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Downregulated Expression of the Cyclase-associated Protein 1 (CAP1) Reduces Migration in Esophageal Squamous Cell Carcinoma

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Objective: Overexpression of cyclase-associated proteins has been associated with poor prognosis in several human cancers. Cyclase-associated protein 1 is a member of the cyclase-associated proteins which contributes to tumor progression. The aim of the present study was to examine the expression of cyclase-associated protein 1 and to elucidate its clinicopathologic significance in a larger series of esophageal squamous cell carcinoma.
Methods: Immunohistochemical and western blot analyses were performed in esophageal squamous cell carcinoma tissues. Survival analyses were performed by using the Kaplan–Meier method. The role of cyclase-associated protein 1 in migration was studied in esophageal squamous cell carcinoma cell lines of TE1 through knocking down cyclase-associated protein 1 with siRNA and overexpression of cyclase-associated protein 1. The regulation of cyclase-associated protein 1 on migration was determined by transwell and wound-healing assays.
Results: Immunohistochemical analysis showed that cyclase-associated protein 1 expression was negatively associated with E-cadherin and significantly associated with lymph node metastases. Survival analysis revealed that cyclase-associated protein 1 overexpression was significantly associated with overall survival ($P = 0.011$). Knock down of cyclase-associated protein 1 in TE1 cells resulted in decreased vimentin and F-actin levels and the capability for migration. In addition, overexpression of cyclase-associated protein 1 promoted the migration of TE1 cells.
Conclusions: These findings suggest that cyclase-associated protein 1 is involved in the metastasis of esophageal squamous cell carcinoma, and that elevated levels of cyclase-associated protein 1 expression may indicate a poor prognosis for patients with esophageal squamous cell carcinoma.

Key words: carcinoma – squamous cell – cell migration

INTRODUCTION

Esophageal squamous cell carcinoma (ESCC) is one of the leading causes of cancer in China (1). Despite surgical treatment and chemotherapy, the prognosis of ESCC had been very poor until recently (2). ESCC often has poor prognosis due to early lymph node metastasis and invasion of neighboring organs, such as the aorta, trachea, bronchus, pericardium and lung (3). Thus, it is extremely necessary to investigate the

molecular mechanisms of the aggressive metastatic phenotype, which is essential for developing an effective treatment for ESCC. Further studies, which aim at identifying novel tumor-related genes and clarifying their roles in ESCC, will help us to elucidate the mechanism of the initiation and progression of ESCC.

Malignant cell transformation requires changes in the ability of cell migration. The disruption of actin cytoskeleton and intercellular adhesions is an important part of the acquisition of invasive properties in epithelial malignancies. The invasive ability of carcinoma cells is associated with reduced expression of adhesion junction molecules and increased expression of mesenchymal markers, frequently referred to as epithelial-to-mesenchymal transition (EMT) (4). Cyclase-associated proteins (CAPs), among the most highly conserved regulators of actin dynamics, exist in organisms from mammals to apicomplexan parasites (5). CAP also binds monomeric actin and, therefore, also possesses a cytoskeletal function (6–10). The actin cytoskeleton is crucial for a number of cellular processes, including morphogenesis, migration, endocytosis and cytokinesis (5). However, only a few studies on CAPs have been reported in mammalian cells (5,11) and human cancer (12,13). The involvement of CAP1 in the aggressive behavior of pancreatic cancer cells has been reported (12). In this study, we will find out the relationship between the expression of CAP1 and the migration of ESCC.

In our research, we examined CAP1 protein levels in ESCC tissue specimens by using immunohistochemistry and western blotting. We determined the correlation between the expression of CAP1 and E-cadherin with clinicopathologic parameters. Here, we used ESCC cell line TE1 as a model and found that siRNA-mediated knockdown of CAP1 decreased TE1 cell migration. Overexpression of CAP1 promoted TE1 cell migration. These findings suggest a novel function for CAP1 in modulating cancer progression by increasing tumor cell migration.

Table 1. CAP1 expression and clinicopathologic parameters in 98 ESCC specimens

Parameters	Total	CAP1		*P*	χ^2
		Score ≤ 4, *n* (%)	Score ≥ 6, *n* (%)		
Age (years)					
<60	37	19 (51.4)	18 (48.6)	0.478	0.070
≥60	61	33 (54.1)	28 (45.9)		
Gender					
Male	71	39 (54.9)	32 (45.1)	0.354	0.361
Female	27	13 (48.1)	14 (51.9)		
Pathologic grading					
Well	17	12 (70.6)	5 (29.4)	0.280	2.546
Moderately	47	23 (48.9)	24 (51.1)		
Poorly	34	17 (50.0)	17 (50.0)		
Lymph node metastasis					
Presence	68	44 (64.7)	24 (35.3)	0.001*	12.094
Absence	30	8 (26.7)	22 (73.3)		
Tumor size (cm)					
<5	75	42 (56.0)	33 (44.0)	0.344	1.108
≥5	23	10 (43.5)	13 (56.5)		
Tumor depth					
T1	11	8 (72.7)	3 (27.3)	0.365	3.175
T2	15	7 (46.7)	8 (53.3)		
T3	25	15 (60.0)	10 (40.0)		
T4	47	22 (46.8)	25 (53.2)		
E-cadherin					
Low	31	26 (83.9)	5 (16.1)	0.000*	17.281
High	67	26 (38.8)	41 (61.1)		

CAPI, cyclase-associated protein; ESCC, esophageal squamous cell carcinoma. Statistical analyses were performed by Pearson's χ^2 test.
*$P < 0.05$ was considered significant.

METHODS

Tissue Specimens

This study was approved by the ethical committees of the Chinese Academy of Medical Sciences Cancer Institute and the Affiliated Hospital of Nantong University, and an informed consent was obtained from each patient. Ninety-eight human ESCC tissue specimens were provided by the Affiliated Hospital of Nantong University (Jiangsu Province, China) from 2006 to 2009. Eight tissues were obtained at the time of surgery and immediately stored in liquid nitrogen until used for western blot analysis. None of the patients had received radiotherapy or chemotherapy before surgery. After surgery, the patients were surveyed every 3 months by physical examination and measurement of serum tumor markers (squamous cell carcinoma antigen and carcinoembryonic antigen), every 6 months by an enhanced computed tomography scan and abdominal ultrasonography, and annually by endoscopy. The mean follow-up period after surgery was 21.7 months. Patients diagnosed with metastasis had lymph node metastasis verified by pathologic analysis. For all the samples, clinicopathologic characteristics (age, gender, differentiation, stage, tumor depth and lymph node metastasis) are shown in Table 1 (14).

Immunohistochemistry

The formalin-fixed and paraffin-embedded (FFPE) sections were dewaxed in xylene and rehydrated in graded ethanol. Endogenous peroxidase activity was blocked by soaking in 0.3% hydrogen peroxide. Thereafter, the sections were processed in 10 mmol/l citrate buffer (pH = 6.0) and heated to 121°C in an autoclave for 20 min to retrieve the antigen. After

rinsing in phosphate-buffered saline (PBS) (pH = 7.2), 10% goat serum was applied for 1 h at room temperature to block any nonspecific reactions. The sections were then incubated overnight at 4°C with anti-CAP1 (diluted 1:100; Santa Cruz Biotechnology) and anti-E-cadherin (diluted 1:100; Santa Cruz Biotechnology). Negative control slides were also processed in parallel using a nonspecific immunoglobulin IgG (Santa Cruz Biotechnology) at the same concentration as the primary antibody. All slides were processed using the peroxidase–antiperoxidase method (Dako, Hamburg, Germany). After rinsing with PBS, the peroxidase reaction was visualized by incubating the sections with diaminobenzidine tetrahydrochloride in 0.05 mol/l Tris buffer (pH = 7.6) containing 0.03% H_2O_2. After rinsing in water, the sections were counterstained with hematoxylin, dehydrated and cover-slipped. Stained sections were observed under a microscope. At least 10 high-power fields were randomly chosen, and at least 400 cells/field were counted.

Evaluation of the Results of Immunohistochemical analysis

The degree of immunostaining of formalin-fixed, paraffin-embedded sections was viewed and scored separately by two independent investigators, who were blinded to the histopathologic features and patient data of the samples, and the scores were determined by combining the proportion of positively stained tumor cells and the intensity of staining. Scores given by the two independent investigators were averaged for further comparative evaluation of the CAP1 and E-cadherin expression. Tumor cell proportion was scored as follows: 0 (no positive tumor cells); 1 (<10% positive tumor cells); 2 (10–35% positive tumor cells); 3 (35–70% positive tumor cells) and 4 (>70% positive tumor cells). Staining intensity was graded according to the following criteria: 0 (no staining), 1 (weak staining = light yellow), 2 (moderate staining = yellow brown) and 3 (strong staining = brown). The staining index (SI) was calculated by multiplying the product of staining intensity score with the proportion of positive tumor cells. Using this method of assessment, we evaluated CAP1 and E-cadherin expression in benign esophageal epithelia and malignant lesions by determining the SI, with scores of 0, 1, 2, 3, 4, 6, 9 or 12. The cutoff value for high and low expression levels was chosen on the basis of a measure of heterogeneity using the log-rank test statistical analysis with respect to overall survival. An optimal cutoff value was identified: a score of $\geq$6 was used to define tumors with high CAP1 and E-cadherin expression, and an SI score of $\leq$4 was used to indicate low CAP1 and E-cadherin expression (15).

Western Blot Analysis

Tissues and cell samples were promptly homogenized in a homogenization buffer containing 1 M Tris–HCl (pH = 7.5), 1% Triton X-100, 1% NP-40 (Nonidet P-40), 10% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 0.5 M EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 1 mM

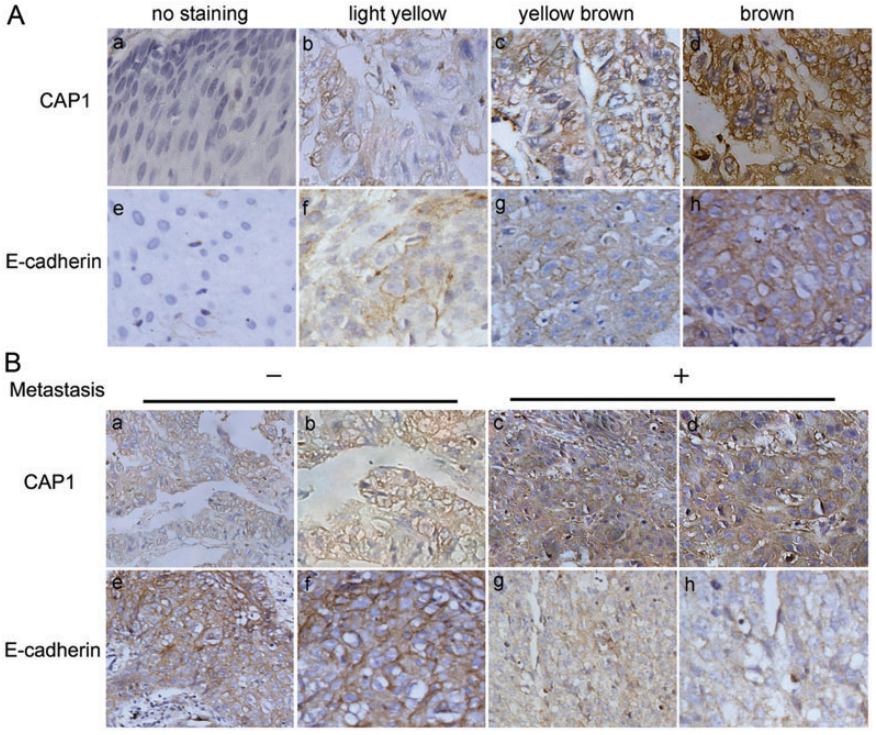

Figure 1. Immunohistochemistry for cyclase-associated protein 1 and E-cadherin was performed as described in the Methods section. (A) Picture examples for each staining intensity criterium (no staining = 0; light yellow = 1; yellow brown = 2 and brown = 3, ×400). (B) (a, b, e, f) Cancer tissues with no lymph node metastasis showed low CAP1 and high E-cadherin expression (×200 and ×400). (c, d, g, h) Cancer tissues with lymph node metastases showed high CAP1 and low E-cadherin expression (×200 and ×400). Details of the experiments are given in the Methods section.

PMSF, then centrifuged at 10 000*g* for 30 min to collect the supernatant. Proteins were mixed with two times loading and DTT (4:5:1), boiled in water for 5–10 min and cooled in ice. Protein concentrations were determined using a Bio-Rad protein assay (Bio-Rad, USA), and then proteins were resolved on 6% sodium dodecyl sulphate-polyacrylamide gel electrophoresis gels and transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% dried skim milk in Tris-buffered saline and Tween 20 (TBST, 2.42 g Tris, 8 g NaC–, 0.05% Tween 20). After 2 h at room temperature, the filters were washed by TBST for three times and then incubated overnight with an affinity purified polyclonal antibody against CAP1 (diluted 1:500; Santa Cruz Biotechnology), E-cadherin (diluted 1:1000; Santa Cruz Biotechnology), vimentin (diluted 1:1000; Abcam) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, diluted 1:1000; Sigma) at 4°C. Finally, the membrane was then incubated with an HRP-labeled secondary antibody for another 2 h at room temperature, and the blots were developed using enhanced chemiluminescence (ECL, Pierce Company, USA). ImageJ (National Institutes of Health) was used to compare the density of bands on western blot analysis.

Cell Cultures

Human esophageal cancer cell lines TE1 were purchased from China Academy of Science cell library and maintained in RPMI 1640 (GibCo BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine and 100 U/ml penicillin–streptomycin mixture (GibCo BRL) at 37°C and 5% CO_2.

Plasmid Constructs and Transfection

The full-length CAP1 (Genbank Accession No. NM_001105530.1) was isolated from the human complementary DNA (cDNA) library and connected to pcDNA3.1-myc. The primers used for CAP1 were as follows: 5′-CGGGATCCATGGCTGACATGCAACAC-3′ (sense) and 3′-GGGGTACCTCCAGCAATTTCTGTCC-5′ (anti-sense). The target sequences for the CAP1 gene were 5′-AGAACCGAGGCAGCAAGAA-3′, 5′-CCGAGGCAGCAAG AAGAAA-3′ and 5′-GAAATGAATGATGCCGCCA-3′. Transfection with the pcDNA3.1-myc vector, myc-CAP1, CAP1/RNAi and the non-specific vector was carried out using lipofectamine 2000 (Invitrogen) and plus reagent in OptiMEM (Invitrogen) as suggested by the manufacturer. Transfected cells were used for the subsequent experiments 48 h after transfection.

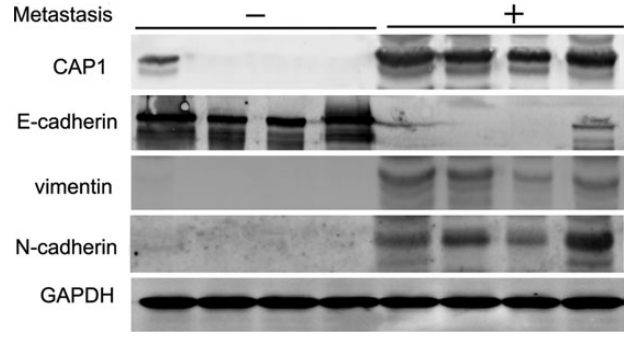

Figure 2. The expression of cyclase-associated protein 1 is high in esophageal squamous cell carcinoma (ESCC) with lymph node metastasis. CAP1, E-cadherin, N-cadherin and vimentin protein levels in human ESCC tissues ($n = 8$ patients) determined by western blot analysis. GAPDH was used as a control for protein load and integrity.

Wound-healing Assay

TE1 cells were transfected with either si-CAP1 or scrambled negative control. When the cell confluence reached ~80% at

Table 2. Survival status and clinicopathologic parameters in 98 ESCC specimens

	Total	Survival status		*P*	χ^2
		Dead, *n* (%)	Alive, *n* (%)		
Age (years)					
<60	37	16 (41.9)	21 (58.1)	0.419	0.171
≥60	61	29 (47.8)	32 (52.2)		
Gender					
Male	71	33 (47.4)	38 (52.6)	0.520	0.330
Female	27	12 (40.6)	15 (59.4)		
Tumor grade					
I	17	4 (20.0)	13 (80.0)	0.120	8.835
II	47	19 (39.2)	28 (60.8)		
III	34	22 (66.7)	12 (33.3)		
Lymph node metastasis					
Presence	68	38 (55.9)	30 (44.1)	0.045*	10.372
Absence	30	7 (23.3)	23 (76.7)		
Tumor size (cm)					
<5	75	33 (42.9)	42 (57.1)	0.326	0.474
≥5	23	12 (53.8)	11 (46.2)		
Tumor depth					
T1	11	4 (36.4)	7 (63.6)	0.852	0.789
T2	15	8 (47.6)	7 (42.3)		
T3	25	11 (40.7)	14 (59.3)		
T4	47	22 (49.0)	25 (51.0)		
CAP1					
Score≤4	52	13 (25.0)	39 (75.0)	0.007*	13.743
Score≥6	46	32 (69.6)	14 (30.4)		
E-cadherin					
Low expression	31	11 (35.5)	20 (64.5)	0.049*	3.507
High expression	67	34 (50.5)	33 (49.5)		

Statistical analyses were performed by Pearson's χ^2 test.
*$P < 0.05$ was considered significant.

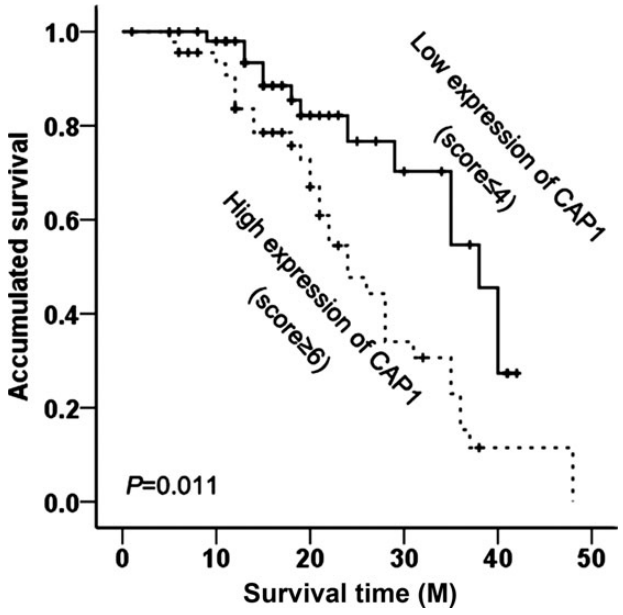

Figure 3. Correlation between cumulative survival with the expression of CAP1. Based on the score of CAP1, patients were divided into high CAP1 expression (score $\geq$6) and low CAP1 expression (score $\leq$4). Patients in the group of high expression of CAP1 had significantly shorter overall survival.

48 h post transfection, wounds were created in confluent cells using a 100 μl pipette tip. The cells were then rinsed with a medium to remove any free-floating cells and debris. The medium was then added, and culture plates were incubated at 37°C. Wound healing was observed at different time points within the scrape line, and representative scrape lines were photographed. Duplicate wells for each condition were examined, and each experiment was repeated three times.

Transwell Assay

For the migration assay, TE1 cells trypsinized and seeded in the medium without serum; then 1×10^5 cells plated into the upper chambers of cell culture inserts (24-well type, 8-mm pore size, Corning, NY, USA), which were placed in the medium containing 20% fetal bovine serum served as a chemoattractant. After 72 h of incubation, the cells attached to the upper side of the filter were mechanically removed, and the cells that had migrated to the undersurface of the membrane were fixed and stained with crystal violet. Digital images were obtained from the membranes, and three random fields were counted. The results were averaged in three independent experiments.

Immunofluorescence

Cells were fixed with 4% formaldehyde for 30 min and permeabilized with 0.1% TritonX-100/PBS for 5 min. Prior to incubation with the respective antibodies, the cells were blocked with PBS containing 3% normal goat serum for 1 h. The cells were incubated at 4°C overnight with polyclonal anti-CAP1 antibodies (diluted 1:100; Santa Cruz Biotechnology), E-cadherin (diluted 1:100; Santa Cruz Biotechnology) and vimentin (diluted 1:100; Abcam). After being washed with PBS, the cells were incubated with AlexFluor-conjugated secondary antibodies (Molecular Probe, Inc.), counterstained with DAPI [one also with filamentous actin (F-actin)] and observed with a fluorescence microscope (Leica CTR 5000).

Table 3. Contribution of various potential prognostic factors to survival by Cox regression analysis in 98 specimens

	Relative ratio	95% confidence interval	*P*
Age (years)	1.467	0.788–2.729	0.227
Gender	0.830	0.425–1.618	0.584
Tumor size	0.990	0.506–1.935	0.976
Lymph node metastasis	1.624	1.047–3.037	0.046*
Pathologic grading	1.974	1.283–3.039	0.012*
Tumor depth	1.024	0.761–1.376	0.878
CAP1	2.384	1.254–4.531	0.008*
E-cadherin	2.031	1.050–4.023	0.049*

Statistical analyses were performed by the Cox test.
*$P < 0.05$ was considered significant.

Statistical Analysis

Statistical analysis was performed using the PASW statistics 18 software package. The association between CAP1 and E-cadherin expression and clinicopathologic features was analyzed using a χ^2 test. For analysis of survival data, Kaplan–Meier curves were constructed, and the log-rank test was performed. Multivariate analysis was performed using Cox's proportional hazards model, with $P < 0.05$ considered statistically significant. The results are expressed as the mean $\pm$ standard error (SE).

RESULTS

Clinical Significance of CAP1 Expression in ESCC

In the present study, we examined the expression and intracellular location of CAP1 in 98 specimens of ESCC by immunohistochemical analysis. The results of immunohistochemistry (IHC) are presented in Fig. 1 and summarized in Table 1. It was found that CAP1 immunoreactivity was localized to the membrane of tumor cells and was weakly expressed in ESCC tissues without lymph node metastases, whereas it was high in tissues with lymph node metastases. CAP1 expression was negatively associated with E-cadherin expression ($P < 0.001$) in all cases of ESCC analyzed. The relationship between clinicopathologic background and CAP1 expression is shown in Table 1. Increased expression of CAP1 exhibited a significant correlation with lymph node metastasis ($P = 0.001$), while

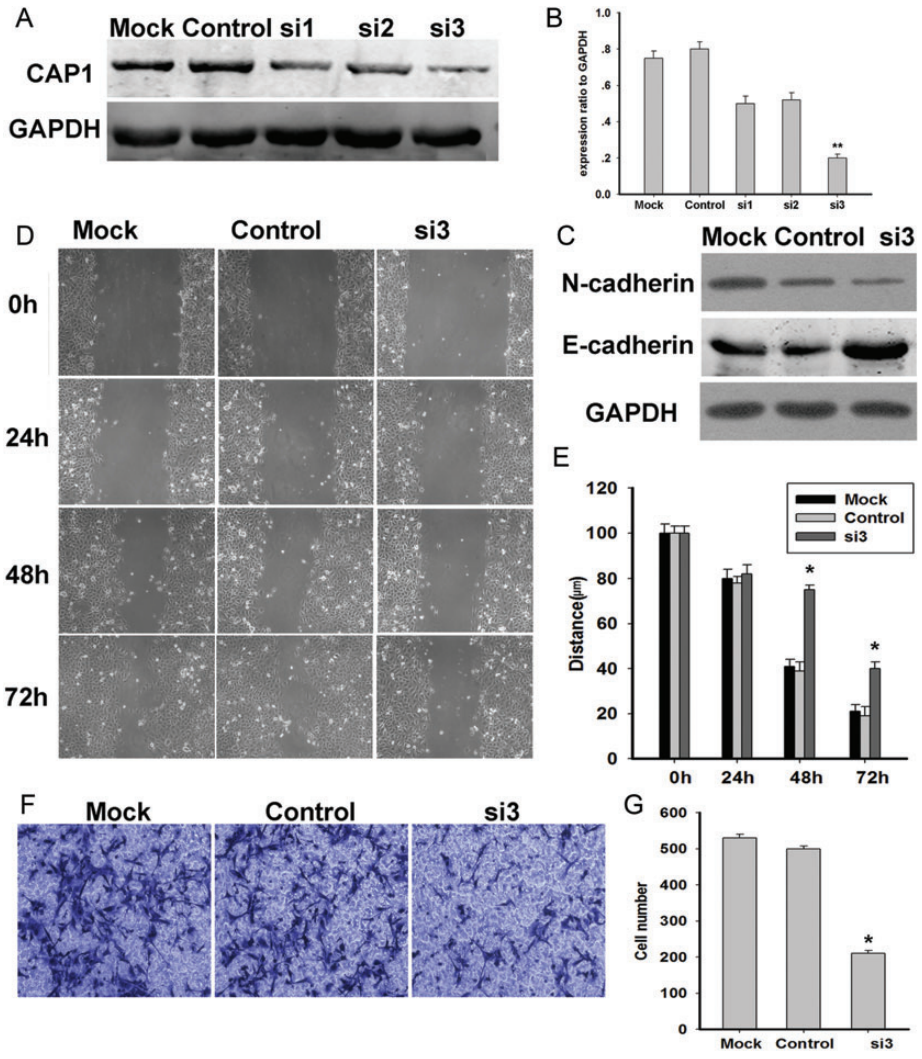

Figure 4. Inhibiting CAP1 expression decreased the migration of TE1 cells. (A) Western blot analysis of CAP1 in mock, control, si1, si2 and si3. (B) The bar chart demonstrates the ratio of CAP1 protein to GAPDH by densitometry. The data are expressed as mean ± SEM (**$P < 0.01$, compared with mock and control). (C) Western blot analysis of E-cadherin, N-cadherin and GAPDH in control. (D) Wound-healing assays with vector and si3 cells (×200 magnification). (E) The bar chart demonstrates the distance for each time point. The data are expressed as means ± SEM ($n = 3$, *$P < 0.01$, compared with mock and control). (F) Knockdown of CAP1 inhibited cell migration by transwell assays. CAP1/negative control (NC) cells showed a higher penetration rate through the membrane compared with CAP1/RNAi cells. (G) Number of cells that invaded through the member was counted in 10 fields under ×40 objective lens. Bars, SD. $n = 3$, *$P < 0.01$.

there were no relationships between CAP1 and age, gender, pathologic grading, tumor size and depth.

Expression of CAP1 in Human ESCC Tissues

To confirm the specificity of the immunohistochemical results, western blot analysis was used in eight ESCC tissues with or without lymph node metastases. And the expression of CAP1 was quantified by densitometry using GAPDH as a loading control. In these cases, CAP1 levels in ESCC tissues without lymph node metastases were significantly lower than those with lymph node metastases (Fig. 2). E-cadherin, N-cadherin and vimentin are the markers of epithelial cells and mesenchymal cells, respectively (16,17), and we examined these markers in the eight tissues. As shown in Fig. 2, expressions of N-cadherin and vimentin were high in the tissues with lymph node metastases. In contrast, the E-cadherin expression was lower in tissues with lymph node metastases than in those without lymph node metastases (Fig. 2).

Survival Analysis

At the end of clinical follow-up, survival information was available in the 98 cases. Of these 98 patients, only 14 of 46 (30.4%) patients in the group of high CAP1 expression were alive versus 39 of 52 (75.0%) in the group of low CAP1 expression (Table 2). When all cases were compared separately with survival status, only CAP1 ($P = 0.007$), E-cadherin ($P = 0.049$) and metastasis ($P = 0.045$) importantly influenced survival (Table 2). In univariate analysis, the Kaplan–Meier survival curves showed that high CAP1 expression resulted in a

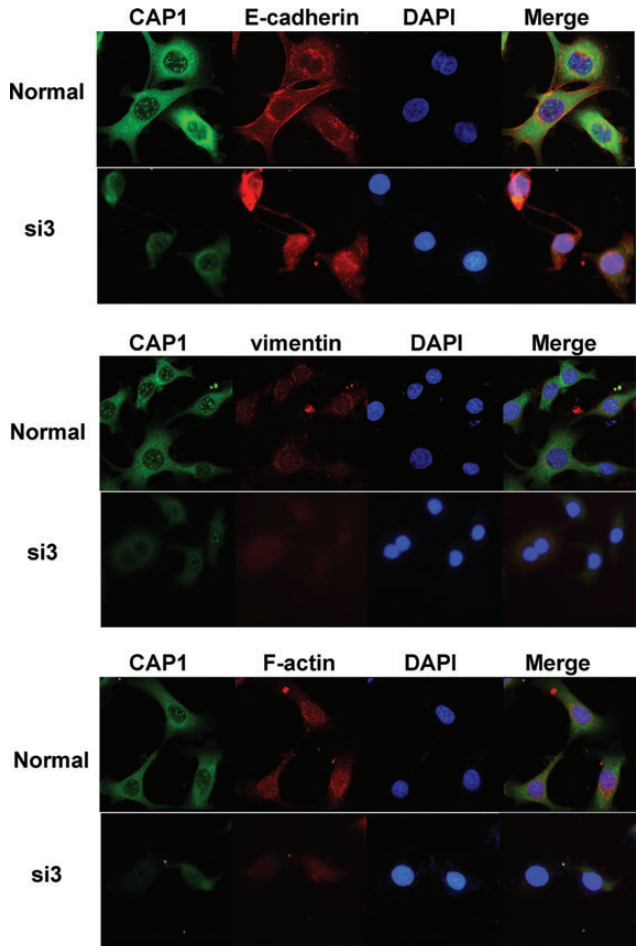

Figure 5. Cells with up-regulated CAP1 expresses epithelial markers and showed loss of mesenchymal markers and F-actin. CAP1/NC and CAP1/RNAi cells were fixed and processed for immunofluorescence with antibodies recognizing E-cadherin, vimentin and F-actin. The results came from three independent experiments.

poor survival with statistical significance (Fig. 3). When a multivariate Cox proportional hazard model was constructed (including age, grade, tumor size, lymph node metastasis, pathologic grading, tumor depth, CAP1 and E-cadherin expression), CAP1, E-cadherin expression, pathologic grading and lymph node metastases were independent prognostic factors in ESCC patients, and CAP1 was the strongest independent predictor of survival ($P = 0.008$, Table 3).

Knockdown of CAP1 Decreases TE1 Cells' Motility

Yamazaki et al. (12) showed that overexpression of CAP1 could affect the aggressive behavior of pancreatic cancers. Overexpression of an oncogene is crucial for the development of tumors as it can promote strong invasion of tumor cells. We chose TE1 cells as a model because TE1 had a high invasion capability among ESCC cell lines (18). To determine the impact of CAP1 expression on the growth of TE1 cells, we constructed a series of siRNA expression vectors (CAP1/RNAi) specific to CAP1 transcripts and transfected them into TE1 cells. The knockdown was confirmed by western blot analysis; CAP1/RNAi was effective compared with the negative control (CAP1/NC) and the normal TE1 cells (Fig. 4A). NIH was used to compare the density of bands on western blot analysis. Of these, the CAP1 siRNA3 reduced the level of CAP1 protein expression by >50% (Fig. 4B). The successful knockdown of the CAP1 gene in TE1 cells provided a useful tool for investigating the function of CAP1 in the growth of TE1 cells. We found that the downregulation of CAP1 caused an increase in the expression of epithelial marker E-cadherin and a decrease in the expression of N-cadherin (Fig. 4C).

Cell motility is indispensable for cancer metastasis (19). We examined the impact of CAP1 expression on the migration of TE1 cells by a wound-healing assay as shown in Fig. 4C. Following incubation of physically wounded cells for 72 h, control cells had traveled a much shorter distance than CAP1/RNAi cells (Fig. 4D and E). Meanwhile, the down expression of CAP1 inhibited cell migration to the bottom chambers compared with control by transwell assays (Fig. 4F and G). The wound-healing and transwell assays indicate that downregulation of CAP1 expression reduces the migration of TE1 cells.

To further address the above-mentioned hypothesis, a CAP1/RNAi was transfected into TE1 cells to examine the role of CAP1. The alteration of CAP1 was accompanied by the upregulation of epithelial marker E-cadherin and downregulation of F-actin and mesenchymal marker vimentin (Fig. 5). These data were confirmed with an immunofluorescence analysis using the same cell lines (Fig. 5).

High Expression of CAP1 Increases the Migration of TE1 Cells

To further confirm the physiological function of CAP1, we performed transient overexpression experiments using a CAP1 expression plasmid in TE1 cells. The results demonstrated that overexpression of CAP1 induced a remarkable increase in cell migration (Fig. 6C and D). Meanwhile, the protein levels of mesenchymal marker N-cadherin dramatically increased in the overexpression of CAP1 TE1 cells, whereas the epithelial marker E-cadherin down regulated (Fig. 6A and B).These gain- and loss-of-function experiments strongly suggested that CAP1 promoted the migration of TE1 cells.

DISCUSSION

ESCC is an aggressive neoplasm in Asia, especially in Southern China (20). Although morbidity and mortality rates have decreased in recent years, the prognosis of ESCC remains unsatisfactory. With the development of cellular biology of tumors and molecular biology, it has been found that the occurrence of esophageal carcinoma is a comprehensive pathologic process with multifunction, multistage and multigene variations (21). A deeper understanding of the molecular events associated with the ESCC is necessary.

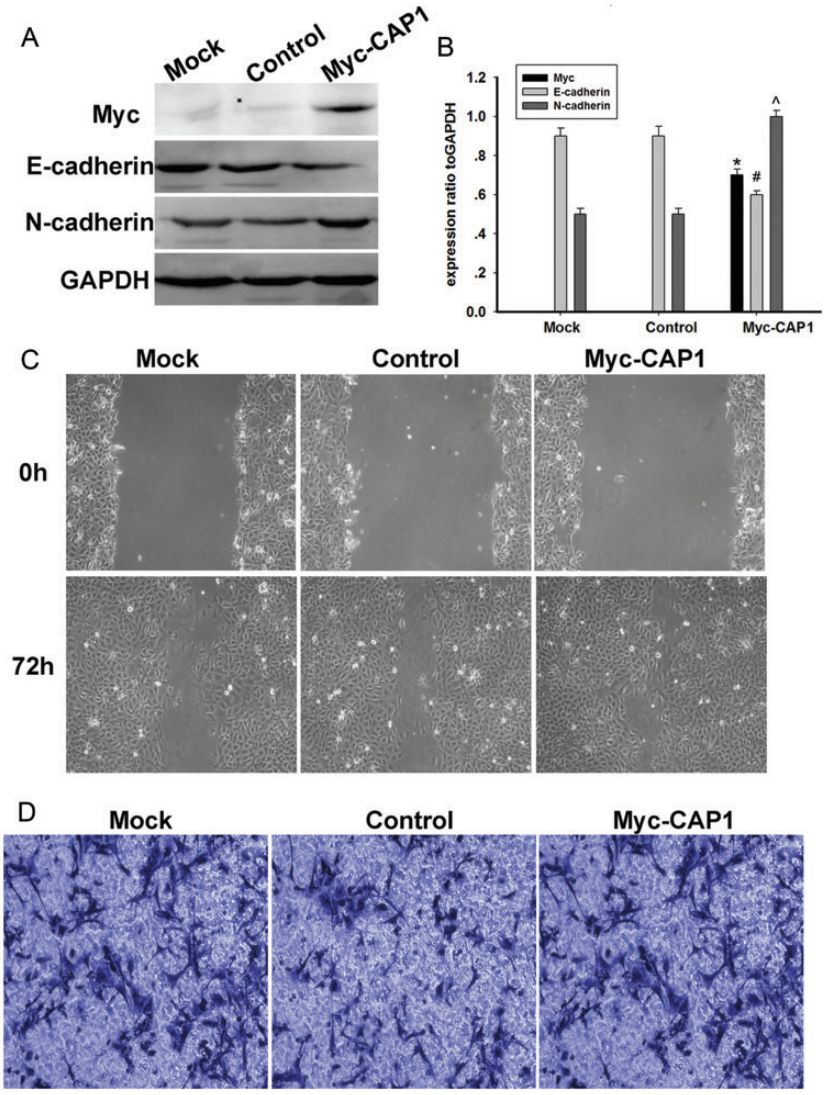

Figure 6. Exogenously expressed CAP1 protein increased TE1 cells' migration ability. (A) Western blot analysis of Myc, E-cadherin, N-cadherin and GAPDH in mock, control and Myc-CAP1 cells. (B) The bar chart demonstrates the ratio of Myc, E-cadherin and N-cadherin protein to GAPDH by densitometry. The data are expressed as mean ± SEM (*, #, ^, $P < 0.01$, compared with mock and control). (C) Wound-healing assays with mock, control and Myc-CAP1 cells (×200 magnification). (D) Overexpression of CAP1 increased cell migration by transwell assays. The results are the mean ± SEM of three independent experiments.

As we know, this is the first report that CAP1 protein is overexpressed in invasive ESCC. In the current study, we investigated CAP1 expression in a series of 98 ESCC tissues and TE1 cells. Upregulation of CAP1 protein level was detected in invasive ESCCs by western blot and IHC analysis. The correlation analysis of CAP1 with clinicopathologic samples demonstrated that upregulation of CAP1 was closely associated with lymph node metastasis ($P = 0.001$) and survival of ESCC patients ($P = 0.007$). It suggested that CAP1 might play an important role in ESCC development and progress. These results match with the phenomenon in pancreatic cancers. Otherwise, we demonstrate that E-cadherin expression is negatively correlated with CAP1, while N-cadherin and vimentin are positively correlated with CAP1 protein levels in ESCC. Immunohistochemistry results show that high expression of CAP1 is correlated with low E-cadherin, while low expression of CAP1 is correlated with high E-cadherin in the ESCC specimen. As a result, we suppose that CAP1 is involved in tumor migration.

Furthermore, to explore the mechanisms involved in the migration function of CAP1, we detected the expression of epithelial marker E-cadherin, mesenchymal marker vimentin and F-actin after knock down of CAP1 in TE1 cells. Using an immunofluorescence analysis, it can be found that down regulation of CAP1 increased the expression of E-cadherin and decreased the expression of vimentin and F-actin. More importantly, we performed both gain- and loss-of-function experiments to demonstrate that CAP1 promoted the migration of TE1 cells *in vitro* by wound-healing assays and transwell migration assays. This might reveal the oncogenic role of CAP1 in ESCC.

EMT is an evolutionarily conserved developmental process which is accompanied by the dissolution of cell–cell junctions and loss of apico-basolateral polarity, resulting in the formation of migratory mesenchymal cells with invasive properties (22,23). Thus, EMT is implicated in tumor progression and metastasis (24). E-cadherin is the main transmembrane adhesion molecule responsible for cell-to-cell interactions and tissue organization in epithelial cells. The basic mechanism involved in the progress of EMT is upregulation of the mesenchymal marker vimentin and downregulation of the epithelial marker E-cadherin (25). It is known that several important signaling molecules reside on the cytoskeleton, which is affected by external stress imparted by the EMT. Deformation of the nucleus can trigger the activation of certain genes (26). EMT is a key process in cancer progress and metastasis, requiring cooperation of the Ras signaling pathway in a multistep process (27). Noegel et al. (28) reported an interaction of CAP with adenylyl cyclase in dictyostelium and an influence on signaling pathways directly as well as through its function as a regulatory component of the cytoskeleton. CAP is involved in regulating the adenylyl cyclase activity in yeast under the control of Ras (29–31); however, its function is not apparent in mammals. Further studies are needed to elucidate the molecular function of CAP1 in the Ras signaling. Considering that expression levels of CAP1 in clinical specimens were associated with lymph node metastasis, it seems that CAP1 overexpression was involved in enhanced cell motility and promoted metastasis of ESCC. Our hypothesis is that CAP1 overexpression increases F-actin and then accumulates more N-cadherin, vimentin and less E-cadherin, which lead to EMT in ESCC.

Taken together, our data show that CAP1 is a novel oncogene in ESCC, and its expression upregulation is associated with lymph node metastasis and poor prognosis in ESCC. Further characterization of the tumor gene function and mechanism of CAP1 will not only facilitate our understanding of ESCC development and progress, but also provide novel therapeutic targets in ESCC treatment.

Conflict of interest statement

None declared.

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