

Overexpression of cofilin correlates with poor survival in breast cancer: A tissue microarray analysis

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Abstract. Cofilin, a key regulator of actin cytoskeleton dynamics, is considered to be involved in cellular migration, tumor invasion and mitosis, and its activity is increased in cancer cells. To address the association between cofilin and breast cancer prognosis, which is unclear at present, cofilin expression was analyzed in tissue microarrays of tumors from 310 patients with breast cancer via immunohistochemistry. In a multivariate Cox regression analysis, a high expression of cofilin in tumor cells correlated significantly with shorter overall survival (hazard ratio, 2.22; 95% confidence interval, 1.35-3.66, $P=0.002$, and with the Nottingham histologic grade, Ki-67 status and human epidermal growth factor receptor 2 status ($P=0.031$, 0.001, and 0.001, respectively). Cofilin expression was not observed as correlated with estrogen or progesterone receptor expression, tumor size or lymph node status. These data demonstrate that cofilin is associated with poor outcome, thereby suggesting that it is a potential prognostic factor in breast cancer.

Introduction

Cancer cell migration and invasion account for the majority of cancer-associated mortalities (1). Increased motility of cancer cells underlies the processes of migration and invasion (2,3) and is an essential step in breast cancer metastasis (4). Targeting tumor cell motility is a potential antitumor strategy (5,6). In response to migratory and chemotactic stimuli, cancer cells form membrane protrusions, which initiate the multi-step migration process. Membrane protrusions result from localized polymerization of sub-membrane actin and consequent formation of actin filaments and the actin framework is widely accepted as the engine driving cell motility; several actin-binding proteins regulate the assembly and disassembly of actin filaments, and thus the dynamic behavior of the actin cytoskeleton (7-9). Of these, the ubiquitous protein cofilin is the most important effector of actin polymerization and depolymerization, generating free barbed ends via pointed-end depolymerization and filament severing (10,11).

The actin-depolymerizing factor (ADF)/cofilin family includes ADF, cofilin and other proteins with similar biochemical activities. Unicellular organisms such as yeasts usually express only one ADF/cofilin isoform, whereas multicellular organisms typically express several. In certain cultured mammalian cell lines and invasive mammary tumor cells, cofilin-1 is the most abundant isoform (12), whereas ADF is expressed at much lower levels. In the present study, cofilin refers to cofilin-1.

Previous studies have suggested that cofilin activity correlates with cancer progression and cancer cell migration and invasion; local activation of cofilin via uncaging induces lamellipodia formation and determines the direction of cell movement (13). siRNA-mediated depletion of cofilin in carcinoma cells inhibits cell motility (12) and the assembly and stability of invadopodia and, consequently, cell invasion (14). Cofilin overexpression increases the rate of cell migration in human glioblastoma cultures (15) and pancreatic cancer (16), and correlates with poor prognosis in human pulmonary adenocarcinoma, gastric cancer, epithelial ovarian cancer and gallbladder carcinoma (17-20). Spontaneous overexpression of cofilin has been detected in invasive subpopulations

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Abbreviations: ADF, actin-depolymerizing factor; ER, estrogen receptor; FISH, fluorescence *in situ* hybridization; Her2, human epidermal growth factor receptor 2; HR, hazard ratio; IHC, immunohistochemistry; NHG, Nottingham histological grade; PR, progesterone receptor

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of mammary tumor cells (21), and is directly associated with the invasion, intravasation and metastasis of mammary tumors (22). Tissue microarray analysis has demonstrated that cofilin staining positively correlates with breast tumor grade (23).

However, to the best of our knowledge there is no direct evidence implicating deregulated cofilin expression in breast cancer prognosis at present. The present study analyzed cofilin expression in tissue microarrays of tumors from 310 patients with breast cancer via immunohistochemistry (IHC). These data provide insight into the role of cofilin in invasive breast cancer and establish correlations between cofilin expression and clinical and pathological parameters.

Materials and methods

Patient material and immunostaining in breast cancer tissue microarrays. Tissue arrays containing samples of invasive breast tumors from 310 patients were purchased from the National Engineering Center for BioChips in Shanghai, China. To prepare the arrays, a 1.5 mm core of tumor tissue was removed from each tumor. Tumors were formalin-fixed for at least 24 h and paraffin-embedded. Cores were taken from the peripheral aspect of the tumor, and necrotic tissue was avoided.

The expression of cofilin, estrogen receptor (ER), progesterone receptor (PR), Ki-67, and human epidermal growth factor receptor 2 (Her2) was determined in the arrays *via* IHC, using the BenchMark ULTRA system (Ventana Medical Systems, Inc., Tucson, AZ, USA) and Leica BOND-MAX system (Leica Microsystems, Ltd., Milton Keynes, UK) according to the manufacturer's protocol. Normal goat serum (10%; Boster Biological Technology, Ltd., Wuhan, China) was used as blocking reagent, and samples were blocked for 20 min at room temperature. UltraView Universal HRP multimer in the DAB Detection Kit (cat. no. 760-500; Ventana Medical Systems, Inc.) was used as the secondary antibody at a ready-to-use dilution and incubated for 30 min at 37°C. For cofilin expression, the cofilin-specific antibody from Abcam (cat. no. ab42824; Cambridge, UK) was used at a 1:1,500 dilution and the incubation time was 8 min at room temperature, while all other primary antibodies required 20 min at 37°C. ER and PR were demonstrated using SP1 (cat. no. 790-4325) and 1E2 (cat. no. 790-4296; both from Ventana Medical Systems, Inc.) antibodies, respectively at a ready-to-use dilution according to the protocol of the manufacturer. Negative expression was defined as <10% positive nuclei (24). Ki-67 was demonstrated using MM1 (cat. no. PA0410; Novocastra; Leica Microsystems, Ltd.) at a ready-to-use dilution according to the protocol of the manufacturer, and the expression was considered positive (>14% immunostained nuclei) or negative (≤14% immunostained nuclei). Her2 expression was assessed semiquantitatively by using a standard protocol (HercepTest; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) (25) and separated into 4 grades (from 0 to 3+).

Fluorescence *in situ* hybridization (FISH) analysis was performed in Her2 2+ samples, using the PathVysion HER-2 DNA Probe kit (Abbott Pharmaceutical Co., Ltd., Lake Bluff, IL, USA) according to the manufacturer's protocol. Her2 expression was designated as weak (IHC grade 0-1+ or FISH)

or strong (IHC grade 3+ or FISH⁺). Lymph node metastasis was staged according to the American Joint Committee on Cancer TNM system (26). Ethical approval for the present study was granted by the Human Research Ethics Committee of the Taizhou Hospital of Zhejiang Province.

Scoring, evaluation and statistical analysis. IHC staining was evaluated by two experienced pathologists blinded to the clinical information. Cofilin staining intensity in the cytoplasm of tumor cells was graded 0-3. The percentage of cofilin-positive cells was scored 0-4 (0-5, 6-25, 26-50, 51-75 and 76-100%, respectively). The final cofilin expression score ranged between 0 and 3 and was based on sum of the intensity and percent positive scores (0-1, 2-3, 4-5 and 6-7, respectively; Fig. 1). The slides were scanned using an Aperio ScanScope slide scanner, and images of representative areas were captured using Image Scope software version 9.0 (Aperio Technologies, Ltd, Oxford, UK) followed by Adobe Illustrator version 16.0 (Adobe Systems, San Jose, CA, USA) (27).

Distributions of pathological and clinical parameters [age, tumor size, Nottingham histological grade (NHG)](28), and lymph node, ER, PR, Her2, and Ki-67 status) according to the final cofilin score were calculated using a one-way ANOVA or the Pearson χ^2 test, as indicated in Table I. Multiple comparisons between the groups was performed using the Student-Newman-Keuls method. Kaplan-Meier analysis and the Breslow test were used to estimate the effect of high cofilin expression on overall survival. For the Kaplan-Meier analysis, final cofilin scores were analyzed in terms of weak (scores of 0, 1, and 2) and strong (a score of 3) expression. Cox regression proportional hazards models were used to estimate hazard ratios (HRs) for mortality from breast cancer according to cofilin expression in univariate and multivariate analyses. The covariates with $P < 0.05$ in the univariate analysis (lymph node, ER, and PR status) were included in the multivariate analysis. All statistical tests were two-sided, and $P < 0.05$ were considered to indicate a statistically significant difference. All calculations were performed using SPSS Statistics version 19 software (IBM Corp., Armonk, NY, USA).

Results

Cofilin expression is associated with clinicopathological variables. The association between cofilin staining intensity and several clinical parameters [age, tumor size, NHG, lymph node metastasis, and ER, PR, Ki-67, and Her2 expression] was determined (Table I). There was a significant association between cofilin staining intensity and NHG ($P = 0.030$). A trend toward a higher NHG in tumors with higher cofilin scores was observed. No tumors exhibited a cofilin score of 3 in combination with the lowest NHG score.

Cofilin expression was also associated with Her2 expression ($P < 0.001$). The distribution of Her2-positive tumors paralleled the distribution of cofilin scores: The majority of Her2-positive tumors exhibited cofilin scores of 2 or 3. A similar association between Ki-67 expression and cofilin expression was observed ($P = 0.001$). As Ki67 positive tumors exhibited a larger percentage of cells with high cofilin expression compared with low cofilin expression, it was hypothesized that positive Ki67 status was associated with high cofilin expression. Cofilin

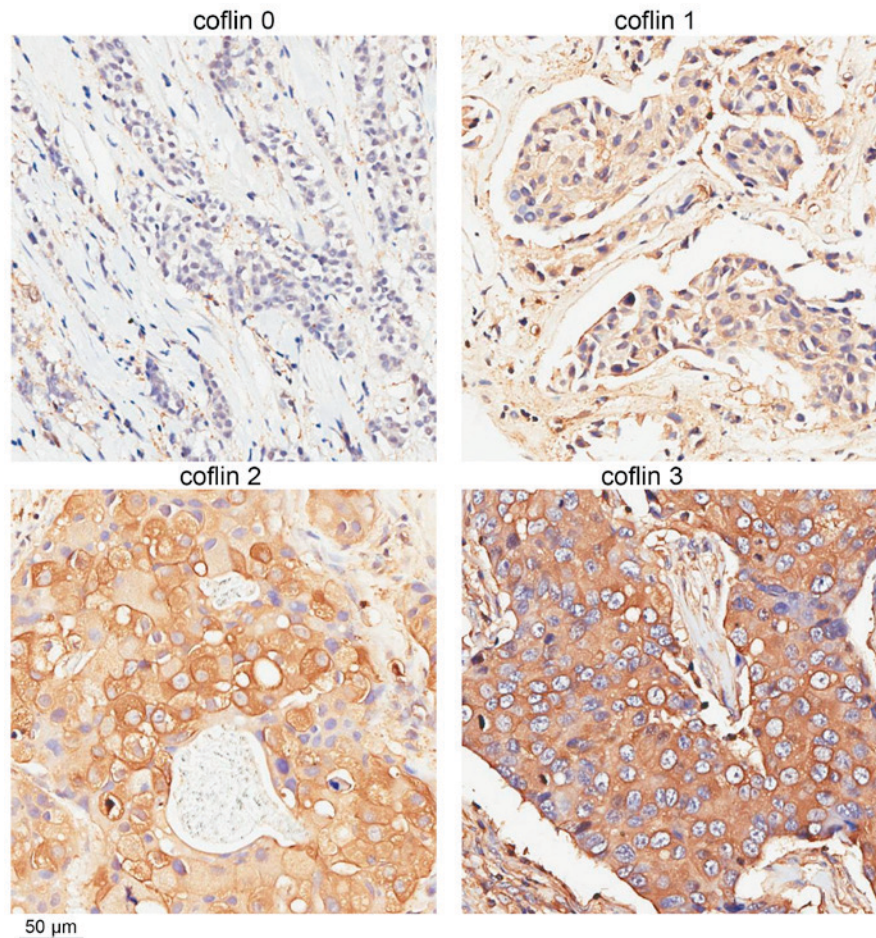


Figure 1. Examples of scoring of immunohistochemistry staining of cofilin expression in breast cancer tissue. Numbers refer to staining intensity.

expression was not significantly associated with age, tumor size, lymph node metastasis or ER or PR expression ($P=0.055$, 0.294 , 0.082 , 0.084 and 0.176 , respectively).

High expression of cofilin is associated with poor survival.

For survival analysis, the cofilin scores were dichotomized: Scores of 0, 1 and 2 denoted weak expression, and a score of 3 denoted strong expression. The rationale for this grouping was the marked difference in cofilin staining intensity between scores 2 and 3 (Fig. 1) and the similarity of the survival curves for patients with scores of 0, 1, or 2 (Fig. 2). Kaplan-Meier analysis demonstrated significant differences in overall survival between patients bearing tumors with weak vs. strong cofilin expression ($P=0.002$; Fig. 2). Univariate and multivariate Cox regression analyses of survival in association with cofilin expression were performed using the same dichotomized variable as in the Kaplan-Meier analysis. The results demonstrated that strong cofilin expression was an independent indicator of reduced overall survival ($P=0.002$; HR, 2.22; 95% confidence interval, 1.35-3.66) when the variables described in Table II were included. Detailed results of the Cox analyses are presented in Table II.

Discussion

Actin is the major component of the cytoskeleton, which serves an important role in tumor cell migration, invasion and

mitosis. The actin-binding protein cofilin, a member of the ADF/cofilin family, is a key regulator of actin polymerization and depolymerization. The activity and output of the cofilin pathway (cofilin and its regulatory proteins) are increased in cancer cells (4,29,30). Cofilin is thought to contribute to at least 3 cancer-associated events: Initial cell transformation (31), increased cell motility during metastasis and cell division (32).

Previous studies have demonstrated that tumors with a higher NHG typically exhibited reduced tubule formation, nuclear atypia and mitoses, and Her2 expression has been associated with tumor cell proliferation and an aggressive phenotype (33-35). In the present study, cofilin staining was associated with NHG, Her2 expression and Ki-67 expression, suggesting that cofilin may be a marker of poor differentiation and high proliferation. In migrating or invading cells, cofilin resides in cell membrane protrusions, for example lamellipodia, invadopodia, and filopodia, which initiate cell movement and determine cell polarity (36,37). This localization is critical for cell movement, endocytosis and cell division, all of which are important for normal cell proliferation, differentiation and cancer development (38). This promotion may be responsible for the positive association between cofilin expression and NHG, Her2 and Ki-67 status.

In agreement with previous studies, the present study identified that cofilin expression did not correlate with ER or PR status (23). There was also no correlation observed between cofilin expression and tumor size. In contrast, another study

Table I. Associations between cofilin expression and clinicopathological features in breast cancer.

Factor	Number	Cofilin staining intensity				P-value
		0	1	2	3	
All, n (%)	310	53 (17)	114 (37)	92 (30)	51 (16)	
Age, years ^a	54 (29-88)	50.5 (29-83)	54 (31-88)	57 (31-87)	56 (37-88)	0.055 ^b
Tumor size, mm ^a	30 (10-150)	30 (10-100)	30 (14-130)	30 (10-150)	35 (10-100)	0.294 ^b
NHG, n (%)						0.030 ^d
I	19 (6)	6 (32)	8 (42)	5 (26)	0 (0)	
II	210 (68)	36 (17)	83 (40)	61 (29)	30 (14)	
III	70 (22)	10 (14)	21 (30)	19 (27)	20 (29)	
Missing	11 (4)					
Nodal status, n (%)						0.082 ^c
N0	141 (45)	21 (15)	48 (34)	49 (34)	23 (16)	
N1	86 (28)	14 (16)	33 (38)	21 (24)	18 (21)	
N2	56 (18)	11 (20)	26 (46)	13 (23)	6 (11)	
N3	21 (7)	7 (33)	2 (10)	8 (38)	4 (19)	
Missing	6 (2)					
ER status, n (%)						0.084 ^c
Positive	191 (62)	26 (14)	76 (40)	62 (32)	27 (14)	
Negative	114 (37)	24 (21)	36 (32)	30 (26)	24 (21)	
Missing	5 (2)					
PR status, n (%)						0.176 ^c
Positive	139 (45)	21 (15)	56 (40)	45 (32)	17 (12)	
Negative	168 (54)	31 (18)	56 (33)	47 (28)	34 (20)	
Missing	3 (1)					
Ki67 status, n (%)						0.001 ^c
>14%	99 (32)	11 (11)	29 (29)	32 (32)	27 (27)	
≤14%	211 (68)	42 (20)	85 (40)	60 (28)	24 (13)	
Missing	0 (0)					
HER2 status, n (%) ^e						0.000 ^d
Strong	77 (25)	2 (2)	29 (38)	28 (36)	18 (23)	
Weak	233 (75)	51 (22)	85 (36)	64 (27)	33 (14)	
Missing	0 (0)					

^aData presented as mean (range). ^bOne-factor analysis of variance. ^cPearson χ^2 test, 2-tailed P-value. ^dFisher's exact test. ^eWeak (score 0-1, or FISH), strong (score 3, or FISH+). NGH, Nottingham histological grade; FISH, fluorescence *in situ* hybridization.

demonstrated a positive association between cofilin expression and tumor stages T0, T1, and T2 (but not T3) in breast cancer (39). Resolution of this discrepancy requires additional study.

Owing to its effects on actin polymerization/depolymerization, cofilin overexpression has been associated with mammary tumor invasion, intravasation, metastasis, lymph node metastasis and a higher nodal stage. Studies on other human malignant tumor types support these associations (20,40,41). However, in the present study, cofilin expression did not correlate with the nodal stage. The present study demonstrated that cofilin expression and the nodal stage are independent prognosis factors in breast cancer. As the number of positive lymph nodes largely depends on the completeness of axillary lymph node dissection, its approximation may not always be accurate (42).

Additionally, the time interval between tumor diagnosis and surgery may affect the nodal stage (43). Consequently, the nodal stage may not reflect a tendency for lymphatic metastasis. These considerations may explain why cofilin expression does not necessarily correlate with the clinical nodal stage. Active cofilin comprises only part of the total level of cofilin in the cytoplasm. The present study measured total cofilin abundance instead of cofilin activity, which is difficult to estimate. Therefore, intensive studies are needed to determine whether cofilin expression or activity is associated with nodal metastasis in human breast cancer.

Notably, high cofilin expression was significantly associated with shorter overall survival. This association remained significant when other clinicopathological factors were included in the COX regression analysis, suggesting that

Table II. Univariate and multivariate COX regression analyses of the effects on overall survival for different patients and characteristics.

A, Variable	Univariate			
	n	HR	95% CI	P-value
Cofilin (strong vs. weak) ^a	310	2.15	1.32-3.49	0.002
ER (positive vs. negative)	305	0.63	0.41-0.96	0.030
PR (positive vs. negative)	307	0.59	0.39-0.92	0.019
Her2 (strong vs. weak)	310	1.37	0.87-2.16	0.177
Ki67 (positive vs. negative)	310	1.22	0.79-1.88	0.374
Size (>20 mm vs. ≤20 mm)	304	1.45	0.85-2.45	0.173
Nottingham histological grade (1-3)	299	1.27	0.83-1.94	0.269
Nodal status (N3, N2, N1, N0)	304	1.39	1.12-1.71	0.002

B, Variable	Multivariate			
	n	HR	95% CI	P-value
Cofilin (strong vs. weak) ^a	299	2.22	1.35-3.66	0.002
ER (positive vs. negative)	299	0.80	0.47-1.36	0.400
PR (positive vs. negative)	299	0.75	0.43-1.32	0.323
Nodal status (N3, N2, N1, N0)	299	1.40	1.13-1.73	0.002

^aCofilin score was dichotomized into weak (0-2) and strong (3). HR, hazard ratio; CI, confidence interval; ER, estrogen receptor; PR, progesterone receptor.

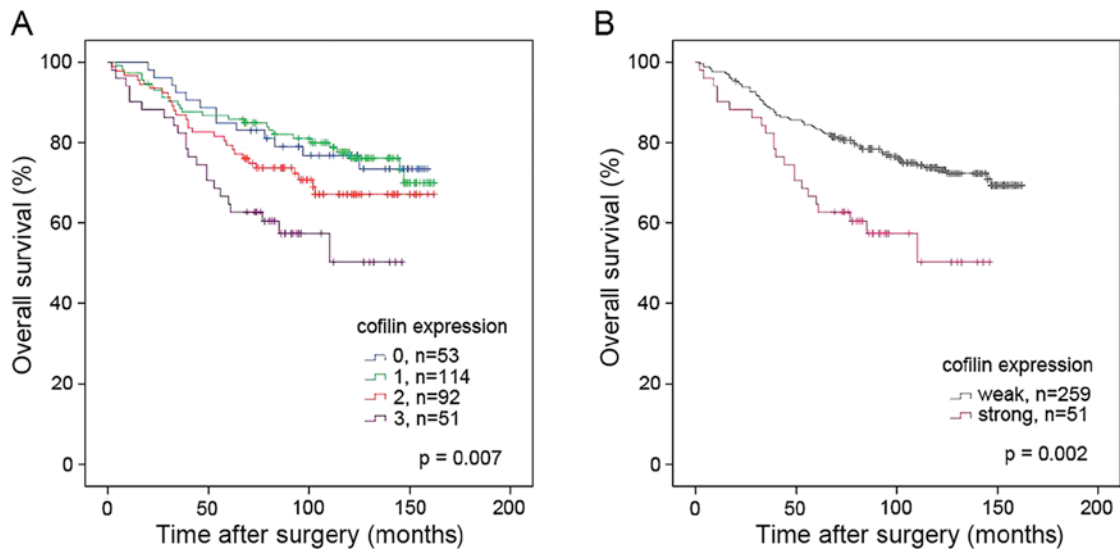


Figure 2. Effects of cofilin expression on overall survival. (A) Kaplan-Meier estimates of survival for cofilin expression demonstrate that higher expression of cofilin in tumor tissue was significantly associated with shorter overall survival. (B) Kaplan-Meier estimates of survival for weak (scores 0, 1, and 2) vs. strong (score 3) cofilin expression in tumor tissue.

cofilin is a potential independent prognostic factor in breast cancer.

In summary, the results of the present study suggest that cofilin may promote the occurrence and development of breast cancer, perhaps via its contribution to cell migration, invasion and/or mitosis. How it does so is beyond the scope of the present study, and requires additional study. The present study suggests that cofilin is a potential independent prognostic

factor in breast cancer, and raises the possibility of targeting cofilin for more effective treatment of breast cancer.

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