PLASMA TISSUE FACTOR LEVEL IN HEPATOCELLULAR CARCINOMA AND CHOLANGIOCARCINOMA PATIENTS

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PLASMA TISSUE FACTOR LEVEL IN HEPATOCELLULAR CARCINOMA AND CHOLANGIOCARCINOMA PATIENTS

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ABSTRACT

Tissue factor (TF) is a primary initiator of the extrinsic pathway of normal blood coagulation. Several studies have previously demonstrated TF expression in malignant tumours. Recently, many investigations demonstrated TF might be involved in tumour angiogenesis and metastasis. However, little is known about the distribution of TF in hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA).

In this study, we examined the significance of plasma TF level in patients with HCC and CCA compared with healthy controls and analysed the correlation among plasma TF level and the clinicopathological features. Blood samples obtained from 57 HCC patients, 31 CCA patients and from 25 healthy individuals who were measured for plasma TF level by enzyme linked immunosorbent assay (ELISA).

HCC and CCA patients showed significantly higher plasma TF level than healthy individuals ($p < 0.05$), and HCC patients showed significantly higher plasma TF level than CCA patients ($p < 0.05$). Moreover, plasma TF levels in HCC was significantly correlated with cirrhosis and patients' age while in CCA, it was significantly correlated with hematocrit (Hct) and prothrombin time (PT) ($p < 0.05$). However, plasma TF level in both HCC and CCA was not significantly associated with other clinical parameters including sex, tumour size, clinical stage, tumour grading, vascular invasion, perineural invasion, lymphatic invasion and the presentation of HBsAg.

This study demonstrated an up-regulation of plasma TF level in HCC and CCA patients compared with healthy individuals. However, no association between plasma TF level and invasiveness was found. The potential clinical relevance of this finding should be further elucidated.

KEY WORDS : TISSUE FACTOR / HEPATOCELLULAR CARCINOMA / CHOLANGIOCARCINOMA / ELISA /

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ระดับทิชชูแฟคเตอร์ในพลาสม่าของผู้ป่วยมะเร็งตับและมะเร็งท่อน้ำดีในตับ
(PLASMA TISSUE FACTOR LEVEL IN HEPATOCELLULAR CARCINOMA AND CHOLANGIOCARCINOMA PATIENTS)

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บทคัดย่อ

ทิชชูแฟคเตอร์เป็นปัจจัยกระตุ้นเริ่มแรกของ extrinsic pathway ของการแข็งเป็นลิ่มของเลือด มีการศึกษาพบการแสดงออกของทิชชูแฟคเตอร์ในมะเร็ง และพบว่าทิชชูแฟคเตอร์อาจเกี่ยวข้องกับกระบวนการสร้างหลอดเลือดใหม่ของเซลล์มะเร็ง (angiogenesis) และการแพร่กระจายของเซลล์มะเร็ง (metastsis) อย่างไรก็ตาม ยังมีรายงานเกี่ยวกับการกระจายของทิชชูแฟคเตอร์ในมะเร็งตับและมะเร็งท่อน้ำดีในตับไม่มากนัก

การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาระดับของทิชชูแฟคเตอร์ในพลาสม่าของผู้ป่วยมะเร็งตับและผู้ป่วยมะเร็งท่อน้ำดีในตับเปรียบเทียบกับคนปกติ และศึกษาความสัมพันธ์ระหว่างระดับของทิชชูแฟคเตอร์ในพลาสม่าของผู้ป่วยกับปัจจัยทางด้านพยาธิกำเนิดของโรค จากการศึกษาระดับของทิชชูแฟคเตอร์ในพลาสม่าของ 3 กลุ่มตัวอย่าง คือ กลุ่มผู้ป่วยมะเร็งตับจำนวน 57 คน, กลุ่มผู้ป่วยมะเร็งท่อน้ำดีตับจำนวน 31 คน และกลุ่มอาสาสมัครที่มีสุขภาพดีจำนวน 25 คน ด้วยวิธี enzyme linked immunosorbent assay (ELISA) พบว่าระดับทิชชูแฟคเตอร์ในพลาสม่าของทั้งผู้ป่วยมะเร็งตับและผู้ป่วยมะเร็งท่อน้ำดีในตับสูงกว่ากลุ่มของคนปกติ และระดับทิชชูแฟคเตอร์ในพลาสม่าของผู้ป่วยมะเร็งตับยังสูงกว่าผู้ป่วยมะเร็งท่อน้ำดีในตับอีกด้วย การเกิดตับแข็งและอายุของผู้ป่วยมีความสัมพันธ์ทางสถิติกับระดับทิชชูแฟคเตอร์ในพลาสม่าของผู้ป่วยมะเร็งตับ ในผู้ป่วยมะเร็งท่อน้ำดีในตับพบว่าระดับทิชชูแฟคเตอร์ในพลาสม่ามีความสัมพันธ์ทางสถิติกับ hematocrit และ prothrombin time แต่ระดับทิชชูแฟคเตอร์ในพลาสม่าของทั้งผู้ป่วยมะเร็งตับและมะเร็งท่อน้ำดีในตับไม่มีความสัมพันธ์ทางสถิติกับปัจจัยทางด้านพยาธิกำเนิดของโรค

จากการวิจัยครั้งนี้มีข้อสรุปว่า ระดับทิชชูแฟคเตอร์ในพลาสม่าของผู้ป่วยมะเร็งตับและมะเร็งท่อน้ำดีในตับสูงกว่าระดับทิชชูแฟคเตอร์ในพลาสม่าของคนปกติ แต่ในการศึกษาครั้งนี้ไม่พบความสัมพันธ์ระหว่างระดับทิชชูแฟคเตอร์และการแพร่กระจายของเซลล์มะเร็ง อย่างไรก็ตาม ยังต้องมีการศึกษาเพิ่มเติมในทางคลินิคต่อไปในอนาคต

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CONTENTS

CONTENTS (cont.)

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AFB1	Aflatoxin B1
AFP	Alpha-Fetoprotein
AFU	Alpha-L-Fucosidase
ALP	Alkaline Phosphatase
ALT	Alanine Transaminase
APC	Adenomatous Polyposis Coli
ASR	Age-Standardised Incidence Rate
bFGF	Basic Fibroblast Growth Factor
BSA	Bovine Serum Albumin
CA-125	Cancer Antigen-125
CA 19-9	Cancer Antigen 19-9
CCA	Cholangiocarcinoma
CDK	Cyclin Dependent Kinase
CEA	Carcinoembryonic antigen
COX-2	Cyclooxygenase-2
CT	Computed Tomography
°C	Degree Celcius
DCP	Des-Gamma-Carboxy Prothrombin
DNA	Deoxyribonucleic Acid
EDTA	Ethylene Diamine tetra Acetic Acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Estrogen Receptor
et al.	*et alii* (and other people)
FVIIa	Activated Cagulation Factor VII
FVII	Coagulation Factor VII
g	Gram

LIST OF ABBREVIATIONS (continued)

GGT	Gamma-Glutamyl Transferase
GTPase	Guanosine Triphosphatase
HCC	Hepatocellular Carcinoma
HBV	Hepatitis B Virus
HBsAg	Hepatitis B Surface Antigen
HCV	Hepatitis C Virus
Hct	Hematocrit
HGF	Hepatocyte Growth Factor
HGFR	Hepatocyte Growth Factor Receptor
HRP	Horseradish Perxidase
H_2SO_4	Sulfuric Acid
ICAM-1	Intracellular Adhesion Molecule-1
ICC	Intrahepatic Chlangiocarcinoma
IGF-I	Insulin-Like Growth Factor-I
IGF-II	Insulin-Like Growth Factor-II
IL-6	Interleukin-6
kDA	Kilo Dalton
KCl	Potassium Chloride
KH_2PO_4	Potassium Dihydrogen Phosphate
L	Litre
LOH	Loss of Heterozygosity
µl	Microlitre
ml	Millilitre
mM	Millimolar
MMP-2	Matrix Metalloproteinase-2
M6P/IGF2R	Mannose-6-Phosphate/Insulin-Like Growth Factor 2 Receptor
mRNA	Messenger Ribonucleic Acid
MVD	Microvessel Density
MW	Molecular Weight
NaCl	Sodium Chloride

LIST OF ABBREVIATIONS (continued)

Na_2HPO_4	Sodium Dihydrogen Phosphate
nm	Nanometre
NSCLC	Non-Small Cell Lung Cancer
pg	Picogram
PAI-1	Plasminogen Activator Inhibitor-1
PBS	Phosphate Buffer Saline
PD-ECGF	Platelet-Derived Endothelial Cell Growth Factor
PSC	Primary Sclerosing Cholangitis
PT	Prothrombin time
PVTT	Portal vein tumour thrombosis
pRB	Retinoblastoma Protein
RB1	Retinoblastoma gene 1
RIA	Radioimmunoassay
RNA	Ribonucleic Acid
rpm	Revolutions per minute
TF	Tissue Factor
TGF-α	Transforming Growth Factor-Alpha
TGF-β	Transforming Growth Factor-Beta
TNM	Tumour-Node-Metastases
TMB	Tetra-Methylbenzidine
TSP-1	Thrombospondin-1
uPA	Urokinase-Type Plasminogen Activator
uPAR	Urokinase-Type Plasminogen Activator Receptor
VEGF	Vascular Endothelial Growth Factor
VTE	Venous thromboembolism
w/v	Weight Per Volume

CHAPTER I
INTRODUCTION

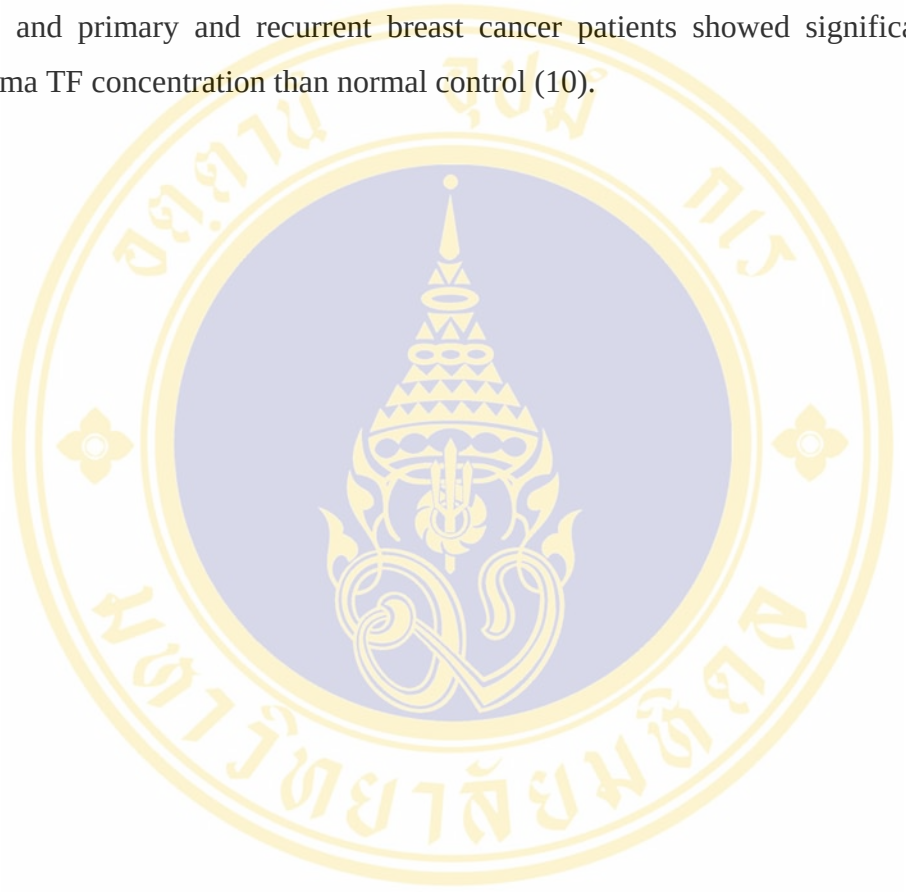
Primary carcinomas of the liver comprise two major types which are hepatocellular carcinoma (HCC), arising from hepatocytes and cholangiocarcinoma (CCA), arising from the bile duct epithelium. HCC accounts for 80% to 90% of primary liver cancers. Virtually, almost all the remainder is CCA while the mixed pattern is uncommon. Liver cancer is the sixth commonest malignant neoplasm and the third commonest cause of death from cancer throughout the world (1). HCC occurs more often in men than women, with an overall ratio of about 3.0:1.0 and mostly in people 40 to 60 years old. It is more common in parts of Asia than Australia, America and Europe. The incidence of HCC varies considerably with the geographic region because of differences in the major causative factors. Abundant epidemiological and experimental evidence indicate that HCC is of multifactorial aetiology. Risk factors of HCC are cirrhosis, hepatitis B virus (HBV) infection, hepatitis C virus (HCV) infection and miscellaneous factors (alcohol, cigarette, oral contraceptive, hemochromatosis, tyrosinemia, glycogen storage disease, alpha-1-antitrypsin deficiency and obstruction of the inferior vena cava). CCA, the second commonest primary liver cancer, is most prevalent in ages 50 to 70. Men are affected 1.5 times more than women. It is common among Southeast Asians, with the world's highest incidence in northeastern Thailand and Laos (2). The etiology of most CCAs remains undetermined. However, liver fluke (*O.viverrini*) is a major predisposing factor for the development of CCA in northeastern Thailand (3, 4).

Tissue factor (TF), a 47 kDa transmembrane receptor protein, is the principal physiological initiator of blood coagulation. The TF gene is localised on chromosome 1p21-22 (5). It encodes for a 263 amino acid single chain polypeptide with three different domains: a 219 amino acid extracellular domain, a 23 amino acid transmembrane domain and a 21 amino acid intracellular domain. TF has also been identified in several tumours and it is a major procoagulant that causes a

hypercoagulable state of malignancy (6). Experimental studies have demonstrated that TF also plays an important role in tumour invasion and metastasis (7, 8). Expression of TF was also found to have a significant correlation with metastatic potential in human lung (9) breast (10) and colorectal (11, 12) carcinomas. The neovessels in a tumour not only provide oxygen and nutrients for tumour growth, but they also provide the route for tumour cell invasion into the circulation. Several studies demonstrated that tumour expression of TF is significantly related to tumour angiogenesis in lung (13) prostate (14) and colorectal (15) carcinomas. Hypervascularity is one of the main characteristics of large and moderately or poorly differentiated HCC (16). In HCC, the mechanisms of angiogenesis remain controversial. Angiogenesis, whether physiological or pathological, is 'switched on' if the balance between proangiogenic factors and angiogenesis inhibitors tilts towards proangiogenic factors. Various studies found that aberrant expression of TF in tumours contributes to the angiogenic phenotype in part by up-regulating the expression of the proangiogenic protein vascular endothelial growth factor (VEGF) and down-regulating the expression of the antiangiogenic protein thrombospondin (15, 17). Recent study of HCC tissues found that TF correlated with VEGF, microvessel density (MVD), the presence of venous invasion, presence of microsatellite nodules, absence of tumour capsule and advanced tumour-node-metastasis (TNM) stage in HCC. Therefore, TF is related to angiogenesis and invasiveness in HCC (18). Evaluation of TF expression may be useful as a prognostic indicator in patients with HCC. It is worthwhile to further investigate the prognostic significance of circulating TF levels in HCC patients, which may be particularly useful in the management of patients. In this study, we quantitatively measured plasma TF level of HCC and CCA patients in comparison with plasma TF level of healthy individuals by using ELISA technique. In addition, the correlation between plasma TF level and the clinicopathological prognostic factors will also be investigated. Although the precise mechanism of TF in angiogenesis and metastasis are not clear, the study of plasma TF level of HCC and CCA patients may be an useful information for further study. One of the possible future modalities in anticancer therapy may be drug targeted to regulate tissue factor expression.

ELISA (enzymed-linked immunosorbent assay) is a highly sensitive and precise method that detects the presence of either antigens or antibodies in the blood by using

enzyme-linked antigens or antibodies matching with interested antigens or antibodies. After substrates are added to the reaction, the results can be read both by eyes and quantified using spectrophotometers. It can handle large numbers of samples that may then be analysed rapidly. By using this method, plasma TF is found to be 67% higher in gastrointestinal and gynaecological cancer patients compare with healthy controls (19) and primary and recurrent breast cancer patients showed significantly higher plasma TF concentration than normal control (10).



Objectives:

1. To study the plasma tissue factor level in hepatocellular carcinoma and cholangiocarcinoma patients compare with healthy individuals by using ELISA technique for quantitative measurement of tissue factor.

2. To study the correlation between the plasma tissue factor level and clinicopathological factors of hepatocellular carcinoma and cholangiocarcinoma.

CHAPTER II
LITERATURE REVIEW

1. Liver cancer

Primary carcinoma of the liver, whether benign or malignant, may arise from hepatocytes, bile duct epithelium, the supporting mesenchymal tissue or more than one of these (Table 1). There are two major types of primary carcinoma of the liver: hepatocellular carcinoma (HCC) arising from hepatocytes and cholangiocarcinoma (CCA) arising from bile duct epithelium. HCC accounts for 80% to 90% of all primary liver cancers. Almost all the remainder is CCA because other primary malignant tumours of the liver are generally rare. The term "cholangiocarcinoma" has been originally intended to refer only to primary tumours of the intrahepatic bile ducts, not those of the extrahepatic bile ducts. Lately, however, the term has been used to include intrahepatic, perihilar and distal extrahepatic tumours of the bile ducts (20). Therefore, CCA is classified into three groups according to the location of the tumour in the hepatobiliary tree: intrahepatic, perihilar and distal extrahepatic (Figure 1). Intrahepatic cholangiocarcinoma (ICC) is the second most common primary liver cancer in the world after HCC. It accounts for about 10-20% of primary liver cancers but its incidence and relative frequency among all liver cancers vary from country to country (21). Because of its high incidence in many countries, its often fulminant course, poor response to conservative treatment, high recurrence after resection and liver transplantation, and grave prognosis, primary carcinoma of the liver is considered to be one of the major malignant diseases in the world today. In recent years, as the understanding of tumour biology and the development of molecular biology techniques, many molecular factors have been shown related to prognosis (22, 23).

2. Epidemiology of liver cancer

Liver cancer is the fifth most common malignancy in men and the eighth in women worldwide. Moreover, it is the third most common cause of death from cancer

Table 1. Classification of primary tumours of the liver (24)

Benign liver tumour	Malignant liver tumour
Epithelial tumours	
Hepatocellular adenoma	Hepatocellular carcinoma
Bile duct adenoma	Cholangiocarcinoma
Biliary cystadenoma	Biliary cystadenocarcinoma
Mesenchymal tumours	
Haemangioma	Angiosarcoma
Haemangioendothelioma	Haemangiosarcoma
Angiomyolipoma	Epithelioid haemangioendothelioma
	Undifferentiated (embryonal) sarcoma
Fibroma	Fibrosarcoma
Leiomyoma	Leiomyosarcoma
	Epitheloid leiomyoma (leiomyoblastoma)
Mixed tumours	
	Hepatoblastoma
	Carcinosarcoma

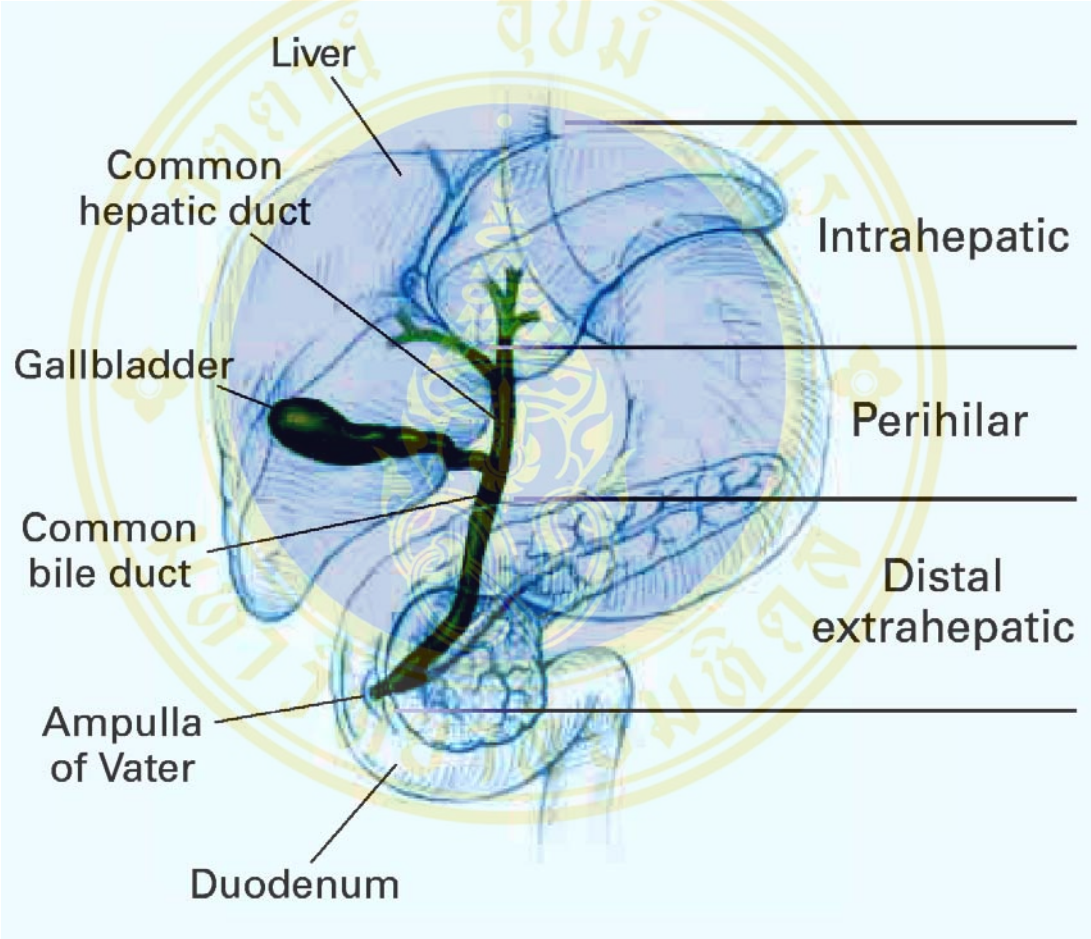


Figure 1. Classification of Cholangiocarcinoma (20)

in men and the sixth in women worldwide (1). The frequency with which liver cancer occurs varies considerably in different geographical regions (Figure 2). The highest incidences occur in Eastern Asia and South-Eastern Asia. China alone accounts for 56.75% of the total cases in the world. The areas of low incidence are Australian continents, Southern Africa, Carribean, Northern Europe and Central America (1).

In comparison to other areas in South-Eastern Asia, the incidence and the mortality rates of liver cancer in Thailand are the highest in South-Eastern Asia (Table 2). Liver cancer in Thailand has been found to be the leading cancer in men (10195 new cases per year or 26.2% of all new cancer cases) and the third in women (4995 new cases per year or 13.3% of all new cancer cases). It is the most common cause of death from cancer both in men (9831 deaths per year or 31.6% of all deaths from cancer) and in women (4821 deaths per year or 20.4% of all deaths from cancer) (Figure 3) (1). HCC, which is associated with hepatitis B virus, is a major problem in all regions of Thailand, with the exception of Khon Kaen and the Northeast. On the other hand, liver flukes related to CCA account for about 89% of all liver cancers in Khon Kaen, which has the highest incidence rate of liver cancer in the world (3, 4).

3. Aetiology and risk factors of HCC

Numerous risk factors for the development of HCC have been identified, including cirrhosis, hepatitis B virus (HBV) infection, hepatitis C virus (HCV) infection, aflatoxins, and miscellaneous factors.

3.1 Cirrhosis

Cirrhosis is the most important risk factor associated with HCC. It is an irreversible result of various disorders that damage liver cells over time. Eventually, the damage becomes so extensive that the normal structure of the liver is distorted and its function is impaired. The causes of cirrhosis include alcohol abuse, chronic hepatitis, prolonged obstruction of the outflow of bile from the liver, and some viral forms of autoimmune liver disease. About 70%-90% of HCCs develop on a background of cirrhosis, and approximately 20% of all patients who die of cirrhosis have evidence of associated HCC at autopsy (25).

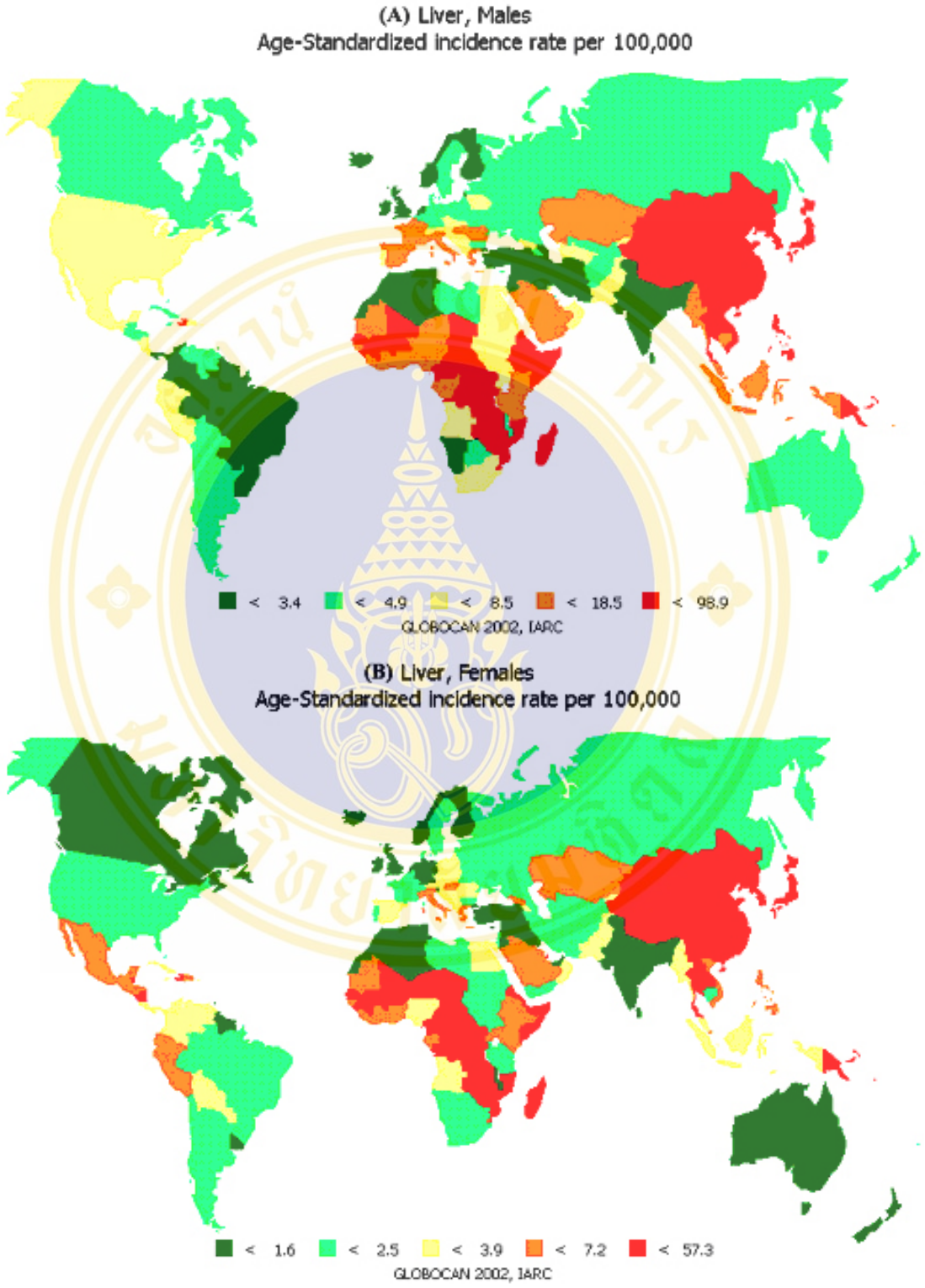


Figure 2. Incidence of liver cancer worldwide (age-standardised rate) in male (A) and female (B) (all age) (1)

Table 2. The incidence rate, mortality rate and prevalence of liver cancer in South-Eastern Asia (1)

(A) Male

Country/Region	Incidence		Mortality		Prevalence	
	Cases	ASR	Deaths	ASR	1-year	5-year
South-Eastern Asia	**35680**	**18.3**	**33504**	**17.2**	**8097**	**17549**
Brunei	11	9.5	11	9	3	5
Cambodia	478	15	450	14.2	110	244
Indonesia	9155	11.3	8632	10.6	2082	4490
Lao	340	22.4	320	21.2	74	162
Malaysia	888	11	839	10.4	201	432
Myanmar	2360	12.7	2213	11.9	543	1198
Philippines	4851	20.3	4225	17.9	1107	2402
Singapore	403	18.4	406	18.6	90	188
Thailand	10195	38.6	9831	37.3	2305	4984
Vietnam	6933	23.7	6515	22.3	1582	3444

(B) Female

Country/Region	Incidence		Mortality		Prevalence	
	Cases	ASR	Deaths	ASR	1-year	5-year
South-Eastern Asia	**12218**	**5.7**	**11552**	**5.4**	**2753**	**5891**
Brunei	4	3.1	4	2.9	0	1
Cambodia	115	2.5	109	2.4	27	60
Indonesia	2334	2.6	2200	2.4	530	1137
Lao	125	7.5	117	7.1	27	58
Malaysia	263	3.1	248	2.9	60	127
Myanmar	735	3.7	690	3.5	169	369
Philippines	1686	6.6	1474	5.8	379	804
Singapore	116	4.8	146	6	25	50
Thailand	4995	17.2	4821	16.6	1120	2390
Vietnam	1827	5.8	1720	5.5	416	895

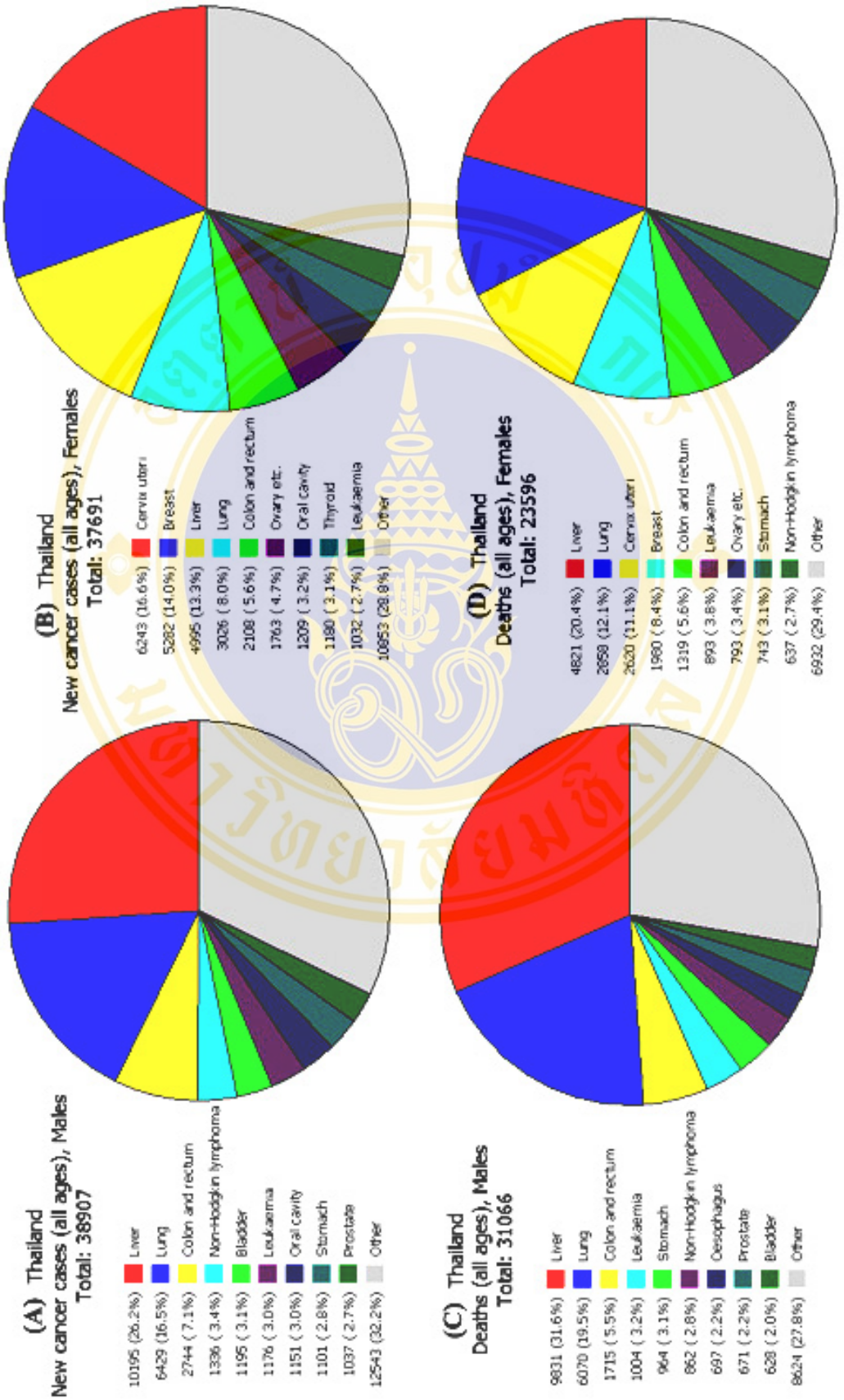


Figure 3. Number of new cancer cases (A, B) and deaths from cancer in Thailand(C, D) (1)

3.2 Hepatitis B virus (HBV)

HBV is the most common risk factor for the development of HCC worldwide. It is an enveloped virus with a very compact, incompletely double-stranded DNA genome. It has a propensity to persist after perinatal infection by a carrier mother or after infection in early life, and after 10-15% of acute HBV infections in adults. Prevalence of chronic HBV infection in the world is shown in Figure 4. Chronic infection with the HBV appears to account for most cases of HCC in high-incidence areas such as eastern Asia (excluding Japan) and Africa (26, 27). In prospective studies, the risk for developing HCC for 3,454 HBsAg carriers is 102 times greater than that of non-carriers (28). Further evidence to support HBV's role in HCC development comes from a vaccination campaign in Taiwan in the 1980s. In Taiwan, this has prompted a significant reduction of the prevalence of HBV carriage in children from 15% to 1% and also a 60% reduction of HCC during childhood (29). In Thailand, chronic infection with HBV is the major risk factor for the development of HCC. Srivatanakul *et al*. has estimated that 41.5% of HCC cases are a consequence of HBV infection (30).

HBV could contribute to hepatocarcinogenesis both directly and indirectly, though the mechanisms remain largely unknown. A well-known direct mechanism is viral genome integration into the hepatocyte chromosomal DNA during the typically long period of infection. HBV replicates via reverse transcription of pregenomic RNA, and the viral genome frequently integrates into multiple sites of the host genome. This process may cause or contribute to genomic instability as a result of point mutations, inversions, translocations and deletions (31). As an indirect mechanism, persistent HBV infection (but not HBV itself) predisposes the hepatocyte to genetic changes resulting from other causes. The virus continuously replicates and causes recurrent episodes of hepatitis. The liver responds to persistent inflammation with continuous regeneration and fibrosis that eventually results in cirrhosis.

3.3 Hepatitis C virus (HCV) infection

Hepatitis C virus is the second most common risk factor for HCC. It is usually transmitted by the parenteral route, the commonest mode of transmission being via blood transfusion and the use of intravenous drugs. The global prevalence of HCV carriers is estimated to average 3%, ranging from 0.1 to 10% or more in different

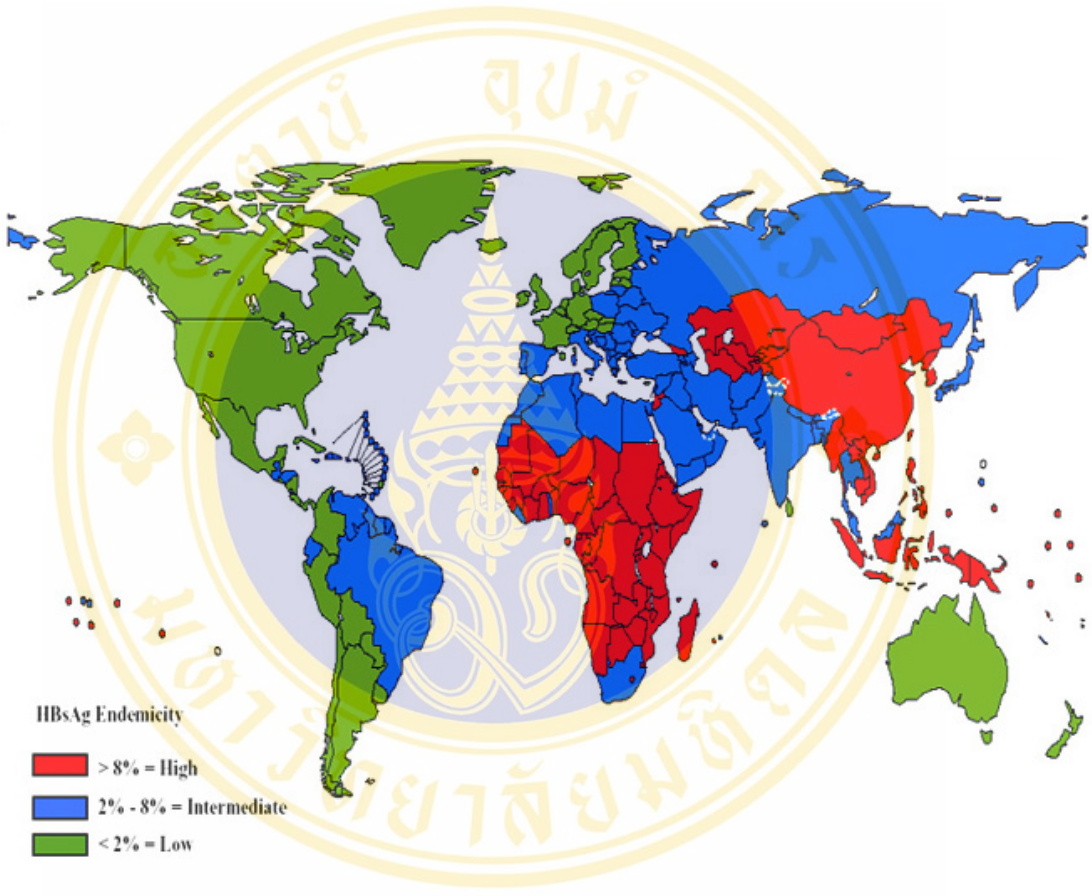


Figure 4. Prevalence of chronic hepatitis B virus (HBV) infection worldwide (32)

countries (Figure 5) (33). Unlike antibodies to hepatitis A and B viruses, antibodies to HCV are not protective and, in most cases, are markers for disease. A high prevalence of anti-HCV has been observed in patients with HBsAg-negative HCC in Italy (70-79%), Spain (77%), and Japan (75-90%). A relatively high prevalence has been observed in France (57%), Florida, U.S. (48%), and a low prevalence in Germany (16%), India (9%), Bangladesh (8%), Taiwan (10-23%), Hong Kong (7%) and South Africa (16%) (34). The prevalence of antibody to HCV seems to be rather low in Thailand (30). Boonmar *et al.* (35) found antibody to HCV in 11.1%of HCC patients negative for HBsAg. Songsivilai *et al.* (36) also found 11.3% of HCV in HCC patients, and concluded that HCV is an important cause of HBV negative liver cancer (out of 80 HCC cases, 8 were infected with HCV and 3 with both HCV and HBV). Long term follow-up studies indicate that the interval between initial HCV infection and the development of cirrhosis and HCC are 20 years and 30 years, respectively (37). HCC develops in 18% of patients with HCV-associated cirrhosis within 2 years and in 75% of patients within 15 years (38). The risk of HCC is increased in patients with HCV who are chronic carriers of HBV or who concomitantly use alcohol. The aetiological role of HCV infection in terms of molecular events in hepatocarcinogenesis is unknown, but it is clearly different from that of HBV infection. HCV is a positive-sense, single-stranded RNA virus and, unlike HBV, is not reverse transcribed to DNA; hence it is not integrated into the host cell DNA. Continuous inflammation, hepatocyte necrosis and ongoing viral replication seem to underlie HCV-related hepatocarcinogenesis. Several clinical and pathological studies have suggested that inflammation is more active in the liver in HCV-associated HCC compared with HBV-associated HCC (39) and liver cell proliferation also is active (40).

3.4 Aflatoxin

Aflatoxin contamination of food probably increases the risk of HCC. Aflatoxins are produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus* and are chemically divided into two groups, aflatoxin B1 (AFB1) and its derivatives and aflatoxin G1 and its series. Whenever food products are found to be contaminated, aflatoxin B1 is present and in larger quantities than other aflatoxins. It is the most toxic and most carcinogenic of all analogues. Epidemiological investigations have

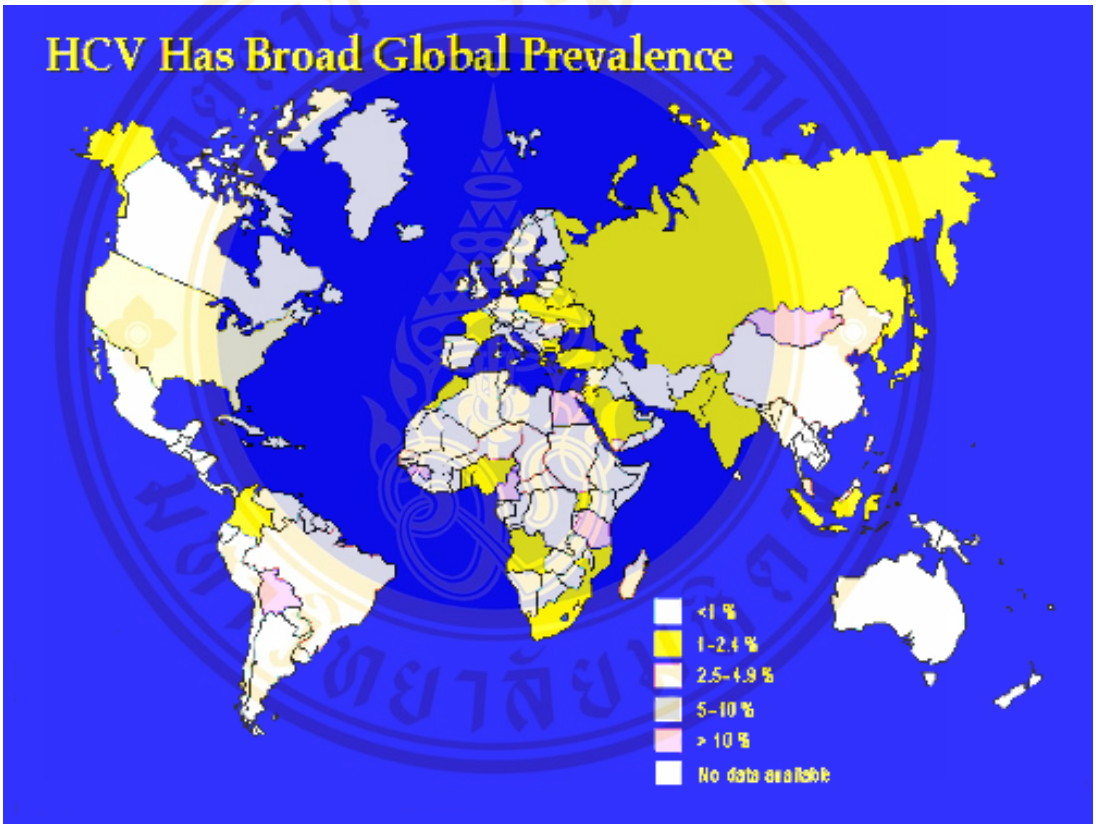


Figure 5. Prevalence of hepatitis C virus (HCV) worldwide (33)

revealed a linear relationship between the content of AFB1 in the diet and the risk of HCC (41, 42). Several evidences has emerged of an association between heavy exposure to aflatoxin and a specific point mutation (guanine to thymine) of the third base of codon 249 of the tumour suppressor gene p53 in HCC, providing a clue to how this mycotoxin may contribute to hepatocellular carcinogenesis (43). When the data on levels of aflatoxins-albumin adducts in sera from Thai subjects are compared with data from other countries in Africa and Southeast Asia indicated that relatively low levels exposure in Thailand (44). This is consistent with the data from sera and liver tissues from Thai patients with HCC, and the low prevalence of G to T mutations at codon 249 of the p53 gene (45).

3.5 Miscellaneous factors

Alcohol is a risk factor for cirrhosis and for HCC. In the United States, where the prevalence of HBV is low, the HCC risk is increased up to 40% by heavy alcohol consumption (25). In London, HCC was seen in 30% of alcoholics with cirrhosis compared with 11% of nonalcoholics with cirrhosis (46). The study of 158 patients with cirrhosis and 79 with HCC, the mean age of HBsAg-positive male patients with cirrhosis who had a drinking habit at the diagnosis of HCC was 38.8 years, 10.5 years younger than the mean age of those without the same habit. A similar difference was noted between HBsAg-negative patients with and without a drinking habit (47). Chronic alcohol use of greater than 80 g/day for more than 10 years increases the risk for HCC approximately 5-fold; alcohol use of less than 80 g/day is associated with a nonsignificant increased risk for HCC (48).

Multiple chemical components of cigarette smoke are hepatic carcinogens in animals (49). Although results are not totally consistent, a number of case-control and cohort studies in diverse populations have implicated cigarette smoking as a causal risk factor for HCC (50, 51). A case-control study measuring DNA adducts of 4-aminobiphenyl, a hepatic carcinogen in animals and a constituent of cigarette smoke, in liver tissues of study subjects showed a statistically significant increase in risk for HCC with increasing levels of adducts (52).

A statistically significant association between the use of oral contraceptive steroids and the occurrence of HCC has been described in woman from populations at low risk of this tumour (53). The relative risk is directly related to the duration of use

(1.5 for < 5 years and 3.9 for > 5 years). Moreover, the increased risk persists for approximately 10 years after stopping this form of contraception (53).

Some forms of inherited metabolic diseases may predispose to HCC. By far the most common of these is hemochromatosis, a disorder of iron metabolism which results in an excessive accumulation of iron in the body. This iron accumulation will eventually lead to cirrhosis and the cirrhosis again provides the right environment for the development of HCC. The comparable figures on the incidence of HCC as a complication of hemochromatosis after 1975 have been greater than 30% (54, 55). A study in Australia suggested a 200-fold excess risk among patients with hemochromatosis (55). Other, rarer, metabolic diseases that are sometimes linked to HCC include tyrosinemia, glycogen storage disease and alpha-1-antitrypsin deficiency.

4. Aetiology and risk factors of CCA

Although the aetiology of most CCAs remains undetermined for most of the cases, liver fluke infestations, primary sclerosing cholangitis (PSC), ulcerative colitis (UC), hepatolithiasis, thorium dioxide exposure and choledochal cysts have been suggested to play roles by inducing hyperplasia, cellular proliferation and ultimately, malignant transformation. The risk factors implied in the development of CCA are given in Table 3.

CCA is more common in areas endemic to liver fluke infection. Liver flukes are strongly associated with this disease. Liver flukes, such as *Opisthorchis viverrini* and *Clonorchis sinensis*, usually enter human's gastrointestinal tract after ingestion of raw fish. *C.sinensis* is mostly seen in China and East Asia while *O.viverrini* is seen in North East Thailand, Laos and Cambodia as well as in Eastern Europe and Russia (56). A prospective case-controlled study conducted in northeastern Thailand demonstrated that patients infected with *O.viverrini* had a higher incidence of CCA than non-infected patients (57).

PSC is a progressive chronic biliary disorder characterized by multiple fibrosing inflammatory lesions involving the intrahepatic and extrahepatic bile ducts. It is a strongly predisposing factor for the development of CCA being detected in 6-14% of the patients. In autopsy series, CCA has been shown in 30-42% of patients with PSC

Table 3. Risk factors for development of CCA (56)

Risk factors in CCA	
Liver fluke infestation	*Opisthorchis viverrini* *Clonorchis sinensis*
Chronic inflammation	Primary sclerosing cholangitis (PSC) Cirrhosis
Biliary calculi	Hepatolithiasis Cholecystolithiasis
Anatomic abnormalities	Caroli's disease Choledochal cyst
Chemicals and carcinogens	Thorium dioxide (Thorotrast) Oral contraceptives Methyldopa Isoniazid Cigarette smoking (in patients with PSC)

(58). In one study of patients followed over 5 years, 8% eventually developed clinically detectable cancer, but occult CCA in patients with PSC has been reported in 36% of autopsy specimens and 40% of explant specimens (59). Ulcerative colitis is another risk factor for CCA. In most of the ulcerative colitis cases, PSC is also present and this association causes further predisposition to the development of CCA. Total colectomy does not prevent CCA development which usually appearing 15-20 years after the onset of ulcerative colitis.

Hepatolithiasis is also strongly associated with this tumour in Asia, and this association was recently reported in a patient in France (60). It is common in East Asia, with a prevalence as high as 20% in Taiwan (61). Cholelithiasis, as well, is a risk factor for CCA. A significant decrease has been reported in the incidence of CCA 10 or more years after cholecystectomy for cholelithiasis (56).

Characterised by cystic dilation of bile ducts, Caroli's disease is a congenital disorder inherited as autosomally recessive manner. CCA is a common complication of this disorder, occurring in about 7% of the cases (56). Another malformation of the biliary tree that predisposes to CCA is congenital bile duct cysts or choledochal cysts that can be seen at any age. They are associated with a 10% risk of developing CCA (62).

Some chemicals and drugs including thorium dioxide, oral contraceptives, anabolic steroids, alpha-methyldopa and isoniazid have also been reported as the aetiology of CCA. In a cohort of patients exposed to thorium dioxide studied in Germany, it was reported that 454 of 2,326 exposed patients developed primary liver tumour (63), and the odds ratio of developing CCA after exposure was calculated to be 316 in this cohort (64). Although smoking alone has not been shown to be a risk factor for CCA, it has been identified as a strong risk factor for developing CCA in PSC.

In contrast to HCC, CCA does not appear to be associated with HBV or HCV infections and is not related to alcoholic liver disease either. However, the incidence of cirrhosis in CCA is reported to be as high as 38% (56). Although an association between cirrhosis and CCA has not been clearly shown, alterations in hormonal levels, impaired metabolism of carcinogens and changes in immunological status can be contributing factors for the development of CCA in patients with cirrhosis.

5. Molecular hepatocarcinogenesis

It is known that carcinogenesis is a multistep process in which a number of mutational genetic alterations occur. In comparison with colonic mucosa cells, hepatocytes have a great deal more metabolic functions affected by many more expressed genes, and much more complicated genetic alterations are expected to occur in the process of carcinogenesis. There have been many studies in which various oncogenes, tumour suppressor genes and growth factors were found to have a variety of changes, but as yet no consistent sequence of genetic changes has emerged.

5.1 Oncogenes

Activation of cellular oncogenes such as *ras* has been reported in spontaneous and chemically induced pre-neoplastic lesions of HCC in animal models (65, 66). The incidence of mutated ras genes varies widely among different tumour types. The ras genes are activated by a single point mutation, and such mutation has been demonstrated in 40% of colon cancer and 90% of pancreatic cancer. In HCC, point mutation of a ras gene is not common (67) but allelic loss and increased methylation may occur as one of the steps (68). Some chemical carcinogens such as aflatoxin B1 and vinyl chloride induced the ras gene mutation in HCC (69, 70). In vitro, activated H-ras oncogene induced metastatic phenotype of a cell line derived from human HCC (71).

Expression of the proto-oncogene *c-myc* has been implicated in liver regeneration and hepatocarcinogenesis. The c-myc gene was amplified in 14 of 42 (33%) human HCCs (72). Amplification of *c-myc* was more frequent in larger and less differentiated tumours (73). Moreover, the disease-free survival in patients showing *c-myc* amplification was significantly shorter than in those without the amplification. The study suggested that *c-myc* amplification is an indicator of malignant potential and poor prognosis in HCC.

β-catenin, a protein associated with the cytoplasmic region of E-cadherin, has been shown to play a role in transcription regulation and in cell-to-cell interactions. It is now apparent that deregulation of β-catenin signaling is an important event in the genesis of a number of malignancies. β-catenin gene mutations in human HCC has shown a prevalence of 18-41% (74). In Chinese population, abnormal

expression of β-catenin gene may contribute importantly to the invasiveness of HCC (75).

5.2 Tumour suppressor genes

The most frequently altered genes in HCC are tumour suppressor genes which have been extensively studied, such as p53, retinoblastoma (RB1), p16, mannose-6-phosphate/insulin-like growth factor 2 receptor (M6P/IGF2R), E-cadherin, adenomatour polyposis coli (APC), and axin gene.

The p53 gene is located on the short arm of human chromosome 17 (17p13.1). Overall, this gene is mutated in about one third of these tumours. In human HCC, loss of heterozygosity (LOH) at chromosome 17p13 has been observed in 25-60% of tumours in different studies (76). The frequency and type of *p53* mutations differ according to the geographic origin and suspected aetiology of HCC. In some parts of the world, mutations are consistent with those caused by aflatoxin exposure and possibly by HBV infection. In HBV infection, there is evidence that the viral transactivating protein HBV X gene inactivates *p53*. It was also found that p53 gene was mutated at codon 249 G to T transversion in HCC from patients in southern Africa and Qidong area of China (77), and an association of this particular mutation and aflatoxin B1 exposure was suggested. This mutation is not found in HCC patients in the USA (78), Japan (79), Germany (80), UK (81), Europe outside UK and Germany (82) and Australia (83) and in only small percentage of patients in Korea (84) and Taiwan (85). Mutational hotspots in the p53 gene other than at codon 249, such as at codon 166 in exon 5 and at codon 286 in exon 8, have also been described in HCCs (86). It remains possible either that each mutation was a result of a different carcinogenic influence or that the different mutations reflect a general genetic instability or repair defect. *p53* mutations may also be late occurrences in hepatocarcinogenesis and may occur in association with dedifferentiation to a more aggressive histological type or the development of metastasis. Various reports show that the *p53* mutations are closely related to the progression of HCC. These mutations are found in 44-50% poorly differentiated HCCs and in 22-36% moderately differentiated HCCs, but in none of the well differentiated HCCs (87). One patient with a long history of chronic HCV hepatitis and ethanol use has been reported in

whom two HCC grade II or III nodules containing two different *p53* mutations were surrounded by a grade I nodule containing only wild-type *p53* (88).

Retinoblastoma (RB1) gene is a tumour suppressor gene located on human chromosome 13 (13q14). LOH at the RB1 gene locus has been found in 25-48% of HCC cases (87, 89), and it has shown that pRB expression is strongly down regulated in 30-50% of HCCs (89). The alteration of pRB expression by HBV X could be a mechanism, contributing to the development of HBV-associated HCC (90). Loss of the retinoblastoma gene or LOH at chromosome 13q was observed in six of eight advanced HCCs carrying a mutated p53 gene. These results suggest the involvement of at least two tumour suppressor genes in a late stage of hepatocarcinogenesis (87). A similar result was reported from HCC patients in China which aflatoxin B1 induced p53 gene mutation also carrying aberration of the RB gene (91).

p16 (*CDKN2/MTS1/p16*^{INK4A}) is a tumour suppressor gene which is located at chromosome 9p. Mutations of the p16 gene occur in HCC at rate of 5-55% (92). Inactivation of p16 gene occurred via different mechanisms, including homozygous deletion, hemizygous deletion, point mutation and hypermethylation of *p16* gene promoter (93, 94). However, there are several studies found that an important mechanism of inactivation is aberrant methylation in the promoter region of the gene (95, 96). *In vitro* experiment found that the transfer of wild-type *p16* gene can inhibit the proliferation and reduce the invasion ability of HCC (97).

M6P/IGF2R gene is located on chromosome 6q26-q27. LOH at the M6P/IGF2R locus has been reported and the M6P/IGF2R gene is mutated in 18-33% of HCCs (98, 99). E-cadherin gene which is located on 16q22.1 was shown to display frequent LOH and methylation in HCCs. Down-regulation of E-cadherin correlated with the size of tumours, high nuclear grade, the mitotic index and survival (100). Adenomatous polyposis coli (APC) gene mutations have been demonstrated not only in colorectal carcinoma but also in a variety of human cancers. However, the possibility of APC as the gene defect in the genesis of human HCC may be rare (101). Axin gene is a new tumour suppressor gene located on 16p. Axin acts as a scaffolding protein in phosphorylation-dependent ubiquitination and degradation of β-catenin (102), and loss of function of axin promotes β-catenin accumulation in the nucleus.

The adenovirus-mediated gene transfer of wild-type *AXIN1* inhibited growth and induced apoptosis in cultured HCC cells (103).

5.3 Growth factors

The interaction of growth factors with specific membrane receptors triggers a cascade of intracellular signals, resulting in the activation or repression of genes associated with cell growth. Unfortunately, little is known about the cellular activity of growth factors during hepatic proliferation and transformation. Some of the growth factors that may be involved in one or more steps in the development of HCC include hepatocyte growth factor (HGF), epidermal growth factor (EGF), transforming growth factor-α (TGF-α), transforming growth factor-β (TGF-β) and insulin-like growth factor-II (IGF-II).

Hepatocyte growth factor (HGF) is a ligand for the c-met protooncogene. It plays a role in regulation of both cell growth and cell motility. Serum HGF level in patients with HCC is significantly higher than normal individuals (104). A similar result has been reported from the rat model of chemically induced hepatocarcinogenesis (105). A recent study found that a close relationship between overexpression of HGF receptor gene (c-met) and tumour metastasis, and the HGF and HGF receptor system plays an important role in regulating tumour growth and metastasis (106). In addition, HCC patients with high c-met in their tumour tissues had a significantly shorter 5-year survival than those with low c-met (107).

Epidermal growth factor (EGF) exerts a wide variety of biological effects including the promotion of proliferation and differentiation of mesenchymal and epithelial cells. Expression of EGF was demonstrated in 14 of 56 (25%) HCCs, while it was totally negative in non-cancerous hepatic tissues (108). The amount of EGF mRNA expression was increased in HCCs compared with the surrounding liver tissues (109). These findings suggest a role for the exclusively produced EGF in HCC cells themselves during the development and progression of the tumour mass. An immunohistochemical study showed a linear localisation of epidermal growth factor receptor (EGFR) along the cell membrane of the HCC cells in 21 of 30 cases of HCC (110). In addition, overexpression of EGFR mRNA was found in poorly differentiated tumours and primarily in patients with early tumour recurrence (111).

Transforming growth factor-α (TGF-α) is a member of a polypeptide growth factors family. It acts in an autocrine or paracrine mode to stimulate directly hepatocyte DNA synthesis (112). Increased production of TGF-α and its receptor has been frequently found in human tumours and transformed HCC cell lines in tissue culture, but not in normal liver tissue (113). Moreover, 65% of patients with HCC have elevated TGF-α levels in urine (114). The detection of greater quantities of TGF-α in the more differentiated portions of HCCs suggests that its increased expression may be an early event in human hepatocarcinogenesis (115).

Transforming growth factor-β (TGF-β) has three major activities in mammals: it inhibits proliferation of most cells, but can stimulate the growth of some mesenchymal cells; it exerts immunosuppressive effects; and it enhances extracellular matrix formation (116). Russell and colleagues (117) showed that hepatic regeneration was inhibited by intravenous administration of TGF-β to partially hepatectomised rats. The negative regulation of proliferation is mediated by the induction of apoptosis in hepatocytes and altered cells in pre-neoplastic hepatic foci (118). TGF-β has been shown to be a potent inhibitor of DNA synthesis in primary hepatocyte cultures and HCC cell lines such as HepG2 and Hep3B (119). Increased expression of TGF-β is found in HCC tissue and elevated serum levels of TGF-β have been found in patients with HCC (120). Moreover, serum TGF-β1 levels were higher in HCV-associated HCC than in chronic hepatitis C ($p<0.05$) (121). By immuno-histochemistry, the high immunoreactivity to TGF-β1 or TGF-β2 is positively related to the recurrence of HCC (122). HCC patients with metastasis showed higher levels of TGF-β1 serum concentrations and stronger expression of TGF-β1 (123). From these studies, TGF-β1 has been suggested to play a role in the development, growth or progression of HCC.

Insulin-like growth factor-II (IGF-II) is a polypeptide hormone and is believed to be a major fetal growth factor. It is structurally and functionally related to insulin and insulin-like growth factor-I (IGF-I) (124). IGF-II is expressed at high levels in fetal liver, and the rate of IGF-II gene transcription declines after birth, to reach the very low level found in normal adult liver. Re-expression of IGF-II has been found in chemically induced hepatocarcinogenesis (125), in transgenic mouse models

of hepatocarcinogenesis, in human HCC and in adjacent uninvolved cirrhotic tissue (126).

6. Molecular cholangiocarcinogenesis

Conversion from normal to malignant bile-duct tissue probably requires a number of successive genomic mutations similar to the sequence of events proposed for other gastrointestinal cancers, although the knowledge of biliary tract cancers is less extensive than that of the more common gastrointestinal cancers. A variety of mutations in oncogenes and tumour suppressor genes have been described in CCA. *C-myc*, *K-ras* and *c-erb-B-2* are the most identified oncogenes in CCA, with *K-ras* mutation being the most extensively studied of all. The most frequent mutation on *K-ras* occur at codons 12, 13 and 61 (127). Activation of *K-ras* occurs by point mutation at codon 12, with changes from glycine (GGT) to aspartic acid (GAT) or, less often, valine (TGT), which is detected in ICC (128). The incidence of *K-ras* mutation in ICC ranges from 0% to 100% as reported in different countries. It is reported to be 100% in English patients (129) and 4% to 60% among Japanese and Thai patients (130).

Allelic loss or point mutation in the tumour suppressor genes p53 and p16 also occurs in a significant portion of CCA. In the nuclei of carcinoma cells, p53 protein was accumulated and immunohistochemically detectable in 25%-75% of ICC cases (131-133), particularly in the well and moderately differentiated CCA. The presence of *p53* mutation or the up-regulation of mdm-2 gene expression in 9 of 13 CCAs strongly supports the idea that impairment of the *p53* pathway is an important and specific step in ICC pathogenesis. Although mutations occur throughout the p53 gene, those in exons 5 to 8 are observed more frequently. The most common mutation is an A to G transition, with missense mutation being more frequent than nonsense mutation (133).

Both *p14* and *p16* share the INK4a-ARF locus, and inactivation of this locus is quite common in CCA (134). *p27* is a cyclin-dependent kinase inhibitor, has an inhibitory effect on the G1 to S phase transition in the cell cycle. ICC patients with low or absent *p27* expression were associated with poor survival compared with the high-expresser group (135).

C-met is a tyrosine kinase transmembrane protein identified as the receptor for HGF. Overexpression of c-met is frequently found in nonneoplastic biliary epithelial cells and also in ICC in humans and is correlated with ICC differentiation, being poorly expressed in poorly differentiated tumours (136). *C-erbB-2*, encoding a transmembrane protein highly homologous to EGFR, is expressed on pathologic biliary epithelial cells. In addition, aberrant expression of *c-erbB-2* is also found frequently in ICC (137), suggesting that the c-erbB-2 oncogene participates in cholangiocarcinogenesis.

TGF-β is generally known to induce growth inhibition of normal as well as neoplastic epithelial cells. Carcinoma cells, including those of biliary tract carcinoma, are thought to gain resistance to such growth inhibitory effects of TGF-β by disrupting the signal cascade of TGF-β, including TGF-β1 receptor, TGF-β2 receptor and *Smad* (138). Transforming growth factor signaling pathway disturbance could be a result of genetic changes in the TGF-β type 1 and 2 receptor gene, followed by genetic inactivation of TGF-β in biliary cancers.

Resistance to apoptosis, especially by the altered expression of Bcl-2 family members, has been implicated as a mechanism contributing to malignant transformation. Resistance to apoptosis by the overexpression of Bcl-2 may be a feature of ICC (139). The rate of Bcl-2 overexpression is about 30%, although this is variable, from 0% to 100%, probably due to the methods applied (140), however, a high content of *bcl-2* mRNA was also found in almost all cases of ICC. In addition, dysregulation of Bcl-2 expression in ICCs may be due to inactivation of tumour suppressor gene, p53, which is known to suppress Bcl-2 expression (141).

The genetic changes that occur during carcinogenesis are reflected in alterations in the expression of mucin glycoproteins on tumour cells (142). Normal bile duct epithelium expresses both MUC3 and MUC6 genes but not MUC1, 2, 4 and 5 genes. As a result of malignant transformation CCA cells express *MUC-1*. Mucins interfere with immune system function and promote metastatic process. Overexpression of *MUC1* was shown to correlate with poorer outcome and with a tendency for lymphatic and perineural invasion and liver metastasis in CCA (142).

The overexpression of cyclooxygenase-2 (*COX-2*) has been observed in chronic cholangitis and biliary tract carcinoma, suggesting that this enzyme may play an

important role in bile duct carcinogenesis and tumour progression (143). Hayashi *et al*. (143) reported that a higher level of *COX-2* was necessary for carcinogenesis in biliary epithelial cells, and that just a moderate level of *COX-2* expression would not be sufficient for carcinogenesis of biliary epithelial cells.

7. Clinical course

The outcome for patients with liver cancer still remains dismal, although it has been improved much in the past few decades, a definitive subset is cured by surgery only, and encouraging long-term survival of patients have been obtained in some clinical centers. The high possibility of intrahepatic and/or extrahepatic recurrence postoperatively remains one major obstacle for further improving the survival and prognosis of liver cancer patients. The life expectancy of liver cancer patients is hard to predict, making it difficult to decide the patient's prognosis. Many factors such as sex, age, background liver disease, tumour size, histological grade, vascular invasion, lymphatic invasion and perineural invasion are found to have prognostic significance.

7.1 Sex and age

Virtually all the epidemiological studies have shown that males are more prone to hepatocarcinogenesis. Many reports indicate that female HCC patient more frequently has a well-encapsulated, less invasive tumour, and longer survival, lower recurrent rate, and better prognosis than male patient. These might be due to the more favorable outcome of cirrhosis itself in this gender (144) or the receptor of sex hormones (145). Estrogen receptor (ER) is found closely related to the prognosis of HCC patients. ER positive HCC has less malignant biologic behavior and a better prognosis than ER negative ones, with higher percentages of single nodule and complete encapsulation. As well as, the ER positive rate of small HCC (62.5%) is higher than that of large HCC (30.4%). However, there is still controversy with the relationship between the better prognosis of female HCC patients and the sex hormone receptors.

Younger HCC patients often have tumours with higher invasiveness and metastatic potentials, higher recurrence possibility, and their survival and prognosis are worse than the older ones. In spite of this, Chedid *et al*. (146) found male, older

patients often had poorly differentiated tumours, and poorer survivals, and the age younger than 45 years was a good prognostic factor.

One study of ICC patients in Thailand found that there were no significant differences in survival time related to age less than 40 years, 40-60 years and over 60 years (p>0.05) and also no significant difference in survival time related to sex (p>0.05) (147).

7.2 Background liver diseases

Co-existing hepatitis status and liver cirrhosis are other important factors influencing the prognosis of HCC patients. The inflammatory activity and hepatic reserve have been confirmed as risk factors for recurrence. Longer disease-free survival is found in patients without active hepatitis, and suppression of co-existing hepatitis is necessary to achieve better disease-free survival (148). The postoperative overall and disease-free survival rates of patients without hepatitis viral infection are better than HBV-related HCC patients and HBV-HCV double infection HCC patients. This is due to HCC patients without hepatitis viral infection have a good liver function reservation.

Functional reserve of the remnant cirrhotic liver is another independent prognostic factor. Hepatic functional damage immediately after hepatectomy is a significant risk factor for early intrahepatic recurrence (149). Some liver functional markers, such as alanine transaminase (ALT), gamma-glutamyl transpeptidase (GGT), and serum albumin level are important predictive markers for disease-free survival of HCC patients (149). The serum albumin level of patients was also an independent risk factor of early recurrence, while liver cirrhosis and serum bilirubin were independent prognostic factors for late recurrence after HCC resection (150).

In CCA, biochemical tests of liver function may reveal a cholestatic picture with elevated bilirubin, alkaline phosphatase and GGT. Prolonged obstruction of the common bile or hepatic duct can cause a reduction in fat-soluble vitamins and increase prothrombin time (20, 151).

7.3 Tumour size

Many studies have confirmed that tumour size is an independent prognostic factor (152, 153). Both of the 5-year overall survival and disease-free survival rates of small HCC (tumour largest diameter ≤5cm) are better than that of large HCC (tumour

diameter >5cm) (22, 23). According to the diameter of the largest nodule of an HCC after hepatic resection, patient survival was significantly higher in those suffering from small size tumours (0-5 cm) in comparison with those with a diameter of the largest nodule exceeding 5 cm (154).In patients with malignant strictures of the common bile duct, the median survival rate was 6.6 months for patients with tumour smaller than 30 mm and 3.2 months for patients with large tumour (p<0.01) (155). On the other hand, there was no significant difference in survival time of ICC patients in Thailand between tumour size 2-5 cm, 5-10 cm and greater than 10 cm (p>0.05) (147).

7.4 Histological grade

Cancer grading is based on the degree of differentiation of tumour cells. In general, a higher grade means that there is a lesser degree of differentiation. The higher the grade, the less the resemblance of the tumor to “normal” liver and the more obvious its morphologic features are to malignant growth. Well-differentiated tumours have a greater resemblance to native tissue than poorly differentiated tumours. The grading system of Edmondson and Steiner is recommended for HCCs. It divides HCCs into four grades, from I to IV, on the basis of histological differentiation (Table 4) (156). Histological grade has been found to have a relationship to tumour size, tumour presentation, and metastatic rate. Low histological grade has been shown to be predictive of disease-free survival but not of overall actuarial survival (157). For CCA, there is one report of a well-differentiated variety associated with long-term survival of 15 or more years (158).

7.5 Vascular invasion

HCC has a propensity to invade the portal vein, and portal vein invasion is one of the determinants of prognosis. Patients who have developed portal invasion at an early stage fare poorly thereafter. HCC also invades large hepatic veins, but less frequently compared with the portal invasion (144). If HCC invades a large hepatic vein, the growth is usually active and quite often extends into the right atrium, or even into the pulmonary vein through the tricuspid valve (144). The prognosis of patients with hepatic vein invasion is much poorer than for those who do not have it. Thus, vascular invasion is the strongest risk factor for recurrence of HCC after liver transplantation (159). In the cirrhosis associated HCC patients after liver transplantation, the highest 10-year survival rate occurred in the group without a

Table 4. Edmanson and Steiner grading system for HCC (156)

Grade I	- Reserved for those HCCs where the difference between the tumour cells and hyperplastic liver cells is so minor that a diagnosis of carcinoma rests upon the demonstration of more aggressive growths in other parts of the neoplasm.
Grade II	- Cells show marked resemblance to normal hepatic cells. - Nuclei are larger and more hyperchromatic than normal cells. - Cytoplasm is abundant and acidophilic. - Cell borders are sharp and clear cut. - Acini are frequent and variable in size. - Lumina are often filled with bile or protein precipitate.
Grade III	- Nuclei are larger and more hyperchromatic than Grade II cells. - The nuclei occupy a relatively greater proportion of the cell (high N:C ratio). - Cytoplasm is granular and acidophilic, but less so than Grade II tumors. - Acini are less frequent and not as often filled with bile or protein precipitate. - More single cell growth in vascular channels is seen than in Grade II.
Grade IV	- Nuclei are intensely hyperchromatic. - Nuclei occupy a high percentage of the cell. - Cytoplasm is variable in amount, often scanty. - Cytoplasm contains fewer granules. - The growth pattern is medullary in character, trabeculae difficult to find, and cell masses seem to lie loosely without cohesion in vascular channels. - Only rare acini are seen. Spindle cell areas have been seen in some tumors. - Short plump cell forms, resembling “oat cell” carcinoma of the lung are seen in some Grade IV tumours.

vascular invasion and was 79% (160). By multivariate analysis of ICC patients who underwent hepatectomy, the presence of vascular invasion was found to be independently associated with poor survival and high recurrence rate (p<0.05) (21, 161).

7.6 Lymphatic invasion

Lymphatic invasion describes cancer cells invading into and present within lymphatic vessels. It is a prognostic factor for some cancers. Intrahepatic lymphatic invasion has been reported as a mode of intrahepatic spread in patients with ICC, and this invasion is thought to adversely affect the prognosis for survival in patients with ICC (162). The absence of lymphatic permeation was found to be associated with significantly better survival in ICC patients having hepatic resection (p<0.02) (163).

7.7 Perineural invasion

Perineural invasion describes cancer cells invading in and around nerves. In variety of tumours, it can be observed and may influence prognosis. This also may be part of the pathology report to help grade the cancer. In an univariate analysis, perineural invasion was determined to be significantly correlated with poor survival of ICC patients after surgery (p<0.05) (164).

8. Staging system

Staging helps in establishing a prognosis and choosing a treatment. It is a crucial variable in treatment outcome because many therapeutic failures have resulted from incorrect patient selection. The TNM staging system of the American Joint Committee on Cancer (AJCC) / International Union Against Cancer (IUCC) applies to all primary carcinomas of the liver, including HCCs, ICCs and mixed tumours (165). The TNM classification depends on the number of tumour nodules, the size of the largest nodule, the presence or absence of vascular or lymphatic invasion, the presence or absence of lymph node metastases and the presence or absence of distant metastasis. A higher stage is usually associated with a poorer prognosis. This staging system can be summarised in Table 5. However, TNM staging system of extrahepatic CCA and perihilar CCA are separately staged from TNM staging system of primary carcinomas of the liver (Table 6).

Table 5. TNM staging system of AJCC and IUCC for HCC (165)

Stage	Primary tumour	Nodal status	Distant metastasis
I	T1	N0	M0
II	T2	N0	M0
III	T1	N1	M0
	T2	N1	M0
	T3	N0, N1	M0
IVA	T4	Any N	M0
IVB	Any T	Any N	M1

T1 : solitary, ≤2 cm, without vascular invasion

T2 : solitary, ≤2 cm, with vascular invasion
multiple, one lobe, ≤2 cm, without vascular invasion
solitary, >2 cm, without vascular invasion

T3 : solitary, >2 cm, with vascular invasion
multiple, one lobe, ≤2 cm, with vascular invasion
multiple, one lobe, >2 cm, with or without vascular invasion

T4 : multiple, more than one lobe, invasion of major branch of portal or hepatic veins

N0 : no regional lymph node metastasis

N1 : regional lymph node metastasis

M0 : no distant metastasis

M1 : distant metastasis

Table 6. TNM staging system for extrahepatic CCA and hilar CCA (56)

Stage	Primary tumour	Nodal status	Distant metastasis
0	Tis	N0	M0
I	T1	N0	M0
II	T2	N0	M0
III	T1-T2	N1-N2	M0
IVA	T3	Any N	M0
IVB	Any T	Any N	M1

Tis : Carcinoma *in situ*

T1 : tumour invades subepithelial connective tissue or fibromuscular layer

T2 : tumour invades perifibromuscular connective tissue

T3 : tumour invades adjacent structures: liver, gallbladder, pancreas, duodenum, colon, stomach

N0 : no regional lymph node metastasis

N1 : metastasis in cystic duct, pericholedochal and/or hilar lymph nodes (i.e. in the hepatoduodenal ligament)

N2 : metastasis in peripancreatic (head only), periduodenal, periportal, celiac, and/or superior mesenteric and/or posterior. Pancreaticoduodenal lymph nodes

M0 : no distant metastasis

M1 : distant metastasis

The survival of 229 HCC patients who did not receive specific treatment is shown in Figure 6. All stage III patients die before 3 months from diagnosis, with a median survival of only 0.7 month. The median survival of stage II patients was 2.0 months, and that of stage I patients 8.3 months (166). Moreover, the survival rates of stage I and II HCC patients after hepatic resection were significantly higher than those of stage III and IV (167).

In ICC, 5-year Kaplan-Meier survival of patients who underwent resection was 35% and tumour stage is a significant prognostic factor when comparing stage I disease with all others stages of the disease (p=0.03) (168).

9. Tumour markers

A tumour marker is defined as a substance present in or produced by a tumour, or the host, that can be used for differentiating neoplastic from normal tissue. The tumour marker can be detected in a solid tumour, in circulating cells in peripheral blood, in lymph nodes, in bone marrow, or in other body fluids (ascites, urine, and stool). Tumours markers are useful in identifying the presence of a cancer, possibly the tissue of origin, establishing the extent of the tumour burden before treatment and monitoring the response to therapy. An ideal tumour marker should be specific for a particular type of cancer. It should be produced only by that type of tumour and not by any non-neoplastic condition. The markers should also be highly sensitive so that the amount produced by a very small tumour is measurable by available laboratory methods. The quantity of the marker should correlate with the tumour load so that it may be used in staging the disease and monitoring response to therapy. The biological half-life of the marker must be short enough so that when the production drops/or ceases, the level falls off rapidly. It should also have prognostic relevance and predict the outcome of symptomatic patients.

9.1 Specificity of tumour markers

9.1.1 Tumour specific proteins

A specific tumour marker is expressed only in tumour cells. The best example is the so called fusion proteins associated with malignant processes in which an oncogene is translocated and fused to an active promoter of another gene. The result is a constantly active production of the fusion protein, leading to the

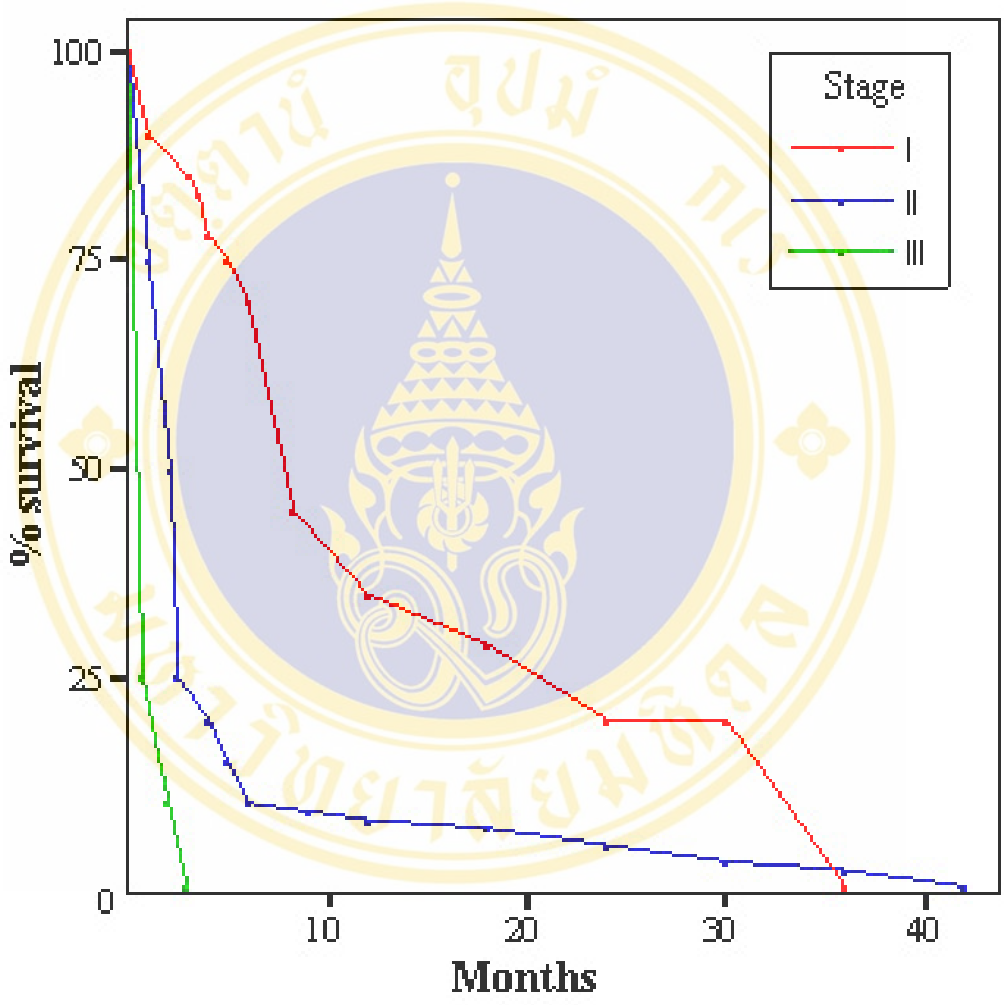


Figure 6. Survival curves for HCC patients who not received specific treatment. The median survival was 0.7 month for stage III patients, 2.0 months for stage II patients and 8.3 months for stage I patients (166).

development of a malignant clone. The Philadelphia chromosome in chronic myeloid leukaemia is the best known example (169). DNA sequences can be recombined not only through translocations but also through inversions and insertions. By recombining DNA in this manner, fusion genes may be created or destroyed, or the regulatory control of genes may be interfered with. These mechanisms frequently occur in haematological malignancies but also in some solid tumours of mesodermal origin.

9.1.2 Nonspecific proteins or markers related to malignant cells

Oncofetal antigens are another kind of marker, less stringent but still very useful. These are expressed in cells during embryological development and in cancer cells. The most commonly used oncofetal antigen, carcinoembryonic antigen, is expressed in all gastrointestinal tumours as well as in many other tumours (170). Alpha-fetoprotein is used to diagnose hepatocellular cancer but is also expressed in testicular and ovarian cancer (171).

9.1.3 Cell specific proteins overexpressed in malignant cells

Some proteins are expressed normally by differentiated cells but are expressed at higher rates in the corresponding tumour cells, which is why a relative increase in serum concentrations can be used as a tumour marker; this is the case with prostate specific antigen concentrations in prostate cancer (172). Cell specific proteins are used for diagnostic purposes, for example, the tyrosinase protein expressed in melanocytes (173).

9.2 Tumour markers of HCC

Alpha-fetoprotein (AFP) is a multifunctional glycoprotein belonging to the family of albumin-like proteins (186). It has been used as a serum marker for HCC in humans for many years and has a sensitivity of 39%–65%, a specificity of 76%–94%, and a positive predictive value of 9%–50% (173, 174). Studies assessing AFP value as a screening tool varied widely in their design and in the characteristics of the patients (type of viral infection, type and severity of liver disease, and so forth). Specificity and sensitivity depend on the prevalence of HCC in the screened population as well as on the AFP cut-off level chosen for the diagnosis. In a case-control study, the higher the AFP cut-off level, the higher the specificity and the lower the sensitivity (174).

AFP may be a useful prognostic indicator for HCC because the median survival rate of HCC patients with markedly elevated AFP was significantly shorter than that of patients with normal or moderately elevated AFP (175). Serum AFP alone has a limited role in the early diagnosis of HCC, because a considerable proportion of HCC patients do not have elevated serum AFP, and serum AFP can increase in patients with diseases other than HCC. Hence, isoforms of AFP have been developed including the lens culinaris agglutinin-reactive fraction of AFP (AFP-L3) and monosialylated AFP (msAFP). A group of investigators from Japan studied the clinical significance of AFP-L3 level in 88 patients with HCC. They showed that those patients with an elevated AFP-L3 had a higher incidence of infiltrative-type and poorly differentiated HCC (176). Another study concerning the significance of msAFP through novel glycosylation immunoabsorbent assay in 36 HCC patients with nondiagnostic AFP, and 28 cirrhosis patients with no tumour showed that the msAFP was able to discriminate HCC from cirrhosis. This is a promising novel tumor marker for the diagnosis of HCC (177).

There are a number of other HCC tumor markers that currently are research tools and not generally available. These include des-gamma-carboxyprothrombin (DCP), ferritin, carcinoembryonic antigen (CEA), GGT, 5′-nucleotide phosphodiesterase and variants of other enzymes (e.g., α-L-fucosidase (AFU), alkaline phosphatase), which are produced by liver. Potentially, these tumour markers used in conjunction with AFP, could be very helpful in diagnosing more cases of HCC than with AFP alone. A correct diagnosis of HCC was made in 38% of patients by measurement of ferritin alone, and in 83.8% by measurement of AFP alone, but in 92.3% by measurement of a combination of these two tumour markers (178). From conjunction of AFU and AFP in Thailand found that the sensitivity was improved to as much as 82.6% (179).

9.3 Tumour markers of CCA

The most widely studied tumour markers of CCA are cancer antigen 19-9 (CA 19-9) and carcinoembryonic antigen (CEA). CA 19-9 has shown some utility as an aid in the diagnosis of CCA, particularly in patients with PSC. CA 19-9 is elevated in up to 85% of patients with CCA (151). In CCA patients with PSC, a CA 19-9 greater than 100 U/ml has been shown in a case-controlled study to have a sensitivity

and specificity of 75% and 80%, respectively (180). In one series of CCA patients with PSC, an index of CA 19-9 + (40 × CEA) > 400 was shown to have a positive predictive value of 100% in predicting progression to CCA, as well as a specificity of 100%, and a sensitivity of 67% (181). However, CA 19-9 could not discriminate between CCA, pancreatic or gastric malignancy and may also be elevated in severe hepatic injury from any causes (151).

CEA is raised in approximately 30% of patients with CCA. It can also be elevated in inflammatory bowel disease, biliary obstruction, other tumours and severe liver injury. Increased levels of CEA can be detected in the bile of patients with perihilar CCA and intrahepatic cholelithiasis compared with patients with benign strictures, whereas patients with sclerosing cholangitis and choledochal cysts having intermediate levels (151).

There has been interest in the use of CA-125 for the diagnosis of CCA. CA-125 is elevated in 40-50% of CCA patients (151). Several other potential serum tumour markers have been linked to CCA including CA-195, CA-242, DU-PAN-2, IL-6 and trypsinogen-2, but their clinical role is currently unclear.

10. Angiogenesis of liver cancer

Blood vessels are fundamentally composed of endothelial cells, which interconnect to form the tubes that direct and maintain blood flow and tissue perfusion. During embryogenesis, blood vessels develop via two processes; vasculogenesis, whereby endothelial cells are born from progenitor cell types; and angiogenesis, in which new capillaries sprout from existing vessels (182, 183). In the adult, new vessels are produced only through angiogenesis. Notably, the vasculature is quiescent in the normal adult mammal, except for highly orderly processes in the female reproductive cycles (ovulation, menstruation, implantation, pregnancy).

Angiogenesis can be regulated both by inducers and inhibitors of endothelial cell proliferation and migration. The balance of inhibitors and inducers governs the angiogenic switch (Figure 7) (184). The prevailing evidence suggests that changes in the relative balance of inducers and inhibitors of angiogenesis can activate the switch. A net balance of inhibitors over activators would maintain the switch in the off position, whereas a shift to an excess of activating stimuli would turn on angiogenesis.

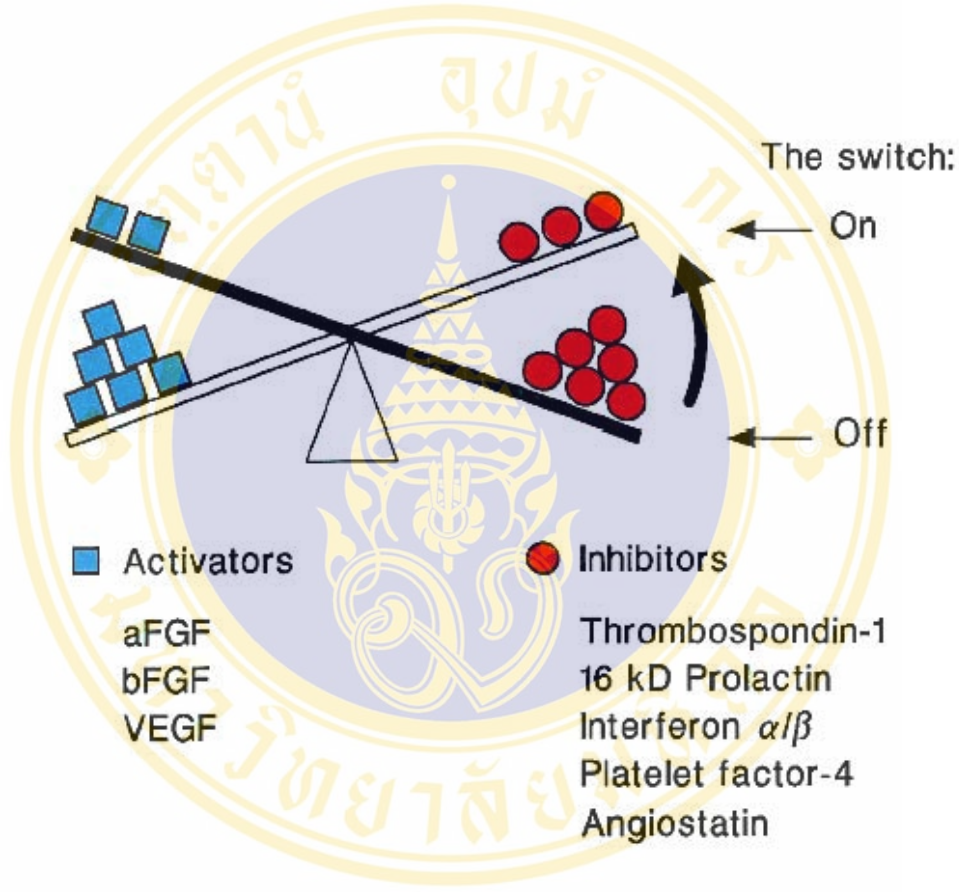


Figure 7. Balance of the angiogenic switch (184)

In general, angiogenesis is “turned off” by the production of more inhibitors than inducers. Outside of female reproductive cycles, angiogenesis in the adult is largely controlled by pathological situations, such as wound healing and tumour growth.

Tumour angiogenesis is the proliferation of a network of blood vessels that penetrates into cancerous growths, supplying nutrients and oxygen and removing waste products. It actually starts with cancerous tumour cells releasing molecules that send signals to surrounding normal host tissue. This signaling activates certain genes in the host tissue that, in turn, make proteins to encourage growth of new blood vessels (Figure 8). Hence, tumour angiogenesis is a prerequisite for tumour growth and metastasis (185).

It is generally accepted that HCC is a hypervascular tumour, and hepatic arterial embolisation has been widely used as an effective therapy for HCC (186). However, HCC develops and progresses from a small-sized and well-differentiated HCC with no developed blood vessels to a larger and moderately or poorly differentiated HCC with a characteristic hypervascularity during the dedifferentiation process (16, 187). In small-sized and well-differentiated HCC, arterial vessels are not well developed, and portal tracts often remain in the tumour. These HCCs often have a blood supply from the portal vein and are not detected by means of angiography (186). On the other hand, larger and poorly differentiated HCCs have well-developed artery-like vessels and are characterised by its hypervascularity. As a result, the angiogenic switch of HCC is on when the tumour develops and progresses to an advanced stage. Studying this angiogenic mechanism and blocking the signals may contribute to the development of effective antitumour therapy, leading to tumour dormancy for HCC (188). Thus far, some angiogenic factors, including vascular endothelium growth factor (VEGF) and angiopoietin, have been reported to promote angiogenesis in HCC (189), but the key factors and detailed mechanisms of the angiogenic switch of HCC are still not well understood at this time.

CCA is relatively hypovascular, in contrast to HCC. CCAs and the surrounding tissue may produce various factors that affect angiogenesis; it depends on the balance of angiogenesis inducers and inhibitors. Enhanced expression of thrombospondin-1 (TSP-1), an antiangiogenetic factor (190), was observed in the fibroblasts of tumor stroma, as well as cancer cells, suggesting that TSP-1 in both cancer cells and tumor

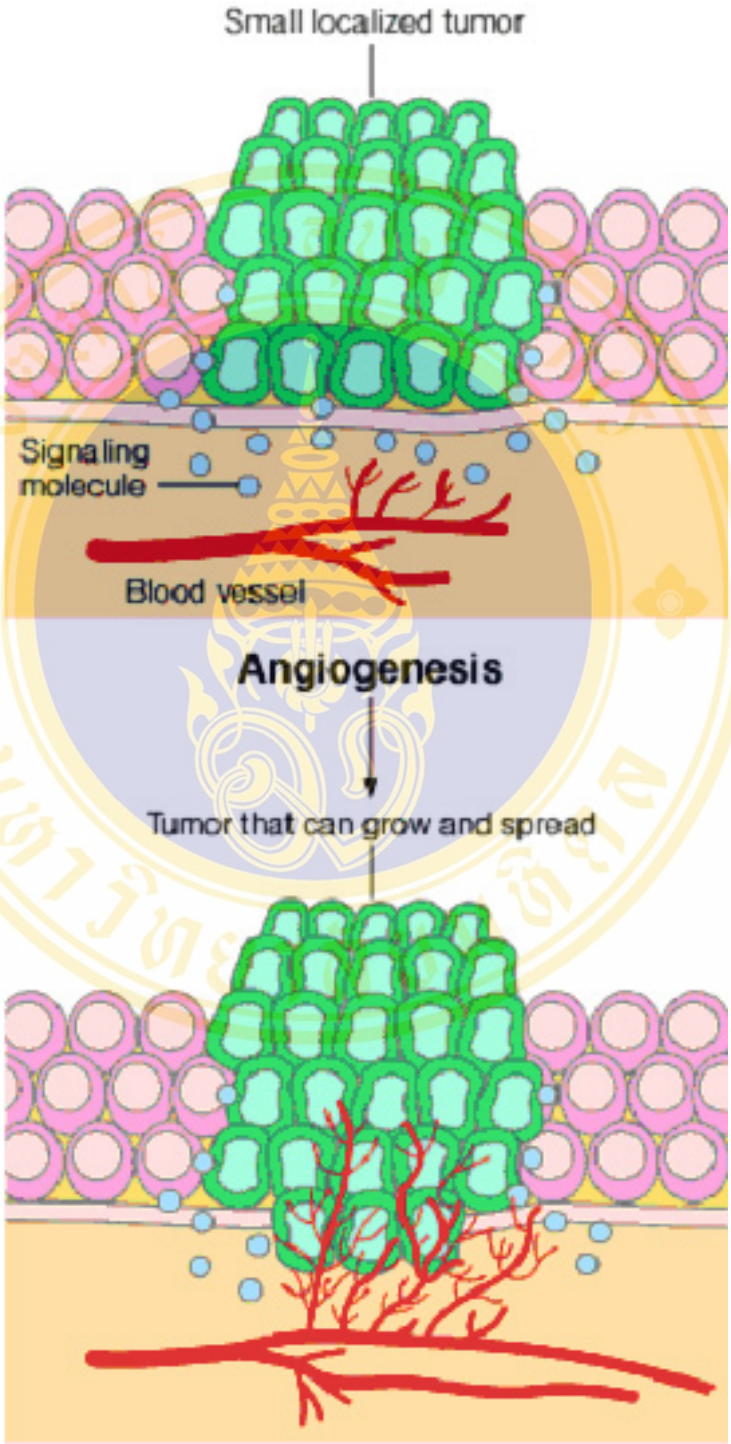


Figure 8. Tumour angiogenesis (191)

stroma may negatively regulate angiogenesis in ICC (190). Up-regulation of TSP-1, together with down-regulation of VEGF in cancer cells, may have a role in the hypovascularity of CCA.

11. Metastatic spread of liver cancer

The detection of metastasis is crucial for patients with liver cancer to receive appropriate therapy, which ultimately determines patient survival. Metastasis of liver cancer has been referred to as a cascade event. Cancer cells, which have multiple genetic abnormalities and grow unregulated, lose their ability to adhere to each other. This, together with their capability to stimulate angiogenesis, provides a means for entry to the blood and lymphatic circulation. In the case of blood circulation, these cells can circulate in the body until settling on the vascular endothelium and leaving the circulation (extravasation) (Figure 9) (192).

In HCCs, intrahepatic metastasis occurs early and more than half of these tumours metastasise to extrahepatic sites, usually the lungs, adrenals or lymph nodes (192). Katyl *et al*. (194) found that lung was both the most frequent site of metastasis (55%) and the most frequent site of the first detectable metastasis (39%). In addition, lymphatic spread of HCC was common (53%). Seventy percent of the patients had involvement of the regional lymph nodes, and the remaining (23%) had distant lymph node involvement. Systemic hematogenous metastases to other sites were documented in the bone (28%) and in the adrenal glands (11%).

In CCA, metastasis is common and occurs to many organs, with 50% of patients at autopsy having hematogenous spread to the lungs, vertebrae, adrenal glands and brain (158). ICC has been reported to metastasise to lymph nodes in 47-58% of cases (195).

From several studies concerning liver cancer metastases in the molecular level show that the molecular events of liver cancer metastasis were similar to that of other solid cancers. Factors that positively related to metastasis included: *p16* and *p53* mutation, *H-ras*, *c-erbB-2*, *mdm2*, TGF-α, EGFR, matrix metalloproteinase-2 (MMP-2), urokinase-type plasminogen activator (uPA), its receptor (uPAR) and inhibitor (PAI-1), intracellular adhesion molecule-1 (ICAM-1), vascular endothelial growth factor (VEGF), platelet-derived endothelial cell growth factor (PD-ECGF), basic

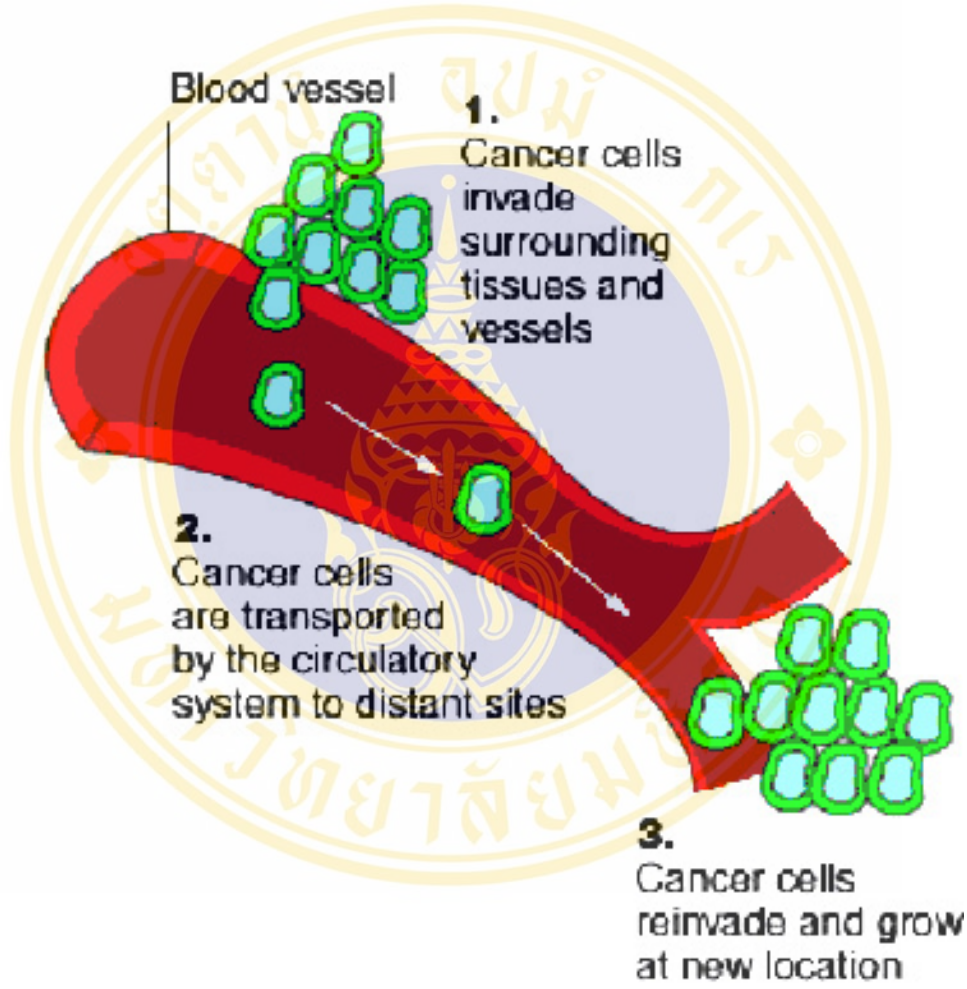


Figure 9. Metastatic spread of cancer cells (192)

fibroblast growth factor (bFGF), etc. These findings provide clues to develop biomarkers for prediction of metastatic recurrence as well as new targets for treatment and prevention of metastatic recurrence.

12. Thrombosis and cancer biology

The association of thrombosis and cancer owes its origin to the observations of Professor Armand Trousseau (196), who noted that patients who present with idiopathic venous thromboembolism (VTE) frequently harbor an occult cancer. Trousseau also observed that patients with known cancer have an increased propensity to acquire VTE. Risk for symptomatic VTE in surgical patients, determined by retrospective analysis of the data from control groups in large, randomised, controlled trials of anticoagulant prophylaxis, appears to be higher in those with cancer than in those without cancer (197). The rates of recurrence of VTE have recently been studied in a large, prospective 11-year trial (198), and shown to be significantly increased in patients with cancer and patients without cancer. The reverse is also true: patients with true idiopathic VTE have a significantly higher risk (fourfold to ninefold) of having an underlying carcinoma diagnosed within 6 months to 1 year of the presentation of symptomatic VTE (199).

In HCC, portal vein tumour thrombosis (PVTT) can be detected in 30% to 62.2% of patients (200). Macroscopic tumour thrombus in portal vein appears to be the terminal stage of HCC, and is associated with the threat of bleeding of the esophageal varices, or liver failure (201). The natural history of untreated HCC with PVTT is very poor. The median survival of such patients was reported to be 2.7 months, whereas survival in those without PVTT was 24.4 months (202). For CCA, there's a report on a CCA patient who developed widespread thromboembolism during disease progression (203).

The pathogenesis of VTE in cancer is complex but relates principally to the procoagulant properties of tumour cells themselves, tumor-associated endothelial cells, and host inflammatory cells. In addition, an unfortunate concatenation of abnormalities of the normal defense mechanisms (*eg*, stasis, vascular defects, reduction in circulating inhibitors, and cell-associated anticoagulants and fibrinolytic activators) predisposes patients with cancer to hypercoagulability (204).

13. Enzyme-linked immunosorbent assay (ELISA)

Serological methods are displaying an increasingly important role in the diagnosis and epidemiology assessment of diseases. There has been the development of methods which use labeled antigens or antibodies, resulting in tests with very high levels of sensitivity and specificity. Enzyme linked immunosorbent assay (ELISA) was first introduced by Engvall and Perlman in 1971 (205), has become the most popular immunoassay used in research laboratories today. This assay is a method for detection and quantitation of either antigens or antibodies in the blood and tissue with great sensitivity and specificity. ELISA can be broadly divided into competitive and non-competitive assays and some of the variations are shown in Figure 10 and 11.

Enzymes used in this technique should have a low relative molecular weight, high stability, they should bind covalently to antibodies and to various functional groups of antigens, the product of the enzyme reaction should be coloured or otherwise readily detectable (206).

The two most popular enzymes for ELISA are alkaline phosphatase and horseradish peroxidase. Both enzymes have excellent stability, high turnover numbers, consistent conjugation, and a variety of substrates. However, each enzyme has its drawbacks. Peroxidase is inhibited by azide, which is used often as a preservative in biochemical buffers. Alkaline phosphatase has a poorly visible chromogenic substrate, *p*-nitrophenyl phosphate, and may occur as an endogenous enzyme in some biological samples. However, alkaline phosphatase has a fluorescent substrate, methyl umbelliferyl phosphate, that increases the sensitivity of phosphatase significantly, but requires an expensive fluorometer. Thus, it normally is used only for high-volume sensitive assays (206).

13.1 Sandwich ELISA

One of the most useful of the non-competitive ELISA is the two-antibody “sandwich” ELISA. The sandwich ELISA measures the amount of antigen between two layers of antibodies (Figure 11b) (206). The antigens to be measured must contain at least two antigenic sites, capable of binding to antibody, since at least two antibodies act in the sandwich.

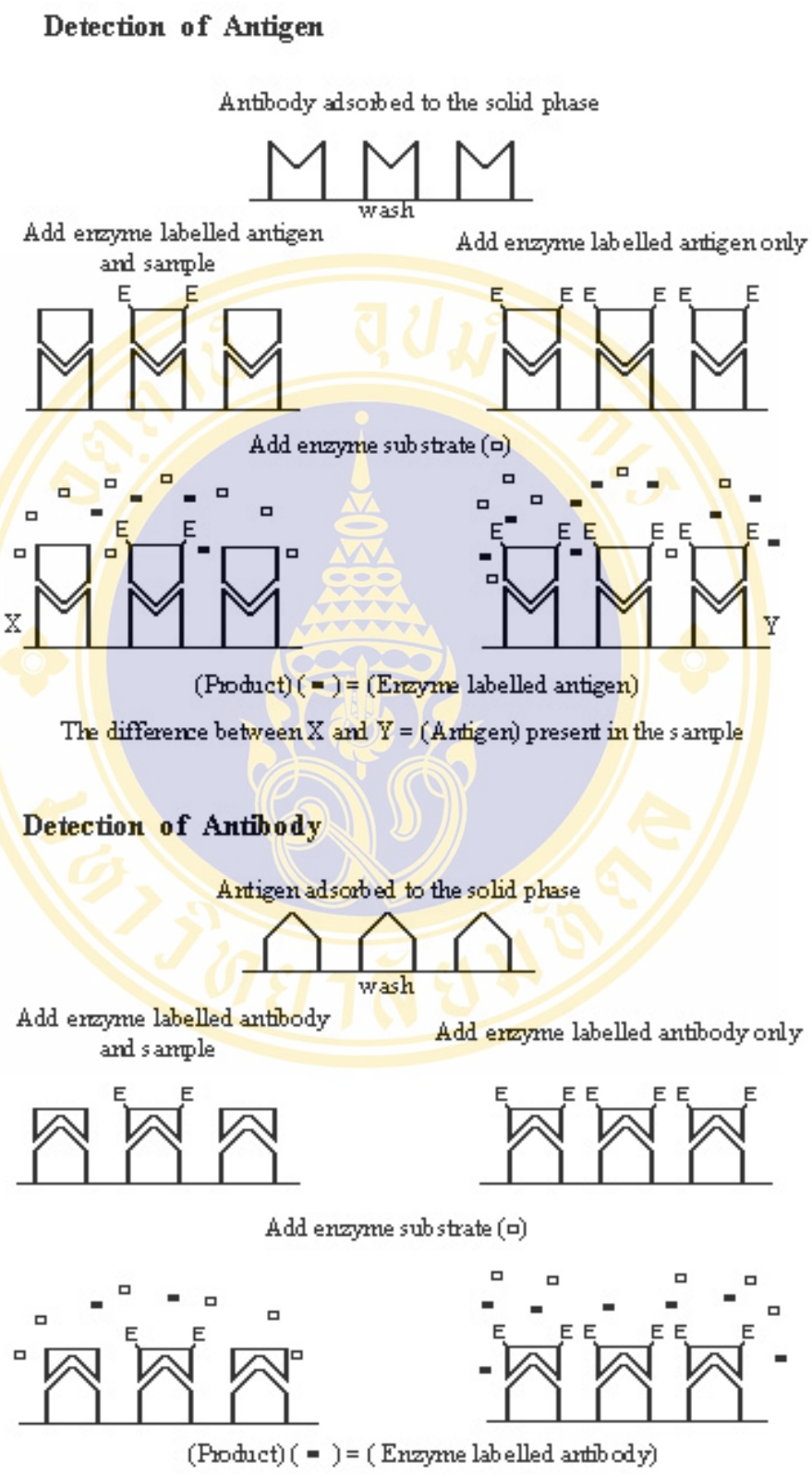


Figure 10. Competitive ELISA for detection of antigen and antibody (206)

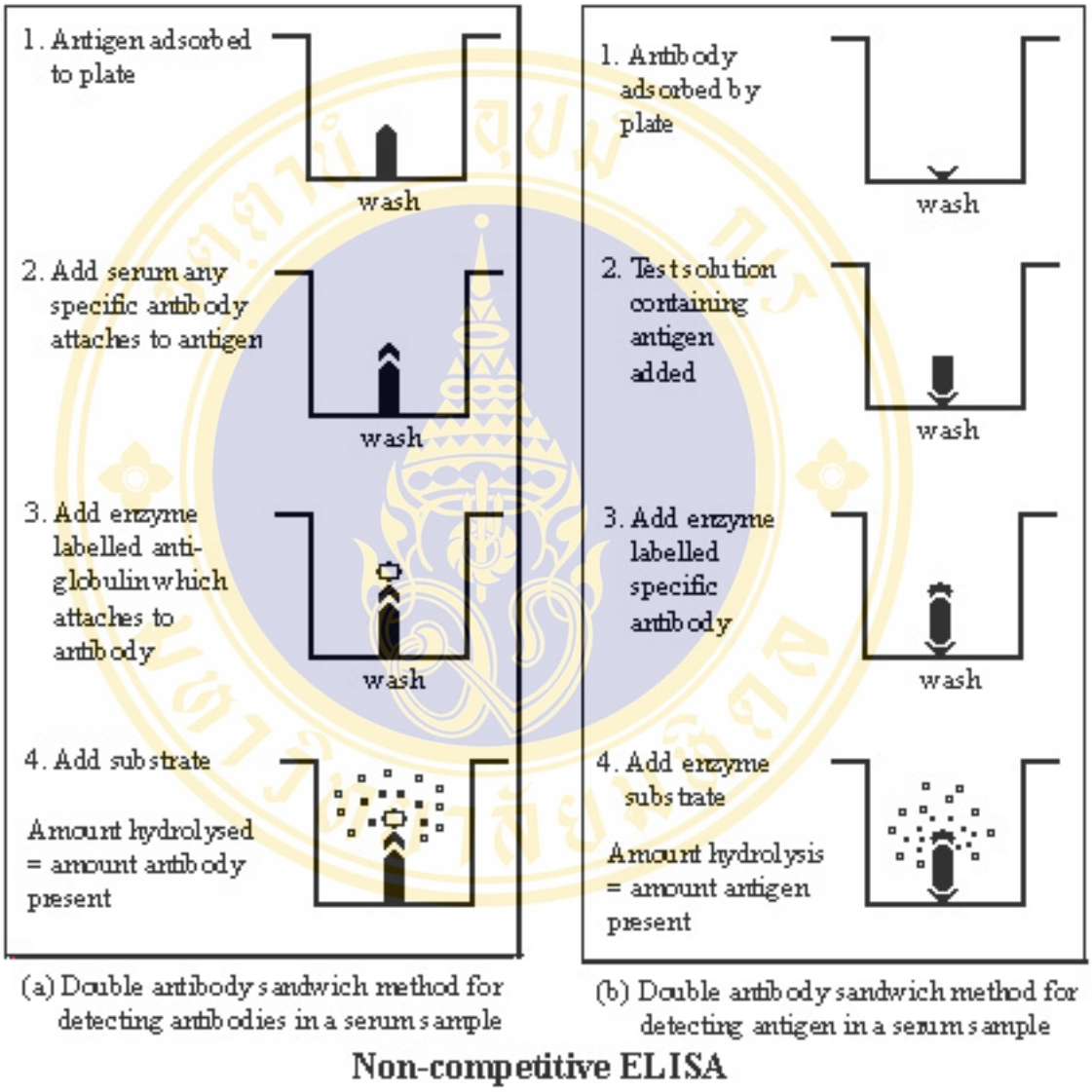


Figure 11. Non-competitive ELISA for detection of antibody (a) and antigen (b) (206)

One antibody (the capture antibody) is purified and bound to a solid phase. Antigen is then added and allowed to complex with the bound antibody. Unbound products are then removed with a wash buffer that prevents nonspecific binding of the serum proteins to any available plastic sites. The enzyme-labeled second antibody (the detection antibody) is allowed to bind to the antigen, thus completing the “sandwich”. The assay is then quantitated by measuring the amount of labeled second antibody bound to the matrix, through the use of colorimetric substrate (206).

Sandwich ELISAs for quantitation of antigens are especially valuable when the concentration of antigens is low and/or they are contained in high concentrations of contaminating protein. The sensitivity of the sandwich ELISA is dependent on four factors: the number of molecules of the first antibody that are bound to the solid phase, the avidity of the first antibody for the antigen, the avidity of the second antibody for the antigen and the specific activity of the second antibody (206).

13.2 Biotin-avidin system

The analysis of tissue or blood cells was frequently done by directly labeled antibody- or double antibody-detection test systems. In the presence of high levels of antigen the analysis presented no problem even with directly labeled antibodies. However, detection of low levels of antigen posed a problem of detection limits. Therefore, the use of a biotin-avidin-enzyme complex has been introduced to increase the sensitivity of ELISA (207).

Avidin is a basic glycoprotein (MW=67,000) found in egg white and tissues of birds, reptiles and amphibians (208, 209). It has an extraordinarily high affinity for biotin and binds four biotin molecules to each molecule of avidin. The four binding sites together with the high affinity of avidin-like proteins for biotin serve as an aid in amplifying the sensitivity of immunoassays, which have become wide-spread as a general tool in all field of biology (210). The association of biotin to tetrameric avidin appears to be mediated by an extensive set of hydrogen bonding and van der Waals interactions, which make the association process practically irreversible (211).

Biotin is a small (MW=244) water-soluble vitamin. Biotin can be conjugated with various proteins (biotinylation), and avidin conjugated with various markers such as the enzymes used in ELISA. Since many biotin molecules can be bound to a single protein (e.g. antibody) molecule, a biotinylated protein will bind to a number of

enzyme-linked avidin molecules and hence, provide amplification of the enzyme signal (207-209). A high degree of biotinylation of antibodies can be achieved without affecting the antigen binding ability of the antibody.

A further modification of the system, using streptavidin in place of avidin, has been described (Figure 12) (212, 213). Streptavidin is a 60 kDa tetrameric protein, obtained from *Streptomyces avidinii*. It is composed of four identical chains, each 159 amino acid residues long (214). Like avidin, streptavidin binds biotin at four binding sites with an extremely high binding constant (215). Streptavidin is more often used than avidin because it is reported to cause less non-specific binding than avidin (212, 213, 216).

14. Tissue factor (TF)

A theory of blood coagulation was established in the 19th century which involved the interaction of prothrombin, calcium ions, fibrinogen and a fourth factor called thrombokinase, today referred to as thromboplastin or, more commonly, tissue factor (TF) (217).

TF is a 47 kDa transmembrane glycoprotein that functions as a cellular receptor for coagulation factor VII (FVII). It plays a pivotal role in initiating the blood coagulation cascade by serving as a cofactor for activated factor VII (FVIIa). The resulting TF-FVIIa complex proteolytically activates coagulation factor IX and X, triggering downstream coagulation pathways that ultimately lead to conversion of fibrinogen to fibrin and clot formation (Figure 13) (5, 218). TF gene spans 12.4 kbp and is located on chromosome 1 (1p21-22) (5). It encodes for a 263 amino acid single chain polypeptide with three different domains: a 219 amino acid extracellular domain, a 23 amino acid transmembrane domain and a 21 amino acid intracellular domain.

In healthy individuals, endothelial cells and monocytes lack detectable TF expression, while extravascular cells in the subendothelial layer of the vessel wall show constitutive TF expression ready to function as a haemostatic barrier to prevent blood loss. During normal haemostasis, TF is not in contact with the circulation, but during tissue injury, the membrane-bound TF is locally expressed and subsequently activates the coagulation cascade (218).

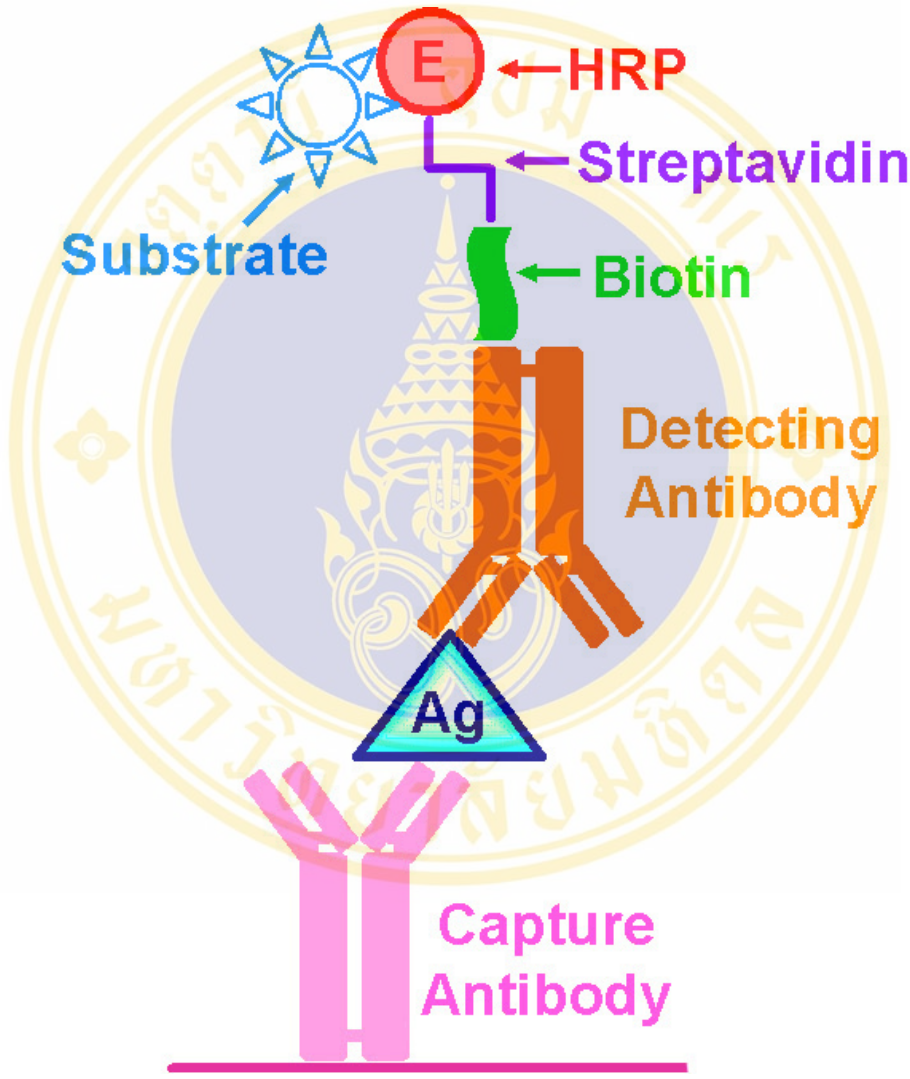


Figure 12. Streptavidin-biotin system in sandwich ELISA

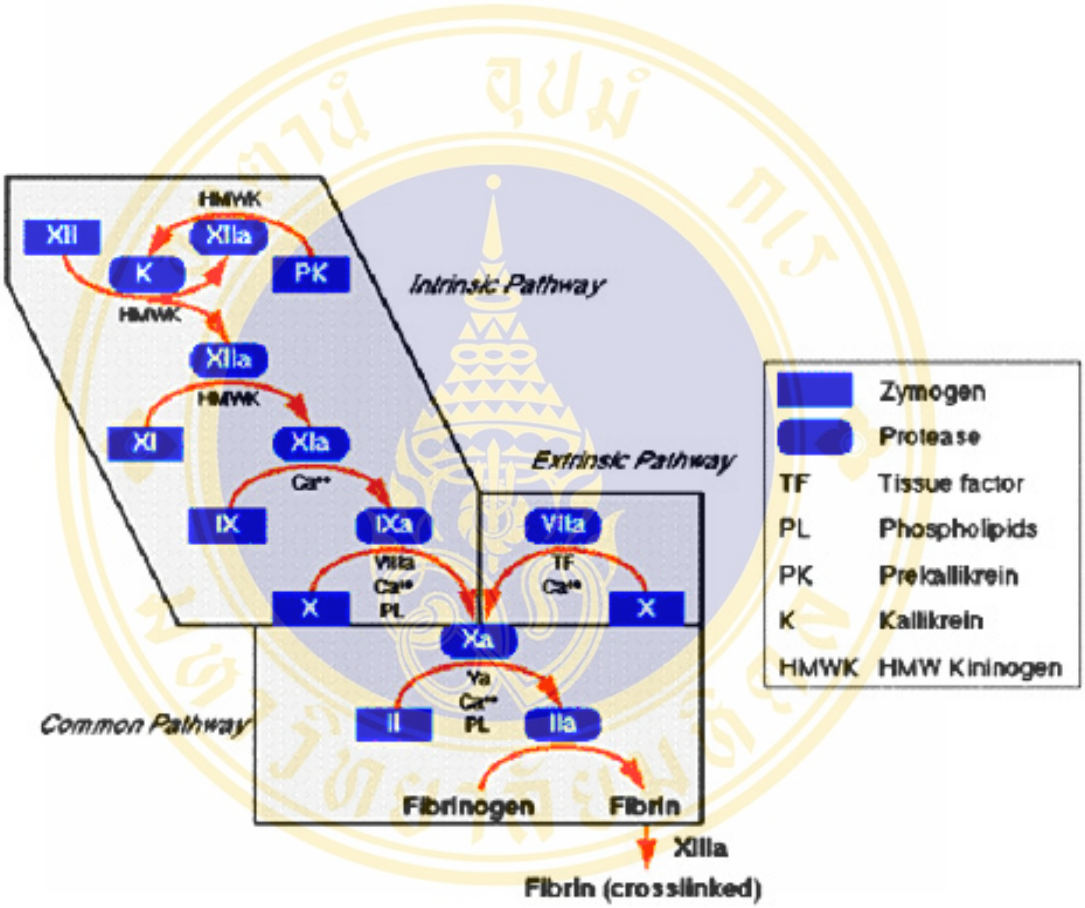


Figure 13. Coagulation cascade (218)

TF plays a role outside haemostasis in vascular development and angiogenesis (both physiologic and pathologic). It is well established that TF deficiency in the transgenic mouse causes embryonic lethality by day 10.5 due to impaired vascular integrity and abnormal development of yolk sac (219). TF is expressed in several types of human cancer such as non-small cell lung cancer (NSCLC), breast cancer, colorectal cancer and prostate cancer (9-15). Moreover, direct correlation between elevated TF expression and advanced stages of malignancy has been confirmed in several types of cancers, including NSCLC (13), breast cancer (220), pancreatic cancer (221), glioma (222), prostate cancer (14) and colorectal cancer (11, 12, 15, 223). The high TF-expression in many tumour cells may be one of the mechanisms leading to hypercoagulable state of malignancy (6).

Experimental studies have demonstrated that TF also plays an important role in tumour invasion and metastasis (7, 8). Expression of TF has been found to have a significant correlation with metastatic potential in human lung (9), breast (10), and colorectal (11, 12) carcinomas. The neovessels in a tumour not only provide oxygen and nutrients for tumour growth, but they also provide the route for tumour cell invasion into the circulation. Several studies demonstrated that tumour expression of TF is significantly related to tumour angiogenesis in lung (13) prostate (14) and colorectal (15) carcinomas. The switch to an angiogenic phenotype requires a shift in balance between proangiogenic factors and antiangiogenic factors that regulate vessel growth and development (17). Aberrant expression of TF in tumours contributes to the angiogenic phenotype in part by up-regulating the expression of the proangiogenic protein VEGF and down-regulating the expression of the antiangiogenic protein thrombospondin (15, 17).

Elevated TF expression in tumours has been correlated with unfavorable prognostic indicators, such as increased angiogenesis, advanced stage of disease, and the multidrug resistant phenotype (224), which contribute to poorer survival rates in cancer patients. However, little is known about TF in HCC and CCA. Only one recent study shows that TF in HCC tissues is found to correlate with VEGF, microvessel density (MVD), the presence of venous invasion, presence of microsatellite nodules, absence of tumour capsule and advanced TNM stage in HCC (18).

CHAPTER III
MATERIALS AND METHODS

1. Materials

Most of the stock solutions used in this study were prepared according to instructions in "Immunology Methods Manual: The Comprehensive Sourcebook of Techniques" (225), unless otherwise stated. Many of the experimental protocols followed can also be found in this manual. Reagents were supplied by sigma, Scientific or Merck BDH. Enzyme conjugate, enzyme conjugate diluent, tetra-methylbenzidinec (TMB), capture antibody, detection antibody and standard TF were supplied from American Diagnostica Inc.

The materials used in each experiment were carefully selected to conform to standard laboratory safety procedures. The processes of cleansing or sterilisation either before or after in vitro experiment were strictly adhered according to the manufactures' instructions and/or the good local laboratory codes of practice. The details of materials and recipes of solutions commonly used in the experiments are described in Appendix A.

2. Study population

Blood samples were provided by Department of Surgery. Details of HCC and CCA patients are listed in Appendix B and C, respectively. The study was performed in adult Thai population. The control group consisted of 25 healthy volunteers. The study groups were 57 HCC patients and 31 CCA patients. Ethical Committee approval was obtained for the study and informed consent was sought from each patient on admission or attending at Siriraj Hospital.

3. Preparation of plasma from blood samples

The EDTA-anticoagulated blood was centrifuged at 3000 rpm for 10 minute and the supernatant was collected. The plasma samples were stored at –20°C until used.

Frozen plasmas were then thawed at 37°C for 15 minutes and diluted 1:4 in sample buffer**.**

3. Enzyme Linked Immunosorbent Assay (ELISA)

To measure the level of TF in plasma, a quantitative sandwich enzyme-linked immunoassay technique was employed (Figure 14).

To coat the capture antibody (murine anti-human TF monoclonal antibody) on ninety six-well microtiter plates, the capture antibody was diluted 1/100 in coating buffer, immediately added 100 µl to every well in the microtiter plates and incubated overnight at 4°C. Then, 150 µl of blocking buffer was added to every well to block nonspecific adsorption of protein and incubated for 90 minutes at room temperature. Standard TF and plasma samples prepared as described in Appendix A was applied to the plate directly, 100 µl/well, in duplicate and the plate was incubated at room temperature for 3 hours. A typical plate format was shown in Figure 15.

The plate was then washed 4 times with washing buffer. Subsequently, 100 µl of detection antibody (biotinylated anti-human TF antibody fragment) was added to each well. The plate was incubated for 60 minutes at room temperature and washed 4 times with washing buffer. Then 100 µl of diluted enzyme conjugate (streptavidin-horseradish peroxidase) was added to each well. The plate was incubated for 60 minutes at room temperature and washed 4 times with washing buffer. After that, 100 µl of substrate (TMB) was added to each well and the plate was incubated for 20 minutes at room temperature. A blue colour developed because we used peroxidase as the enzyme for colour development (Figure 16a). The enzymatic reaction was stopped by adding 50 µl of 0.5 M H_2SO_4 to every well. The solution colour then turned yellow (Figure 16b). The absorbances were read on a Bio-Tek microplate reader at wavelength of 450 nm within 30 minutes. Detection limit of this ELISA was 10 pg/ml (226).

4. Representative standard curve

KinetiCalc was a software program designed to enhance the functionality of the Bio-Tek microplate reader and used for construction of standard curve. The standard

curve was constructed by plotting the mean absorbance value for each tissue factor standard (0 pg/ml, 50 pg/ml, 100 pg/ml, 200 pg/ml, 500 pg/ml and 1000 pg/ml) versus the corresponding concentration of tissue factor in pg/ml. Unknown values were interpolate directly from the standard curve. A standard curve was generated each time the assay was performed.

5. Statistical Analysis

One-way analysis of variance (One-way ANOVA) and unpaired t-test was used to compare plasma TF levels in HCC patients, in CCA patients and in control group. The statistical correlation between continuous variables was tested using the Sperman correlation coefficients. The Mann-Whitney U test and Kruskal-Wallis test were used to assess the statistical correlation between the various clinicopathological factors and plasma TF levels. Probability values (*p* values) less than 0.05 were regarded statistical significant. All statistical analyses were performed using statistical software (SPSS 13.0 for windows).

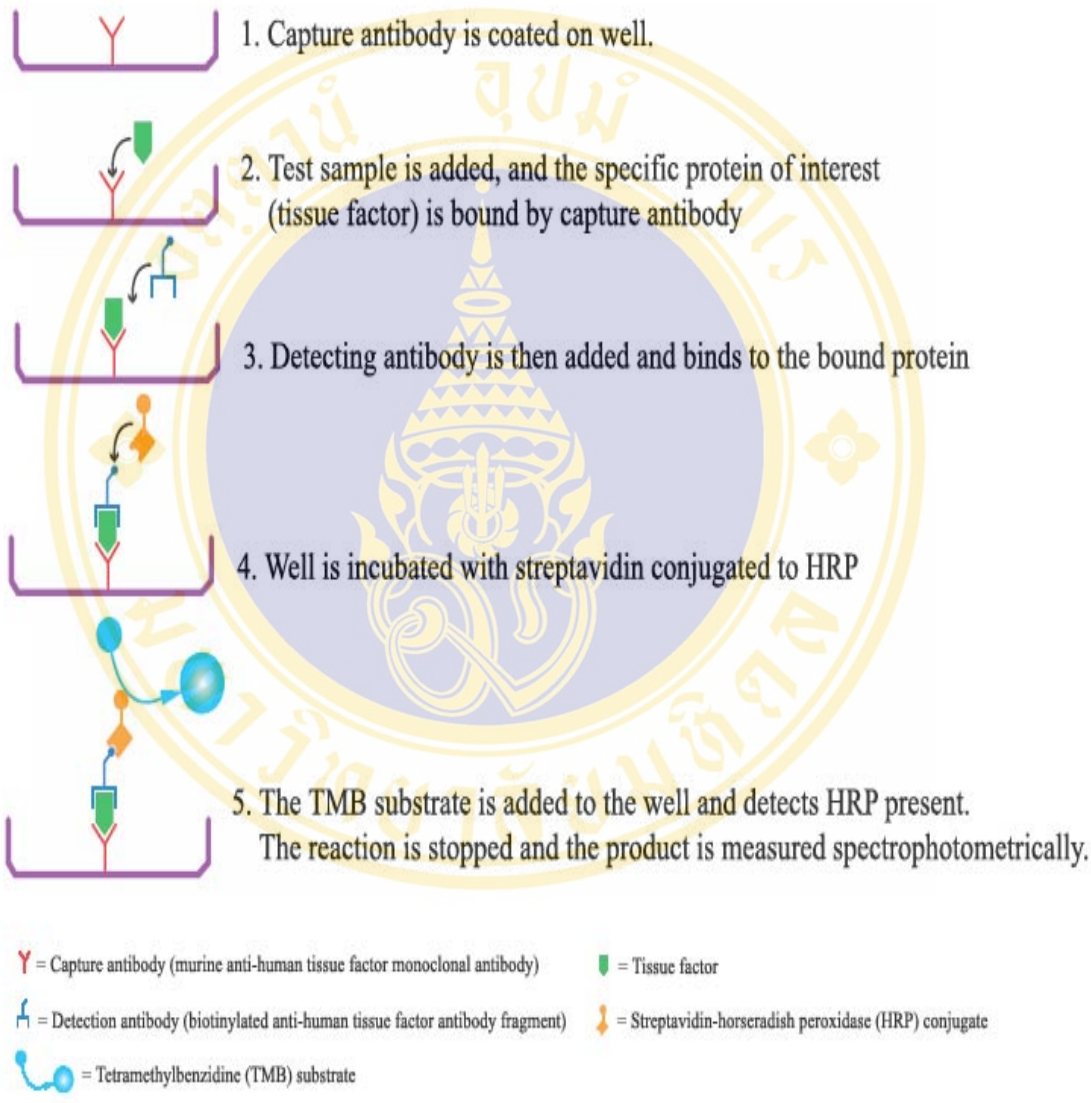


Figure 14. Schematic of sandwich ELISA for determination of plasma TF level

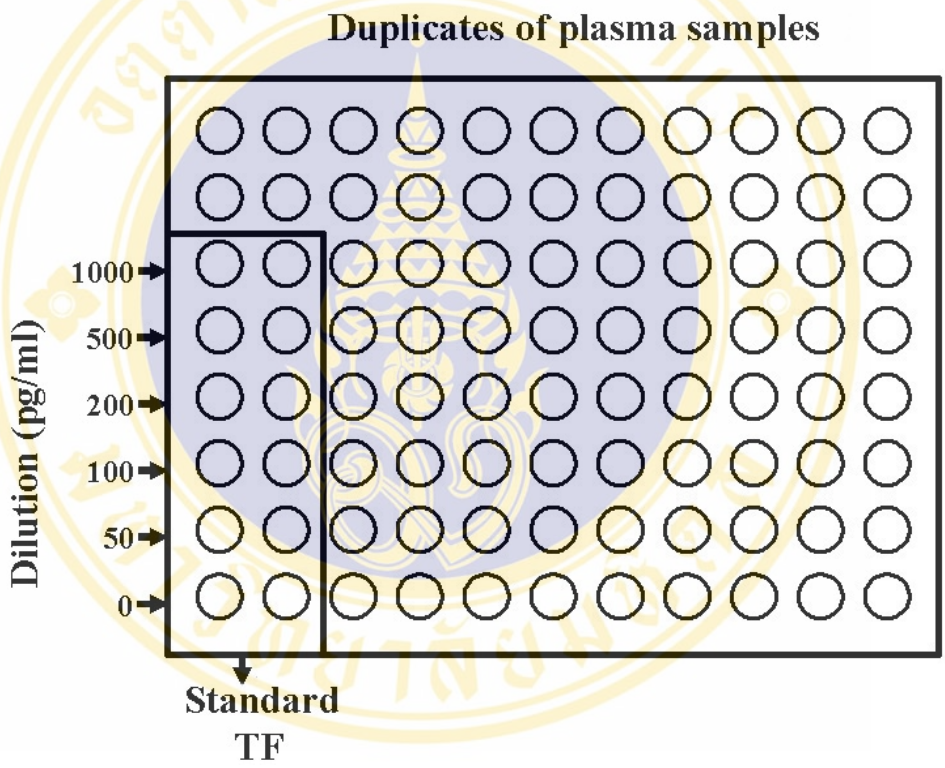
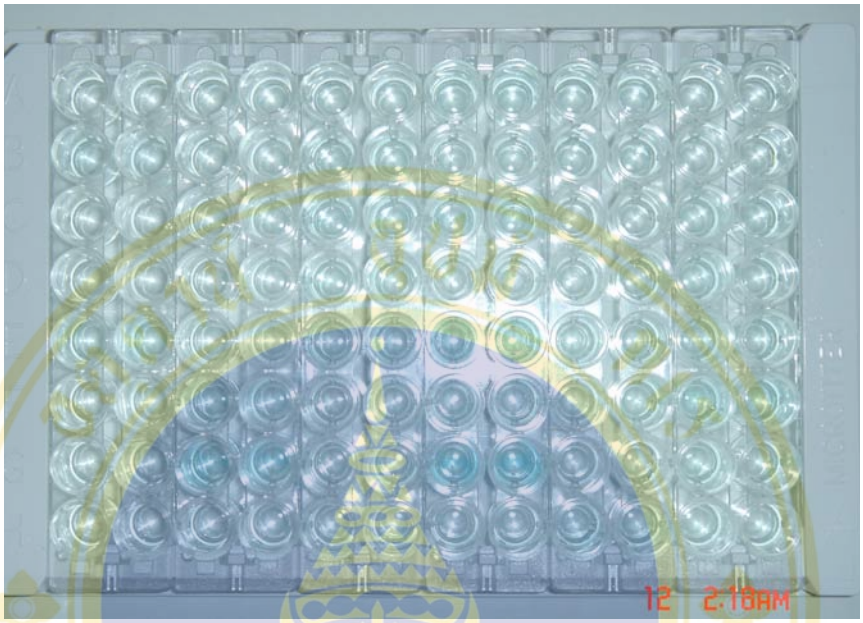


Figure 15. Plate layout for comparison of plasma samples with standard TF

(a)



(b)

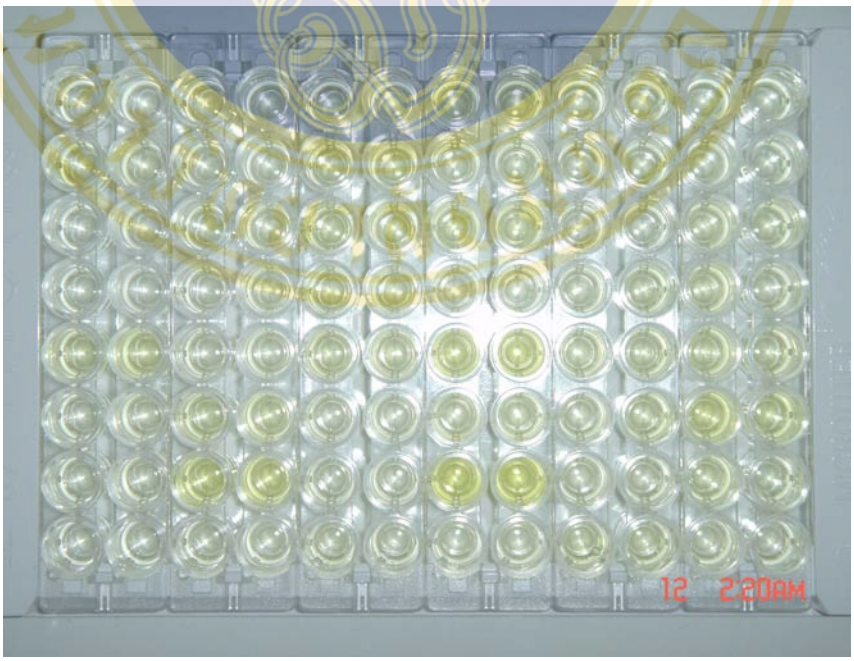


Figure 16. Representation of plate of sandwich ELISA

(a) blue colour developed after peroxidase was added

(b) yellow colour developed after M H_2SO_4 was added

CHAPTER IV
RESULTS

1. Standardisation of the assay

After the absorbances of standard TF were read on the Bio-Tek microplate reader at wavelength of 450 nm, the standard curve was constructed by KinetiCalc software program. The standard curve was the plotting of the mean absorbance value for each TF standard versus the corresponding concentration of TF in pg/ml which, from this experiment, the data produced a straight line (Figure 17). Consequently, absorbance units were converted into TF concentration by this standard curve. To achieve reproducible results, consistent incubation times, identical buffers and reagents, and standard curves had been done for every plate.

2. Plasma TF level in HCC

To determine the significance of circulating TF level in HCC patients, TF level was measured in plasma samples of 25 healthy volunteers and 57 patients with HCC. Since we used diluted samples, we then multiplied the value from the standard curve by the dilutional factor to calculate the corrected sample values.

The median plasma TF level of 57 HCC patients measured by streptavidin-biotin system ELISA was 711.72 pg/ml and ranged from 148.38 pg/ml to 3997.88 pg/ml while that of healthy volunteers was 23.192 pg/ml and ranged from 23.19 pg/ml to 711.72 pg/ml. From statistical analysis, we found a statistically significant difference (p = 0.000) between plasma TF level of HCC patients and healthy volunteers (Table 7).

3. Plasma TF level in CCA

To determine the significance of circulating TF level in CCA patients, TF level was measured in plasma samples of 31 patients with CCA. The median plasma TF level of CCA patients measured by streptavidin-biotin system ELISA was 367.46

pg/ml and ranged from 148.38 pg/ml to 1650.62 pg/ml (Table 7). Statistical analysis demonstrated that CCA patients showed significantly higher plasma TF concentration than normal controls (p=0.000). In addition, plasma TF level of HCC patients was significantly higher than plasma TF level of CCA patients (p = 0.003). Figure 18 showed a box plot of plasma TF level in HCC patients, CCA patients and controls.

4. The correlation between plasma TF level and clinicopathological prognostic factors of HCC

To investigate the correlation between preoperatively plasma TF level of HCC patients and clinicopathological prognostic factors, we performed a statistical analysis using several related prognostic factors of HCC (Table 8, 9). We found that the age of HCC patients was significantly correlated with plasma TF level (p = 0.010, correlation coefficient = 0.336) (Figure 19). The older the patients were, the higher the plasma TF level was. Conversely, other clinical parameters of HCC patients listed in Table 8 were not significantly correlated with plasma TF level. Table 9 showed relationship between plasma TF level and tumour characteristics together with sex, HBsAg and cirrhosis. Plasma TF level was significantly higher in HCC patients with cirrhosis than in those without cirrhosis (p = 0.037). However, plasma TF level was not significantly associated with sex, presence of HBsAg, tumour size, clinical stage and tumour grading. The difference in plasma TF level examined between stage II, III and IV was not significant (p = 0.541), but the mean value of plasma TF level in stage IV tended to be higher than in stage III and II. Similarly, in accordance with tumour grading, the difference in plasma TF level examined between grade I, II, III and IV was not significant (p = 0.263). Plasma TF level of grade IV tumour seemed to be higher than that of grade I, but the number of grade I patients was too small to make an adequate comparison. The prognostic factors representing tumour extension and metastasis (i.e., vascular invasion, perineural invasion and lymphatic invasion) were not associated with plasma TF level either.

5. The correlation between plasma TF level and clinicopathological prognostic factors of CCA

In the same way, to examine whether preoperatively plasma TF level of CCA patients was correlated with clinical prognosis, we performed a statistical analysis including almost all of the related prognostic factors of CCA. Table 10 showed the correlation between plasma TF level and the clinical parameters in CCA. Plasma TF was significantly correlated with hematocrit (Hct) in a positive way (p = 0.027, correlation coefficient = 0.411) (Figure 20). Therefore, the more Hct the patients had, the higher the plasma TF level was. Plasma TF levels were also significantly correlated with prothrombin time (PT) in a negative way (p = 0.027, correlation coefficient = -0.411) (Figure 21). As a result, the lower PT the patients had, the higher the plasma TF level was. Although plasma TF level in CCA patients was significantly higher than normal individuals, it was not associated with sex, tumour size, clinical stage and tumour grading (Table 11). However, plasma TF level is likely to rise in CCA patients with higher stage though they are not statistically significant (p = 0.058). Higher plasma TF level was found in male than female patients but without statistically significant (p = 0.056). The three prognostic factors including lymphatic invasion, vascular invasion and perineural invasion were not related with plasma TF level either. Nevertheless, the number of patients was too small to make an adequate comparison.

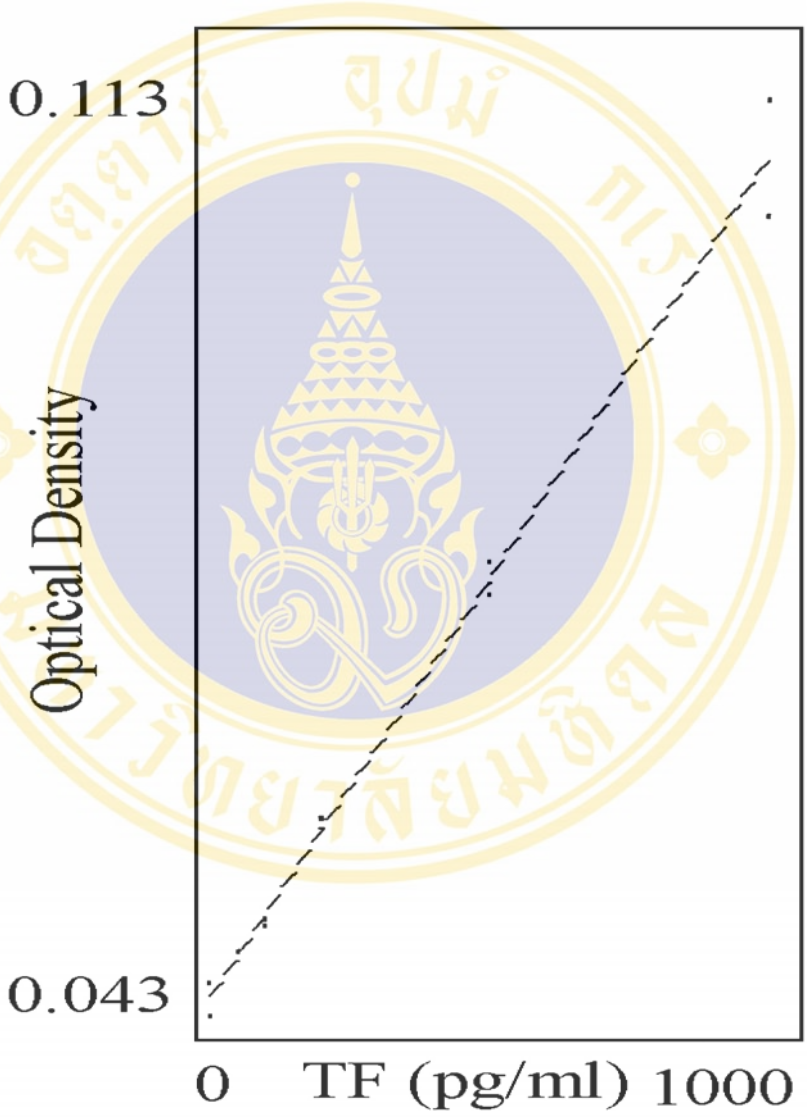


Figure 17. Standard curve from 6 concentrations of standard TF

Table 7. Plasma TF level in HCC patients, CCA patients and control group

	n	Median	Mean±SD	Min	Max	*P*
HCC	57	711.72	904.71±707.87	148.38	3997.88	0.000
CCA	31	367.46	495.67±350.69	148.38	1650.62	0.000
Control	25	23.19	123.34±174.72	23.19	711.72	

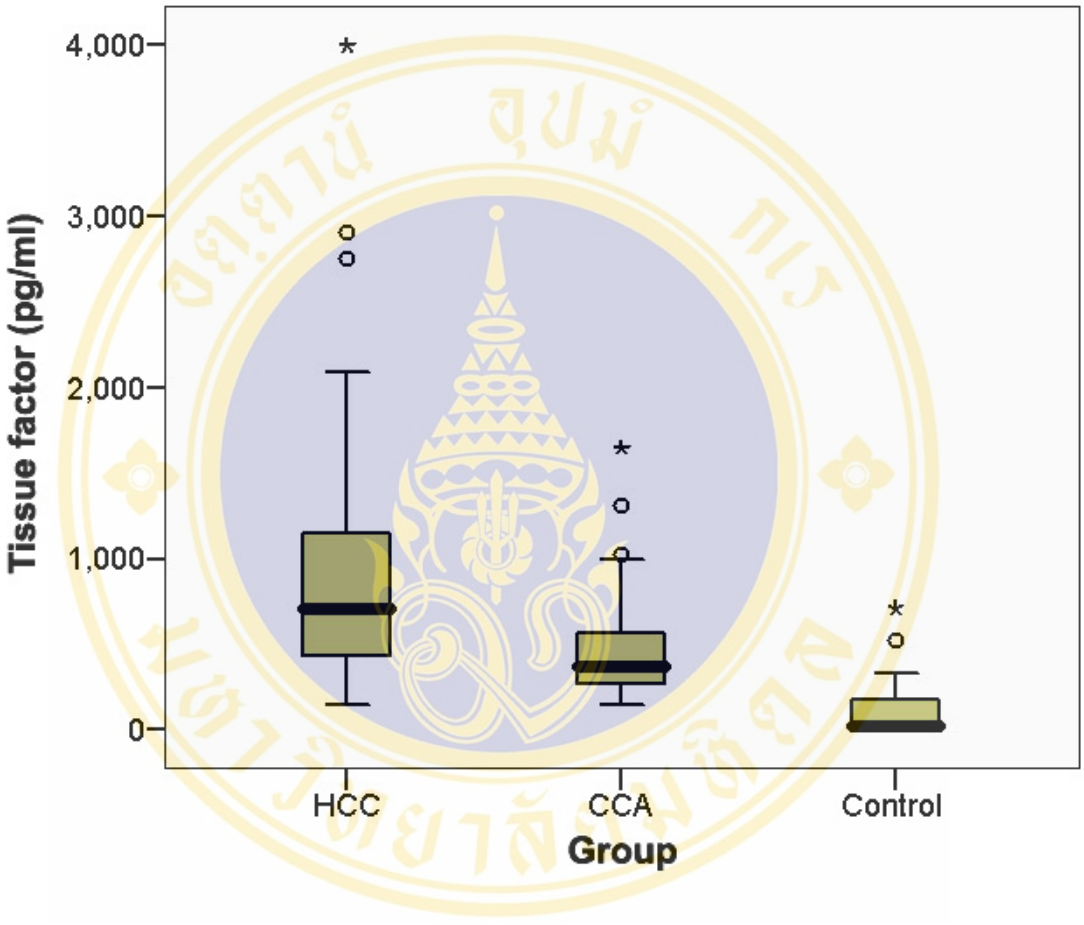


Figure 18. Box plot of plasma TF level in HCC patients, CCA patients and control group

The bold lines within the box indicate the median values. The top and bottom horizontal lines of the box indicate the 25th and 75th percentiles, respectively. The top and bottom horizontal bars indicate data within 1.5 times the interquatile range. The circles indicate data between 1.5 times to 3 times the interquartile range. The asterisks indicate data higher 3 times the interquartile range.

Table 8. Correlation between plasma TF level and clinical parameters in HCC

	Mean±SD	Min-Max	Correlation coefficient	*P*
Patient age (years)	53.30±11.59	23-76	.336	0.010*
AFP (IU/ml)	11,106.65±34,016.28	2-204,800.00	-0.026	0.845
Hct (%)	38.29±6.28	21.00-48.40	0.075	0.577
Platelets (10e3/µl)	205.96±126.78	45.00-745.00	-0.068	0.613
PT (sec)	12.39±1.46	9.70-16.00	0.210	0.117
aPTT (sec)	28.77±3.13	22.80-35.50	-0.016	0.904
TB (mg/dl)	0.94±0.56	0.30-3.10	-0.086	0.523
DB (mg/dl)	0.34±0.35	0.00±1.60	0.056	0.680
SGOT (U/I)	73.70±77.17	21.00-525.00	-0.077	0.571
SGPT (U/I)	62.47±77.90	16.00-577.00	0.019	0.890
ALP (U/I)	136.19±72.26	50.00-420.00	0.038	0.778
GGT (U/I)	174.61±166.47	18.00-960.00	0.029	0.828
Albumin (g/dl)	4.21±4.61	0.40-38.00	-0.137	0.308

* $p < 0.05$

Table 8. Correlation between plasma TF level and clinical parameters in HCC (continued)

	Mean±SD	Min-Max	Correlation coefficient	*P*
Total protein (g/dl)	7.48±1.04	3.40-9.90	0.047	0.726
BUN (mg/dl)	13.23±5.00	4.00-28.00	-0.010	0.942
Cr (mg/dl)	0.94±0.43	0.40-3.20	0.049	0.719

* $p < 0.05$

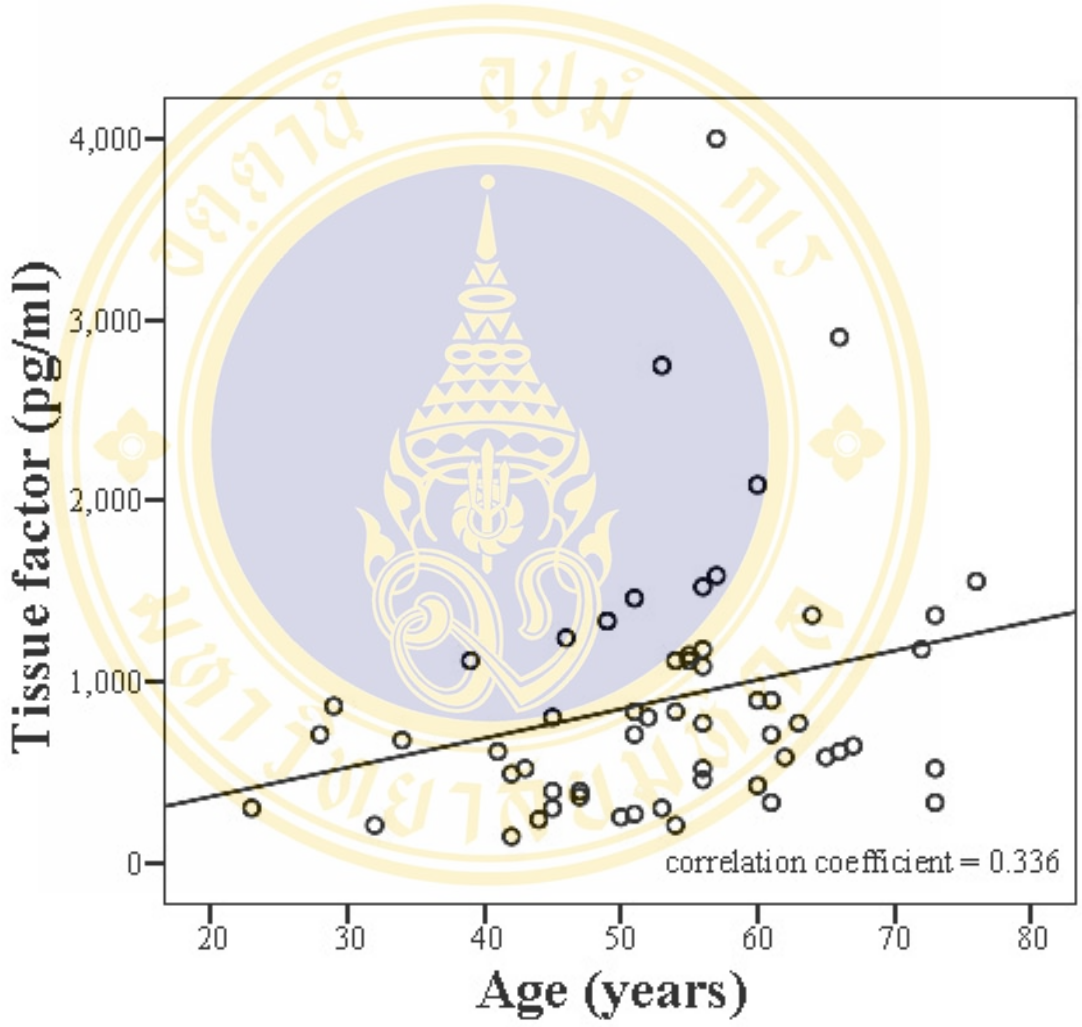


Figure 19. Scatter plot between plasma TF level in HCC patients and age

Table 9. Relationship between plasma TF level in HCC and tumour characteristics together with sex, HBsAg and cirrhosis

	n	Plasma TF level (mean±SD) (pg/ml)	*P*
Sex			
Male	46	957.37±758.94	0.352
Female	11	684.48±384.72	
HBsAg			
Positive	34	891.27±667.50	0.770
Negative	23	924.57±778.76	
Cirrhosis			
No	19	630.06±366.92	0.037*
Yes	36	1051.69±818.04	
Tumour size (cm)			
< 5	15	1020.51±643.23	0.329
5-10	21	816.13±456.73	
>10	19	915.03±992.29	
Tumour grade			
I	1	210.97	
II	26	957.28±789.80	0.254
III	13	954.87±696.44	
IV	2	1259.41±110.65	
TNM stage			
II	28	800.56±479.88	0.515
III	10	937.06±388.05	
IV	17	1061.51±1111.05	

* $p < 0.05$

Table 9. Relationship between plasma TF level in HCC and tumour characteristics together with sex, HBsAg and cirrhosis (Continued)

	n	Plasma TF level (mean±SD) (pg/ml)	*P*
Vascular invasion			
Positive	21	1002.33±898.45	0.665
Negative	34	846.56±592.80	
Perineural invasion			
Positive	2	1760.16±1615.50	0.348
Negative	52	885.95±679.18	
Lymphatic invasion			
Positive	1	617.83	0.748
Negative	53	727.42±148.38	

* $p < 0.05$

Table 10. Correlation between plasma TF level and clinical parameters in CCA

	n	Mean±SD	Min-Max	Correlation coefficient	*P*
Patient Age (years)	31	57.87±10.79	33-73	-0.155	0.405
CEA (ng/ml)	21	155.17±546.95	0.90-2527.90	0.392	0.079
CA19-9 (U/ml)	21	4,809.59±9,234.46	0.60-38,116.00	0.109	0.637
AFP (IU/ml)	20	14.41±45.38	0.80-205.70	0.081	0.733
Hct (%)	29	33.76±4.81	24.90-43.70	0.411	0.027*
Platelets (10e3/µl)	29	310.86±97.89	151.00-562.00	-0.041	0.833
PT (sec)	29	13.16±2.14	10.0-16.9	-0.411	0.027*
aPTT (sec)	29	29.21±4.38	22.5-43.4	0.086	0.657
TB (mg/dl)	29	5.96±10.32	0.30-37.30	-0.063	0.746
DB (mg/dl)	29	4.19±7.95	0.00-30.00	0.011	0.953
SGOT (U/I)	29	66.62±49.17	19.00-205.00	-0.049	0.800
SGPT (U/I)	29	60.55±46.04	12.00-201.00	-0.088	0.651
ALP (U/I)	29	352.76±239.58	72.00-867.00	0.021	0.913

* $p < 0.05$

Table 10. Correlation between plasma TF level and clinical parameters in CCA (continued)

	n	Mean±SD	Min-Max	Correlation coefficient	*P*
GGT (U/I)	28	370.86±276.83	39.00-1053.00	0.135	0.494
Albumin (g/dl)	27	3.50±0.49	2.60-4.40	0.105	0.604
Total protein (g/dl)	25	7.40±1.21	4.70-9.80	0.356	0.080
BUN (mg/dl)	29	12.41±5.49	5.00-30.00	0.138	0.475
Cr (mg/dl)	29	0.83±0.26	0.20-1.40	0.365	0.052

* $p < 0.05$

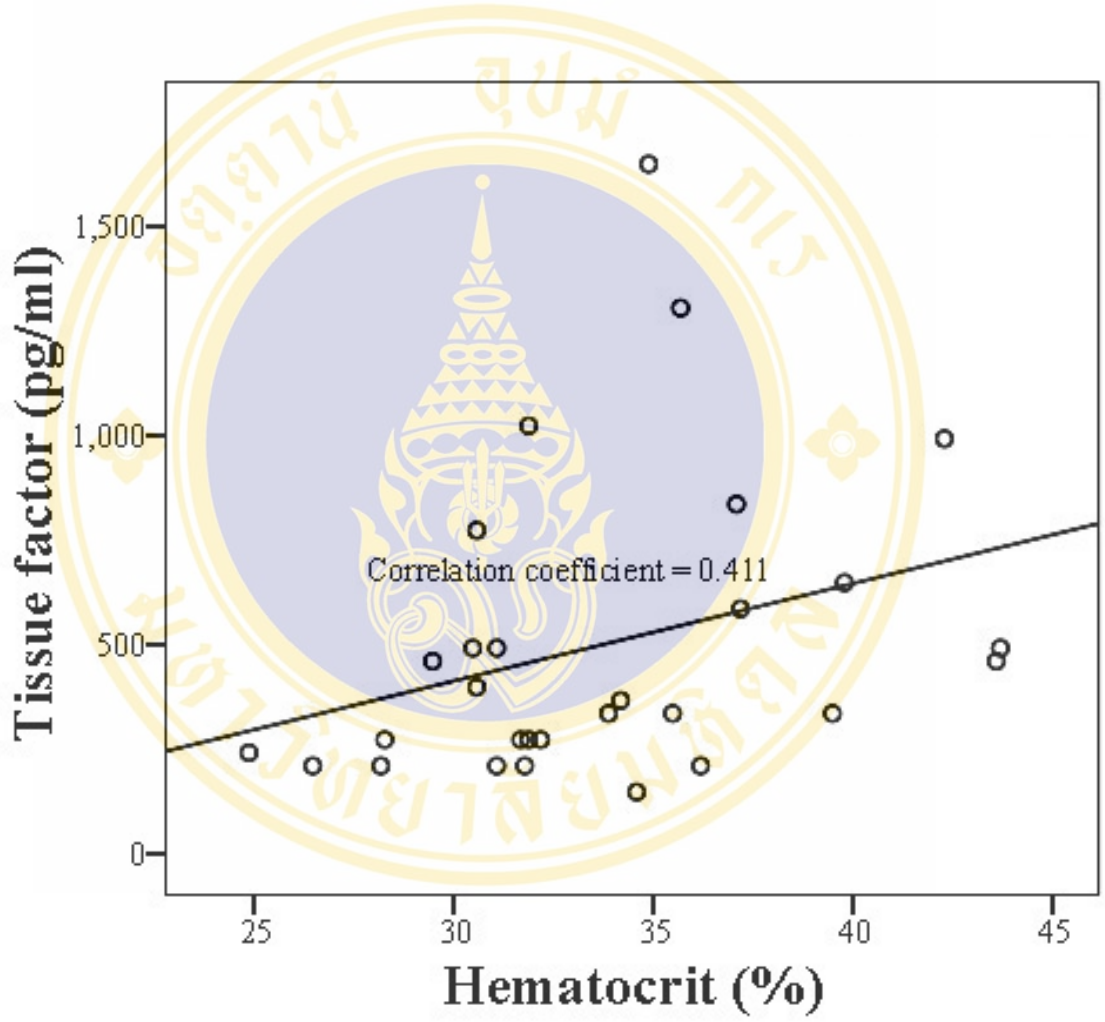


Figure 20. Scatter plot between plasma TF level in CCA patients and hematocrit

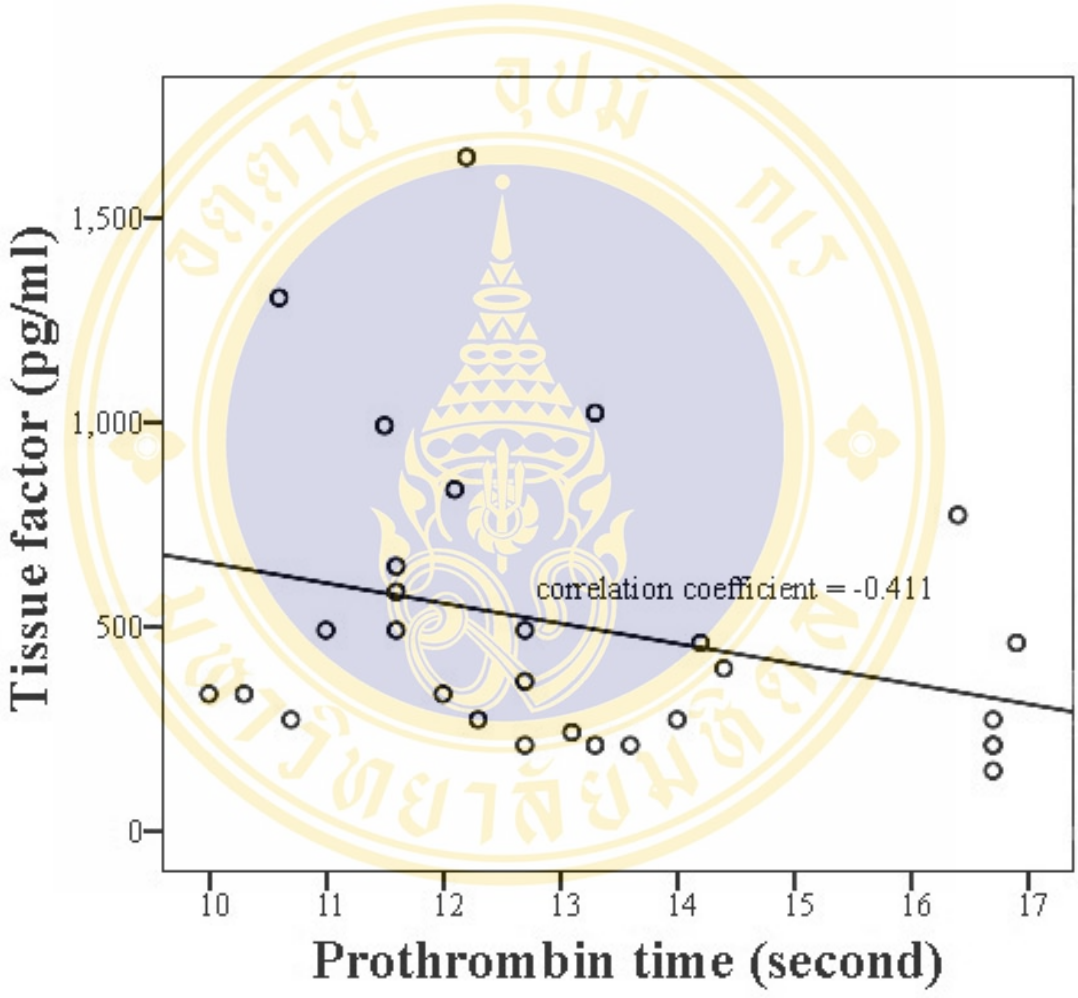


Figure 21. Scatter plot between plasma TF level in CCA patients and prothrombin time

Table 11. Relationship between plasma TF level in CCA and tumour characteristics together with sex

	n	Plasma TF level (mean±SD) (pg/ml)	*P*
Sex			
Male	18	610.87±411.79	0.056
Female	13	336.16±140.54	
Tumour size (cm)			
< 5	11	455.66±334.40	0.231
5-10	14	506.06±397.72	
>10	2	883.85±154.91	
Tumour grade			
Well differentiated	2	304.86±44.26	
Moderately	15	455.09±250.43	0.667
differentiated	2	962.10±973.73	
Poorly differentiated			
TNM stage			
II	3	273.57±62.59	0.058
III	13	405.98±298.32	
IV	12	669.99±412.90	
Vascular invasion			
Positive	5	386.23±247.03	0.742
Negative	7	340.63±102.90	
Perineural invasion			
Positive	10	520.81±431.51	0.874
Negative	1	336.16	
Lymphatic invasion			
Positive	8	351.81±210.28	0.559
Negative	7	349.57±105.39	

CHAPTER V
DISCUSSION

1. The standardisation of ELISA assay

ELISA assay is a safe and simple method of measuring antigens or antibodies with great sensitivity and specificity. It provides three characteristics that often make it the format of choice. First, a solid-phase adsorbent allows quick and thorough washing of unbound reagents. In this assay, a plastic microtiter plate is the solid phase on which the assay is performed. Microtiter plates have 96 wells, allowing 96 samples to be assayed under identical conditions. Second, the enzyme label provides a safe, stable, and sensitive signal compared with another common label, the radionuclide. Finally, ELISA is relatively trouble-free to develop, allowing even novices in the field to create an assay suitable for their needs.

The sandwich streptavidin-biotin system ELISA assay suitable for detecting protein antigens has been successfully established. Since this quantitative ELISA is based on the use of calibrator, it is prudent to realise the importance of the standard in determining the quality of the assay and allowing a comparison of the readings made with those obtained previously. Therefore, the samples and the standards were diluted in similar medium. At the end of the test, labeled antibody was detected by the addition of an enzyme substrate that was converted to a coloured product. The product signal was plotted against antigen concentration to generate a standard curve. The concentrations of the samples could then be read from this curve. The linear region of TF ELISA standard curves was generally obtainable in this series of dilutions of the TF standard, from 1,000 pg/ml to 0 pg/ml.

2. Plasma TF level in HCC and CCA

HCC and CCA are highly malignant tumours with a propensity for invasion and metastasis. Resection is the treatment of choice for both HCC and CCA, but the

prognosis after resection remains unsatisfactory because of a high incidence of recurrence related to tumour metastasis. Lacking of control of metastatic foci and recurrences is the most prevalent cause of death. Hence, it is important to identify the factors that predispose patients to death.

Several studies demonstrated the expression of TF in some malignant tumours, such as colon cancer, breast cancer, small cell lung cancer and prostate cancer (6, 9-11, 14). As a unique member of the superfamily of cytokine receptors, TF expression could be induced by a variety of cytokines and growth factors in malignant conditions (227). Elevated TF level was found in different biological fluids but only few studies reported an increase of serum or plasma TF level in tumour patients.

By using ELISA, a method for detection and quantitation of either antigens or antibodies in the blood and tissue with great sensitivity and specificity, plasma TF was found to be 67% higher in gastrointestinal and gynaecological cancer patients compared with healthy controls (19). Moreover, primary and recurrent breast cancer patients also showed significantly higher plasma TF concentration than normal controls (10).

In our study, TF was detected in plasma of HCC and CCA patients by using streptavidin-biotin system ELISA assay. We demonstrated that plasma TF concentration was up-regulated in both HCC and CCA patients compared with healthy controls. This result was compatible with previous study of TF expression in HCC tissue (18). In addition, we found that plasma TF level of HCC patients was significantly higher than that of CCA patients (p = 0.003). This finding might due to the relatively hypovascularity of CCA, in contrast to HCC, which was often highly vascular (16, 190).

3. The correlation between plasma TF level and clinicopathological prognostic factors of HCC and CCA

Elevated TF expression in tumours has been correlated with many unfavourable prognostic indicators, such as increased angiogenesis, advanced stages of disease and the multidrug resistant phenotype (224) that contribute to poorer survival rates in cancer patients. Hamada *et al.* (222) have previously investigated TF expression in 44 glioma specimens. They found that 1 in 10 (10%) benign gliomas (grade I-II) was

positive for TF, while 13 in 14 (86%) anaplastic astrocytomas (grade III) and 19 in 20 (95%) glioblastomas (grade IV) were moderately or strongly positive for TF. In colorectal cancer, increased TF positivity in high grade tumours has been correlated directly with increased vascular density and VEGF expression, as well as clinical stage, Duke classification and angiogenesis (15). In addition, high TF expression on tumour cells may be related to development of metastasis. Shigemori *et al*. (12) reported that TF expression is detected in 57% of 79 colorectal tumours and in 88% of 17 liver metastatic tumours from primary colorectal cancer. The immunoreactivity for TF in colorectal cancer with lymph node metastasis or with liver metastasis was significantly higher than that in tumour without lymph node metastasis or liver metastasis (15). These findings suggest that there is a close relationship between TF and metastasis in colon cancer.

In this study, plasma TF level was evaluated against several known prognostic clinicopathological factors for correlation. We found that age of HCC patients was significantly correlated with plasma TF level (p = 0.010). An increasing plasma TF level was found in older HCC patients. Chedid *et al* (146) reported that older HCC patients often had poorly differentiated tumours and poorer survivals while age younger than 45 was a good prognostic factor. Furthermore, plasma TF level was also significantly higher in HCC patients with cirrhosis, the most important risk factor associated with HCC, than those without cirrhosis (p = 0.037). However, plasma TF level of HCC patients was not significantly associated with sex, presence of HBsAg, tumour size, clinical stage, tumour grading, vascular invasion, perineural invasion and lymphatic invasion. The reason why plasma TF level failed to show differences in prognostic clinicopathological factors was probably because plasma TF expression is a multifactorial process.

In CCA patients, plasma TF level was significantly correlated with Hct (p = 0.027) and PT (p = 0.027). Our study showed a positive correlation between plasma TF and Hct and a negative correlation between plasma TF and PT. A low preoperative PT was one of the factors associated with recurrence of CCA (228). On the contrary, there was no statistical significant correlation between plasma TF level and the characteristics of patient and tumour, including age, sex, tumour size, clinical stage, tumour grading, vascular invasion, perineural invasion and lymphatic invasion, in

CCA. However, the number of studied CCA patients was too small to make an adequate comparison.

It is well recognised that a growing tumour requires factors that stimulate angiogenesis. TF provides an example of a molecule elaborated by tumour cells to regulate the switch to the angiogenic phenotype by modulating the relative levels of proangiogenic and antiangiogenic molecules. Overexpression of TF in sarcoma cells in mice promotes angiogenesis through the increased production of the positive angiogenic factor VEGF and reduced production of the negative angiogenic factor thrombospondin (17). Unfortunately, we examine neither microvessel density of tumors (which represents angiogenesis) nor other factors associated with angiogenesis, especially VEGF and thrombospondin. Therefore, we could not completely rule out the possibility that plasma TF level of HCC and CCA would enhance angiogenesis and metastasis.

TF may be a novel target for antiangiogenic therapy in HCC and CCA. Blocking of TF activity by monoclonal antibodies could inhibit tumor metastasis in an experimental model (229). TF pathway inhibitor (TFPI), a natural inhibitor of TF mediated coagulation, has been shown to inhibit metastasis in an animal model of melanoma (230). Tumor TF expression can also be suppressed by pentoxyphylline (231) and retinoic acid (232). On the basis of our data, it is worthwhile to explore the efficacy of therapies targeting TF for the treatment of HCC and CCA.

CHAPTER VI
CONCLUSION

Sandwich ELISA assay may be the most versatile and sensitive for the detection of proteins of any ELISA: no purified antigen is required, only a suitable antibody. However, only multivalent antigens with different or repeating epitopes may be detected in this assay, since binding of two antibodies to the antigen is required. This requirement is normally not a limitation for proteins, which are almost always multivalent.

To our knowledge, this is the first study that evaluates the plasma TF level in HCC and CCA patients and its relationship with clinicopathological factors. Although the connection between clotting and malignancy was made over 100 years ago, the clinical importance and therapeutic applications stemming from these intersecting fields have only recently been realised. Results from a number of clinical studies indicated that various molecules of the coagulation and fibrinolysis system participate in the growth and dissemination of tumour cells. It had recently become clear that the primary initiator of coagulation, TF, is expressed in a variety of solid tumours and tumour cell lines. Subsequent results had suggested that TF participated in the growth and dissemination of various cancer types.

In conclusion, this study demonstrates an up-regulation of plasma TF level in HCC and CCA patients compared with healthy individuals. TF may hold the key to specifically targeting tumours for cancer therapy since it is abnormally expressed in many types of tumours and related vascular endothelial cells. However, no association between plasma TF level and invasiveness was found. The prognostic and therapeutic value of TF as a marker for tumour cells, as well as the mechanistic basis for the induction of TF in hepatic cells are areas that warrant further study.

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APPENDIX

Appendix A

Reagents

1. Reagent for sandwich ELISA

1.1 Coating buffer

1.59 g Na_2CO_3

2.93 g $NaHCO_3$

(Dissolve in distilled water; adjusted pH to 9.6 and to final volume of 1 litre)

1.2 Washing buffer

4 ml of 25% TritonX-100

8 g of NaCl

1.15 g of Na_2HPO_4

0.2 g of KH_2 PO_4

0.2 g of KCl

(Made up to a final volume of 1 litre with distilled water)

1.3 Phosphate buffer saline (PBS)

8 g of NaCl

1.15 g of Na_2HPO_4

0.2 g of KH_2 PO_4

0.2 g of KCl

(Dissolve in distilled water; pH adjusted to 7.4 and to final volume of 1 litre)

1.4 Blocking buffer

2.5 g of Bovine serum albumin (BSA)

(Dissolve in PBS; pH adjusted to 7.4 and to final volume of 250 ml)

1.5 Sample buffer

adding BSA to washing buffer to a final concentration of 1%w/v.

1.6 Enzyme conjugate (Streptavidin-horseradish peroxidase)

12 µl of enzyme conjugate

12 ml of enzyme conjugate diluent

(For running all 96 wells at one time)

1.7 Stopping solution (0.5 M H_2SO_4)

18 M stock H_2SO_4 (96%, specific gravity=1.84)

distilled water

1.8 Substrate

Tetra-methylbenzidine (TMB)

2. Antibodies for sandwich ELISA

2.1 Capture antibody

murine anti-human TF monoclonal antibody

2.2 Detection antibody

biotinylated anti-human TF antibody fragment

3. TF standard for sandwich ELISA

TF standard concentrations are 0 pg/ml, 50 pg/ml, 100 pg/ml, 200 pg/ml, 500 pg/ml and 1000 pg/ml.

Appendix B: Clinicopathological data of 57 hepatocellular carcinoma patients

No.	Age	Sex	Tumour size (cm)	Cirrhosis	Staging	Edmanson grading	Vascular invasion	Lymphatic invasion	Perineural invasion	Tissue factor (pg/ml)
1	47	Male	<5	yes	IV	unknown	positive	negative	negative	398.752
2	54	Female	<5	yes	III	II	positive	negative	negative	836.908
3	56	Male	5-10	no	II	III	negative	negative	negative	774.316
4	56	Male	<5	yes	II	III	positive	negative	negative	1087.28
5	45	Female	<5	no	II	II	negative	negative	negative	805.612
6	45	Male	5-10	no	II	II	negative	negative	negative	398.752
7	50	Female	>10	no	II	unknown	negative	negative	negative	255.568
8	53	Male	<5	yes	II	II	negative	negative	negative	304.864
9	61	Male	<5	yes	II	unknown	negative	negative	negative	711.72
10	55	Male	<5	yes	II	unknown	negative	negative	negative	1118.58
11	49	Male	5-10	yes	III	IV	positive	negative	negative	1337.656

Appendix B: Clinicopathological data of 57 hepatocellular carcinoma patients (continued)

No.	Age	Sex	Tumour size (cm)	Cirrhosis	Staging	Edmanson grading	Vascular invasion	Lymphatic invasion	Perineural invasion	Tissue factor (pg/ml)
12	55	Female	5-10	yes	III	II	negative	negative	negative	1149.876
13	63	Male	>10	no	II	II	negative	negative	negative	774.316
14	42	Female	>10	yes	IV	unknown	negative	negative	negative	148.38
15	67	Female	<5	yes	II	II	negative	negative	negative	649.128
16	66	Male	5-10	yes	IV	II	positive	positive	positive	617.832
17	64	Male	5-10	yes	II	II	negative	negative	negative	1368.952
18	51	Male	5-10	no	IV	II	positive	negative	negative	711.72
19	56	Male	<5	yes	II	III	Negative	negative	negative	1525.436
20	57	Male	<5	yes	II	II	negative	negative	negative	1588.028
21	43	Male	5-10	yes	II	III	positive	negative	negative	523.94
22	73	Male	>10	no	IV	II	negative	negative	negative	1368.952
23	56	Male	5-10	no	IV	II	positive	negative	negative	461.348
24	61	Male	>10	yes	II	unknown	negative	negative	negative	336.16

Appendix B: Clinicopathological data of 57 hepatocellular carcinoma patients (continued)

No.	Age	Sex	Tumour size (cm)	Cirrhosis	Staging	Edmanson grading	Vascular invasion	Lymphatic invasion	Perineural invasion	Tissue factor (pg/ml)
25	29	Male	>10	yes	II	III	positive	negative	negative	868.204
26	46	Male	5-10	yes	II	III	negative	negative	negative	1243.764
27	45	Male	>10	no	IV	III	positive	negative	negative	304.864
28	52	Male	5-10	yes	II	III	positive	negative	negative	805.612
29	76	Male	<5	yes	III	II	negative	negative	negative	1556.732
30	41	Male	>10	no	III	unknown	negative	negative	negative	617.832
31	32	Female	5-10	no	IV	I	negative	negative	negative	210.972
32	73	Male	<5	no	II	II	negative	negative	negative	336.16
33	56	Female	<5	yes	II	II	negative	negative	negative	523.94
34	34	Male	5-10	yes	III	unknown	negative	negative	negative	680.424
35	66	Male	>10	yes	IV	III	positive	negative	positive	2902.492
36	56	Female	5-10	yes	III	II	negative	negative	negative	1181.172
37	62	Male	5-10	no	II	III	positive	negative	negative	586.532

Appendix B: Clinicopathological data of 57 hepatocellular carcinoma patients (continued)

No.	Age	Sex	Tumour size (cm)	Cirrhosis	Staging	Edmanson grading	Vascular invasion	Lymphatic invasion	Perineural invasion	Tissue factor (pg/ml)
38	53	Male	<5	yes	IV	unknown	negative	negative	negative	2746.008
39	51	Male	>10	yes	IV	unknown	negative	negative	negative	273.568
40	28	Male	>10	no	IV	II	positive	negative	negative	711.72
41	72	Female	5-10	yes	IV	IV	positive	negative	negative	1181.172
42	60	Male	>10	yes	II	unknown	negative	negative	negative	899.5
43	42	Male	5-10	yes	II	unknown	negative	negative	negative	494.44
44	39	Male	>10	no	IV	unknown	positive	negative	negative	1118.58
45	23	Male	>10	no	IV	III	positive	negative	negative	304.864
46	65	Female	>10	yes	IV	II	positive	negative	negative	586.532
47	73	Male	5-10	no	III	II	negative	negative	negative	523.94
48	47	Male	5-10	yes	III	III	negative	negative	negative	367.456
49	51	Male	>10	no	II	II	positive	negative	negative	1462.844
50	57	Male	>10	yes	IV	II	positive	negative	negative	3997.88

Appendix B: Clinicopathological data of 57 hepatocellular carcinoma patients (continued)

No.	Age	Sex	Tumour size (cm)	Cirrhosis	Staging	Edmanson grading	Vascular invasion	Lymphatic invasion	Perineural invasion	Tissue factor (pg/ml)
51	60	Male	5-10	yes	II	II	negative	negative	negative	2088.78
52	54	Male	>10	yes	II	II	positive	positive	unknown	210.972
53	54	Male	<5	yes	III	III	negative	negative	unknown	1118.58
54	44	Male	>10	no	II	II	positive	unknown	unknown	242.268
55	60	Male	5-10	yes	II	II	unknown	unknown	unknown	430.048
56	61	Male	unknown	unknown	unknown	unknown	unknown	unknown	unknown	899.5
57	51	Male	unknown	unknown	unknown	unknown	unknown	unknown	unknown	836.908

Appendix C

Appendix C: Clinicopathological data of 31 Cholangiocarcinoma patients

No.	Age	Sex	Tumour Size (cm)	Staging	Differentiation	Vascular invasion	Lymphatic invasion	Perineural invasion	Tissue Factor (pg/ml)
1	66	Female	5-10	III	M oderately	positive	positive	positive	242.268
2	54	Male	5-10	III	moderately	negative	negative	positive	461.348
3	40	Male	>10	IV	moderately	positive	positive	positive	774.316
4	60	Female	<5	III	moderately	positive	positive	positive	210.972
5	42	Male	5-10	IV	moderately	positive	unknown	positive	492.644
6	47	Male	5-10	III	moderately	negative	negative	unknown	210.972
7	64	Male	5-10	IV	poorly	unknown	unknown	positive	1650.624
8	56	Female	<5	III	moderately	unknown	unknown	unknown	273.568
9	41	Female	5-10	IV	moderately	unknown	unknown	unknown	555.236
10	58	Male	<5	IV	moderately	unknown	unknown	unknown	836.908
11	51	Male	>10	IV	moderately	unknown	unknown	unknown	993.392

Appendix C: Clinicopathological data of 31 Cholangiocarcinoma patients (continued)

No.	Age	Sex	Tumour Size (cm)	Staging	Differentiation	Vascular invasion	Lymphatic invasion	Perineural invasion	Tissue Factor (pg/ml)
12	62	Female	unknown	IV	moderately	unknown	positive	unknown	273.568
13	62	Male	5-10	IV	unknown	positive	positive	unknown	210.972
14	53	Female	<5	III	unknown	negative	unknown	unknown	336.16
15	56	Female	5-10	III	unknown	unknown	unknown	positive	336.16
16	72	Male	5-10	IV	unknown	unknown	positive	unknown	492.644
17	59	Male	<5	II	unknown	unknown	unknown	unknown	210.972
18	47	Male	<5	III	unknown	unknown	unknown	unknown	1306.36
19	65	Male	5-10	IV	unknown	unknown	unknown	unknown	273.568
20	71	Male	5-10	IV	unknown	unknown	unknown	unknown	1024.688
21	73	Female	<5	III	poorly	negative	negative	positive	273.568
22	71	Female	<5	II	well	negative	negative	positive	273.568
23	69	Male	<5	III	moderately	negative	negative	positive	492.644
24	53	Male	5-10	III	moderately	unknown	negative	unknown	398.752

Appendix C: Clinicopathological data of 31 Cholangiocarcinoma patients (continued)

No.	Age	Sex	Tumour Size (cm)	Staging	Differentiation	Vascular invasion	Lymphatic invasion	Perineural invasion	Tissue Factor (pg/ml)
25	33	Female	<5	IV	moderately	unknown	positive	unknown	461.348
26	53	Female	<5	II	well	negative	negative	negative	336.16
27	70	Female	5-10	III	moderately	unknown	positive	unknown	148.38
28	67	Male	5-10	III	unknown	unknown	unknown	unknown	586.532
29	60	Female	unknown	unknown	unknown	unknown	unknown	unknown	649.128
30	72	Male	unknown	unknown	unknown	unknown	unknown	unknown	367.456
31	47	Male	unknown	unknown	unknown	unknown	unknown	unknown	210.972

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