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Rho/ROCK Pathway Regulates Migration and Invasion of Esophageal Squamous Cell Carcinoma by Regulating Caveolin-1

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ABCDEF 1 Zhaohui Liu*
BCDEF 1 Jing Yu*
BCDEF 2 Ruinuan Wu
BCDEF 1 Shengxin Tang
BCDEF 1 Xiaoman Cai
AG 1 Guanghua Guo
AG 1 Suzuan Chen

1 Department of Gastroenterology, The First Affiliated Hospital of Shantou University Medical College, Shantou, Guangdong, P.R. China
2 Department of Pathology, The First Affiliated Hospital of Shantou University Medical College, Shantou, Guangdong, P.R. China

* Zhaohui Liu and Jing Yu are co-first authors

Corresponding Authors: Guanghua Guo, e-mail: guoguanghuasd@sohu.com, Suzuan Chen, e-mail: chensuzuan@sohu.com
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Background: Esophageal squamous cell carcinoma (ESCC) is a common cancer with poor prognosis. Caveolin-1 (Cav1) and Rho/ROCK pathway play important roles in tumor metastasis, separately. However, less research was focused on the relationship between Cav1 and Rho/ROCK in ECSS metastasis. Therefore, we investigated the relationship between Cav1 and Rho/ROCK pathway in ESCC metastasis.

Material/Methods: Cav1 and phosphorylated Cav1 (PY14Cav1) were examined in ESCC and in adjacent and non-tumorous tissues from ESCC patients by immunohistochemistry (IHC). Small interfering RNA (siRNA) targeting Cav1 or Rho/ROCK inhibitor was used to treat EC109, Eca109, TE1, and TE13 cells. Western blotting (WB) was used to detect Cav1 and PY14Cav1 expression. The wound healing scratch test and transwell assays were used to assess migration and invasion.

Results: Cav1 and PY14Cav1 were gradually expressed at higher levels in ECSS than in adjacent and non-tumor tissues as ESCC stage and lymphatic metastasis increased, and this difference was significant ($P<0.05$). Cav1 was expressed at higher levels in TE1 and TE13 than in EC109 and Eca109, while PY14Cav1 was enhanced in TE1 and TE13 cells but not in EC109 and Eca109, and the difference was significant ($P<0.05$). TE1 and TE13 had significantly ($P<0.05$) stronger motility, migratory, and invasion abilities than EC109 and Eca109 cells. Silencing Cav1 decreased PY14Cav1 expression in TE1 and TE13 cells, as well as suppressing the migration and invasion of all ECSS cells, and these differences were significant ($P<0.05$). Suppressing the Rho/ROCK pathway obviously inhibited Cav1 and PY14Cav1 expressions, as well as significantly ($P<0.05$) decreasing migration and invasion of ESCC cells.

Conclusions: Cav1 and PY14Cav1 were positively correlated with ESCC lymphatic metastasis and cancer stages. Rho/ROCK pathway activation promoted ESCC metastasis by regulating Cav1.

MeSH Keywords: Caveolin 1 • Cell Migration Assays • Neoplasm Invasiveness • Neoplasms, Squamous Cell • Signal Transduction

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Background

Esophageal squamous cell carcinoma (ESCC) is the eighth most common cancer worldwide, and is the sixth most common cause of death from cancer worldwide [1]. Despite surgery with fast therapeutic effect, and adjuvant treatments, the prognosis of ESCC patients is still poor [2]. Since tumor metastasis is one of the main causes for poor prognosis in ESCC, it is imperative to investigate the molecular mechanisms of ESCC.

Many genes play important roles in ESCC metastasis, including Caveolin-1 (Cav1). Cav1 is one of the major components in caveolae membrane structure, and it regulates many biological functions by activating signal pathways, such as regulating cell migration and invasion [3–5]. Increasing evidence suggests that Cav1 is tumor suppressor or tumor promoter in different kinds of cancer [6]. Moreover, the 14th tyrosine in Cav1 is phosphorylated by Src, shown as PY14Cav1 [7], and then PY14Cav1 interacts with various proteins to promote cancer metastasis [8]. In esophageal cancer, Kato and Ando et al. reported that Cav1 expression is positively correlated with lymphatic and distant metastasis, but the molecular mechanism remains unclear [9,10]. However, PY14Cav1 expression in esophageal cancer has not been clearly identified.

The Rho family is critically correlated with cancer metastasis [11,12]. Rho-associated kinase (ROCK) has an essential role in tumor metastasis, and its activation is a potential marker of lymphatic metastasis [13]. According to a previous report [14], ROCK inhibitor reduces the migration of ESCC cells.

To verify the positive association of Cav1 expression level with lymphatic metastasis and distant metastasis in ESCC patients, as well as the role of Rho/ROCK signaling in promoting esophageal cancer metastasis, it is important to investigate whether Cav1 interacts with Rho/ROCK pathway in ESCC. In the present study, we investigated the role of Cav1 and its interaction with the Rho/ROCK pathway in ESCC to explore the potential molecular mechanisms of Cav1 in promoting ESCC metastasis.

Material and Methods

Patients

ESCC and adjacent and non-tumorous tissues were obtained from 60 patients hospitalized at the First Affiliated Hospital of Shantou University Medical College. All patients were diagnosed as being in stages I to III. Clinicopathologic data were collected and analyzed, including age, sex, tumor size, degree of differentiation, TNM stage, and lymphatic metastasis (positive or negative). All data were obtained from patient medical records. The study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Shantou University Medical College. Informed consent was obtained from each patient.

Reagents

Rabbit anti-Cav1 and rabbit anti-PY14Cav1 antibodies were purchased from Cell Signaling Technology, Inc. (CST, Shanghai, China). Mouse anti-PY14Cav1 antibody and Cav1 siRNA (sc-29241 and sc-44202) were purchased from Santa Cruz Biotechnology, Inc. (Shanghai, China). Mouse anti-β-actin antibody was purchased from Proteintech Group, Inc. (Wuhan, China). Goat anti-rabbit and goat anti-mouse secondary antibodies were purchased from Jackson ImmunoResearch Laboratories Inc. (Washington, USA). Rabbit anti-ROCK1 antibody, Y-27632, Tris base, and Tween-20 were obtained from Sigma-Aldrich Co. LLC. (Shanghai, China). Lipofectamine 2000, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), bovine serum albumin (BSA), protein ladder, 3,3'-diaminobenzidine (DAB), protein quant sample lysis kit, and BCA protein assay kit were obtained from Thermo Fisher Scientific, Inc. (Shanghai, China). Transwell system was obtained from Corning Incorporated (Shanghai, China). Matrigel was obtained from Becton, Dickinson and Company (Shanghai, China). Methanol, crystal violet, formalin-fixed solution, xylene, H_2O_2, HCl, and hematoxylin were obtained from Solarbio Life Sciences and Technology Co., Ltd. (Beijing, China). Polyvinylidene fluoride (PVDF) membrane and electrochemiluminescence (ECL) system were obtained from Merck Millipore Corporation (Darmstadt, Germany). Phosphate buffer solution (PBS) and sodium citrate buffer were obtained from Boster Biological Technology Co., Ltd. (Wuhan, China).

Detecting Cav1 and PY14Cav1 expressions in tissues

Immunohistochemistry (IHC) was used for detecting Cav1 and PY14Cav1 expressions in ESCC and in adjacent and non-tumorous tissues. The tissues were fixed in formalin-fixed solution and embedded with paraffin. Then, the tissues were cut into serial slices of 5 μm. After being dewaxed in xylene and hydrated in gradient alcohol, the sections were incubated with antigen retrieval solution (100 mM Tris buffer, pH 9.5) at 100°C for 20 min. Endogenous peroxidase activity was blocked with 30 mL/L H_2O_2 in methanol for 10 min at 25°C. The slides were subjected to antigen retrieval in 0.01 mM sodium citrate buffer for 10 min. Non-specific antibody binding sites were blocked with 4% PBS–BSA for 30 min. Primary anti-Cav1 antibody (1: 1000) was used to incubate at 4°C overnight. Mouse anti-rabbit secondary antibody (1: 500) was used to incubate at 37°C for 1 h. Finally, the slides were incubated with 0.02% DAB and 0.005% H_2O_2 in 0.05 mM Tris-HCl buffer, and the counterstaining was performed with hematoxylin. Negative control slides were incubated with PBS instead of the primary antibody. We observed and analyzed the slides under a microscope (Olympus Corporation, Japan).

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Cell culturing

TE1 and EC109 cell lines were provided by Doctor Hao Zhang (Cancer Hospital of Shantou University Medical College), and Eca109 and TE13 were provided by the Fourth Hospital of Hebei Medical University. All cells were cultured in DMEM containing 10% FBS and placed at 37°C in 5% CO_2 and saturated humidity.

Silencing Cav1

RNA interfering (RNAi) was used for silencing Cav1. Cav1 siRNA1 (sc-29241) and siRNA2 (sc-44202) were transfected into EC109, Eca109, TE1, and TE13 cells with lipofectamine 2000 according to the specifications. Transfecting with empty vector was taken as negative control (NC), while those without transfecting vectors were taken as blank control (BC). After transfecting for 26 h, the medium was discarded and the cells were used for the following experiments.

Suppressing Rho/ROCK pathway

Y-27632 was diluted in medium at the concentration of 10 uM and used to treat the 4 kinds of ESCC cells for 1 h at 37°C. Then, the treated cells were harvested for further experiments.

Western blotting (WB)

Cell lysates were prepared by using a protein quant sample lysis kit. The total proteins were quantified by BCA protein assay kit and then for sodium lauryl sulfonate-polyacrylamide gel electrophoresis (SDS-PAGE). We analyzed 30 μg of protein per sample with 12% separating gel and 5% stocking gel. Subsequently, proteins were transferred to a PVDF membrane and then blocked with 5% non-fat milk in PBST (PBS containing 0.05% Tween-20). Next, the membranes were incubated with rabbit anti-Cav1 (1: 4000), rabbit anti-PY14Cav1 (1: 4000), and mouse anti-β-actin (1: 10000) antibodies at 4°C overnight, separately. Goat anti-rabbit and goat anti-mouse antibodies were used to incubate according binding at 25°C for 1 h. The ECL system was used to detect protein expression.

Wound healing assay

After transfection for 14 h, the treated cells were cultured in 12-well plates at a density of 1.5×10^5 cells/ml until confluence reached 90%. A straight-line wound was made using a 20-ul pipette tip. We removed the cell debris and smoothed the edge of the straight-line wound, then we washed the cells with PBS and cultured them continuously in medium. The wound areas and migrated cells in each group were assessed after 12 h and 24 h.

Transwell assay

After transfection for 14 h, the treated cells were digested and resuspended with DMEM without FBS. We adjusted the concentration as 1×10^5 cells/ml and cultured the cells in the upper chamber of the Transwell system (8μm) with or without Matrigel. DMEM containing 5% FBS was added in the lower chamber. After 12 h (migration) or 24 h (invasion), we fixed the migrated or invasive cells with 4% methanol and cleaned the non-migrated or non-invasive cells on the upper surface of the chamber. We stained the cells with 0.25% crystal violet dissolved in 20% methanol, then observed and counted cells in 5 random 100× fields per well under a microscope.

Statistical analysis

All data are expressed as mean ± standard deviations (X ±SEM) and were analyzed using SPSS 19.0 software. All experiments were performed at least in triplicate. One-way ANOVA was used to analyze the differences among groups. LSD was performed for homogenous data and Dunnett's T3 was performed when data were heterogeneous. $P<0.05$ was considered a significant difference.

Results

Cav1 and PY14Cav1 were positively correlated with TNM stage and lymphatic metastasis of ESCC patients

Clinical characteristics of ESCC patients are shown in Table 1, and IHC results of Cav1 and PY14Cav1 expressions are shown in Figure 1A. According to the analysis, Cav1 or PY14Cav1 expressions were not associated with patient sex, age, tumor size, or degree of tumor differentiation. However, ESCC TNM stage and lymphatic metastasis were significantly ($P<0.05$) positively correlated with Cav1 and PY14Cav1 expressions. There were significantly more patients in stage III with positive Cav1 than those in stage I–II ($P=0.004$), and there were significantly ($P=0.002$) more subjects with positive PY14Cav1 in stage III than subjects in stage I–II. There were significantly ($P=0.025$) more patients with ESCC lymphatic metastasis than those without lymphatic metastasis in the ESCC patients with positive Cav1, and there were significantly ($P=0.008$) more ESCC patients with positive PY14Cav1.

Cav1 and PY14Cav1 expressions enhanced in ESCC tissues

Cav1 and PY14Cav1 expressions in ESCC and in adjacent and non-tumorous tissues are shown in Figure 1A. There was no obvious expression of Cav1 (Figure 1Ac) or PY14Cav1 (Figure 1 Af) in non-tumorous tissues, and there was weak Cav1 (Figure 1 Ab) and PY14Cav1 (Figure 1 Ae) expressions in the adjacent tissues.

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Table 1. Correlation of Cav1 and PY14Cav1 with clinical characteristics of esophageal squamous cell carcinoma patients.

	Total (n)	Cav1		*P* value	PY14Cav1		*P* value
		Positive (n/%)	Negative (n/%)		Positive (n/%)	Negative (n/%)	
N	60	53 (88.3)	7 (11.7)	–	25 (41.7)	35 (58.3)	–
Gender							
Male	47	42 (79.2)	5 (71.4)	0.476	20 (80.0)	27 (77.1)	0.525
Female	13	11 (20.8)	2 (28.6)		5 (20.0)	8 (22.9)	
Age (years)							
≤50	11	10 (18.9)	1 (14.3)	0.621	5 (20.0)	6 (17.1)	0.517
<50	49	43 (81.1)	6 (85.7)		20 (80.0)	29 (82.9)	
Maximum diameter of tumor size (cm)							
≤5	42	37 (69.8)	5 (71.4)	0.651	17 (68.0)	26 (74.2)	0.469
>5	18	16 (30.2)	2 (28.6)		8 (32.0)	10 (28.6)	
Degree of tumor differentiation							
Low	42	37 (69.8)	5 (71.4)	0.651	17 (68.0)	26 (74.2)	0.469
Moderate of high	18	16 (30.2)	2 (28.6)		8 (32.0)	10 (28.6)	
TNM stage							
I–II	29	22 (41.5)	7 (100.0)	0.004	6 (24.0)	23 (79.3)	0.002
III	31	31 (58.5)	0 (0.0)		19 (76.0)	12 (34.4)	
Lymphatic metastasis							
Positive	48	45 (84.9)	3 (42.8)	0.025	24 (96.0)	24 (68.6)	0.008
Negative	12	8 (15.1)	4 (57.2)		1 (4.0)	1 (31.4)	

On the contrary, both Cav1 (Figure 1 Aa) and PY14Cav1 (Figure 1 Ad) expressions were obviously enhanced in ESCC tissues.

Motility, migration, and invasion were enhanced in ESCC cells with higher Cav1 and PY14Cav1 expressions

As shown in Figure 1B, Cav1 expression in EC109 and Eca109 cells was significantly (P<0.05) lower than that in TE1 and TE13 cells, and it was lower in EC109 cells than in Eca109 cells. PY14Cav1 expression was detected in TE1 and TE13 cells, but expression was not obvious in EC109 and Eca109 cells, and that in TE13 cells was significantly (P<0.05) higher than that in TE1 cells.

The results of wound healing assay, including the 4 kinds of migrated cells after 0, 12, and 24 h, are shown in Table 2 and Figure 1C, respectively. In each kind of ESCC cell, wound closure gradually but significantly (P<0.05) increased over time. After 12 h (F=1629.120, P<0.05) and 24 h (F=3306.187, P<0.05), the cell motility among the 4 kinds of ESCC cells were significantly different. After 12 h, wound closure in TE1 and TE13 cells was significantly (P<0.05) greater than that in EC109 and Eca109 cells, and that in TE13 cells was significantly (P<0.05) greater than that in TE1 cells. After 24 h, wound closure in EC109, TE1, and TE13 cells was significantly (P<0.05) greater than in Eca109 cells, and wound closure in TE1 and TE13 cells was significantly (P<0.05) greater than that in EC109 cells.

The results of the transwell assay were shown in Table 2 and Figure 1D. The migration (F=192.701, P<0.05) ability of the 4 kinds of ESCC cells was significantly (F=3.406, P=0.074) different, but the invasion ability was not. The migration ability of TE1 and TE13 cells was significantly (P<0.05) stronger than that of EC109 and Eca109 cells, and the migration ability of TE13 cells was significantly (P<0.05) stronger than that of TE1 cells. However, only the invasion ability of TE13 cells was significantly (P<0.05) stronger than that of EC109.

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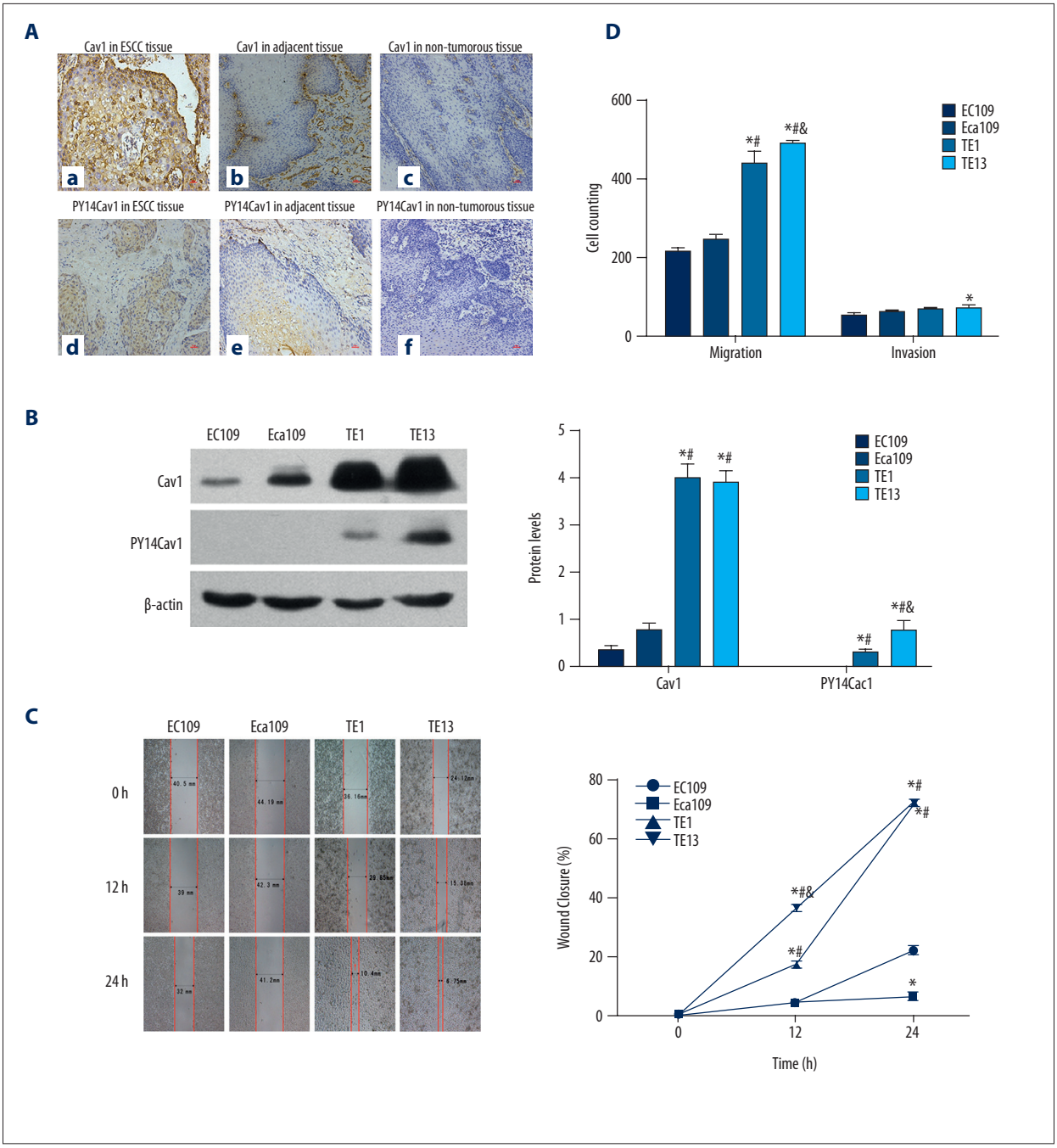

Figure 1. Cav1 and PY14Cav1 expression and esophageal squamous cell carcinoma (ESCC) cell motility, migration, and invasion. (**Aa**) Cav1 in ESCC; (**Ab**) Cav1 in adjacent tissue; (**Ac**) Cav1 in non-tumorous tissue; (**Ad**) PY14Cav1 in ESCC; (**Ae**) PY14Cav1 in adjacent tissue; (**Af**) PY14Cav1 in non-tumorous tissue; (**B**) Cav1 and PY14Cav1 in EC109, Eca109, TE1 and TE13 cells; (**C**) Wound closure of EC109, Eca109, TE1 and TE13 cells; (**D**) Migration and invasion of EC109, Eca109, TE1 and TE13 cells (* compared to EC109, $P<0.05$; # compared to Eca109, $P<0.05$; & compared to TE1, $P<0.05$).

Silencing Cav1 suppressed ESCC migration and invasion

The results of silencing Cav1 are shown in Figure 2A–2D. Compared to NC and BC groups, after silencing, Cav1 levels in EC109 (Figure 2A), Eca109 (Figure 2B), TE1 (Figure 2C), and TE13 (Figure 2D) cells were decreased, while the Cav1 level in NC of EC109 and TE13 cells was significantly ($P<0.05$) lower than in the BC group. In TE1 and TE13 cells, PY14Cav1 levels were significantly ($P<0.05$) reduced compared to the NC and BC groups after silencing Cav1.

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Table 2. Motility, migration and invasion of EC109, Eca109, TE1 and TE13 cells (X±SEM).

	Motility			*F* value	*P* value	Migration	Invasion
	0 h	12 h	24 h				
EC109	0.00±0.00	3.72±0.21	21.93±1.08	1031.177	<0.001	209.00±9.54	46.33±8.50
Eca109	0.00±0.00	4.32±0.14	6.28±0.45*	417.166	<0.001	239.00±14.42	52.33±8.14
TE1	0.00±0.00	17.50±0.87*#	71.54±1.16*#	5928.506	<0.001	436.00±27.62*#	59.33±8.62
TE13	0.00±0.00	36.36±0.95*#&	71.90±1.21*#	4941.364	<0.001	484.33±11.37*#&	66.00±6.56*
F value	–	1629.120	<0.001	<0.001		192.701	3.406
P value	–	3306.187	<0.001			0.074	

* Comparing to EC109, $P<0.05$; # comparing to Eca109, $P<0.05$; & comparing to TE1, $P<0.05$.

Table 3 and Figure 2E show that with siRNA1 ($F=190.062$) and siRNA2 ($F=74.086$) treatments, the migration ability of ESCC cells was significantly different ($P<0.05$). After silencing Cav1, the number of migrating EC109, Eca109, TE1, and TE13 cells decreased significantly ($P<0.05$) compared to the NC and BC groups. Before and after silencing Cav1, there were significantly ($P<0.05$) fewer migrating EC109 and Eca109 cells than migrating TE1 and TE13 cells, and fewer migrating TE1 cells than migrating TE13 cells.

As shown in Table 3 and Figure 2F, with siRNA1 ($F=10.847$) and siRNA2 ($F=6.811$) treatments, the invasive ability of ESCC cells was significantly different ($P<0.05$). After silencing Cav1, the numbers of invasive EC109, Eca109, TE1, and TE13 cells decreased significantly ($P<0.05$) compared to NC and BC groups. Before and after silencing Cav1, there were significantly ($P<0.05$) fewer invasive EC109 and Eca109 cells than TE13 cells. However, there were more invasive TE1 cells than invasive EC109 cells in all groups except for the BC group, and there were significantly ($P<0.05$) more invasive TE1 cells than invasive Eca109 cells only in the NC group.

Suppressing Rho/ROCK pathway inhibits ESCC migration and invasion by targeting Cav1 and PY14Cav1

The results of suppressing the Rho/ROCK pathway are shown in Figure 3A–3D. Compared to the control group, after suppressing the Rho/ROCK pathway with Y-27632 (10 μM), Cav1 expression in EC109 (Figure 3A), Eca109 (Figure 3B), TE1 (Figure 3C), and TE13 (Figure 3-D) cells were all significantly ($P<0.05$) inhibited, as was PY14Cav1 expression in TE1 (Figure 3C) and TE13 (Figure 3D) cells.

As shown in Table 4 and Figure 3E–3F, compared to the control group, the migration and invasion ability of EC109, Eca109, TE1, and TE13 cells all were significantly ($P<0.05$) decreased after suppressing the Rho/ROCK pathway with Y-27632 (10 μM). Additionally, without suppressing the Rho/ROCK pathway, the migration ability of EC109, Eca109, TE1, and TE13 cells increased gradually and significantly ($F=398.628$, $P<0.05$), and a similar tendency was found in the invasion ability of EC109, Eca109, TE1, and TE13 cells ($F=11.191$, $P<0.05$). With suppression of the Rho/ROCK pathway, the migration ability of TE1 and TE13 was greater than the invasion ability of EC109 and Eca109 cells, and the migration ability of TE13 was significantly ($F=79.683$, $P<0.05$) greater than the migration ability of TE1. The invasion ability of TE1 and TE13 cells was significantly ($F=48.125$, $P<0.05$) greater than the invasive ability of EC109 and Eca109 cells, and the invasive ability of Eca109 cells was greater than the invasive ability of EC109 cells.

Discussion

In the present study, on one hand, we collected the clinical characteristics and tumor tissues of ESCC patients to study the correlation between Cav1 and ESCC. By statistical analysis and IHC, we found that Cav1 and PY14Cav1 are positively correlated with ESCC lymphatic metastasis and cancer stages. On the other hand, based on cellular and molecular experiments, we confirmed that higher Cav1 expression promotes ESCC cells metastasis. Additionally, by suppressing Rho/ROCK pathway activity, we found that Cav1 expression is inhibited, as well as suppressing ESCC cells metastasis, suggesting that Rho/ROCK pathway activation promotes ESCC metastasis by enhancing Cav1.

Cav1 is a multifunctional protein, which contributes to cancer progression and metastasis, positively or negatively [15,16]. According to Isik et al. [17,18], Cav1 is associated with poor prognosis with some tumors. In cutaneous squamous cell carcinoma and lung cancer, Cav1 was reported to be a tumor suppressor through inhibiting cell proliferation and/or metastasis [19,20]. According to previous reports, Cav1 is expressed at higher levels in different kinds of tumor [21–23]. Significant correlations between Cav1 and lymphatic metastasis, vein

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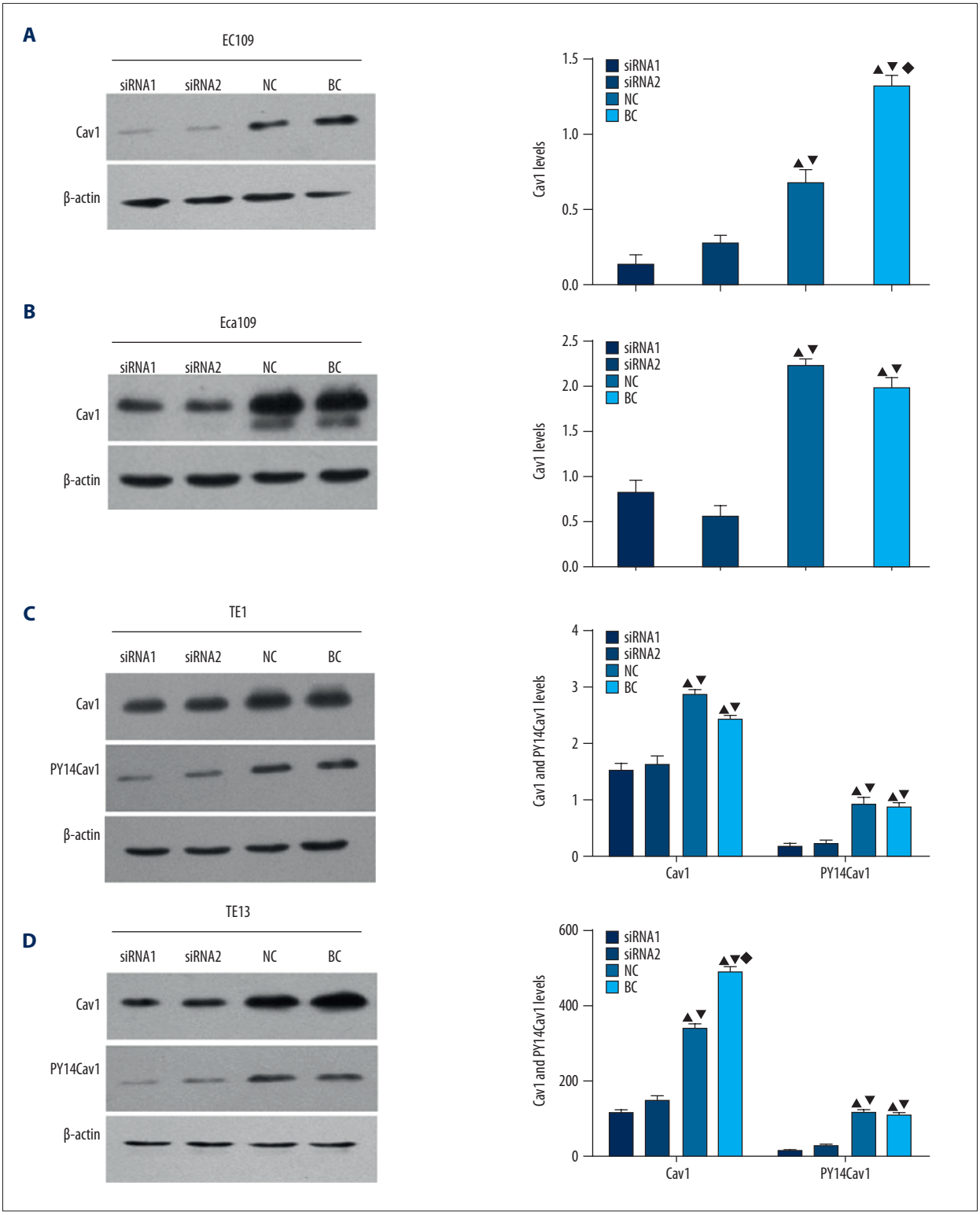

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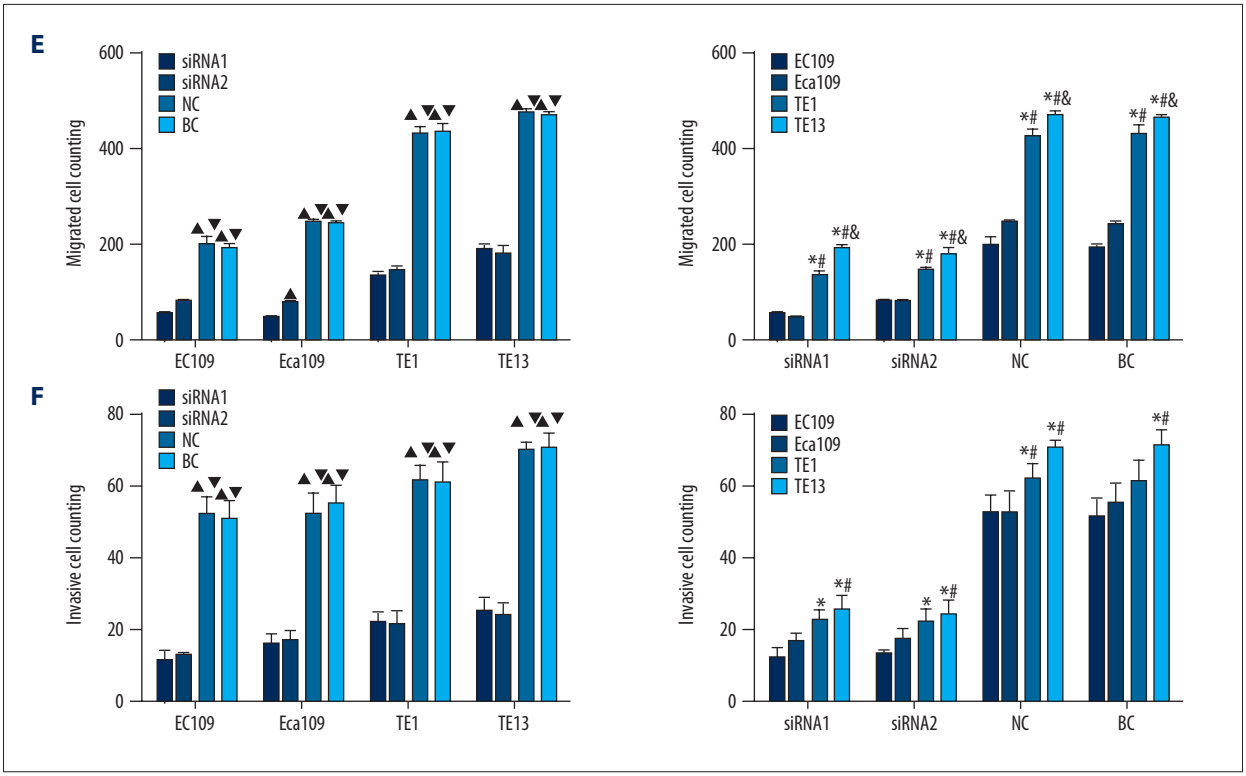

Figure 2. Silencing Cav1 suppressed ESCC migration and invasion. (**A**) Cav1 in EC109 cell; (**B**) Cav1 in Eca109 cell; (**C**) Cav1 and PY14Cav1 in TE1 cell; (**D**) Cav1 and PY14Cav1 in TE13 cell; (**E**) Migrated EC109, Eca109, TE1 and TE13; (**F**) Invasive EC109, Eca109, TE1 and TE13 (NC – negative control; BC – blank control; * comparing to EC109, $P<0.05$; # compared to Eca109, $P<0.05$; & compared to TE1, $P<0.05$; ▲ compared to siRNA1, $P<0.05$; ▼ compared to siRNA2, $P<0.05$).

Table 3. Migration and invasion of EC109, Eca109, TE1 and TE13 cells with or without silencing Cav1 (X±SEM).

	siRNA1	siRNA2	NC	BC	*F* value	*P* value
Migration						
EC109	55.00±6.25	81.67±3.78	197.33±19.22▲▼	192.67±9.50▲▼	61.642	<0.001
Eca109	47.67±3.06	78.33±5.69▲	244.67±7.09*▲▼	241.33±8.50*▲▼	800.222	<0.001
TE1	134.00±10.82*#	144.67±9.07*#	425.00±17.44*#▲▼	430.00±19.92*#▲▼	369.238	<0.001
TE13	188.67±11.02*#&	180.00±16.46*#&	469.00±10.54*#&▲▼	464.33±8.39*#&▲▼	556.146	<0.001
F value	190.062	74.086	254.781	186.753		
P value	<0.001	<0.001	<0.001	<0.001		
Invasion						
EC109	11.67±3.06	13.00±1.00	52.33±5.03▲▼	51.00±5.57▲▼	92.933	<0.001
Eca109	16.33±2.52	17.00±3.00	52.33±6.11▲▼	55.00±5.57▲▼	65.631	<0.001
TE1	22.33±3.06*	21.67±4.04*	61.67±4.51*#▲▼	61.00±6.00▲▼	75.491	<0.001
TE13	25.33±4.04*#	24.00±4.00*#	70.33±2.52*#▲▼	71.00±4.58*#▲▼	141.929	<0.001
F value	10.847	6.811	10.045	7.630		
P value	0.003	0.014	0.004	0.010		

NC – negative control; BC – blank control; * comparing to EC109, $P<0.05$; # comparing to Eca109, $P<0.05$; & comparing to TE1, $P<0.05$; ▲ comparing to siRNA1, $P<0.05$; ▼ comparing to siRNA2, $P<0.05$.

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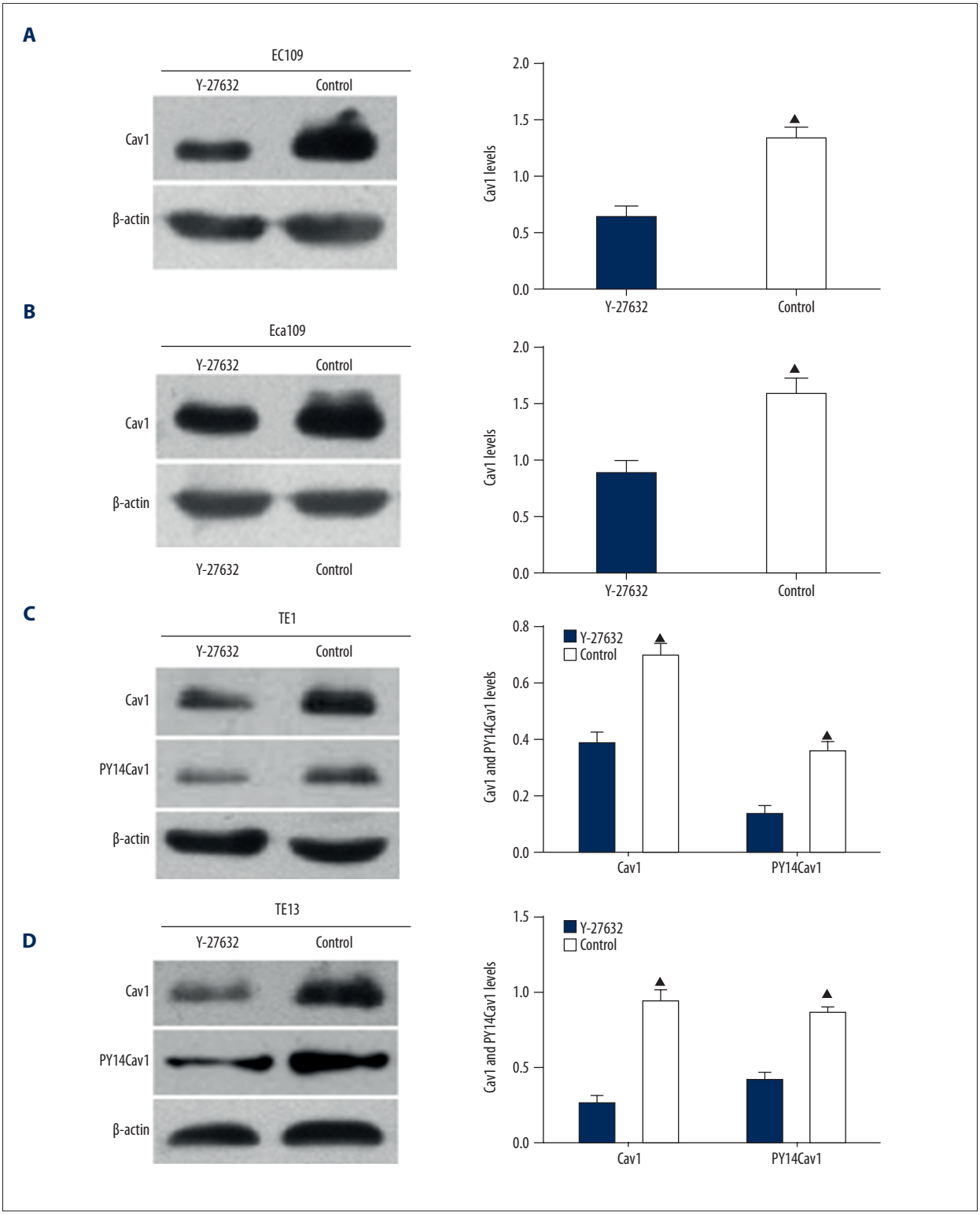

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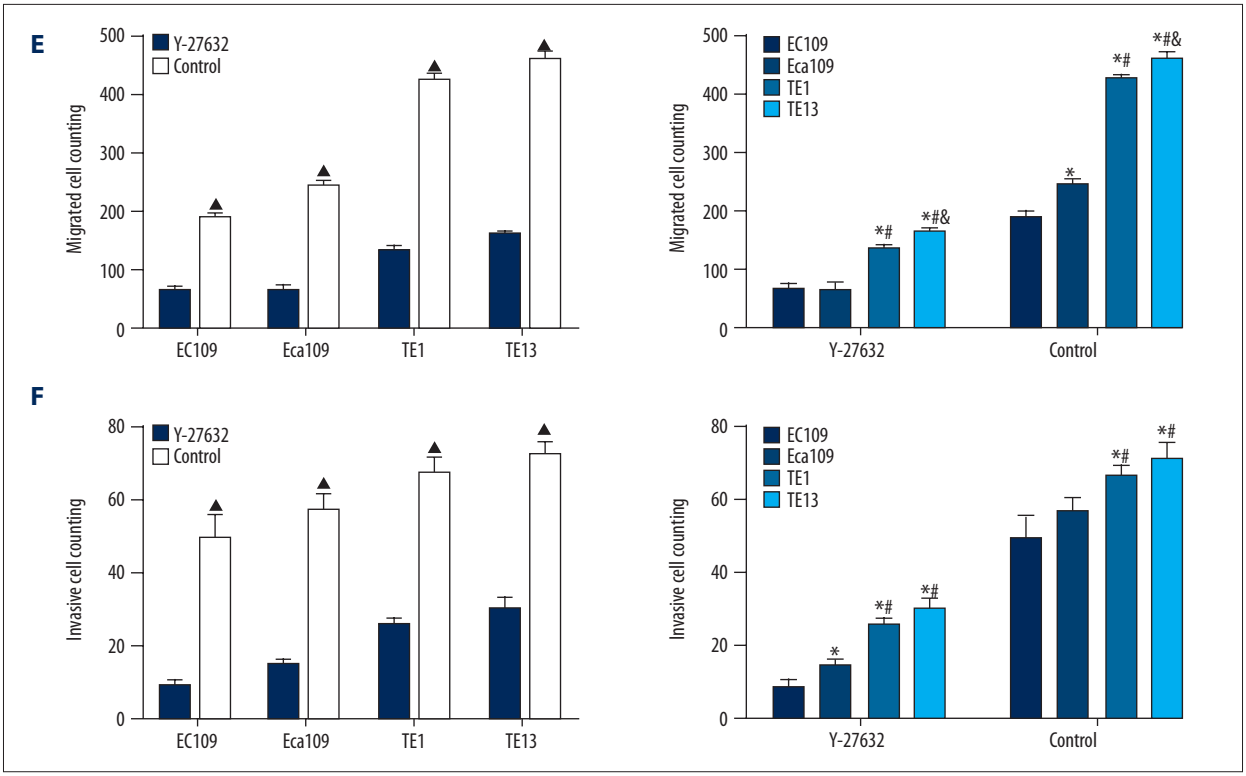

Figure 3. Suppressing the Rho/ROCK pathway inhibited ESCC migration and invasion by targeting Cav1 and PY14Cav1. (**A**) Cav1 in EC109 cell; (**B**) Cav1 in Eca109 cell; (**C**) Cav1 and PY14Cav1 in TE1 cell; (**D**) Cav1 and PY14Cav1 in TE13 cell; (**E**) Migrated EC109, Eca109, TE1 and TE13; (**F**) Invasive EC109, Eca109, TE1 and TE13 (Con. – control group; * compared to EC109, $P<0.05$; # comparing to Eca109, $P<0.05$; & compared to TE1, $P<0.05$; ▲ compared to Y-27632, $P<0.05$).

Table 4. Migration and invasion of EC109, Eca109, TE1 and TE13 cells with or without suppressing Rho/ROCK pathway (X±SEM).

	Y-27632 (10μM)	Control	*F* value	*P* value
Migration				
EC109	66.33±9.45	192.33±9.07▲	277.445	<0.001
Eca109	67.00±13.53	245.33±12.01*▲	291.471	<0.001
TE1	135.33±8.33*#	426.67±10.60*#▲	1401.607	<0.001
TE13	165.33±5.59*#&	461.00±13.75*#&▲	669.798	<0.001
F value	79.683	398.628		
P value	<0.001	<0.001		
Invasion				
EC109	8.67±2.01	49.33±6.51▲	106.314	<0.001
Eca109	14.67±1.53*	57.00±4.36▲	252.016	<0.001
TE1	25.67±2.08*#	66.67±4.51*#▲	204.446	<0.001
TE13	30.00±3.51*#	71.33±4.73*#▲	145.057	<0.001
F value	48.125	11.191		
P value	<0.001	0.003		

* Comparing to EC109, $P<0.05$; # comparing to Eca109, $P<0.05$; & comparing to TE1, $P<0.05$; ▲ comparing to Y-27632, $P<0.05$.

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invasion, and tumor local recurrence after radical surgery were confirmed, such as in pancreatic cancer, bladder cancer, and gastric cancer [24–26]. In ESCC, Cav1 is associated with poor prognosis and survival rate [9,10]. However, the molecular mechanisms of these associations are still unclear.

In the present study, we firstly observed that Cav1 and PY14Cav1 were over-expressed in ESCC tissues compared to the adjacent and non-tumorous tissues. By analyzing the correlation with clinical characteristics, we confirmed that Cav1 and PY14Cav1 are significantly positively correlated with lymphatic metastasis and cancer stage in ESCC patients. These results agree with previous studies [10,27].

According to Kentaro et al. [27], over-expression of Cav1 is positively correlated with lymph node metastasis and pathologic stage. Ando et al. [10] found that Cav1 over-expression is a potentially useful prognostic marker of ESCC. To investigate the molecular mechanisms in the present study, we used 4 kinds of ESCC cells and detected Cav1 and PY14Cav1 expressions. Cav1 and PY14Cav1 expression was higher in TE1 and TE13 cells, which had stronger metastatic ability, while lower Cav1 expression and no PY14Cav1 expression were detected in EC109 and Eca109, which had weaker metastatic ability. Silencing Cav1 expression in ESCC cells not only decreased Cav1 and PY14Cav1 levels, but also suppressed ESCC cell migration and invasion. Therefore, we suggest Cav1 may be a biomarker, as well as a promoter, in ESCC metastasis, which is in agreement with previous studies.

Interestingly, Jia et al. [28] found that the down-regulation of stromal Cav1 expression resulted in the high malignant potential in ESCC, indicating that Cav1 may be an effective prognostic marker for ESCC. The negative correlation between Cav1 in the tumor and Cav1 in the stroma was identified in prostate cancer [29,30] and breast cancer [31]. This suggests that the function of stromal Cav1 is to protect the body from invasion by malignant tumors. In addition, Meltzer et al. [9] showed that hypermethylation of the Cav1 promoter led to gene silencing, which is common in human early esophageal cancer during Barrett's-associated EAC, but this is inconsistent with a previous study [10], as well with as our research. This disagreement may be due the converse role of Cav1 in different cancer stages of ESCC. Shatz et al. [32] showed that Cav1 expression inhibits tumor growth during the early stage of cancer progression, while promoting tumor invasion and metastasis during the later stages.

Evidence of the interactions between Cav1 and Rho GTPases in cancers has been reported in multiple studies [33,34]. Cav1 influences the activation of Rho GTPases, which regulate cell polarity and directional migration [35]. Similarly, Shibu Thomas et al. [26] reported that decreased levels of Cav1 led to decreased activity of RhoA and RhoC, and treatment with a ROCK inhibitor reduced tumor metastasis *in vivo*.

In this study, by using Rho/ROCK inhibitor, we found that Cav1 was downstream of Rho/ROCK signaling, and the metastasis of ESCC cells were also suppressed. Previous reports showed that the Rho family contributes to cancer metastasis [36–38], and our study yielded similar results. Thus, we suggest that the function of Cav1, as an effector of Rho/ROCK signaling, is to promote tumor progression and metastasis, which might be related to its association with poor prognosis in ESCC.

Our study has certain limitations. Firstly, there were only 60 samples in this study, and further investigations with larger sample sizes are needed. Secondly, there was no healthy control group in this study; therefore, the healthy level of related proteins is unknown. Thirdly, we did not detect the activity of Rho/ROCK pathway after silencing Cav1, so their co-interaction is still unclear.

Conclusions

This study is the first to investigate the effect of Cav1 on promoting migration and invasion of ESCC cells, focusing on the relationship with the Rho/ROCK pathway. Cav1 and PY14Cav1 were significantly associated with lymphatic metastasis and cancer stage. Cav1 down-regulation inhibited migration and invasion of ESCC cells. Suppressing the Rho/ROCK signaling pathway down-regulated Cav1 expression and inhibited ESCC migration and invasion. Our results show the association between Cav1 and poor prognosis of ESCC with molecular mechanisms.

Conflict of interest

None.

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