2.1 CANCER BIOLOGY

The word ‘cancer’ comes from the greek word *karkinos* meaning *crab*. Hippocrates, the great greek physician (460-370 B.C.) who is considered the father of modern medicine, gave it this name because the tumors that are the visible evidence of many types of cancer reminded him of a crab with a central body (the tumor or lump) from which several rays-the legs, spread into the surrounding tissue (Moss and Ralph, 2004; Hajdu *et al*., 2011). It is a genetic disorder involving dynamic changes in the genome leading to uncontrolled cell growth, ability to invade and metastasize.

2.1.1 Multi - Step Nature of Cancer

Normally the division and growth of cells are orderly and controlled, but if this process loses its control, the cells will continue to divide into lump, which is called a *tumor*. As long as the neoplastic cells remain clustered together in a single mass, the tumor is said to be benign and complete cure can be achieved by removing the mass surgically. A tumor is considered as cancer only if it is malignant. The development of most of the types of cancers is a step-wise process involving an accumulation of mutation in a number of genes. Perhaps six or seven independent mutations are needed for cancer to be induced. That means the cell has the ability to invade surrounding tissue. Invasiveness usually implies an ability to break loose, enter the blood stream or lymphatic vessels and form secondary tumors or metastasis at other sites in the body. The more widely a cancer metastasizes, the harder it becomes to eradicate. Figure 2.1 illustrates the malignant transformation of a normal cell to cancerous and its subsequent multiplication.

Cancer cells therefore have two heritable properties,

- These cells and their progeny reproduce in defiance of the normal restraints.
- Invade and colonize territories normally required for other cells. These features make the cancer peculiarly dangerous.

Alfred Knudson (1971) proposed two hit mutation model for cancer induction. It states that two mutational events are critical for cancer to develop, one in each allele of the cancer-causing gene. In hereditary cancers one mutation is inherited, predisposing the person to cancer, the other mutation occurs latter in the somatic cells. But in nonhereditary cancers both mutations occur in
somatic cells. This two hit mutation model applies for few cancers. Other cancers involve mutation in many genes.

**Figure 2.1** The malignant transformation of a normal cell and subsequent doublings. After twenty doublings (1 million cancer cells), the cancer is still too small to detect.

Three classes of genes have been shown to be mutated frequently in cancer. They are oncogenes, tumor suppressor genes and mutator genes. Various types of radiation and many chemicals increase the frequency with which cells became cancerous. These agents are called carcinogens. Normally all carcinogens act by causing changes in the genome of the cell. In the case of chemical carcinogens a few act directly on the genome, but most act directly by being converted to active derivatives by cellular enzymes. All carcinogenic forms of radiation act directly.

Chemical carcinogens include both natural and synthetic chemicals. Two major classes of chemical carcinogens are recognized,

1) **Direct acting carcinogens** are chemicals that bind to DNA and act as mutagens. For Example: alkylating agents such as some anti cancer therapeutic chemicals.
2) The second class consists of pro-carcinogens that are converted into metabolically active derivatives, which then bind to DNA and act as mutagens. Examples are polycyclic aromatic hydrocarbons, azo dyes and aflatoxins from fungi. The activation of the pro carcinogens is due to the action of normal cellular enzymes.

Certain viruses such as Human Papilloma virus, Epstein-Barr virus, Hepatitis-B virus are known to cause some types of cancers. Our risk of cancer can be significantly affected by our life habits; for example smoking is responsible for 30% of cancer deaths.

2.1.2 Proliferation, Cell Cycle and Apoptosis in Cancer

The decision of whether a cell survives or dies depends heavily on the impact that external factors have on cell cycle progression and programmed cell death pathways. Under normal circumstances, when the environment either internal or external becomes detrimental to the well being of the cell, the cell responds either by entering a dormant stage or by activating the programmed cell death/apoptosis. However it is well known that cells very occasionally do bypass their own natural response and allow uncontrolled proliferation to occur. This phenomenon is loosely referred to as cancer (Figure 2.2).

![Figure 2.2](image.png)

Figure 2.2 Comparison between programmed cell death/apoptosis in normal cell division and uncontrolled growth in cancer.
Ineffective cellular proliferation, distorted differentiation program, destabilized chromosomal and genetic organization, deregulated programmed cell death or apoptosis all the major four cellular functions that are inappropriately regulated in a neoplasm. To maintain tissue homeostasis and to support normal development each organ maintains tight control over cell division, growth fraction and cell loss. The Cyclin Dependent Kinases (CDKs) which are important in cell cycle check points are inappropriately activated in cancer cell leading to uncontrolled cellular growth and proliferation, or sometimes genetic loss of their inhibitor also lead to cancer.

2.1.3 Types of Cancer
Cancer can be classified according to the type of the cell and the organ in which it originates.

1. **Carcinoma**: About 85% of cancers are carcinoma. They originate in epithelium, which is the converging (or lining) of organ and of the body (skin).
2. **Sarcoma**: These form in the connective tissue of the body such as muscles, bone and fatty tissue (6% of cancers)
3. **Leukemia**: Begin in the blood forming tissues- the bone marrow, the lymph nodes and the spleen all-important components of immune system (2.5%).
4. **Lymphoma**: Lymphoma originates in the cells of lymph system, [the body-circulating network for filtering out impurities (2.5%)]
5. **Other forms**: Brain tumor and other forms of cancer make up the other 4% of cancer.

Cancer can also be classified in terms of how far and to what organs they have spread.

1. An **in situ** cancer is one that is confined to place where it originated.
2. An **invasive** cancer has spread to surrounding tissues.
3. A **metastasize** cancer has invaded distant sites of the body.

2.1.4 Cancer Treatment
Many factors must be considered in making a decision on treatment: the type and location of cancer, the extent to which it has already spread and can be expected to spread, and the patient’s age, sex, general health and personal treatment preferences.
These treatment modes are used either alone or in combination, when one treatment is used to supplement another; the supplementary treatment is called adjuvant.

1. **Surgery:** The removal of malignant tumor in an operation is the oldest and most frequently used cancer treatment, which is effective when a cancer is small and localized. If a tumor has spread, the nearby tissues and lymph glands all removed along with the malignant growth in an operation called radical surgery.

2. **Radiation:** It works by destroying cancer cell with radiation. In external beam radiation, gamma rays or x-rays are used and in case of brachytherapy, radioactive substance like radium is inserted the body. Radiation could be used in conjugation with surgery and chemotherapy.

3. **Chemotherapy:** It kills cancer cells through the use of drugs or hormones, taken orally or through injection. Chemotherapy would also be used alone or in combination with surgery and radiation.

4. **Immunotherapy:** It uses the body’s own immune system to destroy cancer cells. Various immunological agents used in the therapy include substances produced by the body such as interferon, interleukins and Tumor Necrosis Factor and laboratory produced substances like monoclonal antibodies and vaccines.

### 2.2 LUNG CANCER

#### 2.2.1 History

Lung cancer, the most common cause of cancer death in the United States and around the world, was first recognized by French physician, René-Théophile-Hyacinthe Laennec, who first described metastases in the lungs in the early 1800s. In 1815, he published his findings in the "Dictionaire des Sciences Médicales," establishing the disease as a separate entity.

But up until the 20th century, lung cancer was not a widespread disease (Witschi, 2001). In fact, some 150 years ago, it was extremely rare. In 1878, malignant lung tumors represented only 1 percent of all cancers seen at autopsy in the Institute of Pathology of the University of Dresden in Germany. Searches through the medical literature up to the year 1900 reveal a total of a mere 100 cases, and it was not known what actually precipitated the ailment. In the year, 1930 it was duly noted that malignant lung tumors had begun to increase at the turn of the century and perhaps
even more so after World War I and that, possibly, they still were on the increase. It was also noted that while most lung tumors occurred in men, there seemed to be a steady increase in women. Possible etiologic factors such as increased air pollution by gases and dusts, caused by industry; the asphalting of roads; the increase in automobile traffic; exposure to gas in World War I; the influenza pandemic of 1918; and working with benzene or gasoline were postulated as reasons for increasing lung cancer cases. The link between the smoking of cigarettes and lung cancer also began to be suspected by clinicians when they noted the increase of this “unusual” disease. World War I helped to popularize the smoking of cigarettes. Soldiers in the trenches smoked to relieve stress, and so did many civilians, including an increasing number of women at home. It was then also recognized that chemicals encountered in certain occupations could cause lung cancer: arsenic containing compounds in wine growers, asbestos, and nickel and chromium in mine and smelter workers.

2.2.2 Epidemiology: Current scenario in India and worldwide

The global burden of cancer continues to increase largely because of the aging and growth of the world population alongside an increasing adoption of cancer-causing behaviors, particularly smoking, in economically developing countries. About 70% of all cancer deaths occurred in low- and middle-income countries. Deaths from cancer worldwide are projected to continue to rise to over 13.1 million in 2030 (IARC, 2010; Ferlay et al., 2011).

Lung cancer has become one of the world’s leading causes of preventable deaths with high ratio in both males and females with incidence rates declining in both men and women from 2004-2008 (Kumar et al., 2003; Cancer facts and figures, 2012). An estimated 160,340 deaths due to lung cancer, accounting for about 28% of all cancer deaths, are expected to occur in 2012 (Figure 2.3, Cancer facts and figures 2012). From 2004 to 2008, lung cancer incidence rates have decreased by 1.9% per year in men and by 0.3% per year in women. Gender differences in lung cancer mortality patterns reflect historical differences between men women in the uptake and reduction of cigarette smoking over the past 50 years.
India has a National Cancer Control Programme which was established in 1975–76. This has contributed to the development of Regional Cancer Centres (RCCs), oncology wings in medical colleges and support for purchase of teletherapy machines. The District Cancer Control Programme was initiated but did not result in sustainable and productive activity. Leading cancer sites in various cancer registry areas are shown in (Figure 2.4) (Nair et al., 2005). Indian Council of Medical Research’s (ICMR) latest data on the 10 leading sites of cancer in Delhi shows that prevalence of breast cancer among women in Delhi stands at almost 27%, followed by cervix, whose recurrence is almost half of breast cancer cases at 14.6%. Among men, lung cancer is the most common at 10%, while 7% of all cancers are of the prostate.
The present scenario of cancer prevention and treatment in India has many loopholes. Awareness programmes have been undertaken in a few places, but there is no uniform standardized information, education and communication (IEC) strategy for cancer prevention. Diagnostic infrastructure in the country is limited. There are many districts in the country which do not have a pathologist and pathology/cytology services, which are crucial for diagnosing cancer. Treatment results are about 20% less than what is observed for similar conditions in more developed countries, mostly due to late diagnosis and inappropriate treatment. The funds for the cancer programmes are mainly from the Government and needs to be augmented. Private initiatives are few and are unlikely to cater to a large population across different socioeconomic strata, as it is often not a financially viable venture. All elements of cancer control, from surveillance to palliative care, are not linked and coordinated.
2.2.3 Tumor development

Lung cancer is a carcinoma, an abnormal growth that usually starts in the epithelial cells that line the bronchial tubes or the alveoli of the lungs. The role of the lungs is to draw oxygen from the blood and expel toxic CO₂. Because of their nature, lungs are in direct contact with the air. This makes them extremely vulnerable to any pollutants that may be in air. To help protect the lungs against pollutants, the body has developed several defense mechanisms. Mucus (the sticky fluid secreted by the epithelial cells that line the bronchial tubes) and the cilia (microscopic hairs that grow in their walls) are the first line of defense. Every time we breathe, mucus traps dirt and germs from the air, and cilia send them to our throat. Another line of defense that helps in protecting the lungs from the environment is the lymphatic system. The lymph is rich in leukocytes that produce antibodies against germs. Under normal circumstances these defenses are enough to protect the lungs. But when lungs are chronically exposed to pollutants, the glands produce more and more of mucus to get rid of the irritant particles, but the cilia in the airways which have also got damaged by the same pollutants cannot keep up with the excess mucus. As a consequence, mucus accumulates and makes it easy for the infection to develop. This causes the airways and alveoli to become inflamed. The inflammation thickens the walls, making them less elastic and less able to eliminate mucus. Figure 2.5 describes the various stages in the development and spread of lung cancer (Ferreiro and Alcamo, 2007).

The first step in the development of tumor is hyperplasia that is a rapid increase in the number of cells. Such rapid cell division does not lead to tumor formation always, as is the case during development of an embryo or wound healing. Under a microscope, the normal epithelial lung cells look like tall columns. At first the precancerous cells look like the healthy cells, but as their divisions continue, the new cells start to look less and less like the original tall narrow cells. They may be flat and wide and lack cilia. This process of de-differentiation is called dysplasia. Dysplastic cells can no longer perform the tasks normal cells do. They cannot secrete mucus or dispose it off because they don’t have cilia. These two processes, hyperplasia and dysplasia, are considered the premalignant stages of lung cancer, and they can take decades to develop. Eventually the de-differentiated cells form a mass of an abnormal tissue or tumor. To continue growing, the tumor develops a network of new blood vessels through the process called angiogenesis. The cancer may become invasive or spread to other parts of the body via bloodstream or lymphatic system.
Figure 2.5 The development and spread of lung cancer. a. Healthy tissue with normal cells, b. Hyperplasia - genetic damage has occurred and cells begin to divide rapidly, c. Dysplasia - cells have become de-differentiated, d. Tumor formation and angiogenesis have begun, e. The cancer has become invasive and may grow into the chest wall, or space between the lungs, f. Metastatic cancer - cancer has spread to other parts of the body

Mucus accumulation and persistence of infection may lead to blockage of the airways. Various symptoms that may suggest the onset of lung cancer include shortness of breath, hemoptysis (coughing up of blood), wheezing, chest pain, dysphagia (difficulty in swallowing), frequent pneumonias, anorexia (Hamilton et al., 2005; Xu et al., 2012). Other symptoms such as weight loss, fatigue, loss of appetite, bone pain and fever common to other types of cancer are also noticeable.
2.2.4 Classification

On the basis of size and appearance of malignant cells, lung tumors can be histologically classified into two main types; non-small cell lung carcinoma (NSCLC) that accounts for around 80.4% of the total lung cancer types and small cell lung carcinoma (SCLC) that accounts for 16.8% of all lung carcinoma types (Kumar et al., 2003). This classification has important implications for clinical management and prognosis of the disease, as the treatment varies for both the types. NSCLC is sometimes treated with surgery, while SCLC usually responds better to radiotherapy and radiations. NSCLC and SCLC may be categorized further into subtypes (Figure 2.6). The cells in such subtypes remain classified principally by their appearance under the light microscope using simple stains but are grouped together principally because their approach to treatment is similar.

Non small cell lung carcinoma (NSCLC)

1. Squamous cell carcinoma- This is the most common type of lung cancer (accounting for 70-75% of lung cancers), especially found in smokers. It is called so because its cells resemble a type of flat-surfaced cell called a squamous cell. It develops in the major airways, the large bronchi and spreads by invading the local tissues, from where it spreads to lymph nodes and into the bloodstream.

2. Adenocarcinoma- This type accounts for 25-30% of NSCLC. It usually develop beneath the lining (mucosa) of the airways, many start in the periphery of the lung than in the centre of the chest (Horn et al., 2012). Smoking does not seem to predispose to the same extent as other types of lung cancer. A subtype of adenocarcinoma, the bronchoalveolar carcinoma, is more common in female never-smokers, and may have different responses to treatment (Raz et al., 2006).

3. Large cell carcinoma- this accounts for 30-35% of NSCLC. These tumors do not attempt to form recognizable structures, like glands. They are usually found in smokers and may develop in the central or peripheral parts of the lungs.

Small cell lung carcinoma (SCLC)

SCLC accounts for 16.8% of all lung cancer types (Perez-Moreno et al., 2012). The cells of this tumor are small and fragile. Some are called ‘oat cells’ because of their similarity to oat grains.
These cancers grow and spread rapidly through the bloodstream to other organs. They nearly always develop in smokers, usually in the centre part of the lungs.

Figure 2.6 Histopathological classification of lung cancer (25X): a. Normal lung cell (25X), showing many small, bubble like alveoli can be seen in this tissue, as well as a small bronchiole running obliquely along the right side of the specimen. These alveoli have very thin walls and are surrounded by capillaries creating the respiratory membrane where gas exchange takes place between air and blood, b. Squamous cell carcinoma, showing flat scale like cells, c. Adenocarcinoma, at low magnification (25X), a nodular growth pattern with prominent desmoplastic stroma is shown, d. Large cell carcinoma, histological section showing proliferation of atypical cells along the alveolar walls e. Small cell carcinoma, sheets or solid pattern of the cancer cells with round-shaped, hyperchromatic nuclei and scanty cytoplasm.
2.2.5 Possible causes of its spread

Lung cancer is a multifactorial disease — that is, many factors work together to either cause or prevent cancer. Between 80 and 90% of lung cancers are due to smoking, yet 10% of men and 20% of women who develop the disease have never smoked. On the other side of the equation, many people who smoke do not develop lung cancer.

**Tobacco smoking**

The link between cigarette smoking and lung cancer has already been established by the epidemiologic studies as early as in the 1950s (Doll and Hill, 1950). About 90% of lung cancer cases are due to tobacco smoking (Proctor, 2012). The longer a person has been smoking and the more packs per day smoked, the greater is his risk to develop lung cancer (IARC monographs, 2002). Cessation of smoking can decrease the risk of developing lung cancer, but after 15 years, the chance of developing the disease is similar to that of a non-smoker. Research has shown that non-smokers who reside with a smoker have a 24% increase in risk for developing lung cancer when compared with non-smokers who do not reside with a smoker. An estimated 3,000 lung cancer deaths that occur each year in the U.S. are attributable to **passive smoking or second-hand smoke** (U.S. department of health and human services, 2010).

Laboratory evidences have demonstrated that tobacco smoke contains over 7,000 chemical compounds with high levels of carcinogenic and volatile N-nitrosamines such as N-nitrosodimethylamine, N-nitrosopyrrolidine as well as tobacco-specific N-nitrosamines such as N-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (IARC, 2010), responsible for damage to lungs. These agents are formed exclusively from nicotine and from the minor tobacco alkaloids like nornicotine, primarily during the processing, fermentation and aging of tobacco (Figure 2.7, Hoffmann et al., 1995).

**Other risk factors** of developing lung cancer include exposure to radon gas, asbestos, radioactive ores, such as uranium, inhaled chemicals or minerals like arsenic, beryllium, cadmium, vinyl chloride, nickel compounds, chromium compounds, coal products, mustard gas, and chloromethyl ethers, diesel exhaust, air pollution. Prior history of lung cancer in the family increases its risk (U.S. department of health and human services, 2011). The debilitated patients
with compromised immune system and elderly over the age of 65 yrs are more prone to the disease.

![Diagram of nitrosamines formation](image)

**Figure 2.7** Formation of various tobacco-specific nitrosamines. iso-NNAC, 4-(methylnitrosamino)-4-(3-pyridyl)butyric acid; iso-NNAL, 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol; NAB, N’-nitrosoanabasine; NAT, N’-nitrosoanatabine; NNA, 4(methylnitrosamino)-4-(3-pyridyl)-1-butanal; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, N’-nitrosonornicotine

**Tobacco-specific carcinogen [4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol or NNK]**

**Structure**

The carcinogen used for development of our rodent model is NNK, a potent carcinogen whose target organ specificity toward lung has been established in previous studies and is independent of the route of administration (Hecht *et al.*, 1986; Hecht, 2004; Akopyan and Bonavida, 2006). NNK is an aromatic compound with a molecular formula of $C_{10}H_{13}N_3O_2$ ($MW = 207.2316$). This yellow crystalline solid is the most potent carcinogenic compound in the family of nitrosamines. The crystalline structure of NNK has been characterized (Katz *et al.*, 1999) and the chemical structure is illustrated in **Figure 2.7**.
Metabolic activation of NNK and NNAL

NNK has been classified as a human carcinogen by the IARC Working Group on the Evaluation of Carcinogenic Risks to Humans (2007). One of the most intriguing characteristics of NNK is its ability to selectively induce pulmonary tumors in rodents (Hecht, 1998). This lung specificity, along with biochemical and epidemiologic data, implicates NNK as a plausible contributor to the development of lung cancer in smokers (Hecht, 2003; IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2007; Yuan et al., 2009). The carcinogenic effects of NNK are primarily attributed to its metabolic activation (Figure 2.8).

Figure 2.8 A simplified metabolic scheme of NNK and NNAL

NNK does not work in isolation; it requires cellular metabolism and activation to induce tumorigenesis. There are three primary pathways responsible for NNK activation: i) carbonyl reduction, ii) pyridine N-oxidation and iii) α-hydroxylation (Wiener et al., 2004; Proulx et al., 2005). NNK also undergoes reductive metabolism, and in the process produces its metabolized isoform, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), a carcinogenic compound and metabolic product of NNK α-hydroxylation (Hecht et al., 2004). Further metabolic processing and detoxification of NNAL produces non-carcinogenic compounds known as NNAL-Gluces. Nonetheless, the glucuronidation of NNAL guided either by carbonyl reduction or pyridine N-oxidation (part of the reductive metabolic pathway) is an important mechanism of NNK detoxification, activation and consequent development of lung tumors. NNK undergoes cytochrome P450-catalyzed α-hydroxylation by two pathways (either α-methylhydroxylation or
α-methylenehydroxylation), both of which produce carcinogenic intermediates (Proulx et al., 2005). α-Methylene hydroxylation of NNK and NNAL results in the formation of the reactive intermediate methanediazohydroxide, which can form methyl DNA adducts (Peterson et al., 2010). This pathway ultimately results in the formation of the metabolites 4-oxo-4-(3-pyridyl)butyric acid (keto acid) and 4-hydroxy-4-(3-pyridyl)butyric acid (hydroxyl acid). When NNK undergoes α-methyl hydroxylation, the unstable intermediate 4-(3-pyridyl)-4-oxobutanediazohydroxide is formed, which can react with water to yield the metabolite 4-oxo-4-(3-pyridyl)butanol (keto alcohol) or bind to DNA to form pyridyloxobutyl (POB)-DNA adducts. Likewise, the α-methyl hydroxylation of NNAL can produce pyridylhydroxybutyl (PHB)-DNA adducts or the metabolite 4-hydroxy-4-(3-pyridyl)butanol (diol). Previous in vivo studies in which NNK and NNAL were chronically administered in the drinking water of rats showed that the POB and PHB-DNA adducts that developed in the lung included O6-[4-(3-pyridyl)-4-oxobut-1-yl]-2-deoxyguanosine (O6-POB-dGuo), O2-[4-(3-pyridyl)-4-oxobut-1-yl] thymidine (O2-POBdThd), 7-[4-(3-pyridyl)-4-oxobut-1-yl]guanine (7-POB-Gua), O6-[4-(3-pyridyl)-4-hydroxybut-1-yl]-2-deoxyguanosine (O6-PHB-dGuo), O2-[4-(3-pyridyl)-4-hydroxybut-1-yl]thymidine (O2-PHB-dThd), and 7-[4-(3-pyridyl)-4-hydroxybut-1-yl]guanine (7-PHB-Gua) (Lao et al., 2006, 2007; Upadhyaya et al., 2008). The formation of the methyl adduct O6-methylguanine (O6-methyl-Gua) after exposure to NNK was also observed (Upadhyaya et al., 2009, Chiang et al., 2011), which is consistent with previous studies (Belinsky et al., 1986, 1991; Devereux et al., 1991; Hecht, 1998). DNA can be attacked at several positions by metabolites of NNK and NNAL leading to base mispairing and mutagenesis in vitro (Hall and Saffhill, 1983; Eadie et al., 1984; Hecht, 1998), the events that are critical for the initiation of cancer. Of note, concentrations of NNK have been described in tobacco substances such as snuff (1-20 μg/g), cigarette mainstream smoke (20-310 ng/cigarette), and tobacco-exposed areas such as indoor air (≤26 ng/m3) (Katz et al., 1999). Hence, it is to no surprise that both direct and second-hand tobacco smoke exposure leads to quantifiable measures of NNK, NNAL, and NNAL-Glucs in urine (Hecht et al., 2004; Hecht et al., 2004; Tulunay et al., 2005).

**Signaling pathways**

Once activated, NNK initiates a cascade of signaling pathways, resulting in uncontrolled cellular proliferation and tumorigenesis (Figure 2.9). NNK results in mutations in the proto-oncogene K-ras, FasL overexpression and hyperphosphorylation of protein kinase/extracellular signal
regulated kinase 1 (MAPK/ERK1) that cause lung hyperplasia and adenoma formation (Zheng and Takano, 2011).

**Figure 2.9** NNK-mediated activation of signaling pathways.

Further studies demonstrate NNK-activated μ- and m-calpain kinases as necessary ingredients for NNK-induced lung metastasis via the ERK1/2 pathways (Xu and Deng, 2004). Moreover, NNK has been shown to upregulate cellular myelocytomatosis (c-Myc) and B cell leukemia/lymphoma 2 (Bcl₂), two oncoproteins that assist one another in various cellular processes including proliferation, transformation, apoptosis and tumorigenesis. By way of several cellular pathways, such as the protein kinase cascade, protein kinase C-/Raf-1/MAPK/ERK1/2 (PKC-/Raf-1/MEK 1/2), NNK successfully promotes cell survival via phosphorylation and subsequent functional cooperation of c-Myc and Bcl₂ (Jin et al., 2004). Triggering such NNK-dependent events further leads to cellular migration, invasion, and uncontrolled proliferation. ERK1/2 not only induces NNK-specific tumorigenic activities in a direct fashion, but also phosphorylate NFκB that in turn activates cyclin D1 triggering cellular proliferation and lung tumorogenesis (Chen et al., 2011). The phosphoinositide 3-kinase (PI3K/Akt) pathway is also a molecular target of lung cancer; it is constitutively active in NSCLC cells *in vivo* and *in vitro* (West et al., 2004). Significant changes in other cellular agents such as epidermal growth factor receptor (EGFR) and the sulfotransferase 1A1 (*SULT1A1*) gene have also been observed.
2.2.6 Diagnosis

Lung cancer is usually not detected until it has progressed to an advanced stage, because the early-stage disease does not usually cause symptoms (Vollmer et al., 2010). Doctors use a wide range of diagnostic procedures and tests to diagnose lung cancer. The **history and physical examination** may reveal the presence of symptoms or signs that are suspicious for lung cancer. **Chest X-ray** is the most common first diagnostic step and may reveal suspicious areas in the lungs but is unable to determine if these areas are cancerous. **CT (computerized tomography, computerized axial tomography, or CAT) scans** may be performed on the chest, abdomen, and/or brain to examine for both metastatic and lung tumors. A technique called a **low-dose helical CT scan** (or spiral CT scan) is sometimes used in screening for lung cancers. This procedure requires a special type of CT scanner and has been shown to be an effective tool for the identification of small lung cancers in smokers and former smokers. **Magnetic resonance imaging (MRI)** scans may be appropriate when precise detail about a tumor's location is required. The MRI technique uses magnetism, radio waves, and a computer to produce images of body structures. **Positron emission tomography (PET)** scanning is a specialized imaging technique that uses short-lived radioactive drugs to produce three-dimensional colored images of those substances in the tissues within the body. While CT scans and MRI scans look at anatomical structures, PET scans measure metabolic activity and the function of tissues. PET scans can determine whether a tumor tissue is actively growing and can aid in determining the type of cells within a particular tumor. **Sputum cytology** examination, may allow visualization of tumor cells for diagnosis. This is the most risk-free and inexpensive tissue diagnostic procedure, but its value is limited since tumor cells will not always be present in sputum even if a cancer is present. **Fine needle aspiration (FNA)** through the skin, most commonly performed with radiological imaging for guidance, may be useful in retrieving cells for diagnosis from tumor nodules in the lungs. Needle biopsies are particularly useful when the lung tumor is peripherally located in the lung and not accessible to sampling by bronchoscopy. While routine **blood tests** alone cannot diagnose lung cancer, they may reveal biochemical or metabolic abnormalities in the body that accompany cancer. One current focus of research in the area of lung cancer is the development of a blood test to aid in the diagnosis of lung cancer. Researchers have preliminary data that has identified specific proteins, or biomarkers, that are in the blood and may signal that lung cancer is present in someone with a suspicious area seen on a chest X-ray or other imaging study. Unfortunately studies of lung cancer screening with any such tests have failed to lower
mortality rates. These screening techniques also have relatively low sensitivity and specificity as a result many non-malignant diseases are misdiagnosed as false positives and many early-stage cancers are not detected (Bach et al., 2007; Pastorino, 2010).

2.2.7 Prognosis and Treatment
The failure of early diagnosis is the major reason why lung cancer has the highest mortality rate among all cancers. Survival rate for patients with lung cancer is poor, only 15% of patients survive for 5 years after diagnosis (Cancer facts and figures, 2008). The prognosis of lung cancer refers to the chance for cure or prolongation of life (survival) and is dependent upon where the cancer is located, the size of the cancer, the presence of symptoms, the type of lung cancer, and the overall health status of the patient. Accurate staging of the disease is an important part of the management as it provides estimation of patient’s prognosis and identifies treatment strategies (Mirsadraee et al., 2012). Since the introduction of tumor, node, metastasis (TNM) staging by Pierre Denoix between the years 1943 and 1952, the International Association for the Study of Lung Cancer (IASLC) announced a major revision of lung cancer staging which has been included in the seventh edition of the “TNM classification of malignant tumors” published by the UICC in January 2010 (Goldstraw et al., 2007). The new classification is applicable to both NSCLC and SCLC and is based on a larger surgical and non-surgical cohort of patients, and thus more accurate in terms of outcome prediction compared to the previous classification (Table 2.1).

SCLC has the most aggressive growth of all lung cancers, with a median survival time of only two to four months after diagnosis when untreated. (That is, by two to four months, half of all patients have died.) However, SCLC is also the type of lung cancer most responsive to radiation therapy and chemotherapy. Because SCLC spreads rapidly and is usually disseminated at the time of diagnosis, methods such as surgical removal or localized radiation therapy are less effective in treating this type of lung cancer. When chemotherapy is used alone or in combination with other methods, survival time can be prolonged four- to fivefold; however, of all patients with SCLC, only 5%-10% are still alive five years after diagnosis. Most of those who survive have limited-stage SCLC.

In non-small cell lung cancer (NSCLC), the most important prognostic factor is the stage (extent of spread) of the tumor at the time of diagnosis. Results of standard treatment are generally poor
in all but the most smallest of cancers that can be surgically removed. However, in stage I cancers that can be completely removed surgically, five-year survival approaches 75% (Kelsey et al., 2009). Radiation therapy can produce a cure in a small minority of patients with NSCLC and leads to relief of symptoms in most patients. In advanced-stage disease, chemotherapy offers modest improvements in survival although rates of overall survival are poor.

**Table 2.1** Seventh tumor, node, metastasis classification of lung cancer: January 2010.

<table>
<thead>
<tr>
<th>T: Tumour</th>
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<tbody>
<tr>
<td><strong>T0</strong></td>
<td>No evidence of primary tumour</td>
</tr>
<tr>
<td><strong>Tis</strong></td>
<td>Carcinoma in situ</td>
</tr>
<tr>
<td><strong>T1</strong></td>
<td>Tumour &lt; 3 cm in greatest dimension, surrounded by lung or visceral pleura, without bronchoscopic evidence of invasion more proximal than the lobar bronchus (i.e., not in the main bronchus)</td>
</tr>
<tr>
<td><strong>T1a</strong></td>
<td>Tumour &lt; 2 cm in greatest dimension</td>
</tr>
<tr>
<td><strong>T1b</strong></td>
<td>Tumour &gt; 2 cm but &lt; 3 cm in greatest dimension</td>
</tr>
<tr>
<td><strong>T2</strong></td>
<td>Tumour &gt; 3 cm but &lt; 7 cm or tumour with any of the following features (T2 tumours with these features are classified T2a if &lt; 5 cm):</td>
</tr>
<tr>
<td></td>
<td>Involves main bronchus, &gt; 2 cm distal to the carina</td>
</tr>
<tr>
<td></td>
<td>Invades visceral pleura</td>
</tr>
<tr>
<td></td>
<td>Associated with atelectasis or obstructive pneumonitis that extends to the hilar region but does not involve the entire lung</td>
</tr>
<tr>
<td><strong>T2a</strong></td>
<td>Tumour &gt; 3 cm but &lt; 5 cm in greatest dimension</td>
</tr>
<tr>
<td><strong>T2b</strong></td>
<td>Tumour &gt; 5 cm but &lt; 7 cm in greatest dimension</td>
</tr>
<tr>
<td><strong>T3</strong></td>
<td>Tumour &gt; 7 cm or one that directly invades any of the following:</td>
</tr>
<tr>
<td></td>
<td>Chest wall (including superior sulcus tumours), diaphragm, phrenic nerve, mediastinal pleura, parietal pericardium</td>
</tr>
<tr>
<td></td>
<td>Tumour in the main bronchus &lt; 2 cm distal to the carina but without involvement of the carina</td>
</tr>
<tr>
<td></td>
<td>Associated atelectasis or obstructive pneumonitis of the entire lung</td>
</tr>
<tr>
<td></td>
<td>Separate tumour node(s) in the same lobe</td>
</tr>
<tr>
<td><strong>T4</strong></td>
<td>Tumour of any size that invades any of the following:</td>
</tr>
<tr>
<td></td>
<td>Mediastinum, heart, great vessels, trachea, recurrent laryngeal nerve, esophagus, vertebral body, carina</td>
</tr>
<tr>
<td></td>
<td>Separate tumour node(s) in a different ipsilateral lobe</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>N: Nodes</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NX</strong></td>
<td>Regional lymph nodes cannot be assessed</td>
</tr>
<tr>
<td><strong>N0</strong></td>
<td>No regional lymph node metastasis</td>
</tr>
<tr>
<td><strong>N1</strong></td>
<td>Metastasis in ipsilateral peribronchial and/or ipsilateral hilar lymph nodes and intrapulmonary nodes, including involvement by direct extension</td>
</tr>
<tr>
<td><strong>N2</strong></td>
<td>Metastasis in ipsilateral mediastinal and/or subcarinal lymph node(s)</td>
</tr>
<tr>
<td><strong>N3</strong></td>
<td>Metastasis in contralateral mediastinal, contralateral hilar, ipsilateral or contralateral scalene, or supraclavicular lymph node(s)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>M: Metastases</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MX</strong></td>
<td>Distant metastasis cannot be assessed</td>
</tr>
<tr>
<td><strong>M0</strong></td>
<td>No distant metastasis</td>
</tr>
<tr>
<td><strong>M1</strong></td>
<td>Distant metastasis</td>
</tr>
<tr>
<td><strong>M1a</strong></td>
<td>Separate tumour node(s) in a contralateral lobe</td>
</tr>
<tr>
<td></td>
<td>Tumour with pleural nodules or malignant pleural/pericardial effusion</td>
</tr>
<tr>
<td><strong>M1b</strong></td>
<td>Distant metastasis</td>
</tr>
</tbody>
</table>
2.3 PROTEOMICS IN BIOMARKER DISCOVERY

Proteomics is the study of large-scale protein expression patterns, and this can move beyond simple identification of a protein for the study of its isoforms, modifications and interactions (Hoehn et al., 2005). Proteins form the functional components of all metabolic pathways, and are therefore potential candidate targets for diagnosis and therapy of lung cancers. By studying the interrelationship between proteins that occur in healthy and diseased status, proteomics contributes insight into determination of pathophysiological basis for the target identification, and molecular information to study the mechanistic basis for drug action and toxicity.

2.3.1 Proteomics versus genomics

During the last 10 years, both genomic as well as proteomic approaches have been used for biomarker studies. Both approaches are complementary and, along with transcriptomics and metabolomics (which study the transcripts and the metabolites, respectively), integrate the so-called “omic” sciences (Figure 2.10). While epigenetic and genetic alterations are driving carcinogenesis and genomic studies have provided valuable information on lung cancer molecular biology (Chanin et al., 2004), a proteomic approach opens new window into the pathogenesis of lung cancer (Srinivas et al., 2002). Although the genome is stable and gives information about the potential of an organism, the proteome is dynamic and reflects the biological processes that are taking place in that organism (Tunon et al., 2010). There are two main arguments in favor of this new approach. First, the phenotype of a cell is determined by proteins and cannot be predicted by genomics alone. Indeed, protein expression levels are often poorly correlated to messenger RNA expression levels (Simpson et al., 2008). The post-translational modifications such as phosphorylation, glycosylation, and proteolytic processing significantly modify the protein functions and characteristics of the cell or tissue (Sabel et al., 2011). Detecting genetic alterations, such as chromosomal abnormalities, oncogenes and tumor-suppressor genes, can serve in the early diagnosis or prediction of cancer progression. However, proteins represent specific tumor functions and interactions with the tumor microenvironment and can better explain the current status of an individual’s health. Second, while genomic analysis requires DNA extraction from tumor cells that are not easily obtained by non invasive methods, proteomics do not necessarily need a direct access to tumor cells. Proteins can easily and noninvasively be obtained from various sources such as blood and exhaled breath condensate (EBC).
Figure 2.10 Genomics investigates the whole genome (deoxyribonucleic acid [DNA]) and its functional relations. From DNA, ribonucleic acid is transcripted. Transcriptomics studies messenger ribonucleic acid (mRNA). From mRNA transcripts, proteins are translated. Proteomics analyzes the protein expression profile. Endogenous synthesized metabolites are examined by metabolomics.

2.3.2 Technologies involved in proteomics

Proteomics has been used to define tumor subsets in resected lung specimens and has been demonstrated to distinguish primary adenocarcinomas from primary squamous cell carcinomas with 98% accuracy (Ullmann et al., 2004). In addition to lung cancer, proteomics has been applied to early detection and treatment of cancers of the ovary, pancreas, prostate, esophagus, breast, liver and rectum (Farlow et al., 2010; Shah et al., 2010).

A typical proteomic analysis consists of the following steps: 1) sample preparation, 2) optimal separation of protein species, 3) imaging, 4) identification of protein species and their charge variants (posttranslational modification), 5) quantification and 6) data assessment using protein databases (Figure 2.11). In sample preparation, the type of samples and the specific treatment or targeting such as depletion of highly abundant proteins and the enrichment of specific proteins (eg., glycoproteins, phosphoproteins or low-molecular-weight proteins), which depends on the
source of the sample have to be considered. In the discovery phase the proteins are separated using 1D or 2D gel electrophoresis and protein spots are analysed using mass spectrometry methods as MALDI/SELDI-TOF. For verification, intermediate numbers of samples are usually confirmed by an antibody based technology, immunoblotting and by multiple reaction monitoring (MRM) or selective reaction monitoring (SRM). In the last step of validation, further development of antibody-based rapid analysis kits will enable large group validation and clinical screening. Depending on the complexity of the samples to be analysed, there are several variations in each one of the above steps. These details have been reviewed elsewhere (Griffin et al., 2001; Singh et al., 2011).

**Figure 2.11** A schematic workflow of proteomic approach includes sample selection, its preparation and separation and protein identification by mass spectrometry. Biomarker identification through bioinformatics and data processing requires clinical validation by ELISA/Western Blot assays.
Several excellent reviews on various aspects of proteomic techniques are available (Xue et al., 2008; Wright et al., 2012) and will therefore be discussed here only briefly. With the enormous advancement in mass spectrometry (MS), bioinformatics and analytical methods, proteomic approaches greatly promote serum proteomic analysis and biomarker discovery (Figure 2.12).

**Figure 2.12** Proteomic methods used in serum biomarker discovery, serum sample may be analysed by a) Gel- based technologies, b) Shotgun proteomics or c) Image- based technologies.

While gel based applications including, 2D-PAGE and MALDI-TOF are the main but classical technologies used in serum cancer research for identifying the proteins, quantifying changes in protein expression and establishing cancer treatment platforms using novel biomarkers for diagnosis and therapy (Hanash et al., 2010), other gel-free high throughput technologies such as multidimensional protein identification technologies (MudPIT), isotope coded affinity tag
(ICAT), isobaric tagging for relative and absolute quantification (iTRAQ), multiple or selected reaction monitoring (MRM/SRM) also offer great potential for future biomarker discovery in cancer. Shotgun proteomics, 2D-DIGE, SELDI-TOF as well as protein microarrays are applied to obtain patterns of protein expression in tissues, blood, organelles. Large scale western blot assays, chip technologies either coupled with antigens (Lueking et al., 1999; Zhu et al., 2001) or antibodies (Liu et al., 2000; Zhou et al., 2001) have also been used for high throughput analysis.

Two-dimensional electrophoresis was first introduced by O'Farrell and Klose in 1975 and it entails the separation of complex protein mixtures by molecular charge in the first dimension and by mass in the second dimension. Using a non-targeted classical 2D-electrophoresis Bouwman et al. (2011) identified significantly changed protein profile in biofluids and blood cells following extended fasting period. Okano et al. (2007) discovered lung cancer markers that appear in response to treatment with gefitinib. Modified 2D electrophoresis by fluorescent tagging of proteins (DIGE), offers increased throughput, ease of use, reproducibility, and accurate quantitation of protein expression differences (Unlu et al., 1997). This system enables the separation of two or three fluorescently labeled protein samples (Cy2, Cy3 and Cy5) on the same gel. In a recent study proteomic analysis of human saliva from lung cancer patients using two-dimensional difference gel electrophoresis and mass spectrometry revealed 16 candidate biomarkers (Xiao et al., 2011).

Attempts are being made to develop other more efficient and reproducible separation methods that do not use 2DE and are the methods of choice for quantitatively comparing protein levels among biological proteomes. One such method is isotope coded affinity tag (ICAT) involving chemical labeling of isolated proteins and peptides by isobaric tags that facilitates comparison of the proteomes of two samples simultaneously (Kang et al., 2010). While ICAT, a popular in vitro labeling, involves the chemical modification of proteins to tag them before analysis by mass spectrometry, stable isotope labeling with amino acids in cell culture (SILAC) is a simple approach for in vivo incorporation of a label (specific amino acids) into proteins avoiding their chemical manipulations. In addition, the amount of labeled proteins required for analysis using SILAC technique is far less than that with ICAT. The SILAC-based method has broadly been applied in many areas of cell biology and proteomics. Using this technique, several potential
protein biomarkers have been identified from the esophageal squamous cell carcinoma secretome (Kashyap et al., 2010).

Isobaric tag for relative and absolute quantification (iTRAQ) is a gel-free technique that allows relative and absolute quantification of proteins from either cell lines or clinical samples in a single experiment (Maurya et al., 2007). It involves labeling of each peptide pool with an iTRAQ reagent (4-plex, 8-plex) which are then pooled and usually fractionated by nano-LC and analysed by MS/MS. Hu et al. (2010), applied iTRAQ technology for the study of multiple drug reaction mechanisms analyses in gastric cancer, which showed the importance of this technology in identifying differentially expressed proteins in cancer.

Another approach to analyse proteomes without gels is MudPIT. This method involves tryptic digestion of protein mixture and the resultant peptides are separated and identified by strong cation exchange (SCX) and reversed phase (RP) liquid chromatography coupled with mass spectrometry. MudPIT-based proteomics was applied to expressed prostatic secretions obtained from nine men with prostate cancer and resulted in the confident identification of 916 unique proteins (Drake et al., 2010).

Using protein microarrays, it has been possible to detect and identify potential biomarkers for breast cancer (Fan et al., 2010). Protein microarrays use either multiple capture antibodies dotted separately on a slide (forward microarrays) or multiple protein samples dotted and fixed on a single slide (reverse microarrays). These methods can detect the presence of numerous proteins in multiple tissue samples.

Mass spectrometry is considered as the primary tool for protein identification for both gel-based or gel free techniques. Characterization of proteins by MS involves (a) protein ionization and generation of gas-phase ions, (b) separation of ions according to their mass to charge ratio and (c) detection of ions. In gel free approaches such as ICAT and MudPIT samples are directly analysed by MS (liquid chromatography; LCMS and strong cation exchange SCX-LC; reversed phase high performance liquid chromatography RP-HPLC respectively) but in gel-based proteomics like 2DE and 2D-DIGE the protein spots are first excised from the gel and then digested into peptides, followed by separation by LCMS or directly analysed by MALDI/ESI-TOF. Matrix assisted laser
desorption/ionization (MALDI) and Electrospray ionization (ESI) are two main ionization sources and time of flight (TOF), ion trap, quadrapole and fourier transform ion cyclotron (FTIC) are 4 different mass analyzers which are currently used for protein identification and characterization. Since many known cancer biomarkers are glycoproteins such as PSA, CA15-3, CA-125 and CEA, Zheng and Takano (2011) identified 49 candidate serum biomarkers with significant difference between control and the lung cancer cases, using glycoprotein capture and LC-MS/MS.

Surface enhanced laser desorption and ionization (SELDI)-TOF MS is a variation of MALDI in which the protein mixture spotted on a surface modified with a chemical functionality is allowed to crystallize with the matrix. After washing off unbound material, the protein fingerprint is determined and visualized by time-of-flight mass spectrometry. The high-throughput nature and simplicity in its experimental procedures hold out SELDI-TOF MS to be a promising technology for future secretome analysis and biomarker discovery (Poon et al., 2007; Monari et al., 2011).

Unlike traditional mass spectrometry which attempts to detect all proteins in a biological sample in an unfocused fashion, selected reaction monitoring/multiple reaction monitoring (SRM/MRM) is a highly sensitive and selective method for the targeted quantification of an analyte of interest. Such new and modified mass spectrometry based quantification technologies have recently showed that serum amyloid A (SAA2) can be a good biomarker for the detection of lung cancers (Sung et al., 2012).

One of the upcoming and promising technologies to find early stage lung cancer involves the use of synthetic molecules that identifies and probe onto the proteins that are released into the blood in cancer or other diseases. Such a chip- based technology has been envisioned by a clinical diagnostic company, Somalogic, in Colorado that use sets of molecules associated with specific diseases aggregated onto a chip and patients would be easily screened for many diseases at once. Despite various advantages of major proteomic technologies, the inherited limitations of each of them still exist (Table 2.2). As no single method is always suitable for biomarker discovery, often or a combination of these methods is chosen depending on the type of material to be analyzed, the genre of disease and previous knowledge of putative markers.
Table 2.2 Advantages and limitations of proteomic techniques

<table>
<thead>
<tr>
<th>Technology</th>
<th>Application</th>
<th>Strengths</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>2DE</td>
<td>Protein separation, Quantitative expression profiling</td>
<td>Multiple gels can be compared simultaneously, Information on post translational modifications can be discerned</td>
<td>Poor separation of acidic, basic, hydrophobic and low abundant proteins</td>
</tr>
<tr>
<td>DIGE</td>
<td>Protein separation, Quantitative expression profiling</td>
<td>PTM information, Highly sensitive, Reduction of gel to gel variation</td>
<td>Proteins without lysine cannot be labeled, requires special equipment for visualization and fluorophores are very expensive</td>
</tr>
<tr>
<td>ICAT</td>
<td>Chemical isotope labeling of biological samples for quantitative proteomics</td>
<td>Sensitive and reproducible, Detect peptides with low expression levels</td>
<td>Only cysteine containing peptides can be analysed and acidic proteins are not detected.</td>
</tr>
<tr>
<td>iTRAQ</td>
<td>Isobaric tagging of peptides</td>
<td>Protein quantification of multiplex several samples</td>
<td>Increases sample complexity</td>
</tr>
<tr>
<td>MUDPIT</td>
<td>Identification of protein-protein interactions and discovery research</td>
<td>High separation of peptides and requires no chemical labeling</td>
<td>No quantitative analysis</td>
</tr>
<tr>
<td>Protein array</td>
<td>Quantitate specific proteins used in diagnostics (biomarkers or antibody detection)</td>
<td>High sensitivity and throughput, it helps to measure multiple analytes simultaneously</td>
<td>Limited production and specificity of antibody available, May not detect isoforms of analyte</td>
</tr>
<tr>
<td>Mass spectrometry</td>
<td>Primary tool for protein identification and characterization</td>
<td>High sensitivity and specificity. High-throughput. Qualitative and quantitative</td>
<td>No individual method to identify all proteins. Not sensitive enough to identify minor or weak spots. MALDI and ESI do not favor identification of hydrophobic peptides and basic peptides</td>
</tr>
<tr>
<td>Bioinformatics</td>
<td>Analysis of qualitative and quantitative proteomic data</td>
<td>Functional analysis, data mining, and knowledge discovery from mass spectrometric data</td>
<td>No integrated pipeline for processing and analysis of complex data. Search engines do not yield identical results</td>
</tr>
</tbody>
</table>
2.4 BIOMARKER

Biomarkers may be structural molecules, secretory products, proteins and enzymes or non-specific markers of cell turnover that can be used to distinguish normal from abnormal state (Sung et al., 2008). Cancer biomarkers are expected to play an important role in (a) facilitating the early diagnosis of cancer at the molecular level; (b) guiding cancer therapy by verifying cancer stages, molecular characteristics, response to treatment and, therefore presenting clinical end points or surrogate end points. Additionally, early detection would lead to greater success in curing the cancer by starting therapy at an early stage when the tumor mass is smaller and by selecting the appropriate treatment for specific cancer types and (c) helping in selecting patients for adjuvant chemotherapy.

A number of factors play a role in success or failure of a diagnostic screening test. An ideal biomarker should a) be highly sensitive: it should increase pathologically in the presence of the disease; b) be highly specific: it should not express in absence of the disease; c) change in accordance to the clinical evolution, reflecting the current status of disease; d) indicate the presence of relapse before it becomes visible at a clinical level; e) be reproducible and f) be easily determined in the sample and be relatively inexpensive.

Every aspect of proteomics tends to help in the discovery of protein-based biomarkers; however, the development of such biomarkers should proceed in a systematic way and follow the phases of development and evaluation before it reaches from laboratory to clinic (Figure 2.13) (Srivastava and Srivastava, 2005). In the Discovery Phase, a promising substance (biomarker) is identified through basic research in animal models, tissue, or cell cultures. In the Development Phase, a biomarker is identified in human specimens; an assay for the biomarker is developed and/or modified; analytical sensitivity of the assay for the biomarker is tested; and the clinical sensitivity of the assay measured to meet specific biological questions such as detecting mutations for biological events, or specific clinical questions such as detecting precancerous lesions. In the Trial Phase, a biomarker is subjected to broad questions to verify the intended use, such as whether the biomarker is applicable to biological and clinical settings in a variety of conditions. Biomarker is subjected to rigorous evaluation for precision, reproducibility, accuracy and other performance characteristics, including sensitivity and specificity in controlled study designs, such as a case-control or a large prospective trial. In the Application Phase, a biomarker is field-tested
for a specific clinical use, such as screening of cancer, and for its cost and effectiveness in reducing disease burden and mortality due to the disease. An unsuccessful verification or benefit may lead to return to the discovery phase and to cycle re-entry.

Figure 2.13 Different phases of biomarker, this proposed cycle depicts the stages in biomarker development and validation (Modified from Srivastava and Srivastava, 2005).

A specificity of 100%, is desirable in clinical terms (Thomson, 1979), but may not be always practical because of the poor sensitivity that may follow. As a result, some groups have agreed to use a specificity of 95% as the cut-off point (Stieber et al., 1993). The interplay between sensitivity and specificity and the nature of the disease under prediction assigns suitable cut-off points. Sensitivity and specificity calculated at various cut-off points give rise to a receiver operating-characteristic (ROC) curve. A clinically useful biomarker will be one with the largest area under the ROC curve. The alternative use of these receiver operating-characteristic (ROC) curves also helps to preserve a higher sensitivity than when using the 100% specificity as point of reference, and in many cases avoids the burden of 5% false positive cases.

2.4.1 Classification of Cancer Biomarkers
Due to the vast explosion of knowledge over past several decades, different methods have been suggested to classify cancer biomarkers. The following section is an attempt to classify cancer biomarkers according to contemporary findings (Table 2.3).
Table 2.3 Classification of cancer biomarkers

<table>
<thead>
<tr>
<th>Based on Pathological State</th>
<th>Based on Biological Macromolecule</th>
<th>Based on other Types</th>
</tr>
</thead>
<tbody>
<tr>
<td>➢ Prediction Biomarkers</td>
<td>➢ DNA Biomarkers</td>
<td>➢ Imaging Biomarkers</td>
</tr>
<tr>
<td>➢ Detection Biomarkers</td>
<td>➢ RNA Biomarkers</td>
<td>➢ Pathological Biomarkers</td>
</tr>
<tr>
<td>➢ Diagnosis Biomarkers</td>
<td>➢ Protein Biomarkers</td>
<td>➢ In-silico Biomarkers</td>
</tr>
<tr>
<td>➢ Prognosis Biomarkers</td>
<td>➢ Glyco Biomarkers</td>
<td></td>
</tr>
</tbody>
</table>

Diagnostic biomarkers may be present in any stage during cancer development (Verma and Manne, 2006). These facilitate in the diagnosis and histopathological classification of tumor. Based on accurate and early diagnosis of a disease, suitable treatment choices can be made. Recently, the US Food and Drug Administration (FDA) approved some diagnostic markers for bladder cancers based on urine analysis, such as bladder tumor antigen (BTA) and nuclear matrix protein-22 (NMP-22) (Lau et al., 2009). De Petris et al. (2011) in their study confirmed CYFRA21-1 as a diagnostic marker for lung cancer. Prognostic biomarkers can provide valuable information about malignant potential of tumors that facilitates the course of therapy. Examples of clinically useful prognostic markers include hormone receptors, proliferation markers, proteases, markers of angiogenesis, growth factor receptors (Her-2/neu) and p53. It has been reported that upregulation of annexin A3 (ANXA3), a member of a family of calcium and phospholipid-binding proteins, which has been related to cancer metastasis might serve as a novel prognostic biomarker for lung cancer (Indovina et al., 2011). Predictive biomarkers, sometimes also referred to as response markers, help in predicting the treatment response or choosing between different treatment modalities available to cure a particular disease. For example: drugs such as erlotinib or gefitinib work only in lung cancer patients with specific mutations in the epidermal growth factor receptor (EGFR) gene (Sharma et al., 2007). Pemetrexed has been found to be an effective treatment in patients with anaplastic lymphoma kinase-positive NSCLC (Lee et al., 2011).

2.4.2 Promise of Serum Based Biomarkers

For lung cancer biomarker discovery, tissues, sputum and body fluid can be of use. Amongst body fluids, blood is the most commonly used sample as it can be easily collected noninvasively.
and is amenable for repetitive measurements over the time. It is assumed that tissue perfusion of tumors or host responses contribute to modification of circulating proteins or peptides, therefore, blood proteome analysis appears very appealing to researchers addressing the discovery of potential biomarkers that can be helpful in early diagnosis of cancer, monitoring of disease status, development of targeted therapies, evaluation of response to therapy, and survival. Several serum biomarkers have been investigated in lung cancers, but have not been proven very useful in clinical practice because of their limited sensitivity and/or specificity. Serum proteomic analysis of patient specimens has proven to be a formidable challenge, partly due to genetic and environmental differences among patients as well as non-standardized methods of sample preparation and handling (Cho and Sung, 2009). The presence of higher abundance proteins interferes with the identification and quantification of lower abundance proteins (in range of ng/mL in serum). Complexity and dynamic range of protein concentrations can be addressed with a combination of prefractionation techniques that deplete high abundant proteins.

Serum biomarkers can be of three types: 1. serum proteins that are differentially expressed in patients with cancer, or serum proteins that are cleaved or modified in cancer patients, 2. proteins that are secreted by tumor cells into the circulation, 3. intracellular tumor proteins that are released when tumor cells die (Alaiya et al., 2005). Presently, only few blood biomarkers have proven useful for diagnosis of primary cancers. These include serum PSA for prostate cancer; bladder tumor antigen (BTA) and nuclear matrix protein-22 as diagnostic markers for bladder cancer (Konety and Getzenberg, 2001); alpha-fetoprotein (AFP) for hepatocellular carcinoma and testicular cancers; catecholamines for neuroblastoma; immunoglobulins for multiple myeloma (Mountain, 1997); human epididymis protein 4 (HE4) for ovarian cancer (Kim et al., 2011). These are currently approved by FDA but are not sensitive enough for screening and early detection in selected populations.

2.4.3 Lung cancer protein biomarkers in serum: current status

Although their use is not actually recommended or encouraged in lung cancer screening, diagnosis and follow-up, the following biomarkers are frequently used in clinical practice.

Carcinoembryonic antigen (CEA) is a member of a family of cell surface glycoproteins that are produced in excess in essentially all human colon carcinomas and in a high proportion of
carcinomas at many other sites (Benchimol, 1989). Normal blood levels of CEA typically range from 0-5 µg/L while in smokers they typically increase up to 7 µg/l (Sajid et al., 2007). Clinical significance of preoperative values of CEA even if within normal range (0 - 5 ng/ml), are related to worse prognosis (Tomita et al., 2009). Although serum CEA assays have low sensitivity and specificity for diagnosing lung cancers (NIH, 1981), several studies (Arrieta et al., 2009; Tomita et al., 2010; Zaleska et al., 2010; Grunnet et al., 2012) have reported statistical significant evidence for the use of CEA as a prognostic marker in NSCLC patients but not for SCLC patients.

**Cytokeratin fragments** (CYFRA 21-1) are a polygenic polypeptide family that constitutes the main component of keratin filaments, one of the groups of intermediate filaments, which are a crucial part of cytoskeleton. CYFRA 21-1 is a fragment of cytokeratin (CK) 19 expressed in many normal and malignant epithelial cells (Moll et al., 1992). Early clinical studies have shown that elevated levels of serum CYFRA 21-1 were detected in patients with lung cancer (Takada et al., 1995; Molina et al., 2003). Kosacka and Jankowska (2009) concluded that in non-small cell lung cancer patients with strong CK 19 expression (++++) in tumor cells a tendency towards lower CYFRA 21-1 levels in serum is noted. Despite extensive research it is unclear why CYFRA 21-1 is increased only in some lung cancer patients, whereas all lung malignant tumors express CK 19. Studies have shown it was significantly elevated in all types of lung cancer (p< 0.05) with a specificity of 100 %, and sensitivity of 65.7%; significantly elevated in NSCLC as compared to SCLC with a sensitivity of 80% and 40% respectively. In addition, CYFRA 21-1 may not only be a sensitive tumor marker in the diagnosis (El-Nabi et al., 2009), but a high preoperative CYFRA 21-1 level is found to be a significant independent prognostic factor in patients with stage I NSCLC (Hanagiri et al., 2011).

It is substantiated by Tomita et al.’s findings (2010) that the simultaneous use of CYFRA 21-1 and CEA levels may increase the power of prognostic value for lung cancer patients.

**Tissue polypeptide antigen** (TPA) is a circulating complex of polypeptide fragments from cytokeratins 8, 18 and 19. It consists of four protein subunits (A1, B1, B2, C) with molecular weights between 20-45 kDa. TPA is an indicator of high cell proliferation. Increased serum levels of TPA may be present in several non-malignant diseases such as bacterial and viral infections,
acute hepatitis, autoimmune disorders and also during pregnancy. Increased concentrations of the antigen are found in a variety of tumors, including lung cancer. It has been proven that cytokeratins are useful tumor markers for the follow-up, treatment monitoring and prognosis evaluation of lung cancer and among these, tissue polypeptide antigen (TPA) plays an important role (Buccheri and Ferrigno, 1995; Buccheri and Ferrigno, 2001). Since TPA is found to be elevated in several other diseases, it may not be specific for lung cancer. It is suggested by Mumbarkar et al. (2006) that TPA and CYFRA 21-1 are useful serum markers for the diagnosis of NSCLC and their combined use may provide additional information for prognosis.

**Squamous cell carcinoma related antigen (SCC-Ag)** is a purified subfraction of the tumor antigen 4, with a molecular weight of 45,000Da, originally isolated from squamous cell carcinoma of the uterine cervix (Kato and Torigoe, 1977). Elevated serum SCC-Ag levels were also found in patients with squamous cell carcinoma of the bronchus and nasopharynx and occasionally in healthy volunteers and patients with tumors of other histopathological origin (Kato et al., 1979). Kagohashi et al. (2008) revealed that serum SCC levels were elevated in 52.7% of 201 patients with squamous cell lung cancer (SQLC), and in only 14.2% of 438 patients with non-squamous cell lung cancer (NSQLC). Their findings support previous conclusion that the sensitivity of SCC in the detection of NSQLC is low and that assessment of SCC in the clinical management of patients with NSQLC is not recommended (Cheah et al., 1994; Sanchez et al., 1994), although some investigators have reported elevated SCC levels in patients with NSQLC (Lai et al., 1996).

**Soluble interleukin 2 receptor (sIL-2R:IL2)** is a well characterized cytokine, with various immunological functions; the most important one being the capacity to initiate the proliferation of activated T-cells. The sIL-2R may be an important marker of the immune alterations associated with lung cancer. Marino et al. (1990) reported increased levels of sIL-2R in serum samples of patients with untreated lung cancer. Ginns et al. (1990) confirmed that patients with SCC or adenocarcinoma have high values of sIL-2R.

**Acute phase reactant proteins (APRPs)** are produced in response to inflammation. The association between APRP altered levels and cancer has long been established but, only recently, proteomics studies showed that APRP alterations are different in distinct tumor types.
Haptoglobin (Hp) is an acute phase protein that is tetrameric (α2β2) glycoprotein and is mainly synthesized in liver during inflammation and infection. An increase in Hp levels has also been reported in several cancers, such as breast cancer (Hamrita et al., 2009), ovarian cancer (Zhao et al., 2007) and pancreatic cancer (Firpo et al., 2009). The main function of Hp is to remove free plasma hemoglobin but Hp is also involved in angiogenesis (Cid et al., 1993) and cell migration (De Kleijn et al., 2002). In a recent study, serum level of Hp has been compared in patients with lung cancer, other types of solid cancers, and respiratory diseases and healthy donors by LC-ESIMS/MS, Western blotting, and ELISA (Kang et al., 2011). A higher level of Hp was present in the sera of lung cancer patients with respect to healthy controls but only the Hp β chain showed a significant difference between lung cancer and other tumors. Therefore, the Hp β chain seems to be a specific diagnostic marker for lung cancer. However, caution is needed when the Hp β chain is to be used as a marker to differentiate lung cancer from other respiratory diseases because Hp β chain levels overlap between these pathologic states. A search for biomarkers in human lung cancer serum samples using mass spectrometry approaches identified fucosylated Hp significantly increased in serum of each subtype of lung cancer compared to normal donors, thus suggesting its role as a marker to monitor lung cancer progression (Tsai et al., 2011).

**Serum Amyloid A Apolipoprotein (SAA)** are a family of apolipoproteins with several roles, including the transport of cholesterol to the liver, the recruitment of immune cells to inflammatory sites, and the induction of enzymes degrading extracellular matrix (Uhlar et al., 1999). Among the members of this family, SAA1 and SAA2 are synthesized in response to cytokines released by activated monocytes/macrophages. These proteins are produced predominantly by the liver but have been found at elevated levels in several cancers (Biran et al., 1986). Recently, SAA1 and SAA2 were proposed to be specific diagnostic markers for lung cancer as these are expressed at higher levels in blood and cancer tissues from patients with lung cancer compared to samples from healthy donors and patients with other types of cancer or respiratory diseases, as demonstrated by LCMS/MS, ELISA, and immunohistochemistry analyses (Sung et al., 2011). Therefore, SAA1 and SAA2 could also represent new potential therapeutic targets for the inhibition of lung cancer metastasis.
**Marker Enzymes**

**Neuron-specific enolase (NSE)** is a glycolytic enzyme produced in central and peripheral neurons (Greenberg and Lee, 2007). However, NSE activity is increased in SCLC and could be a potential tool for post-therapy monitoring of the disease. NSE was found to be raised in 75% of patients at diagnosis, in 67% of patients with limited disease, and in 86% of patients with extensive disease (Jorgensen et al., 1989). Usually, transient elevations of NSE are seen immediately after chemotherapy followed by a subsequent decline to lower or normal values (Akoun et al., 1985; Splinter et al., 1987). In a study by Mahmood et al. (2011) diagnostic sensitivity of NSE was found to be 86% with 67% specificity suggesting the potential of NSE as a useful tumor marker for SCLC but to a lesser extent for NSCLC.

**Tumor M2-pyruvate** is an isoform of the glycosile enzyme pyruvate kinase, present as an active dimer and less active tetramer. The dimeric isoform is typical of tumor cells and its level can be measured in blood. Choosing a cutoff value at 95% of specificity, sensitivity was 78% of the patients with SCLC and 81% of patients with NSCLC (Oremek et al., 2007). Levels correlate well with tumor progression and remission, making it a valuable tool in disease monitoring, independently from histological subset (Schneider et al., 2002).

**Hormones as cancer biomarker**

**Gastrin releasing peptide (GRP)** consisting of 27 amino acids is able to release gastrointestinal hormones (Wharton et al., 1978). It is closely related to an amphibian skin peptide of 14 amino acids, bombesin (Anastasi et al., 1971). However it has very short half-life and its increased concentrations in the serum are rare (Sorensen et al., 1982; Scagliotti et al., 1989). Using a radioimmunoassay for a GRP precursor, elevated plasma concentrations of pro-GRP have been found in 72% of 71 SCLC patients (Holst et al., 1989). In a recent study by Kim et al. (2011), the diagnostic sensitivity of plasma proGRP was estimated to be 83.8% in distinguishing SCLC from all the other conditions, and 86.5% for discriminating SCLC from the non-malignant cases. The upper normal limit of proGRP in the circulation is 50 pg/ml. Abnormal proGRP results are found in 60-70% and in 75-90% of SCLC patients with local and extensive disease, respectively. ProGRP is more sensitive biomarker than the neuron-specific enolase (NSE) for SCLC, but has
not been found in multivariate analysis to have independent prognostic significance (Molina et al., 2004).

Ectopic secretion of **Adrenocorticotropic hormone (ACTH) and related molecules** was initially observed by Brown et al. (1928) in a patient with Cushing’s syndrome and small cell carcinoma of the lung (SCLC). Ectopic ACTH production has also been described in association with many malignancies, such as ovarian tumors, thymoma, islet cell cancer of the pancreas, medullary cancer of the thyroid, and carcinoid tumor (Azzopardi and Williams, 1968). Elevated serum levels of ACTH have been reported in 25-30% of patients with SCLC (Yalow et al., 1979; Ratcliffe et al., 1982). Conflicting results have been reported concerning a possible correlation between serum levels of the hormone and stage of disease, treatment response, and survival of patients with lung cancer (Hansen et al., 1980). The common precursor to ACTH is pro-opiomelanocortin (POMC), which has been shown to be abnormally elevated in the plasma of lung cancer patients with epidermoid, adenocarcinoma, and small cell type (Chan et al., 1983).

Summing up, ACTH seems to be neither a specific nor a sensitive indicator of lung malignancy, nor does it appear to be reliable in monitoring the response to therapy, or in predicting relapses.

**Antidiuretic hormone** Hyponatremia was observed in association with cancer by Winkler and Krankshaw (1938). Schwartz et al. (1957) postulated its inappropriate secretion as the cause of persistent hyponatraemia in two patients with bronchogenic carcinoma. The concentration of ADH was found to be elevated in 35% of 279 patients with SCLC (Greco et al., 1981), whilst the frequency of the clinical syndrome was quite low (approximately 10% of 596 patients in six different studies (Hainsworth, 1983). Maurer et al. (1983) found that plasma concentrations of ADH-neurophysin were elevated in 65% of 103 patients with SCLC. In patients with initially high values, concentrations were related to the response to therapy, whilst initially normal levels were of no use in disclosing subsequent relapses.

**Calcitonin (CT)** is a 32 amino acid peptide, with a molecular weight of 3,419 Da synthesized by the thyroid C cells (Dilley et al., 1980). Normally, CT is secreted by the thyroid in response to increased plasma calcium concentration, or following the stimulation of certain gastrointestinal hormones. The hormone inhibits the release of calcium and phosphate from bone (Heynen and Franchimont, 1974). Marked elevations of serum calcitonin are usually found in familial
medullary thyroid carcinoma (De Lellis et al., 1978). Elevated levels have also been reported in other malignancies, including breast cancer, carcinoid tumor, hepatoma, renal cell carcinoma, and gastrointestinal cancer (Coombes et al., 1974). Calcitonin was elevated in 59% of 425 SCLC patients (Wallach et al., 1981). Elevated concentrations of CT do not seem to be correlated with stage of disease. However, merging the data from three studies, an increase of calcitonin has been found in 80% of 81 patients with ED, and in only 56% of 71 patients with LD (Roos et al., 1980).

Conflicting reports about the use of calcitonin in monitoring treatment response have so far been published. Wallach et al. (1981) reported a decrease of CT serum levels in SCLC patients responding to chemotherapy, and an increase in progressive diseases. Mulder et al. (1981), on the contrary, showed no such effect. In conclusion, the assay of serum calcitonin seems to be a general indicator of the course of SCLC disease, but it is sufficiently reliable for evaluating the response to treatment.

**Insulin-like growth factors (IGF’s)**, or somatomedin, are polypeptides of about 7.5 kDa, having a structural similarity to pro-insulin (Humbel et al., 1984). There are contrasting reports concerning the utility of IGF as tumor marker (Siwicky et al., 2011). Macauly et al. (1988) measured serum IGF-I concentrations in 42 SCLC patients, and concluded that IGF-I levels do not correlate with the tumor bulk, or with the therapeutic responsiveness of SCLC. Reeve et al. (1990) found elevated levels of these proteins in the serum of patients with both SCLC and NSCLC, and a good correlation between circulating levels and clinical course. Further studies are needed for better evaluation of these substances.

The sensitivity of CEA, CYFRA21-1, TPA, SCCAg, DKK1, NSE, ProGRP in the patients’ serum with lung cancer were 47.50%, 57.00%, 50.93%, 49.00%, 69.50%, 39.73%, 51.48% and the specificity were 92.34%, 80.19%, 90.16%, 88.41%, 91.07%, 92.20%, 89.11%, 94.89% (Lu et al., 2010). All these markers have proved to have a lack of sensitivity and therefore are of little clinical value for screening if used alone. Overall specificity is satisfactory for most of the biomarkers, ranging from 71 to 99%. Several authors have tried to study the value of various combinations of biomarkers to improve overall sensitivity and specificity, especially in screening and early diagnosis, because their level usually correlates with extension of disease. For early stage lung cancers lower cut-off values should be chosen to maintain satisfactory sensitivity,
leading to an overlapping with healthy subjects and consequently loss of specificity. Finally, at high elevated concentrations of cytokeratin 19 fragment, tissue polypeptide antigen and squamous cell carcinoma antigen in squamous cell lung cancer, carcinoembryonic antigen and cancer antigen 125 in adenocarcinoma, as well as progastrin-releasing peptide and neuron-specific enolase in small cell lung cancer are the best suggestive panel of markers available.

**Potential of lung cancer protein biomarkers**

There are some potential lung cancer biomarker molecules, at different stages of development. The potency of serum amyloid A (Cho et al., 2004; 2010), haptoglobin-alpha (Maciel et al., 2005), and a fragment of apolipoprotein A-I (Huang et al., 2006) for lung cancer biomarker is mentioned in some studies but needs further clinical validation. Plasma kallikrein B1 (KLKB1) fragment has potential to be biomarker for diagnosis of lung adenocarcinoma (Heo et al., 2007). KLKB1 has homology with proteins of serine protease-trypsin family and is related to surface-dependent procoagulation, fibrinolysis, kinin generation, and inflammation. It also has homology with KLK3 that produces prostate specific antigen (PSA). About 18 kDa fragment of KLKB1, possibly containing H4 domain of it, showed high levels in lung adenocarcinoma. The finding of KLKB1 fragment supports the ideas of serum peptidomes as great diagnostic potential biomarkers in lung cancer diagnosis (Liotta and Petricoin, 2006; Villaneueva, 2006). Pentraxin-related protein PTX3 also known as TNF-inducible gene 14 (TSG-14) is a protein that in humans is encoded by the *PTX3* gene. PTX3 is rapidly produced and released by several cell types, in particular by mononuclear phagocytes, dendritic cells (DCs), fibroblasts and endothelial cells in response to primary inflammatory signals [e.g., toll like receptors (TLR) engagement, TNFα, IL-1β]. Planque et al. (2009), found serum levels of PTX3 much higher in lung cancer patients (median= 4.91 ng/ml) as compared with healthy individuals (median= 1.52 ng/ml). The diagnostic sensitivity of pentraxin at 100% specificity was found to be 68%. In a recent study, Diamandis et al. (2011) have confirmed Pentraxin-3, but not KLK11 or progranulin, as a new serum biomarker for lung carcinoma. Pentraxin-3 was able to discriminate lung cancer patients from high-risk controls with moderate sensitivity (37% at 90% specificity and 48% at 80% specificity) which was comparable to other currently used lung cancer biomarkers. Dickkopf-1 (DKK1), a secretory protein known as a negative regulator of the Wnt signaling pathway, has been involved in the development of several types of cancers. Investigators have identified Dickkopf-1 (DKK1) as a novel serologic and histochemical biomarker as well as a therapeutic
target for lung cancers (Yamabuki et al., 2007). Sheng et al. (2009) have further confirmed the clinical significance and prognostic value of serum Dickkopf-1 concentrations in patients with lung cancer.

Researchers at Fred Hutchinson Cancer research center have recently discovered some new proteins in the blood that may be associated with early lung cancer development in mice and humans. In-depth plasma proteome analysis of four different mouse models revealed, a protein signature for Titf1/Nkx2-1, a known lineage-survival oncogene in lung cancer, network of dysregulated proteins linked to epidermal growth factor receptor and a distinct plasma signature related to neuroendocrine development (Taguchi et al., 2011). This advancement would bring the promise of a blood test closer to early diagnosis of lung cancer.

2.5 OXIDATIVE STRESS AND LUNG CANCER

2.5.1 Free radicals and oxidative stress
Free radicals or reactive oxygen species are generated by biochemical redox reactions that occur as part of normal cell metabolism. Under normal metabolic conditions 2–5% of the O₂ consumed by mitochondria is converted to reactive oxygen species (Lopaczynski and Zeisel, 2001). Hence, the prevention of excess free radical formation is a vital step for cell survival (Valko et al., 2006). A free radical is a molecule which contains one or more unpaired electrons in its outer orbital (Halliwell et al., 1992) and is therefore highly reactive. Free radicals can act by three different mechanisms, namely addition, donation and removal of electrons.

a) addition x•+y → [x–y]•

b) electron donation x•+y → y•+x+

c) electron removal x•+y → x–+y•+

As their half-life is relatively short, most free radicals cause damage locally, close to their sites of production (Singal et al., 2000). Free radicals are able to bind most cellular components; they can react with unsaturated fatty acids in membrane lipids, damage DNA and denature proteins (Huang et al., 2000).
Lung cancer pathogenesis still remains unknown. Nevertheless, it is postulated that cancer is one of those diseases in which reactive oxygen species (ROS) are involved in its pathogenesis. ROS can be produced endogenously or exogenously. In vivo free radicals are formed during normal aerobic respiration, by activation of phagocytosing cells, in peroxisomes where fatty acids are degraded, and by auto-oxidation of various molecules (Frei et al., 1989; Halliwell, 2007). These endogenous radical production ways account for most of the oxidants produced by cells.

Additional endogenous sources of cellular ROS are neutrophils, eosinophils and macrophages (Valko et al., 2007). But the exogenous or external environment factors such as cigarette smoke, ionizing radiation, pollutants, organic solvents, anesthetic gases, hyperoxic environments and pesticides may intensify the ROS generation. Beside cell-autonomous process involving genetically transformed cancer cells exposed to intrinsic oxidative stress, the importance of stromal cell types populating the tumoral microenvironment is now well established. Indeed, tumor microenvironment may affect evolution of cancers towards aggressiveness and metastatic dissemination through both structure-and function- based (matrix composition, hypoxia, acidity) or cell-based (cancer associated fibroblasts (CAFs) or macrophages (CAMs), endothelial precursors, etc.) mechanisms (Allen and Jones, 2011). Tobacco smoking (First hand or second hand smoking) is one of the major lifestyle risk factors in lung cancer (Huynh et al., 2012). Cigarette smoke is a complex mixture of over 7000 chemical compounds (Rodgman and Perfetti, 2009). Each puff of cigarette smoke contains 1014-16 Reactive oxygen species (ROS) like superoxide (O_2•-), hydrogen peroxide (H_2O_2), hydroxyl (OH•) and peroxyl (ROO•) radicals (Church and Pryor, 1985; Hecht, 2007). Smoke may enhance oxidative stress by following three reasons, first, tobacco is rich in pro-oxidants, which are further supplemented during smoking and chewing, second, these pro-oxidants consume more antioxidants and third, smokers have a tendency for low intake of dietary antioxidants. Oxidative stress induces a cellular redox imbalance which has been found to be present in various cancer cells compared with normal cells, the redox imbalance thus may be related to oncogenic stimulation. DNA mutation is a critical step in carcinogenesis and elevated levels of oxidative DNA lesions (8-OH-G) have been noted in various tumors, strongly implicating such damage in the etiology of cancer. It appears that the DNA damage is predominantly linked with the initiation process (Dizdaroglu, 2012).
Moreover, severe oxidative stress is not only known to cause DNA damage and mutations of tumor suppressor genes which are initial events in carcinogenesis, but can also play an important role in the promotion of multi-step carcinogenesis (Figure 2.14) (Cui, 2012). Hence the study was planned to see the effect of oxidative stress in causation and progression of lung cancer.

![Schematic diagram showing the generation and effects of reactive oxygen species (ROS) in tumor progression. Oncogene activation, macrophage infiltration or hypoxia/reoxygenation in tumors induce the generation of ROS. These ROS have roles in mediating cell proliferation, genomic instability, cell motility, angiogenesis and thus can contribute to tumorigenesis, but also can induce cell cycle arrest, senescence and cell death and thus attenuate tumor growth.](image)

**Figure 2.14** Schematic diagram showing the generation and effects of reactive oxygen species (ROS) in tumor progression. Oncogene activation, macrophage infiltration or hypoxia/reoxygenation in tumors induce the generation of ROS. These ROS have roles in mediating cell proliferation, genomic instability, cell motility, angiogenesis and thus can contribute to tumorigenesis, but also can induce cell cycle arrest, senescence and cell death and thus attenuate tumor growth.

### 2.5.2 Antioxidant defence system

According to the commonly accepted definition by Halliwell and Gutteridge (2007), an antioxidant is “any substance that delays, prevents or removes oxidative damage to a target molecule”. The human body contains a complex antioxidant defense system which depends on the dietary intake of antioxidants as well as the endogenous production of antioxidative compounds such as glutathione (Clarkson and Thompson, 2000; Sotgia *et al.*, 2011).
The damage caused by free radicals can naturally be minimized by a combination of biological antioxidant defence system including enzymatic and non-enzymatic creations (Abdel-Salam et al., 2011; Halliwell, 2012). Important antioxidants enzymes include superoxide dismutase, catalase, and glutathione reductase (Halliwell and Cross, 1994; Mahapatra et al., 2008) and malondialdehyde (MDA) which is a stable end product of free radicals for oxidative damage to tissues. The non-enzymatic part includes a large number of natural and synthetic antioxidant compounds like reduced glutathione.

SOD and CAT are proteins which act primarily in the cell cytoplasm and form the first line defense against oxidants, e.g. superoxide anion and hydrogen peroxide (Gago-Dominguez and Castelao, 2006). In humans, three different forms of SOD are cytosolic-CuZn-SOD, mitochondrial Mn-SOD, and extracellular SOD. Fe-SOD exists in animals but not in humans. Though CuZn-SOD is located in most parts of the cell, Mn-SOD is the most important scavenger of O•–, converting it to hydrogen peroxide and oxygen (Halliwell, 2007). Thus CuZn-SOD and Mn-SOD catalyse the following reaction:

\[
2 \text{O}_2{}^+ + 2 \text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2
\]

CAT neutralizes hydrogen peroxide formed in excess. SOD and CAT also function as anticarcinogenes by inhibiting the initiation and promotion phases in carcinogenesis (Kumaraguruparan et al., 2005; Halliwell, 2007). Glutathione reductase, also known as GSR or GR, reduces glutathione disulfide (GSSG) to the sulphydryl form GSH, where NADPH is used as the reducing agent (Meister, 1988). Glutathione reductase is a flavoenzyme and an important predictor of general oxidant/antioxidant status (Halliwell and Gutteridge, 2007).

Lipid peroxidation is a form of oxidative damage in cell membranes determined as free radicals reacting with polyunsaturated fatty acids (Lopaczynski and Zeisel, 2001). Malondialdehyde (MDA), the main final 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. They are also capable of inducing apoptosis or necrosis in various cells (Lopaczynski and Zeisel, 2001). Measuring MDA levels in the plasma or serum provides a suitable in vivo index of lipid peroxidation (Morabito et al., 2004).
Reduced glutathione (GSH) is a tripeptide with a free thiol group (-SH group) which consists of three amino acids joined together (glutamic acid, cysteine and glycine). It is highly abundant in the cytosol, nuclei and mitochondria (Valko et al., 2006). GSH can directly act as a free radical scavenger by neutralizing HO•, or indirectly by repairing initial damage to macromolecules inflicted by HO•. Glutathione has multiple functions: it modulates cell proliferation and plays a key role in protecting cells against oxidants (Navarro et al., 1999, Kumaraguruparan et al., 2005). GSH has functions in catalysis, metabolism and transport, and is involved in protein synthesis (Meister, 1988). Conjugation with GSH has been suggested as a primary mechanism for the detoxification of lipid peroxidation products. The redox system of GSH consists of primary and secondary antioxidants (glutathione peroxidase, glutathione reductase, glutathione-S-transferase, glucose-6-phosphate dehydrogenase).

Previous studies have suggested that antioxidant activity can be impaired due to production of excessive oxidative stress in lung cancer (Guner et al., 1996; Ho et al., 2001; Peddireddy et al., 2012). The oxidative stress in cancer cells is often associated both with altered expression of molecules involved in ROS metabolism as well as change in sensitivity to various therapeutic modalities. Thus, detail analyses of the biochemical events associated with redox alterations during inflammation and malignant transformation are important in understanding the carcinogenesis processes and may have significant therapeutic implication.