Detection Of Serum Matrix Metalloproteinase (MMP)-7 For Differentiating Cholangiocarcinoma From Benign Biliary Tract Disease Patients: Evaluation Of Diagnostic Accuracy

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การใช้ระดับซีรัม เอ็มเอ็มพี 7 (MMP7) เพื่อการวินิจฉัยแยกโรคมะเร็งทางเดินน้ำดีจากผู้ป่วยทางเดินน้ำดีอุดตัน ที่ไม่ได้เกิดจากมะเร็ง:การประเมินความแม่นยำของการตรวจวินิจฉัย

นายกวิญ ลีละวัฒน์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	DETECTION OF SERUM MATRIX METALLOPROTEINASE (MMP)-7 FOR DIFFERENTIATING CHOLANGIOCARCINOMA FROM BENIGN BILIARY TRACT DISEASE PATIENTS: EVALUATION OF DIAGNOSTIC ACCURACY
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กวิญ ลีละวัฒน์: การใช้ระดับซีรัม เอ็มเอ็มพี 7 (MMP-7) เพื่อการวินิจฉัยแยกโรคมะเร็งทางเดินน้ำดีจากผู้ป่วยทางเดินน้ำดีอุดตัน ที่ไม่ได้เกิดจากมะเร็ง: การประเมินความแม่นยำของการตรวจวินิจฉัย. (Detection Of Serum Matrix Metalloproteinase (MMP)-7 For Differentiating Cholangiocarcinoma From Benign Biliary Tract Disease Patients: Evaluation Of Diagnostic Accuracy) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. นพ. ทวีชัย เตชะพงศ์วรชัย, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. ดร. นพ. อภิวัฒน์ มุทิรางกูร, 50 หน้า.

โรคมะเร็งทางเดินน้ำดีเป็นโรคมะเร็งที่มีความรุนแรงสูง มักมีการลุกลามไปยังอวัยวะข้างเคียงหรืออวัยวะห่างไกล ปัจจุบันยังไม่มีการตรวจหาสารบ่งชี้มะเร็ง (Tumor marker) ในเลือดที่จำเพาะ สำหรับการวินิจฉัยโรคมะเร็งทางเดินน้ำดี การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาระดับ MMP7 ในซีรัม โดยเปรียบเทียบระหว่างผู้ป่วยโรคมะเร็งทางเดินน้ำดี และผู้ป่วยโรคท่อน้ำดีอุดตันที่ไม่ได้เกิดจากมะเร็ง อีกทั้งยังเปรียบเทียบผลที่ได้กับระดับ CA19-9 ในซีรัม การศึกษานี้ถูกแบ่งออกเป็น 2 ส่วน การศึกษาส่วนที่ 1 (การศึกษาย้อนหลังเพื่อเปรียบเทียบระหว่างกลุ่มควบคุมและกลุ่มที่สนใจ) โดยวัดปริมาณ CA19-9 และ MMP7 ในซีรัมจากธนาคารซีรัม (ผู้ป่วยมะเร็งทางเดินน้ำดี 44 ราย และผู้ป่วยท่อน้ำดีอุดตันที่ไม่ได้เกิดจากมะเร็ง 36 ราย) การศึกษาส่วนที่ 2 (การศึกษาชนิดไปข้างหน้า ในผู้ป่วยทุกรายที่มีอาการของโรคทางเดินน้ำดี) โดยศึกษาวัดปริมาณ MMP7 และ CA19-9 ในซีรัมผู้ป่วยทุกรายที่มีอาการท่อน้ำดีอุดตันจำนวน 187 ราย ผลการศึกษาส่วนที่ 1 จากการวิเคราะห์กราฟ Receiver operating characteristic (ROC) พบว่า ปริมาณ MMP7 ในซีรัมมีความแม่นยำในการแยกโรคระหว่างโรคมะเร็งทางเดินน้ำดี และผู้ป่วยโรคท่อน้ำดีอุดตันที่ไม่ได้เกิดจากมะเร็ง (พื้นที่ใต้กราฟ = 0.79, 95%CI = 0.614-0.848) ในขณะที่พื้นที่ใต้กราฟ ROC สำหรับปริมาณ CA19-9 ในซีรัมเท่ากับ 0.63 (95%CI = 0.491- 0.761) ผลการศึกษาส่วนที่ 2 พบว่าปริมาณ MMP7 และ CA19-9 ในซีรัมของผู้ป่วยโรคมะเร็งทางเดินน้ำดีสูงกว่าผู้ป่วยโรคมะเร็งทางเดินน้ำดีอุดตันที่ไม่ได้เกิดจากมะเร็งอย่างมีนัยสำคัญ ($p < 0.001$) มีพื้นที่ใต้กราฟ ROC แสดงว่า ซีรัม MMP7 มีความแม่นยำในการวินิจฉัยแยกโรคมะเร็งทางเดินน้ำดีได้ดีกว่าซีรัม CA19-9 (พื้นที่ใต้กราฟของซีรัม MMP7 เท่ากับ 0.84; CI95% 0.778-0.903 และ ของซีรัม CA19-9 เท่ากับ 0.79; CI95% 0.708-0.868) ความไวและความจำเพาะสำหรับสำหรับซีรัม MMP7 (ที่ Cut-off 5.5 ng/ml) มะเร็งทางเดินน้ำดี เท่ากับ 75% และ 78% ในขณะที่ความไวและความจำเพาะของซีรัม CA19-9 (Cut-off เท่ากับ 100 U/ml) เท่ากับ 68% และ 87% การศึกษานี้สรุปได้ว่าระดับซีรัม MMP7 และ CA19-9 มีประโยชน์ในการวินิจฉัยแยกโรคระหว่างมะเร็งทางเดินน้ำดีและโรคทางเดินน้ำดีอุดตันที่ไม่ได้เกิดจากมะเร็ง

สาขาวิชา การพัฒนาสุขภาพ ลายมือชื่อนิสิต.....
ปีการศึกษา 2552 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....
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KAWIN LEELAWAT: DETECTION OF SERUM MATRIX METALLOPROTEINASE (MMP)-7 FOR DIFFERENTIATING CHOLANGIOCARCINOMA FROM BENIGN BILIARY TRACT DISEASE PATIENTS: EVALUATION OF DIAGNOSTIC ACCURACY.

THESIS ADVISOR: ASSOC. PROF. TAWECHAI TEJAPONGVORACHAI, MD.

THESIS CO-ADVISOR: PROF. APIWAT MUTIRANGURA, MD. Ph.D., 50 pp.

Cholangiocarcinoma is an aggressive tumor with a tendency for local invasion and distant metastases. However, at present, there is no available tumor marker that can differentiate cholangiocarcinoma from benign bile duct disease. This study was designed to determine whether the serum levels of MMP7 can discriminate cholangiocarcinoma patients from benign biliary tract disease patients in comparison to carbohydrate antigen 19-9 (CA19-9). This study was divided into 2 parts. Part 1 (a retrospective nonconsecutive case-control study), we measured the level of CA19-9 and MMP7 in the serum derived from the serum bank (44 cholangiocarcinoma and 36 benign biliary tract diseases patients). Part 2 (prospective consecutive cases from patients with symptoms of biliary tract disease), a total of 187 obstructive jaundice patients was consecutively enrolled and their values of serum MMP7 was assayed and compared with serum CA19-9. For part 1 study, a receiver operating characteristic (ROC) curve analysis revealed that the detection of the serum MMP7 level is reasonably accurate in differentiating cholangiocarcinoma from benign biliary tract disease patients (area under curve=0.73; 95% CI= 0.614-0.848). While the areas under the curve of the ROC curves for CA19-9 were 0.63 (95% CI= 0.491-0.761). For part 2 study, we found that MMP7 and CA19-9 serum levels were significantly elevated in cholangiocarcinoma patients ($p<0.001$). The area under the curve (AUC) from a receiver operating characteristic (ROC) curve analysis for the diagnosis of cholangiocarcinoma, using MMP7 was more accurate than CA19-9 (AUC =0.84; CI 95% 0.778 – 0.903 for MMP7 and AUC = 0.79; CI 95% 0.708 – 0.868 for CA19-9). The sensitivity and specificity of serum MMP7 (cut-off value of 5.5 ng/ml) was 75% and 78%, respectively, while the sensitivity and specificity of serum CA19-9 (cut-off value of 100 U/ml) was 68% and 87%, respectively. We concluded that serum values of MMP7 and CA19-9 appear to be useful biomarkers for differentiating cholangiocarcinoma from benign biliary tract diseases.

Field of Study : Health Development Student's Signature .....

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CHAPTER I

INTRODUCTION

1.1 Background and Rationale

Cholangiocarcinoma is a cancer arising from bile duct epithelium. The incidence of and mortality rate for cholangiocarcinoma varied considerably in different geographic regions, with the incidence highest in Southeast Asia especially in Thailand (1). The causes of lethality of this disease are not only its rapid growth but also the tendency to invade adjacent organs and metastasize(2-5). Therapeutic options for cholangiocarcinoma have been limited due to poor response to chemotherapy and radiation therapy (4, 5). Surgery is perhaps the only effective treatment for cholangiocarcinoma. Three-year survival rates of 35% to 50% are achieved in only a few numbers of patients when negative histological margins are attained at the time of surgery (6-9). Previous studies suggested that the most important prognostic factor is a tumor-free surgical margin while other features that were associated with a poor prognosis include factors connected to the extent of disease that caused by cancer cell invasion, such as bi-lobar distribution, lymph node involvement, vascular invasion and distant metastases (10). To improve the survival rate, the diagnosis and treatment of these patients should be performed as soon as possible. In our clinical setting, most patients with cholangiocarcinoma present with obstructive jaundice. However, there are many benign biliary tract diseases presenting with clinical symptoms like patients with cholangiocarcinoma. The most important issue is to differential patients with cholangiocarcinoma from patients with benign biliary tract diseases because of the different in treatment and in prognosis between these diseases.

It is very difficult to get the tissue for diagnosis of cholangiocarcinoma due to the desmoplastic reaction and the tumor location. In addition, this tumor usually grows along the bile duct without expanding from the bile ducts as a mass forming. Computed tomography (CT), ultrasound, or magnetic resonance imaging (MRI) often missed this lesion (3). Therefore, identification of tumor markers in the serum would be benefit in the clinical managing of this disease. To date, there is a tumor marker for detecting

cholangiocarcinoma; carbohydrate antigen 19-9 (CA19-9). CA19-9 is a mucin-type serum glycoprotein with the immunodeterminant express on the carbohydrate moiety (11). Previously, this marker was studied for the efficacy in diagnosis of cholangiocarcinoma occurring in primary sclerosing cholangitis patients (12, 13). A comparison of CA 19-9, CEA, and combined CA 19-9 and CEA was evaluated in a cohort of patients at Kings College, London. A CA 19-9 cut off of 200 U/mL resulted in a sensitivity of 60% and specificity of 91% for the diagnosis of CCA. A CEA cut off of 5 ng/mL resulted in a sensitivity of 53% and specificity of 86%. An index combining CEA and CA 19-9 ((40xCEA) + CA 19-9, >400 as the threshold value) achieved 100% specificity for the diagnosis of CCA (13). However, this index has been less reliable when evaluated prospectively, with evidence of fluctuation in tumor marker levels and transient elevation in some individuals when followed over time (8). In addition, previous study demonstrated that the level of serum CA19-9 was depended on the severity of bile duct obstruction and the degree of cholangitis. Rising of serum CA19-9 can be detected even in benign bile duct diseases (14). In addition, serum CA19-9 depends on the Lewis phenotype. As many as 10% of the population have been found to be Lewis negative (5), resulting in undetectable CA 19-9 levels. Therefore the novel tumor markers for diagnosis cholangiocarcinoma should be investigated.

Matrix metalloproteinases (MMPs) are zinc dependent endopeptidase. They play roles in the mechanisms of the turnover and degradation of extracellular matrix (ECM) components and basement membranes. MMPs are involved in the processes of fetal development as well as wound healing and inflammation (15). Previous literatures demonstrated that degradation of extracellular matrices by MMPs is a key role in the mechanism of tumor invasion and metastasis (15). MMP7 (matrilysin) is the smallest MMP (28 kd) expressed in the tumor cells but not in the stromal cells. It has been reported that MMP7 is overexpressed in the breast, colorectal, and stomach cancer cells(16-19). Because MMP7 is secreted protein by cancer cells, previous literatures identified that peripheral blood levels of MMP7 are significantly elevated in ovarian cancer patients and renal cell carcinoma patients (19, 20).

Recently, Itatsu k, et al. performed immunohistochemical staining in the resected specimens of cholangiocarcinoma and found that 75.8% of these specimens expressed MMP7 while normal biliary epithelium did not express MMP7. In addition, the expression of MMP7 significantly correlated with the nonpapillary phenotype, poorly differentiated histologic grade, perineural invasion, and advanced cholangiocarcinoma stage (21, 22). Hence, detection of MMP7 in blood circulation may be useful for clinical diagnosis of cholangiocarcinoma. However, until now, there is no study about the detection of MMP7 in blood circulation of cholangiocarcinoma patients. Therefore, we design to do a study about the accuracy of the detection of serum MMP7 for diagnosis of cholangiocarcinoma in our setting.

1.2 Research question

Can serum MMP7 differentiate cholangiocarcinoma patients from benign bile duct disease patients?

1.3 Objectives

1. To investigate the accuracy of serum MMP7 as a diagnostic test for cholangiocarcinoma patients

2. To compare the accuracy of serum MMP7 with serum CA19-9 for diagnosis cholangiocarcinoma

CHAPTER II

LITERATURE REVIEW

Thailand especially in the North-East is the endemic area of cholangiocarcinoma. This disease is one of the most causes of cancer related death in Thailand (1). Only Surgical resection (R0) is the curative treatment for these patients. However, only a small number of patients can be achieved this operation. Most of the patients are presenting with advance stages of disease which beyond surgical treatment. Consequently, diagnosis of cholangiocarcinoma at early stages of disease is very important. Unlike other kinds of solid tumors, a pathological diagnosis of cholangiocarcinoma is very difficult because of the location and the desmoplastic characteristic of this disease (2, 5). For that reason, the diagnosis of cholangiocarcinoma is usually based on radiological imaging and tumor markers.

At present, CA19-9 is an available tumor marker for diagnosis of cholangiocarcinoma. However, most of the literatures studying about the association between cholangiocarcinoma and CA19-9 were performed in the patients with underlying primary sclerosing cholangitis (PSC)(6, 8). While most of the cholangiocarcinoma cases in Thailand are not caused by PSC but caused by chronic infection especially by *Opisthorchiasis viverrini* (1, 23). The utility of serum CA19-9 as the tumor markers for diagnosis of cholangiocarcinoma in this situation is still controversy. Recently, Abraham R. John, et al. reported the sensitivity and specificity of CA19-9 in the diagnosis of cholangiocarcinoma in patients without PSC were 77.9 and 76.3% respectively (cut off point = 100 U/ml) (24). However, most of the cholangiocarcinoma cases in this study were in advance stages of disease when compared with the patients in Patel's study. D.V. Mann, et al. performed a retrospective study in 164 patients with rising of the serum CA19-9 (>33 U/mL). They concluded that the solitary elevated CA19-9 measurement could not be used to discriminate between benign and malignant disease because the level of serum CA19-9 in benign disease is correlated with the level of hyperbilirubinemia ($R=0.41$, $p<0.01$)(25). Anand H. Patel, et al. carried out a prospective diagnostic test study for accuracy of CA19-9 in diagnosis of cholangiocarcinoma in 36 patients with non PSC cholangiocarcinoma, 41 patients

with nonmalignant liver disease, and 26 patients with benign biliary diseases. They demonstrated that the sensitivity of serum CA 19-9 (cut off point = 100 U/ml) in diagnosing cholangiocarcinoma in non PSC patients was only 53%. When compared with the nonmalignant liver disease and the benign bile duct diseases (26). In addition, Leelawat K, et al. reported the sensitivity and specificity of CA19-9 in the diagnosis of cholangiocarcinoma in Thai patients were 60.6 and 80.5% respectively (27). From these data we can concluded that the measurement of serum CA19-9 is not good enough for diagnosis of cholangiocarcinoma. The new tumor marker for diagnosis cholangiocarcinoma is urgently required.

Electronic databases PubMed is searched for citing the articles identified new tumor marker in comparison with CA19-9 as a diagnosis test for cholangiocarcinoma. There are 58 publications for the search terms “tumor marker and cholangiocarcinoma and CA19-9 and blood”. Then we manually reviewed all of these titles and selected only papers that were associated with new tumor marker for diagnosis of cholangiocarcinoma. From the selected papers, the new tumor markers for diagnosis of cholangiocarcinoma are including IL-6, CYFRA21-1, RCAS1, and hTERT mRNA.

Goydos JS, et al. (28) demonstrated that high serum IL-6 marks patients with cholangiocarcinoma and correlates with tumor burden. The participant of this study include 60 patients including 15 cholangiocarcinoma, 14 hepatocellular carcinoma, 26 isolated hepatic colorectal metastasis, 5 benign biliary tract disease and 35 adult healthy volunteers. Their sera were collected in prospective from 1992-1995. The exclusion criteria are the factors known to be associated with elevated IL-6 in serum, including the present of acute infection, chronic inflammatory disease, recent myocardial infarction, surgical procedures within 14 days prior and uremia. Measuring of serum IL-6 was done by using commercial ELISA (Endogen, Cambridge, MA). Statistical analysis for comparing the IL-6 from each tumor type and healthy control was using the Wilcoxon test and independent samples t test for equality of the mean. The results of this study demonstrated that serum IL-6 was detected in all cholangiocarcinoma cases and the positive predictive value was 83.3% and the mean level of IL-6 was significantly high in cholangiocarcinoma patients than in other groups. However, they did not mention the method to measure the sensitivity and specificity for

this test. In addition, the sample size in this study is too small to test the accuracy of IL-6. Cheon, et al. (29) evaluated the usefulness of serum IL-6 in the diagnosis of cholangiocarcinoma and measured changes in serum IL-6 levels following photodynamic therapy (PDT). They found that IL-6 was detected in all patients with cholangiocarcinoma and hepatocellular carcinoma, and in 6 of 23 healthy adults. The level of IL-6 in serum was higher in patients with cholangiocarcinoma than in both other groups ($P < 0.001$). The gold standard for diagnostic of cholangiocarcinoma was done by using ERCP, direct cholangioscopy with biopsy, intraductal ultrasonography, CT, and/ or MRCP. A diagnostic sensitivity of 73% and a specificity of 92%; positive and negative predictive values were 83% and 87%, respectively when using a cutoff point at 25.8 pg/mL of IL-6. However, the authors did not mention the method to get the cutoff concentration of serum IL-6. We suggested that the level of bilirubin in the cholangiocarcinoma patients in this study is quite low (1.54-1.97 mg/dL) while the average of total bilirubin in our patients is more than 10 mg/dL). This factor may be influence with the value of serum IL-6 and they did not mention about the method of blind the result between the value of IL-6 and the diagnosis. The sample size in this study is too small to test the accuracy of IL-6. We suggested that most of our patients with bile duct disease are presenting with cholangitis. This condition can give a false positive result when using IL-6 as a tumor marker.

Takahiro Uenishi, et al. (30) performed a case-control study for test the accuracy of the serum cytokeratin 19 fragment (CYFRA21-1) for diagnosis of intrahepatic cholangiocarcinoma (ICC). They measured the serum level of CYFRA21-1, CA19-9 and CEA in 71 cases of ICC patients and 90 cases of nonmalignant liver diseases patients (case-control study). They analyzed the areas under the receiver operator characteristic (ROC) curves and found that ROC curves demonstrated better discrimination between intrahepatic cholangiocarcinoma and benign liver diseases for CYFRA 21-1 than for CEA or CA 19-9. The sensitivity of 74.7% and specificity of 92.2% were obtained when set the cut-off at 2.7 ng/ml for CYFRA 21-1. However, most of the patients in our clinic are presenting with bile duct obstruction from hilar cholangiocarcinoma. Therefore, the level of CYFRA 21-1 in these patients should be further investigated.

High sensitivity and specificity of serum RCAS1 in diagnosis of cholangiocarcinoma were reported by Enjoji et al (31, 32). They established a threshold value for RCAS1 in collected serum at 10 U/ml by Receiver operating characteristic curves, which allowed differential between cholangiocarcinoma and benign biliary diseases. In addition, they identified that serum RCAS1 was more sensitive for cholangiocarcinoma than CA19-9 (73.9% VS 65.2%), and was not influenced by cholestasis. However, until now, there is no available commercial ELISA kit test for RCAS1 in the market. Therefore, we cannot use RCAS1 as a diagnostic test for cholangiocarcinoma.

Currently, telomerase activity is used as a common molecular tumor marker in the serum and many evidences also found a good correlation between the telomerase activity and the expression of hTERT subunit. Leelawat et al. (27, 33) performed a case-control study demonstrated that hTERT mRNA was detected in almost all of the cholangiocarcinoma patients (84.85% of cases). However, in benign biliary tract disease patients, hTERT mRNA was also detectable (21.9% of cases). Comparison with the common tumor marker, CA19-9 was detected in only 60.60% of cases. The authors used serum from benign biliary tract diseases as a control which is compatible with the real situations that the doctors have to differential cases of benign biliary tract diseases from cholangiocarcinoma cases. This data suggested that hTERT mRNA should be a candidate tumor marker in cholangiocarcinoma patients. However, at this time, the methods to detect hTERT mRNA in serum are too complex and expensive to use in our clinical fields.

The lack of good candidate serum tumor markers for diagnosis of cholangiocarcinoma influences us to investigate a novel tumor marker by explores the molecular biology of cholangiocarcinoma. Typically cholangiocarcinoma cells invade basement membrane of bile duct by secrete enzymes that digest the extracellular matrix protein. These enzymes are known as Matrix metalloproteinase (MMPs).

Matrix metalloproteinases (MMPs) are zinc dependent endopeptidase. They are involved in the mechanisms of the turnover and degradation of extracellular matrix (ECM) components and basement membranes. Increase activity of MMPs can be found in pathological diseases including arthritis, cirrhosis, aortic aneurysms, fibrosis and

cancer progression. At this time, there are 23 human MMPs including interstitial collagenases (MMP-1, -8, -13, and -18), which preferentially digest collagen type I, II and III; gelatinases (MMP-2 and -9), or type IV collagenases; stromelysins (MMP-3, -10, and -11), which can digest laminin; membrane-type MMPs [MMP-14 (MT1-MMP), MMP-15 (MT2-MMP), MMP-16 (MT3-MMP), MMP-17 (MT4-MMP), MMP-24 (MT5-MMP) and MMP-25 (MT6-MMP), matrilysins (MMP7 and-26), the elastase MMP-12, and others (MMP-19,-20,-23, -28)]. There are also endogenous tissue inhibitors of metalloproteinases (TIMPs-1 to -4) (15).

For studying the association between MMPs and cholangiocarcinoma, electronic databases PubMed is searched for citing the articles identified MMPs as a diagnosis test for cholangiocarcinoma. There were 3 papers matched with these criteria. All of these papers identified the expression of MMPs in cholangiocarcinoma specimens derived from surgical resection by immunohistochemical staining. MMP7 is highly found in cholangiocarcinoma specimens. Itatsu K, et al. (21, 22) performed the retrospective analysis study and found that MMP7 is the most frequently expressed in cholangiocarcinoma specimens (MMP-2 (33.9%), MMP7 (75.8%) and MMP-9 (47.5%)). In addition, previous studies identified that MMP7 was found in cholangiocarcinoma but not in hepatocellular carcinoma or normal liver specimens (22, 34). MMP7 is a secreted protein which can be detected in peripheral blood. Previous publications showed that MMP7 can be detected in peripheral blood of renal cell carcinoma and ovarian cancer patients (20, 35). Therefore, MMP7 should be used as a candidate marker for detection of cholangiocarcinoma. However, until now, there is no study about the detection of MMP7 in peripheral blood of cholangiocarcinoma patients.

CHAPTER III

RESEARCH METHODOLOGY

3.1 Research Design

This study was divided into 2 parts

Part I (Discovery Phase): A case-control design of the diagnostic test study was performed by using the serum of 'case' (cholangiocarcinoma) and 'control' (benign obstructive jaundice) from serum bank derived from the patients who treated at Department of Surgery, Rajavithi Hospital during February 2008 to December 2008.

Part II: According to the study of biomarker implementation, it is now widely appreciated that the evaluation of biomarker performance must be separated from biomarker discovery. In discovery research, its performance in those samples may be biased in an overoptimistic direction. To estimate performance without bias, an independent dataset should be investigated (36-39). Therefore, the aim of the Part II study was to evaluate the performance of serum MMP7 and CA19-9 for diagnosis of cholangiocarcinoma in a new independent data set of prospective consecutive cases of patients with evidence of bile duct obstruction from various etiologies. This study was performed according to the PRoBE (a prospective-specimen-collection, retrospective-blinded-evaluation) design (36). We collected the serum from a cohort that represents the target population (consecutive cases of obstructive jaundice patients whom undergone ERCP, PTBD or bile duct surgery) who treated at the Department of Surgery, Rajavithi Hospital during January 2009 – November 2009. After the diagnosis status of these patients was ascertained, the value of serum MMP7 and CA19-9 were assayed in a fashion that blinded to case-control status. The serum of 'true' disease (cholangiocarcinoma) status is obtained for all these patients with reference standard. Therefore there is no referral or partial verification bias. In addition, we implemented the STARD statements (37-39) to ensure standardization and transparency of our study.

3.2 Study Population

Thai obstructive jaundice patients with evidence of intrahepatic bile duct dilatation from radiological study treated at Rajavithi Hospital.

3.3 Target Population

Thai obstructive jaundice patients with evidence of intrahepatic bile duct dilatation from radiological investigation.

3.4 Sample Size Calculation

Part I: There is no previous study about the accuracy of serum MMP7 in diagnosis of cholangiocarcinoma. Therefore, we suggested that the detection of serum MMP7 would be clinically helpful in discrimination between cholangiocarcinoma patients and benign bile duct disease patients, the area under the ROC curve (AUC) of serum MMP7 in diagnosis of cholangiocarcinoma should be higher than 0.70.

We use the PASS 2008 software to calculate the sample size in this study {Hanley, 1983 #59}. The sample size was calculated on the basis of an expected an area under the ROC curve of the serum MMP7 for diagnosis of cholangiocarcinoma of 0.70(40) . To detect a difference of 0.20 between the area under the ROC curve (AUC) under the null hypothesis of 0.50 and an AUC under the alternative hypothesis of 0.70 using a two-sided z-test at a significance level of 0.05. A sample of 41 from the positive group (cholangiocarcinoma cases) and 41 from the negative group (benign bile duct diseases) achieve 90% power were collected. If the values of area under the ROC curve (AUC) of MMP7 is better than 0.7, the Part II study is performed.

Part II: This study was conducted within the Rajavithi Hospital Surgery department located in Bangkok, Thailand. The local ethics committee approved the study protocol. The sample size was calculated on the basis of an area under the ROC curve of the serum MMP7 for diagnosis of cholangiocarcinoma derived from the result on **Part I** (=0.73). (40). By use of a significant level of 0.05 (two sided) and a power of 0.95, a sample of 50 from the cholangiocarcinoma patients was required for the study (41). From previous data, the prevalence of cholangiocarcinoma detection from obstructive jaundice patients treated at our department was shown to be in the range of

27-30%. Therefore, we prospectively included 187 consecutive patients with symptoms of obstructive jaundice who had undergone ERCP, PTBD or bile duct surgery during a period from June of 2008 to July of 2009.

3.5 Reference Standard for Diagnosis of Cholangiocarcinoma

Diagnosis cholangiocarcinoma will be done, if the patients have one of the following criteria:

1. Tissue diagnosis of cholangiocarcinoma by pathologist from Rajavithi Hospital who have experience in diagnosis cholangiocarcinoma for at least 5 years.

2. Cytology diagnosis of cholangiocarcinoma by pathologist from Rajavithi Hospital who have experience in diagnosis cholangiocarcinoma for at least 5 years.

3. Radiological finding (Helical CT scans or MRI with contrast); Detect a tumor (mass lesion from delay enhancement on CT-scan and biliary tract dilatation) at first visit and the progression of tumor is observed when follow up within 3 months or detect tumor at first follow up and the progression of tumor is observed at second follow up.

Gate Criteria*

3.6 Inclusion Criteria:

1. Thai obstructive jaundice patients with evidence of intrahepatic bile duct dilatation from radiological investigation within 6 months and treated at Department of Surgery, Rajavithi Hospital.

2. Age range from 30 – 70 years

3.7 Exclusion Criteria:

1. We exclude the situation that can detect high level of serum MMP7

- Presence of cancer in other organs except in bile duct
- Presence of idiopathic pulmonary fibrosis
- Presence of aortic aneurysm

- Presence of severe arthritis

2. Patients with an inconclusive diagnosis were excluded from this study

3.8 Serum Collection and the Measurement of Serum Biochemistry

Five-milliliter samples of fasting peripheral venous blood from the patients were collected at the time before the procedures (ERCP, PTBD, or bile duct surgery) and their serum was separated and stored at -78°c within 2 h. Serum biochemical tests including albumin, globulin, AST, ALT, total and direct bilirubin, alkaline phosphatase (ALP), CEA and CA19-9 were measured using routine automated methods in the Rajavithi Hospital Pathological Laboratory.

3.9 Detection of Serum MMP7

MMP7 levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN). The diluted serum sample was added in duplicate to 96-well plates coated with the MMP7 antibody and incubated at room temperature for 2 h. After washing six times with washing buffer, the conjugated secondary antibody was added and the plate was further incubated for 2 h. Plates was washed again prior to incubation with the substrate solution for 1 h. The amplifier solution was then added and the plate will be incubated for an additional 30 min. All incubation cycles will be performed at room temperature. Following termination of the reaction with the stop solution (1 N sulfuric acid), the optical density was measured at 490 nm using a spectrophotometric microplate reader. The concentration of MMP7 in each sample was calculated from a standard curve.

3.10 Blinding Methods

The scientists who perform ELISA for MMP7 assay were blinded for the diagnosis of each patient and also blinded for the results of serum CA19-9.

3.11 Outcome Measurement

The primary outcome measure is the accuracy of serum MMP7 for diagnosis of cholangiocarcinoma.

The secondary outcome measure is the comparison between the accuracy of serum MMP7 and CA19-9 for diagnosis cholangiocarcinoma.

3.12 Data Collection

Demographic characteristics, MRI or CT scan findings, blood biochemical tests (AST, ALT, ALP, total bilirubin, direct bilirubin, albumin, globulin, BUN, creatinine, CEA and CA19-9) and clinical data were recorded in a computerized database. Blood tubes for serum MMP7 assay were labeled with serial numbers that represent each patient before sending to the surgical laboratory for MMP7 ELISA procedure.

3.13 Statistical Analysis

Data are presented as the mean ± SD, unless otherwise mentioned. Comparisons between the quantitative variables were performed using *Mann-Whitney U* or Student's t-test, as appropriate. One-way analysis of variance (ANOVA) with the multiple comparisons by Post HOC Scheffe method or Kruskal Wallis test was used to compare each value (MMP7, CA19-9) to the control early and late stage cholangiocarcinoma groups. Qualitative variables were reported as counts, and comparisons between independent groups were performed using Pearson Chi-square tests. Correlations between MMP7 levels and other parameters were examined using the Pearson correlation coefficient. A receiver operating characteristic (ROC) curve was generated by plotting the sensitivity against 1-specificity, and the area under the curve with 95% confidence intervals was calculated. The optimal cutoff points for MMP7 were selected based on the ROC curve analysis. Sensitivity, specificity, positive predictive value and negative predictive values were calculated using a 2 × 2 table of the collected data. The data on various blood chemistries and levels of CA19-9 and MMP7 that were significantly different between the control and cholangiocarcinoma groups were analyzed by multiple logistic regression analysis.

3.14 Ethical Consideration

For Part I study, this study needs the serum from 41 cases of cholangiocarcinoma and 41 cases of benign biliary tract diseases patients. This study used the serum that was collected from the previous associated project “Identification of

MMP-9 in the serum of cholangiocarcinoma and benign biliary tract disease patients" (from February 2008 – January 2009). The study protocol is reviewed and approved by the Institutional Review Board of Rajavithi Hospital. Informed consent is obtained from the patients from February 2009 - November 2009.

3.15 Limitation

It is too invasive to get tissue for diagnosis of cholangiocarcinoma in all patients. In addition the nature of cholangiocarcinoma is infiltrative lesion along the biliary tract. Therefore, many cases are biopsy negative. For that reason, long term follows up in patients who have lesion in the biliary tract but biopsy negative will be added to the reference standard.

Most of the cholangiocarcinoma patients treated at Rajavithi Hospital are in advanced stages. Therefore, the value of MMP7 in the early stage of cholangiocarcinoma may be not appropriate.

Expected benefit and application

To know the accuracy of detection of serum MMP7 may help the physicians to give a proper diagnosis and make a better decision for the management of cholangiocarcinoma patients.

3.16 Conceptual Frame Work

Figure 1 Conceptual of Part I study is demonstrated.

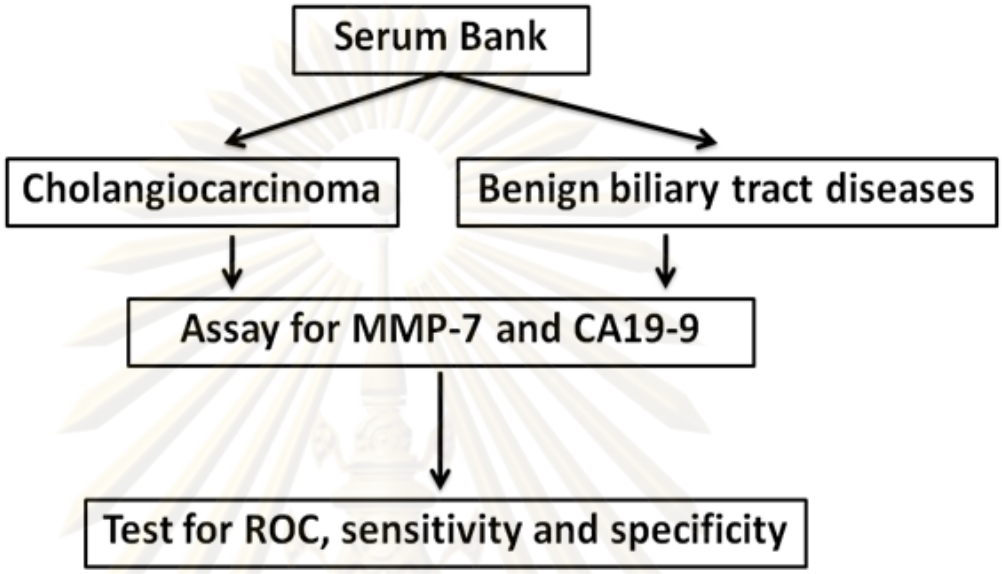
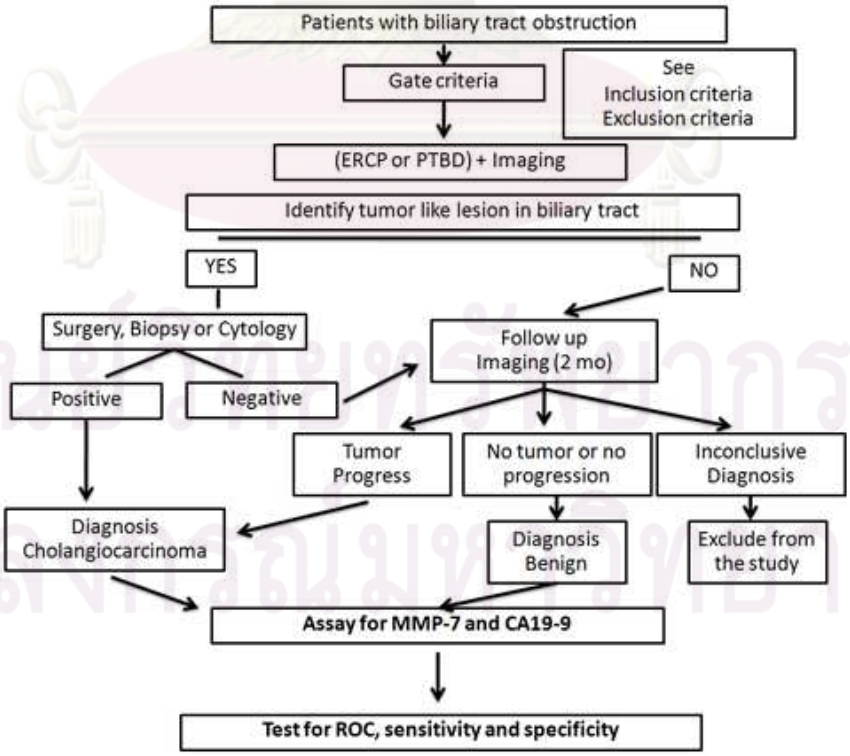


Figure 2 Conceptual of Part II study is demonstrated.



CHAPTER IV

RESULTS

4.1 Part I Results

4.1.1 Patient Characteristics

In cholangiocarcinoma cases, there were 12 cases of intrahepatic cholangiocarcinoma and 32 cases of perihilar cholangiocarcinoma. Primary or secondary common bile duct stones (*78%; n* = 28/33) were the most common diseases in the control patients. The clinical characteristics of the patients in this study are shown in Table 1. No statistically significant differences were found among the data of the patients considered as controls and those with cholangiocarcinoma regarding gender, age, serum albumin, globulin and ALT levels. However, the level of serum AST, bilirubin and alkaline phosphatase were significantly higher in cholangiocarcinoma patients than in controls (*Mann-Whitney U test; p*<0.05).

Table 1 - Clinical characteristics of the patients with benign biliary tract disease (control) and cholangiocarcinoma

	Control (n=33)	Cholangiocarcinoma (n=44)	*p* value
Age (Yr)	54 ±14.5	59±12.9	0.130
Sex (Male:Female)	15:18	26:18	0.258#
Total bilirubin (mg/dL)	4.2±5.53	14.6±11.34	<0.001*
Direct bilirubin (mg/dL)	2.6±3.75	10.3±8.47	<0.001*
Albumin (g/dL)	3.8±0.61	3.1±0.68	0.050
Globulin (g/dL)	3.6±0.73	4.1±0.93	0.253
AST (U/L)	65.4±53.80	183.9±378.82	0.012*
ALT (U/L)	75.0±77.72	101.4±14.49	0.615
ALP (IU/L)	318.6±349.65	551.8±526.04	0.001*

Quantitative variables are presented as the means ± standard deviation. #; Pearson Chi-square was used to compare between two groups, *; the level of serum

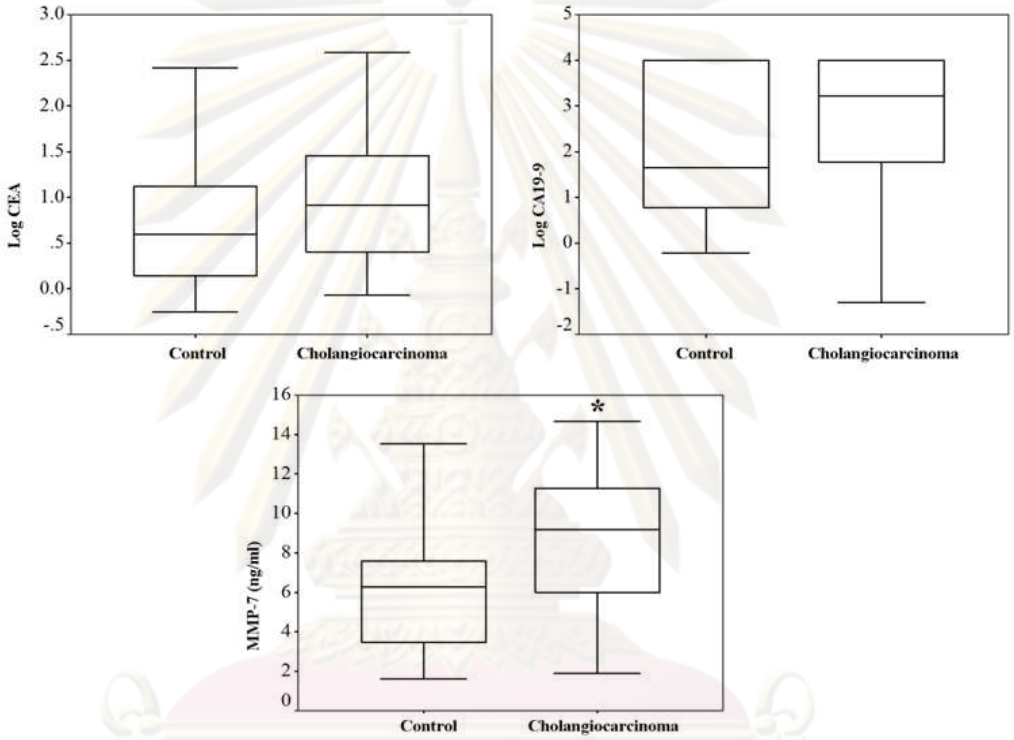
total bilirubin, direct bilirubin, AST and ALP were significantly higher in cholangiocarcinoma patients than in controls (*Mann-Whitney U test; p*<0.05).

4.1.2 Detection of CEA, CA19-9 and MMP7 in serum of cholangiocarcinoma and benign obstructive jaundice patients

The median CEA and CA19-9 values in the control group were 3.96 ng/ml (range; 0.56-260.24) and 45.88 U/ml (range 0.60-10000.00), respectively. The median CEA and CA19-9 values in the cholangiocarcinoma group were 8.27 ng/ml (range; 0.85-131.70) and 2176.00 U/ml (range; 0.50-10000.00), respectively. However, there was no statistically significant difference in the levels of these two markers between the control and cholangiocarcinoma patients (*Mann-Whitney U test; p*=0.057 for CEA and *p*=0.056 for CA19-9). The serum MMP7 values in the cholangiocarcinoma patients (mean±SD; 8.9±3.43 ng/ml) were significantly higher than those in the control patients (mean±SD; 5.9±3.03 ng/ml), (Student's t-test*; p*<0.001, 95% CI 1.34-4.47). These data are shown in Figure 3.

Figure 3 - Serum levels of CEA, CA19-9 and MMP7 in cholangiocarcinoma and control (benign biliary tract disease) patients.

Box plots comparing levels of CEA, CA19-9 and MMP7 are demonstrated. Levels of MMP7 are presented as ng/ml, while CEA and CA19-9 are presented with the log data to accommodate the wide range. *; Only the value for MMP7 between the two groups is significantly different (Student's t-test; $p<0.001$).



We used a CEA cut-off value of 5 ng/ml and a CA19-9 cut-off value of 100 U/ml because these have been the suggested cut-off value for the diagnosis of cholangiocarcinoma (42). Using a CEA cut-off value of 5 ng/ml, the sensitivity was determined to be 58.54% (CI 95% 43.37 - 72.24), and the specificity was determined to be 62.50% (CI 95% 45.25 – 77.07). Using a CA19-9 cut-off value of 100 U/ml, the sensitivity was determined to be 70.45% (CI 95% 55.78 – 81.84), and the specificity was determined to be 63.64% (CI 95% 46.62 – 77.81).

4.1.3 ROC curve analysis for CEA, CA19-9 and MMP7 for diagnosis of cholangiocarcinoma

An ROC curve analysis (Figure 4) was used to calculate an area under the curve (AUC) of 0.63 (CI 95% 0.501 – 0.760) and of 0.63 (CI 95% 0.491 – 0.761) for CEA and CA19-9, respectively. Additionally, an ROC curve analysis was used to calculate an area under the curve of 0.73 (CI 95% 0.614 – 0.848) for MMP7. When comparing the AUC of the ROC curve for CEA, CA19-9 and MMP7 with a chance value equal to 0.5 (the worst value of AUC of ROC), only the AUC of the ROC for MMP7 is significantly higher than 0.5 (p = 0.001). The sensitivity and specificity for CEA, CA19-9 and MMP7 are presented in Table 2.

Figure 4 - ROC curve analyses of CEA, CA19-9 and MMP7 for the diagnosis of cholangiocarcinoma.

The diagnostic accuracy of each biomarker, in terms of its sensitivity and specificity, are presented by receiver operating characteristic (ROC) curve analysis. Figures 2A, 2B, 2C and 2D correspond to **CEA, CA19-9 and MMP7**. Only the area under the curve (AUC) of the ROC for MMP7 is significantly higher than a chance value (0.5).

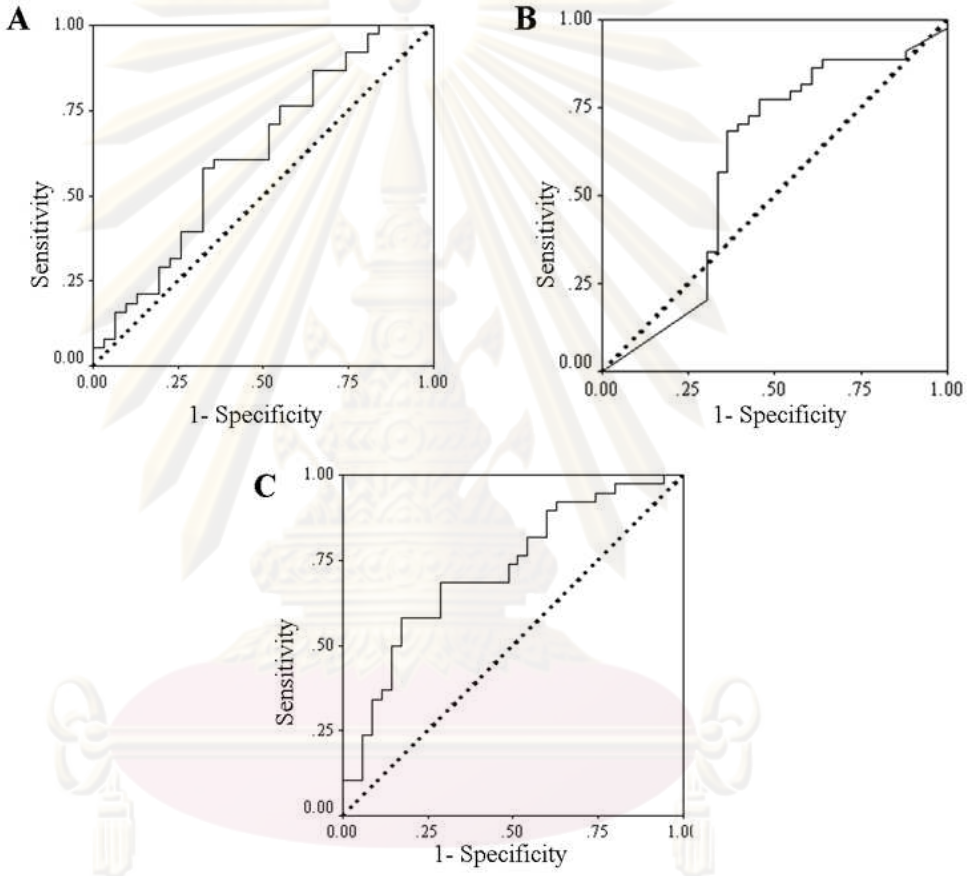


Table 2 - Performance of the biomarkers for the diagnosis of Cholangiocarcinoma

Biomarker (cut-off value)	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	PLR (95% CI)	NLR (95% CI)
CEA (3ng/ml)	70.73 (55.52-82.39)	43.75 (28.17-60.67)	1.26 (0.87-1.81)	0.67 (0.36-1.24)
CEA (5ng/ml)	58.54 (43.37-72.24)	62.50 (45.25-77.07)	1.56 (0.93-2.62)	0.66 (0.42-1.04)
CA19-9 (35 U/ml)	81.82 (68.04-90.49)	48.48 (32.50-64.78)	1.59 (1.11-2.27)	0.38 (0.18-0.77)
CA19-9 (100 U/ml)	70.45 (55.78 - 81.84)	63.64 (46.62-77.81)	1.94 (1.19-3.16)	0.46 (0.28-0.78)
MMP7 (6.0 ng/ml)	76.32 (60.79-87.01)	46.88 (30.87-63.55)	1.44 (0.99-2.08)	0.51 (0.26-1.00)
MMP7 (7.4 ng/ml)	63.16 (47.28-76.62)	71.88 (54.63-84.44)	2.25 (1.23-4.11)	0.51 (0.32-0.82)

The sensitivity, specificity, positive and negative likelihood ratio (LR) as well as their 95% confidence interval (CI) for each marker is presented. The likelihood ratio is the ratio of true and false positives (sensitivity and 1-specificity respectively), where the higher values reflect the probability of a better performance. (PLR, positive likelihood ratio; NLR, negative likelihood ratio; 95% CI, 95% confidence interval)

To determine whether the values of serum MMP7 was predictive of cholangiocarcinoma independently of other tumor markers, we carried out a logistic regression analysis. In a multivariable model using MMP7 (cut-off value=5.5 ng/ml), CEA (cut-off value=5 ng/ml), CA19-9 (cut-off value=100 U/ml), MMP7 (an adjusted odds ratio = 3.1; 95% CI = 1.05-9.03; p=0.041) and CA19-9 (an adjusted odds ratio = 3.3; 95% CI = 1.16-9.34; p=0.025) were the independent predictors of cholangiocarcinoma, whereas CEA was not.

Table 3 Odd Ratios (OR) estimates for diagnosis of cholangiocarcinoma

The significant parameters ($p<0.05$) selected by the model are shown

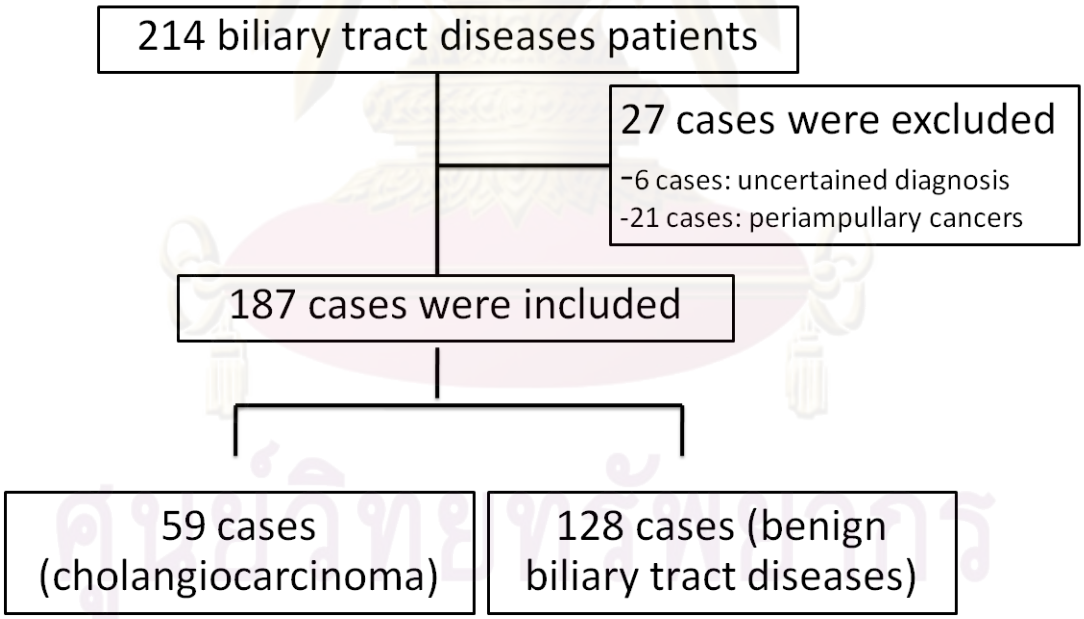
Variables	OR (95% CI)	*p*
CA19-9	3.3 (1.16-9.34)	0.025
MMP7	3.1(1.05-9.03)	0.041
CEA	1.8 (0.62-5.01)	0.287

4.2 Results Part II

4.2.1 Patient characteristics

A total of 214 obstructive jaundice patients were consecutively enrolled. Twenty-one cases were excluded according to their diagnosis of ampullary cancer and duodenum cancer. In addition, six cases were excluded according to their uncertain diagnosis (Figure 5). The 187 subjects studied included 128 patients with benign biliary tract diseases (control group) including intra-hepatic duct stones, common bile duct stones, and benign bile duct strictures, and a total of 59 patients with cholangiocarcinoma. For cholangiocarcinoma, 40 cases were diagnosed as perihilar-cholangiocarcinoma, 16 cases were diagnosed as intrahepatic cholangiocarcinoma and 3 cases were diagnosed as distal common bile duct cholangiocarcinoma.

Figure 5- A flow diagram of a total of 187 obstructive jaundice patients whom were consecutively enrolled in this study



As shown in Table 4, no statistically significant differences in gender, age, serum globulin and ALT levels were identified among the data from the control patients when compared to the cholangiocarcinoma patients. However, the level of serum albumin, AST, bilirubin and alkaline phosphatase (ALP) were significantly higher in cholangiocarcinoma patients than in the control patients (*Mann-Whitney U test; p* < 0.05).

Table 4 Clinical characteristics of patients with benign biliary tract diseases (control) and Cholangiocarcinoma

	Control N=128	Cholangiocarcinoma N=59	*p*
Age (yr)	57 ± 19	60 ± 15	0.287
Sex (male:female)	62:66	36:23	0.118
Albumin (mg/dl)	3.9 ± 0.67	3.1 ± 0.59	<0.001
Globulin (mg/dl)	3.9 ± 0.72	4.1 ± 0.91	0.073
Total bilirubin (U/L)	3.3 ± 3.71	12.0 ± 11.35	<0.001
AST (U/L)	73.4 ± 78.14	91.2 ± 75.91	0.003
ALT (U/L)	76.3 ± 83.32	52.2 ± 43.58	0.884
ALP (U/L)	320.3 ± 230.03	380.5 ± 314.52	<0.001

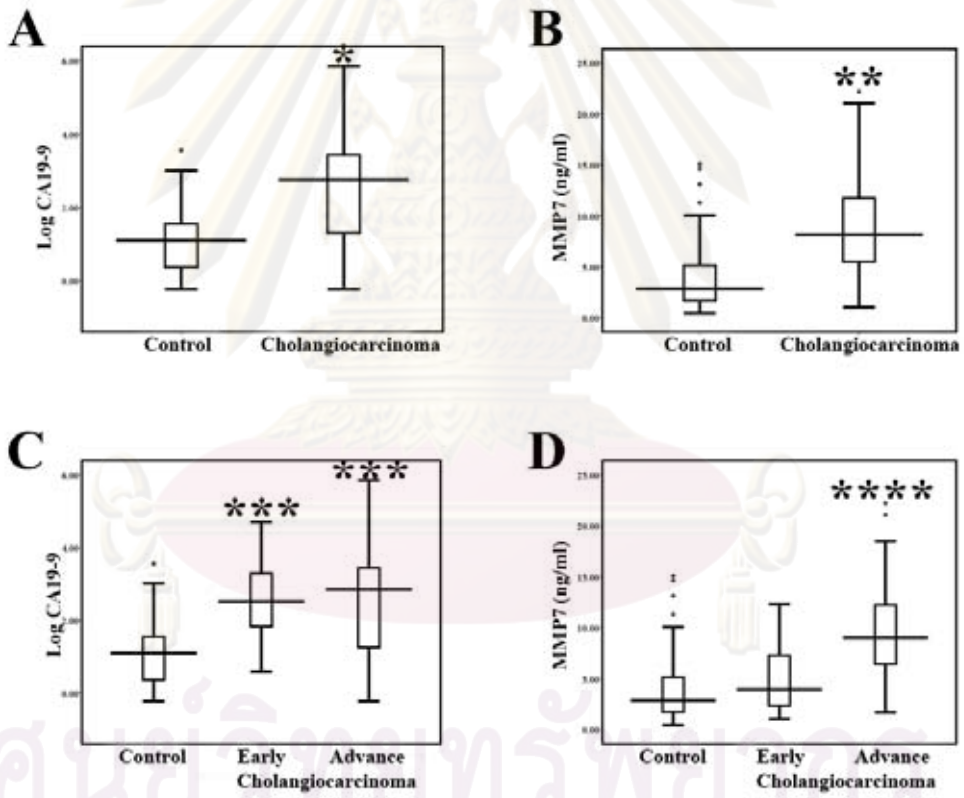
4.2.2 Serum levels of CA19-9 and MMP7*

The serum CA19-9 and MMP7 levels were compared among disease groups. The median values of serum CA19-9 levels were 20.43 U/ml (range: 0.6-71,000 U/ml) in the control group and 571.2 U/ml (range: 0.6-71,000 U/ml) in the cholangiocarcinoma group. The mean values of serum MMP7 levels were 3.7 ± 2.81 ng/ml in the control group and 8.7 ± 4.56 ng/ml in the cholangiocarcinoma group. As shown in Figure 6A and Figure 6B, serum CA19-9 and MMP7 values were significantly higher in cholangiocarcinoma cases when compared to the control patients (CA19-9: *Mann-Whitney U test*; $p<0.001$ and MMP7: Student's t-test; $p<0.001$).

Moreover, we also classified cholangiocarcinoma patients into two groups: early (TNM stage I and II; 11 patients) and advanced (TNM stage III and IV; 48 patients) stages. The data shown in Figure 6C demonstrates that the MMP7 levels tended to increase according to the progression of cholangiocarcinoma. The serum MMP7 values were significantly different between early and late stages of cholangiocarcinoma (ANOVA; $p<0.001$). However, the serum MMP7 values from early stage cholangiocarcinoma were not significantly different from the serum MMP7 values of benign control patients (ANOVA; $p=0.47$). Although the serum CA19-9 values in the early and late stages of cholangiocarcinoma were significantly higher than in the controls (Kruskal Wallis test; $p<0.001$), the values were not significantly different between the early and late stages of cholangiocarcinoma (Figure 6D).

*From Part I study, we found that serum level of CEA is not a predictive factor for diagnosis of cholangiocarcinoma. Therefore, we do not measure the serum level of CEA in Part 2 study

Figure 6 -Serum levels of CA19-9 and MMP7 in cholangiocarcinoma and control (benign biliary tract disease) patients. (A) Box plots comparing levels of CA19-9 and (B) MMP7 between cholangiocarcinoma and control are illustrated. (C) Box plots comparing levels of CA19-9 and (D) MMP7 between early and advance stages of cholangiocarcinoma and control are illustrated. Levels of MMP7 are presented as ng/ml, while CA19-9 is presented with the log data to accommodate the wide range. (*; *Mann-Whitney U*; $p < 0.001$ compare to control, **; Student's t-test; $p < 0.001$ compare to control, ***; Kruskal Wallis test; $p < 0.001$ compare to control, ****; ANOVA; $p < 0.001$ compare to control)



4.2.3 Serum levels of CA19-9 and MMP7 for the diagnosis cholangiocarcinoma

To determine the diagnostic accuracy of serum CA19-9 and MMP7 levels for differentiating cholangiocarcinoma from benign bile duct diseases, an ROC curve analysis was applied to calculate an area under the curve (AUC). These levels were determined to be 0.79 (CI 95% 0.708 – 0.868) and 0.84 (CI 95% 0.778 – 0.903) for CA19-9 and MMP7, respectively (Figure 7). The sensitivity, specificity, positive and negative predictive values for selected cut-off points of CA19-9 and MMP7 are presented in Table 5.

Figure 7 - ROC curve analyses of CA19-9 and MMP7 for the diagnosis of cholangiocarcinoma. The diagnostic accuracy of each biomarker, in terms of its sensitivity and specificity, are presented by receiver operating characteristic (ROC) curve analysis.

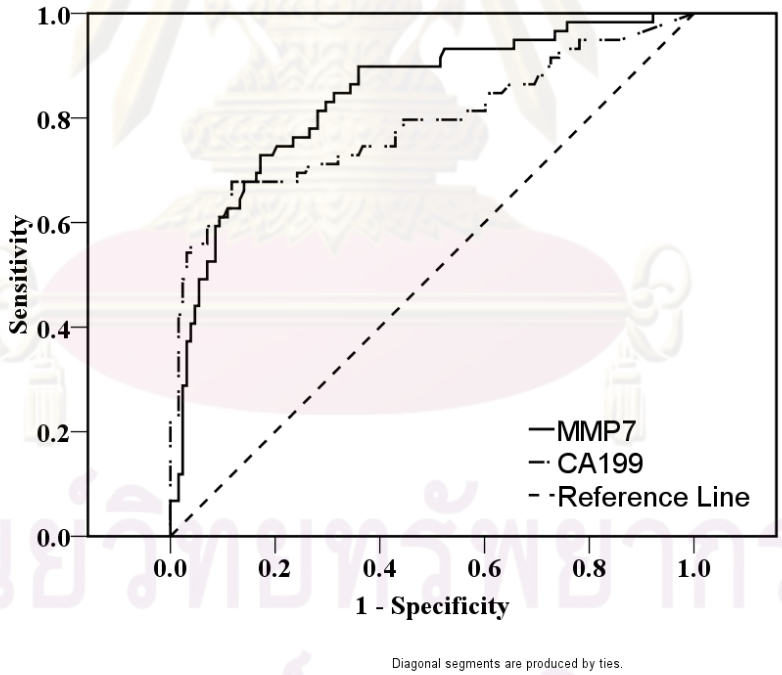


Table 5 Performance of the biomarkers for the diagnosis of cholangiocarcinoma

PPV; positive predictive value, NPV; negative predictive value, LR+; positive likelihood ratio, LR-; negative likelihood ratio, CI; confidence interval

Tumor Markers (cut-off value)	Sensitivity(%) (95% CI)	Specificity (%) (95% CI)	PPV (%) (95% CI)	NPV (%) (95% CI)	LR+(%) (95% CI)	LR-(%) (95% CI)
MMP7 (5.5 ng/ml)	75 (63-86)	78 (71-85)	61 (50-72)	87 (81-93)	3.41 (2.38-4.89)	0.33 (0.21-0.51)
MMP7 (6.5 ng/ml)	63 (50-75)	87 (81-93)	69 (56-81)	83 (77-90)	4.72 (2.91-7.66)	0.43 (0.31-0.60)
MMP7 (7.5 ng/ml)	53 (40-65)	92 (88-97)	76 (62-89)	81 (74-87)	6.73 (3.54-12.70)	0.51 (0.39-0.68)
CA19-9 (35 ng/ml)	71 (60-83)	73 (66-81)	55 (44-66)	85 (78-91)	2.68 (1.93-3.73)	0.39 (0.26-0.59)
CA19-9 (100 ng/ml)	68 (56-80)	87 (81-93)	70 (58-82)	85 (79-91)	5.10 (3.17-8.22)	0.37 (0.25-0.54)
CA19-9 (200 ng/ml)	59 (47-72)	93 (89-97)	80 (68-91)	83 (77-89)	8.44 (4.34-16.40)	0.44 (0.32-0.60)

When the cut-off value of serum MMP7 was set at 5.5 ng/ml and serum CA19-9 values were set at 100 U/ml, the predictive probabilities for the diagnosis of cholangiocarcinoma could then be calculated from logistic regression analysis. As shown in Table 6, if the patients have their serum MMP7 and CA19-9 higher than the cut-off values, they will have a probability for diagnosis of cholangiocarcinoma equal to 86.12%. In addition, if the patients have their serum MMP7 and serum CA19-9 less than the cut-off values, they will have very low probabilities for a positive diagnosis of cholangiocarcinoma (<6.4%).

Table 6 Predicted probability of the combination of serum CA19-9 and MMP7 for diagnosis of Cholangiocarcinoma

CA19-9 (>100 ng/ml)	MMP7 (>5.5 ng/ml)	Predicted probability (%)
-	-	6.40
-	+	36.10
+	-	42.84
+	+	86.12

4.2.4 Correlation between MMP7, CA19-9 and other blood chemistry

The correlation between the values of serum albumin, AST, ALT, ALP, total bilirubin, CA19-9, and MMP7 were investigated. As presented in Table 7, the level of serum MMP7 was significantly correlated with serum albumin, AST, ALP, total bilirubin and CA19-9, although none of these parameters have a high value of Pearson correlation coefficient (> 0.7). We suggest that the significant correlation of these blood chemistries with serum MMP7 is caused by the high number of samples we enrolled in this study.

Table 7 Pearson's correlation coefficients of MMP7, CA19-9, albumin, total bilirubin, AST, ALT and ALP

*Statistically significant; $p < 0.05$

Pearson correlation	CA19-9	Albumin	Total bilirubin	AST	ALT	ALP
MMP7	0.415*	-0.577*	0.328*	0.154*	-0.055	0.268*
CA19-9	0.415*	-0.370*	0.356*	0.064	-0.022	0.139

4.2.5 Evaluation of serum CA19-9 and MMP7 levels for the diagnosis of cholangiocarcinoma: Multiple logistic regression analysis

To determine whether the values of serum CA19-9 and MMP7 were predictive of cholangiocarcinoma independent to the other blood chemistry values that were significantly different between control and cholangiocarcinoma patients, we carried out a logistical regression analysis. In a multivariable model using CA19-9 (cut-off value = 100 ng/ml), MMP7 (cut-off value = 5.5 ng/ml), total bilirubin (cut-off value = 5 U/L), albumin (cut-off value = 4 mg/dl), AST (cut-off value = 100 U/L) and alkaline phosphatase (cut-off value = 200 U/L), CA19-9, MMP7 and albumin were shown to be independent predictors for cholangiocarcinoma. None of the other parameters (total bilirubin, AST and ALP) reached statistical significance (Table 8).

Table 8 Odd Ratios (OR) estimates for diagnosis of cholangiocarcinoma

The significant parameters ($p<0.05$) selected by the model are shown

Variables	OR (95% CI)	*p*
CA19-9	15.2 (5.20-44.56)	<0.001
MMP7	5.5 (1.87-16.03)	0.002
Albumin	0.015 (0.01-0.15)	<0.001
Total bilirubin	2.4 (0.81-7.20)	0.115
AST	1.2 (0.37-4.12)	0.738
ALP	0.3 (0.09-1.05)	0.060

CHAPTER V

DISCUSSION AND CONCLUSION

The need for better tests to diagnose and screen for patients with cholangiocarcinoma is an important issue that must be addressed to improve the treatment results for these patients. Unfortunately, no specific serum tumor markers have been identified for this disease.

Based on the results of our Part I study, the sensitivity and specificity of CEA as a marker for detecting cholangiocarcinoma are 58.54% and 62.50%, respectively. This is consistent with previously published studies that reported that the sensitivity and specificity of CEA for detecting cholangiocarcinoma were 33-84% and 33-100%, respectively (11, 13, 42). Previous articles have addressed the accuracy of CA19-9 in the identification of cholangiocarcinoma. A previous study identified cholangiocarcinoma with a sensitivity of 67.5% and a specificity of 86.8% when a cut-off value of 100 U/ml for CA19-9 was used and a sensitivity of 77.9% and a specificity of 76.3% when a cut-off value of 35 U/ml for CA19-9 was used (24). In our series, we found that the sensitivity was 70.45% and the specificity was 63.64% when using a cut-off value of 100 U/ml for CA19-9. However, the AUC of the ROC curve for CA19-9 was only 0.63 in the discrimination of cholangiocarcinoma in our Part I study. Therefore, when the cut-off value was changed to 35 U/ml, the specificity markedly decreased (81.82% of sensitivity and 48.48% of specificity). We suggest that the differences among the patients should be concerned. In the study published by John, A. R., et al, 25 patients with benign liver tumors and 13 patients with benign bile duct strictures were used as a control group (24). However, in our studies, all the subjects in the control group had been diagnosed with benign bile duct diseases. The reason that we used patients with benign bile duct diseases as a control group was because the symptoms of cholangiocarcinoma are similar to the symptoms of benign bile duct diseases in our clinical setting.

We observed that most of the cholangiocarcinoma patients were suffering from the invasiveness of the cholangiocarcinoma cells into the adjacent organs.

The mechanism by which cancer cells invade the surrounding tissue requires the breakdown of the extracellular matrix and the subsequent migration of the cancerous cells through the degraded structures (43). Because extracellular matrix remodeling is the major activity of a family of enzymes known as matrix metalloproteinases (MMPs), these enzymes were investigated for their contributions to the malignant phenotype in cholangiocarcinoma patients. Previous studies have demonstrated that cholangiocarcinoma specimens frequently express MMP7 (75.8-100%) (21, 22). As far as we are aware, no other published investigation is available that uses the serum MMP7 level to diagnose cholangiocarcinoma. Our study shows that the serum MMP7 level is significantly higher in patients with cholangiocarcinoma than with benign biliary tract diseases.

MMP7 is the smallest of the MMPs and has been demonstrated to degrade or process a variety of matrix and nonmatrix molecules. Unlike most MMPs, which are expressed by stromal cells, MMP7 is principally expressed by epithelial cells (15). A previous study reported that the serum MMP7 level was significantly elevated in patients with ovarian cancer and advanced colorectal cancer (35, 44). We suggest that MMP7 might be detected in many cancers that originate from epithelial cells. In addition, we also found that the accuracy of the serum MMP7 level for the diagnosis of cholangiocarcinoma is better than the serum level of CEA and CA19-9, as observed by calculating the AUC of the ROC curve. Only the AUC of the ROC curve for the serum MMP7 level is significantly higher than a chance value (0.5). Our study demonstrated that use of serum MMP7 could identify cholangiocarcinoma patients from benign biliary tract disease patients. However, further larger prospective studies that evaluated the benefit of serum MMP7 in helping the physician to take decisions on diagnosis cholangiocarcinoma are necessary before the implementation of using serum MMP7 as a marker for cholangiocarcinoma.

According to the study of biomarker implementation, it is now widely appreciated that the evaluation of biomarker performance must be separated from biomarker discovery. In discovery research, its performance in those samples may be biased in an overoptimistic direction. To estimate performance without bias,

an independent dataset should be investigated (36-39). Therefore, the aim of the Part II study was to evaluate the performance of serum MMP7 and CA19-9 for their potentials in the diagnosis of cholangiocarcinoma. We used a new and independent dataset of prospective consecutive cases from patients with evidence of bile duct obstruction from various etiologies. This study was performed according to the PRoBE (a prospective-specimen-collection, retrospective-blinded-evaluation) design (36). We collected the serum from a cohort that is representative of the target population (consecutive cases of obstructive jaundice patients whom undergone ERCP, PTBD or bile duct surgery). After the diagnosis status of these patients was ascertained, the values of serum MMP7 and CA19-9 were assayed in a fashion that blinded the analysis to a case-control status. In addition, we implemented STARD statements (STAndards for the Reporting of Diagnostic accuracy studies) (37-39) to ensure standardization and transparency of our study.

Our study demonstrates that serum MMP7 levels are significantly elevated in patients with a diagnosis of cholangiocarcinoma when compared to patients suffering from benign bile duct diseases. When we compared MMP7 to CA19-9, which is a common clinically-used biomarker of cholangiocarcinoma, the value of AUC of the ROC curve demonstrated that serum levels of MMP7 are better than CA19-9 for the diagnosis of cholangiocarcinoma. These results are consistent with our Part I study, in which serum MMP7 was higher in cholangiocarcinoma than in benign obstructive jaundice patients (40). This suggested that serum MMP7 has the potential to be a tumor marker for cholangiocarcinoma in obstructive jaundice patients.

Previous studies have demonstrated that MMP7 plays a key role in the mechanism of cancer invasion via proteolytic cleavage of the extracellular matrix tissues. It has also been shown to activate other MMPs, such as proMMP-2 and proMMP-9 (45), and inhibit E-cadherin function by ectodomain shedding of E-cadherin (46). The results of several recent studies indicate that MMP7 is over-expressed in a variety of epithelial tumors including those of the esophagus (47), colon (48, 49), pancreas (50), and cholangiocarcinoma tumors (22). In addition, several studies have shown that MMP7 could be detected in the serum of cancer patients, including patients

with ovarian (19), colorectal (44) and gastric cancer (51). This finding suggests that high levels of serum MMP7 are not specific to cholangiocarcinoma. It can be detected in many types of cancer. Therefore, it should be used with other diagnostic modality (clinical presentation and imaging study) before making a diagnosis.

In this Part II study, the values of blood chemistries were shown to be significantly different between control and cholangiocarcinoma groups. Although there were several differences observed, the values of serum CA19-9 and MMP7 levels were shown to be the predictors of cholangiocarcinoma, independent of other blood chemistry values. In addition, the present study is the first to demonstrate the probabilities for the diagnosis cholangiocarcinoma using the combination of serum values of both MMP7 and CA19-9 (Table 3). We suggest that the combination of these markers will aid in the decision of the physician to identify cholangiocarcinoma from benign obstructive jaundice patients.

The values of AUC of the ROC curve for MMP7 and CA19-9 in this study were shown to be much higher than those observed in our Part I study (40). The differences of the designs in each study should be considered. Our Part I study was designed as a retrospective case-control study for diagnostic accuracy. Therefore, some bias from the selection of samples may have occurred. A strength of the Part II study was the implementation of the strategies of PRoBE designs to avoid the problems of bias that may affect the studies of the diagnostic test (36). We collected serum from all obstructive jaundice patients before the diagnosis of cholangiocarcinoma or benign biliary tract diseases had been determined. This procedure assured that biases related to differences in sample collection and handling would be avoided (52). Limitations of this design include the fact that the majority of the study participants were in advancing stages of cholangiocarcinoma. The number of patients with early-stage cholangiocarcinoma was small (n=11), and this number of patients would not have had the statistical power to detect a difference in mean value between these early stages of cholangiocarcinoma and the control group. Further studies, which should include an increased number of early-stage cholangiocarcinoma cases, need to be done before using MMP7 as a screening test for the detection of early stage cholangiocarcinoma.

In addition, this study was performed in the referral center, which has high prevalence of cholangiocarcinoma. As a result, the findings may not be broadly applicable to other hospitals that typically have a low volume of cholangiocarcinoma.

5.1 Conclusions

In conclusion, this study demonstrated that serum MMP7 levels are significantly elevated in cholangiocarcinoma patients. This marker has a potential to be used as a new tumor marker for the discrimination of cholangiocarcinoma patients from benign biliary tract disease patients.

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APPENDICES

APPENDIX A

เอกสารชี้แจงข้อมูลแก่ผู้เข้าร่วมโครงการวิจัย

(Research Subject Information sheet)

ชื่อโครงการวิจัย

การใช้ระดับซีรัม เอ็มเอ็มพี 7 (MMP-7) เพื่อการวินิจฉัยแยกโรคมะเร็งทางเดินน้ำดีจากผู้ป่วยทางเดินน้ำดีอุดตัน ที่ไม่ได้เกิดจากมะเร็ง

วันที่ชี้แจง.....

ชื่อและสถานที่ทำงานของผู้วิจัย:

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ท่านได้รับการเชิญชวนให้เข้าร่วมโครงการวิจัยนี้ แต่ก่อนที่ท่านจะตกลงใจเข้าร่วมหรือไม่ โปรดอ่านข้อความในเอกสารนี้ทั้งหมด เพื่อให้ทราบว่า เหตุใดท่านจึงได้รับเชิญให้เข้าร่วมโครงการวิจัยนี้ โครงการวิจัยนี้ทำเพื่ออะไร หากท่านเข้าร่วมโครงการวิจัยนี้ท่านจะต้องทำอะไรบ้าง รวมทั้งข้อดีและข้อเสียที่อาจจะเกิดขึ้นในระหว่างการวิจัย

ในเอกสารนี้ อาจมีข้อความที่ท่านอ่านแล้วยังไม่เข้าใจ โปรดสอบถามผู้วิจัยหรือผู้ช่วยวิจัยที่ทำโครงการนี้เพื่อให้อธิบายจนกว่าท่านจะเข้าใจ ท่านจะได้รับเอกสารนี้ 1 ชุด กลับไปอ่านที่บ้านเพื่อปรึกษาหารือกับญาติพี่น้อง เพื่อน หรือแพทย์ที่ท่านรู้จัก ให้ช่วยตัดสินใจว่าควรจะเข้าร่วมโครงการวิจัยนี้หรือไม่ การเข้าร่วมในโครงการวิจัยครั้งนี้จะต้องเป็น**ความสมัครใจ**ของท่าน ไม่มีการบังคับหรือชักจูง ถึงแม้ท่านจะไม่เข้าร่วมในโครงการวิจัย ท่านก็จะได้รับการรักษาพยาบาลปกติ การไม่เข้าร่วมหรือถอนตัวจากโครงการวิจัยนี้ จะไม่มีผลกระทบต่อการได้รับบริการ การรักษาพยาบาลหรือผลประโยชน์ที่พึงจะได้รับของท่านแต่อย่างใด

โปรดอย่าลงลายมือชื่อของท่านในเอกสารนี้จนกว่าท่านจะแน่ใจว่ามีความประสงค์จะเข้าร่วมในโครงการวิจัยนี้ คำว่า “ท่าน” ในเอกสารนี้ หมายถึงผู้เข้าร่วมโครงการวิจัยในฐานะเป็นอาสาสมัครในโครงการวิจัยนี้ หากท่านเป็น**ผู้แทนโดยชอบธรรมตามกฎหมาย**ของผู้ที่จะเข้าร่วมในโครงการวิจัย และลงนามแทนในเอกสารนี้ โปรดเข้าใจว่า “ท่าน” ในเอกสารนี้หมายถึงผู้เข้าร่วมในโครงการวิจัยเท่านั้น

โครงการวิจัยนี้มีที่มาอย่างไร และวัตถุประสงค์ของโครงการวิจัย

เนื่องจากผู้ป่วยโรคทางเดินน้ำดีอุดตัน มีสาเหตุทั้งจากมะเร็งทางเดินน้ำดี และโรคทางเดินน้ำดีอุดตันที่ไม่ได้มีสาเหตุจากมะเร็ง เช่น นิ่วในทางเดินน้ำดี ทางเดินน้ำดีตีบจากภาวการณ์อักเสบ และทางเดินน้ำดีตีบจากภาวะแทรกซ้อนของการผ่าตัด การรักษาโรคทางเดินน้ำดีอุดตัน ขึ้นกับโรคของผู้ป่วยเป็นสำคัญ หากมีสาเหตุจากมะเร็ง การรักษาให้ได้ผลดีที่สุด ควรจะเป็นการผ่าตัดใหญ่ หากเป็นโรคทางเดินน้ำดีอุดตันด้วยสาเหตุที่ไม่ได้เกิดจากมะเร็ง การรักษามักจะเป็นการส่องกล้องรักษา หรือการผ่าตัดด้วยแผลขนาดเล็ก อย่างไรก็ตาม การวินิจฉัยแยกโรคมะเร็งกับโรคที่ไม่ใช่มะเร็ง บางครั้งทำได้ยาก ดังนั้นการวิจัยนี้ จึงมีจุดมุ่งหมายเพื่อศึกษาว่า ระดับซีรัมเอ็มเอ็มพี 7 มีความแตกต่างระหว่างผู้ป่วยโรคทางเดินน้ำดีอุดตันที่เกิดจากมะเร็งทางเดินน้ำดี และผู้ป่วยโรคมะเร็งทางเดินน้ำดีอุดตันที่ไม่ได้เกิดจากมะเร็งหรือไม่ หากระดับซีรัมเอ็มเอ็มพี 7 สามารถใช้แยกโรคดังกล่าวได้ ในอนาคตการวินิจฉัยแยกโรคดังกล่าว จะนำซีรัมเอ็มเอ็มพี 7 เพื่อช่วยในการรักษาผู้ป่วยต่อไป

ท่านได้รับเชิญให้เข้าร่วมโครงการวิจัยนี้เพราะคุณสมบัติที่เหมาะสมดังต่อไปนี้

ท่านมีภาวะทางเดินน้ำดีอุดตัน

ท่านไม่สามารถเข้าร่วมโครงการวิจัยได้หากท่านมีคุณสมบัติดังต่อไปนี้

เป็นมะเร็งที่อวัยวะอื่นนอกเหนือจากมะเร็งทางเดินน้ำดี

จะมีการทำโครงการวิจัยนี้ที่ใด และมีจำนวนผู้เข้าร่วมโครงการวิจัยทั้งสิ้นเท่าไร

โครงการนี้ถูกจัดทำที่งานศัลยศาสตร์ทั่วไป โรงพยาบาลราชวิถี โดยรวบรวมซีรัมจากผู้ป่วยโรคมะเร็งทางเดินน้ำดี จำนวน 41 คน และโรคทางเดินน้ำดีที่ไม่ได้เกิดจากมะเร็ง จำนวน 41 คน

หากท่านเข้าร่วมโครงการวิจัยครั้งนี้ ท่านจะต้องปฏิบัติตามขั้นตอน หรือได้รับการปฏิบัติอย่างไรบ้าง

ในขณะที่ท่านได้รับการเจาะเลือด เพื่อตรวจสภาวะการทำงานของตับ และตรวจหาโอกาสที่จะเป็นมะเร็ง) ทูเมอร์ มาร์คเกอร์(Tumor marker โดยทั่วไปจะใช้เลือดประมาณ 20 ซีซี 4) ช้อนโต๊ะ (หากท่านตกลงเข้าร่วมในโครงการวิจัยนี้ ท่านจะถูกเจาะเลือดเพิ่มขึ้นอีก 5 ซีซี) ในเวลาเดียวกัน เลือดของท่านที่ถูกเจาะเพิ่ม จะถูกนำมาแยกเอาส่วนที่เป็นซีรัมมาตรวจหาปริมาณเอ็มเอ็มพี 7 ต่อไป

ประโยชน์ที่คาดว่าจะได้รับจากโครงการวิจัย

หากระดับซีรัมเอ็มเอ็มพี 7 ช่วยการวินิจฉัยแยกโรคมะเร็งทางเดินน้ำดีออกจากผู้ป่วยโรคทางเดินน้ำดีอุดตันได้ จะช่วยให้การรักษาโรคมะเร็งทางเดินน้ำดีดียิ่งขึ้น

ค่าใช้จ่ายที่ผู้เข้าร่วมในโครงการวิจัยจะต้องรับผิดชอบ

ไม่มี

ค่าตอบแทนที่จะได้รับเมื่อเข้าร่วมโครงการวิจัย

ไม่มี

หากเกิดอันตรายที่เกี่ยวข้องกับโครงการวิจัยนี้ จะติดต่อกับใคร และจะได้รับการปฏิบัติอย่างไร

นพ .กวิญ ลีละวัฒน์ โทรศัพท์ 089 4883015 งานศัลยศาสตร์ โรงพยาบาลราชวิถี

หากท่านมีคำถามที่เกี่ยวข้องกับโครงการวิจัยนี้ จะถามใคร ระบุชื่อผู้วิจัยหรือผู้วิจัยร่วม

นพ .กวิญ ลีละวัฒน์ โทรศัพท์ 089 4883015 งานศัลยศาสตร์ โรงพยาบาลราชวิถี

หากท่านรู้สึกว่าได้รับการปฏิบัติอย่างไม่เป็นธรรมในระหว่างโครงการวิจัยนี้ ท่านอาจแจ้งเรื่องได้ที่

นพ .กวิญ ลีละวัฒน์ โทรศัพท์ 089 4883015 งานศัลยศาสตร์ โรงพยาบาลราชวิถี

ข้อมูลส่วนตัวของท่านที่ได้จากโครงการวิจัยครั้งนี้จะถูกนำไปใช้ดังต่อไปนี้

เพื่อการวิจัยระดับซีรัมเอ็มเอ็มพี 7 ในผู้ป่วยโรคทางเดินน้ำดีอุดตัน

ท่านจะถอนตัวออกจากโครงการวิจัยหลังจากได้ลงนามเข้าร่วมโครงการวิจัยแล้วได้หรือไม่

ได้ และการบอกเลิกนี้จะไม่มีผลต่อการรักษาพยาบาลที่ข้าพเจ้าจะพึงได้รับในปัจจุบันและอนาคต

APPENDIX B

หนังสือรับรองเจตนายินยอมเข้าร่วมการวิจัย

ชื่อโครงการวิจัย การใช้ระดับซีรัม เอ็มเอ็มพี 7 (MMP-7) เพื่อการวินิจฉัยแยกโรคมะเร็งทางเดินน้ำดีจากผู้ป่วยทางเดินน้ำดีอุดตัน ที่ไม่ได้เกิดจากมะเร็ง

วันที่ลงนาม.....

ก่อนที่จะลงนามในใบยินยอมให้ทำการวิจัยนี้ ข้าพเจ้าได้รับการอธิบายจากผู้วิจัยถึงวัตถุประสงค์ของการวิจัย วิธีการวิจัย อันตราย หรืออาการที่อาจเกิดขึ้นจากการวิจัย หรือจากยาที่ใช้ รวมทั้งประโยชน์ที่คาดว่าจะเกิดขึ้นจากการวิจัยอย่างละเอียด และมีความเข้าใจดีแล้ว

ผู้วิจัยรับรองว่าจะตอบคำถามที่ข้าพเจ้าสงสัยด้วยความเต็มใจและไม่ปิดบังซ่อนเร้น จนข้าพเจ้าพอใจ

ข้าพเจ้าเข้าร่วมในโครงการวิจัยนี้ด้วยความสมัครใจ โดยปราศจากการบังคับหรือชักจูง

ข้าพเจ้ามีสิทธิที่จะบอกเลิกการเข้าร่วมในโครงการวิจัยเมื่อใดก็ได้ และการบอกเลิกนี้จะไม่มีผลต่อการรักษาพยาบาลที่ข้าพเจ้าจะพึงได้รับในปัจจุบันและอนาคต

ผู้วิจัยรับรองว่าจะเก็บข้อมูลเกี่ยวกับตัวข้าพเจ้าเป็นความลับ และจะเปิดเผยเฉพาะในรูปของสรุปผลการวิจัยโดยไม่มีการระบุชื่อนามสกุลของข้าพเจ้า การเปิดเผยข้อมูลเกี่ยวกับตัวข้าพเจ้าต่อหน่วยงานต่างๆที่เกี่ยวข้อง จะกระทำด้วยเหตุผลทางวิชาการเท่านั้น

ผู้วิจัยรับรองว่าหากเกิดอันตรายใดๆจากการวิจัย ข้าพเจ้าจะได้รับการรักษาพยาบาล และได้รับค่าชดเชย ตามที่ระบุในเอกสารชี้แจงข้อมูลแก่ผู้เข้าร่วมโครงการวิจัย

ข้าพเจ้าจะได้รับเอกสารชี้แจงและหนังสือยินยอมที่มีข้อความเดียวกันกับที่สักวิจัยเก็บไว้เป็นส่วนตัวข้าพเจ้าเอง 1 ชุด

ข้าพเจ้าได้รับทราบข้อความข้างต้นแล้ว มีความเข้าใจดีทุกประการ และลงนามในใบยินยอมด้วยความเต็มใจ

ลงชื่อ.....**ผู้เข้าร่วมโครงการวิจัย**

(……) **ชื่อ-นามสกุล ตัวบรรจง**

ลงชื่อ.....**ผู้ดำเนินโครงการวิจัย**

(……) **ชื่อ-นามสกุล ตัวบรรจง**

ลงชื่อ.....**พยาน**

(……) **ชื่อ-นามสกุล ตัวบรรจง**

ลงชื่อ.....**พยาน**

(……) **ชื่อ-นามสกุล ตัวบรรจง**

หนังสือรับรองเจตนาไม่ประสงค์เข้าร่วมการวิจัย

ชื่อโครงการวิจัย การใช้ระดับซีรัม เอ็มเอ็มพี 7 (MMP-7) เพื่อการวินิจฉัยแยกโรคมะเร็งทางเดินน้ำดีจากผู้ป่วยทางเดินน้ำดีอุดตัน ที่ไม่ได้เกิดจากมะเร็ง

วันที่ลงนาม.....

ก่อนที่จะลงนามในใบยินยอมให้ทำการวิจัยนี้ ข้าพเจ้าได้รับการอธิบายจากผู้วิจัยถึงวัตถุประสงค์ของการวิจัย วิธีการวิจัย อันตราย หรืออาการที่อาจเกิดขึ้นจากการวิจัย หรือจากยาที่ใช้ รวมทั้งประโยชน์ที่คาดว่าจะเกิดขึ้นจากการวิจัยอย่างละเอียด และมีความเข้าใจดีแล้ว

ข้าพเจ้าพิจารณาแล้ว มีความเห็นว่า ไม่ประสงค์เข้าร่วมในโครงการวิจัยนี้

โดยข้าพเจ้าทราบดีว่า การบอกเลิกนี้จะไม่มีผลต่อการรักษาพยาบาลที่ข้าพเจ้าจะพึงได้รับในปัจจุบันและอนาคต

ข้าพเจ้าได้รับทราบข้อความข้างต้นแล้ว มีความเข้าใจดีทุกประการ และลงนามใน**หนังสือรับรองเจตนาไม่ประสงค์เข้าร่วมการวิจัย**

ลงชื่อ.....**ผู้ไม่ประสงค์เข้าร่วมโครงการวิจัย**

(……) **ชื่อ-นามสกุล ตัวบรรจง**

ลงชื่อ.....**ผู้ดำเนินโครงการวิจัย**

(……) **ชื่อ-นามสกุล ตัวบรรจง**

ลงชื่อ.....**พยาน**

(……) **ชื่อ-นามสกุล ตัวบรรจง**

ลงชื่อ.....**พยาน**

(……) **ชื่อ-นามสกุล ตัวบรรจง**

APPENDIX C

Case Record Form

Date of blood collection (Date/Month/Year) …….

H.N. ……**Age** ……Years

Imaging date (Date/Month/Year) …….

Imaging identifies obstructive jaundice ☐CT scan ☐MRI ☐USG

Gate criteria	Yes	No
Presence of cancer in other organs except in bile duct	☐	☐
Presence of idiopathic pulmonary fibrosis	☐	☐
Presence of aortic aneurysm	☐	☐
Presence of severe arthritis	☐	☐

Cholangiogram ☐ERCP ☐PTBD Date ……..

Diagnosis ☐ Benign biliary tract diseases

☐CBD stone ☐IHD stone ☐CBD stricture (chronic) ☐CBD injury

Diagnosis cholangiocarcinoma by (Only for cholangiocarcinoma)

☐ Tissue diagnosis (SN……) ☐ Cytologydiagnosis (SN……)

☐ Radiological finding and the progression of tumor is observed when follow up within 3 months

Blood chemistries (Date/Month/Year) ……/……../……..

AST …….. ALT……..ALP……..

Total Bilirubin……Direct Bilirubin…….

Albumin……..Globulin……

Tumor markers

CEA …….. **(Date/Month/Year)** ……/……../……..

CA19-9 …….. **(Date/Month/Year)** ……/……../……..

MMP-7 …….. **(Date/Month/Year)** ……/……../……..

……

(……)

(Date ……../……../……..)

ผู้บันทึกข้อมูล

APPENDIX D

เอกสารรับรองโครงการวิจัยที่เกี่ยวกับการวิจัยในคน
โรงพยาบาลราชวิถี

เอกสารเลขที่ 7/2552

ชื่อโครงการ (ภาษาไทย) -

(ภาษาอังกฤษ) "The efficacy of serum matrix Metalloproteinase (MMP)-7 for Differentiating Cholangiocarcinoma from Benign Biliary Tract Disease Patient; Evaluation of Diagnostic Accuracy"

ชื่อหัวหน้าโครงการ นายแพทย์กวิญ ลีละวัฒน์
ตำแหน่ง นายแพทย์ชำนาญการพิเศษ
สังกัดหน่วยงาน กลุ่มงานศัลยศาสตร์ โรงพยาบาลราชวิถี

โครงการวิจัยได้ผ่านการพิจารณาและรับรองโดยคณะกรรมการจริยธรรมการวิจัย โรงพยาบาลราชวิถี เมื่อวันที่ 22 เดือน มกราคม พ.ศ. 2552

ลงนาม _____
(รศ.คลินิก (พิเศษ) นพ.อุดม ไกรฤทธิชัย)
ประธานคณะกรรมการจริยธรรมการวิจัย

ลงนาม _____
(นางวารุณี จินารัตน์)
ผู้อำนวยการโรงพยาบาลราชวิถี

BIOGRAPHY

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