Cinnamomum cassia extracts reverses TGF-β1-induced epithelial–mesenchymal transition in human lung adenocarcinoma cells and suppresses tumor growth in vivo

Chin-Yin Lin | Yi-Hsien Hsieh* | Shun-Fa Yang | Shu-Chen Chu | Pei-Ni Chen | Yih-Shou Hsieh

1Institute of Biochemistry, Microbiology and Immunology, Chung Shang Medical University, Taichung City, Taiwan
2Institute of Medicine, Chung Shan Medical University, Taichung, Taiwan
3Institute and Department of Food Science, Central Taiwan University of Science and Technology, Taichung, Taiwan
4Clinical Laboratory, Chung Shan Medical University Hospital, Taichung, Taiwan

Correspondence
Yih-Shou Hsieh, Institute of Biochemistry, Microbiology and Immunology, Chung Shang Medical University, Taichung City, Taiwan.
Email: csmcysh@csmu.edu.tw
Pei-Ni Chen, Institute of Biochemistry, Microbiology and Immunology, Chung Shang Medical University, Taichung City, Taiwan.
Email: peini@csmu.edu.tw

Funding information
Chung Shan Medical University, Grant Number: CSMU-INT-105-02.

Abstract
Metastasis is the most common cause of cancer-related mortality in patients, and epithelial–mesenchymal transition (EMT) is essential for cancer metastasis and antidrug resistance. Cinnamomum cassia has several antioxidative, anti-inflammatory, and anticancer biological effects. However, the anti-EMT effect of C. cassia in human lung carcinoma is rarely reported. In this study, we determined whether C. cassia extracts (CCE) reduces the EMT and tumor growth of human lung adenocarcinoma cells. CCE inhibited the transforming growth factor (TGF)-β1-induced cell motility and invasiveness of A549 and H1299 cells by repressing matrix metalloproteinase-2 and urokinase-type plasminogen activator as well as impaired cell adhesion to collagen. CCE also affected the TGF-β1-induced EMT by downregulating the expression of vimentin and fibronectin and upregulating E-cadherin. The nude mice xenograft model showed that CCE reduced A549 tumor growth. Thus, CCE possesses antimetastatic activity of A549 and H1299 cells by affecting EMT and suppressing A549 tumor growth in vivo. This result suggested that CCE could be used as an antimetastatic agent or as an adjuvant for anticancer therapy.

KEYWORDS
Cinnamomum cassia, epithelial–mesenchymal transition, matrix metalloproteinase, metastasis, lung cancer

1 | INTRODUCTION

Lung carcinoma is the leading cause of cancer mortality worldwide. Resistance to cancer treatment (such as radiation therapy, chemotherapy, and targeted therapy) and cancer metastasis are two major causes of the poor survival of lung cancer patients. Adenocarcinoma is the most common form of lung cancer and classified as a non-small-cell lung cancer (NSCLC). NSCLC accounts for 80% of lung cancers, roughly 50% of which are adenocarcinomas. However, metastasis is the most common cause of mortality, and the high metastatic ability of lung cancer is accountable for poor prognoses and high mortality rates. Therefore, new active compounds should be used for antimetastasis or complementary therapies.

*Yi-Hsien Hsieh contributed equally as first author.
invasion and EMT of human lung cancer and the underlying mechanisms of such effects remain unclear. In this study, we proposed that C. cassia may affect lung adenocarcinoma cells to exert anticancer effects in vitro and in vivo.

2 MATERIALS AND METHODS

2.1 Preparation of CCE

C. cassia was purchased from a store in Taichung, Taiwan. CCE was prepared as previously described. Air-dried branches (100 g) were boiled at 70°C for 24 h with 500 mL of 50% ethanol. The solvent was then removed, and the filtrate was lyophilized and stored at −20°C. The recovery ratio of CCE is 17.25%.

2.2 Cell culture

A549 and H1299 human lung adenocarcinoma cell lines (ATCC, Manassas, VA) were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. All cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂.

2.3 Microculture tetrazolium (MTT) assay

For the cell viability experiment in the concentration range, an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay was performed to determine the cytotoxicity of different concentrations of CCE for 24 and 48 h. After the exposure period, the media were removed and the cells were washed with phosphate-buffered saline. Afterward, the medium was changed and the cells were incubated with MTT reagent (0.5 mg/mL) for 3 h. The viable cell number per dish was directly proportional to the production of formazan, which could be measured spectrophotometrically at 563 nm.

2.4 Colony formation assay

A549 and H1299 cells were plated at 5000 cells as single cells onto a 6-well Petri dish for 8 days. Cells were fed with new culture medium with different concentrations of CCE every 3 days. Colonies were stained with crystal violet. In the end, cells were photographed and counted. A result representing three separate experiments is shown.

2.5 Boyden chamber cell invasion and motility assays

The cells were pretreated with CCE for 2 h prior to stimulation with TGF-β1 (10 ng/mL) for 24 h. Then, the cells were harvested and seeded to Boyden chamber (Neuro Probe, Cabin John, MD) at 1.5 × 10⁴ cells/well in serum-free medium and incubated for another 24 h at 37°C. Matrigel (10 μL) was applied to 8-μm-pore-size polycarbonate membrane filters for the invasion assay, with the bottom chamber of the apparatus containing standard medium. The invaded cells were fixed with methanol and stained with Giemsa. Cell numbers were counted using a light microscope (magnification 100×), while motility assay was conducted as described for the invasion assay, without Matrigel coating.

2.6 Cell–matrix adhesion assay

The cells were placed on 24-well dishes coated with collagen type I or gelatin (10 μL/mL). Nonadherent cells were removed by washing the dishes with phosphate-buffered saline. After staining with 0.1% crystal violet, fixed cells were lysed in 0.2% Triton X-100 and the absorbance was measured at 550 nm.

2.7 Determination of matrix metalloproteinases (MMPs) and urokinase-type plasminogen activator (u-PA) by zymography

For gelatin zymography, collected media were subjected to 0.1% gelatin–8% SDS polyacrylamide gel electrophoresis to determine the MMP-2. The gels were washed with 2.5% Triton X-100 after electrophoresis and incubated in reaction buffer. The gel was then stained with Coomassie brilliant blue R-250. The u-PA activity was visualized by casein zymography. Added were 2% w/v casein and 20 μg/mL plasminogen to 8% SDS-PAGE gels. Samples with a total protein of about 20 μg were then loaded onto the gels. u-PA activity of cells treated or untreated with CCE was measured as described in the gelatin zymography.

2.8 Western blot analysis

The total cell lysates were prepared as described elsewhere. The total cell lysates were incubated with the vimentin, fibronectin, and E-cadherin antibodies (Cell Singling Technology, Inc., Danvers, MA, USA), washed, and monitored by immunoblot assay using specific secondary antibodies. The relative photographic densities were quantitated by scanning the photographic negatives using a gel documentation and analysis system. After measuring the intensity of each band by densitometry, relative intensities were calculated by normalizing to GAPDH from the corresponding sample.

2.9 Bioluminescence imaging (BLI) measurement of tumor growth in nude mice

All procedures involving animals were 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: 1580). For the nude mice xenograft model, 5–6-week-old immunodeficient nude mice (BALB/c AnN.CgFoxn10nu/Crl Narl mice) weighing 17–19 g were used. The mice were housed with a regular 12 h light/12 h dark cycle and ad libitum access to standard rodent chow diet (Laboratory Rodent Diet 5001, LabDiet, St. Louis, MO) and were kept in a pathogen-free environment at the Laboratory Animal Unit. A549 cells (6 × 10⁶ cells/0.1 mL/mouse) were
injected subcutaneously into the right front axilla. Night days post-implantation, the mice were randomly divided into three groups (\( N = 5 \) for each group) and fed by oral gavage with sterile water (control) and CCE (100 and 200 mg/day/kg) suspended in water. The average tumor volume at the start of treatment was approximately 123.24 mm³. In tumor-bearing animals, the growth of tumors was measured every 9 days during the study using vernier calipers. The tumor volumes were calculated using the following formula: tumor volume = 0.5 × long diameter × short diameter × short diameter. BLI was performed using an IVIS50 animal imaging system (Xenogen Corp., Alameda, CA). Tumor growth was monitored by luciferase activity in A549 cells, and the photons emitted from the target site penetrated through the mammalian tissue and could be externally detected and quantified using a sensitive light imaging system.\(^{17}\)

**FIGURE 1** Effects of CCE on cell viability and the colony formation in human lung A549 and H1299 cells. (A) A549 and (B) H1299 cells were pretreated with CCE for 2 h and then cultured in 10 ng/mL TGF-β1 for 24 and 48 h before being subjected to an MTT assay for cell viability. (C) A549 and H1299 cells were treated with various concentrations of CCE for 8 days. The formation of cell colony was measured. A result representing three separate experiments is shown (**, \( P < .01 \); ***, \( P < .001 \) compared with the control group). [Color figure can be viewed at wileyonlinelibrary.com]
2.10 Statistical analysis

Statistical significances were analyzed by one-way ANOVA with post hoc Dunnett’s test. P value < 0.05 was considered statistically significant (Sigma-Stat 2.0, Jandel Scientific, San Rafael, CA).

3 RESULTS

3.1 CCE inhibits the colony formation in human lung A549 and H1299 cells

In the presence of concentrations of 30 and 60 µg/mL of CCE, A549 (Figure 1A) and H1299 (Figure 1B) cells viability was not significantly different to that of controls (0 µM) after treatment with CCE for 24 and 48 h. Colony formation assay is an in vitro cell survival assay based on the ability of a single cell to grow into a colony. We performed colony formation assay and determined the long-term efficacy of CCE on lung cancer cells to investigate further the anticancer activity of CCE. Significant decreases in the number of colonies were observed for A549 and H1299 cells after CCE treatment (Figure 1C).

3.2 CCE reduces TGF-β1-induced invasion and motility

TGF-β1-mediated EMT of human lung cancer cells may contribute to lung cancer metastasis. A549 and H1299 cells were pretreated with CCE prior to stimulation with TGF-β1 to determine whether CCE could affect the TGF-β1-induced scattering. Cells were pretreated with CCE for 2 h prior to stimulation with TGF-β1 (10 ng/mL) for 24 h. We examined whether CCE affected TGF-β1-induced cell invasion. Quantitative analyses by cell invasion assay showed that the invasion of A549 and H1299 cells increased by ~1.2- and 1.2-fold, respectively, upon TGF-β1 treatment, but the TGF-β1-induced invasions were reduced
by CCE treatment dose-dependently (Figure 2A,B). Quantitative analyses by cell motility assay showed that the motility of A549 and H1299 cells increased by 1.2- and 1.3-fold, respectively, upon TGF-β1 treatment, but the TGF-β1-induced motilities were reduced by CCE treatment dose-dependently (Figure 2C,D).

### 3.3 CCE inhibits TGF-β1-induced MMP-2 and u-PA

CCE reduced TGF-β1-induced MMP-2 activity of A549 and H1299 cells in dose- and time-dependent manners (Figure 3A,B). CCE also reduced TGF-β1-induced u-PA activity of both cell lines in dose- and time-dependent manners (Figure 3C,D).

### 3.4 CCE inhibits TGF-β1-induced cell–matrix adhesion

The effects of CCE on TGF-β1-induced cell–matrix adhesion were then evaluated. The results showed that CCE significantly reduced the TGF-β1-induced cell–collagen (Figure 4A,B) and cell–gelatin (Figure 4C,D) interactions of both cell lines.
3.5 | CCE targets signaling molecules that regulate EMT in A549 cells

Western blot analysis was performed to further investigate the molecular mechanisms of CCE. The effects of CCE on the major regulators and markers of TGF-β1-induced EMT were examined. CCE significantly upregulated the epithelial marker E-cadherin but significantly downregulated the TGF-β1-induced mesenchymal markers fibronectin and vimentin in A549 cells (Figure 4E).

3.6 | CCE reduces A549 tumor growth in vivo

A bacterial expression vector was constructed with pGL4.50 [luc2/CMV/Hygro] and transformed into A549 cells to produce the pGL4.50...
Interestingly, our BLI demonstrated that CCE suppressed the luciferase activity, and no luciferase activity was detected in all lungs or other distant sites (Figure 5A). Serial measurements of the volume of the mouse tumor revealed that CCE decreased the tumor volume (Figure 5B). The mice were sacrificed to confirm the changes to validate the results of our IVIS analysis. The CCE reduced the tumor weight (Figure 5C).
FIGURE 6  Effects of cinnamaldehyde on TGF-β1-induced invasion and activities of MMP-2 and u-PA of human lung cells. (A) A549 and (B) H1299 cells were pretreated with cinnamaldehyde for 2 h and then cultured in 10 ng/mL TGF-β1 for 24 and 48 h before being subjected to an MTT assay for cell viability. A549 and H1299 cells were pretreated with cinnamaldehyde for 2 h and then cultured in 10 ng/mL TGF-β1 for 24 h; cell invasion (C and D) were then analyzed. Condition media were subjected to gelatin zymography and casein zymography to analyze the activities of MMP-2 (E and F) and u-PA (G and H), respectively. Data represented the mean ± SD of at least three independent experiments (#, \( P < .05 \); ##, \( P < .01 \); ###, \( P < .001 \) compared with control; *, \( P < .05 \); **, \( P < .01 \); ***, \( P < .001 \) compared with TGF-β1-treated group). [Color figure can be viewed at wileyonlinelibrary.com]
3.7 | Cinnamaldehyde reduces TGF-β1-induced invasion, MMP-2, and u-PA

Cinnamaldehyde is the major constituent of *Cinnamomum cassia*. To substantiate the role for cinnamaldehyde in *Cinnamomum cassia* extracts-reduced invasion and proteinases expression, MTT assay, Boyden chamber invasion assay, gelatin zymography assay, and casein zymography assay were done in both lung cancer A549 and H1299 cells. In the presence of 10 and 20 μM of cinnamaldehyde, A549 (Figure 6A) and H1299 (Figure 6B) cells viability was not significantly different to that of controls (0 μM) after 24 and 48 treatments. Quantitative analyses by cell invasion assay showed that TGF-β1-induced invasions were significantly reduced by cinnamaldehyde treatment in both A549 (Figure 6C) and H1299 (Figure 6D) cells. Cinnamaldehyde also inhibited TGF-β1-induced MMP-2 (Figure 6E,F) and u-PA (Figure 6G,H) activities of both cell lines.

4 | DISCUSSION

Metastasis of lung cancer is the most important cause of patient death. This study was performed to investigate the anticancer effects of CCE on lung cancer both in vitro and in vivo. We clearly showed that CCE reduces the EMT and tumor growth of human lung adenocarcinoma cells. Tumor cell invasion requires both cell invasion and digestion of the basement membrane and the extracellular matrix by MMPs and u-PA. Thus, cell invasion requires both cell invasion and digestion of the basement membrane. Decreasing epithelial markers—such as E-cadherin—and gaining mesenchymal markers—such as N-cadherin, vimentin, fibronectin, and α-smooth muscle actin—are associated with cancer progression, metastasis, and resistance to chemotherapy. In this process, epithelial cells switch to a mesenchymal phenotype by decreasing epithelial markers—such as E-cadherin—and gaining mesenchymal markers—such as N-cadherin, vimentin, fibronectin, and α-smooth muscle actin. EMT is induced by several signaling pathways, such as TGF-β1, Notch, hypoxia, and Hedgehog. EMT-related proteins have been reported to present a close relationship with MMP-2 and MMP-9. TGF-β1 is overexpressed in many malignant human carcinomas such as lung cancer cells. Other reports in cancer progression have indicated that overexpression of TGF-β1 at early stages of carcinogenesis provided tumor-suppressive effects primarily via inhibition of tumor growth, whereas overexpression of TGF-β1 at late stages promoted cancer progression, metastasis, and EMT. This study showed that CCE reversed TGF-β1-induced EMT, cell invasion, and MMP-2 expression in A549 and H1299 cells. Furthermore, we defined that CCE affected EMT by downregulating the expression of vimentin and fibronectin and upregulating the expression of E-cadherin.

Thus, CCE inhibited TGF-β1-induced MMP-2 and u-PA and affected EMT to inhibit the metastasis of human lung A549 and H1299 cells. Moreover, CCE repressed human lung A549 tumor growth in vivo. These results demonstrate the anticancer properties of CCE and its clinical potential as an inhibitor of tumor growth and cancer metastasis in lung cancer cells.

ACKNOWLEDGMENTS

This study was supported by Chung Shan Medical University [CSMU-INT-105-02]. IVIS was performed in the Instrument Center of Chung Shan Medical University, which is supported by National Science Council, Ministry of Education and Chung Shan Medical University.

REFERENCES


