Genetic Polymorphism of *Thymidylate Synthase* gene in Lung Cancer Patients Undergoing Platinum Based Chemotherapy

A Dissertation submitted in partial fulfillment of the requirement for the award

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Submitted by

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DECLARATION

I, the undersigned, hereby declare that the work presented in the M.Sc. dissertation entitled "**Genetic Polymorphism of *Thymidylate Synthase* gene in Lung Cancer Patients Undergoing Platinum Based Chemotherapy**" has been carried out by me under the supervision and guidance of Dr. Siddharth Sharma, Associate Professor, Department of Biotechnology, Thapar Institute of Engineering and Technology, Patiala. Further, I declare that no part of this dissertation has been submitted for a degree or any other qualification of any other university or examining body in India/elsewhere.

Prabhjot Kaur

(Roll no. 301701021)

CERTIFICATE

This is to certified that the dissertation entitled "**Genetic Polymorphism of *Thymidylate Synthase* gene in Lung Cancer Patients Undergoing Platinum Based Chemotherapy**" submitted by degree of Master of Science in the subjects of Biotechnology, Thapar Institute of Engineering and Technology (TIET), Patiala is a bonafied work carried out by Prabhjot Kaur under the supervision of **Dr. Siddharth Sharma**, Ph.D., Associate Professor, Department of Biotechnology, Thapar Institute of Engineering and Technology (TIET), Patiala and that no part of this work has been submitted for any other degree.

The assistance and help received during the course of investigation has been fully acknowledged.

Dr. Siddharth Sharma,

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ABSTRACT

BACKGROUND: This study aimed to explain the role of *TS* (Thymidylate synthase) gene in overall survival of lung cancer patients in North Indian population. TS is involved in folate metabolism. It plays a key role in DNA methylation, synthesis and repair. The SNP used in this study is *TSER* rs34743033. There are polymorphic tandem repeats in *TS* gene in *TSER* (Thymidylate synthase enhancer region). There are variable number of tandem repeats (VNTRs) in *TSER* namely as two repeats (2R), three repeats (3R) and can be up to nine repeats (9R). In the present study, two common polymorphisms have been studied 2R and 3R.

MATERIAL AND METHODS: 150 cases of lung cancer patients were conducted for overall survival of lung cancer patients on the basis of age, gender, smoking status, histology, regimen, pack years, KPS, ECOG and TNM studies. DNA was isolated from peripheral blood and the PCR/RFLP (polymerase chain reaction/restriction fragment length polymorphism) was used to detect the polymorphisms of interest. The amplified product was run on N - PAGE (Native – Polyacrylamide gel electrophoresis) gel.

RESULTS: An analysis of gene polymorphism was performed with respect to the overall survival of 150 lung cancer patients treated with platinum based chemotherapy. Patients with heterozygous (2R/3R) genotype had overall survival of 9.47 months with highly significant value of **0.02** which is more than homozygous (2R) genotype.

CONCLUSION: *TSER* rs34743033 genotype appears to be a potential prognostic factor for advanced NSCLC patients treated with platinum based chemotherapy.

Keywords: *TS* gene, lung cancer, overall survival, platinum based chemotherapy, single nucleotide polymorphism

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ABBREVIATIONS

Abbreviated form	Full Form
ADCC	Adenocarcinoma
AOR	Adjusted Odds Ratio
BSA	Bovine Serum Albumin
CR	Complete Response
DNTPs	Deonucleotide triphosphates
ECOG	Eastern Cooperative Oncology Group
EDTA	Ethylenediaminetetraacetic acid
HR	Hazards Ratio
KPS	Karnofsky Performance status
LC	Lung Cancer
LCC	Large Cell Carcinoma
MST	Median Survival Time
NaCl	Sodium chloride
NSCLC	Non Small Cell Lung Cancer
OR	Odds Ratio
OS	Overall Survival
P:C:I	Phenol:Chloroform:Isoamyl alcohol
PCR	Polymerase Chain Reaction
RFLP	Restricted Fragment Length Polymorphism
PAGE	Polyacrylamide gel electrophoresis
PD	Progressive disease
PGIMER	Post Graduate Institute of Medical Education and Research
PR	Partial Response
SCLC	Small cell lung cancer
SD	Stable disease
SNP	Single Nucleotide Polymorphism
SQCC	Squamous cell carcinoma
Taq	*Thermus aquaticus*
TBE	Tris Borate EDTA
TE	Tris-EDTA
TS	*Thymidylate synthase*

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1.0. INTRODUCTION

Lung cancer is considered as the second most deadly cancer in both males and females in the United States and the world. According to World Health Organization one of the major cause of death is cancer worldwide, which accounts for 9.6 million deaths in 2018. Amongst them the most common cancer is lung cancer (2.09 million case) and breast cancer (2.09 million cases), followed by colorectal cancer (1.80 million cases), prostate cancer (1.28 million cases), skin cancer (1.04 million cases) and stomach cancer (1.03 million cases). About 2,28,150 cases of lung cancer were estimated by American cancer society and 142, 670 deaths from lung cancer are estimated in 2019 (https://www.cancer.org/cancer/non-small-cell-lung-cancer/about/key-statistics.html). It is a disease of modern times, earlier in the 20th century lung cancer accounted for less than 0.5% among all cancers. Several factors affect lung cancer like cigarette and bidi smoking, exposure to radon, asbestos, arsenic in drinking water, occupational hazards and the secondhand smoke. The risk of developing lung cancer is 1 in 15 among males and 1 in 17 among females. The white men are at lower risk of developing lung cancer than the black men. Whereas, the black women has lower risk of developing lung cancer than the white women (according to American cancer society).

Thymidylate synthase gene is a key enzyme of folate metabolism (Uchida *et al*., 2004). It plays an important role in DNA methylation, synthesis and repair. It catalyzes the conversion of dUMP (deoxyuridine monophosphate) to dTMP (deoxythymidine monophosphate) and 5,10-methylenetetrahydrofolate to dihydrofolate. Polymorphism in the *TS* gene has been identified in TSER (TYMS enhancer region) near the initiation site i.e., 5'-UTR. This variant is present in different VNTRs (variable number of tandem repeats). These alleles are 2R, 3R, 4R and can be found upto 9R. 2R and 3R are frequently occurring alleles. The VNTRs are associated with the TS gene expression level. Absolute enzyme activity and gene expression level were thought to be higher in 3R than 2R (Shi *et al*., 2005).

The widely accepted treatment for lung cancer includes surgery, chemotherapy and radiotherapy (Shewach *et al*., 2009). With the advances in regimen, doublet platinum based chemotherapy is given to patient for better survival, but arrival of drug resistance in patients by influx and efflux of chemotherapeutic drugs, cure of lung cancer has become difficult thus affecting the survival of patients. The two polymorphic variants (2R, 3R) of TSER gene (rs 34743033) are liable for affecting the survival of patients. Therefore, to trace the function of above polymorphic variant in overall survival of North Indian population, we evaluated the significance of 2R and 3R polymorphic variants in response rate of patients undergoing platinum based chemotherapy.

Pemetrexed ($C_{20}H_{21}N_5O_6$) is multitargeted antifolate drug. It inhibits several key enzymes of folate pathway such as TS, GARFT, DHFR. TS is main targeted enzyme of pemetrexed. It is approved by United States Food and Drug Administration as a first and second line chemotherapy for the treatment of all types of cancer especially lung cancer. Thymidine is necessary for DNA synthesis and repair. Inhibition of TS by pemetrexed results in decreased amount of thymidine (Krawczyk *et al*., 2014). It is given as a injection into the vein. Patients treated with this drug have to take vitamin B supplements to overcome side effects. Side effects of pemetrexed includes fatigue, nausea, diarrhea, anemia, skin rashes, etc (http://chemocare.com/chemotherapy/drug-info/PEMETREXED.aspx).

2.0. REVIEW OF LITERATURE

2.1. Physiology of lungs

Lung is the major organ of the respiratory system. The main function of the lungs is the exchange of gases (O_2 and CO_2) with air from the atmosphere. Oxygen is inhaled by the lungs from the atmospheric air and carbon dioxide is exhaled by the lungs. The lungs are paired organ that are connected to the trachea by the left and right bronchi. Lungs are pyramid in shape, and are bordered by the diaphragm at the inferior surface. The right lung is shorter and wider than the left lung, but the left lung occupies small volume than the right lung. Each lung is composed of smaller units called lobes. Lobes are separated by fissures. Right lung consists of three lobes i.e., the superior lobe, the inferior lobe and the middle lobe (Canadian cancer society).

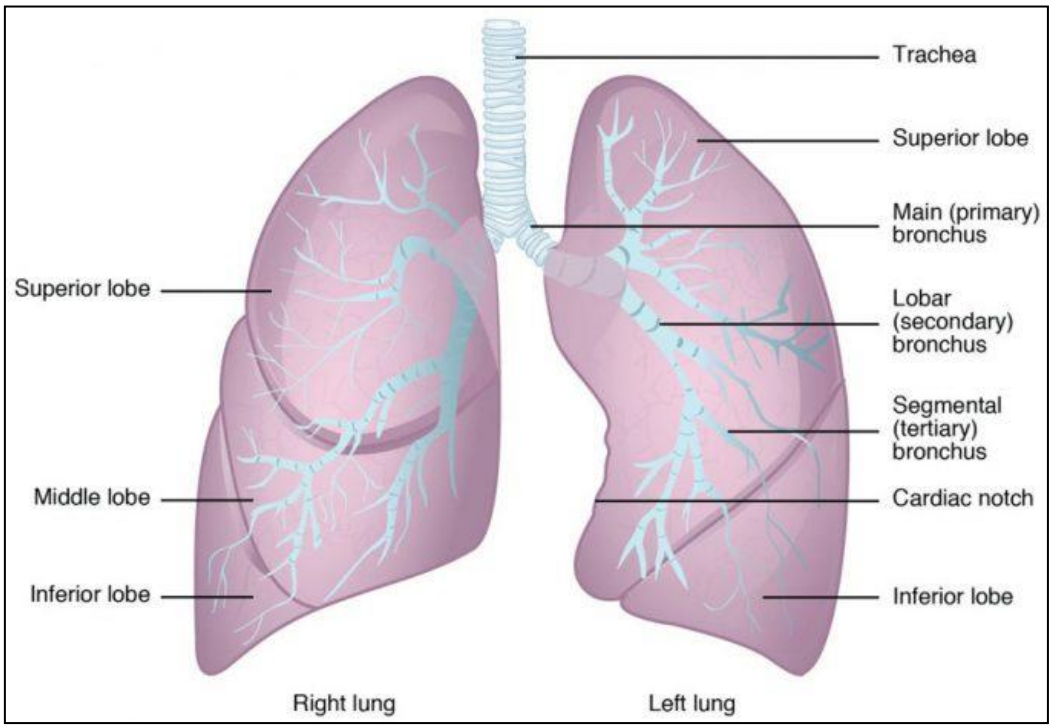

Figure 2.1 Various parts of lungs (Source : Canadian cancer society)

2.2. Lung cancer

Cancer is a multifunctional disease in which abnormal cells divide continuously and may or may not spread to other parts of the body. The cancer which spreads to other parts of the body is known as malignant tumor and which do not spread is known as benign tumor. Lung cancer is the second most common type of cancer diagnosed in both male and females in the United States (de Groot *et al.*, 2018). It is the heterogeneous and complex disease to treat. Tobacco consumption and cigarette smoking is the primary cause of lung cancer (Qasem *et al.,* 2015). The estimated new cases for lung cancer in 2018 are 2,34,030 in which 1,21,680 are male and 1,12,350 are female. Lung cancer accounts for 14% of new cancer for men and 13% for women in the US. Among all cancers, lung cancer has one of the lowest survival rates (de Groot *et al.*, 2018).

2.2.1. Risk factors associated with lung cancer

The different risk factors associated with lung cancer are as follows:-

a) **Tobacco smoking:** It is the primary risk factor for lung cancer. <20% of regular smokers develop lung cancer (Shi *et al.,* 2004). Tobacco smoking produces at least 60 carcinogens. Tobacco smoking includes bidi, cigars, cigarette, pipes. Cigarette smoking consists of cancer causing substances called as carcinogens like nicotine, carbon monoxide, aromatic amines, phenols, formaldehyde, ethyl carbamate, acrolin,etc. Nicotine is a natural alkaloid that acts as acetylcholine agonist in the nervous system and binds to nicotinic acetylcholine receptors (nAChR). Nicotine is an addictive compound of tobacco. Most significant are polycyclicnaromatic hydrocarbons like N-nitrosamines[4-(methylnitrosamino)-1-(13-pyridyl-1-butanone)], benzopyrines, nitrates (de Groot *et al.*, 2018).

   Another compound called bidi is more harmful than cigarette. Bidi is small, thin, hand rolled, unfiltered cigarettes. Bidis are having higher concentrations

of nicotine, tar, carbon monoxide (https://www.cancer.org/cancer/lung-cancer/prevention-and-early-detection/risk-factors.html).

b) **Secondhand or passive smoking:** People who doesn't smoke but breathe in the smoke of others is called secondhand or passive or environmental tobacco smoke or involuntary smoke. Secondhand smoke is a mixture of two types of smoking i.e., sidestream smoking and mainstream smoking. Sidestream smoking is the smoke produced from the burning tip of a cigarette. Mainstream smoking is the smoke exhaled by a smoker.

c) **Radon:** Radon is a naturally occurring colorless, odorless, radioactive gas. It is formed from the breakdown of uranium. Uranium is present in soil and rocks throughout the world. It can move into the air and into underground and surface water. Radon gas present in the air breakdowns into small elements called as radon progeny. This progeny can embed in the lining of the lungs, where they can give off the radiations of radon. These radiations are thus carcinogenic, can damage the lungs and cause lung cancer (American cancer society).

d) **Asbestos:** Asbestos is the naturally occurring mineral as bundles of fibers. It is having serpentine and amphibole subtypes. Serpentine is also known as white asbestos and amphibole are straight and needle like fibers. It is most common carcinogen responsible for lung cancer and other cancers also. It is estimated that it accounts for 5-10% of lung cancer cases (de Groot *et al*., 2018). It is observed that people having daily contact with asbestos have 5-fold excess risk of lung cancer.

e) **Air pollution:** Air pollution was considered as cause of cancer by the International Agency for Research on Cancer (IARC) in 2013. There are two types of air pollution. One is outdoor air pollution and other is indoor air pollution. Outdoor air pollution is a mixture of very small solid and liquid particles such as dust particles, metals, acids, organic chemicals, forest fires, vehicles, wood stoves, etc.

Carcinogens like sulfur dioxide, trace metals, PAH (polycyclic aromatic hydrocarbons) (de Groot *et al*., 2018). Indoor air pollution refers to living habits like passive smoking, fuel use, lacking proper ventilation systems. There is a term called PM (particulate matter) is a complex mixture of solid and liquid particles in air, of different composition and sizes (Lima *et al*., 2013).

f) **Genetic factors:** Average age for lung cancer is 60-80 yrs. At younger age mostly below 50 if someone is having lung cancer this suggests that genetics may be involved. Genes play a major role in the development of cancer. Genes ordered the cells when to divide and when to stop dividing. When genes aren't work properly, some cells grow and divide uncontrollably which leads to cause mutations and cancer persists.

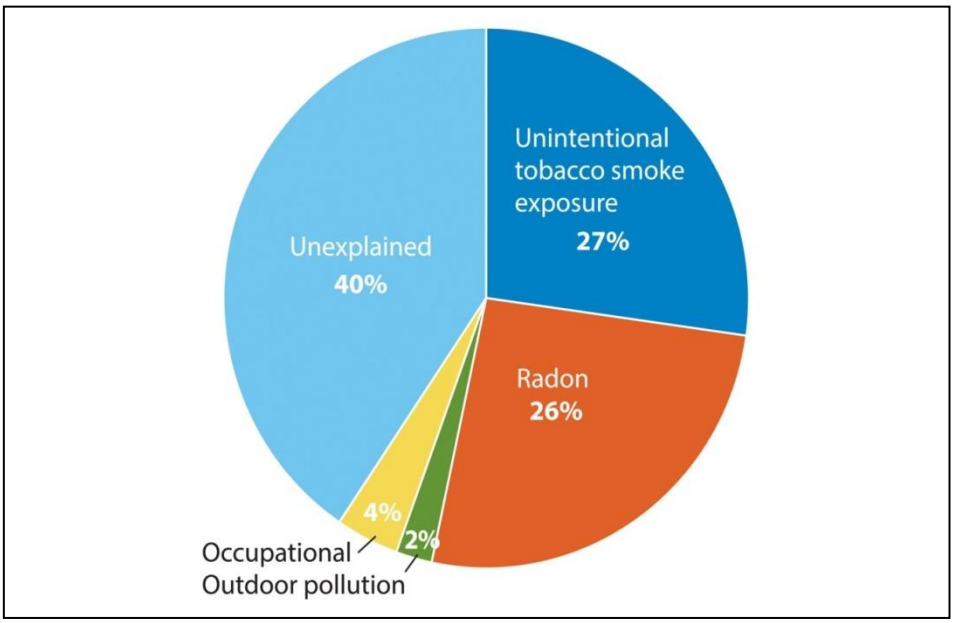

Figure 2.2. Risk factors causing lung cancer (Source : lungcancernews.org)

2.2.2. STAGES

Non small cell lung cancer (NSCLC) is the most common type of cancer. It is diagnosed based on the factor called stage. It is staged using a system called TNM classification.

T – tumor size and location

N – number of lymph nodes involved

M – metastatis means how far the cancer has spreaded

Using the combination of T, N,M classification, NSCLC can be categorized into four stages.

- **Stage I**: It is very rare stage to be diagnosed. At this stage the cancer begins but only in specific part (lung) and has not spread to any lymph node or any other distant organ.
- **Stage II**: The cancer has spread to the lymph nodes but not to the distant organs.
- **Stage III**: It is the prominent stage to be diagnosed. Cancer is widely spread to lymph nodes and to the nearby organs like middle of the chest. Stage III is further of two subtypes; Stage IIIA and Stage IIIB.
- **Stage IV**: Cancer has spread to both lungs and has also spread to the nearby organs.

2.2.3. SIGNS AND SYMPTOMS

Major signs and symptoms of lung cancer are;

- Cough
- Coughing up blood or rust color sputum
- Chest pain
- Hoarseness
- Weight loss and loss of appetite
- Shortness of breath
- Feeling tired or weak
- Yellowing of skin and eyes like in jaundice
- Infections such as bronchitis and pneumonia

2.2.4. DIAGNOSIS:

- Physical examination
- CBC(complete blood count)

- CT scanning for lung cancer staging
- Sputum cytology
- Thoracoscopy
- Bronchoscopy

2.2.5. TYPES OF LUNG CANCER: Based upon morphology of tumor cells, lung cancer is of two types:

1. Non Small Cell Lung Cancer (NSCLC): About 80-85% of lung cancers are non small cell lung cancer. It is of three subtypes classified on the basis of tumor size;

i. **Adenocarcinoma (ADCC):** It is the most common type of nsclc. This type occurs at a rate of 40% among all types. It is common in younger people and women. Also common type for smokers and non-smokers. It develops in the mucus making gland cells in the lining of the airways. It is seen in outer parts of the lungs.

ii. **Squamous cell carcinoma(SQCC):** it is the second common subtype accounts for 25-30%. This develops in the flat cells that covers the surface of airways. It is linked with smoking history and can spread to other parts of the body like lymph nodes, liver, bones, etc if not treated.

iii. **Large Cell Carcinoma(LCC):** It is undifferentiated subtype accounts for 10-12%. It can appears in any part of lung. Treatment of this cancer is quite difficult because it tends to grow at faster rate and metastize quickly.

2. Small Cell Lung Cancer (SCLC): Also called as oat cell carcinoma because of cell morphology. It is a type of malignant cancer. It grows rapidly and quickly.

2.2.6. TREATMENT

The various treatment options available for lung cancer depend on the histological type and clinical stage of the disease. For patients at initial stage i.e., I,II of lung cancer surgery is the vital element, provided the patient should be medically fit for

surgical resection (Pallis *et al*., 2012). Radiotherapy is the choice of treatment for those patients at early stage who refuse to go for surgery. However, the survival rates are lower of radiotherapy when compared to surgery (Rowell *et al*., 2001). In patients with locally advance disease at stage III or IV when tumors are unresectable, chemoradiotherapy is preferred. In this treatment patient is exposed to concurrent chemotherapy and radiotherapy. Chemoradiotherapy is preferred for patients with good performance status so as to be able to sustain the toxicity level of treatment. Chemotherapy is important in cancer treatment basically at stage IV.

2.3. *TS (THYMIDYLATE SYNTHASE)* GENE

Human *thymidylate synthase* gene plays an important role in DNA methylation, synthesis and repair. *TS* gene is located on chromosome 18p11.32 and is comprised of 7 exons. It is of 16 kb in size (Qiao *et al*., 2017). It is a central enzyme involved in metabolism of folic acid as it catalyzes the reductive methylation of deoxyuridine monophosphate (dUMP) by 5,10-methylenetetrahydrofolate to form deoxythymidine monophosphate (dTMP) and dihydrofolate as a secondary product (Curtin *et al.,* 2007). The process of conversion of dUMP to dTMP is essential for the synthesis of thymidine. Thymidine is a nucleotide important in DNA synthesis and repair (Wang *et al*., 2010). dTMP is an important precursor for DNA biosynthesis, replication and repair.

When inhibition of *TS* gene happens, it leads to depletion of dTMP which contributes for the misincorporation of uracil into DNA and thus leads to chromosome instability. *TS* gene expression level is influenced by three polymorphisms in the untranslated regions; first is in the TSER there is a variable number of tandem repeat (VNTR) in 5′-UTR having rs34743033, second is a functional C>G single nucleotide polymorphism (SNP) having rs2853542, third is 6bp insertion/deletion polymorphism having rs34489327 in the 3′-UTR (Lima *et al*., 2014). The two common alleles of TSER are TS*2R and TS*3R. Although more alleles other than these exist but are rare *viz*., 4R, 5R, 7R, 9R. TYMS protein expression and absolute enzyme activity is found to be 3-

fold higher in 3R/3R than 2R/2R (Shi *et al*., 2015). It is reported that African population has higher tandem repeats (Dhawan *et al*., 2017). *TS* gene is 30 to 35 kDa in size.

2.3.1. Functions of *thymidylate synthase* gene

a) TS is the only enzyme of folate metabolism in which 5,10-methylenetetrahydrofolate is oxidized involving one-carbon transfer.
b) It is essential for regulating the balance supply for DNA precursors (adenine, guanine, thymine, cytosine) in normal DNA replication.
c) If there is defect in the enzyme activity due to any reason say environmental factors can cause various genetic and biological abnormalities like thymineless death.
d) It is important target for certain chemotherapeutic drugs.
e) It is induced by transcriptional factor LSF (Late SV40 Factor)/TFCP2.
f) It plays a significant role in liver cancer proliferation, drug resistance.

2.3.2. Role of *TS* gene in folate metabolism

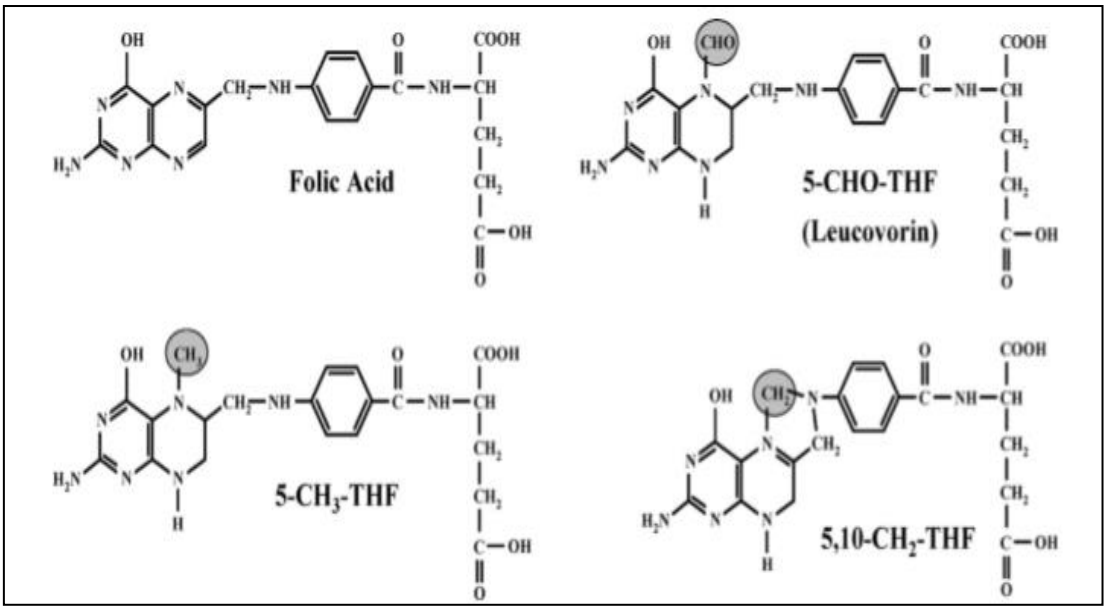

Figure 2.3. Chemical structures of folic acid, leucovorin, 5-methylenetetrahydrofolate and 5,10-methylenetetrahydrofolate. (Source: Gonen *et al*., 2012)

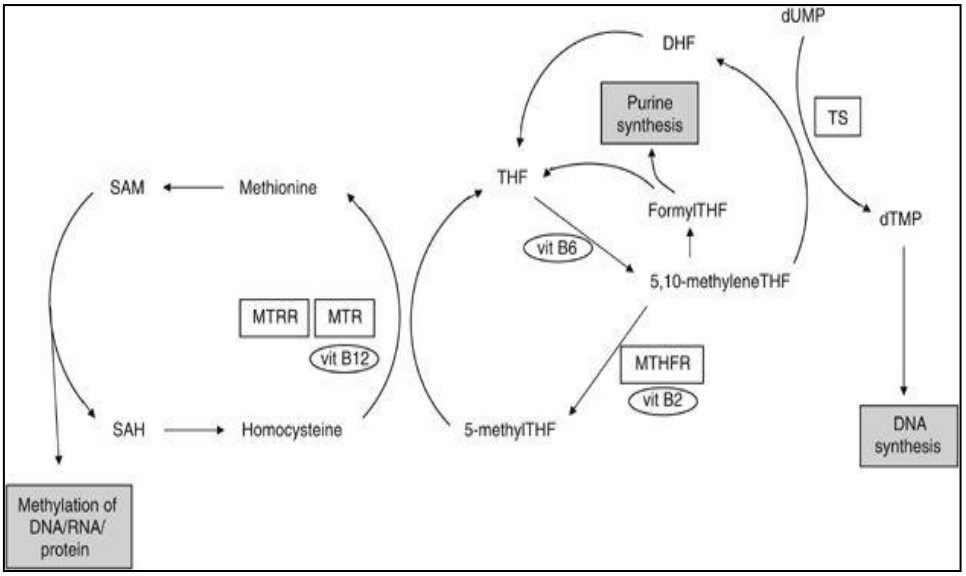

Figure 2.4. Representation of folate metabolism in a schematic view. In boxes there are corresponding enzymes for genes with polymorphisms were investigated. In ovals, there are cofactors for vitamins. TS (thymidylate synthase), MTR (methioine synthase), MTRR (methionine synthase reductase), MTHFR (methylenetetrahydrofolate reductase), DHF (dihydrofolate), THF (tetrahydrofolate), dUMP (deoxyuridine monophosphate), dTMP (deoxythymidine monophosphate), SAH (S-adenosylhomocystiene), SAM (S-adenosylmethionine). (Source: Hubner *et al*., 2006)

Folate is a vitamin-B9 and is found in oxidized form i.e., folic acid (Gonen *et al*., 2012). It is naturally present in various dietary supplements. It is essential in making genetic material, cell division in body. Appropriate folate intake is essential for cell division and homeostatis. Folate is an essential coenzyme in many pathways like amino-acid metabolism, DNA methylation, purine and thymidylate biosynthesis. Folate act as a single carbon donor, converts homocysteine into methionine and also have role in purine and pyrimidine synthesis. Folate metabolism is a complex pathway which involves approximately 30 enzymes in which TS ia a rate limiting enzyme (Zhou *et al*., 2012). The following reaction is catalyzed by *tymidylate synthase* gene in folate metabolism:

5,10-methylenetetrahydrofolate + dUMP =dihydrofolate + dTMP

5,10-methylenetetrahydrofolate is a substrate of thymidylate synthase and a substrate for MTHFR (methylenetetrahydrofolate reductase) enzyme and produce 5-methyltetrahydrofolate, thus this is converted into methionine required for DNA methylation in the presence of TS. This reaction imbalance in the metabolism of folate pathway results in DNA methylation rather than DNA synthesis (Hubner *et al*., 2006). Polymorphism in the *TS* gene is influenced by various genetic changes or dietary factors which alters the function of enzyme, enzyme efficiency, expression level of enzyme may lead to cause any type of cancer in body (Wang *et al*., 2010).

2.3.3. *TS* gene and cancer

In non-hispanic white population, it was observed that *TS* 3'UTR polymorphism could be associated with increased risk of lung cancer and the main cause was found to be alcohol intake. The interaction between *TSER* polymorphism and vitamin B_{12} intake was also observed (Shi *et al*., 2004). The *TS* gene exhibited an association between prognosis of stage I patients of lung cancer. Thus *TS* gene is a prognostic factor in non small cell lung cancer (Takehara *et al*., 2005). TS expression level decreases when treated with flavonoid (flavopiridol) which was due to inhibition of CDKs cells as the cyclin-dependent kinase is active only in G_1 phase of cell cycle. This activity of kinase plays a role in control of TS expression level (Le Francois *et al*., 2007). Association of genotypes of *TS* gene with greater TS expression leads to increase in colon cancer by increasing the mutations in the tumor (Curtin *et al*., 2007). Polymorphism in *TSER* genotype increases the risk of breast cancer in Asian and Caucasian women population (Wang et al., 2010). *TS* gene had genotypes like 2R, 3R, 4R, etc. Though the number is small but 2R/2R genotype polymorphism causes gastric cancer in Asian population (Yim *et al*., 2010). Lower expression level of *TS* gene in NSCLC patients could get benefit in pemetrexed based chemotherapy but not the high expression of *TS* gene (Liu *et al*., 2013). *Thymidylate synthase* gene is highly expressed in non small cell lung cancer patients. No significant association was found between the heterozygous 2R/3R TS polymorphism and the risk for colorectal cancer

in Romanian population (Toma *et al*., 2014). Polymorphism of *TS* gene at 3'UTR position was predicted in Jordian population which showed increased risk of lung cancer (Qasem *et al*., 2015).

2.4. Platinum based Chemotherapy regimens

Platinum complexes are classified under the class of alkylating agents that impair the cell function by alkylating some most important molecules such as DNA, RNA and proteins. They form covalent bonds with the phosphate, amino, carboxyl or sulfyhydryl groups of these molecules. Some of the common platinum based chemotherapeutic agents are described below.

2.4.1. Docetaxel cisplatin/ Docetaxel carboplatin

Docetaxel is the anti-mitotic chemotherapy drug majorly used for the breast, ovarian and NSCLC treatment. It reversibly binds with micrtotubule with high affinity. Docetaxel is a semi-synthetic taxoid derived from 10-deacetyl bacatin III which result into formation of microtubule assembly and inhibition of tubulin depolymerization which leads to stabilization of microtubule. This drug gives 23-33% treatment success and 21% objective response in NSCLC patients as well as new cancer cases respectively. Cisplatin has a mechanism which is different from docetaxel is also used for treatment in NSCLC patients majorly. Studies have shown cisplatin-based chemotherapy in NSCLC shows a superior response rate and survival than those regimens which does not contain cisplatin (Donadieu *et al*., 1991), therefore use of cisplatin regarded as the independent predictor of improved survival (Aihara *et al*., 2002).

2.4.1.1. Mechanism of action

Cisplatin blocks DNA replication which stops the cell proliferation in carcinogenic cells. On administration, one of the two chloride ligands is displaced by water giving the aquo-complex *cis*-[PtCl(NH3)2(H2O)]+, this process is known as aquation. The

aqua ligand in *cis*-[PtCl(NH3)2(H2O)]+ is displaced by the *N*-heterocyclic bases on DNA. Guanine preferentially binds subsequently for the formation of [PtCl(guanine-DNA)(NH3)2]+, leading to DNA cross-linking interfering mitosis. The damaged DNA activates the DNA repair mechanism which activates apoptosis when it appears impossible to repair the damage. Although cisplatin is frequently designated as an alkylating agent but in reality it has no alkyl group and it therefore cannot carry out alkylating reactions. Cisplatin used in combination with another chemotherapy drug has been a cornerstone in treatment of many cancers. Initially platinum responsiveness is high but majority of cancer patients eventually relapse with the cisplatin-resistance. There are many mechanisms which show this resistance which includes cellular uptake and efflux of drug, increased drug detoxification, inhibition of apoptosis and increased DNA repair.

As it is known that docetaxel exerts by promoting and stabilizing the microtubule assembly by preventing microtubule depolymerization and stabilizing microtubule assembly in the absence of GTP (Williamson *et al*., 2005). This results a decrease in number of free tubulin which are required for microtubule formation which inhibits mitotic cell division between metaphase and anaphase preventing cancer cell progeny (Yvon *et al*., 1999). As microtubules do not disassemble in the presence of docetaxel they accumulate inside cell which leads to initiation of apoptosis. Apoptosis has been encouraged by the blocking of apoptosis-blocking bcl-2 oncoprotein. Both *in vitro* and *in vivo* analysis show the anti-neoplastic activity of docetaxel to be effective against a wide range of known cancer cells, cooperate with other anti-neoplastic agents activity, and have greater cytotoxicity. The main mode of therapeutic action of docetaxel is the suppression of microtubule dynamic assembly and disassembly, rather than microtubule bundling leading to apoptosis, or the blocking of bcl-2.

2.4.2. Irinotecan cisplatin/ Irinotecan carboplatin

Irinotecan (Camposter) is a camptothecin-derived compound whose active compound SN-38 interacts with topoisomerase I forms a stabilizing complex with DNA (Fukuda *et al*., 2004). Along with its novel cytotoxic mechanism, irinotecan differs from other chemotherapeutic agents in the multidrug-resistant cell lines which retain irinotecan sensitivity (Tusuro *et al*., 1988). It is used as an approved drug for the metastatic colon cancer in USA. For colon cancer treatment it is either infused alone or with fluorouracil. And for small cell lung cancer treatment it is used in combination with cisplatin. When used as a single agent, the response rate in NSCLC may vary from 15-34% (Pillot *et al*., 2006).

2.4.2.1. Mechanism of action

Irinotecan is a prodrug (CPT-11) that is metabolized into its active form, SN-38, when inside the cell. Irinotecan and SN-38 may be transported out of the cell via multidrug transporters such as ABCB1 (Fukuda *et al*., 2004). Irinotecan is converted into SN-38 via human carboxylesterases but can also be converted to inactive metabolites via CYP3A4 and CYP3A5, and SN-38 can be inactivated through glucuronidation to SN-38G via UGT1A1. This inactivation leads to inhibition of both DNA replication and transcription. The UGT1A1*28 allele provides the strongest support for a role of pharmacogenetics in predicting toxicity from irinotecan therapy (Fukuda *et al*., 2004).

2.5. PEMETREXED BASED CHEMOTHERAPY

Pemetrexed ($C_{20}H_{21}N_5O_6$) is an anti-cancer chemotherapeutic drug. It is approved by United States Food and Drug Administration as a first line chemotherapy for the treatment of cancer especially lung cancer. It is used in the treatment of malignant mesithelioma, metastatic non small cell lung cancer. It is given as a mixture into the vein. Patients treated with this drug have to take vitamin B supplements to overcome the side effects. Side effects of pemetrexed includes fatigue, nausea, diarrhea, anemia, skin rashes, etc (http://chemocare.com/chemotherapy/drug-info/PEMETREXED.aspx).

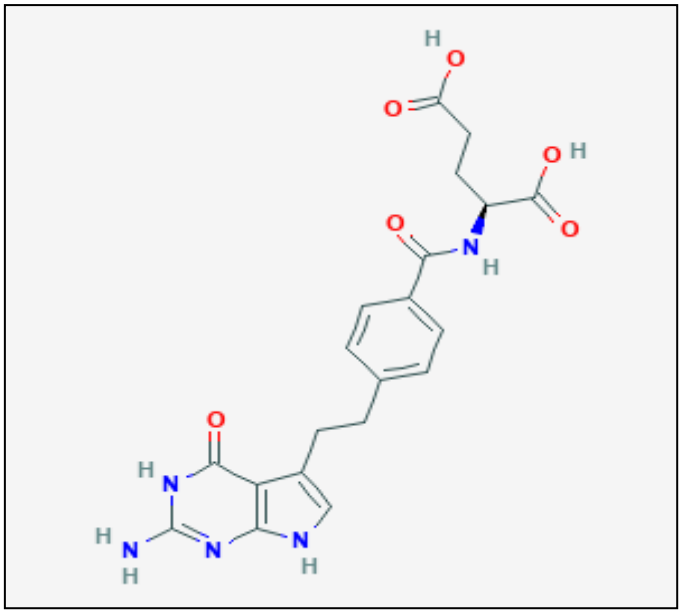

Figure 2.5. Structure of pemetrexed (Source: pubchem.ncbi.nlm.nih.gov)

Pemetrexed is chemically similar to folic acid thus called as folate antimetabolite. It is transported by reduced folate carrier (RFC) into the cells. When pemetrexed enters cells, it is converted into the active pentaglutamate catalyzed by folypolyglutamate synthetase (FPGS). Pemetrexed is one of the best substrate for FPGS. Pemetrexed inhibits multiple enzymes in purine and pyrimidine synthesis. Among these TS is a primary target which is involved in folate metabolism. Inhibition of TS by pemetrexed leads to decrease in thymidine which is important for DNA synthesis. Along with TS DHFR (dihydrofolate reductase) and GARFT (glycinamide ribonucleotide formyltransferase) also gets inhibited. Cytotoxicity of pemetrexed is mediated by TS inhibition (Adjei, 2004). Level of TS mRNA is inversely related with pemetrexed activity in different cancerous cells, but some of the studies suggested that level of TS expression and reduced sensitivity to pemetrexed is higher in lung and colon cancer (Zucali *et al*., 2011). TS mRNA protein expression is higher in SCLC (squamous cell lung cancer) than other types of lung cancer. The poorer response of NSCLC to pemetrexed results in higher level of expression of TS in such tumors (Takezawa *et al*., 2011).

Earlier platinum based chemotherapy was used for almost all types of cancers, but disadvantage of this therapy was that it was used only used for first line treatment of stage 3 and 4 NSCLC. However there was no therapy for second line treatment for advanced NSCLC. Then pemetrexed came into picture. Pemetrexed is used as a vital drug for second line treatment of advanced NSCLC recommended by National Comprehensive Cancer Network in 2006 (Wang *et al*., 2013).

2.6. Importance of the study in context of current status

The considerable reason for cancer susceptibility lies in polymorphism of the different genes. In India, the cases of lung cancer and genetic variations are considerably high. This adds a reason to our population for becoming more susceptible towards lung cancer. On the other hand, only few polymorphism studies are conducted in Indian population creating an unpredictable state. Hence it is necessary to study the association of polymorphism present in genes involved in folate metabolism pathway with lung cancer. Further there has been no study done related to gene polymorphism in *TYMS* gene (rs34743033) in relation to platinum based drugs in North Indian lung cancer population. The study is conducted in order to check the overall survival rate of lung cancer patients, association between polymorphism and the clinical outcomes receiving platinum based chemotherapy.

3.0. AIM OF STUDY

- To study the single nucleotide polymorphic variants of *Thymidylate Synthase* gene TSER (rs 34743033).
- To test for association between genetic polymorphism and clinical outcome in patients receiving platinum based chemotherapy.

4.0. MATERIAL AND METHODS

4.1. Study subjects and sample collection

In current research *TS* gene was studied for 150 patients. Samples were collected from Department of Pulmonary Medicine, Post Graduate Institute of Medical Education and Research (PGIMER) Chandigarh, India. Informed written consent was obtained from all patients. With the help of trained interviewer, the detailed questionnaire was filled by each patient to obtain various details about the patient's demographic and tobacco smoking characteristics. Smokers reported tobacco habits such as smoking of cigarette and bidi. Patients were characterized as light and heavy smokers on the basis of pack years. Pack years was calculated as follows:

[(cigarettes or bidis smoked by an individual per day/20)*number of years smoked]

Medical information of cases including histology, TNM classification, clinical staging, tumor size, regimen, lymph node involvement were obtained from medical records of the hospital. Approximately 3-5 mL of blood was collected from the vein of the patient.

4.2. Isolation of DNA from peripheral blood

Isolation of genomic DNA from whole blood of lung cancer patients was done by Proteinase K digestion, Phenol/Chloroform extraction and ethanol precipitation

Requirements:

a) Washing buffer
b) Lysis buffer
c) Proteinase K
d) Phenol : Chloroform : Isoamyl alcohol (25:24:1)
e) Chloroform : Isoamy alcohol (24:1)
f) Ethanol
g) Tris-EDTA buffer

Preparation of Reagents and Buffers:

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)

LYSIS BUFFER	
Stock concentration	**Working concentration**
1 M Tris-HCl (pH-8.0)	400 mM Tris-HCl (pH-8.0)
10% SDS	1% SDS
0.5 M EDTA(pH-8.0)	60 mM EDTA(pH-8.0)
5 M NaCl	150 mM NaCl
10 mg/mL Proteinase K	100 µg/mL Proteinase K

5X TBE buffer:

5X TBE buffer was prepared by dissolving 54 gm of Tris base, 27.5 gm of boric acid, 20 mL of 0.5 M EDTA. Volume was made upto 1000 mL by adding distilled water.

6X loading dye:

6X loading dye was prepared by dissolving 0.25% bromophenol blue (0.05 gm), 0.25% Xylene cyanol (0.05 gm), 40% sucrose (8 gm). Volume was made upto 20 mL by adding TE buffer.

Procedure of genomic DNA isolation:

- 5 mL of blood was taken and equal volume of cooled washing buffer was added. Centrifuged it at 3500 rpm for 5 minutes.
- Discarded the supernatant and 5 mL of washing buffer was added to the pellet and centrifuged again. Repeated this step thrice.
- Dissolved the pellet in 5 mL of lysis buffer followed by overnight incubation at 46℃.
- Added equal volume of PCI mix in 25:24:1 ratio to the lysed mixture and centrifuged at 8000 rpm for 10 minutes at 4℃.
- After centrifugation took the upper aqueous layer and again added PCI mix and centrifuged.
- To the aqueous layer, added equal volume of CI mix in 24:1 ratio and centrifuged it at 6500 rpm for 5 minutes at 4˚C.
- Again took the aqueous layer, added chilled isopropanol or 2.5 times of absolute ethanol and mixed it gently.
- Freeze it at -20℃ for 1-2 hours.
- Centrifuged it at 12000 rpm for 10 min at 4℃ and discarded the supernatant and the DNA pellet was washed with 70% ethanol twice at 10000 rpm for 5 min at 4℃.
- Dissolve the pellet in 50 µL Tris-EDTA buffer and stored at -20℃.

DNA Quantification

The quality of DNA can be determined by Thermo Scientific Nanodrop Spectrophotometer. It is much better than UV-VISIBLE spectrophotometer because it requires only 1 µL of sample without use of any containment like cuvettes. The sample is placed between two optical surfaces, which determine the path length in vertical orientation. Removal of fixed containment devices from the system allows the path length to change in real time for a sample. This eliminates the need to perform dilutions and hence less cumbersome.

Procedure:

- Cleaned the nanodrop lens using 1 µL of deionised water.
- Opened the nanodrop model and select the nucleic acid module.
- Loaded 1 µL of TE buffer and set blank.
- Loaded 1 µL of DNA sample and select measure to take the reading.
- Concentration and purity of sample was calculated automatically.

DNA concentration can also be calculated as;

DNA concentration (µg/mL)= O.D. at 260nm*50*dilution factor

(Standard DNA sample O.D.= 1 at 50 µg/mL concentration)

Quality/Purity of DNA = O.D. at 260/280

NOTE: A ration of approx 1.8 indicates purity of DNA. A ratio of approx 2.0 indicates pure RNA and below 1.8 ration indicates protein contamination.

4.3. Genotyping by Polymerase Chain Reaction-Restricted Fragment Length Polymorphism (PCR-RFLP)

Polymerase Chain Reaction of TSER genetic variants was carried out to amplify the specific region. PCR is an enzymatic process that helps in detection of specific genes within an environmental DNA sample. Oligonucleotide primers are used in PCR for amplification of required genomic DNA region. The oligonucleotide primers are short, user defined DNA sequences complementary to the target DNA sequences. PCR is a very sensitive process. From a single copy, a multiple copies can be obtained after PCR process. The SNP used in current study is TSER (rs 34743033). To analyze the TSER 28bp repeat polymorphism, fragment was amplified using the following set of primers: forward primer; 5′-GTGGCTCCTGCGTTTCCCCC-3′ and reverse primer; 5′-GGCTCCGAGCCGGCCACAGGCATGGCGCGG-3′ (Qiuling *et al*., 2004). PCR reaction was performed using the total volume of 25 µL.

The detailed information of reagents used in PCR and PCR conditions used for amplifying TSER gene are as follows.

REAGENTS	**STOCK**	**WORKING**
Taq Buffer	10X	1X
Additive 1(BSA)	100X	10X
Additive 2(DMSO)	100%	5%
Forward primer	10 nmol	0.5 nmol
Reverse primer	10 nmol	0.5 nmol
dNTPs	10 mM	0.2 mM
Taq Polymerase	2 units/µL	1.5 units
DNA	100 ng/µL	200ng
Deionised water	-	-

PCR conditions used are described in the following table:

PCR CONDITIONS			
STEPS	**TEMPERATURE**	**TIME**	**CYCLES**
Initial denaturation	95℃	5 minutes	1
Denaturation	94℃	30 seconds	29
Annealing	62℃	45 seconds	
Polymerization	72℃	30 seconds	
Extension	72℃	5minutes	1

The amplified product was run on Native- PAGE gel. PAGE gel is made up of acrylamide and bis-acrylamide. It is highly sensitive method to check the resolution DNA by 1bp. For performing N-PAGE, acrylamide and bis-acrylamide is mixed in a fixed ratio which determines the pore size of the gel. Polymerization in N-PAGE is done with APS (ammonium per sulphate) and TEMED (N,N,N,N-

tetramethylethylenediamine). Acrylamide gels are defined in terms of total percentage of acrylamide and the pore size of the gel. The pore size of the gel can be varied by altering the percentage of acrylamide. Generally 4-12% of gel can be prepared, which have large pore size can be used to separate DNA according to their sizes. For TSER polymorphic variant 10% gel is used. After vertical gel electrophoresis ethidium nromide staining was done for 15 minutes to make the bands visible.

COMPOSITION OF N-PAGE GEL					
Gel	30% acrylamide	Distilled water	5X TBE	10% APS	TEMED
10%	4.0 mL	5.6 mL	2.4 mL	200 µL	10 µL

5.0. RESULTS

5.1. DNA isolation

Genomic DNA of lung cancer patients was isolated from 5 mL of peripheral blood. It was run on 0.7% agarose gel for qualitative analysis. Bands were seen under UV-VIS transilluminator to confirm the presence of DNA bands as shown in figure 5.1. The isolated DNA samples were diluted in TE buffer of concentration 100 ng/µL which can further be used in polymerase chain reaction.

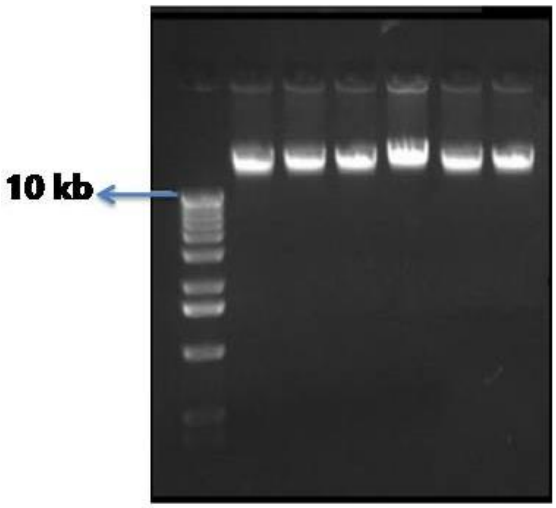

Figure 5.1. Gel picture of genomic DNA run on 0.7% agarose gel

5.2. Polymerase Chain Reaction and Native Polyacrylamide gel electrophoresis for *TSER* rs34743033 gene polymorphism and their amplicons

Polymerase chain reaction of isolated genomic DNA was performed simultaneously to amplify the *TSER* gene using specific primers. The amplicons obtained were run on 10% NATIVE-PAGE gel which was stained in ethidium bromide solution for 15 minutes. The bands were visualized under UV light. Size of the bands were 215bp and 243bp for 2R and 3R, respectively.

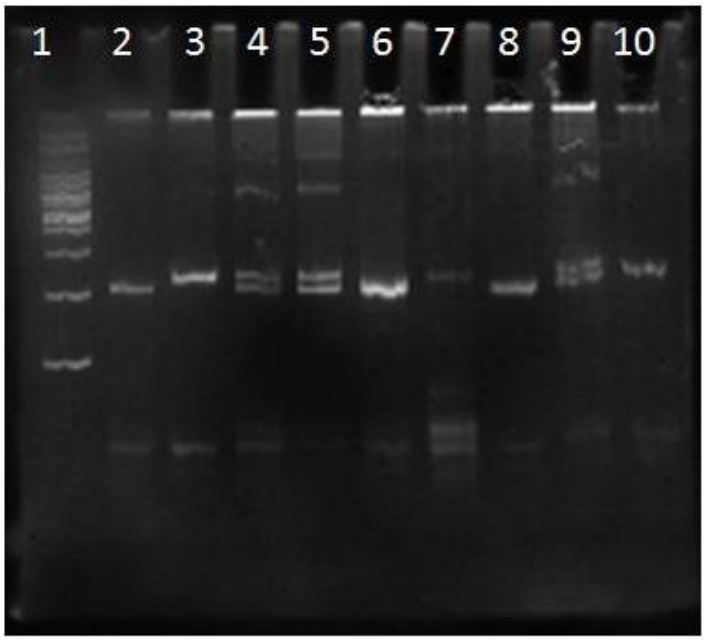

Figure 5.1. 10**%** N-PAGE gel of samples amplified using PCR
(Lane 1: Ladder (100bp); Lane 1,6,8: 3R genotype of *TS* gene; Lane 2,7,10: 2R genotype of *TS* gene; Lane 4,5,9: 2R/3R genotype of *TS* gene)

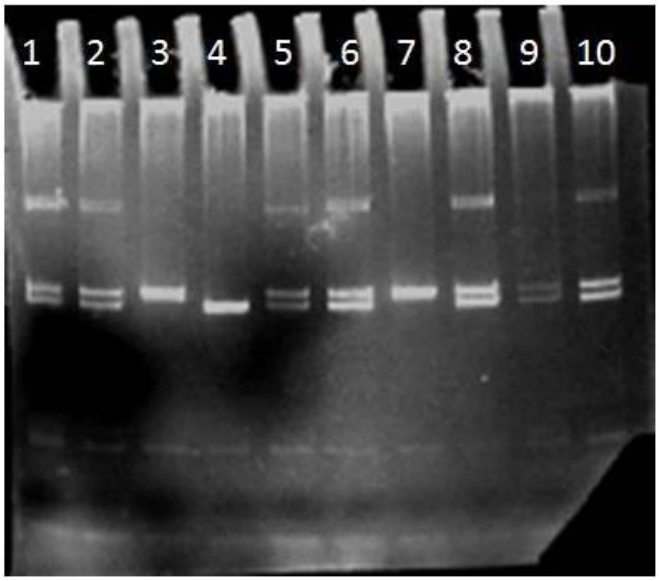

Figure 5.2. 10% N-PAGE gel of samples amplified using PCR
(Lane1,2,5,6,8,9,10: 2R/3R genotype of *TS* gene; Lane 3,7: 2R genotype of *TS* gene; Lane 4: 3R genotype of *TS* gene)

5.3. Demographic characteristics for *TSER* rs34743033 polymorphism

Table 5.1. Distribution of demographic features among lung cancer patients for *TSER* rs34743033 gene polymorphism

Variable	**Cases, n(%) N=150**	**Variable**	**Cases, n(%) N=150**
Age(years)		**Lymph node involvement**	
Mean ± SD	57.51 ± 10.92		
Range	29-80	0	11(7.33)
		1	16(10.66)
		2	60(40.00)
		3	43(28.66)
		4	3(2.00)
		Unknown	17(11.33)
Gender		**Metastatis**	
Male	124(82.66)	0	74(49.33)
Female	26(17.33)	1	59(39.33)
		Unknown	17(11.33)
Smoking status		**KPS**	
Smokers	123(82.00)	<70	13(8.66)
Non smokers	27(18.00)	70-80	73(48.66)
		90-100	57(38.00)
		Unknown	7(4.66)
Pack years		**ECOG**	
Mean ± SD	28.59±37.68	0	21(14.00)
		1	50(33.33)
		2	53(35.33)
		3	16(10.60)
		4	3(2.00)
		Unknown	7(4.66)
Hostological types		**Regimen**	
ADCC	49(32.66)	1	39(26.00)
SQCC	51(34.00)	5	27(18.00)
SCLC	45(30.00)	6	34(22.66)
Others	5(3.33)	Others	12(8.00)
		Unknown	38(25.33)
Tumor size		**TNM Staging**	

T1	5(3.33)	I,II	6(4.00)
T2	13(8.66)	III	69(46.00)
T3	39(26.00)	IV	61(40.66)
T4	74(49.33)	Unknown	14(9.33)
Unknown	19(12.66)		
Objective response			
	1(0.66)		
CR	52(34.44)		
PR	32(21.33)		
SD	8(5.33)		
PD	57(38.00)		
Unknown			

Abbreviations: N = Total no. of lung cancer cases; ADCC = Adenocarcinoma; SQCC = Squamous cell carcinoma; SCLC = Small cell lung cancer; CR = complete response; PR = partial response; SD = stable disease; PD = progressive disease; TNM = Tumor Node Metastatis; KPS = Karnofsky Performance Staus; ECOG = Eastern Cooperative Oncology Group; Regimen 1 = Docetaxel-plus-carboplatin/cisplatin; Regimen5 = Irinotecan-plus-carboplatin/cisplatin; Regimen 6 = Pemetrexed-plus-carboplatin/cisplatin.

The distribution of demographic characteristics in lung cancer patients recruited for the study of *TSER* rs34743033 is mentioned in Table 5.1. The parameters included are age, gender, smoking status, pack per year smoked, histology type, TNM staging and extension, tumor stage, clinical response to treatment, lymph node involvement, metastatic staging, regimen preferred by patients and performance status. The mean age of 150 cases recruited for overall survival study was 57.51 with standard deviation of 10.92 with range 29-80 years. There were more males in the study as compared to females *viz*, 82.66% (124) and 17.33% (26), respectively. Percentage of smokers (82.00%) is much more higher than non-smokers (18.00%) which indicates that smoking is a major factor in acquiring lung cancer. Pack years can be calculated by the formula; cigarettes or beedis smoked per day/20*number of years smoked. The mean of pack years smoked was 28.59 with standard deviation of 37.68. Based on histology it was found that 34.00% patients were SQCC, followed by 32.66% were

ADCC, followed by 30.00% were SCLC and only 3.33% patients belong to unknown histology. Now coming to clinic-pathological parameters such as Tumor Node Metastatic (TNM) staging and extension, 131 patients were available. The size of tumor varies from T1 to T4. Majority of the patients had T4 type of tumor (49.33%), followed by T3 (26.00%), T2 (8.66%), T1 (3.33%). N0, N1, N2 and N3 type lymph node involvement was recognized in 7.33%, 10.66%, 40.00% and 28.66% cases, respectively. Minimal patients reflected N4 type lymph node involvement *i.e.,* 2.00%. Further, no lymph node involvement was observed in 11.33% cases. Then comes metastatic staging, among 133 cases, 74 (49.33%) patients showed distant metastasis. No metastatic staging was shown in 11.33% cases. Significant number of cases showed stage III and stage IV disease (46.00% and 40.66%). Only 4.00% patients were detected in stage I and stage II. Diseased stage of 9.33% was unknown. Among 93 cases whose clinical response was noted, 0.66% showed complete response (CR), 34.44% showed partial response (PR), 21.33% showed stable disease (SD) and 5.33% showed progressive disease (PD). Additionally on categorizing the patients on the basis of regimen they had undergone, it was observed that the majority of the patients were under medication of regimen 1 (26.00%) which is Docetaxel-carboplatin/cisplatin, while 22.66% patients received regimen 6 i.e., Pemetrexed-carboplatin/cisplatin. 18.00% of the cases received regimen 5 i.e., Irinotecan-carboplatin/cisplatin. 8.00% patients were under other medications such as Gemcitabine-carboplatin/cisplatin and others. Regimen of 25.33% cases was not known. Other parameters were studied like KPS (Karnofsky Performance Status) and ECOG (Eastern Cooperative Oncology Group). 8.66% cases had KPS below 70, 48.66% cases had KPS between 70-80 and 38.00% cases had KPS between 90-100 and KPS of 4.66% of the cases was not known. Performance status according to ECOG, better ECOG score was observed in 1 (33.33%) and 2 (35.33%) while in ECOG 0, 3 and 4 14.00%, 10.60% and 2.00% of the cases were reported, respectively. For the remaining 4.66% patients, ECOG was not classified. It was obsereved that the

genotypic distribution of TSER (rs34743033) was consistent with the Hardy-Weinberg equilibrium ($p^2+2pq+q^2=1$; $\chi^2=0.087$; p=0.77).

5.4. Association of *TSER* rs34743033 polymorphism genotypic distribution with overall survival of lung cancer patients

Table 5.2. Genotyping distribution and relationship of *TSER* rs34743033 polymorphism with overall survival of lung cancer patients

***TSER* rs34743033 genotype**	**Dead (127)**	**Alive (23)**	**Median OS Months**	**HR 95% CI**	**Log p**	**Adjusted HR (95% CI)**	**P**
2R	24	2	7.60	1.0 (Reference)	-	1.0 (Reference)	-
2R/3R	62	13	9.47	0.76 (0.46 to 1.27)	0.30	1.20 (0.69 to 2.09)	**0.02**
3R	41	8	9.67	0.78 (0.46 to 1.32)	0.36	0.90 (0.49 to 1.65)	0.89
2R/3R+3R	103	21	9.47	0.77 (0.48 to 1.25)	0.29	1.06 (0.63 to 1.77)	**0.02**

Hazards ratio (HR), 95% Confidence intervals (CI) and their respective p-values were estimated using Kaplan-Meier survival analysis after adjusting for reemission and overall survival (months). Adjusted HR, 95% CI and their respective P-values were determined after adjusting for age, gender, smoking status, stage, KPS, ECOG.

Genotypic frequency of *TSER* rs34743033 was analyzed of the data obtained, classification on the basis of overall survival of lung cancer patients, 84.67% (127) patients were dead and 15.33% (23) patients were alive. Among the patients carrying homozygous wild genotype 2R, 16.00% (24) were dead and 1.33% were alive. Homozygous wild genotype had overall survival months of 7.60 months which was taken as reference. Patients carrying heterozygous genotype 2R/3R, 41.33% (62) were

dead and 8.67% (13) were alive and had overall survival months of 9.47 months which was higher than homozygous wild genotype (9.47 vs. 7.60 months, HR = 0.76, Logrank p = 0.30). Patients carrying mutant genotype 3R, 27.33% (41) were dead and 5.33% (8) were alive. This genotype had overall survival of 9.67 months which was better than homozygous wild genotype (9.67 vs. 7.60 months, HR = 0.78, Logrank p = 0.36). On combining the mutant genotype with heterozygous genotype and comparing with wild genotype 2R/3R+3R, 68.67% (103) were dead and 14.00% were alive. They had overall survival of 9.47 months almost similar to heterozygous genotype (9.47 vs. 7.60 months). However, significant association was found in heterozygous genotype and combined genotype subjects. Significance value was equal to **0.02**. *TSER* rs34743033 polymorphism on evaluation with Kaplan-Meier survival analysis is as observed in figure 5.1. and 5.2.

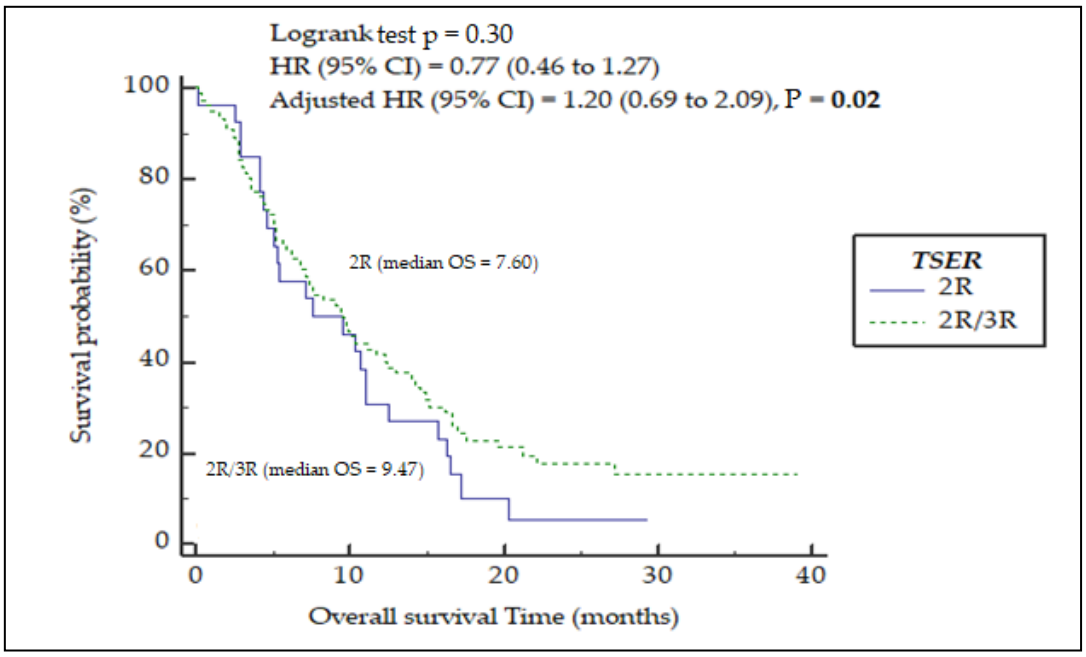

Figure 5.4. Kaplan-Meier survival curve illustrating effect of *TSER* rs34743033 polymorphism on overall survival of lung cancer patients with heterozygous genotype (2R/3R vs. 2R)

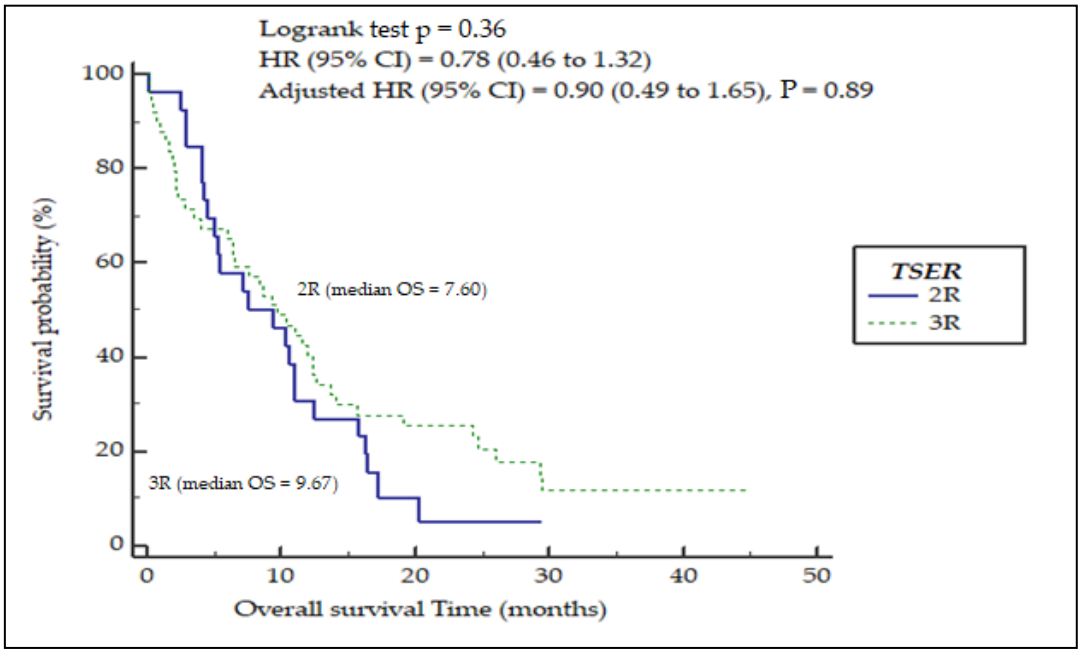

Figure 5.5. Kaplan-Meier survival curve illustrating effect of *TSER* rs34743033 polymorphism on overall survival cancer patients with mutant genotype (2R vs. 3R)

5.5. Association of *TSER* rs34733033 polymorphism genotypic distribution with overall survival of lung cancer patients based on histological subtypes

Table 5.3. Genotypic distribution and relationship of *TSER* rs34743033 polymorphism with overall survival of lung cancer patients on the basis of histological type

ADCC							
TSER rs34743033 genotype	**Dead (40)**	**Alive (9)**	**Median OS months**	**HR 95% CI**	**Log p**	**Adjusted HR 95% CI**	**P**
2R	15	1	7.13	1.0 (Reference)	-	1.0 (Reference)	-
2R/3R	19	5	10.00	0.60 (0.23 to 14.93)	0.18	1.21 (0.50 to 2.98)	0.05
3R	6	3	24.33	0.40 (0.16 to 0.98)	**0.04**	0.73 (0.18 to 2.30)	0.13
2R/3R+3R	25	8	12.23	0.50 (0.24 to 1.03)	0.06		

SCLC							
***TSER* rs34743033 genotype**	**Dead (41)**	**Alive (4)**	**Median OS months**	**HR 95% CI**	**Log p**	**Adjusted HR 95% CI**	**P**
2R	3	0	11.00	1.0 (Reference)	-	1.0 (Reference)	-
2R/3R	22	3	7.33	0.96 (0.28 to 3.30)	0.95	2.21 (0.21 to 23.63)	0.89
3R	16	1	9.67	1.09 (0.32 to 3.73)	0.89		
2R/3R+3R	38	4	8.27	1.00 (0.30 to 3.30)	0.99	1.42 (0.24 to 8.30)	0.70
SQCC							
***TSER* rs34743033 genotype**	**Dead (42)**	**Alive (9)**	**Median OS months**	**HR 95% CI**	**Log p**	**Adjusted HR 95% CI**	**P**
2R	6	1	11.00	1.0 (Reference)	-	1.0 (Reference)	-
2R/3R	18	5	9.73	0.96 (0.37 to 2.49)	0.93		
3R	18	3	7.57	1.05 (0.41 to 2.68)	0.92		
2R/3R+3R	36	8	7.57	0.99 (0.41 to 2.39)	0.99		

Hazard ratios (HR), 95% Confidence intervals (CI) and their respective p-values were estimated using Kaplan-Meier survival analysis after adjusting for reemission and overall survival (months). Adjusted HR, 95% CI and their respective P-values were determined after adjusting for age, gender, smoking status, pack years, stage, KPS, ECOG. ADCC = Adenocarcinoma; SQCC = Squamous cell carcinoma; SCLC = Small cell lung cancer.

In the histological types, cases included for overall survival evaluation for *TSER* rs34743033 polymorphism, 32.66% (49) cases were diagnosed with ADCC, 29.80% (45) cases were diagnosed with SCLC and 34.00% (51) cases were diagnosed with

SQCC. Further, on basis the basis of classification of genotypic frequency, it was found that 32.65% of ADCC, 6.66% of SCLC and 29.41% of SQCC had homozygous wild genotype 2R. 48.98%, 55.55% and 45.09% of ADCC, SCLC and SQCC had heterozygous genotype, respectively. 18.37%, 37.78% and 41.18% of ADCC, SCLC and SQCC had mutant genotype, respectively.

In ADCC, patients with homozygous wild genotype 2R showed MST of 7.13 months which was taken as reference. Heterozygous genotype 2R/3R carrying patients showed an increased survival period of 10.00 months (10.00 vs. 7.13 months, HR = 0.60, Logrank p = 0.18). Mutant genotype 3R had better survival rate than wild genotype as well as heterozygous genotype (24.33 vs. 7.13 months, HR = 0.40, Logrank p = 0.04). The combination of mutant and heterozygous genotype still reflected a better survival period (12.23 vs. 7.13 months, HR = 0.50, Logrank p = 0.06).

In SCLC, patients with homozygous wild genotype 2R showed MST of 11.00 months which was taken as reference. Heterozygous genotype 2R/3R carrying patients showed decreased survival period of 7.33 months (7.33 vs. 11.00 months, HR = 0.96, Log rank p = 0.95). Mutant genotype 3R had survival rate less than homozygous wild genotype (9.67 vs. 11.00 months, HR = 1.09, Logrank p = 0.89). The combination of heterozygous and mutant genotype did not reflected better survival period (8.27 vs. 11.00 months, HR = 1.00, Logrank p = 0.99).

In SQCC, patients with homozygous wild genotype 2R showed MST of 11.00 months which was taken as reference. Heterozygous genotype 2R/3R carrying patients showed decreased survival period of 9.73 months (9.73 vs. 11.00 months, HR = 0.96, Logrank p = 0.93). Mutant genotype 3R had survival period of 7.57 months which was less than both homozygous wild and heterozygous genotype (7.57 vs. 11.00 months, HR = 1.05, Logrank p = 0.92). The combination of mutant and heterozygous

genotype had survival rate same as of mutant genotype (7.57 vs. 11.00 months, HR = 0.99, Logrank p = 0.99).

On the basis of histological types, *TSER* rs34743033 polymorphism on on evaluation with Kaplan-Meier survival analysis is as observed in figure 5.3-5.8.

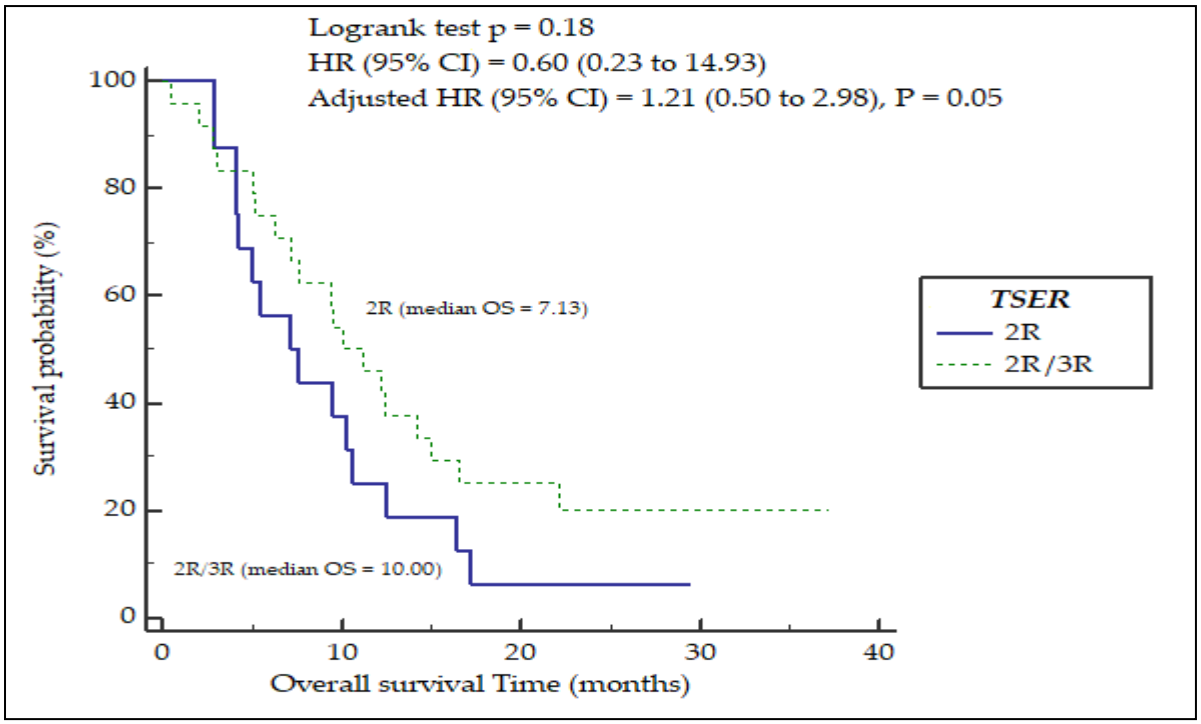

Figure 5.6. Kaplan-Meier survival curve illustrating effect of *TSER* rs34743033 polymorphism on overall survival of ADCC patients with heterozygous genotype (2R/3R vs. 2R)

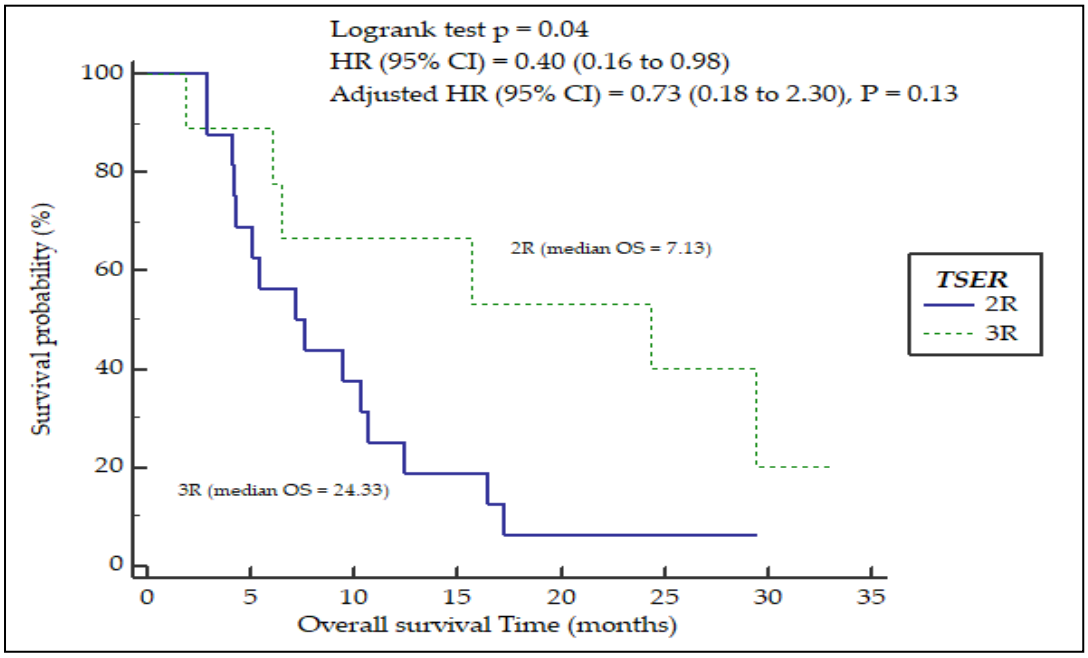

Figure 5.7. Kaplan-Meier survival curve illustrating effect of *TSER* rs34743033 polymorphism on overall survival of ADCC patients with mutant genotype (3R vs. 2R)

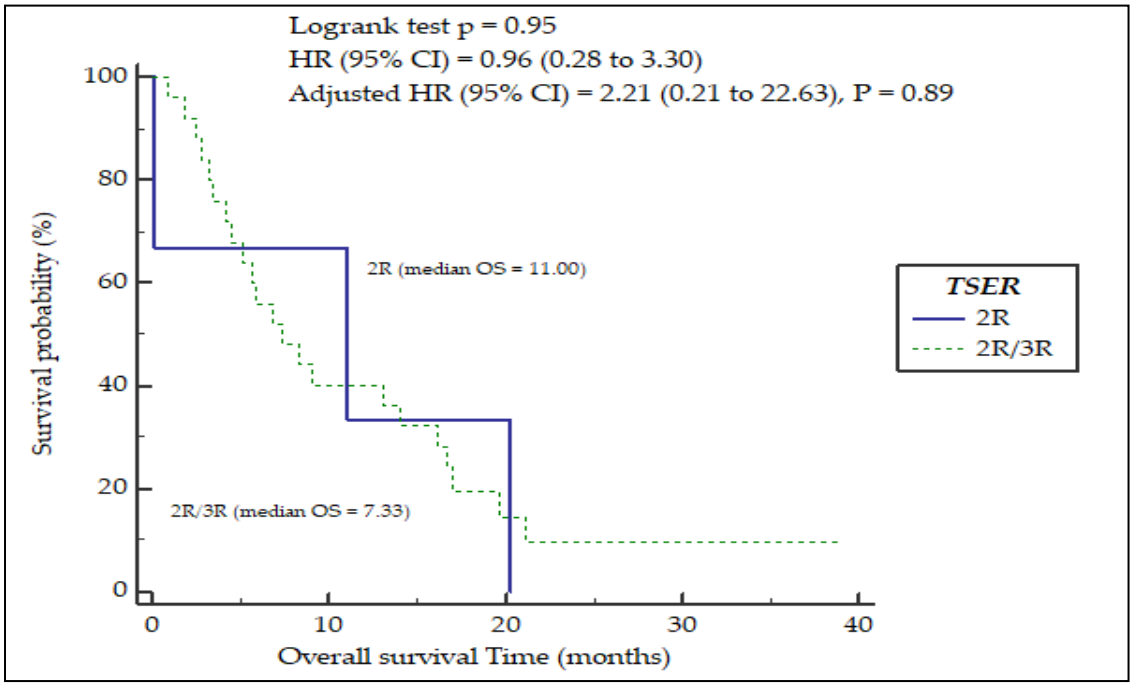

Figure 5.8. Kaplan-Meier survival curve illustrating effect of *TSER* rs34743033 polymorphism on overall survival of SCLC patients with heterozygous genotype (2R/3R vs. 2R)

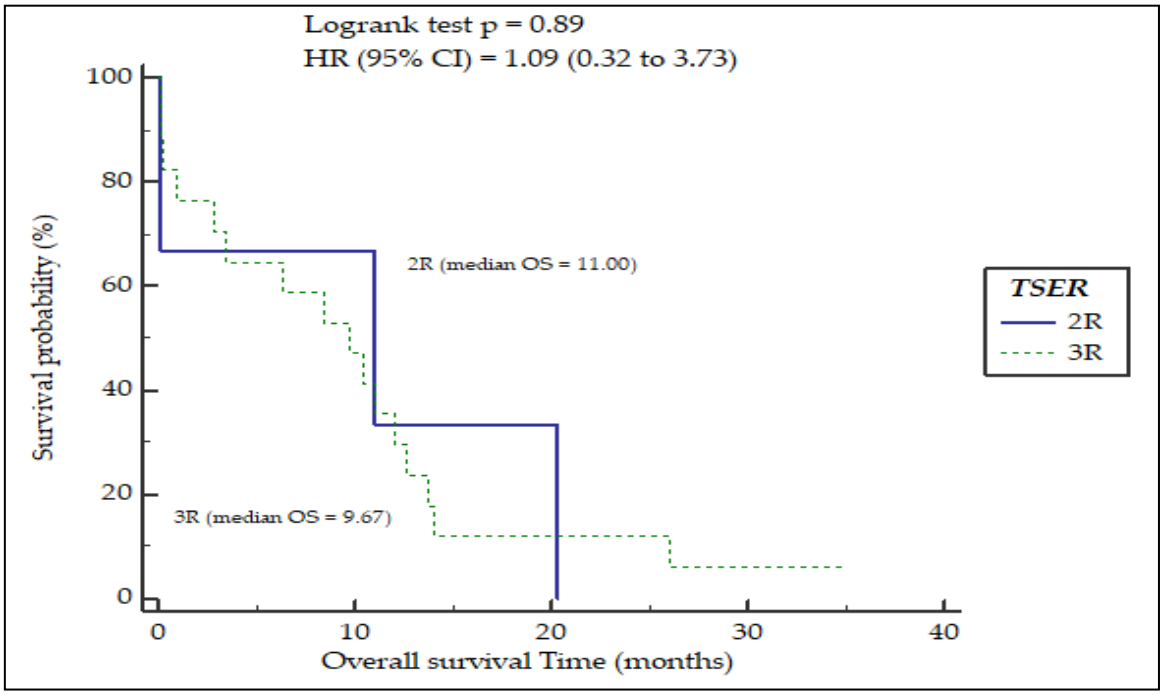

Figure 5.9. Kaplan-Meier survival curve illustrating effect of *TSER* rs34743033 polymorphism on overall survival of SCLC patients with mutant genotype (3R vs. 2R)

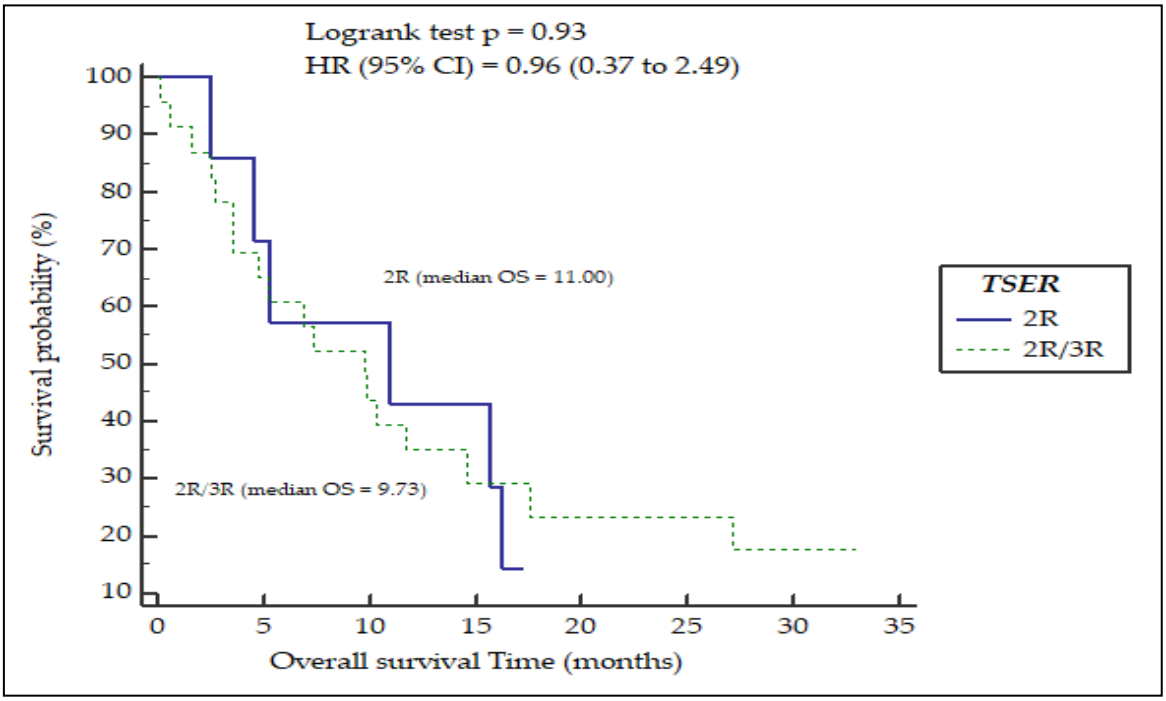

Figure 5.10. Kaplan-Meier survival curve illustrating effect of *TSER* rs34743033 polymorphism on overall survival of SQCC patients with heterozygous genotype (2R/3R vs.2R)

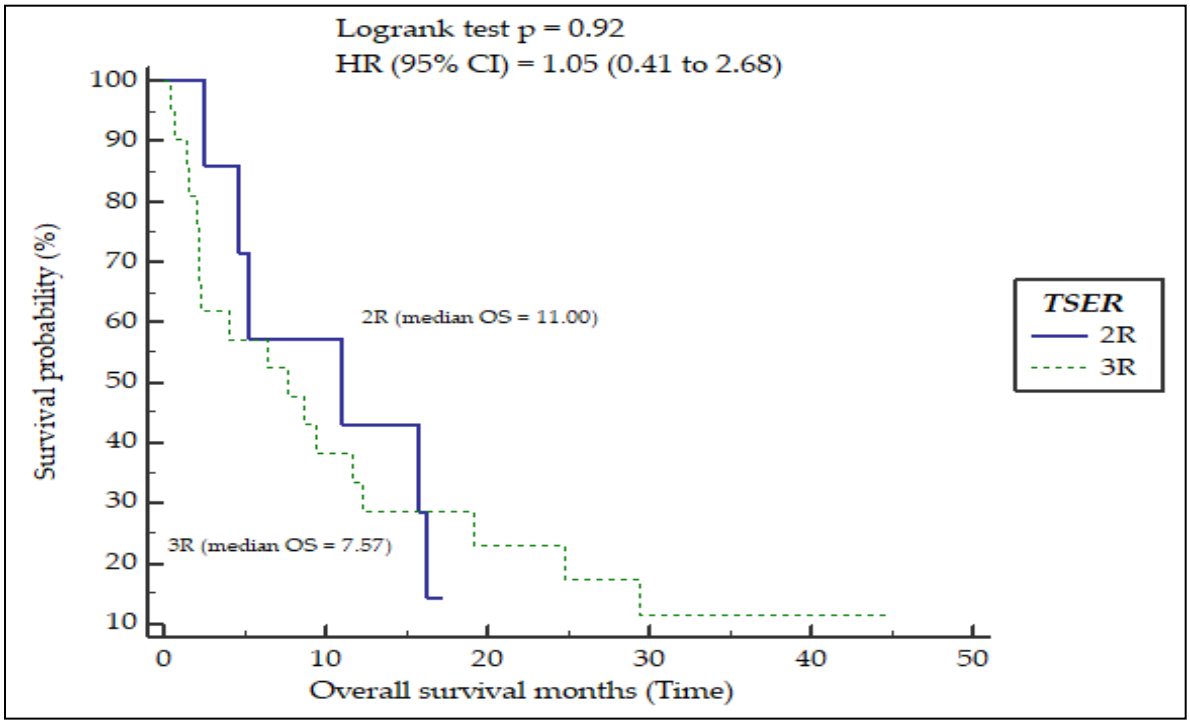

Figure 5.11. Kaplan-Meier survival curve illustrating effect of *TSER* rs34743033 polymorphism on overall survival of SQCC patients with mutant type (3R vs. 2R)

5.6. Association of *TSER* rs34743033 polymorphism genotypic distribution with overall survival of lung cancer patients based on gender

Table 5.4. Genotypic distribution and relationship of *TSER* rs34743033 polymorphism with overall survival of lung cancer patients on the basis of gender

MALE							
***TSER* rs34743033 genotype**	**Dead (102)**	**Alive (22)**	**Median OS months**	**HR (95% CI)**	**Log p**	**Adjusted HR (95% CI)**	**P**
2R	19	1	7.60	1.0 (Reference)	-	1.0 (Reference)	-
2R/3R	50	14	10.00	0.60 (0.33 to 0.2)	0.09	0.92 (0.49 to 1.94)	**0.02**
3R	33	7	9.33	0.70 (0.38 to 1.30)	0.27	0.74 (0.35 to 1.54)	0.85
2R/3R+3R	83	21	9.87	0.64 (0.36 to 1.13)	0.12	0.82 (0.46 to 1.47)	0.07

FEMALE							
TSER rs34743033 genotype	**Dead (24)**	**Alive (2)**	**Median OS months**	**HR (95% CI)**	**Log p**	**Adjusted HR (95% CI)**	**P**
2R	5	1	4.17	1.0 (Reference)	-	1.0 (Reference)	-
2R/3R	11	0	5.13	1.97 (0.69 to 5.65)	0.21	7.77 (0.50 to 121.73)	0.72
3R	8	1	13.70	1.16 (0.37 to 3.55)	0.80	2.43 (0.10 to 58.54)	**0.02**
2R/3R+3R	19	1	6.23	1.50 (0.60 to 3.72)	0.38	2.89 (0.59 to 14.05)	0.55
Hazard ratios (HR), 95% Confidence intervals (CI) and their respective p-values were estimated using Kaplan-Meier survival analysis after adjusted for remission and overall survival (months). Adjusted HR, 95% CI and their respective P-values were determined after adjusting age, ECOG, histology, KPS, pack years, smoking status and stage.							

Further classification was done on the basis of gender and their association was evaluated with various genotype combinations of *TSER* rs34743033 polymorphism. Table 5.4 summarizes the statistical values obtained by performing Kaplan-Meier survival analysis and Cox proportional hazard regression analysis. Of the 124 males, 82.26% were dead and 17.74% were alive. While in case of 26 females, 92.30% were dead and 7.70% were alive.

Homozygous wild genotype 2R of male patients had overall survival of 7.60 months which was taken as reference. Heterozygous genotype 2R/3R of male patients had overall survival of 10.00 months which is better than homozygous wild type (10.00 vs. 7.60 months, HR = 0.60, Logrank p = 0.09). Then mutant genotype 3R of male patients had overall survival of 9.33 months (9.33 vs. 7.60 months, HR = 0.70, Logrank p = 0.27). The combined genotype 2R/3R+3R of male patients had overall survival of 9.87 months (9.87 vs. 7.60 months, HR = 0.64, Logrank p = 0.12). Homozygous wild genotype 2R of female patients had overall survival of 4.17 months which was taken

as reference. Heterozygous genotype 2R/3R had overall survival of 5.13 months (5.13 vs. 4.17 months, HR = 1.97, Logrank p = 0.21). Then mutant genotype 3R had overall survival of 13.70 months (13.70 vs. 4.17 months, HR = 1.16, Logrank p = 0.80) which is far better than homozygous wild and heterozygous genotype.

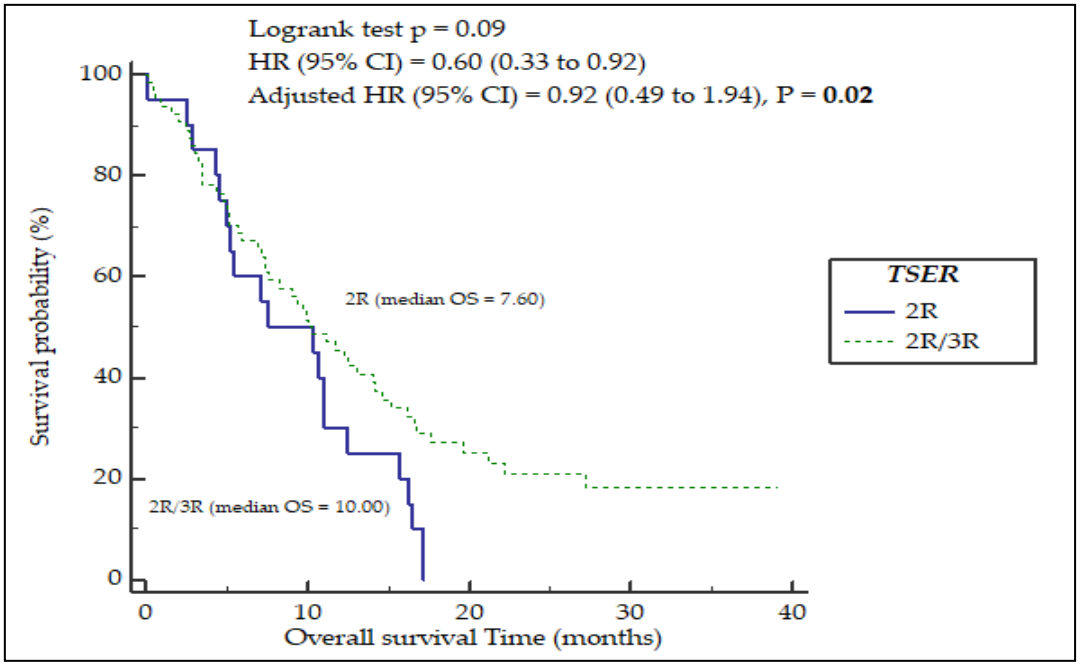

Figure 5.12. Kaplan-Meier survival curve illustrates Effect of *TSER* rs34743033 polymorphism on overall survival of lung cancer patients in male population with heterozygous genotype (2R/3R vs. 2R)

On combining the heterozygous and mutant genotype, it has overall survival of 6.23 months (6.23 vs. 4.17 months, HR = 1.50, Logrank p = 0.38), still better than homozygous wild genotype.

However, significant results were obtained in heterozygous genotype (**0.02**) of male and in mutant genotype (**0.02**) of female for *TSER* rs34743033 polymorphism as mentioned in table 5.4.

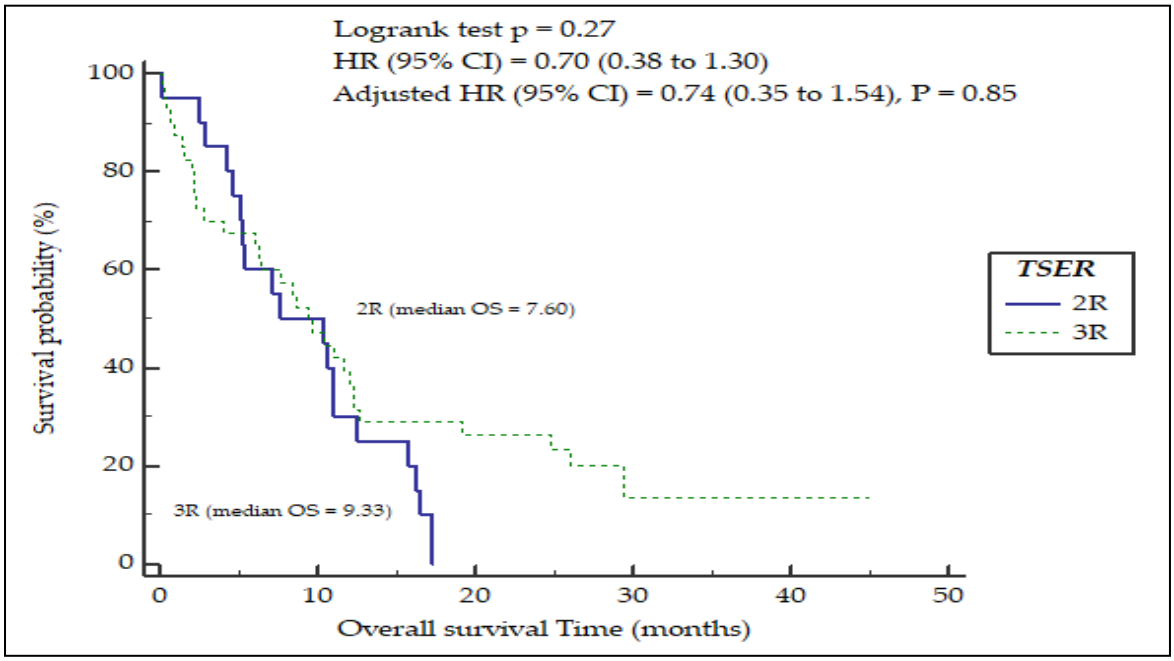

Figure 5.13. Kaplan-Meier survival curve illustrates Effect of *TSER* rs34743033 polymorphism on overall survival of lung cancer patients in male population with mutant genotype (3R vs. 2R)

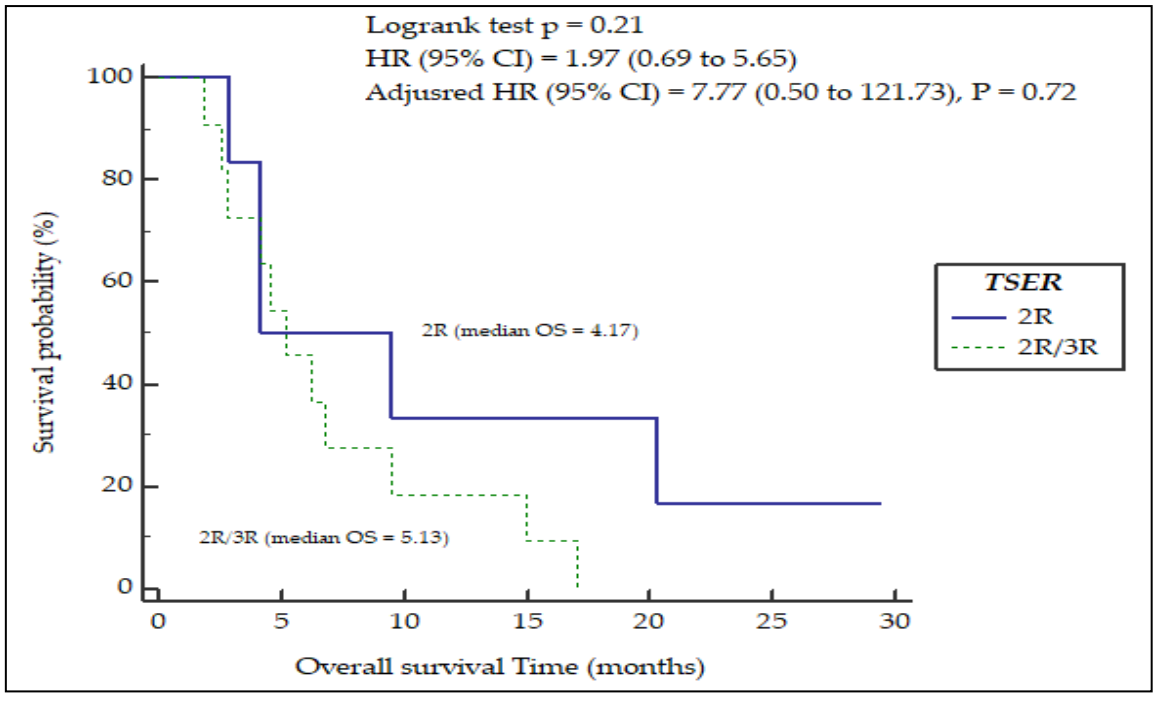

Figure 5.14. Kaplan-Meier survival curve illustrates Effect of *TSER* rs34743033 polymorphism on overall survival of lung cancer patients in female population with heterozygous genotype (2R/3R vs. 2R)

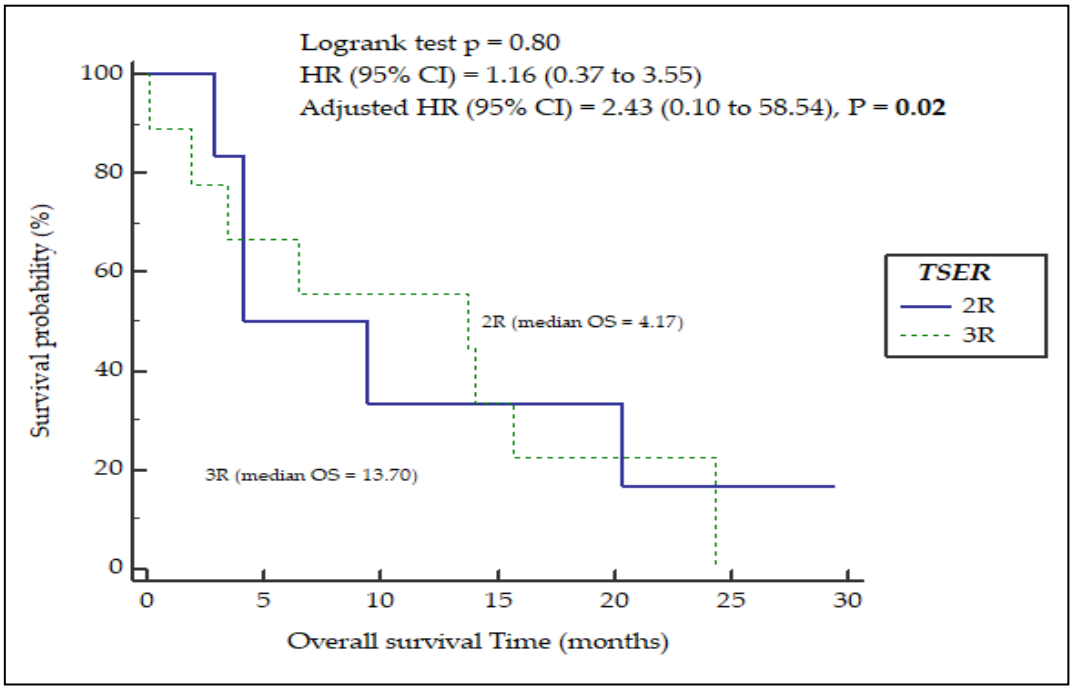

Figure 5.15. Kaplan-Meier survival curve illustrates Effect of *TSER* rs34743033 polymorphism on overall survival of lung cancer patients in female population with mutant genotype (3R vs. 2R)

5.7. Association of *TSER* rs34743033 polymorphism genotypic distribution with overall survival of lung cancer patients based on smoking status

Table 5.5. Genotypic distribution and relationship of *TSER* rs34743033 polymorphism with overall survival of lung cancer patients on the basis of smoking status

SMOKERS							
***TSER* rs34743033 genotype**	**Dead (104)**	**Alive (18)**	**Median OS months**	**HR (95% CI)**	**Log p**	**Adjusted HR (95% CI)**	**P**
2R	17	1	7.60	1.0 (Reference)	-	1.0 (Reference)	-
2R/3R	49	12	9.87	0.70 (0.38 to 1.29)	0.25	1.21 (0.62 to 2.36)	0.07
3R	38	6	8.63	0.81 (0.44 to 1.50)	0.52	0.86 (0.44 to 1.70)	0.90
2R/3R+3R	87	18	9.67	0.75	0.32	0.99	0.15

				(0.42 to 1.33)		(0.55 to 1.81)	
NON-SMOKERS							
***TSER* rs34743033 genotype**	**Dead (22)**	**Alive (5)**	**Median OS months**	**HR (95% CI)**	**Log p**	**Adjusted HR (95% CI)**	**P**
2R	7	1	4.17	1.0 (Reference)	-	1.0 (Reference)	-
2R/3R	12	2	6.23	0.87 (0.33 to 2.26)	0.77		
3R	3	2	24.33	0.38 (0.10 to 1.35)	0.13		
2R/3R+3R	15	4	9.47	0.65 (0.24 to 1.75)	0.40		
Hazard ratio (HR), 95% Confidence intervals (CI) and their respective p-values were estimated using Kaplan-Meier survival analysis after adjusting for remission and overall survival (months). Adjusted HR and 95% CI and their respective P-values were determined after adjusting for age, ECOG, KPS, histology, smoking status, pack years and stage.							

The subjects enlisted for the association study of overall survival of lung cancer patients with genotypic distribution were further categorized on the basis of smoking status as smokers and non-smokers. Of 150 cases recruited for *TSER* rs34743033 polymorphism, 82.00% were smokers and 18.00% were non-smokers. Smoking status can be calculated on the basis of pack years (beedi/cigarette) smoked per day by using the following formula;

$$\text{Pack years} = \frac{\textit{no. of cigarettes/bidis smoked per day} * \textit{no. of years smoked}}{20}$$

Table 5.5 summarizes the statistical values of the association between overall survival of lung cancer patients on the basis of smoking status with genotypic distribution for *TSER* rs34743033 polymorphism. Among smokers, the homozygous wild genotype 2R, showcasing a survival period of 7.60 months in which 84.55% were dead and 5.26% were alive and it was taken as reference. Presence of heterozygous genotype

2R/3R had increased OS than homozygous wild genotype (9.87 vs. 7.60 months, HR = 0.70, Logrank p = 0.25, Adjusted HR = 1.21 and P = 0.07). The mutant genotype 3R had overall survival rate of 8.63 months (8.63 vs. 7.60 months, HR = 0.81. Logrank p = 0.52, Adjusted HR = 0.86, P = 0.90) which is also better than homozygous wild genotype. On combining the heterozygous and mutant genotype, OS showed better results than homozygous wild and mutant type. It had OS of 9.67 months (9.67 vs. 7.60 months, HR = 0.75, Logrank p = 0.52, Adjusted HR = 0.99, P = 0.15).

Among non-smokers, 81.49% patients were dead and 18.51% patients were alive. The homozygous wild genotype 2R had survival period of 4.17 months which was taken as reference. The heterozygous genotype 2R/3R had OS more than homozygous wild genotype i.e., 6.23 months (6.23 vs. 4.17 months, HR = 0.87, Logrank p = 0.77). The mutant genotype 3R had much more overall survival rate of 24.33 months (24.33 vs. 4.17 months, HR = 0.38, Logrank p = 0.13). On combining the heterozygous and mutant genotype, the OS had increased to 9.47 months which is higher than homozygous wild and heterozygous genotype.

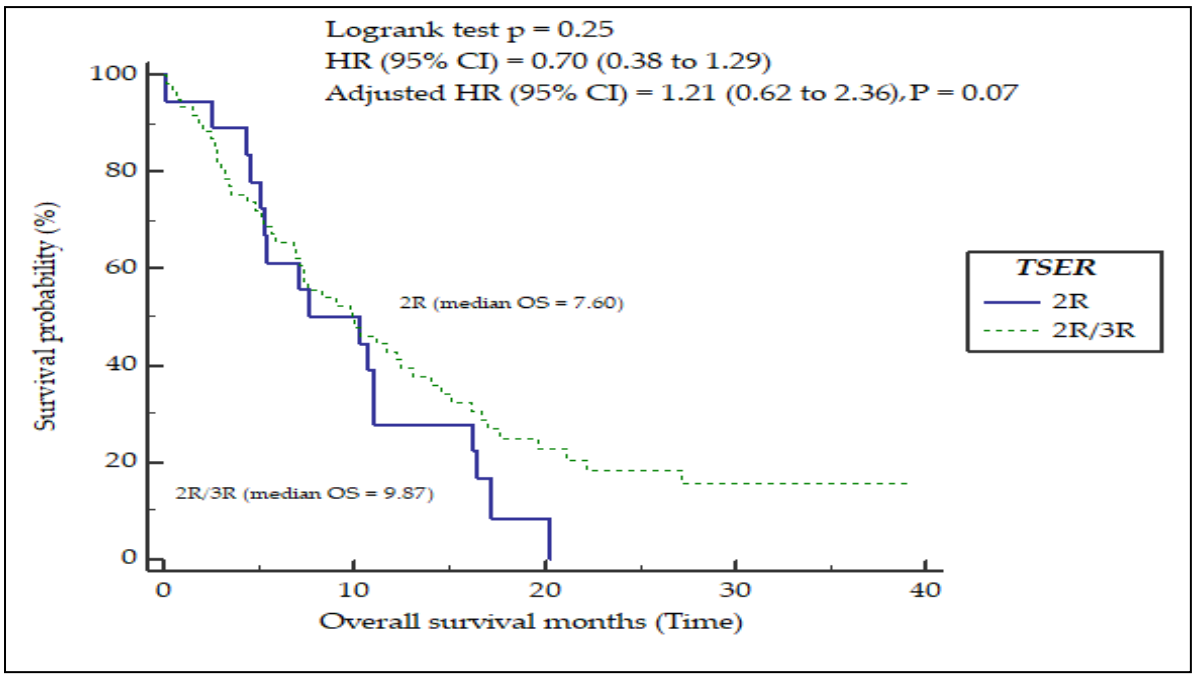

Figure 5.16. Kaplan-Meier survival curve illustrates Effect of *TSER* rs34743033

polymorphism on overall survival of lung cancer patients in smokers with heterozygous genotype (2R/3R vs. 2R)

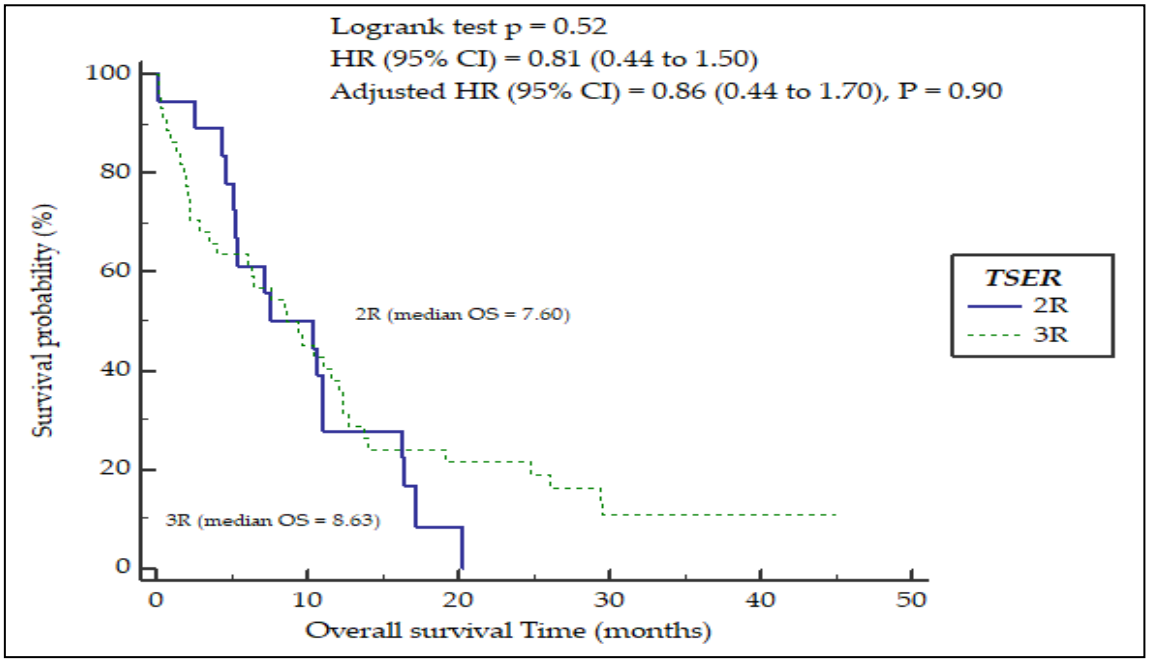

Figure 5.17. Kaplan-Meier survival curve illustrates Effect of *TSER* rs34743033 polymorphism on overall survival of lung cancer patients in smokers with mutant genotype (3R vs. 2R)

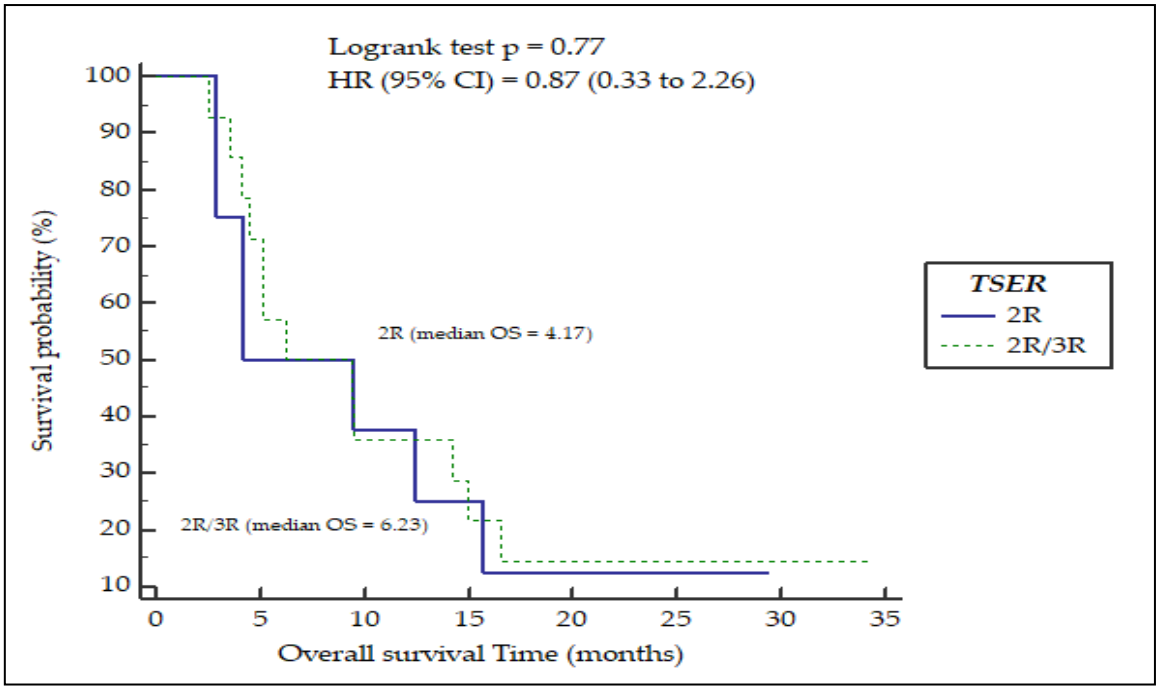

Figure 5.18. Kaplan-Meier survival curve illustrates Effect of *TSER* rs34743033 polymorphism on overall survival of lung cancer patients in non-smokers with heterozygous genotype (2R/3R vs. 2R)

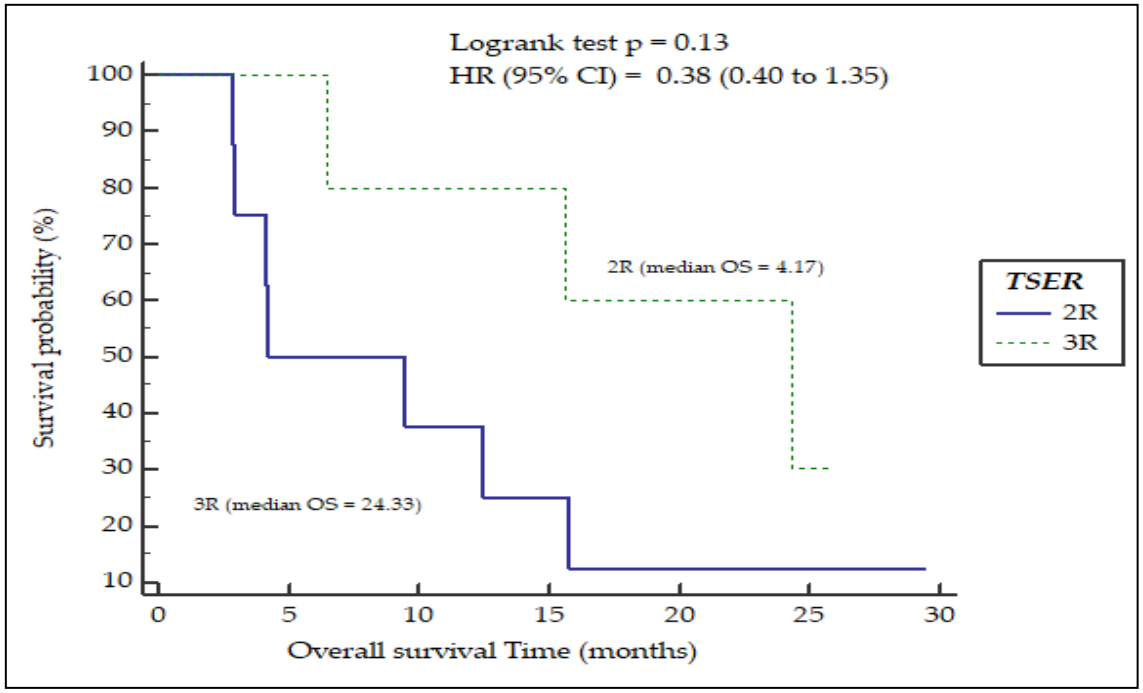

Figure 5.19. Kaplan-Meier survival curve illustrates Effect of *TSER* rs34743033 polymorphism on overall survival of lung cancer patients in non-smokers with mutant genotype (3R vs. 2R)

5.8. Association of *TSER* rs34743033 polymorphism genotypic distribution with overall survival of lung cancer patients on the basis of different regimen

Table 5.6. Genotypic distribution and relationship of *TSER* rs34743033 polymorphism with overall survival of lung cancer patients on the basis of regimen

Docetaxel-plus-Carboplatin/Cisplatin (Regimen 1)							
***TSER* rs34743033 genotype**	**Dead (33)**	**Alive (6)**	**Median OS months**	**HR (95% CI)**	**Log p**	**Adjusted HR (95% CI)**	**P**
2R	8	1	5.07	1.0 (Reference)	-	1.0 (Reference)	-

2R/3R	10	3	8.27	1.06 (0.35 to 3.23)	0.92	3.30 (0.37 to 29.80)	0.55
3R	15	2	12.63	0.96 (0.28 to 3.25)	0.95	7.87 (3.40 to 20.52)	**0.0008**
2R/3R+3R	25	5	9.00	1.02 (0.35 to 2.98)	0.97	3.74 (0.78 to 18.11)	0.09
Irinotecan-plus-Carboplatin/Cisplatin (Regimen 5)							
***TSER* rs34743033 genotype**	**Dead (22)**	**Alive (5)**	**Median OS months**	**HR (95% CI)**	**Log p**	**Adjusted HR (95% CI)**	**P**
2R	2	0	5.27	1.0 (Reference)	-	1.0 (Reference)	-
2R/3R	13	3	7.13	0.35 (0.05 to 2.08)	0.25		
3R	7	2	6.30	0.70 (0.16 to 3.03)	0.63	0.12 (0.00 to 4.18)	0.42
2R/3R+3R	20	5	6.30	0.54 (0.12 to 2.47)	0.42	0.39 (0.05 to 2.66)	**0.01**
Pemetrexed-plus-Carboplatin/Cisplatin (Regimen 6)							
TSER rs34743033 genotype	**Dead (28)**	**Alive (6)**	**Median OS months**	**HR (95% CI)**	**Log p**	**Adjusted HR (95% CI)**	**P**
2R	10	1	4.17	1.0 (Reference)	-	1.0 (Reference)	-
2R/3R	12	4	7.57	0.25 (0.05 to 1.22)	0.08	0.09 (0.00 to 2.77)	0.30
3R	6	1	7.57	0.45 (0.10 to 1.97)	0.29	0.28 (0.01 to 6.17)	0.63
2R/3R+3R	18	5	7.57	0.30 (0.06 to 1.34)	0.11	0.14 (0.01 to 1.55)	0.40

Hazard ratios (HR), 95% Confidence Interval (CI) and their respective p-values were estimated using Kaplan-Meier survival analysis after adjusting for remission and overall survival (months). Adjusted HR, 95% CI and their respective P-values were determined after adjusting for age, gender, pack years, KPS, ECOG, smoking status and stage

In *TSER* rs34743033 genotype, among 39 patients treated with Docetaxel-plus-Carboplatin/Cisplatin (regimen 1), 84.61% were dead and 15.39% were alive. Based on genotypic distribution, it was observed that among individuals carrying homozygous wild (2R) genotype, 24.24% were dead and 3.03% were alive (MST = 5.07 months) which was taken as reference. Overall survival is bit higher in heterozygous (2R/3R) genotype (MST = 8.27, HR = 1.06, Logrank p = 0.55) and in mutant genotype the overall survival is quite higher than other genotypes (MST = 12.63, HR = 0,96, Logrank p = 0.95). And the combine genotype (2R/3R+3R) had overall survival is 9.00 months (MST = 9.00, HR = 1.02, Logrank p = 0.97). However, highly significant value was obtained with multivariate Cox proportional hazards regression analysis showcasing increased survival period in subjects with mutant genotype (MST = 12.63 months, HR = 0,96, P = **0.0008**)

Among 27 patients of Irinotecan-plus-Carboplatin/Cisplatin (regimen 5), 81.48% patients were dead and 18.52% patients were alive. Based on genotypic distribution, it was observed that among homozygous wild genotype, 9.09% were dead and no patient was alive (MST = 5.27 months). Overall survival was higher in heterozygous genotype (MST = 7.13 months, HR = 0.35, Logrank p = 0.25). Mutant genotype and combined genotype had same and decreased OS period (MST = 6.30 months). However, statistically significant value with increased survival period of subjects with combined genotype than subjects with wild genotype (MST = 6.30 months, HR = 0.54, P = **0.01**) was obtained with multivariate Cox proportional hazards regression analysis.

Among 34 patients of Pemetrexed-plus-Carboplatin/Cisplatin (regimen 6), 82.35% cases were dead and 17.65% cases were alive. Based on genotypic distribution it was observed that among individuals carrying homozygous wild type, 35.71% were dead and 16.67% were alive having MST = 4.17 months. Overall survival was same in rest

of the three genotypes (2R/3R, 3R, 2R/3R+3R) i.e., 7.57 months. However, no statistically significant values were obtained with multivariate Cox proportional hazard regression analysis thus denying the association between OS of lung cancer patients on the basis of regimen with genotypic distribution.

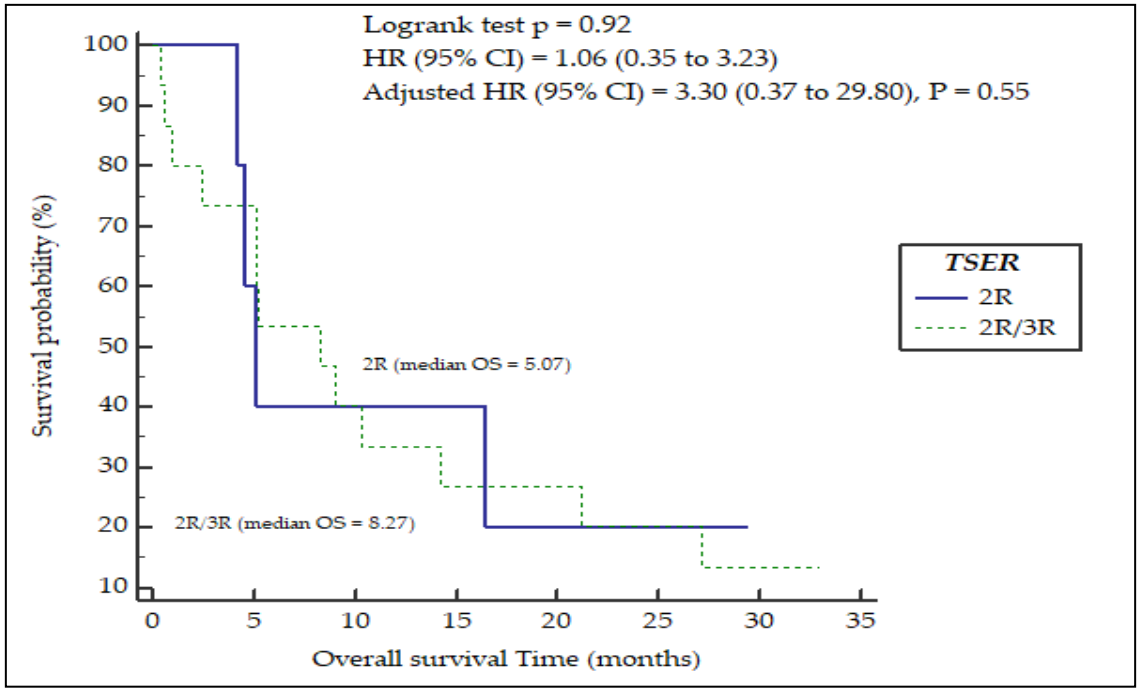

Figure 5.20. Kaplan-Meier survival curve illustrates Effect of *TSER* rs34743033 polymorphism on overall survival of lung cancer patients in regimen 1 with heterozygous genotype (2R/3R vs. 2R)

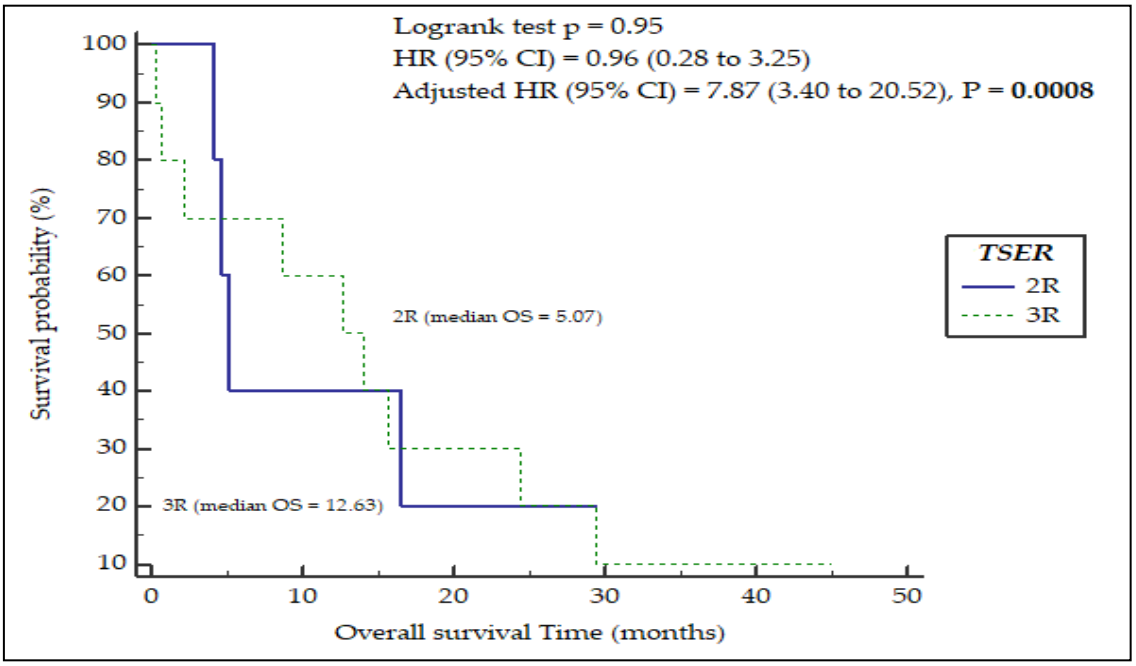

Figure 5.21. Kaplan-Meier survival curve illustrates Effect of *TSER* rs34743033 polymorphism on overall survival of lung cancer patients in regimen 1 with heterozygous genotype (3R vs. 2R)

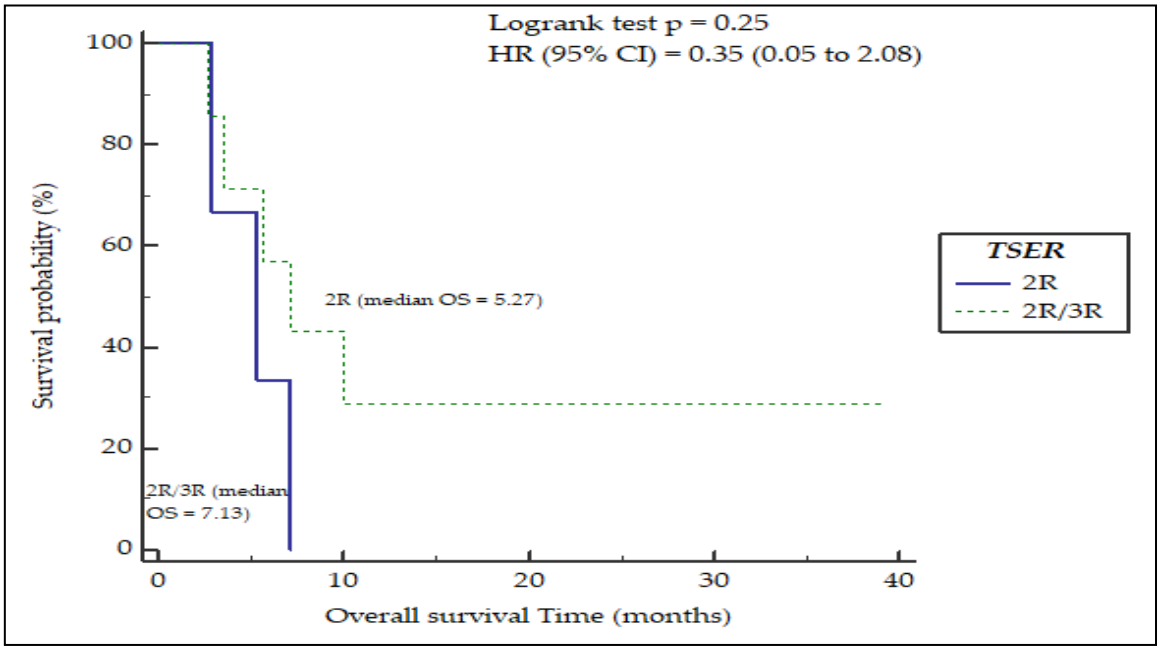

Figure 5.22. Kaplan-Meier survival curve illustrates Effect of *TSER* rs34743033 polymorphism on overall survival of lung cancer patients in regimen 5 with heterozygous genotype (2R/3R vs. 2R)

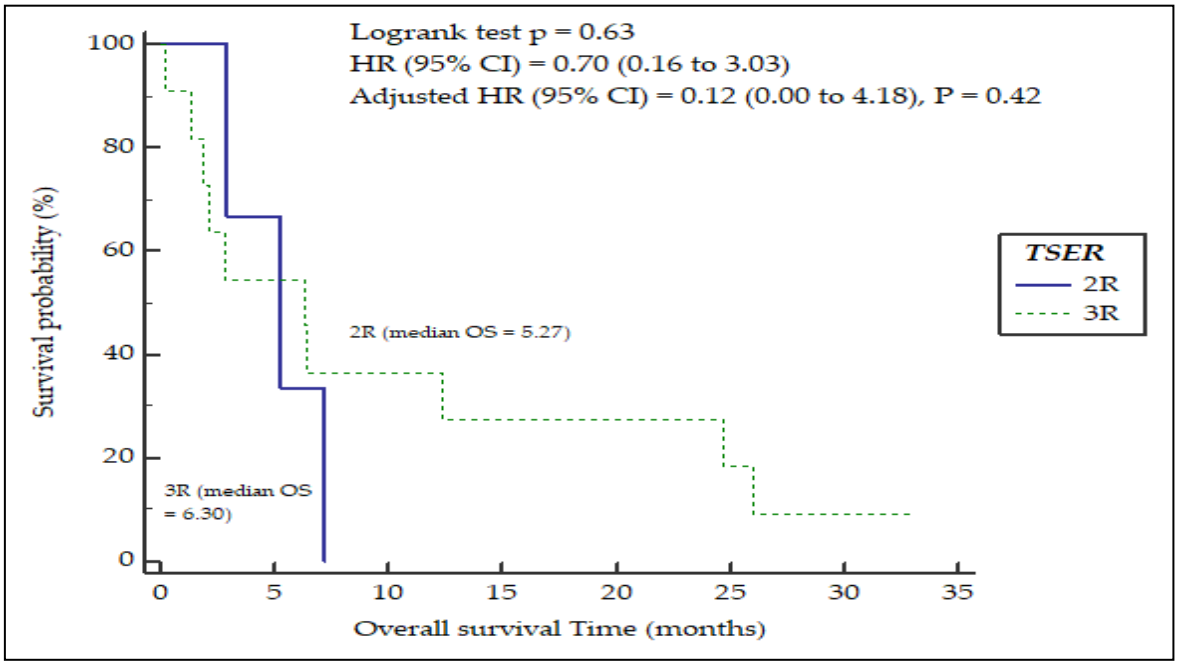

Figure 5.23. Kaplan-Meier survival curve illustrates Effect of *TSER* rs34743033 polymorphism on overall survival of lung cancer patients in regimen 5 with heterozygous genotype (3R vs. 2R)

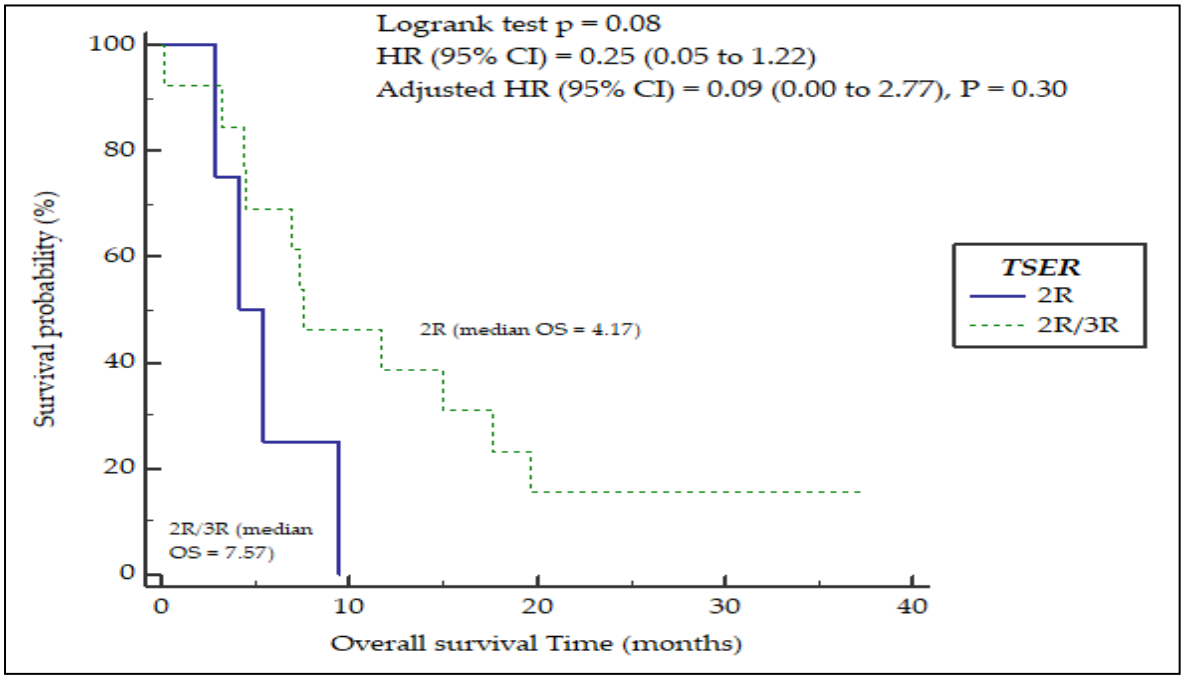

Figure 5.24. Kaplan-Meier survival curve illustrates Effect of *TSER* rs34743033 polymorphism on overall survival of lung cancer patients in regimen 6 with heterozygous genotype (2R/3R vs. 2R)

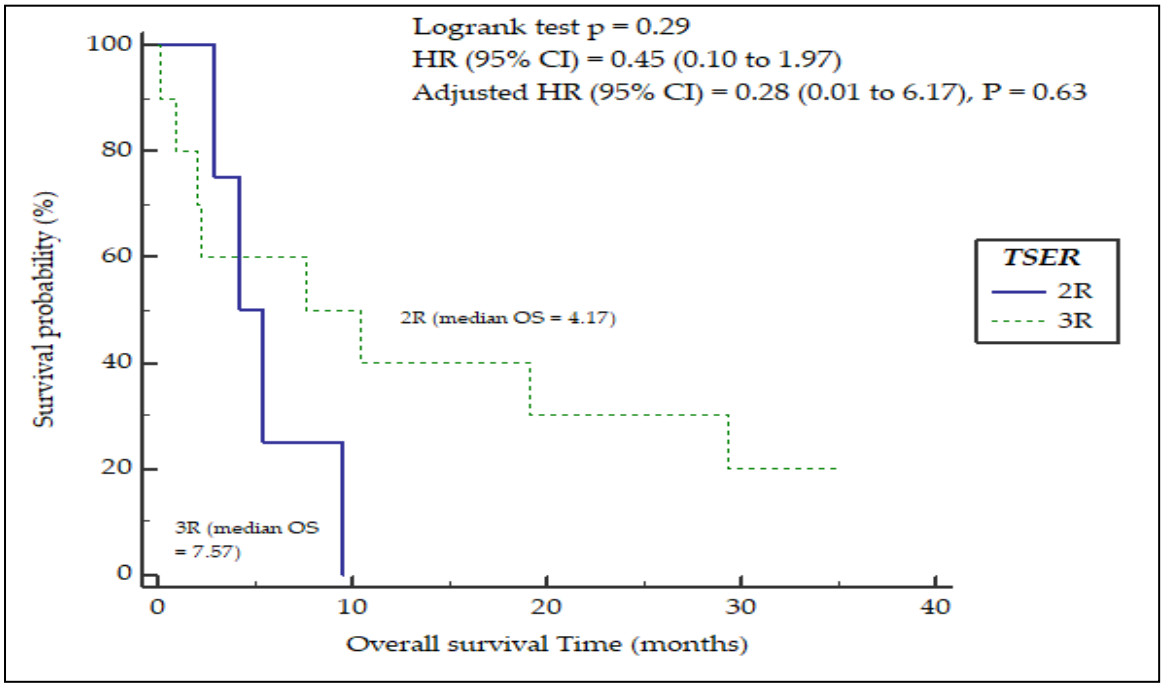

Figure 5.25. Kaplan-Meier survival curve illustrates Effect of *TSER* rs34743033 polymorphism on overall survival of lung cancer patients in regimen 6 with heterozygous genotype (3R vs. 2R)

5.9. Association of *TSER* rs34743033 polymorphism genotypic distribution with overall survival of lung cancer patients based on clinical stage

Table 5.7. Genotypic distribution of *TSER* rs34743033 polymorphism and risk associated with overall survival of lung cancer patients according to clinical stages of lung cancer

Genotype ***TSER*** **rs34743033**	**Clinical stage**		**AOR (95% CI)**	**P-value**
	Stage III N = 66 (n%)	**Stage IV N = 61 (n%)**		
2R	11 (16.66)	11 (18.03)	-	-
2R/3R	33 (50.00)	29 (47.54)	0.72 (0.25 to 2.12)	0.55
3R	22 (33.33)	21 (34.43)	1.40 (0.41 to 4.77)	0.60
2R/3R+3R	55 (83.33)	50 (81.97)	0.95 (0.35 to 2.60)	0.92

Calculated Adjusted Odds Ratio (AOR) and 95% CI by logistic regression analysis with *TSER* rs34743033 respectively, as reference group after adjusting for age, gender, histology and smoking status for clinical stage III and IV. OR > 1 implies that control arm is better than cases and OR < 1 implies that case arm is better than controls provided significance P-value

Table 5.7 demonstrates the statistical values obtained by logistic regression analysis to evaluate the risk associated with overall survival of lung cancer patients on stratification of groups according to clinical stage of the disease with genotype distribution for *TSER* rs34743033 polymorphism. The two group to be evaluated included patients from stage III and stage IV.

Out of 150 cases, 44.00% cases were at stage III and 40.67% cases were at stage IV. As mentioned in the table above, of the 66 cases of stage III, 16.66%, 50.00% and 33.33% were carriers of homozygous wild (2R), heterozygous (2R/3R), mutant (3R) and combine (2R/3R+3R) genotype, respectively. Whereas in case of 61 cases of stage IV, 18.03%, 47.54% and 34.43% were carriers of homozygous wild, heterozygous and mutant genotype, respectively. However, association between genotypic distribution and associated risk with overall survival according to clinical stage cannot be proved as no significant statistical values were obtained by regression logistic analysis.

5.10. Association of *TSER* rs34743033 polymorphism genotypic distribution with overall survival of lung cancer patients based on their Karnofsky Performance Status after receiving chemotherapy

Table 5.8. Genotypic distribution of TSER rs34743033 polymorphism and risk associated with overall survival of lung cancer patients according to KPS

Genotype	**KPS**			
TSER	**KPS 90-100**	**KPS <90**	**AOR (95% CI)**	**P-value**
rs34743033	**N = 57**	**N = 86**		

	(n%)	**(n%)**		
2R	8 (14.03)	16(1.86)	**-**	**-**
2R/3R	28 (49.12)	44 (51.16)	1.00 (0.35 to 2.90)	0.99
3R	21 (36.84)	26 (30.23)	0.49 (0.16 to 1.50)	0.21
2R/3R+3R	49 (85.96)	70 (81.39)	0.82 (0.38 to 1.79)	0.61
Calculated Adjusted Odds Ratio (AOR) and 95% CI by logistic regression analysis with *TSER* rs34743033 genotype respectively, as reference group after adjusting for age, gender, histology and smoking status for KPS. OR > 1 implies that control arm is better than cases and OR < 1 implies that case arm is better than controls				

Table 5.8 demonstrates the statistical values obtained by logistic regression analysis to evaluate the risk associated with overall survival of lung cancer patients on stratification of groups according to Karnofsky Performance Status of the patients with genotype distribution for polymorphism *TSER* rs34743033. The two groups to be evaluated included patient's KPS score 90-100 who are considered as individuals with good performance status and KPS score below 90 who are considered as patients with bad performance status.

Out of 150 cases, 38.00% cases were having KPS 90-100 and 57.33% were having KPS <90. In KPS 90-100, 14.03%, 49.12%, 36.84% and 85.96% of patients had homozygous wild (2R), heterozygous (2R/3R), mutant (3R) and combine (2R/3R+3R) genotype, respectively. Similarly, in KPS below 90, 1.86%, 51.16%, 30.23% and 81.39% of patients had homozygous wild, heterozygous, mutant and combine genotype, respectively. However, no significant statistical values were obtained by logistic regression analysis. Hence association between various genotypic combinations with

associated risk of overall survival in lung cancer patients on the basis of KPS score cannot be proved.

5.11. Association of *TSER* rs34743033 polymorphism genotypic distribution with overall survival of lung cancer patients based on their Eastern Cooperative Oncology Group after receiving chemotherapy

Table 5.9. Genotypic distribution of *TSER* rs34743033 polymorphism and risk associated with overall survival of lung cancer patients according to ECOG

Genotype *TSER* rs34743033	**ECOG**		**AOR (95% CI)**	**P-value**
	ECOG 0+1 N = 71 (n%)	**ECOG 2+3+4 N = 72 (n%)**		
2R	9 (12.68)	15 (20.83)	-	-
2R/3R	40 (56.33)	32 (44.44)	0.51 (0.19 to 1.40)	0.19
3R	22 (30.98)	25 (34.72)	0.93 (0.30 to 2.89)	0.90
2R/3R+3R	62 (87.32)	57 (79.16)	0.60 (0.23 to 1.54)	0.29

Calculated Adjusted Odds Ratio (AOR) and 95% CI by logistic regression analysis with *TSER* rs34743033 respectively, as reference group after adjusting for age, gender, histology and smoking status for ECOG. OR > 1 implies that control arm is better than cases and OR < 1 implies that case arm is better than control arm provided significant P-value

Table 5.9 demonstrates the statistical values obtained by logistic regression analysis to evaluate the risk associated with overall survival of lung cancer patients on stratification of groups according to performance status based on ECOG score of the patients with genotype distribution for polymorphism *TSER* rs34743033. The two groups to be evaluated included patient's ECOG value (0+1) in one group who are considered as individuals with good performance status and ECOG value (2+3+4) who are patients with declined performance status.

Out of 150 patients, 43.00% cases were having ECOG (0+1) and 48.00% cases were having ECOG (2+3+4). In ECOG (0+1), 12.68%, 56.33%, 30.98% and 87.32% of cases had homozygous wild (2R), heterozygous (2R/3R), mutant genotype (3R) and combine (2R/3R+3R) genotype, respectively. Similarly, in ECOG (2+3+4), 20.83%, 44.44%, 34.72% and 79.16% of cases had homozygous wild, heterozygous, mutant and combine genotype, respectively. Nevertheless, no significant statistical values were obtained by logistic regression analysis. Hence, association between various genotypic combinations with associated risk of overall survival in lung cancer patients on the basis of ECOG value cannot be proved.

5.12. Association of *TSER* rs34743033 polymorphism genotypic distribution with clinicopathological parameters

Table 5.10 demonstrates the statistical values obtained by logistic regression analysis to evaluate the risk associated with overall survival of lung cancer patients on stratification of groups according to tumor size and invasion extent based on genotype distribution for polymorphism TSER rs34733033. The two groups to be evaluated according to genotypic distribution included patients with Tumor (T0+T1+T2) in one group giving zero or minimal tumor growth and invasion of Tumor (T3+T4) in another group showcasing extensive tumor size growth and invasion.

Table 5.10. Genotypic distribution of *TSER* rs34743033 polymorphism and risk associated with overall survival of lung cancer patients according to tumor size and invasion

Genotype TSER rs34743033	**Tumor size and invasion**		**AOR (95% CI)**	**P-value**
	T0+T1+T2 N = 18 (n%)	**T3+T4 N = 113 (n%)**		
2R	1	21	-	-

	(5.55)	(18.58)		
2R/3R	8 (44.44)	58 (51.33)	0.20 (0.01 to 2.59)	0.21
3R	9 (50.00)	34 (30.09)	0.10 (0.00 to 1.12)	0.06
2R/3R+3R	17 (94.44)	92 (81.41)	0.18 (0.02 to 1.54)	0.12

Calculated Adjusted Odds Ratio (AOR) and 95% CI by logistic regression analysis with *TSER* rs34743033 respectively, as reference group after adjusting for age, gender, histology and smoking status for tumor size extent of invasion. OR > 1 implies that control arm is better than cases and OR < 1 implies that case arm is better than controls provided significant P-value

Out of 150 cases, 12.00% were cases having T0+T1+T2 and 75.33% were having T3+T4. In case of T0+T1+T2, 5.55%, 44.44%, 50.00% and 94.44% of cases had homozygous wild (2R), heterozygous (2R/3R), mutant (3R) and combined (2R/3R+3R) genotype, respectively. Similarly, in case of T3+T4, 18.58%, 51.33%, 30.09% and 81.41% of cases had homozygous wild, heterozygous, mutant genotype and combine genotype, respectively. No statistically significant values were obtained by logistic regression analysis again. Hence, association between various genotypic combinations with associated risk of overall survival in lung cancer patients on the basis of tumor size and extent of invasion cannot be proved.

Table 5.11. Genotypic distribution of *TSER* rs34743033 polymorphism and risk associated with overall survival of lung cancer patients according to lymph node involvement

Genotype *TSER* rs34743033	**Lymph node involvement**		**AOR (95% CI)**	**P-value**
	N0+N1 N = 87 (n%)	**N2+N3+N4 N = 43 (n%)**		
2R	14 (16.09)	09 (20.93)	-	-

2R/3R	42 (48.27)	21 (48.84)	0.82 (0.30 to 2.27)	0.70
3R	31 (35.63)	13 (30.23)	0.70 (0.22 to 2.28)	0.56
2R/3R+3R	73 (83.91)	34 (79.07)	0.78 (0.30 to 2.03)	0.60
Calculated Adjusted Odds Ratio (AOR) and 95% CI by logistic regression analysis with *TSER* rs34743033 genotype respectively, as reference group after adjusting for age, gender, histology and smoking status. OR > 1 implies that control arm is better than cases and OR < 1 implies that case arm is better than controls				

Table 5.11 demonstrates the statistical values obtained by logistic regression analysis to evaluate the risk associated with overall survival of lung cancer patients on stratification of groups according to lymph node involvement based on genotype distribution for polymorphism *TSER* rs34743033. The two groups to be evaluated included patients Node N0+N1 in one group showcasing absence or minimal lymph node involvement and Node N2+N3+N4 in another group showcasing distant lymph node involvement in another group.

Out of 150 cases, 58.00% were having N0+N1 and 28.67% were having N2+N3+N4. In N0+N1, 16.09%, 48.27, 35.63% and 83.91% of patients had homozygous wild (2R), heterozygous (2R/3R), mutant (3R) and combined (2R/3R+3R) genotype, respectively. In N2+N3+N4, 20.93%, 48.84%, 30.23% and 79.07% of patients had homozygous wild, heterozygous, mutant and combined genotype, respectively. No statistically significant values were obtained by logistic regression analysis again. Hence, association between various genotypic combinations with associated risk of overall survival in lung cancer patients on the basis of lymph node involvement cannot be proved.

Table 5.12. Genotypic distribution of *TSER* rs34743033 polymorphism and risk associated with overall survival of lung cancer according to metastatis

Genotype TSER rs34743033	**Metastatis**		**AOR (95%CI)**	**P-value**
	M0 N = 74 (n%)	**M1 N = 59 (n%)**		
2R	12 (16.21)	11 (18.64)	-	-
2R/3R	39 (52.70)	27 (45.76)	0.70 (0.22 to 2.15)	0.53
3R	23 (31.08)	21 (35.60)	1.14 (0.33 to 3.89)	0.84
2R/3R+3R	62 (83.78)	48 (81.35)	0.86 (0.30 to 2.43)	0.78

Calculated Adjusted Odds Ratio (AOR) and 95% CI by logistic regression analysis with *TSER* rs34743033 respectively, as reference group after adjusting for age, gender, histology and smoking status. OR > 1 implies that control arm is better than cases and OR < 1 implies that case arm is better than controls provided significant P-value

Table 5.12 demonstrates the statistical values obtained by logistic regression analysis to evaluate the risk associated with overall survival of lung cancer patients on classification of groups on the basis of extent of metastatis based on genotype distribution for polymorphism *TSER* rs34743033. The two groups to be evaluated included individuals with metastatis M0 in one group showcasing no metatsatis and metastatis M1 in another group showcasing distant metastatis.

Out of 150 cases, 49.33% were having M0 metastatis and 39.33% were having M1 metastatis. In case of M0 metastatis, 16.21%, 52.70%, 31.08% and 83.78% patients had homozygous wild (2R), heterozygous (2R/3R), mutant (3R) and combine (2R/3R+3R) genotype, respectively. Similarly, in case of M1 metastatis, 18.64%, 45.76%, 35.06% and 81.35% patients had homozygous wild, heterozygous, mutant and combined genotype, respectively. However, no statistically significant values

were obtained by logistic regression analysis again. Hence, association between various genotypic combinations with associated risk of lung cancer patients on the basis of extent of metastatis cannot be proved.

5.13. Association of *TSER* rs34743033 polymorphism genotypic distribution with lung cancer susceptibility on the basis of chemotherapy response

Table 5.13. Genotypic distribution of *TSER* rs34743033 polymorphism and risk associated with overall survival of lung cancer according to chemotherapy response

Genotype *TSER* rs34743033	**Chemotherapy Response**		**AOR (95% CI)**	**P-value**
	Responders CR/PR N = 57 (n%)	**Non-responders SD/PD N = 51 (n%)**		
2R	9(15.79)	9(17.65)	-	-
2R/3R	25(43.86)	24(47.06)	1.62 (0.45 to 5.80)	0.46
3R	23(40.35)	18(35.30)	1.19 (0.30 to 4.68)	0.81
2R/3R+3R	48(84.21)	42(82.35)	1.28 (0.40 to 4.05)	0.67

Calculated Adjusted Odds Ratio (AOR) and 95% CI by logistic regression analysis with *TSER* rs34743033 respectively, as reference group after adjusting for age, gender, smoking status for chemotherapy response. OR > 1 implies that control arm is better than cases and OR < 1 implies that case arm is better than controls provided significant P-value

Chemotherapy response by patients is considered as complete response (CR), partial response (PR), stable disease (SD) and progressive disease (PD). CR and PR are considered as good responders whereas SD and PD are considered as bad responders. Table 5.12 demonstrates the statistical values obtained by logistic regression analysis to evaluate the risk associated with overall survival of lung cancer

patients on classification of groups on the basis of chemotherapy response based on genotype distribution for polymorphism *TSER* rs34743033. The two groups to be evaluated included individuals with good responders (CR/PR) in one group and bad responders (SD/PD) in another group.

Out of 150 cases, 38.00% patients were having good response to chemotherapy and 34.00% were having bad response to chemotherapy. In case of good responders (CR/PR), 15.79%, 43.86%, 40.35% and 84.21% of patients had homozygous wild (2R), heterozygous (2R/3R), mutant (3R) and combined (2R/3R+3R) genotype, respectively. In case of bad responders (SD/PD), 17.65%, 47.06%, 35.30% and 82.35% of patients had homozygous wild, heterozygous, mutant and combined genotype, respectively. Hence, association between various genotypic combinations with associated risk of overall survival in lung cancer patients according to response towards chemotherapy cannot be proved.

6.0. Discussion

Thymidylate synthase is a key enzyme in folate metabolism that catalyzes the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) using 10-methylenetetrahydrofolate as a cofactor, providing the de novo source thymidine. Thymidine is a pyrimidine deoxynucleoside required for DNA replication, synthesis and repair. Polymorphisms in this gene may be associated with various cancers and this gene has prominent role in responding to chemotherapeutic agents (https://www.ncbi.nlm.nih.gov/gene/7298). In the present study, we studied the correlation of *TSER* rs34743033 polymorphism with overall survival of North Indian lung cancer patients, individually, on the basis of age, gender, histology, smoking status, stages, pack years, regimen, KPS and ECOG.

Various studies showed that expression of *TS* gene might play a role in developing cancer. Low expression level of TS expected to be better targeted by inhibitors but high levels induces tumor formation, which shows that *TS* gene is involved in causing cancer (Fernández-Contreras *et al*., 2009). The study conducted by Yim *et al*., (2010), the polymorphism of 2R/2R - 6bp ins/del combination showed poor prognosis and had higher risk of gastric cancer in Korean population but in our study 2R (homozygous wild genotype) allele does not show any risk to lung cancer. Some of the *in-vitro* and *in-vivo* studies have shown that 3R allele of *TSER* was associated with higher TS expression thus *TS* gene is responsible for causing cancer (Zhou *et al*., 2012) but in our study expression level of *TSER* was not demonstrated. Different populations have different effects of *TS* gene in different cancers, this may due to different risk factors. In our study, *TSER* gene is responsible for causing lung cancer in North Indian population, basically alleles of it are responsible and it depends on the patient's overall survival rate but according to Zhou *et al*., (2012) it was demonstrated that there is no association between cancer risk and *TSER* polymorphism in Caucasian population, reason behind this was different linkage patterns in different population or study cannot be interpreted because of small sample size. In the meta-analysis it

was observed that in Hungarian population, 2R/3R (heterozygous genotype) showed less susceptibility to colorectal cancer which correlates with our study. In Asian and Caucasian population, *TSER* had increase susceptibility to cancer, polymorphism may be due to genetic risk factors (Zhou *et al*., 2012).

Sulzyc-Bielicka *et al*., (2013) study demonstrated that different allelic frequency had different ethnicity. Homozygous repeats had 56% allelic frequency, heterozygous occurs in 47% in Polish population and in the present study homozygous wild type 2R is considered as reference for other two alleles and heterozygous contributes 50% of the allelic frequency and mutant genotype contributes 32.67%.

One of the challenges in cancer therapy is to optimize therapeutic options for individual patients because different histological features of cancer show different response to chemotherapy depending upon the tumor features. Pemetrexed, an antifolate drug, most effective inhibitor of *TS* gene for NSCLC patients. Pemetrexed based chemotherapy is the advanced chemotherapy among other chemotherapies. Pemetrexed drug is used based on histopathological types among other chemotherapeutic drugs as most of the therapeutic drugs are biomarkers based nowadays (Liu *et al*., 2013). Polymorphism in the 5'-UTR may result in different progressive and overall survival rate of NSCLC patients treated with pemetrexed. The response rate was higher in 2R/2R genotype which satisfy our results also. *TS* is a potential target gene for many drugs like pemetrexed, capecitabine, raltitrexed. Promoter enhancer region of *TS* gene is prone to polymorphism with two or three repeats in the 5'-UTR, it is the prognostic factor for NSCLC patients treated with pemetrexed, again satisfy our study (Li *et al*., 2013).

Another study conducted by Krawczyk *et al*., (2014), demonstrates that combination of pemetrexed and platinum compounds was registered as first line treatment of NSCLC patients. The patients had higher overall survival rate treated with cisplatin plus pemetrexed (11 months) than in cisplatin plus gemcitabine (10.1 months), but

our study does not correlates with it because the overall survival of patients treated with pemetrexed plus carboplatin/cisplatin based chemotherapy had 4.17 months when compared with docetaxel and irinotecan plus carboplatin/cisplatin based chemotherapy (5.07 and 5.27 OS rate in months). In phase III clinical trials, the death rate for smokers and squamous cell lung cancer patients is higher as compared to non smokers and non small cell lung cancer treated with pemetrexed plus cisplatin which justifies our results also. *TSER* polymorphism might lead to alterations in DNA biosynthesis and methylation, influences the chances of having cancer. 3R allele was related to higher transcription level than 2R allele (Qiao *et al*., 2017), yes it is true because our study also said that 3R allele is having the higher risk of causing lung cancer that 2R allele in North Indian population. According to the opinion of Pastorakova *et al*., (2017), *TSER* genotype was associated with TS protein expression. Cancer cells with 3R genotype had higher protein expression level than 2R/3R genotype and cancer cells with 2R genotype had lowest protein expression level in Slavic population of central Europe (Pastorakova *et al*., 2017). Our study somehow correlates with this study but our main motive is overall survival rate of lung cancer patients. This is also demonstrated that 3R had less overall survival in months than 2R/3R and 2R, similarly 3R genotype had significant risk of cancer than 2R/3R and 2R (Pastorakova *et al*., 2017). In Chinese population, Han *et al*., (2018) demonstrated that *TS* rs34743033 (2R and 2R/3R) genotypes generated poorer overall survival in gastric cancer who had received chemotherapy. In present study, 2R has overall survival rate of 7.60 months and 2R/3R has overall survival rate of 9.47 months in lung cancer patients.

7.0. CONCLUSION

The present case study pertains to patients visiting the Post Graduate Institute of Medical Education and Research (PGIMER), which is a referral center for patients from states like Punjab, Haryana, Uttar Pradesh, Himachal Pradesh, Chandigarh and Jammu & Kashmir. The following points are evident from the present study:

- Our study demonstrated that *TSER* rs347343033 genotype is an important factor contributing to overall survival and clinical outcomes of lung cancer patients in North Indian population.
- However, significant values were obtained in heterozygous (2R/3R) and combined (2R/3R+3R) genotype of overall survival data.
- Another significant value was obtained in gender, heterozygous genotype (male) was **0.02** with median overall survival (months) of 10.00 months.
- Highly significant value was obtained in Docetaxel-plus-Carboplatin/Cisplatin, **0.0008** with median overall survival of 12.63 months.

In conclusion, this study demonstrated that the polymorphisms in *TS* gene, contribute majorly in determining the various factors which affect the prognosis and treatment outcome of lung cancer patients in North Indian population. These genetic polymorphisms can be helpful for studying and framing future diagnostic and therapeutic tools.

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