Curcumin Inhibits the Migration and Invasion of Non-Small-Cell Lung Cancer Cells Through Radiation-Induced Suppression of Epithelial-Mesenchymal Transition and Soluble E-Cadherin Expression

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Abstract
Radiotherapy has been reported to cause cancer metastasis. Thus, a new strategy for radiotherapy must be developed to avoid this side effect. A549 cells were exposed to radiation to induce an epithelial-mesenchymal transition (EMT) cell model. Real-time PCR and western blotting were used to detect mRNA and protein expression levels, and Transwell invasion and wound healing assays were used to detect cell migration and invasion. ELISA was used to detect soluble E-cadherin (sE-cad) secretion. siRNA was used to silence MMP9 expression. The results show that A549R cells exhibited an EMT phenotype with increased E-cadherin, N-cadherin, Snail, Slug, vimentin and Twist expression and decreased pan-keratin expression. sE-cad levels were increased in A549R cells and in the serum of NSCLC patients with distant metastasis. Exogenous sE-cad treatment and sE-cad overexpression promoted A549R and A549 cell migration and invasion. In contrast, blocking sE-cad attenuated A549 cell migration and invasion. Curcumin inhibited sE-cad expression and reversed EMT induced by radiation. Furthermore, curcumin suppressed sE-cad-enhanced A549 and A549R cell migration and invasion. Curcumin inhibited MMP9 expression, and silencing MMP9 suppressed sE-cad expression. Taken together, we found a nonclassic EMT phenomenon induced by radiation. Curcumin inhibits NSCLC migration and invasion by suppressing radiation-induced EMT and sE-cad expression by decreasing MMP9 expression.

Keywords
curcumin, soluble e-cadherin, EMT, MMP9, non-small cell lung cancer

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Introduction
Radiotherapy is widely used as an adjuvant treatment with or without surgery and chemotherapy for non-small-cell lung cancer (NSCLC). During treatment, patients show different responses; some are cured, and some develop recurrence and distant metastasis. Increased evidence has suggested that epithelial-mesenchymal transition (EMT) plays a central role in cancer cell metastasis. Numerous studies indicate that ionizing radiation can enhance the metastatic capabilities of tumor cells by inducing the EMT program. Therefore, potential adjuvant drugs need to be developed to solve this problem. EMT is a normal biological process that occurs during embryonic development and differentiation in which epithelial cells lose polarity and convert to spindle-shaped cells. EMT plays an important role in cancer metastasis, which is characterized by the downregulation of epithelial molecular markers such as E-
cadherin and keratins and the upregulation of mesenchymal molecular markers such as vimentin, N-cadherin and Twist.5

E-cadherin is a membrane glycoprotein that plays an important role in maintaining cell-to-cell adhesion integrity, which is significantly associated with tumor invasiveness and migration.6 Dysfunction or loss of E-cadherin expression has been shown to increase tumor metastasis capacity.7 Increased expression of shiyan were enrolled in the present study. The study population was included 39 adenocarcinoma patients and 44 squamous cell carcinoma patients. All subjects underwent clinical examination, including plain chest radiograph, CT scan of the chest, fiberoptic bronchoscopy and bone scan and so on. All patients were received radiotherapy as the first treatment. A total dose of 50.4 to 61.6 Gy radiotherapy (250 MU/min) was delivered in daily fractions of 2 Gy for 5 consecutive weeks by linear accelerator (X-RAD 320, Precision X-ray Inc., North Branford, CT, USA). Blood samples were collected from the patients before any kind of treatment (surgery, radiation, chemotherapy, and so on). The tumor radiotherapeutic response was assessed by CT according to Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1. This study was carried out in accordance with the principles of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of Taihe Hospital of shiyan Ethical Committee (The Certificate Number: 2019KS021).

Cell Lines, Culture Conditions and Reagents
The lung cancer cell line A549 was purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM (Gibco Invitrogen, Paisley, Scotland) with 10% fetal bovine serum (FBS, BI), 100 U/ml penicillin, and 100 μg/ml streptomycin. (Gibco, USA) at 37°C in a 5% CO2 atmosphere. Trypsin (0.25%, Gibco, USA) was used to passage the cell lines when they reached 90% confluence. The reagents used for cell disposes included recombinant human E-cadherin (rE-cad) (R&D, Minneapolis, MN), curcumin and the E-cadherin ectodomain-blocking antibody DECMA-1 (Sigma, USA).

EMT Cell Model Induction
A549 cells were exposed to fractionated radiation of 2 × 30 Gy X-ray irradiation (total: 60 Gy) (250 MU/min) with a linear accelerator (X-RAD 320, Precision X-ray Inc., North Branford, CT, USA) to induce EMT. Cells (1 × 10^6) were seeded in a T25 culture flask with 10 ml of complete medium before irradiation, and 5 ml of medium was removed after irradiation. Irradiation was performed after the cells recovered proliferation activity.

Plasmid Transfection
The E-cadherin ectodomain sequence was cloned into the CMV-MCS-IRESCGFP-SV40-Neomycin vector (Genechem, Shanghai, China), and the cDNA sequence is shown in Document S1. Cells (2.5 × 10^5 per well) were seeded in 6-well plates and transfected with Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions.

Cell Proliferation Assay
A549R cells were plated in 96-well plates at 3,000/well and allowed to adhere overnight. After the cells were treated with curcumin for 48 h, CCK-8 solution (10 μl) was added to each
well of the plate and incubated for another 2 h. The absorbance was measured using a Multilabel Plate Reader (Monobind Inc, USA) at 450 nm, as previously reported.18

ELISA Assays

Cells were seeded at 2.5 × 10^5 cells/well in a 6-well plate and exposed to different concentrations of curcumin or transfected with sE-cad overexpression plasmid for 72 h. Culture media was analyzed for sE-cad secretion using E-cadherin Quantikine ELISA Kits (R&D Systems) according to the manufacturer’s specifications.

Transwell Invasion Assays

Cell invasion assay was investigated with matrigel-coated Boyden chamber (Costar, Cambridge, MA, USA). Cells were digested and proceed cell counting. A total of 5 × 10^4 cells in 100 μl serum-free medium were added to the upper wells, the lower compartments were added with 500 μl 10% FBS medium allowed cell to migrate for 12 h at 37°C. Using cotton swabs to remove the cells that remained in the upper chamber. The membrane of the upper chamber was fixed with methanol for 20 min and stained with a 0.1% crystal violet solution for 15 min. Then, washing the membrane with PBS for 3 times, invaded cells were counted using ImageJ (National Institutes of Health, USA) and a light microscope.

Wound Healing Assay

Cells were digested and proceed cell counting, cell were seeded in 6-well plates and cultured with DMEM.19 The A549 cells were irradiated after seeding into well plate, the wound creation was performed after irradiation. A 10 μl white micropipette tip was used to create vertical wound in the cell monolayer, then cells were treated with different concentrations of curcumin, sE-cad, DECMA-1 or X-ray irradiation for 48 h. Images of the wound edges were captured at time 0 h and 48 h using a SONY ILCE-A6000L/B camera (Japan).

RNA Extraction and Real-Time PCR

Cell total RNA was extracted with TRIzol reagent (Invitrogen, USA), total RNA was reversed to cDNA with a reverse transcriptase kit (Takara, Japan) according to the manufacturer’s protocol. Real-time quantitative (qPCR) analyses were performed using SYBR Premix Ex Taq™-based detection (Takara, Japan) and a CFX™ real-time system (Bio-Rad, USA). Primer sequences are showed in Table S1. Calculating relative mRNA expression levels using the 2^(-ΔΔCt) method.

Western Blot Assay

Cells were lysed with RIPA lysis buffer containing PMSF protease inhibitor. Whole cell lysates were boiled at 100°C for 5 min after added into 1/4 volume of loading buffer. protein extracts were separated by 10% SDS-PAGE and transferred to PVDF membranes. After blocking 5% non-fat milk, the membranes were cleaved and incubated with primary antibodies, then washed 3 times with TBST and incubated with secondary antibodies. Next, washed membranes for 3 times with TBST, and developing the target molecule using an enhanced chemiluminescence detection kit (Thermo Scientific, Rockford, IL, USA).20 The primary antibodies as following: E-cadherin (1:1000), N-cadherin (1:1000), Vimentin (1:1000), Twist (1:1000), Snail (1:500), Slug (1:1000), and MMP9 (1:1000) (Cell Signaling Technology, MA, USA). Pan-keratin (1:100,000) was purchased from Proteintech (China). GAPDH (1:10,000) antibodies were purchased from Boster (China).

Statistical Analysis

The data were analyses using Student’s t test in GraphPad Prism 5 software (GraphPad Software, La Jolla, CA), and the values are shown as the means ± SD of at least 3 independently conducted experiments. For all analyses, a 2-sided p-value < 0.05 was supposed to statistically significant.

Results

sE-Cad Was Upregulated in Radiation-Induced EMT Cells

To establish an EMT cell model, A549 cells were exposed to X-ray irradiation to induce EMT. When the A549 cell morphology changed from oval epithelium to spindle, the EMT cell model was named A549R (Figure 1A). We examined the expression pattern of epithelial and mesenchymal markers in A549 and A549R cells by real-time PCR and western blot analysis. The expression of the epithelial marker keratin was significantly lower, and the expression of the mesenchymal markers N-cadherin, Snail, Slug, vimentin and Twist was significantly higher in A549R cells than in A549 cells. Interestingly, the epithelial marker E-cadherin was also increased (Figure 1B and 1C). We also detected the migration and invasion potential of A549R cells, and wound healing and Matrigel invasion assays showed that cell migration and invasion were significantly higher in A549R cells than in A549 cells (Figure 1D and 1E).

High Serum Levels of sE-Cad Were Associated With Metastasis in NSCLC

Reports have shown that sE-cad, a cleaved version of full-length E-cadherin, promotes cancer cell EMT and metastasis21 and is upregulated by radiation in cancer cells. Therefore, we wanted to examine whether irradiation affects sE-cad expression in our study. The ELISA data showed that sE-cad expression in A549R cells was higher than that in A549 cells (Figure 2A). We also examined sE-cad expression levels in NSCLC patients with radiotherapy alone. The t-test was used to analyze the differences of each group of clinical characteristics. The results in Table 1 show that the expression levels of sE-cad in serum were not correlated with patient age, T stage or
N stage (P > 0.05) but were correlated with sex, M stage or histopathologic type (P < 0.05). We analyzed the correlation between serum levels of sE-cad and tumor metastasis in NSCLC patients. The data showed that increasing serum levels of sE-cad were correlated with distant metastasis in NSCLC patients (765.7 ± 203.2 ng/mL) compared with nonmetastatic patients (553.2 ± 177.0) (P < 0.05) (Figure 2B).

sE-Cad Enhanced Lung Cancer Cell Migration and Invasion

To examine the effects of sE-cad on A549R cell migration and invasion, A549R cells were pretreated with 400 μg/mL DECMA-1 (an sE-cad blocking antibody). Pretreatment with DECMA-1 reduced A549R cell migration and invasion compared with IgG treatment (invasion: 0.51-fold; migration: 0.34-fold) (Figure 3A and 3B). We also analyzed the effect of sE-cad on A549R cell migration and invasion. The results showed that 800 and 1600 ng/ml rmE-cad (exogenous recombinant sE-cad) significantly promoted cell migration and invasion (1.72-fold and 2.25-fold, respectively) (Figure 3C), and 1600 ng/ml rmE-cad significantly increased the wound healing rate (1.53-fold) (Figure 3D). To determine the effects of sE-cad on nonirradiated cell migration and invasion, A549 cells were transfected with the sE-cad overexpression plasmid. Then, transfection efficiency was detected by

Figure 1. Identification of radiation-induced EMT characteristics in A549R cells. After A549 cells were exposed to fractionated radiation, cell morphology changes were observed with optical microscopy (A), and the expression patterns of epithelial and mesenchymal markers were detected by real-time PCR (B) and western blotting (C). The scale bars indicate 200 μm at 100x magnification and 100 μm at 200x magnification. Wound healing (D) and Transwell invasion (E) assays were used to detect cell migration and invasion. The data are presented as the mean ± SD of 3 independent experiments. *p < 0.05. **p < 0.01.
western blot analysis (Figure 3E), and sE-cad secretion levels were analyzed by ELISA (Figure 3F). sE-cad overexpression significantly enhanced A549 cell migration and invasion (invasion: 1.77-fold; migration: 2.06-fold) (Figure 3G and 3H). Furthermore, sE-cad significantly enhanced A549 cell migration and invasion after pretreatment with 800 and 1600 ng/ml rmE-cad (invasion: 1.55-fold; migration: 1.6-fold) (Figure 3I and 3J). These data indicated that sE-cad enhanced lung cancer cell migration and invasion.

Curcumin Reversed EMT in A549R Cells

Treatment of A549R cells with increasing doses of curcumin for 48 h induced a significant dose-dependent decrease in cell proliferation, with an IC_{50} = 33.06 μM, as indicated by the CCK-8 assay (Figure 4A). To investigate the inhibitory effect of curcumin on irradiation-induced EMT in A549R cells, we examined the expression pattern of epithelial and mesenchymal markers after treatment with different concentrations of curcumin by western blot analysis. E-cadherin, sE-cad, vimentin and Slug expression levels were decreased after exposure to 2, 5, 10, and 20 μM curcumin, with maximum values of 0.44-, 0.60-, 0.52-, and 0.46-fold compared with the levels in control cells. N-cadherin and Snail expression showed a slight decrease after treatment with different concentrations of curcumin. Keratins were upregulated with a maximum 2.04-fold increase; however, curcumin had little effect on Twist expression in A549R cells (Figure 4B and 4C). An inverted optical microscope was used to observe cell shape changes after A549R cells were treated with different doses of curcumin, and the results showed that curcumin reversed cell shape changes from spindle to oval epithelium after pretreatment with 5, 10 and 20 μM curcumin (Figure 4D). These results showed that curcumin reverses EMT induced by X-ray irradiation in A549R cells.

Curcumin Reversed sE-Cad-Enhanced A549 and A549R Cell Migration and Invasion.

Transwell invasion and wound healing assays showed that 10 and 20 μM curcumin significantly decreased cell invasion and migration (invasion: 77% decrease; migration: 61% decrease) (Figure 5A and 5B). The previous data showed that curcumin decreased sE-cad secretion (Figure 4C); therefore, we hypothesized that inhibition of sE-cad secretion may be involved in curcumin-mediated suppression of NSCLC cell migration and invasion. A549 cells treated with 1600 ng/ml rmE-cad had significantly enhanced cell migration and invasion (invasion: 1.38-fold; migration: 1.35-fold). However, compared with rmE-cad treatment alone, curcumin combined with rmE-cad attenuated cell migration and invasion (invasion: 53% decrease; migration: 31% decrease) (Figure 5C and 5D).

Curcumin Decreased sE-cad Expression by Attenuating MMP9 Expression

To study the mechanism of MMP9 production, Real-time PCR was used to examine the expression differences of genes...
involved in E-cadherin cleavage between A549 and A549R. Results showed that the mRNA levels of MMP-2, MMP-9 and plasmin in A549R were higher than in A549 (Figure 6A). Then, we assessed the effect of curcumin on the expression of genes involved in E-cadherin cleavage. The results showed that curcumin significantly decreased MMP9 mRNA levels but did not affect MMP-2, MMP-3, MMP-7, MMP-9, MMP-14, ADAM10, ADAM15, plasmin or kallikrein 7 expression.

**Figure 3.** Effect of sE-cad on lung cancer cell migration and invasion. A549R cells were pretreated with 400 μg/mL DECMA-1 (an sE-cad-blocking antibody) for 4 h and then subjected to Transwell invasion (A) and wound healing (B) assays. A549R cells were pretreated with 0, 800, or 1600 ng/ml rmE-cad for 4 h and then subjected to Transwell invasion (C) and wound healing (D) assays. A549 cells were transfected with the sE-cad overexpression plasmid for 72 h, and western blotting (E) and ELISA (F) were used to detect sE-cad expression. Transwell invasion (G) and wound healing (H) assays were used to detect cell migration and invasion after A549 cells were transfected with the sE-cad overexpression plasmid for 48 h. After A549 cells were pretreated with 0, 800, or 1600 ng/ml rmE-cad for 4 h, Transwell invasion (I) and wound healing (J) assays were used to detect cell migration and invasion. The data are presented as the mean ± SD of 3 independent experiments. *p < 0.05. **p < 0.01. ***p < 0.001.
Curcumin also inhibited MMP-9 protein levels (Figure 6C). To examine whether MMP-9 is involved in sE-cad expression in NSCLC cells, MMP-9-specific siRNA was used to inhibit MMP-9 expression. The results showed that silencing MMP-9 significantly attenuated sE-cad levels \( (p < 0.01) \) (Figure 6D). These data indicate that curcumin inhibits sE-cad expression by attenuating MMP9 expression in NSCLC cells.

**Discussion**

Radiotherapy plays an important clinical role in lung cancer therapy. However, increased evidence has shown that ionizing irradiation may enhance the metastatic capacity of residual cancer cells, including lung cancer. Distant metastasis is the principal cause of radiotherapy failure. Therefore, tumor recurrence and metastasis might be associated with tumor biological behaviors, such as radiation-induced EMT. Reports showed that fractionated radiation treatment obviously induced EMT in cancer cells with downregulation of epithelial molecular markers such as E-cadherin and upregulation of mesenchymal molecular markers. In this study, we examined whether fractionated radiation could induce EMT in A549 cells. Our results showed that fractionated radiation induced spindle cell-like morphologic changes, decreased keratin expression, and upregulated N-cadherin, Snail, Slug, vimentin and Twist expression in A549R cells. Interestingly, E-cadherin expression also increased. Our results showed that the cell migration and invasion rates of A549R cells were significantly higher than those of A549 cells. Compared with classic EMT...
characteristics, our findings may indicate a nonclassical EMT phenomenon in which E-cadherin is increased during radiation-induced EMT in NSCLC cells.

The roles of E-cadherin in cancer are complicated. Membrane-bound E-cadherin plays a critical role in maintaining cell-cell adhesion in epithelial cells. It also inhibits cell transformation and negatively regulates tumor growth in lung, colon and breast cancers.26 Interestingly, E-cadherin has been reported to be found in bone, liver, and other metastatic lesions when protease is abundant.27-30 Yang et al.31 showed that E-cadherin mRNA is expressed in primary gastric tumors, metastatic lymph nodes and peritoneal lavage fluid, but the level was lower than that in primary tumors. Moreover, sE-cad exhibits oncogene characteristics of promoting cancer cell growth, motility and invasion.32 Researchers found increased sE-cad expression in liver, brain and lung metastatic tissues from prostate cancer patients.33 In this study, our data illustrated that sE-cad was upregulated in radiation-treated cells (A549R) in which EMT was induced by radiation. A549R cells showed higher migration and invasion ability than parental A549 cells.

Figure 5. Effect of curcumin on sE-cad-enhanced A549 and A549R cell migration and invasion. A549R cells were exposed to 0, 1, 2, 5, 10, or 20 μM curcumin for 24 h, and Transwell invasion (A) and wound healing (B) assays were used to detect cell migration and invasion. A549 cells were treated with 1600 ng/ml μM rmE-cad and (or) 20 μM curcumin for 48 h. Transwell invasion (C) and wound healing (D) assays were used to detect cell migration and invasion. The data are presented as the mean ± SD of 3 independent experiments. *p < 0.05. **p < 0.01.
and sE-cad promoted A549 cell migration and invasion with rmE-cad treatment or sE-cad overexpression; however, an sE-cad blocking antibody suppressed A549R cell migration and invasion. We also investigated the effects of sE-cad on NSCLC cell EMT; unfortunately, we did not find any changes in cell shape or epithelial and mesenchymal molecular marker expression in A549 or H460 cells after treatment with rmE-cad (data not shown). Previous reports and our current data suggest that sE-cad may play a tumor-promoting role contrary to that of E-cadherin.

Curcumin has been proven to inhibit the initiation, progression and metastasis of multiple tumors, including NSCLC. Curcumin inhibits hypoxia, radiation and TGF-β-induced EMT in pancreatic cancer, breast cancer and ovarian cancer via various signaling pathways. MMP9 plays an important role in cancer cell invasion. Various studies have shown that curcumin prevents cancer invasion by inhibiting MMP9 expression. Several mechanisms by which curcumin regulates MMP9 expression have been elucidated. Li et al. found that curcumin suppresses MMP-9 expression via activation of microRNA-365 in mice. Moreover, curcumin inhibits MMP-9 expression through upregulation of miR-98 and inhibits LIN28A-induced MMP2 and MMP9 expression in the lung cancer cell line A549. In the current study, we found that curcumin shifted the cell shape from spindle to oval epithelium, downregulated E-cadherin, sE-cad, vimentin and Slug expression, and upregulated pan-keratin expression in A549R cells. In addition, curcumin suppressed A549R cell migration and invasion by inhibiting sE-cad expression via MMP9 expression inhibition.

In conclusion, we report a novel strategy for improving radiotherapy-induced NSCLC metastasis. Our study indicates that curcumin inhibits NSCLC cell migration and invasion, likely by suppressing radiation-induced EMT and sE-cad expression. This study may provide a strong rationale for studying the clinical efficacy of anti-E-cad therapies and curcumin to suppress radiation-induced tumor metastasis in NSCLC.

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Figure 6. Effects of curcumin on the MMP9-sE-cad pathway. (A) Real-time PCR was used to examine E-cadherin cleavage-related gene expression in A549 and A549R cells. A549R cells were exposed to 20 μM curcumin for 48 h, (B) Real-time PCR was used to examine E-cadherin cleavage-related gene expression, (C) Western blot analysis was used to detect MMP9 expression, (D) A549R cells transfected with MMP9 siRNA for 48 h, Western blot analysis was used to detect MMP9 expression, and ELISA was used to detect sE-cad expression. The data are presented as the mean ± SD of 3 independent experiments. **p < 0.01.
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Author Contribution
Xinzhou Deng and Chunli Chen are authors Contributed equally

Consent for publication
All authors agreed to the publication of the manuscript.

Declaration of Conflicting Interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethics approval and consent to participate
All patients provided informed consent, and the study was approved by the Ethics Committee of Taihe Hospital of Shiyan Ethical Committee (The Certificate Number: 2019KS021) in accordance with the French laws and the World Medical Association’s Declaration of Helsinki.

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