Role of *DNMT3B* genetic polymorphism (rs1569686) in predicting overall survival and clinic-pathological outcomes in lung cancer patients treated with platinum based doublet chemotherapy

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IN

BIOTECHNOLOGY

Under the supervision of:

Siddharth Sharma, Ph.D

Associate Professor

Dr. Vikas Handa

Assistant Professor

Submitted by:

Manpreet Kaur

Roll No. 301501010

DEPARTMENT OF BIOTECHNOLOGY

THAPAR UNIVERSITY, PATIALA -147004

DECLARATION

I, the under designed, hereby declare that the research work presented in the Msc dissertation entitled **"Role of *DNMT3B* genetic polymorphism (rs1569686) in predicting overall survival and clinic-pathological outcomes in lung cancer patients treated with platinum based doublet chemotherapy"** has been carried out by me under the supervision and guidance of **Dr. Siddharth Sharma** (Associate Professor) and **Dr. Vikas Handa** (Assistant Professor) Department of Biotechnology, Thapar University, Patiala. Further, I declare that no part of this dissertation has been submitted for a degree or any other qualification of any university or examining body in India/ elsewhere.

Manpreet Kaur

Manpreet Kaur
Msc (Biotechnology)
301501010
Thapar University

Date: 17th July 2017
Place: Patiala

CERTIFICATE

This is to certify that dissertation entitled **"Role of *DNMT3B* genetic polymorphism (rs1569686) in predicting overall survival and clinic-pathological outcomes in lung cancer patients treated with platinum based doublet chemotherapy"** submitted by Ms. Manpreet Kaur in partial fulfillment of the requirements for the award of Msc in Biotechnology at Thapar University, Patiala is an authentic work carried out by her under our supervision and guidance.

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

Dr. Moushumi Ghosh
Head and Professor
Department of Biotechnology

Siddharth Sharma, Ph.D
Associate Professor
Dr. Vikas Handa
Assistant Professor
Department of Biotechnology

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Date: 17th July 2017
Place: Patiala

Manpreet Kaur

ABSTARCT

Title: Role of *DNMT3B* gene in predicting overall survival and clinic-pathological outcomes in lung cancer patients treated with doublet chemotherapy. DNA methylation is one of the important epigenetic mechanisms. Any alterations in the methylation mechanism can affect the process of transcription. Basically DNA methylation is a heritable marker which involves the covalent transfer of a methyl group to the C-5 position of the cytosine ring of DNA by DNA methyltransferases (*DNMTs*). *DNMT3B* is essential for the maintenance and establishment of DNA methylation patterns and required for genome-wide de-novo methylation during development.

Objectives: To investigate the role of *DNMT3B* (-579 G>T, rs1569686) polymorphism in modulating the overall survival and clinical outcomes of lung cancer patients.

Results: Lung cancer patients with this polymorphism of *DNMT3B* (-579G>T, rs1569686) found to have significant association in overall survival (HR'=1.45; 95% CI=1.02-2.08; P=0.03). Significant association was also found with overall survival in SCLC patients (HR'=16.27; 95%CI=1.13-232.4; P=0.04) and in SQCC patients (HR'=0.40; 95% CI=0.19-0.86; P=0.01).

Conclusion: Therefore, it is evident that this polymorphism is capable for predicting the overall survival of heterozygous patients and also, shows some interesting relationship of this genotype with overall survival of SCLC and SQCC patients.

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ABBREVIATIONS

PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
BSA	Bovine Serum Albumin
OR	Odds Ratio
OS	Overall Survival
CI	Confidence Interval
SNP	Single Nucleotide Polymorphism
NSCLC	Non-Small Cell Lung Cancer
SCLC	Small Cell Lung Cancer
LC	Lung Cancer
ADCC	Adenocarcinoma

SQCC Squamous Cell Carcinoma

SCLC Small Cell Lung Cancer

KPS Karnofsky Performance Status

ECOG Eastern Cooperative Oncology Group

rs Reference SNP

DNMT3B DNA methyltransferase 3B

TRD Target Recognition Domain

ICF Immunodeficiency, Centromeric heterochromatin instability and facial anomalies

THBS1 Thrombospondin-1

CDH-1 E-Cadherin

TIMP-3 Tissue inhibitor MP3

ER Estrogen receptor

AR Androgen receptor

CHAPTER-1

Introduction

Cancer is being considered as a serious public health issue. Development of cancer may result from inherited mutations in the germ line or from changes in DNA sequences arising in somatic tissues during life. In the development of cancer, genetic factors play an important role like DNA methylation (Lichtenstein *et al.,*2000). Epidemiological studies have contributed continuously to the developing familiarity with the significance of the hereditary and acquired susceptibility variables.

Lung cancer is the preeminent reason for death and is more common in men worldwide. Smoking is strongly associated with the increased risk of lung cancer. The ubiquitousness of lung cancer can be attributed to tobacco smoke and environmental pollutants. Cause of 80-90% of lung cancer is cigarette smoking which contains formaldehyde, acroline, benzene, PAH's, nitrosamines, acetaldehyde which are genotoxic carcinogens. In the environmental surroundings, these carcinogens interact with the tobacco smoke which increases the incidence of lung cancer. This analysis indicates that genetic differences may determine vulnerability to lung cancer. Every individual is differently susceptible to cancer by the complexity of exposure to carcinogen, number of multiple allele present for an enzyme to be encoded and most importantly the predisposed genes for the xenobiotic metabolism in the individual.

There are various genetic factors associated with lung cancer but tobacco smoking is the main leading cause of lung cancer. About 80% of deaths are caused by smoking and many others are caused due to exposure to secondhand smoking. Beside smoking, smokers also exposed to other risk factors such as radon and asbestos are at higher risk.

There are various studies stated that genetic polymorphisms are integrated with cancer risks, and various of them are suggested to use as markers biomarkers for cancer diagnosis. Changes in the DNA methylation patterns are one of the indications if lung cancer and CpG islands which are methylated are proposed to use as biomarkers for the early cancer detection and diagnosis.

Role of DNA Methylation in cancer development:

Carcinogenesis can also result from aberrations of genomic DNA methylation. DNA methylation includes hyper methylation and hypo methylation of promoter or first exon of

cancer related genes. Basically, the process of transfer of covalent methyl group to C-5 position of the cytosine ring of DNA by DNA methyltransferases (DNMTs) is known as DNA methylation which is an epigenetic marker.

Hyper methylation of promoter region of various tumor suppressor genes causes their transcriptional silencing. However hypo methylation of regulatory DNA sequences activates transcription of protooncogenes, reterotransposons, genes encoding proteins involved in genomic instability and malignant cell metastasis. Normally tumor suppressor genes (TSG) products are involved in holding cellular growth at the checkpoint and inhibit expression of the tumorigenic phenotype. Normal proliferation may inhibited by inactivation or loss of TSG products, which may result in malignant transformation.

DNMT3B gene is essential for the establishment of DNA methylation patterns and required for genome-wide de-novo methylation during development. DNA methylation is coordinated with methylation of histones. DNMT3B gene specially methylate nucleosomal DNA within the nucleosome core region. This gene is composed of 23 exons and 22 introns. In the embryonic stem cells, the enzyme is abundantly expressed, but upon differentiation of ES cells the expression of *DNMT3B* is decreased and it remains low in adult somatic tissues. However, various *DNMT3B* splice variants are over expressed that has been reported in tumor cells and suggesting that this enzyme is responsible for epigenetic modifications of DNA.

1.1 POLYMORPHISM:

In total 345 polymorphic sites have reported in DNMT3B gene till date. There are different polymorphisms found of this gene -243T>A, -149C>T and -579 G>T. DNMT3B -243T>A is a novel polymorphism (Gao *et al.,*2016).In case of increased promoter activity, DNMT3B -149 C>T polymorphism is highly associated. DNMT3B (-579 G>T, rs1569686) is localized in the DNMT3B gene promoter. There are two transcriptional start site of DNMT3B gene which are located in exon 1A and 1B. The expression of the gene is regulated by two promoters. One promoter is found in CpG-rich region and other is in CpG-poor region. This DNMT3B polymorphism is located in CpG-poor promoters which is 579 base pair away from exon 1B transcription site. This polymorphism affects the function of the gene but it did not affects transcriptional activity of the DNMT promoter.

As DNMT3B polymorphism (-579 G>T, rs1569686) has not been extensively evaluated for its role in prognosis, evaluation was only seen in a single study of gastric cancer.

However, many studies have focused on analysis the role of this polymorphism in susceptibility toward different cancers. So, in the present study, we are focusing to check the association of DNMT3B -579G>T polymorphism with overall survival and clinical outcomes.

CHAPTER-2

REVIEW OF LITERATURE

2.1 Lung Cancer

Lung cancer is a common type of malignancy that affects both males and Femaless and it becomes the main reason of deaths related to cancer worldwide (Molina *et al.*, 2008). Only 13% of the lung cancer patients survive more than 5 years. Classification of lung cancer is done according to histological types (fig. 2.1) and this classification has important inference for the clinical management and prognosis of the disease. According to histology, there are two main types of groups of lung cancer: Non-small Cell Lung Cancer (NSCLC) and Small Cell Lung Cancer (SCLC).

Approximately 85% of the lung cancers are of NSCLC, which further consists of 3 major histological subtypes: adenocarcinoma, large cell carcinoma and squamous cell carcinoma (fig.2.2) (Balgkouranidou *et al.,* 2013).

Lung carcinoma initiation and further progression is due to the interaction between genetic, epigenetic and environmental factors. Alterations in the epigenetics such as promoter DNA methylation leads to gene silencing are common events in lung cancer. After exposure to different environmental factors such as smoking, chronic inflammation, industrial substances, drugs and asbestos, these epigenetic alterations can take place in defined nuclear positions and chromosome domains (Baylin *et al.*, 2012). According to Epidemiological studies, cigarette smoking has strong association (Alberg *et al.*, 2007) with lung cancer because 80-90% of the lung cancers are attributable to cigarette smoking (Khuder *et al.*, 2001).

2.1.1 Symptoms of lung cancer

- Breathing difficulties like noisy breathing, wheezing or shortness of breath
- Persistent cough that does not go away
- Rust-colored sputum or coughing up blood
- Fatigue
- Weight loss
- Severe chest pain

2.1.2 Histology of Lung cancer

Classification of lung cancer is as follows:

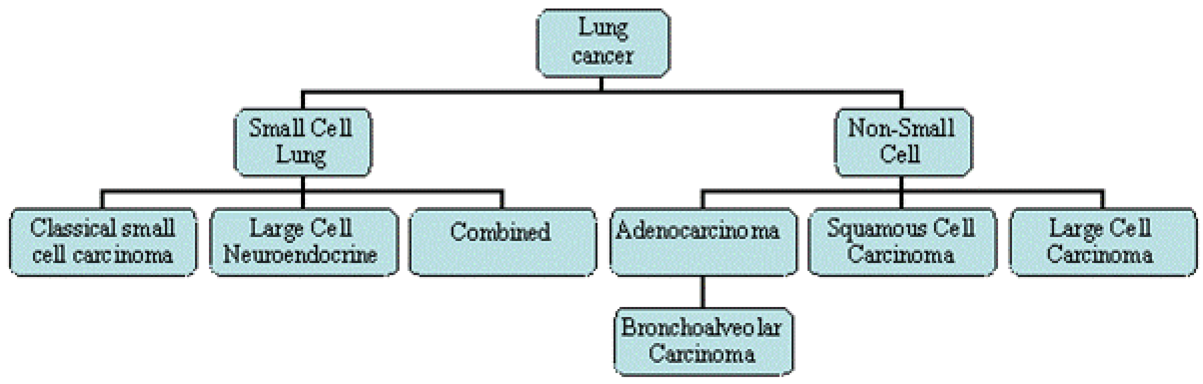

Figure 2.1 Lung Cancer Classification

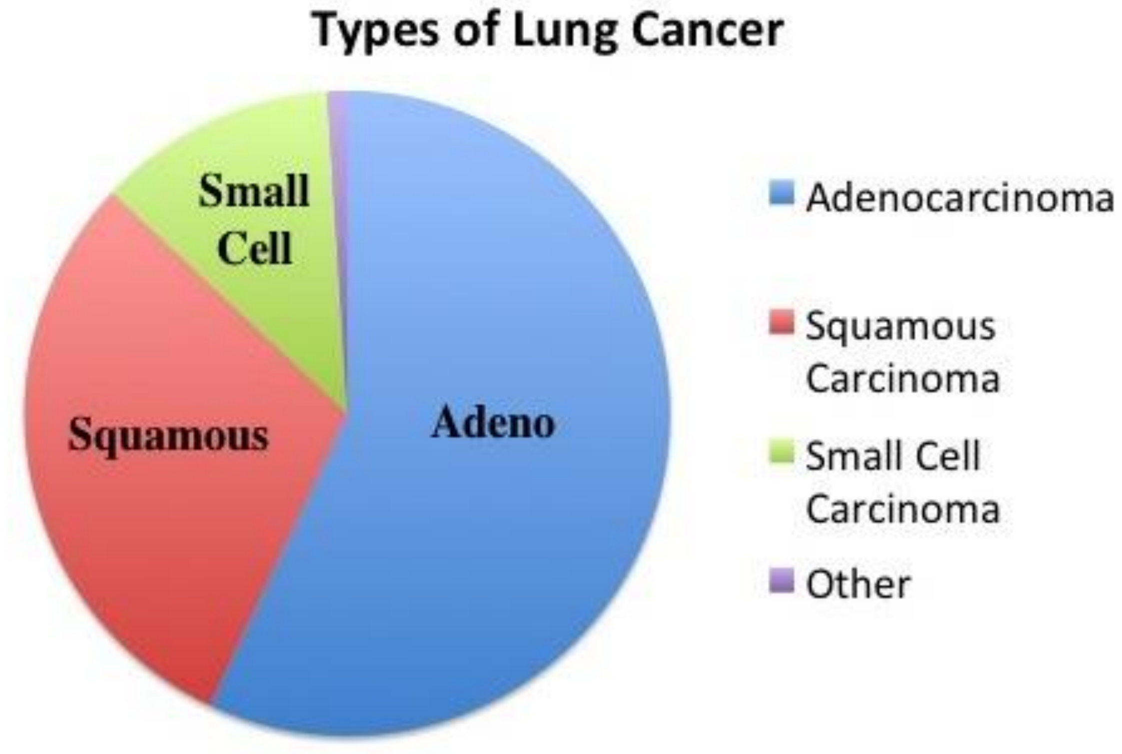

Figure 2.2 Histological subtypes of Lung Cancer (Balgkouranidou *et al.,* 2013).

2.2 Trends of lung cancer worldwide

In the year 2012, there were 14.1 million cancer cases around the world and out of these 7.4 million cases were in men and 6.7 million in women. In 2012, lung cancer was the most common cancer worldwide that contributed to 13% of the total number of new cases diagnosed while in India 6.9% of all new cancer cases diagnosed. A total of 1.8 million Cases were diagnosed in 2012. The principle cause of lung cancer is smoking and it is thought to be responsible for 85%of all types of this cancer. Arsenic present in drinking water and beta-carotene supplements increase the risk of lung cancer.

For both sexes, Hungary had the highest rate of lung cancer. In the less developed countries, about 58% of lung cancer cases occurred. In Northen America and in Europe, lung cancer was at its highest point and the lowest incidence in Caribbean, Latin America and in Africa. For men, In 2012, Hungary has the highest rate of lung cancer and then Armenia and FYR Macedonia. In less developed countries, there were 61% of lung cancer cases present. The highest rate of lung cancer was in Europe and Northen America and the lowest in Africa, Caribbean and Latin America.

For Women, Denmark had the highest incidence of lung cancer followed by USA and Canada. 54% of lung cancer cases occurred in less developed countries. More cases of lung cancer occurred in Northen America and in Oceania and less in Africa, Latin America and Caribbean.

Table 2.1 Estimated Incidence, Mortality and Prevalence Worldwide in 2012 (Globocan, 2012).				
Estimated Numbers (thousands)	**Men**		**Women**	
	Cases	**Deaths**	**Cases**	**Deaths**
World	1242	1099	583	491
More developed regions	490	470	268	210
Less developed regions	751	682	315	281
WHO Africa region (AFRO)	12	11	6	6
WHO Americas region (PAHO)	178	149	146	113
WHO East Mediterranean region (EMRO)	26	23	7	6
WHO Europe region (EURO)	323	283	126	105
WHO South-East Asia region (SEARO)	116	104	46	42
WHO Western Pacific region (WPRO)	588	528	251	220
United States of America	112	92	102	76
China	459	422	193	175
India	54	49	17	15

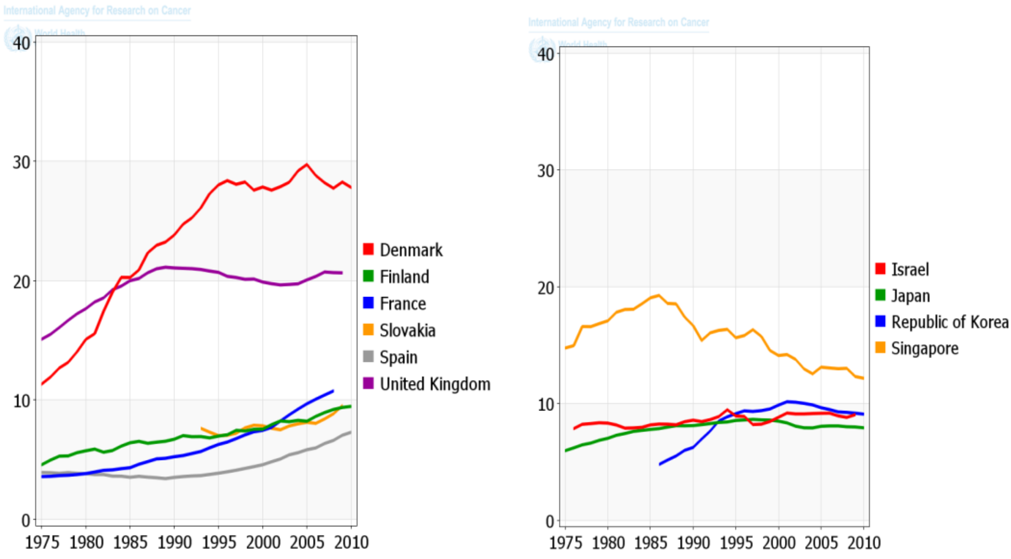

Figure 2.3 Trends in mortality from lung cancer in selected countries (Globacon, 2012).

2.3 Role of DNA methylation in lung cancer

DNMT3B gene is found in humans and mice that is important for the establishment of DNA methylation patterns in these organisms. It is also essential during development in these organisms for genome-wide de-novo methylation. In the mammalian genomic DNA, methylation is catalyzed by DNA methyltransferases (DNMTs).Expression of these enzymes is increased significantly in different types of cancers like colon cancer, breast cancer, prostate cancer, stomach cancer.

2.3.1 Structure: *Dnmt3b* gene consists of 23 exons, 22 introns this gene is located on chromosome 20q11.2. There are five different mRNA isoforms of *DNMT3B* after splitting the primary transcript of *DNMT3B*. These isoforms are:

- *DNMT3B1*
- *DNMT3B2*
- *DNMT3B3*
- *DNMT3B4*
- *DNMT3B5*

2.3.2 FUNCTIONS:

- DNA(cytosine-5) methyltransferase 3 β, abundantly expressed in embryonic stem cells in testis and thymus but rarely detectable in differentiated cells and adult

tissues, with at least two alternatively spliced isoforms essential for de-novo DNA methylation and development.

- This gene is required for the genome –wide de-novo methylation and is essential for the establishment of DNA methylation patterns during development.
- It may function as transcriptional co-repressor by associating with CBX4 and independently of DNA methylation.
- It also seems to be involved in gene silencing.
- This gene is association with *DNMT1*, involved in the activation of BAG1 gene expression by modulating dimethylation of promoter histone H3 at H3K4 and H3K9.
- Isoforms of 4 and 5 are probably not functional due to the deletion of two conserved methyltransferase motifs.

All the highly conserved motifs I, IV, VI, IX, X and target recognition domain sequence in the c terminal domain are present in the *DNMT3B*1 and *DNMT3B2* isoforms. Isoforms *DNMT3B2,* The last three isoforms of *DNMT3B* gene that is *DNMT3B3, DNMT3B4, DNMT3B5* do not contain 21-22, 21 or 22 exons respectively (fig. 2.4) (Luczak *et al*.,2006).

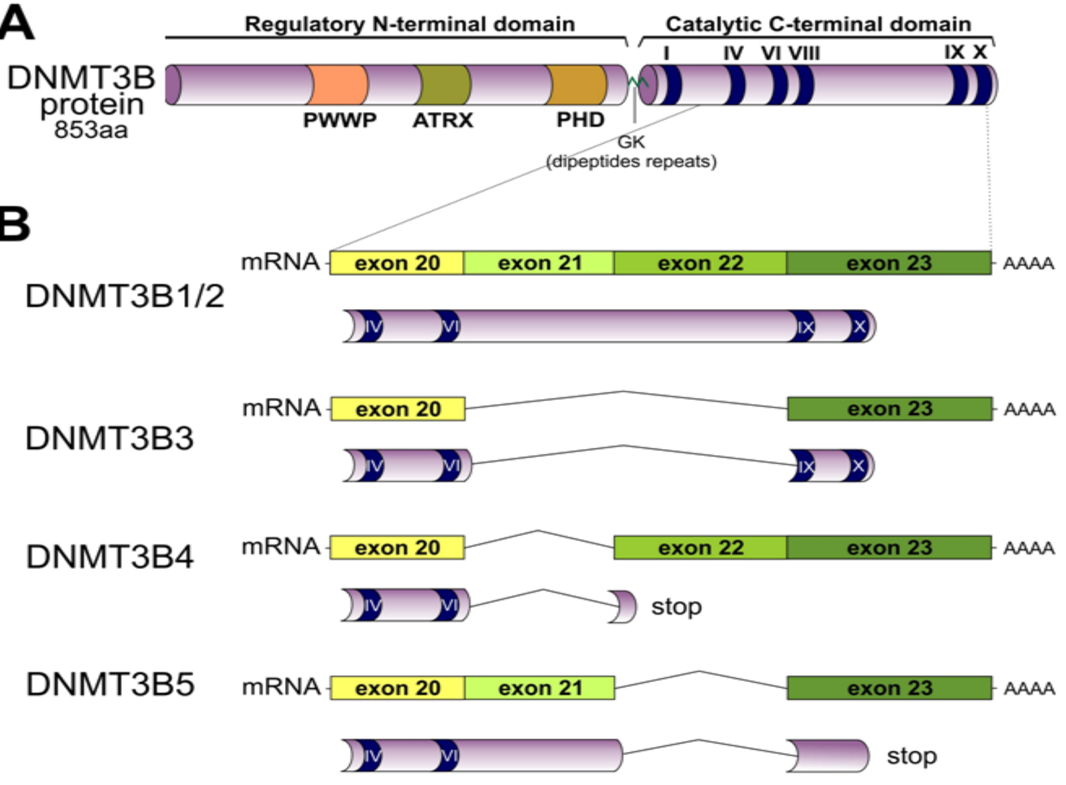

Figure 2.4 *DNMT3B* transcript is spliced into five different isoforms: *DNMT3B1, DNMT3B2*, *DNMT3B3*, *DNMT3B4*, *DNMT3B5,* and *DNMT3B5* (M.W. Luzak and P.P. Jagodzinki 2006)

Huntriss *et al*. and Weisenberger *et al*.,2004 revealed that transcript for *DNMT3B1* mRNA was only present in the embryonic stem cells and these were absent in differentiated somatic cells. This shows that different isoforms of *DNMT3B* are predominantly expressed in human somatic cells and malignant cells. *DNMT3B2* isoform of *DNMT3B* contains all the conserved motifs (I, IV, VI, and IX, X, TRD (Target Recognition Domain)). It is abundantly expressed in breast cancer cell line MCF-7. The third variant of *DNMT3B3* lacks the conserved motif VIII, TRD sequence and the nine amino acids of motif IX. This isoform of *DNMT3B* is enzymatically active and this effect was observed in LD419 fibroblasts and T24 bladder cancer cells. It has been reported that single nucleotide polymorphism(SNPs) in the promoter region of *DNMT3B* gene has been associated with number of cancers , such as colorectal cancer , head and neck cancer, lung cancer, gastric cancer, acute myeloid leukemia (Luczak *et al*.,2006).

Okano *et al.,*1998 reported that *Dnmt3* family consists of two related genes, *Dnmt3a* and *Dnmt3b.* These two classes are abundantly expressed in embryonic stem (ES) cells, but upon differentiation of embryonic stem cells their expression is down-regulated and in adult somatic tissues, their expression remains very low. *Dnmt3a* and *Dnmt3b* proteins which are expressed by baculovirus can methylate cytosine residues in various native and synthetic DNA and it does not show any preference for hemi-methylated oligonucleotide DNA. This shows that *Dnmt3* genes probably encode the de-novo methyltransferase activities that detected in Dnmt1 null mutant ES cells (Robertson *et al*.,2001).

2.3.3 *DNMT3B* gene and its Protein: An essential component of DNA methyltransferase 3B (*Dnmt3b*) is PWWP (Pro-trp-trp-pro) which is responsible for establishing DNA methylation patterns during embryogenesis and gametogenesis. In case of tumorigenesis, DNA methylation by DNA methyltransferase 3B *(DNMT3B)* helps in the inactivation of tumor suppressor genes. In patients with ICF (Immunodeficiency, Centromeric instability and Facial anomalies), a point mutation in the PWWP domain of *Dnmt3b* has been found. PWWP domain is also known as Pro-trp-trp-Pro motif, which is a small domain consisting of 100-150 amino acids. This domain is identified in various proteins that make a part of cell division, growth and differentiation. Most of this domain protein appears to be

nuclear and DNA-binding protein, protein that acts as transcription factors that regulate a variety of developmental processes (Chen *et al.,*2004)

2.3.4 Functional role of *DNMT3B* in maintaining methylation status of tumor suppressor genes and oncogenes:

In mammalian embryogenesis, DNA methylation is an important component. In mice knockout experiment, targetted mutations of DNA methyltransferases and methylation activity are result in early death of mice embryos (Li *et al*.,1992). There are several different genes have been identified that encode DNA methyltransferases. DNMTs exhibit two different roles but these are different in their ability to perform one or the other. The first function of these enzymes is the maintenance of methyltransferase activity and the second one is maintenance of de novo methyltransferase activity. The first function of DNMT is known for methylation of newly formed strands of DNA based on the template of a single parent strand. Methyltransferase activity maintenance identifies the hemi methylated pattern of the parent strand and then recreated this on the daughter strand, allowing this characteristic to be inherited after replication of DNA and cell division. In the second role de novo methyltransferase activity is responsible for new methylation. This activity happens in the early stages of the development of embryo after implantation. After this, the fertilized egg encounters under dimethylation before implantation that removes most of pre-existing patterns of methylation came from parental DNA (Razin *et al.,*1995).

Decreased level of these enzymes ends in less extensive distortion of methylation patterns and is consistent with fetal survival. Mutations of one of the genes coding for a methyltransferase, *DNMT3B*, is executed for specific methylation of Centromeric satellite repeat sequences that can a reason of ICF (Immunodeficiency, centromeric heterochromatin instability and facial anomalies) syndrome (Jeanpierre *et al.,*1993, Okano *et al*., 1998). This function imparts the way by which the cell can regulate its own activity by switching off the expression of certain genes when not needed. This phenomenon in normal cells is the global silencing of genes on the additional X-chromosome in Females (Grant *et al*.,1988).

2.3.5 Role of methylated DNA in carcinogenesis: In the DNA of cancerous cells, altered methylation patterns are known to occur. These patterns are of two types: wide areas of global hypomethylation along the genome and localized areas of hypermethylation at

certain specific sites, the CpG islands within the gene promoter regions (Feinberg *et al.*,1983, Baylin *et al.,* 1983). Depending on these patterns, different theories came out to implicate DNA methylation mechanisms in carcinogenesis (Laird *et al.*,1997). In genetic models of cancer, the amplification of protooncogenes or the silencing of tumor suppressor genes, interrupt the balance that normally controls the cell proliferation and then through the succession of events leading to full malignant state.

Thus, decreased methylation and hence relief of transcriptional silencing, may allow the expression of previously inert protooncogenes to become active and persuade the cell proliferation events as shown in fig. 2.5.

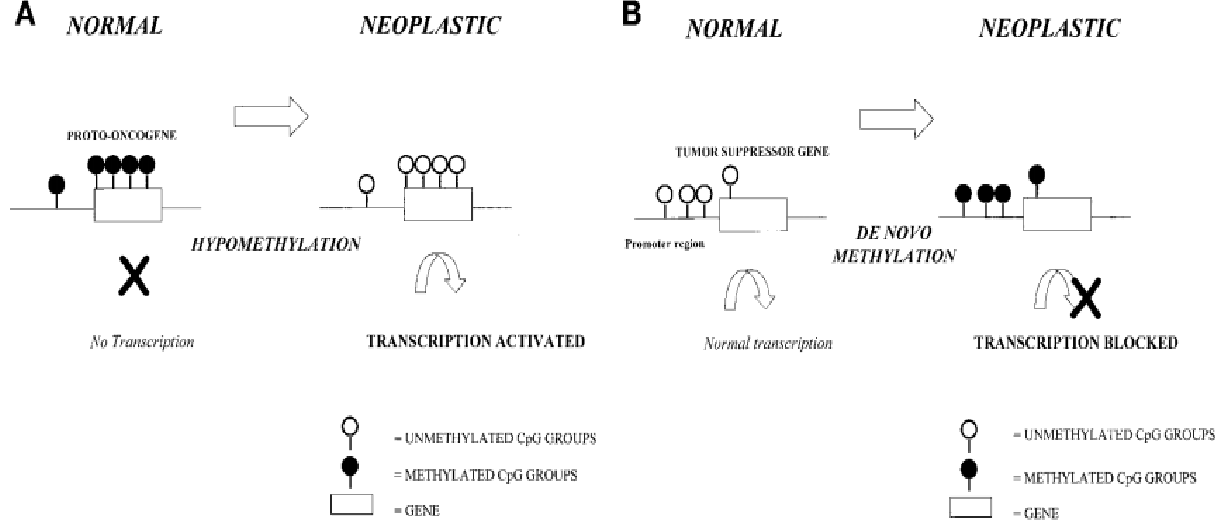

Figure 2.5 Methylation events occurring during carcinogenesis. (A) Activation of previously inactive protooncogenes after hypomethylation. (B) Silencing of tumor suppressor genes after methylation of gene promoter region (Shahjehan A *et al.,*2001).

Alternatively, at previously unmethylated sites, methylation increases, such as the promoter regions of a tumor suppressor gene may result in their silencing by inhibition of transcription and their inability to suppress cell proliferation. With the disruption of the transcription-translation process by increasing the probability for a mutational event to take place and by reducing overall chromosomal stability, abnormal methylation patterns can also indirectly affect gene activity that results in the manufacture of a dysfunctional protein product. Methylated cytosine has a greater propensity to undergo spontaneous deamination and formation of thymine as shown in fig. 2.6. If this happens with a tumor

suppressor gene, then a point mutation develops and loss of control of cell proliferation can occur.

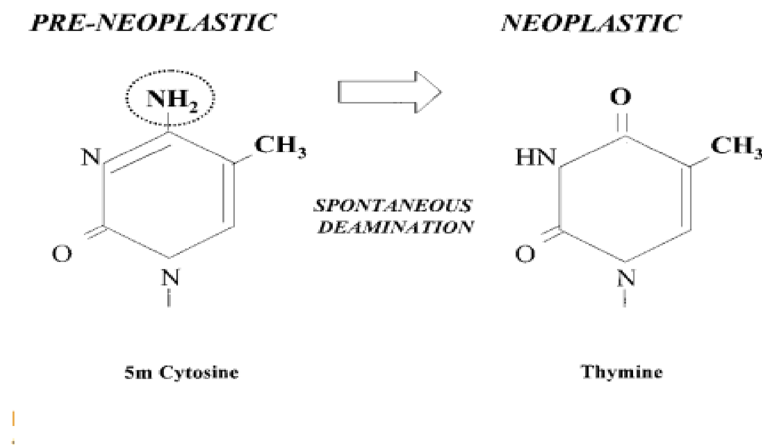

Figure 2.6 Methylation showing a point mutation. Cytosine to thymine point mutation after deamination of methylated cytosine (Shahjehan A *et al.,*2001).

2.3.6 Hypomethylation and gene activation: According to figure 2.5, investigators have failed to implicate a definitive role for hypomethylation and the subsequent activation of proto-oncogenes (Goelz et al.,1985). An example of such a relationship was observed in BCL-2(B- cell lymphoma) gene and human B-cell chronic lymphocytic leukemia but further support for hypomethylation and resultant activation of protooncogenes in this way has not been observed (Hanada *et al.,* 1993). In human tumors, although reduced levels of methylation of genes including C-MYC have been reported, but it has not been possible to show that this is responsible for increased level of gene expression rather than a secondary characteristic observed in cancer cells (Sharrard *et al.,* 1992).

2.3.7 Hypermethylation and gene silencing: The overall ubiquity of CpG dinucleotide pairs is less than the expected statistically in the human genome, the possible combinations available. The expected prevalence is 6% and the actual prevalence is only about 1%. In particular genes associated with tumor suppression and in the promoter regions of many common genes, localized high-density concentrations of CpG repeat sequences between several hundred to a few thousand base pairs exist as islands (Bird *et al.*, 1986). These regions are normally unmethylated but these regions should become methylated and failure to transcribe the downstream gene occurs, causing silencing of that gene as shown in figure 2.5.

This assumption can be confirmed if the reduction of DNA methyltransferase functions through pharmacologic inhibition or gene inactivation results in re expression of previously silenced genes. Evidence shown that localized hypermethylation is responsible for rather than secondary to tumor-related gene inactivation. This evidence has been accumulating from the data obtained from experimental animal models, cell culture technology and observations in human tumor tissues (Laird *et al.*, 1991).

First example of above evidence was observed from the experiment done with mice, mice with heterozygous; multiple intestinal neoplasia mutations of the adenomatous polyposis coli (Apc) gene (Min mice) develop a condition that resembles human familial adenomatous polyposis coli. In the first 6 months of life, these mice develop multiple (>100) intestinal polyps. In Min mice, reduction of DNA methyltransferase activity can be achieved through cross-breeding with mice heterozygous for a DNA methyltransferase gene that express lower levels of endogenous DNA methyltransferase. Further, the addition of 5-aza-2'-deoxycytidine (5-aza-dC) will inhibit the enzyme activity. Polyp formation can be reduced by more than fifty-fold in these mice by this combination of genetic and pharmacologic disruption of enzyme function (Laird *et al.*,1995).

Second example is the hypermethylation of the CpG promoter region of the mismatch repair gene MLH1 (MutL Homolog 1) observed in a subgroup of human colorectal cancers that exhibit microsatellite instability. These sequences are short, polymorphic, repeating segments of DNA between 1 and 4 base pairs distributed across the genome. If there is a deficiency in the cells' ability to their defects in DNA, then alterations to their patter often occurs. If the methylation of MLH1 gene occurs, then the functional protein will not form and diminish the ability of the cell to repair mismatches that occurs in the genome during proliferation and that resulting in an increased mutation rate hundred times greater than that occur in normal cells (Herman *et al.*,1998).

Finally, the inactivation of the CDKN2A (p16) tumor suppressor gene that is located on the short arm of chromosome 9 has been reported in several human cancers. This protein product (p16) is a cyclin-dependent kinase inhibitor and this interferes with the cell cycle and this preventing cellular proliferation. Through point mutations and deletions different genetic alterations have been observed in a wide variety of cancers (Kamb *et al.*,1994). In esophageal adenocarcinoma and in Barrett's metaplastic epithelium, the loss of heterozygosity of p16 is found, the premalignant epithelium from which adenocarcinoma

can evolve (Barrett *et al.*,1996). From the remaining copy of the p16 gene, further mutations and deletions are not common in this cancer (Cairns *et al.*,1994). According to a recent observation, it has been shown that in patients with esophageal adenocarcinoma, the promoter region of p16 is hypermethylated and this epigenetic mechanism can help to inactivate gene fully with genetic event (Wong *et al.,*1997). In the development of human cancers, hypermethylation of the p16 promoter region is the most widely reported epigenetic event to occur (Herman *et al.,* 1995). In patients with Wilms tumors, parental specific gene inactivation or disruption of imprinting, which is a normal function of DNA methylation within the cell, is seen. IGF2 gene which is present in chromosome 11 has the characteristics of autocrine growth factor, H19 gene that appears to act as a tumor suppressor gene is to control cell proliferation. In Wilms tumor, loss of imprinting of these genes can occur. With methylation at normally unmethylated locations upstream to the H19 gene, maternal chromosome reverts to a paternal pattern of gene expression and this result in switching off the H19 gene (Steenman et al., 1994). Table 2.2 shows the examples of genes involved in cell proliferation and methylated in cancer (Shahjehan A et al.,2001).

Table 2.2 Examples of genes involved in cell proliferation and methylated in cancer

Gene	Function	Cancer
p16(CDKN2A)	Cell cycle control	Esophagus, gastric, lung, colorectal, ovary, pancreas, bladder, melanoma, breast
p15(CDKN2B)	Cell cycle control	Leukemia
MLH1(HNPCC)	Mismatch repair	Colorectal, gastric, ovary, endometrium
THBS1(Thrombospondin-1)	Angiogenesis inhibition	Colorectal
CDH-1(E-Cadherin)	Metastasis inhibition	Breast, thyroid
TIMP-3(Tissue inhibitor MP3)	Metastasis inhibition	Kidney, brain, colon, breast, lung
ER(estrogen receptor)	Growth suppression	Breast, colorectal, lung, prostate, leukemia
AR(Androgen receptor)	Growth suppression	Prostate

2.3.8 Expression of *DNMT3B* in normal tissues and tumor cell lines: In most of the tissues, *DNMT3B* transcripts of about 4.2kb are expressed at a very lower level, but these can be easily detected in other tissues like in the testis, thyroid and in bone marrow as shown in fig.2.7. The size difference between these transcripts is too small to be detected by Northern analysis. During sequence analysis of different cDNA clones showed the presence of alternatively spliced transcripts. In tumor cells, hypermethylation of tumor suppressor genes is a common epigenetic lesion. (Laird and Jaenisch,1996; Baylin *et al*.,1998). To check whether that *DNMT3B* are abnormally activated in tumor cells, analysis of *DNMT3* expression was done in various tumor cell lines by using a technique named as northern blot hybridization (Shaoping Xie et al.,1999) As compared to the *DNMT3B*, the expression of *DNMT3A* is at higher level in most tumor cell lines than in adult tissues. The ratio of these transcripts seemed to be variable in different tumor cells lines. Though the expression of *DNMT3B* was very low in normal adult tissues but its expression was dramatically increased in most tumor cell lines examined. As compare to *DNMT1*, the expression level of both *DNMT3A* and *DNMT3B* appeared to be comparable and proportional. During early embryonic development, murine *DNMT3B* gene is highly expressed in undifferentiated embryonic stem (ES) cells and also play a potential role in de novo methylation.(Okano *et al*.,1998).

By gene targeting in embryonic stem cells and in mice, it was investigated that *DNMT3B* acts as de novo methyltransferase in vivo. Since this gene is highly expressed in early embryos, so this was postulating that during early embryogenesis, this gene may have redundant function in global de novo methylation. Because of the clear differences in their expression patterns in adult tissues in both human and mouse suggests that this gene may have distinct function in somatic tissues and it may also methylate different genes or genomic sequences. During tumor formation, due to increased expression of *DNMT1* and *DNMT3* in tumor cell lines, it seeks an enzyme that is responsible for de novo methylation of CpG islands in tumor suppressor genes. In the previous studies, it was not possible to examine *DNMT3* expression in tumor cells, so it was observed that over expression of *DNMT1* was thought to be the main reason of hypermethylation of tumor suppressor genes.

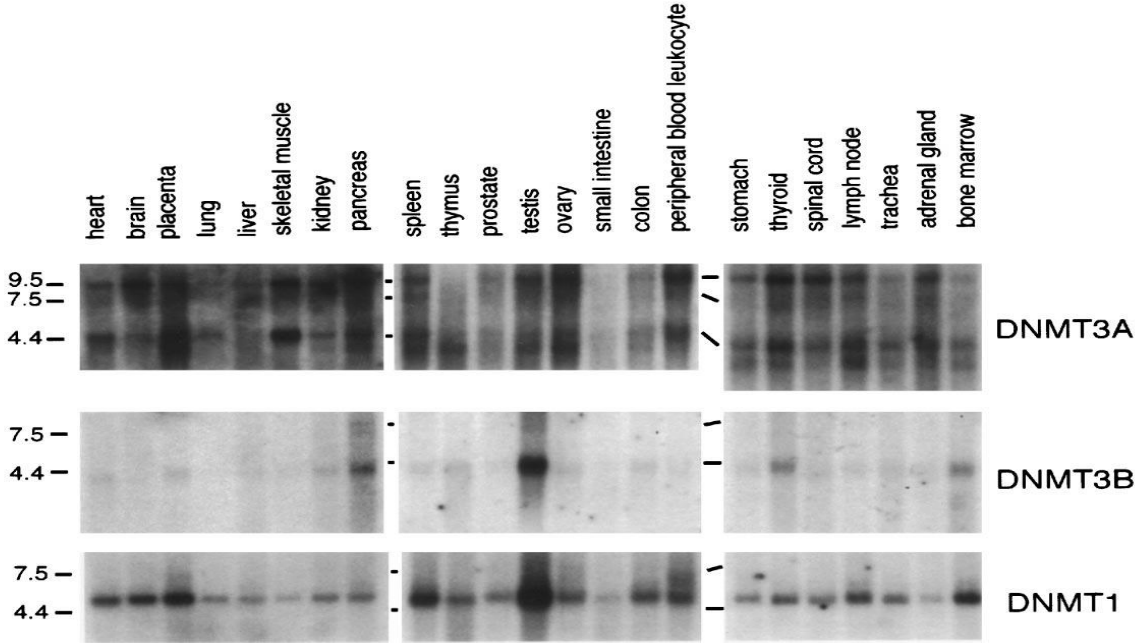

Fig.2.7 Shows the expression of *DNMT3A* and *DNMT3B* in human tissues. Poly A+RNA blots were hybridized with *DNMT3A*, *DNMT3B* and *DNMT1* cDNA probes(S Xie *et al.* /Gene 236 1999).

2.5 *DNMT3B* polymorphism and lung cancer

DNMT3B gene, which is located on chromosome 20q 11.2, it contains several polymorphisms. Two single nucleotide polymorphisms have been confirmed in the promoter region which are important for promoter activity (Shen H *et al.*, 2002), Lee SJ *et al.*,2005), Li Y *et al.,(*2005)). These polymorphisms are -149 C>T (rs2424913) and -579G>T (rs1569686). *DNMT3B*-579G>T is located in the transcription start site of the promoter region and it also effects the gene function. Heterozygous genotypes have been observed to have significantly reduced risk of developing lung cancer and Colorectal cancer (Lee SJ *et al*.,2005), Hong YS *et al.,*2005). The other polymorphism (*DNMT3B* 149 C>T) is located at 149-base pair upstream of the transcription start site of the promoter region and due to this polymorphism promoter activity is increased to 30% . Data has shown that the polymorphism of the *DNMT3B*-149C>T has been shown to increase the susceptibility of an individual to lung cancer (Shen H *et al.,*2002) and breast cancer(.Montgomery KG *et al*.,2005).

Recently, the significance of *DNMT3B* genetic polymorphism for tumorigenesis has been described, however the conclusions were always contradictive. Moreover, the association between colorectal cancer and *DNMT3B* polymorphism remain unclear and therefore a

case study was conducted to know about the association of *DNMT3B*-149C>T and -579G>T polymorphism with colorectal cancer (Fan *et al.*,2008). According to several Genome-wide association studies (GWAS), it has been observed that inherited genetic factors (genetic polymorphisms) increased the risk of lung cancer. In European and American populations, studies report that three chromosomal loci, 15q 24-25.1, 5p15.33 and 6p21 are associated with lung cancer, while four other chromosomal loci, 3q28, 5p15.33, 6p21 and 17q24.2 are associated with ADC risk in Japanese populations.

There are various studies revealed that -579G>T polymorphism is associated with susceptibility to different type of cancers like head and neck cancer, gastric cancer and colon cancer. *DNMT3B* -579 G>T might modify susceptibility to tumors and its association was seen with risk of lung cancer in Korean population (Lee SJ *et al*.,2005).

CHAPTER-3

AIM OF THE STUDY

1. To study the genotypic distribution of DNMT3BG5797 polymorphism in lung cancer patients.
2. To examine the association of this genetic variant with overall survival of lung cancer patients undergoing chemotherapy.
3. To evaluate the role of this genetic variant in predicting prognosis of patients classified on the basis of histology, gender, smoking status, performance and regimens.
4. To investigate the association of this polymorphism with different clinic-pathological parameters.

CHAPTER-4

MATERIALS AND METHODS

4.1 Study subject and sample collection

The current study enrolled a total of 100 lung cancer patients who were recruited from the Department of Pulmonary Medicine, Post Graduate Institute of Medical Education and Research (PGIMER) Chandigarh, India. This study has been revised and accepted by the Institute ethics committee of PGIMER. Informed written agreement was obtained from all enrolled patients or their representatives. All the enlisted patients were histo pathologically diagnosed as having NSCLC or SCLC. Patients under observation having a previous history of cancer were excluded from the study. There was no age, gender, smoking, histological or TNM stage restrictions.

Around,3-6 ml of blood was collected in vacutainers from each individual enrolled in that study. All controls were sub-grouped as sex, age and smoking parameters in order to avoid any sampling bias. Each participant filed up the detailed questionnaire with the help of trained interviewer. The questionnaire compromised information on demographic and smoking characteristics like tobacco habits such as smoking of beedi /cigarette etc. Individuals who smoked regularly were classified as smokers. They were further classified as light and heavy smokers on the basis of pack years (PY) that were calculated by the formula:

[(cigarettes or beedis per day/20)*years smoked]

PY less than or equal to 25 were considered as light smokers and PY greater than 25 were considered as heavy smokers. The medical information of cases such as histology, TNM classification, clinical staging, primary tumor size, involvement of lymph node and metastasis were obtained from medical records of the hospital.

4.2 DNA Extraction:

The genomic DNA was isolated using standard Protein K digestion, phenol/chloroform extraction and ethanol precipitation method from blood samples of both cases and controls (Field *et al* 1999).

Requirements:

1.Washing buffer

2.Lysis buffer

3.Phenol:Chloroform:Isoamylalcohol (25:24:1)

4.Chloroform:Isoamylalcohol (24:1)

5.Isopropanol

6. TE-buffer

Preparation of Buffers:

Washing buffer, Lysis buffer and TE buffer were prepared as shown in tables below.

Table 4.1: Preparation of washing buffer

Stock concentration	Working concentration
1 M sucrose	320mM sucrose
100% Triton X-100	1%Triton X-100
100Mm Magnesium Chloride	5mM magnesium Chloride
100mM Tris-HCI pH (8.0)	10mM Tris-HCl pH (8.0)

Table 4.2: Preparation of Lysis buffer

Stock concentration	Working concentration
1M Tris HCl pH(8.0)	400Mm Tris HCl Ph(8.0)
10%SDS	1% SDS
0.5M EDTA	60Mm EDTA
5M NaCl	150 mM NaCl
10mg/ml Proteinase-K	100µg/ml proteinase-K

Procedure of DNA isolation:

1. Took 3ml of blood and equal amount of washing buffer and then it was added and mixed thoroughly.
2. After mixing, centrifuged it at 3500 rpm for 7 minutes and then for 5 minutes.
3. Decant the upper aqueous layer (supernatant) and then add 3 ml of washing buffer to the pellet.
4. Then after this resuspend the pellet in the washing buffer and centrifuged again.
5. Repeat this step three times.
6. Then dissolved the pellet in 3 ml of lysis buffer and mixed thoroughly.
7. Then it is incubated overnight at 44°c.
8. Then after incubation, added an equal volume of phenol: chloroform: Isoamyl alcohol(PCI)25:24:1(25ml phenol, 24 ml chloroform, 1 ml isoamyl alcohol) and mixed all the contents slowly.
9. Then centrifuged it at 8000rpm for 10 minutes at 4°c.
10. After centrifuge, slowly took the upper aqueous layer and again add PCI mix and then centrifuged at 8000 rpm for 10 minutes.
11. Then took the upper aqueous layer and added equal amount of chloroform: isoamyl alcohol(24:1).
12. Then again centrifuged it at 6500 rpm for 5 minutes and took the upper layer.
13. Then to the aqueous upper layer add the equal volume of chilled isopropanol or 2.5 times volume of absolute ethanol and mixed it slowly.
14. Stored it at -20°C for 1-2 hours.
15. Then Centrifuged it again at 12,000 rpm for 10 min at 4°C.
16. The supernatant was discarded and the pellet of DNA was washed with chilled 70% Ethanol twice at 10,000 rpm for 5 minutes.
17. Decant ethanol and air dry the pellet.
18. Dissolved the pellet in 50µl-150µl Tris-EDTA buffer depending on the size of DNA pellet (Bartlett & White, 2003).

4.3 Qualitative and quantitative estimation of DNA template:

Quantification of DNA is done by using the Thermo scientific Nanodrop spectrophotometer. The advantage of using this is that it uses only 1µl of sample. It was done by using UV spectrophotometer and the absorbance of sample was noted at two

wavelengths A_{260} nm and A_{280} nm. To calculate the purity of DNA, ratio of absorbance at 260nm and 280 nm is used .

If A_{260} nm/ A_{280} ratio is approximately 1.8 then the DNA sample is pure and free from contaminations of RNA and proteins.

If A_{260} nm/ A_{280} ratio is approximately 2.0 or >1.8 it shows RNA contamination in the sample, whereas a ratio less than 1.8 shows protein contamination in the sample.

The concentration of pure double standarded DNA is 50µg/ml and ratio A_{260} nm/ A_{280} is equals to1.0

The DNA concentration in a solution was determined by using this formula given below:

Concentration (µg/ml) = A_{260} X 50 µg/ml X Dilution factor

Procedure: 1. Before using the nanodrop spectrophotometer, firstly clean it by pouring 1µl of deionised water onto the lower optical surface of nanodrop.

2. After that open the Nanodrop software displaying on the screen.

3. Then select the Nucleic acid module.

4. First ,take a blank measurement by loading 1µl of TE and selecting blank from the options.

5. Measured the amount of nucleic acid sample by loading 1µl of DNA sample and then selecting the option- measure.

The Nanodrop Spectrometer automatically calculates the concentration of DNA and its purity.

4.4 Resolution of DNA Fragments On Agarose Gels

Requirements

- Electrophoresis buffer (TAE or TBE)
- Ethidium bromide solution
- Electrophoresis-grade Agarose
- 6X loading dye
- DNA molecular weight markers
- Horizontal gel electrophoresis apparatus
- Gel casting platform

- Gel combs (slot formers)
- DC power supply

Preparing 5X TBE (1000ml)

- Tris base - 54 g
- Boric Acid - 27.5g
- EDTA (0.5M) - 20ml
- Make up final volume with water

Preparing 6X Loading Dye (20ml)

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

Preparation of the Agarose Gel for Electrophoresis

- Prepared an sufficient quantity of electrophoresis buffer.
- Added the required amount of Agarose to a volume of Electrophoresis buffer which is sufficient for preparing the gel. For example, for genomic DNA 0.7% gel (0.7g agarose in 100ml 0.5X TBE) was prepared while for the PCR products 1.7% gel (1.7g agarose in 100ml 0.5X TBE buffer) was prepared.
- Before pouring onto the casting plate, melted agarose was cooled to 55°C in a water bath to prevent deforming of the gel apparatus.
- Before gel casting, Ethidium bromide solution was added to the melted agarose gel to a final concentration of 0.3µg/ml to enable visualization of DNA when seen under UV Transilluminator.
- Poured the melted Agarose onto the gel casting apparatus between 0.5 and 1 cm thick and a comb is inserted after pouring gel, in such a way so that no bubbles are trapped underneath the combs and if bubble were there on the surface of the agarose ,it was removed before the setting of the gel.

Loading and running the gel

- The gel comb was removed slowly with proper care without disrupting the sample wells, after the gel got solidified
- Placed the gel casting tray containing the set gel in the Electrophoresis tank.
- Add sufficient amount of Electrophoresis buffer to cover the gel, till the tops of the wells are submerged and make sure no air pockets were trapped within the wells.
- DNA samples were prepared by mixing 5µl DNA with 2µl of 6X loading dye and 2ul water in case of genomic DNA or by mixing 5µl DNA with 2µl of 6X loading dye in case of PCR product. Then samples were loaded into the wells with micropipette. Care was taken to prevent mixing of the samples between wells.
- A ladder or DNA molecular weight marker is loaded in the first well in case of PCR and digestion products
- Connected the electrodes to a power supply and allowed the Electrophoresis unit to run at 60 V until the dyes migrated the required distance.
- Turned off the power supply, disconnected the leads, and remove the electrophoresis buffer from the electrophoresis tank.
- DNA was visualized by placing the gel on a UV transilluminator and then photographed using Gel Documentation.

4.5 Polymerase Chain Reaction (PCR):

Polymerase Chain Reaction (PCR) is a technique used in molecular biology to amplify small sections of DNA or a gene.

Principle: PCR works on a template DNA for producing multiple copies by using small fragment of DNA sequence called as primer, here which the nucleotides are attached. Enzymes used in the PCR are polymerase enzyme and the amplification is achieved by these polymerases, which binds to DNA to form long strands. For producing several copies of DNA, polymerase adds nucleotides to the primer which serves as a template. PCR takes approximately 2 hours to amplify the product.

There are 3 basic steps involved in the PCR :

- Denaturation
- Annealing
- Extension

In the first step of PCR, the denaturation step, at high temperature (95°c) the dsDNA get converted into ssDNA. After the denaturation, the temperature is again lowered to 50-60°c, which allows the primers to bind to DNA and the polymerase enzyme starts copying the DNA and this step is known as the annealing step. In this step, primer anneals to the template resulting in primer extension. The main function of the extension step is to make complementary copy of DNA strand, hence DNA make multiple number of copies. This will complete the cycle of PCR and this process will repeat again and again which doubles the DNA , after this run the gel on agarose gel and then visualized the results under UV transilluminator.

Requirements for PCR

- Water
- BSA(Bovine Serum Albumin)
- 10X PCR buffer
- Forward Primer (GAGGTCTCATTATGCCTA)
- Backward Primer (GGGAGCTCACCTTCTAGAAA)
- dNTPs
- Taq polymerase
- DNA sample

Table 4.3: Cycling profile of PCR for DNMT3B gene

Steps	Temperature	Time
Initial Denaturation	95°c	5 minutes
Denaturation	94°c	30 seconds
Annealing	46°c	45 seconds
Polymerization	72°c	30 seconds
Final extension	72°c	5 minutes

4.6 Restriction digestion of DNMT3B gene:

Restriction digestion utilizes an enzyme which cleaves the DNA at specific site.Dnmt3B utilizes PvuII enzyme for restriction digestion.

A restriction enzyme isolated from the microorganism Proteus vulgaris, a gram negative bacterium. It recognizes CAG|CTG, GTC|GAC sequence and act perfectly at 37°c in G buffer. This restriction enzyme is supplied with optimized buffer that provide maximum activity during restriction enzyme digestion. Lambda DNA acts as substrate for PvuII restriction enzyme. With the help of this enzyme, cleavage at alternative sequences occurs in the presence of DMSO or high concentrations of glycerol. NAGCTG, CAGCTN, CAGNTG, CAGCNG, CNGCTG and CANCTG are known alternative cleavage sites.

Procedure for Restriction Digestion:

The total reaction mixture for restriction digestion of 20µl consisted of 7.6µl of water, 2.2 µl of 10X buffer G (with BSA), 0.2 µl of PvuII enzyme and 10µl of amplified PCR product. All the samples were incubated at 37°c overnight. The enzyme reaction was stopped at keeping the samples at -20°c and after this the samples were loaded in 2.5% agarose gel and the results were visualized by staining the gel with ethidium bromide.

Statistical Analysis:

For demographic study, variables were categorized into continuous (gender, sex and smoking status) and categorical variables (age and pack years). Chi-square and Paired t-test test was performed for each category respectively. The Hardy-Weinberg equilibrium analysis (p2+2pq+q2=1; where p and q is the frequency of wild and variant type respectively) was evaluated using a goodness-of-fit Chi-square test. The association analysis between *DNMT3B* (-579G>T,rs1569686) genotype and susceptibility towards lung cancer was carried out using logistic regression. OR (odds ratio) along with its confidence intervals (95% CI) was calculated after adjustment with age, gender and smoking status, clinic-pathological features (KPS, ECOG and Tumor stage) and clinical responses to find specific association of *DNMT3B* (-579G>T,rs1569686) with lung cancer risk. Kaplan-Meir and Cox proportional hazard analysis was performed to study overall survival of lung cancer patients. Kaplan-Meir was used to evaluate overall survival time using median OS time and log Rank p-value. Multivariate Cox regression analysis was used to perform secondary analysis which evaluates the independent risk factor of death for each parameter. Probability criteria less than 0.05 was considered significant for all the statistical analysis. All the statistical analysis was performed using Medcalc Software version 15.11.4 (Medcalc Software bvba, Ostend, Belgium; http://www.medcalc.org;

2015). For demographic study, variables were categorized into continuous (gender, sex and smoking status) and categorical variables (age and pack years). Paired t-test and chi-square test was performed for each category respectively. The Hardy-Weinberg equilibrium analysis (p2+2pq+q2=1; where p and q is the frequency of wild and variant type respectively) was evaluated using a goodness-of-fit Chi-square test. The association analysis between *DNMT3B* (-579G>T, rs1569686) genotype and susceptibility towards lung cancer was carried out using logistic regression. OR (odds ratio) along with its confidence intervals (95% CI) was calculated after adjustment with age, gender and smoking status, clinic-pathological features (KPS, ECOG and Tumor stage) and clinical responses to find specific association of *DNMT3B* (-579G>T,rs1569686) with lung cancer risk. Kaplan-Meir and Cox proportional hazard analysis was performed to study overall survival of lung cancer patients. Kaplan-Meir was used to evaluate overall survival time using median OS time and log Rank p-value. Multivariate Cox regression analysis was used to perform secondary analysis which evaluates the independent risk factor of death for each parameter. Probability criteria less than 0.05 was considered significant for all the statistical analysis. All the statistical analysis was performed using Medcalc Software version 15.11.4 (Medcalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2015).

CHAPTER-5

RESULTS

5.1 DNA Isolation

For the isolation of genomic DNA from peripheral blood a simple and efficient procedure was used. Presence of isolated DNA was verified by trans-UV-illuminator and the samples were run on 0.7% agarose gel as shown in fig.5.1.With TE buffer concentrated DNA was further diluted to get a concentration of 100µg/µl and were used as a template in PCR for amplification.

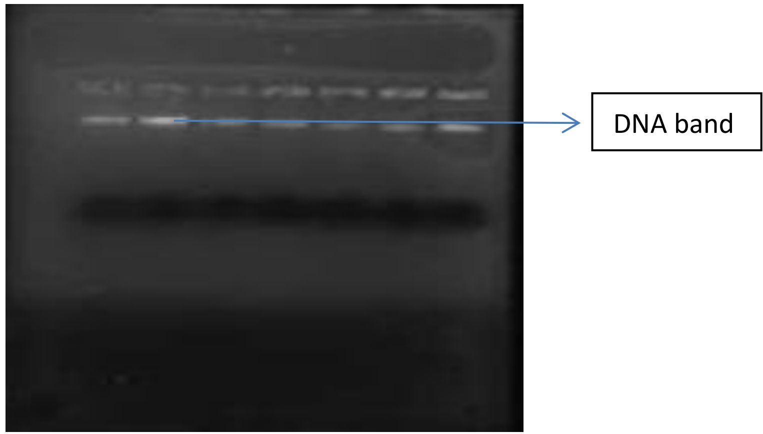

Fig 5.1 Isolated Genomic DNA confirmation by gel electrophoresis

5.2 Polymerase Chain Reaction of *DNMT3B*

DNMT3B gene was amplified by polymerase chain reaction by using forward and reverses primers to get a desired amplicon size of 225bp as shown in fig.5.2. After amplification, the obtained results were analyzed by using 1.7% agarose gel.

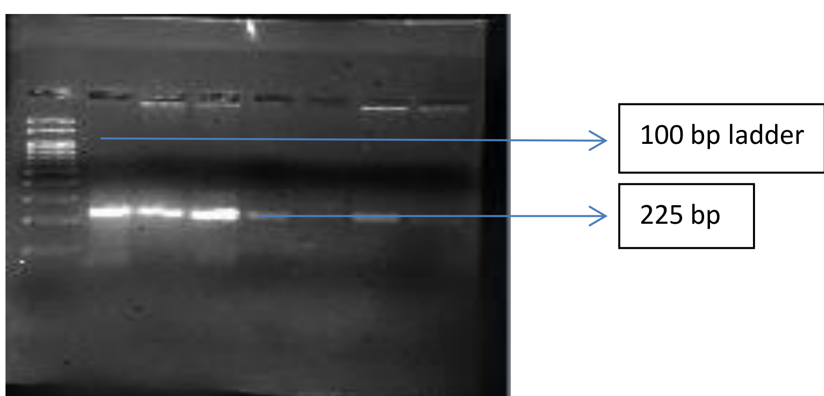

Fig 5.2 Amplified PCR product of *DNMT3B* gene with amplicon size 225 bp

5.3 Restriction Digestion of *DNMT3B* with PvuII enzyme

After PCR, the amplified products were further digested with restriction enzyme PvuII resulting into three fragments of size 225,132 and 93 bp for heterozygous genotype, two fragments of size 132 and 93 bp for mutant genotype and single fragment of size 225 bp for wild genotype as shown in the figure.5.3. Cutting patterns obtained by restriction digestion were checked by 2.5% agarose gel containing EtBr at 80 volts.

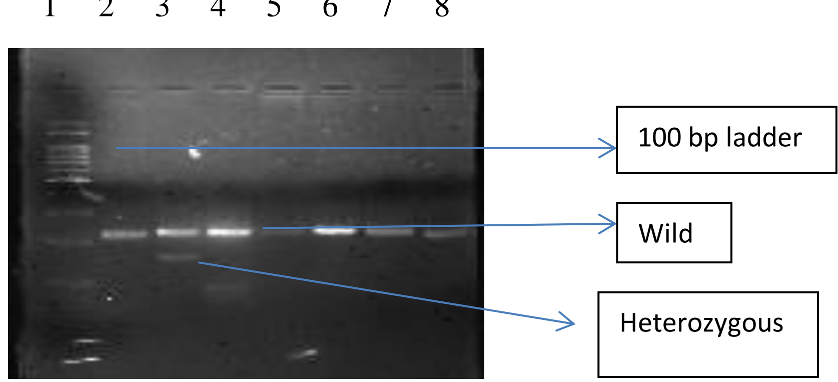

Fig.5.3 showing digestion patterns PCR amplified products of gene *DNMT3B*. Lane 1 represents 100 bp ladder (G. Bioscience); Lane 2, 4, 5, 6, 7, 8: Wild type (GG); Lane 3: Heterozygous
(GT).

5.4 Demographic characteristics

Demographic characteristics of the study groups included gender, age, smoking status, pack years, TNM staging, histological subtypes and other clinical parameters as shown in the table 5.1. The case study conducted consisted of 100 lung cancer cases. The mean age of cases was 59.07± 12.10(range 28-85). The study comprised of 87(87%) maless and 13(13%) Femalesss in the case group. In the present study, 78(78%) of the cases were smokers and 22(22%) were non-smokers, the mean pack years was 33.75± 39.49. Of the 100 lung cancer cases, 40(40%) were squamous cell carcinoma, 44(44%) were adenocarcinoma and 16(16%) were small cell carcinoma. TNM stage was available in form of 4 stages (stage I: 1(1%), II: 2(2%), III: 44(44%), IV: 46(46%). Tumor size T1 and T2 had a very less frequency of 6 and 12% of whereas T3 and T4 had a frequency of 24 and 48%. While during examination of lymph node involvement, N0 had a frequency of 10% while N1, N2, N3 and N4 had frequencies of 10, 47, 27 and 6% respectively. Among all the cases from which TNM data was available 49% of the cases had MO and M1 had a

frequency of 45%. Patients who received chemotherapy named KPS (Karnofsky performance status) in different ranges i.e. 90,100; 80,70 and ≤ 60 had frequencies of 32,51, 15 respectively and also in case of ECOG, the patients who received ECOG in range of 0-1 were 38 and in range 2-4 were 60. Further, after applying different regimens in 100 subjects, patients with regimen-1(doce-cis/doce-carb) were 30, with regimen-2(were 4, with regimen-5(irino-cis/irino-carb) were 15, with regimen-6(peme-cis/peme-carb) were 28 and 23 were unknown.

Table 5.1. Distribution of demographic characteristics of LC cases

Variable	**Cases, n (%)** **N = 100**
Age (years)	
Mean ± SD	59.07±12.10
Range	(28-85)
Gender	
Males	87(87)
Femaless	13(13)
Smoking Status	
Smokers	78(78)
Non – Smokers	22(22)
Pack Years	
Mean ± SD	33.75±39.49
Histologic Types	
SQCC	40(40)
ADCC	44(44)
SCLC	16(16)
TNM Staging	
I	1(1)
II	2(2)

III	44(44)
IV	46(46)
Unclassified	7(7)
Tumor Size	
T1	6(6)
T2	12(12)
T3	24(24)
T4	48(48)
Unknown	10(10)
Lymph Node Involvement	
N0	
N1	10(10)
N2	10(10)
N3	47(47)
N4	27(27)
Unknown	6(6)
Metastasis	
M0	49(49)
M1	45(45)
Unknown	6(6)
KPS	
90,100 (0)	32(32)
80,70 (1)	51(51)
≤60 (2)	15(15)
Unknown	2(2)
ECOG	
0-1	38(38)
2-4	60(60)
Unknown	2(2)
Regimen	

1-doce-cis/doce-carb	30(30)
2-palc-cis/palc-carb	4(4)
5-irino-cis/irino-carb	15(15)
6-peme-cis/peme-carb	28(28)
Unknown	23(23)

5.5 *DNMT3B* genotypic distribution with overall survival of lung cancer patients:

Table 5.2 Genotypic frequency of *DNMT3B* gene among cases and their associated death risk along with overall survival

Genotype	Overall							
DNMT3B	Cases (100) n%	Dead (85) n%	Alive (15) n%	Median OS (months)	HR (95% CI)	Log P	HR' (95%CI)	P value
GG	62(62)	55(64.70)	7(46.66)	6.23	-	-	-	-
GT	37(37)	30(35.29)	8(53.33)	8.20	0.79 (0.51-1.23)	0.31	1.45 (1.02-2.08)	0.03
TT	1(100)	1(1.17)	-	12.06	0.92 (0.13-6.18)	0.93	1.62 (0.51-5.11)	0.40
GT+TT	38(38)	30(35.29)	8(53.33)	8.63	0.79 (0.51-1.23)	0.3 1	0.83 (0.51-1.35)	0.46

Overall survival analysis was performed for 100 lung cancer patients and the results are summarized in Table 5.2. By using univariate Kaplan-Meir analysis, evaluation was done, then it was adjusted by multivariate Cox hazard proportional analysis for various factors such as age, histology, gender, smoking status, stage and performance status while considering the genotype as an independent factor. Among 100 patients, it was observed that 15% (15) patients were alive while 85% (85) were found to be dead due to the disease. Number of patients having wild *DNMT3B* genotype was 62, from these 64.70% (55) was dead and 46.66% (7) were alive. In case of 38 subjects carrying the heterozygous *DNMT3B* genotype, 35.29% (30) were dead and 53.33% (8) were alive. The overall

survival period of patients having *DNMT3B* wild genotype was taken as a reference and it was found that the subjects carrying heterozygous *DNMT3B* genotype had a higher overall survival period (6.23 vs. 8.20 months), HR=0.79; log rank p=0.31. On applying the Cox multivariate hazard proportional analysis, significant association was observed between *DNMT3B* genotype and the overall survival in lung cancer patients (HR'=1.45; 95%CI=1.02-2.08; p=0.03). Only single mutant was found, which was reported to have an MST of 12.06 months. We also carried the overall survival analysis for combined variant genotype patients, they showed a MST of 8.63 months and adjusted HR'=0.83, however, it was not to be statistically significant as shown in fig.5.4.

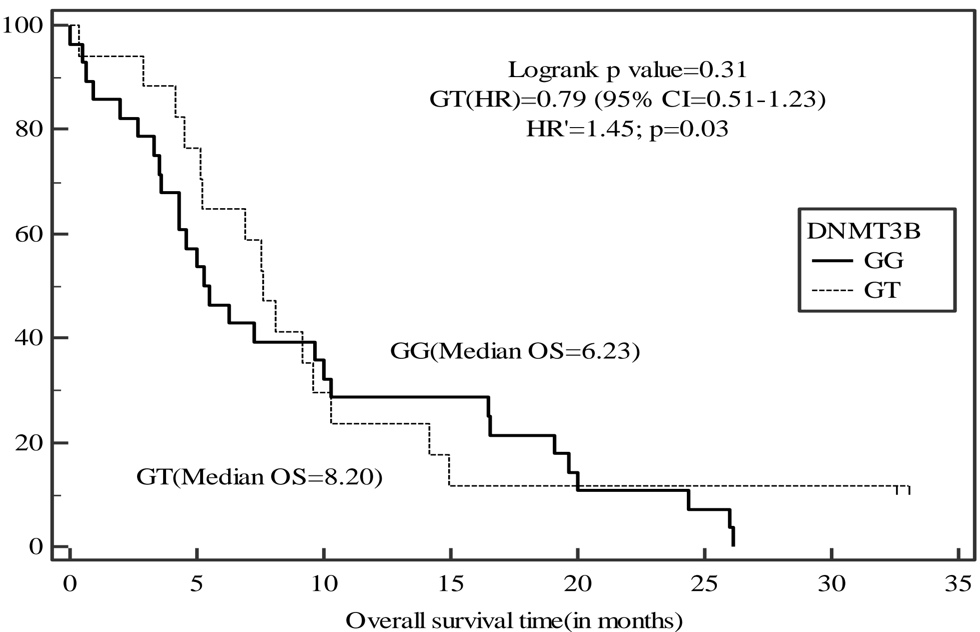

Figure 5.4 Effect of *DNMT3B* polymorphism on overall survival in lung cancer patients

5.5.1 *DNMT3B* genotypic distribution with overall survival of lung cancer patients on the basis of histological subtypes:

Table 5.3.Genotypic frequency of *DNMT3B* gene among cases and their associated death risk along with overall survival for different histological subtype (ADCC).

Genotype	ADCC							
DNMT3B	Cases (44) n%	Dead (37) n%	Alive (7) n%	Median OS (months)	HR (95% CI)	Log P	HR' (95%CI)	P value
GG	26(59.09)	22(59.45)	4(57.14)	8.4	Reference (1.00)	-	Reference (1.00)	-
GT	18(40.90)	15(40.54)	3(42.85)	9.13	1.14 (0.58-2.23)	0.68	2.12 (0.93-4.8)	0.07

When classification of genotype was done on the basis of histology for finding out prognostic effect, in total of 100 cases, 44 ADCC patients were reported, out of which 37(84.09%) were dead and 7(15.90) were alive as shown in table 5.3. Based on their classification according to the genotypic distribution, it was found that among 26 patients had the wild type (GG) *DNMT3B* genotype, 22(59.45) were dead and 4(57.14) were alive. Also, it was found that among 18 patients having the heterozygous type (GT) *DNMT3B* genotype, 15(40.54) were dead and 3(42.85) were alive. In this case, MST of subjects having wild genotype was lower (8.4 months) as compared to those having heterozygous genotype (9.13 months), HR=1.14; log rank p=0.68. After multivariate cox hazard proportional analysis, the adjusted HR was found to be non-significant (HR'=2.12; 95%CI=0.93-4.8; p value=0.07) as shown in the figure 5.5.

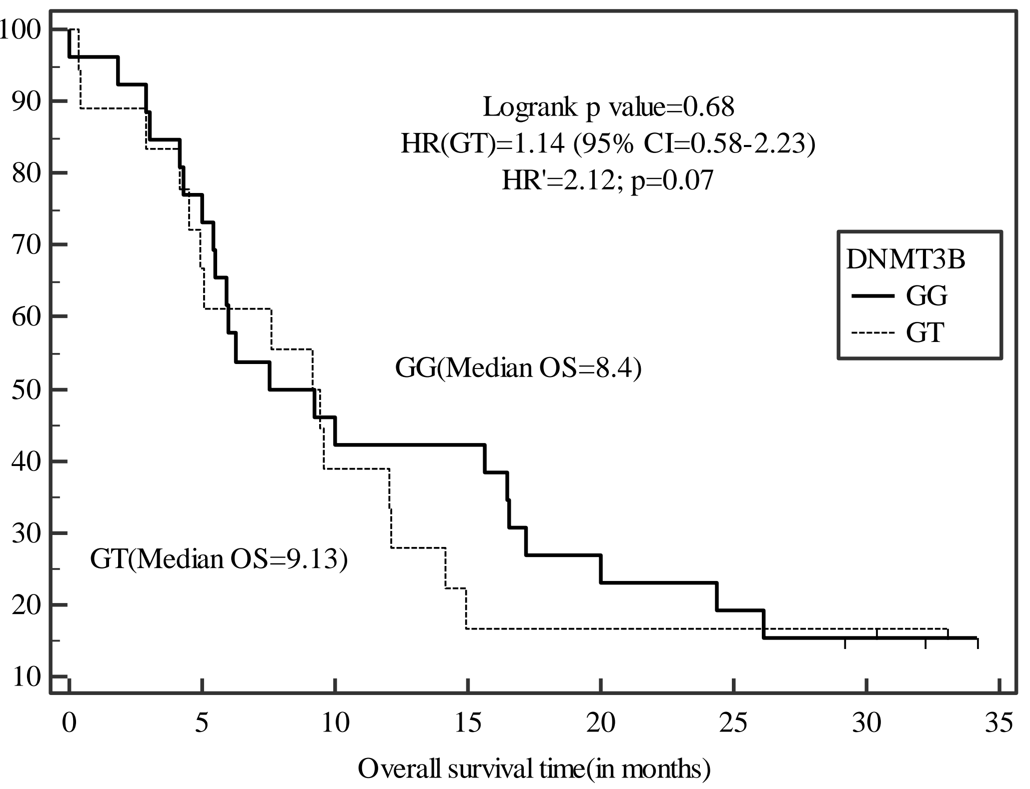

Figure 5.5 Effect of *DNMT3B* polymorphism on OS in ADCC

Table 5.4.Genotypic frequency of *DNMT3B* gene among cases and their associated death risk along with overall survival for different histological subtype SCLC (H+W).

Genotype	SCLC(H+W)							
DNMT3B	Cases (15) n%	Dead (14) n%	Alive (1) n%	Median OS (months)	HR (95% CI)	Log P	HR' (95%CI)	P value
GG	13(86.66)	12(85.71)	1(100)	6.3	Reference (1.00)	-	Reference (1.00)	-
GT	2(13.33)	2(14.28)	-	2.6	1.97 (0.27-13.95)	0.34	16.27 (1.139-232.45)	0.04

Further, among 15 SCLC (H+W) cases, 14 were dead and 1 was alive as shown in table 5.4. Then, on the basis of genotypic distribution it was found that out of 13 patients having wild type genotype(GG) 12(85.71%) were dead and 1(100%) was alive (MST=6.3 months), and out of 2 patients having heterozygous genotype (GT), 2(13.33%) were dead and no one was alive (MST=2.6 months). In this case, the hazard ratio was 1.97; 95% CI=0.27-13.95; p value=0.34 and after multivariate cox hazard proportional analysis, the adjusted HR was 16.27; 95%CI=1.13-232.4 and p value=0.04 which is significant value, therefore, the SCLC patients having GT genotype possess a 16-fold higher mortality rate with p=0.04 as shown in figure 5.6.

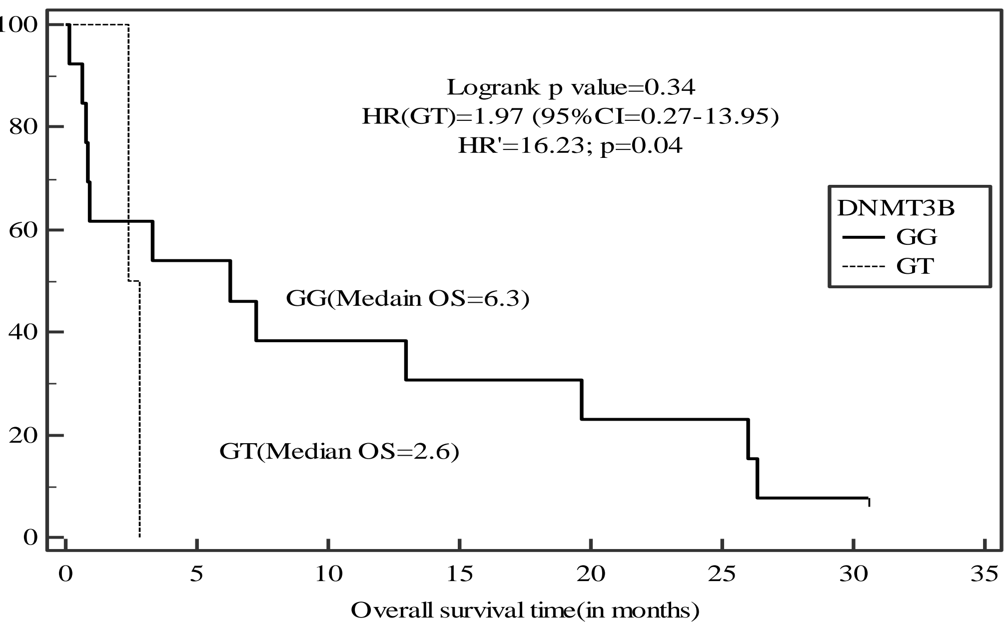

Figure 5.6 Effect of *DNMT3B* polymorphism on OS in SCLC(H+W)

Table 5.5.Genotypic frequency of *DNMT3B* gene among cases and their associated death risk along with overall survival for different histological subtype SCLC (H+M).								
Genotype	**SCLC(H+M)**							
DNMT3B	Cases (16) n%	Dead (15) n%	Alive (1) n%	Median OS (months)	HR (95% CI)	Log P	HR' (95%CI)	P value
GG	13(81.25)	12(80)	1(100)	6.3	Reference (1.00)	-	Reference (1.00)	-
GT+TT	3(18.75)	3(20)	-	2.8	1.84 (0.38-8.77)	0.30	2.91 (0.56-15.11)	0.20

Furthermore, among 16 SCLC (H+M) cases, 15 patients were dead and 1 was alive. Among 13 patients having wild type (GG) genotype, 12(80%) were dead and 1 was alive (100%) (MST=6.3months) and the number of patients having heterozygous genotype were 3, from which 3(20%) were dead and no one was alive. In this case the median survival time was 2.8 months; HR=1.84(95% CI=0.38-8.77); p-value=0.30 and the adjusted hazard ratio after cox analysis was HR'=2.91; 95%CI=0.56-15.11 and p-value=0.20 as shown in figure 5.7.

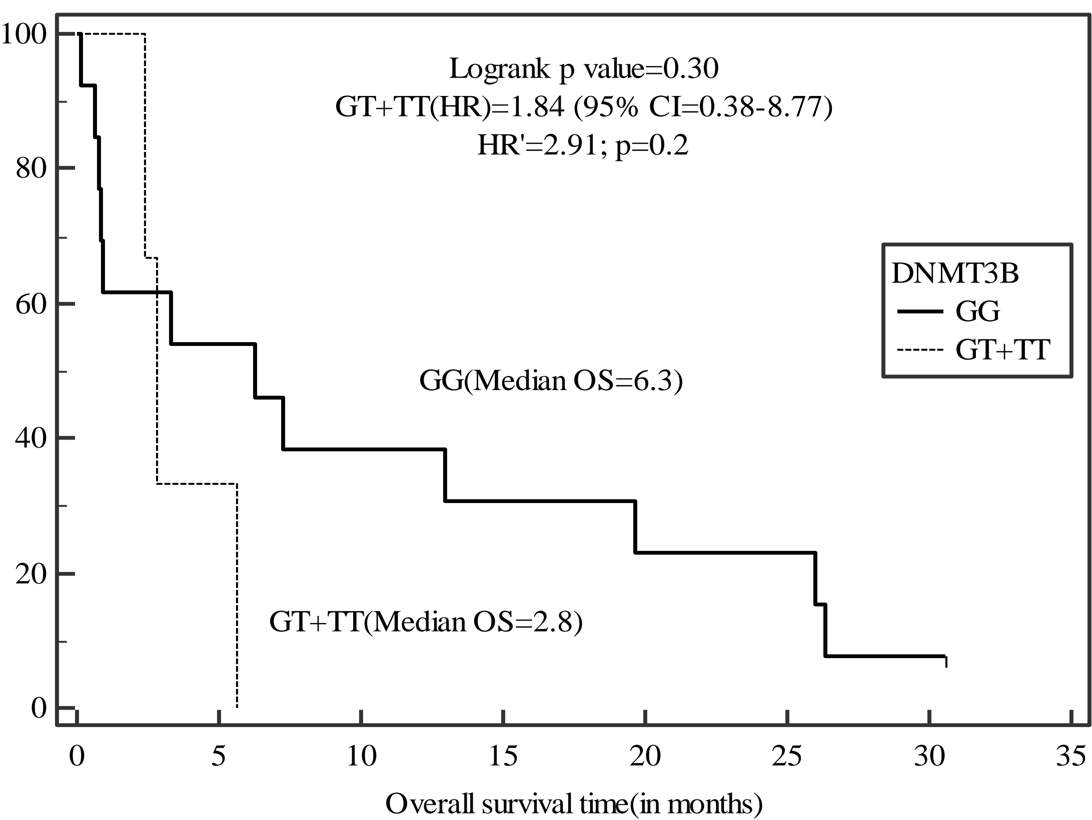

Figure 5.7 Effect of *DNMT3B* polymorphism on OS in SCLC(H+M)

Table 5.6.Genotypic frequency of *DNMT3B* gene among cases and their associated death risk along with overall survival for different histological subtype SQCC

Genotype	SQCC							
DNMT3B	Cases (40) n%	Dead (34) n%	Alive (6) n%	Median OS (months)	HR (95% CI)	Log P	HR' (95%CI)	P value
GG	23(57.5)	21(61.76)	2(33.33)	4.56	Reference (1.00)	-	Reference (1.00)	-
GT	17(42.5)	13(38.23)	4(66.66)	10.26	0.47 (0.24-0.94)	0.03	0.40 (0.19-0.86)	0.01

In the next histological type, among 40 SQCC cases, 34 patients were dead and 6 were alive. Number of patients having wild type (GG) genotype were 23 out of which 21(61.76) were dead and 2(33.33) were alive. Patients having heterozygous (GT) genotype were 17

out of which 13(38.23) were dead and 4(66.66) were alive. MST was much lower in case of wild type genotype (MST=4.56 months) than in the heterozygous genotype (MST=10.26) and in this case HR= 0.47; 95%CI=0.24-0.94; p-value=0.03, the hazard ratio was 0.47, hence showing a significantly lower probability of death in heterozygous SQCC patients. This hazard further decreased on applying cox hazard (0.40) and it was statistically significant, 95%CI=0.19-0.86 and p value=0.01 as shown in figure 5.8.

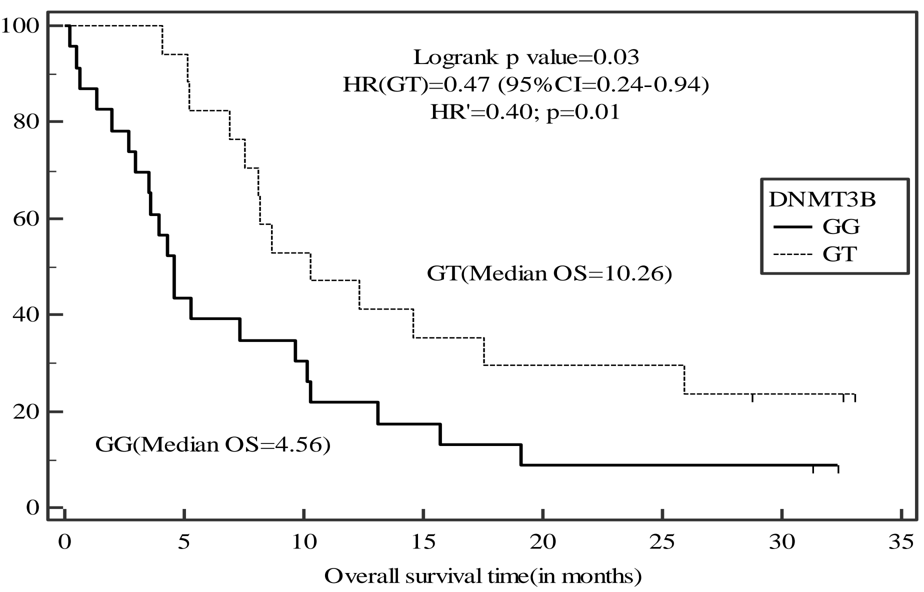

Figure 5.8 Effect of *DNMT3B* polymorphism on OS in SQCC

5.5.2 *DNMT3B* genotypic distribution with overall survival of lung cancer patients on the basis of gender:

Table 5.7.Genotypic frequency of *DNMT3B* gene among cases and their associated death risk along with overall survival for different gender type (Males (H+W)

Genotype	Males(H+W)							
DNMT3B	Cases (86) n%	Dead (73) n%	Alive (13) n%	Median OS (months)	HR (95% CI)	Log P	HR' (95%CI)	P value
GG	54(62.79)	48(65.75)	6(46.15)	6	Reference (1.00)	-	Reference (1.00)	-
GT	32(37.20)	25(34.24)	7(53.84)	8.88	0.70 (0.44-1.12)	0.15	0.72 (0.42-1.22)	0.23

Further, classification of patients was done on the basis of their gender and data has been summarized in table 5.7. It was found that among 86 males (H+W), 84.88 % (73) were dead and15.11 % (13) were alive. Stratifying on the basis of genotype, it was found that among 54 males patients carrying the *DNMT3B* wild genotype, 48 (65.75%) were dead and 6(46.15%) were alive and in 32 patients having the *DNMT3B* heterozygous genotype, 25 (34.24%) were dead and 7 (53.84%) were alive. After comparing the overall survival period between the two, it was observed that the males patients which had the wild *DNMT3B* genotype had a lower MST than the patients carrying the heterozygous *DNMT3B* genotype (6 vs. 8.88 months), HR=0.70; log rank p=0.15. The adjusted HR after applying multivariate Cox hazard proportional analysis was non-significant (HR'=0.72; 95%CI=0.42-1.22; *p*=0.23) as shown in figure 5.9.

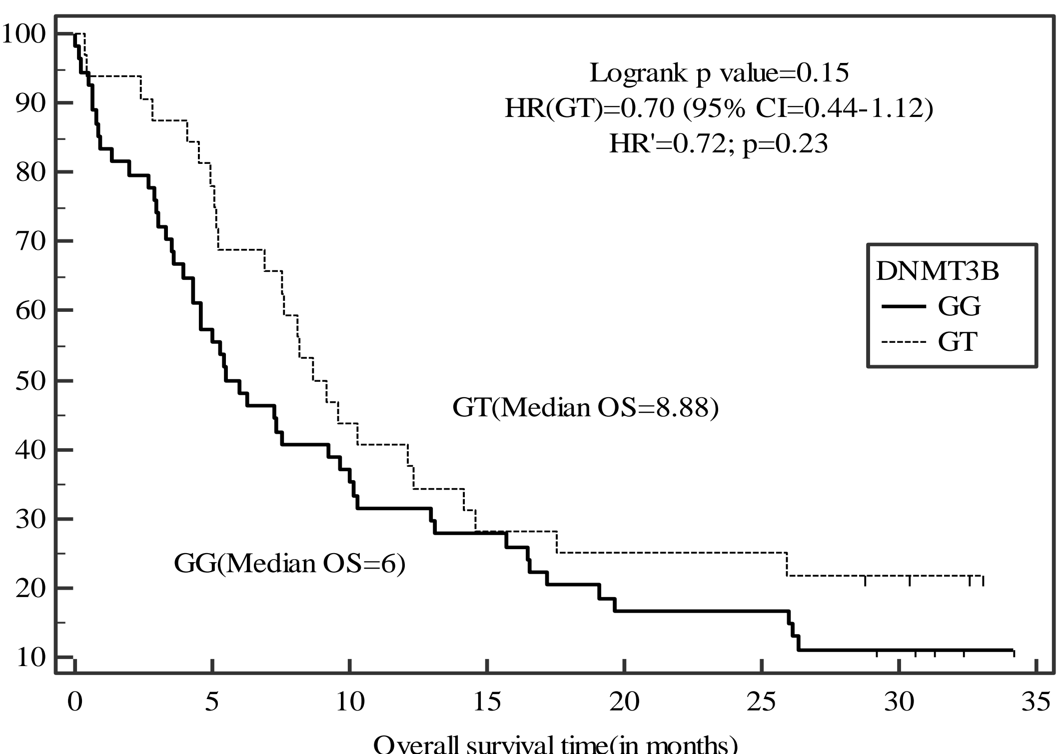

Figure 5.9 Effect of *DNMT3B* polymorphism on OS in males(H+W)

Table 5.8.Genotypic frequency of *DNMT3B* gene among cases and their associated death risk along with overall survival for different gender type (Males (H+M))								
Genotype	**Males(H+M)**							
DNMT3B	Cases (87) n%	Dead (74) n%	Alive (13) n%	Median OS (months)	HR (95% CI)	Log P	HR' (95%CI)	P value
GG	54(62.06)	48(64.86)	6(46.15)	6	Reference (1.00)	-	Reference (1.00)	-
GT+TT	33(37.93)	26(35.13)	7(53.84)	8.63	0.71 (0.45-1.13)	0.17	0.72 (0.43-1.21)	0.22

Further, among 87 males (H+M) patients having the *DNMT3B* gene, 85.05% (74) were dead and 14.94% (13) were alive. Then, among 54 patients having wild *DNMT3B* genotype 64.86%(48) were dead and 46.15%(6) were alive and also in 33 patients having the heterozygous *DNMT3B* genotype, 35.13% (26) were dead and 53.84% (7) were alive. The overall survival period in patients carrying the heterozygous *DNMT3B* genotype was found to be higher than the patients having the wild *DNMT3B* genotype (8.63 vs. 6 months), HR=0.71; log rank p=0.17. After applying the multivariate Cox hazard proportional analysis, the adjusted HR was found to be non-significant. (HR'=0.72, 95%CI=0.43-1.21; *p*=0.22) as shown in figure 5.10.

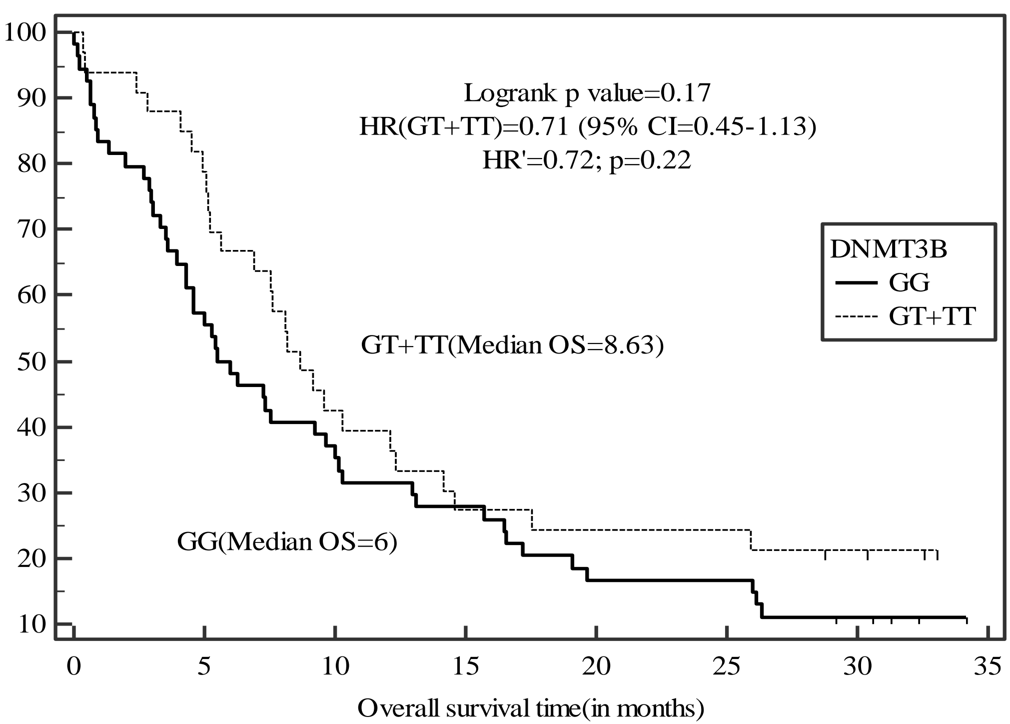

Figure 5.10 Effect of *DNMT3B* polymorphism on OS in males(H+M)

Table 5.9.Genotypic frequency of *DNMT3B* gene among cases and their associated death risk along with overall survival for different gender type (Females)								
Genotype	**Females**							
DNMT3B	Cases (13) n%	Dead (12) n%	Alive (1) n%	Median OS (months)	HR (95% CI)	Log P	HR' (95%CI)	P value
GG	8(61.53)	7(58.33)	1(100)	10.93	Reference (1.00)	-	Reference (1.00)	-
GT	5(38.46)	5(41.66)	-	9.46	1.9 (0.54-6.92)	0.21	2.11 (0.43-10.28)	0.35

In the next gender type, among 13 cases of Femaless patients, 92.30% (12) patients were dead and 7.69% (1) was alive as shown in table 5.9. Number of patients having wild type (GG) *DNMT3B* genotype were 8, from which 58.33 %(7) were dead and 100 %(1) was alive. Patients having heterozygous (GT) *DNMT3B* genotype were 5; from which 41.66% (5) were dead no one was alive. MST was lower in case of subjects carrying heterozygous genotype (MST=9.46 months) than in the wild genotype patients (MST=10.93) and in this

case HR= 1.9; 95%CI=0.54-6.92; p-value=0.21.The adjusted HR after the cox analysis was HR’=2.11; 95%CI=0.43-10.28 and p value=0.35 as shown in figure 5.11.

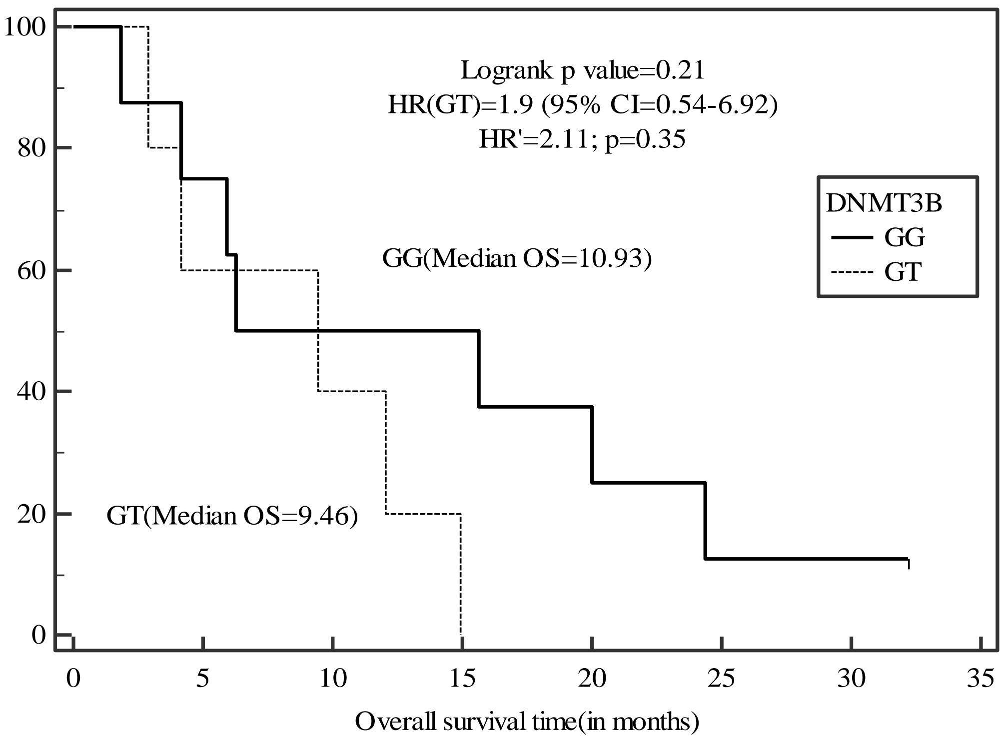

Figure 5.11 Effect of *DNMT3B* polymorphism on OS in Femaless

5.5.3 *DNMT3B* genotypic distribution with overall survival of lung cancer patients on the basis of smoking status:

Table 5.10.Genotypic frequency of *DNMT3B* gene among cases and their associated death risk along with overall survival on basis of smoking status (smokers)

Genotype	**Smokers**							
DNMT3B	Cases (76) n%	Dead (66) n%	Alive (10) n%	Median OS (months)	HR (95% CI)	Log P	HR’ (95%CI)	P value
GG	48(63.15)	43(65.15)	5(50)	5.43	Reference (1.00)	-	Reference (1.00)	-
GT	28(36.84)	23(34.84)	5(50)	8.2	0.74 (0.45-1.21)	0.24	0.76 (0.44-1.30)	0.32

When classification of lung cancer patients was done on the basis of smoking status and overall survival analysis was done and the data shown in table 5.10. It was found that out of 76 patients who smoked, 86.84% (66) were dead and 13.15% (10) were alive. On stratifying them on the basis of genotype it was found that out of 48 patients, who were smokers and carried the wild *DNMT3B* gene, 65.15% (43) were dead and 50% (5) were alive. The overall survival period of smokers having the wild *DNMT3B* genotype was found to be 5.43 months. Similarly, among 28 patients having the heterozygous *DNMT3B* genotype, 34.84% (23) were dead and 50% (5) were alive. The overall survival period in this case was lower than that of the cases having the wild genotype (5.43 vs. 8.2 months). But, this association was not statistically proved as no significant value was obtained after applying the multivariate Cox hazard proportional analysis (HR=0.74; 95%CI=0.45-1.21; log rank p=0.24; HR'=0.76; 95%CI=0.44-1.30; *p*=0.32) as shown in figure 5.12.

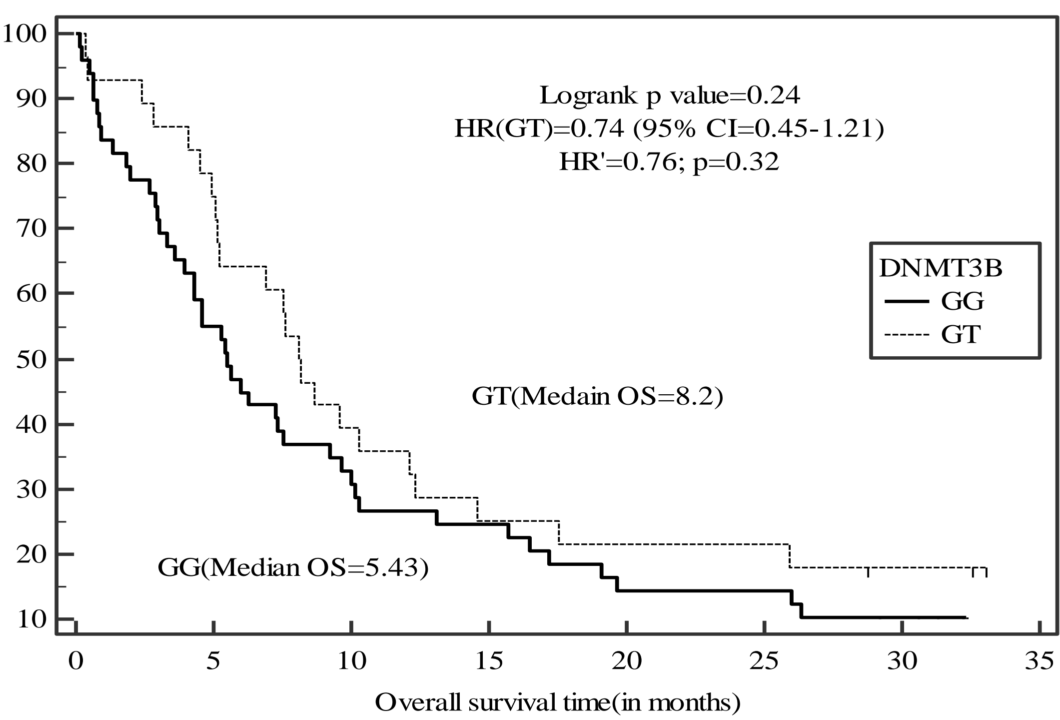

Figure 5.12 Effect of *DNMT3B* polymorphism on OS in Smokers

Table 5.11.Genotypic frequency of *DNMT3B* gene among cases and their associated death risk along with overall survival on basis of smoking status(non-smokers)								
Genotype	**Non-Smokers**							
DNMT3B	Cases (23) n%	Dead (19) n%	Alive (4) n%	Median OS (months)	HR (95% CI)	Log P	HR' (95%CI)	P value
GG	14(60.86)	12(63.15)	2(50)	15.63	Reference (1.00)	-	Reference (1.00)	-
GT	9(39.13)	7(36.84)	2(50)	12.06	0.99 (0.39-2.53)	0.99	1.27 (0.41-3.93)	0.67

Further, among 23 non-smoker patients, 82.60% (19) were dead and 17.39% (4) were alive. Then, on the basis of genotypic distribution it was found that out of 14 patients having wild type *DNMT3B* genotype, 63.15%(12) were dead and 50%(2) were alive (MST=15.63 months), and out of 9 patients having heterozygous genotype, 36.84%(7) were dead and 50%(2) were alive (MST=12.06 months). In this case, the hazard ratio was 0.99; 95% CI=0.39-2.53; p value=0.99 and after multivariate cox hazard proportional analysis, the adjusted HR was 1.27; 95%CI=0.41-3.93 and p value=0.67 which is non-significant value as shown in figure 5.13.

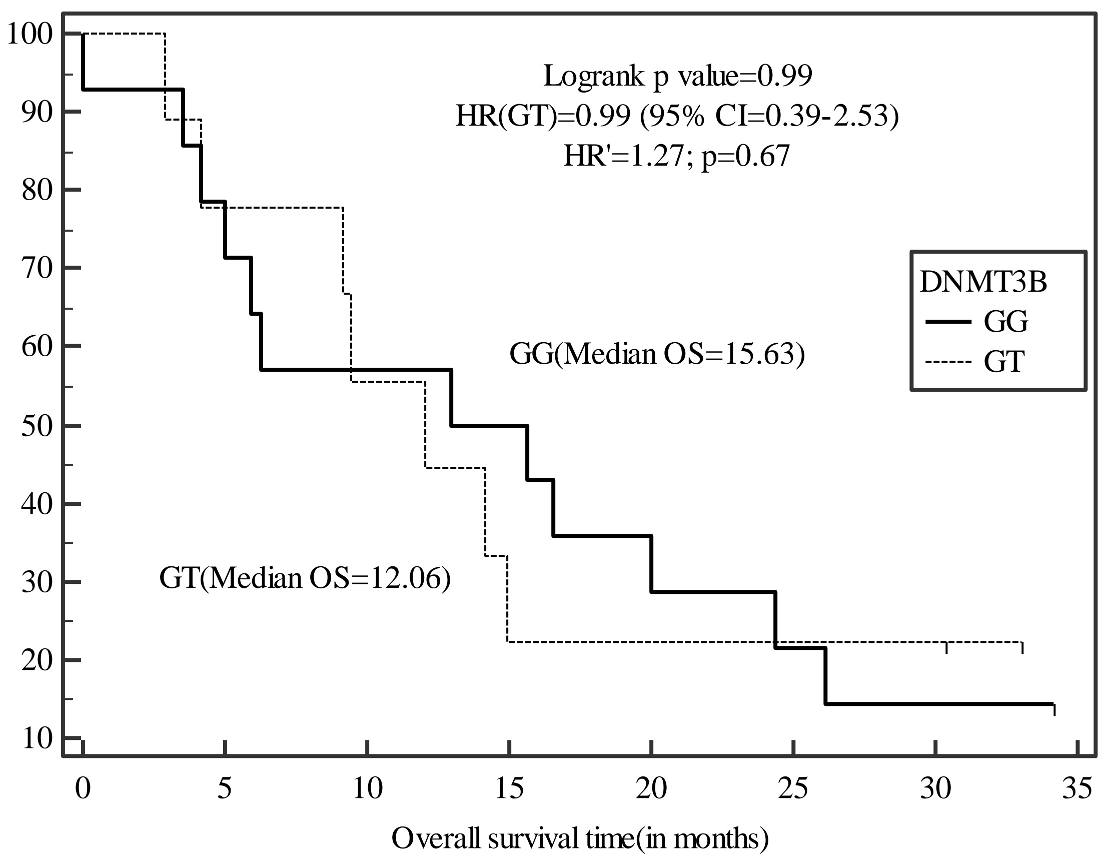

Figure 5.13 Effect of *DNMT3B* polymorphism on OS in non-smokers

5.5.4 *DNMT3B* genotypic distribution with overall survival of lung cancer patients on the basis of their performance status after receiving chemotherapy:

Table 5.12 Genotypic frequency of *DNMT3B* gene among cases and their associated death risk along with overall survival for different performance status after receiving chemotherapy.

Genotype	KPS(0-1)							
DNMT3B	Cases (82) n%	Dead (72) n%	Alive (10) n%	Median OS (months)	HR (95% CI)	Log P	HR’ (95%CI)	P value
GG	50(60.97)	45(62.5)	5(50)	6.11	Reference (1.00)	-	Reference (1.00)	-
GT	32(39.02)	27(37.5)	5(50)	8.35	0.84 (0.52-1.35)	0.48	0.89 (0.53-1.49)	0.66

The patients were stratified on the basis of performance status and on analyzing the patients with KPS (Karnofsky performance status) scale 0-1, it was seen that out of 82 patients, 87.80% (72) were dead and 12.19% (10) were alive. Based on genotypic distribution of *DNMT3B,* it was found that among the 50 patients having the KPS 0-1 and carrying the wild *DNMT3B* gene, 62.5% (45) were dead and 50% (5) were alive. The overall survival period was found to be 6.11 months. In 32 patients carrying the heterozygous *DNMT3B* genotype, 37.5% (27) were dead and 50% (5) were alive, in this case, the overall survival period was found to be 8.35 months and HR=0.84; 95%CI=0.52-1.35; log rank p=0.48). After applying multivariate Cox hazard proportional analysis, the adjusted HR value (HR'=0.89; 95%CI=0.53-1.49; *p*=0.66). This is shown in figure 5.14.

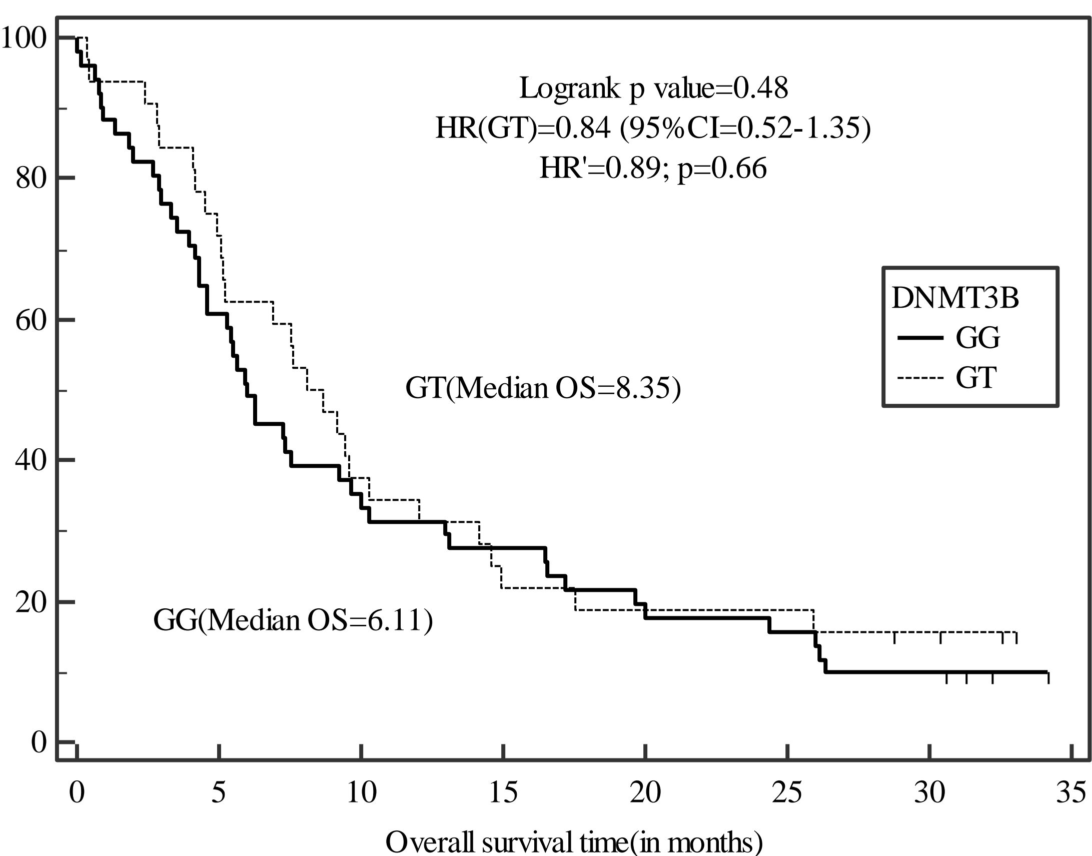

Figure 5.14 Effect of *DNMT3B* polymorphism on OS in KPS(0-1)

Table 5.13 Genotypic frequency of *DNMT3B* gene among cases and their associated death risk along with overall survival for different performance status after receiving chemotherapy.								
Genotype	**KPS (2)**							
DNMT3B	Cases (15) n%	Dead (11) n%	Alive (4) n%	Median OS (months)	HR (95% CI)	Log P	HR' (95%CI)	P value
GG	11(73.33)	9(81.81)	2(50)	4.96	Reference (1.00)	-	Reference (1.00)	-
GT	4(26.66)	2(18.18)	2(50)	-	0.39 (0.11-1.36)	0.21	0.14 (0.003-5.60)	0.30

Then further, considering performance status of patients based on Karnofsky performance status scale (KPS) it has been observed that among 15 patients with KPS range 2, 73.33% (11) were dead and 26.66% (4) were alive as shown in table 5.13. When further stratified on the basis of genotypic distribution of *DNMT3B* it was found that out of 11 patients having the KPS 2 and having the wild *DNMT3B* gene, 81.81% (9) were dead and 50% (2) were alive whereas among 4 patients having the heterozygous *DNMT3B* genotype, 18.18% (2) were dead and 50% (2) were alive. The overall survival period in the patients carrying the wild *DNMT3B* genotype was found to be 4.96 months, HR=0.39; log rank p=0.21. On applying the multivariate Cox hazard proportional analysis, no significant association was found between *DNMT3B* genotype and the overall survival of patients with KPS 2 (HR'=0.14; 95%CI=0.003-5.60; *p*=0.30) as shown in figure 5.15.

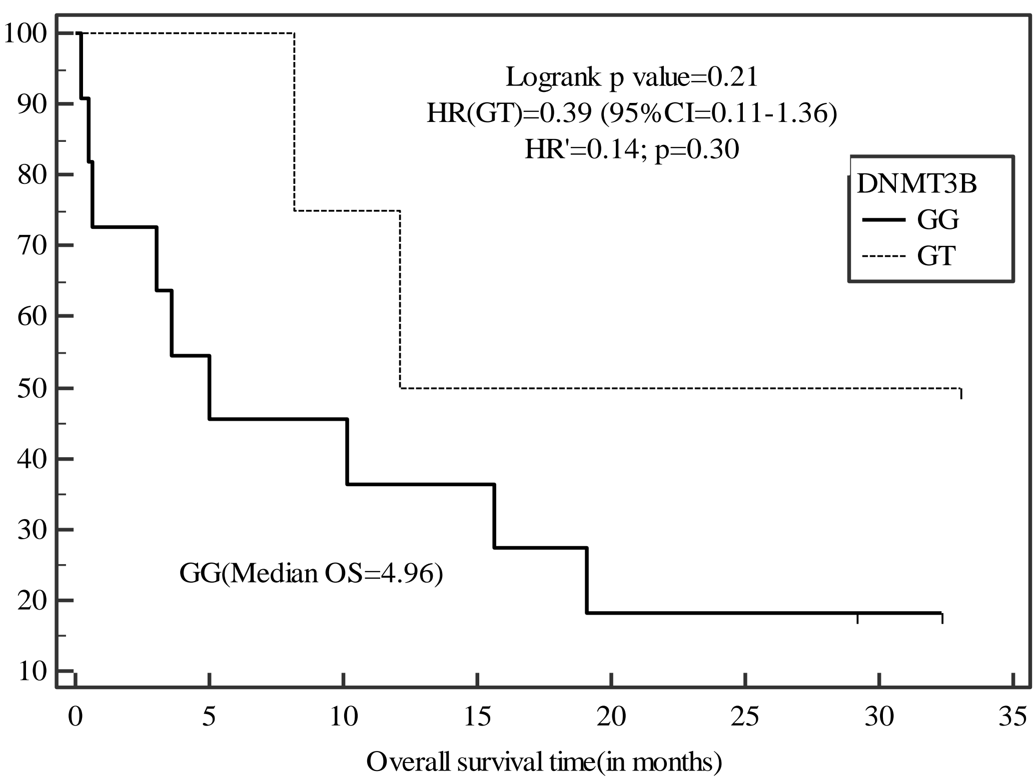

Figure 5.15 Effect of *DNMT3B* polymorphism on OS in KPS (2)

5.5.5 *DNMT3B* genotypic distribution with overall survival of lung cancer patients on the basis of different treatment regimen:

Table 5.14. Genotypic frequency of *DNMT3B* gene among cases and their associated death risk along with overall survival on different treatment regimen.

Genotype	**Regimen-1(docetaxel-cisplatin / docetaxel-carboplatin)**							
DNMT3B	Cases (30) n%	Dead (26) n%	Alive (4) n%	Median OS (months)	HR (95% CI)	Log P	HR' (95%CI)	P value
GG	16(53.33)	16(61.53)	-	4.58	-	-	-	-
GT	14(46.66)	10(38.46)	4(100)	12.3	0.40 (0.18-0.89)	0.01	0.36 (0.14-0.94)	0.03

The patients were analyzed for their OS by stratifying them on the basis of the chemotherapeutic regimen as demonstrated in table 5.14. On analysis of 30 patients treated with docetaxel-cisplatin / docetaxel-carboplatin (regimen 1), 86.66% (26) patients were dead and 13.33% (4) were alive. Based on the genotypic distribution, it was seen that

among 16 patients having the wild *DNMT3B* gene, 61.53% (16) were dead and no one was alive whereas in 14 patients carrying the heterozygous *DNMT3B* genotype, 38.46% (10) were dead and 100% (4) were alive. The overall survival period in patients with the heterozygous *DNMT3B* genotype was found to be higher as compared to patients with the wild *DNMT3B* genotype (12.3 vs. 4.58 months), HR=0.40; log rank p=0.01. A significant association was observed between *DNMT3B* genotype and applying the Cox multivariate hazard proportional analysis (HR'=0.36; 95%CI=0.14-0.94; *p*=0.03). It was observed that these patients have a significantly lower probability of death as compared to wild genotype patients receiving the above mentioned chemotherapeutic regimen as shown in figure 5.16.

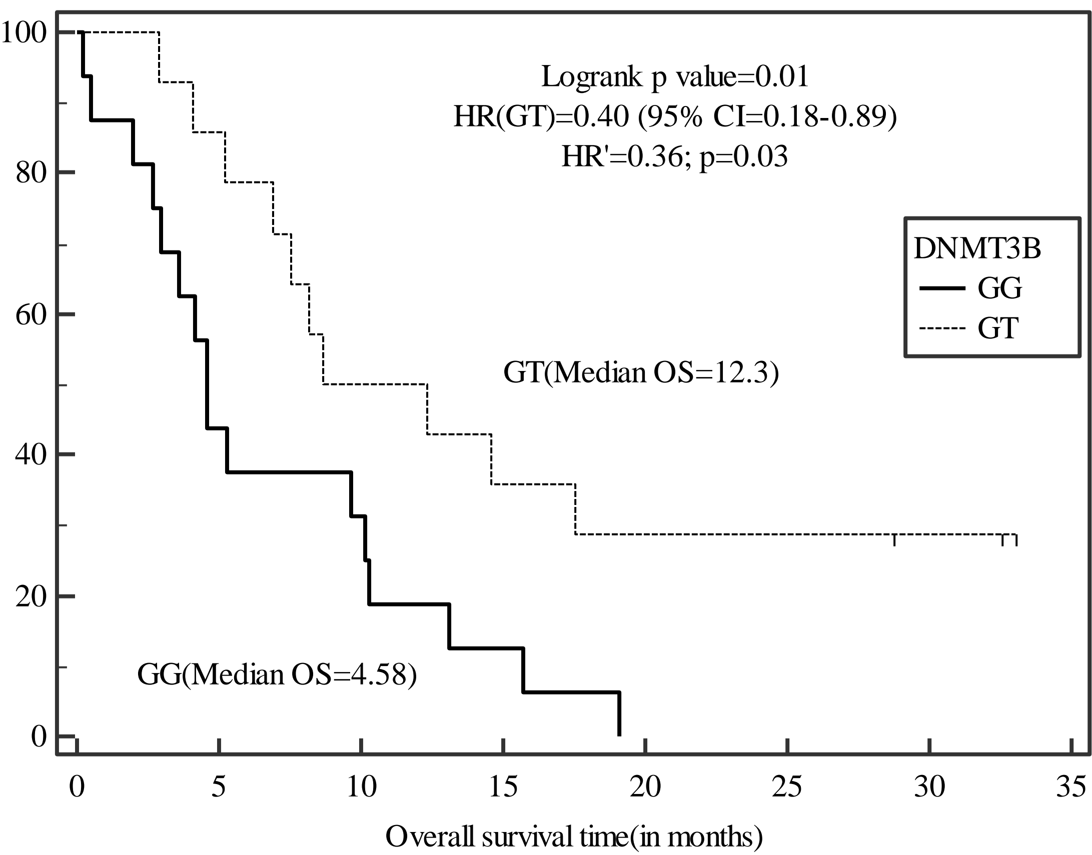

Figure 5.16 Effect of *DNMT3B* polymorphism on OS in patients receving docetaxel-cisplatin / docetaxel carboplatin therapy

Table 5.15. Genotypic frequency of *DNMT3B* gene among cases and their associated death risk along with overall survival on different treatment regimen.								
Genotype	**Regimen-5 (irinotecan-cisplatin / irinotecan-carboplatin)**							
DNMT3B	Cases (15) n%	Dead (13) n%	Alive (2) n%	Median OS (months)	HR (95% CI)	Log P	HR' (95%CI)	P value
GG	13(86.66)	11(84.61)	2(100)	6.3	-	-	-	-
GT	2(13.33)	2(15.38)	-	4.21	1.93 (0.27-13.5)	0.36	3.71 (0.54-25.37)	0.18

Further, Out of 15 patients treated with irinotecan-cisplatin / irinotecan-carboplatin (regimen 5), 86.66% (13) patients were dead and 13.33% (2) were alive. Stratified on the basis of genotypic distribution, it was found that out of 13 patients having the wild *DNMT3B* gene, 84.61% (11) were dead and 100% (2) were alive whereas out of 2 patients carrying the heterozygous *DNMT3B* genotype, 15.38% (2) were dead and no one was alive. The overall survival period in patients treated with irinotecan-cisplatin / irinotecan-carboplatin carrying the heterozygous *DNMT3B* genotype was found to be smaller (4.21 months) than the survival period in patients having the wild *DNMT3B* genotype (4.21 vs. 6.3 months), HR=1.93; log rank p=0.36. However, on applying the Cox multivariate hazard proportional analysis, no association was observed between *DNMT3B* genotype (HR'=3.71; 95%CI=0.54-25.37; *p*=0.18) and the overall survival of patients treated with irinotecan-cisplatin / irinotecan-carboplatin. This is shown in figure 5.17.

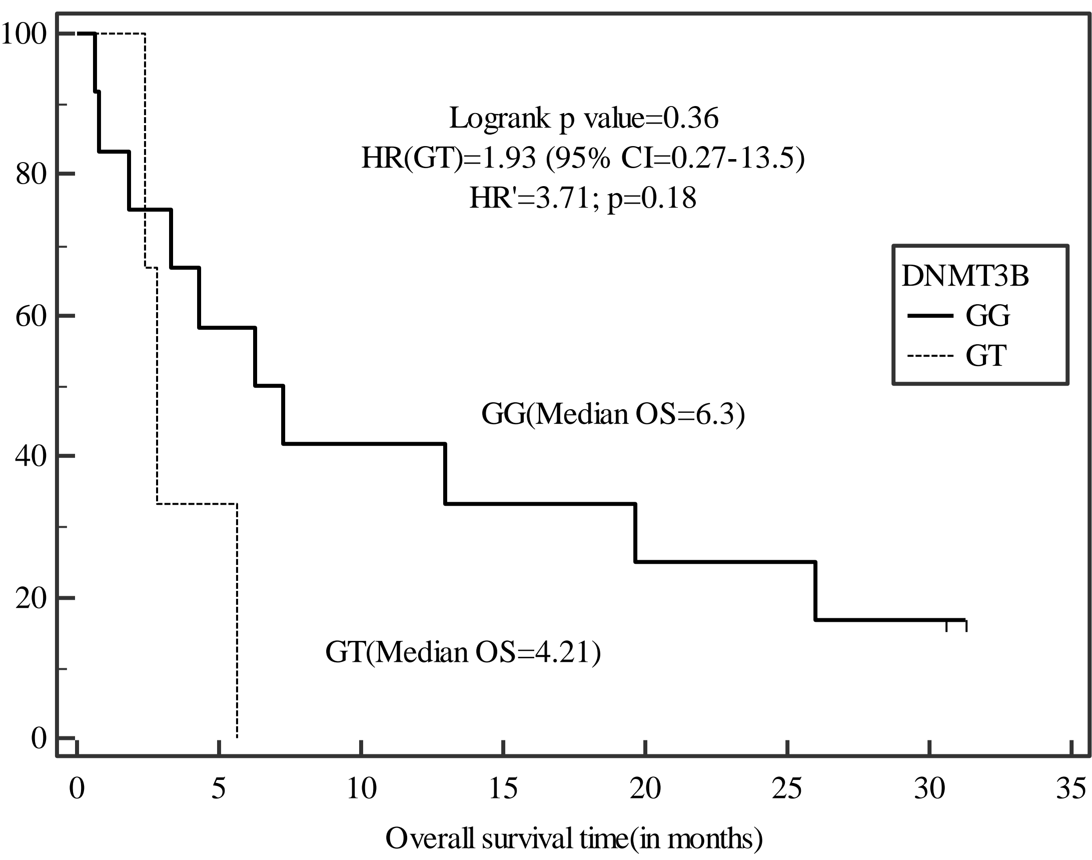

Figure 5.17 Effect of *DNMT3B* polymorphism on OS in patients receving irinotecan-cisplatin /irinotecan- carboplatin.

Table 5.16. Genotypic frequency of *DNMT3B* gene among cases and their associated death risk along with overall survival on different treatment regimen.								
Genotype	**Regimen-6 (pemetrexed-cisplatin / pemetrexed-carboplatin)**							
DNMT3B	Cases (28) n%	Dead (22) n%	Alive (6) n%	Median OS (months)	HR (95% CI)	Log P	HR' (95%CI)	P value
GG	18(64.28)	15(68.18)	3(50)	12.06	-	-	-	-
GT	10(35.71)	7(31.81)	3(50)	9.46	0.91 (0.37-2.22)	0.85	1.67 (0.48-5.82)	0.42

Similar, on analyzing the cases treated with pemetrexed-cisplatin / pemetrexed-carboplatin, it was observed that out of 28 patients, 78.57% (22) were dead and 21.42% (6) were alive. On studying the genotypic effect of *DNMT3B*, it was found that out of 18 patients having the wild *DNMT3B* gene, 68.18% (15) were dead and 50% (3) were alive. Out of 10 patients carrying the heterozygous *DNMT3B* genotype, 31.81% (7) were dead and 50% (3) were alive. The overall survival period of patients treated with regimen 6 and carrying the wild *DNMT3B* genotype was found to be higher wild *DNMT3B* genotype (12.06 months) as compared to the patients carrying the heterozygous *DNMT3B* genotype (12.06 vs. 9.46 months), HR=0.91;5 log rank p=0.85. On applying the Cox multivariate hazard proportional analysis, the adjusted hazard ratio was found to be non-significant (HR'=1.67; 95%CI=0.48-5.82; *p*=0.42) as shown in figure 5.18.

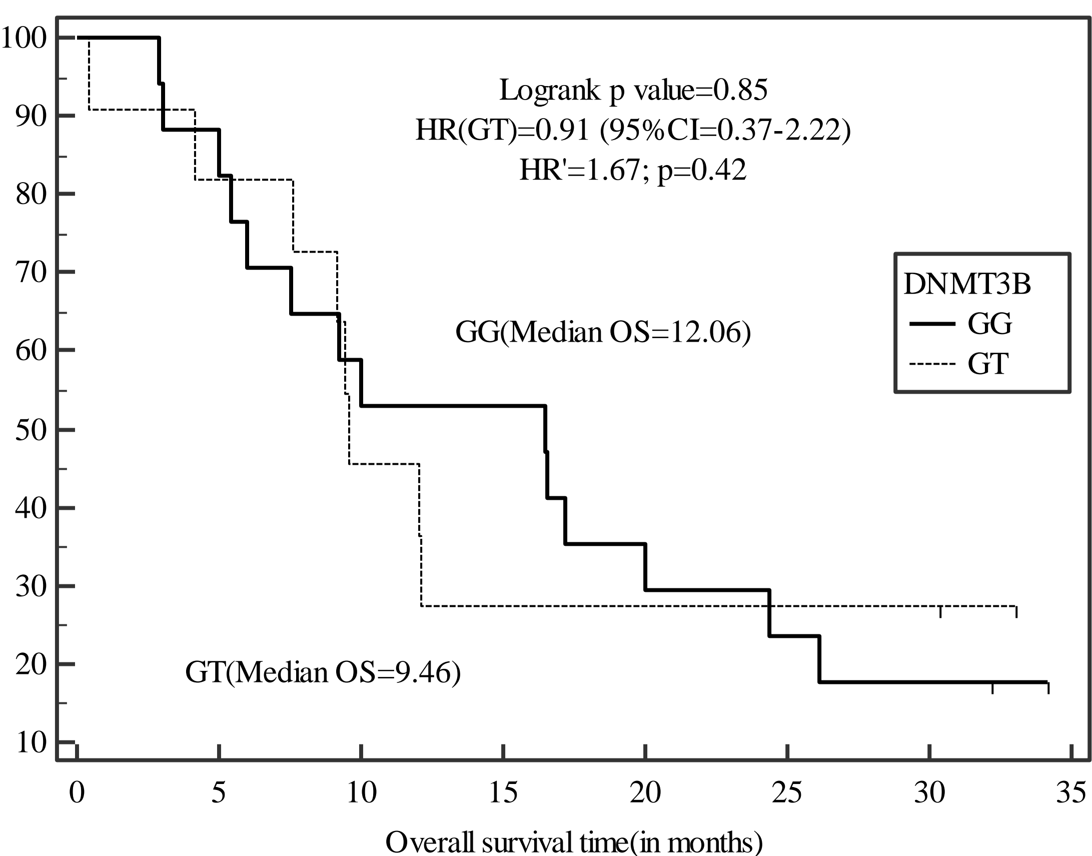

Figure 5.18 Effect of *DNMT3B* polymorphism on OS in patients receving pemetrexed-cisplatin / pemetrexed-carboplatin

5.6 *DNMT3B* genotypic distribution with clinic-pathological parameters

Table 5.17 Representing relationship of *DNMT3B* genotype with clinical-pathological parameters

	Primary Tumor Extension		**AOR (95% CI)**	**P**	**Lymph Node Invasion**		**AOR (95% CI)**	**P**	**Metastasis**		**AOR (95% CI)**	***p***
Genotype	**T_1+T_2 (22) n%**	**T_3+T_4 (73) n%**			**N_0 (10) n%**	**N_1+N_2+N_3 (85) n%**			**Mo (50) n (%)**	**M_1(45) n (%)**		
GG	16 (72.72)	43 (58.90)	Reference		6(60)	53(62.35)	Reference		28 (56)	32(71.11)	Reference	
GT	6 (27.27)	30 (41.09)	2.01 (0.68 - 5.92)	0.20	4(40)	32(37.64)	0.90 (0.22 - 3.69)	0.89	22 (44)	13(28.88)	0.55 (0.20-1.45)	0.22

The patients were analyzed on the basis of clinic-pathological parameters and the data is summarized in table 5.27, it was observed that, while considering 95 patients, 23.15% (22) had moderately differentiated tumors (T0+T1+T2) while 76.84% (73) had fully differentiated tumors (T3+T4). Further stratified on the basis of genotypic distribution of *DNMT3B,* it was found that among 59 patients having wild *DNMT3B* genotype, 72.72% (16) had moderately differentiated tumors and 58.90% (43) of the cases had well differentiated tumors. In 36 patients with the heterozygous genotype, only 27.27% (6) showed moderately differentiated tumors while well differentiated tumors were dominant in 41.09% (30) of the cases. However, no significant relationship was found between the primary tumor extension and the *DNMT3B* genotype (AOR=2.01; 95%CI=0.68-5.92; *p*=0.20).

Among 95 patients, 52.63% (50) were characterized with absence of metastasis whereas 47.36% (45) showed distant metastasis. On the basis of genotypic distribution, it was found that among 60 patients having the wild DNMT3B gene, 56% (28) of the patients had no metastasis whereas 71.11% (32) showed distant metastasis and in 35 patients having the heterozygous DNMT3B genotype, 44% (22) patients showed no metastasis whereas 28.88% (13) showed distant metastasis. This relationship was supported by regression analysis showing that the patients carrying heterozygous DNMT3B genotype had no association of having distant metastasis (AOR=0.55; 95%CI=0.20-1.45=; *p*=0.22).

Further, on characterizing 95 patients on the basis of lymph node invasion it was found that 10.52% (10) were characterized with the absence of lymph node invasion (N0) while 89.47% (85) showed lymph node invasion (N1+N2+N3+N4). When classified on the basis of genotypic distribution, it was found that among 59 patients having the wild DNMT3B gene, 60% (6) showed no invasion of the lymph nodes whereas 62.35% (53) showed lymph node invasion. Also, in 36 patients having the heterozygous DNMT3B genotype, 37.64% (32) showed lymph node invasion while only 40% (4) showed absence of lymph node invasion. However, this relationship could not be justified by regression analysis (AOR=0.90; 95%CI=0.22-3.69; p=0.89).

CHAPTER-6

DISCUSSION

DNA methyltransferase 3B (*DNMT3B*) is an essential enzyme for the maintenance and establishment of genomic methylation patterns. It is also essential during development in these organisms for genome-wide de-novo methylation. In the mammalian genomic DNA, methylation is catalyzed by DNA methyltransferases (*DNMTs*). In several cancers, polymorphism of the *DNMT3B* gene may influence *DNMT3B* activity on DNA methylation. Expression of this DNA methylation enzyme is found to be increased significantly in different types of cancers such as colon cancer, breast cancer, prostate cancer, stomach cancer. There are different variants associated with this gene. Certain polymorphisms in the *DNMT3B* gene can increase the promoter activity in lung cancer. So in the present study, we investigated the influence of *DNMT3B* polymorphism (G579T) on overall survival of lung cancer patients in a hospital-based case study recruiting 100 subjects. This study has been conducted on the north Indian population to check the effect of *DNMT3B* gene polymorphism (G579T) on the overall survival of lung cancer patients based on their histological types, smoking status, gender, clinic-pathological parameters and also to assess the relationship of different clinic-pathological parameters with this polymorphism.

In present study, the prognostic effect of this polymorphism was analyzed. It was evident that median survival time of heterozygous subjects (CT) was more than the reference (CC) (8.20 vs. 6.23). Also, in this case, HR is 0.79 which showcases protective effect; as value of HR is below than 1 with a *p*-value in this case is 0.03. It shows a significant relation with *DNMT3B* genetic polymorphism and the heterozygous subjects exhibit a better survival rate. The stratified analysis on the basis of histology suggested that only SCLC (HR'=16.27; p-value=0.04) and SQCC (HR'=0.04; p value=0.01) patients showed a significant association between the heterozygous (GT) genotype and overall survival. However, there is no association in case of ADCC patients (HR'=2.12; p value=0.07). Further, in the analysis based on gender, it was observed that there was no significant association between the heterozygous genotype neither in males (HR'=0.72; p value=0.23) nor in females (HR'=2.11; p value=0.35) with the overall survival.

Next, for the smoking status i.e. in smokers (HR'=0.76; p value=0.32) and in non-smokers (HR'=1.27; p value=0.67), no significant relation was observed between the heterozygous genotype with overall survival. Similarly, when patients were stratified on the basis of performance status it was observed that there is no significant association between the heterozygous genotype of patients having KPS(0-1) (HR'=0.89; p value=0.66) and patients having KPS(2) (HR'= 0.14; p value=0.30) and also in case of ECOG, no significant association was observed as patients having ECOG(0-1) has HR'=1.08; p value=0.84 while ECOG(2) has HR'=0.76; p value=0.45.

This SNP of *DNMT3B* (-579 G>T, rs1569686) is localized in the *DNMT3B* gene promoter. In exon 1A and1B, two transcriptional start site of *DNMT3B* gene are located. The expression of the gene is regulated by two promoters. Both promoters are found in CpG regions-one in CpG-rich region and other is in CpG –poor region. This *DNMT3B* polymorphism is located in CpG-poor promoters which is 579 base pair away from exon 1B transcription site. This polymorphism did not effect on transcriptional activity of the *DNMT* promoter but this affects the function of the gene (Perfilyeva A1 *et* al).

There are very limited studies which evaluated the role of this polymorphic site in predicting the survival of cancer patients. According to a study conducted by Chuan Wang *et al*., on 422 gastric cancer patients, it was found that the individuals with rs1569686 variant genotype (TG/GG) were significantly associated with poor prognosis in gastric cancer compared to those carrying the TT genotype (HR=1.43,95% CI=1.02-1.99). On the other hand, the findings from the present study determined a better survival rate in case of heterozygous individuals. Similar results were observed for the G allele carriers of rs4911107 and allele carriers of rs4911259. In another SNP rs8118663; GG carriers were found to have a shorter life span than AA/AG genotype (HR=2.72, 95%CI=1.45-5.12). No association was found between each of these SNPs and *DNMT3B* expression.

In an another study conducted by Abul Kalam Azad *et al*., on head and neck cancer patients, it was observed that *DNMT3B* polymorphism 149C>T is associated with survival of head and neck cancer patients (TT(HR)=1.49 (1.15-1.95),p=0.003). This indicates that this gene plays a crucial role in carcinogenesis because by incorporating de novo hyper methylation of promoter in CpG islands, this feature acts as a perfect mechanism for tumor suppressor gene inactivation in human cancer cells.

Also, there are many evidences which support the role of expression of *DNMT3B* gene in predicting the overall survival and affecting the clinical outcomes of the patients. According to Xing *et al.*, mRNA expression of three methylation-regulating gene (*DNMT1, DNMT3B*, *MBD2*) in 148 tumor samples from patients with non-small cell lung cancer (NSCLC) was evaluated and then determined their prognostic values, then it was observed that high level of DNMT1 expression was significantly associated with an increased risk of death in all NSCLC patients (HR =1.74 (1.04–2.90). However, the high level of *DNMT3b* expression was significantly associated with poor prognosis only in young patients (<65 years).

In another study conducted by Niederwieser *et al.*, *DNMT3B* expression was tested in 210 older (≥60 years) adults with primary, cytogenetically normal AML. In this study it was observed that high *DNMT3B* expression associates with lower CR rates and shorter DFS and OS in chemotherapy-treated CN-AML patients aged ≥60 years (Niederwieser et *al.*,2015). Hayette *et al.*, did not find significant differences in OS between high and low *DNMT3B* expressers, high *DNMT3B* expressers had a shorter survival than low *DNMT3B* expressers in 191 AML patients analyzed. Germain *et al.*, have recently demonstrated that DNA methylation levels in leukemic blasts from CN-AML patients are not influenced by *DNMT3B* expression since mainly inactive splice variants of *DNMT3B* are expressed in these cells.

Next, according to Supic *et al.*, Association of *DNMT1* (A201G, rs2228612) and *DNMT3B* (C501T, rs406193) gene polymorphisms with the clinic pathological features and survival Polymorphisms in *DNMT1 (*A201G, rs2228612) and *DNMT3B* (C501T, rs406193) were analyzed in 99 OSCCs. The elevated mRNA levels of *DNMTs* were not significantly associated with gender, age, smoking status, or alcohol use. Higher DNMT3B mRNA levels were found to be more prevalent in females than in males, p=0.092. So, this analysis did not show statistically significant correlation between the DNMT1 and DNMT3B SNPs and corresponding clinical parameters (Supic *et al.*, 2017).

However, there have been many reports about the role of this polymorphism (579G>T) in predicting cancer predisposition. In a study conducted on Colorectal Cancer Risk in Kazakhstan Population, it has been revealed that over expression of *DNMT3b* were significantly associated with a higher incidence of lymph node metastasis. A meta-analysis of 24 case-control studies indicated that *DNMT3B* G39179T polymorphism is associated

with risk of different types of cancer in Asians (OR=0.68, 95% CI=0.53-0.87 for GT vs. TT) but not in Europeans OR=0.82, 95% CI=0.63-1.07 for GT vs. TT). They hypothesized that the mutant T allele can suppress the functions of the methyltransferase enzyme which leads to disruption in the traditional process of tumor suppressor gene methylation in cancer cells. Thus, the T allele can reduce the risk of colorectal cancer development (Perfilyeva A1 *et al.*, 2015).

In the next study by Hong Fan *et al., DNMT3B* polymorphisms on the risk of esophagus cancer in a hospital-based case-control study were tested. They investigated the influence of 579 G>T polymorphism in the *DNMT3B* gene on the risk of esophagus cancer. Individuals carrying G allele in the DNMT3B gene were found to have a nearly consistent risk of EC compared with those carrying T allele. Then we stratified the results by sex and age, patients and controls. Combined GG and GT genotypes showed no significant association between *DNMT3B* 579 G>T polymorphism and risk of esophagus cancer, suggesting that 579 G>T polymorphism in the *DNMT3B* gene cannot be used as a marker of genetic susceptibility to esophagus cancer even in young individuals. This study showed that *DNMT3B* polymorphism was not associated with the risk of esophagus carcinoma (Fan *et al.,* 2008)

Su Jeong Lee *et al*., conducted a study on lung cancer patients and it was observed that carriers with283T and579G alleles were at decreased risk of lung cancer as compared with individuals having 283C and 579T alleles (Lee *et al*., 2005). Shen *et al*., reported an association between *DNMT3B* 46359C4T polymorphism and lung cancer risk in non-Hispanic whites. They found that the heterozygous CT genotype was associated with a significantly increased risk for lung cancer as compared with the homozygous CC genotype.

Present study explores another clinical manifestation of this polymorphic site which is probably the first attempt. Evidence suggests that DNMT activity and aberrant promoter methylation can be modulated differentially in lung cancer by specific carcinogens and cell types exposed to the carcinogen. Hence, there is a great need to assess the interaction of this SNP which affects the activity of this enzyme with various environmental factors and further this study is an preliminary attempt to investigate the role of this variant in predicting the clinical outcome. Further, studies with larger samples size and different ethnicities are required to validate these findings.

CONCLUSION

- In the present study, genotypic distribution of *DNMT3B* (-579 G>T, rs1569686) polymorphism was studied in North Indian population. In the overall distribution, 100 patients, we observed 62% wild, 37% hetero and 1% of mutant genotype.

- A significant association was observed in overall survival with *DNMT3B* genotype (HR'=1.45; 95% CI=1.02-2.08; P=0.03). Significant association was also found with overall survival in SCLC patients (HR'=16.27; 95%CI=1.13-232.4; P=0.04) and in SQCC patients (HR'=0.40; 95% CI=0.19-0.86; P=0.01).

- Therefore, from this study this concluded that this polymorphism is capable for predicting the overall survival of heterozygous patients and also, shows some interesting relationship of this genotype with overall survival of SCLC and SQCC patients.

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APPENDIX-1

1. 0.5M EDTA: Dissolved 9.306g of disodium salt of EDTA in 20ml of deionised water, and then adjusted the pH to 8.0 by 1 M sodium hydroxide. Sterilized the solution by autoclaving.

2. 10% SDS: Dissolved 1g of SDS in 10ml of deionised water.

3. 100mM Tris-Cl (pH 8.0): Dissolved 0.32g of Tris-Cl in 10 ml of deionised water, then adjusted the pH to 8.0 by 1M sodium hydroxide. Sterilized the solution by autoclaving.

4. 10mg/ml Proteinase K: Dissolved 10mg Proteinase K in 1ml of double distilled water. Sterilized the solution by autoclaving.

5. 1mg/ml BSA: Dissolved 100mg of BSA in 100ml of deionised sterile water and kept at 4 C overnight.

6. 5M Sodium chloride (NaCl): Dissolved 5.85g of sodium chloride in 20ml of deionised water. Sterilized the solution by autoclaving.

7. 5X TBE buffer: Dissolved 54g of Tris base and 27.5g of boric acid in 980ml of double distilled water and then added 20ml of 0.5 EDTA. Sterilized the solution by autoclaving.

8. Ethidium Bromide (10mg/ml): Dissolved 1g of ethidium bromide in 100ml of water. Mixed the solution properly.

9. Magnesium chloride (MgCl2) (100mM): Dissolved 0.41gms of MgCl2 in 20ml of deionised water and sterilized by autoclaving.

10. Sucrose (1M): Dissolved 3.41 g of sucrose in 10 ml of deionised water and sterilized by autoclaving.

11. TE buffer (pH 8.0): Added 1ml of 100mM Tris-Cl (pH 8.0) and 200 µl of 0.5M EDTA solution to 8.8 ml of deioinsed water. Sterilized the solution. 12. Triton X- 100 (10%): Took 100 µl of TritonX-100 and mixed with 900 µl of deionised water and mixed properly.

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