Comparison of cofilin-1 and Twist-1 protein expression in human non-small cell lung cancer tissues

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Abstract. Metastasis is the primary cause of mortality in patients with non-small cell lung cancer (NSCLC). Actin cytoskeletal reorganization is usually accompanied by the epithelial-mesenchymal transition (EMT)-induced invasion and metastasis of cancer cells. In the present study, expression levels of the actin-associated protein cofilin-1 and of the pivotal EMT molecule Twist-1 were determined in NSCLC tissues. Using lung cancer tissue arrays, the identification of 67.4% of tissue spots that exhibited reciprocal levels of cofilin-1 and Twist-1 was achieved by immunohistochemical (IHC) staining. This reciprocal expression pattern was also detected in 21 out of 25 clinicopathological NSCLC tissue sections, and in 10 out of 15 NSCLC cell lines. In addition, high levels of cofilin-1 and low levels of Twist-1 accounted for 80 and 71.5% of the reciprocal expression pattern in tissue arrays and clinicopathological tissue samples, respectively. This pattern was also detected in normal lung tissues, stage I and II lung cancer tissues, and adenocarcinoma subtypes of NSCLC tissues. Although cofilin-1 and Twist-1 were expressed inversely, a positive correlation of these two proteins was present in normal lung tissues and lung tumor tissues. Furthermore, enforced expression of cofilin-1 suppressed the expression level of Twist-1 in NSCLC H1299 cells. An on-line Kaplan-Meier survival analytic tool allowed access to a public microarray dataset with a maximum of 1,926 NSCLC samples. The analysis revealed that high expression levels of both cofilin-1 (*CFL1*) and Twist-1 (*TWIST1*) genes were associated with decreased survival of NSCLC patients, notably with regard to the adenocarcinoma subtype. The analysis was conducted using the multivariate Cox regression model. Although the reciprocal association of the expression levels of cofilin-1 and Twist-1 with the survival rate of NSCLC patients requires additional information, it may be a significant indicator of the progression of NSCLC.

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Introduction

According to the 2018 report of the World Health Organization (WHO), lung cancer remains the most common cancer type and is considered as a leading cause of cancer-related mortality in patients worldwide. Non-small cell lung cancer (NSCLC) accounts for 80-85% of all human lung cancer cases (1). According to the histological characteristics, NSCLC can be further divided into several subtypes, including adenocarcinoma, squamous cell carcinoma and large cell carcinoma (2). Over 65% of NSCLC cases are diagnosed as stage III and IV cancers that represent locally advanced malignancy and metastasis status, respectively (3). Enhanced invasive and migratory abilities have been reported to be associated with the epithelial-to-mesenchymal transition (EMT) (4). In addition, the expression level of the EMT-associated transcription factor Twist has been proposed as a poor prognostic marker in lung and breast cancer (5-7).

Several lines of evidence indicate that remodeling of the actin cytoskeleton can induce or regulate EMT in various human cancer types (8-11). Recent reports have also proposed that the expression levels of actin-binding proteins can regulate actin cytoskeleton reorganization and dynamics for the modulation

of EMT (12,13). Cofilin-1 (~19 kDa) is an actin-binding protein that belongs to a member of the actin depolymerizing factor (ADF)/cofilin family. This protein has been shown to accelerate actin dynamics required for cell chemotaxis and increase the migration of non-muscle cells (13). In addition, the activity of cofilin-1 is regulated by the phosphorylation of the protein on serine-3 by the Rho/LIM kinase enzymes (14). Although cofilin-1 is a ubiquitously expressed biomolecule, its expression levels are cell type-dependent. Although cofilin-1 levels are usually increased in advanced human cancers, the etiology and mechanism of these processes remain unclear (15). Recently, the cofilin-1 signaling pathway has been reported to mediate EMT by promoting actin cytoskeletal reorganization and cell-cell adhesion in colorectal and gastric cancer (16,17). The overexpression of cofilin-1 has been reported to induce *let-7* microRNA expression and suppress the growth of NSCLC cells via the downregulation of *TWIST1* (18). In addition, *let-7* microRNA levels were found to regulate EMT by inhibiting the expression levels of high mobility group A2 (HMGA2) and Twist-1 proteins that decrease the development of EMT in cancer cells (19,20). Since overexpression of cofilin-1 can influence the expression level of Twist-1 in cultured cells, it is of considerable interest to further investigate the expression pattern of cofilin-1 and Twist-1 proteins in cancer tissues.

In the present study, the expression levels of cofilin-1 and Twist-1 proteins were examined in human NSCLC tissue arrays and in clinicopathological lung cancer tissue sections by immunohistological staining (IHC). The data demonstrated that 67.4% of lung cancer tissue spots expressed reciprocal levels of cofilin-1 and Twist-1 proteins and 80% of these tissue samples exhibited high levels of cofilin-1 and low levels of Twist-1. The inverse expression levels of cofilin-1 and Twist-1 were also noted in 8 of 15 NSCLC cancer cell lines. Furthermore, overexpression of cofilin-1 directly suppressed Twist-1, whereas disruption of actin cytoskeleton by cytochalasin B did not cause the same effect. Therefore, the reciprocal expression levels of cofilin-1 and Twist-1 proteins are an important characteristic noted in NSCLC tissues, suggesting that cofilin-1 may be a novel factor that influences the expression level of Twist-1.

Materials and methods

Cell lines. Several NSCLC cell lines used in the present study included CL1-0, CL1-5, H661, H596, H1975, H1299, A549, H460, H1563, H2122, H441, PC9, H1355, H23 and H157. The H157 cell line is identical to H1264 as reported by American Type Culture Collection (ATCC) (https://web.expasy.org/cellosaurus/CVCL_0463). These cell lines were maintained in culture media (DMEM or RPMI, can be provided upon request) supplemented with 10% fetal bovine serum and 2 mM L-glutamine (Sigma-Aldrich; Merck KGaA). The protein lysates of these differernt cell lines were extracted for western blot analysis. The addition of 0.1 mg/ml of hygromycine B (Invitrogen; Thermo Fisher Scientific, Inc.) in DMEM medium was used to culture H1299 cells harboring a tetracycline inducible gene expression system for overexpression of *cofilin-1* cDNA (HCOXP). Doxycycline (1 μg/ml) was added to the cells for 24 h in order to induce cofilin-1 expression. The cells were collected for cell lysis and western blot analysis following an additional 4 days of incubation. All of the cell lines were maintained in a humidified incubator with 5% CO_2 at 37˚C and passaged every 48 h.

Reagents. Cytochalasin B was purchased from Sigma-Aldrich/Merck KGaA. The reagents were dissolved in dimethyl sulfoxide (DMSO) to obtain concentrations of 100 mM as stock solutions. The working concentration was 10 mM in the culture medium, and the cells were treated for 24 h prior to extraction.

Tissue arrays, clinicopathological tissue sections, and IHC staining. The lung cancer tissue arrays BC041115b (120 cases/120 cores including 10 normal tissue spots) and 1-OD-CT-RsLug03-002 (62 cases/62 cores including 31 cancer tissues and 31 matched normal adjacent tissue spots) were purchased from the US Biomax Inc.. The two proteins (cofilin-1 and Twist-1) were examined and two pieces of each tissue array type were subjected to IHC staining. IHC staining was further used for the examination of the protein expression in clinicopathological tissue sections. For clinicopathological tissue sections, 25 lung cancer sections were collected from the Division of Pathology, Tao-Yuan General Hospital, Ministry of Health and Welfare, Taiwan from January to December 2016 (Table I). The present study was approved by the Institutional Review Board of Tao-Yuan General Hospital (TYGH104504). The paraffin-embedded tissue sections were maintained at 60˚C for 1 h, and subsequently deparaffinization in xylene (Sigma-Aldrich; Merck KGaA) was performed. The tissue sections were rehydrated in graded ethanol (from 95 to 75%) and finally immersed in phosphate-buffered solution with 0.05% Tween-20 (PBST). For antigen retrieval, the tissue slides were heated in 10 mM citric acid buffer with 0.05% Tween-20 (pH 6.0) for 3 min at 121˚C using a pressure cooker. Following air-cooling, the tissue sections were incubated with peroxidase blocking reagent [RTU, EnVision™+Dual Link System-HRP (DAB+); cat. no. K4065, Dako; Agilent Technologies, Inc.] for 5 min and subsequently blocked with goat serum for an additional 30 min. Thereafter, the tissue sections were incubated with anti-cofilin-1 (1:100 dilution; cat. no. GTX102156) and anti-Twist-1 antibodies (1:250 dilution; cat. no. GTX60776; GeneTex Inc.) at 4˚C overnight followed by horseradish peroxidase (HRP)-conjugated secondary antibodies. The sections were rinsed with PBST, developed in 3',3'-diaminobenzidine (DAB) substrate chromogen [EnVision™+Dual Link System-HRP (DAB+); cat. no. K4065, Dako; Agilent Technologies, Inc.] and finally counterstained with Mayer's hematoxylin (ScyTek Laboratories). All sections were scanned using the Aperio Digital Pathology Slide Scanner (Leica Biosystems). Lepidic growth was excluded in all examined cases of adenocarcinoma containing invasive area for evaluation of the IHC score. Quantification of IHC scores was determined by multiplying the staining intensity (on a scale of 0-3) by the positivity of the staining factor (on a scale of 1-4: 0-25%, 1; 26-50%, 2; 51-75%, 3; and 76-100%, 4). All of the IHC staining tissues were examined and scored by 2 to 3 different individuals in a blinded manner.

Western blot analysis and antibodies. The procedures of lysate extraction, protein electrophoresis and blotting were as described in our previous research (21). For preparation of cell lysates, the monolayers were rinsed with phosphate-buffered saline and subsequently scraped in lysis buffer (0.5% NP-40, 50 mM Tris HCl,

Table I. Features of the clinical resected lung cancer tissue samples examined by immunohistochemistry (IHC).

Case no.	Stage	Age (years)	Sex	Subtype
1	1A	54	F	Adenocarcinoma
2	2A	74	F	Adenocarcinoma
3	3B	77	F	Adenocarcinoma
4	3B	50	M	Adenocarcinoma
5	3B	71	M	Metastatic squamous cell carcinomain
6	4	66	F	Mesothelioma
7	4	69	F	Adenocarcinoma
8	4	61	M	Adenocarcinoma
9	4	71	M	Non-small cell carcinoma favored
10	4	71	M	Small cell lung cancer
11	4	68	F	Adenocarcinoma
12	4	67	M	SCC
13	4	64	F	SCC
14	4	50	F	Mixed adenocarcinoma and rhabdomyosarcoma
15	4	63	M	Adenocarcinoma
16	4	59	M	Adenocarcinoma
17	4	38	M	Adenocarcinoma
18	4	75	M	Adenocarcinoma
19	4	53	M	Adenocarcinoma
20	4	80	F	SCC
21	4	61	M	SCC
22	4	62	F	Adenocarcinoma
23	4	68	M	High grade adenosquamous
24	4	53	M	Pleomorphic carcinoma
25	4	49	M	Adenocarcinoma

F, female; M, male; SCC, squamous cell carcinoma.

120 mM NaCl, and 1% phenylmethylsulfonyl fluoride). The band intensity was determined using densitometry (ImageJ Software version 1.x; National Institutes of Health, Bethesda, MD, USA). The primary antibodies were purchased from GeneTex Inc. and included anti-cofilin-1 (1:1,000 dilution; cat. no. GTX102156), anti-Twist-1 (1:500 dilution; cat. no. GTX60776), anti-SNAIL (1:500 dilution; cat. no. GTX100754), and anti-tubulin (1:1,000 dilution; cat. no. GTX112141) antibodies. Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Thermo Fisher Scientific Inc. (1:4,000 dilution; cat. no. MA515738).

Statistical analysis. The t-test was used for statistical analysis between two groups. The survival probability was determined using an on-line Kaplan-Meier plotter (kmplot.com) tool, which is a meta-analysis based biomarker assessment for 54,675 genes based on the Affymetrix probe set IDs (22). With regard to lung cancer, the maximum sample size of the available patients was 1,926. These data were used for Kaplan-Meier survival and Cox regression analyses. Significant parameters were derived using univariate amd multivariate analyses according to the KM plotter website (23). The gene_ID of cofilin-1 and Twist-1 proteins on the Affymetrix chip were 200021_at and 213943_at, respectively. Cox regression was used to analyze the association between the expression levels of cofilin-1 and Twist-1 proteins and NSCLC according to the results of the Kaplan-Meier method. The significance was determined by the log-rank test. The correlation analysis and scatter diagram were drawn using the MedCalc® software version 18.2.1 (Ostend). A P-value less than 0.05 ($P<0.05$) was considered to indicate a statistically significant difference.

Results

Immunohistological staining of cofilin-1 and Twist-1 proteins in human NSCLC tissue arrays. Human lung cancer tissue arrays (see Materials and methods) were subjected to IHC staining. A total of 2 tissue arrays with identical orders of tissue spots were analyzed using anti-cofilin-1 or anti-Twist-1 antibodies followed by pathological slide scanning. The complete results of IHC staining on these tissues arrays are demonstrated in Fig. 1A. Microscopical investigation was also used to visualize the IHC staining results of cofilin-1 and Twist-1 proteins. T/C was the ratio of Twist-1 staining score divided by cofilin-1 staining scores for each tissue spot (Fig. 1B). The scores of cofilin-1 were compared with those of Twist-1. Both scores exhibited similar levels when the T/C ratio was 1±0.5. The T/C ratios of all lung tumor tissue spots (n=141) are presented as a scatter diagram (Fig. 1C). The results were further summarized and indicated that 67.4% of lung cancer tissue spots expressed reciprocal levels of cofilin-1 and Twist-1 proteins (Fig. 1D). In these tissue spots, 80% of the samples expressed higher levels of cofilin-1 than those of Twist-1 (Fig. 1E).

Comparison of cofilin-1 and Twist-1 expression levels in different stages and subtypes of lung cancer tissues. Subsequently, we compared the results of cofilin-1 and Twist-1 staining in NSCLC samples of different tumor stages and subtypes by calculating the immunostaining scores. The expression levels of both cofilin-1 and Twist-1 proteins were relatively high in stage I, II and III lung cancer tissues when compared with the normal tissues (Fig. 2A). A total of only 130 tissue spots belonging to stage I to III were counted, as the tumor stage of 10 tissue spots in the 1-OD-CT-RsLug03-002 tissue array was mixed or not described. A total of 1 tissue spot was defined as stage IV, so it was not included in the statistics. A correlation analysis was further performed with regard to the regression of cofilin-1 and Twist-1 expression levels in normal lung tissue spots and lung tumor tissue spots. Positive corellations were noted in all of these tissue spots, whereas stage III lung tumor tissues exhibited higher correlation with the expression of the corresponding proteins (Fig. 2B). In contrast to these observations, the mean IHC score of cofilin-1 was higher than that of Twist-1 in normal tissues and in stage I and/or II of lung tumor tissues (Fig. 2C). It is interesting to note that the mean IHC scores of cofilin-1 were higher than those of Twist-1 in the adenocarcinoma subtypes, although this finding was not noted in the squamous cell carcinoma subtype of the lung tumor tissues (Fig. 2D). A considerably

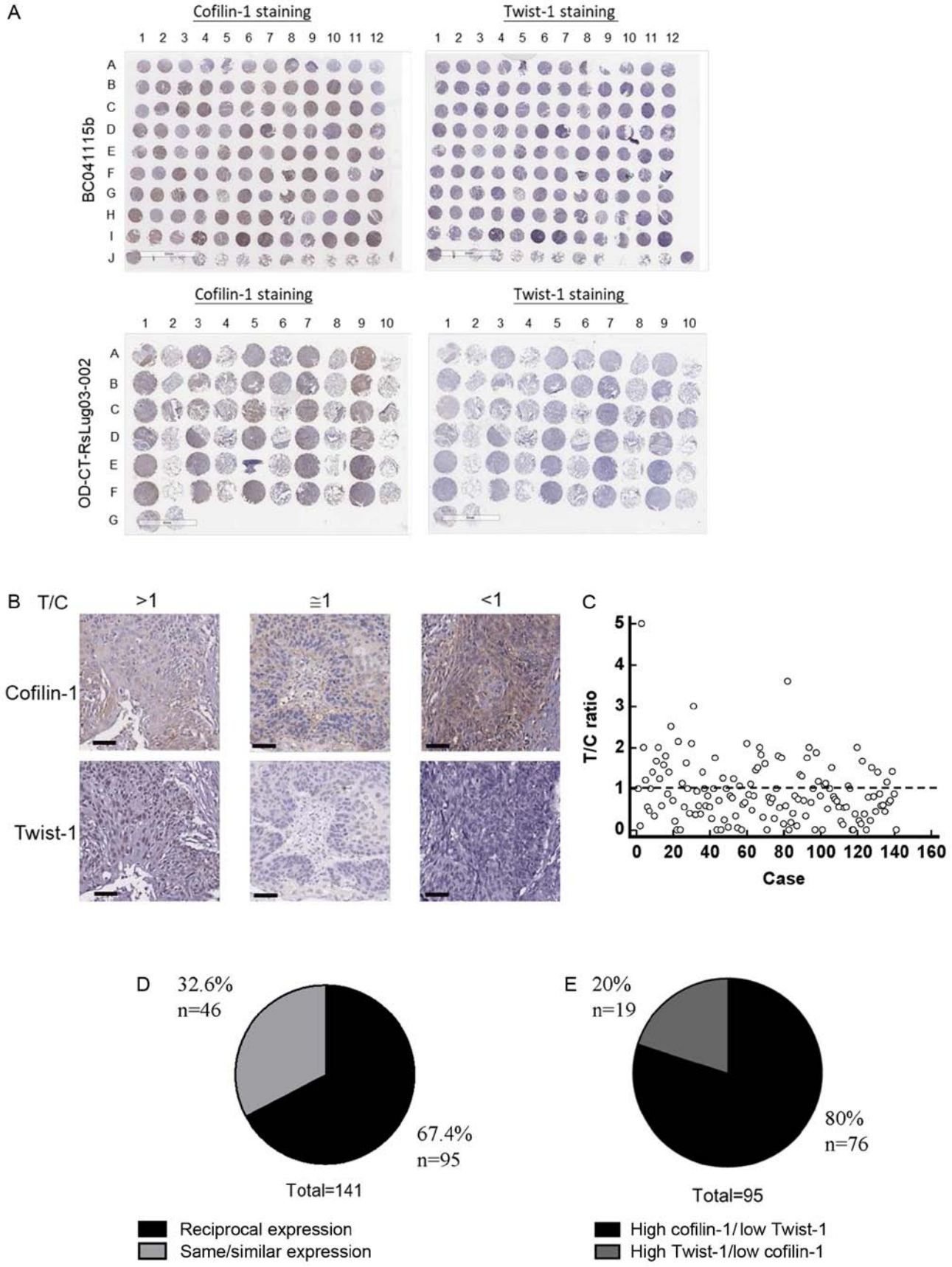

Figure 1. Immunohistochemical (IHC) staining of cofilin-1 and Twist-1 proteins using human lung cancer tissue arrays. (A) A total of 2 sets of tissue arrays, namely BC041115b and 1-OD-CT-RsLug03-002 were stained with anti-cofilin-1 and anti-Twist-1 antibodies. (B) Images of the microscopic examiniation of tissue spots stained with anti-cofilin-1 or anti-Twist-1 antibodies. T/C is the ratio of the Twist-1 IHC score divided by the cofilin-1 IHC score. Scale bar, 60 μm. (C) Scatter plot of the T/C ratio of all lung tumor tissue spots in the tissue arrays (n=141) under investigation. A T/C ratio of 1 (the dotted line) represented the similar IHC scores of cofilin-1 and Twist-1 proteins. (D and E) Pie charts of cofilin-1 and Twist-1 expression patterns in the NSCLC tissue spots. (D) Percentages and numbers of total tissue spots with or without reciprocal expression of cofilin-1 and Twist-1; (E) Percentages and numbers of different reciprocal expression patterns of cofilin-1 and Twist-1 proteins in tissue spots.

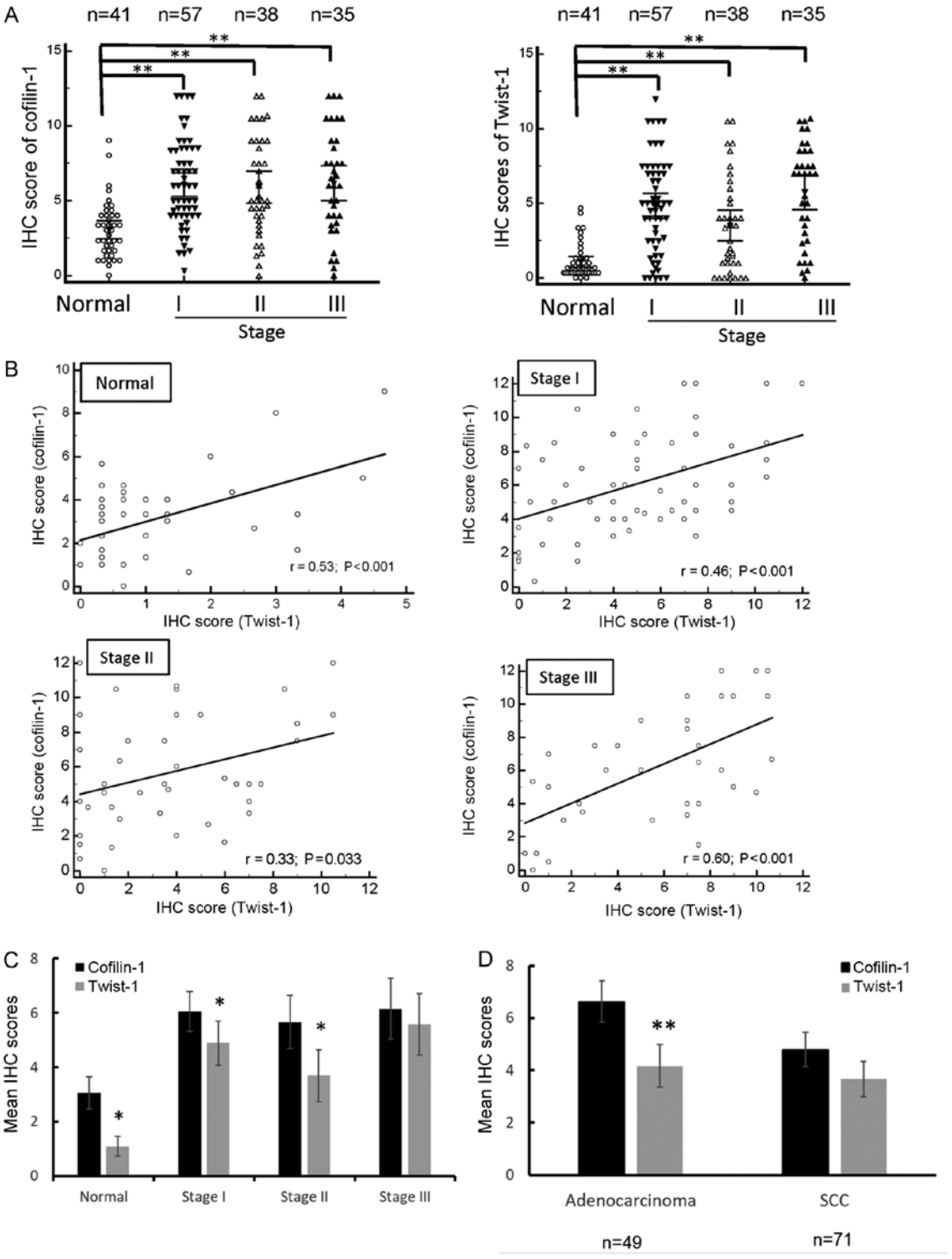

Figure 2. Comparison of cofilin-1 and Twist-1 expression levels in lung cancer of different stages and subtypes. (A) The (IHC) scores of cofilin-1 and Twist-1 in normal lung tissues and stage I, II and III lung cancer tissues from the tissue arrays. The number of each tissue type is shown as n. Error bars represent 95% confidence interval (CI). (B) Correlation analysis of cofilin-1 and Twist-1 in normal lung tissues and different stage lung cancer tissues. (C) Comparison of mean IHC scores between cofilin-1 and Twist-1 proteins in normal and tumor lung tissues. (D) Comparison of the mean IHC scores of cofilin-1 and Twist-1 proteins in adenocarcinoma and SCC tissues of NSCLC. *P<0.05; **P<0.01. IHC, immunohistochemistry; SCC, squamous cell carcinoma; NSCLC, non-small cell lung cancer.

low Twist-1/cofilin-1 ratio was further noted in large cell cancer, bronchioloalveolar carcinoma (or lepidic predominant adenocarcinoma), and mucinous adenocarcinoma, although the total sample size of these subtypes was considerably small (n=4). Taken together, these results suggested that the expression levels of cofilin-1 and Twist-1 proteins were correlated, while the mean expression level of cofilin-1 were higher than those of Twist-1 in normal tissues, low tumor stage tissues, and adenocarcinoma subtype tissues.

Detection of cofilin-1 and Twist-1 expression in resected lung cancer tissues. Hospital-based lung cancer tissue sections

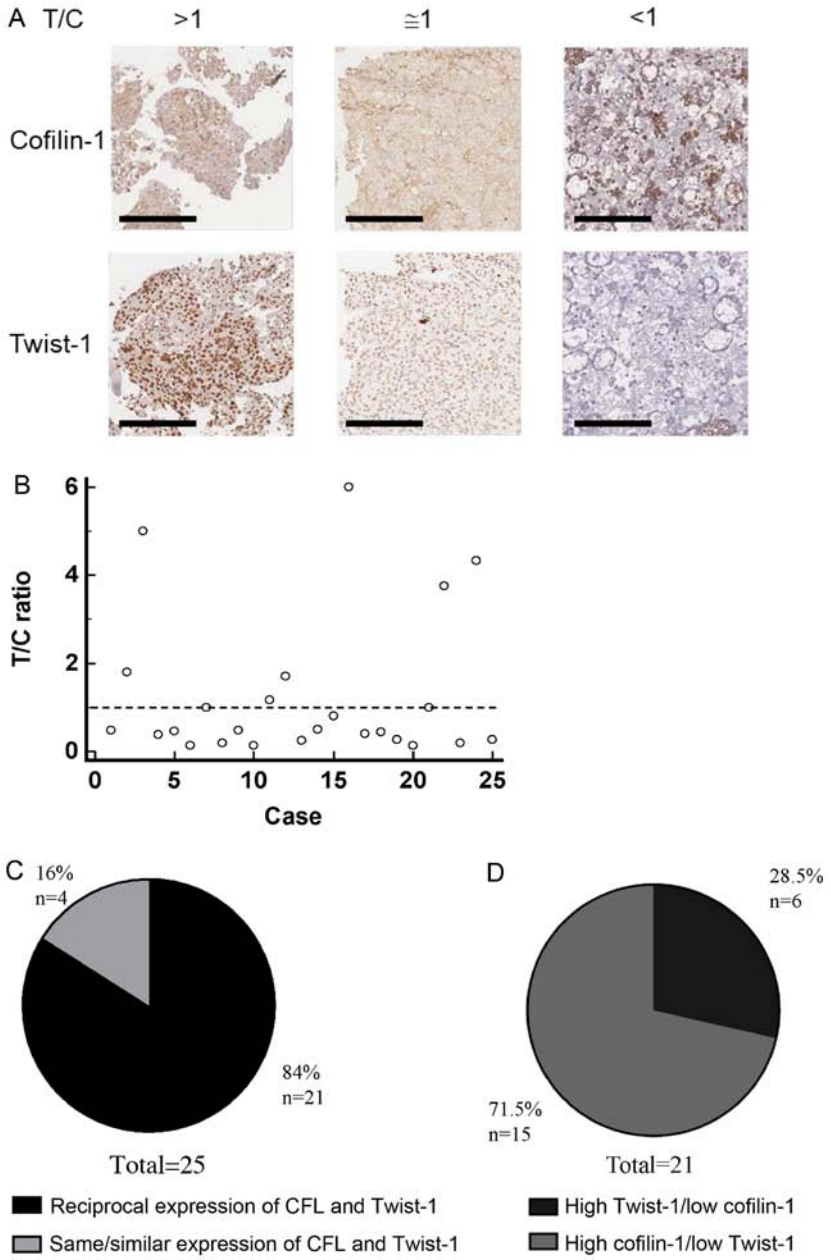

Figure 3. IHC staining of cofilin-1 and Twist-1 proteins in clinicopathological NSCLC tissue sections. (A) Microscopic examination of tissue spots stained with anti-cofilin-1 or anti-Twist-1 antibodies in clinicopathological NSCLC tissue sections. Scale bar, 200 μm. (B) The scatter plot shows the T/C ratio of all clinicopathological NSCLC tissue sections (n=25). The T/C ratio of 1 (the dotted line) represented similar IHC scores for cofilin-1 and Twist-1 proteins. (C and D) Pie charts of cofilin-1 and Twist-1 expression levels. (C) Percentages and numbers of total tissue spots with or without reciprocal expression of cofilin-1 and Twist-1 proteins; (D) Percentages and numbers of different reciprocal expression patterns of cofilin-1 and Twist-1 proteins in tissue sections. IHC, immunohistochemistry; NSCLC, non-small cell lung cancer.

were also examined for the expression of cofilin-1 and Twist-1 proteins. A total of 25 lung cancer tissue samples with consecutive sections were collected and compared for their individual cofilin-1 and Twist-1 IHC scores in order to obtain the T/C ratio as mentioned above (Fig. 3A). The T/C ratio of each tumor tissue section (n=25) was depicted by a scatter diagram (Fig. 3B). A total of 21 of these samples (84%) exhibited reciprocal levels of cofilin-1 and Twist-1 (Fig. 3C). Furthermore, 15 out of 21 (71.5%) exhibited high expression of cofilin-1 and low expression of Twist-1, corresponding to a low T/C ratio (Fig. 3D). In addition, 14 surgical cases were of adenocarcinoma and 4 of squamous cell carcinoma origin, respectively. The remaining 7 cases included various types of lung cancer, such as mesothelioma, metastatic squamous cell carcinoma, mixed adenocarcinoma and rhabdomyosarcoma, high grade adenosquamous, pleomorphic carcinoma, and small cell lung cancer (Table I). Furthermore, the mean IHC score of cofilin-1 was significantly higher than that of Twist-1 in adenocarcinoma, whereas this was not noted in squamous cell carcinoma of these clinicopathological tissues (data not shown). Although the sample size of the clinicopathological tissue sections was small, the expression pattern of cofilin-1 and Twist-1 proteins seemed similar with that noted in the lung cancer tissue arrays.

Comparison of cofilin-1 and Twist-1 expression in human NSCLC cell lines. In addition to NSCLC tissues, we further

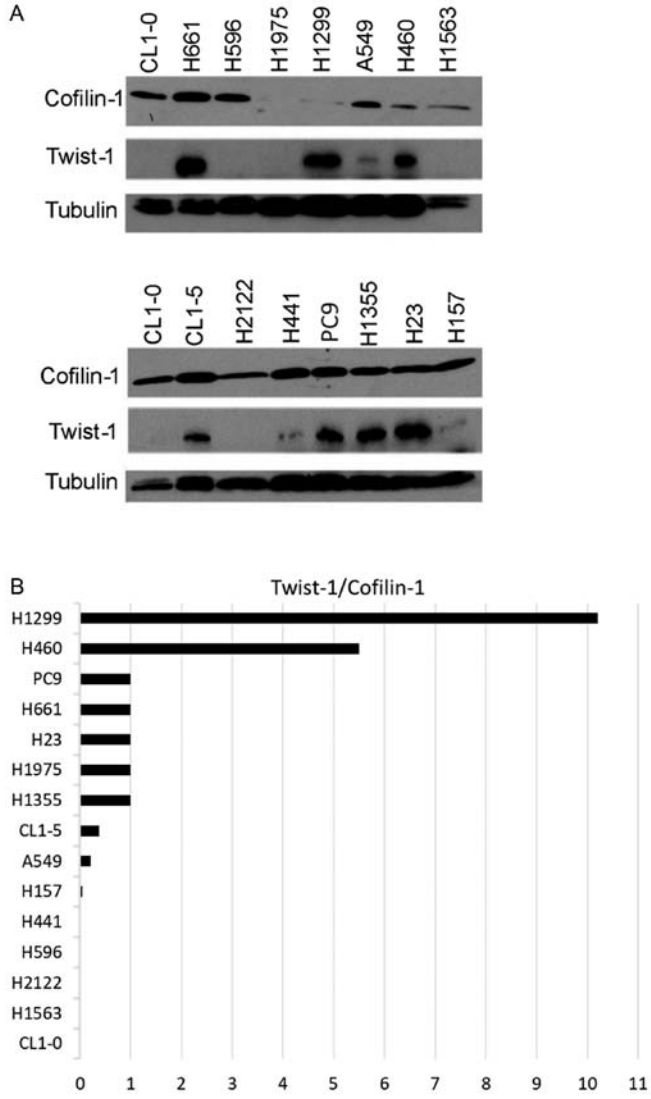

Figure 4. Comparison of cofilin-1 and Twist-1 expression levels in various NSCLC cell lines. (A) Western blot analysis was used to detect the expression levels of cofilin-1 and Twist-1 proteins in 15 NSCLC cell lines. Tubulin was used as a loading control. (B) Comparison of Twist-1/cofilin-1 ratio in selected cell lines using densitometric analysis. NSCLC, non-small cell lung cancer.

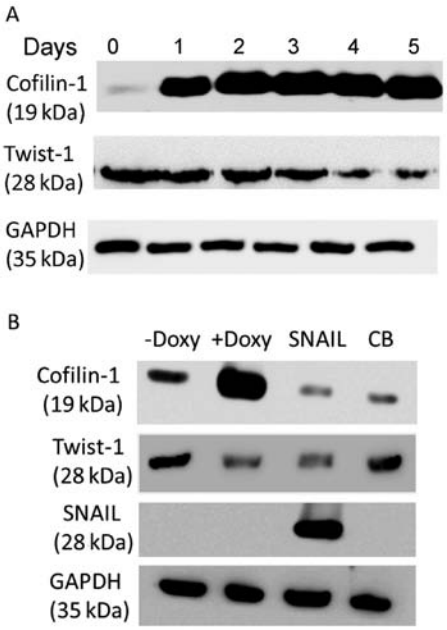

Figure 5. Effects of cofilin-1 on Twist-1 expression following protein overexpression in NSCLC cells. (A) *Cofilin-1* cDNA (HCOXP) stable transfected cells were induced by doxycycline (1 μg/ml) for a total period of 6 days. The expression levels of cofilin-1 and Twist-1 proteins were examined using western blot analysis. (B) The expression levels of Twist-1 following cofilin-1 overexpression, SNAIL overexpression and cytochalasin B (CB), an actin inhibitor (10 μM) treatment in H1299/tet-on-cofilin-1 stable-transfected cells. Doxycycline was administered for 4 days in this experiment. NSCLC, non-small cell lung cancer.

investigated whether reciprocal expression of cofilin-1 and Twist-1 could be detected in various cell lines. A total of 15 NSCLC cell lines were collected for western blot analysis. The results indicated that cofilin-1 and Twist-1 were differentially expressed in these cell lines (Fig. 4A). The expression levels of cofilin-1 in H1975 cells and H1299 cells were significantly lower than those of the other NSCLC cell lines. In contrast to these observations, half of these cell lines exhibited high levels of Twist-1 protein, whereas the other half indicated extremely low expression levels of Twist-1 (Fig. 4A). The T/C ratio was estimated in each cell line using densitometric analysis. A total of 10 out of 15 cell lines exhibited reciprocal expression of cofilin-1 and Twist-1 proteins, whereas a low Twist-1/cofilin-1 ratio was noted in 8 NSCLC cell lines (Fig. 4B). Therefore, the reciprocal expression of cofilin-1 and Twist-1 proteins was also detectable in cultured NSCLC cell lines.

Overexpression of cofilin-1 suppresses Twist-1 levels in NSCLC cells. Since high levels of cofilin-1 were associated with low levels of Twist-1, the potential of cofilin-1 overexpression to suppress the expression level of Twist-1 protein was examined in HCOXP cells (24). The results indicated a time-dependent suppression of Twist-1 due to the overexpression of cofilin-1 using doxycycline induction (Fig. 5A). In contrast to cofilin-1, disruption of the actin cytoskeleton by cytochalasin B (CB), an actin inhibitor did not influence the expression levels of Twist-1 (Fig. 5B). Notably, overexpression of the SNAIL transcription factor suppressed Twist-1 level, whereas the cofilin-1 level was also decreased. These results suggested that cofilin-1 mediates the expression level of Twist-1, although this may not be associated with the destabilization of the actin cytoskeleton.

Effects of cofilin-1 and Twist-1 gene expression on the survival fraction of NSCLC patients. Although the protein expression levels of cofilin-1 and Twist-1 were compared in tissue arrays and clinicopathological tissue sections, the survival data used to analyze the role of these two proteins on the survival of NSCLC patients are limited. Therefore, we adopted the public microarray database and an on-line Kaplan-Meier plot analytical tool in order to evaluate the gene expression levels of cofilin-1 (*CFL1*) and Twist-1 (*TWIST1*) genes on the survival of lung cancer patients (23). High expression levels of the *CFL1* and *TWIST1* genes indicated lower survival rates in NSCLC patients with a univariate Cox regression HR of 1.32 [95% confidence interval (CI): 1.16-1.5] and 1.21 (95% CI, 1.06-1.37), respectively (Fig. 6A). High cofilin-1 and Twist-1 levels were further associated with significantly low survival in the adenocarcinoma subtype of NSCLC patients (HR=2.32 with 95% CI, 1.82-2.96 for cofilin-1, and

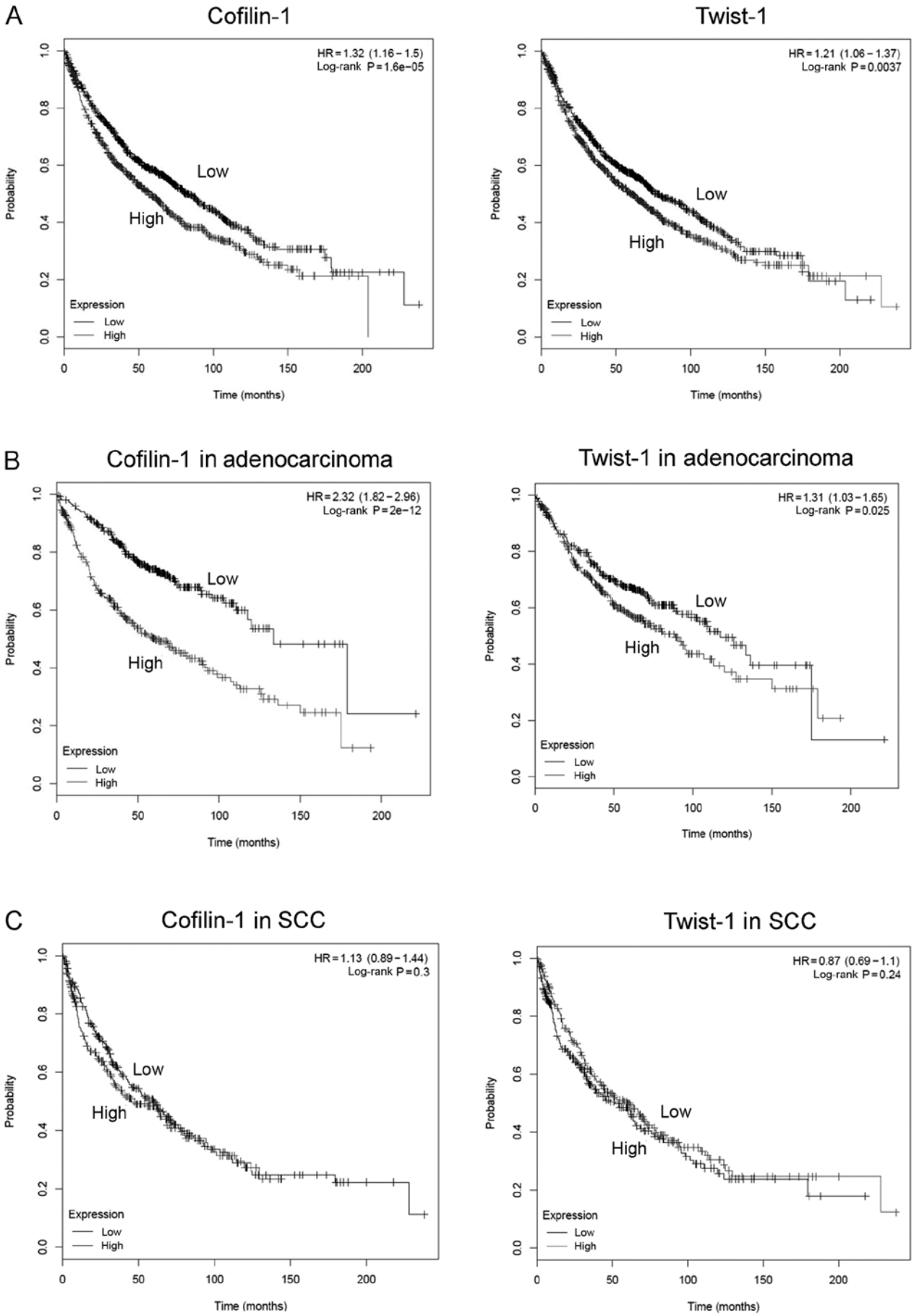

Figure 6. Role of *CFL1* and *TWIST1* gene expression on the survival fraction of NSCLC patients. (A) The Kaplan-Meier method with the log-rank test was used to compare the survival rates in NSCLC patients with different levels of cofilin-1 or Twist-1. The survival rates of patients with (B) adenocarcinoma and (C) SCC subtypes. P<0.05 was considered to indicate a significant difference. NSCLC, non-small cell lung cancer; SCC, squamous cell carcinoma.

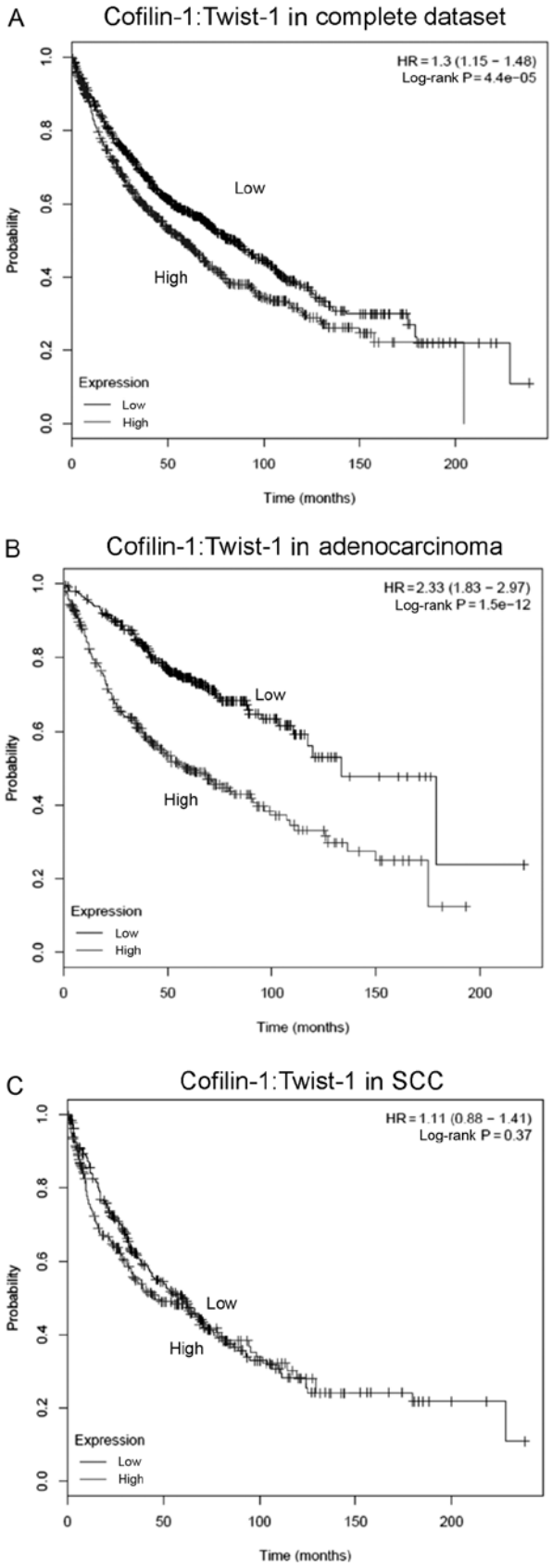

Figure 7. Effects of the co-expression of *CFL1* and *TWIST1* genes on the survival fractions of NSCLC patients. (A) Univariate Cox regression based on the Kaplan-Meier method was used for analysis of the survival fraction in a complete database with 1,926 patients (see Materials and methods). (B) Multivariate Cox regression was used for analysis of the hazard ratio (95% CI) of dual genes in different NSCLC subtypes: (B) adenocarcinoma and (C) SCC. NSCLC, non-small cell lung cancer; SCC, squamous cell carcinoma.

HR=1.31 with 95% CI, 1.03-1.65 for Twist-1). However, this was not noted in squamous cell carcinoma (SCC) lung cancer subtypes (Fig. 6B and C). Therefore, the expression levels of the *CFL1* and *TWIST1* genes may influence the survival rate of patients with different subtypes of NSCLC.

To evaluate the effects of the co-expressed *CFL1* and *TWIST1* genes on the survival of NSCLC patients, a multi-gene classifer with the Cox regression was applied for further anaylsis based on the results of the Kaplan-Meier method. Using the same public microarray database, high expression levels of both cofilin-1 and Twist-1 were associated with reduced survival fraction as determined by a univariate Cox regression HR of 1.3 (95% CI, 1.15-1.48; Fig. 7A). A similar result was also found in the adenocarcinoma subtype samples with a multivariate Cox regression HR of 2.33 (95% CI, 1.83-2.97; Fig. 7B). However, no significant differences were noted in the survival rate of squamous cell carcinoma (SCC) patients with high or low expression of both *CFL1* and *TWIST1* genes (Fig. 7C).

Discussion

Increased cell migration is an important feature of cancer invasion and metastasis. Epithelial-mesenchymal transition (EMT) is responisble for the development of cancer malignancy. However, the mechanisms involved in the association between cancer migration and EMT are not fully understood. We previously showed that overexpression of cofilin-1 could suppress cell invasion of non-small cell lung cancer (NSCLC) cells (25). Cofilin-1 was found to suppress the Twist-1 level following its overexpression in NSCLC cells, whereas overexpression of Twist-1 did not significantly influence the expression level of cofilin-1 (18). To fully understand whether the expression of cofilin-1 and Twist-1 proteins were associated with the clinicopathological characteristics of lung cancer, their expression levels were examined in normal and malignant lung tissues using immunohistochemistry (IHC). It was found that in the majority of the cases, the IHC score of cofilin-1 was higher than that of Twist-1. However, a positive correlation of cofilin-1 and Twist-1 was also detected in these tissues, including normal lung tissues. These findings suggested that even though the co-expression of these two molecules was positively associated, cofilin-1 levels remained higher than Twist-1 in the majority of the tissue samples. This phenomenon was observed not only in lung tumor tissue samples but also in normal lung tissues. Therefore, the expression levels of cofilin-1 were significantly higher than those of Twist-1 in normal lung tissues, and stage I and II lung cancer tissues. However, these findings were not noted in stage III cancer samples (Fig. 2C). The results of the tissue array experiments suggested that reciprocal expression of cofilin-1 and Twist-1 could be detected with regard to different co-expression levels of these proteins, notably in normal and early stages of lung tumor tissues.

The differences noted with regard to the high levels of cofilin-1 expression and the low levels of Twist-1 (low T/C ratio) were observed in the majority of the tissue spots (80%) with regard to the NSCLC adenocarcinoma subtype (Fig. 2B). It has been reported that squamous cell carcinoma expresses higher levels of Twist-1 than those noted in adenocarcinoma (5,26).

Nevertheless, high gene expression levels of both *CFL1* and *TWIST1* were found to only account for the low survival rate of patients with adenocarcinoma and not of patients with squamous cell carcinoma. Since reciprocal expression levels of cofilin-1 and Twist-1 have been mainly detected in adenocarcinoma but not in squamous cell carcinoma tissues, the survival rate of adenocarcinoma may be altered if both genes are expressed inversely. Moreover, the levels of cofilin-1 detected at different tumor stages (I to III) were similar, and they were all higher than those noted in normal lung tissues. Since only one tissue spot was represented as stage IV in this commercial tissue array, this was not included for statistical analysis. In the present tissue spot, the stage IV samples expressed low cofilin-1 and high Twist-1 levels (data not shown). In contrast to these findings, a recent report indicated that sputum cofilin-1 levels were higher in T4 and N stage of lung cancer patients (27). Serum cofilin-1 levels were further reported to be increased in the advanced stage of patients with lung cancer (28). A total of 16 out of 20 stage IV clinicopathological sections exhibited reciprocal expression levels of cofilin-1 and Twist-1, whereas in 10 out of 16 sections the pattern of high cofilin-1/low Twist-1 ratio was noted (Table I). Although the sample size used in the present study was small, the samples were derived from different sources and it appeared that the inverse expression of cofilin-1 and Twist-1 could occur in different cancer stages. The investigation of additional stage IV tumor samples is important to confirm the primary expression pattern of cofilin-1 and Twist-1 proteins in NSCLC tissues.

Since high cofilin-1/low Twist-1 ratio is a predominant phenomenon in the reciprocal expression of cofilin-1 and Twist-1, we further demonstrated that overexpression of cofilin-1 suppressed Twist-1 levels in H1299 cells. Overexpression of cofilin-1 and reduced Twist-1 levels have been reported to induce *let-7* and inhibit tumor growth, invasion and motility *in vivo* and *in vitro* (18). This is in part consistent with a previous report suggesting that Twist-1 could interact with the *BMI1* oncogene to suppress *let-7i*. expression. This interaction was associated with increased tumor invasiveness and poor survival outcome in cancer patients (29). Although overexpression of cofilin-1 can destabilize the actin cytoskeleton (30), cytochalasin B-mediated disruption of the actin cytoskeleton did not reduce the expression levels of Twist-1. Therefore, overexpression of cofilin-1 caused reduction in Twist-1 levels and this effects was not directly associated with actin cytoskeletal destabilization. Moreover, it was previously demonstrated that ectopic expression of Twist-1 did not influence the expression of cofilin-1 (18). In the present study, we further examined whether overexpression of SNAIL could affect the expression levels of cofilin-1. The data indicated that ectopic expression of SNAIL suppressed the expression levels of cofilin-1 and Twist-1 proteins. These differences may be attributed to the different signaling pathways of Twist-1 and SNAIL.

To the best of our knowledge, little is known with regard to the interaction of cofilin-1 and expression of Twist-1. The expression level of Twist-1 can be modulated by a series of upstream regulators, such as the tumor necrosis factor (TNF)-α, the WNT, the receptor tyrosine kinase, transforming growth factor (TGF)-β, the Notch and the hypoxia pathways (31). These pathways are directly or indirectly involved in the remodeling of the actin cytoskeleton (32-37). Since the upregulation of cofilin-1 is essential for the reorganization of actin cytoskeleton, the expression level of Twist-1 may be modulated partially by the activation of these pathways. In contrast to the expression level of the total form of the protein, cofilin-1 phosphorylation is known to be controlled by the Rho small GTPase signaling pathway (38). The inhibitors of Rho kinases have been reported to suppress the nuclear accumulation of Twist-1 (39). Therefore, the Rho signaling pathway may be also associated with the interaction between cofilin-1 and Twist-1. However, the detailed mechanisms of these processes remain to be studied.

The association of *CFL1* and *TWIST1* gene expression with the survival rate of lung cancer patients was analyzed using a public microarray database, since data on the survival information of tissue arrays were insufficient. The expression levels of both genes accounted for the poor prognosis of lung cancer patients, which was consistent with previous studies (40,41). However, in a previous study, the overexpression of cofilin-1 led to the suppression of NSCLC cell grwoth, which was contradictory to our findings (25). Yap *et al* demonstrated that the overexpression of cofilin could either promote or suppress the motility of U373MG glioblastoma tumor cells in a concentration-dependent manner (42). The optimal amount of cofilin-1 overexpression required to promote cell motility was 4.5 times higher than that noted in the control cells. Overexpression of cofilin-1 suppressed the invasion of NSCLC cells at 4.5-fold compared with that of the control cells (25). Using tissue arrays, the mean cofilin-1 IHC score of the NSCLC stage I-IV samples was only 2 times higher than that of normal lung tissues (Fig. 2A and C). Therefore, this discrepancy may be associated with the levels of enforced cofilin-1 overexpression in cell lines and the endogenous levels of cofilin-1 in cancer tissues. However, further investigation is essential in order to interpret these findings.

In summary, the expression levels of cofilin-1 and Twist-1 were investigated using paired lung cancer tissue arrays with consecutive tissue spots. The data demonstrated that 66.6% of the tissue spots exhibited reciprocal expression levels of cofilin-1 and Twist-1. Although high cofilin-1 and low Twist-1 levels were the major characteristics of the pattern of reciprocal expression in normal lung and lung tumor tissues, the results indicated that this pattern may be more useful for the detection of early stage lung adenocarcinoma. Overexpression of cofilin-1 was able to regulate the expression level of Twist-1, whereas this effect was not associated with destabilization of the actin cytoskeleton. Accordiing to the survival analysis of the public microarray dataset, high expression levels of both *CFL1* and *TWIST1* genes were associated with reduced survival of the NSCLC patients. Whether reciprocal expression of cofilin-1 and Twist-1 is able to alter the survival period of NSCLC patients is yet to be discovered.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CYC conducted the experiments and analyzed the data. SLC provided the clinicopathological tissue sections and organized the demographic data. JDL reviewed the manuscript and provided consultant on lung cancer. YCC and MH conceived and performed the western blot analysis. LTL provided background information of lung cancer for manuscript writing and editing. HNL and YJL conceived, designed the study, and wrote the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study was approved by the Institutional Review Board of Tao-Yuan General Hospital (TYGH104504). All of the informed consents had been signed by the tissue donors and stored in Tao-Yuan General Hospital.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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