Delta-Tocotrienol Downregulates Mmp-9 Expression In Nsclc Cells

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DELTA-TOCOTRIENOL DOWN REGULATES MMP-9 EXPRESSION IN NSCLC CELLS

by

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THESIS

Submitted to the Graduate School

of Wayne State University,

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MAJOR: NUTRITION AND FOOD SCIENCE

Approved By:

________________________________________________________________________

Advisor Date
DEDICATION

I dedicate this thesis to my family and friends who have supported me completely no matter what.
ACKNOWLEDGEMENTS

The completion of my thesis could not have been possible without the participation and assistance of many people. It is my proud privilege to owe a deep sense of gratitude to my advisor Dr. Smiti Gupta for her continuous support and sincere guidance throughout the study. I greatly appreciate her patience and motivation in all the time of research and writing this thesis.

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I would like to thank my family for their unconditional love and support and my friends who have been more like family for their continuous love, care and guidance. Above all, I thank the Great Almighty to bestow knowledge and wisdom upon me.
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Chapter 1

INTRODUCTION

1.1 CANCER:

Cancer is the uncontrolled growth of abnormal cells, forming malignant tumors, and invading other parts of the body. Normal cells have constant signals that dictate whether cells should divide and differentiate into another cell or die. Cancer cells develop a degree of anatomy from the signals, resulting in uncontrolled growth and proliferation. If this unregulated proliferation is allowed to continue, it could be fatal.

Cancer remains the second leading cause of death in the United States and the leading cause of death among the most productive age group, i.e., those aged 45–64 y \cite{1}. The causes of cancer are diverse and complex. There are many factors that increase the risk of cancer, which are exposure to radiation, environmental pollutants, certain infections, tobacco use, certain dietary factors, obesity and lack of physical activity \cite{2}. These factors directly damage genes or combine with existing genetic errors within the cells to cause mutations \cite{3}. Tumorigenesis in humans is a multistep process, where normal cells get transformed into malignant cancer cells \cite{4}. Cancer progression or tumorigenesis involves the following steps \cite{5}:

**Initiation**- Initiation is occurrence of mutation on a chromosome, or in a gene segment of DNA. Usually, initiation is not enough to produce cancer as the body’s repair systems can replace damaged sections of DNA. If the cell reproduces with that damaged DNA then more abnormal cells can be made that may develop into cancer.

**Promotion**- The altered cells undergo more changes that may require a *promoter*. A promoter is the one that speeds up the pace of cell division and creates more genetic mutations. A promoter can be a hormone like estrogen or any toxic substance like a chemical in tobacco smoke.
**Progression** - The cells that begin to grow out of control form the basis for all cancers. These out of control cells form tumor. The progression of cancer depends upon the body conditions such as genetic factors and hormones.

Metastasis is the process by which cancer cells spread to other parts of the body and the cancer is called the metastatic cancer [6][7]. Metastatic cancer will have the same name and the same type of cancer cells as the original. Usually cancer is treated by chemotherapy, radiation therapy and surgery but there are also other cancer therapies like immunotherapy, targeted therapy, hyperthermia, stem cell transplant and use of lasers.

**1.2 LUNG CANCER:**

The leading cause of cancer deaths in the United States is lung cancer, and the most common type of cancer diagnosed in men [8]. The primary function of the lungs is to exchange gases. Carbon dioxide is removed from the bloodstream and oxygen enters the bloodstream from the air we breathe. This is performed by the lung. The right lung has three lobes, whereas the left lung has two lobes and a small structure called the lingula. It corresponds to the middle lobe of the right lung. Bronchi are the major airways that enter the lungs and arise from the trachea. The bronchi branch into bronchioles which are smaller airways. Bronchioles end in tiny sacs known as alveoli where the gas exchange takes place [9].

Most cancers that start in lung are carcinomas that are derived from epithelial cells. Lung cancer spreads or metastasizes in very early stages after it forms [9]. It is a very life-threatening cancer and one of the most difficult cancers to treat because it tends to spread in this way even before it can be detected on an imaging test such as a chest x-ray [10]. The most common sites for metastasis of lung cancer are the adrenal glands, brain, bones and liver. But lung cancer can also spread to other parts of the body [9].
1.2.1 Causes of Lung Cancer:

a) Smoking

The most common cause of lung cancer is long-term exposure to tobacco smoke, which causes 80–90% of lung cancers\[^{[11]}\]. Nonsmokers account for 10–15% of the cases\[^{[12]}\], and these cases are often ascribed to a combination of genetic factors, air pollution, radon gas, asbestos and second-hand smoke\[^{[13]}\]–\[^{[16]}\]. Tobacco smoke contains more than 4,000 chemical compounds which are cancer-causing or carcinogenic. Nitrosamines and polycyclic aromatic hydrocarbons are two primary carcinogens present in tobacco smoke. Following smoking cessation new normal cells grow and replace the damaged cells in the lung. This decreases the risk of developing lung cancer each year. In former smokers, the risk of developing lung cancer begins to approach that of a nonsmoker about 15 years after cessation of smoking\[^{[9]}\].

b) Passive smoking

Passive smoking is another risk factor for lung cancer development. Research has shown that there is a 24% increase in risk for developing lung cancer for nonsmokers who reside with a smoker when compared with nonsmokers who do not reside with a smoker\[^{[9]}\]. The risk appears to increase with increase in degree of exposure. 3,000 lung cancer deaths that occur every year in U.S. are attributed to passive smoking\[^{[9]}\].

c) Asbestos Fibers

Mesothelioma is a cancer of the pleura of the lung. It is also cancer of the lining of the abdominal cavity called the peritoneum. Both lung cancer and Mesothelioma have exposure to asbestos as an established risk factor. Asbestos-related lung cancer in workers exposed to
asbestos is drastically increased by cigarette smoking. A fivefold greater risk of developing lung cancer is associated with asbestos workers who do not smoke than nonsmokers, but asbestos workers who smoke have a risk that is fifty- to ninety-fold greater than nonsmokers.

d) **Radon gas**

Radon gas is a known cause of lung cancer. An estimated 12% of lung-cancer deaths are attributed to radon gas or about 20,000 lung-cancer-related deaths annually in the U.S. This makes radon gas the second leading cause of lung cancer in the U.S. Radon gas is a natural radioactive gas formed as a natural decay product of Uranium. As with asbestos exposure, radon exposure coordinated with smoking greatly increases the risk of lung cancer.

e) **Familial Predisposition**

Majority of lung cancers are associated with tobacco smoking, but not all smokers eventually develop lung cancer. This suggests that other factors may play a role in the causation of lung cancer such as individual genetic susceptibility. Numerous studies showed that lung cancer is more likely to occur in both smoking and non-smoking relatives of those who have had lung cancer compared to the general population. Recent research has localized a region on the long (q) arm of the human chromosome number 6 that is likely to contain a gene that confers an increased susceptibility to the development of lung cancer in smokers.

f) **Air pollution**

Air pollution is another important cause of lung cancer. Pollution from vehicles, industry, and power plants, can increase the likelihood of developing lung cancer in individuals exposed to it. Up to 1% of lung cancer deaths are attributable to breathing polluted air, and experts believe
that prolonged exposure to highly polluted air can carry a risk similar to that of passive smoking for the development of lung cancer.

**g) Prior history of lung cancer**

Previous survivors of lung cancer have a greater risk compared to the general population of developing a second lung cancer. An additional risk for of 1-2% per year exists to develop a second lung cancer in survivors of non-small cell lung cancers (NSCLCs). 6% per year is the risk for development of second cancers in survivors of small cell lung cancers (SCLCs).

**1.2.2 Symptoms:**

According to American Cancer Society and Cancer Research UK, symptoms are not seen in most of the lung cancers until they have spread to other parts of the body. Some people show symptoms in early stages. Some common symptoms of lung cancer are cough, chest pain, hoarseness, loss of weight and appetite, coughing up blood or rust-colored sputum (spit or phlegm), shortness of breath, feeling tired or weak, infections such as bronchitis and pneumonia that don’t go away or keep coming back, new onset of wheezing.

If it spreads to distant organs, the most likely symptoms are bone pain, headache, weakness or numbness of arms and legs, dizziness, balance problems, or seizures caused by cancer spread to the brain or spinal cord, yellowing of the skin and eyes caused by cancer spread to the liver. Hoarse voice, difficulty in swallowing, finger clubbing, swelling of the face caused by a blockage of a main blood vessel, swelling in the neck caused by enlarged lymph nodes, pain or discomfort under your ribs on your right side are some less common symptoms of lung cancer.
1.2.3 Lung Cancer Staging:

Staging is essential in the proper selection of treatment for individual patients. The American Joint Committee for Cancer Staging has recommended staging of lung cancer which is more precise definition of the extent of the disease. This classification covers the size and location of the tumor, the extent of the metastases to the lymph nodes, and the presence and extent of distant metastases. TO to T4 classification defines the size and location of primary tumors, NO to N2 classification defines the extent of nodal metastases and whether distant metastases are present. To evaluate the results of therapy and for defining appropriate therapy for the individual patient there is another classification.

T system shows about primary tumors:

<table>
<thead>
<tr>
<th>T0</th>
<th>No evidence of primary tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>TX</td>
<td>Tumor proven by presence of malignant cells in bronchopulmonary secretions but not visualized roentgenographically or bronchoscopically</td>
</tr>
<tr>
<td>T1</td>
<td>Tumor that is 3.0 cm or less in greatest diameter, surrounded by lung or visceral pleura, and without evidence of invasion proximal to lobar bronchus at bronchoscopic</td>
</tr>
<tr>
<td>T2</td>
<td>examination</td>
</tr>
<tr>
<td>----</td>
<td>-------------</td>
</tr>
<tr>
<td></td>
<td>Tumor more than 3.0 cm in diameter or tumor of any size which, with its associated atelectasis or obstructive pneumonitis, extends to hilar region. (at bronchoscopic examination, proximal extent of demonstrable tumor must be at least 2.0 cm distal to carina; any associated atelectasis or obstructive pneumonitis must involve less than an entire lung, and there must be no pleural effusion)</td>
</tr>
<tr>
<td></td>
<td>Tumor of any size with direct extension into adjacent structure (such as chest wall, diaphragm, or mediastinum and its contents) or demonstrable bronchoscopically to be less than 2.0 cm distal to carina, or any tumor associated with atelectasis or obstructive pneumonitis of entire lung or pleural effusion</td>
</tr>
</tbody>
</table>

**Table 1**: T system of Lung Cancer Staging: Peters, R. M. "Staging of lung cancer." *CHEST Journal* 71.5 (1977): 633-634
N system shows about regional lymph nodes:

<table>
<thead>
<tr>
<th>N</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO</td>
<td>Demonstrable metastasis to regional lymph nodes</td>
</tr>
<tr>
<td>N1</td>
<td>Metastasis to lymph nodes in ipsilateral hilar region (including direct extension)</td>
</tr>
<tr>
<td>N2</td>
<td>Metastasis to lymph nodes in mediastinum</td>
</tr>
</tbody>
</table>

**Table 2:** N system of Lung Cancer Staging: Peters, R. M. "Staging of lung cancer." *CHEST Journal* 71.5 (1977): 633-634

M system shows distant metastases:

<table>
<thead>
<tr>
<th>M</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>M0</td>
<td>No distant metastasis</td>
</tr>
<tr>
<td>M1</td>
<td>Distant metastasis, such as In scalene, cervical, or contralateral hilar lymph nodes, brain, bones, lung, liver, etc.</td>
</tr>
</tbody>
</table>

**Table 3:** M system of Lung Cancer Staging: Peters, R. M. "Staging of lung cancer." *CHEST Journal* 71.5 (1977): 633-634

Stage grouping TNM subsets:

<table>
<thead>
<tr>
<th>Stage</th>
<th>TNM subset</th>
</tr>
</thead>
<tbody>
<tr>
<td>Occult carcinoma</td>
<td>Occult carcinoma with bronchopulmonary</td>
</tr>
<tr>
<td>Invasive carcinoma, stage 1</td>
<td>secretions containing malignant cells but without other evidence of primary tumor or evidence of metastasis to regional lymph nodes or distant metastasis</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>-----------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>T1 NO MO</td>
<td>Tumor that can be classified T1 without any metastasis or with metastasis to lymph nodes in ipsilateral hilar region only, or tumor that can be classified T2 without any metastasis to nodes or distant metastasis (note that TX N1 MO and TO N1 MO are also theoretically possible, but such a clinical diagnosis would be difficult, if not impossible, to make; if such a diagnosis is made, it should be included in stage 1)</td>
</tr>
<tr>
<td>T1 N1 MO</td>
<td></td>
</tr>
<tr>
<td>T2 NO MO</td>
<td></td>
</tr>
<tr>
<td>Stage 2</td>
<td></td>
</tr>
<tr>
<td>T2 N1 MO</td>
<td>Tumor classified as T2 with metastasis to lymph nodes in ipsilateral hilar region only</td>
</tr>
<tr>
<td>Stage 3</td>
<td></td>
</tr>
<tr>
<td>T3 with any N or M</td>
<td>Any tumor more extensive than N2 with any T or M T2 or any tumor with metastasis M1 with any T or N to lymph nodes in mediastinum or with distant metastasis</td>
</tr>
<tr>
<td>N2 with any T or M</td>
<td></td>
</tr>
<tr>
<td>M1 with any T or N</td>
<td></td>
</tr>
</tbody>
</table>
### 1.2.4 Types of Lung Cancer:

The main types of lung cancer are small-cell lung carcinoma (SCLC) which is also called oat cell cancer that accounts for about 15% of all lung cancer cases. They are the most aggressive and rapidly growing. They metastasize to many sites in the body and are located after they have spread. Non-small-cell lung carcinoma (NSCLC) that accounts for about 80% of all lung cancer cases, with less than 15% of patients surviving beyond 5 years\(^{[17][18]}\). The major types of NSCLC are adenocarcinoma, squamous cell carcinoma and large cell carcinoma, but there are also several other types which occur less frequently \(^{[19]}\). Adenocarcinoma is a cancer of epithelial tissue which has glandular origin, glandular characteristics, or both. It is the most common form of lung cancer in people \(^{[20]}\). Nearly 40% of all lung cancers in the US are adenocarcinoma that originates in peripheral lung tissue \(^{[21]}\). Inhalation of cigarette smoke results in peripheral lesions which are mostly the case in adenocarcinomas of the lung. Generally, they grow more slowly and form smaller masses than the other subtypes \(^{[22]}\). However, they tend to form metastases widely at an early stage. A549 and H1299 are two cell lines of adenocarcinoma that have been used in this study.

**A549 Cell line:**

The A549 cell line was first developed in 1972 by D.J. Giard, et al. through the removal and culturing of cancerous lung tissue in the explanted tumor of 58-year-old caucasian male \(^{[23]}\). This type of cell, in a normal lung, is squamous and performs the task of diffusing water,
electrolytes and other substances across the surface of the alveoli. They grow adherently, as a monolayer, in vivo [24].

**H1299 Cell line:**

H1299 was initially established from the lung cells of a 43-year-old Caucasian male patient who suffered from non-small cell lung cancer [25]. These cells were derived from the lymph node metastatic site, and exhibit epithelial cell morphology [26]. They lack expression of the p53 protein [27].

**1.2.5 Diagnosis and Treatment:**

Physical examinations and symptoms may show cancer development. Difficulty in breathing, obstruction in airway, or lung infections may also be counted as signs. There are some signs by which chronic lung disease can be indicated. They are cyanosis which is indicated by bluish color of the skin and the mucous membranes caused due to insufficient oxygen in the blood and also clubbing which is associated with changes in the tissue of the nail beds [9]. There are some common techniques that are followed to diagnose lung cancer.

Chest X-ray is the first diagnostic step and the most common way to detect lung cancer. It involves back to front view of the chest also a view from the side. Chest X-ray reveals suspicious areas in the lungs that are abnormal but cannot determine if the areas are cancerous. Particularly, benign tumors or calcified nodules in the lungs called as hamartomas may be identified on a chest X-ray which mimics lung cancer.

CT (computerized tomography) scan is performed to evaluate both metastatic and non-metastatic lung cancer. It is performed on the chest, abdomen, and/or brain. A CT scan is performed particularly of chest, when an X-ray does not show sufficient information about the
location or extent of cancer. CT scans are same as X-ray procedures that combine multiple images with the help of a computer and generate cross-sectional views of the body. In a CT scan the patient is exposed to a minimal amount of radiation. The most common side effect of CT scan may result in itching as a result of an adverse reaction to intravenous contrast material that may have been given prior to the procedure. But that disappears quickly. Severe reactions are rare. CT scans in different parts of the body reveal the locations of metastasis like a scan of abdomen identifies metastasis in the liver or adrenal glands. A CT scan of the head reveals the presence and extent of metastatic cancer in the brain.

Normally, to increase resolution helical CT scan (spiral CT scan) is performed which involves movement in a spiral pattern. This is recommended in low-dose for smokers of ages between 55 and 80 who have at least a 30 year history of smoking. This increases the chances of detecting smaller lung cancers which are more curable.

Magnetic resonance imaging (MRI) scans use magnetism, radio waves, and a computer to produce images. These are used when precise detail about a tumor location is needed. The procedure is similar to CT scanning where the patient is placed on a moveable bed which is inserted into the MRI scanner. There is no chance of exposure to radiation in MRI scanning and no known side effects. The image and resolution built by MRI is in such detail that it can detect tiny changes in structures within the body. MRI cannot be used for patients with medical devices like pace maker because of the risk of the magnet moving the metal parts of these structures.

Positron emission tomography (PET) scanning is a specialized imaging technique which produces three-dimensional colored images of the substances in the tissues within the body. This technique uses radioactive drugs. While CT scans and MRI scans look at anatomical structures, PET scans measure metabolic activity and the function of tissues. PET scans determine if a
tumor tissue is growing actively and aids in ascertaining the type of cells within a particular tumor. In PET scanning, the patient receives a short half-lived radioactive drug, receiving approximately twice amount of radiation exposure as chest X-rays. The drug accumulates in certain tissues more than in others depending on the drug. The drug releases particles known as positrons from tissues that take them up. As the positrons encounter electrons in the body, a reaction takes place releasing gamma rays. A scanner records these gamma rays and maps the area where the radioactive drug has accumulated.

Therapies for lung cancer can be curative (removal or eradication of a cancer) or palliative (measures that are unable to cure a cancer but can reduce pain and suffering). Sometimes more than one type of therapy is prescribed. In such cases, the second therapy is referred to as adjuvant therapy. Chemotherapy or radiotherapy is an example of adjuvant therapy, after surgical removal.

Surgery for removal of the tumor is generally performed at earlier stages (stage I or sometimes stage II) of cancer. It is the treatment of choice for cancer that has not spread beyond the lung. About 10%-35% of lung cancers can be removed surgically, but removal does not always result in a cure, since the tumors may already have spread and can recur at a later time. Surgery is less often performed with SCLC than with NSCLC because these tumors are less likely to be localized to one area that can be removed. The procedure chosen depends on the size and location of the tumor. Surgeons must open the chest wall and may perform a wedge resection of the lung (removal of a portion of one lobe), a lobectomy (removal of one lobe), or a pneumonectomy (removal of an entire lung), lymphadenectomy (removal of lymphnodes). After the surgical procedure, patients may experience difficulty in breathing, pain and weakness. The
risks of surgery include complications due to bleeding, infection, and complications of general anesthesia.

Radiation therapy (RT) is an essential part of lung cancer management [28]. In the past decade, significant improvements have been made in radiation treatment, available to advances in biology, physics and clinical research [23]. Radiation therapy can be used for both NSCLC and SCLC. It uses high-energy X-rays or other types of radiation to kill dividing cancer cells. It can be given as curative therapy, palliative therapy (using lower doses of radiation than with curative therapy), or as adjuvant therapy in combination with surgery or chemotherapy. It can be either external by using a machine that delivers radiation, or internal through radioactive substances in sealed containers administered into the area where the tumor is localized. Radiation therapy is used if surgical removal is impossible due to the spreading of tumor to areas such as the lymph nodes or trachea. Radiation therapy generally shrinks a tumor or limits its growth when given as a sole therapy, yet in 10%-15% of people it leads to long-term remission and palliation of the cancer. Combining radiation therapy with chemotherapy can further prolong survival when chemotherapy is administered. Radiation therapy does not carry the risks of major surgery, but side effects include fatigue and lack of energy. A reduced white blood cell count and low blood platelet levels (leading to excessive bleeding) also can occur with radiation therapy. If the digestive organs are exposed to radiation, patients may experience nausea, vomiting, or diarrhea. Radiation therapy can irritate the skin in the area that is treated, but this irritation generally improves with time after treatment has ended.

Chemotherapy is also given to both NSCLC and SCLC. Chemotherapy is the process of administrating drugs that stop the growth of cancer cells by killing them or preventing them from dividing. Chemotherapy can be given alone or as an adjuvant to surgical therapy, or in
A class of drugs known as the platinum-based drugs has been the most effective in treatment of lung cancers through chemotherapy.

Chemotherapy is the treatment of choice for most SCLC, since these tumors are generally widespread in the body when they are diagnosed. Chemotherapy alone is not particularly effective in treating NSCLC, but when NSCLC has metastasized it can prolong survival in many cases. Chemotherapy may be given as pills, as an intravenous infusion, or as a combination of the two. A combination of drugs is given in a series of treatments, called cycles, over a period of weeks to months, with breaks in between cycles. Unfortunately, the drugs used in chemotherapy also kill normally dividing cells in the body, resulting in unpleasant side effects. Damage to blood cells can result in increased susceptibility to infections and difficulties with blood clotting (bleeding or bruising easily). Other side effects include fatigue, weight loss, hair loss, nausea, vomiting, diarrhea, and mouth sores. The side effects of chemotherapy vary according to the dosage and combination of drugs used and may also vary from individual to individual. Medications have been developed that can treat or prevent many of the side effects of chemotherapy. The side effects generally disappear during the recovery phase of the treatment or after its completion.

Molecular targeted therapy involves the administration of drugs which target tumors with specific genetic changes. For example, the drugs erlotinib (Tarceva) and gefitinib (Iressa) are so-called targeted drugs, which may be used in certain patients with NSCLC who are no longer responding to chemotherapy. Targeted therapy drugs more specifically target cancer cells, resulting in less damage to normal cells than general chemotherapeutic agents. Erlotinib and gefitinib target a protein called the epidermal growth factor receptor (EGFR) that is important in
promoting the division of cells. This protein is found at abnormally high levels on the surface of some types of cancer cells, including many cases of non-small cell lung cancer.

Other examples of targeted therapy include antiangiogenesis drugs that block the formation of new blood vessels within the cancer which leads to deficiency in oxygen-carrying blood and the cancer cells die. Cetuximab is an antibody that binds to the epidermal growth factor receptor (EGFR). In patients with NSCLC whose tumors have been shown to express the EGFR by immunohistochemical analysis, the addition of cetuximab may be considered for some patients. Other targeted therapies include ALK tyrosine kinase inhibitor drugs (for example, crizotinib, ceritinib) that are used in patients whose tumors have an abnormality of the ALK gene.

Despite these improvements in the therapeutic regimes, local recurrence of lung cancer remains a relentless problem [24]. Thus, it is necessary to develop more effective approaches for the treatment of NSCLC.

1.3 Nutrition and Cancer:

Research in 1990’s has recognized that certain nutrients have potent biological effects in addition to their nutritional value [29]. Diet has a major influence in cancer prevention. The World Cancer Research Fund and the American Institute for cancer research have examined the effect of nutrition on cancer incidence in a large study and on a global scale. The study summarized that approximately 30% of the cancer cases could be reduced by changing the dietary habits. More consumption of fruits and vegetables and less meat and dairy products can prevent cancer [30]. Over the recent years, many experiments showed that nutrients act as or on transcriptional factors, that modify gene expression.
Phytochemicals are non-nutritive components in plant based diet, which show anticarcinogenic and antimutagenic properties \[^{31}\]. Some examples of the active components of dietary phytochemicals, which are protective against cancer are curcumin (turmeric), resveratrol (red grapes), lycopene (tomato), catechins (green tea), vitamin C (citrus fruits), beta carotene (carrot), etc. Studies have demonstrated that these dietary agents suppress the inflammatory processes which lead to transformation, hyperproliferation and initiation of carcinogenesis \[^{30}\].

Vitamin E plays a key role in the prevention and inhibition of cancer \[^{31}\][\[^{32}\]. This has been proved by investigating on many human cancers such as colon \[^{33}\], lung \[^{34}\], prostate \[^{35}\] and breast \[^{36}\] cancers. Vitamin E is present in most edible oils to various extents, including those extracted from wheat germ oil, wheat, rice bran (0.035%), barley (0.012% or 44 mg/g oil), oats (0.03%), coconut (0.019%) and palm (0.044%; 0.78–1.08 mg/g oil) \[^{37}\]. The effective role of vitamin E has been also demonstrated against chemotherapy resistant cancer cells. Vitamin E has presented efficiency against specific subhistology types of lung cancer. It is a lipophilic antioxidant that consists of tocopherol (α-, β-, γ-, δ-) and tocotrienol (α-, β-, γ-, δ-) isomers \[^{30}\].

### 1.3.1 Tocopherols and Tocotrienol:

Tocopherols and tocotrienols are amphipathic molecules that are members of the vitamin E family \[^{37}\]. Tocotrienols and tocopherols are collectively known as tocochromanols. They make up the essential nutrient, vitamin E. They are fat soluble nutrients that function as antioxidants in the human body. There are four types of tocotrienols and tocopherols- α, β, γ and δ. All these four types differ in the number and position of the methyl groups in the polar bond \[^{37}\].
The tocopherols are saturated forms of vitamin E, whereas the tocotrienols are unsaturated and possess an isoprenoid side chain. Some evidence suggests that human tissues can convert tocotrienols to tocopherols. Numerous in vitro studies show that tocotrienols and tocopherols manifest anticancer, cardioprotective, and neuroprotective effects. Tocotrienols...
suppress proliferation and induce apoptosis in wide variety of tumor cells including lung,
breast, colon, liver, stomach, skin, pancreas, and prostate. It has been shown that
they can induce apoptosis by inhibiting multiple signaling pathways.

1.3.2 Tocotrienols and Lung Cancer:

Various studies specify that tocotrienols exhibit antioxidant, antiproliferative,
antisurvival, proapoptotic, antiangiogenic, and anti-inflammatory activities. The antiproliferative
activity of tocotrienols is arbitrated through modulation of growth factors such as vascular
endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and
transforming growth factor-beta (TGF-b), HER2/neu, and interleukin-6 (IL-6).
Tocotrienols hinder the survival of various tumor cells by inhibiting the expression of cell
survival proteins like XIAP, IAP-1, IAP-2, bcl-2, bcl-xl, c-FLIP, TRAF-1, survivin and Bfl-
I/A1.

Various studies have shown that tocotrienols can induce apoptosis in a wide variety of
tumor cells, through activation of both extrinsic and intrinsic pathways. The extrinsic pathways
involve induction of death receptors and activation of caspase-8, which leads to caspase-3
activation. The intrinsic pathways involve mitochondrial depolarization, upregulation of
Bax, cleavage of Bid, release of cytochrome C, and activation of caspase-9, which
again leads to activation of caspase-3.

The suppression of angiogenesis by tocotrienols is arbitrated through inhibition of VEGF
expression and VEGF receptor signaling. Suppression of the matrix metalloproteinase
(MMP)-9 gene could also lead to the angiogenesis-suppressive activity.

The results of various experiments indicate that gamma and delta tocotrienol exhibit
greater anticancer activity than alpha or beta tocotrienol. The first detail on the therapeutic
potential of tocotrienols for cancer in animal models was by Kato et al., who showed that tumor-bearing rats administered with tocotrienols had an extended life span \[61\]. The antitumor effects of tocotrienols appear through their ability to suppress angiogenesis \[62\]. Many studies suggest that tocotrienols have potential to both prevent and treat cancer \[63\].

**Figure 3:** Molecular targets of tocotrienols: B.B. Aggarwal et al. / Biochemical Pharmacology 80 (2010) 1613–1631

### 1.4 MMP9 and uPA:

Matrix metallopeptidase 9 (MMP-9), is a matrixin, a class of enzymes that belong to the zinc-metalloproteinases family \[64\]. Matrix metalloproteinase-9 (MMP-9) has been shown to be involved in degradation of the extracellular matrix and promotion of tumor growth and metastasis by its angiogenic properties \[65\]. MMP-9 expression increases with tumor size and its expression is significantly higher in NSCLC cases with metastasis compared to those without
metastasis \cite{66}. In addition, MMP-9 expression is significantly higher in lymph node metastasis than primary lesions. MMP-9 has a metastasis-promoting role \cite{67}. Many studies have shown that MMP family contributes to tumor invasion, metastasis, and angiogenesis \cite{68}. MMP-9 (gelatinase-B) is responsible for cell migration, aggregation, adhesion and invasion in cancer \cite{69}\cite{70}. In order to be capable of this it degrades type IV collagen, the main component of extracellular matrix \cite{71}. The urokinase-type plasminogen activator (uPA) pathway and Notch-1 pathways are major pathways that affect the MMP-9 activities in cancer. uPA is a serine protease which converts inactive plasminogen to active plasmin. The active plasmin is responsible for the activation of MMP-9 which degrades ECM allowing the cell invasion. uPA also cleaves components of the ECM. It is also found to be involved in cell adhesion and migration. uPA receptor (uPAR) is also involved in the pathway responsible for the degradation of extracellular matrix \cite{72}. The uPAR is also entailed in the regulation of cell adhesion and migration \cite{73}. Together, MMP-9 and uPA play an important role in tumor invasion and metastasis.

![Pathway for activation of MMP-9 and degradation of ECM](image)

**Fig 4:** Pathway for activation of MMP-9 and degradation of ECM
**Hypothesis:** Delta tocotrienol shows anti-metastatic properties in MMP-9 mediated mechanisms in lung cancer.

**Specific aim I:** To investigate anti-metastatic properties of delta-tocotrienol on MMP-9 on *invitro* models of NSCLC.

**Specific aim II:** To investigate the underlying mechanism of the metastatic properties of MMP-9 and uPA in NSCLC.
Chapter 2

METHODOLOGY

2.1 Cell Culture and Reagents:

NSCLC cell lines of human A549, H1299 were obtained from ATCC. They were grown in RPMI medium (Mediatech, Manassas, VA) which was supplemented with 10% fetal bovine serum and 1% penicillin streptomycin. 5% CO\textsubscript{2} at 37°C was supplied as growth parameters. Pure δ-tocotrienol was a kind gift from American River Nutrition (DeltaGold® MA, USA). We have treated the cell lines with different concentrations of pure δ-tocotrienol.

2.2 Treatment Media and Basic Protocol:

The treatment media is made by mixing δ-tocotrienol in RPMI medium whereas the control was treated with RPMI media alone. The different concentrations of δ-T used in this study are 10μM, 20μM and 30μM. The percentage of the RPMI media and δ-T depends on the no. of plates to be treated. The basic protocol followed is the confluent cells are first divided into petri dishes and allowed to grow for 24hrs. The optimal growth conditions in the incubator are 5% CO\textsubscript{2} and 37°C. The cells are now treated with desired concentration of δ-T of 10μM, 20μM and 30μM. One of the plates is maintained as control treated only with media. The cells are incubated for 72 hrs at the optimal growth conditions. The cell lysate and the media if required are extracted and stored for further experiments.
2.3 Cell Aggregation Assay:

A single cell suspension was obtained using standard trypsinization procedure. A total of $2 \times 10^5$ cells in 1 ml of RPMI medium with or without different concentrations of $\delta$-T (as the positive control) were placed in polystyrene micro tubes and were gently shaken for every 5 min for 1 hr at $37^\circ$C. At the end glutaraldehyde (at a final concentration 2% (v/v)) was added to stop the aggregation process. Homotypic aggregation was evaluated by Luna automated cell counter (Logos Biosystems, USA). Aggregates were counted per square cm. Significant differences between control and treatments were evaluated using ezANOVA.
2.4 Cell Adhesion Assay:

The cell lines were pre-treated with or without different concentrations of δ-T (control, 10μM, 20μM and 30μM) for 72 hrs. The cell lines were suspended in RPMI medium to form a single-cell suspension and 2 x 10^6 cells/ml (2 x 10^5 cells/well) were seeded into 96-well microtiter cell culture plate pre-coated with Matrigel®. After a 45 min of incubation at 37°C, the wells were washed three times with PBS to remove non-adherent cells and 10μM MTS was added into each well for an additional 2h. As a measure of cell viability, the absorbance at 570 nm was taken on ELx800 plate reader (Bio-Tek, Winooski, VT). Significant differences between control and treatments were evaluated using ezANOVA.

2.5 Cell Invasive Assay:

To test the invasive ability of cells BD Biocoat invasion kit (BD, San Jose, CA) was used. Around 2.5 x 10^5 cells of A549 and H1299 with basal media were seeded in each well of upper chamber. This contains both untreated and treated with δ-tocotrienol (control, 10μM, 20μM and 30μM). 3 ml of culture medium with 10% FBS was added in the mean time to the wells in the lower chamber of the six-well plate. The incubation time allowed was 24h. After incubation the cells on the upper chamber were removed with a cotton stick. The cells from the lower chamber were fixed in 4% Paraformaldehyde and stained with 2% crystal violet and the cells unbound to crystal violet were washed with PBS, before they dried. After that the stained crystal violet (cell bound) was washed with 20 % acetic acids. The results were evaluated microscopically. Also the absorbance of the dissolved crystal violet was measured at 405nm with ELx800 plate reader (Bio-Tek, Winooski, VT). The experimental condition was performed in Triplicates.
2.6 Wound Healing Assay:

A million cells of NSCLC cell lines A549 and H1299 were seeded per well in a six well plate. The cells were incubated overnight and the culture media was removed. A scratch wound was made across each well using pipette tips. The wound area was washed by PBS for three times to remove any loosely held cells. Subsequently, the cells were cultured with or without treatment by δ-tocotrienol and the wound images were taken at 0 h and 20 h as pictures under microscope. Two diagonal parallel lines in each image mark the progress of cells that migrated into the wound.

2.7 Zymogram Gel Assay:

Zymogram gel assay was performed to detect the activity of MMP-9. A549 and H1299 cells were seeded in a six-well plate with or without treatment by δ-tocotrienol. The media of all groups was removed and concentrated by 3KDa Amicon centrifugal filters (EMD Millipore, USA). Zymogram precast gel (Bio-Rad, Hercules, CA) with gelatin was used depending upon the type of the assay. One part sample was mixed with two parts of Zymogram Sample Buffer. Samples were loaded in the gel and the gel was run with 1x Tris-Glycine SDS Running Buffer according to the standard running conditions (100V, constant voltage). Run time was for 90 min or when the bromophenol blue tracking dye reached the bottom of the gel. After running, the Zymogram Renaturing Buffer (10x) was diluted (1:9) with double distilled water and the gel was incubated in the buffer (100 ml for one or two mini-gels) with gentle agitation for 30 minutes at room temperature. Later Zymogram Renaturing Buffer was decanted and replaced with 1x Zymogram Developing Buffer (100 ml for one or two mini-gels). The gel was equilibrated for 30 minutes at room temperature with gentle agitation and then the buffer was replaced with fresh 1x
Zymogram Developing Buffer and again incubated at 37°C overnight for maximum sensitivity. The gel was stained with Coomassie Blue R-250 for 30 minutes to 1 hour the other day. For maximum contrast, stain concentration of 0.5% (w/v in 40% methanol and 10% acetic acid) was used instead of the usual concentration of 0.1%. Gels were destained with an appropriate Coomassie R-250 destaining solution (161-0438) (Methanol: Acetic acid: Water (40: 10: 50). Areas of protease activity appeared as clear bands against a dark blue background where the protease has digested the substrate when measured by chemiluminescent image with chemiDoc XRS (Bio-Rad Laboratories, CA).

2.8 Real-Time Quantitative PCR for Gene Expression Analysis:

One million A549 and H1299 cells were seeded in 100mm dish per plate and incubated for 24 hours. Subsequently, culturing media was replaced with treatment (10μM, 20μM and 30μM) or control medium, incubated for another 72 h. Then, Total RNA was isolated using RNeasy Mini Kit from QIAGEN (Valencia, CA) according to the manufacturer’s protocols. 1000 ng of total RNA from each sample was subjected to first strand cDNA synthesis using High capacity RNA to cDNA master mix (Applied Biosystems, Foster City, CA) in a total volume of 50 ml. qRT-PCR was performed as part of gene expression analysis. Diluted cDNA (2 uL) and 2 uL of each reverse and forward primer and 12.5 uL master mix (SYBR GREEN PCR Master Mix; Applied Biosystems, Warrington, UK) were used in each 25 uL PCR reaction performed in Eppendorf mastercycler realexplex 4 (Eppendorf, Hauppauge, NY) at 25C for 10 min, followed by 48°C for 30 min and 95°C for 5 min. Expression values were normalized with β-actin. Each gene expression was tested in triplicate.
MMP-9  5’-CGG AGT GAG TTG AAC CAG-3’  5’-GTC CCA GTG GGG ATT TAC-3’

2.9 Western Blot for Protein Expression Analysis:

Western blot analysis was performed as part of protein expression analysis with following antibodies β-actin, MMP-9, HES-1 (Cell Signaling Technology, Danvers, MA), uPA (Santa Cruz Biotechnology, USA) and Notch 1 (Life Technologies, USA) in cell signaling pathways. One million A549 and H1299 cells were seeded in 100mm dish per plate and incubated for 24hours, and then they were treated for 72 hours with treatment and control medium, incubated for 72 h. subsequently cells. Cells were lysed in the cold 1X cell lysis buffer (Cell Signaling Technology Danvers, MA) for 30 min on ice with 1X Protease inhibitor (Cell Signaling Technology Danvers, MA). Then Protein concentrations were calculated by using Pierce BSA Protein Assay kit (Bio-Rad Laboratories, CA). Subsequently 50 mg of total cell lysates was mixed with equal amount of 4X lemma buffer (Bio-Rad Laboratories, CA) and 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 2 h at room temperature with 5% nonfat dried milk in 1x TBS buffer containing 0.1% Tween 20(Fisher Scientific, USA). After that, membranes were incubated over night at 4C with primary antibodies (1:1,000). The membranes were washed three times with TBS-T, and subsequently incubated with the secondary antibodies (1:5,000) containing 2% BSA for 2 h at room temperature. The signal intensity was then measured by chemiluminescent image with chemiDoc XRS (Bio-Rad Laboratories, CA).
2.10 Data Analysis:

Statistically significant differences if any between groups were analyzed using ezAnova. Values <0.05 were considered statistically significant.
3.1 Cell Aggregation Assay:

In order to test the effects of delta-tocotrienol on cell aggregation, A549 and H1299 cells were treated with concentrations 10µM, 20µM, 30µM of δ-tocotrienol and control. The cell lines were evaluated microscopically. As shown in the figures (6, 7 and 8) δ-tocotrienol inhibits cell aggregation in a dose dependent manner in both A549 and H1299 cells, respectively.

![Microscopical evaluation (10X) of cell aggregation assay in A549 cell line. The results show inhibition of cell aggregation by δ-tocotrienol.](image)

**Fig 6: Microscopical evaluation (10X) of cell aggregation assay in A549 cell line. The results show inhibition of cell aggregation by δ-tocotrienol.**
Fig 7: Microscopical evaluation (10X) of cell aggregation assay in H1299 cell lines. The results show inhibition of cell aggregation by δ-tocotrienol.
Fig 8: Effect of δ-tocotrienol on cell aggregation of A549 and H1299: Dose dependent reduction in no of cell aggregates in cell lines A549 (A) and H1299 (B). Vertical bars indicate the mean of cell count. All values are significantly different (p < 0.05).
3.2 Adhesion Assay:

To test the effects of delta-tocotrienol on cell adhesion, A549 and H1299 cells were treated with increasing concentration of delta-tocotrienol for 72 h separately followed by adhesion assay. MTT reagent was added and the absorbance at 570/630 was taken. The figure (9) shows the absorbance measured the spectrometer. From the results it can be seen that δ-tocotrienol inhibits cell adhesion in a dose dependent manner in both A549 and H1299 cells.

![Fig 9: Effect of δ-tocotrienol on cell adhesion of A549 and H1299: Dose dependent reduction in cell adhesion in cell lines A549 (A) and H1299 (B). Vertical bars indicate the standard deviation. Absorption is recorded at 570/630 nm. All values are significantly different (p< 0.05).](image)

3.3 Cell Invasion Assay:

Cell invasion assay is used to test the ability of cancer cells to penetrate through extracellular matrix. MMP-9 is an important factor for cell migration and invasion. In order to examine the effect of δ-tocotrienol on the invasive capacity of A549 and H1299 cell lines, Matrigel invasion assay was conducted. Control was tested against treatment of 30μM concentrations. As shown in figures (10 and 11), delta-tocotrienol showed dose dependent decrease in the invasive capability of A549 and H1299 thus, confirming inhibition of MMP-9. Microscopical images and absorbance at 405nm are shown.
Fig 10: Microscopical evaluation (10X) of invasive ability in both cell lines A549 (A) and H1299 (B). Control is tested against treatment with 30μM of δ-tocotrienol.
Fig 11: Effect of δ-tocotrienol on cell invasion of A549 and H1299: A dose dependent decrease in invasive ability of A549 (A) and H1299 (B) cell lines with pure δ-tocotrienol. Absorption is shown against concentrations of treatment. Vertical bars indicate standard deviation. All values are significantly different (p<0.05).

3.4 Wound Healing Assay:

Wound healing assay was performed to determine tumor cell migration ability. A scratch was made with a pipette tip and the results were observed after 18h to evaluate the effect of δ-tocotrienol on the migration ability of A549 and H1299 cell lines. As shown in figures (12, 13), δ-tocotrienol showed dose dependent inhibition in cell migration in both cell lines, A549 and H1299. The images were captured at time point 0 and after 20 h.
**Fig 12:** Microscopical evaluation (10X) of cell migration in A549 cell line. δ-tocotrienol shows inhibition in cell migration in treatment 30μM.

**Fig 13:** Microscopical evaluation (10X) of cell migration in H1299 cell line. δ-tocotrienol shows inhibition in cell migration in treatment 30μM.
3.5 Zymogram Gel Assay:

Zymogram gel assay is used to detect the activity of metalloproteinases. They have the ability to utilize gelatin as substrate. Polyacrylamide gel-based electrophoresis was used to run the samples of control and treatments 10μM, 20μM and 30μM. TNF-α was used as a positive control. A dose dependent reduction in the activity of MMP-9 by δ-tocotrienol is seen as shown in the figure (14).

![Zymogram Gel Assay](image)

**Fig 14:** Effect of δ-tocotrienol on the effect of MMP-9: A dose dependent decrease in the activity of MMP-9 by δ-tocotrienol treatment compared to control TNFα as positive control.
3.6 Real-Time Quantitative PCR for Gene Expression Analysis:

To test the effect of δ-tocotrienol on MMP-9 at gene level, Real-Time PCR was performed to measure the m-RNA expression of MMP-9 in both A549 and H1299 cell lines. Control was tested against the treatments 10μM, 20μM and 30μM. A dose dependent reduction in the MMP-9 gene expression was observed as shown in figure (15).

![Figure 15](image.png)

**Fig 15:** Relative gene expression of MMP-9 in both A549 (A) and H1299 (B) cell lines. The results are dose dependent.
3.7 Western Blot for Protein Expression Analysis:

Western Blot analysis is performed to test the effect of δ-tocotrienol on the protein expression level of MMP-9. Notch-1, HES-1 and uPA are also tested with different concentrations of treatment to evaluate the protein expression level of MMP-9. A dose dependent decrease is observed in the protein expression as shown in figure (16).

![Western Blot Images](image)

**Fig 16:** Effect of δ-tocotrienol on A549 and H1299: Down regulation of protein expressions in MMP-9 pathway with δ-tocotrienol. The decrease in protein expressions is dose dependent
Chapter 4

DISCUSSION

The leading cause of cancer deaths in the United States is lung cancer, and the most common type of cancer diagnosed in men. Non small cell lung cancer (NSCLC) accounts for about 80% of the cases with less than 15% of patients surviving beyond 5 years. Despite many improvements in the therapeutic regimes, local recurrence of lung cancer remains to be a relentless problem. Thus, it is necessary to develop more effective approaches for the treatment of NSCLC.

It has been found out that nutrients act as or on transcriptional factors, that modify gene expression for treating lung cancer. Vitamin E plays a key role in the prevention and inhibition of cancer. This has been proved by investigating on many human cancers such as colon, lung, prostate and breast cancers. Vitamin E is made up of tocopherols and tocotrienols. They are amphipathic molecules that are collectively known as tocochromanols. They are fat soluble nutrients that function as antioxidants in the human body. There are four types of tocotrienols and tocopherols- α, β, γ and δ. All these four types differ in the number and position of the methyl groups in the polar bond. This study focuses on the effect of δ-tocotrienol on the expression of MMP-9 in NSCLC. A549 and H1299 are two cell lines of NSCLC adenocarcinoma in which the effects were observed.

MMP-9 is a class of enzymes that has been shown to be involved in degradation of the extracellular matrix and promotion of tumor growth and metastasis by its angiogenic properties. MMP-9 expression is up regulated in NSCLC cases especially those with metastasis. It is a gelatinase that destroys the basement membrane which is the first barrier to tumor metastasis. Hence elevated expression levels of MMP-9 plays an important role in tumor cell invasion and
metastasis. It is responsible for metastasis by increasing cell migration, aggregation, adhesion and invasion in cancer cells. Cancer metastasis is a complicated multi-step process. It includes migration of cells, aggregation of cells, cell-cell adhesions; cell-matrix interactions, invasion into surrounding tissues. Our previous studies have proved that δ-tocotrienol inhibits cell growth, proliferation and induce apoptosis by inhibiting NF-κB via down regulating Notch-1 in dose and time dependent manner\cite{74}\cite{75}. This study aims at investigating the effect of δ-tocotrienol on the down regulation of MMP-9 and determining the underlying molecular mechanism involved. Anti-metastatic functions of δ-tocotrienol are assessed on cell aggregation, adhesion, invasion, migration, enzyme activity, gene expression and protein expression.

Tumor cells tend to form aggregates. Aggregates exhibit lower levels of apoptosis than single cells. Cell aggregation facilitates metastasis in cancer and promotes higher rate of survival relative to existence as single cells. Another key step in metastasis is the attachment of cancer cells to extracellular matrix. Adhesive interactions between tumor cells and extracellular matrix components, and formation of aggregates play a critical role in cancer invasiveness or metastasis. It can be said that cell aggregation and cell adhesion are the basic or initial steps in cancer metastasis. We have assessed the inhibitory affects of δ-tocotrienol on cell aggregation and adhesion properties of MMP-9 in A549 and H1299 cell lines. Firstly, we observed a dose dependent decrease in cell aggregation in both cell lines for treatments 10µM, 20µM and 30µM when cells were plated as single cell suspension and treated with δ-tocotrienol. Equal amount of cells were used for each treatment. Microscopical examination was supported by the count of cell aggregates. All values were significantly different (p<0.05). Next, Matrigel®-mediated attachment by both cell lines was inhibited by δ-tocotrienol. Equal amount of cells were seeded in 96 well plate coated with Matrigel®, as single cell suspension. Absorbance values were
recorded for treatments 10µM, 20µM and 30µM after incubation. All values were significantly different (p<0.05). Inhibition in cell aggregation and cell adhesion by δ-tocotrienol suggests the inhibition of MMP-9.

Cell invasion is the ability of cells to navigate through the extracellular matrix within a tissue or infiltrate neighboring tissues by degrading the membrane barrier. The ability of the cancer cells to invade and metastasize to other parts of the body causes hurdles to therapeutic intervention. The over expression of MMP-9 in NSCLC promotes invasion. BD Biocoat matrigel invasion chamber was used to evaluate the effect of δ-tocotrienol on the invasive ability of MMP-9. The kit contains matrigel basement membrane matrix which serves as a reconstituted basement membrane in vitro. Upper chamber is seeded with equal amount of cells for control and desired concentration of treatments and the lower chamber is filled with media to support the invading cells. The matrigel layer clogs the pores of the membrane, blocking the non-invasive cells from migrating through the membrane. In contrast, cells with the capability to invade and detach themselves from the upper chamber pass through the matrigel matrix to reach the lower chamber. The incubation time allowed in the study is 24h. The non-invasive cells on the upper chamber are removed with a cotton swab.

A dose dependent decrease in the invasive ability was observed, when control was examined over treatment with 30µM of δ-tocotrienol microscopically and when the absorbance was plotted against control and treatments 10µM, 20µM, 30µM. Cell invasion assay shows inhibition in MMP-9 expression with δ-tocotrienol treatment.

Cell migration, the movement of cells from one area to another is achieved in wound healing, cell differentiation, embryonic development and metastasis of tumors. Cancer combativeness is affected to a great extent by its invasive capability for which cell migration is a
determinant manifestation. Wound healing assay is a convenient method to assess cell migration in vitro. This method basically comprises of creating a scratch on a monolayer cells and observe the changes after a time point. Due to the migration ability of cells increased as a result of over expression of MMP-9, the wound (scratch) closes with the movement of cells across the scratch. This method mimics to some extent migration of cells in vivo. The results were captured by a microscope to discern the migration at time point 0 with migration after 18h. A dose dependent decrease in the migration ability of cells was observed when control was assessed against treatment thus, confirming inhibition of MMP-9 by δ-tocotrienol. The absorbance values recorded for treatments 10µM, 20µM and 30µM also support this will all the values being significantly different (p<0.05). All the above results lead to the conclusion that δ-tocotrienol effectively inhibits several steps which are involved in cell metastasis like aggregation, adhesion, invasion and migration.

MMP-9 has the ability to degrade gelatin, major component of basement membrane. It uses gelatin as substrate. So the activity of this enzyme can be interpreted by the amount of gelatin utilized by the enzyme. Zymogram gel assay is employed for this purpose. This assay applies a sensitive quantifiable polyacrylamide gel-based electrophoretic approach that associates the ability of the proteases with degrading the substrate, gelatin. Clear bands against a dark background are observed as a result, which shows the digested substrate by the proteases. Cell media was used to analyze the MMP-9 activity. The RPMI media used to incubate the cells was not supplemented with FBS as itself contains MMP-9 that could interfere with the Zymogram gel assay.

Tumor necrosis factor alpha (TNFα) is used as positive control. TNFα is a member of TNF cytokine family and is a cell signaling protein that is involved in systemic inflammation.
One of the pivotal chemical mediators embroiled in inflammation related cancers is TNF-α. It is involved in promotion and progression of cancer through activating transcriptional factors like NF-κB. The samples used for performing Zymogram gel assay included those added with TNFα, control and treatments 10μM, 20μM, 30μM. A dose dependent decrease in the band size was observed with δ-tocotrienol which confirms the inhibition in MMP-9 activity.

It has already been shown that MMP-9 over expresses in NSCLC [76]. The purpose of this study was to examine if δ-tocotrienol down regulates MMP-9 expression in lung cancer. In order to understand the underlying mechanism of anti-metastatic properties of δ-tocotrienol on MMP-9, studying its expression at gene and protein level is important. Real-Time PCR analysis and Western blot analysis were performed to examine MMP-9 gene and protein expressions. Expression of β-actin is used as reference. The Ct count for both the cell lines was used to find the relative expression for each treatment. A dose dependent decrease in the relative expression was observed.

Previously we showed that δ-tocotrienol down regulates Notch-1 expression. MMP-9 follows a downstream pathway to Notch-1 and HES-1. Another pathway includes uPA upstream to MMP-9. In order to see effects of δ-tocotrienol on MMP-9 protein expression, its effects on Notch-1, HES-1 and uPA protein expressions were also studied by western blot analysis. β-actin was used as reference protein. A dose dependent reduction in the band size was observed for treatments 10μM, 20μM and 30μM with control for all the genes. This suggests that MMP-9 expression is decreased by the δ-tocotrienol treatment.

In summary, the study shows that δ-tocotrienol shows inhibition in metastatic properties of MMP-9 like cell aggregation, adhesion, invasion, migration and in activity of the enzyme in NSCLC cells. When molecular mechanisms were investigated, there is a decrease in the gene
expression and protein expression levels of MMP-9 by δ-tocotrienol. Further, this study supports the Notch-1 signaling pathway and uPA pathway. Further investigation in animal models and clinical trials need to be done to prove the effect of pure δ-tocotrienol on NSCLC.

**Fig 16:** δ-tocotrienol down regulating genes in MMP-9 pathway
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ABSTRACT

DELTA-TOCOTRIENOL DOWN REGULATES MMP-9 EXPRESSION IN NSCLC CELLS

by

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Lung cancer is the leading cause of cancer death in Unites States. The main types of lung cancer are small cell lung cancer and non small cell lung cancer. NSCLC accounts for about 80% of the cases with less than 15% of patients surviving beyond 5 years. Most common causes of lung cancer are smoking, inhaling asbestos fiber, radon gas, passive smoking, air pollution, familial predisposition, prior history of lung cancer etc. Surgery, chemotherapy, targeted therapy, palliative care are commonly used treatment techniques for lung cancer. Radiation therapy is an important part of lung cancer management. The objective of this study was to investigate if δ-tocotrienol which is an isoform of essential nutrient vitamin E can be successfully used in the treatment of NSCLC. The effect of pure δ-tocotrienol was studied on NSCLC cell lines A549 and H1299. Previously it was proved that Notch-1 pathway could be a therapeutic target for NSCLC and also that pure δ-tocotrienol down regulates Notch-1 via NF -KB. This study mainly focuses on MMP-9 and uPA expression in the NSCLC cell lines via Notch-1 and HES-1. Aggregation and adhesion capability, invasive and migration ability, MMP-9 activity and
molecular mechanisms like gene expression and protein expression were evaluated. Different assays like cell aggregation assay, cell adhesion assay, cell invasion assay, wound healing assay, zymogram gel assay, Real-Time PCR analysis, Western Blot analysis were performed for this purpose. A dose dependent decrease was observed in MMP-9 activity confirming its inhibition. Since there is inhibition in cell aggregation, adhesion, invasion, migration, gene and protein expressions, the study suggests that administration with pure δ-tocotrienol could be an effective approach for the treatment of NSCLC.
AUTOBIOGRAPHICAL STATEMENT

The author, Rohini Sri Harshini Pindiprolu was born in Kakinada, Andhra Pradesh, India on March 11, 1992. In 2013 she earned her Bachelor of Technology degree from TKR College of Engineering and Technology, Jawaharlal Nehru Technological University, Hyderabad, India in the field of Biotechnology. The same year she was accepted to pursue Master of Science degree in Nutrition and Food Science at Wayne State University, Detroit, MI and would be completing her degree in May 2015. During her Masters, in March, 2014 she received the IFT Great Lakes Section Diversity Scholarship. Upon completion of Masters the author intends to be a Food Scientist in Food industry.