaluate P-cofilin expression, we used a P-cofilin-specific antibody (CST, #3313) at a dilution of 1:100. We defined ER and PR negativity according to the current Swedish clinical guidelines (<5% positive nuclei). Ki-67 expression was defined as positive (>14% immunostained nuclei) or negative (≤14% immunostained nuclei). The patient’s HER-2 status was semiquantitatively assessed using a standard protocol (HerceptTest, DakoCytomation Denmark A/S, Glostrup, Denmark), and fluorescence in situ hybridization (FISH) analysis was performed for HER-2-positive samples. HER-2 expression was defined as weak (IHC grade 0–1+ or FISH–) or strong (IHC grade 3+ or FISH+). The patient’s metastasis status was expressed using the American Joint Committee on Cancer TNM staging system.

Scoring for cofilin and P-cofilin

All IHC staining results were evaluated by two experienced pathologists, who were blinded to the patient’s clinical information. Cofilin staining intensity in the cytoplasm of tumor cells was graded using a scale of 0–3, and the percentage of cofilin-positive cells was graded using a scale of 0–4 (0: 0%–5%, 1: 6%–25%, 2: 26%–50%, 3: 51%–75%, and 4: 76%–100%). The final cofilin expression score ranged from 0 to 3 and was based on the sum of the intensity and percent-positive scores (0: total score 0–1, 1: total score 2–3, 2: total score 4–5, and 3: total score 6–7). For the Kaplan–Meier analysis, the final cofilin scores were analyzed as either weak (scores of 0–2) or strong (a score of 3) expression. We graded the percentage of P-cofilin-positive cells as 0 or 1 (0: 0%–10%, 1: >10%) and graded the intensity of P-cofilin staining using a scale of 0–3. The final P-cofilin expression (score 0 or 1) was calculated as the sum of the percent-positive and intensity scores (0: total score 0–2, 1: total score 3–4).

Survival and statistical analysis

Overall survival (OS) was defined as the time from the first operation to death because of any cause, and survivors were censored at the date of last contact. The chi-square test, Student’s *t*-test, and Pearson’s correlation test were used to analyze the patient and clinical characteristics that were associated with the expressions of cofilin and P-cofilin. The Kaplan–Meier method and log-rank test were used to analyze the correlation between OS and dephosphorylated cofilin expression. All statistical analyses were performed using SPSS software (Version 13.0; SPSS Inc., Chicago, IL, USA); all tests were two-sided, and a *P*-value of <0.05 was considered statistically significant.

Results

Pathological information from the invasive ductal cancer tissue microarrays

The tissue microarrays and examples of positive and negative P-cofilin expression are shown in Figure 1. P-cofilin expression was not significantly associated with patient survival in the univariate analysis ($P=0.232$) (Figure 2). When we performed subgroup analyses according to cofilin expression status, we found that patient survival was only significantly associated with negative P-cofilin expression ($P=0.002$) (Table 1). Positive P-cofilin expression was not significantly associated with patient survival ($P=0.355$) (Table 1; Figures 3–4).

We excluded ten patients who had missing information and 50 patients with positive P-cofilin expression. Thus, 240 patients with invasive ductal cancer (IDC) were included in the subsequent analyses, and their clinicopathological characteristics are shown in Table 1. Among the 240 patients with IDC, the mean follow-up was 95.7 ± 2.49 months (range: 2–160 months) and the mean age at diagnosis was 56.5 years (range: 29–88 years). Approximately 17.2% of these patients exhibited positive P-cofilin expression, and ~82.8% of these patients exhibited negative P-cofilin expression. We observed T1, T2, and T3/T4 stages in 27.9%, 60.8%, and 11.3% of the P-cofilin-negative patients, respectively. No lymph

node metastasis (N0) was observed in 44.2% of the cases. The patients with IDC exhibited a TNM stage of 0/I (13.8%), II (53.8%), and III (32.5%), and positive ER expression (60.8%), positive PR expression (45.8%), positive HER-2 expression (25.0%), positive Ki-67 expression (30.0%), and the luminal A subtype (42.1%). When we excluded the interference from P-cofilin, we found that only Ki-67 expression was significantly higher in the cofilin-positive subgroup ($P=0.002$) (Table 2).

Associations of P-cofilin, cofilin, and other clinicopathological features with IDC prognosis

The Kaplan–Meier method and log-rank test were used to evaluate the associations of the various factors with OS (Figure 2; Table 3). The univariate analyses of the patients with IDC revealed that cofilin status, N category, TNM stage, ER status, PR status, and molecular subtypes were associated with OS. Multivariate analyses revealed that cofilin status and TNM stage were independent predictors of OS (Table 3).

Discussion

Actin is the major component of the cytoskeleton, which plays an important role in tumor cell migration, invasion, and mitosis. Cofilin is a ubiquitously expressed actin-binding

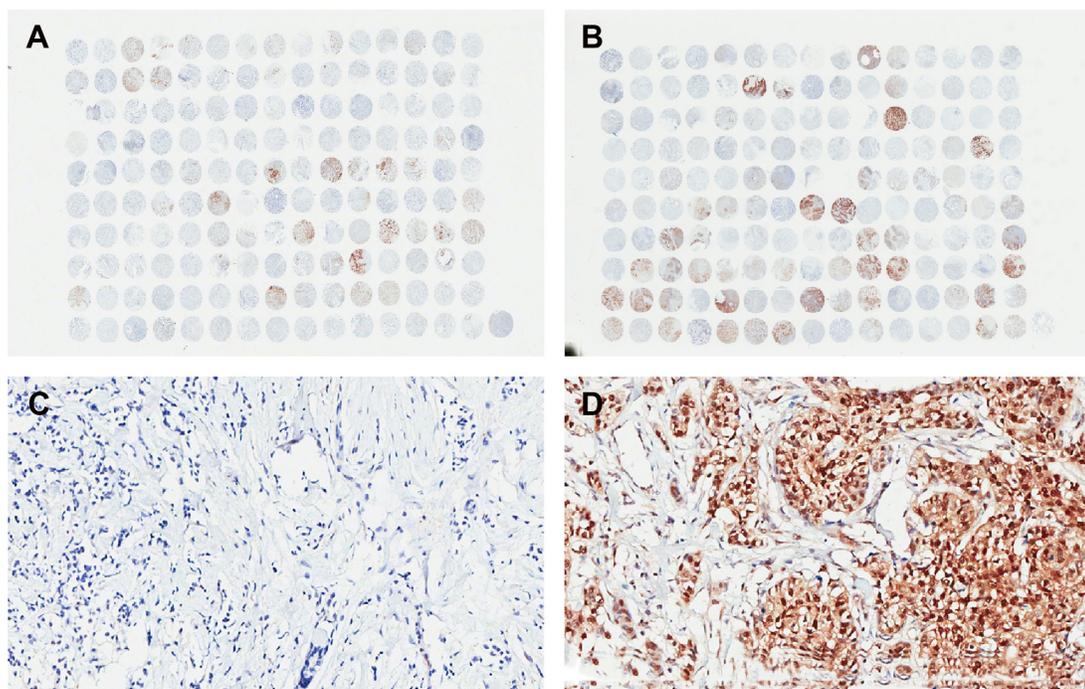


Figure 1 Tissue microarray and immunohistochemistry staining of P-cofilin in invasive ductal breast cancer tumors.

Notes: (A) Tissue microarray 1 (150 cases), 1 \times magnification; (B) tissue microarray 2 (150 cases), 1 \times magnification; (C) negative expression of P-cofilin, 200 \times magnification; and (D) positive expression of P-cofilin, 200 \times magnification.

Abbreviation: P-cofilin, phosphorylated cofilin.

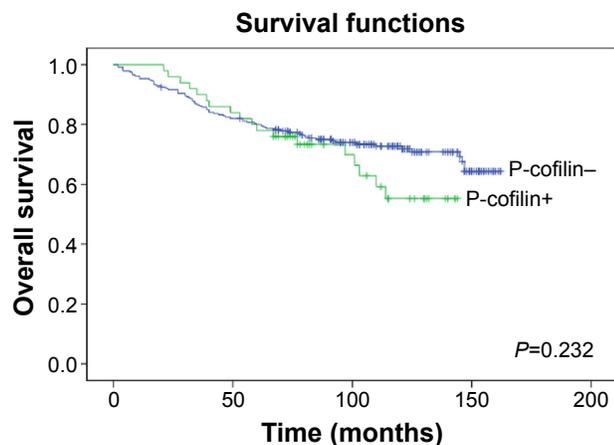


Figure 2 Association of P-cofilin with overall survival among patients with invasive ductal breast cancer.

Abbreviation: P-cofilin, phosphorylated cofilin.

protein, which is responsible for the formation of the actin cytoskeleton and is indispensable for cell cycle control.⁸ Furthermore, cofilin is essential for cell cycle progression and cancer cell migration, intravasation, and invasion.⁹ Cofilin overexpression increases the rate of cell migration in human glioblastoma cultures¹⁰ and is correlated with poor prognosis in other human malignancies.^{11–13} The overexpression of cofilin has also been detected in invasive subpopulations of mammary tumor cells and is directly related to the invasion, intravasation, and metastasis of mammary tumors.^{9,14}

Our previous data revealed that cofilin expression was associated with poor outcomes, although we only measured total levels of cofilin, rather than dephosphorylated cofilin expression. However, recent studies have indicated that the overall activity of the cofilin pathway, and not that of any single gene within the pathway, determines the invasive and metastatic phenotype of tumor cells.¹⁵ In this context, the cofilin pathway is composed of various kinases and phosphatases that regulate cofilin, and phosphorylation of serine 3 in cofilin is a critical step for cofilin recycling and actin polymerization.^{10,16} However, P-cofilin is inactive, and it is assumed that the amount of dephosphorylated cofilin in breast cancer cells is a direct measure of cofilin activity. Therefore, we analyzed the expressions of both cofilin and P-cofilin in

Table 1 Cofilin with or without phosphorylation for predicting overall survival in invasive ductal breast cancer

P-cofilin	Cofilin	Death (n)	Survival (n)	P-value
Negative	Negative	54	154	0.002
	Positive	15	17	
Positive	Negative	11	22	0.355
	Positive	7	10	

Abbreviation: P-cofilin, phosphorylated cofilin.

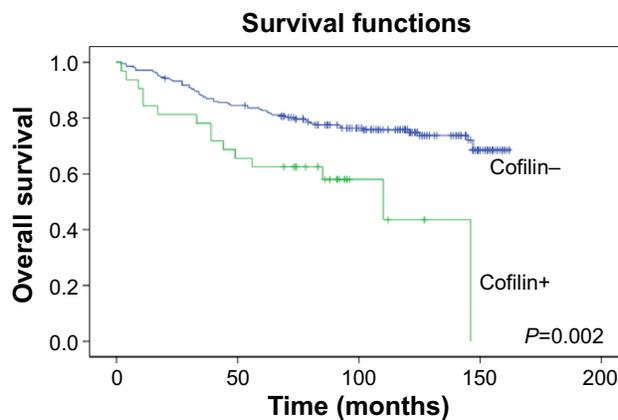


Figure 3 Association of cofilin with overall survival among P-cofilin-negative patients with invasive ductal breast cancer.

Abbreviation: P-cofilin, phosphorylated cofilin.

the tissue microarrays in order to determine whether cofilin activity (dephosphorylated cofilin expression) was correlated with outcomes in cases of human breast cancer.

In this study, we found that elevated cofilin expression in P-cofilin-negative breast cancer tissues predicted poor clinical and survival outcomes. In this context, cofilin expression directly represents dephosphorylated cofilin expression when P-cofilin is not present. However, in the P-cofilin-positive subgroup, the strong and weak cofilin expressions were not significantly associated with OS. Therefore, the presence of P-cofilin may confound any correlation between dephosphorylated cofilin expression and breast cancer prognosis. These results indicate that dephosphorylated cofilin expression, rather than overall expression, might be a preferred biomarker for predicting prognosis in cases of P-cofilin-negative breast cancer.

Because of its effects on actin polymerization and depolymerization, active cofilin has been linked to mammary

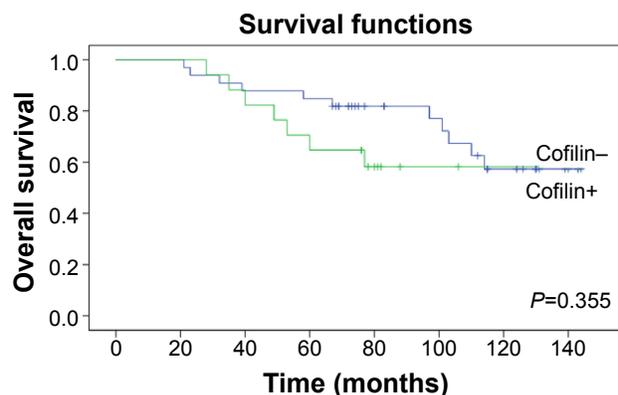


Figure 4 Association of cofilin with overall survival among P-cofilin-positive patients with invasive ductal breast cancer.

Abbreviation: P-cofilin, phosphorylated cofilin.

Table 2 Clinicopathological characteristics of breast cancer with different cofilin expression

Pathological category	Cases, n (%)	Negative cofilin	Positive cofilin	P-value
Age (years)				0.155
<50	87 (36.6%)	79	8	
≥50	153 (63.8%)	129	24	
T category				0.136
T1	67 (27.9%)	62	5	
T2	146 (60.8%)	125	21	
T3/T4	27 (11.3%)	21	6	
N category				0.510
N0	106 (44.2%)	93	13	
N1	67 (27.9%)	55	12	
N2	47 (19.6%)	43	4	
N3	20 (8.3%)	17	3	
TNM stage				0.688
0/I	33 (13.8%)	30	3	
II	129 (53.8%)	110	19	
III	78 (32.5%)	68	10	
ER status				0.177
Negative	94 (39.2%)	78	16	
Positive	146 (60.8%)	130	16	
PR status				0.075
Negative	130 (54.2%)	108	22	
Positive	110 (45.8%)	100	10	
HER-2 status				0.380
Negative	180 (75.0%)	158	22	
Positive	60 (25.0%)	50	10	
Ki-67 status				0.002
Negative	168 (70.0%)	153	15	
Positive	72 (30.0%)	55	17	
Molecular subtypes				0.100
Luminal A	101 (42.1%)	94	7	
Luminal B	52 (21.7%)	43	9	
HER-2	31 (12.9%)	25	6	
TNBC	56 (23.3%)	46	10	

Abbreviations: ER, estrogen receptor; HER-2, human epidermal growth factor receptor 2; PR, progesterone receptor; TNBC, triple-negative breast cancer.

tumor invasion, intravasation, and metastasis, as well as lymph node metastasis and a higher nodal stage. Findings from studies of other human malignant tumor types also support these associations. However, in this study, cofilin expression was not correlated with nodal stage or the number of metastatic lymph nodes in the P-cofilin-negative subgroup. As the number of lymph nodes largely depends on the completeness of the axillary lymph node dissection, this approximation may not always be accurate. Moreover, the time interval between tumor diagnosis and surgery may affect the nodal stage, which may not accurately reflect any tendency toward lymphatic metastasis.^{17,18} Therefore, these factors may explain why dephosphorylated cofilin expression was not correlated with clinical nodal stage in the P-cofilin-negative subgroup. However, most breast cancer-related deaths result from metastasis and/or recurrence, and the correlation between dephosphorylated cofilin expression and poor prognosis may reflect an association with the potential for metastasis.

We also found that Ki-67 expression was significantly higher in the P-cofilin-negative samples that were strongly positive for cofilin. In this context, Ki-67 is a nuclear factor that is expressed in proliferating cells. As actin is the major component of the cytoskeleton, which also plays an important role in tumor cell mitosis, dephosphorylated cofilin expression may be associated with cellular proliferation (as indicated by Ki-67 expression). These results may indicate that dephosphorylated cofilin expression is correlated with

Table 3 Univariate and multivariate analysis of factors that predict overall survival in invasive ductal breast cancer

Pathological category	Univariate analysis			Multivariate analysis		
	P-value	HR	95% CI	P-value	HR	95% CI
Cofilin status	0.002	1.349	1.112–1.637	0.007	1.321	1.079–1.617
Age (years)						
<50 vs ≥50	0.404	0.815	0.503–1.318			
T category						
T1 vs T2 vs T3/T4	0.055	1.476	0.991–2.199			
N category						
N0 vs N1 vs N2 vs N3	0.001	1.956	1.324–2.890	0.686	0.923	0.627–1.359
TNM stage						
0/I vs II vs III	0.001	1.917	1.347–2.729	0.012	2.279	1.199–4.333
ER status						
Negative vs positive	0.030	0.591	0.368–0.950	0.452	1.481	0.532–4.124
PR status						
Negative vs positive	0.012	0.529	0.322–0.869	0.538	0.807	0.408–1.597
HER-2 status						
Negative vs positive	0.444	1.234	0.720–2.116			
Ki-67 status						
Negative vs positive	0.341	1.130	0.879–1.452			
Molecular subtypes						
Luminal A vs Luminal B vs HER-2 vs TNBC	0.004	1.319	1.094–1.592	0.124	1.390	0.914–2.114

Abbreviations: ER, estrogen receptor; HER-2, human epidermal growth factor receptor 2; HR, hazard ratio; PR, progesterone receptor; TNBC, triple-negative breast cancer.

Ki-67 expression and that cofilin levels may be a marker for rapid proliferation in patients who had negative P-cofilin expression.

This study included two important limitations. First, we used a retrospective single-center design. Second, we only evaluated associations with OS, as we did not have access to data regarding recurrence-free and disease-free survivals. Therefore, a prospective multicenter study with long-term follow-up is needed to confirm whether dephosphorylated cofilin expression is an independent prognostic biomarker for patients with P-cofilin-negative breast cancer.

Conclusion

Dephosphorylated cofilin expression might be a useful biomarker for predicting prognosis in patients with breast cancer. Furthermore, inhibitors of the cofilin pathway may provide therapeutic benefits in these patients.

Disclosure

The authors report no conflicts of interest in this work.

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