II. LITERATURE REVIEWS

1. Overview of cancer

Cancer is an abnormal growth of cells. Cancer cells rapidly reproduce despite restriction of space, nutrients shared by other cells, or signals sent from the body to stop reproduction. Cancer cells are often shaped differently from healthy cells, they do not function properly, and they can spread to many areas of the body. Tumors, abnormal growth of tissue, are clusters of cells that are capable of growing and dividing uncontrollably; their growth is not regulated.

In Thailand, the most common cause of death is cancer (Thailand and public health., 2003). The estimated number of deaths was 42,497 cases per year. The leading types of cancers were breast (25%), cervix (20%), lung (16%), liver (12%), colon (7%), esophagus (7%), nasopharynx (5%), rectum (2%), ovary (2%) and other cancers (3%).

2. Colorectal Cancer

Colorectal tumors are often first recognized as a polyp protruding from the wall of the bowel, which may be either hyperplastic (nondysplastic) or dysplastic (adenomatous). Hyperplastic polyps consist of large numbers of cells with normal morphology that do not have a tendency to become malignant (Kent and Mitros, 1991). Adenomatous polyps contain dysplastic cells that fail to show normal intracellular and intercellular organization. Expanding adenomas become progressively more dysplastic and likely to become malignant. The majority of malignant tumors of the colon are thought to be derived from benign polyps. The malignant nature of colorectal tumors are defined by their invasiveness. The major histologic type of colorectal cancer is adenoma, which account for 90-95% of all colorectal tumors (Cooper, 1983; Spjut, 1984), although other rare epithelial tumor types do occur, including squamous-cell carcinomas (SCCs), adenosquamous carcinomas, and undifferentiated carcinomas, which contain no glandular structures or features such as mucinous secretions (Cooper, 1983)

Several studies indicated that certain genetic factors, environmental factors, or dietary factors were probably involved in the etiology of colorectal cancer. Research in epidemiological, clinical and genetic evidence found that a great number of colorectal adenocarcinomas developed from a benign adenomatous polyp progressing though a sequence of events, which might take about 15-20 years (Winawer *et al.*, 1996).

2.1 Genetic alterations during the progression of colorectal cancer

The progression of events leading to the transformation of colonic epithelial cells into a cancer is multistep process that starts with stepwise accumulation of multiple genetic defects (Kinzler and Vogelstein, 1998). There may be as many as twenty genes involved in colorectal carcinogenesis. Especially, the most well defined genetic mutations include those in the adenomatous polyposis coli (APC) gene on chromosome 5, K-ras on chromosome 12, deleted in colon carcinoma (DCC) on chromosome 18, and p53 on chromosome 17 (Fig. 1). Interestingly, mutation of the APC tumor suppressor gene has been detected in 36% to 79% of patients with colorectal cancers and adenomas (Vogelstein *et al.*, 1998; Miyoshi *et al.*, 1992), suggesting that an APC gene mutation may be an early or initiating event in the colorectal tumorigenesis.

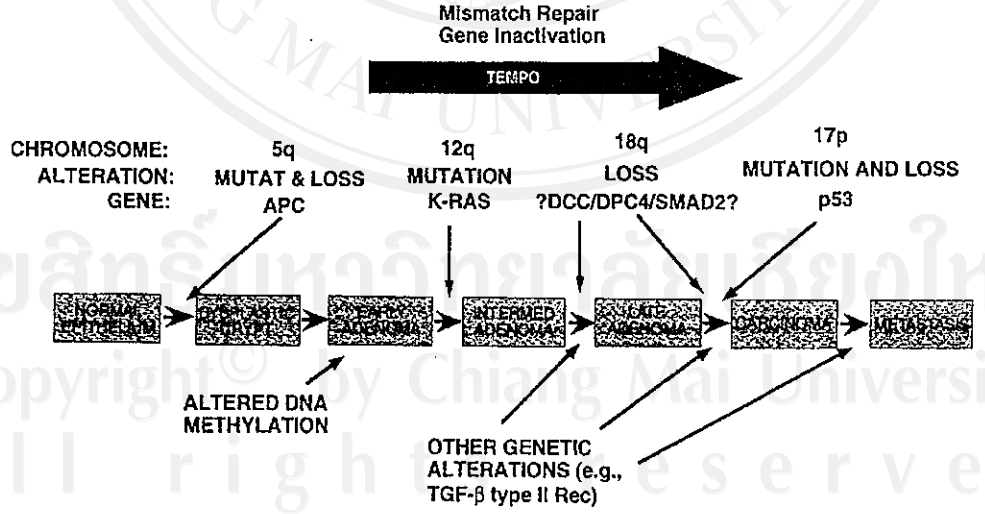

Figure 1. Genetic model for the progression of colorectal cancer.

Additionally, genetic events involve the formation of a very small polyp (adenoma) that latter turn into a cancer as a result from accumulation of these distinct genetic alterations. It has been show that the conversion of late colorectal adenoma to carcinoma was associated with genetic changes of both p53 alleles (Kikuchi-Yanoshita *et al.*, 1992), indicating that p53 is important for the suppression of cellular transformation. p53 is an essential protein for the regulation of genome integrity. Every day of our life, cells face many dangers, including chemical, viruses and ionizing radiation, which can damage the genome and lead to cancer development. p53 binds to many regulatory sites in the genome and arrest cell division until the damage is repaired. Or, if the damage is too severe, p53 initiates the process of apoptosis (Clarke *et al.*, 1993), which directs the cell to commit suicide, permanently removing the damage. Therefore, loss of p53 function will result in a massive accumulation of mutations and instability of the cellular genome, which is the main character of malignant phenotype. This explains why loss of p53 function can converse colorectal adenoma to colorectal carcinoma. Thus, these events require multiple genetic alterations with activation of oncogencs and inactivation of tumor suppressor genes that are essential for the tumorigenesis.

Although, growth factors and cytokines might be important in promoting the development of colorectal cancer is unclear, some specific growth factors are produced by colorectal carcinomas include transforming growth factor alpha (TGFα), insulin-like growth factor II (IGF-II), and human chorionic gonadotropin (hCG) (Goustin *et al.*, 1989). In addition, colorectal carcinoma cells have been demonstrated to possess receptors for epidermal growth factor (EGFR), human chorionic gonadotropin, insulin like growth factors, steroid receptors, and receptors for transferrin and gastrin (Goustin *et al.*, 1989). However, cooperation between TGFβ-1 and the tumor –suppressor gene p53 has been implied in the process of postlactaional mammary gland involution in which these two genes, as well as c-*myc*, are downregulated shortly following weaning (Strange *et al.*, 1992). Loss of TGFβ-1 responsiveness was observed in thyroid follicular cells at the point of transformation from adenomatous cells to a malignant phenotype associated with inactivation of p53 (Wyllie *et al.*, 1991). Since well-differentiated colonic epithelial cells are likewise sensitive to the growth inhibitory actions of TGFβ-1 (Oberhammer *et al.*, 1993; Chuang *et al.*, 1994), the interaction of p53 and TGFβ-1 may have significance in colorectal neoplasia. TGFβ appears to cause its growth inhibitory and apoptotic

effects through regulation of the concentration of a protein $p27^{Kip1}$. The latter binds to cell-cycle-regulatory proteins cyclin E-cdk2 and cyclin D1-cdk4 (Polyak *et al.*, 1994; Toyoshima and Hunter, 1994). This action of TGFβ appears to regulate the quantity and phosphoryaltion state of the retinoblastoma susceptibility protein (pRB) (Yan *et al.*, 1992). Exposure of both a well differentiated and poorly differentiated colorectal carcinoma cell line to TGF resulted in decreased retinoblastoma protein in both tumor types, but enhanced phosphorylation of the retinoblastoma in the undifferentiated and unresponsive cell line, whereas the degree of phosphorylation decreased in retinoblastoma in the responsive cell line (Yan *et al.*, 1992). This action on the phosphorylation state of RB would be expected to cause TGFβ to act as growth inhibit for the differentiated cell line and a growth stimulator for the two undifferentiated cell lines (Yan *et al.*, 1992).

2.2 Risk factors involved in colorectal cancer development

There are several recognized risk factors for development of colorectal cancer, some of which are genetic or related to benign pathological lesions of the colorectum, and others that are related to lifestyle or environment. Approximately 5-10% of colorectal cancers are thought to be related to an inherited predisposition. Familial colorectal cancer can arise in presence or absence of polyposis, which is characterized by the occurrence of multiple polyps lining the walls of the colon. Several polyposis syndromes have been described, the major form of which is familial adenomatous polyposis (FAP) (Kinzler and Vogelstein, 1998). Patients with inflammatory bowel disease (ulcerative colitis) or Crohn's disease (granulomatous clitis) exhibit an increased risk for development of colorectal cancer (Kinzler and Vogelstein, 1998; Hamilton, 1985). The risk of developing colorectal cancer appears to be associated with a diet that is low in fiber and high in calories, protein and fat, especially in red meat. In addition, obesity, sedentary life styles and alcohol consumption have been implicated as potential risk factors (Potter and McMichael, 1986; Giovannucci *et al.*, 1995). A reduced risk of colorectal cancer has recently been linked to the use of nonsteroidal anti-inflammatory drugs (NSAID) such as aspirin (Giovannucci *et al.*, 1995).

3. Liver cancer

Liver cancer or hepatocellular carcinoma (HCC) is one of the most common forms of cancer in the world, with most of the cases occurring in the developing countries. (Waterhouse *et al*, 1987). The incidence of the disease is about 40/100,000 per year in the areas of highest risk in Asia and it is more common in men than in women. The incidence is less than 4/100,000 per year in low-risk areas such as Europe and North America.

Most cases of liver cancer are actually cancers that started in another organ. This is called metastases. Because of its very high blood flow and many biological functions, the liver is one of the most common places for metastases to grow. Tumors that originally arise in the colon, pancreas, stomach, lung or breast can spread to the liver. The geographic distribution of hepatocellular carcinoma closely follows that of hepatitis. It is believed that about 80% of hepatocellular carcinoma is associated with exposure to hepatitis B virus.

3.1 Risk factors involved in liver cancer development

There are two main kinds of liver cancer, hepatoma and cholangiocarcinoma (CCA). Hepatoma is cancer of the hepatocytes. Hepatoma is primary liver cancer. Hepatoma usually grows in the liver as a ball-like tumor, invading the normal tissue surrounding it. A history of infection with the hepatitis B virus puts individuals at risk of developing heptoma. Cancer of the bile duct cells is called cholangiocarcinoma. Cholangiocarcinoma originates in the bile ducts and is often caused by infestation with the liver fluke *Clonorchis* (a parasite).

Certain viruses can infect the liver. The infection may be chronic. The most important risk factor for liver cancer is a chronic infection with the hepatitis B virus or the hepatitis C virus. These viruses can be passed from person to person through blood (such as blood or sexual contact). An infant may catch these viruses from an infected mother. Liver cancer can develop after many years of infection with the virus. These infections may not cause any symptoms, but blood tests can show whether the virus is present. If so, the doctor may suggest treatment. Also, the doctor may discuss ways of avoiding infecting other people. In people who are not already infected with hepatitis B virus, hepatitis B vaccine can prevent chronic hepatitis B infection and can protect against liver cancer. Researchers are now working to develop a vaccine to prevent hepatitis C infection. Cirrhosis is a disease that develops when liver cells are damaged and replaced with scar tissue. Cirrhosis may be caused by alcohol abuse, certain drugs and other

chemicals, and certain viruses or parasites. About 5 percent of people with cirrhosis develop liver cancer. Liver cancer can be caused by aflatoxin, a harmful substance made by certain types of mold. Aflatoxin can form on peanuts, corn, and other nuts and grains. In Asia and Africa, aflatoxin contamination is a problem. However, the U.S. Food and Drug Administration (FDA) does not allow the sale of foods that have high levels of aflatoxin.

Genetic diseases is also correlate to HCC such as, chronic liver injury and development of cirrhosis. Hereditary hemochromatosis is the most common and shows an autosomal recessive pattern of inheritance by linkage analysis using markers on chromosome 6p21 (Gasparini *et al.*, 1993). Recent investigations have led to the discovery of a class of genes with functional properties opposites to those of the oncogenes or tumor-producing genes such as, RB and p53. In addition, the upregulated expression of growth factors are important contributors to the multistep process of hepatocarcinogenesis (Aaronson, 1991). Some of the growth factors that may be involved in one or more step in development of HCC include insulin, insulin-like growth factor II (IGF), TGFα, TGFβ, EGF, aFGF and hepatocyte growth factor (HGF). EGF, HGF, and TGFα appear to be the most potent growth factors with respect to stimulation of hepatocyte proliferation.

3.2 Liver cancer in Thailand

The relationship between infection with the liver fluke, *Opisthorchis viverrini* and CCA has been shown in several reports (Thamavit *et al.*, 1978; Vatanasapt *et al.*, 1990; Parkin *et al.*, 1993; Sriamporn, 1993, Sithithaworn *et al.*, 1994; IARC, 1994). Both exogenous N-nitrosocompounds (Migasena *et al.*, 1980) and endogenous nitrosation (Srivatanakul *et al.*, 1991) are suspected to play a role in parasite-related carcinogenesis. Mutation of p53 tumor suppressor gene has been reported to be present in 35% of cholangiocarcinoma cases in Thailand, similar to the proportion found in Japanese patients (33%) (Kiba *et al.*, 1993). The prevalence rate of opisthorchiasis detected by OV eggs in stool has changed from 40% in 1990-1991 (Vatanasapt *et al.*, 1993) to approximately 25% in 1992-1996 (Vatanasapt, 1997) and 15% in 1998 (Vatanasapt *et al.*, 1998). Infection with Hepatitis B is fairly common in Thailand, with the prevalence of carriers of surface antigen in various reports being 9.4% (Punyagupta *et al.*, 1973), 10% (Srivatanakul *et al.*, 1983), and 8.4% (Srivatanakul *et al.*, 1991). Hepatitis B surface antigen was detected in 61-63.8% of HCC patients, which is higher than in blood donors (5.28%) and in CCA patients (16.7%) (Petchclai et al., 1992; Songsivilai et al., 1996). Hepatitis

C infection is also a recognised risk factor for liver cancer, but the prevalence of antibody to Hepatitis C seems to be rather low in Thailand (Srivatanakul *et al.*, 1991). Boonmar *et al.*, (1990) found antibody to Hepatitis C virus in 11.1% of HCC patients negative for hepatitis B surface antigen. Songsivilai *et al.*, (1996) also found 11.3% of Hepatitis C virus in HCC patients, and also showed that Hepatitis C virus is an important cause of Hepatitis-B negative liver cancer (out of 80 HCC cases, 8 were infected with HCV, and 3 with both HCV & HBV).

4. Lung cancer

Lung cancer is a disease in which the cells of the lung tissues grow uncontrollably and form tumors. Lung cancer is the leading cause of death from cancer among both men and women in the United States (Greenlee *et al.*, 2000). There are two kinds of lung cancers. The two main kinds are primary and secondary. Primary lung cancer starts in the lung itself. Primary lung cancer is divided into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), depending on cell size under the microscope. Each type of primary lung cancer grows and spreads in different ways. Secondary lung cancer is cancer that starts somewhere else in the body (for example, the breast or colon) and spreads to the lungs.

4.1 Histologic types of lung cancer

Small cell lung cancer was formerly called oat cell cancer, because the cells resemble oats in their shape. About a fourth of all lung cancers are small cell cancers. This type is a very aggressive cancer and spreads to other organs within a short time. It is generally found in people who are heavy smokers (http:// www.cancer.org/docroot/CRI).

Non-small cell cancer account for about 75% of lung cancers. This type of lung cancer grows and spreads more slowly than small cell cancer. There are 5 types of non-small cell lung cancer. The 5 types of non-small cell lung cancer have different kinds of cancer cells. The cancer cells of each type grow and spread in different ways. The types of non-small cell lung cancer are named for the kinds of cells found in the cancer and how the cells look when viewed under a microscope:

- Squamous cell carcinoma (SCC): Cancer that begins in squamous cells, which are thin, flat cells that look like fish scales. This is also called epidermoid carcinoma.

- Adenocarcinoma: Cancer that begins in cells that have glandular (secretory) properties.
- Large cell carcinoma: Cancer in which the cells are large and look abnormal when viewed under a microscope.
- Adenosquamous carcinoma: Cancer that begins in cells that look flattened when viewed under a microscope. These cells also have glandular (secretory) properties.
- Undifferentiated carcinoma: Cancer cells that do not look like normal cells and multiply uncontrollably.

Cancers of the lung and bronchus represent 91% of all respiratory system cancer (Landis *et al.*, 1999). The remainder of respiratory system cancers include tumors of the larynx and nasal cavities. SCCs account for approx 35% of lung cancers (Faber, 1991). This histologic subtype of lung cancer is closely correlated with cigarette smoking and represents the most common type of lung cancer among men. SCCs display varying levels of differentiation, from tumors consisting of well-differentiated keratinized sequamous epithelium to tumors consisting of undifferentiated anaplastic cells. Adenocarcinomas have increased in frequency in recent year and now account for nearly 35% of lung cancers (Faber, 1991). These tumors grow faster than SCCs and frequently metastasize to the brain. Pulmonary adenocarcinomas can present as well-differentiated tumors consisting of well-differentiated glandular epithelium, or as undifferentiated tumors composed of highly mitotic anaplastic cells. Large-cell undifferentiated carcinomas account for approx 15% of all lung cancer (Faber, 1991). These tumors lack squamous-or glandular-cell characteristics and are typically composed of large anaplastic cells with frequent mitotic figures. Clinically, these tumors metastasize early and have a poor prognosis. Small-cell lung carcinomas (SCLC) make up the majority of the remaining cancers (10%) (Faber, 1991). These tumors are also associated with smoking history. SCLCs tend to produce a variety of neuroendocrine substances that can cause symptoms related to the biological activity of the hormonal substance. About 10% of SCLCs display a paraneoplastic phenotype related to production of these neuroendocrine effectors (Bunn *et al.*, 1985). These tumors grow rapidly, metastasize early, and have a very poor pronosis.

4.2 Risk factors involved in lung cancer development

The majority of lung cancers are attributable to exposure to known carcinogenic agents, particularly cigarette smoke. Several lines of evidence strongly link cigarette smoking to lung cancer. Smokers have a significantly increased risk (11-fold to 22-fold) for development of lung cancer compared to nonsmokers (Shopland *et al.*, 1991), and cessation of smoking decreases the risk for lung cancer compared to continued smoking (Shopland *et al.*, 1991; Garfinkel and Silverberg, 1991). Furthermore, heavy smokers exhibit a greater risk than light smokers, suggesting a dose-response relationship between cigarette consumption and lung-cancer risk (Shopland *et al.*, 1991; Garfinkel and Silverberg, 1991). Numerous mutagenic and carcinogenic substances have been identified as constituents of the particulate and vapor phases of cigarette smoke, including benzo[*a*]pyrene, dibenza[*a*]anthracene, nickel, cadmium, polonium, urethane, formaldehyde, nitrogen oxides, and nitrosodiethylamine (Public Health Service., 1982). There is also evidence that smoking combined with certain environmental (or occupational) exposures results in potentiation of lung-cancer risk. Urban smokers exhibit a significantly higher incidence of lung cancer than smoker from rural areas, suggesting a possible role for air pollution in development of lung cancer (Haenszel *et al.*, 1962). Occupational exposure to asbestos, bis(chloromethyl)ether, and chromium have been associated with increased risk for development of lung cancer (Hammond *et al.*, 1979; IARC, 1980). Exposure to the radioactive gas radon has been suggested to increase the risk of lung cancer development. This gas is ubiquitous in the earth's atmosphere, creating the opportunity for exposure of vast number of people. However, passive exposure to the background levels of radon found in domestic dwellings and other enclosures are not sufficiently high to increase lung-cancer risk appreciably (Blot *et al.*, 1990). High-level radon exposure has been documented among miners working in uranium, iron, zinc, tin, and fluorspar mines (Archer *et al.*, 1976; Harley and Harley, 1990). These workers show an excess of lung cancer (compared to nonminers) that varies depending on the radon concentration encountered in the ambient air of the specific mine (Archer *et al.*, 1976; Harley and Harley, 1990).

5. Staging of colorectal, liver, and lung cancer

The stage of these cancers is not finally revealed until after the resection when the removed specimen has been analyzed by the pathologist. It is a process that tells the physician how widespread a cancer may be and this will show if the cancer has spread and how far. The treatment and prognosis for the cancer depend, to a large extent, on the patient's stage at diagnosis.

The stage system for colorectal, liver, and lung cancer used in the United Stages is the international TNM system developed by the American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC) (Fleming *et al.*, 1997). The characteristics that form the basic of the staging system are based on the assessment of three components including T stands for the extent of the primary tumor, N stands for the absence or presence and extent of regional lymph node metastasis, and M is for the absence or presence of distant metastasis. The use of numerical subsets of the TNM component indicates the progressive extent of the malignant disease. In TNM staging, information about the tumor, lymph nodes, and metastasis is compound in a process called stage grouping (Table 1, 2 and 3). The stage of cancer is described in Roman numerals from I to IV.

Table 1. Staging systems for colorectal cancer.

Primary Tumor (T)

TX Primary tumor cannot be assessed
T0 No evidence of primary tumor
TIS Carcinoma in situ: intraepithelial tumor or invasion of lamina propria*
T1 Tumor invades or submucosa
T2 Tumor invades muscularis propria
T3 Tumor invades through the muscularis propria into the subserosa, or into nonperitonealized pericolic or perirectal tissues
T4 Tumor directly invades other organs or structures, and/or perforates visceral peritoneum**

Regional Lymph nodes (N)

NX Regional lymph nodes cannot be assessed
N0 No regional lymph node metastasis
N1 Metastasis in 1 to 3 regional lymph nodes
N2 Metastasis in 4 or more regional lymph nodes

Distant Metastasis (M)

MX Distant metastasis cannot be assessed
M0 No distant metastasis
M1 Distant metastasis

Stage grouping

AJCC/UICC				**Dukes'**
Stage 0	Tis	N0	M0	A
Stage I	T1	N0	M0	A
	T2	N0	M0	A
Stage II	T3	N0	M0	B
	T4	N0	M0	B
Stage III	Any T	N1	M0	C
	Any T	N2	M0	C
Stage IV	Any T	Any N	M1	D

*Note: TIS includes cancer cells confined within the glandular basement membrane (intraepithelial) or lamina propria (intramucosal) with no extension through the muscularis mucosae into the submucosa.

**Note: Direct invasion in T4 includes invasion of other segments of the colorectum by way of the serosa; for example, invasion of the sigmoid colon by carcinoma of the cecum.

(The original source for this material is the AJCC® cancer Staging Manual, 5th edition (1997) published by Lippincott-Raven Publishers, Philadelphia, Pennsylvania.)

Table 2 Staging systems for liver cancer.

Primary Tumor (T)

TX Primary tumor cannot be assessed

T0 No evidence of primary tumor

T1 Solitary tumor $\leq$ 2 cm in greatest dimension without vascular invasion

T2 Solitary tumor $\geq$ 2 cm in greatest dimension with vascular invasion ; or multiple tumors limited to one lobe, none > 2 cm in greatest dimension, without vascular invasion ; or solitary tumor > 2 cm in greatest dimension without vascular invasion

T3 Solitary tumor > 2 cm in greatest dimension with vascular invasion or multiple tumors limited to one lobe, none > 2 cm in greatest dimension, with vascular invasion ; or multiple tumors limited to one lobe, any > 2 cm in greatest dimension, with or without vascular invasion

T4 Multiple tumors in more than one lobe, or tumor (s) involve (s) a major branch of the portal or hepatic vein (s), or invasion of adjacent organs other than the gallbladder, or perforation of the visceral peritoneum

Regional Lymph nodes (N)

NX Regional lymph nodes cannot be assessed

N0 No regional lymph node metastasis

N1 Regional lymph nodes

Distant Metastasis (M)

MX Distant metastasis cannot be assessed

M0 No distant metastasis

M1 Distant metastasis

Stage grouping

AJCC/UICC

Stage 0	TIS	N0	M0
Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage IIIA	T3	N0	M0
Stage IIIB	T1-3	N1	M0
Stage IVA	T4	Any N	M0
Stage IVB	Any T	Any N	M1

Adapted from : Fleming ID, Cooper JS, Henson DE, et al, eds, AJCC cancer staging manual. 5th ed. Philadelphia:LippinocottRaven, 1997:98-9.

Table 3. Staging systems for lung cancer

Primary Tumor (T)

TX Primary tumor cannot be assessed, or tumor proven by presence of malignant cells in sputum or bronchial washing but not visualized by imaging or bronchoscopy

T0 No evidence of primary tumor

TIS Carcinoma in situ

T1 Tumor 3 cm or less in greatest dimension, surrounded by lung or visceral pleura, without bronchoscopic evidence of invasion more proximal than the lobar bronchus

T2 Tumor with any of the following features of size or extent :

More than 3 cm in greatest dimension

Involves main bronchus, 2 cm or more distal to the carina

Invades the visceral pleura

Associated with atelectasis or obstructive pneumonitis which extends to the hilar region but dose not involve the entire lung

T3 Tumor of any size that directly invades any of the following : chest wall (including superior sulcus tumors), diaphragm, mediastinal pleura, parietal pericardium ; or tumor in the main bronchus less than 2 cm distal to the carina but without involvement of the carina; or associated atelectasis or obstructive pneumonitis of the entire lung

T4 Tumor of any size that invades any of the following : mediastinum, heart, great vessels, trachea, esophagus, vertebral body, carina ; or tumor with a malignant pleural effusion

Regional Lymph nodes (N)

NX Regional lymph nodes cannot be assessed

N0 No regional lymph node metastasis

N1 Metastasis in ipsilateral peribronchial and / or ipsilateral hilar lymph nodes, and intrapulmonary node including direct extension

N2 Metastasis in ipsilateral mediastinal and/or subcarinal lymph node (s)

N3 Metastasis in contralateral mediastinal, contralateral hilar, ipsilateral or contralateral scalene or supraclavicular lymph node (s)

Distant metastases (M)

MX Presence of distant metastasis cannot be assessed

M0 No distant metastasis

M1 Distant metastasis

Stage grouping

Occult carcinoma	TX	N0	M0
0	Tis	N0	M0
I	T1	N0	M0
	T2	N0	M0
II	T1	N1	M0
	T2	N1	M0
IIIA	T1	N2	M0
	T2	N2	M0
	T3	N0	M0
	T3	N1	M0
	T3	N2	M0
IIIB	Any T	N3	M0
	T4	Any N	M0
IV	Any T	Any N	M1

6. Tumor markers currently used for diagnosis of cancer

Tumor markers are substances that can be detected in higher than normal amounts in the blood, urine or body tissues of some people with certain type of cancer. A tumor marker may be produced by the tumor itself, or by the body in response to a cancer presence. When diagnosing cancer, blood and pieces of tumor tissue are tested, these tests help to determine the characteristics of the tumor such as aggressiveness, rate of growth, and degree of abnormality. Tumor markers may be proteins, antigens, or hormones. Tumor markers tests are not used alone in diagnosis because most markers can be found in elevated levels in people who have benign conditions, and because no tumor marker is specific to a particular cancer. Not every tumor will cause an elevation in tumor marker test, especially in the early stages of cancer. Physicians can use changes in tumor marker levels to follow the course of the disease, to measure the effect of treatment, and to check for recurrence.

At the present time, several markers are used in the follow-up of cancer patients to detect a recurrence of metastasis. In the diagnosis of cancer, tumor markers have not been effective except in specific instances. These included CEA for colorectal cancer (Thompson *et al.*, 1969), Alpha-fetoprotein (AFP) for liver cancer (Bellet *et al.*, 1984).

6.1 Tumor markers for colorectal cancer

The tumor markers commonly used to monitor patient with colorectal cancer such as CEA. The CEA was one of the first oncofetal antigens to be described and exploited clinically. It is associated with the plasma membrane of tumor cells, from which it may be released into the blood. Although CEA was first identified in colorectal cancer, an abnormal CEA blood level is specific neither for colorectal cancer nor for malignancy in general (Fletcher, 1986). Elevated CEA levels are found in a variety of cancers other than colonic, including pancreatic, gastric, lung, and breast. It is also detected in benign conditions including cirrhosis, inflammatory bowel disease, chronic lung disease, and pancreatitis. The CEA was found to be elevated in up to 19 percent of smokers and in 3 percent of a healthy control population (Alexander *et al.*, 1976; Bates and Longo, 1987). The half-life of CEA in plasma appears to be about 6-8 days. This estimate is based on the observation that elevated CEA levels return to normal in about two months following successful removal of a CEA-producing primary tumor. Thus, the test for CEA cannot substitute for a pathological diagnosis. As a screening test, the CEA is also inadequate. Since cancer prevalence in a healthy population is low, an elevated CEA has an

unacceptably low positive predictive value, with excess false positives. Also, since elevated CEA occurs in the advanced stage of incurable cancer but is low in the early, curable disease, the likelihood of a positive result affecting a patient's survival is diminished.

6.2 Tumor markers for liver cancer

Alpha-fetoprotein is an α_1-globulin and is a product of the fetal liver, gastrointestinal tract, and yolk sac (Ruoslahti and Terry., 1976). The protein is normally present in the fetal circulation. There is good evidence to suggest that AFP functions as the fetal counterpart of adult albumin because there is considerable homology between the two proteins. Again, the protein gradually disappears from plasma during neonatal life to be replaced by albumin, but never entirely disappears in the adult. Marked increases in plasma AFP concentration are observed in about 80% of patients with hepatocellular carcinoma and about 60% of patients with nonseminomatous testicular tumors. Like CEA, AFP levels may be elevated in plasma in the presence of nonmalignant diseases, especially cirrhosis. AFP has a half-life in plasma of about 5.5 days. A slower rate of disappearance of the protein, or failure of serum levels to return to normal following surgery, are strongly suggestive of the presence of residual disease (Abelev., 1989).

6.3 Tumor markers for lung cancer

The markers used to monitor small cell cancer of the lung, the enzyme neuronspecific enolase (NSE) is produced by neuroendocrine tumors and plasma concentrations are raised above 12.5 μg/L in 50-60% of patients with SCLC confined to the lung and 86-90% of those with more advanced disease. At this cut-off, up to 45% of NSCLC may be positive but concentrations in SCCL tend to be higher. It may also be mildly elevated in patients with benign lung diseases. NSE is of some prognostic value in those patients with levels > 25 μg/L on diagnosis have a shorter survival than those with levels < 25 μg/L. Plasma NSE concentrations fall to < 8 μg/L with successful chemotherapy. Another enzyme, creatine kinase BB may also be elevated in SCCL but offers no advantage over NSE. It is elevated in less than 5% of patients with localized disease. Non-small cell carcinoma of the lung, CEA is elevated in about a third of cases and is of some prognostic value in that the higher the concentration, the greater the tumor burden and the worse the prognosis. An assay that is being increasingly is CYFRA 21-1 (Cytokeratin fragments) using antibodies 21 and 1. It detects cytokeratin 19 which is abundant in NSCCL. At a specificity of 95% against benign lung disease (a level of 3.3 μg/L) the overall

sensitivity for NSCCL is 51% (62% for squamous cell carcinoma). It is elevated in about 20% of SCCL. Other causes, or contributing factors to elevated concentrations, are renal failure and liver disease. In malignancy values correlate with tumor burden and contribute to staging of the disease. In monitoring, rises in CYFRA 21-1 have preceded clinical recurrence by 2-6 months. CYFRA 21-1 is a non-specific marker and elevated values have been noted in malignancy of the gastrointestinal tract, and of the genitourinary tract including the prostate.

7. Cancer metastasis

Cancer cells can spread along tissue planes and into various tissues spaces and cavities, but the two major routes of metastatic spread are via lymphatic vessels or blood vessels. Indeed, for the purpose of clinical staging, metastases are subdivided into two groups: those in regional lymph nodes, which are usually regarded as having disseminated via the lymphatic circulation, and those which arise at more distant sites and organs, which have usually spread via the blood vascular system. It used to be thought that these two routes were independent options and sarcomas were regarded as most likely to spread via the blood vascular system, while carcinoma spread initially via lymphatics to lymph nodes, where the cells could be arrested before disseminating more widely. However, as these two circulation systems are widely interconnected; therefore, they cannot be regarded as independent routes of spread (Glaves, 1983).

Different types of tumors have different patterns of spread. Tumors of the head and neck, for example, usually spread initially to regional lymph nodes and only when more advanced to distant sites; thus localized therapy that includes treatment of regional neck nodes can be effective. In contrast, tumors of the breast can spread early to distant sites. Involvement of axillary lymph nodes at the time of primary treatment is correlated with the presence of distant metastases, but about 25% of patients with no evidence of lymph node disease at the time of primary treatment are later found to have widespread metastases. Thus, the lack of lymph-node metastases does not rule out the possibility that the cancer has disseminated via the blood vascular system (Glaves, 1983).

8. Angiogenesis and angiogenic factors

Angiogenesis is a fundamental process by which new blood vessels are formed (Folkman and Hanahan, 1991). It occurs normally in the human body at specific times in development and growth. For example, a developing child in a mother's womb must create the vast network of arteries, veins, and capillaries that are found in the human body. A process called vasculogenesis creates the primary network of vascular endothelial cells that will become major blood vessels. Later on, angiogenesis remodels this network into the small new blood vessels or capillaries that complete the child's circulatory system. Proliferation of new blood vessels also takes place in adults, although it is a relatively infrequent event. In women, angiogenesis is active a few days each month as new blood vessels form in the lining of the uterus during the menstrual cycle. Also, angiogenesis is necessary for the repair or regeneration of tissues during wound healing.

However, many diseases are driven by permanent unregulated angiognesis. In arthritis, new capillary blood vessels invade the joint and destroy cartilage. In diabetes, new capillaries in the retina invade the vitreous, bleed, and cause blindness (Folkman and Klagsbrun, 1987). Angiogenesis is a complex process that is mediated by the endothelial cells that line blood vessels (Daniel and Abrahamson., 2000). Unlike quiescent endothelial cells that rarely divide, angiogenic endothelial cells undergo a complex sequence of events that includes the secretion of metalloproteases and other matrix-degrading enzyme, cell migration into the newly created space, endothelial cell division and proliferation, and vessel formation. These are well-regulated processes involving a number of stimulators such as fibroblast growth factor (FGF) (Nugent and Iozzo, 2000)

The healthy body controls angiogenesis through a series of "on" and "off" switches: The main "on" switches are known as angiogenesis-stimulating growth factors and the main "off" switches are known as angiogenesis inhibitors. When angiogenic growth factors are produced in excess of angiogenesis inhibitors, the balance is tipped in favor of blood vessel growth. When inhibitors are present in excess of stimulators, angiogenesis is stopped. The normal, healthy body maintains a perfect balance of angiogenesis modulators. In general, angiogenesis is "turned off" by the production of more inhibitors than stimulators. In many serious diseases states, the body loses control over angiogenesis. Angiogenesis-dependent diseases result when new blood vessels either grow excessively or insufficiently.

Angiogenesis process was involving extensive interplay between cells, soluble factors and extracellular matrix (ECM) components. The construction of a vascular network requires different sequential steps including: (i) the release of proteases from "activated" endothelial cells, (ii) degradation of the basement membrane surrounding the existing vessel, (iii) migration of the endothelial cells into the interstitial space, (iv) endothelial cell proliferation, (v) lumen formation, (vi) generation of new basement membrane with the recruitment of pericytes, (vii) fusion of the newly formed vessels, and (vii) initiation of blood flow.

The best angiogenic factors are VEGF and basic fibroblast growth factor (FGF-β). Platelet-derived growth factor (PDGF) also participates in vessel generation and maturation (Gerhardt and Betsholtz, 2003). Other contributors to angiogenesis, such as the angiopoietins Ang-1/Ang-2 and their receptor Tie-2 as well as ephrinB2 and its receptor EphB4, are attracting the interest of investigators, and other approaches focus on unique properties of endothelial cells in new vessels to reverse tumor neovascularization. The principal stimulus for VEGF production by tumor cells appears to be oxygen deprivation, although other stimuli (eg, transforming growth factor-β, epidermal growth factor, insulin-like growth factor, oncogenic mutations, hyperglycemia, mechanical stress, and inflammation) are recognized contributors (Acker and Plate, 2004).

When a growing tumor senses a falling oxygen concentration, the tumor cells stabilize and accumulate hypoxia-inducible factor I-α (HIF-Iα), which joins its constitutively produced β subunit to activate hypoxia response elements in DNA within the nucleus (Pugh and Ratcliffe, 2003; Semenza, 2003). This activates the transcription of numerous genes for proteins that regulate energy metabolism, erythropoiesis, cell growth, cell survival, vascular remodeling, and vasomotor responses. Among these is VEGF, which increases the permeability of blood vessels, allowing passage of fibrin into the extravascular space. VEGF and other growth factors stimulate endothelial cells in these nearby capillaries to grow, divide, and produce additional angiogenesis-related growth factor (such as PDGF-B). Secretion of proteolytic enzymes is induced in tumor endothelial cells and infiltration of myeloid cells, resulting in local degradation of the basement membrane of the precursor blood vessel and in weakening of intercellular interactions in the blood vessel wall. Basement membrane degradation is necessary for the generation of new blood vessels. Urokinase-type plasminogen activator (uPA)/plasmin and matrix metalloproteinases (MMPs) are the most important enzymes involved in the proteolysis

of the basement membrane (Heissig *et al.*, 2003). The cells involved in tumorigenesis secrete enzymes that activate latent forms of metalloproteinase and provide a high level of metalloproteinase activity. Against this background, local proteolytic is generated, resulting in partial proteolysis of the extracellular matrix and promoting the endothelial and tumor cell invasion of adjacent tissue. The proteolytic enzymes can also release additional growth factors, such as members of the VEGF and FGF families, from the pericellular matrix. The proliferating endotheliai cells migrate through the MMP-digested matrix toward the growth factor stimulus. Adhesion molecules, most importantly integirns, mediate the migration of the new endothelial cells toward the growth factor stimulus, and additional enzymes are released to dissolve the surrounding tissue. Integirns $\alpha_v\beta_3$ and $\alpha_v\beta_1$ present on the surface of activated endothelial cells are critical for the differentiation, maturation, and survival of angiogenic blood vessels (Eliceiri and Cheresh, 1999).

9. Vascular endothelial growth factor (VEGF): The most studied angiogenic factor

VEGF is angiogenic factor which was activate the vascular endothelial cell by tyrosine kinase receptor. All VEGF isoforms can bind either of the tyrosine kinases receptor, VEGFR-1 (or Flt-1) or VEGFR-2 (or KDR/Flk-1) (Meyer *et al.*, 1999). Neuropilin-1 binds the VEGF164 isoform and can potentiate VEGFR-2 activity, acting as a co-receptor (Soker *et al.*, 1998). The expression of VEGF is potentiated in response to hypoxia (Shweiki *et al.*, 1992), by activated oncogenes, and by a variety of cytokines (Pages *et al.*, 2000; Park *et al.*, 1993). VEGF induces endothelial cell proliferation, promotes cell migration, and inhibits apoptosis (D'Arcangelo *et al.*, 2000). *In vivo* VEGF induces angiogenesis as well as permeabilization of blood vessels (Roberts and Palade., 1995), and plays a central role in the regulation of vasculogenesis (Millauer *et al.*, 1993). VEGF contributes to the vascular remodelling that occurs during the ovarian cycle and embryonic implantation (Hazzard *et al.*, 1999; Shweiki *et al.*, 1993) and disorders like endometriosis (Lebovic *et al.*, 2000; Mahnke *et al.*, 2000). Deregulated VEGF expression contributes to the development of solid tumor by promoting tumor angiogenesis and to the etiology of several addition diseases that are characterized by abnormal angiogenesis like diabetes mellitus (Hovind *et al.*, 2000), rheumatoid arthritis (Cho *et al.*, 2000) and psoriasis (Bhushan *et al.*, 1999). Consequently, inhibition of VEGF signaling abrogates the development

of a wide variety of tumors (Kim *et al.*, 1993; Melnyk *et al.*, 1996; Millauer *et al.*, 1994). In the light of novel findings VEGF seems to act as oncogenic factor in endothelial cells when overexpressed (Arbiser *et al.*, 2000).

VEGF is widely expressed in normal adult human tissues, the highest levels were found in normal lung, kidney, heart, and adrenal gland by Northern analysis (Berse *et al.*, 1992). *In situ* hybridization the signals were strongest in the alveolar walls of the lung and in the renal glomeruli, in the outer cortex epithelium of the adrenal gland and cardiac myocytes. A novel approach, in which VEGF expression was tagged with LacZ, also provided evidence for expression of VEGF in the endothelial cells of the outflow tract of the heart and endocardium (Miquerol *et al.*, 1999).

VEGF is a mitogen for vascular endothelial cells derived from arteries, veins, and lymphatics, but it lacks significant mitogenic activity for other cell types (Ferrara and Davis-Smyth, 1997). VEGF is the most important factor for various angiogenic processes in normal and pathological. The important role of it about tumors growth, metastasis and invasion. It was produced by cancer cell.

The human VEGF gene is organized in eight exons, separated by seven introns. The coding region spans approximately 14 kb (Houck *et al.*, 1991; Tischer *et al.*, 1991). The human VEGF gene is localized in chromosome 6p21.3 (Vincenti *et al.*, 1996). Alternative exon splicing of a single VEGF gene results in the generation of at least six different molecular species, having respectively 121, 145, 165, 183, 189, and 206 amino acids, following signal sequence cleavage ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$, $VEGF_{206}$) (Fig. 2). $VEGF_{165}$ lacks the residues encoded by exon6, while $VEGF_{121}$ lacks the residues encoded by exons 6 and 7. Compared to $VEGF_{165}$, $VEGF_{121}$ lacks 44 amino acids, $VEGF_{189}$ has an insertion of 24 amino acids highly enriched in basic residues, and $VEGF_{206}$ has an additional insertion of 17 amino acids (Houck *et al.*, 1991).

$VEGF_{165}$ is the predominant molecular species produced by a variety of normal and transformed cells. Transcripts encoding $VEGF_{121}$ and $VEGF_{189}$ are detected in the majority of cells and tissues expressing the VEGF gene (Houck *et al.*, 1991). In contrast, $VEGF_{206}$ is a rare form (Houck *et al.*, 1991).

Native VEGF is a basic, heparin-binding, homodimeric glycoprotein of 45 kDa (Ferrara and Henzel, 1989). These properties correspond to those of $VEGF_{165}$, the major isoform (Hock *et al.*, 1992). $VEGF_{121}$ is a weakly acidic polypeptide that fails to bind to heparin (Hock *et al.*, 1992). $VEGF_{189}$ and $VEGF_{206}$ are more basic and bind to heparin with greater affinity than $VEGF_{165}$ (Hock *et al.*, 1992). $VEGF_{121}$ is a freely diffusible protein; $VEGF_{165}$ is also secreted although a significant fraction remains bound to the cell surface and the extracellular matrix. In contrast, $VEGF_{189}$ and $VEGF_{206}$ are almost completely sequestered in the extracellular matrix (Park *et al.*, 1993).

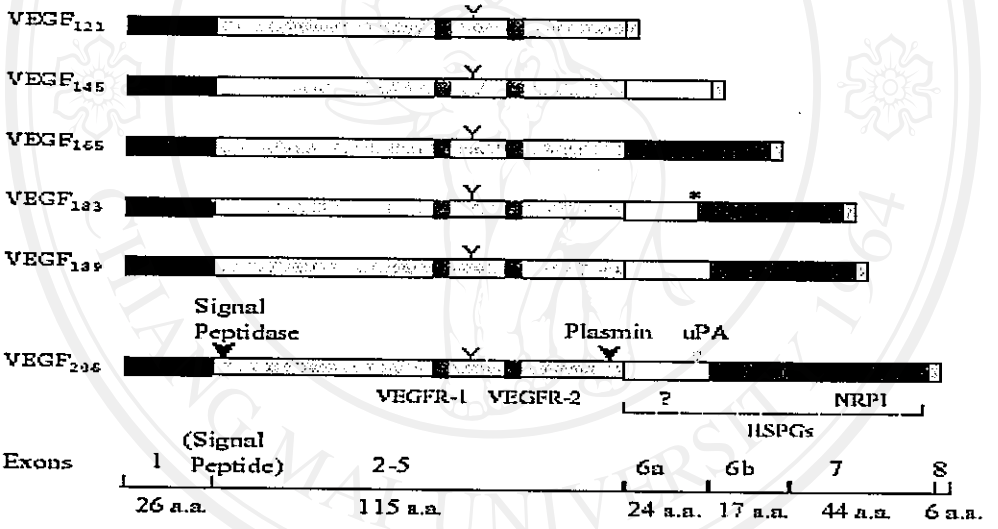

Figure 2. Schematic diagram showing important domains different alternatively spliced variants of human VEGF

10. The VEGF receptors

10.1 VEGFR-1

VEGF-1 receptor is expressed predominantly in endothelial cells but it is found in trophoblast cells, monocytes (Barleon *et al.*, 1996) and renal mesangial cells (Takahashi *et al.*, 1995). In addition, there are tumorigenic cell types that express VEGFR-1 (Cohen *et al.*, 1995). The receptor is probably activated by all VEGF ioforms but fulfills somewhat different functions *in vivo*, as targeted gene disruption experiments revealed (Shalaby *et al.*, 1995). VEGFR-1 can transduce signals of other growth factors belonging to the VEGF family, but only the VEGF isoforms can bind to VEGFR-1. The transcription of VEGFR-1 is enhanced by hypoxia (Gerber *et al.*, 1997). Alanine-scanning mutagenesis has revealed that Asp (63), Glu (64), and Glu (64) and Glu (67) are required for the binding of VEGF to VEGFR-1. VEGFR-1 does not induce cell proliferation in response to VEGF. MAP kinase is not activated by VEGF in cells expressing recombinant VEGFR-1 (Seetharam *et al.*, 1995). Therefore it is possible that VEGFR-1 dose not induce cell proliferation because it dose not activate MAP kinase. Finally, activation of VEGFR-1 results in the generation of proteases that are required for the breakdown of the basement membrane of blood vessels in the first steps of angiogenesis (Olofsson *et al.*, 1998; Unemori *et al.*, 1992).

10.2 VEGFR-2

VEGFR-2 is not only expressed predominantly in endothelial cells, but also in hematopoietic stem cells, megakaryocytes, and retinal progenitor cells (Katoh *et al.*, 1995). In the retina, two functional VEGFR-2 forms are expressed as a result alternative splicing (Wen *et al.*, 1998). Malignant melanoma cells usually express VEGFR-2. Only the final glycosylated form of VEGFR-2 is capable of undergoing autophosphorylation in response to VEGF (Takahashi and Shibuya, 1997). Transcription of VEGFR-2 is not enhanced by hypoxia. VEGFR-2 production is also up regulated under hypoxic conditions, but the mechanism responsible for the induction seems to be post-transcriptional (Waltenberger *et al.*, 1996). Activation of the VEGFR-2 receptor by VEGF in cells devoid of VEGFR-1 results in a mitogenic response (Kondo *et al.*, 1998). When VEGFR-2 is activated by VEGF, endothelial cells migration is obtained (Yoshida *et al.*, 1996).

10.3 NEUROPILIN-1

Endothelial cells were also found to contain VEGF receptors possessing a lower molecular weight than either VEGFR-2 or VEGFR-1 (Gitay-Goren *et al.*, 1992). It was subsequently found that these smaller VEGF receptors bind to $VEGF_{165}$ but, but not to $VEGF_{121}$. Therefore these receptors are not related to the VEGFR-1 or VEGFR-2 that bind both VEGF isoforms (Gitay-Goren *et al.*, 1996), but instead neuropilin-1, a receptor for several types of semaphorins that were initially characterized as repellents of nerve growth cones (He and Tessier-Lavigne, 1997; Soker *et al.*, 1998). Neuropilin-1 also functions as a receptor for the heparin binding form of PIGF, PIGF-2, but not for PIGF-1 (Migdal *et al.*, 1998). The neuropilins have a short intracellular domain and thus are unlikely to function as independent receptors. On the other hand, gene distruption studies indicate that mouse embryos lacking a functional neuropilin-1 gene die because their cardiovascular system fails to develop properly (Kitsukawa *et al.*, 1997).

Neuropilin-1 is considered a $VEGF_{165}$ co-receptor, because VEGFR-2 has been shown to bind $VEGF_{165}$ more efficiently in cells expressing neuropilin-1. In addition, neuropilin potentiates effect endothelial cell migratory response to $VEGF_{165}$.

10.4 VEGFR-3

VEGFR-3 is a highly glycosylated, relatively stable, cell surface associated tyrosine kinase of approximately 180 kDa. Its cDNA was cloned from human erythroleukemia cell and placental libraries (Aprelikova *et al.*, 1992; Galland *et al.*, 1993). On the basis of structural similarities VEGFR-1 and VEGFR-3 receptors constitute a subfamily of class III tyrosine kinases. Two isoforms of VEGFR-3, have been identified differing in their C-terminal ends.

In the early stages of development VEGFR-3 is widely expressed in the vascular endothelial cells (Kukk *et al.*, 1997). Disruption of the VEGFR-3 gene causes disorganization in the large vessels, resulting in defective lumina. As a result, the pericardial cavity is filled with fluid and the embryo dies of cardiovascular failure (Dumont *et al.*, 1998). Further studies with this knockout model have shown that the embryos suffer from severe anemia (Hamada *et al.*, 2000). After organogenesis, the VEGFR-3 becomes restricted to lymphatic endothelial cells (Kaipainen *et al.*, 1993) and a missense mutation in VEGFR-3 which creates an inactive tyrosine kinase causes primary human lymphedema (Irrthum *et al.*, 2000; Karkkainen and Petrova, 2000). In the neovasculature of tumors VEGFR-3 is upregulated, which limits the use of the

receptor in defining lymphatic vessels (Valtola *et al.*, 1999). Recently, it has been shown that inactivation of VEGFR-3 by blocking it with a monoclonal antibody suppresses tumor growth by inhibiting the neoangiogenesis of tumor-bearing tissues (Kubo et al., 2000). In the light of these findings, VEGFR-3 may be needed for maintaining the integrity of the endothelial lining during angiogenesis.

11. Other VEGF –related genes

There are many VEGF-related genes has been identified. VEGF is also classified as one of member of this gene family as VEGF-A. This group of genes are classified according to the differences in their roles and binding to receptor on different cell type. For example, VEGF-A is the most potent direct-acting angiogenic protein and specific to vascular endothelial cell. VEGF-B is important for angiogenesis in heart. VEGF-C expression is regulated by growth factors and inflammatory cytokines but not hypoxia.

11.1 VEGF-B

VEGF-B has two known isoforms of 167 and 186 amino acid residues, but it has only very low mitogenic potency (Olofsson *et al.*, 1996). The expression of VEGF-B is not regulated by hypoxia (Joukov *et al.*, 1997a). VEGF-B is a ligand for VEGFR-1 and neuropilin-1 (Makinen *et al.*, 1999; Olofsson *et al.*, 1999). Although originally cloned from human tumor cell libraries, it has been shown that VEGF-B is expressed in a variety of normal human tissues, primarily in the developing myocardium (Enholm *et al.*, 1997; Joukov *et al.*, 1997b). In human tumors, VEGF-B is commonly present in both benign and malignant tumors (Donnini *et al.*, 1999; Salven *et al.*, 1998), although in colon neoplasms VEGF-B mRNA levels have been found unchanged (Andre *et al.*, 2000).

11.2 VEGF-C

VEGF-C was found in the growth medium of PC-3 prostatic adenocarcinoma cells (Joukov *et al.*, 1996; Lee *et al.*, 1996). It is synthesized as a disulfide-liked prepropeptide dimmer of 61 kDa subunit size and by proteolytic maturation a homodimer of 21 kDa is formed. Partially processed and mature forms of VEGF-C bind VEGFR-3 with high affinity, while only the fully processed form binds VEGFR-2.

VEGF-C stimulates the migration and proliferation of endothelial cells *in vitro* and *in vivo* (Taipale *et al.*, 1999). Recently, it was shown to stimulate the vasculogenesis and suppress the hematopoiesis in a dose-dependent manner like VEGF-A (Hamada *et al.*, 2000). Compared to VEGF, VEGF-C is 4-5 times less potent in the vascular permeability assay (Joukov *et al.*, 1997b). VEGF-C mRNA levels are increased by serum and its component growth factors, platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) as well as transforming growth factor-β (TGF-β) and the tumor promoter phorbol myristate 12, 13-acetate (PMA) stimulation (Enholm *et al.*, 1997). Conversely, hypoxia, Ras oncoprotein and mutant p53 tumor suppressor do not have an influence on VEGF-C mRNA levels. IL-1 and TNF-α have been shown to stimulate VEGF-C expression in human lung fibroblasts and in human umbilical vein endothelial cells (HUVEC) (Ristimaki *et al.*, 1998). Further, the anti-inflammatory glucocorticoid dexamethasone inhibits IL-1-induced VEGF-C mRNA expression. It appears that VEGF-C could be a mediator in inflammatory reactions (Narko *et al.*, 1999). VEGF-C has a dual biological role being able to induce both angiogenesis and lymphangiogenesis (Oh *et al.*, 1997; Pepper *et al.*, 1998). The latter has been shown by overexpressing VEGF-C in the murine skin under K 14 promoter (Jeltsch *et al.*, 1997). Under ischemic conditions *in vivo* VEGF-C induces also angiogenesis in a rabbit hindlimb model (Witzenbichler *et al.*, 1998).

VEGF-C mRNA is weakly expressed in lymph nodes, heart, placenta, skeletal muscle, ovary, and small intestine tissues (Joukov *et al.*, 1996). Its mRNA levels do not correlate with the neoplastic progression of human colonic mucosa (Andre *et al.*, 2000). In contrast, some evidence has been presented for VEGF-C association with lymph node metastasis of colorectal carcinoma (Akagi *et al.*, 2000). In breast cancer, VEGF-C has been found in the cytoplasm of intraductal and invasive cancer cells and its receptor in blood vessels, suggesting an angiogenic role for VEGF (Valtola *et al.*, 1999).

11.3 VEGF-D

Human VEGF-D was isolated as a VEGF-related transcript (Yamada *et al.*, 1997) and the mouse homologue, so-called c-fos-induced growth factor (FIGF) was cloned independently (Orlandini *et al.*, 1996). VEGF-D is a ligand for VEGFR-2 and VEGFR-3 and a mitogen for endothelial cells (Achen *et al.*, 1998). VEGF-D is lymphangiogenic although less potent compared to VEGF-C (Carmeliet., 2000). Human VEGF-D mRNA is expressed prominently in

the lung, heart and small intestine. Two *in situ* analyses of both mouse fetal (E17) and young adult tissues displayed intense VEGF-D signals in the lung (Avantaggiato *et al.*, 1998; Farnebo *et al.*, 1999). On the basis of these studies VEGF-D seems to be down-regulated in the course of development.

11.4 VEGF-E

Orf virus is a linear double-stranded DNA virus that causes contagious pustular dermatitis which is characterized histologically by vascular and edematous lesions (Ogawa *et al.*, 1998). As the virus was only found in the keratinocytes and the vascular response in the dermis was VEGF-like, the observations led to the discovery of VEGF-E from the genome of Orf virus strain NZ-7. VEGF-E induces tissue-factor (TF) expression, the proliferation, migration and sprouting of cultured vascular endothelial cells and angiogenesis *in vivo* (Meyer *et al.*, 1999).

12. Regulation of VEGF gene expression

12.1 Oxygen tension

Oxygen tension is a key regulator of VEGF gene expression. VEGF mRNA expression is induced by exposure to low pO_2 (hypoxia) in a variety of normal and transformed cultured cell types (Minchenko *et al.*, 1994; Shima *et al.*, 1995). Hypoxia induces a rapid and strong increase in VEGF mRNA levels, which is particularly noticeable around necrotic areas of tumors (Shweiki *et al.*, 1992; Minchenko *et al*, 1994). A hypoxia response element (HRE) acts upstream of the *VEGF* gene as an enhancer (Levy *et al*, 1995; Liu *et al*, 1995). This HRE contains a consensus binding site for hypoxia-inducible factor-1 (HIF-1), a heterodimer of the bHLH(helix-loop-helix)-type transcription factors HIF-1α and ARNT (aryl hydrocarbon receptor nuclear translocator) (Madan and Curtin, 1993; Forsythe *et al*, 1996). Low oxygen tension increases HIF-1 levels at a post-transcriptional level and increases its DNA-binding ability (Jiang *et al*, 1996; Salceda *et al*, 1996; Semenza *et al*, 1997).

In addition, the von Hippel-Lindau tumor suppressor (VHL) negatively regulates hypoxia-induced genes, including *VEGF* (Siemester *et al*, 1996; Iliopoulos *et al*, 1996). VHL cytoplasmically sequesters PKCζ and PKCδ, preventing their translocation to the cell membrane, subsequent MAPK activation and induction of VEGF (Pal *et al*, 1997). Changes in

cell signalling through differentiation might also influence VEGF expression through control of PKC and cAMP/PKA pathways (Claffey *et al*, 1992; Garrido *et al*, 1993). The *VEGF* promoter contains potential binding sites for the transcription factors Sp1, AP-1 and AP-2, through which PKC and PKA can influence gene expression (Tischer *et al*, 1991).

Hypoxia increases the half-life of VEGF mRNA, which is intrinsically labile owing to the presence of three synergistic sequence elements within the 5′and 3′ untranslated regions (Dibbens, 1999). Binding of a hypoxia-induced stability factor increases the half-life of this mRNA three- to eight-fold (Levy *et al*, 1998). An alternative transcription-initiation site allows VEGF mRNA translation from a downstream ribosomal entry site. This might be advantageous under hypoxic stress, when cap-dependent translation can be inhibited (Stein *et al*, 1998; Akiri *et al*, 1998).

12.2 Hormones and cytokines

A number of cytokines, hormones, and growth factors are able up-regulate VEGF mRNA expression in various cell types. Epidermal growth factor, TGF-β, or keratinocyte growth factor result in a marked induction of VEGF mRNA expression (Frank *et al.*, 1995). Epidermal growth factor also stimulates VEGF release by cultured glioblastoma cells (Goldman *et al.*, 1993). In addition, treatment of quiescent cultures of epithelial and fibroblastic cell line with TGF-β resulted in induction of VEGF mRNA and release of VEGF protein in the medium (Pertovaara *et al.*, 1994). Both interleukin 1α and prostaglandin E_2 induce expression of VEGF in cultured synovial fibroblasts, suggesting the participation of such inductive mechanisms in inflammatory angiogenesis (Ben-Av *et al.*, 1995). Interleukin 6 has also been shown significantly to induce VEGF expression in several cell lines (Cohen *et al.*, 1996). Insulin-like growth factor 1 has also been shown to induce VEGF mRNA and protein in cultured colorectal carcinoma cells (Warren *et al.*, 1996). Thyroid-stimulating hormone also induces VEGF expression in several thyroid carcinoma cell lines (Soh *et al.*, 1996). Moreover, VEGF expression in the midgestation (16-20 weeks) human fetal adrenal cortex (Shifren *et al.*, 1998). ACTH was able to induce VEGF expression in cultured human fetal adrenal cells, suggesting that VEGF may be a local regulator of adrenal cortical angiogenesis and a mediator of the tropic action of ACTH (Shifren *et al.*, 1998).

13. Expression pattern of VEGF in cancer

It has been demonstrated that VEGF mRNA isoform is differently in cancer. While $VEGF_{121}$ appeared to predominate in melanoma (Potgens *et al.*, 1995; Redondo *et al.*, 2000), breast carcinoma (Zhang *et al.*, 1995), bladder cancer (Li *et al.*, 2001), and lung cancer tissues (Ohta *et al.*, 1997), $VEGF_{165}$ is the major splice variant present in glioblastoma (Berkman *et al.*, 1993). In addition, other report showed that the expression of $VEGF_{189}$ in non-small cell lung carcinoma (Cheung *et al.*, 1998; Oshika *et al.*, 1998; Yuan *et al.*, 2001) was correlated with tumor angiogenesis, shorter postoperative relapse time, and shorter survival of the patients. Other also found that the expression of $VEGF_{189}$ was correlated with poor prognosis in colon cancer (Tokunaga *et al.*, 1998a), and esophageal cancer (Tokunaga *et al.*, 1998b).

14. Diagnostic and prognostic value of circulating VEGF for malignant disease

Measurements of circulating free VEGF are useful for study of malignant diseases, which are associated with both genetically and hypoxia-induced overproduction of VEGF. The VEGF isoform specificity of the antibodies is also critical because both $VEGF_{121}$ and $VEGF_{165}$ are secreted. Apart from the investigation of tissues VEGF expression, yet the circulating level of VEGF in serum from cancer patients as well as from health normal controls has long been determined. The examination of patients with all types demonstrated an aberrant increase in the circulating level of VEGF (Yamamoto *et al.*, 1996). Primary breast cancer patients showed an aberrant increase in VEGF levels (Yamamoto *et al.*, 1996). This aberrant expression of VEGF in serum was significantly associated with the progression of the disease, and with VEGF protein expression in tumor tissues. In addition, a Western blot confirmed the presence of the VEGF165 form in serum from a patient with recurrent breast cancer (Yamamoto *et al.*, 1996). Other studies also demonstrated an increase level of circulating VEGF in patients with various types of tumor, which included brain (Takano *et al.*, 1996), renal (Takahashi *et al.*, 1994), melanoma (Claffey *et al.*, 1996), breast (Toi *et al.*, 1996), gastrointestinal (Chung *et al.*, 1996), and head and neck squamous cell carcinoma (Riedel *et al.*, 2003). From the above literature review, it can be concluded that: 1) Although increased expression of certain VEGF transcripts has been demonstrated to correlate with the progression of various tumors, the actual protein level of the different VEGF isoform and their significance during cellular transformation is

unknown. Moreover, it has been suggested that elevated protein expression in tumor tissues was mediated by both enhanced transcription and translation (Scott *et al.*, 1998). In order to understand the role of VEGF in tumor progression, thus it is important to investigate expression of different VEGF isoform at the protein level during tumorigenesis. To our knowledge, no studies focusing on the VEGF isoform pattern at protein level has been reported. 2) Although numerous publications dealing with the measurement of circulating VEGF for diagnostic and therapeutic monitoring have been published over the last 10 years, the relationship between the production of tissue VEGF and its concentration in blood is still insufficient.

Therefore, the aims of this study were to determine: 1) The protein expression pattern of VEGF isoforms in colorectal, lung and liver tumors in comparison to the corresponding adjacent normal tissues in order to understand whether which VEGF protein isoforms play an important role during tumorigenesis. 2) The relationship between the expression pattern of VEGF and level of total circulating VEGF in the blood. 3) Comparing level of circulating VEGF in patients and healthy volunteers.