Lung cancer has become one of the leading causes of cancer deaths in developed countries and is also rising at an alarming rate in developing countries. Despite the advances made in diagnosis and treatment in the last few decades, the prognosis of lung cancer is still very poor, with a 5-yr overall survival generally <10% of the cases in many countries (Parsons et al., 2010). On the basis of this conception, numerous reliable and lung specific serologic protein biomarkers have been characterized. These proteins may play role in the pathophysiology of lung cancer and may have potential for diagnostic and prognostic biomarker development that may ultimately lead to better patient care. Exploring the presence of such markers secreted by cancer cells into the bloodstream would involve minimal invasive surgeries and would open new vistas in clinical oncology. In addition to smoking, occupational exposure to carcinogens, indoor air pollution and dietary factors have been implicated in the causation of lung cancer. Molecular genetics of lung cancer has opened up new vistas of research in carcinogenesis. Lung carcinogenesis is a multistep process characterized by the accumulation of successive molecular genetic and epigenetic abnormalities, structural aberrations including deletions and translocations resulting in selection of clonal cells with uncontrolled growth capacities throughout the whole respiratory tract. These mutations include activation of the dominant cellular protooncogenes (which promote oncogenesis) of the ras and myc family (c-myc, L-myc, N-myc, c-raf, K-ras, N-ras, H-ras) and inactivation of the recessive or tumor suppressor genes like p53, p16 and Rb genes (these genes help suppression of tumor development) (Behera, 2012). Defects in the p53 and Rb pathways are common to many of the histological types of lung cancer. Approximately 50% of all lung cancers harbour dysfunctional p53 protein with p53 mutations being detected in almost 90% of SCLC and 50% of NSCLC (Rodin and Rodin, 2005; Viktornsson et al., 2005). A large proportion of tobacco-associated mutations in tumor protein p53 are G:C → T:A transversions, and are most commonly seen in squamous and small cell carcinomas, whereas in non-smokers the transversions are G to A (Pfeifer et al., 2002). Rb is also inactivated in 90% of SCLC and 15% of NSCLC. Disruption of the Rb pathway in NSCLC seems to be associated with inactivation of the p16INK4A gene which encodes a cyclin-dependent kinase inhibitor (Motadi et al., 2007).

Due to the short life span of rodents, it’s easy to study cellular transformation in them from its initiation to development of full-blown malignant cancer. This makes them a widely used animal model for experimental studies. With lung carcinogenesis models, it may be helpful to gain insights
into basic biology of lung tumors, find out markers for early diagnosis, and validate anti-lung cancer prevention and therapies. A number of carcinogens for lung cancer induction in rats include benzopyrene, cigarette smoke, vinyl carbamate and DEN but all these carcinogens cause multiple organ carcinogenesis. The incidence of lung adenocarcinoma has been remarkably increasing in recent years due to the introduction of filter cigarettes and secondary-hand smoking because the people are being exposed to higher amounts of nitrogen oxides, especially 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), which is widely applied in animal model of lung tumors. At the same time amounts of benzo[a]pyrene, a cause for squamous-cell carcinoma of the lung in rodents, have decreased. Collectively, these observations support the role of NNK as a significant cause of lung cancer in smokers (Wynder and Hoffmann, 1994).

The basic goal is to provide a biochemical foundation for exploring the relation between exposure to environmental tobacco smoke and lung cancer. Environmental tobacco smoke is the term used to describe the material in indoor air that results from tobacco smoking. Research in this area has focused on NNK. This substance is present in both unburned tobacco (1–2 μg/g) and in cigarette smoke (100–200 ng per cigarette), as confirmed in many international studies (Spiegelhalder and Bartsch, 1996). It is not found in materials other than those related to tobacco. NNK is a powerful pulmonary carcinogen, inducing lung tumors in rats, mice, and hamsters whatever the route of administration (Hecht et al., 1994).

In our laboratory, we therefore developed a rodent model by injecting NNK. Male Wistar rats were given a combination of low and two different doses of NNK; a single dose of 2.5 mg/kg bwt followed by 1.5 mg/kg bwt) doses 3 days/week for a total dose of 100mg/kg (Figure 4.1). During all these years, many researchers have examined the dosimetry resulting from exposure to different carcinogens for cytotoxicity and DNA adducts formation (Belinsky et al., 1987). Our laboratory has been interested in understanding the tissue selectivity of tobacco specific nitrosamine, NNK. In rodent carcinogenicity studies (Hoffmann et al., 1984), treatment with high doses of NNK resulted in a similar high incidence of lung, liver and nasal cavity tumors whereas in our experiment after administering a single high dose of 2.5 mg/Kg of NNK and combining it with lower doses of NNK (1.5 mg/Kg) the prevalence of malignant tumors in rats was specifically in lungs than in other target tissues as evident by histopathology. This kind of multiple dosing of NNK simulating chain
smoking in humans leads to a steady-state DNA adduct level to be achieved by the opposing effects of damage and repair. The accumulation and persistence of such DNA adducts in the lung may be a contributing factor to the induction of tumors associated with carcinogenesis. An etiological role of NNK in the induction of human lung cancer is supported by evidence showing that NNK requires activation catalyzed by multiple forms of cytochrome P450 isoenzymes, forming damaging adducts and free radical intermediates that covalently bind to or oxidize cellular macromolecules such as DNA, protein and lipid initiating or promoting inflammatory, toxic or carcinogenic processes (Hecht, 1998; Hussain et al., 2003).

The successful development of the animal model was further validated by histopathological observation of the lungs (Figures 4.2A,B). In the present study, NNK administration induced the onset of dysplasia or preneoplastic lesions in three months that became the precursors of tumor formation in lungs in nine months as evidenced by histopathological reports. After three months of administration of NNK, the principal changes seen were dysplasia of the bronchial epithelial cells resulting in multilayering and heaping up of the bronchial epithelium. There was also associated inflammatory cell infiltration in the bronchial wall. However no tumor islet formation or presence of atypia was noted in the bronchial epithelial cells at this period. The subsequent lot of lung biopsies taken at 9 and 12 months of NNK treatment clearly showed tumor islet formation in the lung parenchyma along with significant nuclear hyperchromasia in the bronchiolar epithelial cells confirming neoplastic transformation in the lung tissues in the treated animals at this stage. The tumor cells were 122x62 microns large in size and the major organs like kidney and liver were found to be normal at this stage. Such kind of slow progression of tumor formation also gives sufficient time to analyze significant changes in sera at proteome level (Bhatnagar et al., 2012).

A variety of clinical alterations such as loss of hair, loss of appetite and water were also noticed within a month of NNK administration (Table 4.1). In our study we also observed that rats which received NNK showed a significant decrease in their body weights when they were compared to control rats of the same age. This effect was attributed to protein malnutrition (the treated animals showed decreased appetite and reduced food intake) and chemical carcinogenesis (Figure 4.3). Experimentally, the role of dietary fat and its relationship to carcinogenesis has been explored in various settings (Reddy, 1992; Welsch, 1995). Epidemiological studies indicate that the fat content
of the diet plays an important role in the development of NNK-induced adenoma, adenocarcinoma, and adenosquamous carcinoma in rat lung (Wynder et al., 1987; Goodman et al., 1992; Hoffmann et al., 1993). In our study we modified the dietary fat by increasing its content to 26.15% in order to promote the chances of tumor formation.

The pathogenesis of cancer has not yet been clarified, but substantial evidence suggests that free radicals, particularly oxygen radicals, play an important role in the complex course of multistep carcinogenesis. Given the long term evolutionary development of cancer, these conditions are not normally expected to cause cancer unless they are the source of primary mutagenic event. Some important exogenous causes of oxidative stress involved in carcinogenesis are tobacco smoke in bronchogenic carcinoma, ultraviolet light in skin cancer, fatty acids in food for colorectal cancer and ethanol for hepatocellular carcinoma (Diplock et al., 1994; Batcioglu et al., 2006). Antioxidants constitute the foremost defence system that limit the toxicity associated with free radicals. In aerobic life cycle, oxygen free radicals (OFR) are formed in normal cell metabolism from molecular oxygen. Despite antioxidant defenses, these OFR cause constant damage to oxidisable molecules which are repaired or replaced in a dynamic equilibrium. Oxidative stress arises either due to the overproduction of OFR or from the deficiency of antioxidant (enzymatic or non enzymatic) defence or repair mechanisms and results in reversible or irreversible tissue injury (Cerutti and Trump, 1991; Beltowski et al., 2000).

Exposure to NNK has been reported to result in the development of neoplastic lesions in experimental animals (Akopyan and Bonavida, 2006; Ye et al., 2007). By studying the biochemical alterations that take place in an animal model, it is possible to gain more insight into the mechanisms leading to the altered metabolic process in human lung cancer. Although NNK can form an electrophilic reactive intermediate, studies have shown that NNK may also initiate the formation of ROS. Radical “scavengers” reduced the amount of NNK-initiated DNA single strand breaks in cultured human lung cells, suggesting that at least part of the genotoxicity of NNK was ROS-mediated (Weitberg and Corvese, 1993). However it has been reported that NNK disrupts the mitochondrial respiratory chain leading to an increased generation of superoxide anion and hydrogen peroxide (Chuang and Hu., 2006). This may lead to increased lipid peroxidation and
oxidative damaged macromolecules including lipid, DNA, RNA, antioxidant enzymes in subsequent cell through disruption of cellular functions and integrity (Freeman and Crapo, 1982).

In our study we found increased TBARS (thiobarbituric acid reactive substances) concentration in serum (Figure 4.4) from NNK treated rats, indicating that there is a non-specific over production of free radicals in some phases of diseases. The data suggest ongoing lipid peroxidation within the influenced tissues, and subsequent release of lipid peroxidation products into the circulation, possibly induced by enhanced generation of oxygen radicals or deficiency of antioxidant defence mechanisms. The present findings are in agreement with previous studies which suggested that accumulation of ROS may result in significantly increased lipid peroxidation at cellular and molecular level and may be involved in tumor promotion (Zieba et al., 2000; Kaynak, 2002; Kayanar et al., 2005). It has been reported that malondialdehyde- the main and ultimate product of lipid peroxidation as well as other products of polyunsaturated fatty acid damage might react with amino acid residues of proteins and lead to their oxidative modification. This aldehyde also has the ability to increase oxidative stress by promoting the cellular consumption of glutathione (Marnett, 2002; Niederhofer et al., 2003).

SOD is the first enzyme in antioxidant defence that eliminates superoxide radicals to form H$_2$O$_2$ and hence protect the cells from the toxic effects induced by free radicals. Decreased activity of SOD has been reported in pathological conditions. In our study, the activity of the antioxidant enzyme SOD in serum of NNK treated rats was also found to be decreased significantly (Fig 4.5). It has been reported that free radicals attack proteins, mainly enzymes (Comporti et al., 1985). Hence it is assumed that the decrease in activities of SOD in our present study might be due to the increased free radical attack i.e. due to elevated lipid peroxidation products (MDA) in sera of cancer patients.

Catalase has been implicated to play an important role in the protection of cells by detoxification of high concentrations of H$_2$O$_2$. In present study, the decrease in catalase activity (Figure 4.6) may have relationship with excess H$_2$O$_2$ production following NNK treatment or due to SOD inhibition.

GSH (reduced glutathione), a widely distributed cellular reductant is a metabolic regulator and putative indicator of health. It is a key molecule in redox body homeostasis. Blood glutathione
levels are believed to be predictors of morbidity and mortality (Lang et al., 1992). GSH can directly act as a free radical scavenger by neutralizing HO•, or indirectly by repairing initial damage to macromolecules inflicted by HO•. A decrease of blood GSH in circulation has been reported in several diseases including malignancies (Della et al., 2000; Lin and Yin, 2007). But there are reports where the increase in its concentration has been shown in some cancer patients (Boisio et al., 1990; Abou-Ghalia and Found, 2000). In our study, the GR and GSH levels were found to be significantly decreased (Figures 4.7 & 4.8 respectively) in serum of NNK treated rats over control rats. The decreased GSH level may be due to increased level of lipid oxidation products which may be associated with the less availability of NADPH required for the activity of glutathione reductase (GR) to transform oxidized glutathione to GSH (Sarkar et al., 1995) due to increased production of ROS at a rate exceeding the ability to regenerate GSH. Oxidative stress may cause changes in the glutathione redox state in cancer tissues. Alterations in GSH status (GR, GSH levels) in blood serum may reflect changes in tumors. Tumor cells have been reported to sequester essential antioxidants such as GSH to meet the demands of growing tumor (Buzby et al., 1980; Kumaraguruparan et al., 2002).

Blood serum contains a large number of secreted low abundance proteins that are critical for signaling cascades and regulatory events. During necrosis, apoptosis and hemolysis, contents of cells may be released into serum. The presence of these components in blood reinforces the benefits of using a proteomic approach for identifying biomarkers for disease states. For medical practitioners, the measurement of serum proteins may be a powerful clinical assessment tool for detecting, diagnosing and monitoring of diseases and pathophysiological processes. A disturbance in the interrelationship among these proteins can indicate the presence of infection, inflammation, malnutrition or other types of autoimmune diseases. In NNK-induced lung tumors, genetic mutation, chromosome instability, gene methylation, and activation of oncogenes have been found so as to disrupt the expression profiles of some proteins or enzymes in various cellular signal pathways. As serum protein determinations can provide valuable information at early stage during the course of a disease, patient outcomes can be improved and the cost of patient care can be reduced.
In the present study, we compared the protein profiles between the serum of control and lung cancer induced rats using 1-D, 2-DE and LC-MS approaches (Figures 4.9-4.13). Using this approach we have observed a number of proteins that were differentially expressed in control and treated rat sera. Four of these proteins that were characterized have been identified as

- **a. Transferrin** (Spot no:2, Molecular weight:78.5kDa, pI:6.94, MOWSE score:167; present only in control sera samples),
- **b. Apolipoprotein A-I precursor** (Spot no:A, Molecular weight:30.75, pI:5.2, MOWSE score:71; present only in control sera samples),
- **c. Serum alpha1-antitrypsin** (Spot no:16; Molecular weight:52, pI:5.5, MOWSE score:108; present only in treated sera samples),
- **d. Immunoglobulin Gamma1 heavy chain constant region** (Spot no:15, Molecular weight:55, pI:7.12, MOWSE score:208; present only in treated serum samples).

Tumor markers are secreted, released, or leaked into the interstitial fluids, and thus into the lymph, and finally (or directly) into the bloodstream, where they become detectable in serum samples. In order to validate these four proteins in rats’ sera, Western blotting was performed with protein specific antibodies (Figure 4.14). The expression of all these proteins with the course of disease over the period of 1,3,6 and 9 months was also studied in NNK treated rats. It was found that the expression of transferrin and apolipoprotein A-I precursor was depleted/decreased in treated rats’ sera samples in comparison to control over the period of nine months with progression of disease (Figures 4.14A,B). The serum alpha-1 antitrypsin levels were increased in treated rats’ sera in comparison to control over the period of time (Figure 4.14C). The level of immunoglobulin gamma1 heavy chain (IgG1H) was also found to increase with time (Figure 4.14D). Western blot confirmed the upregulation/downregulation of these proteins in accordance to the data obtained from PD QUEST of 2-D gels (Table 4.3)

**Transferrin** is a plasma protein that transports iron through the blood to the liver, spleen and bone marrow. It is a glycoprotein that binds iron very tightly but reversibly. It is an 80kDa protein with homologous N-terminal and C-terminal iron binding domains (Huebers and Finch, 1987). The liver is the main tissue for transferring synthesis but other sources such as the brain also produce this molecule. The main role of transferrin is to deliver iron from absorption centre in the duodenum and red blood cells macrophages to all tissues. Transferrin plays a key role predominantly in tissues where erythropoiesis and active cell division occur. Transferrin is also associated with the innate immune system, creating an environment of low free iron, where few bacteria are able to survive. The serum transferrin receptor (sTfR) is a sensitive indicator of iron deficiency erythropoiesis that
is not affected by inflammation. Concentrations of this molecule are inversely correlated with body iron-stores (Kuvibidila et al., 2004). In fact, increases in body iron stores have been reported in patients with liver and lung cancers. Specifically, various investigators have shown that tissue and serum ferretin levels are abnormally high, while serum iron and serum transferrin levels (Faulk et al., 1980) are low in breast cancer and other types of cancers. These latter conclusions were made by assessing the saturation of transferrin with iron and by examining the serum levels of the iron-storage protein, ferretin (Lamoureux et al., 1982). The transferrin and its receptor have also been explored to cause reduction in tumor cells by using the receptor to attract antibodies. It has been shown that the NNK treatment enhances the expression of fatty acid synthase, transketolase, pulmonary surfactant-associated protein C, L-plastin, annexin A1, and haptoglobin, but the expression of transferrin and apolipoprotein A-I decreased (Stevens et al., 1994). To be able to enter the bloodstream directly, larger molecules, often proteins, are cleaved into truncated forms or fragments, which are sometimes specific to the protease micro-environment of the tumor. Though the molecular weight of transferrin is 80 kDa, we identified its presence on 2-D gel (9\textsuperscript{th} month control serum sample) in low molecular weight range. In our study, based on apparent molecular weight of putative transferrin, it may be possible that its truncated derivative is present in the spot.

**Apolipoprotein A-I (apoA-I)** is the major protein constituent of high density lipoproteins (HDL) and lymph hylomicrons (Nofer et al., 2005). In human, proapoA-I is synthesized as a precursor protein, preproapoA-I, of 267 amino acids and is thought to occupy a surface position on the lipoprotein. ApoA-I activates lecithin-cholesterol acyltransferase, which is the cholesterol-esterifying enzyme of plasma involved in the production of mature circulating HDL (Ghiselli et al., 1985). Even though only \~5% of the total circulating apoA-I is found in lipid-free or lipid-poor forms (Obici et al., 2006) it is thought that the highly dynamic catabolism of HDL yields this protein conformation which subsequently acquires lipids, enhancing cholesterol removal in both physiological (Rye and Barter, 2004) and proatherogenic conditions (Curtiss et al., 2006). In addition to its role in lipid homeostasis, apoA-I has been shown to exhibit antioxidant and anti-inflammatory properties (Gharavi et al., 2007). ApoA-I has been suggested to be linked with pro-inflammatory cytokines such as tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) (Simpson et al., 2010). Calabresi et al. (2003) showed that HDL reduces free TNF-\(\alpha\) resulting in reduced tissue damage, reduced infiltration of macrophages and neutrophils, and attenuated tumor formation. Thus, HDL and apoA-
I reductions may contribute to an inflammatory process that is linked to lung cancer biology. In our study, apolipoprotein (apo) A-I was found to be down-regulated in lung cancer. Low levels of apo A-I are associated with low levels of HDL and impaired clearance of excess cholesterol from the body. Biological mechanisms that might link low plasma levels of HDL-cholesterol with cancer are not well understood (Boyd and McGuire, 1990; Fiorenza et al., 2000; Mc Growder et al., 2011). Inflammation reduces HDL-cholesterol (Esteve et al., 2005) and likely increases risk of lung cancer (Engels, 2008). However, it is also plausible that this association reflects the effect of other lifestyle factors such as body mass index (BMI), cigarette smoking, alcohol intake, diabetes, hypertension, or dyslipidemia may also influence the circulating levels of HDL and apoA-I. The biological and clinical implications of these metabolic markers should therefore, be further investigated.

**Serum alphal-antitrypsin (AAT)** is a 52-kD protease inhibitor, is produced mainly by the liver, but also by extrahepatic cells, including neutrophils and certain cancer cells (Du Bois et al., 1991; Paakko et al., 1996). AAT is the archetype member of the SERPIN (SERine Proteinase Inhibitor) super family of structurally related proteins, which have remarkable structural homology characterized by a dominant A β-sheet and a mobile reactive center loop that presents a peptide sequence as the pseudosubstrate for the target proteinase (Elliott et al., 2000). The literature concerning AAT in lung cancer has had conflicting observations. Some groups have shown a positive correlation between high levels of AAT in plasma and lung cancer risk (Zelvyte et al., 2004) whereas others have shown that a genetic deficiency in AAT is associated with an increased risk for lung cancer development (Yang et al., 2008). Our experiments showed that AAT was significantly increased in the serum of NNK treated rats. Several types of cancer, including non-small cell lung adenocarcinoma, have been associated with increased serum levels of AAT (Nash et al., 1980; Kataoka et al., 2002). AAT is an acute phase protein and its concentration rises up to 3–4-fold above normal during acute inflammation (Molmenti et al., 1993). Elevated level of AAT is suggested as cancer marker that discriminates cancer from chronic benign diseases, and clinical remission from relapse (Varela and Lopez, 1995). Results of the study by Sawaya et al. (1987) supported a hypothesis that production of AAT by tumor cells correlates with the regional proteolytic and inflammatory activity, which are probably involved in the protection of tumor cells. Future investigations should clarify a role for AAT in carcinogenesis and identify pro-inflammatory or some still unknown factors that lead to increased susceptibility to lung cancer associated with
AAT. Identifying these mechanisms will contribute to a better understanding of carcinogenesis, as well as to develop possible preventive measures (Topic et al., 2012).

Another protein that was identified in the sera of treated rats was **Immunoglobulin Gamma1 heavy chain (constant region)**. Immunoglobulin Gamma (IgG) is an important component of the adaptive immune system and constitutes 75–80% of total immunoglobulin. Immunoglobulin biology was originally characterized in lymphoid cells. Immunoglobulin molecules are composed of two identical light (L) chains of molecular weight 22,500 and two heavy (H) chains of molecular weight 50,000 to 75,000, which are linked by non-covalent interactions and disulfide bridges to form a structure with twofold symmetry. Each chain is characterized by a unique (or nearly unique) sequence in their C-terminal region that contributes to determining antigen specificity. Immunoglobulin L chains are classified into two isotypes (or classes), κ and λ. The relative proportions of κ and λ vary considerably with species, from κ to λ ratio of 65% to 35% in humans to a ratio of 97% to 3% in mice (Davies et al., 1975). There are five immunoglobulin isotypes, IgG, IgM, IgA, IgD, and IgE. Although each isotype can possess either κ or λ light chains, their H chains (called γ, μ, α, δ and ε respectively) are all different, and each is specific to its immunoglobulin class (Amzel and Polijak, 1979; Chen et al., 2010). Traditionally it was believed that the only source of immunoglobulin gamma (IgGs) was mature B lymphocytes but some researchers have reported that IgG could also be detected in carcinoma cells derived from epithelium (Haynes et al., 1998; Zheng et al., 2007). The studies by Gianazza and co-workers on rat serum identified 34 proteins with human homologues and concluded that even abundant proteins could be markers for disease states (Knekt et al., 1994; Eberini et al., 1999; 2000). Elevated levels of IgG, IgM or IgA antibodies are frequently observed in patients with cancers of epithelial origin, including carcinomas of breast, colon and liver (Kimoto, 1998). IgG-specific monoclonal or polyclonal antibodies were used in immunohistochemistry to determine the expression of IgG in malignant epithelial tumors. In all evaluated cancer tissues, including those from breast cancer (n=10), liver cancer (n=14), colon cancer (n=6) and lung cancer (n=12), positive staining was detected in the cytoplasm of all cancer tissues. In contrast, IgG was not detected in similar number of normal tissues of breast, colon, liver and lung (Qiu et al., 2003). IgG with unidentified specificity would also be secreted directly from cancer cells and these antibodies are involved in their survival and
growth (Olubuyide et al., 1993). In the serum and tissue of lung squamous carcinoma different levels of immunoglobulin protein fragments were detected which might present evidences for IgG secretion (Ouyang et al., 2007). This characteristic pattern of cancer IgG expression may serve as a potential marker for malignant cell transformation. However, as yet, because of scarcity of reports on the gene expression of IgG in cancer cell lines, cancer tissues and serum, there is still a controversy regarding the expression of IgG protein by them. The discovery of immunoglobulin heavy chain proteins and their corresponding genes in cancer cells is relatively new. Qiu et al. (2003) reported that human cancers of epithelial origin including carcinomas of breast, colon, liver, and lung, could produce IgG. In their study they identified mRNA of IgG heavy chain in these tumor cells, and also found corresponding expression of IgG. Immunohistochemistry analysis showed that IgG was localized in the cytoplasm or on the plasma membrane of these cells. In 2006, Babbage et al. analyzed immunoglobulin heavy chain (IgH) gene expression by nested RT-PCR in six well-defined breast cancer cell lines (BT474, MDAMB-231, MCF-7, SKBR3, T47D, and ZR75-1). IgH gene transcripts were identifiable in four of these six cell lines. Lung cancer patients have been reported to have generalized immune dysfunction of the cell-mediated immune response. In contrast, little is known about the humoral immune function in these patients. The exact function of IgG in cancers has not yet been elucidated, but IgG might be involved in the promotion of tumor growth based on preliminary in vitro evidence of its capacity to stimulate cell proliferation and invasion (Lee et al., 2009). Another observation supporting a growth-promoting role of IgG include the observed correlation between the level of IgG expression and tumor grade and stage in soft tissue tumors (Chen et al., 2010). It is also necessary to determine the level of the subclasses of IgG (IgG1, IgG2, IgG3, and IgG4) for a particular disease and not just the whole IgG because IgG subclasses are unique for different illnesses.

More studies are needed to answer a number of key questions regarding this discovery. The molecular mechanism, biological function and clinical significance of immunoglobulin production in non-lymphoid cells, particularly cancer cells, warrants in-depth investigation. It will be intriguing to discover whether immunoglobulins derived from non-lymphoid cells have the same primary structure or is modified and whether the cancer specific immunoglobulins have the traditional function as normal immunoglobulins produced by lymphoid lineage cells, whether these immunoglobulins can bind to their parent cells, and what are the antigen specificities of these
cancer derived immunoglobulins. Attention should be paid to transcriptional control of immunoglobulin expression and analysis of the character of rearranged DNA sequences in cancer cells for comparison with sequences from normal B lymphocytes. By doing so we hope to understand whether the same mechanism governs immunoglobulin production in both B lymphocytes and cancer cells, or whether the mechanism governing the latter is distinctly different. Since there appears to be association between immunoglobulin expression and cell transformation, the question as to whether immunoglobulin expression is a cause or a result of cell transformation awaits investigation. It also remains to be seen whether there is any correlation between immunoglobulin expression level and tumor differentiation. It would be of interest to examine whether immunoglobulin expression can be used as a diagnostic and prognostic marker or a therapeutic target. It is likely that the answers to these questions will give us new clues about carcinogenesis and cancer therapy. As immunoglobulins not only plays a pivotal role in the physiology and pathology of the human body, but is also a crucial tool in diagnosis and treatment of diseases by pathologists and other physicians, the possible unveiling of an unknown aspect of this class of molecules warrants urgent attention. Due to limited literature about the function of immunoglobulins expressed in cancer cells, it is still a question whether cancer derived immunoglobulins has similar function as normal immunoglobulins, like complement activation, binding to FcRs, antibody-dependent cell-mediated cytotoxicity, and inducing phagocytosis. The antigen specificities of cancer derived immunoglobulins are also unknown. Those questions provide directions for future research.

In this study, immunoglobulin gamma1 heavy chain showed the maximum MOWSE score and was therefore chosen to further validate its corresponding gene expression in NNK induced lung cancer tissue samples by RT-PCR (Reverse Transcriptase) analysis. This study was designed to evaluate IgG1 gene expression and its possible significance in lung tissue samples. The total RNA was isolated from treated rat’s lungs by TRI reagent. Two clear bands of ribosomal RNA (28S and 18S) were visible, showing the RNA isolated was pure and intact (Figure 4.15).

Based on our NCBI-BLAST complementation analysis, the amino acid sequence of the 55kDa protein (Immunoglobulin gamma1 heavy chain, constant region with the highest MOWSE score of 208) was used to deduce the primary nucleotide sequence of the gene encoding the protein in rat
DISCUSSION

The sequence analysis revealed that the gene corresponding to this protein corresponds to a 1.4kb fragment (Figure 4.16). In this study, we cloned IgG1 heavy chain of *Rattus norvegicus* in an attempt to determine its role in lung cancer. The gene encoding the 55kDa protein was sequenced, cloned and expressed in an *E. coli* strain. Using the gene sequence data, the 31 base oligonucleotide primers were designed from 5' and 3'-end regions of this 1.4kb gene from *Rattus norvegicus*. The *Bam* HI and *Hind* III restriction sites were engineered into the 5'-end of forward and reverse primers respectively to facilitate directional cloning into plasmid pRSETc. cDNA was prepared from total RNA and was further analysed by PCR amplification using sequence specific forward and reverse nucleotide primers. The RT-PCR amplification generated a product of 1.4 kb, which was in agreement with that estimated from the sequence of the gene available in databank. The amplified product was sequenced by TCGA (The Centre for Genomic Application) and was purified to remove free primers as these may reduce the efficiency of ligation of PCR product to vector during subsequent steps (Figures 4.17A,B). The pRSET vectors were used for cloning purposes and they are pUC-derived expression vectors designed for high-level expression of His-tagged proteins (Figure 4.18). The 1.4kb gene was cloned in vector pRSETc between *Bam* HI and *Hind* III sites in MCS region. The vector and the PCR product were double digested with *Bam* HI and *Hind* III enzymes. The digested vector was checked on 0.8% agarose gel, eluted, purified (Figure 4.19) and ligated. As the cloning was done in directional manner there was no need to check the orientation. Therefore, the ligation mixture was directly transferred into *E. coli* DH5α cells. The cells were screened for the presence of cloned gene on the basis of antibiotic selection pressure. As the plasmid pRSETc carried ampicillin resistance gene, only those cells carrying the recombinant plasmid would be able to survive ampicillin selection pressure (Figure 4.20). The positive cells were picked from the culture plate, reseeded in fresh growth media and grown to confluency. The transformants were checked for the presence of the insert by plasmid isolation and double digestion with *Bam* HI and *Hind* III showing the release of 1.4kb fragment (Figure 4.21). The recombinant plasmids of 4.3kb molecular weight that gave strong positive signals were selected for expression studies. In the resulting construct pSJ-4.3, the inserted gene is fused in frame with ATG codon and 6x His tag coding sequence (at 5’end) and MCS (multiple cloning site) at 3’ end, present in the vector pRSETc (Figure 4.22). Representative clone containing ~1.4kb insert (Clone No. 5) was confirmed through DNA sequencing using forward primer (Figure 4.23A). It had 92% homology with the reported nucleotide sequence of IgG1H. The deduced amino acid sequence of the protein...
immunoglobulin gamma1 heavy chain obtained from translated nucleotide sequence of the clone, showed 75% homology (Figure 4.23B). These results suggest that there is a modification in protein during tumor development and the secreted protein is a modified form of immunoglobulin gamma1 heavy chain. Changes/ modification in this gene might have resulted in over-expression or accumulation of IgG1H protein in NNK treated rat sera samples in comparison to control, which is one of the most frequent consequences of carcinogenesis (Lutz and Nowakowska-Swirta, 2002; Wu et al., 2009).

The pRSETc vector is designed for high-level prokaryotic expression regulated by strong bacteriophage T7 promoter/lac operator. Expression is induced by the production of T7 RNA polymerase in BL21(DE3) E. coli. These cells also produce T7 lysozyme to reduce basal expression of target genes. The pRSETc vector enables placement of the 6xHis tag at N-terminus of the recombinant protein and allows for rapid purification with nickel-chelating resin and detection with an Anti-HisG Antibody. After the fragments were cloned in expression vector and transformed into E. coli cells, the clone pSJ-4.3 was further expressed in E. coli BL21(DE3) cells. For protein expression, cultures were incubated at 37°C until the absorbance at 600nm reached 0.6; IPTG (1mM) was added at this stage for the induction of the recombinant protein production. It was evident from the comparison of the polypeptide profiles obtained in the absence and presence of IPTG that induction of gene expression resulted in the appearance of a new (approximately 55kDa) band, which is consistent with the predicted size of recombinant protein (Figure 4.24). IPTG, an inducer of lac repressor-regulated promoters was used to induce the lac operon because, in contrast to allolactose, which is the natural inducer of the operon, IPTG cannot be hydrolyzed and broken down by the cell. Hence its concentration does not change during an experiment. In case of E. coli BL21(DE3) system, addition of 1 mM IPTG was found to be optimal for achieving high level expression of 55kDa protein. An increase in the IPTG concentration above 1mM did not have any significant improvement in the expression of 55kDa protein. Based on optimized conditions, large-scale expression of the protein was carried out.

To obtain functionally active protein, 55kDa induced protein was purified from E. coli via the 6xHis tag expressed on the amino (N) terminus of the protein using nickel-NTA chromatography. For purification and in vivo studies, 200mL of LB medium containing ampicillin (100 µg/mL) was
inoculated with BL21(DE3) cells transformed with the recombinant expression vector pSJ-4.3. When the OD$_{600}$ of the culture reached approximately 0.6-0.8 it was induced with IPTG and induction was allowed to proceed for four hours before harvesting the cells. Aliquots of the induced culture were lysed separately using urea lysis buffer and separated into supernatant and pellet. Analysis of the urea lysate confirmed the presence of the major proportion of the 55kDa protein band in the urea supernatant demonstrating that the protein expressed in *E. coli* is predominantly insoluble.

Solubilization is a critical step towards obtaining maximal amount of the desired protein in solution without inducing any chemical or deleterious modifications to it. Wide panels of detergents are available for the solubilization of inclusion bodies including strong denaturants like urea, guanidinium salts and detergents such as sodium dodecyl (Stockel *et al*., 1997), n-cetyl trimethylammonium bromide (CTAB) (Cardamone *et al*., 1995) and sarkosyl (Burgess, 1996). In our study imidazole of varying concentrations and urea as denaturant were used for solubilization. After solubilization, refolding is accomplished by controlled removal of the excess of the reagents, thus creating an appropriate environment where protein can fold spontaneously (Singh and Panda, 2005). Ideally transfer of protein molecules from denaturant solution to aqueous solvent will force them to collapse into a compact structure, leading to a misfolded, aggregated protein. Usually, an intermediate concentration of the denaturant (Urea-6M) is taken, which is low enough to force protein molecules to collapse, yet can allow them to stay in solution and be flexible to reorganize their structure. The column was washed with a low concentration (80 mM) of imidazole, which will compete with low-affinity histidine–column interactions to remove from the column any, perhaps histidine-rich, proteins that are non-specifically bound. Finally, the tagged protein itself is removed from the column by increasing the concentration of imidazole to a high level (500 mM). This process results in the single-step purification of the tagged protein to yield a very pure, almost homogenous sample. The purified fractions were analyzed by SDS-PAGE (Figure 4.25). Approximately 80-90\% of the induced protein bound to the column and it also appears that there was no significant non-specific binding to the affinity matrix. Elution with a buffer containing 500 mM imidazole resulted in the emergence of highly purified (> 95\%) recombinant protein. Thus it became clear that the *Rattus norvegicus* gene for 55kDa protein was expressed in *E. coli* using an
IPTG-inducible vector and purified to near homogeneity by a single step Ni-NTA affinity chromatography with a yield of approximately 40 mg/litre of culture.

To confirm that the over expressed band seen in SDS-PAGE is recombinant protein with N-terminal His-tag, Western blot was performed with monoclonal anti-His antibody as primary antibody (GenScript, USA) and goat anti mouse IgG-peroxidase (Sigma) as secondary antibody. The blots were developed using DAB/H₂O₂ substrate. A band was observed only in induced culture suggesting the presence of His tagged protein (Figure 4.26).

Our results suggest that tumor-derived IgG or the presence of IgG1H in lung cancer cells might contribute in cancer initiation in the precancerous stage when the epithelial cells are actively proliferating. Lung cancer cell produced IgG1H is involved in the biological behavior of this cancer and may serve as useful marker for cancer cell differentiation and prognosis. Locally produced IgG could also be a potential target for therapy. Thus, the determination of the level of IgG1 by itself and/or in combination with conventional markers may provide relevant information regarding the noninvasive detection of early stages of lung cancer. Development of methods aiming at selective blockade of tumor-derived IgG thus may constitute a potentially new approach for cancer therapy and prevention.

Antibodies have several properties which make them excellent indicators of disease and their detection forms the basis of many *in vitro* diagnostic tests. Sometimes, in addition to producing antibodies against foreign molecules, the immune system generates antibodies to self-proteins (“autoantibodies”) in response to many pathological processes. For example, studies have identified autoantibodies associated with several types of cancers including, cancers of the lung, breast, head and neck, colon, ovary and prostate, offering the potential of improved diagnosis of these important cancers (Tan and Zhang, 2008; Gnjatic *et al.*, 2009). The immune response occurs early during tumor development and as a result the presence of autoantibodies against tumor antigens in serum might provide an effective means for cancer screening and early diagnosis (Qiu and Hanash, 2009). It is believed that autoantibodies are generated through over-expression, mutation, release of proteins from damaged tissues, mis-folding or mis-presentation of proteins which leads to their recognition by the immune system. Modification of the gene leading to accumulation of its
expressed protein due to increased stability can be measured through immunoassay. The accumulated protein then acts as an antigen, with subsequent development of antibodies which are detectable in tissues, sloughed cells, blood, and other body fluids.

To validate the presence of antibodies against the recombinant protein immunoglobulin gamma1 heavy chain in rat sera, Western blot analysis was carried out. It was confirmed that the antibodies for IgG1H were present in treated rat sera at 1 month and increased with progression of cancer when compared to the control group. No such antibodies were present in control animals (Figure 4.27). This result suggests that immune system of the animal may have responded to the modified IgG1H protein by recognizing it as a foreign protein or the upregulation of this modified protein in cancer might have triggered the formation of antibodies detectable in serum of NNK treated rats, thus invoking the humoral response in them (Caron et al., 2007; Wu et al., 2010).

Before any biomarker can enter into clinical practice, it needs to be validated with patient samples. Detection of pre-clinical changes, which precede the development of the overt form of a disease and identification of measurable indicators signaling such changes in people exposed to occupational and environmental pollutants, constitutes one of the principal research aims of molecular epidemiology. The latter makes use of suitable biological indicators (biomarkers) as basic research tools to monitor pathological processes during the latent stage of the disease in people exposed to harmful agents (Indulski and Lutz, 1997). Subsequently, clinical trials would be required to show that these biomarkers can be used to detect early lung cancers and, importantly, that early detection translates into improved survival. In virtue of this belief, we tried to study the pattern of expression of four proteins (transferrin, apolipoprotein A-I precursor, serum alpha-1 antitrypsin, immunoglobulin gamma1 heavy chain constant region) in healthy individual and lung cancer patient sera samples. 1-D protein profiles of both control and lung cancer patient were obtained and many proteins were found to be differentially expressed (Figure 4.28). ELISA assay was performed to compare the expression levels of three proteins and a significant decrease of 23.65% and 19% was observed in the levels of transferrin and apolipoprotein A-I respectively in sera of lung cancer patients when compared with healthy donors. Alpha-1 antitrypsin levels were increased significantly by 26.66% in sera of lung cancer patients when compared with healthy donors (Figure 4.29). To validate the presence and study the expression levels of the protein immunoglobulin
gamma heavy chain (IgG1H) in human sera, Western blot analysis was carried out. It was confirmed that IgG1H was upregulated in lung cancer patient sera in comparison to control (Figure 4.30).

The pattern of expression of these four proteins (transferrin, apolipoprotein A-I, serum alpha-1 antitrypsin, immunoglobulin gamma heavy chain constant region) in human sera (healthy individual and lung cancer patient) were in accordance to those found in rat sera and therefore our attempt to validate them was successful.

**Future challenges and prospects**

The devastating incidence of diseases and their clinical seriousness have stimulated innumerable research studies. One of the thrust areas of health management research is to develop new markers for early detection of diseases when it can easily be managed as well as the markers for prognosis, progression and response to treatment. Therefore, a major challenge would be the determination of a reliable serologic marker reflecting the disease behavior before it becomes obvious in clinical level, be easily reproducible and feasible to be measured serially, meaning to be acceptable by the patient. On the basis of this conception, a number of serum markers that are lung-specific proteins have been delineated. The proteins may play role in the pathophysiology of lung cancer and may have potential for diagnostic and prognostic biomarker development that may ultimately lead to better patient care. Nevertheless, substantial challenges still remain with pressing need of standardization, sample handling, reproducibility and marker quantitation/ ratiometric measurements. Moreover, use of different proteomic platforms may create some discrepancies in proteomics pattern across different research and clinical centres.

The major challenge lies not only in the discovery but in the proper evaluation of candidate biomarkers. For example, before any biomarker can enter into clinical practice, it needs to be validated with patient samples. Subsequently, clinical trials would be required to show that these biomarkers can be used to detect early lung cancers and, importantly, that early detection translates into improved survival. The effectiveness and the diagnostic accuracy of the biomarkers and definition of its discriminative cut-off levels by applying ROC curve analysis need to be established. These observations coupled with the evidence that the pathogenesis of lung cancer still
remain poorly understood and ambiguous may explain the limitations in the clinical use of serum markers. In order to establish diagnostic, prognostic and therapeutic importance of biomarkers leading to a better understanding of the biological characteristics of lung cancer may involve certain ethical and legal issues that need to be addressed.

Although, the currently available or potential lung cancer biomarkers are not sensitive or specific enough to be used clinically in the diagnosis, stratification, prognosis, or drug responses, we can envision that this will be greatly improved in the future. For the development of useful lung cancer biomarkers, we think three aspects need to be considered. First, we need to analyze currently available or potential biomarkers in a large set of clinical samples including other cancer types and other diseases conditions, especially inflammatory diseases. This will reveal the usefulness of these biomarkers. Second, with further improvements of technology, we need to look for more specific and low abundant biomarkers focused on specific subtypes of lung cancers. Third, one specific biomarker may not be enough to predict or monitor lung cancer, thus, several good biomarkers need to be combined, with quantitative information, to be really useful in the clinical purposes. For clinical implications, a biomarker needs to be validated and followed by approval from the FDA. The financial support can be arranged by public and private resources. Thus, the collaboration among investigators in universities and institutes, clinicians, industrial participants and FDA is a must to bring a biomarker from the lab to clinic. Currently, the application status for most of these biologic markers is still in its infancy and remains exploratory.

The speed of evolution of the field of proteomics is dramatic and much better tools are being designed and incorporated in the effort to enhance both protein/peptide mass coverage as well as peak resolution for identification precision. Eventually, it is hoped that utilizing those serum biomarkers that can reflect the disease activity or predict/monitor response to therapy would positively impact on the clinical outcome of lung cancer patients. Ultimately, successful assimilation of research disciplines, including comparative genomics, functional genomics, functional proteomics and bioinformatics would greatly facilitate the clinical, radiological and physiological monitoring of the disease and identify high risk patients who would benefit from aggressive management of established risk factors and help developing novel targeted anti-cancer drugs.