DADS REPRESSED EMT IN A549 LUNG CANCER CELLS BY MODULATION OF BOTH CANONICAL AND NON-CANONICAL TGF-β SIGNALING PATHWAYS

Dissertation submitted in partial fulfilment for the degree of

Master of Science in Biotechnology

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CERTIFICATE

This is to certify that the dissertation entitled "**DADS repressed EMT in A549 lung cancer cells by modulation of both canonical and non-canonical TGF-β signaling pathway**" submitted by Ms Priyanka Das, Roll No-1661043, Registration No-16647251469 to the KIIT School of Biotechnology, KIIT to be deemed University, Bhubaneswar-751024, for the degree of Master of Science in Biotechnology is his original work, based on the results of the experiments and investigations carried out independently by her during the period from 8th January to 15th May,2018 of study under my guidance. She has shown keen interest in performing the experiment , participating in daily laboratory events and preparing for her project report.

This is also to certify that the above said work has not previously submitted for the award of any degree, diploma, fellowship in any Indian or foreign University.

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CERTIFICATE

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During this period she actively took part in the project entitled"DADS repressed EMT in A549 lung cancer cells by modulation of both canonical and non-canonical TGF-β signaling pathways ".

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Declaration

I hereby declare that the work embodied in this project entitled " DADS repressed EMT in A549 lung cancer cells by modulation of both canonical and non-canonical tgf-β signaling pathways" submitted by me,under the guidance of Dr. Dona Sinha, Senior Scientiific Officer (SSO-I grade), Head of Dept. Of Receptor Biology and Tumor Metastasis, Chittaranjan National Cancer Research Institute, Kolkata.I submited this reseach work for the degree of Master of Science on Biotechnology in School of Biotechnology, KIIT to be deemed University. Orissa.

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Abstract

Problem: Lung cancer is the leading cause of cancer related mortality. The global lung cancer incidence in 2012 was 1.8 million and mortality rate was 1.6 million. Metastasis and invasion of cancer are associated with epithelial mesenchymal transition (EMT), a process in which a polarized epithelial cell loses its apical basal polarity and acquires mesenchymal phenotype, with enhanced migratory capacity, invasiveness, and elevated resistance to apoptosis. Hence, modulation of EMT can be an attractive strategy to prevent lung cancer metastasis. The standard chemotherapeutic regimes are associated with high toxicity to normal cells, various side effects and chemoresistance. Owing to low systemic toxicity natural compounds have gained attention as anticancer agents. Diallyl disulphide (DADS) is a component of garlic oil which is known for its anti tumor potential.

Objective: The present study was aimed to decipher the role of DADS against TGF-β-induced EMT in A549 lung cancer cells.

Methodology: The effect of DADS against TGF-β-induced EMT in A549 lung cancer cells was investigated with cell scattering assay, immunoblotting and semi quantitative reverse transcriptase PCR.

Achievement: DADS treatment resulted in a significant down regulation of TGF-β induced mesenchymal markers [N-cadherin] and up regulation of epithelial marker [E-cadherin]. DADS reduced the expression of Smad4 as well as Ras, Raf, pERK1/2 and ERK1/2 which were up regulated by TGF-β in A549 cells. Thus, DADS was capable of modulating TGF-β-induced canonical and non-canonical pathways which in turn caused reversal of EMT. Therefore, DADS may be proposed as a potential natural compound to confront EMT in lung cancer. However, more pre-clinical studies and clinical trials are needed to establish DADS as an effective chemotherapeutic agent against lung cancer.

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Abbreviations

EMT	Epithelial mesenchymal transition
NSCLC	Non small cell lung cancer
SCLS	Small cell lung cancer
ZEB	Zinc finger E-box-binding-homeobox
TCF/LEF	T cell factor/lymphoid enhance factor
ECM	Extracellular matrix
MMP	Matrix metalloproteinase
GM-CSF	Granulocyte macrophage colony stimulating factor
FBS	Fetal Bovine Serum
RPMI	Rosewell Park Memorial Institute Medium
PBS	Phosphate buffer saline
DADS	Diallyl disulphide
APS	Alkaline phosphatase
RIPA	Radioimmunoprecipitation buffer
TBST	Tris buffer saline with tween 20
SDS	Sodium dodecyl sulphate
APS	Ammonium persulfate
TEMED	Tetramethylethylenediamine
TRIS	Tris (hydroxymethyl) aminomethane

EGCG	Epigallocatechin-3-gallate
AXIN	Axis inhibition protein
GSK-3β	Glycogen synthase kinase 3-beta
APC	Adenomatus polyposis coli
FZD	Frizzled
LRP	Lipoprotein receptor-related protein
DVL	Disheveled
TCF	Transcription factor
TGF	Transforming growth factor
R-Smad	Receptor regulated smad
Co-Smad	Common mediator smad
ERK	Extracellular signal-regulated kinase
MAPK	Mitogen-activated protein kinases
RTK	Receptor tyrosine kinase
SH2	Src homology 2
Grb	Growth factor receptor binding protein
BSA	Bovine serum albumin
SNAIL	Zinc finger protein
SMAD	Suppressor of mothers against decapentaplagic

CHAPTER-1

1.Introduction

1.1 Background and Context

In recent times cancer has emerged as one of the deadliest disease worldwide. In India, next to cardiovascular diseases cancer ranks as the second cause of mortality [1]. A study by Aggarwal (2016) et al, in 2013 indicated a total number of 14.9 million cancer cases with a mortality of 8.2 million [2]. Cancer cell possess various biological characteristic i.e., rapid proliferation, resistance to cell death promote angiogenesis, inhibit growth suppressor, induce invasiveness and metastasis [3].

Lung Cancer

Lung cancer is one of the common cancer and it is the second most cause of cancer-related death all over the world [1]. Lung cancer appears to arise in the bronchi in response to repetitive carcinogenic stimuli, inflammation, and irritation. Disruption of cell development occurs in the mucosal lining and progresses to elevate or erode the basal membrane. The tumor then spreads throughout the lung and eventually metastasizes to the lymph nodes and other parts of the body (Figure 1).

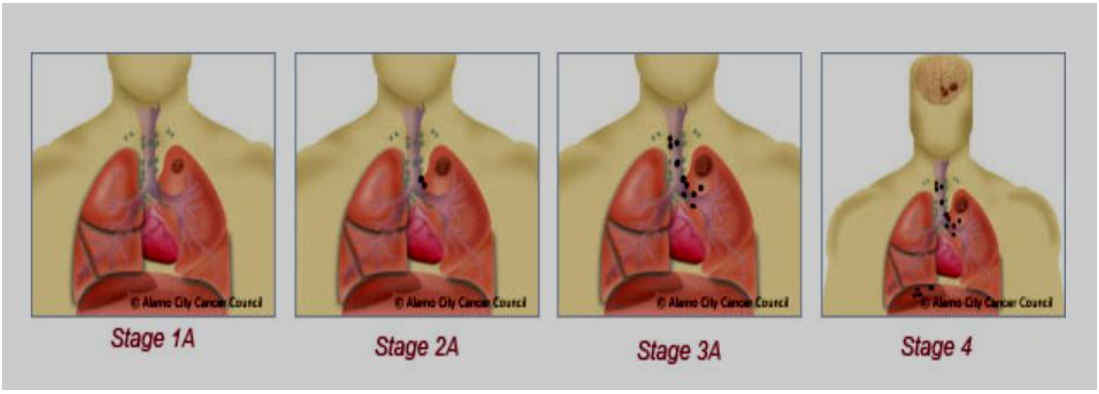

Figure 1: Lung cancer stages

Lung Cancer Statistics

Occurrence wise most common cancers all over the world are lung (1.61 million, 12.7% of the total), breast (1.38 million, 10.9%), and colorectal cancers (1.23 million, 9.7%). The most common causes of cancer deaths are lung cancer (1.38 million, 18.2% of the total), stomach cancer (738,000 deaths, 9.7%) and liver cancer (696,000 deaths, 9.2%) [4]. Lung cancer report shows that the 13 per cent of all new cancer cases and 19 per cent of cancer related deaths worldwide.

Occurrence wise in India, lung cancer represents 6.9 per cent of all new cancer cases and 9.3 per cent of all cancer related deaths in both male and female [5]. According to the GLOBOCAN report of 2012 shows that the number of lung cancer case and number of lung cancer death were 70000 and 65000 respectively [Table-1]. The highest reported incidence of lung cancer in both sexes is from Mizoram, in India [5].

Table 1: Lung cancer statistics (2012)

LUNG CANCER	INCIDENCE	MORTALITY
MEN	54,000	49,000
WOMEN	17,000	15,000
BOTH SEXES.	70,000	64,000

Causes of Lung Cancer

A) Smoking

Worldwide 80% lung cancers are caused due to smoking [6].In India, smoking is very common in 29% of adult males; 11.5 % in male collegians; 2.5% in adult females and 8.1 % in school children. Tobacco (bidi or cigarette) consumption killed people at the age of 25 to 69 years, losing 20 year of life expectancy [7, 8].

B) Other Causes

The incidence of lung cancer among non-smokers is estimated to be 15%. These cases occur due to genetic factor, pesticides, radon gas, [9] air pollution, passive and static smoking. Farmers use pesticides for the production of the crop annually in developing country. In pesticides, 85% of the 2.6 million metric ton of active ingredients are present which are responsible for lung cancer [9].

Lung Cancer Types

The type of lung cancer depends upon the cell type from where it originates. It is classified as:

1) Non-small cell lung cancer (NSCLS): About 7 in every 8 people diagnosed with lung cancer are with the non small cell lung cancer. It does not grow and spread as fast as small cell lung cancer (SCLC) cells [10]. NSCLC are classified as follows.

a. Adenocarcinoma, appears from cell lining of the alveoli.

b. Squamous cell carcinoma appears from cell lining of the bronchi.

c. Large Cell Carcinoma is a form of adenocarcinoma but the cells are larger in appearance.

2) Small cell lung cancer(SCLC): The cells of SCLC appear small under microscope. About 1 in every 8 people diagnosed with lung cancer are with small cell lung cancer. Small cell lung cancer spread more quickly and tends to start in the middle of the lung [11].

Epithelial mesenchymal transition (EMT) in cancer progression:

Epithelial mesenchymal transition (EMT) is a process in which epithelial cells gradually acquire a mesenchymal (fibroblast-like) cell phenotype [12]. Epithelial cells show apical basal polarity adhere and communicate with each other through specialized intracellular junction and are positioned at a base membrane that help in physiology. The mesenchymal cells are unique spindle shaped that exhibit end to end polarity and also have some characteristic feature like migratory capacity, invasiveness and resistance to apoptosis [13]. Epithelial to mesechymal transition is a most essential biological process for embryonic development, gastrulation, and the neural crest development, for development of heart and other organs.

EMT is also involved in tissue repairing system, organ fibrosis and cancer progression process. EMT is related to the development of many type of tumors and it is also involved in cancer cell metastasis and treatment resistance [14] (Figure-2).

During EMT, epithelial cells undergo some changes i.e,

1) Change in transcriptional regulation
2) Changes in cytoskeleton and mortality
3) Changes in cell adhesion
4) Changes in synthesis of extracellular matrix

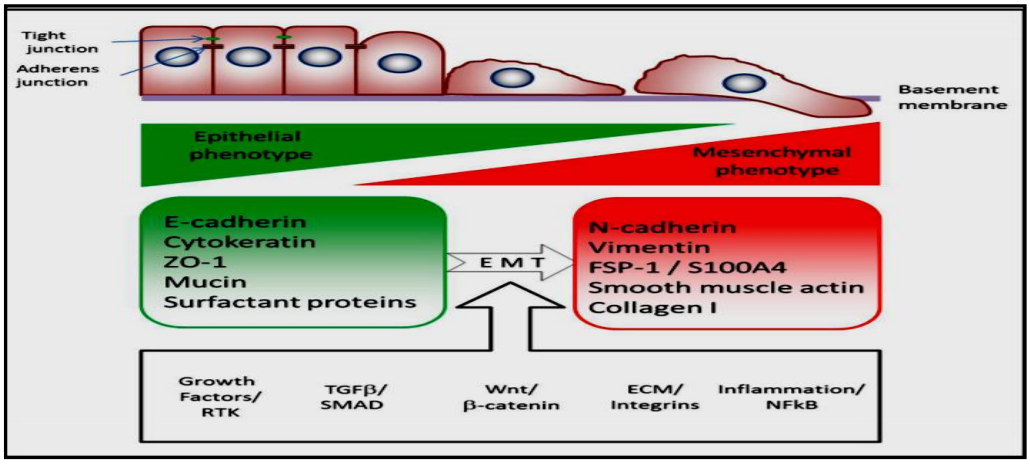

Figure 2: The process of EMT

Snail, Slug ZEB 1, ZEB 2, Twist, β-catenin and T-cell factor/lymphoid enhance factor (TCF/LEF) are the transcriptional factor are identified as the key regulators of EMT. In epithelial cell, cytokeratins are the characteristic intermediate filament. During EMT cell are reprogrammed to express vimentin. The first molecular sign of EMT involves split of cell-cell junction by reduction of junction component i.e, occludin, ZO-1, claudins, and E-cadherin. The deprivation of E-cadherin is a key component of the EMT followed by induction of the N-cadherin which is a key component of mesenchymal cells. When cells

undergo EMT, synthesis of some extracellular matrix (ECM) component like fibronectin and collagen type 1 also takes place[12].

Types of EMT:

In biological system three different types of EMT are found to operate and controls different physiological activities.

"Type 1" EMT: It is associated with implantation, embryogenesis and organ development(Figure-3A).

"Type 2" EMT: It is associated with wound healing, tissue regeneration and organ fibrosis. Type 2 EMT is associated with generation of fibroblast to reconstruct and repair tissue following inflammatory injury or trauma(Figure-3B).

"Type 3" EMT: It occurs in the context of tumor growth and cancer progression. The type 3 EMT is promoted by the genomic modification obtained by cancer cell and generate cell which has invasive property that permit cancer cell to pass into the blood stream and spread to the other organ.(Figure-3C)

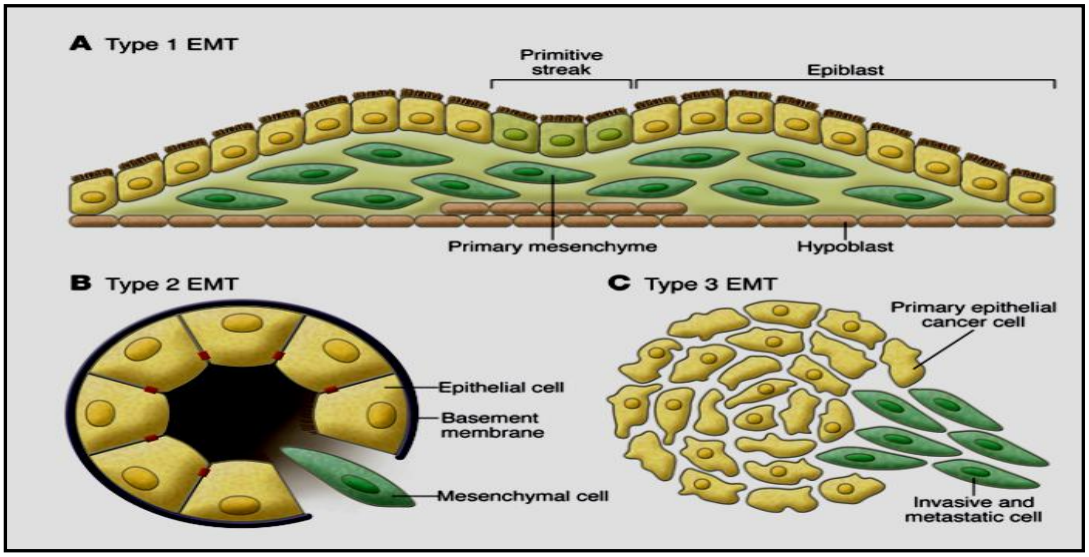

Figure 3: Three different types of EMT

WNT signaling and EMT (Canonical):

In the canonical pathway, in the absence of the of WNT a β-catenin destruction complex is formed which consist of axis inhibition protein (AXIN), adenomatus poliposis coli (APC), glycogen synthase kinase 3-beta (GSK-3β), whereby β-catenin is phosphorylated at serine 9 and then proteolytically degraded. In the presence of WNT it binds to the one of the ten frizzled (FZD) receptor then a receptor complex between WNT, FZD, lipoprotein receptor-related protein (LRP), disheveled (DVL) and AXIN are formed. In this active complex DVL gets phosphorylated and eventually inhibit GSK-3β, resulting is the reduced phosphorylation and consequently stop the proteolytic destruction of β-catenin. β-catenin subsequently accumulates in the cytoplasm. This cytoplasmic β-catenin translocates to the nucleus where it binds with the promoter region of EMT inducing genes leading to the transcription of EMT related proteins [15](Figure-4).

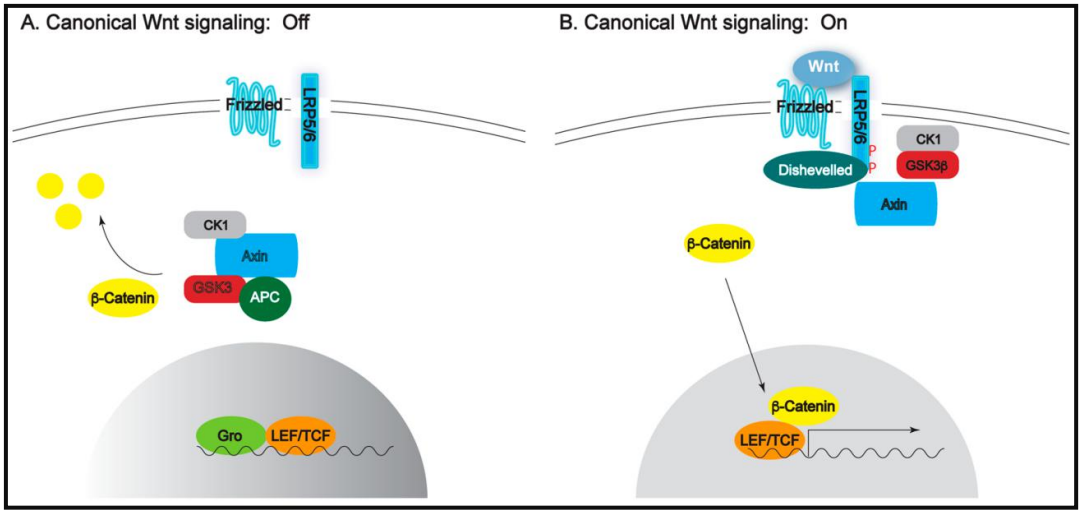

Figure 4: WNT signaling(Canonical)

Smad dependent TGF-β pathway (Canonical):

TGF-β is most important factor in EMT and cancer cell distribution. A study by Katsuno et al in 2013 showed that the TGF-β pathway promoted invasiveness and metastasis by induced expression of transcription factors Snail, Slug, twist, ZEB1 and TCF3. These factors inhibited E-cadherin expression and upregulated the expression of N-cadherin, vimentin and promote the secretion of the matrix metalloproteinases [16].

TGF-β receptor is membrane bound receptor with serine threonine kinase activity. TGF-β bind as a ligand to the type II receptor, TGFβ-RII, with the assistance of type III receptor, TGFβ-RIII. When TGFβ-RIII bound tightly to TGF-βRII, forms a heterotetrametric complex with the type I receptor, TGFβ-RI which is phosphorylated. Then the type I receptor phosphorylates receptor-regulated smad (R-smad) such as smad 2 and smad 3 protein at two C terminal [17]. The activated Smad 2 and Smad 3 come together and combine with common mediator smad (co-Smad) i.e smad 4 and form a trimeric Smad complex, which can be transfered from cytosol to into the nucleus. In the nucleus this heterotrimeric complex bind to the DNA specific sequence transcription factor to induce transcription of target gene such as Snail, Slug etc [18] (Figure-5).

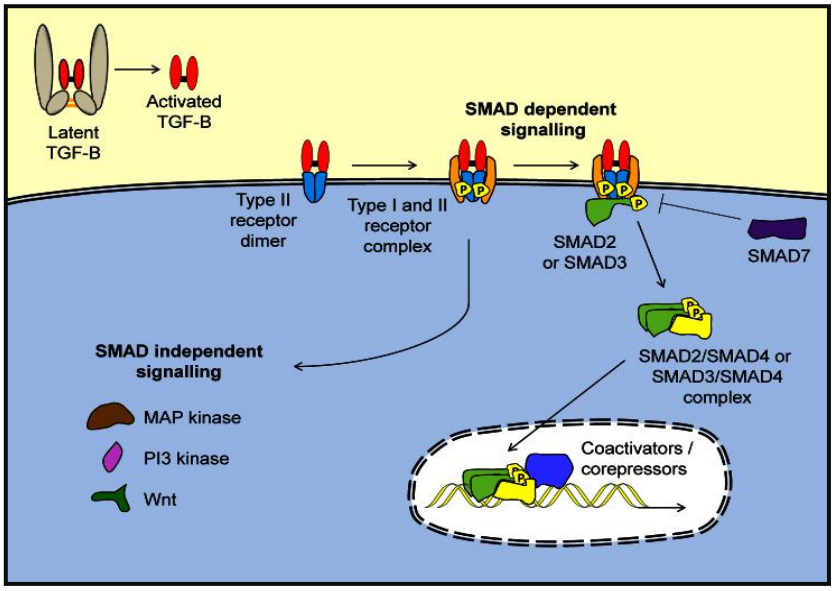

Figure 5: Smad dependent pathway

Smad independent pathway (Non-canonical):

The TGF-β can activate the ERK MAPK pathway by rapid activation of Ras. The rapid GTP loading Ras may require Raf and MAPK and lead to the activation Erk through MEK1. Activation of ERK by TGF-β was observed in epithelial cell, breast cancer cell and fibroblast. In receptor tyrosine kinase (RTK) /Ras/Erk signaling pathway, growth factor binds to the RTK which induce dimerization and activation of RTK. Once phosphorylated these tyrosine residues serve as a docking site for signaling molecule such as Src homology 2(SH2) OR phosphor tyrosin binding domain i.e, Src and growth factor receptor binding protein 2(Grb2). It is an adapter protein which binds to the SOS in the cytoplasm in absence of the ligand. Grb2/SOS is required by the RTK. It brings SOS to the plasma membrane, where it activates Ras by catalyzing the exchange of GDP to GTP. In the GTP bound state Ras bind to the Raf and activate MAPK cascade which include Erk and MEK (Figure-6).

Erk activation is one of the non smad pathway which is necessary for the TGF-β mediated EMT. Erk is required for the disassembly cell adhesion junction and induction of cell motility by TGF-β [19].

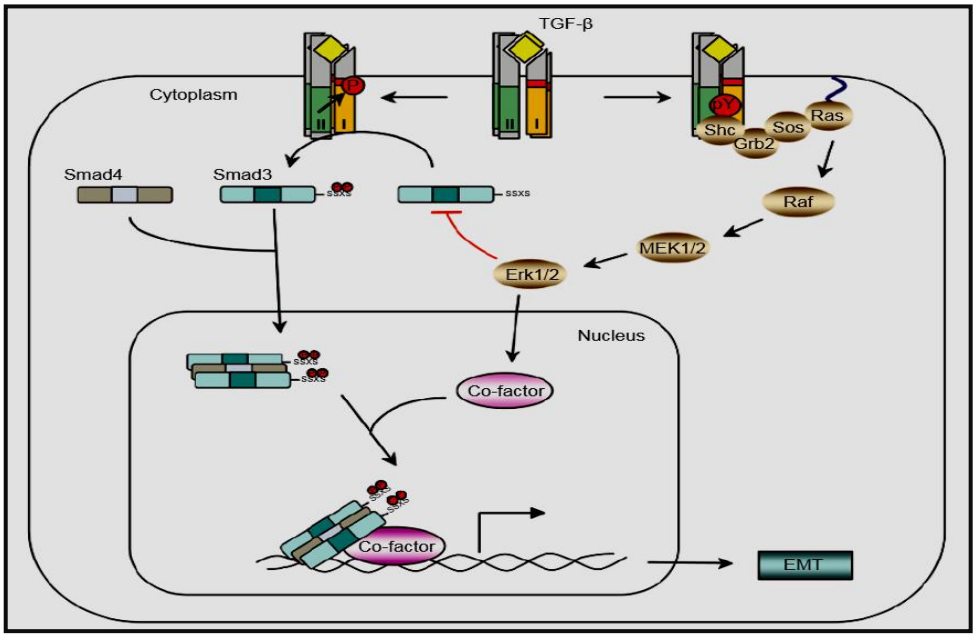

Figure 6: TGF-β mediated non-smad dependent signaling

Inhibition of EMT in lung cancer for therapeutic implication:

- Inhibition of EMT transition and tumor metastasis by miRs-33a and miR-124 respectively, compute in treatment of lung cancer [20, 21].
- Treatment of human lung carcinoma cell line (A549) with CX-4945 (small molecule inhibitor of CK-2) inhibited the signaling of TGF-β and down regulated EMT markers; where CX-4945 inhibited TGF-β1 cadherin switch preventing progression towards the mesenchymal phenotype [22].

Drawbacks of synthetic drugs

- There is a lack of specificity; the drugs employed target too many enzymes. A wrong time of inhibition against tumor progression at certain developmental stages can lead to increased tumor aggressiveness [23,24].

- Clinical trials of first and second generation small molecule matrix metalloproteinase (MMP) inhibiting drugs in breast and other form of cancer were plagued by the serious side effects of musculoskeletal syndrome. This is a dose-limiting toxicity frequently resulting in failure to achieve targeted plasma levels.

Phytochemicals in EMT regulation:

EMT play an important role in tumor metastasis. So, prevention of EMT is an important tool to reduce tumor progression. A novel preventive therapy is treatment with the phytochemicals i.e, the natural dietary substances which are nontoxic to normal human cell but effective to inhibiting cancer cell. Phytochemicals, derived from plant are mostly found in the vegetables, soy and fruits and considered as strong source of cancer preventive phytonutrition to inhibit development and progression of many types of cancer [25] (Figure-7).

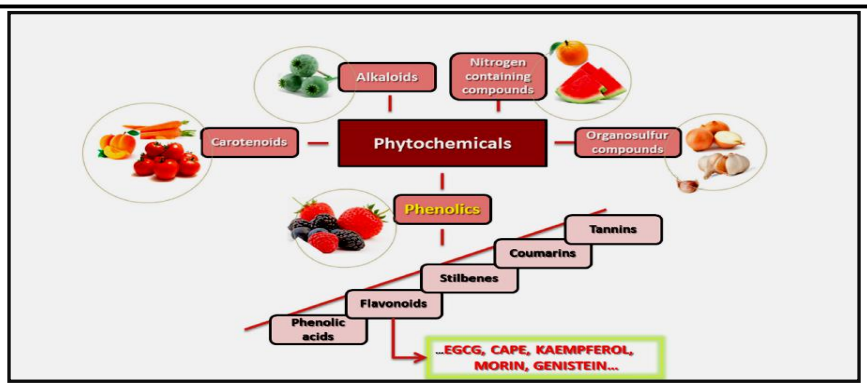

Figure 7: Type of phytochemicals

Some important phytochemicals which have been reported for inhibition of EMT:

Resveratrol: (trans 3,4,5-trihydroxystilbene)(Figure-8), is a stilbene phytoalexin. It was first found in the root of the oriental medicine plant *Polygonum cuspidatum*. It is also a polyphenolic compound found in skin of the red fruit. Resveratrol has antioxidative, antiproliferative and also anti-inflammatory properties [25]. Resveratrol inhibited granulocyte macrophage colony stimulating factor (GM-CSF) and NF-κB in A549 cells[26]. Resveratrol inhibited TGF-β1 triggered EMT development in A549 lung cancer cell. It also inhibited the transcription factors Snail and Slug[27].

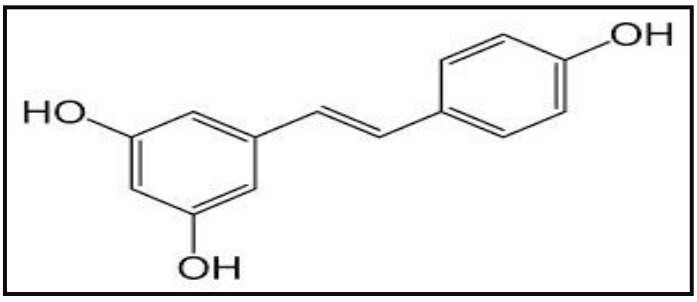

Figure 8: Structure of Resveratrol

Epigallocatechin-3-gallate (EGCG): is the principal polyphenolic component in green tea (*Camellia sinensis*) [28](fig-9). EGCG, upregulated the expression of E-cadherin and downregulated the expression of vimentin in A549 cells. EGCG inhibited TGF-β, by the downregulation of phosphorylated Smad2 and Erk1/2 in A549 non-small cell lung cancer[29].

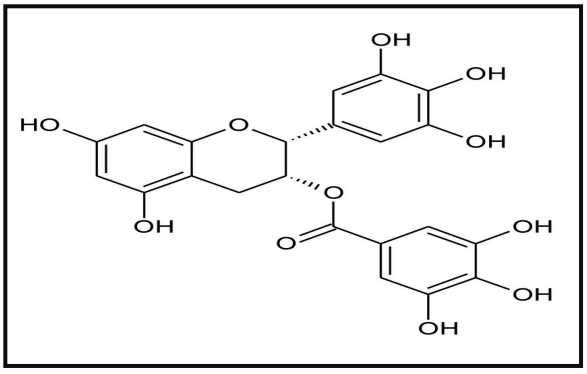

Figure 9: Structure of EGCG

Curcumin: (Diferuloylmethane). It is a phenolic compound derived from rhizome of the plant *Curcuma longa [30](10)(Figure-10)*. Curcumin exhibits strong anti-inflammatory, antioxidant, and anticarcinogenic properties [31]. Previous evidences showed that curcumin blocked cancer cell proliferation, transformation and invasion. Curcumin also inhibited migratory and invasive capacity, reduced tumor growth and had significant effect on the expression of matrix metalloproteinase (MMPs) of A549 cells [32].

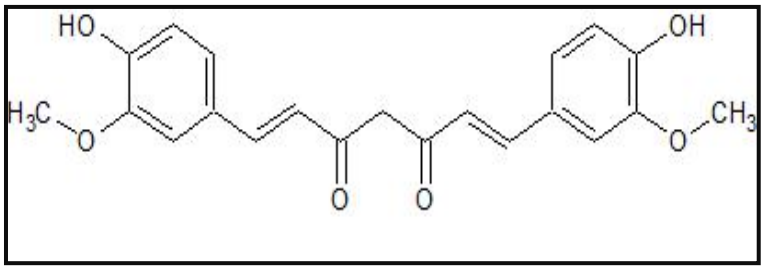

Figure 10: Structure of Curcumin

Kaempferol: (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4-H-1-benzopyran-4-one) is a flavanoid, found in many edible plant like tea, broccoli, cabbage, beans and tomato(Figure-11). It is derived from the rhizome of *Kaempferi galangal* L[25]. Kaempferol strongly inhibited TGF-β1 and MMP-2 activation in A549 cells. It also inhibited Smad3 binding to the promoter of snail [33].

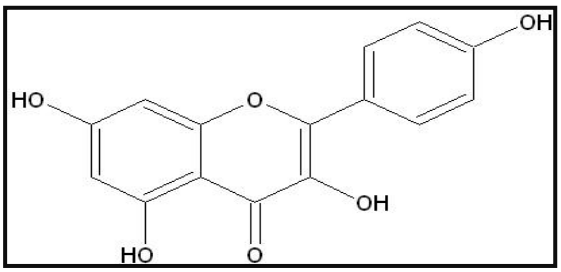

Figure 11: Structure of Kaempferol

DIALLYL DISULPHIDE (DADS): (3-[(Prop-2-en-1-yl)disulfyl]prop-1-en)(fig-12)

Sources and Chemistry: Garlic (*Allium sativum*)(Figure-12) is consider as one of the 20th most important vegetables throughout the world with various uses such as a raw vegetable, or as an ingredient in traditional or modern medicine [34]. DADS is an organosulfur stable oil soluble bioactive component found in garlic, has been reported to demonstrate a variety of pharmacological and biological activities including anti-inflammatory, anti-microbial and anti-metastatic activity [35]. DADS is produced by the decomposition of the alicin, which is an unstable bioactive compound found in garlic. Hence DADS could be easily oxidized to the alicin with the hydrogen peroxidase(Figure-13).

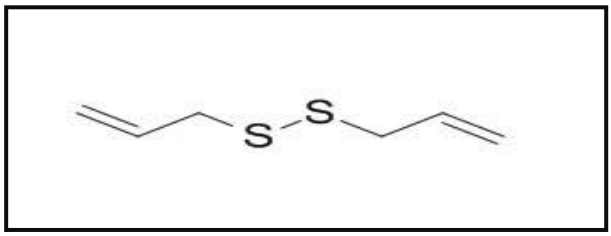

Figure 12: Structure of Diallyl disulfide

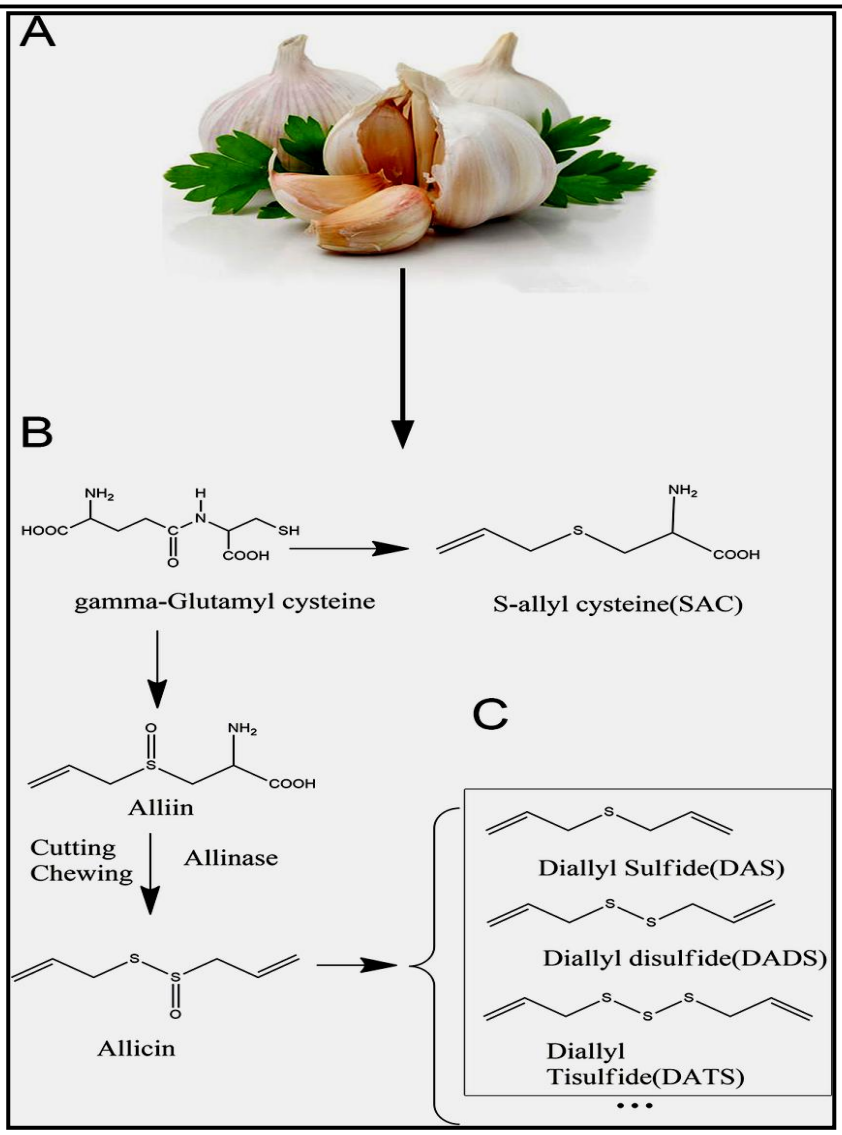

Figure 13: Chemistry of diallyl disulfide

Role of DADS in cancer metastasis:

DADS have also shown multi-targeted anti-tumor activities. The mechanism of action of DADS includes-activation of metabolizing enzymes of cell cycle arrest, induction of apoptosis and differentiation, histone modification and inhibition of angiogenesis and invasion. DADS inhibited 90.3% of lung tumor nodule formation when the drug was administered simultaneously in a in C57BL/6 mice [36]. A study by Lai et.al showed that DADS inhibited the migration and invasion of highly metastatic colo 205 human colon cancer cells in vitro. DADS exhibited anti-proliferative effect in colon cancer HT-29 [37] and anti invasive activity through

tightening of tight junctions and inhibition of MMP activities in LNCaP prostate cancer cells [38].

1.2 Scope and Objective:

The existing therapeutic regimes against lung cancer metastasis are confronted with a number of challenges like- high dose toxicity, non-specificity and various side effects. Under such circumstances, our study aims at studying the regulatory effect of DADS on lung cancer metastasis by targeting EMT, an event that plays integral role in cancer metastasis.The results obtained by this study may be useful for paving more detailed pre-clinical and clinical studies to establish DADS as an effective chemotherapeutic drug for lung cancer management.

1.3 Achievements:

a. DADS was able to inhibit TGFβ-induced cell scattering and could also restore the cell morphology in A549 cells.

b. Modulation of TGF-β-induced m-RNA expression of epithelial and mesenchymal markers by DADS was also observed.

c. Reversal of TGF-β-induced EMT by DADS was found to be regulated by both cannonical and non-cannonical TGF-β signaling.

1.4 Overview of Dissertation:

Cancer is the leading cause of human death. Non small cell lung cancer is a leading cause of death worldwide with a survival rate of 1%.Metastasis and invasion of cancer are associated with Epithelial –mesenchymal Transition (EMT), a process in which a polarized epithelial cell loses its apical basal polarity and acquires mesenchymal phenotype, with enhanced migratory capacity, invasiveness, and elevated resistance to apoptosis. Hence, modulation of EMT can be an attractive strategy to prevent lung cancer metastasis. This present study aim at evolution of the role of diallyl disulfide (DADS) on the cell morphology and cell scattering in TGF-β treated

cell. Also decipher the effect of DADS in cell signalling pathway in the TGF-β treated A549 cell.DADS treatment resulted in a significant down regulation of TGF-β induced mesenchymal markers [N-cadherin] and up regulation of epithelial marker [E-cadherin]. Western Blot result shows that DADS reduce the expression of Smad 4, Ras, Raf which was upregulated by TGF-β in A549 cell. Teken together this result indicate the efficacy of DADS in amelioraion of TGF-β induce EMT in A549 cell line

CHAPTER-2

2.Review of Literature:

In recent year lung cancer is the leading cause of cancer worldwide[39]. The survival rate of non small cell lung cancer is less than 1%. These cases are often caused by a combination of genetic factor and exposure to the carcinogenic molecule, radon gas, asbestos, smoke and other form of air pollution[40]. The existing treatment regimens including radiotherapy and chemotherapy are not completely effective for management of non small cell lung cancer. Despite recent advances in therapy of lung cancer, side effects of treatment and multidrug resistance remain the main obstacle of lung cancer treatment. The spreading of cancer occurs through metastasis and invasion, which mainly involves the process of EMT. EMT is a process by which epithelia cell lose their polarity and cell-cell junction and obtain migratory and invasive property to become mesenchymal stem cell. Hence targeting EMT could be an attractive strategy in the treatment of cancer.

In prostate cancer cell TGF-β induce EMT as evidence by loss of epithelial marker (E-cadherin, cytokeratins, ZO-1) and up regulation of mesenchymal maeker (N-cadherin, Vimentin)[41]. In rat intestine or mink lung epithelial cell TGF-β also induce EMT by the activation of Erk/MAPK pathway showing rapid activation of Ras.

Synthetic drug has a lack of specificity; the drugs employed target too many enzymes. A wrong time of inhibition against tumor progression at certain developmental stages can lead to increased tumor aggressiveness[23,24]. Since the therapeutic drug are not that effective in treating the tumor and has various side effect so to tackle these problem use phytochemical. Recently some dieatry phytochemical like resveratrol, EGCG, curcumin and kaempferol have been found to prevent or delay the progression of cancer by targeting EMT via inhibiting the expression of Snail, Slug, MMP and also down regulated the expression of the phosphorylated smad2 . So in this study we used DADS, which is a phytochemical found in garlic. Garlic is most important vegetables throughout the world with various uses such as a raw vegetable, or as an ingredient in traditional or modern medicine[34].Garlic is a rich source of sulpher containing antioxidant that are also used in cancer chemo preservation. DADS representing 60% of garlic oil is reported to be the most stable antioxidant among the organo sulfur compound of garlic.

DADS possess anti metastatic and anti tumorigenic properties. DADA appeared effective at reducing the growth of human tumor cells originating from colon, skin, and lung[42]. DADS cause cell cycle arrest and induce apoptosis in PC3[43] and MDA-MB-231 cells by decrease in Bcl-2: BAX ratio and activation of casepase[44].

The mechanism of action of DADS includes-activation of metabolizing enzymes of cell cycle arrest, induction of apoptosis and differentiation, histone modification and inhibition of angiogenesis and invasion. DADS inhibited 90.3% of lung tumor nodule formation when the drug was administered simultaneously in a in C57BL/6 mice [36]. A study by Lai et.al showed that DADS inhibited the migration and invasion of highly metastatic colo 205 human colon cancer cells in vitro. DADS exhibited anti-proliferative effect in colon cancer HT-29 [37] and anti invasive activity through tightening of tight junctions and inhibition of MMP activities in LNCaP prostate cancer cells [38].

There are also few report on the modulatory effects of DADS in lung cancer. DADS has been reported to inhibit lung metastasis of B16F-10 melanoma cells in mice,[45] induced apoptosis in non small cell lung cancer cell line by modulating the expression of Bcl-2, Bax, and p53[46]. DADS also induce G2/M cell cycle arrest and apoptosis A549 lung cancer cells[47]. However the study of the efficacy of DADS in regulation of EMT induced metastasis in human lung adenocarcinoma epithelial cells (A549) is still elusive. With this background it would be interesting to investigate the role of DADS in regulating the molecules of canonical Wnt signaling as well as both canonical and non-canonical pathway of Smad that plays a vital role in EMT progression.

CHAPTER-3

3 Aim and Objective:

1) To induce EMT in A549 lung cancer cell by TGF-β (10ng/ml).

2) To study the effect of DADS on cell morphology, scattering and EMT markers in TGF-β treated cells.

3) To decipher the signaling pathway underlying the regulatory effect of DADS on TGF-β in induced EMT in A459 cells.

CHAPTER-4

4. MATERIALS AND METHODS:

4.1 Chemicals:

DADS was purchased from Sigma Aldrich. Rosewell Park Memorial Institute Medium (RPMI), fetal bovine serum (FBS), Penicillin/Streptomycin was purchased fro1m Gibco Life Technologies Corporation. Antibodies were purchased from Santa Cruz. All the other chemicals were obtained from Sisco Research Laboratory

4.2 Cell Culture:

A549 cells were procured from National Center for Cell Sciences (Pune) and maintained in RPMI supplemented with 10% FBS, Penicillin (100 U/ml) and Streptomycin (100mg/ml) at 37° C in a humified atmosphere of 5% CO_2 incubator.

4.3 Treatment of Cells:

A549 cells were grown to 90% confluency. These confluent cells were subjected to serum starvation for 24 hours and then treated with DADS (7.5 uM) alone or with TGF-β (10 ng/ml) or left untreated. (Dose and time of DADS treatment was determined prior to this project).

4.4 Cells scattering assay

Cell scattering assay was performed according to the protocol described by Frams et al., [39] with slight modifications. Briefly the cells were seeded onto coverslips in 1 ml growth medium at a density of 1×10^4/ml. The cells were then incubated for 48 h to form small colonies. Then the growth media was removed, the cells were washed with sterile PBS and were serum starved for 24 h. The serum starved cells were then insulted with TGF-β (10ng/ml) alone or TGF-β along with DADS (7.5μM) for 24 h. After 24h, the cells were washed with PBS, fixed in ice cold methanol and stained with Gill's haematoxylin for 10min. The cells were viewed under inverted microscope (Olympus CX40, Tokyo, Japan) equipped with Magnus Pro 3.7 software. The number of colony forming cells were counted and expressed as percentage.

4.5 Protein extraction from cells:

Principle:

To perform analysis, a protein should be purified away from other cellular components. This process usually begins with cell lysis, in which the cell's membrane is disrupted and its internal contents are released into a solution. The primary purpose of lysis buffer is isolating protein of interest and keeping them in a stable environment.

Protocol:

Both control and treated cells were trypsinized and harvested by spinning at 5,000 rpm for 5 minutes. The media was discarded and to the cell pellet RIPA buffer was added in ratio of 1:2. The pellet was mixed properly with the RIPA. The cell pellets were incubated at -80^{O}C for 1 hour for the cell membrane to rupture. The samples were then thawed at 4°C. The supernatant was collected and stored at -80°C for further protein analysis. The total protein content of each protein extract was estimated by Lowry's method of Protein estimation.

4.6 Protein estimation:

Principle:

The total protein content of each protein extract was estimated by Lowry's method of protein estimation. The Lowry method is based on the reaction of Cu^{+}, produced by the oxidation of peptide bonds, with Folin–Ciocalteu reagent. The total protein concentration is exhibited by a color change of the sample solution in proportion to protein concentration, which can then be measured using spectrophotometric techniques.

Protocol:

For the estimation of the extract protein by Lowey's method prepare standard solution, sample solution and blank. The standard solution contain 20µl BSA, 80µL 0.1(N) NaOH,500µl alkaline solution and 50µl folin reagent. The sample solution contain 10µl sample ,80µL 0.1(N) NaOH,500µl alkaline solution and 50µl folin

reagent and blank was also prepare which contain ,100µL 0.1(N) NaOH,500µl alkaline solution and 50µl folin reagent. These sample were vortexed and kept in dark for 30 min.

After that OD was measured using spectrophotometer at 750 nm. Then protein contain were calculated with respect to standerd.

4.7 Western Blotting:

Principle:

Western blot is often used to separate and identify proteins. In this technique a mixture of proteins is separated based on molecular weight, and thus by type, through gel electrophoresis. These results are then transferred to a membrane producing a band for each protein. The membrane is then incubated with labels antibodies specific to the protein of interest.

The unbound antibody is washed off leaving only the bound antibody to the protein of interest. The bound antibodies are then detected by developing the film. As the antibodies only bind to the protein of interest, only one band should be visible. The thickness of the band corresponds to the amount of protein present; thus, a loading standard can indicate the amount of protein present.

Protocol:

Briefly protein (70µg) obtained from control and treated cell lysate was subjected to SDS-PAGE (10%) and blotted onto nitrocellulose membranes, blocked using 5% BSA in TBS-T (50m Tris, 150mM NaCl, 0.05% Tween-20), incubated with Anti-β-actin,

Anti-E-cadherin, Anti –N-cadherin, Anti-Snail, Anti- Twist, anti-monoclonal antibodies (1:1000 dilution) for 90 min at 37˚C, washed with TBS-T and incubated with respective alkaline phosphatase coupled secondary antibody for 90 min at 37˚C (1:1000). Bands were visualized in a developing solution containing BCIP/NBT in 1.5mM tris HCl (8.8) in dark for 10 mins. The band intensity was calculated using Image J Launcher (version 1.4.3.67) software.

4.8 RT-PCR

Total RNA from TGF-β-induced A549 cells treated with or without DADS, was isolated using TRIzol reagent and the isolated RNA was quantified spectrophotometrically. cDNA was synthesized from 2 μg of total RNA using using RetroScript kit. The cDNAs were amplified with specific primers for E-cadherin (forward-TTGACGCCGAGAGCTACAC, reverse-AATTCACTCTGCCCAGGACG), N-cadherin (forward-CCTTTCAAACACAGCCACGG, reverse-TGTTTGGGTCGGTCTGGATG) and β-actin (forward-GTCCACCTTCCAGCAGATGTG, reverse-GCATTTGCGGTGGACGAT). The PCR conditions included an initial denaturation step for 7 min at 95°C and 35 cycles of 40 sec at 95°C, annealing at 49.7°C for E-cadherin, 49.5°C for N-cadherin and 52.5°C for β-actin and extension at 72°C for 30s. After the last cycle, a final extension was performed at 72°C for 7 min. β-actin was used as an internal control. Densitometric quantitation of products was determined using Image-J (version 1.4.3.67) software. The relative intensity was expressed as the ratio of the intensity of the band for object gene to that of β-actin.

4.9 Statistical analysis

All quantitative data are reported as mean ± SEM obtained from at least three independent experiments (unless stated otherwise). Comparisons between the two groups of samples were performed using unpaired Student's *t*-test, whereas multiple comparisons of more than two groups of samples were performed using one-way analysis of variance (ANOVA) with Dunnett's post-hoc test. *P* values less than 0.05 were considered statistically significant.

CHAPTER-5

5. Result:

5.1. Effect of DADS on TGF-β induced cell scattering in A549 cells:

Stimulation with several mitogenic growth factors like hepatocyte growth factor (HGF) have been reported to induce cell scattering in colony forming cells [48]. Hence, we sought to investigate the effect of TGF-β on scattering of A549 colonies and also the effect of DADS in inhibition of TGF-β induced cell scattering. Experimental results indicated that TGF-β(10ng/ml) treated A549 cells had are 6.9% of colony forming cells. However, the colony forming cells increased to 13.7% when the TGF-β treated cells were co-treated with 7.5µM DADS. Also a close scrutiny of the cell morphology revealed that the cells underwent typical morphological changes in response to TGF-β. Results showed that TGF-β treated cells acquired a spindle shaped morphology whereas the cells treated with both TGF-β and DADS showed a more rounded morphology (Figure.14)

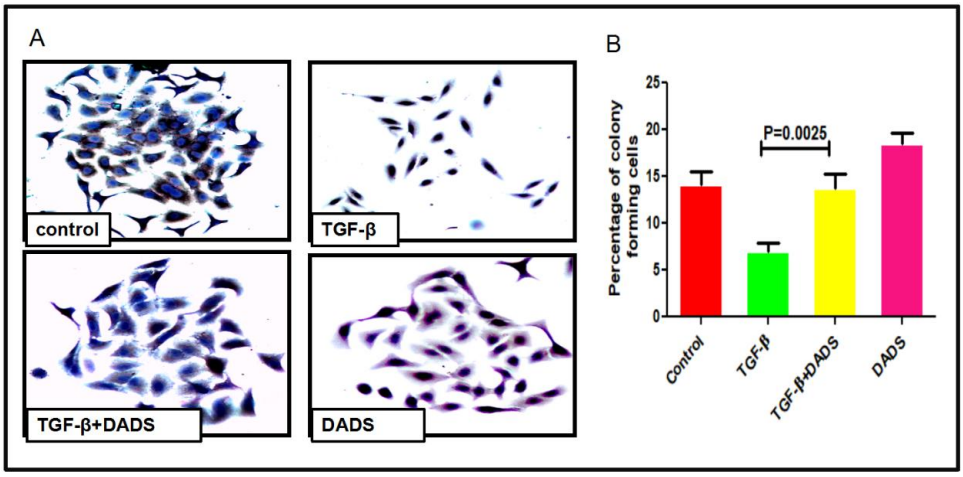

Figure 14: Effect of DADS on TGF-β induced cell scattering effect in A549:

Cell scattering assay to investigate the effect of DADS on TGF-β induced A549 cells exhibit enhanced colony forming ability of the cells in comparison to the TGF-β induced cells. A. Pictorial representation of the colony forming cells to show enhanced colony forming properties in the TGF-β induced cells; B. Graphical representation of the obtained results to show significant enhancement in the colony formation of the TGF-β induced A549 cells.

5.2. Modulation of TGF-β induced E-cadherin and N-cadherin expression by DADS

Biochemical hallmarks of EMT reversal include gain of expression of epithelial marker proteins such as E-cadherin with a concomitant decrease in mesenchymal marker like N-cadherin expression [49]. Our RT-PCR results indicated that on TGF-β treatment to A549 cells, the mRNA level of mesenchymal phenotype marker N-cadherin increased significantly whereas E-cadherin mRNA expression is downregulated. Interestingly, treatment of A549 cells with DADS (7.5µg/ml) for 24h resulted in suppression of mesenchymal marker N-cadherin and upregulation of epithelial marker E cadherin (Figure- 15,16). These results points towards the efficacy of DADS in suppression of EMT by modulating different EMT markers.

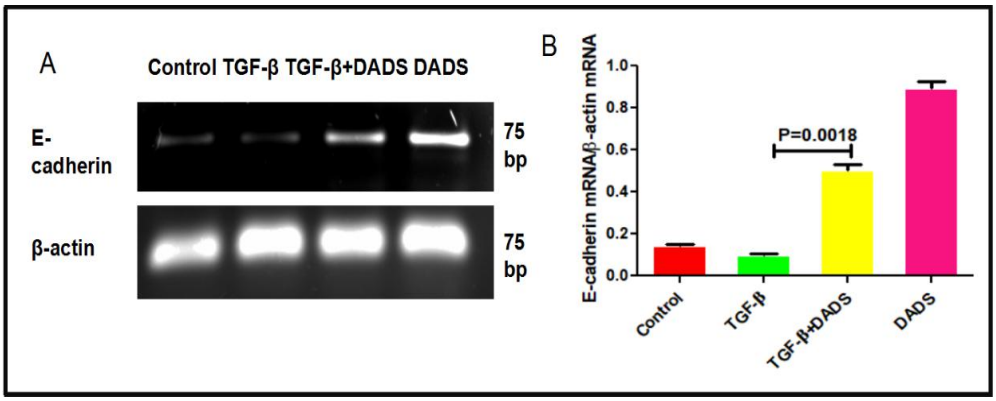

Figure 15: Modulation of TGF-β induced E cadherin transcript profile by DADS:

DADS could enhance the formation of E- cadherin as evident from the transcript profile of the A549 cells induced with TGF-β. A. Pictorial representation of the mRNA transcript profile shows enhanced E-cadherin in TGF-β induced DADS treated cells with respect to the TGF-β cells; B. Graphical representation of the band intensities calculated by ImageJ Launcher (version 1.4.3.67) depicting significant enhanced expression of the E-cadherin molecule.

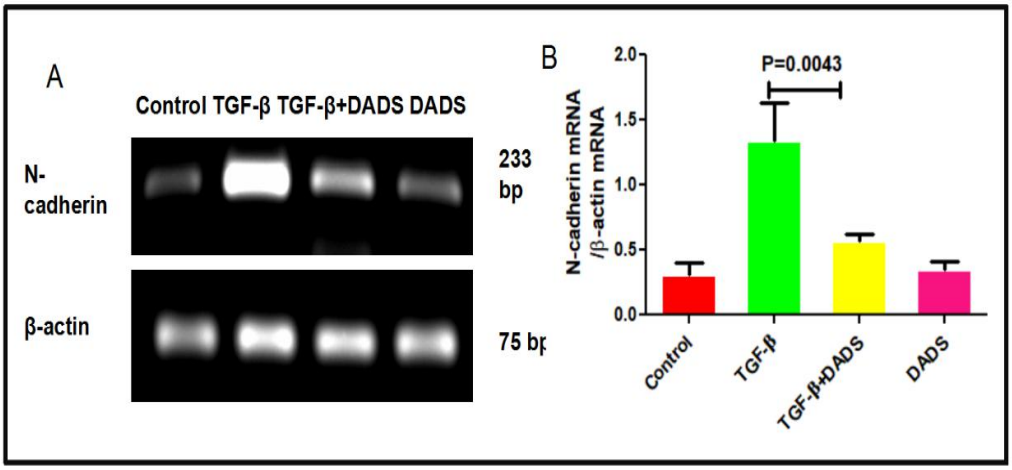

Figure 16: Modulation of TGF-β induce N cadherin transcript profile by DADS:

DADS could induce the formation of N- cadherin as evident from the transcript profile of the A549 cells induced with TGF-β. A. Pictorial representation of the mRNA transcript profile shows suppress N-cadherin in TGF-β induced DADS treated cells with respect to the TGF-β cells; B. Graphical representation of the band intensities calculated by ImageJ Launcher (version 1.4.3.67) depicting significant reduced expression of the N-cadherin molecule.

5.3. Effect exert no modulatory effect on TGF-β-induced Wnt signaling in A549 cells:

Aberrant activation of the Wnt β-catenin signaling pathway (canonical) is frequently involved in cancer development and progression due to modulation of multiple related molecules [50]

Hence, we sought to investigate the role of DADS in modulation of Wnt/β-catenin pathway in TGF-β insulted A549 cells. Interestingly, western blot results showed no significant upregulation in the expression of dvl2, p-GSK3β, β-catenin and LEF in TGF-β (10ng/ml) treated cells. Also, there was no significant change in the expressions of these proteins when the TGF-β treated cells were co-treated with 7.5 µM DADS (Figure-17). These results indicated that Wnt/β-catenin pathway might not be operating in TGF-β treated A549 cells.

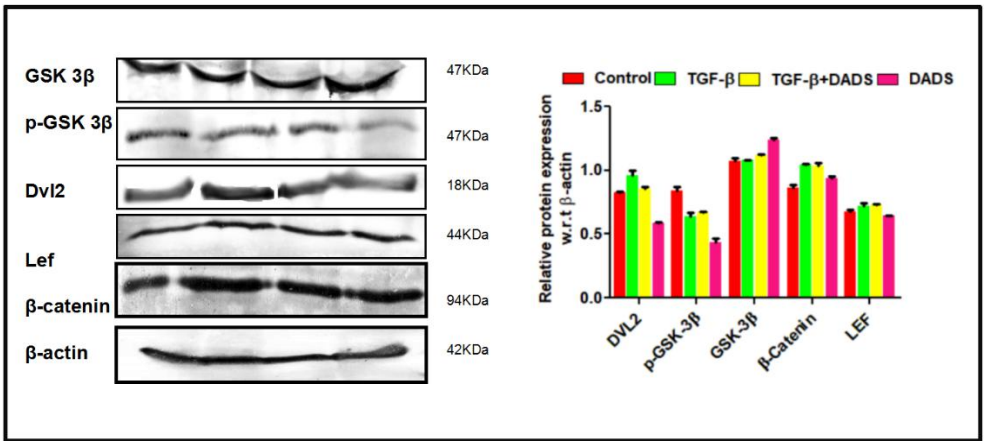

Figure 17: Effect on TGF-β-induced Wnt signaling in A549 cells:

DADS may not operating Wnt/β catenine pathway in TGF-β treated A549 cell. A. Pictorial representation of the westen blot shows no significance upregulation of dvl2, p-GSK3β, β-catenin and LEF in A549 cell treated TGF-β(10ng/ml) alone or along with DADS(7.5µM) ; B. Graphical representation of the band intensities calculated by ImageJ Launcher (version 1.4.3.67) depicting no significant alteration in the dvl2, p-GSK3β, β-catenin and LEF.

5.4.DADS modulated TGF-β induced EMT via both smad dependent (canonical) and smad independent (non-canonical) TGF-β signaling:

The previous results indicating that DADS has almost no effect on Wnt/β- catenin signaling, instigated us to determine whether the anti EMT effect of DADS is targeting smad signaling. Consequently, we sought to investigate the expressions of smad2/3 and 4 (canonical pathway) in A549 cells treated with TGF-β (10ng/ml) alone or TGF-β along with DADS (7.5µM). Western blot results showed that TGF-β treatment lead to upregulation of smad4 expression which was significantly subdued in cells co-treated with DADS. However, there was no significant change in the expression of smad 2/3 in both the treatment modes. Further investigation of the non-cannonical TGF-β signaling revealed that TGF-β treatment lead to upregulation of Ras, Raf expression and increased the phosphorylation of Erk1/2. However, these effects were subdued by DADS treatment (Figure-18,19)

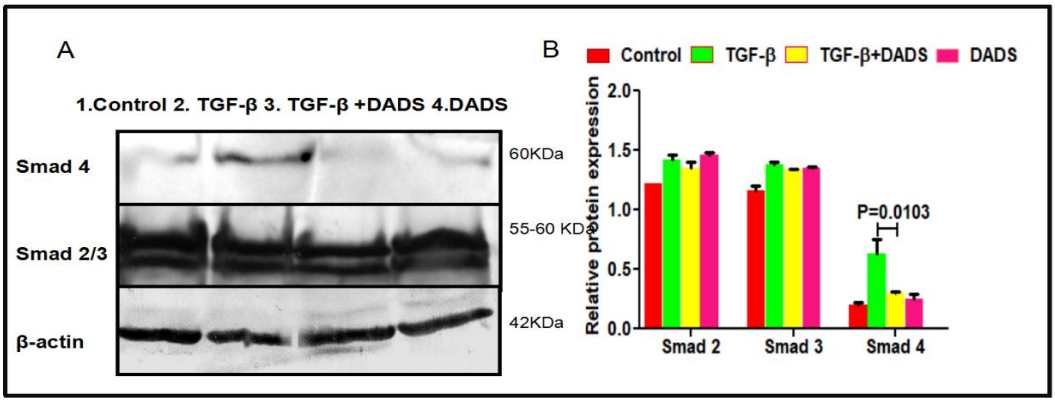

Figure 18: DADS modulated TGF-β induced EMT via canonical TGF-β signaling (smad dependent pathway)

DADS may operate through the canonical TGF-β pathway in TGF-β induced A549 cell. A. Pictorial representation of the westen blot shows upregulated smad4 in TGF-β(10ng/ml) treated A549 cell that was consequently downregulated when co-treated with DADS. But the expression of smad2/3 has no significant changes in both TGF-β and DADS co-treated A549 cell ; B. Graphical representation of the

band intensities calculated by ImageJ Launcher (version 1.4.3.67) depict significant reduction only in the smad4.

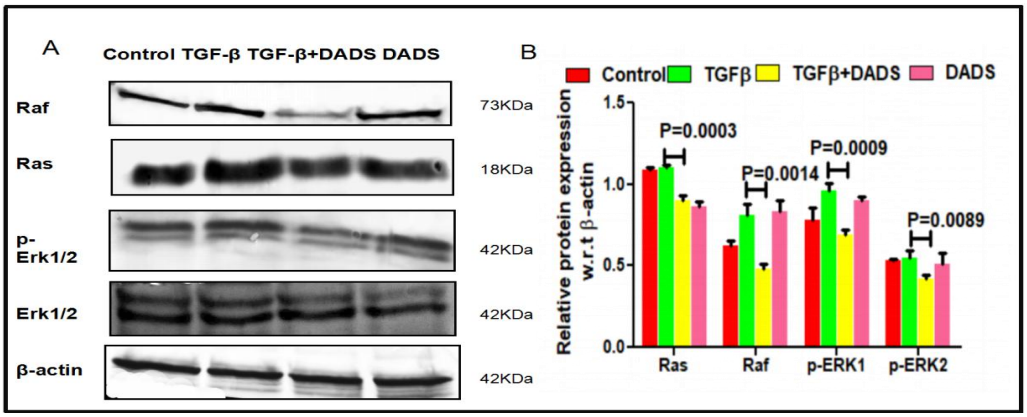

Figure19: DADS modulated TGF-β induced EMT via non-canonical TGF-β signaling (smad independent pathway)

DADS may be operating also through the non-canonical TGF-β pathway to regulate EMT induced by TGF-β in A549 cell. A. Pictorial representation of the westen blot shows upregulation of Raf, Ras, pErk1/2and Erk1/2 in TGF-β(10ng/ml) treated A549 cell and Raf, Ras, pErk1/2 and Erk1/2 also downregulated when co-treated with DADS.; B. Graphical representation of the band intensities calculated by ImageJ Launcher (version 1.4.3.67) depicting significant changes in the Raf,Ras, pErk1/2 and Erk1/2.

CHAPTER-6

6.Discussion:

Lung cancer is the leading cause of cancer-related morbidity and mortality worldwide among both men and women [51]. Despite the advancement in the understanding and treatment, the overall 5-year survival for all patients diagnosed with lung cancer remains less than 15 %, a rate that has barely changed over the last 30 years. Clearly more efforts are needed to improve our understanding of lung cancer biology in order to improve treatment and prevention strategies. Lung cancer is sometimes sensitive to either chemotherapy or radiation therapy, depending on types and stages. Despite recent advances in therapy of lung cancer, side effects of treatment and multidrug resistance remain the main obstacle of lung cancer treatment. Exploring novel agents with minimum side effects and maximum sensitivity attracts attention for lung cancer research. The spreading of cancer occurs through metastasis and invasion, which mainly involves the process of EMT. The EMT is a highly conserved cellular program that allows polarized, immotile epithelial cells to convert to motile mesenchymal cells[52] Hence targeting EMT could be an alternative therapy for curing as in lung cancer in metastatic stage.

Phytochemicals are listed as secondary metabolites that are naturally found in plants with roles involved in the restoration of damaged cells [53]. Phytochemicals were used in cancer prevention and therapy in traditional medicine due to their safety, lack of side effects and their bioavailability, from a wide range of natural sources. Diallyl disulphide, one of the bioactive component of garlic, possesses multiple biological activities including antimicrobial, hypolipidemic, antithrombotic, and antitumor activities[54]. Though DADS exhibited potential anti-metastatic activity, the efficacy of DADS in regulation of EMT in lung cancer still remains unknown.

EMT requires modifications in cell shape and substratum adhesions and these events are dependent on the reorganisation of the actin cytoskeleton. Several mitogenic growth factors are well known to induce such a conversion, termed “cell scattering”.Cell scattering is used to describe the dynamic dispersion of compact colonies of epithelial cells initiated by membrane ruffling following which some cells within the colony begin to separate from their surrounding cells and show a shape resembling that of motile fibroblasts. These cells continue to migrate, finally leading to a "scatter" phenomenon. Since the scattering of epithelial colonies have

characteristics of EMT, such as the reduce of epithelial cell-cell junctions and the asset of a motile mesenchymal cell phenotype [55]. Hence inhibition of cell scattering could be looked upon as an important step towards suppression of EMT. DADS treatment to TGF-β insulted A549 cells not only modulated the cell scattering but also restored the epithelial phenotype of the cells.

Members of the transforming growth factor-β (TGF-β) superfamily have been implicated as major induction signals of EMT during almost all of the morphogenetic events, which thus leads to upregulation of mesenchymal marker expression[52]. TGF-β promotes EMT by a combination of Smad dependent transcriptional events and Smad-independent effects on cell junction complexes. One of the key targets for repression during EMT is the cell-cell adhesion receptor, E cadherin which is commonly downregulated in many cancers, and its over-expression can suppress invasion by tumor cells. TGF-β induced EMT often coincides with loss of E-cadherin expression. The degradation of E cadherin is subsequently balanced by the up regulation of mesenchymal neural cadherin (N-cadherin) and results in a "cadherin switch" that alters cell adhesion. In present study, RT-PCR results showed that TGF-β resulted in the decreased mRNA expression of E cadherin[56] and an increased expression of N-cadherin which was subsequently reversed by DADS treatment.

There are several reports delineating the pro-migratory role of β-catenin protein in TGF-β treated cells. Moreover, activation of β-catenin pathway has been reported in cancer stem cells (CSCs) [57]. Under normal conditions, β-catenin exists in physical association with membrane-bound E-cadherin. However, if unbound with surface E-cadherin, β-catenin becomes free to translocate to the nucleus and transcriptionally activates several pro-migratory genes necessary for EMT in association with the TCF/LEF transcription factors [58]. However when we examined the expression of wnt related signaling molecules like p-GSK-3β, dvl2,LEF,β-catenin in TGF-β treated cells, we found that there was no significant upregulation of these proteins. Moreover DADS treatment also did not bring about any significant change in the expression of these proteins. These results negate the possibility of participation of wnt signaling in modulation of EMT by DADS.

Transforming growth factor-β signaling pathway plays a pivotal but complex role in tumor development and progression depending on tumor types and stages[59].

Alterations in TGF-β [60] signaling are linked to a variety of human diseases including cancer, inflammation, and tissue fibrosis. TGF-β signals through a heteromeric complex of two type I and two type II transmembrane serine-threonine kinase receptors. In

response to TGF-β, the type II receptor kinases phosphorylate the type I receptors, which then leads to activation of the cellular responses to TGF-β [61]. Ligand binding to TGF-β receptors initiates Smad2/3/4 complex formation and translocation to the nucleus (Smad pathway) to control gene expression. TGF-β signaling can also activate MAPK pathways (non-Smad pathway) in concert with other growth factors [62]. Various studies have explored the roles of TGF-β-activated Smads in EMT. Increased expression of Smad2 or Smad3 with Smad4 induces EMT or enhances the induction of EMT by the activated form of TβRI, in NMuMG cells [63.64]. RNA interference-mediated knockdown of Smad4 expression or expression of a dominant negative mutant of Smad4 results in preserved E-cadherin expression [64,65,66,67], suppression of fibrotic type I collagen synthesis in vitro [66], and decreased bone metastasis in vivo. Furthermore, genetic ablation of Smad4 leads to preservation of epithelial markers and a lower degree of EMT in adenocarcinoma [68]. In our study we also found that smad4 expression is upregulated in TGF-β treated cells which was modulated by TGF-β treatment. A study by Mao et al also indicated that valproic acid, an anticonvulsant drug inhibited EMT in renal cell carcinoma by downregulation of smad 4 expression. They also showed that silencing of SMAD4 expression decreased the expression of EMT markers in renal cell carcinoma [69].Hence downregulation of Smad4 is indispensable for suppression of EMT. However interestingly there was no significant change in smad 2/3 expression in TGF-β treated cells with respect to untreated control. Also DADS treatment could not bring about any significant change in smad 2/3 expression. However further experiments needs to be conducted to check the phosphorylation status of smad2/3 to predict whether smad2/3 are activated in A549 cells in response to TGF-β insult.

TGF-β also elicits signaling responses through pathways that are generally considered as important effector pathways for tyrosine kinase receptors in response to ligands that do not belong to the TGF-β family. The rapid activation of these non-smad signaling pathways by

TGF-β often follows similar kinetics as smad signaling, and attenuation of Smad signaling does not generally affect the activation of these pathways [61].Among the non-Smad signaling responses, activation of Ras, Raf and ERK MAP kinase pathway in response to TGF-β has been linked to TGF-β-induced EMT through their regulation of distinct processes, such as cytoskeleton organization, cell growth, survival, migration or invasion.A study supports a cooperation between ERK MAP kinase signaling and TGF-β/smad signaling in TGF-β-induced

gene expression [70]. The activation of ERK1/2 signaling increase TGFβ-induced EMT, followed by the morphological changes and the upregulation of EMT markers(N-cadherin) and ECM components. In the Ras/Raf/ERK signaling pathway, binding of growth factors to their receptor tyrosine kinase (RTK) increase its dimerization and activation. This results in auto- and trans-phosphorylation of multiple tyrosine residues in the cytoplasmic domain of RTK. Once phosphorylated, these tyrosine residues serve as docking sites for numerous signaling molecules with either Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains, such as Src and growth factor receptor binding protein 2 (Grb2). Grb2 is an adaptor protein that is bound to Sos in the cytoplasm in the absence of ligand stimulation. Upon RTK activation, Grb2/Sos complex is recruited to the RTK, which brings Sos to the plasma membrane, where it activates Ras by catalyzing the exchange of GDP for GTP. In its GTP-bound state, Ras can bind Raf and activate a MAPK cascade that includes ERK [19]. In our study we found that TGF-β stimulation in A549 cells upregulated the expression of Ras as well as Raf which further leads to the phosphorylation and hence activation of ERK. However the TGF-β induced upregulation in the expression of these proteins were counteracted on DADS treatment. These results pointed out to the fact that TGF-β promoted EMT through a combination of Smad-dependent and Smad-independent pathways in A549 cells which was counteracted by DADS. ERK is also required for disassembly of cell adherens junctions and induction of cell motility by TGF-β [19]. ERK activation is one of the non-Smad pathways necessary for TGF-β-mediated EMT.

7.Conclusion:

Taken together, our results suggest that DADS may inhibit TGF-β-induced EMT in A549 cells by modulation of both canonical and non-canonical TGF-β signaling pathways. We propose that DADS could be an effective supplement to traditional chemotherapies for lung cancer patients.

7.1 Summary:

DADS was able to revert the EMT chracteristics in TGFβ-induced A549 cells as evident from the cell scattering assay where colony forming characteristics had increased as well cellular morphology was restored. Besides, the epithelial and mesenchymal markers also had modulatory effects as evident from the mRNA profiling. Moreover, the Reversal of TGF-β-induced EMT by DADS was found to be regulated by both cannonical and non-cannonical TGF-β signaling.

7.2 Evaluation

1.10ng/ml TGF-β treatment for 24 hr could induce EMT in A549 lung cancer cell line.

2.TGF-β (10ng/ml) treatment for A549 lung cancer cell induced cells scattering and resulted in a spindle shaped morphology of A549 cells which were significantly reversed by treatment with 7.5μM DADS for 24 hr.

3.TGF-β upregulated the m-RNA expression of the mesenchymal marker N-cadherin which was suppressed by DADS treatment(7.5μM) for 24 hr. Moreover TGF-β(10ng/ml) treatment in A549 cells resulted in a downregulation of epithelial marker E-cadherin which was restored by the DADS(7.5 μM) treatment for 24 hr.

4.Further investigation of molecular mechanism indicated that DADS (7.5μM) inhibited TGF-β induced EMT via canonical smad signaling by suppressing Smad4 expression . Also DADS (7.5μM) had potential role in modulation of TGF-β induced non canonical signaling pathway via suppression of Ras, Raf expression and ERK phosphorylation.

Hence, the adverse side effect of synthetic drug by using natural compound with low toxicity could be bypassed by DADS.

7.3 Future Work

Our finding shows DADS plays a significant role in regulation of TGF-β induced EMT in A549 cell line via both canonical and non-canonical TGF-β pathway.

- However, further studies needs to be carried out by using specific inhibitors to reconfirm the participation of above mention signaling pathway in regulation of TGF-β induced EMT by DADS in A549 cell line.
- Moreover further investigation of efficacy of DADS in vivo and clinical trial are also warranted.

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