

# Delta-tocotrienol inhibits non-small-cell lung cancer cell invasion via the inhibition of NF- $\kappa$ B, uPA activator, and MMP-9

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**Background:** Delta-tocotrienol ( $\delta$ T), an isomer of vitamin E, exhibits anticancer properties in different cancer types including non-small-cell lung cancer (NSCLC). Yet, anti-invasive effects of  $\delta$ T and its underlying cellular mechanism in NSCLC have not been fully explored. Matrix metalloproteinase 9 (MMP-9)-based cell migration and invasion are critical cellular mechanisms in cancer development. The current evidence indicates that MMP-9 is upregulated in most patients, and the inhibition of MMPs is involved in decreasing invasion and metastasis in NSCLC. Therefore, its suppression is a promising strategy for attenuating cell invasion and metastasis processes in NSCLC.

**Purpose:** The aim of this study was to evaluate the possibility of MMP-9 inhibition as the underlying mechanism behind the antimetastatic properties of  $\delta$ T on NSCLC cells.

**Methods:** The effects of  $\delta$ T on cell proliferation, migration, invasion, adhesion, and aggregation capabilities were investigated using different cell-based assays. An inhibitory effect of MMP-9 enzyme activity with  $\delta$ T was also identified using gel zymography. Using real-time PCR and Western blot analysis, a number of cellular proteins, regulatory genes, and miRNA involved in the Notch-1 and urokinase-type plasminogen activator (uPA)-mediated MMP-9 pathways were examined.

**Results:** The study found that  $\delta$ T inhibited cell proliferation, cell migration, invasion, aggregation, and adhesion in a concentration-dependent manner and reduced MMP-9 activities. Real-time PCR and Western blot analysis data revealed that  $\delta$ T increased miR-451 expressions and downregulated Notch-1-mediated nuclear factor- $\kappa$ B (NF- $\kappa$ B), which led to the repressed expression of MMP-9 and uPA proteins.

**Conclusion:**  $\delta$ T attenuated tumor invasion and metastasis by the repression of MMP-9/uPA via downregulation of Notch-1 and NF- $\kappa$ B pathways and upregulation of miR-451. The data suggest that  $\delta$ T may have potential therapeutic benefit against NSCLC metastasis.

**Keywords:** metalloproteinases, miR-451, lung cancer, A549, H1299, metastasis, cell migration, vitamin E

## Introduction

Lung cancer is the leading cause of estimated cancer deaths in the USA.<sup>1</sup> Non-small-cell lung cancer (NSCLC) accounts for 85% of all lung cancer cases and can be classified into three subtypes: squamous cell carcinoma, large cell carcinoma, and adenocarcinoma. The initial stage of NSCLC has a 5-year survival rate of 55%, but this rate reduces to <4% for cases diagnosed with distant metastasis.<sup>1</sup> With current advances in the understanding of mechanisms of cancer invasion and metastasis, it is becoming clear that matrix metalloproteinases (MMPs), an enzyme with 21 subtypes in humans,<sup>2,3</sup> have a strong association with local invasion or distant metastasis.<sup>2</sup> Several studies ranging from cell culture<sup>4</sup> to clinical investigations<sup>5-7</sup> have reported

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the inhibition of MMPs in conditions of decreasing invasion and metastasis in NSCLC.

Matrix metalloproteinase 9 (MMP-9), a subtype of MMPs, regulates cell migration, angiogenesis, adhesion, aggregation, and immune response in cancer.<sup>8–10</sup> In this process, MMP-9 is mainly responsible for degrading collagen type IV and elastin in basal membranes, facilitating lung cancer metastasis. High levels of MMP-9 have also been reported in the serum of lung carcinoma patients.<sup>11</sup> Therefore, the modulation of MMP-9 protein expressions and their activities would be excellent therapeutic targets for the inhibition of invasion and metastasis processes in NSCLC. Urokinase-type plasminogen activator (uPA), a serine proteinase, binds to the urokinase-type plasminogen activator receptor (uPAR) and transforms inactive plasmin and other proteases, including MMP-9, into their active forms. Regulating uPA is one of the major approaches that can directly modulate MMP-9 activities in cancer.<sup>12</sup> The uPA pathway includes several proteins such as serine protease, uPAR, and the endogenous inhibitors, plasminogen activator inhibitors 1 and 2.<sup>13</sup> The uPA system enables transformation of zymogen plasminogen into plasmin in the process of extracellular matrix (ECM) degradation.<sup>14</sup> The plasmin, then, facilitates the conversion of inactive pro-MMP-9 into active MMP-9. Increased expression of the uPA system has been reported in NSCLC tissue as compared to normal lung tissue.<sup>15</sup> Using antisense technology, Rao et al<sup>16</sup> showed that the inhibition of uPA and MMP-9 might be an excellent anti-invasion and antimetastatic approach for cancer gene therapy in lung cancer. Although the inhibition of uPA and/or MMP-9 is a possible therapeutic target for preventing local invasion or distant metastases in lung cancer, uPA and MMP-9 pathways have shown cross talks with external factors, namely transcription factors (TFs) and miRNA. These cross talks have made it more complex to modulate MMP-9 directly.

Tong et al<sup>17</sup> showed that nuclear factor- $\kappa$ B (NF- $\kappa$ B), a TF involved in cancer initiation and progression, directly binds with the uPA promoter *in vitro*. The same study showed that the inhibition of NF- $\kappa$ B activities decreased cell invasion and uPA synthesis in NSCLC cells. The MMP-9 promoter has binding sites for NF- $\kappa$ B.<sup>18</sup> Inability of NF- $\kappa$ B to bind with the MMP-9 promoter has been shown to decrease MMP-9 synthesis.<sup>18–21</sup> Moreover, it has been suggested that the NF- $\kappa$ B signaling pathway contributes to the progression of metastasis by regulating MMP-9 in colorectal cancer,<sup>22</sup> prostate cancer,<sup>23</sup> renal cancer,<sup>24</sup> ovarian cancer,<sup>25</sup> and head and neck cancer.<sup>26</sup>

In addition, elevated miR-451, a small noncoding RNA that controls gene expression through sequence-specific binding to target mRNA, was found to decrease cell invasion and metastasis, with corresponding decrease in MMP-9 expression levels in primary liver cancer.<sup>27</sup> Elevated miR-451 expressions were also found to suppress cell proliferation and metastasis in A549 lung cancer cell lines.<sup>28</sup> However, the role of NF- $\kappa$ B and/or uPA in mediating MMP-9 function and their interaction with miR-451 in cell invasion and metastasis in lung cancer remains generally unclear.

Previous studies from our laboratory showed that delta-tocotrienol ( $\delta$ T), an isomer of vitamin E found naturally in food sources, inhibits NF- $\kappa$ B signaling pathways via the downregulation of Notch-1, thereby decreasing the proliferation and metastatic/invasion potential, while inducing apoptosis of NSCLC cells in a concentration- and time-dependent manner.<sup>28–33</sup> This study investigated the antimetastatic effect of  $\delta$ T on NSCLC cells with the hypothesis that the MMP-9-dependent invasion and metastasis of NSCLC cells are inhibited by the suppression of Notch-1-mediated NF- $\kappa$ B and uPA pathways and induction of miR-451.

## Materials and methods

### Reagents and antibodies

American River Nutrition (Delta Gold, MA, USA) donated  $\delta$ T for this experiment. Dimethyl sulfoxide (DMSO), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), bovine serum albumin (BSA), and bicinchoninic acid (BCA) assay were purchased from Thermo Scientific Pierce (St Louis, MO, USA). Two NSCLC cell lines, A549 and H1299, were purchased from American Type Culture Collection (Manassas, VA, USA). Roswell Park Memorial Institute (RPMI) medium (Mediatech, Manassas, VA, USA) was used as a growth medium for cells. Antibodies  $\beta$ -actin, MMP-9, Hairy and Enhancer of Split-1 (HES-1), Notch-1 (Cell Signaling Technology, Danvers, MA, USA), and uPA (Santa Cruz Biotechnology Inc., Dallas, TX, USA) were used for Western blot analysis.

### Cell culture and treatments

A549 and H1299 were cultured in RPMI medium and supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin in 5% CO<sub>2</sub> at 37°C. The cell growth medium was changed every 2–3 days. Adherent cells were detached using trypsin–EDTA and centrifuged at 1,500 rpm. The treatment media were prepared by mixing  $\delta$ T (10, 20, or 30  $\mu$ M <0.01% DMSO as a vector) in the RPMI medium, whereas the control was grown in RPMI media containing <0.01% DMSO.

## Cell viability assay

A 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay was conducted to analyze the antiproliferative effects of  $\delta T$  on the above NSCLC cell lines;  $5 \times 10^4$  of A549 and H1299 cells were grown in 96-well plates overnight. Afterward, the media were changed, and the cells were treated with fresh cell growth medium containing  $<0.01\%$  DMSO (control) or treatment medium containing 10, 20, 30, and 40  $\mu M$  of  $\delta T$ . After 72 hours of treatment, 20  $\mu L$  of CellTiter 96<sup>®</sup> Aqueous Reagent from Promega (Madison, WI, USA) was mixed into each well and incubated for 2 hours. The absorbance at 490 nm was then measured using the Bio-Tek ELx800<sup>™</sup> plate reader (Winooski, VT, USA). Each variant of the experiment was performed with six replicates.

## Cell invasion assay

The ability of A549 and H1299 lung cancer cells to pass through filters coated with Matrigel<sup>®</sup> was assessed by a BD Biocoat<sup>™</sup> invasion kit (BD Biosciences, San Jose, CA, USA);  $2.5 \times 10^5$  of both cell lines were prepared in an aqueous environment with PBS and resuspended into each upper chamber of 6-well plates with a serum-free medium in the presence or absence of treatment; 3 mL of growth medium with 15% FBS was added to lower chambers and incubated for 20 hours. After incubation, the attached cells on the upper side of the Matrigel surface (in the upper chamber) were wiped with a cotton swab, and 50  $\mu L$  of MTS reagent was added to the lower chamber. The absorbance of the media contained in the lower chamber was measured at 450 nm using the Bio-Tek ELx800 plate reader. Each experiment was carried out in triplicate.

Again, the same procedure was continued up to 20 hours of incubation in a BD Biocoat invasion kit. The cells attached to the Matrigel upper surface were then wiped using a cotton swab, and the cells invading through the Matrigel to the lower surface were fixed with 4% paraformaldehyde and stained with 2% crystal violet. The invading cells on the Matrigel lower surface were counted and photographed with the camera attached to the light microscope. The data were shown in pictures as stained cells attached to the bottom of Matrigel surface from randomly selected areas.

## Cell migration assay

To detect the migration rate of cells using a wound healing assay, A549 and H1299 cells were grown in a 6-well dish at a concentration of  $1 \times 10^6$  cells per well. After the cells had been incubated for 30 hours, the growth media were replaced with or without treatment media. Scratches were made using a

pipette tip (100  $\mu L$ ) and photographed immediately (time 0). After a 20-hour incubation with treatment media at 0, 10, 20, and 30  $\mu M$ , all the wound areas were washed with PBS three times to ensure that no loosely held cells were attached. The plate was imaged, and the width of the scratch was measured by using a Nikon H 600L microscope (Nikon Corporation, Tokyo, Japan) at 10 $\times$  objective.

## Cell aggregation assay

To investigate the antiaggregation capabilities of  $\delta T$ , a single-cell suspension was created using cell trypsinization technique;  $2 \times 10^5$  cells in 1 mL of RPMI growth medium with or without  $\delta T$  (10, 20, and 30  $\mu M$ ) concentration were placed in polystyrene microtubes and were gently shaken every 5 minutes for 1 hour at 37°C. After 1 hour, glutaraldehyde was added at a final concentration of 2% (v/v) to halt the cell aggregation. Homotypic aggregation was assessed and photographed by a LUNA<sup>™</sup> automated cell counter (Logos Biosystems, Gyeonggi-do, South Korea). The aggregates were counted per  $cm^2$ , and significant differences between control and treatments were evaluated using ezANOVA software.

## Cell adhesion assay

The cell lines A549 and H1299 (1,000,000) were pretreated with or without  $\delta T$  concentrations (control, 10, 20, and 30  $\mu M$ ) for 72 hours. The cell lines were suspended in cell growth medium to form a single-cell suspension, and  $2 \times 10^6$  cells/mL ( $2 \times 10^5$  cells/well) were seeded into a 96-well cell culture plate precoated with Matrigel. After 1 hour of incubation, the wells were washed three times with PBS to eliminate nonadherent cells, and 100  $\mu L$  of RPMI medium with 20  $\mu M$  MTS reagent was added into each well for an additional 2 hours. As a measure of a number of cells, the absorbance at 570 nm was taken on an ELx800 plate reader. Significant differences between control and treatments were evaluated using ezANOVA software.

## Zymogram gel assay

To investigate the activity of MMP-9, A549 and H1299 cells were plated in a six-well plate with or without treatment by  $\delta T$  in FBS-free RPMI media. The media of all groups were removed and concentrated into 10 $\times$  media by using 3-KDa Amicon<sup>®</sup> centrifugal filters (MilliporeSigma, Burlington, MA, USA). Next, the total protein concentrations of the concentrated media were estimated by a Pierce BCA protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) and

normalized to 2,000 mg in the sample. Zymogram precast gel (Bio-Rad Laboratories) with gelatin coating was used as a separation gel. One part of sample was mixed with two parts of zymogram sample buffer. Samples were loaded in the gel, and the gel was run with 1× Tris–glycine–sodium dodecyl sulfate (SDS) buffer at 200 V. When the tracking dye (bromophenol blue) touched the end of the gel, the gel was removed from the apparatus. Then, the gel was incubated in the renaturing buffer (100 mL for one or two mini-gels) with gentle shaking for 30 minutes at 25°C. Afterward, the zymogram renaturing buffer was exchanged with 1× developing buffer, and the gel was equilibrated for 30 minutes at room temperature with gentle agitation, followed by incubation with fresh 1× zymogram developing buffer at 37°C overnight for maximum sensitivity. The gel was stained with Coomassie blue R-250 for 30 minutes to 1 hour the next day. Stain concentration of 0.5% (w/v in 40% methanol and 10% acetic acid) was used, and the gels were destained with Coomassie R-250 destaining solution (methanol/acetic acid/water, 40:10:50). Where the MMP-9 had digested the substrate, zones of MMP-9 activity appeared as clear bands against a dark blue background.

## Western blot analysis

To investigate the protein expression in signaling pathways of A549 and H1299 cells with  $\delta$ T exposure, 1,000,000 cells were plated per 100-mm dish and incubated for 24 hours. The cells were then grown for 72 hours with treatment and control medium. Next, the cells were lysed in the cold 1× cell lysis buffer (Cell Signaling Technology) for 30 minutes on ice with 1× protease inhibitor (Cell Signaling Technology), and protein concentrations were calculated using a Pierce BCA protein assay kit. Subsequently, 50 mg of total cell lysates was mixed with same amounts of 4× Laemmli buffer (Bio-Rad Laboratories), and the samples were loaded onto a 10% SDS–polyacrylamide gel. The gel was electrophoretically transferred to a polyvinylidene difluoride (PVDF) transfer membrane (Whatman, Clifton, NJ, USA) using a transfer buffer (25 mM Tris, 190 mM glycine, and 20% methanol) after gel electrophoresis. The PVDF membranes were incubated for 2 hours at room temperature with 5% BSA in 1× Tris-buffered saline buffer containing 0.1% Tween (TBS-T) and then incubated overnight at 4°C with primary antibodies (1:1,000). The membranes were washed three times with TBS-T and incubated with the secondary antibodies (1:5,000) containing 2% BSA at room temperature for 2 hours. The signal strength was then measured in an image with Chemi-Doc™ XRS system (Bio-Rad Laboratories).

## Quantitative real-time PCR (qRT-PCR)

For this process, 1,000,000 A549 and H520 cells were seeded per 100-mm dish per plate and incubated for 24 hours. Subsequently, the culturing media were replaced with treatment or control medium and incubated for another 72 hours. The RNA was then extracted using RNeasy® Mini Kit from Qiagen (Valencia, CA, USA); 1,000 ng of total RNA from each sample was subjected to first-strand cDNA synthesis using high-capacity RNA to cDNA master mix (Applied Biosystems, Foster City, CA, USA) in a total volume of 50 mL. qRT-PCR was performed as part of gene expression analysis. The appropriate primers – sense of MMP-9, 5'-CAACATCACCTATTGGATCC-3', antisense of MMP-9, 5'-CGGGTGTAGAGTCTCTCGCT-3', sense of  $\beta$ -actin, 5'-TGGTCTCTGGGCATCTCAGGC-3', antisense of 5'-GGTGAACCTGCTGTTGCCCTCA-3' – were used for PCR amplification. Diluted cDNA (2  $\mu$ L) and 2  $\mu$ L of each reverse and forward primer and 12.5  $\mu$ L master mix (SYBR GREEN PCR Master Mix; Applied Biosystems, Warrington, UK) were used in each 25  $\mu$ L PCR performed in Eppendorf MasterCycler realplex 4 (Eppendorf, Hauppauge, NY, USA) at 25°C for 10 minutes, followed by 48°C for 30 minutes, and 95°C for 5 minutes. Expression values were normalized with  $\beta$ -actin. Each gene expression was tested in triplicate.

## miRNA-451 expression analysis

To examine the miRNA-451 expression, the total RNA was extracted from the cultured cells using RNeasy Mini Kit from Qiagen according to the manufacturer's protocols. The concentration and purity of the RNA were determined using the NanoDrop 2000 system (Thermo Fisher Scientific, Tokyo, Japan). SYBR GREEN PCR Master Mix (Applied Biosystems, Warrington, UK) was used for the qRT-PCR according to the manufacturer's instructions. U-6 primers from miScript miRNA PCR (Qiagen) were used as an endogenous reference, and its levels were used for normalization.

## NF- $\kappa$ B-binding activity

To determine the NF- $\kappa$ B TF-binding activity, A549 and H1299 cells were seeded in Petri dishes and incubated for 24 hours. The cells were then treated with or without  $\delta$ T. After 72 hours of treatment, they were collected and washed with the PBS. The nuclear protein extraction was performed with an NE-PER Nuclear and Cytoplasmic Extraction Reagent kit (Pierce Biotechnology, Rockford, IL, USA) based on the manufacturer's protocols. Nuclear protein concentrations were determined using the Pierce BCA protein assay kit (Pierce Biotechnology), and an NF- $\kappa$ B filter plate assay



kit (Signosis, Sunnyvale, CA, USA) was used to determine the NF- $\kappa$ B DNA-binding ability. The assay was performed according to the manufacturer's protocol. The biotin-labeled DNA sequence of NF- $\kappa$ B was mixed with 3  $\mu$ g of nuclear extract to make an NF- $\kappa$ B–DNA-binding complex. Then, 10  $\mu$ L TF-binding buffer mix, 2  $\mu$ L NF- $\kappa$ B probe, and 3  $\mu$ g of distilled water were added to each sample, bringing the total volume to 20  $\mu$ L. The filter plate was used to retain the bound NF- $\kappa$ B probe, while the unbound NF- $\kappa$ B probe was filtered out. The bound, prelabelled NF- $\kappa$ B probe was then eluted from the filter, collected, and transferred to a hybridization plate for quantitative analysis. The NF- $\kappa$ B probe was further detected using streptavidin–horseradish peroxidase, and the luminescence of the probe was measured using Bio-Tek Synergy Microplate Reader.

### Statistical analysis

The data were expressed as the mean  $\pm$  SD of the mean. Differences were evaluated by using the one-way analysis of variance followed by Tukey honestly significant difference multiple comparison tests using the ezANOVA software.  $P < 0.05$  was considered to specify a statistically significant change.

## Results

### Antiproliferative effect of $\delta$ T

The MTS assay showed a concentration-dependent reduction in cell proliferation in both A549 and H1299 cells with the addition of  $\delta$ T. A reduction of ~5%, 40%, 91%, and 92% of cell growth was observed after 72 hours of incubations with 10, 20, 30, and 40  $\mu$ M of  $\delta$ T, respectively, compared with control A549 cells. Similarly, the H1299 cell line showed ~20%, 16%, 53%, and 93% cell growth inhibition, respectively, under the same conditions and same doses of  $\delta$ T and (Figure 1A).

### Inhibition of cell invasion and migration by $\delta$ T

We observed dose-dependent anti-invasive effects of  $\delta$ T in both A549 and H1299 cells at concentration levels ranging from 0 to 30  $\mu$ M in the Matrigel invasion assay (Figure 1B). At 10, 20, and 30  $\mu$ M concentrations of  $\delta$ T, cell invasion was reduced by ~10%, 35%, and 65% in A549 cell lines, respectively, after 72-hour incubation. Similarly, H1299 cells showed ~35%, 45%, and 70% inhibition of cell invasion under the same conditions. For further confirmation, microscopic images of the migrated cells through the membrane were taken, stained, and analyzed. In A549, as compared to the untreated cells, a small number of treated cells were observed

to migrate through the membrane. Hardly any invaded cells could be observed upon the treatment of H1299 cells at 30  $\mu$ M of  $\delta$ T ( $P < 0.05$  at 30  $\mu$ M treatment; Figure 1B). The wound healing assay was completed to explore the migration of A549 and H1299 cells after  $\delta$ T treatment. As shown in Figure 1C,  $\delta$ T significantly inhibited the migration of cells after 20 hours of treatment with 30  $\mu$ M  $\delta$ T.

### Inhibition of cell aggregation and adhesion capabilities by $\delta$ T

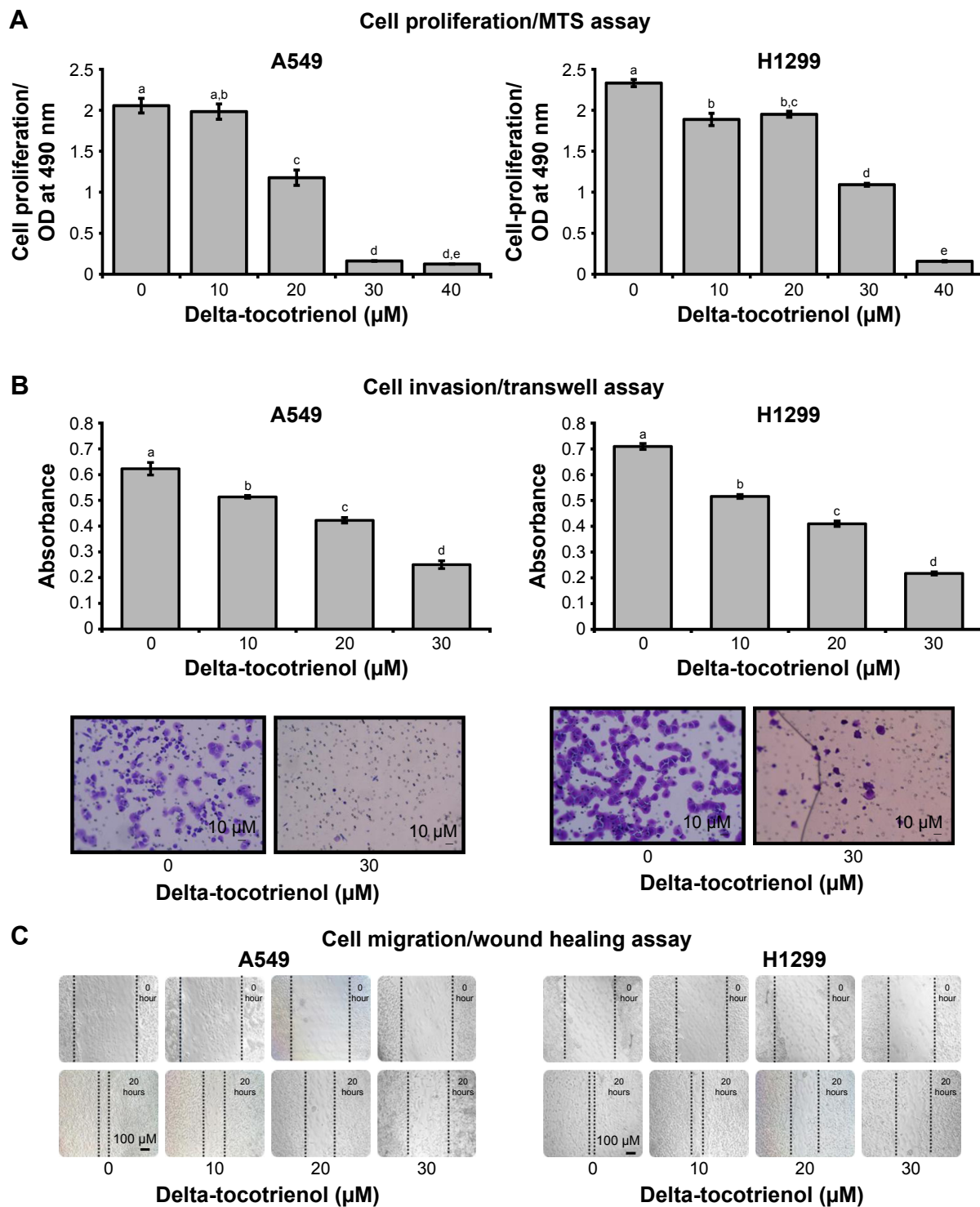
As shown in Figure 2A,  $\delta$ T inhibited cell aggregation at 30  $\mu$ M of  $\delta$ T in both A549 and H1299 cells. To test the effects of  $\delta$ T on cell adhesion to the Matrigel surface, A549 and H1299 cells were exposed with  $\delta$ T for 72 hours, and adhesion capacities to Matrigel surface were measured. We observed a dose-dependent inhibition of cell adhesion in both A549 and H1299 cells (Figure 2B).

### $\delta$ T suppressed expression of MMP-9

Since metalloproteinase plays a central function in cell invasion, we then performed the zymography assay to compare the action of MMPs in treated and control cells. A dose-dependent decrease in the MMP-9 activity resulted in the zymography assay with  $\delta$ T (Figure 3B) at concentrations of 0, 10, 20, and 30  $\mu$ M. TNF- $\alpha$ -treated cells served as a positive control. The results from the Western blot analysis indicate that  $\delta$ T inhibits MMP-9 protein expressions in a concentration- and time-dependent manner compared with the control in both A549 and H1299 cells (Figure 3A). In order to verify whether the suppression of MMP-9 protein expression resulted from decreasing m-RNA expression, real-time PCR analysis was performed. PCR is a gold standard for gene expression analysis, and it has been widely used for gene expression analysis in a variety of study models including cells, microbes,<sup>34–36</sup> and animals.<sup>37</sup> We observed that the mRNA level of MMP-9 was reduced significantly in a dose-dependent manner, compared with the control group (Figure 3D), in both A549 and H1299 cells after the treatment with  $\delta$ T for 72 hours. The  $\delta$ T-mediated change in the mRNA levels of MMP-9 was correlated with the protein level expression of MMP-9, as indicated by the results from the Western blot analysis, suggesting that  $\delta$ T may regulate MMP-9 expression at transcription levels from TFs, upstream signaling pathways, or other factors such as miRNA.

### $\delta$ T increases expression of miR-451

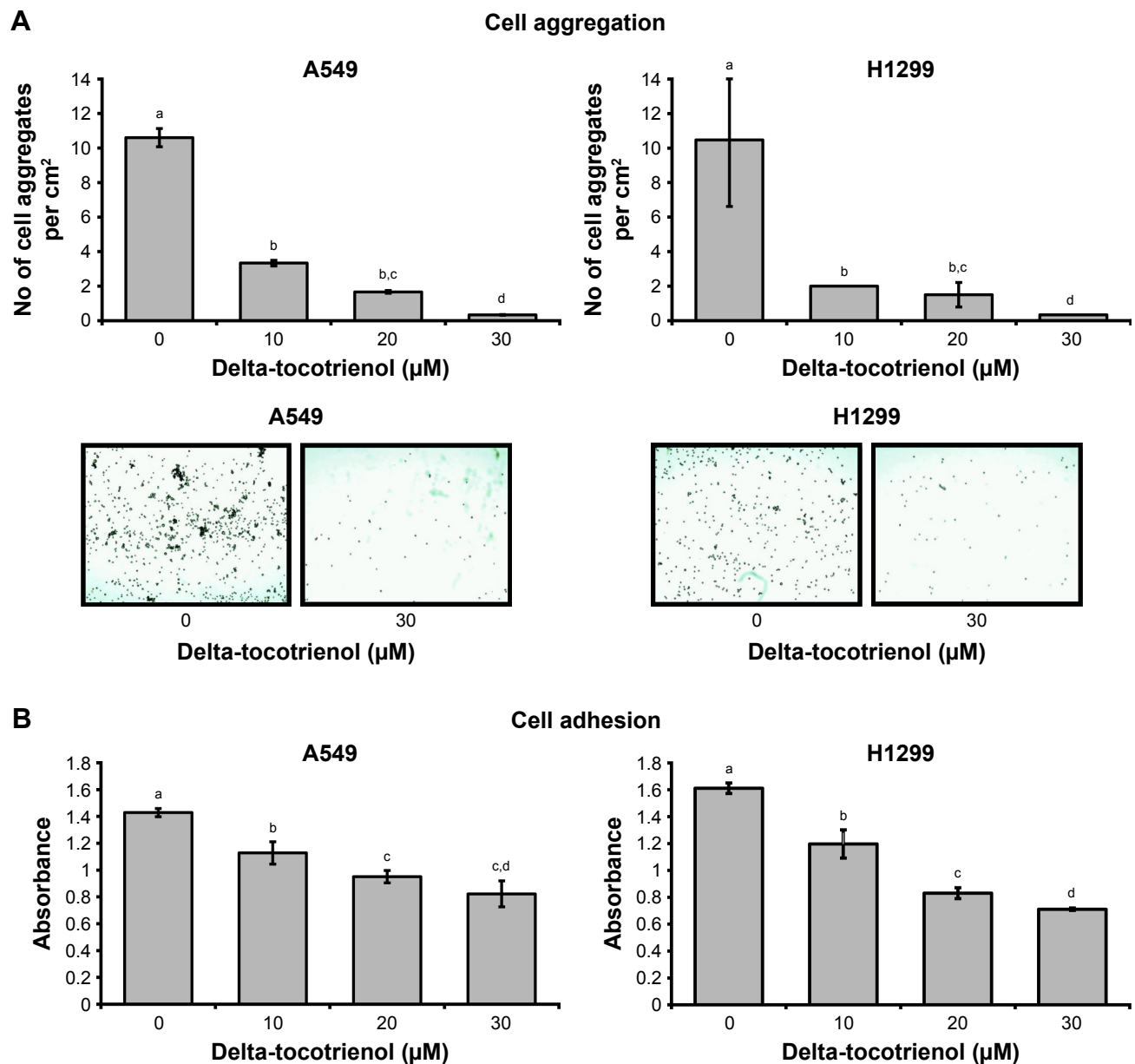
Accumulating evidence showed that miR-451 plays a key role in different aspects of carcinogenesis in lung cancer



**Figure 1**  $\delta$ T inhibits cell proliferation, invasion, and cell migration in NSCLC cells.

**Notes:** (A) A549 and H1299 cells were treated with various concentrations of  $\delta$ T (0–40  $\mu$ M) for 72 hours; then, cell viability was measured by MTS assay; vertical bars indicate the mean absorbance  $\pm$  SD (n=6). (B) Cell invasion was examined by transwell assay. After incubation for 20 hours, the cells that had invaded to the lower chamber were measured from MTS-based color development (upper panel); vertical bars indicate the mean absorbance  $\pm$  SD (n=3). Invaded cells also were fixed and stained with crystal violet dye and captured by the microscope (lower panel); representative images are presented (100 $\times$ ); scale bars represent 10  $\mu$ m. Purple color patches indicate invaded cells, whereas small dots indicate the pores in the Matrigel surface. (C) Cell migration was examined by wound healing assay; representative images at 20 hours after scratching are presented (10 $\times$ ); scale bars represent 100  $\mu$ m. Different superscript letters on bar graph indicate significant difference ( $P < 0.05$ ) compared with the other treatment groups as assessed by one-way ANOVA followed by Tukey HSD method as a multiple comparison test. In contrast, no significant difference between the treatment groups is indicated by the letters shared in common.

**Abbreviations:** ANOVA, analysis of variance;  $\delta$ T, delta-tocotrienol; HSD, honestly significant difference; MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium); NSCLC, non-small-cell lung cancer.



**Figure 2**  $\delta$ T inhibits cell aggregation and adhesion in A549 and H1299 cells.

**Notes:** (A) Cell aggregation capabilities of A549 and H1299 cells upon the  $\delta$ T treatment were examined by cell aggregation assay. Vertical bars indicate the cell aggregates per cm<sup>2</sup>  $\pm$  SEM (n=3); photographic views of A549 and H1299 cell aggregates after treating with/without  $\delta$ T are shown in the lower panel. Representative images are presented (2 $\times$ ). (B) Cell adhesion properties of A549 and H1299 cells were examined by measuring the percentage of adhering cells into Matrigel surface upon the  $\delta$ T treatment; vertical bars indicate the mean absorbance (number of cells on Matrigel surface upon the  $\delta$ T treatment)  $\pm$  SD (n=3). Different superscript letters on bar graph indicate significant difference ( $P < 0.05$ ) compared with the other treatment groups as assessed by one-way ANOVA followed by Tukey HSD method as a multiple comparison test. In contrast, no significant difference between the treatment groups is indicated by the letters shared in common.

**Abbreviations:** ANOVA, analysis of variance;  $\delta$ T, delta-tocotrienol; HSD, honestly significant difference; SEM, standard error of the mean.

including tumor growth, invasion, radioresistance, and chemoresistance.<sup>38–41</sup> A recent case analysis with 370 NSCLC incidents revealed that miR-451 expression was lower in carcinoma tissues than in paired background lung tissues.<sup>42</sup> The same study also revealed that the miR-451 expression was inversely correlated with macrophage migration inhibitory factor expression, a factor that induces the expression

of MMP-9.<sup>42</sup> In another study, A549 cells transfected with miR-451 showed reduced cell invasion and metastasis compared with the control by MTT transwell invasion assay and wound healing assay, respectively. Cancer metastasis factors (MMP-9, VEGF, and CXCR4) showed corresponding reduction in protein expression by Western blot analysis.<sup>43</sup> This evidence suggests the influence of miR-451 on MMP-9

expression. Therefore, we evaluated the differences in miR-451 expression upon  $\delta$ T treatments in A549 and H1299 cells. Indeed, we found that miR-451 expression increased by 2- and 3.5-fold upon treatment with  $\delta$ T in A549 and H1299 cells, respectively, as compared to control cells (Figure 3E). These results suggest that  $\delta$ T possibly inhibits the cell invasion of NSCLS via increased expression of miR-451, which may further mediate the expression of MMP-9 in A549 and H1299 cells.

### $\delta$ T inhibits expression uPA/Notch-1 pathway proteins

In the cell metastasis process, MMP-9 and/or their activators (uPA) play a major role in making the interaction between cells and the ECM.<sup>44</sup> Elevated expression levels of the uPA and MMP-9 were reported in NSCLC tissue as compared to normal lung tissue,<sup>15</sup> indicating that they may be facilitators for the metastasis process. Infection with Ad-uPAR-MMP-9 caused a decrease in the expression of uPA and MMP-9 by >90% with

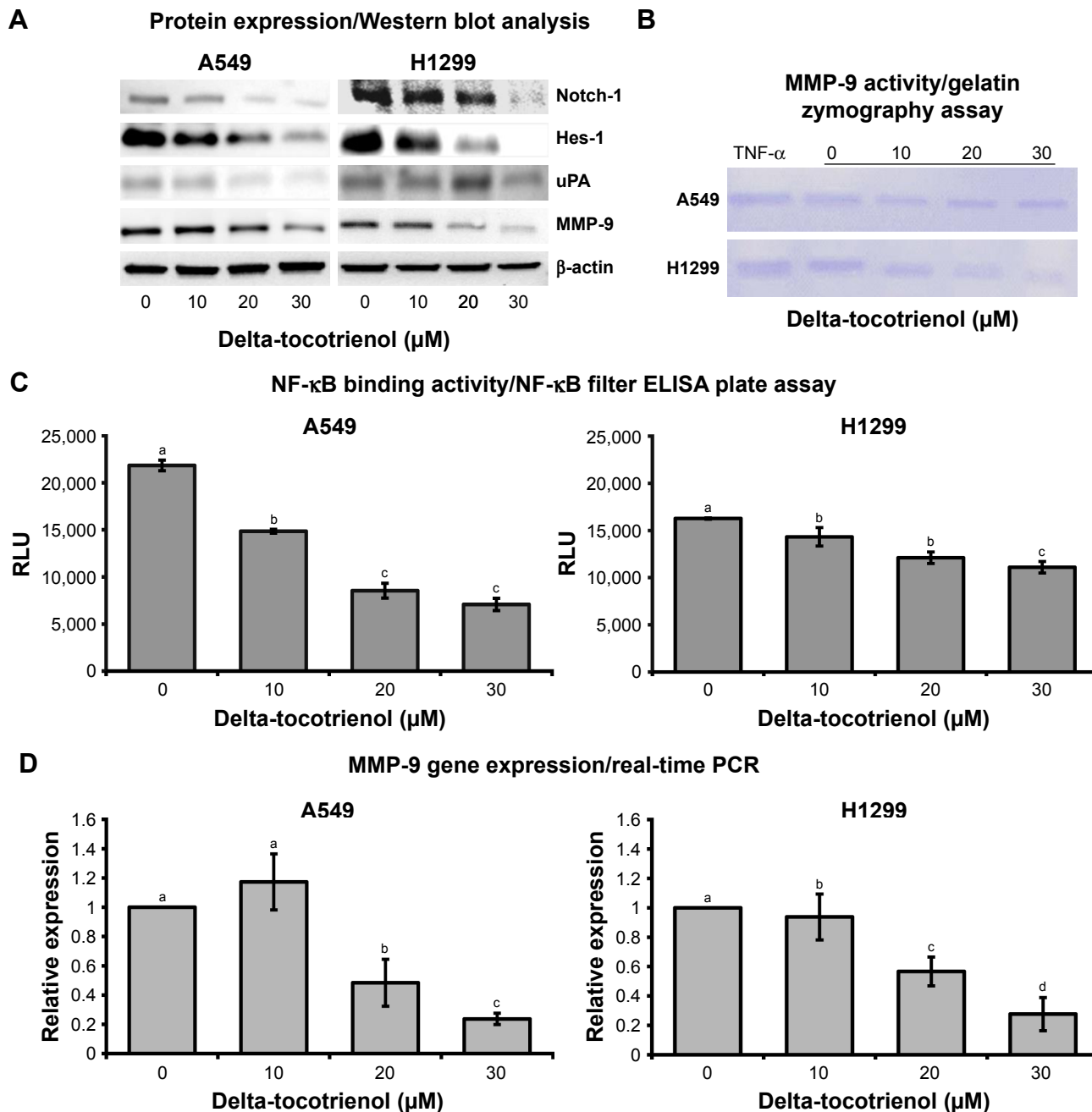
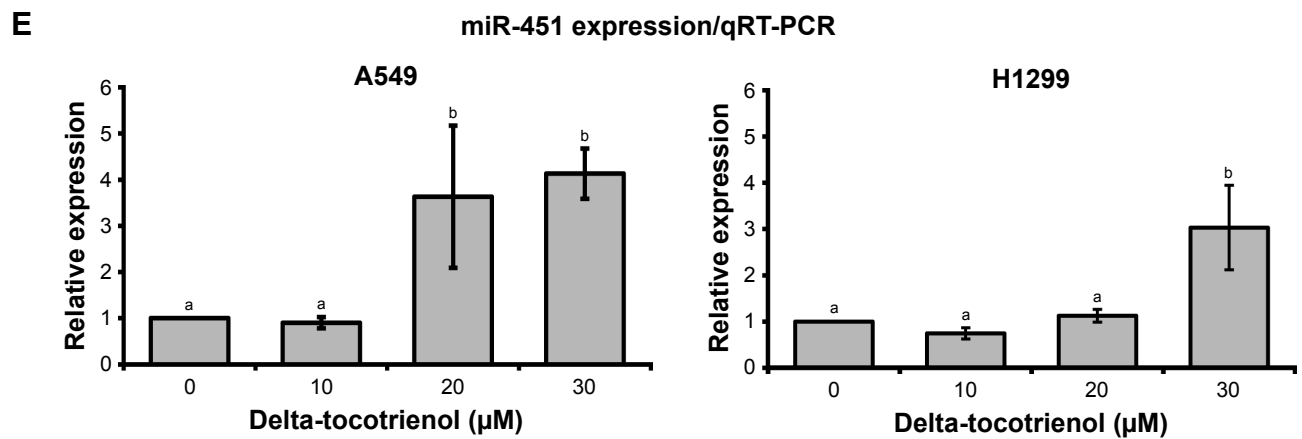


Figure 3 (Continued)





**Figure 3**  $\delta$ T inhibits MMP-9 activity and expression via the inhibition of Notch-1, NF- $\kappa$ B, and uPA.

**Notes:** (A) The expressions of Notch-1, HES-1, uPA, MMP-9, and  $\beta$ -actin proteins were detected by Western blot analysis in A549 and H1299 after treating with 0, 10, 20, and 30  $\mu$ M concentrations of  $\delta$ T for 72 hours. (B) Functional MMP-9 activities with or without  $\delta$ T treatment were determined by gelatin zymography in A549 and H1299 cells. TNF- $\alpha$ -treated cells were used as a positive control for this study. (C) NF- $\kappa$ B binding activity by  $\delta$ T in A549 and H1299 cells detected by NF- $\kappa$ B filter plate assay kit. The vertical bars indicate the mean absorbance  $\pm$  SD ( $n=4$ ); qRT-PCR analysis was carried out after the cells were treated with or without different doses of  $\delta$ T to observe the (D) MMP-9 gene expression (relative to  $\beta$ -actin) and (E) miR-451 expression (relative to U-6). The vertical bars indicate the mean relative expression  $\pm$  SD ( $n=3$ ) of MMP-9 and miR-451 in A549 and H1299. Different superscript letters on bar graph indicate significant difference ( $P<0.05$ ) compared with the other treatment groups as assessed by one-way ANOVA followed by Tukey HSD method as a multiple comparison test. In contrast, no significant difference between the treatment groups is indicated by the letters shared in common.

**Abbreviations:** ANOVA, analysis of variance;  $\delta$ T, delta-tocotrienol; HSD, honestly significant difference; MMP-9, matrix metalloproteinase 9; qRT-PCR, quantitative real-time PCR; TNF- $\alpha$ , tumor necrosis factor-alpha; NF- $\kappa$ B, nuclear factor- $\kappa$ B; uPA, urokinase-type plasminogen activator.

a concomitant reduction in cell invasion by H1299 cells.<sup>16</sup> In breast cancer cells, silencing of uPA and MMP-9 reduced their wound healing, migratory, invasive, and adhesive capacity.<sup>45</sup> We observed similar effects upon treatment of NSCLC cells with  $\delta$ T. This evidence strongly suggests the possible relationship between MMP-9 and uPA expressions in cancer cells. Therefore, we explored the protein expressions of uPA and MMP-9 using Western blot analysis. Our data showed a dose-dependent inhibition of both MMP-9 and uPA expressions in A549 and H1299 cells upon  $\delta$ T treatment, suggesting that  $\delta$ T inhibits cell invasion and metastasis via the inhibition of uPA and MMP-9 in A549 and H1299 cells (Figure 3A).

Aberrant Notch-1 expressions have been reported in a variety of human malignancies. It appears to regulate many basic processes essential for cancer development and progression, including cell growth, survival, apoptosis, migration, and invasion along with implicated MMP-9 expressions. Inhibiting Notch signaling using gamma-secretase inhibitor resulted in reduced expression of MMP-9 induced by DLK1 in H1299 and A549 cell lines.<sup>46</sup> Moreover, silencing of Notch-1 protein led to downregulation and inactivation of VEGF and MMP-9, thereby inhibiting invasion and metastasis of pancreatic cancer cells.<sup>47</sup> Since these studies indicate a direct relationship between Notch-1 and MMP-9 proteins, we tested Notch-1 expression in whole-cell lysates by Western blotting to determine whether  $\delta$ T downregulates

MMP-9 expression in a Notch-signaling-dependent manner in NSCLC. Our results showed that  $\delta$ T did downregulate Notch-1 expression in both A549 and H1299 cells (Figure 3A) along with the expression of MMP-9. Furthermore, the expression of HES-1, a Notch-1 downstream target gene,<sup>48</sup> was also examined by Western blot in A549 and H1299 cells to validate downstream involvement of Notch-1 signaling in the regulation of MMP-9 expression. Our results showed that  $\delta$ T downregulated HES-1 expression along with Notch-1 expression in both A549 and H1299 cells (Figure 3A). The simultaneous inhibition of Notch-1/HES-1 and MMP-9 with  $\delta$ T suggests a mechanistic interplay between MMP-9 and uPA/Notch-1 in metastasis.

### Inhibition of NF- $\kappa$ B DNA-binding activity with $\delta$ T

MMP-9 promoter is one of the most intensively studied promoters<sup>49</sup> containing several TF-binding elements, including binding sites for NF- $\kappa$ B.<sup>50</sup> Since NF- $\kappa$ B plays important roles in the regulation of MMP-9 expression, we evaluated whether  $\delta$ T affected the DNA-binding activity of the nuclear translocated NF- $\kappa$ B in these cells. Our data showed a dose-dependent inhibition of NF- $\kappa$ B-binding activities in A549 and H1299 upon  $\delta$ T treatment, suggesting that the inhibition of MMP-9 expression lowers DNA-binding activity of translocated NF- $\kappa$ B (Figure 3C).

## Discussion

The metastasis process is a multistep process, involving the migration/invasion of cells through the ECM/basement membrane into the circulatory system, survival without contact with the ECM, adhesion to the wall of lymphatic or blood vessels, and detachment from the vessels (extravasation), followed by survival and proliferation in target tissues.<sup>51</sup> The effect of  $\delta$ T on the cascade of steps involved in metastasis was tested in NSCLC cells using in vitro assays: wound healing, cell invasion, cell adhesion assay, aggregation assay, and the MTS for cell proliferation. Based on our findings,  $\delta$ T inhibited all the metastasis functions in the above in vitro assays, suggesting its potential as an antimetastasis facilitator in A549 and H1299 cells. Furthermore, our previous studies, using histone enzyme-linked immunosorbent assay and Annexin-V stain-based flow cytometry analysis, demonstrated that the 0–30  $\mu$ M range of  $\delta$ T was not necrotic to A549 or H1299 cells and instead induced apoptosis in a dose-dependent manner.<sup>30</sup> Therefore, antimetastasis functions of  $\delta$ T in in vitro assays are not due to necrotic effects.

The ECM is proteolytically degraded, facilitating the migration and invasion of cancer cells. Single cells detached from a tumor and released to the intercellular junctions during metastasis<sup>52</sup> are capable of making cell aggregates. Inherently, nontumor single cells are prone to anoikis, a form of apoptosis which is encouraged by insufficient or inappropriate cell–ECM interactions or attachments.<sup>53</sup> However, cancer cells showed resistance to anoikis, and as a result, they can survive after detachment from their main location.<sup>51</sup> Cancer cell aggregates exhibit lower levels of anoikis compared with regular apoptosis. Therefore, the formation of cell aggregates may induce regular cell apoptosis in cancer cells. However, we observed a dose-dependent decrease in cell aggregate formation as well as a reduction in cell adhesion capabilities with  $\delta$ T treatment. This may have occurred due to the stimulation of anoikis in nonadhesive cancer cells/single migrating cells and an induction of regular apoptosis in cell aggregates. Future studies are needed to explain  $\delta$ T-based apoptosis approaches in A549 and H1299 cells.

The Notch-1 signaling pathway plays a significant function in regulating cancer cell invasion and metastasis. Notch-1 expression was shown to increase in some NSCLC patients and is found to be associated with a TNM stage in histological grading,<sup>54</sup> suggesting that Notch-1 may play a fundamental role in the metastasis of NSCLC. Notch-1 inhibition decreases the development of brain metastases from breast cancer.<sup>55</sup> Abnormal Notch-1 expression is also strongly correlated with metastatic tumor disease in hepatocellular carcinoma patients.<sup>56</sup> Silencing of Notch-1 inhibited the invasion of

human pancreatic cancer cells by inhibiting the expression of MMP-9 and uPA.<sup>57</sup> Interestingly, our study found that  $\delta$ T dose dependently inhibited the Notch-1 expressions compared with the control. To further validate its activity, we investigated the expression of Notch-1 downstream protein HES-1, as it is well documented that the activated Notch-1 pathway can increase the expression of its downstream HES-1 activities. Indeed, we observed that  $\delta$ T downregulated HES-1 protein expression dose dependently along with Notch-1 expression in A549 and H1299 cells, which suggests that  $\delta$ T is a potential inhibitor of Notch-1 signaling pathway.

Many studies have established that miR-451 is widely dysregulated in human malignancies, including lung cancer,<sup>38,58</sup> gastric cancer,<sup>59</sup> breast cancer,<sup>60</sup> glioma,<sup>61</sup> and leukemia,<sup>62</sup> indicating the important role of miR-451 in oncogenesis. The downregulation of miR-451 has been reported in gastric and colon cancers and childhood B-cell precursor acute lymphoblastic leukemia.<sup>63</sup> miR-451 functions as a tumor suppressor and regulates survival in NSCLC cells.<sup>55</sup> It is also one of the most downregulated miRs in lung cancer patients.<sup>55</sup> Our results demonstrated that  $\delta$ T treatment induced the expression of miR-451 in a dose-dependent manner in both A549 and H1299 NSCLC cell lines. Moreover, the induction of miR-451 expression by  $\delta$ T occurred with a suppression of the Notch-1 signaling pathway. These findings reveal possible cross talk between Notch-1 signaling and miR-451 for regulation of cell migration and invasion in A549 and H1299 cell lines. This corroborates previous findings where the suppression of miR-451 was shown to induce Notch-1-induced oncogenesis in T-cell acute lymphoblastic leukemia.<sup>62</sup>

Although a variety of stimuli from different signaling pathways are known to activate NF- $\kappa$ B, Notch-1 is considered as one of the key pathways in the activation of NF- $\kappa$ B via its ligands.<sup>64</sup> The downregulation of Notch-1 has been shown to inhibit invasion by the inactivation of NF- $\kappa$ B and MMP-9 in pancreatic cancer cells,<sup>47</sup> lung cancer,<sup>29</sup> and prostate cancer.<sup>65</sup> In this study, the data showed a dose-dependent decrease in NF- $\kappa$ B DNA-binding activity in A549 and H1299 cells with different concentrations of  $\delta$ T. NF- $\kappa$ B has multiple binding targets including the MMP-9 promoter in cancer cells.<sup>66</sup> Regulatory elements in the 670-bp promoter region of MMP-9 include activator protein (AP)-1, AP-2, specificity of protein 1, and NF- $\kappa$ B.<sup>18</sup> Mutation of the NF- $\kappa$ B binding site of MMP-9 promoter leads to a dramatic reduction in MMP-9 promoter activation and MMP-9 expression.<sup>18–21</sup> Therefore, NF- $\kappa$ B DNA-binding activities are very crucial for the expression of MMP-9 genes. Modulating NF- $\kappa$ B activation using several natural and artificial agents was shown to alter MMP-9 expression in lung cancer cell lines.

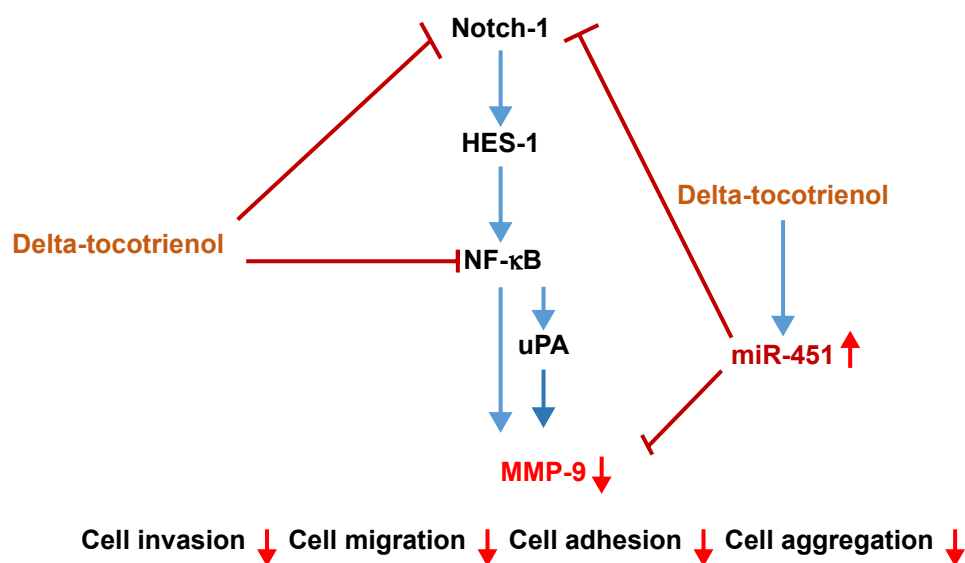
For instance, stromal cell-derived factor 1 was reported to increase the invasiveness of A549 cells through activation of ERK/NF- $\kappa$ B signaling, which is responsible for the increase of MMP-9 expression of the cells.<sup>67</sup> Nifedipine exerts its NF- $\kappa$ B inhibiting activity by suppressing IKK-mediated I $\kappa$ B degradation, subsequently suppressing MMP-9 expression and activity.<sup>68,69</sup> In lung cancer cells, radiotherapy-induced MMP-9 expression and lung metastasis were reduced after blocking NF- $\kappa$ B using arsenic trioxide.<sup>70</sup> In this study, we observed a dose-dependent inhibition of MMP-9 expression at the protein and mRNA levels along with reduced NF- $\kappa$ B DNA-binding activities upon the  $\delta$ T treatment, suggesting that the binding of TF NF- $\kappa$ B was reduced at the MMP-9 promoter upon  $\delta$ T treatment. Further, several other studies have shown that  $\delta$ T inhibits the expression of NF- $\kappa$ B in different cells lines.<sup>71–73</sup> Therefore, it is reasonable to suggest that the lower binding activities of NF- $\kappa$ B are due to the lower expression of NF- $\kappa$ B upon  $\delta$ T treatment.

$\delta$ T has been shown to have a very broad range of therapeutic potentials; it is used as an antioxidant, analgesic, anti-inflammatory, antibacterial, antipyretic, antithrombotic, anticancer agent and has cardioprotective, hepatoprotective, hypoglycemic, and nephroprotective properties.<sup>74,75</sup> Multiple downstream targets of  $\delta$ T have been summarized in several reviews.<sup>74–76</sup> In our previous studies, we reported that  $\delta$ T has multiple downstream targets, namely HES-1, VEGF, BCL-2, BCL-XL, Cyclin D1, Survivin, P-mTOR, mTOR, P-AKT, AKT, S6K, LAT-1, ASCT2, and c-MYC.<sup>28–33</sup> However, in this study, we targeted MMP-9 as a key metastasis mediator in NSCLC.<sup>77</sup>

Other studies have reported that the antimetastatic effect of nutraceuticals is individually linked to different metastatic pathways. For example, the nutraceuticals carnosic acid, silibinin, curcumin, epigallocatechin gallate, quercetin, and baicalin inhibited cell migration and invasion by downregulating uPA and/or MMP-9 expression in cervical cancer cells, melanoma cells, human breast epithelial cells, oral cancer cells, and prostate and liver cancer cells, respectively.<sup>78–82</sup> Liu et al<sup>83</sup> showed that  $\gamma$ -tocotrienol inhibits cell invasion and metastasis of the human gastric system by downregulating MMP-9 and MMP-2. Interestingly, our results demonstrated that the antimetastatic functions of  $\delta$ T on the inhibition of invasion and migration in lung cancer cells are due to the suppression of Notch-1, NF- $\kappa$ B, MMP-9, or uPA along with induced miR-451. Thus, our data suggested that  $\delta$ T is an effective and efficient antimetastatic compound with multiple therapeutic targets.

## Conclusion

Our results provide mechanistic insight into the modulation of in vitro invasion and metastasis in lung cancer cells by  $\delta$ T. The data showed that  $\delta$ T suppressed cell migration, invasion, and adhesion in NSCLC cells by significantly lowering protease activity of MMP-9, inhibiting Notch-1-mediated NF- $\kappa$ B and uPA pathways, which led to the downregulated expression of MMP-9 with simultaneous increased expression of miR-451. Based on our findings, we propose that  $\delta$ T attenuates tumor aggressiveness, invasion, and metastasis by the downregulation of MMP-9 via Notch-1 and uPA pathways (Figure 4). This study suggested consideration of  $\delta$ T as an effective potential natural therapeutic approach



**Figure 4** Proposed mechanism by which delta-tocotrienol suppresses cell migration and invasion in A549 and H1299 cells.

**Notes:** Red ↓, downregulation; red ↑, upregulation; red ⊥, inhibition; blue →, activation.

**Abbreviations:** MMP-9, matrix metalloproteinase 9; NF- $\kappa$ B, nuclear factor- $\kappa$ B; uPA, urokinase-type plasminogen activator.

against lung cancer invasion and metastasis. Further studies to establish the efficacy of  $\delta T$  in preventing lung cancer migration and invasion at the animal and clinical levels are highly recommended.

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## Disclosure

The authors report no conflicts of interest in this work.

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