Duchesnea indica extract suppresses the migration of human lung adenocarcinoma cells by inhibiting epithelial–mesenchymal transition

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Abstract
Epithelial–mesenchymal transition (EMT) is a process through which epithelial cells are transformed into mesenchymal cells; EMT diminishes cell polarity and cell–cell adhesion in cancer cells, leading to enhanced migratory and invasive properties. In this experiment, zymography, cell invasion, and migration assays were performed. Results indicated that Duchesnea indica extracts (DIE) inhibited highly metastatic A549 and H1299 cells by reducing the secretions of matrix metalloproteinase-2 and urokinase-type plasminogen activator. Cell adhesion assay also demonstrated that DIE reduced the cell adhesion properties. Western blot analysis showed that DIE down-regulated the expression of N-cadherin, fibronectin, and vimentin, which are mesenchymal markers, and enhanced that of E-cadherin, which is an epithelial marker. In vivo study showed that tumor growth was significantly reduced in BALB/c nude mouse xenograft model administered with oral gavage of DIE. Therefore, DIE could be exhibits potential as a phytochemical-based platform for prevention and treatment of lung cancer.

KEYWORDS
Duchesnea indica, epithelial–mesenchymal transition, lung adenocarcinoma, matrix metalloproteinase, metastasis, urokinase-type plasminogen activator

1 | INTRODUCTION

Cancer chemoprevention uses natural or synthetic agents or their combination. This prevention strategy has been increasingly used in recent years.1–3 Duchesnea indica is a peculiar natural plant belonging to the Rosaceae family and has been traditionally used for medicinal purposes; D. indica is widely distributed in Europe, the America, Japan, and China, indicating the favorable environmental adaptability of this plant. Previous studies investigated herpes simplex virus-induced inflammatory injury on neurons in RAW264.7 cell line; results indicated that the ethanol extract of D. indica reduced apoptosis by inducing microglia4 and activating the anti-inflammatory mechanism.5 The methanol extract of D. indica provides protection against oxidative stresses in vitro and in vivo.6 D. indica contains compounds that can inhibit growth of cervical and ovarian cancer cells by inducing apoptosis and cell cycle arrest. However, the effect of D. indica on the metastasis of lung cancer has not been fully explored yet.

About 90% of cancer-related deaths are caused by transfer of cancer cells. This phenomenon, which is also known as cancer metastasis, occurs when cancer cells enter the systemic blood circulation, survive,
Extravasate, and grow back in distant organs.7 Cancer cells are medi-
ated by proteases to degrade the extracellular matrix (ECM). In particu-
lar, protease-like matrix metallo proteinases (MMPs) and urokinase-
type plasminogen activator (u-PA) play important roles in cancer metast-
asis. MMPs are serine proteinases that belong to a unique family of
zinc-binding endopeptidases and comprise metalloproteinase-9 (MMP-
9) and MMP-2. u-PA is also a serine proteinase associated with cancer metastasis.8 Therefore, MMPs and u-PA or their associated regulatory pathways could be the target of chemopreventive agents.9

Epithelial–mesenchymal transition (EMT) is a de-differentiation pro-
gram that converts adherent epithelial cells into individual migratory
cells; this phenomenon occurs during embryogenesis, tissue regener-
ation, and wound healing. In pathology, EMT is related to fibrosis, carci-
nogenesis, cancer metastasis, and drug resistance.10,11 EMT was found
to be correlated with lung cancer metastasis.12,13 E-Cadherin is an epi-
thelial molecule that regulates cell-to-cell contact, cell polarity, and epi-
thelial pattern. E-cadherin is considered a biomarker for poor prognosis
of lung cancer,14 considering that reduced E-cadherin expression is
positively correlated with tumor stage and grade.15 EMT enhances the
expression of mesenchymal markers, such as vimentin, N-cadherin, and
fibronectin but decreases that of epithelial markers, such as E-
cadherin.16

Thus far, the effect of D. indica on human lung cancer invasion and
EMT has not been investigated. This study aims to explore the effects
of D. indica extract (DIE) on human lung cancer cell lines A549 and
H1299 in terms of anti-metastatic and anti-EMT capability.

2 MATERIALS AND METHODS

2.1 Preparation of DIE

D. indica leaves were purchased from local herb stores in Taichung, Tai-
wan. Dried D. indica leaf peduncle extracts were prepared by conden-
sation, followed by lyophilization, using previously described
methods.17 Briefly, 100 g of air-dried D. indica leaves were boiled twice
with 500 mL of 50% ethanol at 50°C for 12 hours. The solvent was
removed, and the filtrate was lyophilized and stored at −20°C.

2.2 Cell culture and DIE treatment

Human lung adenocarcinoma cell line A549 and H1299 purchased
from ATCC (Manassas, VA). A549 and H1299 were cultured in DMEM
medium (Gibco, St. Louis, MO) supplemented with 10% fetal bovine
serum (FBS) (Gibco, St. Louis, MO), 100 μg/mL streptomycin (Sigma, St.
Louis, MO), and 100 U/mL penicillin (Sigma, St. Louis, MO). The cell
cultures were maintained at 37°C in a humidified atmosphere of 5%
CO2. The culture medium was added with DIE (25, 50, 75, and 100 μg/
ml) to achieve the indicated concentrations and incubated for 24 or 48
hours. DMSO (Sigma, St. Louis, MO)(final concentration 0.1%) without
DIE was used as blank reagent.

2.3 Determination of cell viability

Cell viability was determined using MTT (Sigma, St. Louis, MO) and col-
orimetric assay to evaluate the cytotoxicity of DIE.18 The cells were
seeded in 24-well plates (4 × 10⁴ cells/well) and treated with DIE (0,
25, 50, 75, and 100 μg/mL) at 37°C. After incubation for 24 and 48
hours, the medium was removed and the cells were washed with phosphate-buffered saline (PBS). The medium was changed, and the
cells were incubated with MTT reagent (final concentration 0.5 mg/mL)
for 4 hours. The viable cell number/dish was directly proportional to
the amount of formazan crystals; formazan was produced through sol-
ubilization with isopropanol (Sigma, St. Louis, MO) and measured with
a Hitachi U-1900 spectrophotometer (Hitachi, Tokyo, Japan) at
563 nm.

2.4 Determination of MMP-2 and u-PA by
zymography

The activity of MMP-2 on the conditioned medium was assessed
through gelatin-zymogram protease assays.19 Samples were prepared
with a standard SDS gel-loading buffer without β-mercaptoethanol and
were not boiled prior to loading. The prepared samples were subjected
to 8% SDS-PAGE (0.75 mm, containing 0.1% gelatin; Sigma, St. Louis,
MO). Electrophoresis was performed at 150 V in an OWL P-1 (Alpha
Multi services, Inc., Conroe, TX) apparatus for 3 hours. The gels were
washed twice with 100 mL of distilled water containing 2% Triton X-
100 (Sigma, St. Louis, MO) on a gyration shaker at room temperature
for 30 minutes to remove the SDS. The gel was then incubated in
50 mL of reaction buffer (40 mM Tris-HCl, pH 8.0 [USB Corp, Taipei,
Taiwan], 20 mM CaCl₂ [Sigma, St. Louis, MO], and 0.02% NaN₃ [Sigma,
St. Louis, MO]) at 37°C overnight. The gel was stained with Coomasie
brilliant blue R-250 (Sigma, St. Louis, MO) and de-stained with metha-
nol (Sigma, St. Louis, MO)/acetic acid (Sigma, St. Louis, MO)/water (50/
75/875, v/v/v). u-PA activity was evaluated using previously described
methods.19 The SDS-PAGE gels (8%) were added with 2% w/v casein
and 20 μg/mL plasminogen (Sigma, St. Louis, MO). Electrophoresis and
zymography analyses were then performed.

2.5 Wound healing migration assay

Wounds were introduced into the confluent monolayer of cells with
culture inserts (Ibidi, Am, Klopferspitz, Germany) to create a cleared
line. Each culture insert was loaded with 2 × 10⁴ cells. After 24 hours,
the medium was removed and replaced with DMEM (Gibco, St.
Louis, MO) containing 1% FBS (Gibco, St. Louis, MO). The culture
was then added with DIE (0, 25, 50, 75, and 100 μg/mL) and incubated at
37°C. Cell migration into the wound area was recorded at 0, 24, and
48 hours using a microscope CKX41 (Olympus, Shinjuku, Tokyo,
Japan).

2.6 Cell invasion and migration assays

A549 and H1299 cells were pre-treated with DIE (0, 25, 50, 75, and
100 μg/mL) for 48 hours. In the invasion assay, the cells were
FIGURE 1 Effects of DIE on cell viability and activities of MMP-2 and u-PA protein. A549 and H1299 cells were treated with DIE for (A) 24 hours and (B) 48 hours and then subjected to MTT assay for cell viability analysis. (C) Gelatin zymography analysis of the activity of MMP-2. (D) Casein zymography analysis of the activity of u-PA. Data represent the mean ± SD of at least three independent experiments (compared with control, * \(P < .05\); ** \(P < .01\); and *** \(P < .001\)).

FIGURE 2 Effects of DIE on cell migration, invasion, motility, adhesion, and spreading. (A) A549 and H1299 cells were subjected to cell migration analysis by wound-healing assay. The migration ability of A549 and H1299 cells was quantified relative to that of the control being 100% (without DIE for 24 h and 48 h). A549 and H1299 cells were pre-treated with DIE at indicated concentrations for 48 hours; equal number of cells was subjected to analyses of (B) invasion and (C) motility by Boyden chamber; (D) adhesion was also analyzed by cell adhesion assay. (E) Cell spread assay were photographed for morphological studies. Data represent the mean ± SD of at least three independent experiments (compared with control, * \(P < .05\); ** \(P < .01\); and *** \(P < .001\)). [Color figure can be viewed at wileyonlinelibrary.com]
harvested, seeded (1.5 × 10^4 cells/well) in serum-free medium, and incubated in a Boyden chamber (Neuro Probe, Cabin John, MD) at 37°C for 24 hours. Briefly, 10 μL of Matrigel (BD Biosciences, Bedford, MA) (25 mg/50 mL) was applied to polycarbonate membrane filters (Neuro Probe, Cabin John, MD), with a pore size of 8 mM; the bottom chamber of the apparatus contained the standard medium. After incubation, the filters were air dried in a laminar flow hood for 5 hours. The invaded cells were fixed with methanol and stained with Giemsa (Sigma, St. Louis, MO). Cell number was counted on a light microscope CKX41 (Olympus, Shinjuku, Tokyo, Japan). Migration assay was performed.

**FIGURE 3** Effects of DIE on EMT-related protein and inhibitory effect on TGF-β1-induced EMT. Western blot analysis of (A) FAK pathway and (B) cytoskeleton related protein, with β-actin as internal control, in A549 and H1299 cells after 48 hours of treatment with DIE. (C) Nuclear extracts were subjected to Western blot analysis with anti-c-Jun and c-Fos antibodies. A549 cells were treated with DIE for 48 hours and co-stimulated with TGF-β1; the cells were then subjected to (D) cell spread and (E) immunofluorescence analyses. [Color figure can be viewed at wileyonlinelibrary.com]
using similar methods; however, the cells were initially incubated for 8 hours and no Matrigel coating was used.20

2.7 | Cell adhesion assay

A549 and H1299 cells were pre-treated with DIE (0, 25, 50, 75, and 100 µg/mL) for 48 hours. The cells (1 × 10^5 cells/well) were plated in 24-well plates coated with 10 µg/mL type I collagen (Worthington Corp, NY). Non-adherent cells were removed by washing with PBS. After staining with 0.1% crystal violet, the fixed cells were lysed in 0.2% Triton X-100; absorbance was determined at 550 nm.21

2.8 | Cell spreading assay

A549 and H1299 cells were pre-treated with DIE (0 and 100 µg/mL) for 48 hours. The cells (2 × 10^5 cells/well) were plated in six-well plates coated with type I collagen (10 µg/mL). Cell morphology was photographed at 1, 3, and 5 hours.22

2.9 | Immunoblotting

Samples of cell lysates or nuclear fractions were separated by 12.5% SDS-PAGE and transferred onto nitrocellulose membrane (GE Healthcare, Taipei, Taiwan) using previously described methods.20 The blot was treated using standard procedures and probed with antibodies (c-Jun, c-Fos, total-paxillin, phospho-paxillin Ser 178, phospho-FAK Tyr397, phospho-paxillin Tyr118, E-cadherin, N-cadherin, vimentin, fibronectin, phospho-ERK1/2, and total-ERK1/2 [Cell Signaling, Danvers, MA]; β-actin, total-FAK, and C23 [Santa Cruz, CA] [1:1000 dilution, monoclonal]). Protein expression was detected by chemiluminescence with an Enhanced Chemiluminescence Plus Detection Kit (Amersham Life Sciences, Inc., Piscataway, NJ).

2.10 | Immunofluorescence assay

A549 cells (1 × 10^4 cells/well) were seeded in eight-well glass and treated with DIE (0 and 50 µg/mL) at 37°C and incubated for 48 hours. The cells were removed from the medium and fixed in 4% paraformaldehyde (Sigma, St., Louis, Mo) at room temperature for 12 minutes. The cells were then washed in PBS, blocked, and permeabilized in PBS containing 4% bovine serum albumin (Sigma, St., Louis, Mo) and 0.1% Triton X-100 at room temperature for 90 minutes. The slides were incubated overnight at 4°C with anti-vimentin antibodies, followed by incubation with TRITC-conjugated anti-rabbit Ig. Nuclei were counterstained with 4',6-Diamidino-2-phenylindole (DAPI) (Sigma, St., Louis, Mo) at room temperature for 1 hours. The cells were viewed and photographed under upright fluorescence microscope Axioskop 2 Plus (Carl Zeiss AG, Oberkochen, Germany).

2.11 | Bioluminescence imaging of tumor growth in nude mice

All procedures involving animals were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) of the
institutional animal welfare guidelines of the Chung Shan Medical University (IACUC Approval Number: 822). Immunodeficient nude mice (BALB/cAnN.CgFoxn nu/Crl Narl mice), aged 5-6 weeks and weighing 17-19 g, were used to establish a xenograft model. The mice were housed with a regular 12 h light/12 h dark cycle and given ad libitum access to standard rodent chow diet (Laboratory Rodent Diet 5001, LabDiet, St. Louis, MO). The animals were kept in a pathogen-free environment at the Laboratory Animal Unit. A549 cells (5 × 10⁶ cells/0.1 mL/mouse) mixed with matrix gel (Corning Inc., NY) (cell:matrix gel = 4:1) were injected subcutaneously into the right front axilla of the mouse. After 8 days of implantation, the mice were randomly divided into three groups (N = 6 for each group) and fed by oral gavage with sterile water (control) and DIE (250 and 500 mg/kg/day) suspended in water. Bioluminescence imaging was performed with an IVIS50 animal imaging system (Xenogen Corp, Alameda, CA). Tumor growth was monitored by luciferase activity in A549 cells; photons emitted from the target site penetrated through the mammalian tissue and could be externally detected and quantified with a sensitive light imaging system.²

2.12 Immunohistochemistry analysis
Paraffin-embedded slides were deparaffinized, and antigen unmasking was carried out by microwave heating in citrate buffer for 20 minutes. Slides were incubated with primary anti-MMP-2, anti-N-cadherin, and anti-E-cadherin antibodies. Enzyme-conjugated secondary anti-mouse antibodies were also added and the specific staining can be visualized after adding the enzyme-specific substrate.²

2.13 High-performance liquid chromatography (HPLC) analysis
HPLC analysis (Waters 600 with a 2998 photodiode array detector) was conducted with a LiChroCART RP-18 (Merck KGaA, Darmstadt, Germany) reversed phase column (200 mM × 4 mm, 5 μm). The mobile phase consisted of water/acetic acid (Sigma, St. Louis, MO) (0.05%, v/v) (solvent A) and acetic acid/water/acetonitrile (Sigma, St. Louis, MO) (0.05%, v/v) (solvent B). Elution was carried out in a programmed gradient as follows: 0-30 minutes with 0%-100% B.

2.14 Statistical analysis
Differences were calculated by Student’s t-test (SigmaStat 2.0). Differences at P < .05 were considered statistically significant.

3 RESULTS
3.1 DIE inhibits the activity of MMP-2 and u-PA in human lung cancer cells
DIE was extracted from D. indica using 50% ethanol and incubated with lung cancer A549 and H1299 cells. The effect of DIE on the growth of A549 and H1299 cells was determined. The results of MTT
assay showed that A549 treatment with 100 μg/mL DIE inhibited A549 cell proliferation by about 20% at 24 hours (Figure 1A) and 48 hours (Figure 1B), while H1299 cell viability was not significantly different to that of controls (0 μM) after treatment with 100 μg/mL DIE for 24 hours (Figure 1A) and 48 hours (Figure 1B). We then treated A549 and H1299 cells with different concentrations of DIE and evaluated using gelatin and casein zymography. The activity of MMP-2 and u-PA, which are correlated with cancer invasion, were evaluated. Analysis of cells treated with 100 μg/mL DIE through gelatin zymography showed that MMP-2 activity decreased by 57% and 77% in A549 and H1299 cells, respectively (Figure 1C). Moreover, analysis through casein zymography demonstrated that u-PA activity decreased by 98.2%, and 61% in A549 and H1299 cells, respectively (Figure 1D).

3.2 | DIE inhibits metastasis in human lung cancer cells

The anti-metastatic ability of DIE was assessed through wound healing assays. After 48 hours of wounding, the wound area produced a marked cell migration and DIE significantly delayed wound healing. DIE inhibited the migration of A549 and H1299 cells (Figure 2A). We used Boyden chamber to assess the ability of DIE to inhibit the invasion and motility of both A549 and H1299 cells. The results indicated that treatment with 100 μg/mL DIE reduced the invasion and motility of A549 cells by 40% (Figure 2B) and 62% (Figure 2C), respectively. In H1299 cells, 100 μg/mL DIE reduced invasion and motility by 59% (Figure 2B) and 63% (Figure 2C), respectively. When invading host tissues, lung cancer cells adhere to ECM to create new links. Therefore, cell–matrix adhesion was assessed to determine the effect of DIE. In A549 and H1299 cells treated with DIE, cell–matrix interactions significantly decreased (Figure 2D). In the cell spreading assay, the majority of A549 and H1299 cells exhibited spread morphology after 5 hours. At the same time point, DIE treatment decreased the number of A549 and H1299 cells with spread morphology (Figure 2E).

3.3 | Effect of DIE on signaling molecules regulating EMT in human lung cancer cells

We evaluated the expression of p-ERK, p-FAK, and p-paxillin in A549 and H1299 cells to elucidate the anti-metastatic and anti-invasive mechanism of DIE. DIE treatment significantly down-regulated the expression of p-ERK, p-FAK Tyr397, and p-paxillin Tyr118 but did not affect the expression of p-paxillin Ser178 (Figure 3A). Therefore, DIE possibly inhibited the ERK1/2 pathway to reduce the activity of MMP-2 and u-PA and the invasion of A549 and H1299 cells. We also analyzed the main EMT regulators and markers. The result showed that DIE increased the expression levels of epithelial markers, such as E-cadherin, but decreased those of mesenchymal markers, such as N-cadherin, fibronectin, and vimentin, in A549 and H1299 cells (Figure 3B). Considering that transcription factors, such as c-Jun and c-Fos, are involved in regulating the activity of EMT, we used Western blot for further analysis. DIE decreased the expression levels of mesenchymal-related transcription factors, such as c-Jun and c-Fos, in both A549 and H1299 cell lines (Figure 3C). We then used TGF-β1 induced A549 into mesenchymal phenotype. Treatment with DIE did not significantly change the A549 phenotype (Figure 3D). However, in confocal microscopy analysis, DIE inhibited TGF-β1 induced-vimentin expression of the mesenchymal phenotype A549 (Figure 3E).

3.4 | DIE reduces p-FAK expression to inhibit invasion and metastasis of A549 cells

The FAK inhibitor 14 was used to confirm the mechanism of DIE in reducing p-FAK expression in A549 cells. The FAK inhibitor 14 strongly reduced...
the expression of p-FAK Tyr397 and p-paxillin Tyr118 (Figure 4A). Treatment with DIE and FAK inhibitor 14 strongly suppressed the invasion (Figure 4B) and motility (Figure 4C) of A549 cells. In particular, treatment with 50 μg/mL DIE and 5 μM FAK inhibitor 14 significantly inhibited cell invasion and motility (Figure 4A). Hence, DIE inhibited the invasion of A549 cells, but the underlying mechanism must be confirmed.

3.5 | Anti-tumor effects of DIE in vivo

We determined luciferase expression in A549-bearing nude mice treated with 0, 250, and 500 mg/kg/day DIE by oral gavage. Compared with the control, cells treated with DIE orally for 40 days showed decreased tumor volume (Figure 5A), and weight (Figure 5B). Luciferase showed the same result. DIE inhibited the tumor growth in A549 cells (Figure 5C). Moreover, analysis of the body of nude mice showed that DIE exhibited no significant toxicity (Figure 5D). Hence, DIE inhibited the growth of A549 cells in vivo. Histochemical analysis of the pathologic sections of these tumors showed that DIE treated tumors had low levels of MMP-2, N-cadherin, and high levels of E-cadherin compared with control A549 tumors (Figure 5E).

3.6 | DIE contains epigallocatechin

We used HPLC chromatography to analyze the components of DIE. The spectra showed peaks corresponding to retention time, and absorbance was monitored at 280 nm. We compared DIE (Figure 6A) with 13 standard compounds, including gallic acid, gallicatechin (GC), protocatechuic acid (PCA), epigatecitatechin (EGC), catechin, methyl gallate, epicatechin (EC), gallicatechin gallate (GCG), epigatecitatechin gallate (EGCG), rutin, epicatechin gallate (ECG), naringin, and quercetin (Figure 6B). The results showed that ECG is a component of DIE. Moreover, the peak of DIE perfectly overlapped with that of ECG (Figure 6C). These results confirmed our inference.
3.7 | ECG inhibits the metastasis of human lung cancer cells

Treatment with 25 μM (P < .001) and 50 μM (P < .001) ECG for 24 hours significantly delayed wound healing (Figure 7A). Analysis using the Boyden chamber demonstrated that 25 μM (P < .05) and 50 μM (P < .01) ECG strongly inhibited A549 cell invasion (Figure 7B). The same results were observed for motility by treatment with 25 μM and 50 μM ECG (P < .001) (Figure 7C). In summary, we confirm that DIE can down-regulate the ERK1/2 pathway, affect the expression of transcription factors such as c-Jun and c-Fos, reduce the activity of MMP-2 and u-PA, and inhibit A549 cell invasion, leading to increased E-cadherin expression and reverse EMT (Figure 8).

4 | DISCUSSION

Lung cancer is one of the most commonly diagnosed cancer. The mortality rate of lung cancer is higher than that of other cancers worldwide. Metastasis is the main cause of lung cancer-related deaths and poor prognosis of this disease. Therefore, the correlation between EMT and metastasis has been the focus of anti-cancer research. Cancer metastasis is a complex signaling pathway comprising several steps, such as cell proliferation, division, proteolysis digestion of ECM, changes in the adhesion capability between the cell and the ECM, invasion through the basement membrane to reach the circulation system, extravasation, and growth of tumor at metastasis site. This study used a complex strategy, and we found that DIE inhibited the activity of MMP-2 and u-PA (Figure 1).

In addition to secretion of MMPs and u-PA, EMT activation decreased the expression level of E-cadherin and increased those of vimentin and fibronectin. Studies have shown that reduced E-cadherin expression is associated with progression and poor prognosis of prostate and breast cancers. Moreover, noninvasive cancer cells can be activated by blocking the expression or knocking down of E-cadherin; meanwhile, invasive cancer cells can be inactivated by over-expressing E-cadherin. Similarly, a previous study on various types of human cancer cells showed that E-cadherin is lost in the early stage of tumor development, thereby maintaining the mesenchymal phenotype. The present study demonstrated that DIE suppressed the invasion, motility, and adhesion of human lung cancer cell line A549 and H1299 (Figure 2). Furthermore, DIE did not only reduce MMP-2 and u-PA secretion (Figure 1) to inhibit the invasion of A549 and H1299 cells but also increase the expression of E-cadherin and decrease that of vimentin and fibronectin (Figure 3B). Thus, we
confirmed that DIE enhances E-cadherin expression in A549 and H1299 cells, and this phenomenon could be one of the anti-metastasis mechanisms of DIE.

Paxillin is associated with FAK, a non-receptor tyrosine kinase, and is also involved in principal cellular processes.\(^\text{35}\) Paxillin mainly binds to FAK and is a known as a focal adhesion molecule that participates in cell migration and adhesion.\(^\text{36}\) Moreover, paxillin plays a role in adhesion changes associated with the EMT in human carcinoma.\(^\text{37}\) The paxillin phosphorylation site contains several serine/threonine and tyrosine. Extracellular stimulation phosphorylates these sites and recruits adhesion-related signaling molecules to activate the pathway, leading to cell migration and invasion.\(^\text{38}\) In the present study, Western blot analysis showed that DIE could inhibit FAK Tyr 397 and paxillin Tyr 118 phosphorylation in human lung cancer cell line A549 and H1299 (Figure 3A). Treatment with the FAK inhibitor 14 was confirmed to perform that DIE inhibits FAK (Figure 4A). We proved that treatment with both DIE and FAK inhibitor 14 suppressed p-FAK Tyr 397 expression and the invasion and motility of A549 cells (Figure 4). However, reduced E-cadherin expression was also associated with epithelial growth factor receptor (EGFR) and integrin; hence, whether these molecules are located upstream of FAK and affect FAK activity must be further investigated.\(^\text{39}\)

In vivo studies showed that DIE inhibited the growth of A549 cells and downregulated EMT related protein expression in tumor (Figure 5). Composition analysis revealed that DIE contained ECG (Figure 6). Previous research demonstrated that ECG could inhibit EMT in oral cancer.\(^\text{22}\) In the present experiment, ECG exhibited similar effect, namely, suppression of the invasion and metastasis in A549 cells (Figure 7). Therefore, ECG could be one of the components of DIE that suppress EMT in A549 cells. However, the contents of DIE and their actual mechanism must be further identified.

In this study, DIE in human lung cancer cell line A549 can down-regulate ERK1/2 to decrease the activity of MMP-2 and u-PA. DIE inhibited the FAK pathway to enhance the expression of E-cadherin and reduce the expression of vimentin and fibronectin. Finally, DIE suppressed cell invasion, motility, and adhesion. In addition, treatment with DIE reversed the EMT and inhibited the growth of A549 cells in vivo. Future studies should develop novel anti-cancer drugs to reverse EMT and suppress cancer cell invasion and metastasis.

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