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Inhibition of Cell Growth and Induction of Apoptosis in Non-Small Cell Lung Cancer Cells by Delta-Tocotrienol is Associated With Notch-1 Down-Regulation

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ABSTRACT

Lung cancer is the leading cause of death among all cancers. Non-small cell lung cancer accounts for 80% of lung cancer with a 5-year survival rate of 16%. Notch pathway, especially Notch-1 is up-regulated in a subgroup of non-small cell lung cancer patients. Since Notch-1 signaling plays an important role in cell proliferation, differentiation, and apoptosis, down-regulation of Notch-1 may exert anti-tumor effects. The objective of this study was to investigate whether delta-tocotrienol, a naturally occurring isoform of Vitamin E, inhibits non-small cell lung cancer cell growth via Notch signaling. Treatment with delta-tocotrienol resulted in a dose and time dependent inhibition of cell growth, cell migration, tumor cell invasiveness, and induction of apoptosis. Real-time RT-PCR and western blot analysis showed that antitumor activity by delta-tocotrienol was associated with a decrease in Notch-1, Hes-1, Survivin, MMP-9, VEGF, and Bcl-XL expression. In addition, there was a decrease in NF-κB-DNA binding activity. These results suggest that down-regulation of Notch-1, via inhibition of NF-κB signaling pathways by delta-tocotrienol, could provide a potential novel approach for prevention of tumor progression in non-small cell lung cancer. J. Cell. Biochem. 112: 2773–2783, 2011. © 2011 Wiley-Liss, Inc.

KEY WORDS: DELTA-TOCOTRIENOL; NOTCH-1; NF-κB; LUNG CARCINOMA; APOPTOSIS

Notch proteins, the transmembrane receptors, are highly conserved in the development and the determination of cell fate [Artavanis-Tsakonas et al., 1999]. This is because the ligand-receptor signaling pathway, Notch, plays critical roles in mediating cell proliferation, survival, and apoptosis [Ohishi et al., 2003]. To date, four Notch receptors, namely Notch 1–4 have been identified in mammals. [Mumm and Kopan, 2000]. Additionally, five Notch ligands including DLL-1, DLL-3, DLL-4, Jagged-1, and Jagged-2 have been recognized [Bigas et al., 1998; Mumm and Kopan, 2000]. All the Notch receptors and their ligands have been shown to be related to cancer [Miele et al., 2006]. Once the ligands bind to the extracellular domain, Notch receptors undergo a series of proteolitic cleavages, releasing the intracellular Notch which then translocates into the nucleus [Oswald et al., 2001]. Inside the nucleus, the active forms of Notch along with other transcription factors regulate the expression of target genes such as Hes-1, Bcl-XL, and Survivin [Wang et al., 2006a; Wang et al., 2006c]. Since notch signaling regulates critical cell fate decision, alterations in Notch signaling are associated with tumorigenesis. Notch expression is known to be up-regulated in different types of cancers including colon, lung, head and neck, and pancreatic [Buchler et al., 2005; Reedijk et al., 2008; Westhoff et al., 2009; Lin et al., 2010]. Overexpression of Notch-1 has been shown to inhibit apoptosis in different cancer types [Miele and Osborne, 1999; Jundt et al., 2002], suggesting its potential as a therapeutic target.

Lung cancer is the major cause of death among malignant diseases, of which Non-small Cell Lung carcinoma (NSCLC) with a 16% 5-year survival rate, accounts for 80% of all lung cancers [Wu et al., 2009]. Clinical data have demonstrated that 30% of NSCLC has increased Notch activity and 10% of NSCLC has gain-of-function mutation on Notch-1 gene [Westhoff et al., 2009]. Recently, it has been reported that Notch-1 stimulates survival of NSCLC cells during hypoxia by activating the IGF pathway [Eliasz et al., 2010]. Another key apoptotic regulator, nuclear factor-kappaB (NF-κB), plays important roles in cancer cell transformation and development [Karin, 2006]. More and more data show that Notch-1/Hes-1 pathways cross-talk with the NF-κB pathway. Notch ligands induce NF-κB activation in leukemia cells, and decreased Notch-1 lowers

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NF-κB DNA binding activity [Itoh et al., 2009]. Moreover, Notch-1 has been found to induce sustained NF-κB activity by facilitating its nuclear retention [Shin et al., 2006]. Specifically, NF-κB2 promoter activity has been shown to be activated by Notch-1 pathway [Oswald et al., 1998]. Recently, Notch-1/Hes-1 pathways have been reported to be upstream to the maintenance of NF-κB activation in leukemia in vivo and in vitro [Espinosa et al., 2010].

However, the mechanisms by which Notch-1 induces cell growth and inhibits apoptosis in NSCLC are still unclear. Since Notch-1 down-regulation has shown anti-neoplastic effects in vivo and in vitro [Miele and Osborne, 1999; Jundt et al., 2002; Nickoloff et al., 2003], the potential for treating certain cancers could be achieved by inhibiting Notch signal transduction. Tocotrienols, components of naturally occurring vitamin E exist as four chemical isoforms (alpha, beta, gamma, and delta) and are rich in cereal grains and palm oils. Tocotrienol have been shown to have anti-tumor effects on different human cancer cells including prostate, breast, colon, melanoma, and lung cancers [Mcanally et al., 2003; Sylvester and Shah, 2005; Kumar et al., 2006]. Additionally, tocotrienols can induce apoptosis by inhibiting multiple signaling pathways such as EGFR, NF-κB, MAPK, and PI3K/AKT pathways [Shirode and Sylvester, 2010]. Despite recent progress, the effect of tocotienols on Notch signaling in NSCLC remains to be elucidated. In this study, we investigated the effect of delta tocotrienol on NSCLC cell growth and apoptosis. Our working hypothesis was that tocotrienols, specifically delta-tocotrienol, would inhibit NSCLC cell growth and induce apoptosis by inhibition of Notch-1 signaling via the NF-kB pathway.

MATERIALS AND METHODS

CELL CULTURE, REAGENTS, AND ANTIBODIES

Human NSCLC cell lines, including A549, H1299 obtained from ATCC were grown in DMEM medium (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin in 5% CO_2. Pure delta-tocotrienol was a kind gift from American River Nutrition, Inc (American River Nutrition, Hadley, MA). Protease inhibitor cocktail was obtained from Sigma (St. Louis, Mo). Primary antibodies for Poly (ADP-ribose) polymerase (PARP), β-actin and cell lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin) were purchased from Cell Signaling Technology (Danvers, MA). Primary antibodies against Notch-1, Hes-1, Survivin, Bcl-XL were bought from Santa Cruz Biotechnology (Santa Cruz, CA). The secondary antibodies were bought from Bio-Rad Laboratories (Hercules, CA).

CELL VIABILITY STUDIES BY MTS ASSAY

The A549 and H1299 cells (5×10^3) were seeded in a 96-well culture plate after overnight incubation medium was removed and replaced with a fresh medium containing DMSO (vehicle control) or different concentrations of delta-tocotrienol diluted from a 20 mM stock solution. After 72 h of incubation, 20 μl of CellTiter 96 AQueous One Solution Reagent (Promega, Madison, WI) was added to each well. After 2 h incubation at 37°C in a humidified, 5% CO_2 atmosphere, the absorbance at 490 nm was recorded on ELx800 plate reader (Bio-Tek, Winooski, VT). Each variant of the experiment was performed in triplicate.

HISTONE/DNA ELISA FOR DETECTION OF APOPTOSIS

The Cell Death Detection ELISA Kit (Roche, Palo Alto, CA) was used to detect apoptosis in NSCLC cells. Briefly, 10^5 cells were seeded in six well plates. After 24 h incubation, cells were treated with delta-tocotrienol or control for 72 h. The cells were then lysed, and cytoplasmic histone/DNA fragments were extracted and incubated in microtiter plate modules coated with anti-histone antibody. In order to detect the immobilized histone/DNA fragment, peroxidase-conjugated anti-DNA antibody was used before color development with ABTS substrate for peroxidase. The spectrophotometric absorbance of the samples was determined by using ELx800 plate reader (Bio-Tek, Winooski, VT) at 405 nm.

CLONGENIC ASSAY

One million cells were seeded in 100 mm dish per plate, incubated overnight. Subsequently, the cells were cultured with delta-tocotrienol or control, grown for 72 h. Later, the viable cells were counted and plated in 100 mm dishes in a range of 1,000 cells per plate. The cells were then incubated for 21 days at 37°C in a 5% CO_2 incubator. All the colonies were fixed in 4% Paraformaldehyde and stained with 2% crystal violet.

FLOW CYTOMETRY AND CELL CYCLE ANALYSIS

Cells were seeded in 100 mm dish per plate, incubated overnight. Subsequently, all the cells were starved for another 24 h. The cells were released to control or delta-tocotrienol treatment and grown for 72 h. Later, cells were collected and fixed with ice-cold 70% (v/v) ethanol for 24 h. After centrifugation at 3,000*g* for 5 min, the cell pellets were washed with PBS (pH 7.4) and resuspended in PBS containing propidium iodide (50 μg/ml), and DNase-free RNase (1 μg/ml). Samples were then incubated at room temperature for 2 h, and DNA content was determined by flow cytometry using a FACScan flow cytometer (BD, San Jose, CA).

ANNEXIN V-FITC METHOD FOR APOPTOSIS ANALYSIS

Annexin V-FITC apoptosis detection kit (BD, San Jose, USA) was used to measure the apoptotic cells. Briefly, A549 and H1299 cells were incubated in the presence or absence of delta-tocotrienol for 48 h. Cells were trypsinized, washed twice with ice-cold PBS and re-suspended in 1× binding buffer at a concentration of 10^5/ml cells in a total volume of 100 μl. After that, 5 μl of Annexin V-FITC and 5 μl of PI (Propidium Iodide) were added. All the samples were kept in the dark for 20 min at room temperature. Finally, 400 μl of 1× binding buffer was then added to each tube and the number of apoptotic cells was analyzed by flow cytometry (BD, San Jose, CA).

WOUND HEALING ASSAY

A549 and H1299 were seeded in a six well plate at the concentration of 4×10^5 cells per well. After overnight incubation, the culture media was removed and a scratch wound across each well was made using fine tips. All the wound areas were washed by PBS for three times to make sure no loosely held cells were attached. Subsequently, the cells were cultured in presence or absence of

delta-tocotrienol and the wound images were taken as 0 h. After 20 h, pictures were taken under microscope. Two diagonal parallel lines in each image mark the progress of cells that migrated into the wound.

CELL INVASIVE ASSAY

BD Biocoat invasion kit (BD, San Jose, CA) was used to evaluate the tumor invasive ability. Briefly, around 2.5×10^5 cells of A549 and H1299 with basal media were transferred in each six well upper chamber in the presence or absence of delta-tocotrienol. In the meantime, 3 ml of culture medium with 10% FBS was added into each lower chamber of six-well plate. After 20 h incubation, the cells on the upper chamber were removed using cotton stick. Each experimental condition was performed in duplicates. The cells were fixed in 4% Paraformaldehyde and stained with 2% crystal violet. To determine the cells number, cells were counted under a microscope in five random fields.

PROTEIN EXTRACTION AND WESTERN BLOTTING

A549 and H1299 cell lines were treated with or without delta-tocotrienol for 72 h to evaluate the effects of treatment on Notch-1, Hes-1, PARP, Survivin, Bcl-XL, and β-actin expressions. Cells were lysed in the cold lysis buffer for 30 min on ice. Protein concentrations were determined using the Bradford protein assay kit (Bio-Rad Laboratories, CA). Each sample contained 50 μg of total cell lysates. The samples were loaded on 10% SDS-polyacrylamide gel electrophoresis. After electrophoresis, the gel electrophoretically was transferred to a nitrocellulose membrane (Whatman, Clifton, NJ) using transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol) in Hoefer TE70XP transfer apparatus (Holliston, MA). The membranes were incubated for 1 h at room temperature with 5% nonfat dried milk in 1 × TBS buffer containing 0.1% Tween. After that, membranes were incubated over night at 4°C with primary antibodies (1:1,000). The membranes were washed three times with TBS-T, and subsequently incubated with the secondary antibodies (1:5,000) containing 2% BSA for 2 h at room temperature. The signal intensity was then measured by chemiluminescent image with chemiDoc XRS (Bio-Rad Laboratories, CA).

REAL-TIME QUANTITATIVE PCR FOR GENE EXPRESSION ANALYSIS

Total RNA was isolated using RNeasy Mini Kit from QIAGEN (Valencia, CA) according to the manufacturer's protocols. Two microgram 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) in a total volume of 20 μl. Reverse transcription reaction were performed at 25°C for 10 min, followed by 48°C for 30 min and 95°C for 5 min. Real-time PCR analysis was performed using Eppendorf realplex 4 system (Hauppauge, NY). The sequences of the primers sets used for this analysis are as follows: Notch-1, forward primer (5′-CAC TGT GGG CGG GTC C-3′) and reverse primer (5′-GTT GTA TTG GTT CGG CAC CAT-3′); Hes-1, forward (5′-GAC AGC ATC TGA GCA CAG AAA TG-3′) and reverse primer (5′- GTC ATG GCA TTG ATC TGG GTC AT-3′); MMP-9, forward primer (5′-CGG AGT GAG TTG AAC CAG-3′) and reverse primer (5′-GTC CCA GTG GGG ATT TAC-3′); VEGF, forward primer (5′-GCC TTG CCT TGC TGC TCT AC-3′) and reverse primer (5′-TTC TGC CCT CCT CCT TCT GC-3′); GAPDH, forward primer (5′-CAG TGA GCT TCC CGT TCAG-3′) and reverse primer (5′-ACC CAG AAG ACT GTG GAT GG-3′). Primers were verified by running them on virtual PCR, and primer concentrations were optimized to avoid primer dimer formation. Real-time PCR amplifications were performed using 2 × SYBR Green PCR Master Mix (Applied Biosystems). Two microliter of RT reaction was used for a total volume of 25 μl quantitative PCR reactions. The thermal profile for SYBR real-time PCR was 95°C 10 min followed by 50 cycles of 95°C 15 s and 60°C 1 min. Data were analyzed according to the comparative fold increase or decrease in gene expression determined by ct values and normalized by GAPDH expression in each sample.

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA) FOR MEASURING NF-κB ACTIVITY

EMSA was conducted to measure the activity of NF-κB in delta-tocotrienol-treated and -untreated cells. Briefly, A549 and H1299 cells were treated with or without of delta-tocotrienol. After 48 h treatment, nuclear protein was extracted from each sample using nuclear protein extraction kit according to the protocol (Pierce, Rockford, IL). Five mircrograms of nuclear protein of each sample was incubated with IRDye-700 labeled NF-κB oligonucleotide. The incubation mixture included 2 μg of poly (deoxyinosinic-deoxycytidylic acid) in the binding buffer. The DNA-protein complex formed was separated by running on 8.0% native polyacralyamide gel using buffer containing 50 mmol/L Tris, 200 mmol/L glycine (pH 8.5), and 1 mmol/L EDTA. In the end, the gel was visualized by Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).

DATA ANALYSIS

Results were expressed as means ± SEM and analyzed using GraphPad Prism 4.0 (Graph pad Software, La Jolla, CA). Statistical comparisons between groups were done using one-way ANOVA. Values of $P < 0.05$ were considered to be statistically significant and individual P-values are reported in the figures, separately.

RESULTS

EFFECTS OF DELTA-TOCOTRIENOL ON GROWTH OF NSCLC CELLS

In order to test the effects of delta-tocotrienol on cell growth, A549 and H1299 cells were treated with increasing concentration of delta-tocotrienol for 72 h separately followed by MTS assay. As shown in Figure 1A and B, delta-tocotrienol inhibits cell growth in a dose dependent manner in both A549 and H1299 cells, respectively. In A549 cell line, treatment with 10, 20, and 30 μM of delta-tocotrienol for 72 h resulted in 8, 29, and 77% of cell growth inhibition relative to control, respectively. Similarly, treatment of H1299 cell line with 10, 20, and 30 μM of delta-tocotrienol for 72 h resulted in 11, 45, and 87% of cell growth inhibition, respectively, relative to control. These results indicate that delta-tocotrienol was an effective inhibitor of NSCLC cell growth as a single agent.

To confirm the effects of delta-tocotrienol on cells growth, clonogenic assay was performed. Figure 5C and D show a dose dependent inhibition of colony formation by delta tocotrienol as compared to the control. For both cell lines, colony formation was

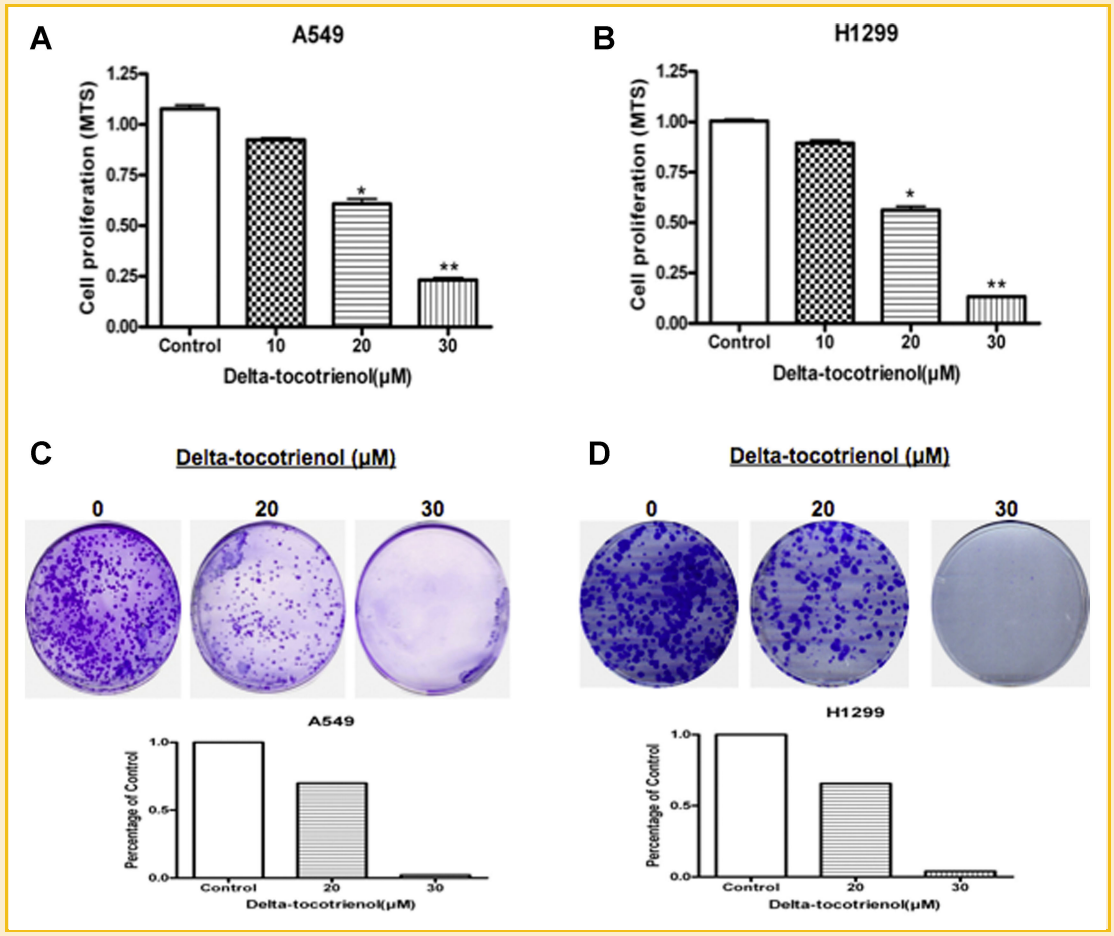

Fig. 1. Antiproliferative effects of delta-tocotrienol on NSCLC cells. Cell viability of human NSCLC cell lines A549 (A) and H1299 (B) cells. Both A549 and H1299 cells were initially plated at a density of 5×10^3 cells/well (3 wells/group) in 96-well plates and grown in experimental medium containing 0, 10, 20, 30 μM of delta-tocotrienol for 72 h. Viable cell number was determined using the MTS colorimetric assay. Vertical bars indicate the mean cell count ± SEM (n = 3). $^*P < 0.05$ is considered as significant as compared with vehicle-treated controls. Cell survival of human NSCLC cell lines A549 (C) and H1299 (D) cells. A549 and H1299 cells treated with different concentration of delta-tocotrienol (0, 20, 30 μM) were evaluated by the clonogenic assay. Photomicrographic difference in colony formation in A549 and H1299 cells untreated and treated with delta-tocotrienol are shown. There was a significant reduction in the colony formation in A549 and H1299 cells treated compared with cells untreated ($P < 0.05$).

barely seen at 30 μM delta-tocotrienol treatments. Overall, the results from the clonogenic assay were consistent with the MTS data shown in Figure 1A and B, confirming that delta-tocotrienol significantly inhibits the growth of NSCLC cells ($P < 0.05$ at 30 μM treatment).

INDUCTION OF APOPTOSIS BY DELTA-TOCOTRIENOL

Since inhibition of cell growth could also result from apoptosis induced by delta-tocotrienol, we further investigated whether delta-tocotrienol could induce apoptosis in both cell lines by two different approaches, histone/DNA ELISA and the Annexin V/PIstaining. As demonstrated in Figure 2A and B, delta-tocotrienol induced apoptosis in both cell lines, A549 (Fig. 2A) and H1299 (Fig. 2B) in dose dependent manner. Annexin V/PI staining confirmed apoptosis-inducing effect of delta-tocotrienol in both cell lines tested (Fig. 2C and D), respectively. Figure 2C and D represents quantitation of apoptotic cells, as detected by Annexin V staining after treatment with 20 μM delta-tocotrienol. Our results clearly show that delta-tocotrienol treatment resulted in a statistically significant ($P < 0.05$) increase in the percentage of apoptotic cells in both NSCLC cell lines.

ANALYSIS OF CELL CYCLE DISTRIBUTION AFTER TREATMENT WITH DELTA-TOCOTRIENOL

To further investigate cell growth inhibition by delta-tocotrienol, cell cycle distributions were performed using propidium iodide staining by flow cytometry. Both A549 and H1299 cells, treated in the absence or presence of delta-tocotrienol at 20 μM for 48 h were analyzed. Figure 3A and B show increasing G_0-G_1 arrest patterns in delta-tocotrienol treated cells compared to the control in both cell lines, A549 and H1299, respectively. Both cell lines showed a dose-dependent G_0-G_1 phase arrest induced by delta-tocotrienol. For A549 cells (Fig. 3A), there were about 76% cells in G_0-G_1 phase in treatment group (30 μM tocotrienol) compared to 70% in control cells. A similar response was observed in the H1299 cells (Fig. 3B)

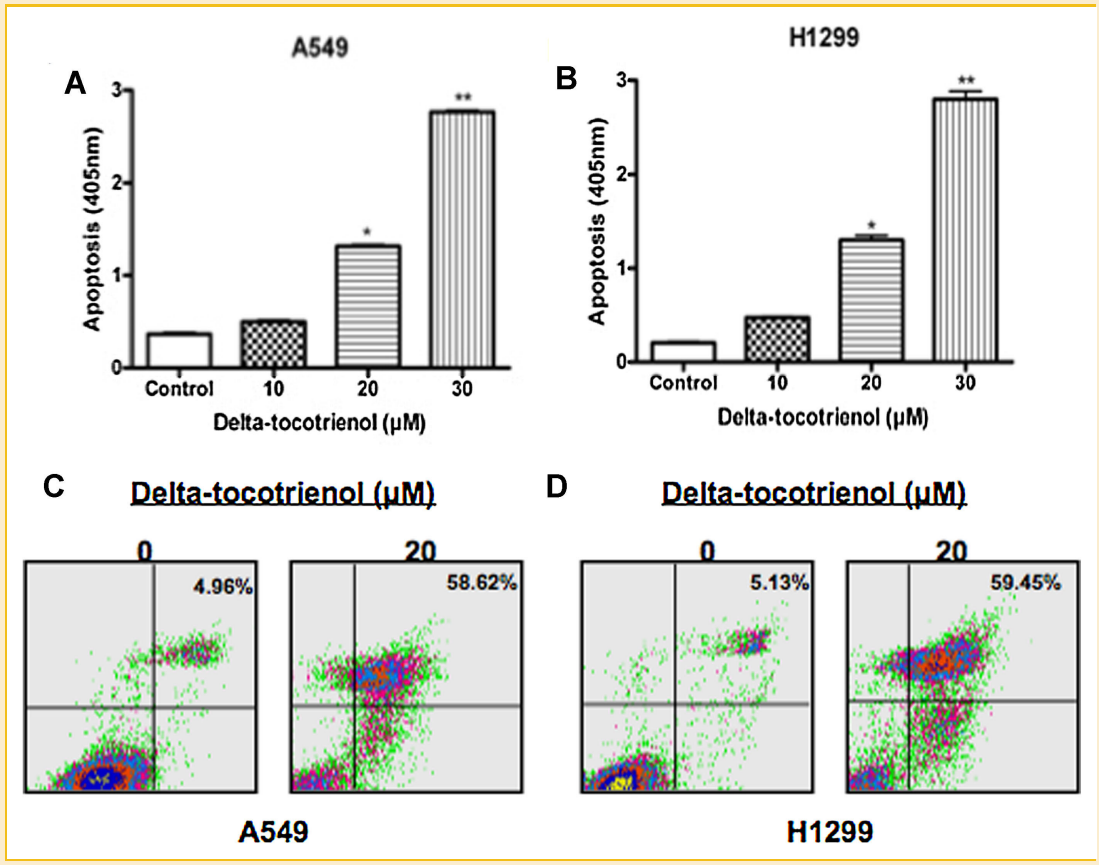

Fig. 2. Induction of apoptotic effects of delta-tocotrienol in NSCLC cells. A549 (A) and H1299 (B) cells were treated with increasing concentration of delta-tocotrienol for 72 h. Apoptosis was determined by histone/DNA ELISA. $^{*}P < 0.05$, $^{**}P < 0.01$. A549 (C) and H1299 (D) cells were treated with 20 μM of delta-tocotrienol for 72 h. Apoptosis of both cell lines was determined by Annexin V-FITC.

with 67% of cells in G_0-G_1 phase in treatment group (30 μM tocotrienol) compared to 54% in control cells.

DOWN-REGULATION OF THE NOTCH-1 AND ITS TARGET GENES EXPRESSIONS BY DELTA-TOCOTRIENOL

Thus far, our results have shown that delta-tocotrienol inhibited cell growth and induced cell apoptotic death in NSCLC cells. In order to further understand the molecular mechanism involved in delta-tocotrienol -induced apoptosis of NSCLC cells, modifications in the cell death pathway were investigated. Given that Notch signaling and its gene products are known to regulate cell proliferation cell cycle distribution and apoptosis, we explored whether delta-tocotrienol could regulate Notch signaling pathway. Real-time PCR and western blotting were used to measure Notch-1 mRNA and protein expressions in NSCLC cell lines treated with or without delta-tocotrienol at different time points. As shown on Figure 4A and B, the mRNA expression of Notch-1 gene was decreased after delta-tocotrienol treatment in both cell lines, suggesting that delta-tocotrienol exerted a transcription inhibition on Notch-1 gene expression. Furthermore, western blotting data (Fig. 4) demonstrated that delta-tocotrienol inhibited the protein expression of Notch-1 in a dose dependent manner in both NSCLC cell lines. These results show that delta-tocotrienol regulates the transcription and translation of Notch-1 gene.

To further confirm our results, we also conducted real-time PCR and western blotting to assess Notch-1 target genes such as Hes-1 and Survivin in NSCLC cells after delta-tocotrienol treatment. A dose dependent decrease in Hes-1 and Survivin protein levels with delta-tocotrienol treatment was observed (Fig. 4 A and B). Taken together, our findings strongly suggest that delta-tocotrienol suppressed transcription and translation of Notch-1 and its target genes such as Hes-1, Survinin possibly giving rise to reduced proliferation, and enhanced apoptosis in NSCLC cells.

INHIBITION OF NF-κB DNA BINDING ACTIVITY WITH DELTA-TOCOTRIENOL

The NF-κB pathway plays important roles in cancer cell transformation, cell invasion, and apoptosis. Further, NF-κB has been shown to cross-talk with Notch signaling [Wang et al., 2006b]. The effect of delta-tocotrienol on NF-κB DNA-binding activity in NSCLC cells was determined by subjecting nuclear extracts from delta-tocotrienol treated A549 and H1299 cells to EMSA. As shown in the Figure 5A and B, compared to the control, delta-tocotrienol significantly inhibited the DNA-binding activity of NF-κB in dose

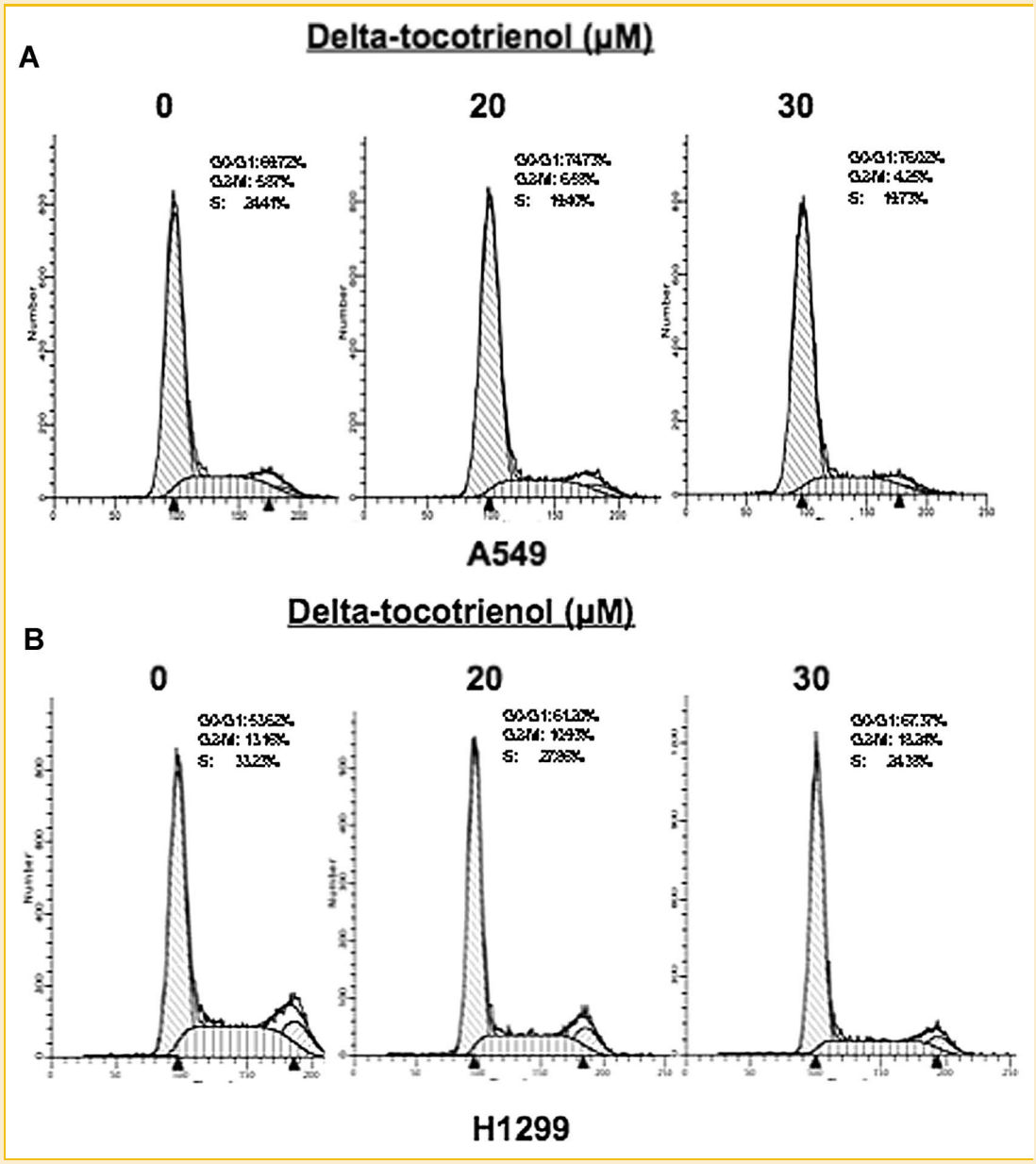

Fig. 3. Delta-tocotrienol induces cell cycle arrest at G_0-G_1 phase A549 (A) and H1299 (B) cells were seeded at a density of 1×10^6 cells in 100-mm dishes without serum for 24 h. After that, different concentrations (0, 20, 30 μM) of delta-tocotrienol were added and incubated for 72 h. Cell cycle distributions were evaluated by flow cytometry.

dependent manner for both cell lines. Given that NF-κB is a master point for multiple pathways involved in proliferation, survival, and invasion, inhibition of NF-κB activity by delta-tocotrienol confirms the latter's potential benefit as an anti-cancer agent.

The effect of delta-tocotrienol on the expressions of VEGF and MMP9, downstream target genes of NF-κB, responsible for cell migration and invasion, were evaluated by real-time PCR. As shown in Figure 5C and D, the expressions of MMP9 and VEGF in both cell lines were significantly inhibited on treatment with delta-tocotienol. The results clearly demonstrate that delta-tocotrienol inhibited NF-κB activity and its target genes' expressions.

INHIBITION OF CELL INVASION AND MIGRATION BY DELTA-TOCOTRIENOL

Although effect of delta-tocotrienol on anti-proliferation and induction of apoptosis in certain cancers has been shown, its effects on tumor cells migration and invasion has not been evaluated thus far. Since delta-tocotrienol inhibited MMP-9 and VEGF, important factors for cell migration and invasion, we conducted a Matrigel invasion assay in order to assess its effect on the invasive capacity of A549 and H1299 NSCLC cells. As shown in Figure 6A and B, delta-tocotrienol-treated A549 and H1299 cells depict a decrease in their invasive capability by at least three times as compared with the

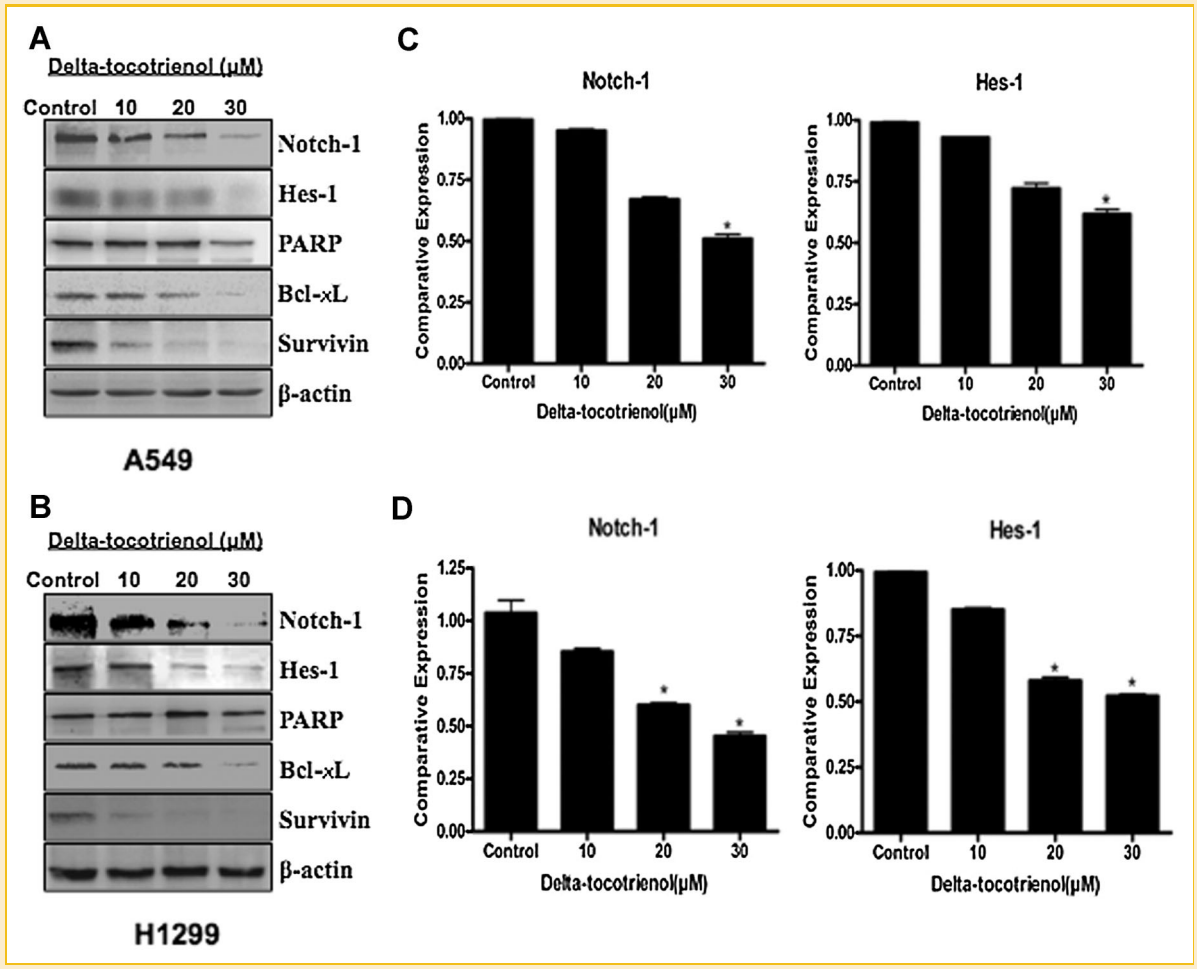

Fig. 4. Down-regulation of Notch-1 and its target genes by delta-tocotrienol A549 (A) and H1299 (B) cells were treated with varied concentrations of delta-tocotrienol for 72 h. Left panel: The expressions of Notch-1, Hes-1, PARP, Bcl-XL, and Survivin protein were detected by western blotting analysis. Middle and right panel: Notch-1 mRNA and Hes-1 mRNA were detected by Real-time RT-PCR, respectively.

untreated control. Wound healing assay was performed to determine tumor cell migration ability. As demonstrated in Figure 6C and D, delta-tocotrienol inhibited cell migration in a dose dependent manner in both cell lines, A549 and H1299.

DISCUSSION

Lung cancer, especially NSCLC, is the leading cause of death among all types of cancers with a 5-year survival rate of 16% [Wu et al., 2009]. Abnormal Notch pathway has been found in 30% of NSCLC patients, and is implicated in their higher mortality rate [Westhoff et al., 2009]. A549 and H1299 cell lines are representatives of such abnormal notch expression cells. As the Notch pathway, especially Notch-1 signaling plays an important role in the determination of cell fate its inhibition may provide a promising target for cancer therapy. Gamma (γ)-secretase plays a crucial role in the Notch pathway by activating it as a result of preteolytic cleavage of the notch receptor from the membrane [Shih Ie and Wang, 2007]. The importance of its inhibition as a target for cancer therapy is reflected in the number of γ-secretase inhibitors (GSI) under preclinical investigations [Shih Ie and Wang, 2007]. Acute toxicity is a major barrier for the usage of most of the GSI compounds [Shih Ie and Wang, 2007]. Identification of compounds targeting Notch signaling in NSCLC with minimal toxicity might provide new impetus in this area.

Bioactive dietary agents such as delta-tocotrienol might have a significant impact in lung cancer prevention and/or therapy as a single agent or agent in combinatorial therapy. Although delta-tocotrienol, an isomer of vitamin E, has demonstrated its anti-cancer effects in a few cancer models [Constantinou et al., 2009], there is no report regarding the molecular mechanism by which delta-tocotrienol may induce apoptosis in NSCLC cells [Mcanally et al., 2003; Kumar et al., 2006]. We hypothesized that delta-tocotrienol could be effective against NSCLC cell growth via the Notch-1 pathway. In the current study, we investigated the effects and molecular mechanisms of delta-tocotrienol in cell lines with over expression of Notch-1. We found that delta-tocotrienol was efficient in inhibiting the growth and proliferation of cells. In the cell proliferation (MTS) assay (Fig. 1A and B), we found that delta-

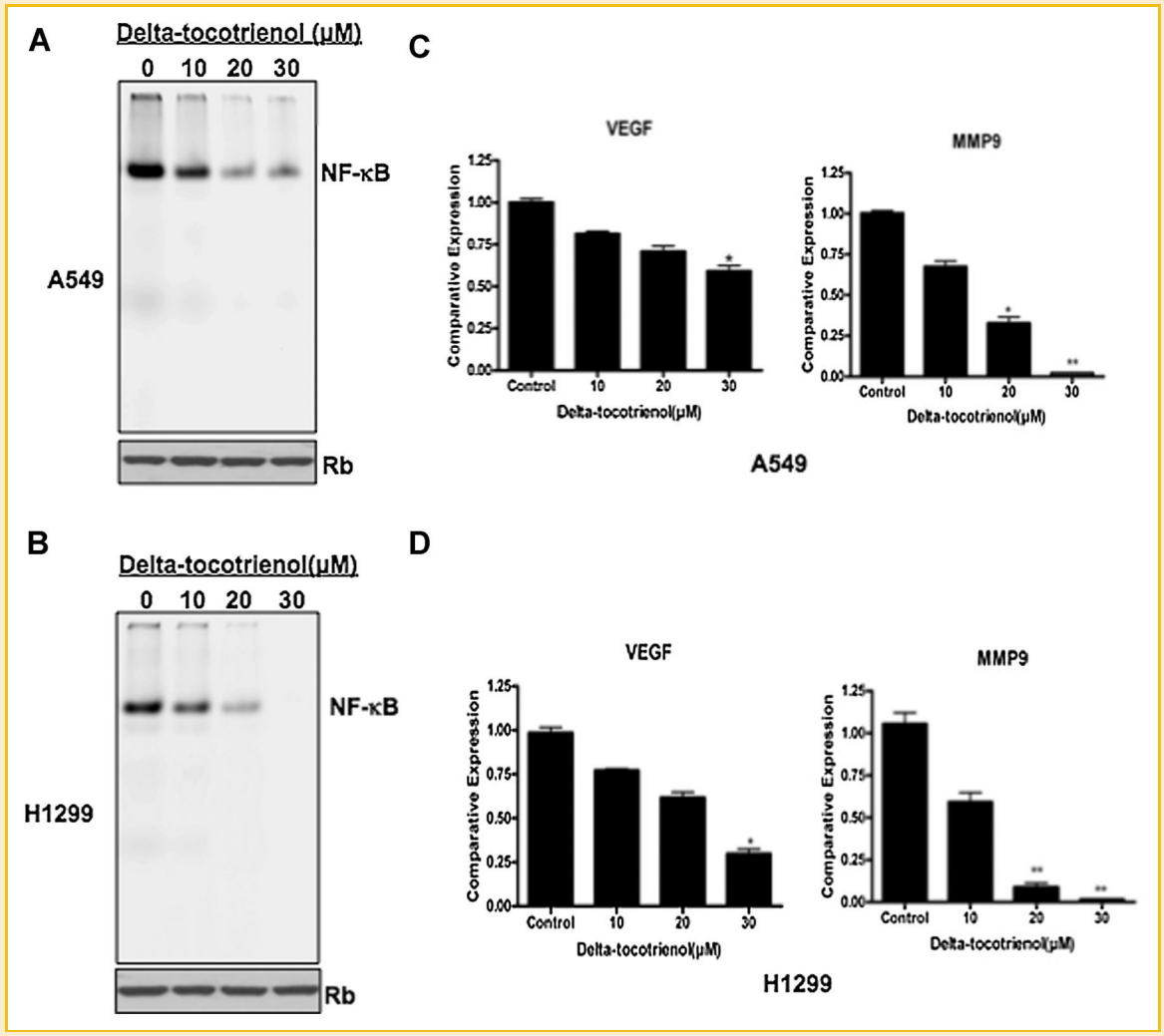

Fig. 5. Dose-dependent down-regulation of NF-kB activity and its down-stream genes by delta-tocotrienol. A549 (A) and H1299 (B) cells were incubated with increasing concentrations of delta-tocotrienol or DMSO-control for 72 h, and nuclear proteins were subjected to gel shift assay for the evaluation of NF-κB DNA binding activity. A549(C) and H1299 (D) were treated with or without of delta-tocotrienol. NSCLC cells for 48 h. The expressions of VEGF and MMP9 were analyzed by real-time-RT-PCR. Relative gene expressions were presented as means ± SEM of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

tocotrienol was effective against A549 and H1299 cell growth. In line with the MTS test, the clonogenic assay (Fig. 1C and D) demonstrated that delta-tocotrienol efficiently inhibited cells growth in a dose dependent fashion.

Most anti-cancer agents inhibit cancer cell proliferation and tumor progression by inducing apoptosis. Therefore, we assessed apoptosis-inducing effects of delta-tocotrienol in both NSCLC cell lines. An earlier publication reported that delta-tocotrienols induced apoptosis and cell cycle arrest in pancreatic cancer cells [Hussein and Mo, 2009]. In our study, delta-tocotrienol elicited a dramatic induction of apoptotic processes in NSCLC cells, as shown by DNA/histone fragmentation analysis and Annexin V staining analysis. Because inhibition of cell growth is associated with the cell cycle arrest, we investigated whether or not delta-tocotrienol could induce cell cycle arrest. Indeed, our results establish that delta-tocotrienol induced cell cycle arrest in the G_0-G_1 phase for both cell lines in a dose dependent manner.

Recent reports have shown that Notch-1 expression regulates cell death through both apoptosis and cell cycle arrest [Guo et al., 2009]. Moreover, as both A549 and H1299 have higher Notch-1 expression, we wanted to determine if delta-tocotrienol induces apoptosis, anti-metastasis, and cell cycle arrest by inhibiting the Notch-1 pathway. In order to explore the molecular mechanisms induced by delta-tocotrienol, we examined the protein expressions such as Notch-1, Hes-1 and apoptosis pathway proteins such as PARP, Survivin, and Bcl-XL. In the present study, we clearly demonstrate that delta-tocotrienol induced apoptosis in NSCLC by reducing expression of Notch-1, Hes-1. Because Survivin and Bcl-XL expression prevent cells from apoptosis, our results suggest that decreased Survivin and Bcl-XL expression may also participate in apoptosis induced by delta-tocotrienol in NSCLC. These results, along with dose-dependent PARP cleavage indicate the inhibition of cell growth observed in NSCLC treated with delta-tocotrienol may be due to the increase in apoptosis.

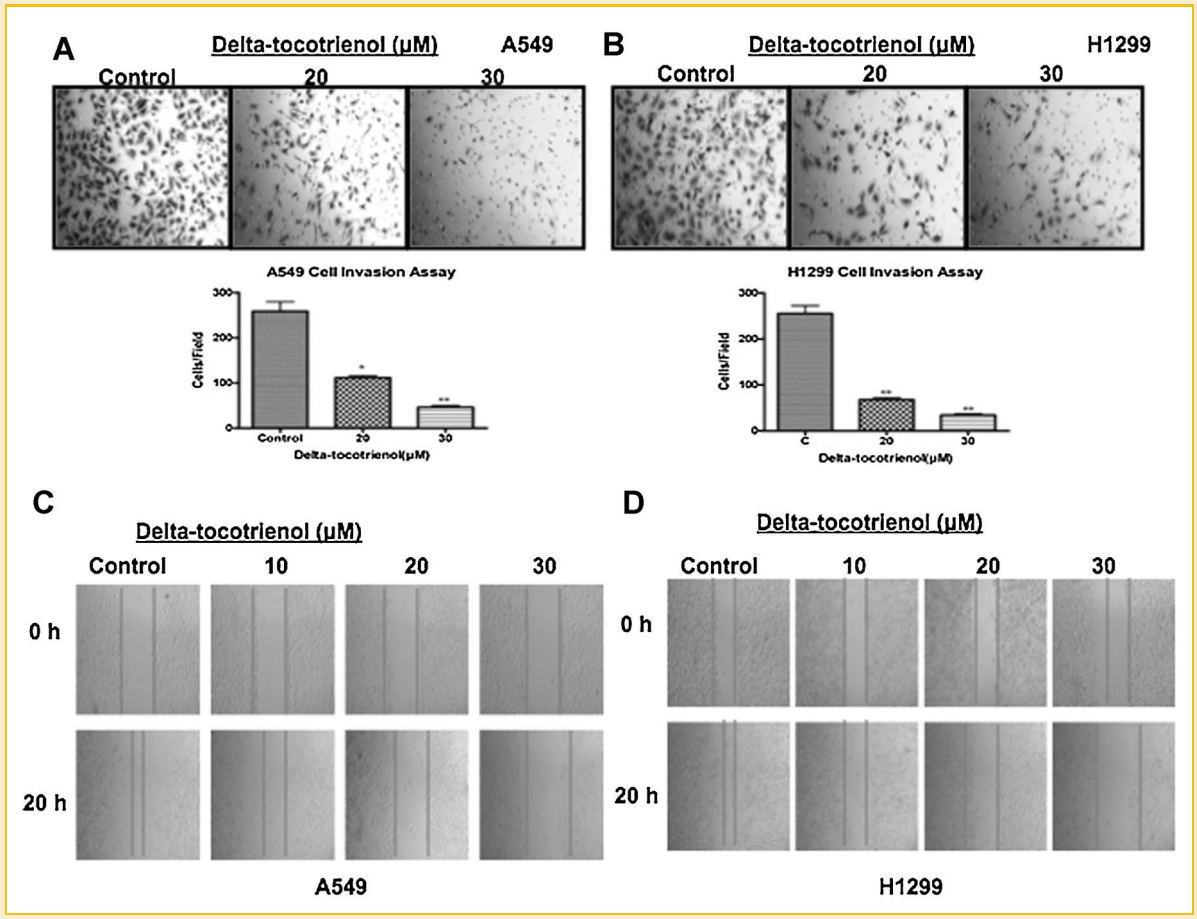

Fig. 6. Delta-tocotrienol inhibits cell migration and invasion dose-dependent inhibition of NSCLC cells invasion by delta-tocotrienol. A549 (A) and H1299 (B) cells were seeded treated seeded into Matrigel-coated inserts with delta-tocotrienol or DMSO. Cells that invaded to the lower surface of the insert over a period of 20 h were stained with crystal violet dye. Five random fields were counted for the number of invaded NSCLC cells. Cell invasion were presented as means ± SEM of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$. (C and D), dose-dependent inhibition of NSCLC cells migration by delta-tocotrienol using the wound healing assay. Uniform wounds were done by scratching in confluent cultures which were treated with delta-tocotrienol over 20 h. After that, the wound healing images were captured using a microscope at 10× objective.

NF-κB plays important roles in many cellular processes including cell proliferation, invasion, and angiogenesis all of which are crucial for cancer development and progression. Recently, Notch-1 pathway has been reported to cross-talk with the NF-κB pathway [Wang et al., 2006b]. A previous report showed that mice with reduced Notch pathway had significantly decreased NF-κB activity [Wang et al., 2004]. However, there is no report till date showing the inhibition of NF-κB biding activity through notch-1 pathway by delta-tocotrienol. Since delta-tocotrienol can inhibit the Notch-1 pathways and Notch-1 can cross-talk with NF-κB, we further performed the EMSA to investigate the activity of NF-κB upon delta-tocotrienol treatment. So, consistent with the literature documentation on the overlap between the Notch and NF-κB pathway, in addition to inhibition of Notch signaling, our results clearly support the idea of simultaneous inactivation of NF-κB binding in NSCLC cells (Fig. 5 A and B). In addition, we wanted to explore the anti-metastatic effect of delta-tocotrienol act in NSCLC cells. Indeed, we showed that in both A549 and H1299 cells, migration and invasiveness were significantly reduced under treatment of delta-tocotrienol (Fig. 6). Shibata et al. [2008] found that delta-tocotrienol suppressed hypoxia-induced VEGF and IL-8 expression at both mRNA and protein levels which in turn suppressed tumor angiogenesis. Consistent with the previous study, our study confirmed that the anti-metastatic effects induced by delta-tocotrienol were associated by a decrease in VEGF and MMP-9 (Fig. 5C and D) expressions.

In summary, we have provided experimental evidence that indicates that delta-tocotrienol inhibited Notch-1 signaling, cell proliferation, invasion and induced apoptosis in NSCLC cells. Moreover, our current data provide mechanistic information showing that delta-tocotrienol exerts its pro-apoptotic effects on NSCLC cells, at least in part due to inactivation of Notch-1, Hes-1 and NF-κB signaling (Fig. 7). On the basis of our results, we propose a hypothetical pathway by which delta-tocotrienol inhibits cell growth of NSCLC cells. Further in-depth experiments are needed to ascertain the specific mechanisms by which delta-tocotrienol regulates these pathways. However, previous studies on the effect of vitamin E, mainly tocopherols on cancer types in cell and animal or clinical studies have shown inconsistent results. This may be attributed to their low bioavailability leading to decreased

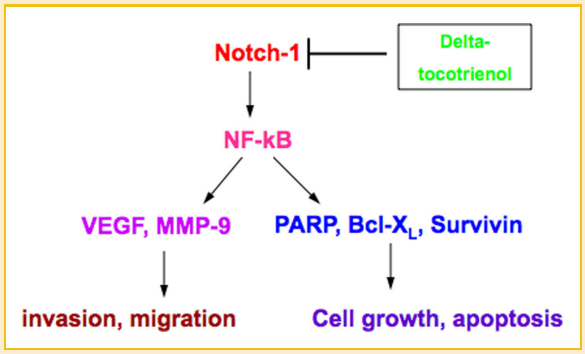

Fig. 7. Molecular pathways induced by delta-tocotrienol in NSCLC cells.

concentrations in the target tissues [Ju et al., 2010; Sylvester et al., 2010]. Thus additional in vivo studies, for example in transgenic mice models, and future clinical trials will be needed to establish whether delta-tocotrienol could be useful in combination with conventional chemotherapeutics or conventional targeted agents for the treatment of NSCLC for which at present, there is no effective and curative therapy.

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