Torilis japonica extract fraction compound, EGFR-targeted inhibition of cancer abnormal metastasis in A549 lung cancer cells

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Abstract. The number of patients who die from lung cancer is steadily increasing. According to the 2012 statistics, lung cancer accounts for the highest percentage of death from cancer in both sexes. Many research studies found that lung cancer can be caused not only by smoking but by outdoor pollution, and it leads to over-activation of various surface proteins in cancer cells. The over-activity of epidermal growth factor receptor (EGFR) is implicated as a crucial factor in inducing abnormal metastasis of lung cancer cells. In this study, we investigated the inhibitory effect of *Torilis japonica* extract (TJE) major fraction compound in A549 lung cancer cells by inhibiting EGFR activity. We confirmed that inhibitory effect of TJE on the abnormal metastasis using invasion assay and 3D cell culture method, as well as the inhibition of EGFR signaling pathway, co-binding with Stat3 and dimer formation for translocation to the nucleus. We confirmed the EGFR targets inhibition of TJE when compared with EGFR knockdown group using siRNA transfection. The CAM assay confirmed once again the efficacy of the TJE. We suggest that TJE is a new potential reagent for EGFR-targeted therapy and anti-abnormal metastasis in A549 lung cancer cells.

Introduction

The recorded incidence of cancer is increasing because of the increasing number of aging populations, as well as increasing prevalence of risk factors such as smoking, overweight and changing dietary patterns (1,2). Smoking is the most common reason for cancer incidence and deaths. In 2012, lung cancer patients due to smoking and other causes were the leading cause of cancer death worldwide both in males and females (3). According to this tendency, there is a movement to reduce smoking in the world. However, apart from a decrease in smoking, the number of patients and death rate caused by lung cancer has not decreased. Recent studies have shown that smoking does not simply induce an increase in lung cancer patients. Other known risk factors for lung cancer include exposure to outdoor pollution and this is the largest cause of lung cancer patients in China and East Asia (4-6).

Many of the factors involved in outdoor pollution induce cancerization through gene mutation, as well as induction of overgrowth and abnormal-metastasis of cancer cells leading to over-activity of surface proteins such as the growth factor receptors (7,8). Over-activation of epidermal growth factor receptor (EGFR) leads to not only abnormal proliferation by inducing Erk and Akt activation, which is a common cancer factor but increases abnormal metastasis of cancer cells through the JAK-STAT signaling pathways (9-11). The active EGFR directly translocates into the cancer cell nucleus and induces expression of factors involved in cancer cells metastasis. Recent studies have shown that EGFR-activated Stat3 induces expression of the SNAIL family and induces abnormal metastasis of cancer cells (12,13). Moreover, the EGFR co-binding with Stat3 and EGFR dimers translocate into the cancer cell nucleus and it leads to expression of factors related to cancer cell proliferation and invasion (14-16). Therefore, to inhibit abnormal growth and metastasis of cancer cells, it is very important to find a target substance capable of inhibiting the EGFR activation. Recently, we discovered a novel substance that can inhibit the abnormal metastasis of MCF-7 breast cancer cells by targeting EGFR (17).

We found that *Torilis japonica* extract (TJE) major fraction substance not only inhibits the activity of EGFR but regulate the expression of factors involved in cancer cell abnormal metastasis. However, we found that the substance targeted EGFR, but could not confirm that exact mechanism. In this study, we investigated the mechanism by which TJE targets EGFR and inhibit the abnormal metastasis in A549 lung cancer cells. In addition to the inhibition of surface of EGFR,

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we also examined the mechanism of expression suppression of abnormal metastasis-related factors through translocation to the cancer cell nucleus.

Materials and methods

Plant material and preparation of TJE. Dried whole fruit of TJE was purchased from Na-num Pharmacy (Kyung-buk, Korea). Plant material (200 g) was extracted two times with 95% ethanol (800 ml) at room temperature for 3 days and it was subsequently filtered (raw compound). For the active compound fraction, raw compound was mixed with compound:Methanol (9:1), and 200 μl aliquots were injected into a series 1100 HPLC system (Agilent Technologies, Inc., Santa Clara, CA, USA). A symmetry C-18 column (4.6x2.5 cm; Waters, Milford, MA, USA) was used. The mobile phase consisted of acetonitrile:water solution (3:1, v/v) pumped at a rate of 1 ml/min. Major peaks was recorded and same fractions were combined. The combined filtrate was concentrated under vacuum at 60˚C, and completely dried by freeze drying. TJE powder was dissolved in DMSO and filtrated by 0.2-μm pore size filter for *in vitro* and *ex vivo* studies.

Reagent. Phalloidin, EGF were from Sigma-Aldrich (St. Louis, MO, USA). Specific antibodies that recognized p-EGFR, p-Stat3, Stat3, p-JAK2, JAK2, p-Akt, p-Erk, Akt, Erk, β-actin were obtained from Cell Signaling Technology (Beverly, MA, USA) and EGFR, E-cadherin, N-cadherin from Santa Cruz Biotechnology (Dallas, TX, USA).

Cell culture. A549 and fibroblast cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were grown in DMEM medium (HyClone, Waltham, MA, USA) containing 10% fetal bovine serum (HyClone) and 1% antibiotics (100 mg/l streptomycin, 100 U/ml penicillin) at 37˚C in a 5% CO_2 atmosphere. Cells were suspended by Trypsin-EDTA (HyClone) and separated $1.5x10^5$/ml at each plate, every 48 h.

Invasion assay. Quantitative cell invasion assays were performed using a modified Boyden chamber (Costar, Corning Inc., Corning, NY, USA) with 8.0 μm pore polycarbonate membrane inserts with Matrigel-coated 24-well plates as described previously. The lower chamber was filled with the complete medium for control and complete medium with TJE at the indicated dose and EGF (see Figures). The A549 cells ($5x10^4$ cells/ml) in serum-free medium were added into the upper chamber. The cells were allowed to invade for 24 h at 37˚C. The non-invasive cells were removed from the upper surface of the membrane by scraping with a cotton swab, and the invasive cells were stained with crystal violet and photographed under a bright field microscope (Carl Zeiss, Thomwood, NY, USA).

Immunofluorescence staining with wound healing assay. Cells were seeded $2.5x10^6$/ml in 12-well plate with the cover glass, and incubated to 100% confluence. After the incubation, the wound in the cell monolayer in the center of the well was treated with TJE. After treatment, at the indicated time and dose at 37˚C in a 5% CO_2 atmosphere, the cells were fixed with 3.7% formaldehyde for 20 min and permeabilized with 0.2% Triton X-100 for 20 min for stress fiber staining. For staining of specific proteins, cells were fixed and permeabilized with 95% methyl alcohol for 15 min. Cells were washed with PBS twice and reacted with specific antibody overnight at 4˚C. Cells were washed with PBS twice and reacted to secondary antibody and stained with 0.1% Phalloidin-FITC for 40 min. Fluorescence was detected by confocal microscopy (Carl Zeiss).

3D cell culture (organotypic cell culture) for invasive cell detection. Human fibroblast cells were seeded on 0.3-μm pore size cell culture insert plate and incubation with Matrigel and type I collagen mixture. After cell mixture was detached from the insert plate, $2x10^5$/ml A549 cancer cells were placed in the mixture and incubated for 1 week in the complete medium. After incubation, the 3D cell formation medium was placed in the bottom well and cultured for 3 weeks while changing the medium every 2 days. The appropriate amount of TJE and EGF was added to the new medium, and place in the bottom well, and incubate for 1 week. Metastatic cancer cells were identified using hematoxylin and eosin (H&E) staining.

Egg preparation and cancer cell inoculation. Fertilized chicken eggs were purchased from a local hatchery and incubated for 8 days after breeding at 37˚C with 45% humidity. Eggs were cleaned with pre-warmed 70% ethanol and a small hole was drilled into the eggshell where the air sac is located. A 2-cm window was carefully opened for inoculation. The hole was then vacuumed to exclude air, thus creating space for the CAM. A total of $1x10^6$ cells re-suspended in 20 μl serum and antibiotics-free DMEM medium with Matrigel was added on the CAM. The window was then covered with medical film and the egg was placed back into the incubator. After five-day inoculation, silicon ring was implanted on micro-tumor tissue and TJE treated with or without EGF. Seven more days after incubation, micro-tumors were removed from CAMs, for metastasis study, specific tissue of developing chickens was harvested and stored at -80˚C before DNA extraction.

Subcellular protein fraction. A subcellular protein fraction kit was used (Thermo Fisher Scientific, Waltham, MA, USA). Cells were seeded at $1x10^6$/ml in 100-mm dish and incubated for 24 h. After the incubation, treated with the test compound for the indicated times (see Figures) at 37˚C in a 5% CO_2 atmosphere. After the incubation, subcellular proteins were separated according to the manufacturer's instructions. A separate protein was analyzed by western blotting.

Immunoprecipitation. We used sure-bead protein G magnetic beads kit (Bio-Rad, Hercules, CA, USA). Cells were seeded at $1x10^6$/ml in 100-mm plate and incubated for 24 h. After incubation, cells were treated with the test compound for 24 h at 37˚C in a 5% CO_2 atmosphere. Whole lysate and nucleus fraction proteins were incubated with specific antibody bound magnetic beads. Beads were washed using a magnetizer and PBS. Target proteins were eluted in 1X non-reducing sample buffer and analyzed by western blotting.

Protein dimerization. We used a BS3 (Thermo Fisher Scientific) protein crosslinker. Cells were seeded at $1x10^6$/ml

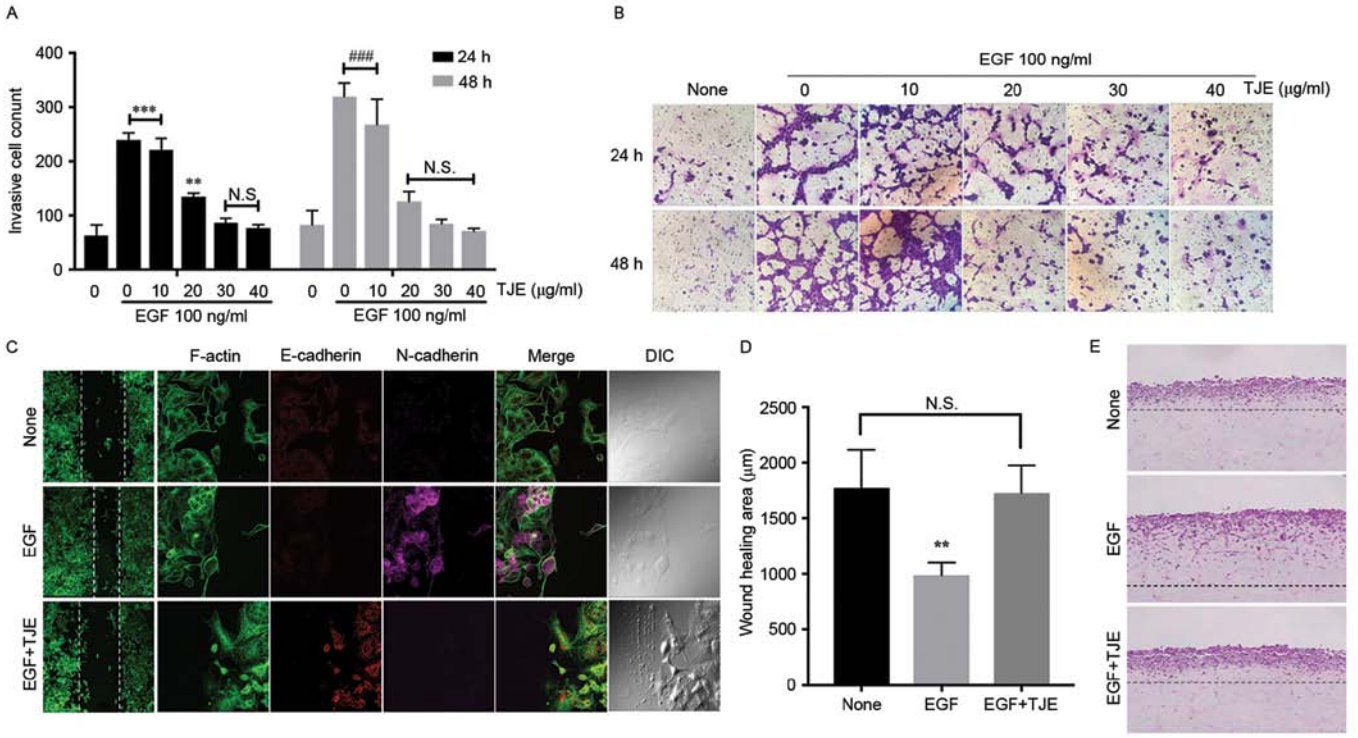

Figure 1. TJE suppresses cell migration and invasion in EGF-stimulated A549 cells. (A and B) Invasive cells were measured by invasion assay. Invasive cells were determined by counting cells in microscopic fields per sample. Compared to control; **P<0.01, ***P<0.001 in 24 h and compared to control in 48 h; $^{###}$P<0.001 (each experiment's n=3). N.S., not significant. (C and D) Anti-migration abilities were detected by wound healing assay with immunofluorescence staining. F-actin (green), E-cadherin (red), N-cadherin (violet). For the counter staining, we using a DIC image. The wound healing area was quantified using Prism after confirming the degree of reduction by using imageJ. Compared to control; **P<0.01 (each experiment n=3). N.S., not significant. (E) Invasive cancer cells were qualitatively identified using 3D cell culture.

in 100-mm dish and incubated for 24 h. After the incubation, treated with the test compound for the indicated times at 37˚C in a 5% CO_2 atmosphere. After the incubation, proteins were extracted with non-denaturing lysis buffer, and reacted with BS3 according to manufacturer's instructions. Protein dimerization was analyzed by western blotting.

Transient transfection with small interfering RNA. Small interfering RNA (siRNA) was purchased by Dharmacon (Chicago, IL, USA), and a Nucleofector (Lonza, Basel, Switzerland) for transfection. For transient transfection, $2x10^6$/ml cells were re-suspended in a transfection reagent with targeting siRNA. The siRNA was inserted by electric shock according to the manual provided by the manufacturer. After incubation for 24 h, cells were treated with the compound.

Western blotting. Cells were seeded at $1x10^5$/ml in 6-well plate and incubated for 24 h, and after the incubation, treated with the test compound for indicating times at 37˚C in a 5% CO_2 atmosphere. Cells were rinsed twice with ice-cold PBS and scraped with lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 1 mM PMSF) and subjected to the western blot analysis. The 1st antibody reaction was overnight at 4˚C and 2nd antibody for 75 min at room temperature with slow agitation.

Quantitative polymerase chain reaction (PCR). Total RNA was extracted using RiboEx (GeneAll Biotechnology, Seoul, Korea) according to the manufacturer's instructions, and cDNA was generated using ReverseAids cDNA synthesis kit (Thermo Fisher Scientific) according to the manufacturer's instructions. RT-PCR was performed with the following temperature profile: a pre-denaturation step of 10 min at 95˚C, followed by 35 cycles of 95˚C for 30 sec, annealing temperature for 30 sec and 72˚C for 30 sec and a final exposure at 72˚C for 10 min. qPCR was performed using qPCR greenstar master-mix (Bioneer, Seoul, Korea) and StepOne™ (Applied Biosystems, Foster city, CA, USA) for amplification and detection. A pre-denaturation step of 10 min at 95˚C, followed by 42 cycles of 95˚C for 20 sec, annealing and extension for 60 sec. Specific primer sequence for amplification was: forward, TAGATGCCCCCAAATCTCAG; reverse, GAGCT GCTCCATCTGTAGGG.

Statistical analysis. Invasive cells, wound healing area and gene expression were statistically analyzed using ANOVA test (Prism version 7; GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

TJE suppresses cell migration and invasion in EGF-stimulated A549 cells. To confirm the abnormal metastasis inhibition effect of TJE, the number of invasion cancer cells was counted after TJE treatment in EGF-stimulated A549 lung cancer cells. The concentration was increased, the number of metastatic cancer

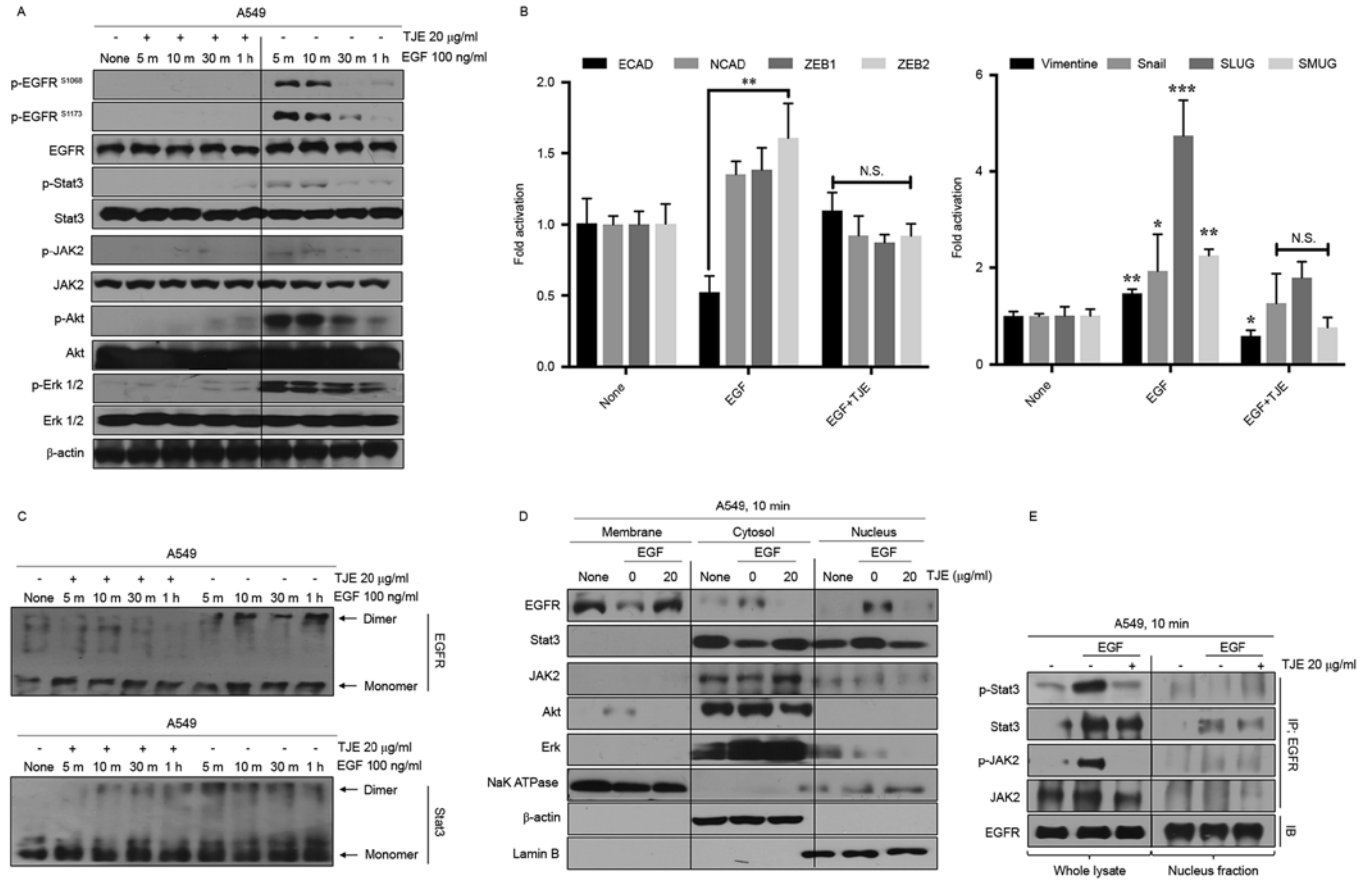

Figure 2. TJE regulates expression of EMT marker and activation of EGFR downstream signaling pathways. (A) The change of EGFR phosphorylation level was examined and its downstream protein phosphorylation levels were detected by western blotting. (B) EMT marker gene expression levels were examined by qPCR. Compared to control; *P<0.05, **P<0.01, ***P<0.001 in 24 h (each experiment's n=3). N.S., not significant. (C) The dimerization of EGFR and Stat3 was confirmed by western blotting after the reaction with Bs3. (D and E) The nucleus translocation of EGFR and JAK2-stat3 was confirmed by western blotting after protein fractionation to membrane-cytosol-nucleus. Co-binding of EGFR with JAK2-Stat3 was confirmed by western blotting via co-IP.

cells was reduced (Fig. 1A and B). Moreover, it was confirmed through 3D cell culture method that the extent of cancer cells that were transferred to the normal cell layer was reduced by the TJE treatment (Fig. 1E). Immunofluorescence analysis of E-cadherin, N-cadherin and F-actin with or without TJE treatment in EGF-stimulation. Despite the EGF-stimulation, TJE not only upregulated expression of E-cadherin but reduced N-cadherin expression. Moreover, EGF-induced cell migration activities, but there was a decrease in the migration area in TJE co-treated group (Fig. 1C and D).

TJE regulates expression of EMT marker and activation of EGFR downstream signaling pathways. To confirm the EGFR signaling regulation by TJE, we analyzed the activity of EGFR and its downstream signaling proteins such as Akt, Erk, Stat3 and JAK2 in EGF was treated with time after TJE treatment. Fig. 2A shows that the activity of EGFR and its downstream proteins were decreased by TJE treatment. Moreover, the expression of specific factors related to cancer cell metastasis such as SNAIL family, N-cadherin and vimentin was reduced while the expression of E-cadherin was found to be normal in TJE treatment group (Fig. 2B). In the case of EGFR and Stat3, which form a dimer in EGF-stimulation, dimer formation was inhibited in TJE treatment group (Fig. 2C). We confirm that TJE inhibited the translocation to the nucleus of EGFR and Stat3, and the co-binding of EGFR with Stat3 was also decreased (Fig. 2D and E).

TJE suppresses abnormal metastasis through the EGFR target pathways. As a result of comparing the inhibitory effect of the TJE against cancer cell abnormal metastasis in the state where a specific protein is knocked down using a siRNA transfection, the inhibition of invasive cells, metastasis-related gene expression and the change of wound healing area in TJE treatment group were not different compared with EGFR knockdown group. There was no difference in the JAK2 knockdown group. However, in the Stat3 knockdown group, the change was similar to that of the EGF only treatment group (Fig. 3B-D). In addition, the formation of stress fibers, which indicate the movement of cancer cells, was not significantly different from that of the EGFR knockdown group in the TJE treated group (Fig. 3A).

TJE inhibits cancer abnormal metastasis in CAM. To confirm the suppressed abnormal metastasis activity by TJE in an *ex vivo* model, we made a cancer cell implantation model in CAM. As shown in Fig. 4, EGF treatment group formed blood vessels in the cellular matrix with cancer cells. However, TJE co-treatment group did not form blood vessel or contract the cellular matrix. From quantification PCR

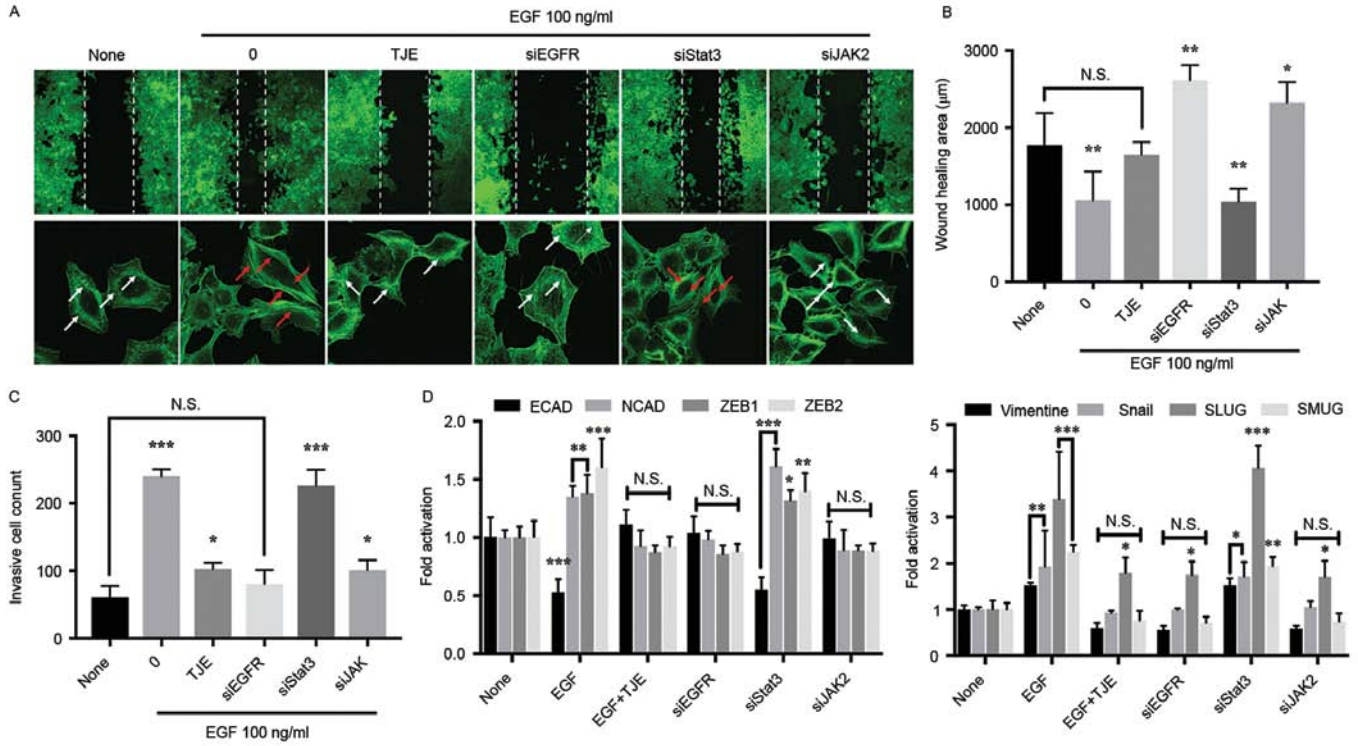

Figure 3. TJE suppresses abnormal metastasis through the EGFR target pathways. The specific protein was knocked down using siRNA and treated with the TJE in EGF-stimulated A549 lung cancer cells. (A and B) Anti-migration abilities were detected by wound healing assay with immunofluorescence staining. F-actin (green) and stress fiber are indicated by red arrows. The wound healing area was quantified using Prism after confirming the degree of reduction by using imageJ. Compared to control; *P<0.05, ***P<0.001 (each experiment's n=3). N.S., not significant. (C) Invasive cells were measured by invasion assay. Invasive cells were determined by counting cells in microscopic fields per sample. Compared to control; *P<0.05, **P<0.01, ***P<0.001 (each experiment n=3). N.S., not significant. (D) EMT marker gene expression levels were examined by qPCR. *P<0.05, **P<0.01, ***P<0.001 (each experiment n=3). N.S., not significant.

Table I. Primer sequences for the amplification of target genes.

Gene	Primer sequence (5'-3')	Amplification size (bp)	Annealing temp. (˚C)
E-cadherin	F: CGGACGATGATGTGAACACC R: TTGCTGTTGTGCTTAACCCC	213	60.0
N-cadherin	F: GACAATGCCCCTCAAGTGTT R: CCATTAAGCCGAGTGATGGT	179	59.5
Vimentin	F: GAGAACTTTGCCGTTGAAG R: TCCAGCAGCTTCCTGTAGGT	170	59.5
SNAIL	F: CCCCAATCGGAAGCCTAACT R: ACAGAGTCCCAGATGAGCA	157	60.0
SLUG	F: CTTTTTCTTGCCCTCACTGC R: GCTTCGGAGTGAAGAAATGC	224	59.0
SMUG	F: GTCCGCAGTCTTACGAGGAG R: CCAGCTTGAGGGTCTGAATC	159	60.0
ZEB1	F: TGGACTGAGTGTGGAAAAGC R: TGGTGATGCTGAAAGAGACG	237	60.0
ZEB2	F: TTCCTGGGCTACGACCATAC R: GCCTTGAGTGCTCGATAAGG	393	60.0
GAPDH	F: CAAGGTCATCCATGACAACTTTG R: GTCCACCACCCTGTTGCTGTAG	496	58.0

F, forward, R, reverse.

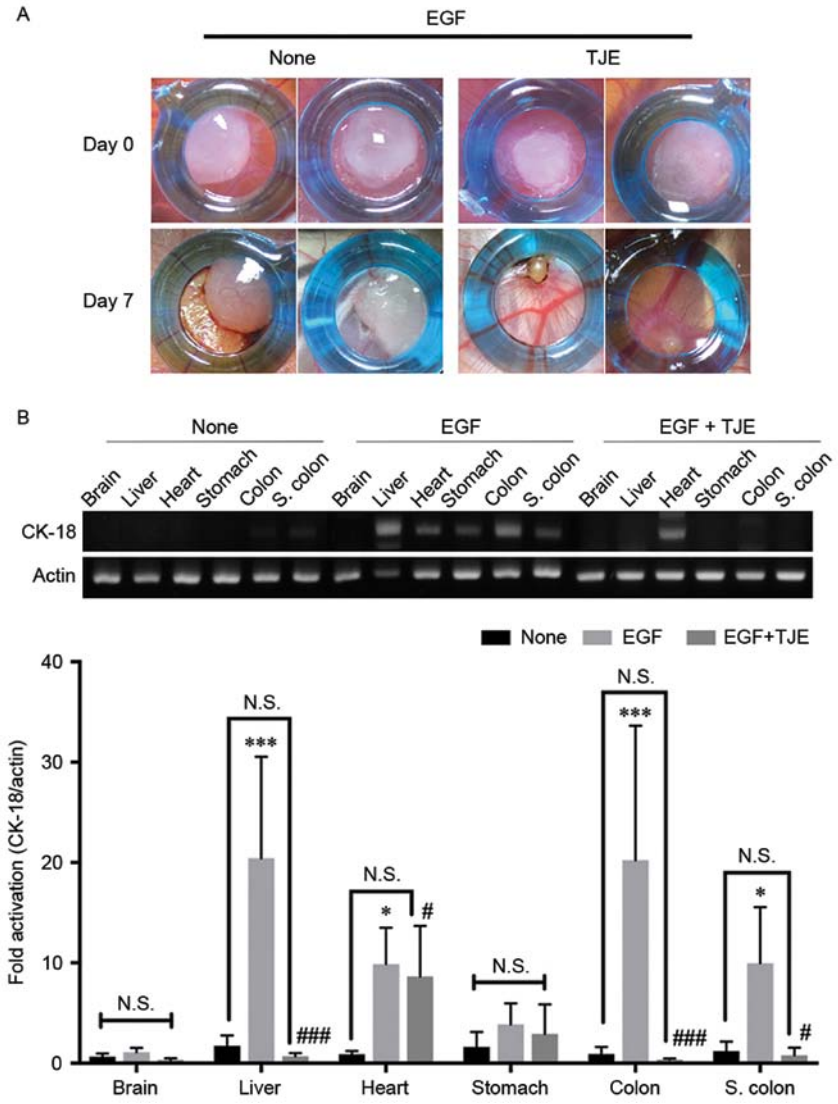

Figure 4. TJE inhibits cancer abnormal metastasis in CAM. (A) After the cancer cells had been placed on the CAM, the TJE and EGF were treated using O-ring. The image was taken 7 days after the treatment with the TJE and EGF. (B) RNA extracted from Chick's organ was quantitatively analyzed by qPCR using human-specific gene primer. Compared to control; *P<0.05, ***P<0.001 and compared to EGF-stimulated group; $^{\#}$P<0.05, $^{\#\#\#}$P<0.001 (each experiment n=3). N.S., not significant.

using Chick's organ tissue, the EGF treatment group cancer cells were detected with human-specific genes especially the brain and heart tissue. Despite the EGF stimulation in cellular matrix, TJE co-treatment group had reduced detection of human-specific genes and it is not detected similarly to that in normal Chick's organ tissue.

Discussion

The number of patients who die from lung cancer is steadily increasing worldwide. Previous studies have shown that smoking is the leading cause of lung cancer, and smoking cessation has become active worldwide (1-3). However, recent research found that lung cancer incidence can not be suppressed simply by quitting, and they found that another reason for lung cancer is outdoor pollution. It was found that many factors in the air induce over-activation of surface proteins such as growth factor receptors, and induce cancerization (4-6). In particularly, the over-activation of EGFR has been shown to induce abnormal proliferation and metastasis to normal organ resulting in the death of the patient. Thus, finding a substance that can inhibit the EGFR activation has become a very important research topic. In this study, we investigated the mechanism of inhibiting the cancer cell abnormal metastasis by EGFR inactivation through the TJE treatment. First of all, we examined the inhibitory effect of the TJE in the abnormal metastasis of cancer cells. We confirmed that the number of the metastatic cells was reduced concentration-dependently. In addition, the range of the wound healing area and metastasis-related protein expression were not different in the TJE treatment group when compared with the normal condition (Fig. 1). The activity of EGFR and its downstream proteins was also reduced when compared with the EGF-stimulated group (Fig. 2A).

Recent studies have shown that dimer formation of EGFR and Stat3 play a crucial role in nucleus translocation (18-20). We showed that the formation of dimer by EGF-stimulation was inhibited by the TJE treatment. We

confirmed that the intranuclear translocation of EGFR and the co-binding with Stat3 were inhibited by TJE treatment in EGF-stimulated A549 lung cancer cells (Fig. 2C and D). We examined the expression of cancer cell abnormal metastasis and metastasis-related factor compared with EGFR, Stat3 and JAK2 knockdown group using a siRNA transfection. The cancer cell metastasis and expression of related factors was decreased in EGFR knockdown group and TJE treated group when compared with EGF-stimulated group (Fig. 3). Moreover, we confirm that the formation of stress fiber, which is a cell metastasis marker, decreased. However, in the knockdown group of Stat3, the inhibition of cancer metastasis and the expression of related factors did not appear. This indicates that EGFR can induce cancer metastasis without going through Stat3. Previous studies have found that the activity of EGFR can induce cancer cell metastasis and proliferation through its own dimer formation without co-binding with Stat3 (18,21,22). Base on the above results, it was confirmed that the TJE that we secured not only regulates the EGFR signaling pathway but inhibits the cancer cell metastasis due to the EGFR-exclusive activity. In addition, the inhibitory effect of the TJE on cancer cell metastasis was confirmed by CAM assay. As a result, it was confirmed that human cancer cells were invasive to CAM organs in the EGF-stimulated group, whereas it did not appear in the TJE treated group even by EGF-stimulation (Fig. 4).

In conclusion, we suggest that the inhibitory effect of the TJE major fraction substance in the cancer abnormal metastasis is indicated by regulation of EGFR signaling pathway and suppression of its own activity through the targeting of EGFR. Therefore, we demonstrated that TJE has potential as an anticancer metastasis agent and may provide a substitute for chemotherapeutic drugs.

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