2.1 Cancer

Cancer remains a global killer with a shifting burden from the developed to the developing countries (Jemal et al., 2008). According to the WHO (fact sheet no. 297), cancer accounted for 7.6 million deaths in 2008 which was around 13% of all deaths that year and this death toll is ever increasing. Cancer related deaths worldwide are continuing to rise and speculated to be 13.1 million at the end of 2030.

Cancer is widely assumed as a heterogeneous group of disorders, in which an altered/abnormal cell is characterized by uncontrolled growth and spread. The spread of tumors is called metastasis and is the major cause of cancer related deaths; it is a process by which a malignancy spreads from a primary to a distant site. As per metastatic characteristics, tumor may be non-cancerous (benign) or cancerous (malignant). Cancer cells break away from the primary site of tumor and travel in the body through circulatory or lymphatic system and assist the tumor growth in another area of the body this process is known as metastasis and invasion of tumor cell to other body parts (Raubenheimer & Noffke, 2006).

Development of cancer is not a single step process but it occurs through a series of clonally selected ‘genetic’ or ‘epigenetic’ changes in key oncogenes and TSGs. These genetic and epigenetic abnormalities give limitless potential of a cancer cell for evading normal pathway of division, differentiation, apoptosis and inhibition. Whatever be the origin or cause of cancer development, cancer cells have 6 hallmarks which are the common feature for all cancer types. These include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. Reprogramming of energy metabolism and evading immune destruction are the two more hallmarks recently proposed for cancer cell. Along with these abnormal behaviours of cancer cell a tumor increases the complexity in having stock of recruited cells that creates the “tumor microenvironment” and help to acquire hallmark traits (Gerhauser, 2013; Hanahan & Weinberg, 2011; Szic, 2011).
The induction of cancer depends on inherited and acquired susceptibility factors, on the exposure to initiation factors (exogenous and endogenous carcinogens), and on the promotion and progression factors. Two types of factors are responsible for occurrence of cancer (carcinogenesis). External factors (tobacco, chemicals, radiation, and infectious organisms) and internal factors (inherited/acquired mutations genetic and epigenetic, hormones, immune conditions, and mutations that occur from metabolism) both are causes of cancer. These causal factors may act together or in sequence to initiate or promote the development of cancer. Several years often pass between exposure to external factors and detectable cancer. Aging is also a fundamental cause of cancer development (Anand et al., 2008; Rosman-Urbach et al., 2006; Kikuchi et al., 2004; Peltomaki et al., 2001).

2.2 **Lung cancer**

Lung cancer is among the most common and lethal forms of cancer in both male and female. Tobacco use is a single major cause for the development of lung cancer. Epidemiological data show that 80% of all lung cancers patients worldwide have smoking history (Ezzati & Lopez, 2003). Only a 15% of lung cancer cases occur in non-smoker and these cases are often attributed to a combination of genetic factors, radon gas, asbestos, pesticides and air pollution including passive and static smoking (Behera, 2012; Ezzati & Lopez, 2003). Near about 5 million deaths in the world every year are attributed to tobacco use with 2.41 million deaths occurring in developing countries and 2.43 million in developed countries. In Indian context 1 million of total lung cancer death of the current 5 million deaths in the world, and 2.41 million deaths in developing countries are contributed by India (Behera, 2012; Thankappan & Thresia, 2007; Ezzati & Lopez, 2003) and, this death toll is estimated to be 1.5 million in 2020. Lethality of lung cancer and low survival rate pertains to its late diagnosis, low clinical response or development of multiple drug resistance. However, the reasons behind it are not fully understood (Incoronato et al, 2011).

2.2.1 **Types of lung cancer**

The lung is a spongy tissue. Its significant portion of solid mass is made up of the bronchi, bronchioles and alveoli. There are 300 million alveoli and over a million bronchioles, and
over 95% of lung cancer is alveolar or bronchogenic carcinoma. There are 40 types of cells in the lung. Four amongst these are unique for the lung and include non ciliated bronchiolar cells (Clara cells), squamous cells (Type I), great alveolar cells (Type 2), and alveolar macrophages. The Clara and squamous cells – present in bronchial epithelium, contribute to the secretion of airway mucus. Depending upon its origin from a certain cell type, Lung cancer can be divided into two major histopathological groups non small cell lung carcinoma & small cell lung carcinoma (NSCLC and SCLC) (Travis et al., 2011; Sutherland & Berns, 2010; Herbst et al, 2008; Collins et al., 2007; Schiller, 2001; Van Zandwijk et al., 1995).

2.2.1.1 Non small cell lung cancer (NSCLC)

NSCLC divided into the following types (Travis et al., 2011, 2002)

- **Squamous cell carcinoma:** it arises from the cells lining the bronchi (Squamous cells). Squamous cell carcinoma used to be more common, but now accounts for roughly 30% of non-small cell lung cancers in western countries. It usually starts in the bronchial tubes, centrally in the lungs. It has been speculated that filtered cigarettes have caused the decline in squamous cell lung cancer and the adenocarcinoma is more common now since toxins are inhaled deeper into the lungs.

- **Adenocarcinoma:** it arises from glands and cells lining the alveoli (Type II cells). Adenocarcinoma is the most common form of non-small cell lung cancer accounting for up to 50% of cases lung cancer (Figure-1). It starts in the periphery of the lungs and can be present for a long time before it is detected. A less common form of adenocarcinoma is bronchioloalveolar carcinoma (BAC). This type of lung cancer arises in the small air sacs in the lungs. It responds fairly well to some of the new lung cancer treatments called targeted therapies (Travis et al., 2011).

- **Large cell carcinoma:** It accounts for only 10% of total lung cancers (Figure-1). It is a form of adenocarcinoma but the cells appear much larger on the microscope. (Travis et al., 2011; Herbst et al, 2008; Collins et al., 2007; Van Zandwijk et al., 1995).
Figure 1: Incidence of different types of lung cancer, WHO report (fact sheet no 297).
2.2.1.2 Small cell lung cancer (SCLC)

Small cell lung cancer (also called oat cell carcinoma) accounts for 15-20% of lung cancers (Figure-1). Unlike the more common non-small cell lung cancers, small cell lung cancer grows rapidly, but often responds well to chemotherapy initially. Unfortunately, it tends to become more resistant to treatment as the disease progresses. Small cell lung cancers usually begin in the large airways (bronchi) of the lungs, but spread early, often to the brain. They are broken down into only two stages, limited and extensive stage small cell lung cancer. 60 to 70% of people already have extensive stage disease at the time of diagnosis. Most cases of small cell lung cancer are due to smoking. In India SCLC is prevalent over NSCLC but in big cities NSCLC is slowly prevailing (Travis et al., 2011; Herbst et al, 2008; Collins et al, 2007; Schiller, 2001).

2.2.2 Lung cancer in non-smokers

Lung cancer in non-smokers is more common than many people realize. In fact, lung cancer in never-smokers is now considered the 6th most common cause of cancer deaths. Though we lump smokers and non-smokers together when discussing lung cancer, but lung cancer in non-smokers are a different disease in many ways. Overall, 10-15% of lung cancers occur in non-smokers. Two-thirds of the non-smokers who get lung cancer are women, and 20% of lung cancers in women occur in individuals who have never smoked. This percentage is significantly higher in Asian women (Yano et al, 2011; Samet et al., 2009; Subramanian & Govindan, 2008). Usually adenocarcinoma found in non-smokers, and is the most common type seen in women. At genetic, cellular, and molecular level lung cancer biology in non-smokers especially in women are different than lung cancer in smokers. This means that the changes in cells that make them lung cancer cells are different at all levels, from the genetic blueprint that tells cells when to divide and grow, to the way that the cells function and communicate with other cells.

Currently, a similar treatment is adopted for lung cancer in smokers and non-smokers. As we learn more about the differences between cancer in smokers and non-smokers, this could change the strategy and outcome of lung cancer treatment in smokers and non-
It is likely that estrogen plays a role in the development and progression of lung cancer and research is being done to define this further. Ovaries removal and treatment with estrogen and progesterone (hormone replacement therapy) after menopause may increase the risk lung cancer death in women (but not increase the risk of lung cancer) (Yano et al, 2011; Samet et al., 2009; Rudin et al., 2009; Subramanian & Govindan, 2008; Bryant & Jams, 2007).

### 2.2.3 Current scenario of lung cancer control

Various treatments for lung cancer are available today that depends upon the stage and type of lung cancer. Early detected lung cancer may be cured with available treatments. When lung cancer detected at advanced stage treatment is more palliative than curative which can improve survival and decrease the pain and discomfort of cancer (Molina et al., 2008; Sher et al., 2008; Collins et al., 2007).

Lung cancer is dealt with basically three kinds of treatments:

- **Surgery**: Surgery is done when lung cancer is detected before it metastasizes beyond the lungs. Mostly surgery can be curative in case of non-small cell lung cancer.

- **Radiation therapy**: Use of high-energy radiation to kill cancer cells and shrink tumors is called as radiotherapy. SCLC and NSCLC both are treated with radiation therapy, which is often combined with chemotherapy, surgery or both. Radiation damage DNA and cause cytotoxic or cytostatic effect. The cancer cell is more susceptible to damage due to their high dividing nature than normal cell. Healthy cells are also affected but are better able to repair the damage. There are side effects of this therapy which includes irritation, hair loss, cardiac toxicity and even secondary cancers (Tyldesley et al., 2001).

- **Chemotherapy**: Chemotherapy is the use of cytotoxic drug to kill cancer cells or makes them quiescent. There are various side effects of chemotherapy due to its
cytotoxic effect but still it is a potent means to reduce the cancer cells. For lung cancer some common medications used are: Platinol (cisplatin), Paraplatin (carboplatin), Taxotere (docetaxel), VePesid (etoposide), Adriamycin (doxorubicin) etc (Gerber et al., 2012; Stinchcombe & Socinski, 2009).

Targeted therapies for lung cancer: Overall 5 year survival with lung cancer is less than 15% and this situation has not changed since last 30 years with conventional therapeutic approaches (Cagle & Chirieac, 2012; Larsen et al., 2011; Jemal et al., 2008). After the discovery of specific small molecule inhibitors of various pathways conceptualize targeted therapy which brought hope to reduce suffering and mortality in patients with lung cancer.

In targeted therapy a small molecule is used specifically to inhibit a biomolecule of interest. Selection of therapeutic agents is based on genetic mutations and signaling pathways altered in lung cancer. In the last 10 years, 4 targeted therapies have been approved for treatment of lung cancer: gefitinib in 2002 (EGFR inhibitor), erlotinib in 2003 (EGFR inhibitor), bevacizumab in 2006 (vascular endothelial growth factor-A inhibitor), and crizotinib in 2011 (anaplastic lymphoma kinase and ROS1 inhibitor). Researchers are ongoing to increasing the number of such agents in the near future (Cagle & Chirieac, 2012; Thomas et al., 2012; Larsen et al., 2011; Heist & Christiani, 2009). There is tremendous potential in targeted therapies for treating cancer in combination with conventional therapies. Personalized medicine may help to solve the purpose. But for that there is need to explore more molecular targets and abnormalities in lung cancer or any cancer. Each and every cancer type or their sub types are different from each other in having different molecular abnormalities. More knowledge about the biology of cancer is the only way which could be helpful in deciding therapy or prevention of a particular cancer. Intervention with chemopreventive agent, either natural or synthetic origin, is also used to search the newer targets for cancer control. The majority of cancer researches, now a day, are focusing on this point and searching different pathways
and their involvement in the various processes of tumor development (Cagle & Chirieac, 2012; Thomas et al., 2012; Larsen et al., 2011; Heist & Christiani, 2009).

2.2.4 New pathways in cancer control

Newer pathways involving epigenetics (Ho et al., 2013), inflammation (Rayburn et al., 2009) and cell signaling (Cagle & Chirieac, 2012) are being targeted for cancer therapeutic purposes. Cell signaling molecule receptor tyrosine kinase (RTKs) serve as potential therapeutic targets in several solid tumors, including lung cancer. Epigenetic inhibitor of DNA methylation (Azacytidine DNMT inhibitor), Histone modifiers (Vorinostat HDAC inhibitor) is under clinical trial or under use for treating cutaneous T cell lymphoma (CTCL) (Azad et al., 2013; Ho et al., 2013). Non steroidal anti inflammatory drugs (NSAIDs) drug have been used in some part of cancer treatment for reducing pain. Research in the recent past, invivo, in vitro or epidemiological research revealed that epigenetic and inflammation strongly associated with cancer development and advancement. Their inhibition is showing a promising future of cancer detection, therapeutics, prognosis and prevention (Rayburn et al., 2009). Combination therapies are now emerging field for fighting against cancer (Juergens et al., 2011).

2.3 Epigenetics

Conrad Hal Waddington introduced the term epigenetics as the study of those processes involved in the unfolding of development (Waddington, 1942). Epigenetics was redefined again in the late 1980s as the study of heritable traits that are not dependent on the primary sequence of DNA. The field of epigenetics grew rapidly after knowing its importance in regulating chromatin structure and gene expression (Goldberg et al, 2007; Cavalli, 2006). Researches in last decades reveal that epigenetics does not only affect the individual gene expression but also plays important roles in the maintenance and global shaping of developmental patterning of an organism. This involves both maintenance of the cellular memory, required for developmental stability, and cell type specific changes during development of patterning in organogenesis (Feinberg et al., 2006). Epigenetic changes are important for development, maintenance and survival of tissue and specific functions of a
cell. Each and every cell has its epigenetic blue print which was inherited by its progeny (Lund & Lohuizen, 2004).

Epigenetic changes involve methylation at 5th position of cytosine in DNA and modification of histone protein by phosphorylation, acetylation and methylation. These covalent changes in DNA sequence and histone protein alter the cellular gene expression, by modifying chromatin structure and transcription factor binding, without changing DNA sequences (Chuang et al, 2007). Epigenetic mechanisms tightly regulate gene expression via chromatin remodeling. Modulation between DNA methylation and histone modification can turn on or off gene transcription by allowing or disallowing transcription factor binding (Goldberg et al, 2007).

### 2.3.1 Molecules that regulate epigenetic changes

Various molecules are involved in maintaining the epigenetic of a cell. DNA methyltransferases are the enzymes which methylated DNA at CpG sites. These are of three types DNMT1, DNMT3a, and DNMT3b (Plass & Soloway, 2002). Methyl binding domain proteins sense methylation mark of CpG sites and help to make repressor complexes. The methyl CpG binding domain proteins are MBD1-6 and MeCP2 (Ballestar & Wolffe, 2001; Wade, 2001). Histones and their modifying enzymes are important for making and maintaining chromatin structure. Histone modifying enzymes modify histone at lysine and serine amino acids and change its affinity toward chromatin for maintaining chromatin dynamics. These are histone acetylases, HDACs, HMTs, phosphorylases and sumoylating enzymes. Polycomb proteins that were identified in Drosophila make Polycomb repressor complex (PRC1 and PRC2) which modifies histone at specific positions and direct DNA methylation. Enhancer of zest 2 (EZH2) is an important member of PRC2 and functions as histone methyl transferases (Handy et al., 2011; Grau et al., 2011; Ducasse & Brown, 2006; Fischle et al., 2003). A pictorial representation of epigenetic regulation is given in figure-2.
Figure-2: Transcriptional repression by epigenetic mechanism
2.3.2 **Epigenetic pathways are interlinked**

DNA methylation and histone modifications are important and major change of epigenetic but does not function independently. DNA methylation works as a signal for other gene silencing proteins and events which function to render a gene inactive via a complex network. The experimental system revealed that proteins recruited on the methylated promoter sequences bring the gene silencing and change the chromatin conformation. Methyl CpG binding proteins bind to the methylated CpG sequences of DNA and recruited the HDACs and HP1 proteins. HDACs deacetylate histones, which condense the chromatin and form a repressor complex (Zou, X. et al., 2011; Gronbeak et al., 2007; Rolof, T.C. et al., 2003; Jones et al., 1998; Nan et al., 1997). Repressor complex inhibits interaction of transcription factors with their respective transcription sites. It is also evident from various reports that histone methylation directs DNA methylation through HP1 protein and DNMT1 (Jin et al., 2011). Various mechanistic studies have been performed to show the link between HMTs, DNMTs, histone methylation and DNA methylation.

It has been shown that a decrease in suppressor of Variegation 3-9 Homolog 1 (SUV39H1) can decrease in DNA methylation. G9a, HMT of H3K9, has also been linked to affect the methylation of DNA. H4R3 methylation interacts with DNMT3b and direct de novo methylation (Jin et al., 2011; Henckel A., et al., 2009; Esteve et al., 2006). Use of HDAC inhibitors revealed a tight link between HDAC regulation and DNA methylation (Sarkar et al., 2011). DNMT1, DNMT3a and DNMT3b recruited by Polycomb protein EZH2 on methylated H3K27 site and target corresponding DNA for methylation. SETBD1 interacts with DNMT3a/b and causes DNA methylation in cancer cells (Komashko & Farnham, 2010).

2.4 **Epigenetics and cancer**

It has been reported that the disturbance in epigenetic regulation can have pathological outcomes, which include immune disorders and cancer (Feinberg et al., 2006). Near about all types of human cancer have abnormal epigenetic, which evolves with genetic changes and progresses in cancer development. DNA hypomethylation was initially reported in
human tumor, but soon after hypermethylation of TSG, histone modification and mi RNAs were also found to be involved in human cancer (Esteller, 2008). Various TSGs and oncogenes have been shown to be under tight regulation of epigenetics. There is ever growing knowledge that showed interlink between genetic and epigenetic changes in cancer (Jones & Baylin, 2007; Ting et al., 2006). We cannot say which would come first, genetic or epigenetic, to initiate and progress cancer (Brower et al., 2011). We will sequentially review different aspect of epigenetic and their relation with cancer.

2.5 DNA methylation

The architecture of the nucleus and control of gene activity is critically regulated by DNA methylation. DNA methylation varies from species to species and between tissues. DNA methylation controls certain tissue specific genes and germ line genes like MAGE, which are not expressed in any tissue except malignant tumors (Sharma et al., 2010). 60-90% of cytosine residues within CpG dinucleotides are methylated in the genomic DNA of mammalian cells. In higher eukaryotes, spontaneous deamination of 5 methyl cytosine to thymine (T) make CpG pairs major mutational hotspots (Plewa & Jagodziński, 2005). The first exon and promoter of numerous genes contain CpG islands which are GC-rich (60-70%) DNA regions (Takai et al., 2003).

CpG methylation compacts the chromosome and causes the inactive state of chromatin. The compact chromosomal state is beneficial because it stabilizes DNA and not allow transposable element to break and translocate DNA. In contrast CpG Island of euchromatin remains unmethylated which facilitate transcription factor binding and transcription of housekeeping genes and regulatory proteins. In cancer, this controlled methylation state got disrupted and aberrant methylation pattern arise, which may be hypo or hyper methylation, and resulted in different consequences (Smet & Loriot, 2010; Wade, 2001; Pogribny & Beland, 2009; Kanai & Hirohashi, 2007; Pennings et al., 2005).
2.5.1 DNA hypomethylation and cancer

DNA hypomethylation has unpredictable consequences of cell fate. DNA hypomethylation is worthy to be studied because near about all types of cancer have this epigenetic phenomenon, but still its significance is not well understood. Now a day’s various strategies are under use, which employed the demethylating agent as cancer therapy. Genomic DNA hypomethylation and promoter hypermethylation do not have any correlation between them in cancer cell. Both the epigenetic events are independent and have different roles in cancer development and progression. Mostly repetitive DNA sequences get hypomethylated in cancer tissue, which increase from benign to metastatic cancer (Smet & Loriot, 2010; Ehrlich, 2009). Hypomethylation promotes early precancerous lesions through the genomic deletion. Hypomethylation promotes carcinogenesis by genomic instability, loss of imprinting, reactivation of transposable elements and oncogene. Hypomethylated DNA promotes chromosomal rearrangement and mitotic recombination which induce deletion and translocation. Epidemiological and experimental research showed that the loss of genomic imprinting increases the risk of cancer development like Willms tumor and Wiedemann syndrome (Smet & Loriot, 2010; Ehrlich, 2009; El-Osta et al., 2004). Loss of imprinting of Insulin-like growth factor 2 (IGF2) increase the risk of cancer. Hypomethylation reactivate genes like paired box 2 (PAX2) and mir-let-7 a-3 which take part in various cancers (Pogribny & Beland, 2009).

2.5.2 Hypermethylation of promoters and cancer

Mechanisms behind hypermethylation in a cancer cell are not fully understood. It is reported that tumor cells which have genomic hypomethylation are densely hypermethylated at CpG island regions (Rountree et al., 2001). Various reports have shown the presence of many regional hot spots for hypermethylation on chromosomes 3p, 9p, 11p and 17p in a variety of human tumors. These chromosomal regions contain many TSGs like Von Hippel-Lindau, fragile histidine triad protein (FHIT), p16, p15, retinoic acid receptor-β, MLH1 and P53. Genes that are not present in these regions are also under tight control of CpG methylation and implicated in cancer some of these are MGMT, Cyclo oxygenase-2, Ras-association domain family 1, isoform a (RASSF1a), p21 etc.
Promoter CpG islands which contains 5' regulatory regions are the primary target for aberrant hypermethylation in tumor cells. TSGs transcription suppression by hypermethylation is a major event in the origin of many types of cancer. Hypermethylation totally inhibits the transcription of a gene. Epigenetically suppressed genes behave like gene that has lost its function by deletion or mutation (Lewandowska & Bartoszek, 2011; Sharma et al., 2010; Danesi et al., 2003; Laird & Jaenisch, 1994). Deamination of 5 methyl cytosine to thymine is an endogenous process of cells and catalyzed by the DNMT enzymes when it is over expressed (Kangaspeska et al., 2008; Me´tivier, 2008). There has been an ever growing list of genes which got hypermethylated not only in cancer but also in other diseases. Profiling of methylated genes could be helpful in deciding the therapeutic strategy, prognosis, evaluation of risk and diagnosis (Lewandowska & Bartoszek, 2011; Sharma et al., 2010).

2.5.3 Genes involved in DNA methylation

Genomic DNA methylation in mammalian genome is catalyzed by DNA methyltransferases (DNMTs). The mammalian DNMTs are DNMT1, DNMT3A and DNMT3B, which together with accessory proteins, like DNMT3L, are responsible for acquisition of methylation pattern during various vital cellular processes of embryogenesis, gametogenesis, and somatic tissue development. DNMTs are important for the initiation of chromatin remodelling and gene expression regulation (Miyake et al., 2010; Plewa & Jagodziński, 2005).

Activation or inactivation of a gene transcription is regulated via tight reversible epigenetic changes by the utilization of selective genome information during gametogenesis, embryogenesis and cell differentiation (Teitell & Richardson 2003). As the role of DNMTs investigated in DNA methylation and the role of their alteration become clear in the development of various disorders, including cancer, these molecules open a new way of thinking in respect of cancer therapy (Plewa & Jagodziński, 2005; Salle et al., 2004).
DNMTs are categorized as maintenance and de novo methyltransferase (Margot et al., 2003; Bestor, 2000). DNMT1, maintenance DNMT, add a methyl group to hemimethylated DNA during replication. De novo DNMTs are DNMT3a and DNMT3b which can effectively modify cytosine to 5 methyl cytosine post-replicatively in unmethylated DNA.

### 2.5.3.4 DNMT1 is a major maintenance DNA methyltransferase

DNMT1 is responsible for maintenance of the DNA methylation pattern during replication. Replication generates hemimethylated eukaryotic genomic DNA. These hemimethylated CpGs are precisely methylated to maintain the original DNA methylation pattern by DNMT1 enzyme. Along with the PCNA and DNA polymerase DNMT1 is present at the replication fork, which methylated the newly biosynthesized DNA strands (Hermann et al., 2004). DNMT1 displays a 5 to 40 fold higher activity in vitro for hemimethylated DNA than for unmethylated DNA (Hermann et al., 2004; Bestor, 2000). However, this enzyme also exhibits very weak de novo methylation activity which is stimulated by DNMT3A (Plewa & Jagodziński, 2005; Fatemi et al., 2002).

### 2.5.3.2 DNMT3a and DNMT3b are denovo DNA methyltransferase

These methyltransferases are responsible for the de novo methylation of DNA and methylate CpG dinucleotides without preference for hemimethylated DNA, particularly during embryogenesis. DNMT3A and DNMT3B activity is reduced after the differentiation of ES cells and remains low in adult somatic tissues. The expression of DNMT3A is ubiquitous, while DNMT3B is expressed at very low level in most tissues except the testis, thyroid and bone marrow (Xie et al., 1999). DNMT3a/b level is profoundly increased in various tumor cell lines, indicating that it plays an important role in tumorigenesis (Hermann et al., 2004; Robertson et al., 1999).

### 2.5.3.3 DNMTs and cancer

The fundamental role of DNMT activity has been shown in the initiation and progression of lung cancer (Belinsky et al., 1996). DNMT over-expression not only blocks normal
differentiation but also assists proliferation (Tang et al., 2009). The levels of DNMT1, 3A, and 3B mRNA have shown to be elevated in various malignancies, including hepatomas, prostate, colorectal, and breast tumors (Kanwal & Gupta, 2010; Fabbri et al., 2007; Girault et al., 2003; Saito et al., 2003). Recently, the mRNA levels of DNMT1 and DNMT3B have been found to be elevated in NSCLCs along with DNMT1 level independently correlated with prognosis in NSCLC patients (Kim et al., 2006). DNMT1, 3a, and 3b protein expression have been shown to be highly expressed in lung tumors of smokers (Talikka et al., 2012; Lin et al., 2007). In lung SCC, elevated DNMT1 expression has been shown to predict a poorer prognosis and elevated expression of both DNMT1 and DNMT3B have been shown to be correlated with hypermethylation of TSG promoters (Lin et al., 2007). DNMT3B promoter polymorphism, which significantly increases promoter activity, has been correlated with an increased risk of lung cancer in a hospital-based case-control study (Shen et al., 2002).

The inhibition of DNMT1 mediated DNA methylation reduced tobacco carcinogen-induced lung cancer in mice by more than 50% (Belinsky et al., 2007). Multiple studies reveal that cancer state can be altered by inhibiting epigenetic molecules. DNMT inhibition by pharmacologically active compounds or by antisense oligonucleotides inhibits cell growth in vitro and in vivo (Ganeshan et al., 2009). Various compounds which have shown to have DNMT inhibitor properties broadly fall into two categories A- nucleoside analogues, which incorporated into DNA sequences and inhibit DNMT activity. B- non-nucleoside analogues which do not incorporate into DNA sequences but affects the DNMT activity by other means (Amatori et al., 2010). Azacytidine, a nucleoside analogue, is under phase I/II clinical trial for NSCLC and other solid tumors in combination therapy. Decitabine used for AML clinical trial phase II. 5-fluoro-2’-deoxycytidine is under phase II clinical trial for the head and neck, lung, bladder and breast neoplasm. Dietary non nucleoside analogue inhibitors are genistein, hydralazine, curcumine and EGCG are under phase I/II trial for various cancers (National Cancer Institute, Clinical trial- http://www.cancer.gov/clinicaltrials). Other DNMT inhibitors which are under investigation are zebularine, RG108, procanamides, and psammaplin A (Reuter et al., 2011; Tang et al., 2009).
2.5.4 Methyl binding domain proteins (MBD) read the DNA methylation mark

The methylation of DNA and its components work as a signal for binding of various proteins which are functional aspect of it. These proteins are named as methyl-CpG binding proteins (MBPs). MBP proteins bind to methyl CpGs and recruit protein complexes. Histone-modifying enzymes are present in these complexes, which leads to gene silencing via heterochromatin formation (Fuks, 2005). The first protein discovered was methyl CpG binding protein2 (MeCP2) and currently the number of MBPs are 15 (Parry & Clarke, 2011).

MBP family is divided into 3 groups: Methyl binding domain (MBD) containing proteins, methyl-CpG binding zinc fingers, and the SRA domain containing proteins (Hung & Shen, 2003). Among these proteins MBD protein has been shown to be deregulated in various types of cancer, including lung cancer, though other MBPs also altered in various cancers but at a lesser extent. According to conserved domain database MBDs further divided into 3 classes HMT-MBD, HAT-MBD and MeCP2-MBD. Among the 3 groups MeCP2-MBD involved in lung cancer development and pathogenesis. There are seven members in this protein group which includes MeCP2 and MBD 1-6 (Parry & Clarke, 2011).

2.5.4.1 MeCP2

It is a 50 kDa protein, works as a global transcriptional repressor and encoded by a gene on the X chromosome. It can bind to a single methylated CpG and recruit repressor complex to silence transcription via histone deacetylation (Nan et al., 1998). Mostly this protein studied in respect to Rett syndrome, a neuro developmental disorder (Amir et al., 1999). It is less understood in respect of tumorigenesis. In spite of that it has been shown to involve in various cancer types in myeloma, hematological malignancies, and breast, colorectal, lung, liver, and prostate cancer (Parry & Clarke, 2011). As we understand further regulatory processes of MeCP2 it would lead to targets for therapy in cancer.
2.5.4.2 MBD1

This protein is the largest member of the family in having 13 isoforms of the gene expressed from chromosome (Cooper et al., 1983). Polymorphisms of MBD1 gene have been strongly correlated with lung cancer risk (Liu et al., 2008). The role of MBD1 in transcriptional silencing of genes in cancer has been investigated in vitro. Acute promyelocytic leukemia (Villa et al., 2006) pancreatic cancer (Liu et al., 2008) and colon (Mc Gough et al., 2008) etc. cancer cell lines have been shown to have MBD1 role in gene silencing. MBD1 silenced genes have been associated with drug resistance and the immune system interactions of cancers (Liu et al., 2008).

1.5.4.3 MBD2

MBD2 mediates the methylated DNA binding functions for transcriptional repressor complexes (Parry & Clarke, 2011). 2 different transcriptional repressor complexes, MECP1 and Mi2/ NuRD complexes use MBD2 to direct HDACs and chromatin remodelers to methylated promoters, where they effect transcriptional repression (Feng and Zhang, 2000). This protein has been shown to silence genes in a variety of cancers: colorectal, (Park et al., 2007) lung, (Zhuravel et al., 2008) and various other cancers. It has been shown to be an attractive target for colorectal cancer (Kanwal and Gupta, 2010).

2.5.4.4 MBD3

There are multiple splice variants of MBD3, which lack methylated CpGs binding due to alterations within the MBD. It is an important part of the Mi2/NuRD repression complex. Roles in human cancer that are specific to MBD3 are not commonly observed. It works together with MBD2 (Parry & Clarke, 2011; Kanwal and Gupta, 2010).

2.5.4.5 MBD4

This MBD protein has shown to interact with the DNA repair machinery. MBD4 plays a key role in maintaining methylated DNA gene regulation and suppressing mutation at CpG sites. The significance of MBD4 in cancer is largely indirect as deficiency results in a mutator phenotype and subsequent alterations to bona fide TSG. MBD5 and MBD6 are less
characterized proteins. No data available for their role in human cancers (Parry & Clarke, 2011; Kanwal and Gupta, 2010).

Clinically MBD proteins have been shown to be important in the prognosis and diagnosis of cancer. MBD2 protein has been studied as the potential target for the cancer treatment by many groups. Antisense therapy has been suggested for MBD2 in cancer cell lines (Parry & Clarke, 2011; Szyf, 2009; Slack et al., 2002).

2.6 Histone modifications and cancer

In eukaryotes, one of the most abundant and highly conserved proteins is histone. Histone plays important roles in the regulation of many vital biological processes of DNA transcription, replication, repair, and the cell cycle by forming the orderly structure of chromosomes and compact DNA into it. Compartmentalization of chromatin into active and inactive domains is attributed to histone modifications (Jayani et al., 2010). Histone modifications have been shown to maintain the poised transcriptional state of important genes (Bernstein et al., 2006). Histone modifications reported to date include acetylation, phosphorylation, methylation, ADP ribosylation, and ubiquitination (Ellis et al., 2009).

Acetylation and methylation of histone and the enzymes involved in the process have been clinically associated with cancer and identified as a marker of tumor cells (Ellis et al., 2009). Histone deacetylases (HDAC), histone acetyl transferase (HATs), histone methyl transferases (HMTs), histone demethylases (HDM) are the enzymes which bring about acetylation and methylation of histones. There are five classes of histone proteins H1, H2a, H2b, H3, and H4. Among histones, H3 and H4 are of importance in cancer biology because their N terminal tail modification could turn on or off gene transcription permanently (Imai & Ochiai 2011; Wang et al, 2007). In general, active state of gene transcription related to histone acetylation. But histone methylation could cause active or repressive transcription depending on lysine position and number of methyl groups (Fraga et al, 2005; Seligson et al, 2005).
2.6.1 Histone deacetylases

Histone acetylation and deacetylation are the reversible and regulatory processes and are most abundant post-translational modifications in eukaryotic cells. Two families of enzymes, histone acetyl transferases (HATs) and histone deacetylases (HDACs) mediate this phenomenon. Aberrant expression of HDACs has been found in multiple types of cancer along with protein and histones acetylation. HDACs have emerged as promising targets in cancer therapeutics, and the development of HDAC inhibitors (HDACi) (Barneda-Zahonero & Parra, 2012; Ouaissi et al., 2011; Nakagawa et al., 2007).

Eighteen mammalian HDACs have been identified and categorized in 4 groups (Ouaissi et al., 2011). Eleven of the HDACs are zinc dependent, classified on the basis of homology to yeast (Marks & Xu, 2009). HDACs Class I and II are similar with yeast deacetylases RPD3 and HDA1; Class III shows homology to yeast silent information regulatory protein (SIR2p). Class IV is specific class, including only one HDAC11 which have some similarity with Class I and II enzymes (Bolden et al., 2006). Class I HDACs 1, 2, 3, and 8; Class II comprises HDACs 4, 5, 6, 7, and 9. HDAC class III consists SIRT 1-7 proteins. HDAC6 and HDAC10 are carriers of two catalytic sites and are therefore grouped in subclass IIB (Ouaissi et al., 2011; Marks & Xu, 2009). Class I Histone deacetylases (HDACs) play a central role in controlling cell cycle regulation, cell differentiation, and tissue development and are expressed almost exclusively in the nucleus of all cell types (Reichert & Choukrallah, 2012). HDAC1 and HDAC3 have been shown to involve in the regulation of proliferation and survival of cancer cells (Glaser et al., 2003). Overexpression of class I HDACs have been reported in several cancer tissues, such as stomach, esophagus, colorectal, prostate, breast and lung (Nakagawa et al., 2007). HDAC1 has been shown to be a potential target for cancer therapeutics because inhibition of HDAC1 resulted in differentiation, apoptosis and cytostatic effect on cancer cell (Marks & Xu, 2009).

HDAC1 has been shown to modulate breast cancer progression through inhibiting estrogen receptor alpha protein expression and transcriptional activity (Kawai et al., 2003). Elevated
HDAC1 expression found to be present in highly proliferative tissues, embryonic stem (ES) cells, and several transformed cell lines, suggesting a link between HDAC1 function and proliferation (Senese et al., 2007).

Inhibition of HDAC has been shown promising in cancer therapeutics and prevention. Various drugs and active compounds of synthetic and natural origin have been used as HDAC inhibitors. Most of the HDAC inhibitors are in clinical trial as a combination therapy some of that are valproic acid (VPA), Vorinostat, Belinostat and panobinostat under phase II trial for the AML, CLL, NSCLC, SCLC, B-CLL, brain tumor, glioma, breast, thyroid, pancreatic cancer, HNSCC and various solid malignancies (National Cancer Institute, Clinical trial - http://www.cancer.gov/clinicaltrials). Dietary compounds such as butyrate, EGCG, curcumin, selenium, isothiocyanate, I3C, genistein, parthenolide, quercetin diallyldisulfide (DADS) and sulforaphane (SFN) act as ligands for HDAC and exhibit HDAC inhibitory activity (Rajendran et al., 2011; Dashwood & Ho, 2008).

2.6.2 Histone methyltransferases

Protein methylation marks are generated by protein methyl transferases (PMT), protein lysine methyl transferases (PKMTs) and protein arginine methyl transferases (PRMTs) which are specific in generating methylation mark on histone tail. EZH2, SUV39H1, G9a are methylation writers on lysine (PKMTs) and are altered in various types of human cancers, including lung cancer (Kondo et al, 2008; Chi et al, 2010; Yost et al, 2011). SUV39H1 and G9a are H3K9 methyltransferases which transfer methyl group on lysine at ninth position of H3 tail. Specifically G9a generates dimethylation whereas SUV39H1 generates di and tri methylation marks on H3K9. EZH2 methylates lysine on 27 position in H3 tails. SUV420H1 brings about tri methylation in lysine at position 20 in H4, H4K20 (Kondo et al, 2008; Yost et al, 2011; He et al, 2012; Wongtawan et al, 2011). Histone methylation marks, get miswritten in cancer due to altered expression of specific methyltransferase (Chang & Hung, 2012; Yost et al, 2011; Chi et al, 2010).
2.6.2.1 EZH2

EZH2 is highly conserved histone methyltransferase that targets lysine-27 of histone H3 and tri methylate it. It is a part of polycomb repressive complex 2 (PRC2). Methylated H3-K27 chromatin mark is commonly associated with silencing of different genes in organisms ranging from plants to flies to humans. Overexpression of EZH2 is reported in various types of cancers, including lung, liver, prostate, breast, bladder, colon, skin, hematopoietic malignancies etc. suggesting its involvement in proliferation, invasion, metastasis and angiogenesis (Fussbroich et al., 2011; Reuter et al., 2011).

EZH2 has been shown to be functionally linked with DNA methylation and histone deacetylation (Reuter et al., 2011). EZH2 is needed for DNMT binding and CpG methylation of target genes but, conversely, DNMTs is not needed for EZH2 chromatin association (Vire et al., 2006). Histone methyltransferase activity of PRC2 showed EZH2 and HDACs are physically and functionally linked (Tie et al., 2001). Although the mechanistic contribution of EZH2 to cancer progression is not yet fully determined, functional links between EZH2 mediated histone methylation and DNA methylation suggest partnership with the gene silencing machinery implicated in tumor suppressor loss (Reuter et al., 2011).

2.6.2.2 SUV39H1

SUV39H1 is the human homolog of the Drosophila Su (var) 3-9 histone methyltransferase. The SUV39H1 and SUV39H2 enzymes specifically trimethylate H3K9 and use mono methylated H3K9 as a preferred substrate. SUV39H1 plays a critical role in the establishment of constitutive heterochromatin especially at pericentric heterochromatin (Cohen et al., 2011). However, recent studies also suggested that SUV39H1 may direct the transcriptional repression within the euchromatin promoter (Kang et al., 2007). SUV39H1 has been shown to control cell-cycle genes in S phase through H3K9 methylation in differentiating cells (Ali et al., 2004).
Mouse with double knockout Suv39h1/ Suv39h2 shows the reduced level of H3K9 methylation, which shown to be associated with genome instability and predisposition to cancer (Ozdag et al., 2006). SUV39H1 directly interact with DNMT1 and HP1 protein in colorectal cancer (Kang et al., 2007). It also interacts with HDAC1 and causes gene silencing. Interaction of SUV39H1, MBD1 and HP1 take part in DNA methylation based transcriptional repression (Carbone et al., 2006; Fujita et al., 2003).

2.6.2.3 G9a

G9a is identified as Su (var), Enhancer of Zeste, Trithorax (SET) domain–containing protein, which is responsible for dimethylation of H3K9 (H3K9me2) (Tachibana et al., 2002). Available reports showed the oncogenic potential of G9a and its validity for therapeutic target. Suppression of G9a reduces cell proliferation and anchorage-independent colony growth while inducing apoptosis in immortalized normal human bronchial epithelial cells (Watanabe et al., 2008). G9a is required for the maintenance of the malignant phenotype. G9a has shown to promote lung cancer invasion and metastasis (Chen et al., 2010). It is over expressed in various human cancers, including leukemia, prostate carcinoma, hepatocellular carcinoma, and lung cancer and has been shown to play a role in mental retardation, inflammation, drug addiction and HIV-1 latency maintenance (Yost et al., 2011).

It has been shown in various reports that targeting EZH2, SUV39H1 or G9a in different cancer cell lines blocked the proliferation of cells, more or less by affecting the expression of TSGs like p16^{INK4}, RASSF1a, P21^{Kip1}, p15^{INK4B} and E-cadherin (Fussbroich et al, 2011; Lakshmikuttyamma et al, 2010; Kondo et al, 2008; Ougolkov et al, 2008). These reports suggested the potential of HMTs as therapeutic targets in cancer. Various synthetic or natural chemicals of polyphenolic nature are shown to possess therapeutic or chemopreventive properties and have been shown to exert antitumor effect by inhibiting histone methyl transferases. Some synthetic chemicals like GSK126, DNZeP inhibit EZH2 (McCabe et al, 2012; Crea et al, 2012) or BIX 01294 inhibits G9a (Yost et al, 2011). Dietary components like poly unsaturated fatty acids (PUFA) and ω-3 inhibit EZH2,
chaetocin inhibits SUV39H1, EGCG and curcumin have potential to inhibit the activity and expression of other HMTs (Gerhauser, 2013; Berghe, 2012).

### 2.6.3 Histone methylation marks in cancer

As oppose to histone acetylation marks, histone methylation marks have some special meaning in respect of gene transcription. H3K4me3 and H3K4me2 are highly enriched on gene promoters which are transcriptionally competent or active. H3K9me1, H3K27me1, H3K36me3, H3K79me2/3 and H2BK5me1 methylation marks are also present on active gene promoters. Generally H3K27me3, H3K9me3 and H3K20 methylation are present on transcriptionally repressed promoters. Silent pericentric heterochromatin is marked by H3K9me3 (Varier & Timmers, 2011; Ellis et al., 2009; Kouzarides, 2007). Histone modification marks get miswritten in various cancers. H3K9me2 found to be decreased in pancreatic adenocarcinoma, prostate, kidney cancers (Chervona & Costa, 2012; Kouzarides, 2007). H3K9me3 increased in gastric adenocarcinoma, lung cancer and breast cancer (Varier & Timmers, 2011). H3K27me3 mark has been shown to be increased in paragangliomas breast, ovarian, pancreatic colorectal adenocarcinoma, breast carcinomas (Chervona & Costa, 2012; Varier & Timmers, 2011).

### 2.7 TSGs affected by epigenetic alteration in lung cancer

A TSG, or anti-oncogene, is a gene that protects a cell from one step on the path to cancer. When this gene is mutated to cause a loss or reduction in its function, the cell can progress to cancer, usually in combination with other genetic or epigenetic changes. TSGs may include class of genes which works as a cell cycle regulators, DNA repair, xenotoxin metabolism or immune regulators (Lodish et al., 2000). Various reports are there which showed the involvement of promoter hypermethylation in lung cancer. p16\(^{\text{INK4}}\) is a well characterized and well-studied example in lung cancer which got methylated at promoter region and has been correlated with gene silencing as an early event in tumorigenesis (Risch & Plass, 2008). Other genes include H-cadherin, death-associated protein (DAP) kinase 1 (DAPK1), 14-3-3 sigma (Risch & Plass, 2008) and the candidate TSG RASSF1A (Dammann et al., 2000).
Numerous genes, including RARβ, tissue inhibitor of metalloproteinase 3 (TIMP3), p16, MGMT, DAPK1, E-cadherin (ECAD), p14ARF, p16 and glutathione S-transferase P1 (GSTP1) were found to be methylated at various degrees in primary non small cell lung cancers (Zochbauer-Muller et al. 2001). Moreover, gene promoter methylation has been proposed as a biomarker for early detection of lung cancer and monitoring the prevention trials. Methylation in p16 and/or MGMT promoters were used to diagnose squamous cell lung carcinoma in smokers, up to 3 years prior to clinical diagnosis (Palmisano et al., 2000). Epigenetic biomarkers are being investigated for lung cancer detection in sputum or plasma (Hsu et al., 2007). Genes which are involved in other function like DNA repair or drug metabolism are also silenced by epigenetic alteration in cancer. Few examples are MLH1, MGMT and GSTP1 (Radhakrishnan et al, 2011).

2.7.1 p16 (CDKN2A)

TSG p16 is an important cell cycle regulator in the cyclin D-Rb pathway. It controls cellular proliferation by acting as a cyclin/ cyclin dependent kinase inhibitor to prevent Rb phosphorylation (Agarwal et al., 2012). Basically p16 inhibits CDK4 and CDK6 by binding near catalytic sites which change the confirmation of CDKs (Russo et al., 1998). Its ARF product stabilizes p53 protein by sequestering MDM2 protein (Boehme & Blattner, 2009). This protein is important for G1 cell cycle progression and works as G1/S cell cycle checkpoint. Inactivation of p16 has been shown to be an important aspect of tumor development. In tumor development process, this gene gets mutated, methylated or deleted as early event of NSCLC. Inactivation by methylation of this gene predicts risk of lung cancer development. The re-expression of p16 has also been used as a prognostic marker in epigenetic therapy of lung cancer (Agarwal et al., 2012; Witcher & Emerson, 2009; Liu et al., 2006).

2.7.2 MLH1

MutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli), also known as MLH1, is a human gene located on chromosome 3 (NCBI data base). It is a human homolog of the E. coli DNA mismatches repair (MMR) gene mutL. MLH1 along with MSH proteins involve
in post replicative MMR. During MMR single-strand breaks are introduced by MLH1 near the mismatch which further identified by exonuclease EXO1 to degrade the strand containing the mismatch. Methylation of parent DNA strand do not let its degradation by preventing cleavage and therefore assure the correctness of newly synthesized mutated DNA strand. MLH1 plays a role to recruit the DNA polymerase III to the site of the MMR (Kadyrov et al., 2006). MLH1 also implicated in DNA damage signaling, a process which induces cell cycle arrest and can lead to apoptosis in case of major DNA damages (Kadyrov et al., 2006; Stojic et al., 2004). MLH1 has different alternative splice variants. This gene commonly associated with hereditary nonpolyposis colorectal cancer. This gene was identified as a locus frequently mutated in hereditary nonpolyposis colon cancer (HNPCC) (Radhakrishnan et al., 2011; Varley et al., 2009). MLH1 promoter polymorphism has been shown to involve in various cancers, including lung, breast and colorectal cancer (Raptis et al., 2007). Methylation of the MLH1 gene has been correlated with high microsatellite instability (MSIH) and mismatch repair defect in sporadic colorectal cancer, where this phenotype is associated with better patient survival. MLH1 methylation has been reported in NSCLC with frequencies ranging from 7 to 59%. The reduced MLH1 expression has also been reported in 59% of lung cancers (Seng et al., 2008).

2.7.3 **DAPK1**

Death-associated protein kinase 1 is a160-kD candidate TSG. It is a microfilament-bound kinase, which positively mediates gamma-interferon induced programmed cell death (Esteller, 2002). DAPK1 has been shown to suppress the metastatic ability of carcinoma cells (Inbal et al., 1997). Its expression has been shown to be absent or decreased in various cancers and cancer cell lines (Botezatu et al., 2008). DAPK1 has been reported to be suppressed by promoter methylation in malignant tissue, but its methylation was not found significant in benign tumors or at early stages (Mir et al., 2013; Zorko et al., 2010; Botezatu et al., 2008).
2.7.4 MGMT

MGMT is a DNA-repair protein that removes alkyl adducts from the O6 position of guanine and the O4 position of thymine, effectively restoring these DNA bases and preventing alkylating agent induced cell death (Shah et al., 2011). This protein gets auto inactivated after the removal of alkylating agent from DNA base. Promoter hypermethylation has been shown to inhibit the transcription of MGMT and inhibit its gene expression. MGMT methylation is commonly found in various cancers, including lung cancer, colorectal cancers, gliomas, head and neck cancers, and lymphomas (Lai et al., 2009; Esteller, 2000). With the help of a transgenic mouse model it has been suggested that MGMT inactivation by promoter methylation plays an important role in lung carcinogenesis because K-ras mutation in lung tumors significantly reduced in MGMT transgenic mice compared to that of non-transgenic mice (Liu et al., 2006). MGMT promoter methylation has been used as a predictive marker in cancers; it indicates those individuals who are likely to respond to chemotherapy with alkylating agents (Candiloro & Dobrovic, 2009).

2.8 Inflammation involved in the genesis and advancement of cancer

Inflammation is a pathological process of an organism for responding to external harmful stimuli or pathogen. Classically inflammation is characterized by pain, swelling, redness and loss of function. There are many key processes in the inflammatory response, including increasing vascular permeabilization, releasing of eicosanoids, and the production of cytokines, chemokines, and amines (histamine) by immune cells. These processes are intended to eradicate the stimulus or pathogen from the organism (Ferrero-Miliani et al., 2006). Inflammation is a beneficial event as it is involved in host defense, repair, and regeneration of tissue after any internal or external insult, microbial infections and tissue damage. Even it is involved in tumor inhibition through immune surveillance and tumor specific antigen recognition (Mantovani et al., 2008, Rakoff-Nahoum, 2006).

Inflammation and cancer are tightly linked. Epidemiological reports showed that chronic infection or other types of chronic inflammation are causal agents for occurrence of near
about 25% of all cancer (Hussain et al., 2007). Chronic inflammation is detrimental and frequently predisposes cells for an oncogenic transformation along with other deleterious effects. Various mechanisms are involved in oncogenic transformation by chronic inflammation which might be induction of genomic instability, increasing angiogenesis, altering the genomic epigenetic state and increasing cell proliferation (Schetter et al., 2010). In neoplastic transformation inflammation can alter the expression of oncogenes and TSGs (Coppe et al., 2008).

It has been shown that inflammatory pathways are interlinked with the epigenetic pathways. Inflammatory molecules affect the expression of epigenetic molecules. There are reports which showed that IL6 can induce the DNMT1 expression and could bring the promoter methylation of the SOCS gene in colorectal cancer. NF-κB along with HDAC1 can silence miR-29b (Li et al., 2012; Lin & Chen, 2010; Liu & Chen 2011). Deregulation in inflammatory cytokines (ILs) and chemokine expression, reactive oxygen and nitrogen species (RONS) over-production, increased nuclear factor kappa B (NF-κB), STAT3 and cyclooxygenase-2 (COX-2), expressions are just some of the molecular factors that contribute to inflammation induced carcinogenesis (Schetter et al., 2010).

2.8.1 **NF-κB**

NF-κB is a heterodimeric transcription factor, expressed in the cytoplasm of virtually all cell types. There are five NF-κB/Rel family members: RelA (p65), c-Rel, RelB, NF-κB1 (p50; p105), and NF-κB2 (p52; p100) (Hayden & Ghosh, 2009). RelB expression is limited to the thymus, lymph nodes, and Peyer’s patches while c-Rel expressed only in hematopoietic cells and lymphocytes. A wide variety of cells in mammals express RelA and p50 heterodimer. There is a consensus sequence GGGRNYYCC for each NF-κB dimer binding but each dimer has a different DNA-binding affinity for κB sites bearing. Homo dimers of NF-κB complexes lack transactivation domains, such as p50 homodimer, but may impose transcriptional repression (Nishikori, 2005). There are two NF-κB regulatory pathways one is classical other is alternative pathway but both pathways have different regulatory circuits. The classical pathway involved p50/RelA and activated by
ligand binding to tumor necrosis factor type 1/2 receptors (TNFR1/2), T-cell receptors (TCR), B-cell receptors (BCR, or the Toll-like receptor (TLR), interleukin-1 receptor (IL-1R) super family members. Target genes of classical pathway involved chemokine, cytokines, and adhesion molecules which perpetuate inflammatory responses, and promote cell survival. The alternative pathway involved p52/RelB is triggered by the activation of certain TNF receptor family members, including lymphotoxin b receptor (LTbR), B-cell-activating factor belonging to the TNF family receptor (BAFF-R), CD40, and CD30. This pathway regulates the development of lymphoid organs and the adaptive immune system (Oeckinghaus et al., 2011; Oeckinghaus & Ghosh, 2009; Nishikori, 2005). NF-kB is expressed in cell types, where its activity is controlled by a family of regulatory proteins, called inhibitors of NF-kB (IκB) (Karin, 2009).

Role of NF-kB classical pathway has been correlated with tumor promotion and metastasis. NF-kB targets many genes that facilitate tumor progression, inflammation, cellular immortality, cell survival, angiogenesis, proliferation, tumor promotion, and metastasis. These includes IL-6, COX-2, cyclin D1, IL-1, TNF, chemokines, telomerase, BCL-XL, cIAP, VEGF, TNF, IL-8, c-MYC, iNOS, MMP-9, etc (Fan et al., 2013).

2.8.2 **STAT3**

There are seven Stat proteins (STAT1, STAT2, STAT3, STAT4, STAT5 (STAT5α and STAT5β), and STAT6 act as latent transcription factors that primarily mediate signalling from cytokines and growth factor receptors (Yue & Turkson, 2009). STAT proteins are activated through phosphorylation on the carboxy-terminally located conserved tyrosine residues. Phosphorylation of STAT proteins forms stable homo and/or heterodimers in the cytoplasm by reciprocal SH2 domain interaction. STAT dimmers translocate to the nucleus and bind to DNA in a sequence-specific manner that regulate transcription of target genes. Different STAT proteins show preferred specificity for individual cytokine family receptors (Jarnicki et al., 2010).
STAT3 protein is a member of the STAT family activated by Janus kinase (JAKs) by inducing the phosphorylation of STAT3 at tyrosine residue 705 (Y705). The STAT3 proteins involve in regulating many aspects of growth, survival, proliferation, migration, invasion, and differentiation in cells (Buettner et al., 2002). STAT3 has been shown to activate frequently in various human cancers and established human cancer cell lines, including multiple myeloma, glioblastoma, colorectal and hepatocellular carcinoma (Lin et al., 2010). Small interfering RNAs (siRNAs) or antisense oligonucleotides against STAT3 signaling in carcinoma cells inhibit cancer cell growth, invasion and metastasis, and induce apoptosis (Li et al., 2006). Elevated level of STAT3 phosphorylation has been correlated with the tumor invasion, metastasis, and worse prognosis in colorectal, hepatocellular and other carcinoma (Lin et al., 2010).

2.8.3 IL6

IL6 is a major pleiotropic, pro-inflammatory cytokine which plays a role in immune response, hematopoiesis, cell differentiation, wound repair, and bone remodelling (Tawara et al., 2011). IL6 is mainly produced by T cells along with a variety of cells including macrophages, fibroblasts, synovial cells, endothelial cells, glia cells, and keratinocytes. A variety of stimuli can induce IL6 expression, including bacterial and viral infection and microbial components such as lipopolysaccharide (LPS) other cytokines such as IL1, tumor necrosis factor (TNF), and platelet-derived growth factor (PDGF) are also potent inducers of IL6 (Kishimoto, 2005).

IL6 signals by a common signaling receptor, gp130, expressed in many cell types. Binding of IL6 to the soluble IL6R receptor (gp80) induces dimerization of gp130 chains resulting in activation of the associated Janus kinases (JAKs). JAKs phosphorylated gp130, leading to the recruitment and activation of the STAT3 andSTAT1 transcription factors as well as other molecules (SHP2, Ras-MAPK, and PI3K). The importance of IL6 signaling in mediating tumorigenesis has been examined in various studies, where it promotes the in vivo growth of tumors in models of prostate, breast, and lung cancers. It has also been
linked with the epigenetic changes and suppression of TSG in cancer (Bromberg & Wang, 2009).

2.8.4 COX-2

COX or prostaglandin G/H synthase was elucidated as the target of aspirin and other NSAIDs (Vane, 1971). Since then COX pathways as well as its functions in both physiological and pathological conditions were identified. It is a key player in the biosynthesis of prostaglandins from arachidonic acid following its release from the plasma membrane by the action of phospholipase-A2. Prostaglandins are important for many normal physiological processes, including modulation of immune responses, protection of the gastrointestinal mucosa, maintenance of renal homeostasis and the regulation of blood clotting in a variety of tissues. Furthermore, prostaglandins also function in pathological conditions where they can promote inflammation, swelling, pain and fever (Greenhough et al., 2009; Urade, 2007).

There are two types of COX enzymes COX-1 and COX-2. COX-1 is considered a housekeeping enzyme responsible for maintaining basal prostaglandin levels important for tissue homeostasis. In contrast, COX-2 is an inducible enzyme most tissues do not normally express it (Divvela et al., 2010; Urade, 2007). COX-2 expressed in some special conditions like inflammations it also expressed in some tissues constitutively like brain and kidney (Divvela et al., 2010).

Overexpression of COX-2 has been reported in a variety of cancers, few examples are colon, stomach, breast, lung, esophagus, and prostate (Fosslien, 2001). COX-2 is induced by stimuli such as mitogens, cytokines, growth factors and tumor promoters, and has been elucidated to be involved in cancer development and pathogenesis (Urade, 2007).

2.8.5 Cyclin D1

Cyclins are small molecules involved in cell cycle regulation and progression. These are four major cyclin A, B, D, E. Among these cyclin D is solidly established as an oncogene
with an important pathogenic role in many human tumors. There are three highly homologous and almost indistinguishable biochemically D-type cyclins (D1, D2 and D3) (Stamatakos et al., 2010).

The cyclin D1 is a proto-oncogene, which plays an important role in regulating G1 to S phase progression in different cell types. Cyclin D1 and its binding partners cyclin dependent kinase 4 and 6 (CDK4 and CDK6) form active complexes that phosphorylate and inactivate the retinoblastoma protein (RB) and promote cell cycle progression (Alao, 2007; Lundberg & Weinberg, 1998). Cyclin D1 is important for the development and progression of several cancers including breast, oesophagus, bladder and lung (Alao, 2007; Fu et al., 2004). Cyclin D1 over expression is a common event in cancer but does not occur solely as a consequence of gene amplification. Rather, increased level of cyclin D1 frequently results from its defective regulation at the post-translational level (Stamatakos et al., 2010).

2.8.6 Targeting inflammation in cancer control

Information about the inflammatory state of a tissue may serve as a beneficial end point in cancer therapeutics in deciding therapeutic regime and developing diagnostic measures. Chemoprevention strategies may also be developed by using anti-inflammatory drugs to reduce cancer incidences. Manipulation of the local inflammatory states surrounding tumors may also constitute a therapeutic option (Schetter et al., 2010). Selective inhibition of NF-κB pathway by BMS-345541 (Madonna et al., 2012) or STAT3 by FLLL32 (Lin et al., 2010) shows potential for targeting various cancers. Various dietary and natural compounds have been shown to possess anti-inflammatory property by inhibiting NF-κB, STAT3 or COX-2 pathways. Curcumin, resveratrol, guggulsterone, zerumbone, Emodin, Boswellic acids, Sulforaphane, indole-3-carbinol, Caffeoylquinic acids, Flavopiridol, Linalool, monoterpenes, Anethole, Withonalides, Ellagic acid, Genistein, Sesqiterpene lactones, Flavonoids, catechins, Silybinin etc are few dietary active ingredient which have been shown to target various pathways including inflammation for their antitumor activity (Aggarwal & Shishodia, 2006).
2.9 **Modeling lung cancer in mouse**

Development of cancer can roughly be divided in four stages initiation, promotion, progression and metastasis. A better understanding and characterization of molecular changes behind the development and advancement cancer could be of use in detecting, curing and preventing this disease (Yuspa, 2000). An animal model of cancer presents an opportunity for understanding this disease in molecular context. Majority of phase II clinical chemoprevention trials are performed on patients with precancerous lesions in the lung. In such case development of a mouse model to screen for potential chemopreventive or chemotherapeutic agents for human lung cancer becomes one of the highest priorities for cancer chemoprevention of lung cancer (Wakamatsu et al., 2007; Meuwissen and Berns, 2005). The carcinogenesis studies that have investigated molecular alterations in mouse lung tumors have provided the basis for new hypothesis driven studies (Wakamatsu et al., 2007).

Spontaneous lung tumorigenesis and susceptibility vary largely between mouse-inbred strains. It has been shown that the higher susceptible mouse strains to spontaneous lung tumor are also very responsive to chemical induction of lung tumors (Shimkin and Stoner, 1975). Most susceptible mouse strains are A/J and SWR mice, whereas O20 and BALB/c are intermediate susceptible. CBA and C3H are more resistant than BALB/c but C57BL/6 and DBA are almost completely resistant. BALB/cJ and susceptible A/J strains have \textit{K-Ras} polymorphism in intron 2 which influence \textit{K-Ras} expression and sensitivity to lung cancer (Pandey & Gupta 2012; Meuwissen & Berns, 2005). BALB/cJ and susceptible A/J strains show a polymorphism in candidate TSG \textit{Cdkn2a}, which encodes the tumor suppressor p16\textsuperscript{ink4a} (Herzog et al., 1999). Being intermediate susceptible strain BALB/c mice have been used in studying mouse lung tumor development, molecular alteration and chemoprevention (Pandey & Gupta 2012; Wakamatsu, 2007; Meuwissen & Berns, 2005).

Chemical carcinogenesis in mouse lung mimics human lung carcinogenesis in various aspects. Due to the molecular and histological similarities between murine and human
adenocarcinoma, carcinogen induced NSCLCs model in mice is often employed in tumor development and chemopreventive studies (Stathopoulos et al., 2007; Meylan et al., 2009). Induction of lung tumor with chemical carcinogens is very reproducible and almost invariably results in pulmonary adenoma and adenocarcinoma (Malkinson, 1989; Shimkin & Stoner, 1975). Polycyclic aromatic hydrocarbons and nitrosamines derived from tobacco and ethyl carbamate (urethane) are very potent carcinogens has been used extensively in mouse lung tumorigenesis (Meuwissen & Berns, 2005).

2.10 **Urethane**

Urethane is a chemical compound first prepared in the nineteenth century. Structurally, it is an ester of carbamic acid having molecular formula C₃H₇NO₂ (Figure-3). It is naturally present in fermented food and alcoholic beverages in trace amount 2 ppb to 12 ppm (Matsudo et al., 1993). It has been used as an antineoplastic agent and for other medicinal purposes, but this ended after it was discovered to be carcinogenic. In the mid 1940s and 50s, various reports showed urethane health hazard and neoplastic property in experimental system (Nomura, 1975).

2.10.1 **Mechanism of urethane induced lung tumorigenesis**

Urethane metabolized by cytochrome p450 2E1 (CYP2E1) inside lung and form two compounds vinyl carbamate and its epoxy derivative. These compounds are ultimate electrophillic metabolites responsible for genotoxicity and carcinogenicity of urethane (Avanzo et al., 2005). Epoxy ethyl carbamate interacts with DNA to form 7-(2-oxoethyl) guanine adducts. It has been shown that lung tumors chemically induced by urethane originate from APTII cells are of both type solid and papillary murine pulmonary adenomas (Avanzo et al., 2005; Mason et al., 2000).
Figure-3: Chemical structure of urethane ($C_3H_7NO_2$)

Figure-4: Mechanisms of urethane induced mutations in lung tumorigenesis
After carcinogen treatment there is a transient decrease in the number of proliferating Clara and alveolar type 2 cells. The cell numbers recover and then soon surpass the number of cells of control mice (Shimkin et al., 1969). In an early phase multiple hyperplastic foci in bronchioles and alveoli can be detected (Foley et al., 1991). Many of these foci, but not all, then develop further into adenomas and finally, after several months, into adenocarcinoma with in situ invasiveness. Latency and tumor number depend on the susceptibility of the strain and can be increased by transplacental carcinogenesis (Meuwissen & Berns, 2005). However, benign papillary and solid adenomas are also found, that do not further develop into malignant adenocarcinoma. The malignant adenocarcinoma rarely metastasizes. Histological analysis of adenocarcinoma showed an equal distribution between papillary and solid subtypes (Nikitin et al. 2004; Malkinson, 2001). Mechanism of urethane induced mutations in lung tumorigenesis is shown in figure-4.

2.11 Chemoprevention

It is an old saying that “prevention is better than cure” which signifies its importance in controlling disease. The work of Lee Wattenberg on the selective inhibition of carcinogenesis during initiation, promotion or progression founded the field of cancer chemoprevention (Mehta et al., 2011). Dr. Michael B. Sporn in 1976 coined and defined the term cancer chemoprevention. He defined the term chemoprevention as the use of natural or synthetic compounds to inhibit, suppress or reverse the development and progression of carcinogenesis before invasion and metastasis occur (Mehta et al., 2011; Sporn & Suh 2000). Chemoprevention may be applied at different time of carcinogenesis to reduce occurrence of in situ or invasive cancers (primary intervention at earlier stages in the process) or cancer morbidity and/or mortality (secondary intervention at later stages in the process) (Walaszek et al., 2004). Depending on the time when preventive measures were taken to stop the disease, chemoprevention may be primary, secondary or tertiary prevention. In particular, primary prevention means preventing the occurrence of diseases. Secondary prevention means early detection and intervention, preferably before the condition is clinically apparent and has the aim of reversing, halting or at least retarding the progress of a condition. Tertiary prevention means minimizing the effect of diseases by
preventing complications and premature deteriorations (Steward & Brown, 2013; Flora et al., 2001).

Chemopreventive compounds act via reversal of altered states of neoplastic lesion, block the replication of altered cells and induce the apoptosis. It may also act by prevention of metabolic activation of carcinogens or their subsequent binding to DNA. Whatever be the mode of action of a chemopreventive agents they all have certain molecular targets which may be related to various cellular pathways (Gerhauser, 2013; Hong & Sporn, 1997). Chemoprevention of cancer is a young discipline that is progressively emerging from its pioneer stage. Use of dietary compounds is now emerging as a new strategy for cancer chemoprevention (Gerhauser, 2013). Natural dietary agents, including fruits, vegetables, and spices have drawn a great deal of attention from both the scientific community and the public owing to their demonstrated ability to suppress cancers. Various stages of chemoprevention are shown in figure-5.

Various active ingredients of dietary products for example curcumin, resveratrol, guggulsterone, zerumbone, Emodin, Boswellic acids, sulforaphane, indole-3-carbinol, caffeoylquinic acids, flavopiridol, linalool, monoterpenes, anethole, withonalides, ellagic acid, genistein, sesqiterpene lactones, flavonoids, catechins, silybinin etc have been shown to target various pathways including epigenetic and inflammation for their antitumor activity (Gerhauser, 2013; Aggarwal & Shishodia, 2006).
Figure- 5: - Chemoprevention at various stages of carcinogenesis
2.11.1 Inositol hexaphosphate a chemopreventive agent

Inositol, a 6 carbon carbohydrate is known to be an essential nutrient and a member of the vitamin B complex (Figure-6). It is abundantly present in cereals and legumes (0.4- 6.4%) and it is typically the most abundant inositol phosphate found in eukaryotic cells with its concentrations ranging from 10 μM to 100 μM. As an ingredient of dietary fiber IP6 is physiologically present in the human large gut at levels reaching 4 mM (Weglarz et al., 2008).

IP6 has been shown to possess various activities involving antioxidant, anti inflammatory, antitumor activities. IP6 exhibiting its tumor suppressing effect through its anti-angiogenic, anti-oxidant and anti inflammatory properties (Gupta et al., 2003; Raina et al., 2008; Vucenik et al., 2004). IP6 exert its effect via lower IP phosphates because lower IP1-4 has been shown to involve in signal transduction pathways of various cellular functions including proliferation, growth, cell cycle regulation differentiation. IP6 causes reversible phosphorylation of specific proteins and affect PI3K, MAPK, PKC pathways to exert its antitumor activity. IP3 and IP4 are involved in Ca+ mobilization and homeostasis (Weglarz et al., 2008; Vucenik & Shamsuddin, 2006; Vucenik et al., 2004). IP6 augments NK cell activity in vitro and normalizes the carcinogen-induced depression of NK cell activity in vivo (Baten et al., 1989; Zhang et al., 2005).

It can form tight insoluble complexes with a variety of polyvalent nutritionally important mineral cations, e.g. calcium, iron and zinc (Vucenik & Shamsuddin, 2006). It has also been reported that a high IP6-containing diet did not negatively affect rat plasma copper and zinc concentrations and no relation of zinc deficiency with IP6 has been observed in women who ingested vegetarian or meat-based diet with equal IP6 contents (Kristensen et al., 2006).

Over the past few years interest in IP6 has stemmed mostly from its potentially important anti neoplastic activity against various types of cancer, including lung cancer (Pandey and Gupta, 2012; Vucenik & Shamsuddin, 2006). In vitro studies have indicated that IP6
inhibits the growth of human breast, colon, prostate, liver cancer cells, erythroleukemia cells and rhabdomyosarcoma (Vucenik & Shamsuddin, 2006). IP6 inhibits cell transformation in mouse epidermal JB6 cells and reverses the transformed phenotype of HepG2 liver cancer cells (Raina et al., 2008; Vucenik & Shamsuddin, 2006; Vucenik et al., 2004). With in vivo studies IP6 has been shown to reduce prostate cancer in TRAMP mice, suppress dimethyl hydrazine induced large intestinal cancer in CD-1 mice, inhibit growth of 7,12-dimethylbenz(a)anthracene–induced skin and mammary tumorigenesis, reduces urethane induced lung carcinogenesis, (Pandey & Gupta, 2012; Raina et al., 2008; Vucenik & Shamsuddin, 2005; Vucenik et al., 2004). One more beneficial aspect of IP6 is it acts synergistically with other chemopreventive and therapeutic compounds like doxorubicin and tamoxifen (Vucenik & Shamsuddin, 2003).

![Chemical structure of Inositol hexaphosphate](image)

*Figure-6: - Chemical structure of Inositol hexaphosphate (C_{6}H_{18}O_{24}P_{6}).*

### 2.11.2 Sulindac in cancer chemoprevention

The levels of prostaglandins and the activity of COX-2 both are elevated in tumor cells (Fosslien, 2001). Suppression of COX-2 activity through the administration of non-steroidal anti-inflammatory drugs (NSAIDs) represents a viable mechanistic approach to cancer prevention (Rao et al., 1996). The rationale for the use of NSAIDs for cancer chemoprevention is further strengthened by the long history of human use of these agents, resulting in the development of extensive toxicological profiles for many members of this
drug class (Singh et al., 1994). Although significant toxicity is associated with high doses of these drugs, many years of clinical experience have resulted in the identification of dose levels of several NSAIDs that can be administered chronically to humans without adverse side effects. Studies from a number of laboratories have demonstrated that several commonly prescribed NSAIDs (aspirin, indomethacin and piroxicam) can suppress cancer induction in various sites in experimental animals, including breast, colon, urinary bladder, esophagus and skin (Rao et al., 1996; Kellogg et al., 1994).

Sulindac is one of the most promising pharmaceutical agent from the group of NSAIDs. Sulindac is reported to inhibit carcinogenesis in experimental tumor models and shown to act against tumor cells in vitro directly. Sulindac sulfide and inactive sulindac sulfone are the metabolite of Sulindac which are inhibitors of the COX-2 enzyme. Sulindac itself does not inhibit COX-2. Sulindac and its derivatives, alone or in combination with other chemotherapeutic, have been found to induce growth suppression and apoptosis in cultures of tumor cells (Jakubowska-Mucka et al., 2011). Sulindac has been evaluated as a chemopreventive or therapeutic agent in several clinical trials (NCT00755976, NCT00299195 and NCT00118365 available at http://www.clinicaltrials.gov). There are also attempt to use sulindac sulfone, known as exisulind, in combination treatments of various types of cancer (Govindan et al., 2009; Sinibaldi et al., 2006).

In light of previous knowledge we designed a periodic study to understand the involvement of epigenetic changes in the development of lung tumours. Here we used urethane induced mouse lung tumour development as experimental model and IP6 as a chemopreventive agent. Sulindac was used as a reference chemopreventive agent. Different time points were selected to understand the sequential molecular event of lung tumour development. Along with epigenetic changes we studied other molecular parameter related inflammation, repair, proliferation for correlating and justifying our findings.