

## CHAPTER I

### I. INTRODUCTION

Cancer is a group of more than 100 diseases. Although each kind differs from the others in many ways, every type of cancer is a disease of some of the body's cells. Healthy cells that make up the body's tissues, grow, divide, and replace themselves in an orderly way. This process keeps the body in good repair. Sometimes, however, some cells lose the ability to limit and direct their growth. They grow too rapidly and without any order. Too many cells are produced, and tumors are formed and uncontrolled growth. Benign tumors do not spread to other parts of the body and are seldom a threat to life. Benign tumors can often be removed by surgery, and they are not likely to return. In the other hand, malignant tumors (cancer) can invade and destroy nearby healthy tissues and organs. Cancerous cells can also spread, or metastasize, to other parts of the body and form new tumors. If the spread is not controlled, it can result in death. Cancer is caused by both external factors (tobacco, chemicals, radical, and infectious organisms) and internal factors (inherited mutations, hormones, immune conditions, and mutation that occur from metabolism). These causal factors may act together or in sequence to initiate or promote carcinogenesis. The major discovery in cancer biology has been that tumorigenesis is a multistep process associated with accumulated genetic alterations in somatic cells. These genes, termed oncogenes, encode for proteins that are qualitatively and/or quantitatively aberrant in tumor cells relative to normal cells. Therefore, detection

of these proteins, many of which are involved in the initiation of cancer, may allow for early detection of the malignant state. Many oncogenic proteins are immunogenic, that is why patients whose tumors express a particular oncogenic protein can have detectable antibody immunity directed against the protein. Over the last decade, antibody immunity directed against proteins involved in the malignant transformation has been described for almost every human tumor. Studies have progressed from observational assessments describing the ability to detect immunity to investigation designed to determine whether the measurement of tumor-specific antibody immunity might be helpful in diagnosis and management of human cancer. Cancer is potentially curable if diagnosed when localized. Unfortunately, many cancers are diagnosed at an advanced stage. Despite aggressive surgery and chemotherapy, the overall survival of advanced-stage cancer patients is poor. Early diagnosis is essential to make progress in the treatment and, ultimately, the survival of patients with cancer. Tumor markers currently used to help in screening or diagnosing of cancer are those circulating tumor proteins such as: (Lindblom *et al.*, 2000; Salgia *et al.*, 2001; Wu *et al.*, 1997)

**1. Carcinoembryonic Antigen (CEA)** is a protein found in many types of cells but associated with tumors and the developing fetus. It is a complex glycoprotein of molecular weight 20000 that is associated with the plasma membrane of tumor cells, from which it may be released into the blood. Although CEA was first identified in colon cancer, an abnormal CEA blood level is specific neither for colon cancer nor for malignancy in general. Elevated CEA levels are found in a variety of cancers including colonic, 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 % of smokers and in 3 %

of a healthy control population. Thus, the test for CEA cannot substitute for a pathological diagnosis. As a screening test, the CEA was 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. The CEA was often positive in malignancies other than colonic. In cancer of the breast, lung, pancreas, stomach, and ovary the CEA may be elevated and can be used to monitor the progress of disease or response to treatment.

**2. Alpha-Fetoprotein (AFP)** is a normal fetal serum protein synthesized by the liver, yolk sac, and gastrointestinal tract that shares sequence homology with albumin. It is a major component of fetal plasma. However, it clears rapidly from the circulation following birth. AFP is a marker for hepatocellular and germ cell (nonseminoma) carcinoma. AFP is elevated in normal pregnancy, benign liver disease (hepatitis, cirrhosis), as well as in cancer. AFP is also elevated in testicular germ cell tumors containing embryonal or endodermal sinus elements. A definitive positive marker value is highly sensitive in indicating relapse or response to treatment. The AFP is rarely elevated in healthy persons, and a rise is seen in only a few disease states. Elevation occurs in certain liver diseases, especially acute viral or drug induced hepatitis and conditions associated with hepatic regeneration. In general, the elevations are under 500 ng/ml and do not denote hepatocellular carcinoma. Is also elevated in ataxia-telangiectasia and in hereditary tyrosinosis. Thus, AFP is a useful marker in hepatocellular carcinoma and germ cell tumors, the only conditions associated with extreme elevations greater than 500 ng/ml. In both tumors it has value

in diagnosis and monitoring of therapy. In the former, which is one of the most common tumors worldwide, AFP may be of use in screening.

**3. Cancer antigen 125 (CA 125)** was produced by a number of cell types; CA 125 is primarily produced by ovarian cancer cells. Eighty percent of women with ovarian cancer have increased CA 125 levels. Although the test is not sensitive or specific enough to be used for screening, it contributes to a diagnosis when combined with an ultrasound and pelvic examination. Blood levels of CA 125 are used primarily to monitor the treatment of ovarian cancer. A falling CA 125 level usually indicates that cancer is responding to the treatment. After diagnosis and treatment, serial measurements help detect remaining or recurrent cancer. A negative or normal result, however, does not guarantee the absence of cancer. Women may have increased CA 125 levels during menstruation and pregnancy. Increased levels are also found in pelvic inflammatory disease, endometriosis, pancreatitis, and liver disease. Elevated levels are also associated with non-ovarian cancers including cancers of the uterus, cervix, pancreas, liver, colon, breast, lung or digestive tract.

**4. Cancer antigen 19-9 (CA 19-9)** has been identified in patients with digestive tract or intra-abdominal carcinomas such as colorectal cancer, pancreatic cancer, stomach cancer and bile duct cancer. In pancreatic cancer, higher levels are associated with more advanced disease. After diagnosis, levels help predict the success of surgery and monitor the course of the cancer. Not all people with pancreatic cancer have increased CA 19-9 levels. This antigen is related to the Lewis blood group and so only patients positive for the Lewis blood group antigen will test positive for CA 19-9. It is also increased in liver and gastrointestinal cancers and in noncancerous diseases, including pancreatitis, gallstones and jaundice.

**5. Prostate-Specific Antigen (PSA)** is the most commonly tested tumor marker for the prostate gland. It is normally present in low levels in the blood of all adult men. The normal range is 0 - 4 ng/ml. PSA is prostate-specific, not cancer-specific. A variety of conditions can raise PSA levels: prostatitis (prostate inflammation), benign prostatic hypertrophy (prostate enlargement), and prostate cancer. PSA levels can also be influenced by a number of other factors. Some prostate glands normally produce more PSA than others. PSA levels tend to increase with age and PSA levels can vary with race: African Americans often have higher PSA levels, while Asian men often have lower PSA levels. PSA seems to have the capability of achieving at least one of the characteristics of ideal tumor marker- tissue specificity; it is found in normal prostatic epithelium and secretions but not in other tissues. It is a glycoprotein, whose function may be to lyse the seminal clot. PSA is highly sensitive for the presence of prostate cancer. The elevation correlated with stage and tumor volume. It is predictive of recurrence and response to treatment. Finally, the antigen has prognostic value in patients with very high values prior to surgery are likely to relapse. Unfortunately, PSA is detectable in normal men and often is elevated in benign prostatic hypertrophy, which may limit its value as a screening tool for prostate cancer. A recent study has shown that PSA combined with rectal exam is a better method of detecting prostate cancer than rectal exam alone.

**6. Human Chorionic Gonadotropin (HCG)** is a glycoprotein consisting of subunits  $\alpha$  and  $\beta$ , which are noncovalently linked. The hormone is normally produced by the syncytiotrophoblastic cells of the placenta and is elevated in pregnancy. Its most important uses as a tumor marker are in gestational trophoblastic disease and germ cell tumors. All gestational trophoblastic tumors produce HCG, and it is a valuable marker in these tumors, screening reliably in all cases and indicating

poor responses to treatment. The level correlates with tumor mass and thus has prognostic value. HCG is extremely sensitive, being elevated in women with minute amounts of tumor. The patient is followed weekly during treatment, and at the completion of treatment indefinite follow up is advised to detect recurrence. HCG is essential in managing trophoblastic neoplasms. The level of HCG is occasionally elevated in other cancers including those of breast, lung, and gastrointestinal tract, but in these diseases it has found little clinical application.

**7. Neuron-specific enolase (NSE)** is a protein found mainly in neurons and neuroendocrine cells. It is elevated in tumors derived from these tissues, including neuroblastoma and small cell lung cancer. It can give information about the extent of the disease, the patient's prognosis and the patient's response to treatment. NSE can also be elevated in medullary thyroid cancers, carcinoid tumors, pancreatic endocrine tumors, and melanoma.

**8. Cytokeratin-19 fragments (CYFRA 21-1)** have been found in the presence urological, gastrointestinal, and gynecological cancers. It has also been found in various benign diseases. Although some study results are promising for the use of this marker in detecting squamous cell tumors, additional research is needed to determine if its specificity can be useful in the diagnosis and treatment of patients with lung cancer (NACB, 2006).

However, these tumor markers are proteins that shed from the surface of growing tumors and, in general, are associated with bulky or advanced disease. A serologic marker that is prevalent in early-stage disease would be a more optimal candidate to develop as a diagnostic tool. Antibody immunity to tumor-associated proteins may be a more appropriate serologic measure of cancer exposure. The assessment of an antibody immune response to a protein is quite different from the direct measurement of the protein

itself because; antibody responses can be generated against proteins that are expressed on the surface of cells and do not circulate, antibody responses can be detected even when small amounts of the immunogenic protein are present and antibody responses can indicate exposure to tumor-related protein. Antibody immunity has been used for decades to identify individuals exposed to infectious disease proteins. The immune system can respond to minimal amounts of protein by mounting an amplified antibody response that is readily detected in serum. Immunogenic tumor antigens, such as p53, are common to a number of different cancers and, therefore, provide a logical starting point for serological studies of cancer diagnosis. The presence of p53 autoantibodies seems to be associated with more progressive cancers and reduced disease-free survival of (surgically) treated patients. There is accumulating evidence that p53 autoantibodies may be indicative for a poor prognosis and a higher risk of tumor relapse. p53 autoantibodies have a useful potential in patient monitoring during therapeutically follow-up. A postoperative significant drop in p53 autoantibodies may be the result of complete tumor resection and successful (adjuvant) chemotherapy. The percentage of positive sera for cancer patients varied over a wide range, which might be due to different diagnostic accuracy of several tests. In addition, established immunological techniques are now available to improve the ability of immunologic diagnostics to discern patients with cancer from non-affected individuals. As the detection of immunity to oncogenic proteins becomes more routine, studies, such as those described in this thesis, have evolved to begin to address specific clinical questions such as the role of antibody immunity as a marker for patients exposed to cancer, as a tool to monitor therapy, or as an indicator of disease prognosis.

## II. LITERATURE REVIEW

### 1. p53 tumor suppressor : the guardian of the genome

For a cell, maintaining the integrity of its genome is of paramount importance. If it fails in this task and manages to divide anyway, both of its daughter cells may inherit an abnormal chromosome complement, with potentially dire consequences. In addition to subtle genetic mutations, most cancer cells show dramatic karyotypic changes, including gains and losses of chromosomes, gross chromosomal rearrangements, and amplification or deletion of genetic material, although scientists are still wrestling with the issue of whether this genome instability causes cancer or merely arises after a cell is already well along the path to malignancy. One of the most serious threats to genome stability is unrepaired DNA damage. For the reason the tumor suppressor protein p53 which is a transcription factor that can trigger DNA damage response has been described as “the guardian of the genome”. The p53 gene is localized on the short arm of chromosome 17 and it encodes a 393-amino acid phosphoprotein, which is presented at very low levels in normal cells. This molecule appears to play a major role in the maintenance of genomic integrity. Following DNA damage, p53 can either arrest the cells at the G1 phase of the cell cycle, thus providing time for the damage to be repaired (Lane *et al.*, 1993; Hollstein *et al.*, 1991; Levine *et al.*, 1991), or induce apoptosis (Caron de Fromental *et al.*, 1992). Both pathways prevent replication of damaged DNA and further accumulation of mutations. When DNA damage has occurred, p53 induces the p21 protein to bind with (and inactivate) cdk2, thereby preventing transition from G1 phase to S phase until DNA repair occurs. If effective repair is not possible, enhanced p53 protein will be causing apoptosis. To cause cell death, p53 induces transcription of a number of genes, including



those for APF-1 (Apoptosis Protease-activating Factor) and Bax protein. Bax (BAX) protein translocates to the mitochondria where it triggers cytochrome c release, causing when its level increases, the caspase cascade leading to apoptosis. The ability to trigger cell cycle arrest and apoptosis of p53, when its level increase, make p53 a very dangerous protein. Therefore, normal cell possess a regulator so called MDM2 to do down regulate p53 protein level under normal condition. MDM2 oncogene protein binds to p53 preventing its transactivation function and promoting p53 ubiquitination and proteosome degradation.

## **2. The p53 mutation and p53 accumulation are biomarker for cancer**

Mutations in the p53 gene represent a common genetic hallmark of human cancer and are associated with a loss of normal p53 function. Mutations of the p53 gene result in non-functional p53 protein occur. Cells containing biologically inactive p53 protein are devoid of such protective mechanisms and they are genetically unstable. Genetic mutation is the most common pathway for p53 inactivation. Being mutated in approximately 50% of all tumors, p53 is currently considered the most frequently altered gene in human tumorigenesis (Crawford *et al.*, 1982; Winter *et al.*, 1992). Since the discovery of this gene, more than 4000 abnormalities have been reported, the majority of them being missense point mutations that result in single amino acid substitutions. These mutations cluster between exon 5–9, which correspond to highly conserved domains of the protein (Schlichtholz *et al.*, 1994). Mutations in these domains prevent p53 from binding properly to DNA. Allowing cells to entry into S phase despite the presence of DNA damage results in the higher rate of mutation that is necessary for the progression stage of cancer. Loss of p53 function also increases the probability of cellular immortalization. Malignancies with p53 mutations, such as ErbB2 in breast cancer, are extremely resistant to chemotherapy

intended to induce apoptosis or cell cycle arrest of cancer cells. Conversely, malignancies that rarely contain p53 mutations (such as testicular teratomas) are very sensitive to chemotherapeutic treatment. Many p53 mutants have a different conformation and a longer lifetime compared to the wild-type protein. Increased lifetime causes mutant p53 protein accumulation in the tumor cells, which is detectable by conventional immunohistochemical or other immunologic methods. p53 mutations and their implications in human tumorigenesis attracted much attention over the past 10 years. Every aspect concerning the p53 gene and its protein product has been thoroughly studied, in efforts aiming to clarify the role of this molecule in malignant transformation. Studies in several types of cancer provide evidence that the p53 gene is a potentially useful biomarker for predicting prognosis and patient's response to treatment. Research shows that either mutation in the p53 gene or accumulation of the p53 protein can be used to predict the following kinds of information on prognosis and outcome of patient with various types of cancer:

- Risk stratification (i.e., classifying individual patients by their degree of risk for poor outcome) (Schlichtholz, *et al.*, 1994; Lubin, *et al.*, 1995).
- Resistance of individual patient to certain chemotherapy drugs (Lubin, *et al.*, 1995; Zalcman *et al.*, 1998).
- Probability of early relapse and shortened survival of patient with lung, breast, bladder, colorectal, esophageal, ovarian, and head and neck cancers (Rosenfeld *et al.*, 1997; Angelopoulou, *et al.*, 1996; Vogl *et al.*, 2000).

However, DNA sequence analysis to determine the status of the p53 gene, or immunohistochemical procedures to determine the status of the p53 protein in a cell, is labor intensive, time-consuming, costly, invasive and too complex for routine hospital use.

### 3. The presence of autoantibodies against p53 in serum

Until recently, the discovery that some patients with cancer develop an immune response against p53 did not attract much attention. Crawford *et al.* (Crawford *et al.*, 1983) reported for the first time the presence of autoantibodies against p53 in serum of patients with breast cancer. During the past few years, analysis of a large number of sera from patients with various malignancies revealed that the most immunogenic tumors are those of the lung (Angelopoulou *et al.*, 1994; Guinee *et al.*, 1995; Wild *et al.*, 1995; Green *et al.*, 1995; Angelopoulou *et al.*, 1996; Angelopoulou *et al.*, 1997), ovary (Guinee *et al.*, 1995; Wild *et al.*, 1995., Houbiers *et al.*, 1995; Davidoff *et al.*, 1992; Schlichtholz *et al.*, 1992), colon (Guinee *et al.*, 1995; Wild *et al.*, 1995; Peyrat *et al.*, 1995), breast (Angelopoulou *et al.*, 1993; Guinee *et al.*, 1995; Wild *et al.*, 1995; Mudenda *et al.*, 1994) and head and neck (Guinee *et al.*, 1995; Wild *et al.*, 1995; Bourhis *et al.*, 1996). The positivity rates for autoantibodies correlate with the frequency of p53 mutations in these tumors. Recently, it was suggested that p53 antibodies may represent a new and sensitive tool for early diagnosis. Furthermore, it has been shown that these serum antibodies represent indicators of unfavorable prognosis in patients with various cancers. Although much work has been done towards identifying the clinical value of p53 antibodies in serum, the molecular mechanisms that render this self protein immunogenic, leading to generation of autoantibodies in some patients still remain unclear.

### 4. p53 antibodies as a cancer diagnostic tool

Several recent studies highlight both the potential as well as the problems associated with using antibodies to p53 as a cancer diagnostic. Head and neck cancer is a good model for testing the utility of a biomarker, as premalignant precursor lesions are easily assessable and well characterized. Mutations in p53 have been reported in 53–93% of head and neck squamous cell carcinoma. In addition, it has been shown that p53

protein is over expressed in both primary and recurrent oral cancers as well as premalignant lesions (Ralhan *et al.*, 1998). Ralhan and colleagues (Ralhan *et al.*, 1998) evaluated sera from 183 patients with premalignant and malignant oral lesions and normal controls for circulating p53 antibodies. The results of the serum antibody assays were correlated with accumulation of p53 protein in patients' matched oral specimens. Circulating p53 antibodies were detected in 34% of cancer patients and 30% of patients with premalignant lesions. There was a significant association of the presence of p53 antibodies with increased tumor size and the anaplastic nature of the tumor, both factors indicative of a poor prognosis. The expression of p53 was analyzed in 43 matched tissue specimens, 18 premalignant and 25 oral cancers. All 18 patients with p53 antibody seropositivity, 7 premalignant and 11 cancer cases, showed p53 accumulation in their oral lesions. However, the total number of patients positive for p53 antibodies was less than that of patients with detectable p53 protein in their lesions. These data highlight one of the problems with the development of p53 antibodies as a single serologic test to diagnose malignancy: although the specificity of the approach can be high, the sensitivity is generally low. p53 antibodies were detectable only in patients with p53 protein over expression in their tumors, and presence of p53 antibodies correlated with tumor stage and grade and shortened overall survival and relapse-free survival. Patients with both colorectal and lung cancer have detectable p53 antibodies which correlate with a high rate of p53 mutation in these cancers. Alterations in the *p53* gene are found in about 60% of both colorectal and lung cancer and the prevalence of p53 antibodies in both cancers are in the range of 25–30% (Lubin *et al.*, 1995; Hammel *et al.*, 1997; Zalcmann *et al.*, 1998; van der Burg *et al.*, 2001) The use of p53 antibodies as a diagnostic test in lung cancer has shown limited success due to the low sensitivity (reviewed in Soussi, 2000). Rosenfeld and colleagues (Rosenfeld *et al.*, 1997) found p53 antibodies to be present in

only 27 (16%) of 170 patients with small cell lung cancer (SCLC). However, none of the 50 control sera was positive for p53 antibodies, and all patients were studied at the time of SCLC diagnosis, demonstrating that p53 antibodies can be detected relatively early in the course of the disease. Investigations in colon cancer have found 14 (26%) of 54 patients with colorectal cancer to have detectable serum p53 antibodies (Hammel *et al.*, 1997). There were no detectable p53 antibodies in the 24 control patients who had non-malignant digestive disease. In this study both CEA and CA 19.9, tumor markers used to follow the course of colon cancer, had a higher sensitivity (37% and 28%, respectively) for the diagnosis of colorectal cancer than p53 antibody measurements. Of note, there was a simultaneous increase in CEA, CA 19.9 and p53 antibodies in only 20% of patients (Hammel *et al.*, 1997). Furthermore, 30% of patients with normal CEA and CA 19.9 had significant p53 antibody concentrations, suggesting that CEA, CA 19.9 and p53 antibody testing may be complementary methods in identifying patients with colorectal cancer. Indeed, the use of antibodies to p53 to detect cancer may be of greater benefit as a member of a panel of biomarkers than as a “stand alone” test. The concept of panels of markers to diagnose malignancy, including the use of antibody response to p53, is being tested in hepatocellular carcinoma (HCC) where mutations in the *p53* tumor suppressor gene are present in up to 37% of cases (Raedle *et al.*, 1998). Alphafetoprotein (AFP) is the only established tumor marker with reasonable specificity for the detection of HCC. However, the sensitivity of this screening test is low at 60–69% (Edis *et al.*, 1998; Raedle *et al.*, 1998). Two recent studies have evaluated the use of p53 antibody testing in combination with AFP in detection of HCC. Raedle *et al.* (Raedle *et al.*, 1998) found p53 antibodies to be present in 22% of HCC-positive patients with corresponding elevated AFP levels in 69% of HCC patients. By combining the two measurements, serological HCC screening was improved to 76% sensitivity, however there was a decrease in

specificity from 96% to 88%. Edis *et al.* (Edis *et al.*, 1998) found p53 antibodies in 13% of HCC positive patients with corresponding elevated AFP levels in 33% of patients. In this study, adding the p53 antibody test to AFP screening of patients with HCC induced by viral hepatitis increased the sensitivity from 60% to 80%. In both these studies, p53 antibodies were found in patients with chronic liver disease but without detectable HCC, 4% and 21%, respectively. The high levels of p53 antibodies in these patients without evidence of cancer might be secondary to liver cirrhosis and an increased HCC risk, or possibly these patients may have had a clinically undetectable cancer. This observation raises the possibility that the presence of p53 antibodies may precede the clinical manifestation of cancer by several months.

#### **5. p53 Antibodies and population at high risk of cancer**

p53 accumulation is the major component in the appearance of these p53 autoantibodies. These antibodies are usually IgG indicating a secondary response after a prolonged immunization before the diagnosis of the disease (Lubin *et al.*, 1995). All of the studies described up until now used sera taken at the time of diagnosis before any treatment. Thus, it is reasonable to presume that such p53 antibodies could be used as an early indicator of p53 mutations in tumors in which such alterations occur early during tumoral progression. One good model for testing this hypothesis is that of lung cancer and heavy smokers. It is well established that p53 accumulation is an early event in lung cancer and that such cancer is strongly associated with tobacco smoking. Indeed, in 1994, p53 antibodies were found in two heavy smokers who were negative for any detectable lung cancer (Schlichtholz, *et al.*, 1994). One of the patients could not be followed but died 8 months later from a rapidly growing lung tumor. The second patient, PT37, was placed under surveillance, with regular assay for p53 antibodies and thoracic X-rays. Two years later, lung cancer was detected in this patient before any clinical manifestations of

the disease (Lubin *et al.*, 1995). The patient showed good response to therapy that paralleled the total disappearance of p53 antibodies. (Lubin, *et al.*, 1995; Zalcman *et al.*, 1998). Since 1996, this patient has been tumor-free, and a recent check-up indicated that neither the tumor nor p53 antibodies had reappeared. To our knowledge, this is the only prospective study that addressed the importance of p53 antibodies in individuals at high risk for cancer, and that used such assays for clinical management of the patient. Since that work, several studies have demonstrated that p53 antibodies can be found in the sera of high-risk individuals.

Angiosarcoma of the liver is an extremely rare cancer in humans; it is found in individuals, including workers in several types of industries, who have been exposed to several carcinogens such as vinyl chloride. p53 antibodies were detected in the sera of individuals several years before the diagnosis of the tumors (Trivers *et al.*, 1995). This work is of importance because it is known that p53 mutations are frequent in individuals exposed to various carcinogens, and such mutations usually occur early in the transforming process (Bennett *et al.*, 1993). Therefore, this assay could be useful for early identification of cancer in individuals occupationally exposed to carcinogens. Similarly, p53 antibodies have been detected in the sera of patients with chronic obstructive

There exist certain clinical situations in which nonmalignant lesions can predate their progression toward cancer. This is the case in Barrett's esophagus, the histopathological sequence for metaplasia which develops as a consequence of chronic reflux to dysplasia and then to carcinoma is well established for these tumors. In Barrett's esophagus, a variety of molecular changes have been characterized and correlated with tumor initiation and progression. Mutations and accumulation of p53 are found mainly in the transition from low- to high-grade dysplasia and are associated with an increased risk of cancer. The finding of p53 autoantibodies in patients with Barrett's esophagus may be

promising if confirmed in a larger population because it may predate clinical diagnosis of esophageal ADC (Cawley *et al.*, 1998). A similar situation occurs in individuals with premalignant oral lesions (leukoplakia) due to tobacco or betel nut chewing. Such individuals are at high risk of developing oral cancer (5–10%). p53 antibodies have been found at high frequency in patients with premalignant and malignant lesions, which suggests that such antibodies could be used for early detection of cancer (Ralhan *et al.*, 1998). Unfortunately, no follow-up has been performed on these patients. Due to the high frequency of this type of cancer in countries such as India or Pakistan, this kind of diagnosis could be of importance (Tavassoli *et al.*, 1998).

#### **6. p53 antibodies and follow-up of patients during therapy**

Because p53 accumulation is the main trigger of this humoral response, it was of interest to examine the behavior of this p53 antibody during therapy to see whether there was a relationship between tumor disappearance and a decrease in p53 antibodies. Several studies have addressed this question in various types of cancer (Angelopoulou *et al.*, 1994; Angelopoulou *et al.*, 1997; Hammel *et al.*, 1997; Zalcman *et al.*, 1998; Saffroy *et al.*, 1999; Polge *et al.*, 1998; Gadducci *et al.*, 1998; Shimada *et al.*, 1998). Such studies can only be performed using a quantitative assay, but this has not been taken into account in many reports. Using immunoprecipitation and two different ELISA formats, Zalcman *et al.* (Zalcman *et al.*, 1998) showed that there was a good correlation between the specific evolution of the p53 autoantibodies titer and the response to therapy in patients with lung cancer. A similar situation was described in colorectal (Hammel *et al.*, 1997) and ovarian cancer (Angelopoulou *et al.*, 1994).

Iizasa T. and colleagues (Iizasa *et al.*, 2005) analyzed the correlation among serum anti-p53 autoantibodies, immunohistochemical staining for p53, and clinical features (age, gender, smoking history, histological type, differentiation, stage, T factor,



tumor size, and N factor) in resected non-small-cell lung carcinomas. A total of 62 cases of resected NSCLC were studied (43 men and 19 women; 33 adenocarcinomas, 21 squamous cell carcinomas, 8 large-cell carcinomas). Preoperative serum titers of anti-p53 autoantibodies were detected in 13/62 cases (21.0%). A correlation between histological type and positive titers of serum p53 autoantibodies was seen (large-cell carcinoma versus squamous cell carcinoma and adenocarcinoma,  $P = 0.031$ ). Out of 25 cases, 10 (40%) with positive immunohistochemical staining for p53 had positive titers, whereas 3 positive titers were found in 37 patients with negative immunohistochemical staining for p53 ( $P = 0.0025$ ). Serum titers of anti-p53 autoantibodies were present in approximately 20% of the cases of NSCLC, and over expression of p53 protein in tumor cells was detectable in approximately 40%. Serum anti-p53 autoantibodies may be a clinical parameter for the presence of p53 mutations and p53 over expression in NSCLC patients.

In other studies, clinical data were not available. In several patients, the disappearance of p53 antibodies was very rapid, nearly as rapid as the half-life of human IgG. (Zalcman *et al.*, 1998; Lubin *et al.*, 1995) which lead to the suspecting that the reduction of p53 antibodies may due to the suppression of the immune system by radiation or chemotherapy during treatment. However, several arguments demonstrate the specificity of p53 antibodies variation during therapy as follows: (a) there is no variation in total serum immunoglobulins; (b) there is no variation in the amount of antibodies directed toward other antigens; and (c) a decrease of p53 antibodies can occur in patients who have been treated by surgery without any chemo- or radiotherapy. All of these observations indicate that constant stimulation of the immune system is necessary to maintain a high level of p53 autoantibodies. Removing the tumor would prevent such stimulation.

## 7. The reappearance of p53 antibodies and a relapse of tumor

The use of serum p53 antibodies as a prognostic tool in various cancers has had mixed results. In SCLC, the presence of p53 antibodies in patients with newly diagnosed disease was not associated with any clinical characteristics or prognostic markers (Rosenfeld *et al.*, 1997). In unvaried analysis, Angelopoulou, *et al.* (Angelopoulou, *et al.*, 1996) found p53 antibody-positive patients with ovarian cancer to be at increased risk for relapse but not death. In multivariate analysis, however, the difference in disease-free and overall survival between patients who were p53 antibody-positive or negative was not statistically significant. Vogl *et al.* (Vogl *et al.*, 2000) found the presence of p53 antibodies in patients with ovarian cancer to positively correlate with tumor stage and grade, as well as, decreased relapse-free and overall survival. These results were statistically significant suggesting that p53-antibody testing does have prognostic value in the clinical management of patients with ovarian cancer. The presence of p53 antibodies in patients with colon cancer has been shown to be an indicator of poor prognosis. Two of the three studies found an association between p53 antibodies and shortened survival (reviewed in Soussi, 2000). One investigation found the presence of p53 antibodies correlated with several prognostic factors including histological differentiation, grade, shape of tumor and tumor invasion into blood vessels (Houbiers *et al.*, 1995). Patients with p53 antibodies were shown to have both decreased disease-free and overall survival. In breast cancer, several studies have demonstrated the presence of p53 antibodies to be indicative of worse prognosis. (Lenner *et al.*, 1999; reviewed in Soussi, 2000). A recent population based epidemiological study showed the presence of p53 antibodies to be significant for both the risk of having breast cancer and decreased overall survival suggesting that p53 antibodies be regarded as a marker for more aggressive disease

(Lenner *et al.*, 1999). The association between p53 antibodies and shortened survival in oral cancer has been demonstrated in several studies (reviewed in Soussi, 2000). A recent study by Ralhan and colleagues (Ralhan *et al.*, 1998) in the evaluation of oral cancers, demonstrated an association of p53 antibody seropositivity and increase in tumor size and poor.

There was also significant decreased overall survival in the patients with p53 antibodies compared to the seronegative patients with oral cancer suggesting that detection of p53 antibodies may be a useful marker for identifying oral tumors having poor prognosis. These studies suggest an association between p53 antibodies and poor prognosis, specifically in tumors with poor differentiation. There is also a suggestion that p53 antibodies may be of value in determining both disease-free and overall survival, however, this has not been clearly established. One of the problems with interpretation of many of these retrospective analyses is that the patient populations evaluated are associated with diseases that, in general, have an overall poor prognosis. For example, in ovarian cancer, most women are diagnosed in advanced stages of disease. Although their tumors are initially responsive to standard treatment, the majority of patients will relapse within five years and eventually succumb to their cancer. Thus, to have the statistical power to truly stratify patients for prognosis based on the p53-antibody response, the absolute number of the total population must be quite large to include a significant number of patients who do not die of their disease. The same problem occurs in the evaluation of p53 as a prognostic marker in other malignancies that are associated with an overall poor prognosis such as SCLC and oral carcinomas. The use of p53 antibodies for monitoring cancer patients during their therapy has been studied in several types of cancer. Zalcman *et al.* (Zalcman *et al.*, 1998) monitored 32 patients with lung cancer, 16 individuals positive for p53 antibodies and 16 negative, over a period of 30 months. A

decrease greater than 50%, compared to the initial titer, in p53 antibodies was seen in 12 of 16 antibody-positive patients during chemotherapy that led to partial or complete remission of disease. The specificity of these p53 antibodies was confirmed by two different ELISA procedures and immunoprecipitation. Of the patients with a decrease in antibody titer, eight had a complete response to therapy and four obtained a partial response. Of the five patients without any variation in their antibody titer, two showed a partial response and no response was seen in the other three. No patient with a complete response had a stable level of p53 antibodies, whereas patients without response always maintained an unvarying level of p53 (Zalcman *et al.*, 1998). The correlation between the specific evolution of p53 antibody titer and response to treatment suggests that p53 antibodies could be a useful tool in monitoring response to therapy and relapse before it is clinically detectable. Other studies have found the ratio of p53 antibodies in patients with colon cancer to decrease within the first months after surgery, including those with Dukes' C cancer and hepatic metastasis who underwent palliative resection (Hammel *et al.*, 1997). In two patients the variations in p53 antibody ratio strongly correlated with tumor relapse or progression suggesting that monitoring of p53 antibodies may help in the early diagnosis and treatment of relapse in asymptomatic patients (Hammel *et al.*, 1997). In breast cancer, the reappearance of p53 antibodies can be detected two years after initial therapy. These increases in p53 antibodies have been detected several months before the detection of relapse (reviewed in Soussi, 2000). Thus, p53 antibodies appear to be a useful tool in monitoring the response to therapy as well as monitoring for early relapse before it becomes clinically evident in lung, colon and breast cancer.

## 8. p53 antibodies and short survival

In breast cancer, several studies indicate that p53 antibodies were found in patients with tumors that have high grades and/or that were negative for steroid hormone receptors (Crawford *et al.*, 1982; Angelopoulou *et al.*, 1994; Schlichtholz *et al.*, 1992; Peyrat *et al.*, 1995; Mudenda *et al.*, 1994), two clinical parameters already known to be associated with p53 mutations and bad prognosis. Two studies, on 353 and 165 patients, found an association between p53 antibodies and short survival (Peyrat *et al.*, 1995; Lenner *et al.*, 1999), whereas one study (82 patients) did not find any association (Willsher *et al.*, 1996) and another study (50 patients) found an association with good survival (Porzolt *et al.*, 1994). In gastric cancer, three of four studies found an association between p53-autoantibodies and poorly differentiated tumors and short survival (Maehara *et al.*, 1999; Wu *et al.*, 1999; Shiota *et al.*, 1998). In colon cancer, two of three studies also found an association between p53 autoantibodies and short survival (Kressner *et al.*, 1998; Houbiers *et al.*, 1995; Angelopoulou *et al.*, 1997). In lung cancer, as for p53 mutations, controversies exist concerning the clinical value of p53 antibodies. In NSCLC, p53 antibodies seem to be associated with poor survival, especially in squamous cell carcinoma (Angelopoulou *et al.*, 1997; Kressner *et al.*, 1998; Houbiers *et al.*, 1995), whereas in SCLC, the studies are very divergent (Angelopoulou *et al.*, 1997; Laudanski *et al.*, 1998). In oral cancer, two studies have also demonstrated an association between p53 antibodies and short survival (Lai *et al.*, 1998; Komiya *et al.*, 1997). Taken together, in all of these studies, there is a trend toward an association between p53 antibodies and tumors with poor differentiation, a feature already observed with p53 mutations. The value of p53 antibodies in terms of survival is promising.

## 9. p53 autoantibodies comparison with established tumor markers

Cioffi M. and colleague (Cioffi *et al.*, 2001) assayed serum p53 antibodies using ELISA method and all positive sera were confirmed by Western-blot method. Level of serum CEA, TPA, CYFRA21-1 and NSE were also determined using IRMA (Immunoradiometric Assay). Serum p53 antibody were detectable (p53 antibody positive) in 35/109 (32.1%) patients with lung cancer. About 17/57 (29.8%) patients affected with NSCLC and 18/52 (34.6%) with SCLC were p53 antibody positive. CEA, TPA, CYFRA21-1 and NSE sensitivity in lung cancer patients (NSCLC+SCLC) is 50.5%, 58.7%, 42.2%, 35.8%, respectively. The lower sensitivity (32.1%) of serum p53 antibody is connected with the higher specificity and diagnostic accuracy (100% and 69%, respectively). Out of 35 patient's p53 antibody positive, five (14.3%) exhibit only serum p53Ab, while serum values of the established tumor markers were lower than cut-off. The result from this study suggested that serum p53 antibody assessment is a simple and a low-cost assay with a good specificity and diagnostic accuracy that in lung cancer patients can be used at least in combination with the established tumor markers.

## 10. The current methods of p53 antibodies detection

The high specificity of p53 autoantibodies serological analysis is due to the truly rare of the antibodies in the normal population. It has been estimated that the specificity of this assay attain around 95%. However, one of the disadvantages of p53 autoantibodies detection is the lack of sensitivity. It has been reported that only 20–40% of patients with p53 mutations developed p53 autoantibodies. This may be partly explained by the observation that only p53 mutations that were localized in exon 5 and 6 with an altered protein conformation used to bind to HSP70 were associated with production p53 antibodies in cancer patients. However, this may also due to a lack of sensitivity of the

current methods used to detect autoantibodies. Jacques Rand colleagues (Jacques *et al.*, 1999) compared diagnostic accuracy of three commercial ELISAs for anti-p53 antibodies using ROC curve analysis. The anti-p53 antibody value in each serum sample was assessed by three different ELISAs: test A, a solid-phase ELISA using eukaryotically expressed wildtype p53 (PharmaCell; distributed by Coulter-Immuno-2014 Technical Briefstech); test B, a solid-phase ELISA using prokaryotically expressed wild-type p53 (Steinbeis Transfer Center; distributed by Dianova); and test C, a two-site sandwich ELISA using native p53 extracts from tumor cells (Orga-Med). The assay protocol was performed according to the manufacturer recommendation. In addition, the positivity of p53 antibody in patient serum assayed by ELISA was confirmed by Western blot analysis. It was found that the solid-phase ELISA using prokaryotically expressed wild-type p53 (Dianova) showed the highest diagnostic accuracy, with a significant difference from other tests. This study demonstrated that prokaryotically expressed wild type p53 was the best antigen for p53 antibodies detection. Therefore, production of wild type p53 recombinant protein in prokaryotic cells is a suitable approach. *Escherichia coli* is one of the most widely used hosts for the production of recombinant proteins and its genetics are far better characterized than those of any other microorganism. Recent progress in the fundamental understanding of transcription, translation, and protein folding in *E. coli*, together with the availability of improved genetic tools are making this bacterium more valuable than ever for the expression of complex eukaryotic proteins. Although, the reason why prokaryotically expression system which believed to have limited post-translational modification produced the best antigen to detect autoantibodies is unclear, it is possible that the miss fold confirmation of protein produced from bacteria is similar to the misfold p53 that activate immune response in cancer patients in the first place. *E. coli*-produced proteins are a common way to produce large amounts of antigen, do not

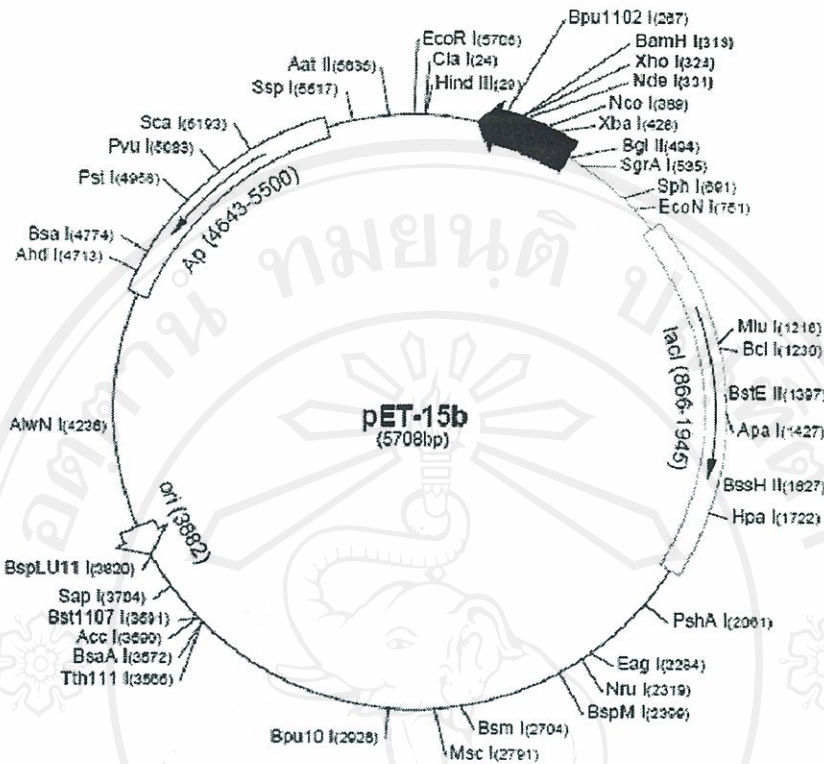
recapitulate the posttranslational modifications that are seen in eukaryotic cells (Savopoulos *et al.*, 2000; Webley *et al.*, 2000; Bukhtiyarova *et al.*, 2004). Several recombinant proteins which were used to be antigen in ELISA were tagged with another protein to facilitate detection and purification such as hexahistidine, GST and BCCP (biotin carboxyl carrier peptide).

### 11. Production of recombinant protein using pET system

pET system is the system developed for the cloning and expression of recombinant protein in *E. coli*. In this system, fusion sequences which are small in size and can be used for affinity purification was tagged with target protein to facilitate detection and purification. Histidine-tag, most commonly known as "His-tag", is the most used tag world wide for tagging recombinant proteins. (His)<sub>6</sub> generally refers to 6 histidine residues present in a protein (usually fusion protein). These hexahistidine allow the purification of "(His)<sub>6</sub>" recombinant protein to be purified using a Nickel column or Nickelresin etc. (the histidine binds the Nickel and then can be washed and eluted with specific buffers). (His)<sub>6</sub> is mostly used for facilitation of the purification of expressed recombinant proteins, but also for detection purposes. As an example, Atsuhito Y. and colleagues (Atsuhito *et al.*, 2001) produced (His)<sub>6</sub> survivin to establish ELISA for anti-survivin antibodies.

The pET-15b vector carries an N-terminal His•Tag sequence followed by a thrombin site and three cloning sites containing *NaeI*, *XhoI* and *BamHI* restriction sites, respectively. The vector carries an ampicillin resistance gene. The cloning/expression region of the coding strand transcribed by T7 RNA polymerase is shown below.





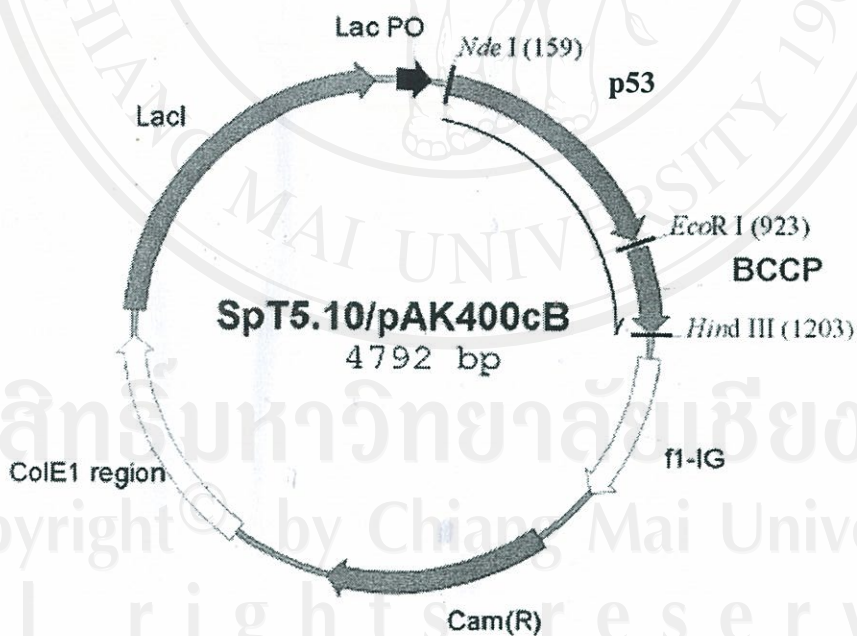
**Figure 1.** A schematic presentation illustrating the main features of the pET-15b expression vector.

## 12. Production of a biotinylated recombinant protein using pAK400 vector

The biotin-avidin/streptavidin system is used in numerous biotechnological and diagnostic application, primarily due to the high affinity of proteins avidin and streptavidin to a small biotin molecule. The biotin carboxyl carrier protein (BCCP) is a subunit of the acetyl-CoA carboxylase, and the biotinylated BCCP serves as a carrier of an activated carboxyl group in a metabolic carboxylate transfer process in the *E. coli* cell. This domain can be fused to recombinant proteins and render them to be biotinylated *in vivo* by the endogenous biotin ligase of *E. coli*. (Chapman-Smith *et al.*, 1994; Nenortas *et al.*, 1996). Schatz (Schatz *et al.*, 1993) generated a short artificial peptide substrate (13 amino acid) for the *E. coli* biotin ligase. This peptide has been used for the enzymatic biotinylation of various recombinant proteins both as an N and a C-terminal fusion (Tsao

*et al.*, 1996; Tucker *et al.*, 1996; Duffy *et al.*, 1998; Saviranta *et al.*, 1998; Smith *et al.*, 1998). Unlike the chemical biotinylation methods, the enzymatic biotinylation of recombinant proteins guarantees the site-specific attachment of only one biotin molecule per protein, a property which can be utilized, for example, for the immobilization of protein molecules in a defined orientation.

The pAK400 vector encodes the BCCP which is intracellularly produced and biotinylated. The gene encoding the BCCP-domain was cloned from the *E. coli* genome and joined to the C-terminus of the target gene. The expression is controlled by the lac promoter-operator (Lac PO) which is repressed by the plasmid-encoded lac repressor (LacI) in the absence of the inducer. The restriction sites used for the construction of plasmid are *NaeI*, *EcoRI* and *HindIII* restriction sites, respectively. The vector carries a chloramphenicol resistance gene.



**Figure 2.** A schematic presentation illustrating the main features of the pAK400 expression vector. ColE1 region, the region containing the plasmid origin of replication; f1-IG, the region containing a filamentous phage origin of replication; Cam(R), chloramphenicol resistance gene.

### III. OBJECTIVES

1. To generate expression DNA constructs harboring p53 encoding DNA using pAK400 vector and pET-15b vector in order to produce p53 recombinant protein.
2. To optimize culture conditions in order to obtain the best quality and quantity of (His)<sub>6</sub>-p53 fusion protein and p53-BCCP fusion protein.
3. To compare and characterize p53 recombinant proteins produced from these 2 expression systems (pET-15b and pAK400) as an antigen to detect p53 autoantibodies.
4. To optimize ELISA conditions in order to develop a p53 autoantibody detection kit.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่

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