Role of *TGF-β* and Wnt antagonist gene *sFRP4* in predicting overall survival and clinic-pathological outcomes in lung cancer patients treated with platinum based doublet chemotherapy

A Thesis
Submitted in the partial fulfillment of the requirement
for the award of the degree of

MASTER OF TECHNOLOGY

IN

BIOTECHNOLOGY

Under the supervision of:
Dr. Siddharth Sharma
Assistant Professor

Submitted by:
Sheetal Vats
Roll No. 601504009

DEPARTMENT OF BIOTECHNOLOGY

THAPAR UNIVERSITY, PATIALA -147004

DECLARATION

I, the under designed, hereby declare that the research work presented in the Master of Technology dissertation entitled **"Role of *TGF beta* and Wnt antagonists gene *sFRP4* in predicting overall survival and clinic-pathological outcomes in lung cancer patients treated with platinum based doublet chemotherapy"** has been carried out by me under the supervision and guidance of **Dr. Siddharth Sharma**, Department of Biotechnology, Thapar University, Patiala. Further, I declare that no part of this dissertation has been submitted for a degree or any other qualification of any university or examining body in India/ elsewhere.

Sheetal Vats
M.Tech (Biotechnology)
601504009
Thapar University

Date: 21 August, 2017
Place: Patiala

CERTIFICATE

This is to certify that dissertation entitled **"Role of *TGF beta* and Wnt antagonists gene *sFRP4* in predicting overall survival and clinic-pathological outcomes in lung cancer patients treated with platinum based doublet chemotherapy"** being submitted by **Ms. Sheetal Vats** towards the partial fulfillment of the requirements for the award of the degree of Maters in Technology in Biotechnology of the department Biotechnology at Thapar University, Patiala is an authentic work carried out by her under my supervision and guidance.

To the best of our knowledge, the matter embodied in this dissertation has not been submitted to any other university/ institute for award of any degree or diploma.

Dr. Siddharth Sharma
Associate professor
Department of Biotechnology
Thapar University
Patiala, Punjab

Ms. Sheetal Vats
M.Tech Biotechnology
(601504009)
Thapar University
Patiala, Punjab

ACKNOWLEDGEMENT

Thanks to Lord Almighty who bestowed me the divine guidance to embark upon this task of keeping in realms of facts and events. The period of this valuable thesis has proved to be extremely important for me as there is no possible way otherwise to feel this much confident for my laboratory skill& management. I am extremely thankful for the guidance and help of several individuals who in one way or another contributed and extended their valuable assistance in the preparation and completion of the study. I would express my genuine, heartfelt gratitude to my guide **Dr. Siddharth Sharma**, Associate professor, Department of Biotechnology, Thapar University, Patiala for his valuable support, thoughtful encouragement and careful supervision without which this work would not have been possible. I would also like to thank him for greatly enriching my knowledge and giving constant inspiration.

I would like to render my sincere thanks to **Dr. Moushumi Ghosh**, Head of the Department of Biotechnology, Thapar University, Patiala, Punjab.

My deep sense of gratitude lies for **Charu Bahl** who spared most of her valuable time in guiding me for this work. Her constant support and guidance has been so fruitful for my thesis. Her thoughtful management, constructive criticism and expedient advice will always inspire me for better laboratory handling and for positive attitude toward work.

My eternal gratitude goes to my affectionate parents and my friends for their never ending support.

Sheetal Vats

Date: 21 August 2017

Place: Patiala.

ABSTARCT

Title: Role of *TGF beta* and Wnt antagonists gene *sFRP4* in predicting overall survival and clinic-pathological outcomes in lung cancer patients treated with platinum based doublet chemotherapy. *TGF-β1* gene is located at chromosome no. 19q13.1-13.39. *TGF-β1* maintains balance between cell renewal and cell differentiation by inhibiting cell cycle progression through G1 arrest. Depending upon the tumor type and stage, *TGF-β* has been reported for tumor suppression as well as for tumor activation properties. *sFRP4* is 346 amino acid long sequence, 10.99 Kb long and located on 7p14.1. They are extracellular glycoproteins which act as Wnt antagonists as they bind with Wnt proteins and block Wnt signaling pathways. Objectives: To investigate the role of *TGF-β1* polymorphism in modulating the survival and clinical outcomes of lung cancer patients and to examine the role of *sFRP4* polymorphisms in affecting the overall survival and prognosis of lung cancer patients. Materials and methods: A total of 186 and 340 cases were genotyped in case of *TGF beta* and *sFRP4* respectively using PCR-RFLP. The overall survival was done using Kaplan-Meier and Cox regression analysis. Results: Subjects with *CA* and *CA+AA* genotype for *sFRP4* rs1802073 and ECOG (0-1) showed significant association with overall survival when analyzed by using univariate analysis (HR=1.97; 95%CI=1.06-3.66; P=0.004 and HR=1.93; 95%CI=1.04-3.56; P=0.005), whereas analysis by Cox regression showed significant association in genotypes *AA* (HR=0.42; 95%CI=0.24-0.72; P=0.001), *CA* (HR=0.43; 95%CI=0.20-0.92; P=0.03) and *CA+AA* (HR=0.46; 95%CI=0.27-0.79; P=0.005). Subjects with ECOG (2-4) *sFRP4* rs1802073 with Cox regression model showed significant for mutant *AA* genotype (HR=2.54; 95%CI=1.33-4.86; P=0.004). Conclusion: Our study demonstrated that *TGF-β1* rs1800469 C/T polymorphism is not an important factor contributing to survival and clinical outcomes of lung cancer patients in north Indian population. On the other hand *sFRP4* rs1802073 ($Pro^{320}Thr$) is significantly contributing in increasing survival and clinical outcomes of lung cancer.

TABLE OF CONTENTS

List of Tables

List of Figures

Abbreviations

PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
BSA	Bovine Serum Albumin
OR	Odds Ratio
OS	Overall Survival
CI	Confidence Interval
SNP	Single Nucleotide Polymorphism
NSCLC	Non-Small Cell Lung Cancer
SCLC	Small Cell Lung Cancer
LC	Lung Cancer
ADCC	Adenocarcinoma
SQCC	Squamous Cell Carcinoma
SCLC	Small Cell Lung Cancer
KPS	Karnofsky Performance Status
ECOG	Eastern Cooperative Oncology Group
CR	Complete Response
PR	Partial Response
SD	Stable Disease
PD	Partial Disease
TNF	Tumor Necrosis Factor
TGF-β	Transforming Growth Factor
CDK	Cyclin Dependent Kinase
ATM	Ataxia Talengiectasia Mutagenesis
EMT	Epithelial to Mesenchymal Transition
ALK	Anaplastic Lymphoma Kinase
JNK	c-Jun N-terminal Kinases
BMP	Bone Morphogenetic Proteins
MSI	Microsatellite Instability
MAPK	Mitogen Activated Protein Kinase
rs	Reference SNP
AP	Activator Protein
HIF	Hypoxia Inducible Factor

Fz	Frizzled
Lrp	Lipoprotein Receptor Related Protein
Dvl	Dishevelled Protein
TCF	T cell factor
WIF	Wnt Inhibitory Factors
sFRP	Secreted Frizzled Receptor Protein
CRD	Cysteine Rich Domain
NLD	Nertin like Domain

CHAPTER 1

INTRODUCTION

Lung cancer (Small cell and non-small cell) is considered as second most common cancer in both males and females. American cancer society estimates about 222,500 lung cancer new cases in 2017. Several risk factors are responsible for occurrence of lung cancer such as exposure of radon, asbestos, arsenic in drinking water, occupational hazards, second hand smoke and the most common factor is smoking (La Vecchia C *et al.,* 2003). Cigarette smoke consists of 60 carcinogenic compounds such as polycyclic aromatic hydrocarbons (benzopyrene), carbon monoxide and phenols. Acrolin has been reported as one of the most abundant aldehyde, reactive, mutagenic compound in cigarette smoking. Research carried out in 20th century revealed that factors at genetic level are casually associated with lung cancer, thus further studies at genetic level would help us to identify determinants of susceptibility of these factors. Signaling pathways plays an important role in pathogenesis of lung cancer and by understanding these pathways we may determine treatment strategies to target molecular aberrations. Depending upon the tumor type, this gene is reported in both tumor suppression as well as in tumor progression activities (Meulmeester E *et al.,* 2011; Tang B *et al.,* 1999). *TGF-β* helps in tumor suppression by regulating cell proliferation through G1 arrest, maintains genomic stability by sensing damaged DNA, apoptosis and senescence impaired phosphorylated factors and by modulating different growth factors in tumor microenvironment .On the basis of cellular and extracellular type, presence of this gene has been reported in tumor activation as well, where it helps in EMT (epithelial to mesenchymal transition), immune invasion and angiogenesis (formation of new blood vessels) (Kretzschmar M *et al.*, 1998).

TGF-β1 is a cytokine belonging to *TGF-β* superfamily and reported as oncogene. *TGF-β* superfamily includes *TGF-β* (1-3), activin, BMP (bone morphogenic proteins), inhibins and many growth differentiation factors such as myostatin, nodal etc. The protein encoded by *TGF-β* is crucial for various functions including apoptosis, cellular homeostasis, cell growth, maintaining balance between cell renewal and differentiation and strongly activated in response to tissue injury (Massague et. al., 1998).

TGF-β1 siganling may takes place in both Smad dependent and Smad independent manner (Massagué J. 2000). *TGF-β1* superfamily bind with high affinity cell surface receptors (Type I and Type II). In Smad dependent pathway, signaling takes place by activating *TGF-β* type I receptor

which promote internalization of receptor in endosome, activate Smad proteins and phosphorylate two serine residues of R-Smads. Association of R-Smads with Smad protein (Smad-4) transport it in the nucleus where GALA-Smad2 complex would activate various transcription factors like Oct-4, Sox-2 and regulate cellular proliferation and differentiation (Liu F *et al.,* 1997). Whereas in Smad independent pathway ration of type I and type II receptor is responsible for compromised tumor suppressor activity. Smad independent pathway is activated through some other pathways like Erk, p38 MAPK kinase and JNK pathways (Yu L *et al.,* 2002). Alteration in *TGF-β* signaling takes place due to mutation or expression loss of TβRII (*TGF-β* receptor II) gene and due to Smad4 mutation in MH2 domain .Alteration in signaling pathway is reported in various human disorders such as homeostatsis of many organs, fibrotic disorders and in improper development of organs and in various human cancers such as breast cancer (Xiao-ou shu et al.,2004), lung cancer (NSCLC and SCLC) (Xiaomin Niu *et al.,* 2012), pancreatic cancer (Wu Gy *et al.,* 2001) and gastric cancer (Shi *et al.,* 2011). TGF β1 has been reported in the radiation related fibrosis development in lung cancer patients.

TGF-β1 is a 25 KDa dimeric polypeptide present in the promoter region. Amino terminal domain of *TGF-β1* is non-covalently attached with mature COOH domain of the protein. The gene is located on chromosome 19q13.1-13.39. Common SNP's of *TGF-β1* which are reported for cancer occurrence are rs1800469, rs1982073, rs4444903, rs4803455 and rs1800470. In the present study, we aim to study the effect of *TGF-β1* rs1800469 on the overall survival of lung cancer patients. *TGF-β1* rs1800469 also known as SNP -1347 C>T or -509 C>T is present in the negative regulatory promoter region of *TGF-β*1. Presence of this genetic polymorphism in the promoter region may change production amount of protein but does not affect nature of protein. Due to genetic changes, T allele is replaced by allele C because of which molecular changes takes place such as recruitment of AP1 binding sites. These molecular changes leads dysregulated blood plasma levels responsible for occurrence of various other human disorders. Increased T allele may elevate *TGF-β1* production which in turn bind with higher number of receptors and dysregulate Smad dependent signaling pathways. Other than lung cancer, this polymorphism has been reported in occurrence of breast cancer in Chinese population, Sweden and Germany population (Jin Q. *et al*., 2004); gastric cancer in German, Chinese (Yuan X *et al.,* 2009) population and pancreatic cancer in Iranian (Faegheh Behboudi Farahbakhsh *et al.,* 2017). Five polymorphisms of *TGF-β1*

have been studied which are located: two at position +869 and +915 in the signal sequence, two at position -800 and -509 in the promoter region and one at position +72 in non-translated region.

Other than *TGF-β,* Wnt signaling is also reported in controlling various cellular processes. Wnt proteins are characterized by their highly insoluble nature due to N- terminal signal peptide and highly conserved cysteine residues that control various functions such as cell fate determination, stem cell homeostasis, embryogenesis, proliferation and differentiation (Storm EE *et al*., 1994). The term Wnt was coined by merging two names, Wingless (Drosophila segment polarity gene) and MMTV (mouse mammary tumor virus) proto-oncogene Int-1. Till date 19 different members of Wnt family has been identified, play common and major role in development and cellular processes. Major role of Wnt proteins is reported in stem cell maintenance where it maintains self-renewable nature of cells by affecting microenvironment through autocrine and paracrine signaling; in embryogenesis by formation of primitive streak and in determining cell fate during skeletal development (Guo X *et al.,* 2004).

Wnt ligands act as growth factors by using any of the three pathways including; Canonical wnt/β catenin pathway, Wnt/planar cell pathway or Wnt/Ca^{2+} pathway. Wnts contain N-terminal signal sequence at their amino termini that target them to endomembrane/ secretary compartment. Among all three, Canonical Wnt/ β catenin pathway is best studied for Wnt signaling in which Wnt ligands bind with LRP (low density receptor related protein) or with Frizzled (Fz) receptor complex (Reya, T *et al.,* 2001), phosphorylate LRP and destruct β catenin which further accumulate in nucleus and activate various transcription genes such as c-Myc, Cox-2 and cyclin D1. Other proteins responsible for activation of canonical pathway are R-spondins. Depending upon the nature different proteins are reported in Wnt signaling pathway act as agonists (Wnt, Norrin and R-spondin) and antagonists (*WIF, sFRP* and *DKK*). In the absence of Wnt signaling, β catenin resides in its complex form consisting *APC, GSK3β, axin* and *CK1* in the cytoplasm, whereas in presence of Wnt signaling pathway Wnt ligands bind with its extracellular transmembrane receptor, destruct and activate β catenin to regulate various cellular functions.

Like all other pathways, genetic changes in the in sFRP may alter signaling pathway which is consider as one of the most important factor of tumor occurrence. Mutation in β-catenin and axin (Dahmen, R. P. *et al.* 2001) are reported in occurrence of medulloblastoma due to dysregulated Wnt signaling pathway. Studies carried out in Wnt1 transgenic mice found that dysregulation in

Wnt signaling pathway may develop mammary tumor. David H. Stewart (2013) use murine models and found role of Wnt pathway in NSCLC development. Various genes were reported responsible for increase (Wnt family, β-catenin, Fz8, TCF-4 etc) and decrease (Sfrp, WIF, DKK, COX-2) in NSCLC expressions. *In vitro* analysis on co-expression of Wnt2 and Fz-8 are reported in Wnt pathway activation.

sFRPs (secreted frizzled receptor proteins) are extracellular proteins act as negative regulator. Till date five sFRPs (sFRP 1-5) has been discovered having 300 amino acid long signal sequence. sFRP 4 is 10.99 Kb long, sited on short arm of chromosome 7 (7p14.1). This gene incorporates 6 coding exons and 6 conserved cysteine residues. All five members of sFRP family shows structural similarity with CRD (cysteine rich domain) ligand binding domain of frizzled family. CRD is one of the most important character of sFRP molecular structure which is characterized by the presence of ten cysteine residues at conserved positions. Presence of sFRP in Wnt signaling pathway may cause morphological alterations by sequestering Wnt through NTR and CRD domain which may increase cytoplasmic level of β-catenin and inhibit activation of various expression genes. Both CRD and NTR are reported in activating Wnt/Ca^{2+} signaling pathway by increasing intracellular Ca^{2+} levels This gene is considered as having tumor suppression potential by decreasing cell cycle progression (Longman D *et al.*, 2012).

Variation at genetic and molecular level in genome generates SNPs (Single nucleotide polymorphism), identified as primary cause of cancer occurrence. Two SNPs are studied for this gene are rs1802073 (Pro^{320} Thr) and rs1802074. sFRP4 rs1802073 is less explored, however a study conducted by Hiroshi Hirata *et al.*, 2014 found that sFRP4 rs1802073 is responsible for renal cell carcinoma occurrence and characterized by polyphen software as probably damaging protein confirmation.

So, the aim of the present study is to understand the relevance of *TGF-β1* rs1800469 (-509 *C>T*) and sFRP4 rs1802073 (*Pro^{320} Thr*) on the overall survival of lung cancer patients among north Indian population.

CHAPTER 2

Review of literature:

2.1 Overview of Lung Cancer

Cancer is a multifunctional disease in which abnormal cells divide continuously and may spread (malignant tumor) or may not spread (benign tumor) to other parts of body. Abnormal cell growth takes place in the malignant cells responsible for causing lung cancer with a potential to spread various other parts of the body. Lung cancer is characterized as abnormal cell growth causing formation of extensive cell mass within the lung tissues with a capability to spread in nearby cells and affect their functions. Risk factors associated with occurrence of lung cancer are:

A) **Tobacco smoking:** 90% male patients and 79% female patients of lung cancer cases are reported due to tobacco smoking. Tobacco smoking includes cigarette, pipes, cigars and bidi (La Vecchia C *et al.,* 2003). Cigarette smoke consist 4000 active chemical compounds out of which more than 60 compounds are carcinogens (Hoffmann D *et al.,* 2001) such as polycyclic aromatic hydrocarbons (benzopyrene), carbon monoxide, aromatic amines (nicotine, ABP), phenols, formaldehyde, ethyl carbamate and acrolin. Acrolin has been reported as one of the most reactive, mutagenic and abundant aldehyde in cigarette smoking (Pleasance *et al.,* 2010).

   Another tobacco containing compound is bidi which is even more harmful than cigarette. Bidi contains abundance of many toxic agents such as nitrosonornicotine (NNN) and 4(methyl-nitrosoamino)-1-(3 pyridol) NNK than cigarette, which contains (6.2-12 µg/g v/s 1.3-58.0 µg/g) (Thorgeirsson *et al.,* 2010).

B) **Occupational hazards:** Various physical, chemical and biological agents along with their mixture are responsible for lung cancer occurrence. Various chemical agents used for industrial purposes as chemical intermediates and monomers causing lung cancer are arsenic compounds), acrylonitrile, Benz[a]anthracene and Benzo[o]pyrene, asbestos, Bis (chloromethyl ether.

   According to IARC (International agency for research in lung cancer) aluminium producing, radon exposing, iron and steel founding industries are responsible for occuence of lung cancer.

C) **Secondhand smoke** (exposure of smokers) also known as passive smoke and involuntary smoke takes place due to either by inhaling smoke exhaled by smokers

(mainstream smoke) or due to smoke given off by a burning tobacco product (side stream smoke). 7000 chemicals has been identified in second hand smoke out of which 250 chemicals are known to be harmful such as ammonia, carbon monoxide, hydrogen cyanide etc.

Table2.1 : Representing various lung cancer causing agents in humans and their exposure types (Source: International agency for research in lung cancer IARC)

Agents	Primary exposure types
Ionizing radiations	
➢ Alpha particles	Environmental, occupational
➢ X-radiation, gamma radiation	Environmental, occupational
Chemicals and mixtures	
➢ Bis (chloromethyl ether)	occupational
➢ Coal-tar pitch	occupational
➢ Diesel exhausts	Environmental, occupational
Occupation	
➢ Aluminium production	Occupational
➢ Coal gasification	Occupational
➢ Coke production	Occupational
➢ Painting	Occupational
➢ Rubber production industry	Occupational
Metals	
➢ Arsenic compounds	Environmental, occupational
➢ Cadmium compounds	Occupational
➢ Nickel compounds	Occupational
Dust and fibers	
➢ Asbestos	Environmental, occupational
➢ Silica dust, crystalline	Environmental, occupational

Personal habits ➢ Coal, indoor emission ➢ Secondhand smoke	 Environmental, occupational Environmental, occupational

2.1.2 Worldwide scenario of Lung cancer:

The cases of lung cancer in last few decades has been increasing immensely all around the world due to various factors such as smoking, chemical exposure etc. According to worldwide scenario of lung cancer shown in figure 2.1, approximately 1.8 million new cases were reported in year 2012. A report by American cancer society report mortality rate in developed (22%) and developing (14%) countries. Out of this 1.8 million population, 58% of lung cancer cases were reported in less developed countries with highest estimated rates in northern America (33.8), Northern Europe (23.7) and with a higher relative rate in Northern Asia (19.2). As compared to all, lower estimated rates were reported in Africa (.9%). Lung cancer is reported as the most common cancer in males, while in females rates are comparatively less.

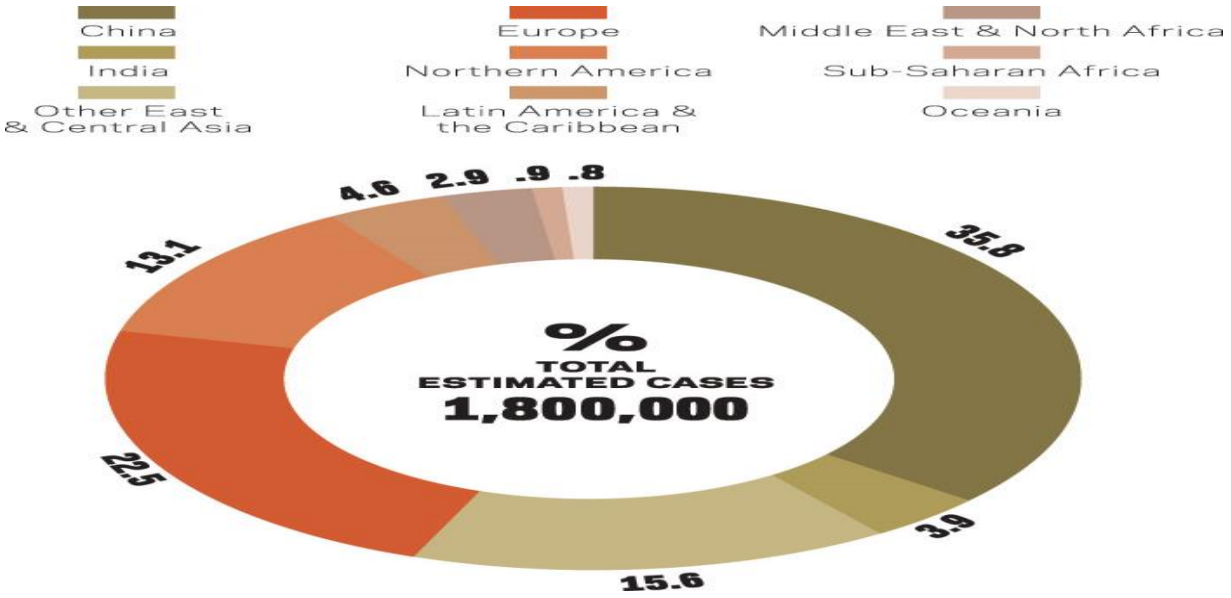


Figure 2.1: Estimated lung cancer cases and their percentage by region (Source: http://canceratlas.cancer.org/the-burden/lung-cancer/)

2.1.3 Signs and symptoms of lung cancer:

Due to abnormal cell growth in lung tissues, formation of solid tumor takes which leads to improper functioning of lung tissues. Major clinical signs and symptoms which may implicate occurrence of lung cancer are:

- ✓ Cough, chest pain, shortness of breath
- ✓ Wheezing
- ✓ Weight loss and loss of apetite, weakness
- ✓ Recurring infections such as pneumonia, bronchitis

2.1.2 Diagnosis of lung cancer:

In order to detect lung cancer and to found stage of cancer, several tests are recommended which includes:

- ✓ CT scan for lung cancer staging
- ✓ Physical examination, CBC, chest X-ray
- ✓ Bronchoscopy, sputum cytology, thoracoscopy, mediastinoscopy to confirm diagnosis

2.1.3 Treatment of lung cancer:

Treatment for lung cancer basically depends upon type and stage of lung cancer. Treatments used for lung cancer are described below:

- ✓ Surgery (lobectomy, wedge resection, pneumonectomy)
- ✓ Chemotherapy
- ✓ Radiation

2.1.4 Types of Lung Cancer

Based upon the morphology of tumor cells there are two main types of lung cancer:

I) Non-small cell lung cancer (NSCLC): Classified on the basis of size of tumor. NSCLC is considered as the most common type of cancer occurs with a rate of 85%. NSCLC consists three subtypes:

a) **Adenocarcinoma:** Most common cancer occurs at a rate of 40% of all lung cancer. This type is more common in females and in younger people. Most common type for smokers as well as for nonsmokers. ADCC takes place in mucus secreting cells and tends to grow slower as compared to other cancer types. People diagnose with adenocarcinoma *in situ* tend to have better prognosis than other patients.

b) **Squamous cell carcinoma:** This subtype of NSCLC accounts for 25%-30% of all lung cancer and becomes second largest histological subtype. Squamous cells are flat cells as shown in figure 2.2, which line airways of lungs and the cancer occurs near a bronchus in the middle of lungs (epidermoid tumor). SQCC is often linked with smoking history and may spread to various other parts of body such as bones, lymph nodes, liver if not treated.

c) **Large cell carcinoma:** This is undifferentiated form of lung cancer accounts for 10-15% of NSCLC includes all other forms of NSCLC other than ADCC and SQCC. This type of carcinoma is difficult to treat as it grows with a faster rate and metastize quickly.

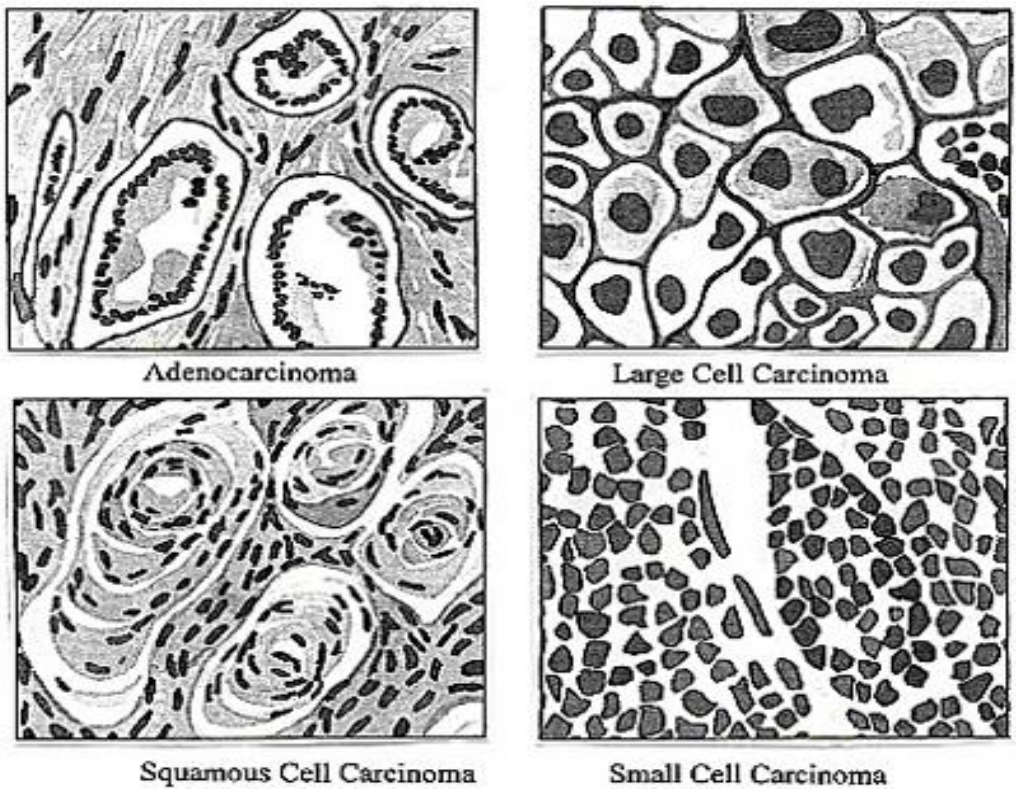


Figure 2.2: Representing microscopic images of different histology of lung cancer (Source: http://www.immunerecovery.net/lung_cancer.html)

II) Small cell lung cancer (SCLC):

Classified as small cell on the basis of morphology because of the size of cancer cells under microscope observation. It is also known as oat cell carcinoma and small cell undifferentiated carcinoma. This form of cancer is very rarely associated with nonsmoking persons. SCLC commonly starts in bronchi region and spreads very quickly.

2.2 Transforming Growth Factor-β1 (*TGF-β1*) gene:

Transforming growth factor beta is a cytokine type that belongs to *TGF-β1* superfamily. This superfamily includes *TGF-β1, TGF-β2* and *TGF-β3.* It controls various cellular functions such as cellular proliferation and differentiation, embryonal development, various immune functions, cell adhesion and interactions with extracellular matrix (Massague et. al., 1998). TGF-β1 is a 25 KDa dimeric polypeptide. Amino terminal domain of TGF-β1 is non-covalently attached with mature COOH domain and it is located on chromosome 19q13.1-13.39.

2.2.1 Functions of *TGF-β1* gene:

a) Controls tissue homeostasis
b) Proliferation, maintain balance between cell renewal and cell differentiation (Massague et. al., 1998)
c) Universal characteristics of human epithelial tumors
d) Strongly activate in response to tissue injury
e) Inhibit growth of ectodermally derived cells (Nørgaard P *et al.*, 1996, P Nørgaard et al 1994)
f) Arrest cell cycle in hematopoietic and mesenchymal cells

2.2.2 Mechanism *TGF-β1* signaling in tumor suppression:

The genetic evidence from human tumors shows that *TGF-β1* plays its role in tumor suppression and tumor promotion. *TGF-β1* achieves its tumor suppressive effects by:

a) **Cell proliferation regulation arm:** By inhibiting cell cycle progression through G1 arrest, *TGF-β* regulates cell cycle proliferation. This process in epithelial cells takes place in coordination with CDK inhibitors (p21cip1 and p15Ink4b) and by suppressing proliferative drivers such as c-Myc and ID. In the whole process of cell proliferation, *p21cip1* and *p15Ink4b* inhibits interaction between cdk-2-cyclin E/A. This complex further results in dephosphorylation of *pRB* and inhibits progression of G1 to S phase (Meulmeester E *et al.*,

2011). Along with p21cip1 and p15Ink4b, Smad 3 and 4 form a complex with various other transcription factors such as Fox O and Sp1 which inhibits process of cell proliferation (Pardali K *et al.,* 2000). *TGF-β* gene inhibits nuclear factors ID1, 2 and 3 which plays important role in cell differentiation and progression.

b) Genomic stability: *TGF-β* maintains genomic stability and thus become important in tumor suppressor activities. *TGF-β* sense DNA damage and functions as an extracellular sensor of DNA. Inhibition of *TGF-β1* receptor has been reported in impaired phosphorylation which may affect various checkpoints including ATM, chk2 and p53. These unregulated checkpoints increase radiosensitivity of cells as compared to *TGF-β* competent cells. On the other hand, Smad-4 demonstrates a significant role in maintaining genomic stability by regulating BRCA/DNA repair pathway (Bornstein S *et al.,* 2009).

c) Apoptosis and senescence: Role of *TGF-β* has been reported in both induction as well as in suppression of apoptosis, on the basis of cellular and extracellular factors. *In vitro* studies were performed to prove this cell dependent characteristic of *TGF-β* and found that in hematopoietic cell lines this gene suppress apoptosis through PI3K-AKT pathway whereas in HCC cell lines, it induce death associated protein kinases and thus cause apoptosis . Various other genes affected by *TGF-β* are DAXX, FAS, BIM and various other members of BCL2 family (Siegel PM *et al.,* 2003).

d) **Tumor microenvironment:** Role of *TGF-β* in tumor suppressive activities by modulating growth factor has been reported in mammary glands. *In vitro* studies shows that overexpression of dominated negative T*βRII* increase expression of hepatocyte growth factor in stroma of mammary gland which results in proliferation of mammary cells by increasing lateral branching of adjacent mammary ducts (Joseph H *et al.,* 1999).During tumorigenesis, *TGF-β* suppress immune and inflammatory process and inhibit CD^{4+} and T_H cells, CD^{8+} and T_C cells, dendritic cells, macrophages and NK cells. Due to immune tolerance, *TGF-β* disruptive signaling is considered as molecular mechanism in pre-cancerous inflammatory disease pathogenesis.

2.2.3 Mechanism *TGF-β1* signaling in tumor progression: Genetic changes takes place due to tumor are responsible for escaping tumor suppressive mechanisms and thus results in tumor metastasis and progression. Various factors responsible for tumor progression are:

a) **Epithelial-to-mesenchymal transition:**

EMT is a reversible method in which cells loose epithelial properties and start attaining mesenchymal characteristics. This process takes place during wound healing, tumorigenesis and in pathological process of fibrinogenesis. *In vitro* studies has been done to find correlation between Smads (Smad-3, 4 and Smad-7). In response to *TGF-β,* reduced expressions of Smad 3&4 (co-smads) shows significantly reduced EMT expressions, while higher Smad-7 (Smad inhibitor) along with higher EMT has been studied (Tang B *et al.,* 1999).

The reversibility of EMT process depends upon cytokines and *TGF-β* level. Besides acquiring properties of mesenchymal cells, epithelial cells does acquire stem cell characteristics under *TGF-β* influence (Kretzschmar M *et al.,* 1998), where stem cells increase proliferation and progression of tumor cells.

b) **Immune evasion:** *TGF-β* results in immuneosuppression which results in inhibition of various immune cells. After genetic changes in cells, *TGF-β* suppresses transcription of pro-apoptotic factors, FAS ligand, interferons and many other immunologically active cells. Due to inhibition of antigen processing cells, T cells become immunosuppressive which results in inhibition of apoptosis and thus enhance proliferation and metastasis of tumor cells. *TGF-β* inhibition has been reported in increasing NK-cells activity and in suppression of metastasis of cancer cells (Lee JC *et al.,* 2004).

c) **Metastasis and angiogenesis:** *TGF-β* promotes angiogenesis through vascular endothelial growth factors (VEGF) and connective tissue growth factor (CTGF) (Kang Y,*et al.,* 2003) where amplification of angiogenesis results in impairment of *TGF-β* signaling process.

Tumor metastasis involves tumor vascularization, cellular growth, cell survival at distant sites, interaction with the microenvironment and intravasation into blood or lymphatic vessels. Genetic changes in tumor cells leads to unregulated signaling pathway and occurrence of all these factors further results in metastasis of tumor cells.

2.3. Signaling of Transforming Growth Factor beta 1 gene:

Irregular signaling of genes may upregulate or downregulate the signaling pathway which is responsible for various human diseases. Role of *TGF-β1* gene is reported for both tumor suppressor and tumor enhancing activities, where upregulation of this gene may cause cancer. *TGF-β1*

superfamily signaling may take place in both Smad dependent and Smad independent manner [Zhou Y *et al.*, 2016]. Alteration of *TGF-β1* signaling pathway is reported for various human disorders such as fibrotic disorders, inflammatory disorders affect development and homeostasis of many organs.

2.3.1 Smad dependent *TGF-β1* signaling pathway:

There are eight Smads (Smad 1-8) which are intracellular proteins. The product of *Drosophila* gene *Mad (mothers against dpp*) is the founding member of SMAD family which further helped in identification of various other related genes. Smad 1, 5 and 8 are activated by ALK1, ALK2, ALK3 and RIB/ALK6. Smad 2 and 3 are activated by activin and *TGF-β* receptors ActRIB and TβR1 (Itoh *et al.*, 2000; Moustakas *et al.*, 2001; Derynck *et al.*, 1997) and with transforming growth factor beta1 to activate downstream gene transcription and transduce extracellular signals. Smad 6 and Smad 7 are inhibitory Smads which inhibit phosphorylation of effector Smads through their binding of MH2 domain with type I receptor as shown in figure 2.3. Various studies were done to find the interaction between *TGF-β* and Smads and the most coercive evidence came in sight was found in response to *TGF-β*, Smads are phosphorylated and become transcriptionally active (Zhang Y *et al.*, 1996).

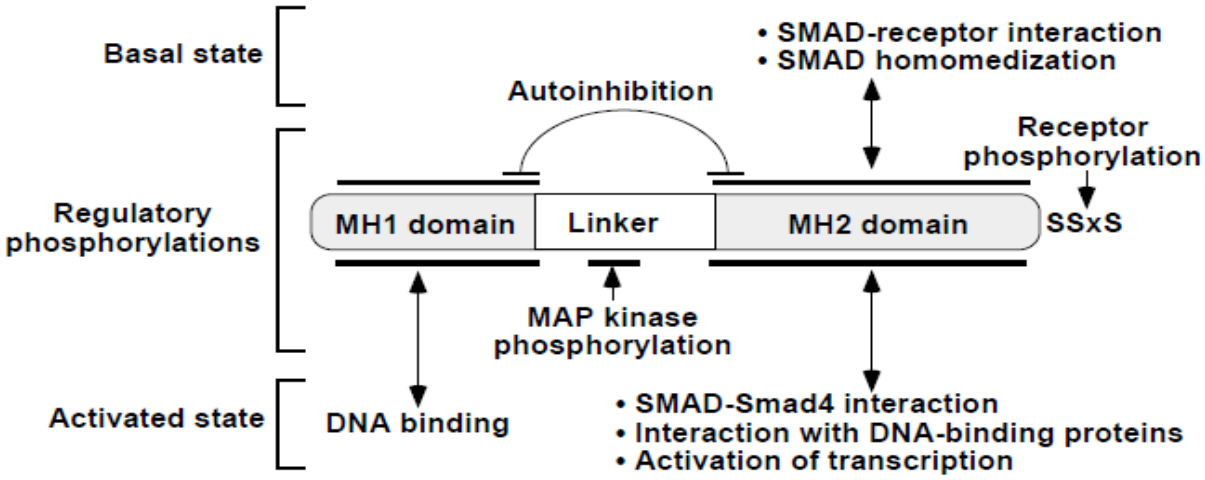


Figure 2.3: In the basal state, SMAD remains inactive where MH1 domain and MH2 domain are interacting with each other. Receptor mediated phosphorylation takes place at C-terminal motif when activated type I receptors interact with receptor regulated SMAD which results in activation

of SMAD. In the activated state SMADs associate with Smad4 (co-Smad) and various DNA binding proteins and performs transcriptional activities (J. Massague; 1998)

a) Activation of Type I receptors:

TGF-β superfamily ligands bind with high affinity Type I and Type II cell surface serine-threonine kinase receptors to produce cellular effects such as cellular proliferation and differentiation. Type I (55 KDa) and Type II (70 KDa) receptors are categorized as glycoproteins of having 500 and 570 amino acids respectively (ten Dijke P *et al.,* 1993). As shown in figure 2, binding of ligand of dimeric nature induces heterotetrameric receptor complex formation between type I and type II receptors (Ebner R *et al.,* 1993). Type II receptors remains constitutively active and transphosphorylate type I receptors by phosphorylation in GS rich domain of juxtamembrane of TβR1 at serine and threonine in TTSGSGSGLP sequence. Signaling takes place only when type I and type II receptors are brought together (Okadome T *et al.,* 1994).

b) Smad activated *TGF-β* signaling:

TGF-β ligands promotes internalization of receptor in the endosomes for Smad dependent signaling pathway activation (Penheiter *et al.,* 2002). Smad binding specificity and receptor signaling is mainly determined by type I receptor kinase L45 loop having nine amino acids sequence which interacts with MH2 L3 loop of R-Smads. Activated Type I receptor kinases phosphorylate R-Smads and 2, 3 Smad protein receptor residues at the carboxy terminus SSXS motif. After phosphorylation, dissociation of R-Smad and type I receptor takes place which allows conformational changes and transition from unphosphorylated monomeric R-Smad to oligomeric complex (Wu, J. W. et al 2001; Chacko, B. M. et al 2001).

Activated Smads associate with co-Smad (Smad4) and translocate this complex to the nucleus, *TGF-β* phosphorylated receptors remains active for 3-4 hours which maintains this complex in the nucleus and regulate gene expressions. R-Smads keeps dephosphorylated in the continuous manner in the nucleus which results in Smad complex dissociation and keep exporting inactive Smad to the cytoplasm (Xu L et al.,2002).In the nucleus Smad activate transcription process by GAL4-Smad2 complex formation in response to *TGF-β* which also require Smad4.

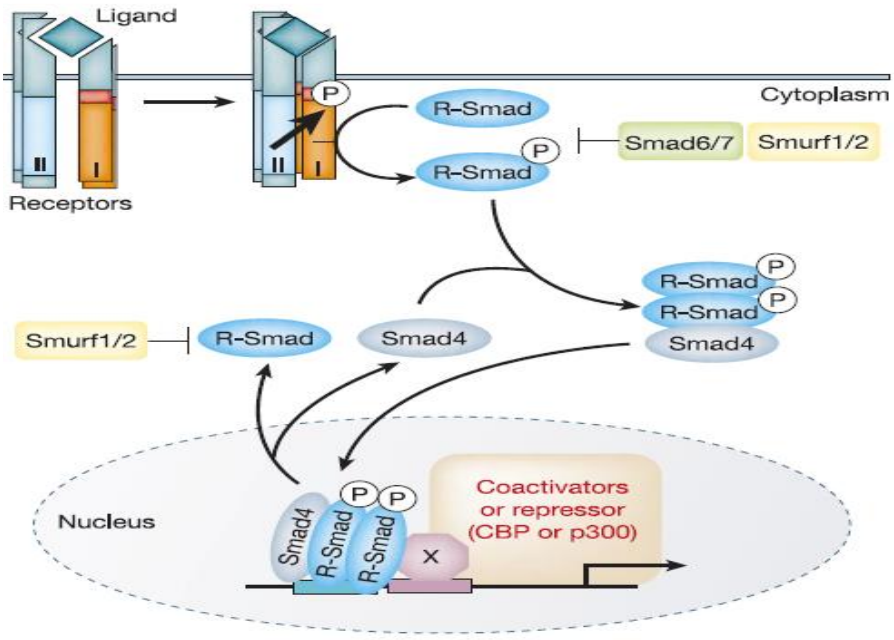


Figure 2.4: Ligand binds with serine-threonine transmembrane kinase receptors, where type II receptors induces transphosphorylation in type I receptors. Activated type I receptor further phosphorylate selected Smads (depend upon the ligand) and these receptor activated R-Smads form a complex with Smad4 which further translocate to nucleus, bind with different DNA binding transcription factors, p300 and CBP coactivators and regulate transcription of various genes (Derynck R *et al.*, 1997).

2.3.2 Smad independent *TGF-β1* signaling pathway:

Other than Smad, *TGF-β* can also activate some other pathways such as Erk, p38 MAPK kinase and JNK pathways. MAPK signaling pathway activation takes place in response to mutated *TGF-β* Type I receptors, defective in Smad activation (Yu L *et al.*, 2002). Rapid activation by *TGF- β* takes place in 5-15 minutes in Smad independent manner through MAPK pathway (Massagué J. 2000). In epithelial cells *TGF-β* rapidly activate Ras which further initiate Erk MAPK signaling pathway. In response to many stimuli such as BMP and *TGF-β* activate MAPK kinase kinases (MAPKKKs) which further activate p38 MAPK and JNK signaling pathways as shown in figure 2.5.TAK1 is a serine threonine protein kinase which regulates the activities of nuclear factor-kappa beta and MAPK. Role of TAK1 is reported in NSCLC development and progression.

TGF-β induced activation of all above mentioned signaling pathways results in induction of *TGF-β1*expression which amplify *TGF-β* response and induce secondary *TGF-β* responses. The dual ability of *TGF-β* plays important role in ETS (epithelial to mesenchymal transformation) which also depends upon Erk and p38 MAPK signaling pathways (Zavadil, J. *et al* 2001).

Binding of Smads also affect *TGF-β* responses such as binding of Smad 7 with JNK affects its activity by enhancing JNK signaling pathway. JNK signaling regulate interaction of c-Jun and co-repressors, whereas this complex inhibit Smad2 signaling (Pessah, M. *et al* 2002). Thus for cellular response of *TGF- β* proper balance is desired between MAPK pathways and direct activation of Smads.

Other than MAPK, *TGF- β* can rapidly activate some other pathways (as shown in figure 2.5) such as Rho-like GTPase's, plays very important role in mediating pro-tumeriogenic effects of *TGF- β*.

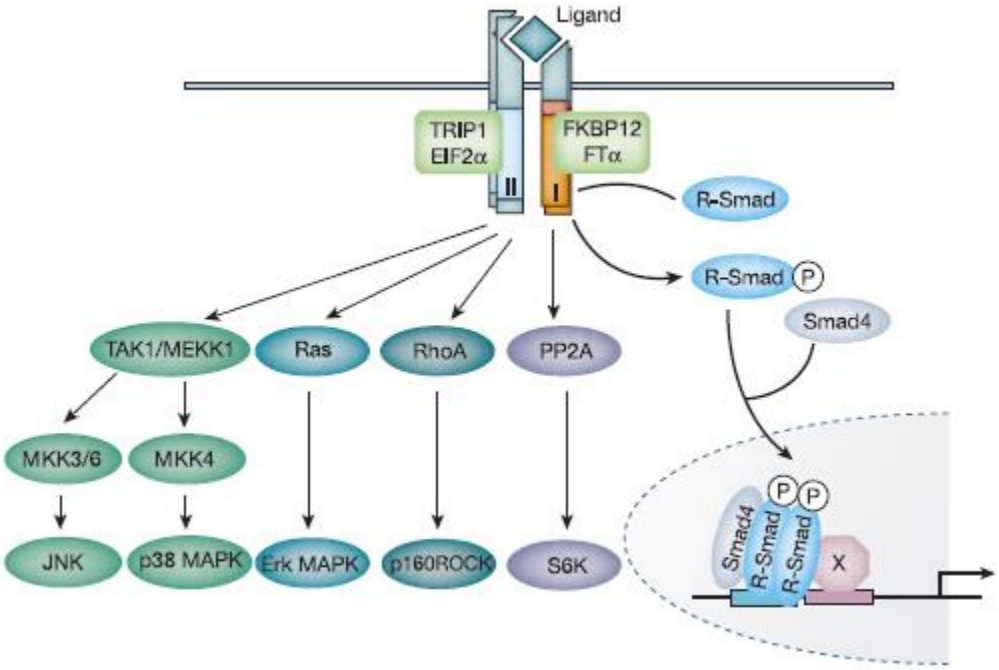


Figure 2.5: In Smad independent signaling pathway activation of *TGF-β* ligand-receptor interaction takes place by the means of various others signaling pathways, such as TAK1/MEKK1, Ras, RhoA, MAPK and PP2A.

2.4 *TGF-β* signaling pathway alterations and lung cancer:

TGF-β receptor alteration is one of the major factor for tumor occurrence (Laurence Levy *et al.*, 2006). *TGF- β* type II receptor (TβRII) gene alteration is responsible for causing non-small cell lung cancer as well as for small cell lung cancer (Tani M *et al.*, 1997). Most commonly mutated *TGF-β* gene causing lung cancer are TβRII and Smad 2, 4.

2.4.1. Alteration due to TβRII gene:

TβRII gene consists of 10 base pair poly-adenine repeats called as BAT-RII which act as hotspot for mutation in this gene (Markowitz S *et al.,* 1995). This gene has been mapped on 3p. Loss of heterozygosity in 3p is frequently observed in various cancers including NSCLC, SCLC and gastric cancer (Guo RJ *et al.*, 1998). Mutation in this gene has been observed because of mutation mismatch repair system (MMR) in microsatellite region and cause microsatellite instability (MSI). MMR inactivates those protein complexes which perform their function during DNA replication and helps in repairing base pair mismatches. This replication error started accumulating short sequences and affects mono, di- and tri nucleotide tracts and thus BAT-RII. Due to these replication errors, non-sense mutation will takes place in which either one or two adenine get inserted or deleted, or mutation occurs in both serine-threonine kinase or in transmembrane domain (Parsons R *et al.,* 1995). Defective TβRII lacks both of them, hence affect phosphorylation and whole signaling process which results in altered i.e. truncated protein formation.

Besides mutation in the TβRII coding region, expression loss of TβRII due to A-G mutation at 5'untranslated region at position -364 results in reduced TβRII mRNA, decreased transcriptional activity and thus downregulated TβRII expressio. In NSCLC, CpG methylation in TβRII promoter region accounts for downregulation and loss of expression of TβRII (Zhang HT *et al.*, 2004).

2.4.2 Smad alteration and lung cancer:

Smad4 mutation takes place in MH2 domain (codon clustered after 200 in C terminal) of protein which helps in oligomerization of Smad4 with R-Smads (Shi Y et al 1997). Loss of heterozygosity on chromosome 18q21 does possess at least 2 lost regions, DPC4 and MADH4. Most frequent mutation occurs in SMAD4 is non-sense and frameshift mutation results in truncated protein generation which lowers down SMAD4 protein stability. This abnormal protein will be unable to bind with Co-Smad, hence affect signaling process (Maurice D *et al.*, 2001)

Missense mutation is also reported in N-terminal of MH1 domain and MH2 domain which cause:

a) Homo-oligomerization of Smad4 (Hata A *et al.*, 1997)
b) Generate a dominant and autoinhibited form of Smad4 which disrupt interaction of Smad4 with Smad2
c) Generation of unstable protein which will degrade rapidly without completing signaling pathway (Xu J *et al.*, 2000)
d) Disrupt signaling pathway by inhibiting DNA binding and by inhibiting nuclear translocation (Moren A et al 2000)

2.5 *TGF-β1* rs1800469 polymorphism mechanism and splicing:

rs1800469 also known as SNP -1347 C>T or -509 C>T is present in the negative regulatory promoter region of *TGF-β1* gene. Presence of this genetic polymorphism in promoter region may change the production amount of protein but not the nature of protein. Due to replacement of allele T by allele C, molecular changes takes place. C version normally downregulate production of protein, whereas in T version increased *TGF-β1* amount produced due to recruitment of AP1 binding sites. It depends upon cancer type, whether increased *TGF-β1* is good or bad as this gene act as both tumor suppression and tumor activation.

Activator protein1 (AP1) and hypoxia inducible factor (HIF-1α) are the key regulators of *TGF-β1*. AP1 is comprised of heterodimers of c-Fos with either JunB, JunD, c-Jun or it may be comprised of homodimers of JunD. AP1 along with JunD cause downregulation of -1347C and expression of other genes such as p19, IL-6, and transglutaminase1. In healthy conditions, AP1 bound with -1347C, however polymorphism of allele T activate HIF-1α responsible for competition between HIF-1α and AP1 for binding sites. Various studies demonstrate that it is recruitment of AP1 responsible for expression difference between -1347T and -1347C.

AP1 binding takes place in the promoter region palindromic sequence ACTCAGT and nucleotides act as binding site for HIF-1α are similar in the reverse orientation. Replacement of allele C with allele T may cause molecular changes, recruit AP1, allows binding of HIF-1α and increase production of *TGF-β1* in the promoter region. Variations are reported among individuals with risk, outcome of numerous diseases due to two fold difference in the plasma levels like immune system disorders, during transplantation and reported in the progression of diseases such as in carcinoma.

To confirm the molecular process, Shah *et. al.*, 2006 use -1347C and -1347T labelled probes and antibody mediated competition was performed. The data outcomes suggest competition between AP1 and HIF-1α for overlapping binding sites on -1347 probes.

Table 2.2: Polymorphism of gene *TGF-β1* rs1800469

Population	**Type of cancer**	**Functional effects**	**Reference**
Chinese population	Breast cancer	Increased Overall survival	D.J. *et al*.,2007
German	Gastric cancer	Improvement in TNM staging	Guan, X *et al.,* 2009
Southern Chinese population	Non-small cell lung cancer	No significant association was observed in radiation pneumonitis risk among NSCLC cases treated with radiotherapy	Xiaomin Niu *et al.*, 2012
Chinese population	Non-small cell lung cancer	Lower radiation pneumonitis probability after chemotherapy for NSCLC	Yuan *et al.*, 2009
Iranian population	Pancreatic cancer	No significant association was found between geme polymorphism and risk of cancer occurrence	Faegheh Behboudi Farahbakhsh *et al.*, 2017
German population	Pancreatic head cancer	Polymorphism of this gene does not influence risk of pancreatic head cancer development	Wu Gy *et al.*, 2010

2.6 Wnt protein secretion:

Wnt proteins consist high number of cysteine residues conserved pattern along with an N-terminal signal peptide which targets Wnt to endomembrane/ secretary compartment. Wnt proteins are characterized by their highly insoluble nature and for activation of Wnt proteins particular modification i.e. cysteine palmitoylation takes place. Wnt signaling is highly conserved system that controls various functions such as tissue and stem cell homeostasis, embryogenesis, proliferation, differentiation, cell fate determination, normal physiology processes in adult tissues

and control cancer by the means of signaling pathways (M. Kleber *et al.*, 2004; W.J. Nelson *et al.*, 2004).

The term Wnt was coined by merging two names, first is Wingless (Drosophilla segment polarity gene) and another is MMTV (moue mammary tumor virus) proto-oncogene Int-1. To date, 19 members of Wnt protein family with highly conserved cysteine residues are identified which is thought to play major role in developmental and cellular processes. Secreted Wnt ligands act as growth factor by using three different pathways:

a) Canonical Wnt/ β catenin pathway
b) Wnt/ Ca^{2+} pathway
c) Wnt/ planar cell pathway (PCP)

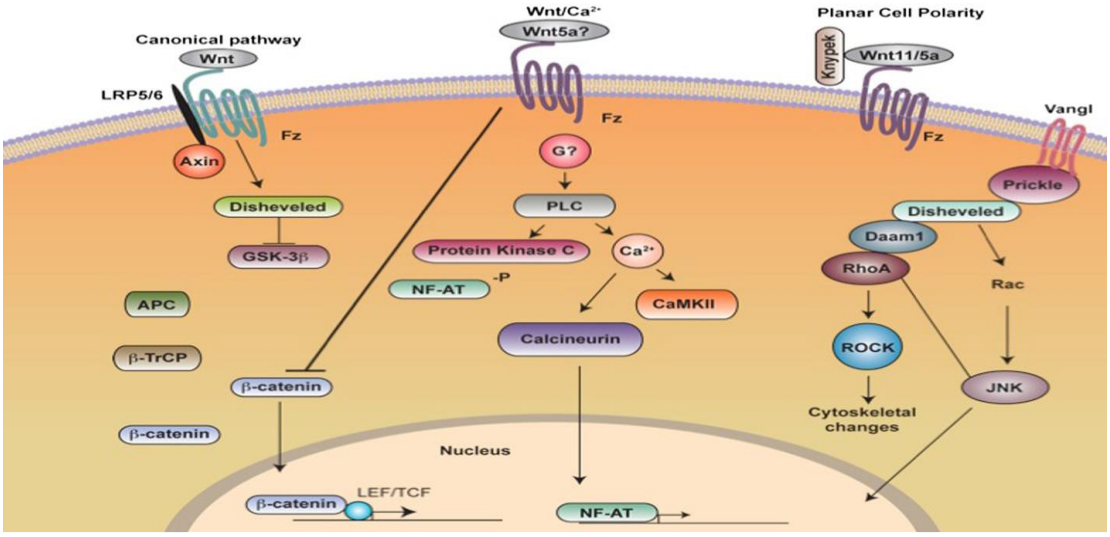


Figure 2.6: Depicting three pathways involved in Wnt signaling (Yingzi Yang 2012)

Out of these three, Canonical Wnt/ β catenin signaling pathway is best studied for Wnt signaling pathway.

2.6.1 Canonical Wnt/ β catenin pathway:

In the Canonical Wnt/ β catenin pathway, Wnt ligands bind with Frizzled (Fz) or low density lipoprotein receptor related protein (Lrp) receptor complex.

i) In the absence of Wnt signaling:

Intracellular levels of β catenin are regulated by APC, GSK3β and CK1 (casein kinase 1), axin1 and axin2 (altogether known as destruction complex) in the absence of Wnt signaling. This destruction complex binds and phosphorylates β catenin residues (serine and threonine) and promotes its ubiquitination and proteolytic degradation as shown in figure 2.7.

ii) In the presence of Wnt signaling:

In the presence of Wnt signaling, Wnt ligands bind with Frizzled and LRP receptor complex, stimulates Dvl (Dishevelled protein) and Fz binding in cytoplasmic receptor domain which further leads to phosphorylation of Lrp. Phosphorylated Lrp promotes β catenin consisting axin, APC and Gsk3β destruction. After this destruction, β catenin accumulates in cytoplasm and then translocated in the nucleus where it replace Groucho and bind with TCF.β catenin and TCF act as transcriptional activator and promotes expression of of Wnt target genes as shown in figure 2.6 genes such as c-myc, cox-2 and cyclin D1.

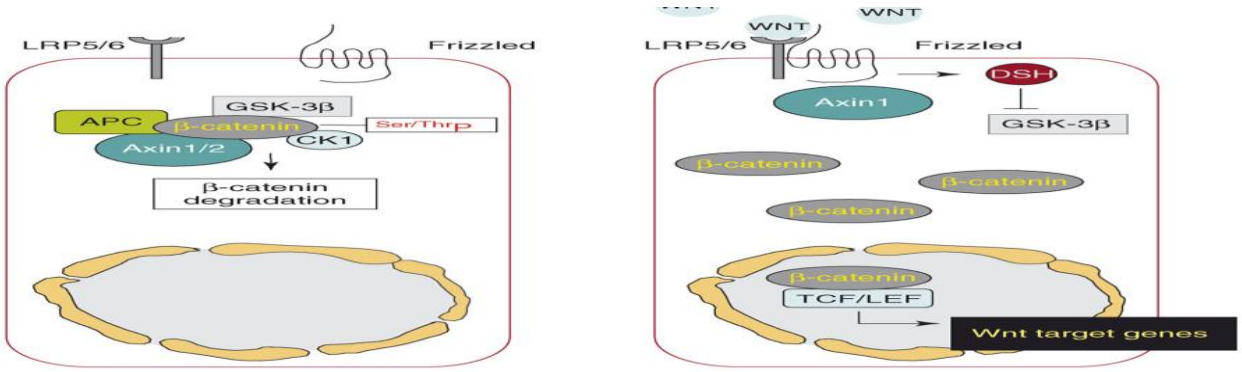


Figure 2.7: canonical Wnt signaling pathway in absence and presence of Wnt protein (Riccardo fodde *et al.,* 2007)

2.6.2 Receptors, agonists and antagonists of gene:

Wnts bind with **Frizzled (Fz)** proteins via single pass transmembrane molecule of LRP family known as LRP-5 &6. Frizzled proteins are characterized as seven pass transmembrane receptors having an extracellular N- terminal cysteine rich domain. Other than Fz, **Derailed** also act as

receptor for DWnt5 which is a transmembrane tyrosine kinase receptor and binds with Wnt via an extracellular WIF domain.

Other factor which promote activation of Canonical Wnt/ β catenin pathway is thrombospondin domain containing **R-spondin** proteins. Kim *et al* identify human R-spondin as a strong Wnt pathway activating protein to stabilize β catenin by promoting proliferation of intestinal crypt cells. **DKK** proteins act as antagonist by inhibiting Wnt signaling pathway (Glinka *et al.,* 1998). DKK1 promotes internalization and inactivation of LRP6 by their crosslinking with kremens (another class of transmembrane molecule).

sFRP (secreted Frizzled receptor proteins) are found to be structurally similar with CRD ligand binding domain of Frizzled family of Wnt receptors. Likewise **WIF** (Wnt inhibitory factors) proteins are similar to Derailed extracellular portion and act as antagonists for Wnt signaling pathway.

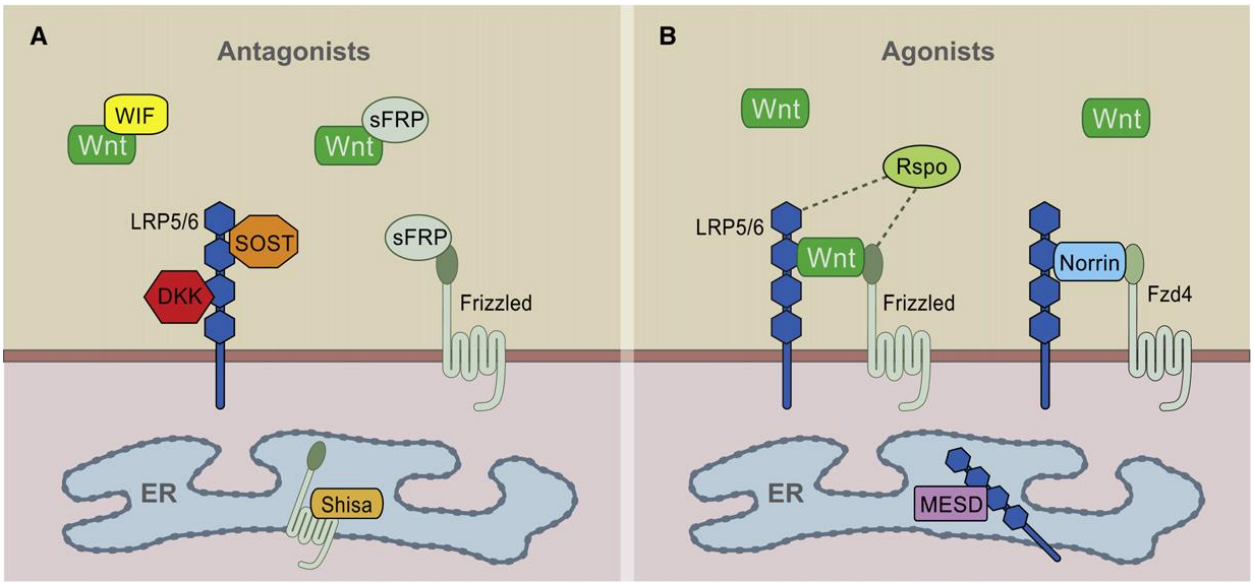


Figure 2.8: representing agonists and antagonists of secreted Wnt

(Antagonists: WIF, *sFRP*, DKK) and (Agonists: Wnt, norrin, R-spondin)

2.7 Functions of Wnt protein: Canonical Wnt signaling pathway control various cellular processes by differentiation and proliferation of genes.

i) Role of Wnt signaling in stem cell maintenance:

Stem cell possess capacity of multi-differentiation and self-renewal capacity of most adult epithelial tissues. Stem cells are present in the specialized niche, and Wnt signaling pathway maintains self-renewable properties by effecting microenvironment in the form of autocrine and paracrine signaling (Taipale, J. & Beachy *et al.*, 2001; Reya, T *et al.*, 2001). Indeterminate property of stem cells makes them key component for mutation accumulation, which may further lead to tumor initiation and metastasis. Wnt signaling pathway plays important role in promoting self-renewable properties of embryonic stem cells (ESC's) which are pluripotent in nature and give rise to all the cell organs.

ii) Role of Wnt signaling in embryogenesis:

The primitive streak, a transient structure marks the initiation of gastrulation process which is ultimately responsible for whole developmental process by embryogenesis. Formation of primitive streak is regulated by various signaling pathways such as Notch, bone morphogenetic proteins and Wnt signaling pathway. Wnt signaling create gradients and various transcription factors such as c-myc, cyclinn D1 and cox2 (as shown in figure 2.5) for regulation of embryogenesis.

iii) Role of Wnt signaling in cell fate determination during skeletal development:

Canonical Wnt signaling pathway plays an important role in osteoblast differentiation and joint formation. To understand these functions, Yingzi Yang, 2012 used top Gal Wnt signaling reporter mouse strain, where lac Z represents Wnt signaling activity. They found that during joint formation, Gdf5 (joint marker) marked X-gal is upregulated (Storm EE et al.,1994) shows how the gradients of β catenin maintains endochondral ossification and joint formation (Guo X, Day TF *et al.*, 2004). To promote differentiation of chondrocytes during endochondral ossification Wnt signaling kept low while upregulated for membranous ossification as shown in figure 2.9.

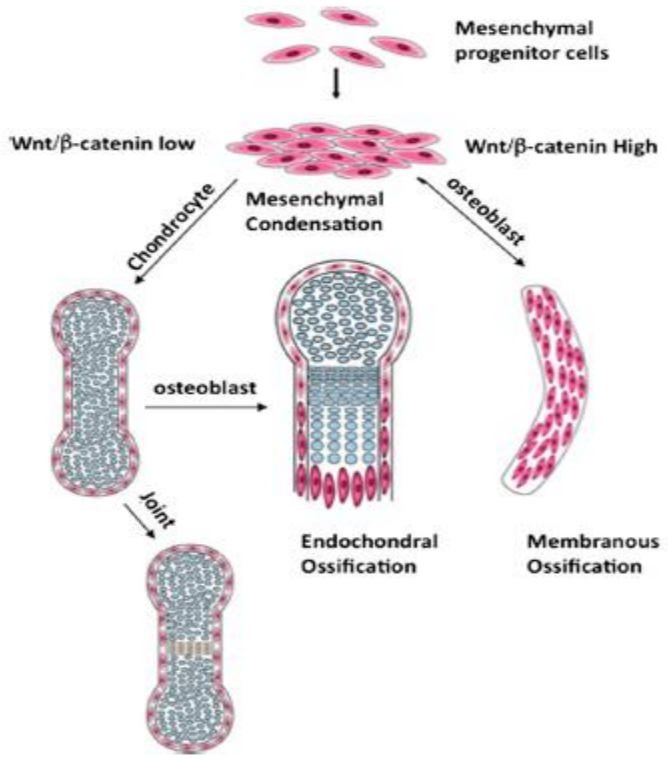


Figure 2.9: Depicting importance of gradients of Wnt signaling pathway in joint formation and membrane ossification during skeletal development (Yingzi Yang 2012)

2.8 Wnt signaling and cancer:

As we have discussed above, Wnt signaling pathway is a key element for cell maintenance by controlling various cell cycle regulating genes expressions. Any genetic change in genes responsible for regulation of this pathway may leads to unregulated gene expressions which is consider as one of the important factor for tumor occurrence.

Dysregulated Wnt signaling and occurrence of cancer is supported by fact that mutation in β catenin (Zurawel, R. H *et al*., 1998) and axin (Dahmen, R. P. *et al.* 2001) are responsible for occurrence of medulloblastoma (a cerebellum brain tumor), by increasing cycling and expansion of neuronal progenitor cells. Furthermore, studies were carried out in Wnt1 transgenic mice, where dysregulated Wnt in mammary progenitor cells develop mammary tumors, where these tumors possess very high frequency of progenitors and stem cell properties (Liu, B. Y. *et al.*, 2004). Hence we can opt, Wnt signaling pathway involving β catenin as a key element in maintenance of variety of systems, and how dysregulation in Wnt pathway cause cancer as shown in figure 2.10.

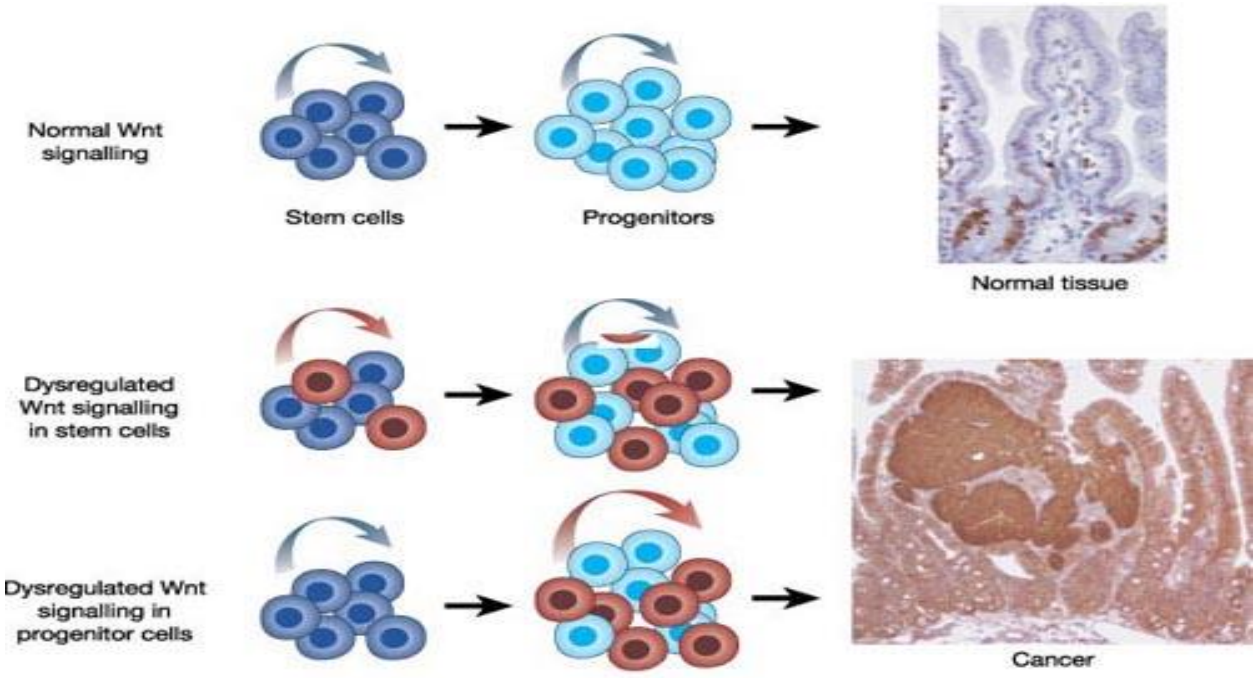


Figure 2.10: Normal Wnt signaling as shown in blue arrow leads to normal proliferation and formation of normal tissue, but dysregulated Wnt signaling as shown in red arrow may cause constitutive renewal in stem cells and in progenitor cells for long term survival which may lead to occurrence of cancer (Tannishtha Reya *et al.*, 2013)

2.9 Wnt signaling and lung cancer:

The key element considered as occurrence of lung cancer is change at genetic or molecular level in the genes involved in regulation of cell signaling pathways. Studying cancer at molecular level, would help us to identify primary causes of genes which could be targeted by drugs, furthermore it may help us in designing of drugs to modify genes expressions (Vaugha AE Halbert *et al.*, 2012**).** David H. found role of Wnt pathway in NSCLC development by using murine models. Role of Wnt pathway genes in tumorigenesis is reported by using Kras transgenic mice and by using Wnt mutant mice.

Various genes responsible for increase and decrease in expressions of NSCLC are shown in table 2.3.

Table 2.3 Wnt pathway alterations in NSCLC			
Increased expressions in resected NSCLC	**References**	**Decreased or inhibitory expressions in resected NSCLC**	**References**
Wnt 1, 2, 3, 11	Teng Y *et al.,* 2010	Wnt-7a	Barker *et al.,* 2006
β catenin	W. J. Nelson *et al.,* 2000	*sFRP*1, 2, 4, 5	Zhou J *et al.,* 2016
TCF-4	Xu HT *et al.,* 2007	DKK-1, 3	Jung IL *et al.,* 2010
Porcupine	Bartling B *et al.,* 2007	Axin	Huang SM *et al.,* 2010
Pygopus-2	Liu Y *et al.,* 2013	APC	Pecina-slaus N *et al.,* 2011
Dishevelled 1, 2 3	Ma L *et al.,* 2010	COX-2	Barker N. *et al.,* 2006

Other than genes promoting lung cancer by promoting Wnt pathway are Sulf-1, Sulf-2, mi-RNA and percostin. *In vitro* analysis of NSCLC cell lines states that co-expression of Wnt2 and FZD-8 activate Wnt pathway. Wnt3 work along with Ki67 and increase c-myc and survivin expressions which results in poor prognosis. Wnt-5a works in non-canonical manner decrease cadherin expressions, cellular adhesion and block canonical Wnt signaling pathway. Wnt-5a expressions are reported in smokers where this pathway cause squamous cell carcinoma. Cigarette smoking exposure causing lung adenocarcinoma is regulated by mi-RNA which in turn affect expression of Wnt-5a as shown in figure 2.10. Wnt-5a may block Wnt canonical pathway but in turn activate process of differentiation and proliferation by activating other genes

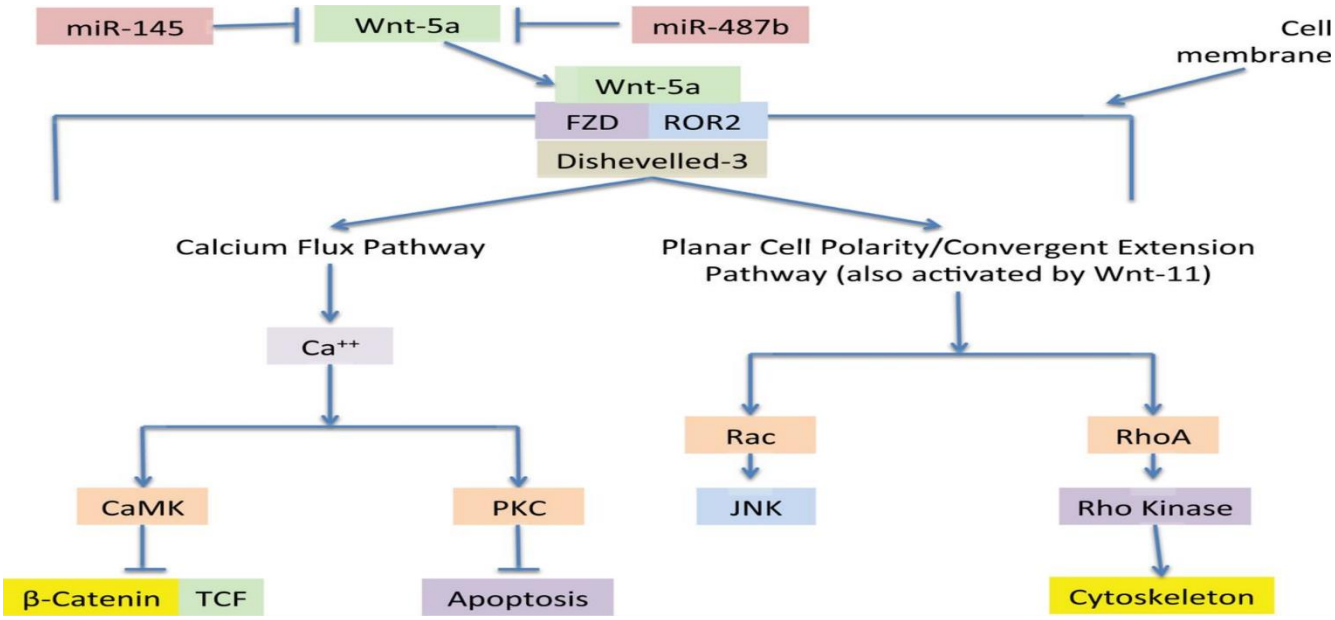


Figure 2.11: Depicting Wnt-5a non canonical Wnt signaling via planar cell polarity and calcium flux pathway (W. J. Nelson *et al.*, 2000)

2.10 *sFRP* (Secreted Frizzled related receptor) and signaling pathway:

SFRP's are extracellular glycoproteins which act as Wnt antagonists, as they bind with Wnt proteins and block Wnt signaling pathways as shown in figure 2.12. Two classes of Wnt antagonists are reported, first block signaling via FZD receptors (Surana R *et al.*, 2014) which include *sFRP*'s and WIF while in second class Dickkopf bind with LRP5/6 (He B *et al.*, 2005) and block signaling. *sFRP*'s shows homology with CRD (Cysteine rich domain) region of Frizzled receptors. To date five mammalian *sFRP*'s have been identified (*sFRP* 1-5), 300 amino acids long with a signal sequence. All five members share a common molecular property of having a FZD like CRD region. Cysteine rich domain is a major part of *sFRP* Wnt signaling pathway, which includes various conserved regions such as 10 conserved cysteine sequence homologous to CRD of FZD receptors. CRD constitute a C-terminal and N-terminal domain. C-terminal domain of *sFRP* is characterized od positively charged residues to confer heparin binding properties, and six cysteine residues present in this domain form 3 disulphide bridges. N-terminal domain is characterized by a signal peptide sequence. Various studies conducted to confirm the binding pattern of Wnt and *sFRP* and role of *sFRP* and found that it act as antagonist for Wnt signaling by various morphological alterations which may increase level of β catenin. *sFRP* molecules decrease

cell cycle proliferation by affecting cell cycle progression, thus considered as having potential role for tumor suppressor activities.

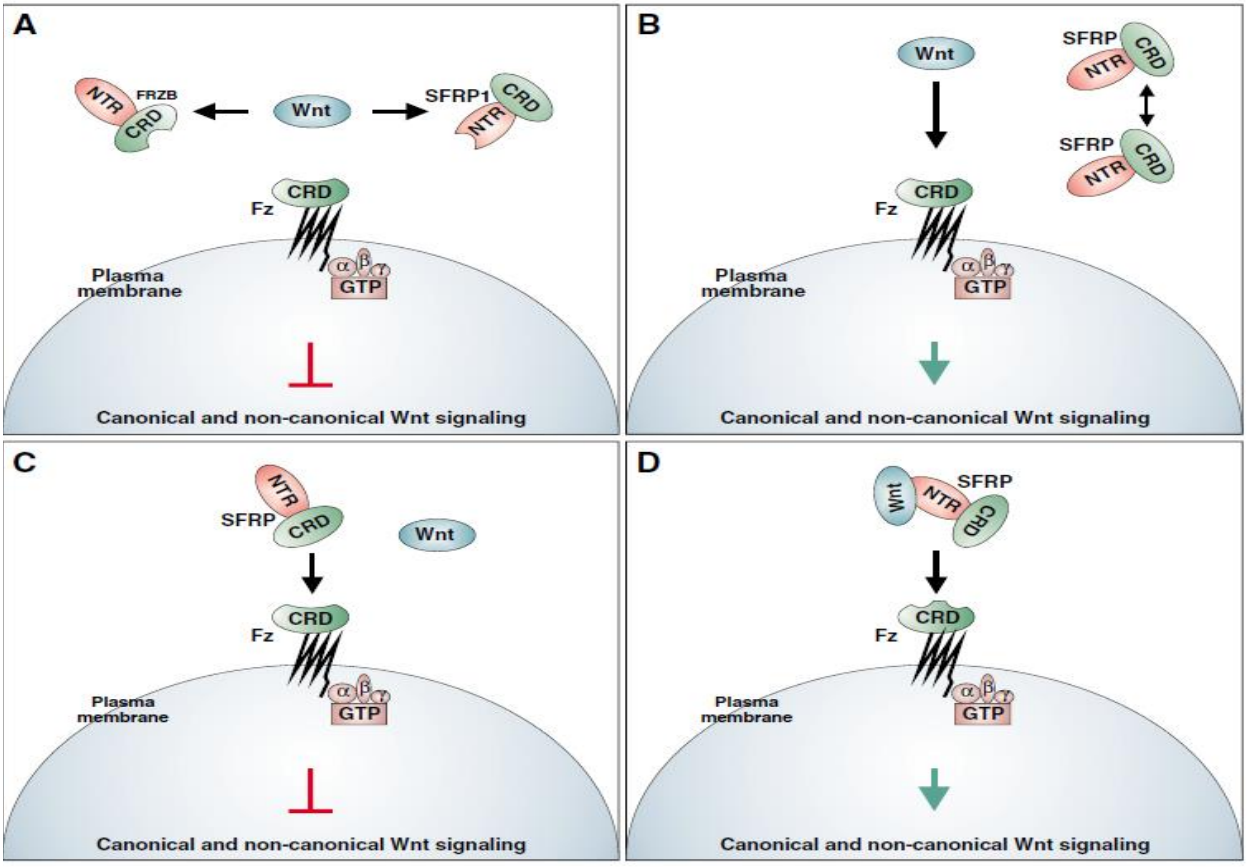


Figure 2.12: Depicts all the possible mechanisms how *sFRP* affects Wnt signaling through various mechanisms. A) Act as classical antagonists by sequestering Wnt through NTR and CRD domain. B) By titrating with each other, it may enhance Wnt signaling. C) Prevent signal transduction of Wnt by working in dominant negative fashion. D) They may promote Wnt signaling by favouring Wnt-FZD interaction (Paola Bovolenta *et al.*, 2008).

2.11 Secreted Frizzled related receptor 4 (*sFRP4*) and genetic variants:

sFRP4 gene belongs to *sFRP* family result in blocking of Wnt signaling pathway. As compared to other members, this gene shows different structural homology with highest molecular weight (39.9 KDa) and 346 amino acid long sequence (Jones SE *et al.*, 2002). *sFRP4* is 10.99 Kb long, sited on the short arm of chromosome 7 (7p14.1). This gene incorporates 6 coding exons along with 6 conserved cysteine residues in the C-terminal. Cysteine residues are linked with each other via a

disulphide bridge (Chong JM *et al.*, 2002). Similar to all other family members of *sFRP*, the key structure for Wnt signaling pathway blocking is CRD (Cysteine rich domain), which resides in N-terminus comprised of 120 amino acids, along with 20-30 amino acids long signal peptide sequence. In N-terminus, NLD (Nertin like domain) resides reported in induction of apoptosis (Longman D *et al.*, 2012). Like CRD region, NLD also consist six conserved cysteine residues, whereas both of them are reported for Wnt/ Ca^{2+} signaling pathway activation by increasing intracellular Ca^{2+} levels.

Presence of *sFRP4* sequester Wnt and prevents its binding with FZD as shown in figure 2.13. Complex of *Axin/ APC/ GSK3 β* phosphorylate β catenin, degrade by ubiquitination and degrade it. No further translocation of β catenin in nucleus hence no expression of target genes results in inhibition of angiogenesis (Longman D *et al.*, 2012).

Genetic alteration in any gene may cause dysregulation of gene expressions which may cause various diseases. Variations in SNP rs1802073 at genetic and molecular level has been identified as primary cause of various diseases. *sFRP4* rs1802073 (Pro^{320} Thr) is less explored. It has been found by Hiroshi Hirata *et al.*, in 2014 that *sFRP4* rs1802073 (Pro^{320} Thr) has been related with renal cell carcinoma , characterized as polyphen score and adjusted as probably damaging. Polyphen score is a software used to predict SNP effect on protein confirmation. One other genetic variant of this gene [*sFRP4* rs1802074] has been explored for its role in occurrence of renal carcinogenesis. A study done by in Turkish population report that rs1802074 polymorphisms showed a decreased risk of lung cancer.

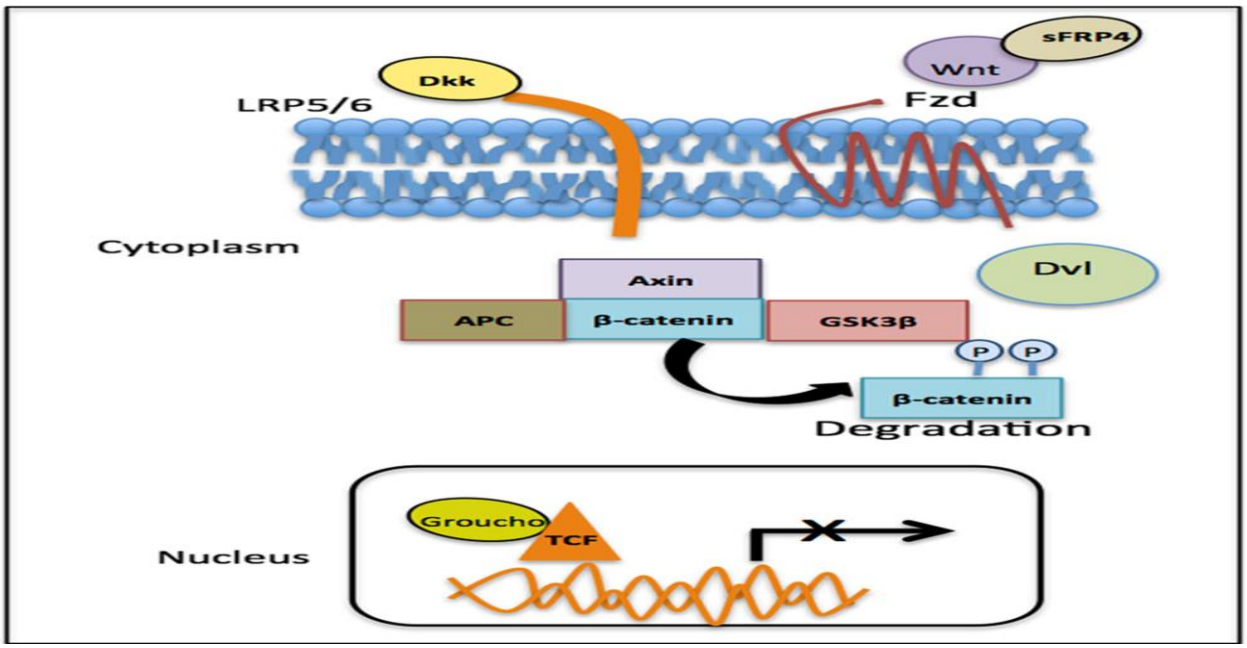


Figure 2.13: Depicts *sFRP4* presence act as antagonists for Wnt signaling pathway where β catenin is phosphorylated by axin and leads it in inactive state (Sebastian Pohl *et al.*, 2014).

CHAPTER 3

Aim of Study

1. To study the genotypic distribution of *TGF-β1* rs1800469 and *sFRP4* rs1802073 polymorphism in lung cancer patients.
2. To investigate the role of *TGF-β1* polymorphism in modulating the survival and clinical outcomes of lung cancer patients.
3. To examine the role of *sFRP4* polymorphism in affecting the overall survival and prognosis of lung cancer patients.
4. To evaluate the role of both rs1800469 and rs1802073 in predicting the clinical outcomes of patients stratified on the basis of histology, gender, smoking, performance status and regimen.
5. To find out the association of rs1800469 and rs1802073 with clinic pathological parameters.

CHAPTER 4

MATERIALS AND METHODS

4.1. Sample collection:

In current research two genes were studied included 186 and 340 patients. Samples were recruited from Department of Pulmonary Medicine, Post Graduate Institute of Medical Education and Research (PGIMER) Chandigarh (India) along with written consent of all the patients. With the help of trained interviewer, each participant filled up a detailed questionnaire to obtain various details about the patients demographic and tobacco smoking characteristics. Detailed information of age, gender, histology, TNM staging and smoking status with cumulative smoking exposure were gathered from patients. Patients were characterized as light and heavy smokers on the basis of pack years and pack years of smokers were calculated as [cigarettes or beedis per day/20*number of years smoked]. All the patients recruited for current study were histopathologically diagnosed with lung cancer different types as NSCLC (Adenocarcinoma, Squamous cell carcinoma), SCLC and LC. Medical records include age, gender, smoking status, histology, TNM staging, Tumor size, regimen, performance status, lymph node involvement and clinicpathological records.

4.2. DNA extraction from peripheral blood:

Isolation of genomic DNA from whole blood samples was done by using Proteinase K digestion, phenol/chloroform extraction and ethanol precipitation method .

- **Requirements**

- Washing buffer
- Lysis buffer
- Proteinase K
- Phenol: Chloroform: Isoamyl alcohol (25:24:1)
- Chloroform: Isoamyl alcohol (24:1)
- Isopropanol
- Ethanol
- Tris-EDTA buffer

- **Preparation of Buffers:**

i) Preparation of washing buffer (50 ml) was done as shown in table 4.1.

Table 4.1. Representing requirements for preparation of washing buffer	
Stock Concentration	**Working Concentration**
1 M Sucrose	320 mM Sucrose
100% Triton X-100	1% Triton X-100
100 mM Magnesium chloride	5 mM Magnesium chloride
100 mM Tris-HCl (pH-8.0)	10 mM Tris-HCl (pH-8.0)

ii) Lysis buffer (50ml) preparation for DNA extraction was done as shown in table 4.2.

Table 4.2. Representing requirements for preparation of lysis buffer	
Stock Concentration	**Working Concentration**
1 M Tris HCl (pH-8.0)	400mM Tris HCl (pH-8.0)
10 % SDS	1 % SDS
0.5 M EDTA	60 mM EDTA
5 M Nacl	150 mM Nacl
10 mg/ml Proteinase-K	100 µg/ml Proteinase-K

iii) Preparation of TE buffer (50ml) was done as shown in table 4.3.

Table 4.3. Representing requirements for preparation of TE buffer	
Stock Concentration	**Working Concentration**
1 M Tris HCl (pH-8.0)	10 mM Tris HCl (pH-8.0)
0.5 M EDTA	1 mM EDTA

- **Procedure of genomic DNA isolation:**

1. 5ml venous blood was isolated from patients and add equal amount (5ml) of washing buffer in it and centrifuge at 3500rpm for 5 minutes.

2. Discard the supernatant and add equal volume of washing buffer in pellet which comprise (1.6ml 1M Sucrose, 0.5 ml Triton X-100, 0.25ml $MgCl_2$, 0.5 ml 100mM Tris HCl and 0.26ml of water conc. for every single reaction) and centrifuge it at 3500 rpm for 5 minutes to resuspend the pellet. Repeat step 2 for two times more.
3. Dissolved the pellet in 5ml of Lysis buffer (1 M Tris HCl 2ml,10% SDS 0.5ml ,0.5 M EDTA 0.6ml, 5M NaCl 0.15ml,10mg/ml Proteinase-K 0.05ml and water 1.7ml) and incubated at 44 °C overnight.
4. Add P:C:I (Phenol 25ml: Chloroform 24ml: Isoamylalcohol 1ml) in equal volume of incubated reagent and slowly mix the contents.
5. Centrifuge the mixed contents at a speed of 8000rpm for 10 minutes at a temperature of 4°C.
6. Separate out upper aqueous layer, add PCI mix and again centrifuge to separate upper aqueous layer.
7. Add equal volume of C: I (Chloroform 24ml: Isoamyl alcohol 1ml) in the separated aqueous layer and centrifuge at 6500 rpm for 5 minutes.
8. Separate out upper aqueous layer and add equal volume of chilled isopropanol or absolute ethanol (100%) in 2.5 volumes of aqueous layer and mix it gently.
9. Freeze the mixture at -20°C for 1-2 hours and then centrifuge at 4°C for 10 minutes at 12,000 rpm.
10. Discard the supernatant and wash the pellet with 70% ethanol by centrifugation at 10,000 rpm for 5 minutes. Repeat this washing step for twice.
11. Decant ethanol and air dry the pellet.
12. Depending on the size of DNA pellet, dissoloved it in 50µl-150µl Tris-EDTA buffer to increase the shelf life of DNA by avoiding DNA degradation.

4.3. Quantitative estimation of DNA samples:

Thermo scientific Nanodrop spectrophotometer (Thermo Fisher Scientific, USA) was used to quantify and assess purity the DNA, RNA and protein samples. It utilize 1µl sample with xenon flash lamp light source. It allows direct pipetting of samples onto an optical measurement surface that define the path length (1mm) in vertical orientation. Absorbance of samples was recorded at two wavelengths A_{260} nm and A_{280} nm and the ratio of absorbance at 260nm and

280 nm is used to assess the purity of DNA. Ratio 1.8 represents pure DNA without any RNA or protein contamination whereas >1.8 means RNA contamination and <1.8 means sample is contaminated with proteins.

- **concentration of DNA can be calculated as**:
  {DNA concentration (µg/ml) = O.D at 260nm × 50 × Dilution factor} where 50µg/ml of DNA is equal to 1 O.D

1. To clean lower optical surface of Nanodrop (Thermo Fisher Scientific, USA), 1ml deionized water was pipetted onto it.
2. Nanodrop software was opened and nucleic acid module was selected.
3. Loaded 1µl sample on the surface to take a blank measurement by selecting "blank" on the screen.
4. 1µl sample was loaded on the surface and by selecting "measure" on the screen, nucleic acid quantification was done which measure purity and concentration of DNA.

4.4 Agarose gel method qualitative estimation of DNA:

- Autoclaved water
- Electrophoresis buffer (TBE)
- Electrophoresis-grade Agarose
- Ethidium bromide
- Gel casting trays and combs
- Gel casting platform
- 6X loading dye
- DNA molecular weight markers
- Horizontal gel electrophoresis apparatus
- DC power supply

<u>Procedure</u>

1. **Preparation of 5X TBE (1000 ml)**
   - Tris base-54 g

- Boric Acid-27.5 g
- EDTA (0.5M)-20 ml
- Make up final volume with water

2. Preparation of 6X Loading Dye (20ml)

- 0.25% Bromophenol blue- 0.05 g
- 0.25% Xylene Cyanol- 0.05 g
- 40% Sucrose-8 g
- Make up final volume with TE buffer

3. Preparation of agarose gel for electrophoresis:

- Prepared an adequate volume of electrophoresis buffer.
- Added the desired amount of Electrophoresis-grade Agarose to a volume of Electrophoresis buffer sufficient for preparing the gel i.e.
  - For genomic DNA 0.7% gel (0.7g agarose in 100ml 0.5X TBE) was prepared
  - For the PCR products 1.7% gel (1.7g agarose in 100ml 0.5X TBE buffer) was prepared.
- To prevent warping of the gel apparatus, melted agarose was cooled to 55°C in a water bath before pouring onto the gel platform.
- Before pouring gel into gel tray, Ethidium bromide solution was added to the melted well mixed agarose gel to a final concentration of 0.3µg/ml (EtBr facilitate visualization of DNA when seen under UV Transilluminator).
- Insert gel comb in the gel casting apparatus and poured the melted Agarose onto the apparatus between 0.5 and 1 cm thickness, making sure no bubbles presence underneath the combs or on the surface of agarose.

4. Loading and running the gel

- After solidification, combs were removed from gel carefully so that no sampling wells would not disrupt.
- Placed the settled gel casting tray in the electrophoresis tank and added sufficient amount of electrophoresis buffer to cover the gel.
- DNA samples were added by mixing 5µl DNA with 2µl of 6X loading dye and 2ul water.
- Samples were loaded in the wells by micropipette.

- Allowed the Electrophoresis apparatus to run at 60 V until the marker dyes migrated the desired distance.
- Visualized the presence of DNA by placing the gel on a UV transilluminator and then photographed using Gel Documentation.

4.5 PCR (Polymerase Chain Reaction):

Polymerase chain reaction ia a molecular technique developed by Karry B. Mullis in 1983. This technique is used to amplify a specific segment of DNA region *in vitro*. It relies on thermostable *Taq* polymerase that makes new DNA strands using template. Template DNA is the main component which need to be copied. *Taq* polymerase rely on primers which are short nucleotide sequences and provides DNA synthesis starting points. Forward and reverse primers are designed in such a way that they flank the target region which should be copied. Binding of primers with tempelate takes place via complementary base pairing. Repeated cycles of heating and cooling in PCR allows DNA to be synthesized.

Requirements for PCR:

- Autoclaved Milli Q water
- Tris-EDTA buffer
- BSA
- PCR buffer
- Taq DNA polymerase
- Forward primer
- Reverse primer
- dNTPs
- DNA sample

4.6 PCR amplification of *TGF β1* rs1800469 C/T:

To analyze C to T polymorphism in *TGF β1* gene a technique called PCR-RFLP (Polymerase chain reaction-Restriction Fragment Length Polymorphism) was used. PCR amplification of 808 bp long DNA fragment was done in 15µl reaction mixture comprises 100µg/ml BSA (bovine serum albumin), 1x PCR buffer with 1.5 mM $Mgcl_2$ (Thermo Fisher Scientific, USA), 0.5µl each of the forward (5'-CCCGGCTCCATTTCCAG-3') and reverse primers (5'-GGTCACCAGAGAAAGAGGAC-3'), 200µM dNTPs, 1U *Taq* polymerase (Thermo Fisher Scientific, USA), 300 ng of DNA and volume makeup with PCR grade water. PCR conditions used for

amplification were 95°C for 5 min and 94°C for 1 min (denaturation), 58°C for 1 min (annealing) followed by 72°C (extension) for 1 min and 7 min final extension for 35 cycles to obtain product size of 808 bp. Table 4.3 depicts total volume used for 10 reactions where individual reaction consist volume of 15 µl. finally, amplification of product of desired band size of 808bp was confirmed by using agarose gel electrophoresis.

Forward primer:- 5'-CCCGGCTCCATTTCCAG-3'

Reverse primer:- 5'-GGTCACCAGAGAAAGAGGAC-3'

Band size:- 808 bp

Table 4.4. Representing requirements for Polymerase Chain Reaction (PCR)

Reagents	Stock Concentration	Working Concentration	Volume Used
Additive 1 (BSA)	1000µg/ml	100 µg/ml	16.50 µl
PCR Buffer (Mg^{2+} concentration)	10X (15mM)	1X (1.5mM)	16.50 µl
TGF β1 rs1800469 C/T Forward Primer	10 µM	0.5 µM	8.25 µl
TGF β1 rs1800469 C/T Reverse Primer	10 µM	0.5 µM	8.25 µl
Taq Polymerase	2.0 U	1.0 U	2.20 µl
dNTPs	10 mM	0.2 mM	3.30 µl
PCR Grade Water	-	-	77 µl
DNA template	100 ng/ µl	300 ng	3 µl

Table 4.5. Representing steps of PCR along with their specified temperature

S. No.	Steps	Temperature	Time
1.	Initial Denaturation	95°C	5 min
2.	Denaturation	94°C	1 min
3.	Annealing	58°C	1 min
4.	Polymerization	72°C	1 min
5.	Final Extension	72°C	7 min

4.7 PCR amplification of *sFRP4* rs1802073 C/A:

Analysis of gene *sFRP4* rs1802073 C/A was performed using technique Nested PCR-RFLP in which specific primers were used. To avoid non-specific binding due to amplification of unexpected primer binding sites "Nested PCR" was performed in which two sets of primers were used for primary and secondary PCR amplification. PCR amplification of 246 bp long DNA fragment was done in 20µl reaction mixture comprises 100µg/ml BSA (bovine serum albumin), 1x PCR buffer with 1.5 mM $Mgcl_2$ (gene direX), 0.5µl each of the forward and reverse primers , 200µM dNTPs, 1U Taq polymerase (gene direX), 300 ng of DNA and PCR grade water. PCR was performed with initial denaturation step of 5 minutes at 95ºC then followed by 30 cycles of 30 sec at 94ºC, 45 seconds at 52ºC and 58ºC as annealing step for 1st and 2nd run respectively and 30 seconds at 72ºC followed by final extension step of 5mins at 72ºC. The amplification was confirmed by using agarose gel electrophoresis. Table 4.5 depicts total volume used for 10 reactions where individual reaction consist volume of 20 µl.

1st PCR: Forward primer:- 5'-GAGCACCATAAAGGGGTGAG-3'

Reverse primer:-5'-GGGCACATGGCCTTACATAG-3'

2nd PCR: Forward primer:-5'-ACAGCGGAGAACAGTTCAGG-3'

Reverse primer:-5'-TGGCCTTACATAGGCTGTCC-3'

Band size: 246 bp

Table 4.6. Representing requirements for Polymerase Chain Reaction (PCR)

Reagents	Stock Concentration	Working Concentration	Volume Used
Additive 1 (BSA)	1000µg/ml	100 µg/ml	22 µl
PCR Buffer (Mg concentration)	10X (15mM)	1X (1.5mM)	22 µl
sFRP4 rs1802073 C/A Primer Forward	10 µM	0.5 µM	4.4 µl
sFRP4 rs1802073 C/A Primer Reverse	10 µM	0.5 µM	4.4 µl
Taq Polymerase	5.0 U	1.0 U	1.76 µl
dNTPs	10 mM	0.2 mM	4.4 µl
PCR Grade Water	-	-	114.84 µl
DNA template	100 ng/µl	300 ng	3 µl

Table 4.7. Representing steps of primary PCR along with their specified temperature			
S. No.	**Steps**	**Temperature**	**Time**
1.	Initial Denaturation	95^{o}C	5 min
2.	Denaturation	94^{o}C	30 sec
3.	Annealing	52^{o}C	45 sec
4.	Polymerization	72^{o}C	30 sec
5.	Final Extension	72^{o}C	5 min

Table 4.8. Representing steps of secondary PCR along with their specified temperature			
S. No.	**Steps**	**Temperature**	**Time**
1.	Initial Denaturation	95^{o}C	5 min
2.	Denaturation	94^{o}C	30 sec
3.	Annealing	58^{o}C	45 sec
4.	Polymerization	72^{o}C	30 sec
5.	Final Extension	72^{o}C	5 min

4.8. Restriction Digestion of *TGF β1* rs1800469 C/T:

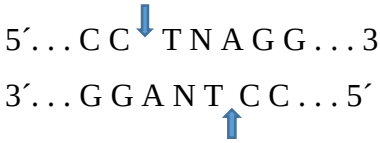
Restriction digestion enzymes were used to cleave amplified DNA amplicons at desired site to get DNA fragments which defines polymorphism of the gene. Restriction enzyme used for *TGF β1* rs1800469 C/T is *Bsu361* (Thermo Fisher Scientific, USA) 10U/µl. This enzyme is isolated from an *E. coli* strain that carries the cloned *Bsu36I* gene from *Bacillus subtilis 36*.

4.8.1 Procedure:

a) Total reaction mixture of 20 µl was made which consist 2.2 µl of 10X NEB buffer, 0.2 µl (2U) of 10 U/µl *Bsu361* (New England Biosciences, USA), 10 µl PCR product and 7.6 µl of water.
b) Reaction mixture was incubated at 37^{o} C for overnight.

c) After overnight incubation samples were kept at -20ºC to stop the reaction.
d) Digested samples were run at 2.5% agarose gel containing EtBr at a speed of 80 V.
e) The results were visualized by using UV transilluminator and captured the pictures by using another instrument called Gel Doc (Bio-Rad, Berkeley, California).

Recognition site for *Bsu361*:



4.8.2 Cutting pattern of *TGF β1* rs1800469 C/T:

Wild genotype (TT) – 808bp

Mutant genotype (CC) – 617bp /191bp

Heterozygous genotype (CT) – 808bp/ 617bp/ 191bp

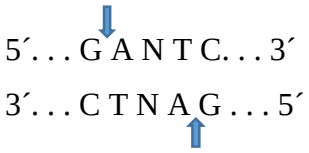
4.9. Restriction Digestion of *sFRP4* rs1802073 C/A:

The PCR product of gene *sFRP4* rs1802073 C/A was subjected to restriction digestion with units of *Hinf1I*. This product has been isolated from an *E. coli* strain that carries the *HinfI* gene from *Haemophilus influenza*e.

4.9.1 Procedure:

a) Total reaction mixture of 20 µl was made which consist 2.2 µl of 10X buffer R, 0.2 µl (2U) of 10 U/µl *Hinf1I*. (Thermo Fisher Scientific, USA), 10 µl PCR product and 7.6 µl of water.
b) Reaction mixture was incubated at 37º C for overnight.
c) After overnight incubation samples were kept at -20ºC to stop the reaction.
d) Digested samples were run at 2.5% agarose gel containing EtBr at a speed of 80 V.
e) The results were visualized by using UV transilluminator and captured the pictures by using another instrument called Gel Doc (Bio-Rad, Berkeley, California).

Recognition site for *Hinf1I*:



4.9.2 Cutting pattern of *sFRP4* rs1802073 C/A :

Wild genotype (CC) – 246bp

Mutant genotype (AA) – 165bp / 81bp

Heterozygous genotype (CA) – 246bp/ 165bp/ 81bp

4.10. Statistical Analyses:

Medcalc software version 17.5.3 (Medcalc software, Ostend, Belgium) was used to evaluate difference in the distribution of demographic and genotypic characteristics of cases and for all statistical data analysis. Chi-square test ($\chi 2$ test) and *t*-test were done for categorical data and for continuous variables respectively. For demographic studies, variables were categorized into continuous (gender, sex, smoking status) and categorical variables (age and pack years. Risk of lung cancer due to allelic variants was identified by calculating odds ratio along with 95% CI (Confidence Interval) and P-values with adjustment of Age, gender and smoking status, clinic-pathological features (KPS, ECOG and tumor stage) and clinical responses to find specific association of both the genes towards lung cancer susceptibility . For all the cases genotypic frequency of both the polymorphism was calculated by using Hardy-Weinberg equilibrium theory (p2+2pq+q2=1; where p is the frequency of the wild-type allele and q is the frequency of the variant allele).

To study overall survival Kaplan-Meier and Cox proportional hazard analysis were performed. Overall survival and *p*-value were evaluated by using Kaplan-Meier hazard analysis, whereas Multivariate Cox regression analysis was used to evaluate independent risk factors for each parameter. While considering both the analysis, *p*-value less than 0.05 was considered as significant for statistical analysis.

CHAPTER 5

RESULTS

5.1 DNA Isolation

Peripheral blood was used for isolation of genomic DNA. Presence of isolated DNA was affirmed by trans-UV-illuminator using 0.7% agarose gel as shown in Fig.5.1. Concentrated DNA was further diluted with TE to get a concentration of 100 ng/µl and were used as template for Polymerase chain reaction for amplification

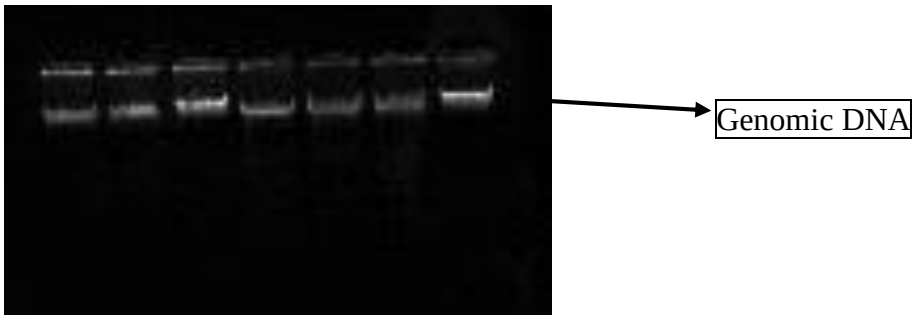


Fig 5.1 Isolated Genomic DNA confirmation by Gel electrophoresis

5.2 Polymerase Chain Reaction of *TGF β1* rs1800469 C/T

TGF β1 gene was amplified by polymerase chain reaction by using forward and reverse primers to get a desired amplicon size of 808 bp as shown in figure 5.2. Obtained amplified results were analyzed by using 1.7% agarose gel.

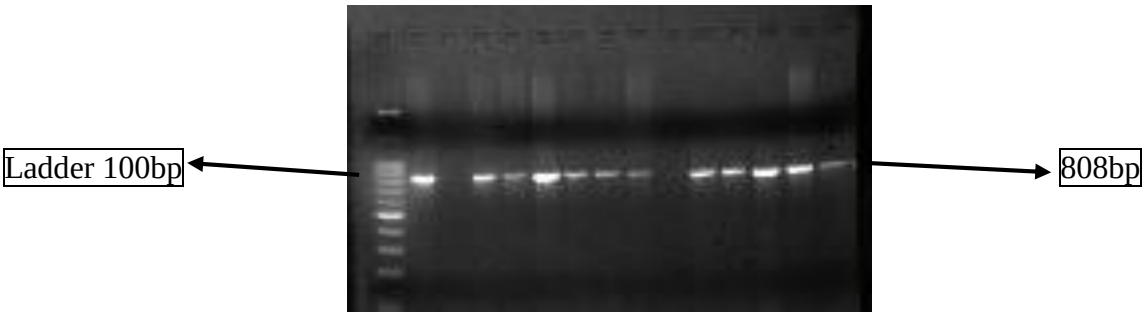


Fig 5.2 Amplified PCR product of *TGF β1* gene with amplicon size 808

5.3 Polymerase Chain Reaction of *sFRP4* rs1802073 C/A

Polymerase chain reaction was performed for *sFRP4* gene. Desired size amplicons of size 246bp were analyzed using 1.7% agarose by agarose gel electrophoresis as shown in fig 5.3.

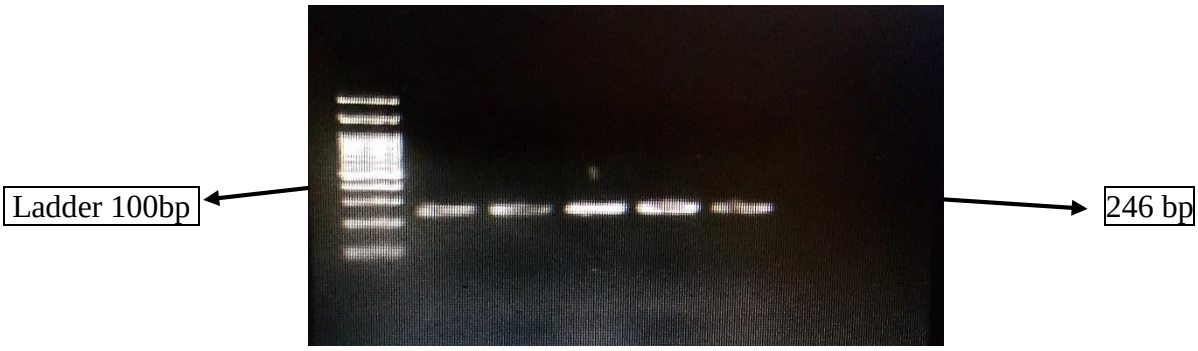


Fig 5.3 Showing amplified product of *sFRP4* rs1802073 C/A with amplicon size of 246 bp

5.4 Restriction digestion of *TGF β1* rs1800469 C/T with enzyme *Bsu361*

The amplified products were further digested with restriction enzyme *Bsu361* resulted into three fragments of size 808, 617 and 191 bp for heterozygous genotype, two fragments of size 617 and 191 bp if genotype is mutant and single fragment of size 808 bp for wild genotype as shown in figure 5.4. Cutting patterns obtained by digestion were checked by 2.5 % agarose gel containing EtBr at 80 volts.

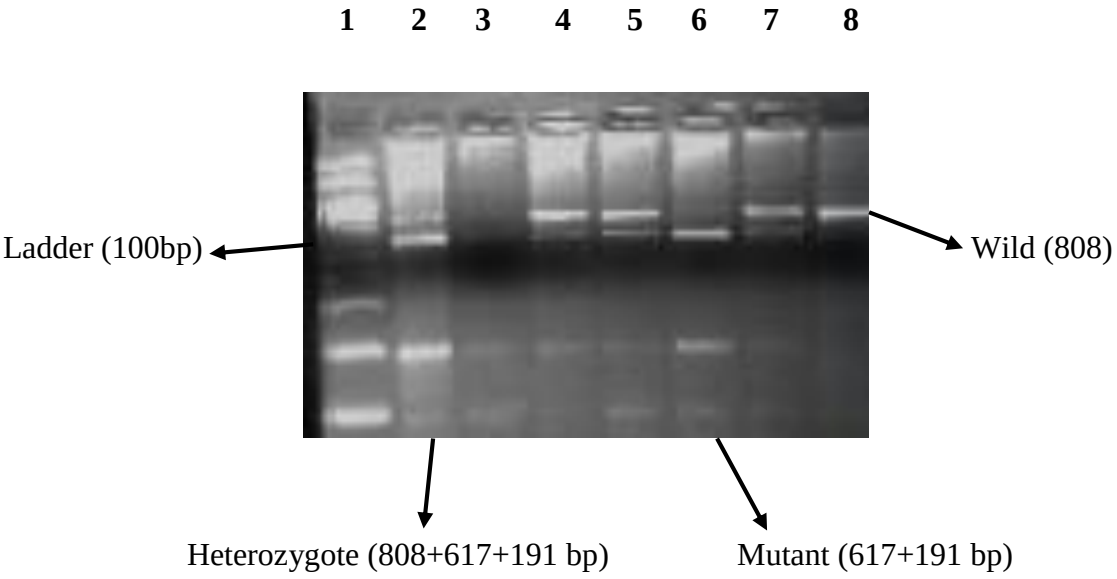


Fig 5.4 Showing digestion pattern of PCR amplified products of gene *TGF β1* rs1800469 C/T .Lane 1 represents 100bp ladder (G. Bioscience); Lane2,4,5,7 : Heterozygote type (*CT*) ; Lane6: Mutant type (*CC*); Lane8: wild type (*TT*).

5.5 Restriction digestion of *sFRP4* rs1802073 C/A with restriction enzyme *Hinf1*

Digestion of amplified product of size 246 bp was done by using *Hinf1* enzyme by incubating the sample at 37° C. Digested pattern was observed by 2.5% agarose gel to analyze genotype patterns. Three bands of size 246, 165 and 81 bp were observed in heterozygous (*CA*) genotype, two in mutant (*AA*) genotype of size 165 and 81 bp while single band of size 246 bp was observed in wild (*CC*) genotype, where ladder of 100 bp was used as shown in fig. 5.5.

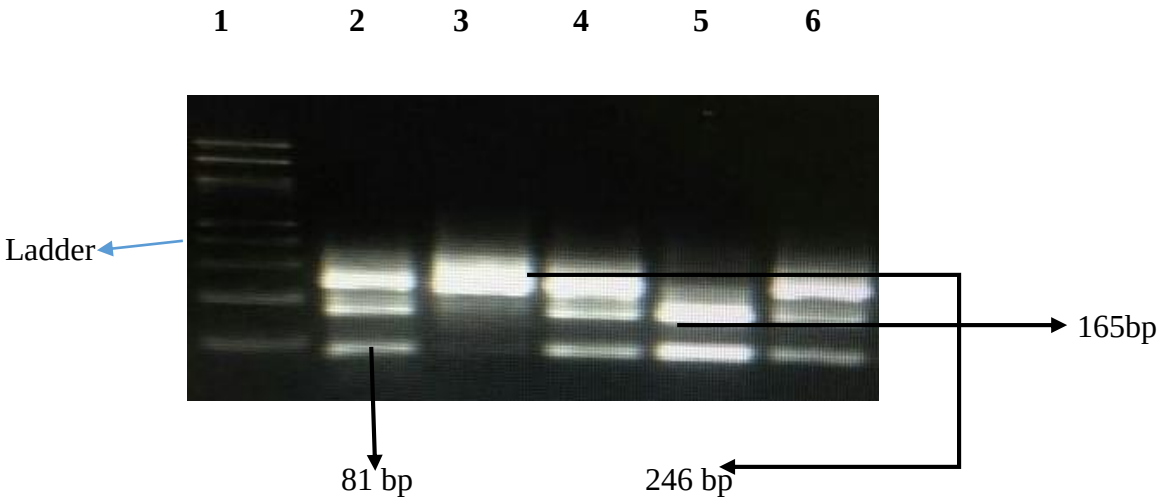


Fig 5.5 Ethidium bromide stained gel showing different polymorphism of *sFRP4* gene. Lane 1 shows 100 bp ladder (G.Bioscience), lane 2, 4 and 6 : heterozygous (246+165+81 bp) genotype; lane 3: wild (246 bp); lane 5: mutant (165+81 bp) genotype.

5.6. Demographic distribution of lung cancer patients for *TGF β1* gene:

The demographic distribution for parameters in cases of lung cancer patients (*TGF β1* gene) is summarized in Table5.1. Study group includes age, gender, smoking status, KPS, ECOG and regimen for cases. The clinical parameters of lung cancer patients included histologic types, TNM staging, tumor size extension, lymph node development, metastasis along with clinical response parameter (complete response (CR), partial response (PR), stable disease (SD), progressive disease (PD)) for cases. The study enlisted total of 186 cases having mean age of 57.97(±10.61). This study comprised total 153(87.63%) males and 23(12.36%) females without any significant level

of distribution in population. The study constitute higher number 153 (82.25%) of smoking patients than non-smoker patients 33 (17.74%) which shows smoking as a significant parameter for occurrence of lung cancer. Furthermore, number of pack years in smokers is 28.55(±37.56). Among all histologic types SQCC was found to be the most common one 82(44.08) whereas, ADCC, SCLC and LCC were 42(22.58%), 1(0.53%) and 61(32.79%) respectively. TNM stage data was available for 178 patients among which stage IV cases was highly proportionated with 94 (50.53) cases, 77 (41.39%) cases of stage III, 6 (3.22%) cases of stage II and 1(0.53%) case of stage I. During metastasis study of 165 cases, M_0 were overrepresented (54.30%) than M_1 64 (34.40%). Out of 186 patients 112 cases were estimated for clinical response among which 54 (29.03%) patients showed progressive response, 41 (22.04%) showed partial response towards chemotherapy treatment. Tumor size T1 and T2 had less frequency of 5.37% and 23% respectively whereas T3 and T4 had a frequency of 27.73% and 47.84 %. Total 186 cases were studied on KPS performance scale and 186 for ECOG performance scale. While examining lymph node involvement, N0 had a frequency of 13.97% whereas N1, N2, N3 and N4 had frequency of 10.75%, 44.62%, 22.58% and 0.53% respectively.

Table 5.1 Distribution of demographic characteristics for gene *TGF β1* rs1800469 *C/T* of LC cases

VARIABLE	**CASES, n (%) N=186**	**VARIABLE**	**CASES, n (%) N=186**
Age(years)		**Lymph node involvement**	
Mean ± SD	57.97±10.61	N0	26(13.97)
Range	28-86	N1	20(10.75)
		N2	83(44.62)
		N3	42(22.58)
		N4	1(0.53)
			14(7.52)
Gender		**Metastasis**	
Male	163(87.63)	M0	101(54.30)
Female	23(12.36)	M1	64(34.40)
Smoking status		**KPS**	
Smokers	153(82.25)	10-70	66(35.48)
Non-smokers	33(17.74)	80-100	120(64.51)
Pack years		**ECOG**	
Mean ± SD	28.55±37.56	0-1	91(48.92)
		2-4	95(51.07)
Histologic types		**Regimen**	
SQCC	82(44.08)	**Docetaxel+ cisplatin/carboplatin**	52(27.95)
ADCC	61(32.79)		
SCLC	42(22.58)	**irinotecan+ cisplatin/carboplatin**	37(19.89)
Others	1(0.53)		

		pemetrexed+ cisplatin/carboplatin	42(22.58)
Tumor size T1 T2 T3 T4 Unknown	 10(5.37) 23(12.36) 46(27.73) 89(47.84) 18(9.67)	**TMN staging** I II III IV Unknown	 1(0.53) 6(3.22) 94(50.53) 77(41.39) 8(4.30)
Objective Response CR PR SD PD	 3(1.61) 54(29.03) 41(22.04) 14(7.52)		
Abbreviations: SD=Standard Deviation, n=total number of case patients or controls subjects .[a] *p*-values were derived from Pearson Chi-square test except age; Student t-test was used for age. All *p*-values are two-sided. $p < 0.05$ was considered statistically significant.			

5.7 Association of *TGF-β1 rs1800469 C/T* on the overall survival of lung cancer patients:

Table (5.2) summarized overall survival of gene *TGF β* performed for total 186 lung cancer patients. Statistical analysis of all samples was done by using univariate Kaplan-Meier analysis and then reaffirmed by multivariate Cox hazard analysis to determine adjusted and non-adjusted hazard ratio (HR), 95% confidence interval (CI), log rank *p* values and Kaplan Meier curves. It correlates overall survival (in months) and effect of gene by considering various prognostic factors (age, gender, smoking status, histology, performance status, remission) considering genotype as an independent factor. We evaluated patients from day of diagnosis to the last follow up date and found that out of 186 patients, 161 (86.55) patients were dead and 25 (13.44) patients were alive. By considering wild (*TT*) genotype as reference, we found that the median survival time (MST) with mutant (*CC*) genotype was higher than wild (*TT*) and heterozygote (*CT*) genotype (8.30 *vs* 8.20 and 6.43 months) as shown in [fig.5.6 (A, B, C)]. Mutant (*CC*) variant was proved to have good prognostic effect as compare to other two genotypes (HR=1.11; 95% CI=0.68-1.82; *p*=0.65) by using univariate Kaplan Meier analysis and further with multivariate Cox hazard proportional analysis by adjusting different covariates (HR'=1.03; 95% CI=0.60-1.78; *p*=0.90) in patients with better survival. Heterozygote (*CT*) variant with MST 6.43 months exhibited no significant association neither with Kaplan Meier analysis (HR'=0.87; 95% CI=0.60-1.26; *p*=0.48) nor after adjusting different covariates and performing Cox hazard proportional analysis (HR'=1.19; 95% CI=0.80-1.76; *p*=0.37). Similarly combined genotype (*CT+CC*) with MST 7.13 months showed

no significant association by univariate (HR'=0.92; 95% CI=0.65=1.32; *p*=0.69) or multivariate (HR'=1.11; 95% CI=0.76-1.62; *p*=0.58) analysis.

Table 5.2 Representing relationship of *TGF-β* genotype with overall survival of lung cancer patients

	Overall						
Genotype	**Dead (161) n%**	**Alive (25) n%**	**Median OS months**	**HR (95% CI)**	**Log *p***	**HR[b] (95% CI)[b]**	***p***
TT	38 (88.37)	5 (11.63)	8.20	-	-	-	-
CT	96 (88.07)	13 (11.93)	6.43	0.87 (0.60-1.26)	0.48	1.19 (0.80-1.76)	0.37
CC	27 (79.41)	7 (20.59)	8.30	1.11 (0.68-1.82)	0.65	1.03 (0.60-1.78)	0.90
CT+CC	123 (86.01)	20 (13.99)	7.13	0.92 (0.65-1.32)	0.69	1.11 (0.76-1.62)	0.58

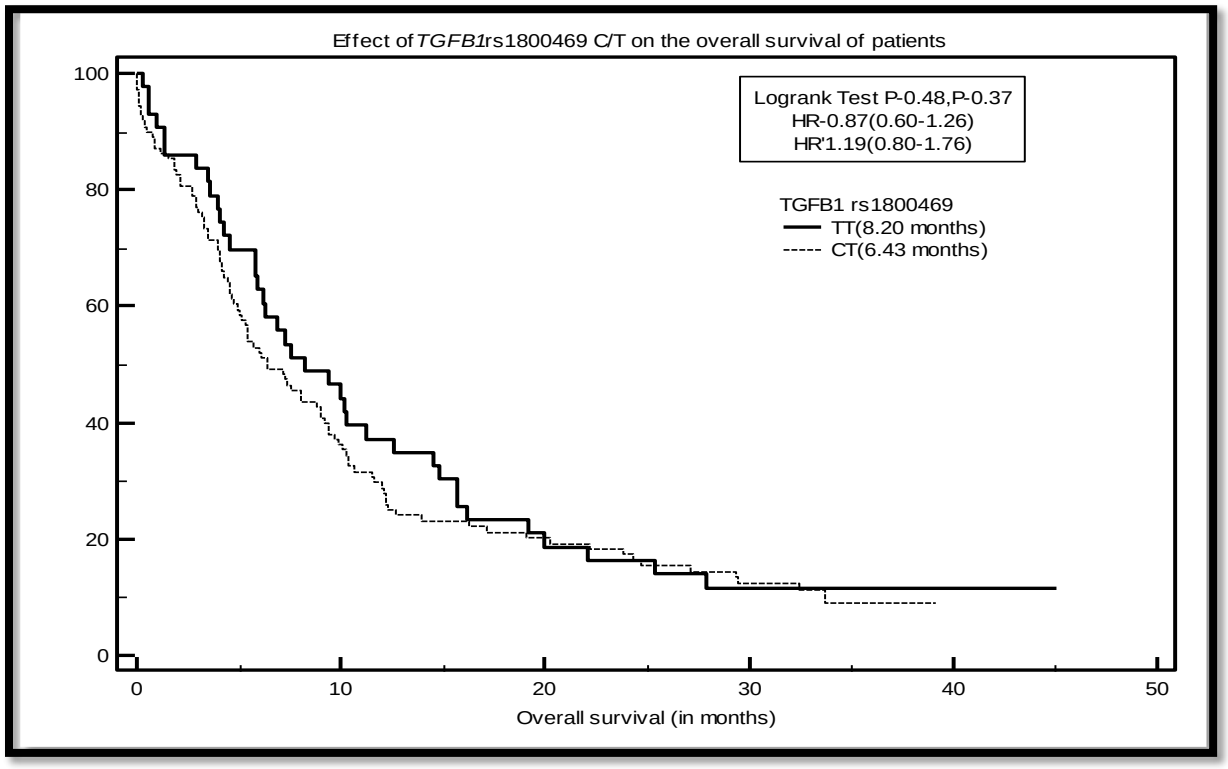


Fig.5.6 (A) Kaplan-Meier curves of *TGF β1* rs1800469 *C/T* polymorphism showing overall survival of patients having (*TT* and *CT*) genotype

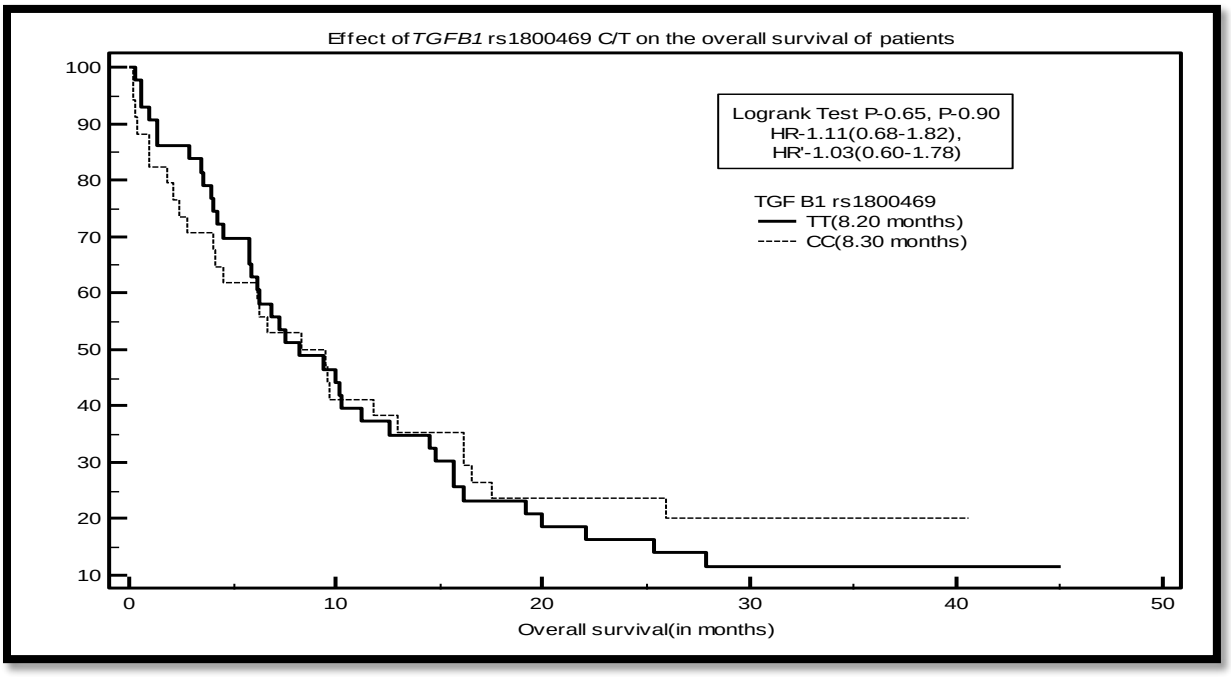


Fig.5.6 (B) Kaplan-Meier curves of *TGF β1* rs1800469 *C/T* polymorphism showing overall survival of patients having (*TT* and *CC*) genotype

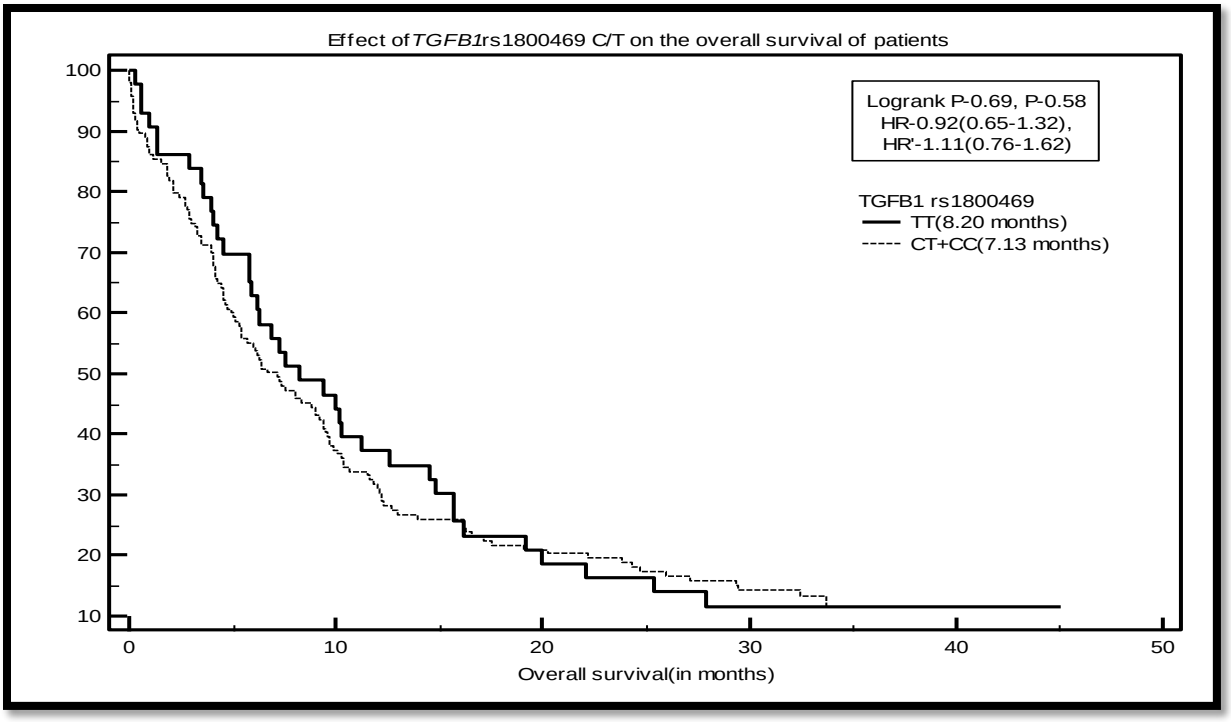


Fig.5.6 (C) Kaplan-Meier curves of *TGF β1* rs1800469 *C/T* polymorphism showing overall survival of patients having (*TT* and *CT+CC*) genotype

5.8 Genotypic distribution and association of *TGF-β1 rs1800469 C/T* with overall survival of lung cancer patients on the basis of histological subtypes:

After dividing data on the basis of histological subtypes total 185 cases were studied for analysis of *TGF-β1* gene association with overall survival. Longer survival period was found in patients with mutant (*CC*) genotype in SCLC subgroup (11.83 months) and a lower death rate, however showed no significant association by univariate analysis (HR=0.59, 95% CI=0.19-1.85, *p*=0.31) but significant association was observed by multivariate Cox hazard analysis after adjusting different covariates (HR'=0.00, 95% CI=0.00-0.01, *p*=**0.01**) as shown in table (5.3). In AD*CC* and SQ*CC* patients, longer survival period is reported in wild (*TT*) genotype when compared with heterozygote (*CT*) and mutant (*CC*) genotype (9.40 and 8.20 months). Significant association was observed in SCLC mutant (*CC*) genotype by applying Cox hazard analysis after adjusting different covariates. Among all three subgroups, combined variant genotype (*CT+CC*) of AD*CC* patients showed highest survival period as compare to SQ*CC* and SCLC patients (9.40 *vs* 6.40 and 7.23). However, the difference was not found to be statistically significant. Comparison of overall survival for ADCC genotypes is shown in figures 5.7 A, B and C, for SQCC in 5.8 A, B and C and for SCLC comparison is shown in figure 5.9 A, B and C.

Table 5.3 Representing relationship of *TGF-β* genotype with overall survival of lung cancer patients on the basis of histology

	1. ADCC						
Genotype	**Dead (51) n%**	**Alive (10) n%**	**Median OS months**	**HR (95%CI)**	**Log *p***	**HR[b] (95% CI)[b]**	***p***
TT	13 (81.25)	3 (18.75)	9.40	-	-	-	-
CT	27 (90)	3 (10)	6.10	0.66 (0.35-1.24)	0.21	1.66 (0.78-3.53)	0.18
CC	11 (73.33)	4 (26.67)	8.30	0.90 (0.40-2.03)	0.10	0.31 (0.07-1.25)	0.81
CT+CC	38 (84.44)	7 (15.56)	9.40	0.74 (0.41-1.34)	0.35	1.24 (0.59-2.57)	0.55

	2. SQCC						
Genotype	**Dead (70) n%**	**Alive (12)**	**Median (OS months)**	**HR (95%CI)**	**Log *p***	**HR[b] (95% CI)[b]**	***p***

		n%					
TT	18 (90)	2 (10)	8.20	-	-	-	-
CT	44 (84.62)	8 (15.38)	6.40	1.01 (0.58-1.76)	0.95	0.85 (0.47-1.54)	0.61
CC	8 (80)	2 (20)	6.23	1.24 (0.55-2.78)	0.59	0.85 (0.25-2.90)	0.80
CT+CC	52 (83.37)	10 (16.13)	6.40	1.04 (0.60-1.80)	0.86	0.91 (0.50-1.65)	0.77

3. SCLC

Genotype	**Dead (45) n%**	**Alive (3) n%**	**Median (OS months)**	**HR (95%CI)**	**Log *p***	**HR[b] (95% CI)[b]**	***p***
TT	6 (100)	0 (0)	5.80	-	-	-	-
CT	25 (92.59)	2 (7.41)	7.23	1.03 (0.42-2.54)	0.94	1.51 (0.53-4.29)	0.43
CC	14 (93.33)	1 (6.66)	11.83	0.59 (0.19-1.85)	0.31	0.00 (0.00-0.01)	**0.01**
CT+CC	39 (95.12)	3 (4.88)	7.23	0.95 (0.39-2.30)	0.91	1.44 (0.54-3.81)	0.45

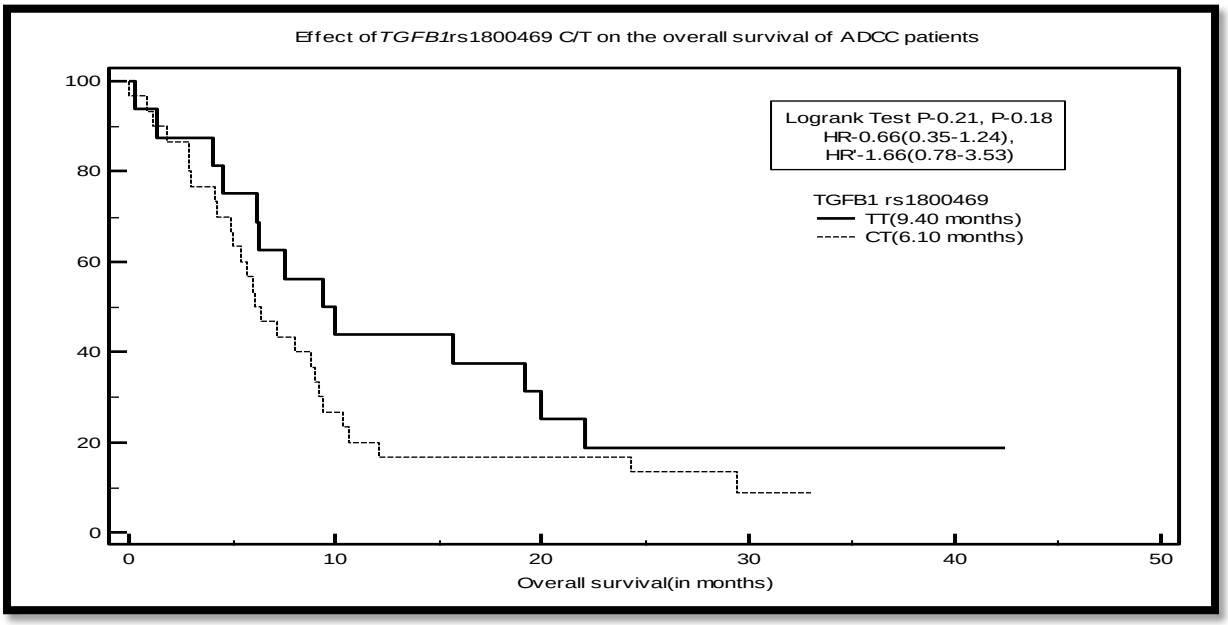


Fig.5.7 (A) Kaplan-Meier curves of *TGF β1* rs1800469 *C/T* polymorphism showing overall survival of ADCC patients having (*TT* and *CT*) genotype

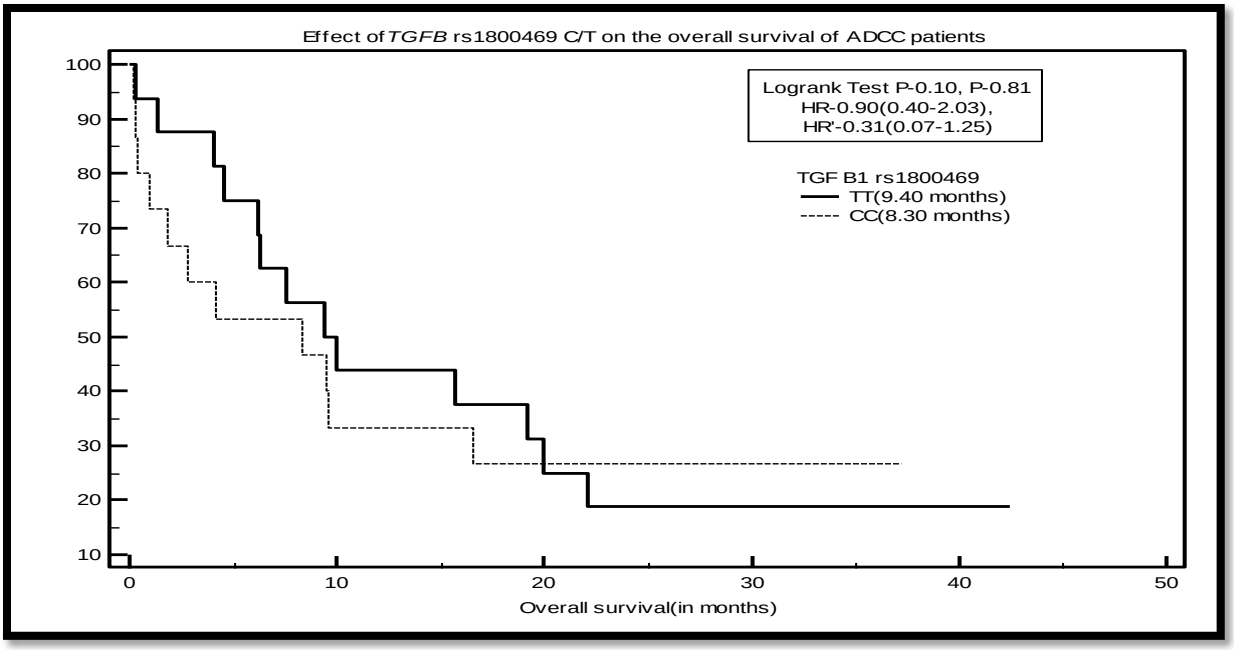


Fig.5.7 (B) Kaplan-Meier curves of *TGF β1* rs1800469 *C/T* polymorphism showing overall survival of ADCC patients having (*TT* and *CC*) genotype

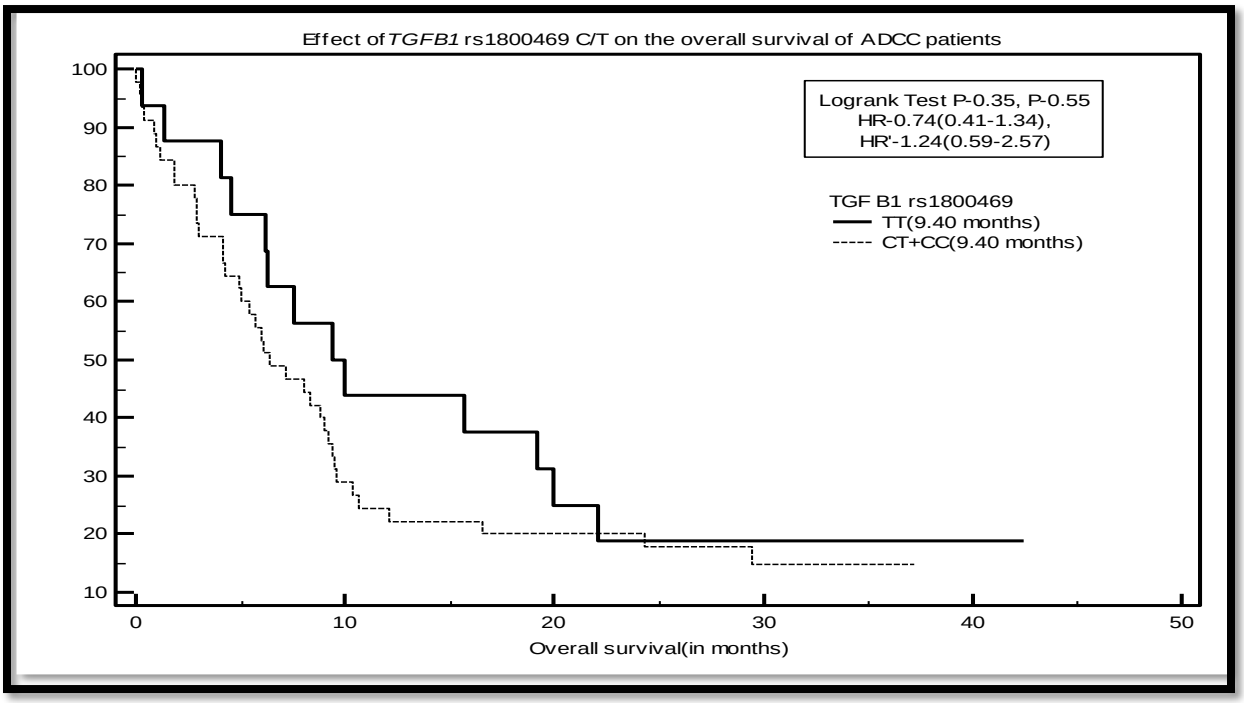


Fig.5.7 (C) Kaplan-Meier curves of *TGF β1* rs1800469 *C/T* polymorphism showing overall survival of ADCC patients having (*TT* and *CT+CC*) genotype

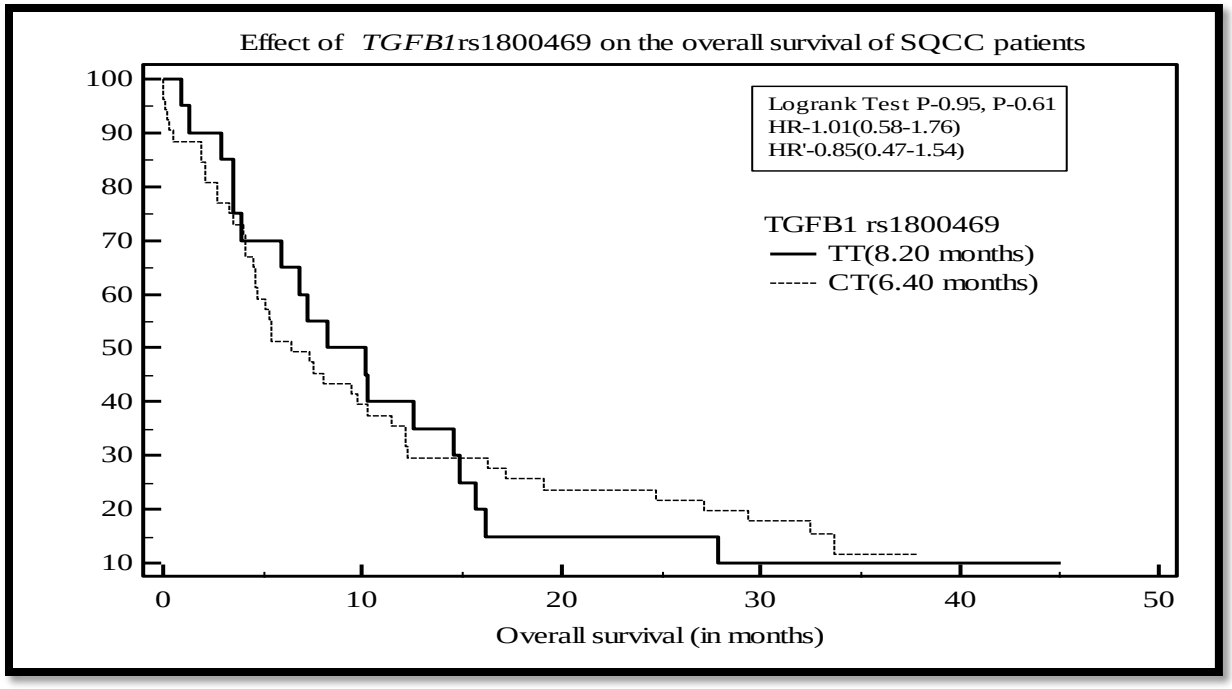


Fig.5.8 (A) Kaplan-Meier curves of *TGF β1* rs1800469 *C/T* polymorphism showing overall survival of SQCC patients having (*TT* and *CT*) genotype

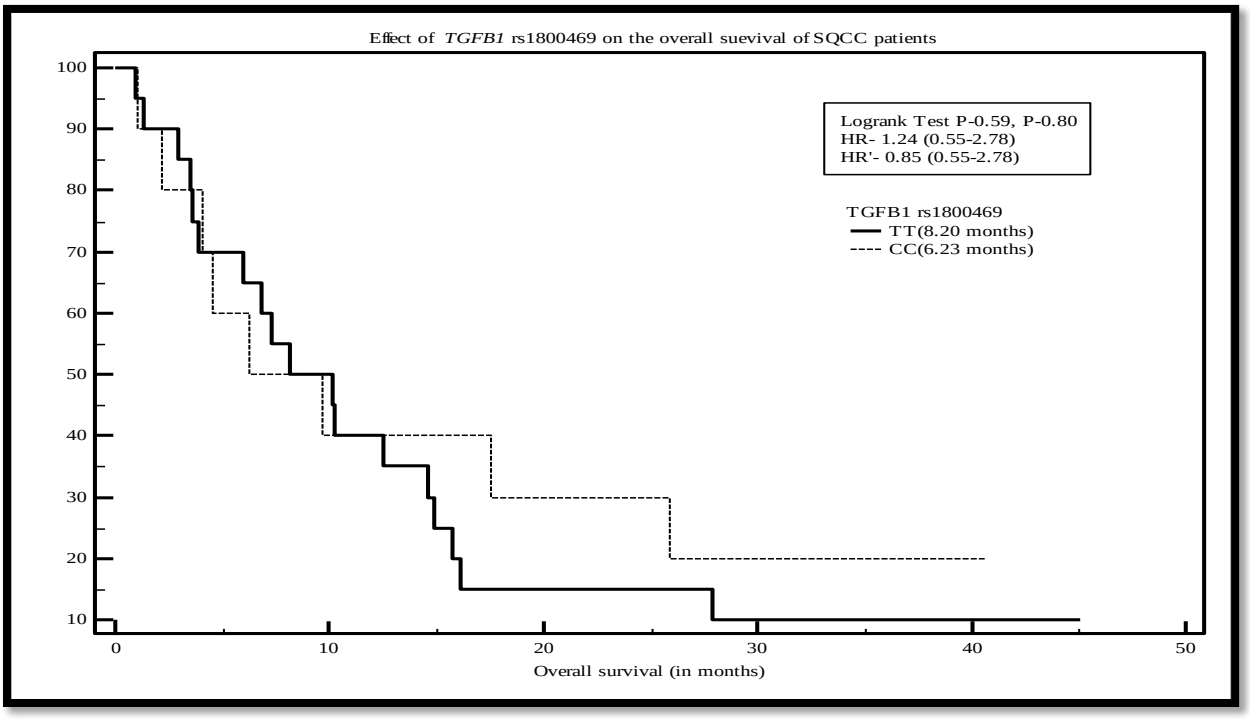


Fig.5.8 (B) Kaplan-Meier curves of *TGF β1* rs1800469 *C/T* polymorphism showing overall survival of SQCC patients having (*TT* and *CC*) genotype

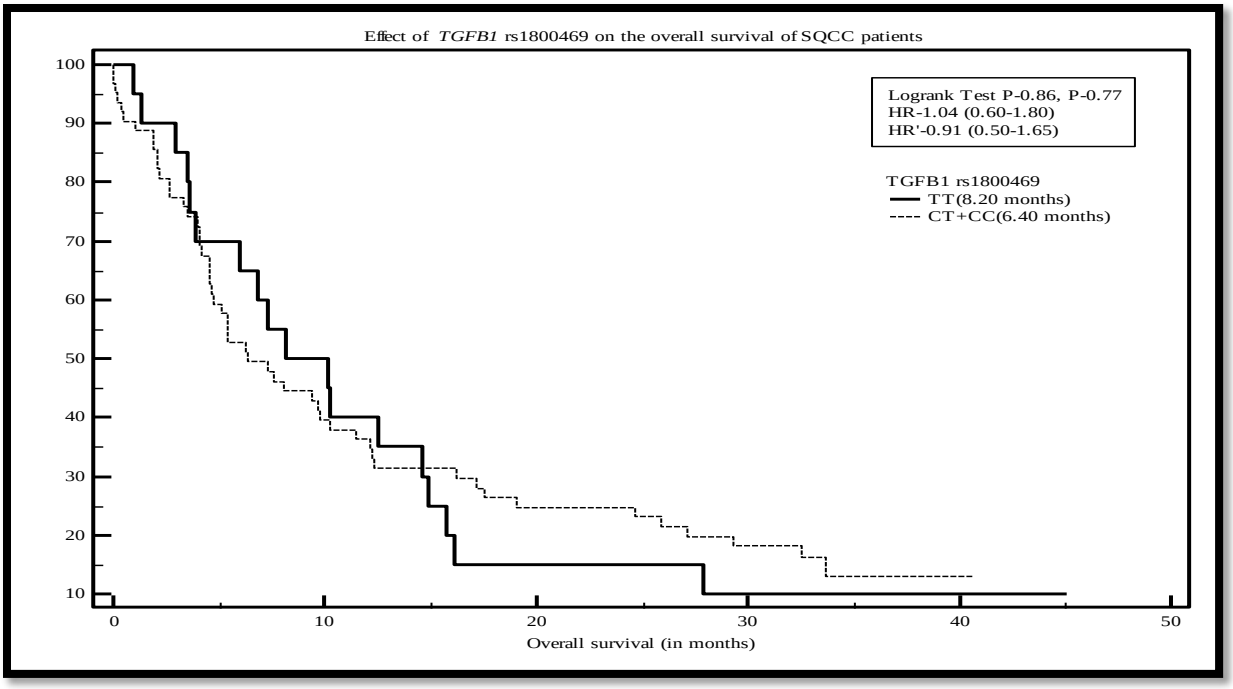


Fig.5.8 (C) Kaplan-Meier curves of *TGF β1* rs1800469 *C/T* polymorphism showing overall survival of SQCC patients having (*TT* and *CT+CC*) genotype

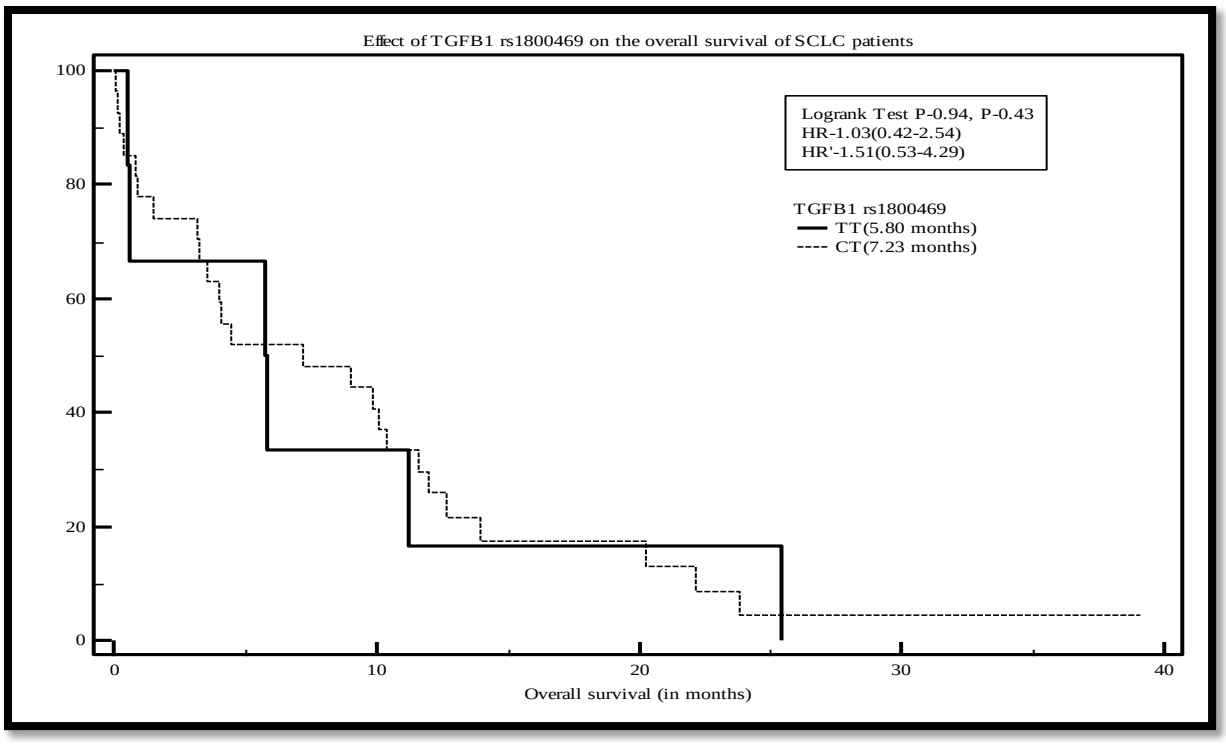


Fig.5.9 (A) Kaplan-Meier curves of *TGF β1* rs1800469 *C/T* polymorphism showing overall survival of SCLC patients having (*TT* and *CT*) genotype

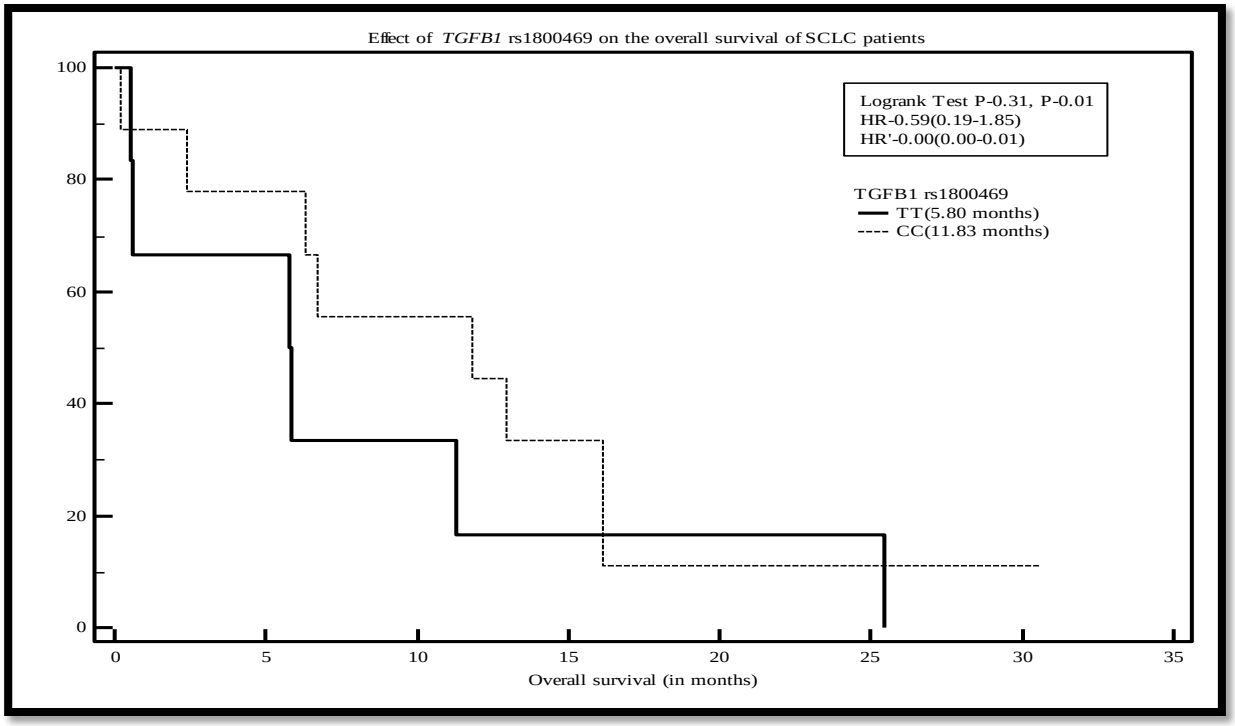


Fig.5.9 (B) Kaplan-Meier curves of *TGF β1* rs1800469 *C/T* polymorphism showing overall survival of SCLC patients having (*TT* and *CC*) genotype

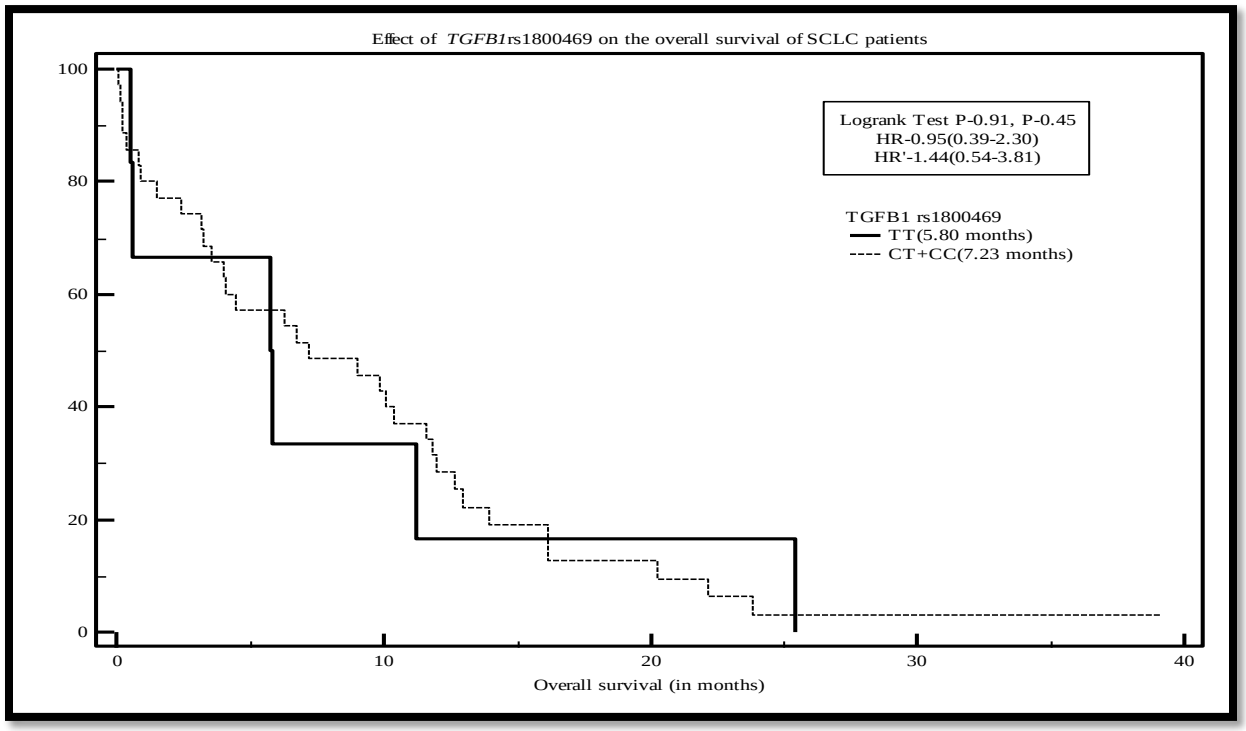


Fig.5.9 (C) Kaplan-Meier curves of *TGF β1* rs1800469 *C/T* polymorphism showing overall survival of SCLC patients having (*TT* and *CT+CC*) genotype

5.9 Genotypic distribution and association of *TGF-β1 rs1800469 C/T* with overall survival of lung cancer patients on the basis of gender:

Further stratifying patients on the basis of gender, lower death rate was observed in mutant (*CC*) genotype in males (9.66 months) for *TGF-β1* gene which was not statistically significant. However female's wild (*TT*) genotype analyzed for decreased death rate as compared to heterozygote (*CT*) and mutant (*CC*) genotype (20 *vs* 8.8 and 4.13 months). No significant probability for lung cancer patients even with less mortal rate was observed in males (HR=0.73; 95% CI=0.43-1.23; *p*=0.25) or females (HR=1.34; 95% CI=0.35-5.15; *p*=0.69) as shown in table 5.4.

Table 5.4 Representing relationship of *TGF-β* genotype with overall survival of lung cancer patients on the basis of gender

	Males							Females						
Genotype	**Dead (141) n%**	**Alive (22) n%**	**Median (OS months)**	**HR (95% CI)**	**Log *p***	**HR[b] (95% CI)[b]**	***p***	**Dead (20) n%**	**Alive (3) n%**	**Median (OS months)**	**HR (95% CI)**	**Log *p***	**HR[b] (95% CI)[b]**	***P***
TT	36 (90)	4 (10)	7.56	-	-	-	-	2 (66.67)	1 (33.33)	20	-	-	-	-
CT	83 (88.30)	11 (11.70)	6	1.11 (0.76-1.63)	0.57	1.09 (0.73-1.64)	0.65	13 (86.67)	2 (13.33)	8.8	1.34 (0.35-5.15)	0.69	7.34 (0.78-68.62)	0.81
CC	22 (75.86)	7 (24.14)	9.66	0.73 (0.4-1.23)	0.25	0.72 (0.4-1.31)	0.29	5 (100)	0 (0)	4.13	2.80 (0.6-12.3)	0.15	1.34 (0.35-5.15)	0.81
CT+CC	105 (85.37)	18 (14.63)	7.13	1.01 (0.6-1.47)	0.94	0.97 (0.6-1.44)	0.91	18 (90)	2 (2)	6.73	1.63 (0.4-5.50)	0.50	4.08 (0.73-22.5)	0.11

5.10 Association of *TGF-β1 rs1800469 C/T* with overall survival of lung cancer patients on the basis of their smoking status:

By demarcating patients on the basis of smoking status no significant association was observed for *TGF-β1* gene. Non-smoking patients shows higher MST (9.46 months) as compare to smoking patients (8.3 months). In smoking patients mutant (*CC*) genotype shows higher survival period than wild (*TT*) and heterozygote (*CT*) genotype (8.3 *vs* 8.2 and 6.2 months). Similarly in non-smoking patients also mutant (*CC*) genotype has been analyzed for higher MST than wild (*TT*) and heterozygote (*CT*) genotype (9.46 *vs* 5.83 and 8.03). No significant prognosis for lung cancer patients was observed in smoking and non-smoking patients even with higher survival period

mutant (*CC*) genotype (HR=1.00; 95% CI=0.57-1.74; *p*=0.98 and HR=1.73; 95% CI=0.53-5.63; *p*=0.32) respectively, as shown in table 5.5.

Table 5.5 Representing relationship of *TGF-β* genotype with overall survival of lung cancer patients on the basis of smoking status

	Smokers							Non smokers						
Genotype	**Dead (133) n%**	**Alive** (20) **n%**	**Median (OS months)**	**HR (95% CI)**	**Log *p***	**HR[b] (95% CI)[b]**	***p***	**Dead (50) n%**	**Alive (5) n%**	**Median (OS months)**	**HR (95% CI)**	**Log *p***	**HR[b] (95% CI)[b]**	***p***
TT	32 (86.49)	5 (13.51)	8.2	-		-	-	6 (100)	0 (0)	5.83	-		-	-
CT	80 (88.89)	10 (11.11)	6.2	1.20 (0.81-1.78)	0.37	1.25 (0.82-1.92)	0.28	16 (84.21)	3 (15.79)	8.03	1.39 (0.50-3.88)	0.47	1.10 (0.35-3.45)	0.85
CC	21 (80.77)	5 (19.23)	8.3	1.00 (0.57-1.74)	0.98	1.19 (0.79-1.78)	0.17	6 (75)	2 (25)	9.46	1.73 (0.53-5.63)	0.32	0.41 (0.07-2.30)	0.31
CT+CC	101 (86.93)	15 (13.07)	6.3	1.15 (0.78-1.69)	0.47	1.19 (0.78-1.79)	0.17	22 (81.48)	5 (18.52)	8.80	1.49 (0.53-4.16)	0.37	0.63 (0.21-1.82)	0.39

5.11 Genotypic distribution and association of *TGF-β1 rs1800469 C/T* with overall survival of lung cancer patients on the basis of regimen:

Further classification was done for *TGF-β1* gene by stratifying patients on the basis of regimen (Docetaxel+ cisplatin/carboplatin, Irinotecan+ cisplatin/carboplatin and Pemetrexed+ cisplatin/carboplatin). It has been analyzed that all three genotypes (*TT, CT* and *CC*) of docetaxel+ cisplatin/carboplatin classification possess approximately same median survival rate (10.13, 9.43 and 9.66 months) as shown in table 5.6 (A). In irinotecan+ cisplatin/carboplatin heterozygote genotype (*CT*) has been analyzed for lower risk of death with higher median survival rate (7.23 months) as compared to wild and mutant genotype (5.80 and 6.73 months).Similarly patients with pemetrexed+ cisplatin/carboplatin showed higher survival period among all subjects with wild (*TT*) genotype than heterozygotes (*CT*) and mutant (*CC*) genotype i.e. (10.00 *vs* 6.10) and (10.00 *vs* 9.46) months with higher survival rate and less mortality, as shown in table 5.6 (B).

Table 5.6 (B) Representing relationship of *TGF-β* genotype with overall survival of lung cancer patients on the basis of regimen

Irinotecan+ cisplatin/carboplatin						Pemetrexed+ cisplatin/carboplatin					
Genotype	**Dead (32) n%**	**Alive (5) n%**	**Median (OS months)**	**HR (95%CI)**	**Log *p***	**Genotype**	**Dead (34) n%**	**Alive (8) n%**	**Median (OS months)**	**HR (95%CI)**	**Log *p***
TT	8 (80)	2 (20)	5.80	-	-	**TT**	7 (77.78)	2 (22.2)	10	-	-
CT	18 (90)	2 (10)	7.23	0.79 (0.35-1.76)	0.57	**CT**	21 (84)	4 (16)	6.10	0.67 (0.30-1.48)	0.36
CC	6 (85.71)	1 (14.29)	6.73	1.03 (0.36-2.98)	0.94	**CC**	6 (75)	2 (25)	9.46	0.84 (0.28-2.55)	0.76
CT+CC	24 (88.89)	3 (11.11)	7.23	0.83 (0.38-1.79)	0.65	**CT+CC**	27 (81.82)	6 (18.1)	6.43	0.71 (0.33-1.53)	0.43

Table 5.6 (A) Representing relationship of *TGF-β* genotype with overall survival of lung cancer patients on the basis of regimen

Docetaxel+ cisplatin/carboplatin					
Genotype	**Dead (44) n%**	**Alive (8) n%**	**Median (OS months)**	**HR (95%CI)**	**Log *p***
TT	11 (100)	0 (0)	10.13	-	-
CT	27 (84.37)	5 (15.63)	9.43	1.38 (0.64-2.96)	0.34
CC	6 (66.67)	3 (33.33)	9.66	1.80 (0.69-4.67)	0.22
CT+CC	33 (80.49)	8 (9.66)	9.66	1.43 (0.67-3.05)	0.28

5.12 Genotypic distribution and association of *TGF-β1 rs1800469 C/T* with overall survival of lung cancer patients on the basis of performance status after receiving chemotherapy:

Considering *TGF-β1* gene and after classifying patients on the basis of performance status [KPS (80-100) and (<70 KPS), ECOG (0-1) and (2-4)] it has been analyzed that mutant (*CC*) genotype of KPS (80-100) and wild (*TT*) genotype of ECOG (0-1) suggest a trend of good improvement among patients after chemotherapy treatment but no significant association with overall survival was observed, as shown in table 5.7 (a) and 5.7 (b). Subjects with KPS score 80-100 with mutant (*CC*) genotype had a higher MST (12.96 months) as compared to wild (*TT*) genotype (7.56 months) and heterozygote (*CT*) genotype (6.40 months), as shown in table 5.7. Patients having wild (*TT*) genotype and ECOG score 0, 1 showed higher probability rate than mutant (*CC*) and heterozygotes (14.56 *vs* 9.00 and 11.83 months). Heterozygote variant (*CT*) exhibited no significant association with overall survival (HR=0.59; 95% CI=0.34-1.02; p=0.09) even after adjusting with different covariates (HR=1.68; 95% CI=0.86-3.27; p=0.12).

Table 5.7 (a) Representing relationship of *TGF-β* genotype with overall survival of lung cancer patients on the basis of performance status

KPS (80-100)								KPS (<70)							
Genotype	Dead (66) n%	Alive (10) n%	Median (OS months)	HR (95%CI)	Log p	HRb (95% CI)b	p	**Genotype**	Dead (95) n%	Alive (15) n%	Median (OS months)	HR (95%CI)	Log p	HRb (95% CI)b	P
TT	18 (85.71)	3 (14.29)	7.56	-	-	-	-	**TT**	20 (90.91)	2 (9.09)	8.20	-	-	-	-
CT	36 (87.80)	5 (12.20)	6.40	0.86 (0.49-1.49)	0.60	1.31 (0.73-2.36)	0.36	**CT**	60 (88.24)	8 (11.76)	7.23	0.91 (0.56-1.50)	0.74	1.07 (0.63-1.83)	0.78
CC	12 (85.71)	2 (14.29)	12.96	1.20 (0.58-2.46)	0.61	0.94 (0.37-2.38)	0.90	**CC**	15 (75)	5 (25)	6.23	0.99 (0.50-1.93)	0.98	0.99 (0.47-2.07)	0.98
CT+CC	48 (87.27)	7 (12.73)	8.80	0.93 (0.54-1.60)	0.81	1.14 (0.65-2.01)	0.62	**CT+CC**	75 (85.23)	13 (14.77)	6.30	1.05 (0.57-1.51)	0.82	1.05 (0.63-1.76)	0.82

Table 5.7 (b) Representing relationship of *TGF-β* genotype with overall survival of lung cancer patients on the basis of performance status

ECOG (0-1)								ECOG (2-4)							
Genotype	**Dead (77) n%**	**Alive (14) n%**	**Median (OS months)**	**HR (95%CI)**	**Log *p***	**HR[b] (95% CI)[b]**	*p*	**Genotype**	**Dead (84) n%**	**Alive (11) n%**	**Median (OS months)**	**HR (95%CI)**	**Log *p***	**HR[b] (95% CI)[b]**	***p***
TT	13 (76.47)	4 (23.53)	14.56	-	-	-	-	**TT**	25 (96.15)	1 (3.85)	6.16	-	-	-	-
CT	49 (90.74)	5 (9.26)	9.00	0.59 (0.34-1.02)	0.09	1.68 (0.86-3.27)	0.12	**CT**	47 (85.45)	8 (14.55)	5.43	1.10 (0.67-1.80)	0.68	0.93 (0.55-1.57)	0.81
CC	15 (75)	5 (25)	11.83	0.94 (0.45-1.99)	0.89	0.81 (0.33-1.99)	0.65	**CC**	12 (85.71)	2 (14.29)	4.03	0.99 (0.49-1.97)	0.97	1.19 (0.47-3.04)	0.70
CT+CC	64 (88.49)	10 (13.51)	9.00	0.68 (0.40-1.16)	0.21	1.43 (0.76-2.70)	0.26	**CT+CC**	59 (85.51)	10 (14.49)	5.43	1.07 (0.66-1.72)	0.75	0.89 (0.54-1.46)	0.69

5.13 Genotypic distribution and association of *TGF β1* rs1800469 C/T with clinic pathological parameters:

Table (5.8) precisely describe regression analysis data which shows association of *TGF β1* gene clinic-pathological parameters and risk of occurrence of lung cancer. Out of 186 patients 135 (72.58%) were found to be highly differentiated while 33 (17.74%) were moderately differentiated. No statistical significant correlation was observed to determine susceptibility of occurrence of lung cancer with any of the genotype of *TGF β1* gene. Combined variant (*CT+CC*) was found to be predominant factor in both highly differentiated tumor (T_3+T_4) and moderately differentiated tumor (T1+T_2) without any significant value. Based upon TNM staging criteria, 94 patients were stratified in stage III (50.53%) while 77 were stage IV patients (41.39).Among both the stages (III and IV) heterozygote population was found in the highest frequency (57.44% and 33.09%). Lymph node invasion was studied for 26 (15.20%) N_0 and 145 (84.79%) N_1+N_2+N_3 patients. No significant association was found when N_0 *vs* N_1+N_2+N_3 were studied. Heterozygote (*CT*) genotype showed slightly lower *p* value (0.71) as compared to mutant (*CC*) genotype (0.75).

Table 5.8 Representing relationship of different *TGF β1* genotypes with the clinic-pathological parameters

	Clinical Stage		**[b]AOR (95% CI)[b]**	**P**	**Primary Tumor Extension**		**[b]AOR (95%CI) [b]**	**P**	**Lymph Node Invasion**		**[b]AOR (95% CI)[b]**	**P**
Genotype	**III (94) n%**	**IV (77) n%**			**T_1+T_2 (33) n%**	**T_3+T_4 (135) n%**			**N_0 (26) n%**	**N_1+N_2+ N_3 (145) n%**		
TT	25 (26.65)	15 (19.48)	_	_	7 (21.21)	34 (25.18)	_	_	6 (23.07)	35 (24.13)	_	_
CT	54 (57.47)	46 (59.75)	1.34 (0.62-2.89)	0.44	18 (54.54)	78 (57.77)	0.88 (0.33-2.36)	0.81	16 (61.53)	83 (57.24)	0.82 (0.29-2.33)	0.71
CC	15 (15.95)	16 (20.77)	1.66 (0.61-4.50)	0.31	8 (24.24)	23 (17.03)	0.82 (0.00-0.00)	0.99	4 (15.38)	27 (18.62)	1.29 (0.26-6.30)	0.75
CT+CC	69 (73.41)	62 (80.51)	1.42 (0.68-2.97)	0.34	26 (78.78)	101 (74.81)	0.82 (0.32-2.08)	0.67	20 (76.92)	110 (75.86)	0.89 (0.30-2.60)	0.84

5.14 Genotypic distribution and association of *TGF β1* rs1800469 C/T with clinic-pathological parameters on the basis of gender and metastasis:

Association of gene with clinic-pathological parameters was studied on the basis of gender also with higher population of males (87.63%) than females (12.36%), but no significant association was found. Based upon metastasis criteria 101 (61.21%) patients were classified as M_0 while 64 (38.78%) were studied as M_1 patients without showing any association (5.9). So we can conclude that *TGF β1* gene was not found to play any role in lung cancer progression as no significant association was observed in different clinic-pathological parameters and different genotypes.

Table 5.9 Representing relationship of different *TGF β1* genotypes with the clinic-pathological parameters

	Sex		**[b] AOR (95% CI) [b]**	***p***	**Metastasis**		**[b] AOR (95% CI) [b]**	***P***
Genotype	**F (23) n (%)**	**M (163) n (%)**			**Mo (101) n (%)**	**M_1 (64) n (%)**		
TT	3 (13.04)	40 (24.53)	-	-	25 (24.75)	10 (15.62)	-	-
CT	15 (65.21)	94 (57.66)	1.00 (0.00-0.00)	1.00	59 (58.41)	40 (62.5)	1.12 (0.51-2.45)	0.76
CC	5 (21.73)	29 (17.79)	1.00 (0.00-0.00)	1.00	17 (16.83)	14 (21.87)	1.32 (0.46-3.79)	0.59
CT+CC	20 (86.95)	123 (75.46)	1.00 (0.00-0.00)	1.00	76 (75.24)	54 (84.37)	1.15 (0.54-2.46)	0.70

[b] Adjusted Odds ratios, 95% confidence intervals and their corresponding *p*-values were calculated by unconditional logistic analysis after adjusting for age, gender and smoking.

5.15 Genotypic distribution and Association of *TGF β1* rs1800469 C/T with lung cancer on the basis of chemotherapy response:

Association of *TGF β1* with lung cancer considering chemotherapy response is summarized in table (5.10). Stratification was done on the basis of good responders (CR+PR) and bad responders (SD+PD). Out of 186 patients, 57 (30.64%) were characterized as good responders, while 55 (29.56%) patients as bad responders. CR+PR *vs* SD+PD studies evaluated almost similar *p* values for heterozygous (*CT*) and mutant (*CC*) genotype (0.70 and 0.77). However no significant

association was found in lung cancer patients undergoing chemotherapy with any of the genotype of *TGF β1* gene.

Table 5.10 Representing relationship of different *TGF β1* genotypes with the chemotherapy response

	Response of chemotherapy		[b]AOR (95% CI)[b]	*p*
Genotype	**CR+PR (57) n (%)**	**SD+PD(55) n (%)**		
TT	14 (24.56)	12 (21.81)	–	–
CT	31 (54.38)	33 (60)	1.20 (0.47-3.05)	0.70
CC	12 (21.05)	10 (18.18)	1.19 (0.35-3.99)	0.77
CT+CC	43 (75.43)	43 (78.18)	1.17 (0.47-2.86)	0.72

[b] Adjusted Odds ratios, 95% confidence intervals and their corresponding *p*-values were calculated by unconditional logistic analysis after adjusting for age, gender and smoking.

5.16 Demographic distribution of lung cancer patients for *sFRP4* gene:

The demographic distribution of lung cancer patients' cases is summarized in table (5.11). Our study comprised age, gender, smoking status, pack years. The clinical parameter for lung cancer patients include histological types (ADCC, SQCC, SCLC), TNM staging (Stage I-IV), tumor size extension (T1-T4), lymph node development (N_0-N_4), Metastasis (M_0-M_1) along with clinical response i.e. CR, PR, SD and PD. Our study enlisted total 340 cases having mean age of 57.96 (±10.61). Our data for *Sfrp4* gene comprised total 295 (86.76%) males and 45 (13.23%) females without any significant level of distribution. Our study constitute 282 (82.94%) smokers and 58 (17.05%) non-smoker patients which proves smoking as a significant criteria for lung cancer occurrence. Moreover number of pack years in smokers is (37.70%). Our study consist three histologic types among which SQCC histology 125 (36.76%) is the most common type as compared to SCLC and ADCC i.e. 101 (29.70%) and 108 (31.76%) respectively. Tumor size T_3 and T_4 has very high frequency of 78 (22.94%) and 169 (49.70%) as compared to T_1 and T_2 i.e. 14 (4.11%) and 38 (11.17%). Out of 340, total 233 patients were reported for clinical response in which 112 (32.94%) showed partial response, 10 (2.94%) complete response, 88 (25.88%) stable disease and 23 (6.76%) patients showed progressive disease. While examining Lymph node development N_2 had a frequency of 146 (42.94%) whereas N_0 has 37 (10.88%), N_1, N_3 and N_4 has

32 (9.41%), 89 (26.17%) and 3 (0.88%) respectively. During Metastasis total 307 cases were studied out of which M_0 were found in higher proportion i.e. 174 (51.17%) whereas, M_1 were 133 (39.11%). Total 331 cases were studied on Karnofsky (KPS) performance scale and 331 for ECOG performance scale. TNM stage data was accessible for total 313 patients out of which Stage III has been reported in highest proportion 164 (48.23%), stage IV comprised total 139 (40.88%), stage II 7 (2.05%) and stage I comprised 3 (0.88%) cases.

Table 5.11 Distribution of demographic characteristics of gene *sFRP4* rs1802073 *C/A* LC cases

VARIABLE	CASES, n (%) N=340	VARIABLE	CASES, n (%) N=340
Age(years)		**Lymph node involvement**	
Mean ± SD	57.96±10.61	N0	37(10.88)
Range	26-86	N1	32(9.41)
		N2	146(42.94)
		N3	89(26.17)
		N4	3(0.88)
		Unknown	33(9.70)
Gender		**Metastasis**	
Male	295(86.76)	M0	174(51.17)
Female	45(13.23)	M1	133(39.11)
		Unknown	33(9.70)
Smoking status		**KPS**	
Smokers	282(82.94)	30-60	44(12.94)
Non-smokers	58(17.05)	70-80	154(45.29)
		90-100	133(39.11)
		Unknown	9(2.64)
Pack years		**ECOG**	
Mean ± SD	37.70±28.65	0-1	165(48.52)
		2-4	166(48.82)
		Unknown	9(2.64)
Histologic types		**Regimen**	
SQCC	125(36.76)	1	82(24.11)
ADCC	101(29.70)	5	80(23.52)
SCLC	108(31.76)	6	71(20.88)
Others	6(1.76)	Unknown	107(31.47)
Tumor size		**TMN staging**	
T1	14(4.11)	I	3(0.88)
T2	38(11.17)	II	7(2.05)
T3	78(22.94)	III	164(48.23)
T4	169(49.70)	IV	139(40.88)
Unknown	41(12.05)	Unknown	27(7.94)

Objective Response		
CR	10(2.94)	
PR	112(32.94)	
SD	88(25.88)	
PD	23(6.76)	
Unknown	107(31.47)	
Abbreviations: SD=Standard Deviation, n=total number of case patients or controls subjects .[a] *p*-values were derived from Pearson Chi-square test except age; Student t-test was used for age. All *p*-values are two-sided. $p < 0.05$ was considered statistically significant.		

5.17 Association of *sFRP4 rs 1802073 C/A* on the overall survival of lung cancer patients:

Table (5.12) outlined OS (overall survival) of gene *sFRP4* performed for total 340 lung cancer patients' cases. After evaluating patients from diagnosis date to last follow up, we concluded that out of 340 patients, 289 (85%) were dead while only 51 (15%) were alive. We considered wild (*CC*) genotype as reference, and found that heterozygote (*CA*) genotype median survival time is higher than mutant (*AA*) and wild (*CC*) genotype (9.46 *vs* 7.6 and 5.83 months) as shown in (fig 5.10 A, B, C). No significant association was found with any of the genotype neither by using Kaplan Meier univariate analysis nor by Cox hazard proportional multivariate analysis. Likewise, combined genotype with MST 8.80 months showed no significant association neither by univariate analysis (HR=1.11; 95% CI=0.78-1.58; *p*=0.53) nor by multivariate Cox (HR=1.02; 95% CI=0.71-1.47; *p*=0.88).

Table 5.12 Representing relationship of s*FRP4* genotype with overall survival of lung cancer patients

Overall							
Genotype	**Dead (289) n%**	**Alive (51) n%**	**Median OS months**	**HR (95%CI)**	**Log *p***	**HR[b] (95% CI)[b]**	***p***
CC	39 (84.78)	7 (15.22)	7.6	-	-	-	-
CA	188 (84.68)	34 (15.32)	9.46	1.18 (0.82-1.70)	0.33	0.90 (0.62-1.32)	0.61
AA	62 (86.11)	10 (13.89)	5.83	0.93 (0.62-1.39)	0.74	1.29 (0.80-2.07)	0.28
CA+AA	250 (85.03)	44 (14.97)	8.80	1.11 (0.78-1.58)	0.53	1.02 (0.71-1.47)	0.88

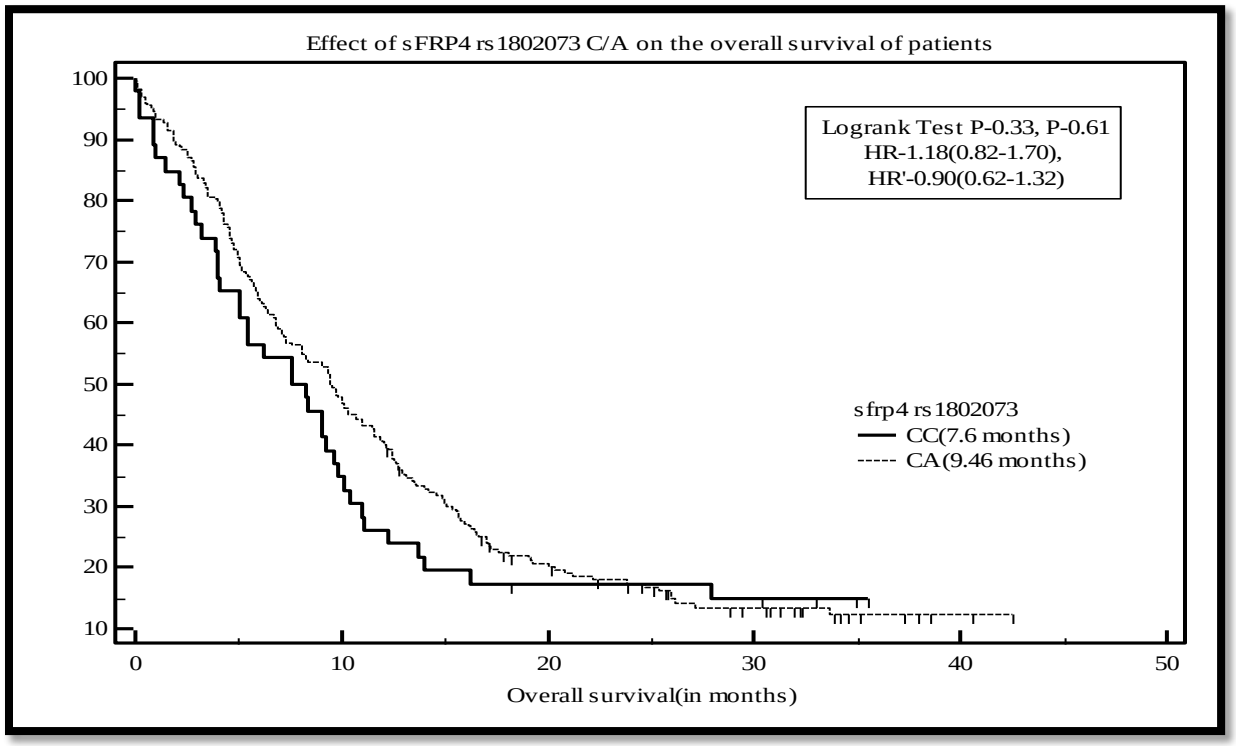


Fig.5.10 (A) Kaplan-Meier curves of *sFRP4* rs1802073 *C/A* polymorphism overall survival of patients having (*CC* and *CA*) genotype

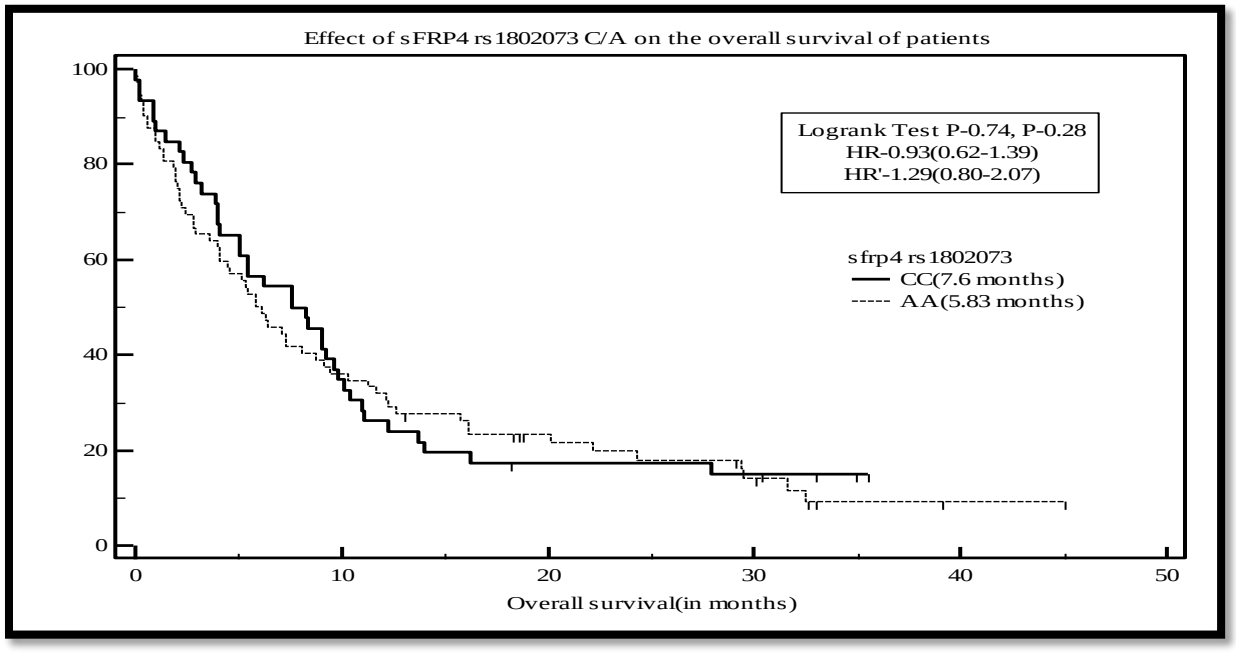


Fig.5.10 (B) Kaplan-Meier curves of *sFRP4* rs1802073 *C/A* polymorphism overall survival of patients having (*CC* and *AA*) genotype

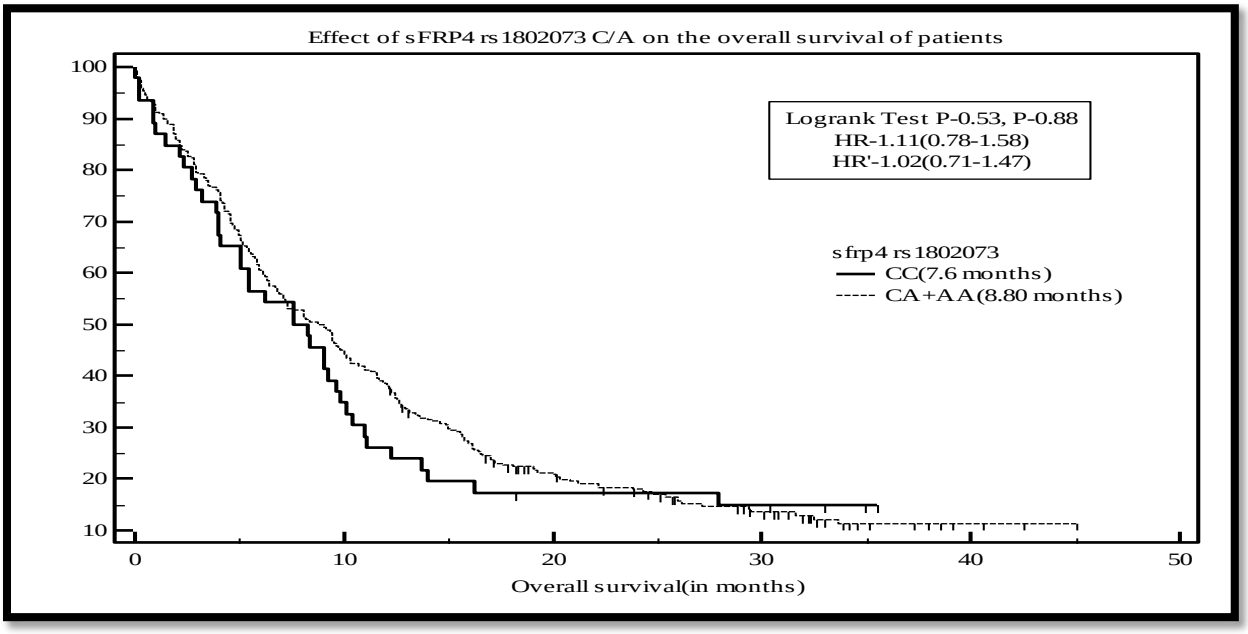


Fig.5.10 (C) Kaplan-Meier curves of *sFRP4* rs1802073 *C/A* polymorphism overall survival of patients having (*CC* and *CA+AA*) genotype

5.18 Genotypic distribution and association of *sFRP4 rs 1802073 C/A* with overall survival of lung cancer patients on the basis of histological subtypes:

Total 334 cases were studied for *sFRP4* gene association with overall survival. SCLC patients with heterozygote (*CA*) genotype showed significant association by applying univariate analysis (HR=1.65, 95% CI=0.89-3.06, *p*=**0.05**) as compared to mutant (*AA*) genotype (HR=1.05, 95% CI=0.52-2.12, *p*=0.87). Higher survival period (10.66 month) was found in heterozygote (*CA*) genotype as compared to wild (*CC*) and mutant (*AA*) genotype (8.26 and 5.80 months). In ADCC and SQCC patients' higher survival period is reported in heterozygote (*CA*) genotype (9.46 and 8.20 months) without showing any statistical significance. Furthermore combined variant (*CA+AA*) genotype presents marginally significant values (MST=9.00, HR=0.84-2.76, *p*=0.09), as shown in table 5.13. Comparison of overall survival for ADCC genotypes is shown in figures C.

Table 5.13 Representing relationship of *sFRP4* genotype with overall survival of lung cancer patients on the basis of histology

1. ADCC

Genotype	Dead (99) n%	Alive (21) n%	Median OS months	HR (95%CI)	Log *p*	HR[b] (95% CI)[b]	*p*

CC	10 (83.33)	2 (16.67)	7.60	-	-	-	-
CA	58 (80.56)	14 (19.44)	9.46	1.24 (0.60-2.58)	0.51	0.80 (0.34-1.87)	0.61
AA	21 (87.50)	3 (12.50)	4.13	0.67 (0.32-1.37)	0.29	1.64 (0.56-4.75)	0.36
CA+AA	89 (82.41)	19 (17.59)	9.13	1.06 (0.54-2.08)	0.85	0.90 (0.41-1.99)	0.81

2. SQCC

Genotype	**Dead (102) n%**	**Alive (23) n%**	**Median (OS months)**	**HR (95%CI)**	**Log *p***	**HR[b] (95% CI)[b]**	***p***
CC	11 (73.33)	4 (26.67)	6.23	-	-	-	-
CA	65 (82.28)	14 (17.72)	8.20	0.87 (0.47-1.61)	0.69	1.00 (0.46-2.17)	0.98
AA	26 (83.37)	5 (16.13)	7.33	0.81 (0.41-1.60)	0.55	1.05 (0.45-2.44)	0.89
CA+AA	91 (82.73)	19 (17.27)	8.06	0.84 (0.46-1.51)	0.59	1.07 (0.56-2.19)	0.84

3. SCLC

Genotype	**Dead (94) n%**	**Alive (7) n%**	**Median (OS months)**	**HR (95%CI)**	**Log *p***	**HR[b] (95% CI)[b]**	***p***
CC	18 (94.74)	1 (5.26)	8.26	-	-	-	-
CA	62 (92.54)	5 (7.46)	10.66	1.65 (0.89-3.06)	**0.05**	0.66 (0.34-1.26)	0.21
AA	14 (93.33)	1 (6.67)	5.80	1.05 (0.52-2.12)	0.87	1.11 (0.46-2.68)	0.81
CA+AA	76 (92.68)	6 (7.32)	9.00	1.52 (0.84-2.76)	0.09	0.83 (0.45-1.53)	0.55

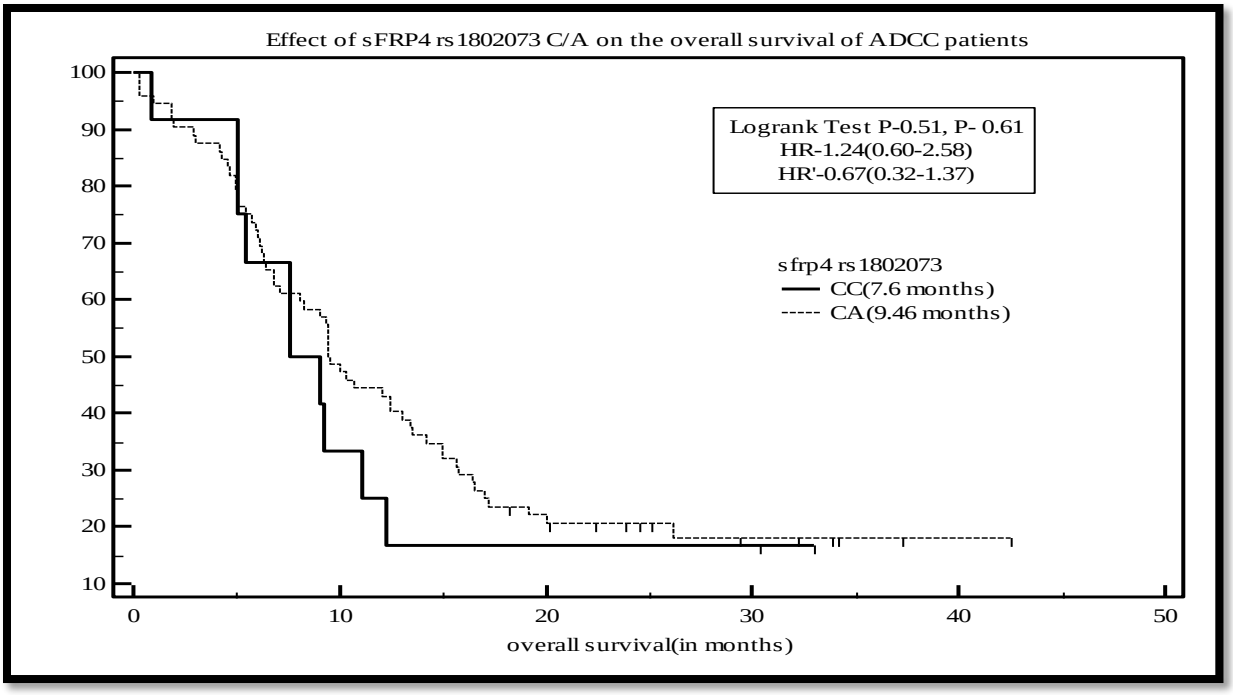


Fig.5.11 (A) Kaplan-Meier curves of *sFRP4* rs1802073 *C/A* polymorphism overall survival of ADCC patients having (*CC* and *CA*) genotype

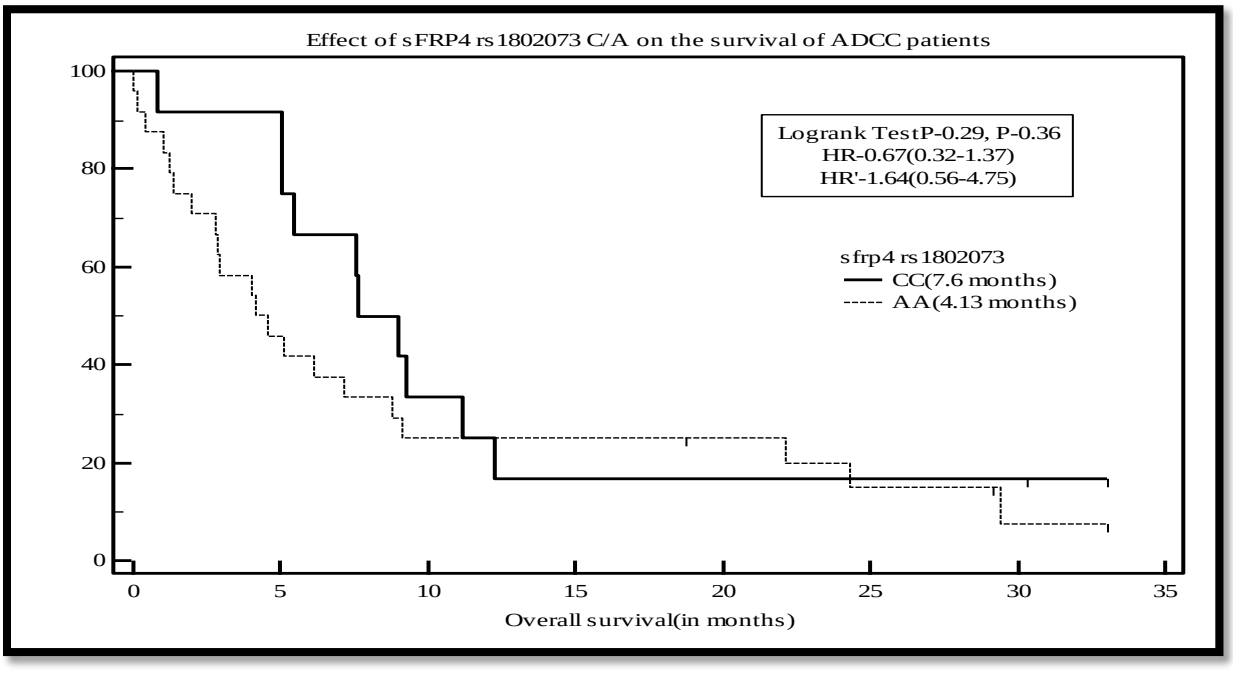


Fig.5.11 (B) Kaplan-Meier curves of *sFRP4* rs1802073 *C/A* polymorphism overall survival of ADCC patients having (*CC* and *AA*) genotype

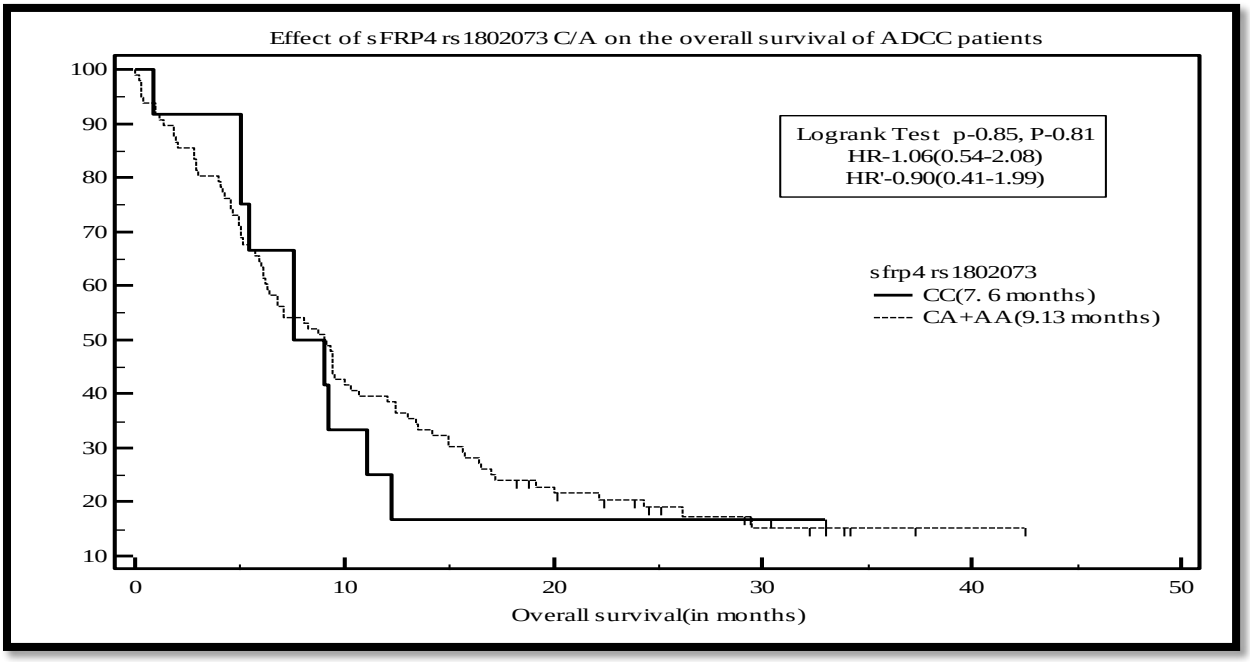


Fig.5.11 (C) Kaplan-Meier curves of *sFRP4* rs1802073 *C/A* polymorphism overall survival of patients having (*CC* and *CA+AA*) genotype

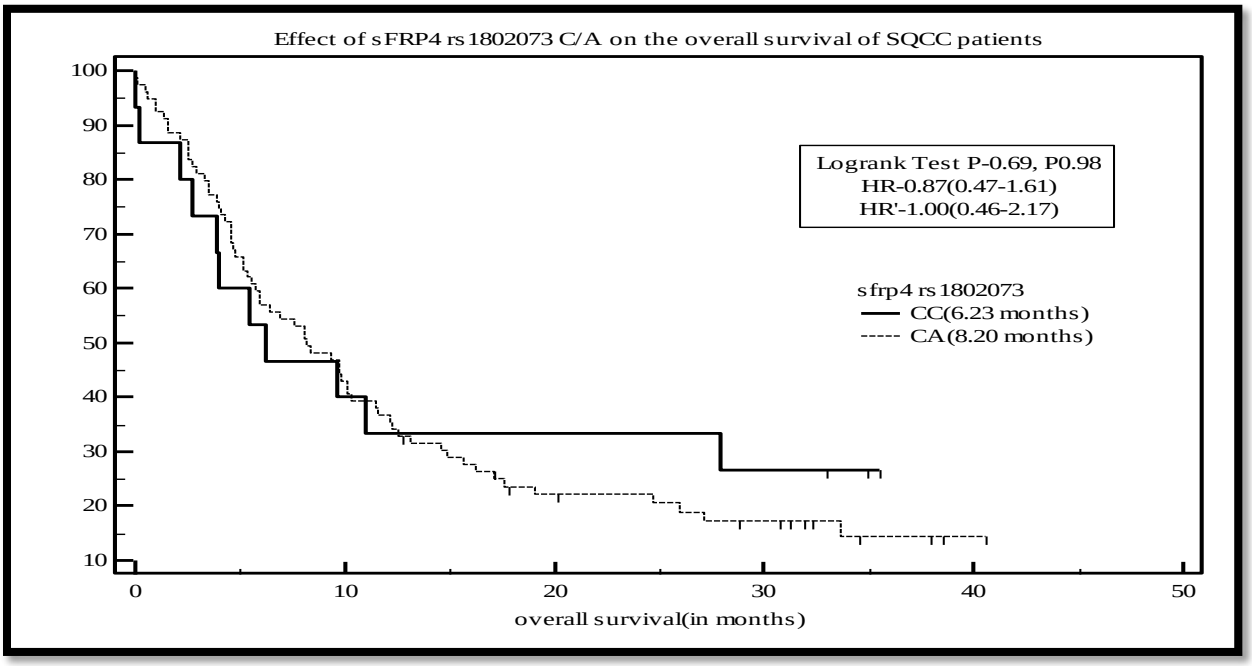


Fig.5.12 (A) Kaplan-Meier curves of *sFRP4* rs1802073 polymorphism overall survival of SQCC patients having (*CC* and *CA*) genotype

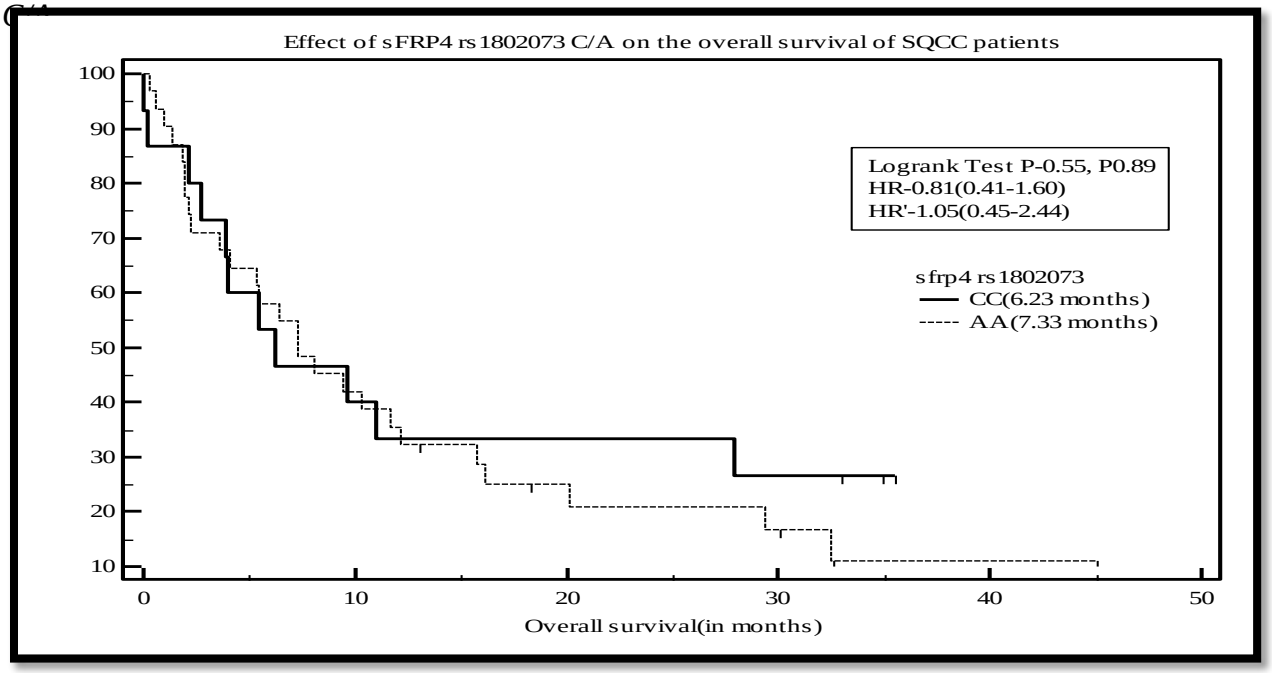


Fig.5.12 (B) Kaplan-Meier curves of *sFRP4* rs1802073 *C/A* polymorphism overall survival of SQCC patients having (*CC* and *AA*) genotype

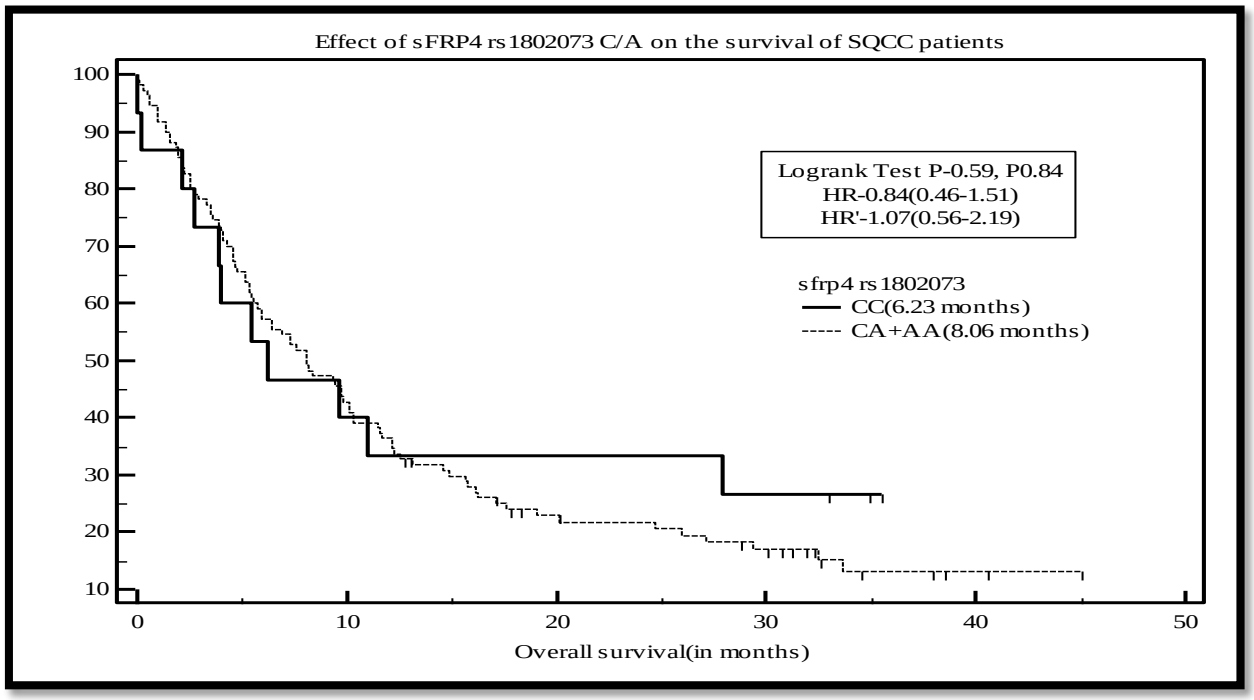


Fig.5.12 (C) Kaplan-Meier curves of *sFRP4* rs1802073 *C/A* polymorphism overall survival of SQCC patients having (*CC* and *CA+AA*) genotype

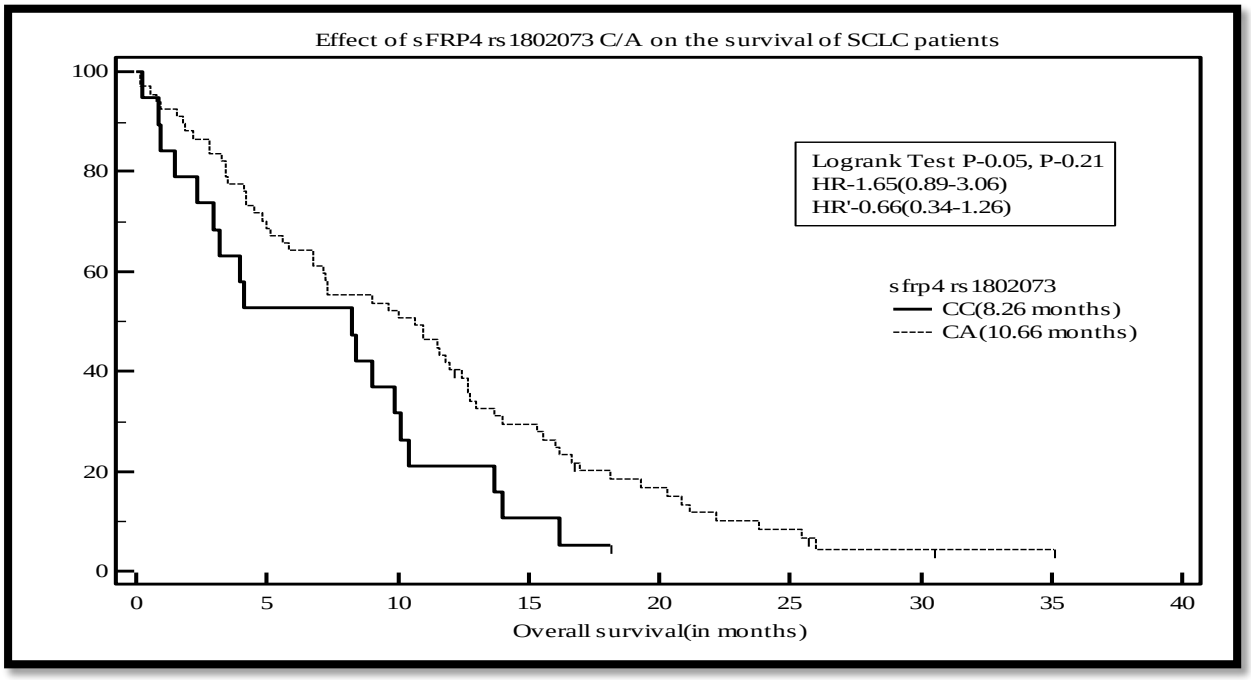


Fig.5.13 (A) Kaplan-Meier curves of *sFRP4* rs1802073 *C/A* polymorphism overall survival of SCLC patients having (*CC* and *CA*) genotype

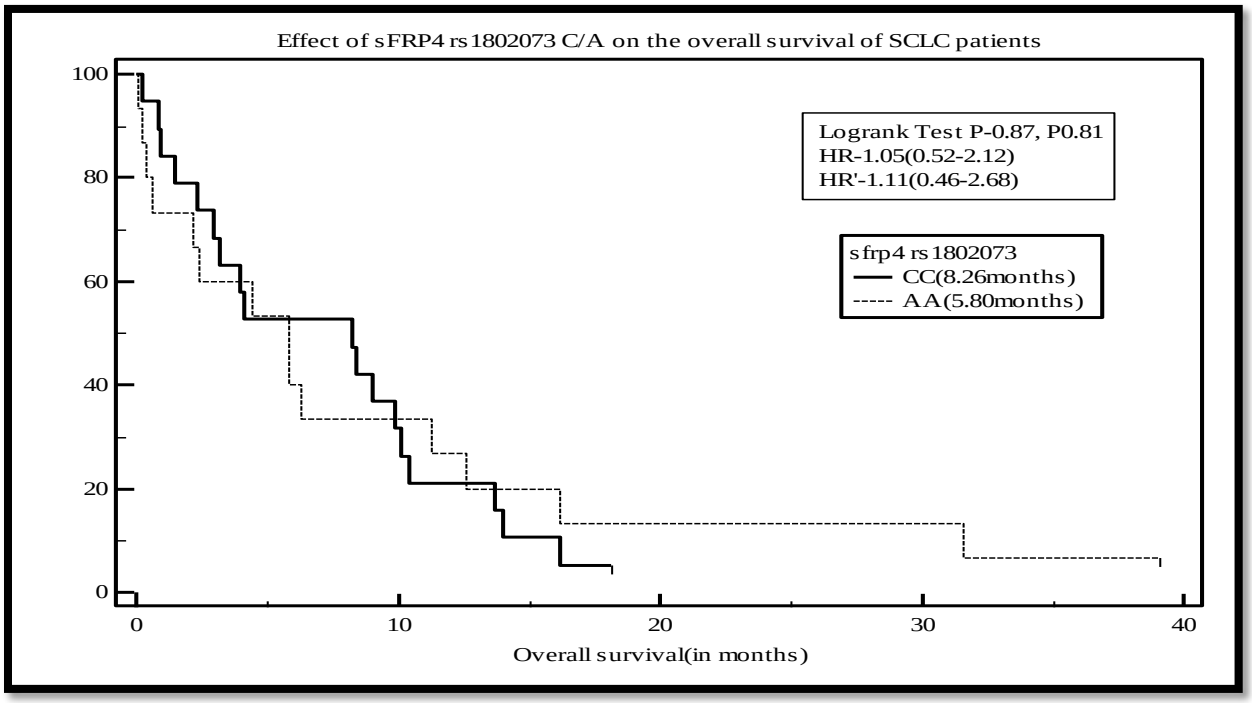


Fig.5.13 (B) Kaplan-Meier curves of *sFRP4* rs1802073 *C/A* polymorphism overall survival of SCLC patients having (*CC* and *CA+AA*) genotype

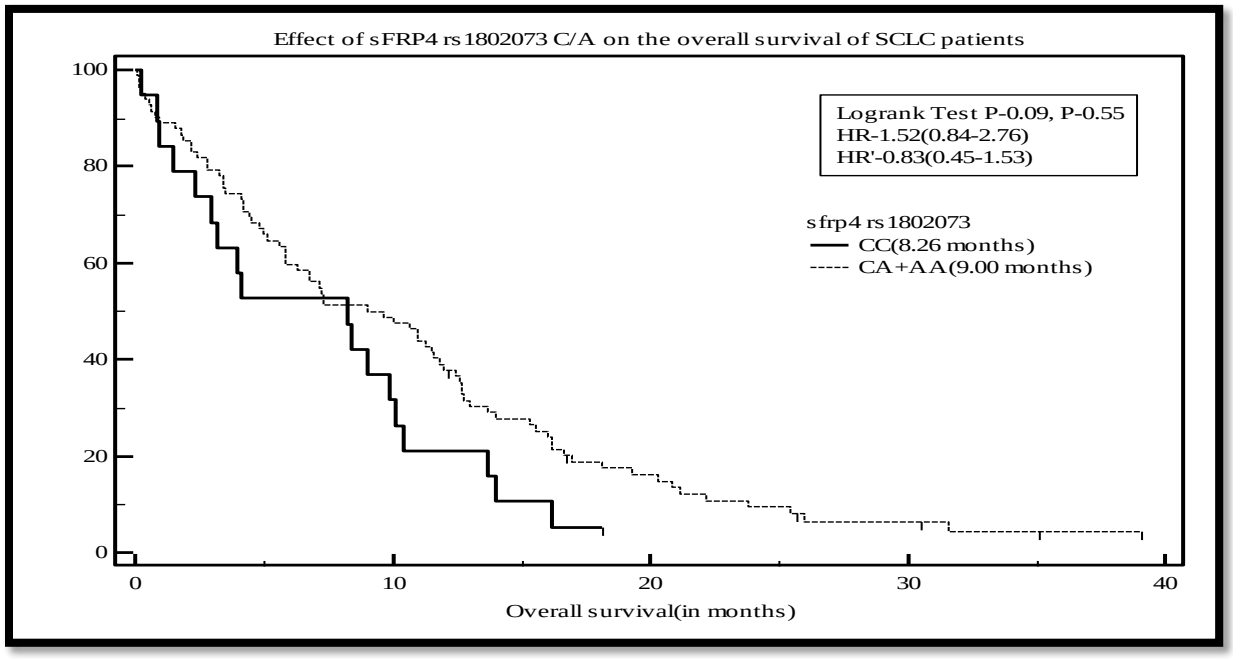


Fig.5.13 (C) Kaplan-Meier curves of *sFRP4* rs1802073 *C/A* polymorphism overall survival of SCLC patients having (*CC* and *CA+AA*) genotype

5.19 Genotypic distribution and association of *sFRP4 rs1802073 C/A* with overall survival of lung cancer patients on the basis of gender:

sFRP4 gene studied for north Indian population was further categorized on the basis of gender which include 295 (86.76%) males and 45 (13.23%) females. It was observed that male heterozygote (*CA*) genotype had a higher survival rate (9.73 months) without showing any statistical significance (HR=1.17, 95%CI=0.79-1.72, *p*=0.40). In females survival rate of wild (*CC*) genotype is greater than heterozygote (*CA*) and mutant (*AA*) genotype (13.70 *vs* 8.03 and 4.13 months). No statistical significance was observed for any of genotype (*CC, CA* and *AA*) in females when analyzed by univariate Kaplan analysis or with multivariate Cox hazard analysis representing in table 5.14.

Table 5.14 Representing relationship of *sFRP4* genotype with overall survival of lung cancer patients on the basis of gender

	Males							Females						
Genotype	**Dead (249) n%**	**Alive (46) n%**	**Median (OS months)**	**HR (95% CI)**	**Log *p***	**HR[b] (95% CI)[b]**	***p***	**Dead (40) n%**	**Alive (5) n%**	**Median (OS months)**	**HR (95%CI)**	**Log *p***	**HR[b] (95% CI)[b]**	***P***
CC	34 (82.93)	7 (17.07)	7.60	-		-	-	5 (100)	0 (0)	13.70	-	-	-	-
CA	162 (84.82)	29 (15.18)	9.73	1.17 (0.79-1.72)	0.40	0.89 (0.59-1.34)	0.60	26 (83.87)	5 (16.13)	8.03	1.39 (0.47-4.08)	0.48	0.69 (0.15-3.01)	0.62
AA	53 (84.13)	10 (15.87)	6.40	1.00 (0.65-1.54)	0.99	1.89 (0.71-1.97)	0.50	9 (100)	0 (0)	4.13	0.68 (0.23-1.95)	0.46	47.88 (2.63-871.52)	0.009
CA+AA	215 (84.65)	39 (15.35)	9.33	1.12 (0.7-1.63)	0.53	0.97 (0.66-1.44)	0.90	35 (87.50)	5 (12.50)	6.43	1.18 (0.43-3.23)	0.71	1.18 (0.37-3.72)	0.77

5.20 Association of *sFRP4 rs1802073 C/A* with overall survival of lung cancer patients on the basis of their smoking status:

While this demarcation for *sFRP4* analyses total 282 smokers and 58 nonsmokers. No statistical significance was observed in any of population. MST is almost similar for heterozygote (*CA*) genotype of smoker and nonsmoker population (9.36 and 9.46 months). Furthermore combined variant (*AA+CA*) genotype showed higher survival rate in both smokers (8.20 months) and nonsmokers (9.43 months) with statistical analysis (HR=1.11, 95 %CI=0.77-1.61, *p*=0.53 *vs* HR=0.97, 95 %CI=0.30-3.10, *p*=0.96), as shown in table 5.15.

Table 5.15 Representing relationship of *sFRP4* genotype with overall survival of lung cancer patients on the basis of smoking status

	Smokers							Non-smokers						
Genotype	**Dead (241) n%**	**Alive** (41) **n%**	**Median (OS months)**	**HR (95% CI)**	**Log *p***	**HR[b] (95% CI)[b]**	***p***	**Dead (47) n%**	**Alive (10) n%**	**Median (OS months)**	**HR (95% CI)**	**Log *p***	**HR[b] (95% CI)[b]**	***p***
CC	36 (85.71)	6 (14.29)	7.60	-		-	-	3 (75)	1 (25)	4.10	-		-	-
CA	156 (85.71)	26 (14.29)	9.36	1.17 (0.80-1.72)	0.37	0.86 (0.58-1.27)	0.45	31 (79.49)	8 (20.51)	9.46	1.04 (0.31-3.51)	0.93	2.06 (0.51-8.38)	0.30
AA	49	9	5.43	0.95	0.84	1.50	0.12	13	1	6.10	0.82	0.75	1.05	0.96

	(84.48)	(15.52)		(0.62-1.47)		(0.89-2.51)		(92.86)	(7.14)		(0.25-2.69)		(0.13-8.42)	
CA+AA	205 (85.42)	35 (14.58)	8.20	1.11 (0.77-1.61)	0.53	0.99 (0.68-1.45)	0.99	44 (83.02)	9 (16.98)	9.43	0.97 (0.30-3.10)	0.96	1.76 (0.46-6.64)	0.40

5.21 Genotypic distribution and association of *sFRP4 rs1802073 C/A* with overall survival of lung cancer patients on the basis of regimen:

Moreover studies were carried out for another gene *sFRP4* on the basis of regimen. Out of regimen (Docetaxel+ cisplatin/carboplatin, Irinotecan+ cisplatin/carboplatin and Pemetrexed+ cisplatin/carboplatin) heterozygote (*CA*) genotype showed higher survival rate (10.33 months) without any significant association with overall survival of lung cancer patients (HR=1.08, 95% CI=0.47-2.47, *p*=0.84), as shown in table 5.16 (a, b). Almost similar MST was observed for regimen Docetaxel+ cisplatin/carboplatin genotypes *CC*, *CA* and *AA* (8.40, 9.73 and 8.40 months) for wild (*CC*), heterozygote (*CA*), and mutant (*AA*) genotype respectively.

Table 5.16 (a) Representing relationship of *sFRP4* genotype with overall survival of lung cancer patients on the basis of regimen

Docetaxel+ cisplatin/carboplatin

Genotype	Dead (68) n%	Alive (14) n%	Median (OS months)	HR (95%CI)	Log *p*	HR[b] (95% CI)[b]	*p*
CC	10 (76.92)	3 (23.08)	8.40	-	-	-	-
CA	43 (84.31)	8 (15.69)	9.73	0.92 (0.47-1.80)	0.81	1.06 (0.43-2.57)	0.88
AA	15 (83.33)	3 (16.67)	8.40	0.96 (0.43-2.13)	0.92	0.61 (0.33-1.13)	0.12
CA+AA	58 (84.06)	11 (15.94)	9.73	0.92 (0.47-1.76)	0.80	1.06 (0.43-2.57)	0.88

Table 5.16 (b) Representing relationship of *sFRP4* genotype with overall survival of lung cancer patients on the basis of regimen														
Irinotecan+ cisplatin/carboplatin								**Pemetrexed+ cisplatin/carboplatin**						
Genotype	**Dead (70) n%**	**Alive (10) n%**	**Median (OS months)**	**HR (95%CI)**	**Log *p***	**HR[b] (95% CI)[b]**	***p***	**Dead (55) n%**	**Alive (16) n%**	**Median (OS months)**	**HR (95%CI)**	**Log *p***	**HR[b] (95% CI)[b]**	***p***
CC	10 (90.91)	1 (9.09)	8.26	-		-		7 (77.78)	2 (22.22)	9.23	-		-	-
CA	46 (86.79)	7 (13.21)	10.00	1.64 (0.72-3.72)	0.14	0.76 (0.31-1.83)	0.54	38 (77.55)	11 (22.45)	10.33	1.08 (0.47-2.47)	0.84	0.94 (0.38-2.30)	0.90
AA	14 (87.50)	2 (12.50)	5.83	1.36 (0.58-3.18)	0.43	1.06 (0.34-3.26)	0.91	10 (76.92)	3 (23.08)	6.10	0.77 (0.24-2.00)	0.60	3.10 (0.62-15.3)	0.16
CA+AA	60 (86.96)	9 (13.04)	9.66	1.58 (0.71-3.52)	0.16	0.76 (0.31-1.83)	0.54	48 (77.42)	14 (22.58)	9.46	1.00 (0.45-2.22)	0.99	0.98 (0.40-2.40)	0.97

5.22 Genotypic distribution and association of *sFRP4 rs1802073 C/A* with overall survival of lung cancer patients on the basis of performance status after receiving chemotherapy:

For *sFRP4* gene, no significant association was reported for KPS performance status. Heterozygote (*CA*) genotype of KPS (90-100) shows comparatively lesser death rate as compared to wild (*CC*) and mutant (*AA*) genotype (11.50 *vs* 5.06 and 6.10 months) as shown in table 5.17 (a) and 5.17 (b). ECOG (0, 1) and ECOG (2-4) patients were analyzed for estimation of overall survival and the data is shown n table 5.17 (c). Lesser probability of death was optimized with heterozygote (*CA)* and combined variant (*CA+AA*) genotype of ECOG (0, 1) patients with univariate analysis (MST= 11.50 months, HR=1.97, 95% CI=1.06-3.66, *p*=**0.004** and MST=11.50 months, HR=1.93, 95% CI=1.04-3.56, *p*=**0.005**). Further multivariate studies scrutinized wild (HR'=0.42, 95% CI=0.24-0.72, *p*=**0.001**), heterozygote (HR'=0.43, 95% CI=0.20-0.92, *p*=**0.03**) and mutant (HR'=0.46, 95% CI=0.27-0.79, *p*=**0.005**) genotype to be statistically significant with as shown in fig. (5.14. A, B and C) and in table 5.17 (c). Furthermore multivariate analysis of ECOG (2-4) mutant (AA) genotype predicted significant association (MST= 5.13 months, HR'=2.54, 95% CI=1.33-4.86, *p*=**0.004**).

Table 5.17 (a) Representing relationship of *sFRP4* genotype with overall survival of lung cancer patients on the basis of performance status (KPS)	
KPS (30-60)	**KPS (70-80)**

Genotype	Dead (33) n%	Alive (11) n%	Median (OS months)	HR (95% CI)	Log *p*	HR[b] (95% CI)[b]	*p*	Dead (134) n%	Alive (20) n%	Median (OS months)	HR (95% CI)	Log *p*	HR[b] (95% CI)[b]	*p*
CC	7 (70)	3 (30)	9.00	-		-	-	21 (91.30)	2 (8.70)	7.60	-	-	-	-
CA	22 (78.57)	6 (21.43)	8.20	0.77 (0.34-1.71)	0.54	0.76 (0.37-1.56)	0.46	79 (86.81)	12 (13.19)	7.23	1.27 (0.75-2.14)	0.31	0.79 (0.47-1.33)	0.38
AA	4 (66.67)	2 (33.33)	3.56	0.92 (0.26-3.20)	0.89	0.93 (0.39-2.20)	0.87	34 (85.00)	6 (15)	5.80	1.12 (0.64-1.94)	0.67	1.32 (0.70-2.48)	0.38
CA+AA	26 (76.47)	8 (23.53)	8.20	0.79 (0.36-1.75)	0.59	0.84 (0.42-1.68)	0.63	113 (86.26)	18 (13.74)	7.13	1.22 (0.74-2.02)	0.38	0.90 (0.55-1.48)	0.69

Table 5.17 (b) Representing relationship of *sFRP4* genotype with overall survival of lung cancer patients on the basis of performance status (KPS)

KPS(90-100)							
Genotype	**Dead (114) n%**	**Alive (19) n%**	**Median (OS months)**	**HR (95%CI)**	**Log *p***	**HR[b] (95% CI)[b]**	*p*
CC	10 (83.33)	2 (23.53)	5.06	-	-	-	-
CA	81 (84.37)	15 (9.26)	11.50	1.44 (0.67-3.10)	0.26	1.09 (0.41-2.90)	0.84
AA	23 (92)	2 (25)	6.10	0.94 (0.45-1.97)	0.88	0.75 (0.18-3.09)	0.69
CA+AA	104 (85.95)	17 (13.51)	9.86	1.31 (0.63-2.71)	0.40	1.08 (0.43-2.71)	0.86

Table 5.17 (c) Representing relationship of *sFRP4* genotype with overall survival of lung cancer patients on the basis of performance status (ECOG)

ECOG (0-1)							
Genotype	**Dead (146) n%**	**Alive (20) n%**	**Median (OS months)**	**HR (95%CI)**	**Log *p***	**HR[b] (95% CI)[b]**	***p***
CC	18 (78.26)	5 (5)	9.66	-	-	-	-
CA	89 (89)	11 (11)	6.86	0.72 (0.45-1.14)	0.20	1.39 (0.79-2.44)	0.24
AA	39	4	5.13	0.59	0.06	2.54	**0.004**

	(90.70)	(9.30)		(0.35-1.00)		(1.33-4.86)	
CA+AA	128 (89.51)	15 (10.49)	6.30	0.68 (0.44-1.04)	0.12	1.55 (0.91-2.65)	0.10
CC	20 (90.91)	2 (9.09)	5.06	-	-	-	-
CA	93 (80.87)	22 (19.13)	11.50	1.97 (1.06-3.66)	**0.004**	0.42 (0.24-0.72)	**0.001**
AA	22 (78.57)	6 (21.43)	8.80	1.69 (0.89-3.20)	0.07	0.43 (0.20-0.92)	**0.03**
CA+AA	115 (80.42)	28 (19.58)	11.50	1.93 (1.04-3.56)	**0.005**	0.46 (0.27-0.79)	**0.005**

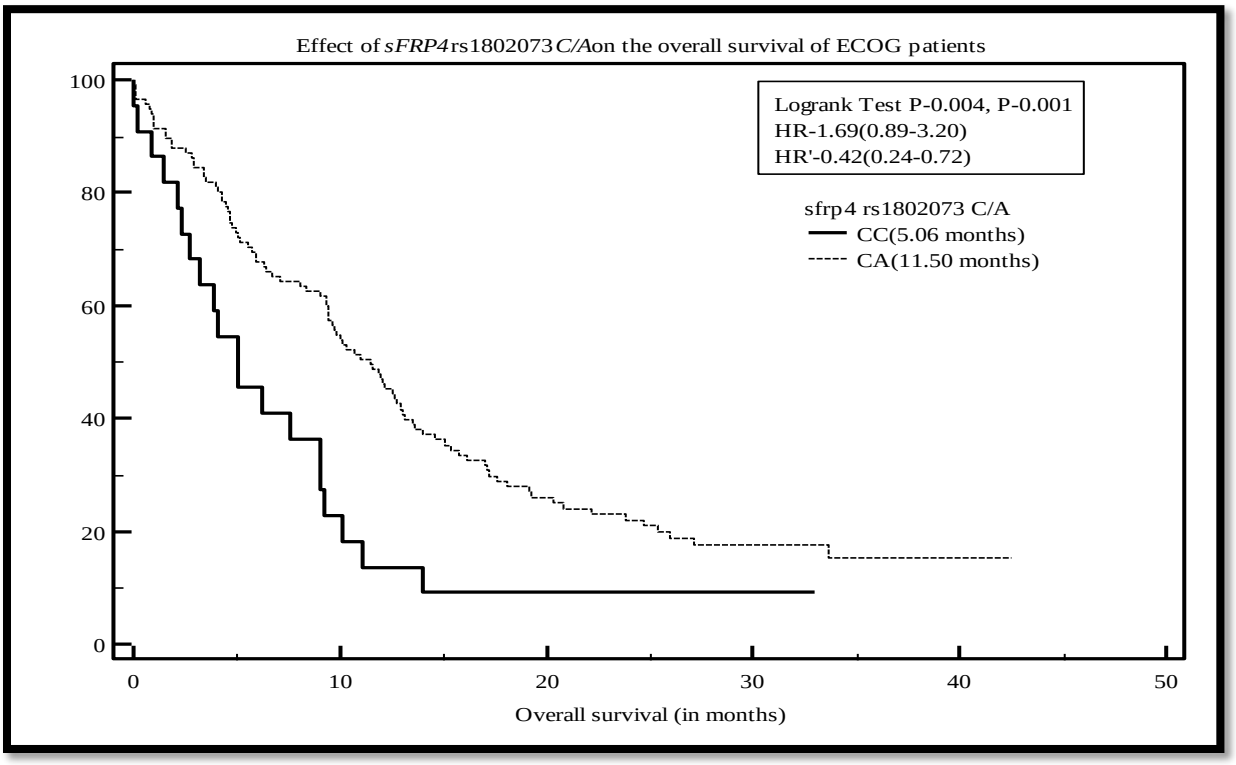


Fig.5.14 (A) Kaplan-Meier curves of *sFRP4* rs1802073 *C/A* polymorphism overall survival of ECOG patients having (*CC* and *CA*) genotype

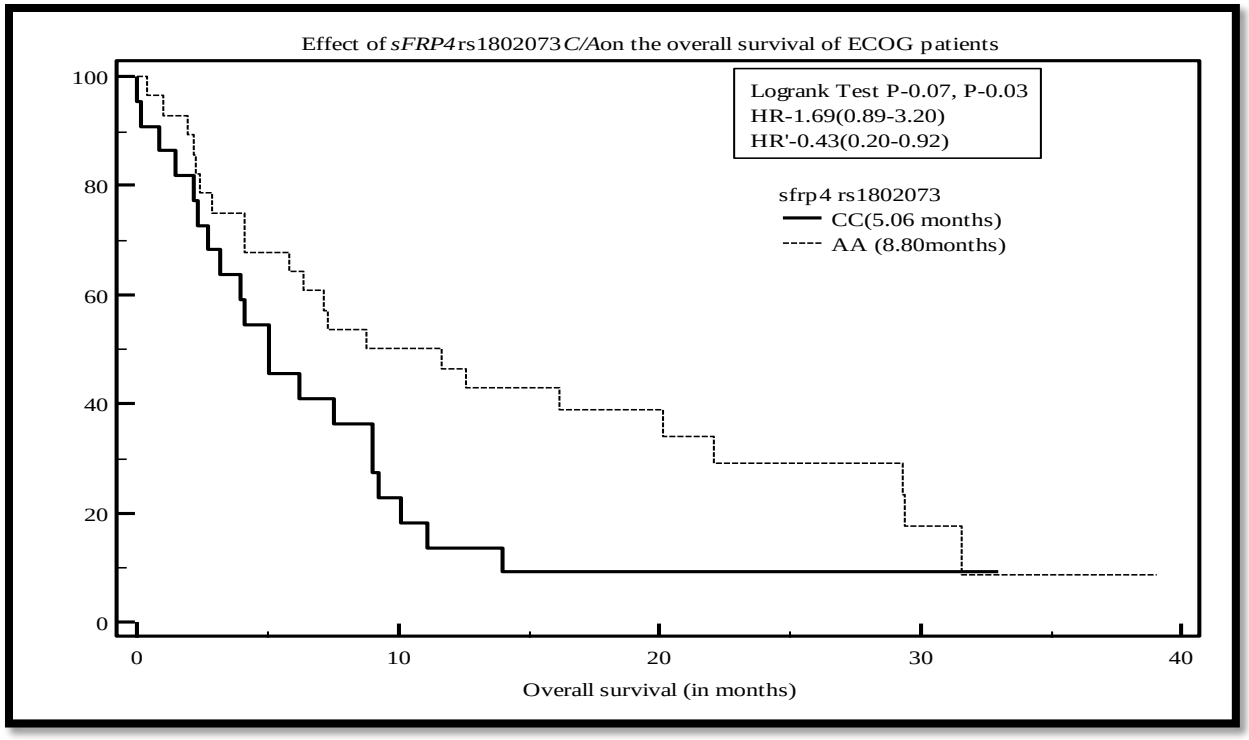


fig.5.14 (B) Kaplan-Meier curves of *sFRP4* rs1802073 *C/A* polymorphism overall survival of ECOG patients having (*CC* and *CA+AA*) genotype

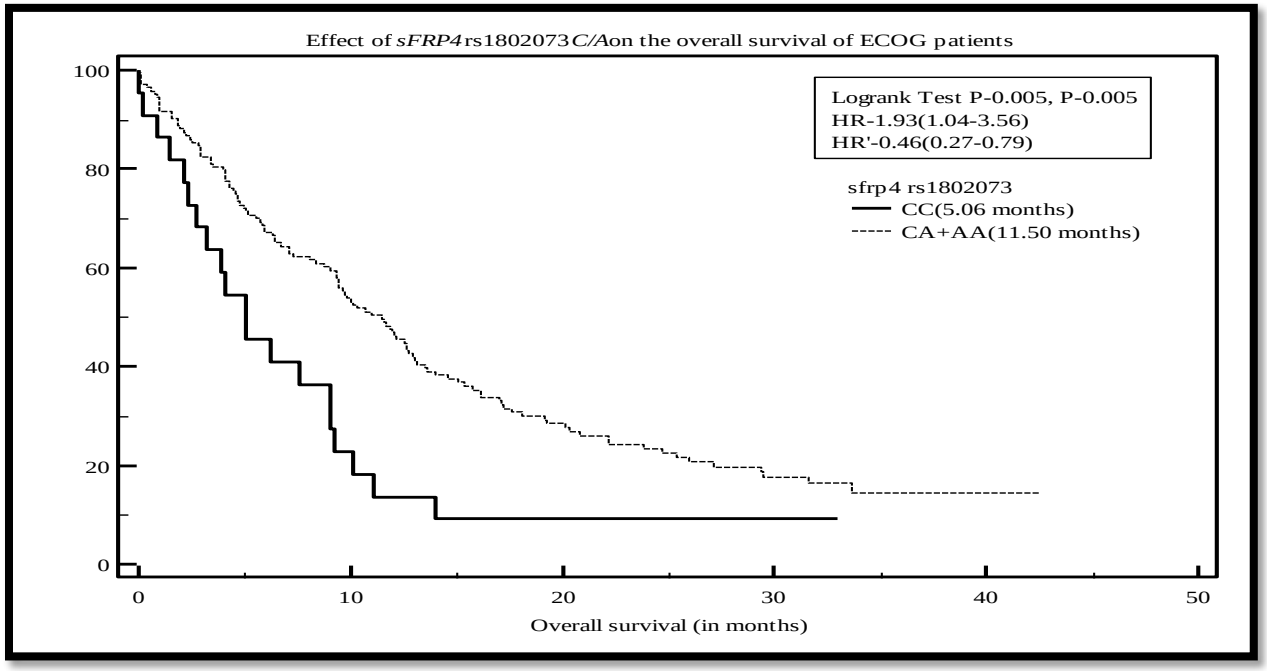


Fig.5.14 (C) Kaplan-Meier curves of *sFRP4* rs1802073 *C/A* polymorphism overall survival of ECOG patients having (*CC* and *CA+AA*) genotype

5.23 Genotypic distribution and Association of *sFRP4* rs1802073 *C/A* with lung cancer the basis of chemotherapy response:

Association of *sFRP4* and lung cancer patients with chemotherapy response is summarized in table (5.18). Out of 340 patients 10 (2.94%) patients exhibiting complete response, 112 (32.94%) partial response, 88 (25.88%) showed stable disease while 23 (6.76%) showed progressive disease. CR+PR patients tend to show a higher genotypic frequency of heterozygous (*CA*) genotype than wild (*CC*) and mutant genotypes (*AA*) towards chemotherapy response (21.47% *vs* 2.35% and 7.05%). Significant association was observed for variant (*CA+AA*) genotype toward chemotherapy response with a significant lower *p* value **0.02.**

Table 5.18 Representing relationship of different *sFRP4* genotypes with the chemotherapy response

	Response of chemotherapy		**[b]AOR (95% CI)[b]**	***p***
Genotype	**CR+PR (105) n (%)**	**SD+PD(91) n (%)**		
CC	8 (2.35)	21 (6.17)	-	-
CA	73 (21.47)	51 (15)	0.44 (0.16-1.24)	0.12
AA	24 (7.05)	19 (5.58)	0.29 (0.06-1.25)	0.09
CA+AA	97 (28.52)	70 (20.58)	0.36 (0.15-0.86)	**0.02**

[b] Adjusted Odds ratios, 95% confidence intervals and their corresponding *p*-values were calculated by unconditional logistic analysis after adjusting for age, gender and smoking.

5.24 Genotypic distribution and association of *sFRP4* rs1802073 *C/A* with clinic-pathological parameters:

Association of *sFRP4* gene clinic-pathological parameters and risk of occurrence of lung cancer is demarcated in table 5.19(a) and (b).No significant association was found when data was studied for lymph node invasion (N_0 *vs* $N_1+N_2+N_3$). Further studies were done by classifying patients on the basis of different stages comprises 164 (48.23%) stage III and 139(40.88%) stage IV patients. Subjects carrying *C* and *A* allele showed significant association with a significant lower *p* value **0.01.** Almost similar *p* values were obtained for mutant (*AA*) and heterozygote (*AA+CA*) genotype without any significant association (0.54 and 0.44) when studies were made on the basis of tumor size. Out of 340 patients 51.17% cases were stratified as M_0 and 39.11% cases as M_1. Study of M_0 *vs* M_1 shows significant association between heterozygote (*CA*) genotype and variant (*CA+AA*) genotype with lung cancer progression (*p* value **0.0003** and **0.002)** as shown in table 5.19 (b).

Table 5.19 (a) Representing relationship of different *sFRP4* genotypes with the clinic-pathological parameters

	Clinical Stage		[b]AOR (95%CI)[b]	P	Primary Tumor Extension		[b]AOR (95%CI)[b]	P	Lymph Node Invasion		[b]AOR (95%CI)[b]	P
Genotype	**III (164) n%**	**IV (139) n%**			**T_1+T_2 (52) n%**	**T_3+T_4 (247) n%**			**N_0 (37) n%**	**N_1+N_2+N_3 (267) n%**		
CC	21 (6.17)	22 (6.47)	_	_	8 (2.35)	32 (9.41)	_	_	3 (0.88)	40 (11.76)	_	_
CA	107 (31.47)	89 (26.17)	0.37 (0.16-0.84)	**0.01**	32 (9.41)	168 (49.41)	0.68 (0.20-2.31)	0.54	25 (7.35)	176 (51.76)	1.21 (0.44-3.32)	0.71
AA	36 (10.58)	28 (8.23)	0.79 (0.22-2.81)	0.72	12 (3.52)	47 (13.82)	0.42 (0.04-3.76)	0.44	9 (2.64)	51 (15)	0.92 (0.20-4.08)	0.91
CA+AA	143 (42.05)	117 (34.41)	0.79 (0.22-2.81)	0.72	44 (12.94)	215 (63.23)	0.77 (0.27-2.19)	0.62	34 (10)	227 (66.76)	2.06 (0.95-4.44)	0.06

[b] Adjusted Odds ratios, 95% confidence intervals and their corresponding *p*-values were calculated by unconditional logistic analysis after adjusting age, gender and smoking

Table 5.19 (b) Representing relationship of different *sFRP4* genotypes with the clinic-pathological parameters

	Sex		[b] AOR (95% CI) [b]	*p*	Metastasis		[b] AOR (95% CI) [b]	*p*
Genotype	**F (45) n (%)**	**M (295) n (%)**			**Mo (156) n (%)**	**M_1 (133) n (%)**		
CC	5 (1.47)	41 (12.05)	-	-	19 (5.58)	22 (6.47)	-	-
CA	31 (9.11)	191 (56.17)	1.00 (0.00-0.00)	1.00	108 (31.76)	84 (24.70)	0.29 (0.15-0.56)	**0.0003**
AA	9 (2.64)	63 (18.52)	1.00 (0.00-0.00)	1.00	29 (8.52)	27 (7.94)	0.45 (0.15-1.32)	0.15
CA+AA	40 (11.76)	254 (74.70)	1.00 (0.00-0.00)	1.00	137 (40.29)	111 (32.64)	0.30 (0.13-0.65)	**0.002**

[b] Adjusted Odds ratios, 95% confidence intervals and their corresponding *p*-values were calculated by unconditional logistic analysis after adjusting for age, gender and smoking.

CHAPTER 6

Discussion

TGF-β is a cytokine which is integrated for many signaling pathways and control various functions such as proliferation and differentiation, arrest cell cycle, inhibit ectodermal derived cell growth etc. Depending upon the type of cancer, dysregulation in this gene has been reported in both tumor suppressor as well as in tumor progression activities. It is also known that *TGF-β* act as tumor suppressor gene in early stage of cancer, but switch to tumor enhancer in later stages. It helps in tumor suppression by inhibiting cell cycle progression through G1 arrest, maintain genomic stability by sensing DNA damage and by modulating growth factors in tumor microenvironment (Joseph H *et al.,* 1999). This gene has been reported in mechanism of tumor progression, where it helps in epithelial to mesenchymal transition, immunosuppression, and promoter metastasis and in angiogenesis using vascular endothelial growth factor (Kretzschmar M *et al.,* 1998).

Several SNPs of *TGF-β* have been reported in modulating protein function and modulating gene expression. In present study, we had evaluated the overall survival of lung cancer patients in north Indian population. [Li, T et al., 2008] studied that -509 T allele is significantly associated with higher level of *TGF-β* than C allele and suggest protective effect on gastric cancer whether Guo RJ *et al.,* 1998 did not find any significant association between *TGF-β* and overall survival in gastric cancer. However significant association between rs1800469 and hepatocellular carcinoma in Chinese population was reported by [Guo, Y *et al.,* 2013]. In the present study, in codon -509 C>T, the genotype frequency are 0.21, 0.10 and 0.35 in genotypes *Thr/Thr*, *Cys/Thr* and *Cys/Cys* respectively which is not associated with improved survival rate in lung cancer patients. Our result shows similar findings with Park *et al.,* 1999, but different to the findings of Kang *et al.,* Meta-analysis performed by Yi Liu *et al.,* 2012 report that this polymorphism is not associated with occurrence of colorectal cancer. Similar findings were reported by Tamizifar *et al.,* that this genotype and allelic frequency did not show any significant association in ulcerative colitis. Guangfu *et al.,* 2008 found that the mutant (TT) and heterozygote (CT) genotype of *TGF-β*1 rs1800469 were significantly associated with decreased risk of esophageal squamous cell carcinoma as compared with wild (CC) genotype. Farahbakhsh *et al.,* 2017 found significant association of *TGF-β*1 509 C>T with pancreatic carcinoma.

Studies conducted by Yang demonstrate association of *TGF-β* high expression with the three year survival rate in patients with glioma [Joana Vieira de Castro *et al.,* 2015], whereas similar results

were reported in gastric cancer [Guan, X *et al.,* 2009], breast cancer [Qianren *et al.*, 2004], hepatocellular carcinoma [Huang *et al.*, 2013] and in renal cancer.

Signaling of *TGF-β* gene takes place in both Smad dependent as well as in Smad independent manner where translocation of GALA-Smad2 complex in nucleus activate various DNA binding transcription factors such as p300 and CBP co-activators . On the other hand, some other pathways such as Erk, JNK, MAPK kinase and Rho-like GTPase's transcription factors activation takes place in Smad independent signaling (Yu L *et al.,* 2002).

Subgroup analysis was also performed based on univariate and multivariate analytical methods. The HR (Hazard ratio) in both the analysis differ, which shows no statistical association. Contradictory results were observed in pancreatic cancer which suggest that *TGF-β*1 rs1800469 is significantly associated with overall survival [Behboudi Farahbakhsh *et al.,* ; 2015]. No significant difference in the overall survival of patients in the different genotype of *TGF-β* was observed, once the tumor had grown bigger and become metastatic. The present study has been carried out in North India population to evaluate association of *TGF-β*1 gene with lung cancer patients by considering parameters such as gender, age, smoking status, histological subtypes, performance status, clinicpathological parameters and response of patients to platinum based chemotherapy. In the present study no significant association was evaluated between *TGF-β* genetic variants and individual susceptibility towards lung cancer. Similar results were reported by Joana cieira de castro *et al.,* 2015 where none of the *TGF-β* polymorphism is significantly associated with survival of glioblastoma. Dysregulated signaling pathways of *TGF-β* gene due to genetic and molecular changes has been reported in various cancers like breast cancer, pancreatic cancer, gastric, non-small cell lung cancer and small cell lung cancer (Laurence Levy *et al.*, 2006; Maurice D *et al.,* 2001).

An attempt was made in this study to classify patients on the basis of histological subtypes and find out the influence of this variant on the different. When the patients were stratified on the basis of histological subtypes (ADCC, SQCC and SCLC) no significant clinical outcomes were observed by univariate analysis. However, significant association was found with SCLC subtypes when the data was analyzed using multivariate Cox regression analysis (P=0.01).

In the current study no significant association was found in ADCC histology in any of the genotype. Ren Y. *et al.,* 2015 report significant association of ADCC and exposure of cooking oil

fumes, where they report dose manner protective effects and conclude that polymorphism of *TGF-β* could be related with overall survival. In the current study genotypes showing P-values are 0.21, 0.10 and 0.35 in genotypes *Thr/Thr*, *Cys/Thr* and *Cys/Cys* respectively which is not associated with improved survival rate in lung cancer patients. Our result shoes similar findings with Park *et al.,* and Ren Y. *et al*., 2015, but different to the findings of Kang *et al.,* Guan *et al.,* did not found any significant association between *TGF-β* 509 C>T polymorphism and gastric cancer. However, SCLC (small cell lung cancer) histology no significant association were observed by using univariate Kaplan-Meier analysis (HR=0.59; CI=0.19-1.85; P=0.31) but shows significant association with multivariate Cox analysis (HR=0.00; CI=0.01-1.85; P=0.01) of lung cancer patients with overall survival with median survival time of 11.83 months.

In Chinese population as well, study of *TGF-β* rs1800469 by Hu S *et al.,* 2012 did not reveal any significant association with nasopharyngeal carcinoma (NPC). Faeghah Behboudi Farahbaksh *et al.,* in 2017 report no significant association of pancreatic cancer in Iranian population.

Due to signaling dysregulation, several studies reported increase in blood plasma level with T allele of C509T and report highest plasma levels with TT homozygote and conclude that because of increased transcriptional activities, variant T allele is responsible for irregulated blood plasma levels. However contradictory results were also reported to show that it is C allele instead to T allele responsible for increased plasma levels due to genetic heterogeneity and ethnic differences.

Further studies were carried out on the basis of clinic-pathological parameters considering metastasis, lymph node invasion, stages, tumor extension, objective response and gender. Our findings does not show any significant association with overall survival. Similar findings were reported by Yuan *et al.,* in 2013 *TGF-β*1 rs1800469 association with overall survival (OS) and distant metastasis free survival (DMFS) in NSCLC patients treated with radiotherapy (with or without chemotherapy). By doing multivariate analysis, they found association of gene with poor overall survival (HR=1.46; CI= 1.01-2.11; P=0.04), after adjusting various factors.

Wnt signaling is extremely complex pathway associated with various biological functions such as embryogenesis, cell fate determination during skeletal development, stem cell maintenance, cell proliferation and differentiation, during normal physiological processes in adult tissues and in defining polarity (Reya, T *et al.,* 2001). Various antagonists including *sFRP* (secreted Frizzled receptor proteins) affects signaling pathway of Wnt either by accumulating and increasing Ca^{2+}

concentration or by phosphorylating β-catenin (Glinka *et al.*, 1998). *sFRP4* and Frizzled related receptor share structural homology at N-terminal. In the absence of *sFRP4* proteins, Fz receptors bind with Wnt, β-catenin translocated to nucleus ultimately expression of various transcription factors such as c-myc, cyclin D1 and cox-2 takes place, but the presence of *sFRP4* protein affects signaling pathway by inhibiting binding of Wnt and Fz receptors thus no activation of Wnt pathway. Aberrant Wnt signaling due to genetic and molecular changes have been reported for occurrence of lung cancer. The present study has been carried out in North India population to evaluate association of *sFRP4* gene with lung cancer patients by considering parameters such as gender, age, smoking status, histological subtypes, performance status, clinicpathological parameters and response of patients to platinum based chemotherapy. The present study describe significant association between *sFRP4* genetic variants and individual susceptibility towards lung cancer. Upregulated expressions of *sFRP4* are reported in various cancers including breast, colorectal and prostate due to increase in cytoplasmic β-catenin level, which suggest role of *sFRP4* in good prognosis of various cancers. Findings from the current study suggested no significant association between *sFRP4* rs1802073 and overall survival of lung cancer patients. A study suggested significant association of *sFRP4* rs1802073 with the colorectal cancer. David H. Stewart (2013) found role of Wnt pathway in NSCLC development by using murine models. David H. Stewart (2013) found role of Wnt pathway in NSCLC development by using murine models.

Lung cancer is a complex and entangled solid tumor in which different histological subtypes initiate and progress through different pathological pathways. So, an attempt was made in this study to find out the influence of this variant on the different histological subtypes. When the patients were stratified on the basis of histological subtypes (ADCC, SQCC and SCLC) significant clinical outcomes were observed. Our study conferred the minimum median survival rate of *Ala/Ala* of ADCC histological subtype when compared to SQCC and SCLC. A significant association was found in SCLC histological subtype (HR' =1.65, P =0.05) in *Cys/Ala* genotype.

It has been evident by numerous scientific findings (6) that tobacco smoke is the leading cause of lung cancer owing to the various types of carcinogens present in it. These carcinogens are reported as major cause for genetic and molecular changes. A study done by Miral Yimlaz *et al.*, 2015 in Turkish population report that rs1802074 polymorphisms showed a decreased risk of lung cancer.

sFRP4 rs1802073 (*Pro*^{320} *Thr*) is less explored. It has been found by Hiroshi Hirata *et al.*, in 2014 that *sFRP4* rs1802073 (*Pro*^{320} *Thr*) has been related with renal cell carcinoma.

However, considering smoking as a cofactor for prognosis, no significant association was found either in univariate or in multivariate analysis. . When effect of *sFRP4 Pro320 Thr* genotype was correlated with gender, no significant association was found in any of gender. But, on applying the Cox multivariate hazard proportional analysis, a statistically significant association was observed in females in *Ala/Ala* genotype (HR"=47.88; 95%CI=2.63-871.52; P =0.009).

Further studies were carried out on the basis of performance status by considering KPS and ECOG. The choice of lung cancer chemotherapy depends on the disease stage and is largely influenced by patients' performance status. Platinum drugs, mainly cisplatin or carboplatin, are considered to be the first-line chemotherapy for most metastatic cancers including lung cancer. These drugs interact with the DNA to form DNA adducts which leads to inhibition of nuclear functions and hence apoptosis. Secondly, the platinum complexes also interact with glutathione in cytoplasm, thus preventing the drugs from binding to DNA. This renders the cell resistant. An imbalance in the host's detoxification system results in ineffective or rapid excretion of platinum drugs.

When progression of patient's disease is diagnosed by ECOG performance status, significant correlation were found in *Cys/Ala, Cys /Cys* genotypes with univariate Kaplan Meier (HR'=11.50; 95%CI=1.06-3.66; P=0.004**,** HR'=1.93; 95%CI=1.04-3.56; P=0.005) whereas significant values were found in all three genotypes in *Cys/Ala, Cys /Cys*, *Ala*/ *Ala* with multivariate Cox hazard proportional analysis (P=0.001, P=0.03, P=0.005). On the other hand progression of disease by Karnofsky performance scale (KPS) in lung cancer patient's showed no significant association.

In this study, various clinic-pathological parameters, such as clinical stage, tumor extension, lymph node invasion, metastasis and gender were analyzed in association with the two genotypes and it was found that this gene is highly associated with clinical stage *Cys/Ala + Ala/Ala* genotype (P=0.02), metastasis in *Cys/Ala* (P=0.002) and *Cys/Ala + Ala/Ala* genotype (P=0.003) and in association with stages in *Cys/Ala* genotype (P=0.01).

CHAPTER 7

CONCLUSION

In conclusion, our study demonstrated that *TGF-β1* rs1800469 polymorphism is not an important factor contributing to survival and clinical outcomes of lung cancer patients in North Indian population. However, significant values were reported in mutant genotype (*CC*) of SCLC histology.

On the other hand we conclude that sFRP4 rs1802073 (Pro^{320} *Thr*) is not significantly contributing in increasing survival and clinical outcomes of lung cancer. However, patients categorized as ECOG (*CC*) and (*CA*) genotype, and females (*AA*) genotype showed significant association with overall survival.

Validation of these findings with functional evaluation and larger studies with more rigorous study designs are needed. Studies are also required to assess the carcinogenesis role of passive smoking, diet and cooking, pollution, occupational exposure, other environmental factors, in addition to genetic factors, so to establish a risk profile for each individual or sub-group in affirmation of the prevention scope.

CHAPTER 8

References

- Antonia, S., Munoz-Antonia, T., Li, X., Reiss, M., and Jackson, R. (1996). A mutation in the transforming growth factor β type II receptor gene promoter associated with loss of gene expression. *Cancer research*; 56(21), 4831-4835.
- Bakin, A. V., Rinehart, C., Tomlinson, A. K. & Arteaga, C. L. (2002). p38 mitogen-activated protein kinase is required for TGFβ-mediated fibroblastic transdifferentiation and cell migration. *J. Cell Sci.;* 115: 3193−3206.
- Barker, N., & Clevers, H. (2006). Mining the Wnt pathway for cancer therapeutics. *Nature reviews Drug discovery; 5*(12): 997-1014.
- Bartling, B., Rehbein, G., Simm, A., Silber, R. E., & Hofmann, H. S. (2010). Porcupine expression is associated with the expression of S100P and other cancer-related molecules in non-small cell lung carcinoma. *International journal of oncology; 36*(4): 1015-1021.
- Behboudi Farahbakhsh, F., Nazemalhosseini Mojarad, E., Azimzadeh, P., Goudarzi, F., Mohammad Alizadeh, A. H., Haghazali, M. Asadzadeh Aghdaei, H. (2017). TGF-β1 polymorphisms -509 C>T and +915 G>C and risk of pancreatic cancer. *Gastroenterology and Hepatology From Bed to Bench; 10*(1): 14–20.
- Bornstein, S., White, R., Malkoski, S., (2009). Smad4 loss in mice causes spontaneous head and neck cancer with increased genomic instability and inflammation. *J. Clin. Invest.*; 119(11): 8–19.
- Bovolenta, P., Gorny, A.K., Esteva, P., Steinbeisser, H. (2014). Secreted Wnt inhibitors or Modulators Wnt Signaling in development and Disease. *J. Cell Sci.;* 121(6): 177-193.
- Brugge, W. R., Lauwers, G. Y., Sahani, D., Fernandez-del Castillo, C., & Warshaw, A. L. (2004). Cystic neoplasms of the pancreas. *New England Journal of Medicine; 351*(12): 1218-1226.
- Chacko, B. M. *et al.* (2001). The L3 loop and C-terminal phosphorylation jointly define Smad protein trimerization . *Nature Struct. Biol.;* 8: 248

- Chesarone, M. A., DuPage, A. G., & Goode, B. L. (2010). Unleashing formins to remodel the actin and microtubule cytoskeletons. *Nature reviews Molecular cell biology; 11*(1): 62-74.
- Chong, J. M., Uren, A., Rubin, J. S., Speicher, D. W. (2002). Disulfide bond assignments of secreted Frizzled-related protein-1 provide insights about Frizzled homology and netrin modules. *J. Biol. Chem.*; 277: 5134–44.
- Crisan, M., Yap, S., Casteilla, L., Chen, C. W., Corselli, M., Park, T. S., & Norotte, C. (2008). A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell stem cell; 3*(3): 301-313.
- Dahmen, R. P. *et al. (2001).* Deletions of AXIN1, a component of the WNT/wingless pathway, in sporadic medulloblastomas. *Cancer Res.;* 61: 7039−7043.
- De Castro, J. V., Gonçalves, C. S., Costa, S., Linhares, P., Vaz, R., Nabiço, R., & Costa, B. M. (2015). Impact of TGF-β1-509C/T and 869T/C polymorphisms on glioma risk and patient prognosis. *Tumor Biology; 36*(8): 6525-6532.
- Derynck, R. & Feng, X.-H. (1997) TGF-β receptor signaling. *Biochim. Biophys. Acta.;* 1333: 105−150.
- Ebner, R., Chen, R. H., Shum, L., Lawler, S., Zioncheck, T. F., Lee, A., & Derynck, R. (1993). Cloning of type I TGF-beta receptor and its effect on TGF-beta binding to the type II receptor. *Science*; *260*(5112): 1344-1349
- Fodde, R., & Brabletz, T. (2007). Wnt/β-catenin signaling in cancer stemness and malignant behavior. *Current opinion in cell biology*; *19*(2): 150-158.
- Glinka, A., Wu, W., Delius, H., Monaghan, A. P., Blumenstock, C., & Niehrs, C. (1998). Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. *Nature; 391*(6665): 357-362.
- Guan, X., Zhao, H., Niu, J., Tang, D., Ajani, J. A., & Wei, Q. (2009). The VEGF-634G> C promoter polymorphism is associated with risk of gastric cancer. *BMC gastroenterology; 9*(1): 77-86.

- Guo RJ, Wang Y, Kaneko E, et al. (1998). Analyses of mutation and loss of heterozygosity of coding sequences of the entire transforming growth factor b type II receptor gene in sporadic human gastric cancer. *Carcinogenesis*;19(15): 39–44.
- Guo, X., Day, T. F., Jiang, X., Garrett-Beal, L., Topol, L., Yang, Y. (2004). Wnt/beta-catenin signaling is sufficient and necessary for synovial joint formation. *Genes Dev.*; 18(19): 2404–2417.
- Guo, Y., Zang, C., Li, Y., Yuan, L., Liu, Q., Zhang, L., & Li, S. (2013). Association between *TGF-β1* Polymorphisms and Hepatocellular Carcinoma Risk: A Meta-Analysis. *Genetic Testing and Molecular Biomarkers*, *17*(11); 814–820.
- Hata, A., Lo, R. S., Wotton, D., Lagna, G., Massague, J. (1997). Mutations increasing autoinhibition inactivate tumour suppressors Smad2 and Smad4. *Nature*; 388: 82–97.
- He, B., Lee, A. Y., Dadfarmay, S., You, L., Xu, Z., Reguart, N., (2005). Secreted frizzled-related protein 4 is silenced by hypermethylation and induces apoptosis in beta-catenin-deficient human mesothelioma cells. *Cancer Res.*; 65: 743–800.
- Hirata, H., Hinoda, Y., Nakajima, K., Kikuno, N., Yamamura, S., Kawakami, K., Dahiya, R. (2009). Wnt antagonist gene polymorphisms and renal cancer. *Cancer*; *115*(19): 4488–4503.
- Hoffmann, D., Hoffmann, I., El-Bayoumy, K., (2001). The less harmful cigarette: a controversial issue atribute to Ernst L. Wynder. *Chem. Res. Toxicol.*; 14: 767-790.
- Hu, S., Zhou, G., Zhang, L., Jiang, H., & Xiao, M. (2012). The effects of functional polymorphisms in the TGFβ1 gene on nasopharyngeal carcinoma susceptibility. *Otolaryngology--Head and Neck Surgery*; *146*(4): 579-584.
- Huang, J. F., Guo, Y. J., Zhao, C. X., Yuan, S. X., Wang, Y., Tang, G. N., & Sun, S. H. (2013). Hepatitis B virus X protein (HBx)- related long noncoding RNA (lncRNA) down- regulated expression by HBx (Dreh) inhibits hepatocellular carcinoma metastasis by targeting the intermediate filament protein vimentin. *Hepatology*; *57*(5): 1882-1892.

- Huang, S. M. A., Mishina, Y. M., Liu, S., Cheung, A., Stegmeier, F., Michaud, G. A.,& Hild, M. (2009). Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling. *Nature; 461*(7264): 614-620.
- I.S. Kim, F. Otto, B. Zabel, S. (1999). Mundlos Regulation of chondrocyte differentiation by Cbfa1 Mech. *Dev.*; 80: 159–170.
- Inman, G. J., Nicolas, F. J. & Hill, C. S. (2002). Nucleocytoplasmic shuttling of Smads 2, 3, and 4 permits sensing of TGF-β receptor activity. *Mol. Cell;* 10: 283−294.
- Itoh, S., Itoh, F., Goumans, M. J. & ten Dijke, P. (2000). Signaling of transforming growth factor-β family members through Smad proteins. *Eur. J. Biochem.;* 267: 6954−6967.
- Jin, Q., Hemminki, K., Grzybowska, E., Klaes, R., Söderberg, M., Zientek, H., & Försti, A. (2004). Polymorphisms and haplotype structures in genes for transforming growth factor β1 and its receptors in familial and unselected breast cancers. *International journal of cancer*; *112*(1): 94-99.
- Jones, S. E., Jomary, C. (2002). Secreted Frizzled-related proteins: searching for relationships and patterns. *BioEssays*; 24: 811–820.
- Joseph, H., Gorska, A. E., Sohn, P., (1999). Overexpression of a kinase-deficient transforming growth factor-beta type II receptor in mouse mammary stroma results in increased epithelial branching. *Mol. Biol. Cell*; 10(4): 1221–1234.
- Jung, H. Y., Jun, S., Lee, M., Kim, H. C., Wang, X., Ji, H., & Park, J. I. (2013). PAF and EZH2 induce Wnt/β-catenin signaling hyperactivation. *Mol. cell; 52*(2): 193-205.
- Kang, H. G., Chae, M. H., Park, J. M., Kim, E. J., Park, J. H., Kam, S., & Kim, Y. L. (2006). Polymorphisms in TGF-β1 gene and the risk of lung cancer. *Lung cancer; 52*(1): 1-7.
- Kang, Y., Siegel, P. M., Shu, W., (2003). A multigenic program mediating breast cancer metastasis tobone. *Cancer Cell*; 3(6): 537–549.
- Kleber, M., and Sommer, L. (2004). Wnt signaling and the regulation of stem cell function, Curr. Opin. *Cell Biol.*; 16: 681–687.

- Kraft, D. J., Jacobs, P., Hunter, K. B., Cox, D. G., Yeager, M., Hankinson, S. E., & Wang, J. (2007). A genome-wide association study identifies alleles in FGFR2 associated with risk of sporadic postmenopausal breast cancer. *Nature genetics; 39*(7): 870-874.
- Kretzschmar, M, Massague, J. (1998). SMADs: mediators and regulators of TGF-beta signaling. *Curr. Opin. Genet. Dev.*; 8(1): 103–111.
- La Vecchia, C., Franceschi, S., Levi F. (2003). Epidemiological research on cancer with a focus on Europe. *Eur. J. Cancer Prev.*; 12: 5-14.
- Laurence Levy and Caroline S. Hill. (2006). Alterations in components of the TGF-b superfamily signaling pathways in human cancer. *Cytokine & Growth Factor Reviews*; 17: 41–58.
- Laurent Kodjabachian, Martha, L. Rebbert, Amir Rattner, Philip M. Smallwood, Cynthia Harryman Samos, Roel Nusse, Igor B. Dawid & Jeremy Nathans (1999). A new secreted protein that binds to Wnt proteins and inhibits their activites. *Nature*; 398: 431-436.
- Lee, J. C., Lee, K. M., Kim D. W., Heo DS. (2004). Elevated TGF-beta1 secretion and down-modulation of NKG2D underlies impaired NK cytotoxicity in cancer patients. *J. Immunol*; 172(12): 7335–7340.
- Li, T., Cao, B.-W., Dai, Y., Cui, H., Yang, H.-L. and Xu, C.-Q. (2008). Correlation of *transforming growth factor beta-1*gene polymorphisms C-509T and T869C and the risk of gastric cancer in China. *Journal of Gastroenterology and Hepatology*; 23: 638–642.
- Liu, B. Y., McDermott, S. P., Khwaja, S. S. & Alexander, C. M. (2004). The transforming activity of Wnt effectors correlates with their ability to induce the accumulation of mammary progenitor cells. *Proc. Natl. Acad. Sci. USA*; 101: 4158−4163.
- Liu, Y., Dong, Q. Z., Wang, S., Fang, C. Q., Miao, Y., Wang, L., & Wang, E. H. (2013). Abnormal expression of Pygopus 2 correlates with a malignant phenotype in human lung cancer. *BMC cancer; 13*(1): 346-355.

- Longman, D., Arfuso, F., Viola, H. M., Hool, L. C., Dharmarajan, A. M. (2012). The role of the cysteine-rich domain and netrin-like domain of secreted frizzled-related protein 4 in angiogenesis inhibition in vitro. *Oncol. Res*.; 20: 1–6.
- Markowit, Z., Wang, J., Myeroff, L., (1995). Inactivation of the type II TGF-b receptor in colon cancer cells with microsatellite instability. *Science*; 268: 13-36.
- Massague, J. (1998). TGF-β signal transduction. *Annu. Rev. Biochem.*; 67: 753–791.
- Massague, J. (2000). How cells read TGF-β signals. *Nature Rev. Mol. Cell Biol.;* 1: 169−178.
- Maurice, D., Pierreux, C. E., Howell, M., Wilentz, R. E., Owen, M. J., Hill, C. S. (2001). Loss of Smad4 function in pancreatic tumors: C-terminal truncation leads to decreased stability. *J. Biol. Chem.*; 276: 75– 81.
- Mazars, A. (2001).Evidence for a role of the JNK cascade in Smad7-mediated apoptosis. *J. Biol. Chem.;* 276: 97−115.
- Meulmeester, E., Ten Dijke, P. (2011). The dynamic roles of TGF-beta in cancer. A comprehensive review on TGFβ signaling dealing with its dual role in cancer. *J. Pathol.*; 223(2): 205–218.
- Moren, A., Itoh, S., Moustakas, A., Dijke, P., Heldin, C. H. (2000). Functional consequences of tumorigenic missense mutations in the aminoterminal domain of Smad4. *Oncogene*; 19: 4396–3404.
- Moustakas, A., Souchelnytskyi, S. & Heldin, C. H. (2001). Smad regulation in TGF-β signal transduction. *J. Cell Sci.;* 114: 4359−4369.
- Nelson, W. J., Nusse, R. R. (2004). Convergence of Wnt, beta-catenin, and cadherin pathways. *Science*; 303: 1483–1487.
- Norgaard, P., Damstrup, L., Rygaard, K., Spang-Thomsen, M. and Skovgaard Poulsen, H. (1994). Growth suppression by transforming growth factor β_1 of human small-cell lung cancer cell lines is associated with expression of the type II receptor. *British Journal of Cancer;* 69: 802–808.

- Norgaard, P., Spang-Thomsen, M., Poulsen, H. S. (1996). Expression and autoregulation of transforming growth factor beta receptor mRNA in small-cell lung cancer cell lines. *British Journal Cancer*; 73(9): 1037-1048.
- Okadome, T., Yamashita, H., Franzen, P., Moren, A., Heldin, C. H. (1994).transforming growth factor $\beta_1$signaling transduction. *J. Biol. Chem.*; 269: 753–759.
- Pardali, K., Kurisaki, A., Moren, A. (2000). Role of Smad proteins and transcription factor Sp1 in p21(Waf1/Cip1) regulation by transforming growth factor-beta. *J. Biol. Chem.*; 275(38): 244–256.
- Park, E. B., Shim, K. S., Kim, K. H., & Han, W. S., (1999). Elevated serum levels of transforming growth factor- β1 in patients with colorectal carcinoma. *Cancer; 85*(3): 554-561.
- Parsons, R., Myeroff, L. L., Liu, B. (1995). Microsatellite instability and mutations of the transforming growth factor b type II receptor gene in colorectal cancer. *Cancer Res.*; 55: 48–50.
- Pecina-Slaus, N., Nikuseva Martic, T., Kokotovic, T., Kusec, V., Tomas, D., & Hrascan, R. (2011). AXIN-1 protein expression and localization in glioblastoma. *Collegium antropologicum; 35*(1): 101-106.
- Penheiter, S. G. *et al.* (2002). Internalization-dependent and -independent requirements for transforming growth factor β receptor signaling via the Smad pathway. *Mol. Cell. Biol.;* 22: 4750−4759.
- Pessah, M. (2002). c-Jun associates with the oncoprotein Ski and suppresses Smad2 transcriptional activity. *J. Biol. Chem;* 277: 94−100.
- Pleasance, E. D., Stephens, P. J., O'meara, S., McBride, D. J., Meynert, A., Jones, D. & Varela, I. (2010). A small-cell lung cancer genome with complex signatures of tobacco exposure. *Nature;* 463(7278): 184-190
- Qianren Jin, Kari Hemminki, Ewa Grzybowska, Rüdiger Klaes, Magnus Söderberg, Helena Zientek, Jadwiga Rogozinska-Szczepka, Beata Utracka-Hutka, Jolanta Pamula,

Wioletta Pekala, *et al.* (2004). Polymorphisms and haplotype structures in genes for transforming growth factor beta1 and its receptors in familial and unselected breast cancers. *Int. J. Cancer*; 112(1): 94–99.

- Ren, Y., Yin, Z., Li, K., Wan, Y., Li, X., Wu, W., Zhou, B. (2015). TGFβ-1 and TGFBR2 polymorphisms, cooking oil fume exposure and risk of lung adenocarcinoma in Chinese nonsmoking females: a case control study; *BMC Medical Genetics;*11(6): 16-22.
- Reya, T., Duncan, A. W., Ailles, L., Domen, J., Scherer, D. C., Willert, K., & Weissman, I. L. (2003). A role for Wnt signalling in self-renewal of haematopoietic stem cells. *Nature; 423*(6938): 409-414.
- Reya, T., Morrison, S. J., Clarke, M. F. & Weissman, I. L. (2001). Stem cells, cancer, and cancer stem cells. *Nature*; 414: 105−111.
- Rohde, H., Gebbensleben, B., Bauer, P., Stützer, H., & Zieschang, J. (1989). Has there been any improvement in the staging of gastric cancer? Findings from the German gastric cancer TNM study group. *Cancer; 64*(12): 2465-2481.
- Shi, Y., Hata, A., Lo, R. S., Massague, J., Pavletich, N. P. (1997). A structural basis for mutational inactivation of the tumour suppressor Smad4. *Nature*; 388: 87–93.
- Shi, Y., Hu, Z., Wu, C., Dai, J., Li, H., Dong, J & Zhang, H. (2011). A genome-wide association study identifies new susceptibility loci for non-cardia gastric cancer at 3q13. 31 and 5p13. 1. *Nature genetics*; *43*(12): 1215-1218.
- Siegel, P. M., Massague, J. (2003). Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. *Nat. Rev. Cancer*; 3(11): 807–821.
- Stewart, D. J., Chang, D. W., Ye, Y., Spitz, M., Lu, C., Shu, X., Wu, X. (2014). Wnt Signaling Pathway Pharmacogenetics in Non-Small Cell Lung Cancer. *The Pharmacogenomics Journal*; *14*(6): 509–522.
- Storm, E. E., Huynh, T. V., Copeland, N. G., Jenkins, N. A., Kingsley, D. M., Lee, S. J. (1994). Limb alterations in brachypodism mice due to mutations in a new member of the TGF beta-superfamily. *Nature*; 368(6472): 639–643.

- Surana, R., Sikka, S., Cai, W., Shin, E. M., Warrier, S.R., Tan, H. J. (1845). Secreted frizzled related proteins: implications in cancers. *Biochim. Biophys. Acta.*; 2014:53–65.
- Taipale, J. & Beachy, P. A. (2001). The Hedgehog and Wnt signalling pathways in cancer. *Nature*; 411: 349−354.
- Tang, B., de Castro, K., Barnes, H. E. (1999). Loss of responsiveness to transforming growth factor beta induces malignant transformation of nontumorigenic rat prostate epithelial cells. *Cancer Res.;* 59(19): 34–42.
- Tang, Y., Katuri, V., Srinivasan, R. (2005). Transforming growth factor-beta suppresses nonmetastatic colon cancer through Smad4 and adaptor protein ELF at an early stage of tumorigenesis. *Cancer Res.*; 65(10): 28–37.
- Tani, M., Takenoshita, S., Kohno, T, (1997). Infrequent mutations of the transforming growth factor b-type II receptor gene at chromosome 3p22 in human lung cancers with chromosome 3p deletions. *Carcinogenesis*; 18: 19–21.
- Ten Dijke, P., Ichijo, H., Franzen, P., Schulz, P., Saras, J. (1993). Oncogene; 8: 79– 87.
- Teng, Y., Wang, X., Wang, Y., & Ma, D. (2010). Wnt/β-catenin signaling regulates cancer stem cells in lung cancer A549 cells. *Biochemical and biophysical research communications; 392*(3): 373-379.
- Thorgeirsson, T. E., Gudbjartsson, D. F., Surakka, I., Vink, J. M., Amin, N., Geller, F., & Gieger, C. (2010). Sequence variants at CHRNB3-CHRNA6 and CYP2A6 affect smoking behavior. *Nature genetics; 42*(5): 448-453.
- Vaugha, A. E., Halbert, C. L., Wootton, S. K., Miller, A. D. (2012). Lung cancer in mice induced by the jaagsiekte sheep retrovirus envelope protein is not maintained by rare cancer stem cells, but tumorigenicity does correlate with Wnt pathway activation. *Mol. Cancer Res.*; 10(1): 86– 95.
- Wu, J. W. *et al.* (2001). Crystal structure of a phosphorylated Smad2. Recognition of phosphoserine by the MH2 domain and insights on Smad function in TGF-β signaling. *Mol. Cell;* 8: 1277−1289.

- Xiaomin Niu , Hongxuan Li , Zhiwei Chen , Yun Liu , Mengyuan Kan , Daizhan Zhou , Ziming Li , Xiangyun Ye , Shengping Shen , Changxing Lv , Shun Lu. (2012). A Study of Ethnic Differences in TGFβ1 Gene Polymorphisms and Effects on the Risk of Radiation Pneumonitis in Non-Small-Cell Lung Cancer. *J. Thorac. Oncol.*; 7 (11): 1668-1675.
- Xu J, Attisano L (2000). Mutations in the tumor suppressors Smad2 and Smad4 inactivate transforming growth factor b signaling by targeting Smads to the ubiquitin–proteasome pathway. *Proc. Natl. Acad. Sci. USA*; 97: 20–35.
- Xu, L., Kang, Y., Col, S. & Massagué, J. (2002). Smad2 nucleocytoplasmic shuttling by nucleoporins CAN/Nup214 and Nup153 feeds TGF-β signaling complexes in the cytoplasm and nucleus. *Mol. Cell;* 10: 271−282.
- Yi Liu, Wei Zhou, De-Wu Zhong. (2012). Meta-analyses of the associations between four common TGF-β1 genetic polymorphisms and risk of colorectal tumor. *Tumor biology*; 33: 1191-1199.
- Yilmaz, M., Donmez, G., Kacan, T., Sari, I., Babacan, N.A., Sari, M. and Kilickap, S., 2015. Significant association between polymorphisms of Wnt antagonist genes and lung cancer. *J. Investig. Med;* 63: 935-941.
- Yingzi Yang. (2012). Wnt signaling in development and disease.*Cell & Bioscience;* 2: 14-20.
- Yu, L., Hebert, M. C. & Zhang, Y. E. (2002). TGF-β receptor-activated p38 MAP kinase mediates Smad-independent TGF-β responses. *EMBO J;* 21: 3749−3759.
- Yuan, X., Liao, Z., Liu, Z., Wang, L. E., Tucker, S. L., Mao, L., & Milas, L. (2009). Single Nucleotide Polymorphism at rs1982073: T869C of the TGF β 1 Gene Is Associated with the Risk of Radiation Pneumonitis in Patients With Non–Small-Cell Lung Cancer Treated With Definitive Radiotherapy. *Journal of clinical oncology; 27*(20): 3370-3378.
- Zavadil, J. *et al.* (2001). Genetic programs of epithelial cell plasticity directed by transforming growth factor-β. *Proc. Natl. Acad. Sci. USA;* 98: 6686−6691.

- Zhang, H. T., Chen, X. F., Wang, M. H., et al. (2004). Defective expression of transforming growth factor b receptor type II is associated with CpG methylated promoter in primary non-small cell lung cancer. *Clin. Cancer Res.*; 10(23): 59–67.
- Zhou, Y., Yang, S., Zhang, P. (2016). Effect of Exogenous Fetuin-A on TGF-β/Smad Signaling in Hepatic Stellate Cells. *Biomed. Res. Int.*; 84(6): 22-37.
- Zurawel, R. H., Chiappa, S. A., Allen, C. & Raffel, C. (1998). Sporadic medulloblastomas contain oncogenic β-catenin mutations. *Cancer Res.;* 58: 896−899.

CHAPTER 9

APPENDIX-1

1. 0.5M EDTA: Dissolved 9.306g of disodium salt of EDTA in 20ml of deionised water, and then adjusted the pH to 8.0 by 1 M sodium hydroxide. Sterilized the solution by autoclaving.
2. 10% SDS: Dissolved 1g of SDS in 10ml of deionised water.
3. 100mM Tris-Cl (pH 8.0): Dissolved 0.32g of Tris-Cl in 10 ml of deionised water, then adjusted the pH to 8.0 by 1M sodium hydroxide. Sterilized the solution by autoclaving.
4. 10mg/ml Proteinase K: Dissolved 10mg Proteinase K in 1ml of double distilled water. Sterilized the solution by autoclaving.
5. 1mg/ml BSA: Dissolved 100mg of BSA in 100ml of deionised sterile water and kept at 4 C overnight.
6. 5M Sodium chloride (NaCl): Dissolved 5.85g of sodium chloride in 20ml of deionised water. Sterilized the solution by autoclaving.
7. 5X TBE buffer: Dissolved 54g of Tris base and 27.5g of boric acid in 980ml of double distilled water and then added 20ml of 0.5 EDTA. Sterilized the solution by autoclaving.
8. Ethidium Bromide (10mg/ml): Dissolved 1g of ethidium bromide in 100ml of water. Mixed the solution properly.
9. Magnesium chloride (MgCl2) (100mM): Dissolved 0.41gms of MgCl2 in 20ml of deionised water and sterilized by autoclaving.
10. Sucrose (1M): Dissolved 3.41 g of sucrose in 10 ml of deionised water and sterilized by autoclaving.
11. TE buffer (pH 8.0): Added 1ml of 100mM Tris-Cl (pH 8.0) and 200 µl of 0.5M EDTA solution to 8.8 ml of deioinsed water. Sterilized the solution.
12. Triton X- 100 (10%): Took 100 µl of TritonX-100 and mixed with 900 µl of deionised water and mixed properly.

Project Semester | Thesis - DUE 16-Jul-2017 | Roadmap | Paper 1 of 1

Originality GradeMark PeerMark

M.tech
BY SHEETAL VATS

turnitin

7%
SIMILAR

OL

14

Match Overview

1	TGF-β in Human Disea… Publication	2%
2	pharmaceuticalintellige… Internet source	1%
3	Smad Signal Transduct… Publication	1%
4	"TGF- SIGNAL TRANS… Publication	<1%
5	Fodde, R.. "Wnt/@b-ca… Publication	<1%
6	Liliana Attisano. "Regul… Publication	<1%
7	www.dominicanhospital… Internet source	<1%
8	docplayer.net Internet source	<1%

Text-Only Report