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Intervention of non-small cell line cancer oncogenic pathways with delta-tocotrienol

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INTERVENTION OF NON-SMALL CELL LINE CANCER ONCOGENIC PATHWAYS WITH DELTA-TOCOTRIENOL

by

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DEDICATION

This dissertation is dedicated in loving admiration to my father and mother, Yanqi Ji and Xiaolan Du, whose solid support and continued encouragement gave me the foundation and strength to pursue my research. Words cannot express my thanks to my wife, Yan Wang and daughters Catherine and Charity, for their love, support, trust and more than anything else for their patience, over these past few years. I would like to extend my thanks to my parents-in-law, Yumin Wang and Yafang Chen, for their enthusiasm and support. Finally, I would like to express my thanks to my uncle, Chen Ji and his family for their constant motivation in my endeavor.

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TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

Introduction

Cancer:

The modern paradigm that mutations in somatic cells cause the uncontrolled cell proliferation called cancer is now well established (1). It is known that cells normally divide in a controlled way, but abnormal cells keep on re-dividing, forming an expanding “lump”. This cluster of abnormal cells is called a tumor. Some tumors are benign or harmless and often don't need treatment. But malignant tumors, the cancers, can spread (2). Cancer is the uncontrolled growth and spread of cells that may affect almost any tissue of the body. Among men, lung , prostate and colon cancer are the most common cancers worldwide. For women, the most common cancers are lung, breast and cervical cancer. More than 10 million people are diagnosed with cancer every year. Worldwide, it is estimated that there will be 15 million new cases of cancer every year by 2020. Cancer causes 6 million deaths every year—or 12% of all deaths are due to cancer worldwide (3). Over the past decade, many treatment strategies has been implemented to improve the survival of patients diagnosed with lung cancer including chemotherapy, radiation therapy, target therapy, and immunotherapy; however, the outcome has been very disappointing. With few exceptions, the development of cancer in adult humans involves a complex succession of events that can accumulate over many decades. The cells in the emerging neoplastic clone accumulate within them a series of genetic or epigenetic changes that lead to changes in gene activity, and thus altered phenotypes which are subject to selection (4). Douglas Hanahan and Robert A. Weinberg suggest that the vast catalog of cancer cell genotypes is a manifestation of six essential alterations in cell physiology that collectively dictate malignant growth (5): self-

sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. Each of these physiologic changes—novel capabilities acquired during tumor development—contributes to the successful breaching of anticancer defense mechanisms hardwired into cells and tissues.

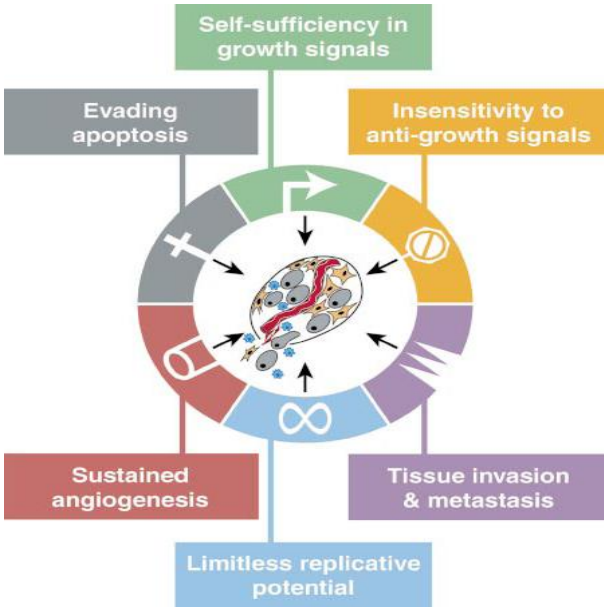

Fig.1.1 The Hallmarks of Cancer (5)

Oncogenes and Tumor Suppressor Genes

It is widely accepted that cancer is a genetic disease and DNA alteration is an important step in every tumorgenesis. Somatic mutations that have been selected during tumorigenesis are, by definition, causally related to tumor formation, and therefore represent legitimate targets for anti-cancer drugs (6). Accumulation of mutation in these genes will leads to cancer phenotype. These cancer genes are involved in molecular pathways supporting cellular mechanisms such as DNA

repair, cell proliferation, apoptosis and cell-cell interaction. These cancer arises through the accumulation of mutations in specific classes of genes within the cell (6). A simplistic interpretation divides cancer genes into two broad categories and their functions are as follows:

- **Oncogenes** promote cell growth and when mutated result in constitutively or abnormally active proteins; such mutations are mainly of the dominant type.
- **Tumor suppressors genes** are negative controllers of cell cycle progression and the mutations that affect them are typically recessive.

Lung cancer:

Cancer, the uncontrolled growth and spread of abnormal cells, results from the accumulation of numerous sequential mutations and alterations in nuclear and cytoplasmic molecules (7). Cancer progression or tumorigenesis is considered to involve three key steps: initiation, in which a normal cell is transformed into an initiated or abnormal cell, promotion, by which the initiated cell is converted into a preneoplastic cell, and progression, the process whereby the cells become neoplastic (8). Cancer may be initiated due to multiple factors including exposure to carcinogens, repeated genetic damage by oxidative stress, chronic inflammation or hormonal imbalance. This followed by a cascade of reactions, triggered by multiple signaling molecules makes it difficult to target a specific molecule responsible for the disease and thereby retard progression.

Lung cancer is the leading cause of death among all malignant diseases. It has been estimated that 226,160 (116,470 men and 109,690 women) will be diagnosed with, and 160,340 will die of cancer of the lung and bronchus in 2012 (9). There are two main types of lung cancer: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC account for

80% of all lung cancer reported with 16% of five-year survival rate (10). NSCLC consists of three histological types: squamous-cell carcinoma (SCC, 28%), large-cell carcinoma (LCC, 42%) and adenocarcinoma (AC, 48%) (11). The leading cause of lung cancer is direct and second hand smoking which accounts for 75% of all lung cancer (12). The remaining 25% of lung cancers are attributable to exposure to substances such as arsenic, asbestos, radioactive dust (radon), other environmental factors and genetic changes (12). Based on the clinical and molecular characteristics of never smoking versus smoking induced lung cancers, it has been suggested that they are separate entities (13).

The major obstacle in improving the treatment outcome of lung cancer, especially NSCLC, is that it progresses undetected (asymptomatic) till it has metastasized. The common symptoms for lung cancer include persistent cough, chest pain, coughing up blood, hoarseness, weight loss, fatigue and recurrent respiratory infections (such as pneumonia). All these symptoms can be related to other common respiratory disease and not specifically to lung cancer (14). The invasive NSCLC establishes distant metastases in organs including the bones, contralateral lung, liver and brain ahead of diagnosis which are lethal for the patients rather than the primary tumors in lung themselves (15). According to the American Society of Clinical Oncology Clinical Practice, platinum combinations with non-platinum therapy remain the first line of the agents to treat the NSCLC patients (12). These standard chemotherapy drugs or their combinations showed no significant improvement in overall survival long term in NSCLC patients (16). Therefore, novel and effective chemotherapeutic strategies to target NSCLC are under in-depth investigations. One promising treatment strategy involves in sub-group of NSCLC patients based on their expression of clinically relevant molecules. These molecules are encoded by the mutation of specific genes, in this case oncogenes, tumor suppressor genes, and

microRNAs, which play crucial roles in tumorigenesis and its progression, which are discussed focusing on several potential targets and targeted agents for the treatment of NSCLC.

Lung cancer and EGFR

Epidermal growth factor (EGFR) is the cell-surface growth factor receptor which exists as an inactive monomer from inside the cell membrane. Upon ligand binding, EGFR may form homodimers of EGFR-EGFR or heterodimers with other ErbB family members, such as ErbB2 or ErbB3 by dimerization. EGFR dimerization stimulates its intrinsic intracellular tyrosine kinase activity. As a result, the c-terminal tyrosine residues of EGFR are autophosphorylated by these tyrosine kinases. This autophosphorylation activates the downstream signaling of EGFR via protein-protein interaction. The downstream signaling initiates signal transduction cascades, such as the activation of PI3K-AKT pathway, leading to DNA synthesis, cell proliferation, and migration. EGFR has been found to be overexpressed in many kinds of cancers including lung, breast, ovarian, head and neck cancers (13-15, 17). Moreover, the overexpression and activation of EGFR is a predictor of disease progression, and sensitivity to the kinase inhibitors (TKI) in NSCLC patients (13). Thus inhibition of EGFR is a logical approach for cancer treatment or slowing down its progression.

Gefitinib and Erlotinib were the first two approved small molecules EGFR-TKI for the treatment of NSCLC. These TKI competitively binds the EGFR at the ATP binding site in order to inhibit EGFR activation. In clinical trials, American and Japanese NSCLC patients responded differently to TKI. The rate of response between American and Japanese was 10% vs. 30%, respectively (18, 19). Overall, Asian, female, non-smoking patients showed better drug response than other patients (20). Further gene sequencing data showed that somatic gene mutations coded

for EGFR tyrosine kinase are responsible for TKI efficacy. Most of the EGFR beneficial mutations of NSCLC belong to two types: LREA deletion in exon 19 and L858R point mutation in exon 21 (20, 21).

However, some responsive patients who initially respond to TKI show an acquired resistance, associated with secondary somatic mutation in T790M on exon 20 or by amplification of MET, another cell membrane receptor (22, 23). Clinical data shows that about 50% of patients who developed gefitinib-resistant have T790M somatic mutation (24-26). Additionally, MET amplification accounted for 20% of patients who developed gefitinib-resistant, through ERBB3 (a membrane of the EGFR family)-dependent activation of PI3K pathway (27). New strategies to overcome the acquired resistance to TKI are being explored. Second generation irreversible EGFR inhibitors have been shown to beat the TKI resistance with secondary somatic mutation in T790M in preclinical models (28, 29). Recently, data has been shown that simultaneous targeting of the MET and EGFR pathways can provide synergistic inhibitory effects for the treatment of cancers in which both pathways are activated (30). However, the percentage of patients with EGFR-MET double mutation is very low, so only a few patients will benefit from new drug.

Lung cancer and Notch-1 pathway:

Notch proteins, the transmembrane receptors, are highly conserved in the development and the determination of cell fate (31). As the ligand-receptor singaling pathway, Notch signaling plays critical roles in mediating cell proliferation, survival, apoptosis (32). Until now, four Notch receptors have been found in mammals, namely Notch-1-4 (33). Additionally, five Notch ligands including DLL-1, DLL-3, DLL-4, Jagged-1, Jagged-2 have been identified (33, 34). All the Notch receptors and their lignads have been shown to be related to cancer (35). Once

their ligands bind to the extracellular domain, Notch receptors undergo a series of proteolytic cleavages, releasing the intracellular Notch which translocates into the nucleus (36). Inside the nucleus, the active forms of Notch combining with other transcription factors regulate the expression of target genes, such as Hes-1, Bcl-X_L, Survivin (37, 38). Since notch signaling regulates critical cell fate decision, alterations in Notch signaling are associated with tumorigenesis. It has been found that notch signaling is frequently dysregulated with up-regulated expression in different types of cancers such as lung, colon, head and neck, pancreas (39-42). Overexpression of Notch-1 has been shown to inhibit apoptosis in different types of cancers (43, 44), suggesting that Notch could be considered as a therapeutic target. Clinical data has demonstrated that 30 % of NSCLC patients have increased Notch activity among which 10% of NSCLC has gain-of-function mutation on the *Notch-1* gene (39). Recently, it has been reported that Notch-1 stimulates survival of NSCLC cells during hypoxia by activating the IGF pathway (45). As γ-secretase is the last and obligatory step to activate the Notch pathways, a group of small-molecule inhibitors of the γ-secretase complex (GSIs), which prevent intracellular Notch-1 (ICN1) release into nucleus, are being tested in the clinical trials. As down-regulation of Notch-1 showed anti-neoplastic effects in vivo and in vitro (43, 44, 46), the potential for treating certain cancers could be achieved by inhibiting Notch signal transduction. However, the major obstacle to the use of these compounds is due to their high systemic toxicity (39). Therefore novel compounds that could target the Notch pathway would be a promising strategy for cancer therapy.

Lung cancer and NF-κB pathway:

Nuclear factor-kappaB (NF-κB), another key apoptotic regulator, which plays important roles in cancer cell transformation and development (47). Accumulating data shows that there is a cross-talk between the Notch-1/Hes-1 pathways and the NF-κB pathway. Notch ligands induced NF-κB activation in leukemia cells, and decreased Notch-1 expression was accompanied with concomittent decrease in NF-κB binding activity (48). Moreover Notch-1 has been found to induce sustained NF-κB activity by facilitating its nuclear retention (49). Specially, NF-κB2 promoter activity is activated by the Notch-1 pathway (50). Recently, Notch-1/Hes-1 pathways were found as the upstream mechanisms for maintainance of NF-κB activation in leukemia in vivo and in vitro (51). Recently, clinical data showed that increased expression of the NF-κB inhibitor IκB predicted for improved response and survival in EGFR-mutant lung cancer patients treated with EGFR-TKI (52). Taken together, these results suggest that NF-κB could be considered as a potential target that might provide synergistic effects upon treatment of NSCLC with other chemo-drugs.

Lung Cancer and microRNA:

MicroRNAs (miR) are small non-coding RNAs that are involved in post-transcriptional gene regulation (53). These molecules silence their target gene expression by directly interacting with the 3'-untranslated region (3'-UTR) of mRNA, promoting RNA degradation and/or inhibiting transcription. Accumulating data demonstrates that miRNA plays important roles in cancers by regulating the expression of various oncogenes and tumor suppressors genes (54). More importantly, it has been suggested that dysregulations of specific miRNAs and their targets in various types of cancer is associated with the development and progression of cancers (55, 56). For example, reduced expression of let-7 microRNAs, the tumor suppressor gene, has been

shown to be associated with shortened postoperative survival in lung cancer patients (57). In addition, miR-107 and miR-185 were found to localize in frequently altered chromosomal regions in human lung cancer and over-expression of miR-107 and miR-185 significantly reduced the proliferation of A549 and H1299 NSCLC cells (58). Clinical data has demonstrated that the miR-34 family was down-regulated in tumor compared with normal tissue, and low levels of miR-34a expression were correlated with a high probability of relapse in lung cancer (59). Therefore, it is important to unravel the relationship between miRNA expression and oncogene such as Notch-1 signaling. It is also important to find novel agents that could regulate the specific miRNA expressions and Notch-1 pathway which could then be potentially useful for the treatment of NSCLC in the future.

Tocotrienols and cancer prevention:

Tocotrienols are chemical isoforms of natural vitamin E (alpha, beta, gamma, and delta) which are rich in cereal grains and palm oil. Compared to tocopherols, tocotrienols has unsaturated side chain with three double bonds in their farnesyl isoprenoid tail. Unlike tocopherols, tocotrienols only account for 1% of total vitamin E research because of their lower availability in nature. Found in wheat germ, barley, some grains and vegetable oils, palm oil represents one of the richest natural sources of tocotrienols with 70% of its Vitamin E in the form of tocotrienols (60, 61).

Identification of α-tocotrienol as an inhibitor of cholesterol biosynthesis led to a spur of activity in tocotrienol research (62). Later, tocotrienols were found to regulate cholesterol production in mammalian cells by post-transcriptional suppression of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (63). Studies indicate that delta and gamma-

tocotrienol enhance HMG-CoA reductase ubiquitination and degradation. In addition, delta-tocotrienol could block the processing of sterol regulatory element-binding proteins (SREBPs) (64). This suppression of HMG-CoA activity is corroborated with a decrease in total cholesterol and low density lipoprotein plasma levels in hamsters (65). Taken together, these reports suggest that dietary supplement of tocotrienols may represent a novel approach for the treatment of hypercholesterolemia.

In comparison to the tocopherols, tocotrienols have more potent antioxidant properties due to the presence of three double bonds in the hydrocarbon tail (66, 67). Overproduction of reactive oxygen and nitrogen species (RO/NS), leading to oxidative stress is a well established contributor to a multitude of chronic diseases, for example, cardiovascular, cancer, neurodegeneration and to the normal process of aging as well (68). Thus agents that help to lower the burden of oxidative stress may be of benefit in preventing or delaying onset of these diseases. In line with their antioxidant capabilities, administration of tocotrienols but not tocopherols reduced the accumulation of protein carbonyls (indicators of oxidative damage during aging) and consequently extended the mean life span in C. elegans (69). Very recently, dietary supplementation with tocotrienols demonstrated improved T- cell function in old mice (70).

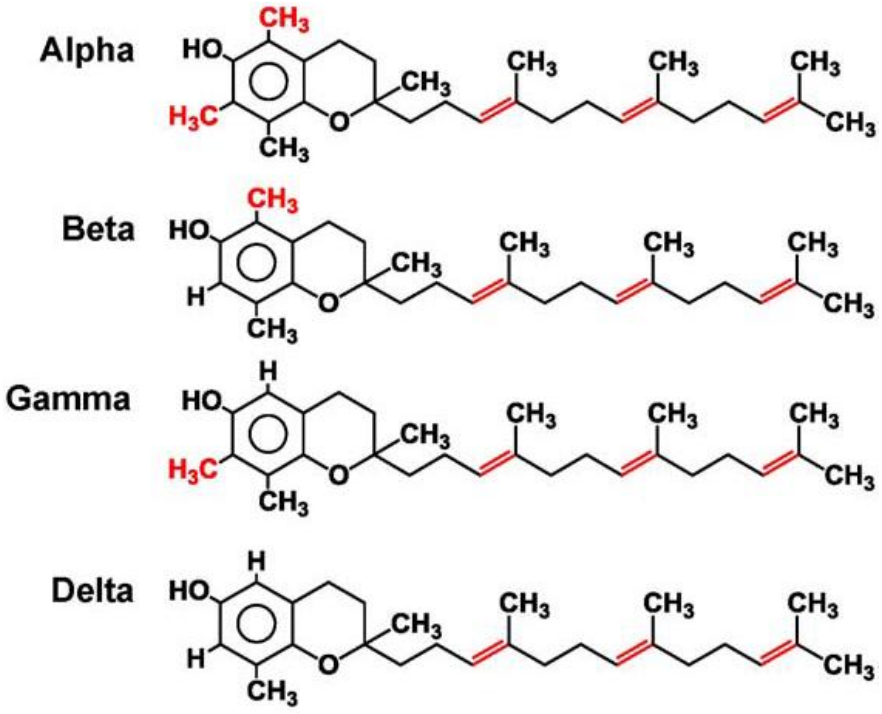

Fig.1.2 Chemical Structure of Tocotrienols (71)

Although α-tocotrienol seems to offer the most neuroprotection, among all the vitamin E isomers, γ and δ-tocotrienols have been shown to be superior to the other isomers in cancer prevention. One of the earlier studies investigating the anti-cancer effect of tocotrienols, showed that alpha- and gamma-tocotrienols were effective against sarcoma 180, Ehrlich carcinoma, and invasive mammary carcinoma. In addition, gamma-tocotrienol showed a slight life-prolonging effect in mice with Meth A fibrosarcoma. However, no antitumor activity of tocotrienols was observed against P388 leukemia at doses of 5-40 mg/kg/d (72). Since then, there has been a tremendous interest in investigating the anti-cancer effects of tocotrienols. As such, tocotrienols have now been shown to have anti-tumor effects on different human cancer cells including prostate, breast, colon, melanoma, and lung cancers (73-75). Additionally, it has been shown that

tocotrienols can induce apoptosis by inhibiting multiple signaling pathways such as EGFR, NF-kB, MAPK, and PI3K/AKT pathways (76).

CHAPTER 2

Background and Specific Aims

Lung cancer is the leading cause of death among all the cancers. NSCLC accounts for 80% of lung cancer with a five-year survival rate of 16%. Since Notch-1 signaling plays an important role in cell proliferation, differentiation, and apoptosis; and thus down-regulation of Notch-1 may exert anti-tumor effects. Gamma-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. The importance of its inhibition as a target for cancer therapy is reflected in the number of gamma-secretase inhibitors (GSI) under preclinical investigations. Acute toxicity is a major barrier for the usage of most of the GSI compounds. Identification of compounds targeting Notch signaling in NSCLC with minimal toxicity might provide new impetus in this area. *We hypothesize that delta–tocotrienol will reduce NSCLC cell growth via inhibition of Notch-1 signaling pathway which in turn can be used as a sensitizer to cisplatin.*

Specific Aim 1: To determine the anti-cancer activity of delta -tocotrienol against different NSCLC cells and elucidate its in vitro effect on Notch-1 and NF-κB signaling pathways. We characterized anti-cancer effects of delta -tocotrienol on NSCLC cell lines overexpression Notch-1. Since there is lack of data showing the effect of delta-tocotrienol on NSCLC cell lines overexpressing Notch-1, we identified molecular mechanisms on these cell lines. Specifically, we investigated the Notch-1 cellular pathway and also the transcription factor NF-κB pathway. First, we determined the delta –tocotrienol IC_{50} (half inhibition concentration) on these three cell lines by MTS

assay. Second, we tested delta –tocotrienol's ability for the inhibition of cell growth of these cell lines by flow cytometry. Third, we detected gene expression regulation effect of delta –tocotrienol on the nuclear transcription factor. NF-κB activity was measured by electrophoretic mobility shift assay (EMSA). Lastly, we measured the protein expression of effectors of Notch-1 by western blotting.

Specific Specific Aim 2: To identify the alterations in microRNA expression profile of NSCLC cells after treatment with delta-tocotrineol. Utilizing MicroRNA (miRNA) microarray, we found the inhibition of NSCLC cell growth and induction of apoptosis by delta-tocotrienol due to modulation of Notch-1 pathway mediated through alteration of specific miRNA expression. Pre-miRNA transfection was utilized to investigate the molecular mechanism induced by delta-tocotrienol. By using transfection, we knock-down specific miRNA and elucidated its effects on cell proliferation, apoptosis and invasion.

Specific Aim 3: To elucidate synergistic effects of delta - tocotrienol and cisplatin (DNA damage) on drug resistant cell models. We investigated the growth inhibitory effects of a low dose treatment of cisplatin alone or in combination with delta -tocotrienol and investigated the intracellular signaling mechanisms. Since delta tocotrienol inhibit the cell proliferation by different mechanisms, we identified the additive effects of cisplatin and delta-tocotrienol on these cell lines. First, we identified the delta –tocotrienol IC_{50} (half inhibition concentration) alone or in combination with different concentration of erolitinib on these cell lines by MTS assay. Second, we tested delta –tocotrienol and cisplatin separately or combination with cisplatin for inhibitory their

growth effects on these cell lines by flow cytometry. lastly, the nuclear transcription factor NF-κB activity was measured by EMSA assay. Last, we investigated changes in protein expression by western blotting.

CHAPTER 3

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 the cancers. Non-small cell lung cancer accounts for 80% of lung cancer with a five-year survival rate of 16%. Notch pathway, especially Notch-1 is upregulated 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.

Introduction

Notch proteins, the transmembrane receptors, are highly conserved in the development and the determination of cell fate (31). This is because the ligand-receptor signaling pathway, Notch, plays critical roles in mediating cell proliferation, survival, and apoptosis (32). Until now, four Notch receptors, namely Notch1-4 have been found in mammals. (33). Additionally, five Notch ligands including DLL-1, DLL-3, DLL-4, Jagged-1, Jagged-2 have been identified (33, 34). All the Notch receptors and their ligands have been shown to be related to cancer (35). Once their ligands bind to the extracellular domain, Notch receptors undergo a series of proteolitic cleavages, releasing the intracellular Notch which translocates into nucleus (36). Inside the nucleus, the active forms of Notch combining with other transcription factors regulate the expression of target genes, such as Hes-1, Bcl-xL, Survivin (37, 38). Since notch signaling regulates critical cell fate decision, alterations in Notch signaling are associated with tumorigenesis. Notch expression has is known to be up-regulated in different types of cancers including colon, lung, head and neck, and pancreatic (39-42). Overexpression of Notch-1 has been shown to inhibit apoptosis in different cancers types (43, 44), suggesting its potential as a therapeutic target.

Lung cancer is the major cause of death among malignant diseases, and Non-small Cell Lung carcinoma (NSCLC) accounts for 80% of lung cancer, with a 16% five-year survival rate (10). Clinical data has demonstrated that 30 % of NSCLC has increased Notch activity and 10% of NSCLC has gain-of-function mutation on Notch-1 gene (39). Recently, it has been reported that Notch-1 stimulates survival of NSCLC cells during hypoxia by activating the IGF pathway (45). Another key apoptotic regulator, nuclear factor-kappaB (NF-κB) plays important roles in

cancer cell transformation and development (47). More and more data shows 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 NF-κB DNA binding activity (48). Moreover, Notch-1 has been found to induce sustained NF-κB activity by facilitating its nuclear retention (49). Specifically, NF-κB2 promoter activity has been shown to be activated by Notch-1 pathway (77). 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* (51).

However, the mechanisms by which Notch-1 inhibits cell growth and induced apoptosis in NSCLC are still unclear. Since Notch-1 down-regulation has shown anti-neoplastic effects *in vivo* and *in vitro* (43, 44, 46), the potential for treating certain cancers could be achieved by inhibiting Notch signal transduction. Tocotrienols, components of naturally occurring vitamin E exit 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 (73-75). Additionally, tocotrienols can induce apoptosis by inhibiting multiple signaling pathways such as EGFR, NF-κB, MAPK, and PI3K/AKT pathways (76). 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 will 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 24, 48 and 72 h of incubation, 20µl 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 490nm 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 3000× g for 5 min, the cell pellet 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) were 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 X 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 X 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 $4x10^5$ cells per well. After overnight incubation, the culture media were 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 attached. Subsequently, the cells were culture in presence or absence of delta-tocotrienol and the wound images were taken as 0 h. After 20h, wound healing pictures were taken under microscope.

Cell invasive assay:

BD Biocoat invasion kit (BD, San Jose, CA) was used to evaluate the tumor invasive ability. Briefly, around 2.5 x 10^5 cells of A549 and H1299 at with basal media was transferred in each 6-well upper chamber in the presence or absence of delta-tocotrienol. In the meantime, three milliliter of culture medium with 10% FBS was added into each lower chamber of 6-well plate. After 20 h incubation, the cells on the upper chamber were removed using cotton stick. Each of experimental conditions 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 of delta-tocotrienol for 72 hours 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 minutes on ice. Protein concentrations were determined using the Bradford protein assay kit (Bio-Rad Laboratories, CA). Each sample contained 50μg 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 (25mM Tris, 190mM glycine, 20% methanol) in Hoefer TE70XP transfer apparatus (Holliston, MA). The membranes were incubated for 1 hour at room temperature with 5% nonfat dried milk in 1 x TBS buffer containing 0.1% Tween. After that, membranes were incubated over night at 4°C

with primary antibodies (1:1000). The membranes were washed 3 times with TBS-T, and subsequently incubated with the secondary antibodies (1:5000) containing 2% BSA for 2 hours 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, USA) according to the manufacturer's protocols. Two microgram of total RNA from each sample was subjected to first strand cDNA synthesis using TaqMan reverse transcription reagents kit (Applied Biosystems, Foster City, CA) in a total volume of 20 µl. Reverse transcription reaction were performed at 25ºC for 10 min, followed by 48ºC for 30 min and 95ºC for 5 min. Real-time PCR analysis were 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'); All these primers are checked by running them on virtual PCR, and primer concentration are optimized to avoid primer dimer formation. Real-time PCR amplifications will be performed using 2 × SYBR Green PCR Master

Mix (Applied Biosystems). Two microliter of RT reaction will be used for a total volume of 25 microliter quantitative PCR reactions. The thermal profile for SYBR real-time PCR was 95ºC 10 min followed by 50 cycles of 95ºC 15 s and 60ºC 1 min. Data were analyzed according to the comparative fold increases or decrease in gene expression determined by quantitation of 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 mircrogram of nuclear proteins 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 cell 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 3.1 A 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, 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, 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. Fig. 3.1 C 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 barely seen at 30 μM delta-tocotrienol treatments. Overall, the results from the clonogenic assay were consistent with the MTS data shown in Fig. 3.1 A and 3.1B, 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 Fig. 3.2A and 3.2B, delta-tocotrienol induced apoptosis in both cell lines, A549 (Fig. 3.2 A) and H1299 (Fig. 3.2 B) in dose dependent manner. Annexin V/PI staining confirmed apoptosis-inducing effect of delta-tocotrienol in both cell lines tested (Fig. 3.2 C and D), respectively. Fig. 3.2 C 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 staning by flow cytometry. Both A549 and H1299 cells were treated in the absence or presence of delta-tocotrienol at 20 μM for 48 h were analyzed. Fig. 3.3 A 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 were showed a dose-dependent G_0-G_1 phase arrest induced by delta-tocotrienol. For A549 cells (Fig. 3.3 A), there was 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. 3.3

B) 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 Fig. 3.4 (A and B), the mRNA expression of Notch-1gene 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. 3.4) demonstrated that delta-tocotrienol inhibited the protein expression of Notch-1 in a dose dependent manner in both NSCLC cell lines. These results showed 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. 3.4 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 (78). 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 Fig. 3.5 A and B, compared to the control, delta-tocotrienol significantly inhibits the DNA-binding activity of NF-κB in dose 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 Fig. 5 C 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-kB 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 has 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 Fig. 6 A and B, delta-tocotrienol-treated A549 and H1299 cells depict a decrease in their invasive capability by at least 3 times as compared with the untreated control. Wound healing assay was performed to determine tumor cell migration ability. As demonstrated in Fig. 3.6 C 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 five-year survival rate of 16% (10). Abnormal Notch pathway has been found in 30% of NSCLC patients, and is implicated in their higher mortality rate (39). 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 (79). The importance of its inhibition as a target for cancer therapy is reflected in the number of γ-secretase inhibitors (GSI) under preclinical investigations (79). Acute toxicity is a major barrier for the usage of most of the GSI compounds(79). 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 (80) , there is no report regarding the molecular mechanism by which delta-tocotrienol may induce apoptosis in NSCLC cells(73, 75). 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-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 (81). 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 (82). 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.

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 (78). A previous report showed that mice with reduced Notch pathway had significantly decreased NF-κB activity (83). There is no report regarding the molecular mechanism by which deltatocotrienol may induce apoptosis in NSCLC cells. 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-kB 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 A *et al* found that delta-tocotrienol suppressed hypoxia-induced VEGF and IL-8 expression at both mRNA and protein levels which in turn suppressed tumor angiogenesis (84). 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. 5 C 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. 3.7). 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 concentrations in the target tissues (85). 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.

Figure Legends:

Figure 3.1: Antiproliferative effects of Delta-tocotrienol on NSCLC cells.

Cell viability (A and B) of human NSCLC cell lines A549 and H1299 cells. Both A549 (left) and H1299 (Right) cells were initially plated at a density of 5×10^3 cells/well (3wells/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 and H1299 cells. A549 (C) and H1299 (D) 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. There was a significant reduction in the colony formation in A549 and H1299 cells treated compared with cells untreated.

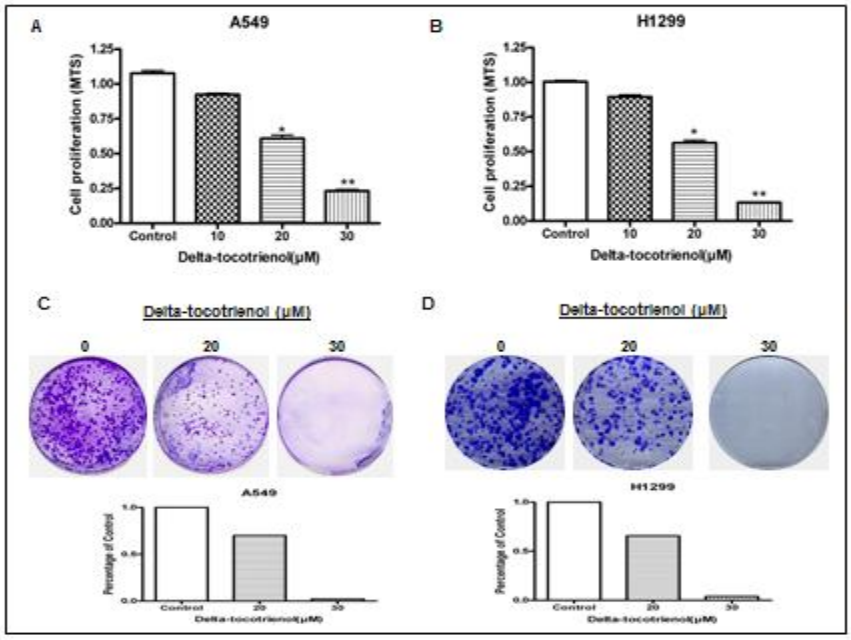

Fig-1

Figure 3.2: Induction of apoptotic effects of delta-tocotrienol and cell cycle analysis on NSCLC cells.

A and B, A549 and H1299 cells were treated with increasing concentration of delta-tocotrienol for 72 h. After that, the apoptosis of both cell lines were determined by histone/DNA ELISA. .*P<0.05, **P< 0.01.

C and D, A549 and H1299 cells were treated with 20 μM of delta-tocotrienol for 72 h. After that, the apoptosis of both cell lines were determined by Annexin V-FITC.

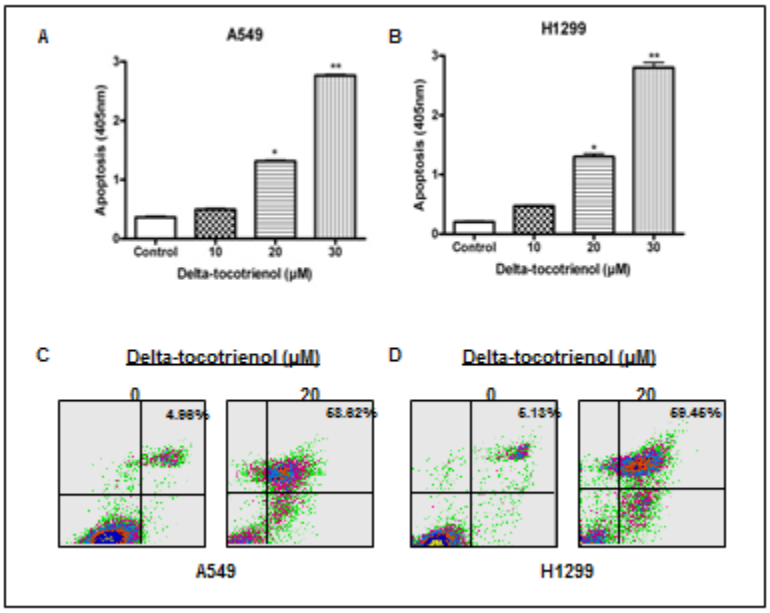

Fig-2

Figure 3.3: Delta-tocotrienol induces cell cycle arrest at G_0-G_1 phase

A and B, A549 and H1299 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, 30μM) of delta-tocotrienol were added and incubated for 72 h. Cell cycle distributions were done by flow cytometry.

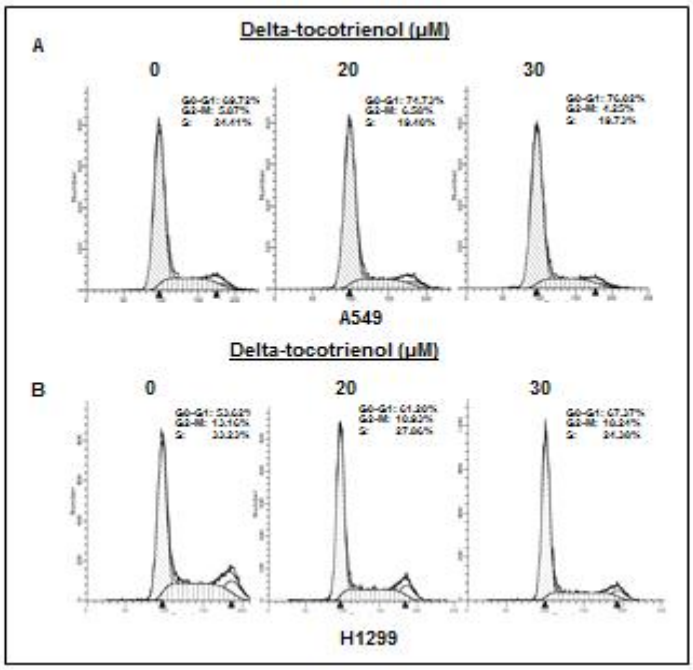

Fig-3

Figure 3.4: Dow-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 hours. Left panel: the expressions of Notch-1, Hes-1, PARP, Bcl-xL, 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.

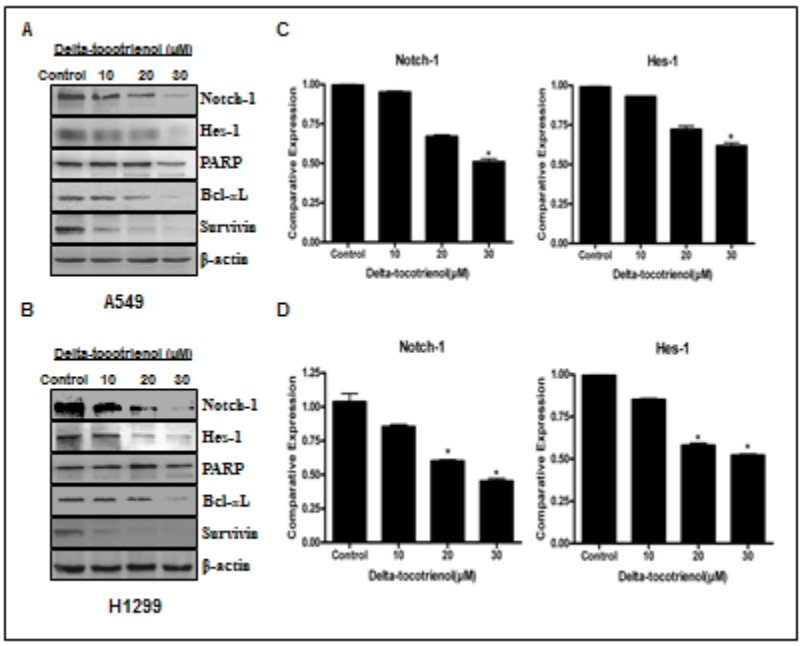

Fig-4

Figure 3.5: 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 ± S.E.M of three independent experiments.*P<0.05, **P< 0.01.

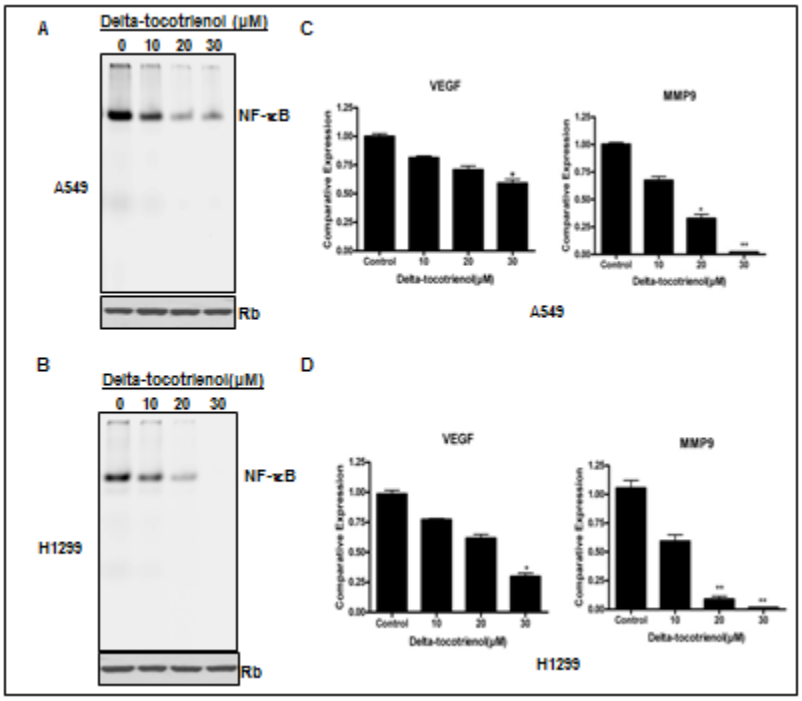

Fig-5

Figure 3.6: Delta-tocotrienol inhibits cell migration and invasion

A and B, Dose-dependent inhibition of NSCLC cells invasion by delta-tocotrienol. A549 and H1299 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 ± S.E.M 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.

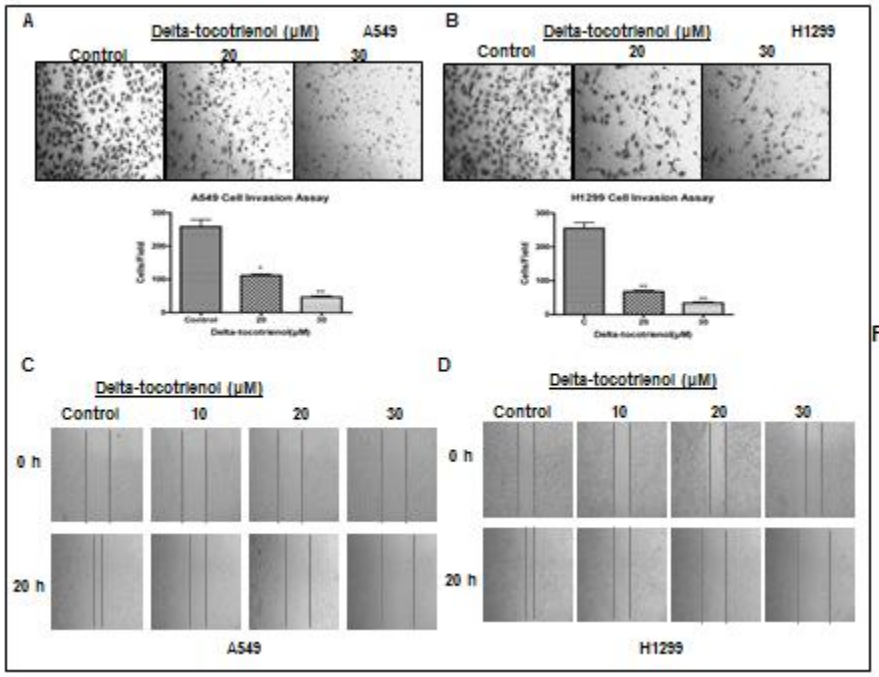

Fig-6

Figure 3.7: Molecular pathways induced by delta-tocotrienol on NSCLC cells.

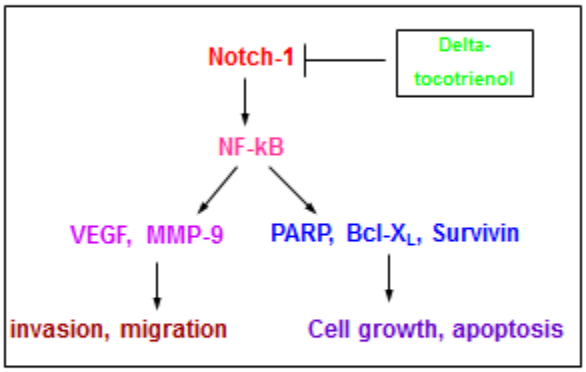
Notch-1
Delta-tocotrienol
NF-kB
VEGF, MMP-9
PARP, Bcl-X$_L$, Survivin
invasion, migration
Cell growth, apoptosis

Fig-7

CHAPTER 4

Delta-Tocotrienol Suppresses Notch-1 Pathway by Up-regulating miR-34a in Non-Small Cell Lung Cancer Cells

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KEY WORDS: Delta-tocotrienol; miR-34a; Notch-1; Non-small Cell Lung cancer.

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Abstract

MicroRNAs (miRNAs) are small non-coding RNAs that play critical roles in regulating various cellular functions by transcriptional silencing. MiRNAs can function as either oncogenes or tumor suppressors (oncomirs), depending on cancer types. In this study, using miRNA microarray, we observed that down-regulation of the Notch-1 pathway, by delta-tocotrienol, correlated with up-regulation of miR-34a, in non-small cell lung cancer cells (NSCLC). Moreover, re-expression of miR-34a by transfection in NSCLC cells resulted in inhibition of cell growth and invasiveness, induction of apoptosis and enhanced p53 activity. Furthermore, cellular mechanism studies revealed that induction of miR-34a decreased the expression of Notch-1 and its downstream targets including Hes-1, Cyclin D1, Survivin, and Bcl-2. Our findings suggest that delta-tocotrienol is a non-toxic activator of mir-34a which can inhibit NSCLC cell proliferation, induce apoptosis, and inhibit invasion, thus offering a potential starting point for the design of novel anticancer agents.

Introduction

Lung cancer is the leading cause of death among all malignant diseases, with non-small cell lung carcinoma (NSCLC) reported to have a five-year survival rate of only 16%, accounting for 80% of all lung cancer cases(10). Clinical data has demonstrated that 30% of NSCLC cases have increased Notch activity while 10% have gain-of-function mutation of the Notch-1 gene(39). After a series of proteolytic cleavages, the active form of Notch translocates from the cell membrane into the nucleus(36). Subsequently, Notch combines with other transcription factors to regulate the expression of its target genes, such as cyclin D_1, Bcl-2, and Survivin(37, 38). Since Notch signaling regulates critical cell fate decisions, alterations in Notch signaling are associated with tumorigenesis. Indeed, Notch expression has been reported to be up-regulated in different types of cancers including colon, lung, head and neck, and pancreatic cancers(39, 40, 42, 86). Overexpression of Notch-1 has been shown to inhibit apoptosis in many human cancers(43, 44), suggesting its potential as a therapeutic target. Recently, Notch-1 has been reported to stimulate survival of NSCLC cells during hypoxia by activating the IGF pathway (45). As a Notch downstream target, cyclin D_1 expression is another indicator of poor prognosis in resectable NSCLC (87). Cyclin D_1 is cell cycle regulator protein expressed during the G_1 phase and drives the G_1/S phase transition. Likewise, overexpressions of cyclin D_1 have been found in other types of cancers such as breast, bladder, and colorectal cancers (86-88).

MicroRNAs (miRNAs) are small non-coding RNAs that are involved in posttranscriptional gene regulation (53). These molecules silence expression of their target genes by directly interacting with the 3'-untranslated region (3'-UTR) of mRNA and promoting RNA degradation as well as inhibiting transcription. Accumulating data demonstrates that miRNAs play important roles in

cancers by regulating the expression of various oncogenes and tumor suppressors genes [54],[88]. For example, reduced expression of let-7 has been shown to be associated with shortened postoperative survival in lung cancer patients(57). Therefore, it is important to investigate the relationship between miRNA and the Notch signaling pathway. It is also important to find novel agents that could regulate the miRNA and Notch-1 pathway which could be useful for the treatment of NSCLC in the future.

It has been demonstrated that tocotrienols can induce apoptosis by inhibiting multiple signaling pathways such as the EGFR, NF-κB, MAPK, and PI3K/AKT pathways (76). Previously, we provided experimental evidence showing that delta-tocotrienol inhibited Notch-1 signaling, cell proliferation, invasion and induced apoptosis in NSCLC cells(89). In this study, we report that inhibition of NSCLC cell growth and induction of apoptosis by delta-tocotrienol due to modulation of Notch-1 pathway occurs via alteration of specific miRNA expression.

Materials and Methods

Cell culture, reagents and antibodies:

Human NSCLC cell lines (A549 and H1650) obtained from ATCC were grown in RPMI1640 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 cyclin D_1, 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-2, and p53 were bought from Santa Cruz Biotechnology (Santa Cruz, CA). The secondary antibodies were purchased from Bio-Rad Laboratories (Hercules, CA).

MicroRNA microarray analysis:

MicroRNA expression of 84 miRNA was measured using the RT^2 miRNA PCR array system (SABiosciences, MD) according to the manufacturer's instructions. The Eppendorf realplex 4 system (Hauppauge, NY) was used for all PCR reactions. Data analysis was performed using the RT^2 Profiler PCR Array Data Analysis (SABiosciences, MD). The expressions of all the miRNAs were normalized to hsa-SNORD-44. Further, DIANA-microT was used to predict the target genes.

MiRNA real-time reverse transcriptase-PCR:

To validate the altered expression of the miRNA (miR-34a) that was found by miRNA array analysis, we first converted the miRNA to cDNA using RT^2 First-Stand cDNA Synthesis Kit

(SABiosciences, MD). This was followed by real-time miRNA reverse transcriptase-PCR (RT-PCR) analysis using miR-34a and snord-44 primers from SABiosciences (SABiosciences, MD) to validate data from the microarrays.

MiRNA-34a transfection:

A549 and H1650 cells were seeded in six-well plates at a density of two million per well for 24h and then transfected with pre-miRNA-34a (miR-34a), miRNA-negative control (negative control) or miRNA-34 inhibitor (AS-miR-34a) at a final concentration of 10 nmol/L using DharmaFect Transfection Reagent (Dharmacon, CO). For the combination treatment of miRNA-34 inhibitor and delta-tocotrienol, A549 and H1650 cells were transfected with AS-miR-34a for 6 h, and then delta-tocotrienol stock solution was added to each well for a final concentration of 20 μM. After 72 h of incubation, the cells were subjected to different experiments as outlined below.

Cell viability studies by MTS assay:

The A549 and H1650 cells (5×10^3) were seeded in a 96-well culture plate. After overnight incubation, the medium was removed and replaced with transient transfection medium containing either negative control, miR-34a, delta-tocotrienol or the combination of delta-tocotrienol and Anti-sense (AS)-miR-34a. 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 quantify apoptosis in NSCLC cells. Briefly, 2 x10^5 cells were seeded in six-well plates. After 24 h incubation, cells were treated with transient transfection medium containing either negative control, miR-34a, delta-tocotrienol or the combination of delta-tocotrienol and AS-miR-34a 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 at 405 nm was determined by using ELx800 plate reader (Bio-Tek, Winooski, VT).

Clonogenic assay:

Cells (2x10^5) were seeded in six-well plates for 24h. Subsequently, the cells were cultured with transfection medium containing either negative control or miR-34a for 72 h. This was followed by counting of the viable cells which were then plated in 100 mm dishes at 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.

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 H1650 cells were incubated in the presence of either negative control, miR-34a, delta-tocotrienol or the combination of delta-tocotrienol and AS-miR-34a for 72 h. Cells were trypsinized, washed twice with ice-cold PBS and re-suspended in 1 X 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 X binding buffer was added to each tube and the number of apoptotic cells was analyzed by flow cytometry (BD, San Jose, CA).

Flow cytometry and cell cycle analysis:

Four million cells were seeded in 100 mm dish incubated overnight. Subsequently, all the cells were starved for another 24 h. After that, the cells were released to transient transfection medium containing either negative control, miR-34a, delta-tocotrienol or the combination of delta-tocotrienol and AS-miR-34a for 72 h, followed by collection and fixing with ice-cold 70% (v/v) ethanol for 24 h. After centrifugation at 3000× g for 5 min, the cell pellet 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 the DNA content was determined by flow cytometry using a FAC Scan flow cytometer (BD, San Jose, CA).

Cell invasive assay:

BD Biocoat invasion kit (BD, San Jose, CA) was used to evaluate the tumor invasive ability. Two million cells were seeded in six-well plates. Cells were then cultured with transient transfection medium containing negative control or miR-34a for 72 h. Subsequently, 0.5 x 10^5 cells of A549 and H1650 with basal media were transferred into the upper chamber of each 6-well plate. In the meantime, three milliliter of culture medium with 10% FBS was added into each lower chamber of the 6-well plate. After 20 h incubation, the cells in the upper chamber were removed using a cotton swab. Each experimental condition was performed in triplicate. The cells were fixed in 4% Paraformaldehyde and stained with 2% crystal violet for 10 mins. The

stain in the cells was then dissolved in 20% acetic acid and the absorbance measured using ELx800 plate reader (Bio-Tek, Winooski, VT) at 570 nm.

Protein extraction and western blotting:

A549 and H1650 cells were treated with negative control, miR-34a, delta-tocotrienol, AS-miR-34a or the combination of delta-tocotrienol and AS-miR-34a for 72 h to evaluate the effects of treatment on Notch-1, Hes-1, Bcl-2, cyclin D1, and β-actin expressions. Cells were lysed in the cold lysis buffer for 30 minutes on ice. Protein concentrations were determined using the Bradford protein assay kit (Bio-Rad Laboratories, CA). Each sample contained 50μg of total cell lysates. The samples were subjected to 10% SDS-polyacrylamide gel electrophoresis. After electrophoresis, the proteins were transferred to a nitrocellulose membrane (Whatman, Clifton, NJ) using transfer buffer (25mM Tris, 190mM glycine, 20% methanol) in a Hoefer TE70XP transfer apparatus (Holliston, MA). The membranes were incubated for 1 hour at room temperature with 5% nonfat dried milk in 1 x TBS buffer containing 0.1% Tween (TBS-T) Subsequently, the membranes were incubated over night at 4°C with primary antibodies (1:1000). The membranes were washed 3 times with TBS-T, and subsequently incubated with the secondary antibodies (1:5000) containing 2% BSA for 2 hours at room temperature. The signal intensity was measured by chemiluminescent imaging using a chemiDoc XRS imager (Bio-Rad Laboratories, CA).

Immunostaining assay and confocal microscopy

Single cell suspensions of A549 and H1650 cells were prepared and plated in Millicell® EZ slide (Milipore, MA). After transfection with negative control or miR-34a as described above, the cells were washed with 1× PBS, and fixed with 4% paraformaldehyde for

immunofluorescence staining. After washing 3 times with 1x PBS, cells were blocked in PBS containing 1% BSA for 2 h at room temperature and incubated with a mouse anti-p53 in blocking buffer for 2 h at room temperature. Cells were then incubated with Alexa Fluor 488 - conjugated anti-mouse IgG (1:50 dilution) for 1 h at room temperature and mounted with 30 µl of the ProLong Gold antifade reagents (Invitrogen, CA). The p53 labeled cells were photographed under Nikon Eclipse 80i confocal microscope (Nikon, CA) using software Nikon Elements built in the microscope.

Data Analysis:

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

Results

Treatment of NSCLC cells with delta-tocotrienol showed increased expression of miR-34a

In order to investigate the differences in miRNA expression in NSCLC cell line (H1650) upon treatment by delta-tocotrienol, we conducted a miRNA array analysis using the RT^2 miRNA PCR array system (SABiosciences, MD). We found that miR-34a expression was 4-fold higher in the H1650 cells treated with delta-tocotrienol compared with untreated H1650 cell (Fig. 4.1 A). The results from the miRNA array were validated by miRNA RT-PCR analysis upon treatment of delta-tocotrienol (Fig. 4.1 B and 4.1 C). As indicated in Fig 1B, there was a significant ($p<0.05$) increase in miR-34a expression in the delta-tocotrienol treated A549 cells compared to controls in dose dependent and time dependent manner. Similarly, a significant increase in miR-34a expression was also observed in H1650 cells in a dose and time dependent manner (Fig 4.1 C). Since delta-tocotrienol has been shown to have anti-cancer effects in different cancer cell lines(89, 90), these results suggest that miR-34a could be an inhibitory molecule for cancer development and progression and that delta-tocotrienol could inhibit the progression of NSCLC through induction of miR-34a in NSCLC cells. Based on the results of these PCR data, a 20 µM concentration of delta-tocotrienol was selected for evaluations of its effects in further experiments.

The efficiency of re-expression of miR-34a in NSCLC cells

In order to elucidate the role of miR-34a in the proliferation of NSCLC, we tested the transfection efficiency of miR-34a in NSCLC cells. As shown in the Fig 4.2 A and 4.2 B, the relative expression of miR-34a was induced after 4 h transfection in both A549 and H1650 cell

lines. The relative expressions of miR-34 after 72 h transfection, were about 118 and 120 fold-higher than the controls in the A549 and H1650 cell lines, respectively.

Re-expression of miR-34a inhibited proliferation of NSCLC cells

To investigate the role of miR-34a in the regulation of cell proliferation, we transfected A549 and H1650 cells with negative control, miR-34a, delta-tocotrienol or the combination of delta-tocotrienol and AS-miR-34a for 72 h followed by MTS assay. We found that re-expression of miR-34a significantly inhibits cell proliferation in A549 and H1650 cells. The re-expression of miR-34a for 72 h resulted in approximately 60% of cell growth inhibition relative to negative control in both A549 and H1650 cell lines. As shown in Figs 4.2 C and 4.2 D, there was no significant difference in cell viability of cells transfected with the negative control and the control cells. The negative control, was therefore used as the control for further experiments. Conversely, transfection of both A549 and H1650 cells with AS-miR-34a, knockdown of miR-34a, resulted in a loss of sensitivity to delta-tocotrienol treatment. In A549 cells, cell proliferation was inhibited by 74% with delta-tocotrienol alone while the combination treatment of AS-miR-34a and delta-tocotrienol reduced it by only 57%. Similarly, for the H1650 cells, inhibition of proliferation decreased from 80% induced by delta-tocotrienol alone to 69% induced by combination treatment of AS-miR-34a and delta-tocotrienol. Taken together, these results indicate that re-expression of miR-34a in NSCLC cells can inhibit cell proliferation as compared with the controls.

To confirm the effects of miR-34 re-expression on cells growth, clonogenic assays on A549 and H1650 were performed. Fig.4.2 E and 4.2 F show significant inhibition of colony formation by miR-34a reexpression compared to the negative control. Overall, the results from the clonogenic

assay were consistent with the MTS data shown in Fig. 4.2 C and 4.2 D, confirming that miR-34a significantly inhibits the proliferation of NSCLC cells

Induction of apoptosis by re-expression of miR-34a

Since inhibition of cell growth could also result from apoptosis induced by re-expression of miR-34a, we further investigated whether re-expression of miR-34a could induce apoptosis by two different approaches. As shown in Fig. 4.3 A, our histone/DNA ELISA data demonstrates that apoptosis induced by re-expression of miR-34 in A549 cells is about 1.8 folds greater than that induced in the control. Likewise, in the H1650 cell line (Fig. 4.3B), the re-expression of miR-34a induced approximately three times the amount of apoptosis as compared with the control.

The ELISA data, was further confirmed by Annexin V/PI staining analysis in H1650 cells (Fig. 4.3 C). Consistent with our ELISA data, the re-expression of miR-34a initiated about 18% apoptosis as compared to 10% in the control cells. Conversely, a decrease in apoptosis from 61% in control cells treated with delta tocotrienol to 55% in the miR-34a knockdown cells, under treatment with delta-tocotrienol was observed. Collectively, our results suggest that miR-34 reexpression caused a statistically significant ($p < 0.05$) increase in the percentage of apoptotic cells in NSCLC cell lines.

Analysis of cell cycle distribution after re-expression of miR-34a

In order to further investigate cell growth inhibition by reexpression of miR-34a, cell cycle distributions were examined using propidium iodide staining followed by flow cytometry. As demonstrated in Fig. 4.4 A and 4.4 B, both A549 and H1650 cell lines, showed increased G_0-G_1

arrest patterns after reexpression of miR-34a. For A549 cells (Fig. 4.4 A), there were about 63 % cells in the G_0-G_1 phase in the miR-34 overexpression group compared to 55 % in control cells. A similar response was observed in the H1650 cells (Fig. 4.4 B) with about 75 % of cells in the G_0-G_1 phase in the miR-34a overexpression group as compared to 57 % in control cells. In an effort to confirm our results, we treated both, the A549 and H1650 cells with delta-tocotrienol alone or with the combination of delta-tocotrienol and AS-miR-34a. As shown in Fig 4A, we found that knockdown of miR-34a deceased the proportion of cells in the G_0-G_1 phase from 77% to 65% upon treatment with delta-tocotrienol in A549 cells. Similarly, knockdown of miR-34a caused the decease in the fraction of cells in G_0-G_1 arrest from 62% to 50% upon treatment with delta-tocotrienol in the H1650 cell line (Fig 4.4 B).

MiR-34a transfection or delta-tocotrienol treatment suppressed Notch-1 and its downstream gene expression

Since previous data from our group demonstrated that delta-tocotrienol inhibited cell growth by down-regulation of the Notch-1 pathway(89), we used DIANA-microT to predict whether Notch-1 pathway is the target genes of miR-34a. To investigate the role of miR-34a in the regulation of cellular signaling, A549 and H1650 cells were transfected with miR-34a for 72 h. As shown in Fig 4.5 A and 4.5 B, we found that reexpression of miR-34a in A549 and H1650 cells resulted in the downregulation of Notch-1 and its downstream molecules such as Hes-1, Bcl-2 and Survivin. We also found that the expression of cyclin D1, a key regulator of G_1-S cell cycle transition, was reduced.

In order to gain further molecular insight, we assessed whether inactivation of miR-34a by its specific inhibitor could lessen the effects of delta-tocotrienol. We found that down-regulation of

miR-34a opposes the effects induced by delta-tocotrienol. As shown in Fig. 4.5 A and 4.5 B, addition of delta tocotrienol to miR-34a knockdown cells only partially restored delta-tocotrienols ability to decrease Notch-1 and its downstream signaling molecules, such as Hes-1, Surivivin and Bcl-2. Taken together, our findings suggest that delta-tocotrienol inhibited cell proliferation and induced apoptosis by downregulating the Notch-1 pathway through miR-34a overexpression in NSCLC cells.

Inhibition of cell invasion by overexpression of miR-34a

Although effect of miR-34 reexpression on anti-proliferation and induction of apoptosis have been shown(91), its effects on tumor cell invasion have not been evaluated so far. Using invasion assay, we found that the invasive capacity of A549 ($p<0.01$) and H1650 ($P<0.05$) cells was significantly decreased by the reexpression of miR-34a compared with the controls. As showed in Fig. 4.5 C, reexpression of miR-34a in A549 inhibits invasive capability by 28%. Similarly, reexpression of miR-34a in H1650 cells (Fig. 4.5 D) decreases its invasive ability by 20%.

Re-expression of miR-34a promotes p53 activity

P53, a tumor suppressor gene, has been shown to play important roles in tumor progression(92) and drug responses(93). To determine the effects of miR-34 reexpression on the transcriptional activities of the p53, A549 and H1650 cells were transiently transfected with miR-34a or negative control (Fig 4.6 A and 4.6 B). We determined the subcellular co-localizations of p53 by immunofluorescence and confocal microscopy. Consistent with our apoptosis analysis, DAPI staining demonstrated that reexpression of miR-34 induced greater apoptosis in both NSCLC cell lines. In addition, confocal microscopy data showed that p53 activity was increased and co-localized in the nucleus by reexpression of miR-34a as compared with the control in both cell

lines. Taken together, these results suggest that miR-34 induced apoptosis via the activation of the p53 pathway.

Discussion

Cancer is a genetic disease resulting from the failure in the regulation of cell growth. For diseases to occur, the genes which regulate cell growth and differentiation must be altered so as to transform a normal cell to a cancer cell (94). Accumulating evidence reveals that pathogenesis of cancer is a multistep process of sequential alterations in several, often many, oncogenes, tumor-suppressor genes, or miRNA in human cancers including lung cancer(95, 96). MiR-34a has been shown to be associated with cancer cell proliferation and drug resistance through E2F in colon cancer cells (97). In addition, miR-34a was reported to be down-regulated in different cancer cell lines including neuro, melanoma, kidney, breast, and pancreatic cancer cells(98-100). Recently, a study showed that NSCLC patients with upregulated miR-34a had better prognosis for survival(101). In the current study, we found that miR-34 can be induced by the treatment of delta-tocotrienol in NSCLC cells. We also found that reexpression of miR-34a in A549 and H1650 NSCLC cells inhibits proliferation, induces apoptosis, and initiates G_0/G_1 cell cycle arrest. Following from our previous data that demonstrated the anti-cancer potential of delta-tocotrienol in NSCLC cell lines(89), the current results confirm the activity of miR-34a as a tumor suppressor and its potential role as one of the key players in the inhibition of NSCLC cells by delta-tocotrienol. Moreover, the anti-cancer effects were associated with depressed Notch-1 signaling. We therefore propose that delta-tocotrienol suppresses the Notch-1 pathway by up-regulating miR-34a in NSCLC cells.

The molecular mechanisms involved in the miR-34 mediated inhibition of cell proliferation and invasion are still unclear. From our results, we believe that miR-34a inhibits cell proliferation and invasion partly through the regulation of Notch-1 signaling pathway. From the DIANA-

microT database, we found that Notch-1 is the predicted target of miR-34a. Although miR-34 has been reported to suppress the glioma cell proliferation(91), regulation of cancer proliferation and invasion in NSCLC by Notch-1 is unknown. In our study, we found that the reexpression of miR-34a by transfection suppressed the expression of Notch-1 and its target genes including Hes-1, Survivin and Bcl-2 in NSCLC cells.

As a key G_1-S cell cycle transition regulator, overexpression of cyclin D_1 has been shown to promote cell growth and is associated with chemotherapeutic drug resistance(102, 103). In the current study, we found that treatment with delta-tocotrienol can reduce the expression of cyclin D_1 (Fig 5A and B) in NSCLC cells. Moreover, transfection with miR-34a also inhibited the cyclin D_1 expression in both NSCLC cell lines (Fig 5A and B). Since cyclin D_1 is required for G_1-S transition, our cell cycle data consistently showed that reexpression of miR-34a in NSCLC cells induces G_1-S arrest. Specifically, in the lower cyclin D1 expression, the higher percentage of cells was arrested in G_0-G_1 phase compared to control.

Loss of function of p53, a well-recognized tumor suppressor gene, is associated with the pathogenesis of different types of human malignancies (92, 93). Previous studies have shown that p53 controls cell-cycle progression, apoptosis, DNA repair, and angiogenesis through up-regulating miR-34a (98). Other studies have demonstrated that suppression of p53 pathway is associated with the activated Notch-1(101, 104). In combination, these studies suggest that there might be cross-talk between the p53 and Notch-1 pathways which need further investigations. In our study, we found that up-regulation of miR-34a by transfection could induce the activation of p53, which translocates into the nucleus and promotes apoptosis in NSCLC (Fig. 6). In addition, this activation of p53 is associated with down-regulation of Notch-1 expression. We found that

delta-tocotrienol could down-regulate Notch-1 expression and up-regulate the miR-34a expression. Thus we propose that delta-tocotrienol suppresses the Notch-1 pathway by up-regulating miR-34a in NSCLC cells.

In conclusion, our results demonstrate that delta-tocotrienol can upregulate miR-34a expression, inhibit cancer cell proliferation, induce apoptosis, and reduce cancer cell invasion, at least in part due to downregulation of Notch-1, the molecular target that is predominately activated in NSCLC. Very recently, miR-34a was found to be downregulated in glioblastoma multiforme cells and was shown to inhibit cell growth by targeting the Notch-1 pathway. Moreover knockdown of notch-1 showed similar cellular functions as overexpression of miR-34a both *in vitro* and *in vivo*(104). These data corroborate some of work presented here. However further in vivo studies in appropriate animal models for NSCLC are needed to establish whether delta-tocotrienol could be useful in combination with conventional chemotherapeutics or conventional targeted agents such as cisplatin and erolitinib for the treatment of NSCLC.

Figure Legends:

Figure 4.1: Delta-tocotrienol induces the overexpression of miR-34a on NSCLC cells

A, MicroRNA microarray data (Fig 1A) of H1650 cell treated with or without of delta-tocotrienol. The cut-off lines represent 4-fold change between the control and delta-tocotrienol treated H1650 cell. The plot was automatically generated by uploading the CT value to the Qiagen website. Differences in the relative expression of microRNA were analyzed between control and delta-tocotrienol treated H1650 cell.

B-C, The microarray data was validated in NSCLC cells, A549 (Fig. 1 B) and H1650 (Fig. 1C) using RT-PCR. The left panel in figures 1B and 1C show a time dependent comparative expression of miR-34a with 15µM delta-tocotrienol treatment. The right panels in both figures represent a dose dependent response of comparative expression of miR-34a at 72 hour time point. *p < 0.05, **p < 0.01.

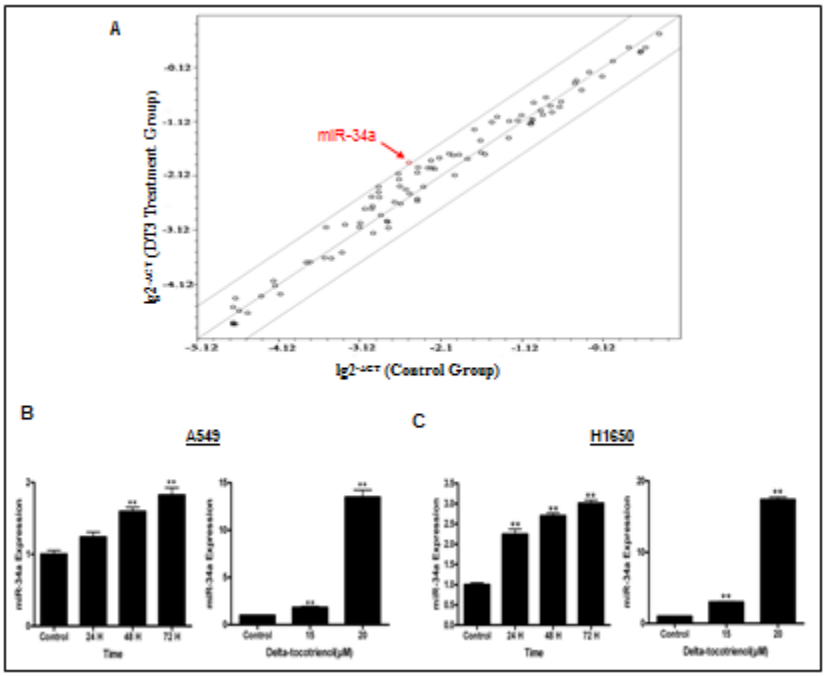


Figure 4.2: Anti-proliferative effects by miR-34a re-expression in the NSCLC cells

A-B, Time response of Transfection efficiencies of miRNA-34a at a final concentration of 10 nmol/L, normalized to Snord-44 in A549 (Fig.2A) and H1650 (Fig.2B) cell lines.

C-D, Cell viability of human NSCLC cell lines A549 (Fig.2C) and H1650 (Fig.2D) cells using the MTS colorimetric assay. NC: negative control; 34a: pre-miR-34a; AS-34a: Antisense miR-34a; DT3: delta-tocotrienol. Vertical bars indicate the mean cell count ± SEM (n = 3). *$p < 0.05$ is considered significant as compared with negative controls.

E-F, Photomicrographic differences in colony formation by clonogenic assay depicting cell survival of human NSCLC cell lines A549 and H1650 cells. NC: negative control; 34a: pre-miR-34a; AS-34a: Antisense miR-34a; DT3: delta-tocotrienol.

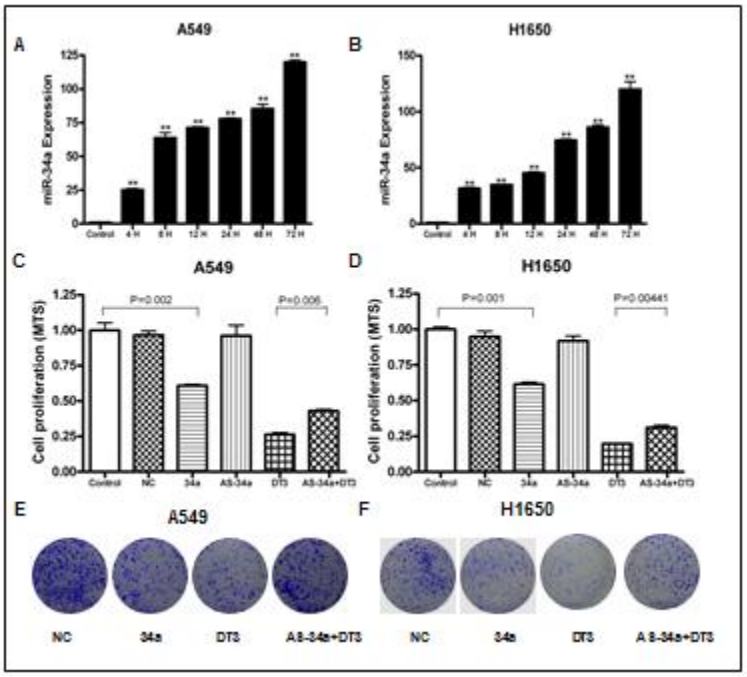


Figure 4.3: Induction of apoptotic effects by reexpression of miR-34a in the NSCLC cells

A and **B**, A549 and H1650 cells were transfected with containing negative control, miR-34a, delta-tocotrienol or the combination of delta-tocotrienol and AS-miR-34a for 72 h. The apoptosis of both cell lines were determined by histone/DNA ELISA.

C, H1650 cell was transfected with negative control, miR-34a, delta-tocotrienol or the combination of delta-tocotrienol and AS-miR-34a for 72 h. Apoptosis was determined by Annexin V-FITC analysis. The percentage of dead cells (upper left quadrant), live cells (lower left quadrant), cells in late apoptosis (PI+/Annexin V+; upper right quadrant) and cells in early apoptosis (PI-/Annexin V+; lower right quadrant) are indicated.

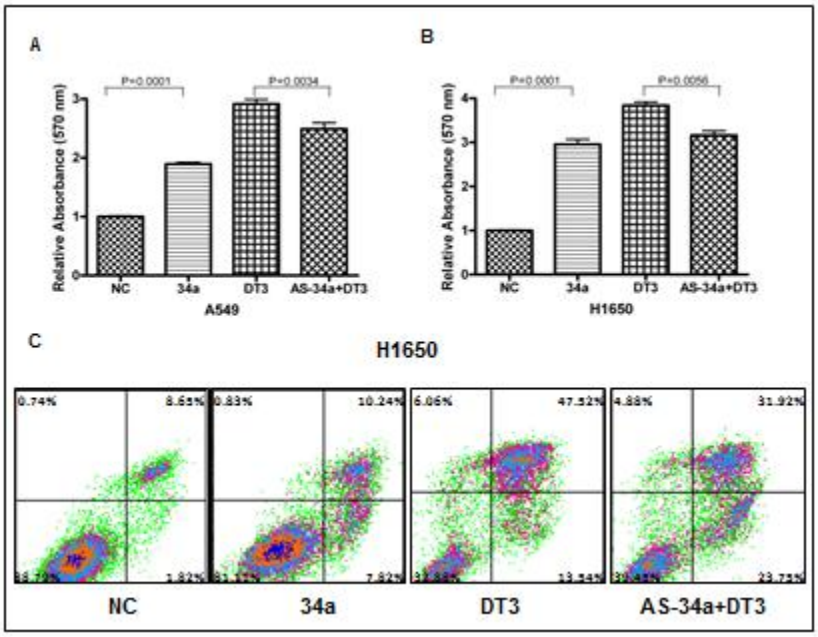


Figure 4.4: Re-expression of miR-34a induces cell cycle arrest at G_0-G_1 phase

A-B, Cell cycle distributions analyzed by using flow cytometry in A549 (A) and H1650 (B) cells after 72 hours incubation. NC: negative control; 34a: pre-miR-34a; AS-34a: Antisense miR-34a; DT3: delta-tocotrienol.

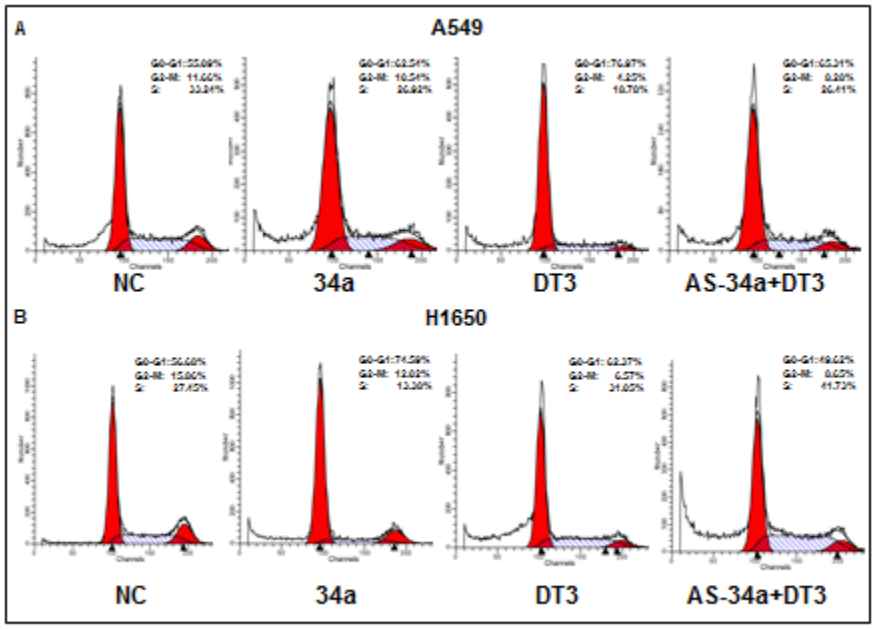


Figure 4.5: Down-regulation of Notch-1 and its target genes by Re-expression of miR-34a and cell invasion

A-B, The expressions of Notch-1, Hes-1, Cyclin D1, Survivin, Bcl-2 and β-actin protein were analyzed by western blotting analysis followed by chemiluminescence detection in A549 (A) and H1650 (B) cells after 72 h treatment. NC: negative control; 34a: pre-miR-34a; AS-34a: Antisense miR-34a; DT3: delta-tocotrienol.

C-D, Inhibition of NSCLC cells invasion ability by miR-34 re-expression using Matrigel-coated inserts in A549 (Fig. 5C) and H1650 (Fig. 5D) cells. NC: negative control; 34a: pre-miR-34a; 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 is presented (lower panels) as means ± S.E.M of three independent experiments.*P<0.05, **P< 0.01.

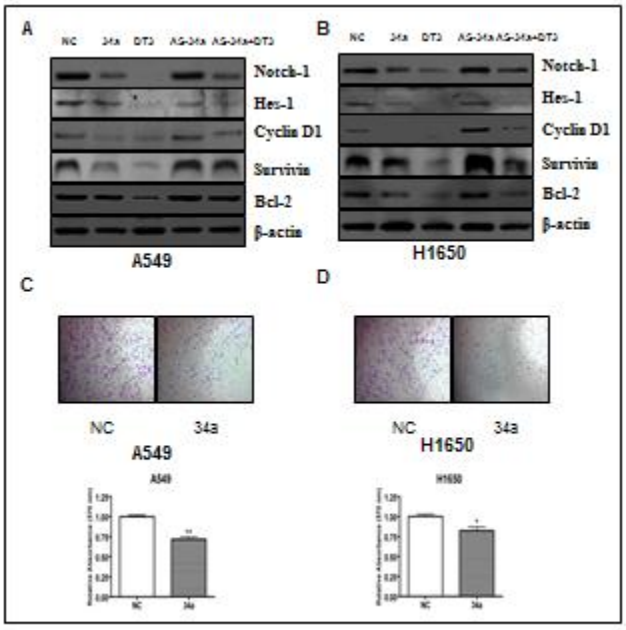
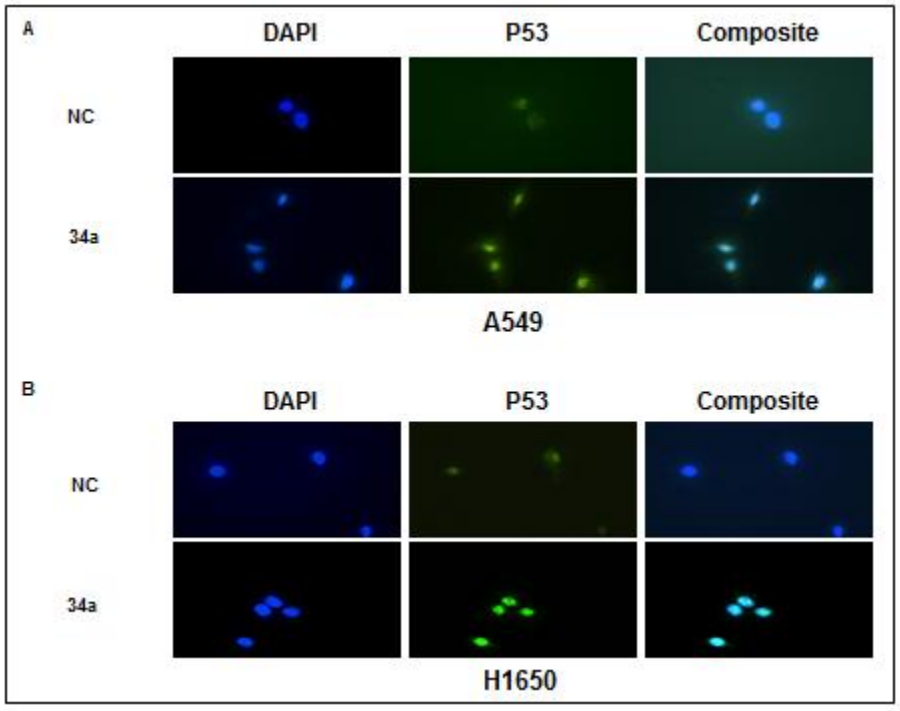


Figure 4.6: Immunoreactivity with p53 by fluorescent immunocytochemistry

MiR-34a reexpression induces the p53 expression and apoptosis in NSCLC cells. Fluorescence microscopy analysis showing p53 expressions in A549 (A) and H1650 (B) cells upon transfection with miR-34a or negative control. The cells were mounted with antifade mounting medium and analyzed by confocal microscopy (40X resolution).



CHAPTER 5

Delta-tocotrienol augments cisplatin induced suppression of non-small cell lung cancer cells via inhibition of the Notch-1 pathway.

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***Key Words*:** Delta-tocotrienol, Cisplatin, Notch-1; Non-small Cell Lung carcinoma; Apoptosis;

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Abstract

Non-small cell lung cancer (NSCLC), accounts for 80% of lung cancer, the leading cause of all cancer deaths. Previously, we demonstrated that delta-tocotrienol inhibits NSCLC cell proliferation, invasion and induces apoptosis by down-regulation of the Notch-1 signaling pathway. The objective of this study was to investigate whether delta-tocotrienol, could enhance the anticancer effects of the drug, cisplatin, Treatment with a combination of delta-tocotrienol and cisplatin resulted in a dose dependent, significant inhibition of cell growth, migration, invasiveness, and induction of apoptosis in NSCLC cells, as compared to the single agents. This was associated with a decrease in NF-κB-DNA binding activity, decrease in Notch-1, Hes-1, Bcl-2 and increase in cleaved Caspase-3 and PARP expressions. These results suggest that down-regulation of Notch-1, via inhibition of NF-κB signaling pathways by delta-tocotrienol and cisplatin, in combination, could provide a potential novel approach for tumor arrest in NSCLC, while lowering the effective dose of cisplatin.

Introduction

Lung cancer is the leading cause of death among all malignant diseases. It is estimated that 226,160 people (116,470 men and 109,690 women) will be diagnosed with this disease and of these 160,340 will die of cancer of the lung and bronchus in 2012 (105). Non-small cell lung cancer (NSCLC) accounts for 80% of all lung cancer reported and has a poor five-year survival rate of only 16% (10). This is partly attributed to the fact that NSCLC progresses undetected (asymptomatically) till it has metastasized. Some of the common symptoms for lung cancer include persistent cough, chest pain, coughing up blood, hoarseness, weight loss, fatigue and recurrent respiratory infections, all of which can be related to other respiratory disease in addition to lung cancer and hence are not specific (14). The invasive NSCLC rapidly establishes distant metastases in organs including the bones, contralateral lung, liver and brain ahead of diagnosis which are lethal for the patients rather than the primary tumors in lung themselves (15).

Another factor contributing to the poor prognosis of NSCLC patients is the lack of effective therapy to battle the aggressive disease. According to the American Society of Clinical Oncology Clinical Practice, platinum (cisplatin and carboplatin) and non-platinum combination therapies are the standard first line of agents used to treat NSCLC patients (12) with Cisplatin being the most frequently used chemotherapeutic agent for the treatment of NSCLC. However, the utility of Cisplatin for the clinical management of NSCLC patients is limited by its dose related drug resistance. This disappointing outcome strongly suggests that innovative research is required to manage this fatal disease. Among the different mechanisms proposed to be involved in cisplatin resistance, are changes in cellular uptake and efflux of the drug, increased

detoxification of the drug, inhibition of apoptosis and increased DNA repair (106). Also, it has been reported that the Notch pathway may play a role in cisplatin induced drug resistance. For example, it has been shown that the Notch-1 expression is negatively correlated to cisplatin-sensitivity of head and neck squamous cell carcinoma, and could be used to predict cisplatin-sensitivity (107). Moreover, Notch-1 was highly expressed in cisplatin-resistant head and neck squamous cell carcinoma patients suggesting that the overexpression of Notch-1 crosstalk induces the reprogrammed survival pathways in head and neck squamous cell carcinoma responding to chemotherapy (108). Similarly, up-regulation of Notch-1 is associated with the cisplatin resistance in ovarian cancer cell lines (109). In addition, concurrent inhibition of Notch-1 pathway and use of cisplatin elicits a striking induction of colorectal cancer cell death (110). These results support the notion that inactivation of Notch pathway could sensitize the patients who are likely to respond to cisplatin.

Notch signaling plays an important role in cell proliferation and apoptosis (31). Since Notch signaling regulates critical cell fate decision, alterations in Notch signaling are associated with tumorigenesis. It has been found that Notch signaling is frequently dysregulated with up-regulated expression in different types of cancers such as lung, colon, head and neck, pancreatic (39-42). Overexpression of Notch-1 has been shown to inhibit apoptosis in different types of cancers (43, 44). Clinical data has demonstrated that 30 % of NSCLC has increased Notch activity and 10% of NSCLC has gain-of-function mutation on Notch-1 gene (39). These data suggest Notch could be considered as a therapeutic target.

Previously, we demonstrated that treatment of NSCLC cells with delta-tocotrienol resulted in a dose and time dependent inhibition of cell growth, cell migration, tumor cell

invasiveness, and induction of apoptosis (89). Real-time RT-PCR and Western blot analysis showed that antitumor activity by delta-tocotrienol was associated with a decrease in expression of Notch-1, Hes-1, Survivin, MMP-9, VEGF, and Bcl-2. In addition, there was a decrease in nuclear factor-kappaB (NF-κB) DNA binding activity (89). It is of interest to see whether a combination of delta tocotrienol and cisplatin may be an effective therapy against NSCLC, while sensitizing the cells against acquired drug resistance. The objective of this study was to investigate the growth inhibitory effects of a low dose treatment of cisplatin in combination with delta-tocotrienol and further to demonstrate the effect of the combination on intracellular signaling mechanisms.

Materials and Methods

Cell culture, reagents and antibodies:

Human NSCLC cell lines, including A549, H1650 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 cleaved Caspase 3, 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, Bcl-2 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 H1650 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), delta-tocotrienol alone, cisplatin alone, or the combination of delta-tocotrienol and cisplatin. After 72 h of incubation, 20µl 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 490nm was recorded on ELx800 plate reader (Bio-Tek, Winooski, VT). Each variant of the experiment was performed in triplicate.

Clongenic assay:

One million cells were seeded in 100 mm dish per plate, incubated overnight. Subsequently, the cells were cultured in the presence of control medium, delta-tocotrienol (15µM) alone, cisplatin (4 µM) alone, or the combination of delta-tocotrienol (15µM) and cisplatin (4 µM), 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.

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 in the presence of control medium, delta-tocotrienol (15µM) alone, cisplatin (4µM) alone, or the combination of delta-tocotrienol (15 µM) and cisplatin (4 µM) 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.

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 H1650 cells were incubated in the presence of control medium, delta-tocotrienol (15 µM) alone, cisplatin (4 µM) alone, or the combination of delta-tocotrienol (15 µM) and cisplatin (4 µM) for 72 h. Cells were trypsinized, washed twice with ice-cold PBS and re-suspended in 1 X 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 X 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 H1650 were seeded in a six well plate at the concentration of $4x10^5$ 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 attached. Subsequently, the cells were cultured in the presence of control medium, delta-tocotrienol (15 µM) alone, cisplatin (4 µM) alone, or the combination of delta-tocotrienol (15 µM) and cisplatin (4 µM). The wound images were taken as 0 h. After 20h, wound healing pictures were taken under microscope.

Cell invasive assay:

BD Biocoat invasion kit (BD, San Jose, CA) was used to evaluate the tumor invasive ability. Briefly, around 2.5 x 10^5 cells of A549 and H1650 with basal media were transferred in each 6-well upper chamber in the presence of control medium, delta-tocotrienol (15 µM) alone, cisplatin (4 µM) alone, or the combination of delta-tocotrienol and cisplatin. In the meantime, 3 ml of culture medium with 10% FBS was added into each lower chamber of 6-well plate. After 20 h incubation, the cells on the upper chamber were removed using cotton stick. Each of experimental conditions was performed in duplicates. The cells were fixed in 4% paraformaldehyde and stained with 2% crystal violet. The spectrophotometric absorbance of the samples was determined by using ELx800 plate reader (Bio-Tek, Winooski, VT) at 570 nm.

Protein extraction and western blotting:

A549 and H1650 cell lines were treated in the presence of control medium, delta-tocotrienol (15 µM) alone, cisplatin (4 µM) alone, or the combination of delta-tocotrienol (15

μM) and cisplatin (4 μM) for 72 hours to evaluate the effects of treatment on Notch-1, Hes-1, PARP, Survivin, Bcl-2, and □-actin expressions. Cells were lysed in the cold lysis buffer for 30 minutes on ice. Protein concentrations were determined using the Bradford protein assay kit (Bio-Rad Laboratories, CA). Each sample contained 50μg of total cell lysates. The samples were loaded on 10% SDS-polyacrylamide gel electrophoresis. After that, the gel was transferred to a nitrocellulose membrane (Whatman, Clifton, NJ) using transfer buffer (25mM Tris, 190mM glycine, 20% methanol) in Hoefer TE70XP transfer apparatus (Holliston, MA). The membranes were incubated for 1 hour at room temperature with 5% nonfat dried milk in 1 x TBS buffer containing 0.1% Tween. After that, membranes were incubated over night at 4°C with primary antibodies (1:1000). The membranes were washed 3 times with TBS-T, and subsequently incubated with the secondary antibodies (1:5000) containing 2% BSA for 2 hours 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, USA) according to the manufacturer's protocols. Two microgram of total RNA from each sample was subjected to first strand cDNA synthesis using TaqMan reverse transcription reagents kit (Applied Biosystems, Foster City, CA) in a total volume of 20 μl. Reverse transcription reaction were performed at 25ºC for 10 min, followed by 48ºC for 30 min and 95ºC for 5 min. Real-time PCR analysis were performed using Eppendorf Realplex 4 system (Hauppauge, NY). The sequences of the primers sets used for this analysis are as follows: 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'); All these primers are checked by running them on virtual PCR, and primer concentration was 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 microliter quantitative PCR reactions. The thermal profile for SYBR real-time PCR was 95ºC 10 min, followed by 50 cycles of 95ºC 15 s and 60ºC 1 min. Data were analyzed according to the comparative fold increases or decrease in gene expression determined by quantitation of normalized by GAPDH expression in each sample.

Microwell colorimetric NF-κB assay for measuring NF-κB activity:

TransAM™ Transcription Factor ELISAs kit for P65 (Avtive Motif, Carlsbad, CA) was used to evaluate the binding activity of NF-κB according to the protocol. Briefly, one million of A549 and H1650 Cells were seeded in 100 mm dish. After 24 h incubation, cells were treated in the presence of control medium, delta-tocotrienol (15 μM) alone, cisplatin (4 μM) alone, or the combination of delta-tocotrienol (15 μM) and cisplatin (4 μM) for 72 h. After that, nuclear protein was extracted from each sample using nuclear protein extraction kit according to the protocol (Pierce, Rockford, IL). Two microgram of each sample was incubated in the microplate coated with anti-p65 DNA sequence. In order to dectect the p65-DNA binding complex, peroxidase-conjugated anti-DNA antibody was used before color development with ABTS

substrate for peroxidase. The chemillumiance of the samples was determined by using chemiDoc XRS (Bio-Rad Laboratories, CA). The volume of each sample was determined by Quantity One software (Bio-Rad Laboratories, CA).

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. Calcusyn (Biosoft, United Kingdom) was used to analyze the combination effect of delta-tocotrienol and cisplatin.

Results:

Delta-tocotrienol enhances the effects of cisplatin to inhibit the growth of NSCLC Cells.

In order to test the effects of delta-tocotrienol, cisplatin and their combination on cell growth, A549 and H1650 cells were treated with control medium, delta-tocotrienol alone, cisplatin alone, or the combination of delta-tocotrienol and cisplatin for 72 h separately followed by MTS assay. As shown in figure 5.1 A (A549) and B (H1650), a significant potentiation in the inhibition of cell growth was observed by combination of delta-tocotrienol and cisplatin compared to single agents in both A549 and H1650 cells respectively. In A549 cell line, combination treatment with 7.5 µM of delta-tocotrienol and 2 µM of ciplatin, 15 µM of delta-tocotrienol and 4 µM of ciplatin, and 30 µM of delta-tocotrienol and 8 µM of ciplatin for 72 h resulted in 25%, 55%, and 92% of cell growth inhibition relative to control, respectively. Similarly, treatment of H1650 cell line with these combinations for 72 h resulted in 32%, 56%, and 91% of cell growth inhibition, respectively, relative to control. Based on the MTS results, we select 15 µM of delta-tocotrienol and 4 µM of ciplatin to perform further experiments.

Delta-tocotrienol and/or cisplatin inhibit clonogy formation in NSCLC cells.

In order to confirm the effects of delta-tocotrienol and cisplatin on cells growth, A549 and H1650 cells were treated with each of the single agents or their combination and assessed for cell viability by clonogenic assay. As shown in the Fig. 5.1 C (A549) and D (H1650), the combination treatment of delta-tocotrienol and cisplatin resulted in a significant inhibition of colony formation compared to either agent alone or the control in both NSCLC cell lines.

Overall, the results from the clonogenic assay were consistent with the MTS shown in Fig. 1A and 1B. The molecular mechanisms understanding NSCLC cell growth inhibition were further investigated, and the results are presented in the following sections.

Delta-tocotrienol and/or cisplatin induced Apoptosis in NSCLC cells

Since inhibition of cell growth could also result from apoptosis induced by delta-tocotrienol and cisplatin, we further investigated whether delta-tocotrienol, cisplatin and in combination could induce apoptosis in both cell lines by two different approaches, histone/DNA ELISA and the Annexin V/PI staining. The effects of delta-tocotrienol (15 μM), cisplatin (4 μM) individually and in combination were tested using ELISA in both cell lines. As shown in Fig. 5.2 A (A549) and Fig. 2B (H1650), exposure of A549 and H1650 to delta-tocotrienol (15 μM) and cisplatin (4 μM) for 72 h significantly enhanced apoptosis. In addition, the combination of delta-tocotrienol (15 μM) and cisplatin (4 μM) further increased apoptosis in both cell lines.

The Annexin V/PI staining data confirmed apoptosis-inducing effect of delta-tocotrienol and/ cisplatin treatment in both cell lines tested (Fig. 5.2 C and D), respectively. In A549 cell line (Fig. 5.2 C), the combination treatment of delta-tocotrienol and cisplatin induced 48.06% apoptosis as compared with 14.35% with delta-tocotrienol (15 μM) and 16.20% in cisplatin (4 μM) treatments alone. Similarly, in H1650 cell line (Fig. 5.2 D), the combination treatment of delta-tocotrienol and cisplatin induced 44.59% apoptosis as compared with 17.68% with delta-tocotrienol (15 μM) and 19.79% in cisplatin (4 μM) treatments alone. These results are consistent with those from the MTS assay, suggesting that the potentiation in overall cell growth inhibition by the combination could in part be due the induction of apoptosis in both NSCLC cell lines.

Isobologram analysis.

As both delta-tocotrienol and cisplatin alone induce apoptosis in various cancer cells, we wanted to verify the effect of the combination of delta-tocotrienol and cisplatin treatment in A549 and H1650 cell lines. As shown in Fig. 6.3 A and Fig. 6.3 B, the combination index as calculated by the calsusyn software of ED_{75} found to be 1.06 (A549) and 1.05 (H1650) respectively. According to the combination definitions, these results confirm the additive effects between delta-tocotrienol and cisplatin in NSCLC cells.

Delta-tocotrienol and/or cisplatin attenuate the Notch-1 signaling pathways.

Thus far, our results have shown that delta-tocotrienol, cisplatin and their combination inhibited cell growth and induced apoptotic cell death in NSCLC cells. Our previous data demonstrated that delta-tocotrienol induced apoptosis through the Notch-1 pathway in NSCLC cells (89). In order to further understand the molecular mechanism involved in delta-tocotrienol and cisplatin induced apoptosis of NSCLC cells, modifications in the cell death pathway were investigated. Using western blotting analysis, we found that combination treatment of delta-tocotrienol and cisplatin significantly suppressed the protein expression of Notch-1 and its downstream signaling molecule Hes-1 in NSCLC A549 (Fig. 6.4 A) and H1650 (Fig. 6.4 B) compared to treatment by either delta-tocotrienol or cisplatin alone. Given that Notch signaling and its gene products are known to regulate cell proliferation, cell cycle distribution and apoptosis, we further explored the apoptosis related genes in both NSCLC cell lines. Our data shows that the combination treatment of delta-tocotrienol and cisplatin suppressed the Notch-1

pathway as compared with either delta-tocotrienol or cisplain alone in A549 and H1650 cell line respectively. This suppression of Notch-1 was associated with the higher expression of cleaved caspase 3, cleaved PARP and inhibition of Bcl-2 expression.

To further confirm the result on changes in protein expression, we also conducted real-time PCR to assess Notch-1 and its target genes such as Hes-1 in NSCLC cells upon the treatment of delta-tocotrienol, cisplatin and their combination. Our data clearly demonstrated that the combination treatment of delta-tocotrienol and cisplatin was a more potent suppress of Notch-1 signaling pathway in A549 (Fig. 6.4 C) and H1650 (Fig. 6.4 D) cells as compared to either compound or control. Taken together, our findings strongly suggest that the combination treatment of delta-tocotrienol and cisplatin suppressed transcription and translation of Notch-1 and its target genes such as Hes-1, Bcl-2 leading to reduced proliferation, and enhanced apoptosis in NSCLC cells.

Delta-tocotrienol and cisplatin combination inhibits NF-κB activation and downstream pathways

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 (78). Cisplatin, a DNA damage drug, is known to increase the NF-κB activity (111, 112). This increasing activity of NF-κB is associated with drug resistance. Interestingly, our previous data demonstrated that delta-tocotrienol can successfully reduce NF-κB DNA binding activity in NSCLC cells (89). The effect of combination treatment of delta-tocotrienol and cisplatin on NF-κB DNA-binding activity in NSCLC cells was determined by subjecting nuclear extracts from

treated A549 and H1650 cells to p65 ELISA. As shown in the Fig. 5 A and B, compared to the control, the combination treatment significantly inhibits the DNA-binding activity of NF-κB for both cell lines. These results suggest that the treatment with delta-tocotrienol decreased the cisplatin-induced NF-κB activation which further confirms the potential benefit of delta-tocotrienol as a sensitizing agent to cisplatin.

Since VEGF and MMP9 are known to be the downstream target genes of NF-κB signaling pathway, the relative expression of VEGF and MMP9 were evaluated by RT-PCR. As shown in Fig. 6.5 C (A549) and 6.5 D (H1650), the combination treatment of delta-tocotrienol and cisplatin significantly inhibited the expression of VEGF and MMP9 in both cell lines, respectively. The results clearly demonstrate that delta-tocotrienol sensitized the NSCLC cells to cisplatin by inhibiting NF-kB activity and its target genes expressions.

Delta-tocotrienol and/or cisplatin reduce cell invasion and migration

Although the effect of delta-tocotrienol and cisplatin on anti-proliferation and induction of apoptosis has been shown in certain cancers, their effects on tumor cells migration and invasion has not been evaluated thus far. Since the combination treatment of delta-tocotrienol and cisplatin inhibited MMP-9 and VEGF expression, which are 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 H1650 NSCLC cells. As shown in Fig. 6.6 A and B, the combination treatment of delta-tocotrienol and cisplatin in A549 and H1650 cells significantly

decrease their invasive capability as compared with either the untreated control, or delta-tocotrienol, and ciplatin alone.

In order to determine the combination effect of delta-tocotrienol and cisplatin on tumor migration ability in NSCLC cells, we performed the wound healing assay. As demonstrated in Fig. 6.6 C (A549) and D (H1650), the combination of delta-tocotrienol and cisplatin inhibited cell migration compared with the untreated control, delta-tocotrienol and cisplatin alone.

Discussion:

Cisplatin, is one of the most widely used chemotherapy drugs, and has been approved for treatment of different types of human solid carcinoma including lung, ovarian, bladder, and testicular cancers. Unfortunately, drug resistance and toxicity during chemotherapy remains a major hurdle and challenge for the usage of cisplatin in cancer therapy. Our previous data showed that treatment of NSCLC cells with delta-tocotrienol results in a dose dependent inhibition of cell growth, cell migration, tumor cell invasiveness, and induction of apoptosis (89). Real-time RT-PCR and Western blot analysis showed that antitumor activity of delta-tocotrienol was associated with a decrease in Notch-1, Hes-1, Survivin, MMP-9, VEGF, and Bcl-2 expression (89). The aim of this study was to determine whether delta-tocotrienol has potential in combinational therapy with cisplatin for the treatment of NSCLC and further, to elucidate its molecular mechanism. The data from the current study, demonstrates that treatment of NSCLC cells with delta-tocotrienol and cisplatin in combination resulted in a dose dependent inhibition of cell proliferation. Also, this is the first study to report the effectiveness of delta-tocotrienol in inducing apoptosis in NSCLC cell lines, when used in combination with cisplatin. Abnormal overexpression of Notch-1 pathway has been found in a subset of NSCLC patients (39). Interestingly, activated forms of Notch-1 and its down-stream molecule Hes-1 can be stimulated by cisplatin. Delta-tocotrienol mitigated the activation of Notch-1, leading to greater expression of apoptotic proteins such as cleaved caspase 3, cleaved PARP and the inhibition of Bcl-2 expression as compared with either delta-tocotrienol or cisplain alone in A549 and H1650 cell line, respectively. Therefore, the inhibition of Notch-1 signaling by delta-tocotrienol augmentation is a rational strategy against NSCLC cells to reduce the escape from cell death phenomenon by cisplatin.

Our previous data also demonstrated that delta-tocotrienol can decrease NF-κB-DNA binding activity. 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 (47). Growing data indicates that there is cross-talk between the Notch-1/Hes-1 pathways and the NF-κB pathway. Notch ligands induce NF-κB activation in leukemia cells, and decreased Notch-1 expression in these cells has been shown to be accompanied with concomittent decrease in NF-κB binding activity (48). Moreover Notch-1 has been found to induce sustained NF-κB activity by facilitating its nuclear retention (49). Recently, Notch-1/Hes-1 pathways were found to be upstream mechanisms for maintainence of NF-κB activation in leukemia in vivo and in vitro (51). Interestingly, cisplatin, a DNA damaging agent, has been shown to induce the NF-κB activity in *vitro* and in *vivo* (113, 114). The activated NF-κB plays important roles in drug resistance and tumor relapse. Consistent with previous data, we found that treatment with delta-tocotrienol can reduce the NF-κB activity (Fig 5A and B) in NSCLC cells. Moreover, delta-tocotrienol also suppressed the NF-κB activity induced by cisplatin in both NSCLC cell lines (Fig 6.5 A and B). These molecular findings lend support in favor of simultaneous targeting of Notch-1 and NF-κB for effective sensitizing NSCLC cells to cisplatin.

Furthermore, we wanted to explore the anti-metastatic effect of delta-tocotrienol and cisplatin action in NSCLC cells. Indeed, we showed that in both A549 and H1650 cells, migration and invasiveness were significantly reduced under treatment of delta-tocotrienol and cisplatin (Fig. 6.6). Previous, it has been reported that delta-tocotrienol suppressed hypoxia-induced VEGF and IL-8 expression at both mRNA and protein levels which in turn suppressed tumor angiogenesis (84). Consistent with previous studies, our study confirmed that the anti-

metastatic effects induced by delta-tocotrienol and cisplatin were associated with a decrease in VEGF and MMP-9 (Fig. 6.5 C and D) expressions.

In conclusion, in this study, we provided evidence to support that delta-tocotrienol can enhance sensitivity of cisplatin in NSCLC cells. The combined treatment of delta-tocotrienl and cisplatin significantly suppressed both NSCLC cell growth, colony formation, cell migration and invasion. Delta-tocotrienol suppressed cisplatin-caused activation of NF-κB pathway. Although the data needs to be substantiated further in a valid *in vivo* animal model for lung cancer, our findings indicate the potential of this combination of delta-tocotrienol and cisplatin as a novel therapeutic strategy for NSCLC.

Figure Legends:

Figure 5.1: Antiproliferative effects of Delta-tocotrienol and/or cisplatin on NSCLC cells.

Cell viability (A and B) of human NSCLC cell lines A549 and H1650 cells. Both A549 (left) and H1650 (Right) cells were initially plated at a density of 5×10^3 cells/well (3wells/group) in 96-well plates and grown in experimental medium with delta-tocotrienol (D), cisplatin (C) and the combination 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 and H1650 cells. A549 (C) and H1650 (D) cells treated with delta-tocotrienol (15 μM), cisplatin (4 μM), and the combination were evaluated by the clonogenic assay. Photomicrographic difference in colony formation in A549 and H1650 cells untreated and treated. There was a significant reduction in the colony formation in A549 and H1650 cells treated compared with cells untreated.

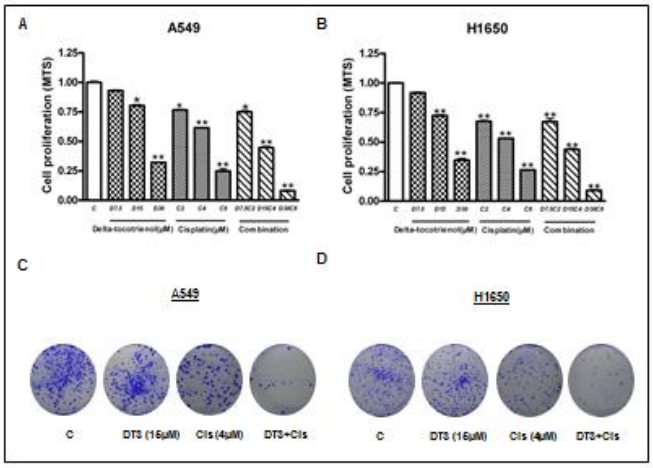


Figure 5.2: Induction of apoptotic effects of delta-tocotrienol and cisplatin NSCLC cells.

A and B, A549 and H1650 cells were treated with delta-tocotrienol (15 µM), cisplatin (4 µM), and the combination for 72 h. After that, the apoptosis of both cell lines were determined by histone/DNA ELISA. .*P<0.05, **P< 0.01.

C and D, A549 and H1650 cells were treated with delta-tocotrienol (15 µM), cisplatin (4 µM), and the combination for 72 h. After that, the apoptosis of both cell lines were determined by Annexin V-FITC.

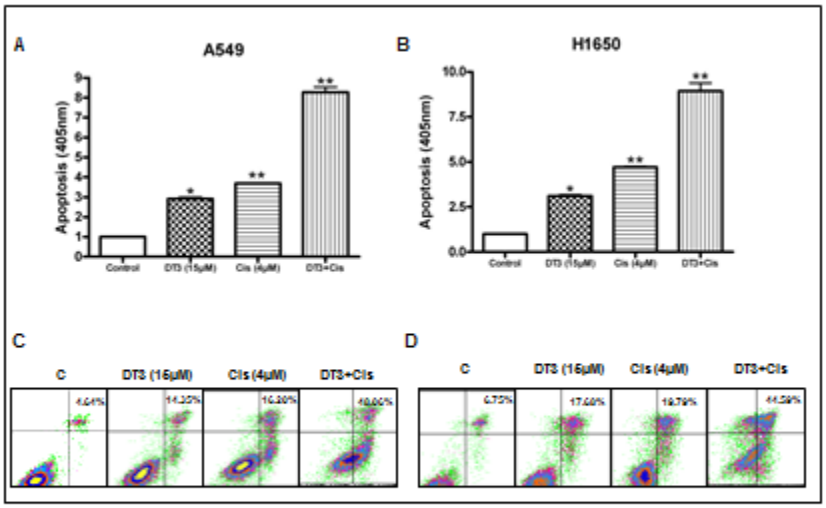


Figure 5.3: Isobologram plots for the combination analysis of delta-tocotrienol and cisplatin

Isobologram plots for combination treatments with delta-tocotrienol (7.5, 15, 30 µM) and cisplatin (2, 4, 8 µM) in A549 (A) and H1650 (B) were evaluated by the MTT assay. CI, combination index.

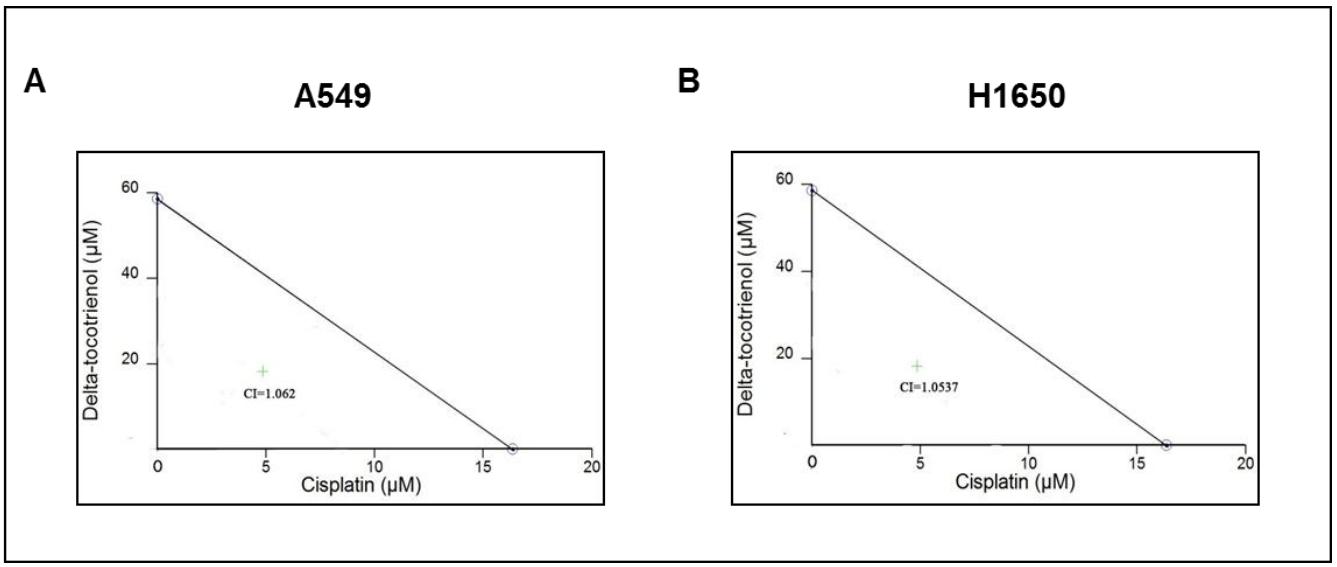


Figure 5.4: Dow-regulation of Notch-1 and its target genes by delta-tocotrienol and/or cisplatin

A549 (A) and H1650 (B) cells were treated with delta-tocotrienol (15 µM), cisplatin (4 µM), and the combination for 72 h. Upper panel: the expressions of Notch-1, Hes-1, PARP, caspase-3, Bcl-2 protein were detected by western blotting analysis in A549 (A) and H1650 (B) NSCLC cells. Lower panel:: Notch-1 mRNA and Hes-1 mRNA were detected by Real-time RT-PCR in A549 (C) and H1650 (D) , respectively.

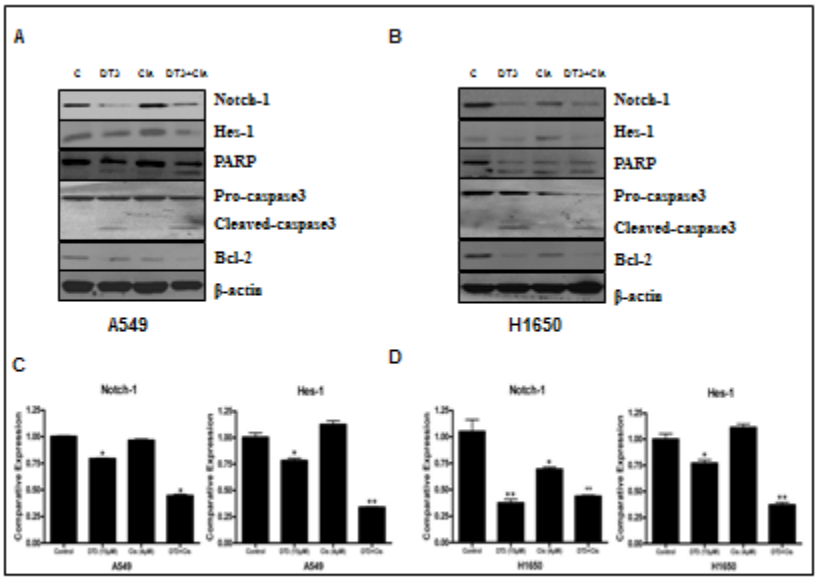


Figure 5.5: Dose-dependent down-regulation of NF-kB activity and its down-stream genes by delta-tocotrienol and/or cisplatin

A549 (A) and H1650 (B) cells were incubated with delta-tocotrienol (15 µM), cisplatin (4 µM), and the combination or DMSO-control for 72 h, and nuclear proteins were subjected to ELISA assay for the evaluation of NF-κB DNA binding activity.

A549(C) and H1650 (D) were treated delta-tocotrienol (15 µM), cisplatin (4 µM), and the combination for 72 h. The expressions of VEGF and MMP9 were analyzed by real-time-RT-PCR. Relative gene expressions were presented as means ± S.E.M of three independent experiments.*P<0.05, **P< 0.01.

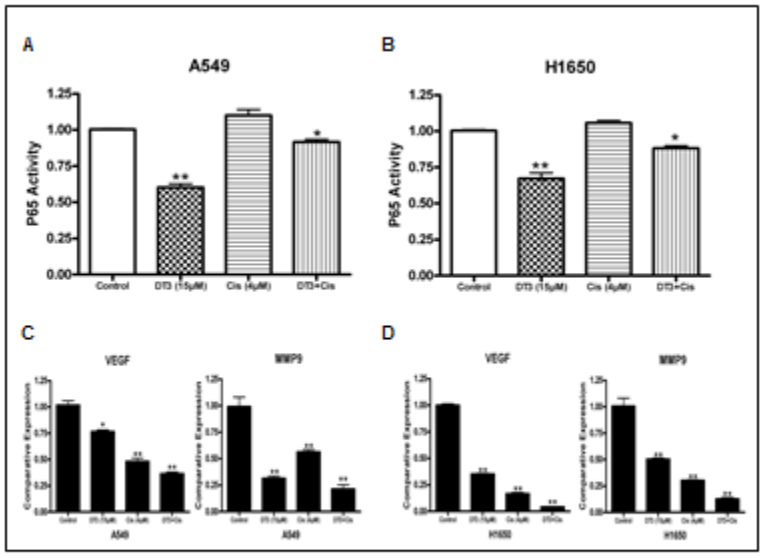
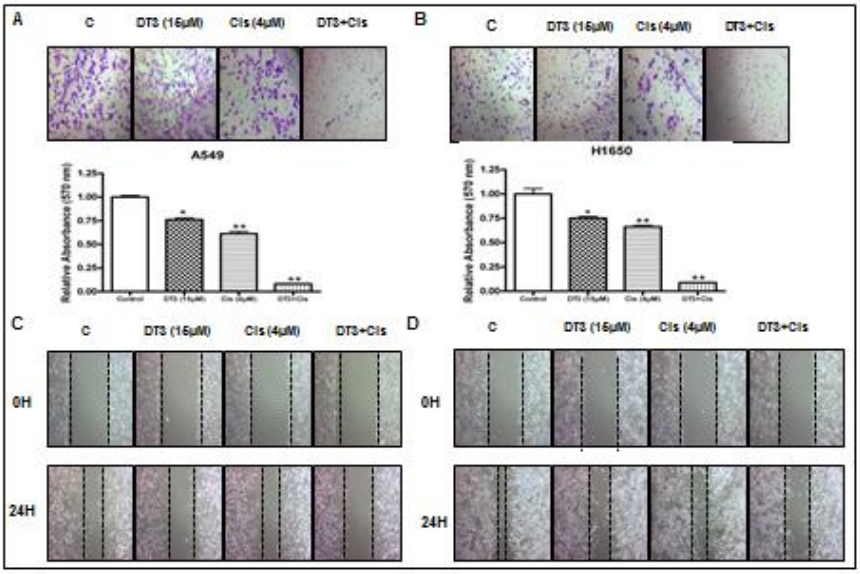


Figure 5.6: Delta-tocotrienol and/or cisplatin inhibits cell migration and invasion

A and B, Inhibition of NSCLC cells invasion by delta-tocotrienol, cisplatin, and the combination for 72 h. Upper panel: A549 (A) and H1650 (B) cells were seeded treated seeded into Matrigel-coated inserts with delta-tocotrienol (15 µM), cisplatin (4 µM), the combination or DMSO. Cells that invaded to the lower surface of the insert over a period of 20 h were stained with crystal violet dye. Lower panel: The photometric intensity of the invaded cells was determined for the number of A549 (A) and H1650 (B) cells. Cell invasion were presented as means ± S.E.M of three independent experiments.*P<0.05, **P< 0.01.

C and D, Inhibition of NSCLC cells migration by delta-tocotrienol (15 µM), cisplatin (4 µM), the combination or DMSO 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.



CHAPTER 6 CONCLUSION

Taken together, we have presented evidence that delta-tocotrienol could inhibit cell proliferation, clogonecity, cell invasion and migration and induce apoptosis in a dose dependent manner in NSCLC cell lines. Delta-tocotrienol could also arrest the NSCLC in G_0-G_1 phase. Protein expression analysis demonstrates that the effect of delta-tocotrienol on NSCLC cell lines was mediated by down-regulation on Notch-1 signaling pathway. EMSA analysis indicates that delta-tocotrienol reduce the NF-kB DNA binding activity.

Furthermore, our microRNA microarray data showed that treatment of NSCLC cells by delta-tocotrienol induced the expression of miR-34a. Utilizing the transfection of pre-miR-34a and antisense of miR-34a, we demonstrated that delta-tocotrienol inhibit cell proliferation, clogonecity, cell invasion and induce the apoptosis through the induction of miR-34a in NSCLC cell lines. The confocal immunochemistry analysis showed that the apoptosis initiated by miR-34a was in part mediated through the expression of p53 pathway.

Finally, we showed that delta-tocotrienol could sensitize NSCLC cell lines to cisplatin. This effect was due the suppression of NF-kB upon treatment of cells with delta-tocotrienol. The ability of delta-tocotrienol and cisplatin within therapeutic range to induce apoptosis in vitro suggests that this strategy could be an active and attractive regimen for patients diagnosed with NSCLC.

SUMMARY AND FUTURE DIRECTIONS

Study 1: Lung cancer is the leading cause of death among all types of carcinomas and NSCLC account for 80% of lung cancers. Clinically, 30% of NSCLC patients were found to have up regulation of Notch-1 pathway. In the current study, we have provided experimental evidence showing that delta-tocotrienol could inhibit Notch-1 signaling, cell proliferation, invasion and also induced apoptosis in NSCLC cells. Moreover, our current data provide mechanistic insight 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-kB signaling. On the basis of our results, we propose a hypothetical pathway as shown by which delta-tocotrienol inhibits cell growth of NSCLC cells.

Future Directions: Further in-depth experiments are needed to ascertain the specific mechanisms by which delta-tocotrienol regulates oncogenic pathway for example EGFR, PI3K, MAPK. 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 concentrations in the target tissues. 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.

Study 2: Accumulating data demonstrate that microRNA play important roles in the pathogenesis of tumor progression. Some microRNAs are known to regulate carcinogenesis as a tumor suppressors while others are working as oncogenes. Our results demonstrate that delta-

tocotrienol can upregulate miR-34a, expression and inhibit cancer cell proliferation, induce apoptosis, and reduce cancer cell invasion, at least in part due to downregulation of Notch-1, the molecular target that is predominately activated in NSCLC. Very recently, miR-34a was found to be downregulated in glioblastoma multiforme cells and was shown to inhibit cell growth by targeting the Notch-1 pathway. Moreover knockdown of Notch-1 showed similar cellular functions as overexpression of miR-34a both in vitro and in vivo. These data corroborate some of two work presented here.

Future Directions: Besides the data we published, the effects of delta-tocotrienol on the expression to microRNA need to be further explored. In addition, in vivo studies in appropriate animal models for NSCLC are needed to establish whether delta-tocotrienol could be useful in combination with conventional chemotherapeutics or conventional targeted agents such as cisplatin and erolitinib, respectively for the treatment of NSCLC.

Study 3: Although the development of target therapy, the response rate to EGFR-TKI for the NSCLC patients is still 10%. Many attempts to improve the survival of patients with NSCLC remain disappointing, suggesting newer treatment strategies must be developed. Based on foregoing discussion and our initial conviction that delta-tocotrienol may have therapeutic advantage, its effect in combination with another potent chemo agent- cisplatin was investigated We provided evidences in this study that delta-tocotrienol can enhance the sensitivity of cisplatin in NSCLC cells. The combined treatment of delta-tocotrienl and cisplatin significantly suppressed both NSCLC cells growth, colony formation, cell migration and invasion. Delta-tocotrienol suppressed cisplatin-induced activation of NF-κB pathway. Assessment of the

combination index values along with apoptosis induction by this combination proved to be additive with greater inhibition of cell viability and induction of apoptosis through the inhibition of Notch-1 pathway. These findings indicate the potential of this combination of delta-tocotrienol and cisplatin as a novel therapeutic strategy for NSCLC.

Future Directions: The future studies will include experimental design to determine the effects of delta-tocotrienol and other drugs on more oncogenic pathways such as EGFR, MAPK, mTOR. In addition, in vivo studies in appropriate animal models for NSCLC are needed to establish whether delta-tocotrienol could be useful in combination with conventional chemotherapeutics or conventional targeted agents such as gemicitabine and erolitinib, respectively for the treatment of NSCLC.

REFERENCES

1. McWhorter JE. Malignant Epithelial Tumors of the Neck of Unknown Origin. Ann Surg. 1929;90:1-14.
2. Scott G. Discussion on the Radiographic Appearances of Bone Tumours, Benign and Malignant. Proc R Soc Med. 1914;7:66-71.
3. Proctor RN. Tobacco and the global lung cancer epidemic. Nat Rev Cancer. 2001;1:82-6.
4. Nowell PC. The clonal evolution of tumor cell populations. Science. 1976;194:23-8.
5. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144:646-74.
6. Stern DF, Heffernan PA, Weinberg RA. p185, a product of the neu proto-oncogene, is a receptorlike protein associated with tyrosine kinase activity. Mol Cell Biol. 1986;6:1729-40.
7. Gescher AJ, Sharma RA, Steward WP. Cancer chemoprevention by dietary constituents: a tale of failure and promise. Lancet Oncol. 2001;2:371-9.
8. Thangapazham RL, Sharma A, Maheshwari RK. Multiple molecular targets in cancer chemoprevention by curcumin. AAPS J. 2006;8:E443-9.
9. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. CA: a cancer journal for clinicians. 2012;62:10-29.
10. Wu X, Piper-Hunter MG, Crawford M, Nuovo GJ, Marsh CB, Otterson GA, et al. MicroRNAs in the pathogenesis of Lung Cancer. J Thorac Oncol. 2009;4:1028-34.
11. Tuveson DA, Jacks T. Modeling human lung cancer in mice: similarities and shortcomings. Oncogene. 1999;18:5318-24.

12. Visvanathan K, Chlebowski RT, Hurley P, Col NF, Ropka M, Collyar D, et al. American society of clinical oncology clinical practice guideline update on the use of pharmacologic interventions including tamoxifen, raloxifene, and aromatase inhibition for breast cancer risk reduction. J Clin Oncol. 2009;27:3235-58.

13. Hirsch FR, Varella-Garcia M, Cappuzzo F. Predictive value of EGFR and HER2 overexpression in advanced non-small-cell lung cancer. Oncogene. 2009;28 Suppl 1:S32-7.

14. Chrysogelos SA, Dickson RB. EGF receptor expression, regulation, and function in breast cancer. Breast Cancer Res Treat. 1994;29:29-40.

15. Maihle NJ, Baron AT, Barrette BA, Boardman CH, Christensen TA, Cora EM, et al. EGF/ErbB receptor family in ovarian cancer. Cancer Treat Res. 2002;107:247-58.

16. Schiller JH, Harrington D, Belani CP, Langer C, Sandler A, Krook J, et al. Comparison of four chemotherapy regimens for advanced non-small-cell lung cancer. N Engl J Med. 2002;346:92-8.

17. Rogers SJ, Harrington KJ, Rhys-Evans P, P OC, Eccles SA. Biological significance of c-erbB family oncogenes in head and neck cancer. Cancer Metastasis Rev. 2005;24:47-69.

18. Fukuoka M, Yano S, Giaccone G, Tamura T, Nakagawa K, Douillard JY, et al. Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer (The IDEAL 1 Trial) [corrected]. J Clin Oncol. 2003;21:2237-46.

19. Kris MG, Natale RB, Herbst RS, Lynch TJ, Jr., Prager D, Belani CP, et al. Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in

symptomatic patients with non-small cell lung cancer: a randomized trial. JAMA. 2003;290:2149-58.

20. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med. 2004;350:2129-39.

21. Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science. 2004;304:1497-500.

22. Kobayashi S, Boggon TJ, Dayaram T, Janne PA, Kocher O, Meyerson M, et al. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. N Engl J Med. 2005;352:786-92.

23. Pao W, Miller VA, Politi KA, Riely GJ, Somwar R, Zakowski MF, et al. Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. PLoS Med. 2005;2:e73.

24. Kosaka T, Yatabe Y, Endoh H, Yoshida K, Hida T, Tsuboi M, et al. Analysis of epidermal growth factor receptor gene mutation in patients with non-small cell lung cancer and acquired resistance to gefitinib. Clin Cancer Res. 2006;12:5764-9.

25. Balak MN, Gong Y, Riely GJ, Somwar R, Li AR, Zakowski MF, et al. Novel D761Y and common secondary T790M mutations in epidermal growth factor receptor-mutant lung adenocarcinomas with acquired resistance to kinase inhibitors. Clin Cancer Res. 2006;12:6494-501.

26. Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. Science. 2007;316:1039-43.

27. Bean J, Riely GJ, Balak M, Marks JL, Ladanyi M, Miller VA, et al. Acquired resistance to epidermal growth factor receptor kinase inhibitors associated with a novel T854A mutation in a patient with EGFR-mutant lung adenocarcinoma. Clin Cancer Res. 2008;14:7519-25.

28. Riely GJ. Second-generation epidermal growth factor receptor tyrosine kinase inhibitors in non-small cell lung cancer. J Thorac Oncol. 2008;3:S146-9.

29. Engelman JA, Zejnullahu K, Gale CM, Lifshits E, Gonzales AJ, Shimamura T, et al. PF00299804, an irreversible pan-ERBB inhibitor, is effective in lung cancer models with EGFR and ERBB2 mutations that are resistant to gefitinib. Cancer Res. 2007;67:11924-32.

30. Zhang YW, Staal B, Essenburg C, Su Y, Kang L, West R, et al. MET kinase inhibitor SGX523 synergizes with epidermal growth factor receptor inhibitor erlotinib in a hepatocyte growth factor-dependent fashion to suppress carcinoma growth. Cancer Res. 2010;70:6880-90.

31. Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: cell fate control and signal integration in development. Science. 1999;284:770-6.

32. Ohishi K, Katayama N, Shiku H, Varnum-Finney B, Bernstein ID. Notch signalling in hematopoiesis. Semin Cell Dev Biol. 2003;14:143-50.

33. Mumm JS, Kopan R. Notch signaling: from the outside in. Dev Biol. 2000;228:151-65.

34. Bigas A, Martin DIK, Milner LA. Notch1 and Notch2 inhibit myeloid differentiation in response to different cytokines. Molecular and Cellular Biology. 1998;18:2324-33.

35. Miele L, Golde T, Osborne B. Notch signaling in cancer. Curr Mol Med. 2006;6:905-18.

36. Oswald F, Tauber B, Dobner T, Bourteele S, Kostezka U, Adler G, et al. p300 acts as a transcriptional coactivator for mammalian Notch-1. Molecular and Cellular Biology. 2001;21:7761-74.

37. Wang Z, Zhang Y, Li Y, Banerjee S, Liao J, Sarkar FH. Down-regulation of Notch-1 contributes to cell growth inhibition and apoptosis in pancreatic cancer cells. Mol Cancer Ther. 2006;5:483-93.

38. Wang Z, Banerjee S, Li Y, Rahman KM, Zhang Y, Sarkar FH. Down-regulation of notch-1 inhibits invasion by inactivation of nuclear factor-kappaB, vascular endothelial growth factor, and matrix metalloproteinase-9 in pancreatic cancer cells. Cancer Res. 2006;66:2778-84.

39. Westhoff B, Colaluca IN, D'Ario G, Donzelli M, Tosoni D, Volorio S, et al. Alterations of the Notch pathway in lung cancer. Proc Natl Acad Sci U S A. 2009;106:22293-8.

40. Reedijk M, Odorcic S, Zhang H, Chetty R, Tennert C, Dickson BC, et al. Activation of Notch signaling in human colon adenocarcinoma. Int J Oncol. 2008;33:1223-9.

41. Lin JT, Chen MK, Yeh KT, Chang CS, Chang TH, Lin CY, et al. Association of High Levels of Jagged-1 and Notch-1 Expression with Poor Prognosis in Head and Neck Cancer. Ann Surg Oncol. 2010.

42. Buchler P, Gazdhar A, Schubert M, Giese N, Reber HA, Hines OJ, et al. The Notch signaling pathway is related to neurovascular progression of pancreatic cancer. Ann Surg. 2005;242:791-800, discussion -1.

43. Jundt F, Anagnostopoulos I, Forster R, Mathas S, Stein H, Dorken B. Activated Notch1 signaling promotes tumor cell proliferation and survival in Hodgkin and anaplastic large cell lymphoma. Blood. 2002;99:3398-403.

44. Miele L, Osborne B. Arbiter of differentiation and death: Notch signaling meets apoptosis. J Cell Physiol. 1999;181:393-409.

45. Eliasz S, Liang S, Chen Y, De Marco MA, Machek O, Skucha S, et al. Notch-1 stimulates survival of lung adenocarcinoma cells during hypoxia by activating the IGF-1R pathway. Oncogene. 2010;29:2488-98.

46. Nickoloff BJ, Osborne BA, Miele L. Notch signaling as a therapeutic target in cancer: a new approach to the development of cell fate modifying agents. Oncogene. 2003;22:6598-608.

47. Karin M. Nuclear factor-kappaB in cancer development and progression. Nature. 2006;441:431-6.

48. Itoh M, Fu L, Tohda S. NF-kappaB activation induced by Notch ligand stimulation in acute myeloid leukemia cells. Oncol Rep. 2009;22:631-4.

49. Shin HM, Minter LM, Cho OH, Gottipati S, Fauq AH, Golde TE, et al. Notch1 augments NF-kappaB activity by facilitating its nuclear retention. EMBO J. 2006;25:129-38.

50. Oswald F, Liptay S, Adler G, Schmid RM. NF-kappaB2 is a putative target gene of activated Notch-1 via RBP-Jkappa. Mol Cell Biol. 1998;18:2077-88.

51. Espinosa L, Cathelin S, D'Altri T, Trimarchi T, Statnikov A, Guiu J, et al. The Notch/Hes1 pathway sustains NF-kappaB activation through CYLD repression in T cell leukemia. Cancer Cell. 2010;18:268-81.

52. Bivona TG, Hieronymus H, Parker J, Chang K, Taron M, Rosell R, et al. FAS and NF-kappaB signalling modulate dependence of lung cancers on mutant EGFR. Nature. 2011;471:523-6.

53. Lee RC, Ambros V. An extensive class of small RNAs in Caenorhabditis elegans. Science. 2001;294:862-4.

54. Caldas C, Brenton JD. Sizing up miRNAs as cancer genes. Nat Med. 2005;11:712-4.

55. Gironella M, Seux M, Xie MJ, Cano C, Tomasini R, Gommeaux J, et al. Tumor protein 53-induced nuclear protein 1 expression is repressed by miR-155, and its restoration inhibits pancreatic tumor development. Proc Natl Acad Sci U S A. 2007;104:16170-5.

56. Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci U S A. 2006;103:2257-61.

57. Takamizawa J, Konishi H, Yanagisawa K, Tomida S, Osada H, Endoh H, et al. Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. Cancer Res. 2004;64:3753-6.

58. Takahashi Y, Forrest AR, Maeno E, Hashimoto T, Daub CO, Yasuda J. MiR-107 and MiR-185 can induce cell cycle arrest in human non small cell lung cancer cell lines. PLoS One. 2009;4:e6677.

59. Gallardo E, Navarro A, Vinolas N, Marrades RM, Diaz T, Gel B, et al. miR-34a as a prognostic marker of relapse in surgically resected non-small-cell lung cancer. Carcinogenesis. 2009;30:1903-9.

60. Sundram K, Sambanthamurthi R, Tan YA. Palm fruit chemistry and nutrition. Asia Pac J Clin Nutr. 2003;12:355-62.

61. Elson CE. Tropical oils: nutritional and scientific issues. Crit Rev Food Sci Nutr. 1992;31:79-102.

62. Qureshi AA, Burger WC, Peterson DM, Elson CE. The structure of an inhibitor of cholesterol biosynthesis isolated from barley. J Biol Chem. 1986;261:10544-50.

63. Parker RA, Pearce BC, Clark RW, Gordon DA, Wright JJ. Tocotrienols regulate cholesterol production in mammalian cells by post-transcriptional suppression of 3-hydroxy-3-methylglutaryl-coenzyme A reductase. J Biol Chem. 1993;268:11230-8.

64. Song BL, DeBose-Boyd RA. Insig-dependent ubiquitination and degradation of 3-hydroxy-3-methylglutaryl coenzyme a reductase stimulated by delta- and gamma-tocotrienols. J Biol Chem. 2006;281:25054-61.

65. Raederstorff D, Elste V, Aebischer C, Weber P. Effect of either gamma-tocotrienol or a tocotrienol mixture on the plasma lipid profile in hamsters. Ann Nutr Metab. 2002;46:17-23.

66. Serbinova E, Kagan V, Han D, Packer L. Free radical recycling and intramembrane mobility in the antioxidant properties of alpha-tocopherol and alpha-tocotrienol. Free Radic Biol Med. 1991;10:263-75.

67. Serbinova EA, Packer L. Antioxidant properties of alpha-tocopherol and alpha-tocotrienol. Methods Enzymol. 1994;234:354-66.

68. Golden TR, Hinerfeld DA, Melov S. Oxidative stress and aging: beyond correlation. Aging Cell. 2002;1:117-23.

69. Adachi H, Ishii N. Effects of tocotrienols on life span and protein carbonylation in Caenorhabditis elegans. J Gerontol A Biol Sci Med Sci. 2000;55:B280-5.

70. Ren Z, Pae M, Dao MC, Smith D, Meydani SN, Wu D. Dietary supplementation with tocotrienols enhances immune function in C57BL/6 mice. J Nutr. 2010;140:1335-41.

71. Aggarwal BB, Sundaram C, Prasad S, Kannappan R. Tocotrienols, the vitamin E of the 21st century: its potential against cancer and other chronic diseases. Biochemical pharmacology. 2010;80:1613-31.

72. Komiyama K, Iizuka K, Yamaoka M, Watanabe H, Tsuchiya N, Umezawa I. Studies on the biological activity of tocotrienols. Chem Pharm Bull (Tokyo). 1989;37:1369-71.

73. Kumar KS, Raghavan M, Hieber K, Ege C, Mog S, Parra N, et al. Preferential radiation sensitization of prostate cancer in nude mice by nutraceutical antioxidant gamma-tocotrienol. Life Sci. 2006;78:2099-104.

74. Sylvester PW, Shah S. Intracellular mechanisms mediating tocotrienol-induced apoptosis in neoplastic mammary epithelial cells. Asia Pac J Clin Nutr. 2005;14:366-73.

75. McAnally JA, Jung M, Mo H. Farnesyl-O-acetylhydroquinone and geranyl-O-acetylhydroquinone suppress the proliferation of murine B16 melanoma cells, human prostate and colon adenocarcinoma cells, human lung carcinoma cells, and human leukemia cells. Cancer Lett. 2003;202:181-92.

76. Shirode AB, Sylvester PW. Synergistic anticancer effects of combined gamma-tocotrienol and celecoxib treatment are associated with suppression in Akt and NFkappaB signaling. Biomed Pharmacother. 2010;64:327-32.

77. Oswald F, Liptay S, Adler G, Schmid RM. NF-kappaB2 is a putative target gene of activated Notch-1 via RBP-Jkappa. Molecular and Cellular Biology. 1998;18:2077-88.

78. Wang Z, Zhang Y, Banerjee S, Li Y, Sarkar FH. Notch-1 down-regulation by curcumin is associated with the inhibition of cell growth and the induction of apoptosis in pancreatic cancer cells. Cancer. 2006;106:2503-13.

79. Shih Ie M, Wang TL. Notch signaling, gamma-secretase inhibitors, and cancer therapy. Cancer Res. 2007;67:1879-82.

80. Constantinou C, Hyatt JA, Vraka PS, Papas A, Papas KA, Neophytou C, et al. Induction of caspase-independent programmed cell death by vitamin E natural homologs and synthetic derivatives. Nutr Cancer. 2009;61:864-74.

81. Hussein D, Mo H. d-Dlta-tocotrienol-mediated suppression of the proliferation of human PANC-1, MIA PaCa-2, and BxPC-3 pancreatic carcinoma cells. Pancreas. 2009;38:e124-36.

82. Guo D, Ye J, Dai J, Li L, Chen F, Ma D, et al. Notch-1 regulates Akt signaling pathway and the expression of cell cycle regulatory proteins cyclin D1, CDK2 and p21 in T-ALL cell lines. Leuk Res. 2009;33:678-85.

83. Wang Y, Chan SL, Miele L, Yao PJ, Mackes J, Ingram DK, et al. Involvement of Notch signaling in hippocampal synaptic plasticity. Proc Natl Acad Sci U S A. 2004;101:9458-62.

84. Shibata A, Nakagawa K, Sookwong P, Tsuduki T, Tomita S, Shirakawa H, et al. Tocotrienol inhibits secretion of angiogenic factors from human colorectal adenocarcinoma cells by suppressing hypoxia-inducible factor-1alpha. J Nutr. 2008;138:2136-42.

85. Ju J, Picinich SC, Yang Z, Zhao Y, Suh N, Kong AN, et al. Cancer-preventive activities of tocopherols and tocotrienols. Carcinogenesis. 2010;31:533-42.

86. Lin JT, Chen MK, Yeh KT, Chang CS, Chang TH, Lin CY, et al. Association of high levels of Jagged-1 and Notch-1 expression with poor prognosis in head and neck cancer. Annals of surgical oncology. 2010;17:2976-83.

87. Keum JS, Kong G, Yang SC, Shin DH, Park SS, Lee JH, et al. Cyclin D1 overexpression is an indicator of poor prognosis in resectable non-small cell lung cancer. Br J Cancer. 1999;81:127-32.

88. Calin GA, Croce CM. MicroRNA signatures in human cancers. Nat Rev Cancer. 2006;6:857-66.

89. Ji X, Wang Z, Geamanu A, Sarkar FH, Gupta SV. 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. J Cell Biochem. 2011;112:2773-83.

90. Zhang JS, Li DM, He N, Liu YH, Wang CH, Jiang SQ, et al. A paraptosis-like cell death induced by delta-tocotrienol in human colon carcinoma SW620 cells is associated with the suppression of the Wnt signaling pathway. Toxicology. 2011;285:8-17.

91. Guessous F, Zhang Y, Kofman A, Catania A, Li Y, Schiff D, et al. microRNA-34a is tumor suppressive in brain tumors and glioma stem cells. Cell Cycle. 2010;9:1031-6.

92. Reich NC, Levine AJ. Growth regulation of a cellular tumour antigen, p53, in nontransformed cells. Nature. 1984;308:199-201.

93. Gerwin BI, Spillare E, Forrester K, Lehman TA, Kispert J, Welsh JA, et al. Mutant p53 can induce tumorigenic conversion of human bronchial epithelial cells and reduce their responsiveness to a negative growth factor, transforming growth factor beta 1. Proc Natl Acad Sci U S A. 1992;89:2759-63.

94. Croce CM. Oncogenes and cancer. N Engl J Med. 2008;358:502-11.

95. Vosa U, Vooder T, Kolde R, Fischer K, Valk K, Tonisson N, et al. Identification of miR-374a as a prognostic marker for survival in patients with early-stage nonsmall cell lung cancer. Genes Chromosomes Cancer. 2011;50:812-22.

96. Saito M, Schetter AJ, Mollerup S, Kohno T, Skaug V, Bowman ED, et al. The association of microRNA expression with prognosis and progression in early-stage, non-small cell lung adenocarcinoma: a retrospective analysis of three cohorts. Clin Cancer Res. 2011;17:1875-82.

97. Tazawa H, Tsuchiya N, Izumiya M, Nakagama H. Tumor-suppressive miR-34a induces senescence-like growth arrest through modulation of the E2F pathway in human colon cancer cells. Proc Natl Acad Sci U S A. 2007;104:15472-7.

98. Chang TC, Wentzel EA, Kent OA, Ramachandran K, Mullendore M, Lee KH, et al. Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. Mol Cell. 2007;26:745-52.

99. Welch C, Chen Y, Stallings RL. MicroRNA-34a functions as a potential tumor suppressor by inducing apoptosis in neuroblastoma cells. Oncogene. 2007;26:5017-22.

100. Lodygin D, Tarasov V, Epanchintsev A, Berking C, Knyazeva T, Korner H, et al. Inactivation of miR-34a by aberrant CpG methylation in multiple types of cancer. Cell Cycle. 2008;7:2591-600.

101. Mudduluru G, Ceppi P, Kumarswamy R, Scagliotti GV, Papotti M, Allgayer H. Regulation of Axl receptor tyrosine kinase expression by miR-34a and miR-199a/b in solid cancer. Oncogene. 2011;30:2888-99.

102. Biliran H, Jr., Wang Y, Banerjee S, Xu H, Heng H, Thakur A, et al. Overexpression of cyclin D1 promotes tumor cell growth and confers resistance to cisplatin-mediated

apoptosis in an elastase-myc transgene-expressing pancreatic tumor cell line. Clin Cancer Res. 2005;11:6075-86.

103. Henriksson E, Baldetorp B, Borg A, Kjellen E, Akervall J, Wennerberg J, et al. p53 mutation and cyclin D1 amplification correlate with cisplatin sensitivity in xenografted human squamous cell carcinomas from head and neck. Acta Oncol. 2006;45:300-5.

104. Li WB, Ma MW, Dong LJ, Wang F, Chen LX, Li XR. MicroRNA-34a targets notch1 and inhibits cell proliferation in glioblastoma multiforme. Cancer Biol Ther. 2011;12:477-83.

105. Siegel R, Ward E, Brawley O, Jemal A. Cancer statistics, 2011: the impact of eliminating socioeconomic and racial disparities on premature cancer deaths. CA Cancer J Clin. 2011;61:212-36.

106. Stordal B, Davey M. Understanding cisplatin resistance using cellular models. IUBMB Life. 2007;59:696-9.

107. Zhang ZP, Sun YL, Fu L, Gu F, Zhang L, Hao XS. Correlation of Notch1 expression and activation to cisplatin-sensitivity of head and neck squamous cell carcinoma. Ai Zheng. 2009;28:100-3.

108. Gu F, Ma Y, Zhang Z, Zhao J, Kobayashi H, Zhang L, et al. Expression of Stat3 and Notch1 is associated with cisplatin resistance in head and neck squamous cell carcinoma. Oncol Rep. 2010;23:671-6.

109. Zhang S, Balch C, Chan MW, Lai HC, Matei D, Schilder JM, et al. Identification and characterization of ovarian cancer-initiating cells from primary human tumors. Cancer Res. 2008;68:4311-20.

110. Aleksic T, Feller SM. Gamma-secretase inhibition combined with platinum compounds enhances cell death in a large subset of colorectal cancer cells. Cell Commun Signal. 2008;6:8.

111. Watanabe K, Inai S, Jinnouchi K, Bada S, Hess A, Michel O, et al. Nuclear-factor kappa B (NF-kappa B)-inducible nitric oxide synthase (iNOS/NOS II) pathway damages the stria vascularis in cisplatin-treated mice. Anticancer Res. 2002;22:4081-5.

112. Lee JE, Nakagawa T, Kita T, Kim TS, Iguchi F, Endo T, et al. Mechanisms of apoptosis induced by cisplatin in marginal cells in mouse stria vascularis. ORL J Otorhinolaryngol Relat Spec. 2004;66:111-8.

113. Li Y, Ahmed F, Ali S, Philip PA, Kucuk O, Sarkar FH. Inactivation of nuclear factor kappaB by soy isoflavone genistein contributes to increased apoptosis induced by chemotherapeutic agents in human cancer cells. Cancer Res. 2005;65:6934-42.

114. Banerjee S, Zhang Y, Ali S, Bhuiyan M, Wang Z, Chiao PJ, et al. Molecular evidence for increased antitumor activity of gemcitabine by genistein in vitro and in vivo using an orthotopic model of pancreatic cancer. Cancer Res. 2005;65:9064-72.

ABSTRACT

INTERVENTION OF NON-SMALL CELL LINE CANCER ONCOGENIC PATHWAYS WITH DELTA-TOCOTRIENOL

by

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Major: Nutrition and Food Science

Degree: Doctor of philosophy

Non-small cell lung cancer (NSCLC) is most hostile and leading cause of cancer deaths in the United States. Clinical data has demonstrated that 30 % of NSCLC patients have increased Notch activity and 10% of NSCLC have gain-of-function mutation on Notch-1 gene. Our data demonstrated that delta-tocotrienol could inhibit NSCLC cells proliferation, invasion and induce apoptosis by down-regulation of the Notch-1 signaling pathway. Using microRNA microarray and microRNA transfection, our findings further suggest that delta-tocotrienol is a non-toxic activator of miR-34a which can inhibit NSCLC cell proliferation, induce apoptosis, and inhibit invasion. Last but not the least, We observed that delta-tocotrienol individually or in combination with cisplatin exhibited potent anticancer abilities in NSCLC cells by down-regulating oncogenic pathways such as Notch-1 and NF-κB pathways and up-regulating the tumor suppressor pathways such as Bcl-2, caspase-3. Decreased NF- κB activity was observed in both cell lines in combination treatment along with significantly reduced levels of angiogenic

factors MMP-9, VEGF thus offering a potential starting point for the design of novel anticancer agents. Overall, our results suggest that delta-tocotrienol could be a promising approach for designing tailored novel combination therapies for the treatment of human NSCLC.

AUTOBIOGRAPHICAL STATEMENT

EDUCATION

Wayne State University **2007~Present** Detroit, MI

Pursing a PhD in nutrition/cancer prevention

Free University of Brussels **2003~2005** Brussels, Belgium

Master of Biomedical Sciences, Cell and Gene Therapy

Dalian Ocean University **1998~2002** Dalian, China

Bachelor of Food Science

PUBLICATIONS

Peer-Review Papers

1. Vermeij J, Teugels E, Bourgain C, Xiangming J, in 't Veld P, Ghislain V, et al. Genomic activation of the EGFR and HER2-neu genes in a significant proportion of invasive epithelial ovarian cancers. **BMC Cancer** 2008;8:3.
2. Ji X, Wang Z, Geamanu A, Sarkar FH, Gupta SV. 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. **J Cell Biochem** 2011;112:2773-2783.
3. Xiangming Ji, Wang Z, Geamanu A, Sarkar FH, Gupta SV. Delta-tocotrienol suppresses Notch-1 pathway by up-regulating miR-34a in non-small cell lung cancer cells. **Int J Cancer** 2012; Mar 21
4. Andreea Geamanu, Nadia Saadat, Xiangming Ji and SVGupta etal ProAlgaZyme and its sub-fractions increase plasma HDL-cholesterol via up regulation of ApoA1, ABCA1 and SRB1 and inhibition of CETP in hypercholestereolemic hamsters. **Food and Dietary Supplement 2011**
5. Arvind Goja, Andreea Geamanu, Nadia Saadat, Xiangming Ji, Doina David and Smiti V Gupta. Change in metabolomic profiles due to cancer progression in Golden Syrian Hamsters: Preventative effect of curcumin. **Cancer Prevention** 2012 (in press)
6. Xiangming Ji, Zhiwei Wang, Arvind Goja, Andreea Geamanu, Fazlul H Sarkar and Smiti V. Gupta. Delta-tocotrienol enhances the effect of cisplatin in suppression of non-small cell lung cancer cells via inhibition of Notch-1 pathway. **Anticancer Research.** 2012 (in press)

Book Chapter

Potential of Tocotrienols in Lung Cancer. Xiangming Ji, Arvind Goja and Smiti V. Gupta, 2011.

AWARDS

1. EB 2012, Diet and Cancer second monetary prizes, 2012
2. NIH Dietary Supplement Practicum Travel Award, 2012
3. Graduate Summer Dissertation Fellowship, 2012