

## RESEARCH ARTICLE

# Serum Cathepsin B to Cystatin C Ratio as a Potential Marker for the Diagnosis of Cholangiocarcinoma

Ammala Monsouvanh<sup>1</sup>, Tanakorn Proungvitaya<sup>1,2</sup>, Temduang Limpai boon<sup>1,2</sup>, Chaisiri Wongkham<sup>2</sup>, Sopit Wongkham<sup>2</sup>, Vor Luvira<sup>2</sup>, Siriporn Proungvitaya<sup>1,2\*</sup>

### Abstract

Cholangiocarcinoma (CCA) is a cancer of the bile duct epithelial cells. The highest incidence rate of CCA with a poor prognosis and poor response to chemotherapy is found in Southeast Asian countries, especially in northeastern Thailand and Lao PDR. Cathepsin B is a lysosomal cysteine protease which is regulated by cysteine proteinase inhibitors such as cystatin C. Elevation of cathepsin B levels in biological fluid has been observed in patients with inflammatory diseases and many cancers. We aimed to investigate the serum cathepsin B and cystatin C levels of CCA patients to evaluate the feasibility of using cathepsin B and cystatin C as markers for the diagnosis of CCA. Fifty-six sera from CCA patients, 17 with benign biliary diseases (BBD) and 13 from controls were collected and the cathepsin B and cystatin C levels were determined. In addition, cathepsin B expression was investigated immunohistochemically for 9 matched-pairs of cancerous and adjacent tissues of CCA patients. Serum cathepsin B, but not cystatin C, was significantly higher in CCA and BBD patient groups compared to that in the control group. Consistently, all cancerous tissues strongly expressed cathepsin B while adjacent tissues were negative in 7 out of 9 cases. In contrast, serum cystatin C levels were comparable between CCA and control groups, although serum cystatin C levels in the BBD group was higher than that in the control or CCA groups. When the serum cathepsin B to cystatin C ratio was calculated, that of the CCA group was significantly higher than that of the control group, and, although statistically not significant, the ratio of CCA group showed a trend to be higher than that of the BBD group. Thus, the cathepsin B to cystatin C ratio might be used as an alternative marker for aiding diagnosis of CCA.

**Keywords:** Cathepsin B - cystatin C - cholangiocarcinoma

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### Introduction

Cholangiocarcinoma (CCA) arises from malignant transformation of cholangiocytes and currently classified as intra-hepatic and extra-hepatic CCA (Lazaridis and Gores, 2005; Gatto et al., 2010). The highest incidence rate of CCA is found in Southeast Asian countries especially in Khon Kaen province of Thailand (118.8/100,000), where intrahepatic CCA is commonly found. The CCA in this endemic area is closely related with *Opisthorchis viverrini* (OV) infection. The prevalence of OV infection in the endemic area among Thai adult population is 24.5% and is 58.5% among Laos adult population in Saravane province, Lao PDR. The prognosis of CCA patients is usually poor and no response to chemotherapy (Lazaridis and Gores, 2005; Sayasone et al., 2007; Sripa et al., 2011).

Cathepsin B is a lysosomal cysteine protease. There are two forms of cathepsin B, namely, non-glycosylated or

single-chain form (27 kDa) and glycosylated or two-chain form (28-30 kDa) (Mach et al., 1992; Iacobuzio-Donahue et al., 1997). It is secreted and detected in the biological fluid of inflammatory diseases and many cancers (Claus et al., 1998; Nagai et al., 2003; Chu et al., 2004; Strojan et al., 2004; Tsai et al., 2009). Cathepsin B is regulated by cysteine protease inhibitors, including cystatin C and stefin A (Sloane, 1990). Imbalances between proteinases and their inhibitors correlated with tumor progression (Calkins and Sloane, 1995), and the measurement of cathepsin B and cystatin C levels has been suggested to be beneficial for prognosis or diagnosis of diseases (Nagai et al., 2003). The serum level of cathepsin B may serve as a prognostic factor for patients with advanced melanoma (Kos et al., 1997). Compared to the healthy controls, the cathepsin B and cyatatin C levels were significantly increased in the sera of lung cancer patients (Chen et al., 2011).

In spite of extensive search for biomarkers related

<sup>1</sup>Centre for Research and Development of Medical Diagnostic Laboratories, Faculty of Associated Medical Sciences, <sup>2</sup>Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand \*For correspondence: sirpat@kku.ac.th

to CCA, reliable specific diagnostic biomarkers have not yet been identified. Tremendous efforts are still paid to find new markers appropriate for using in diagnosis of malignancies (Wongkham and Silsirivanit, 2012). As mentioned above, cathepsin B and cystatin C have been currently studied as biomarkers in several diseases including cancers. However, little is known about the levels of cathepsin B and cystatin C in CCA. Therefore, in this preliminary study, we aimed to determine cathepsin B and its inhibitor (cystatin C) levels in the sera of CCA and controls.

## Materials and Methods

### *Serum and tissue samples*

Fifty-six CCA sera and 17 benign biliary diseases (BBD) sera were kindly provided from the Liver Flukes and Cholangiocarcinoma Research Center (LFCRC), Faculty of Medicine, Khon Kaen University. The normal control sera were collected from 13 healthy persons who came to check-up at the Faculty of Associated Medical Sciences (AMS), Khon Kaen University. The data of serum ALT, AST, ALP (U/L) and creatinine (mg/dL) of 56 CCA and 15 healthy persons were collected from LFCRC and AMS.

The sera were kept at  $-20^{\circ}\text{C}$  until use. This project was approved by the Ethical Committee of Khon Kaen University, Thailand (HE551283).

Nine cancerous tissues including adjacent tissues from 9 patients were kindly provided from LFCRC. These are fresh frozen and formalin-fixed tissues. All tissue samples were sectioned for immunostaining technique.

### *Measurements of cathepsin B and cystatin C levels in the sera*

Cathepsin B levels in the serum samples were measured using human cathepsin B ELISA kit (Biorbyte Ltd, Bartholomew's court, U.K). The sera were diluted 1:20 and assayed according to the manufacturer's instructions. The absorbance was measured at 450 nm in a microplate reader. Serum cathepsin B levels were quantified using human cathepsin B as a standard and a calibration curve was constructed using MasterPlex® curve-fitting software (version 2.0.0.73, Hitachi Solutions America, South San Francisco, CA).

Cystatin C level was determined using 3  $\mu\text{L}$  of each serum and Cystatin C immunoturbidimetric assay (Diazyme, California, USA) on a Beckman Synchron CX4 clinical chemistry analyzer (Beckman Coulter, USA). This was an end point assay, where 5 calibrators, 2 levels of controls (High and low internal controls) were included.

### *Immunohistochemical detection of cathepsin B in CCA and adjacent tissues*

Paraffin-embedded CCA sections of 4  $\mu\text{m}$  thicknesses were deparaffined by soaking each for 2 minutes in xylene, absolute ethanol, 95% ethanol and 70% ethanol, respectively. The sections were boiled in 1X citrate buffer (pH 6.0) for 10 minutes and washed in 1X phosphate buffer saline. Then, the endogenous peroxidase activity was blocked with 3%  $\text{H}_2\text{O}_2$  in methanol for 30 minutes

in the dark and non-specific background binding was blocked with 10% fetal bovine serum for 30 minutes. The sections were incubated with 170  $\mu\text{L}$  of rabbit anti-cathepsin B polyclonal antibody (1:200 dilution, Biorbyte Ltd, Bartholomew's court, UK) at  $4^{\circ}\text{C}$  overnight. The sections were washed in 1XPBS and incubated with 170  $\mu\text{L}$  of anti-rabbit antibody using EnVision System (DakoCytomation, Inc, California, USA) for 60 minutes and the signal was developed by diaminobenzidine (DakoCytomation, Inc, California, USA) for 5 minutes in the dark. The sections were washed with running tap water until clear and counterstained with hematoxylin for 5 minutes. The sections were dehydrated in an ascending series of ethanol and finally immersed in xylene. The sections were mounted with Permount® and sealed with a cover glass.

The immunohistochemical (IHC) staining scores for cathepsin B expression were graded into 4 categories; 0, 1+, 2+ and 3+ based on stained multiplied by intensity of stained cells. The percentage of the stained cells such as grade 0 was 0%, grade 1 was 1-25%, grade 2 was 26-50% and grade 3 was >50%, respectively (Yang et al., 2010). The intensity of stained cells was graded as follows; 0: negative, 1: weak, 2: moderate, and 3: strong.

### *Statistical analysis*

The test for statistical significance of serum cathepsin B level between CCA, BBD patients and controls was done by Mann-Whitney test. The data were presented as the median with quartile deviation for two or more independent experiments.

## Results

### *Serum cathepsin B and cystatin C in CCA, benign biliary diseases and control groups*

Serum cathepsin B levels of the CCA, BBD and control groups were presented as median with quartile deviation (Figure 1). Serum cathepsin B levels of CCA ( $88\pm 43$  ng/mL) and BBD ( $128\pm 36$  ng/mL) were significantly higher than that of the control group ( $50\pm 17$  ng/mL). Serum cystatin C level of the BBD group ( $1950\pm 145$  ng/mL) was significantly higher than that of CCA ( $1400\pm 250$  ng/mL) or the control group ( $1230\pm 190$  ng/mL).

When the serum cathepsin B to cystatin C ratios were taking in account, that of the CCA ( $0.07\pm 0.03$ ) group was significantly higher than that of the control group ( $0.05\pm 0.02$ ), and, although statistically not significant, showed a trend to be higher than that of the BBD group ( $0.07\pm 0.02$ ) (Figure 1). The cut-off value of the serum cathepsin B to cystatin C ratio determined by the ROC curve analysis of CCA was  $\geq 0.06$ . The sensitivity was 63.0% and the specificity was 100.0%. The number of CCA patients having the cathepsin B to cystatin C ratio above the cut-off value was 35 out of 56.

When the CCA group were divided into 2 sub-groups according to age, sex, pathological type and survival time, only the age was significantly correlated with the cathepsin B to cystatin C ratio. The CCA patients of  $\geq 59$ -year-old showed lower ratio than the CCA patients <59-year-old (Table 1).

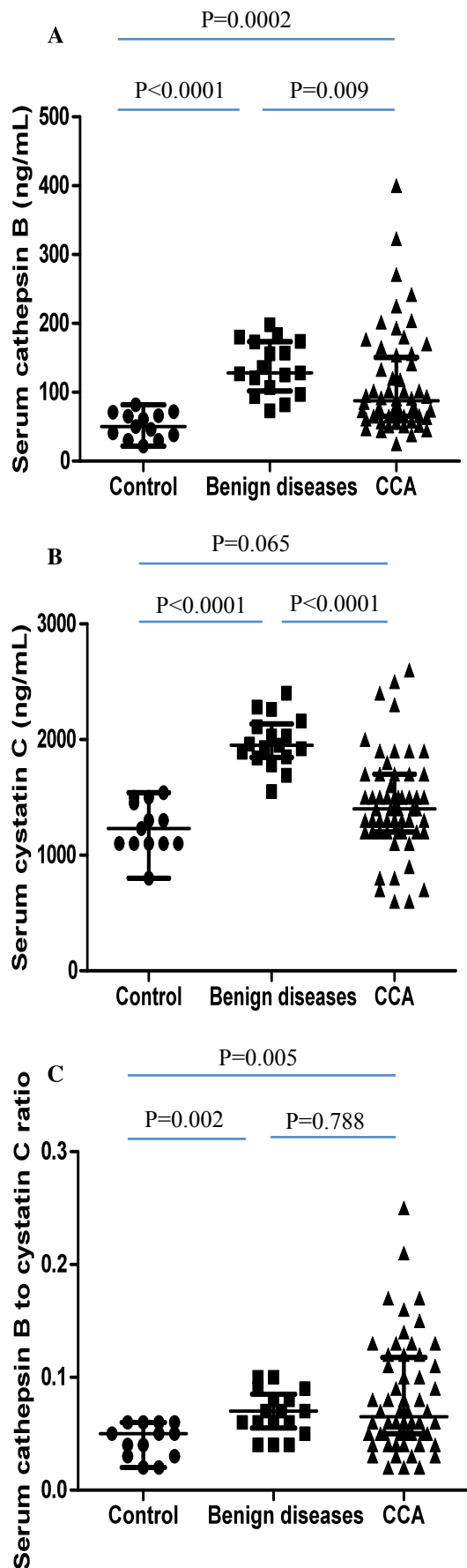


Figure 1. Comparison of Serum Cathepsin B and Cystatin C and its Ratio in CCA, Benign Diseases and Control Group. (A) serum cathepsin B, (B) serum cystatin C, (C) Ratio of serum cathepsin B to cystatin C

*Expression of cathepsin B in CCA tissues*

When the expression of cathepsin B in CCA tissues was examined, cathepsin B was strongly expressed in all 9 cancerous tissues of CCA patients. According to the IHC scores for cathepsin B expression, the samples from 4 cases (45%) were 3+, 1 case (10%) was 2+, 4 cases (45%) were 1+. In contrast, cathepsin B expression was seen in

**Table 1. The Comparison between Serum Cathepsin B to Cystatin C Ratio and Clinical Parameters in 56 CCA**

Clinical parameters (n)	Cathepsin B to cystatin C ratio	p value
Age		
≥59 (28)	0.06±0.03	0.018
<59 (28)	0.08±0.04	
Sex		
Male (38)	0.08±0.04	0.132
Female (18)	0.06±0.02	
Pathological type		
Non-papillary CCA (18)	0.06±0.05	0.263
Papillary CCA (38)	0.07±0.03	
Survival time		
Long time ≥63.43 Wks (28)	0.07±0.04	0.961
Short time <63.43 Wks (28)	0.06±0.03	

The statistical significance is p-value < 0.05 (Mann-Whitney test)

**Table 2. The Expression of Cathepsin B in Cancerous and Adjacent Tissues of CCA Patients**

Patients number	Immunohistochemistry (IHC) score		Serum Cathepsin B (ng/mL)
	Cancerous tissues	Adjacent tissues	
1	3+	0	66
2	3+	0	63
3	3+	1+	45
4	3+	0	25
5	2+	1+	102
6	1+	0	102
7	1+	0	102
8	1+	0	61
9	1+	0	38

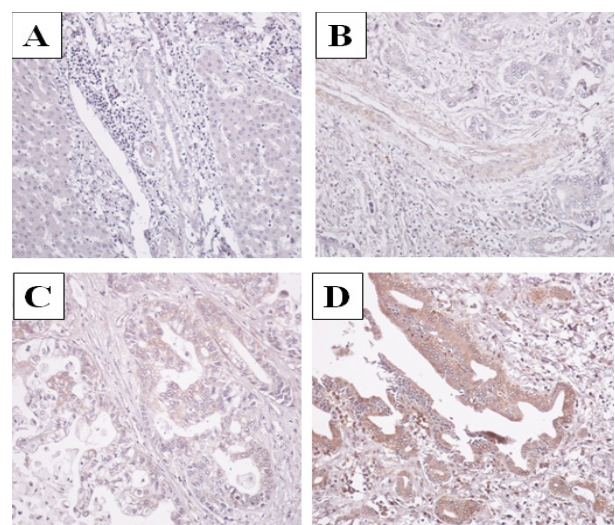


Figure 2. IHC Staining for Cathepsin B in The Cancerous and Adjacent Tissues from CCA Patients. A: negative stain (score 0) in the adjacent tissue. B, C and D: positive stain in cancerous tissues with scores 1, 2 and 3, respectively

the adjacent (non-cancerous) tissues of 2 out of 9 cases (22%) (Figure 2, Table 2). The intensity of cathepsin B staining did not directly related to the cathepsin B level in the serum (Table 2).

## Discussion

We measured cathepsin B levels in the serum samples from CCA patients, BBD patients and controls using an ELISA kit. The results show that the serum cathepsin B level in the CCA and BBD patients group was significantly ( $p < 0.05$ ) higher than that of the control group. Cathepsin B is normally located in lysosomes and involved in the turnover of proteins and plays various roles in maintaining the normal metabolism of cells (Uchiyama and Anderson, 1994). An increased expression and release of cathepsin B has been observed in various tumor tissues as well as in the sera of various tumor patients (Kos et al., 1998). As can be seen in Figure 1, we recognized that CCA patients can be divided into 2 sub-groups, high and low cathepsin B. Whether this is related to any biochemical nature of CCA or to prognosis of patients should be explored using a large scale samples.

In the present study, immunohistochemical study revealed that cathepsin B protein was detected in all cancerous tissues with negative reactivity in 7 out of 9 adjacent (non-cancerous) tissues. The intensity of cathepsin B staining in tumor tissue did not match to the serum cathepsin B level (Table 2), suggesting that serum cathepsin B level is not primarily determined by tumor. Retrospective immunohistochemical analysis revealed that the postoperative survival time of the lung cancer patients who had cathepsin B-positive tumors were significantly shorter than those having cathepsin B-negative tumor (Kayser et al., 2003). Whether such a correlation would be observed in CCA should be analysed in future.

Cathepsin B is over-expressed in several cancers such as oral squamous cell carcinoma and colorectal cancer (Hirai et al., 1999; Yang et al., 2010). Our results added that cathepsin B is over-expressed in CCA. Cathepsin B expression by human colorectal cancers and surrounding noncancerous cell components is assumed to contribute to both local invasion at the early stage and remote metastasis without influence of cystatin C (Hirai et al., 1999). The elevated serum cathepsin B level may be a reflection of increased cathepsin B production in the intracellular or extracellular responses of CCA. However, the results of the cathepsin B immunostaining did not correlate with the serum cathepsin B level (Table 2). The regulation mechanism for cathepsin B level needs further investigation.

Cystatin C, a member of the cystatin family, is a non-glycosylated 13 kDa basic protein. It is a potent inhibitor for lysosomal cysteine proteinases such as cathepsin B, H, L and S (Abrahamson et al., 2003). The mean serum cystatin C level of intrahepatic cholestasis patients, was reported to be remarkably higher than that of extrahepatic cholestasis patients (Buyukberber et al., 2010). However, in this study, CCA were all intrahepatic type, and serum cystatin C levels were not significantly higher in the CCA

compared to the control group. While BBD is a diffuse disease of the liver, CCA is rather a focal change. Such difference might affect the serum cystatin C levels.

In the present study, both serum cathepsin B and cystatin C levels in the patients with BBD are remarkably higher than those in the CCA and control groups. However, cathepsin B and cystatin C ratio of the CCA group was significantly higher than that of the control group without significant difference between CCA and BBD group. After statistical analysis, we could not find any specific parameters related to the elevated cathepsin B to cystatin C ratio. Also, the arbitrarily set cut-off value gave only 63.0% sensitivity. Whether the elevation of cathepsin B to cystatin C ratio is related to any subtype of CCA or to any specific clinical manifestation should be explored in future.

In conclusion, serum cathepsin B to cystatin C ratio may be useful for the diagnosis of CCA.

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