Cancer, a terminal pathological disorder, is life threatening as compared to heart and other diseases, if not treated in time (WHO-fact sheet no. 297; Jemal et al., 2008). Lung cancer is the most fatal form among all the cancer types. A total 28% of all cancer deaths are occurring due to lung cancer (Behera, 2012; Ezzati & Lopez, 2003). The survival rate of lung cancer patients is very low which is less than 5 years (Risch & Plass, 2008). Late diagnosis, poor prognosis and development of multiple drugs resistant worsen the survival conditions (Incoronato et al., 2011). Low survival and large number of deaths due to lung cancer pertains to the lack of knowledge about the molecular events involved in the development of lung cancer. Lack of molecular markers and non invasive procedure of detection hampered the lung cancer treatment and prognosis (Incoronato et al., 2011; Risch & Plass, 2008). Understanding the molecular alterations in the inception and the establishment of cancer could be of help in controlling the disease. Though, the development of tumors is a result of altered pathways and cellular developmental stages, the recognition and understanding of the molecular signatures before and after the onset of tumors could provide the insights in the etiology of cancer that can be useful for early detection, diagnosis, and treatment (Bhatt et al., 2010).

Tumor development stages consist of both genetic and epigenetic alterations (Tsai & Baylin, 2011; Karikas, 2011). Since genetic modifications are irreversible, reversible modifications in the epigenetics have more potential for the exploitation in targeting the cancer control. DNA methylation and histone modification, are the major events in epigenetics. Epigenetics govern cell fate and alteration in epigenetics causes serious consequences like development of cancer. Gene promoter CpG methylation and histone modifications are the epigenetic signatures that have been correlated with the TSG silencing in tumors. Epigenetic silencing of TSGs affects various cellular pathways, including DNA repair and cell death (Lewandowska & Bartoszek, 2011; Kanwal & Gupta, 2011). Various cancer types have been shown to posses a certain epigenetic signature which can be exploited as a marker of detection, prognosis and chemoprevention (Tsai & Baylin, 2011; Lund & Lohuizen, 2004). Epigenetic changes have also been linked with other cellular pathways like inflammation, which are tightly linked to cancer development (Li et al., 2012; Li & Tollefsbol, 2010; Liu et al., 2010; Steele et al., 2009). Inflammation assists in neoplastic and/or malignant progression of cancer (Li & Tollefsbol,
Available reports suggest that lung inflammation could facilitate the chemically induced lung tumor development.

Different strategies have been used to control or manage the development of tumors but none of them proved to be optimum. Therefore, the chemoprevention concept of delaying or preventing cancer remains a viable and attainable goal for the future. Successful implementation of nontoxic, safe compounds in clinical trials for preventing the cancer in high-risk populations suggests that chemoprevention is a rational and appealing strategy (Brenner & Gescher, 2005; Tsao et al., 2004). A number of natural or synthetic compounds have been used or are under investigation as a chemopreventive agent to retard or prevent the development of tumors by intervening the critical cellular pathways (Karikas, 2011; Tsao et al., 2004).

Epigenetics and other related molecular alterations have been shown in the established tumors. But there is scarcity of information about what kind of molecular alterations are involved during the course of tumor development. Moreover, involvement of epigenetic changes during the period of the development of tumors is not well understood (Sharma et al., 2010). We have evaluated the epigenetic changes and have correlated them with the development of tumors at different end points from start to finish in mouse model. The development of tumors and all the molecular changes at different end points were sensitive towards the presence of chemopreventive agent, IP6. Along with epigenetic changes we have also analyzed the alteration in pro-inflammatory events and TSGs related to DNA repair and cell death. (Li & Tollefsbol, 2010; Steele et al., 2009)

Urethane exposure resulted in the development of tumors as a function of time. At 1 week time point, lung histology remained normal, suggesting the safer period after carcinogen exposure. But the appearance of hyperplasia and lymphocytic infiltration without tumors in the lungs at the later end points of 4 weeks suggested the pre neoplastic status before the onset of tumors. This statement was supported by the appearance of tumors at the later end point of 12 weeks. This is in agreement with earlier reports on the progression of carcinogen induced hyperplasia into tumors (Foley et al. 1991; Shimkin et al. 1969). The pathological alterations culminated in the development of well defined tumors at the later end points of 24 or 36
weeks. Thus, in the line of earlier reports, we suggested the relationship between the development of chemically induced pulmonary adenomas and the alterations in tissue histology (Stathopoulos, et al. 2007; Dutt & Wong 2006; Meuwissen, 2005; Shimkin & Stoner 1975; Malkinson 1989). Urethane induced mouse lung tumor model is an excellent model to study NSCLCs and elucidating events that influence development of tumors, because lung adenomas developed by urethane have similar histology and molecular features to human adenocarcinoma (Stathopoulos et al., 2007; Dutt & Wong, 2006; Meuwissen, 2005). Moreover, urethane induced tumors showed RAS and p53 mutation which is a common and similar phenomenon in human adenocarcinoma development (Meuwissen, 2005). Further, the molecular analysis projected a correlation between the degree of alterations and the state of the tumor development from start to finish, the early and late stage of tumorigenesis are also useful in screening the compounds for their chemopreventive potential.

The alterations in the epigenetic events were observed during the development of tumors even before the onset of tumors. Progression of DNMT1 upregulation with the time of urethane exposure from 1 to 36 weeks suggested the critical involvement of DNMT1 deregulation and its downstream events before and after the onset of tumors (Kanwal & Gupta et al., 2010; Tang et al., 2009; Fabbri et al., 2007). The upregulation of DNMT1 appeared to be a function of time with the maximum levels after the establishment of well defined tumors at 24 or 36 weeks. A significant (p<0.05) overexpression of DNMT1 even at the earliest end point of 1 week time (Figure-9) showed its involvement in the early stages of the development of tumor as suggested by others (Belinsky, 1996). We also showed that along with maintenance DNMT1, de-novo methyl transferases DNMT3a and DNMT3b also got deregulated during the course of tumor development. Upregulation of DNMT3b expression appeared to be slightly higher than DNMT3a. DNTM1 is mainly involved in the maintenance of DNA methylation and has been implicated in tumors as well. However, DNMT1 functions better in association with DNMT3a and DNMT3b (Chen et al., 2007). Our observations supported existing reports on the established tumors (Beaulieu et al., 2002; Linhart et al., 2007; Tang et al., 2009) by showing the upregulation in DNMT3b at early time points before the onset of tumors. This event could be responsible for generating the aberrant pattern of DNA methylation during the development of tumors after urethane exposure.
The overexpressed DNMT protein was functionally active and its enzymatic activity supported the upregulated protein expression at all the end points in a time dependent manner after urethane exposure. Though, statistically insignificant, the enzyme activity of DNMT at 1 week was 20% higher than that of control and this suggests the involvement of DNMTs as a critical player even before the onset of tumors. Up regulation of different DNMTs at as early as 1 or 4 week time point suggest their involvement in the early process of cell proliferation and transformation. The continued increase in the DNMT enzyme activity during the tumor genesis and development in our study is supported by others as well (Jin & Robertson, 2013; Belinsky et al., 1996).

Upregulation of DNMT proteins were envisaged in term of promoter CpG methylation. Available reports suggest that along with other cellular factors, DNMT1 can preferentially methylate specific genes (Jair et al., 2006). Gene promoter CpG methylation represents a potential means for TSG silencing in cancer and has been correlated with up regulated DNMTs expression in tumors (Lewandowska & Bartoszek, 2011). The mechanism of gene promoter hypermethylation has not been fully understood. However, it has been shown that the different types of cancer have hypermethylation in some TSGs (Bowman et al., 2006; Egger et al., 2004; Esteller, 2008; Lund & Lohuizen, 2004). We showed the impact of the altered epigenetic events on the expression of certain tumor suppressor genes by analyzing the promoter CpG methylation of genes, including p16, MLH1, MGMT, DAPK1 and COX-2 during the process of tumor development from start to finish.

Promoter CpG hyper methylation corresponded to the down regulated expression of p16 and MLH1 from start to finish the development of tumors. Inactivation of p16 is very well correlated with the tumors and p16 methylation is an important phenomenon in tumors that can be used in evaluation of tumors (Agarwal et al., 2012; Witcher & Emerson, 2009; Liu et al., 2006). We showed the progression of p16 gene promoter CpG methylation with the time, before and after the onset of tumors. This also suggested the deregulation in cell cycle before and after the onset of tumors. Lack of promoter CpG methylation at 1 week time could be due to the early time of initiation where clones of transformed cells were not selected to a detectable level. Promoter CpG methylation at 4 week end point was well correlated with the
p16 down regulation and suggested its relevance in the beginning of the development of tumors. Increasing pattern of promoter CpG methylation from 12 to 36 weeks end points and the down regulation of p16 at respective time points showed their involvement in the development of tumors. Likewise, we showed the involvement of promoter CpG methylation in MLH1 gene in time dependent progression of the tumors. Promoter CpG methylation in MLH1 gene could not be detected at the time of no tumor, 1 or 4 week end point, suggesting the active DNA damage repair as a protective measure in developing the earlier lesion of tumor development. However, the appearance of this methylation at 12 weeks that increased further at 24 and 36 week suggested the ineffective DNA damage repair enzyme leading to the progressive development of tumors (Seng et al., 2008; Raptis et al., 2007).

Unlike p16 and MLH1 genes, MGMT and DAPK1 promoter CpGs were found to be methylated only in tumor tissues. We did not observe promoter CpG hyper methylation of MGMT at early end points of our study, but the CpG hyper methylation was observed at 24 weeks with the presence of benign adenomas. Hyper methylation increased further at 36 weeks which showed the predictive importance of promoter CpG hyper methylation in MGMT in NSCLCs (Candiloro & Dobrovic, 2009). Our observation of the promoter CpG hypermethylation in DAPK1 genes only at the time of well defined tumors at 36 weeks end point in accordance with earlier reports where it was found to be methylated in malignant tumors but not in benign or in early stage tumors (Mir et al., 2013; Zorko et al., 2010; Botezatu et al., 2008). We did not observe promoter CpG hypermethylation in the COX-2 gene as it is not frequently regulated via promoter CpG methylation. Moreover, available reports suggest COX-2 promoter CpG methylation is not a frequently occurring phenomenon in cancer (A sting et al., 2011; Toyota & Issa, 2005). Analysis of promoter CpG methylation of genes like p16, MGMT or GSTP in the established tumors has been suggested as biomarkers of detection, prevention and prognosis (Esteller, 2008; Palmisano et al., 2000) and shown to be useful in non-invasive detection of NSCLCs. Whereas, our study showed the involvement of the gene promoter CpG hypermethylation even before the onset of tumors and its progression till the establishment of tumors. Promoter CpG methylation of p16 or MLH1 seems to have predictive value in lung tumor development as the CpG methylation present at early time end points. Whereas, promoter CpG methylation of DAPK1 and MGMT was seems
to be involved in advancement of tumors (Esteller, 2008; Palmisano et al., 2000). Occurrence of promoter CpG hypermethylation confirmed the functional activity of the upregulated DNMTs in mouse lungs as others have also shown the DNMT3b caused de novo methylation of TSG favoring the tumor development (Beaulieu et al., 2002; Linhart et al., 2007; Tang et al., 2009).

DNMTs do not function in isolation to inactivate the gene transcription. They work along with other genes forming a transcription repressor complex. DNMT1 interacts with MBDs, HDAC, E2F1 and other proteins which make it an important player of TSG suppression (Tang, 2009). MBD proteins have been linked to the repression of TSG via promoter CpG methylation in human cancers with HDACs (Gronbaek et al., 2007; Jones et al., 1998; Rolof et al., 2003; Lopez-Serra & Esteller, 2008; Zou et al., 2011). Deregulated MBDs in established cancers have been reported by others (Parry & Clarke, 2011; Kanwal & Gupta et al., 2010; Liu et al., 2008; Zhuravel et al., 2008). We showed that the upregulation of MBD proteins was a function of time after urethane exposure and further increased at 24 or 36 weeks end point of established tumors showing their positive correlation with the development of tumors. Among all the MBD proteins studied, MeCP2 and MBD2 found to be upregulated significantly at earlier end points. A slight upregulation of MBD1 at earlier end points, when tumors were not well defined, suggested its involvement and regulation in later stages of the development of tumors. Overall, the deregulation of MBD proteins as early as 4 or 12 weeks end points suggested their involvement in establishing the developed tumor in mouse lung. Others have also suggested that elevated levels of MBDs have tumorigenic potential and are indicative of gene silencing (Parry, 2011; Zhuravel, 2008; Liu, 2008; Park, 2007; Muller, 2003). Based on our observation, we can suggest that the upregulation of MBDs, a component of the transcription repressor complex, could be helping the upregulated DNMTs in executing the methylation followed by gene silencing during the development of tumors (Gronbaek et al., 2007; Jones et al., 1998; Pandey & Gupta, 2011). It has been reported that along with DNA methylation, MBD proteins are also clinically important for diagnosis and prognosis of cancer (Kanwal & Gupta, 2010). That is how MBDs could be considered as a potential target for cancer prevention and therapy.
Having shown the alteration in DNA CpG methylation and associated molecules, we analyzed histone modifications, the other component of epigenetics. DNA methylation and histone modifications both are interlinked (Jin et al., 2011). DNA methylation, the best-known epigenetic marker, occurs in the context of nucleosome positioning, DNA sequence composition and histone modifications (Chodavarapu et al., 2010; Jones & Liang, 2009; Lee & Mahadevan, 2009; Vaissiere et al., 2008). Therefore, we showed the status of histone modification in terms of modified histone, H3K9me2 and H3K27me3 and expression of HDAC1 and histone methyl transferases, EZH2, SUV39H1 and G9a during the genesis of lung tumors between 1-36 weeks.

Time dependent upregulation of HDAC1 from 1-36 weeks before and after the onset of tumors, suggested its critical role in the progression and the development of the tumors. HDAC1 upregulation at early time points 1 or 4 weeks before the onset of tumors, (Figure-14) showed its importance in early neoplastic transformations of the cells for the development of tumors. Overexpression of HDAC1 by urethane exposure at 1 week end point in lung cells or at 4 weeks end point in lung hyperplasia itself suggested a link between HDAC1 function and proliferation. IHC analysis showing the higher expression of HDAC1 in tumor tissue than surrounding and hyperplastic tissue supports its role in the formation of tumors. Elevated HDAC1 has been shown to be present in highly proliferating tissues, embryonic stem (ES) cells and several transformed cell lines (Senese, 2007). HDAC1 over expression has been variably linked with prognosis of solid tumors (Bowman et al., 2006). These results suggest HDAC1 as a potential target for the prevention and control of tumor development as its inhibitors are among the prominent candidates used for cancer control (Witt et al., 2009) possibly by inducing the apoptosis as HDAC1 has an anti apoptotic role in the development of lung cancer (Bowman et al., 2006; Witt et al., 2009).

Over expression of EZH2, a histone methyl transferase is reported in various types of cancers and its inhibition blocks cell proliferation in cancer cell lines suggesting its involvement in proliferation along with invasion, metastasis and angiogenesis (Fussbroich et al, 2011). EZH2 got upregulated significantly just in a week after urethane exposure and increased during the time of well defined tumors as a function of time suggesting EZH2 modulation as an early
event involved in the cell proliferation, pre neoplastic transformation and establishment of the tumors. H3K27 hypermethylation suggested the functional activity of EZH2 up regulation. Though, the degree of H3K27 modulation in terms of H3K27me3 levels was little at 1 week, it increased significantly thereafter at 4, 12, 24 or 36 weeks time when the tumor growth progressed from start to finish. This aberrant tri methylation of H3K27, a result of EZH2 up regulation could suppress the transcription of genes whose transcription sites are near H3K27me3. This has been shown to be an important phenomenon in the development of different malignancies (Sneeringer et al, 2010; Garcia & Licht, 2010).

G9a and SUV39H1 are responsible for di and tri methylation of H3K9 and repress the gene transcription crucial for supporting and maintaining the growth of malignant cells (Kondo et al, 2008). G9a, present at the silent domain of euchromatin region, causes mono or di methylation of H3K9 (Rice et al, 2003). A significant G9a upregulation at 12, 24 or 36 weeks, when tumor development progressed in mouse lung, indicated its role in promoting the growth of tumor. A slight down regulation of G9a at early end points could be due to the maintenance of the related events. Our observations are supported by the reports showing the involvement of G9a in liver, prostate, kidney and lung cancer invasion and metastasis (Chen et al, 2010; Kondo et al, 2007). Methylation at Lys-9 on histone H3 is specifically catalyzed by SUV39H and has recently been shown to be a marker of heterochromatin (Noma et al, 2001; Peters et al, 2001). On the other hand, SUV39H1, present in heterochromatin region, causes H3K9 trimethylation (Rice et al, 2003). A time dependent upregulation of SUV39H1 expression in our study was supported by the IHC analysis that showed the overexpression of SUV39H1 in the hyperplastic region of lung tissues. SUV39H1 expression increased with the progression of tumor growth suggesting the possible role of SUV39H1 in the urethane induced transformation in the course of the development of tumors.

On the contrary, trimethylated histone, H3K9me2 is shown to be reduced in different cancers (Chen et al, 2010; Seligson et al, 2009). Our data are in agreement with the available reports as we showed an overall decrease in the dimethylation status of H3K9me2. However, in contrast to the overall decrease in the level of H3K9me2, IHC analysis showed the increased
levels of the H3K9me2 in certain tumors and hyper plastic regions at 12, 24 or 36 weeks endpoints as compared to surrounding or control tissue.

Presence of H3K27me3 and H3K9me2 mark on gene promoters is an important phenomenon for altered gene expression (Kondo et al, 2003). Having shown the alteration in histone modification and histone modifying proteins, we analyzed the presence of H3K27me3 and H3K9me2 marks on promoters of p16 and MLH1 genes. On the basis of our ChIP analysis data, we suggest that histone methylation is a critical modification responsible for maintenance of promoter DNA methylation-associated gene silencing in lung tumor development. Our study provided the evidence in support of the presence of H3K27me3 or H3K9me2 mark on the promoters of p16 and MLH1 which increased with tumor progression at the end points of 12, 24 or 36 weeks. However, the presence of H3K27me3 mark on p16 gene promoter could be seen even at 4 weeks which was not evident for H3K9me2 at the same time. At the same time the degree of interaction between promoter and modified histone was more for p16 than MLH1. Level of H3K27me3 was more than H3K9me2 on p16 or MLH1 promoter. With these results we suggest that H3K27me3 and H3K9me2 are closely related to DNA methylation and acts as an epigenetic mark of silencing in the tumor suppressor genes like P16 or MLH1 (Varier & Timmers, 2011; Ellis et al., 2009; Kouzarides, 2007). The interaction of methylated histones with gene promoters in a time dependent manner suggest their involvement in the clonal expansion of the initiated cells progressing towards the development of tumors by suppressing the TSGs.

We have shown and discussed how epigenetic scenario changes in the inception and in the establishment of urethane induced lung tumors and how gene promoters of TSGs are altered by the methylation and histone modifications. These covalent modifications affect the gene expression as a downstream event and this is reflected in the expression level of p16, MLH1, MGMT and DAPK1 in our study. Expression of p16 and MLH1 was not affected much at 1 week time point, the early phase of tumor development. But as the tumor progressed with time from 4 to 36 weeks, these genes started showing downregulation at the proteins and mRNA level. The down regulation of p16 and MLH1 observed at 4 weeks time, whereas it was maximum at 24 or 36 weeks when the tumors were well established. IHC data confirmed the
higher alterations in the tumorous tissues. Gene promoter CpG methylation and the presence of methyl histone marks on the gene promoters corresponded to the downregulation of the gene expression suggesting the epigenetic silencing of these genes during the period of tumor development. Inactivation of the p16 gene by aberrant methylation could represent a critical step in the genesis of NSCLC by allowing the uncontrolled clonal expansion of some of these premalignant lesions to cancer (Kim et al., 2001; Esteller et al., 2001). Similarly, MLH1 expression has been shown to be reduced and downregulated by methylation in NSCLC (Seng et al., 2008).

MGMT, another DNA damage repair gene, has been shown to be inactivated by promoter methylation in established lung cancer (Lai et al., 2009; Herman et al., 2000) and we also showed that MGMT was significantly modulated at the later stages of the tumor development than the earlier stages. However, the frequency of MGMT methylation was lower than that of p16 as suggested by others (Liu et al., 2006). Altered p16 during the entire period of tumor development suggested the altered cell cycle and its progression to the ‘S’ phase. But the unaffected MGMT status during the early stage of tumor development reflects the active DNA damage repair that prevented the onset of tumors.

Downregulation of MGMT at the 24 or 36 weeks corresponded to its promoter methylation with respect to time. These observations suggest the role of methylation in inactivation of MGMT and its role in the advancement and the progression of urethane induced lung tumors by altering the DNA damage repair system. Suppression of DAPK1 by methylation has been shown in tumors but not in the early stage of tumor development (Mir et al., 2013; Zorko et al., 2010; Botezatu et al., 2008) and we also showed the downregulation of DAPK1 at 24 or 36 weeks, the later stages of tumor development, which is very well correlated with its promoter methylation. This indicated the active apoptotic machinery at the earlier stages of tumor development. The lack of methylation and down regulation at early end points suggested unaltered functions of these TSGs but not at the later end points following carcinogen exposure.
Lung inflammation could facilitate the chemically induced lung tumor development and have been linked with epigenetic changes (Li et al., 2012; Pandey & Gupta, 2012; Aggarwal et al., 2006). Here we tried to answer the question if the deregulation in epigenetic pathway after urethane exposure is linked with the molecules involved in inflammation and cell cycle progression. NF-κB along with HDAC1 could affect the DNMT expression in neoplastic progression (Li & Tollefsbol, 2010; Liu et al., 2010; Steele et al., 2009). Whereas, IL6 can induce the DNMT1 expression and could facilitate promoter methylation (Li et al., 2012). Status of the molecules involved in the inflammation and in the cell cycle is correlated with the status of epigenetic changes taking place during the development of lung tumors.

Over expression and activation of STAT3 mediates the tumorigenesis through inflammation (Jarnicki et al., 2010) and we have shown the over expression and activation of STAT3 as a function of time, before and after the onset tumors. STAT3 gets activated after its phosphorylation. Over expression of phosphorylated STAT3 (Tyr 705) at all the end points from 1- 36 weeks supported the functional activity of the STAT3 and its involvement in the genesis of tumors. Further, upregulation of the downstream targets of STAT3 like cytokine IL6 confirmed the functionality of the upregulated and active STAT3. Upregulation and activation of STAT3 and IL6 overexpression at 1 or 4 weeks suggest their crucial role in the initiation of the process of tumor development by affecting the inflammatory process as shown by others (Grivennikov et al., 2009; Hong et al., 2007; Kishimoto, 2005) and thereafter in the establishment of well defined tumors.

Upregulation of NF-κB was a function of time during the development of lung tumors by urethane suggesting its role in the different stages of tumor development described by others (Meylan et al., 2009; Stathopoulos et al., 2007). Overexpression of NF-κB at 1, 4 or 12 weeks, the early stages of tumor development could be involved in triggering the inflammatory responses by carcinogenic insults that gets hyped with the time, duration and help in tumor development (Mantovani et al., 2008; Stathopoulos et al., 2007; Nishikori, 2005). p50 and RelA, the subunits of NF-κB heterodimer, showed the similar pattern. Data obtained by IHC analysis for p50 indicated the localization of p50/RelA complex. We could say this on the basis of the distinct p50 localization and over expression in nucleus of air ways epithelium
after urethane treatment with a significant increase in tumor tissues. Translocation of p50/RelA complex from cytoplasm to the nucleus and transcription of its target genes has been shown by others (Nishikori, 2005). NF-κB and STAT3 regulates each other through cytokines and transcription of genes like Bcl protein family and cyclin D1 (Gao et al., 2007). Cyclin D1, a transcriptional target of NF-κB and STAT3 transcription factors, regulates cell cycle progression from G1 to S phase and involved in cell proliferation and cancer (Stamatakos et al., 2010; Jarnicki et al., 2010; Nishikori, 2005). NF-κB and cyclin D1 appeared to be more responsive toward the urethane exposure with the passage of time. A significant increase in cyclin D1 expression could be due to the over expression of transcription factors NF-κB and STAT3 and thus, potentiating the cell proliferation. The continued over expression of COX-2, a target gene of NF-κB (Schafer & Brugge, 2007; Kishimoto, 2005), is in agreement with the functional activity of over expressed NF-κB. Moreover, COX-2 itself has been shown to be upregulated in various tumors and shown to be involved in tumor development (Urade, 2007). Upregulation of COX-2 at all the time points could be for its involvement in neoplastic transformation and establishment of tumors by altering the inflammatory and related processes. Hence, deregulation in inflammatory pathways corresponded to the altered epigenetic scenario after urethane exposure.

We have provided the evidences about the status and the involvement of epigenetic and associated events during the course of the development of tumors from start to finish. In order to exploit the molecular modulations as targets for the control of tumors, they should be responsive to the antitumor agents. The study of the development of tumors and the molecular modulations in presence of IP6, an antitumor agent, fulfilled this aspect also. Study on chemoprevention has grown rapidly with the significant suppression of tumors by chemopreventive agents by affecting signal transduction, hormone/growth factor activity, oncogenes/TSGs, terminal differentiation, apoptosis, immune response, angiogenesis or inflammation (Hursting et al, 1999; Kelloff et al, 2000). Chemopreventive interventions by compound with any molecular change are likely to give an insight for targeted cancer therapies and prevention (Gupta et al., 2003; Raina et al., 2008). Certain dietary compounds have been shown to possess the anticancer or chemopreventive properties. Tea polyphenols, butyric acid, ω-3 PUFA, chaetocin, EGCG, curcumin have potential to inhibit the activity and
expression of DNMTs, HDACs and HMTs for manifesting their chemopreventive activity (Gerhauser, 2013; Berghe, 2012). IP6 is a naturally occurring sugar phosphate, exhibiting tumor suppressing effect through its anti-angiogenic, anti-oxidant and anti inflammatory properties (Gupta et al, 2003). We used sulindac as a reference compound to assess the relative effects of IP6. Sulindac is a synthetic prodrug which converted into sulindac sulfide in liver. Sulindac sulfide is a potent inhibitor of COX-1 and COX-2 enzyme. Chemopreventive efficacy has been established in tumor models including lung tumors (Jakubowska-mucka et al., 2011; Marchetti et al., 2009; Castonguay and Rioux, 1997; Reddy et al., 1999). Along with anti inflammatory potential sulindac possess anti HDAC activity as well. By inhibiting HDAC1 sulindac can alter the epigenetic state of a cell (Liu et al., 2005).

Similar to sulindac, IP6 inhibited the tumor developed by urethane in terms of size and number (Figure-8, Table- 4 or 5) suggesting it’s potential in preventing the development of tumors. IP6 affected the early stage of tumor progression by inhibiting the hyperplasia and lymphocytic infiltration in 4 weeks time.

The analysis of the effects of IP6 or sulindac on the urethane caused molecular modulations suggested the possible mechanisms of chemoprevention by these compounds. IP6 and sulindac protected the urethane induced molecular changes at all the time points, but the reduction in molecular changes at early end points of study were not significant statistically. That might be due to the early stage of the development of tumors where changes were not enough to be statistically significant. Prevention was more pronounced at the later end points at 4 or 12 weeks due to higher degree of molecular alterations leading to the development of the tumor.

Prevention of DNMT1/3a/3b overexpression at almost all the end points by IP6 showed its potential to affect the CpG hypermethylation, a covalent modification, in silencing the genes during the development of tumors. As per the results of IHC analysis and DNMT enzyme activity, IP6 could protect the DNMT status even at 1 week time when the tumor pathology was unaltered. Sulindac also protected the urethane induced DNMT overexpression but not to the degree of IP6. Other naturally occurring compounds like tea polyphenols have been shown
to retard the tumor development by modulating DNMT expression (Gerhauser, 2013; Berghe, 2012). IP6 also protected the p16, MLH1, MGMT and DAPK1 gene promoter CpG hypermethylation caused by DNMT overexpression. Thus, IP6 could protect overexpression of DNMT and its downstream effect on the methylation as well, suggesting its chemopreventive action through the modulation of CpG methylation. It has already been reported that inhibition of DNMTs decreased the promoter CpG methylation and reexpression of TSGs (Li et al., 2012; Garzon et al., 2009). Preventive effects of IP6 or sulindac on the overexpressed MBDs proteins suggested that the presence of IP6 could prevent the associated molecules as well involved in the CpG gene promoter hypermethylation in gene silencing and this effect was more pronounced as tumor progressed.

Further, we showed that the presence of IP6 or sulindac could equally protect the alterations in the histone modifications in terms of histone methyl transferases and methylation of histones representing the other component of the epigenetics. Protection of upregulated HDAC1, EZH2, G9a and SUV39H1 was more pronounced during the later phases of the development of tumors in the presence of IP6 confirming the involvement of these molecules in the progression of tumors and its control. Our observations are supported by others showing the inhibition of activity and expression of HDACs and HMTs by natural compounds like ω-3 PUFA, chaetocin, EGCG, curcumin (Gerhauser, 2013; Berghe, 2012).

Increased levels of H3K9me2 and H3K27me are the results of the functional activity of the overexpressed histone methyltransferases. We showed that IP6 prevented the increase in H3K9me2 and H3K27me and their presence on the p16 and MLH1 gene promoters as a result of the prevention of histone methyltransferases overexpression. Sulindac exerted its effects in a similar fashion.

Epigenetics also affects the inflammatory events and we confirmed this by showing the protective effects of IP6 or sulindac on the upregulated inflammatory molecules. Urethane has been shown to induce inflammation in mouse lungs (Pandey & Gupta, 2012; Stathopoulos, et al. 2007) and IP6 or sulindac are shown to have strong anti inflammatory properties (Jakubowska-mucka et al., 2011; Marchetti et al., 2009; Liao et al., 2007; Castonguay and
It has been shown that IP6 inhibits dextran sulfate sodium (DSS) induced colon inflammation (Liao et al., 2007). We supplemented this by showing the protective effects of IP6 or sulindac on the urethane induced STAT3 and NF-κB overexpression from start to finish the development of tumors. We can suggest that IP6 or sulindac exert their chemopreventive effects by involving the important inflammatory pathways. Further, the prevention of the overexpression of IL6, COX-2 and cyclin D1, the downstream targets of STAT3 and NF-κB, confirmed the involvement of inflammatory molecules in chemoprevention by IP6 or sulindac. A crosstalk between the inflammatory modulation and epigenetic changes has been suggested (Li et al., 2012) as 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 microRNA-29b and can enhance DNMT expression (Li et al., 2012; Lin & Chen, 2010; Liu & Chen 2011; Pandey & Gupta, 2013).

With our results and available reports we suggest that IP6 or sulindac could modulate epigenetic changes via modulating inflammatory pathways involving cell cycle regulators, DNA damage repair system in reducing the development of tumors after urethane exposure. IP6 seems to have a little higher potential as chemopreventive agent than sulindac under our experimental condition and dose. This might be due to higher dose of IP6 (2%) than sulindac dose (0.008%).

In summary, we can say that our study provided with a valuable insight of the involvement of epigenetic events during the tenure of the development of tumor by showing the alterations in the epigenetics and associated molecules with the passage of time for the development of tumors. Alteration in DNMTs, HDAC, EZH2 and HMTs supported by the gene promoter CpG methylation and histone methylation marks showed the importance of the deregulated epigenetics at early stages after carcinogen exposure. Simultaneous alteration in inflammatory pathways involving associated molecules like STAT3, NF-κB, COX-2, cyclin D1, IL6 after the urethane exposure in preneoplastic lesions suggests a relationship between epigenetics and the inflammation even before the onset of tumors. IP6 has the potential to prevent the development of tumors at all the stages even before the onset of tumors by preventing the epigenetic and inflammatory changes. Modulations of the epigenetic pathways and associated events at early end points after urethane exposure and then in the tumor tissues in presence or
absence of IP6 confirmed their involvement even in the pre neoplastic progression making these events a potential candidates for the exploitation in the development of agents for cancer control.

Our observations are novel in the sense that we provided the insight of the development of tumors from its inception to its establishment by showing the altered epigenetics along with inflammation, cell cycle regulation, DNA damage repair system before and after the onset of tumors in the presence or absence of antitumor agent showing their relevance in the advancing tumors and use in planning the strategies for cancer control.