

RESEARCH ARTICLE

Identification of Biliary Bile Acids in Patients with Benign Biliary Diseases, Hepatocellular Carcinoma and Cholangiocarcinoma

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Abstract

Bile acids are implicated as aetiological factors in many types of gastrointestinal tract cancer including cholangiocarcinoma (CCA). Alterations in bile acid concentrations may affect the pathogenesis of these different types of cancer. Our aim was to determine the bile acid profile in gallbladder bile from patients who underwent liver resection. Thirty-seven patients with cholangiocarcinoma, 5 with hepatocellular carcinoma, and 7 with benign biliary diseases were studied. High pressure liquid chromatography was used to analyze conjugated and unconjugated bile acids. CCA patients with low (≤ 2 mg/dl) and high (> 2 mg/dl) levels of total serum bilirubin had significantly higher total bile acid and conjugated bile acid concentrations than the benign biliary disease group. Markedly elevated levels of cholic and chenodeoxycholic acid were found in CCA cases with high levels of total serum bilirubin. Concentrations of total bile acids and primary bile acid were correlated with serum cholesterol, bilirubin and ALP in CCA. Notably, correlation of the carcinoembryonic antigen, a tumor marker, was found with level of total bile acids and chenodeoxycholic acid. These findings suggest a different pattern of bile acid concentration in cancer patients compared to patients with benign biliary diseases. Thus, accumulation of certain bile acids may be involved in carcinogenesis.

Keywords: Bile acids - cholangiocarcinoma - hepatocellular carcinoma

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Introduction

Bile acids are sterol-derived compounds synthesized and secreted by hepatocytes into bile canaliculi (Plaa et al., 1982). Bile acids are actively secreted by the liver into bile and discharged into the intestinal lumen upon ingestion of a meal. Bile acids confer detergent-like properties that are important for their physiological functions in absorption of dietary lipids and fat-soluble vitamins from the small intestine (Chiang, 2009). Bile acids that are synthesized from cholesterol in hepatocytes are termed primary bile acids. The most abundant primary C24 bile acids are chenodeoxycholic acid (CDCA) and cholic acid (CA). Bile acids that are formed by bacterial modification by removal, oxidation, or epimerization of the nuclear hydroxyl groups of primary bile acids are termed secondary bile acids. The most common C24 7-deoxy bile acids are lithocholic acid (LCA), formed by bacterial 7-dehydroxylation of CDCA, and deoxycholic acid (DCA), formed by bacterial 7-dehydroxylation of CA.

Bile acids are also implicated as causative agents in cancers of the gastrointestinal tract, including cancers

of the esophagus, stomach, small intestine, biliary tract, pancreas and colon (Kuwahara et al., 1989; Reveille et al., 1990; Ross et al., 1991; Bayerdorffer et al., 1995; Kauer and Stein, 2002; Tucker et al., 2004). As bile fluid contains major pool of bile acids, the interaction of bile acids with cholangiocytes leads to alterations of cholangiocyte secretion, proliferation, apoptosis and differentiation (Alpini et al., 1999; Alpini et al., 2001; 2002a). Deleterious effects of bile acid exposure that are thought to be related to carcinogenesis include induction of reactive oxygen and reactive nitrogen species; induction of DNA damage, mutations, apoptosis in the short term, and selection for apoptosis resistance in the long term (Craven et al., 1987; Booth et al., 1997; Crowley-Weber et al., 2002). Bile acids may promote carcinogenesis by stimulating a variety of kinase signaling pathways; for example proliferation of cholangiocytes induced by bile acids occurs by a phosphatidylinositol 3-kinase-dependent pathway (Alpini et al., 2002b). Transactivation of the epidermal growth factor receptor by bile acids has been shown to stimulate cholangiocyte proliferation (Yoon et al., 2002a).

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Although studies have shown that bile acids can be carcinogenic, there are few reports evaluating bile acid composition in biliary tract cancer, especially for liver fluke-associated CCA. Increased levels serum bile acids such as CA and CDCA have been reported in human opisthorchiasis (Migasena et al., 1983). N-nitrosotaurocholic and N-nitrosoglycocholic acid are mutagenic in the Ames test (Puju et al., 1982). DCA is also a potent promoter of diethylnitrosamine-initiated hepatocarcinoma (Cameron et al., 1982). Most previous studies have reported bile acid levels in serum but not in bile or tumor tissue. The objective of this study is to compare bile acid concentrations and composition between subjects with cholangiocarcinoma, hepatocellular carcinoma and benign biliary diseases. This information may be useful to elucidate the contribution of certain bile acids in cancer development in future studies.

Materials and Methods

Patients and bile collection

Bile samples were collected from patients who were undergone liver resection at Srinagarind Hospital, Khon Kaen, Thailand. Forty-nine bile samples were successfully collected. There were 37 patients with cholangiocarcinoma, 5 patients with hepatocellular carcinoma, and 7 patients with benign biliary diseases, including cholangitis and cystadenoma. The diagnosis was based on clinical findings, ultrasonography and histological data. The study protocol has been approved by the Khon Kaen University Ethics Committee for Human Research (HE 531163). Informed consents were obtained from all subjects. Ten to fifteen ml of bile was collected via gallbladder puncture. Samples were supplemented with 100 ppm of butylated hydroxytoluene, flushed with nitrogen, and stored at -70°C until analyses. All biochemical analyses that were used in this study were obtained from the Clinical Chemistry Laboratory Unit of Srinagarind Hospital.

Bile acid analysis

Samples were centrifuged at 3500 rpm for 10 min. The bile samples (100 μl each) were transferred to a glass tube containing 500 μg of internal standard (5 β -cholanic acid). Samples were dried under nitrogen at 50°C and re-dissolved in 1 ml of distilled water (DW). Samples were then subjected to solid-phase extraction using a Bond-Elute C18 cartridge (Varian Assoc, Walnut creek, CA). The C18 cartridge was preconditioned prior to loading with successive elutions by 2 ml of chloroform – methanol 2:1 (v/v), 5 ml of methanol and 5 ml of (DW). After loading each sample, the column was washed with 2 ml of DW and 2 ml of n-hexane. The column was left for 10 minutes to remove excess solvents. Bile acids were recovered from the cartridge by elution with 5 ml of methanol. The eluents were then evaporated to dryness under nitrogen at $\sim 50^{\circ}\text{C}$ and the residue was dissolved in 1 ml of methanol.

High pressure liquid chromatography (HPLC) analysis for conjugated and free bile acids was carried out with a three pump Rainin Dynamax system (Rainin Inst., Woburn, MA). The sample was applied to a 4.6x250

mm Spherisorb ODS2, 5 μM (Phase Separations Ltd., Clwyd, U.K) and eluted with gradient from 65% methanol/37% 0.03 M ammonium acetate, pH 4.5 to 90% methanol/10% ammonium acetate buffer, followed by 100% methanol. Detection was by an evaporative light-scattering detector (ELSD Mk III, Altech, Deefield, IL), which had been calibrated with 14 standard bile acids. The bile acid standards were tauroursodeoxycholic acid (TUDC), glycoursocholic acid (GUDC), taurocholic acid (TCA), glycocholic acid (GCA), taurochenodeoxycholic acid (TCDC), taurodeoxycholic acid (TDC), tauroolithocholic acid (TLC), glycolithocholic acid (GLC), free ursodeoxycholic acid (UDCA), free lithocholic acid (LCA) (Calbiochem, Gibbstown, NJ), glycochenodeoxycholic acid (GCDC), glycodeoxycholic acid (GDCA), free cholic acid (CA), free chenodeoxycholic acid (CDCA) and free deoxycholic acid (DCA) (Sigma-Aldrich, St. Louis, MO).

Statistical analysis

Results are presented as mean \pm standard deviation. The data were analyzed by using Kruskal-Wallis one-way analysis. P-value <0.05 was considered statistically significant.

Results

Demography of patients and laboratory characteristics

Forty-nine total cases were analyzed: 37 with CCA; 5 with hepatocellular carcinoma; and 7 with benign biliary disease, including cholangitis and biliary cystadenoma. The male to female ratio was 2.5:1 and the average age was 58 years (range 35-96 years). Of the 37 patients with CCA, seven had a level of total serum bilirubin >2 mg/dl (HB). In addition, 30 of CCA patients had levels of total serum bilirubin ≤ 2 mg/dl (LB). Significant differences in total and direct serum bilirubin between the CCA with the LB group and the benign biliary disease group were not observed. Levels of cholesterol, total bilirubin, direct bilirubin, albumin and globulin in the hepatocellular carcinoma group were not different compared to other groups. Globulin levels in all patients were slightly higher than the normal range. There was no difference in cholesterol levels between patients (Table 1).

Liver function tests were also performed. Alanine aminotransferase (ALT), alkaline phosphatase (ALP) and aspartate aminotransferase (AST) levels were significantly higher in the CCA with HB group compared to the benign biliary disease and the CCA with LB groups. Levels of ALT and ALP in the hepatocellular carcinoma group were significantly lower compared to the levels in the CCA with HB group (Figure 1).

Bile acid profile in human gallbladder bile

Comparison of total bile acids, total conjugated bile acids (glycine and taurine conjugated) as well as total unconjugated bile acids in the CCA, hepatocellular carcinoma and benign biliary diseases groups are shown in Figure 2.

Total concentrations of bile acids in the benign biliary diseases, hepatocellular carcinoma, CCA with LB and

Table 1. Biochemical Parameters in Serum of Patients with Benign Biliary Diseases, Hepatocellular Carcinoma and CCA

Parameters (normal range)	Benign biliary disease: BD (n=7)	Hepatocellular carcinoma: HCC (n=5)	CCA (n=37)	
			Total serum bilirubin ≤ 2 mg/dl (LB) (n=30)	Total serum bilirubin >2 mg/dl (HB) (n=7)
Cholesterol (127-262 mg/dl)	172 \pm 25 (132-199)	149 \pm 55 (110-228)	183 \pm 50 (124-280)	231.0 \pm 68.0 (130.0-327.0)
Total bilirubin (0.25-1.5 mg/dl)	1.5 \pm 2* (0.3-5.3)	2.4 \pm 3.2 (0.4-7.2)	0.4 \pm 1.9* (0.2-1.0)	7.8 \pm 8.7 (2.1-26.6)
Direct bilirubin (0-0.5 mg/dl)	0.8 \pm 1.4* (0.1-3.7)	1.5 \pm 2.6 (0.1-5.4)	0.13 \pm 0.08* (0.1-0.3)	5.8 \pm 8.0 (1.0-23.7)
Albumin (3.8-5.4 g/dl)	3.5 \pm 0.7 (2.7-4.7)	3.8 \pm 0.8 (2.7-4.6)	4.1 \pm 0.6 (2.4-5.0)	3.7 \pm 0.6 (2.9- 4.8)
Globulin (2.6-3.4 g/dl)	4.2 \pm 0.9 (3.4-5.9)	4.5 \pm 1.1 (3.1-5.5)	4.2 \pm 0.8 (2.7-6.0)	4.0 \pm 0.6 (3.3- 4.9)

*indicates significant differences compared to the CCA with total serum bilirubin >2 mg/dl (HB) along the same horizontal line, Each value represents mean \pm SD with range in parenthesis

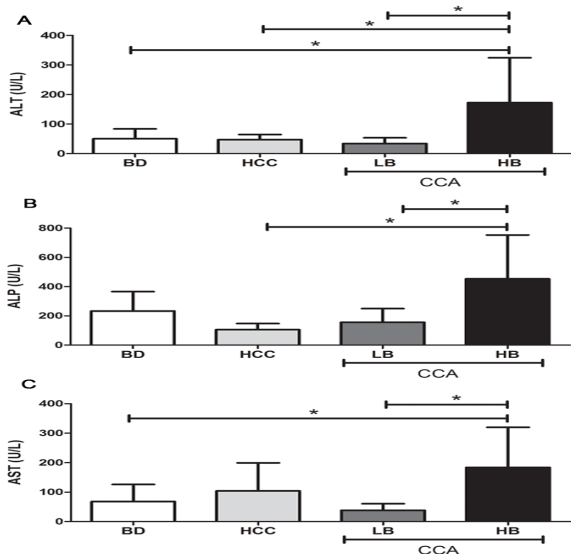


Figure 1. Bar Graphs Represent Serum Biochemical Parameters. Mean serum ALT (A), ALP (B) and AST (C) in patients with benign biliary disease (BD), hepatocellular carcinoma (HCC) and CCA patients with total serum bilirubin ≤ 2 mg/dl (LB) and >2 mg/dl (HB). P-values were obtained by One-Way ANOVA test. * indicates significantly different (P<0.05)

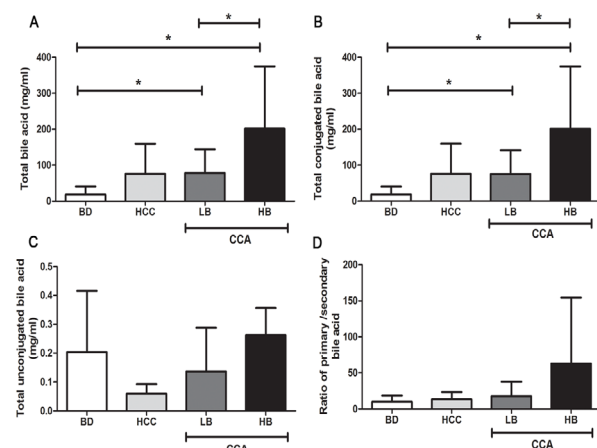


Figure 2. Mean Levels of Total Bile Acid (A), Total Conjugated Bile Acids (B) Total Unconjugated Bile Acids (C) and Ratios of Primary to Secondary Bile Acids in Gallbladder Bile from Patients with Benign Biliary Disease (BD), Hepatocellular Carcinoma (HCC) and CCA Patients with Total Serum Bilirubin ≤ 2 mg/dl (LB) and >2 mg/dl (HB). *Indicates significantly different (P<0.05)

HB groups were 18.7 \pm 21.7 mg/ml, 76.1 \pm 83.4 mg/ml, 78.2 \pm 65.5 mg/ml and 201.8 \pm 173.2, respectively. The CCA groups with LB and HB had significantly higher total bile acid concentrations than the benign biliary disease group (P<0.05). Moreover, total bile acid concentrations in the CCA with HB group was increased compared to the CCA with LB group (P<0.05).

Levels of total conjugated bile acids in the benign biliary disease, hepatocellular carcinoma, CCA with LB and HB groups were 18.4 \pm 21.7 mg/ml, 76.0 \pm 83.3 mg/ml, 75.5 \pm 66.0 mg/ml and 201.5 \pm 173.1 mg/ml, respectively. Total conjugated bile acid concentrations in the CCA with LB and HB groups were significantly increased compared to the benign biliary disease group (P<0.05). Total unconjugated bile acid concentrations in the benign biliary disease, hepatocellular carcinoma, CCA without bile duct obstruction and CCA with LB and HB groups were 0.20 \pm 0.2 mg/ml, 0.06 \pm 0.03 mg/ml, 0.14 \pm 0.15 mg/ml and 0.3 \pm 0.1 mg/ml, respectively. No significant differences were observed between all groups.

The ratios of primary (CA, TCA, GCA, CDCA, TCDC and GCDC) to secondary (DC, TDC, GDC, UDCA, TUDC, GUDC, LCA, TLC and GLC) bile acids were also analyzed. Ratios of primary/secondary bile acids in the benign biliary diseases, hepatocellular carcinoma, CCA with LB and HB groups were 9.9 \pm 8.2 mg/ml, 13.2 \pm 10.0 mg/ml, 17.7 \pm 20 mg/ml and 62.98 \pm 91.16 mg/ml, respectively. The increasing primary:secondary ratio in CCA with HB was noted but this was not significantly different.

Levels of the primary bile acids CA and CDCA are shown in figure 3. Markedly elevated levels of CA were found in CCA patients with HB. Levels of CA in patient with benign biliary disease, hepatocellular carcinoma, CCA with LB and HB were 8.8 \pm 9.2 mg/ml, 11.7 \pm 5.3 mg/ml, 35.2 \pm 31.5 mg/ml and 122.3 \pm 144.0 mg/ml, respectively. Significant differences in CA concentration were observed in patients with CCA with LB and HB compared to patients with benign biliary disease (P<0.05). Levels of CA in the CCA with HB group were higher than in the hepatocellular carcinoma group, but this difference was not significant. Moreover, the level of CDCA was also significantly increased in patients with CCA with LB and HB compared to patients with benign biliary disease (P<0.05).

The concentrations of secondary bile acids including DCA, UDCA and LCA were analyzed and results are

Table 2. Bivariate Correlation between Bile Acid Levels and Biochemical Tests

		Cholesterol	Total bilirubin	Direct bilirubin	ALP	CEA
Total bile acid	Pearson Correlation	0.460*	0.514*	0.536*	0.510*	0.353*
	P value	0.004	0.001	0.001	0.001	0.050
Total primary bile acid	Pearson Correlation	0.456*	0.531*	0.554*	0.543*	0.369*
	P value	0.005	0.001	0	0.001	0.041
Total secondary bile acid	Pearson Correlation					
	P value	ns	ns	ns	ns	ns
Total CA	Pearson Correlation	0.402*	0.456*	0.477*	0.643*	
	P value	0.014	0.005	0.003	0.000	ns
Total CDCA	Pearson Correlation	0.404*	0.490*	0.509*		0.430*
	P value	0.013	0.002	0.001	ns	0.016

*indicates the significant correlation, ns indicates no significant correlation

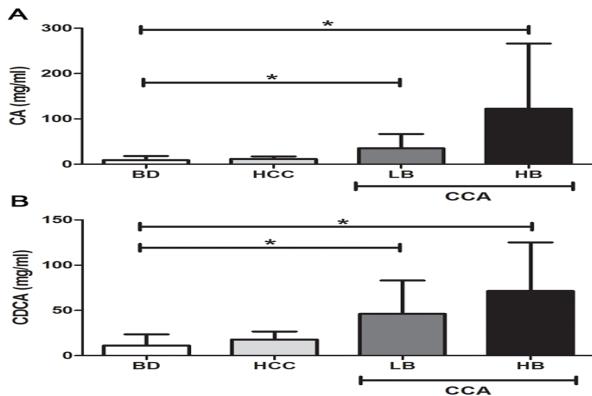


Figure 3. Levels of the Primary Bile Acids A; Cholic Acid (CA) and B; Chenodeoxycholic Acid (CDCA) in Gallbladder Bile from Patients with Benign Biliary Disease (BD), Hepatocellular Carcinoma (HCC) and CCA Patients with Total Serum Bilirubin ≤ 2 mg/dl (LB) and > 2 mg/dl (HB). Data are expressed as mean \pm SD. *indicates significantly different ($P < 0.05$).

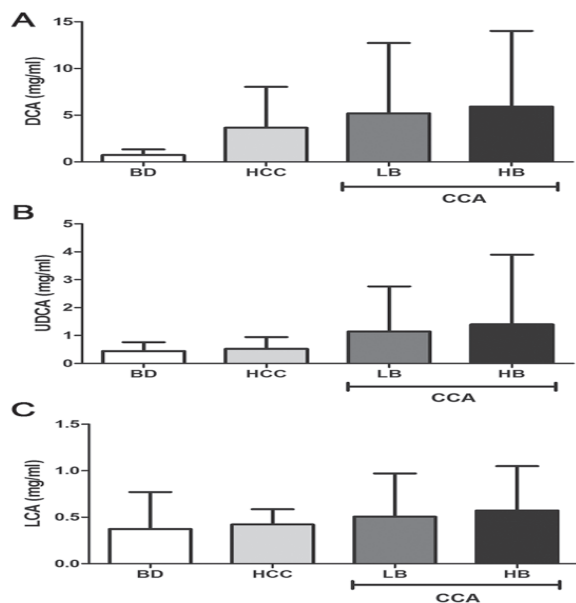


Figure 4. Levels of the Secondary Bile Acids, A) Deoxycholic Acid (DCA), B) Ursodeoxycholic Acid (UDCA) and C) Lithocholic Acid (LCA) in Patients with Benign Biliary Disease (BD), Hepatocellular Carcinoma (HCC) and CCA Patients with Total Serum Bilirubin ≤ 2 mg/dl (LB) and > 2 mg/dl (HB). Data are expressed as mean \pm SD.

shown in Figure 4. The level of DCA tended to be increased in patient with hepatocellular carcinoma and CCA with LB and HB compared to patients with benign biliary diseases, but this was not significantly different. Significant differences in UDCA and LCA were not observed in any group.

Correlation of bile acids and biochemical characteristics

To elucidate the effect of bile duct obstruction, the correlations between bile acid levels and liver function tests were analyzed in the CCA group by using bivariate analysis (Table 2). Levels of total bile acids and total primary bile acids were correlated with cholesterol, total bilirubin, direct bilirubin and ALP levels in CCA patients ($P < 0.01$). No significant differences were found in levels of total secondary bile acids and these liver biochemical tests. The primary bile acids CA and CDCA correlated with cholesterol, total and direct bilirubin and ALP levels ($P < 0.05$). In contrast, levels of the secondary bile acids including DCA, UDCA and LCA were not significantly correlated with cholesterol, total and direct bilirubin and ALP levels (data not shown). The correlation between bile acid and tumor markers was also investigated. Interestingly, the levels of total bile acid, total primary bile acid and chenodeoxycholic acid were significantly correlated ($P < 0.05$) with the serum level of carcinoembryonic antigen (CEA). However, a significant correlation was not observed between levels of carbohydrate antigen (CA) 19-9 and alpha-fetoprotein (AFP) (data not shown).

Discussion

Many studies have also found that there is an excess risk of forming malignant tumors in some organs exposed to high concentration of bile acids, such as in the gastrointestinal tract (Bernstein et al., 2009). Alteration of bile acid composition increased the incidence of cholangiocarcinoma and colorectal adenocarcinoma (Kinami et al., 1993; Debruyne et al., 2002). In this study, we determined the bile acid profiles in bile from patients with CCA and hepatocellular carcinoma, and compared these profiles to those found in the bile of patients with benign biliary diseases. Higher levels of total bile acids were found in CCA and hepatocellular carcinoma patients. Markedly increased levels of total bile acids were observed in samples from patients with CCA with

HB. This finding suggests that bile duct obstruction may alter bile acid levels. Higher levels of primary bile acids resulting in an increased primary:secondary bile acid ratio in samples from patients with CCA with HB were also found. A positive correlation between total bile acids or total primary bile acids and biliary tract obstruction was also found, with total bilirubin and direct bilirubin representing markers of bile obstruction. CA and CDCA were also increased in the CCA group compared to the benign biliary disease group.

A previous study showed that bile duct obstruction increases bile acid synthesis. Bile duct ligation in rats increased serum, liver and urine bile acid levels (Kinugasa et al., 1981). The mechanism by which bile duct obstruction induces bile acid synthesis remain unclear but accelerated hepatic cholesterol synthesis and increased serum cholesterol levels have been proposed (Cooper and Ockner, 1974). A correlation between total bile acids and primary bile acids and cholesterol concentrations was also found in this study. Therefore, increased cholesterol synthesis is consistent with increased bile acid production. Park et al. (2006) also reported increased CA in bile from patients with biliary tract cancer compared to patients' bile with biliary tract stones and normal bile. In contrast, they found decreased total and secondary bile acids in biliary tract cancers. Low levels of secondary bile acids may be caused by primary bile acids that cannot reach the regions in bowel where secondary bile acids are produced. Production of secondary bile acids also depends on bacterial enzymes, so infection could be a contributing factor. Recently, *Helicobacter pylori* was found in 66% of bile samples in patients with CCA (Boonyanugomol et al., 2012). This finding supports higher production of secondary bile acids in CCA compared to benign biliary diseases. Although we used benign biliary disease as a control, significantly different results compared to the cancer groups were obtained. Sharif et al. (2010) reported different patterns of bile acids in bile from patients with CCA compared to bile from patients with benign biliary disease. They also suggested bile acids as potential disease biomarkers for distinguishing malignant from benign biliary disease. Although the differences were not significantly different, we also found increasing levels of total bile acids, total conjugated bile acids and primary bile acids in bile from patients with CCA with HB compared to bile from patients with hepatocellular carcinoma. Lower levels of ALT, AST and ALP were found in the bile from patients with hepatocellular carcinoma compared to the bile from patients with CCA and high levels of serum bilirubin. This suggested that liver function affected the bile acid concentration.

Alterations in bile acid concentrations in CCA were also previously reported in serum. Increased levels of CA and CDCA was found in human opisthorchiasis which is a CCA-associated liver fluke (Migasena et al., 1983). Elevations of total bile acids and total conjugated bile acids were reported in the serum of patients in the CCA group compared with normal controls (Changbumrung et al., 1990). Increased bile acids in serum may be caused by biliary obstruction (Hong et al., 1973). Chronic exposure to bile acids may play an important role in

cholangiocarcinogenesis (Jansen, 2007). Concurrent with the present study, the positive correlation of total bile acids and CDCA with levels of serum CEA was observed in CCA patients. This result indicates the possible association of bile acid and cancer development. Previous studies suggested a role for bile acids in cholangiocarcinogenesis (Sirica, 2005). DCA, a potent promoter in the CCA hamster model, was shown to induce COX-2 in cultured immortalized human cholangiocytes via an EGFR-dependent mechanism and to block protein degradation of myeloid cell leukemia protein 1, a potent anti-apoptosis protein (Yoon et al., 2002a; 2002b). The underlying mechanism of bile acids on cholangiocarcinogenesis may involve their receptors. Adding the antagonist of farnesoid x receptor (FXR), a nuclear receptor for bile acid enhanced cholangiocarcinogenesis induced by free bile acids (Dai et al., 2011). Moreover, in bile duct obstruction, increased biliary bile acid concentration initiated enhanced cholangiocyte proliferation in an animal model (Alpini et al., 2002b).

In conclusion, total bile acids, conjugated bile acids, unconjugated bile acids and individual bile acids were analyzed in this study. Elevated levels of total bile acids, conjugated bile acids and primary bile acids were found in CCA. Bile duct obstruction may contribute to an alteration of bile acid concentration. Levels of CA, CDCA and DCA were increased in cancer groups. Moreover, levels of total bile acids and CDCA were also related to the level of CEA. These findings suggest a different bile acid concentration profile in the cancer groups compared to benign biliary disease groups. Alteration of bile acid concentration may be a reflection of the disease process. Since cholangiocytes are directly exposed to carcinogens in bile, profiling of bile acids in this study provides useful information about which bile acids may contribute to pathogenesis. Further studies are needed to determine the mechanisms underlying the correlation between bile acid composition and carcinogenesis.

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