CHAPTER I

Introduction

Cancer is the most dangerous disease which destroys not only the patient but also the hope and the feeling of all the involving people. Medical scientists have tried to find out the ways to remove and control this harmful disease for many decades. But little pleasure and success have been found. In Thailand, the rate of cancer death is a positive slope in every years, for example, in 1968, it was the seventh of the cause of death in Thailand. But in the next 15 years later, in 1983, it changed to be the third after the accident and heart disease (1). The World Health Organization(WHO) has designated 3 issues to prevent and get rid of this disease. They are 1) Primary prevention ; removing the cause and hence prevention the development of new cases. 2) Secondary prevention ; early detection on a symptomatic stage of the disease process when condition is reversible. 3) Tertiary prevention ; the treatment of the disease to prolong life or limit disability (2). Besides these, the follow up of the patients is also very important to insure the efficiency of all WHO issues (3).

It looks like that the first issue should be the best method to eradicate the disease. However, it is also the most difficult method (4,5). And the success of the

third issue is also dependent on the early diagnosis of the second issue which should be emphasized. It was estimated that the symptoms of cancer patient will appear when the malignant cells are increase to 10^9 cells (1 cm diameter), calculated as 30 doubling are required from a single malignant cell (A doubling is defined as the change of cell number from n cells to 2n cells). The estimation of the doubling time for most solid tumours are vary between 7 to 120 days. For example, the doubling time for breast cancer has been estimated to be about 90 days. Therefore, 2,700 days, more than 7 years, will begin symptoms. However, the most sensitive laboratory test can pick up approximately 10^4 to 10^6 cells growth which is faster than physical examination and x-rays (3) (figure 1).

The 5 leading of cancer incidences in Thailand are : cancers of the liver, lung, oral cavity, colon and rectum, and the stomach in male, and cancers of the cervix, breast, liver, oral cavity, and the ovary in female, respectively(6,7). The 10 leading form of cancer registered is shown in table 1. The best method for diagnosis and follow-up cancer patients should be simple, easy, safe, and finally, it should be useful for diagnosis the cancer as early as it can (3).

At the present time, the most promissing method for the diagnosis of neoplasm is the detection of tumour marker (3,8). The tumour markers are specific substances which are secreted into body fluid, and detected by suitable laboratory technique. The tumour marker may be any substances which release from tumours and should associate with tumour growth (3,9). Tumour markers were divided into four groups, hormones (10), enzymes (9,11,12), onco-faefal protein (9,13) and others, such as polyamine, neucleoside, etc (14,15,16).

The most interesting tumour markers are onco-faetal proteins, especially, the well known markers are alpha-1-faetoprotein(AFP) (8,17) and carcinoembryonic antigen(CEA) (18). The usefulness of AFP for diagnosis in hepatocellular carcinoma varies between 50 - 80%, as reported by many investigators dependings on the laboratory technique, cause of the disease and population (8,17,19). The second well known marker, serum CEA can be of value in the evaluation of patients when cancer is suspected or carcinoma is diagnosed. Monitoring CEA values before and after cancer therapy allows early detection of tumour recurrences or the presence of undetected metastasis. But, CEA is not suitable for screening. Neither proof of malignancy nor of specific type of malignancy is provided by the CEA values (18,20,21, 22,23,24,25). However, both popular markers were also elevated in other diseases, such as AFP also elevated in cirrhosis (19), severe combined immunodeficiency(26), and

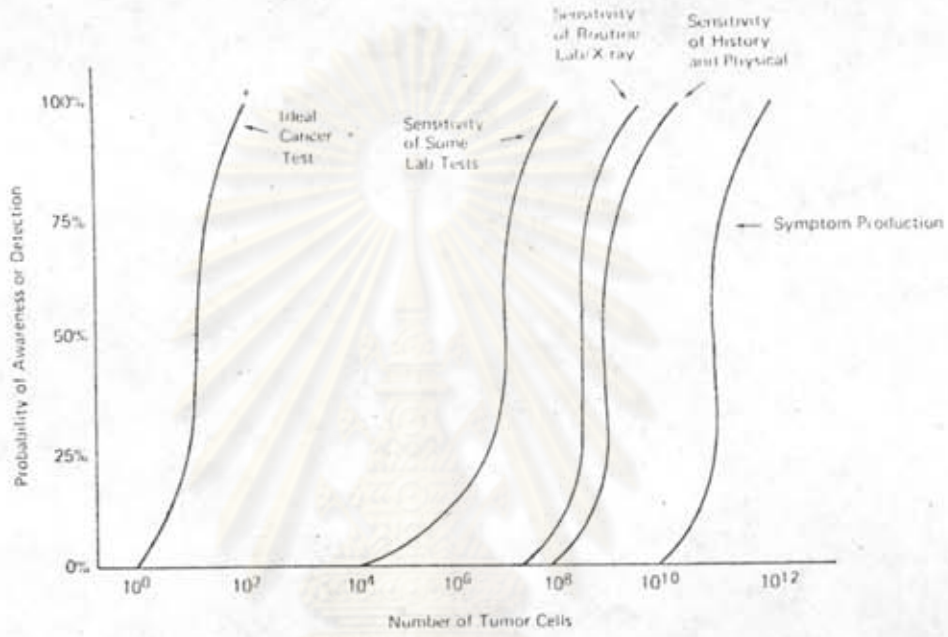

Figure 1(3) The relationships between tumour burden (or number of cancer cells) and the probability of establishing a diagnosis of cancer in various real and hypothetical studies.

Table 1(7) The 10 leading sites of cancer in male and female

site/in male	No. of cases	%
All sites	6,773	100.00
Liver	1,081	15.96
Lung	890	13.14
Oral Cavity	517	7.63
Colon and Rectum	448	6.61
Stomach	355	5.24
Skin	338	4.99
Esophagus	302	4.46
Larynx	279	4.12
Nasopharynx	269	3.97
Penis	248	3.66

site/in female	No. of cases	%
All sites	7,966	100.00
Cervix Uteri	2,317	29.09
Breast	912	11.45
Liver	509	6.39
Oral Cavity	504	6.33
Ovary	383	4.81
Colon and rectum	333	4.18
Lung	327	4.10
Thyroid	293	3.68
Skin	289	3.63
Uterus	254	3.19

some of other cancer patients (17). CEA was also raised in many other diseases and some normal individuals (20,21,22).

Also, other groups of oncofaetal proteins were found such as Bs - faetoprotein , Gamma faetoprotein , Faetal sulphoglycoprotein and alpha-2-faetoprotein(21). And one of the interesting oncofaetal protein is "Ferritin" which is classified as a member of alpha-2-H faetoprotein (27,28,29). It has been reported to be a tumour marker for some cancers such as leukemia (30,31), Hodgkins disease (32,33), pancreatic cancer (34), liver cancer (35,36), lung cancer (37,38), and others (39,40,41,42,43,44,45,46).

Liver and lung cancer are the first and second commonest ones of cancer in Thai males respectively (6). AFP may be useful for some types of liver cancer, but not all. In the mean time, tumour marker of lung cancer has not been found yet. The previous research of serum ferritin in both types of cancer are taken for the basis of this study.

Lung cancer

Lung cancer is the leading form of cancer in many countries, particularly in Western Europe and North America (47). In Thailand, lung cancer has been the second leading form of cancer in males for the last ten years. Its incidence is related to ones who have history of prolong contact with some chemical reagents, such as benzopyrene (1), asbestos, chromium, and others (1,47,48). It is also closely related to habit of continuous smoking for more than 20 years. In Thailand, 80% of lung cancer patients have smoked usually at least 1 pack per day for more than 20 years (1,48).

Virtually, all types of lung cancer are derived from epithelial tissue (49). Although, there are several histologic types, squamous cell(epidermoid) carcinoma is the most common form, predominately in males, and closely linked to smoking habits. The next most common one is adenocarcinoma which constitutes high percentage of cases in low risk countries, such as several Latin American and African countries, and females, and is less closely related to smoking. A disproportionate recent increase in adenocarcinoma has been noted but may be related to changing diagnostic practices (48). The third most common types is small cell carcinoma(47), which is more common in males and closely related to smoking, however, in Thailand small cell carcinoma is more common than adenocarcinoma

(50). Other cell types, such as large cell carcinoma, alveolar cell type and malignant carcinoid are relatively uncommon (2,32,51).

The steps in making a diagnosis of lung cancer in order are as follows :1) history 2) physical examination 3) chest x-ray study 4) cytologic sputum examination 5) bronchoscopy 6) biopsy of easily accessible lesion 7) mediastinoscopy and 8) thoracotomy (51,52,53). However, the common method used is composed of steps 1-6. There are three choices for lung cancer therapy, each method depends on the condition of patients and determination of physicians, they are surgical resection, radiation and chemotherapy, or the combination of methods.

Lung cancer is the most difficult one to treat when compared with other cancers. As shown in Figure 2, it demonstrates the incidence of lung cancer in USA, in 1981, in male. The incidence was 22% but their mortality rate raised to be 34 %. In which, the incidence is 9% in female but its death was 16 % (54). The data displays that the mortality rate was much higher when compared with the incidence. One of the reasons for this difference is always too late diagnosis for lung cancer depending on the detection method.

According to the "Cancer Statistics 1981"(7) of the National Cancer Institute (Thailand). The numbers of lung cancer registered are 890 cases (13.14% among all male cancer) and 327 cases (4.10% among all female cancer). Its

incidence can be calculated as the third leading form of cancer in both sexes (2.56 per 100,000 population). Besides these, this malignant cancer is also causing major problems for both possible protection and success of the treatment. The incidence may still be rising in Thailand since the country is being developed into the industrial country.

To cope with this dangerous cancer, the developement of diagnosis shoud be strongly emphasized.

Liver cancer

Liver cancer is one of the most frequent malignancy of males and a leading cause of dealth in parts of Africa and Asia (55), including Thailand(6). Three major etiologic factors are thought to be associated with the majority of cases of liver cancer are hepatitis B virus. alcohol, and aflatoxin; world wide, about 75 percent of cases occur in patients with cirrhosis (56).

The method for liver cancer diagnosis begins with history and physical examination. Diagnostic procedures using ultrasound are painless, harmless, and have no contra indications at the intensities used. Computed tomography is an important advances in diagnostic imagine. This method utilizes X-radiation to produce an display of the body in cross-section. The main advantage of the system is the ability to detect very small differences in attenuation of

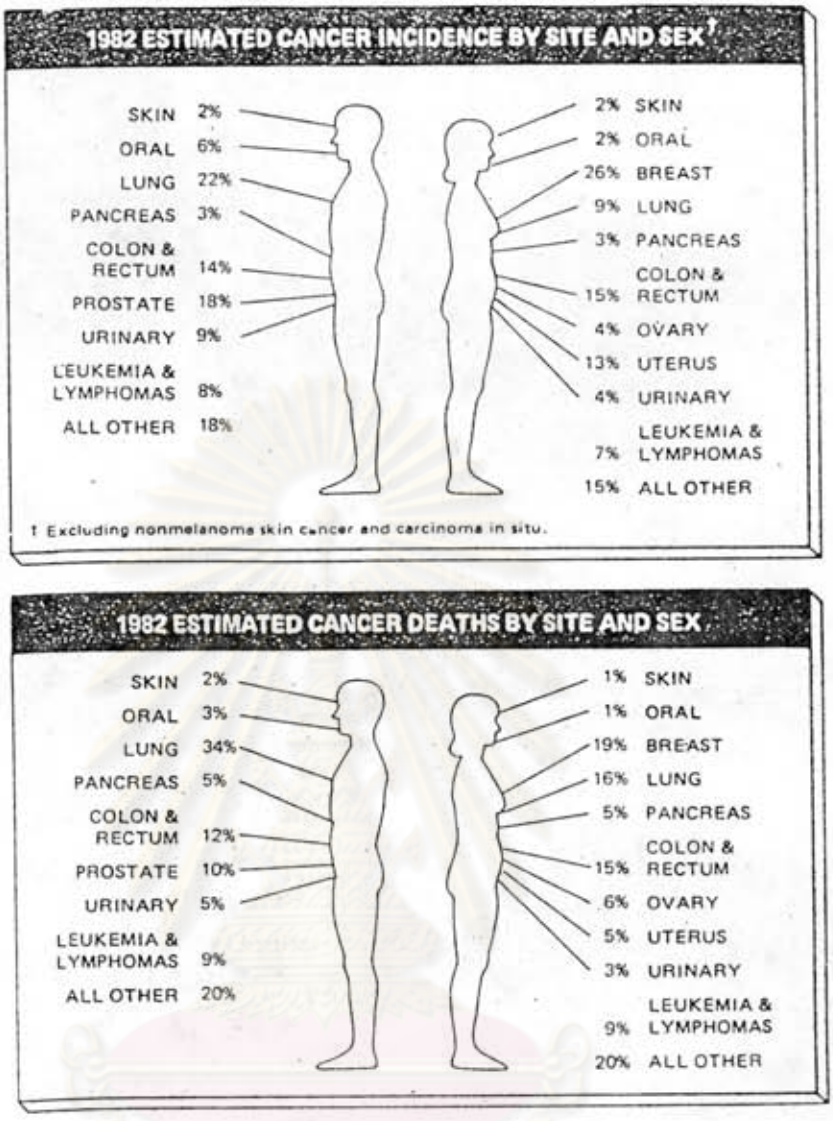

Figure 2(54) The estimation of incidence and mortarity of each cancer, by site and sex. the data from The National Cancer Institute's Surveillance, Epidemiology and End Results (SEER) Programs (Nonmelanoma skin cancer and carcinoma in situ have not been included in the statistics).

X-Radiation so that structures not visible on conventional radiographs are clearly identified (57). There are also other methods together such as liver scan, arteriogram and others. However, biopsy for pathological examination is still the confirmatory method (58).

AFP is the well known tumour marker of liver cancer. It can be used for early screening diagnosis (8,17), but it is not satisfactory to diagnose all types of liver cancer. So, other tumour markers for this cancer have been being searched. Kew et al(62) suggested that ferritin is possibly the second tumour marker for liver cancer.

Ferritin

Structure and Properties of Ferritin

Ferritin is an ubiquitous water soluble polypeptide protein. It is widely distributed in plasma and many organs of mammalian animals (59,60) and some plants (61). This polypeptide protein is composed of 24 subunits which is formed as a pentagonal trioctahedron (62) (Figure 3).

Ferritin is a large spherical protein shell with a hollow core(63). It is capable of binding up to 4,000 atoms of iron (0.023 to 0.067 pg Fe /pg of ferritin)(64). The apoferritin (precursor of ferritin) has a molecular weight of approximately between 430,000 to 480,000 daltons (63, 65,66).

Ferritin possesses two types of subunits which have been designated as H(Heart) and L(liver) subunit (40,67,68). These have been shown to differ in molecular weights (H; 21,000 daltons and L; 19,000 daltons)(69) amino acid composition (70), immunologic activities (68,71,72,73) and isoelectric points (71,73). The H-subunit is composed of 22,600 ± 200 amino acids and the L-subunit is composed of 20,300 ± 600 amino acids (70,74,75).

The isoelectric point of H-subunit is less than 5.20 while L-subunit's is more than 5.20 (71,73). Proportion of these two types of subunit bring to the different types of ferritin, each of these different ferritin is called "isoferritin" (68,72,73,76,77) (Figure 4 and Table 2) .

Each of ferritin differs in some properties such as the composition of subunit (Figure 5 and Table 3) and the antigenic property. The difference of antigenic property between basic ferritin (such as liver ferritin) and acid ferritin (such as HeLa cell ferritin) is demonstrated in Figure 6. It showed that each ferritin reacted more specific with its homologous antiserum according to the difference of their antigenic property (40,68).

Figure 3(62) Structure of ferritin (24 subunits of ferritin in a pentagonal trioctahedron model).

Figure 4(68) Different motives of each ferritin in IEF (isoelectric focusing technique, pI range 4-7) From H (Heart) and He (HeLa cell) ferritin which were more acidic ferritins, with L (liver) and Lc (crystallized liver) ferritin which were more basic ferritin.

Table 2(72) The pI values of normal human serum and tissue ferritins as different source (*denotes major isoferritin peak).

Serum	Liver	Spleen	Heart	Kidney	Pancrease
	6.14	6.14			
			6.13		
			6.00		
	5.95	5.95			
	5.85				
		5.80			
5.62*					5.55
5.56*	5.56				
	5.54*	5.54			
5.45	5.45	5.45			
		5.36*		5.36	
5.35*	5.35*		5.35*		
					5.34*
		5.30*			5.30*
	5.29		5.29*		
5.28					
				5.25*	5.25*
			5.23*		
				5.22*	
					5.19
			5.18*		
5.16					
				5.12*	
5.04			5.06		

Figure 5(40) Different motives of subunit composition of each ferritin (L = liver, T = tumour of lung metastasis liver cancer, H = heart, He = HeLa cell). Each of ferritin was dissociated the multimeric shell with 67% acetic acid. After run with acid-urea gel electrophoresis, the gel was stained with coomasie brilliant blue, after washed. The relative amount of the separated subunits were estimated from gel scan at 550 nm. (Bars on the left are motives of each ferritin before dissociated to be subunit. The right ones are after dissociated.).

Table 3(40) The subunit composition of each isoferritin from liver, heart, HeLa cell and tumour of lung metastasis liver cancer after estimation from gel scan, as shown in Figure 5.

	H-subunit (%)	HL-subunit(%)
liver	10	90
tumour	15	85
heart	40	60
HeLa cell	70	30

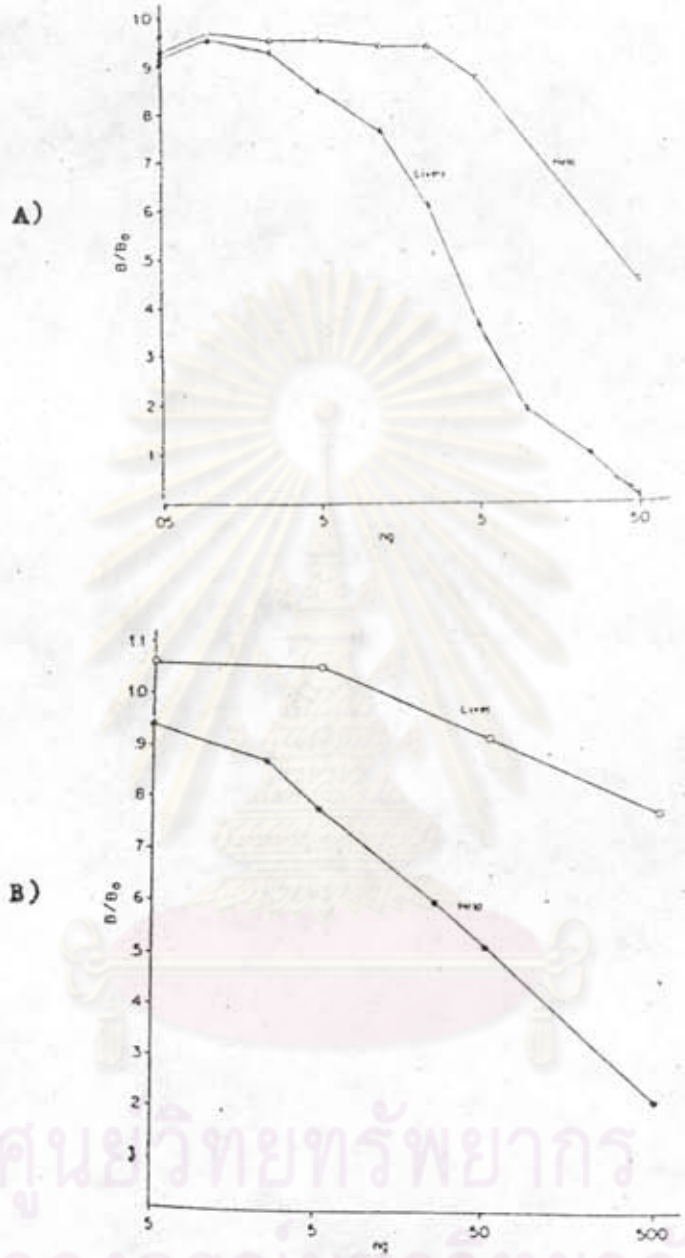

Figure 6(68) Difference in immunologic activity of different ferritins by different antiferritins, the RIA technique was used. (A) anti-liver ferritin (basic ferritin) was used and (B) anti-HeLa cell ferritin (acid ferritin) was used.

Isoferritins have been divided into two groups. One is more basic isoferritin (pI range 5.4 - 5.7), such as isoferritin from liver and spleen, the other is more acid isoferritin (pI range 4.8 - 5.3), such as isoferritin from heart, kidney and Hela cell (68,72).

However, each of isoferritin has almost the same physiologic and chemical properties. One importance of this protein property is heat stable at 80°C. This property make it relatively easy to be extracted from tissue and other proteins (62,78).

Synthesis and Function of Ferritin

Ferritin appears to be primarily synthesized on free polysomes within the cell as would be expected for its role as a storage protein (62,79,80). However, there are some evidences that some synthesis may also occur in membrane bound polysomes which are known to be active in the biosynthesis of secretory protein (62,79,80,81). But it is uncertain as yet whether the plasma ferritin is synthesized on membrane bound polysomes and actively secreted or whether it is merely a product of cellular damage. However there are some evidences to expect that the plasma ferritin is a product of membrane bound polysomes, such as, the low iron content of serum ferritin, even in iron overload states(82), and the presence of carbohydrate in the serum ferritin molecule (83).

The process of iron transfer from transferrin to ferritin appears to involve 3 steps procedure. Those are 1) reduction, ascorbic acid is the best reducing agent 2) chelation, one of the important chelating agent is adenosine triphosphate (ATP), not the source of energy, and 3) oxidation, ferrous iron is assumed to penetrate into the apoferritin shell where it is oxidized to ferric hydroxide (62).

Apoferritin + Fe^{++} --------> ferritin

Previous Study of Ferritin and Malignancies

As stated earlier, ferritin synthesis is increased in several malignant states. Elevating circulating ferritin levels have been often found in the serum or tissue of many kinds of cancers, including lung cancer and liver cancer (30,32,34,36,37,39,40,41,45,46).

Veltri et al, 1977, had isolated 3 tumour associated antigen (TAA) from human squamous cell lung carcinoma. They were identified as ferritin, alpha-1-globulin, and lactoferrin. They showed that ferritin cross-reacts with monospecific antisera to human liver ferritin (38). The other investigators, Groupp et al, 1978 used anti-placental ferritin to study serum ferritin level by the Laurell

electrophoresis technique. They reported that 58 out of 81 in all stages of lung cancer showed high serum ferritin level. However, in non-metastatis lung cancer, ferritin was detected only 14 out of 37 (37.9 %) patients(43). Other investigators had also studied serum ferritin level using other antiferritins in lung and other cancers. These studies have shown that some cancer patients, including lung cancer, have high serum ferritin level(43,44,45,46).

Serum ferritin level was raised in 34 out of 35 (97%) with hepatocellular carcinoma, reported by Melia et al (36). But, serum ferritin level was also raised in 20 out of 23 (87 %) with uncomplicated cirrhosis.

However, the serum ferritin level in hepatocellular carcinoma was negatively correlate with alpha-1-Faetoprotein, and may be used as a second marker of primary hepatocellular carcinoma in the negative alpha-1-faetoprotein liver cancer patient (35).

Serum ferritin in other diseases

There were some reports about high serum ferritin level in other diseases, such as, haemochromatosis,(84,85) Thallasemia, myocardial infarction (78,86,87,88,89) and slightly increased in inflammation (89). The low ferritin level was also reported in iron deficiency anemia. Serum ferritin level was not changed in some abnormal haemoglobin patients, such as haemoglobin H-disease (78).

Normal serum ferritin level

Many investigators have examined the serum ferritin level in the people who have no evidence of serum ferritin involving diseases. Their data are closely in the same range and mean. However, serum levels of ferritin in male and female are different, mean level of ferritin in male was higher than female (84,90,91,92). In Thailand, Suwanik et al, 1979, have studied serum ferritin level of normal population in Bangkok. The study showed that in 45 males, mean is 81 ng/ml (range 21-314 ng/ml) and in 200 females, mean is 45 ng/ml (range 13-173 ng/ml) (93). Serum ferritin level is not different by age in normal adult, but in female after menopause, the serum ferritin level is slightly raised (78).

It has been demonstrated that serum ferritin contains carbohydrate. The presence of this carbohydrate appears to influence the clearance of injected serum ferritin as compared with tissue ferritin which do not contain carbohydrate. The half life of tissue ferritin is very short (T 1/2 = 1-14 minutes) while the half life of serum ferritin is longer by several hours (78,83).

Direction for study

This work aims to study serum ferritin level of cancer patients, especially in lung cancer, by using anti-liver ferritin. The study follows the work of Veltri (1977) and Jones (1980). Veltri et al (38) identified ferritin in tissue of squamous lung cancer which was cross reacted with liver ferritin. Jones et al(44) isolated ferritin from lung cancer tissue and found that the amount of spleen ferritin is greater than HeLa cell ferritin. In the same study, serum ferritin was tested in 15 lung cancer patients using anti-HeLa cell ferritin by competitive RIA technique, only 3 cases whose serum ferritin was 2-3 ng/ml. The other 12 cases, serum ferritin was found even less than 2 ng/ml. On the other hand, these 15 patients had the value of serum ferritin level as 25-1190 ng/ml when tested by anti-spleen ferritin.

From Jones's study (44), it might conclude that high amount of basic ferritin (spleen ferritin) were found in lung cancer tissue and appreciable level was also present in serum. However, the immunological reaction of spleen ferritin and liver ferritin was very similar (68,72). Other investigators using anti - liver ferritin in the investigation of serum ferritin level had included lung cancer for their study. Therefore, specificity of anti-liver ferritin was also tested in this study.

Apart from lung cancer, in other cancers including liver cancer which is highly common in Thai male, serum ferritin level were also tested. In order to find a possibility of serum ferritin as a tumour marker for other kinds of cancers, anti-liver ferritin will be used for the test.

The ELISA method was chosen for the study due to the sensitivity in the level of nanogram per millilitre; which the normal level of serum ferritin was approximately 0-350 ng/ml, as previously reported (91). However, this technique requires expensive instruments and the technique was rather complicate for small laboratories when compared to other methods such as Counter Immunoclectrophoresis(CIEP) and Radial Immunodiffusion (RID) methods.

The CIEP was qualitatively known to be less sensitive than the ELISA, but it may be sensitive enough to detect very high level of serum ferritin in cancer patients. Quantitatively, serum ferritin level of cancer patients may be measured by the RID method but very low level of serum ferritin in normal individuals may not be detected. This study both method will be also used to compare with the expected ELISA technique.

Purposes of this study

1. To modify the ELISA technique for testing serum ferritin level from the original Anderson and Kelly's technique. The precision and accuracy of the test were also evaluated.

2. To study the possibility for test serum ferritin level by Radial Immunodiffusion (RID) in comparison with Enzyme Linked Immunoadsorbent Assay (ELISA). The sensitivity of Counter Immunoelectrophoresis (CIEP) method is evaluated for the detection of serum ferritin in cancer patients.

3. To study the possibility of ferritin as a tumour marker for diagnosis of cancers , especially in lung cancers.