รายงานวิจัยฉบับสมบูรณ์

โครงการ การตรวจหาเซลล์มะเร็งทางเดินน้ำดีในกระแสเลือด (circulating tumor cells; CTCs) โดยการ วัดปริมาณhTERT mRNA เพื่อนำมาใช้ทางคลินิกสำหรับวินิจฉัยและประเมิน ผู้ป่วยโรคมะเร็งทางเดินน้ำดี

โดย

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มค 2554

สัญญาเลขที่ **RSA5280002**

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ผู้วิจัย สังกัด

กวิญ ลีละวัฒน์

งานศัลยศาสตร์ทั่วไป โรงพยาบาลราชวิถี

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

กิตติกรรมประกาศ

ขอขอบพระคุณ ภาควิชาศัลยศาสตร์ โรงพยาบาลราชวิถี ที่เอื้อเฟื้อสถานที่ วัสดุอุปกรณ์ต่าง ๆ สำหรับทำโครงงาน ขอขอบพระคุณ สำนักงานกองทุนสนับสนุนการวิจัย (สกว) ที่ได้ให้เงินทุนสำหรับสนับสนุนในการทำโครงงานนี้ ขอขอบคุณ อาจารย์ เพื่อน และ คุณศิริลักษณ์ นารอง จากภาควิชาศัลยศาสตร์ โรงพยาบาลราชวิถี ซึ่งกรุณาสละเวลา ให้ความรู้และคำแนะนำ และความช่วยเหลือตลอดการทำโครงงาน

ท้ายที่สุด ขอกราบขอบพระคุณ คุณพ่อ คุณแม่ และ ภรรยาผู้เป็นที่รัก ผู้ให้กำลังใจและคำแนะนำตลอดการทำโครงงาน

ผศ. ดร. นพ. กวิญ ลีละวัฒน์
20 มค 2555

บทคัดย่อ

รหัสโครงการ: RSA5280002

ชื่อโครงการ: การตรวจหาเซลล์มะเร็งทางเดินน้ำดีในกระแสเลือด (circulating tumor cells; CTCs) โดยการวัดปริมาณhTERT mRNA เพื่อนำมาใช้ทางคลินิกสำหรับวินิจฉัยและประเมินผู้ป่วยโรคมะเร็งทางเดินน้ำดี

ชื่อนักวิจัย: กวิญ ลีละวัฒน์ งานศัลยศาสตร์ทั่วไป โรงพยาบาลราชวิถี

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ระยะเวลาโครงการ: 1 มีค 2552 - 1 มีค 2555

วัตถุประสงค์: เพื่อศึกษา

1) ระดับ Circulating tumor cells ระดับซีรั่ม MMP7 และ MMP9 สามารถใช้วินิจฉัยแยกโรคมะเร็งทางเดินน้ำดี จากผู้ป่วยโรคทางเดินน้ำดีอุดตันที่ไม่ใช่มะเร็ง
2) Tumor marker ที่วัดจากเลือดผู้ป่วย ที่ดีที่สุดจากการศึกษาใน ข้อ 1 จะสามารถใช้วินิจฉัยแยกโรคมะเร็งทางเดินน้ำดี ในการศึกษาแบบไปข้างหน้า ในผู้ป่วยโรคมะเร็งทางเดินน้ำดีอีกกลุ่ม
3) ระดับ CTCs สามารถใช้ทำนายอัตราการรอดชีวิตในผู้ป่วยโรคมะเร็งทางเดินน้ำดีระยะลุกลามเพื่อเลือกการรักษาที่เหมาะสม

วิธีทดลอง:

1) วัดระดับ circulating tumor cells (CTCs) (โดยการวัดระดับ hTERT และ CK19 mRNA ในเม็ดเลือดขาวชนิดเซลล์เดี่ยว) ซีรัม matrix metalloproteinase (MMP)-7 MMP-9 CEA และ CA19-9 ในเลือดผู้ป่วยโรคมะเร็งทางเดินน้ำดี และ ผู้ป่วยโรคทางเดินน้ำดีที่ไม่ใช่มะเร็ง วิเคราะห์ค่า ความแม่นยำในการวินิจฉัย
2) วัดระดับ Tumor markers ที่คัดเลือกได้จากการทดลองที่ 1 ในเลือดผู้ป่วยโรคมะเร็งทางเดินน้ำดี และ ผู้ป่วยโรคทางเดินน้ำดีที่ไม่ใช่มะเร็ง ทุกรายแบบไปข้างหน้า โดยผู้ป่วยในการศึกษานี้ไม่เกี่ยวกับการศึกษาที่ 1 และนำผลที่ได้มาวิเคราะห์ค่า ความแม่นยำในการวินิจฉัย ความไว และความจำเพาะ
3) วัดระดับ circulating tumor cells (CTCs) ซีรัม matrix metalloproteinase (MMP)-7 และ CA19-9 ในเลือดผู้ป่วยโรคมะเร็งทางเดินน้ำดีระยะลุกลามที่จำเป็นต้องได้รับการรักษาด้วยการใส่ท่อน้ำดีเทียม นำค่าที่ได้มาวิเคราะห์หาปัจจัยทำนายการรอดชีวิต (prognostic values) ในผู้ป่วยกลุ่มนี้

ผลการทดลอง:

1) จากการวัดระดับ circulating tumor cells (CTCs) ซีรัม matrix metalloproteinase (MMP)-7 MMP-9 CEA และ CA19-9 พบว่ามีเพียง MMP7 เท่านั้นที่แตกต่างอย่างมีนัยสำคัญทางสถิติ เมื่อเทียบระหว่างผู้ป่วยโรคมะเร็งทางเดินน้ำดี และ ผู้ป่วยโรคทางเดินน้ำดีที่ไม่ใช่มะเร็ง
2) ผู้วิจัยคัดเลือก MMP7 มาตรวจในผู้ป่วยโรคมะเร็งทางเดินน้ำดี และ ผู้ป่วยโรคทางเดินน้ำดีที่ไม่ใช่มะเร็งทุกรายแบบไปข้างหน้า พบว่ามีความไว 75% และ ความจำเพาะ 78% (ค่า cut-off = 5.5 ng/ml)
3) ผู้ป่วยที่มีค่า CK19 mRNA ในเลือดเป็นบวก มีการรอดชีวิตต่ำกว่า ผู้ป่วยที่มีค่า CK19 mRNA ในเลือดเป็นลบ อย่างมีนัยสำคัญทางสถิติ (p = 0.009) การวิเคราะห์แบบ multivariable analysis พบว่า CK19 mRNA ในเลือดเป็นบวก อายุผู้ป่วย และระดับบิลิรูบิน เป็นปัจจัยที่สัมพันธ์กับการรอดชีวิตของผู้ป่วย

สรุปและวิจารณ์ผลการทดลอง:

ระดับซีรัม MMP7 น่าจะเป็น tumor marker ที่ดีสำหรับผู้ป่วยโรคมะเร็งทางเดินน้ำดี ในขณะที่ ระดับ CK19 mRNA สามารถใช้ทำนายการรอดชีวิตของผู้ป่วยโรคมะเร็งทางเดินน้ำดีระยะลุกลามที่จำเป็นต้องได้รับการรักษาด้วยการใส่ท่อน้ำดีเทียมได้ ควรมีการศึกษาชนิดไปข้างหน้าเพื่อทดสอบว่า ระดับ MMP7 และ CK19 mRNA มีผลต่อการช่วยตัดสินใจในการรักษาผู้ป่วยโรคมะเร็งทางเดินน้ำดีหรือไม่

คำหลัก:

โรคมะเร็งทางเดินน้ำดี circulating tumor cells, CK19 mRNA, hTERT mRNA , MMP-7

ABSTRACT

Project Code: RSA5280002
Project Title: Detection of circulating tumor cells by identify hTERT mRNA in cholangiocarcinoma patients; is it clinically useful?
Investigator: Kawin Leelawat MD, PhD. Department of Surgery, Rajavithi Hospital, Bangkok, Thailand
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Project Period: 1March 2009 - 1 March 2012

Objectives

1) To determine whether the circulating tumor cells (CTCs), serum levels of matrix metalloproteinase (MMP)-7 and MMP-9 can discriminate cholangiocarcinoma patients from benign biliary tract disease patients, 2) To determine whether the best selected marker from objective 1 has the potential to diagnosis cholangiocarcinoma in an independent prospective-consecutive study and 3) To determine the role of CTCs in prediction of the overall survival of patients with advanced malignant biliary tract obstruction.

Methods

1) We measured the level of CTCs (by examining two markers, cytokeratin (CK) 19 and human telomerase reverse transcriptase (hTERT) mRNA from peripheral blood-mononuclear cells (PBMC)), carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA19-9), MMP-7 and MMP-9 in the blood samples of 44 cholangiocarcinoma and 36 benign biliary tract diseases patients.
2) A total of 187 patients with obstructive jaundice were consecutively enrolled. After the diagnostic status of these patients was ascertained, their levels of selected marker were assayed.
3) We investigated the prognostic value of CTCs, in 40 patients diagnosed with advanced malignant biliary tract diseases.

Results

1) Among the blood levels of CTCs, CEA, CA19-9, MMP-7 and MMP-9, only the serum MMP-7 levels were significantly higher in the patients with cholangiocarcinoma compared to benign biliary tract disease patients ($p < 0.001$).
2) The independent prospective study showed that the sensitivity and specificity of serum MMP7 for diagnosis cholangiocarcinoma 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.
3) Positive CK19 mRNA expression was significantly associated with worse overall survival ($P = 0.009$). Multivariable analysis determined that positive CK19 mRNA expression, patient's age and serum bilirubin was each independently associated with overall survival.

Conclusion

Serum MMP-7 appears to be a valuable diagnostic marker in the discrimination of cholangiocarcinoma from benign biliary tract disease. Positive CK19 mRNA levels in peripheral blood appear to provide a valuable marker to predict the overall survival of patients with advanced malignant biliary tract obstruction.

Keywords: Cholangiocarcinoma, circulating tumor cells, CK19 mRNA, hTERT mRNA , MMP-7

INTRODUCTION

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]. In United state, the most commonly recognized risk factors for cholangiocarcinoma is primary sclerosing cholangitis (PSC)[2]. However, in Southeast Asia especially in Thailand, infection with hepatobiliary flukes (*Opisthorchis viverrini*) is the most common risk factor for cholangiocarcinoma [1, 3, 4]. Therapeutic options for cholangiocarcinoma have been limited due to poor response to chemotherapy and radiation therapy. Surgery is perhaps the only effective treatment for cholangiocarcinoma. Five-year survival rates of 32% to 50% are achieved in only a few numbers of patients when negative histological margins are attained at the time of surgery [5-7]. To improve the survival rate, the diagnosis and treatment of these patients should be performed as soon as possible. In this project, we used the basic molecular methods to identify the novel tumor markers for diagnosis cholangiocarcinoma. In addition, the prognostic markers for cholangiocarcinoma patients were demonstrated.

For diagnosis of cholangiocarcinoma, it is very difficult to get the tissue due to the tumour location and the desmoplastic reaction. In addition, this tumour 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 [8]. Therefore, identification of tumor markers in the serum would be benefit in the clinical managing of this disease. To date, there are two common tumor markers for detecting cholangiocarcinoma; carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9). CEA is unspecific and can be elevated in the setting of other gastrointestinal or gynecologic malignancies or other bile duct pathology such as cholangitis

and hepatolithiasis [9]. Previous studies demonstrated that the sensitivity and specificity for CA 19-9 value >100 U/ml for cholangiocarcinoma in primary sclerosing cholangitis (PSC) was 89% and 86%, respectively [3, 10]. However, A CA 19-9 cut off of 100 U/ml resulted in a sensitivity of only 53.0-67.5% for the diagnosis of cholangiocarcinoma in patients without PSC [11, 12]. 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. The rising of serum CA19-9 can be detected even in benign bile duct diseases [13, 14]. Therefore the novel tumour markers for diagnosis cholangiocarcinoma in patients without with PSC should be investigated. Typically tumour cells invade basement membrane by secrete enzymes that digest the extracellular matrix protein. These enzymes are known as Matrix metalloproteinase (MMPs). MMPs are zinc dependent endopeptidase. They are involved in the mechanisms of the turnover and degradation of extracellular matrix (ECM) components and basement membranes [15]. Recently, Itatsu k, et al. examined the expression of MMPs in surgically resected specimens of cholangiocarcinoma by immunohistochemical and found that 47.5 and 75.8% of these specimens expressed MMP-9 and MMP-7 respectively [16]. Previous studies demonstrated that MMP-9 can be detected in serum of gastric cancer patients and MMP-7 was increased in colorectal, ovarian and renal cancer patients [17-20]. Therefore, detection of MMP-9 and MMP-7 in blood circulation may be useful for clinical diagnosis of cholangiocarcinoma. However, until now, there is no study about the detection of serum MMP-9 and MMP-7 in cholangiocarcinoma patients.

Circulating tumor cells (CTCs) detection in peripheral blood of epithelial cancer patients is found to be a novel indicator for the presence of primary tumors and/or metastasis[21]. Previous study indicated that micrometastases of gastric cancer can be detected in circulating peripheral

blood using quantitative real-time RT-PCR. In addition, CK19 is a better marker for CTCs than CK18, CK20 and CEA[22]. Our group also suggested that the level of hTERT mRNA is significantly high in cholangiocarcinoma patients[23]. Until now, however, there have been no studies concerning the role of the detection of CTCs by using CK19 and hTERT gene in diagnosis cholangiocarcinoma patients. Therefore, the first objective of this study is to study the accuracy of the detection of serum MMP-9 and MMP-7 and CTCs for diagnosis of 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 [24-26]. Therefore, the second objective study was to evaluate the performance of selected marker (the best marker derived from the first objective) 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 [24]. 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). After the diagnosis status of these patients was ascertained, the value of selected markers were assayed in a fashion that blinded to case-control status. In addition, we implemented the STARD statements [27-29] to ensure standardization and transparency of our study.

The prognostic markers were studied in advance malignant biliary tract patients. Malignant biliary tract obstruction is a condition that can result from tumors of the biliary tract, ampulla of

vater, duodenum or head of the pancreas. In Thailand, cholangiocarcinoma (CCA) is the most common cause of malignant biliary tract obstruction [1]. Despite recent advances in the diagnosis and treatment of this disease, patient outcome remains poor. The high mortality rate arising from malignant biliary tract obstruction is due to the aggressiveness of tumors that are often discovered at a late-stage of disease progression [30]. Palliative therapeutic approaches to endoscopic biliary drainage, such as the use of endoprosthesis stents, are generally recommended for these patients. The two major types of endoprosthesis stents are plastic or polyethylene (PE) stents and self-expanding metal stents (SEMS). Previous studies have demonstrated that partial or total occlusion of PE stents usually occurs 3 to 4 months after insertion [31]. Four randomized controlled studies (RCTs) demonstrated that SEMS exhibit significantly higher patency rates compared to PE stents (9 VS 1.5 months) [32-34]; however, SEMS are much more expensive than PE stents (1500 USD versus 80 USD, in Thailand). A recent study indicated that patients who have a predicted survival duration of greater than 4.5 months should use SEMs for their palliative biliary drainage [35]. In this instance, the higher cost of the SEMs is balanced by a decreased need for repeat intervention that is often necessary in patients who have received PE stents. Therefore, identification of reliable prognostic factors that allow for an accurate prediction of survival duration in patients suffering from advanced malignant biliary tract obstruction is extremely important.

One of the major mechanisms required for tumor metastasis is the dissemination of tumor cells from the primary tumor into circulating blood [36]. Previous studies have indicated that detection of circulating tumor cells (CTCs) in the peripheral blood can be used in staging and prognosis stratification for breast and colon cancer patients [37] [38]. Until now, however, there have been no studies concerning the role of the detection of CTCs as a prognostic factor in malignant biliary tract disease patients.

To date, the most common CTCs detection method is quantitative real-time reverse transcription (RT) -PCR, a process that can detect mRNA expression levels of the genes coding for these tumor antigens [39]. A high-quality detection marker is required for efficient quantitative real-time RT-PCR-mediated detection of CTCs. Therefore, identification of a good target marker is of the utmost importance for CTC detection. Several gene markers, such as human cytokeratin (CK) 19 and telomerase reverse transcriptase (hTERT), have been evaluated as tumor-specific markers for the detection of CTCs in gastrointestinal cancers[23, 40].

Human telomerase reverse transcriptase (hTERT) mRNA can be detected in 85% of all cancer cells, including cholangiocarcinoma cells[41]. This is in contrast to most normal cells, which exhibit little or no expression. Our previous study demonstrated that high levels of hTERT mRNA can be detected in the blood circulation of cholangiocarcinoma patients, and it has also been suggested that hTERT mRNA is a promising marker for the detection of cancer cells[42].

Cytokeratin 19 (CK19) is generally expressed in ductal epithelium (bile ducts, pancreas, and renal collecting tubules) and in the mucosa of the gastrointestinal tract. CK19 immunohistochemistry is used in diagnostic pathology mainly to confirm epithelial immunophenotype in undifferentiated tumors or to establish biliary, pancreatic or renal ductular origin [43]. Most of adenocarcinomas arising within the gastrointestinal tract are CK19 positive, including cholangiocarcinoma and pancreatic cancer [44]. Many investigators have used the detection of CK19 mRNA in peripheral blood as a target gene to investigate CTCs [44, 45]; however, until now there have been no studies involving the detection of hTERT and CK19 in the peripheral blood of malignant biliary tract disease patients.

In this study, our aim was to evaluate if the levels of CTCs could be used to predict the overall survival of patients diagnosed with advanced malignant biliary tract obstruction disease.

Cytokeratin (CK)-19 and hTERT were selected as the target genes for CTCs. In addition, this study was performed in accordance with the REporting recommendations for tumor MARKer prognostic studies (REMARK) criteria [46] to ensure standardization and transparency of our study.

METHODS

1) Detection of MMP7, MMP9, CTCs as a diagnostic marker for cholangiocarcinoma patients

Patients and samples

Pre-treatment fasting blood samples (n = 80) were collected from obstructive jaundice patients underwent endoscopic retrograde cholangiography (ERCP) or biliary tract surgery at Rajavithi Hospital. All patient serum and clinical information were obtained with patient consent after approval by Rajavithi Ethics Committee. 36 patients were diagnosis of benign biliary tract diseases and 44 patients were diagnosed as having cholangiocarcinoma without primary sclerosing cholangitis by one of these criteria; 1) tissue biopsy (n=7), 2) cytology (n=17), and 3) radiological finding (helical CT scan or MRI) and the clinical observation identify the progression of tumour at follow up (n=20). Serum samples from these patients were separated by centrifugation within 2 h and frozen at -80°C. The biochemical studies of serum samples, including AST, ALT, total and direct bilirubin, alkaline phosphatase (ALP), CEA and CA19-9 were measured using routine automated methods in the Pathological Laboratory at Rajavithi Hospital.

Measurement of serum MMP-7 and MMP-9

Serum MMP-9 and MMP-7 levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN). The diluted serum samples were added in duplicate to 96-well plates coated with the MMP-9 or MMP-7 antibody and incubated at room temperature for 2 h. After washing three times with washing buffer, the conjugated secondary antibody was added and the plate was further incubated for 2 h. Plates will be washed again prior to incubation with the substrate solution for 1 h. The amplifier solution was then added and the

plate was incubated for an additional 30 min. All incubation cycles were 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 MMP-9 and MMP-7 in each sample was calculated from a standard curve.

Detection of Circulating tumor cells (CTCs)

Total RNA of peripheral blood mononuclear cells (PBMC) fraction samples was extracted using the RNeasy mini kit (Qiagen, GmbH, Germany) following the protocol provided by the manufacturer. RNA integrity was checked by electrophoresis and quantified by absorption at 260 and 280 nm using a spectrophotometer (Beckman Coulter Du® 800, Fullerton, CA). Total RNA was reverse-transcribed using random primers and the IscriptTM cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) following the protocol provided by the manufacturer. cDNA was stored at -80°C until use.

Detection of CK-19 and hTERT mRNA by quantitative real-time PCR

Expression of CK19 and hTERT genes was analyzed using specific primers. In this assay, the housekeeping gene β-actin was used as an internal control to normalize variations in integrity and total amount of cDNA. Quantitative real-time PCR assays were performed in triplicate using SYBR Green master mix (Superarray, Frederick, MD, USA) on the Chromo 4™ System (MJ Opticon Monitor ver. 3.1) (Bio-Rad, USA). The conditions began with 20 min at 50°C. After this, 42 cycling steps for amplification of PCR products were performed (15 s, 94°C for denaturation; 30 s, 60°C for annealing; and 30 s, 72°C for extension). Melting curve analysis was used to assess the specificity of the amplified products. The expression levels of CK19 and hTERT genes were measured from the cDNA by quantitative real time PCR using the relative quantification method (2^{-}

$\Delta\Delta_{Ct}$ method)[47]. The fold-change in gene expression was normalized to a housekeeping gene (β-actin).

Primer	Forward	Reverse
hTERT	GCGGAAGACAGTGGTGAACT	AGC TGGAGTAGT CGCTCT GC
CK19	CCCGCGACTACAGCCACTA	GCTCATGCGCAGAGCCT
β-Actin	GTGGGGCGCCCCAGGCACCA	GTCCTTAATGTCACGCACGATTTC

Statistical analysis

Comparison between the quantitative variables was performed by using Mann-Whitney U or Student's t-test, as appropriate. Qualitative variables were reported as count and comparisons between independent groups were performed by using by Pearson Chi-square. The diagnostic accuracy of each of the candidate biomarkers was evaluated using receiver operating characteristic (ROC) curve analysis , which correlate true- and false-positive rates [sensitivity and (1–specificity)], was constructed. In addition, an area under the ROC curve (AUC) with 95% confidence intervals (CI) was calculated for each marker. The optimal cut-off points for MMP-9 and MMP-7 were selected base on ROC curve analysis. Sensitivity, specificity, positive predictive value and negative predictive value were calculated on the 2 x 2 table of the collected data.

2) Prospective study for evaluation of selected marker from the first objective as a diagnostic marker for cholangiocarcinoma patients

Patients and study design

This study was conducted at the Department of Surgery, Rajavithi Hospital, Bangkok, Thailand. The study protocol was approved by the local ethic committee. 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[48]. By use of a significant level of 0.05 (two sided) and power of 0.95, a sample of 50 from the cholangiocarcinoma patients was required. From the previous data, the prevalence of cholangiocarcinoma detection from obstructive jaundice patients treated at our department is 30%. Therefore we prospectively included 200 consecutive patients who had the symptoms of obstructive jaundice and undergone ERCP, PTBD or bile duct surgery during June 2008 to July 2009. Exclusion criteria included present of other cancers except biliary tract cancer (cholangiocarcinoma, gall bladder cancer and periampullary cancer), age lesser than 20 years and present of severe pulmonary fibrosis[20]. All patients gave their written inform consent. Diagnosis of cholangiocarcinoma was performed as previously described by one of the following criteria [48]; 1) tissue biopsy, 2) cytology, and 3) radiological finding (helical CT scan or MRI) and clinical observation to identify the progression of the tumor at follow up at least 2 months. The patients whom their diagnosis was not conclusive were excluded from this study.

Serum collection and measurement of serum biochemistry

The 5 ml of fasting peripheral venous blood from these patients were collected at the time before the procedures (ERCP, PTBD, or bile duct surgery) and their serum were separated and stored at -78°c within 2h. Serum biochemical test including albumin, globulin, AST, ALT, total and

direct bilirubin, alkaline phosphatase (ALP) and CA19-9, were measured using routine automated methods in the Pathological Laboratory at Rajavithi Hospital.

Measurement of serum MMP7

For the evaluation of serum MMP7 as a marker for the diagnosis cholangiocarcinoma, the value of serum MMP7 was studied in a fashion that blinded to case-control status after the diagnosis status of these patients was ascertained. The serum level of MMP7 was measured by using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) as previously described [48]. Briefly, the diluted serum samples were added in duplicate to 96-well plates coated with the MMP7 antibody and incubated at room temperature for 2 h. After washing, the conjugated secondary antibody was added, and the plate was further incubated for 2 h. Plates were washed again prior to incubation with the substrate solution for 1 h. 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. The scientist examining these serum specimens was not aware of the patient's diagnosis. In addition, the test results of MMP7 had no influence on clinical diagnosis of these patients.

Statistical analysis

Data are presented as the mean ± SD, unless otherwise mentioned. Comparison between the quantitative variables was performed by using Mann-Whitney U or Student's t-test, as appropriate. Qualitative variables were reported as counts, and comparisons between independent groups were performed by using by Pearson Chi-square. Correlations between MMP7 levels and other parameters were checked with 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 value were calculated using a 2 × 2 table of the collected data.

3) To evaluate if the levels of CTCs could be used to predict the overall survival of patients diagnosed with advanced malignant biliary tract obstruction disease

Patients and samples

We prospectively included the patients with advanced malignant biliary tract diseases who had undergone palliative ERCP or PTBD at Department of Surgery, Rajavithi Hospital during a period from January 2008 to December 2009. The cutoff date for data analysis was December 31, 2010. For inclusion in the study, it was required that patients present with malignant bile duct obstruction that was not amenable to curative resection and that patients submit to a follow-up examination after at least 1 month. All patient blood and clinical information was obtained with patient consent after approval by the Rajavithi Hospital Ethics Committee.

Pre-treatment fasting blood samples were collected from the peripheral vein into EDTA-containing tubes. The first 3 mL of blood were discarded to prevent epidermal contamination (2 syringe technique). Sample processing was performed within 1 hour of blood withdrawal. Blood was transferred into a 30-mL falcon tube and centrifuged at 1,800 rpm at room temperature for 20 minutes. Plasma was removed, and the peripheral blood mononuclear cell (PBMC) fraction was stored at -80°C until used.

RNA extraction and cDNA synthesis

Total RNA of PBMC fraction samples was extracted using the RNeasy mini kit (Qiagen, GmbH, Germany) following the protocol provided by the manufacturer. RNA integrity was checked by electrophoresis and quantified by absorption at 260 and 280 nm using a spectrophotometer (Beckman Coulter Du® 800, Fullerton, CA). Total RNA was reverse-transcribed using random

primers and the IscriptTM cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) following the protocol provided by the manufacturer. cDNA was stored at -80°C until use.

Detection of CK-19 and hTERT mRNA by quantitative real-time PCR

Expression of CK19 and hTERT genes was analyzed as previously described The expression levels of CK19 and hTERT genes were measured from the cDNA by quantitative real time PCR using the relative quantification method ($2^{-\Delta\Delta Ct}$ method)[47]. The fold-change in gene expression was normalized to a housekeeping gene (β-actin) and relative to a calibrator sample. A pool of cDNA derived from PBMCs of 30 cases of benign (common bile duct stone and gall stone) biliary tract disease patients was used as the calibrator source[49]. Evaluation of the $2^{-\Delta\Delta Ct}$ indicates the fold change in gene expression relative to the calibrator. In this study, we set the positive value as a fold change in gene expression that was greater than 1.5 times relative to the calibrator and the negative value was set as a fold change in gene expression that was lesser than or equal to 1.5 times relative to the calibrator.

Determination of blood chemistries in serum samples

Biochemical studies of serum samples, including AST, ALT, total and direct bilirubin, alkaline phosphatase (ALP), CEA and CA19-9, were measured using routine automated methods in the Pathological Laboratory at Rajavithi Hospital.

Cell lines and cell spiking experiments

The human cholangiocarcinoma cell line RMCCA1[50] was incubated in Ham's F12 medium (Invitrogen -Gibco, Carlsbad, CA, USA) containing 10% fetal calf serum (Euroclone - Celbio, Pero, MI) at 37°C in 5% CO2. To determine the sensitivity of quantitative real-time PCR for detecting cancer cells in PBMCs, cell spiking experiments was performed. The PBMCs obtained from healthy volunteers were counted and diluted in Ham's F12 medium. RMCCA1 cells were serially diluted

from 1 × 10^6 cells/milliliter to 1 cell/milliliter and added to the PBMCs. Quantitative real-time PCR was then performed to detect CK19 and hTERT mRNA.

Statistical analysis

The primary endpoint of this study was the overall survival of the patients. Survival curves were estimated using the Kaplan-Meyer method, and univariable survival comparisons were calculated according to the log rank test. Multivariable survival analysis was performed using the Cox proportional hazards regression model. Comparison between the quantitative variables was performed by using Mann-Whitney U or Student's t-test, as appropriate. Qualitative variables were reported as counts, and comparisons between independent groups were performed using the Pearson Chi-square test. All tests of significance were two sided and $P < 0.05$ was considered statistically significant.

RESULTS

1) Detection of MMP7, MMP9, CTCs as a diagnostic marker for cholangiocarcinoma patients

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) was the most common diseases in this control patients. The clinical characteristics of the patients in this study are shown in Table 1. No statistically differences were found among the data of 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

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).

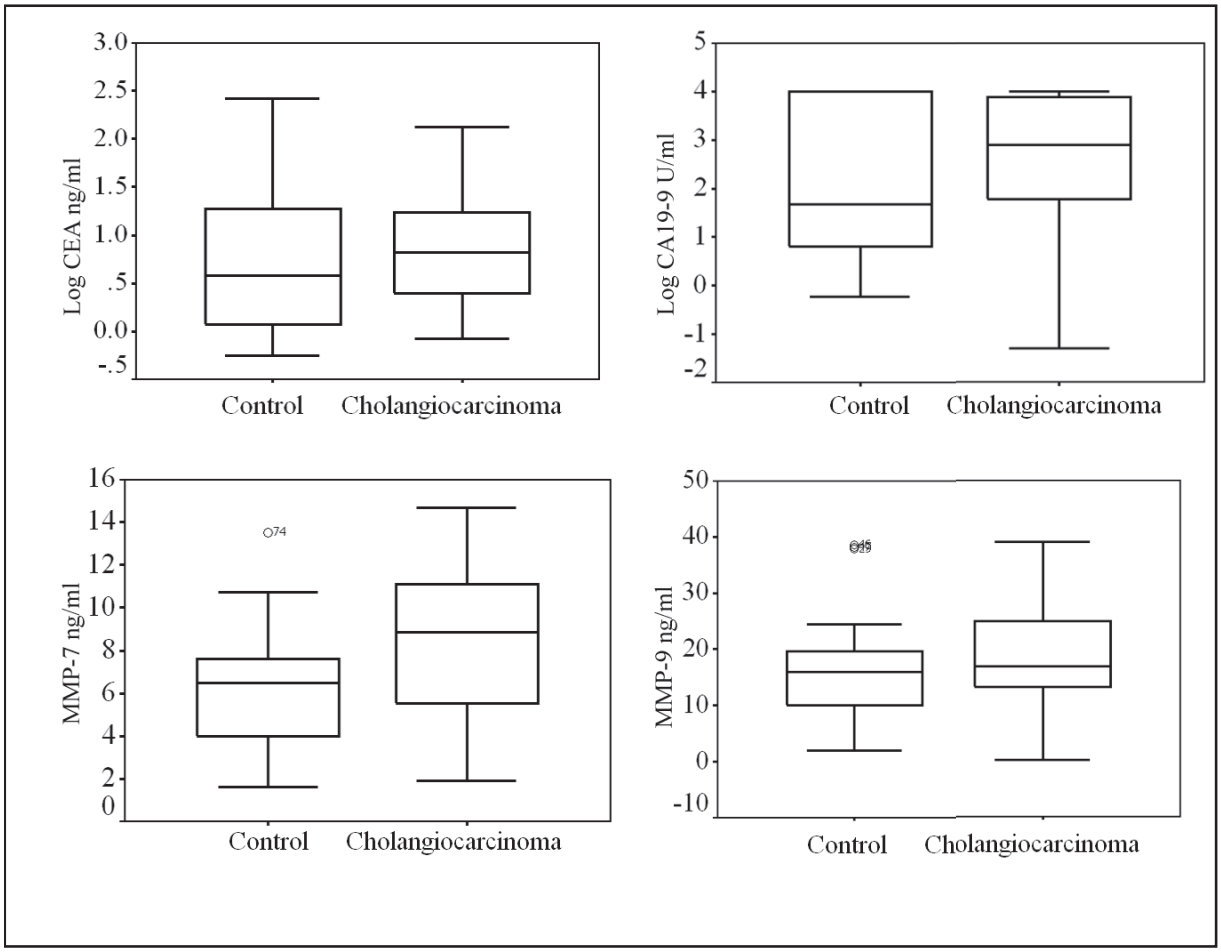
	Control (n=36)	Cholangiocarcinoma (n=44)	*p* value
Age (Yr)	54 ±14.5	59±12.9	0.130
Sex (Male:Female)	15:16	26:18	0.248#
Total bilirubin (mg/dL)	4.2±5.53	14.6±11.34	<0.001*
Direct bilirubin (mg/dL)	2.6±3.75	10.3±8.47	<0.001*
Albumin (g/dL)	3.8±0.61	3.1±0.68	0.050
Globulin (g/dL)	3.6±0.73	4.1±0.93	0.253
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*

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

The median CEA and CA19-9 value determined in control group were 3.81ng/ml (range; 0.56-260.24) and 47.42 U/ml (range 0.60-10000.00) respectively. The median CEA and CA19-9 value determined in cholangiocarcinoma group were 6.61 ng/ml (range; 0.85-131.70) and 789.25 U/ml (range; 0.50-10000.00) respectively. However, there was no statistically difference between the levels of these 2 markers in control and cholangiocarcinoma patients (Mann-Whitney U test; p=0.234 for CEA and p=0.155 for CA19-9). These data are demonstrated in Figure 1. We used a CEA cut-off value of 5 ng/ml and CA19-9 cut-off value of 100 U/ml because these have been the cut-off value suggested for the diagnosis of cholangiocarcinoma [7]. Using the CEA cut-off value of 5 ng/ml, gave the sensitivity was 57.58% (CI 95% 52.66 - 82.62) and the specificity was 61.50% (CI 95% 28.76 – 64.54). Using the CA19-9 cut-off value of 100 U/ml gave a sensitivity of 70.45% (CI95% 55.78 – 81.84) and a specificity of 61.29% (CI 95% 43.82 – 76.27).

Figure 1. **Serum levels of CEA, CA19-9, MMP-7 and MMP-9 in cholangiocarcinoma and control (benign biliary tract disease) patients**.

Box plots comparing levels of CEA, CA19-9, MMP-7 and MMP-9 are demonstrated. Levels of MMP-7 and MMP-9 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 MMP-7 between the two groups is significantly different (Student's t-test*; p*<0.001).



Detection of MMP-9 and MMP-7 in serum of cholangiocarcinoma and benign obstructive jaundice patients

There was no statistically difference between the levels of MMP-9 in the control (median, 16.04; range, 2.06-38.54) and in cholangiocarcinoma patients (median, 16.85; range, 0.18-39.02), (Mann-Whitney U test; p=0.193). In contrast, serum MMP-7 values in cholangiocarcinoma patients (median, 8.87; range, 1.90-14.66) were significantly higher than those in the control patients (median, 6.49; range, 1.62-13.53), (Mann-Whitney U test; p=0.003).

ROC curve analysis for CEA, CA19-9, MMP-9 and MMP-7 for diagnosis of cholangiocarcinoma

The ROC curve analysis (Figure 2) demonstrated an area under curve (AUC) of 0.54 (CI 95% 0.361 – 0.717) and of 0.54 (CI 95% 0.352 – 0.722) for CEA and CA19-9 respectively. The ROC curve analysis demonstrated an area under curve of 0.63 (CI 95% 0.468 – 0.805) and of 0.75 (CI 95% 0.606 – 0.896) for MMP-9 and MMP-7 respectively. When compare the AUC of ROC curve of CEA, CA19-9, MMP-9 and MMP-7 with a chance value equally to 0.5 (the worst value of AUC of ROC). Only the AUC of ROC of MMP-7 is significantly higher than 0.5 ($p = 0.005$).

For MMP-9, with a cut-off value of 15 ng/ml gave a sensitivity of 63.64% (CI 95% 48.87% -76.22%) and a specificity of 41.94% (CI 95% 26.42 – 59.23) while for MMP-7, with a cut-off value of 7.4 ng/ml gave a sensitivity of 63.64% (CI95% 46.62 -77.81) and a specificity of 71.43% (CI 95% 52.94 – 84.75) for diagnosis cholangiocarcinoma. The sensitivity and specificity for other cut-off values of CEA, CA19-9, MMP-9 and MMP-7 were present in Table 2.

Owing to the significant different of the serum AST, ALP, total bilirubin and direct bilirubin between control and cholangiocarcinoma patients, we investigated the correlation between the values of these blood chemistries and the values of CEA, CA19-9, MMP-9 and MMP-7. The results

showed that none of these parameters was significant correlation ($p>0.05$) (Table 3 and Figure 3). To determine whether the values of serum MMP-9 and MMP-7 were predictive of cholangiocarcinoma independently of other tumour markers or blood chemistries, we did logistic regression analysis. In a multivariate model using MMP-9, MMP-7, CEA, CA19-9, AST and total bilirubin; MMP-9 (an adjusted odd ratio = 1.10; 95% CI = 1.01-1.19; $p=0.038$), MMP-7 (an adjusted odd ratio = 1.33; 95% CI = 1.04-1.76; $p=0.021$) and total bilirubin (an adjusted odd ratio = 1.11; 95% CI = 1.02-1.21; $p=0.011$) were the independent predictors of cholangiocarcinoma whereas CEA, CA19-9 and AST were not.

Figure 2. **ROC curve analyses of CEA, CA19-9, MMP-9 and MMP-7 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, MMP-7 and MMP-9**. Only the area under the curve (AUC) of the ROC for MMP-7 is significantly higher than a chance value (0.5).

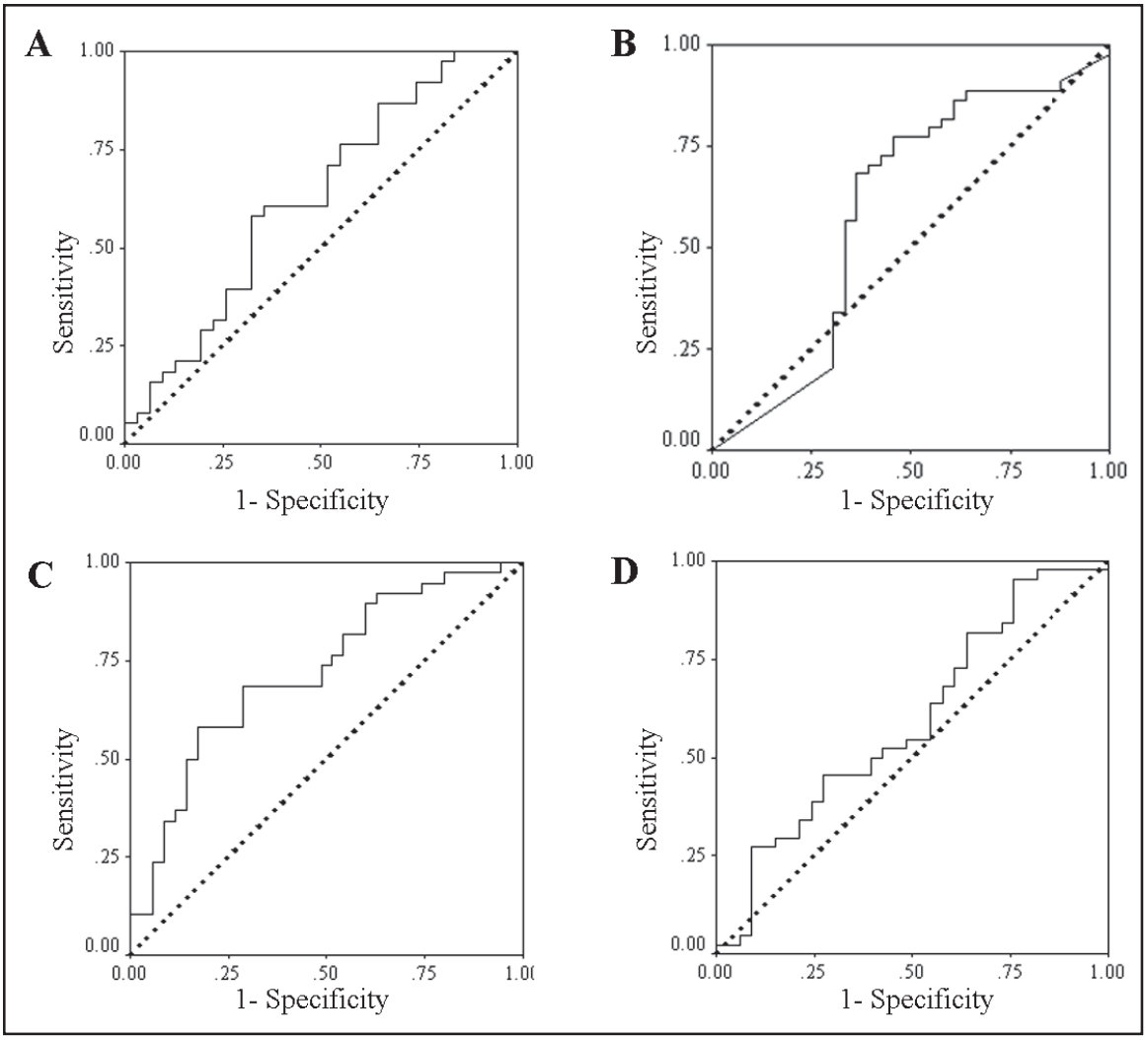


Table 2 - Performance of the biomarkers for the diagnosis of cholangiocarcinoma

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)

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)
MMP-9 (15.0 ng/ml)	63.64 (48.87-76.22)	41.94 (26.42-59.23)	1.67 (0.93-3.01)	0.59 (0.35-0.98)
MMP-9 (20.0 ng/ml)	34.10 (21.88-48.86)	74.19 (56.75-86.30)	1.32 (0.64-2.73)	0.89 (0.66-1.20)
MMP-7 (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)
MMP-7 (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)

Figure 3. A scatter plot was used to identify the correlation between the blood chemistry values (total bilirubin, AST, ALP, Log CEA and Log CA19-9) and MMP-9 or MMP-7 in the control and cholangiocarcinoma patients.

This figure demonstrates that there is no significant correlation (p>0.05) between the blood chemistry values and MMP-9 or MMP-7 in both groups.

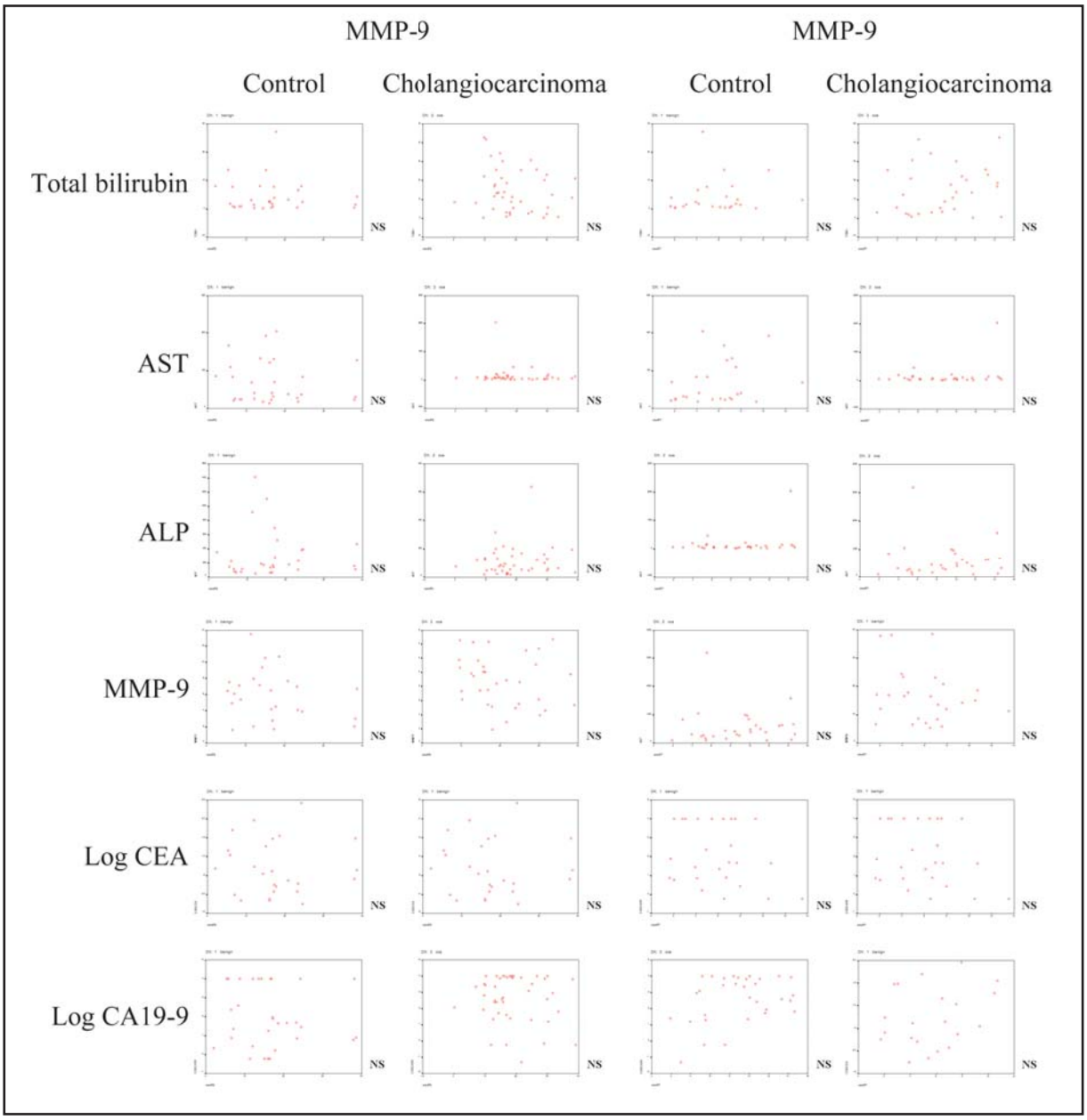


Table 3: The correlation between the blood chemistry values (total bilirubin, AST, ALP, Log CEA and Log CA19-9) and MMP-9 or MMP-7 in the control and cholangiocarcinoma patients. No significant correlation is identified (p>0.05).

Control			Total Bilirubin	Albumin	Globulin	AST	ALT	ALP	LOG CA19-9	LOG CEA	MMP-9	MMP-7
	LOG CA19-9	Pearson Correlation	-0.18	0.03	-0.33	-0.13	-0.21	-0.42	1.00	0.30	-0.07	-0.25
		Sig. (2-tailed)	0.34	0.89	0.07	0.49	0.28	0.02	.	0.14	0.70	0.22
	LOG CEA	Pearson Correlation	0.11	-0.54	-0.11	0.02	-0.04	0.34	0.30	1.00	-0.02	0.27
		Sig. (2-tailed)	0.61	0.01	0.62	0.92	0.86	0.10	0.14	.	0.93	0.24
	MMP-9	Pearson Correlation	-0.08	0.10	0.11	-0.10	0.03	0.00	-0.07	-0.02	1.00	-0.23
		Sig. (2-tailed)	0.66	0.61	0.57	0.62	0.88	0.99	0.70	0.93	.	0.26
	MMP-7	Pearson Correlation	0.10	-0.34	0.06	0.27	0.26	0.53	-0.25	0.27	-0.23	1.00
		Sig. (2-tailed)	0.64	0.10	0.77	0.19	0.22	0.01	0.22	0.24	0.26	.
Cholangiocarcinoma												
			Total Bilirubin	Albumin	Globulin	AST	ALT	ALP	LOG CA19-9	LOG CEA	MMP-9	MMP-7
	LOG CA19-9	Pearson Correlation	0.03	-0.14	0.20	-0.01	-0.09	-0.02	1.00	0.14	-0.11	0.31
		Sig. (2-tailed)	0.86	0.36	0.20	0.94	0.57	0.90	.	0.44	0.46	0.07
	LOG CEA	Pearson Correlation	0.17	0.04	-0.05	-0.35	0.02	-0.04	0.14	1.00	-0.08	-0.07
		Sig. (2-tailed)	0.34	0.82	0.79	0.05	0.90	0.81	0.44	.	0.66	0.75
	MMP-9	Pearson Correlation	-0.19	0.00	0.25	-0.10	-0.09	0.10	-0.11	-0.08	1.00	-0.17
		Sig. (2-tailed)	0.23	0.99	0.11	0.51	0.57	0.51	0.46	0.66	.	0.33
	MMP-7	Pearson Correlation	0.20	-0.04	0.15	0.24	-0.04	0.02	0.31	-0.07	-0.17	1.00
		Sig. (2-tailed)	0.28	0.84	0.42	0.19	0.82	0.91	0.07	0.75	0.33	.

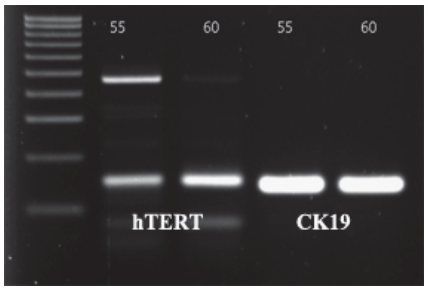
Detection of CTCs in peripheral blood of cholangiocarcinoma and benign obstructive jaundice patients

In this study, we used CK19 and hTERT genes as targets for the detection of CTCs. There was no statistically difference between the levels of CK19 mRNA in the control and in cholangiocarcinoma patients (Mann-Whitney U test; p=0.122). In contrast, hTERT mRNA values in cholangiocarcinoma patients were significantly higher than those in the control patients (Mann-Whitney U test; p=0.005) (Figure 4).

However, when compare the AUC of ROC curve of CK19, hTERT mRNA and CA19-9 with a chance value equally to 0.5 (the worst value of AUC of ROC). Only the AUC of ROC of CA19-9 is significantly higher than 0.5 (p = 0.002) (Figure 5).

Figure 4. A) amplification of hTERT and CK19 genes was demonstrated on agarose gel. B) SD bar graph comparing levels of hTERT and CK19 mRNA are demonstrated. Only the value for hTERT mRNA between the two groups is significantly different (Mann-Whitney U test; p=0.005).

A)



B)

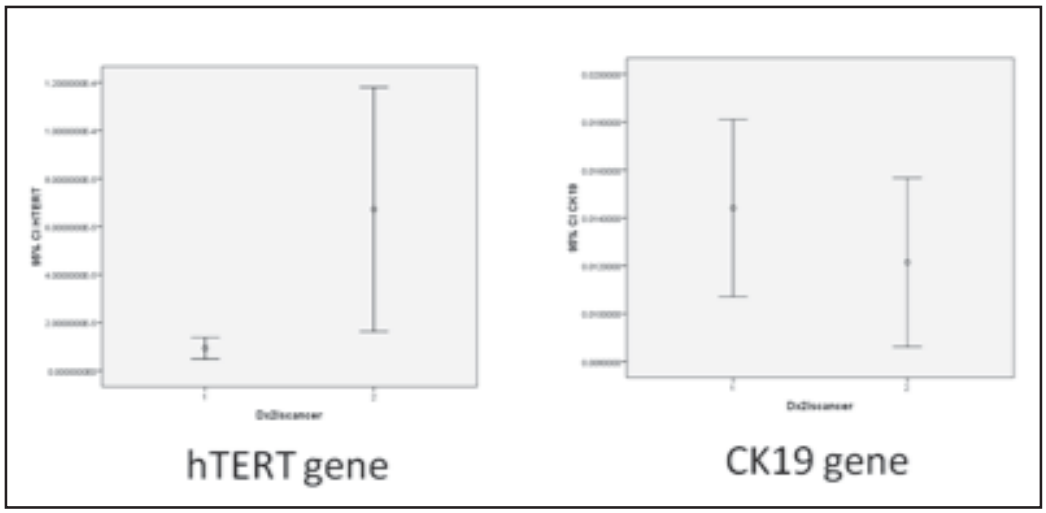
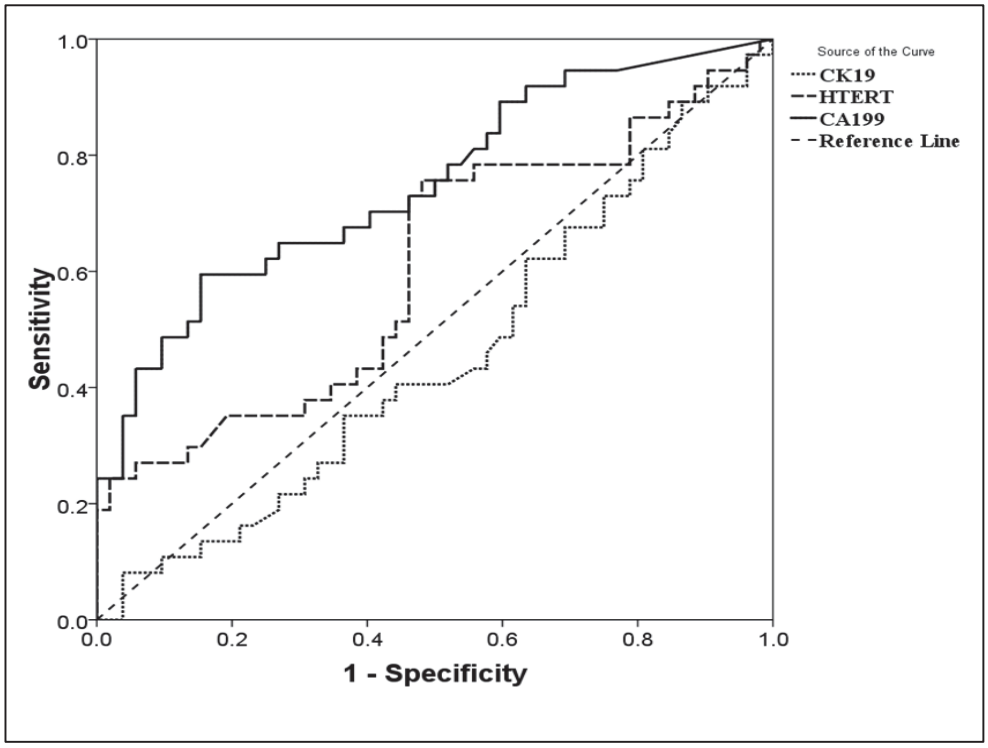


Figure 5. ROC curve analyses of CK19 and hTERT mRNA and CA19-9 for the diagnosis of cholangiocarcinoma.

The diagnostic accuracy of each biomarker, in terms of its sensitivity and specificity, are presented by receiver operating characteristic (ROC) curve analysis. Only the area under the curve (AUC) of the ROC for CA19-9 is significantly higher than a chance value (0.5).



2) Prospective study for evaluation of MMP7 as a diagnostic marker for cholangiocarcinoma patients

Patient characteristics

A total of 214 obstructive jaundice patients were consecutively enrolled. Twenty-seven cases were excluded according to their diagnosis of ampullary cancer (7 cases), pancreatic cancer (9 cases), gall bladder cancer (3 cases), duodenum cancer (2 cases), metastatic cancer from ovarian cancer (1 case) and hepatocellular carcinoma (2 cases). In addition, nineteen cases were excluded according to their uncertain diagnosis. 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 6).

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; $p < 0.05$).

Figure 6. A flow diagram of a total of 187 obstructive jaundice patients whom were consecutively enrolled in this study

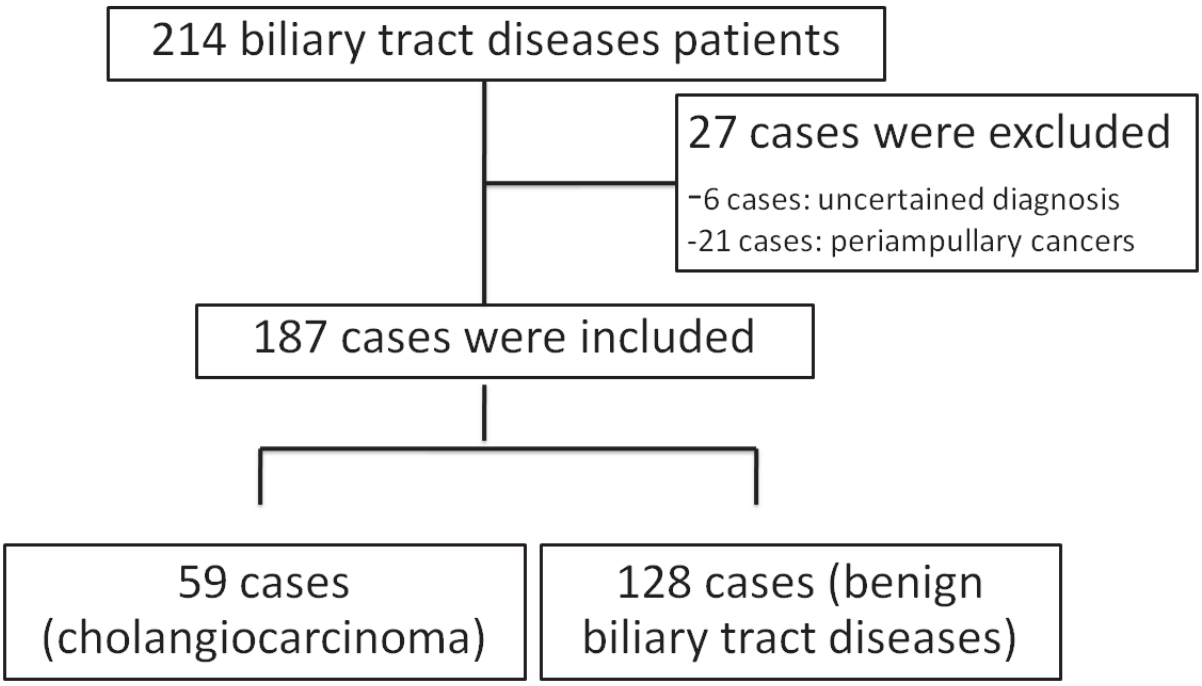


Table 4. Clinical characteristics of patients with benign biliary tract diseases (control) and cholangiocarcinoma

	Control N=128	Cholangiocarcinoma N=59	*P*
Age (yr)	61 ± 7	67 ± 5	0.451
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 (mg/dL)	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

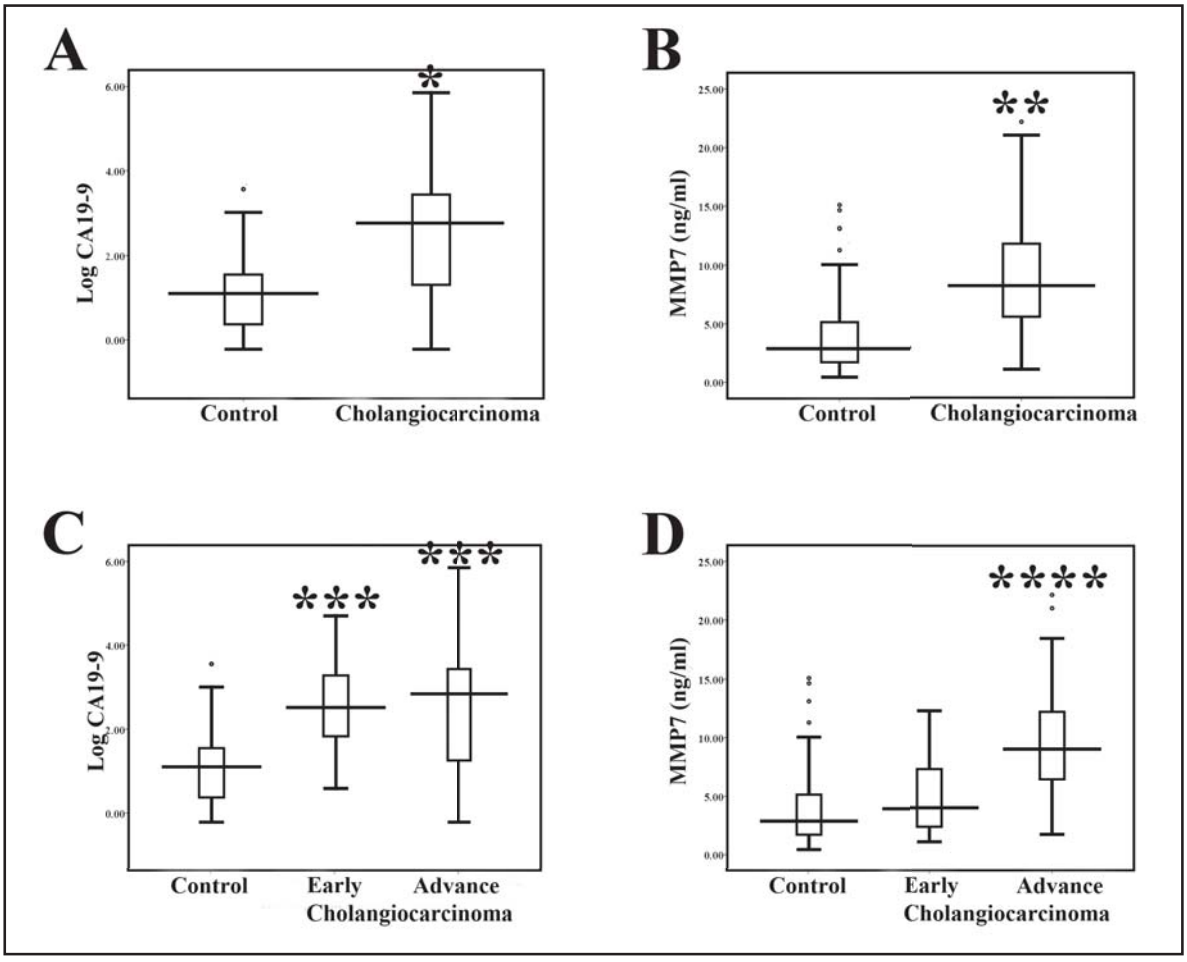
Serum levels of CA19-9 and MMP7

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 7A and B, 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. 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 7C). The data shown in Figure 7D 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; $P=0.47$).

Figure 7. Serum levels of CA19-9 and MMP7 in cholangiocarcinoma and control (benign biliary tract disease) patients.

(A) Box plots comparing levels of CA19-9; (B) MMP7 between cholangiocarcinoma and control are illustrated; (C) Box plots comparing levels of CA19-9; (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)



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 (95% CI: 0.708 – 0.868) and 0.84 (95% CI: 0.778 – 0.903) for CA19-9 and MMP7, respectively (Figure 8). The sensitivity, specificity, positive and negative predictive values for selected cut-off points of CA19-9 and MMP7 are presented in Table 5.

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%).

Figure 8. 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.

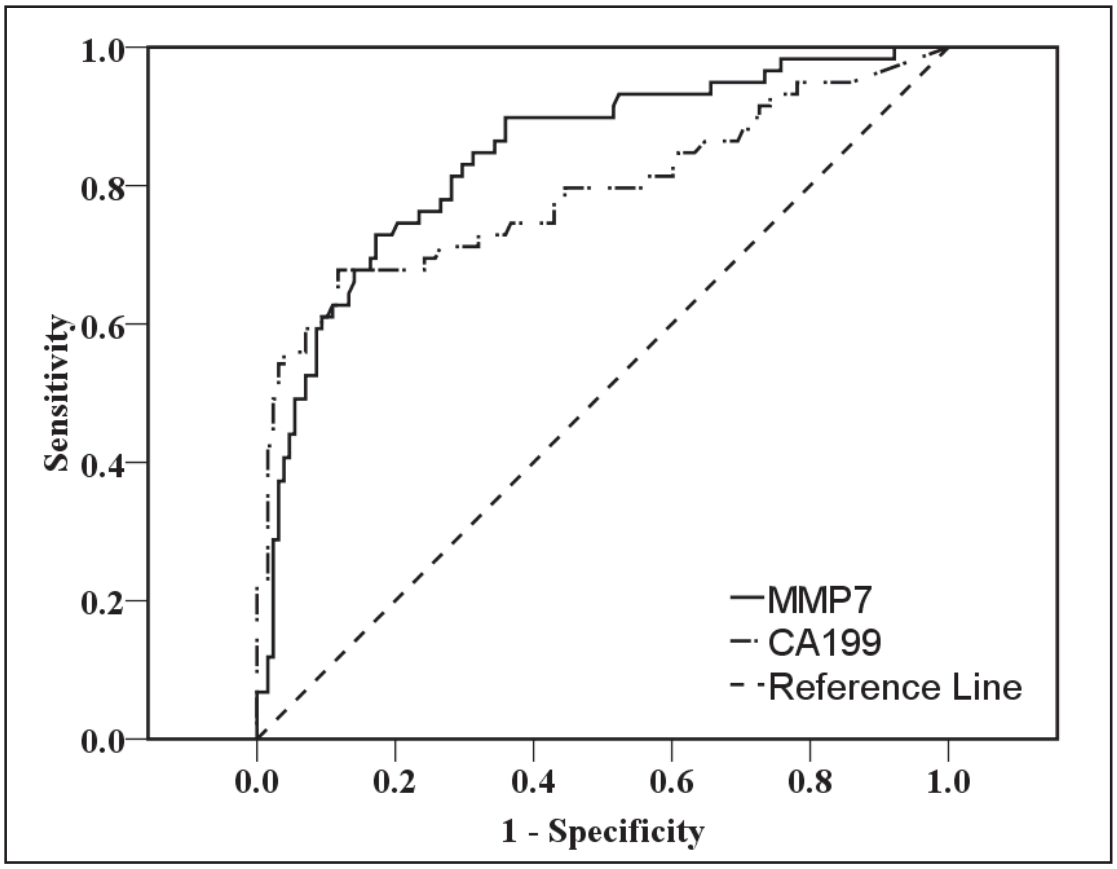


Table 5. Performance of the biomarkers for the diagnosis of cholangiocarcinoma

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 U/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 U/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 U/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)

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

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

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

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 mg/dL), 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

3) To evaluate if the levels of CTCs could be used to predict the overall survival of patients diagnosed with advanced malignant biliary tract obstruction disease

Patient Characteristics

Forty-two malignant biliary tract disease patients were included in this study. Two patients were excluded from this study because the quality of the RNA extracted from their peripheral blood was poor. The average age of these patients was 62 years (range 41–82 years). With regard to cancer-type, 6 (15.0%) were pancreatic head cancer, 2 (5.0%) were ampullary cancer, 2 (5.0%) were gall bladder cancer, 2 (5.0%) were middle and distal common bile duct cancer and 28 (70.0%) were hilar cholangiocarcinoma. The clinical characteristics of the patients examined in this study are shown in Table 9.

Table 9. Clinical characteristics of patients diagnosed with advanced malignant biliary tract obstruction

	Parameters	N (%)
Gender (Male/Female)	Male	23
	Female	17
Age	< 60 yr	18
	> 60 yr	22
Type of cancers	Hilar cholangiocarcinoma	28
	Pancreatic cancer	6
	CBD cancer	2
	Ampullary cancer	2
	Gall bladder cancer	2

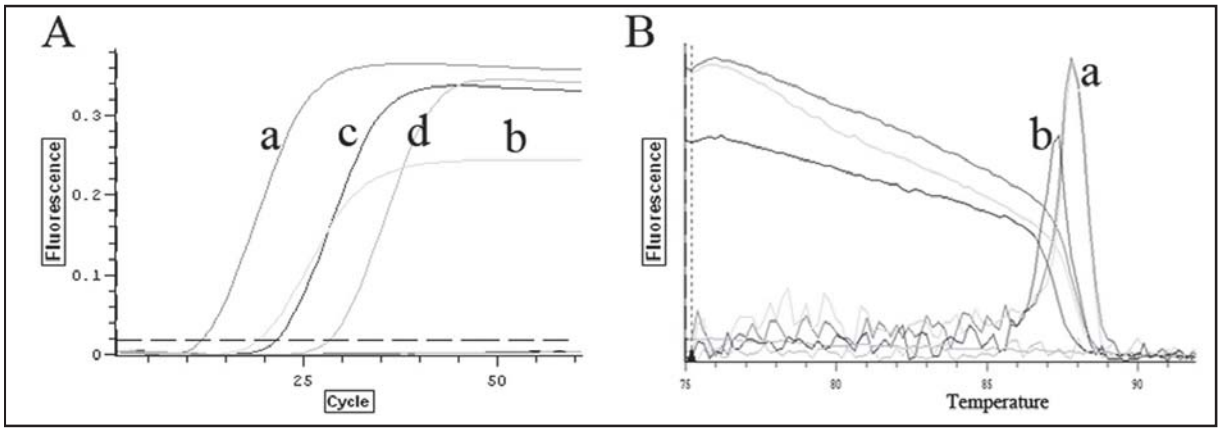
Cell spiking assay

CK19 and hTERT mRNA levels were elevated in the RMCCA1 cell line (Figure 9); therefore, we decided to use this cell line as a positive control for our study. Detection sensitivity of the quantitative real-time PCR assay was determined by serial 10-fold dilutions of RMCCA1 cells in PBMCs. The results demonstrated that CK19 and hTERT mRNA could be detected at levels up to 1,000 cells per 10^{10} PBMC dilutions.

Figure 9. Gene expression levels of CK19 and hTERT in RMCCA1 cells (as measured by quantitative real time PCR).

A. Amplification plot of CK19 mRNA [from 10,000 RMCCA1 cells (a) and 1,000 RMCCA1 cells (b)] and amplification plot of hTERT mRNA [from 10,000 RMCCA1 cells (c) and 1,000 RMCCA1 cells (d)] are demonstrated.

B. SYBR Green melting curve for quantitative real time RT-PCR. The melting curves from quantitative real time PCR for CK19 (a) and hTERT (b) consistently gave a single peak with no evidence of non-specific amplification or primer-dimerisation.



Detection of hTERT and CK19 mRNA in PBMCs of malignant biliary tract disease patients

PBMC samples from 40 patients were evaluated for the expression of hTERT and CK19 mRNA. The expression was positive (gene expression more than 1.5 times relative to the calibrator) in 45% (18/40) of samples for CK19 mRNA and 60% (24/40) of samples for hTERT. Figure 10 illustrates the distribution of CK19 mRNA and hTERT mRNA expression in the peripheral blood of these patients.

Relationship between CK19 and hTERT mRNA expression in peripheral blood and clinic pathological features of patients

No statistically significant differences were found among the data obtained from the patients considered as negative or those who were positive for hTERT or CK19 mRNA expression in PBMCs. Factors evaluated included gender, age, serum albumin, globulin bilirubin AST and ALT and alkaline phosphatase levels (Table 10).

Figure 10. The distribution levels of CK19 and hTERT genes measured in the peripheral blood of 40 patients. The positive value is determined as a fold change in gene expression of more than 1.5 times relative to the calibrator

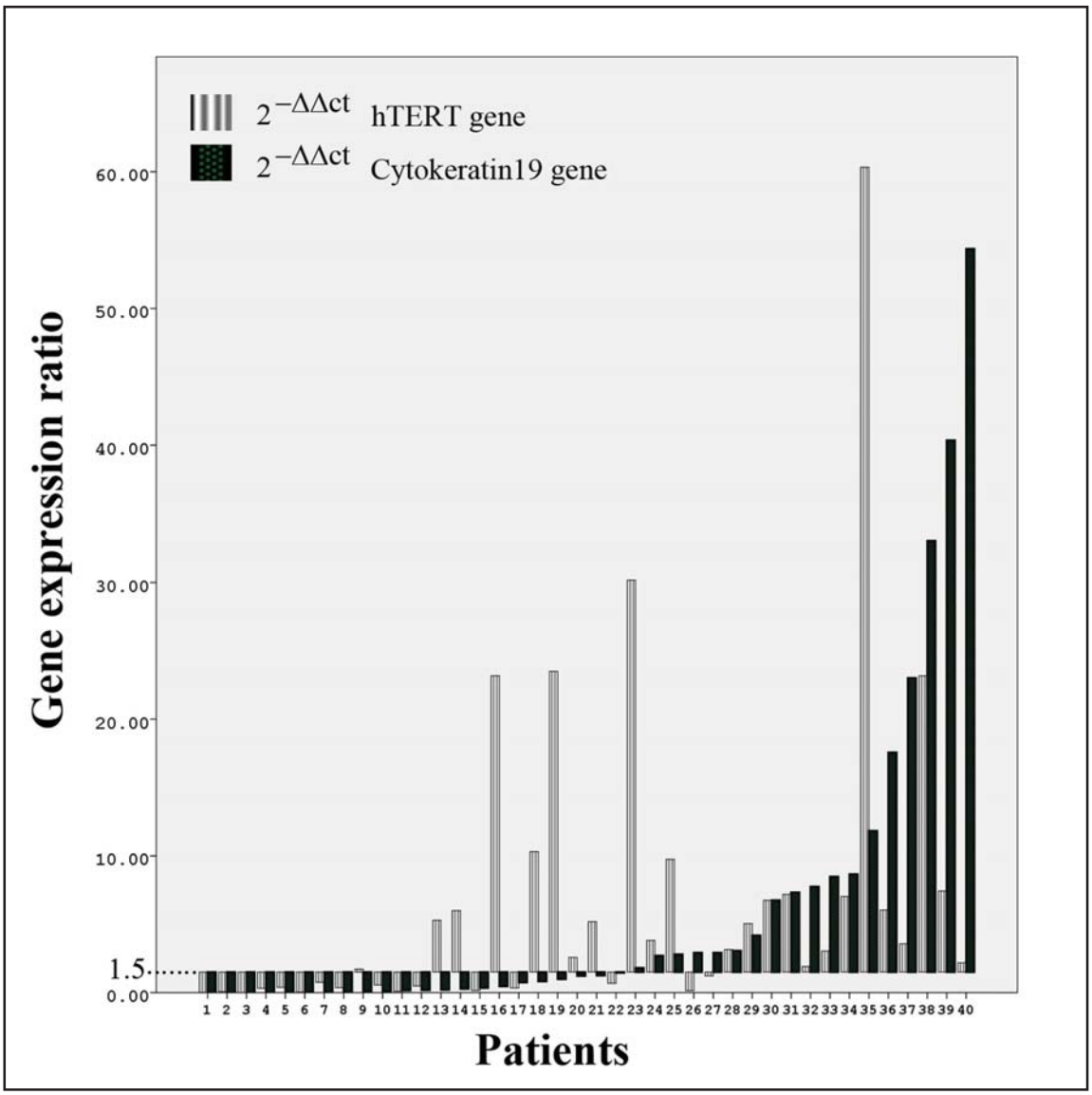


Table 10 Clinical characteristics of patients with negative and positive CK19 and hTERT gene expression

	CK19 gene		*p* value	hTERT gene		*p* value
	Negative	Positive		Negative	Positive	
Age (Yr)	63.80	61.38	0.42	65.26	60.88	0.16
Sex (Male:Female)	11:11	12:6	0.35#	8:8	15:9	0.52#
Total bilirubin (mg/dl)	16.21	16.42	0.95	17.73	15.47	0.53
Albumin (g/dl)	2.88	2.97	0.62	2.83	2.99	0.36
Globulin (g/dl)	3.98	3.76	0.42	3.92	3.84	0.79
AST (U/L)	84.57	112.80	0.23	69.27	117.48	0.12
ALT (U/L)	42.47	65.85	0.12	34.87	66.68	0.28
ALP (IU/L)	449.68	528.85	0.56	436.88	421.91	0.98
BUN (mg/dl)	13.77	26.58	0.13	12.25	23.52	0.18
Creatinine (mg/dl)	0.75	1.30	0.20	0.61	1.19	0.22
CA19-9 (U/ml)	570.20	594.30	0.62*	1818.00	259.15	0.11*
CEA (ng/ml)	7.47	5.68	0.50*	7.21	5.82	0.23*

Quantitative variables are presented as the mean value, with the exception of CA19-9 and CEA, which are presented as median values. *; Mann-Whitney U test was used to compare groups. #; Pearson Chi-square was used to compare two groups.

Survival analysis

At the time of data analysis, only 1 patient was alive and 39 patients had died. The median overall survival for these patients was 4.0 months (CI 95%; 2.56-4.56). The median survival time was 1.7 months in patients with positive CK19 mRNA expression, whereas the survival time was 5.3 months in patients with negative CK19 mRNA expression (Log Rank; p=0.009). We found that the median survival time in patients with a negative hTERT mRNA expression was not significantly different from patients with positive hTERT mRNA expression (5.9 months vs. 3.2 months; Log Rank; p=0.183) (Figure 11).

We also examined the roles of CA19-9 and MMP7 as a prognostic factors for these patients. The results showed that the median survival time in patients with high levels of CA19-9 (> 200 ng/ml) was not significantly different from patients with low levels of CA19-9 (< 200 ng/ml) (6.16 months vs. 4.59 months; Log Rank; p=0.270). Moreover, we demonstrated that the median survival time in patients with high levels of MMP7 (> 7.5 ng/ml) was not significantly different from patients with low levels of MMP7 (< 7.5 ng/ml) (4.94 months vs. 5.38 months; Log Rank; p=0.943). (Figure 12).

Figure 11. Kaplan-Meier survival curves of patients with positive or negative expression of A) CK19 and B) hTERT genes measured in the peripheral blood. *; Log-rank test *p* value = 0.009. **; Log-rank test *p* value = 0.183.

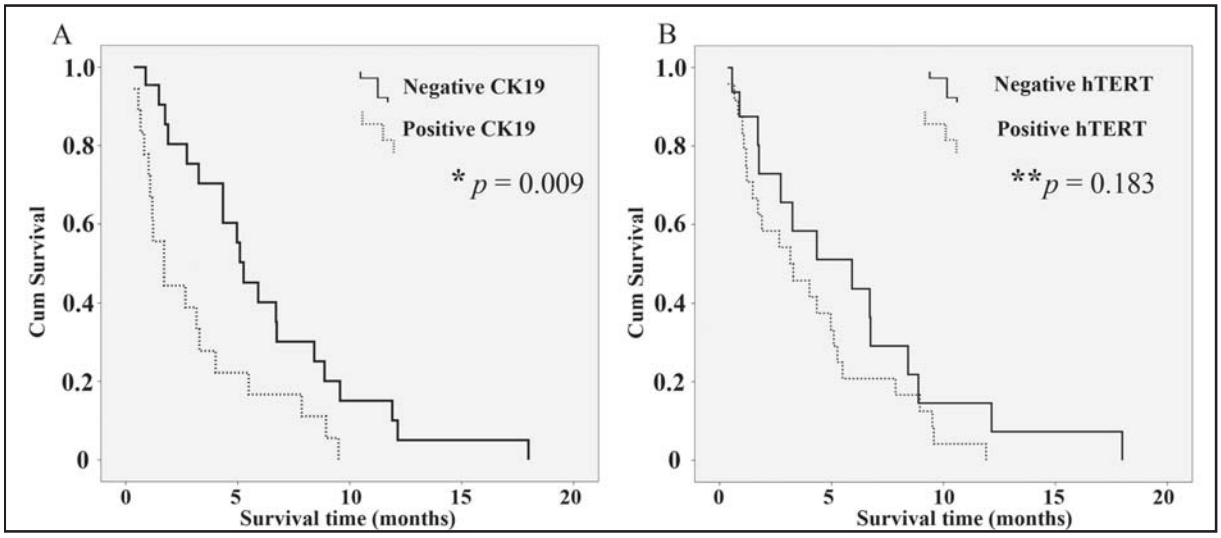
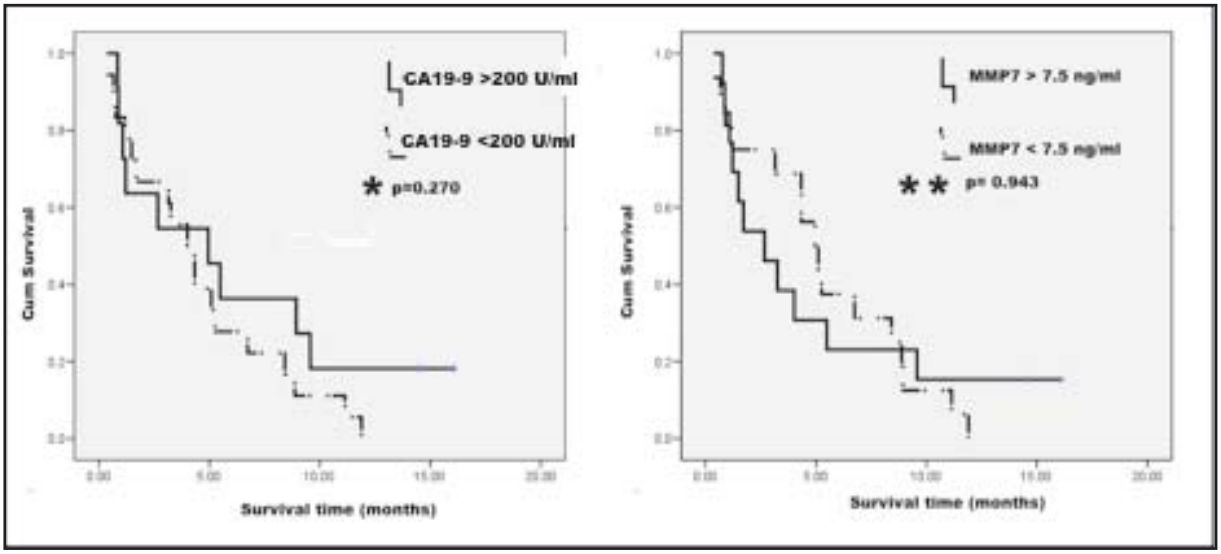


Figure 12. Kaplan-Meier survival curves of patients with high or low levels of A) CA19-9 and B) MMP7 measured in the peripheral blood. *; Log-rank test *p* value = 0.270. **; Log-rank test *p* value = 0.943.



To identify variables that could be of potential prognostic significance in patients diagnosed with advanced malignant biliary tract disease, univariable and multivariable analyses were performed using the Cox proportional hazard model to compare the impact of the mRNA expression levels of CK19 and hTERT. Other clinical parameters such as positive or negative hTERT expression,), CK19 expression (positive or negative), CEA (cut-off value=5 ng/ml), CA19-9 (cut-off value=500 U/ml), total bilirubin (cut-off value= 15 mg/dl) and albumin (cut-off value=3 g/dl) were also examined. Univariable analysis indicated that only CK19 mRNA expression showed significance as a prognostic factor. Multivariable analysis demonstrated that CK19 mRNA expression (p=0.024), age (p=0.026) and serum bilirubin (p=0.002) were all independent risk factors for survival. The relative risk for patients that were determined to be CK19 mRNA positive was 3.2 times greater than that of patients who were CK19 mRNA negative (Table 11).

Table 11 Survival analysis using clinical parameters measured by univariable and multivariable analysis

Variables	Univariable analysis		Multivariate analysis	
	p-Value	Hazard ratio (95% CI[#])	*p*-Value	Hazard ratio (95% CI[#])
CK19 expression	0.011*	2.42 (1.22-4.78)	0.024*	3.20 (1.17-8.75)
hTERT expression	0.188	1.60 (0.80-3.22)	0.580	1.34 (0.47-3.82)
Age	0.541	0.82 (0.42-1.57)	0.026*	0.38 (0.16-0.89)
Total bilirubin	0.106	1.72 (0.89-3.31)	0.002*	3.97 (1.69-9.36)
Albumin	0.360	0.73 (0.37-1.35)	0.213	0.59 (0.26-1.35)
CA19-9	0.374	0.73 (0.37-1.43)	0.478	0.74 (0.32-1.70)
CEA	0.381	1.39 (0.68-2.88)	0.124	1.86 (0.84-4.12)

*Statistically significant

[#]95% CI; 95% Confidence interval

DISCUSSION

1) Detection of MMP7 as a diagnostic marker for cholangiocarcinoma patients

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 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 [9, 51]. 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 [11]. 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 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 [11]. 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 demonstrated that hTERT mRNA is significantly higher in cholangiocarcinoma than in control patients. However, the diagnostic accuracy of hTERT mRNA is not good enough to use as a tumor marker for diagnosis cholangiocarcinoma. The role of CTCs in cholangiocarcinoma should be further study. 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 [15]. 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 the expression of MMP-9 and MMP-7 can be detected in cholangiocarcinoma specimens [16, 52, 53]. Therefore, in our study, the accuracy of serum MMP-9 and MMP-7 levels were investigated in an effort to find a reliable serum marker that can discriminate the benign biliary tract diseases from cholangiocarcinoma.

There are numerous studies that demonstrate that the serum level of MMP-9 is significantly elevated in many types of cancers, including breast cancer, esophageal cancer, and lung cancer [54-56], but previous reports have shown that the incidence of MMP-9 expression in cholangiocarcinoma specimens is only 9-47.5% [16][52]. Our study demonstrated that there is no statistically significant difference in the serum MMP-9 levels between cholangiocarcinoma patients and control patients. Previous studies revealed that detection of MMP-9 in serum is an artifact

representing the release of MMP-9 from leukocytes during the clotting process in the blood collection tube[57, 58]. The role of circulating MMP-9 in diagnosing cholangiocarcinoma should be further investigated by collecting the plasma instead of serum and the assay should be performed without long delay [59].

As far as we are aware, no other published investigation is available that uses the serum MMP-7 level to diagnose cholangiocarcinoma. Our study shows that the serum MMP-7 level is significantly higher in patients with cholangiocarcinoma than with benign biliary tract diseases. MMP-7 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, MMP-7 is principally expressed by epithelial cells [60]. A previous study reported that the serum MMP-7 level was significantly elevated in patients with ovarian cancer and advanced colorectal cancer [17, 61]. We suggest that MMP-7 might be detected in many cancers that originate from epithelial cells. In addition, we also found that the accuracy of the serum MMP-7 level for the diagnosis of cholangiocarcinoma is better than the serum level of MMP-9, CEA and CA19-9, as observed by calculating the AUC of the ROC curve. Only the AUC of the ROC curve for the serum MMP-7 level is significantly higher than a chance value (0.5). Our study demonstrated that use of serum MMP-7 could identify cholangiocarcinoma patients from benign biliary tract disease patients. However, further larger prospective studies that evaluated the benefit of serum MMP-7 in helping the physician to take decisions on diagnosis cholangiocarcinoma are necessary before the implementation of using serum MMP-7 as a marker for cholangiocarcinoma. Previous studies determined that expression of MMP-7 in cholangiocarcinoma is an unfavorable postoperative prognostic factor for cholangiocarcinoma patients [53]. However, in this study, most of the cholangiocarcinoma patients had been diagnosed with unresectable tumors; only five patients

underwent curative resection (R0). Therefore, an analysis for a prognostic factor of cholangiocarcinoma could not be clarified. Further studies that include many cases of resectable cholangiocarcinoma need to be completed before the serum MMP-7 level can be used as a prognostic factor for cholangiocarcinoma.

2) Prospective study for evaluation of MMP7 as a diagnostic marker for cholangiocarcinoma patients

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 previous study, in which serum MMP7 was higher in cholangiocarcinoma than in benign obstructive jaundice patients[48]. 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, and inhibit E-cadherin function by ectodomain shedding of E-cadherin[62]. The results of several recent studies indicate that MMP7 is over-expressed in a variety of epithelial tumors including those of the esophagus[63], colon[64], pancreas[65], and cholangiocarcinoma tumors[16]. In addition, several studies have shown that MMP7 could be detected in the serum of cancer patients, including patients with ovarian[66], colorectal[61] and gastric cancer[67]. 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 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 previous study[48]. The differences of the designs in each study should be considered. Our previous 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 present 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[24]. 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[68]. 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.

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.

3) To evaluate if the levels of CTCs could be used to predict the overall survival of patients diagnosed with advanced malignant biliary tract obstruction disease

The highest incidence of cholangiocarcinoma occurs in Thailand, and the majority of patients included in this study were diagnosed with this disease. In this study, the median survival was 4.0 months, a finding that is not significantly different from the 3.6 to 5.0 month survival time observed in previous studies of advanced malignant biliary tract disease where the majority of patients were diagnosed with pancreatic cancer[69, 70]. These results indicate that all cancers that lead to malignant biliary tract obstruction are highly lethal. Most advanced malignant biliary tract disease patients can only be treated with palliative biliary tract drainage.

The choice of stents (PE or SEMs) for endoscopic palliation of jaundice due to malignant biliary tract obstruction is dependent upon the estimation of patient survival[35]. Therefore, there is a need for more accurate tests to predict the survival of patients with advance malignant biliary tract diseases, as this could significantly improve the treatment results for these patients. This is

the first cohort paper that studies the level of CTCs as a prognostic factor for overall survival of patients diagnosed with advanced malignant biliary tract obstruction.

We used quantitative real-time RT-PCR to detect CTCs. As a result of the PCR-based methods, we cannot identify exactly the cell source of the measured markers. These methods assess the expression of target genes from mRNA extracted from the lysates of cells harvested from the peripheral blood of patients. As such, these samples contain not only CTCs but also PBMCs, circulating endothelial cells (CEC) and skin cells (e.g. keratinocytes, fibroblasts and melanocytes) that contaminate the sample during blood withdrawal and provide alternate potential sources for the PCR-detected genes[39, 71]. Therefore, strict selection of target genes used for detection of CTCs is very important. In this study, we used CK19 and hTERT genes as targets for the detection of CTCs. Previous studies have suggested that CTCs are likely to be the principal cell source for CK19 gene expression, as CK19 expression is mainly restricted to epithelial cells and is limited in normal peripheral blood cells[72]. Additionally, we used the 2-syringe technique during blood collection to avoid epithelium contamination from injected site.

Based on the results of our study, patients with positive CK19 expression exhibit significantly shorter overall survival compared to patients with negative CK19 expression (5.3 months VS 1.7 months; p=0.009). Additionally, multivariable analysis using the Cox regression model also demonstrated that the levels of CK19 expression in peripheral blood, the levels of serum total bilirubin and the age of the patients can all function as independent prognostic factors in patients diagnosed with advanced malignant biliary tract disease. This is consistent with previously published studies that reported that positive CK19 mRNA expression in peripheral blood was independently associated with a reduction in disease-free survival in patients diagnosed with breast cancer[45]. In addition, positive CK19 mRNA expression in peripheral blood following

chemo-radiation was an independent, unfavorable prognostic factor for both overall survival and progression-free survival in patients diagnosed with non-small cell lung cancer[44].

In this study, we demonstrated that the number of patients with positive hTERT mRNA expression in peripheral blood is higher than the number of patients with positive CK19 mRNA expression in peripheral blood (60% VS 45%); however, detection of hTERT mRNA in the peripheral blood is not identified as an independent prognostic factor in this study. We suggested that the detection of hTERT mRNA expression levels is not a good candidate for use as a prognostic factor for patients with advanced malignant biliary tract disease. It may be suitable for the distinction between malignant and benign biliary tract diseases in combination with other tumor specific markers.

In this study, neither univariable nor multivariable analysis indicated that serum levels of CA19-9 or MMP7 could be used as a prognostic factor for patients diagnosed with advanced malignant biliary tract disease. This finding is inconsistent with a previous study that indicated that the levels of serum CA19-9 are of prognostic relevance in patients with biliary tract cancer[73]. We suggested that differences among the patients should be considered. Overall survival in the previous study was 16.1 months[74], whereas the overall survival in observed in our study was 4.0 months. In addition, the majority of patients in the previous study received chemotherapy, while only two patients in our study received this treatment. This finding reflects a higher disease severity in the patients examined in our study.

Our study demonstrated that the expression of CK19 mRNA in PBMCs prior to palliative procedures was significantly associated with overall survival in advanced malignant biliary tract disease patients. We therefore recommend PE stents for patients with positive CK19 mRNA expression in their peripheral blood. The more expensive SEMS should be reserved for patients

with negative peripheral blood expression of CK19 mRNA. Further cost-effectiveness studies should be performed to evaluate the benefit of using CK19 mRNA in helping the physician make decisions regarding the selection of stent-type and the need for endoscopic repeat intervention in advanced malignant biliary tract disease patients.

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Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. **ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า)**

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2. **การนำผลงานวิจัยไปใช้ประโยชน์**

- เชิงพาณิชย์ (มีการนำไปผลิต/ขาย/ก่อให้เกิดรายได้ หรือมีการนำไปประยุกต์ใช้โดยภาคธุรกิจ/บุคคลทั่วไป)
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  - นำองค์ความรู้จากงานวิจัย ไปใช้เพื่อประกอบการรักษาผู้ป่วยโรคมะเร็งทางเดินน้ำดี

3. **อื่น ๆ (เช่น ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ การเสนอผลงานในที่ประชุมวิชาการ หนังสือ การจดสิทธิบัตร)**

นำเสนอผลงาน งานประชุมวิชาการประจำปี 2553 และ 2554 ของราชวิทยาลัยศัลยกรรมประเทศไทย

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v18.i2.175

World J Gastroenterol 2012 January 14; 18(2): 175-181
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Prognostic relevance of circulating CK19 mRNA in advanced malignant biliary tract diseases

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Supported by Rajavithi Hospital Project Grant and Thailand Research Fund, No. RSA52
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Received: April 9, 2011 Revised: June 17, 2011
Accepted: June 24, 2011
Published online: January 14, 2012

Abstract

AIM: To determine the role of circulating tumor cells (CTCs) in prediction of the overall survival of patients with advanced malignant biliary tract obstruction.

METHODS: We investigated the prognostic value of CTCs by examining two markers, cytokeratin (CK) 19 and human telomerase reverse transcriptase (hTERT) mRNA, in 40 patients diagnosed with advanced malignant biliary tract diseases. Quantitative real-time reverse transcription polymerase chain reaction was used to detect CK19 and hTERT mRNA in the peripheral blood of these patients. Overall survival was analyzed using the Kaplan-Meier method and Cox regression modeling.

RESULTS: Positive CK19 and hTERT mRNA expression was detected in 45% and 60%, respectively, of the 40 patients. Univariable analysis indicated that positive CK19 mRNA expression was significantly associated with worse overall survival (P = 0.009). Multivariable analysis determined that positive CK19 mRNA expression, patient's age and serum bilirubin were each independently associated with overall survival.

CONCLUSION: CK19 mRNA expression levels in peripheral blood appear to provide a valuable marker to predict the overall survival of patients with advanced malignant biliary tract obstruction.

Key words: Circulating tumor cells; Cytokeratin 19; Human telomerase reverse transcriptase; Malignant biliary tract obstruction; Overall survival

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INTRODUCTION

Malignant biliary tract obstruction is a condition that can

result from tumors of the biliary tract, ampulla of vater, duodenum or head of the pancreas. In Thailand, cholangiocarcinoma (CCA) is the most common cause of malignant biliary tract obstruction[1]. Despite recent advances in the diagnosis and treatment of this disease, patient outcome remains poor. The high mortality rate arising from malignant biliary tract obstruction is due to the aggressiveness of tumors that are often discovered at a late stage of disease progression[2]. Palliative therapeutic approaches to endoscopic biliary drainage, such as the use of endoprosthesis stents, are generally recommended for these patients. The two major types of endoprosthesis stents are plastic or polyethylene (PE) stents and self-expanding metal stents (SEMS). Previous studies have demonstrated that partial or total occlusion of PE stents usually occurs 3-4 mo after insertion[3]. Four randomized controlled studies demonstrated that SEMS exhibited a significantly higher patency rate as compared with the PE stents (9 mo *vs* 1.5 mo)[4-7]; however, SEMS is much more expensive than PE stents (1500 USD *vs* 80 USD, in Thailand). A recent study indicated that patients who have a predicted survival duration of more than 4.5 mo should use SEMs for their palliative biliary drainage[8]. In this instance, the higher cost of the SEMs is balanced by a decreased need for repeat intervention that is often necessary in patients who have received PE stents. Therefore, identification of reliable prognostic factors that allow for an accurate prediction of survival duration in patients with advanced malignant biliary tract obstruction is extremely important.

One of the major mechanisms for tumor metastasis is the dissemination of tumor cells from the primary tumor into circulating blood[9]. Previous studies have indicated that detection of circulating tumor cells (CTCs) in the peripheral blood can be used in staging and prognosis stratification for breast and colon cancer patients[10-12]. Until now, however, there has been no study concerning the role of the detection of CTCs as a prognostic factor in patients with malignant biliary tract diseases.

To date, the most common CTCs detection method is quantitative real-time reverse transcription polymerase chain reaction (RT-PCR), a process that can detect mRNA expression levels of the genes coding for these tumor antigens[13]. A high-quality detection marker is required for efficient quantitative real-time RT-PCR-mediated detection of CTCs. Therefore, identification of a good target marker is of the utmost importance for CTC detection. Several gene markers, such as cytokeratin (CK) 19 and human telomerase reverse transcriptase (hTERT), have been evaluated as tumor-specific markers for the detection of CTCs in gastrointestinal cancers[14,15].

hTERT mRNA can be detected in 85% of all cancer cells, including cholangiocarcinoma cells[16]. This is in contrast to most normal cells, which exhibit little or no expression. Our previous study demonstrated that high levels of hTERT mRNA can be detected in the blood circulation of cholangiocarcinoma patients, and it has also been suggested that hTERT mRNA is a promising marker for the detection of cancer cells[17].

CK19 is generally expressed in ductal epithelium (bile ducts, pancreas, and renal collecting tubules) and in the mucosa of the gastrointestinal tract. CK19 immunohistochemistry is used in diagnostic pathology mainly to confirm epithelial immunophenotype in undifferentiated tumors or to establish biliary, pancreatic or renal ductular origin[18]. Most adenocarcinomas arising from the gastrointestinal tract are CK19 positive, including cholangiocarcinoma and pancreatic cancer[18]. Many investigators have used the detection of CK19 mRNA in peripheral blood as a target gene to investigate CTCs[14,19,20]; however, until now there has been no study focusing on the detection of hTERT and CK19 in the peripheral blood of patients with malignant biliary tract diseases.

This study was aimed to evaluate if the levels of CTCs could be used to predict the overall survival of patients with advanced malignant biliary tract obstruction. *CK-19* and *hTERT* were selected as the target genes for CTCs. In addition, this study was performed in accordance with the REporting recommendations for tumor MARKer prognostic studies[21] to ensure the standardization and transparency of the study.

MATERIALS AND METHODS

Patients and samples

We prospectively included the patients with advanced malignant biliary tract diseases who underwent palliative endoscopic retrograde cholangiopancreatography or percutaneous transhepatic biliary drainage at Department of Surgery, Rajavithi Hospital from January 2008 to December 2009. The cutoff date for data analysis was December 31, 2010. The inclusion requirements included patients present with malignant bile duct obstruction that was not amenable to curative resection and patients who were followed up for at least one month after biliary tract drainage. All blood and clinical information was obtained with patient informed consent after approval by the Rajavithi Hospital Ethics Committee.

Pre-treatment fasting blood samples were collected from the peripheral vein into ethylenediaminetetraacetic acid-containing tubes. The first 3 mL blood was discarded to prevent epidermal contamination (2-syringe technique). Sample processing was performed within 1 h of blood withdrawal. Blood was transferred into a 30-mL falcon tube and centrifuged at 1800 r/min at room temperature for 20 min. Plasma was removed, and the peripheral blood mononuclear cell (PBMC) fraction was stored at -80 ℃ until use.

RNA extraction and cDNA synthesis

The total RNA of PBMC fraction samples was extracted using the RNeasy mini kit (Qiagen, GmbH, Germany) following the protocol provided by the manufacturer. RNA integrity was checked by electrophoresis and quantified by absorption at 260 and 280 nm using a spectrophotometer (Beckman Coulter Du® 800, Fullerton, CA). Total RNA was reversely transcribed using random primers and

Table 1 Primer sequences

Primer	Forward	Reverse
hTERT	GCGGAAGACAGTGGTGAACT	AGC TGGAGTAGT CGCTCT GC
CK19	CCCGCGACTACAGCCACTA	GCTCATGCGCAGAGCCT
β-Actin	GTGGGGCGCCCCAGGCACCA	GTCCTTAATGTCACGCACGATTTC

hTERT: Human telomerase reverse transcriptase; CK19: Cytokeratin 19.

Table 2 Clinical characteristics of patients with advanced malignant biliary tract obstruction

	Parameters	*n* (%)
Gender	Male	23 (57.5)
	Female	17 (42.5)
Age (yr)	< 60	18 (45.0)
	> 60	22 (55.0)
Type of cancers	Hilar cholangiocarcinoma	28 (70.0)
	Pancreatic cancer	6 (15.0)
	Common bile duct cancer	2 (5.0)
	Ampullary cancer	2 (5.0)
	Gall bladder cancer	2 (5.0)

the IscriptTM cDNA synthesis kit (Bio-Rad, Hercules, CA, United States) following the protocol provided by the manufacturer. cDNA was stored at -80 ℃ until use.

Detection of CK-19 and hTERT mRNA by quantitative real-time PCR

Expression of *CK19* and *hTERT* genes was analyzed using specific primers (Table 1). In this assay, the housekeeping gene *β-actin* was used as an internal control to normalize variations in integrity and the total amount of cDNA. Quantitative real-time PCR assays were performed in triplicate using SYBR Green master mix (Superarray, Frederick, MD, United States) on the Chromo 4™ System (MJ Opticon Monitor ver. 3.1) (Bio-Rad, United States) for 20 min at 50 ℃. After this, 42 cycling steps for amplification of PCR products were performed (15 s, 94 ℃ for denaturation; 30 s, 60 ℃ for annealing; and 30 s, 72 ℃ for extension). Melting curve analysis was used to assess the specificity of the amplified products. The expression levels of *CK19* and *hTERT* genes from the cDNA were measured by quantitative real-time PCR using the relative quantification method ($2^{-\Delta\Delta Ct}$ method)[22]. The fold-change in gene expression was normalized to a housekeeping gene (*β-actin*) and relative to a calibrator sample. A pool of cDNA derived from the PBMCs of 30 cases of benign (common bile duct stone and gall stone) biliary tract diseases was used as the calibrator source[23]. Evaluation of the $2^{-\Delta\Delta Ct}$ indicates the fold change in gene expression relative to the calibrator. In this study, we set the positive value as a fold change in gene expression that was greater than 1.5 times relative to the calibrator and the negative value was set as a fold change in gene expression that was lesser than or equal to 1.5 times relative to the calibrator.

Determination of blood chemistries in serum samples

Biochemical studies of serum samples, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), total and direct bilirubin, alkaline phosphatase, carcinoembryonic antigen (CEA) and cancer antigen (CA)19-9, were measured using routine automated methods in the Pathological Laboratory at Rajavithi Hospital.

Cell lines and cell spiking experiments

The human cholangiocarcinoma cell line RMCCA1[24] was incubated in Ham's F12 medium (Invitrogen-Gibco, Carlsbad, CA, United States) containing 10% fetal calf serum (Euroclone-Celbio, Pero, MI) at 37 ℃ in 5% CO_2. To determine the sensitivity of quantitative real-time PCR for detecting cancer cells in PBMCs, cell spiking experiments was performed. The PBMCs obtained from healthy volunteers were counted and diluted in Ham's F12 medium. RMCCA1 cells were serially diluted from 1 × 10^6 cells/mL to 1 cell/mL and added to the PBMCs. Quantitative real-time PCR was then performed to detect CK19 and hTERT mRNA.

Statistical analysis

The primary endpoint of this study was the overall survival of the patients. Survival curves were estimated using the Kaplan-Meyer method, and univariable survival comparisons were calculated according to the log rank test. Multivariable survival analysis was performed using the Cox proportional hazards regression model. The quantitative variables were compared using Mann-Whitney *U* or Student's *t* test, as appropriate. Qualitative variables were reported as counts, and comparisons between independent groups were performed using the Pearson χ^2 test. All tests of significance were two sided and $P < 0.05$ was considered statistically significant.

RESULTS

Patient characteristics

Forty-two patients with malignant biliary tract disease were included in this study. Two patients were excluded because of the poor quality of the RNA extracted from their peripheral blood. The average age of these patients was 62 years (range, 41-82 years). With regard to cancer type, 6 (15.0%) were pancreatic head cancer, 2 (5.0%) were ampullary cancer, 2 (5.0%) were gall bladder cancer, 2 (5.0%) were middle and distal common bile duct cancer and 28 (70.0%) were hilar cholangiocarcinoma. The clinical characteristics of the patients are shown in Table 2.

Cell spiking assay

CK19 and hTERT mRNA levels were elevated in the RMCCA1 cell line (Figure 1); therefore, we decided to use this cell line as a positive control for our study. Detection sensitivity of the quantitative real-time PCR assay was determined by serial 10-fold dilutions of RMCCA1 cells in PBMCs. The results demonstrated that CK19 and hTERT mRNA could be detected at levels up to 1000 cells per 10^{10} PBMC dilutions.

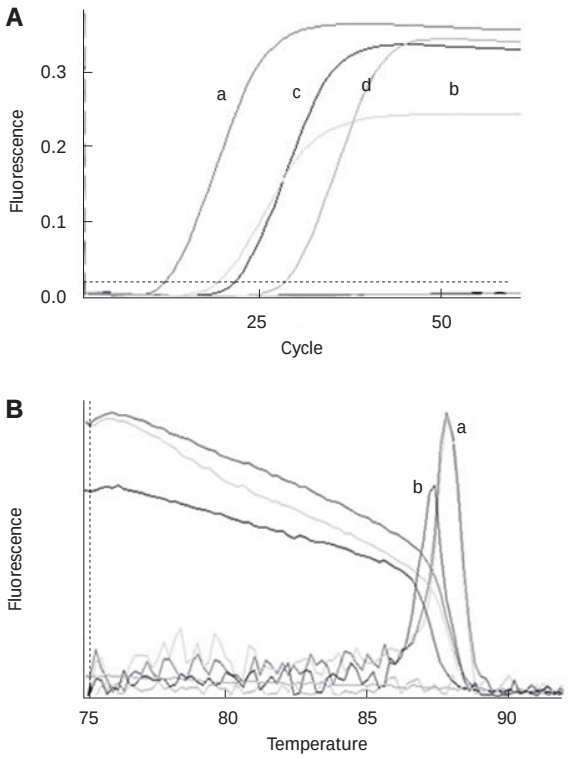

Figure 1 Gene expression levels of cytokeratin 19 and human telomerase reverse transcriptase in RMCCA1 cells (as measured by quantitative real time polymerase chain reaction). A: Amplification plot of cytokeratin (CK)19 mRNA from 10 000 RMCCA1 cells. (a) 1000 RMCCA1 cells; (b) amplification plot of human telomerase reverse transcriptase (hTERT) mRNA from 10 000 RMCCA1 cells; (c) 1000 RMCCA1 cells; and (d) are demonstrated; B: SYBR Green melting curve for quantitative real time reverse transcription polymerase chain reaction (RT-PCR). The melting curves from quantitative real time PCR for CK19 (a) and hTERT (b) consistently gave a single peak with no evidence of non-specific amplification or primer-dimerisation.

Detection of hTERT and CK19 mRNA in PBMCs of malignant biliary tract disease patients

PBMC samples from 40 patients were evaluated for the expression of hTERT and CK19 mRNA. The expression was positive (gene expression more than 1.5 times relative to the calibrator) in 45% (18/40) of samples for CK19 mRNA and 60% (24/40) of samples for hTERT. Figure 2 illustrates the distribution of CK19 mRNA and hTERT mRNA expression in the peripheral blood of these patients.

Relationship between CK19 and hTERT mRNA expression in peripheral blood and clinic pathological features of patients

No statistically significant difference was found among the data obtained from the patients considered as negative or those who were positive for hTERT or CK19 mRNA expression in PBMCs. Factors evaluated included gender, age, serum albumin, globulin bilirubin AST and ALT and alkaline phosphatase levels (Table 3).

Survival analysis

At the time of data analysis, only 1 patient was alive and 39 patients had died. The median overall survival for these patients was 4.0 mo (95% CI: 2.56-4.56). The median survival time was 1.7 mo in patients with positive CK19 mRNA expression, whereas the survival time was 5.3 mo in patients with negative CK19 mRNA expression (Log Rank, $P = 0.009$). We found that the median survival time in patients with a negative hTERT mRNA expression was not significantly different from patients with positive hTERT mRNA expression (5.9 mo *vs* 3.2 mo, Log Rank, $P = 0.183$) (Figure 3).

To identify variables that could be of potential prognostic significance in patients with advanced malignant biliary tract disease, univariable and multivariable analyses were performed using the Cox proportional hazard model to compare the impact of the mRNA expression levels of CK19 and hTERT. Other clinical parameters such as positive or negative hTERT expression, CK19 expression (positive or negative), CEA (cut-off value = 5 ng/mL), CA19-9 (cut-off value = 500 U/mL), total bilirubin (cut-off value = 15 mg/dL) and albumin (cut-off value = 3 g/dL) were also examined. Univariable analysis indicated that only CK19 mRNA expression showed significance as a prognostic factor. Multivariable analysis demonstrated that CK19 mRNA expression ($P = 0.024$), age ($P = 0.026$) and serum bilirubin ($P = 0.002$) were all independent risk factors for survival. The relative risk for CK19 mRNA positive patients was 3.2 times greater than that for CK19 mRNA negative patients (Table 4).

Table 3 Clinical characteristics of patients with negative and positive cytokeratin 19 and human telomerase reverse transcriptase gene expression

	CK19 gene		*P*	*hTERT* gene		*P*
	Negative	Positive	value	Negative	Positive	value
Age (yr)	63.80	61.38	0.42	65.26	60.88	0.16
Sex (male: female)	11:11	12:6	0.35[1]	8:8	15:9	0.52[1]
Total bilirubin (mg/dL)	16.21	16.42	0.95	17.73	15.47	0.53
Albumin (g/dL)	2.88	2.97	0.62	2.83	2.99	0.36
Globulin (g/dL)	3.98	3.76	0.42	3.92	3.84	0.79
AST (U/L)	84.57	112.80	0.23	69.27	117.48	0.12
ALT (U/L)	42.47	65.85	0.12	34.87	66.68	0.28
ALP (IU/L)	449.68	528.85	0.56	436.88	421.91	0.98
BUN (mg/dL)	13.77	26.58	0.13	12.25	23.52	0.18
Creatinine (mg/dL)	0.75	1.30	0.20	0.61	1.19	0.22
CA19-9 (U/mL)	570.20	594.30	0.62[2]	1818.00	259.15	0.11[2]
CEA (ng/mL)	7.47	5.68	0.50[2]	7.21	5.82	0.23[2]

Quantitative variables are presented as the mean value, with the exception of cancer antigen (CA)19-9 and carcinoembryonic antigen (CEA), which are presented as median values. [1]Pearson χ^2 was used to compare two groups; [2]Mann-Whitney *U* test was used to compare groups. CK19: Cytokeratin 19; hTERT: Human telomerase reverse transcriptase; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; BUN: Blood urea nitrogen.

DISCUSSION

The highest incidence of cholangiocarcinoma occurs in Thailand, and the majority of patients included in this study were diagnosed with this disease[1]. In this study,

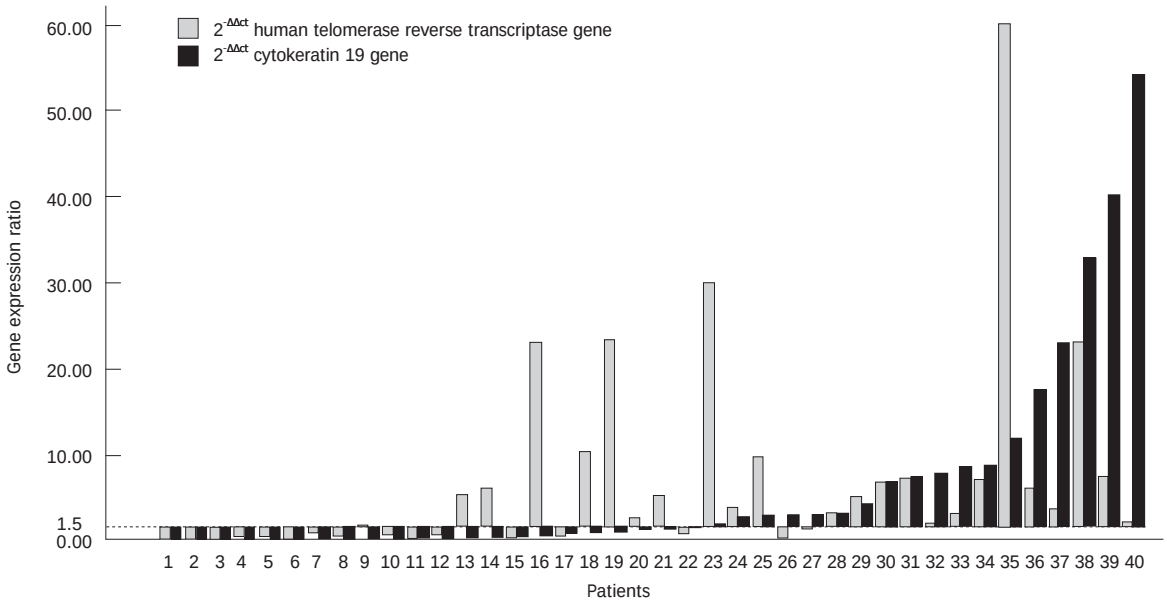

Figure 2 The distribution levels of cytokeratin 19 and human telomerase reverse transcriptase genes in the peripheral blood of 40 patients. The positive value is determined as a fold change in gene expression of more than 1.5 times relative to the calibrator.

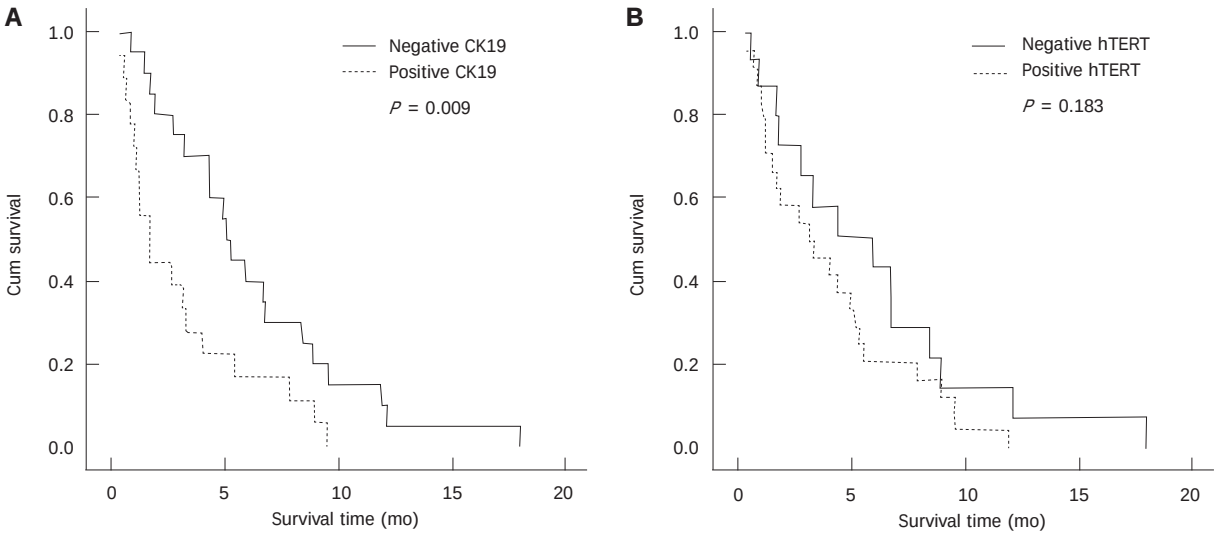

Figure 3 Kaplan-Meier survival curves of patients with positive or negative expression of cytokeratin 19 (A) and human telomerase reverse transcriptase (B) genes measured in the peripheral blood. CK19: Cytokeratin 19; hTERT: Human telomerase reverse transcriptase.

the median survival was 4.0 mo, a finding that is not significantly different from the survival time (3.6-5.0 mo) observed in previous studies of advanced malignant biliary tract disease where the majority of patients were diagnosed with pancreatic cancer[7,8,25]. These results indicate that all cancers that lead to malignant biliary tract obstruction are highly lethal. Most advanced malignant biliary tract disease patients can only be treated with palliative biliary tract drainage.

The choice of stents (PE or SEMs) for endoscopic palliation of jaundice due to malignant biliary tract obstruction is dependent upon the estimation of patient survival[8]. Therefore, there is a need for more accurate tests to predict the survival of patients with advanced malignant biliary tract diseases, as this could significantly improve the treatment outcome for these patients. This is the first cohort paper that studies the level of CTCs as a prognostic factor for overall survival of patients with advanced malignant biliary tract obstruction.

We used quantitative real-time RT-PCR to detect CTCs. As a result of the PCR-based methods, we cannot identify exactly the cell source of the measured markers. Quantitative real-time RT-PCR assesses the expression of target genes from mRNA extracted from the lysates of cells harvested from the peripheral blood of patients. As such, these samples contain not only CTC but

Table 4 Survival analysis using clinical parameters measured by univariable and multivariable analysis

Variables	Univariable analysis		Multivariate analysis	
	P value	Hazard ratio (95% CI)	*P* value	Hazard ratio (95% CI)
CK19 expression	0.011[1]	2.42 (1.22-4.78)	0.024[1]	3.20 (1.17-8.75)
hTERT expression	0.188	1.60 (0.80-3.22)	0.580	1.34 (0.47-3.82)
Age	0.541	0.82 (0.42-1.57)	0.026[1]	0.38 (0.16-0.89)
Total bilirubin	0.106	1.72 (0.89-3.31)	0.002[1]	3.97 (1.69-9.36)
Albumin	0.360	0.73 (0.37-1.35)	0.213	0.59 (0.26-1.35)
CA19-9	0.374	0.73 (0.37-1.43)	0.478	0.74 (0.32-1.70)
CEA	0.381	1.39 (0.68-2.88)	0.124	1.86 (0.84-4.12)

[1]Statistically significant. CI: Confidence interval; CK19: Cytokeratin 19; hTERT: Human telomerase reverse transcriptase; CA: Cancer antigen; CEA: Carcinoembryonic antigen.

also PBMC, circulating endothelial cells and skin cells (e.g., keratinocytes, fibroblasts and melanocytes) that contaminate the sample during blood withdrawal and provide alternate potential sources for the PCR-detected genes[13,26]. Therefore, strict selection of target genes for detection of CTCs is very important. In this study, we used *CK19* and *hTERT* genes as targets for the detection of CTCs. Previous studies have suggested that CTCs are likely to be the principal cell source for *CK19* gene expression, as CK19 expression is mainly restricted to epithelial cells and is limited in normal peripheral blood cells[20,27]. Additionally, we used the 2-syringe technique during blood collection to avoid epithelium contamination from injected site.

In our study, the patients with positive CK19 expression exhibited significantly shorter overall survival compared with the patients with negative CK19 expression (5.3 mo *vs* 1.7 mo; $P = 0.009$). Additionally, multivariable analysis using the Cox regression model also demonstrated that the levels of CK19 expression in peripheral blood, the levels of serum total bilirubin and the age of the patients can all function as independent prognostic factors in patients with advanced malignant biliary tract disease. This is consistent with previously published studies that reported that positive CK19 mRNA expression in peripheral blood was independently associated with a reduction in disease-free survival in patients with breast cancer[20]. In addition, positive CK19 mRNA expression in peripheral blood following chemoradiation was an independent, unfavorable prognostic factor for both overall survival and progression-free survival in patients diagnosed with non-small cell lung cancer[19].

In this study, there were more patients with positive hTERT mRNA expression in peripheral blood than the patients with positive CK19 mRNA expression (60% *vs* 45%); however, detection of hTERT mRNA in the peripheral blood was not identified as an independent prognostic factor in this study. We suggested that the detection of hTERT mRNA expression levels was not a good candidate as a prognostic factor for patients with advanced malignant biliary tract disease. It may be suitable for the distinction between malignant and benign biliary tract diseases in combination with other tumor specific markers.

In this study, neither univariable nor multivariable analysis indicated that serum levels of CA19-9 could be used as a prognostic factor for patients with advanced malignant biliary tract disease. This finding is inconsistent with a previous study that indicated that the levels of serum CA19-9 are of prognostic relevance in patients with biliary tract cancer[28,29]. We suggested that differences among the patients should be considered. Overall survival in a previous study was 16.1 mo[29], whereas the overall survival in our study was 4.0 mo. In addition, the majority of patients in the previous study received chemotherapy, while only two patients in our study received this treatment. This finding reflects a higher disease severity in the patients examined in our study.

Our study demonstrated that the expression of CK19 mRNA in PBMCs prior to palliative procedures was significantly associated with overall survival of the patients with advanced malignant biliary tract disease. We therefore recommend PE stents for patients with positive CK19 mRNA expression in their peripheral blood. The more expensive SEMS should be reserved for patients with negative peripheral blood expression of CK19 mRNA. Further cost-effectiveness studies should be conducted to evaluate the benefit of using CK19 mRNA in helping the physician make decisions regarding the selection of stent-type and the need for endoscopic repeat intervention in patients with advanced malignant biliary tract disease.

COMMENTS

Background

In Thailand, cholangiocarcinoma is the most common cause of malignant biliary tract obstruction. Despite recent advances in the diagnosis and treatment of this disease, patient outcome remains poor. Palliative therapeutic approaches to endoscopic biliary drainage, such as the use of endoprosthesis stents, are generally recommended for these patients. Identification of reliable prognostic factors that allow for an accurate prediction of survival duration in patients suffering from advanced malignant biliary tract obstruction is extremely important.

Research frontiers

This study demonstrated that the levels of circulating tumor cells (CTCs) could be used to predict the overall survival of patients with advanced malignant biliary tract obstruction.

Innovations and breakthroughs

The expression of cytokeratin (CK)19 mRNA in peripheral blood mononuclear cells prior to palliative procedures was significantly associated with overall survival of patients with advanced malignant biliary tract disease.

Applications

This study recommends polyethylene stents for patients with positive CK19 mRNA expression in their peripheral blood. The more expensive self-expanding metal stents should be reserved for patients with negative peripheral blood expression of CK19 mRNA.

Terminology

CTCs is the dissemination of tumor cells from the primary tumor into circulating blood. The detection of CTCs can be used in staging and prognosis stratification for cancer patients.

Peer review

This study provides evidences that high levels of CTCs in advanced malignant biliary tract obstruction patients might be used as a prognostic factor. This is a well performed and clearly presented study.

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S- Editor Sun H **L- Editor** Ma JY **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i37.4697

World J Gastroenterol 2010 October 7; 16(37): 4697-4703
ISSN 1007-9327 (print)

BRIEF ARTICLE

Prospective study of MMP7 serum levels in the diagnosis of cholangiocarcinoma

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Received: April 24, 2010 Revised: July 6, 2010
Accepted: July 13, 2010
Published online: October 7, 2010

Abstract

AIM: To determine whether the serum level of matrix metalloproteinase-7 (MMP7) has the potential to diagnosis cholangiocarcinoma from benign biliary tract diseases.

METHODS: This study was performed according to the PRoBE (a prospective-specimen-collection, retrospective-blinded-evaluation) design. A total of 187 patients with obstructive jaundice were consecutively enrolled. After the diagnostic status of these patients was ascertained, their levels of serum MMP7 were assayed and compared with serum carbohydrate antigen 19-9 (CA19-9). This was conducted in a blinded case (cholangiocarcinoma)-control (benign biliary tract disease) setup.

RESULTS: 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, 95% CI: 0.778-0.903 for MMP7 and AUC = 0.79, 95% CI: 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.

CONCLUSION: Serum values of MMP7 and CA19-9 appear to be useful biomarkers for differentiating cholangiocarcinoma from benign biliary tract obstructive diseases.

Key words: Carbohydrate antigen 19-9; Cholangiocarcinoma; Matrix metalloproteinase-7; Sensitivity; Specificity; Tumor marker

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Leelawat K, Narong S, Wannaprasert J, Ratanashu-ek T. Prospective study of MMP7 serum levels in the diagnosis of cholangiocarcinoma. *World J Gastroenterol* 2010; 16(37): 4697-4703 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i37/4697.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i37.4697

INTRODUCTION

Cholangiocarcinoma is one of the most common causes of cancer-related mortality in Thailand[1]. The high mortality rate of cholangiocarcinoma is due to the aggressiveness of tumors that are often discovered at a late-stage

of disease progression[2]. To improve the survival rate, the diagnosis and treatment of these patients should be performed as soon as possible. Bile duct obstruction is the focal symptom that the vast majority of patients with cholangiocarcinoma present with at hospital. However, there are many cases of benign biliary tract diseases including common bile duct stone and bile duct stricture. These other conditions often present with clinical symptoms similar to those of patients with cholangiocarcinoma. In addition, it is very difficult to obtain pathological tissue for the diagnosis of cholangiocarcinoma due to both the desmoplastic reaction and the tumor location[3,4]. Brush cytology has a sensitivity of only 62.5% for detecting cholangiocarcinoma[5]. Owing to the differences in treatment and prognosis between cholangiocarcinoma and benign biliary tract diseases, the most important issue is to obtain a reliable method to differentially diagnosis patients with cholangiocarcinoma from those with benign biliary tract diseases. Identification of tumor markers in the serum would aid in the accurate diagnosis of cholangiocarcinoma.

To date, carbohydrate antigen 19-9 (CA19-9) is used as a tumor marker for detecting cholangiocarcinoma. The sensitivity and specificity of CA19-9 in diagnosing cholangiocarcinoma were shown to be 53%-89% and 80.5%-86%, respectively[6-9]. Unfortunately, elevated serum levels of CA19-9 have also been found in patients with benign obstructive jaundice[10,11]. Due to this, CA19-9 is a not a reliable marker for differentiating cholangiocarcinoma from benign obstructive jaundice.

Previous studies demonstrated that high expression of matrix metalloproteinase-7 (MMP7) could be readily detected in cholangiocarcinoma specimens[12-14]. We previously performed research to study the serum levels of CA19-9, CEA, MMP9 and MMP7 in patients with obstructive jaundice[6]. This previous study was performed using a case-control design and serum collected from a serum bank. The results showed that only the level of MMP7 was significantly higher in patients with cholangiocarcinoma compared to patients suffering from benign biliary tract disease. In addition, when comparing the areas under the curve of the receiver operating characteristic (ROC) for CEA, CA19-9 and MMP9, a ROC curve analysis demonstrated that the detection of MMP7 in serum was the most accurate for differentiating cholangiocarcinoma from benign biliary tract disease. This finding indicated that serum MMP7 should be used as a tumor marker for the diagnosis of cholangiocarcinoma in patients with obstructive jaundice.

According to the study of biomarker use, it is now widely appreciated that the evaluation of biomarker performance must be separated from biomarker discovery. In discovery research, its performance in samples may be biased in an overoptimistic direction. To estimate performance without bias, an independent dataset should be investigated[15-18]. Therefore, the aim of the present study was to evaluate the performance of serum MMP7 and CA19-9 for their potential in the diagnosis of cholangiocarcinoma. We used a new and independent dataset of prospective consecutive cases with evidence of bile duct obstruction due to various etiologies. This study was performed according to the PRoBE (a prospective-specimen-collection, retrospective-blinded-evaluation) design[15]. We collected serum from a cohort that was representative of the target population (consecutive cases of obstructive jaundice who had undergone ERCP, PTBD or bile duct surgery). After the diagnostic status of these patients was ascertained, the levels 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)[16-18] to ensure standardization and transparency of our study.

MATERIALS AND METHODS

Patients and study design

This study was conducted within the Rajavithi Hospital Surgery Department located in Bangkok, Thailand. The local ethics committee approved the study protocol. Sample size was calculated on the basis of an expected area under the ROC curve of MMP7 serum levels (= 0.70) for the diagnosis of cholangiocarcinoma[6]. Using a significance level of 0.05 (two-sided) and a power of 0.95, a sample of 50 cholangiocarcinoma patients was required for the study[19]. From previous data, the prevalence of cholangiocarcinoma detection in patients with obstructive jaundice treated at our department was shown to be in the range of 27%-30%. Therefore, we prospectively included consecutive patients with symptoms of obstructive jaundice who had undergone ERCP, PTBD or bile duct surgery during the period from June of 2008 to July of 2009. Exclusion criteria included presence of other cancers, age less than 20 years and the presence of severe pulmonary fibrosis[20]. All patients gave written informed consent. The diagnosis of cholangiocarcinoma was carried out using one of the following tests[6]: (1) tissue biopsy; and (2) cytology plus radiological (helical CT scan or MRI) and clinical observation to identify tumor progression at a follow-up of at least two months. Patients with an inconclusive diagnosis were excluded from this study.

Serum collection and the measurement of serum biochemistry

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

Measurement of serum MMP7 levels

The serum levels of MMP7 were measured using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Sys-

tems, Minneapolis, MN, USA), as previously described[6]. Briefly, the diluted serum samples were added in duplicate to 96-well plates coated with MMP7 antibody and then incubated at room temperature for 2 h. After washing, the conjugated secondary antibody was added, and the plate was further incubated for another 2 h. Plates were washed again prior to incubation with the substrate solution for 1 h. 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. The scientist examining these serum samples was unaware of the patient's diagnosis. In addition, the MMP7 test results had no influence on the clinical diagnosis of the patients in the study.

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 multiple comparisons by the 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 ROC curve was generated by plotting the sensitivity against 1-specificity, and the area under the curve with 95% confidence intervals (CI) 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.

RESULTS

Patient characteristics

A total of 230 patients with obstructive jaundice were consecutively enrolled. Twenty-four cases were excluded according to their diagnosis of ampullary cancer (7 cases), pancreatic cancer (9 cases), gall bladder cancer (3 cases), duodenum cancer (2 cases), metastatic cancer from ovarian cancer (1 case) and hepatocellular carcinoma (2 cases). In addition, nineteen cases were excluded according to their uncertain diagnosis. The 187 subjects studied included 128 patients with benign biliary tract diseases (control group) which included 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 1).

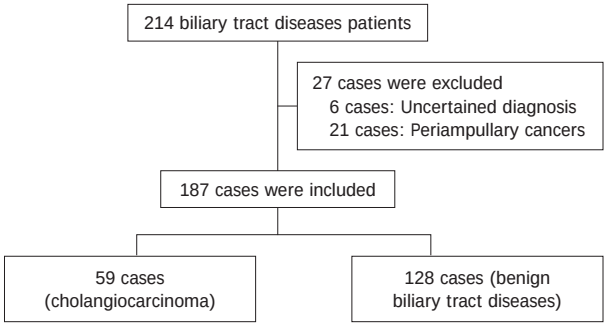

Figure 1 A flow diagram of 187 obstructive jaundice patients who were consecutively enrolled in this study.

Table 1 Clinical characteristics of patients with benign biliary tract diseases (control) and cholangiocarcinoma

	Control *n* = 128	Cholangiocarcinoma *n* = 59	*P*
Age (yr)	61 ± 7	67 ± 5	0.451
Sex (M:F)	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 (mg/dL)	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

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase.

As shown in Table 1, 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 ALP were significantly higher in cholangiocarcinoma patients than in the control patients (Mann-Whitney *U* test, $P < 0.05$).

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: 0.6-71000 U/mL) in the control group and 571.2 U/mL (range: 0.6-71000 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 2A and B, 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. 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

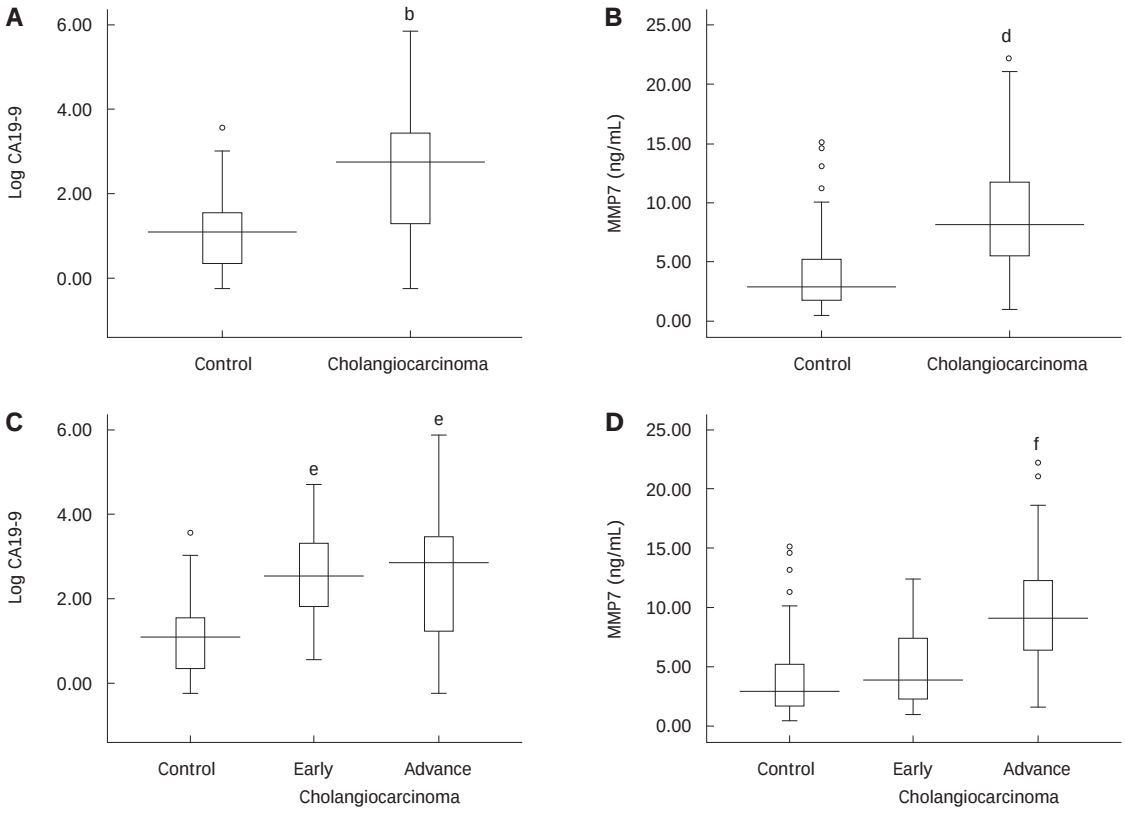

Figure 2 Serum levels of carbohydrate antigen 19-9 and matrix metalloproteinase-7 in cholangiocarcinoma and control (benign biliary tract disease) patients. A: Box plots comparing levels of carbohydrate antigen 19-9 (CA19-9); B: Matrix metalloproteinase-7 (MMP7) between cholangiocarcinoma and control patients are illustrated; C: Box plots comparing levels of CA19-9; D: MMP7 between early and advanced stages of cholangiocarcinoma and controls are illustrated. Levels of MMP7 are presented as ng/mL, while CA19-9 is presented with the log data to accommodate the wide range. ([b]Mann-Whitney *U*, $P < 0.001$ *vs* control; [d]Student's *t*-test, $P < 0.001$ *vs* control; [e]Kruskal Wallis test, $P < 0.001$ *vs* control; [f]ANOVA, $P < 0.001$ *vs* control).

and late stages of cholangiocarcinoma (Figure 2C). The data shown in Figure 2D demonstrates that the MMP7 levels tended to increase according to the progression of cholangiocarcinoma. The serum MMP7 levels were significantly different between early and late stages of cholangiocarcinoma (ANOVA, $P < 0.001$). However, the serum MMP7 levels in early stage cholangiocarcinoma were not significantly different from the serum MMP7 levels in benign control patients (ANOVA, $P = 0.47$).

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, a ROC curve analysis was applied to calculate the area under the curve (AUC). These levels were determined to be 0.79 (95% CI: 0.708-0.868) and 0.84 (95% CI: 0.778-0.903) for CA19-9 and MMP7, respectively (Figure 3). The sensitivity, specificity, positive and negative predictive values for selected cut-off points of CA19-9 and MMP7 are presented in Table 2.

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 3, if the patients had a serum MMP7 and CA19-9 level higher than the cut-off values, the probability of a diagnosis of cholangiocarcinoma was equal to 86.12%. In addition, if the patients had a serum MMP7 and serum CA19-9 level less than the cut-off values, the probability of a positive diagnosis of cholangiocarcinoma was very low (< 6.4%).

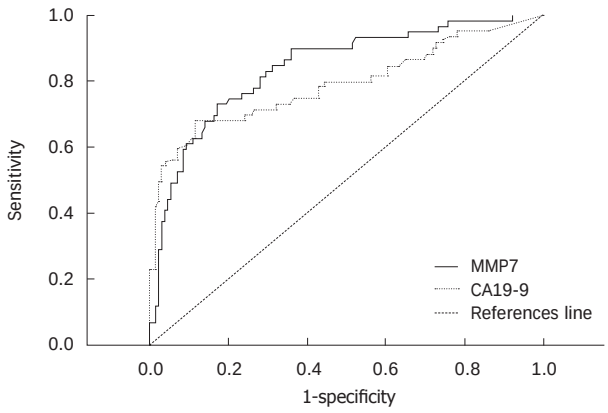

Figure 3 Receiver operating characteristic curve analyses of carbohydrate antigen 19-9 and matrix metalloproteinase-7 for the diagnosis of cholangiocarcinoma. The diagnostic accuracy of each biomarker, in terms of its sensitivity and specificity, are presented by receiver operating characteristic curve analysis. MMP7: Matrix metalloproteinase-7; CA19-9: Carbohydrate antigen 19-9.

Table 2 Performance of the biomarkers for the diagnosis of cholangiocarcinoma (%) (95% CI)

Tumor markers (cut-off value)	Sensitivity	Specificity	PPV	NPV	LR+	LR-
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 U/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 U/mL)	68 (56-80)	87 (81-93)	70 (58-82)	85 (79-91)	5.1 (3.17-8.22)	0.37 (0.25-0.54)
CA19-9 (200 U/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)

PPV: Positive predictive value; NPV: Negative predictive value; LR+: Positive likelihood ratio; LR-: Negative likelihood ratio; CI: Confidence interval; MMP7: Matrix metalloproteinase-7; CA19-9: Carbohydrate antigen 19-9.

Table 3 Predicted probability of the combination of serum carbohydrate antigen 19-9 and matrix metalloproteinase-7 for diagnosis of cholangiocarcinoma

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

MMP7: Matrix metalloproteinase-7; CA19-9: Carbohydrate antigen 19-9.

Table 4 Pearson's correlation coefficients of matrix metalloproteinase-7, carbohydrate antigen 19-9, albumin, total bilirubin, aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase

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

[a]Statistically significant, $P < 0.05$. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; MMP7: Matrix metalloproteinase-7; CA19-9: Carbohydrate antigen 19-9.

Correlation between MMP7, CA19-9 and other blood chemistry

The correlations between the levels of serum albumin, AST, ALT, ALP, total bilirubin, CA19-9, and MMP7 were investigated. As presented in Table 4, the level of serum MMP7 was significantly correlated with serum albumin, AST, ALP, total bilirubin and CA19-9, although none of these parameters had a high Pearson correlation coefficient value (> 0.7). We suggest that the significant correlation of these blood chemistries with serum MMP7 was caused by the high number of samples analyzed in this study.

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

To determine whether the levels of serum CA19-9 and MMP7 were predictive of cholangiocarcinoma independent of the other blood chemistry levels 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 mg/dL), albumin (cut-off value = 4 mg/dL), AST (cut-off value = 100 U/L) and ALP (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 5).

Table 5 Odd Ratios estimates for diagnosis of cholangiocarcinoma

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

The significant parameters ($P < 0.05$) selected by the model are shown. AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; MMP7: Matrix metalloproteinase-7; CA19-9: Carbohydrate antigen 19-9.

DISCUSSION

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 from 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 previous study, in which serum MMP7 was higher in cholangiocarcinoma than in benign obstructive jaundice patients[6]. This suggested that serum MMP7 has the potential to be a tumor marker for cholangiocarcinoma in patients with obstructive jaundice.

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[21], and inhibit E-cadherin function by ectodomain shedding of E-cadherin[22]. The results of several recent studies indicate that MMP7 is over-expressed in a

variety of epithelial tumors including those of the esophagus[23], colon[24,25], pancreas[26], and cholangiocarcinoma tumors[12]. In addition, several studies have shown that MMP7 could be detected in the serum of cancer patients, including patients with ovarian[27], colorectal[28] and gastric cancer[29]. 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 modalities (clinical presentation and imaging study) before making a diagnosis.

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

The values of AUC from the ROC curve for MMP7 and CA19-9 in this study were shown to be much higher than those observed in our previous study[6]. The differences in the designs of these studies should be considered. Our previous 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 present study was the implementation of the strategies of the PRoBE designs to avoid the problems of bias that may affect the studies of the diagnostic test[15]. We collected serum from all obstructive jaundice patients before the diagnosis of cholangiocarcinoma or benign biliary tract diseases was determined. This procedure assured that biases related to differences in sample collection and handling would be avoided[30]. Limitations of this design include the fact that the majority of the study participants were in advanced 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 carried out before using MMP7 as a screening test for the detection of early stage cholangiocarcinoma. In addition, this study was performed in a referral center, which has a 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.

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

ACKNOWLEDGMENTS

We thank Associate Professor Dr. Tawechai Tejapongvorachai and Professor Dr. Apiwat Mutirangura for their valuable contributions, and Dr. Chulaluk Komoltri, Dr. Somrat Lertmaharit and Thai CERTC staff members for help with statistical analyses.

COMMENTS

Background

To date, carbohydrate antigen 19-9 (CA19-9) is used as a tumor marker for detecting cholangiocarcinoma. Unfortunately, elevated serum levels of CA19-9 have also been found in patients with benign obstructive jaundice. Previous studies demonstrated that cholangiocarcinoma cells express a high level of matrix metalloproteinase (MMP)-7.

Research frontiers

High expression of MMP7 was detected in cholangiocarcinoma specimens. In addition, the authors' previous nonconsecutive case-control study demonstrated that the serum level of MMP7 is higher in cholangiocarcinoma than in benign biliary tract disease patients. However, a prospective consecutive study of the evaluation of serum MMP7 as a diagnostic marker for cholangiocarcinoma has not been established. In this study, the authors collected a new and independent dataset of prospective consecutive cases with evidence of bile duct obstruction due to various etiologies, and demonstrated that the serum level of MMP7 could be a potential tumor marker for differentiating cholangiocarcinoma from benign biliary tract obstruction.

Innovations and breakthroughs

This is the first consecutive prospective study to report that the serum level of MMP7 was significantly higher in patients with cholangiocarcinoma than in those with benign biliary tract obstruction. The authors suggest that the serum level of MMP7 may be a potential tumor marker for differentiating cholangiocarcinoma from benign biliary tract obstruction.

Applications

This study may represent a future strategy for diagnosing patients with cholangiocarcinoma by the detection of serum level of MMP7 and CA19-9.

Peer review

This is a prospective-specimen-collection and retrospective-blinded-evaluation study of 187 patients with obstructive jaundice where a novel serum marker, MMP7, for the diagnosis of cholangiocarcinoma was investigated. In general, it's a nicely designed and accomplished study with sound conclusion, hopefully of interest for a wide range of readers and researchers.

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S- Editor Wang JL **L- Editor** Webster JR **E- Editor** Ma WH

BMC Gastroenterology

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Detection of serum MMP-7 and MMP-9 in cholangiocarcinoma patients: evaluation of diagnostic accuracy

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Published: 30 April 2009

BMC Gastroenterology 2009, **9**:30 doi:10.1186/1471-230X-9-30

Received: 18 January 2009
Accepted: 30 April 2009

This article is available from: http://www.biomedcentral.com/1471-230X/9/30

Abstract

Background: Cholangiocarcinoma is an aggressive tumor with a tendency for local invasion and distant metastases. Timely diagnosis is very important because surgical resection (R0) remains the only hope for a cure. However, at present, there is no available tumor marker that can differentiate cholangiocarcinoma from benign bile duct disease. Previous studies have demonstrated that matrix metalloproteinase (MMP)-7 and MMP-9 are frequently expressed in cholangiocarcinoma specimens.

Methods: This study was designed to determine whether the serum levels of MMP-7 and MMP-9 can discriminate cholangiocarcinoma patients from benign biliary tract disease patients in comparison to carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9). We measured the level of CEA, CA19-9, MMP-7 and MMP-9 in the serum of 44 cholangiocarcinoma and 36 benign biliary tract diseases patients.

Results: Among the serum levels of CEA, CA19-9, MMP-7 and MMP-9, only the serum MMP-7 level was significantly higher in the patients with cholangiocarcinoma (8.9 ± 3.43 ng/ml) compared to benign biliary tract disease patients (5.9 ± 3.03 ng/ml) ($p < 0.001$). An receiver operating characteristic (ROC) curve analysis revealed that the detection of the serum MMP-7 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 CEA, CA19-9 and MMP-9 were 0.63 (95% CI = 0.501–0.760), 0.63 (95% CI = 0.491–0.761) and 0.59 (95% CI = 0.455–0.722), respectively.

Conclusion: Serum MMP-7 appears to be a valuable diagnostic marker in the discrimination of cholangiocarcinoma from benign biliary tract disease. Further prospective studies for serum MMP-7 measurement should be carried out to further investigate the potential of this molecule as a biomarker of cholangiocarcinoma.

Background

The incidence of and mortality rate for cholangiocarcinoma varies considerably among different geographic regions, with the highest incidence being observed in Southeast Asia, especially in Thailand [1]. In the United States, the most commonly recognized risk factor for cholangiocarcinoma is primary sclerosing cholangitis (PSC) [2,3]. However, in Southeast Asia and especially in Thailand, infection with hepatobiliary flukes (*Opisthorchis viverrini*) is the most common risk factor for cholangiocarcinoma [4]. Therapeutic options for cholangiocarcinoma have been limited since this type of cancer responds poorly to chemotherapy and radiation therapy. Surgery is perhaps the only effective treatment for cholangiocarcinoma. Five-year survival, which typically has a rate between 32% and 50%, is achieved by only a small number of patients when negative histological margins are attained at the time of surgery [5]. To improve the survival rate, patients must be diagnosed and treated as early in the disease onset as possible.

To properly diagnose cholangiocarcinoma, it is very difficult to get to the tissue due to the tumor location and the desmoplastic reaction. In addition, this tumor typically grows along the bile duct without expanding from the bile ducts as a forming mass. Computed tomography (CT), ultrasound, and magnetic resonance imaging (MRI) often miss this lesion [6]. Therefore, identification of tumor markers in the serum would be beneficial in the clinical management of this disease. To date, there are two common tumor markers used for detecting cholangiocarcinoma, carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9). CEA is unspecific and can be elevated in the setting of other gastrointestinal or gynecologic malignancies or other bile duct pathologies, such as cholangitis and hepatolithiasis [7]. Previous studies have demonstrated that the sensitivity and specificity of a CA 19-9 value >100 U/ml for cholangiocarcinoma in primary sclerosing cholangitis (PSC) are 89% and 86%, respectively [8,9]. However, a cut-off of the CA 19-9 value at 100 U/ml resulted in a sensitivity of only 53.0–67.5% for diagnosing cholangiocarcinoma in patients without PSC [10,11]. In addition, a previous study demonstrated that the level of serum CA19-9 is dependent on the severity of the bile duct obstruction and the degree of cholangitis. An increase in the serum level of CA19-9 can be detected even in benign bile duct diseases [12,13]. Therefore, novel tumor markers should be investigated to better diagnose cholangiocarcinoma in patients with or without PSC.

Typically, tumor cells invade the basement membrane by secreting enzymes that digest the extracellular matrix proteins. These enzymes are known as matrix metalloproteinase (MMPs). MMPs are zinc-dependent endopeptidases. They are involved in the turnover and degradation of the extracellular matrix (ECM) components and basement membranes [14]. Recently, Itatsu K, et al. examined the expression of MMPs in surgically resected specimens of cholangiocarcinoma using an immunohistochemical method and found that 47.5 and 75.8% of these specimens expressed MMP-9 and MMP-7, respectively [15]. Previous studies have demonstrated that MMP-9 can be detected in the serum of gastric cancer patients and MMP-7 is increased in colorectal, ovarian and renal cancer patients [16-19]. Therefore, detection of MMP-9 and MMP-7 in the blood circulation may be useful for the clinical diagnosis of cholangiocarcinoma. To date, there is no published study on the detection of serum levels of MMP-9 and MMP-7 in cholangiocarcinoma patients. The objective of this study was to determine the accuracy of detecting serum levels of MMP-9 and MMP-7 for the diagnosis of cholangiocarcinoma in patients without primary sclerosing cholangitis.

Methods

Patients and samples

Pre-treatment fasting serum samples (n = 80) were collected from obstructive jaundice patients who underwent endoscopic retrograde cholangiography (ERCP) or biliary tract surgery at Rajavithi Hospital. All patient sera and clinical information were obtained with patient consent after approval by Rajavithi Ethics Committee. Thirty-six patients were diagnosed with benign biliary tract diseases, and 44 patients were diagnosed as having cholangiocarcinoma by one of the following criteria: 1) tissue biopsy (n = 7), 2) cytology (n = 17), and 3) radiological finding (helical CT scan or MRI) and clinical observation to identify the progression of the tumor at follow up (n = 20). Serum samples from these patients were separated by centrifugation within 2 h and frozen at -80°C. The biochemical studies of serum samples, including AST, ALT, total and direct bilirubin, alkaline phosphatase (ALP), CEA and CA19-9, were measured using routine automated methods in the Pathological Laboratory at Rajavithi Hospital.

Measurement of serum MMP-7 and MMP-9

Serum MMP-9 and MMP-7 levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN). The diluted serum samples were added in duplicate to 96-well plates coated with the MMP-9 or MMP-7 antibody and incubated at room temperature for 2 h. After washing three times with washing buffer, the conjugated secondary antibody was added, and the plate was further incubated for 2 h. Plates were washed again prior to incubation with the substrate solution for 1 h. The amplifier solution was then added, and the plate was incubated for an additional 30 min. All incubation cycles were 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 MMP-9 and MMP-7 in each sample was calculated from a standard curve.

Statistical analysis

Comparison between the quantitative variables was performed by using *Mann-Whitney U* or Student's t-test, as appropriate. Qualitative variables were reported as counts, and comparisons between independent groups were performed by using by Pearson Chi-square. The diagnostic accuracy of each of the candidate biomarkers was evaluated using receiver operating characteristic (ROC) curve analysis, which correlates true- and false-positive rates [sensitivity and (1-specificity)]. In addition, an area under the ROC curve (AUC) with 95% confidence intervals (CI) was calculated for each marker. The optimal cut-off points for MMP-9 and MMP-7 were selected based on the ROC curve analysis. Sensitivity, specificity, positive predictive value and negative predictive value were calculated using a 2 × 2 table of the collected data.

Results

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) 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).

Detection of CEA and CA19-9 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). These data are shown in Figure 1. 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 [7]. 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).

Detection of MMP-9 and MMP-7 in serum of cholangiocarcinoma and benign obstructive jaundice patients

There was no statistically significant difference in the levels of MMP-9 between the control (mean ± SD; 16.5 ± 9.30 ng/ml) and cholangiocarcinoma patients (mean ± SD; 18.9 ± 8.55 ng/ml), (Student's t-test; *p* = 0.251, 95% CI -1.74–6.55). In contrast, the serum MMP-7 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).

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

	Control (n = 36)	Cholangiocarcinoma (n = 44)	*p* value
Age (Yr)	54 ± 14.5	59 ± 12.9	0.130
Sex (Male:Female)	15:16	26:18	0.248#
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).

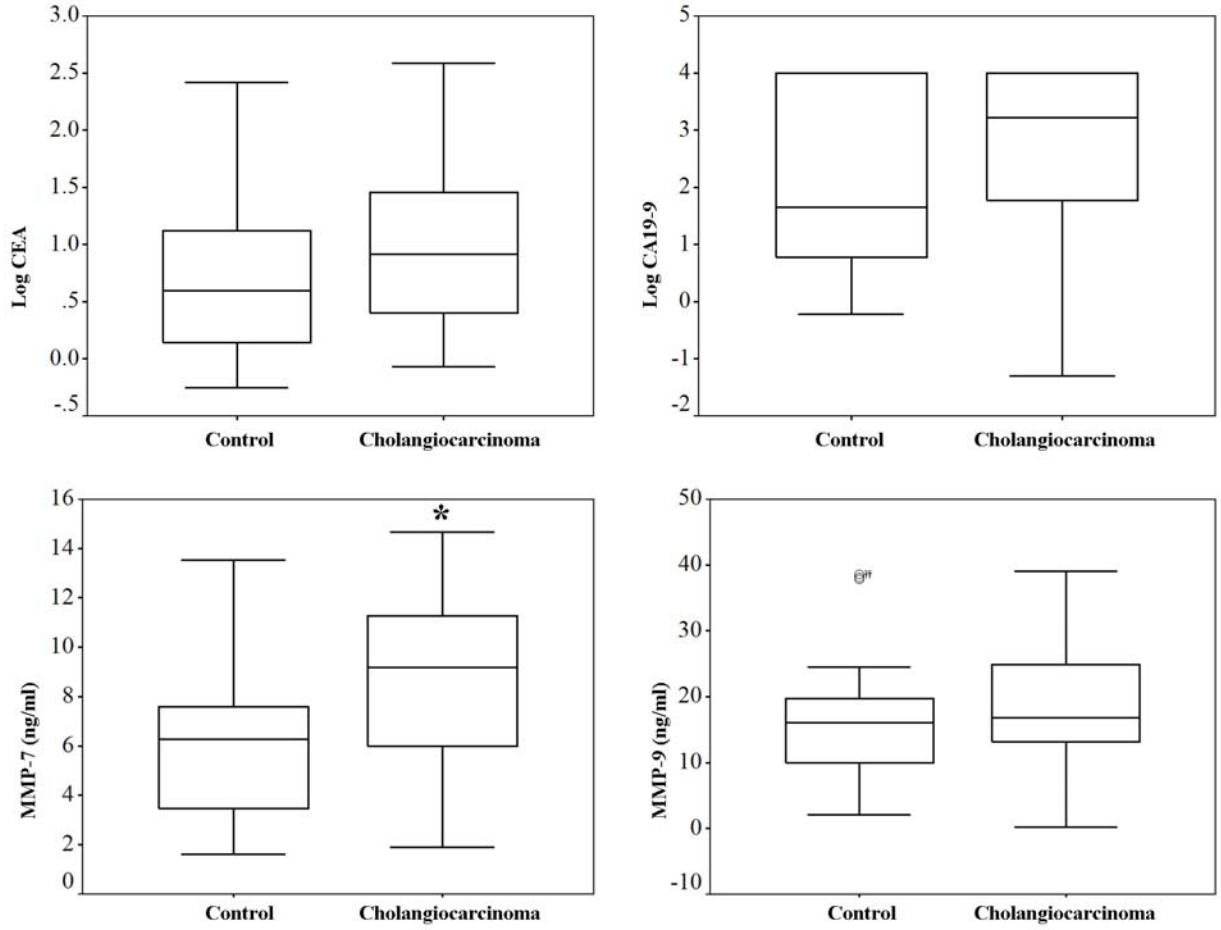

Figure 1
Serum levels of CEA, CA19-9, MMP-7 and MMP-9 in cholangiocarcinoma and control (benign biliary tract disease) patients. Box plots comparing levels of CEA, CA19-9, MMP-7 and MMP-9 are demonstrated. Levels of MMP-7 and MMP-9 are presented as ng/ml, while CEA and CA19-9 are presented with the log data to accommodate the wide range. *; Only the value for MMP-7 between the two groups is significantly different (Student's t-test; $p < 0.001$).

ROC curve analysis for CEA, CA19-9, MMP-9 and MMP-7 for diagnosis of cholangiocarcinoma

An ROC curve analysis (Figure 2) 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 0.59 (CI 95% 0.455 – 0.722) and of 0.73 (CI 95% 0.614 – 0.848) for MMP-9 and MMP-7, respectively. When comparing the AUC of the ROC curve for CEA, CA19-9, MMP-9 and MMP-7 with a chance value equal to 0.5 (the worst value of AUC of ROC), only the AUC of the ROC for MMP-7 is significantly higher than 0.5 ($p = 0.001$). The sensitivity and specificity for CEA, CA19-9, MMP-9 and MMP-7 are presented in Table 2.

Due to the significant difference of the serum AST, ALP, total bilirubin and direct bilirubin between the control and cholangiocarcinoma patients, we investigated the correlation between the values of these blood chemistries and the values for CEA, CA19-9, MMP-9 and MMP-7. The results showed that none of these parameters was significantly correlated ($p > 0.05$) (see Addition file 1 and Addition file 2). To determine whether the values of serum MMP-9 and MMP-7 were predictive of cholangiocarcinoma independently of other tumor markers, we carried out a logistic regression analysis. In a multivariable model using MMP-9 (cut-off value = 15 ng/ml), MMP-7 (cut-off value = 7.4 ng/ml), CEA (cut-off value = 5 ng/ml), CA19-9 (cut-off value = 100 U/ml), MMP-9 (an adjusted odds ratio = 3.76; 95% CI = 1.05–13.47; $p = 0.04$), MMP-7 (an

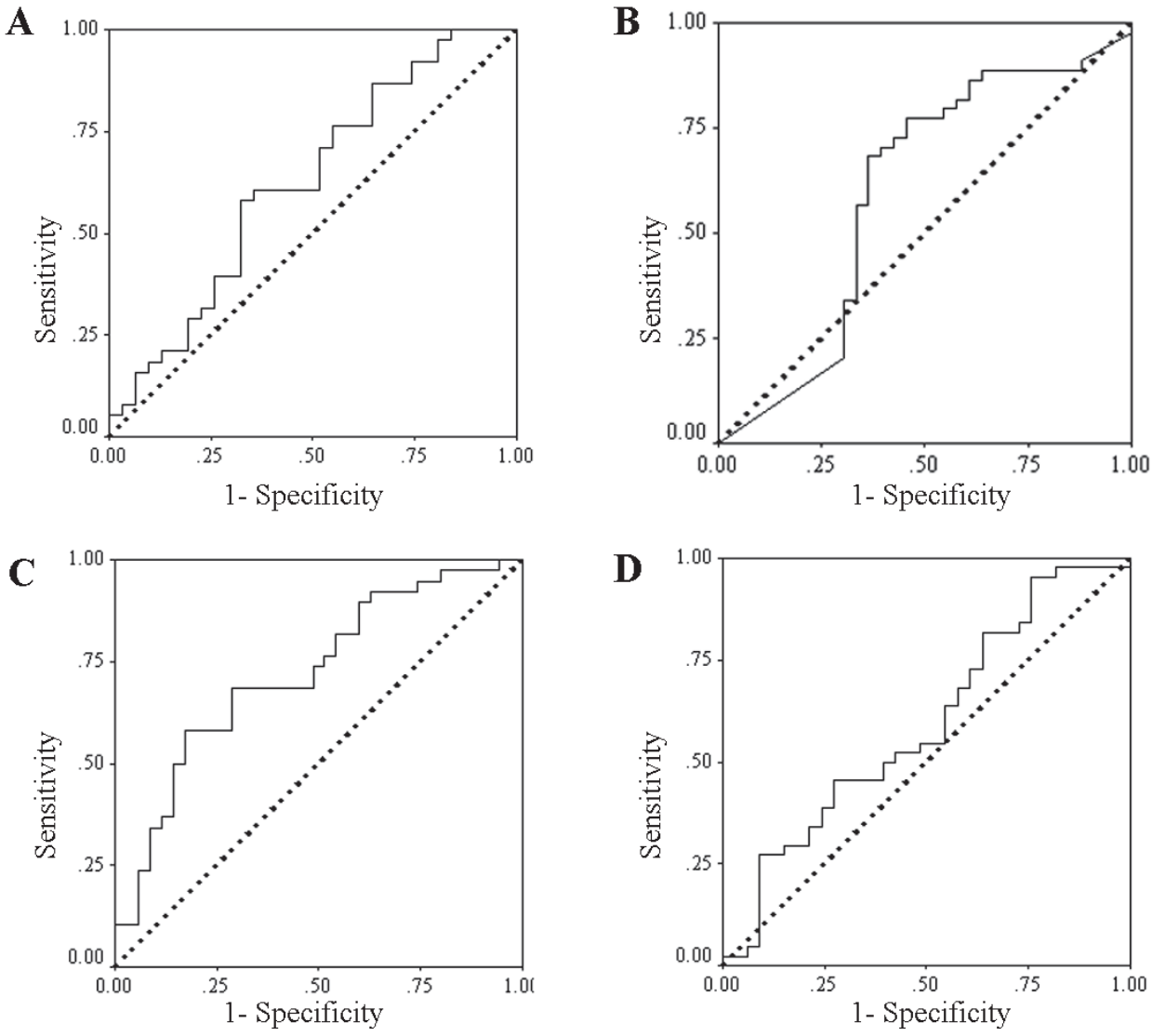

Figure 2
ROC curve analyses of CEA, CA19-9, MMP-9 and MMP-7 for the diagnosis of cholangiocarcinoma. The diagnostic accuracy of each biomarker, in terms of its sensitivity and specificity, are presented by receiver operating characteristic (ROC) curve analysis. Figures 2A, 2B, 2C and 2D correspond to **CEA, CA19-9, MMP-7 and MMP-9**. Only the area under the curve (AUC) of the ROC for MMP-7 is significantly higher than a chance value (0.5).

adjusted odds ratio = 5.33; 95% CI = 1.55–18.31; p = 0.008) and CA19-9 (an adjusted odds ratio = 4.60; 95% CI = 1.23–17.30; p = 0.02) were the independent predictors of cholangiocarcinoma, whereas CEA was not.

Discussion

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 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%, respec-

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	70.73	43.75	1.26	0.67
(3 ng/ml)	(55.52–82.39)	(28.17–60.67)	(0.87–1.81)	(0.36–1.24)
CEA	58.54	62.50	1.56	0.66
(5 ng/ml)	(43.37–72.24)	(45.25–77.07)	(0.93–2.62)	(0.42–1.04)
CA19-9	81.82	48.48	1.59	0.38
(35 U/ml)	(68.04–90.49)	(32.50–64.78)	(1.11–2.27)	(0.18–0.77)
CA19-9	70.45	63.64	1.94	0.46
(100 U/ml)	(55.78 – 81.84)	(46.62–77.81)	(1.19–3.16)	(0.28–0.78)
MMP-9	63.64	41.94	1.67	0.59
(15.0 ng/ml)	(48.87–76.22)	(26.42–59.23)	(0.93–3.01)	(0.35–0.98)
MMP-9	34.10	74.19	1.32	0.89
(20.0 ng/ml)	(21.88–48.86)	(56.75–86.30)	(0.64–2.73)	(0.66–1.20)
MMP-7	76.32	46.88	1.44	0.51
(6.0 ng/ml)	(60.79–87.01)	(30.87–63.55)	(0.99–2.08)	(0.26–1.00)
MMP-7	63.16	71.88	2.25	0.51
(7.4 ng/ml)	(47.28–76.62)	(54.63–84.44)	(1.23–4.11)	(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)

tively [7,20]. 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 [10]. 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 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 [10]. 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 [14]. 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 the expression of MMP-9 and MMP-7 can be detected in cholangiocarcinoma specimens [21-23]. Therefore, in our study, the accuracy of serum MMP-9 and MMP-7 levels were investigated in an effort to find a reliable serum marker that can discriminate the benign biliary tract diseases from cholangiocarcinoma.

There are numerous studies that demonstrate that the serum level of MMP-9 is significantly elevated in many types of cancers, including breast cancer, esophageal cancer, and lung cancer [24-26], but previous reports have shown that the incidence of MMP-9 expression in cholangiocarcinoma specimens is only 9–47.5% [15,22]. Our study demonstrated that there is no statistically significant difference in the serum MMP-9 levels between cholangiocarcinoma patients and control patients. Previous studies revealed that detection of MMP-9 in serum is an artifact representing the release of MMP-9 from leukocytes during the clotting process in the blood collection tube [27,28]. The role of circulating MMP-9 in diagnosing cholangiocarcinoma should be further investigated by collecting the plasma instead of serum and the assay should be performed without long delay [29].

Previous studies have demonstrated that cholangiocarcinoma specimens frequently express MMP-7 (75.8–100%) [15,21]. As far as we are aware, no other published investigation is available that uses the serum MMP-7 level to diagnose cholangiocarcinoma. Our study shows that the serum MMP-7 level is significantly higher in patients with cholangiocarcinoma than with benign biliary tract dis-

eases. MMP-7 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, MMP-7 is principally expressed by epithelial cells [30]. A previous study reported that the serum MMP-7 level was significantly elevated in patients with ovarian cancer and advanced colorectal cancer [16,31]. We suggest that MMP-7 might be detected in many cancers that originate from epithelial cells. In addition, we also found that the accuracy of the serum MMP-7 level for the diagnosis of cholangiocarcinoma is better than the serum level of MMP-9, CEA and CA19-9, as observed by calculating the AUC of the ROC curve. Only the AUC of the ROC curve for the serum MMP-7 level is significantly higher than a chance value (0.5). Our study demonstrated that use of serum MMP-7 could identify cholangiocarcinoma patients from benign biliary tract disease patients. However, further larger prospective studies that evaluated the benefit of serum MMP-7 in helping the physician to take decisions on diagnosis cholangiocarcinoma are necessary before the implementation of using serum MMP-7 as a marker for cholangiocarcinoma. Previous studies determined that expression of MMP-7 in cholangiocarcinoma is an unfavorable postoperative prognostic factor for cholangiocarcinoma patients [15]. However, in this study, most of the cholangiocarcinoma patients had been diagnosed with unresectable tumors; only five patients underwent curative resection (R0). Therefore, an analysis for a prognostic factor of cholangiocarcinoma could not be clarified. Further studies that include many cases of resectable cholangiocarcinoma need to be completed before the serum MMP-7 level can be used as a prognostic factor for cholangiocarcinoma.

Conclusion

The elevation of serum MMP-7 levels could be a very useful tool for the detection of cholangiocarcinoma, especially in those patients with obstructive jaundice.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

KL conceived of, designed, and coordinated the study and the statistical analysis and drafted the manuscript. SS coordinated the study and helped with the statistical analysis. SN carried out the MMP-7 and MMP-9 assays and helped with the statistical analysis and JW coordinated the study.

Additional material

Additional file 1

The correlation between the blood chemistry values and MMP-9 or MMP-7. *This table demonstrates the correlation between the blood chemistry values (total bilirubin, AST, ALP, Log CEA and Log CA19-9) and MMP-9 or MMP-7 in the control and cholangiocarcinoma patients. No significant correlation is identified* ($p > 0.05$).

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Additional file 2

Scatter plot of the correlation between the blood chemistry values and MMP-9 or MMP-7. *A scatter plot was used to identify the correlation between the blood chemistry values (total bilirubin, AST, ALP, Log CEA and Log CA19-9) and MMP-9 or MMP-7 in the control and cholangiocarcinoma patients. This figure demonstrates that there is no significant correlation* ($p > 0.05$) *between the blood chemistry values and MMP-9 or MMP-7 in both groups.*

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Acknowledgements

This study was funded by Thailand Research Fund (RSA52) and Rajavithi Hospital. We thank Assoc. Prof. Dr. Tawechai Tejapongvorachai and Prof. Dr. Apiwat Mutirangura for their clinical guidance, and Dr. Chulaluk Komoltri, Dr. Somrat Lertmaharit and Thai CERTC staffs for help with statistics.

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Pre-publication history

The pre-publication history for this paper can be accessed here:

http://www.biomedcentral.com/1471-230X/9/30/prepub